John Geigert

The Challenge of CMC Regulatory Compliance for Biopharmaceuticals

Fourth Edition

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Preface

Preparing the fourth edition of my book was again a most humbling experience for me. My primary purpose for updating the previous edition was to continue to provide relevant insight and practical suggestions for a common sense, cost-effective, risk-managed approach to meeting the Chemistry, Manufacturing and Controls (CMC) regulatory compliance requirements and expectations for biopharmaceuticals as human medicinal products. But the scope of this approach was almost overwhelming as there was so much that could not be included in this latest updated edition. I trust that my choices will be of the most benefit to the upcoming next generation of Project Management, Process Development, Manufacturing, Analytical Development, Quality Control, Quality Assurance, and Regulatory Affairs staff who take responsibility for ensuring the quality, efficacy, and safety of these biopharmaceutical medicines for patients.

So many changes continue in the advancement of the protein-based biopharmaceuticals. Over 250 recombinant proteins, monoclonal antibodies, bispecific antibodies, fusion proteins, antibody Fab and Fc fragments, antibody-drug conjugates (ADCs) are now in the marketplace in both the United States of America (USA) and European Union (EU). And not to forget that over 80 biosimilars are also now available across all major classes of off-patent recombinant proteins and monoclonal antibodies.

The increasing entrance into the marketplace over the past 5 years of the gene therapy-based biopharmaceuticals. Using genetically engineered viruses, genes (DNA) are delivered either directly to the patient or to collected patient cells that then get administered back to the patient, so that the patient becomes the "bioreactor" producing the needed protein product. About 20 gene therapy-based biopharmaceuticals are now market-approved, with an estimate of about 10 new therapies to be approved every year going forward.

Another measure of the rapid degree of change occurring in the biopharmaceutical field is reflected in over 400 CMC regulatory compliance references listed in this book that were either issued or updated since the release of the last edition.

I am indebted to two major regulatory authorities: the United States Food and Drug Administration (FDA) and the European Medicines Agency (EMA). These

two regulatory authorities publish on their websites an abundance of CMC regulatory guidances (recommendations and expectations) to help the developing and changing biopharmaceutical industry. In addition, they both upload (after a biopharmaceutical medicine is market-approved) discussions, reviews, and meeting minutes during the biopharmaceutical review process, thus providing insights into how the regulatory authority currently views the current application of manufacturer's biopharmaceutical CMC regulatory compliant strategy. Many of the references listed in this book are from this information that is readily downloaded from their websites. Thanks also goes to the International Council of Harmonisation (ICH), in their harmonizing of consensus CMC guidelines. ICH has issued harmonized CMC guidelines not only for the content to be included in biopharmaceutical submissions seeking market approval (e.g., for viral safety evaluation, comparability of biopharmaceuticals after a manufacturing process change, etc.) but also for the strategic control for the complex biopharmaceutical manufacturing processes (e.g., for pharmaceutical development, quality risk management, pharmaceutical quality system, etc.). While the initial focus for ICH was on the protein-based biopharmaceuticals, as their guidances are updated, aspects for the gene-therapy biopharmaceuticals are being included. The ICH regulatory guidelines have driven the biopharmaceutical industry to a higher standard of manufacturing excellence and quality control, introducing the principles of Quality by Design (QbD), Quality Risk Management (QRM), Pharmaceutical Quality Systems (PQS), Knowledge Management (KM), Established Conditions (EC), and Post-Approval Change Management Protocols (PACMPs). It is for this reason that I have provided the FDA, EMA, and ICH website locations (listed at the end of each chapter) for the many regulations, guidance documents, and case examples that I have used in the preparation of this book.

Expediting clinical pathways have shortened the time in clinical development – Breakthrough Therapy designation (BTD) and Regenerative Medicines Advanced Therapy (RMAT) designation in the USA; PRIME (PRIority MEdicine) designation in the European Union. This shortening of the clinical study time from First-in-Human (FIH) entry studies to market approval (estimated to be at least a 2–3 year savings) has placed great demands upon the CMC regulatory compliance strategy with ever-decreasing time to complete all of the required development, optimization, validation, site changes, etc. for the challenging biopharmaceutical manufacturing processes and complex products. This enhanced pressure on the CMC teams has not gone unnoticed by the regulatory authorities and they are keenly aware of the potential delays in biopharmaceutical product market approval that now can be due to CMC issues. Discussion on how to effectively manage the CMC regulatory compliance strategy under clinical expedited pathways is provided in this book.

Thanks also go to the companies who stumbled in their CMC regulatory compliance strategy, resulting in delay or rejection of their biopharmaceutical, so that we can learn from their mistakes. At times, an effective CMC regulatory compliance strategy can seem like a mystery. Sometimes this mystery is self-induced in our companies, when the CMC team is not aware that an effective CMC regulatory

compliance strategy can be at hand. Through means of this fourth edition, I want to show clearly the "good news" that CMC regulatory compliance no longer has to be a mystery. But I also want to caution against the "bad news" that there can be too much CMC regulatory compliance information available, "an information overload." This is where this book becomes invaluable (along with the help of a good CMC consultant of course) in sifting through all of the public guidance available to determine which pieces are relevant for each specific biopharmaceutical manufacturing processes and product types. To reinforce that no commercial proprietary information is revealed in this book, I have provided internet website addresses for the comments on the various companies and their CMC biopharmaceutical issues, mentioned in this book.

Throughout this book, I use the terms "biologic" or "biological" whenever I am discussing CMC issues that apply across the board to pharmaceuticals that are (1) derived from living organisms, (2) have challenging manufacturing processes, and (3) are complex products. But, I use the terms "biopharmaceutical" or "recombinant DNA-derived" whenever I am discussing CMC issues specific for biologics manufactured from genetically engineered living organisms.

In Chap. 1, defining the terminology used in CMC regulatory compliance of biopharmaceuticals is paramount to being able to effectively communicate not only throughout our industry but also with the regulatory authorities. Also, the increasing diversity of biopharmaceutical product types is unveiled, with a discussion of the four major "waves" of product types that have entered the marketplace from the early 1990s through today. Today, there are the protein-based biopharmaceuticals (the recombinant proteins and the monoclonal antibodies) and there are the ever-increasing number of gene therapy-based biopharmaceuticals (the viral vectors and the genetically modified patient cells). In addition, a non-viral vector (mRNA) is now being pursued in the clinic. Time will tell how abundant these human patient "bioreactors" will become. In Chap. 2, the various regulatory pathways for initiating a clinical trial, for maintaining the clinical trial during its development and then for seeking market approval for biopharmaceuticals, is examined, within the USA and European Union regions. Differences in the regulatory pathways are discussed. In Chap. 3, biopharmaceuticals are shown to be definitely different from chemical drugs. This is not a perception, but a reality, and it is reflected by the statements on regulatory authority websites and in the wording of the regulatory guidances that they issue. Differences due to starting materials, differences due to the ability to control the manufacturing process, and differences due to the molecular complexity of the products are examined across four product types. Avoidance of the word "biogeneric" for biosimilars is discussed. In Chap. 4, why the risk-based approach is absolutely necessary to effectively manage the minimum CMC regulatory compliance continuum, due to the challenge in manufacturing and complexity of the resulting biopharmaceuticals, is examined. The ICH-recommended risk-based approach for biopharmaceuticals – Quality by Design (QbD)/Quality Risk Management (QRM) – is also discussed and shown to be an invaluable tool for establishing and

maintaining adequate and appropriate control for all biopharmaceutical types. In Chap. 5, the four primary adventitious agents of concern for biopharmaceuticals are examined in detail – prions, viruses, mycoplasma, bacteria/fungi. While each manufacturing process type has a different level of risk due to adventitious agent contamination, there is no biopharmaceutical manufacturing process that carries no risk of adventitious agent contamination. In Chap. 6, the significant differences between the starting materials for the protein-based biopharmaceuticals (Master Cell Bank) and the many starting materials for the gene therapy-based biopharmaceuticals (i.e., the viral vectors, transduced patient cells, mRNA non-viral vector) are evaluated. A problem here can carry all the way through the manufacturing process to the final administered biopharmaceutical product. In Chaps. 7 and 8, the risk-based requirements and expectations for an adequate and appropriate design and control of the biopharmaceutical drug substance manufacturing stages are examined. Similarities and differences between what is expected and what is doable for the drug substance manufacture, across the four types of biopharmaceuticals (recombinant proteins and monoclonal antibodies, viral and non-viral vectors, and genetically modified patient cells), are compared and contrasted. In Chap. 9, the risk-based requirements and expectations for an adequate and appropriate design and control of the biopharmaceutical drug product manufacturing stages – formulation, container closure, and aseptic processing of filling/sealing – are examined. Sometimes between the purified drug substance and formulation, the drug substance is conjugated (e.g., antibody-drug conjugates, PEGylation). In Chaps. 10 and 11, compared to chemical drugs, biopharmaceuticals have a large, complex biomolecular structure, seemingly endless structural variants, and, in addition, a highly complex process-related impurity profile, primarily due to the use of living systems involved in their manufacturing process. The challenges for the physicochemical and functional characterization of the different biopharmaceutical types – recombinant proteins, monoclonal antibodies, genetically engineered viruses, genetically engineered cells, mRNA non-viral vector – are examined, along with the use of a risk-based approach for process-related impurity control (and hopefully reduction or removal through the purification process). In Chap. 12, it is shown that because of the size and complexity of a biopharmaceutical functional/therapeutic activity, assays are required for strength/ potency measurement. In this chapter, the three types of functional activity assays for measuring potency are examined: bioassay, surrogate, and assay matrix. In Chap. 13, the seven major categories of Critical Quality Attributes (CQAs) are explored. Specific testing to meet the requirements of each of these quality attributes, for each biopharmaceutical type, is discussed. In Chap. 14, the art of specification setting for biopharmaceuticals is examined – both for the release of batches and for setting the shelf-life. The use of a risk-based approach to set the limits or ranges through clinical development and into market approval for a

biopharmaceutical is discussed. The concept of an interim regulatory specification for a to-be-marketed biopharmaceutical, especially when so few batches are available today to statistically set specifications, is explored. In Chap. 15, the three risk-based concerns that need to be addressed by an effective comparability study are examined. Demonstrating comparability for a biopharmaceutical after a manufacturing process change is no easy task, whether it be for a recombinant protein, monoclonal antibody, viral vector, or genetically modified patient cells. In Chap. 16, the critical importance of communicating with the regulatory authorities on the CMC regulatory compliance strategy is stressed. Finally, in this chapter, an encouragement is given to senior management to take advantage of CMC-focused meetings available with the regulatory authorities.

CMC regulatory compliance strategy does not determine the direction of the clinical development program; its primary purpose is to support it, but that does not mean that we in CMC should avoid the tough decisions that scientists must make when advancing the applications of genetic engineering toward human. Francis S. Collins, the former Director of the United States National Institutes of Health (NIH) and the Human Genome Project (HGP) laid out this thought for all to consider [1]:

> Is the science of genetics and genomics beginning to allow us to "play God"? That phrase is the one most commonly used by those expressing concern about these advances, even when the speaker is a nonbeliever. Clearly the concern would be lessened if we could count on human beings to play God as God does, with infinite love and benevolence. Our track record is not so good. Difficult decisions arise when a conflict appears between the mandate to heal and the moral obligation to do no harm. But we have no alternative but to face those dilemmas head-on, attempt to understand all of the nuances, include the perspectives of all the stakeholders, and try to reach a consensus. The need to succeed at these endeavors is just once more compelling reason why the current battles between the scientific and spiritual worldviews need to be resolved – we desperately need both voices to be at the table, and not to be shouting at each other.

Learning never ceases in the area of biopharmaceutical CMC regulatory compliance strategy. After 40 years in the biopharmaceutical industry, I would have thought by now that there would be "nothing new under the sun" to learn. But I am constantly amazed at the energy and creativity by my colleagues continually developing new manufacturing process technologies and new product types, which demand challenging CMC strategies to effectively manage and ensure their regulatory compliance. It is my sincere desire that this book will be of help to those who work in these biopharmaceutical companies both today and for years to come. I encourage the users of this book to seek to learn more on their own about CMC regulatory compliance strategy for biopharmaceuticals.

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Reference

1. Collins FS. The language of god – a scientist presents evidence for belief. Free Press/Simon & Schuster, Inc; 2007.

Photo courtesy of Nicki Geigert Photography

Acknowledgments

Many colleagues during my 50 years of service in the biopharmaceutical industry have impacted my understanding of CMC regulatory compliance strategy and have indirectly contributed to the writing of this book. I would like to especially acknowledge my friends and colleagues at my former companies (all of which now have been acquired by larger biopharmaceutical companies and no longer exist as separate entities) – Cetus Corporation, Immunex Corporation, and IDEC Pharmaceuticals – for the insights and experiences that we shared. I would also like to acknowledge my new friends and colleagues in the many biopharmaceutical companies that I now serve as their consultant – for the many CMC regulatory compliance strategies that we wrestle with.

A special expression of appreciation goes to my wife, Nicki, who understood the time commitment and mental exhaustion that comes along with trying to update a book of this magnitude and for her patient support and strong encouragement again throughout this entire lengthy process.

Quoting from Albert Einstein, Nobel laureate in Physics, "The most beautiful thing we can experience is the mysterious. It is the source of all art and science. He to whom this emotion is a stranger, who can no longer pause to wonder and stand rapt in awe, is as good as dead; his eyes are closed." I have been in awe watching my fellow scientists unravel the ever-increasing intricate complexity and design of life – carrying out genetic engineering on living cells to re-design them either to over-produce recombinant proteins or monoclonal antibodies or to become a living biopharmaceutical product itself such as a genetically engineered T-cell. As a scientist who believes that God is the ultimate genetic engineer, I trust that scientists will eventually comprehend and appreciate His original creative work.

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Abbreviations

AAV	Adeno-associated virus
ACF	Animal component-free
ADC	Antibody drug conjugate
ADCC	Antibody-dependent cellular cytotoxicity
AEX	Anion exchange chromatography
ANDA	Abbreviated New Drug Application
API	Active pharmaceutical ingredient
ARM	Alliance for Regenerative Medicine
ASO	Antisense oligonucleotide
ATMP	Advanced Therapy Medicinal Product
AUC	Analytical ultracentrifugation
BHK	Baby hamster kidney
BLA	Biologic License Application
BPCI	Biologics Price Competition and Innovation Act of 2009
BSA	Bovine serum albumin
BSE	Bovine spongiform encephalopathy
BsUFA	Biosimilar User Fee Act
BTD	Breakthrough Therapy Designation
CAR	Chimeric antigen receptor
CAT	Committee for Advanced Therapies
CBER	Center for Biologics Evaluation and Research
CBMP	Cell-based medicinal product
CBP	Cells beyond production
CD	Cluster of differentiation
CDC	Complement-dependent cytotoxicity
CDC	Center for Disease Control and Prevention
CDER	Center for Drug Evaluation and Research
CDRH	Center for Devices and Radiological Health
CDSCO	Central Drugs Standard Control Organization
CFDA	China Food and Drug Administration
CFR	Code of Federal Regulations

CFU	Colony-forming unit
CGE	Capillary gel electrophoresis
CGMP	Current good manufacturing practice
CGTP	Cell and Gene Therapy Product
CHMP	Committee for Medicinal Products for Human Use
CHO	Chinese hamster ovary
CI	Confidence interval
CJD	Creutzfeldt-Jakob disease
CMC	Chemistry, Manufacturing and Controls
CMO	Contract manufacturing organization
CP	Comparability protocol
CPP	Critical process parameter
CQA	Critical quality attribute
CRL	Complete Response Letter
CTA	Clinical Trial Authorisation
CTD	ICH common technical document
CTIS	Clinical Trials Information System
CTL	Contract testing laboratory
CTN	Clinical Trial Notification
Da	Dalton
DF	Diafiltration
DNA	Deoxyribonucleic acid
DOE	Design of experiments
DP	Drug product
d-PCR	Digital-PCR
DS	Drug substance
E&L	Extractables and leachables
EC	Established Condition
EC	European Commission
eCTD	Electronic common technical document
EDQM	European Directorate for the Quality of Medicines and Healthcare
ELA	Establishment License Application
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
EOP2	End of phase 2
EPAR	European Public Assessment Report
EPC	End of production cells
EU	Endotoxin unit
EU	European Union
Fab	Antigen-binding fragment
FBS	Fetal bovine serum
Fc	Fragment crystallizable
FD&C	Food, Drug, and Cosmetics Act
FDA	Food and Drug Administration
FIH	First-in-human

FMEA	Failure mode effects analysis
FOI	Freedom of information
gc	Genome copy
GMP	Good manufacturing practice
GOI	Gene of interest
HC	Health Canada
HC	Heavy chain
HCP	Host cellular protein
HPLC	High-performance liquid chromatography
HSA	Human serum albumin
ICH	International Council for Harmonisation
Ig	Immunoglobulin
IMPD	Investigational Medicinal Product Dossier
IND	Investigational New Drug
INN	International non-proprietary name
IPC	In-process control
IS	International standard
IU	International unit
IV	intravenous
IVT	In vitro transcription
kDa	Kilodalton
kg	Kilogram
KM	Knowledge management
KPP	Key process parameter
LAL	Limulus Amebocyte Lysate
LC	Light chain
LIVCA	Limit of in vitro cell age
LNP	Lipid nanoparticle
LoQ	List of Questions
LRF	Log10 reduction factor
LV	Lentivirus
LVV	Lentiviral vector
MAA	Marketing Authorisation Application
mAb	Monoclonal antibody
MAH	Marketing authorization holder
MCB	Master Cell Bank
MFDS	Ministry of Food and Drug Safety (Korea)
mg	Milligram
MHLW	Ministry of Health, Labor, and Welfare
MHRA	Medicines and Healthcare Products Regulatory Agency
mL	Milliliter
MMV	Mouse minute virus
mRNA	Messenger RNA
MS	Mass spectrometry
NAT	Nucleic acid test

NCA	National Competent Authority
NDA	New Drug Application
NDS	New Drug Substance
ng	Nanogram
NGS	Next-generation sequencing
NIH	National Institutes of Health
NMWCO	Nominal molecular weight cutoff
NIIMBL	National Institute for Innovation in Manufacturing Biopharmaceuticals
OTP	Office of Therapeutic Products
ORA	Office of Regulatory Affairs
PACMP	Post-approval change management protocol
PAGE	Polyacrylamide gel electrophoresis
PAI	Pre-approval inspection
PAT	Process analytical technology
PCR	Polymerase chain reaction
PDA	Parenteral Drug Association
PDMA	Pharmaceutical and Medical Devices Agency (Japan)
PDUFA	Prescription Drug User Fee Act
pg	Picogram
PHA	Preliminary hazards analysis
Ph.Eur.	European Pharmacopeia
PhRMA	Pharmaceutical Research and Manufacturers of America
PHS	United States Public Health Service Act
PLI	Product License Inspection
PMDA	Pharmaceutical and Medical Devices Agency
ppm	Part per million
PPQ	Process performance qualification
PQS	Pharmaceutical Quality System
PRIME	PRIority MEdicine
PrP	Prion protein
PrP^{TSE}	Abnormal isoform of prion protein
PV	Process validation
QA	Quality Assurance
QbD	Quality by Design
QC	Quality Control
q-PCR	Quantitative-PCR
QRM	Quality Risk Management
QTPP	Quality Target Product Profile
QU	Quality Unit
RAPS	Regulatory Affairs Professional Society
RCA	Replication-competent adeno-associated virus
RCL	Replication-competent lentivirus
RCV	Replication-competent virus
RLD	Reference listed drug
RMAT	Regenerative Medicine Advanced Therapy

RMM	Rapid microbiological method
RNA	Ribonucleic acid
ROW	Rest of the world
RP	Reference product
RP-HPLC	Reversed-phase HPLC
RPM	Regulatory program manager
RRF	Risk ranking filtering
SAWP	Scientific advice working party
SBP	Similar biotherapeutic product
SC	Subcutaneous
scFv	Single-chain variable fragment
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate-PAGE
SEC-HPLC	Size exclusion HPLC
si-RNA	Small-interfering RNA
TEM	Transmission electron microscopy
TFF	Tangential flow filtration
TGA	Australian Therapeutics Goods Administration
TSE	Transmissible spongiform encephalopathy
UF	Ultrafiltration
UF/DF	Ultrafiltration/diafiltration
USA	United States of America
USDA	United States Department of Agriculture
USP	United States Pharmacopeia
vCJD	Variant Creutzfeldt-Jakob disease
VCN	Vector copy number
VHP	Voluntary Harmonisation Program
WCB	Working Cell Bank
WHO	World Health Organization
XRPD	X-ray powder diffraction

Chapter 1
Biopharmaceutical Landscape

Abstract This chapter traces the rapidly expanding clinical-developing and market-approved biopharmaceutical portfolio. Although biological medicines have been developed over the past century (e.g., immune serums, vaccines), only in the last three decades have biopharmaceuticals started to reach the marketplace. From the early 1990s through today, biopharmaceuticals have entered the marketplace in four major 'waves'. The protein-based biopharmaceuticals were first – 1st wave recombinant proteins, 2nd wave monoclonal antibodies, and 3rd wave biosimilars. The protein-based biopharmaceuticals all have been manufactured by applying the Central Dogma of Molecular Biology: DNA → mRNA → amino acid (protein). This one way flow of genetic information was applied to living microorganisms, to produce the biopharmaceuticals in bioreactors. Today, the 4th wave of biopharmaceuticals is entering the marketplace. These are the gene therapy-based biopharmaceuticals (viral vectors and genetically modified patient cells), which are also being manufactured by applying the Central Dogma of Molecule Biology: DNA → mRNA → amino acid (protein). But the twist, is that the gene therapy-biopharmaceuticals use humans to produce the proteins inside their bodies. Now, the human patient is the 'bioreactor'. In this chapter, the ever-increasing diverse landscape of biopharmaceutical types included in each of the four waves will be examined, along with the Chemistry, Manufacturing & Control (CMC) terminology used in describing their regulatory compliance.

Keywords Biologic · Biopharmaceutical · Recombinant · rDNA-derived · Protein · Monoclonal · Bispecific · ADC · Biosimilar · CGTP · ATMP · Virus · Vector · Cell · Nanobody · Gene · Dogma

1.1 Introduction

Understanding the language used in any discipline or industry is paramount to being able to effectively communicate with others in field. For example, those who work in the computer science industry speak of 'terabytes and gigabytes' and 'C and

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JavaScript'; those who work in the financial investment field speak of 'ETFs' and 'collateralized debt obligations.' So too, in the field of Chemistry Manufacturing & Control (CMC) regulatory compliance, language is most important. For the biopharmaceutical industry that is developing over a thousand medicines for over a hundred human diseases, our communication challenge is to appreciate the regulatory implications and expectations associated with our CMC language. 'Biologic', 'biological', 'biopharmaceutical', 'rDNA-derived', 'ATMP', and 'CGTP' are all terms commonly used in our industry, and their regulatory meaning needs to be fully appreciated.

Although biological medicines have been developed over the past century (e.g., immune serums, vaccines), only in the last three decades have biopharmaceuticals reached the market-approval stage. From 1990s through today, biopharmaceuticals have entered the marketplace in four major 'waves':

$$\begin{gathered}(\text{1st wave})\,\text{recombinant proteins}\\ \downarrow\\ (\text{2nd wave})\,\text{monoclonal antibodies}\\ \downarrow\\ (\text{3rd wave})\ \text{biosimilars}\\ \downarrow\\ (\text{4th wave})\,\text{gene therapies}\end{gathered}$$

The landscape of biopharmaceutical products included in each of the four waves, when examined, reveals the tremendous skill of molecular biologists in applying genetic engineering tools to the manufacture of these diverse human medicines.

1.2 What's in a Name

'Biologic', 'biological', 'biopharmaceutical', 'rDNA-derived', 'ATMP, and 'CGTP' are all terms commonly used in our industry, but their regulatory compliance meaning may not be fully understood. The regulatory compliance terminology landscape will be painted so that a proper and full understanding of these terms can be gained.

1.2.1 Terms: 'Biologic' and 'Biological'

'Biologic' and 'biological' are, by definition, products connected to living systems. According to the United States Food & Drug Administration (FDA) and the European Medicines Agency (EMA), the following general regulatory definitions for these products are found:

FDA [1]

Biological products include a wide range of products such as vaccines, blood and blood components, allergenics, somatic cells, gene therapy, tissues, and recombinant therapeutic proteins. Biologics can be composed of sugars, proteins, or nucleic acids or complex combinations of these substances, or may be living entities such as cells and tissues. Biologics are isolated from a variety of natural sources - human, animal, or microorganism - and may be produced by biotechnology methods and other cutting-edge technologies. Gene-based and cellular biologics, for example, often are at the forefront of biomedical research, and may be used to treat a variety of medical conditions for which no other treatments are available.

EMA [2, 3]

Biological medicine: A medicine whose active substance is made by a living organism.

Assuring the quality of biological medicinal products is challenging, as they often consist of a number of product variants and process related impurities whose safety and efficacy profiles are difficult to predict. However, unlike chemical entities, toxic impurities are generally not an issue, and the safety issues of biological/biotechnological products are more often related to the mechanism of action of the biological product or to immunogenicity.

Combining these general definitions together, the following three-fold consensus regulatory definition of a biologic/biological is derived:

1. *must be either produced by or extracted from a living source*
2. *involves a challenging manufacturing process*
3. *is a complex product*

It is important to stress that there are three specific requirements in the definition of a biologic/biological. Just because a drug product is produced by a living organism is not sufficient to make it a biologic; the product must also meet the other two requirements, i.e., a challenging manufacturing process and being a complex product. For example, the majority of the antibiotic drugs on the market are produced by fermentation using living microorganisms (e.g., bacteria or fungi to manufacture penicillins, cephalosporins, tetracyclines, etc.); however, these medicines are regulated as chemical drugs and not biologics owing, in the opinion of the regulatory authorities, to the lack of challenge in the manufacturing process and to the lack of complexity of the product. Therefore, it is important to emphasize that all three requirements of the consensus definition are necessary to be a biologic/biological.

Natural biological natural treatments have been around for millennia, with the use of natural tree barks, plants and herbs, discovered by local inhabitants. However, biological natural medicines have been around for only a century, and some biological medicines continue today even in the age of genetic engineering.

Animal-derived immune serums (also referred to as anti-toxins) were first made available in the early twentieth century [4]:

The late 19th century was one of the most exciting times imaginable for physicians and scientists working in biological research arenas around the world. Robert Koch in Germany was investigating and isolating the bacterial organisms responsible for anthrax, rabies, tuberculosis and cholera… This burgeoning science of immunology began rapidly developing new vaccines and anti-toxins that promised to prevent and cure some of the most dangerous and dreaded epidemic diseases afflicting mankind. Researchers Emil von Behring and Shibasaburo Kitasato in Robert Koch's lab, for example, discovered that animals

> injected with diphtheria and tetanus toxins produced anti-toxins which could be inoculated into other animals to both cure and provide future immunity from these dread diseases. Their serum therapy was tested at Berlin's Charite` hospital at the end of 1891 and the chemical company Hoechst began commercial antitoxin serum production soon after. Mortality rates from diphtheria in Europe dropped dramatically and laboratories in the United States quickly rushed to begin production of these new life-saving biological products.

The passive use of animal-derived immune serums has been replaced by the active use of prophylactic vaccines. However, the use of animal-derived anti-toxins (antidotes) for poisonous bites (e.g., snake, scorpion, spider, etc.) continues today.

Vaccines are intended to induce or increase an antigen-specific immune response for prophylactic immunization. The age of vaccines is credited to Edward Jenner in the late eighteenth century for applying cowpox as a vaccine against smallpox. Vaccines have greatly reduced or eliminated many infectious diseases that once routinely killed or harmed infants, children, and adults. The US Center for Diseases Control and Prevention (CDC) explains the history of the development of the measles vaccine [5]:

> In the decade before 1963 when a vaccine became available, nearly all children got measles by the time they were 15 years of age. It is estimated 3 to 4 million people in the United States were infected each year. Also, each year, among reported cases, an estimated 400 to 500 people died, 48,000 were hospitalized, and 1000 suffered encephalitis (swelling of the brain) from measles… In 1954, John F. Enders and Dr. Thomas C. Peebles collected blood samples from several ill students during a measles outbreak in Boston, Massachusetts. They wanted to isolate the measles virus in the student's blood and create a measles vaccine. They succeeded in isolating measles in 13-year-old David Edmonston's blood. In 1963, John Enders and colleagues transformed their Edmonston-B strain of measles virus into a vaccine and licensed it in the United States. In 1968, an improved and even weaker measles vaccine, developed by Maurice Hilleman and colleagues, began to be distributed. This vaccine, called the Edmonston-Enders (formerly "Moraten") strain has been the only measles vaccine used in the United States since 1968. Measles vaccine is usually combined with mumps and rubella (MMR), or combined with mumps, rubella and varicella (MMRV).

Today, biologic vaccines have been raised against many harmful bacteria (e.g., diphtheria, pertussis, tetanus, etc.) and viruses (e.g., chickenpox, influenza, hepatitis, measles, mumps, etc.). Vaccines have eliminated the threat of the smallpox virus, and hopefully soon the threat of the polio virus. Although the regulatory authorities have market approved over 80 vaccines to date [6], vaccine development continues as there seems to be an endless struggle against ongoing and new infectious viruses (e.g., HIV, Chikungunya virus, Zika virus,, etc.). It should be noted that a number of viral vaccines, in recent years, are now being produced using genetic engineering methods (e.g., Hepatitis B virus, Dengue virus, COVID-19, etc.).

Human plasma-derived proteins are another type of natural biologic medicines. Blood plasma contains a mixture of many different kinds of proteins, of which a few are of therapeutic interest. During World War II, Edwin Joseph Cohn, an American chemist, led a team that devised a method called the 'Cohn fractionation process' that separated the individual proteins out of plasma [7]. Human plasma-derived protein products are of considerable commercial interest as indicated by regulatory

authorities market approval of many fractionated plasma products, which include human albumin (for replenishing and maintaining blood volume after traumatic injury or surgery), immune globulins (for passive prophylaxis and immune disease disorders), antihemophilic factor (Factor VIII), coagulation factors (e.g., Factor VII, Factor IX, Factor X), and protease anticoagulation factors (e.g., antithrombin, fibrinogen) [8]. It should be noted that a number of human plasma-derived proteins, in recent years, are now also being produced using genetic engineering methods (e.g., Factors VII, VIII, IX and X; C1 esterase inhibitor; etc.).

Therapeutic hormonal proteins are also biologic medicines which can be isolated from a variety of biological sources – from slaughtered animals (bovine or porcine insulin), from human cadavers (human growth hormone) and from the urine of menopausal women (gonadotropins). The discovery of insulin was a profound biologic treatment for diabetes. In 1922, Frederick Banting and Charles Best extracted the hormone insulin, which controls blood sugar levels, from the pancreases of dogs, and administered the extract to a 14-year-old boy suffering from type I diabetes mellitus, saving his life and proving insulin's efficacy in treating human diabetes. Following their discovery, virtually all insulin for human use was harvested from animal pancreases from stockyards that slaughtered pigs and cows. It is estimated that it took two tons of pig pancreases to yield only 8 ounces (~200 grams) of porcine insulin [9]. Human insulin, not bovine insulin, is today produced using genetic engineering methods.

The discovery of human growth hormone at the start of the twentieth century led to treatment of children who were unusually short because of pituitary disorders. It was used conservatively, primarily due to its short supply since it had to be isolated from human cadavers. However, by the mid 1980s, critical concerns about the safety of human growth hormone derived from the pituitary glands of human cadavers arose. Safety reports appeared of a fatal neurological disease in young people receiving human growth hormone. This disease known by its eponym Creutzfeldt-Jacob (C-J) syndrome is caused by a sub-viral particle called a prion which also causes mad-cow disease. It is extremely rare occurring as a cause of death in only one in a million individuals and almost never diagnosed in children. Regulatory authorities responded by mandating the market withdrawal of cadaver-derived human growth hormone. Human growth hormone is today produced using genetic engineering methods.

1.2.2 Terms: 'Biopharmaceutical' and 'rDNA-Derived'

In 1953, a landmark series of papers on DNA structure were published by Watson and Crick, and others. The work of these scientists firmly established that DNA was a double helix with anti-parallel nucleotide chains and specific base pairings. This scientific insight led to great advances in biochemistry and gave birth to the new discipline of 'molecular biology'. An explosion of scientific research and discovery followed, which opened up the door to genetically adding human genes to living

microorganisms for manufacture of biopharmaceutical proteins, and even today to adding human genes to either replace faulty or missing genes or to add new genes into humans.

According to the Merriam-Webster dictionary, the term 'biopharmaceutical' is defined as "*a pharmaceutical derived from biological sources and especially one produced by biotechnology*", and then further 'biotechnology' is defined as "*the manipulation (as through genetic engineering) of living organisms or their components to produce useful commercial products*". Thus, the term 'biopharmaceutical' defines a subset of biologics. Biopharmaceuticals meet the three-fold consensus definition of all biologics, but in addition they must be produced or extracted from living organisms that have been altered by genetic engineering technology:

1. *must be either produced by or extracted from a* ***genetically engineered*** *living source*
2. *involves a challenging manufacturing process*
3. *is a complex product*

Unfortunately, the term 'biopharmaceutical' has since been hijacked by the popular press, and is now being loosely defined as 'biohealth'. For example, Wikipedia defines biopharmaceuticals as '*any pharmaceutical drug product manufactured in, extracted from, or semisynthesized from biological sources … including vaccines, whole blood, blood components, allergenics,….*' Pharmaceutical companies (just check their websites) and pharmaceutical associations have responded and also use the term 'biopharmaceutical' loosely to even cover chemically synthesized pharmaceuticals (e.g., the chemically synthesized antiviral medicines for treating AIDS and hepatitis C are commonly described as biopharmaceuticals). Regulatory authorities are aware of this hijacking [10]:

> Broad definitions of "biotechnology-derived" incorporate all products biologically sourced or using biological systems in their manufacturing; some definitions expand the concept to include peptides and nucleic acid oligomers that are chemically synthesized; whereas a limited definition might restrict products to those prepared using recombinant DNA procedures or "modern" cell culture techniques.

Therefore, they have rarely used this term. In fact, the only inclusion of the term biopharmaceutical in a regulatory document is in an International Council of Harmonisation (ICH) guidance document on preclinical safety studies (ICH S6(R1)) where the terms 'biotechnology-derived pharmaceuticals (biopharmaceuticals)' and 'biopharmaceuticals' are used over 25 times [11].

'Recombinant DNA' or 'rDNA-derived' or 'genetically modified' are the terms preferred by the regulatory authorities for this subset of biologic products. These terms are being used in the guidances and regulations that are issued by the FDA, EMA, ICH, and the World Health Organization (WHO).

When the term 'biopharmaceutical' is used in this book, it will have the original definition – i.e., *must be either produced by or extracted from a genetically engineered living source*. Therefore, the terms 'biopharmaceutical', 'recombinant DNA-derived', and 'rDNA-derived' will be used interchangeably herein.

1.2.3 Terms: 'ATMP' and 'CGTP'

In the late-2000s, two new terms started appearing in regulatory authority guidances and communications: ''ATMP' (Advanced Therapy Medicinal Product) and 'CGTP' (Cell & Gene Therapy Product). Here are the regulatory authorities' definition of these terms:

EMA [12]

Advanced therapy medicinal products (ATMPs) are medicines for human use that are based on genes, tissues or cells. They offer groundbreaking new opportunities for the treatment of disease and injury. ATMPs can be classified into three main types:

- *gene therapy medicines:* these contain genes that lead to a therapeutic, prophylactic or diagnostic effect. They work by inserting 'recombinant' genes into the body, usually to treat a variety of diseases, including genetic disorders, cancer or long-term diseases. A recombinant gene is a stretch of DNA that is created in the laboratory, bringing together DNA from different sources;
- *somatic-cell therapy medicines***:** these contain cells or tissues that have been manipulated to change their biological characteristics or cells or tissues not intended to be used for the same essential functions in the body. They can be used to cure, diagnose or prevent diseases;
- *tissue-engineered medicines***:** these contain cells or tissues that have been modified so they can be used to repair, regenerate or replace human tissue.

FDA [13]

Cellular and Gene Therapy Products. Cellular therapy products include cellular immunotherapies, cancer vaccines, and other types of both autologous and allogeneic cells for certain therapeutic indications, including hematopoetic stem cells and adult and embryonic stem cells. Human gene therapy is the administration of genetic material to modify or manipulate the expression of a gene product or to alter the biological properties of living cells for therapeutic use.

While ATMPs and CGTPs essentially cover the same type of biological medicinal products, EMA prefers the ATMP term while the FDA currently prefers the CGTP term. More than likely, regulatory authorities in the future will settle on the term 'advanced therapies'.

The FDA has recently introduced another term: 'RMAT'. Regenerative Medicine Advanced Therapy (RMAT) is a designation that is defined as follows [14].

A drug is eligible for regenerative medicine advanced therapy (RMAT) designation if:

(a) The drug is a regenerative medicine therapy, which is defined as a cell therapy, therapeutic tissue engineering product, human cell and tissue product, or any combination product using such therapies or products, except for those regulated solely under Section 361 of the Public Health Service Act and part 1271 of Title 21, Code of Federal Regulations;
(b) The drug is intended to treat, modify, reverse, or cure a serious or life-threatening disease or condition; and
(c) Preliminary clinical evidence indicates that the drug has the potential to address unmet medical needs for such disease or condition

This CMC regulatory compliance book is focusing on biopharmaceuticals. The gene therapies (i.e., gene-based biopharmaceuticals) fit into this scope. If the cellular therapies and tissue-engineered therapies include genetic engineering of the

cells/tissues, then they are defined as gene therapies and are discussed in this book (e.g., *ex vivo* CAR T-cell gene therapies). But many of the CMC regulatory compliance requirements discussed in this book, also apply as well for the cellular therapies that are not genetically modified (i.e., they only meet the requirement of being cells that are substantially manipulated).

1.3 Diversity of the Biopharmaceutical Product Landscape

With the advent of molecular biology, the understanding of the components involved in the Central Dogma of Molecular Biology (i.e., the transcription of genetic information from DNA to mRNA that is then translated to produce protein), and the introduction of genetic engineering capabilities, biopharmaceuticals started entering clinical development in the late 1970s and entering the marketplace in the late-1980s. Biopharmaceutical products have entered the marketplace in four major 'waves' – similar to the waves in the ocean building in size until they land on the shoreline. These four 'waves' of biopharmaceuticals are:

(1st wave) recombinant proteins
(2nd wave) monoclonal antibodies
(3rd wave) biosimilars
(4th wave) gene therapies

1.3.1 1st Wave – Recombinant Proteins

The 'first wave' of biopharmaceuticals to enter the marketplace consisted of manufactured recombinant human proteins (rhu proteins). Utilizing the cellular energy of living microorganisms and the introduction of foreign DNA (i.e., the gene), the Central Dogma of Molecular Biology is carried out: DNA (deoxyribonucleic acid) is transcribed into mRNA (messenger ribonucleic acid) which is then translated into proteins (linked together amino acids through an amide bond into a polypeptide). Since the introduced DNA is combined with a natural microorganism, the manufactured protein product is referred to as 'recombinant' protein.

In 1982, the first recombinant DNA-derived protein – human insulin – was approved for the marketplace. As far as polypeptide chains go, it is a small protein. Figure 1.1 presents the amino acid sequence for recombinant human insulin: 51 amino acids spread over two polypeptides (21 amino acid A chain and 30 amino acid B chain), including three disulfide bonds between cysteines.

Today, much larger manufactured recombinant proteins are in the marketplace. Figure 1.2 presents the 570 amino acid linear schematic for the recombinant human protein, Xenpozyme, which is used as an enzyme replacement therapy for patients with acid sphingomyelinase deficiency (ASMD) [15]. Starting from the N-terminus

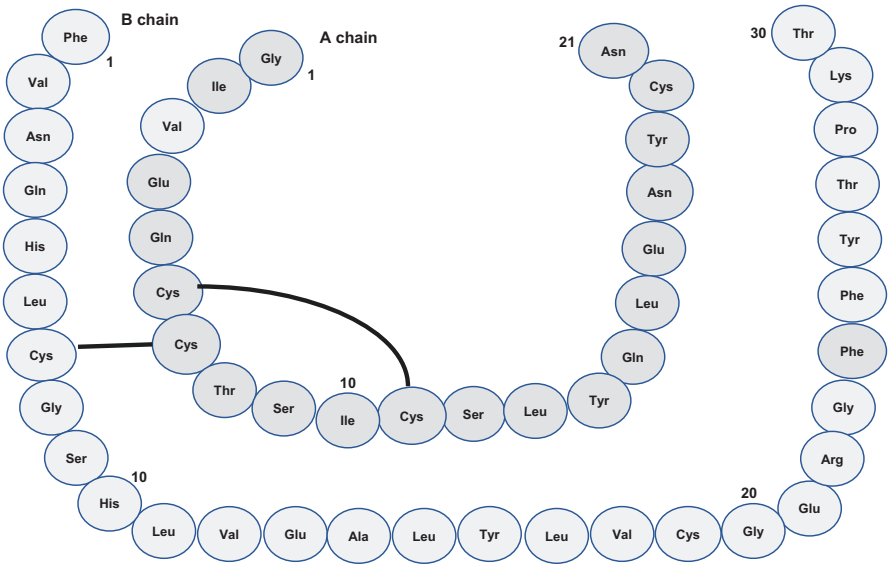

Fig. 1.1 Amino acid sequence for the 51 amino acid recombinant human insulin

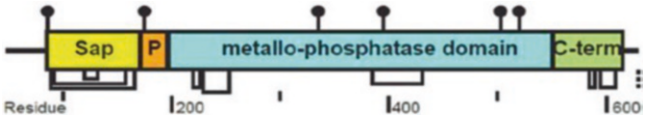

Fig. 1.2 Amino acid linear schematic for the 570 amino acid recombinant human Xenpozyme

of the olipudase alfa polypeptide (i.e., left to right): (1) N-terminal saposin (Sap) domain, (2) a proline (P) rich linker region, (3) a catalytic metallophosphatase domain, and (4) a helical C-terminal structural domain ending in a cysteine.

While recombinant proteins are frequently described in linear two-dimension terms (i.e., linked sequence of amino acids in the polypeptide), proteins are three-dimension structures, as illustrated in Fig. 1.3, for the 497 amino acid X-ray structure of the recombinant human protein, Vpriv (velaglucerase alfa), used as an enzyme replacement therapy for patients with acid sphingomyelinase [16].

Molecular biologists can not only transfer natural DNA sequences into microorganisms, but they can also, through genetic engineering methods, alter the natural DNA gene sequence to be inserted into the microorganism, which in turn alters the expressed protein amino acid sequence. This can result in potential desirable changes in the therapeutic activity for the recombinant protein. For example, for recombinant human insulin, several genetically engineered amino acid sequence changes have been made in the insulin sequence. Several of these market-approved biopharmaceuticals are described below (sequence comparison can be made to human insulin in Fig. 1.1):

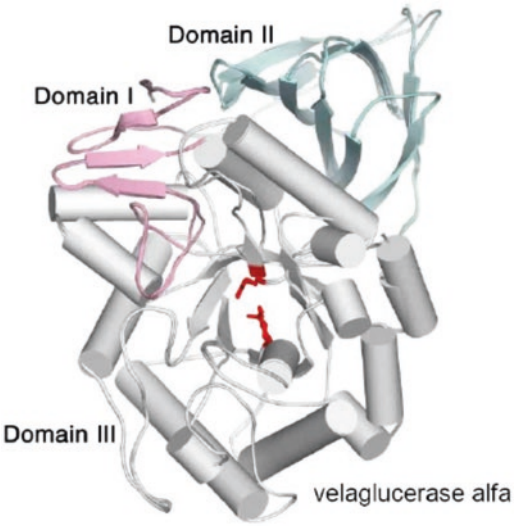

Fig. 1.3 X-ray crystal 3-dimension structure of the 497 amino acid recombinant human Vpriv

INSULIN ASPARATE – rapid acting insulin; differs from the amino acid polypeptide sequence of human insulin in that the amino acid proline, Pro, at position B28 is replaced by aspartic acid, Asp.

INSULIN LISPRO – rapid acting insulin; differs from the amino acid polypeptide sequence of human insulin in that the amino acid proline, Pro, at position B28 is replaced by lysine, Lys; and the amino acid lysine, Lys, at position B29 is replaced by proline, Pro.

INSULIN GLARGINE – long-acting insulin; differs from the amino acid polypeptide sequence of human insulin in that the amino acid asparagine, Asn, at position A21 is replaced by glycine, Gly; and two arginine amino acids, Arg-Arg, added to the C-terminus of the B-chain at positions B31 and B32.

Genetically engineered *Escherichia coli* (*E. coli*) bacteria cells were used to produce the first recombinant proteins, and this microorganism remains a common production cell type. But, production options for recombinant proteins have increased to include genetically engineered yeast cells, insect cells, plant cells, animal cells and human cells, and even transgenic animals.

Today, over 120 different recombinant protein medicines are in the marketplace. The general description of some of these market-approved recombinant proteins [17] is presented in Table 1.1.

1.3.2 2nd Wave – Monoclonal Antibodies

The ‘second wave’ of biopharmaceuticals to enter the marketplace consisted of monoclonal antibodies (mAbs). Monoclonal antibodies are a form of recombinant proteins referred to as immunoglobulins (Igs), with IgG being the most common

Table 1.1 General description of some commercial recombinant proteins.

Recombinant Protein	General Description
Proleukin (aldesleukin)	**PROLEUKIN is produced by a genetically engineered *E. coli* strain containing an analog of the human interleukin-2 gene. Genetic engineering techniques were used to modify the human IL-2 gene, and the resulting expression clone encodes a modified 132 amino acid human interleukin-2 recombinant protein:** **• the molecule has no N-terminal alanine - the codon for this amino acid was deleted during genetic engineering** **• the molecule has serine substituted for cysteine at amino acid position 125, accomplished by site specific manipulation during genetic engineering**
Jetrea (ocriplasmin)	**JETREA is a recombinant truncated form of human plasmin produced in a methylotrophic yeast (*Pichia pastoris*). The recombinant protein consists of two peptide chains – the first is 19 amino acids long and the second is 230 amino acids long, linked together by two disulfide bonds.**
Mepsevii (vestronidase)	**MEPSEVII is a recombinant human lysosomal beta glucuronidase which is a purified human enzyme produced by recombinant DNA technology in a Chinese hamster ovary (CHO) cell line. The recombinant protein exists as a homotetramer, with each monomer consisting of 629 amino acids.**
Elelyso (taliglucerase alfa)	**ELELYSO is produced by recombinant DNA technology using genetically modified plant cells (carrot root). The recombinant protein of 506 amino acids differs from native human glucocerebrosidase by a signal peptide of two amino acids at the N terminus and seven amino acids at the C terminus.**
Kanuma (sebelipase alfa)	**KANUMA is produced by recombinant DNA technology expressed in genetically engineered chickens (*Gallus gallus*) and purified from the egg white of transgenic hens. The recombinant protein has the same 378 amino acid sequence as the human lipase.**

isotype. The IgG mAb has a familiar 'Y shape' protein structure, and consists of approximately 1330 amino acids, resulting in a molecular weight of approximately 146 kDa (calculated from the primary amino acid sequence, non-glycosylated). The Y shape represents 4 polypeptides held together by disulfide bonding. There are two identical light (L) chain polypeptides designated as L chains, and two identical heavy (H) chain polypeptides designated as H chains. Each light chain polypeptide consists of a variable region [V_L] and a constant region [C_L]. Each heavy chain polypeptide consists of a variable region [V_H] and 3 constant regions [C_H1, C_H2 and C_H3]. The heavy and light chains are held together through disulfide bonds. The upper Y portion is referred to as the fragment antigen-binding (Fab) region and confers antigen specificity. The lower Y portion is the fragment crystallizable (Fc) region and causes effector functions. Figure 1.4 presents the 2-dimensional schematic of the IgG monoclonal antibody (note, each box represents a polypeptide).

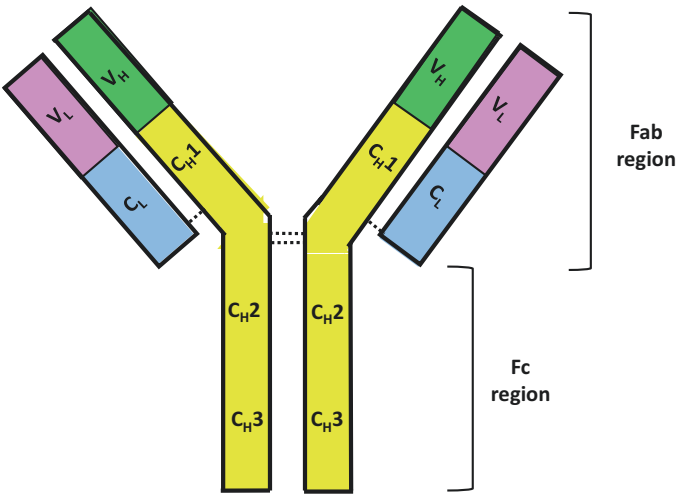

Fig. 1.4 Two-dimensional schematic of the IgG monoclonal antibody

The first monoclonal antibody (mAb) approved for the marketplace was Orthoclone OKT3 (muromonab), in 1986, which had medical application in preventing rejection of kidney organ transplants. It was viewed as a technological success (i.e., the ability to target specific receptors on cells) and opened the door for exploration of many other monoclonal antibody medical applications. The first monoclonal antibodies were 'murine' mAbs (i.e., both the variable region and the constant region amino acids are mouse-derived), developed using the mouse hybridoma technology derived back in the mid-1970s. As molecular biologists explored with their genetic engineering techniques, the next step in mAb generation involved genetic fusion of human DNA with mouse DNA which resulted in 'chimeric' mAbs (i.e., the variable region amino acids are animal-derived but the constant region amino acids are human-derived). This then led to further genetic engineering approaches which resulted in 'humanized' mAbs (i.e., 90% or greater of the variable region amino acids and the constant region amino acids are human-derived). Today, through ongoing molecular cloning capability, 'human' mAbs (i.e., 100% of both the variable region and the constant region amino acids are human-derived) are in the marketplace. The general description of some market-approved monoclonal antibodies [18] is presented in Table 1.2.

Monoclonal antibodies are homodimeric, monovalent affinity molecules, as shown in Fig. 1.4 (i.e., there are two identical light chain polypeptides and two identical heavy chain polypeptides attached by disulfide bonding). However, if each Fab binding region is needed to bind to a different antigen, then heterodimeric, divalent affinity antibodies can be manufactured, referred to as bispecific antibodies (BsAbs). A common molecular structure for a bispecific antibody is illustrated in Fig. 1.5, where each Fab binding region binds to a different antigen.

Table 1.2 General description of some commercial monoclonal antibodies

Monoclonal Antibody	General Description
MabThera (rituximab)	MABTHERA is a recombinant chimeric mouse/human IgG1 kappa mAb that is produced in mammalian cell culture using Chinese Hamster Ovary (CHO) cells. The mAb consists of murine light and heavy chain variable regions, and human gamma 1 heavy chain and kappa light chain constant regions.
Unituxin (dinutuximab)	UNITUXIN is a glycosylated chimeric IgG1 human/mouse monoclonal antibody, produced in a murine myeloma cell (SP2/0 hybridoma cell), that incorporates human constant regions for the heavy chain IgG1 and the kappa light chain, along with the mouse variable regions
Uplizna (enebilizumab)	UPLIZNA is a recombinant humanized IgG1 kappa mAb that binds to the B cell-specific surface antigen CD19. The Chinese Hamster Ovary (CHO) cell line used for mAb expression has been designed to be fucosyltransferase-deficient (i.e. lost the ability to transfer fucose), and the N-linked oligosaccharides attached at the residue Asn-301 are therefore homogenously afucosylated without core fucosylation thereby increasing the antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP) activities.
Beyfortus (nirsevimab)	BEYFORTUS is a fully human, anti-RSV neutralising monoclonal antibody (IgG1/kappa isotypes for the heavy/light chains), isolated from memory B cells from human donors. It is produced in Chinese hamster ovary (CHO) cells by recombinant DNA technology. Nirsevimab was engineered with 9 amino acid substitutions to increase affinity for the F protein and reduce antigenicity, and a triple amino acid substitution (M257Y/S259T/T261E, referred to as "YTE") in the Fc region to extend serum half-life. Binding to human Fc receptors is maintained, and the mAb is expected to exhibit normal Fc-mediated effector functions.

Two manufacturing approaches are currently applied to bispecific antibodies. One way bispecific antibodies can be produced is by chemical conjugation: two monoclonal antibodies are first produced independently in separate cell cultures; prior to purification, the two mAbs are chemically reduced, mixed, then re-oxidized which allows for re-assembly as either homodimer or heterodimer; the mixture is then purified selecting for the heterodimer antibody. A second way bispecific antibodies can be produced is through recombinant techniques in which the genes for 2 different heavy chains and 2 different lights chains are inserted into the same cell line. Tecvayli in is manufactured the first way and Vabysmo is manufactured the second way [19], see Table 1.3. In addition, the Fc region is frequently genetically engineered to enhance heterodimeric matching (i.e., genetic mutation of the C_H3 domains of the 2 different heavy chain genes – a 'knob' mutation in one and a 'hole' mutation in the other to promote heavy chain heterodimerization).

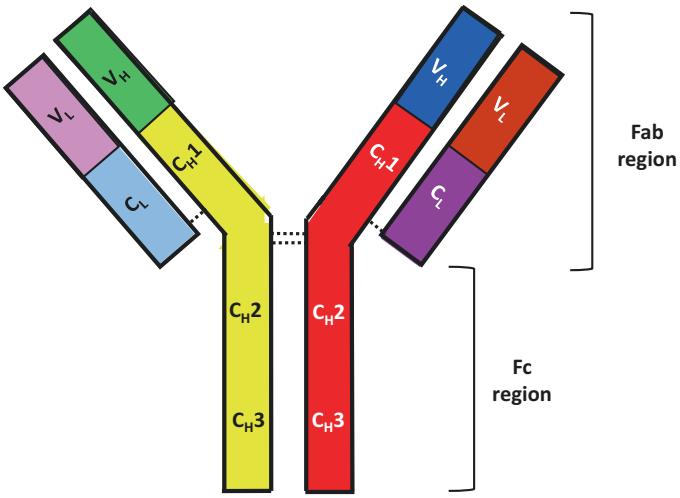

Fig. 1.5 Two-dimensional schematic of a bispecific antibody

Table 1.3 General description of two commercial bispecific antibodies (bsAbs)

Bispecific Antibody	General Description
Tecvayli (teclistamab)	TECVAYLI is a humanised immunoglobulin G4 (IgG4) bispecific antibody against B-cell maturation antigen (BCMA) and cluster of differentiation 3 (CD3) receptors. It is produced by cultivation of two separate recombinant CHO cells. The bispecific antibody is prepared by controlled reduction and oxidation of the two mAbs. The Fab arm exchange was facilitated by amino acid substitutions at positions F410L and R414K in the C_H3 domain of the parental anti-CD3 HC to enable preferential refolding of the heterodimer.
Vabysmo (faricimab)	VABYSMO is a recombinant bispecific antibody produced in Chinese hamster ovary (CHO) cells and consists of two different heavy chains (452 amino acid residues and 462 amino acid residues) and two different light chains (214 amino acid residues and 213 amino acid residues) with inter- and intra-chain disulfide bonds, that are typical for IgG1 antibodies plus an additional disulfide bridge in the CH3-CH3 interface. To enforce heterodimerisation of the two different heavy chains, several point mutations were introduced ("knobs into holes"). Exchange of CH1 and CL domains in the Ang-2 binding Fab promotes the correct assembly of the two different light chains, known as the "CrossMAb approach". Modification of faricimab neonatal Fc receptor (FcRn) and Fc gamma receptor (FcγR) binding sites disables the antibody's Fc-mediated effector functions.

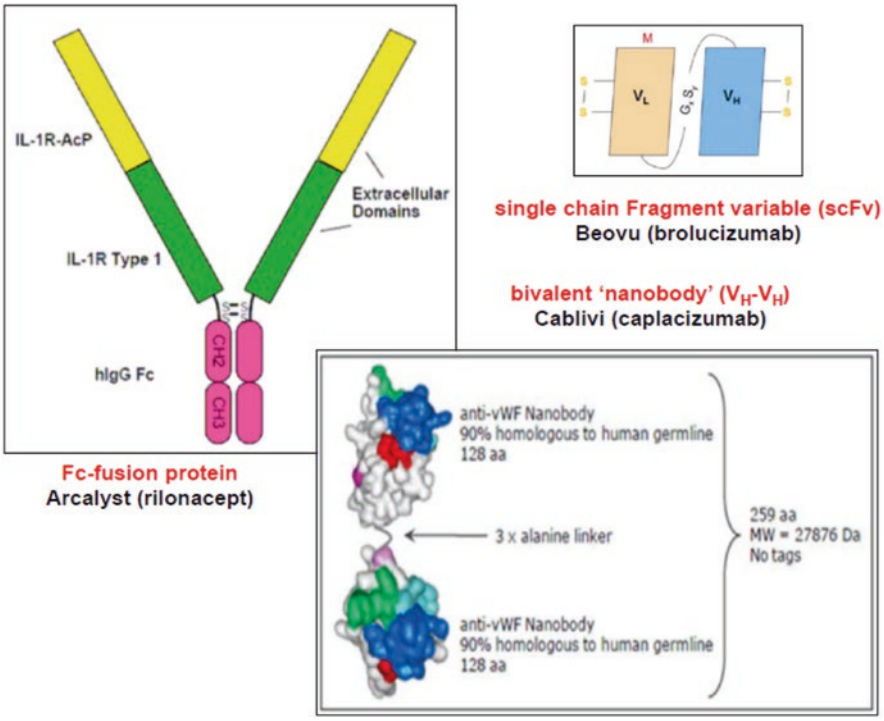

Fig. 1.6 Molecular schematic of some market-approved engineered antibody fragments

Monoclonal antibodies have two major biological activities: (1) the Fab region that binds to specific antigenic sites, and (2) the Fc region for cell-mediated activities. But both activities are not necessarily needed for all medical treatments. The development of smaller, fragments of a monoclonal antibody has been growing, taking advantage of one or the other biological activity of a monoclonal antibody. These genetically engineered antibody fragments can be used on their own (e.g., sc-Fv) or linked to other recombinant proteins (Fc-fusion proteins). Figure 1.6 presents representative structures for some market-approved antibody fragments, and their general descriptions [20] are presented in Table 1.4.

Finally, as if monoclonal antibodies were not already complex enough, they are now also being chemically linked to cytotoxic chemical drugs, forming antibody-drug conjugates (ADCs). ADCs take advantage of the targetability of a mAb to deliver a cytotoxic chemical drug directly to specific cells, minimizing general cell death (i.e., 'kill cancer cells not healthy cells'). The manufacture of these ADCs consists of numerous variables: (1) the choice of monoclonal antibody, (2) the choice of cytotoxic chemical drug, (3) the choice of hydrolysable chemical linker (4) the mechanism and sites of chemical drug-linker attachment to the mAb, and (5)

Table 1.4 General description of some commercial engineered antibody fragments

Fc-Fusion Protein	General Description
Arcalyst (rilonacept)	ARCALYST is a fusion protein consisting of human cytokine receptor extracellular domains and the Fc portion of human IgG1. It incorporates in a single molecule the extracellular domains of both receptor components required for IL-1 signalling. The molecule was created by fusing the sequences encoding the extracellular domains of the IL-1R-AcP, IL-1R Type I, and human IgG Fc in-line without any intervening linker sequences. The dimer is covalently linked by disulphide bonds in the Fc region. It is produced in Chinese hamster ovary (CHO) cells.
Fab Fragment	**General Description**
Beovu (brolucizumab)	BEOVU is a humanised single-chain Fragment variable (scFv) which inhibits vascular endothelial growth factor-A (VEGF-A) binding to its receptors. It is produced in *E. coli* cells, with a molecular weight of ~26 kDa. The molecule consists of a light-chain fragment (V_L) and a heavy-chain fragment (V_H), with both chains connected via a flexible glycine/serine linker.
Cablivi (caplacizumab)	CABLIVI is a humanised bivalent nanobody targeting the A1-domain of von Willebrand factor. It is produced in *E. coli* cells, with a molecular weight of ~28 kDa. The molecule consists of two identical genetically linked heavy-chain fragments (V_H -V_H), joined by a tri-alanine linker.

the DAR (drug-to-antibody ratio). The increased CMC regulatory compliance challenges associated with the ADCs will be addressed in Chap. 9. Figure 1.7 presents representative structures for two commercial ADCs described in Table 1.5. The general description of some market-approved ADCs [21] is presented in Table 1.5.

Today, over 140 monoclonal antibodies, bispecific antibodies, Fc-fusion proteins, Fab antibody fragments, and ADCs are in the marketplace. In fact, the best-selling therapeutic pharmaceutical in the world continues to be the monoclonal antibody Humira (adalimumab), a fully human IgG mAb produced in CHO cells – $20+ billion annual sales [22]. Also included in the top 10 best-selling pharmaceuticals in the world are two other monoclonal antibodies and a Fc-fusion protein: (1) Keytruda (pembrolizumab), humanized IgG4 mAb produced in CHO cells, (2) Stelara (ustekinumab), humanized IgG1 mAb produced in murine Sp2/0 cells, and (3) Eylea (aflibercept) Fc-fusion protein produced in CHO cells.

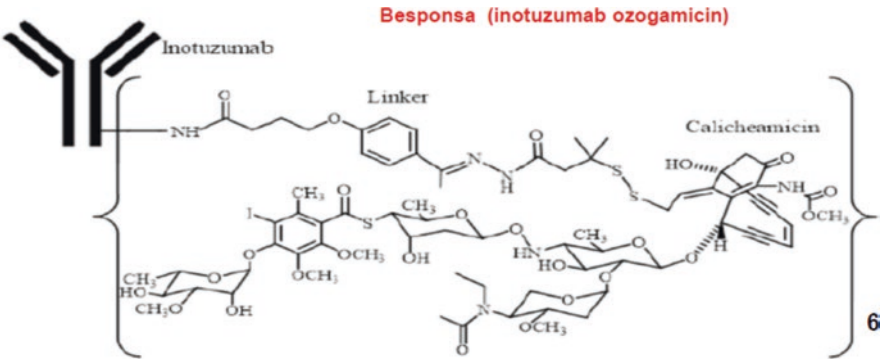

Fig. 1.7 Molecular schematic of some commercial antibody-drug conjugates (ADCs)

Table 1.5 General description of some commercial antibody-drug conjugates (ADCs)

ADC	General Description
Trodelvy (sacituzumab govitecan)	TRODELVY is an ADC composed of a humanised IgG1 mAb (anti-Trop-2) which is produced in Sp2/0 murine cells and a potent topoisomerase inhibitor small molecule SN38 (a camptothecin-derived agent) which is conjugated through thioether bonds to the mAb by a hydrolysable linker. The ADC has an average molar drug to antibody ratio (DAR) of 7 to 8.
Kadcycla (trastuzumab emtansine)	KADCYLA is an ADC composed of a recombinant humanized IgG1 mAb (anti-Her-2) produced in Chinese hamster ovary (CHO) cells and the cytotoxic microtubule-inhibitory maytansinoid, DM1. DM1 is linked to the lysine residues on the antibody via the heterobifunctional reagent *trans*-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC). The ADC has an average molar drug to antibody ratio (DAR) of 3 to 4.
Mylotarg (gemtuzumab ozogamicin)	MYLOTARG is an ADC composed of a humanised IgG4 mAb (anti-CD33) produced in NS0 murine cells and a cytotoxic agent N-acetyl gamma calicheamicin. The linker forms an amide bond with the antibody and forms a disulfide bond with the calicheamicin. The linker also contains an internal hydrazone bond, which is acid-labile. the ADC has an average molar drug to antibody ratio (DAR) of 2 to 3.

1.3.3 3rd Wave – Biosimilars

The 'third wave' of biopharmaceuticals to enter the marketplace consisted of biosimilars. Biosimilars are not new biopharmaceuticals but instead are 'highly similar' versions of already market-approved innovator biopharmaceuticals. Manufacturers of innovator biopharmaceuticals have two blockages that prevent competition for

their commercial products. First, market exclusivity which is granted at the time of market approval by the regulatory authorities for innovator medicines. For biologics, this is currently 12 years by the FDA and up to 11 years by EMA. Second, patent coverage which can be issued anywhere along the development lifetime of the biologic. For biologics, this is currently 20 years from date of filing through both the U.S. Patent Office and the European Patent Office. Both time periods, market exclusivity and patent coverage, run concurrently. At some point, the time clock runs out, so that now these biopharmaceuticals can be manufactured by others than the innovator manufacturer. To date, only the highly purified, thoroughly characterized recombinant proteins and monoclonal antibodies have been market-approved as biosimilars.

The following are the regulatory authority descriptions of biosimilars:

EMA [23]

A biosimilar medicine is developed to be highly similar to an existing biological medicine. This existing biological medicine is a medicine that has already been approved and is used in the EU and referred to as the reference medicine. After the reference medicine comes off patent and finishes its exclusivity term, the biosimilar medicine is allowed to come onto the market. Highly similar means that the biosimilar and its reference medicine are essentially the same, though there may be minor differences in their active substances. These minor differences are due to the fact that these active substances are usually large and complex molecules and that they are made by living cells. Some degree of variability is inherent to all biological medicines and minor differences may occur among different batches of the same biological medicine. Differences may also be observed following changes in the manufacturing process of a biological medicine. Such changes are carefully regulated by the European Medicines Agency. Any differences between the biosimilar and its reference medicine are kept within strict limits to ensure that both work in the same way. The biosimilar and its reference medicine can be compared to leaves on a tree: they appear the same and serve the same purpose, but under the microscope, there will be a very small degree of difference due to the fact they are based on biological processes. However, biosimilar medicines go through an intensive scientific assessment before marketing to ensure that, despite these small differences, they can be expected to be as safe and effective as the reference medicine.

World Health Organization (WHO) [24]

Biotherapeutic products (biotherapeutics) have a successful record in treating many life-threatening and chronic diseases. The expiry of patents and/or data protection periods for a number of such biotherapeutics has ushered in an era of products that are designed to be highly "similar" to the corresponding licensed "originator" product. Based on a comprehensive head-to-head comparison and demonstrated high similarity, such products can partly rely for their licensing on safety and efficacy data obtained for the originator products. A variety of terms have been used to describe these products, including "biosimilars", "similar biotherapeutic products", "similar biological medicinal products" and "biosimilar products".

FDA [25]

FDA's description of biosimilars is presented in Fig. 1.8.

A consensus regulatory definition for a biosimilar is: *a biosimilar is highly similar (but not identical) to an already market approved biopharmaceutical; it will have minor CMC differences from the reference biopharmaceutical but these differences cannot be clinically meaningful in terms of safety and potency.* It is most important to take note that a biosimilar is not a generic drug. There are several significant

Fig. 1.8 FDA's description of a biosimilar

CMC regulatory compliance differences between biosimilars and chemical generics, and these will be discussed further in Chap. 3.

EMA was the first to approve a biosimilar in 2006, with the FDA not approving its first biosimilar until 2015. The FDA delay was due to the need to obtain a change in the US Public Health Service Act to allow for a regulatory approval abbreviated pathway for biosimilars. An additional delay for the FDA was the limitation imposed on over 100 natural proteins and recombinant proteins (including recombinant human insulins) that were not under the coverage of the US Public Health Service Act. Until 2020, these proteins had to be approved as 'follow-on proteins', not biosimilars, under the Food, Drug & Cosmetics Act (see Chap. 2 for further discussion on this). While Europe had an initial lead on approving biosimilars for the marketplace, the USA is rapidly catching up.

Today, over 80 biosimilars (recombinant proteins and monoclonal antibodies) are in the marketplace. The general description of two market-approved biosimilars, compared with the general description of the reference innovator biopharmaceutical [26], is presented in Table 1.6. As can be seen, apart from the branded name, the CMC descriptions are identical.

Table 1.6 Comparison of general biosimilar description compared to innovator

Type	General Description	
	Reference Innovator Product	**Biosimilar Product**
monoclonal antibody	**HERCEPTIN (trastuzumab) is a recombinant DNA-derived humanized monoclonal antibody that selectively binds with high affinity to HER2 (the extracellular domain of the human epidermal growth factor receptor 2 protein). Trastuzumab is produced by Chinese Hamster Ovary (CHO) cells.** ***[Genentech/Roche received market approval in 1998/2000 (FDA/EMA)]***	**KANJINTI (trastuzumab) is a recombinant DNA-derived humanized monoclonal antibody that selectively binds with high affinity to HER2 (the extracellular domain of the human epidermal growth factor receptor 2 protein). Trastuzumab is produced by Chinese Hamster Ovary (CHO) cells.** ***[Amgen received market approval in 2019/2018 (FDA/EMA]***
Fab fragment	**LUCENTIS (ranibizumab) is a recombinant humanized IgG1 kappa monoclonal antibody Fab fragment that binds to human vascular endothelial growth factor A (VEGF-A). It lacks an Fc region and has a molecular weight of approximately 48 kDa and is produced by *E. coli* cells.** ***[Genentech/Roche received market approval in 2006/2007 (FDA/EMA)]***	**BYOOVIZ (ranibizumab) is a recombinant humanized IgG1 kappa monoclonal antibody Fab fragment that binds to human vascular endothelial growth factor A (VEGF-A). It lacks an Fc region and has a molecular weight of approximately 48 kDa and is produced by *E. coli* cells.** ***[Samsung Bioepis received market approval in 2021 (both FDA and EMA)]***

1.3.4 Transitioning to the 'Fourth Wave' of Biopharmaceuticals

What are the two things in common across the first three waves of biopharmaceuticals – recombinant proteins, monoclonal antibodies, biosimilars? First, these biopharmaceuticals are all proteins (consisting of amino acids linked into polypeptide chains). Second, they all have been manufactured by applying the Central Dogma of Molecular Biology: DNA → mRNA → amino acid (protein). And applying it to living microorganisms.

Since the early 1990s, transfer of human genes (human DNA) into microorganisms (e.g., *E. coli*, yeast, CHO, etc.) has been the foundation of biopharmaceuticals. The gene transfer yields a recombinant cell line. The genetic machinery of the recombinant living cell line transcribes the human DNA into human mRNA, which is then translated into human protein production. This DNA to protein process takes place in bioreactors. The cell cultures containing the induced recombinant proteins or monoclonal antibodies are harvested, and then the biopharmaceutical purified. After final formulation and filling into a container closure, the protein-based biopharmaceutical (recombinant protein, monoclonal antibody, biosimilar) is finally ready to be administered to the patient (see Fig. 1.9).

Now along comes the 4th wave of biopharmaceuticals, referred to as gene therapy-biopharmaceuticals. What is the one thing that distinguishes the gene therapy-biopharmaceuticals from the protein-based biopharmaceuticals? The gene therapy-based biopharmaceuticals are nucleic acid-based (either DNA or mRNA genes contained within genetically engineered viral or non-viral vectors, or patient

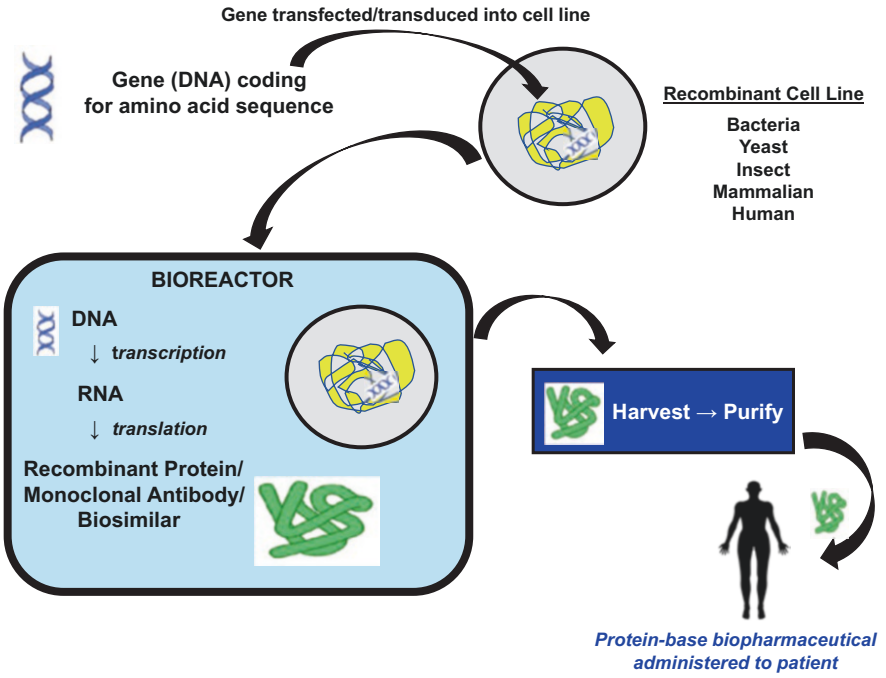

Fig. 1.9 Manufacture of protein-based biopharmaceuticals (waves 1, 2, 3)

cells). What is the one thing that is the same between the gene therapy-biopharmaceuticals and the protein-based biopharmaceuticals? They both have been manufactured by applying the Central Dogma of Molecular Biology: DNA → mRNA → amino acid (protein). But the twist, is that the gene therapy-biopharmaceuticals use humans to produce the proteins inside their bodies. Now, the human patient is the 'bioreactor' for producing directly *in situ* the gene-derived protein, without the need for purification, formulation or delivery device, to obtain the desired medical benefit (see Fig. 1.10).

1.3.5 4th Wave – Gene Therapy-Based Biopharmaceuticals

The most recent wave, the 'fourth wave' of biopharmaceuticals to enter the marketplace consists of the nucleic acid-based or gene therapy-based biopharmaceuticals. The following regulatory authority guidances help explain what these are:

FDA [27]

Human gene therapy seeks to modify or manipulate the expression of a gene or to alter the biological properties of living cells for therapeutic use. Gene therapy is a technique that modifies a person's genes to treat or cure disease. Gene therapies can work by several mechanisms:

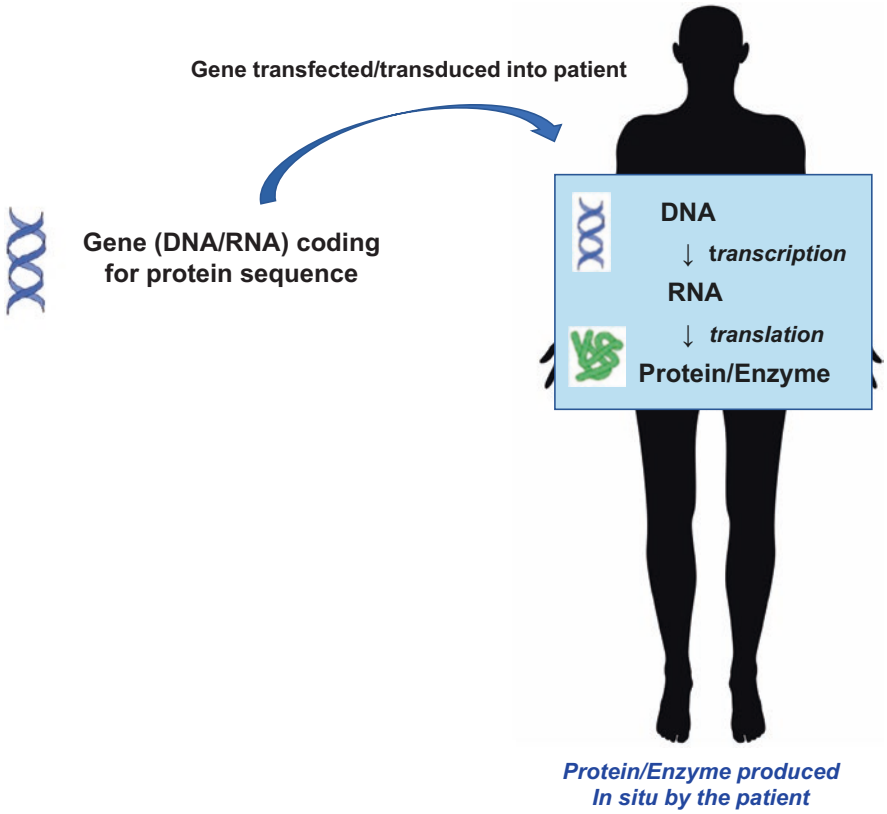

Fig. 1.10 Manufacture of gene therapy-based biopharmaceuticals (wave 4)

- Replacing a disease-causing gene with a healthy copy of the gene
- Inactivating a disease-causing gene that is not functioning properly
- Introducing a new or modified gene into the body to help treat a disease

There are a variety of types of gene therapy products, including:

- ***Plasmid DNA*:** Circular DNA molecules can be genetically engineered to carry therapeutic genes into human cells.
- ***Viral vectors*:** Viruses have a natural ability to deliver genetic material into cells, and therefore some gene therapy products are derived from viruses. Once viruses have been modified to remove their ability to cause infectious disease, these modified viruses can be used as vectors (vehicles) to carry therapeutic genes into human cells.
- ***Bacterial vectors*:** Bacteria can be modified to prevent them from causing infectious disease and then used as vectors (vehicles) to carry therapeutic genes into human tissues.
- ***Human gene editing technology*:** The goals of gene editing are to disrupt harmful genes or to repair mutated genes.
- ***Patient-derived cellular gene therapy products*:** Cells are removed from the patient, genetically modified (often using a viral vector) and then returned to the patient.

EMA [28]

Gene therapy medicinal products (GTMPs) generally consist of a vector or delivery formulation/system containing a genetic construct engineered to express a specific transgene ('therapeutic sequence') for the regulation, repair, replacement, addition or deletion of a genetic sequence. The active substance is the nucleic acid sequence(s), or genetically modified microorganism(s), virus(es) or cells. The active substance may be composed of multiple elements. By using such gene therapy constructs, in vivo genetic regulation or genetic modification of somatic cells can be achieved. Vectors used in GTMPs can be engineered to target specific tissues or cells or to ensure the safety of the GTMP (deletion of genes associated with virulence, pathogenicity, immunotoxicity or replication-competence).

Health Canada (HC) [29]

Gene therapy is using "genes as medicine". It is an experimental approach to treating genetic disease where the faulty gene is fixed, replaced or supplemented with a healthy gene so that it can function normally. To get a new gene into a cell's genome, it must be carried in a molecule called a vector. The most common vectors currently being used are viruses, which naturally invade cells and insert their genetic material into that cell's genome. To use a virus as a vector, the virus' own genes are removed and replaced with the new gene destined for the cell. When the virus attacks the cell, it will insert the genetic material it carries. A successful transfer will result in the target cell now carrying the new gene that will correct the problem caused by the faulty gene. Viruses that can be used as vectors include retroviruses like HIV, adenoviruses (one of which causes the common cold), adeno-associated viruses and herpes simplex viruses. There are also many non-viral vectors being tested for gene therapy uses. These include artificial lipid spheres called liposomes, DNA attached to a molecule that will bind to a receptor on the target cell, artificial chromosomes and naked DNA that is not attached to another molecule at all and can be directly inserted into the cell. The actual transfer of the new gene into the target cell can happen in two ways: ex vivo and in vivo. The ex vivo approach involves transferring the new gene into cells that have been removed from the patient and grown in the laboratory. Once the transfer is complete, the cells are returned to the patient, where they will continue to grow and produce the new gene product. The in vivo approach delivers the vector directly to the patient, where transfer of the new gene will occur in the target cells within the body.

As shown in Fig. 1.11, there are two main approaches to inserting the gene therapy-base biopharmaceuticals – *in vivo* using viral or non-viral vectors and *ex vivo* genetically modifying collected patient cells which are then returned to the patient [27]:

For *in vivo* gene therapy-based biopharmaceuticals, adeno-associated virus (AAV) viral vectors have been the dominant method for delivering the genes to the patient. Recombinant AAV (rAAV) carries a single-stranded DNA molecule (the gene of interest, GOI) within its protein capsid. A schematic illustrating the structure of recombinant AAV is presented in Fig. 1.12. The structure in this figure is of the gene therapy-based biopharmaceutical, Glybera (alipogene tiparvovec), in which the protein capsid contains the gene LPL (lipoprotein lipase) [30].

Although recombinant AAV viral vector is currently the vector of choice for *in vivo* delivery, non-viral vectors (e.g., mRNA encapsulated in lipids) are under consideration.

For *ex vivo* gene therapy, lentivirus (LV) viral vectors have been the dominant method for delivering the genes to the collected patient's cells. Recombinant lentivirus (rLV), a retrovirus, carries two copies of single-stranded mRNA (the gene of interest, GOI) within its protein capsid. The lentivirus also carries enzymes to

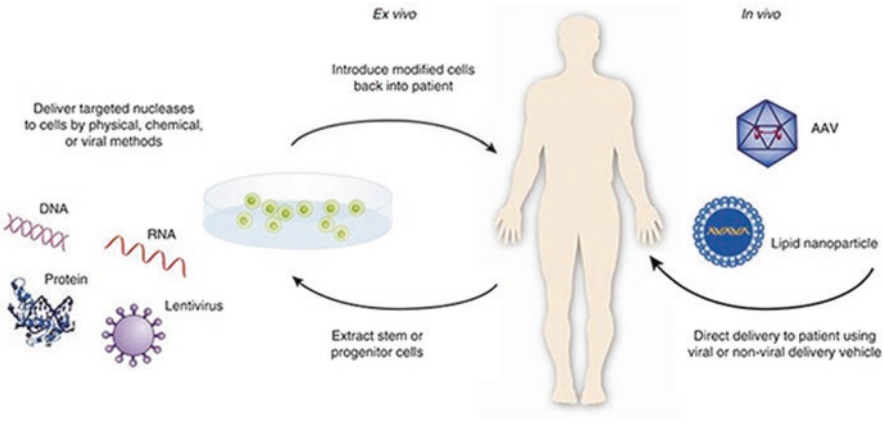

Fig. 1.11 Two main approaches to inserting gene therapy-base biopharmaceuticals into patients

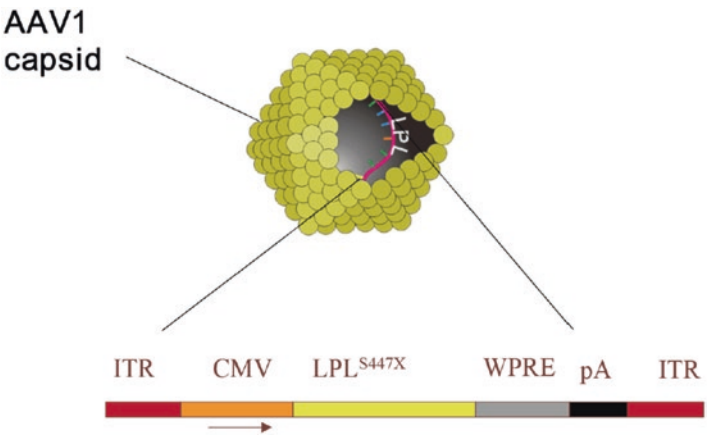

Fig. 1.12 Schematic illustrating the structure of a recombinant AAV viral vector

reverse transcriptase the mRNA into DNA, and then integrate the DNA into the patient cell genome. The transduced patient cells, which is the gene therapy-based biopharmaceutical, is then administered back to the patient. A schematic illustrating a new gene, anti-CD19 chimeric antigen receptor (CAR), delivered to the patient T cell by transduction with rLV, is presented in Fig. 1.13. The CAR T cells are Breyanzi (lisocabtagene maraleucel) [31].

Although LV is currently the vector of choice for *ex vivo* delivery, non-viral vectors (e.g., mRNA encapsulated in lipids) and electroporation are under consideration. Gene restoration to stem cells and gene addition to T cells have currently been the primary focus of *ex vivo* gene therapy, but both genome editing and additional cell types (e.g., natural killer, NK, cells) are being pursued.

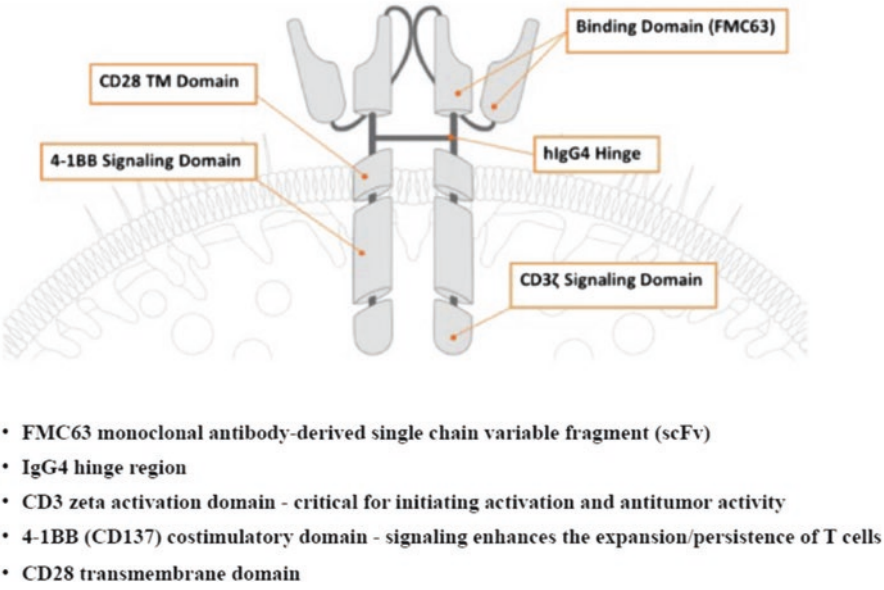

Fig. 1.13 Schematic illustrating the anti-CD19 CAR transduced onto a T-cell

It is important to recognize that two areas, what might look like involvement of gene therapy-biopharmaceuticals, are excluded from the correct definition. For example, oligonucleotide therapeutics such as antisense oligonucleotides (ASOs) or small-interfering RNA (siRNA) that bind to a target mRNA sequence to alter mRNA or protein expression, are not gene therapy-based biopharmaceuticals (they interact with a gene but they do not modify or replace a gene: they are chemical drugs). Also, great interest has been raised due to the SARS-CoV-2 (also referred to as COVID-19) pandemic. A number of 'genetic vaccines' have been developed and are now market-approved including both mRNA in lipid nanoparticle (mRNA-LNP) emulsions and genetically engineered adenoviruses. These are vaccines, and were market-approved under biologic vaccine regulations. They are not included under the definition of gene therapy. In fact, the European Commission directive 2001/83/EC specifically excludes vaccines against infectious diseases from being defined as gene therapies [32]. And, FDA reviewed the genetic vaccines for market-approval under its Office of Vaccines Research & Review (OVRR), not its Office of Tissues & Advanced Therapies (OTAT) currently renamed the Office of Therapeutic Products (OTP). But, it should be pointed out that the principles of developing and manufacturing these genetic vaccines are in common with those used to develop and manufacture gene therapy-based biopharmaceuticals.

This book will focus on those gene therapy-based biopharmaceuticals that have achieved market-approval status: the rAAV viral vectors for *in vivo* delivery, and the genetically modified patient cells transduced *ex vivo* by the rLV viral vectors. Some market-approved gene therapy-based biopharmaceuticals are presented in Table 1.7

Table 1.7 General description of some commercial rAAV vectors

rAAV Viral Vectors	General Description
Zolgensma (onasemnogene abeparvovec)	**ZOLGENSMA is a non-replicating, self-complementary AAV9 vector, produced by transient triple plasmid transfection of HEK293 cells. It contains the human survival motor neuron gene (*SMN1*) under the control of the cytomegalovirus (CMV) enhancer/chicken-β-actin-hybrid promoter (CB). One of the two adeno-associated vector (AAV) inverted terminal repeats (ITRs) has been modified to promote intramolecular annealing of the transgene, thus forming a double-stranded transgene ready for transcription. The size of the packaged self-complementary vector genome is ~4.6 kb. The capsid is comprised of 60 viral proteins (VP1, VP2, VP3) in a ratio of approximately 1:1:10 that are derived from the AAV serotype 9. The capsid proteins are produced by alternate splicing such that VP2 and VP3 are two truncated forms of VP1, all with common C-terminal sequences.**
Roctavian (valoctocogene roxaparvovec)	**ROCTAVIAN is a non-replicating recombinant AAV5 vector, produced by co-infection of Spodoptera frugiperda (Sf9) insect cells with recombinant baculovirus. The vector genome is contained within an icosahedral capsid composed of three AAV structural proteins, VP1, VP2, and VP3, of approximately 25 nm in diameter. While VP2 and VP3 are essential for capsid formation, VP1 is essential for infectivity of the capsid. VP1 contains a phospholipase A2 (PLA2) domain required for endosomal escape of the capsid and subsequent trafficking to the nucleus. The vector genome includes double-stranded inverted terminal repeats (ITRs) at its 5' and 3' ends and single-stranded DNA encoding a hybrid human liver-specific promoter (HLP), a BDD hFVIII gene and a synthetic polyadenylation signal. The hFVIII-SQ coding DNA sequence includes a codon optimised nucleic acid sequence encoding the A1, A2, A3, C1 and C2 FVIII protein domains. The wild-type B domain between the A2 and A3 domains is replaced by a 14 amino acid "SQ" linker sequence, from the normal B domain sequence.**

(rAAV viral vectors) [33, 34] and Table 1.8 (genetically modified patients cells transduced by rLV) [35, 36].

Also in this book, comments will also be made on the up-and-coming non-viral vector (mRNA) in clinical studies for therapeutic use, especially dealing with its manufacture and characterization (noted in Chaps. 6, 7, 8, 9, 10 and 11).

While the number of market-approved gene therapy-based biopharmaceuticals is currently under 20 products, according to various industry forecasts, it is entirely possible that this group of biopharmaceuticals might become a dominate medicine type of the future. Dr. Scott Gottlieb, former Commissioner of the FDA, made the following statement in 2019 [37]:

> The FDA is witnessing a surge of cell and gene therapy products entering early development, evidenced by a large upswing in the number of investigational new drug (IND)

Table 1.8 General description of some commercial genetically modified patient cells

Genetically Modified Patient Cells	General Description
Skysona (elivaldogene autotemcel)	**SKYSONA is an autologous CD34+ cell-enriched population that contains hematopoietic stem cells transduced with lentiviral vector (LVV) encoding *ABCD1* cDNA for human adrenoleukodystrophy protein (ALDP). (The LVV is produced by transient plasmid transfection of HEK293T cells).**
Abecma (idecabtagene vicleucel)	**ABECMA is a genetically modified autologous T cell immunotherapy product consisting of T cells transduced with an anti-BCMA chimeric antigen receptor (CAR) lentiviral vector (LVV). The autologous T cells transduced *ex vivo* with the anti-BCMA CAR LVV to express the anti-BCMA CAR on the T cell surface. Binding of ide-cel to BCMA-expressing target cells results in CAR+ T cell proliferation, cytokine secretion, and subsequent cytolytic killing of BCMA-expressing cells. (The LVV is produced by transient four plasmid transfection of HEK293T cells).**
Zynteglo (betibeglogene autotemcel)	**ZYNTEGLO is an autologous CD34+ cell-enriched population that contains hematopoietic stem cells transduced with lentiviral vector (LVV) encoding the $\beta^{A\text{-}T87Q}$-globin gene. (The LVV is produced by transient plasmid transfection of HEK293T cells).**
Tecartus (*brexucabtagene autoleucel)*	**TECARTUS is a genetically modified autologous T cell immunotherapy product consisting of T cells transduced with an anti-CD19 chimeric antigen receptor (CAR) gamma-retroviral vector (γRV). (The γ-retrovirus vector is produced constitutively from a stably-transduced PG13 cell line).**

applications. Based on this activity, we anticipate that the number of product approvals for cell and gene therapies will grow in the coming years, reflecting significant scientific advancement and the clinical promise of these new innovations. We anticipate that by 2020 we will be receiving more than 200 INDs per year, building upon our total of more than 800 active cell-based or directly administered gene therapy INDs currently on file with the FDA. And by 2025, we predict that the FDA will be approving 10 to 20 cell and gene therapy products a year based on an assessment of the current pipeline and the clinical success rates of these products.

His predicted annual rate of new cell and gene therapy approvals by 2025 is the same annual rate expected for new innovator recombinant protein/monoclonal antibody market approvals. In 2022, there were a total of seven new gene therapy-based biopharmaceuticals market-approved, so the yearly rate of market-approval is close to the number 10, the minimum number projected just 2 years from now in 2025. However, the major activity with the cell and gene therapies is ‘under the radar’ (i.e., currently in clinical development). At last report, the FDA had over 3000 INDs for these product type under review, which indicates that many more market-approvals are to come.

Another sign of the possibility of a future 'tsunami' of market-approved gene therapy-based biopharmaceuticals is the financial investment by many biopharmaceutical companies both startup as well as established protein-based biopharmaceutical companies (e.g., Roche, Novartis, Gilead, Bristol Myers Squibb, Johnson & Johnson, Amgen, etc.). In addition, the financial investment by many contract development & manufacturing organizations (CDMOs) (e.g., Catalent, Lonza, Fujifilm, etc.), as well as by many component vendors (e.g., Pall, Sartorius, etc.) providing the support needed for these products. 'Follow the money' points to considerable activity for gene therapy-based biopharmaceuticals in the future.

As mentioned previously in Sect. 1.2.3, the terms 'Cell & Gene Therapy Product' (CGTP) and 'Advanced Therapy Medicinal Product' (ATMP) define a group of biologics that include both gene therapies as well as cellular therapies and tissue-engineered therapies. Only if the cellular therapies and tissue-engineered therapies include genetic engineering are they defined as gene therapy-based biopharmaceuticals and examined in this book (e.g., *ex vivo* CAR T-cell gene therapies). But it should be pointed out that the many principles discussed in this book related to cell source materials, cell characterization and cell culturing manufacturing control also apply to the cellular therapy products.

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Chapter 2
Regulatory Pathways Impacting Biopharmaceuticals

Abstract To obtain market approval from a regulatory authority for a new medicine, manufacturers must first initiate and then complete human clinical trials. Each regulatory authority has a prescribed regulatory pathway for initiating the clinical trial, for maintaining the clinical trial during its development and then for seeking market approval. But not all pathways are the same or appropriate for all types of biopharmaceuticals, even within the same regulatory region. In this chapter, the pathways for regulatory approval specific for all biopharmaceutical types (recombinant proteins, monoclonal antibodies, biosimilars, viral vectors, genetically modified patient cells) both within the United States (regulated by the U.S. FDA) and the European Union (regulated by EMA) are discussed. Similarities and differences in the CMC regulatory compliance requirements in these pathways as applied to biopharmaceuticals are examined.

Keywords FDA · FD&C · PHS · IND · NDA · BLA · CFR · NCA · EMA · Directive · Regulation · IMPD · CTA · MAA · QTP · Protein · Bioqualifier

2.1 Different Regulatory Pathways

Clinical trials are research studies in which people volunteer to help find answers to specific health questions. When carefully conducted, they are the safest and fastest way to find new treatments and ways to improve health. Clinical trials are conducted according to a regulatory authority approved plan, called a protocol, which describes the following:

- the types of patients who may enter the study
- the schedules of tests and procedures
- the drugs involved
- the dosages, or amount of the drug
- the length of the study
- what the researchers hope to learn from the study

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Volunteers who participate in the clinical study must agree to the rules and terms outlined in the protocol. Similarly, researchers, doctors, and other health professionals who manage the clinical trials must follow strict rules set by the regulatory authority. So also must the drug manufacturer follow strict rules set by the regulatory authority to ensure that their developing medicine is safe for the patients. All of these rules, when appropriately followed, make sure that those who agree to participate are treated as safely as possible.

Each regulatory authority has a prescribed regulatory pathway for manufacturers (i.e., sponsors) to initiate a clinical trial, to maintain the clinical trial during its development, and then to seek market approval. Differences between regulatory regions and differences within a regulatory region exist. In the United States, all biopharmaceuticals are covered under the Public Health Services Act (but only since 2020), and regulated both in clinical development and for market approval by the U.S. Food and Drug Administration (FDA). In the European Union, all biopharmaceuticals are covered under various regulations or directives, but regulated by National Competent Authorities (NCAs), that is individual countries within the EU, during clinical development; and then by the European Medicines Agency (EMA) for market approval. These differences in the pathways between regulatory regions and differences in the pathways within a regulatory region, as well as differences in the pathways over time, can lead to confusion for those responsible for maintaining CMC regulatory compliance for the biopharmaceuticals manufactured by their company.

2.2 Navigating United States Regulation for Biopharmaceuticals

In the United States, laws are initiated/amended and approved by the Legislative Branch (Congress), and then handed over to the Executive Branch (President) for final approval. If the law impacts how pharmaceuticals are to be regulated, the FDA has the responsibility of first interpreting the intent of the new/amended law and then implementing and enforcing it, as shown in Fig. 2.1.

In the United States, there are two pharmaceutical laws (the Food, Drug, & Cosmetic Act – FD&C Act, and the Public Health Services Act – PHS Act), each of which sets up a regulatory pathway, referred to as the NDA pathway and the BLA pathway, respectively, see Fig. 2.2. Within the FDA, there are two major review centers for pharmaceuticals (the Center for Drug Evaluation and Research – CDER and the Center for Biologics Evaluation and Research – CBER). Needless to say, this 2x2 matrix can be somewhat confusing for biopharmaceuticals, especially due to changes over time concerning which pharmaceutical law covers which biopharmaceuticals, and due to changes over time of which FDA review center is responsible for which biopharmaceuticals.

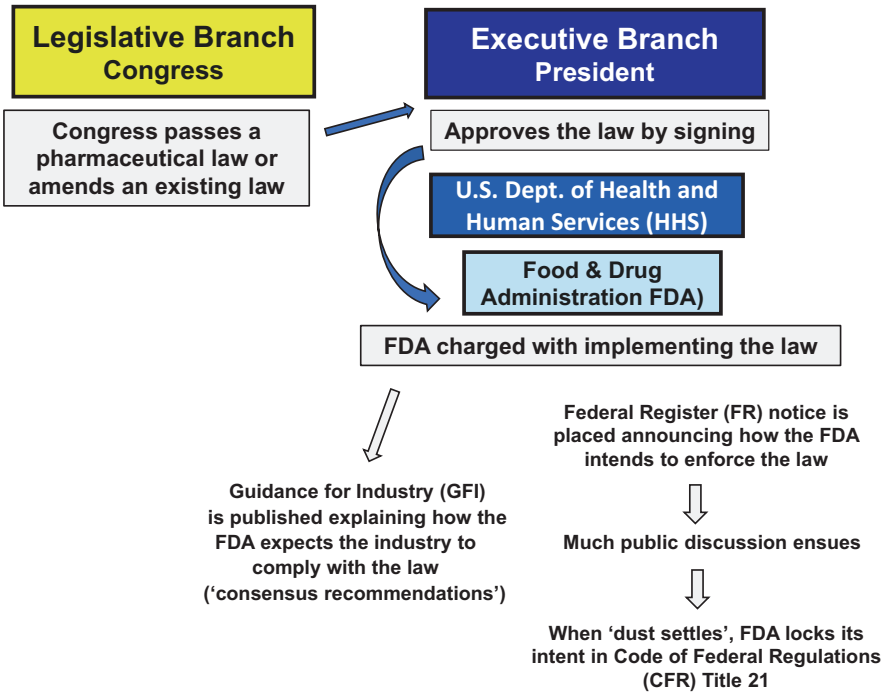

Fig. 2.1 The pharmaceutical regulatory system in the United States

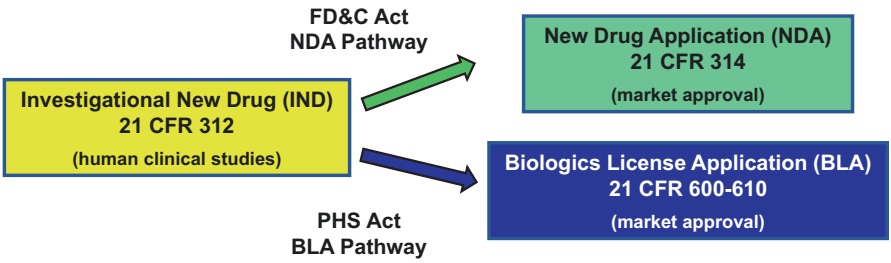

Fig. 2.2 Regulatory drug development pathways in the USA

2.2.1 Food, Drug, & Cosmetic (FD&C) Act

The Food, Drug, & Cosmetic Act (FD&C Act), originally passed by Congress in 1938, established the Investigational New Drug (IND) to New Drug Application (NDA) pathway. The general requirements for initiating and then maintaining human clinical trials under an IND are described in the FDA's Code of Federal

Table 2.1 Partial outline of 21 CFR Part 312 IND clinical development requirements

Part 312 Investigational New Drug Application

Subpart A – General Provisions
- **312.1 – Scope**
- **312.2 – Applicability**
- **312.3 – Definitions and interpretations**

Subpart B – Investigation New Drug Application (IND)
- **312.20 – Requirements for an IND**
- **312.21 – Phases of an investigation**
- **312.22 – General principles of the IND submission**
- **312.23 – IND content and format**
- **312.31 – Information amendments**
- **312.33 – Annual reports**
- **312.38 – Withdrawal of an IND**

Subpart C – Administrative Actions
- **312.42 – Clinical holds and requests for modification**
- **312.47 – Meetings**

Table 2.2 Partial outline of 21 CFR Part 314 NDA market approval requirements

Part 314 Applications for FDA Approval to Market a New Drug

Subpart A – General Provisions
- **314.1 – Scope of this part**
- **314.2 – Purpose**
- **314.3 - Definitions**

Subpart B - Applications
- **314.50 – Content and format of an NDA**
- **314.55 – Pediatric use information**
- **314.70 – Supplements and other changes to an approved NDA**
- **314.71 – Procedures for submission of a supplement**
- **314.72 – Change in ownership of an application**
- **314.81 – Other postmarketing reports**
- **314.90 – Waivers**

Regulations (CFR) Title 21 – Food and Drugs: Part 312 [1]. Table 2.1 presents a partial outline of these IND requirements.

The general requirements for seeking market approval after completing the required clinical studies by submitting the NDA are described in FDA's Code of Federal Regulations (CFR) Title 21 – Food and Drugs: Part 314 [2]. Table 2.2 presents a partial outline of the NDA requirements.

This regulatory drug development pathway from IND to NDA, required under the FD&C Act, is termed the New Drug Application (NDA) pathway (see Fig. 2.2).

Over time, the FD&C Act has been amended many, many times either to further strengthen its regulation over pharmaceutical medicines or to modify elements of its pathway. One of the major amendments established the regulatory abbreviated pathway for generic chemical drugs: Abbreviated New Drug Application (ANDA) in 1984.

The regulation of biopharmaceuticals by the FD&C Act has changed dramatically over time. At the time of the FD&C Act passage in 1938, all chemical drugs (both natural-sourced and chemically synthesized) were regulated under this law. However, in 1941, Congress passed the Insulin Amendment Act to ensure that the protein hormone, porcine insulin, could also be regulated by the FDA under the FD&C Act. This amendment to the law, allowed over time, the 'grandfathering in' of over 100 other proteins (both natural-sourced and recombinant), specifically proteins that were hormones or enzymes. It was not until March 23, 2020, that all of these proteins were transferred out of the FD&C law and moved over into the Public Health Services (PHS) law [3]. Market-approved biopharmaceuticals such as recombinant human insulin, recombinant human growth hormone, recombinant human hyaluronidase, etc., were included in this transfer. Today, there are no longer any proteins remaining under the FD&C Act.

As a point of reference, 'proteins' are defined as polypeptides consisting of greater than 40 amino acids, and are considered biologicals regulated under the PHS Act. 'Peptides' on the other hand are defined as polypeptides consisting of no more than 40 amino acids, and are considered chemical drugs regulated under the FD&C Act [4]. However, if the peptide is to be used as a vaccine, it will be regulated under the PHS Act.

2.2.2 Public Health Services (PHS) Act

After the FD&C Act was passed in 1938, Congress became concerned about a group of pharmaceutical medicines considered 'biologicals', which were a group of medicines that typically were not very pure, needed more oversight over the manufacturing process, and required extra release testing – compared to the typical chemical drug. In 1944, only 6 years after the FD&C Act, Congress passed the Public Health Services Act (PHS Act). This Act established the Investigational New Drug (IND) to Biologics License Application (BLA) pathway.

The general requirements for obtaining and maintaining human clinical trials under an IND are described in the FDA's Code of Federal Regulations (CFR) Title 21 – Food and Drugs: Part 312. These are the same requirements listed in Table 2.1 for pharmaceutical medicines under the FD&C Act. The general requirements for seeking market approval after completing the required clinical studies by submitting the BLA are described in the FDA's Code of Federal Regulations (CFR) Title 21 – Food and Drugs: Parts 600–610 [5]. Table 2.3 presents a partial outline of the BLA requirements.

Table 2.3 Partial outline of 21 CFR Parts 601–610 BLA market approval requirements

Part 601 Licensing

Subpart A – General Provisions
- **601.2 – Applications for biologics license**
- **601.3 – Complete response letter to the applicant**

Subpart C – Biologics Licensing
- **601.12 – Changes to an approved application**
- **601.14 – Regulatory submission in electronic format**
- **601.20 – Biologics licenses; issuance and conditions**

Part 610 General Biological Products Standards
- **610.1 – Tests prior to release required for each lot**
- **610.2 – Requests for sample and protocols**
- **610.10 - Potency**
- **610.12 - Purity**
- **610.14 - Identity**

The regulatory pathway required under the PHS Act is termed the Biologics License Application (BLA) pathway (see Fig. 2.2). It should be noted that the original market approval in the PHS Act was called the Product License Application/Establishment License Application (PLA/ELA), but in 1996 it was shortened to the current name, Biologics License Application (BLA).

The PHS Act in 1944 stated which specific products types would be included in the law:

> The term "biological product" means a virus, therapeutic serum, toxin, antitoxin or analogous product, or arsphenamine or derivative of arsphenamine (or any other trivalent organic arsenic compound), applicable to the prevention, treatment, or cure of a disease or condition of human beings.

Overtime additional product types were listed in the law, so that today, the current definition of 'biological product' in the PHS Act (CFR 21 Part 600.3(h)) includes the following [6]:

> The term "biological product" means a virus, therapeutic serum, toxin, antitoxin, vaccine, blood, blood component or derivative, allergenic product, protein or analogous product, or arsphenamine or derivative of arsphenamine (or any other trivalent organic arsenic compound), applicable to the prevention, treatment, or cure of a disease or condition of human beings.

Recombinant proteins, monoclonal antibodies and biosimilars all easily fit under the definition of 'protein' covered by the PHS Act, However, gene therapy-based biopharmaceuticals are currently embraced under the definition of 'analogous products' (i.e., as showing a likeness that permits one to draw an analogy) in the PHS Act.

Two interesting aspects of the PHS definition of 'biological product':

1. Surprisingly, the chemically synthesized arsenic-containing organic compound, arsphenamine (also known as Compound 606), still remains today in the legal definition of a biological product in the PHS Act. Most likely it was included in

the 1944 definition of a biological because it was the first chemically synthesized antibiotic, as well as the only effective treatment for syphilis at that time.
2. In 2010, when 'protein' was added to the list of biological products in the PHS Act, it had the phrase 'protein (except any chemically synthesized polypeptides)'; today, the phrase is only 'protein'. This leaves open the possibility that if a protein could be commercially chemically synthesized it would be included under the PHS Act umbrella. It should be pointed out that 'protein' means any alpha amino acid polymer with a specified amino acid sequence that is greater than 40 amino acids [4].

2.2.3 Similarity in CMC Regulatory Requirements Between the Two FDA Laws

All pharmaceuticals in the USA, whether under the FD&C Act or the PHS Act, have certain CMC regulatory requirements that must be met: (1) expectation that the manufacturing facility operations and the product release/stability testing are carried out under current Good Manufacturing Practices (cGMPs), and (2) required submission of CMC information to the FDA for their independent assessment of adequate and appropriate patient safety protection before proceeding with human clinical trials.

Both the FD&C Act and the PHS Act are linked together by FDA's requirements for current Good Manufacturing Practices (cGMPs). These minimum requirements help ensure the quality and safety of all pharmaceutical medicines. The Code of Federal Regulations Title 21 Parts 210–211 describes the cGMPs requirements, and states clearly in Part 211.1(b) that the regulations apply to both chemical drugs in the FD&C Act and biologics in the PHS Act [7].

Both the FD&C Act and the PHS Act are also linked together by FDA's requirements for CMC content that needs to be submitted to them. The Code of Federal Regulations Title 21 Part 312 describes FDA's requirements for the conduct of human investigational clinical studies, and states clearly in Part 312.2(a) that the CMC content requirement applies to both chemical drugs in the FD&C Act and biologics in the PHS Act [8].

To initiate a human clinical trial or to make changes during the clinical development period, the manufacturer (sponsor) is required to submit to the FDA Form 1571 – Investigational New Drug Application (IND), that is filled in using the ICH electronic Common Technical Document (eCTD) format. The CMC content to be submitted along with the form follows the general information listed in Code of Federal Regulations Title 21 Part 312.23(a)(7) [9]. See Tables 2.4 (general guidance) and 2.5 (specifics for drug substance and drug product) for the outline of required CMC information in the IND.

Table 2.4 CFR Title 21 Part 312.23(a)(7) – General guidance for CMC information in an IND

(7) Chemistry, manufacturing, and control information.

(i) As appropriate for the particular investigations covered by the IND, a section describing the composition, manufacture, and control of the drug substance and the drug product. Although in each phase of the investigation sufficient information is required to be submitted to assure the proper identification, quality, purity, and strength of the investigational drug, the amount of information needed to make that assurance will vary with the phase of the investigation, the proposed duration of the investigation, the dosage form, and the amount of information otherwise available. FDA recognizes that modifications to the method of preparation of the new drug substance and dosage form and changes in the dosage form itself are likely as the investigation progresses. Therefore, the emphasis in an initial Phase 1 submission should generally be placed on the identification and control of the raw materials and the new drug substance. Final specifications for the drug substance and drug product are not expected until the end of the investigational process.

(ii) It should be emphasized that the amount of information to be submitted depends upon the scope of the proposed clinical investigation. For example, although stability data are required in all phases of the IND to demonstrate that the new drug substance and drug product are within acceptable chemical and physical limits for the planned duration of the proposed clinical investigation, if very short-term tests are proposed, the supporting stability data can be correspondingly limited.

(iii) As drug development proceeds and as the scale or production is changed from the pilot-scale production appropriate for the limited initial clinical investigations to the larger-scale production needed for expanded clinical trials, the sponsor should submit information amendments to supplement the initial information submitted on the chemistry, manufacturing, and control processes with information appropriate to the expanded scope of the investigation.

Table 2.5 CFR Title 21 Part 312.23(a)(7) – Required CMC information in an IND

(iv) Reflecting the distinctions described in this paragraph (a)(7), and based on the phase(s) to be studied, the submission is required to contain the following:

(*a*) *Drug substance.* A description of the drug substance, including its physical, chemical, or biological characteristics; the name and address of its manufacturer; the general method of preparation of the drug substance; the acceptable limits and analytical methods used to assure the identity, strength, quality, and purity of the drug substance; and information sufficient to support stability of the drug substance during the toxicological studies and the planned clinical studies. Reference to the current edition of the United States Pharmacopeia - National Formulary may satisfy relevant requirements in this paragraph.

(*b*) *Drug product.* A list of all components, which may include reasonable alternatives for inactive compounds, used in the manufacture of the investigational drug product, including both those components intended to appear in the drug product and those which may not appear but which are used in the manufacturing process, and, where applicable, the quantitative composition of the investigational drug product, including any reasonable variations that may be expected during the investigational stage; the name and address of the drug product manufacturer; a brief general description of the manufacturing and packaging procedure as appropriate for the product; the acceptable limits and analytical methods used to assure the identity, strength, quality, and purity of the drug product; and information sufficient to assure the product's stability during the planned clinical studies. Reference to the current edition of the United States Pharmacopeia - National Formulary may satisfy certain requirements in this paragraph.

(*c*) A brief general description of the composition, manufacture, and control of any placebo used in a controlled clinical trial.

(*d*) *Labeling.* A copy of all labels and labeling to be provided to each investigator.

(*e*) *Environmental analysis requirements.* A claim for categorical exclusion under § 25.30 or 25.31 or an environmental assessment under § 25.40.

Table 2.6 Module 3.2.S – Outline of required CMC information for market approval of DS

3.2.S.1 General Information
- **3.2.S.1.1 Nomenclature**
- **3.2.S.1.2 Structure**
- **3.2.S.1.3 General Properties**

3.2.S.2 Manufacture
- **3.2.S.2.1 Manufacturer(s)**
- **3.2.S.2.2 Description of Manufacturing Process and Process Controls**
- **3.2.S.2.3 Control of Materials**
- **3.2.S.2.4 Control of Critical Steps and Intermediates**
- **3.2.S.2.5 Process Validation and/or Evaluation**
- **3.2.S.2.6 Manufacturing Process Development**

3.2.S.3 Characterization
- **3.2.S.3.1 Elucidation of Structure and Other Characteristics**
- **3.2.S.3.2 Impurities**

3.2.S.4 Control of Drug Substance
- **3.2.S.4.1 Specification**
- **3.2.S.4.2 Analytical Procedures**
- **3.2.S.4.3 Validation of Analytical Procedures**
- **3.2.S.4.4 Batch Analysis**
- **3.2.S.4.5 Justification of Specifications**

3.2.S.5 Reference Standards or Materials

3.2.S.6 Container Closure System

3.2.S.7 Stability
- **3.2.S.7.1 Stability Summary and Conclusions**
- **3.2.S.7.2 Post-Approval Stability Protocol and Stability Commitment**
- **3.2.S.7.3 Stability Data**

For requesting market approval, whether for a chemical drug or a biologic, the manufacturer (sponsor) is required to submit to the FDA Form 356 h – Application to Market a New or Abbreviated New Drug or Biologic for Human Use, that is filled in using the ICH electronic Common Technical Document (eCTD) format. The CMC content to be submitted along with the form follows the general information listed in ICH M4Q(R1) – The Common Technical Document for the Registration of Pharmaceuticals for Human Use: Quality [10]. See Tables 2.6 (drug substance) and 2.7 (drug product) for the outline of required CMC information in the BLA filing.

Note, the eCTD format and the outline of CMC content to provide, is the same for both the IND and BLA submissions. The main difference is in the level of CMC detail that is required – during clinical development being far less than that needed for market approval.

Table 2.7 Module 3.2.P – Outline of required CMC information for market approval of DP

3.2.P.1 Description and Composition of the Drug Product
3.2.P.2 Pharmaceutical Development
- **3.2.P.2.1 Components of the Drug Product**
- **3.2.P.2.2 Drug Product**
- **3.2.P.2.3 Manufacturing Process Development**
- **3.2.P.2.4 Container Closure System**
- **3.2.P.2.5 Microbiological Attributes**
- **3.2.P.2.6 Compatibility**

3.2.P.3 Manufacture
- **3.2.P.3.1 Manufacturer(s)**
- **3.2.P.3.2 Batch Formula**
- **3.2.P.3.3 Description of Manufacturing Process and Process Controls**
- **3.2.P.3.4 Controls of Critical Steps and Intermediates**
- **3.2.P.3.5 Process Validation and/or Evaluation**

3.2.P.4 Control of Excipients
- **3.2.P.4.1 Specifications**
- **3.2.P.4.2 Analytical Procedures**
- **3.2.P.4.3 Validation of Analytical Procedures**
- **3.2.P.4.4 Justification of Specifications**
- **3.2.P.4.5 Excipients of Human or Animal Origin**
- **3.2.P.4.6 Novel Excipients**

3.2.P.5 Control of Drug Product
- **3.2.P.5.1 Specification(s)**
- **3.2.P.5.2 Analytical Procedures**
- **3.2.P.5.3 Validation of Analytical Procedures**
- **3.2.P.5.4 Batch Analysis**
- **3.2.P.5.5 Characterization of Impurities**
- **3.2.P.5.6 Justification of Specifications**

3.2.P.6 Reference Standards or Materials
3.2.P.7 Container Closure System
3.2.P.8 Stability
- **3.2.P.8.1 Stability Summary and Conclusions**
- **3.2.P.8.2 Post-Approval Stability Protocol and Stability Commitment**
- **3.2.P.8.3 Stability Data**

2.2.4 Significant Differences in CMC Regulatory Requirements Between the Two FDA Laws

Congress originally passed the PHS Act because they felt that tighter control and more FDA oversight were necessary for the subset of pharmaceuticals referred to as ‘biologicals.’ Over the years, the FDA has realized that certain extra controls were no longer necessary for the biologics:

1. FDA eliminated in 2012 the extra sterility test requirement at the bulk sterile filtration process step for commercial biologics stated in 21 CFR Part 610.12 [11]
2. FDA eliminated in 2015 the general safety test that was required for release of all commercial biologic batches stated in 21 CFR Part 610.11 [12]

However, currently, there are three significant CMC regulatory requirements for market-approved biologics under the PHS Act that are not required for market-approved chemical drugs under the FD&C Act:

1. Commercial Batch-to-Batch Biologic Product Release – 21 CFR Part 610.2
2. Identity Testing of Commercial Finished Drug Product After Labeling – 21 CFR Part 610.14
3. Extra 4-Letter 'Bioqualifier' Suffix Added to INN Assigned to Commercial Biologics

It is important to stress that the impact of these remaining extra, tighter regulatory controls and testing on biopharmaceuticals only take place after market approval, and not during clinical development.

2.2.4.1 Commercial Batch-to-Batch Biologic Product Release

FDA, as part of their real-time, ongoing monitoring of the quality of commercial biologic products, can require a pre-release protocol, as stated in 21 CFR Part 610.2. This FDA lot release mechanism allows the FDA, on a batch-to-batch approach, to review the test results and/or to confirm the test results in their own laboratories before the manufacturer's Quality Unit (QU) is permitted to release a batch into commercial distribution. Because both CBER (for viral vectors and genetically modified patient cells) and CDER (for recombinant proteins, monoclonal antibodies, and biosimilars) are responsible for biopharmaceuticals, Part 610.2 addresses both centers [13]:

> (a) *Licensed biological products regulated by CBER.* Samples of any lot of any licensed product together with the protocols showing results of applicable tests, may at any time be required to be sent to the Director, Center for Biologics Evaluation and Research (see mailing addresses in § 600.2 of this chapter). Upon notification by the Director, Center for Biologics Evaluation and Research, a manufacturer shall not distribute a lot of a product until the lot is released by the Director, Center for Biologics Evaluation and Research: Provided, That the Director, Center for Biologics Evaluation and Research, shall not issue such notification except when deemed necessary for the safety, purity, or potency of the product.
>
> (b) *License biological products regulated by CDER.* Samples of any lot of any licensed product together with the protocols showing results of applicable tests, may at any time be required to be sent to the Director, Center for Drug Evaluation and Research (see mailing addresses in § 600.2) for official release. Upon notification by the Director, Center for Drug Evaluation and Research, a manufacturer shall not distribute a lot of a biological product until the lot is released by the Director, Center for Drug Evaluation and Research: Provided, That the Director, Center for Drug Evaluation and Research shall not issue such notification except when deemed necessary for the safety, purity, or potency of the product.

This procedure is in sharp contrast with the FD&C Act, which does not require FDA commercial batch-to-batch pre-release for chemical drugs. Under that Act, the manufacturer's Quality Unit has the responsibility of releasing commercial drug product after it completes its review of (1) all of the required release test results, and (2) all manufacturing batch records along with the manufacturing facility support documentation. The Quality Unit can then issue a Certificate of cGMP compliance and release the batch into commercial inventory.

The level of FDA's involvement in commercial batch-to-batch release is clearly stated in the BLA market approval letters that the FDA sends to a manufacturer, and then publishes on their website. For example, all vaccines, including the recombinant antigens produced by genetic engineering, are under the FDA pre-release requirement: '*please submit final container samples of the product in final containers together with protocols showing results of all applicable test*' [14].

For some biopharmaceutical types, the FDA has waived their extra involvement in commercial batch-to-batch release, but not all. The following published FDA BLA market approval letters indicate which biopharmaceuticals are under a waiver:

Human Plasma-Derived Proteins The FDA pre-release requirement is in place for natural-sourced human plasma-derived proteins, but is waived if the plasma-derived protein is a recombinant protein derived by genetic engineering.

> **SEVENFACT, Coagulation Factor VIIa (Recombinant) – April 1, 2020** [15]
> FDA LOT RELEASE
> You are not currently required to submit samples or protocols of future lots of coagulation factor VIIa (recombinant)-jncw to the Center for Biologics Evaluation and Research (CBER) for release by the Director, CBER, under 21 CFR 610.2(a). We will continue to monitor compliance with 21 CFR 610.1 requiring completion of tests for conformity with standards applicable to each product prior to release of each lot.

Recombinant Proteins, Monoclonal Antibodies, Biosimilars Commercial recombinant proteins and monoclonal antibodies, as well as biosimilars, have had a FDA automatic waiver of the pre-release requirement since 1995 [16]:

> FDA is also announcing that FDA is eliminating lot-by-lot release for licensed well characterized therapeutic recombinant DNA-derived and monoclonal antibody biotechnology products. After approval, manufacturers of such products are no longer requested to submit samples and protocols for individual lots of products to the Center for Biologics Evaluation and Research (CBER) for routine lot-by-lot release. Manufacturers may begin distributing products affected by this policy after notification by CBER and without awaiting approval of a supplement to their product license applications. This notice is intended to reduce unnecessary burdens for industry without diminishing public health protection.

> **ENJAYMO, Sutimlimab-jome – February 4, 2022** [17]
> FDA LOT RELEASE
> You are not currently required to submit samples of future lots of Enjaymo to the Center for Drug Evaluation and Research (CDER) for release by the Director, CDER, under 21 CFR 610.2. We will continue to monitor compliance with 21 CFR 610.1, requiring completion of tests for conformity with standards applicable to each product prior to release of each lot.

Gene Therapy Viral Vectors The FDA pre-release requirement is in place for all viral vectors used for *in vivo* gene therapy.

> **HEMGENIX, Etanacogene Dezaparvovec – November 22, 2022** [18]
> FDA LOT RELEASE
> You are required to submit lot release protocols for future lots of etranacogene dezaparvovec-drlb to the Center for Biologics Evaluation and Research (CBER) for release by the Director, CBER, under 21 CFR 610.2(a). We will continue to monitor compliance with 21 CFR 610.1 requiring completion of tests for conformity with standards applicable to each product prior to release of each lot**.**

Genetically Modified Patient Cells The FDA pre-release requirement is currently being waived on a product-by-product basis.

> **CARVYKTI, Ciltacabtagene Autoleucel – February 28, 2022** [19]
> FDA LOT RELEASE
> You are not currently required to submit samples or protocols of future lots of ciltacabtagene autoleucel to the Center for Biologics Evaluation and Research (CBER) for release by the Director, CBER, under 21 CFR 610.2(a). We will continue to monitor compliance with 21 CFR 610.1 requiring completion of tests for conformity with standards applicable to each product prior to release of each lot.

From internal FDA documents, an understanding of where the FDA stands on this pre-release requirement for all viral vectors can be found. An internal FDA team discussion occurred April 10, 2019 over the need to have this pre-release requirement for the ZOLGENSMA (onasemnogene abeparvovec-xioi) *in vivo* gene therapy. A summary of that internal team meeting discussion has been placed on the FDA website, and provides background on FDA's reasoning to continue to require the pre-release for the *in vivo* gene therapy-based biopharmaceuticals [20]:

> Andrew Byrnes explained DCGT's preference for quarterly surveillance instead of lot release due to the large number of lots (approximately 1 per week) and the risk to commercial supply that could be caused by delays in release. Andrew explained that given the relatively short shelf life (effectively only 8 months), routine lot release could delay distribution of the product.
>
> Jay Eltermann expressed that all products are subject to lot release, but case by case exemptions have been granted, e.g., CAR-T cells. Jay explained that this product has attributes that support the need for routine lot release - it is not a patient specific product, it is a novel product from a manufacturer with little experience, and there appear to be testing issues. It therefore cannot be under surveillance. AveXis will need to establish an acceptable lot release history (longer than 5 years), accumulate stability data, and demonstrate the manufacturing process is well controlled before submitting a supplement to request surveillance as an alternative to routine lot release.
>
> Maryna Eichelberger explained that lot release would give CBER confidence with the product, and regardless if the protocols are electronic or paper, they come to DPMQ/PRB. They are reviewed by the Product Office (PO) and DBSQC reviewers. Paper protocols are physically routed to sequential reviewers and therefore if paper protocols are submitted, it could delay the release. AveXis could send electronic protocols after BLA approval. The Testing Plan (TP), a CBER internal document, determines the LRS routing. There are no PDUFA time lines for lot release. However, the Lot Release Branch (LRB) is

> committed to releasing lots within 30 business days of protocol receipt. Jay mentioned that LRS captures tests which are released, but no test data is captured in LRS.
>
> Maryna suggested that OTAT decide if additional information regarding lot release tests need to be included in the Lot release protocol. Andrew mentioned some additional information may be useful like chromatograms. Jay remarked that in the future if the product were to be considered for surveillance, this additional data may be helpful in the decision making.

Also, as noted above, commercial genetically modified patient cells biopharmaceuticals are being waived from the mandatory pre-release batch requirement. This waiver for the *ex vivo* gene therapy-based biopharmaceuticals is on a case-by-case basis, most likely because the current commercial batches of these products have autologous use (i.e., the cells taken from a patient, after genetic engineering transduction, are administered back to only the same patient). Should allogeneic (i.e., the cells taken from a donor, after genetic engineering transduction, are administered back to multiple patients) *ex vivo* gene therapy-based biopharmaceuticals receive market-approval, the FDA may impose the pre-release requirement on them.

2.2.4.2 21 CFR 610.14 Identity Testing of Commercial Biologic Drug Product After Labeling

The PHS Act of 1944 originally contained additional release testing requirements for commercial biologicals that were not required for commercial chemical drugs under the FD&C Act. Over the years, the FDA has eliminated these extra release testing requirements, except for one – the identity test after labeling of finished drug product stated in 21 CFR Part 610.14.

21 CFR Part 610.14 is not the confirmation of identity that is required for all drug substance and drug product release, covered by both the FD&C Act and the PHS Act, as part of batch release. Also, 21 CFR Part 610.14 is not the confirmation of label identity that is required for all drug product labeling operations. Strict labeling control of the drug product operation is mandatory under cGMPs, with the requirement of a careful examination of the labels to confirm identity and conformity to the labeling specified in the batch production records, and to perform a label accountability. These two identity controls described above – identity test for release of drug product batch and label control – are acceptable for batch release for commercial chemical drugs under the FD&C Act. However, in the PHS Act, there is an extra identity test requirement for releasing commercial biological products – 21 CFR Part 610.14 [21]:

> The contents of a final container of each filling of each lot shall be tested for identity after all labeling operations shall have been completed. The identity test shall be specific for each product in a manner that will adequately identify it as the product designated on final container and package labels and circulars, and distinguish it from any other product being processed in the same laboratory. Identity may be established either through the physical or chemical characteristics of the product, inspection by macroscopic or microscopic methods, specific cultural tests, or in vitro or in vivo immunological tests.

This identity test involves collecting a single labeled finished drug product unit (i.e., vial, syringe) and performing an identity test on the content inside that labeled unit. This identity test does not need to be highly specific of the product, just specific enough to confirm that the content in the labeled unit is the intended product listed on the label and not another product present in the manufacturing area that could have been mixed up with the intended product. Simple identity assays such as immunological dot blots (antibody reagents specific for the intended protein), IEF peak profiles, or HPLC peak profiles can suffice.

Since it is the law, this extra identity test is a legal requirement to obtain and maintain FDA market approval for all biologics, which includes all biopharmaceuticals. Surprisingly, the FDA continues to have to remind new manufacturers seeking market approval of their biopharmaceuticals of this legal requirement:

Trogarzo (Ibalizumab-uiyk) Monoclonal Antibody – During FDA Mid-Cycle BLA Meeting (August 18, 2017) [22]

The BLA submission does not contain information regarding identity testing of labeled ibalizumab drug product vials. 21 CFR 610.14 requires that identity testing be performed on each filled DP lot after all labeling operations have been completed. The identity test method for the labeled drug product should be appropriately validated for its intended use. Update your BLA with the following information: • a description of the identity test method for the labelled drug product • appropriate method validation, or if applicable, method transfer data • revise FDA-356h form to include testing facility information • revise Section 3.2.P.3.1 of Module 3 to include the testing facility information.

Idacio (Adalimumab-aacf) Monoclonal Antibody – Stated in Market Approval Letter (December 2022) [23]

We remind you of your postmarketing commitments: To implement identity test(s) for final MSB11022 drug product assembled in prefilled syringe with the autoinjector devices after labeling and secondary packaging per 21 CFR 610.14. The final identity test and supporting information will be submitted to the BLA per 21 CFR 601.12. Final report submission: June 2023.

Zolgensma (Onasemnogene Abeparvovec-xioi) Recombinant AAV Viral Vector – During FDA Late-Cycle BLA Meeting (March 18, 2019) [24]

On February 6, 2019, you informed FDA inspectors that a single DP lot may be for … different markets. FDA inspectors informed you that each lot … of DP intended for the US market must be tested for identity after completion of labeling operations, to comply with 21 CFR 610.14. Please confirm that you will perform identity testing in this manner. Please submit to the BLA an updated labeling MBR.

Discussion: FDA noted that identity testing should be performed on all lots and … after labeling. The applicant stated that they will provide the requested information.

2.2.4.3 'Bioqualifier' Suffix Added to Assigned INN for Commercial Biologics

The FDA has determined that there is a need for biological products licensed under the PHS Act to bear not only the expected international nonproprietary name (INN) but in addition a FDA-designated 4-letter suffix, referred to as a 'bioqualifier'. The

Table 2.8 Added bioqualifiers for some market-approved biopharmaceuticals

Biopharmaceutical Type	Commercial Biopharmaceutical Product		
	Brand Name	International Nonproprietary Name (INN)	Added Bioqualifier
Recombinant Protein	Palynziq	pegvaliase	-pqpz
Monoclonal Antibody	Enspryng	satralizumab	-mwge
Antibody-Drug Conjugate	Zynlonta	loncastuximab tesirine	-lpyl
Biosimilar mAb	Yusimry	adalimumab	-aqvh
	Hulio		-fkjp
	Hadlima		-bwwd
In Vivo AAV Viral Vector	Zolgensma	onasemnogene abeparvovec	-xioi
Genetically Modified Patient Cells	Breyanzi	lisocabtagene maraleucel	

suffix selected is to be devoid of meaning and composed of four lowercase letters. FDA intends to apply the bioqualifier to new biologics approved for market, and eventually will be applied retrospectively to previous market approval biologics. The FDA's justification for doing this is summarized below [25]:

> The Agency considers appropriate pharmacovigilance fundamentally important for biological products. Although safety of biological products is rigorously assessed before approval, safety issues that are specific to a manufacturer may arise after approval with any marketed product. To help ensure patient safety and allow the Agency and the manufacturer to swiftly identify and address a problem, FDA aims to track adverse events to a specific manufacturer (and as appropriate, to a lot or manufacturing site for a particular biological product) and allow surveillance systems to detect safety signals throughout the life cycle of a product. Identifying a biological product's manufacturer can help target remedial action (including recall) to avoid implicating a broader set of products for which no such problem exists.\ Nonproprietary names that include distinguishing suffixes can serve as a key element to identify specific products in spontaneous adverse event reporting and to reinforce accurate product identification in billing and claims records used for active pharmacovigilance. Other product specific identifiers, such as proprietary names or NDCs, may not be available or could change over time. A distinguishing suffix will also support the tracking of product-specific events over time, thereby enhancing the accurate attribution of product-specific adverse event reports.

FDA requests that the manufacturer (sponsor) submit to them up to 10 different bioqualifier suffixes of four lowercase letters either during the IND clinical stage or in the BLA submission.

Bioqualifiers are not assigned to any FD&C Act chemical drug or generic chemical drug. It is also most interesting that no other regulatory authority around the world is assigning a bioqualifier to any of their market-approved pharmaceuticals, including the EMA. Table 2.8 presents the added bioqualifiers for some commercial

biopharmaceuticals [26]. Note, *ex vivo* genetically modified patient cells market-approved biopharmaceuticals have not received bioqualifiers. This is most likely due to this biopharmaceutical type currently being autologous, resulting in small patient-specific batches.

2.2.5 CDER, CBER and CDRH

The United States Food and Drug Administration currently consists of six 'Centers', of which, two Centers are primarily involved in the regulatory review and oversight of biopharmaceuticals: Center for Biologics Evaluation and Research (CBER) and Center for Drug Evaluation and Research (CDER). A third Center, the Center for Devices and Radiological Health (CDRH), enters into the review, typically for consultation, when the biopharmaceutical is part of a combination product (i.e., the product is a biologic combined with a device).

One would intuitively think that CBER, because of the word 'biologics' in its name, would be the Center responsible for all biopharmaceuticals regulated under the PHS Act, and that CDER would regulate only FD&C Act chemical drugs. But things started changing in the mid-2000's:

- In June 2003, FDA transferred the regulatory review and oversight of recombinant proteins, monoclonal antibodies and biosimilars from CBER to CDER. In CDER, these PHS Act biopharmaceuticals are referred to as 'therapeutic biological products' [27].
- In March 2020, FDA transferred 100+ protein hormones and enzymes, both natural sourced and recombinant proteins, from the FD&C Act over to the PHS Act, but kept the regulatory review under the control of CDER [3].

CDER CDER's approach to regulatory review and oversight of the protein-based biopharmaceuticals (recombinant proteins, monoclonal antibodies, and biosimilars) that they are responsible for is based on a multidiscipline review team managed by specific medical-oriented Offices within the Office of New Drugs (OND). Specific Offices associated with the intended medical application for the product (e.g., Office of Oncologic Diseases if biopharmaceutical is for treating cancer, Office of Immunology and Inflammation if biopharmaceutical is for treating autoimmunity, etc.). Support for the CMC review of PHS Act biopharmaceuticals within CDER is provided by the Office of Biotechnology Products (OBP) [28], and if pursuing a biosimilar, the Office of Therapeutic Biologics and Biosimilars (OTBB) [29].

CBER CBER's approach to regulatory review and oversight of the biopharmaceuticals that they are responsible for is now based in the Office of Therapeutic Products (OTP). OTP, previously called the Office of Tissues and Advanced Therapies (OTAT), contains the Office of Gene Therapy CMC (for the gene-based biopharmaceuticals – viral vectors and genetically modified patient cells), the Office of Cellular

Therapy and Human Tissues CMC, and the Office of Plasma Protein Therapeutics CMC (for recombinant proteins) [30].

CDRH CDRH provides consulting services to both CDER and CBER when a medical device is in combination with a biopharmaceutical. CDRH reviews the device requirements. Device concerns such as device cGMP manufacturing, human factor studies, stability studies for the functioning of the device through the shelf life, have to be addressed for market approval [31].

2.3 Navigating the European Union Regulation for Biopharmaceuticals

In the European Union (EU), pharmaceutical-related laws are initiated/amended by the European Commission and finally approved by the European Parliament (EP). If the law impacts how pharmaceuticals are to be regulated, the EMA has the responsibility of first interpreting the intent of the new/amended law and then implementing and enforcing it, as shown in Fig. 2.3.

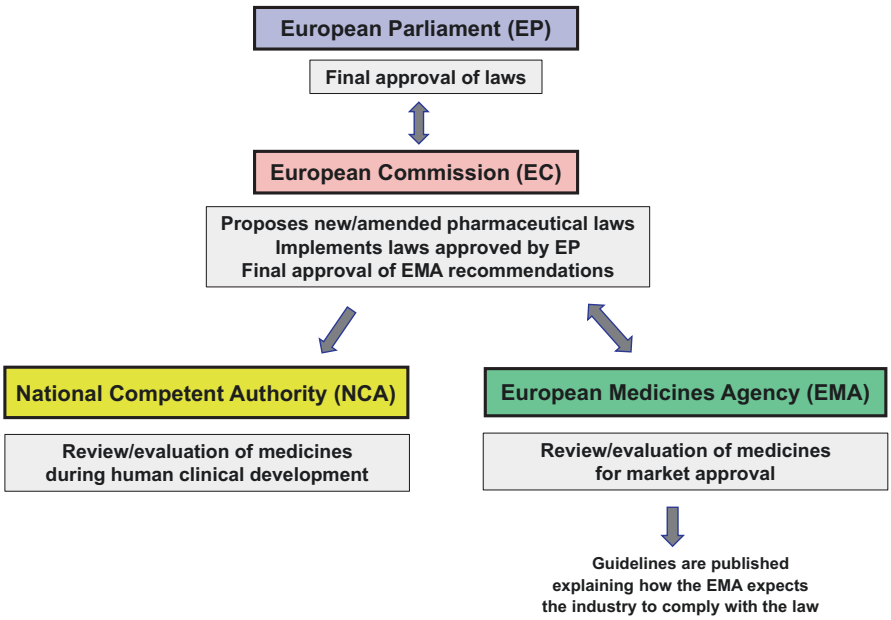

Fig. 2.3 The pharmaceutical regulatory system in the European Union

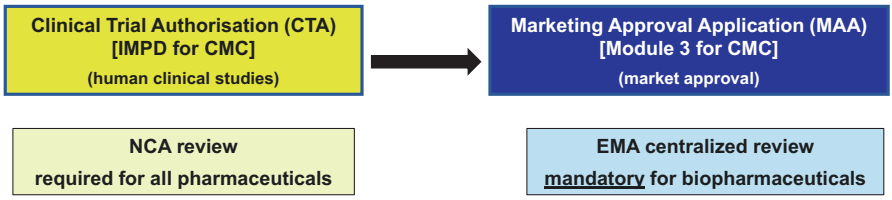

Fig. 2.4 Regulatory drug development pathway in the European Union

The EU pharmaceutical legal system requires individual Member States (i.e., National Competent Authorities, NCAs, in each country) to review and control pharmaceutical medicines used in human clinical studies; while for market approval, the centralized European Medicines Agency (EMA) has regulatory review and oversight of most pharmaceutical medicines. For all biopharmaceuticals, it is mandatory that they are under the centralized review of the EMA (see Fig. 2.4)

2.3.1 NCA Review and Approval During Clinical Development

To initiate a human clinical trial in the EU requires submission of a Clinical Trial Application (CTA) to a National Competent Authority (or Authorities), then wait for their review and approval to proceed. One element of the CTA is the Investigational Medicinal Product Dossier (IMPD) which contains the required CMC documentation. The CMC content to be submitted in the IMPD [32] follows the general outline listed in ICH M4Q(R1) – The Common Technical Document for the Registration of Pharmaceuticals for Human Use: Quality (see Tables 2.6 and 2.7).

Prior to 2022, investigational medicinal product studies were under Directive 2001/20/EC. A 'directive' is an EU 'legislative act that sets out overall goals and objectives.' The directive allowed individual Members States to adapt the act into their legal system, as appropriate. Thus, unlike the United States system where a single IND is submitted to the FDA for a clinical study to be conducted anywhere in the 50 states, the EU system required the CTA/IMPD to be submitted individually to each Member State that a clinical study was intended to be carried out. Thus, each country was not aware of the requests made by the other countries to initiate a clinical trial. From a CMC perspective, this led occasionally to differing and sometimes conflicting requirements imposed on the manufacture and testing of the clinical products.

Beginning in January 2023, investigational medicinal product studies are now under Regulation 536/2014. A 'regulation' is an EU legislative act that is binding across the entire European Union region. The new Clinical Trials legislation has taken the legal form of a Regulation and will replace national law. This helps ensure that the rules for assessing clinical trial applications and for conducting

clinical trials are identical throughout the EU; which is vital to ensure that Member States, in authorizing and supervising the conduct of a clinical trial, base themselves on the same rules. Some of the main advantages of the new Regulation are [33]:

- A streamlined application procedure via a single-entry point – an EU portal and database, for all clinical trials conducted in EEA (registration via the portal will be a prerequisite for the assessment of any application)
- A single authorization procedure for all clinical trials, allowing a faster and thorough assessment of an application by all Member States concerned, and ensuring one single assessment outcome and authorization per Member State
- Strictly defined deadlines for the assessment of clinical trial application;
- Simplified reporting procedures which will spare sponsors from submitting broadly identical information separately to various bodies and different Member States

2.3.2 EMA Review and Approval at Market Approval

In the European Union, there are two ways of obtaining market approval: either (1) the centralized procedure involving the EMA, or (2) the national authorization procedure involving the NCAs of the Member States. However, for biopharmaceuticals, the centralized procedure is compulsory [34]:

- medicines derived from biotechnology processes, such as genetic engineering (recombinant proteins, monoclonal antibodies, biosimilars)
- advanced-therapy medicines, such as gene-therapy, somatic cell-therapy or tissue-engineered medicines (viral vectors, genetically modified patient cells, mRNA non-viral vector)

The manufacturer submits a Market Authorisation Application (MAA) to EMA. The MAA is in the same eCTD format used for the IMPD submission. The main difference is in the level of CMC detail that is required – during clinical development being far less than that needed for market approval. Upon submission to the EMA, the Committee for Medicinal Products for Human Use (CHMP) carries out a scientific assessment of the application and then gives a recommendation on whether the medicine should be marketed or not. For review of submitted MAAs involving advanced therapy medicinal products (ATMPs), such as the gene therapy-based biopharmaceuticals, the CHMP utilizes the expertise of the Committee for Advanced Therapies (CAT) [35].

EMA submits its recommendation to the European Commission which makes a legally binding decision based on EMA's recommendation within 67 days of receipt. Once granted by the European Commission, the centralized marketing authorization is valid in all EU Member States as well as in the European Economic Area (EEA) countries Iceland, Liechtenstein and Norway.

Table 2.9 Comparison of handling of market-approved biopharmaceuticals

Difference in Handling of Market-Approved Biopharmaceuticals		
	FDA	**EMA**
Batch-to-Batch Regulatory Authority Pre-Release	**Yes (Section 2.2.4.1)**	**No**
Identity Test After Final Labeling of Drug Product	**Yes (Section 2.2.4.2)**	**No**
Addition of a 'Bioqualifier' to the suffix of the INN	**Yes (Section 2.2.4.3)**	**No**

2.3.3 CMC Regulation Differences Between EMA and FDA for Biopharmaceuticals

During clinical development, both EMA and FDA have comparable requirements and expectations, for the most part, for the amount and type of CMC information needed for their review to allow human clinical studies to proceed. One area of difference is in the expectation for the amount of stability data necessary to initiate the clinical study:

1. EMA requires a proposed shelf life to be assigned to biopharmaceutical batches used in the clinical studies [32].
2. FDA does not require a shelf life assignment in the CMC information provided to them [36], but instead expects the Quality Unit within the company to monitor on-going stability studies within its Pharmaceutical Quality System (PQS).

A comparison between EMA and FDA, on how market-approved biopharmaceuticals are handled, is presented in Table 2.9. Aside from these three major differences, the CMC requirements and expectations for obtaining market approval of biopharmaceuticals are comparable between EMA and the FDA. Much thanks goes to the efforts of the International Council of Harmonisation (ICH) in bringing the USA, EU and Japanese regulatory authorities together to reach consensus guidelines for the CMC of biopharmaceuticals.

2.4 Embrace the CMC Regulatory Compliance Complexity

The world beyond the USA and EU is a large place, and biopharmaceutical companies operate across many geographical areas. But, keeping track of each country's or region's CMC regulatory compliance review procedures and oversite for

biopharmaceuticals is challenging. Therefore, it is important to continually check the information available on the appropriate country-specific regulatory authority website. The names of some country-specific regulatory authorities are listed below:

Australia – Therapeutic Goods Administration (TGA)
Canada – Health Canada (HC)
China – National Medical Products Administration (NMPA)
Brazil – National Health Surveillance Agency (ANVISA)
India – Central Drugs Standard Control Organization (CDSCO)
Japan – Pharmaceuticals and Medical Devices Agency (PMDA)

Other resources can also be helpful, such as the Regulatory Affairs Professional Society (RAPS, www. RAPS.org), which publishes numerous articles and books available to its members on regulatory compliance inside, as well as outside, the USA and EU regions.

The United Kingdom (UK) deserves a comment. UK completed its exit from the European Union in December 2020. As a result, the UK regulatory authority, Medicines & Healthcare Products Regulatory Agency (MHRA), uncoupled from the centralized procedures of the EMA. Fortunately, to date, the UK regulatory review system still closely follows the EMA system. Initiation of a clinical study requires the filing of the CTA along with the IMPD for CMC documentation. Filing for market approval requires the filing of a MAA [37].

The chapters in this book will address the 'embracing' of risk. A risk-based approach is necessary for biopharmaceuticals. The goal is to establish an acceptable CMC regulatory compliance in whatever stage the biopharmaceutical is in, during clinical development and post-market-approval. The risk-based process is straightforward:

> Identify Risks → Evaluate Level of Each Risk → Lean Into (Prioritize) the Risks → Develop an Effective Plan of Action to Mitigate/Eliminate the Highest Risks → Monitor against plan and modify as necessary

The risk-based approach described in this book is phrased: 'Minimum CMC Regulatory Compliance Strategy Continuum'; which has two primary stages: early clinical (e.g., First-in-Human) and late clinical (e.g., pivotal).

[Others use the risk-based phrase 'Clinical Phase-Appropriate', but great care is necessary in identifying the exact clinical phase with this phrase: e.g., some manufacturers have a traditional Phase 1, Phase 2, Phase 3 – while others have hybrid Phases – e.g., Phase 1/2; some manufacturers have a traditional Phase 3 pivotal – while others complete Phase 3 after market approval].

Abandon the urge to simplify everything and embrace the CMC complexity of biopharmaceutical regulation. Regulatory affairs professionals play a pivotal role in educating their respective senior management and company staff to the CMC regulatory compliance differences between the various pharmaceutical laws and the regulatory authorities that execute them. Especially for biopharmaceuticals, regulatory affairs professionals are the navigational guides through the CMC regulatory labyrinth.

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35. EMA website: Committees – Committee for Advanced Therapies (CAT); www.ema.europa.eu/en/committees/committee-advanced-therapies-cat
36. FDA CFR Code of Federal Regulations: Title 21 Part 211.137(g) Current Good Manufacturing Practice for Finished Pharmaceuticals, Subpart G – Packaging and Label Control – Expiration Dating; https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm?fr=211.137
37. UK Medicines & Healthcare Products Regulatory Agency (MHRA) website; www.gov.uk/government/organisations/medicines-and-healthcare-products-regulatory-agency

Chapter 3
Differences in CMC Regulatory Compliance: Biopharmaceuticals Versus Chemical Drugs

Abstract Regulatory compliance is essential for patient protection, and it applies both to Clinical as well as to Chemistry, Manufacturing & Controls (CMC). For CMC, there is no 'one size fits all' approach to regulatory compliance for all pharmaceutical types. Biopharmaceuticals are definitely different from chemical drugs. And among the different biopharmaceutical types there are major differences. This is not a perception, but a reality, accepted by regulatory authorities. In this chapter, four pharmaceutical types will be compared: (1) chemical drugs, (2) protein-based biopharmaceuticals – recombinant proteins and monoclonal antibodies, (3) viral vector biopharmaceuticals, and (4) genetically modified patient cells biopharmaceuticals. Four major CMC regulatory compliance areas will be examined: (1) non-living versus living source material, (2) impact of the manufacturing process on product consistency, (3) complexity of the manufactured product, and (4) biosimilars are not bio-generics. These differences emphasize the need for a risk-base approach toward CMC regulatory compliance to protect patients.

Keywords Chemical · Biopharmaceuticals · Protein-based · Vector · Cells · Plasmids · Biosimilars · Generics

3.1 Regulatory Authorities Agree

Regulatory compliance is essential for patient protection, and it applies both to Clinical (i.e., human clinical studies) as well as to Chemistry, Manufacturing & Controls (CMC). CMC encompasses all of the following:

Chemistry: starting materials, drug substance, and drug product

characterization
impurity profiles
Critical Quality Attributes (CQAs)
release criteria
stability profile

J. Geigert, *The Challenge of CMC Regulatory Compliance for Biopharmaceuticals*, https://doi.org/10.1007/978-3-031-31909-9_3

Manufacturing: the process from starting material(s) through final drug product

facility and utilities
raw materials and starting materials
process design and operation of the upstream and downstream drug substance process
process design and operation of the formulation and filling drug product process
Critical Process Parameters (CPPs)

Control: applied Pharmaceutical Quality System (PQS)

documentation issuance, review, and control
Quality Unit oversight
cGMP compliance
internal auditing and external vendor auditing
Control Strategy (CS)

'CMC Regulatory Compliance' is accomplished through a strategy that ensures that all regulatory authority requirements and expectations for CMC are achieved. For CMC, there is no 'one size fits all' strategy to regulatory compliance for all pharmaceuticals. The strongest argument that biopharmaceuticals have differences in their CMC regulatory compliance compared to the chemical drugs is from the statements made by the regulatory authorities themselves. In the eyes of regulatory authorities, biopharmaceuticals are definitely different from chemical drugs. This is not a perception, but a reality, and it is reflected by the statements on the FDA, EMA and ICH websites, and in the wording of the regulatory guidances that they issue. A glance at the statements on the respective regulatory authority websites readily shows this:

FDA [1]

How do biological products differ from conventional drugs? In contrast to most drugs that are chemically synthesized and their structure is known, most biologics are complex mixtures that are not easily identified or characterized. Biological products, including those manufactured by biotechnology, tend to be heat sensitive and susceptible to microbial contamination. Therefore, it is necessary to use aseptic principles from initial manufacturing steps, which is also in contrast to most conventional drugs.

EMA [2]

Biological medicines ('biologicals') contain active substances from a biological source, such as living cells or organisms … The manufacture of biological medicines tends to be more complex than for chemically-derived molecules. Most biological medicines are made by biotechnology, often using sophisticated cell systems and recombinant DNA technology … Compared with small chemical substances, biological medicines consist of large and often complex molecular structures. Sophisticated analytical methods (e.g., peptide mapping, mass spectrometry and assays in cells) are used to study their physicochemical and functional properties such as molecular structure, protein modifications and biological activity. Biological medicines are made by living organisms, which are naturally variable. Thus, the active substance in the final biological medicine can have an inherent degree of minor variability ('microheterogeneity'). This minor variability must fall within the acceptable range to ensure consistent safety and efficacy. This is done by adjusting the manufacturing process to guarantee that the active substance fits

into the desired specifications range. This degree of minor variability can be present within or between batches of the same biological medicine, particularly when manufacturing processes are modified during the commercial life of the medicine (e.g., increasing production scale).

While the International Council on Harmonisation (ICH) is not a regulatory authority, they do issue 'consensus' guidelines that are accepted by the U.S. FDA, EU EMA, Japan PMDA, and many other regulatory authorities. As ICH developed these guidelines, they had to face the reality of the CMC regulatory compliance differences between biopharmaceuticals (i.e., recombinant proteins and monoclonal antibodies) and chemical drugs. The ICH consensus guideline on 'Specifications: Test Procedures and Acceptance Criteria' consists of two documents – one for chemical drugs (ICH Q6A) and one for biopharmaceuticals (ICH Q6B):

ICH Q6A [3]

This guideline may be applicable to synthetic and semi-synthetic antibiotics and synthetic peptides of low molecular weight; however, it is not sufficient to adequately describe specifications of higher molecular weight peptides and polypeptides, and biotechnological/biological products.

ICH Q6B [4]

The principles adopted and explained in this document apply to proteins and polypeptides, their derivatives, and products of which they are components (e.g., conjugates). These proteins and polypeptides are produced from recombinant or nonrecombinant cell-culture expression systems and can be highly purified and characterized using an appropriate set of analytical procedures. The principles outlined in this document may also apply to other product types such as proteins and polypeptides isolated from tissues and body fluids. To determine applicability, manufacturers should consult with the appropriate regulatory authorities. This document does not cover antibiotics, synthetic peptides and polypeptides, heparins, vitamins, cell metabolites, DNA products, allergenic extracts, conventional vaccines, cells, whole blood, and cellular blood components. A separate ICH Guideline, "Specifications: Test Procedures and Acceptance Criteria for New Drugs Substances and New Drug Products: Chemical Substances" addresses specifications, and other criteria for chemical substances.

In addition, the ICH consensus guideline on 'Stability Testing' also consists of two documents – one for chemical drugs (ICH Q1A(R2)) and one for biopharmaceuticals (ICH Q5C):

ICH Q1A(R2) [5]

The guidance addresses the information to be submitted in registration applications for new molecular entities and associated drug products … Further guidance on new dosage forms and on biotechnological/biological products can be found in ICH guidances Q1C and Q5C, respectively.

ICH Q5C [6]

The guidance stated in this annex applies to well-characterised proteins and polypeptides, their derivatives and products of which they are components, and which are isolated from tissues, body fluids, cell cultures, or produced using rDNA technology. Thus, the document covers the generation and submission of stability data for products such as cytokines (interferons, interleukins, colony stimulating factors, tumour necrosis factors), erythropoietins, plasminogen activators, blood plasma factors, growth hormones and growth factors, insulins, monoclonal antibodies, and vaccines consisting of well-characterised proteins or polypeptides. In addition, the guidance outlined in the following sections may apply to other types of products, such as conventional vaccines, after

> consultation with the appropriate regulatory authorities. The document does not cover antibiotics, allergenic extracts, heparins, vitamins, whole blood, or cellular blood components.

Notice that the two ICH biopharmaceutical-specific guidelines above address only the protein-based biopharmaceuticals (recombinant proteins and monoclonal antibodies). Consideration was given to developing guidelines for the gene therapy-based biopharmaceuticals (viral vectors, genetically modified patient cells, mRNA non-viral vectors) but because of limited resources, ICH abandoned the effort in 2011 [7].

3.2 Four Major CMC Regulatory Compliance Differences

The regulatory authorities state clearly that biopharmaceuticals are not like chemical drugs. Because of these differences, regulatory authorities review and regulate the CMC regulatory compliance of each product type differently. The following four major CMC regulatory compliance differences gives an appreciation of why regulatory authorities manage the biopharmaceuticals so differently than chemical drugs, and will be examined in the following sections:

Section 3.2.1 Difference: due to type of starting material
Section 3.2.2 Difference: due to inconsistency of manufactured product
Section 3.2.3 Difference: due to complexity of molecular structure
Section 3.2.4 Difference: biosimilars are not 'bio-generics'

Furthermore, there are differences between the protein-based biopharmaceuticals (recombinant proteins and monoclonal antibodies) and the gene therapy-based biopharmaceuticals (viral vectors, genetically modified patient cells) – which also leads to some differences in CMC regulatory compliance.

To illustrate all of these differences in CMC regulatory compliance, a comparison between four product categories will be made: (1) chemical drug, (2) protein-based biopharmaceutical (recombinant proteins, monoclonal antibodies), (3) vector-based biopharmaceutical (viral vectors), and (4) genetically modified patient cells biopharmaceutical. Note, comments about non-viral vectors (e.g., mRNA encapsulated in lipids) will also be made.

3.2.1 Difference: Due to Type of Starting Material

Chemical drugs are typically synthesized using non-living reagents, with reactions run under harsh environments (e.g., high temperatures, high pressures, organic solvents, metal catalysts). On the other hand, biopharmaceuticals are dependent upon living systems for their biosynthesis, which require mild temperatures and aqueous environments. Protein-based biopharmaceuticals (recombinant proteins and

monoclonal antibodies) are biosynthesized by living genetically modified microorganisms (e.g., bacteria, yeast, insect, mammalian, human cells). Viral vector-based biopharmaceuticals are infectious viruses that are biosynthesized typically from human cells transfected with DNA plasmids that were expressed by recombinant *E. coli* cells. Genetically modified patient cell biopharmaceuticals are living cells that are transduced by a viral vector (typically lentivirus) which are biosynthesized typically from human cells transfected with DNA plasmids that were expressed by recombinant *E. coli* cells. Figure 3.1 summarizes these main differences in starting materials across the four product types.

The significant differences in the use of non-living versus living starting materials across the four product categories highlight the need for differences in their CMC regulatory compliance. A non-viral vector-based biopharmaceuticals being pursued in clinical development is mRNA encapsulated with lipids for protection. mRNA is a large biomolecule biosynthesized by *in vitro* transcription cell-free enzymatic reaction of a linearized DNA plasmid that was expressed by recombinant *E. coli* cells, so it is in the same category box as the viral vector biopharmaceuticals.

The use of living organism starting materials in the biosynthesis adds in extra requirements and controls. Living systems must maintain their viability to be functional. Living systems require nutrition, oxygen, and a habitable environment to carry out their biosynthetic task. The habitable environment allows the living organism to thrive, but also can allow adventitious agent contaminants to thrive. Therefore, the CMC regulatory compliance focus for the biopharmaceuticals must adequately address the following three concerns of all living organisms: (1) keep 'alive', (2) keep 'happy', (3) keep 'healthy'.

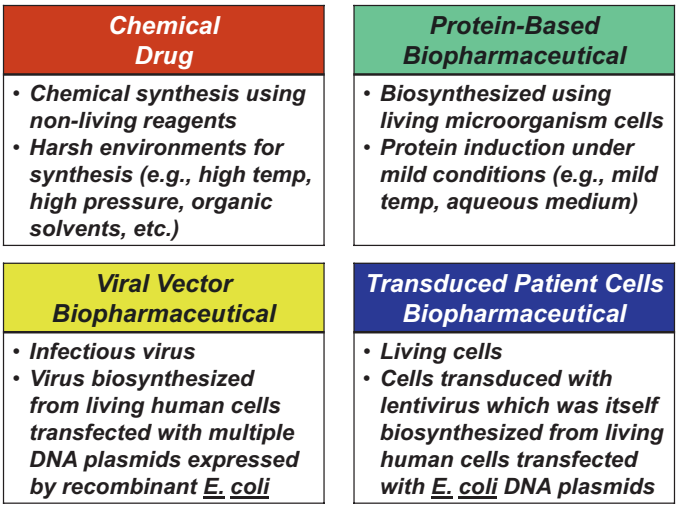

Fig. 3.1 Difference due to type of starting material across the four product types

3.2.1.1 Keep 'Alive'

Living organisms must be kept 'alive'. Around the clock, 24/7, for as long as needed to either biosynthesize the product (e.g., a monoclonal antibody) or to be the product (e.g., a viral vector). Dead living systems cannot produce or be the desired biopharmaceutical.

Today, there is an abundance of choice in living system starting materials for producing protein-based biopharmaceuticals: bacterial cells (e.g., *Escherichia*), yeast cells (e.g., *Saccharomyces*), plant cells (e.g., carrot root), insect cells (e.g., *Spodoptera*), animal cells (e.g., CHO), and human cells (e.g., HEK293). Today, there is also choice in living system starting materials for production of viral vector-based biopharmaceuticals: bacterial cells (e.g., *Escherichia coli*) for producing DNA plasmids, and insect cells (e.g., baculovirus infected *Spodoptera)* for producing viral seeds. And we must not forget the choices in the patient's own cell starting material for viral vector transduction, which today are T cells and stem cells, but tomorrow could be NK cells, etc.

Whatever is the living organism starting material linked to production of the biopharmaceutical. it will eventually lose its power to divide and to grow (i.e., undergo senescence), and die. So, in order to slow this process down, living cells are typically stored frozen in the liquid nitrogen vapor phase. The cells are not dead, just hibernating. But the freezing of cells and the subsequent thawing of the frozen cells can be challenging. Usually a slow, controlled freezing cycle is used (to minimize ice crystal formation from harming the cells) for putting the cells into hibernation. Then a fast thawing cycle is used to bring the frozen cells up to unfrozen conditions. Since cell death (i.e., loss of viability) can occur even in the frozen state, it is most important that the cells are monitored periodically for any loss in viability. Further details on the CMC regulatory compliance controls for cell-based starting materials are discussed in Chap. 6.

3.2.1.2 Keep 'Happy'

Living organisms must be kept 'happy'. Apologies for the non-technical term used – 'happy' – but it is used to emphasize the importance of the manufacturing process design. It is desired that the living organism over-produce the desired biopharmaceutical be it a monoclonal antibody or a transgene-carrying adeno-associated virus. But for that to happen, the living organism requires adequate nutrients, a friendly oxygen/carbon dioxide gas environment, pH balance, correct temperature control, etc. Considerable studies are carried out to evaluate the impact of numerous process parameters on cell metabolism, biopharmaceutical induction and post-translational impacts. Process development scientists go to great care into designing the production process to ensure that the living system is optimized for overproduction of the desired biopharmaceutical. Details on the extensive studies that process engineers carry out for the upstream cell culture process of biopharmaceutical manufacturing are discussed further in Chap. 7.

3.2.1.3 Keep 'Healthy'

Living organisms must be kept 'healthy'. Adventitious agents (i.e., bacteria, fungi, mycoplasmas, viruses, and transmissible spongiform encephalopathy agents) abound in the environment and in the raw materials and components used in manufacturing. It is critical that adventitious agents are not inadvertently exposed to the living organism involved in biopharmaceutical manufacturing. Once an adventitious agent infects a living production system, the manufacturing process and the biopharmaceutical produced have a serious safety problem. It is a nasty world outside in the environment, and multiple barriers (e.g., sterile handling and aseptic processing) must be erected to protect the living system from these adventitious agents during the entire production process. In contrast to biopharmaceutical production, the harsh environment of chemical synthesis (e.g., high temperatures, high pressures, organic solvents, etc.) tends to effectively destroy adventitious agents that might be present in those manufacturing processes. Further discussion of adventitious agents occurs in Chap. 5.

Since life generates life, it is important to know the heritage of the living organism being used in biopharmaceutical production. Cells (specifically insect, animal, and human cells), due to past exposures to viruses, may have a latent virus present in their genome, which may be transmitted vertically from one cell generation to the next. Upon stress of the living organism in the production process (e.g., due to cell aging, nutrient depletion, etc.), a latent viral contaminant can be shocked into activity, producing infectious particles. One of the best examples of a latent virus induction in a living system is with children exposed to varicella zoster virus (chickenpox). After suffering 1–2 weeks of misery, children recover from the initial virus infection, and forget about the event. However, the chickenpox virus lies dormant in ganglia nerve cells in their body. Chickenpox virus can then later in life (typically after the age of 50) re-awake and turn into infectious shingles, which is characterized by a rash of blisters that generally develop in a band on one side of the body and can cause severe pain that may last for weeks and, in some adults, for months or years after the episode. Details on the need to show the absence of latent viruses in cell lines by means of genetic stability studies are discussed in Chap. 7.

3.2.2 Difference: Due to Inconsistency of Manufactured Product

Chemical drug synthesis is typically considered highly consistent. Biopharmaceutical manufacture on the other hand, due to their linkage with a living system for production, yields a much more variable product, as shown in Fig. 3.2.

The use of a living organism in the manufacturing process adds, to varying degrees, the inherent variability encountered in the manufactured product. The

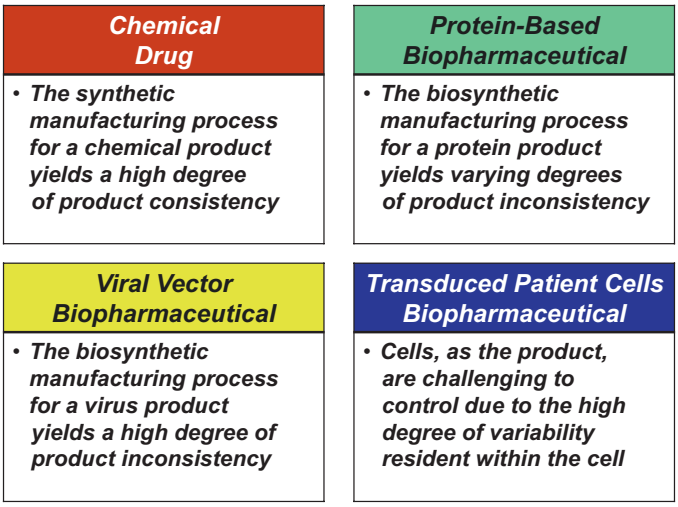

Fig. 3.2 Difference due to inconsistency of manufactured product across the four product types

following are comments from regulatory authorities on the inherent, and sometimes unintended sources of, variation in the product manufactured:

EMA (Recombinant Proteins and Monoclonal Antibody Process) [8]

The manufacture of biological medicinal products involves certain specific considerations arising from the nature of the products and the processes. The ways in which biological medicinal products are manufactured, controlled and administered make some particular precautions necessary. Unlike conventional medicinal products, which are manufactured using chemical and physical techniques capable of a high degree of consistency, the manufacture of biological medicinal substances and products involves biological processes and materials, such as cultivation of cells or extraction of material from living organisms. These biological processes may display inherent variability, so that the range and nature of by-products may be variable. As a result, quality risk management (QRM) principles are particularly important for this class of materials and should be used to develop their control strategy across all stages of manufacture so as to minimise variability and to reduce the opportunity for contamination and cross-contamination. Since materials and processing conditions used in cultivation processes are designed to provide conditions for the growth of specific cells and microorganisms, this provides extraneous microbial contaminants the opportunity to grow. In addition, many products are limited in their ability to withstand a wide range of purification techniques particularly those designed to inactivate or remove adventitious viral contaminants. The design of the processes, equipment, facilities, utilities, the conditions of preparation and addition of buffers and reagents, and training of the operators are key considerations to minimise such contamination events … Control usually involves biological analytical techniques, which typically have a greater variability than physico-chemical determinations. A robust manufacturing process is therefore crucial and in-process controls take on a particular importance in the manufacture of biological active substances and medicinal products.

EMA (Genetically Modified Patient Cells Process) [9]

The manufacturing risks may differ according to the type of product, nature/characteristics of the starting materials and level of complexity of the manufacturing process. The

> risk-based approach, according to the relevant ATMP guideline (EMA/CAT/CPWP/686637/2011), should be applied for the design of the manufacturing process in order to assess the criticality of the quality attributes and manufacturing process parameters and to increase the assurance of routinely producing batches of the intended quality. Unintended variability, for example in culture conditions, activation steps, transduction /transfection media and conditions or vector concentration/transduction efficiency/ Multiplicity of Infection (MOI) during production may result in quantitative and/or qualitative differences in the quality of the product or the impurities present.
>
> ***FDA (CAR T Cell Process)*** [10]
>
> CAR T cell manufacturing involves multiple biological materials and complex multi-step procedures, which are potential sources of variability among product lots. Thus, control of the manufacturing process and appropriate in-process and lot release testing are crucial to ensure CAR T cell safety, quality, and lot-to-lot consistency. In addition, changes to the manufacturing process are common during product development. It is essential to understand the effects of such changes on product quality. The same type of CAR T cells may be manufactured at several facilities. Multisite manufacturing may shorten the timeline from cellular starting material collection to administration for autologous products; however, differences between manufacturing facilities may contribute to product variability. Transduction efficiency can differ from lot to lot, resulting in variation in the percentage of transduced cells. This variation can lead to substantial differences in the active cell dose administered to different subjects, even when the same total cell dose is administered. Ideally, manufacturers should work to control variability in the transduction process. However, even with a consistent manufacturing process, such variations in transduction efficiency are expected to occur.

A non-viral vector-based biopharmaceuticals being pursued in clinical development is mRNA encapsulated with lipids for protection. mRNA is a large biomolecule biosynthesized by *in vitro* transcription cell-free enzymatic reaction of a linearized DNA plasmid that was expressed by recombinant *E. coli* cells, so the level of inconsistency in the produced product would be in the same category box as the viral vector biopharmaceuticals.

Ways of controlling the inherent variability impact of the manufacturing process on the produced biopharmaceutical will be further discussed in Chap. 7.

3.2.3 Difference: Due to Complexity of Molecular Structure

Chemical drug molecular structures can vary from the very simple (e.g., aspirin which is a chemically synthesized drug of 9 carbons and a molecular weight of only 180 daltons) to the somewhat complex (e.g., Spinraza (nusinersen) which is a chemically synthesized antisense oligonucleotide – also referred to as an ASO – 18 nucleotide residues, molecular formula of $C_{234}H_{323}N_{61}O_{128}P_{17}S_{17}Na_{17}$ and a molecular weight of 7501 daltons [11]. But the molecular structure complexity of a chemical drug comes nowhere near the complexity of a biosynthesized biopharmaceutical.

As shown in Fig. 3.3, the molecular complexity across the four product types increases: chemical drugs → protein-based biopharmaceuticals → vector-based biopharmaceuticals → genetically modified patient cell biopharmaceuticals.

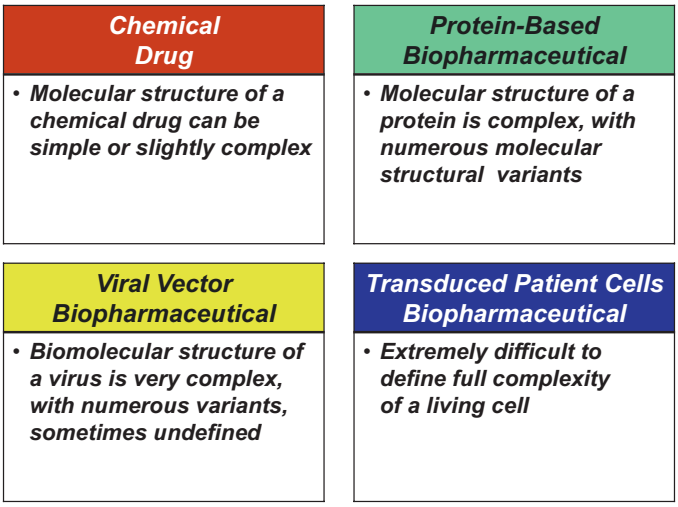

Fig. 3.3 Difference due to complexity of molecular structure across the four product types

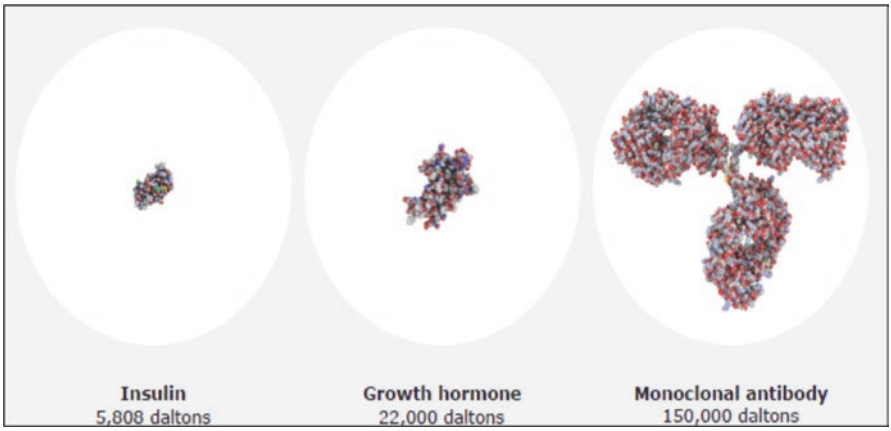

Fig. 3.4 Illustration of size of recombinant proteins and monoclonal antibodies

3.2.3.1 Molecular Structure Complexity of Protein-Based Biopharmaceuticals

Compared to the molecular structure of chemical drugs, recombinant proteins and monoclonal antibodies are much larger in size – ranging from small proteins (e.g., recombinant human insulin, 52 amino acids) to monoclonal antibodies (~1330 amino acids) [2], as shown in Fig. 3.4.

Compared to chemical drugs, recombinant proteins and monoclonal antibodies, not only are much larger in size, but also due to their amino acid chain structure, are much more complex. Amino acid chains are subject to change and degradation, and

are not 'rock solid'. Modifications can occur on both the N-terminus (e.g., glutamine cyclization to pyroglutamate) and the C-terminus (e.g., lysine truncation) of the polypeptide chain, as well as within the polypeptide chain (e.g., hydrolysis of peptide bond, oxidation of methionine, disulfide scrambling among cysteine, deamidation of glutamine). Glycan moieties (i.e., the carbohydrate moieties) that are attached to different sites on the protein chain introduce considerable heterogeneity with different types of monosaccharides linked in different sequences, length, and branching of carbohydrate chains. Furthermore, the higher order structure possibilities (e.g., from dimers to visible inherent protein aggregates) all adds to the complexity of recombinant proteins and monoclonal antibodies.

Taken together, by theoretically calculating all the possible variations that can occur to the amino acids and to the glycan moieties, it has been estimated that approximately 100 million possible molecular variants of a monoclonal antibody molecule could potentially occur [12]. These possible molecular variants cannot be taken lightly, since there are potential clinical safety concerns associated with them:

EMA [13]

Important factors influencing the immunogenicity of therapeutic proteins include the origin (e.g. foreign or human) and nature of the active substance (endogenous proteins, post-translational modifications), significant modifications of the therapeutic protein (e.g. pegylation and fusion proteins), product-related (e.g. degradation products, impurities, aggregates) and process-related impurities (host cell proteins, lipids or DNA, microbial contaminants), formulation (excipients) and the interactions between the drug and/or formulation with the primary product packaging (e.g. containers, closures)… Therapeutic protein analogues to human endogenous proteins may trigger an immune response due to variations in the amino acid sequence or changes to the protein structure compared to the endogenous protein as a result of post-translational modifications, or other changes during all steps of the drug substance and/or drug product manufacturing process, storage and administration… Glycosylation can influence both the physicochemical and biological properties of a protein. The presence or absence, as well as the structure of carbohydrate moieties may have both a direct or indirect impact on the immunogenicity of therapeutic proteins; the glycan can induce an immune response itself (e.g. glycans of non-human origin), or its presence may affect the conformation of the protein in such a way that the protein becomes immunogenic.

FDA [14]

Because therapeutic proteins are made in living systems, there may be heterogeneity in certain quality attributes of these products. Heterogeneity in therapeutic proteins may arise in a number of ways and may affect the expected clinical performance of a protein product. Replication errors in the DNA encoding the protein sequence and amino acid misincorporation may occur during translation, although the level of these errors is typically low. In addition, most protein products undergo posttranslational modifications that can alter the functions of the protein by attaching other biochemical groups such as phosphate and various lipids and carbohydrates; by proteolytic cleavage following translation; by changing the chemical nature of an amino acid (e.g., formylation); or by many other mechanisms. Such modifications can result from intracellular activities during cell culture or by deliberate modification of the protein (e.g., PEGylation). Other posttranslational modifications can be a consequence of manufacturing process operations; for example, glycation may occur with exposure of the product to reducing sugars. Also, certain storage conditions may be more or less permissive for certain degradation pathways such as oxidation, deamidation, or aggregation. All of these product-related variants may alter the biological properties of the expressed recombinant protein.

3.2.3.2 Molecular Structure Complexity of Vector-Based Biopharmaceuticals

Compared to the complex molecular structure of recombinant proteins and monoclonal antibodies, the molecular structure of the vector-based biopharmaceuticals are even more complex.

The non-viral vector, messenger ribonucleic acid (mRNA), for gene therapy, consists of a long, single-stranded chain of ribonucleic acids. An illustration of the increasing molecular structure complexity of mRNAs can come by looking at the mRNA used in the SARS-Cov-2 vaccines for COVID-19 – a mRNA of over 3800 nucleotides [15]. Using an average molecular weight of 320 Daltons for each mRNA nucleotide, 3800 nucleotides equal an approximate molecular weight of over one million Daltons.

The viral vector, recombinant live adeno-associated virus (rAAV) is the most common viral vector used for human *in vivo* gene therapy. The adeno-associated virus consists of a protein capsid (i.e., viral particle) which contains within a long single-stranded chain of DNA (deoxyribonucleic acid). An AAV protein capsid is approximately 20 nanometers in diameter (compared to about 10 nanometers for the size of a typical monoclonal antibody). Since the capsid is a protein, it is subject to all the molecular variations that impact recombinant proteins. An illustration of the further increase in molecular complexity of viruses can come by looking at the single strand of DNA within the capsid of Glybera (alipogene tiparvovec) – a DNA strand of about 3600 nucleotides [16]. Using an average molecular weight of 300 Daltons for each DNA nucleotide, 3600 nucleotides equal an approximate molecular weight of over one million Daltons.

3.2.3.3 Molecular Structure Complexity of Transduced Patient Cell Biopharmaceuticals

Genetically modified patient cell biopharmaceuticals are at the extreme end of complexity. The significantly increased size of human cells (e.g., T-cells are approximately 7 microns in diameter) compared to the size of a monoclonal antibody (about 0.01 microns) and their increased complexity (each human cell contains approximately 20,000 genes) becomes an extra challenge.

The major complexity of cell-based biopharmaceuticals has elevated the importance of addressing CMC regulatory compliance concerns early in the development of the manufacturing process. Former FDA Commissioner Dr. Scott Gottlieb speaking about the challenges associated with this group of biopharmaceuticals stated [17]:

> In contrast to traditional drug review, where 80 percent of the review is focused on the clinical portion of that process, and maybe 20 percent is focused on the product issues, I'd say that this general principal is almost completely inverted when it comes to cell and gene therapy. The initial clinical efficacy is often established early, and sometimes in small series of patients. It's the product questions that are more complex and uncertain. The more challenging questions relate to product manufacturing and quality, or questions like how much

you can change, or enlarge, the gene cassette that you load into a vector before the gene insert will change the conformation of the vector in ways that also fundamentally alter the entire product's safety or performance.

3.2.4 Difference: Biosimilars Are Not 'Bio-Generics'

There are generic chemical drugs, and there are biosimilar biopharmaceuticals, but there are no 'bio-generic' biopharmaceuticals. As shown in Fig. 3.5, the ability to unlink the manufacturing process from the produced product decreases: chemical drugs → protein-based biopharmaceuticals → vector-based biopharmaceuticals → genetically modified patient cell biopharmaceuticals.

3.2.4.1 Generic Chemical Drugs

Although chemical drug manufacturing has its challenges, for many chemical drugs, the manufacturing process and the chemical drug product can be unlinked, which forms the basis of generic chemical drugs. A generic chemical drug product is one that meets the requirement of being 'equivalent' to an innovator drug product in dosage form, strength, route of administration, quality, performance characteristics, and intended use.

The generic chemical drug manufacturer must meet the following core CMC regulatory compliance requirements [18]:

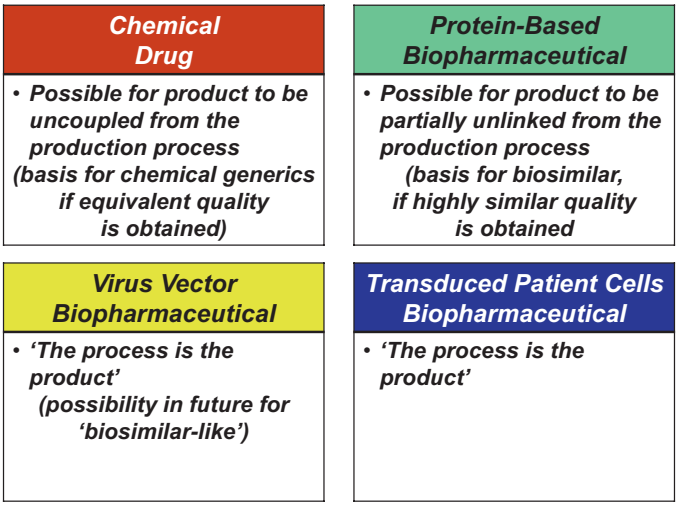

Fig. 3.5 Linkage between manufacturing process and product across the four product types

- *The manufacturer is capable of making the drug correctly***.** Often different companies are involved (such as one company manufacturing the active ingredient and another company manufacturing the finished drug). Generic drug manufacturers must produce batches of the drugs they want to market and provide information about the manufacturing of those batches for FDA to review.
- *The manufacturer is capable of making the drug consistently***.** Generic drug manufacturers must explain how they intend to manufacture the drug, and provide evidence that each step of the manufacturing process will produce the same result each time. FDA scientists review those procedures and FDA inspectors go to the generic drug manufacturer's facility to verify that the manufacturer is capable of making the drug consistently and to check that the information the manufacturer has submitted to FDA is accurate.
- *The "active ingredient" is the same as that of the brand***.** An active ingredient in a medicine is the component that makes it pharmaceutically active - effective against the illness or condition it is treating. Generic drug companies must provide evidence that shows that their active ingredient is the same as that of the brand-name drug they copy, and FDA must review that evidence.

From the CMC perspective, the way the manufacturer of a generic chemical drug demonstrates that the product is 'the same' as the innovator's chemical drug product is by meeting the quality standards listed in a published pharmacopeia quality monograph. For the United States, the United States Pharmacopeia (USP) – which is an independent, scientific organization – reviews, accepts and publishes quality standards for chemical drugs. These quality standards are legally recognized by the FDA [19]. For the European Union, the European Directorate for the Quality of Medicines & Healthcare (EDQM) – which is under the Council of Europe – reviews, accepts and publishes quality standards for chemical drugs. Conformity to a product quality monograph in the European Pharmacopeia (Ph.Eur.) is mandatory across the European Union [20]. Thus, for a generic chemical drug, the key CMC focus is on the quality of the drug product compared to the innovator chemical drug product, rather than comparison of the two manufacturing processes. Since a generic chemical drug is equivalent to the innovator's chemical drug, the two are interchangeable for patient administration.

3.2.4.2 Biosimilar Biopharmaceuticals

The comprehensive and comparative studies (CMC, Non-clinical and Clinical studies) needed to demonstrate 'highly similar' for a protein-based biopharmaceutical far exceed the limited comparative studies needed to demonstrate 'equivalence' for a generic chemical drug (i.e., must be chemically identical to the innovator drug and must demonstrate bioequivalence). The use of the term 'bio-generic' for biopharmaceuticals implies that not much effort is needed to establish highly similar. This leads manufacturers to assume that biosimilar manufacturing is as straightforward as generic chemical drug manufacturing. This viewpoint leads to confusion and discouragement; and the regulatory authorities have spoken out against the use of the term 'bio-generics':

FDA [21]

Are biosimilars the same as generic drugs? Biosimilars and generic drugs are versions of brand name drugs and may offer more affordable treatment options to patients. Biosimilars and generics are each approved through different abbreviated pathways that avoid duplicating costly clinical trials. But biosimilars are not generics, and there are important differences between biosimilars and generic drugs. For example, the active ingredients of generic drugs are the same as those of brand name drugs. In addition, the manufacturer of a generic drug must demonstrate that the generic is bioequivalent to the brand name drug. By contrast, biosimilar manufacturers must demonstrate that the biosimilar is highly similar to the reference product, except for minor differences in clinically inactive components. Biosimilar manufacturers must also demonstrate that there are no clinically meaningful differences between the biosimilar and the reference product in terms of safety and effectiveness

EMA [22]

Why biosimilars are not considered generic medicines. A biosimilar is not regarded as a generic of a biological medicine. This is mostly because the natural variability and more complex manufacturing of biological medicines do not allow an exact replication of the molecular microheterogeneity. Consequently, more studies are needed for regulatory approval of biosimilars than for generics to ensure that minor differences do not affect safety or efficacy.

World Health Organization (WHO) [23]

Biotherapeutic products (biotherapeutics) have a successful record in treating many life-threatening and chronic diseases. The expiry of patents and/or data protection periods for a number of such biotherapeutics has ushered in an era of products that are designed to be highly "similar" to the corresponding licensed "originator" product. Based on a comprehensive head-to-head comparison and demonstrated high similarity, such products can partly rely for their licensing on safety and efficacy data obtained for the originator products. A variety of terms have been used to describe these products, including "biosimilars", "similar biotherapeutic products", "similar biological medicinal products" and "biosimilar products". The term "generic medicine" is usually used to describe chemical, small-molecule medicinal products that are structurally identical to an originator product whose patent and/or data protection period has expired. Demonstration of the analytical sameness and bioequivalence of the generic medicine to a reference product is usually appropriate and sufficient proof of therapeutic equivalence between the two. However, the approach established for generic medicines is not suitable for the development, evaluation and licensing of relatively large and complex proteins such as biosimilars.

A generic chemical drug must meet the equivalency of the quality standard compared to the innovator chemical drug, but they are generally not required to include preclinical (animal) and clinical (human) data to establish safety and effectiveness. Instead, generic applicants must scientifically demonstrate that their product is bioequivalent (i.e., performs in the same manner as the innovator drug). A way scientists demonstrate bioequivalence is to measure the time it takes the generic drug to reach the bloodstream in a small set of healthy, volunteers. This gives them the rate of absorption, or bioavailability, of the generic chemical drug, which they can then compare to that of the innovator chemical drug. The generic version must deliver the same amount of active ingredients into a patient's bloodstream in the same amount of time as the innovator drug.

While the innovator protein-based biopharmaceutical manufacturer must carry out CMC, Non-Clinical and Clinical studies to statistically confirm efficacy and safety of the biopharmaceutical, the biosimilar biopharmaceutical manufacturer must carry out comparative CMC, Non-Clinical and Clinical studies, see Fig. 3.6

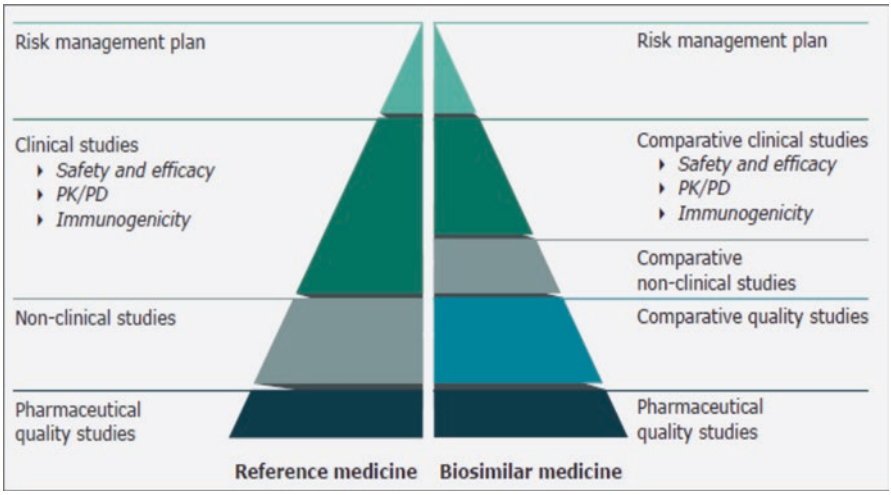

Fig. 3.6 Comparison of studies for market-approval – innovator (reference) versus biosimilar

(left side of the triangle is for the innovator manufacturer and the right side of the triangle is for the biosimilar manufacturer) [22]. For a biosimilar, the key foundation step is an extensive CMC comparative analysis, also referred to as comparative quality studies. This analysis is carried out between the biosimilar and the innovator's biopharmaceutical (also referred to as 'reference product' in USA or 'reference medicinal product' in EU).

Due to the depth of analytical/functional characterization tools available for recombinant proteins and monoclonal antibodies, and the number of batches to be compared (at least ten batches each of reference and the biosimilar), the CMC comparative analytical testing will be extensive. The market-approved biosimilar Aybintio (Samsung Bioepis biosimilar to the Genentech/Roche Avastin, bevacizumab) illustrates this [24]:

> A total of 46 EU-sourced Avastin lots have been purchased from the market and have been used for the similarity range establishment. A list including the exact lot number, strength and the expiry data of each single Avastin lot is provided. The expiry dates of the Avastin lots cover the period from February 2014 until September 2018 … The characterisation of the reference medicinal product and the subsequent side-by-side comparison, using 18 SB8 [Aybinto] lots and 9 Avastin lots, included a broad panel of standard and state-of-the-art methods which covered relevant physicochemical as well as biological quality attributes. In particular, quantity, primary structure (molecular weight, amino acid sequence, N- and C-terminal sequence, peptide mapping, methionine oxidation, deamidation, glycation), purity and impurities (SE-HPLC, reducing and non-reducing CE-SDS), charged variants (CEX-HPLC, icIEF), hydrophobic variants (HI-HPLC), carbohydrate structure (identification of the N-glycan site, N-glycan identification, N-glycan profile), and higher order structure (CD-, intrinsic, extrinsic, and Fourier Transform Infrared spectroscopy, Hydrogen/Deuterium exchange, differential scanning calorimetry, SE-HPLC/MALLS, analytical ultracentrifugation, dynamic light scattering, and micro-flow imaging) have been addressed. Regarding the biological characteristics cell-based potency assays, binding assays, and Fc related activities, and additional assays have been used.

The large number of manufactured batches is typical in these biosimilar CMC comparisons. Since the biosimilar manufacturer will have only limited access to the innovator's manufacturing process and active ingredient, the expectation is that the biosimilar manufacturer will collect commercial drug product samples from numerous released batches over an extended period of time (typically years) for this CMC comparative analysis. The FDA is very specific on this recommendation [14]:

> Reference Product. To ensure that the full range of product variability is accurately captured, sponsors should acquire multiple reference product lots throughout the development program of a proposed biosimilar in sufficient quantity to conduct multiple physiochemical and functional assays. Considering the inherent heterogeneity present in protein products and the expected lot-to-lot variability stemming from manufacturing processes, the Agency recommends that a sponsor include at least 10 reference product lots (acquired over a time frame that spans expiration dates of several years), in the analytical assessment to ensure that the variability of the reference product is captured adequately. The final number of lots should be sufficient to provide adequate information regarding the variability of the reference product. In cases where limited numbers of reference product lots are available (e.g., for certain orphan drugs), alternate flexible comparative analytical assessments plans should be proposed and discussed with the Agency.
>
> 1. Proposed Product. The Agency recommends that a sponsor include at least 6 to 10 lots of the proposed product in the comparative analytical assessment, to ensure 1) adequate characterization of the proposed product and understanding of manufacturing variability, and 2) adequate comparison to the reference product. These should include lots manufactured with the investigational- and commercial-scale processes, and may include validation lots, as well as product lots manufactured at different scales, including engineering lots. These lots should be representative of the intended commercial manufacturing process.

Even though extensive, the CMC comparison alone will not remove all residual uncertainty about whether the biosimilar is truly highly similar or not. Therefore, Non-clinical and Clinical comprehensive comparisons are also necessary for market approval of biosimilars.

While generic chemical drugs are automatically interchangeable, 'biosimilarity' is not always equivalent to 'interchangeability' across all regulatory regions. The following illustrates the contrast between the FDA and EMA on this issue:

> ***FDA*** [25]
>
> What is the difference between a biosimilar and an interchangeable product?
>
> As mentioned above, an interchangeable product, in addition to being biosimilar, meets additional requirements based on further evaluation and testing of the product. A manufacturer of a proposed interchangeable product will need to provide additional information to show that an interchangeable product is expected to produce the same clinical result as the reference product in any given patient. Also, for a product that is administered to a patient more than once, a manufacturer will need to provide data and information to evaluate the risk, in terms of safety and decreased efficacy, of alternating or switching between the products.
>
> As a result, a product approved as an interchangeable product means that FDA has concluded it may be substituted for the reference product without consulting the prescriber. For example, say a patient self-administers a biological product by injection to treat their rheumatoid arthritis. To receive the biosimilar instead of the reference product, the patient may need a prescription from a health care prescriber written specifically for that biosimilar. However, once a product is approved by FDA as interchangeable, the patient may be able

> to take a prescription for the reference product to the pharmacy and, depending on the state, the pharmacist could substitute the interchangeable product for the reference product without consulting the prescriber. Note that pharmacy laws and practices vary from state to state.
>
> ***EMA*** [26]
>
> EMA and the Heads of Medicines Agencies (HMA) have issued a joint statement confirming that biosimilar medicines approved in the European Union (EU) are interchangeable with their reference medicine or with an equivalent biosimilar. While interchangeable use of biosimilars is already practiced in many Member States, this joint position harmonises the EU approach. It brings more clarity for healthcare professionals and thus helps more patients to have access to biological medicines across the EU.
>
> A biosimilar is a biological medicine highly similar to another already approved biological medicine (the 'reference medicine'). Interchangeability in this context means that the reference medicine can be replaced* by a biosimilar without a patient experiencing any changes in the clinical effect. *"EMA has approved 86 biosimilar medicines since 2006. These medicines have been thoroughly reviewed and monitored over the past 15 years and the experience from clinical practice has shown that in terms of efficacy, safety and immunogenicity they are comparable to their reference products and are therefore interchangeable", says Emer Cooke, EMA's Executive Director. "This is good news for patients and healthcare professionals, who have wider access to important therapeutic options to treat serious diseases such as cancer, diabetes and rheumatoid arthritis."*
>
> EMA's position is based on the experience gained in clinical practice, where it has become common that doctors switch patients between different biological medicinal products. Approved biosimilars have demonstrated similar efficacy, safety and immunogenicity compared with their reference medicines, and analysis of more than one million patient-treatment years of safety data did not raise any safety concerns. Thus, EU experts considered that when a biosimilar is granted approval in the EU, it can be used instead of its reference product (or vice versa) or replaced by another biosimilar of the same reference product.
>
> Decisions regarding substitution at pharmacy-level (the practice of dispensing one medicine instead of another without consulting the prescriber) are managed by individual Member States

Regulatory authorities, at least for now, have approved for commercial distribution, biosimilars that are well-controlled and highly purified, such as is the case for the recombinant proteins and monoclonal antibodies [27]:

> In principle, the concept of similar biological medicinal product is applicable to any biological product. However, in practice, the success of such a development approach will depend on the ability to characterise the product and therefore to demonstrate the similar nature of the concerned products.

3.3 The Times Are Changing

Biopharmaceuticals are definitely different from chemical drugs, and therefore the regulatory authorities will have different and extra controls to ensure their CMC regulatory compliance. This is not a perception, but a reality, in full agreement with regulatory authorities. When I entered the biopharmaceutical industry 45 years ago in the late 1970's, the dogma of the regulatory authorities was very clear as follows: "the biologic manufacturing process defines the biologic product." Unlike chemical

drugs which had a risk-based assessment for allowing manufacturing process changes, biologics at that time were placed in a fixed high-risk category, and required regulatory authority preapproval for almost all manufacturing process changes. Then, by the 2000's, the regulatory authorities had the opportunity to review numerous recombinant DNA-derived protein and monoclonal antibody biopharmaceuticals for market approval. This helped shape their current regulatory authority dogma which is as follows: "the biologic manufacturing process may impact the biologic product; whatever impact needs to be assessed". Today, the potential impact of a biopharmaceutical manufacturing process, like a chemical drug manufacturing process, is now assessed using a risk-based approach. And it is now the responsibility of the biopharmaceutical manufacturer to demonstrate to the regulatory authority what impact, if any, a manufacturing process might have on the product.

Today, biosimilars are only being considered for recombinant proteins and monoclonal antibodies because of the available analytical and functional capability to thoroughly and comprehensively characterize these biopharmaceuticals. As the characterization tools for the nucleic acid-based biopharmaceuticals (gene therapy) advance, maybe one day in the future, these might also be considered for biosimilars.

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Chapter 4
Risk Management of the Minimum CMC Regulatory Compliance Continuum

Abstract The challenge of control of the biopharmaceutical manufacturing process coupled with the complexity of the resulting biopharmaceutical products create pressure on meeting the CMC regulatory compliance requirements expected by regulatory authorities. Fortunately, the regulatory authorities accept a strategy that embraces a 'minimum CMC regulatory compliance continuum' for biopharmaceuticals (both protein-based and gene therapy-based). In this chapter, it will be discussed how the minimum CMC regulatory compliance continuum can be achieved by applying a strategic risk-based approach toward three interactive components – (1) CMC Regulatory, (2) cGMPs, and (3) the Quality System. A strategic risk-based approach recommended by the regulatory authorities – Quality by Design/Quality Risk Management (QbD/QRM) – will also be examined in detail.

Keywords Minimum · Continuum · Risk · cGMPs · QbD · QRM · QTPP · Critical · CQA · CPP · Strategy

4.1 Strategic Risk Management Is Essential

'Strategy' is 'a plan of action designed to lead to an overall defined goal.' For CMC regulatory compliance, the strategy is the course of activities that lead to a successful regulatory goal (e.g., initiating first-in-human clinical studies, obtaining market approval, etc.). Due to the challenge of the control of the biopharmaceutical manufacturing process coupled with the complexity of the resulting biopharmaceutical products (both protein-based and nucleic acid-based), a risk-based approach is absolutely necessary for an effective strategy. Fortunately, the regulatory authorities accept a strategy that embraces a 'minimum CMC regulatory compliance continuum' for biopharmaceuticals. By applying a risk-based approach toward three interactive components, CMC regulatory compliance can be achieved during the changing clinical development stages:

1. CMC Regulatory

J. Geigert, *The Challenge of CMC Regulatory Compliance for Biopharmaceuticals*, https://doi.org/10.1007/978-3-031-31909-9_4

2. Current Good Manufacturing Practices (cGMPs)
3. Quality System

Regulatory authorities recommend a risk-based approach embedded within the International Council on Harmonisation (ICH) quality guidelines – Quality by Design (QbD, ICH Q8(R2) and Quality Risk Management (QRM, ICH Q9(R1)) – that serves biopharmaceuticals well.

4.2 Minimum CMC Regulatory Compliance Continuum

Every activity, every decision, every change, carries risk (i.e., both a probability that an event might occur and a degree of harm should that event occur). But not all risks carry the same level of concern; hence, a risk-based approach is necessary to sort through all of the identified risks, and then prioritize the risks so that the focus of limited resources can be applied to addressing and controlling the more critical identified risks. The complexity involved with control of the biopharmaceutical manufacturing process coupled with control of the produced biopharmaceutical, introduces an abundance of CMC regulatory compliance risks, which need to be effectively managed. Regulatory authorities are well aware that it is not possible to scientifically fit these risks into a one-size-fits-all risk assessment. Therefore, they acknowledge, and even encourage a flexible risk-based strategy referred to in this book as the 'minimum CMC regulatory compliance continuum.' This risk-based strategy is also referred to as the 'clinical phase-appropriate strategy', referring to the various traditional clinical phases in clinical development. But the traditional clinical phases (i.e., phase 1, phase 2, phase 3) today are fluid, with various hybrids (e.g., phase 1/2, pivotal phase 2, etc.) occurring, and even a transition to what can be considered a 'seamless' clinical development program. This is why in this book, I prefer to use the beginning to the end approach, the minimum – continuum risk-based approach, to describe the management of CMC regulatory compliance. Clinical 'stages' will be referred to, but only at a very elementary level: early stage clinical (starts with first-in-human, FIH, study) and late stage clinical (typically the pivotal clinical program to confirm efficacy).

'Minimum' is defined as 'the least quantity assignable.' From a CMC regulatory compliance perspective, this refers to a threshold of compliance that must be achieved – cannot go below – at given stages of clinical development. 'Continuum' is defined as 'a coherent whole characterized as a progression of values or elements varying by degrees.' From a CMC regulatory compliance perspective, this refers to the threshold of compliance that must keep rising as clinical development advances, as illustrated in Fig. 4.1.

This minimum CMC regulatory compliance continuum risk-based approach is recognized by the regulatory authorities and implemented across the biopharmaceutical industry because it allows for the needed flexibility throughout clinical development. But flexibility should not be translated into avoidance or inaction. The

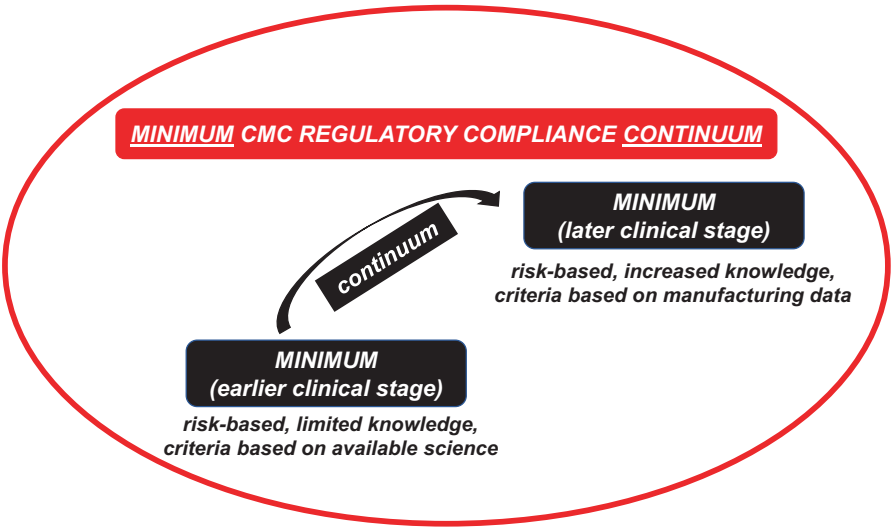

Fig. 4.1 Minimum CMC regulatory compliance continuum

risk-based approach has to be actively managed. The minimum CMC regulatory compliance continuum must be adequately and appropriately maintained throughout the entire clinical development period.

4.3 Three Interactive CMC Regulatory Compliance Components

Protecting patients from risks associated with the control of the biopharmaceutical manufacturing process and the produced biopharmaceutical is paramount. Three interactive CMC regulatory compliance components lead to an effective minimum CMC regulatory compliance continuum strategy: (1) CMC Regulatory, (2) cGMPs and (3) the Quality System:

CMC Regulatory refers to the CMC content necessary to be submitted to the regulatory authorities for them to appropriately and adequately review and assess patient safety concerns related to the manufacturing process and the biopharmaceutical product

cGMPs refer to current good manufacturing practices identified by regulatory authorities, that are required to be carried out in the manufacturing facility during the manufacture and testing of the biopharmaceutical to protect patients

Quality System refers to the Quality Unit (e.g., Quality Assurance, Quality Control, GMP Compliance) required to be a check-and-balance to Manufacturing to ensure the required cGMPs are actually carried out during the manufacturing

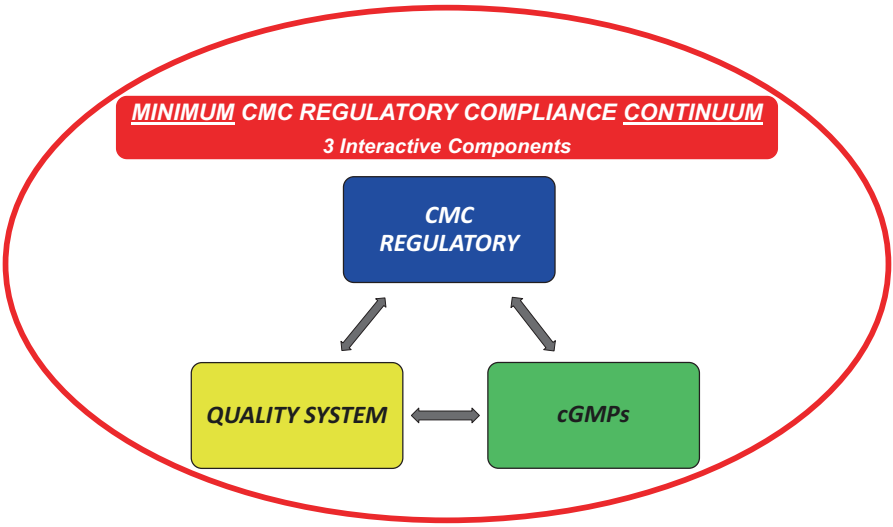

Fig. 4.2 Three interactive CMC regulatory compliance components

process and that the requirements for the information to be submitted to the regulatory authority under CMC Regulatory are met

Each of the three components, as shown in Fig. 4.2, work together, and when applied across the different clinical development stages, can provide an effective and flexible risk-based approach that can help ensure both the 'minimum' and the 'continuum' of CMC regulatory compliance for biopharmaceuticals are achieved.

4.3.1 CMC Regulatory

'CMC Regulatory' refers to the information about the manufacturing process and the product that must be communicated to the regulatory authorities in the regulatory submissions, for clinical development and eventually market approval. Overall, CMC Regulatory is to be managed in a risk-based and clinical stage-appropriate approach, as illustrated in Fig. 4.3.

4.3.1.1 CMC Content to Be Submitted – Regulatory Authority Guidance

The basic CMC information to be submitted in regulatory submissions is consistent across the regulatory authorities, and follows the general content shown in Table 4.1. (See Chap. 2, Sect. 2.2.3 for more detailed information on the general CMC content expected by regulatory authorities).

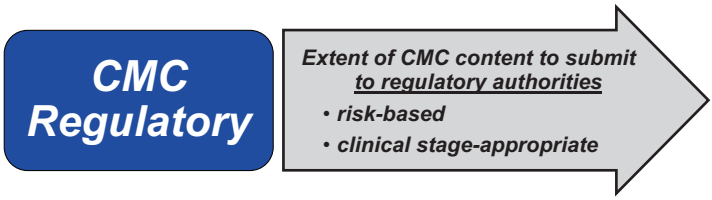

Fig. 4.3 CMC Regulatory component

Table 4.1 Basic CMC Regulatory information to be submitted to regulatory authorities

Drug Substance (DS, API)	Drug Product (DP)
Manufacturer & Sites of Manufacture	Manufacturer & Sites of Manufacture
Manufacturing Process Description	Manufacturing Process Description
Manufacturing Process Controls	Manufacturing Process Controls
Source Material(s)	Excipients
Characterization of Product	Formulation
Release Testing of DS	Release Testing of DP
Stability Testing of DS	Stability Testing of DP
Adventitious Agent Control (TSE, Virus, Mycoplasma, Microbial)	

FDA (in the form of Guidance for Industry, GfI) and EMA (in the form of guidelines) communicate the specific requirements and expectations for the CMC Regulatory content to be included in the various regulatory submissions. Table 4.2 presents a number of these regulatory guidances/guidelines that describe the required and expected CMC content to be included in the regulatory submissions for protein-based biopharmaceuticals (e.g., monoclonal antibodies, fusion proteins, Fab fragments, bispecific antibodies, etc.), and the required and expected CMC content to be included in the submissions for gene therapy-based biopharmaceuticals (e.g., viral vectors, genetically modified patient cells).

The International Council on Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) has and continues to play a significant role in developing CMC Regulatory content in submissions related to the protein-based biopharmaceuticals. ICH has come a long way from the initial three regulatory authorities (FDA, EMA, and PMDA) that were the founding members in 1990. ICH now includes 11 other regulatory authority members from around the world (e.g., MHRA – UK, ANVISA – Brazil, NMPA – China, etc.), as well as 20 regulatory authority observers [1]. The ICH 'Q' regulatory guidelines that lay out the CMC Regulatory content in submissions specifically for protein-based biopharmaceuticals are listed in Table 4.3. Consideration was given by ICH to developing CMC

Table 4.2 FDA/EMA guidance on CMC Regulatory content to be included in submissions

FDA website (www.FDA.gov)	EMA website (www.EMA.Europe.eu)
PROTEIN-BASED BIOPHARMACEUTICALS	
Guidance for Industry For the Submission of Chemistry, Manufacturing, and Controls Information for a Therapeutic Recombinant DNA-Derived Product or a Monoclonal Antibody Product for In Vivo Use (1996)	Guideline on Development, Production, Characterisation and Specification for Monoclonal Antibodies and Related Products (2016)
Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use (2017)	Guideline on the Requirements for Quality Documentation Concerning Biological Investigational Medicinal Products In Clinical Trials (2022)
GENE THERAPY-BASED BIOPHARMACEUTICALS	
Guidance for Industry: Chemistry, Manufacturing & Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs) (2020)	Guideline on the Quality, Non-Clinical and Clinical Aspects of Gene Therapy Medicinal Products (2018)
Guidance for Industry Human Gene Therapy Products Incorporating Human Genome Editing (2022)	Guideline on the Quality, Non-Clinical and Clinical Requirements for Investigational Advanced Therapy Medicinal Products in Clinical Trials (2019)
Guidance for Industry Considerations for the Development of Chimeric Antigen Receptor (CAR) T Cell Products (2022)	Guideline on Quality, Non-Clinical and Clinical Aspects of Medicinal Products Containing Genetically Modified Cells (2020)

regulatory guidelines for submissions related to the gene-based biopharmaceuticals, but because of limited resources abandoned the effort in 2011 [2].

4.3.1.2 CMC Content – Risk-Based and Clinical Stage-Appropriate

Regulatory authorities concur that for CMC Regulatory the extent of CMC content to be included in the submissions needs to be risk-based and clinical stage-appropriate. FDA in the Code of Federal Regulations 312.23 states clearly that a risk-based and clinical stage-appropriate submission of CMC content is appropriate for all pharmaceuticals during clinical development [3]:

> Chemistry, manufacturing, and control information. As appropriate for the particular investigations covered by the IND, a section describing the composition, manufacture, and control of the drug substance and the drug product. Although in each phase of the investigation sufficient information is required to be submitted to assure the proper identification, quality, purity, and strength of the investigational drug, the amount of information needed to make that assurance will vary with the phase of the investigation, the proposed duration of the

Table 4.3 ICH 'Q' guidelines on CMC Regulatory content to be included in submissions

PROTEIN-BASED BIOPHARMACEUTICALS
ICH website (www.ICH.org)
Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin Q5A(R2)
Analysis of the Expression Construct in Cells Used for Production of r-DNA Derived Protein Products Q5B
Quality of Biotechnological Products: Stability Testing of Biotechnological/ Biological Products Q5C
Derivation and Characterization of Cell Substrates Used for Production of Biotechnological/Biological Products Q5D
Comparability of Biotechnological/Biological Products Subject to Changes in Their Manufacturing Process Q5E
Specifications: Test Procedures and Acceptance Criteria for Biotechnological/ Biological Products ICH Q6B

investigation, the dosage form, and the amount of information otherwise available. FDA recognizes that modifications to the method of preparation of the new drug substance and dosage form and changes in the dosage form itself are likely as the investigation progresses. Therefore, the emphasis in an initial Phase 1 submission should generally be placed on the identification and control of the raw materials and the new drug substance. Final specifications for the drug substance and drug product are not expected until the end of the investigational process.

As drug development proceeds and as the scale or production is changed from the pilot-scale production appropriate for the limited initial clinical investigations to the larger scale production needed for expanded clinical trials, the sponsor should submit information amendments to supplement the initial information submitted on the chemistry, manufacturing, and control processes with information appropriate to the expanded scope of the investigation.

Both FDA and EMA in their respective guideline/guidance re-confirm that a risk-based and clinical stage-appropriate submission of CMC content is appropriate for all biopharmaceuticals, both protein-based and gene therapy-based biopharmaceuticals, during clinical development:

EMA [4]

In determining the content of the IMPD, a risk-based approach can be applied. The content of the dossier can be adapted having regard to the identified risks. In particular, the applicant can perform at the beginning of product development an initial risk analysis based on existing knowledge on the type of product and its intended use. Aspects to be taken into consideration include the origin of the cells, the type of vector and/or the method used for the genetic modification, the manufacturing process, the non-cellular components and the specific therapeutic use as applicable.

The risk analysis should be updated by the applicant throughout the product life cycle as new data become available. Key points relevant to the understanding of the product development approach chosen, should be summarized in the IMPD.

> The level of effort and documentation should be commensurate with the level of risk.
> ***FDA*** [5]
>
> You are not required to complete all CTD sections in your original IND submission. The amount of CMC information to be submitted in your IND depends on the phase of investigation and the scope of the clinical investigation proposed (21 CFR 312.23(a)(7)). The emphasis for CMC review in all phases of development is product safety and manufacturing controls. We expect that sponsors may need to make modifications and additions to previously submitted information as clinical development proceeds and additional product knowledge and manufacturing experience is collected (21 CFR 312.31).

EMA has published two CMC Regulatory information guidelines for the CMC content to be submitted in Investigation Medicinal Product Dossier (IMPD) submissions during the clinical development period – one guideline specifically addresses the CMC Regulatory content for protein-based biopharmaceuticals (recombinant proteins and monoclonal antibodies) [6], the other guideline specifically addresses the CMC Regulatory content for the gene therapy-based biopharmaceuticals (viral vectors and genetically modified patient cells) [4]. Both guidelines illustrate the risk-based, clinical stage-appropriate approach providing the necessary flexibility in the management of the extent of CMC Regulatory information to be included in the IMPD submissions, see Table 4.4. Notice how similar the risk-based flexibility is between the protein-based biopharmaceuticals and the gene therapy-based biopharmaceuticals guidelines.

FDA has published a CMC Regulatory information guidance addressing the risk-based, clinical stage-appropriate CMC content to be submitted in Investigational New Drug (IND) submissions during the clinical development period for DNA/mRNA-based biopharmaceuticals [5]. Both the drug substance section and the drug product section of the IND are discussed from a clinical stage-appropriate approach, see Table 4.5. Notice how similar the risk-based flexibility is between the drug substance section and the drug product section. Also notice how similar the risk-based flexibility for the DNA/mRNA-based biopharmaceuticals is between this FDA guidance and the previous EMA guideline of Table 4.4.

And finally, the FDA has also published a CMC Regulatory information guidance addressing the CMC content to be submitted in Investigational New Drug (IND) submissions during the clinical development period for CAR T cell biopharmaceuticals. This guidance also re-confirms the risk-based, clinical stage-appropriate approach providing the necessary flexibility in the management of the extent of CMC Regulatory information to be included in the IND submissions of these genetically modified patient cells [7]:

> The emphasis for CMC in all phases of development is product safety and manufacturing control. We recommend that CAR T cells be developed following a life cycle approach where information may be gathered over the course of product development and submitted in a stage-appropriate manner. The amount of CMC information to be submitted in your IND depends on the phase and the scope of the clinical investigation proposed (21 CFR 312.23(a)(7)). Therefore, you may not need to complete all CTD sections in your original IND submission. Similarly, CAR T cells and vectors are to be manufactured under Good

Table 4.4 Risk-based and clinical stage-appropriate CMC content flexibility in IMPD submissions

CMC Content in IMPD	PROTEIN-BASED BIOPHARMACEUTICALS	GENE THERAPY-BASED BIOPHARMACEUTICALS
S.2.4 Control of Critical Steps & Intermediates	**Tests and acceptance criteria for the control of critical steps in the manufacturing process should be provided… It is acknowledged that due to limited data at an early stage of development (phase I/II) complete information may not be available.**	**Critical steps in the manufacturing process should be identified as appropriate for the stage of development and all available data and acceptance criteria should be provided… It is acknowledged that due to limited data at an early stage of development complete information may not be available.**
S.2.6 Manufacturing Process Development	**Manufacturing processes and their control strategies are continuously being improved and optimised, especially during the development phase and early phases of clinical trials. Changes to the manufacturing process and controls should be summarized.**	**Manufacturing processes and their control strategies are continuously being improved and optimised, especially during early phases of clinical trials and development. Changes to the manufacturing process and controls should be summarized and the rationale for changes should be presented.**
S.4.1 Specification	**As the acceptance criteria are normally based on a limited number of development batches and batches used in non-clinical and clinical studies, they are by their nature inherently preliminary and may need to be reviewed and adjusted during further development.**	**As the acceptance criteria are normally based on a limited number of development batches and batches used in non-clinical and clinical studies, they are by their nature preliminary and need to be subject to review during development.**

Manufacturing Practice (GMP) conditions that are appropriate for the stage of development (section 501(a) (2) (B) of the Federal 218 Food, Drug, and Cosmetic Act (FD&C Act) (21 U.S.C. 351(a)(2)(B)). Additional CMC information may be needed to align product development with the clinical development, especially when the latter is rapidly progressing under an expedited development program.

For CAR T cells in the early stages of clinical development, very few specifications are finalized, and some tests may still be under development. Cellular characterization data collected during early studies can inform release criteria used in later development to ensure product and process consistency. Thus, characterization studies are crucial to support product development and comparability assessments. For studies in which a primary objective is to gather meaningful data about product efficacy, we recommend that acceptance criteria be refined to ensure batches are well-defined and consistently manufactured.

In summary, the CMC Regulatory component, working together with the other two components, provides an effective risk-based, clinical stage-appropriate, flexible approach that helps ensure that both the ‘minimum’ and the ‘continuum’ of CMC regulatory compliance for biopharmaceuticals is achieved.

Table 4.5 Risk-based and clinical stage-appropriate CMC content flexibility in IND submissions

CMC Content in IND	GENE THERAPY-BASED BIOPHARMACEUTICALS	
	Drug Substance (S)	Drug Product (P)
3.2.S.2.4 3.2.P.3.4 Control of Critical Steps & Intermediates	You should describe the control of critical steps and intermediates in the manufacturing process... The Agency acknowledges that this information may be limited in the early phases of development and recommends that sponsors provide additional information and updates as product development proceeds.	You should describe the control of critical steps and intermediates in the manufacturing process… The Agency acknowledges that this information may be limited in the early phases of development and recommends that sponsors provide this information at the appropriate stage.
3.2.S.2.5 3.2.P.3.5 Process Validation and/or Evaluation	Process validation studies are generally or typically not required for early stage manufacturing, and thus, most original IND submissions will not include performance qualification.	Process validation is not required for early stage manufacturing, and thus, most original IND submissions will not include this information.
3.2.S.4.1 3.2.P.5.1 Specifications	For products in the early stages of clinical development, very few specifications are finalized, and some tests may still be under development. However, the testing plan submitted in your IND should be adequate to describe the physical, chemical, or biological characteristics of the DS necessary to ensure that the DS meets acceptable limits for identity, strength (potency), quality, and purity (21 CFR 312.23(a)(7)(iv)(a)).	You should list DP specifications in your original IND submission…. DP specifications should be further refined as a part of product development under an IND. We recommend that sponsors establish or, in some cases, tighten acceptance criteria, based on manufacturing experience as clinical development proceeds.

4.3.2 cGMPs

'cGMPs' refer to the current good manufacturing practices enforced by regulatory authorities that ensure proper design, monitoring, and operation of the manufacturing facility, control over the manufacturing process, and adequate and appropriate procedures for the release of the final drug product – focused on patient safety. Overall, cGMPs are to be managed in a risk-based and clinical stage-appropriate approach, as illustrated in Fig. 4.4.

4.3.2.1 GMPs Required by Regulatory Authorities

FDA (in the form of Guidance for Industry, GfI) and EMA (in the form of guidelines) communicate the specific requirements and expectations for the cGMPs that are to be carried out. Some of the required and expected good manufacturing practices for protein-based biopharmaceuticals (e.g., monoclonal antibodies, Fc-fusion

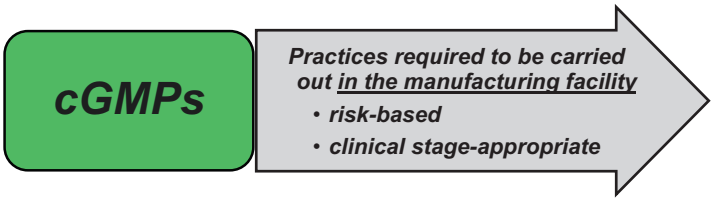

Fig. 4.4 cGMPs component

Table 4.6 FDA/EMA guidance on cGMPs for biopharmaceutical manufacturing and control

PROTEIN-BASED BIOPHARMACEUTICALS	
ICH website (www.ICH.org)	EMA website (www.EMA.Europe.eu)
Good Manufacturing Practice Guide for Active Pharmaceutical Ingredients – ICH Q7 (2000)	EC EudraLex- Volume 4 – Good Manufacturing Practice (GMP) Guidelines – Annex 2 – Manufacture of Biological Active Substances and Medicinal Products for Human Use (2018)
GENE THERAPY-BASED BIOPHARMACEUTICALS	
PIC/S website (PICScheme.org)	EMA website (www.EMA.Europe.eu)
Guide to Good Manufacturing Practice for Medicinal Products Annexes – Annex 2A – Manufacture of Advanced Therapy Medicinal Products for Human Use (2022)	EC EudraLex – Volume 4 – Good Manufacturing Practice (GMP) Guidelines – Guidelines on Good Manufacturing Practice Specific for Advanced Therapy Medicinal Products (2017)
	Questions and Answers on the Principles of GMP for the Manufacturing of Starting Materials of Biological Origin Used to Transfer Genetic Material for the Manufacturing of ATMPs (2021)
ALL BIOPHARMACEUTICALS	
FDA website (www.FDA.gov)	
Guidance for Industry: CGMP for Phase 1 Investigational Drugs (2008)	

proteins, Fab fragments, bispecific antibodies, etc.), and the required and expected manufacturing practices for gene therapy-based biopharmaceuticals (e.g., viral vectors, genetically modified patient cells.), are listed in Table 4.6.

Keep in mind that GMPs are minimum requirements of the needed control. It is also very clear that GMPs are required from first-in-human (FIH) clinical studies onwards:

FDA [8]

Consistent with the FD&C Act (§ 501(a) (2) (B)), CGMP must be in effect for the manufacture of each batch of investigational drug used during phase 1 clinical trials. Manufacturers should establish manufacturing controls based on identified hazards for the manufacturing setting that follow good scientific and QC principles.

EMA [9]

Compliance with good manufacturing practice ("GMP") is mandatory for all medicinal products that have been granted a marketing authorisation. Likewise, the manufacture of investigational medicinal products must be in accordance with GMP.

ICH [10]

Appropriate GMP concepts should be applied in the production of APIs for use in clinical trials with a suitable mechanism of approval of each batch. A quality unit(s) independent from production should be established for the approval or rejection of each batch of API for use in clinical trials.

There are basic GMPs related to the appropriate and adequate design and control of all biopharmaceutical manufacturing facilities [9]:

Premises must be suitable for the operations to be carried out. In particular, they should be designed to minimise the opportunity for extraneous contamination, cross-contamination, the risk of errors and, in general, any adverse effect on the quality of products. It is important that the following general principles are implemented:

(i) Premises should be kept clean (disinfection to be applied as appropriate).
(ii) Premises should be carefully maintained, ensuring that repair and maintenance operations do not present any hazard to the quality of products.
(iii) Lighting, temperature, humidity and ventilation should be appropriate for the activities performed and should not adversely affect the ATMPs or the functioning of equipment.
(iv) Appropriate measures to monitor key environmental parameters should be applied.
(v) Premises should be designed and equipped so as to afford maximum protection against the entry of insects or other animals.
(vi) Steps should be taken to prevent the entry of unauthorised people. Production, storage and quality control areas should not be used as a transit area by personnel who do not work in them. When such passage is unavoidable, appropriate control measures should be applied.
(vii) The manufacture of technical poisons, such as pesticides and herbicides, should not be allowed in premises used for the manufacture of ATMPs.

There are basic GMPs related to the appropriate and adequate design and operation of all biopharmaceutical manufacturing process equipment [9]:

Equipment used in production or control operations should be suitable for its intended purpose and it should not present any hazard to the product. Parts of production equipment that come into contact with the product should not have unwanted reactive, additive, adsorptive or absorptive properties that may affect the quality of the product. In addition, parts of the equipment that come into contact with cells/tissues should be sterile.

Major equipment (*e.g.* reactors, storage containers) and permanently installed processing lines should be appropriately identified to prevent mix-ups; the integrity of the equipment's components should be verified as appropriate having regard to the specific risk of the product and the intended manufacturing process (*e.g.* ensuring structural integrity during freeze and thawing); the location and installation of the equipment should be adequate to minimise risks of errors or contamination; connections that are to be made in aseptic conditions should be performed in a critical clean area of grade A with a background clean area of grade B, unless there is subsequent sterilisation by steam-in-place or the connection is made by means of a validated sterile system (*e.g.* sterile tube welders, aseptic connection with a sterile septum); balances and measurement equipment should be of appropriate range and precision to ensure the accuracy of weighing operations; equipment should be adequately maintained; adequate cleaning and storage of the equipment is essential in order to avoid the risk of contamination for the products.

4.3.2.2 GMPs – Risk-Based and Clinical Stage Appropriate

Regulatory authorities concur that cGMPs for manufacturing process and product control need to be risk-based, clinical-stage appropriate and flexible:

FDA [11]

The CGMP requirements were established to be flexible in order to allow each manufacturer to decide individually how to best implement the necessary controls by using scientifically sound design, processing methods, and testing procedures. The flexibility in these regulations allows companies to use modern technologies and innovative approaches to achieve higher quality through continual improvement. Accordingly, the "C" in CGMP stands for "current," requiring companies to use technologies and systems that are up-to-date in order to comply with the regulations. Systems and equipment that may have been "top-of-the-line" to prevent contamination, mix-ups, and errors 10 or 20 years ago may be less than adequate by today's standards.

FDA [8]

This guidance describes an approach manufacturers may use to implement manufacturing controls that are appropriate for the phase 1 clinical trial stage of development. The approach described in this guidance reflects the fact that some manufacturing controls and the extent of manufacturing controls needed to achieve appropriate product quality differ not only between investigational and commercial manufacture, but also among the various phases of clinical trials. Consistent with FDA's CGMP for the 21 Century initiative, where applicable, manufacturers are also expected to implement manufacturing controls that reflect product and manufacturing considerations, evolving process and product knowledge, and manufacturing experience.

The following manufacturing controls are applicable to the manufacture of phase 1 investigational drugs and in some specific manufacturing situations. These recommendations provide flexibility to the manufacturers in implementing CGMP controls appropriate to their specific situation and application.

ICH [10]

The controls used in the manufacture of APIs for use in clinical trials should be consistent with the stage of development of the drug product incorporating the API. Process and test procedures should be flexible to provide for changes as knowledge of the process increases and clinical testing of a drug product progresses from pre-clinical stages through clinical stages. Once drug development reaches the stage where the API is produced for use in drug products intended for clinical trials, manufacturers should ensure that APIs are manufactured in suitable facilities using appropriate production and control procedures to ensure the quality of the API.

Having this cGMP risk-based flexibility has been especially important for gene therapy-based biopharmaceuticals due to the rapidly-evolving technology and product types in this field [9]:

ATMPs are at the forefront of scientific innovation and the field is experiencing rapid technological change that also impacts on the manufacturing processes. For instance, new manufacturing models are emerging to address the specific challenges of ATMPs (*e.g.* decentralised manufacturing for autologous products). Additionally, ATMPs are also often developed in an academic or hospital setting operating under quality systems different to those typically required for the manufacture of conventional medicinal products. It follows that, in laying down the GMP requirements applicable to ATMPs, it is necessary to recognise a certain level of flexibility so that the ATMP manufacturer can implement the mea-

> sures that are most appropriate having regard to specific characteristics of the manufacturing process and of the product. This is particularly important in the case of investigational ATMPs, especially in early phases of clinical trials (phase I and phase I/II), due to the often incomplete knowledge about the product (*e.g.* potency) as well as the evolving nature of the routines (in order to adjust the manufacturing process to the increased knowledge of the product).
>
> The risk-based approach permits the manufacturer to design the organisational, technical and structural measures that are put in place to comply with GMP – and thus to ensure quality according to the specific risks of the product and the manufacturing process. While the risk-based approach brings flexibility, it also implies that the manufacturer is responsible to put in place the control/mitigation measures that are necessary to address the specific risks of the product and of the manufacturing process.

FDA has published a guidance addressing the cGMP requirements and expectations for the first-in-human (FIH) clinical studies (referred to as Phase 1). This regulatory document provides some general guidance on how the risk-based approach can provide the necessary flexibility to ensure proper design, monitoring, and operation of the manufacturing facility, control over the manufacturing process, and adequate and appropriate procedures for the release of the product at such an early clinical stage [8], see Table 4.7.

EMA has published a cGMP guideline that provides some specific examples of cGMP risk-based flexibility for the gene therapy-based biopharmaceuticals during clinical development [9], see Table 4.8. It is important to note that while the primary focus of this regulatory document is towards commercial product, the document also includes guidance for clinical development (i.e., the term 'investigational ATMP' is used over 50 times in the text).

In summary, the cGMP component, working together with the other two components, provides an effective risk-based, clinical stage-appropriate, flexible approach that helps ensure that both the 'minimum' and the 'continuum' of CMC regulatory compliance for biopharmaceuticals is achieved.

4.3.3 Quality System

'Quality System' refers to the management systems that ensure appropriate documentation and quality control of the manufacturing process and the product release, including detecting and investigating process and product deviations. The Quality System is to ensure that the required CMC Regulatory commitments and the required cGMPs are appropriately and adequately carried out by the manufacturing and quality control staff. Overall, the Quality System is to be managed in a risk-based, clinical stage-appropriate approach, as illustrated in Fig. 4.5.

Table 4.7 General guidance on flexible risk-based cGMPs during early clinical stage development

Adherence to CGMP during manufacture of phase 1 investigational drugs occurs mostly through: **• Well-defined, written procedures** **• Adequately controlled equipment and manufacturing environment** **• Accurately and consistently recorded data from manufacturing (and testing)**
In applying appropriate CGMP, we recommend that manufacturers consider carefully the hazards and associated risks from the manufacturing environment that might adversely affect the quality of a phase 1 investigational drug, especially when the phase 1 investigational drug is manufactured in laboratory facilities that are not expressly or solely designed for their manufacture. For example,of particular importance is the susceptibility of a phase 1 investigational drug to contamination or cross contamination with other substances (e.g. chemicals, biologicals, adventitious agents) that may be present from previous or concurrent research or manufacturing activities.
We recommend the following steps to establish the appropriate manufacturing environment for phase 1 investigational drugs: **• A comprehensive and systematic evaluation of the manufacturing (i.e., product environment, equipment, process, personnel, materials) to identify potential hazards** **• Appropriate actions prior to/ during manufacturing to eliminate/ mitigate potential hazards to safeguard the quality of the phase 1 drug**
A number of technologies and resources are available that can facilitate conformance with CGMP and streamline product development. Some examples include: Use of disposable equipment and process aids to reduce cleaning burden and chances of contamination; Use of commercial, prepackaged materials (e.g., Water For Injection (WFI), pre-sterilized containers and closures) to eliminate the need for additional equipment or for demonstrating CGMP control of existing equipment; Use of closed process equipment (i.e., the phase 1 investigational drug is not exposed to the environment during processing) to alleviate the need for stricter room classification for air quality; Use of contract or shared CGMP manufacturing facilities and testing laboratories (including specialized services).

4.3.3.1 Quality System Required by Regulatory Authorities

The FDA guidances and EMA guidelines discussed under the cGMPs section (see Table 4.6) communicate the specific requirements and expectations for the Quality System that is to be established. The Quality System is required from first-in-human (FIH) clinical studies onwards:

FDA [8]

During product development, the quality and safety of phase 1 investigational drugs are maintained, in part, by having appropriate QC procedures in effect. Using established or standardized QC procedures and following appropriate CGMP will also facilitate the manufacture of equivalent or comparable IND product for future clinical trials as needed.

EMA [9]

It is important to ensure that data obtained from the early phases of a clinical trial can be used in subsequent phases of development. Therefore, a functional quality system should be in place for the manufacturing of investigational ATMPs.

Table 4.8 Flexible, risk-based cGMPs for gene-based biopharmaceuticals in clinical development

cGMP Area	Specific Examples of cGMP Risk-Based Flexibility for Gene Therapy-Based Biopharmaceuticals
Manufacturing Facility and Process Equipment	In early phases of clinical research (clinical trial phases I and I/II) when the manufacturing activity is very low, calibration, maintenance activities, inspection or checking of facilities and equipment should be performed at appropriate intervals, which may be based on a risk-analysis. The suitability for use of all equipment should be verified before it is used.
Cleaning Validation	For investigational ATMPs, cleaning verification is acceptable. In such cases, there should be sufficient data from the verification to support a conclusion that the equipment is clean/ available for use
Manufacturing Process Controls	In case of investigational ATMPs, the knowledge and understanding of the product may be limited, particularly for early phases of clinical trials (phase I and I/II). It is therefore acknowledged that the manufacturing process (including quality controls) may need to be adapted as the knowledge of the process increases. In the early phases of development, it is critical to carefully control and document the manufacturing process. It is expected that the manufacturing process and quality controls become more refined as development progresses. Manufacturing processes and their control strategies should be reviewed regularly, and they should be improved as appropriate.
Manufacturing Process Validation	The manufacturing process for investigational ATMPs is not expected to be validated but appropriate monitoring and control measures should be implemented to ensure compliance with the requirements in the clinical trial authorisation. Additionally, it is expected that the aseptic processes (and, where applicable, sterilising processes) have been validated.
Source Materials	Following the establishment of cell banks and master and viral seed lots, quarantine and release procedures should be followed. Evidence of the stability and recovery of seeds and banks should be documented and records should be kept in a manner permitting trend evaluation. In the case of investigational ATMPs, a gradual approach is acceptable.

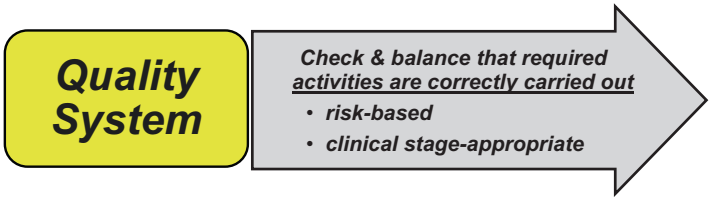

Fig. 4.5 Quality System component

There are basics of the Quality System that need to be in place throughout clinical development for all biopharmaceutical manufacturing and product release operations, according to both the FDA [8] and EMA [9], see Table 4.9.

Table 4.9 Basics for the Quality System during clinical development

FDA	EMA
Every manufacturer should establish a written plan that describes the role of and responsibilities for QC functions. For example, a written plan should provide, at a minimum, for the following functions. **• Responsibility for examining the various materials used in the manufacture of a phase 1 investigational drug (e.g.,containers, closures, in-process materials, raw materials, packaging materials, and labeling) to ensure that they are appropriate and meet defined, relevant quality standards** **• Responsibility for review and approval of manufacturing procedures, testing procedures, and acceptance criteria** **• Responsibility for releasing or rejecting each batch of phase 1 investigational drug based on a cumulative review of completed manufacturing records and other relevant information (e.g., procedures were followed, product tests performed appropriately, acceptance criteria met)** **• Responsibility for investigating unexpected results or errors that occur during manufacturing or from complaints received and initiation of corrective action, if appropriate.**	**Through the pharmaceutical quality system it should be ensured that:** **(i) the personnel are adequately trained and there is clear allocation of responsibilities;** **(ii) the premises and equipment are suitable for the intended use and that there is appropriate maintenance thereof;** **(iii) there is an adequate documentation system that ensures that appropriate specifications are laid down for materials, intermediates, bulk products and the finished product, that the production process is clearly understood, and that appropriate records are kept;** **(iv) the manufacturing process is adequate to ensure consistent production (appropriate to the relevant stage of development), the quality of the product, and the compliance thereof with the relevant specifications;** **(v) there is a quality control system which is operationally independent from production;** **(vi) arrangements are in place for the prospective evaluation of planned changes and their approval prior to implementation taking into account regulatory requirements … and for the evaluation of changes implemented….**

4.3.3.2 Quality Unit Independence from Manufacturing

Independence of the Quality Unit from Manufacturing, even during clinical development, is essential to provide an effective, adequate and appropriate 'check-and-balance' that satisfies regulatory authorities over necessary patient safety concerns:

FDA [8]

Although quality is the responsibility of all personnel involved in manufacturing, we recommend that you assign an individual(s) to perform QC functions independent of manufacturing responsibilities, especially for the cumulative review and release of phase 1 investigational drug batches.

FDA [5]

We recommend that you include a description of your Quality Unit whose duties should include establishing procedures to qualify reagents and critical materials, prevent microbial contamination, cross-contamination, and product mix-ups. In addition, your Quality Unit

should have procedures in place to investigate lot failures, out-of-specification results, and ways to implement corrective actions as product development progresses. We recommend that your IND include a summary of your Quality Unit, including the manner in which quality control testing and oversight are separated from the manufacturing unit.

EMA [9]

Because of their essential role in the quality system, the person responsible for production, the person responsible for quality control and the Qualified Person ("QP") should be appointed by senior management…. The roles and responsibilities of key personnel should be clearly defined and communicated within the organisation.

However, for small start-up biopharmaceutical manufacturers, there is an allowable exemption, but only under very limited circumstances, to the required independence of the Quality Unit from Manufacturing:

FDA [8]

However, in very limited circumstances and depending on the size and structure of an organization, all QC functions may be performed by the same individual(s) performing manufacturing. For example, in some small operations, it may be necessary to have the same individual perform both manufacturing and QC functions, including release or rejection of each batch. However, in such circumstances, we strongly recommend that another qualified individual not involved in the manufacturing operation conduct an additional *periodic* review of manufacturing records and other QC activities.

When activities such as testing, commonly performed by dedicated QC personnel in commercial manufacture, are performed by manufacturing personnel in phase 1 studies, adequate controls should be in place (e.g., segregation of testing from manufacturing) so as to not contaminate testing or negatively affect test results.

EMA [9]

However, responsibility for production and for quality control cannot be assumed by the same person. In small organisations, where teams are multi-skilled and trained in both quality control and production activities, it is acceptable that the same person is responsible for both roles (production and quality control) with respect to different batches. For any given batch, the responsibility for production and quality control of the batch must be vested on two different persons. Accordingly, it becomes particularly important that the independency of the quality control activities from the production activities for the same batch is clearly established through appropriate written procedures.

4.3.3.3 Critical Importance of Training

Due to the challenge of controlling biopharmaceutical manufacturing processes and the complexity of the biopharmaceutical products, staff training takes on an extremely important role. It is required that there be an adequate number of personnel with appropriate qualifications and appropriate practical experience relevant to the intended operations. The Quality Unit needs to ensure that such training is taking place. For all biopharmaceuticals, the first three areas of training below are required; for the nucleic acid-based biopharmaceuticals, the fourth area of training is also required [9]:

1. All personnel should receive training on the principles of GMP that affect them and receive initial and periodic training relevant to their tasks.

2. There should be appropriate (and periodic) training in the requirements specific to the manufacturing, testing, and traceability of the product.
3. Personnel working in clean areas should be given specific training on aseptic manufacturing, including the basic aspects of microbiology. Prior to participating in routine aseptic manufacturing operations, personnel should participate in a successful process simulation test.
4. In addition, there should be appropriate training to prevent the transfer of communicable diseases from biological raw and starting materials to the operators and vice versa. Personnel handling genetically modified organisms ("GMOs") require additional training to prevent cross-contamination risks and potential environmental impacts.

This training needs to be documented by the Quality Unit and kept up to date. Because training is time-intensive and expensive, senior management must be supportive of this requirement.

4.3.3.4 Quality Unit Flexibility – Market-Approval vs Clinical Development

EMA has published a guideline that explains how the risk-based approach provides to the Quality Unit the necessary flexibility it needs to ensure an adequate and appropriate Quality System for manufacturing of ATMPs, post-market approval versus during clinical development [9]. Table 4.10 presents this comparison.

In summary, the Quality System component, working together with the other two components, provides an effective risk-based, clinical stage-appropriate, flexible approach that helps ensure that both the 'minimum' and the 'continuum' of CMC regulatory compliance for biopharmaceuticals is achieved.

4.3.4 Industry Embracing the Three Interactive Regulatory Components

Not only the regulatory authorities, but also the biopharmaceutical industry, embraces the three interactive components that lead to an effective minimum CMC regulatory compliance strategy. The Parenteral Drug Association (PDA) in Technical Report No. 56 *Application of Phase-Appropriate Quality System and cGMP to the Development of Therapeutic Protein Drug Substance (API or Biological Active Substance)* [12] discusses in detail the relationship among the three interactive regulatory components – CMC Regulatory, cGMPs and the Quality System – mentioned above. While specific to protein-based biopharmaceuticals, the Technical Report is also applicable to gene-based biopharmaceuticals.

The value of Technical Report No. 56 is that it represents a consensus position across the biopharmaceutical industry, not only prepared by various representatives

Table 4.10 Risk-based Quality Unit flexible expectations for ATMP manufacturing

Quality System Area	Adequate and Appropriate Risk-Based Quality Unit Expectations for ATMP Manufacturing and Control	
	Post-Market Approval	Flexibility During Clinical Development
QU Expectation ATMP Batch Records & Reports	Records should be made or completed at the time each action is taken. (i) Receipt records for each delivery of raw materials, starting material, bulk, intermediate, packaging material (i) A batch processing record (ii) Results of release testing (iii) Environmental monitoring records (iv) On-going stability (v) Self-inspections recorded	The level of documentation will vary depending on the product and stage of development. The records should enable the entire history of a batch to be traced. Additionally, the records/reports should form the basis for assessment of the suitability for certification and release of a particular batch.
QU Expectation Documentation on the Control of the ATMP Manufacturing Process	There should be appropriate documentation of policies and procedures to be applied by the manufacturer with a view to safeguard the quality of the product: (i) Qualification of premises and equipment. (ii) Validation of manufacturing process (iii) Validation of relevant analytical methods. (iv) Maintenance and calibration of equipment. (v) Cleaning procedures. (vi) Environmental monitoring. (vii) Investigations into deviations and non-conformances. (viii) Procedures for handling of quality complaints and recalls.	The documentation of the above policies and procedures should be adjusted to the stage of development. The documentation for phase I and I/II clinical trials can be more limited but it is expected that it becomes more comprehensive in later phases of development. The manufacturing process for investigational ATMPs is not expected to be validated but appropriate monitoring and control measures should be implemented to ensure compliance with the requirements in the clinical trial authorisation. For investigational ATMPs, cleaning verification is acceptable. In such cases, there should be sufficient data from the verification to support a conclusion that the equipment is clean and available for further use.

across the biopharmaceutical industry but also peer-reviewed by leaders in the biopharmaceutical industry as well as several regulatory authority reviewers. The report provides tables, figures and details on applying the risk-based and clinical stage-appropriate approach to supply safe clinical materials for studies in humans while maintaining manufacturing and control flexibility at clinical study scales, and during scale-up and process transfer to commercial facilities. The following is a sample of the consensus discussion in the Technical Report:

> Quality System (such as Good Laboratory Practice, Good Research Practice, etc.) and cGMP should be implemented in a graded manner throughout the development and investigative stages of animal and clinical product testing. During the R&D and toxicity study stages, cGMP does not apply in the strict sense. During these stages, the key quality system principles are sufficient documentation of production and characterization of the protein molecule batches in order to support subsequent clinical trials. The material used for preclinical safety studies, such as cross reactivity and toxicity studies, must be similar to the material to be used in human subjects. This enables the use of the preclinical data to support the use of the drug substance/active pharmaceutical ingredient for investigational studies in humans. There should be documentation about the testing performed on the test article and

a CoA. Characterization, source documentation, and adequate reserves of a Reference Standard (RS) used to release clinical batches are typically established at this stage. A clearly documented pedigree for RS material must be available before Phase 1 material can be released. Documented maintenance and calibration of laboratory equipment is also highly recommended, and the qualification of toxicology laboratories and animal facilities is required by good laboratory practices (GLP). It is also important to be able to assess the level of endotoxin in the toxicity study materials due to the potential impact on study results.

During the clinical testing phases, the emphasis is on product safety and activity. Implementation of cGMP principles should begin early in the investigative stages when small amounts of protein products are produced for use in human studies. Documented calibration, maintenance, and cleaning of process and analytical equipment are required. As more product batches are made to satisfy clinical testing requirements, and as product and process understanding is acquired, cGMP should be implemented with increasing stringency in order to ultimately establish readiness for commercial production. It is possible to use data from pre-validation test lots as supportive data, but full implementation of cGMP should be in place when manufacturing the formal validation lots used to support commercial launch (such as for the registration stability batches) and for any materials purposed for commercial distribution.

For Phase 1, principles of cGMP should be followed in terms of fundamental documentation and equipment operation, but the detail of these activities will, of necessity, be less than that for Phase 3 or for BLA/MAA submission. For example, because the final process will not be set at Phase 1, batch records do not necessarily require acceptance criteria for yields or processing times. There is, however, the expectation that there is documented evidence of yield and product quality as the process transitions from the bench scale to the pilot scale. It is highly recommended to analyze demonstration lots at the bench scale and the pilot scale with qualified release methods and approved standard operating procedures (SOP). This information is useful for setting initial technical specifications for release of GMP lots. The goal is to show early on that quality has/is being designed into the process.

4.3.5 Consequences of Inadequate Senior Management Support

The three interactive regulatory components – CMC Regulatory, cGMPs and the Quality System – when adequately and appropriately implemented – effectively risk manage the minimum CMC regulatory compliance continuum, and provide the necessary patient protection. But in order to be effective, it especially requires strong senior management support for the time and expense needed to carry out the required and expected tasks.

A core strategic regulatory guideline is ICH Q10, *Pharmaceutical Quality Systems* [13]. The document covers areas such as Quality Risk Management (QRM), the Quality Manual (QM), Knowledge Management (KM), and the role of the Quality Unit (QU). But a key emphasis of this core guideline is the significant role that senior management plays in the success of the CMC regulatory compliance strategy:

> Leadership is essential to establish and maintain a company-wide commitment to quality and for the performance of the pharmaceutical quality system.

(a) *Senior management* has the ultimate responsibility to ensure an effective pharmaceutical quality system is in place to achieve the *quality objectives,* and that roles, responsibilities, and authorities are defined, communicated and implemented throughout the company.
(b) Management should:
    1. Participate in the design, implementation, monitoring and maintenance of an effective pharmaceutical quality system;
    2. Demonstrate strong and visible support for the pharmaceutical quality system and ensure its implementation throughout their organisation;
    3. Ensure a timely and effective communication and escalation process exists to raise quality issues to the appropriate levels of management;
    4. Define individual and collective roles, responsibilities, authorities and interrelationships of all organisational units related to the pharmaceutical quality system. Ensure these interactions are communicated and understood at all levels of the organisation. An independent quality unit/structure with authority to fulfil certain pharmaceutical quality system responsibilities is required by regional regulations;
    5. Conduct management reviews of process performance and product quality and of the pharmaceutical quality system;
    6. Advocate continual improvement;
    7. Commit appropriate resources.

Senior management, as defined by ICH Q10, are "person(s) who direct and control a company or site at the highest levels with the authority and responsibility to mobilize resources within the company or site". "Senior management have the ultimate responsibility to ensure an effective pharmaceutical quality system is in place to achieve the quality objectives, and that roles, responsibilities, and authorities are defined, communicated and implemented throughout the company."

Senior management set the stage for the level of risk tolerance accepted by the company and controls the resource allocation – both forces that impact the effectiveness of a minimum CMC regulatory compliance continuum strategy. Senior management lead, and when senior management speak or act, their staff listens and responds. If senior management are too risk tolerant (i.e., 'try to get by' or 'keep pushing ahead, will fix that later'), they may incorrectly believe that the appropriate biopharmaceutical CMC regulatory strategy is to try to do as little as possible. That philosophy trickles down to middle management and staff, which eventually develops into a corporate culture that under-appreciates the important 'check-balance' role of Quality Assurance, Quality Control and Regulatory Compliance, which leads to an unhealthy balance across the members of the CMC team.

Senior management never have to issue any directives to the effect that 'CMC regulatory compliance should be sacrificed for production.' But in reality, by their lack of attention, they may signal within the company what is more important (e.g., the management of the clinical strategy) versus was is less important (e.g., management of the CMC regulatory compliance strategy). The annual budget discussions also send a message to the company. Frequently in CMC regulatory support budgets, the question surfaces: 'What CMC activities can be postponed until later?' A far better wording of that question would be: 'What CMC activities can be postponed until later, *without incurring an unacceptable CMC regulatory compliance risk to the project and to the patients?*'

Inappropriate or inadequate oversight by senior management towards CMC regulatory compliance, or lack of commitment of appropriate resources, can be a recipe for significant delays either in advancing clinical development for biopharmaceuticals or keeping them out of the marketplace. A case example of senior management apparently not fully comprehending the potential seriousness of a CMC regulatory compliance problem, occurred at Genzyme Corporation in 2009. Genzyme, which commercially manufactured several lifesaving recombinant protein enzymes for children, encountered a viral contamination (identified as Vesivirus 2117) in one of their six bioreactors at their Massachusetts manufacturing site. Senior management announced in a press release on June 16, 2009 that they were 'temporarily' halting production at the site to sanitize the facility. Senior management also stated that they 'expect the manufacturing facility to be fully operational within about a month.' However, the press release also mentioned that Genzyme had two similar contamination problems previously in their large-scale bioreactors, one in Belgium and the other in the USA, just 1 year earlier. In those two 2008 contaminations, Genzyme never identified the root cause of the problem, but at least manufacturing operators observed decline in cell productivity so they immediately locked down the bioreactor so that the contents would not be released into the purification area but instead could be decontaminated. There was no discussion of any root cause analysis being seriously initiated in the 2008 incidents. Unfortunately, in the June 2009 contamination, there was no decline in cell productivity so manufacturing was unaware of the virus-contaminated bioreactor contents. The virus-contaminated bioreactor contents were not quarantined but instead passed into the purification suites, which then allowed the virus contamination to spread around the manufacturing facility. Only then was a full root cause analysis initiated, but unfortunately the damage was already done. In sum total, the manufacturing facility was shut down for approximately 6 months (not 1 month), a severe product shortage of the critical lifesaving recombinant proteins resulted, and significant revenue loss due to discarding of work-in-process batches. The extent of the facility recovery effort was described in a September 2009 press release by Genzyme: *"This effort required replacement of many fixtures at Allston Landing…. Five miles of insulation, one mile of copper tubing and fittings, and 660 feet of sanitary tubing and fittings were sanitized or replaced. Several key vessels were replaced during this period also. More than 700 fluorescent light lenses were removed and replaced. In addition, approximately 3253 valve diaphragms, 36,625 gaskets, 267 HEPA filters, 233 ball valves and 358 rebuild kits were used."* Subsequent inspection by the FDA led to a loss of confidence in the senior management leadership of the company and Genzyme was placed under a Consent Decree. Finally, Sanofi purchased Genzyme and took over the senior management leadership role [14].

A more recent case example of apparent inappropriate or inadequate oversight by senior management towards CMC regulatory compliance occurred during the COVID-19 pandemic and the rush to manufacture COVID-19 vaccines. Emergent BioSolutions was approved to manufacture two genetically engineered adenovirus vaccines: Johnson & Johnson's human adenovirus and AstraZeneca's chimpanzee adenovirus. Although these genetic vaccines are not regulated as gene-based

biopharmaceuticals, they are manufactured and controlled using similar CMC Regulatory, cGMPs and Quality System principles. In April 2021, Johnson & Johnson detected AstraZeneca's chimpanzee adenovirus contamination in a batch of their human adenovirus drug substance. This caused a flurry of reactions from the FDA, including rejection of specific Johnson & Johnson vaccine batches and removing completely the AstraZeneca manufacturing off of the manufacturing site. In May 2022, the U.S. Congress released a report entitled '*The Coronavirus Vaccine Manufacturing Failures of Emergent BioSolutions*', which was a scathing report on Emergent's senior management and their inability to effectively take responsibility and provide adequate support for the CMC regulatory compliance in their manufacturing facility [15]:

> *Emergent executives promoted the company's manufacturing capabilities despite being warned of severe deficiencies*. Documents obtained by the Committees reveal that before Emergent finalized manufacturing agreements with Johnson & Johnson and AstraZeneca, Emergent's then-Executive Vice President of Manufacturing and Technical Operations privately acknowledged that he had warned Emergent senior executives "for a few years" about the company's deficient quality systems, including that "room to improve is a huge understatement." Despite these internal warnings, Emergent entered into contracts with Johnson & Johnson and AstraZeneca to manufacture coronavirus vaccines for $482 million and $174 million, respectively. After manufacturing started, internal Emergent communications reveal that the Senior Director of Quality at the Bayview manufacturing facility stated, "Our risk is high!" and, "we lack commercial GMP [good manufacturing practices] compliance maturity."
>
> *FDA, Johnson & Johnson, and AstraZeneca identified multiple deficiencies at Bayview, which Emergent failed to remediate despite urgent warnings.* Documents reveal that the Trump Administration was aware, prior to awarding the contract in May 2020, of serious deficiencies at Emergent's Bayview facility that could impact manufacturing. In July 2020, AstraZeneca personnel raised concerns to Emergent about the need to remediate these deficiencies before starting manufacturing, noting that they were "concerned that the FDA observation was that Emergent isn't prepared for commercial manufacturing as things stand currently, and yet we will start commercial manufacture [sic] there very soon." Internal Johnson & Johnson communications from October 2020 show that Emergent had struggled to maintain quality standards and that it was "unclear" if the site was ready for commercial manufacturing and to "effectively manage all the remediation efforts." An outside consultant to Emergent provided a stark warning in November 2020 with regards to manufacturing: "I am stating very loudly that this work is NON-CGMP compliant. And a direct regulatory risk."
>
> Inexperienced staff and high staff turnover contributed to vaccine contamination. The investigation revealed that Emergent acknowledged in July and August 2020 that their staff were insufficiently trained, noting that "most temporary employees [have] little or no pharmaceutical experience." In November and December 2020, following persistent issues with contamination, AstraZeneca sent teams to Bayview because Emergent "lacked the appropriate level of knowledge or expertise." Ultimately, AstraZeneca concluded that "poor cleaning was part of the root cause." Internally, one Emergent executive posed questions on the state of the Bayview facility, asking, "When will all these trash going to be out of here? Trash are piling up." During a staff briefing, FDA acknowledged, "Clearly, in retrospect, they hired a lot of individuals not as familiar with vaccine manufacturing, that did not have adequate training to do so."

By the time it was all over nearly 400 million doses of Johnson& Johnson coronavirus vaccine had to be destroyed.

4.4 QbD/QRM Risk-Based Approach

Regulatory authorities have recommended a strategic risk-based approach that prioritizes the efforts for controlling a manufacturing process and ensuring that the product obtained has adequate and appropriate quality. Through the International Council on Harmonization (ICH), two published consensus regulatory guidelines address this risk-based CMC regulatory compliance strategy: ICH Q8(R2), *Pharmaceutical Development* and ICH Q9(R1), *Quality Risk Management*, see Fig. 4.6.

The focus of ICH Q8(R2) is on how "to design a quality product and its manufacturing process to consistently deliver the intended performance of the product" [16]. The guidance introduces the concept of Quality by Design (QbD), which is defined as 'a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management'. QbD consists of four major steps: (1) Quality Target Product Profile, (2) Critical Quality Attributes, (3) Critical Process Parameters, and (4) Control Strategy. It is important to stress that QbD's central message is to the development groups in the company (i.e., Process Development and Analytical Development). For it is from these groups that the 'sound science' of the manufacturing process and the product is determined.

The focus of ICH Q9(R1) is on developing "a systematic process for the assessment, control, communication, and review to the quality of the drug product across the product lifecycle" [17]. This guidance focusing on the concept of Quality Risk Management (QRM) encourages the application of statistical analysis tools (e.g., Design of Experiments, DOE) and the use of recognized risk management tools such as Failure Mode Effects Analysis (FMEA) and Risk Ranking and Filtering (RRF) to prioritize the elements of critically associated with the Quality Attributes and Process Parameters, into critical and non-critical categories.

Considering the challenges and complexities of biopharmaceuticals, the principles laid down by QbD/QRM are strategically important for an effective risk-based

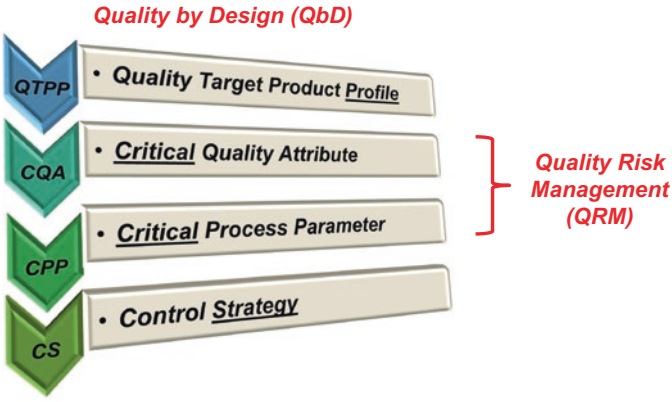

Fig. 4.6 QbD/QRM risk-based approach

management. There is no lack of papers and books written on how to apply QbD/QRM to biopharmaceuticals, but five references that I personally recommend are:

1. Quality by Design for Biopharmaceuticals: Principles and Case Studies; A.S. Rathore and R. Mhatre [18]
2. Quality by Design for Monoclonal Antibodies: Description of an Integrated System; series of papers by Genentech and Roche in *Biologicals* Volume 44 Issue 5 [19]
3. PDA Technical Reports on QRM and QbD [20]
4. NIIMBL: N-mAb, a Case Study [21]
5. ARM: Project A-Gene [22]

It should be noted that although the QbD/QRM risk-based approach is highly recommended by the regulatory authorities, it is not mandatory. However, as will be seen in the following sections, the terminology associated with QbD/QRM is the current language of communicating with the regulatory authorities in our CMC regulatory compliance submissions.

4.4.1 Quality Target Product Profile (QTPP)

The Quality Target Product Profile (QTPP), see Fig. 4.7, as defined in ICH Q8(R2), is '*a prospective summary of the quality characteristics of a drug product that ideally will be achieved to ensure the desired quality, taking into account safety and efficacy of the drug product*.' The QTPP is linked to the Target Product Profile (TPP) which is the corporate medical vision for the product. The important role of the QTPP is as a project management tool to keep all CMC disciplines (e.g., Development, Manufacturing, Quality Control, Quality Assurance, CMC Regulatory Affairs, etc.) moving together in the same direction toward a common target. The QTPP is a dynamic project management tool, being revised and updated either if the TPP changes or as the developing science requires change in what is possible from the manufacturing process or the product.

Fig. 4.7 Quality target product profile (QTPP)

The National Institute for Innovation in Manufacturing Biopharmaceuticals (NIIMBL), working in collaboration with over 20 biopharmaceutical companies, issued *N-mAb – A Case Study to Support Development and Adoption of Integrated Continuous Bioprocesses for Monoclonal Antibodies* [21]. In that report, a model TPP/QTPP was developed for protein-based biopharmaceuticals (specifically monoclonal antibodies). The corporate medical vision for the monoclonal antibody (TPP) was linked to the needed quality characteristics of the product (QTPP); the 'must have' and 'nice to have' became the target that the CMC team members had to work together on to achieve. Illustration of a general TPP/QTPP for a monoclonal antibody is found in Table 4.11.

The Alliance for Regenerative Medicine (ARM), working in collaboration with over 20 biopharmaceutical companies, issued *Project A-Gene – A Case Study-Based Approach to Integrating QbD Principles in Gene Therapy CMC Programs* [22]. In that report, a model QTPP was prepared for a gene therapy-based biopharmaceutical), specifically an *in vivo* AAV viral vector. Illustration of a general QTPP for a viral vector is found in Table 4.12.

Table 4.11 Illustration of a general TPP/QTPP for a monoclonal antibody

MONOCLONAL ANTIBODY	
Target Product Profile (TPP)	
Indication	**N-mab is a humanized IgG1 antibody intended as a treatment for indolent non-Hodgkin's Lymphoma (NHL) in an adult population**
Mechanism of Action (MOA)	**The mechanism of action for N-mab is through binding to a tumor cell surface antigen, Lymph-1, and stimulating B cell killing.**
Route of Administration	**Initial: IV administration Future: SC injection**
Quality Targe Product Profile (QTPP)	
Dosage Form	**Sterile liquid formulation Initial: single-use glass vial Future: single-use glass syringe**
Dosage Strength	**1 mL Initial: 75 mg/mL Future: 150 mg/mL**
Shelf-Life	**2-3 year stability refrigerated 2-4 week stability at room temperature**
CQAs to Control	**Glycosylation (N-glycans) Deamidation (Asn325) Aggregation Residual HCP impurity**

Table 4.12 Illustration of a general QTPP for a viral vector (AAV)

VIRAL VECTOR (AAV)	
Quality Target Product Profile (QTPP)	
Dosage Form	**Sterile frozen liquid formulation Single-use polymer vial**
Dosage Strength	**Under study**
Shelf-Life	**1-2 year stability frozen**
CQAs to Control	**Appearance (free of particulates) Identity of transgene (sequenced) Identity of capsid (≥ 90% SDS-PAGE) Content (vector genome titer) Content (virus infectivity) Purity (% empty capsids) Aggregation Residual endotoxin (within safety limit) Residual host cellular DNA Residual plasmid DNA Residual host cell protein**

The actual QTPP that was used as a guide in the development of market-approved monoclonal antibody, Phesgo (pertuzumab/trastuzumab) [23], was published in the EMA European Public Assessment Report (EPAR), and is shown in Table 4.13.

Since the QTPP is the product quality target pulling together all of the disciplines under the CMC team, it makes sense to prepare this target sooner than later. The QTPP is a living document, undergoing change either top down (i.e., change in the TPP, the corporate direction for the product) or bottom up (i.e., realization that some prospective characteristic for the desired product may not be scientifically feasible and needs to be changed).

4.4.2 Critical Quality Attribute (CQA)

A Critical Quality Attribute (CQA), see Fig. 4.8, as defined in ICH Q8(R2), is '*a physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality*'.

Looking at a biopharmaceutical, there are many attributes (also referred to as properties or characteristics) that can describe the quality of the product – descriptors either about the product itself (e.g., purity, potency), or product-related impurities (e.g., aggregates, oxidized variants), or process-related residual impurities (e.g., host cellular nucleic acids, host cell proteins), or obligatory pharmacopeial testing

Table 4.13 QTPP used as a guide for development of Phesgo (pertuzumab/trastuzumab)

Product Attribute	Target
Molecule Type	Two humanized monovalent monoclonal antibodies (based on IgG1 framework)
Indication	HER-2-positive early breast cancer (neoadjuvant and adjuvant) and first-line metastatic breast cancer
Patient Population	Adults
Patient Weight	Average 70 kg
Mechanism of Action Pertuzumab	A recombinant humanized monoclonal antibody that binds to the dimerization domain of HER2 (sub-domain II of the ECD). Upon binding to HER2, it inhibits the ability of HER2 to dimerize with other HERs (HER1, HER3, and HER4), thereby inhibiting multiple HER signaling pathways that mediate cancer cell proliferation and survival. Pertuzumab may also work by activating ADCC.
Mechanism of Action Trastuzumab	A recombinant humanized monoclonal antibody that binds specifically and with high affinity to sub-domain IV of the ECD of HER2 and inhibits the proliferation of human tumor cells overexpressing HER2 both *in vitro* and *in vivo*. Trastuzumab-mediated ADCC is preferentially exerted on HER2-overexpressing cancer cells compared with cancer cells that do not overexpress HER2.
Dosage Form and Strength	Single-dose, sterile, preservative-free solution for subcutaneous injection containing: LD: 80 mg/mL pertuzumab + 40 mg/mL trastuzumab with 2000 U/mL rHuPH20 MD: 60 mg/mL pertuzumab + 60 mg/mL trastuzumab with 2000 U/mL rHuPH20
Protein Content per Vial	LD: net quantity 1200 mg pertuzumab/600mg trastuzumab (15 mL nominal fill volume, 20 mL vial size) MD: net quantity 600 mg pertuzumab/600 mg trastuzumab (10 mL nominal fill volume, 15 mL vial size)
Dose	LD: 1200 mg pertuzumab/600 mg trastuzumab MD: 600 mg pertuzumab/600 mg trastuzumab
Administration	Subcutaneous, undiluted
Co-Administered Drugs	Concomitantly with chemotherapy or as a single agent (without chemotherapy)
Drug Product Container Closure System	Type I borosilicate glass vials, fluororesin-laminated stopper, aluminum seal with plastic flip-off cap
Shelf Life	Minimal claim at submission drug product shelf life ≥ 18 months (target ≥ 21 months) at 2°C–8°C
Stability during Administration	Physicochemically stable for 28 days at 2°C–8°C and up to 24 hours at 9°C–30°C in disposable syringes.

requirements (e.g., pH, concentration), or safety from adventitious agent contaminants (e.g., bacterial endotoxin, mycoplasma). For a biopharmaceutical, the list of quality attributes that define quality (referred to as quality attributes or QA) can easily be over 30 for either a monoclonal antibody or an *in vivo* AAV viral vector. The challenge is in narrowing down which QAs are actually CQAs. ICH Q11,

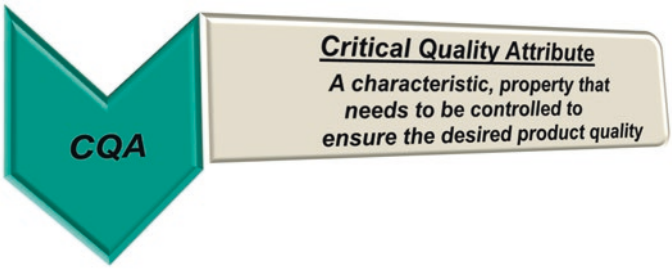

Fig. 4.8 Critical Quality Attribute (CQA)

Development and Manufacture of Drug Substances (Chemical Entities and Biotechnological/Biological Entities), provides practical guidance on handling the challenge of determining CQAs for biopharmaceuticals [24]:

> Drug substance CQAs typically include those properties or characteristics that affect identity, purity, biological activity and stability. When physical properties are important with respect to drug product manufacture or performance, these can be designated as CQAs. In the case of biotechnological/biological products, most of the CQAs of the drug product are associated with the drug substance and thus are a direct result of the design of the drug substance or its manufacturing process.
>
> Impurities are an important class of potential drug substance CQAs because of their potential impact on drug product safety… For biotechnological/biological products, impurities may be process-related or product-related (see ICH Q6B). Process-related impurities include: cell substrate-derived impurities (e.g., Host Cell Proteins (HCP) and DNA); cell culture-derived impurities (e.g., media components); and downstream-derived impurities (e.g., column leachables). Determining CQAs for biotechnology/biological products should also include consideration of contaminants, as defined in Q6B, including all adventitiously introduced materials not intended to be part of the manufacturing process (e.g., adventitious viral, bacterial, or mycoplasma contamination).
>
> The identification of CQAs for complex products can be challenging. Biotechnological/biological products, for example, typically possess such a large number of quality attributes that it might not be possible to fully evaluate the impact on safety and efficacy of each one. Risk assessments can be performed to rank or prioritise quality attributes. Prior knowledge can be used at the beginning of development and assessments can be iteratively updated with development data (including data from nonclinical and clinical studies) during the lifecycle. Knowledge regarding mechanism of action and biological characterisation, such as studies evaluating structure-function relationships, can contribute to the assessment of risk for some product attributes.

In general, there is a three-step process in sorting out the QAs to identify the CQAs. The first step is the obvious one – identify and list all of the quality attributes associated with the biopharmaceutical. The second step is to carry out a risk assessment on all of the listed QAs and determine the risk score/risk priority number for each QA. ICH Q9 (Quality Risk Management) provides the risk assessment tools that are most useful (e.g., Risk Ranking and Filtering [RRF], Failure Mode Effects Analysis [FMEA]). Finally in the third step, set a threshold for the risk score/risk priority number, in which everything above the threshold will be listed as a CQA and everything below the threshold will be listed as a non-CQA.

Early in clinical development, the identification of CQAs, actually potential CQAs (pCQAs), is necessary to guide manufacturing process and product development efforts. The potential CQA list can either increase (pCQA moves up to CQA) or decrease (pCQA moves down to non-CQA) during the course of clinical development as the science of the process and product are investigated and better understood. In the market approval submission, the list of potential CQAs become CQAs.

General guidance on carrying out the QA ↔ CQA risk assessment for monoclonal antibodies has been published by the CMC Biotech Working Group (development scientists from seven major biopharmaceutical companies) – *A-Mab: A Case Study in Bioprocess Development* [25]. The QAs for product-related impurities and the process-related impurities were criticality ranked, using three different risk-ranking tools: (1) Risk Ranking and Filtering (RRF), (2) Preliminary Hazards Analysis (PHA), and (3) Safety Assessment. The higher the risk score/risk priority number, the more likely the decision would be to assign that QA as a CQA. Table 4.14 presents a summary of their QA ↔ CQA risk assessment, using the Risk Ranking and Filtering tool.

General guidance on carrying out the QA ↔ CQA risk assessment for viral vector biopharmaceuticals has been published by the Alliance for Regenerative Medicine, in their issued *Project A-Gene – A Case Study-Based Approach to*

Table 4.14 Example of a risk assessment to assign criticality to QAs for a monoclonal antibody

MONOCLONAL ANTIBODY Risk Ranking and Filtering CQA RISK ASSESSMENT			
Product Quality Attribute	**Impact**	**Uncertainty**	**Risk Score**
Non-Glycosylated Heavy Chain	16	5	80
High Mannose Content	16	5	80
Sialic Acid Content	12	5	60
Afucosylation	20	3	60
Aggregation	12	5	60
Galactose Content	16	3	48
Residual Host Cell Proteins	12	3	36
CQA ↑		Non-CQA ↓	
Residual Protein A	16	1	16
Residual Methotrexate	16	1	16
Oxidation	4	3	12
Residual Host Cellular DNA	2	3	6
C-Terminal Lysine	2	2	4
Deamidated Isoforms	2	2	4

Integrating QbD Principles in Gene Therapy CMC Programs [22]. The QAs for product-related impurities and the process-related impurities were criticality ranked, using Preliminary Hazards Analysis (PHA). The higher the risk score, the more likely the decision would be to assign that QA either as a potential CQA (pCQA) or a CQA. Note how many pCQAs and CQAs are present, and so few non-CQAs, due to the increased complexity of a viral particle (compared to a protein) and due to the increased severity of the process-related impurities for a gene therapy-based biopharmaceutical. Table 4.15 presents a summary of their QA ↔ pCQA ↔ CQA risk assessment, using the Preliminary Hazards Analysis tool.

The risk assessment approach used in determining CQAs for the market-approved monoclonal antibody, Gazyvaro (obinutuzumab), was published in the EMA European Public Assessment Report (EPAR) [26]. The manufacturer used several different risk assessment tools to prioritize criticality for different quality attribute groups:

1. ***Product-related and residual process-related impurities***. Risk prioritized by Risk Ranking and Filtering (RRF)

Table 4.15 Example of a risk assessment to assign criticality to QAs for an *in vivo* AAV product

ViRAL VECTOR (AAV) Risk Ranking and Filtering CQA RISK ASSESSMENT			
Product Quality Attribute	**Severity**	**Uncertainty**	**Risk Score**
Potency	10	3	30
Vector Genome Titer	10	2	20
Capsid Protein Purity	10	2	20
Residual Endotoxin	10	1	10
Sterility	10	1	10
Visible Particulates	10	1	10
Capsid Identity	10	1	10
Genome Identity	10	1	10
Replication Competent Virus	10	1	10
CQA ↑	Potential CQA ↓		
Capsids (% Full)	3	3	9
Residual Plasmid DNA	3	1	3
Residual Host Cellular DNA	3	1	3
Residual Transfection Reagent	3	1	3
Residual Host Cell Protein	1	1	1

Table 4.16 Multiple risk assessment approaches used to determine CQAs for Gazyvaro mAb

Product Quality Attribute	Risk Assessment Approach	Rational for Risk Approach Applied
Product Variants Structural Variants Glycosylation	CQA RRF	Impact to patient safety and product efficacy
Process-Related Impurities Host cell DNA Host Cell Proteins	CQA RRF	Impact to patient safety and product efficacy
Raw Materials Cell culture and purification components (nutrients, trace elements, salts, buffers, etc.)	Toxicological Comparison (EDI_o and ADE)	CQA if EDI_o is > ADE
Leachables From process equipment (e.g., filters) and/or primary packaging materials (e.g., stoppers)	Trace Analysis	CQA if detected above trace level
Adventitious Agents Viral purity, Microbiological purity, Endotoxin	None Required	Patient safety risk: Obligatory CQAs
Drug Substance **and/or Drug Product** Protein content Visible Particles Appearance Sterility	None Required	Patient safety and product efficacy risk: Obligatory CQAs

2. ***Residual raw material-related impurities***. Risk prioritized by toxicological safety assessment (i.e., estimated daily intake – EDI_o; and acceptable daily intake – ADE)
3. ***Leachable-related impurities***. Risk prioritized only if detectable
4. ***Obligatory CQA assignments***. Required safety and pharmacopeial testing requirements. Automatically assigned as CQAs.

A summary of these four separate risk assessments is presented in Table 4.16.

4.4.3 Critical Process Parameter (CPP)

A Critical Process Parameter (CPP), see Fig. 4.9, as defined in ICH Q8(R2), is '*a process parameter whose variability has an impact on a critical quality attribute and therefore should be monitored or controlled to ensure the process produces the desired quality*'.

Looking at the biopharmaceutical manufacturing process, there are many process steps (e.g., cell expansion, production, harvest, chromatographic purification,

Fig. 4.9 Critical Process Parameter (CPP)

etc.), and each process step has numerous sub-steps (e.g., for a chromatographic purification step there is column prep, equilibration, loading, wash, eluting, strip, recycle), and each sub-step has various process parameters (e.g., for eluting from a chromatographic sub-step, there is pH, conductivity, resin use age, etc.). The challenge is to identify which of the many process parameters across all of the sub-steps across all of the process steps are critical process parameters (CPPs). CPPs need to be monitored and controlled to ensure that the manufacturing process produces the biopharmaceutical having the desired quality, safety and efficacy.

To determine a CPP, in principle, seems fairly straightforward, using a similar three step process that was used previously for determining CQAs. Step 1, make two lists; one to list all of the manufacturing process steps and one to list all of the identified CQAs. From these two lists, determine which CQAs most likely might be impacted by which manufacturing process steps. For example, the CQA for glycosylation profile. Carbohydrates are added post-translationally to a monoclonal antibody during the upstream expression process step. The glycosylation profile may or may not be impacted by any of the upstream cell culturing steps prior to expression, and from experience will not be impacted by any of the downstream purification process steps. So, the process parameters for only the upstream cell culturing and expression steps would need to be closely examined for impact on the glycosylation profile CQA. In another example, the CQA for residual host cell proteins (HCPs). HCPs (typically form cells that have lysed) are generated during the cell culturing processes of the upstream production steps. So, the process parameters for the cell culturing production steps would need to be closely examined to see which parameters might increase the starting level of HCP, and the process parameters for the downstream purification process steps (at least up to the nanofiltration process step) would need to be closely examined to see which parameters might increase the removal of residual host cell proteins.

Then Step 2, vary each identified process parameter and measure the impact on the identified CQA(s). Design of experiment (DOE) studies will be needed in Step 2 due to the magnitude of interactions that need to be assessed. Critical decisions

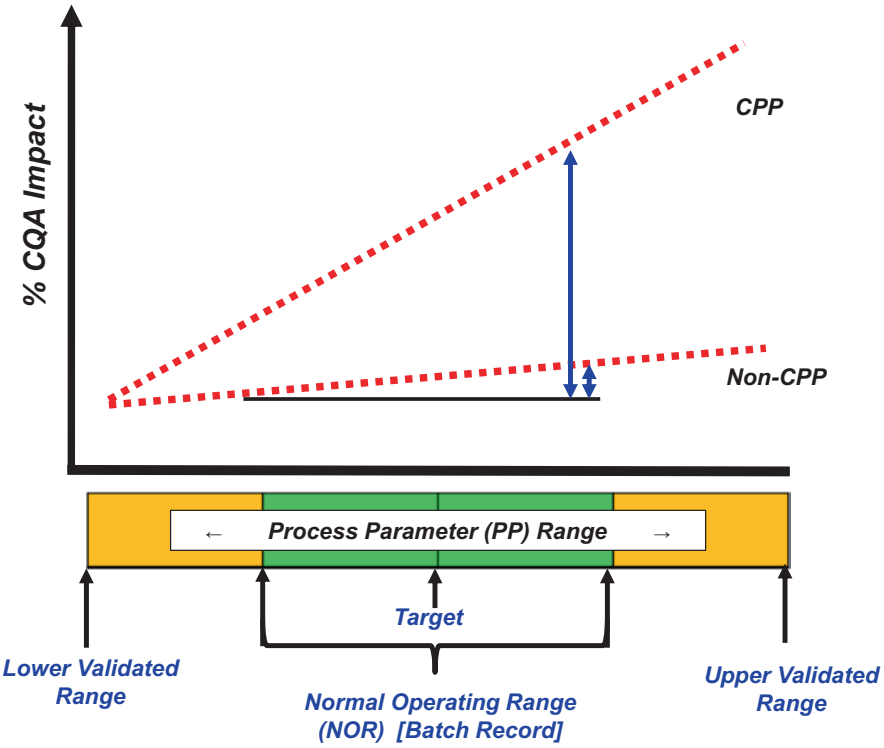

Fig. 4.10 Determining a CPP versus a non-CPP by the degree of % CQA impact

need to be made: (1) how many of the numerous process parameters should be examined, (2) what process parameter ranges should be evaluated to measure the impact on the CQAs, and (3) what are the relevant CQAs to examine for impact by each process parameter. As illustrated in Fig. 4.10, not all CQA impacts rise to the level of a CPP. Sometimes the phrase 'statistically significant' or 'practically significant' is assigned to the level of % CQA change that leads to defining a CPP. A must read before attempting a CQA impact study is an updated and improved mathematical approach to determining the impact ratio (i.e., the degree of change on a CQA) published by Hoffmann-La Roche/Genentech – Lamerz, J, et al., in the PDA Journal of Pharmaceutical Science and Technology, 2022; 76(6), 497–508 [27].

Finally, Step 3, determine how much impact on a CQA value is necessary for a specific process parameter to be considered a CPP.

A CPP will have a specified range listed in the production batch record in which operational control must be maintained. Falling outside of the range will result in an investigation and may likely lead to a rejected batch due to potential effect on a CQA. Some manufacturers, in addition to assigning CPPs and non-CPPs, also assign key process parameters (KPPs). A KPP does not significantly impact a CQA but it impacts manufacturing process performance (e.g., step yield). A KPP will also

Table 4.17 FDA recommended table format for communicating CPPs

Process Parameter/ Operating Parameter/ In-Process Control (IPC)	Proposed Range for Commercial Manufacturing	Criticality Classification	Range Assessed During Process Development Studies	Validated Range	Clinical Study Range	Justification of the Proposed Commercial Acceptable Range

have a control range assigned to it in the production batch record, and falling outside of the control range will trigger a quality action to determine if there was any quality impact at all, and the final outcome of the batch.

Communicating CPPs and non-CPPs (and KPPs) uniformly to various regulatory authority reviewers has been challenging, with each manufacturer using their own charting system for capturing all of the decisions that went into the final list. The FDA is now providing a recommended table to assist in this effort for the BLA submission [28, 29], see Table 4.17. (Note, there are slight variations in the headers of the columns, which is why two references are provided.) The instructions provided with the tabular format are: (1) use a separate table for each process unit operation (therefore there will be many tables – e.g., a table for harvesting, a table for Protein A affinity, a table for nanofiltration, etc.), (2) process parameter information listed in the tables is expected to be summarized from the Module 3 content (not replace the content in Module 3), (3) the tables should be submitted either in Module 1 or Module 3R of the BLA. Also:

1. For each process parameter listed in column 1 (the yellow column), a critically classification (the green column, 3) is to be listed – CPP, non-critical CPP, KPP
2. The various ways of calculating the process parameter ranges are to be listed in columns 2, 4–6
3. In the last column (column 7) of the table, the FDA recommends just a brief justification for the acceptable range (e.g., 'development range', 'validation range', 'platform experience').

4.4.4 Control Strategy (CS)

Control Strategy (CS), see Fig. 4.11, as defined in ICH Q10, is '*a planned set of controls, derived from current product and process understanding, that assures process performance and product quality. The controls can include parameters and attributes related to drug substance and drug product materials and components, facility and equipment operating conditions, in-process controls, finished product specifications, and the associated methods and frequency of monitoring and control*.'

Fig. 4.11 Control Strategy (CS)

According to ICH Q11, there are at least four main components in a biopharmaceutical control strategy [24]:

> A control strategy can include, but is not limited to, the following:
>
> - Controls on material attributes (including raw materials, starting materials, intermediates, reagents primary packaging materials for the drug substance, etc.);
> - Controls implicit in the design of the manufacturing process (e.g., sequence of purification steps [biotechnological/biological drug substances], or order of addition of reagents [chemical entities]);
> - In-process controls (including in-process tests and process parameters);
> - Controls on drug substance (e.g., release testing).

1. **Controls on raw material attributes**. Critical raw materials carry a significant risk to the quality of the biopharmaceutical under manufacture. Biological-based raw materials carry the extra risk of adventitious agent contamination and frequently they are mixtures presenting potential batch-to-batch variation. Various cell culture media components, for example, can contain heavy metal ion levels that can impact glycosylation results on the biopharmaceutical. Such was the case during the manufacture of the antibody-drug conjugate, Polivy (polatuzumab vedotin), which had its glycosylation impacted by the manganese levels in the media [30]:

   > Process validation was performed following a two-tiered approach. Four consecutive process performance qualification (PPQ) batches were produced at full-scale using the commercial v1.0 process and six additional clinical batches produced according to the same v1.0 manufacturing process were compared to the four PPQ batches. Evaluation of CQAs, IPCs, and performance parameters demonstrated that the manufacturing process is consistent throughout the process. A shift in glycosylation profile (sum of afucosylation and G0F) was observed between v1.0 clinical and v1.0 PPQ batches. This shift was assigned to increased levels of trace element manganese impurity. As a result, a manganese acceptance criterion was implemented and a verification batch was produced, showing glycosylation levels comparable to previous v1.0 clinical manufacturing runs.

2. **Controls implicit in the design of the manufacturing process**. This could involve cell culture production (e.g., length of time in production phase in bioreactor might be extended to allow protein/virus to be produced, although at lower productivity; however, cells are dying, lysing, releasing extra cellular impurities into the solution, which might eventually overburden the capacity of the down-

stream purification steps) and/or purification (e.g., there are multiple choices for a final 'polishing' chromatography step in purification – anion exchange, cation exchange, hydrophobic interaction, size exclusion – but what is the correct order of steps for the specific biopharmaceutical?)

3. **Controls on in-process tests**. These tests are used to provide assurance that the manufacturing process is performing as expected. They are typically assigned action limits rather than specifications. However, for biopharmaceuticals, sometimes the in-process tests have specified limits (e.g., the testing of the bioreactor culture prior to harvest to demonstrate the absence of adventitious agent contamination).
4. **Controls on the product (using CQAs) and the process (using CPPs).** These have already been discussed in the previous sections (Sects. 4.4.2 and 4.4.3).

As indicated above, the control strategy for the biopharmaceutical should be a combination of these four main components. All too often, product specifications (CQAs) only are considered, rather than building in more quality reliability through the design and control of the manufacturing process and the control over the raw materials introduced into the manufacturing process.

The challenge is how to present the complete control strategy to a regulatory authority, especially for their review when seeking market approval. The FDA is now providing a recommended table to assist in this effort for the BLA submission [28, 29], see Table 4.18. (Note, there are slight variations in the headers of the columns, which is why two references are provided.) The instructions provided with the tabular format are: (1) CQAs listed in the tables are expected to be summarized from the Module 3 content (not replace the content in Module 3), and (2) the tables should be submitted either in Module 1 or Module 3R of the BLA. Also:

1. For each CQA listed in column 1 (the yellow column), the proposed control strategy for that CQA (the green column, 5) is to be listed – e.g., in-process, validation removal, release testing, shelf-life testing
2. In column 2, the area of impact of the CQA is to be listed – e.g., contributes to potency, immunogenicity, safety, efficacy

Table 4.18 FDA recommended table format for communicating the Control Strategy

Critical Quality Attributes (Including Process and Product Related Impurities for DS and DP)	Impact	Source	Analytical Mehtod	Proposed Control Strategy	Justification of the Proposed Control Strategy

3. In column 3, the source of the CQA – e.g., intrinsic to molecule, fermentation, purification column.
4. In column 4, all methods that can test the CQA are to be listed – e.g., in-process, release, shelf-life
5. In the last column (column 6) of the table, the FDA recommends just a brief justification for the proposed control strategy

4.4.5 Design Space for Biopharmaceuticals

Design space, as defined in ICH Q8(R2), is '*the multidimensional combination and interaction of input variables (e.g., material attributes) and process parameters that have been demonstrated to provide assurance of quality. Working within the design space is not considered as a change. Movement out of the design space is considered to be a change and would normally initiate a regulatory post approval change process. Design space is proposed by the applicant and is subject to regulatory assessment and approval*.'

Design space can be applied to individual manufacturing process steps or to an entire manufacturing process. ICH Q11 presents an example of design space applied to an individual purification process step. A graphical representation of removal of three different process-related impurities (i.e., viruses, host cell DNA, and host cell proteins) by an anion exchange chromatography step [24] is shown in Fig. 4.12. In the figure, the three boxes on the right represent test ranges for the three process-related impurities across the same two chromatography process parameters (i.e., conductivity and pH). Within the process parameter ranges, a satisfactory clearance for each of three individual process-related impurities was obtained (viral clearance, DNA, host cell protein) – as denoted by the white box within each box. In the figure, the large box on the left represents the superimposed overlay of the three process-related impurity boxes from the right. The white box within the large box on the left is labeled "design space". Design space is the range of the two chromatography process parameters, which yield satisfactory clearance of all three process-related impurities. While the "sweet spot" (i.e., the design space) in the figure looks impressive, and is scientifically instructive, obtaining that design space came only with a major investment in resources for exploring the operational conditions of the anion exchange chromatography process step.

Obtaining regulatory authority freedom to operate within a design space requires the manufacturer to convince the regulatory authority that there is no 'residual uncertainty' – that all aspects of understanding a specific process step are known and under control by the manufacturer – if granted that freedom. Due to the manufacturing challenges and the complexity of biopharmaceuticals, obtaining design space has been most difficult, if not possible, to date. Only one manufacturer has succeeded in obtaining market-approval for their biopharmaceutical with a design space designation: Genentech's monoclonal antibody obinutuzumab, referred to as Gazyva in the USA and as Gazyvaro in the EU:

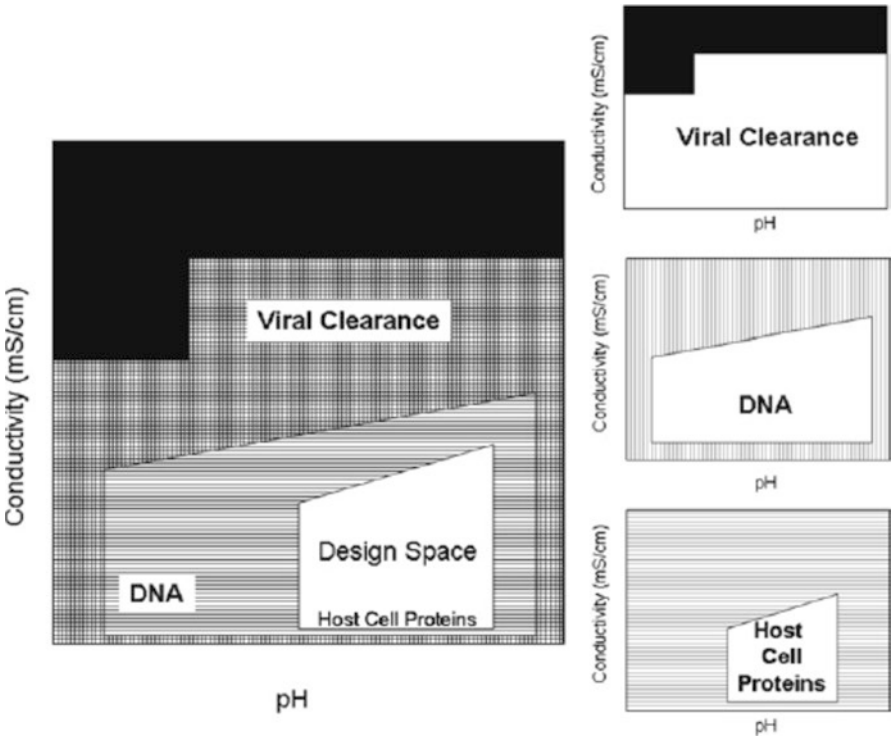

Fig. 4.12 Illustration of design space applied to an anion exchange chromatography step

FDA market approval of Gazyva November 2013 [31]

Upon review of the supporting date, the design space as proposed in BLA 125486 was found to be acceptable. The Agency would like to reiterate that in addition to the information described in the application, it is our expectation that plans for implementation of the design space for the commercial process are documented within the firm's Quality System. Such quality systems may include plans for handling movements within the design space (e.g., change control procedures, plans for updating batch records). In accordance with ICH Q8(R2), while the Agency does not expect any regulatory notification for movements within the design space, any other changes in the manufacturing, testing, packaging, or labeling or manufacturing facilities for GAZYVA (obinutuzumab) will require the submission of information to your biologics license application for our review and written approval, consistent with 21 CFR 601.12.

EMA market approval of Gazyvaro May 2014 [26]

Quality by Design (QbD) principles have been applied during the development of obinutuzumab. The design space of obinutuzumab includes all the unit operations, the process parameters describing the operation of each of the unit operations, and the raw materials used. The design space is limited by the Multivariate Acceptable Ranges (MARs) for all process parameters (CPPs and non-CPPs) described in the dossier. Changes to the targets for all process parameters within their MARs are considered to be movement within the design space. Changes to the MARs of CPPs or non-CPPs would be considered to be movement outside the design space. Even though a huge quantity of data was provided by the Applicant, a sum of uncertainties at all steps of the building of the design space led to doubts, at Day 120, on its suitability. Following the Applicant's Day 120 and Day 180

> responses, the management of remaining uncertainties was sufficiently addressed. Especially the final proposed control strategy, which does take into account remaining uncertainties led to the overall conclusion that the claimed design space is considered acceptable.

Surprisingly, Genentech has not talked much about this success. Possibly, because they would have to explain that unique success against the return-on-investment (ROI) to get it.

Today, to ensure that regulatory authority reviewers do not mix up a biopharmaceutical company's intent of filing under QbD principles without seeking design space freedom, various phrases are used in the submitted market application dossiers such as 'using principles of QbD' or 'applying QbD elements'.

4.5 Limitations of Risk-Based CMC Regulatory Compliance

Quality by Design (QbD) needs to be applied during clinical development, sometimes when it is uncertain or too early to determine if the biopharmaceutical product will ever make it into the marketplace. Since QbD costs money to effectively put in place, the need to implement it during this clinical development period can be stumbling block to senior management in terms of their willingness to support the risk-based CMC efforts. A critical thought for senior management – if not willing to invest in QbD, what are the options? QbD is investing in success. If not QbD, then what? QbC (Quality by Chance) is not a viable option!

The weakest link in any risk-based CMC regulatory compliance strategy is typically the staff involved with Quality Risk Management (QRM) in making the risk criticality assignments. Inexperienced or incompetent or fatigued staff are not going to lead to any kind of useful strategic CMC guidance; but, instead will lead to a document that will sit on a shelf rather than used to guide the CMC team members. QRM requires teams working together to reach a consensus decision. The current draft of ICH Q9(R1) brings to the forefront the critical concern of 'subjectivity' (versus objectivity) in making these consensus risk assignment determinations [32]:

> Quality risk management activities are usually, but not always, undertaken by interdisciplinary teams. When teams are formed, they should include experts from the appropriate areas (e.g., quality unit, business development, engineering, regulatory affairs, production operations, sales and marketing, supply chain, legal, statistics and clinical) in addition to individuals who are knowledgeable about the quality risk management process. Subjectivity can impact every stage of a quality risk management process, especially the identification of hazards and estimates of their probabilities of occurrence, the estimation of risk reduction and the effectiveness of decisions made from quality risk management activities. Subjectivity can be introduced in quality risk management through differences in how risks are assessed and in how hazards, harms and risks are perceived by different stakeholders. Subjectivity can also be introduced through the use of tools with poorly designed risk scoring scales. While subjectivity cannot be completely eliminated from quality risk management activities, it may be controlled by addressing bias, the proper use of quality risk management tools and maximising the use of relevant data and sources of knowledge (see ICH Q10, Section II.E.1). All participants involved with quality risk management activities should acknowledge, anticipate, and address the potential for subjectivity.

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Chapter 5
Ever-Present Threat of Adventitious Agent Contamination

Abstract If there is one serious patient safety concern that permeates every aspect of biopharmaceutical manufacturing, it is the ever-present threat of introducing and then further propagating adventitious agent contamination. Adventitious agents can enter into the manufacturing process through raw materials, starting materials, and operation of the upstream cell/seed culture manufacturing steps; but also, even the operation of the downstream and drug product manufacturing process steps. Because biopharmaceutical manufacture is linked to living systems, if adventitious agents are introduced into the biological process, they have the potential of being propagated and causing even greater patient harm. In this chapter, the four primary adventitious agents of concern for biopharmaceuticals are examined in detail – prions, viruses, mycoplasmas, and bacteria/fungi. The various complementary risk mitigating approaches for controlling for these contaminating agents are discussed. The strength of these risk mitigating approaches varies between the protein-based biopharmaceutical processes (recombinant proteins and monoclonal antibodies) and the gene therapy-based biopharmaceutical processes (viral vectors and genetically modified patient cells). Where it may be appropriate to use a risk-based minimum CMC regulatory compliance continuum approach in controlling for adventitious agent contamination is also considered. While each manufacturing process type has a different level of risk due to adventitious agent contamination, there is no biopharmaceutical manufacturing process that carries no risk of adventitious agent contamination.

Keywords Adventitious · Microbiological · Prion · TSE · Virus · Clearance · Competency · Mycoplasma · Bacteria · Fungi · NAT · RMM · PCR · Replication-competent · NGS

J. Geigert, *The Challenge of CMC Regulatory Compliance for Biopharmaceuticals*, https://doi.org/10.1007/978-3-031-31909-9_5

5.1 Risk-Managing the Ever-Present Threat

If there is one serious patient safety concern that permeates every aspect of biopharmaceutical manufacturing, it is the ever-present threat of introducing and then further propagating adventitious agent contamination. An adventitious agent is defined as "*contaminating microorganisms of the cell culture or source materials including bacteria, fungi, mycoplasmas/spiroplasmas, mycobacteria, Rickettsia, protozoa, parasites, transmissible spongiform encephalopathies (TSE) agent, and viruses that have been unintentionally introduced into the manufacturing process of a biological product*" [1]. The four primary adventitious agents of concern for biopharmaceuticals are examined in detail – (1) prions, (2) viruses, (3) mycoplasmas, and (4) bacteria/fungi.

These contaminates can enter into the manufacturing process through numerous portals – raw materials, starting materials, operational conditions of the upstream cell/seed culture production steps, operational conditions of the downstream purification steps, and even operational conditions of the drug product manufacturing steps. ICH Q9(R1) *Quality Risk Management* [2] lays out a three-step risk-managed approach for minimizing this threat of adventitious agent contamination:

Step 1: *Risk Assessment*. This first step involves the identification, analysis, and prioritization of the risks associated with potential exposure of the adventitious agents to the biopharmaceutical manufacturing process. It attempts to answer the questions of (1) what are the portals of entry in the manufacturing process for each of the different types of adventitious agents, (2) what might go wrong during the manufacturing process that could allow adventitious agent entry, and (3) what might be the seriousness of the patient safety consequence if something does go wrong and the adventitious agent enters the manufacturing process.

↓

Step 2: *Risk Control*. This second step requires a thorough and scientific decision-making process to determine (a) what actions are necessary to prevent the different types of adventitious agents from entering into the manufacturing process (i.e., erecting checkpoints and barriers to entry), (b) what testing might be necessary throughout the manufacturing process to confirm the absence of entry by these adventitious agents, and (c) what potential inactivation or removal actions might be possible should adventitious agents enter.

↓

Step 3: *Risk Review*. This third step addresses the need to re-evaluate the initial risk assessment and established risk controls whenever new knowledge about adventitious agents is obtained or new experiences with adventitious agent contaminations occur in the biopharmaceutical manufacturing process.

Not every biopharmaceutical manufacturing process carries the same identical risk of contamination from each of the four primary adventitious agents. But there is no biopharmaceutical manufacturing process that carries no risk of adventitious agent contamination.

The following adventitious agents will be examined in detail to illustrate the approaches that biopharmaceutical manufacturers can use to control for the risks:

Section 5.2 Adventitious prions
Section 5.3 Adventitious viruses
Section 5.4 Adventitious mycoplasmas
Section 5.5 Adventitious bacteria/fungi

5.2 Adventitious Prions

Prions (a term coined by blending the words 'protein' and 'infection') are proteinaceous infectious agents. The accumulation of prions can cause a rare but fatal neurological illness, referred to as transmissible spongiform encephalopathy (TSE). According to the U.S. Centers for Disease Control and Prevention (CDC), several prion diseases have been identified in both animals and humans [3], see Table 5.1.

Prions remain a mystery even today. Animals and humans have a normal cellular glycoprotein known called PrP (proteinaceous infectious particle). The theory holds that PrP is normally in a stable shape (pN) that does not cause disease. The protein can be flipped, however, into an abnormal shape (pD) that does cause disease. pD is infectious because it can associate with pN and convert it to pD, in an exponential process – each pD can convert more pN to pD. Exponential amplification of the prion (converting pN into pD in the body) would then result in disease. All of the TSE diseases have a long incubation period, and eventually lead to dementia and death. There is no treatment and, thus, no cure. Infectious prions continue to remain a mystery. Appearing to be in violation of the central dogma of molecular biology in that genetic information passes from nucleic acids to protein, no hypothesis has been proven to explain either the protein-only composition of infectious prions or the mechanism for their formation in the neurons of infected hosts [4].

It should be noted that prions have also been discovered in yeast and filamentous fungi. Although these yeast and fungal proteins share common characteristics with mammalian prion protein (e.g., infectious self-perpetuating formation of β-sheet aggregates), they are not considered harmful to their host and do not appear to be associated with any human disease state [5].

Table 5.1 Known prion diseases

Human Prion Diseases	Animal Prion Diseases
Creutzfeldt-Jakob Disease (CJD)	Bovine Spongiform Encephalopathy (BSE)
Variant Creutzfeldt-Jakob Disease (vCJD)	Feline Spongiform Encephalopathy
Fatal Familial Insomnia	Chronic Wasting Disease (CWD)
Kuru	Scrapie

Due to the potential risk to patient safety and the inability of knowing if infectious prions are present, prions must be prevented from entering any portal of the manufacturing process.

5.2.1 Prion Risk Assessment

The level of patient safety risk from prion contamination is a function of three factors:

(1) *Probability of prions being present*. There are only two expected pathways of entry of prions into a biopharmaceutical manufacturing process: (1) specific starting materials (e.g., transgenic animals as starting materials for recombinant proteins, human donor cells as starting material for allogeneic genetically engineered cells), and (2) exposure to animal- or human-derived raw materials during the manufacturing process (e.g., fetal bovine serum).

(2) *Potential severity if prions contaminate the biopharmaceutical*. If the manufactured biopharmaceutical contains prions, the patient runs the potential risk of encountering significant neurological problems and eventual death. Unfortunately, this has occurred, in the past, from natural protein isolated from human cadaveric pituitary starting material. The natural human growth hormone isolated from this starting material was contaminated with prions, in which some of the patients acquired iatrogenic Creutzfeldt-Jakob disease (iCJD) [6]:

> The general term CJD comprises sporadic CJD (sCJD), iatrogenic CJD (iCJD), and familial CJD (fCJD) … a small percentage (less than 1%) of CJD cases are iatrogenic (iCJD) and are acquired through transplantation of dura mater from donors with CJD or through injections of cadaveric pituitary human growth hormone (hGH) from contaminated preparations. Among the nearly 7,700 people exposed to cadaveric pituitary hGH in the National Hormone and Pituitary Program (NHPP) in the United States (U.S.), 33 cases of iCJD have been reported. All the NHPP iCJD cases to date have occurred among about 2,600 people who began cadaveric pituitary hGH treatment prior to 1977, with an average treatment duration of 8.2 years. The average incubation period between the start of NHPP cadaveric pituitary hGH exposure and onset of CJD symptoms is 28 years and the time between the start of NHPP cadaveric pituitary hGH treatment and the first sign of CJD symptoms ranges from 14 to 45 years.

(3) *Detectability of prions*. For all biopharmaceutical manufacturers, infectious prion detection presents a nightmarish situation. There is currently no rapid nor easy test to distinguish a normal protein versus an abnormal protein. So, the manufacturer may not even be aware that prions are present. The most reliable tests for measuring prion infectivity are the *in vivo* mouse or hamster bioassays that require intracranial inoculation of the biological sample into the animal, followed by a multi-month incubation, and finally a necropsy on their brains. It

is important to point out that the lack of detectability also hinders confirmation of removal of prions from manufacturing process equipment exposed to prion-containing materials. TSE agents are quite resistant to most disinfecting regimens, and there is no current consensus on specific details of decontamination requirements. However, some methods of decontamination include steam autoclaving at 132 °C for 1–4 h, or treatment with 1N or 2N NaOH for at least 1 h. These treatments are known to diminish, but may not eliminate, infectivity. The most reliable method of clearing prion contamination is by removing the contacted equipment out of the manufacturing facility or to use single-use equipment (i.e., disposable). Hence, biopharmaceutical manufacturers are extremely cautious about exposing their facility or manufacturing process equipment to potentially prion-containing materials.

5.2.2 Prion Risk Control

Risk control for prions, which must be in place from manufacture of biopharmaceutical batches for first-in-human (FIH) clinical studies onward, considers the necessary steps to either eliminate or mitigate prion contamination of the biopharmaceutical from those manufacturing process steps identified in the risk assessment as being a potential contributor of prions. Risk control for prion contamination involves choices. If the manufacturer choses to use raw materials that are animal- or human-derived, then the biopharmaceutical manufacturer must carry out appropriate risk controls to minimize the risk of exposure to prion contamination. If the manufacturer choses to use starting materials directly-derived from an animal or human source, then a higher prion risk is present.

There are three complementary risk mitigating approaches used for controlling adventitious agent contaminations – (1) barriers to entry, (2) testing to confirm absence, and (3) inactivation/removal to ensure absence in a biopharmaceutical solution. For prions, testing to confirm absence is not feasible and inactivation/removal to ensure absence has only been applied to recombinant protein manufacturing processes from transgenic animals. Therefore, that pretty much leaves barriers to entry as the primary approach of protection from infectious prions in biopharmaceuticals.

The following high prion risk raw materials/starting materials will be examined to illustrate the approaches that manufacturers can use to control for the risk:

Section 5.2.2.1 Raw materials/excipients: animal- or human-derived
Section 5.2.2.2 Starting material: transgenic animal manufacture of recombinant proteins
Section 5.2.2.3 Starting material: allogeneic human cells for *ex vivo* gene therapy

5.2.2.1 Raw Materials/Excipients: Animal- or Human-Derived

Animal-derived raw materials (which also include product-contacting materials and components like chromatography column resins) and excipients can be a potential source of prion contamination. The best defense against prion contamination is to not use such animal-derived materials. If that is not possible, then the next best defense is to minimize the risk by the choice of which animal-derived raw materials are used.

The biopharmaceutical industry over the past decade to minimize prion exposure has been weaning itself from the use of animal-derived raw materials, both primary (i.e., material derived directly from animals, e.g., FBS obtained from blood drawn from bovine fetuses) and secondary (i.e., material processed from animal tissues used in the manufacture of the raw material, e.g., polysorbate derived from fatty acids obtained from hydrolyzed beef tallow). This has resulted in a mix of acronyms:

ACF – animal component-free (i.e., no primary animal-derived raw materials used)
AOF – animal origin-free (i.e., no primary or secondary animal-derived raw materials used)
CD – chemically-defined (i.e., only raw materials of known chemical structure used)

Human-derived materials are rarely used today in biopharmaceutical manufacturing processes, except occasionally in one location – formulation, and except for one specific human-derived excipient – human serum albumin. If human serum albumin is used in the biopharmaceutical formulation, it is market-approved grade.

But, despite the desire to not use animal- or human-derived raw materials, these materials still are necessary in some commercial biopharmaceutical manufacturing processes, primarily being used in the development of the recombinant cell line and early stages of cell culture manufacturing, as indicated in the examples below:

Recombinant Protein (Besremi, Ropeginterferon Alfa-2b) [7]

The applicant provided information on two materials of animal origin – IPTG used for induction of protein expression and stearate, which is used during manufacture of the cation exchange resin matrix. IPTG is manufactured using bovine milk from India; the stearate (from tallow origin) has been manufactured by rigorous processes in accordance with the requirements of the European guideline EMA/410/01 rev.3. The applicant's risk assessment regarding the risk for TSE transmission for both materials is considered adequate.

Monoclonal Antibody (Kesimpta, Ofatumumab) [8]

No raw materials of animal- or human origin are used during the manufacture of ofatumumab. During early steps of the generation of the production cell line, animal-derived raw materials were used. A risk assessment has been conducted evaluating the risk of transmitting TSE from these raw materials, considering the species and/or geographical origin and the manufacturing process of the materials in question. Based on the above considerations, it is concluded that the risk of transmitting infective TSE is negligible…. The only raw materials of biological or recombinant origin used for the manufacture of the active substance are foetal bovine serum (FBS), used for cryopreservation of the master cell bank (MCB), and recombinant Protein A, used for the affinity chromatography step of the purification process. The FBS is accompanied by a Ph. Eur. CEP for TSE safety. The Protein A is expressed in *E. coli*, fermented in an animal-material free medium.

Viral Vector rAAV (Zolgensma, Onasemnogene Abeparvovec) [9]

The listed animal derived materials used in the cell bank generation and cell culture are serum, cell disassociation agent, and production media containing human transferrin. The serum used is sourced from animals in New Zealand and gamma irradiated. A certificate of suitability was provided for the serum used at MCB and WCB manufacture and used for production. Production medium used as cell culture medium after transfection contains human transferrin. The source plasma is collected from U.S. donors in FDA approved centres, thus the TSE risk is deemed negligible. The information provided for human transferrin with regard to plasma testing (HIV 1/2 Ab, HCV Ab, HbsAg; NAT testing for HAV, HBV, HCV, HIV-1 and PB19) and virus inactivation procedures (heat treatment of the product for 10 h at 60 °C) is noted. Upon request, only transferrin for which information on quality and control is provided in line with Chap. 10 of the Guideline on plasma-derived medicinal products (EMA/CHMP/BWP/706271/2010) will be used in Zolgensma manufacture.

Genetically Modified Patient T-Cells (Tecartus, Autologous Anti-CD19-Transduced CD3+ Cells) [10]

Donors of the T-cells are of autologous origin, therefore, defined selection criteria with regard to Creutzfeldt-Jakob disease (CJD) do not apply according to Directive 2006/17/EC. Foetal bovine serum (FBS) is used during production of the retroviral vector PG13-CD19-CAR-H3 and has been used during production of the vector producing cell banks. For all FBS valid certificates of suitability issued by the EDQM are provided.

A TSE risk minimization evaluation is required whenever animal- or human-derived raw materials are used in a biopharmaceutical manufacturing process. The evaluation examines three primary complementary parameters: (1) the source animals and their geographical origin, (2) nature of the animal material used in its manufacture and any procedures in place to avoid cross-contamination with higher risk materials, and (3) production processes including the quality assurance system in place to ensure product consistency and traceability. Some of the scientific evaluation considerations for the TSE risk minimization evaluation, as recommended by EMA [11] include:

> Since the use of animal-derived materials is unavoidable for the production of some medicinal products and that complete elimination of risk at source is rarely possible, the measures taken to manage the risk of transmitting animal TSEs via medicinal products represent risk minimisation rather than risk elimination. Consequently, the basis for regulatory compliance should be based on a risk assessment, taking into consideration all pertinent factors as identified in this chapter…. When manufacturers have a choice the use of materials from 'non-TSE-relevant animal species' or non-animal origin is preferred. The rationale for using materials derived from 'TSE-relevant animal species' instead of materials from 'non-TSE-relevant species' or of non-animal origin should be given. If materials from 'TSE-relevant animal species' have to be used, consideration should be given to all the necessary measures to minimise the risk of transmission of TSE…. In a TSE infected animal, different organs and secretions have different levels of infectivity. If materials from 'TSE-relevant animal species' have to be used, consideration should be given to use materials of the lowest category of risk…. As the TSE infectivity accumulates in bovine animals over an incubation period of several years, it is prudent to source from young animals.

This EMA guidance also provides prescribed conditions for use of several animal-derived raw materials in biologic manufacturing: collagen, gelatin, bovine blood and blood derivatives, tallow derivatives, animal charcoal, milk and milk derivatives, wool derivatives, amino acids and peptones.

Biopharmaceutical manufacturers today rely heavily upon the European Directorate for the Quality of Medicines and Healthcare (EDQM) for certification of TSE suitability of animal-derived raw materials [12]. Under the EDQM procedure, raw material vendors, after paying a fee, send in a full dossier describing in detail the manufacturing method of their animal-derived raw material and the impurities that are associated with it, the countries of origin, the type of animal tissues and the quality assurance, so that the reference to the European Pharmacopoeia 5.2.8 can be validated. The dossier is processed according to a procedure that guarantees its confidentiality and it is assessed by independent experts whose impartiality is guaranteed by their status and a confidentiality agreement. If the documentation matches the EMA TSE risk minimization requirements, the EDQM issues a Certificate of TSE Suitability. The certificate is provided to the vendor. The vendor can then provide this to the biopharmaceutical manufacturer so that in a submission to a regulatory authority the manufacturer can demonstrate TSE risk minimization compliance for that specific animal-derived material.

Human-derived materials are infrequently used today in biopharmaceutical manufacturing processes, except occasionally in one location – formulation, and except for one specific human-derived excipient – human serum albumin. For genetically modified patient cells biopharmaceuticals, human serum albumin can be found in some of the formulations:

Breyanzi (Lisocabtagene Maraleucel) [13]

The BREYANZI formulation contains 75% (v/v) Cryostor CS10 [containing 7.5% dimethylsulfoxide (v/v)], 24% (v/v) Multiple Electrolytes for Injection, Type 1, 1% (v/v) of 25% albumin (human).

Tecartus (Autologous Anti-CD19-Transduced CD3+ Cells) [10]

The excipients used in the formulation of finished product are sodium chloride injection (Ph. Eur.), albumin (human serum albumin, HSA) (Ph. Eur.), and CryoStor CS10 which is a cryopreservative agent containing DMSO (Ph. Eur.).

When human albumin (as an excipient) or human AB serum (to culture human cells) are used, regulatory authorities generally have a preference for the material to be from their own regulatory region:

FDA [14]

If human albumin is used, you should use FDA-licensed products and have procedures in place to ensure that no recalled lots were used during manufacture or preparation of the product. If human AB serum is used (e.g., for ex vivo genetically modified cells), you should ensure the serum is processed from blood or plasma collected at FDA licensed facilities.

EMA [15]

The ATMP manufacturer should verify compliance of the supplier's materials with the agreed specifications. The level of supervision and further testing by the ATMP manufacturer should be proportionate to the risks posed by the individual materials. Reliance on the certificate of analysis of the supplier is acceptable if all the risks are duly understood and measures are put in place to eliminate the risks or mitigate them to an acceptable level (*e.g.* qualification of suppliers). For raw materials that are authorised as medicinal products in the EU (*e.g.* cytokines, human serum albumin, recombinant proteins) the certificate of analysis of the supplier is not required. Where available, the use of authorised medicinal products is encouraged.

Responding to questions submitted to them, FDA CBER subject matter experts on gene therapy held a 'town hall' meeting in 2022. Although the published transcript is not official FDA guidance, it does provide insight into CBER's current thought process on several subjects, including why they insist on human serum albumin being FDA-licensed:

> ***On what basis does the FDA require the use for U.S.-licensed HSA and not allow the use of EU- licensed HSA, as an ancillary material in the manufacturing of gene and cell therapy products? Neither HSAs are manufactured from plasma collected in countries with TSE- related risk (UK, France, and Ireland), and both utilize a similar questionnaire to verify if the donor stayed in TSE- related risk countries in the relevant years.***
>
> This is a question that we get a lot. While we do consider other factors – so not just TSE risk but factors such as viral inactivation reduction processing steps, whether FDA-approved tests were used to test the donor material, and whether testing was performed in CLIA-certified labs, other than – like I said, other than just TSE risk, the FDA is becoming more flexible, particularly if the HSA is used upstream in the manufacturing process. We do continue to recommend that you use the safest, highest-quality HSA available, which in most cases would be a version that's licensed in the U.S. This is particularly the case whenever the HSA is used as an excipient, since it will be directly administered to the patient. However, if you do choose to use a version of human blood-derived HSA that is not licensed in the U.S., in upstream manufacturing you may be able to do so, provided that you're able to submit information supporting that donor eligibility, including donor screening and donor testing. The albumin manufacturing and the appropriate product standards do conform with that of U.S.-licensed HSA product, as described in 21 CFR 640.80 through 83.
>
> [The published transcript can be found at FDA CBER OTAT Town Hall: Gene Therapy Chemistry, Manufacturing, and Controls (September 29, 2022); https://www.fda.gov/news-events/otat-town-hall-gene-therapy-chemistry-manufacturing-and-controls-09292022]

Biopharmaceutical manufacturers are looking for ways to further reduce their dependence on animal- and human-derived materials. A number of non-animal-derived materials are now available to manufacturers for consideration as a substitute for the animal-derived materials (see Table 5.2).

Table 5.2 Non-animal-derived material substitutes for animal-derived materials

Animal-Derived Material	Non-Animal-Derived Material Substitute
Bovine insulin	Recombinant human insulin from bacteria/yeast cell cultures
Bovine or human transferrin	Recombinant human transferrin from yeast cell cultures
Porcine trypsin	Recombinant human trypsin from yeast cell cultures

5.2.2.2 Starting Material: Transgenic Animal Manufacture of Recombinant Proteins

Manufacturers have a choice for the type of transgenic animal (i.e., animals that have been genetically engineered with the introduction of new protein-coding DNA) in which to produce a recombinant protein product – either they can select a TSE-relevant animal species (e.g., goat, sheep, cow, etc.) or they can select a non-TSE-relevant animal species (e.g., rabbit, pig, birds, etc.). Non-TSE-relevant animal species are not susceptible to TSE infection naturally; while TSE-relevant animal species are susceptible to TSE infection naturally. EMA has issued TSE guidance for use of transgenic animals to manufacture recombinant proteins [16]:

> From a TSE point of view, the use of a TSE irrelevant species of transgenic animal is desirable; however, where a TSE relevant species is used, justification should be provided and the most current version of the EC Note for Guidance on Minimising the Risk of Transmitting Animal Spongiform Encephalopathy Agents via Human and Veterinary Medicinal Products, EMA/410/0115 should be applied. Steps should be taken to minimise TSE contamination, such as embryo washing at the level of production of the transgenic founder, and a detailed history of all animals used in establishing the transgenic animal line as well as the production animals themselves, a history of the premises where the animals are kept, the use of a closed herd and the measures taken when introducing new animals into the herd, the monitoring and testing of animals, the TSE category of the harvested material from the involved species and any ability of downstream processing steps to remove or inactivate TSE agents should be documented.

Currently, only four biopharmaceuticals manufactured by transgenic animal starting materials have been market approved. One biopharmaceutical has been manufactured from a TSE-relevant animal species, for which prion risk controls are a major concern:

> ***Transgenic Goats – ATryn (Recombinant Human Antithrombin)*** [17]
> Antithrombin (Recombinant) is produced by recombinant DNA technology using genetically engineered goats into which the DNA coding sequence for human antithrombin has been introduced along with a mammary gland specific DNA sequence, which directs the expression of the antithrombin into the milk. The goats in which antithrombin (Recombinant) is produced are USDA certified scrapie-free, and controlled for specific pathogens… In addition, although the goats are from a closed, USDA certified scrapie-free herd, the purification process was challenged to remove prions. The manufacturing steps were shown capable of achieving the following $\log_{10}$ reductions: 2.0 (tangential filtration), 2.2 (affinity column), ≥3.3 (ion exchange column), ≥3.8 (hydrophobic interaction column).

Three biopharmaceuticals have been manufactured from non-TSE-relevant animal species (one from transgenic chickens and two from transgenic rabbits), for which prion risk controls are of some concern:

> ***Transgenic Chickens – Kanuma (Sebelipase Alfa)*** [18]
> A line of transgenic hens and males has been established containing the gene encoding hLAL. For the creation of transgenic animals a retroviral vector was developed encoding the hLAL sequence within non-coding, regulatory elements of the gene for tissue specificity. Replication deficient viral particles carrying the hLAL-encoding retroviral vector were generated via transfection of an immortalised chicken cell line. For integra-

tion of the hLAL sequence into the chicken genome the viral particles were injected in chicken embryos. One male with sufficient level of transgene cassette content in its semen as determined by PCR was chosen as the founder animal, generation zero (G0), to generate G1 hemizygotic transgenic animals, that all carried one copy of the transgene. G1 hen was selected due to highest levels of hLAL in EW. Subsequent genetic characterisation of this hen confirmed the correct size of the transgene, integration of a single copy of the transgene into Gallus gallus and the correct hLAL sequence. Of the transgene G1 generation, 3 males with the same genetic characteristics of the transgene were selected for generation of the G2 transgene progeny. Transgene G2 hens were the first animals to constitute the Production Line, after a match of the genetic characteristics with that of G1 hen had been reconfirmed for a proportional number of animals. The production line includes all hens of G2 and following generations either hemizygous or homozygous for the transgene. Since generation G5, propagation of further generations has occurred only via breeding campaigns between hLAL positive hens and males. Breeding campaigns are conducted only via artificial insemination of hens. Sebelipase alfa is produced by recombinant DNA technology in egg white (EW) of transgenic chicken Gallus gallus. The eggs are defined as the starting material.

Trangenic Rabbits – Cevenfacta (Eptacog Beta) [19]

Cevenfacta (LR769) is an activated recombinant human coagulation rhFVIIa produced by recombinant deoxyribonucleic acid (DNA) technology employing site-directed expression of the human FVII gene in the mammary gland of transgenic rabbits. The transgene containing the FVII has been stably integrated into the transgenic rabbit genome. The recombinant human FVII gene is exclusively expressed by the mammary gland under the control of a beta-casein specific promoter. Milk from these transgenic rabbits is collected and the FVII protein expressed is subsequently purified and activated during the purification process to FVIIa. The glycoprotein produced (FVII) consists of 406 amino acid residues (molecular weight 50 KDa) which is structurally similar to human plasma derived coagulation FVIIa and has similar functional properties to human plasma-derived FVIIa and to another recombinant FVIIa (eptacog alfa).

Rabbits are non-TSE-relevant animal species (according to the 'Note for guidance on minimising the risk of transmitting animal spongiform encephalopathy agents via human and veterinary medicinal products' (EMA/410/01, current revision). Additional safety measures have been implemented at the rabbit facilities e.g. the rabbits have no possible contact with other animal species and the rabbit feed is devoid from any animal-derived material.

Note, that for biopharmaceuticals manufactured by transgenic animals, it is possible to perform a prion clearance safety evaluation of the capability of the purification process steps to remove/inactivate putative prion contamination. However, it has only been done for biopharmaceuticals from a TSE-relevant animal species (e.g., goats).

In December 2020, the FDA announced its approval of the use of transgenic pigs, a non-TSE relevant animal species, as a future potential starting material source for recombinant protein manufacture [20]:

> Today, the U.S. Food and Drug Administration approved a first-of-its-kind intentional genomic alteration (IGA) in a line of domestic pigs, referred to as GalSafe pigs, which may be used for food or human therapeutics. This is the first IGA in an animal that the FDA has approved for both human food consumption and as a source for potential therapeutic uses. The IGA in GalSafe pigs is intended to eliminate alpha-gal sugar on the surface of the pigs' cells. People with Alpha-gal syndrome (AGS) may have mild to severe allergic reactions to alpha-gal sugar found in red meat (e.g., beef, pork, and lamb).

5.2.2.3 Starting Material: Allogeneic Genetically Modified Donor Cells

For genetically modified human cells as biopharmaceuticals, if allogeneic for *ex vivo* use, the prion concern is from both Creutzfeldt-Jakob Disease (CJD) and the variant CJD (vCJD) which might be present in the collected human donor cells. This is most important as allogeneic cells after being genetically modified can be used in multiple patients. Thus, the primary line of defense against prions with this starting material is the selection and screening process of the donor of the human cells:

EMA [21]

Most of the cell based medicinal products currently under clinical investigation or already in use in some members states are from autologous donors, therefore, no specific considerations regarding CJD or vCJD risk are required (except if additional components of human origin are used in their preparation, and for which a risk assessment for potential TSE contamination should be considered). For cell based products from allogeneic donors, the WHO classification and guidelines on tissue infectivity (WHO Guidelines on Tissue Infectivity Distribution in Transmissible Spongiform Encephalopathies 2010) should also be considered as a part of the benefit-risk assessment of the medicinal product. Tissue infectivity in CJD seems mainly confined to the central nervous system and tissues anatomically associated with it. Regarding vCJD, infectivity has also been shown associated with blood and lymphoreticular tissues so precautionary measures should be considered if any of those tissues are used as the starting material for a cell based product. Where relevant, the recommendations of the CHMP Position statement on Creutzfeldt-Jakob disease and plasma-derived and urine-derived medicinal products should be taken into account. For human cells contained in ATMPs, there is no manufacturing process to add a further barrier to transmission of a TSE agent. In any case, the final risk-benefit for the therapeutic use of these medicinal products derived from human cells and tissues will have to be decided on a case-by-case basis.

FDA [14]:

Autologous Cells. You are not required to make a donor eligibility determination or to perform donor screening on autologous cells or tissues (21 CFR 1271.90(a)(1)). However, you should determine based on donor information whether your manufacturing procedures increase the risk to the patient by further propagation of pathogenic agents that may be present in the donor, as applicable. You should also describe precautions to prevent the spread of viruses or other adventitious agents to persons other than the autologous recipient.

Allogeneic Cells. For allogeneic cells or tissues, you must perform donor screening and testing, as required in 21 CFR Part 1271, Subpart C, except for those cells and tissues that meet the exceptions in 21 CFR 1271.90(a). Donors of all types of cells and tissues must be screened for risk factors and clinical evidence of relevant communicable disease agents and diseases, including: human immunodeficiency virus (HIV); hepatitis B virus (HBV); hepatitis C virus (HCV); human TSE, including Creutzfeldt-Jakob disease (CJD) and variant CJD (vCJD); and *Treponema pallidum* (syphilis) (21 CFR 1271.75)….

5.2.3 Necessity of Ongoing Prion Risk Review

The ultimate solution to infectious prion risk probability is to not expose the manufacturing process equipment or facility or biopharmaceutical product, if at all possible, to infectious prions. Since complete elimination of TSE risk is impossible for

most biopharmaceutical manufacturing processes, appropriate and adequate risk assessment and risk control measures to minimize TSE exposure are important. Prion control is not appropriate for a minimum CMC regulatory compliance continuum risk-based approach, since the full prion control measures need to be in place from first-in-human (FIH) clinical studies onwards through market approval for patient safety.

An effective TSE risk management strategy must also include risk review, which incorporates the need for a reassessment of the previous risk assessment and the implemented risk controls, as new scientific information becomes available. For example, if 1 day, a fast and reliable infectious prion assay becomes available, the previous prion risk controls will have to be reconsidered. Consideration of an added test would then need to be incorporated into the overall risk management plan for a biopharmaceutical manufacturing process.

5.3 Adventitious Viruses

Viruses (a term coined by the Dutch microbiologist Martinus Beijerinck in the 1890s to describe the infectious agent of tobacco mosaic disease) are ultramicroscopic (20–300 nm in diameter) infectious agents that replicate only within the cells of living hosts. Viruses consist of nucleic acid (either DNA or RNA, but not both) surrounded by a protein coat called a capsid. In addition, many viruses have an envelope, which is a membranous lipid structure that surrounds the nucleic acid genome enclosed in the capsid. RNA-based viruses have an enzyme called reverse transcriptase that permits the usual sequence of DNA to RNA to be reversed, so the virus can make a DNA version of itself.

Viruses are a well-known threat to all living organisms, including plants, insects, animals and humans (see Table 5.3 for a list of some viruses that are known to harm humans).

For biopharmaceutical manufacturers, adventitious viruses present a major concern. First, there is no universal, rapid assay to detect all possible contaminating viruses that could be present in a biopharmaceutical manufacturing process. Second, if a virus enters into a cell culture manufacturing process, virus proliferation can occur, increasing the potential impact on the entire manufacturing facility if the contaminated biopharmaceutical solution is accidentally released and passed from

Table 5.3 List of some viruses known to harm human health

List of Some Viruses Known to Harm Human Health	
Coronavirus	Herpes simplex virus
Dengue virus	HIV
Ebola virus	Monkeypox (mpox)
Hantavirus	Poliovirus
Hepatitis A, B, C	Yellow fever virus

the closed integrity of a bioreactor into the surrounding environment; thus, impacting not only that batch but subsequent manufactured batches.

5.3.1 Virus Risk Assessment

The level of patient safety risk from virus contamination is a function of three factors:

(1) *Probability of viruses being present*. Like prions, viruses carry a probability of presence from certain types of materials used in biopharmaceutical manufacturing. But unlike prions, the probability risk is higher for viruses simply because of the abundance of different viruses that could potentially be present. Exposure to animal- or human-derived raw materials during the manufacturing process (e.g., fetal bovine serum), use of starting materials directly derived from animal or human sources (e.g., transgenic animals, human donor cells) and biopharmaceuticals derived from insect, animal or human cell lines, all carry a higher risk of introducing virus contamination. But it should be emphasized that since viruses can also be introduced into the biopharmaceutical manufacturing process from (a) non-biological raw materials, (b) the operators involved in manufacturing, and (c) the facility environment itself, most manufacturing processes involving cell culture steps have some level of risk of potential virus contamination.

(2) *Potential severity if virus contaminates the biopharmaceutical*. If the manufactured biopharmaceutical contains adventitious virus, the patient runs a potential risk of significant harm. Unfortunately, this has occurred, in the past, from starting material of human plasma. Significant patient harm and eventual death has resulted in patients exposed to human plasma-derived proteins contaminated with viruses, such as was the case with natural human factor VIII and factor IX contaminated with human immunodeficiency virus (HIV) and hepatitis C virus (HCV) [22]:

> In 1965, Dr. Judith Graham Pool, a researcher at Stanford University, published a paper on cryoprecipitate. In a major breakthrough, she discovered that the precipitate left from thawing plasma was rich in factor VIII. Because cryoprecipitate contained a substantial amount of factor in a smaller volume, it could be infused to control serious bleeding. Blood banks could produce and store the component, making emergency surgery and elective procedures for patients with hemophilia patients much more manageable. By the 1970s, freeze-dried powdered concentrates containing factor VIII and IX became available…. By the mid-1980s, it was confirmed that HIV/AIDS could be transmitted through the use of blood and blood products, such as those used to treat hemophilia. Approximately half of the people with hemophilia in the US eventually became infected with HIV through contaminated blood products; thousands died. The overwhelming impact of HIV on the bleeding disorders community was felt into the next few decades. The hepatitis C virus (HCV) infection was also transmitted through contaminated factor products, pooled from the blood of hundreds of thousands of donors. Before testing for HCV began in 1992, an estimated 44% of all people with hemophilia had contracted it. With the advent of more

sophisticated screening methods and purification techniques, the risk of contracting HCV through factor products is virtually nil.

It should be noted that the severity of patient harm will vary due to the type and amount of the contaminating infectious virus. But in all cases, adventitious virus contaminated biopharmaceuticals are not to be administered to patients. Assurance that the biopharmaceutical is free of adventitious viruses is essential for patient safety.

(3) *Detectability of viruses*. For biopharmaceutical manufacturers, infectious viruses present a challenging situation. While there are a variety of virus test methods available, there is currently no universal, rapid assay to detect all possible contaminating viruses that might be present. Therefore, the biopharmaceutical manufacturer will have to apply numerous virus assays at key points along the manufacturing process, especially for the choice of raw materials, the manufacture of the starting materials, and the cell culture process steps where contaminating viruses can propagate, to provide acceptable assurance of absence of contaminating viruses. In addition, the viral vectors used in the manufacture of gene therapies have the additional challenge of detecting a contaminating virus in the presence of a product that is itself a virus.

5.3.2 Virus Risk Control

Risk control for viruses, which must be in place from manufacture of biopharmaceutical batches for first-in-human (FIH) clinical studies onward, considers the necessary steps to either eliminate or mitigate virus contamination of biopharmaceuticals from those manufacturing process steps identified in the risk assessment as being a potential contributor of contaminating viruses. Risk control for virus contamination involves choices. If the manufacturer chooses to utilize raw materials that are animal-derived, then the manufactured biopharmaceutical carries an increased risk of being exposed to virus contamination. If the chosen starting material is directly derived from human sources (e.g., human donor cells), then virus risk is present. If the biopharmaceutical is derived from cell lines of insect, animal or human origin, then virus risk is present. Since the facility environment, the manufacturing staff, non-biological origin raw materials and components, and the design of the manufacturing process, also all could contribute to a risk of virus contamination, the unknown risk associated from each of these must also be evaluated and controlled.

There are three complementary risk mitigating approaches used for controlling adventitious agent contaminations – (1) barriers to entry, (2) testing to confirm absence, and (3) inactivation/removal to ensure absence in a biopharmaceutical solution. For adventitious viruses, most biopharmaceutical manufacturing processes can apply all three barriers (e.g., recombinant proteins, monoclonal antibodies, viral vectors). But some other biopharmaceutical manufacturing processes can apply only the first two of the risk mitigating approaches (e.g., genetically modified patient cells).

The following high virus risk areas will be examined to illustrate the approaches that manufacturers can use to control for the risk:

Section 5.3.2.1 Raw materials: animal-derived – FBS and trypsin
Section 5.3.2.2 Starting material: patient/donor cells for *ex vivo* gene therapy
Section 5.3.2.3 Cell culture manufacturing process: protein-based biopharmaceuticals
Section 5.3.2.4 Cell culture manufacturing process: viral vector biopharmaceuticals
Section 5.3.2.5 Cell transduction/expanding: genetically modified patient cells

5.3.2.1 Raw Materials: Animal-Derived – FBS and Trypsin

Two of the most common, but also high virus risk, animal-derived raw materials used in biopharmaceutical manufacturing are (1) fetal bovine serum, and (2) porcine trypsin:

Fetal Bovine Serum. Bovine serum stimulates animal and human cells to grow and multiply and helps to keep the cells normal and healthy over time. The largest proportion of bovine serum used to support the growth of cells in cell culture is fetal bovine serum (FBS, blood from bovine fetuses). FBS fulfils the function especially well because it contains particularly high levels of substances that promote cell growth. It also has favorably low levels of certain other molecules, including immunoglobulins, which are found in the blood of older animals and may interfere with production processes. Regulatory authorities require a number of viruses to be tested for in each batch of FBS to be used in manufacturing: bluetongue and related orbiviruses, bovine adenovirus, bovine parvovirus, bovine respiratory syncytial virus, bovine viral diarrhea virus, rabies virus, and reovirus 3. It is strongly recommended, in addition to direct testing for viruses, that FBS be inactivated for viruses by a validated and efficacious treatment such as gamma irradiation. Serum suppliers and users are also cautioned to be aware of emerging bovine viruses, and are similarly encouraged to investigate the presence of such agents in bovine serum and to take appropriate action to eliminate or reduce the presence of any novel virus in serum [23].

Porcine Trypsin. Porcine trypsin is a reagent widely used during the manufacture of biological medicinal products. The main application is the detachment of cells from culture vessels for passaging. Porcine trypsin, an animal derived material extracted from the pancreas of pigs, carries the risk of contamination with adventitious agents. This may especially be the case for certain viruses that are widespread among pigs and which are difficult to eliminate due to their high resistance to physicochemical treatment. A comprehensive literature-based risk analysis of potential porcine viruses that may contaminate porcine trypsin and could pose a risk to humans identified 55 porcine virus species from 17 different families. Inactivation/removal of microbiological agents is considered as a major factor contributing to adventitious agent safety of trypsin. Therefore, selected irradia-

tion, virus filtration and low pH process steps should be carefully validated with respect to their virus inactivation/removal capacity [24]. Regulatory authorities require a number of viruses to be tested for in each batch of porcine trypsin to be used in manufacturing: porcine parvovirus, bovine viral diarrhea virus (BVDV), reovirus, rabies virus, porcine adenovirus, transmissible gastroenteritis virus (TGE), porcine hemagglutinating encephalitis virus, and porcine parvovirus. Porcine trypsin suppliers and users are also cautioned to be aware that other porcine viruses might be of concern, depending upon exposure history and species of origin of the cell substrate or reagent. Such additional viruses include circoviruses, enteroviruses, porcine respiratory and reproductive syndrome virus (PRRS), porcine cytomegalovirus, porcine influenza viruses, pseudorabies virus, swine pox virus, swine fever virus (hog cholera virus, African), vesicular stomatitis virus (VSV), Nipah virus and porcine retroviruses [25].

It is because of the high virus contaminating risk that for these two animal-derived raw materials each has a specific and extensive list of viruses that must be tested for and found to be absent prior to their use in manufacturing. Thus, the primary virus risk mitigation approach for these raw materials is the barrier to entry, by means of testing for absence of adventitious virus.

5.3.2.2 Starting Material: Patient/Donor Cells for *Ex Vivo* Gene Therapy

Either autologous cells (i.e., cells originally sourced from the same patient that is receiving the administered cells) or allogeneic cells (i.e., cells originally sourced from a donor that is not the patient receiving the administered cells), must be appropriately aseptically handled from time of collection to time of administration to patient in order to prevent introduction of adventitious viral contamination. An adventitious virus that gets into the manufacturing process of a genetically modified patient cells biopharmaceutical will not disappear.

Autologous human cells will be returned to the same patient that donated them. Although the regulatory authorities recommend virus testing for donor eligibility, it is not required. However, the manufacturer must determine if their cell culture methods could propagate infectious virus already present in the donor and whether adventitious virus contamination could occur [14]:

> Autologous Cells. You are not required to make a donor eligibility determination or to perform donor screening on autologous cells or tissues (21 CFR 1271.90(a)(1)). However, you should determine based on donor information whether your manufacturing procedures increase the risk to the patient by further propagation of pathogenic agents that may be present in the donor, as applicable. You should also describe precautions to prevent the spread of viruses or other adventitious agents to persons other than the autologous recipient.

On the other hand, allogeneic human cells will be used for multiple patients, so the risk of patient harm due to virus contamination is considerably higher. Manufacturers must apply the full scope of virus contamination assessment on the donor, both screening and testing for presence of viruses [14]:

> Allogeneic Cells. For allogeneic cells or tissues, you must perform donor screening and testing, as required in 21 CFR Part 1271, Subpart C, except for those cells and tissues that meet the exceptions in 21 CFR 1271.90(a). Donors of all types of cells and tissues must be screened for risk factors and clinical evidence of relevant communicable disease agents and diseases, including: human immunodeficiency virus (HIV); hepatitis B virus (HBV); hepatitis C virus (HCV); human TSE, including Creutzfeldt-Jakob disease (CJD) and variant CJD (vCJD); and *Treponema pallidum* (syphilis) (21 CFR 1271.75). In addition, donors of viable leukocyte-rich cells or tissues should be screened for human T-lymphotropic virus (HTLV). You must also test a specimen of donor cells or tissue for evidence of infection due to relevant communicable disease agents, including: HIV-1; HIV-2; HBV; HCV; syphilis; and if the material is leukocyte-rich cells or tissue, HTLV-1, HTLV-2, and cytomegalovirus (21 CFR 1271.85). For donor eligibility testing, you must use appropriate FDA-licensed, approved, or cleared donor screening tests (21 CFR 1271.80(c)). Moreover, the required testing must be performed by a laboratory that either is certified to perform such testing on human specimens under the Clinical Laboratory Improvement Amendments of 1988 (42 U.S.C. 263a) and 42 CFR Part 493 or has met equivalent requirements as determined by the Centers for Medicare and Medicaid Services (21 CFR 1271.80(c)). You should also refer to recent Center for Biologics Evaluation and Research guidance documents on donor eligibility for additional information on testing for emerging relevant communicable disease agents and diseases (e.g., West Nile virus (WNV), Zika virus)…. Allogeneic cells from a single donor or source tissue may sometimes be expanded and stored for greater consistency and control in manufacturing. In these situations, we generally recommend that you qualify allogeneic master and working cell banks in the same way as cell banks used for production of viral vectors …, provided that you have sufficient material for this testing. In these situations, we are concerned about the introduction of adventitious agents (e.g., viruses, bacteria, mycoplasma) during the bank manufacturing process, especially from human, bovine or porcine materials, animal feeder cells, other animal-derived reagents, or human AB serum, if used.

The same requirement for manufacturers to apply the full scope of virus contamination assessment on donors, both screening and testing for presence of viruses, is required in the European Union under the European Commission Directive 2006/17/EC [26]. Aside from rigorously testing the donor cells for adventitious virus, there are no other virus controls for cells.

The following example from a market-approved genetically modified patient cells biopharmaceutical illustrates the adventitious virus controls for the manufacture of these products:

> ***Carvytki (Ciltacabtagene Autoleucel)*** [27]
>
> The autologous PBMCs are obtained from the patients by leukapheresis. Each patient/PBMC donor is tested according to EU guidelines 2002/98/EC, 2004/23/EC and their daughter directives as well as national and local guidelines, policies, and procedures. The testing procedure for a second apheresis, if needed, is also performed in compliance with Dir 2006/17/EC. Since HIV-positive patients are allowed for treatment with Carvykti, the risk of recombination and trans-complementation and thus reactivation of the LV in T cells derived from these patients has been discussed. Despite the fact that the risk cannot be finally excluded, there are several measures in place for risk minimisation, including the design of the LV and testing of the finished product for replication competent lentiviruses (RCL). Furthermore, there is a medical need for treating also HIV-positive patients with Carvykti and patients are advised to continue antiretroviral therapy following Carvykti treatment. Finally, due to the current missing experience with manufacturing Carvykti for patients testing positive for HIV, active HBV, or active

HCV, the applicant will impose additional pharmacovigilance activities into the risk-management system for such patients as conditions to the marketing authorisation.

Of the three complementary risk mitigating approaches used for controlling adventitious virus contaminations, only two are effective for human cells for *ex vivo* gene therapy – (1) barriers to entry (i.e., aseptic cell handling procedures), and (2) testing of raw materials and starting materials to confirm absence. With collected patient cells, there is neither final product testing nor inactivation/removal procedure to support the absence of adventitious virus.

5.3.2.3 Cell Culture Manufacturing Process: Protein-Based Biopharmaceuticals

Each type of cell line used to manufacture a protein-based biopharmaceutical carries a different level risk of virus contamination and adventitious virus susceptibility. There are cell lines of high viral contamination risk (e.g., insect, animal and human cell lines), and there are cell lines of low viral contamination risk (e.g., bacteria, yeast, plant cell lines). The low viral contamination risk cell lines will be examined first. Then, the high viral contamination risk cell lines will be examined in greater detail.

5.3.2.3.1 Low Risk Viral Contamination Cell Lines – Bacteria, Yeast, Plant Cells

Each type of cell line used to manufacture a recombinant protein or monoclonal antibody carries a different level risk of virus contamination and adventitious virus susceptibility. The following are cell lines considered to be of low-level viral contamination risk:

Bacteria Cell Lines. Viruses do not infect nor replicate in bacterial cell cultures; however, a virus-like component, called bacteriophage ("phage" meaning "to eat"), can infect and replicate in bacterial cells, which can adversely affect cell line stability and biologic productivity. Each bacteriophage type exhibits a defined bacterial host range. Some bacteriophages are very specific for one or two closely related bacterial species, while others can infect and replicate in a variety of bacterial cells. Upon infecting a bacterial host cell, some bacteriophages, known as lytic or virulent phages, release the replicated phage particles by lysing (bursting) the host cell. Other types of bacteriophages, known as lysogenic or temperate, integrate their nucleic acid into the host's chromosome to be replicated during cell division. During this time, they are not virulent. The phage genome may later become active, initiating production of phage particles and destruction of the host cell. The lysed cells release proteins into the culture which can cause foaming and clog air filters resulting in pressure buildup in a bioreactor. The increased pressure can lead to leakage out of the bioreactor which can contaminate the manufacturing facility.

The following case example of a market-approved recombinant protein from a bacteria cell line manufacturing process illustrates the limited adventitious virus controls needed:

Besremi (Ropeginterferon Alfa-2b) [7]

As the production cell substrate for ropeginterferon alfa-2b is *E. coli*, this represents a major barrier to the transmission of viral adventitious agents. Viruses do not infect or replicate in *E. coli* cells and only a virus-like component, bacteriophage, can infect and replicate there. The strategy for controlling the risk of bacteriophage contamination is inactivation of bacteriophage from the potential source. It is concluded that the risk assessment on viral safety shows very low risk of transmission of viral adventitious agents. The risk is appropriately minimised with the control strategy.

Yeast Cell Lines. Viruses do not infect or replicate in yeast cells; therefore, viral control or reduction measures are not necessary. The following case example of a market-approved monoclonal antibody from a yeast cell line manufacturing process illustrates the limited adventitious virus controls needed:

Vyepti (Eptinezumab) [28]

Vyepti is expressed in the yeast *Pichia pastoris*. *Pichia pastoris* is not a potential host for the amplification of viruses that are infectious for human or animal cells. Therefore, no virus safety testing on cell banks and unprocessed bulk has been performed and the purification process was not validated for its virus reducing capacity. This approach is in compliance with current guidelines. No materials of human or animal origin are used in the whole manufacturing process and none of the excipients are of human or animal origin. In summary, the viral safety of Vyepti has been sufficiently demonstrated.

Plant Cell Lines. Plant viral diseases can cause great harm to wild plants and crops, so there is concern that plant-specific viruses might impact a plant cell culture manufacturing process. However, known viruses harmful to humans have not been found to replicate in cultured plant cells. The following case example of a market-approved recombinant protein from a plant cell line manufacturing process illustrates the limited adventitious virus controls needed:

Elelyso (Taliglucerase Alfa) [29]

MCB vials were also tested for plant specific carrot viruses and found negative…Taliglucerase alfa is produced by a proprietary innovative technology where transformed carrot plant root cells, cultured in suspension in a closed bioreactor system, express the protein. The plant cell culture system is free of mammalian derived components which are not required for efficient plant cell growth and protein production. The carrot plant cells cultures are naturally and biologically protected from being infected by human or mammalian viruses or other pathogen due to host-pathogen specificity. Furthermore, plant viruses cannot be propagated in plant cells cultured in suspension. Finally, plant viruses pose no risk to humans. Based on this rationale and on the current scientific knowledge, the carrot cell culture line used for the production of taliglucerase alfa cannot be a host for viruses.

Keep in mind that ‘low’ viral contamination risk does not mean ‘no’ viral contamination risk. The manufacturer still needs to complete a documented risk assessment across the entire manufacturing process to confirm that adequate controls are in place to protect the biopharmaceutical during manufacture from adventitious virus contamination.

5.3.2.3.2 High Risk Viral Contamination Cell Lines – Insect, Animal, Human Cells

Recombinant proteins, monoclonal antibodies produced using either animal or human cell lines, are highly susceptible to adventitious virus contamination. ICH Q5A(R2) *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* [30] serves as the primary guidance for the minimization and prevention of adventitious virus contamination in these manufacturing processes. It should be noted that this ICH guideline describes not only adventitious virus patient safety concerns, but also 'endogenous virus' (i.e., viral DNA sequence embedded in the germline of the cell) patient safety concerns.

Some of the general scientific risk-based considerations for minimization of viral contaminations in these manufacturing processes stated in the ICH guideline are:

> Adventitious viruses may contaminate the production process by several routes including, but not limited to, (1) the use of contaminated biological raw materials or reagents such as animal serum components during cell culture, (2) the use of a virus or viral vector (including helper viruses used in their production) to induce expression of specific genes encoding a desired protein, (3) the use of a contaminated raw material or reagent used during downstream purification, such as a monoclonal antibody coupled affinity resin for product selection or purification, (4) the use of a contaminated excipient during formulation, and (5) contamination from the environment, including storage of non-biological raw materials or during cell culture and medium handling. Monitoring cell culture parameters can be helpful in the early detection of potential adventitious viral contamination. Manufacturers should avoid using human- and animal-derived raw materials (e.g., human serum, bovine serum, porcine trypsin) in their manufacturing processes when possible. When this is not possible, the use of animal-derived raw materials should be supported by the relevant documentation or qualification of the material, commensurate with risk. Information such as the country of origin, tissue of origin, virus inactivation or removal steps applied during the manufacturing process of the material, and the types of virus testing that have been performed on the raw material should be provided. When possible, cell culture media or media supplement treatments such as gamma irradiation, virus filtration, high temperature short time processing, or ultraviolet C irradiation can be used as additional virus risk mitigation measures.

Additionally, this ICH guideline emphasizes the importance of three (3) principle complementary approaches to control the potential viral contamination of these biopharmaceuticals, which is discussed in the next sections.

5.3.2.3.3 Complementary Virus Control: Absence in the Recombinant Production Cell Line

One of the three complementary virus controls to protect the recombinant protein or monoclonal antibody during its manufacture is the selecting, preparation and, testing of the recombinant production cell line for the absence of undesirable infectious virus contaminants.

Animal and human cell lines can either contain endogenous virus or become contaminated with adventitious virus. These cell lines can harbor endogenous retroviruses (i.e., viruses transmitted vertically from one cell generation to the next since

the viral genome persists within the cell). For example, hamster cell lines (such as CHO, BHK) and rat cell lines typically express noninfectious, defective retroviral particles; while mouse myeloma and hybridoma cell lines (such as NS0 and Sp2/0) can express infectious retroviral particles; the retroviral particles could be expressed either constitutively or latently [1]:

> Cell lines such as CHO, BHK-21, NS0, and Sp2/0 have frequently been used as substrates for drug production with no reported safety problems related to virus contamination of the products and may be classified as "well-characterized" because the endogenous retrovirus particles have been studied extensively. Furthermore, the total number of retrovirus-like particles present in the harvest is evaluated quantitatively (TEM or quantitative PCR) on a representative number of lots and retrovirus clearance is demonstrated with significant safety factors. In these situations, testing for infectious retrovirus may be reduced (*e.g.*, test one lot then discontinue testing, but repeat when there is a significant change in the cell-culture process such as a change in scale).

For production cell lines, there are three locations where the presence of contaminating viruses is expected to be tested, and confirmed to be absent:

- Master Cell Bank (MCB) – Extensive screening for both endogenous and non-endogenous viral contamination should be performed
- Working Cell Bank (WCB) – Each Working Cell Bank (WCB) as a starting cell substrate for drug production should be tested for adventitious virus either by direct testing or by analysis of cells at the limit of in vitro cell age, initiated from the WCB
- Cells at the Limit of In Vitro Cell age (LIVCA) used for production – LIVCA should be based on data derived from production cells expanded under pilot-plant scale or commercial-scale conditions to the proposed in vitro cell age or beyond; these cells should be evaluated once for those endogenous viruses that may have been latent (i.e., non-infectious) in the MCB and WCB

The challenge in detecting contaminating viruses is the absence of a universal virus assay. There are so many viruses – several thousand viruses have been reported – and there is no universal virus test detection method. Each virus test detects only specified virus types with varying degrees of sensitivity. Therefore, to provide adequate assurance that contaminating viruses are indeed absent in a production cell bank, multiple virus testing approaches need to be considered, for example:

- In vivo tests in animals – observation of the health of an animal after being inoculated with the biologic
- In vitro tests in cell culture – observation of the effect on the cells, typically cytopathic, after being exposed to the biologic
- Antibody production tests in pathogen-free animals – generation of antibodies to specific viruses after being inoculated with the biologic
- Visual detection of viral particles – by transmission electron microscopy (TEM)
- Nucleic acid tests – detection of specific viral DNA or RNA sequences
- Enzymatic tests – detection of specific viral enzymes (e.g., reverse transcriptase in RNA-based viruses)

Table 5.4 Virus tests recommended to be performed once for animal/human cell lines

	Master Cell Bank (MCB)	Working Cell Bank (WCB)	Cells at the Limit of *In Vitro* Cell Age (LIVCA)
Test for Retroviruses and Other Endogenous Viruses			
Infectivity	+	-	+
Electron Microscopy	+	-	+
Reverse Transcriptase	+	-	+
Other Virus-Specific Tests	as appropriate	-	as appropriate
Tests for Non-Endogenous or Other Adventitious Viruses			
In Vitro Assays or NGS	+	+	+
In Vivo Assays or NGS	+	-	+
Antibody Production Tests or Specific Molecular Assay	+	-	-
Other Virus-Specific Tests	+	-	-

- Next Generation Sequencing (NGS) – also referred to as high throughput sequencing (HTS) or massive parallel sequencing (MPS) or deep sequencing, multi-step nucleic acid-based technology with broad capabilities for agnostic detection of known and unknown adventitious agents

Due to the high susceptibility to viral contamination, extensive viral testing of the animal and human production cell lines is required prior to their release as cell banks for the manufacture of protein-based biopharmaceuticals [30], as presented in Table 5.4.

5.3.2.3.4 Complementary Virus Control: Testing at the Appropriate Manufacturing Stage

A second of the three complementary virus controls to protect the recombinant protein or monoclonal antibody during its manufacture is the testing of the product at appropriate step(s) during production for the absence of contaminating infectious viruses.

The unprocessed bulk constitutes one or multiple pooled harvests of cells and culture media. A representative sample of the unprocessed bulk, removed from the production bioreactor before further processing, represents one of the most suitable levels at which the possibility of adventitious virus contamination can be determined with a high probability of detection. After this point in the manufacturing process, cells are removed, so further proliferation of virus cannot continue.

Adventitious virus testing should be routinely applied to each unprocessed bulk. This may include *in vitro* screening assays using several cell lines or broad molecular virus detection methods such as Next Generation Sequencing (NGS). Based on the risk assessment (considering the cell substrate, use of animal-derived raw

materials or reagents, and level of virus clearance of the process), the indicator cell cultures should be observed for at least 2 weeks. Detection for specific viruses or families of viruses may also be appropriate to include based on risk assessment (e.g., Minute virus of mice if CHO cells are used in manufacturing). When appropriate, a PCR or other molecular method may also be selected, as rapid test methods can facilitate real-time decision making. Note, adventitious virus testing is required for each batch of cell culture production, whether for commercial product or clinical trial material [31]:

> Independent of the stage of development, each batch of unprocessed bulk material that will be used to manufacture clinical trial material should be tested as per Q5A. The sample to be tested should include cells, when appropriate, and tests should include in vitro and PCR-based screening tests for adventitious agents and an estimation of retroviral particles, where applicable. No further testing is required for bulks deriving from CHO cell lines. For manufacture based upon NS0 or Sp2/0 cell lines, tests for infectious retroviruses should be applied on a one-off basis but should be repeated if there is a significant change in production cell culture, e.g. manufacturing scale. For manufacture based upon any other cell line, tests for infectious retroviruses and in vivo tests … should be applied on a one-off basis, but should be repeated if there is a significant change in production cell culture, e.g. manufacturing scale…. Consideration should be given to the inclusion of a test for MMV if the cell line is permissive for this virus.

If any adventitious viruses are detected at the unprocessed bulk stage, the harvest should not be used for product manufacture unless justified. The process should be carefully checked to determine the root cause and extent of the contamination, and appropriate actions should be taken.

5.3.2.3.5 Complementary Virus Control: Clearance Capacity of the Purification Process

The third of the three complementary virus controls to protect the recombinant protein or monoclonal antibody during its manufacture is to assess the capacity of the purification process to clear infectious virus should it be present.

Although appropriate virus testing is performed at several locations throughout the culturing stage of the manufacturing process to show the absence of virus contamination, unfortunately, there is no universal assay that can readily detect the presence of all possible viruses. So, what happens if an unknown, unexpected (i.e., putative) virus is present, but just not detected? How can the patient be protected from such an event? To provide a safety net, the regulatory authorities require an assessment of viral clearance through the purification process as an added measure of protection for the patient. Some of the general scientific considerations on how to properly conduct these viral clearance studies are provided in the ICH guideline [30]:

> Evaluation and characterisation of the virus removal or inactivation procedures are important for establishing the safety of biotechnology products. Past instances of contamination have occurred with agents whose presence was not known or even suspected. Though this happened to biological products derived from various source materials

other than fully characterised cell lines, it reinforces that assessment of viral clearance provides a measure of confidence that any unknown, unsuspected, and harmful viruses may be removed. Studies should be carried out in a well-documented and controlled manner.

The objectives of viral clearance studies are (1) to assess process steps that effectively inactivate or remove viruses and (2) to estimate quantitatively the overall level of virus reduction obtained by the process. These should be achieved by the deliberate addition (i.e., "spiking") of significant amounts of a virus to the crude material or to different fractions obtained during the various process steps and demonstrating its removal or inactivation during the subsequent steps. It is not necessary to evaluate or characterise every step of a manufacturing process if adequate clearance is demonstrated by the use of fewer steps. It should be considered that other steps in the process may have an indirect effect on the viral inactivation or removal achieved. Manufacturers should explain and justify the approach used in studies to evaluate virus clearance. In general, in order to determine the amount of endogenous virus particles that enter the purification process, quantification should be performed on three cell cultures campaigns, lots or batches. This data should be submitted as part of the marketing application or registration package.

The reduction of virus infectivity may be achieved by removing virus particles or by inactivating viral infectivity. For each production step assessed, the possible mechanism of loss of viral infectivity should be described with regard to whether it results from inactivation or removal. For inactivation steps, the study should be planned so that samples are taken at different times and an inactivation curve is constructed…. Reduction factors are normally expressed on a logarithmic scale to show that, while residual virus infectivity will never be reduced to zero, it may be greatly reduced mathematically.

In addition to clearance studies for viruses known to be present, studies to characterise the ability to remove or inactivate other viruses should be conducted. The purpose of studies using viruses with a range of unknown or unexpected biochemical and biophysical properties is to characterise the robustness of the procedure rather than to achieve a specific inactivation or removal goal. A demonstration of the capacity of the production process to inactivate or remove viruses is desirable. Such studies are not performed to evaluate a specific safety risk. Therefore, achieving a specific clearance value is not needed.

These viral clearance studies encompass a variety of viruses chosen both to resemble viruses which may contaminate the product and to represent a wide range of physico-chemical properties in order to test the ability of the system to eliminate viruses in general. Some recommendations on the viruses to consider are provided:

A major issue in performing a viral clearance study is to determine which viruses should be used. Such viruses fall into three categories: (1) "relevant" viruses, (2) specific "model" viruses, and (3) non-specific "model" viruses.

"Relevant" viruses are used in the process evaluation of viral clearance studies which are the identified viruses or of the same species as the viruses that are known, or likely to contaminate the cell substrate or any other reagents or materials used in the production process. The process for purification and/or inactivation should demonstrate the capability to remove and/or inactivate such viruses.

When a "relevant" virus is not available or when it is not well adapted to the process evaluation of viral clearance studies (e.g., it cannot be grown *in vitro* to sufficiently high titers), a specific "model" virus should be used as a substitute. An appropriate specific "model" virus can be a virus which is closely related to the known or suspected virus (same genus or family), having similar physical and chemical properties to the observed or suspected virus. [Some examples of specific model viruses – murine leukemia virus

for murine cells, CHO-derived endogenous virus particles for CHO cells, pseudorabies virus for human cells].

When the purpose is to characterise the capacity of the manufacturing process to remove and/or inactivate viruses in general, i.e., to characterise the robustness of the clearance process, viral clearance characterisation studies should be performed with non-specific "model" viruses with differing properties.

In Chap. 4, the risk-based approach referred to as the minimum CMC regulatory compliance continuum was introduced. This risk-base approach can be adapted to the required viral clearance study. Comparing two guidance documents: (1) An EMA guideline addressing the minimum viral clearance requirements during the clinical development stage (especially in order to enter the clinic at FIH) [32] and (2) the ICH Q5A(R2) guideline addresses the minimum requirements for the market approval stage [30]. The comparison of the viral clearance requirements, on the one hand to enter clinical studies and on other hand to enter market approval, is presented in Table 5.5 (*italicized* indicate my added comments).

Therefore, a complete and thorough viral clearance study is not needed to enter the clinical development stage, it is needed for market approval. And this makes sense, since these viral clearance studies are expensive, and the manufacturer needs time to define and then lock down their final production and purification manufacturing processes.

An appropriate and adequate viral clearance study can only be as meaningful and relevant as the design of the study, and its execution. That is why ICH Q5A(R2) emphasizes the importance of the scientific and technical expertise of those who carry out these viral clearance studies [30]:

Therefore, viral clearance studies should be conducted in a separate laboratory equipped for virological work and performed by staff with virological expertise in conjunction with production personnel involved in designing and preparing a scaled-down version of the purification process.

However, two other guidances on viral clearance studies – the FDA Guidance for Industry on Process Validation and the PDA Technical Report #60 on Process Validation – not only emphasize the scientific and technical expertise needed, but also emphasize the need for Quality Unit oversight:

FDA [33]

Although often performed at small-scale laboratories, most viral inactivation and impurity clearance studies cannot be considered early process design experiments. Viral and impurity clearance studies intended to evaluate and estimate product quality at commercial scale should have a level of quality unit oversight that will ensure that the studies follow sound scientific methods and principles and the conclusions are supported by the data.

PDA [34]

The Quality Unit should provide appropriate oversight and approval of process validation studies required under GMPs. Although not all process validation activities are performed under GMPs, it is wise to include the Quality and Regulatory representatives on the cross-functional team. The degree and type of documentation required varies during the validation lifecycle, but documentation is an important element of all stages of process validations. Documentation requirements are greatest during the process qualifica-

Table 5.5 Minimum requirements for viral clearance safety evaluation – clinical versus for market

Minimum Requirements for Viral Clearance Safety Evaluation	
Clinical Development Stage (FIH onward)	**Market Approval Stage**
Validation of virus clearance to be completed and included in clinical trial application	**Full validation of virus clearance to be completed and included in market application**
Studies should include two orthogonal steps that complement each other in their mode of action studies; the reproducibility to be demonstrated by at least two independent studies	**Studies should include two distinct effective orthogonal steps that complement each other in their mode of action – an 'effective' virus removal step gives reproducible reduction of virus load in the order of 4 logs or more, shown by at least two independent studies**
Clearance to be demonstrated for more than one manufacturing process step	**Clearance to be demonstrated across the manufacturing process steps; one of the manufacturing steps should effectively clear non-enveloped viruses** **[*typically, all process steps that may contribute significantly to virus clearance are studied*]**
Clearance to be demonstrated for both an enveloped and a non-enveloped virus (preferably a parvovirus)	**Clearance to be demonstrated for a range of other potential virus types – different genomes (DNA, RNA), different physical sizes, enveloped/non-enveloped** **[*typically, 4 virus types studied*]**
N/A	**Chromatography media/resin lifetime use defined** **[*confirmed by reduced scale; concurrent validation at full scale*]**

tion and verification stages. Studies during these stages should conform to GMPs and be approved by the Quality Unit

The following case examples of market-approved protein-based biopharmaceuticals from an animal/human cell line manufacturing process illustrate how adventitious virus is effectively controlled:

CHO-Produced Monoclonal Antibody (Imjudo, Tremelimumab) [35]

A comprehensive programme, in accordance with ICH Q5A, is employed to test, evaluate and eliminate the potential risks of adventitious and endogenous viral agents. The programme includes control of raw materials used in the manufacturing, viral testing and characterisation of the cell banks (MCB, WCB, LIVCA) used in the GMP process, virus testing of UPB and viral clearance and inactivation assessment of the purification process. Viral clearance capability of the active substance purification process was evaluated in scale-down experiments using 4 model viruses. The viral clearance experiments were performed matching pre-defined acceptance ranges for process parameters and

performance outputs. The level of purification of the scaled-down version was shown to be representative of the production procedure.

All viral clearance experiments were performed in duplicate. The lower log10 reduction value (LRV) from the duplicate experiments was used to calculate cumulative LRV. The viral clearance experiments demonstrated that the purification process provides a cumulative LRV of ≥21.16, ≥18.28, ≥17.05, and ≥16.49, respectively, for the 4 model viruses. For the chromatography steps, the used chromatography resin provided LRVs either comparable to (within 0.5 log10) or better than the new chromatography resin, demonstrating that resin reuse has no negative impact on the viral clearance capacity of the chromatography steps. The resin sanitisation and storage studies demonstrated that the solutions used for the sanitisation and storage of the resins meet acceptable levels of antimicrobial efficacy and that the risk of cross contamination is minimal.

Endogenous retrovirus-like particles (RLPs) may be present in the cell line used to produce the tremelimumab active substance. These particles are measured by TEM analysis of the UPB. A safety factor for the removal of RLPs was calculated, resulting in a factor of greater than 9.0 log10 for the removal of endogenous virus, which is equivalent to less than 1 retrovirus-like particle for every 1.0 × 109 doses of tremelimumab. The results are considered adequate.

CHO-Produced Recombinant Fc Fusion Protein (Reblozyl, Luspatercept) [36]

Raw materials used in the luspatercept manufacturing process do not contain materials of animal origin. All the cell banks were extensively tested for non-viral adventitious agents. All testing specification were met and no bacterial, fungi, and mycoplasma contamination was detected. Appropriate characterisation and safety testing of the cell banks is in place; data derived thereof do not raise a concern. Unprocessed bulk from the cell culture is tested for adventitious agents, mycoplasma and mouse minute virus (MMV). The manufacturing process includes two dedicated virus inactivation/reduction steps, but also the three chromatography steps were included into the virus clearance studies. Virus clearance studies have been conducted with four model viruses in small scale models. The scale-down models have been appropriately qualified. The data demonstrate that the luspatercept purification process provides substantial clearance of viruses with a wide range of physicochemical properties through a combination of inactivation and removal. In summary, the provided information for both non-viral and viral adventitious agents is satisfactory, and no issues arise.

CHO-Produced Monoclonal Antibody (Qarziba, Dinutuximab Beta) [37]

The process steps C10 (Benzonase/Triton X-100 inactivation wash step), V10 (low pH inactivation), C20 (chromatographic step) and I20 (filtration step) were tested for their potential to clear viral contaminants. Only low clearance of Reo3 and MVM were observed in the initial study report for Protein A, therefore further analysis was performed separately for low pH inactivation and the Benzonase/Triton X-100 wash step, evaluated by incubation with these reagents for 30 min at 18 °C. This resulted in acceptable clearance of pseudorabies virus (PRV) (≥5.36 $\log_{10}$), but lower viral reduction of xenotropic murine leukemia virus (X-MuLV) (≥2.99 $\log_{10}$) under these conditions. Similar results were obtained for low pH, with clearance of PRV (≥5.03 $\log_{10}$), but lower clearance of X-MuLV (≥2.86 $\log_{10}$). Good clearance was shown for all viruses with the virus filtration step (I20) and the Q-Sepharose chromatography (flow-through). The combination of virus safety data from the cell banks (both MCB and EPC), data from TEM analysis of crude bulk harvest and the current safety margin obtained for X-MuLV during virus validation shows that the production process of APN311 is suitable for clearance of potential viral contamination. Overall $\log_{10}$ reduction factors were ≥17.09 for X-MuLV, ≥13.65 for MVM, ≥23.89 for PRV and ≥13.32 for Reo3. The safety margin (based on X-MuLV) is ≥9.14 $\log_{10}$…. Virus validation has not been performed using aged resin for Q-Sepharose chromatography, but this will be limited to a

maximum of 10 runs based on the small scale column lifetime studies, or until the chromatography resin expiry date.

5.3.2.4 Cell Culture Manufacturing Process: Viral Vector Biopharmaceuticals

Currently, the two viral vectors that are used in gene therapy are the recombinant adeno-associated virus (rAAV) used as a drug product for *in vivo* gene therapy, and the recombinant lentivirus (rLV) used as a starting material for *ex vivo* gene therapy. The three-fold virus control considerations that were applied to the protein-based biopharmaceutical manufacturing mentioned in Sect. 5.3.2.3, also have similar application to the viral vector biopharmaceutical manufacturing.

Viral vectors for gene therapy must be free of adventitious virus contamination. So testing is performed on all cell banks, the unprocessed bulk (considered the best location for detection) and as part of the batch release testing. Testing for adventitious virus in a viral vector has its challenges. The viral vector could cause a cytopathic effect with cell-based assays, in which case, the viral vector needs to be 'neutralized' in order to specifically measure for adventitious virus. Nucleic acid amplification tests (NATs or NAATs), such as polymerase chain reaction (PCR), measure the presence of virus DNA/RNA, not whether the virus present is infectious. Next Generation Sequencing (NGS) is attractive in its ability to detect unknown viral contaminations, but it also cannot determine from the nucleic acid sequence if the virus is infectious or not. Viral vectors that are manufactured using the insect cell-baculovirus system need to be tested for the absence of rhabdovirus.

Viral vectors must also be free of replication competent virus. Viral vectors are genetically designed to be non-replicating; therefore, the presence of a replication-competent virus is a serious patient safety concern. Even though the viral vectors are designed to be non-replicating, through recombinant events during culturing, viral vectors can become replication competent. So testing is performed on cell banks (only required on the MCB, not the WCB), the unprocessed bulk (considered the best location for detection) and as part of the batch release testing.

ICH Q5A(R2) *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* addresses some of the challenges in assessing the capacity of a viral vector purification process to clear adventitious viruses and other unwanted viruses from the desired viral product, and provides helpful guidance in designing the viral safety evaluation study [30]:

- Virus clearance should be validated using representative and qualified scale-down systems
- The physicochemical characteristics of the viral vector will determine how virus clearance will apply within the product purification scheme
- Virus clearance validation should include model viruses representative of adventitious, endogenous, and if possible, the relevant helper virus
- Common virus inactivation steps such as treatment with detergent or solvent/detergent may be suitable, when the product is compatible, such as non-enveloped viral

vectors. Alternatively, virus filtration may be more suitable for small viral vector such as AAV when virus removal can be based on the size exclusion.

- When appropriate, viral clearance studies should be performed to determine virus reduction factors for the relevant step(s) of the production process. Some viral-vector products such as AAV are amenable to robust viral clearance steps, ensuring adventitious and helper virus clearance inactivation or removal.
- Since virus clearance steps during production may not achieve the same robustness as for recombinant proteins, the viral safety of these products relies also on closed processing, testing and other preventative controls.

Always best to check with the contract testing laboratories that carry out he viral clearance safety evaluations for the latest on what is expected for viral vectors.

The following two case examples of market-approved viral vector biopharmaceuticals from both an insect cell line and a human cell line manufacturing process illustrate the total adventitious virus controls:

Recombinant AAV – Zolgensma (Onasemnogene Abeparvovec) [9]

rAAV vector manufactured using HEK293 human cells transfected with three plasmids.

Cell bank testing for viruses is described in CTD section 3.2.S.2.3. The commercial MCB is tested in line with ICH Q5A, including NAT test panels for human viruses and AAV, and *in vitro* assays for bovine and porcine viruses. WCBs are tested for viral contaminants *in vitro* using 3 cell lines.

All cell banks were found negative for all viruses tested except for a low signal with the PERT assay. Even though such result is not unexpected for this highly sensitive assay, the applicant was asked to clarify if an infectivity assay has been performed as required by Ph. Eur. 5.2.3 under such conditions. In response, the applicant justified the retrovirus testing scheme and committed to perform an infectivity assay for the next MCB in case of positive Q-PERT assay. Upon request, the applicant also provided data on virus testing on end-of-production (EOP) cells derived from the current three WCBs.

Production control cells, pre-lysed harvest and the intermediate are tested for viral contaminants *in vitro*, which is in line with Ph. Eur. and ICH Q5A. The Active Substance release tests include a test for rcAAV. A discussion of the risk of replication competent AAV is included in section 3.2.A.

Virus inactivation and clearance studies were performed. All three steps were validated for virus inactivation/removal in down scaled spiking studies. X-MuLV, PRV, HAV, and MVM were used as model viruses whose choice is acceptable. The down scaling was adequate. The results of the virus validation studies suggest that the manufacturing process is capable to effectively inactivate/remove enveloped viruses (log reduction >8).

Recombinant AAV – Roctavian (Valoctocogene Roxaparvovec) [38]

AAV vector manufactured by co-infection of Spodoptera frugiperda (Sf9) insect cells with recombinant baculovirus (rBV).

Analysis of raw materials, cell bank preparation and testing, adventitious agent control by environmental and other controls during manufacturing and viral clearance have been performed for the AAV5-hFVIII-SQ manufacturing process. Potential adventitious agent contamination is controlled through appropriate sourcing and screening of raw materials, testing of the cell banks and genetic starting materials, appropriate equipment cleaning, and a robust system of inactivation, removal, and in-process testing during the manufacturing process. The controls, precautions, testing, and demonstrated clearance of multiple virus types in the manufacturing process collectively demonstrate that the AAV5-hFVIII-SQ manufacturing process is robust and reproducible and provides adequate protection against contamination from adventitious agents in AAV5-hFVIII-SQ FBDS.

No materials of direct human or animal origin are used in the manufacture of AAV5-hFVIII-SQ. Several raw materials of indirect animal origin were used at the early stage. The information provided for raw materials confirms a negligible risk in relation to adventitious agents.

Cell banks and genetic starting materials used for AAV5-hFVIII-SQ production were screened for adventitious agents contamination, following the principles of Ph. Eur. 5.14 monograph.

The manufacturing process includes efficient steps to clear adventitious viruses according to viral validation studies performed.

Overall, adventitious agents safety is considered sufficiently assured.

Non-viral vectors such as mRNA encapsulated by lipids do not need to be tested for viral contamination due to their method of manufacture. First, the linearized DNA plasmid template is produced by recombinant *E. coli*. Bacteria do not propagate viruses (see Sect. 5.3.2.3.1). Second, the mRNA is enzymatically, but cell-free, transcribed from the linearized DNA plasmid template. Cells are required to propagate viruses. Therefore, incoming control of the materials and components used in the manufacturing process of mRNA non-viral vector is the barrier for protection against adventitious viruses.

5.3.2.5 Cell Culture Transduction/Expansion: Genetically Modified Patient Cells

As the drug substance and drug product for the genetically modified patient cells biopharmaceutical are both cells, the design of the manufacturing process contains no steps capable of inactivating or removing viruses. Therefore, virus safety for the transduction of patient cells with a viral vector (e.g., rLV) primarily relies on the adequate control (i.e., assurance of absence of viral contamination) of starting materials (patient cells and the lentivirus vector) and raw materials.

Two case examples from market-approved genetically modified patient cells biopharmaceuticals demonstrate the importance of the tight control of the starting materials and raw materials that are brought into these manufacturing processes:

Carvytki (Ciltacabtagene Autoleucel) [27]

No adventitious virus testing is done on the finished product and no virus inactivation steps are implemented in the ciltacabtagene autoleucel manufacturing process due to the nature of the product which consists of living cells and which is in line with current guidelines. Virus safety, therefore, relies on the selection, qualification, testing and control of the starting and raw materials. In summary, virus safety of Carvykti has been demonstrated.

Abecma (idecabtagene vicleucel) [39]

Virus safety

The virus safety of the ide-cel final product relies solely on the selection and quality of the raw materials, testing of starting materials and adherence to GMP. The anti-BCMA02 lentiviral vector is produced by transient transfection of HEK293T cells. The cell line genealogy has been sufficiently described and tested sufficiently for adventitious viruses according to Ph. Eur. 5.2.3 and ICH Q5A. No viruses were found by any assay in any cell bank. The autologous PBMCs are obtained from the patients by leukapheresis. Each patient/PBMC donor is tested at minimum according to Directive 2006/17/EC with CE-

marked test kits. No materials of direct animal origin are used in the manufacture of ide-cel. None of the excipients are of animal or human origin. Sufficient information on virus safety is provided for the other raw materials of biological origin and is supported by respective certificates. In summary, virus safety has been sufficiently assured.

5.3.3 No Room for Complacency with Adventitious Virus

From the examples of adventitious virus control illustrated in the previous section – animal-derived raw materials, starting materials involving human cells, protein-based biopharmaceuticals manufactured from many different cell lines, and viral vectors manufactured from insect/human cell lines – the challenge for biopharmaceuticals is enormous. Quality risk management to minimize or prevent adventitious virus contamination is a major commitment of resources both in terms of the involvement of Manufacturing and Quality personnel and in terms of the expense required to put in place and then carry out all of the needed virus controls. So once the virus risk assessment has been completed and the virus controls are operational, it is a natural response to relax and feel comfortable that everything necessary is now in place. But an effective adventitious virus risk management strategy must also include risk review, especially a reassessment of the risk controls as new scientific or technical information becomes available.

On the one hand, the number of adventitious virus contaminations that have occurred with market-approved biologics and biopharmaceuticals is low – only 12 adventitious virus contamination events with Chinese Hamster Ovary (CHO) cells have been reported in about 40 years [40]. However, there have been many thousands of biopharmaceutical batches manufactured over those years without any adventitious virus concerns. These numbers might lead some manufacturers into downplaying the adventitious virus risk to their biopharmaceutical processes. But that would be dangerous, as evidenced by the examples provided in Chap. 4, Sect. 4.3.5, which in one case example demonstrated how a Vesivirus 2117 contamination in a Chinese hamster ovary (CHO) manufacturing process shut the entire manufacturing facility down for over 3 months, and in a second case example demonstrated how a genetically engineered virus being manufactured in one biologic process became cross-contaminated with another genetically engineered virus being manufactured in a second biologic process.

The biopharmaceutical industry has a long history of supplying safe and effective therapies to patients owing to the extensive controls in place to ensure product safety. Despite these controls, adventitious virus contamination is a real risk with severe consequences. Although testing is a key component of viral safety, testing alone is not enough to ensure that a given product is free of a viral contaminant. A holistic, multifaceted approach must be taken.

Since it might be a matter of ‘not if, but ‘when’, an adventitious virus contamination might impact a biopharmaceutical manufacturing process and subsequently its facility’s operations, the motto of ‘always be prepared’ is important to apply. Having

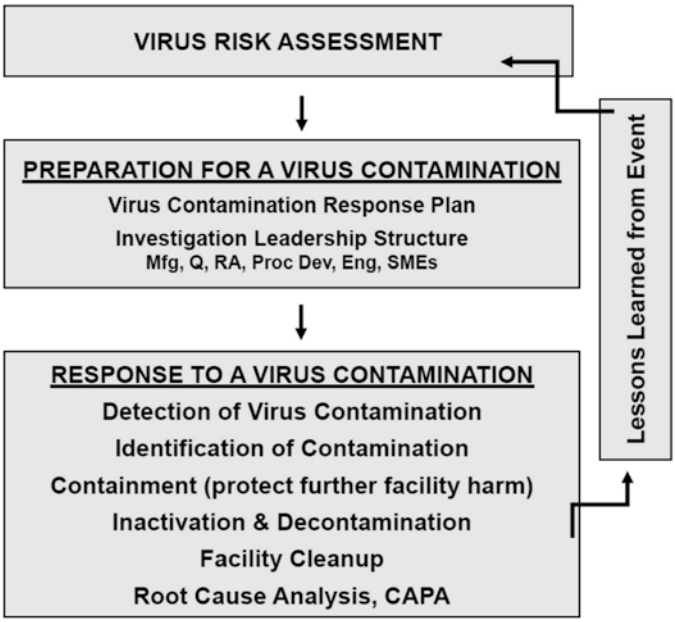

Fig. 5.1 Risk mitigation/risk preparation/response recovery for a facility virus contamination

a proactive virus contamination preparation and response plan in place makes good business and regulatory sense. The Parenteral Drug Association (PDA) has prepared an industry consensus Technical Report No. 83 entitled *Virus Contamination in Biomanufacturing: Risk Mitigation, Preparedness, and Response* [41]. Elements of the recommended risk mitigation, risk preparation and response recovery for dealing with an adventitious virus contamination is presented in Fig. 5.1.

5.4 Adventitious Mycoplasma

Mollicutes, best known genus being mycoplasma, is a class of bacteria with unique properties that creates unique challenges for biopharmaceutical manufacturers [42]:

> The genus Mycoplasma represents a group of minute bacteria which have no cell walls. The genus comprises more than 120 species. They are the smallest self-replicating prokaryotic organisms. The cells vary in size and morphology and cannot be Gram stained, but impressions of colonies on solid agar can be stained with methylene blue or equivalent. Mycoplasma are parasites and commensals, and some may be pathogenic to a variety of animal and plant hosts. In humans, Mycoplasma are usually surface parasites that colonize the epithelial lining of the respiratory and urogenital tracts. Mycoplasma are common and may cause serious contamination in cell and/or tissue cultures used to generate compendial articles. They may also cause contamination of filter sterilized soybean casein digest broth. A cell culture may persist for an extended period of time without causing apparent cell damage. Infection of cells in a culture can affect nearly every pathway of cell metabolism,

including alteration of the cells' phenotypical characteristics and normal growth. The presence of Mycoplasma species does not always result in turbid growth in cultures or visible alteration of the cells.

For biopharmaceutical manufacturers, infectious mycoplasma's unique properties of small size (0.1–0.8 microns in diameter) and pliable cell shape (due to lack of rigid cell wall) present a major four-fold challenge:

(1) Mycoplasmas can penetrate 0.22 μm-rated sterilizing-grade filters which is a key process step extensively used in the biopharmaceutical manufacturing industry to prevent bacterial intrusion into product solutions
(2) Routine quality control microbial test methods, that readily detect the presence of other bacteria, are not suitable for detecting presence of mycoplasmas – a specialized media (Hayflick media) is necessary for its growth
(3) A cell culture contaminated with infectious mycoplasma may or may not show any indication of cell growth property changes that can provide an early warning of contamination to manufacturing when monitoring the cell culture process
(4) If infectious mycoplasma gets into a cell culture manufacturing process, mycoplasma proliferation can occur, increasing the impact on the entire manufacturing facility should the contaminated biologic solution be passed from the closed bioreactor into the more open purification process

Mycoplasmas, due to their small genome size, require amino acids, cholesterol and long-chain fatty acids for growth. Eukaryotic cells (e.g., insect, animal or human), and the media that they are grown in, provide mycoplasmas with these needed biochemicals. Six mycoplasma species have been reported previously in mammalian cell culture contaminations [43], see Table 5.6.

5.4.1 Mycoplasma Risk Assessment

The level of patient safety risk from mycoplasma contamination is a function of three factors:

(1) *Probability of mycoplasma being present*. Like viruses, mycoplasmas carry a probability of presence from certain types of materials used in biopharmaceutical manufacturing. Exposure to animal- or human-derived raw materials during the manufacturing process, as well as biopharmaceuticals derived from insect,

Table 5.6 Mycoplasma species that have contaminated mammalian cell cultures

Mycoplasma Species Reported to Contaminate Mammalian Cell Culture Manufacturing	
Mycoplasma hyorhinis	*Mycoplasma orale*
Mycoplasma salivarium	*Mycoplasma fermentans*
Mycoplasma arginini	*Acholeplasma laidlawii*

animal or human cell lines, all carry a higher risk of introducing mycoplasma contamination. But it should be emphasized that since mycoplasmas can also be introduced into the biopharmaceutical manufacturing process from (a) non-biological raw materials, (b) the operators involved in manufacturing, and (c) the facility environment itself, most manufacturing processes involving cell culture steps have some level of risk of potential mycoplasma contamination.

(2) *Potential severity if mycoplasma contaminates the biopharmaceutical*. Severity from mycoplasma-contaminated biologic medicines is unknown. Fortunately, risk to human health from mycoplasmas contaminating biologic medicines is still only theoretical. To date, due to the rigorous testing required of all biologics, mycoplasma infections have not been implicated in any administered biologics. If mycoplasma is detected in a biopharmaceutical batch, that batch is rejected for patient safety reasons.

(3) *Detectability of mycoplasmas*. For biopharmaceutical manufacturers, infectious mycoplasmas present a somewhat tricky detection situation. Mycoplasma can induce changes to cell cultures producing the biopharmaceutical which include altered growth rates, morphological changes, chromosomal aberrations, and altered cell metabolism. The impact of these changes is not easy to predict as sometimes the cells are noticeably negatively impacted and sometimes the impact is covert. Testing for the presence of mycoplasmas requires either (1) up to 28 days if a culture method is used or (2) only hours if a nucleic acid amplification test (NAT), such as PCR, is used.

5.4.2 Mycoplasma Risk Control

Risk control for mycoplasma, which must be in place from manufacture of biopharmaceutical batches for first-in-human (FIH) clinical studies onward, considers the necessary steps to either eliminate or mitigate mycoplasma contamination of biopharmaceuticals from those manufacturing process steps identified in the risk assessment as being a potential contributor of contaminating mycoplasma. As seen in the previous section with viruses, risk control for mycoplasma contamination involves choices. If the chosen raw material is animal-derived, then the manufactured biopharmaceutical carries an increased risk of being exposed to mycoplasma contamination. If the chosen starting material is directly derived from human sources (e.g., patient cells, human donor cells), then mycoplasma risk is present. If the biopharmaceutical is derived from cell lines of insect, animal or human origin, then mycoplasma risk is present. Since the facility environment, the manufacturing staff, non-biological origin raw materials and components, and the design of the manufacturing process, also all could contribute to a risk of mycoplasma contamination, the unknown risk associated for each of these must also be evaluated and controlled.

Similar to the multiple complementary risk mitigating approaches used for controlling adventitious virus contamination, a multiple complementary approach is

used also for controlling adventitious mycoplasma contamination. The two complementary risk mitigating approaches used for controlling adventitious mycoplasma contaminations are: (1) erecting barriers to prevent entry of mycoplasma into the biopharmaceutical manufacturing process, and (2) testing the product at appropriate steps of production for the absence of contaminating infectious mycoplasmas. These two principle complementary approaches for adventitious mycoplasma control, which are applicable to all biopharmaceutical types are discussed in the next sections.

5.4.2.1 Two Complementary Mycoplasma Controls: Erecting Barriers and Testing

Mycoplasma contamination is well-known in laboratories that handle cell cultures [44]:

> Mycoplasmas are frequent contaminants of cell cultures and bioprocessing fluids. It is well established that mycoplasma contamination of continuous cell cultures ranges from 15% to 35%, with primary cell cultures exhibiting a minimum 1% contamination rate. Mycoplasma contamination can be caused by poor culturing practices or malfunctioning laboratory equipment. As an example of poor culture practice, reusing pipet tips may transfer mycoplasma-infected media into otherwise sterile media, propagating the spread of the contaminant. On the other hand, faulty laminar flow could disperse mycoplasma-containing dust and aerosols throughout a biosafety cabinet, thereby contaminating all of the media and cells within.

Biopharmaceutical manufacturers are also aware of mycoplasma contamination in their bioreactors, but there is a hesitancy for them to publicly discuss mycoplasma contaminations, almost like it is the industry's 'dark secret.' But it does occur, with estimates as high as 6% within the biopharmaceutical industry [45].

There are several barriers to mycoplasma entry that can be considered, depending upon the type of biopharmaceutical manufacturing process:

(1) Confirming that select raw materials and source materials that are brought into the manufacturing environment are tested first for absence of mycoplasmas
(2) Treating select raw materials with either gamma irradiation or UV irradiation or High Temperature Short Time (HTST)
(3) Ensuring that human operator exposure to a biopharmaceutical solution does not occur by following cGMP procedures
(4) For select biopharmaceutical solutions and buffers, apply 0.1 micron filtration.

All biopharmaceuticals either manufactured by insect/animal/human cell cultures or derived from human cells must be tested to ensure the absence of mycoplasma contamination. This is true regardless of whether the material is produced for clinical testing or as a marketed product. On the other hand, biopharmaceuticals derived from bacteria/yeast/plant cell cultures are not required to be tested for mycoplasma contamination, because the cell culture media does not support mycoplasma proliferation.

The following will be examined to illustrate the approaches that manufacturers have to take to control for the risk of adventitious mycoplasma:

- *Section 5.4.2.2* Test methods available for mycoplasma detection
- *Section 5.4.2.3* Cell culture manufacturing process: protein-based biopharmaceuticals
- *Section 5.4.2.4* Cell culture manufacturing process: viral vector biopharmaceuticals
- Section 5.4.2.5 Cell transduction/expansion: genetically modified patient cells

5.4.2.2 Test Methods Available for Mycoplasma Detection

The standard mycoplasma test is the 28-day culture method in which the appearance of 'fried egg' yellow colonies on a milky white medium background is confirmation of mycoplasma presence. The culture method detects all known mycoplasmas and at a very sensitive level. Mycoplasma testing via the cell culture method is describe in the pharmacopeias: United States Pharmacopeia <63> [42] and European Pharmacopeia 2.6.7 [46].

Although the culture-based method can sensitively detect all known mycoplasmas, the major concern for this test is that it requires a minimum 28-day incubation to complete. Nucleic acid amplification tests (NATs) such as the various polymerase chain reaction (PCR) assays amplify contaminating mycoplasma DNA and can produce a result within hours. Mycoplasma testing via NATs is described in the pharmacopeias: United States Pharmacopeia <77> [43] and European Pharmacopeia 2.6.7 [46]. Rapid test result turnaround is critical for time-sensitive mycoplasma contamination testing such as is encountered in the testing of (1) biopharmaceutical unprocessed bulks prior to breaking containment of a bioreactor and (2) cell-based biopharmaceutical medicines which may only have a shelf life of a few days.

Knowing that a bioreactor does not contain infectious mycoplasma prior to breaking containment is important for biopharmaceutical manufacturers of recombinant proteins, monoclonal antibodies, and viral vectors. Biopharmaceutical manufacturers cannot hold their unprocessed bulks for 28-days for the mycoplasma culture test, so they release the bioreactor contents into purification at manufacturer's risk. Should the culture test come back "positive" at the later date, the manufacturer has created a potential disaster, exposing both the manufacturing facility and its personnel to a significant mycoplasma contamination risk. NATs allow the manufacturer to rapidly test the unprocessed bulk for mycoplasmas within a typical work shift. The PCR testing typically starts about 20–24 h prior to harvest. If no mycoplasma DNA is detected in the PCR assay, then the bioreactor containment can be broken and the unprocessed bulk released to purification. If there is a question about the PCR assay, there is enough time to repeat the assay. If infectious mycoplasma was present in the bioreactor when the first sample was taken, there should be a further amplification of mycoplasma DNA due to the additional culture time in the bioreactor. If infectious mycoplasma DNA is confirmed in the PCR assay, then

the bioreactor containment is not broken, and the culture material is inactivated and properly disposed, and all of the bioreactor equipment and components are either sanitized or replaced.

NATs can replace the culture mycoplasma test method, but only after they have been appropriately validated to ensure that the NAT has the ability to detect the breadth of known mycoplasmas at the appropriate level of detection. See the pharmacopeia references mentioned above for how the NATs can be successfully validated to replace the 28-day culture test method. A word of caution about NATs – they only can measure mycoplasma DNA present; they cannot tell the difference between infectious mycoplasma and non-infectious (dead) mycoplasma.

5.4.2.3 Cell Culture Manufacturing Process: Protein-Based Biopharmaceuticals

Tests are required for the absence of mycoplasma contamination in insect/animal/human Master Cell Banks (MCBs), Working Cell Banks (WCBs), and every batch of the cell culture unprocessed bulk material (this is the stage in manufacturing where mycoplasma detection is the most sensitive) [47], see Table 5.7.

The following case example of a market-approved protein-based biopharmaceutical shows the testing for mycoplasma absence throughout the manufacturing process:

Enhertu (Trastuzumab Deruxtecan) Antibody-Drug Conjugate [48]

Cell banks for mAb

The master, working, and limit-of-in-vitro-age (LIVCA) cell banks have been tested according to ICH Q5A and Q5D for absence of non-viral (mycoplasma, bacteria, fungi) and viral adventitious agents, and endogenous retroviruses.

Bulk harvest of mAb

Bulk harvest is routinely tested for the bioburden level and the absence of mycoplasma and adventitious viruses (IPCs). The bioburden and mycoplasma testing are conducted according to Ph. Eur. Testing for viral contaminants is conducted using a 28 days *in vitro* assay for adventitious viruses with MRC-5, Vero, CHO, and 324K cells as detector cells. Results have been provided from three commercial scale batches, verifying the absence of mycoplasma, bioburden, and viral contamination.

Table 5.7 Mycoplasma testing to be performed across the protein-based manufacturing process

Biopharmaceutical Process Type	MCB/WCB	Unprocessed Bulk (Harvest)	Drug Substance	Drug Product
Protein-based biopharmaceuticals (recombinant proteins, monoclonal antibodies, biosimilars)	+	+	-	-

5.4.2.4 Cell Culture Manufacturing Process: Viral Vector Biopharmaceuticals

Viral vectors, whether for *in vivo* gene therapy or for *ex vivo* gene therapy, must meet the same absence of mycoplasma standard – recombinant adeno-associated virus (rAAV) is an *in vivo* viral vector used as the drug product, recombinant lentivirus (rLV), is an *ex vivo* viral vector us as the starting material. Tests are required for the absence of mycoplasma contamination in Master Cell Banks (MCBs), Working Cell Banks (WCBs), and every batch of the unprocessed virus bulk material (this is the stage in manufacturing where mycoplasma detection is the most sensitive) [14], see Table 5.8. Note if insect cells coupled with baculoviruses are used to manufacture the viral vector, testing for spiroplasma (a genus like mycoplasma in Mollicutes) is required in the Master Virus Banks (MVBs) and Working Virus Banks (WVBs).

The following case example of a viral vector (*ex vivo* rLV as starting material), used in the manufacture of a genetically modified patient cells biopharmaceutical, shows the testing for mycoplasma absence throughout the manufacturing process:

Tecartus (Autologous Anti-CD19-Transduced CD3+ Cells) [10]

Starting Materials – Retroviral Vector

A single vial of the PG13-CD19-CAR-H3 MCB was used to produce vials of WCB. The WCB has been shown to be free of bacterial, fungal and mycoplasma contamination in compliance with ICH Q5A (R1) and Ph. Eur. (Sect. 5.2.3).

Each harvest of PG13-CD19-H3 vector is considered a unique lot, and testing is conducted to assure sterility, while safety tests for mycoplasma, adventitious virus and replication competent retrovirus (RCR) are performed only on material in the last harvest from the production campaign. The last harvest is considered a worst-case condition and testing at this stage assures that the entire production campaign remains free of adventitious agents and that replication competent retrovirus is not present.

Verification of the compendial analytical methods for sterility, endotoxin and mycoplasma are performed in accordance with Ph. Eur. Summaries of the validation and verification reports for each method are provided.

Table 5.8 Mycoplasma testing to be performed across the viral vector manufacturing process

Biopharmaceutical Process Type	MCB/WCB	Unprocessed Bulk (Harvest)	End Use
rLV vectors for *ex vivo* gene therapy	+	+	- (starting material)
rAAV vectors for *in vivo* gene therapy	+	+	- (drug product)

5.4.2.5 Cell Transduction/Expansion Patient Cells: Genetically Modified Patient Cells

Due to the product being cells, concern for mycoplasma contamination throughout the manufacturing process has to be considered. There are two starting materials for *ex vivo* gene therapy: the viral vector and the patient/donor cells. Whether for *ex vivo* use or *in vivo* use, viral vectors are required to be tested for the absence of mycoplasma contamination during their manufacture (as noted above). Patient cells for autologous use are tested for mycoplasma absence. Donor cells for allogeneic use are required to be tested for absence of mycoplasma, as well as any Master Cell Banks (MCBs) and Working Cell Banks (WCBs) prepared. There is also a recommendation to perform mycoplasma testing on the transduced cell product at the manufacturing stage when the test is most likely to detect contamination, such as after pooling of the transduced cultures prior to cell washing [49]. Also, mycoplasma testing is required for the cellular biopharmaceutical drug product, see Table 5.9.

The following two case examples of market-approved genetically modified patient cells biopharmaceuticals show the testing for mycoplasma absence throughout the manufacturing process:

Abecma (Idecabtagene Vicleucel) [39]
Drug Substance – Transduced T-Cells
Microbial contamination control within the manufacturing process is ensured by using aseptic techniques and closed system manipulations, whenever possible. A mycoplasma release test at harvest day is performed. Manufacturing facilities are designed to prevent microbial contamination, and microbial contamination controls are in place. Raw materials are procured sterile or sterile filtered prior to use in manufacture.

Libmeldy (Autologous CD34+ cell encoding ARSA gene) [50]
Starting materials – Autologous cells (bone marrow or mobilised peripheral blood)
Procurement of bone marrow (BM) or mobilized peripheral blood (mPB) by qualified centres and compliance with relevant directives is confirmed. Virus screening, mycoplasma testing and compliance with EC 2004/23/EC is confirmed by verification of documentation prior to the start of manufacturing. Acceptance criteria for appearance and quantity of the cell suspension were included upon request.
Drug Product – Transduced Cells

Table 5.9 Mycoplasma testing to be performed across ex vivo gene therapy manufacturing process

Biopharmaceutical Process Type	Viral Vector	Patient/Donor Cells	After Transduction Prior to Washing	Drug Product
autologous *ex vivo* gene therapy	+	+	+	+
allogeneic *ex vivo* gene therapy	+	+ (also on MCB/WCB, if any	+	+

Microbiological control (BactAlert), endotoxin, and mycoplasma are tested at the finished product release, and the results will be available prior to administration to the patient.

5.4.3 CMC Strategy Tip: Improvements Sometimes Lead to Other Problems

The heightened safety concerns due to animal-derived materials potentially contaminating a biopharmaceutical with infectious prions have motivated manufacturers to try to substitute plant-derived proteins for animal-derived proteins. The move to plant-derived protein was considered "safe." What was not adequately considered was that there are no regulations that govern farming practices as it relates to plant-derived materials to be used for biopharmaceutical manufacturing. Plant-derived materials are exposed to rodents and insects and manure fertilizer (animal and/or human). Therefore, plant-derived materials could be a major carrier of other adventitious agents such as mycoplasmas.

A surprising result occurred at a manufacturer when "sterilized" TSB (a plant peptone-enriched medium) to be used in a media fill study was prepared by 0.2-μm filtration rather than by the typical steam autoclaving process. The mycoplasma contamination event that occurred was reported by the FDA [51]:

> A firm recently had multiple media fill failures. The media fill runs, simulating the filling process during production, were conducted inside an isolator. The firm used TSB (non-sterile bulk powder) from a commercial source, and prepared the sterile solution by filtering through a 0.2 micron sterilizing filter. An investigation was launched to trace the source of contamination. The investigation was not successful in isolating or recovering the contaminating organism using conventional microbiological techniques, including the use of selective (e.g., blood agar) and nonselective (e.g., TSB and tryptic soy agar) media, and examination under a microscope. The contaminant was eventually identified to be *Acholeplasma laidlawii* by using 16S rRNA gene sequence. The firm subsequently conducted studies to confirm the presence of *Acholeplasma laidlawii* in the lot of TSB used. Therefore, it was not a contaminant from the process, but from the media source.
>
> Resolution: For now, this firm has decided to filter prepared TSB, for use in media fills, through a 0.1 micron filter (note: we do not expect or require firms to routinely use 0.1 micron filters for media preparation). In the future, the firm will use sterile, irradiated TSB when it becomes available from a commercial supplier. (Firm's autoclave is too small to permit processing of TSB for media fills, so this was not a viable option.) The firm will continue monitoring for *Mycoplasma* and has revalidated their cleaning procedure to verify its removal. In this case, a thorough investigation by the firm led to a determination of the cause of the failure and an appropriate corrective action.

Every change carries a risk. Be careful about exchanging one adventitious agent risk for another.

5.5 Adventitious Bacteria/Fungi

The fourth and most ubiquitous adventitious agent is the bacteria/fungi. Bacteria are prokaryotic organisms, typically range in size from 0.5 to 5 microns and have a wide range of shapes (e.g., spherical, rod, coiled), cell walls (e.g., Gram-positive thick cell wall, Gram-negative thin cell wall), and different nutrient growth requirements. Fungi are eukaryotic organisms, typically grow as hyphae, which are cylindrical, threadlike structures of 2–10 microns in diameter and up to several centimeters in length.

For biopharmaceutical manufacturers, infectious bacteria and fungi ubiquitous presence and their ability to survive, if not proliferate, in a variety of solutions present a five-fold challenge:

(1) Bacteria/fungi are everywhere throughout the entire manufacturing facility, in the raw materials, starting materials, and components used for biopharmaceutical manufacturing, and in the staff that are present.
(2) If bacteria/fungi enter into a cell culture manufacturing process, microbial proliferation can occur due to the rich nutrient media used, increasing the impact on the entire manufacturing facility if a contaminated biopharmaceutical solution is passed from the closed bioreactor into the more open purification process.
(3) Bacteria/fungi contamination can also occur and possibly proliferate even in the downstream purification process buffers and solutions.
(4) Bacteria/fungi contamination can also occur during formulation and the drug product filling operations. And if there is a breach in the integrity of the container closure, bacteria/fungi contamination can enter into the biopharmaceutical product on the shelf.
(5) Bacteria/fungi can excrete endotoxins and exotoxins that are harmful to patients, and they can also excrete protein hydrolyzing proteases that can affect a protein-based biopharmaceutical's shelf life. (Concerns and testing for the excreted bacteria/fungi components are discussed in Chap. 10 under process-related impurities).

5.5.1 Bacteria/Fungi Risk Assessment

The level of patient safety risk from bacteria/fungi contamination is a function of three factors:

(1) *Probability of bacteria/fungi being present*. Probability of presence of infectious bacteria/fungi can occur throughout the entire manufacturing process, from raw materials to the source materials to the drug substance upstream and downstream processes and all the way to the biopharmaceutical drug product being filled into a container closure. However, probability of proliferation of infectious bacteria/fungi is greatest either during a cell culture manufacturing

process or during the handling of patient cells for genetic transformation and subsequent culturing.

(2) *Potential severity if bacteria/fungi contaminate the biopharmaceutical*. Severity to human health from bacteria/fungi contaminating biologic medicines is not just theoretical. A therapeutic serum was contaminated with *Clostridium tetani* (which causes tetanus, lockjaw) resulting in the death of children [52]:

> Jim was an ordinary horse, but he had an extraordinary effect on public health. Some say this retired milk wagon horse spurred the passage of the law that eventually gave the Food and Drug Administration its regulatory authority over vaccines and other biological products. In 1901, diphtheria patients were routinely treated with antitoxin derived from the blood serum of horses. After 13 children died of tetanus because of contaminated antitoxin, Congress passed the 1902 Biologics Control Act, giving the government its first regulation of vaccine and antitoxin production. Jim's prominence stemmed from a tragedy in St. Louis in 1901. At that time, the standard treatment for children with diphtheria was an antitoxin serum made from the blood of horses. Jim had produced over 30 quarts of antitoxin in three years, but the horse was destroyed after contracting tetanus. The serum from Jim's tainted blood was accidentally bottled and used to treat diphtheria patients, causing the death of 13 children. The serum had been manufactured in local establishments with no central or uniform controls in place to ensure potency and purity. Nor were there inspections or testing of the final product.

3. *Detectability of bacteria/fungi*. For biopharmaceutical manufacturers, infectious bacteria/fungi are generally easy to detect, since the bulk of Quality Control's microbiology testing services are committed to this. However, confirmation of bacteria/fungi absence requires up to 14 days if a culture method is used ('no growth') or absence can be determined within a few days if a rapid microbiological method (RMM) is used. See the sections below for more information on testing.

5.5.2 Bacteria/Fungi Risk Control

Risk control for bacteria/fungi, which must be in place from manufacture of biopharmaceutical batches for first-in-human (FIH) clinical studies onward, considers the necessary steps to either eliminate or mitigate contamination of biopharmaceuticals from those conditions, components and manufacturing process steps that are potential contributors of contaminating bacteria/fungi.

Similar to the multiple complementary risk mitigating approaches used for controlling adventitious virus and mycoplasma contamination, a multiple complementary approach is used also for controlling adventitious bacterial/fungal contamination. The two complementary risk mitigating approaches used for controlling adventitious bacterial/fungal contaminations are: (1) erecting barriers to prevent entry of bacteria/fungi into the biopharmaceutical manufacturing process, and (2) testing the product at appropriate steps of production for the absence of contaminating infectious bacteria/fungi. These two principle complementary approaches for adventitious bacterial/fungal control are discussed in the next sections.

5.5.2.1 Two Complementary Bacteria/Fungi Controls: Erecting Barriers and Testing

The United States Pharmacopeia (USP) is preparing a general monograph for considering microbial risk control in a holistic way – <1114> *Microbial Control Strategies for Cell Therapy Products* [53]. While specific for cell therapy products, it is also applicable to all biopharmaceuticals, see Table 5.10. Basically, from a holistic risk-based control strategy, every portal that bacteria/fungi could enter the biopharmaceutical manufacturing process needs to be first risk level-assessed, then the assigned risk levels prioritized according to the potential of allowing contamination into the process or product. Based on this prioritization, if need be, additional barriers can be added to protect the process and the product further from bacteria/fungi contamination. Barriers such as (1) the selection and testing of raw materials (especially those that are animal-derived) and source materials (e.g., cell banks) before they are brought into the manufacturing environment, and (2) the liberal use of 0.2 micron microbial-reduction filtration, are all very important barriers.

Another holistic approach to microbial risk control can be found in the PDA Technical Report Number 90 *Contamination Control Strategy Development in*

Table 5.10 A holistic approach to microbial risk control

MICROBIAL RISK CONTROL CONSIDERATIONS			
FACTORS	**Manufacturing Operations**	**Manufacturing Facilities**	**Materials**
CONTROLS	**Process Design** **Process Validation** **Qualified Personnel** **Gowned Personnel**	**Facility Design Optimized** **Equipment Design/ Maintenance** **Utilities System Qualification** **Product/Process Flow**	**Single Use & Sterilized Materials** **Integrity/ Compatibility** **Supplier Qualification/ Certification**
MONITORING / TESTING	**In-Process Control Testing** **Final Product Testing** **Aseptic Process Simulation** **Quality Assurance Oversight** **Personnel Training & Qualification Program**	**Microbiological Environmental Monitoring** **Disinfectant Effectiveness Validation** **Air Filter Integrity Test** **Utilities Monitoring (water, air, etc.)**	**Supplier Initial & Recertification Program** **Container Closure Integrity Test** **Material Microbiology Testing**

Pharmaceutical Manufacturing (2023) [54]. This document covers the elements of the contamination control strategy (CCS) ranging from process design, facilities and utilities, raw materials, environmental monitoring, personnel training, process equipment design and validation, etc.

In addition to erecting barriers of entry for adventitious bacterial/fungi, testing for this contamination is needed across the entire biopharmaceutical manufacturing process:

- Raw materials (especially animal-derived – typically performed by the vendor)
- Source materials (e.g., cell banks, plasmids, viral and non-viral vectors, patient cells, etc.)
- Unprocessed bulk material (i.e., the cell culture immediately prior to harvest of a bioreactor)
- Purification (each process step)
- Drug substance release
- Drug product release

It is extremely challenging to keep adventitious bacteria/fungi out of the biopharmaceutical manufacturing processes; therefore, All biopharmaceuticals must be tested to ensure the absence of contamination. This is true regardless of whether the material is produced for clinical testing or as a marketed product. The following will be examined to illustrate the approaches that manufacturers have to take to control for the risk of adventitious bacteria/fungi:

- *Section 5.5.2.2* Test methods available for bacteria/fungi detection
- *Section 5.5.2.3* Bacteria/fungi control; protein-based biopharmaceuticals
- *Section 5.5.2.4* Bacteria/fungi control; gene therapy-based biopharmaceuticals

5.5.2.2 Test Methods Available for Bacterial/Fungi Detection

Detection and/or quantitation of adventitious bacteria/fungi presence can be done using culture-based test methods that employ visual confirmation of turbidity or colony enumeration: sterility test, culture purity test and microbial enumeration test. Culture-based test methods are described in pharmacopeias, such as the United States Pharmacopeia (USP), the European Pharmacopoeia (Ph. Eur.) and the Japanese Pharmacopeia (JP).

- ***Sterility Test***. Sterility testing involves broth immersion (either the test sample is directly inoculated into the broth or the test sample is passed through a size-exclusion membrane capable of retaining microbes, followed by washing and then immersion of membrane into the broth). Incubation is for 14 days in two specified broth media. The ICH harmonized sterility test procedure is found in the pharmacopeias: <71> USP; *Section 2.6.1* Ph. Eur.; and *Section 4.06* JP. Detection of viable microbes is by visual examination for turbidity formation in the liquid broths, and the acceptance criterion for the sterility test is “no growth.”

- ***Culture Purity Test***. This test is a modification of the sterility test to be used with prokaryote cultures (e.g., bacteria and yeast cells). Determination of "no growth" in the standard growth-based sterility test is not possible due to the interference from the bacteria/yeast production cells. An alternate test, referred to as a "culture purity test" or a "nonhost contamination test," is used whenever the production cells are present. Test samples are plated on agar plates containing specified media and incubated at specified temperatures. After incubation, the colonies on the plate are microscopically examined to look for morphological differences. With a trained microbiologist, the ability to detect contaminating microbes is typically better than 1 contaminating microbe per 50,000 colonies or better.
- ***Microbial Enumeration Test***. This test, also commonly known as a 'Bioburden Test', is a quantitative test that determines the Total Aerobic Microbial Count (TAMC) and Total Yeast and Mold Count (TYMC) present. Testing involves agar plates – either the test sample is directly spread over the agar plate surface, or the test sample is passed through a size-exclusion membrane capable of retaining microbes, followed by washing and then placing the membrane on the agar plate surface. Incubation for a minimum of 3–5 days. The ICH harmonized test procedure is found in the pharmacopeias: <61> USP; *Section 2.6.12* Ph. Eur.; and *Section 4.05* JP. Quantitation of viable microbes is by visual counting of the colony-forming units (CFUs) on the agar surfaces. For bioburden, modifications of the microbial enumeration test can include use of only a single agar plate medium, different incubation times, volume of test solution, etc.

Although the historical culture-based test methods can sensitively detect a broad spectrum of microbes, the major challenge is that the tests require 3–5 days (bioburden) or 14 days (sterility) of incubation to complete. Such long incubation time periods are not appropriate either for short shelf-life biopharmaceuticals which require short manufacturing turnaround times or when there is limited sample available (e.g., patient specific cells). Various rapid microbiological methods (RMMs) have been developed to more rapidly test for and detect bacteria/fungi, for example: (1) growth-based high-magnification imaging systems to visually detect colonies appearing from solutions plated on microbial growth plates (results typically within 24–48 h), and (2) growth-based adenosine triphosphate (ATP) bioluminescence using ATP-luciferin-luciferase detection of colonies appearing from solutions plated on microbial growth plates (results typically within 24–48 h). A list of current RMMs can be found on the Rapid Microbiology Methods website (*rapidmicromethods.com/*). Validation of RMMs is described in <1233> USP and Section 5.1.6 Ph. Eur. Validation of RMMs is also described in industry technical reports: PDA Technical Report 33 [55] and PDA Points to Consider for Microbial Control in ATMP Manufacturing [56]. In addition, some vendors of RMMs provide validation protocol and report templates, as well as advice on how to validate their specific RMM.

5.5.2.3 Bacteria/Fungi Control: Protein-Based Biopharmaceuticals

Regulatory authorities are very concerned that biopharmaceutical manufacturers provide adequate and appropriate control over microbial contamination, not only at the end of the manufacturing process but also throughout the entire process, and not only for commercial manufacturing but also during clinical development manufacturing. The following comments and referenced guidances express their concern for the protein-based biopharmaceuticals (recombinant proteins and monoclonal antibodies).

A clinical development guidance from the FDA, Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use [57], states clearly where bacteria/fungi testing is required through a monoclonal antibody manufacturing process:

- Sterility testing for the Master Cell Bank and the Working Cell Bank is required
- Either sterility testing (no growth) or bioburden testing with acceptable limits is required for the unprocessed bulk stage
- Either sterility testing (no growth) or bioburden testing with acceptable limits is required for the drug substance
- Sterility testing is required for the drug product (injectable)

Table 5.11 summarizes the regulatory authority requirements and recommendations, and combines it with some industry experience, for bacteria/fungi testing across a typical recombinant protein or monoclonal antibody manufacturing process. Required acceptance criteria or that which is recommended is also included.

The PDA Technical Report Number 90 *Contamination Control Strategy Development in Pharmaceutical Manufacturing* (2023) [54] lists the following in-process bioburden levels 'currently expected' for recombinant protein and monoclonal antibody manufacturing:

- Bioreactor harvest: NMT 10 CFU/10 mL
- Purification process steps: NMT 100 CFU/10 mL
- Prior to sterile filtration of DP: NMT 10 CFU/100 mL

These limits agree with Table 5.11, except the bioreactor harvest (unprocessed bulk) is a ten-fold higher threshold.

FDA uses a meeting-minutes-approach to stress to protein-based biopharmaceutical manufacturers the seriousness of their concern to have adequate control over bacteria/fungi. When a manufacturer approaches them for a pre-BLA submission meeting, the FDA frequently includes in the written meeting minutes, additional specific guidance on their expectations for the bacteria/fungi control information to be included in the planned filing. The following three tables contain the information on bacteria/fungi control, provided to the manufacturer of a recombinant protein seeking market approval, Nexviazyme (avalglucosidase alfa-ngpt) [58]: Table 5.12 covers the Drug Substance (Module 3.2.S), Table 5.13 covers the Drug Product control (Module 3.2.P.3.3/.4), and Table 5.14 cover the Drug Product process validation (Module 3.2.P.3.5).

Table 5.11 Bacteria/Fungi testing requirements/recommendations for a mAb process

MONOCLONAL ANTIBODY MANUFACTURING PROCESS			
Process Step	**Bacteria/Fungi Testing**	**Required**	**Recommended**
MCB/WCB	Sterility	Sterile	
Cell Culture Expansion Production	Microscopic Visual Check		No unexpected microorganisms
Unprocessed Bulk (prior to harvest)	Sterility or Bioburden	Sterile or NMT 1 CFU/10 mL	
Purification	Bioburden		Low, but set on manufacturing process capability
Drug Substance	Sterility or Bioburden	Sterile or Bioburden NMT 1 CFU/10 mL (if to be held refrigerated)	
Prior to Sterile Filtration	Bioburden	NMT 10 CFU/100 mL	
Drug Product (Injectable)	Sterility	Sterile	

Guidance is provided on monitoring bioburden and endotoxin across both the drug substance and the drug product manufacturing process steps, ensuring that they are included in hold times studies (along with the stability testing of the biopharmaceutical product) to validate the holds, the critical nature of the low bioburden level loaded onto a sterilizing filter to get the correct sterility assurance level (SAL).

5.5.2.4 Bacteria/Fungi Control: Gene Therapy-Based Biopharmaceuticals

Regulatory authorities are just as concerned, if not more so, for bacteria/fungi control during gene therapy-based biopharmaceutical manufacturing since the products are either infectious viral vectors or living transduced patient cells. FDA's guidance [59] is included in Table 5.15 for the drug substance and Table 5.16 for the drug product.

Guidance is provided on monitoring bioburden and endotoxin across the starting material manufacture (i.e., the DNA plasmids), and both the drug substance and the drug product manufacturing process steps; and that microbial testing take place at the most appropriate location(s) in the manufacturing process.

Table 5.12 Bacteria/fungi control data to be included in BLA filing – Module 3.2.S

The CMC Drug Substance section of the 351(a) BLA (Section 3.2.S) should contain information and data summaries for microbial and endotoxin control of the drug substance. The information should include, but not be limited to, the following: **• Bioburden and endotoxin levels at critical manufacturing steps should be monitored using qualified bioburden and endotoxin tests. Bioburden sampling should occur prior to any 0.2 µm filtration step. The pre-established bioburden and endotoxin limits should be provided (3.2.S.2.4).** **• Bioburden and endotoxin data obtained during manufacture of three process qualification (PPQ) lots (3.2.S.2.5).** **• Microbial data from three successful product intermediate hold time validation runs at manufacturing scale. Bioburden and endotoxin levels before and after the maximum allowed hold time should be monitored and bioburden and endotoxin limits provided (3.2.S.2.5).** **• Chromatography resin and UF/DF membrane lifetime study protocols and acceptance criteria for bioburden and endotoxin samples. During the lifetime studies, bioburden and endotoxin samples should be taken at the end of storage prior to sanitization (3.2.S.2.5).** **• Information and summary results from the shipping validation studies (3.2.S.2.5).** **• Drug substance bioburden and endotoxin release specifications (3.2.S.4).** **• Summary reports and results from bioburden and endotoxin test method qualification studies performed for in-process intermediates and the drug substance. If compendial test methods are used, brief descriptions of the methods should be provided in addition to the compendial reference numbers (3.2.S.4).**

Both FDA and EMA sound warnings on the challenges of the sterility test for these viral vectors and genetically modified patient cells biopharmaceuticals: Table 5.17 (for FDA [14]) and Table 5.18 (for EMA, [60]). The allowance, if needed, to take samples prior to the final drug product for sterility testing, and also using gram stains, as an early surrogate of sterility, followed by the official sterility test (sometimes completed after patient administration). The warning of being careful with the use of antibiotics – antibiotics can reduce bioburden, but they can not only interfere with the sterility testing, but also as a residual become a patient safety concern.

The following two case examples of market-approved gene therapy-based biopharmaceuticals illustrate bacteria/fungi control throughout their manufacturing process:

Viral Vector (rAAV) – Zolgensma (Onasemnogene Abeparvovec) [9]
Non-viral adventitious agents

Table 5.13 Bacteria/fungi control data to be included in BLA filing – Module 3.2.P.3.3/.4

The CMC Drug Product section of the 351(a) BLA (Section 3.2.P) should contain validation data summaries to support the aseptic processing operations. For guidance on the type of data and information that should be submitted, refer to Guidance for Industry for the Submission Documentation for Sterilization Process Validation in Applications for Human and Veterinary Drug Products.
The following information should be provided in Sections 3.2.P.3.3 and/or 3.2.P.3.4, as appropriate.
- **Identification of the manufacturing areas and type of fill line (e.g., open, RABS, isolator), including area classifications.**
- **Description of the sterilizing filter (supplier, size, membrane material, membrane surface area, etc.); sterilizing filtration parameters (pressure and/or flow rate), as validated by the microbial retention study; wetting agent used for post-use integrity testing of the sterilizing filter and post-use integrity test acceptance criteria.**
- **Parameters for filling and capping for the vials.**
- **A list of all equipment and components that contact the sterile drug product (i.e. the sterile-fluid pathway) with the corresponding method(s) of sterilization and depyrogenation, including process parameters. The list should include single-use equipment.**
- **Processing and hold time limits, including the time limit for sterilizing filtration and aseptic filling.**
- **Sampling points and in-process limits for bioburden and endotoxin. Bioburden samples should be taken at the end of the hold time prior to the subsequent filtration step. Pre-sterile filtration bioburden limits should not exceed 10 CFU/100 mL**

Control of contamination of the manufacturing facility (e.g. environmental monitoring, cleaning procedures) are briefly described in section 3.2.A.1; Media fill studies are described in section 3.2.P.3. and have been successfully performed.

With regard to the testing performed for non-viral adventitious agents, for cell bank testing, and the Active substance manufacturing process reference is made to CTD section 3.2.S.2.3. The cell banks tested negative for bacteria, fungi and mycoplasma. In process controls for bioburden and endotoxin are in place. The Active substance release tests include bioburden; Finished product is tested for sterility and endotoxin. The provided information suggests adequate control of non-viral adventitious agents.

Recommendation(s) for future quality development

In the context of the obligation of the MAHs to take due account of technical and scientific progress, the CAT recommends the following points for investigation: … 8. The applicant commits to revalidate the sterility test method to demonstrate absence of interference for Ph. Eur. compliant sampling volumes by June 2020.

Ex Vivo Gene Therapy – Libmedy (Autologous CD34+ Cell Encoding ARSA Gene) [50]

Starting materials (Lentivirus Vector)

The information provided for plasmid manufacturing and control is generally in line with the expectations. The plasmids are manufactured in accordance with the principles of GMP and testing is in agreement with Ph. Eur. 5.14 requirements. (including identity, genomic integrity, plasmid DNA, host cell DNA, endotoxin and sterility).

Control of critical steps and intermediates (Active Substance)

In process controls for the active substance manufacturing process are in place. Microbiological control is performed on the starting materials. The finished product is tested for mycoplasma, endotoxin, and sterility.

Table 5.14 Bacteria/fungi control data to be included in BLA filing – Module 3.2.P.3.5

The following study protocols and validation data summaries should be included in Section 3.2.P.3.5, as appropriate:
- **Bacterial filter retention study for the sterilizing filter. Include a comparison of validation test parameters with routine sterile filtration parameters.**
- **Sterilization and depyrogenation of equipment and components that contact the sterile drug product. Provide summary data for the three validation studies and describe the equipment and component revalidation program.**
- **In-process microbial controls and hold times. Three successful product intermediate hold time validation runs should be performed at manufacturing scale, unless an alternative approach can be scientifically justified. Bioburden and endotoxin levels before and after the maximum allowed hold time should be monitored and bioburden and endotoxin limits provided.**
- **Isolator decontamination summary data and information, if applicable.**
- **Three successful consecutive media fill runs, including summary environmental monitoring data obtained during the runs. Describe the environmental and personnel monitoring procedures followed during media fills and compare them to the procedures followed during routine production.**
- **Information and summary results from shipping validation studies.**
- **Validation of capping parameters, using a container closure integrity test.**
- **Lyophilizer sterilization validation summary data and information.**

Non-viral adventitious agents

The microbial control is sufficiently described. Appropriate in process controls are in place in the LVV manufacturing process. Microbiological control is performed on the mPB or BM. Upon request a method description and validation were provided. As the manufacturing process is short and includes several wash steps, it is agreed that no further in process tests are in place for microbial control. The final product is tested for mycoplasma, endotoxin, and sterility.

A short note on bacteria/fungi control for non-viral vectors, such as mRNA encapsulated in lipids. The *in vitro* transcription of the linearized DNA plasmid template is cell-free; and the biopharmaceutical product is not an infectious virus or a living cell. Therefore, the control of bacteria/fungi for mRNA is very similar to the control of bacteria/fungi used with the protein-based biopharmaceuticals (from the harvest through the downstream purification process onto the drug product formulation and sterile filling into a container closure).

Table 5.15 Bacteria/fungi control for the drug substance (Module 3.2.S)

Description of Manufacturing Process and Process Controls (3.2.S.2.2)
The description of your manufacturing process should include a process flow diagram(s) and a detailed narrative. Your description should clearly identify any process controls and in-process testing (e.g., titer, bioburden, viability, impurities) as well as acceptable operating parameters (e.g., process times, temperature ranges, cell passage number, pH, CO_2, dissolved O_2, glucose level).
Cell Culture (for Vector Production)
The description of all cell culture conditions should contain sufficient detail to make understandable any of the process steps that apply, process timing, culture conditions, hold times and transfer steps, and materials used (e.g., media components, bags/flasks). You should describe whether the cell culture system is open or closed and any aseptic processing steps. If extensive culture times are needed, you should outline the in-process controls you have in place to monitor cell quality (e.g., viability, bioburden, pH, dissolved O_2).
Control of Drug Substance (3.2.S.4)
Safety testing on the DS should include microbiological testing, such as bioburden (or sterility, as appropriate), mycoplasma, and adventitious viral agent testing, to ensure product quality. Guidelines and/or procedures for many safety tests have been described in detail, elsewhere (e.g., bioburden, sterility, mycoplasma, adventitious agent testing, and tests for specific pathogens. Analytical procedures different than those outlined in the United States Pharmacopeia (USP), FDA guidance, or CFR may be acceptable under an IND if you provide adequate information about your test method, including specificity, sensitivity, and robustness. Examples of alternative methods, which may be needed for live cells, include rapid sterility tests, rapid mycoplasma tests (including PCR-based tests), and rapid endotoxin tests. For these non-compendial tests we recommend that you qualify/validate them to ensure they are fit for their intended use.

5.5.2.5 Message Received

Because adventitious bacteria/fungi are so prevalent and the potential of so many portals of entry into a biopharmaceutical manufacturing process, there is so much regulatory authority guidance provided to ensure that patients will be protected from this adventitious contamination. Quality risk management to minimize or prevent adventitious bacterial/fungi contamination of biopharmaceuticals is a major commitment of resources. Commitment of Manufacturing and Quality personnel involvement, commitment in terms of resources to carry out of the necessary validated aseptic processing conditions, and all of the expenses for the in-process testing and final release testing.

A major, very important barrier, in the control of bacteria/fungi, is to ensure that current good manufacturing practices (cGMPs) are followed. Paying attention to what is brought into the biopharmaceutical manufacturing facility (e.g., avoid cardboard and wood which can have high microbial bioburdens), how the manufacturing personnel are dressed (e.g., adequate and appropriate gowning), and monitoring

Table 5.16 Bacteria/fungi control for the drug product (Module 3.2.P)

Pharmaceutical Development (3.2.P.2)
Microbiological Attributes
We recommend, for products intended to be sterile, that you provide details on measures taken to ensure aseptic processing, describe the final product microbial testing, and address how the integrity of the container closure system to prevent microbial contamination will be assessed.
Drug Product Manufacture (3.2.P.3)
For ex vivo genetically modified cells that are administered immediately after manufacturing, we recommend a negative test result from an in-process sterility test (on a sample taken 48 to 72 hours prior to final harvest) for release of the DP.
Control of Drug Product (3.2.P.5)
Specifications
We recommend that product release assays be performed at the manufacturing step at which they are necessary and appropriate. For example, mycoplasma and adventitious agents release testing is recommended on cell culture harvest material, as discussed in section V.A.4.a., "Specification (3.2.S.4.1)," of this guidance. In addition, sterility, endotoxin, and identity testing are recommended on the final container product to ensure absence of microbial contamination or to detect product mix-ups that might have occurred during the final DP manufacturing steps (e.g., buffer exchange, dilution, or finish and fill steps).
If you freeze the DP before use, we recommend that you perform sterility testing on a sample of the product prior to cryopreservation so that results will be available before the product is administered to a patient. However, if the product undergoes manipulation after thawing (e.g., washing, culturing), particularly if procedures are performed in an open system, you may need to perform additional release testing including sterility and identity testing to ensure product quality.
We recommend that you incorporate the results of in-process sterility testing into your acceptance criteria for final product specifications.

the environment in which the biopharmaceutical is being handled (e.g., environmental monitoring programs) are basic cGMPs. Following cGMPs are especially critical during aseptic processing conditions where sterility of the biopharmaceutical is the intended requirement. FDA's guidance on aseptic processing [61] and EMA's Annex 1 on aseptic processing [62] provide the biopharmaceutical industry with specific requirements and recommendations on how aseptic processing is to be performed, especially the importance of the process simulations.

It is because of this importance that regulatory authorities carry out thorough and comprehensive cGMP inspections of the manufacturer's biopharmaceutical operations. And sometimes, the manufacturer comes up short on meeting the required cGMP standard, as is revealed in the following FDA 483 deficiencies noted upon inspection of biopharmaceutical manufacturers:

Rentschler Biopharma (Dates of FDA Inspection – February 07-15, 2022 [63]
Drug Substance Manufacturer – Recombinant Proteins, Monoclonal Antibodies
Deficiencies in aseptic process simulation for filling process

Table 5.17 FDA caution about sterility testing challenges for gene therapy-based biopharmaceuticals

Analytical Procedures - Sterility

We recognize that the compendial sterility tests (USP <71>; 610.12) may not be suitable for all products (e.g., those with limited shelf life). As mentioned in section V.A.4.b., "Analytical Procedures (3.2.S.4.2)," of this guidance, rapid sterility tests may be acceptable for ex vivo genetically modified cells administered fresh or with limited hold time between final formulation and patient administration.

For ex vivo genetically modified cells that are administered immediately after manufacturing, in-process sterility testing on sample taken 48 to 72 hours prior to final harvest is recommended for product release. For such products, aside from an in-process sterility test, we also recommend that sponsors perform a rapid microbial detection test, such as a Gram stain, on the final formulated product and a sterility test, compliant with 21 CFR 610.12, on the final formulated product.

Under this approach, the release criteria for sterility would be based on a negative result of the Gram stain and a no-growth result from the 48 to 72 hour in-process sterility test. Although the results of the sterility culture performed on the final product will not be available for product release, this testing will provide useful data. A negative result will provide assurance that an aseptic technique was maintained. A positive result will provide information for the medical management of the subject and trigger an investigation of the cause of the sterility failure. The sterility test on the final formulated product should be continued for the full duration (e.g.14 days for USP <71> method) to obtain the final sterility test result, even after the product has been administered to the patient.

In addition, please be aware that a product may sometimes interfere with the results of sterility testing. For example, a product component or manufacturing impurities (e.g., antibiotics) may have mycotoxic or anti-bacterial properties. Therefore, we recommend that you assess the validity of the sterility assay using the bacteriostasis and fungistasis testing, as described in USP <71> Sterility Tests.

Equipment and facility systems in support of manufacture have not been adequately validated

Inadequate system for monitoring environmental conditions of critical process equipment.

Procedural controls in support of manufacture are not followed

Eli Lilly and Company (Dates of FDA Inspection – February 18-March 05, 2021) [64]

Parenteral Manufacturing Operations – Monoclonal Antibodies

Inadequate system for monitoring environmental conditions in aseptic processing areas

Failed to establish and follow appropriate written procedures that are designed to prevent microbiological contamination of drug products purporting to be sterile, and that include validation of all aseptic and sterilization processes

Employees engaged in the manufacture, processing, packing and holding of a drug product lack the training required to perform their assigned functions

Lonza Houston (Dates of FDA Inspection – December 03-10, 2020) [65]

Manufacturer – Viral Vector

Material contamination controls are not adequate

Written procedures are not always followed

Table 5.18 EMA caution about sterility testing challenges for gene therapy-based biopharmaceuticals

2.39. The application of the sterility test to the finished product in accordance with the European Pharmacopoeia (Ph. Eur. 2.6.1) may not always be possible due to the scarcity of materials available, or it may not be possible to wait for the final result of the test before the product is released due to short shelf-life or medical need. In these cases, the strategy regarding sterility assurance has to be adapted. For example, the use of alternative methods for preliminary results, combined with sterility testing of media or intermediate product at subsequent (relevant) timepoints could be considered.
2.40. The use of validated alternative rapid microbiological methods may also be considered. For example, sole reliance on alternative microbiological methods according to Ph. Eur. 2.6.27 may be acceptable when this is justified having regard to the specific characteristics of the product and the related risks, and provided that the suitability of the method for the specific product has been demonstrated.
7.11. The use of antimicrobials may be necessary to reduce bioburden associated with the procurement of living tissues and cells. However, it is stressed that the use of antimicrobials does not replace the requirement for aseptic manufacturing. When antimicrobials are used, they should be removed as soon as possible, unless the presence thereof in the finished product is specifically foreseen in the marketing authorisation/clinical trials authorisation (e.g. antibiotics that are part of the matrix of the finished product). Additionally, it is important to ensure that antibiotics or antimicrobials do not interfere with the sterility testing, and that they are not present in the finished product (unless specifically foreseen in the marketing authorisation/clinical trial authorisation).
7.30. Where the results from the test(s) required to release the starting materials take a long time (e.g. sterility test), it may be permissible to process the starting materials before the results of the test(s) are available. The risk of using a potentially failed material and its potential impact on other batches should be clearly assessed and understood. In such cases, the finished product should only be released if the results of these tests are satisfactory, unless appropriate risk mitigation measures are implemented.
Investigational ATMPs
10.50. First-in-man and exploratory clinical trials: Sterility and microbial assays should be validated.

When it comes to the potential of bacterial/fungal contamination, the message from the regulatory authorities is loud and clear: protect the process to protect the product to protect the patient.

5.5.3 CMC Strategy Tip: Not All Discoveries Have Been Made Yet

One would think that after over three decades of bacteria/fungi risk control operations for biopharmaceutical processes that nothing ‘new under the sun’ would appear. But the necessity of an ongoing risk review is still very important.

Genentech reported discovering a novel bacterial contamination in their 20 L seed train bioreactors for the Chinese Hamster Ovary (CHO) cell culture manufacture of the monoclonal antibody MabThera (rituximab) [66]. The contamination was observed during routine microscopic visual examination of the cell culture, but it was not observed upon Gram staining, and it was not detected in the standard Quality Control 5-day bioburden colony count on agar plate test method. Also, there was no indication from the cell culture manufacturing process controls (e.g., pH, dissolved oxygen, productivity) of anything unusual. Furthermore, this bacterium was able to pass through not only 0.2 micron filters but also 0.1 micron filters. Finally, from bacterial DNA sequencing, the bacterial contamination was identified as *Leptospira licerasiae*.

An exhaustive root cause analysis was performed and Genentech concluded that the most likely potential source of *L. licerasiae* was either raw materials (although no evidence was found; it was very difficult to test conclusively) or environment (this bacterium was found in untreated water source used in site cooling tower) or personnel (no direct correlation with people, but personnel could be carrier from environment). As preventative actions, Genentech implemented non-routine culture testing in specialized medium to enhance detection in the following two samples: (1) Working Cell Bank (WCB) ampoule thaw and (2) preharvest cell culture fluid (the unprocessed bulk). In addition, they optimized and implemented a *Leptospira*-specific PCR assay to enhance detection sensitivity (estimated LOD to be 10^2 organisms/mL vs. visual examination LOD of 10^6 organisms/mL) [67].

This *Leptospira licerasiae* event that occurred was reported by the FDA on their website, and then proceeded to remind the biologic industry why an ongoing risk review is absolutely necessary for adventitious bacterial/fungal contamination [68], see Table 5.19.

5.6 ‘Not Detected’ Is Not Confirmation of Absence

Absolute freedom from adventitious agents is a myth. While ‘not detected’ may be reported in testing, such a result really means ‘not detected at the level of sensitivity and within the inclusion criteria of detecting a specific adventitious agent’. One detects only what one knows to look for. Therefore, in biopharmaceutical manufacturing, the ever-present threat from these adventitious agents exist. However, three basic strategic steps can be taken to continue to minimize their risk to our biopharmaceutical products:

- Remove or replace, where possible, animal-derived and human-derived materials from the biopharmaceutical manufacturing process. While this is important for control of viruses and mycoplasmas, it is especially important for prions, where the only effective option of risk reduction from TSEs is to not have these materials in the manufacturing process.

Table 5.19 FDA background on *Leptospira licerasiae* cell culture contamination

14. Can Leptospira species penetrate sterilizing-grade filters? If so, what should manufacturers keep in mind in their ongoing lifecycle risk management efforts to ensure microbial control?
FDA is aware of a report of *Leptospira licerasiae* contamination in cell cultures (Chen, Bergenvin, et al. 2012). There is no indication that this bacterium ultimately contaminated either the finished drug substance or drug product. This bacterium has been found to pass through 0.1 µm pore size rated sterilizing-grade membrane filters. While this specific species was the identified contaminant in this case, other *Leptospira* species also are capable of passing through 0.1 µm pore size rated filters (see Faine 1982). Compendial microbiological test methods typically used in association with upstream biotechnology and pharmaceutical production are not capable of detecting this type of bacteria. Whether this apparently rare contamination risk may be more widespread is unknown, and we are sharing this information so that manufacturers can consider whether this hazard may be relevant to their operations. *Leptospira* are Gram-negative aerobic spirochetes that are flexible, highly motile, and spiral-shaped with internal flagella. The bacteria measure 1µm in diameter and 10-20 µm in length. While some of the *Leptospira* are harmless fresh-water saprophytes, other species are pathogenic and can cause leptosporosis, a significant disease in humans and animals. Based on current information, *Leptospira* contamination does not appear to occur frequently, and purification steps that follow cell culture in a typical biotechnology operation would be expected to prevent carryover to the finished drug substance. Testing of bulk drug substances produced in the reported cases did not detect the *Leptospira* species, and no evidence of deleterious effects on in-process product were observed in the known case study. As a general principle, manufacturers should use sound risk management and be aware of unusual microbiota reported in the literature that may impact their manufacturing processes (e.g., cell culture biotechnology, conventional sterile drug manufacturing). Manufacturers should assess their operations, be aware of potential risks, and apply appropriate risk management based on an understanding of possible or emerging contamination risks.

- Keep an eye on the spectrum of adventitious agents that can impact your specific biopharmaceutical manufacturing process – both the current known ones and emerging infections. Consider advanced techniques (e.g., massively parallel sequencing (MPS) or next generation sequencing (NGS)) for understanding the extent of adventitious agent contamination that could threaten the biopharmaceutical manufacturing process. Track the literature for reports of new adventitious agents.
- Apply the principle of continuous process improvement from ICH Q10. Overcome the natural reluctance to further improve risk minimization steps that seem to have worked well in the past.

The current risk minimization plans mentioned in this chapter have worked to greatly reduce the safety risk to patients of adventitious agent contamination in biopharmaceuticals. There have been no reported cases to date of patient harm due to an infectious adventitious prion, virus or mycoplasma transmission. Let's keep it that way.

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Chapter 6
Starting Materials for Manufacturing the Biopharmaceutical Drug Substance

Abstract Starting materials (also referred to as source materials) are the starting point for cGMP manufacturing of the biopharmaceutical drug substance. Some of the key attributes desired for any starting material are to be homogeneous, fully characterized, free of adventitious agents, free of undesired impurities, and readily available for manufacturing. Problems with the starting material can create problems all the way through the manufacturing process to the finished biopharmaceutical drug product. The focus of this chapter is on the typical single starting material for the protein-based biopharmaceuticals (i.e., the recombinant cell bank) and on the numerous starting materials for the gene therapy-based biopharmaceuticals (i.e., the cell banks, plasmids, viral vectors, patient cells, etc.). Regulatory authorities understand the critical importance of the starting materials and have high expectations, as well as major requirements, for the biopharmaceutical manufacturer's control over and use of these starting materials, which will be examined in this chapter. Misunderstandings about starting materials will also need to be corrected.

Keywords Cells · Banks · MCB · WCB · Gene-of-interest · Vector · Plasmids · Development · Genetics · Recombinant · Master · Working · Clone · Expression · Constructs

6.1 In the Beginning

There are three stages involved in the manufacture of a biopharmaceutical drug substance: (1) obtaining/manufacturing the starting materials, (2) carrying out the upstream production of the biopharmaceutical, and (3) completing the downstream purification of the biopharmaceutical, see Fig. 6.1. Starting materials (also referred to as source materials) are the starting point for the manufacture of the biopharmaceutical drug substance (also referred to as an active pharmaceutical ingredient, API). The critical importance of this first stage in the manufacture of all biopharmaceutical types (recombinant proteins, monoclonal antibodies, viral vectors,

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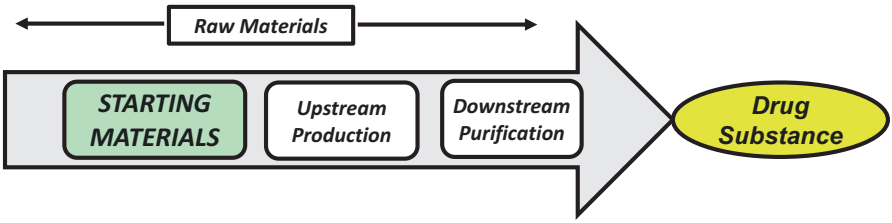

Fig. 6.1 Starting Materials: first stage in the manufacture of the drug substance

genetically modified patient cells, mRNA non-viral vector) will soon become evident in this chapter.

Some of the key attributes desired for any starting material are to be homogeneous, fully characterized, free of adventitious agents, free of undesired impurities, and readily available for manufacturing. Problems with the starting material can create problems all the way through the manufacturing process to the finished biopharmaceutical drug product.

Each manufactured pharmaceutical type requires its own type of starting material:

Chemical Drugs [1]

A starting material should be a substance of defined chemical properties and structure. Non-isolated intermediates are usually not considered appropriate starting materials. A starting material is incorporated as a significant structural fragment into the structure of the drug substance. "Significant structural fragment" in this context is intended to distinguish starting materials from reagents, solvents, or other raw materials.

Recombinant Proteins and Monoclonal Antibodies [2]

Cell banks are the starting point for manufacture of biotechnological drug substances and some biological drug substances. In some regions, these are referred to as source materials; in others, starting materials.

Gene Therapy-Based Biopharmaceuticals [3]

In the case of products consisting of viruses or viral vectors, the starting materials shall be the components from which the viral vector is obtained, i.e., the master virus vector seed or the plasmids used to transfer the packaging cells and the master cell bank of the packaging cell line.

In the case of genetically modified cells, the starting materials shall be the components used to obtain the genetically modified cells, i.e., the starting materials to produce the vector, the vector and the human or animal cells.

Starting materials for both the protein-based biopharmaceuticals (i.e., recombinant cell banks used to manufacture the recombinant proteins and monoclonal antibodies) and the gene therapy-based biopharmaceuticals (i.e., the cell banks, plasmids, viral vectors, patient cells, etc. used to manufacture the viral vectors and the genetically modified patient cells) will be examined in this chapter. Regulatory authorities understand the critical importance of the starting materials and have high expectations, as well as major requirements, for the biopharmaceutical manufacturer's control over and use of these starting materials, which will be examined in this chapter. Misunderstandings about starting materials will also need to be corrected.

6.2 Starting Material for Recombinant Proteins and Monoclonal Antibodies

A cell source that is homogeneous, fully characterized, free of adventitious agents and undesired impurities, and readily available when needed for manufacturing, is necessary for manufacturing recombinant proteins, monoclonal antibodies, and biosimilars. That source of cells is most commonly achieved by means of a 'cell bank' [4]:

> Cell banking assures that an adequate supply of equivalent, well-characterized cells exists for production over the expected lifetime of the product. In addition to providing a constant supply of biological starting material, cell banking provides you with the opportunity to undertake a comprehensive characterization of the cell substrate and to minimize the chance of adventitious agent contamination and/or to maximize the chance of detection of a contaminant.

Because recombinant cells have been banked since the beginning of recombinant protein manufacture in the early-1990's, there is a wealth of knowledge of how to properly prepare, characterize and maintain these banks, which will be examined in this chapter. Other starting materials are occasionally used in the manufacture of protein-based biopharmaceuticals – virus seed banks (used to manufacture recombinant proteins from genetically engineered baculovirus infected insect cells), plant seed banks (used in the manufacture of recombinant proteins from transgenic plants), and animal founders (used in the manufacture of recombinant proteins from transgenic animals). These occasionally manufacturing processes are outside the discussion of this chapter, but many of the principles discussed for starting materials still apply.

6.2.1 Development Genetics – Steps Prior to Cell Banking

Prior to the manufacture of the recombinant cell bank for a protein-based biopharmaceutical, the chosen cell line needs to first undergo genetic construction. Genetic construction involves carrying out gene therapy on a microorganism. A 'human gene' is first linked to a plasmid vector (this combination is referred to as the 'expression construct'), and then the expression construct is transferred into a living host cell to provide the energy to drive the DNA → mRNA → amino acids, linked together in a protein.

The 'human gene' is the piece of DNA that encodes for the sequence of the amino acids of the desired biopharmaceutical recombinant protein or monoclonal antibody. Many manufacturers modify the human gene coding to improve the performance of some specific effect of the eventual produced biopharmaceutical (e.g., elimination of disulfide scrambling by exchanging the nucleic acid codon for cysteine with serine, the insertion of human sequences into genes for humanized monoclonal antibodies, etc.).

The ‘vector’ is a larger DNA molecule (typically a plasmid) into which the gene of interest is inserted to yield the ‘expression construct’ (the gene plus vector). The vector contains promoters to control when and where the host cell transcription will occur, enhancers to increase the likelihood that gene transcription will occur, and other proprietary pieces of DNA that contain the manufacturer’s genetic tricks and trade secrets for the desired function of the gene in a host cell.

The ‘living host cell’ provides the life chemistry to drive the genetic mechanisms. Manufacturers of recombinant proteins and monoclonal antibodies have a wide choice for their selected host cell (e.g., bacteria, yeast, plant, insect, animal, or human host cells). Some of the major drivers that enter into a manufacturer’s choice of host cell line include:

- A cell line that can produce the desired type of biopharmaceutical product (e.g., need for glycosylation or complex tertiary refolding of the expressed protein)
- Cell line expertise in-house (i.e., development genetics experience of existing technical staff in assembling one type of recombinant cell line over another)
- Prior capital investments (e.g., design of an existing installed bioreactor type)
- Corporate image (e.g., cell line patent or proprietary ownership; technology platform communicated when raising venture capital funds)
- Acquisition/purchase of a biopharmaceutical from another company (i.e., staying with the existing cell line and manufacturing process to maintain clinical development momentum)

The first two drivers above are science-based, while the last three drivers are business-based.

The final choice of the host cell line has a major impact not only on the way the biopharmaceutical will have to be manufactured but also on the characteristics of the manufactured biopharmaceutical. The choice of the host cell line impacts the expectations from the cell culture production (e.g., expression levels, post-translational modifications, impurity profiles). The choice of the host cell line determines the amount of required adventitious agent control and safety testing (e.g., insect, animal, human cell lines require extensive virus screening and viral clearance evaluation through purification). Each cell line type has advantages and disadvantages as shown in Table 6.1, for a comparison of bacterial vs mammalian host cell lines. Therefore, the chosen host cell line is an important strategic decision for the biopharmaceutical manufacturer.

Genetic construction involves linking a ‘human gene’ first onto a plasmid vector (this combination is referred to as the ‘expression construct’), and then genetically inserting the expression construct into a host cell. This insertion of the expression construct into a cell can be (1) by transduction with a virus or (2) by transfection with a DNA plasmid or (3) by transformation via electroporation. This process forms a ‘recombinant’ cell. The basic schematic of this genetic construction process is illustrated in Fig. 6.2.

The end result of genetic construction is the recombinant cell; but, not just one recombinant cell, but thousands of recombinant cells. In this mixture of recombinant cells, some cells may have a single copy of the gene integrated into its genome,

Table 6.1 Some advantages/disadvantages between different host cell lines

	Bacterial Cell Line (*E. coli*)	Mammalian Cell Line (CHO)
Advantages	• Fast growing • Low cost (simple media) • Low risk safety concerns from adventitious agents	• Proteins secreted into medium • Proper folding of proteins • Glycosylation similar to humans • Low endotoxin levels
Disadvantages	• Proteins in inclusion bodies • Issues with refolding • No glycosylation • High endotoxin levels	• Slow growing • High cost (complex media) • High risk safety concerns from adventitious agents

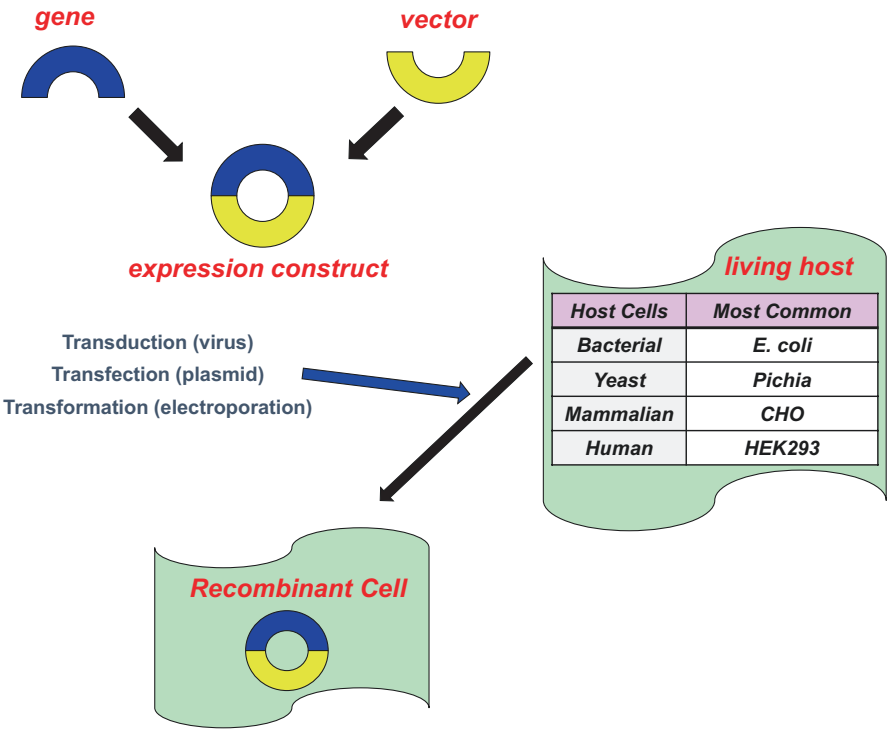

Fig. 6.2 Schematic of the genetic construction process for a recombinant cell

while others may have tens of gene copies, and some may have no copies of the gene. In this mixture of recombinant cells, some cells may have fragments of the gene copy integrated into its genome. From these thousands of recombinant cells, a single recombinant cell, having all of the desired properties, is selected (called the cloning process). The single recombinant cell selected is expanded, so that it can be used to lay down first a Master Cell Bank (MCB), and if needed a Working Cell

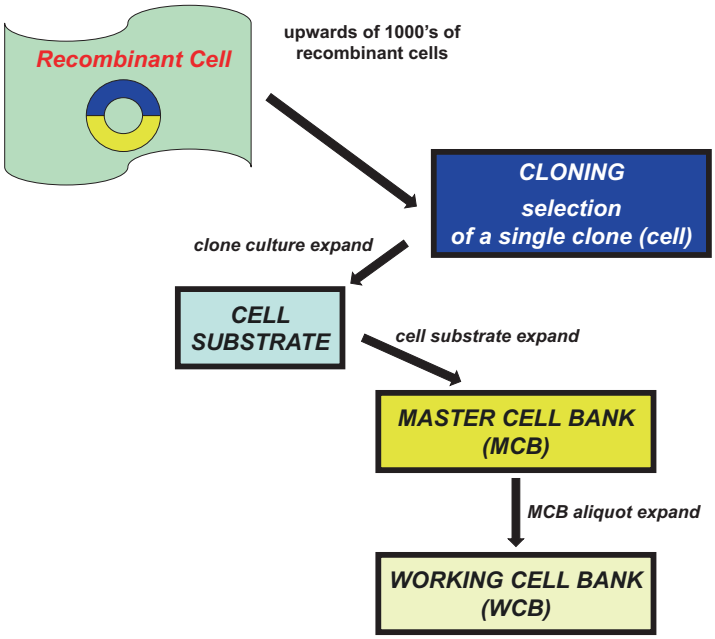

Fig. 6.3 Schematic of the process of selecting a single recombinant cell for the MCB/WCB

Bank (WCB), as shown in the schematic in Fig. 6.3. The activities from the expanding of the cell substrate through to the MCB and WCB laying down, are required to be carried out under current Good Manufacturing Practices (cGMPs).

6.2.2 Importance of Documenting Development Genetics

It is most important that adequate documentation occurs during development genetics – gene DNA origin, plasmid origin, genetic construction, cloning, preparation of cell substrate, etc.. This documentation is important not only from a business perspective (i.e., traceability of what was actually done for patent purposes), but also from a regulatory authority CMC compliance review perspective (i.e., understanding if any patient safety issues might be associated with these early steps). Development genetics is not required to be under the formality of current Good Manufacturing Practices (cGMPs), but principles of GMP (described in Sect. 6.3.2) should be considered, and sound documentation of what is being carried out is a part of that.

The amount of development genetics documentation that needs to be provided to a regulatory authority to initiate human clinical studies (referred to as first-in-human, FIH) is far less than the amount of documentation that needs to be provided to a regulatory authority to obtain market approval. This is a clear illustration of the

risk-based approach, referred to as the minimum CMC regulatory compliance continuum, discussed in Chap. 4. The reason for the request of the different amounts of information on development genetics will become clear in Sect. 6.2.6.

To initiate a human clinical study, the amount of information on development genetics that needs to be provided in the IND or IMPD regulatory submission is a 'brief description':

Recombinant Proteins and Monoclonal Antibodies [5]

A brief description of the source and generation (flow chart of the successive steps) of the cell substrate, analysis of the expression vector used to genetically modify the cells and incorporated in the parental/host cell used to develop the Master Cell Bank (MCB), and the strategy by which the expression of the relevant gene is promoted and controlled in production should be provided, following the principles of ICH Q5D.

Monoclonal Antibodies [6]

The following information should be provided in the IND …:

(a) Source, name, and characterization of the parent cell line, including any immunoglobulin heavy or light chains that it synthesizes and/or secretes, the fusion partner in the case of hybridomas, or the host cell line in the case of transfected cells producing recombinant mAb.
(b) Species, animal strain, characterization, and tissue origin of the immune cell.
(c) Description of immortalization procedures, if any, used in generating the cell line.
(d) Identification and characterization of the immunogen.
(e) Description of the immunization scheme.
(f) Description of the screening procedure used.
(g) Description of the cell cloning procedures.

However, when seeking market approval, regulatory guidances have clearly communicated that 'full and comprehensive information' on development genetics needs to be provided in the BLA or MAA regulatory submission:

ICH Q5B [7]

The manufacturer should describe the origin of the nucleotide sequence coding for the protein. This should include identification and source of the cell from which the nucleotide sequence was originally obtained. Methods used to prepare the DNA coding for the protein should be described.

The steps in the assembly of the expression construct should be described in detail. This description should include the source and function of the component parts of the expression construct, e.g., origins of replication, antibiotic resistance genes, promoters, enhancers, whether or not the protein is being synthesised as a fusion protein. A detailed component map and a complete annotated sequence of the plasmid should be given, indicating those regions that have been sequenced during the construction and those taken from the literature. Other expressed proteins encoded by the plasmid should be indicated. The nucleotide sequence of the coding region of the gene of interest and associated flanking regions that are inserted into the vector, up to and including the junctions of insertion, should be determined by DNA sequencing of the construct. A description of the method of transfer of the expression construct into the host cell should be provided. In addition, methods used to amplify the expression construct and criteria used to select the cell clone for production should be described in detail.

ICH Q5D [8]

The source of cells (laboratory or culture collection) from which the cell substrate was derived should be stated, and relevant references from the scientific literature should be cited. Information obtained directly from the source laboratory is preferred. When this is not available, literature references may be utilised. For human cell lines, it is relevant

to describe the following characteristics of the original donor: Tissue or organ of origin, ethnic and geographical origin, age, sex and general physiological condition. If known, the state of health or medical history of the donor should be reported along with the results of any tests of the donor for pathogenic agents. Specifically, for human diploid fibroblasts, the age of the donor may influence the in vitro lifespan of the cell line and this information should be provided if available. For animal cell lines, relevant descriptions of the source include species, strains, breeding conditions, tissue or organ of origin, geographical origin, age and sex, the results of tests for pathogenic agents, and general physiological condition of the original donor. For microbes, manufacturers should describe the species, strain, and known genotypic and phenotypic characteristics of the organism from which the cell substrate was derived. Manufacturers should also describe the pathogenicity, toxin production, and other biohazard information, if any.

The cultivation history of the cells should be documented. The method originally used for the isolation of the cells should be described as well as the procedures used in the culturing of the cells in vitro and any procedures used to establish cell lines (for example, use of any physical, chemical, or biological procedure, or added nucleotide sequences). A description of any genetic manipulation or selection should be provided. All available information regarding the identification, characteristics, and results of testing of these cells for endogenous and adventitious agents should be provided.

Regarding the generation of cell substrates, applicants should provide a thorough discussion of procedures which would provide exposure to infectious agents. Constituents of the culture medium should be described, in particular, information regarding exposure of the cells to materials of human or animal origin such as serum, enzymes, hydrolysates, or other living cells. The description should include the source, method of preparation and control, test results, and quality assurance. Relevant literature on these points may be referenced when available. This information will allow a detailed analysis of potential entry routes for adventitious agents from these sources, and will be part of the risk-benefit analysis of the product.

During the generation of the cell substrate, one or more specific procedures may be utilised in the ultimate development of the desired characteristics. These may include, for example, cell fusion, transfection, selection, colony isolation, cloning, gene amplification, and adaptation to specific culture conditions or media. Information regarding the methodologies ***utilised*** in developing the cell substrate can help to provide a clear understanding of the history of the cell substrate. Some cell substrates such as human diploid fibroblasts may not need extensive manipulation or cloning prior to cell banking.

FDA [9]

The submission should include a detailed description of the host cell and expression vector system and their preparation as delineated below:

Host Cells – A description of the source, relevant phenotype, and genotype should be provided for the host cell used to construct the biological production system. The results of the characterization of the host cell for phenotypic and genotypic markers, including those that will be monitored for cell stability, purity, and selection should be included.

Gene Construct – A detailed description of the gene which was introduced into the host cells, including both the cell type and origin of the source material, should be provided. A description of the method(s) used to prepare the gene construct and a restriction enzyme digestion map of the construct should be included. The complete nucleotide sequence of the coding region and regulatory elements of the expression construct, with translated amino acid sequence, should be provided, including annotation designating all important sequence features.

Vector – Detailed information regarding the vector and genetic elements should be provided, including a description of the source and function of the component parts of the vector, e.g. origins of replication, antibiotic resistance genes, promoters,

> enhancers. A restriction enzyme digestion map indicating at least those sites used in construction of the vector should be provided. The genetic markers critical for the characterization of the production cells should be indicated.
> *Final Gene Construct* – A detailed description should be provided of the cloning process which resulted in the final recombinant gene construct. The information should include a step-by-step description of the assembly of the gene fragments and vector or other genetic elements to form the final gene construct. A restriction enzyme digestion map indicating at least those sites used in construction of the final product construct should be provided.
> *Cloning and Establishment of the Recombinant Cell Lines* – Depending on the methods to be utilized to transfer a final gene construct or isolated gene fragments into its host, the mechanism of transfer, copy number, and the physical state of the final construct inside the host cell (i.e. integrated or extrachromosomal), should be provided. In addition, the amplification of the gene construct, if applicable, selection of the recombinant cell clone, and establishment of the seed should be completely described.

Why do the regulatory authorities want to review all of this development genetics information in the submission for market approval? The primary reason is that they have the responsibility to adequately assess the patient safety of the prepared cell banks; and the required documentation on development genetics provides them a piece of that assurance [8]:

> It is important to provide supportive documentation which describes the history of the cell substrate that is used in the manufacture of a biotechnological/biological product, as well as any parental cell line from which it was totally or partially derived. Events during the research and development phases of the cell substrate may contribute significantly to assessment of the risks associated with the use of that particular cell substrate for production. The information supplied in this regard is meant to facilitate an overall evaluation which will ensure the quality and safety of the product. Careful records of the manipulation of the cell substrate should be maintained throughout its development. Description of cell history is only one tool of many used for cell substrate characterisation.

From a CMC regulatory compliance strategy perspective, development genetics activities present a threefold challenge: (1) these activities may be carried out by R&D personnel where detailed documentation may not be foremost on their minds, (2) R&D personnel do not operate under GMPs, and (3) these activities are performed long before the biopharmaceutical even enters human clinical trials. Unfortunately, over time, records get lost, and R&D staff come and go in a company, which can lead to a loss of this information. A useful CMC regulatory compliance strategy to overcome this record keeping challenge is to prepare the thorough and comprehensive written description of the development genetics – origin, genetic construction, cloning, and cell substrate preparation – sooner than later, preferrable at the same time that the clinical development submission (IND for FDA and IMPD for Europe). Although the full CMC description is not required until the market application dossier is submitted, I personally recommend to manufacturers that they prepare this CMC information and submit it anyway in their Phase 1 IND/IMPD submission (typically as an appendix – in that way the reviewer doesn't have to look at if they don't have the time). The inclusion of this information accomplishes three purposes:

1. Forces the manufacturer to record what occurred – What better time to secure written R&D documentation of what actually happened than close to the time it occurred. It won't be any easier to compile this documentation in the future; in fact, with people coming and going in companies, some CMC information may actually get lost if not compiled early.
2. Forces a manufacturer to review what occurred – When the report is being written, it also can be risk assessed. This can be an invaluable time to ensure that nothing has been missed, or to identify any concerns with the traceability of the origins of the host, vector, and gene of interest, or to determine if there is an anomaly with any of the test results. Thus, early detection of a CMC problem allows time to address or correct any identified issues. During the preparation of the market application dossier is not the time to find a CMC surprise in the development genetics.
3. Serves as an excellent repository – When the market application dossier is prepared in the future, the CMC content needed will already be present in the IND/IMPD submission file, where a regulatory affairs group can readily locate it.

On the other hand, frequently today, the genetic construction, cloning and manufacturing of the recombinant cell bank is contracted out to contract manufacturing organizations (CMOs) that specialize in these services. A thorough documentation package suitable for regulatory authority submission is expected to be provided as part of the contacted work.

6.2.3 Importance of the Single Clone

Regulatory authorities have clearly stated for decades that a genetically engineered Master Cell Bank (MCB), is to be derived from a single clone:

> ***FDA*** [4]
> MASTER CELL BANK (MCB): A bank of a cell substrate from which all subsequent cell banks used for vaccine production will be derived. The MCB represents a characterized collection of cells derived from a single tissue or cell.
>
> ***EMA*** [5]
> ***The*** MCB and/or WCB if used should be characterised and results of tests performed should be provided. Clonality of the cell banks should be addressed for mammalian cell lines.
>
> ***ICH Q5D*** [8]
> MCB (Master Cell Bank). An aliquot of a single pool of cells which generally has been prepared from the selected cell clone under defined conditions, dispensed into multiple containers and stored under defined conditions. The MCB is used to derive all working cell banks. The testing performed on a new MCB (from a previous initial cell clone, MCB or WCB) should be the same as for the MCB unless justified.

Clonality is not an academic issue but a significant manufacturing process control issue. Individual clones can impact the biopharmaceutical manufacturing process performance and/or the expressed biopharmaceutical product:

FDA [4]

In addition, a cell substrate that has been derived by cell cloning might have different characteristics from the parental cell line. Because it is derived from one or a few cells, it might not have characteristics representative of the original population from which it was cloned. Alternatively, a clone might be selected as the cell substrate because of its particular outlier characteristics, such as rapid propagation in culture or adaptation to particular cell culture conditions that modify its growth properties to enhance vaccine virus replication (e.g., development of suspension cell cultures from adherent cells). It is important that you thoroughly evaluate the characteristics of derivative or engineered cell substrates, as it cannot be assumed that the parental cell characteristics were maintained following the manipulations used to generate the production cell substrate.

USP <1042> [10]

The reason for generating a clonally derived cell line relates to the ability of a controlled process to produce a consistent product with minimal heterogeneity…. In contrast, use of an entirely non-clonal cell population as a starting point may give rise to outgrowth of a subpopulation of cells that generate products with different CQAs. For instance, this could affect glycosylation, which could then impact the mechanism of action if the product is an antibody that functions by antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC). Likewise, a different population with a different integration site might have altered expression levels, growth metrics, and stability, which could have the potential to lead to drug shortages if a cell bank is no longer performing as expected. Such adverse end points could be exacerbated in conditions where cell culture parameters or raw materials have been altered in a way that places selective pressure on the system.

Choosing clones must be done correctly. You get what you select for. "*A clone of Einstein wouldn't be stupid, but he wouldn't necessarily be any genus, either*," a quote ascribed to James D. Watson, co-discoverer of the structure of DNA, illustrates this point. The World Health Organization (WHO) has published a guideline on recommended cloning practices [11]:

The most promising cell/vector combination will then be used to generate a large number of clones (100s–1000s) after transfecting the culture with rDNA. Typically, these clones will be screened These WHO best practices for the clone selection the highest productivity (10–50) will be taken forward for further evaluation. Further testing will then be used to select a small number (1–5) for establishment as small pre-master cell banks, and a final selection will be made, often based on stability characteristics and amenability to scale-up, before finally generating a MCB and WCB.

In the process of cloning a cell culture, single cells should be selected for expansion. The cloning procedure should be carefully documented, including the provenance of the original culture, the cloning protocol, and reagents used. Cloning by one round of limiting dilution will not necessarily guarantee derivation from single cells; additional subcloning steps should be performed. Alternatively or in addition to limiting dilution steps the cloning procedure can include more recent technology such as single cell sorting and arraying, or colony picking from dilute seeds into semisolid media. In any case, the cloning procedure should be fully documented, accompanied by imaging techniques and/or appropriate statistics.

The recommended cloning procedure, which involves a double pass using the limiting dilution approach, is illustrated in Fig. 6.4.

The United States Pharmacopeia (USP) is developing a general informational chapter on Cell Banking, Article <1042>, which covers clonality methods, including using some of the modern imaging and clone isolating approaches [10]:

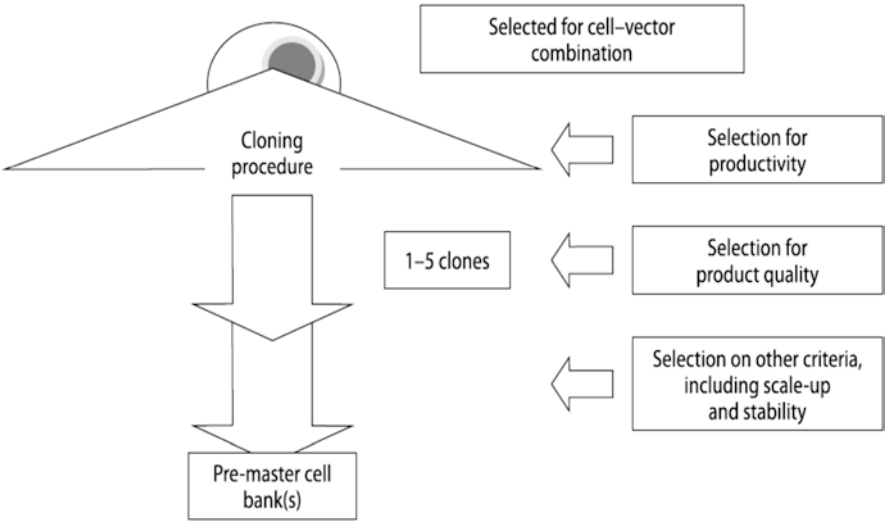

Fig. 6.4 Threefold screening steps to select the desired clone

Limiting Dilution Cloning (LDC). A procedure whereby cells are plated at an appropriately justified low cell density in a 96-well plate. Some wells will be devoid of cells. This is achieved by preparing a set of increasingly greater dilutions of the non-clonal starting population and visually verifying the number of cells initially deposited per well. Clones that appear to be good performers can be expanded through sequentially larger wells until sufficient cells are generated to establish a cell bank. Two rounds of LDC provide an approximately 99% probability that the cell line will be monoclonal.

Single-Round LDC with Imaging. Rather than relying on the visual acuity of the operator, after LDC, the stained cells are deposited into imaging-quality well plates where day 0 images are acquired in both fluorescence and brightfield imaging.

Fluorescence-Activated Cell Sorting.

Automated Clone Picking.

What is the risk if the cloning process was not done or not documented correctly? Manufacturing consistency requires homogenous cells every time a production batch is initiated. If the cells are not clonal, they are not homogenous, and this could lead to overly broad setting of manufacturing process limits. Furthermore, manufacturing process change control is dependent upon homogenous cells. If the cells are not clonal, a process change may cause a different distribution of cells after the change that might lead to different process performance or quality attributes in the produced biopharmaceutical. Since manufacturing scale-up is typically required prior to commercialization, having a change in product quality at this late stage of clinical development could be a disaster. Therefore, it is risky to proceed with non-clonal cells that can result in approval delay or rejection by the regulatory authorities if the non-clonality results in failure to demonstrate product consistency during process validation and/or failure to demonstrate product comparability during scale-up or site transfer. Biopharmaceutical manufacturers have encountered this concern

from regulatory authorities during review of their marketing approval application submissions:

Monoclonal Antibody from CHO (Crysvita, Burosumab) [12, 13]

The establishment of burosumab MCB includes multiple selection procedures for the cells that produce burosumab with adequate growth profiles. However, a formal cloning procedure was conducted only once. Therefore, there is residual uncertainty for the monoclonality of burosumab MCB. The goal of the study is to demonstrate consistent genetic profiles for the subclones of burosumab MCB to ensure the monoclonality of burosumab MCB.

The specifications for burosumab drug substance and drug product are acceptable to ensure adequate quality and safety for the initial marketed product. Assurance of the monoclonality of the burosumab MCB will reduce the risk of the generation of product variants and ensure the consistency of product quality throughout the product life cycle.

Conduct studies to further characterize the burosumab master cell bank (MCB) and to support the monoclonality of the MCB.

Fortunately, lack of proof of clonality does not necessarily preclude market approvability. As shown in the example above, if the regulatory authority is concerned about proof of clonality, they can rely upon 'augmentation' of CMC quality control strategies such as those impacting future WCBs (e.g., tighter controls for qualification), future process changes (e.g., moving non-CPP process parameters up to CPPs), and/or product specifications (e.g., adding additional ones or tightening existing ones). This augmentation approach by the regulatory authorities to clonality, rather than a strict accept/reject of a recombinant cell bank, is more in line with the biopharmaceutical industry viewpoint. A 'white paper', co-authored by scientists of leading US-based biopharmaceutical companies (with help from European-based contributors), has been published to express the value of the multi-control approach versus a strong emphasis on proof of clonality [14].

6.2.4 CGMPs for Manufacture and Maintenance of Cell Banks

Once the developmental genetics are complete and the cell substrate from the chosen clone prepared, the remaining process step is to manufacture of the Master Cell Bank (MCB). The cell substrate is expanded until a desired volume and a desired viable cell density are reached, followed by concentration and formulation in cryogenic medium to a final fixed viable cell density. The culture is then aliquoted into cryogenic vials and frozen. Typically, 250 vials containing 1 mL aliquots are prepared of the MCB. Assuming 200 MCB vials remain after release and stability testing, and after retains are pulled, that would permit the initiation of 200 cell culture production runs (assuming 1 MCB vial is thawed per run). For some biopharmaceuticals, such as those under orphan drug designation, having only a MCB may be sufficient for the lifetime of the product manufacturing. However, most biopharmaceuticals will eventually require many more production runs, so Working Cell Banks (WCBs) are manufactured. From each MCB aliquot, using essentially the

same culture expansion procedure to obtain the desired final fixed vial cell density as was used from the cell substrate, followed by the same aliquoting procedure into cryogenic vials, 250 frozen aliquots of a WCB can be prepared. Thus, assuming the availability of 200 WCB vials from each MCB aliquot and assuming one WCB vial is thawed per run, a theoretical total of 40,000 production runs could be initiated.

The old phrase 'garbage in, garbage out' aptly applies to recombinant cell banks, which is why adherence to basic current good manufacturing practices (cGMPs) is so crucial during preparation of the MCB and WCB [15]:

> As part of product lifecycle management, establishment of seed lots and cell banks, including master and working generations, should be performed under circumstances which are demonstrably appropriate. This should include an appropriately controlled environment to protect the seed lot and the cell bank and the personnel handling it. During the establishment of the seed lot and cell bank, no other living or infectious material (e.g. virus, cell lines or cell strains) should be handled simultaneously in the same area or by the same persons. For stages prior to the master seed or cell bank generation, where only the principles of GMP may be applied, documentation should be available to support traceability including issues related to components used during development with potential impact on product safety (e.g. reagents of biological origin) from initial sourcing and genetic development if applicable.

A problem caused during the preparation of the MCB or WCB does not go away, but potentially manifests itself throughout the cell culture process impacting the produced biopharmaceutical.

The ongoing maintenance of the MCB/WCB is also of utmost importance:

EU GMP Annex 2 [15]

Storage containers should be sealed, clearly labelled and kept at an appropriate temperature. A stock inventory must be kept. The storage temperature should be recorded continuously and, where used, the liquid nitrogen level monitored. Deviation from set limits and corrective and preventive action taken should be recorded.

It is desirable to split stocks and to store the split stocks at different locations so as to minimize the risks of total loss.

Once containers are removed from the cell bank management system, they should not be returned.

ICH Q7 GMP for APIs [16]

Access to cell banks should be limited to authorized personnel.

Cell banks should be maintained under storage conditions designed to maintain viability and prevent contamination.

Records of the use of the vials from the cell banks and storage conditions should be maintained.

Where appropriate, cell banks should be periodically monitored to determine suitability for use.

No regulatory authority wants a commercial biopharmaceutical shortage due to poor management of the MCB resulting in its total consumption or loss. So, (1) an acceptable level of the MCB inventory needs to be demonstrated, and (2) catastrophic event planning is required [8]:

> Manufacturers should describe their strategy for providing a continued supply of cells from their cell bank(s), including the anticipated utilization rate of the cell bank(s) for production, the expected intervals between generation of new cell bank(s). To ensure continuous, uninterrupted production of pharmaceuticals, manufacturers should carefully consider the

> steps that can be taken to provide for protection from catastrophic events that could render the cell bank unusable. Examples of these events include fires, power outages and human error. Manufacturers should describe their plans for such precautions; for example, these may include redundancy in the storage of bank containers in multiple freezers, use of back-up power, use of automatic liquid nitrogen fill systems for storage units, storage of a portion of the MCB and WCB at remote sites, or regeneration of the MCB.

This is why cGMPs for bank maintenance require controlling access to the banks and keeping records of inventory levels. How much inventory is expected? From personal experience, no questions were received from a regulatory authority reviewer when a 20+ year cell bank inventory could be documented, but a question was received when the inventory level could only be documented for a 5 year inventory. Bottom line, the more inventory, the better. Catastrophic event planning for the cell banks is also very important to the regulatory authorities. Read the newspapers, natural and man-made disasters happen all the time (e.g., hurricanes on the East Coast of the USA, earthquakes on the West Coast of the USA). Any of these natural disasters could affect delivery supply of liquid nitrogen to storage freezers or electricity to backup units. Manufacturing sites can also be hit with fires or explosions which can cut off access to storage areas. It is a wise CMC strategy to store the cell banks in at least two geographically different locations to prevent the total loss of a cell bank.

Keep in mind that this ongoing maintenance of MCBs/WCBs is subject to regulatory authority inspections. A check-off list of questions prepared for inspection of these cell banks [17] is shown in Table 6.2.

6.2.5 Characterization of Recombinant Cell Banks

Following the establishment of the Master and Working Cell Banks, quarantine and release procedures are required to be followed. The characterization of the cell banks for their release to manufacturing use is most important. A recombinant cell bank needs to be tested for the following characteristics:

Identity – (1) correct gene, (2) correct vector, (3) correct host
Purity – (1) absence of other host cells, (2) absence of adventitious agents
Suitability for Use – (1) viability, (2) gene copy number, (3) productivity and fidelity of expressed protein-based biopharmaceutical during manufacturing
Genetic Stability – (1) productivity and fidelity of expressed protein-based biopharmaceutical over many cell culturing passages, (2) retention of gene copy number, (3) changes (if any) in sites of integration, (4) absence of latent virus induction (for animal/human cell lines)
Other Safety Concerns – specific issues associated with the chosen host (e.g., tumorigenicity for a new animal/human cell line)

The specific characterization testing program for these five elements will vary for any given cell bank according to the nature of the chosen cell line (e.g., growth

Table 6.2 PIC/S regulatory authority aid for GMP inspecting cell bank maintenance

Maintenance of Master and Working Cell Banks	
Notes	**Crucial Questions**
Access for authorized personnel	**Procedures, names**
Storage and storage conditions	**Records (limits, correction action procedure) Alarm system (records, 24 hr link) Risk of contamination (control related to the N2 level, validation) Risk of confusion (dedicated tanks, mapping of containers) Identical treatment of all containers during storage (procedure)**
Protection from catastrophic events	**Back up power Automatic liquid Nitrogen fill systems (alarm system, contract with the supplier) Redundancy, remote sites (procedure, description)**
Records of use of vials	**Inventory Once removed no return of the containers**
Periodical monitoring	**Suitability for use**

requirements, susceptibility to virus infection), its history of preparation (including use of any animal- and human-derived materials), and available testing procedures. Tumorigenicity (the capacity of a cell population inoculated into an animal model to produce a tumor by proliferation at the site of inoculation and/or at a distant site by metastasis) is a safety concern for new continuous cell lines (CCLs) that have not previously been reviewed by the regulatory authorities. Testing for tumorigenicity is not required for CCLs known already to be tumorigenic [11]:

> Many CCLs (*e.g.*, BHK-21, CHO, HeLa) are classified as tumourigenic because they possess the capacity to form tumors in immunosuppressed animals such as rodents…. If the CCL has already been demonstrated to be tumourigenic (*e.g.*, BHK-21, CHO, HEK293, Cl27), or if the class of cells to which it belongs is tumourigenic (*e.g.*, hybridomas, SCLs), it may not be necessary to perform additional tumourigenicity tests on cells used for the manufacture of therapeutic products…. A new cell line (DCL, SCL or CCL) should be presumed to be tumorigenic unless data demonstrate that it is not.

For animal and human cell banks, there will be the additional issue of determining the presence of endogenous viruses (i.e., a virus whose genome is already present in an integrated form in the cell line). For example, hamster cell lines (such as CHO and BHK-21) typically express non-infectious, defective endogenous retroviral particles (Type A and Type C), while mouse myeloma and hybridoma cells (such as

NS0 and Sp2/0) can express infectious endogenous retroviral particles. These cell lines have been used frequently for biopharmaceutical manufacturing with no reported safety problems. The total number of retrovirus-like particles present in the harvest can be readily quantitated (TEM or qPCR) and retrovirus clearance determined with significant safety factors [11].

Many biopharmaceutical manufacturers contract out the characterization testing of their MCB/WCB. Most Contract Testing Labs (CTLs) track changes to the regulatory authority's expectations for cell bank characterization across the various types of cell lines (e.g., bacterial, yeast, insect, mammalian) and can recommend to their clients a suggested list of current characterization tests for the MCB and or the WCB. Table 6.3 illustrates some of the suggested characterization testing for a bacterial MCB and its WCB. Table 6.4 illustrates some of the suggested characterization testing for a mammalian MCB and its WCB.

It is also a GMP requirement that cell banks be periodically assessed to determine ongoing stability (especially if the manufacturer is claiming decades of use life for the banks) [8]:

> Evidence for banked cell stability under defined storage conditions will usually be generated during production of clinical trial material from the banked cells. Data from the determination of cell viability when the preserved cells are reconstituted for production of clinical trial supplies will verify that the revived cells have survived the preservation process. Available data should be clearly documented in the application dossiers, plus a proposal for monitoring of banked cell stability should be provided. The proposed monitoring

Table 6.3 Some suggested characterization testing for the *E. coli* MCB and WCB

Characterization Testing	BACTERIAL (*E. coli*)	
	Master Cell Bank (MCB)	Working Cell Bank (WCB)
Culture Identity	Sequencing of 25-28S rRNA	Sequencing of 25-28S rRNA
Gene Identity & Integrity	Sequence Analysis Gene Copy Number	Gene Copy Number
Plasmid Identity & Integrity	Retention of Selectable Markers Restriction Endonuclease Analyses	
Viability	Colony Count: CFU/mL or CFU/vial	Colony Count: CFU/mL or CFU/vial
Culture Purity	Cellular Morphology by Gram stain (No contaminating microbial cells)	Cellular Morphology by Gram stain (No contaminating microbial cells)
Non-Viral Adventitious Agents	Absent of Bacteriophage	Absence of Bacteriophage

Table 6.4 Some suggested characterization testing for the CHO MCB and WCB

Characterization Testing	MAMMALIAN (CHO)	
	Master Cell Bank (MCB)	Working Cell Bank (WCB)
Culture Identity	CO1 Barcoding for CHO	CO1 Barcoding for CHO
Gene Identity & Integrity	Sequence Analysis Gene Copy Number	Gene Copy Number
Viability	Viable Cells/mL or Viable Cells/vial	Viable Cells/mL or Viable Cells/vial
Non-Viral Adventitious Agents	Absence of Mycoplasma Sterility	Absence of Mycoplasma Sterility
Virus Tests	*In Vitro* Adventitious Agents *In Vivo* Inapparent *Virus* *In Vivo* Biosafety (MAP, HAP) Reverse Transcriptase Transmission Electron Microscopy (TEM, viral particle count) Bovine 9CFR Porcine 9CFR Minute Mouse Virus (MMV)	In Vitro Adventitious Agents Minute Mouse Virus (MMV) Transmission Electron Microscopy (TEM, viral particle count)

can be performed at the time that one or more containers of the cryopreserved bank is thawed for production use, when the product or production consistency is monitored in a relevant way, or when one or more containers of the cryopreserved MCB is thawed for preparation of a new WCB (and the new WCB is properly qualified), as appropriate. In the case when production does not take place for a long period of time, viability testing on the cell bank used as a source of the production substrate should be performed at an interval described in the marketing application. If the viability of the cell substrate is not significantly decreased, generally no further testing of the MCB or WCB is considered necessary.

As noted above, the ongoing stability of the WCB can be confirmed every time a WCB aliquot is thawed, since viability is measured upon thaw. Then the WCB is used to initiate manufacturing a drug substance batch of the recombinant protein or monoclonal antibody, which can be compared in release testing to previous drug substance batches. However, the ongoing stability of the MCB needs to be on a formal stability program since it will infrequently be used to manufacture a WCB. There is no regulatory authority guidance on the frequency of stability testing for a MCB, so consultants have typically recommended every 4–5 years. The FDA did indicate their preference for the MCB frequency of stability testing in a communication to Genentech, during the BLA review of the CHO-produced monoclonal antibody, Perjeta (pertuzumab). Genentech proposed conducting stability tests on the MCB every 10 years, while the FDA stated clearly, they wanted stability tests to be conducted every 4 years for at least a 20-year period [18].

6.2.6 *Recombinant Cell Bank Myth Busting*

A myth is "*a traditional or legendary story, with or without a determinable basis of fact that explains some practice*." As with all myths, there is always an element of truth. The following is a myth: "*a Master Cell Bank used to manufacture biopharmaceuticals for clinical trials is perfectly acceptable for use in the manufacture of commercial biopharmaceuticals*."

The Master Cell Bank (MCB) used in the manufacture of recombinant proteins and monoclonal antibodies is established before human clinical trials are initiated. Information on its preparation and characterization of the MCB is summarized in the IND/IMPD submission and reviewed by the regulatory authorities [5]:

> A MCB should be established prior to the initiation of phase I trials. It is acknowledged that a Working Cell Bank (WCB) may not always be established. Information on the generation, qualification and storage of the cell banks is required. The MCB and/or WCB if used should be characterised and results of tests performed should be provided. Clonality of the cell banks should be addressed for mammalian cell lines. The generation and characterisation of the cell banks should be performed in accordance with the principles of ICH Q5D. Cell banks should be characterised for relevant phenotypic and genotypic markers so that the identity, viability, and purity of cells used for the production are ensured. The nucleic acid sequence of the expression cassette including sequence of the coding region should be confirmed prior to the initiation of clinical trials. As for any process change, the introduction of a WCB may potentially impact the quality profile of the active substance and comparability should be considered. The safety assessment for adventitious agents and qualification of the cell banks used for the production of the active substance should be provided in A.2, if appropriate.

Regulatory authority reviewers, like those of us who work in the biopharmaceutical manufacturing industry, are overworked and have resource time limitations to carry out their assigned tasks. They must prioritize the depth of their review of submissions and apply a minimum CMC regulatory compliance risk-based approach. Hence, their primary review focus of the MCB/WCB section in the IND/IMPD is patient safety [19]:

> Although CDER acknowledges its review responsibilities, it does not have unlimited resources to review all submissions with the highest level of scrutiny in short time frames. CDER review staff must prioritize their workload and evaluate individual submissions in the context of their place in drug development.
>
> When CDER review staff and their team leaders receive submissions for their assigned IND applications, they are responsible for screening the submissions to determine the extent of review needed, the need for consultation, and the prioritization for content review. For submissions without required responses, CDER review staff will prioritize submission review based on: (1) relative importance to subject safety followed by (2) the importance of the submission to the sponsor's development program.
>
> Because review of a new IND focuses primarily on safety, CDER review divisions should hold an internal IND safety meeting, timed so that the division may provide requests to and receive responses from the sponsor for relatively easy fixes (e.g., minor protocol changes for added safety monitoring) that if not discussed might otherwise lead to a clinical hold.

But, when it is time for their review of the MCB/WCB section in the BLA/MAA, not only is patient safety re-evaluated, but now also the review includes an evaluation of the ability of the MCB/WCB to yield a stable, continuous, homogeneous source for ongoing biopharmaceutical commercial manufacturing. The detailed information in the filed market application dossier on the development genetics, the MCB characterization and its long-term stability are now thoroughly reviewed. Surprises appear during this extra level of review of the cell banks – lack of clonality, cell bank instability, inventory depletion, etc. – and manufacturers are expected to appropriately respond to them, including taking the time to prepare a new MCB, if necessary.

What might be acceptable or not identified as a major concern during the initial clinical development stages, might be deemed unacceptable to a regulatory authority reviewer at the market approval stage. The following are some examples of MCB/WCB concerns raised not during the regulatory authority review at the initial clinical development stage, but only after the pivotal clinical development studies were completed and the MCB/WCB data submitted in the market application dossier:

Recombinant Protein, Elelyso (Taliglucerase Alfa) [20]

We are reviewing the chemistry, manufacturing, and controls section of your submission and we have the following comments and information requests. In order to continue our evaluation of your NDA, we request a prompt written response to the following: According to … ICH Q5B, the purpose of analyzing the expression construct is to establish that the correct coding sequence of the product has been incorporated into the host cell and is maintained during culture to the end of production. You have provided nucleic acid sequencing data. indicating that only … of the sequenced clones had the expected deoxyribonucleic acid (DNA) sequence, with some of the changes in DNA sequence altering the protein sequence. You attributed this result to matrix effects and polymerase chain reaction (PCR) artifacts but provided no data to support this conclusion. Additionally, no information was provided demonstrating that the protein coding sequence is maintained during culture to the end of production. Please submit the following information to the NDA: Genome sequencing results for the master cell bank (MCB) and for end-of-production cells…. All unexpected sequences should be confirmed by a suitable method.

Recombinant Protein, Vimizim (Elosulfase Alfa) [21]

In Section 3.2.A.2.1 of your BLA, you specify that …. The master file you reference … does not provide sufficient information to assess the adequacy of virus testing of this human sourced component and your master cell bank has not been tested for the presence of any human viruses. This raises a concern that human virus may be present in your cell bank and this could impact the safety of your final drug product. Therefore, provide a risk assessment and relevant data (literature reference, etc.) on human virus infection and propagation in your CHO-K-1 cell line. Specific human viruses that you should consider in your evaluation include hepatitis A, B, C viruses, enteroviruses, human HIV-1, HIV-2, HTLV-1, HTLV-3, circoviruses, parvovirus B19, papillomaviruses, human polyomaviruses, human adenoviruses, Epstein-Barr virus, human cytomegalovirus, human herpes viruses 6, 7, 8, and simian viruses that could potentially infect humans (SV40, SFV, SIV, SRV, STLV). Based on this information, you should provide a risk assessment and propose and justify a strategy to test your master cell bank for the most relevant human viruses, or justify why testing for the presence of human viruses is not necessary.

See also the issue of lack of clonality in the MCB of a monoclonal antibody that was a concern to the regulatory reviewer at the BLA stage, but not the IND stage, mentioned in Sect. 6.2.3.

Therefore, this MCB myth is wrong. It is one of the reasons that a CMC regulatory compliance gap analysis should be done on the MCB during clinical development to ensure that there will be no surprises or delays in obtaining commercial market approval by not meeting the regulatory authority's expectation for a commercial MCB.

6.2.7 *Meeting CMC Regulatory Compliance for Cell Bank Starting Materials*

The following two case examples of a market-approved monoclonal antibody and a Fc-fusion protein, indicate the complete CMC documentation for the cell banks in the market application dossier that meet regulatory authority compliance expectations:

Monoclonal Antibody, Beyfortus (Nirsevimab) [22]

Nirsevimab is produced in Chinese hamster ovary (CHO) cells by recombinant DNA technology. The generation of the nirsevimab cell substrate and master cell bank (MCB) and WCB establishment has been adequately described. The chosen cell line was expanded and stored as the research cell bank (RCB). Vials from the RCB were tested for the presence of bacteria, fungi, mycoplasma, and virus contamination, and no contaminating microorganisms or viruses were detected. MCB, WCB and end-of-production cell bank (EOPCB) and limit-of-in-vitro-cell-age (LIVCA) cell bank have been prepared and tested for identity, purity and safety in accordance with ICH Q5A, Q5B and Q5D and the CHMP guidance on *Virus Safety Evaluation of Biotechnological Investigational Medicinal Products*. No bacterial, microplasma, fungi or viral contamination has been detected in the cell banks. All test methods for cell bank identity, purity and safety testing have been briefly described and results have been provided. Tests for bacteriostasis and fungistasis, mycoplasma and sterility are conducted according to current compendial methods. Non-compendial analytical methods were evaluated to ensure that appropriate controls are incorporated into the assay. The methods were confirmed to be suitable for the characterisation of the cell banks. Genetic and phenotypic stability of the cell banks have been analysed, confirming that the cells can stably express nirsevimab during culture after MCB thaw. All raw materials used in the active substance manufacturing process, including cell banking and cell culturing are purchased from Quality-approved suppliers. Upon receipt supplier certificates of analysis are reviewed and materials are inspected, tested and released according to specifications. No material of human origin was used in host cell culture, cell line development, banking of the MCB and WCB, or in active substance manufacturing. No material of animal origin was used in cell line development or cell banking after this. Certificate of analysis, certificate of origin and TSE certificate equivalent information has been submitted and is found acceptable.

Fc-Fusion Protein, Nepexto (Etanercept) [23]

The target fusion protein TNFR:Fc is expressed in a CHO-dhfr cell line. Sufficient information on the host cell line in terms of origin, culture and storage conditions has been provided. The generation of the expression plasmid has been described in sufficient

detail and information on the generation of the parental cell line has also been provided. Additional experiments have been carried out to demonstrate the clonality and genetic stability of the cell line. The cloning strategy has been sufficiently described. A two-tiered cell bank system, comprising master cell bank (MCB) and WCB was established. Release specifications and characterisation data of MCB and WCB were provided. Adequate tests to confirm the genetic stability of the MCB and end of production cell bank (EPCB) have been performed. A protocol for future qualification of new WCBs has been provided. A scale down production process was used to analyse end of production cells. The limit of *in vitro* cell age (LIVCA) for production is defined. Representativeness of scale down study for the production process is considered demonstrated and stability of EPCB is considered confirmed.

Sufficient information on raw materials used in the active substance manufacturing process has been submitted. Compendial raw materials are tested in accordance with the corresponding monograph, while specifications (including test methods) for non-compendial raw materials are presented.

6.3 Starting Materials for Gene Therapy-Based Biopharmaceuticals

Starting materials, whether for the recombinant proteins and monoclonal antibodies or the gene therapy-based biopharmaceuticals (viral vectors, genetically modified patient cells) are expected to be homogeneous, fully characterized, free of adventitious agents and undesired impurities, and readily available when needed for manufacturing. However, starting materials for gene therapy-base biopharmaceuticals have many differences from the protein-based biopharmaceutical starting materials:

- Rather than a typical recombinant master cell bank (MCB) starting material for a recombinant protein, there are numerous possible starting materials for gene therapies – cell banks, DNA plasmids, viral and non-viral vectors, patient cells, etc.
- Rather than decades of regulatory authority experience with recombinant cell bank starting materials, there is limited regulatory authority experience with the newer gene therapy starting materials
- A starting material for one gene therapy manufacturing process, can be a drug substance for another gene therapy manufacturing process (e.g., a viral vector is a starting material for an *ex vivo* gene therapy, but a drug substance for an *in vivo* gene therapy)

6.3.1 Development Genetics

Similar to the protein-based biopharmaceuticals described in Sect. 6.2.1, development genetics for gene therapy biopharmaceuticals are required to obtain the desired gene of interest genetic code that will be transferred into humans. The following

general regulatory guidance is provided on the development genetics information expected to be included in clinical development submissions (IND/IMPD) [24]:

> Development Genetics
> For all vectors, full documentation of the origin where applicable, history and biological characteristics of the parental virus or bacterium should be provided.
> All the genetic elements of the GTIMP should be described including those aimed at therapy, delivery, control and production and the rationale for their inclusion should be given. For helper virus, the same level of detail should be provided.
> For plasmid DNA, full sequence should be provided.
> DNA elements used for selection should be justified. The presence of antibiotic resistance genes in a GTIMP finished product should be avoided given the burden of bacterial multi-resistance to antibiotics and the existence of alternatives methods for selection. If unavoidable a risk analysis should be made.
> Data on the control and stability of the vector and the therapeutic sequence(s) during development should be provided. The degree of fidelity of the replication systems should be ensured as far as possible and described. Evidence should be obtained to demonstrate that the therapeutic sequence remains unmodified and is stably maintained during any amplification.
> Cells used for the amplification of the genetic material should be characterised.
> Details of the construction of any packaging/producer cell line or helper virus should be provided.
> When GTIMP consists of genetically modified cells, both the required information on the viral vector plus information on the modified cellular component should be provided following the recommendations above.

As will be discussed in the next section, activities related to development genetics are to be carried out under the 'principles of GMP' rather than cGMPs.

6.3.2 CGMPs Versus 'Principles of GMP'

Gene therapy-based biopharmaceuticals have a diverse set of starting materials for their manufacture. For each starting material used, the risks presented to the quality, safety and function from its source through its incorporation into the final drug product must be evaluated. Based on that risk assessment, either current good manufacturing practices (cGMPs) or the 'principles of GMP' are to be applied. How does the manufacturer determine which level of GMP is to be applied to the manufacture of their starting materials? A risk-based assessment of the importance of the manufacturing process step and its location in the overall manufacturing process for the starting material is to be applied [25]:

> How does the ATMPs manufacturer ascertain which sections of Part IV of the GMP Guide are relevant for the manufacturing of starting material? Application of a RBA to starting material manufacturing is a critical part of the process to understand the risks to material quality…. In laying down the principles of GMP applicable to starting materials, it is necessary to recognise a certain level of flexibility for investigational ATMPs based on a risk based approach (RBA), especially in early phases of clinical trials (phase I and phase I/II), due to the often incomplete knowledge about the product as well as the evolving nature of the routines.

> For each material used, the risks presented to the quality, safety and function from its source through its incorporation in the finished pharmaceutical dose form must be identified. Risk factors for consideration should include, but are not limited to:
>
> (i) transmissible spongiform encephalopathy;
> (ii) potential for viral contamination and cross contamination with other vectors or other genetical material;
> (iii) replication competent virus (in case of replication-deficient viral vector). It should be demonstrated the absence of formation of replication competent virus at the level of the viral production system used;
> (iv) potential for microbiological (e.g. Mycoplasma) or endotoxin/pyrogen contamination;
> (v) potential, in general, for any impurity originating from the raw materials, or generated as part of the process and carried over;
> (vi) sterility assurance for materials claimed to be sterile;
> (vii) potential for any impurities carried over from other processes, in absence of dedicated equipment and/or facilities (for instance residual DNA (antibiotic resistance gene, residual DNA from potentially tumorigenic cell lines etc.), substance of animal origin, antibiotic etc.);
> (viii) environmental control and storage/transportation conditions including cold chain management if appropriate;
> (ix) stability;
> (x) supply chain complexity and integrity of packages.

In case that significant risks to the product are identified, measures for risk control and mitigation should be defined and implemented.

'Principles of GMP' are applied for activities related to development genetics leading to the manufacture of the recombinant cell banks for all biopharmaceuticals [15]:

> For stages prior to the master seed or cell bank generation, where only the principles of GMP may be applied, documentation should be available to support traceability including issues related to components used during development with potential impact on product safety (e.g. reagents of biological origin) from initial sourcing and genetic development if applicable.

Master/working cell banks (MCB/WCB) to prepare starting materials are required to be manufactured and maintained cGMPs, whether they are to be used for manufacturing recombinant proteins or monoclonal antibodies or the gene therapy-based biopharmaceuticals. As previously mentioned in Sect. 6.2.4, 'garbage in, garbage out', applies to all of these cell banks.

'Principles of GMP' are to be applied to certain components used in the manufacture of gene therapy starting materials (e.g., DNA plasmids containing gene of interest sequences, linearized DNA plasmid containing an mRNA sequence, etc.) [25]:

> For certain starting materials of biological origin, (such as e.g. linear DNA used as template for *ex vivo* transcription into mRNA, plasmids to generate viral vectors and/or mRNA, and vectors) used to transfer genetic material for the manufacturing of ATMPs it is, however, mandatory that the principles of GMP are complied with.

Because starting materials for the gene therapy biopharmaceuticals are so diverse, the next sections will discuss in greater detail where and when the principles of GMP can be applied versus adherence to cGMPs required.

6.3.3 CMC Information on Starting Materials in Regulatory Submissions

Regulatory authorities, over the past several years, are becoming clearer in their guidances on both the type and the amount of CMC information for gene therapy starting materials that they expect to receive from manufacturers in regulatory submissions in order for them to adequately complete their patient safety review. Some general regulatory guidance is provided on the starting material information expected to be included in clinical development submissions (IND/IMPD) of gene therapy manufacturing processes:

EMA ATMPs [26]

Control of Materials (S.2.3)

Raw and Starting Materials

Materials used in the manufacture of the active substance (starting materials and raw materials) should be listed and their acceptance criteria for production should be provided, identifying where each material is used in the process. The manufacturing materials and reagents need to be qualified from the perspective of safety prior to human clinical trials…. The quality of starting and raw materials is a key factor in the production of ATMPs. Therefore avoiding contamination, minimising variability of starting and raw materials is vital for the manufacturing process. Where transport conditions impact their quality, the specific conditions of transport should be described and their suitability verified. Adequate precautions need to be set to ensure proper handling. For viral safety aspects the principles laid down in the general text of the Ph. Eur. 5.1.7. on viral safety should be followed for every substance of animal and human origin that is used during the production. Measures should be taken to reduce the risk of transmissible spongiform encephalopathy according to the relevant European legislation and guidelines.

Starting Materials for GTIMP

Viral vectors are starting materials, also when used to transduce cells and not remaining in the active substance. Information on the vector should be provided in the starting material section. The same level of information that is needed for the vector as active substance should be provided in this situation.

Genome editing tools used ex-vivo to generate genetically modified cells are by analogy also considered as starting materials.

For in vitro-transcribed (m)RNAs used as active substances, the linearized template plasmid DNA should be considered as a starting material.

Complexing materials (a substance used to form a complex with DNA which facilitates transfer of that DNA into a cell, for example, calcium phosphate, lipids or proteins) for formulating the drug substance are considered as starting materials and have to be qualified for their intended purpose. The level of information to be provided will depend on nature of the complexing material and resulting DS.

In the case of gene therapy *ex vivo* (i.e. genetically modified cells), the active substance is composed of the modified cells. The unmodified cells, the viral or non-viral vectors and any other nucleic acid and/or protein used in the genetic modification of the cells are considered starting material. The requirements for the gene/vector component should additionally be taken into consideration. In this case of *ex vivo* use, viral vectors, plasmids, recombinant proteins and recombinant mRNA, the components to produce them (e.g. plasmids, cells) are also considered starting materials. In this case, the principles of GMP, as provided in the General Principles in the Guidelines for GMP for ATMP, should be applied from the cells bank systems used to produce the starting materials, when applicable.

EMA Gene Therapy [27]

By far the most common vector systems used for gene therapy to date have been viral vectors and plasmid DNA vectors. Viral vectors may be replication defective, replication competent or replication-conditional, each type requiring specific consideration with regard to design and safety. Plasmid DNA vectors may be administered either in a simple salt solution (referred to as "naked" DNA) or may be complexed with a carrier or in a delivery formulation. The same vectors can be used as starting materials for the manufacture of genetically modified cells. In that situation, full information on the vector should be provided and the information should be presented accordingly in the Module 3.

Starting materials

All starting materials used for manufacture of the active substance should be listed and information on the source, quality and control of these materials must be provided. The establishment of bacterial/cell/virus seed or bank(s) is expected for starting materials which are bankable. The source and history of the cells or bacterial or virus seeds used for generation of the respective banks should be described and genetic stability of the parent material demonstrated. All starting materials, including master and working cell banks and viral seeds should be appropriately characterised and monitored (e.g. according to the concepts outlined in ICH guideline Q5D). Evidence of freedom from contamination with adventitious agents is essential. For all starting materials, the absence of microbial/viral and fungal contaminants should be ensured through testing after expansion to the limit of in vitro cultivation used for production (see ICH guidelines Q5A, Ph.Eur. 5.14 and cross-reference to Ph.Eur. 5.2.3 and Ph.Eur. 2.6.16). Where materials of ruminant origin are used in preparation of the master and working seeds or cell banks, compliance with relevant Transmissible Spongiform Encephalopathy (TSE) note for guidance is required. The guideline on the use of bovine serum should also be consulted, where appropriate. Applicants should also have regard to the guideline on the use of porcine trypsin used in the manufacture of human biological medicinal products where applicable. Where applicable, genetic stability of the starting materials should be demonstrated at the beginning and the end of the culturing process.

Further CMC regulatory compliance recommendations on the starting materials for the current two major types of gene therapy-based biopharmaceuticals are discussed in the following sections:

Section 6.3.3.1 Starting materials: rAAV viral vector for *in vivo* gene therapy
Section 6.3.3.2 Starting materials: *ex vivo* genetically modified patient cells using rLV

Regulatory authority guidance is beginning to be available for the following two types of gene therapy-based biopharmaceutical starting materials and are discussed in the following sections:

Section 6.3.3.3 Starting materials: mRNA non-viral vector for gene therapy
Section 6.3.3.4 Starting materials: genome editing

6.3.3.1 Starting Materials: rAAV Viral Vector for *In Vivo* Gene Therapy

Recombinant adeno-associated viruses (rAAVs) are non-enveloped DNA viruses that package single-stranded DNA (i.e., gene of interest, GOI) to be used for transferring genes directly (*in vivo* gene therapy) into humans. Three manufacturing

processes can be used for producing a rAAV vector: (1) transient recombinant DNA plasmid transfection of a human cell line, (2) transient recombinant baculovirus transduction of an insect cell line, and (3) production using a virus-infected producer/packaging recombinant cell line. The manufacturing process incorporating transient recombinant DNA plasmids has led to several market-approved biopharmaceuticals, and will be examined in this section.

For the transient plasmid transfection manufacturing process of the rAAV viral vector, four starting materials need to be manufactured: (1) three separate recombinant DNA plasmids (over which the various genes for AAV assembly and the GOI are distributed), and (2) a Master/Working human cell bank to assemble and propagate the virus, as shown in Fig. 6.5.

As shown in Fig. 6.5, one of the starting materials is the human cell bank (typically, human embryonic kidney cells, HEK293 or the daughter cell line HEK293T). This human cell line is used to assemble and propagate rAAV. For this cell bank, the same CMC regulatory compliance manufacturing concerns discussed in Sect. 6.2 (i.e., documentation of development genetics, cloning, cGMP maintenance for cell bank) apply here [28]:

> Control of Materials (3.2.S.2.3)
> *Banking Systems (Starting Materials)*
> A banking system improves control and consistency in the manufacturing of many biologics. Banking assures an adequate supply of equivalent, well-characterized material for production over the expected lifetime of production. For these reasons, banked materials are a common starting point for many routine production applications. We outline our current thinking for the qualification of different banking systems below, including

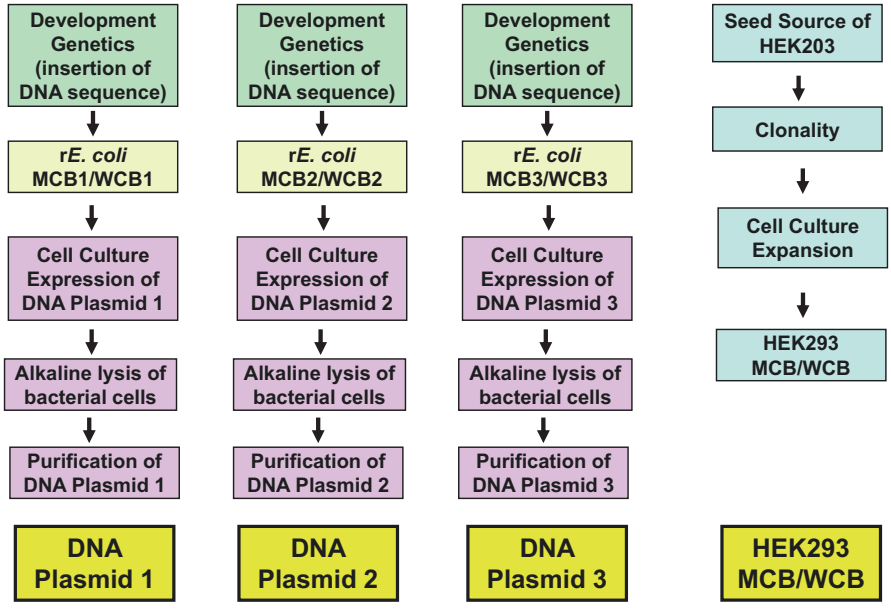

Fig. 6.5 Manufacture of the four starting materials for rAAV viral vector

banks of cell substrates for production of viral vectors, banks of bacterial/microbial cells, allogeneic donor cell banks, and banks of viral vectors. We recommend that you provide a summary of the testing in this section, and COAs in section 3.2.A.2 of the CTD. Information on bank qualification and adventitious agent testing should also be included in your comprehensive "Adventitious Agents Safety Evaluation (3.2.A.2)" section of the CTD.

In your IND, you should provide a description of the history and detailed derivation of the source

material for the cell bank. Your description should include information on cell source (including species of origin); how the bank was generated (e.g., from a single colony isolate or through limiting dilution); testing performed to characterize the bank; and if applicable, materials used to genetically modify the source material (e.g., packaging cell line).

When a cell substrate has been genetically modified (e.g., to provide viral proteins to allow virus replication or packaging), you should provide a description of the materials used for the genetic modification, including information on the quality of the materials (e.g., plasmids, viruses, gene editing components) used to introduce the genetic changes. Materials used to generate cell substrates for production of viral vectors should be sufficiently characterized to ensure safety and purity of the final gene therapy product.

In addition, we recommend that you provide information on how the cell banks are stored and maintained as well as detailed information on qualification to adequately establish the safety, identity, purity, and stability of the cells used in your manufacturing process.

Additional regulatory guidance is provided on the qualification of this human MCB/WCB [28]:

Cell bank qualification should include tests to:

- Ensure absence of microbial contamination, including sterility, mycoplasma (and spiroplasma for insect cells), and adventitious viral agents. For cell lines used for production of viral vectors, we recommend that you test for retroviral contamination, using reverse transcriptase assays and transmission electron microscopic analysis.
- The presence of an adventitious viral agent in your bank should be vigorously investigated, and re-derivation of the bank should be considered. In some instances, robust viral clearance studies may be necessary to remove and inactivate adventitious agents.
- Ensure absence of species-specific pathogens.
  - For human cells, this may include testing for cytomegalovirus (CMV), HIV-1 & 2, HTLV-1 &-2, human herpesvirus-6, -7 and -8 (HHV-6, -7 & -8), JC virus, BK virus, Epstein-Barr virus (EBV), human parvovirus B19, HBV, human papillomavirus (HPV), and HCV, as appropriate.
  - For other animal or insect cells, we recommend tests for species-specific viruses, as appropriate. For instance, for Vero cells, we recommend testing for simian polyomavirus SV40 and simian retrovirus.
  - For insect cells, you should evaluate the presence of arboviruses in a susceptible cell line, such as baby hamster kidney (BHK21) cells. Insect cell lines with known viral contamination should be avoided.
- Identify cells. You should identify your cells through tests that distinguish them from other cell lines used in your facility. For cell lines that you have purchased from a type collection, vendor, or received from another investigator, we recommend master cell bank (MCB) testing to confirm the purity of the cells by genetic analysis (i.e., short tandem repeat analysis or other profiling analysis).

- Establish stability of the cell bank. Stability can be assessed by measuring viability of cells over time after cryopreservation. We also recommend a one-time test of end of production cells (EOP) or mock production cells of similar passage history, to be tested for their suitability to produce your vector. For stable retroviral vector producer cells, we recommend that you test the genetic stability of the gene insert in the EOP cells.
- Assess the ability of new cell lines to form tumors. We recommend that you perform tumorigenicity tests for cell lines that have not been previously characterized for their potential to form tumors. This test would not be necessary for cells known to form tumors.

Bacterial cell banks are not always required for generation of plasmids, but when a bacterial cell bank is needed, as in the case shown in Fig. 6.5, each of the three recombinant DNA plasmids require a recombinant *E. coli* MCB/WCB for their manufacture. The old phrase 'garbage in, garbage out' aptly applies as well to these recombinant *E. coli* cell banks, which is why adherence to cGMPs is not required, but the best choice, when laying down the cell banks and during their maintenance. From the FDA's perspective, the same amount of information that would be provided to them for a protein-based biopharmaceutical MCB/WCB is expected for these bacterial cell banks for the DNA plasmids [28]:

Bacterial or Microbial Master Cell Banks

Bacterial MCBs are frequently used as the starting material to generate plasmid DNA, which can be used as a gene therapy DS or used as a manufacturing intermediate to generate a DS for other gene therapy products, such as AAV or lentiviral vectors…. We generally recommend the establishment of a bacterial MCB, as it can provide a consistent starting material for the manufacture of plasmids or microbial vectors. However, MCBs may not be necessary for all manufacturing situations if the plasmid intermediate is appropriately qualified (e.g., for early phase studies when the plasmid is used to make a vector for ex vivo modification of cells). We recognize the diversity of uses for bacterial MCBs, and recommend that you appropriately qualify the bank, and submit sufficient detailed information for the qualification of the banked material regardless of use. You should provide a description of the history and derivation of the materials used to generate the cell bank, including information on how plasmid vectors were designed and constructed. For the bank material, itself, you should describe the genotype and source of the microbial cells, provide information on how the material was generated, and how the bank is stored and maintained as well as information on the qualification of the bank (including cell bank COAs) to adequately establish the safety, identity, purity, and stability of the microbial cell preparation used in the manufacturing process.

For bacterial cell banks used to manufacture a DNA plasmid, we recommend the testing include:

- Bacterial host strain identity;
- Plasmid presence, confirmed by bacterial growth on selective medium, restriction digest, or DNA sequencing;
- Bacterial cell count;
- Bacterial host strain purity (no inappropriate organisms, negative for bacteriophage);
- Plasmid identity by RE analysis;
- Full plasmid sequencing. We recommend that you fully sequence plasmids and submit an annotated sequence for the vector, as described in more detail in the section below on viral vector banks; and
- Transgene expression and/or activity, as applicable.

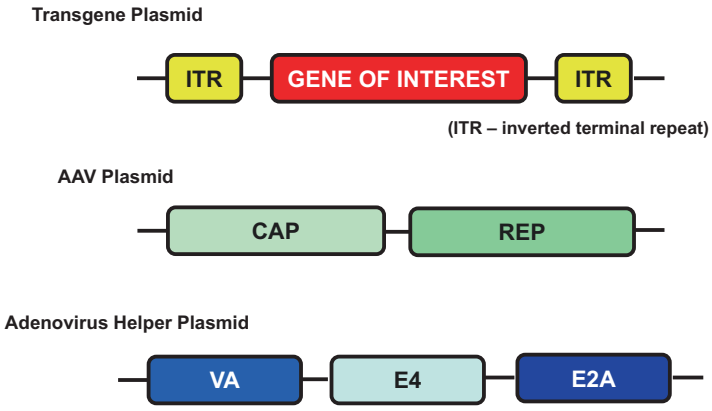

Fig. 6.6 The three DNA plasmid starting materials for transient rAAV vector manufacture

Then, as shown in Fig. 6.5, the remaining three starting materials are the recombinant DNA plasmids. The AAV genome is 4.7 kb long and contains 2 genes, referred to as Rep and Cap, which are required for viral replication and integration. Deleting Rep and Cap, and inserting them on a separate DNA plasmid, serves three purposes: (1) it eliminates the ability of a helper virus to permit AAV-infected cells to produce new AAV virions, (2) it permits insertion of the gene of interest (GOI) up to approximately 4 kb long, and (3) it virtually eliminates the ability of AAV to integrate. A typical gene assembly of the three separate recombinant DNA plasmids is shown in Fig. 6.6.

CMC regulatory guidance for the DNA plasmid starting materials (note, the FDA refers to these recombinant DNA plasmids as 'intermediates') [28] is provided below:

> Control of Critical Steps and Intermediates (3.2.S.2.4)
> Intermediates in gene therapy manufacturing may also include DNA plasmids that are used in the manufacture of other gene therapy products, such as AAV or lentiviral vectors. We recommend that DNA plasmid intermediates be derived from qualified banks, as described in more detail above and in section V.A.2.c., "Control of Materials (3.2.S.2.3)," of this guidance. In addition, we recommend that you provide information on the plasmid manufacturing procedures, reagents, and plasmid specifications for use, regardless of whether they were made by the IND sponsor or a contract manufacturer. In general, we recommend that this testing include assays to ensure the identity, purity, potency, and safety of the final product. For a DNA plasmid, this may include sterility, endotoxin, purity (including percent of supercoiled form and residual cell DNA, RNA, and protein levels), and identity testing (restriction digest and sequencing if sequencing was not performed on the bacterial bank). A COA documenting plasmid quality testing should be included in the IND (section 3.2.A.2 of the CTD).
> *Regional Information (3.2.R)*
> Information that is specific to a regulatory region can be put in the regional section of eCTD. This would include … plasmid sequencing information ….

Additional CMC regulatory guidance for characterization and testing of the DNA plasmids can be found in the FDA regulatory guidance for plasmid DNA used as

Table 6.5 Starting materials – manufacture of rAAV for *in vivo* gene therapy

Product Type	Starting Materials		Active Substance
***In Vivo* Gene Therapy: Viral Vectors**	**Plasmids manufacture**	**Cell Banks establishment of MCB/WCB**	**Viral Vector Manufacture**
Yellow – Principles of GMP		**Green – cGMPs**	

vaccines [29], in the European Pharmacopoeia 5.14 general chapter on *Gene Transfer Medicinal Products for Human Use* [30], which includes plasmid vectors for human use, and BioPhorum's discussion on establishing plasmid specifications using a risk-based approach [31].

The EMA guideline on *Questions and Answers on the Principles of GMP for the Manufacturing of Starting Materials of Biological Origin Used to Transfer Genetic Material for the Manufacturing of ATMPs* brings together all of the starting materials for this rAAV manufacturing process, and states the level of GMP compliance expected [25], see Table 6.5.

FDA CBER concurs with EMA on this delineation of the level of GMP requirements as stated in comments at a public town hall meeting with gene therapy subject matter experts [32]:

> ***Could OTAT comment on the expectation for starting material plasmids used to make factors such as drug product or for genetically modified cells in relation to GMP status at the time of BLA, including whether the level of details to be provided in the BLA is equivalent to what is expected for a drug substance and whether qualification of analytical method used for release of plasmids would be deemed acceptable?***
>
> The U.S. regulations do not require that plasmid starting material be made under strict GMPs, nor that the level of detail for these materials is the same as a drug substance. For instance, you put the plasmids' starting material information into a subsection of Module 3 for the drug substance in the controlled materials. This just kind of gives you a hint about how much detail would be there. You need to keep in mind that the plasmid quality is critical to drug product manufacturing, and the individual manufacturer should set standards as needed for all the materials that come into their facility. This helps them ensure that they can reproducibly make their product…While the plasmid itself does not need to be made in a GMP environment, the vectors do. Therefore, the vector manufacturing site must comply with GMPs, and the starting material coming into the facility to manufacture the drug substance/drug product must be tested to verify identity and conformity with specifications for purity, strength, and quality.

The manufacture of the *in vivo* viral vector (rAAV as drug substance) is discussed in the next chapter.

The following are two case examples of starting materials for market-approved *in vivo* gene therapy biopharmaceuticals, using rAAV biopharmaceuticals manufactured via the transient three plasmid transfection process, that met regulatory authority CMC regulatory compliance requirements:

Luxturna (Voretigene Neparvovec) [33]

The Starting Materials for manufacture of voretigene neparvovec active substance consist of a mammalian cell substrate and three purified recombinant DNA plasmids. AAV2 is produced in cells through transient transfection with three plasmids that contain the genetic information to produce the coded viral vector.

Master cell bank

The development and characterisation of the MCB and the three purified recombinant DNA plasmids has been adequately described. Screening for a range of specific human, bovine and porcine viruses is performed in accordance with ICH Q5D. The product is currently manufactured directly from vials of the MCB, which has been accepted. The applicant plans to implement a Working Cell bank (WCB), which is currently being qualified, by post-approval variation.

A description of the derivation, characterisation and manufacture of these plasmids has been provided. Tests and specifications for the three plasmids consist of manufacturer's specifications for testing of new lots of plasmid and additional controls performed after receipt for confirmation of new lots of plasmid prior to release. All plasmids must pass manufacturer's specifications and internal testing criteria to be released by Quality Assurance for use in manufacture. The proposed tests and specifications for all three plasmids are considered adequate.

[Note, specific details about the Transgene Plasmid were provided in an Advisory Committee meeting with the FDA [34] – see Figure 6.7.]

Zolgensma (Onasemnogene Abeparvovec) [35]

The Starting Materials for onasemnogene abeparvovec consist of a mammalian cell bank and three recombinant plasmids. An overview of raw materials used in the manufacturing of the active substance has been provided including information on their intended use, whether they are compendial or non-compendial, and specifications for non-compendial material. Information on the vendors of critical raw materials is provided. Specifications and representative certificates of analysis are provided.

For the starting materials, information on the source, history, and generation of the plasmids and the cell banks has been provided. The applicant used a vial of HEK 293 cells to

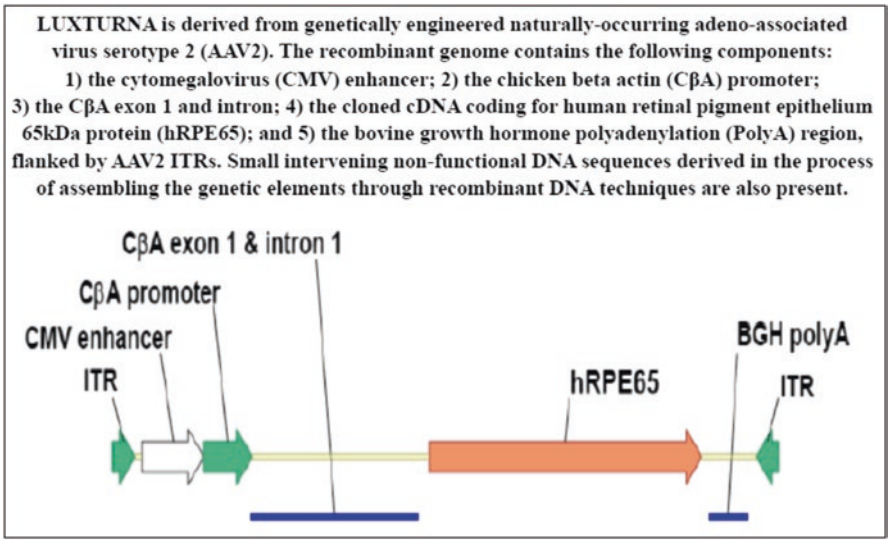

Fig. 6.7 Transgene plasmid for Luxturna rAAV *in vivo* gene therapy

create a pre-MCB (master cell bank). Subsequently a MCB and three WCBs (working cell banks) were manufactured under GMP. All three generated WCBs will be used for manufacture of clinical and commercial material.

Zolgensma AS is produced by co-transfection of HEK293 cells with three plasmids:

- Vector Plasmid (pSMN)
- AAV Plasmid (pAAV2/9) containing the AAV rep2 and cap9 wild-type genes
- Adenovirus Helper Plasmid (pHELP).

Plasmid DNA maps are provided. The plasmid manufacturing and testing sites are provided. The tests and/or acceptance criteria for the plasmid DNA batches manufactured by the two plasmid production sites show some differences, which have been sufficiently justified. An overview was provided of the plasmid DNA batches (manufacturer and batch number) that have been used for the production of the different commercial AS batches listed in the MAA.

6.3.3.2 Starting Materials: Manufacture of Genetically Modified Patient Cells (Using rLV)

The manufacturing process for *ex vivo* gene therapy using a viral vector requires two starting materials: (1) the viral vector and (2) the collected patient cells.

Recombinant retroviruses, such as gammaretrovirus and lentivirus (LV), are enveloped RNA viruses that package two copies of single-stranded RNA to be used for transferring genes into collected patient cells (*ex vivo* gene therapy). Two manufacturing processes can be used for producing the recombinant lentivirus (rLV) starting material: (1) transient recombinant DNA plasmid transfection of a human cell line, and (2) production using a virus-infected producer/packaging recombinant cell line. The manufacturing process incorporating transient recombinant DNA plasmids has led to several market-approved biopharmaceuticals, and will be examined in this section.

The manufacturing process for the rLV viral vector starting material requires the manufacture of (1) four separate recombinant *E. coli* cell banks, (2) four separate DNA plasmids over which the various genes for LV assembly (Gag/Pol packaging plasmid, Rev. regulatory element plasmid, Envelope plasmid, etc.) and the GOI are distributed, and (3) a Master/Working human cell bank, as shown in Fig. 6.8.

The following case example of market-approved Skysona (elivaldogene autotemcel) [36] illustrates the use of not four, but five, separate recombinant DNA plasmids in the transient plasmid manufacture of rLV starting material:

A multi-plasmid system, consisting of a plasmid transfer vector (pLBP100) containing the ABCD1 "therapeutic" transgene, and 4 packaging plasmids containing viral packaging genes, including HIV-1-derived gag/pol, tat, rev, and the vesicular stomatitis virus derived glycoprotein G (VSV-G) envelope, are used to produce Lenti-D LVV. The multi-plasmid system was designed to prevent recombination and emergence of replication competent lentivirus (RCL).

Importantly, the viral packaging genes encoding these viral proteins are only present on the plasmids. No viral packaging genes are included in the Lenti-D LVV particle and thus it is replication incompetent. In HEK293T cells the viral protein components produced from the plasmids lead to LVV particle formation and the packaging of the viral RNA

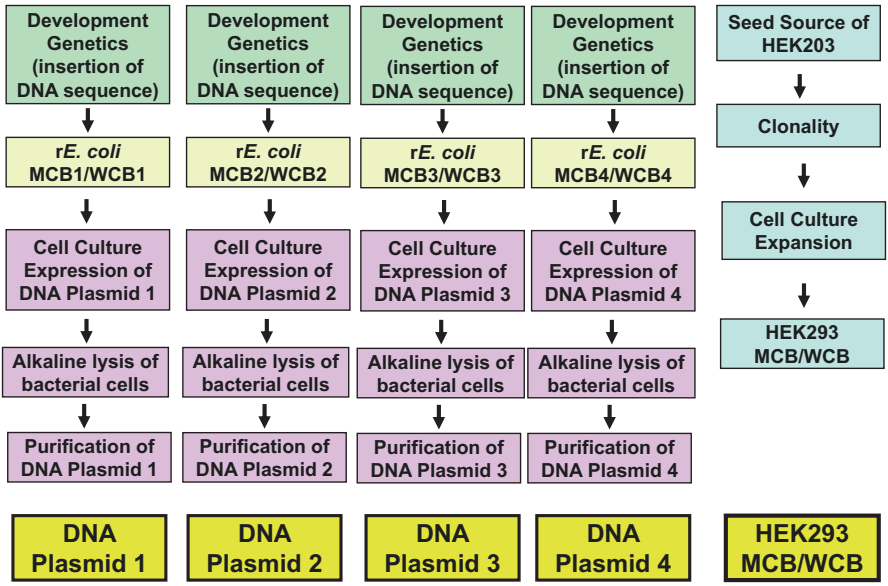

Fig. 6.8 Manufacture of the five starting materials for rLV viral vector

genome, which is encoded by the pLBP100 transfer vector. HIV-1 viral genes that are dispensable were removed from the plasmid system, and include those that encode HIV envelope, vpr, vpu, and nef proteins. Notably, all these deleted genes are required for HIV pathogenesis.

As with the case of the manufacture of the rAAV viral vector and the human cell bank in Sect. 6.3.3.1, the same level of concern applies here for the manufacture of the rLV viral vector and the manufacture of the HEK293 human cell bank. If *E. coli* bacterial cell banks are used for plasmid manufacture (as in Fig. 6.8), then they also should be laid down and maintained under at least the principles of cGMPs, but because of their importance, consideration should be given to doing this under cGMPs. FDA refers to the DNA plasmids used in the manufacture of the rLV vector starting material as 'intermediates' [28] Some biopharmaceutical companies refer to the components used in the manufacture of the rLV vector starting material as 'critical materials', which gives the same emphasis as starting materials.

The manufacture of the rLV vector starting material is from the transient plasmid transfection of the HEK293 cells. The level of GMP control for the rLV vector manufacturing is expressed differently between the EMA ('under principles of GMP') and the FDA ('under cGMP'):

EMA [37]

For *ex vivo* gene transfer, the tools used to genetically modify the cells shall be, as appropriate, the vector (e.g. viral or non-viral vector) and the components to produce them. Principles of good manufacturing practice (GMP) shall apply from the bank system used to produce the vector onwards.

> Starting materials used for the production of genetically modified cells … shall be carefully qualified to assure a consistent manufacturing process. The amount of data to be provided for each starting material is the same as required for, respectively, the drug substance of a cell-based medicinal product and the drug substance of an *in vivo* gene therapy medicinal product.
>
> ***FDA*** [38]
>
> *Vector Manufacturing and Testing.* The vector should be well-characterized prior to initiation of clinical studies. For licensure, the vector must be manufactured according to CGMP standards (21 CFR Parts 210 and 211) and analytical assays must be validated (21 CFR 238 211.165(e)). During CAR T cell Biologics License Application (BLA) review, vector manufacturing facilities are subject to inspection. Vector quality directly contributes to the quality and consistency of the CAR T cells. We recommend that sponsors describe the vector structure, characterization and testing of the Master and Working Cell Banks, characterization of reference materials, vector manufacture and testing, and vector stability. Vector lot release testing should include measures of safety, identity, purity, and potency. A potency assay that assesses the biological activity of the transgene may be developed in coordination with the CAR T cell potency assay. Transgene expression alone as a measure of potency may be sufficient to support early-phase IND studies; however, additional measures of biological potency will likely be requested for clinical study(s) intended to provide primary evidence of effectiveness to support a marketing application. Additionally, we recommend vector lot release testing include assays to determine the vector concentration that can be used to normalize the amount of vector used for transduction during CAR T cell manufacturing. For example, we recommend testing viral vectors for transducing units per milliliter (mL) in a suitable cell line or healthy donor cells. Subsequently, T cell transduction can then be optimized to determine the amount of vector that is added per cell to achieve the target percentage of CAR-positive cells in the CAR T cell DP. Vector safety testing should include microbiological testing such as sterility, mycoplasma, endotoxin, and adventitious agent testing to ensure that the CAR T cell DP is not compromised. Additional testing may be recommended depending on the type of transgene vector being used. For example, there are additional safety concerns related to the use of retroviral-based vectors and additional testing expectations. The recommendations for long term follow-up of patients generally depends on the safety concerns associated with the vector and the propensity for the vector to integrate.

The manufacture of the *ex vivo* viral vector (rLV as starting material) is discussed further in the next chapter.

As shown in Fig. 6.8, the second starting material for *ex vivo* gene therapy using rLV vector is the collected patient cells. The patient cells could be for autologous use (i.e., collected and returned to the same patient) or for allogeneic use (i.e., collected from a donor and administered to multiple patients). The collection of the patient cells is not under a GMP requirement, but that does not mean that control over this cell collection process is not necessary. The following are some regulatory comments specifically about the collection and handling of the patient cells starting material for *ex vivo* gene therapy [28]:

> Control of Materials (3.2.S.2.3)
>
> *Cells – Autologous and Allogeneic Cells or Tissue.* For autologous or allogeneic cells or tissue, you should provide a description of the cell source, the collection procedure, and any related handling, culturing, processing, storage, shipping, and testing that is performed prior to use in manufacture. Your description should include the following information:

- materials used for collection (including devices, reagents, tubing, and containers);
- method of cell collection (i.e., standard blood draw, bone marrow aspiration, or apheresis);
- enrichment steps, cryopreservation, if performed;
- labeling and tracking of collected samples;
- hold times; and
- transportation conditions to the manufacturing facility.

As an example, for cells collected by leukapheresis: you should provide a summary description of the collection device(s); operating parameters; volumes or number of cells to be collected; and how the collected material is labeled, stored, tracked, and transported to the manufacturing facility. Establishing well-designed process controls and standard operating procedures (SOPs) for manipulating and handling starting and in-process materials can help reduce variability in the manufacturing process and ultimately in the DS and DP. This is especially important for multi-center clinical trials, where establishing standardized procedures for cell collection and handling across all collection sites is critical to assuring the quality and safety of the final product as well as ensuring control of the manufacturing process. In your IND, you should include a list of collection sites, their FDA Establishment Identifier, and any accreditations for compliance with established standards (e.g., Foundation for the Accreditation of Cellular Therapy (FACT)), if applicable.

The FDA has provided regulatory comments specific to the *ex vivo* CAR T cell perspective on the collection and handling of patient cells [38]:

Collection, Handling, and Testing of Cellular Starting Material. The nature of the cells used as starting material may be critical for CAR T cell quality and function. Due to patient or donor variability, the cellular starting material can represent a major source of lot-to-lot variability. Here, we describe considerations for cellular starting material, using starting material obtained from leukapheresis (referred to as "leukapheresis starting material") as an example. The recommendations in this section may be applicable to other types of cellular starting material as well.

You should have appropriate procedures in place to ensure adequate control of the leukapheresis starting material during shipping to the manufacturing facility (e.g., temperature control), and information regarding shipping containers and temperature monitoring should be provided. Validation of the shipping process and any hold or cryopreservation steps, including assessment of leukapheresis starting material stability under the intended conditions, should be included for licensure.

The probability of manufacturing success may be increased by establishing acceptance criteria for the leukapheresis starting material used in CAR T cell manufacturing. For example, you may specify a minimum cell number, viability, and percent CD3+ cells. We recommend that you test the leukapheresis starting material for microbial contamination (e.g., sterility or bioburden) prior to initiating CAR T cell manufacturing or that you retain a sample for post hoc testing in the event of a DP sterility test failure. Additional characterization of the leukapheresis starting material (e.g., for percent and absolute number of CD4+ and CD8+ T cells, NK cells, monocytes, B cells) may inform the CAR T cell manufacturing process as these characteristics may influence T cell selection and expansion and final CAR T cell quality.

The EMA guideline on *Questions and Answers on the Principles of GMP for the Manufacturing of Starting Materials of Biological Origin Used to Transfer Genetic Material for the Manufacturing of ATMPs* brings together the two starting materials for the genetic modification of patient cells, and states the level of GMP compliance expected [25], see Table 6.6.

Table 6.6 Starting materials – manufacture of genetically modified cells (using rLV)

Product Type	Starting Materials			Active Substance
***Ex Vivo*: Genetically Modified Cells**	**Tissues/Cells donation, procurement, testing**	**Cell Banks establishment (MCB, WCB) for plasmid and/or vector expansion**	**Plasmid manufacture Vector manufacture**	**Genetically Modified Patient Cells Manufacture**
Blue – not under GMPs		**Yellow – Principles of GMP**		**Green – cGMPs**

FDA disagrees with placing the manufacture of the vector under the principles of GMP in this table, rather than under cGMPs. Their quote in the discussion over Table 6.5, at the public town hall meeting with gene therapy subject matter experts [32] stated: "*While the plasmid itself does not need to be made in a GMP environment, the vectors do. Therefore, the vector manufacturing site must comply with GMPs, and the starting material coming into the facility to manufacture the drug substance/drug product must be tested to verify identity and conformity with specifications for purity, strength, and quality*." Then in reference [38] listed earlier, the FDA guidance document states: '*Vector Manufacturing and Testing. The vector should be well-characterized prior to initiation of clinical studies. For licensure, the vector must be manufactured according to CGMP standards (21 CFR Parts 210 and 211) and analytical assays must be validated (21 CFR 238211.165(e)).*' In practice, the difference might not be that major, but it is an area where clear agreement (if possible) needs to be stated between EMA and FDA.

Skysona (elivaldogene autotemcel) is a case example of a market-approved *ex vivo* genetically modified patient cells that illustrates the CMC regulatory compliance information, provided to EMA, for their two starting materials (collected patient cells and the rLV viral vector) [39]:

Collected Patient Cells Starting Material

Procurement of cell starting material is performed in compliance with European Union (EU) Directive 2004/23/EC and implementing Directives 2006/17/EC, 2006/86/EC (as amended by Directive 2015/565), and 2015/566 (referred to as "Tissue Directives"). The program is additionally designed to comply with EU Directive 2002/98/EC and implementing directives ("Blood Directives"). The collection of autologous cells for commercial manufacture of eli-cel is performed by Qualified Treatment Centres (QTCs), which may include an Apheresis Collection Centre (ACC), a Cell Therapy Lab (CTL) and/or a finished product cryostorage location and infusion centre. QTCs are certified or licensed as either Tissue or Blood Establishments in accordance with national legislation implementing relevant EU Directives. QTCs are additionally qualified by the applicant following a programme sufficiently described in the application and involving apheresis assessment, quality audits, quality agreements, on-site training and a follow-up and maintenance programme. The approach is considered adequate. Information on donor selection is provided. Donor screening and testing of prospective eli-cel patients is performed for the presence of specific infectious diseases prior to the collection of HPC-A. Infectious disease testing is performed by labs that are appropri-

ately qualified using appropriately certified or approved test kits (CE-marked). Since HPC-A is for autologous use, positive test results may not necessarily prevent its use for the manufacture of eli-cel. In certain cases, additional tests for infectious disease markers may be performed to rule in or out persisting infection. The infectious disease testing strategy to determine acceptance of HPC-A is sufficiently described and deemed acceptable. A brief description of the HPC-A collection process is provided. Collection of peripheral blood mononuclear cells (PBMCs) is performed following standard operating procedures. The apheresis collection devices and kits are specified, and CE-declarations of conformity are provided. The HPC-A from a single mobilisation cycle is used to support production of one batch of finished product. If necessary, an additional mobilisation cycle may be subsequently completed following a pre-defined time interval to collect additional cells that will support manufacture of a supplemental finished product batch

to achieve the finished product patient dose. Characterisation data for HPC-A material used in the manufacturing of batches used in the clinical development (studies ALD-102 and ALD-104) are provided. The shipping system for HPC-A has been qualified using a risk-based approach through both Operational Qualification (OQ, performed by both the vendor and by the applicant) and Performance Qualification (PQ). In addition, shipping qualification studies were performed for worst-case simulated conditions, including atmospheric condition, shock (dropping) and vibration test regimes posed upon HPC-A samples.

Recombinant Lentivirus Vector Starting Material

HEK293T Cells

The modified, human, embryonic, kidney cell-line, designated HEK293T, is the mammalian packaging cell line used as the cell substrate in the Lenti-D LVV transfection process. Information on the source, history and generation of the cell line is provided. A master cell bank (MCB) and WCBs of HEK293T cells have been established under GMP and in accordance with Ph.Eur. 5.2.3 and ICH Q5D. Information on the manufacture and qualification of the MCB and WCBs is provided. WCBs were tested in accordance with ICH Guideline Q5D with regards to identity, viability, and absence of viral and non-viral contaminants. Brief but adequate descriptions of the analytical methods applied for qualification of the cell banks are provided. All test results met specification and the WCBs were shown to be free of contamination by bacteria, fungi, mycoplasma, and virus as summarised in the dossier. The results support their use in the manufacturing of the LVV. Assessment of adventitious agents is done through test methods utilising *in vitro* and *in vivo* techniques. Foetal bovine serum is tested by PCR for absence of adventitious viruses in accordance with CFR 9 (Code of Federal Regulations, USA) prior to gamma-irradiation, which is deemed an acceptable approach. No significant trends were identified over the *in vitro* age range with respect to cell viability, total viable cells, population doubling time, or cell morphology. These results demonstrate the stability of the cell bank system with respect to manufacturing consistency across the *in vitro* age range used in manufacturing. Also, the stability and performance of HEK293T WCBs are monitored for each Lenti-D LVV manufacturing run. HEK293T cells were expanded during an engineering run and collected prior to transfection to generate an end of production cell bank (EOPCB). Testing in accordance with ICH Q5D demonstrated that the HEK293T EOPCB was free of detectable bacteria, fungi and adventitious viruses and support the use of the WCB.

Plasmids (and E. coli Cell Banks)

A multi-plasmid system, consisting of a transfer plasmid (pLBP100) that carries the *ABCD1* (ATP-binding cassette, sub-family D, member 1) gene, which encodes the human adrenoleukodystrophy protein (ALDP), and packaging plasmids is used to manufacture Lenti-D LVV by transient transfection of HEK293T cells. Information on the structural elements, associated plasmid maps and full sequence information for the plasmids is

provided. The plasmid production site is qualified and managed by the applicant, in accordance with their quality management system and the plasmids are manufactured according to the applicant's specifications. Adherence to principles of GMP is ensured by the quality system in place, including audits being performed regularly. A flow diagram and brief narrative description of the plasmid manufacturing process have been provided, including information of process parameters and in-process controls. Individual WCBs are used for the manufacture of each of the plasmids. The manufacturing process consists of fermentation and harvest, a downstream purification process, ending up with final filtration, filling and storage. Stability has been verified for the proposed shelf-life. The manufacturing process performance has been evaluated with regards to process control and consistency, aseptic manufacturing, sterilising grade filter validation, cleaning validation, and shipping validation. The information provided on the manufacturer and the manufacturing process is considered adequate. Certificates of Analysis have been provided for all cell banks. The generation and qualification of the cell banks, including master, working and end of production cell banks, is in accordance with the requirements of Ph. Eur. 5.14.

6.3.3.3 Starting Materials: mRNA to Be Used in Gene Therapy

Messenger RNA (mRNA), when encapsulated in lipids for protection from nucleases, is a non-viral vector that can be used for gene delivery. The manufacturing process for the mRNA vector requires two starting materials: (1) a linearized recombinant DNA plasmid that contains the mRNA sequence for the gene of interest (GOI), and (2) nucleoside triphosphates (NTPs).

The first starting material is the recombinant template, the linearized recombinant DNA plasmid. This starting material is prepared from the recombinant DNA plasmid which is manufactured using a recombinant *E. coli* cell bank, as shown in Fig. 6.9.

The recombinant DNA plasmid development genetics requires decisions not only on the complete and correct mRNA coding region for the gene of interest (GOI), but also on the necessary promoters (e.g., bacteriophage T7 promoter upstream of the GOI), capping methodology, sequence optimization, and polyadenylation method for the mRNA to be transcribed.

The recombinant DNA plasmid CMC regulatory compliance concerns and expectations for the manufacture of the recombinant DNA plasmid from *E. coli* cell banks are the same as those discussed in the previous section for the recombinant DNA plasmids for the viral vectors.

The recombinant DNA plasmid is a circular structure where the mRNA gene coding is less accessible; which is why it needs to be linearized using restriction endonuclease prior to *in vitro* transcription (IVT). IVT will be discussed in the next chapter.

The use of a mRNA non-viral vector for either *in vivo* gene therapy or *ex vivo* gene therapy (either autologous or allogeneic patient cells) has not yet reached market-approved stage. But, using the experience from the development and manufacturing of two market-approved COVID-19 mRNA vaccines, an understanding of

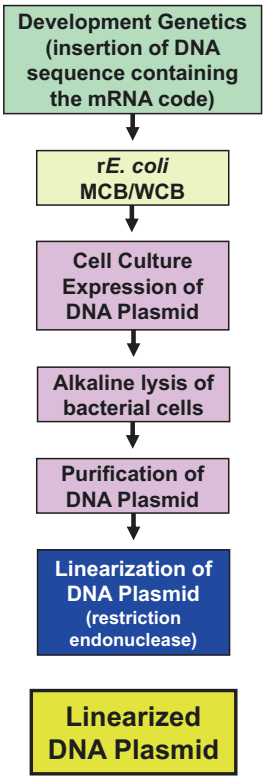

Fig. 6.9 Manufacture of the linearized DNA plasmid starting material for mRNA production

CMC regulatory compliance requirements and expectations for the linearized recombinant DNA plasmid starting material can be gained:

Comirnaty (COVID-19 mRNA vaccine (nucleoside-modified)) [40]

The BNT162b2 active substance is manufactured by in vitro transcription using a linear DNA template, produced via plasmid DNA from transformed *Escherichia coli* cells. The cell banks involved in the plasmid manufacturing process are described. Master cell bank (MCB) and working cell bank (WCB) qualification tests are listed. Relevant specifications are set and data from the current MCB and WCB are provided. The plasmid MCBs and WCBs are enrolled in a cell bank stability program. The strategy is considered adequate, noting that the dossier will be updated as appropriate. A protocol for establishment of future WCBs is provided. Following fermentation, the cells are harvested and chemically lysed to recover the plasmid DNA. After this lysis step, the circular plasmid DNA is purified. The circular plasmid DNA is filtered and stored frozen. The strategy for establishing the initial shelf-life is endorsed and data provided support the proposed shelf life. A list of the raw materials as well as other materials used in the manufacture of the linear DNA template is provided. All materials used are animal origin free and sourced from approved suppliers. Specifications for the circular plasmid DNA as well as for the DNA linear template are provided. Process- and product-related impurities including host cell genomic DNA, RNA, proteins, endotoxins, bioburden and plasmid isoforms, for the plasmid DNA, are routinely quantified. The reference material is described.

Spikevax (COVID-19 mRNA vaccine (nucleoside-modified)) [41]

Starting material: The starting materials in the manufacture of CX-031302 mRNA are the linearised plasmid template and the nucleotides ATP, CTP, GTP, N1-Me-ΨTP. The nucleotides are the same nucleotides as used for manufacture of CX-024414 mRNA.

Linearised Plasmid Template: A unique linearised DNA plasmid template specific for CX-031302 mRNA was manufactured at ModernaTX, Inc. (Norwood, MA, USA). The features of the plasmid template specific for CX-031302 mRNA are consistent with CX-024414 mRNA, with the exception of the specific sequence of the coding region. The full plasmid DNA sequence and the plasmid map are provided. The host cell line used for manufacture of PL-028274 for CX-031302 mRNA is the same as described for CX-024414 mRNA. The cell banking system is two-tiered, including a master cell bank (MCB) and a working cell bank (WCB). The manufacturers involved in cell bank production are listed. Manufacture and testing of MCB and WCB was conducted as for the original CX-024414 containing plasmid. Release results for MCB and WCB are provided including for culture purity, lytic and lysogenic bacteriophages, viability, marker retention, strain identity (for MCB only), plasmid identify, plasmid integrity and plasmid copy number. The analytical procedures used to perform release are also described. Qualification of MCB and WCB have also been described. The MCB and WCB stability protocol and all available data are provided. The test methods and acceptance criteria are the same as for release testing. All available data show compliance to specification. The CX-031302 mRNA plasmid, PL-028274, is manufactured for CX-031302 mRNA using the same procedure as described for CX-024414 mRNA. The same approach to characterisation testing and kanamycin risk assessment described for CX- 024414 mRNA was taken for CX-031302 mRNA. The specification for the linearised plasmid includes: appearance, concentration, plasmid identity, % linear plasmid, residual genomic DBA, residual RBNA, residual protein, bacterial endotoxin and bioburden. The final filtered bulk long-term storage condition for the linearised plasmid is −20 °C ± 5 °C, with a formal shelf-life of three years. A shelf life of 3 years under long-term storage condition of −20 °C ± 5 °C is requested for the linearised plasmid based on the prototype vaccine and supported by limited data collected in an on-going stability study that have been initiated linearised plasmid. Considering that no changes are included in the manufacturing process of the DNA template as compared to the original variant, the shelf-life is considered sufficiently supported by the original data.

The second starting material is the nucleoside triphosphates (NTPs; A,C,G,U). However, instead of uridine nucleoside (U), N1-methyl-pseudouridine (N1-methyl-Ψ) is added to minimize indiscriminate recognition of exogenous mRNA by pathogen-associated molecular pattern regions.

The EMA guideline on *Questions and Answers on the Principles of GMP for the Manufacturing of Starting Materials of Biological Origin Used to Transfer Genetic Material for the Manufacturing of ATMPs* identifies the starting materials and summarizes the level of GMP compliance expected for the linearized DNA plasmid containing the mRNA coding for the gene of interest [25], see Table 6.7.

The nucleoside triphosphates (NTPs) are a starting material and should be listed here. Also not listed is the recombinant *E. coli* cell bank used to manufacture the DNA plasmid. The same argument for preparing this bacterial cell bank under cGMPs, although not required, is the same one used for the *E. coli* cell banks used for viral vectors: the old phrase ‘garbage in, garbage out’.

Table 6.7 Starting materials – manufacture of the mRNA non-viral vector

Product Type	Starting Materials	Active Substance
In Vivo Gene Therapy: mRNA	Plasmid Manufacturing and linearization	mRNA In vitro transcription
ATMP starting materials are underlined Yellow – Principles of GMP Green – cGMPs		

6.3.3.4 Starting Materials: Genome Editing

In the previous three sections, Sects. 6.3.3.1, 6.3.3.2 and 6.3.3.3, gene therapies involving either replacement of an existing dysfunctional gene or addition of a new gene (e.g., CAR T cells) using either a viral or non-viral vector were discussed. But it is also possible to either disable or edit an existing human gene. This process referred to as 'genome editing' can be applied to human gene therapies. The ability to edit human genomes has been available for many years but the process of carrying it out with nucleases such as ZNF (zinc-finger nuclease) or TALEN (transcription activator-like effector nuclease) has been challenging. Enter CRISPR (clustered regularly interspaced short palindromic repeats). With CRISPR, a short RNA template is manufactured that matches a target DNA sequence in the genome. Strands of RNA and DNA can bind to each other when they have matching sequences. The RNA portion of the CRISPR, called a guide RNA, directs Cas9 enzyme to the targeted DNA sequence. Cas9 cuts the genome at this location to make the edit. CRISPR can make deletions in the genome (e.g., gene silencing) and/or be engineered to insert new DNA sequences into the genome.

Genome editing has not yet reached market approval stage. But the regulatory authorities have already provided CMC regulatory compliance requirements for the starting materials of genome editing of human cells:

EMA [37]

For genome editing approaches, the tools used to genetically modify the cells shall be, as appropriate, the vector (viral or non-viral vector) carrying the nucleic acid sequences encoding the modifying enzyme, the mRNA expressing the modifying enzyme, the modifying enzyme itself, the genetic sequence for modification of the cell genome (e.g. a regulatory guide RNA) or a ribonucleoprotein (e.g. Cas9 protein pre-complexed with gRNA), the repair template (e.g. linear DNA fragment or a plasmid), and the components to produce them. When vectors, mRNA or proteins are used, the principles of GMP shall apply from the bank system used to produce these materials onwards.

Starting materials used for the production of genetically modified cells and genome edited products shall be carefully qualified to assure a consistent manufacturing process. The amount of data to be provided for each starting material is the same as required for, respectively, the drug substance of a cell-based medicinal product and the drug substance of an *in vivo* gene therapy medicinal product. When using pre-complexed ribonu-

cleoprotein, as might take place during some genome editing procedures, the amount of data to be provided for each starting material (e.g. recombinant protein and guide RNA) is also the same as required for the drug substances of a biologic medicinal product and a chemical medicinal product, respectively. Detailed information should be provided on the manufacturing process, control of materials, characterisation, process development, control of critical steps, process validation, analytical procedures, and stability. Information on starting materials should be included in the Common Technical Document (CTD) under the heading of "control of materials", either when produced in house or supplied by another manufacturer. However, for the vector and the cells separate 3.2.S Modules can be considered.

FDA [42]

Genome Editing (GE). A GE technology may be composed of a single or multiple GE component(s). These GE components may include the nuclease, DNA targeting elements (i.e., elements used to dictate the target DNA sequence, such as guide RNA) and a donor DNA template (i.e., DNA sequence provided to repair the target sequence), if applicable.

Component Manufacture and Testing. GE components can be administered in vivo using nanoparticles, plasmids, or viral vectors, or they can be used to modify cells ex vivo. When administered *in vivo* in the form of DNA, RNA and/or protein via nanoparticles, the GE components are considered the active pharmaceutical ingredients or drug substances…. If used to modify cells ex vivo, GE component quality is considered critical for the manufacture of the final product because without these components, the resulting cell product would not have the same pharmacological activity.

Detailed descriptions of how each GE component is manufactured, purified and tested must be provided in the IND (21 CFR 312.23(a)(7)). We recommend a description of the manufacturing process and any in-process controls for each GE component include a flow diagram(s) and a detailed narrative. We recommend sponsors provide lists of the reagents used during these processes and certificates of analysis. Descriptions of the following should be provided in the IND for each GE component manufacturing site:

- The quality control and quality assurance programs in place;
- Procedures in place to ensure product tracking and segregation;
- Procedures in place to prevent, detect and correct deficiencies in the manufacturing process;
- Procedures for shipping of the GE component from the component manufacturing site to the final product manufacturing site.

This information is needed even if the GE component is manufactured by a contract manufacturer and may also be cross-referenced if it is present in an existing IND or Master File. For most Phase 1 clinical investigations, sponsors should follow the recommendations in FDA's Guidance for Industry: CGMP for Phase 1 Investigational Drugs for the manufacture of these components (see 21 CFR 210.2(c)). However, for later Phase studies and for licensure, GE components must be manufactured according to CGMP standards (21 CFR Parts 210 and 211), with particular consideration for control of reagent quality, manufacturing process, and analytical methods.

We recommend each GE component be tested appropriately. In addition to evaluating the sterility, identity, purity and functionality of each component, as applicable, additional testing, such as that for process residuals, should be included, depending on the manufacturing process. Descriptions of the analytical procedures utilized for GE component testing, including the sensitivity and specificity of the procedures, should be included in the IND. Sponsors should also outline any in-process testing performed to ensure the quality of the components, as appropriate.

We also recommend GE components be assessed for stability. Outlines of stability study protocols and any available stability data should be provided in the IND. Stability studies should be conducted on all GE components (e.g., lyophilized and reconstituted

materials, if applicable). Stability studies should include stability-indicating tests assessing critical product attributes, such as purity and functionality, that may be affected during storage.

6.3.4 Planning for the Logistical Challenges

The importance of the starting materials in gene therapy cannot be under-estimated. The ability to proceed forward in manufacturing depends upon their availability. As discussed in this chapter, there are multiple product-specific starting materials needed to be manufactured in order to prepare each individual viral vector for gene delivery. Months, if not years, can be lost waiting for all needed starting materials (e.g., needing four specific recombinant DNA plasmids for rLV starting material manufacture but only three specific DNA plasmids are on hand), as some companies have learned the hard way, if not planned for accordingly. Logistics are especially important for gene therapies.

Since many of the starting materials are supplied to a gene therapy manufacturer via a contract manufacturing organization (CMO) supplier, it is important that the obligations between the supplier and the manufacturer are clearly delineated and achieved. Regulatory authorities have much to say about the CMC regulatory compliance aspects of these contract relations [25]:

> The ATMP manufacturer should have access to information about the starting materials that is relevant to ascertain the impact thereof on the quality, safety and efficacy profile of the finished product. To this end, the contract/technical agreement between the supplier of the starting material(s) and the ATMP manufacturer should provide for the transfer of information about the starting material that is relevant to the quality, safety and/or efficacy of the finished product…appropriate principles of GMP should be determined in the agreement between the ATMP manufacturer and the manufacturer/QC testing site of the relevant starting material. This should cover aspects of the quality management system, documentation, raw materials, cell banks, production, specification, testing and control, storage, and other aspects of handling and distribution as appropriate having regard to the relevant risks for the quality, safety and efficacy of the finished product. The extent of the requirements should be proportionate to the potential impact of the starting material in the quality, safety and efficacy of the finished medicinal product.

On an additional point, the heavy reliance on DNA plasmids in the manufacturing of the gene therapy-based biopharmaceuticals, has sprouted a new set of terminology: research grade plasmids, GMP-like plasmids, and cGMP plasmids. As expected, the higher the GMP grade, the higher the cost.

- *Research grade DNA plasmids*. These R&D plasmids will be whatever paperwork, that is provided by the vendor, says it is. 'Research' conveys no expected level of information, or quality grade. You get whatever the paperwork says. This lack of any accepted definition is the reason that regulatory authorities are not comfortable with research grade. But this grade can be suitable for process development studies.
- *GMP-like grade DNA plasmids*. There is no unified definition of 'GMP-like', so the manufacturer needs to pay close attention to what was actually carried out. But there is the expectation that these plasmids will be manufactured under controlled conditions in appropriately designed facilities and have paperwork that describes the controls in place and the testing performed.

- *cGMP DNA plasmids*. Here there is the expectation that these plasmids will be manufactured according to the published cGMP requirements and expectations (see Chapter 4, Section 4.3.2), and have the paperwork documentation that describes the controls that were in place and the testing that was performed. Also, that there was Quality Unit oversight and accountability for what is reported.

Most companies migrate to the highest cGMP DNA plasmids grade for human clinical studies (even First-in-Human) to avoid any regulatory authority concerns, and to avoid the comparability challenge of switching starting materials at a later date in the clinical development program.

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Chapter 7
Upstream Production of the Biopharmaceutical Drug Substance

Abstract Upstream production of the biopharmaceutical drug substance involves living cells – whether it is living recombinant cells that express a recombinant protein or monoclonal antibody, or living cells that assemble and then propagate a viral vector, or living patient cells that are to be genetically modified. Tight control and cGMP adherence over the handling of these living cells determine the outcome of the upstream production – the yield of the intended biopharmaceutical, the type and amount of biomolecular structural variants formed, and the type and amount of process-related impurities that must be managed in purification. The focus of this chapter is on the upstream production process for both the protein-based biopharmaceuticals (recombinant proteins and monoclonal antibodies) and the gene therapy-based biopharmaceuticals (viral vectors and genetically modified patient cells, mRNA non-viral vector). Regulatory authorities have high expectations, as well as major requirements for the upstream production process, which will be examined in this chapter. Application, where appropriate, for the minimum CMC regulatory compliance continuum risk-based approach, will be discussed. In addition, the upstream production process for mRNA non-viral vector – using cell-free *in vitro* transcription production – will also be examined.

Keyword API · Upstream · Expression · Expansion · Production · Transient · Transfection · Transduction · Bioreactors · Propagation · Induction · IVT · Harvest · Genetic stability · Reduced-scale · DOE

7.1 At the Start of the DS Manufacturing Process

There are three stages involved in the manufacture of a biopharmaceutical drug substance: (1) obtaining/manufacturing the starting materials, (2) carrying out the upstream production of the biopharmaceutical, and (3) completing the downstream purification of the biopharmaceutical, see Fig. 7.1. The upstream production

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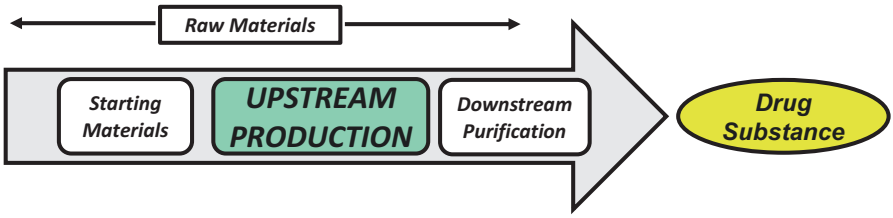

Fig. 7.1 Upstream production: second stage in the manufacture of the drug substance

process incorporates the starting materials into the biosynthesis of the biopharmaceutical drug substance. The critical importance of this second stage in the manufacture of all biopharmaceutical types (recombinant proteins, monoclonal antibodies, viral vectors, genetically modified patient cells, mRNA non-viral vector) will soon become evident in this chapter.

While the obtaining/manufacturing of starting materials discussed in Chap. 6 required adherence either to the principles of GMP or cGMPs, the upstream production for manufacturing of the biopharmaceutical drug substance requires strict adherence to cGMPs. In addition, tight control of the upstream production process (referred to as critical process parameters, CPPs) is both necessary and challenging in order to ensure that the correct and desired sequence (either amino acid or nucleic acid), along with any necessary required modifications (e.g., post-translational glycosylation), are present on the produced biopharmaceutical. The tight control is also necessary to control the type and amount of biomolecular structural variants formed, and the resulting type and amount of process-related impurities that will need to be managed during the purification stage. The focus of this chapter is on the upstream production process for both the protein-based biopharmaceuticals (recombinant proteins and monoclonal antibodies) and the gene therapy-based biopharmaceuticals (viral vectors and genetically modified patient cells). Regulatory authorities have high expectations, as well as major requirements for the upstream production process, which will be examined in this chapter. Application, where appropriate, for the minimum CMC regulatory compliance continuum risk-based approach will be discussed. In addition, the upstream production process for mRNA non-viral vector – using cell-free *in vitro* transcription production – will also be examined.

Figure 7.2 presents a general overview of the upstream production processes of four types of biopharmaceutical drug substances. For each upstream production process, risk-based decisions must be made, for example, (1) for cell culture production of the recombinant proteins, monoclonal antibodies and viral vectors, a decision on the bioreactor mode of production is needed, (2) for genetically modifying patient cells, a decision on the degree of cell transduction to occur, and (3) for mRNA production, a decision on the cell-free *in vitro* transcription reaction conditions.

CMC regulatory compliance requirements and recommendations on the upstream production processes of four types of biopharmaceuticals are discussed in the following sections:

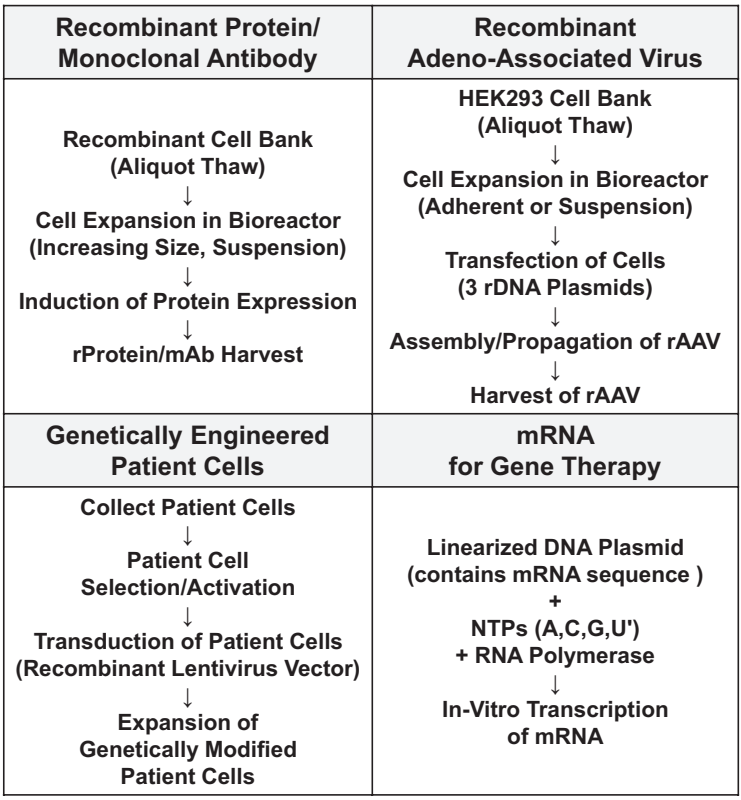

Fig. 7.2 Overview of upstream production processes for four types of biopharmaceuticals

– *Section 7.2* Upstream cell culture production of recombinant proteins and mAbs
– *Section 7.3* Upstream cell culture production of viral vectors
– *Section 7.4* Upstream production of genetically modified patient cells
– *Section 7.5* Upstream IVT production of non-viral vector (mRNA)

7.2 Upstream Cell Culture Production of Recombinant Proteins and Monoclonal Antibodies

The critical step prior to the initiation of the upstream cell culture production process for recombinant proteins and monoclonal antibodies is the GMP manufacture, characterization, and release of the recombinant master cell bank (MCB) and the working cell bank (WCB), as discussed in Chap. 6, Sect. 6.2. In general, the upstream cell culture production process for recombinant proteins and monoclonal antibodies occurs as follows: (1) thaw of an aliquot of the recombinant working cell bank (WCB), (2) inoculation of cells into flasks/vessels/bioreactors and passaged to

an ever-increasing number of cells (ultimately to a sufficient number of viable cells for the last-stage large-scale bioreactor), (3) at the last-stage bioreactor, the production bioreactor, the cells are induced to express the recombinant protein or monoclonal antibody, and (4) harvesting of the bioreactor contents and delivery for purification of the expressed protein biopharmaceutical.

7.2.1 Assembling the rProtein/mAb Upstream Production Process

The design of the upstream cell culture production process requires choices that need to be made, especially (1) the expression system for production, and (2) the mode of bioreactor operation.

7.2.1.1 Expression Systems for Recombinant Protein/Monoclonal Antibody Production

The construction of the recombinant cell bank, as discussed in Chap. 6, Sect. 6.2, determined the selection of the expression system for the biosynthesis of the recombinant protein or monoclonal antibody. Seven (7) different expression systems can be selected for production:

1. ***Bacterial Cell Expression***: The *E. coli* recombinant bacterial cell expression system has a proven track record of over three decades, being the expression system for the first biopharmaceutical approved for market – recombinant human insulin. This gram-negative bacterium typically requires only a few days of fermentation to produce the intended biopharmaceutical, with typical expression levels in the grams per liter. The expressed recombinant protein typically exists as insoluble inclusion or refractile bodies, but through genetic engineering, the bacterial cell can be designed to express the soluble protein in the periplasmatic space of the cell. Production of the biopharmaceutical is controlled by promotor DNA sequences included on the expression construct of the recombinant *E. coli* cells. When the promotor is turned off through a growth phase, high cell densities can be achieved. When the promotor is turned on (frequently by causing a temperature shift in the fermentation), a high rate of protein expression occurs. The major limitation of *E. coli* cells is that they cannot express posttranslational modified proteins (e.g., glycosylated forms).
2. ***Yeast Cell Expression***: The *Saccharomyces cerevisiae* and *Pichia pastoris* yeast expression systems provide short doubling times yielding high cell densities. Like the bacterial cells, yeast cells typically require only a few days of fermentation to produce the intended biopharmaceutical, with typical expression levels up to grams per liter. Yeast cells are easy to grow in large scale with simple nutritional demands that lower the media cost. *Pichia* can grow on methanol as

a carbon source. In contrast to the bacterial cells, yeast cells can express correctly folded proteins directly to the medium. The rigid cell wall allows the use of all sorts of bioreactors possible regardless of stirring and shaking mechanisms. The monoclonal antibody, Vyepti (eptinezumab-jjmr), is market-approved using *Pichia* cell expression [1].

3. ***Insect Cell Expression***: The lepidopteran insect cell infected with a genetically engineered baculovirus (referred to as BEVS) is a high-yielding expression system for recombinant proteins, including those requiring complex post-translational modifications (e.g., glycosylation). Advances in more stable cell lines, better expression cassettes and better understanding of the cell culture conditions, have opened up the caterpillar insect cells, for example, the fall armyworm *Spodoptera frugiperda* and the cabbage looper *Trichopulsia ni*, as an alternative to the bacterial and yeast expression systems. Viral vaccine manufacturers have taken advantage of the insect cell expression systems in the manufacture of commercial recombinant proteins (viral antigens), e.g., Cervarix (HPV, human papillomavirus vaccine) [2].
4. ***Plant Cell Expression***: Various plant cell expression systems have been studied for decades, especially the tobacco plant cell *Nicotiana tabacum*. As plant cell media lack any mammalian components that are susceptible to the transmission of mammalian viruses or prions, such as those related to bovine spongiform encephalopathy, plant cell systems naturally do not carry the risk of infection by, or transmission of, human or other animal pathogens. However, the glycosylation pattern with plant cells is different than with mammalian cells. The only commercial biopharmaceutical to date from plant cells is Elelyso (taliglucerase alfa), a recombinant protein, which is manufactured using carrot root cells [3].
5. ***Mammalian Cell Expression***: Compared to microbial cell systems, producing biopharmaceuticals in mammalian (animal or human) cell systems is generally more expensive. However, it is in the mammalian cell systems that complex proteins can be readily expressed. Chinese hamster ovary (CHO) cells have a proven track record of three decades in producing both recombinant proteins and monoclonal antibodies. CHO cells were first isolated in 1956 from a spontaneously immortalized population of fibroblast cells from the cultured ovarian cells of a partially inbred Chinese hamster – CHO-K1. Chemical mutagenesis of the CHO cell line produced a cell line with a deletion of one dihydrofolate reductase (DHFR) gene and inactivation of the other DHFR gene – CHO-DXB11. Ionizing radiation of the CHO cell line produced a cell line with both DHFR genes deleted – CHO-DG44. DHFR deficiency allows the use of methotrexate, a chemical inhibitor of DHFR, to increase the transgene copy number and thus the productivity of the CHO cells. By combining the gene of interest with a selectable gene, such as the DHFR gene, methotrexate resistance can be used to select for CHO cells that have increased copies of the DHFR gene and therefore higher levels of biopharmaceutical production [4]. Other mammalian cell lines that are being used to manufacture commercial biopharmaceuticals include baby hamster kidney (BHK) cells and murine myeloma/hybridoma cells (such as NS0 and Sp2/0).

6. ***Transgenic Animal Expression***: Complex posttranslational modified proteins can be successfully expressed in their native biologically active form using a transgenic animal expression system. However, it can take almost 3 years from transgene introduction into the animal to production at a usable level. Animal husbandry procedures are known technologies. The recombinant protein is usually expressed in the mammary gland, often at high gram of protein per liter of milk concentrations. With transgenic chickens, the recombinant protein is expressed in the eggs. Several transgenic recombinant proteins are currently market-approved using either goats, rabbits, or chickens: transgenic goats (ATryn, recombinant antithrombin) [5], transgenic rabbits (Cevenfacta, recombinant Factor VIIa) [6], and transgenic chickens (Kanuma, recombinant sebelipase alfa) [7].
7. ***Transgenic Plant Expression***: Transgenic plant expression systems (e.g., tobacco, corn, rice, etc.) for producing of biopharmaceuticals holds promise. But to date, no biopharmaceutical has been market-approved using this transgenic system.

Seven expression systems to choose from; however, two of these expression systems currently dominate the production of commercial biopharmaceuticals: recombinant *E. coli* bacterial cells and recombinant Chinese hamster ovary (CHO) mammalian cells.

The choice of the needed expression system is important to obtain the production of the desired recombinant protein or monoclonal antibody. Cell productivity is important, but so is the need for the biopharmaceutical to be produced in its correct form (i.e., full amino acid sequence, correct folding, and higher order structure) and with the correct post-translational modifications (e.g., glycosylation). Biosimilar manufacturers understand better, than any other group, the importance of choice in expression systems. A biosimilar manufacturer must have a highly similar biopharmaceutical product to that of the innovator manufacturer's reference product (RP). Hence, they give serious attention to matching the expression system of the innovator manufacturer. Different expression systems can lead to significant differences in the expressed biopharmaceutical; ultimately resulting in non-comparability [8]:

> It is understood that a manufacturer developing a biosimilar will not normally have access to confidential details of the RP manufacturing process – thus, the process will differ from the licensed process for the RP. In order to produce a high-quality product as similar as possible to the RP, the biosimilar manufacturer should assemble all available knowledge on the RP regarding the type of host cell, product formulation and the container closure system used for marketing. Although the biosimilar does not need to be expressed in the same type of host cell as that used for the RP, it is recommended that a similar host cell type is used (for example, Escherichia coli, Chinese hamster ovary cells, etc.). This will reduce the potential for critical changes in the quality attributes of the protein, or in post-translational modifications, product-related impurities or the process-related impurity profile, that could potentially affect clinical outcomes and immunogenicity. If a different host cell is used (for example to avoid unwanted and potentially immunogenic glycan structures present in the RP) then changes introduced in terms of product-related substances, as well as product- and process-related impurities, need to be carefully considered.

But, this does not mean that a biosimilar manufacturer cannot choose a different expression system than that of the innovator manufacturer; just that they need to be very careful in their selection, and must be able to confirm that their biosimilar is highly similar to the innovator's biopharmaceutical. Two such successful different expression system examples, between the biosimilar upstream process and the innovator upstream process, are the following:

Recombinant Protein: Semglee (Insulin Glargine) [9]

The active substance, recombinant insulin glargine, is a structurally modified insulin analogue. Semglee has the same amino acid sequence as Lantus and, in contrast to Lantus which is produced in *E. coli*, is produced in *Pichia pastoris* (a yeast). The primary, secondary and tertiary structures have been found comparable to the profiles of EU and US Lantus reference.

Monoclonal Antibody: Flixibi (Infliximab) [10]

The host cell line used in Flixabi manufacturing is the Chinese hamster ovary (CHO) cell line instead of SP2/0 cells, which are used by the reference product. This is acceptable because the CHO cell line is widely used for the manufacture of biotherapeutics.

7.2.1.2 Mode of Bioreactor Operations

The cell culture production process occurs in a closed vessel (referred to here in the generic sense as a 'bioreactor') and involves a complex set of conditions. Bioreactor operations are required to be performed under current Good Manufacturing Practices (cGMPs). It should be noted that some manufacturers make a distinction between a fermenter (which is used for microbial cell systems) and a bioreactor (which is used for mammalian cell systems). In this CMC book, the terms 'fermenter' and 'bioreactor' will be used interchangeably, as well as the terms 'fermentation' and 'cell culturing'.

The performance of the bioreactor is governed by thermodynamics (e.g., solubility of oxygen in the medium), kinetics (e.g., cell growth and product formation), and transport of materials (e.g., moving nutrients into the cells and removing waste products away from the cells). Optimal mixing ensures effective oxygen transfer, heat transfer, and dispersal of materials. Minor deficiencies in circulation of the production medium can have major effects on growth and production. Because of the differences in characteristics between microbial and mammalian cells, the performance required from the bioreactor will be different for each cell type. For example, a mammalian cell requires an extended processing time compared to a microbial cell, thus requiring the bioreactor to perform under longer stringent aseptic operating conditions. And mammalian cells are more easily damaged by shear than microbial cells, thus requiring the bioreactor to provide a gentler circulation system. The faster-growing bacterial cells are more challenging for mass, oxygen, and heat transfers than the slower-growing mammalian cells.

Cell culture production media, frequently proprietary, are made up of many components, essential for supporting the productivity and longevity of the cell culture production:

Inorganic salts (sodium, phosphate) to maintain osmotic balance
Amino acids for cell growth
Carbohydrates as a main source of energy for the cells
Fatty acids and lipids for cell membrane synthesis
Vitamins for cell growth
Trace elements for the growth and biological functions of cells
Anions (phosphate, nitrate, sulfate, chloride) as sources of energy
Buffering agents to maintain correct pH conditions to support optimum growth
Growth factors to promote cell growth
Peptones and hydrolysates to enhance cell growth and titer
L-glutamine to support cell growth and protein synthesis
Antibiotics to minimize contamination

An orchestrated balance between the upstream production process and the downstream purification process needs to be achieved. For example, what sense is it to design a biopharmaceutical production process that produces a high yield of the active pharmaceutical ingredient (API) and then link it to a purification process that is either incapable of handling the amounts or uneconomical to perform. Since the amounts of a biopharmaceutical needed are constantly changing over the course of clinical development (i.e., typically increasing amounts needed from early phase to late stage), and then may need to be further scaled-up for commercial manufacture, this orchestrated balance requires dynamic adjustments.

Today, a number of choices in bioreactor design are available. For small-scale manufacture, the cell cultures can be expanded in shake flasks, spinners, roller bottles, wave bags, or small-scale bioreactors. For large-scale manufacture, the cell cultures can be expanded in stainless steel bioreactors (up to 15,000 L scale, and even larger) or in platform-rocker wave bags (up to 500 L scale) or even in disposable single-use bioreactor (SUB) plastic bags (up to 2000 L scale). Circulation designs to move nutrients into the cells and remove waste products away from the cells include stirring (motor driven impellor), airlift (gas sparged into bottom of tank) or wave motion (rocking). Three bioreactor operational modes are available:

- *Batch Mode* – The bioreactor is operated in a closed system with a fixed culture volume in which the cells grow until maximum cell density depending on medium nutrients, product toxicity, waste product toxicity, and other essential factors are reached; the cells typically follow classic kinetics with a log phase of rapid proliferation and a stationary phase where the amount of cells does not change and where the protein-based biopharmaceuticals are produced; when the batch culture is terminated, the entire batch is harvested in one operation.
- *Fed-Batch Mode* – Fresh culture medium is added to the bioreactor in fixed volumes throughout the process thus increasing the volume of the cell culture with time, while neither cells nor medium leave the bioreactor; this permits the nutri-

ent levels to be kept more or less constant for an extended time and allows the possibility to switch from one substrate to another thus rendering the use of inducible promotors possible; the feed rate can be subjected to feedback control strategies using, for example, measurement of the glucose concentration, dissolved oxygen (DO), biomass production, or heat generation.

- *Perfusion Mode* – Fresh culture medium is continuously added to the bioreactor while removing an equivalent amount of medium (with or without cells); perfusion mode offers tight control of the growth conditions, and cells can be kept in their productive phase for several months, if required; although the productivity might be lower per liter of culture medium, the constant flow of product, which can be harvested at short intervals, often compensates for the extra media costs.

Of the three modes, the fed-batch mode is currently the most common bioreactor operation. A schematic of fed-batch bioreactor operations is presented in Fig. 7.3. Steps 1 and 2 are seed culture expansion steps. One container of the Working Cell Bank (WCB) is expanded to a volume of culture that contains enough cells to meet a target initial cell density of the production bioreactor. To provide flexibility in the manufacturing schedule, the seed cultures can be maintained for additional culture passages or used to generate additional inoculum trains. Step 3 is the production bioreactor operated in a fed-batch mode. The production bioreactor is inoculated and cultivated at controlled conditions for temperature, pH, and dissolved oxygen

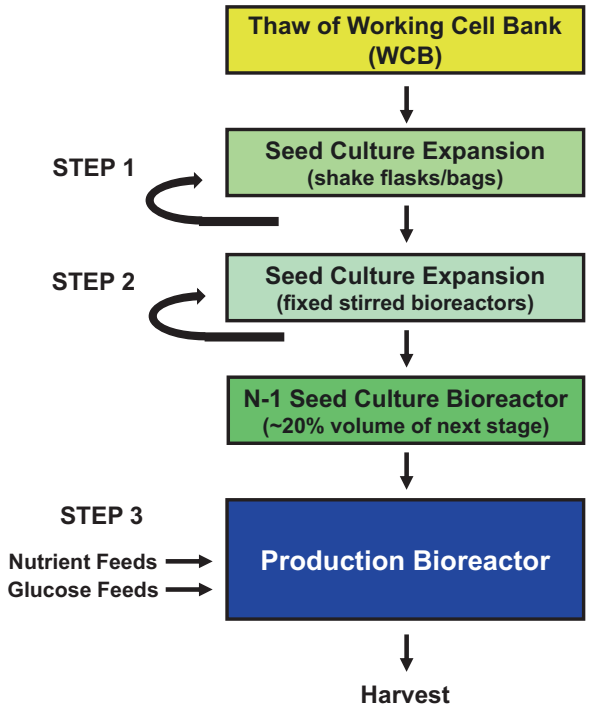

Fig. 7.3 Schematic of fed-batch mode bioreactor operations

(DO). A bolus addition of nutrient feed is added at defined times post-inoculation and multiple discrete glucose feeds are used to maintain the glucose concentration at a set value. Antifoam solution is added as required for foam control. Viable cell concentration (VCC), culture viability, induced recombinant protein or monoclonal antibody concentration, and residual glucose concentration, at a minimum are monitored periodically.

Today, a bioreactor could be a permanent stainless-steel vessel or a disposable single-use (SUB) plastic bag. A major benefit of SUBs is the reduction of effort and expense in cleaning validation between manufactured batches of the same biopharmaceutical, especially if a multi-product production operation. On the other hand, SUBs are single-use so there is the cost of disposal, and there could be a potential sensitivity to certain cell lines due to leachables from the plastic. Some Contract Manufacturing Organizations (CMOs) offer stainless steel bioreactor production, other CMOs offer SUB production, and some CMOs offer a mix. Samsung Biologics (www.Samsungbiologics.com) has in-place large-scale stainless-steel bioreactors for mammalian expression systems, having installed thirty-four 15,000 L bioreactors (over 500,000 L of capacity) at its manufacturing site in South Korea. WuXi Biologics (www.Wuxibiologics.com) has over 200,000 L of SUB capacity at its manufacturing sites in China. SUBs have become especially attractive for those CMO sites that carry out short manufacturing campaigns of multiple recombinant proteins and/or monoclonal antibodies.

7.2.2 Applying the Minimum CMC Regulatory Compliance Continuum

As discussed in Chap. 4, Sect. 4.3.1, the regulatory authorities accept a minimum CMC regulatory compliance continuum risk-based approach for biopharmaceutical manufacturing of recombinant proteins and monoclonal antibodies. For the upstream production process of the protein-based biopharmaceutical drug substance, the following guidance is provided during early-stage (including FIH) clinical development [11]:

S.2.2. Description of manufacturing process and process controls

The manufacturing process and process controls should be adequately described. The manufacturing process typically starts with one or more vials of the cell bank and includes cell culture, harvest(s), purification, modification reactions and filling.

A flow chart of all successive steps including relevant process parameters and in-process-testing should be given. The control strategy should focus on safety relevant in-process controls (IPCs) and acceptance criteria for critical steps (e.g. ranges for process parameters of steps involved in virus removal) should be established for manufacture of phase I/II material. These in-process controls (process parameters and in process testing as defined in ICH Q11) should be provided with action limits or preliminary acceptance criteria. For other IPCs, monitoring might be appropriate and acceptance criteria or action limits do not need to be provided. Since early development control limits are normally based on a limited number of development batches, they are inherently pre-

liminary. During development, as additional process knowledge is gained, further details of IPCs should be provided and acceptance criteria reviewed.

S.2.5. Process validation

Process validation data should be collected throughout development, although they are not required to be submitted in the IMPD.

S.2.6. Manufacturing process development – Process improvement

Manufacturing processes and their control strategies are continuously being improved and optimised, especially during the development phase and early phases of clinical trials. Changes to the manufacturing process and controls should be summarized. This description should allow a clear identification of the process versions used to produce each batch used in non-clinical and clinical studies, in order to establish an appropriate link between pre-change and post-change batches. Comparative flow charts and/or list of process changes may be used to present the process evolution.

Increasing control over the upstream production process is expected to evolve as the biopharmaceutical moves from early-stage clinical development into late-stage clinical development. As the stage of clinical development advances, critical process parameters (CPPs) are identified for the upstream production process of the protein-based biopharmaceutical (typically prior to entering the pivotal clinical trials). This identification can typically be accomplished through design of experiment (DOE) type studies, using small reactors (e.g., Ambr® 15 mL microbioreactors) to vary upstream production parameters across a range of values and to measure upstream production outputs, as illustrated in Table 7.1. Both the impact of individual production parameters and the correlation of effects across multiple production parameters can be obtained.

At the time of submission of a dossier for market approval, the validation/evaluation of the upstream production process for the protein-based biopharmaceutical is expected to be completed and the CMC documentation summarized in the submission:

ICH M4Q(R1) [12]

3.2.S.2.2 Description of Manufacturing Process and Process Controls

Biotech: Information should be provided on the manufacturing process, which typically starts with a vial(s) of the cell bank, and includes cell culture, harvest(s), purification and modification reactions, filling, storage and shipping conditions.

Cell culture and harvest

Table 7.1 Some upstream cell production bioreactor parameters and output measurements

Upstream Production Parameters to Vary	Upstream Production Outputs to Measure
Cell passage number	Viable cell density
Initial seeding density	Viability
Nutrient feed amount	Recombinant protein titer
Nutrient feed timing	Glucose level
pH operating range	Lactate level
Temperature operating range	Ammonia level
Temperature shift timing	Product characteristics (e.g., glycosylation)
Dissolved oxygen level	

A flow diagram should be provided that illustrates the manufacturing route from the original inoculum (e.g. cells contained in one or more vials(s) of the Working Cell Bank up to the last harvesting operation. The diagram should include all steps (i.e., unit operations) and intermediates. Relevant information for each stage, such as population doubling levels, cell concentration, volumes, pH, cultivation times, holding times, and temperature, should be included. Critical steps and critical intermediates for which specifications are established (as mentioned in 3.2.S.2.4) should be identified. A description of each process step in the flow diagram should be provided. Information should be included on, for example, scale; culture media and other additives (details provided in 3.2.S.2.3); major equipment (details provided in 3.2.A.1); and process controls, including in-process tests and operational parameters, process steps, equipment and intermediates with acceptance criteria (details provided in 3.2.S.2.4). Information on procedures used to transfer material between steps, equipment, areas, and buildings, as appropriate, and shipping and storage conditions should be provided.

3.2.S.2.5 Process Validation and/or Evaluation

Biotech: Sufficient information should be provided on validation and evaluation studies to demonstrate that the manufacturing process (including reprocessing steps) is suitable for its intended purpose and to substantiate selection of critical process controls (operational parameters and in-process tests) and their limits for critical manufacturing steps (e.g., cell culture, harvesting, purification, and modification). The plan for conducting the study should be described and the results, analysis and conclusions from the executed study(ies) should be provided. The analytical procedures and corresponding validation should be cross-referenced (e.g., 3.2.S.2.4, 3.2.S.4.3) or provided as part of justifying the selection of critical process controls and acceptance criteria.

FDA for BLA [13]

A detailed description of the process of inoculation, cell growth and harvesting should be submitted. The composition of the medium, equipment preparation and sterilization, as well as fermentation medium sterilization, should be described. For all stages of any fermentation process the procedures which prevent contamination with adventitious agents should be described. The stages of cell growth should be described in detail including the selection of inoculum, scale-up for propagation, and established and proposed (if different) production batch size. All operating conditions and in-process controls should also be described and appropriate ranges for operating and control parameters, such as fermentation time, cell doubling time, cell culture purity, cell viability, pH, CO2, etc., established. If induction is required for production of protein, detailed information including induction conditions and controls employed should also be described. The submission should include the process used to inactivate cells utilized in the production of a drug substance prior to their release into the environment… If the culture supernatant or cell pellet is stored prior to processing, data supporting its stability during storage should be provided. The manipulation of more than one cell line in a single area or the use of any piece of equipment for more than one cell line should be indicated and measures to ensure prevention of cross contamination should be discussed.

Validation Studies for the Cell Growth and Harvesting Process. A description and documentation of the validation studies which identify critical parameters to be used as in-process controls, to ensure the success of routine production should be submitted.

EMA for MAA [14]

Process evaluation activities should demonstrate that the cell culture steps, from the initiation of the manufacturing process (e.g. thaw of a WCB vial) up to and/or beyond the PDL defined by termination criteria, are capable of consistently delivering inocula, harvest(s) and ultimately an active substance of appropriate quality after downstream processing. Several aspects should be considered when validating cell culture. The level of detail provided should support the criticality assignment of process parameters. These activities could include evaluation of specific cell traits or indices (e.g. morpho-

> logical characteristics, growth characteristics (population doubling level), cell number, viability, biochemical markers, immunological markers, productivity of the desired product, oxygen or glucose consumption rates, ammonia or lactate production rates, process parameters and operating conditions (e.g. time, temperatures, agitation rates, working volumes, media feed, induction of production). Evaluation of any critical conditions for the control of expression of the desired product in the production bioreactor is crucial. The conditions utilised to end fermentation/cell culture cycle and initiate harvest should be appropriately defined. Termination criteria should be defined and justified based on relevant information (e.g. yield, maximum generation number or population doubling level, consistency of cell growth, viability, duration and microbial purity and, ultimately, consistency of the quality of the active substance).

The ongoing validation/evaluation of the upstream production process control for the protein-based biopharmaceutical is subject to GMP compliance inspections from various regulatory authorities. Table 7.2 presents some questions, prepared by PIC/S, that regulatory authority reviewers can consider for such an inspection [15]. Note, the Pharmaceutical Inspection Co-operation Scheme (PIC/S) is a non-binding, informal co-operative arrangement between regulatory authorities in the field of GMP of medicinal products. PIC/S presently comprises 52 participating authorities coming from all over the world (Europe, Africa, America, Asia, and Australia).

7.2.3 Genetic Instability During Production of Protein-Based Drug Substances

All living systems, including cell lines producing a biopharmaceutical drug substance, undergo the potential for genetic change. When foreign genes are inserted into cell lines, the foreign genetic content can undergo change over time – either loss of the genetic content (e.g., loss of gene copy number) or rearrangement of the inserted genetic content – referred to as ‘genetic instability’.

As the recombinant cells are passaged in the seed stage and then in the bioreactor stage, genetic change can occur compromising the quality and/or the safety of the produced biopharmaceutical recombinant protein or monoclonal antibody. Regulatory authorities are concerned about this potential genetic instability, and they require for market approval a confirmation of genetic stability [16]:

> For the evaluation of stability during cultivation for production, at least two time points should be examined, one using cells which have received a minimal number of subcultivations, and another using cells at or beyond the limit of *in vitro* cell age for production use described in the marketing application. The limit of *in vitro* cell age for production use should be based on data derived from production cells expanded under pilot plant scale or commercial scale conditions to the proposed limit of *in vitro* cell age for production use or beyond. Generally, the production cells are obtained by expansion of cells from the WCB; cells from the MCB could be used with appropriate justification. This demonstration of cell substrate stability is commonly performed once for each product marketing application.

The limit of *in vitro* cell age (LIVCA) time period begins with the thaw of the WCB, continues through the seed and bioreactor production stages, and then prior to

Table 7.2 PIC/S regulatory aid for GMP inspection of the DS upstream production process

Process	Crucial Questions
Fermentation System	Are there single harvest or continuous harvest (simultaneous fermentation and harvesting)? Are the construction, the material, and the material finish (surface, roughness, polish, weld seam processing, etc.) of the following components and fittings adequate and confirm cGMP rules: fermenter (open, closed, or a contained system?) pipe work (dead legs…) valves, vent filters manometers pH-/oxometers thermocouples, temperature sensors pipes and valves for charge and discharge
Controlling	How is the addition of the following objects documented? Water Media Buffers, acids, lye's Cell substrates Induction agent Gases Antifoam
Inoculation of Process	Is information on seed culture/cell bank management available? Where is the cell substrate produced? Is there an expansion of culture (in pre culture /intermediate fermenter or main fermenter)? Is an inoculation procedure in place? Is there an assessment on the risk of contamination during inoculation? Is bioburden of seed material measured? Are endotoxins of seed material measured? Is the virus content of seed material controlled?
Fermentation Process	Is there a correspondence between process specifications (e.g. number of cell doublings, yield etc.) and the data of the inspected batch? Is there a proof that sampling does not pose a risk of contamination? Is there an inactivation process? Are intermediate products stored? Is there a proof that harvesting does not pose a risk of contamination? Do all critical operation parameters are monitored during process? process time temperature pH pO2 pCO2 pressure agitation rates addition of gases addition of buffers, acids, lye's bioburden viral content endotoxins viscosity Are the further parameters of the fermentation process monitored? contamination cell identification cell growth cell productivity cell viability cell ratio (co-cultivation of two different cells) cell aggregate formation

harvest, production cells are collected from the bioreactor and transferred to Process Development for a continuing of cell culture passaging (at reduced bioreactor scale), as illustrated by Fig. 7.4 One question always asked is 'how long does the extended culturing in Process Development have to occur?' While there is no

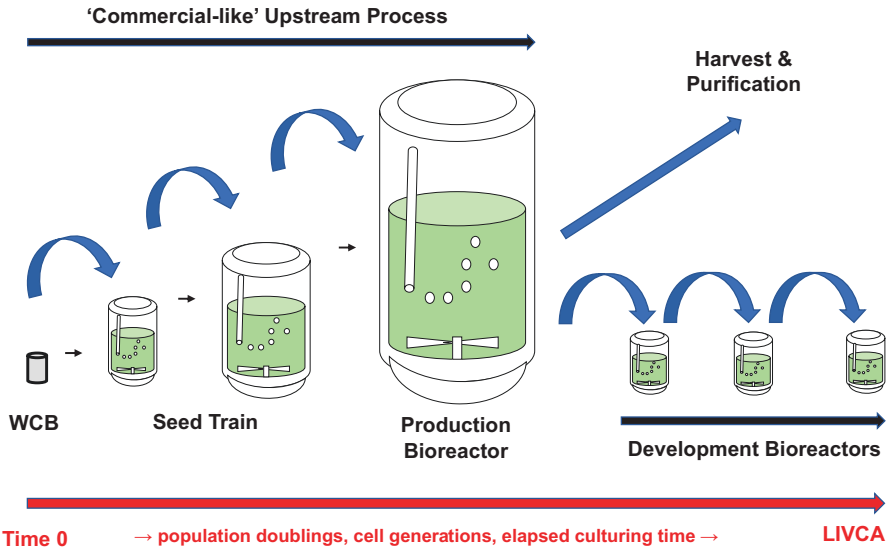

Fig. 7.4 Illustration of the recommended cell culturing conditions for genetic stability assessment

official number of passages provided in the regulatory guidances, the United States Pharmacopeia (USP) in general monograph Article <1042> Cell Banking states '*at a minimum, LIVCA should have 10 population doubling levels (PDLs) beyond the typical manufacturing window*' [17].

Testing for genetic stability involves evaluating over the defined LIVCA time period both the amino acid sequence of the biopharmaceutical recombinant protein or monoclonal antibody and the nucleic acid sequence of the transgene in the recombinant cells, as well as the retention of the copy number of this transgene [16]:

> Evaluation of the cell substrate with respect to the consistent production of the intended product of interest should be the primary subject of concern. The type of testing and test article(s) used for such assessments will depend on the nature of the cell substrate, the cultivation methods, and the product. For cell lines containing recombinant DNA expression constructs, consistency of the coding sequence of the expression construct should be verified in cells cultivated to the limit of *in vitro* cell age for production use or beyond by either nucleic acid testing or product analysis, as described in the relevant ICH guideline.

For mammalian recombinant cell lines, genetic stability also includes testing at the end of the LIVCA time period for induction of latent endogenous virus [18]:

> The limit of *in vitro* cell age used for production should be based on data derived from production cells expanded under pilot-plant scale or commercial-scale conditions to the proposed *in vitro* cell age or beyond. Generally, the production cells are obtained by expansion of the WCB; the MCB could also be used to prepare the production cells. Cells at the limit of *in vitro* cell age should be evaluated once for those endogenous viruses that may have been undetected in the MCB and WCB.

While science-based approaches are encouraged by the regulatory authorities, being creative with the traditional genetic stability study design illustrated in Fig. 7.5

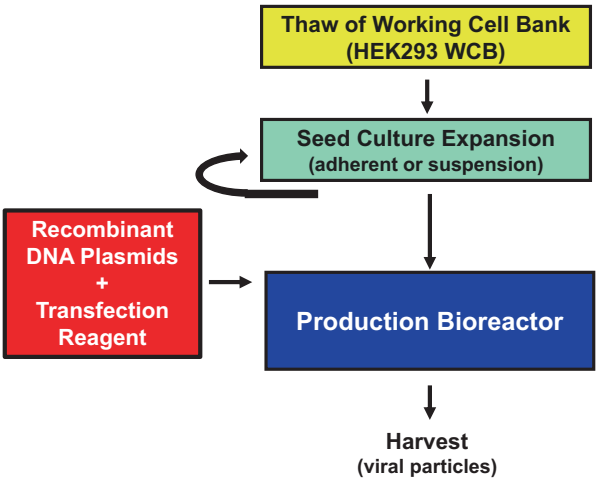

Fig. 7.5 Schematic of the viral vector upstream production process

apparently is not. Genentech in seeking market approval in 2004 for Avastin (bevacizumab) carried out the LIVCA genetic stability study using only small-scale bioreactors as a mimic of the commercial manufacturing process. FDA felt that the small-scale study was not fully representative of the commercial-like cell culturing scale, and required the company to repeat the genetic stability study at full scale [19]. Then, in seeking market approval for Perjeta (pertuzumab) in 2012, Genentech again carried out the LIVCA genetic stability study using small-scale bioreactors as a mimic of the commercial manufacturing process. And again, the FDA took issue with the small-scale study not being fully representative of the commercial-like cell culturing scale, even requesting an internal FDA biostatistical consult that identified multiple issues regarding the assessment of equivalency between the small-scale models and the full-scale process. The FDA again required the company to repeat the genetic stability study at full scale: '*Because of concerns regarding the models not being representative of the commercial process, it was determined that this testing would need to be done on cells from the commercial process*' [20].

When the LIVCA genetic stability studies have been done, genetic instability has occasionally been found. The following two case examples illustrate the interaction between EMA and the biopharmaceutical manufacturer over genetic instability issues presented in their MAA submissions:

Monoclonal Antibody, Murine Sp2/0 Myeloma Cell Line: Inflectra (Infliximab) [21]
Cells at the limit of in vitro cell age were characterised from the EPCB and acceptable testing results for the EPCB are provided. Retrovirus particles have been identified, as expected for this cell line. Genetic stability testing for the EPCB compared with the MCB indicated a significant reduction in gene copy number, but although this affects productivity, the quality of CT-P13 from the EPCB was shown to be acceptable.

Monoclonal Antibody, CHO Cell Line, Qarziba (Dinutuximab Beta) [22]
Comparative genetic characterisation included sequence analysis of APN311-specific cDNA derived from MCB, WCB and EPC (RT-PCR and cDNA sequencing), showing

> heavy chain and light chain is identical in MCB, WCB and EPC (0, 5 and 10 days, also 19 days at small scale) and corresponds to the reference sequence, confirming genetic stability of the cell line… Determination of the transgene copy number showed 6 copies per cell for light chain and 2 - 3 copies per cell for heavy chain (MCB and WCB), with a slightly lower copy number for the day 19 extended culture samples (5 copies for light chain and 2 copies for heavy chain). While these results might indicate some instability over extended production, no reduction in productivity was detected up to 10 days in the production bioreactor…. Genetic stability of the WCB and EPCs at mRNA level (in comparison to the MCB) for the intended period of use was confirmed.

In the first case example, copy number was loss during cell culture passaging resulting in a loss of overall productivity, but no impact on the quality of the biopharmaceutical produced. In the second case example, copy number was loss during cell culture passaging, but no impact either on overall productivity or on quality of the biopharmaceutical produced.

Scientists at Merck reported on a genetic instability finding in the process of qualifying a new WCB from the existing commercial approved MCB for an unidentified commercial recombinant protein manufactured in CHO cells [23]:

> During the validation of an additional working cell bank derived from a validated master cell bank to support the commercial production continuum of a recombinant protein, we observed an unexpected chromosomal location of the gene of interest in some end-of-production cells. This event – identified by fluorescence in situ hybridization and multicolour chromosome painting as a reciprocal translocation involving a chromosome region containing the gene of interest with its integral coding and flanking sequences – was unique, occurred probably during or prior to multicolour chromosome painting establishment, and was transmitted to the descending generations. Cells bearing the translocation had a transient and process-independent selective advantage, which did not affect process performance and product quality. However, this first report of a translocation affecting the gene of interest location in Chinese Hamster Ovary cells used for producing a biotherapeutic indicates the importance of the demonstration of the integrity of the gene of interest in end-of-production cells.

The bottom line is that genetic instability does occur, and it needs to be evaluated across the LIVCA time period in recombinant cell line production processes for recombinant proteins and monoclonal antibodies. Instability happens, but as long as it can be confirmed that there is no significant impact on the quality of the manufactured protein-based biopharmaceutical, the genetic instability can be justified as acceptable.

7.2.4 Meeting CMC Regulatory Compliance for Upstream Production

The following case examples of market-approved monoclonal antibodies and engineered antibodies, indicate the complete CMC documentation for the upstream production process in the market application dossier that meet regulatory authority compliance expectations:

Single-Chain Fv Antibody Fragment: Beovu (Brolucizumab) [24]

Brolucizumab is a recombinant humanised single-chain Fv (scFv) antibody fragment … expressed in an *E. coli* BL21(DE3). Brolucizumab active substance is manufactured according to a fed batch mode culture. The upstream process starts with the thawing of one vial of working cell bank (WCB) followed by expansion steps and then culture in the production bioreactor (15000 L). At a defined cell density, the induction of protein expression is triggered. After incubation, the cells are separated from the fermentation media by disc stack centrifugation. The inclusion bodies are then isolated by various washes followed by a centrifugation step each time. The isolated IBs can be stored at 4 – 12°C for up to 168 hours prior to solubilisation.

The genetic cell bank stability of the production cell line was appropriately demonstrated.

Monoclonal Antibody: Vyepti (Eptinezumab) [25]

Eptinezumab is produced in a yeast-based (*Pichia pastoris*) expression system using conventional fermentation and downstream purification processes. The upstream process of eptinezumab bulk drug substance (BDS) comprises 3 individual steps, which are all related to the *Pichia pastoris*-based microbial fermentation procedure: the inoculum preparation step, the seed fermentation step, and the production fermentation step. *Pichia pastoris* cells are first removed by centrifugation, and the supernatant is treated with a flocculating agent resulting in the flocculation of impurities. These are removed by a second centrifugation step followed by filtration of the resulting supernatant. Stability of the cell banks and the genetic integrations were demonstrated through End of Production (EOP) and Limit of *In Vitro* Cell Age End-of-Production Cell (LIVCA) testing. The validated LIVCA duration includes pilot scale fermentation process duration plus a duplicated seed expansion process. The validated limit of *in vitro* cell age for the MCB and is population doublings from the thaw of a vial from MCB through the end of production has been established.

Bispecific Antibody: Hemlibra (Emicizumab) [26]

Emicizumab is produced using a suspension-adapted CHO cell line. The source is the working cell bank (WCB), which is derived from the master cell bank (MCB). The cell culture process for the production of emicizumab active substance involves three stages: the seed train, the inoculum train, and the production culture. The seed train is used to provide a continuous source of cells for the production of multiple batches and is started by thawing one vial of the working cell bank (WCB). The inoculum train is used to expand the cell population for introduction into the production stage. The production stage is used to produce emicizumab, which is secreted into the culture fluid. During production, cell viability and productivity are enhanced by addition of nutrients. The production culture is harvested by separating the secreted molecule from cells and cell debris. From each production run, a single batch of harvested cell culture fluid (HCCF) is produced and can be traced back to the WCB vial used to initiate the manufacturing process. Following the cell culture steps, harvest is initiated from the production bioreactor. Following completion of the cell culture production stage, the secreted emicizumab in the pre-harvest cell culture fluid (PHCCF) is separated from cells and cellular debris. The harvested cell culture fluid (HCCF) is cooled and stored. Once the contents of the bioreactor are processed, water for injections may be flushed to recover any residual product.

Monoclonal Antibody: Qarziba (Dinutuximab Beta) [22]

The monoclonal antibody ch14.18/CHO is expressed in a recombinant CHO cell line. The production of one batch APN311 active substance commences with thawing of one vial APN311 working cell bank (WCB). During a series of four sub-cultivations, the cells are propagated in shake flasks and subsequently in 20 L, 100 L and 500 L bioreactors to achieve cell expansion for subsequent inoculation of the production bioreactor. Cultivation of the cells in the production bioreactor is performed in fed-batch mode, using a fixed feeding strategy. The cultivation is terminated when a viability is reached,

followed by harvesting and clarification of the supernatant. The biomass is separated by centrifugation and the supernatant is depth filtered followed by a 0.2 μm filtration.

Comparative genetic characterisation included sequence analysis of APN311-specific cDNA derived from MCB, WCB and EPC (RT-PCR and cDNA sequencing), showing heavy chain and light chain is identical in MCB, WCB and EPC (0, 5 and 10 days, also 19 days at small scale) and corresponds to the reference sequence, confirming genetic stability of the cell line.

7.3 Upstream Cell Culture Production of Viral Vectors

Both AAV and LV viral vectors will be discussed in this section:

- Adeno-associated virus (AAV) is the most widely used viral vector for direct patient injection *in vivo* genetic modification, while lentivirus (LV) is the most widely used viral vector for *ex vivo* genetical modification of collected patient cells
- Recombinant AAV (rAAV) delivers the gene to the cell nucleus where it remains episomal, while recombinant LV (rLV) delivers the gene to the cell nucleus where it is integrated into the cell genome.
- rAAV is a drug substance for *in vivo* use, while rLV is a starting material for *ex vivo* use.

For upstream cell culture production of viral vectors, there are two modes of manufacture. The first mode is through a transient production system, involving either transfection with multiple recombinant DNA plasmids of a human cell line or transduction with multiple recombinant viral seeds of an insect cell line (the *baculovirus* expression vector system). The second mode is through a recombinant stable producer/packaging cell line. In this chapter, the focus will be on the transient upstream cell culture production method that uses multiple recombinant DNA plasmids.

7.3.1 Assembling the Viral Vector Production Process

The critical step prior to the upstream cell culture production process for the viral vectors is the manufacture, characterization, and release of the cell banks and the recombinant DNA plasmid starting materials, as discussed in Chap. 6, Sects. 6.3.3.1 and 6.3.3.2. With these starting materials, the upstream manufacturing process follows the following general pathway: (1) thaw of an aliquot of the host human working cell bank (WCB), (2) inoculation of the cells into flasks/vessels/bioreactors and passaged to an ever increasing number of cells (ultimately to have sufficient cells for the last-stage large-scale bioreactor), to provide flexibility in the manufacturing schedule, the seed cultures can be maintained for additional culture passages or used to generate additional inoculum trains, (3) at the last-stage bioreactor the cells (either adherent or in suspension) are transfected with the multiple recombinant

DNA plasmids (each containing a piece of the viral vector genome and the gene of interest, GOI), (4) over 2–4 days, within the human cells, the recombinant viral vector is assembled and propagated, and (5) finally the viral vector particles harvested. A schematic of the viral vector upstream production process is presented in Fig. 7.5.

7.3.1.1 Cell Culture Process

The upstream cell culture process for the viral vectors is similar to the cell culture process of the protein-based biopharmaceuticals in Sect. 7.2. Human embryonic kidney cells, HEK293 or HEK293T (derived from HEK293), are the predominant host cells used for production of viral vectors. A Master Cell Bank (MCB) and a Working Cell Bank (WCB) are prepared for this host cell system. Chap. 6, Sect. 6.3.3.1 describes the controls, qualifications, characterization testing and GMPs to be carried out for this host cell starting material. The FDA does express a caution for the use of HEK cells [27]:

> If you are using cells that are tumor-derived (e.g., Hela) or have tumorigenic phenotypes (e.g., HEK293, HEK293T) or other characteristics that may give rise to special concerns, the limitation of specific residual DNA quantities may be needed to assure product safety. In addition to controlling host cell DNA content and size, as described above, you should also control the level of relevant transforming sequences in your product with acceptance criteria that limit patient exposure. For example, products made in 293T cells should be tested for adenovirus E1 and SV40 Large T antigen sequences, similarly products made in Hela cells should be tested for E6/E7 genes. Your tests should be appropriately controlled and of sufficient sensitivity and specificity to determine the level of these sequences in your product.

HEK293 cells can be grown either adherently or in suspension mode. Adherent cells are attached to a solid surface either in flasks, roller bottles or fixed-bed bioreactors. After each cell expansion stage, the cells are detached from the solid surface typically using trypsin and transferred to a larger vessel for additional cell expansion, until the final fixed-bed bioreactor. Suspension cells utilize standard stirred-tank bioreactors. Suspension cells can take advantage of the extensive technical knowledge about cell expansion from the use of bioreactors for the protein-based biopharmaceuticals. But suspension cells can also clump together during expansion stages.

7.3.1.2 Transient Transfection with Recombinant DNA Plasmids

After sufficient cell expansion to obtain the target cell density, whether adherent or suspension cells, the next step, a most critical step, is the transient transfecting of the HEK293 cells with multiple recombinant DNA plasmids – three for rAAV and four for rLV.

Transfection is a biological reaction that incorporates foreign genetic material into a cell, in this case, recombinant DNA plasmids. In transient transfection, the foreign genetic material exists in the cell for only a short time period and is not

integrated into the cell genome. The transfection process also requires a transfection reagent. This critical reagent is typically a cationic lipid, polymer, or liposome that interacts with or encapsulates negatively charged nucleic acids. Transfection reagents overcome electrostatic repulsions between the DNA plasmids and cell membranes to overcome the plasma membrane barrier. Some transfection reagents also incorporate functional groups that facilitate release from endosomal membranes. For example, the polyethyleneimine (PEI) transfection reagent forms an ionic interaction with the negatively charged backbone of the DNA plasmid to form a positively charged transfection complex. The positively charged transfection complex then can interact with negatively charged lipids and proteoglycans present on the external cell membrane lipid bilayer, which triggers cellular uptake by the HEK293 cells.

Transient transfection of HEK293 cells with the multiple recombinant DNA plasmids is a complex biological reaction that requires tight control over various critical process parameters (CPPs):

1. Ratio of the three (if AAV) or four (if LV) DNA plasmids in the transfection reagent mixture
2. Ratio of total DNA plasmids to transfection reagent in the transfection reagent mixture
3. Ratio of transfection mixture to HEK293 cells
4. Timing during cell culture for the transfection of the HEK293 cells
5. Number of transfection cycles on the HEK293 cells

Transfected HEK293 cells are then allowed to assemble and propagate the viral vector for 2–4 days. The goal is to maximize the production of the viral vector with the DNA gene of interest (GOI) encapsulated within the viral protein capsid, and minimize the production of viral vector that does not contain the GOI (referred to as 'empty capsids'), which will be discussed in Chap. 11. Regulatory authorities are also concerned with formation of viral vector particles that contain contaminating nucleic acids [27]:

> Some vectors, including AAV, can package a large amount of non-vector DNA (e.g., plasmid DNA, helper virus sequences, cellular DNA), and it may not be possible to remove or reduce this DNA from the product to a level to assure safety based on current guidance. Therefore, we strongly recommend that the cell lines and helper sequences used to make viral vectors that package non-vector DNA, such as AAV, be carefully chosen to reduce the risks of the product. Sponsors should provide necessary quality data, risk assessments, and/or details of their process and product control strategy to address and mitigate potential risks posed by the manufacturing systems used.

7.3.1.3 Viral Particle Harvest

Virus harvesting is dependent upon the type of bioreactor mode and the type of virus that was propagated:

1. **AAV:** rAAV viral vector accumulates both in the culture solution and inside the cells. Therefore, if adherent cells were used in production, the cells are first dislodged from the fixed-bed bioreactor, and then the bioreactor contents harvested. If suspension cells were used in production, the bioreactor contents are directly harvested. The HEK293 cells in the collected bioreactor contents are lysed with a surfactant to release any contained virus particles.
2. **LV:** rLV viral vector particles accumulate in the culture solution.

The harvested contents are then clarified typically by depth filtration to remove cells and cell fragments. The solution is treated with nuclease to digest both host cell DNA and residual plasmid DNA. If need be, the clarified harvest solution containing the viral vector is concentrated by ultrafiltration/diafiltration (UF/DF) and then held for purification.

7.3.1.4 Resources for a More in-Depth Comparison Between AAV and LV Production

For a more in-depth understanding of the viral vector upstream production process, and a comparison between the adeno-associated virus (AAV) and lentivirus (LV) processes, check out two consensus publications, prepared through input from the biopharmaceutical manufacturing industry:

Adeno-Associated Virus (AAV) Vector

A-Gene (2021): A case study-based approach for *in vivo* gene therapy CMC programs

– a over 60 individuals volunteered their time [28]

Chapter 5: Upstream and Downstream Processing

Lentivirus (LV) Vector

A-Cell (2022): A case study-based approach for CAR T cell-based therapy CMC programs

– over 40 individuals volunteered their time [29]

Chapter 7: Lentiviral *Vector Manufacturing Process*

Also, the various contract manufacturing organizations (CMOs) and processing equipment vendors have considerable information on the upstream production process for viral vectors, readily available on their websites.

7.3.2 Applying the Minimum CMC Regulatory Compliance Continuum

As discussed in Chap. 4, Sect. 4.3.1, the regulatory authorities accept a minimum CMC regulatory compliance continuum risk-based approach for manufacturing of biopharmaceuticals. Since the upstream production processes for the protein-based biopharmaceuticals and the gene therapy viral vectors are similar, these two production processes share similar minimum CMC regulatory compliance continuum principles (see the principles for the protein-based biopharmaceuticals in Sect. 7.2.2).

For the upstream production process of viral vectors, the following guidance is provided during early-stage (including FIH) clinical development:

EMA [30]

S.2.2. Description of manufacturing process and process controls

The manufacturing process of an ATIMP and process controls should be carefully designed and described concisely and step-by-step. The suitability of the controls for the intended purpose needs to be proven. A flow chart of all successive steps of the drug substance manufacturing process should be provided starting from biological fluid/tissue/organ or from cell banks/viral seeds. Critical steps and intermediate products should be indicated as well as relevant process parameters, in-process controls (IPCs) and acceptance criteria. IPC testing (for early phase developments) should focus at the minimum on safety aspects. Critical steps should already be identified for the manufacture of early clinical trial material and adequate acceptance criteria for these critical steps established, for other IPCs, monitoring might be appropriate.

For GTIMP the following aspects should be considered as applicable:

- Batch(es) and scale should be defined, including information on any pooling of harvests or intermediates.
- Any reprocessing during manufacture of the active substance (e.g. filter integrity test failure) should be described and justified.
- The applicant should establish that the vector sequence remains stable throughout cell culture. Where sufficient manufacturing experience permits, a maximal passage number for the cells should be established.
- The rationale for the use of a particular cell substrate should be provided.
- Manufacturers should seek to control unintended variability as far as possible, for example in culture conditions or inoculation steps during production.
- The manufacturing process must be set up to minimise the risk of microbiological contamination.

S.2.5. Process validation

Process characterisation/evaluation data should be collected throughout the development. It is acknowledged that some degree of variability of the active substance due to the characteristics of the starting materials is intrinsic to ATMPs. In this regard, it is recommended that critical process parameters, critical quality attributes and the associated acceptance criteria should be set based on the development data and current knowledge. This is achieved through implementation of appropriate monitoring and control measures. Summaries of the process characterisation and verification studies need to be provided, but the reports themselves are not required to be submitted as part of the IMPD. In addition the process characterisation/ evaluation summaries, validation of the aseptic process and the viral removal/inactivation steps are expected to be validated prior to the FIH clinical trials.

FDA [27]

Description *of Manufacturing Process and Process Controls (3.2.S.2.2)*

Your description of the DS manufacturing process and process controls should include the following, as applicable: cell culture; transduction; cell expansion; harvest(s); purification; filling; and storage and shipping conditions. Your description should also accurately represent your process and process controls. We acknowledge that information on process controls may be limited early in development and recommend that sponsors provide additional information and updates as product development proceeds.

i. Batch and Scale

A description of how you define each manufacturing run (i.e., batch, lot, other) should be submitted with an explanation of the batch (or lot) numbering system. You should clearly state whether any pooling of harvests or intermediates occurs during manufacturing. If pooling is necessary during production, we recommend that you control the storage conditions (e.g., time, temperature, container closure system) for each pool and that you describe the testing performed prior to pooling to ensure the quality of each pool. We also recommend that you provide an explanation for how the batch scale is defined (e.g., bioreactor volume, cell processing capacity) and how the DS is quantified (e.g., vector genomes, transducing units, infectious particles, mass, number of gene modified cells) to facilitate review and allow a better understanding of the manufacturing process. When known, please include the yield expected per batch.

ii. Manufacturing Process

The description of your manufacturing process should include a process flow diagram(s) and a detailed narrative. Your description should clearly identify any process controls and in-process testing (e.g., titer, bioburden, viability, impurities) as well as acceptable operating parameters (e.g., process times, temperature ranges, cell passage number, pH, CO2, dissolved O2, glucose level). We acknowledge that this information may be gathered over the course of product development and may be submitted in a stage-appropriate manner. We recommend that you monitor process performance parameters for process consistency. Process trend analysis and evaluation of process parameters and materials will help to determine and establish process control strategies. You should clearly describe any controls for cleaning and change over as well as tracking and segregation procedures that are in place to prevent cross-contamination throughout the manufacturing process.

a. Cell Culture (for Vector Production)

The description of all cell culture conditions should contain sufficient detail to make understandable any of the process steps that apply, process timing, culture conditions, hold times and transfer steps, and materials used (e.g., media components, bags/flasks). You should describe whether the cell culture system is open or closed and any aseptic processing steps.

b. Vector Production

For the manufacture of gene therapy vectors (e.g., virus, bacteria, plasmids), you should provide a description of all production and purification procedures. Production procedures should include the cell culture and expansion steps, transfection or infection procedures, harvest steps, hold times, vector purification (e.g., density gradient centrifugation, column purification), concentration or buffer exchange steps, and the reagents/components used during these processes.

Increasing control over the viral vector upstream production process is expected to evolve as the gene therapy viral vector biopharmaceutical moves from early-stage clinical development into late-stage clinical development. There will be the manufacturing challenges due not only to the complexity of the biologic process that is carried out but also to the limited number of batches that might be manufactured prior to seeking market approval [30]:

> *S.2.2. Description of manufacturing process and process controls*
>
> During development, as process knowledge is gained, further details of in-process testing should be provided and acceptance criteria reviewed. As development proceeds, manufacturing consistency needs to be demonstrated. For a marketing authorisation, the manufacturing process needs to be validated.
>
> *S.2.6. Manufacturing process development – Process improvement*
>
> Manufacturing processes and their control strategies are continuously being improved and optimised, especially during early phases of clinical trials and development.
>
> It is recognised that in particular for GTIMPs, only a limited number of batches may be produced prior to MAA. Therefore, it is particularly important to gather sufficiently detailed manufacturing process and batch analytical data throughout the development process as these can be used as supportive information during a licence application.

At the time of submission of a dossier for market approval, the validation/evaluation of the upstream production process for the viral vector biopharmaceutical drug substance is expected to be completed and the CMC documentation summarized in the submission. The CMC information expected is similar to that for protein-based biopharmaceuticals as discussed in Sect. 7.2.2 and follows the outline of Module 3 in the ICH common technical document (CTD). FDA CBER concurs with using ICH Module 3 for gene therapies as stated in comments at a public town hall meeting with gene therapy subject matter experts [31]:

> ***In the interest of improving the quality of the submissions submitted, would OTAT consider publishing mock IND Module 3 sections for those sections where they may see common deficiencies — for example, 3.2.S.2.3: Control of Materials?***
>
> I would like to direct people to the gene therapy CMC guidance. Not to sound like a broken record, but that is set up to be comprehensive for all of the different gene therapy products. It's broken down into the CTD format. And it does, for example, for the control of materials, talk about the types of information that are needed: CQAs, quality information related to safety testing, for instance, of human or animal-derived materials, cell bank qualification — all the things that we would expect to have in the Control Materials section. But when we're thinking about BLA submissions, it's important to have all of your ducks in a row. One thing that we might consider you to think about is to give us a more granular submission of the table of contents for your submitted or expected BLA. Right now, oftentimes, we get the headings – the CTD headings – with no information on what's actually going to be provided in each of those different sections. It's good to know not only that you were going to include control of materials but exactly what different document headings will be included in there for us to be able to give you better feedback as you're preparing for a BLA submission.

7.3.3 Genetic Instability During Production of Viral Vectors

All living systems, including cell lines and viruses, undergo the potential for genetic change. When foreign genes are inserted into cell lines or viruses, the foreign genetic content can undergo change over time – either loss of the genetic content (e.g., loss of gene copy number) or rearrangement of the inserted genetic content – referred to as 'genetic instability'. The importance of confirming genetic stability of recombinant cell lines and the amino acid sequence of the biopharmaceutical proteins produced by them, was discussed in Sect. 7.2.4. It is also importance to confirm the genetic stability of the human cell line used to assemble and propagate the viral vector, and the fidelity of the nucleic acid sequence of the gene of interest (GOI) over the upstream production process time period.

Genetic mutations can take place at multiple locations during the upstream production process for a viral vector. A genetic mutation could take place in the non-recombinant HEK293 cell line, that could impact the ability of the cell line to assemble and/or propagate rAAV. A genetic mutation could take place in the virus gene code that assembles the protein capsid. A genetic mutation could take place in the gene of interest (GOI) code. Therefore, it is important that the fidelity of the upstream production process for viral vector manufacturing be confirmed as remaining unchanged [30]:

> Data on the control and stability of the vector and the therapeutic sequence(s) during development and in production should be provided. The degree of fidelity of the replication systems should be ensured as far as possible and described in order to ensure integrity and homogeneity of the amplified nucleic acids. Evidence should be obtained to demonstrate that the correct sequence has been made and that this has been stably maintained during any amplification so that the therapeutic sequence remains unmodified.
>
> Cells used in amplification of the genetic material should be fully characterised; the history of the cell line, its identification, characteristics and potential viral contaminants should be described. Special attention should be given to the possibility of contamination with other cells, bacteria, viruses or extraneous genetic sequences. Appropriate process validation studies will contribute to demonstration of genetic stability during production.

7.3.4 Meeting CMC Regulatory Compliance for Upstream Production

The following case examples, of market-approved rAAV viral vectors for *in vivo* gene therapy, indicate the complete CMC documentation for the upstream production in the market application dossier that meet regulatory authority compliance expectations:

Luxturna (Voretigene Neparvovec-rzyl) [32]

The voretigene neparvovec-rzyl Drug Substance (DS) is manufactured by Spark Therapeutics, Inc. The manufacturing process is based on cell culture and transient transfection of adherent human embryonic kidney epithelial cells (HEK293) with three plasmid constructs encoding: an expression cassette for normal human RPE65, helper

virus-derived sequences, and AAV2 capsid and rep sequences required for packaging of the RPE65 cassette into recombinant AAV2 particles. To generate the DS in cell culture, HEK293 cells from a qualified Master Cell Bank (MCB) are expanded in roller bottles and transfected with the plasmid DNAs.

Zolgensma (Onasemnogene Abeparvovec) [33]

The AS Upstream manufacturing process consists of five steps, or unit operations: (1) Cell Expansion, (2) Bioreactor Operations, (3) Bioreactor Harvest, (4) Harvest Clarification, and (5) Intermediate. During Upstream manufacturing, one vial of the human embryonic kidney cells (HEK293) working cell bank (WCB) is thawed, and cells are expanded. The expanded cells are harvested and used to inoculate the bioreactor. The cells expanded are transfected with a triple DNA plasmid solution. After cell culture, the cells are harvested and clarified. The clarified harvest is processed to Intermediate and frozen. All open cell/product manipulations in the Upstream manufacturing process occur inside a biosafety cabinet (BSC) within an ISO 7 area. Media, solutions, and buffers that are prepared by AveXis are prepared in an ISO 8 area.

Zolgensma AS is produced by co-transfection of HEK293 cells with three plasmids:

- Vector Plasmid (pSMN)
- AAV Plasmid (pAAV2/9) containing the AAV rep2 and cap9 wild-type genes
- Adenovirus Helper Plasmid (pHELP).

CMC information for market approval of the upstream production of rAAV using the *Baculovirus* insect cell production system can be found in the manufacture of market-approved Roctavian (valoctocogene roxaparvovec) [34].

7.4 Upstream Production of Genetically Modified Patient Cells

The critical step prior to the upstream *ex vivo* transduction of patient cells involves the preparation of the two starting materials – the viral vector (typically recombinant lentivirus (rLV)) and the collected patient cells, as discussed in Chap. 6, Sect. 6.3.3.2. With these starting materials, the upstream manufacturing process follows the following general pathway: (1) activation of select patient cells, (2) transduction using a rLV viral vector, and (3) expansion of transduced patient cells. A schematic of the upstream *ex vivo* transduction of patient cells production process is presented in Fig. 7.6.

7.4.1 Assembling the Transduction Process

The process for genetic modification of patient cells follows the following general pathway: (1) specific cells are selected from the patient cell starting material mix and activated, (2) the selected patient cells are transduced with a recombinant lentivirus vector starting material, and (3) the genetically modified patient cells are expanded.

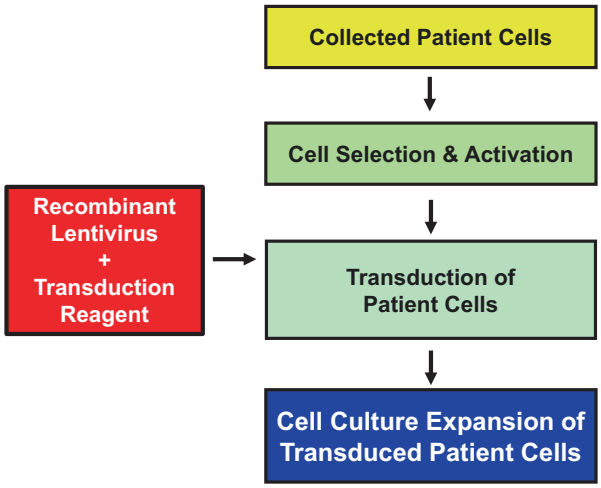

Fig. 7.6 Schematic of the ex vivo transduction of patient cells process

7.4.1.1 Selection and Activation of Collected Patient Cells

One starting material for *ex vivo* gene therapy is the collected patient, as discussed in Chap. 6, Sect. 6.3.3.2. The patient cells could be for autologous use (i.e., collected and returned to the same patient) or for allogeneic use (i.e., collected from a donor and administered to multiple patients). Leukapheresis utilizes a medical device in which a patient's blood is separated into leukocytes (white blood cells) which are collected and red blood cells and platelets which are returned to the patient. The collected leukocytes from the patient are cell mixtures (e.g., B cells, T cells, NK cells, residual red blood cells, etc.). However, for efficient genetic modification, a specific cell type, such as T cells, is desired. One way to obtain a specific cell type from the patient cell mixture is by mixing the patient cells with magnetic beads that are coated with antibodies that bind to specific cell receptors on specific cells. For example, for the selection of T cells, magnetic beads coated with two antibodies (anti-CD3 and anti-CD28) that bind to receptors on T cells are mixed with the patient cells. The magnetic bead-bound T cells are then separated using a magnetic cell separator. Figure 7.7 illustrates the effectiveness of this cell separation for the market-approved Kymriah (tisagenlecleucel) [35]. For T cells, an additional advantage of using the magnetic beads with the two antibodies is that binding to the CD3 and CD28 receptors on the T cells mimics their natural activation by dendritic cells.

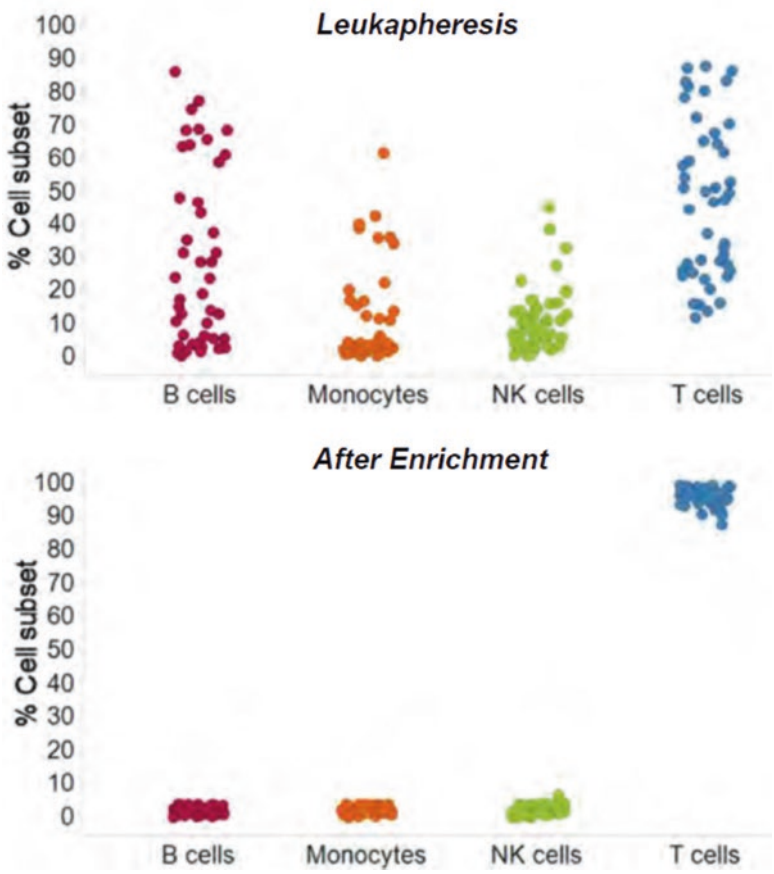

Fig. 7.7 T cell selection from patient cells – before and after enrichment

7.4.1.2 Transduction of Selected Patient Cells with Recombinant Lentivirus Vector

Transduction is a biological reaction that incorporates foreign genetic material into a cell, using a viral vector, in this case recombinant lentivirus (rLV) viral vector, a starting material discussed earlier in Sect. 7.3. The viral vector transduction process is assisted with a transduction reagent, that assists with the entry of the viral vector into the patient cell.

Transduction of patient cells with a viral vector is a complex biological reaction that requires tight control over various critical process parameters (CPPs):

1. Ratio of viral vector to transduction reagent in the transduction reagent mixture
2. Ratio of transduction mixture to patient cells
3. Number of transduction cycles on patient cells
4. Time interval between multiple transductions

5. Target gene copies per patient cells

Transduced patient cells are then expanded for 2–3 days.

There has been some discussion about the target number of gene copies per patient cells. FDA CBER addressed this at a public town hall meeting with gene therapy subject matter experts [31]:

> ***Many companies, websites, and literature indicate the FDA recommends controlling vector copy numbers for lentiviruses to less than five copies for transduced cells. However, a primary reference has never been located. Does the FDA still recommend sponsor limiting VCN to less than five copies for transduced cells? If not, what is the recommendation?***
>
> And so we do recommend that vector copy number is reported as copies per transduced cell. And we recommend that you set your release criteria based on the manufacturing experience that you have at the time. And this is supported for phase 1 INDs with information from preclinical studies, and from developmental lots, we really recommend that you do some process development in order to optimize the transduction efficiency to maximize that in most cases while keeping the vector copy number as low as possible for safety reasons. And then you should propose the release criteria based on that and give justification from that information. In most cases, there's not a lot of information to initiate phase 1 studies. And so we are generally comfortable with five copies per transduced cell. However, that is not a hard rule. And then as you continue to gain manufacturing experience and clinical experience through the clinical study, these release criteria along with all your release criteria can be refined to better reflect product safety and consistency.

7.4.1.3 Resources for a More in-Depth Understanding of the Transduction Process

For a more in-depth understanding of the upstream production process for genetic modification of patient cells, check out a consensus publication, prepared through input from the biopharmaceutical manufacturing industry:

CAR T Cells

A-Cell (2022): A case study-based approach for CAR T cell-based therapy CMC programs

– over 40 individuals volunteered their time [29]

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Also, the various contract manufacturing organizations (CMOs) and processing equipment vendors have considerable information on the upstream production process for genetically modified patient cells (especially CAR T cells), readily available on their websites.

7.4.2 *Applying the Minimum CMC Regulatory Compliance Continuum*

As discussed in Chap. 4, Sect. 4.3.1, the regulatory authorities accept a minimum CMC regulatory compliance continuum risk-based approach for manufacturing of biopharmaceuticals. The transduction of patient cells is required to be performed under current Good Manufacturing Practices (cGMPs. For the upstream production process of transduced patient cells, the following FDA guidance is provided during early-stage (including FIH) clinical development [36]:

CAR T cell manufacturing process control

CAR T cell manufacturing often requires specialized ancillary materials, including selection reagents, activation reagents, antibodies, cytokines, serum, and growth factors. The safety and quality of such materials can vary widely depending on factors such as source or vendors. Lot-to-lot variability and stability of reagents can also be problematic. We recommend sponsors qualify ancillary materials for quality, safety, and potency through vendor qualification programs and incoming material qualification programs, including quarantine, Certificate of Analysis (COA) and Certificate of Origin (COO) assessment, visual inspection, and testing, as appropriate.

To assure product safety, CAR T cells should be free of viable contaminating microorganisms; however, the final DP cannot be terminally sterilized as cells need to be fully viable and functional. Therefore, manufacturing should be conducted by using validated aseptic processing under current good manufacturing practice (CGMP) conditions.

The IND should contain information demonstrating the ability to produce CAR T cells according to the proposed manufacturing process through the production of developmental or engineering batches. To support process development, sponsors may cross reference information from highly-related CAR T cell manufacturing (e.g., same manufacturing process but with a different CAR construct) at the same facility. Generally, starting material from a healthy donor is appropriate for manufacturing process developmental batches. However, patient-derived starting material may have intrinsic properties that affect CAR T cell manufacturing because of disease state, prior treatment, or other inherent patient characteristics. Therefore, in some cases, when using patient-derived starting material, additional manufacturing process development may be recommended for autologous CAR T cells.

We do not require use of approved or cleared medical devices as equipment in CAR T cell manufacturing after collection of the cellular starting material. The suitability of manufacturing equipment (such as centrifugation/washing, selection, or incubation equipment, including automated equipment) should be qualified by assessing the CQAs of the product under the chosen mode of operation and specific equipment settings. This qualification is the responsibility of the IND sponsor, not the medical device or equipment manufacturer. Manufacturing equipment operating parameters should be validated to support the BLA.

Throughout development, CPPs should be identified and used to establish in-process controls. Examples include:

- Using a fixed bead:cell ratio at the activation stage.
- Using a constant amount of vector per cell (e.g., a fixed multiplicity of infection for viral vectors) and a fixed duration at the gene transfer step.
- Using fixed electroporation settings.
- Monitoring cell expansion in culture and maintaining an optimal cell density by addition of media.

Appropriate in-process testing at relevant time points is vital to achieve and maintain control of the manufacturing process. In-process testing regimens for CAR T cells typically assess multiple parameters (e.g., viability, cell number, cell phenotype, CAR expression). Results from in-process tests can be used to guide manufacturing decisions at critical steps, such as when to change culture media or to determine when the CAR T cells are ready to harvest.

Increasing control over the transduction of patient cells production process is expected to evolve as the genetically modified patient cells biopharmaceutical moves from early-stage clinical development into late-stage clinical development:

EMA [37]

4.2. Manufacturing Process

The procedures for any manipulation should be documented in detail and closely monitored according to specified process controls (including process parameters and operating ranges, in-process controls/tests and materials' attributes). Unintended variability, for example in culture conditions, activation steps, transduction /transfection media and conditions or vector concentration/transduction efficiency/ Multiplicity of Infection (MOI) during production may result in quantitative and/or qualitative differences in the quality of the product or the impurities present.

4.2.1. Cell preparation and culture

Additional manufacturing steps on the starting material may follow (e.g. organ/tissue dissociation, enrichment/isolation/selection of the cell population of interest, activation/stimulation) for which a comprehensive description is expected. In addition, full details of all process parameters and in-process tests and corresponding numeric operating range/set point and acceptance criteria/action limits to ensure the desired product critical quality attributes (CQAs) should be provided. Special consideration should be given to the cell characteristics that potentially impinge on the subsequent gene transfer steps.

4.2.2. Genetic modification

The genetic modification of the cells is a manufacturing step that is affected by a variety of inputs and therefore its control is critical. Genetic modification efficiency may depend on different factors such as target cell features (primary cells or cell lines, adherent or in suspension, dividing or quiescent), features of the cell culture (culture system such as flasks or bags, cell seeding density or concentration), type and amount of vector and/or modifying enzyme, transduction/transfection reagent, time of incubation and culture media components.

Genetic modification can be achieved by a number of approaches (see above). Regardless of the system used, all conditions and processing steps should be developed and validated for the intended clinical functions and the associated risks of the genetically modified cells.

A detailed description of any manipulation procedure should be provided. Genetic modification should be carried out using a validated manufacturing process. When using integrating vectors (e.g. LV and RV), multiplicity of infection should be kept at the minimum shown to be effective by transduction efficiency studies and clinical studies. For genome editing protocols, generation of on- and off-target modifications should be addressed as part of process development and characterisation. A risk assessment should be presented to address the potential appearance of off-target modifications during manufacturing.

At the time of submission of a dossier for market approval, the validation/evaluation of the upstream production process for the genetically modified patient cell biopharmaceutical drug substance is expected to be completed and the CMC

documentation summarized in the submission. The CMC information expected in the market application dossier follows the outline of Module 3 in the ICH common technical document (CTD). FDA CBER concurs with using ICH Module 3, see comment in Sect. 7.3.2.

A caution about applying the minimum CMC regulatory compliance continuum to the manufacturing process for genetically modified patient cells. FDA CBER addressed the potentially severe short time limitations for this group of biopharmaceuticals at a public town hall meeting with gene therapy subject matter experts [31]:

> ***What is the "must-have" and "good-to-have" CMC information for a gene therapy product going into phase 3 IND studies or late- phase studies that intend to support a marketing approval?***
>
> In addition to safety, which is our main concern during early-phase studies, late-phase studies should reflect the planned commercial setting so that you can support interpretation of the clinical study data in order to assess the product's efficacy. The gene therapy CMC guidance does provide some insight into these phase-specific expectations. But I'm going to highlight what you might consider some must-haves and good-to-haves here. First and foremost, you must have qualified assays, including a potency assay, in place prior to initiating studies that are intended to provide the primary efficacy data for licensure. As many times, our products don't have what would be called a formal phase 3 study. And so for these late-stage studies, you should also have appropriate CGMPs in place in order to support the product quality and facility control. Our general advice is that you should determine where the variability and risk are possible in your process. And then, through that risk assessment, you should ensure that you have the controls in place in order to reduce the variability of your product. And this is going to be assessed on a product-by-product basis. For instance, for a fresh cell-based gene therapy, shipping validation should be conducted in order to support that the product quality is not affected from the time of release to the time of delivery and administration at the clinical site. For an AAV vector, the product should be formulated using a nominal titer so that you can gain experience with the commercial dosing strategy. And that can be part of the interpretation of the efficacy assessment. Overall, we recommend that you move to the expected commercial configuration prior to conducting this pivotal study. And this will reduce the risk in your developmental process. This includes using the intended commercial manufacturing process at the intended manufacturing facility and using the expected lot release testing strategy. And this will position you to have the maximal data at your disposal to use for your license application. And it's also going to reduce the complications related to comparability assessments that may occur during the BLA review.

The potential for a rapid clinical development pace can occur with these biopharmaceuticals, places a strong emphasis on having a high degree of CMC regulatory compliance control over the manufacturing process sooner than later. Protein-based biopharmaceuticals typically take about 10 years in clinical development to reach market-approval [38]. Table 7.3 illustrates, with three case examples of market-approved genetically modified patient cells, a much faster clinical development pace [39].

Table 7.3 Elapsed time from clinical start (IND) to seeking market approval (BLA)

Ex Vivo Genetically Modified Patient Cells	IND Submitted	BLA Submitted	Elapsed Time
Tecartus (brexucabtagene autoleucel) Kite Pharma	October 2015	December 2019	4 years
Breyanzi (lisocabtagene maraleucel) Juno Therapeutics	June 2015	December 2019	4 years
Abecma (idecabtagene vicleucel) Bristol-Myers Squibb	October 2015	July 2020	5 years

7.4.3 Meeting CMC Regulatory Compliance for Upstream Production

The following case examples, of market-approved *ex vivo* transduced patient cell biopharmaceuticals, indicate the complete CMC documentation for the upstream production in the market application dossier that meet regulatory authority compliance expectations:

Kymriah (Tisagenlecleucel) [35]

Key steps in the manufacturing process

Step 1. Patients undergo leukapheresis to collect their blood mononuclear cells; these are cryopreserved and shipped to the manufacturing facility using a dedicated courier service (and stored at ≤ -120°C). After thawing, cells undergo a procedure to remove cells detrimental to CAR transduction and growth (i.e. monocytes and B-lineage lymphoblasts) and to enrich for T cells

Step 2. Enrichment and activation

At Day 0, T cell enrichment is performed based on the cellular composition of the patient leukapheresis material. Percentage of monocytes and percentage of B lineage cells are measured by flow cytometry. The percentage of monocytes and B lineage cells dictates the choice of pathway for T cell enrichment. The stimulation of T cells is performed using immunomagnetic beads bearing anti-CD3/CD28 monoclonal antibodies, Dynabeads® CD3/CD28 Cell Therapy Systems (CTS)™. The cell-bead suspension then undergoes magnetic separation, retaining the bead-bound CD3+ /CD45+ T cell fraction.

Step 3. Transduction

The bead-bound cells in this positive fraction are advanced to lentiviral vector transduction. Lentiviral vector transduction utilizes a self-inactivating minimal lentiviral vector that encodes the CD19-targeting CAR; transduction is performed twice, over 2 successive days. (Note: vector is produced by a third-party provider.)

Step 4. Expansion

On Day 3, following the second incubation period, the cell culture is washed to remove non-integrated vector and residual vector particles. The washed cells are seeded into a disposable culture system. The culture is continued over a period of several days until the cell number is sufficient to enable harvest.

Abecma (Idecabtagene Vicleucel) [40]

ABECMA is prepared from the patient's peripheral blood mononuclear cells (PBMCs), which are obtained via a standard leukapheresis procedure. The mononuclear cells are enriched for T cells, through activation with anti-CD3 and anti-CD28 antibodies in the presence of IL-2, which are then transduced with the replication-incompetent lentiviral vector containing the anti-BCMA CAR transgene. The transduced T cells are expanded in cell culture, washed, formulated into a suspension, and cryopreserved.

Skysona (Elivaldogene Autotemcel) [41]

SKYSONA (elivaldogene autotemcel) is an autologous HSC-based gene therapy prepared from the patient's HSCs, which are collected via apheresis procedure(s). The autologous cells are enriched for CD34+ cells, then transduced ex vivo with Lenti-D LVV, and cultured with growth factors overnight. Lenti-D LVV is a replication-incompetent, self-inactivating LVV carrying *ABCD1* cDNA that encodes normal ALDP. The *ABCD1* gene is under the control of an internal MNDU3 promoter, which is a modified viral promoter and has been shown to control expression of the transgene in HSCs and their progeny in all lineages. The transduced CD34+ cells are washed, formulated into a suspension, and then cryopreserved.

7.5 Upstream IVT Production of Non-Viral Vector (mRNA)

Messenger RNA (mRNA) encapsulated in lipid is a non-viral vector that can be used for gene delivery. *In vitro* transcription (IVT) is a cell-free, biochemical process that allows for the production of mRNA. IVT requires the following four components:

1. starting material: linearized recombinant DNA plasmid (contains coding for mRNA)
2. starting material: nucleoside triphosphates (four NTPs)
3. critical material: RNA polymerase (a bacteriophage (T7, SP6) enzyme that transcribes DNA into RNA)
4. a reaction buffer that provides appropriate salt and pH balance

As discussed in Chap. 6, Sect. 6.3.3.3, the linearized recombinant DNA plasmid is derived from the recombinant DNA plasmid (containing the specific mRNA code) expressed by a recombinant *E. coli* cell line. On the linearized DNA plasmid template, the coding sequence for the specific protein of interest is flanked by 5′ cap and 3′ poly(A) tail untranslated regions designed to maximize the translational efficiency and cytoplasmic stability of the mRNA. The open reading frame encoding the specific protein of interest is marked by start and stop codons and is flanked by untranslated regions.

7.5.1 Assembling the Non-Viral mRNA Production Process

The upstream production process for the mRNA non-viral vector requires two starting materials: the linearized DNA plasmid and the nucleoside triphosphates (NTPs; ATP, GTP, CTP, and UTP). However, instead of uridine triphosphate (UTP), N1-methyl-pseudouridine triphosphate (N1-methyl-ΨTP) is added to minimize

indiscriminate recognition of exogenous mRNA by pathogen-associated molecular pattern regions. The *in vitro* transcription (IVT) reaction also requires a critical raw material: RNA polymerase.

The genetic information packed into the linearized DNA plasmid determines the mRNA that is produced. The linearized recombinant DNA plasmid template must have the following nucleic acid sequences: (1) RNA promoter sequence, upstream of the mRNA of interest, (2) sequence for the mRNA of interest, and (3) sequence for the poly(A) tail at the 3′ end of the mRNA. The nucleic acid sequence for the 5′ cap structure can be incorporated into the linearized recombinant DNA plasmid template or added to the produced mRNA enzymatically at a later time.

The enzymatic reaction, the *in vitro transcription* of mRNA, is run involving the linearized recombinant DNA plasmid, the added nucleoside triphosphates, and the added RNA polymerase. The enzymatic reaction is stopped by adding a proteinase. The linearized DNA plasmid is digested away with the addition of a deoxyribonuclease (DNase). Adding EDTA quenches the reaction.

The *in vitro* transcription (IVT) cell-free reaction results in a mRNA that contains five regulatory regions as shown in the schematic in Fig. 7.8:

1. 5′ cap structure – the cap region at the 5′ end of the sequence is essential for mRNA maturation; it allows the ribosome to recognize and efficiently translate the sequence and protects the molecule from nuclease digestion
2. 5′ untranslated region (UTR) – impacts translation efficiency, localization and stability
3. Coding region – the open reading frame or coding sequence (CDS) region contains the gene of interest (GOI) to be expressed; codon optimization and modification of nucleotides can contribute to translation efficiency
4. 3′ untranslated region (UTR) – impacts translation efficiency, localization and stability
5. 3′ poly(A) tail – crucial for protein translation and mRNA stability by preventing digestion via 3′ exonuclease; a dependency of translational efficiency and stability on the length of the poly(A) tail

7.5.2 *Meeting CMC Regulatory Compliance for Upstream Production*

At present, there are no market-approved therapeutic products using a mRNA non-viral vector. However, IVT production of mRNA non-viral vector was successful in rapid development of two COVID-19 vaccines (less than 2 years from coding region

Fig. 7.8 Schematic of manufactured mRNA viral vector

identification to market approval). Such potential rapid clinical development for mRNA places a strong emphasis on having a high degree of CMC regulatory compliance control over the manufacturing process prior to entering the initial clinical development stage for therapeutic product development.

7.6 Looking into the Future

The upstream production process for the protein-based biopharmaceuticals (recombinant proteins and monoclonal antibodies) has been developing for over three decades. What was once considered proprietary in this manufacturing process, knowledge held closely by the few companies that were developing these biopharmaceuticals, is now widely discussed and published in the public domain. Biopharmaceutical companies have joined together to publish consensus documents illustrating, in detail, the CMC regulatory compliance concerns for the upstream production of protein-based biopharmaceuticals:

A-mAb (2009): A case study in bioprocess development – company representatives from Abbott, Amgen, Eli Lilly & Company, Genentech, GlaxoSmithKline, MedImmune, and Pfizer were brought together [42]

N-mAb (2022): A case study to support development and adoption of integrated continuous bioprocesses for monoclonal antibodies – 55 individuals from 22 organizations volunteered their time [43]

This clarity and openness among the protein-based biopharmaceutical manufacturers has facilitated a high level of awareness of the CMC regulatory compliance issues that need to be effectively handled in protecting the patients from problems with the upstream production of these products.

For the upstream production processes of the viral vectors (rAAV for *in vivo* use and rLV for *ex vivo* use) and the genetically modified patient cells, the biopharmaceutical industry is still in the early developing stage, where there is a shroud of secrecy over the development and control of these manufacturing upstream production processes. But, attempts are being made to be more open, as for example, the biopharmaceutical companies that have recently joined together to publish consensus documents communicating, at least on a general level, some of the CMC regulatory compliance concerns for the upstream production of these gene therapy-based biopharmaceuticals:

A-Gene (2021): A case study-based approach to integrating QbD principles in gene therapy CMC programs – over 60 individuals volunteered their time [28]

A-Cell (2022): A case study-based approach to integrating QbD principles in CAR T cell-based therapy CMC programs – over 40 individuals volunteered their time [29]

And contract manufacturing organizations (CMOs) that are doing the bulk of viral vector upstream production for the biopharmaceutical industry, as well the vendors

that supply the needed components and reagents, are also leading in communicating non-proprietary information on how these manufacturing processes are performed and what is most important from a CMC regulatory compliance perspective.

One can hope that in the near future, more openness on the CMC regulatory compliance challenges, and the manufacturer's solutions to them, will be discussed for these new gene therapy-based biopharmaceuticals, especially for the upstream production processes, where so many biological and biochemical events take place. Not having a complete picture of what manufacturers are learning about this stage, working in the dark, not knowing if there are any patient safety issue that could be occurring, is unacceptable. After all, our motto is to protect the patient!

It took the industry three decades to be open about the recombinant protein and monoclonal antibody upstream production stage. If projections are correct about the gene therapy-biopharmaceuticals, we won't have three decades to wait for such openness with the viral vectors.

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Chapter 8
Downstream Purification of the Biopharmaceutical Drug Substance

Abstract The upstream production process has been designed to yield the intended biopharmaceutical. But the upstream design carries with it a complex impurity profile that needs to be separated away from the biopharmaceutical – many product-related impurities, process-related impurities, and adventitious agent contaminants, some of which are unique to the type of biopharmaceutical that was expressed. The downstream purification process has to be correspondingly designed to the hand-off from the upstream cell culture bioreactor, and then purify the intended biopharmaceutical from that mixture. It is no mean feat to accomplish this and end up with a regulatory complaint acceptable level for each of the residual impurities. Downstream purification requires adherence to cGMPs and tight control of the process parameters to obtain the desired biopharmaceutical purity. The focus of this chapter is on the downstream purification process for both the protein-based biopharmaceuticals (recombinant proteins and monoclonal antibodies) and the gene therapy-based biopharmaceuticals (viral vectors and genetically modified patient cells, mRNA non-viral vector). Regulatory authorities have high expectations, as well as major requirements for the downstream purification process, which will be examined in this chapter. Application, where appropriate, for the minimum CMC regulatory compliance continuum risk-based approach, will be discussed.

Keywords API · Downstream · Purification · Chromatography · Filtration · Nanofiltration · Concentration · Impurities · Reduced-scale · Residuals · HCPs · Process-related · Clearance · Variants

8.1 At the End of the DS Manufacturing Process

There are three stages involved in the manufacture of a biopharmaceutical drug substance: (1) obtaining/manufacturing the starting materials, (2) carrying out the upstream production of the biopharmaceutical, and (3) completing the downstream purification of the biopharmaceutical, see Fig. 8.1. The downstream purification process removes or minimizes product-related and process-related impurities, and

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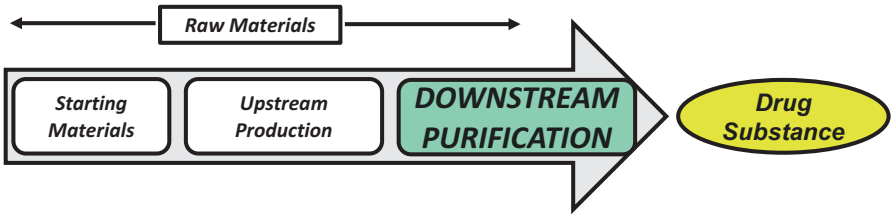

Fig. 8.1 Downstream purification: third stage in the manufacture of the drug substance

adventitious agent contaminants from the upstream biosynthesized biopharmaceutical. The critical importance of this third stage in the manufacture of all biopharmaceutical types (recombinant proteins, monoclonal antibodies, viral vectors, genetically modified patient cells, mRNA vector) will soon become evident in this chapter.

As with the upstream production, the downstream purification process requires adherence to cGMPs and tight control over the process parameters. This tight control of the upstream manufacturing process (referred to as critical process parameters, CPPs) is both necessary and challenging in order to ensure that the various types of impurities (product-related, product-related, and adventitious agents) are either eliminated or minimized in the purified biopharmaceutical drug substance. The focus of this chapter is on the risk-based requirements and expectations for control of the biopharmaceutical downstream purification process, across the clinical development lifecycle and into market approval. Regulatory authorities have high expectations and major requirements for the downstream purification process for both the protein-based biopharmaceuticals (recombinant proteins and monoclonal antibodies) and the gene therapy-based biopharmaceuticals (viral vectors and genetically modified patient cells, mRNA non-viral vector). Application, where appropriate, for the minimum CMC regulatory compliance continuum risk-based approach will be discussed.

Figure 8.2 presents a general overview of the downstream purification processes of four types of biopharmaceutical drug substances. For each downstream purification process, risk-based decisions must be made, for example, (1) the specific biomolecular variants product-related impurities of concern, and (2) the necessary degree of purification to achieve a regulatory compliant safety level for the residual process-related impurities.

Many biopharmaceuticals today, during the last purification process step, typically UF/DF, while being concentrated, also have their buffer solutions exchanged for a buffer solution that contains the drug product excipients; thus, becoming a formulated bulk drug substance. Since formulation and excipients are discussed in the drug product sections of regulatory submissions, the formulation of biopharmaceutical drug substances will be discussed in *Chap. 9* on the manufacture of the drug product. Also discussed in the drug product section, *Chap. 9*, will be the conjugation of the purified drug substances (e.g., PEGylation, ADCs).

Recombinant Protein/ Monoclonal Antibody	Recombinant Adeno-Associated Virus
Harvested rProtein/mAb ↓ Chromatography/Filtration ↓ Concentration/Buffer Exchange ↓ 0.2 Micron Filtration ↓ Bulk Drug Substance	Harvested Recombinant AAV ↓ Chromatography/Filtration ↓ Concentration/Buffer Exchange ↓ 0.2 Micron Sterile Filtration ↓ rAAV Bulk Drug Substance
Genetically Engineered Patient Cells	**mRNA for Gene Therapy**
Expanded Transduced Patient Cells ↓ Cell Washing/Bead Removal ↓ Genetically Modified Patient Cells Bulk Drug Substance	In Vitro Transcribed mRNA ↓ Chromatography/Filtration ↓ Concentration/Buffer Exchange ↓ 0.2 Micron Filtration ↓ mRNA Bulk Drug Substance

Fig. 8.2 Overview of downstream purification processes for four types of biopharmaceuticals

CMC regulatory compliance requirements and recommendations on the downstream purification processes of four types of biopharmaceuticals are discussed in the following sections:

Section 8.2 Downstream purification of recombinant proteins and mAbs
Section 8.3 Downstream purification of viral vectors
Section 8.4 Downstream purification of transduced patient cells
Section 8.5 Downstream purification of non-viral vector (mRNA)

8.2 Downstream Purification of Recombinant Proteins and Monoclonal Antibodies

In general, the downstream purification process for recombinant proteins and monoclonal antibodies involves taking the upstream bioreactor harvested, clarified bulk material, and passing the biopharmaceutical containing solution through a carefully chosen set of chromatographic and filtration steps, the order of which is critically important in order to achieve the desired purification level. At the last step, the purified biopharmaceutical may or may not be mixed with formulation excipients

through buffer exchanges. The aliquoted purified biopharmaceutical material is referred to as the bulk drug substance.

8.2.1 Assembling the rProtein/mAb Downstream Purification Process

The design of the downstream purification process is dependent upon the complex mixture of the upstream production harvested material, and requires choices that need to be made, especially the type and order of arrangement of various chromatography and filtration systems.

8.2.1.1 Harvest Step – The Link Between Production and Purification

The harvest step is the link between the upstream production process and the downstream purification process. The design of the harvesting step from the bioreactor is important to effectively separate the cells and cell debris from the biosynthesized biopharmaceutical product:

1. If expression yields the typical extracellular biopharmaceutical, the solution in the bioreactor is collected for further processing. The primary separation of the produced biopharmaceutical from cells is accomplished by centrifugation or depth filtration (works on the principles of mechanical sieving and adsorption) or tangential flow filtration (TFF; also known as crossflow filtration; works on the principle that continuously recirculated cells pass along membrane surfaces while the liquid filtrate, which contains the product, is collected).
2. If expression yields intracellular dense particles (commonly referred to as 'refractile bodies' or 'inclusion bodies'), which occurs with recombinant proteins produced by *E. coli*, the cells in the bioreactor are first collected, then disrupted by chemical, enzymatic, or physical methods. Following disruption, cellular debris is removed from the protein inclusion bodies by centrifugation or filtration.

8.2.1.2 Chromatographic Systems for Proteins

The downstream purification process for a recombinant protein or monoclonal antibody involves taking a large volume of crude product (i.e., the harvested solution) and converting it into a smaller volume of pure product. Chromatographic systems are well established for purifying recombinant proteins and monoclonal antibodies:

1. ***Size Exclusion Chromatography (SEC)***: SEC separates proteins by molecular size, by taking advantage of column resins having a specified pore diameter.

Proteins and impurities too large in size to enter the resin pores are excluded and move through the column rapidly, eluting at the beginning of the chromatogram, the void peak. Proteins having access to the pores are said to be 'included'. Separation characteristics are determined by the pore size distribution within the pore volume. Proteins at the upper molecular size end are included only in the larger pores, which gives them a shorter path length through the column and earlier elution. Proteins at the lower molecular size end interact with all of the pores, which give them a longer path length through the column and later elution. Compared to the other chromatographic processes, size exclusion chromatography provides lower resolution, lower capacity and is longer cycles.

2. ***Ion Exchange Chromatography*** **(IEC)**: IEC separates proteins by their charged residues. Charged residues on protein surfaces include the side groups of amino acids, the α-amino and α-carboxyl termini of the chains, and the sialic acid residues on glycoproteins. These residues are amphoteric, making the sign and net charge on proteins a function of pH. The pH at which a protein's positive charge balances its negative charge is its isoelectric point (pI). A protein binds to an anion exchanger (resin bearing negative charge) at pH values above its pI. A protein binds to a cation exchanger (resin bearing positive charge) at pH values below its pI. The bound proteins are eluted either by altering the pH of the column buffer, by addition of competing ions, or both. Membrane-based chromatography is a form of IEC. Rather than a column packed with resin beads, polymeric ion exchange membranes contained in cassettes are used. These membranes are single-use and disposable. Ion exchange is regarded as a non-denaturing chromatographic method with high recoveries of protein mass and biological specific activity.
3. ***Reversed-Phase Chromatography*** **(RPC)**: RPC separates proteins by their hydrophobicity (i.e., water repelling). The column resin, containing covalently bonded alkyl hydrocarbons or aromatic ligands, provides the hydrophobic surface to bind the proteins. Upon increasing the concentration of the organic solvent and decreasing the concentration of water in the column buffer, the bound proteins elute according to their hydrophobicity, from lower to higher. RPC is regarded as a denaturing chromatographic method with varying recoveries of protein mass and impact on biological specific activity.
4. ***Hydrophobic Interaction Chromatography*** **(HIC)**: HIC utilizes two fundamental principles. The first principle is that salts are preferentially excluded from both protein surfaces and chromatography resins. Thus, with increasing salt concentration in the column buffer, it becomes favorable for proteins to bind to the resin. The second principle is that hydrophobic interactions are a strong attractive force in salt solutions. Therefore, strongly hydrophobic proteins bind more strongly to the resin than weakly hydrophobic proteins. By removing the salt from the column buffer, the bound proteins elute according to their hydrophobicity, from lower to higher. Sometimes the addition of organic solvents to the column buffer is necessary to elute the bound strongly hydrophobic proteins. HIC is regarded as a denaturing chromatographic method with varying recoveries of protein mass and impact on biological specific activity.

5. ***Affinity Chromatography*** **(AC)**: AC exploits the interaction of bound ligands on a chromatographic resin with proteins in solution. The ligands can be chemical or biochemical. Chemical ligands include chelated metals (e.g., nickel to bind to histidine sites on the protein) and dyes (e.g., Cibacron Blue, also known as blue-2, to bind to strongly basic proteins). Biochemical ligands include Protein A, Protein G, and lectins. The most widely used biochemical ligand is Protein A (a cell wall component of *Staphylococcus aureus* that binds strongly to IgG monoclonal antibodies). The protein solution is passed through the affinity column, the specific proteins bind to the column resin, the column resins are washed, and then the bound proteins are eluted. Affinity chromatography is regarded as a non-denaturing chromatographic method with high recoveries of protein mass and biological specific activity and excellent removal of nonbinding impurities such as DNA and host proteins. Possible leaching of the bound ligands into the protein solution must be addressed.

8.2.1.3 Filtration Systems for Proteins

Filtration (including concentration) methods are also well established for purifying recombinant proteins and monoclonal antibodies:

1. **Normal Flow Filtration (NFF)**: In NFF (also referred to as 'dead end filtration'), the solution flows perpendicularly through a filter membrane with the size of the pores determining which portion of the feed is allowed to pass through and which will remain trapped in the filter membrane. Sterilizing filters (0.2 micron pores), mycoplasma retention filters (0.1 micron pores) and virus reduction nanofilters (0.05 or smaller micron pores) are examples of NFF in which the biopharmaceutical protein flows through the filter and the respective adventitious agent is trapped in the filter membrane.
2. **Tangential Flow Filtration (TFF)**: In TFF (also referred to as 'cross flow filtration'), the feed stream flows tangentially across the filter membrane at positive pressure. As it passes across the membrane, the solutes in the feed stream that are smaller than the membrane's pore size pass through the membrane. As a general rule, the molecular weight cut-off (MWCO) of the membrane should be 1/3rd to 1/6th the molecular weight of the solute molecule to be retained (3-6X Rule) in order to assure complete retention. TFF operates in two modes: ultrafiltration (UF), where fresh solution is not added, is for biopharmaceutical concentration; while diafiltration (DF), where fresh solution is added, is for desalting and exchanging of buffers.

While filtration may seem like a straightforward operational process step, product recovery and efficiency of performance of the intended separations can be challenging with biopharmaceutical solutions. Following the vendors instructions for the filters purchased is always prudent. Other invaluable resources for proper filtration operations are *PDA Technical Report No. 15 (Validation of Tangential Flow Filtration in Biopharmaceutical Applications, 2009)*, and *PDA Technical Report 41 (Virus Retentive Filtration, 2022)*; available for purchase at www.PDA.org.

8.2.1.4 The Downstream Purification Process

Process Development scientists assemble the various available chromatographic and filtration methods into a purification process that meets the needs for the specific biopharmaceutical recombinant protein or monoclonal antibody – (1) the need to remove or minimize the residuals of concern in the harvested material, and (2) the need to purify increasing amounts of harvested recombinant protein/mAb either as the size of the bioreactor increases or the induced protein expression level increases within the bioreactor. For IgG monoclonal antibodies, the downstream purification process is well established, as presented in Fig. 8.3.

8.2.2 Applying the Minimum CMC Regulatory Compliance Continuum

As discussed in *Chap. 4, Sect. 4.3.1*, the regulatory authorities accept a minimum CMC regulatory compliance continuum risk-based approach for biopharmaceutical manufacturing of recombinant proteins and monoclonal antibodies. For the downstream purification process of the protein-based biopharmaceutical drug substance, the following guidance is provided during early-stage (including FIH) clinical development [1]:

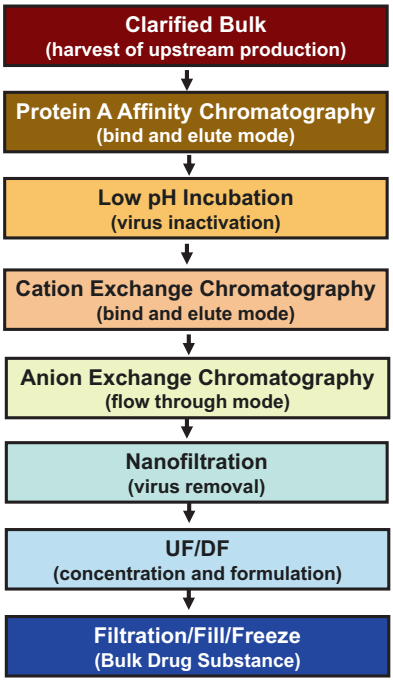

Fig. 8.3 Typical downstream purification process for IgG monoclonal antibodies

S.2.2. Description of manufacturing process and process controls

The manufacturing process and process controls should be adequately described. The manufacturing process typically starts with one or more vials of the cell bank and includes cell culture, harvest(s), purification, modification reactions and filling.

A flow chart of all successive steps including relevant process parameters and in-process-testing should be given. The control strategy should focus on safety relevant in-process controls (IPCs) and acceptance criteria for critical steps (e.g. ranges for process parameters of steps involved in virus removal) should be established for manufacture of phase I/II material. These in-process controls (process parameters and in process testing as defined in ICH Q11) should be provided with action limits or preliminary acceptance criteria. For other IPCs, monitoring might be appropriate and acceptance criteria or action limits do not need to be provided. Since early development control limits are normally based on a limited number of development batches, they are inherently preliminary. During development, as additional process knowledge is gained, further details of IPCs should be provided and acceptance criteria reviewed.

S.2.5. Process validation

Process validation data should be collected throughout development, although they are not required to be submitted in the IMPD. For manufacturing steps intended to remove or inactivate viral contaminants, the relevant information should be provided in the section A2, Adventitious agents safety evaluation.

S.2.6. Manufacturing process development – Process improvement

Manufacturing processes and their control strategies are continuously being improved and optimised, especially during the development phase and early phases of clinical trials. Changes to the manufacturing process and controls should be summarized. This description should allow a clear identification of the process versions used to produce each batch used in non-clinical and clinical studies, in order to establish an appropriate link between pre-change and post-change batches. Comparative flow charts and/or list of process changes may be used to present the process evolution. If process changes are made to steps involved in viral clearance, justification should be provided as to whether a new viral clearance study is required, or whether the previous study is still applicable.

Increasing control over the downstream purification process is expected to evolve as the biopharmaceutical moves from early-stage clinical development into late-stage clinical development. As the stage of clinical development advances, critical process parameters (CPPs) are identified for each step of the downstream purification process of the protein-based biopharmaceutical (typically prior to entering the pivotal clinical trials). This identification is typically accomplished by carrying out reduced-scale studies (see *Sect. 8.2.3*) to explore the process parameters that impact the operational limits of each downstream purification process step. Some typical CPPs identified for the monoclonal antibody purification process [2] shown in Fig. 8.3, are presented in Fig. 8.4.

At the time of submission of a dossier for market approval, the validation/evaluation of the downstream purification process for the protein-based biopharmaceutical is expected to be completed and the CMC documentation summarized in the submission:

ICH M4Q(R1) [3]

3.2.S.2.2 Description of Manufacturing Process and Process Controls

Biotech: Information should be provided on the manufacturing process, which typically starts with a vial(s) of the cell bank, and includes cell culture, harvest(s), purification and modification reactions, filling, storage and shipping conditions.

Purification and Modification Reactions

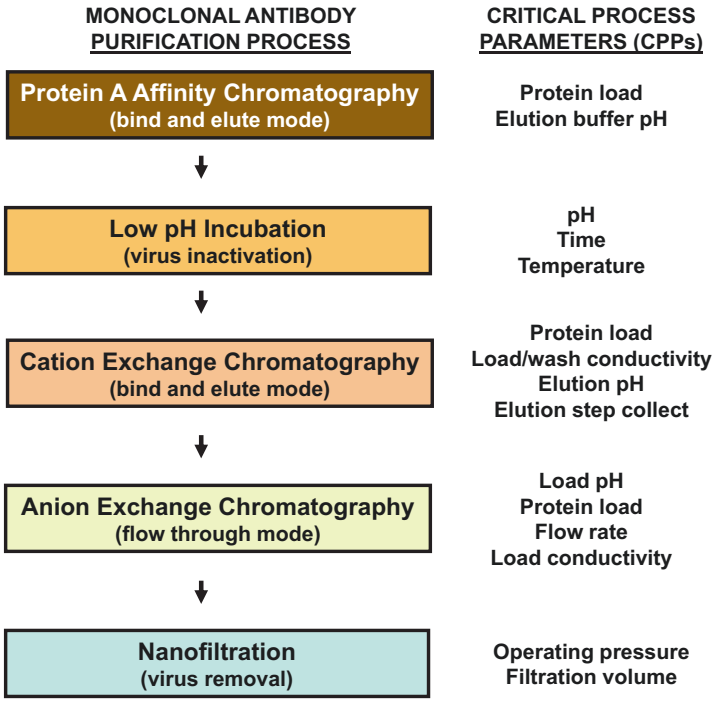

Fig. 8.4 Some typical CPPs for a downstream purification process for a monoclonal antibody

A flow diagram should be provided that illustrates the purification steps (i.e., unit operations) from the crude harvest(s) up to the step preceding filling of the drug substance. All steps and intermediates and relevant information for each stage (e.g., volumes, pH, critical processing time, holding times, temperatures and elution profiles and selection of fraction, storage of intermediate, if applicable) should be included. Critical steps for which specifications are established as mentioned in 3.2.S.2.4 should be identified. A description of each process step (as identified in the flow diagram) should be provided. The description should include information on, for example, scale, buffers and other reagents (details provided in 3.2.S.2.3), major equipment (details provided in 3.2.A.1), and materials. For materials such as membranes and chromatography resins, information for conditions of use and reuse also should be provided. (Equipment details in 3.2.A.1; validation studies for the reuse and regeneration of columns and membranes in 3.2.S.2.5.) The description should include process controls (including in-process tests and operational parameters) with acceptance criteria for process steps, equipment and intermediates. (Details in 3.2.S.2.4.) Reprocessing procedures with criteria for reprocessing of any intermediate or the drug substance should be described. (Details should be given in 3.2.S.2.5.) Information on procedures used to transfer material between steps, equipment, areas, and buildings, as appropriate, and shipping and storage conditions should be provided (details on shipping and storage provided in 3.2.S.2.4.).

3.2.S.2.5 Process Validation and/or Evaluation

Biotech: Sufficient information should be provided on validation and evaluation studies to demonstrate that the manufacturing process (including reprocessing steps) is suitable for its intended purpose and to substantiate selection of critical process controls (operational parameters and in-process tests) and their limits for critical manufacturing steps

(e.g., cell culture, harvesting, purification, and modification). The plan for conducting the study should be described and the results, analysis and conclusions from the executed study(ies) should be provided. The analytical procedures and corresponding validation should be cross-referenced (e.g., 3.2.S.2.4, 3.2.S.4.3) or provided as part of justifying the selection of critical process controls and acceptance criteria. For manufacturing steps intended to remove or inactivate viral contaminants, the information from evaluation studies should be provided in 3.2.A.2.

FDA for BLA [4]

A detailed description of the purification and downstream processing, including a rationale for the chosen methods, and the precautions taken to assure containment and prevention of contamination or cross contamination should be provided. In-process bioburden and endotoxin limits should be specified where appropriate. Any reprocessing using a validated reprocessing method and the conditions for batch eligibility should be described. If applicable, indication should be made as to the multiuse nature of areas and equipment (e.g. campaigning vs. concurrent manufacture; dedicated vs. shared equipment) used for these procedures. A brief description of the controls employed to ensure segregation and prevent cross contamination, or reference to another section containing this information, should be provided.

Validation Studies for the Purification Process. A description and documentation of the validation of the purification process to demonstrate adequate removal of extraneous substances such as chemicals used for purification, column contaminants, endotoxin, antibiotics, residual host proteins, DNA, and viruses, where appropriate, should be provided.

EMA for MAA [5]

The capacity of the proposed purification procedures to deliver the desired product and to remove product and process-related impurities (e.g. unwanted variants, HCPs, nucleic acids, media components, viruses and reagents used in the modification of the protein) to acceptable levels should be thoroughly evaluated. This generally includes establishment of adequate analytical methods required for respective impurity detection and an estimation of the concentrating or removing capacity for each unit operation followed by the determination of appropriate acceptance criteria. For certain process-related impurities (e.g. HCP, DNA, antibiotics) scale-down spiking experiments may be required to determine the removal capacity of the individual purification steps. Evaluation of purification steps for which high impurity clearance are claimed, operating in worst case and/or non-standard conditions (e.g. process hold times, spiking challenge) could be performed to document the robustness of the process. For some components (e.g. low-molecular weight media components), a risk-based approach is acceptable showing that no safety concerns like immunogenicity or toxicity are present. Evaluation of steps where viral clearance is claimed should be performed as described, according to ICH Q5A (R1). Process conditions (e.g. column loading capacity, flow rate, length of column, elution/washing and/or regenerating conditions) and performance parameters/indicators (e.g. yield, chromatographic profiles) should be appropriately evaluated. Columns should also be evaluated throughout the expected lifetime of the column regarding purification ability (e.g. clearance, peak resolution in separation of isoforms), leaching of ligands (e.g. dye, affinity ligand) and/or chromatographic material (e.g. resin). Absence of specific leaching studies may be acceptable for some resins, but requires appropriate justification. Considering the number of purification cycles required for this evaluation, small scale studies are considered appropriate to estimate and set the maximum number of cycles at the time of the regulatory submission, provided that commercial scale verification is performed on an ongoing basis to confirm the column performance and integrity, in accordance with a protocol approved at the time of marketing authorisation application.

Verification of downstream process. Verification activities should confirm the intended performance of the entire downstream process (e.g. regarding purity, impurity clearance, correct refolding and formation of intended modifications) to consistently generate the targeted quality of process intermediates and active substance (i.e. appropriate purity/impurity profile for the given stage). This should be supported by in-process testing results of process parameters and process outputs.

Hold time, storage and transportation. Where process intermediates are held or stored, the impact of the hold times and conditions on the product quality from a structural and microbial point of view should be appropriately evaluated. The evaluation should be conducted as real-time, real-condition studies, usually on commercial scale material.

The ongoing validation/evaluation of the downstream purification process control for the protein-based biopharmaceutical drug substance is subject to GMP compliance inspections from various regulatory authorities. Table 8.1 presents some questions, prepared by PIC/S, that regulatory authority reviewers can consider for such an inspection [6]. Note, the Pharmaceutical Inspection Co-operation Scheme (PIC/S) is a non-binding, informal co-operative arrangement between regulatory authorities in the field of GMP of medicinal products. PIC/S presently comprises 52 participating authorities coming from all over the world (Europe, Africa, America, Asia, and Australia).

During manufacturing facility inspections, regulatory authorities have identified some serious process validation issues, as illustrated by the case example for Andexxa (recombinant coagulation factor Xa). During its pre-license inspection (PLI), the FDA identified serious deficiencies in the process validation, especially

Table 8.1 PIC/S regulatory aid for GMP inspection of the DS downstream purification process

Process	Crucial Questions
Chromatography Systems	Is the size of the column resin volume defined or is it calculated? Are the flow and pressure during packing defined? Preparation, use and dismantling of the system? Specifications for critical parameters? (e.g. linear liquid flow, column bed height, gradient slope, temp) Are product collection criteria strictly defined? Are cleaning procedure and used cleaning agents described What are the storage conditions? (e.g. temp, time, storage solutions)
Column Resins	Are resins tested regarding chemical/biological, physical and functional aspects? Is life time of resins/maximum number of runs defined and what is the basis? Are HETP and asymmetric measurements performed? Are leachables tested? Is consistency of purification profiles a performance criteria? Are resins dedicated to one manufacturing step of one product?
Viral Removal Steps	Are critical process steps performed within their validated parameters? Are pre and post viral removal steps performed in separated areas with separate air handling units? Is the equipment dedicated to pre and post virus removal steps? Do workers pass from pre viral to post viral areas?
Cleaning Validation	How is the equipment cleaned and how is it validated? Are product specific assays performed? Are these assays validated? Is the holding time of dirty and clean equipment defined and covered by cleaning validation studies?

the control of residual impurities through the purification process, which led to a delay in BLA approval [7]:

> We acknowledge that ANDEXAA is a breakthrough therapy developed for an indication that addresses an urgent unmet medical need. As such, FDA is committed to working with Portola to advance your manufacturing program. We have submitted multiple requests for information (IRs), and we have received your responses. We have determined that these responses to our IRs are incomplete. The data you provided in your responses to the Form FDA 483 issued on do not adequately address the deficiencies in the validation of the ANDEXXA manufacturing process that were identified during the Pre-License Inspection (PLI) of the facility. The ANDEXXA process is not validated to assure reasonable control of sources of variability that could affect production output and to assure that the process is capable of consistently delivering a product of well-defined quality. Complete the validation studies for the clearance of all impurities and submit the final study reports to demonstrate identification and control of these impurities. This is needed to assure process consistency and establish a process control strategy which will ensure the quality of the commercially manufactured product. Please note that impurity clearance studies are considered critical to the process qualification stage of process validation (reference is made to the 2011 *FDA Guidance on Process Validation*) and therefore prior to submission to FDA these studies should be reviewed and approved by your quality assurance unit to document the use of sound scientific methodology and principles with adequate data to support the conclusions.

8.2.3 Value/Limitation of Reduced-Scale Purification Studies

Reduced-scale studies are important and necessary for characterizing and validating the downstream purification process steps. Not only is there the advantage in cost-savings (development lab operational costs versus commercial-scale manufacturing operational costs), but also a number of the reduced-scale studies cannot be carried out at commercial-scale. For example, virus clearance evaluation studies examine the virus inactivation and/or clearance capability of the individual purification process steps. Virus is spiked into the protein-based solution at the beginning of a process step and then the virus detected in the protein-based solution at the end of the process step is measured, resulting in a $\log_{10}$ reduction value. If the study were to be performed at commercial-scale, worker safety issues would occur due to the potential exposure of handling large quantities of infectious virus; not to mention the violation of cGMPs in bringing an infectious virus into a GMP manufacturing facility. Therefore, such studies are carried out at reduced-scale, usually in a development laboratory.

A variety of reduced-scale studies need to be carried out, as presented in Table 8.2.

As with any model design, it is most important to recognize their limitations. How the reduced-scale study is designed and executed impacts the ultimate justification of its representation of the commercial-scale process. George Box, a noted British statistician, is often quoted for stating the following: *"Now it would be very remarkable if any system existing in the real world could be exactly represented by*

Table 8.2 Some reduced-scale studies for the downstream purification process of rproteins

Downstream Purification Process for Recombinant Proteins and Monoclonal Antibodies	
Reduced-Scale Study	**Comments**
Virus Clearance Evaluation (inactivation, removal)	**Virus spiking studies across the individual chromatography columns and nanofiltration step; obtaining log_{10} virus reduction factors** ***(Chapter 5, Table 5.5 describes the requirements for clinical development versus market approval)***
Process-Related Impurities	**Impurity spiking studies across the individual chromatography columns, obtaining reduction factors** • **Host cell DNA** • **Host cell protein (HCP)** • **Protein A leachables** • **Media components of concern**
Product-Related Impurities	**Tracking clearance across the individual chromatography columns, obtaining reduction factors** • **Aggregates** • **Molecular variants of concern**
Intermediate Hold Times	**Product stability upon holding** ***(will have to be confirmed at full commercial-like scale, both for protein stability as well as control of endotoxin and bioburden buildup)***
Chromatographic Column Resin Use Life	**Determination of maximum number of re-uses for each chromatography resin in the purification process** ***(will have to be confirmed the end of commercial column resin use cycle)***

any simple model. However, cunningly chosen parsimonious models often do provide remarkably useful approximations." [8]. Therefore, the head-to-head comparability of the reduced-scale study design with the commercial-scale process design is critical.

Regulatory authorities have much to say about these reduced-scale studies, ranging from the need to justify the study, to the importance of Quality Unit oversight for some of these studies, and to the possibility of the need to repeat at commercial-scale for confirmation:

ICH Q11 [9].

Small-scale models can be developed and used to support process development studies. The development of a model should account for scale effects and be representative of the proposed commercial process. A scientifically justified model can enable a prediction of quality, and can be used to support the extrapolation of operating conditions across multiple scales and equipment.

For biotechnological/biological drug substances, the information provided in the dossier in support of process validation usually contains both commercial-scale process validation

studies and small-scale studies. Process validation batches should be representative of the commercial process, taking into account the batch definition as detailed in the process description.

The contribution of data from small-scale studies to the overall validation package will depend upon demonstration that the small-scale model is an appropriate representation of the proposed commercial-scale. Data should be provided demonstrating that the model is scalable and representative of the proposed commercial process. Successful demonstration of the suitability of the small-scale model can enable manufacturers to propose process validation with reduced dependence on testing of commercial-scale batches. Data derived from commercial-scale batches should confirm results obtained from small-scale studies used to generate data in support of process validation. Scientific grounds, or reference to guidelines which do not require or specifically exclude such studies, can be an appropriate justification to conduct certain studies only at small-scale (e.g., viral removal).

Studies should be conducted to demonstrate the ability of the process to remove product-related impurities, process-related impurities (ICH Q6B) and potential contaminants (such as viruses in processes using material from human or animal origin, see ICH Q5A). Studies carried out to demonstrate the lifetime of chromatography columns can include experimental studies carried out in small-scale models but should be confirmed during commercial-scale production.

EMA for MAA [5]

Small scale models are important tools in the development and evaluation of biopharmaceutical manufacturing processes. During process evaluation, small scale models enable evaluation of input material and parameter variability to an extent that may not be feasible at manufacturing scale.

A small scale model must be designed and executed, and ultimately justified, as an appropriate representation of the manufacturing process.

When used, small scale models should be described and their relevance for the commercial scale should be justified, in terms of objective, design, inputs and outputs. When validation studies are highly dependent on the small scale model studies (e.g. design space claimed), it may be necessary to demonstrate that when operating under the same conditions using representative input materials, the outputs resulting from the commercial scale process match those of the small scale model. Any difference in operating conditions, inputs or outputs should be appropriately justified. Depending on the differences observed and their understanding, approaches to managing these differences (e.g. use of correction factors in cases where Design of Experiments is used) could be acceptable if well documented and justified. The use of such an approach requires appropriate management of the risks linked to this uncertainty (e.g. managed through the control strategy).

The contribution of data from small scale studies to the overall validation package will depend upon demonstration that the small scale model is an appropriate representation of the proposed commercial scale. Successful demonstration of the suitability of the small scale model could reduce data requirements for process verification (e.g. reduced number of batches) and/or impact on the control strategy (e.g. alternative approach to end product testing, ongoing process verification) by evaluation and understanding of the sources of variability of CQAs.

FDA for BLA [10]

Although often performed at small-scale laboratories, most viral inactivation and impurity clearance studies cannot be considered early process design experiments. Viral and impurity clearance studies intended to evaluate and estimate product quality at commercial scale should have a level of quality unit oversight that will ensure that the studies follow sound scientific methods and principles and the conclusions are supported by the data.

> The degree and type of documentation required by CGMP vary during the validation lifecycle. Documentation requirements are greatest during Stage 2, process qualification, and Stage 3, continued process verification. Studies during these stages must conform to CGMPs and must be approved by the quality unit in accordance with the regulations (see §§ 211.22 and 211.100). Viral and impurity clearance studies, even when performed at small scale, also require quality unit oversight.

Expect that the regulatory authorities will thoroughly review these reduced-scale studies included in the market application dossiers. If they have serious concerns about their design and comparability to the commercial-scale process, the manufacturer will be required to address the concerns. The following case example illustrates this for a report submitted to the FDA during the BLA review of a recombinant Fc-fusion protein [11]:

> Process characterization studies used to determine the regulatory commitments in the BLA, including the process parameters and in-process controls were inadequate. These studies relied upon the use of small scale models that were not appropriately qualified. For example, the qualifications did not include all CQAs relevant to the unit operations, and the criteria used to evaluate the models were not sufficient. In addition, the process characterization studies themselves were not adequate. For example, all relevant CQAs were not included, and the process parameters studies were, in some cases, too narrow. To address this issue, at the request of the Agency, the sponsor updated sections 3.2.S.2.2, 3.2.S.2.4, 3.2.P.3.3, and 3.2.P.3.4 of the BLA with additional regulatory commitments.

8.2.4 Meeting CMC Regulatory Compliance for Downstream Purification

Case examples were presented in *Chap. 7, Sect. 7.2.4*, for four upstream production processes of market-approved monoclonal antibodies and engineered antibodies. The following case examples indicate the complete CMC documentation in the market application dossier that meet regulatory authority compliance expectations for the downstream purification processes for these same four market-approved biopharmaceuticals:

Single-Chain Fv Antibody Fragment: Beovu (Brolucizumab) [12]

> The protein (IB) is then solubilised and reduced followed by renaturation of the unfolded protein by dilution into a refolding buffer. The refolded protein may be stored at 15–25 °C for up to 48 h. After the refolding step the protein solution is filtered through a depth and bioburden reduction filter before starting the first purification step. The downstream manufacturing process (DSP) includes 3 chromatography steps, 2 ultrafiltration/diafiltration steps and one last ultrafiltration to further concentrate the protein solution. At the end of the manufacturing process, the active substance is pre-formulated with sodium citrate, sucrose and 0.01 g/L polysorbate 80. It is then filtered through a 0.2 μm filter into PETG bottles, stored at ≤−65 °C and shipped to the finished product manufacturing sites.

Monoclonal Antibody: Vyepti (Eptinezumab) [13]

> Cell-free harvested solution: Capture of the monoclonal antibody by protein A purification represents the final isolation step in the manufacturing process of eptinezumab BDS. Further purification of eptinezumab is accomplished by the use of 2 additional,

consecutive chromatography steps. Finally, the resulting solution is ultrafiltered/diafiltered followed by eptinezumab bulk drug substance (BDS) filled into bottles and stored frozen.

Bispecific Antibody: Hemlibra (Emicizumab) [14]

The emicizumab purification process consists of chromatography steps and additional steps for removal and inactivation of potential viral contaminants. The final step in the active substance purification process is concentration of the product and buffer exchange using ultrafiltration and diafiltration (UFDF). Protein concentration and buffer composition are adjusted to the active substance specification by addition of a stock solution containing histidine and arginine buffer and poloxamer 188. The active substance solution is filtered into appropriate storage containers.

Monoclonal Antibody: Qarziba (Dinutuximab Beta) [15]

The harvest of one production bioreactor is ultra-filtered for concentration of the harvest and capture of dinutuximab beta is performed in up to three Protein A chromatography runs, depending on product concentration of the ultrafiltration harvest. After washing, the bound antibody is eluted from the column using a pH-shift and is collected as a single fraction per cycle. Every Protein A capture elution is followed immediately by a low pH virus inactivation step. The virus-inactivated intermediates are pH adjusted then filtered, pooled and further purified in one anion exchange flow-through chromatography cycle (Q Sepharose Fast Flow), to reduce endotoxins, host cell proteins (HCP), DNA and Protein A leachables. The anion exchange chromatography is also defined as a virus removal step. The flow-through fraction is ultrafiltered and diafiltered, followed by a mixed mode flow-through chromatography step (Capto Adhere), to reduce product-related impurities. The last steps are the third ultrafiltration/diafiltration step, performed to adjust the product concentration and to exchange the matrix in the final formulation buffer. This is followed by the virus nanofiltration step for retention and removal of potential viral particles, with filtration through a cascade of a 0.2/0.1 μm filter and finally a virus reduction filter. The final formulation step is to adjust the final active substance excipient composition and protein concentration. A final 0.2 μm filtration is performed before aliquoting the APN311 active substance in single use containers (bags), which results in a single batch of APN311 active substance. The labelled active substance bags are stored at for up to 9 months until further processing,

8.3 Downstream Purification of Viral Vectors

Adeno-associated virus (AAV) is the most widely used viral vector for direct patient injection *in vivo* genetic modification, while lentivirus (LV) is the most widely used viral vector for *ex vivo* genetical modification of collect patient cells. rAAV is a drug substance for *in vivo* use, while rLV is a starting material for *ex vivo* use. The design of the downstream purification process requires not only choices in the arrangement of the chromatography and filtration systems; but also, recognizing handling issues with each viral vector (e.g., no surfactants with rLV).

8.3.1 Assembling the Viral Vector Downstream Purification Process

On the one hand, the downstream purification process viral vectors bears similarity to the downstream process of a monoclonal antibody. For example, both purification processes use chromatographic resins designed to separate out common process-related impurities (e.g., host cell proteins, host cellular DNA, endotoxin). But on the other hand, because a viral vector is much larger and more complex (e.g., vectors contain a protein capsid and a transgene, and are infectious) than a monoclonal antibody, extra care in the handling of a viral vector through the purification process is necessary.

The two viral vectors may seem to have similar properties, but they have different purification process designs. A major distinction between the two types of viral vectors, is the propensity of AAV to contain empty capsids (i.e., absent the transgene), which is not a problem with LV vectors. Removal of empty capsids with rAAV is a major chromatographic challenge. Affinity chromatography removes many impurities but captures both full and empty capsids. A polishing step is needed to separate the full and empty capsids using anion exchange or multimodal chromatography. But, even after optimization of the chromatographic step, the peaks that represent the full and empty capsids typically overlap to some degree, which means some undesired materials remain. Depending on how the full capsid fraction is collected, some viral genomes may be lost.

The downstream purification process for viral vectors involves a variety of chromatographic and filtration steps, the order of which is critically important in order to achieve the desired purification level.

8.3.1.1 Chromatographic and Filtration Systems for Viral Vectors

The large volume of harvested, clarified, nuclease-treated bulk from the upstream production process, needs to be converted into a smaller volume of pure viral vector using chromatographic and filtration process methods.

Affinity chromatography (AC) is a powerful separation tool that relies on the interaction of the viral particle with a ligand (typically a camelid-derived ligand) that has a high affinity for specific viruses, but not for impurities. This bind/elute chromatographic method is commonly applied to AAV vectors, but not LV vectors.

Anion exchange chromatography (AEX) is a simple, versatile, and cost-effective technique that has emerged as a powerful bind/elute polishing step in the purification of both AAV and LV viral vectors. For AAV, anion exchange is also one of the few methods that can separate full from empty AAV capsids due to a difference in the charge of full particles from the presence of the full-length DNA (the transgene).

Tangential flow filtration (TTF), UF/DF, is used in concentration and buffer exchange of viral vector solutions, but also to remove small sized process-related impurities.

8.3.1.2 The Downstream Purification Process

Process Development scientists assemble the various available chromatographic and filtration methods into a purification process that meets the needs for the specific biopharmaceutical viral vector – (1) the need to remove or minimize the residuals of concern from the harvested material, and (2) the need to purify increasing amounts of harvested viral vector either as the size of the bioreactor increases or the viral vector expression level increases within the bioreactor.

For a rAAV vector, produced using a transient triple DNA plasmid transfection process, a typical downstream purification process is presented in Fig. 8.5. (For the rLV vector, the downstream purification process is similar, but no affinity column is used, and possibly a size exclusion chromatographic step would be added to improve purity further).

8.3.1.3 Resources for a More In-Depth Comparison Between AAV and LV Purification

For a more in-depth understanding of the viral vector downstream purification process, and a comparison between the adeno-associated virus (AAV) and lentivirus (LV) processes, check out two consensus publications, prepared through input from the biopharmaceutical manufacturing industry:

Adeno-Associated Virus (AAV) Vector

A-Gene (2021): A case study-based approach for *in vivo* gene therapy CMC programs

- a over 60 individuals volunteered their time [16]
- *Chapter 5: Upstream and Downstream Processing*

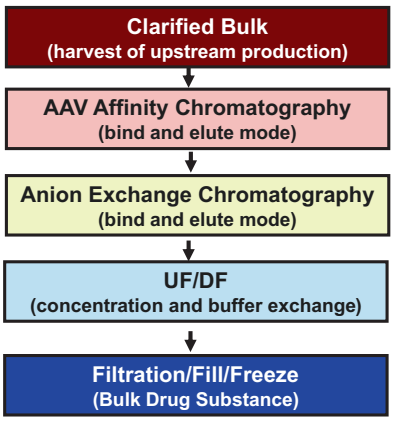

Fig. 8.5 Typical downstream purification process for rAAV viral vector

Lentivirus (LV) Vector

> *A-Cell* (2022): A case study-based approach for CAR T cell-based therapy CMC programs
>
> – over 40 individuals volunteered their time [17]
>
> – *Chapter 7: Lentiviral Vector Manufacturing Process*

Also, the various contract manufacturing organizations (CMOs) and processing equipment vendors have considerable information on the downstream purification process for viral vectors, readily available on their websites.

8.3.2 Applying the Minimum CMC Regulatory Compliance Continuum

As discussed in *Chap. 4, Sect. 4.3.1*, the regulatory authorities accept a minimum CMC regulatory compliance continuum risk-based approach for manufacturing of biopharmaceuticals.

For the downstream purification process of viral vectors, the following guidance is provided during early-stage (including FIH) clinical development:

> ***EMA*** [18]
> *S.2.2. Description of manufacturing process and process controls*
> The manufacturing process of an ATIMP and process controls should be carefully designed and described concisely and step-by-step. The suitability of the controls for the intended purpose needs to be proven. A flow chart of all successive steps of the drug substance manufacturing process should be provided starting from biological fluid/tissue/organ or from cell banks/viral seeds. Critical steps and intermediate products should be indicated as well as relevant process parameters, in-process controls (IPCs) and acceptance criteria. IPC testing (for early phase developments) should focus at the minimum on safety aspects. Critical steps should already be identified for the manufacture of early clinical trial material and adequate acceptance criteria for these critical steps established, for other IPCs, monitoring might be appropriate.
>
> > For GTIMP the following aspects should be considered as applicable:
> > Batch(es) and scale should be defined, including information on any pooling of harvests or intermediates.
> > Any reprocessing during manufacture of the active substance (e.g. filter integrity test failure) should be described and justified.
> > A purification process should be in place to reduce impurities. Impurities include hybrid viruses in the case of virus vector production, host cell-DNA and protein, residual plasmid DNA, lipids and polysaccharides in the case of production systems which involve bacterial fermentations, and RNA and chromosomal DNA in the case of plasmid purification. Ideally steps should be taken over time, in design, construction and production to minimise or eliminate these.
> > The manufacturing process must be set up to minimise the risk of microbiological contamination.
>
> ***FDA*** [19]
> *Description of Manufacturing Process and Process Controls (3.2.S.2.2)*

Your description of the DS manufacturing process and process controls should include the following, as applicable: cell culture; transduction; cell expansion; harvest(s); purification; filling; and storage and shipping conditions. Your description should also accurately represent your process and process controls. We acknowledge that information on process controls may be limited early in development and recommend that sponsors provide additional information and updates as product development proceeds.

i Batch and Scale

A description of how you define each manufacturing run (i.e., batch, lot, other) should be submitted with an explanation of the batch (or lot) numbering system. You should clearly state whether any pooling of harvests or intermediates occurs during manufacturing. If pooling is necessary during production, we recommend that you control the storage conditions (e.g., time, temperature, container closure system) for each pool and that you describe the testing performed prior to pooling to ensure the quality of each pool. We also recommend that you provide an explanation for how the batch scale is defined (e.g., bioreactor volume, cell processing capacity) and how the DS is quantified (e.g., vector genomes, transducing units, infectious particles, mass, number of gene modified cells) to facilitate review and allow a better understanding of the manufacturing process. When known, please include the yield expected per batch.

ii. Manufacturing Process

b. Vector Production

For the manufacture of gene therapy vectors (e.g., virus, bacteria, plasmids), you should provide a description of all production and purification procedures. Production procedures should include the cell culture and expansion steps, transfection or infection procedures, harvest steps, hold times, vector purification (e.g., density gradient centrifugation, column purification), concentration or buffer exchange steps, and the reagents/components used during these processes.

d. Control of Critical Steps and Intermediates

You should define manufacturing intermediates and provide information on the quality and control of intermediates. Intermediates may include material from collection or hold steps, such as temporary storage of bulk harvest, concentration steps, or purification intermediates (e.g., column fractions or eluate). The duration of production steps and hold times should be controlled and recorded to facilitate the establishment of process limits and to allow for future validation of each step and hold time within the proposed limits in support of a license application.

Increasing control over the viral vector downstream purification process is expected to evolve as the viral vector biopharmaceutical moves from early-stage clinical development into late-stage clinical development. There will be manufacturing challenges not only due to the complexity of the biopharmaceutical process that is carried out but also due to the limited number of batches that might be manufactured prior to seeking market approval [18].

S.2. Manufacture

S.2.2. Description of manufacturing process and process controls

During development, as process knowledge is gained, further details of in-process testing should be provided and acceptance criteria reviewed. As development proceeds, manufacturing consistency needs to be demonstrated. For a marketing authorisation, the manufacturing process needs to be validated.

S.2.6. Manufacturing process development – Process improvement
Manufacturing processes and their control strategies are continuously being improved and optimised, especially during early phases of clinical trials and development.
It is recognised that in particular for GTIMPs, only a limited number of batches may be produced prior to MAA. Therefore, it is particularly important to gather sufficiently detailed manufacturing process and batch analytical data throughout the development process as these can be used as supportive information during a license application.

At the time of submission of a dossier for market approval, the validation/evaluation of the downstream purification process for the viral vector biopharmaceutical drug substance is expected to be completed and the CMC documentation summarized in the submission. The CMC information expected is similar to that for protein-based biopharmaceuticals as discussed in *Sect. 8.2.2* and follows the outline of Module 3 in the ICH common technical document (CTD).

8.3.3 Value/Limitation of Reduced-Scale Purification Studies

Reduced-scale studies are important and necessary for characterizing and validating the downstream purification process steps. Not only is there the advantage in cost-savings (development lab operational costs versus commercial-scale manufacturing operational costs), but also a number of the reduced-scale studies cannot be carried out at commercial-scale.

A variety of reduced-scale studies need to be carried out, as presented in Table 8.3. Note the similarity to protein-based biopharmaceuticals for the reduced scale purification studies in Table 8.2.

8.3.3.1 Meeting CMC Regulatory Compliance for Downstream Purification

There is limited specific information on the downstream purification of market-approved rAAV viral vector, but three case examples are included here. The following two case examples are from HEK293-triple DNA plasmid transfection manufacturing process:

Zolgensma (Onasemnogene Abeparvovec) [20]
The AS Downstream manufacturing process includes thawing and pooling of the intermediate, clarification process, chromatographic purification step, filtration and centrifugation steps and filling of the AS. No reprocessing is allowed.
Luxturna (Voretigene Neparvovec) [21]

The downstream purification process separates empty AAV capsids from full AAV capsids, and primarily full particles are administered in the final product, which is formulated in a physiologic buffer containing a surfactant to help prevent loss of vector on product contact surfaces.

Table 8.3 Some reduced-scale studies for the downstream purification process of viral vectors

Downstream Purification Process for Viral Vectors	
Reduced-Scale Study	**Comments**
Virus Clearance Evaluation (inactivation, removal)	**Risk-based** ***(if HEK293-transient plasmid)*** **Virus spiking studies** ***(if insect-baculovirus)***
Process-Related Impurities	**Impurity spiking studies across the individual chromatography columns, obtaining reduction factors** • **Host cell DNA** • **Host cell protein (HCP)** • **Affinity column leachables (if rAAV)** • **Media components of concern**
Product-Related Impurities	**Tracking clearance across the purification process** • **Aggregates** • **Empty capsids (if rAAV)**
Intermediate Hold Times	**Product stability upon holding** ***(will have to be confirmed at full commercial-like scale, both for viral vector stability as well as control of endotoxin and bioburden buildup)***
Chromatographic Column Resin Use Life	**Determination of maximum number of re-uses for each chromatography resin in the purification process** ***(will have to be confirmed the end of commercial column resin use cycle)***

CRUDE CELL HARVEST

↓

Concentration by Tangential Flow Filtration

↓

Microfluidization and Filtration

↓

Cation Exchange Chromatography

↓

CsCl Density Centrifugation

↓

Sub-lot Pooling, Buffer Exchange and Formulation

Note, the cesium chloride density gradient centrifugation is designed to separate full from empty AAV capsids.

The following case example is from the insect cell-*Baculovirus* transduction manufacturing process:

Roctavian (valoctocogene roxaparvovec) [22]

Results of cell culture, transfection and harvest steps comply with targets and expected ranges. At the purification stage, most results fall within expected ranges. Clearance of product- and process-related impurities was studied during process validation lots. The data show acceptable clearance of product-related impurities. Hold times have been validated. The manufacturing process includes efficient steps to clear adventitious viruses according to viral validation studies performed.

8.4 Downstream Purification of Transduced Patient's Cells

Collected patient cells are transduced with the recombinant lentivirus vector. While chromatography and filtration process steps are well established for purification of recombinant proteins, monoclonal antibodies, and even viral vectors, such process steps are not appropriate for any type of cells. Cells are large (micron size) which eliminates the use of chromatographic process methods. Cells are fragile which means extra care needs to be in place when either concentrating cell solutions or changing buffer solutions.

The limited purification process for transduced patient cells is cell washing, and if magnetic beads were employed in the upstream production process, the removal of these beads. Cell washing simultaneously fulfills three main purposes:

- Remove/reduce cellular impurities, additives, and non-target media components below acceptable release levels
- Reduce the overall sample volume to increase the overall cell concentration to meet downstream formulation requirements
- Perform a media exchange to transition the cells from the harvested culture pool to a basal formulation media

The following case examples summarize the downstream purification process for market-approved *ex vivo* transduced patient cell biopharmaceuticals:

Kymriah (Tisagenlecleucel) [23]

The culture is continued over a period of several days until the cell number is sufficient to enable harvest. When the cell count reaches the required minimum number of total viable cells, the cells are separated from the beads using a magnetic separation device, harvested, and washed.

Abecma (Idecabtagene Vicleucel) [24]

The transduced T cells are expanded in cell culture, washed, formulated into a suspension, and cryopreserved.

This severe limitation on purification of cells highlights the important emphasis that is placed on the quality of the raw materials, starting materials and product-contact components that are used in the manufacture of transduced patient cells, as whatever is added to the manufacturing process may end up in the cells to be administered to the patient.

For a more in-depth understanding of the restricted downstream purification process for transduced patient cells, check out the consensus publication, prepared through input from the biopharmaceutical manufacturing industry:

Lentivirus (LV) Vector

> *A-Cell* (2022): A case study-based approach for CAR T cell-based therapy CMC programs
>
> – over 40 individuals volunteered their time [17]
>
> – *Chapter 8: Manufacturing of Cell-Based Therapies*

Also, the various contract manufacturing organizations (CMOs) and processing equipment vendors have some information on the restricted downstream purification process for transduced patient cells, readily available on their websites.

8.5 Downstream Purification of Non-viral mRNA

The cell-free *in vitro* transcribed (IVT) biosynthesis of the non-viral vector messenger RNA (mRNA), in principle, should require a straightforward downstream purification process. On the one hand, there are no cells to separate nor purification of any complex cell culture media raw materials. But on the other hand, there is residual linearized DNA plasmid starting material, as well as residual polymerase and NTPs (nucleoside triphosphates) used in the *in vitro* transcription reaction. In addition, IVT reactions typically contain a substantial proportion of incomplete transcripts and posttranscriptional double-stranded RNA (dsRNA) variants.

8.5.1 Assembling the Non-viral Vector Downstream Purification Process

Non-viral vector mRNA presents challenges to purification. The mRNA molecule is large, and the negatively charged backbone repels itself forcing the molecule to elongate in size; therefore, only chromatographic matrixes with large pore sizes can be used. Furthermore, mRNA is more sensitive to sheer forces than proteins. And there is the ever-present concern of ribonucleases (RNases) in any of the purification solutions or components that can digest the mRNA.

As with the purification of recombinant proteins, monoclonal antibodies, and viral vectors, the design of the downstream purification process for the *in vitro* transcribed mRNA requires choices on the selection of the chromatographic method and the operation of the filtration methods to be used. A typical purification process for mRNA is presented in Fig. 8.6.

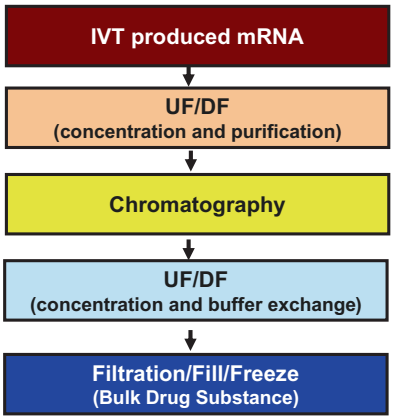

Fig. 8.6 Typical downstream purification process for mRNA non-viral vector

The first step in purification is the tangential flow filtration (TFF, also known as UF/DF) step which is to accomplish two purposes: (1) to concentrate the mRNA solution and exchange buffers for the following chromatographic step, and (2) to remove small-sized impurities from the IVT mixture (e.g., EDTA, NTPs, etc.).

The second step is chromatography. No one chromatographic method has become an industry standard, and the progress being made with various chromatographic media is typically covered under the proprietary umbrella. But, different modes of chromatography are employed for purification of mRNA, including anion exchange and hydrophobic interaction monolithic chromatography (matrices with large channels and low turbulence that will allow the mRNA molecules to easily pass through and avoid shearing), and affinity chromatography. The latter exploits the hybridization affinity between the poly-A tail on the mRNA and a poly-dT chain coupled to a chromatographic stationary phase, which can be destabilized when salt is removed, releasing the mRNA.

The third step is a second UF/DF step for mRNA concentration and to prepare the purified mRNA for formulation, which is then followed by 0.2 micron filtration.

8.5.2 Meeting CMC Regulatory Compliance for Downstream Purification

At present, there are no market-approved therapeutic products using a mRNA non-viral vector. However, purification of IVT-produced mRNA non-viral vector was successful in rapid development of two COVID-19 vaccines (less than 2 years from coding region identification to market approval). Such potential rapid clinical development for mRNA places a strong emphasis on having a high degree of CMC regulatory compliance control over the manufacturing process prior to entering the initial clinical development stage.

Due to a lack of regulatory authority guidance specifically for mRNA for therapeutic use, manufacturers to date have followed the regulatory guidance for vaccine development.

8.6 Can We Speed Up Filling in the Knowledge Gap

After three decades of developing downstream purification processes for over 200 market-approved recombinant proteins and monoclonal antibodies, the scientific toolbox available to development scientists for this type of biopharmaceutical is full. And continuously increasing as new knowledge, new chromatographic modalities, new analytical methods, confirm that these designed purification processes are delivering what they claim to do. Patients are being well protected by the open communication across the protein-based biopharmaceutical industry, leading to robust purification process designs.

For the gene therapy-biopharmaceuticals (especially the viral vectors), much is still needed to learn about these downstream purification processes. The knowledge base is growing, but still under a proprietary umbrella as manufacturers begin to establish their toolboxes. Trust that in the near future, a more open communication and sharing of this downstream purification information will become available, If projections are correct about the gene therapy-biopharmaceuticals, we won't have three decades to build robust purification process designs.

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Chapter 9
Manufacturing the Biopharmaceutical Drug Product

Abstract Manufacture of the finished drug product is the last process step (from starting materials to drug substance and now to drug product) for the biopharmaceutical. Biopharmaceuticals are typically packaged as sterile injectables; therefore, biopharmaceutical drug product manufacturing requires strict adherence to cGMPs, appropriate and adequate aseptic processing, and tight control of the filling process parameters. At times, before the drug product process begins, the purified bulk drug substance is conjugated (e.g., antibody drug-conjugates (ADCs), PEGylation), and these bioconjugation manufacturing process steps are also examined in this chapter. The two main process stages for the drug product are discussed in this chapter: (1) formulation (if excipients are not already present in the bulk drug substance), and (2) aseptic filling and sealing into an appropriate container closure system. Drug product manufacturing for both the protein-based biopharmaceuticals (recombinant proteins and monoclonal antibodies) and the gene therapy-based biopharmaceuticals (viral vectors, genetically modified patient cells) is the focus of this chapter. Comments will also be made on combination products, which involve the use of a device as a container closure. Application, where appropriate, for the minimum CMC regulatory compliance continuum risk-based approach will be discussed.

Keywords Pegylation · Conjugation · ADC · Formulation · Compounding · Container · Closure · Processing · Aseptic · Compatability · Extractables · Leachables · Sterile · Filtration · Filling

9.1 The Drug Product Manufacturing Process

The process stages from the bulk drug substance to the final drug product varies across the different types of biopharmaceuticals. For the protein-based biopharmaceuticals (recombinant proteins and monoclonal antibodies) and the viral vectors, many times the drug substance is formulated during the last purification step (UF/DF allows exchange of buffer solution including introduction of excipients) prior to being held as the bulk drug substance. At times, for the protein-based

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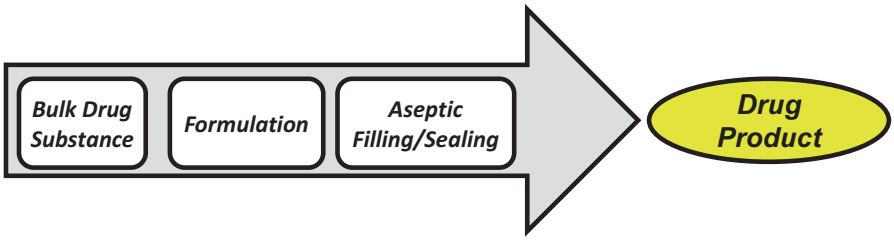


Fig. 9.1 Manufacture of the biopharmaceutical drug product

biopharmaceuticals, the purified drug substance is conjugated with either PEGylation or a chemical drug (i.e., ADC), and then formulated prior to being held as the bulk drug substance. For the genetically modified patient cells, the process is continuous from the drug substance to the drug product stage; but arbitrarily, after the final washing of the cells, prior to formulation, is considered the drug product stage.

According to the regulatory submission templates for a drug product (i.e., ICH Common Technical Document), there are two main stages between the bulk drug substance and the final sterile drug product – formulation and the aseptic filling/sealing step, see Fig. 9.1.

Biopharmaceuticals are packaged as sterile injectables; therefore, biopharmaceutical drug product manufacturing requires strict adherence to cGMPs, appropriate and adequate aseptic processing, and tight control of the filling process parameters. The CMC regulatory compliance focus in this chapter covers both the clinical development lifecycle and into market approval. Application, where appropriate, for the minimum CMC regulatory compliance continuum risk-based approach will be discussed.

In this chapter, conjugation of the purified drug substance prior to formulation is also examined. And even though the bulk drug substance may already be final formulated with excipients, formulation is discussed in this chapter. Due to the current absence of any therapeutic market-approved non-viral vector (such as mRNA), limited specific comments on that drug product manufacture will be made, but this biopharmaceutical type should be closely matched to the protein-based biopharmaceutical drug product manufacturing concerns.

And finally, in this chapter, comments will also be made on combination products, which involve the use of a device as a container closure.

9.2 Conjugation of the Purified Protein Drug Substance

To enhance a specific clinical benefit, a recombinant protein or a monoclonal antibody purified drug substance, prior to formulation, as shown in Fig. 9.2, may undergo conjugation: (1) antibody-chemical drug (ADC) conjugation to deliver cytotoxicity to specific sites in the patient's body, or (2) PEGylation to increase *in*

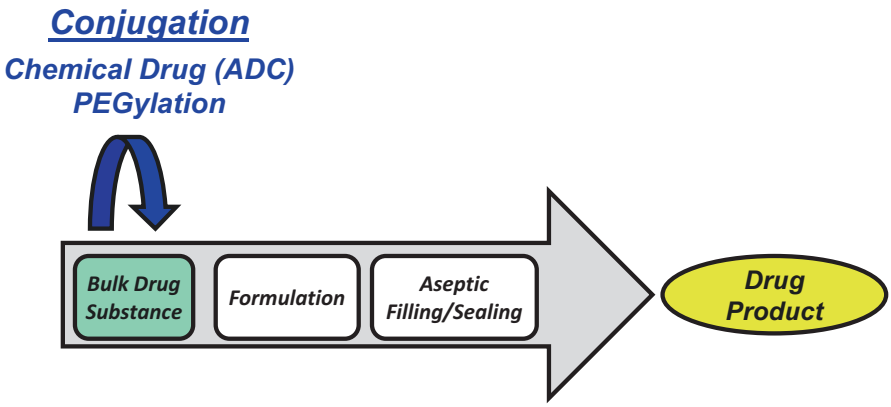


Fig. 9.2 Conjugation of recombinant proteins and monoclonal antibodies

vivo duration of bioavailability. 'Conjugation' is the chemical linking together of two molecules. Upon conjugation, the biopharmaceutical purified drug substance is referred to as 'starting material' or 'intermediate'. Upon subsequent purification, the conjugated biopharmaceutical is the drug substance, which then can be processed forward as the drug product.

9.2.1 Antibody-Drug Conjugates (ADCs)

Antibody-drug conjugates (ADCs) are complex, multi-component biopharmaceuticals. ADCs aim to take advantage of the specificity of the monoclonal antibody (mAb) to deliver a potent cytotoxic chemical drug selectively to antigen-expressing tumor cells (referred to as target-directed cancer therapy). ADCs require the assembly of three components:

- Targeting agent: the monoclonal antibody biopharmaceutical
- Warhead: the cytotoxic chemical drug
- Linker: the chemical component used to conjugate the mAb targeting agent and the chemical drug warhead together by a covalent bond; sometimes including a 'spacer arm'

In the manufacture of the ADC drug substance, there are two intermediates that are reacted together. One intermediate is the purified monoclonal antibody drug substance. Although an 'intermediate', regulatory authorities expect that the same CMC regulatory compliance documentation will be provided to them as if the monoclonal antibody was a drug substance. The other intermediate is the drug-linker (the linker chemically combined to the chemical drug warhead). During the manufacture of the ADC drug substance, the drug-linker is chemically attached to specific amino acids on the monoclonal antibody, through either thiols (e.g.,

cysteine) or amines (e.g., lysine). After the chemical reaction, the ADC is purified yielding the ADC bulk drug substance.

Cysteine-linked ADCs are common due to their controllable drug-to-antibody ratio (DAR). These ADCs are manufactured by partial reduction of the 4 pairs of interchain disulfide bonds on the mAb, which are not critical for structural stability, while keeping the 12 intrachain disulfide bonds intact. That reduction can generate 0 to 4 pairs of free thiols, which can be conjugated with the drug-linker. The free thiols react with a maleimide (i.e., an unsaturated imide) structure on the drug-linker to form a stable covalent bond. Depending on the degree of reduction of the interchain disulfide bonds on the mAb, DAR values of 0, 2, 4, 6, and 8 can be obtained.

Lysine-linked ADCs provides non-specific conjugation. The IgG mAb contains over 80 lysine amino acids (this amino acid has a free amine group), most of which are exposed on the surface of the mAb. Among these surface lysines, more than 20 can serve as potential ADC conjugation sites. The free amino group on the lysine amino acids react with an activated ester compound (e.g., N-hydroxysuccinimide (NHS)) structure on the drug linker to form a stable covalent bond. The chemical reaction results in a heterogeneous mixture of ADCs with both different number and conjugation sites of drug payloads per mAb.

Ongoing development and modification of the drug-linker (e.g., modifying the warhead to increase or decrease its toxicity level; modifying the linker to be either cleavable or non-cleavable by hydrolysis) continues. Fine tuning of the drug-antibody ratio (DAR) is necessary to obtain the appropriate narrow therapeutic range, but not at a cost of losing mAb tumor cell-binding capability.

Manufacture of the ADC requires detailed attention to the CMC regulatory compliance concerns associated with (1) the monoclonal antibody manufacturing process, (2) the chemical drug and the chemical linker manufacturing processes, (3) the chemical reaction of assembling the drug-linker, and (4) the chemical reaction of attaching the drug-linker to the mAb. Some of these CMC concerns are summarized in Table 9.1.

There are over ten market-approved ADC biopharmaceuticals, and many more under clinical development. The following case example illustrates the CMC regulatory compliance concerns that were addressed for a market-approved ADC biopharmaceutical:

Trodelvy (Sacituzumab Govitecan), Cysteine-Linked ADC [1]
Sacituzumab govitecan is composed of the following 2 intermediates:

1. The humanised monoclonal antibody, hRS7 IgG1κ, that binds to Trop-2, a transmembrane calcium signal transducer that is overexpressed in many epithelial cancers, including triple-negative breast cancer (TNBC) – [see Table 9.2]
2. CL2A-SN38, a drug linker comprised of SN-38, a camptothecin-derived agent (topoisomerase I inhibitor) and CL2A, a hydrolysable linker – [see Fig. 9.3]

Manufacture of sacituzumab govitecan drug substance – [see Fig. 9.4].

Table 9.1 Some CMC regulatory compliance concerns associated with the manufacture of ADCs

CMC Concerns for the Manufacture of the Monoclonal Antibody Intermediate			
Process Stage	***CMC Manufacturing/Quality Concerns***		
Starting Material	**Recombinant Master Cell Bank (MCB)** (see *Chapter 6)*		
Purified Drug Substance	**Cell culture production of mAb** (see *Chapter 7)* **Purification of mAb drug substance** (see *Chapter 8)*		
CMC Concerns for the Manufacture of the Drug-Linker Intermediate			
Process Stage	***CMC Manufacturing/Quality Concerns***	***Chemical Linker***	***Chemical Toxin***
Starting Materials	**Starting material consistency** **Control of the chemical manufacturing process** **Chemical-related residual impurities** (Organic Solvents, Elements, Mutagenic)	✓ ✓ ✓	✓ ✓ ✓
Linker-Toxin Intermediate	**Control of the chemical reaction of Drug-Linker** **Chemical drug related residual impurities** (free toxin)	✓ ✓	
CMC Concerns for the Manufacture of the ADC Drug Substance			
Process Stage	***CMC Manufacturing/Quality Concerns***		
Chemical Reaction	**Control of the chemical reaction of mAb + Drug-Linker** (DAR – drug-to-antibody ratio)		
Purified ADC Drug Substance	**Control of the purification of the synthesized ADC** (removal of unbound drug and drug-linker)		

Table 9.2 Monoclonal antibody intermediate for manufacture of Trodelvy ADC

The monoclonal antibody intermediate is manufactured in accordance with EU GMP.

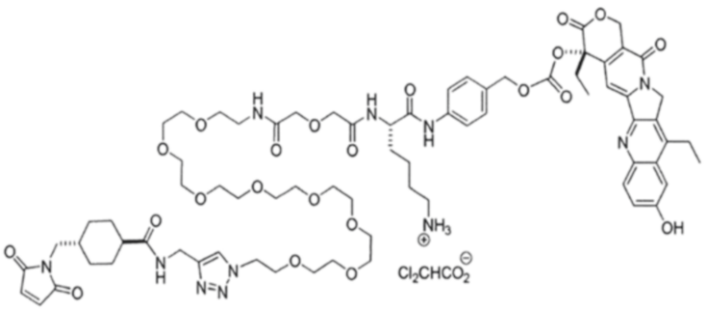
hRS7 IgG1κ antibody intermediate (hRS7 IgG1κ) is manufactured from a Sp2/0-AG14 cell line using a fed-batch bioreactor process, consisting of thaw and inoculum expansion, cell culture expansion and production in a bioreactor, followed by harvest. The intact IgG is purified from the cell culture broth by a series of column chromatography and filtration steps.

Process validation

A lifecycle approach has been used for validation of the commercial manufacturing process of hRS7 IgG1κ; Process Design, Process Verification (also referred to as PPQ) and ongoing process verification. These batches were manufactured under a pre-approved protocol and acceptance criteria to show that the manufacturing process can consistently produce product meeting quality criteria. The performance parameter results obtained during process validation demonstrate that the cell culture and purification processes are under control and can be considered successfully validated. Deviations to the PPQ protocol are described in sufficient detail and were determined not to adversely affect the product or the process, and therefore not to impact the PPQ. Commercial scale process equipment cleaning validation was successfully executed with no process deviations.

The commercial process for the manufacture of hRS7 IgG1κis considered validated. The applicant follows a comprehensive control strategy linking the control provided by each unit operation with control provided by raw materials, procedural elements, environmental factors, process parameters, in-process and release testing and stability monitoring.

CL2A-SN38 drug-linker intermediate



Control of critical steps and intermediates

The review of process parameters and operating ranges concluded that appropriate process monitoring, and process control mechanisms have been designed into the manufacturing process to ensure production of CL2A-SN38 of acceptable quality. In addition to the quality control of the starting materials and key reagents, these mechanisms include the incorporation of IPC tests and processing instructions that define actions to take based upon observations during batch production.

Process validation

The CL2A-SN38 manufacturing process does not involve aseptic processing or sterilisation so no process validation information is required for this intermediate. Only shipment conditions have been adequately validated. The results have been presented.

Fig. 9.3 Drug-linker intermediate for manufacture of Trodelvy ADC

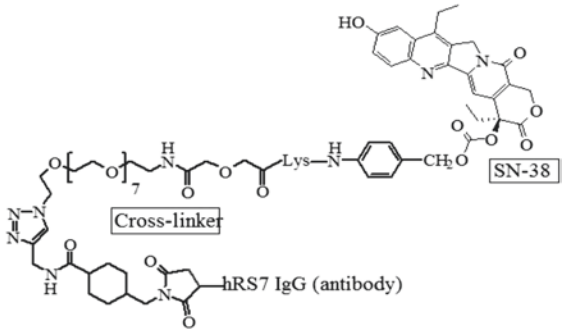
9.2.2 PEGylation

PEGylation is the covalent binding of one or more polyethylene glycol molecules to another molecule. PEGylation of recombinant proteins and monoclonal antibodies typically enhance their stability and/or diminish their immunogenicity when administered to patients. Polyethylene glycols (PEGs) are inert, nontoxic, and typically contain one terminal hydroxyl group (e.g., mPEG has a structure of $CH_3O–(CH_2CH_2O)_n–CH_2CH_2–OH$) that can be chemically activated. The free hydroxyl group on PEG is activated by chemical replacement with a more reactive functional group (e.g., succinimidyl, maleimidyl, aldehyde, sulfhydryl). Depending upon the activated functional group present, the PEG covalently attaches to the respective amines, thiols, or oxidized carbohydrates present on the recombinant protein or monoclonal antibody. The PEGylation reaction, depending upon the conditions, can place one or more PEG molecules onto the biopharmaceutical.

In the manufacture of the PEGylated drug substance, there are two intermediates that are reacted together. One intermediate is the purified recombinant protein or monoclonal antibody drug substance. Although an ‘intermediate’, regulatory authorities expect that the same CMC regulatory compliance documentation will be provided to them as if the recombinant protein or monoclonal antibody was a drug

Sacituzumab govitecan results from the conjugation via thioether bonds of the following intermediates:
- Sacituzumab, a humanised monoclonal antibody (hRS7 IgG1κ);
- CL2A-SN38, a drug linker comprised of SN-38, a camptothecin-derived agent (topoisomerase I inhibitor) and CL2A, a hydrolysable linker.

The ADC has an average molar drug to antibody ratio (DAR) of approximately 7 to 8 drug molecules per antibody, and a molecular weight of approximately 160 kDa.



Process validation

The sacituzumab govitecan active substance process validation strategy was designed to demonstrate that the commercial process is capable of consistently delivering active substance with the required quality. The process validation strategy included consecutive PPQ batches of active substance, manufactured according to the commercial process. The process performance parameter results obtained during process qualification demonstrate that the active substance manufacturing process consistently meets criteria for process performance and product quality specifications for active substance.

Fig. 9.4 Manufacture of Trodelvy ADC bulk drug substance

substance. The other intermediate is the activated PEGylation agent (e.g., PEG that has been oxidized to yield an aldehyde functional group).

During the manufacture of the PEGylated drug substance, the activated PEG molecule is chemically attached to specific amino acids on the biopharmaceutical (e.g., lysine). Amino group-linked PEGylation is common because of the number of accessible primary amino groups on the surface of recombinant proteins and biopharmaceuticals. The N-terminal amino acid, with the alpha NH_2, is a typical target. Because PEGylation is a chemical reaction, a manufacturer must exercise careful control not only over the quality of the PEG raw material (e.g., range of molecular weight species, impurities), but also over the reaction conditions to control the site(s) and extent of PEGylation. In design, the attached PEG molecules should be distant from the binding site of the biopharmaceutical. After the chemical reaction, the PEGylated biopharmaceutical is purified (typically by ion exchange chromatography), separating out the free PEG and, if need be, selecting for the desired degree of PEGylation; finally yielding the PEGylated bulk drug substance.

PEGylation requires detailed attention to the CMC regulatory compliance concerns associated with (1) the recombinant protein or monoclonal antibody manufacturing process, (2) the activated PEG molecule manufacturing process, and (3) the PEGylation reaction.

There are over 20 market-approved PEGylated biopharmaceuticals, including numerous biosimilars. The following two case examples illustrate the CMC regulatory compliance concerns that were addressed for a market-approved PEGylated biopharmaceutical:

Nyvepria (Pegfilgrastim) [2]

Nyvepria (PF-06881894) (pegfilgrastim) is a covalent conjugate of recombinant methionyl human granulocyte-colony stimulating factor (G-CSF) (referred to as filgrastim) and a 20 kDa monomethoxypolyethylene glycol propionaldehyde (mPEG-p).

Filgrastim is expressed in *Escherichia coli* (*E. coli*) as a 175 amino acid protein with a theoretical average mass of 18,799 Da.

Pegfilgrastim is synthesised by Schiff-base reduction of a 20 kDa mPEG-p with the N-terminal amine of filgrastim at Met1. The mPEG-p used for pegylation is a heterogeneous mixture with varying numbers (approximately 412 to 536) of ethylene oxide units.

The filgrastim intermediate (FI) is thawed and pegylated, where 20 kDa mPEG-p is covalently bound to FI. Powdered mPEG-p critical intermediate is used to prepare mPEG-p solution which is used on the same day the pegylation reaction is performed. The pegylated FI is further purified by CEX chromatography prior to concentration and buffer exchange by UF/DF. The protein concentration is adjusted to a target concentration. Polysorbate 20 is then added and the formulated bulk solution is processed by a final (0.2 μm) filtration to produce the AS which is stored in PETG bottles at the proposed storage condition until finished product (FP) manufacture. Reprocessing conditions for specific steps have been defined. The process has been sufficiently described and in-process controls are adequately set to control the process.

Besremi (Ropegomterferpm Alfa-2b) [3]

The process is divided into upstream process, downstream process-1 (proline-interferon alfa-2b AS intermediate purification), synthesis of the 40 kDa PEG intermediate and downstream process-2 (ropeginterferon alfa-2b purification).

Interferon alfa-2 is a non-glycosylated polypeptide chain of 165 amino acid residues. Different types of alfa-2 interferon exist, varying in the amino acid residue at position 23. The selected one for this product is interferon alpha-2b, with arginine at position 23. Proline-interferon alfa-2b contains an identical amino acid sequence to interferon alpha-2b plus an extra proline at the N-terminus (166 amino acid residues).

Ropeginterferon alfa-2b is a PEGylated proline-interferon alfa-2b synthesized by conjugating a single 40 kDa PEG molecule to the N-terminal proline residue.

The process starts with fermentation in *E. coli*. The product is expressed as an intracellular protein in the form of inclusion bodies. The product is extracted by lysing the cells followed by washing with buffers, and then solubilizing the inclusion bodies that contain the product. Downstream processing steps include protein refolding and chromatography and ultrafiltration/diafiltration purification steps to produce one proline-interferon alfa-2b intermediate batch. This intermediate is stored in a specified Ph.Eur. compliant container. The route of synthesis as well as the process parameters and in-process controls of the 40 kDa PEG intermediate are suitably described in the dossier. The specification of the 40 kDa PEG intermediate was justified and completed with the requested parameters during the procedure. The fate and purge of impurities generated throughout the production of the intermediate were also satisfactorily addressed. An appropriate QP declaration has been provided confirming appropriate audit of the sites and that the manufacture of the intermediate complies with the principles and guidelines of good manufacturing practice. The shelf-life for the PEG intermediate under the specified storage condition is agreed. Proline-interferon alfa-2b is then PEGylated by attaching a 40 kDa two-arm branched PEG intermediate to the N-terminal proline. It is then further purified via chromatography and UF/DF steps to produce the formulated active substance.

9.3 Formulation

Formulation – the addition of excipients to the purified bulk drug substance – is prior to the aseptic filling and sealing in the manufacture of the drug product, see Fig. 9.5.

As mentioned previously, for the protein-based biopharmaceuticals (recombinant proteins and monoclonal antibodies) and the viral vectors, many times the drug substance is formulated during the last purification step (UF/DF allows exchange of buffer solution including introduction of excipients) prior to being held as the bulk drug substance. The formulated material is referred to as the 'formulated bulk drug substance' if further compounding needs to take place prior to filling. 'Compounding' could involve thawing and pooling of either the frozen bulk drug substance or the frozen formulated bulk drug substance, or compounding could involve adjusting excipient levels in the formulated bulk drug substance. But, the formulated material is referred to as the 'formulated bulk drug product' if no further compounding needs to take place prior to filling.

The formulation scientist has a major challenge in designing the optimum formulation for the different biopharmaceutical types. The most critical requirement for the formulation is that it maintains the functional activity of the biopharmaceutical during the drug product manufacturing process and over its shelf life. Another important requirement for the formulation is that it should minimize physicochemical change to the biomolecular structure (e.g., oxidation of methionine oxidation on proteins, aggregation of viral vector capsids, etc.).

Each excipient added into the formulation carries a patient safety risk since it will be administered along with the biopharmaceutical:

- Pharmacopeia excipients are considered lowest risk (due to specific quality testing required in the monographs)
- Animal-derived excipients introduce the potential risks of contaminating adventitious agents (see Chap. 5)
- 'Novel excipients' are either (1) an ingredient that is used for the first time in a drug product in a specific regulatory region, or (2) a substance that is used for the first time in the intended route of patient administration; novel excipients are considered the highest risk because of the unknown safety risk to patients

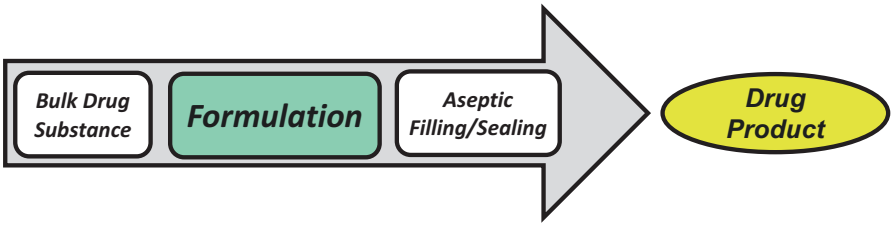


Fig. 9.5 Formulation stage in the drug product manufacturing process pathway

The higher the perceived risk of the excipient, the more detailed CMC information in the submissions required by the regulatory authorities for their safety review. In addition, novel excipients frequently require extended animal toxicology studies. Inhalation administration of Afrezza, a market-approved inhaled formulation of recombinant human insulin illustrates this. To effectively penetrate into the lung, the particle size of the drug product needed to be between 0.5–5.8 microns. Larger particle size impacts in the back of the throat and fails to get into the lungs; smaller particle size gets exhaled before it can get into the lungs. A novel excipient, beads of fumaryl diketopiperazine (FDKP), was required to be in the formulation to achieve this required particle size. Since FDKP is novel, the excipient required a two-year non-clinical toxicological safety assessment study (rat carcinogenicity test) [4].

Because of the diversity of formulations across the different types of biopharmaceuticals, each of the following types will be examined:

Section 9.3.1: Formulation of recombinant proteins and monoclonal antibodies
Section 9.3.2: Formulation of viral vectors
Section 9.3.3: Formulation of genetically modified patient cells
Section 9.3.4: Formulation of non-viral vectors (mRNA)

9.3.1 Formulation of Recombinant Proteins & Monoclonal Antibodies

For recombinant proteins and monoclonal antibodies, Fig. 9.5 illustrates the manufacturing pathway from the bulk drug substance to the drug product, with formulation between the two stages. But, today, frequently, for recombinant proteins and monoclonal antibodies, Fig. 9.6 is the preferred pathway. The last downstream purification process step for the drug substance is tangential flow filtration (TFF, or UF/DF), which can introduce formulation excipients into the purified drug substance during the process; thus, yielding the formulated bulk drug product.

Regardless of whether the formulation approach of Fig. 9.5 or Fig. 9.6 is used, recombinant proteins and monoclonal antibodies need to be formulated. Protein-based biopharmaceuticals have been around for decades, and their formulation science is well established. Recombinant proteins and monoclonal antibodies are either lyophilized or liquid solution preparations, typically administered by injection. Typical formulation excipients reflect this: pH buffer, ionic strength/osmolality salts, surfactants, etc.

With the advent of biosimilars, the degree of flexibility in formulations has become more visible. Biosimilar manufacturers, while seeking to manufacture a formulated drug product highly similar to that of the innovator manufacturer, at times, based on their own formulation studies, slightly change the formulation from that of the innovator's biopharmaceutical, whose formulation most likely was developed sometimes 20 years earlier. The case example for recombinant adalimumab

Table 9.3 Formulation of Humira (adalimumab) and its biosimilars

Formulations of Adalimumab, HUMIRA and Its Many Biosimilars			
HUMIRA	***ABRILADA***	***IDACIO***	***HULIO***
Adalimumab *Sodium chloride* *Sodium phosphate* *Sodium citrate* *Mannitol* *Polysorbate 80* *pH 5.2*	*Adalimumab* *EDTA* *L-Histidine* *L-Methionine* *Sucrose* *Polysorbate 80* *pH 5.5*	*Adalimumab* *Sodium chloride* *Glacial acetic acid* *Trehalose* *Polysorbate 80* *pH 5.2*	*Adalimumab* *L-Methionine* *Na Glutamate* *Sorbitol* *Polysorbate 80* *pH 5.2*

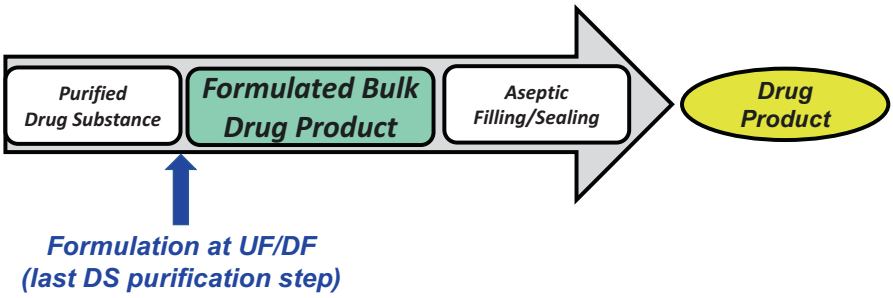


Fig. 9.6 Formulated bulk drug product in the drug product manufacturing process pathway

monoclonal antibody (Humira), and its many market-approved biosimilars [5], illustrates this (see Table 9.3).

A case example from another biosimilar of Humira, in this case Imraldi (which has a formulation consisting of sodium citrate, L-histidine, sorbitol and polysorbate 20), illustrates the type of formulation studies carried out to determine the final formulation for the protein-based biopharmaceuticals [6]:

> In the developmental stage, formulation development studies were performed to confirm the effects of pH, buffer, excipient, and protein concentration on the stability of Imraldi finished product. The formulation development studies and the results were presented. From the results of the developmental studies above, the following conclusions were drawn for optimised Imraldi formulation. Finished product formulation robustness study was done to assess the formulation robustness of Imraldi finished product with variation of protein concentration, pH, L-histidine concentration and sorbitol concentration. Additionally, optimal formulation composition range was identified through this study. Results of the developmental robustness study showed that the Imraldi finished product formulation is robust within range of protein concentration, pH, and L-histidine concentration. The overall results of the formulation robustness study indicate that the formulation may be sufficiently robust at the proposed storage conditions, and that the protein concentration and pH are important factors to ensure acceptable quality of the finished product throughout the shelf-life.

A number of manufacturers are designing their recombinant protein and monoclonal antibody formulations for ease-of-self-administration, i.e., subcutaneous

Table 9.4 Formulations of rituximab

Formulations of Rituximab	
Rituxan/MabThera IV Administration	***Rituxan Hycela/MabThera SC SC Administration***
Rituximab (10 mg/mL)	***Rituximab (120 mg/mL)***
Sodium chloride	***Hyaluronidase human***
Sodium citrate	***L-Histidine***
Polysorbate 80	***L-Methionine***
pH 6.2 - 6.8	***Trehalose***
Osmolality 324-396 mOsmol/kg	***Polysorbate 80***
	pH 5.2 - 5.8
	Osmolality 300-400 mOsmol/kg

syringe injection. For subcutaneous injection, a delivered volume less than 1.5 mL is desired, which means that frequently high protein concentrations (greater than 100 mg/mL) are required. The challenge is that protein viscosity generally increases exponentially with concentration due to molecule-to-molecule interactions. Increasing solution viscosity creates challenges for the filling process and for the syringe delivery administration. Therefore, formulation development strives to reduce the viscosity. A case example of a market-approved monoclonal antibody requiring a different formulation when switching from intravenous injection (IV) to subcutaneous injection (SC) is illustrated by the formulations for rituximab [7], see Table 9.4. Note, the addition of hyaluronidase human, a recombinant protein, into the formulation is to increase permeability of the subcutaneous tissue by temporarily depolymerizing hyaluronan.

The minimum CMC regulatory compliance continuum risk-based approach can be applied to formulation development of recombinant proteins and monoclonal antibodies. For entering into clinical studies (FIH), it is expected that in the IND/IMPD submission that a short description of formulation development, including justification of any new pharmaceutical form or excipient, is provided [8]:

> *Pharmaceutical Development*
>
> For early development there may be only limited information to include in this section. A short description of formulation development, including justification of any new pharmaceutical form or excipient, should be provided. It should be documented that the combination of intended formulation and packaging material does not impair correct dosing, ensuring for example that the product is not adsorbed to the wall of the container or infusion system. This is particularly relevant for low dose and highly diluted presentations.
>
> *Manufacturing Process Development*
>
> Changes in the manufacturing process including changes in formulation and dosage form compared to previous clinical trials should be described. An appropriate comparability exercise should support significant changes, e.g. formulation changes. In this regard, expectations are similar to those described in S.2.6. This data should be sufficiently detailed to allow an appropriate understanding of the changes and assessment of possible consequences to the safety of the patient.

Any changes in the formulation during the clinical phases should be documented and justified with respect to their impact on quality, safety, clinical properties, dosing and stability of the medicinal product.

Formulation development typically continues through clinical development. When seeking market approval, it is expected that in the BLA/MAA submission that a detailed justification is included for both the excipients present and the levels used in the final formulation [9, 10]:

Formulation Development

A summary should be provided describing the development of the formulation, including identification of those attributes that are critical to the quality of the drug product, taking into consideration intended usage and route of administration. Information from formal experimental designs can be useful in identifying critical or interacting variables that might be important to ensure the quality of the drug product. The summary should highlight the evolution of the formulation design from initial concept up to the final design. This summary should also take into consideration the choice of drug product components (e.g., the properties of the drug substance, excipients, container closure system, any relevant dosing device), the manufacturing process, and, if appropriate, knowledge gained from the development of similar drug product(s). Any excipient ranges included in the batch formula (3.2.P.3.2) should be justified in this section of the application; this justification can often be based on the experience gained during development or manufacture. A summary of formulations used in clinical safety and efficacy and in any relevant bioavailability or bioequivalence studies should be provided. Any changes between the proposed commercial formulation and those formulations used in pivotal clinical batches and primary stability batches should be clearly described and the rationale for the changes provided.

3.2.P.1 Description and Composition of the Drug Product

A description of the drug product and its composition should be provided. The information provided should include, for example: Description of the dosage form: Composition, i.e., list of all components of the dosage form, and their amount on a per-unit basis (including overages, if any) the function of the components, and a reference to their quality standards (e.g., compendial monographs or manufacturer's specifications).

9.3.2 Formulation of Gene Therapy Viral Vectors

Although gene therapy viral vectors (e.g., rAAV and rLV) are nucleic acid-base rather than amino acid-based, the challenges and controls for their formulation are comparable to those of the protein-based biopharmaceuticals:

- The formulation must maintain functional activity of the vector and minimize physicochemical change to the molecular structure over the shelf life
- Risk-based considerations for the choice of excipients to use (e.g., compendial, animal-derived, novel)
- The last downstream purification process step, the ultrafiltration/diafiltration (UF/DF) step, typically prepares the purified bulk drug substance with the required excipients for the formulation, as shown in Fig. 9.6
- Typical formulation excipients are pH buffer, ionic strength/osmolality salts, and surfactants, etc.

Table 9.5 Formulations of commercial rAAV vectors for *in vivo* administration

Formulations of Viral Vectors (In Vivo rAAV)	
ROCTAVIAN ***(valoctocogene roxaparvovec)***	***ADSTILADRIN*** ***(nadofaragene firadenovec-vncg)***
Mannitol ***Poloxamer 188*** ***Sodium chloride*** ***Sodium phosphate***	***Sucrose*** ***Polysorbate 80*** ***Sodium phosphate*** ***Sodium citrate*** ***Tromethamine*** ***Magnesium chloride*** ***Glycerol*** ***Hydroxypropyl-beta-cyclodextrin*** ***Syn3 [N-(3-cholamidopropyl)-N-(3-lactobionamidopropyl)]-cholamide***

From the market-approved rAAV vector biopharmaceuticals, one can obtain an insight into the typical excipients used in these formulations [11, 12], see Table 9.5.

In market-approved Adstiladrin, one of the formulation excipients is Syn 3, which is added as a transduction enhancing agent for the rAAV vector. Syn3 ([N-(3-cholamidopropyl)-N-(3-lactobionamidopropyl)]-cholamide) is a novel excipient. Syn 3 underwent safety pharmacology, pharmacokinetics, toxicology and genotoxicity studies to be approved in the formulation [13].

The minimum CMC regulatory compliance continuum risk-based approach can be applied to the formulation development of recombinant rAAV vectors. For entering into clinical studies (FIH), it is expected that in the IND/IMPD submission that a short description of formulation development, including justification of any new pharmaceutical form or excipient, is provided [14]:

> *Pharmaceutical Development*
>
> For early development there may be only limited information to include in this section. It should be documented that the combination of intended formulation and packaging material does not impair correct dosing, ensuring for example that the product is not adsorbed to the wall of the container or infusion system. This is particularly relevant for low dose and highly diluted presentations.
>
> *Manufacturing Process Development*
>
> Changes in the manufacturing process including changes in formulation and dosage form compared to previous clinical trials should be described. An appropriate comparability exercise should support significant changes, e.g. formulation changes. In this regard, expectations are similar to those described in S.2.6. This data should be sufficiently detailed to allow an appropriate understanding of the changes and assessment of possible consequences to the safety of the patient.

However, as with the protein-based biopharmaceuticals, when seeking market approval, it is expected that in the BLA/MAA submission that a detailed justification is included for both the excipients present and the levels used in the formulation (see Sect. 9.3.1, referencing ICH).

9.3.3 *Formulation of Genetically Modified Patient Cells*

Genetically modified patient cells are typically manufactured in a continuous process from cell harvesting through formulation with suitable cryopreservation excipients, to filling into the final container; frequently employing the same manufacturing facility area and staff. Often the distinction between drug substance and drug product is not always easy to separate, but most manufacturers report in their CMC submissions, that the drug product manufacturing process for their cell-based biopharmaceutical begins with formulation step and continues through the filling operation.

Formulation involves the mixing of the expanded transduced patient cells, immediately after the cells are harvested, with a cryoprotectant, other excipients as needed, and a diluent to arrive at the desired concentration of cells. Formulation is a temperature-dependent and time-sensitive step since the harvested cells during this step are held in suboptimal environmental conditions and without nutrients. Appropriate formulation is needed to stabilize the cells so they can withstand stress factors such as temperature excursions, pH changes, and mechanical stress caused by handling, storage, shipment, and bedside preparation. The cryoprotectant (i.e., dimethyl sulfoxide, DMSO) addition rate and the temperature at which this occurs must be carefully determined and rigorously controlled, since DMSO can lyse cells. Human serum albumin (HSA) is a common excipient because it not only is the most ubiquitous protein in blood, but also is known to create an optimal microenvironment for sustained cell viability.

From the market-approved genetically modified patient cells biopharmaceuticals, one can obtain an insight into the typical excipients used in these formulations [15], see Table 9.6.

The FDA recommends that careful consideration be given to CAR T cell formulations due to the logistics involved between a fresh patient infusion and a cryopreservation [16]:

> CAR T cells may be formulated for fresh infusion or cryopreserved for later administration. The choice of formulation depends on the product development strategy and practical constraints. Fresh CAR T cells have a limited shelf life before product quality degrades. We recommend that the maximum time between formulation and infusion be defined and supported by stability studies. Additionally, the timeframe in which release tests can be performed is limited. Therefore, it is crucial to develop and implement well-designed logistics, which may include: timing for sampling and testing for lot release; reporting Quality Control (QC) testing results and Quality Assurance (QA) review for lot release; scheduling product shipping; and receiving and handling of the fresh product at the clinical site. On the other hand, cryopreservation allows sufficient time for full release testing and flexibility in scheduling patients for infusion. We generally recommend cryopreservation when CAR T cells are manufactured at a central location and shipped to clinical sites for administration. For cryopreserved CAR T cells, the risks associated with infusion of the cryoprotectant should be assessed, and controlled thawing of the product at the clinical site may be critical to maintain product quality. Regardless of the formulation, there should be appropriate procedures to ensure adequate control of the CAR T cells during shipping to the clinical

Table 9.6 Formulations of commercial transduced patient cells for administration

Formulations of Genetically Modified Patient Cells	
BREYANZI ***(lisocabtagene maraleucel)***	***ABECMA*** ***(idecabtagene vicleucel)***
75% (v/v) Cryostor CS10 ***24% (v/v) Multiple Electrolytes for Injection, Type 1*** ***1% (v/v) albumin (human)*** ***(final DMSO concentration 5%)*** ***(final HSA concentration of 0.25%)***	***50% Plasma-Lyte A*** ***50% CryoStor® CS10*** ***(final DMSO concentration 5%)***

site. These procedures should be described in the IND, in place before initiating clinical studies, and validated prior to licensure

9.3.4 Formulation of mRNA Non-viral Vector

For mRNA, Fig. 9.5 illustrates the manufacturing pathway from the bulk drug substance to the drug product, with formulation between the two stages. Formulation (encapsulation) is critical for the unstable mRNA and to move the negatively charged and hydrophilic mRNA across cell membranes.

The formulation of mRNA with lipids typically consists of three process steps. Step 1 involves rapid mixing of the mRNA (in acidic solution) and the lipids (in ethanol) to spontaneously create encapsulated mRNA. Since physical properties of the LNPs such as size and morphology are tied to their biodistribution and function, control over both the chemistry and the mixing environment in which self-assembly occurs is vital to ensuring the uniformity and quality of the particles. Development scientists design and optimize the right combination and proportions of lipid species, buffers, and organic solvents, to reproducibly manufacture the desired uniform population of LNP particles. (Take note that Step 1 requires the use of organic solvents; worker and flammability safety standards are required in the handling of these organic solvents). Step 2 involves tangential flow filtration (TFF) to remove residual solvent (ethanol) by diafiltration, and to concentrate the mRNA-LNPs formulated bulk product by ultrafiltration in the final buffer formulation. Since mRNA-LNP is shear sensitive, the selection of hollow fiber or flat sheet cassettes, the molecular weight cut off threshold need to be evaluated together with the processing conditions of each operational step. Step 3 is sterile filtration.

No mRNA non-viral vector for either *in vivo* gene therapy or *ex vivo* gene therapy has yet reached market-approved stage. However, mRNA-LNP has been used in two market-approved COVID-19 vaccines, from where an idea of the probable type of formulation components used can be obtained [17, 18], see Table 9.7.

Table 9.7 Formulations of market-approved COVID-19 mRNA vaccines

FORMULATION COMPONENT	mRNA-LNP COVID-19 Vaccines	
	SPIKEVAX	COMIRNATY
Cationic Lipid (drives lipid to electrostatically interact with the phosphate backbone of the mRNA)	heptadecan-9-yl 8-((2-hydroxyethyl) (6-oxo-6-(undecyloxy) hexyl) amino)octanoate	((4-hydroxybutyl)azanediyl) bis(hexane-6,1-diyl)bis(2-hexyldecanoate)
Phospholipid (found in cell membranes)	1,2-distearoyl-sn-glycero-3-phosphocholine	1,2-distearoyl-sn-glycero-3-phosphocholine
Cholesterol (found in cell membranes)	cholesterol	cholesterol
Synthetic PEGylated Lipid (imparts steric stabilization to LNP particle)	1,2-dimyristoyl-rac-glycero-3-methylpolyoxyethylene	2-[(polyethylene glycol)-2000]-N,N-ditetradecylacetamide
Sugar (promotes product stability during freeze-thaw)	sucrose	sucrose
Other	tromethamol HCl sodium acetate	sodium/potassium chloride sodium/potassium phosphate

9.3.5 Formulation Changes – Tread Carefully

Changes in formulation occur throughout clinical development, although it is recommended that such changes should occur in early clinical stages rather than in later clinical stages (where potential impact on gathering efficacy data might occur). Changes in formulation also occur successfully in the commercial stage.

But not all biopharmaceutical formulation changes are successful. Despite all of the product comparability studies that a manufacturer can carry out on a new formulation, sometimes subtle differences are missed. The case example of Bayer Healthcare Pharmaceuticals with a minor formulation change to their commercial biopharmaceutical product Leukine (recombinant GM-CSF) illustrates the risk. To their established commercial liquid Leukine formulation in 2006, a 'dash' of EDTA (a chelator known to trap metal ions enhancing protein stability) was added to the formulation. In January 2008, Bayer voluntarily withdrew the product after post-marketing safety reports indicated an upward trend in adverse events, in particular, that of transient syncope (i.e., patient fainting after injection). In some patients, the addition of EDTA appeared to increase the absorption rate of GM-CSF, and may have resulted in a temporary increase in plasma concentration of GM-CSF shortly after administration. The unanticipated boost to the pharmacodynamic properties of Leukine likely contributed to the transient adverse events observed. By May 2008 (5 months off market), Bayer returned to the market with the original non-EDTA liquid Leukine formulation. Bayer's market department came up with the most interesting slogan: *'Back to the Future: Original Liquid Leukine Coming Soon'* [19].

Because of patient safety concerns such as above, and the fact that it can sometimes take years to confirm that a new low percentage adverse event profile in patients is developing, regulatory authorities view biopharmaceutical formulation changes as potentially 'high risk'. FDA states that *'a change in the composition or formulation (e.g., pH, ionic strength, molarity) of solutions (e.g., buffers, reagents) used in processing'* requires a prior approval supplement [20]; WHO states that *'addition of a dosage form or change in the formulation (e.g. lyophilized powder to liquid, change in the amount of excipient, new diluent for lyophilized product)'* is a major change requiring a prior approval supplement; [21]; the European Commission states that any minor adjustment of the quantitative composition of the finished product with respect to excipients if the *change relates to a biological/immunological product* is a Type II variation requiring a prior approval supplement [22].

9.4 The Container Closure System

A container closure system refers to the sum of packaging components that together contain and protect the dosage form. This includes primary packaging components and secondary packaging components, if the latter are intended to provide additional protection to the drug product. For biopharmaceuticals, the desired container closure system is one that (1) is compatible with the formulated biopharmaceutical, and (2) maintains the functional activity, quality and safety of the biopharmaceutical.

Since biopharmaceutical drug products are primarily administered parenterally (i.e., by injection), the most common container closures for these products are either glass/plastic vials with rubber closures, prefilled glass syringes, or plastic patient infusion bags (typically for genetically modified patient cells). Table 9.8 presents some typical container closures used for market-approved biopharmaceuticals.

Table 9.8 Some container closures used for market-approved biopharmaceutical drug products

Container Closure Systems for Market-Approved Biopharmaceutical Drug Products	
BIOPHARMACEUTICAL	***CONTAINER CLOSURE SYSTEM***
Recombinant Protein Monoclonal Antibody	***type I glass vial with a chlorobutyl rubber stopper*** ***single-use prefilled syringe (PFS) comprised of a glass syringe barrel with a staked needle and a rigid needle shield, a plunger stopper, a plunger rod***
Viral Vector (rAAV)	***cyclic olefin polymer vial with a chlorobutyl rubber stopper***
Genetically Modified Patient Cells	***ethylene vinyl acetate (EVA) cryostorage bags designed for storage of blood***

Regardless of the container closure type, the regulatory authorities have two main patient safety concerns: (1) the ability of the container closure to ensure the sterility of the contained biopharmaceutical solution or lyophilized solid through its shelf life, and (2) potential interactions between the drug product and the container closure.

9.4.1 Close Encounters Not Wanted: Product – Container-Closure Interactions

Interactions between the product and the container closure system can go both ways: (1) the product-contact surfaces of the container closure system can negatively impact the biopharmaceutical, or (2) the biopharmaceutical and/or its formulation components can negatively impact the container closure system.

A case example of product-contact surfaces of the container closure system negatively impacting a biopharmaceutical is the effect of residual tungsten ions in prefilled syringes. A prefilled syringe is composed of several product-contacting materials: glass barrel, rubber plunger and a stainless-steel needle. During manufacture of a prefilled syringe, a tungsten pin is used to shape and maintain the hole in the glass barrel (which is heated to around 1200 °C) where the stainless-steel needle will eventually be glued in. It has been discovered that during the pin removal, residual tungsten ions can remain in the glass barrel. Residual tungsten ions causes rapid protein aggregation [23]. Residual tungsten ions are not a problem with glass vials.

A case example of the biopharmaceutical and/or its formulation components negatively impacting the container closure system is the delamination of the inner glass walls of a glass vial. Applying the newly developed technique of micro-flow imaging (MFI), Amgen discovered extremely thin glass flakes in its Epogen/Procrit (recombinant human erythropoietin) product present in a glass vial/rubber stopper container closure system. This glass delamination could have potentially serious adverse events such as embolic, thrombotic and other vascular safety concerns upon injection into a patient [24]. Amgen in 2010 initiated a massive product recall of glass vial batches of Epogen/Procrit [25]. Fortunately, Amgen had invested heavily in its prefilled syringe platform for the container closure of Epogen/Procrit before the event. Delamination is not a problem with prefilled syringes.

Because of patient safety concerns such as above, regulatory authorities view biopharmaceutical formulation changes as potentially 'high risk'. FDA states that *'a change in the Container Closure System (CCS) used for storage and/or shipping of a stored intermediate that includes a change in the product-contact material, or dimensions (size and shape)'* requires a prior approval supplement [20]; WHO states that *'addition of a new presentation (for example, addition of a new pre-filled syringe where the approved presentation is a vial for a biotherapeutic in a liquid dosage form)'* is a major change requiring a prior approval supplement [21]; the European Commission states that '*any change in immediate packaging of the finished product if the change relates to a biological/immunological product*' is a Type II variation requiring a prior approval supplement [22].

Changes in container closure systems frequently occur during clinical development, although it is recommended that such changes should occur in early clinical stages rather than in later clinical stages (where potential impact on gathering efficacy data might occur). The challenge is to achieve the desired container closure system within the timeframe of the clinical development program prior to market approval. Manufacturers may start the clinical program using a simple glass vial/rubber closure system, but then realize that a more suitable container closure system (e.g., prefilled syringe) may be necessary for commercial applications. Surprises can happen when changing out the container closure system for a biopharmaceutical. The case example of Palynziq (pegvaliase-pqpz) illustrates this. During clinical development, a glass vial/rubber (VS) closure system was changed out to a prefilled syringe (PFS) closure system, but with the PFSs an unexpected higher pharmacokinetic (PK) product exposure to the patient resulted. This became a major focus of discussion at the pre-BLA submission meeting with the FDA [26]:

> *Question 4:*
> Does the Agency agree that the proposed drug product presentation (pre-filled syringe) is acceptable for licensure?
> *FDA Response:*
> No, we cannot agree at this time that the proposed to-be-marketed presentation (pre-filled syringe, PFS) is acceptable for licensure. A determination about the acceptability of the PFS presentation will be made after the review of the analytical and pharmacokinetic (PK) comparability between the vial-and-syringe (VS) presentation and the PFS presentation, as well as efficacy and safety data supporting the use of PFS drug product provided in your upcoming BLA submission. We note that you switched from the VS presentation to the PFS presentation during the pegvaliase clinical development program. The VS presentation was used in the phase 1 and phase 2 studies, whereas VS and PFS presentations were both used in your phase 3 studies. You have concluded that pegvaliase PK was not comparable between the VS presentation and the PFS presentation based on results from your study 165-302 Part 3, which showed that PK exposure was 64% - 68% higher in the PFS presentation than in the VS presentation. Despite the lack of PK comparability, you are proposing the PFS presentation as the to-be-marketed product. Therefore, you need to comprehensively evaluate the pharmacodynamics, efficacy, and safety of the PFS presentation in your clinical studies and provide adequate data to support commercialization of the PFS drug product.

In the end, the PFS container closure system was market approved. The FDA concluded that because patient dosing was individually titrated, the PK difference with the PFSs was not a significant patient safety issue [27].

9.4.2 Applying the Minimum CMC Regulatory Compliance Continuum

The minimum CMC regulatory compliance continuum risk-based approach can be applied to the amount of CMC information on the container closure that is expected from regulatory authorities in the IND/IMPD submissions. The following CMC regulatory compliance guidance is provided during early-stage (including FIH) clinical development:

Recombinant Proteins/mAbs [8]

The intended primary packaging to be used for the IMP in the clinical trial should be described. Where appropriate, reference should be made to the relevant pharmacopoeial monograph. If the product is packed in a non-standard administration device, or if non-compendial materials are used, description and specifications should be provided. For products intended for parenteral use where there is potential for interaction between product and container closure system, more details may be needed (e.g. extractable/leachable for phase III studies).

Gene Therapy-Based Biopharmaceuticals [14]

The intended primary packaging to be used for the IMP in the clinical trial should be described and compatibility with the product should be justified. Where appropriate, reference should be made to the relevant pharmacopoeial monograph. If non-compendial materials are used, description and specifications should be provided. For any device used in/as the container closure system, evidence of CE mark for the intended use should be provided. If the product is packed in a non-certified administration device, a description and specifications should be provided. For parenteral products with a potential for interaction between product and container closure system more details regarding biocompatibility may be needed. Where applicable, information on the sterilisation procedures of the container and the closure should be provided.

All Biopharmaceuticals [28]

Control of Components, and Containers and Closures

You should establish written procedures describing the handling, review, acceptance, and control of material (i.e., components, containers, closures) used in the manufacture of a phase 1 investigational drug. Materials should be controlled (e.g., segregated, labeled) until you have examined or tested the materials, as appropriate, and released them for use in manufacturing. It is important to handle and store such materials in a manner that prevents degradation or contamination.

At the time of submission of a dossier for market approval, studies to support the justification of the choice of container closure and confirmation that it is appropriate and adequate for the to-be-marketed biopharmaceutical drug product are required to be submitted in the BLA/MAA:

Recombinant Proteins/Monoclonal Antibodies [10]

A description of the container closure system(s) should be provided, including the identity of materials of construction of each primary packaging component, and their specifications. The specifications should include description and identification (and critical dimensions with drawings, where appropriate). Non-compendial methods (with validation) should be included, where appropriate. For non-functional secondary packaging components (e.g., those that do not provide additional protection), only a brief description should be provided. For functional secondary packaging components, additional information should be provided. The suitability should be discussed with respect to, for example, choice of materials, protection from moisture and light, compatibility of the materials of construction with the drug substance, including sorption to container and leaching, and/or safety of materials of construction.

All Biopharmaceuticals [29]

Suitability for the Intended Use

Every proposed packaging system should be shown to be suitable for its intended use: it should adequately protect the dosage form; it should be compatible with the dosage form; and it should be composed of materials that are considered safe for use with the dosage form and the route of administration. If the packaging system has a performance feature in addition to containing the product, the assembled container closure system should be shown to function properly. Information intended to establish suitability may be generated by the applicant, by the supplier of the material of construction or the

component, or by a laboratory under contract to either the applicant or the firm. An adequately detailed description of the tests, methods, acceptance criteria, reference standards, and validation information for the studies should be provided.

(a) Protection

A container closure system should provide the dosage form with adequate protection from factors (e.g., temperature, light) that can cause a degradation in the quality of that dosage form over its shelf life. Common causes of such degradation are: exposure to light, loss of solvent, exposure to reactive gases (e.g., oxygen), absorption of water vapor, and microbial contamination. A drug product can also suffer an unacceptable loss in quality if it is contaminated by filth.

Protection from microbial contamination is provided by maintaining adequate container integrity after the packaging system has been sealed. An adequate and validated procedure should be used for drug product manufacture and packaging.

(b) Compatibility

Packaging components that are compatible with a dosage form will not interact sufficiently to cause unacceptable changes in the quality of either the dosage form or the packaging component.

(c) Safety

Packaging components should be constructed of materials that will not leach harmful or undesirable amounts of substances to which a patient will be exposed when being treated with the drug product. This consideration is especially important for those packaging components which may be in direct contact with the dosage form, but it is also applicable to any component from which substances may migrate into the dosage form (e.g., an ink or adhesive). The approach for toxicological evaluation of the safety of extractables should be based on good scientific principles and take into account the specific container closure system, drug product formulation, dosage form, route of administration, and dose regimen (chronic or short-term dosing).

9.4.3 Combination Products

A combination product is '*a product comprised of two or more different types of medical products (i.e., a combination of a drug, device, and/or biological product with one another)*' [30]. A glass vial containing a biopharmaceutical is not a combination product because it merely holds the biopharmaceutical. However, a prefilled syringe that not only holds the biopharmaceutical but also delivers the biopharmaceutical is a combination product.

The 'primary mode of action' (PMOA) determines how the combination product will be regulated. For biopharmaceuticals in prefilled syringes, the biopharmaceutical typically has the PMOA, and the combination product is regulated as a biologic. This is referred to as a biologic-led combination product. In order to receive market approval, cGMPs have to be met both for the biopharmaceutical and for the device. The cGMPs for biopharmaceuticals were introduced in Chap. 4, Sect. 4.3.2, and then discussed throughout this book. Devices have cGMPs also, which must be met. FDA's device cGMPs are described in 21 CFR Part 820.

The FDA has issued a cGMP guidance on combination products that walks through the requirements for a drug-led prefilled syringe combination product [31]. The FDA requires the drug manufacturer to meet all of the 21 CFR 211 cGMPs for market-approval of the drug product. But the FDA permits a 'streamlining' approach

Table 9.9 FDA suggestions for meeting design controls of prefilled syringe cGMPs

Design Input/User Needs	*Design Output*
Required minimum/maximum delivery dose for drug	Drawing/specification for syringe minimum/maximum volume
Drug viscosity and desired/required delivery rate	Drawing/specification for needle bore, glide force, for example
Expected use condition (e.g., expected user experience/education level)	Content, reading level, for example, for the prefilled syringe's labeling
Maximum allowable temperature of drug	Packaging/labeling specifications for the prefilled syringe
No degradation of drug or syringe over the expected shelf-life as a result of contact with one another	Specifications for drug-contacting syringe materials
Expected shipping method and appropriate storage conditions	Design drawings/specifications for primary and secondary packaging
Drug delivery method (e.g., needle or needleless delivery)	Drawing/specification for needle and/or other associated syringe components

for the device (i.e., the prefilled syringe), so that it needs to meet only four key portions of the device cGMPs:

21 CFR 820.20 Management responsibility
21 CFR 820.30 Design controls (input, output, validation)
21 CFR 820.50 Purchasing controls
21 CFR 820.100 Corrective and preventive action (CAPA)

Table 9.9 presents some design inputs/outputs that the FDA used to illustrate the design controls for the prefilled syringe example.

The FDA also provided the following design verification/validation studies to complete the design control for the prefilled syringe example: (1) bench testing of the delivery of the drug from the syringe to ensure repeatable and accurate drug delivery; (2) shock and vibration testing of the packaged prefilled syringe to ensure no damage or loss of integrity in shipping; (3) human factor studies to confirm that expected users can adequately follow the instructions for use; (4) biocompatibility testing; (5) drug and syringe compatibility studies; (6) leachables and extractables testing; and (7) verification that the prefilled syringe works with all expected delivery methods (i.e., needle, needleless).

9.5 Stringent Aseptic Processing During the Filling/Sealing Process

The final stage in the drug product manufacturing process for all biopharmaceutical types (recombinant protein, monoclonal antibody, rAAV viral vector, genetically modified patient cells, mRNA non-viral vector) is to fill the formulated bulk drug into the selected container closure system, and securely seal it, see Fig. 9.7.

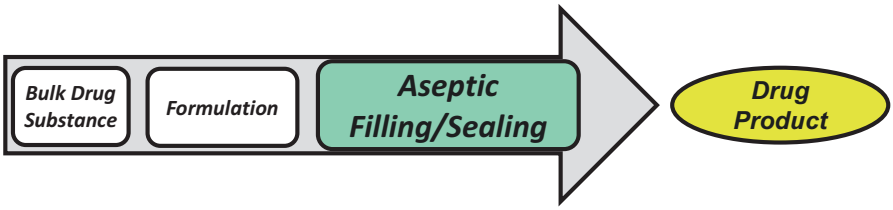


Fig. 9.7 Aseptic processing stage in the drug product manufacturing process

9.5.1 Sterile Formulated Bulk Drug

The sterility of the formulated biopharmaceutical bulk drug prior to the filling operation is a critical component of aseptic processing. Recombinant proteins, monoclonal antibodies, and both viral and non-viral vectors, can be sterilized using a sterilizing grade filter (nominal pore size of 0.22 micron). Statistically, in order to achieve a sterility assurance level (SAL) of 10^{-6} (i.e., a probability of a non-sterile unit of one in a million), the formulated bulk drug should have a bioburden level at NMT 10 CFU/100 mL prior to the filtration sterilization [8]:

> For sterilisation by filtration the maximum acceptable bioburden prior to the filtration must be stated in the application. In most situations NMT 10 CFU/100 ml will be acceptable. Test volumes of less than 100 ml may be used if justified.

To achieve the SAL, manufacturers typically run redundant or dual 0.22 micron sterilizing filters in series – the first 0.22 micron filter is treated as a microbial reduction filter and the second 0.22 micron filter is considered the filter for sterilization.

Regulatory authorities have much to say about this important filter sterilization process step:

> ***FDA*** [32]
>
> Filtration is a common method of sterilizing drug product solutions. A sterilizing grade filter should be validated to reproducibly remove viable microorganisms from the process stream, producing a sterile effluent. Currently, such filters usually have a rated pore size of 0.2 µm or smaller. Use of redundant sterilizing filters should be considered in many cases. Whatever filter or combination of filters is used, validation should include microbiological challenges to simulate worst-case production conditions for the material to be filtered and integrity test results of the filters used for the study. Product bioburden should be evaluated when selecting a suitable challenge microorganism to assess which microorganism represents the worst-case challenge to the filter. The microorganism *Brevundimonas diminuta* (ATCC 19146) when properly grown, harvested and used, is a common challenge microorganism for 0.2 µm rated filters because of its small size (0.3 µm mean diameter). A challenge concentration of at least 10^7 organisms per cm^2 of effective filtration area should generally be used, resulting in no passage of the challenge microorganism. The challenge concentration used for validation is intended to provide a margin of safety well beyond what would be expected in production. Factors that can affect filter performance generally include (1) viscosity and surface tension of the material to be filtered, (2) pH, (3) compatibility of the material or formulation components with the filter itself, (4) pressures, (5) flow rates, (6) maximum use time, (7) temperature, (8) osmolality, (9) and the effects of hydraulic shock. When designing the

validation protocol, it is important to address the effect of the extremes of processing factors on the filter capability to produce sterile effluent. Filter validation should be conducted using the worst-case conditions, such as maximum filter use time and pressure. Filter validation experiments, including microbial challenges, need not be conducted in the actual manufacturing areas. However, it is essential that laboratory experiments simulate actual production conditions.

EMA [33]

Suitable bioburden reduction prefilters and/or sterilising grade filters may be used at multiple points during the manufacturing process to ensure a low and controlled bioburden of the liquid prior to the final sterilising filter. Due to the potential additional risks of a sterile filtration process, as compared with other sterilisation processes, an additional filtration through a sterile sterilising grade filter, as close to the point of fill as possible, should be considered as part of an overall Contamination Control Strategy. The selection of components for the filtration system and their interconnection and arrangement within the filtration system, including pre-filters, should be based on the critical quality attributes of the product, justified and documented. The filtration system should minimize the generation of fibres and particles, not cause or contribute to unacceptable levels of impurities, or possess characteristics that otherwise alter the quality and efficacy of the product. Similarly, the filter characteristics should be compatible with the fluid and not be adversely affected by the product to be filtered. Adsorption of product components and extraction/leaching of filter components should be evaluated. Bioburden samples should be taken from the bulk product and immediately prior to the final sterile filtration. In case where a redundant filtration set-up is used, it should be taken prior to the first filter. Systems for taking samples should be designed so as not to introduce contamination.

Proteins, viral vectors and non-viral vectors can be sterilized by filtration, but genetically modified patient cells cannot be sterilized by filtration [16]:

To assure product safety, CAR T cells should be free of viable contaminating microorganisms; however, the final DP cannot be terminally sterilized as cells need to be fully viable and functional. Therefore, manufacturing should be conducted by using validated aseptic processing under current good manufacturing practice (CGMP) conditions. Product safety is further supported by the use of sterility testing (21 CFR 610.12) per United States Pharmacopeia (USP) Chapter 71 or an appropriately qualified and validated test method.

Therefore, stringent aseptic process handling of cells is especially critical for cell-based biopharmaceuticals, as mentioned in the next section.

9.5.2 Aseptic Filling/Sealing Process Step

Since the vast majority of biopharmaceutical drug products are administered by injection, these drug products must be sterile (absence of contaminating microorganisms). While many chemical drug products can be terminally treated by heat or irradiation to ensure that the contents in the sealed container closure are sterile, biopharmaceutical drug products cannot. Instead, the formulated bulk drug biopharmaceutical and the container closure must be sterile prior to the filling/sealing process, and the filling/sealing process must be done under stringent aseptic processing control [32]:

> There are basic differences between the production of sterile drug products using aseptic processing and production using terminal sterilization. Terminal sterilization usually involves filling and sealing product containers under high-quality environmental conditions. Products are filled and sealed in this type of environment to minimize the microbial and particulate content of the in-process product and to help ensure that the subsequent sterilization process is successful. In most cases, the product, container, and closure have low bioburden, but they are not sterile. The product in its final container is then subjected to a sterilization process such as heat or irradiation. In an aseptic process, the drug product, container, and closure are first subjected to sterilization methods separately, as appropriate, and then brought together. Because there is no process to sterilize the product in its final container, it is critical that containers be filled and sealed in an extremely high-quality environment. Aseptic processing involves more variables than terminal sterilization. Before aseptic assembly into a final product, the individual parts of the final product are generally subjected to various sterilization processes. For example, glass containers are subjected to dry heat; rubber closures are subjected to moist heat; and liquid dosage forms are subjected to filtration. Each of these manufacturing processes requires validation and control. Each process could introduce an error that ultimately could lead to the distribution of a contaminated product. Any manual or mechanical manipulation of the sterilized drug, components, containers, or closures prior to or during aseptic assembly poses the risk of contamination and thus necessitates careful control. A terminally sterilized drug product, on the other hand, undergoes final sterilization in a sealed container, thus limiting the possibility of error.

Under aseptic processing operations, whether for the manufacture of a chemical drug product or any of the biopharmaceutical drug product types, the following five areas are closely interrelated to achieve the desired sterile drug product: (1) the sterility of the formulated bulk drug, (2) the sterility of the container and closure components, (3) the cleanliness environment of the manufacturing facility where filling and sealing takes place, (4) the cGMP performance of the manufacturing operators carrying out the process step, and (5) confirmation of drug product sterility by compendial test methods. When it comes to the drug product manufacture of sterile injectables, patients who receive biopharmaceuticals in first-in-human (FIH) clinical studies must be protected as much as patients who purchase a commercial drug product. Regulatory authorities are very clear on this patient safety point:

> ***All Biopharmaceuticals*** [28]
>
> Because product sterility is a critical element of human subject safety, you should take special precautions for phase 1 investigational drugs that are intended to be sterile. You should give thorough consideration to implementing appropriate controls for aseptic processing to ensure a sterile phase 1 investigational drug. The guidance issued by FDA on aseptic processing is a good reference when using aseptic processing.
>
> ***Recombinant Proteins/Monoclonal Antibodies*** [8]
>
> The state of validation of aseptic processing and lyophilisation should be briefly described, if applicable. Taking into account EudraLex Vol. 4, Annex 13, the validation of sterilising processes should be of the same standard as for product authorised for marketing. The dossier should particularly include information directly relating to the product safety, i.e. on bioburden and media fill runs.
>
> ***Gene Therapy-Based Biopharmaceuticals*** [14]
>
> Validation of the aseptic process and the viral removal/inactivation steps are expected to be validated prior to the FIH clinical trials

Validation of the drug product manufacturing aseptic process using process simulations is a critical component of aseptic processing of ensuring sterile drug products.

All it takes is one step in the aseptic filling/sealing process to fail for microbial contamination to enter the drug product. Process simulation, also known as a media fill, includes exposing microbiological growth medium to product contact surfaces of process equipment, container closure systems, critical manufacturing environments, and process manipulations to closely simulate the same potential exposure that the biopharmaceutical drug product itself will undergo. The sealed containers filled with the medium are then incubated to detect microbial contamination. Results are then interpreted to assess the potential for a unit of drug product to become contaminated during actual operations (e.g., filling line start-up, sterile ingredient additions, aseptic connections, filling, closing). Regulatory authorities have provided thorough guidance on the critical aspects of the design of aseptic processing simulation studies:

FDA [32]

To ensure the sterility of products purporting to be sterile, sterilization, aseptic filling and closing operations must be adequately validated (§ 211.113). The goal of even the most effective sterilization processes can be defeated if the sterilized elements of a product (the drug formulation, the container, and the closure) are brought together under conditions that contaminate any of those elements. An aseptic processing operation should be validated using a microbiological growth medium in place of the product. This *process simulation*, also known as a *media fill*, normally includes exposing the microbiological growth medium to product contact surfaces of equipment, container closure systems, critical environments, and process manipulations to closely simulate the same exposure that the product itself will undergo. The sealed containers filled with the medium are then incubated to detect microbial contamination. Results are then interpreted to assess the potential for a unit of drug product to become contaminated during actual operations (e.g., start-up, sterile ingredient additions, aseptic connections, filling, closing). Environmental monitoring data from the process simulation can also provide useful information for the processing line evaluation.

Study Design. A media fill program should incorporate the contamination risk factors that occur on a production line, and accurately assesses the state of process control. Media fill studies should closely simulate aseptic manufacturing operations incorporating, as appropriate, worst-case activities and conditions that provide a challenge to aseptic operations. FDA recommends that the media fill program address applicable issues such as:

- Factors associated with the longest permitted run on the processing line that can pose contamination risk (e.g., operator fatigue)
- Representative number, type, and complexity of normal interventions that occur with each run, as well as nonroutine interventions and events (e.g., maintenance, stoppages, equipment adjustments)
- Aseptic assembly of equipment (e.g., at start-up, during processing)
- Number of personnel and their activities
- Representative number of aseptic additions (e.g., charging containers and closures as well as sterile ingredients) or transfers • Shift changes, breaks, and gown changes (when applicable)
- Type of aseptic equipment disconnections/connections
- Aseptic sample collections
- Line speed and configuration
- Weight checks
- Container closure systems (e.g., sizes, type, compatibility with equipment)

EMA [33]

Aseptic process simulation (APS) (also known as media fill)

Periodic verification of the effectiveness of the controls in place for aseptic processing should include an APS using a sterile nutrient media and/or surrogate in place of the product. The APS should not be considered as the primary means to validate the aseptic process or aspects of the aseptic process. The effectiveness of the aseptic process should be determined through process design, adherence to the pharmaceutical quality system and process controls, training, and evaluation of monitoring data. Selection of an appropriate nutrient media and/or surrogate should be made based on the ability of the media and/or surrogate to imitate physical product characteristics assessed to pose a risk to product sterility during the aseptic process. Where processing stages may indirectly impact the viability of any introduced microbial contamination, (e.g. aseptically produced semi-solids, powders, solid materials, microspheres, liposomes and other formulations where product is cooled or heated or lyophilized), alternative procedures that represent the operations as closely as possible should be developed. Where surrogate materials, such as buffers, are used in parts of the APS, the surrogate material should not inhibit the growth of any potential contamination. The APS should imitate as closely as possible the routine aseptic manufacturing process and include all the critical manufacturing steps

Another useful source of guidance on aseptic processing simulation are the Parenteral Drug Association (PDA) Points to Consider documents, which can be purchased on the PDA website (www.PDA.org): *Points to Consider for Aseptic Processing Part 1* (January 2015), and *Points to Consider for Aseptic Processing Part 2* (May 2016).

Keep in mind that for genetically modified patient cells, aseptic process simulation becomes critical for two reasons: (1) the added protection of a filtration sterilization step is not possible, and (2) the large number of manual manipulation process steps involved in cell culture manufacturing. Bags are commonly used as the final container for genetically modified patient cells due to the existing abundance of infrastructure for processing, freezing, and storing bag container systems from the long history of bag usage in blood banking systems. Many filling operations start out as manual operations in a biological safety cabinet (BSC). As batch sizes increase when the overall manufacturing process is scaled out/up, manual operations quickly become a bottleneck. Several devices have been launched into the marketplace that are capable of rapidly filling bags with formulated cell products. While capable of quickly filling multiple bags or vials and greatly reducing contact time in DMSO, some devices lack the capability to cool formulated cell products and some must be used in an isolator.

9.6 Applying the Minimum CMC Regulatory Compliance Continuum

As discussed in Chap. 4, Sect. 4.2, the regulatory authorities accept a minimum CMC regulatory compliance continuum risk-based approach for biopharmaceutical manufacturing. Regulatory guidance for the manufacture of the drug product for use in early-stage (including FIH) clinical development, on the one hand, requires

adequate control of the drug product manufacturing process, but on the other hand recognizes the limitations of process knowledge and understanding on the control strategy at this stage:

Recombinant Proteins/Monoclonal Antibodies [8]

P.3.3. Description of manufacturing process and process controls

A flow chart showing all steps of the manufacturing process, including relevant IPCs (process parameters and in-process-tests), should be provided accompanied by a brief process description. The IPCs may be recorded as action limits or reported as preliminary acceptance criteria and the focus should be on safety relevant attributes. For other IPCs, monitoring might be appropriate and acceptance criteria and action limits do not need to be reported. During development, as additional process knowledge is gained, further details of IPCs should be provided and acceptance criteria reviewed. Most products containing recombinant proteins and monoclonal antibodies are manufactured by an aseptic process, which is considered to be non-standard. Non-standard manufacturing processes or new technologies and new packaging processes should be described in sufficient detail. Reprocessing may be acceptable for particular manufacturing steps (e.g. re-filtration) only if the steps are adequately described and appropriately justified.

P.3.4. Control of critical steps and intermediates

Tests and acceptance criteria for the control of critical steps in the manufacturing process should be provided. It is acknowledged that due to limited data at an early stage of development (phase I/II) complete information may not be available. If holding times are foreseen for process intermediates, duration and storage conditions should be provided and justified by data in terms of physicochemical, biological and microbiological properties. For sterilisation by filtration the maximum acceptable bioburden prior to the filtration must be stated in the application. In most situations NMT 10 CFU/100 ml will be acceptable. Test volumes of less than 100 ml may be used if justified.

P.3.5. Process validation

The state of validation of aseptic processing and lyophilisation should be briefly described, if applicable. Taking into account EudraLex Vol. 4, Annex 13, the validation of sterilising processes should be of the same standard as for product authorised for marketing. The dossier should particularly include information directly relating to the product safety, i.e. on bioburden and media fill runs.

Gene Therapy-Based Biopharmaceuticals [14]

P.3.3. Description of manufacturing process and process controls

A flow chart of all successive steps including in-process-testing should be given. The results of in process testing may be recorded as action limits or reported as preliminary acceptance criteria. During development, as process knowledge is gained, further detail of in-process testing and the criteria should be provided and acceptance criteria reviewed.

P.3.4. Control of critical steps and intermediates

Tests and acceptance criteria for the control of critical steps in the manufacturing process should be provided. It is acknowledged that due to limited data at an early stage of development complete information may not be available. The critical manufacturing steps required to ensure a given stage of cellular differentiation necessary for the intended use should be controlled with relevant markers. Considerations on the manufacturing process should also take into account the product-associated risk profile. If holding times are foreseen for process intermediates, periods and storage conditions should be provided and justified by data in terms of physicochemical, biological and microbiological properties. For sterilisation by filtration the maximum acceptable bioburden prior to the filtration must be provided in the application. Reprocessing may be acceptable for particular manufacturing steps only if the steps are adequately described and appropriately justified.

P.3.5. Process validation and/or evaluation

> Process characterisation / evaluation data should be collected throughout the development preparing for Marketing Authorisation Application. At that stage the entire manufacturing process, storage etc. should be validated. Refer to S.2.5 for further details on the extent of evaluation/validation data required throughout development.

Regulatory authorities recognize that drug product manufacturing process knowledge will be acquired during clinical development, and that this additional knowledge should lead to an improved and tightened control strategy for the drug product manufacturing process. Figure 9.8 illustrates some of the identified critical process parameters (CPPs) for the various manufacturing process steps from the formulated bulk drug to the drug product. At the end of the drug product filling process a 100% visual check is done which can be challenging for some biopharmaceutical types (e.g., presence of proteinaceous particles, translucent solution due to lipids present with the mRNA non-viral vector, etc.).

For market approval, a validated control strategy for the drug product manufacturing process is required to be documented in the submitted Common Technical Document (CTD) market authorization application:

> ***ICH M4Q(R1)*** [10]
> A flow diagram should be presented giving the steps of the process and showing where materials enter the process. The critical steps and points at which process controls, intermediate tests or final product controls are conducted should be identified. A narrative description of the manufacturing process, including packaging, that represents the sequence of steps undertaken and the scale of production should also be provided.

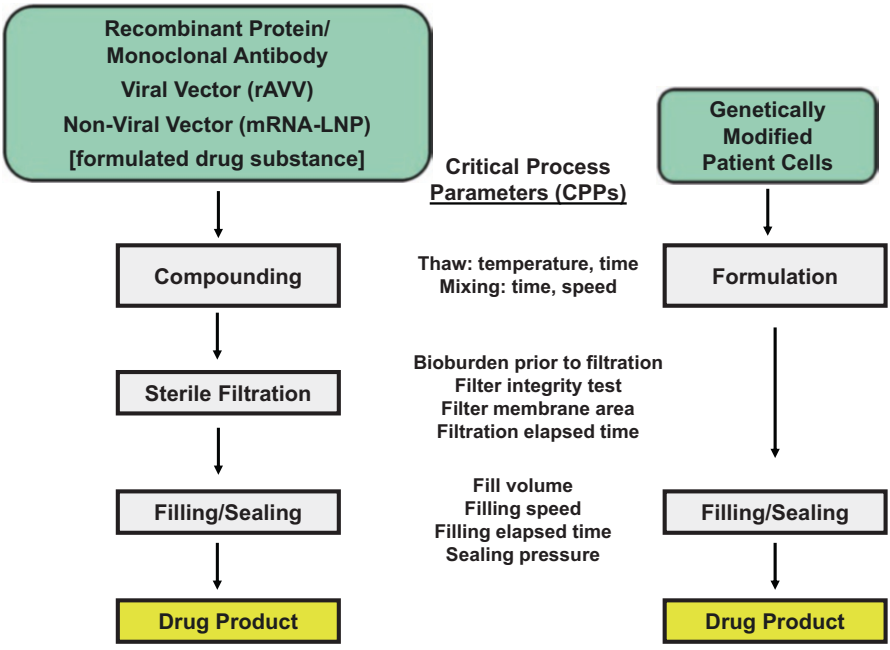


Fig. 9.8 Typical drug product manufacturing process for the four biopharmaceutical types

> Novel processes or technologies and packaging operations that directly affect product quality should be described with a greater level of detail… Steps in the process should have the appropriate process parameters identified, such as time, temperature, or pH. Associated numeric values can be presented as an expected range. Numeric ranges for critical steps should be justified in Sect. 3.2.P.3.4… Proposals for the reprocessing of materials should be justified. Any data to support this justification should be either referenced or filed in this section (3.2.P.3.3)… Description, documentation, and results of the validation and/or evaluation studies should be provided for critical steps or critical assays used in the manufacturing process (e.g., validation of the sterilisation process or aseptic processing or filling).

FDA for BLA [34]

> A complete description of the manufacturing process flow of the formulated bulk and finished drug product should be provided. This discussion should include a description of sterilization operations, aseptic processing procedures, lyophilization, and packaging procedures. Accompanying this narrative, a flow chart should be provided that indicates the production step, the equipment and materials used, the room or area where the operation is performed (may reference the simple diagram in II. B. 2.) and a listing of the in-process controls and tests performed on the product at each step. This flow diagram or narrative should also include information on the methods of transfer of the product between steps, i.e. Sterile, SIP connection, sanitary connection, open transfers under laminar flow units, etc. Such transfers should be described for movement of product between equipment, areas/rooms, buildings and sites.

It is important to emphasize that the regulatory authority expectation is that complete drug product documentation is to be provided in the market application dossier. The FDA has recognized that not every biopharmaceutical drug product manufacturer fully understands the definition of 'complete' manufacturing documentation. FDA uses a meeting-minutes-approach to stress to protein-based biopharmaceutical manufacturers the seriousness of their concern to have adequate control over the drug product manufacturing process. When a manufacturer approaches them for a pre-BLA submission meeting, the FDA frequently includes in the written meeting minutes, additional specific guidance on their expectations for the control information to be included in the planned filing. The following two tables contain the information, provided to the manufacturer of a recombinant protein seeking market approval, Tecvayli (teclistamab-cqyv) [35]: Table 9.10 covers the Drug Product control (Module 3.2.P.3.3/.4), and Table 9.11 cover the Drug Product process validation (Module 3.2.P.3.5). Although the focus of this FDA provided information is emphasizing the control necessary over adventitious agent contamination (especially bacterial/fungal contaminants), it does include the other validation/evaluation activities necessary for control of the drug product process.

Table 9.10 Drug product process control data to be included in BLA filing – 3.2.P.3.3/.4

The following information should be provided in Sections 3.2.P.3.3 (Description of Manufacturing Process and Process Control) and/or 3.2.P.3.4 (Controls of Critical Steps and Intermediates, as appropriate:
• **Identification of the manufacturing areas and type of fill line (e.g. open, RABS, isolator), including area classifications**
• **Description of the sterilizing filter (supplier, size, membrane material, membrane surface area, etc.); sterilizing filtration parameters (e.g., differential pressure if a pump is used) as validated by the microbial retention study; wetting agent used for post-use integrity testing of the sterilizing filter and post-use integrity test acceptance criteria.**
• **Parameters for filling and capping for the vials**
• **A list of all equipment and components that contact the sterile drug product (i.e. the sterile-fluid pathway) with the corresponding method(s) of sterilization and depyrogenation, including process parameters. The list should include single-use equipment**
• **Processing and hold time limits, including the time limit for sterilizing filtration and aseptic filling**
• **Sampling points and in-process limits for bioburden and endotoxin. Bioburden samples should be taken at the end of the hold time prior to the subsequent filtration step. Pre-sterile filtration bioburden limits should not exceed 10 CFU/100 mL**

9.7 Meeting CMC Regulatory Compliance for Drug Product Manufacturing

The following three case examples of market-approved biopharmaceuticals indicate the complete CMC documentation in the market application dossier that meet regulatory authority compliance expectations for the drug product manufacturing process:

Ximluci (Ranibizumab), Monoclonal Antibody Fragment [36]

The manufacturing process is standard and includes thawing of active substance, compounding with formulation buffer followed by sterile filtration through two sterile filters in series and filling into pre-sterilised vials. There is 100% visual inspection of all filled vials The active substance thaw and hold time is considered acceptable. The time out of refrigerator during visual inspection and secondary packaging is adequately justified. No reprocessing is proposed. The manufacturing process is controlled by CPPs at the buffer preparation, sterile filtration, filling, inspection and labelling stages. The process is controlled by IPCs at the buffer preparation, sterile filtration and filling stages.

Process validation Data are provided for consecutive, commercial scale batches manufactured and filled into vials. The transport validation/verification system is considered acceptable.

Roctavian (Valoctocogene Roxaparvovec), rAAV Viral Vector [37]

The compatibility of FBDS with all contact parts included in BioMarin fill-finish line intended to be used for the commercial finished product manufacturing was confirmed. As regards determination of hold times, data are presented to support the hold steps. CPPs were defined at different steps of the manufacturing process.

Table 9.11 Drug product process validation/evaluation to be included in BLA filing – 3.2.P.3.5

The following study protocols and validation data summaries should be included in Section 3.2.P.3.5 (Process Validation and/or Evaluation), as appropriate:

- **Bacterial filter retention study for the sterilizing filter. Include a comparison of validation test parameters with routine sterile filtration parameters**
- **Sterilization and depyrogenation of equipment and components that contact the sterile drug product. Provide summary data for the three validation studies and describe the equipment and component revalidation program**
- **In-process microbial controls and hold times. Three successful product intermediate hold time validation runs should be performed at manufacturing scale, unless an alternative approach can be scientifically justified. Bioburden and endotoxin levels before and after the maximum allowed hold time should be monitored and bioburden and endotoxin limits provided**
- **Isolator decontamination summary data and information, if applicable.**
- **Three successful consecutive media fill runs, including summary environmental monitoring data obtained during the runs. Describe the environmental and personnel monitoring procedures followed during media fills and compare them to the procedures followed during routine production**
- **Information and summary results from shipping validation studies**
- **Validation of capping parameters, using a container closure integrity test**
- **Container closure integrity testing. System integrity should be demonstrated initially and during stability. Container closure integrity method validation should demonstrate that the assay is sensitive enough to detect breaches that could allow microbial ingress (≤ 20 microns). Container closure integrity testing should be performed *in lieu* of sterility testing for stability samples every 12 months (annually) until expiry**

Process Validation. Process validation batches were executed. For all batches, all CPPs were within the normal operating ranges (NORs). Finished product manufacturing limits were assessed and were adequately validated. Finished product uniformity was checked. Process simulation (also known as media fill) runs were performed. All process simulation runs were successful and validate the number of filled vials. The applicant has given a rationale to justify this maximum batch size. Concerning shipping qualification, a summary confirming the physical integrity of the shipping container and vials and the maintenance of the temperature during the transport at ≤ -60 °C is provided for finished product and finished goods.

Zynteglo (Betibeglogene Autotemcel), Genetically Modified Patient Cells [38]

To manufacture Zynteglo FP, the AS cells are resuspended in CryoStor CS5 at a concentration of 1.2×10^6 to 20×10^6 cells/mL and filled into cryopreservation bags. Each bag is placed in a secondary container and a chilled metal cassette, and frozen before being placed in the vapour phase of liquid nitrogen for storage.

Process validation and/or Evaluation. The PPQ study was performed using healthy donor cells. This approach has been accepted. Three consecutive FP batches were manufactured from apheresis material. The PPQ study results suggest that the intended commercial manufacturing process is capable to consistently yield a product meeting the acceptance criteria.

Aseptic Process Simulation Studies. Aseptic process simulation (media fill simulation) was successfully completed for three consecutive process simulation runs. It was confirmed that the media fill simulation covers all the aseptic processing steps.

Shipping Validation. The shipping system used for transporting Zynteglo FP from the manufacturing facility to FP infusion centres within the European Union Regions has been qualified, taking a risk-based approach through Operational Qualification and Performance Qualification under worst case conditions.

But not every biopharmaceutical manufacturer meets the required complete CMC documentation in the submitted market application dossier for the drug product manufacturing process. Two case examples from biopharmaceuticals that eventually reached market approval follow. The first case example is for the following recombinant protein, which received a Complete Response Letter from the FDA after ten months of review of the submitted BLA:

Fylnetra (Pegfilgrastim-pbbk), Recombinant Protein [39]

We have completed our review of this application and have determined that we cannot approve this application in its present form. We have described our reasons for this action below and, where possible, our recommendations to address these issues.

MICROBIOLOGY

1. Information regarding media fill studies is inadequate. Please update Sect. 3.2.P.3.5 of the BLA with the following:
   (a) Summarized results (media fill date, container closure, filled volume, duration, number of units filled/incubated/rejected, positive) from the three initial media fill validation runs and the latest requalification run that was performed to validate the syringe line filling process relevant to the drug product.
   (b) Description of the hold periods (date, temperature, duration) simulated in each media fill run.
   (c) Description of confirmatory growth promotion test. Include a list of microorganisms used in the test.
2. The bacterial retention study for the sterilizing-grade filter was performed using the drug substance, which is not adequate. Please update BLA sect. 3.2.P.3.5 with the following:
   (a) Protocol and data from the validation studies using three different lots of the sterilizing filter intended for commercial production using the final drug product solution.
   (b) Study/report# and the date of the study.
   (c) Comparison of validation test parameters with those used during routine operation (i.e., temperature, filtration time, filtration pressure, flow volume, and flow rate, etc.).
   (d) Description of the challenge microorganism, membrane lot numbers, pore size rating, pre- and post-filtration bubble point, challenge CFU/cm2).
   (e) Demonstration of viability of the challenge organism in the presence of drug product.

The biopharmaceutical manufacturer resubmitted the BLA three months later, and received market approval six months after that – a total CMC delay of nine months [40].

The second case example is for the following viral vector biopharmaceutical, which received a Complete Response Letter from the FDA after seven months of review of the submitted BLA:

Adstiladrin (Nadofaragene Firadenovec-vncg), Viral Vector [41]

After our complete review, we have concluded that we cannot grant final approval because of the deficiencies outlined below.

1. CBER conducted a Pre-License Inspection (PLI) of the FinVector Oy facility from January 20–25 and January 27–28, 2020, and issued a Form FDA 483, List of Inspectional Observations. Your responses to the FDA 483 received through March 2, 2020, do not sufficiently address the concerns noted during the inspection as your corrective actions do not appear to be comprehensive enough to address the systemic issues. Examples include:
   - Investigations of deviations do not include a comprehensive evaluation to determine the impact on product safety, and initiation of corrective actions to prevent recurrence of issues is not consistently performed.
   - Your manufacturing procedures are not sufficiently detailed to provide consistent lot-to-lot reproducibility of your finished product.
   - The cleaning and disinfection are not fully validated to demonstrate that the cleaning agents are effective, and that fumigation decontamination is effective for viral inactivation.
   - There is lack of assurance of the accuracy of certain test results, including sterility. Your storage and shipping conditions of the sterility samples are not controlled or validated to sufficiently demonstrate that the sample has not been altered prior to testing.
   - The observations described in the Form FDA 483 issued at the close of the inspection referenced above are an indication of your quality unit not fulfilling its responsibility to assure the identity, strength, quality, and purity of nadofaragene firadenovec. Approval of a biologics license application or issuance of a biologics license constitutes a determination that the establishment(s) and the product meet applicable requirements to ensure the continued safety, purity, and potency of such products. Applicable requirements for the maintenance of establishments for the manufacture of a product include, but are not limited to, the good manufacturing practice requirements. Your corrective actions need to be more comprehensive with respect to addressing the underlying quality oversight issues, and a second PLI will be necessary to verify the corrective actions once they have been fully implemented.
2. Aseptic process validation successful media fills was not completed prior to manufacture of your Process Performance Qualification lots. Please repeat these runs after the media fill acceptance criteria have been updated and any operator re-training as required based on changes to the aseptic process procedures. Please submit the media fill report following completion of the media fill runs to support your commercial manufacturing process.
3. Sterility assurance of the product contact equipment and surfaces has not been demonstrated based upon the review of the sterilizing autoclave final report Validation Summary Report GMP, GMP Revalidation 2018 and associated data run sheets. The testing documentation in the report is inadequate to ensure that all surfaces have been adequately sterilized. Please provide documentation and data demonstrating exact placement during testing. The information should include diagrams or photos and placement, as well as detailed descriptions of how the items were prepared (i.e., size and type of bag used, double bag, quantity of items inside, any wrapping, tubing diameter and length, etc.). The detailed item description from validation testing should be added to the operating procedure to ensure items are prepared and in the same manner as validated.
4. Equipment operating parameters established after qualification are not detailed in the manufacturing procedures and/or batch records to ensure consistent operation of equipment within qualified parameters. In addition, equipment qualification deviations have not been resolved to ensure there is no impact on performance validation or process validation testing. The use of inconsistent operating parameters across multiple pieces of equipment may impact product quality.

To address this concern, please establish operating parameters for your equipment based upon the capabilities as demonstrated during your equipment qualification. Please submit the pertinent manufacturing procedure or relevant section of the batch record to demonstrate the establishment of the equipment operating parameters.

5. The manufacturing process requires a maximum duration from the time of drug substance to drug product filled vial freezer storage. However, based on batch records reviewed, this requirement is not tracked to insure the limit is not surpassed. Please ensure this critical process parameter is adequately documented. Please provide documentation demonstrating this process time duration is met during production.

The biopharmaceutical manufacturer resubmitted the BLA twenty-six months later, and received market approval six months after that – a total CMC delay of thirty-two months [42].

After market-approval, the biopharmaceutical drug product manufacturing process is subject to cGMP compliance inspections from various regulatory authorities. Adequate and appropriate control of the biologic drug product manufacturing process is challenging, but doable. Yet, things wrong happen! According to Murphy's Law, *"If there are two or more ways to do something, and one of those ways can result in a catastrophe, then someone will do it."* Whether due to a staff member not following, and sometimes not even knowing, the required GMP requirements or a lack of management support for the Quality Unit, serious control problems can develop with the biopharmaceutical drug product manufacturing process. This usually leads to either a product recall or a major warning from a regulatory authority's GMP inspectors. A case example is the FDA inspection of Celltrion. Celltrion is a manufacturer of commercial biosimilar drug products. From a May 2017 FDA inspection of its South Korean drug product manufacturing site, it received a Form FDA 483 that stated the following [43]:

The current inspection found the firm continues to manufacture a drug substance and lyophilized injectable drug product for the US market. At the conclusion of the inspection a 12-item FDA 483 was issued including observations for: investigations of discrepancies were not thorough or timely; procedures for aseptic processing were not established and followed; validation of the aseptic process was deficient; appropriate procedures for environmental monitoring of the aseptic processing areas were not established; cleaning procedures for the aseptic processing areas were not adequate; equipment in the aseptic processing areas was not of an appropriate design; process validation studies did not evaluate intra-batch variability; complete testing records are not maintained and reviewed; controls over electronic records are not established; document issuance and use is not controlled; data is not documented contemporaneously; and batch records do not contain complete information related to the production of a batch.

The cGMP inspection, followed by the inadequate written response of the company, resulted in a subsequent FDA Warning Letter issued on January 2018 [44].

Bottom line, the strength of the chain holding together the successful drug product manufacturing process for a biopharmaceutical is only as strong as the weakest link!

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Chapter 10
Complex Process-Related Impurity Profiles

Abstract Every manufactured human medicine contains residual impurities associated with its manufacturing process. Patient safety risk associated with the process-related impurities needs to be assessed. Compared to chemical drugs, biopharmaceuticals (whether recombinant proteins, monoclonal antibodies, viral vectors, genetically modified patient cells, or mRNA non-viral vectors) have a highly complex process-related impurity safety profile due to the biological manufacturing processes employed. In this chapter, the risk-based control strategy for prioritizing the concerns associated with the numerous process-related impurities produced by the different biopharmaceutical manufacturing processes, will be discussed. Application of the minimum CMC regulatory compliance continuum risk-based approach will be examined. In addition, four specific process-related impurities will be discussed in detailed – residual DNA, residual host cell proteins, endotoxin, and leachables.

Keywords Impurities · Process-Related · Upstream · Detection · Quantitation · DNA · HCPs · Downstream · Antibiotics · Endotoxin · Minimum · Leachables · Nitrosamines · Risk-Based

The design and operation of the entire biopharmaceutical manufacturing process – from starting material(s) → drug substance upstream production → drug substance downstream purification → formulation → drug product filling/sealing in a container closure system – contributes to the presence and levels of residual process-related impurities in the biopharmaceutical final drug product. For example, for a recombinant protein, the length of cell culture production run time, impacts not only the total yield of the produced biopharmaceutical, but also the level of host cell proteins present in the harvested solution due to increased lysing of production cells with age (typically tracked by the decrease in measured % cell viability). Such an increase in the amount of host cell proteins increases the pressure on the purification process to effectively remove them from the purified biopharmaceutical. If the host cell protein level is too high, it may overload the purification capacity. Therefore,

J. Geigert, *The Challenge of CMC Regulatory Compliance for Biopharmaceuticals*, https://doi.org/10.1007/978-3-031-31909-9_10

the effective control of process-related impurities, requires the entire biopharmaceutical manufacturing process – from starting material(s) through to the final drug product – to be evaluated from a risk-based perspective.

Compared to chemical drugs, biopharmaceuticals (whether recombinant proteins, monoclonal antibodies, viral vectors, genetically modified patient cells or mRNA non-viral vectors) have a highly complex process-related impurity safety profile, especially due to the biological manufacturing processes employed. Each biopharmaceutical manufacturing process type generates multiple, different process-related impurities, which need to be identified, appropriately measured, and adequately managed for patient safety concerns. The risk-based control strategy for prioritizing the concerns associated with the numerous process-related impurities produced by the different biopharmaceutical manufacturing processes, will be discussed. Application of the minimum CMC regulatory compliance continuum risk-based approach will be examined. In addition, specific process-related impurities will be discussed in detailed – residual DNA, residual host cell proteins, endotoxin, and leachables.

10.1 Is It a Process-Related Impurity?

"Not everything that can be counted counts, and not everything that counts can be counted", is a maxim ascribed to the sociologist William Bruce Cameron. Applied to process-related impurities, there seems an endless list of impurities ('whatever goes into the pot that does not become the final drug product can be considered an impurity'). Also, not all process-related impurities have the same level of patient safety risk, with a corresponding degree of residual level control needed.

Terminology can be confusing. There are 'process-related impurities', 'contaminants', and 'product-related impurities' [1]:

> *Process-Related Impurities*: Impurities that are derived from the manufacturing process.
>
> *Contaminants*: Any adventitiously introduced materials not intended to be part of the manufacturing process of the drug substance or drug product.
>
> *Product-Related Impurities*: Molecular variants of the desired product which do not have properties comparable to those of the desired product with respect to activity, efficacy, and safety.

'Process-related impurities' are the subject of this chapter.

'Contaminants', such as adventitious agents, were discussed in Chap. 5.

'Product-related impurities' are discussed in Chap. 11.

Process-related impurities are an important class of critical quality attributes (CQAs) because of their potential impact on patient safety. Chemical pharmaceuticals have two major groups of process-related impurities: organic impurities and inorganic impurities, while biopharmaceuticals have a much more diverse group of process-related impurities [2]:

Impurities are an important class of potential drug substance CQAs because of their potential impact on drug product safety. For chemical entities, impurities can include organic impurities (including potentially mutagenic impurities), inorganic impurities e.g., metal residues, and residual solvents (see ICH Q3A and Q3C). For biotechnological/biological products, impurities may be process-related or product-related (see ICH Q6B). Process-related impurities include: cell substrate-derived impurities (e.g., Host Cell Proteins (HCP) and DNA); cell culture-derived impurities (e.g., media components); and downstream-derived impurities (e.g., column leachables). Determining CQAs for biotechnology/biological products should also include consideration of contaminants, as defined in Q6B, including all adventitiously introduced materials not intended to be part of the manufacturing process (e.g., adventitious viral, bacterial, or mycoplasma contamination).

10.2 Process-Related Impurities Based on Manufacturing Process Type

Each biopharmaceutical manufacturing process type needs to be risk-assessed, across each of its process steps, for contribution toward formation of process-related impurities, as illustrated in the Ishikawa diagram (also known as fishbone diagram) presented in Fig. 10.1.

Specifics on each of the biopharmaceutical manufacturing process steps listed in Fig. 10.1, can be found in Chap. 6 (starting materials), Chap. 7 (upstream production), Chap. 8 (downstream purification) and Chap. 9 (formulation, container closure, filling/sealing).

A risk-based assessment of the various process-related impurities, from the manufacturing processes for each of the four biopharmaceutical types, will be performed to understand regulatory authority patient safety concerns:

Section 10.2.1 Recombinant proteins and monoclonal antibodies
Section 10.2.2 Viral vectors
Section 10.2.3 Genetically modified patient cells
Section 10.2.4 mRNA non-viral vector

Application of the minimum CMC regulatory compliance continuum from early-stage clinical development through late-stage clinical development, and into market approval, will be examined for control of these process-related impurities.

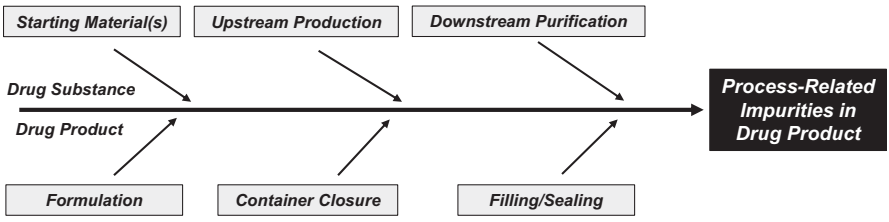

Fig. 10.1 Potential sources of process-related impurities in a biopharmaceutical process

Since the following four process-related impurities are common to all four biopharmaceutical types, they will be examined in greater detail:

Section 10.3.1 Residual DNA
Section 10.3.2 Residual host cell proteins
Section 10.3.3 Endotoxins
Section 10.3.4 Leachables

10.2.1 Recombinant Proteins and Monoclonal Antibodies

With over 200 market-approved recombinant proteins and monoclonal antibodies, much is known about the type of process-related impurities that can be encountered with this biopharmaceutical manufacturing process type [1]:

> *Process-related impurities and contaminants*
> These are derived from the manufacturing process and are classified into three major categories: cell substrate-derived, cell culture-derived and downstream-derived.
>
> (a) Cell substrate-derived impurities include, but are not limited to, proteins derived from the host organism, nucleic acid (host cell genomic, vector, or total DNA). For host cell proteins, a sensitive assay e.g., immunoassay, capable of detecting a wide range of protein impurities is generally utilized. In the case of an immunoassay, a polyclonal antibody used in the test is generated by immunization with a preparation of a production cell minus the product-coding gene, fusion partners, or other appropriate cell lines. The level of DNA from the host cells can be detected by direct analysis on the product (such as hybridization techniques). Clearance studies, which could include spiking experiments at the laboratory scale, to demonstrate the removal of cell substrate-derived impurities such as nucleic acids and host cell proteins may sometimes be used to eliminate the need for establishing acceptance criteria for these impurities.
> (b) Cell culture-derived impurities include, but are not limited to, inducers antibiotics, serum, and other media components.
> (c) Downstream-derived impurities include, but are not limited to, enzymes, chemical and biochemical processing reagents (e.g., cyanogen bromide, guanidine, oxidizing and reducing agents), inorganic salts (e.g., heavy metals, arsenic, non metallic ion), solvents, carriers, ligands (e.g., monoclonal antibodies), and other leachables.

A summary of the origin of potential process-related impurities, across the various process steps of a recombinant protein/monoclonal antibody manufacturing process, is presented in Table 10.1.

10.2.1.1 DS Upstream Production

The drug substance upstream production process is the major contributor to the process-related impurities in a protein-based biopharmaceutical. Potential upstream production impurities arise from the many raw materials in the cell culture media. Cell culture media are complex – providing the various amino acids, vitamins,

Table 10.1 Potential process-related impurities from a rprotein/mAb manufacturing process

Recombinant Protein/mAb Manufacturing Process	
Manufacturing Process Step	**Origin of Potential Residual Process-Related Impurities**
Starting Material (Master/Working Cell Bank)	(so little MCB/WCB used; such a major dilution through the upstream production process)
DS Upstream Production	Medium components Antibiotics Inducers Antifoam Host cell DNA Host cell protein (HCP)
DS Downstream Purification	Column resin leachable (e.g., Protein A ligand) Buffers
Conjugation	Elemental impurities from the chemical drug
Formulation	Impurities in the excipients
Container Closure System	Leachables from container Leachables from closure
DP Filling/Sealing Process	Leachables from product-contact surfaces (filing line tubing)

sugars and inorganic salts for cells. In addition, the cell culture media may also include serum (e.g., fetal bovine serum), growth factors (e.g., insulin-like growth factor), lipids (e.g., cholesterol), selective agents (e.g., methotrexate), antibiotics (e.g., gentamicin), antifoam (e.g., Pluronic F-68), etc. Each of these raw materials can become a process-related impurity. Potential upstream production impurities also arise from the cell cultures themselves (e.g., host cell DNA and host cell proteins). These are discussed in greater detail in Sect. 10.3.

Fetal bovine serum (FBS) stimulates animal and human cells to grow and multiply, and helps to keep the production cells normal and healthy over time. Fetal bovine serum contains bovine serum albumin (BSA), immunoglobulins (IgG), as well as a rich mixture of other proteins. However, FBS brings in not only the risk of prions (see Chap. 5), but also when present as a residual impurity, the concern for immunogenicity.

Insulin and insulin-like growth factor-1 (IGF-1) are widely used growth factors to delay apoptosis in mammalian cell culture, which leads to increased productivity. These growth factors suppress cell death in serum-free culture by means of lowering intracellular reactive oxygen species levels via an increase in the specific glucose consumption rate. But growth factor residuals in a biopharmaceutical could become a concern for their hormonal activity.

Gene amplification systems are used to increase productivity of a recombinant protein or monoclonal antibody. For mammalian cells, either a dihydrofolate reductase (DHFR) system using methotrexate (MTX) resistance (CHO) or a glutamine

synthetase (GS) system using methionine sulfoximine (MSX) resistance (CHO and NS0) can be used to maintain selective pressure. Methotrexate, while used medically for treatment of certain autoimmunity diseases and cancers, has the potential for various organ toxicities; methionine sulfoximine is a potential neurotoxin. Therefore, methotrexate or methionine sulfoximine, while used only in the early seed cultures, could be a residual in a biopharmaceutical and thus become a concern for toxicity.

Antibiotics are used in cell culture production of biopharmaceuticals either as a genetic selective agent or to help prevent bacterial and mycoplasma contamination during cell culture production. With recombinant bacteria cell lines (e.g., *E. coli*), the antibiotics tetracycline and kanamycin are used as a genetic selective agent during production of recombinant proteins The plasmid in a recombinant cell line plasmid, in addition to containing the gene for the protein, also contains a gene segment imparting antibiotic resistance. If a recombinant cell loses the plasmid, protein expression ends, but so does its antibiotic resistance. The antibiotic then shuts down cellular protein synthesis vital to the cell's survival. In this way, the media nutrients are preserved for those cells that are continuing to produce the recombinant protein. With mammalian cell lines (e.g., CHO), on occasion, antibiotics such as gentamicin are added into the cell culture medium during production of recombinant proteins or monoclonal antibodies as prophylactic protection to prevent either microbial or mycoplasma contamination. Note, beta-lactams (e.g., penicillin) are restricted from use in cell culture production due to the potential of a severe allergic reaction if residuals are present in the administered drug product [3]. When an antibiotic is added to the cell culture production of a recombinant protein or monoclonal antibody, its removal must be accomplished [4], as shown in Table 10.2. In addition, there needs to be a justification for the use of any antibiotic in the manufacturing process, as illustrated by the postmarketing commitment for the use of the antibiotic kanamycin in an *E. coli*-produced recombinant protein, Palynziq (pegvaliase-pqpz) [5]:

> Perform a study to evaluate the impact of the removal of kanamycin during the pegvaliase fermentation process. If the data support removal of kanamycin, then submit a plan for the removal of kanamycin from the pegvaliase manufacturing process.

Also important to note that host cell wall components (e.g., polysaccharides) can also be process-related impurities. For market-approval of the *Pichia pastoris*-produced recombinant protein, Jetrea (ocriplasmin), the FDA imposed a post-market approval commitment to evaluate the potential impact of the cell wall components in the drug substance [6]:

> To evaluate drug substance for the presence of yeast cell wall components (i.e. chitin, mannans, and β-glucans). Provide a risk assessment of the potential impact these product related impurities may have on the quality, safety and efficacy of ocriplasmin and propose an appropriate control strategy.

Table 10.2 Antibiotic use in cell culture manufacturing of recombinant proteins/mAbs

Antibiotics Used in the Recombinant Protein/Monoclonal Antibody Cell Culture Manufacturing Process			
Biopharmaceutical	**Culture**	**Antibiotic**	**FDA Package Insert**
Monoclonal Antibody Fab Fragment Byooviz (ranibizumab-nuna)	*E. coli*	Tetracycline	Produced by an *E. coli* expression system in a nutrient medium containing the antibiotic tetracycline. Tetracycline is not detectable in the final product.
Fab Fragment Fusion Protein Lumoxiti (moxetumomab pasudotox-tdtk)	*E. coli*	Kanamycin	During the *E. coli* manufacturing process, fermentation is carried out in nutrient medium containing the antibiotic kanamycin. However, kanamycin is cleared in the manufacturing process and is not detectable in the final product.
Monoclonal Antibody Perjecta (pertuxumab)	CHO	Gentamicin	Produced in a mammalian cell (Chinese Hamster Ovary) culture that may contain the antibiotic, gentamicin. Gentamicin is not detectable in the final product.

10.2.1.2 DS Downstream Purification

The drug substance downstream purification process is designed to meet the challenges of the process-related impurities arising from the upstream production process, whether it is using a cell culture process (as discussed above) or from a transgenic animal (removal of milk proteins that could cause a severe immune response, including anaphylaxis) or from a transgenic plant (removal of toxicants from the plant biomass, such as alkaloids or pesticides). But the downstream purification process can also introduce additional process-related impurities into the biopharmaceutical product. Potential downstream impurities such as enzymes, processing reagents (e.g., cyanogen bromide, guanidine, oxidizing and reducing agents), leached metallic elements (e.g., leaching from a metal-complex chromatographic resin), leached ligands (e.g., leaching from a Protein A affinity column resin), etc., all need to be evaluated.

Protein A affinity chromatography is an important purification step for monoclonal antibodies. Protein A is a cell wall protein deriving from *Staphylococcus aureus*, which exhibits unique binding properties for the Fc domains of IgG molecules. Leached Protein A from the affinity resin into the biopharmaceutical solution may trigger an immune response. Since these affinity columns are expensive,

manufacturers try to obtain several hundred cycles of use for them. Thus, the amount of leaching that occurs at the beginning cycle of resin use and at the end of lifetime cycle of resin use must also be studied [7]:

> Leaching of mAb or impurities from the solid support into the final product should be considered when specifications are established for the drug substance. The amount of column leachables is not uniform over the column lifespan and depends on several factors (e.g., length of storage, solutions used in the regeneration and/or sanitization steps, column operating parameters). A variety of methods can be used to test for leachables such as sampling the buffer flow-through prior to the load of the drug substance intermediate, in-process testing of the intermediate bulk, or testing the final drug substance. Alternatively, if documentation is available that the production steps that follow the use of the reagent mAb reduce the maximum amount of column leachables to appropriate levels, this documentation can be provided in lieu of routine testing for leachables.

Fortunately, the vendors of Protein A immobilized resin provide the ELISA test kits for measuring the residual level of leached Protein A in the purified biopharmaceutical.

Organic solvents are occasionally used with chromatographic purification, for example, isopropanol with reversed-phase HPLC processes. When organic solvents are used, the principles of ICH Q3C(R5) *Impurities – Guideline for Residual Solvents* [8] apply. Since there is no therapeutic benefit from residual solvents, all residual solvents should be removed to the extent possible to meet product specifications, good manufacturing practices, or other quality-based requirements.

10.2.1.3 Conjugation of DS

For biopharmaceuticals, the safety risk of residual elemental impurities is considered low, except for the case of antibody-drug conjugates (ADCs) where elemental impurities can be introduced through the conjugated chemical drug [9]

> For biotechnology-derived products, the risks of elemental impurities being present at levels that raise safety concerns at the drug substance stage are considered low. This is largely because: a) elements are not typically used as catalysts or reagents in the manufacturing of biotech products; b) elements are added at trace levels in media feeds during cell culture processes, without accumulation and with significant dilution/removal during further processing; c) typical purification schemes used in biotech manufacturing such as extraction, chromatography steps and dialysis or Ultrafiltration-Diafiltration (UF/DF) have the capacity to clear elements introduced in cell culture/fermentation steps or from contact with manufacturing equipment to negligible levels. As such, specific controls on elemental impurities up to the biotech drug substance are generally not needed. In cases where the biotechnology-derived drug substance contains synthetic structures (such as antibody-drug conjugates), appropriate controls on the small molecule component for elemental impurities should be evaluated.

10.2.1.4 DP Formulation

The formulation process can introduce process-related impurities into a biopharmaceutical product. Biopharmaceuticals incorporate a wide range of excipients in their formulations as discussed in Chap. 9. Sourcing for compendial quality (e.g., USP or

Ph. Eur.), establishing vendor quality/supply chain agreements, and preventing degradation, are important for excipients.

As an example, polysorbates which are mixtures of polyethoxylated sorbitan fatty acid monoesters in which the fatty acid composition is heterogeneous. This excipient has proven highly effective in preventing protein aggregation in formulations of commercial recombinant proteins and monoclonal antibodies. However, numerous factors, including impurities from raw materials, storage temperature, other excipients, and exposure to light, can affect the quality of polysorbate excipients in formulations. Oxidation and hydrolysis are the two main degradation pathways of polysorbate. A variety of host cell proteins (enzymes found in *E. coli*., CHO cells, and yeast) can cause polysorbate to undergo rapid degradation involving hydrolysis of the fatty acid chain leading to sub-visible and visible particle formation in polysorbate-containing recombinant protein and monoclonal antibody formulations [10].

10.2.1.5 Impurities from Filling/Sealing of the Drug Product

The drug product filling/sealing manufacturing process can introduce process-related impurities into a biopharmaceutical product, such as residual hydrogen peroxide and excess silicon oil.

Aseptic processing is essential for biopharmaceutical filling processes. Vapor phase hydrogen peroxide (VPHP), as a bio-decontamination agent for isolators and cleanrooms, has become standard where filling operations are carried out. However, residual levels of hydrogen peroxide can oxidize recombinant proteins and monoclonal antibodies (especially the methionine, tryptophan and cysteine amino acids). The migration of the biopharmaceutical solution through silicone tubing on the filling line, and open, filled glass vials in the aseptic environment, are considered important sources of residual hydrogen peroxide uptake into a drug product [11].

Silicone oil is needed to coat the rubber stoppers for sealing the glass vials or the rubber plunger to move the liquid out of a pre-filled syringe container closure system. Excess silicone oil on the stoppers/plungers can result in silicone oil droplets in the product solution that could enhance aggregation of proteins. As discussed in Chap. 9, leachables can arise where the biopharmaceutical product contacts a surface.

For residual elemental impurities in the final drug product, a risk-based assessment is prudent [9]:

> However, potential elemental impurity sources included in drug product manufacturing (e.g., excipients) and other environmental sources should be considered for biotechnologically-derived drug products. The contribution of these sources to the finished product should be assessed because they are typically introduced in the drug product manufacture at a step in the process where subsequent elemental impurity removal is not generally performed. Risk factors that should be considered in this assessment should include the type of excipients used, the processing conditions and their susceptibility to contamination by environmental factors (e.g., controlled areas for sterile manufacturing and use of purified water) and overall dosing frequency.

10.2.1.6 Applying the Minimum CMC Regulatory Compliance Continuum

As discussed in Chap. 4, Sect. 4.3.1, the regulatory authorities accept a minimum CMC regulatory compliance continuum risk-based approach for biopharmaceutical manufacturing. For the control of the recombinant protein and monoclonal antibody process-related impurities, the following guidance is provided during early-stage (including FIH) clinical development [12]:

S.3.2. Impurities

Process related impurities (e.g. host cell proteins, host cell DNA, media residues, column leachables) and product related impurities (e.g. precursors, cleaved forms, degradation products, aggregates) should be addressed. Quantitative information on impurities should be provided including maximum amount for the highest clinical dose. For certain process-related impurities (e.g. antifoam agents), an estimation of clearance may be justified. In case only qualitative data are provided for certain impurities, this should be justified.

S.4.1. Specification

Upper limits, taking into account safety considerations, should be set for the impurities.

P.5.1. Specification

Upper limits, taking safety considerations into account, should be set for impurities. They may need to be reviewed and adjusted during further development. For the impurities not covered by the active substance specification, upper limits should be set, taking into account safety considerations.

Increasing understanding and control over the process-related impurities is expected to evolve as the biopharmaceutical moves from early-stage clinical development into late-stage clinical development. At the time of submission of the market approval dossier for the protein-based biopharmaceutical drug product, it is expected by the regulatory authorities that (1) the control strategy for the various process-related impurities is defined, and (2) the assigned residual level limit for the process-related impurities can be justified:

ICH M4Q(R1) [13]

Impurities

In addition to evaluating the purity of the drug substance and drug product, which may be composed of the desired product and multiple product-related substances, the manufacturer should also assess impurities which may be present. Impurities may be either process or product-related. They can be of known structure, partially characterized, or unidentified. When adequate quantities of impurities can be generated, these materials should be characterized to the extent possible and, where possible, their biological activities should be evaluated. Process-related impurities encompass those that are derived from the manufacturing process, i.e., cell substrates (e.g., host cell proteins, host cell DNA), cell culture (e.g., inducers, antibiotics, or media components), or downstream processing. Further, the acceptance criteria for impurities should be based on data obtained from lots used in preclinical and clinical studies and manufacturing consistency lots. Individual and/or collective acceptance criteria for impurities (product-related and process-related) should be set, as appropriate.

Under certain circumstances, acceptance criteria for selected impurities may not be necessary. For certain impurities, testing of either the drug substance or the drug product may not be necessary and may not need to be included in the specifications if efficient control or removal to acceptable levels is demonstrated by suitable studies. This testing can

include verification at commercial scale in accordance with regional regulations. It is recognized that only limited data may be available at the time of submission of an application. This concept may, therefore, sometimes be implemented after marketing authorization, in accordance with regional regulations.

FDA for BLA [14]

Impurities Profile. A discussion of the impurities profiles, with supporting analytical data, should be provided. Profiles of variants of the protein drug substance (e.g., cleaved, aggregated, deamidated, oxidized forms, etc.), as well as non-product related impurities (e.g., process reagents and cell culture components), should be included.

EMA for MAA [15]

The capacity of the proposed purification procedures to deliver the desired product and to remove product and process-related impurities (e.g. unwanted variants, HCPs, nucleic acids, media components, viruses and reagents used in the modification of the protein) to acceptable levels should be thoroughly evaluated. This generally includes establishment of adequate analytical methods required for respective impurity detection and an estimation of the concentrating or removing capacity for each unit operation followed by the determination of appropriate acceptance criteria. For certain process-related impurities (e.g. HCP, DNA, antibiotics) scale-down spiking experiments may be required to determine the removal capacity of the individual purification steps. Evaluation of purification steps for which high impurity clearance are claimed, operating in worst case and/or non-standard conditions (e.g. process hold times, spiking challenge) could be performed to document the robustness of the process. For some components (e.g. low-molecular weight media components), a risk-based approach is acceptable showing that no safety concerns like immunogenicity or toxicity are present.

The following is a case example of the process-related impurity profile for a market-approved single-chain Fv antibody fragment, Beovu (brolucizumab) [16]:

To assess the depletion capacity of the manufacturing process for process-related impurities (HCP, host cell DNA, culture media and buffer components removal), validation studies for removal of those impurities were performed and samples taken from different process steps were monitored. Spiking studies were also performed for the HCP and host cell DNA. The current manufacturing process was found capable of effectively and consistently reducing these impurities to low levels. Most of the process-related impurities were below the LOQ and below the safety concern threshold (SCT) of 1.5 µg/day.

As regards the extractables and leachables, a risk assessment including a toxicological assessment was performed for the materials that are in contact with the product, process pools, buffers, media or solutions used for the manufacturing of brolucizumab active substance. Based on the comprehensive risk assessment, and the simulated in-use leachable study, it can be concluded that materials used in the manufacturing and storage of brolucizumab active substance have no risk of leachables in the active substance.

10.2.2 Viral Vectors

With a handful of *in vivo* viral vector biopharmaceuticals (i.e., recombinant adeno-associated virus, rAAV), now market-approved, an understanding of the regulatory safety concerns for their specific process-related impurity profile is now available. A summary of the origin of potential process-related impurities, across the various process steps of the viral vector manufacturing process, is presented in Table 10.3.

Table 10.3 Potential process-related impurities for the viral vector manufacturing process

Viral Vector Manufacturing Process	
Manufacturing Process Step	**Origin of Potential Residual Process-Related Impurities**
Starting Materials **MCB/WCB** **rDNA Plasmids**	**(so little MCB/WCB used; such a major dilution through the upstream production process)** **Process-related residuals from *E, coli* manufacture of DNA plasmids**
DS Upstream Production	**Medium components** **Host cell DNA** **Host cell protein (HCP)** **Transfection rDNA plasmid DNA** **Transfection reagent (e.g., PEI)**
DS Downstream Purification	**Nuclease** **Column resin leachable (e.g., affinity ligand)** **Buffers**
Formulation	**Impurities in the excipients**
Container Closure System	**Leachables from container** **Leachables from closure**
DP Filling/Sealing Process	**Leachables from product-contact surfaces (filling line tubing)**

Since the viral vector manufacturing process is similar to that for the protein-based biopharmaceutical manufacturing process (especially the upstream cell culture production and downstream chromatographic/filtration purification process steps), many of the process-related impurity types are also in common.

10.2.2.1 Sources of Process-Related Impurities

Each of the six process steps listed in Table 10.3 for the manufacture of the viral vectors contribute toward the total process-related impurity profile:

10.2.2.1.1 Starting Materials

The starting materials for the manufacture of a viral vector product contribute to the overall impurity profile. The manufactured starting materials (i.e., the multiple recombinant DNA plasmids manufactured by *E. coli*) may contain residual host cell DNA and host cell proteins, and antibiotics (e.g., kanamycin), and bring these impurities into the viral vector manufacturing process.

10.2.2.1.2 Upstream Production Process

The drug substance cell culture upstream production process for viral vectors is the major contributor to the process-related impurity profile. Potential upstream production impurities arise from the many raw materials in the cell culture media. Cell culture media are complex; containing the various amino acids, vitamins, sugars and inorganic salts for cells. In addition, the cell culture media can also include bovine serum albumin (BSA) or serum (e.g., fetal bovine serum), growth factors (e.g., insulin-like growth factor), lipids (e.g., cholesterol), antifoam (e.g., Pluronic F-68), etc. Each of these raw materials can become a process-related impurity. Residual transfecting plasmid DNA and transfection reagent (e.g., PEI) are other upstream production potential impurities. Also, potential upstream production impurities also arise from the transfected cell culture (e.g., HEK293) used to assemble and propagate the virus (e.g., host cell DNA and host cell protein).

10.2.2.1.3 Downstream Purification Process

The drug substance downstream purification process for viral vectors can introduce additional process-related impurities into the viral vector product. Potential downstream impurities such as enzymes (e.g., nuclease to lyse cells), leached ligands (e.g., leaching from an affinity column resin), all need to be evaluated.

AAV affinity chromatography is an important purification step for viral vectors. This method utilizes camelid single-domain antibodies (V_HH) that can be engineered to recognize different variations of the AAV vector. Leached V_HH from the affinity resin into the biopharmaceutical solution may trigger an immune response. Since these affinity columns are expensive, manufacturers try to obtain many cycles of use for them. Thus, the amount of leaching that occurs over the column lifetime use must also be studied.

10.2.2.1.4 Formulation, Container Closure System, DP Filling/ Sealing Process

Similar issues of process-related impurities as discussed in Sects. 10.2.1.4–10.2.1.5 for protein-based manufacturing process, apply for these viral vector drug products.

While the above six process steps apply to potential process-related impurities for manufacturing of an *in vivo* recombinant AAV viral vector, the potential process-related impurity concerns also apply to recombinant LV viral vector used as a starting material for *ex vivo* transduction of patient cells.

10.2.2.2 Applying the Minimum CMC Regulatory Compliance Continuum

As discussed in Chap. 4, Sect. 4.3.1, the regulatory authorities accept a minimum CMC regulatory compliance continuum risk-based approach for biopharmaceutical manufacturing. For the control of the viral vector process-related impurities, the following guidance is provided during early-stage (including FIH) clinical development:

FDA for IND [17]

Impurities (3.2.S.3.2)

We recommend that your manufacturing process be designed to remove process- and product-related impurities and that you have tests in place to measure levels of residual impurities. You should describe your test procedures in the IND and set appropriate limits. Your initial specification, including acceptance limits, for impurities may be refined with additional manufacturing experience. We recommend that you measure impurities throughout product development, as this will help ensure product safety, contribute to your understanding of the manufacturing process, and provide a baseline for comparing product quality after manufacturing changes, if needed.

Process-Related Impurities

We recommend testing for process-related impurities. These include, but are not limited to, residual cell substrate proteins, extraneous nucleic acid sequences, helper virus contaminants (i.e., infectious virus, viral DNA, viral proteins), and reagents used during manufacture, such as cytokines, growth factors, antibodies, selection beads, serum, and solvents.

FDA for IND Neurodegenerative Diseases [18]

Process-related impurities, such as host cell proteins, may contribute to unwanted immunogenic reactions in the study subject. For this reason, we recommend that the residual host cell protein levels be as low as can be reasonably achieved based on manufacturing experience. Depending on the location of product administration and the expected low turnover in the neuronal tissue, administered host-cell DNA impurity may be expected to persist for a prolonged period of time, and may contribute to the development of adverse events. As such, we recommend that sponsors carefully consider characteristics of the cell lines used in the manufacture of viral vectors that may impact the safety of the final product (such as presence of tumorigenic sequences) and limit residual host cell-DNA levels and DNA size. For additional information, refer to the CMC guidance (Ref. 1). The endotoxin levels should be kept to less than 0.2EU/kg body weight/hour when the drug product is administered by the intrathecal route. Lastly, plasmids can also be a source of process-related contaminants in viral vector (e.g., adeno-associated virus (AAV))-based GT products. Plasmids used to generate recombinant viral vectors should meet acceptable limits for purity, and manufacturing controls should be in place to avoid cross-contamination of plasmids. If the plasmids are manufactured in a multi-product manufacturing facility, they should be tested for the presence of other contaminating plasmids that may have been co-purified. Alternatively, a risk assessment may be conducted to provide assurance of freedom from other contaminating plasmids that may have been co-purified.

EMA for IMPD [19]

4.2.3.3 Impurities

Potential impurities in the DS and/or DP will be influenced by the nature of the product and the choice of production/manufacturing process. These include host cell proteins, host cell DNA, helper viruses/sequences, packaging viruses or sequences, residues of biological materials introduced during productions such as bovine serum or albumin, anti-

biotics, leachables from equipment, endotoxins, replication competent vector, and any proteins co-expressed with the transgene. Additional impurities needing consideration may include hybrid viruses in the case of virus vector production, lipids and polysaccharides in the case of production systems which involve bacterial fermentations, and RNA and chromosomal DNA in the case of plasmid purification.

Process-related impurities include residues of starting materials (residual DNA and residual host cell protein from each cell bank), raw materials (culture reagents, purification reagents and equipment materials, helper viruses and helper virus nucleic acid used in production), adventitious agents and leachables and extractables from the process.

Increasing control over the process-related impurities is expected to evolve as the biopharmaceutical viral vector moves from early-stage clinical development into late-stage clinical development. At the time of submission of the market approval dossier for the biopharmaceutical viral vector drug product, it is expected by the regulatory authorities that (1) the control strategy for the various process-related impurities is defined, and (2) the assigned residual level limit for the process-related impurities can be justified (as discussed previously for the protein-based biopharmaceuticals in Sect. 10.2.1.6).

There is limited specific information on the process-related impurities of market-approved rAAV viral vectors, but there are two case examples – one from the HEK293-triple DNA plasmid transfection manufacturing process (Luxturna) and the other from the insect cell-*Baculovirus* transduction manufacturing process (Roctavian):

Luxturna (Valoctocogene Roxaparvovec) [20]

LUXTURNA may also contain residual components of HEK293 cells including DNA and protein and trace quantities of fetal bovine serum.

Roctavian (Valoctocogene Roxaparvovec) [21]

Product- and process-related impurities were thoroughly evaluated. Process-related impurities include the various components of cell culture process, harvest and purification processes. Most impurities are present at levels below the limit of detection/quantitation (LOD/LOQ) or at very low levels. Characterisation of DNA impurities is extensive.

10.2.3 Genetically Modified Patient Cells

With an increasing number of genetically modified patient cells biopharmaceuticals for *ex vivo* application now market-approved, an understanding of the regulatory safety concerns for their specific process-related impurity profile is now available. A summary of the origin of potential process-related impurities, across the various process steps of the genetically modified patient cells manufacturing process, is presented in Table 10.4.

Table 10.4 Potential process-related impurities for the genetically modified patient cells process.

Genetically Modified Patient Cells Manufacturing Process	
Manufacturing Process Step	**Origin of Potential Residual Process-Related Impurities**
Starting Materials	
Patient Cells	**Unwanted cell types and fragments**
Viral Vector	**Viral vector impurities see Table 10.3**
DS Upstream Production	**Activating reagent Magnetic beads Residual transduction viral vector DNA Transduction reagent (e.g., cationic) Cell expanding components**
DS Downstream Purification	**Impurities in the wash solutions/buffers**
Formulation	**Impurities in the excipients**
Container Closure System	**Leachables from container Leachables from closure**
DP Filling/Sealing Process	**Leachables from product-contact surfaces**

10.2.3.1 Sources of Process-Related Impurities

Each of the six process steps listed in Table 10.4 for the manufacture of the genetically modified patient cells, contribute toward the total process-related impurity profile:

10.2.3.1.1 Starting Materials

The two starting materials for the manufacture of a genetically modified patient cells product contribute to the overall impurity profile. One starting material, the manufactured recombinant lentivirus vector using four recombinant DNA plasmids manufactured by *E. coli*, may contain residual host cell DNA and host cell proteins, and antibiotics (e.g., kanamycin) if used, and carry these into the viral vector manufacturing process. The total process-related impurities arising from the manufacture of the viral vector was discussed in Sect. 10.2.2.1. And in addition, the residual viral vector itself is a potential process-related impurity in the cell product. The second starting material, the patient cells, can introduce process-related impurities (e.g., the non-desired patient cell types, cell fragments). The second starting material is the collected patient cells. Patient cells are mixtures, that not only contain leukocytes (T, B, NK cells) but also cellular fragments.

10.2.3.1.2 Upstream Production Process

The drug substance upstream production process for genetically modified patient cells is the major contributor to the process-related impurity profile. Potential upstream production impurities arise from the culturing components added to the patient cells such as antibiotics, cytokines, magnetic beads bound with cell activation antibodies in the cell culture media. Cell culture media are complex, providing the various amino acids, vitamins, sugars and inorganic salts for cells. Residual transducing virus DNA and transduction reagent (e.g., PEI) are other upstream production potential impurities.

10.2.3.1.3 Downstream Purification Process

Keep in mind that the purification process for cells is primarily washing, but impurities in the washing solutions can introduce additional process-related impurities into the cellular product.

10.2.3.1.4 Formulation, Container Closure System, DP Filling/Sealing

Similar issues of process-related impurities as discussed in Sect. 10.2.1.4–10.2.1.5 for protein-based manufacturing process, apply for these genetically modified patient cells drug products.

10.2.3.2 Applying the Minimum CMC Regulatory Compliance Continuum

As discussed in Chap. 4, Sect. 4.3.1, the regulatory authorities accept a minimum CMC regulatory compliance continuum risk-based approach for biopharmaceutical manufacturing. For the control of the genetically modified patient cells process-related impurities, the following guidance is provided during early-stage (including FIH) clinical development:

> ***EMA for IMPD*** [22]
> After the genetic modification procedure, cells are generally subject to one or more additional manufacturing steps. Examples of such steps are washes to eliminate any possible stable or transient genetic modification system-related impurities (such as viral vector, plasmids, modifying enzymes, etc.), enrichment/isolation/purification/selection and culture for further expansion (to allow sufficient cell growth and achievement of a target dose) before being formulated and filled into the final containers.

A biopharmaceutical industry-wide (over 40 individuals contributed) guidance document, referred to as A-Cell, published in 2022, addresses control of the genetically modified patient cells process-related impurities [23]:

> Provisions to remove possible contaminants or impurities should be made early on in CMC development. As CMC development progresses, an emphasis should be placed on validating manufacturing processes that either remove or introduce contaminants. Contaminants should be removed or prevented in accordance with limits set based on safety data from relevant preclinical and/or clinical studies. Common contaminants in final cell-based therapy products include product-related impurities (which originate from the heterogeneous nature of the starting cellular material for cell-based therapies) such as of non-T cells, residual tumor cells, non-viable and non-transduced T cells, and extracellular vesicles; as well as process-related impurities such as cell culture media (especially in cases in which animal-derived supplements such as fetal bovine serum are used), matrix components, residual beads, residual reagents (e.g. DMSO), cellular DNA, antibodies, product isoforms, helper viruses, viral vectors or associated components (including empty capsids), and leachables and extractables from manufacturing components.

The A-Cell guidance also addresses residual beads and residual media components [23]:

> *Residual beads process impurities*
>
> During the manufacture of some cell-based therapies, such as CAR-T cells, reagent-coated beads are commonly used. These beads are considered ancillary materials used in the cell production process. Thus, sufficient bead removal or reduction has to be demonstrated prior to administration of the final product to patients. Residual beads could elicit a pharmacological or toxicological effect in the patient, e.g., due to unwanted T-cell activation *in vivo,* thus posing a risk to patient safety. Therefore, limits are often set for residual bead counts as part of the control strategy. While there are no standard acceptance criteria for residual bead impurity in the final DP due to the various types of beads (and reagents), broad range of indications, and method of delivery, studies for CAR-T production typically report >5-log depletion of beads throughout the manufacturing process to establish proof of safety.
>
> *Media supplement impurities*
>
> Effective cell therapies are dependent on optimal cell culture conditions, including the culture media selection. Choosing an appropriate medium that is conducive to the activation and expansion of T cells, as well as transduction with the CAR construct, is key to maintaining cell health and producing efficacious CAR-T products. Traditionally, media supplemented with serum (from animal or human origin) are widely used in the manufacturing process, but these supplements come with a range of challenges. Serum can vary in quality (critical material attributes) between batches due to its complex composition of a large number of constituents, and possibly result in inconsistency of the final CAR-T product efficacy. In some cases, high concentrations of serum have been shown to inhibit cell growth. Additionally, components of serum may also introduce adventitious agents into the production process, inhibit genetic modification such as transduction, resulting in adverse effects in the patient such as hypersensitivity reactions.

Increasing control of the process-related impurities is expected to evolve as the biopharmaceutical genetically modified patient cells moves from early-stage clinical development into late-stage clinical development. At the time of submission of the market approval dossier for the biopharmaceutical genetically modified patient cells drug product, it is expected by the regulatory authorities that (1) the control strategy for the various process-related impurities is defined, and (2) the assigned residual level limit for the process-related impurities can be justified:

> ***EMA for MAA*** [24]
>
> *4.2.5. Process validation*

> In addition to the requirements described for process validation in the Guideline on human cell-based medicinal products (EMEA/CHMP/410869/2006), the following aspects should be addressed, as applicable: absence of adventitious contaminants, absence of modifying enzymes and nucleic acids, removal of infectious particles, … removal or elimination of the target nucleic acid sequences when appropriate, removal or reduction of impurities associated with the genetic modification.

The following is a case example of the process-related impurity profile for a market-approved genetically modified patient cells for ex vivo use, Kymriah (tisagenlecleucel) [25, 26]:

> *Step 4. Expansion*
>
> On Day 3, following the second incubation period, the cell culture is washed to remove nonintegrated vector and residual vector particles. The washed cells are seeded into a disposable culture system. The culture is continued over a period of several days until the cell number is sufficient to enable harvest.
>
> *Step 5. Formulation*
>
> When the cell count reaches the required minimum number of total viable cells, the cells are separated from the beads using a magnetic separation device, harvested, and washed.
>
> There are significant levels of antibody conjugated beads (CD3/CD28) at the end of the cell expansion step before harvest (at maximum in 10 mL solution). The antibody conjugated beads (CD3/CD28) are actively removed to meet the acceptance criterion … Reduction of process-related residuals is a consequence of a series of washes and volume replacement processes. There are multiple washing and bead removal steps in the manufacturing process that are capable of removing impurities. Overall, this represents up to a 5000-fold reduction of residual carry-over by volume replacement.

10.2.4 mRNA Non-Viral Vector

While no mRNA non-viral vector therapeutic biopharmaceutical has been market-approved, lessons on the regulatory safety concerns for their specific process-related impurity profile can be learned from the mRNA non-viral vector for the COVID-19 mRNA vaccines.

A summary of the origin of potential process-related impurities, across the various process steps of the mRNA non-viral vector manufacturing process, is presented in Table 10.5.

10.2.4.1 Sources of Process-Related Impurities

Each of the six process steps listed in Table 10.5 for the manufacture of the mRNA non-viral vector, contribute toward the total process-related impurity profile:

Table 10.5 Potential process-related impurities for the mRNA non-viral vector process

mRNA Non-Viral Vector Manufacturing Process	
Manufacturing Process Step	**Origin of Potential Residual Process-Related Impurities**
Starting Materials **Linearized Plasmid** **NTPs**	**Process-related residuals from *E. coli* manufacture of DNA plasmids** **Enzyme used to linearized DNA** **Impurities in NTPs**
DS Upstream Production	**Residual linearized plasmid template DNA** **Impurities in polymerase**
DS Downstream Purification	**Column resin leachable** **Buffers**
Formulation	**Impurities in the manufactured lipids (elemental, organic solvents, mutagenic)** **Organic solvents used for mRNA encapsulaton**
Container Closure System	**Leachables from container** **Leachables from closure**
DP Filling/Sealing Process	**Leachables from product-contact surfaces**

10.2.4.1.1 Starting Materials

The linearized recombinant DNA plasmid starting material, which was enzymatically prepared from the recombinant DNA plasmid manufactured by *E. coli*, may contain residual host cell DNA and host cell proteins, and antibiotics (e.g., kanamycin), and carry these into the mRNA *in vitro* transcription (IVT) manufacturing process. Impurities in the second starting material, the nucleoside triphosphates (NTPs), can be brought into the reaction.

10.2.4.1.2 Upstream Production Process

The drug substance upstream production process for the mRNA non-viral vector is the major contributor to the process-related impurity profile. Potential upstream production impurities arise from the enzymatic IVT reaction on the linearized DNA plasmid (e.g., residual nucleoside triphosphates, residual polymerase). Residual linearized recombinant DNA plasmid itself is a potential process-related impurity in the non-viral vector product.

10.2.4.1.3 Downstream Purification Process

The drug substance downstream purification process for mRNA can introduce additional process-related impurities (e.g., leached resins), which need to be evaluated. Oligo(dT) affinity chromatography is an important purification step for mRNA, binding to the 3′ polyA tail. Leaching from the affinity resin into the biopharmaceutical solution may trigger an immune response. Since these affinity columns are expensive, manufacturers try to obtain many cycles of use for them. Thus, the amount of leaching that occurs over the column lifetime use must also be studied.

10.2.4.1.4 Formulation, Container Closure System, DP Filling/ Sealing Process

For mRNA non-viral vectors, formulation is another major contributor to the process-related impurity profile. The various lipids can both carry in impurities as well as degrade during the manufacturing process. The use of an organic solvent (e.g., ethanol) in the formulation process requires its removal.

10.2.4.2 Applying the Minimum CMC Regulatory Compliance Continuum

As discussed in Chap. 4, Sect. 4.3.1, the regulatory authorities accept a minimum CMC regulatory compliance continuum risk-based approach for biopharmaceutical manufacturing. While there is no formal regulatory guidance issued yet for the control of the mRNA non-viral vector process-related impurities, the basic principles for all process-related impurities apply.

The process-related impurities from the mRNA non-viral vector process need to be identified (see Table 10.5), and then an increasing control established over the residual levels as the clinical development moves from early-stage clinical development into late-stage clinical development. At the time of submission of the market approval dossier for the biopharmaceutical mRNA non-viral vector, it is expected by the regulatory authorities that (1) the control strategy for the various process-related impurities is defined, and (2) the assigned residual level limit for the process-related impurities can be justified.

The use of the mRNA non-viral vector for either *in vivo* gene therapy or *ex vivo* gene therapy (either autologous or allogeneic patient cells) has not yet reached market approval stage. But it can be safely assumed that the process-related concerns for mRNA non-viral vector manufacture will be similar to those for the viral vectors manufacture.

Lipids will be involved in the formulation of mRNA non-viral vectors. An idea of the regulatory authority concerns of process-related impurities arising from the use of lipids is found in the following case example of a market-approved COVID-19 mRNA vaccine:

Spikevax (COVID-19 mRNA vaccine (nucleoside-modified), Moderna [27]
SM-102, a novel, ionisable lipid excipient
The information provided on potential impurities in SM-102 comprise product related substances and process related impurities (elemental impurities, residuals solvents, peroxides, water content and inorganic impurities). The applicant will provide an evaluation of mutagenic impurities based on ICH M7. A test on benzene, which might be present in e.g. toluene or acetone should be performed on the final excipient or on a suitable intermediate if not otherwise justified.
PEG2000-DMG, the polyethylene glycol-lipid conjugate novel excipient
The following attributes have been included in the specification of the novel excipient PEG2000-DMG: …, residual solvents by GC, bacterial endotoxins, bioburden, residual heavy metals.

10.3 Specific Process-Related Impurities

While all process-related impurities need to risk-assessed from a patient safety perspective, several of these impurities are not only considered high risk, but the concern applies across all four of the biopharmaceutical drug product types (recombinant proteins/monoclonal antibodies, rAAV viral vectors, genetically modified patient cells, mRNA non-viral vectors):

Section 10.3.1 Residual DNA
Section 10.3.2 Residual host cell proteins
Section 10.3.3 Endotoxins
Section 10.3.4 Leachables

10.3.1 Residual DNA

Biopharmaceuticals are linked to biological manufacturing processes, which means that nucleic acid sequences, DNA/RNA, from cells or plasmids or vectors are involved; and therefore, there will be the need to control the residual DNA levels in the final biopharmaceutical. Different biopharmaceutical types have different residual DNA impurity concerns:

Recombinant proteins and monoclonal antibodies. The DNA impurity of concern is the residual host cell DNA from the recombinant cell line used in manufacturing.
Viral vectors: The DNA impurity of concern is both the residual host cell DNA from the *E. coli* cell line used in manufacturing and the residual plasmid DNAs from the transfection of the cells
Genetically modified patient cells. The DNA impurity of concern is the residual viral vector DNA remaining after *ex vivo* transduction of the patient cells.
mRNA non-viral vector.: The DNA impurity of concern is both the residual host cell DNA from the *E. coli* cell line used in manufacturing and the residual linearized DNA plasmid from the IVT manufacturing.

Residual host cell DNA in both recombinant proteins/monoclonal antibodies and viral vectors will be examined in this section. Residual starting material DNA (from plasmids or virus seeds) will also be commented on.

10.3.1.1 Recombinant Proteins and Monoclonal Antibodies

In the 1980s, when the first cell culture-produced recombinant proteins and monoclonal antibodies approached market-approval, concerns arose that some of the residual DNA might contain oncogenic or tumor-causing sequences, which would be a danger to patients. At that time, being cautious and having little safety data to rely on, the World Health Organization (WHO) recommended a host cellular DNA limit of not more than 100 picograms (pg) DNA per dose for biopharmaceuticals that were produced by continuous recombinant cell lines for parenteral administration. In 1998, WHO recommended that the DNA limit be reset to not more than 10 nanograms (ng) DNA per dose (a 100-fold increase in allowance) [28]. This new limit was based on additional data that showed that milligram amounts of DNA containing an activated oncogene from human tumor cells did not cause tumors in nonhuman primates during an evaluation period of 10 years and the understanding that human blood transfusions contain substantial amounts of DNA in plasma (75-450 μg per unit of blood). Today, both the recommended limit, plus a nucleic acid sequence size limit, are considered important in residual host cellular DNA control [29]:

> Residual risk might be a risk to your final product because of oncogenic and/or infectivity potential. There are several potential mechanisms by which residual DNA could be oncogenic, including the integration and expression of encoded oncogenes or insertional mutagenesis following DNA integration. Residual DNA also might be capable of transmitting viral infections if retroviral proviruses, integrated copies of DNA viruses, or extra chromosomal genomes are present. The risks of oncogenicity and infectivity of your cell substrate DNA can be lessened by decreasing its biological activity. This can be accomplished by decreasing the amount of residual DNA and reducing the size of the DNA (e.g., by DNAse treatment or other methods) to below the size of a functional gene (based on current evidence, approximately 200 base pairs).

These residual host cellular DNA upper limits were designed originally for recombinant proteins and monoclonal antibodies, where the source of nucleic acid residual is from the cellular DNA of the recombinant cell line used in the upstream production process.

10.3.1.2 Viral Vectors

With the clinical development and market-approval of the viral vector biopharmaceuticals (i.e., rAAV for *in vivo* use), two sources of residual DNA can result in the final product – (1) residual host cell DNA from the cell line used to propagate the virus, and (2) residual plasmid DNA from the multiple DNA plasmids used to

transfect the cell line. Both types of residual DNA are important to control and minimize.

The regulatory authorities have provided recommendations on the control and allowable levels for the residual DNA impurity arising from the multiple sources of DNA impurities in these biopharmaceuticals [17]:

> A common process-related impurity for many vector preparations is residual nucleic acid, such as cell substrate DNA, which can copurify with the vector. Some vectors, including AAV, can also package (i.e., inside the viral capsid) a large amount of plasmid DNA sequences (used during transfection) as well as cellular DNA. The presence of these impurities may have adverse effects on product quality and safety. We recommend that you optimize your manufacturing process to reduce non-vector DNA contamination in your product. Additionally, you should monitor and control the amount of extraneous nucleic acid sequences in your product.
>
> Since some cell substrates also harbor tumorigenic genetic sequences or retroviral sequences that may be capable of transmitting infection, we recommend that you take steps to minimize the biological activity of any residual DNA associated with your vector. This can be accomplished by reducing the size of the DNA to below the size of a functional gene and by decreasing the amount of residual DNA. We recommend that you limit the amount of residual DNA for continuous non-tumorigenic cells to less than 10 ng/dose and the DNA size to below approximately 200 base pairs.
>
> If you are using cells that are tumor-derived (e.g., Hela) or have tumorigenic phenotypes (e.g., HEK293, HEK293T) or other characteristics that may give rise to special concerns, the limitation of specific residual DNA quantities may be needed to assure product safety. In addition to controlling host cell DNA content and size, as described above, you should also control the level of relevant transforming sequences in your product with acceptance criteria that limit patient exposure. For example, products made in 293 T cells should be tested for adenovirus E1 and SV40 Large T antigen sequences, similarly products made in Hela cells should be tested for E6/E7 genes. Your tests should be appropriately controlled and of sufficient sensitivity and specificity to determine the level of these sequences in your product.

Note, that the same residual host cell DNA limit set for the protein-based biopharmaceuticals in Sect. 10.3.1.1, also applies here: less than 10 ng/dose and the DNA size to below approximately 200 base pairs. But the regulatory authorities are aware of the challenges in sometimes meeting this limit, as addressed by FDA CBER at a public town hall meeting with gene therapy subject matter experts [30]:

> ***For viral vector production, can cell lines such as HEK293 or 293 T cells that are derived from tumors be employed as long as the proper testing is performed to assess the amount, size, and level of the transforming gene products fall within the FDA guidance as being less than 10 nanograms per patient dose?***
>
> In general, yes, you can use these cell lines to make vectors. We recommend that you do a risk assessment to make sure what you would need to test for those cells and then that you monitor for the amount of host cell DNA for the amount of specific oncogenes from the cell - say, if it's HeLa cells, that you would monitor E6 or E7; for these 293 T cells, that you look for the T antigen and things like that. We understand that you may not be able to meet the WHO standard for certain gene therapy products such as AAVs at 10 nanograms per dose, and we recommend that you measure the amount you have, you report it, and you determine levels that are shown to be safe in lots that have been administered, and we'll evaluate the data as you go through.

10.3.1.3 Measuring Residual Host Cell DNA

While several methods are available to measure low levels of residual DNA, the two prominent methods are quantitative polymerase chain reaction (qPCR) and digital polymerase chain reaction (dPCR). Both PCR methods target a nucleic acid sequence (specific to the cell line used in manufacturing) and carry out thermal cycling steps with primers, probes, and a polymerase enzyme to amplify the targeted DNA sequence for detection. Prior to amplification, for qPCR the test sample is placed in a single vessel, while for dPCR the test sample is partitioned into thousands of individual vessels. qPCR data are collected during the exponential phase of the amplification reaction, while dPCR data are collected at the end of the limiting dilution amplification reaction. The qPCR test method is described in the European Pharmacopeia 2.6.35, *Quantitation and Characterization of Residual Host-Cell DNA*.

At least for the protein-based biopharmaceuticals, regulatory authorities also embrace a process validation approach, in lieu of QC batch-to-batch release testing, for residual host cell DNA [31]:

> Regarding residual DNA, it is already accepted for bacteria- and yeast-derived products that there is no need for routine testing provided that acceptable levels in the final product are achieved and, adequate validation data are submitted in the dossier. As far as DNA from continuous mammalian cell lines (CCLs) is concerned, this impurity was considered, in the past, as a risk factor because of concerns that residual host DNA may be tumorigenic. Further information, however, now suggests that CCL DNA poses much less of a risk than previously thought and accordingly should be considered as a general impurity. Validation studies (e.g., spiking experiments using an adequate size distribution of DNA) should be performed in an attempt to identify the major steps capable of reducing the DNA burden and to document the capacity of those steps in reducing residual cellular DNA content in the final product, to an acceptable and defined level. In addition to the validation studies, results of DNA quantitation on a minimum number of production batches (e.g., 5 consecutive batches) should be provided to demonstrate the reproducibility of the production process in reducing residual DNA to the level expected from the validation studies. Based on satisfactory validation data and consistent results on a limited number of production batches, its seems reasonable not to perform routinely CCL DNA tests at the purified bulk level (or other appropriate steps).

If the validation study demonstrates that the residual host cell DNA levels are acceptably low, and the recombinant protein or monoclonal antibody manufacturing process can be shown to robustly continue to maintain the residual DNA impurity levels at the patient-safe level, then a request to eliminate routine batch-to-batch QC release testing can be submitted to the regulatory authority.

10.3.1.4 Measuring Residual Plasmid or Viral Seed DNA in Transfected/Transduced Cells

Viral vectors can be manufactured either by recombinant plasmid transfection of HEK293 cells or by recombinant *Baculovirus* transduction of insect cells. Residual plasmid or baculovirus DNA can be measured either by qPCR or dPCR, using specific nucleic acid target sequences.

The measured levels will be of concern to the regulatory authorities. For example, FDA placed Sarepta Therapeutics' gene therapy clinical program on clinical hold when it was discovered that residual DNA plasmid fragments from the transient production process were present at an unacceptable level in their *in vivo* recombinant viral vector [32]. In another example, EMA had issues with the level of residual baculovirus (used in the transduction of insect cells) cellular DNA in the manufactured rAAV viral vector, Glybera (alipogene tiparvovec), that was under MAA review [33]:

> Of major concern however, was the carry over of baculovirus DNA. Residual baculovirus DNA was not measured in the three clinical lots administered in the first clinical AMT-011 trial. Residual baculovirus DNA varied in the 6 lots of the commercial process raising the question of whether these baculovirus sequences can be transcribed and corresponding proteins translated. The applicant was requested to investigate further whether this was possible. It was observed that following administration the recombinant virus is distributed to many different tissues and organs, as such the diversity of cell lines to be used in this evaluation needed to be carefully justified. A detailed risk assessment regarding the clinical consequences of administering significant amounts of baculovirus sequence, and the subsequent expression of proteins (even if theoretical), was also requested. The applicant was asked to take into consideration the fact that the virus is likely to remain in the patient for a considerable amount of time, and so therefore, will the baculovirus sequences. It was also noted, that the extent of co-packaged baculovirus DNA sequences could be underestimated due to the design of residual DNA assay.

10.3.2 Residual Host Cell Proteins (HCPs)

Living systems that produce the biopharmaceuticals consist of thousands of proteins that are part of their life function. During manufacturing, some amount of host cell-derived material will inevitably be introduced into the process stream, this includes host cell proteins (HCPs), that must be targeted for clearance through the purification process.

Residual HCPs have the potential to affect product safety, efficacy, and/or quality. The primary safety concern with HCPs in biopharmaceutical products is their immunogenic potential to induce anti-HCP antibodies that could induce a clinical effect in patients. Co-purifying lipase and esterase HCPs can degrade excipients such as polysorbate. Thus, regulatory agencies consider the presence of HCPs to be a critical quality attribute (CQA). Sufficient clearance of HCP impurities is essential.

Residual HCP profiles are complex, unique and specific to the particular host cells under specific culture conditions and manufacturing processes. HCPs can vary in pI (~3–11) and hydrophobicity, and HCPs display a wide range of molecular weights (from ~5 kDa to at least ~250 kDa), depending on the host cell and manufacturing process used. The number of HCPs in upstream samples can run anywhere from several hundred to over a thousand, depending on the host cell and culture conditions.

Two biopharmaceutical types involve cell culturing, thus they have residual host cell protein impurity concerns:

Recombinant proteins and monoclonal antibodies. Residual host cell proteins from the recombinant cell line used in manufacturing.

Viral vectors: Residual host cell proteins from the cell line used to assemble and propagate the virus.

10.3.2.1 Recombinant Proteins and Monoclonal Antibodies

The primary safety concern with HCPs in the recombinant proteins and monoclonal antibodies is their immunogenic potential to induce anti-HCP antibodies to neutralize the proteins in patients. A case example from a market-approved recombinant protein and a case example from a EMA MAA-filed monoclonal antibody, illustrate this safety concern:

Omnitrope (Recombinant Human Growth Hormone) [34]

In clinical studies EP2K-99-PhIII and EP2K-99-PhIIIFo with the earlier product Somatropin Sandoz powder (API Covance) up to 60% of the enrolled patients had developed anti-GH antibodies without showing any influence on growth rate. Careful investigation revealed high concentrations of host cell proteins (leading to development of anti-HCP antibodies in all patients treated with this product), which are known to enhance the antibody reaction against GH. Therefore, the manufacturing process for Omnitrope has been slightly modified by introducing additional purification steps during the development process of the product. The concentrations of host cell proteins in the subsequent formulations (API Sandoz and liquid (API Sandoz)) were within the range known from other authorised GH-containing products. Anti-GH antibody formation with Omnitrope and Somatropin Sandoz liquid (API Sandoz) was within the range known from other GH-containing products.

Monoclonal Antibody (Lebrikizumab) Clinical Material [35]

Following initial phase III studies, the material used in lebrikizumab clinical trials was found to have a process-related impurity identified as Chinese hamster ovary phospholipase B-like 2 (PLBL2) which co-purified with lebrikizumab. The immunogenic potential of PLBL2 and its potential impact on the immunogenicity of lebrikizumab in clinical studies were therefore evaluated. Data from the clinical studies demonstrated that ~90% of subjects developed a specific and measurable immune response to PLBL2. Given the high incidence of antibodies to PLBL2 as well as the comparable safety profile observed between placebo- and drug-treated subjects, no correlation between safety events and anti-PLBL2 antibodies could be made. Additionally, no impact on the incidence of anti-lebrikizumab antibodies was observed, suggesting the lack of an adjuvant effect from PLBL2. Interim analysis from ongoing phase III studies using material with substantially reduced levels of PLBL2 with patients having had longer exposure shows significantly less and dose-dependent frequency of immune responses to PLBL2.

In addition, residual HCPs can also have a direct effect on the quality of the biopharmaceutical itself. Copurifying HCPs proteases can compromise the yield and stability of the produced biopharmaceutical. Copurifying lipase and esterase HCPs can degrade excipients such as polysorbate forming particles [36].

In sharp contrast with their position on residual host cellular DNA limit, regulatory authorities have not set an official upper limit for residual HCP levels in any type of biopharmaceutical:

EMA [31]

Regarding the acceptable limits to be set, it should be stressed that it is impossible to set a common limit of HCP contamination for all biotechnology products. Indeed, host cell proteins are impurities that vary qualitatively and quantitatively from one product to another and even from one production/purification system to another. In the same manner, standardization of the analytical methods would be problematic as the reagents used in the tests are product- and production system-related. For HCP, it is difficult to identify the material sufficiently representative of the impurities to be followed at the relevant steps or to be used in a validation approach ("spiked material").

Unofficially, the target for recombinant protein and monoclonal antibody manufacturing has been not more than or equal to 100 ng/mg (ppm) during clinical development, with a lower level based on actual manufacturing process performance when seeking market approval [37].

For recombinant protein and monoclonal antibodies, Regulatory authorities also accept a process validation approach, in lieu of QC batch-to-batch release testing, for these residuals, but on a conservative 'case-by-case' basis [31]:

Finally elimination of HCPs, in most cases, makes use of chromatographic columns for which the selectivity and yield of the procedures depend not only on the quality of the material but also on the way to columns are used and re-used, storage conditions, sanitization and life span. The validation approach cannot cover all these critical parameters. For HCP, the validation approach should only be considered for each product on a "case by case" basis depending on i) the quality of the analytical method used to identify and quantitate the HCP impurities, ii) the design and quality of the validation studies and, iii) the intended use of the product (dose, treatment, duration, etc.).

10.3.2.2 Viral Vectors

HCP process-related impurities are a major concern not only for the recombinant proteins and monoclonal antibodies, but also for the cell culture-derived viral vectors:

FDA [17]

We recommend testing for process-related impurities. These include, but are not limited to, residual cell substrate proteins, ….

EMA [19]

Potential impurities in the DS and/or DP will be influenced by the nature of the product and the choice of production/manufacturing process. These include host cell proteins, … and any proteins co-expressed with the transgene. For the release specifications, tests should be developed and relevant (upper) limits set to monitor the residual levels of contaminants of cellular origin, e.g. host cell protein (including helper virus protein) ….

As there is no official residual HCP target for the recombinant proteins and monoclonal antibodies, there is no official residual HCP target for the viral vectors. To fill the void, biopharmaceutical manufacturer's consensus HCP target recommendations have been developed for the viral vector biopharmaceuticals:

- Plasmid starting material [38]: NMT 2% HCP

- Viral vectors [39]: Less than or equal to 100 ng HCP/$10^{12\text{-}13}$ vector genomes (For mRNA non-viral vector, the *in vitro* transcription mRNA production is cell-free, so no concern for host cell proteins).

10.3.2.3 Measuring Residual Host Cell Proteins

Two methods are available to measure trace amounts of residual HCPs in biopharmaceuticals. Enzyme-linked immunosorbent assays (ELISAs) remain the biopharmaceutical industry's standard method for HCP detection. As illustrated in Fig. 10.2, if HCPs are present, they will bind to the capture antibody attached to the plastic plate, and the reporting antibody with a label will then bind to the captured HCP; the stronger the label signal, the higher the level of residual HCPs present. Biopharmaceutical manufacturers at the early clinical development stage frequently start out using a vendor purchased (also referred to as commercially-available or generic) HCP ELISA kit to measure the residual level of HCPs in their product, but by the late clinical development stage they typically switch over either to their own custom, in-house, product-specific, proprietary HCP ELISA test method or a platform proprietary HCP ELISA test method (the HCP standard and antibodies used are in common with the company's specific cell line used for other similar biopharmaceuticals). It is most critical that the chosen HCP test method reagents have the appropriate breadth for detecting all of the host cell proteins present (which can be determined using two-dimensional gel analysis or mass spectrometry). Both pharmacopeias have general chapters on the preparation of the critical reagents and

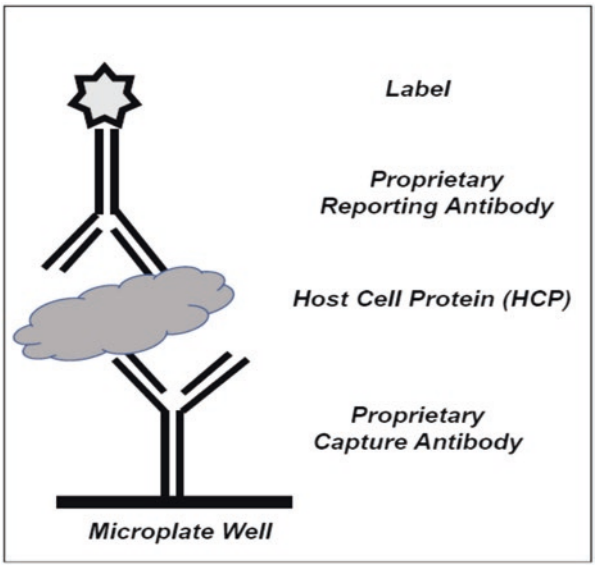

Fig. 10.2 Illustration of the ELISA test method for residual host cell proteins

validation of HCP ELISAs: European Pharmacopeia 2.6.34, *Host-Cell Protein Assays;* United States Pharmacopeia <1132> *Residual Host Cell Protein Measurement in Biopharmaceuticals*.

An orthogonal test method to measure trace amounts of specific residual HCPs in biopharmaceuticals is liquid chromatography/mass spectrometry (LC/MS). By searching the LC/MS data against protein databases containing the amino acid sequences from, e.g., Chinese hamster, human, *E. coli*, yeast, insects, etc., it is now possible to identify any HCP from the microorganism or eukaryotic cell line used for biopharmaceutical production [40].

Due to the safety concern, the reliability of the residual HCP measurement, along with the measured test results, will be evaluated by the regulatory authority in the submitted market approval application. The following EMA negative response, upon reviewing the HCP data submitted in the MAA dossier for Ixinity (recombinant coagulation factor IX) produced by CHO cells, illustrates the high level of concern that a regulatory authority places on residual HCP levels [41]:

> Based on the review of the data on quality, it is considered that the application for IB1001 cannot be recommended for positive opinion at present time since a major quality objection and other quality concerns are raised, which preclude a recommendation for marketing authorisation. The major objection is related to the insufficient reduction and control of HCP which led to a high number of patients developing anti-CHO protein antibodies and therefore is considered as a quality related safety concern. Further action to reduce the HCP content in the DS is required. To address this major objection from a quality point of view, the MAH should, for example, either show that this can be achieved by improving the performance and control of the current chromatographic steps or add an additional purification step to the manufacturing process. Clearance of HCP throughout the purification process should be shown by appropriate validation studies. In case an additional purification step is introduced comparability and characterization data will be required besides process validation data, as this change might alter the quality attributes of the active substance and the efficacy and safety profile of the product. Comparability of the post-change product with previously used clinical trial material should be investigated in order to address the question whether additional clinical studies will be needed.

The FDA also reacted negatively to the high HCP levels in Ixinity (recombinant coagulation factor IX) presented in the submitted BLA dossier, especially when 23% of the patients developed antibodies against CHO HCPs. The FDA issued a Complete Response (CR) Letter to the manufacturer which required an improved purification process to be developed. Three (3) years later, after a resubmission of the updated BLA, the FDA finally approved the biopharmaceutical incorporating the improved purification process and a more sensitive HCP test method [42].

10.3.3 Residual Endotoxin – LAL and LER

Endotoxin (also referred to as bacterial endotoxin), derived from the cell walls of gram-negative bacteria, is a pyrogenic substance that causes fever which can lead to septic shock. Unfortunately, its presence is almost everywhere, from raw materials

to starting materials, to chromatography columns, to processed solutions, to container closures, etc. Its residual level present in all biopharmaceutical types must be tested for patient safety:

> ***Recombinant Proteins/Monoclonal Antibodies*** [43]
> Appearance, solubility, pH, osmolality, extractable volume, sterility, bacterial endotoxins, stabiliser and water, should be assessed where appropriate.
> ***Viral and Non-Viral Vectors*** [17]
> Purity testing includes assays for pyrogenicity or endotoxin and residual manufacturing impurities. Although the rabbit pyrogen test method is the current required method for testing certain licensed biological products for pyrogenic substances (21 CFR 610.13(b)), we generally accept alternative test methods, such as the Limulus Amebocyte Lysate (LAL).
> ***Genetically Modified Patient Cells*** [22]
> In addition to general pharmaceutical tests (e.g. sterility, endotoxin, appearance etc.), release testing should include analysis of quantity, identity, purity, impurities (product- and process-related) and potency.

The Limulus Amoebocyte Lysate (LAL) assay uses the blood of horseshoe crabs, which contains an enzyme cascade and co-factors leading to either gel clotting or chromogenic peptide cleavage, in the presence of endotoxin. Pharmacopeias (e.g., USP <85>; Ph.Eur. <2.6.14>; JP 4.01) provide the details on how the harmonized LAL test is to be performed, and the appropriate setting of system suitability criteria. The regulatory authority recommended residual target levels are [17]:

> For any parenteral drug, except those administered intrathecally or intraocularly, we recommend that the upper limit of acceptance criterion for endotoxin be 5 Endotoxin Unit (EU)/kg body weight/hour. For intrathecally-administered drugs, we recommend an upper limit of acceptance be set at 0.2 EU/kg body weight/hour. For intraocularly-administered drugs, we recommend acceptance criterion of not more than (NMT) 2.0 EU/dose/eye for injected or implanted DPs or NMT 0.5 EU/mL for ophthalmic irrigation products.

Testing for endotoxin alone might not be sufficient to verify the absence of pyrogenic substances, e.g., microbial contaminations by gram-positive bacteria cause pyrogenicity that is not detectable with the endotoxin assay. EP 5.1.10 requires verifying the absence of non-endotoxin pyrogens during the production process and in the final product as a prerequisite for using the endotoxin assay for batch release, while 21 CFR 610.13 requires demonstrating the equivalence of the chosen test method to the rabbit pyrogen test, which effectively also means that the absence of non-endotoxin pyrogens must be demonstrated. The FDA typically adds this requirement into the meeting minutes from a pre-BLA meeting with a manufacturer, as a way of reminding them that the test has to be done [44]:

> Summary report and results of the Rabbit Pyrogen Test conducted on three batches of drug product in accordance with 21 CFR610.13(b).

The term ‘low endotoxin recovery’ (LER), describes the failure to detect spiked endotoxin into a biological drug product solution. LER potentially represents a public health concern because endotoxin contaminations in LER positive sample matrices may yield false-negative results when tested by compendial endotoxin assays. Regulators have taken note of these concerns; and are requesting LAL hold time

studies in the submitted marketing application dossiers. Design of LER studies has been published by the BioPhorum Operations Group (BPOG), involving 14 biopharmaceutical companies in the testing [45]. The pharmaceutical association, PDA, in concert with FDA subject matter experts, has also published Technical Report 82 on the design of LER studies [46]. In addition, the FDA typically adds this LER requirement into the meeting minutes from a pre-BLA meeting with a manufacturer, as a way of reminding them that the testing has to be done [44]:

> Low endotoxin recovery studies. Certain product formulations have been reported to mask the detectability of endotoxin in the USP <85> *Bacterial Endotoxin Test* (BET). The effect of hold time on endotoxin detection should be assessed by spiking a known amount of standard endotoxin (RSE or purified CSE) into undiluted drug product and then testing for recoverable endotoxin over.

time.

10.3.4 Leachables

In the development of any drug product, careful consideration needs to be given to impurities that may originate from manufacturing equipment, process components, and packaging materials. These impurities could either have a direct impact on patient safety (i.e., toxicity) or interact with the drug itself impacting its quality or stability. Extractables are chemical species that migrate from packaging or container materials into the contents when exposed to certain solvents under exaggerated temperature and time conditions. Leachables are chemical species that make their way into the product under normal application conditions. There is generally an overlap such that the leachables involved may be classified as a subset of the extractables; however, if the leachable interacts with the drug product or packaging materials, new components can be present.

Leachables from process equipment and container closure systems are of major concern to regulatory authorities:

> ***FDA*** [47]
> 21 CFR 600.11 Physical establishment, equipment, animals, and care.
> (b) *Equipment.* All surfaces that come in contact with products shall be clean and free of surface solids, leachable contaminants, and other materials that will hasten the deterioration of the product or otherwise render it less suitable for the intended use.
> (h) *Containers and closures.* All final containers and closures shall be clean and free of surface solids, leachable contaminants and other materials that will hasten the deterioration of the product or otherwise render it less suitable for the intended use.
> ***EMA*** [48]
> When single use equipment is used in evaluation studies, consideration should be given to leachables and extractables. Information should be provided on the nature and amount of potential leachables, and the removal of such impurities. Besides data, this normally includes a risk assessment. Data do not necessarily need to be generated under actual process conditions, for example supplier data or data generated under representative model conditions may be suitable. During process evaluation, small scale studies are acceptable to assess leachable profiles, leachable removal and the impact of such impu-

rities on cell culture performance. For verification studies, commercial scale equipment should be used. Various batches of disposable components should be used, as appropriate, in the manufacturing of verification batches in order to assess their impact on the product quality.

Because of the large number of product-contacting surfaces that are encountered in biopharmaceutical manufacturing, prioritization of the risk from leachables is essential. Some of the risk assessment factors that should be considered are:

- Nature of the material – inherent resistance to leaching
- Nature of the product solution – its leaching power
- Nature of the leachables – toxicity, reactivity with biopharmaceutical
- Processing conditions – duration of product contact, surface contact area, pH, temperature
- Proximity to final drug product stage – further downstream is less opportunity to purify away

10.3.4.1 Highest Risk of Leachables for Biopharmaceuticals

For many biopharmaceuticals, the two highest risk areas for leachables are (1) single-use plastic bioreactors and bags that are used in the manufacturing of the drug substance, as well as the product solutions and buffers used during drug product manufacturing, and (2) the drug product container closure system.

The implementation of single-use bioreactors (SUBs) and plastic bags in biopharmaceutical manufacturing processes has increased significantly over the past few years. These plastic bags are manufactured from a variety of polymeric materials and additives using proprietary formulations and methods. A cross-section of the container wall typically consists of several distinct co-extruded layers, including a fluid-contacting layer, a gas barrier layer, adhesive layers, and an outer layer to provide mechanical strength and protection. While the fluid-contacting layer may be constructed of an inert material, leachables from the other layers could potentially migrate into the storage solution during the manufacturing process. Chemical additives used to manufacture plastic films are common sources of leachables. These include anti-oxidants, lubricants, adhesives, anti-statics, colorants, light stabilizers, and plasticizers. Degradation of the container material and/or additives during sterilization by gamma irradiation is another significant source of leachables. Extractable assessments of single-use plastic bags are available through the venders; however, venders cannot possibly be expected to conceive of every single condition under which their plastic bags will be used in a manufacturing setting; therefore, the responsibility of evaluating leachables in the context of a specific biopharmaceutical manufacturing setting falls squarely upon the shoulders of the manufacturer. Since the biopharmaceutical drug substance can be stored in the plastic bag up to several years at temperatures up to refrigeration temperature depending upon its established shelf life, determining the impact, if any, of leachables may be important to study on both fresh and aged drug substance batches. The BioPhorum

Operations Group (BPOG) have published two key guidance documents to help control potential risks associated with leachable impurities released by single use components. These BPOG documents include '*Best Practices Guide for Evaluating Leachables Risk from Polymeric Single-Use Systems Used in Biopharmaceutical Manufacturing*' [49] and '*Standardized Extractables Testing Protocol for Single-Use Systems in Biomanufacturing*' [50], and are widely used by biopharmaceutical manufacturers and venders, as best practice for extractables and leachables testing.

Biopharmaceutical drug product container closure systems consist of glass or polymer vials/syringes/bags, stainless steel needles, and rubber stoppers or septums. Extractable assessments of individual container closure components are available through the venders; however, venders cannot possibly be expected to conceive of every biopharmaceutical drug product formulation under which their components will be used; therefore, the responsibility of evaluating leachables in the context of a specific biopharmaceutical container closure system falls squarely upon the shoulders of the manufacturer. Since the biopharmaceutical drug product can be held in the container closure system up to several years at temperatures up to room temperature depending upon its established shelf life, determining the impact, if any, of leachables is important to study on both fresh and aged drug product batches.

10.3.4.2 Applying the Minimum CMC Regulatory Compliance Continuum

As discussed in Chap. 4, Sect. 4.3.1, the regulatory authorities accept a minimum CMC regulatory compliance continuum risk-based approach for biopharmaceutical manufacturing. Since the clinical outcome is closely monitored, regulatory authorities do not have a major concern for extractables and leachables (E&L) during the early clinical stages. Manufacturers primarily identify in their IND/IMPD submissions the product-contacting equipment and components, and if available, extractable information provided by the supplier. Specifically for the container closure of the drug substance and the drug product, any concerns associated with leachables are mentioned:

EMA for IMPD of Recombinant Proteins/Monoclonal Antibodies [12]

S.6. Container closure system

The immediate packaging material used for the active substance should be stated. Possible interactions between the active substance and the immediate packaging should be considered.

P.7. Container closure system

The intended primary packaging to be used for the IMP in the clinical trial should be described.

EMA for IMPD of Gene Therapy-Based Biopharmaceuticals [51]

S.6. Container closure system

The immediate packaging material used for the active substance should be stated. Information on the sterilisation procedures of the container and the closure should be provided. A possible interaction between the immediate packaging and the active substance should be considered.

P.7. Container closure system

> The intended primary packaging to be used for the IMP in the clinical trial should be described and compatibility with the product should be justified. For parenteral products with a potential for interaction between product and container closure system more details regarding biocompatibility may be needed.

Regulatory authorities encourage the manufacturer during late stage clinical development to consider the potential impact of possible E&L on the biopharmaceutical drug product in their choice of product-contacting equipment and container closure components, to avoid surprises before the container closure for the commercial product is finalized [12]:

> For products intended for parenteral use where there is potential for interaction between product and container closure system, more details may be needed (e.g. extractable/leachable for phase III studies).

To obtain market approval, justification of the container closure system for both the drug substance and the drug product, including any E&L concerns, is required in the submitted Common Technical Document (CTD) for market authorization [13]:

> *Drug Substance 3.2.S.6 Container Closure System*
> The suitability should be discussed with respect to, for example, choice of materials, protection from moisture and light, compatibility of the materials of construction with the drug substance, including sorption to container and leaching, and/or safety of materials of construction.
> *Drug Product 3.2.P.2.4 Container Closure System*
> The suitability of the container closure system (described in 3.2.P.7) used for the storage, transportation (shipping) and use of the drug product should be discussed. This discussion should consider, e.g., choice of materials, protection from moisture and light, compatibility of the materials of construction with the dosage form (including sorption to container and leaching) safety of materials of construction, and performance (such as reproducibility of the dose delivery from the device when presented as part of the drug product).

The FDA expects leachable studies to be conducted on the biopharmaceutical final drug product both on a fresh drug product batch and on a drug product batch that is at or near its expiry date. The shelf-life assessment of leachables is sometimes forgotten by manufacturers so the FDA has been granting BLA market approval, but with a post-market approval commitment to complete the leachable study:

> ***Monoclonal Antibody, Tremfya (Guselkumab)*** [52]
> Perform a leachable study to evaluate the drug product container closure system through the end of shelf-life when stored under the recommended conditions. Testing will be performed at regular intervals and will include appropriate methods to detect, identify, and quantify organic non-volatile (e.g., HPLC-UV-MS), volatile (e.g., headspace GC-MS) and semi-volatile (e.g., GC-MS) species and metals (e.g., ICP-MS). Study results will be updated annually in the BLA Annual Report. Submit complete data and the risk evaluation for potential impact of leachables on product safety and quality to the BLA. The timeline you submitted on June 20, 2017, states that you will conduct this study according to the following schedule:
> Final Protocol Completion: 09/2017.
> Study Completion: 01/2020
> Final Report Submission: 06/2020.
> ***Monoclonal Antibody, Margenza (Margetuximab-cmkb)*** [53]

Conduct a DP leachables study to evaluate the DP container closure system through the end of shelf-life when stored under the recommended conditions of 2-8 °C. Analysis will include appropriate methods to detect, organic non-volatile (e.g., HPLC-UV-MS), volatile (e.g., headspace GC-MS) and semi-volatile (e.g., GC-MS) species and metals (e.g., ICP-MS) including their chemical identification and quantitation. Testing will be performed at regular intervals throughout the shelf life with study results to be updated annually in the BLA Annual Report. The final report to submitted to the BLA will include the complete data and risk evaluation for the potential leachables on product safety and quality. The timetable you submitted on November 19, 2020, states that you will conduct this study according to the following schedule:
Study Results: submission in annual report.
Study Completion: 09/2024
Final Report Submission: 03/2025.

10.4 Unknown Unknowns

Donald Rumsfeld, while serving as US Secretary of Defense in 2002, is quoted for his response about unknown unknowns [54]:

> There are things we know that we know. There are known unknowns. That is to say there are things that we now know we don't know. But there are also unknown unknowns. There are things we don't know we don't know.

Many laughed at his response, even though Mr. Rumsfeld was not the first to historically state it (there is reference to 'unknown unknowns' going back decades and even centuries). But for process-related impurities, there is a critical element of truth here.

A biopharmaceutical manufacturer knows what they know, and those are the process-related impurities that they will consider testing for during the manufacturing process and/or the drug product. A biopharmaceutical manufacturer also knows that there are unknowns (e.g., impurities that lack an appropriate test method), and those are the process-related impurities for which a risk-based safety assessment might be carried out to determine if further toxicology studies are needed. It is the unknown unknowns, gaps in our process-related impurity knowledge that we don't know exist, that could create patient safety concerns. Genuine surprises arise out of this category. It is so easy to become complacent and believe that the manufacturing process is under full control. But if the right impurity tests are not being done, or the analytical methods are insufficient to reliably measure the process-related impurities, or a thorough process-related impurity profile assessment is lacking, then one enters into the unknown unknowns.

The current state of concern for carcinogenic nitrosamine impurities in biopharmaceuticals is a perfect example of unknown unknowns. Nitrosamine (also called N-nitrosamine) impurities became a source of concern to regulatory authorities in 2018 when these compounds were surprisingly discovered in certain chemical drug products. Nitrosamines [having the chemical structure (R1)(R2)-N-N=O] occur naturally in very small quantities, but they are a potential carcinogen. Regulatory

requirements to test all medicines for nitrosamines became a mandate, and additional chemical drugs, especially those with an exposed amine functional group were found to also be of concern. Biologicals are now also required to be evaluated for the risk for nitrosamines [55]:

> Following the conclusion of the review under Article 5(3), the CHMP considered that there is also a risk of presence of nitrosamines in biological medicinal products, in particular for the biological medicines with the following risk factors:
>
> - biologicals containing chemically synthesised fragments, where risk factors similar to chemically synthesised active substances are present;
> - biologicals using processes where nitrosating reagents are deliberately added;
> - biologicals packaged in certain primary packaging material, such as blister packs containing nitrocellulose.

Two case examples from market approved biopharmaceuticals show the type of nitrosamine review required today:

Lunsimio (Mosunetuzumab) [56]

Mosunetuzumab is a CHO-produced humanised full-length anti-CD20/CD3 T-cell-engaging bispecific antibody of isotype immunoglobulin G1 (IgG1).

A risk assessment regarding the potential presence of nitrosamines in Lunsumio was provided where it is concluded that the risk is negligible. Based on the information provided it is accepted that no risk was identified on the possible presence of nitrosamine impurities in the active substance or the related finished product. Therefore, no additional control measures are deemed necessary.

Abecma (Idecabtagene Vicleucel) [57]

The patient's T cells are engineered *ex vivo* to express the anti-BCMA CAR on the T cell surface.

Based on a review of the manufacturing process, a risk analysis and the information received from both the raw material and the single use component suppliers, no actionable risk for the presence of nitrosamine impurities in ide-cel finished product was identified. The applicant will assess the risk for the presence of nitrosamines in the event of process and/or material changes and will initiate confirmatory testing as required if a risk is identified.

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Chapter 11
Seemingly Endless Biomolecular Structural Variants

Abstract Compared to chemical drugs, biopharmaceuticals have a highly complex, seemingly endless, biomolecular structural variant profile, especially due to the biological cell-based manufacturing processes employed in their biosynthesis. And each type of biopharmaceutical – be it a protein, a viral vector or a genetically modified patient cell – will have not only multiple structural variants but also different types of structural variants. Sometimes a molecular structural variant will have the same activity, safety and efficacy as the intended biopharmaceutical, but sometimes not. Therefore, knowing the type of biomolecular structural variants that could be present, and how the manufacturing process contributes to their presence, are important first steps in a patient safety risk assessment. In this chapter, the numerous biomolecular structural variants associated with four different biopharmaceutical types (recombinant proteins and monoclonal antibodies, viral vectors, genetically modified patient cells, mRNA non-viral vectors), will be examined. Application of the minimum CMC regulatory compliance continuum risk-based approach towards the level of understanding expected for the biomolecular structural variants will be discussed.

Keywords Structure · Primary · Secondary · Tertiary · Posttranslational · Aggregation · Deamidation · Oxidation · Genomic · Truncation · Glycosylation · Proteomic · Biomolecular · Capsids · Empty

Compared to chemical drugs, biopharmaceuticals have a highly complex, seemingly endless, biomolecular structural variant profile, especially due to the biological cell-based manufacturing processes employed in their biosynthesis. And each type of biopharmaceutical – be it a protein, a viral vector or a genetically modified patient cell – will have not only multiple structural variants but also different types of structural variants. Sometimes a molecular structural variant will have the same activity, safety and efficacy as the intended biopharmaceutical, but sometimes not. Therefore, it is important to know the type of biomolecular structural variants that could be present.

J. Geigert, *The Challenge of CMC Regulatory Compliance for Biopharmaceuticals*, https://doi.org/10.1007/978-3-031-31909-9_11

The entire biopharmaceutical manufacturing process – from starting material(s) → drug substance upstream production → drug substance downstream purification → bulk drug substance formulation → drug product filling/sealing in a container closure system → shelf-life storage – contributes to the total presence and levels of biomolecular structural variants in the biopharmaceutical final drug product. In addition, how each of these individual manufacturing process steps are designed and carried out, can further impact the type and amount of biomolecular structural variants present in the final drug product. For example, for a recombinant protein, if a recombinant DNA sequence variant of the intended DNA sequence is present in the genome of the recombinant Master Cell Bank, then during the upstream production manufacturing process, both the intended recombinant protein and the alternate sequence variant protein can be expressed, which results in a biomolecular structural variant ending up in the final drug product. Therefore, the effective control over the biomolecular structural variants, requires the entire biopharmaceutical manufacturing process – from starting material(s) to drug product – to be considered.

In this chapter, the numerous biomolecular structural variants associated with four different biopharmaceutical types (recombinant proteins and monoclonal antibodies, viral vectors, genetically modified patient cells, mRNA non-viral vectors), will be examined. Application of the minimum CMC regulatory compliance continuum risk-based approach towards the level of understanding expected for the biomolecular structural variants will be discussed.

Considerations for assigning an appropriate specification limit to the various identified biomolecular structural variants will be discussed in Chap. 14.

11.1 Is It a Biomolecular Structural Variant?

Owing to the typical small size of chemical molecules and the availability of abundant, sophisticated analytical methodology such as nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS), and X-ray powder diffraction (XRPD), the detection and identification of molecular structural variants in a chemical drug product is fairly straightforward. With the ever-increasing sophistication of the characterization tools today for protein analysis, we are getting a glimpse of the almost endless list of biomolecular structural variants possible with these large, complex biopharmaceutical drug products. But for the much larger size biopharmaceuticals (e.g., protein, virus particle, living cell), detection and identification of biomolecular structural variants is much more challenging.

Terminology can be confusing. There are ‘product-related substances’ and ‘product-related impurities’ [1]:

> **Product-Related Substances.** Molecular variants of the desired product formed during manufacture and/or storage which are active and have no deleterious effect on the safety and efficacy of the drug product. These variants possess properties comparable to the desired product and are not considered impurities.

Product-Related Impurities. Molecular variants of the desired product (e.g., precursors, certain degradation products arising during manufacture and/or storage) which do not have properties comparable to those of the desired product with respect to activity, efficacy, and safety.

A 'biomolecule' is a component related to living cells – proteins, DNA, RNA, other cellular components, and the cells themselves. 'Biomolecular structural variants' include both product-related substances and product-related impurities. Whether the biomolecular structural variant is comparable or not comparable to the desired biopharmaceutical product with respect to activity, efficacy, and safety, requires further evaluation (e.g., see discussion on potency in Chap. 12).

Each biopharmaceutical manufacturing process type needs to be risk-assessed, across each of its process steps, for contribution toward formation of biomolecular structural variants, as illustrated in the Ishikawa diagram (also known as fishbone diagram) of Fig. 11.1.

Specifics on each of the biopharmaceutical manufacturing process steps listed in Fig. 11.1, can be found in Chap. 6 (starting materials), Chap. 7 (upstream production), Chap. 8 (downstream purification) and Chap. 9 (formulation, container closure, filling/sealing).

The various biomolecular structural variants, from the manufacturing processes for each of the four biopharmaceutical types will be examined:

Section 11.2 Recombinant proteins and monoclonal antibodies
Section 11.3 Viral vectors
Section 11.4 Genetically modified patient cells
Section 11.5 mRNA non-viral vector

Application of the minimum CMC regulatory compliance continuum from early-stage clinical development through late-stage clinical development, and into market approval, will be examined for these biomolecular structural variants to understand regulatory authority patient safety concerns.

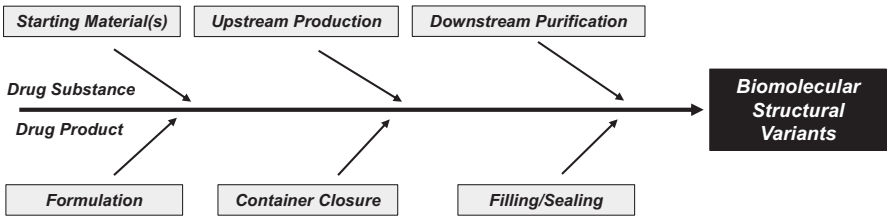

Fig. 11.1 Potential sources of biomolecular structural variants in a biopharmaceutical process

11.2 Recombinant Proteins and Monoclonal Antibodies

With over 200 market-approved recombinant proteins and monoclonal antibodies, much is known about the type of frequently encountered biomolecular structural variants with this biopharmaceutical manufacturing process type [1]:

> The following represents the most frequently encountered molecular variants of the desired product and lists relevant technology for their assessment. Such variants may need considerable effort in isolation and characterization in order to identify the type of modification(s). Degradation products arising during manufacture and/or storage in significant amounts should be tested for and monitored against appropriately established acceptance criteria.
>
> (a) Truncated forms: Hydrolytic enzymes or chemicals may catalyze the cleavage of peptide bonds. These may be detected by HPLC or SDS-PAGE. Peptide mapping may be useful, depending on the property of the variant.
> (b) Other modified forms: Deamidated, isomerized, mismatched S-S linked, oxidized or altered conjugated forms (e.g., glycosylation, phosphorylation) may be detected and characterized by chromatographic, electrophoretic and/or other relevant analytical methods (e.g., HPLC, capillary electrophoresis, mass spectroscopy, circular dichroism).
> (c) Aggregates: The category of aggregates includes dimers and higher multiples of the desired product. These are generally resolved from the desired product and product-related substances, and quantitated by appropriate analytical procedures (e.g., size exclusion chromatography, capillary electrophoresis).

11.2.1 Origin of the Protein Variants in the Manufacturing Process

Recombinant protein and monoclonal antibody molecular structure can be described in terms of a primary structure (i.e., polypeptide chain of amino acids with attached chains of carbohydrate), a secondary structure (i.e., intra-strand α-helix, inter-strand ß-sheet), and a tertiary structure (i.e., three-dimensional (3D) structure). The term 'higher order structure' (HOS) refers to secondary and tertiary structures as a group.

A summary of the origin of potential biomolecular structural variants across the various process steps of the recombinant protein/monoclonal antibody manufacturing process, is presented in Table 11.1.

11.2.1.1 Starting Material

Biomolecular structural variants can be caused by genetic variations at the starting material stage. The recombinant cell lines (MCB/WCB) will contain the intended gene of interest (GOI) sequence, but also may contain genetic mutated and/or truncated gene sequences. A survey of biopharmaceutical manufacturers uncovered that 5–20% of mammalian cell line clones carry genetic mutations of the transgene [2]. For example, the market-approved bispecific antibody, Lunsumio

Table 11.1 Origin of biomolecular structural variants across the recombinant protein/mAb process.

Recombinant Protein/mAb Manufacturing Process	
Manufacturing Process Step	**Origin of Potential Biomolecular Structural Variants**
Starting Material (Master/Working Cell Bank)	Genetic mutation in transgene sequence Truncation of inserted transgene sequence
Cell Culture DS Upstream Production	Misreading of mRNA codon Misincorporation of amino acids (due to deficient medium components) Post-translational modifications
DS Downstream Purification	Instability of protein-based molecule
Conjugation	New variants (e.g., DAR)
Formulation	Instability of protein-based molecule
Container Closure System	Instability of protein-based molecule (1^o, 2^o, 3^o structural changes)
DP Filling/Sealing Process	

(mosunetuzumab), has a gene sequence variant present, which fortunately did not impact the clinical safety profile [3]:

> A sequence variant is present at low levels that were within clinical experience. Based on the risk evaluation conducted by the Applicant, it can be agreed that the sequence variant at the levels observed is unlikely to impact the clinical safety profile, bioactivity, immunogenicity, or PK of mosunetuzumab, and it can also be agreed that no routine testing is required. This position is in line with the CHMP/EMA Scientific Advice. In conclusion, the approach taken by the Applicant to control the level of the sequence variant is endorsed.

11.2.1.2 DS Upstream Production

Biomolecular structural variants can be caused by genetic variations and/or post-translational variations during the drug substance upstream production stage.

The process of expressing the protein by the recombinant MCB/WCB can cause genetic biomolecular structural variants:

1. Biomolecular structural variants can arise due to misincorporation of amino acids into the protein sequence due to deficiencies in the medium nutrients. For example, when *E. coli* is starved of methionine (Met) and/or leucine (Leu) during its recombinant protein production phase, the recombinant organism can biosynthesize norleucine (Nle) and incorporate it into polypeptide chain at the

amino acid position normally occupied by methionine, yielding an analogue of the wild-type protein [4].

2. Biomolecular structural variants can arise due to misreading of the gene of interest (GOI) sequence (either DNA → mRNA or mRNA codon → amino acid). A survey of biopharmaceutical manufacturers uncovered that 5–30% of mammalian cell line bioreactor samples submitted for sequence variant analysis contained misincorporations of amino acids in the protein product [2].

The process of expressing the protein in living cells yields post-translational biomolecular structural variants:

3. The greatest contribution to biomolecular structural variants is the post-translational modification of the recombinant protein or monoclonal antibody while still within the production cell. Glycosylation, attachment of oligosaccharide chains to the recombinant protein or monoclonal antibody, is not a template-driven process (i.e., not like the straightforward synthesis of the protein from the gene). Glycosylation of a polypeptide chain is highly variable: (a) it is cell line dependent (e.g., bacteria cannot yield glycosylated proteins, yeast can produce hypermannosylation, etc.) [5], (b) it varies due to changes in the expression of hundreds of glycosyltransferases and glycosidases located in the cell line, and (c) it is impacted by the composition of the cell culture medium and the operating parameters of the production process. Glycosylation of the polypeptide chain via the amino acid asparagine (Asn) adds N-linked oligosaccharides, referred to as N-glycans. Glycosylation of the polypeptide chain via either the amino acid threonine (Thr) or the amino acid serine (Ser) adds O-linked oligosaccharides, referred to as O-glycans. Figure 11.2 illustrates the complex N-glycan profiles for two manufacturers of the market-approved monoclonal antibody trastuzumab produced by CHO cells [6]. In the example, Mylan (the biosimilar manufacturer)

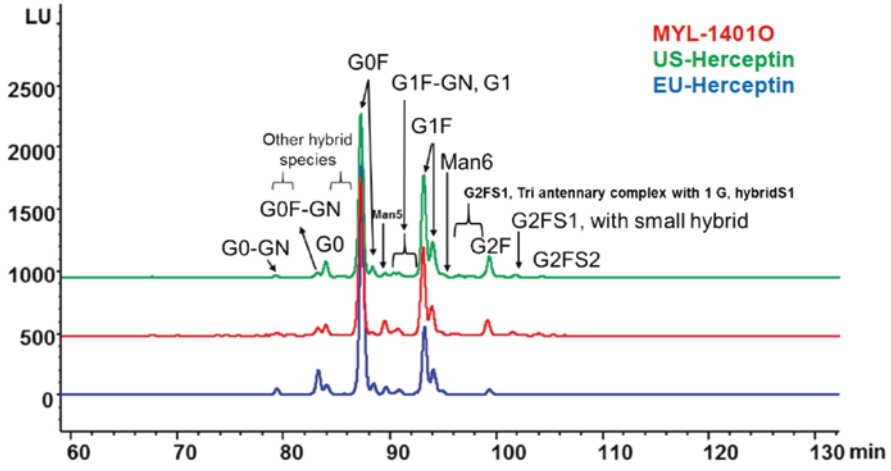

Fig. 11.2 N-glycan profiles of two market-approved highly similar trastuzumab mAbs

had to match the glycosylation profile on Herceptin (Genentech, the innovator manufacturer)
4. The produced biopharmaceutical can undergo further change within the bioreactor. For example, lysine (Lys) residues found at the C-termini of monoclonal antibodies can be cleaved by endogenous carboxypeptidases, resulting in either both heavy chains or just one of the two heavy chains losing a lysine.

11.2.1.3 DS Downstream Purification → DP Filling/Sealing

Once the biopharmaceutical has been produced by the upstream process, further biomolecular structural variants can be caused by instability of the recombinant protein or monoclonal antibody that can occur anywhere downstream of production – downstream purification through drug product manufacture through the shelf-life of the biopharmaceutical.

11.2.2 Structural Variants Due to the Nature of Proteins

Proteins are composed of amino acids linked together in a polypeptide chain. Specific amino acids in the polypeptide chain are especially sensitive to change. The following are some potentially unstable amino acids:

- Methionine (Met) can be oxidized to methionine sulfoxide [$-H_2C-S-CH_3 \rightarrow -H_2C-S(=O)-CH_3$]; tryptophan can be oxidized to hydroxytryptophan [$-H_2C$-indole → $-H_2C$-indole-(OH)]
- Cysteine (Cys) can react with a free thiol group on a second cysteine, and upon oxidation, yield a disulfide bond; correct disulfide bond formation is required for appropriate protein folding which impacts both functional activity and thermodynamic stability, but disulfide bond scrambling does occur
- Asparagine (Asn) can deamidate to aspartic acid (Asp) at neutral and basic pH values; since Asn deamidation occurs through formation of a five-member succinimide ring, isomerization occurs with formation of two isomers (Asp and iso-Asp)

Furthermore, the higher order structure (HOS) of recombinant proteins and monoclonal antibodies is also subject to change, with the most noticeable structural change being the formation of protein aggregates, either subvisible or visible particles. Proteins have a natural propensity to aggregate due to the dynamic nature of their structure, which is held together by a combination of Van der Waals forces, hydrogen bonds, disulfide linkages, and hydrophobic interactions. Disruption of this delicate balance can expose internal hydrophobic regions of the polypeptide chain, which may then interact with areas on other proteins to form larger complexes of misfolded proteins. This aggregation can be ‘native,’ in which the protein structure is maintained and the aggregation is largely reversible, or ‘non-native,’

where denaturation and structural changes mean this effect is largely irreversible. Aggregates may continue to grow and form over a wide-size range, including up to and beyond the formation of visible particles, and ultimately this leads to precipitation. Subvisible particles are usually defined as particles that are not visible to the naked eye and have a size of less than 100 microns. The pharmacopeias have compendial requirements relating to particulate matter in injections, which requires the quantification of subvisible particles that are ≥10 micron and ≥25 micron in size, usually using light obscuration and flow imaging techniques. With the use of size exclusion chromatography (SEC-HPLC), protein monomer and smaller aggregates (<0.1 micron in size), typically caused by oligomerization, are quantitated.

Protein aggregation can have an impact on product quality in terms of efficacy and/or immunogenicity. Therefore, the impact of the manufacturing process and subsequent handling of the protein needs to be risk assessed to minimize the potential impact of aggregation.

11.2.3 Totality of Possible Biomolecular Structural Variants

Summing up the biomolecular structural variants that can arise from the sources listed above. Table 11.2 presents a list of known structural variants typical for the protein-based biopharmaceuticals.

Characterization analysis for the presence of these biomolecular variant types that could be present in the recombinant protein or the monoclonal antibody is necessary for entry into human clinical studies, with ongoing studies to confirm the full list of variants, and eventually a risk-assessment plan for their control.

Table 11.2 Known structural variants for protein-based biopharmaceuticals

Known Structural Variants for Protein-Based Biopharmaceuticals	
Protein Sequence Sequence variants Mis-incorporated amino acids N-terminal variants C-terminal variants Protein hydrolysis variants	**Molecule Protein Properties** Acidic charge variants Basic charge variants Low molecular weight variants High molecular weight variants Monomer/Aggregation size variants
Individual Amino Acid Instability Oxidation variants Deamidation variants Disulfide scrambling variants	**Higher Order Structures** Secondary structural variants Tertiary structural variants
Molecule Carbohydrate Properties Site variants Site occupancy variants N-glycan structural variants	**Glycosylation** Afucosylation variants Galactosylation variants Sialyation variants

The presence or level of a single, specific biopharmaceutical structural variant can become a major point of discussion with regulatory authorities when seeking market approval. For example, during FDA's Advisory Committee review of the biosimilarity between Sandoz's etanercept (Erelzi) and Amgen's etanercept (Enbrel), the level of concern for a specific disulfide scrambling variant, shown in Fig. 11.3, was raised (the cysteines in the red outlined box indicate the site of disulfide scrambling).

Etanercept is a Fc-fusion protein, a homodimer of two 249 amino acid polypeptides. Disulfide scrambling among cysteine amino acids at positions 74, 78, 88 and

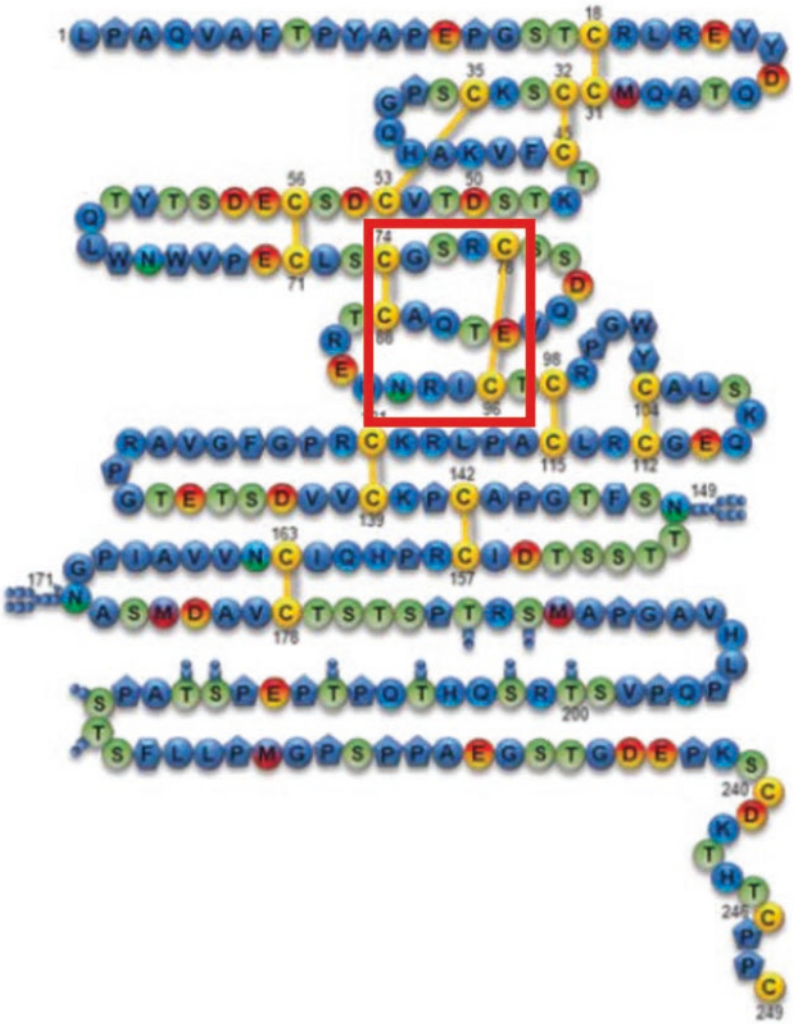

Fig. 11.3 Site of disulfide scrambling in the Fc-fusion protein etanercept

96 led to a measurable reduction in the biological activity of the molecule (indicated by the red lined box in Fig. 11.3), which raised questions about the reliability of the measurement and the need for comparable levels in the reference biopharmaceutical and the biosimilar [7]. Acceptable levels were found, and the biosimilar received market-approval.

So how many biomolecular structural variants are there for a recombinant protein or monoclonal antibody? Many, many. It has been calculated that for a monoclonal antibody, that the total potential biomolecular structural variants can be up to 100 million (assuming that each variant consists of one changed amino acid or one changed carbohydrate at a time) [8]. Not that it is possible to detect so many structural variants with today's analytical tools, but the ever-increasing sophistication of analytical methodology (e.g., capillary resolution, MS/MS) allows for more and more of these variants to be accounted for [9].

For a full appreciation of the depth of biomolecular structural variants encountered with recombinant proteins and monoclonal antibodies, check out the FDA Advisory Committee Meeting Packages and see the extensive analysis of numerous biomolecular structural variants, that biosimilar manufacturers encounter in bringing their biosimilars into the market:

- Amgen's Biosimilar to Avastin [10]
- Celltrion's Biosimilar to Rituxan [11]
- Amgen's biosimilar to Humira [12]

11.2.4 Applying the Minimum CMC Regulatory Compliance Continuum

As discussed in Chap. 4, Sect. 4.3.1, the regulatory authorities accept a minimum CMC regulatory compliance continuum risk-based approach for biopharmaceutical manufacturing. For the characterization and control of the recombinant protein and monoclonal antibody biomolecular structural variants, the following guidance is provided during early-stage (including FIH) clinical development [13]:

> *S.3.1. Elucidation of structure and other characteristics*
> Characterisation of a biotechnological or biological substance (which includes the determination of physico-chemical properties, biological activity, immuno-chemical properties, purity and impurities) by appropriate techniques is necessary to allow a suitable specification to be established. Reference to literature data only is not acceptable, unless otherwise justified by prior knowledge from similar molecules for modifications where there is no safety concern (e.g. C-terminal lysine for monoclonal antibodies). Adequate characterisation should be performed in the development phase prior to phase I and, where necessary, following significant process changes. All relevant information available on the primary, secondary and higher-order structure including post-translational (e.g. glycoforms) and other modifications of the active substance should be provided. Details should be provided on the biological activity (i.e. the specific ability or capacity of a product to achieve a defined biological effect). Usually, prior to initiation of phase I studies, the biological activity should be determined using an appropriate, reliable and

qualified method. Lack of such an assay should be justified. It is recognised that the extent of characterisation data will increase during development. The rationale for selection of the methods used for characterisation should be provided and their suitability should be justified.

To enter clinical development, 'adequate' understanding of the biomolecular structural variants present is expected, but as clinical development advances, so to is the expectation that knowledge about the biomolecular structural variants present will advance (*It is recognised that the extent of characterisation data will increase during development*).

At the time of submission of the market approval dossier for the protein-based biopharmaceutical drug product, it is expected by the regulatory authorities that (1) the biomolecular structural variants are known, (2 an assessment of activity, efficacy and patient safety has been made for the detected variants, and (3) a justified limit for those biomolecular product-related impurities of concern:

ICH Q6B [1]

Extensive characterization is performed in the development phase and, where necessary, following significant process changes. At the time of submission, the product should have been compared with an appropriate reference standard, if available. When feasible and relevant, it should be compared with its natural counterpart. Also, at the time of submission, the manufacturer should have established appropriately characterized in-house reference materials which will serve for biological and physicochemical testing of production lots. New analytical technology and modifications to existing technology are continually being developed and should be utilized when appropriate.

2.1.1 Physicochemical properties

A physicochemical characterization program will generally include a determination of the composition, physical properties, and primary structure of the desired product. In some cases, information regarding higher-order structure of the desired product (the fidelity of which is generally inferred by its biological activity) may be obtained by appropriate physicochemical methodologies. An inherent degree of structural heterogeneity occurs in proteins due to the biosynthetic processes used by living organisms to produce them; therefore, the desired product can be a mixture of anticipated post-translationally modified forms (e.g., glycoforms). These forms may be active and their presence may have no deleterious effect on the safety and efficacy of the product. The manufacturer should define the pattern of heterogeneity of the desired product and demonstrate consistency with that of the lots used in preclinical and clinical studies. If a consistent pattern of product heterogeneity is demonstrated, an evaluation of the activity, efficacy and safety (including immunogenicity) of individual forms may not be necessary. Heterogeneity can also be produced during manufacture and/or storage of the drug substance or drug product. Since the heterogeneity of these products defines their quality, the degree and profile of this heterogeneity should be characterized, to assure lot-to-lot consistency. When these variants of the desired product have properties comparable to those of the desired product with respect to activity, efficacy and safety, they are considered product-related substances. When process changes and degradation products result in heterogeneity patterns which differ from those observed in the material used during preclinical and clinical development, the significance of these alterations should be evaluated.

FDA for BLA [14]

Physicochemical Characterization

A description and the results of all the analytical testing performed on the manufacturer's reference standard lot and qualifying lots to characterize the drug substance should be included. Information from specific tests regarding identity, purity, stability and consistency of manufacture of the drug substance should be provided. Examples of analyses

for which information may be submitted include, but are not necessarily limited to the following: • amino acid analysis • amino acid sequencing, entire sequence or amino- and carboxy-terminal sequences • peptide mapping • determination of disulfide linkage • Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) (reduced and non-reduced) • isoelectric focusing • Conventional and High Pressure Liquid Chromatography (HPLC) e.g., reverse-phase, size exclusion, ion-exchange, etc. • mass spectroscopy • assays to detect product-related proteins including deamidated, oxidized, cleaved, and aggregated forms and other variants e.g., amino acid substitutions, adducts/derivatives.

Additional physicochemical characterization may be required for products undergoing post-translational modifications, for example, glycosylation, sulfation, phosphorylation, or formylation. Additional physicochemical characterization may also be required for products derivatized with other agents, including other proteins, toxins, drugs, radionuclides, or chemicals. The information submitted should include the degree of derivatization or conjugation, the amount of unmodified product, removal of free materials (e.g., toxins, radionuclides, linkers, etc.), and the stability of the modified product.

The following is a case example of the biomolecular structural variant profile for a market-approved monoclonal antibody, Oyavas (bevacizumab) [15]:

Characterisation

The Applicant comprehensively characterised the structure and biological properties of MB02 using orthogonal, state-of-the art analytical methods. The amino acid sequence of MB02 was experimentally confirmed by peptide mapping with 100% sequence coverage. Presence of C- and N-terminal variants (pyroglutamate, Lys-clipping), oxidation, and deamidation is presented. The higher order structure was evaluated by a combination of disulphide bridge mapping, far-UV CD, hydrogen/deuterium exchange mass spectrometry, μDSC, and DLS. Charge and size variants were determined using complementary analytical methods (CEX-HPLC, cIEF and CE-SDS, SE-HPLC, respectively). Glycoanalysis comprised the identification of the oligosaccharide pattern and site occupancy by peptide mapping and HILIC-UPLC-FLR; in addition, sialic acid content (NeuAc and NeuGc) was determined by UHPL-FLR. The absence of alpha-1,3-galactose structures has been demonstrated. Product-related variants and impurities detected by SEC- and CEX-HPLC were identified and characterised using relevant orthogonal methods. Functional characterisation of the variants/impurities included determination of VEGF binding by competitive ELISA. The levels of HHL fragments, as observed by non-reducing CE-SDS, were demonstrated to be higher in MB02 active substance as compared to Avastin. Detailed analyses by peptide mapping and mass spectrometry revealed amino acid substitutions, with relevant substitution levels (>1%) only occurring at position HC226. Extensive experiments support the Applicant's conclusion that the amino acid substitution is metabolic. Absolute quantitation based on stable isotope labelling revealed relative levels of amino acid substitution at HC226 of 1.29 ± 0.23%. The chemistry behind the change and the related information has been sufficiently discussed by the Applicant. The Applicant described future options to minimise the amino acid substitution in the commercial process and submitted a corresponding post approval change management protocol (PACMP) for implementation of an optimised active substance manufacturing process.

11.3 Viral Vectors

Viral vectors consist of both proteins (the capsid) and nucleic acid strands (the DNA or RNA inside the capsid):

- Recombinant adeno-associated virus (rAAV) vector consists of an icosahedral protein capsid built by three proteins (VP1, VP2, VP3). The protein capsid contains one single-strand of DNA, containing both the gene of interest (GOI) and other genes for function.
- Recombinant lentivirus (LV) vector consists of an icosahedral phospholipid enveloped protein capsid built by at least ten proteins (one of which is the enzyme reverse transcriptase). The protein capsid contains two single-strands of RNA, containing the gene of interest (GOI) and other genes for function.

With a handful of *in vivo* viral vector biopharmaceuticals (i.e., rAAV) now market-approved, and a larger set of *ex vivo* viral vectors (i.e., rLV) successfully used as starting material for market-approved genetically modified patient cell biopharmaceuticals, a limited understanding of the type of frequently encountered biomolecular structural variants with this biopharmaceutical manufacturing process type is now becoming available.

11.3.1 Sources of Biomolecular Structural Variants

Viral vectors consist of both proteins (the capsid) and nucleic acid (genes for functioning and the gene of interest inside the capsid). Both proteins and nucleic acids contribute to the total biomolecular variants for the viral vectors. The type of changes that proteins encounter (variations due to genetic issues, unstable individual amino acids, post-translational modifications, general instability) was discussed in Sect. 11.2. These same type of changes can occur with the protein capsid. The DNA strand inside the capsid also leads to biomolecular structural variants.

A summary of the origin of potential biomolecular structural variants, across the various process steps of the viral vector manufacturing process, is presented in Table 11.3.

Each of the six process steps listed in Table 11.3, apply to both common types of viral vectors – rAAV (DNA strand) for *in vivo* use and rLV (RNA strand) for *ex vivo* use.

11.3.1.1 Starting Materials

The integrity and genetic identity of each of the recombinant DNA plasmid starting materials is most important, as these gene sequences determine the outcome of the assembled viral vector.. The DNA plasmids are used in the transient transfection

Table 11.3 Origin of biomolecular structural variants across the viral vector process.

Viral Vector Manufacturing Process	
Manufacturing Process Step	**Origin of Potential Biomolecular Structural Variants**
Starting Materials **MCB/WCB** **rDNA Plasmids**	**Condition of HEK293 cells** **Genetic mutation in transgene sequence** **Truncation of transgene sequence**
Cell Culture **Plasmid Transfection** **DS Upstream Production**	**Capsid protein variants** **Genetic variant in viral genes** **Genetic variant in transgene** **DNA strand not inserted into capsid (empty capsids)** **Replication competent virus**
DS Downstream Purification	**Instability of capsid proteins** **Loss of DNA strand from capsid** **Change in virus function gene (loss of infectivity)**
Formulation	
Container Closure System	
DP Filling/Sealing Process	

process, and the resulting assembled viral vector will incorporate not only the intended gene sequences but also any gene sequence variants present, potentially resulting in either protein-related variants or gene of interest variants or both. Cross-contamination among DNA plasmids must be avoided [16]:

> Plasmids used to generate recombinant viral vectors should meet acceptable limits for purity, and manufacturing controls should be in place to avoid cross-contamination of plasmids. If the plasmids are manufactured in a multi-product manufacturing facility, they should be tested for the presence of other contaminating plasmids that may have been co-purified. Alternatively, a risk assessment may be conducted to provide assurance of freedom from other contaminating plasmids that may have been co-purified.

11.3.1.2 DS Upstream Production

Through transient transfection with multiple recombinant DNA plasmids, the viral particle is assembled and propagated in the HEK293 cell culture process. As was discussed with the protein-based biopharmaceuticals (see Sect. 11.2), mis-readings and mis-incorporations of the amino acids into the polypeptides of the capsid can occur.

During the virus assembly within the transfected cells, the DNA (if AAV) /RNA (if LV) strands that are inserted inside the capsid may be complete (the desired sequence outcome) or may have incomplete strands inside the capsid. Or even, no DNA/RNA strand.

Another major structural variant is the safety issue of replication competent virus (which is discussed in Chap. 13). It is a variant of the virus (can occur with both rAVV and rLV) that recombines with genes from the host cell to restore virus competency.

11.3.1.3 DS Downstream Purification → DP Filling/Sealing Process

During the drug substance downstream purification process through the drug product filling/sealing process, additional biomolecular structural variants can arise in the assembled virus particle. The proteins making up the virus capsid have certain inherently unstable specific amino acids (see Sect. 11.2 for specific amino acids subject to change). The gene of interest inside the capsid can be expelled resulting in additional empty capsids. And the viral vector might lose its infectivity.

11.3.2 Issue of Empty Capsids

One of the major challenges in the manufacturing of viral vectors is the effort required to optimize for full capsid enrichment, a problem prevalent with rAAV but not rLV. Full capsids are viral particles that contain the DNA strand with the gene of interest, while empty capsids are viral particles without the gene of interest. rAAV empty capsids increase the overall antigenic load and potentially exacerbate capsid-triggered innate and adaptive immune responses. rAAV empty capsids may compete with full capsids for receptor binding on target cells, which could necessitate an increase in the required vector dose.

Some gene therapy upstream production processes yield a high empty capsid load (e.g., up to 90% empty) which challenges the downstream purification process to purify away the empty capsid product-related component. For example, Spark Therapeutics used cesium chloride ultracentrifugation to remove the empty capsids [17]. Various manufacturers are exploring ion exchange chromatography, including anion exchange membranes, taking advantage of the slight physical difference between capsids with versus without the gene of interest present.

FDA's Cellular, Tissue and Gene Therapies Advisory Committee held a two-day meeting in September 2021 to discuss the toxicity risks of rAAV vector-based gene therapy products. One question asked of the committee was '*Please discuss whether an upper limit should be set for the total vector genome dose per subject, and, given that many AAV products contain significant amounts of empty capsids, please discuss whether an upper limit should be set on the total capsid dose'*. The following conclusions were reached by the Advisory Committee [18]:

> Preparations of AAV vector products can contain impurities, including empty capsids, that lack vector DNA. The AAV protein found in empty capsids might trigger antibodies, complements, and T cells. Although it is possible to remove empty capsids during the manufacturing process, doing so makes manufacturing more complex and expensive. Many manufacturers either do not remove empty capsids or only partially remove them.

As to the empty capsids, or total capsid dose, again the committee did not discuss support for arbitrary capsid limits, but instead some standardization and measures of vector QC, including vector titer, the number of empty capsids, capsids around other contaminants in the preparation. So, measures of those are evolving, but need more standardization and discussion in order to really know what's being measured and then to better consider limits, if any.

11.3.3 Applying the Minimum CMC Regulatory Compliance Continuum

As discussed in Chap. 4, Sect. 4.3.1, the regulatory authorities accept a minimum CMC regulatory compliance continuum risk-based approach for biopharmaceutical manufacturing. The level of knowledge and control for the biomolecular structural variants for a virus vector to enter human clinical trials is higher than that for the protein-based biopharmaceuticals – especially the knowledge about the capsid contents (what is inside them). The following guidance is provided during early-stage (including FIH) clinical development:

> ***FDA for IND*** [19]
> *Structure (3.2.S.1.2)*
> For viral-based gene therapies, you should include a description of the composition of the viral capsid and envelope structures, as appropriate, and any modifications to these structures (e.g., modifications to antibody binding sites or tropism-changing elements). We recommend that you include biophysical characteristics (e.g., molecular weight, particle size) and biochemical characteristics (e.g., glycosylation sites). You should also describe the nature of the genome of viral vectors, whether single-stranded, double-stranded, or self-complementary, deoxyribonucleic acid (DNA) or RNA, and the copy number of genomes per particle.
> *Impurities (3.2.S.3.2)*
> We recommend that your manufacturing process be designed to remove process- and product-related impurities and that you have tests in place to measure levels of residual impurities. You should describe your test procedures in the IND and set appropriate limits.
> *Product-Related Impurities*
> For viral vectors, typical product-related impurities may include defective interfering particles, non-infectious particles, empty capsid particles, or replicating recombinant virus contaminants. These impurities should be measured and may be reported as a ratio, for example, full:empty particles or virus particles:infectious units.
> ***FDA for IND Neurodegenerative Diseases*** [16]
> Drug product purity should be carefully evaluated early in product development. Purity assessment generally includes the evaluation of residual product-related impurities

(e.g., incomplete viral particles, cellular subtypes) and process-related impurities (e.g., residual host cell proteins, host cell DNA, endotoxin).

Product-related impurities in GT viral vector-based products used to treat neurodegenerative conditions include empty and wild-type viral particles, and replication-competent viruses. We recommend that qualified assays for evaluation of empty particles (where applicable), product-related variants (mutations in the viral genome, transgene, etc.), and non-recombinant viral particles (e.g., replication-competent viruses, wild-type viruses) be established early in the product development cycle.

EMA for IMPD [20]

4.2.3.1 Elucidation of structure and other characteristics

The data confirming the sequence of the therapeutic gene and genetic elements required for selectivity/regulation/control of the therapeutic sequence should be provided. Mapping data, e.g. via restriction endonucleases, should be provided to complement sequence data and transcription/translation elements and open reading frames analysed. It should be demonstrated that there is no inclusion of known oncogenic/tumourigenic sequences. Tests should be included to show integrity and homogeneity of the recombinant viral genome or plasmid and the genetic stability of the vector and therapeutic sequence. Phenotypic identity and analysis of the therapeutic sequences and selectivity/regulatory elements delivered by the vector should be included. Physicochemical characteristics such as refractive index, particle or molecular size average and distribution, and aggregation levels should be determined in characterization studies. For viral vectors the tissue tropism, infectivity (in a variety of cell cultures), virulence, replication capacity, ratio of infectious to non-infectious particles, and immunological characteristics where appropriate should be documented. Mean particle size and aggregates should be analysed. For viral vectors, insertion sites should be determined where appropriate and the potential for insertional mutagenesis established and associated risks fully evaluated.

4.2.3.3 Impurities

Product-related impurities, such as vectors with deleted, rearranged, hybrid or mutated sequences should be identified and their levels quantified. The possibilities for co-packaged extraneous DNA sequences being present in the vector should be explored. Reference should be made to potential degradation during the manufacturing process affecting key properties of the vector such as infectivity/non-infectious forms, plasmid forms with reduced transduction efficacy, or degradation of nucleic acid complexes through, for example, oxidation or depolymerisation. In the case of vectors designed to be replication deficient or conditionally replicating, the absence of replication competent vector should be demonstrated and/or conditional replication demonstrated.

Increasing control of the biomolecular structural variants is expected to evolve as the biopharmaceutical viral vector moves from early-stage clinical development into late-stage clinical development [19]:

> Your initial specification, including acceptance limits, for impurities may be refined with additional manufacturing experience. We recommend that you measure impurities throughout product development, as this will help ensure product safety, contribute to your understanding of the manufacturing process, and provide a baseline for comparing product quality after manufacturing changes, if needed.

At the time of submission of the market approval dossier for the biopharmaceutical viral vector drug product, it is expected by the regulatory authorities that (1) the control strategy for the various biomolecular structural variants is defined, and (2) the assigned limits for the biomolecular product-related impurities can be justified.

The following is a case example of the biomolecular structural variant profile for a market-approved viral vector, Zolgensma (onasemnogene abeparvovec), recombinant adeno-associated (rAAV) viral vector for *in vivo* use [21]:

Characterisation

Elucidation of structure and other characteristics

The active substance has been sufficiently characterised by physicochemical and biological state-of-the-art methods revealing that the active substance has the expected structure. The analytical results are consistent with the proposed structure. The characterisation study commences with a purification by gradient centrifugation of one Process B FP batch to produce a band profile. Each band of proposed full capsid material was isolated and further characterized by SDS-PAGE analysis, genomic (RNA/DNA) isolation, *in vitro* SMN expression analysis, residual DNA analysis, genomic titre by PCR, total protein content, analytical ultracentrifugation (AUC) and sequencing. The analysis of the capsid proteins suggests slight differences in residual DNA levels and capsid protein ratio among the bands. The isolated bands are shown to be capable of transducing cells and expressing the encoded SMN gene indicating the presence of functional and infectious particles. It remains to be demonstrated whether ratio of the bands and capsid protein ratio are comparable among batches and how the ratio is affected by the production process. The applicant committed to performing additional studies to characterize bands.

11.4 Genetically Modified Patient Cells

From proteins to virus vectors, now to patient cells – the size and complexity of the biopharmaceutical product increases. For cells, many of the tools to observe changes in cell function and to measure phenotypic drift have already been developed. For genetically modified patient cells, now an additional feature of cell characterization is observing how the transferred RNA strand via the recombinant lentivirus (rLV) transduction has changed the function of the cells.

11.4.1 Sources of Biomolecular Structural Variants

The patient cells are genetically modified by transduction with a recombinant lentivirus (rLV) viral vector. The goal of the transduction is to impart a new or improved genetic capability to the patient's own cells. The genetic capability can be measured by the cells expressing the new function. The hardest part is determining if any other cell functionality has been impacted.

A summary of the origin of potential biomolecular structural variants, across the various process steps of the genetically modified patient cells manufacturing process, is presented in Table 11.4.

Each of the six process steps listed in Table 11.4 for the manufacture of the genetically modified patient cells, contribute toward the total biomolecular structural variant profile:

Table 11.4 Origin of biomolecular structural variants across the genetically modified patient cells.

Genetically Modified Patient Cells Manufacturing Process	
Manufacturing Process Step	**Origin of Potential Biomolecular Structural Variants**
Starting Materials **Patient Cells** **rLV Viral Vector**	**Condition/functioning of incoming cells** **Non-target cells** **Transgene sequence variants**
Viral Vector Transduction DS Upstream Production	**Over-transduced cells** **Non-transduced cells** **Dead target cells** **Impact on other cell functions** **Replication competent virus**
DS Downstream Purification	**Cell instability or phenotypic drift** **(loss of cell viability, loss of cell functionality)** **Instability of transduced gene**
Formulation	
Container Closure System	
DP Filling/Sealing Process	

11.4.1.1 Starting Materials

For genetically modified patient cells, there are two starting materials: the incoming patient cells and the recombinant lentivirus (rLV) vector. The incoming patient cells are variable, being a reflection of the sickness of the patient at the time of collection. Processing of the collected patient cells may be variable in terms of cell types present. If the rLV vector starting material has any sequence variants in the transgene, those sequence variants can end up in the genetically modified cells.

11.4.1.2 DS Upstream Production

The upstream production transduction reaction is the largest contributor to biomolecular structural variants of the patient cells. Cells are transduced with the viral vector. Some cells will take up the transgene (with varying copy number), some will not. Some patient cells might be impacted in other cell functionalities (e.g., cell morphology, cell phenotype, cell metabolism, cellular responses, etc.). Another major structural variant is the safety issue of replication competent virus (which is discussed in Chap. 13).

11.4.1.3 DS Downstream Purification → DP Filling/Sealing

During the downstream handling, washing and packaging, the transduced cells may not be stable, and undergo change leading to loss of viability or some other cellular functional activity. It is possible for the transduced cells to be genetically instability leading to loss of the vector copy number.

11.4.2 VCN – Not Too Much, Not Too Little

A key characteristic of genetically engineered cells – whether recombinant mammalian cells (like CHO) for producing recombinant proteins or genetically modified patient cells – is copy number. The copy number is the average number of transgenes per transduced cells. For the recombinant mammalian cells used to manufacture recombinant proteins there is not pre-set target for what that copy number should be. Depending upon the goals of the development scientists, the copy number could be 1 or 10 or larger. The manufacturer makes the choice. But for human patient cells that are to be transduced, the copy number (referred to as vector copy number, VCN) takes on a potential safety concern.

Vector copy number (VCN) is a measure of vector integration into the human cell genome, a surrogate measurement of lentivirus transduction. But the VCN number also represents a safety concern, as lentiviral transduction presents a theoretical safety risk of insertional oncogenesis.

The FDA has much to say about the VCN [22]:

> Transgene integration can potentially alter expression of cellular genes and contribute to tumorigenicity. Therefore, transgene integration in the DP is an important safety parameter to measure for CAR T cell release. If the vector system directs transgene integration, the average number of integrations per CAR-positive cell, generally referred to as VCN, should be determined and reported on the Certificate of Analysis (COA) for each lot. Determining VCN as a function of total cells, includes non-transduced cells in the denominator and lowers the reported vector integration rate. Using the percentage of CAR-positive cells, the average VCN per CAR-expressing cell can be calculated. VCN as a function of CAR-expressing cells will provide a more accurate representation of the VCN in transduced cells and thus a more accurate representation of product risk for insertional mutagenesis. We recommend that the transduction process be optimized to control VCN while meeting target transduction frequency.

In that same guidance document, the FDA also provides recommendations on what the VCN value should be:

> We recommend that the VCN release criterion be determined through experience and justified based on a risk assessment. The risk assessment may include supporting data from studies such as insertion site analysis, clonal dominance, dose, indication, study population, etc. Supporting experimental data may be obtained from multiple engineering manufacturing runs. In some cases, such as CAR T cells manufactured without extended culture, determining the stably integrated VCN at the time of lot release testing may be difficult (e.g., due to persistence of episomal copies of non-integrated vectors). In this case, an interim VCN

assessment at the time of lot release, followed by subsequent VCN assessment(s) on cultured CAR T cells, may be needed to determine the stably integrated VCN.

But the regulatory authorities do not provide a target number for the VCN. However, an industry-wide study indicated that manufacturers of CAR T cell-based therapy typically keep the VCN below five [23].

11.4.3 Applying the Minimum CMC Regulatory Compliance Continuum

As discussed in Chap. 4, Sect. 4.3.1, the regulatory authorities accept a minimum CMC regulatory compliance continuum risk-based approach for biopharmaceutical manufacturing. For the control of the genetically modified patient cells biomolecular structural variants, the following guidance is provided during early-stage (including FIH) clinical development:

EMA for Genetically Modified Cells [24]

Rigorous characterisation of the genetically modified cell medicinal product (either alone or in combination with a medical device) is essential.

The use of a range of appropriately qualified molecular, biological and immunological methods for the following characteristics should be addressed, as appropriate:

- cell identity and viability
- cell phenotype / morphology
- heterogeneity of the cell population (e.g. percentage of sub-populations)
- proliferation and/or differentiation capacity of the genetically modified cells
- cell functionality (other than proliferation/differentiation, when applicable)
- transduction/transfection efficiency (e.g. percentage of transduced cells)
- sequence and integrity of transgene
- genetic stability upon *in vitro* proliferation and/or differentiation
- identity and activity of the expressed gene product
- vector copy number per transduced/transfected cell
- vector integration profile (when applicable)
- vector/transgenes removal or elimination (when applicable)
- vector release from cells

Tests should be applied to determine levels of cellular impurities such as other cell types including those unintendedly modified, non-transduced/non-transfected or unmodified genome edited target cells and cell fragments.

Increasing control of the biomolecular structural variants is expected to evolve as the biopharmaceutical genetically modified patient cells moves from early-stage clinical development into late-stage clinical development. At the time of submission of the market approval dossier for the biopharmaceutical genetically modified patient cells drug product, it is expected by the regulatory authorities that (1) the control strategy for the various biomolecular structural variants is defined, and (2) the assigned limits for the biomolecular product-related impurities can be justified:

The following is a case example of the biomolecular structural variant profile for a market-approved genetically modified patient cells (CAR T cell), Breyanzi (lisocabtagene maraleucel) [25]:

> Characterisation data has been collected throughout development. Overall, the characterisation data provided show homogeneity between the two cell components and has been provided for a relevant number of batches. Shared considerations were provided for the CD8+ and CD4+ active substances on the structural and functional characterisation of the CAR. The information provided, covering the CAR amino acid sequence, size and structure determination including primary structure as well as functional features of the CAR binding and activation domains, reassure about the CAR intended structure and functions. In terms of phenotypic and functional characterisation, the data suggests that the two cell components show a high degree of purity. The applicant has provided the requested information on the materials' phenotypic composition. Extensive characterisation has been provided also in terms of (VCN)/transduced T cell, CAR protein expression on the surface of cells and %CD3 + CAR+. The claim of a consistent CAR expression/cell is supported by characterisation data. As far as the product-related impurities are concerned, the approach adopted by the applicant takes into account residual cell types. Overall, the approach is extensive and endorsed. It is recognised that the characterisation has been performed on a relevant number of batches.

11.5 mRNA Non-Viral Vector

While no mRNA non-viral vector therapeutic biopharmaceuticals have been market-approved, lessons on the biomolecular structural variant profile have been learned from mRNA for the COVID-19 mRNA vaccines:

- mRNA is enzymatically produced from a recombinant linearized DNA by *in vitro* transcription (IVT). The identity and integrity of the linearized DNA plasmid starting material is critical, as the cell-free enzymatic reaction will transcribe not only the intended gene sequence into the mRNA but also any variant sequences.
- Intact mRNA is necessary for functionality, so the enzymatic reaction conditions (e.g., adequate nucleotide triphosphates, with modified UTP; adequate polymerase) must have the capability to driving the reaction to completion.
- Biomolecular structural variants of mRNA can consist of variants in the 5′ end of the molecule and/or the polyA tail at the 3′ end.

It is also known that the IVT reaction can cause aborted (short) transcripts, double-stranded RNA, and aggregates. In addition, mRNA is known to be unstable due to sheer forces during purification. The presence of contaminating RNases leads to mRNA degradation.

A summary of the origin of potential biomolecular structural variants, across the various process steps of the mRNA non-viral vector manufacturing process, is presented in Table 11.5.

Table 11.5 Origin of biomolecular structural variants across the mRNA non-viral vector process

Non-viral Vector (mRNA) Manufacturing Process	
Manufacturing Process Step	**Origin of Potential Biomolecular Structural Variants**
Starting Materials **Linearized DNA Plasmid**	**Genetic mutation in linearized DNA plasmid**
NTPs	**Impurities in NTPs**
In Vitro Transcribed DS Upstream Production	**Aborted mRNA incomplete transcripts** **5′ cap variant** **3′ poly(A) variant** **Double-stranded mRNA**
DS Downstream Purification	**Instability of mRNA molecule (sheer, RNases)**
Formulation	**Instability of mRNA molecule (aggregates, sheer, RNases)**
Container Closure System	
DP Filling/Sealing Process	

11.6 Variants – A Journey, Not a Destination

"*As soon as you go into any biological process in any real detail, you discover it's open-ended in terms of what needs to be found out about it*" is a quote ascribed to Joshua Lederberg, American molecular biologist and Nobel Prize winner. There seems to be an endless amount of biomolecular structural variants, especially as new and/or improved characterization tools become available.

Biopharmaceutical structural variant characterization is a journey, not a destination.

Comprehensive and extensive identification, characterization, and control of the biomolecular structural variants is a regulatory requirement for market approval of biopharmaceuticals. The CMC descriptions, in the submitted market application dossiers, are thoroughly reviewed by the regulatory authorities. Most of the time, the reviewers are satisfied with the level of information provided. But, at times, the manufacturer misses the mark. In that case, the manufacturer is notified of the deficiency and must respond with more sufficient characterization of the biomolecular structural variants. The following is a case example for a market-approved recombinant protein, Oxervate (cenegermin, recombinant nerve growth factor) [26]:

> The analytical package to characterise cenegermin includes SDS-PAGE, peptide mapping, SE-HPLC, RP-HPLC, IEX-HPLC, potency assay, N-terminal sequencing by Edman degradation, amino acid composition and protein concentration by amino acid analysis, secondary structure determination by Circular Dichroism (CD) and intact molecular weight determination by mass spectroscopy. Data of some reference standard assays were initially not described or presented for review. This was summarized during the procedure as a major objection on characterisation. A more thorough characterisation study was requested and, specifically, further information was requested on the purity profile, functional characterisation, protein modifications and secondary/tertiary structure of the active substance. During the procedure the Applicant provided the data from the analyses by SDS-PAGE gels, peptide mapping, SE-HPLC, RP-HPLC and IEX-HPLC, performed on rhNGF reference standards RS1213 and RS0515, and on rhProNGF reference standard RS0115. In addition, data obtained from the detection of aggregates by AUC and from SDS-PAGE in reducing and non-reducing conditions in gels silver stained were provided. The Applicant has provided a brief discussion to support adequate characterisation of potential post-translational modifications and has confirmed the suitability of the proposed RP-HPLC method to detect relevant post-translational modifications. The results of the assays performed for the characterisation of the secondary and tertiary structure were presented. The following analytical methods were selected for this purpose: disulphide bond mapping, far and near UV circular dichroism, free sulphydryls by Ellman's assay, intrinsic tryptophan fluorescence, FT-IR (Fourier Transformed Infrared) spectroscopy, Differential Scanning Calorimetry: the analysis is ongoing, to be provided before MA. The Applicant has committed to provide several reports, including data derived from the ongoing Differential Scanning Calorimetry analyses before marketing authorization with the closing sequence.

Comprehensive and extensive identification, characterization, and control of the biomolecular structural variants is more than of academic interest. An awareness of their presence alongside the desired biopharmaceutical product, and an increased understanding of how these product-related variants function, provide further assurance of the identity, quality, potency and safety of the biopharmaceuticals, be they either recombinant proteins/monoclonal antibodies, viral or non-viral vectors, or genetically modified cells.

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Chapter 12
Indispensable Potency (Biological Activity)

Abstract The measurement of potency (strength) is a regulatory requirement for all pharmaceuticals. For chemical drugs, the potency is determined by measuring the amount and purity of the active pharmaceutical ingredient that is present. Except for a few compendial small recombinant proteins (e.g., recombinant human insulin), measuring only the amount and purity is insufficient for determining the potency (i.e., biological activity) of biopharmaceuticals. The multiple factors that contribute to the challenge of measuring potency of a biopharmaceutical (whether recombinant protein, monoclonal antibody, viral vector, or genetically modified patient cells) will be examined in this chapter. The three major types of potency assays for biopharmaceuticals will be discussed: bioassay, surrogate assay, and assay matrix. Application of the minimum CMC regulatory compliance continuum risk-based approach will also be examined for the development, optimization, and validation of biopharmaceutical potency assays.

Keywords Strength · Potency · Activity · Therapeutic · Biological · Functional · Bioassay · Surrogate · Matrix · ADCC · CDC · Apoptosis · ELISA · Validation · Three-dimensional

Biopharmaceuticals are not like chemical drugs where potency (i.e., strength) can be determined directly by measuring the amount and purity of the active pharmaceutical ingredient (API) that is present. Except for a few compendial small recombinant proteins (e.g., recombinant human insulin), measuring only the amount and purity are insufficient for determining the potency (i.e., biological activity) of biopharmaceuticals. Multiple factors contribute to the measured potency of a biopharmaceutical (whether a recombinant protein, monoclonal antibody, viral vector, or genetically modified patient cells). First, a biopharmaceutical has a three-dimensional conformation to its higher order biomolecular structure that impacts the potency. Second, biopharmaceuticals have multiple biomolecular structural variants, which may or may not contribute to the total biological activity. Third, the biopharmaceutical molecule/virus particle/cell might have multiple biological

J. Geigert, *The Challenge of CMC Regulatory Compliance for Biopharmaceuticals*, https://doi.org/10.1007/978-3-031-31909-9_12

activities. For example, the typical IgG monoclonal antibody has at least three potential biological activities: antibody-dependent cell cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), and apoptosis/programmed cell death (PCD). Therefore, the measurement of potency for a biopharmaceutical is challenging. In this chapter, the three major types of assays for measuring the potency of biopharmaceuticals will be discussed: bioassay, surrogate assay, and assay matrix. Application of the minimum CMC regulatory compliance continuum risk-based approach will also be examined for the development, optimization, and validation of these biopharmaceutical potency assays.

12.1 Is It Active?

For all medicines, the 'strength' of the active pharmaceutical ingredient (API) must be determined. Terminology can be confusing with several words used to describe strength: 'therapeutic activity', 'biological activity', and 'potency'.

For a chemical drug, the strength of API present is its measured content and purity expressed either as (1) mass amount (mass/mass), or (2) concentration (mass/volume), or (3) units (e.g., mass amount of an antibiotic):

FDA [1]

210.3(b)(16). Strength means: (i) The concentration of the drug substance (for example, weight/weight, weight/volume, or unit dose/volume basis), and/or (ii) The potency, that is, the therapeutic activity of the drug product as indicated by appropriate laboratory tests or by adequately developed and controlled clinical data (expressed, for example, in terms of units by reference to a standard).

European Commission [2]

Strength of the medicinal product: The content of the active substances expressed quantitatively per dosage unit, per unit of volume or weight according to the dosage form.

Different strengths for the same chemical drug (e.g., different API amounts per tablet; 25 mg, 100 mg, etc.) are referred to as therapeutic activity or potency.

For a biopharmaceutical, however, the amount and purity of the API present, is not a sufficient measure of its strength (potency). Potency for a biopharmaceutical is the measurement of the biological activity in the amount of API present:

FDA [3]

600.3(s). The word potency is interpreted to mean the specific ability or capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended, to effect a given result.

EMA [4]

Potency: The measure of the biological activity using a suitably quantitative biological assay (also called potency assay or bioassay), based on the attribute of the product which is linked to the relevant biological properties.

ICH [5]

Biological Activity: The specific ability or capacity of the product to achieve a defined biological effect. Potency is the quantitative measure of the biological activity.

12.2 Importance of the Potency Assay

Determination of potency (biological activity) is one of the most important critical quality attributes (CQAs) for a biopharmaceutical. Multiple factors contribute to the challenge of measuring the potency of a biopharmaceutical (whether a recombinant protein, monoclonal antibody, viral vector, or genetically modified patient cells): (1) a three-dimensional higher order conformation of the biomolecular structure, (2) the presence of multiple biomolecular structural variants that may or may not contribute to the potency, and (3) multiple biological activities that may be present. Therefore, the importance of an adequate and appropriate biological activity measurement of biopharmaceuticals cannot be emphasized enough. There are five areas, throughout the life cycle of clinical development, where the potency measurement makes important contributions:

Section 12.2.1 Characterization
Section 12.2.2 QC batch-to-batch release
Section 12.2.3 QC stability program
Section 12.2.4 Product comparability after manufacturing process changes
Section 12.2.5 Demonstration of biosimilarity

12.2.1 Characterization

Regulatory authorities are clear on the importance of potency measurement as part of the characterization of a biopharmaceutical, including the type of biological activities that need to have assays developed for their measurement:

> ***Recombinant Proteins and Monoclonal Antibodies*** [5]
> *Biological activity*
> Assessment of the biological properties constitutes an equally essential step in establishing a complete characterization profile. An important property is the biological activity that describes the specific ability or capacity of a product to achieve a defined biological effect. A valid biological assay to measure the biological activity should be provided by the manufacturer. Examples of procedures used to measure biological activity include: Animal-based biological assays, which measure an organism's biological response to the product; Cell culture-based biological assays, which measure biochemical or physiological response at the cellular level; Biochemical assays, which measure biological activities such as enzymatic reaction rates or biological responses induced by immunological interactions. Other procedures such as ligand and receptor binding assays, may be acceptable. Potency (expressed in units) is the quantitative measure of biological activity based on the attribute of the product which is linked to the relevant biological properties, whereas, quantity (expressed in mass) is a physicochemical measure of protein content. Mimicking the biological activity in the clinical situation is not always necessary. A correlation between the expected clinical response and the activity in the biological assay should be established in pharmacodynamic or clinical studies. Often, for complex molecules, the physicochemical information may be extensive but unable to confirm the higher-order structure which, however, can be inferred from the biologi-

cal activity. In such cases, a biological assay, with wider confidence limits, may be acceptable when combined with a specific quantitative measure.

Viral Vectors [6]

Characterisation studies of GTIMP

Characterisation of a gene therapy active substance (which includes the determination of physico-chemical, biological and functional properties, purity and impurities) by appropriate techniques is necessary to allow relevant specifications to be established. The intended action of regulating, repairing, replacing, adding or deleting a genetic sequence should be demonstrated. The potency assay should normally encompass an evaluation of the efficiency of gene modification (infectivity/transduction efficiency/delivery efficiency) and the level and stability of expression of the therapeutic sequence or its direct activity or deletion. Where possible the potency assay should include a measure of the functional activity of the therapeutic sequence or the product of it.

Biological activity

The intended action of regulating, repairing, replacing, adding or deleting a genetic sequence should be demonstrated. The *in vitro* biological activity of all transgene(s) and any other expressed sequences should be determined. The level of transgene expression, associated biological activity, and factors associated with the proposed mechanism of action of the vector/delivery system including maintenance of the therapeutic sequence in the target cell should be analysed.

Genetically Modified Patient Cells [7]

Potency

To estimate the potency of the genetically modified cells, biological tests should be applied to determine the functional properties of the cells, where applicable, and those achieved by the genetic modification. The potency test(s) should provide, as far as possible, quantitative information on the intended function of the cell and the transgene product. The choice of the potency assay for release should be justified based on the characterisation studies and its feasibility as release assay, taking into account practical limitations (e.g. material available or limited shelf life). Due to their inherent variability, the limited predictability for the human situation and 3R considerations, biological potency tests in animal tissues, maintained *ex vivo* or in whole animals, should only be considered in situations where a suitable *in vitro* method cannot be developed. The potency testing should not be limited to cell functionality, but also include other relevant tests, e.g. cell viability. Furthermore, where relevant, release tests for the potential to proliferate, differentiate and persist after administration should be in place.

Genome-Edited (GE) Patient Cells [8]

When establishing potency tests for *ex vivo*-modified human GE DP, we recommend assays be developed that measure the properties of the cells and the intended functional outcomes of the genomic modifications resulting from GE. For example, we recommend that potency assays for a genome-edited CD34+ hematopoietic stem/progenitor cell product measure both the stem/progenitor cell activity and the functional outcome of the GE. In some instances, surrogate potency tests may be acceptable; however, it is critical that the data provided supports a correlation between the output of the surrogate potency test and the functional outcome of the GE.

It should be noted, that for some small-sized recombinant proteins (e.g., recombinant human insulin), typically compendial, where the physicochemical structure and higher order structure (e.g., three-dimensional conformation) are clearly known, measuring only the amount and purity of those proteins is possible for determining the potency (i.e., biological activity) [5]:

Importantly, a biological assay to measure the biological activity of the product may be replaced by physicochemical tests only in those instances where: sufficient physicochemical information about the drug, including higher-order structure, can be thoroughly estab-

lished by such physicochemical methods, and relevant correlation to biologic activity demonstrated; and there exists a well-established manufacturing history. Where physicochemical tests alone are used to quantitate the biological activity (based on appropriate correlation), results should be expressed in mass.

12.2.2 QC Batch-to-Batch Release

Regulatory authorities, emphasizing the importance of potency measurement as part of batch-to-batch QC release, provide the following guidance:

Recombinant Proteins and Monoclonal Antibodies [5]

A relevant, validated potency assay should be part of the specifications for a biotechnological or biological drug substance and/or drug product. When an appropriate potency assay is used for the drug product, an alternative method (physicochemical and/or biological) may suffice for quantitative assessment at the drug substance stage. In some cases, the measurement of specific activity may provide additional useful information.

Viral Vectors [6]

Generally the biological activity measurement will become the potency test for DS and DP. From the characterisation and evaluation of the biological activities, the quality attribute(s) relevant for the potency should be identified. Potency is the quantitative measure of biological activity, which is inked to the relevant biological properties and the claimed mechanism of action. The potency assay should be developed based on the biological activity (i.e. the specific ability or capacity of a product to achieve a defined biological effect).

Genetically Modified Patient Cells [9]

Potency

Both the vector and the CAR T cell DP must be tested for potency.

Discussion of how to set the potency specification occurs in Chap. 14.

12.2.3 QC Stability Program

Not only is potency foundational to the batch-to-batch QC release process, but also potency is foundational for demonstrating the stability of the biopharmaceutical over its shelf life:

Recombinant Proteins and Monoclonal Antibodies [10]

When the intended use of a product is linked to a definable and measurable biological activity, testing for potency should be part of the stability studies. For the purpose of stability testing of the products described in this guideline, potency is the specific ability or capacity of a product to achieve its intended effect. It is based on the measurement of some attribute of the product and is determined by a suitable quantitative method. Potency studies should be performed at appropriate intervals as defined in the stability protocol and the results should be reported in units of biological activity calibrated, whenever possible, against nationally or internationally recognized standard. Where no national or international reference standards exist, the assay results may be reported in in-house derived units using a characterised reference material.

Viral Vectors [6]

Stability-indicating methods should be included in this stability protocol to provide assurance that changes in the purity / impurity profile and potency of the active substance would be detected. A potency assay should be included in the stability protocol, unless otherwise justified. For GTIMP, vector integrity, biological activity (including transduction capacity) and strength are critical product attributes which should always be included in stability studies. It is appreciated, however, that during early development the potency assay may not be fully developed. Where feasible forced degradation studies may also provide important information on degradation products and identify stability indicating parameters to be tested.

12.2.4 Product Comparability After Manufacturing Process Changes

Manufacturing process changes occur, and when they do, it is important that the biopharmaceutical is confirmed to be highly similar to the previous product. Regulatory authorities emphasize the role of the potency comparison in this challenge:

Recombinant Proteins and Monoclonal Antibodies [11]

Biological Activity

Biological assay results can serve multiple purposes in the confirmation of product quality attributes that are useful for characterisation and batch analysis, and, in some cases, could serve as a link to clinical activity. The manufacturer should consider the limitations of biological assays, such as high variability, that might prevent detection of differences that occur as a result of a manufacturing process change. In cases where the biological assay also serves as a complement to physicochemical analysis, e.g., as a surrogate assay for higher order structure, the use of a relevant biological assay with appropriate precision and accuracy might provide a suitable approach to confirm that change in specific higher order structure has not occurred following manufacturing process changes. Where physicochemical or biological assays are not considered adequate to confirm that the higher order structure is maintained, it might be appropriate to conduct a nonclinical or clinical study. When changes are made to a product with multiple biological activities, manufacturers should consider performing a set of relevant functional assays designed to evaluate the range of activities. For example, certain proteins possess multiple functional domains that express enzymatic and receptor mediated activities. In such situations, manufacturers should consider evaluating all relevant functional activities.

Genetically Modified Patient Cells [12]

A potency assay is an extremely valuable tool to provide assurance of unaltered biological characteristics of the product throughout the development of the product. This is especially important when changes to the manufacturing process are introduced after production of material for non-clinical studies or pivotal clinical studies.

Detailed discussion on how to appropriately and adequately manage product comparability after manufacturing process changes occurs in Chap. 16.

12.2.5 Demonstration of Biosimilarity

Biosimilar manufacturers of recombinant proteins and monoclonal antibodies face the major challenge of demonstrating that their manufactured biosimilar is highly similar to that of the innovator's biopharmaceutical. While an extensive analytical comparison is the foundation of demonstrating this comparability, the potency comparison plays the central role in this confirmation. Comparative potencies can cover for a multitude of minor biomolecular structural differences between the two biopharmaceuticals:

> *Recombinant Proteins and Monoclonal Antibodies* [11]
> Sponsors can use functional assays to provide additional evidence that the biologic activity and potency of the proposed product are highly similar to those of the reference product and/or to support a conclusion that there are no clinically meaningful differences between the proposed product and the reference product. Such assays also may be used to provide additional evidence that the MOA of the two products is the same to the extent the MOA of the reference product is known. Functional assays can be used to provide additional data to support results from structural analyses, investigate the consequences of observed structural differences, and explore structure-activity relationships. These assays are expected to be comparative so they can provide evidence of similarity or reveal differences in the performance of the proposed product compared to the reference product, especially differences resulting from variations in structure that cannot be detected using current analytical methods.

12.3 Measurement of Biological Activity for Biopharmaceuticals

Measurement of biological activity applies to all biopharmaceuticals whether they be recombinant proteins, monoclonal antibodies, viral vectors, or genetically modified patient cells. The earlier in the clinical development program that the biological activity assay can be developed, optimized, and determined to be scientifically sound and appropriate, the greater its value in guiding the development of the manufacturing process.

Ideally, the selected potency assay for measurement of biological activity of a biopharmaceutical should have the following general properties:

1. Reflective of the mechanism of action (MOA) of the biopharmaceutical to ensure a meaningful response linked to human clinical activity
2. Sensitive to structural/molecular/cellular changes in the biopharmaceutical that might signal clinically meaningful impact on safety and/or efficacy
3. Ease of test method validation (e.g., accurate, precise, linear) to ensure a consistent measurement over time
4. Stability-indicating to be able to reliably detect changes in the biopharmaceutical over shelf life
5. Easy to perform by the analysts in a Quality Control (QC) laboratory

But realistically, there will be compromises in what can be obtained from any designed potency assay. In order to be truly reflective of the clinical MOA, many times multiple product-specific test methods needs to be developed. Major investment in assay design and development will be needed to see how closely the assay matches the clinical MOA; followed by significant resources and expense to see if the potency assay can be optimized sufficiently to function as a release assay in Quality Control. Quality Control staff will be inconvenienced by the bioassays because of the need to care for the living cells over weekends and holidays.

With the advanced therapy medical products (cell and gene therapy products), additional significant challenges to potency measurement can arise: (1) limited batch size and limited material for testing, (2) lack of appropriate reference standards, and (3) complex mechanism of action.

Potency assay measurement uses an array of techniques, including cell-based bioassays, surrogate (typically, non-biological analytical) assays, and flexible multi-assay approaches. Consistent performance of a development potency assay requires a controlled environment, skilled QC analysts, and well-developed and characterized methods.

The following three major types of assays for measuring the potency of biopharmaceuticals will be examined:

Section 12.3.1 Bioassay
Section 12.3.2 Surrogate Assay
Section 12.3.3 Assay Matrix

12.3.1 Bioassay

A 'bioassay' determines pharmaceutical strength by measuring the effect of the biopharmaceutical on a living system. Bioassays can be either *in vivo* (i.e., measurement of the effect when the biopharmaceutical is injected into a whole animal) or *in vitro* (i.e., measurement of the effect when the biopharmaceutical is exposed to cells growing in mini-wells in the laboratory). All bioassays can be broken down into a basic three-step outline:

Step 1: expose a living system to the biopharmaceutical
Step 2: allow the biopharmaceutical to interact with the living system to cause some type of functional or therapeutic change of the living system
Step 3: measure the amount of the functional or therapeutic change of the living system

In vivo bioassays are highly variable and expensive, but they have a long history of use and they can do the job in assigning potency to a biopharmaceutical. However, regulatory authorities encourage the responsible limitation of animal use whenever possible (i.e., 3R's – Replacement, Reduction, Refinement) [4]:

> *In vitro* biological potency tests should be developed. If not feasible, biological potency tests in animal tissues maintained *ex vivo* or in whole animals can be considered. Transgenic animals or animals with transplanted human tissues or systems, e.g. a suitable xenograft model, may be suitable for this purpose. In order to reduce the use of animals in accordance with the 3R principles a validated *in vitro* method is generally preferred over animal testing wherever possible (Directive 2010/63/EU).

In vitro bioassays are typically cell-based bioassays that determine the relative potency of a biopharmaceutical by comparing the biological response/activity related to its mechanism of action with that of a control/reference material. That comparison quantifies the product's efficacy and ability to achieve a defined biological effect. Living cells can undergo a number of reactions when exposed to the biological activity of the biopharmaceutical including receptor binding, receptor activation, cell signaling, and drug internalization. In a typical cell-based *in vitro* bioassay, a fixed amount of the appropriate culture medium and a fixed concentration of cells are placed in individual wells within a plastic microtiter plate. A fixed amount of the biopharmaceutical (or reference standard or QC reference control) is placed at one end of the plate followed by serial dilution across the tray. Upon incubation at a fixed temperature for a defined period of time, the biopharmaceutical interacts with the cells, causing a biologic effect (e.g., cell proliferation, cell death, cell differentiation, cytokine production, etc.). The serial dilution across the tray permits a dose-response curve to be generated of the biological response, which can then be appropriately measured.

In vitro bioassays can also be ligand- and receptor-binding assays, which directly measure a biopharmaceutical's affinity to its target. Note, these binding assays are bioassays if that is the intended biological response/activity related to the biopharmaceutical's clinical mechanism of action; if not, then they are surrogate assays, which are discussed in the next section.

In vitro bioassays are more variable and more expensive than the physicochemical structural assays typically performed by Quality Control (e.g., HPLC, electrophoresis), due to the potential contribution of each of the three major scientific disciplines involved with bioassays:

- *Physics* (e.g., tight control of temperature distribution and water evaporation rates across the plastic microtiter plate; accurate and precise transfer of microliter volumes)
- *Biology* (e.g., responding cells need to be maintained, sometimes for extended periods of time, in a uniform environment that allows a consistent response to the biological activity of the biopharmaceutical)
- *Mathematics* (e.g., repetitive dose–response curves to minimize tray position variation; statistics to confirm parallelism and calculate the potency value)

But it is a misconception that *in vitro* bioassays need to be so much more variable than the other QC assays. Edge effects related to different conditions for cells in the inner and outer wells of a microplate, can be counteracted by using plates with water reservoirs along the edge wells. The cells themselves are a large driver of variability (e.g., due to genetic drift, mycoplasma contamination, etc.). But clonal cell

lines tested for the absence of mycoplasma can be derived. Such cells can be banked and used with an expectation of uniform response through some number of continuous passages. Layout scrambling helps to minimize intraassay variability that comes from positional bias associated with edge effects, multichannel pipetting, and plate readers. Using the scientific principles of Quality by Design (QbD) – that is using a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management (discussed in Chap. 4, Sect. 4.4) – reproducible and reliable bioassays are possible. The United States Pharmacopeia (USP) in its general chapters – <1032> *Design and Development of Biological Assays*, <1033> *Biological Assay Validation*, and < 1034> *Analysis of Biological Assays* – provide much guidance on the design, development, optimization, and validation of bioassays.

Bioassays should try to mimic as close as possible the biopharmaceuticals mechanism of action (MOA). The case example in Fig. 12.1 illustrates the *in vitro* cell-based bioassay potency release assay for the market-approved biopharmaceutical, Mvasi (bevacizumab-awwb), a monoclonal antibody (referred to as AB 215) [13]. Vascular endothelial growth factors (VEGFs) and their receptors (VEGFRs) are involved in the formation of new blood vessels, a process referred to as angiogenesis. Cancer cells are dependent upon this formation of new blood vessels. Blocking VEGF from interacting with VEGFR is a mechanism of slowing down cancer cell growth. The bioassay is based on the monoclonal antibody targeting human

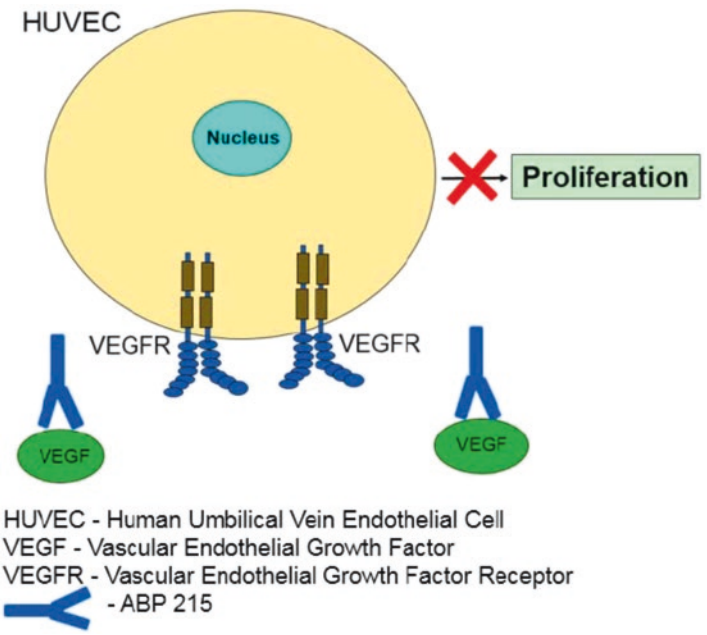

Fig. 12.1 A cell proliferation inhibition cell-based bioassay for a market-approved mAb

vascular endothelial growth factor (VEGF) and preventing its interaction with the VEGF receptor (VEGFR) on the cell surface. Without this interaction cell proliferation is inhibited. The bioassay thus measures the decrease in cell proliferation of VEGFR-containing cells due to the level of biological activity of the monoclonal antibody present.

12.3.2 Surrogate Assay

In cases where interaction with a cell-based system is complicated by properties of the biopharmaceutical product and/or technical limitations of assays so that development of a suitable bioassay is not feasible, it will be necessary to identify a surrogate measurement of the biological activity. Surrogate assays are typically non-biological analytical assays and include test methods that measure immunochemical (e.g., quantitative flow cytometry; enzyme-linked immunosorbant assay (ELISA)), molecular (e.g., reverse transcription polymerase chain reaction (RT-PCR); quantitative polymerase chain reaction, qPCR) or biochemical (e.g., enzymatic reaction) properties of the biopharmaceutical outside of a living system.

A key component for regulatory authority acceptance of any surrogate assay for therapeutic activity measurement is that the surrogate measurement must be substantiated by correlation to a relevant product-specific biological activity. This correlation may be established using various approaches, including comparison to preclinical/proof of concept data, *in vivo* animal or clinical data, or *in vitro* cellular or biochemical data. As part of the correlation relationship, the surrogate assay must be able to discriminate between active product and an inactive or degraded form of the product.

Ultimately, the regulatory authority needs to be convinced of the appropriateness of the surrogate assay, and this amount of convincing should not be underestimated [14]:

> *What is Necessary to Establish a Correlation between Biological Activity and a Non-Biological Analytical Assay(s)?*
>
> To demonstrate potency using an analytical assay or assay matrix as a surrogate measurement of biological activity, you should provide sufficient, scientifically sound data (i.e., based on suitably qualified assays, an appropriate number of replicates, multiple lots or various patient samples, etc.) to establish a correlation between the surrogate measurement(s) and the biological activity related to potency. We recommend that you consult with your CBER review team prior to design of correlative studies.
>
> The correlative relationship between the surrogate measurement and biological activity may be established using various approaches, including comparison to preclinical/proof of concept data, in vivo data (animal or clinical), or in vitro cellular or biochemical data. If you choose to use an analytical assay as a surrogate measurement of biological activity to meet the potency requirements for licensed biological products, you will need to meet the criteria listed above. You should also show that the assay can discriminate between active product and an inactive or degraded form of the product; and perform sufficiently controlled studies and/or employ a validated analytical assay.

The suitability of data used to support the correlative relationship between the surrogate assay and the biological activity of the product is evaluated on a case-by-case basis and depends on or is influenced by the following:

Type and relevance of the correlation(s) being made;
The amount of product information you have accumulated;
How well the biological activity of the product is understood; and
How well the surrogate measurement(s) reflects biological activity.

As with any potency assay, you should start collecting product and assay characterization data to support your choice of assay during early investigational phases.

The further the chosen potency assay is from the clinical mechanism of action pathway of the biopharmaceutical, the riskier is becomes from a regulatory authority standpoint. At a minimum, it will be necessary to demonstrate that the surrogate potency assay does impact somewhere along the mechanism of action pathway.

Regulatory authorities have recommendations (and reservations) concerning the use of potency surrogate assays:

Monoclonal Antibodies [15]

Because a binding assay demonstrates binding between the mAb or therapeutic protein and its target, it is generally sufficient to serve as a potency assay at the early stages of drug development. However, a binding assay assesses only one aspect of the potency of a product. Therefore, sponsors should subsequently develop methods that more comprehensively monitor the proposed mechanism(s) of action of the products. These methods should be incorporated into drug substance and drug product release testing and stability protocols. Potency assays should be described, justified, qualified, and validated to support a BLA.

For the purposes of this guidance, *binding assays* are defined as assays that quantify the binding between the mAb or other therapeutic protein and its target. These assays are established early in product development, typically in the form of a direct binding assay such as an enzyme-linked immunosorbent assay (ELISA) or a surface plasmon resonance (SPR) assay. Product lots should be compared to an appropriately qualified in-house reference material and activity should be expressed as a percentage of the reference material value. Although helpful in the initial phases of development, these assays do not directly confirm the product's ability to inhibit the target protein's activity and should not be used in lieu of methods that confirm potency.

Monoclonal Antibodies [16]

A relevant potency assay should be part of the specifications for drug substance and/or drug product, and should ideally reflect the biological activity in the clinical situation. For antibodies for which the clinical activity is only dependent on binding/neutralising properties, a potency assay that measures binding to the target (i.e. binding assay) may be deemed acceptable, if appropriately justified. Where effector functions are relevant for clinical activity, a cell-based bioassay or another assay that takes effector functions into account should be performed. A combination of two separate methods, one measuring the specificity and one giving an indication of an effector function (e.g. complement activation, C1q binding, Fc gamma receptor binding) may be acceptable if a cell-based assay is not feasible or if the combination of two methods gives more precise results. Although the two types of potency assays (binding or cell-based) often yield comparable results, these assays cannot be deemed interchangeable, because there are product attributes that may not affect binding to target (e.g. glycosylation, fragmentation) but may affect further signalling or receptor expression.

Viral Vectors [4]

The potency assay should normally encompass an evaluation of the efficiency of gene transfer (infectivity/transduction/delivery) and the level of expression of the therapeutic

sequence or its direct activity. Where possible the potency assay should include a measure of the functional activity of the therapeutic sequence or the product of it. This functional test may be supplemented with immunochemical methods to determine the integrity and quantity of an expressed protein product if appropriate.

For release testing simpler surrogate assays (e.g. based on nucleic acid amplification) may be acceptable, provided a correlation to the more functional test or the clinical outcome has been established in bridging studies.

Genetically Modified Patient Cells [7]

Potency testing for products containing genetically modified T-cells against tumour cells (e.g. CAR-T cells) is preferably based on the cytotoxic potential of the T-cells. Assay read-outs could, therefore, include actual death of target tumour cells or induction of intracellular pathways and loss of membrane integrity (with leakage of intracellular components) shown to lead to irreversible target cell death.

Surrogate read-outs for biological activity of CAR-T cell products could be the secretion of specific cytokines/cytotoxic molecules or expression of activation/degranulation markers by T-cells, provided that relation with target cell death is shown. When no autologous tumour material can be used as target, the relevance of surrogate target cells should be justified.

The two case examples of the market-approved Alzheimer's disease monoclonal antibodies (Leqembi, lecanemab-irmb [17]; Aduhelm, aducanumab-avwa [18]), illustrate the use of surrogate assays for potency. The mechanism of action (MOA) of these monoclonal antibodies: a human monoclonal antibody targeting Aβ-aggregates, Aβ-soluble oligomers, and Aβ-insoluble fibrils, to reduce amyloid plaques that accumulate in the brains of people with Alzheimer's disease.

Since it is very difficult to develop a QC bioassay that can timely measure the reduction in Alzheimer's disease for batch release, two binding assays are used to measure potency. Leqembi is used as the case example:

Potency Assay: The sponsor uses two potency assays in DS and DP release and stability and has also implemented control over the allowable glycosylation of lecanemab-irmb in order to assure consistent control of potency and safety.

Aβ binding assay: This antigen binding ELISA measures the relative binding potency (RBP%) of lecanemab DS and DP binding to amyloid beta (Aβ) compared to the reference standard. Streptavidin-coated microtiter plates are used to capture biotinylated lecanemab antigen, Aβ (1-16), which are recognized by lecanemab DS/DP and reference standard. The capture complex is detected using alkaline phosphatase (AP) conjugated goat anti-human IgG (H+L) and visualized using 4-methyl umbelliferyl phosphate (MUP) substrate. The generated fluorescence is measured using a fluorescent plate reader.

FcɣRIIa binding assay: Binding of lecanemab to FCɣRIIa (CD32a) is measured relative to that of the reference standard by Surface Plasmon Resonance (SPR). A Cytiva Series S Sensor Chip CM5 is coated with immobilized anti-His antibody, which in turn captures the His-tagged FcɣRIIa receptor. Lecanemab samples are injected and flow over the chip; complex formation is measured as resonance units based on the change in resonance observed using polarized light directed at the chip. The relative response is directly proportional to the amount of lecanemab bound to the FcɣRIIa receptor. Results are reported relative to a reference standard and expressed as a relative binding potency.

The case example of the market-approved Mepsevii (recombinant vestronidase alfa) illustrates the use of an enzymatic potency release assay that employs a surrogate substrate (4-methylumbelliferyl-glucuronide) in place of the natural (but highly

complex) substrate glycosaminoglycans. The cleavage of the fluorescent 4-methylumbelliferyl is the measure of enzymatic activity of this recombinant protein [19].

12.3.3 Assay Matrix

Frequently, a single bioassay may not provide an adequate measurement of potency for a biopharmaceutical. This can occur, for example, if (1) the biopharmaceutical has a complex mechanism of action, (2) the biopharmaceutical has multiple biological activities, or (3) the existing bioassay/surrogate is not fit for use (i.e., not quantitative, not sufficiently robust, or lacks adequate precision). If one assay is not sufficient to measure the potency, then an alternative approach is used; that is, developing multiple complementary assays that measure different product attributes associated with quality, consistency and stability. When used together and when results are correlated with a relevant biological activity, these complementary assays provide an adequate measure of potency. Such a collection of assays (referred to as an assay matrix) might consist of a combination of bioassays, bioassay and analytical assays, or a combination of analytical assays.

Monoclonal antibodies typically require an assay matrix for potency measurement. They exhibit multiple activities across the molecular structure, as illustrated by the market-approved monoclonal antibody, rituximab, for the elimination of CD20+ B-cells [20], see Fig. 12.2. Because of these multiple activities, rituximab, as a typical monoclonal antibody, has four common mechanisms of biological activity [20], see Fig. 12.3:

CDC (Complement-Dependent Cytotoxicity): Binding of rituximab to CD20 on B-cell surface causes activation of the complement cascade, which generates the membrane attack complex (MAC) that can directly induce B-cell lysis

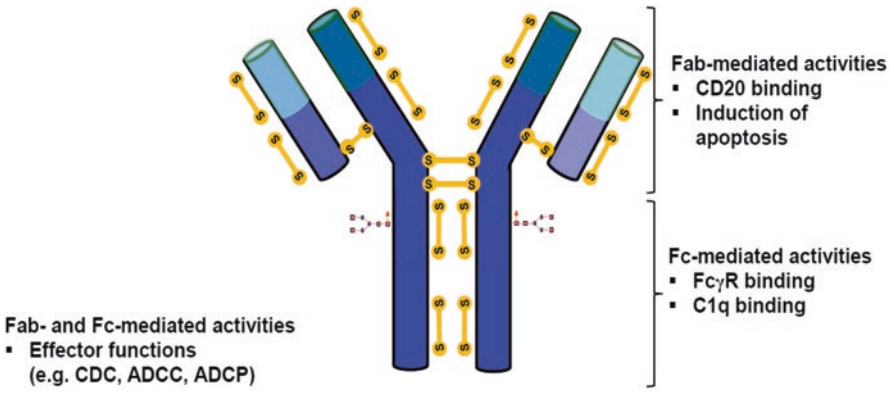

Fig. 12.2 Functional activities across the molecular structure of rituximab, a monoclonal antibody

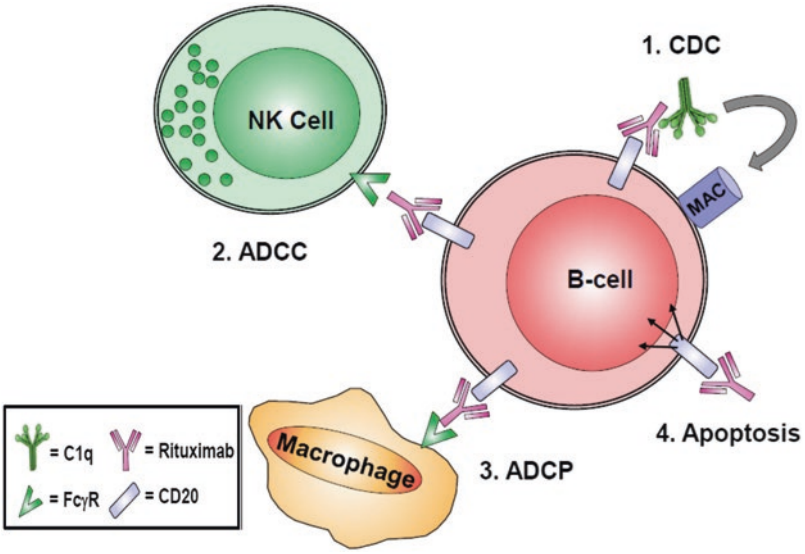

Fig. 12.3 Four mechanisms of rituximab-mediated B-cell death

ADCC (Antibody-Dependent Cellular Cytotoxicity): Binding of rituximab allows interaction with effector cells such as natural killer (NK) cells via Fc gamma receptors (FcɣR), which can lead to release of perforin and granzyme by the effector cell, resulting in lysis of the B-cell

ADCP (Antibody-Dependent Cellular Phagocytosis): Cells that are opsonized by the CD20 antibodies may be subject to ADCP, mediated by binding to Fc receptors on macrophages resulting in phagocytosis of the B-cell

Apoptosis: The crosslinking of several molecules of rituximab and CD20 in the lipid raft may initiate the interaction of these complexes with signaling pathways that can weakly mediate direct apoptosis (cell death)

For the above monoclonal antibody, Truxima (rituximab-abbs), two of the four biological activities were set as the 'potency' assay [21]: (1) Complement-Dependent Cytotoxicity (CDC) Assay – a cell-based cytotoxicity assay, and (2) FcγRIIIa Binding Affinity using Surface Plasmon Resonance (SPR) – a binding assay to measure an attribute which modulates Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC).

Another example of potency assay matrix is the monoclonal antibody, Idacio (adalimumab-aacf), also known as MSB11022, which has three biological activities as the 'potency' assay [22]:

Mechanism of Action

MSB11022 binds specifically to TNF-α and neutralizes its biological function by blocking its interaction with TNFR1 (p55) and TNFR2 (p75) cell surface receptors. TNF-α, expressed by immune cells and other cells in response to infection or inflammation, is expressed in soluble form (sTNF-α) and membrane-bound form (tm-TNF-α). MSB11022 binds to sTNF- α and tm-TNF-α and neutralizes its biological activity, which in turn reduces the inflammatory response. MSB1102 can also induce multiple Fc-dependent effector functions that depend on binding to tm-TNF-α, including induc-

tion of regulatory macrophages, antibody mediated reverse signaling, ADCC, and CDC. These activities have been suggested to contribute to inflammatory bowel disease indications, although the significance of the contribution of any of these individual mechanisms is not well established.

In Vitro Bioassay: This assay reflects the primary mechanism of action of adalimumab. It is based on the ability of MSB11022 to inhibit cytotoxicity induced by recombinant human TNF in a dose-depended manner on the murine fibroblast cell line A9. Cells are incubated in the presence of cycloheximide, to sensitize the cells to TNF-alpha-induced apoptosis. The Applicant indicates the A9 cell line is derived from the L-929 parental cell line that is less sensitive to TNF-induced cytotoxicity. The reported potency value is the average of results from three independent assays. Biological activity of the sample is expressed as % activity compared to the reference standard and is calculated from the dose-response curve of the sample and reference standard using a 4-PL regression analysis.

ADCC Reporter Bioassay: ADCC activity is a potential MoA for the CD and UC indications. This bioassay measures induction of the ADCC signaling pathway to determine ADCC activity of MSB11022. It measures cross-linking of target and effector cells by adalimumab and subsequent activation of the effector cells. The effector cells are a recombinant Jurkat T cell line that expresses the FcγRIIIA complex and the luciferase reporter gene under the control of a nuclear factor of activated T-cells (NFAT) response element. The target cells are tmTNF expressing CHO-K1 cells. Binding of the antibody to both tmTNF (via the Fab arm) and the FcγRIIIa receptor (via the Fc region) cross-links the cells, activating a biological signal, which is quantified through the luciferase activity readout from the NFAT pathway activation in the effector cells.

CDC Bioassay: The assay is based on the ability of MSB11022 to lyse tmTNF expressing cell line (tmTNF CHO-K1) via CDC by baby rabbit complement. The tmTNF CHO-K1 cells are incubated with a dose response curve of MSB11022 and a fixed concentration of complement. At the end of incubation, cell viability is measured by a luminescence assay reagent. Cell death is evaluated by the amount of ATP released by cells, which correlates with the decrease in luminescence.

And, of course, bispecific monoclonal antibodies, by their very nature, will have a potency assay matrix, as illustrated by the Vabymso (faricimab-svoa) [23]:

Description: Faricimab-svoa is a humanized bispecific IgG1 antibody that binds vascular endothelial growth factor A (VEGF-A) with one arm and angiopoetin-2 (Ang-2) with the other arm.

Mechanism of Action (MoA): The clinical efficacy of faricimab-svoa for its indications is mediated by binding both Ang-2 and VEGF-A and preventing the interactions of these two angiogenic factors with their respective receptors. Ang-2 and VEGF-A synergistically increase vascular permeability and stimulate neovascularization. By dual inhibition of Ang-2 and VEGF-A, faricimab-svoa reduces vascular permeability and inflammation, inhibits pathological angiogenesis and restores vascular stability.

Anti-Ang-2 by Tie-2 Phosphorylation Assay: The faricimab-svoa dose-dependent inhibition of Ang-2 induced Tie-2 receptor phosphorylation is quantified as the surrogate measurement for the neutralization of Ang-2. Specifically, serial dilutions of faricimab-svoa standard, control and samples are pre-incubated with a fixed concentration of recombinant human Ang-2 ligand. The faricimab-svoa/Ang-2 solutions are then incubated with the HEK293_Tie-2 cells that stably express Tie-2. Phosphorylation of the Tie-2 receptor in the cell lysate is then measured using a homogeneous time-resolved fluorescence (HTRF) detection system. The results, expressed in fluorescence ratios, are plotted against the faricimab-svoa concentrations. The relative potency of a sample is calculated based on the concentration shift between reference and sample dose-response curve fits.

Anti-VEGF-A by VEGF Reporter Gene Assay: The faricimab-svoa dose-dependent inhibition of VEGF-A induced activation of the Luciferase reporter gene is quantified as the surrogate measurement for the neutralization of VEGF-A. Specifically, serial dilutions of faricimab-svoa standard, control and samples are pre-incubated with a fixed concentration of recombinant human VEGF-A ligand (the 165 isoform). The faricimab-svoa/VEGF-A solutions are then incubated with the NFAT-RE-luc2P/KDR HEK293 cells that stably express VEGFR2 and a NFAT-RE-luc2P Luciferase reporter gene. After addition of a luminescent Luciferase substrate, the NFAT-Luciferase reporter gene expression is quantified by measuring luminescence. The results, expressed in relative luminescence units, are plotted against the faricimab-svoa concentrations. The relative potency of a sample is calculated based on the shift between reference and sample dose-response curve fits.

Viral vectors and genetically modified patient cells also typically require an assay matrix for potency measurement:

Viral Vectors [14]

Mechanism of Action (MOA)

Ideally, the potency assay will represent the product's mechanism of action (i.e., relevant therapeutic activity or intended biological effect). However, many CGT products have complex (e.g., rely on multiple biological activities) and/or not fully characterized mechanisms of action (MOA), making it difficult to determine which product attributes are most relevant to measuring potency. Nonetheless, all attempts should be made to develop potency measurements that reflect the product's relevant biological properties. For example, a gene therapy vector relies on at least two biological activities for its potency: the ability to transfer a genetic sequence to a cell; and the biological effect of the expressed genetic sequence. Therefore, the potency assay should incorporate both a measure of gene transfer and the biological effect of the transferred gene.

Genetically Modified Patient Cells [9]

Potency

Both the vector and the CAR T cell DP must be tested for potency. Upon antigen engagement, CAR T cells kill target cells using multiple mechanisms. Therefore, a matrix approach may be recommended to measure potency (e.g., cell killing assay, transduction efficiency measure, and cytokine secretion assays). We recommend using orthogonal methods to characterize CAR T cell function during product development. This approach will support comparability studies and will allow you to determine the best matrix of assays to use for commercial lot release. If the CAR T cells express multiple transgene elements, there should be a potency assay to measure activity of each functional element. For example, if the CAR T cell includes a cytokine transgene in addition to the CAR, you should develop a potency assay to assess the activity of that cytokine, in addition to the potency assay(s) to assess CAR activity.

Responding to questions submitted to them, FDA CBER subject matter experts on gene therapy held a 'town hall' meeting in 2022. Although the published transcript is not official FDA guidance, it does provide insight into CBER's current thought process on several subjects, including the use of the assay matrix for the gene therapy biopharmaceuticals [24]:

Has the agency shifted away from the potential to utilize a potency matrix that can be used as a surrogate for function to a firm stance that a quantitative functional assay is required? If not, how could a sponsor leverage a potency matrix in place of a quantitative functional assay? Would this approach be acceptable for BLA submission?

General recommendations for potency testing and requirements are outlined in detail in the 2011 potency guidance that we published. The guidance outlines phase-appropriate

flexible approaches, but it tries not to be too prescriptive, so we couldn't give too many examples about specific potency testing because of the diversity of cell and gene therapy products. One thing to keep in mind is that potency is required at all phases of product development, according to the Food, Drug, and Cosmetic Act. We generally accept quantitative measure of gene expression for first-in-human studies – early-phase studies. As your product advances in clinical development, the expectations are that the potency tests should be refined to measure a biological activity of the product per the regulations. The test should demonstrate that the product is capable of affecting a given result, and for this, we interpret that to mean that the product is functional and based on what you expect it to do. We understand that some sponsors may have difficulties establishing a single suitable test for products with very complex mechanism action or number of activities in the product, which is why we recommend that you begin thinking about product characterization and potency assay development during your early clinical development. Ideally, during your preclinical development when you're designing your proof-of-concept studies, you really need to start thinking about this from the beginning. A quantitative test that measures the biological function of the product is expected prior to the initiation of clinical studies meant to support efficacy for a marketing application. And the validation data should be submitted with your BLA. How this test is implemented, whether it's a single test or a product function or a matrix, as outlined in the question, is very product-specific and should be discussed with your review team early in development. The suitability of a surrogate? Well, we don't absolutely require only functional tests. We do think that that's the ideal, and that is actually what's expected. If you want to use a surrogate approach to support your phase 3 studies or licensure, you have to comply with a number of product-specific considerations. Some of these include whether – that you should think about whether the tests and the matrix measure meaningful CQAs. How relevant is the functional test that you're using? If this is a semi-quantitative test, does it measure meaningful data that contributes to the overall potency assessment? It's important that this actually be a real measurement of the function. Whether your tests are quantitative, whether they're precise and specific to the product – these are all put into the consideration. How well the tests are correlated to the expected functional activity of the product – and what kind of data are you planning to submit for the statistical analysis that would be used to support correlation studies? If you're not planning to submit a quantitative functional test in your BLA, you need to have very strong correlation studies to support any other matrix approach. These questions should all be considered when you're designing your matrix approach for potency testing. If there's insufficient supporting data, if the assays in the matrix are not controlled or have exceptionally wide acceptance criteria that provide no meaningful measure of activity, we're much less likely to accept this approach.

12.4 Applying the Minimum CMC Regulatory Compliance Continuum

Bioassay development is a complex process that must be undertaken with great rigor and attention to detail. Consistency and reliability of results over time are paramount. Well-developed and characterized methods are the end result of much phase-appropriate development work. Anticipate long months for the recombinant proteins/monoclonal antibodies, but even years for the gene therapy-based biopharmaceuticals, of effort to ready a bioassay for market-approval use.

For recombinant proteins and monoclonal antibodies, biological activity is a requirement for releasing the biopharmaceutical for human use [25]

> Details should be provided on the biological activity (i.e. the specific ability or capacity of a product to achieve a defined biological effect). Usually, prior to initiation of phase I studies, the biological activity should be determined using an appropriate, reliable and qualified method. Lack of such an assay should be justified. It is recognised that the extent of characterisation data will increase during development.

Typically, in the early clinical stage, the potency assay is a binding surrogate assay, because binding assays are relatively fast and simple to develop. During clinical development, a cell-based bioassay should be under development. By the pivotal clinical study, most manufacturers will have a cell-based bioassay as their measure of potency. The cell-based bioassay needs to be validated for the submission of the market approval dossier.

For the gene therapy-based biopharmaceuticals (viral vectors and genetically modified patient cells), there is greater urgency in a faster maturity of the potency cell-based assay development:

> ***Viral Vector*** [9]
>
> Vector lot release testing should include measures of safety, identity, purity, and potency. A potency assay that assesses the biological activity of the transgene may be developed in coordination with the CAR T cell potency assay. Transgene expression alone as a measure of potency may be sufficient to support early-phase IND studies; however, additional measures of biological potency will likely be requested for clinical study(s) intended to provide primary evidence of effectiveness to support a marketing application. Additionally, we recommend vector lot release testing include assays to determine the vector concentration that can be used to normalize the amount of vector used for transduction during CAR T cell manufacturing. For example, we recommend testing viral vectors for transducing units per milliliter (mL) in a suitable cell line or healthy donor cells. Subsequently, T cell transduction can then be optimized to determine the amount of vector that is added per cell to achieve the target percentage of CAR-positive cells in the CAR T cell DP.
>
> ***Gene Therapy-Based Biopharmaceuticals*** [6]
>
> It is strongly recommended that the development of a suitable potency assay be started as soon as possible. Preferably, a suitable potency assay should already be in place when material for the FIH clinical trial is produced and it should be validated prior to confirmatory clinical trials unless otherwise justified. Surrogate potency markers can be considered for release tests, but appropriate justification on their relevance in the context of the intended action of the ATIMP is needed.

Because the ability to measure potency is a most important critical quality attribute (CQA), the recommendation from the regulatory authorities is to initiate potency assay development by way of product characterization during preclinical and early clinical investigations to obtain as much product information as possible [14]:

> Measuring potency during early product development has a number of advantages, such as allowing you to:
>
> Demonstrate product activity, quality and consistency throughout product development;
> Generate a collection of data to support specifications for lot release;
> Provide a basis for assessing manufacturing changes;
> Evaluate product stability;

Recognize technical problems or reasons a different assay might be preferable;
Evaluate multiple assays; and
Collect sufficient data to support correlation studies, if necessary.

The FDA has introduced the phrase '*progressive potency assay implementation*' to match the risk-based approach of the minimum CMC regulatory compliance continuum, with three clinical development timeline milestones: (1) initiation of first-in-human (FIH) clinical studies, (2) advancing into pivotal clinical studies, and (3) seeking market approval [14]:

Potency at Initiation of FIH Clinical Studies

For some products in pre-clinical, Phase 1 and early Phase 2 studies, limited quantitative information on relevant biological attributes may be sufficient. Assay acceptance criteria should be set as a numerical range and should be adjusted throughout the product development stages to reflect manufacturing and clinical experience. Potency assays performed on product lots used for early clinical studies are likely to have wider acceptance ranges than assays used in later phase investigations. As clinical study progresses and product knowledge increases, you should develop and implement improved potency measurement(s) that quantitatively assesses relevant biological product attribute(s) (see 21 CFR 312.23(a)(7)).

Potency to Advance into Pivotal Clinical Studies

The primary objective of later phase investigational studies (i.e., Phase 3, pivotal) is to gather meaningful data about product efficacy, which is determined by adequate and well-controlled clinical trial(s). One aspect of an adequate and well controlled trial is administering product lots with similar potency, in that conformance to established limits for potency is necessary to provide reasonable confidence that product lots will perform as expected at a given dose in patients. Therefore, your potency assay or assay matrix design and acceptance criteria should establish appropriate limits for potency to assure that product lots are well-defined, biologically active, and consistently manufactured. If you do not provide sufficient assurance of potency of product lots to be used in your pivotal trial(s), your trial may be considered "deficient in design to meet its stated objectives" and may be placed on clinical hold (21 CFR 312.42(b)(2)(ii)).

Potency When Seeking Market Approval

To obtain a biologics license, a validated potency assay or assay matrix with defined acceptance criteria must be described and justified in the BLA (21 CFR 601.2(a) and 211.165(e). The acceptance criteria should be based on knowledge gained through manufacturing experience and data collected from assays performed during all phases of product development and clinical investigation. As you evaluate product conformance lots or lots manufactured explicitly for use in your pivotal clinical studies, acceptance criteria should be refined to reflect these data. The potency assay acceptance criteria defined in your BLA, which are intended for subsequent lot release testing, should reflect the potency limits established for product lots used in the pivotal clinical studies demonstrating clinical effectiveness (see FDC Act, Section 505(d), 21 U.S.C. 351).

Responding to questions submitted to them, FDA CBER subject matter experts on gene therapy held a 'town hall' meeting in 2022. Although the published transcript is not official FDA guidance, it does provide insight into CBER's current thought process on several subjects, including the timing during clinical development for when the potency test of the viral vector (specifically a lentivirus starting material for genetically modified patient cells) is needed [24]:

Could CBER elaborate on the current expectations for potency tests for viral vectors used for ex vivo modified gene therapy products? Specifically, when during clinical devel-

opment does CBER expect that potency should be included as a part of lot release testing rather than as characterization?

We do believe that transgene vectors used for ex vivo modified gene therapy products are critical to the activity of the drug product. And we do consider them critical components because of this, and this has really described in the gene therapy CMC guidance – that without the vector, the resulting product would not have the same pharmacological activity. And therefore, the activity of the vectors should be demonstrated as part of the vector lot release testing….we do have some flexibility in when you would introduce a biological potency assay for these factors. And so for early phase studies, you may use a transgene expression assay in lieu of the biological potency assay. However, similar to the drug product expectations, potency assays should be established and qualified prior to initiating the pivotal studies. And really, for the timing of implementing your potency assay for the transgene vector, we really would recommend that you implement it as early as possible so you can gain experience with it and gain information in order to inform lot release testing or specifications for the commercial setting. And so that could mean that you could implement your potency assay earlier on in development and during those early-stage studies, have it as report results, and then refine that acceptance criteria as you move on to the later-phase studies.

12.5 Missing the Target

As noted above, regulatory authorities allow considerable flexibility in determining the appropriate measurement(s) of potency for each biopharmaceutical. Unfortunately, this flexibility can lead to complacency. Sometimes unless specific discussion about potency is held with the regulatory authorities during the clinical development program, it might appear that the potency assay acceptable during the early clinical development program is also acceptable for obtaining market approval of the biopharmaceutical. Receiving a 'surprise response' from the regulatory authority reviewer about deficiencies in one's potency assay, either in the choice of the potency assay or its level of validation, becomes a major setback. Biopharmaceutical manufacturers sometimes underestimate the amount of effort (both in time and resources) necessary to 'fix' the potency concern. Several examples follow to illustrate this problem.

Recombinant Protein. During the FDA BLA submission review for Oxervate (cenegermin-bkbj), which is a recombinant nerve growth factor (NGF), the reliability of the cell-based potency bioassay came under concern. The bioassay was a typical cell-based cell proliferation bioassay – plate cells (using TF-1 human bone marrow erythroblast cells), expose to test samples, incubate cell culture, measure cell proliferation by a viable dye and UV absorbance at 490 nm. FDA questioned whether the bioassay was appropriately designed and sufficiently reliable for market approval. In the FDA market approval letter, the manufacturer was issued two post-marketing commitments related to the potency bioassay, with the final reports to be submitted to the FDA within 13 months and 16 months, respectively [26]:

To conduct structure-function studies to better understand whether all critical aspects of NGF biological function relevant to receptor binding are adequately controlled by the current TF-1 cell based assay, that only assesses NGF activity through binding the TrkA.

> To implement a control reference material for the potency assay to improve control over the assay variability and provide additional assurance that the RS is performing as expected during routine potency testing. The potency assay control material should perform within established acceptance criteria relative to the reference standard.

Biosimilar. During the EMA MAA submission review for Fulphila (pegfilgrastim), which was to be a biosimilar of pegylated granulocyte-colony stimulating factor (G-CSF), the reliability of the cell-based potency bioassay came under serious concern. One of the reasons that led to the manufacturer withdrawing the MAA at Day 120 was the lack of appropriate validation of their potency assay [27]:

> The validation data for the potency assay is considered incomplete. It needs to be justified why Neulasta was used for validation and not the product to be authorized. Also, the validation of the potency assay does not comprise an analysis of the assay robustness which is of significant importance for method transfer. Parameters such as cell passage, cell number and days of pre-culture, incubation time, temperature are not covered. In addition, critical reagents are not identified and no specifications are provided and the impact of different batches is not addressed. It should also be clarified which version of the software was/is used. Information should be provided on how and when the method was transferred. Some other concerns were identified regarding the incomplete validation of the potency assay.

Genetically Engineered Patient Cells. During the EMA MAA submission review for Skysona (elivaldogene autotemcel), an autologous CD34+ cell-enriched population containing cells transduced with lentiviral vector, the reliability of the potency assay (measurement of reduction of very long chain fatty acids due to the functional protein produced by the transduced cells) was questioned [28]:

> During the procedure, a major objection was raised in relation to the proposed potency assay (% VLCFA reduction assay), covering the lack of established specification limits, a request for further justifications to support the stability and validation data packages, a request for further data on the first step in the assay, and a request for data demonstrating the successful transfer of the potency assay to the batch release testing site. Additional data and justifications provided in response were considered acceptable and the major objection is considered resolved, with a recommendation to further review the acceptance criteria (discussed above) and to provide the final transfer validation report.

Quite frankly, there is no excuse for these major potency concerns being discovered and discussed after the market application dossier is submitted to the regulatory authority. The regulatory authorities offer abundant opportunities for manufacturers to discuss the suitability and reliability of their potency assays with them in advance of submitting the market application dossier (see Chap. 16 on holding CMC-focused meetings with a regulatory authority).

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Chapter 13
Biopharmaceutical Critical Quality Attributes

Abstract Manufacturers must ensure that the quality of their medicines, both for the drug substance (DS) and for the drug product (DP), meet all of the regulatory requirements with respect to product safety and efficacy. Critical quality attributes (CQAs) define the needed product quality. CQAs are important to generate reliable clinical data, demonstrate manufactured batch-to-batch consistency, support meaningful specifications for release, confirm the shelf life, and show product comparability after manufacturing process changes. Biopharmaceutical CQAs address biomolecular structure and structural variants, biological and immunochemical properties, the safety limits for process-related impurities and adventitious agents, and required compendial requirements for the drug substance and the drug product. The seven major CQA categories, applicable for the three types of biopharmaceuticals (recombinant proteins/monoclonal antibodies, viral vectors, genetically modified patient cells, mRNA non-viral vectors), will be examined in this chapter: (1) appearance/description, (2) identity, (3) purity/impurities, (4) potency, (5) quantity, (6) safety, and (7) general.

Keywords Molecular · Appearance · Description · Identity · Purities · Impurities · Endotoxin · Adventitious potency · Quantity · Intrinsic · Particulates · Proteinaceous · Particles · Analytical · mRNA

As previously discussed in Chap. 4, Sect. 4.4.2, a Critical Quality Attribute (CQA) is '*a physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality*'. Critical quality attributes (CQAs) define the needed product quality. CQAs are important to generate reliable clinical data, demonstrate manufactured batch-to-batch consistency, support meaningful specifications for release, confirm the shelf life, and show product comparability after manufacturing process changes. Biopharmaceutical CQAs address biomolecular structure and structural variants, biological and immunochemical properties, the safety limits for process-related impurities and adventitious agents, and required compendial requirements for the drug substance and the drug product.

J. Geigert, *The Challenge of CMC Regulatory Compliance for Biopharmaceuticals*, https://doi.org/10.1007/978-3-031-31909-9_13

Seven major CQA categories apply across the types of biopharmaceuticals (recombinant proteins/monoclonal antibodies, viral vectors, genetically modified patient cells, mRNA non-viral vectors). These CQA categories will be examined in the following order:

Section 13.1 Appearance
Section 13.2 Identity
Section 13.3 Purity/impurities
Section 13.4 Potency
Section 13.5 Quantity
Section 13.6 Safety
Section 13.7 General

How to assign specifications to each of the CQAs will be discussed in Chap. 14.

13.1 Appearance

'Appearance' is the quality attribute that consists of physical state, color and clarity. It is a regulatory requirement for all medicines, including biopharmaceuticals.

13.1.1 Common Descriptors of Appearance

- ***Physical state*** is a visual descriptor (i.e, description is based on the visual observation. Biopharmaceuticals are described as either solid (e.g., freeze dried powder), liquid, suspension or frozen.
- ***Color*** is a visual descriptor, sometimes employing a qualitative visible comparison to a reference color solution (i.e., European Pharmacopeia <2.2.2 > Degree of Coloration of Liquids). Recombinant proteins and monoclonal antibodies, if lyophilized, take on the color of the bulking agent(s) (which is frequently sucrose) in the formulation (i.e., typically white to off-white). Recombinant proteins and monoclonal antibodies, if liquid solution, are typically visually described as either colorless or colorless to some shade of yellow (typically due to the concentration of protein present). Viral vectors, typically frozen, are visually described as colorless. Genetically modified patient cells can be visually described running the color range from white to reddish (depending upon the mixture of cells present), upon thawing. mRNA non-viral vectors, typically encapsulated in lipids, are visually described as a white to off-white color of the suspension.
- **Clarity** is a visual descriptor, sometimes employing a qualitative visible comparison to a reference opalescence solution (i.e., European Pharmacopeia <2.2.1 > Clarity and Degree of Opalescence of Liquids). Recombinant protein, monoclonal antibody and viral vector solutions are typically visually described

as either clear or clear with some proteinaceous particles present. Genetically modified patient cells, upon thawing, are visually described as clear or cell clumps present. mRNA non-viral vectors, typically encapsulated in lipids, exists as a translucent suspension.

The range of appearance descriptions for market-approved biopharmaceutical drug products (as reported in their FDA labeling-package inserts) [1, 2], is presented in Table 13.1.

13.1.2 Intrinsic Visible Particles

If the biopharmaceutical is an injectable, visual particles may be present, and their presence raises a potential patient safety concern if in the administered drug product [3]:

Table 13.1 Some descriptions for appearance with market-approved biopharmaceuticals

Descriptions of APPEARANCE for Biopharmaceutical Drug Products	
Biopharmaceutical Type	**Appearance CQA**
Recombinant Protein Besremi (ropeginterferon alfa-2b-njft)	clear and colorless to slightly yellowish solution
Monoclonal Antibody Ocrevus (ocrelizumab)	clear or slightly opalescent, and colorless to pale brown solution
Monoclonal Antibody Fasenra (benralizumab)	clear to opalescent, colorless to slightly yellow solution, a few translucent or white to off-white particles may be present
Antibody-Drug Conjugate Zynlonta (loncastuximab tesirine-lpyl)	white to offwhite, lyophilized powder, which has a cake-like appearance
Viral Vector (rAAV) Adstiladrin (nadofaragene firadenovec-vncg)	clear to opalescent suspension
Genetically Modified Patient Cells Skysona (elivaldogene autotemcel)	thawed product is colorless to white to red, including shades of white or pink, light yellow, and orange suspension of cells and may contain small proteinaceous particles; due to the presence of cells, the solution, may be clear to slightly cloudy and may contain visible cell aggregates

Clinical Risk of Visible Particulates

The clinical manifestations of adverse events caused by particulate contamination vary and may depend on the route of administration (e.g., intravascular, intravisceral, intramuscular), patient population, and nature or class of the particulates themselves (e.g., physical size or shape, quantity, chemical reactivity to certain cells or tissues, immunogenicity, infectivity, carcinogenicity). Particulates in intravascular or intravisceral injections generally can cause more adverse events than those in subcutaneous or intramuscular injections. According to published case reports, serious adverse events involving injectable products contaminated with visible particulates have included:

- At the systemic level, infection and venous and arterial emboli (thrombotic or nonthrombotic).
- Microscopic emboli, abscesses, and granulomas in visceral organs.
- Phlebitis, inflammatory reactions, granulomas, and infections at injection sites.

There are two main groupings for visible particles:

- *Extrinsic Particles*: These are contaminant visible foreign particles that are coming from outside the manufacturing process; derived from either the environment, process equipment, primary packaging or personnel (e.g., fibers, paint flakes, insect parts, human hair, etc.). These particles are considered adulteration.
- *Intrinsic Particles*: These are visible particles that are coming from within the manufacturing process; derived from either the drug product processing (e.g., silicone oil droplets), the formulation (fatty acid particles from degradation of polysorbates), or interactions between the primary packaging (e.g., glass vial delamination fragments). But these visible particles can also be product-related (e.g., aggregation of proteins, clumping of cells), as discussed in Chap. 11. Intrinsic particles can increase during the shelf life of the biopharmaceutical.

The European Pharmacopoeia (Ph.Eur.) and United States Pharmacopeia (USP) monographs for parenteral preparations require drug products for injection administration to be “practically free” or “essentially free” of visible particulates, respectively. Both USP and Ph.Eur. provide recommendations on how to properly carry out visual particulate measurements: USP <790> *Visible Particulates in Injections*; Ph.Eur. 5.17.2 *Recommendations on Testing of Particulate Contamination: Visible Particles.*

Recombinant proteins, monoclonal antibodies, and viral vectors, have an inherent molecular property to self-associate or aggregate and form visible intrinsic proteinaceous particles despite formulation, manufacturing process, and container closure development to minimize visible protein particles. This propensity is a basic thermodynamic property of a protein molecule that cannot always be totally overcome. Proteins (including the proteins in the viral vector capsid) naturally aggregate, starting from the nanometer size of the subvisible dimers and oligomers all the

way up to the 100+ micron size of the visible protein particles. Genetically modified patient cells also clump together.

Biopharmaceutical manufacturers carry out a risk-based assessment of both the type and amount of visible particles present in their specific product, and then evaluate the potential patient harm that can be caused. Using the minimum CMC regulatory compliance continuum risk-based approach, 'essentially free' is defined loosely in early stage clinical development, but becomes more definitive in late stage clinical development as manufacturing experience is gained with the specific biopharmaceutical. If there are concerns about intrinsic visible particulates such as protein aggregates, the use of an in-line filter during patient administration may be considered.

13.2 Identity

'Identity' is the quality attribute of the entity being what it is stated to be. For example, a fingerprint or biometric eye scan confirms the identity of a specific individual. Identity, a regulatory requirement for all medicines, is confirming that the molecular structure of the product is what it claims to be.

13.2.1 Difference in Identity Between Chemical Drugs and Biopharmaceuticals

Identity confirmation is required by regulatory authorities for all pharmaceuticals, but the level of confirmation is different between a chemical drug and a biopharmaceutical. For a chemical drug, identity testing must be '*specific*' and must be '*able to discriminate between chemical compounds of closely related structure which are likely to be present*' [4]:

Identification: identification testing should optimally be able to discriminate between compounds of closely related structure which are likely to be present. Identification tests should be specific for the new drug substance, e.g., infrared spectroscopy. Identification solely by a single chromatographic retention time, for example, is not regarded as being specific. However, the use of two chromatographic procedures, where the separation is based on different principles or a combination of tests into a single procedure, such as HPLC/UV diode array, HPLC/MS, or GC/MS is generally acceptable.

For a biopharmaceutical, identity testing must be '*highly specific*' and must be '*based on unique aspects of its molecular structure and/or other specific properties*' [5]:

Identity The identity test(s) should be highly specific for the drug substance and should be based on unique aspects of its molecular structure and/or other specific properties. More than one test (physicochemical, biological and/or immunochemical) may be necessary to establish identity. The identity test(s) can be qualitative in nature. Some of the methods typically used for characterization of the product … may be employed and/or modified as appropriate for the purpose of establishing identity.

As described previously in detail in Chap. 11, biopharmaceuticals have complex biomolecular structures as well as numerous structural variants. This is one of the main reasons that more than one test may be necessary to satisfy the criteria for identity.

13.2.2 Regulatory Guidance on Identity

The following regulatory authority guidance is provided for identity confirmation of the various types of biopharmaceuticals:

Monoclonal Antibodies [6]

Identity

The identity test(s) should be highly specific and should be based on unique aspects of the product's molecular structure and/or other specific properties (e.g. peptide map, anti-idiotype immunoassay, or other appropriate method). Considering the great similarity of the constant domains of different antibodies, more than one test (physicochemical, biological and/or immunochemical) may be necessary to establish identity, and such test(s) should be able to discriminate other antibodies that may be manufactured in the same facility.

Viral Vectors [7]

Identity and integrity

The genetic identity and integrity of the drug substance should be assured using tests that identify both the therapeutic sequence and the vector. Such tests might include DNA sequencing or restriction enzyme mapping and immunological assays. The identity of the drug substance may also be confirmed through infection/transduction assays and detection of expression/activity of the therapeutic sequence(s) (see potency assay section). This identity test is especially important for complexed nucleic acid sequences.

Genetically Modified Patient Cells [8]

Identity

Identity testing should include an assay to detect the presence of the specific cell population as well as the intended genetic modification (at DNA level or an assay to detect the presence of the intended product translated from the genetic modification on protein level). The test methods should be specific for those components.

Table 13.2 Some identity tests for biopharmaceutical products

IDENTITY Tests for Biopharmaceutical Products	
Biopharmaceutical Type	**Some 'Highly Specific & Unique to Structure' Test Methods**
Recombinant Protein/ Monoclonal Antibody	Peptide mapping or ELISA
Viral Vector	Capsid: Peptide mapping or ELISA Transgene: qPCR or Sequencing
Genetically Modified Patient Cells	Transduced Cells: Flow cytometry
mRNA Non-Viral Vector	Sequence: reverse transcriptase – PCR

Some typical identity tests are shown in Table 13.2. Therefore, the identity test for a biopharmaceutical is typically some combination of one or more of the characterization test methods and/or purity test methods (see Chap. 11), and occasionally with an added potency test method (see Chap. 12).

13.2.3 Meeting the Criteria

Achieving the identity standard for a biopharmaceutical is not always straightforward, and regulatory authorities continue to remind manufacturers that their identity test must be highly specific and measure a unique aspect of the biopharmaceutical's molecular structure and/or other specific properties. This is illustrated by the following FDA written response provided to a recombinant protein manufacturer seeking to initiate their pivotal clinical trial [9]:

> The current identity tests are inadequate for both your drug substance (DS) and drug product (DP) because these tests do not measure a unique structural aspect of the DS and DP. For example, the molecular weight (measured by SDS-PAGE gel) is not a unique feature, i.e., many other proteins can have the same molecular weight. Similarly, IEF and potency tests are not proper identity tests because they do not measure a unique feature of your product. In addition, the ICH Q6B guidance does not state that a combination of assays may be used; rather, it states more than one assay may be 'necessary' to demonstrate a unique structural feature. Please develop a definitive identity assay such as peptide mapping or an immune-based assay.

Note, for biopharmaceuticals under the FDA Public Health Services (PHS) Act, there is also the regulatory requirement for carrying out an additional identity test. That is the content identity test performed after the finished drug product is labeled, according to FDA Title 21 CFR 610.14 (see discussion in Chap. 2).

13.3 Purity/Impurities

'Purity' is the quality attribute that describes the percentage of component(s) in the total amount that are desired to be present, while impurities describe the levels of component(s) in the total amount that are not desired to be present in the medicine. Measurement of purity and impurities is a regulatory requirement for all biopharmaceuticals.

13.3.1 What Is Purity for a Biopharmaceutical?

Each biopharmaceutical type (recombinant proteins/monoclonal antibodies, viral vectors, genetically modified patient cells) contains not only the desired biomolecular structure, but also many biomolecular structural variants, as discussed in detail in Chap. 11. These biomolecular structural variants may be either product-related substances or product-related impurities. Each of the manufacturing processes for the different types of biopharmaceuticals contribute to the diversity and level of process-related impurities, as discussed in detail in Chap. 10.

For biopharmaceuticals, there is no one test method that can truly be called the 'purity test method' – that is, measure all possible biomolecular structural variants. For biopharmaceuticals, there is no one test method that can truly be called the 'impurity test method' – that is, measure all possible process-related impurities. Test methods are designed to measure specific factors. Purity and impurity therefore are relative terms [5]:

The absolute purity of biotechnological and biological products is difficult to determine and the results are method-dependent. Consequently, the purity of the drug substance is usually estimated by a combination of methods. The choice and optimization of analytical procedures should focus on the separation of the desired product from product-related substances and from impurities.

Some typical purity/impurities tests are shown in Table 13.3. It is the composite of all of the purity and impurity test methods that are used to analyze the biopharmaceutical that ultimately define the value.

13.3.2 Regulatory Guidance on Purity/Impurities

The following regulatory authority recommendations provide insights on purity/impurity for the different biopharmaceutical product types:

Monoclonal Antibodies [10]
Purity, impurity and contaminants

Table 13.3 Some purity/impurities tests for biopharmaceutical products

PURITY/IMPURITIES Tests for Biopharmaceutical Products	
Biopharmaceutical Type	**Some Purity/Impurities Test Methods**
Recombinant Protein/ Monoclonal Antibody	Size Variants: CE-SDS Charge Variants: IEX-HPLC pI Variants: cIEF % Monomer: SEC-HPLC Residual Host Cell DNA: qPCR Residual HCPs: ELISA
Viral Vector	Capsid Protein Purity: CE-SDS % Full Capsids: AEX-HPLC % Aggregation: SEC-HPLC Residual Host Cell DNA: qPCR Residual Plasmid DNA: qPCR Residual HCPs: ELISA
Genetically Modified Patient Cells (CAR T Cells)	% CAR+ Cells: Flow cytometry % Viable Cells: Cell counter % Non-T Cells: Flow cytometry % Residual Beads: Microscopy
mRNA Non-Viral Vector	% Intact mRNA: CGE 5´ Cap: HPLC 3´ Poly(A): HPLC Double-Stranded RNA: ELISA Residual DNA template: qPCR

Monoclonal antibodies commonly display several sources of heterogeneity (e.g. C-terminal lysine processing, N-terminal pyroglutamate, deamidation, oxidation, isomerisation, fragmentation, disulfide bond mismatch, N-linked oligosaccharide, glycation), which lead to a complex purity/impurity profile comprising several molecular entities or variants. Potential process-related impurities (e.g. HCP, host cell DNA, cell culture residues, downstream processing residues) should be identified, and evaluated qualitatively and/or quantitatively, as appropriate.

Gene Therapies [11]

During the production of an ATIMP, variable amounts of impurities, product- and process-related, may be introduced into the active substance. The aim should be to maximise the active components and minimise features which do not contribute, or may negatively impact on therapeutic activity/safety. The setting of purity specifications should be based on characterisation studies conducted as part of product development. Purity does not necessarily imply homogeneity, however, product consistency needs to be demonstrated.

Genetically Modified Patient Cells [8]

Purity

Purity is generally related to the intended cell type and to the transduction/transfection and genome editing efficiency, i.e. percentage of genetically modified cells. The degree of purity should be defined taking into account the nature and intended use of the product, the method of its production and also the degree of consistency of the production process.

A significant portion of the total test methods within the seven CQAs, will be in the purity/impurities CQA for biopharmaceuticals.

13.4 Potency

'Potency' is the quality attribute for the quantitative measure of biological activity, and is a regulatory requirement for all biopharmaceuticals. Potency was discussed in detail in Chap. 12.

Descriptors of potency vary by biopharmaceutical type as presented in Table 13.4, for some market-approved biopharmaceuticals [12–14].

Table 13.4 Some descriptions of potency found in market-approved biopharmaceuticals

Descriptions of POTENCY for Biopharmaceutical Drug Products	
Biopharmaceutical Type	**Potency CQA**
Recombinant Protein Releuko (filgrastim-ayow)	liquid containing filgrastim-ayow at a specific activity of $1.0 \pm 0.6 \times 10^8$ U/mg (measured by a cell bioassay)
rAAV Viral Vector (rAAV) Adstiladrin (nadofaragene firadenovec-vncg)	nominal concentration of 3×10^{11} viral particles (vp)/mL)
Hemgenix (etranacogene dezaparvovec-drlb)	nominal concentration of 1×10^{13} genome copies (gc)/mL
Genetically Modified Patient Cells Tecartus (brexucabtagene autoleucel)	CAR+ T-cells in approximately 68 mL per bag
Skysona (elivaldogene autotemcel)	between 3.6 to 30×10^6 CD34+ cells/mL (cells transduced with ALDP gene)

13.5 Quantity

'Quantity' is the quality attribute that describes the total amount/content of the medicine present in the container closure, and it is a regulatory requirement for all biopharmaceuticals.

13.5.1 Common Descriptors of Quantity

Quantity (or content) for biopharmaceuticals is expressed as 'potency x total amount'. For recombinant proteins and monoclonal antibodies, potency can be expressed as 'mg', when compared to a reference standard. The range of quantity descriptions, illustrated by the descriptions found in market-approved biopharmaceutical drug products [15, 16], is presented in Table 13.5.

Table 13.5 Some descriptions for quantity found in market-approved biopharmaceuticals

Descriptions of QUANTITY for Biopharmaceutical Drug Products	
Biopharmaceutical Type	**Quantity CQA**
Monoclonal Antibody Fasenra (benralizumab)	each single-dose prefilled syringe delivers 1 mL containing 30 mg benralizumab [30 mg in syringe]
Antibody-Drug Conjugate Zynlonta (loncastuximab tesirine-lpyl)	10 mg of loncastuximab tesirine-lpyl as a lyophilized powder in a single-dose vial [10 mg in vial]
Viral Vector (rAAV) Adstiladrin (nadofaragene firadenovec-vncg)	vials have a nominal concentration of 3×10^{11} viral particles (vp)/mL), each vial contains not less than 20 mL [NLT 6 x 10^{12} viral particles]
Viral Vector (rAAV) Hemgenix (etranacogene dezaparvovec-drlb)	vials have a nominal concentration of 1×10^{13} gene copies (gc)/mL, each vial contains no less than 10 mL [NLT 1 x 10^{14} viral particles]
Genetically Modified Patient Cells Skysona (elivaldogene autotemcel)	each bag contains between 4×10^6 and 30×10^6 cells/mL (3.6 to 30×10^6 CD34+ cells/mL), frozen in approximately 20 mL of solution. [7.2-60 x 10^7 CD34+ cells]

13.5.2 Regulatory Guidance on Quantity

The following regulatory authority recommendations provide insights on quantity for the different biopharmaceutical product types:

Recombinant Proteins and Monoclonal Antibodies [5]

Quantity, usually measured as protein content, is critical for a biotechnological and biological product and should be determined using an appropriate assay, usually physicochemical in nature. In some cases, it may be demonstrated that the quantity values obtained may be directly related to those found using the biological assay. When this correlation exists, it may be appropriate to use measurement of quantity rather than the measurement of biological activity in manufacturing processes, such as filling.

The quantity of the drug substance in the drug product, usually based on protein content (mass), should be determined using an appropriate assay. In cases where product manufacture is based upon potency, there may be no need for an alternate determination of quantity.

Monoclonal Antibodies [10]

Quantity should be determined using an appropriate physicochemical and/or immunochemical assay. It should be demonstrated that the quantity values obtained are directly related to those derived using the biological assay. When this correlation exists, it may be appropriate to use measurement of quantity rather than the measurement of biological activity in the product labelling and manufacturing processes, such as filling. The quantity of the drug substance, usually based on protein content (mass), should be determined using an appropriate assay.

Viral Vectors [7]

Content

The quantity of the drug substance should be established. For viral vectors, infectious titre should be quantified; the number of particles (infectious/non-infectious, empty/genome containing) should also be determined. Particle to infectivity ratio should be included to define the content of the drug substance.

Genetically Modified Patient Cells [17]

If your final product is genetically modified cell-based gene therapy, you should have an acceptance criterion for the minimum number of genetically modified cells in a product lot. We recommend that the product dose for such products be based on the total number of genetically modified cells.

13.5.3 Measurement of Quantity

Measurement of quantity requires different assays for the different biopharmaceuticals:

1. *Recombinant proteins and monoclonal antibodies*: Ultraviolet (UV) absorbance at 280 nm is the standard test method. Absorbance is due to the presence of aromatic amino acids, mainly tyrosine and tryptophan in the protein sequence. UV spectroscopy follows the Beers-Lamberts law which converts absorbance (A) to protein concentration (c): $c = A/\varepsilon \times l$. With traditional UV-Vis spectroscopy, the path length (l) is a fixed value, while with variable pathlength spectroscopy, the pathlength is varied, allowing measurement of high protein concentrations (up to 300 mg/mL without dilution).

The reliability of the UV protein concentration measurement is dependent upon the accuracy of the assigned extinction coefficient (ε) value. This ε value can be either calculated from the protein's known amino acid structure or experimentally determined from amino acid analysis (AAA), but regulatory authorities recommend the justification of the assigned ε value by experimental method. Unfortunately, manufacturers sometimes do not pay enough attention to the accuracy of the ε value. For example, a discrepancy in clinical dosing occurred due to an incorrectly assigned extinction coefficient value [18]:

The protein content determination for dinutuximab manufactured by NCI/SAIC was measured using the wrong extinction coefficient for absorbance. Therefore the apparent differences in dosing in the clinical trials conducted with NCI-sourced material and UTC-sourced material are not true differences but reflect a change to a more accurate calculation of protein content in the UTC product. The dose of NCI-sourced dinutuximab was based on protein concentrations determined by an absorbance assay in which the extinction coefficient was not experimentally determined. The dose of "25 mg" is actually 17.5 mg dinutuximab, based on UTC's validated absorbance assay using the correct extinction for this protein.

Also an incorrect extinction coefficient was the cause for the strange specific activity assignment for Proleukin (aldesleukin, recombinant interleukin-2) [19]: 18 million Units in 1.1 mg. Specific activity is typically expressed per 1 mg.

2. *Viral Vectors*. Measurement of the vector genome titer (e.g., gene copies or virus particles) requires the use of a polymerase chain reaction, either quantitative or digital. The UV260nm/UV280nm ratio is a measurement of the percentage of full viral particles (i.e., particles containing the gene of interest).
3. *Genetically Modified Patient Cells*. The viable cell concentration is typically measured by means of an automatic cell counter. Genetic probes are necessary to determine the percentage of transduced cells.
4. *Excipients.* The quantity of each excipient that is present in the drug product is typically controlled through the manufacturing batch records where detailed instructions are provided on how to prepare the concentrations of the excipients in the formulation. However, quantity measurement is necessary for excipients that have a functional purpose towards the biopharmaceutical, where a specified limit or range must be controlled. For example, polysorbate-20 and polysorbate-80, surfactants, are commonly used in protein formulations. An adequate amount

must be present (that is, above the critical micelle concentration) to protect the protein from insolubility or minimize aggregation.

5. *mRNA Non-Viral Vectors*. Measurement of the non-viral vector requires the use of a reverse transcriptase – polymerase chain reaction (RT-PCR), either quantitative or digital. The UV260nm is a measurement of mRNA present. The extinction coefficient of mRNA is 0.025 $(mg/mL)^{-1}\ cm^{-1}$.

13.6 Safety

'Safety' is the quality attribute that describes the absence of adventitious agent contamination in the manufactured injectable medicine, and it is a regulatory requirement for all biopharmaceuticals. Gene therapy-based biopharmaceuticals have extra safety requirements. Because of potential genetic mutations during manufacturing, gene therapy biopharmaceuticals are required to demonstrate the absence of replication competent virus (RCV). Because of a theoretical safety risk of insertional oncogenesis, the vector copy number (VCN) is considered a safety test (see Chap. 11, Sect. 11.4.2).

13.6.1 Safety from Adventitious Agents

For all biopharmaceutical types, the major safety concerns relate to the following: (1) adventitious agent contamination (e.g., TSE, virus, mycoplasma), (2) microbial contamination (controlled either by means of sterility testing or bioburden control), and (3) endotoxin. These safety sources were discussed in detail in Chap. 5. For recombinant proteins and monoclonal antibodies, the control over adventitious agent contamination is confirmed at the end of the upstream production cell culture stage of the manufacturing process. For the gene therapy biopharmaceuticals, adventitious agent concerns carry through the entire manufacturing process. The genetically modified patient cells have the extra burden of needing to be manufactured completely under aseptic conditions as cells cannot be sterile filtered at the end.

To maximize the sensitivity of safety testing, it is important that each test is performed at the stage of manufacturing at which contamination is most likely to be detected. For example, tests for mycoplasma or adventitious viruses should be performed on cell culture harvest material (cells and supernatant) prior to further processing, e.g., prior to clarification, filtration, purification, and inactivation. In addition, alternative methods, which may be needed for live cells, include rapid sterility tests, rapid mycoplasma tests (including PCR-based tests), and rapid endotoxin tests should be qualified/validated to ensure they are fit for their intended use [17].

13.6.2 Replication Competent Virus

What sets the viral vectors and the genetically modified patient cells apart is the concern for a genetic mutation that can result in a replication competent virus (RCV): RCA is replication competent adenovirus; RCR is replication competent retrovirus (like lentivirus). Regulatory authorities have provided guidance on this important patient safety concern:

Replication Competent Virus (RCV) [7]

For replication deficient viral vectors, demonstration of replication incompetence begins with a clearly documented strategy to render the viral vector replication incompetent. The possibility of any recombination events leading to RCV or replication via *trans* regulation should be discussed. The absence of RCV is then tested on the drug substance, intermediates where appropriate, as well as any packaging/producer cell lines. Screening for RCVs should be in accordance with Pharmacopoeial recommendations, using a suitably sensitive detector cell line and appropriate passage numbers. Based on the application of the risk-based approach, e.g. taking into consideration the experience with the same cell line and vector, the applicant can justify the stage of production at which RCV testing will be performed.

Replication Competent Virus (RCA and RCR) [17]

Replication Competent Virus

For non-replicating gene therapy viral vectors, we recommend specific testing, due to the potential for these vectors to recombine or revert to a parental or wild-type (WT) phenotype at a low frequency. Tests for replication-competent, parental, or wild-type viruses that may be generated during production (e.g., replication-competent adenovirus (RCA) and replication-competent retrovirus (RCR)) should be performed on material collected at the appropriate stage of the manufacturing process. For example, we recommend testing banked material for the presence of replication-competent viruses and as a specification for in-process or release testing of the DS or DP, as appropriate.

(a) *Replication-Competent Retrovirus (RCR) Testing*

Most retroviral-based products (including lentivirus and foamy virus-based products) used for gene therapy applications are designed to be replication defective. To ensure the absence of RCR, you should perform testing for RCR at multiple points during production of a retroviral vector.

(b) *Replication-Competent Adenovirus (RCA) Testing*

Most adenoviral-based products used for gene therapy applications are designed to be replication defective. RCA may be generated at a low frequency as a result of homologous recombination between viral vector sequences and viral sequences present in the cell substrate during manufacturing. Therefore, for adenoviral-based gene therapy products, we recommend that you qualify your MVB for RCA and test either the DS or DP of each production lot for RCA. We recommend a maximum level of 1 RCA in 3×10^{10} viral particles.

(c) *Replication-Competent AAV (rcAAV) Testing*

Preparations of AAV vectors can be contaminated with helper virus-dependent rcAAV, also referred to as wild-type AAV or pseudo wild-type AAV. These rcAAV are generated through homologous or non-homologous recombination events between AAV elements present on the vector and AAV rep and cap sequences that are present during manufacture. While wild-type AAV has no known associated pathology and cannot replicate without helper virus, expression of cap or rep genes in infected cells can result in unintended immune responses, which can reduce effectiveness and may have unintended safety risks. Therefore, we recommend that you test for rcAAV, which could potentially replicate in the presence of helper virus, and report these results in the IND. A number of methods have been published for evaluating the level of rcAAV, including amplification of AAV in the presence of helper virus, followed by PCR for rep/inverted terminal repeats (ITR) junctions, and PCR for rep and cap sequences, following DNase digestion of the vector preparation. We do not recommend a specific method for determining rcAAV in this guidance. You should describe your test method and assay sensitivity in the IND.

Replication Competent Retrovirus (RCR) [20].

Design of the Viral Vector. Retroviral vectors have been designed to reduce the likelihood of generating RCR during the manufacturing process. For instance, the likelihood that recombination will generate RCR is reduced by manufacturing vectors using a split plasmid design, where the vector genome is on a separate plasmid from the envelope gene and packaging functions. RCR generation can be further reduced by using more than two plasmids for vector production. Lentiviral vectors have been further modified to remove genes encoding viral accessory and regulatory proteins, which would cripple the functionality of an RCR in the event an RCR may be generated.

Test Sensitivity. we are recommending that you test a sufficient amount of vector to demonstrate that your vector contains <1 RCR per patient dose, when applicable to your clinical manufacturing practice. Additionally, we are recommending that all retroviral vector transduced cell products be tested for RCR, including those cultured for 4 days or less. We have found no convincing evidence that the length of culture time influences the likelihood of RCR development in transduced cells.

Ex Vivo Transduced Cells. If the retroviral vector is used for ex vivo genetic modification of cells, it is possible that RCR may be present in your vector at undetectable levels, which could be amplified during the manufacture of ex vivo transduced cells. Therefore, we recommend that each lot of ex vivo retroviral transduced cells be tested for RCR. This recommendation applies regardless of the length of time that the cells are cultured after transduction, because the length of culture time (e.g., greater than 4 days) has not been shown to strongly influence the likelihood of RCR development.

Probability of Detection. Current manufacturing experience indicates that <1 RCR/dose equivalent is an achievable level for retroviral vector preparations intended for clinical use. Therefore, we now recommend that sufficient supernatant be tested to ensure a 95% probability of detection of RCR if present at a concentration of 1 RCR/dose equivalent.

13.7 General

'General' is the quality attribute that describes the properties that are associated with the dose form for the medicine, and it is a regulatory requirement for all biopharmaceuticals.

General quality attributes are those tests required by pharmacopeias for biopharmaceuticals. Measurement of general quality attributes include, depending upon the biopharmaceutical type, formulation, and its intended dose form: pH, osmolarity, volume of injection in container, reconstitution time (if lyophilized), etc. Test methods for each of these general quality attributes are described in the pharmacopeias. If the biopharmaceutical is primarily packaged in a product-contact delivery device (e.g., pre-filled syringe, auto-injector pen, etc.), then confirming the functional aspects of the delivery device is also required.

13.8 Compiled Tables of CQAs for Different Biopharmaceutical Types

A biopharmaceutical manufacturer's list of the test methods included within the seven CQA categories for their biopharmaceutical, is typically proprietary. However, once a biopharmaceutical is market-approved by the FDA, the FDA uploads to their website their internal review of the information submitted in the BLA. From these uploaded reviews, on occasion, the list of the test methods within the seven CQAs are publicly released. Note that in these public domain releases for commercial biopharmaceutical products, some test methods may be missing within a CQA category, especially for the process-related impurity CQAs (which may be managed through process validation control rather than batch-to-batch testing).

Another source of information on testing is the the biopharmaceutical industry itself which has developed their own proposals for what the CQAs should be. These are general proposals but they do help reflect what manufacturers are considering as they set up their CQA lists of tests. These consensus proposals have been issued for monoclonal antibodies, viral vectors and genetically modified patient cells.

And finally, the United States Pharmacopeia (USP) has published a draft guideline on the CQA testing and test methodology for mRNA non-viral vectors.

13.8.1 FDA Released CQA Test Results

FDA thoroughly reviews the CMC information in Module 3 of the common technical document (CTD) provided by the manufacturer in their BLA submission to seek market-approval. Upon acceptance of the biopharmaceutical description and control, and after completing the entire CMC document review, they make the decision

to accept or not the submitted BLA. Upon approval, the manufacturer receives a market approval letter allowing them to market the biopharmaceutical. A couple of months later, the FDA uploads to their approved product website, some of their review documents, allowing the public to better understand their approval process. From these uploaded reviews, on occasion, the list of the test methods within the seven CQAs of the approved biopharmaceutical are included. Note that in these public domain releases for commercial biopharmaceutical products, some test methods may be missing within a CQA category, especially for the process-related impurity CQAs (which may be managed through process validation control rather than batch-to-batch testing).

Table 13.6 presents the testing across the seven CQA categories for a market-approved monoclonal antibody, Nucala (mepolizumab), for both the drug substance and the drug product [21]. Notice, the testing specific to the drug substance: (1) adventitious agent mycoplasma and virus testing which takes place at the unprocessed bioreactor bulk stage, and (2) the testing for the upstream production

Table 13.6 Testing in the seven CQA categories for a market-approved monoclonal antibody

Testing in Each CQA Category for a Monoclonal Antibody		
CQA	**Drug Substance**	**Drug Product**
Appearance	Visual Observation (color, clarity, visible particles)	Visual Observation (color, clarity, visible particles)
Identity	IL-5 Binding (Surface Plasmon Resonance) Capillary Isoelectric Focusing (cIEF)	IL-5 Binding (Surface Plasmon Resonance) Capillary Isoelectric Focusing (cIEF)
Purity/ Impurities	Size Exclusion Chromatography (SEC-HPLC) Capillary Gel Electrophoresis (CGE) Capillary Isoelectric Focusing (cIEF) Residual Host Cell Proteins	Size Exclusion Chromatography (SEC-HPLC) Capillary Gel Electrophoresis (CGE) Capillary Isoelectric Focusing (cIEF)
Potency	IL-5 Binding (Surface Plasmon Resonance)	IL-5 Binding (Surface Plasmon Resonance) IL-5 Neutralization Bioassay
Quantity	Protein Concentration (UV)	Protein Concentration (UV)
Safety	Bioburden Endotoxin (LAL) Mycoplasma (unprocessed bulk stage) In Vitro Adventitious Virus (unprocessed bulk stage)	Sterility Endotoxin (LAL) (Container Closure Integrity for shelf-life testing)
General	pH	pH Particulate Matter (HIAC) Residual Moisture Reconstitution Time Weight Variation Osmolality

Table 13.7 Testing in the seven CQA categories for a market-approved viral vector drug product

Testing in Each CQA Category for a Viral Vector	
CQA Category	**Drug Product**
Appearance	Visual Observation (color, clarity, visible particles)
Identity	Vector DNA Identity (PCR)
Purity/Impurities	Subvisible Particles Capsid Content
Potency	Vector Infectivity Potency (functional FIX protein produced in human hepatocyte cell line when infected with vector, test sample compared to reference)
Quantity	Vector Genome Concentration Total Particle Concentration
Safety	Sterility Endotoxin (LAL)
General	Sucrose Concentration Polysorbate-20 Concentration Extractable Volume

process-related impurities (e.g., HCP) takes place at the drug substance stage. Notice, the testing specific to the drug product: testing due to the final dose form.

Table 13.7 presents the testing across the seven CQA categories for a market-approved viral vector (rAAV), Hemgenix (etranacogene dezaparvovec), for the drug product [22].

13.8.2 Project A-Gene

Project A-Gene, released in 2021, is a biopharmaceutical case study-based approach on how to integrate Quality by Design (QbD) principles into gene therapy CMC programs. It is important to understand that the proposals on CQAs in this document for gene therapy products (specifically, rAAV *in vivo* viral vectors) were driven by CMC subject matter experts from within the biopharmaceutical manufacturing companies. It is not a regulatory authority guidance, but it provides recommendations based on the biopharmaceutical industry's experience [23]:

A-Gene is not intended to represent a standard to be rigidly applied. It is a hypothetical case study representing an archetype of an AAV vector for gene therapy. Therefore, it is a snapshot in time of current best principles in a rapidly evolving

field. The data cited in the document are non-proprietary, and are intended to be for illustrative purposes only. Where appropriate the authors have borrowed formatting and structure from the A-Mab case study. While we have attempted to be as comprehensive as possible, and have subjected the document to rigorous review, it is not a "recipe book" for AAV manufacture. Some aspects of process development (e.g., facility design), were deliberately omitted for the sake of brevity. Importantly, A-Gene is not an example of a mock regulatory submission, nor should it be interpreted as regulatory advice, or cited as regulatory guidance. As a final point, we wish to thank those who contributed to this effort. The Alliance for Regenerative Medicine is grateful to the innumerable thought leaders, subject matter experts, and researchers who have helped to make this project a reality. We also wish to extend our deep appreciation to the National Institute for Innovation in Manufacturing Biopharmaceuticals for their support and contributions to this effort, and for working with ARM to make this project a reality. We look forward to continuing our work with our members, key opinion leaders, and the numerous innovators who have made gene therapies a reality for the thousands of patients who rely on biotherapeutic developments to improve their quality of life. We intend to continue our work with these groups to maintain the relevance and accuracy of this document as the industry advances.

The hypothetical gene therapy product that was selected was a recombinant adeno-associated virus (rAVV), manufactured using the transient triple DNA plasmid process. Table 13.8 is a compilation of the testing recommendations across the seven CQA categories for a viral vector biopharmaceutical, for both the drug substance (DS) and the drug product (DP).

A couple of comments about Table 13.8 for the testing of a viral vector:

Quantity – Viral Particle Content

Viral particle count includes both full capsids (i.e., transgene present) and empty capsids (i.e., transgene absent). Since some manufactured processes can yield up to 90% empty capsid viral particles, further analysis is expected. Further detail on the empty-fill capsid ratio can be obtained by ion exchange chromatography (IEX-HPLC), analytical ultracentrifugation (AUC) or transmission electron spectroscopy (TEM).

Purity/Impurities – Process-Related Impurities

Not all process-related impurities will appear on the release testing list. Those that can be controlled through process validation will not (e,g,, antifoam, PEI, etc.).

Potency

There are three biological activities for a viral vector to be effective: (1) it must infect a cell and transfer the transgene into the cell, (2) the transferred transgene needs to produce the intended protein, and (3) the expressed protein must be functional. The desire is to develop a cell-based potency assay that measures this mechanism of action (MOA). But until such a bioassay is in place, there might need to be two or more assays to ensure that all three activities of the viral vector are measured.

Table 13.8 Proposed testing in each CQA category for a viral vector

Proposed Testing in Each CQA Category for a Hypothetical Viral Vector (rAAV)				
CQA Category	**Test Method**	**Measurement**	**DS**	**DP**
Appearance	Visual Observation (Compendial)	Color Clarity Visible particles	√ √	√ √ √
Identity	Peptide Map qPCR	Capsid Identity Vector genome identity	√ √	√ √
Purity/ Impurities	Purity rCGE, RP-HPLC or SDS-PAGE UV260/UV280 Ratio SEC-HPLC or DLS	 Capsid protein purity Particle content Particle aggregation	 √ √ √	 √ √ √
	Process-Related Residuals ELISA qPCR ELISA ELISA ELISA qPCR	 HCP Host cell DNA BSA Affinity ligand Benzonase Plasmid DNA	 √ √ √ √ √ √	
Potency	Infectivity qPCR [Cell-Based bioassay]	TCID50 Transgene expression [Mechanism of Action (MOA)]	√ √ √	√ √ √
Quantity	qPCR ELISA	Vector genome titer Viral particle count	√ √	√ √
Safety	Microbial Growth Microbial Growth LAL Cell-Based Assay	Bioburden Sterility Endotoxin Replication competent AAV	√ √ √	 √ √
General (compendial)	pH Meter Osmolality Extractable Volume	pH value Osmolality Extractable volume (mL)	√ √	√ √ √

Assays must be done to assess the functional activity of the vectors. In earlier stages of development, ELISA may be used to demonstrate transgene protein expression in a dose-dependent manner, whereas later stages of development (e.g., phase 3 clinical trial) require the establishment of a bioassay that quantifies the functional activity of the transgene product. Additionally, quantification of infectivity may be used as a supplemental approach to assess the functional activity of AAV vectors. Potency assays should take into account the product's MOAs. However, this tends to be a large hurdle for gene therapies due to the multifaceted nature of MOAs. Many gene therapies have complex MOAs that rely on multiple biological activities (transfection/infection, gene transcription, translation, action of translated protein), and the MOAs of many are not fully characterized. Thus, several stages of MOA must be captured within a single potency assay.

Safety
Adventitious virus and mycoplasma are tested at the unprocessed bulk production cell culture stage (which is the most sensitive point in the process for detection). Results must be negative.

13.8.3 Project A-Cell

Project A-Cell, released in 2022, is a biopharmaceutical case study-based approach on how to integrate Quality by Design (QbD) principles into cell-based therapy CMC programs. It is important to understand that the proposals on CQAs in this document for cell-based therapy products (specifically, *ex vivo* CAR T cells) were driven by CMC subject matter experts from within the biopharmaceutical industry (contributions from more than 50 industry experts from more than 30 leading therapeutics developers and service/technology providers). It is not a regulatory authority guidance, but it provides recommendations based on the biopharmaceutical industry's experience [24]:

A-Cell is not intended to represent a standard to be rigidly applied. It is a hypothetical case study of the development of a cell-based therapy product. Therefore, it is a snapshot in time of current best practices in a rapidly evolving field. The data cited in this document are non-proprietary, and are intended for illustrative purposes only. While we have attempted to be as comprehensive as possible, and have subjected the document to rigorous review, it is not a "recipe book" for cell-based therapy manufacture. Importantly, A-Cell is not an example of a mock regulatory submission, nor should it be interpreted as regulatory advice, or cited as regulatory guidance.

The hypothetical cell-based therapy product that was selected was collected patient cells, transduced with a recombinant lentivirus (rLV), to manufacture genetically modified chimeric antigen receptor (CAR) T cells. Table 13.9 is a compilation of the testing recommendations across the seven CQA categories for a genetically modified patient cells biopharmaceutical, for both the drug substance (DS) and the drug product (DP).

A couple of comments on Table 13.9 for the testing of genetically modified patient cells:

Drug Substance vs Drug Product
Cell-based therapies are not like other biopharmaceutical manufacturing processes in which there is a distinct drug substance stage, where the product is held, release tested, before processing into the drug product. Cell-based therapies are continuous manufacturing processes. Fitting information about genetically modified patient cell biopharmaceuticals into the ICH common technical document (CTD) structure can be challenging for a continuous manufacturing process due to the need for clear separation between drug substance and drug product. For continuously

Table 13.9 Proposed testing in each CQA category for genetically modified patient cells

Proposed Testing in Each CQA Category for a Hypothetical CAR T Cells		
CQA Category	**Test Method**	**Measurement**
Appearance	Visual Observation (Compendial)	Color Clarity Visible Particles
Identity	Flow Cytometry	CAR+ T cells
Purity/ Impurities	Purity Cell Counter with Dye Flow Cytometry	Viable cells CAR+ T cells
	Process-Related Residuals Microscopy Flow Cytometry ELISA	Beads Unwanted cell types Serum/BSA
Potency	ELISA Cytotoxicity Bioassay	IFN-ɣ cytokine release [Mechanism of Action (MOA)]
Quantity	Cell Counter Flow Cytometry	Total Cells CAR+ T cells
Safety	Gram Staining Microbial Growth Microbial Growth LAL qPCR qPCR VSV-G Gene	Bacteria Sterility Mycoplasma Endotoxin Vector copy number Replication competent LV
General (compendial)	pH Meter Osmolality Extractable Volume	pH value Osmolality Extractable volume (mL)

manufactured cells, the DS section of the CTD would typically describe the process steps from harvesting cells to just prior to formulation, with the DP section of the CTD typically describing the formulation and filling process steps. The DP release test sample would typically be pulled after formulation but prior to filling, and it would be stored under conditions identical to the final product (typically frozen) until tested. Testing should be done at the furthest point in the manufacturing process unless assay interference occurs (e.g., due to low concentration or formulation excipients). The following is a typical DS specification section of the CTD [25]:

> The ide-cel active substance immediately enters the finished product process, the transition from active substance to finished product does not include any hold steps. Therefore, there are no specifications, batch analyses or justification of specification or description of container closure systems for ide-cel active substance. Ide-cel is controlled at the level of the finished product. Considering the nature of the product, the applicant's approach is considered acceptable.

Potency
In early-stage clinical development, quantity can be used for potency. But for late-stage and market approval, a bioassay to measure functionality of the CAR (i.e., the mechanism of action, MOA) is required.

Safety
Vector copy number (VCN) is listed under safety. VCN is a surrogate measure of lentiviral transduction, which detects vector transgene integration into the host cell genome. It is intrinsically required for the CAR protein expression and efficacy. Lentiviral transduction presents a theoretical safety risk of insertional oncogenesis, thus VCN is also considered a safety assay.

13.8.4 mRNA (USP)

The United States Pharmacopeia (USP) recognizing the need for direction on mRNA quality prepared a draft guideline on analytical procedures for this biopharmaceutical type [25]:

> A common set of methods is needed. Since the successful application of mRNA technology is relatively new, regulatory guidelines and industry standards to guide non-proprietary aspects of mRNA quality during development and manufacturing are still evolving. These include areas such as verifying the identity of the drug substance, controlling impurities and measuring content for dosing. Without a common set of methods for determining quality, developers and manufacturers of mRNA products must develop their own in-house methods and protocols, taking attention and resources away from a company's successful application of mRNA technology unique to the medical product.

The USP guideline addressed future vaccine efforts with mRNA non-viral vectors, but it has application to therapeutic uses of mRNA also. Table 13.10 is a compilation of the testing recommendations across the seven CQA categories for a mRNA non-viral vector biopharmaceutical; but, for the drug product (DP) only, and only after the mRNA has been extracted from lipids. Note, the USP table does not address the testing for potency; also *E. coli* HCP may need to be tested (if not tested for in linearized DNA plasmid starting material).

Table 13.10 Proposed USP testing in each CQA category for mRNA non-viral vectors

Proposed USP Testing in Each CQA Category for a Hypothetical mRNA Non-Viral Vector		
CQA Category	**Measurement**	**USP Test Method**
Appearance	Visual Observation	USP <1> USP <790>
Identity	Sequence Confirmation	Next generation sequencing (NGS) Sanger sequencing Reverse transcriptase-PCR
Purity/ Impurities	Purity % intact mRNA 5´ Cap 3´ Poly(A)	Capillary gel electrophoresis (CGE) HPLC HPLC
	Process-Related Residuals Residual DNA template Residual dsRNA	qPCR immunoblot
Quantity	mRNA Content	Reverse transcriptase-PCR (q or d) UV
Safety	Bioburden Sterility Endotoxin	USP <61>, <62>, <1115> USP <71> USP <85>
General	pH Meter	USP <791>

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Chapter 14
The Art of Setting Biopharmaceutical Specifications – Release and Shelf-Life

Abstract Quality Control testing, coupled with the control over the manufacturing process, provides assurance of a biopharmaceutical's identity, purity, quality, potency, and safety. Each test method performed has an assigned limit, or range, or descriptor, referred to as specification. Specifications are required for release and shelf-life testing of the biopharmaceutical drug substance and drug product. In this chapter, the challenge of setting these specifications for biopharmaceuticals will be discussed – a challenge that should not be under-estimated. The need to apply the minimum CMC regulatory compliance continuum risk-based approach to specification assignments, from the start of clinical development through market approval, will be stressed. Regulatory guidance and industry practice on setting release and shelf-life specifications across the different types of biopharmaceuticals (protein-based, viral vectors and genetically modified patient cells) will be examined and compared.

Keywords Specifications · Release · Shelf life · Risk-based · Development · Justification · Commercial · Biosimilars · Potency · Purity · Limit · Range · Descriptor · Instability · In-use

A patient has to trust that the biopharmaceutical product being administered is what it claims to be. The manufacturer's claim for the identity, purity, quality, potency, and safety of their biopharmaceutical is based on the control over the manufacturing process coupled with Quality Control's testing that is performed for both the drug substance and drug product, both for release and shelf-life. Each test is associated with one of the seven critical quality attribute (CQA) categories discussed in Chap. 13: (1) appearance, (2) identity, (3) purity/impurities, (4) potency, (5) quantity, (6) safety, and (7) general. Each test has an assigned specification. In this chapter, the challenge of setting these specifications for a biopharmaceutical will be discussed. A challenge that should not be under-estimated for biopharmaceuticals, especially due to the large number of biomolecular structural variants and the complexity of the potency assay(s) needed.

J. Geigert, *The Challenge of CMC Regulatory Compliance for Biopharmaceuticals*, https://doi.org/10.1007/978-3-031-31909-9_14

The need to apply the minimum CMC regulatory compliance continuum risk-based approach to specification assignments, from the start of clinical development through market approval, will be stressed. Regulatory guidance and industry practice on setting release and shelf-life specifications across the different types of biopharmaceuticals (recombinant proteins, monoclonal antibodies, viral vectors, genetically modified patient cells) will be examined and compared.

14.1 What Is a Specification?

A specification is much more than just being a limit or a range value, that is set by the manufacturer at their convenience. A specification has a multi-facet regulatory meaning. A 'specification' is defined as [1]:

> *Specification:* A list of tests, references to analytical procedures, and appropriate acceptance criteria which are numerical limits, ranges, or other criteria for the tests described. It establishes the set of criteria to which a drug substance or drug product should conform to be considered acceptable for its intended use. "Conformance to specifications" means that the drug substance and/or drug product, when tested according to the listed analytical procedures, will meet the listed acceptance criteria. Specifications are critical quality standards that are proposed and justified by the manufacturer and approved by regulatory authorities.

By definition, a specification has four components: (1) *the listed test* – the critical quality attribute (CQA) that is to be measured, (2) *reference to a analytical procedure* – the specific test method designed to measure the CQA, (3) *appropriate acceptance criteria* – the boundaries or limits that the test results must meet, and, especially important, (4) *approved by a regulatory authority* – the manufacturer has to justify the value set.

14.1.1 Connections Between Testing Categories

There is much testing that is required for a biopharmaceutical. The testing can be separated into four categories:

(1) ***'Characterization testing'*** is precisely deciphering and describing a biopharmaceutical's physicochemical and functional activity properties. Product characterization is performed to learn about the science of the biopharmaceutical molecule, to understand where it may be physico-chemically weak and unstable, and to try to understand what changes might occur that could impact clinical efficacy or patient safety. Product characterization utilizes a vast availability of analytical, biological, immunological and microbiological test methods. Characterization testing typically is performed by Analytical Development. Characterization testing does not have specifications assigned, and when the

results are reported to a regulatory authority, the acceptance criteria set are typically 'report value'.

(2) ***'Release testing'*** is that testing performed by Quality Control (QC) in order to release a specific batch (lot) of biopharmaceutical drug substance or drug product. Based on the knowledge obtained during characterization testing, a subset of scientifically sound and appropriate QC release tests is chosen. For each release test, there is to be an assigned specification that must be met.

(3) ***'Shelf-life testing'*** is that testing performed by QC in order to assure that the released biopharmaceutical drug substance or drug product batch continues to meet its defined required properties through its assigned shelf-life. Based on the knowledge obtained during the release testing, as well as accelerated and stress stability studies, a subset of scientifically sound and appropriate QC stability-indicating tests is chosen. For each product shelf-life test, there is an assigned specification that the product is expected to remain within over the set time period.

(4) ***'Administration testing'*** (also called 'in-use' testing) is that testing performed by QC to support the handling and preparation of the drug product to be administered in the clinic to the patient. Based on the knowledge obtained during the release and shelf-life testing, a subset of QC stability tests are run to provide guidance on what can and cannot be done in the clinic to prepare the drug product for final administration to the patient. Specifications are not assigned, but dilution procedures, diluents to use or not use, and time limits for holding are defined.

The interrelationship between all four testing categories is illustrated in Fig. 14.1. Each category of testing has its purpose and value, and this purpose shouldn't get confused. The flow of selected test methods is from product characterization testing to batch release testing to shelf-life testing to administered drug testing. And here is the challenge. Unless product characterization testing of the biopharmaceutical has been comprehensive and thorough, the subset of QC release tests selected may not be appropriate or even scientifically meaningful; and then, the following subset of QC shelf-life tests and for the handling of the administered drug product may also not be appropriate or even scientifically meaningful.

Appropriate and adequate setting of specifications are important both to the manufacturer and to the patient:

– to the biopharmaceutical manufacturer who does not want to reject a manufactured batch that is safe for the patient
– to the patient who must trust that the appropriate and adequate quality, safety, potency, and purity of the biopharmaceutical that they are taking is met.

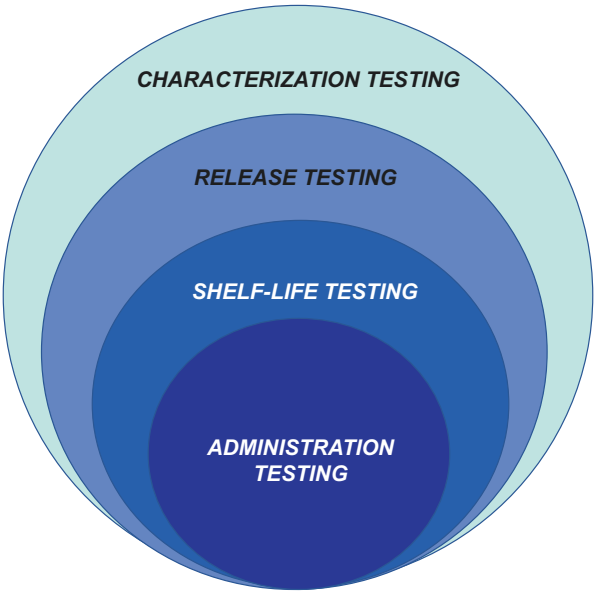

Fig. 14.1 Interrelationship between the four testing categories

14.1.2 Specifications – Only as Reliable as the Test Method Used

A test method is performed, the test result is obtained and matched against the assigned specification, the result meets the requirement – everything is ok. In the real world, that is not always true. The reliability of the test result is only as strong as the reliability of the chosen test method used to obtain it.

At all times, whether during clinical development or seeking market approval, test methods are expected to be 'scientifically sound and appropriate'. The regulatory authorities clearly understand the importance of applying a risk-based approach, such as the minimum CMC regulatory compliance continuum, to improve upon the scientific understanding and obtain the documented evidence of the reliability (i.e., validation) of the test methods over the clinical development period:

> ***Recombinant Proteins and Monoclonal Antibodies*** [2]
> Validation of analytical procedures during clinical development is seen as an evolving process. For phase I and II clinical trials, the suitability of the analytical methods used should be confirmed. The acceptance limits (e.g. acceptance limits for the determination of the content of impurities, where relevant) and the parameters (specificity, linearity, range, accuracy, precision, quantification and detection limit, as appropriate) for performing validation of the analytical methods should be presented in a tabulated form. If validation studies have been undertaken for early phase trials, a tabulated summary of the results of analytical method validation studies could be provided for further assurance.
> *Information for phase III clinical trials*

> Validation of the analytical methods used for release and stability testing should be provided. A tabulated summary of the results of the validation carried out should be submitted (e.g. results or values found for specificity, linearity, range, accuracy, precision, quantification and detection limit, as appropriate). By the end of phase III full method validation must be completed, including confirmation of robustness. It is not necessary to provide a full validation report.
>
> ***Viral Vectors and Genetically Modified Patient Cells*** [3]
>
> *S.4.3. Validation of analytical procedures*
>
> Validation of analytical procedures during clinical development is an evolving process. An appropriate degree of method qualification should be applied at each stage to demonstrate the methods are suitable for their intended use at that time. For exploratory clinical trials, the suitability of the analytical methods used should be confirmed and preliminary acceptance limits defined (e.g. acceptance limits for the determination of the content of impurities). The parameters for performing qualification of the analytical methods (specificity, linearity, range, accuracy, precision, quantitation and limit of detection, as appropriate) should be presented in tabulated form. It is not necessary to provide full interim validation report. If validation studies have been undertaken for early phase trials, a tabulated summary of the results of analytical method validation studies could be provided for further assurance.
>
> *Information for confirmatory clinical trials*
>
> For confirmatory clinical trials, the guidelines applicable to Marketing Authorisation Applications do apply. Validation of analytical methods for batch release and stability testing is expected. It is not necessary to provide full validation reports. A tabulated summary of the results of the validation carried out should be provided.

Validation of a biopharmaceutical test method follows the same principles for assessing assay characteristics that are used for chemical drugs. ICH Q2(R2) *Validation of Analytical Procedures* [4] and the FDA Guidance for Industry entitled *Analytical Procedures and Methods Validation for Drugs and Biologics* [5] lay out the approach to be used for validation of the various assay characteristics (e.g., specificity, accuracy, precision, linearity, etc.). Most likely the most challenging assay to validate for the biopharmaceutical will be the potency assay discussed in Chap. 12.

During the biopharmaceutical's lifecycle, QC test methods are frequently transferred from lab-to-lab (e.g., to a new testing lab within the organization, to a new contract testing laboratory, to a new contract manufacturing organization) which creates a challenge on its own. The regulatory expectation is that test method transfer would follow a similar approach required for manufacturing process transfer (i.e., a formal test method transfer protocol is to be prepared, then signed off with the required cGMP signatures, the protocol is executed, and all required studies completed, followed by preparation of the formal transfer report, and final sign off) [6, 7].

Don't under-estimate the resources and time needed to complete the validation of the QC test methods. Manufacturers frequently find that their test methods are not adequately or appropriately validated when seeking market approval. Regulatory authorities must then impose a post-marketing approval CMC commitment on the manufacturer to complete the necessary validation of their test methods. This is illustrated in the example of the viral vector, Zolgensma (onasemnogene abeparvovec), which was EMA market-approved in March 2020 [8]:

In the context of the obligation of the MAHs to take due account of technical and scientific progress, the CAT recommends the following points for investigation:

1. Control of vector genome integrity: The applicant should develop and implement a release test and define scientifically justified acceptance criteria. When results of the first 30 batches tested with the in-house method are available, the continued need for a release test for vector genome integrity may be re-evaluated.
4. The applicant will develop a more sensitive and precise method for analysis of protein impurity by end of 2020 and will submit a variation in 2021. Additionally, the specifications of impurities will be assessed in conjunction with the development of this method and modified as appropriate.
5. The applicant will perform LC-MS analysis in order to attempt to identify the impurities listed in the specification. A progress update will be provided by June 2020.
6. The applicant will validate an aggregation assay and, once validated, submit a variation to add this method to the stability program for finished product in 2021.
7. The applicant commits to further characterize the aggregation for multiple batches ensuring that end of shelf-life samples are included in the characterisation. Data will be provided with the first application for renewal of the conditional marketing authorisation application.
8. The applicant commits to revalidate the sterility test method to demonstrate absence of interference for Ph. Eur. compliant sampling volumes by June 2020.

Bottom line, no matter where the biopharmaceutical is in clinical development, the test methods need to be 'fit for use', because the decision to release a biopharmaceutical batch is dependent upon the results. As clinical development advances, follow the test method validation guidances to ensure that the validation is completed in the appropriate timely manner. And finally, ensure that the test method validation information submitted to the regulatory authority is thorough, clearly described, and meets the required confirmation of test method reliability.

14.2 Setting Release Specifications

To have meaningful release specifications, it is most important to recognize the linkage of the assigned acceptance criteria to the test method, the manufacturing process, biopharmaceutical product, and the medical application. Four primary approaches are used to set the release specification acceptance criteria. Statistical analysis is required to justify the acceptance criteria for release specifications that are based on manufactured batch data.

14.2.1 Spec Linkages

Release specifications are linked to (1) the specific test method, (2) the specific biopharmaceutical manufacturing process, (3) specific manufactured biopharmaceutical, and (4) the intended medical application:

(1) ***Linked to the Test Method***

Different test methods to measure the same parameter yield different outcomes, depending upon the mechanism of the test method and its sensitivity. (For example, the increased number of pI peaks when switching from isoelectric focusing (IEF) to capillary IEF).

(2) ***Linked to the Manufacturing Process***

Each manufacturing process generates in addition to the intended biopharmaceutical a set of biomolecular structural variants and a set of process-related impurities. This specificity needs to be addressed in the setting of specifications for the biopharmaceutical.

(3) ***Linked to the Manufactured Product***

A biopharmaceutical is a large biomolecule. What changes can occur to a recombinant protein or monoclonal antibody, will differ for a viral vector or for a genetically modified patient cells. (Which is why different tests are including in the stability programs for each biopharmaceutical type).

(4) ***Linked to the Intended Medical Application***

A therapeutic biopharmaceutical might be administered to patients in micrograms, or milligrams or even grams (e.g., monoclonal antibodies). For a number of the patient safety tests, the increasing patient dose amounts change the specification level that needs to be set for the biopharmaceutical.

14.2.2 Approaches to Setting Specs

Specifications need an assigned acceptance criteria. There are four primary approaches used to set that criteria: (1) upper limit based primarily on patient safety concerns, (2) upper limit below patient safety concerns based on the performance of the manufacturing process, (3) upper limit or range solely based on the performance of the manufacturing process, and (4) use of a descriptor:

(1) ***Upper Limit Based Solely on Patient Safety Concerns***

Specifications in the Safety CQA category typically fit into this specification setting approach. Upper limits for safety specifications are not negotiable – neither during clinical development nor for market approval. For the most part, safety specifications are very specific with definition of what ‘absent’ or ‘negative’ means. For example, sterility is mandatory for all injectable biopharmaceutical types. The required sterility test must meet the specification of ‘no growth’ at a limit of detection of at least 100 colony forming units per test sample, confirmed by compendial bacteriostasis/fungistasis testing.

(2) ***Upper Limit Below Safety Concern Based on Manufacturing Performance***

Some of the specifications in the Purity/Impurities CQA category fit into this specification setting approach. For example, both residual host cellular DNA and residual endotoxin have upper safety limits that cannot be exceeded. As the manufacturer gains experience with the consistency of the biopharmaceutical manufacturing process, the batch data are used to set the upper specification which is typically well below the patient safety level.

(3) ***Upper Limit/Range Set Solely on Manufacturing Performance***

Most of the specifications for the Purity/Impurity CQA category fit into this specification setting approach. The uniqueness of the various biomolecular structural variants and process-related impurities for each of the biopharmaceutical types make it difficult to set any predefined specifications. Test data must be compiled across the manufactured batches.

(4) ***Use of Descriptors***

The specification for the Appearance CQA category fits into this specification setting approach. Visual appearance, determined by either eyesight or with qualitative reference color and opalescent standards, is described by color and clarity. Also, in the chromatographic and electrophoretic methods, a reference material is run alongside the test samples, with a specification set as 'comparable to reference material'. The definition of what comparable stands for (e.g., certain number or type of peaks) is defined in the test procedure.

While it's recognized that the amount of information available, for use in each of these four specification setting approaches, will be limited at the early clinical stage, the information that can be used is expected to increase through clinical development moving towards market approval (see Sect. 14.5). Statistical analysis is required to justify the final acceptance criteria for release specifications that are based on manufactured batch data.

14.2.3 A Time for Everything – Including Statistics

As clinical development advances, especially looking toward the pivotal clinical program or toward market approval, an adequate number of manufactured biopharmaceutical batches should be available to perform a statistical analysis for justifying the acceptance criteria for a release specification. Two key factors play into the relevance of using statistics in setting meaningful release specification acceptance criteria based on manufacturing process performance: (1) the batches included in the analysis, and (2) the statistical tool used.

One of the two key factors in setting a range or a limit for a release specification based on manufacturing process performance, is the choice of the batches to include (or not include) in the calculation. Manufactured batches used in the clinic setting

are especially important in this analysis, as they support that the acceptance criteria of the release specification are within the human safety and efficacy data collected on the biopharmaceutical. It is one of the reasons that regulatory authorities prefer the assigned range or limit to not be wider or higher than what was actually tested for these product batches used in the clinical program. However, typically, few manufactured batches are actually used in the pivotal clinical studies where efficacy is evaluated; therefore, it is necessary to add in batches: (1) manufactured but not used in clinical trials, (2) batches across the entire clinical development program, and (3) at times, even batches from the preclinical program or small-scale development studies. But, whatever batches are included in the analysis, need to be defended. Not all manufactured batches may be justified, especially if there have been significant manufacturing process changes or analytical method changes during the early stages of clinical development, or if there were quality or safety reasons why a batch was not used in the clinical study. Expect regulatory authorities to question the choice of manufactured batches used in the statistical analysis to set the acceptance criteria, especially if batches outside of the clinical studies are included.

The second of the two key factors in setting a range or limit based on manufacturing process performance is the choice of analysis tool. There are three main tools – one is non-statistical and the other two are statistical comparisons:

- ***Min, Max***. A range or limit based on minimum/maximum is set by looking across the values of all of the batches included in the analysis, and selecting the two extremes. This is not a statistical approach, and this approach is the least accepted by the regulatory authorities. But when displayed, it helps to appreciate the full range encountered in manufacturing.
- ***Reference Interval***. Also known as the 'sigma rule'. Sigma, or standard deviation, is a measure of the dispersion (spread) of batch data around a mean value. A mean ± 2 standard deviations covers 95.5% of the batch data; a mean ± 3 standard deviations covers 99.7% of the batch data. Ranges established using the mean ± 3 standard deviations seem to be the most favored of the statistical analyses by the regulatory reviewers.
- ***Tolerance Interval***. This statistical analysis is similar to the reference interval approach, but it incorporates probabilities (i.e., % confidence) into the range calculation. This approach recognizes that the uncertainty of a range assignment increases as the data set size decreases. Using published tolerance interval tables, typically, a range is determined that incorporates a 95.5% confidence into the 99.7% of the manufactured batch data. Thus, a range established with only 10 batches available would be equal to about a mean ± 4 standard deviations; while a range established with 30 batches in the set would be equal to about a mean ± 3 standard deviations. Ranges established using this tolerance interval approach is favored by the biopharmaceutical industry, but it can meet resistance from some regulatory reviewers.

Do not blindly except the range set by either of the three approaches listed above. The validity of each approach is based on the data set being uniformly (i.e., normally) distributed around the mean. Expect regulatory authorities to question the

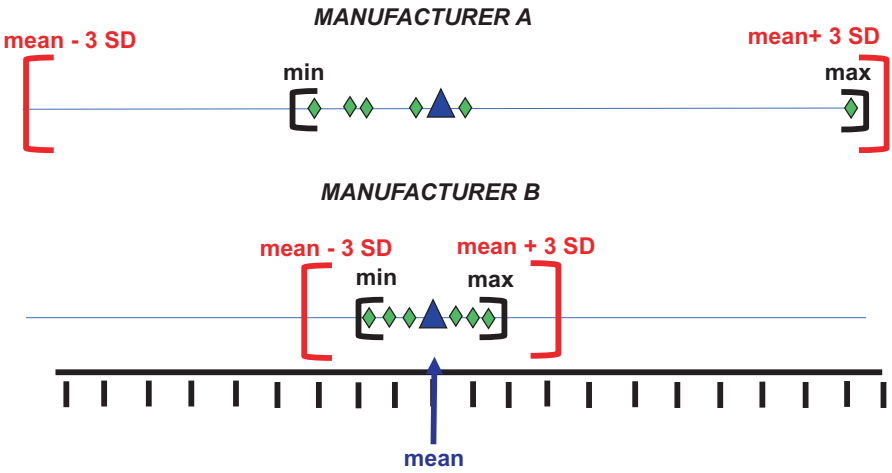

Fig. 14.2 Illustration of visually viewing data – not just looking at the mean values

distribution of the batch data used in the analysis to set the range. As an example, consider manufacturer A who produces six biopharmaceutical batches with the following potency values: 15, 12, 13, 30, 10, and 17 IU/mg. Then consider manufacturer B who produces the same product and ends up with six biopharmaceutical batches with the following potency values: 12, 19, 18, 14, 17, and 15 IU/mg. Both manufacturers set the potency range using the mean ± 3 standard deviations calculation, and both result in a mean potency value of 16 IU/mg. But the potency range from manufacturer A's batches is from 0 to 43 IU/mg (unacceptably out of control manufacturing process), while the potency range from manufacturer B's batches is from 9 to 22 IU/mg (under control manufacturing process), as illustrated in Fig. 14.2. Regulatory authorities do not reward poor manufacturing performance, which is why proposed specifications are thoroughly examined by them.

Statisticians from the FDA's Office of Biostatistics have published their thoughts on the strengths and weaknesses of each of these three approaches [9]. While not really liking any of these simple approaches, they do emphasize that *"all specifications need to be scientifically meaningful."*

14.3 Setting Shelf-Life Specifications

Shelf-life specifications are linked to the release specifications. Those CQA test methods that show change over storage time for the biopharmaceutical are the ones that will be evaluated as shelf-life specifications. Therefore, appropriate and adequate stability studies are required.

14.3.1 Stability Assessment – A Regulatory Requirement

Stability studies are required by the regulatory authorities for all biopharmaceutical types in order to determine which CQA release specifications might be changing with storage. The release specifications that are confirmed to change, as well as the degree of change measured over time, will serve as the justification of the shelf-life specification limits/range that will need to be set for the biopharmaceutical.

Regulatory authorities are very clear that a stability program is to be put in place from the beginning of the clinical development program, so that as clinical development proceeds, confirmation of the shelf-life can be appropriately and adequately set, for all biopharmaceutical types:

Recombinant Proteins and Monoclonal Antibodies [10]

Biotechnological/biological products do have distinguishing characteristics to which consideration should be given in any well-defined testing program designed to confirm their stability during the intended storage period. For such products, in which the active components are typically proteins and/or polypeptides, maintenance of molecular conformation and, hence of biological activity, is dependent on noncovalent as well as covalent forces. The products are particularly sensitive to environmental factors such as temperature changes, oxidation, light, ionic content, and shear. In order to ensure maintenance of biological activity and to avoid degradation, stringent conditions for their storage are usually necessary. The evaluation of stability may necessitate complex analytical methodologies. Assays for biological activity, where applicable, should be part of the pivotal stability studies. Appropriate physicochemical, biochemical and immunochemical methods for the analysis of the molecular entity and the quantitative detection of degradation products should also be part of the stability program whenever purity and molecular characteristics of the product permit use of these methodologies. With the above concerns in mind, the applicant should develop the proper supporting stability data for a biotechnological/biological product and consider many external conditions which can affect the product's potency, purity and quality. Primary data to support a requested storage period for either drug substance or drug product should be based on long-term, real-time, real-condition stability studies. Thus, the development of a proper long-term stability program becomes critical to the successful development of a commercial product.

Viral Vectors [11]

Stability protocols, stability data, justifications for the container-closure system used, and proposed shelf-lives and storage conditions, should be presented for the drug substance, drug product and any intermediate product stored during production (i.e. intermediates for which a holding time is scheduled on the production process scheme). The principles outlined in ICH stability guidelines (and particularly *ICH guideline Q5C*) should be followed. Real time stability studies should be undertaken, in particular for the DS and DP intended for marketing. However, it is acknowledged that accelerated stability studies (e.g. at elevated temperatures or under other stress conditions relevant for the product of interest) may provide complementary supporting evidence for the stability of the product and help to establish the stability profile. Forced degradation studies provide important information on degradation products and identify stability indicating test. In general, the shelf-life specifications should be derived from the release specifications, with additional emphasis on the stability-indicating features of tests used and tests/limits for degradation products. Vector integrity, biological potency (including transduction capacities) and strength are critical product attributes which should always be included in stability studies.

Genetically Modified Patient Cells [12]
Stability studies, including in-use stability studies, should be conducted according to the principles described in the Guideline on human cell-based medicinal products (EMEA/CHMP/410869/2006). Quality attributes to be followed during stability studies should be defined on the basis of characterisation studies. They should be stability indicating (and quantitative) and be able to detect clinically meaningful changes in the product.

14.3.2 Key Basics of the Stability Program

ICH Q5C lays out the key basics for the stability program of all biopharmaceutical types. But it is important to recognize that the stability requirements for a biopharmaceutical (as laid out in ICH Q5C) are not exactly the same for a chemical drug (as laid out in ICH Q1A(R2)):

Minimum stability data required for label-claim conditions –
Chemical drug: 12 months
Biopharmaceutical: 6 months
Definition of 'significant change' –
Chemical drug: 5% change
Biopharmaceutical: case-by-case
Extrapolation used to extend shelf-life –
Chemical drug: Yes (Arrhenius Plotting)
Biopharmaceutical: Not predictive

While ICH Q5C specifically designs stability programs for recombinant proteins and monoclonal antibodies, the same four design principles are universal to all biopharmaceutical types: (1) selection of the batches to have test samples placed on study, (2) the storage conditions for the test samples on study, (3) the CQA test methods to use in the testing, and (4) the frequency of testing the stored test samples:

(1) ***Batch Selection***

During the clinical development period, typically, up to three batches of the biopharmaceutical drug substance and drug product each are added to an ongoing stability program, as each batch is manufactured. When major manufacturing process changes occur during clinical development, the batch count might need to be restarted. Whether the previous batches remain on stability study is dependent upon whether the previous product is still being used in the clinical program and whether the previous product can be justified as comparable to the new manufacturing process. When seeking market approval, the ICH Q5C recommendation is for a minimum of three (3) batches each of the biopharmaceutical drug substance and drug product to have completed the desired shelf-life time period.

It is key that the biopharmaceutical batches placed onto the stability program be 'representative' of the biopharmaceutical either currently under clinical development or the to-be-marketed product. This means representative of (1) the actual manufacturing process, (2) the container closure (although at a reduced scale for the drug substance), and (3) the quality intended for the biopharmaceutical. It cannot be emphasized strongly enough the need for the product batches on stability study to be adequately 'representative'. If a regulatory authority deems the product in a study to not be representative, the acquired stability data may be declared invalid.

(2) ***Storage Conditions***

A minimum of two storage condition are needed: long-term and accelerated. Biopharmaceuticals need to be maintained over their shelf life at a defined storage temperature (e.g., frozen, refrigerated, etc.) that is stated on the label for the product – this is referred to as the long-term study. To increase the ability to earlier detect product change, the stability study also includes an 'accelerated storage condition', which primarily means holding the product at an elevated temperature above the label claim, see Table 14.1. Note, biopharmaceuticals are in sealed vials, syringes and/or bags, so they are protected from environmental humidity. Therefore, unlike chemical drugs which are frequently tablets, the relative humidity (RH) requirement can be justified as not being necessary.

(3) ***CQAs to Test***

Based on a scientific risk assessment, the CQA release test specifications that are likely to change over the shelf-life are placed into the testing profile of the stability program. For example, typically, biomolecular structural variants are likely to increase over shelf-life, while process-related impurities are not expected to change. While sterility is not expected to change over the shelf-life of the biopharmaceutical, it is a required test in the stability program. But, instead of the culture sterility test method, a container closure integrity testing (CCIT) is performed over the shelf-life study. Note, if the biopharmaceutical is in a device (e.g., prefilled syringe), then confirming the functioning of the device (e.g., plunger not sticking) is also a required test in the stability program.

(4) ***Frequency of Testing***

ICH Q5C recommends the minimum testing frequency shown in Table 14.2.

Table 14.1 Stability test samples storage conditions

STABILITY STORAGE CONDITIONS		
Label Claim	Long-Term Storage Condition	Accelerated Storage Condition
Liquid N_2 vapor	≤ -120°C	-20°C ± 5°C
Frozen	-20°C ± 5°C	5°C ± 3°C
Refrigerated	5°C ± 3°C	25°C ± 2°C
Ambient	25°C ± 2°C	40°C ± 2°C

Table 14.2 Recommended minimum testing frequency in stability program

Recommended Minimum Testing Frequency in Stability Program	
Intended Shelf Life	**Minimum Testing Frequency**
≤ 12 months	0, 1, 2, 3, 6, 9, 12 months
≥ 12 months	0, 3, 6, 9, 12, 18, 24, 36 months

14.4 In-Use Guidance for the Administered Drug

Drug product release specifications confirm that the biopharmaceutical is acceptable for human use. Shelf-life specifications confirm that as long as the drug product is within the approved expiry date, it is acceptable for human use. Guidance provided to the patient, physician, hospital pharmacy is now necessary to ensure that the quality and safety specifications of the biopharmaceutical are not voided by their handling for patient administration. Which means to the biopharmaceutical manufacturer, it is of utmost importance, to know how their biopharmaceutical is intended to be used in the clinic setting. It might be diluted (so which diluents should be used), it might be held for extended periods of time at different temperatures (so which time and temperature conditions are appropriate), etc.

Therefore, regulatory authorities expect that in-use studies are performed:

Recombinant Proteins and Monoclonal Antibodies [2]

In-use stability data should be presented for preparations intended for use after reconstitution, dilution, mixing or for multidose presentations. These studies are not required if the preparation is to be used immediately after opening or reconstitution.

Viral Vectors [11]

Where relevant, the in-use stability of the drug product (after reconstitution or after thawing) should be properly investigated including its compatibility with any diluents used in reconstitution and if appropriate, devices used for administration. The recommended in-use time period should be justified.

Genetically Modified Patient Cells [3]

For preparations intended for use after reconstitution, dilution or mixing, a maximum shelf life needs to be defined and supported by in-use stability data.

The in-use instructions provided with the drug product package insert of several market-approved biopharmaceuticals, illustrate the type of information that a manufacturer generates to provide direction in the clinic setting [13, 14], see Table 14.3.

Microbial growth in sterile biopharmaceutical solutions, after they are withdrawn from the drug product container closure, is a safety concern of regulatory authorities, due to the holding times and temperatures used in the clinic. Manufacturers need to perform the required microbial growth study with their drug product solution and likely dilutions, and anticipated holding times in the clinic. The following describes the FDA proposed study [15]:

Microbiological studies in support of the post-reconstitution and/or post-dilution storage conditions. Describe the test methods and results that employ a minimum countable inoculum (10-100 CFU) to simulate potential microbial contamination that may occur during dilution. The test should be run at the label's recommended storage conditions, be con-

Table 14.3 In-use handling instructions for market-approved biopharmaceuticals

In-Use Stability Conditions in Package Insert	
Biopharmaceutical Type	**Instructions Provided**
Monoclonal Antibody Fc Fragment Vyvgart (efgartigimod alfa-fcab)	Dilute the withdrawn VYVGART with 0.9% Sodium Chloride Injection, USP.. Administer immediately after dilution and complete the infusion within 4 hours of dilution. If immediate use is not possible, the diluted solution may be stored refrigerated at 2°C to 8°C (36°F to 46°F) for up to 8 hours. Do not freeze.
Viral Vector (rAAV) Adstiladrin (nadofaragene firadenovec-vncg)	Upon receipt, cartons of ADSTILADRIN can be stored as • In a freezer ≤ -60°C (≤ -76°F) until expiry date. The vials may be stored for up to 24 hours at room temperate or refrigerated once it is taken out of the freezer. Connect the syringe to the vial adaptor and withdraw the contents of the vial into the syringe. Use ADSTILADRIN within 1 hour of drawing into syringe.
Genetically Modified Patient Cells Carvykti (ciltacabtagene autoleucel)	Store and transport below -120°C. Thaw CARVYKTI at 37°C±2°C using either a water bath or dry thaw method until there is no visible ice in the infusion bag. Total time from start of thaw until completion of thawing should be no more than 15 minutes. Gently mix the contents of the bag to disperse clumps of cellular material. Do not pre-filter into a different container, wash, spin down, or resuspend CARVYKTI in new media prior to infusion. Once thawed, the CARVYKTI infusion must be completed within 2.5 hours at room/ambient temperature (20°C to 25°C).

ducted for twice the recommended storage period, bracket the drug product concentrations that would be administered to patients, and use the label-recommended reconstitution solutions and diluents. Periodic intermediate sample times are recommended. Challenge organisms may include strains described in USP <51> *Antimicrobial Effectiveness Testing*, plus typical skin flora or species associated with hospital-borne infections. *In lieu* of this data, the product labeling should recommend that the post-reconstitution and/or post-dilution storage period is not more than 4 hours.

14.5 Applying the Minimum CMC Regulatory Compliance Continuum

Borrowing a quote from Lewis Carroll's book Alice's Adventure in Wonderland: *"Would you tell me please, which way I ought to go from here?" "That depends a good deal on where you want to get to," said the Cat. "I don't much care where," said Alice. "Then it doesn't matter which way you go," said the Cat. "– so long as I get somewhere," Alice added as an explanation*. Setting specifications during

clinical development can seem sometimes as confusing and directionless. Hastily setting a limit or range for a specification, without scientific support, does not serve our patients adequately, especially if the specification is incorrectly set or not justifiable. This can lead on the one hand to a false sense of quality and patient safety and on the other hand to safe product batches being rejected and not available to meet the patient's need. The goal of a risk-based approach is to always add value where and when necessary, and always to do what is appropriate and adequate to protect the patient at the right time.

14.5.1 Minimum … Continuum Applied to Release Specifications

As discussed in Chap. 4, Sect. 4.3.1, the regulatory authorities accept a minimum CMC regulatory compliance continuum risk-based approach for biopharmaceutical manufacturing. For release specifications, the regulatory requirements and expectations can be divided into three stages: (1) initiation of clinical development stage (FIH), (2) later clinical development stage (initiation of pivotal study) and (3) seeking market approval stage.

14.5.1.1 Initiation of Clinical Development Stage (FIH)

For release specifications, at the initiation of clinical development stage (e.g., First-in-Human, FIH), regulatory authority expectations are, in summary, as follows:

- Release specifications for both the drug substance and drug product are to be established across the seven groups of CQAs (see Chap. 13)
- The selection of tests are driven by the characterization studies on the biopharmaceutical
- Specifications should have actual ranges/limits – use of 'record value' or 'report results' is discouraged; however, for product characterization tests not yet completely defined (e.g., glycosylation), it is acceptable to use 'report results'

  [Note, this point is clearly stated by EMA for the IMPD, but FDA does not mention it for the IND; however, in practice, FDA reviewers do expect this to be followed]

- Recognition that these specifications are preliminary, not finalized
- Recognition that the limits set may be wide and not reflect ultimate process capability – using whatever meaningful batch data are available (including development batches)
- Recognition that more CQA test methods will need to be done to cover for the uncertainty in the specifications

- Safety tests (especially for adventitious mycoplasma and virus contamination) with their specifications are to be at the appropriate location through the manufacturing process
- Emphasis on establishing a potency assay sooner than later (for the gene therapies)
- Consideration will be given to continuous manufacturing processes (e.g., genetically modified patient cells) to test either at DS or DP, but not necessary both
- Product batches that do not meet the specifications are not to be released

For release specifications, at the initiation of clinical development stage, three major regulatory guidance documents, covering the different biopharmaceutical types, have been provided:

EMA for the IMPD – Recombinant Proteins and Monoclonal Antibodies [2]

S.4.1. Specification

The specification for the batch(es) of active substance to be used in the clinical trial should define acceptance criteria together with the tests used to exert sufficient control of the quality of the active substance. Tests and defined acceptance criteria are mandatory for quantity, identity and purity and a limit of 'record' or 'report results' will not be acceptable for these quality attributes. A test for biological activity should be included unless otherwise justified. Upper limits, taking into account safety considerations, should be set for the impurities. Microbiological quality for the active substance should be specified. As the acceptance criteria are normally based on a limited number of development batches and batches used in non-clinical and clinical studies, they are by their nature inherently preliminary and may need to be reviewed and adjusted during further development. Product characteristics that are not completely defined at a certain stage of development (e.g. glycosylation, charge heterogeneity) or for which the available data is too limited to establish relevant acceptance criteria, should also be recorded. As a consequence, such product characteristics could be included in the specification, without pre-defined acceptance limits. In such cases, a limit of 'record' or 'report results' is acceptable. The results should be reported in the Batch Analyses section (S.4.4).

P.5.1. Specification

The same principles as described for setting the active substance specification should be applied to the medicinal product. In the specification, the tests used as well as their acceptance criteria should be defined for the batch(es) of the product to be used in the clinical trial to enable sufficient control of quality of the product. Tests for content, identity and purity are mandatory. Tests for sterility and endotoxins are mandatory for sterile products. A test for biological activity should be included unless otherwise justified. Upper limits, taking safety considerations into account, should be set for impurities. They may need to be reviewed and adjusted during further development. Acceptance criteria for IMP quality attributes should take into account safety considerations and the stage of development. Since the acceptance criteria are normally based on a limited number of development batches and batches used in non-clinical and clinical studies, their nature is inherently preliminary. They may need to be reviewed and adjusted during further development. The analytical methods and the limits for content and bioactivity should ensure a correct dosing. For the impurities not covered by the active substance specification, upper limits should be set, taking into account safety considerations.

S.4.5. Justification of specification

A justification for the quality attributes included in the specification and the acceptance criteria for purity, impurities, biological activity and any other quality attributes which may be relevant to the performance of the medicinal product should be provided. The justification should be based on relevant development data, the batches used in non-

clinical and/or clinical studies and data from stability studies, taking into account the methods used for their control. It is acknowledged that during clinical development, the acceptance criteria may be wider and may not reflect process capability. However, for those quality attributes that may impact patient safety, the limits should be carefully considered taking into account available knowledge (e.g. process capability, product type, dose, duration of dosing etc.). The relevance of the selected potency assay and its proposed acceptance limits should be justified.

FDA for the IND – Viral Vectors and Genetically Modified Patient Cells [16]

Control of Drug Substance (3.2.S.4) Specification (3.2.S.4.1)

You should list DS specifications in your original IND submission. Specifications are defined as a list of tests, references to analytical procedures, and appropriate acceptance criteria used to assess quality. Acceptance criteria should be established and justified, based on data obtained from lots used in preclinical and/or clinical studies, data from lots used for demonstration of manufacturing consistency, data from stability studies, and relevant development data. For products in the early stages of clinical development, very few specifications are finalized, and some tests may still be under development. However, the testing plan submitted in your IND should be adequate to describe the physical, chemical, or biological characteristics of the DS necessary to ensure that the DS meets acceptable limits for identity, strength (potency), quality, and purity (21 CFR 312.23(a)(7)(iv)(*a*)). Your IND should include specifications with established acceptance criteria for safety testing at Phase 1. Safety testing includes tests to ensure freedom from extraneous material, adventitious agents, microbial contamination, and replication competent virus. To maximize the sensitivity of safety testing, it is important that you perform each test at the stage of production at which contamination is most likely to be detected. For example, tests for mycoplasma or adventitious viruses (in vivo or in vitro) should be performed on cell culture harvest material (cells and supernatant) prior to further processing, e.g., prior to clarification, filtration, purification, and inactivation. Your IND should also include specifications for measuring an appropriate dose level (i.e., strength or potency) at Phase 1. To ensure consistent dosing in your clinical investigations, assays used to determine dose (e.g., vector genome titer by quantitative polymerase chain reaction (qPCR), transducing units, plaque-forming units, flow cytometry for transduced cells) should be qualified as suitable for use prior to initiating clinical studies. Additional testing will depend on the type of gene therapy product and the phase of clinical development. These tests may include assays to assess product characteristics, such as identity, purity (including endotoxin and contaminants, such as residual host cell DNA, bovine serum albumin (BSA), DNase), and potency/strength. Please note that the same types of tests listed in this section of the guidance may not be necessary for the release of both the DS and DP. In certain situations, the DS and DP may not be readily distinguishable due to the design of manufacturing process and control. In such cases, the sponsor may inquire with FDA on how to define DS and DP and meet release requirements. In some cases, repeat testing may be good practice; however, redundant testing may not always be feasible or provide additional information. In this case, we recommend that you provide a rationale to support the selection of testing performed for release of either DS or DP.

Control of Drug Product (3.2.P.5) Specifications (3.2.P.5.1)

You should list DP specifications in your original IND submission. Your testing plan should be adequate to describe the physical, chemical, or biological characteristics of the DP necessary to ensure that the DP meets acceptable limits for identity, strength (potency), quality, and purity (21 CFR 312.23(a)(7)(iv)(*b*)). Product lots that fail to meet specifications should not be used in your clinical investigation without FDA approval. For early phase clinical studies, we recommend that assays be in place to assess safety (which includes tests to ensure freedom from extraneous material, adventitious agents, and microbial contamination) and dose (e.g., vector genomes, vector particles, or geneti-

cally modified cells) of the product. We recommend that product release assays be performed at the manufacturing step at which they are necessary and appropriate. For example, mycoplasma and adventitious agents release testing is recommended on cell culture harvest material. In addition, sterility, endotoxin, and identity testing are recommended on the final container product to ensure absence of microbial contamination or to detect product mix-ups that might have occurred during the final DP manufacturing steps (e.g., buffer exchange, dilution, or finish and fill steps).

EMA for the IMPD – Viral Vectors and Genetically Modified Patient Cells [3]

S.4.1. Specification

The specifications for the batch(es) of the active substance to be used in the clinical trial should be defined. The acceptance criteria together with the tests used should ensure sufficient control of the quality of the active substance. The release specification of the active substance should be selected on the basis of parameters defined during the characterisation studies. The selection of tests is product-specific and needs to be defined and justified by the applicant. During early phases of clinical development specification can include wider acceptance criteria based on the current knowledge of the risks. As the acceptance criteria are normally based on a limited number of development batches and batches used in non-clinical and clinical studies, they are by their nature preliminary and need to be subject to review during development. Product characteristics that are not completely defined at a certain stage of development or for which the available data is too limited to establish relevant acceptance criteria, should also be recorded. As a consequence, such product characteristics could be included in the specification, without pre-defined acceptance limits. The results should be reported in the Batch Analyses section (S.4.4). It is nevertheless stressed that these parameters cannot replace existing and sufficient specification. If certain release tests cannot be performed on the active substance or finished product, but only on key intermediates and/or as in-process tests, this needs to be justified. Specifications should be meaningful and quantitative and a limit of 'record' or 'report results' should be avoided whenever possible. For test parameters relevant to safety, the absence of defined limits is not acceptable. Tests and defined acceptance criteria are expected for quantity, identity, purity, microbiological assays and biological activity. For a FIH trial the absence of quantitative limits for potency/biological activity would have to be justified by the applicant. Upper limits, taking safety considerations into account, should be set for impurities. Microbiological safety testing of the active substance should be specified. When development and validation was performed using cells from healthy volunteers, acceptance criteria should be revised when sufficient data with patient material is available. In case of GTIMP, the genetic identity and integrity of the drug substance should be assured. Test should identify both the therapeutic sequence, the vector and, if applicable and possible, the complexed nucleic acid sequences. In addition to sequencing data, the identity of the drug substance may also be confirmed through infection/transduction assays and detection of expression/activity of the therapeutic sequence(s).

S.4.5. Justification of specification

A justification for the quality attributes included the specification and the acceptance criteria for purity, impurities, biological activity and any other quality attributes which may be relevant to the performance of the medicinal product is required already for an exploratory clinical study. Early selection of a potency assay and its proposed acceptance limits is recommended. The justification of specifications should be based on sound scientific knowledge supported by the available development data, the batches used in non-clinical and/or clinical studies and data from stability studies, taking into account the methods used for their control. The justification should address how the respective quality attributes and acceptance criteria are relevant for the performance of the medicinal product. It is acknowledged that during early clinical development when there is only limited experience, the acceptance criteria may be wide. However, for

those quality attributes that may impact patient safety, the limits should be carefully considered taking into account available knowledge (e.g. impurities).

P.4.1. Specification

References to the Ph.Eur., the pharmacopoeia of an EU Member State, USP or JP may be applied. For excipients not covered by any of the aforementioned standards, an in-house specification should be provided. Acceptance criteria should be presented preferably as quantitative limits, ranges, or other attributes or variables for the tests described. Release criteria may be refined as product development progresses toward the marketing authorisation application.

14.5.1.2 Later Clinical Development Stage (Pivotal Clinical Studies)

For release specifications, at the later clinical development stages (e.g., pivotal clinical studies), regulatory authority expectations are increased compared to the earlier clinical development stage, in summary, as follows:

- Release test methods will be modified – some added, some deleted
- Recognition that the specification ranges/limits will change based upon additional batch data being available – expectation that the specifications will tighten
- Higher confidence in the quality measurements being linked to the clinical outcome

For release specifications, at this later clinical development stage, the following regulatory guidance is provided:

EMA for the IMPD – Recombinant Proteins and Monoclonal Antibodies [2]

S.4.1. Specification

Additional information for phase III clinical trials

As knowledge and experience increases, the addition or removal of parameters and modification of analytical methods may be necessary. Specifications and acceptance criteria set for previous trials should be reviewed and, where appropriate, adjusted to the current stage of development.

S.4.5. Justification of the Specification

Changes to a previously applied specification (e.g. addition or removal of parameters, widening of acceptance criteria) should be indicated and justified.

FDA for the IND – Viral Vectors and Genetically Modified Patient Cells [16]

Batch Analysis (3.2.S.4.4)

We recommend that you annually update this section of your IND as new batches are produced. You should indicate any batches that fail to meet release specifications and any action taken to investigate the failure according to your Quality Unit procedures.

Justification of Specification (3.2.S.4.5)

You should provide justification for the DS specifications in your IND. We recognize that acceptance criteria may be adjusted throughout the product development stages, based on both manufacturing and clinical experience. For early stage clinical studies, assays used to characterize production lots may be more variable than those used in later phase investigations.

For later stage investigational studies in which the primary objective is to gather meaningful data about product efficacy, we recommend that acceptance criteria be tightened to ensure batches are well-defined and consistently manufactured.

Specifications (3.2.P.5.1)

DP specifications should be further refined as a part of product development under an IND. We recommend that sponsors establish or, in some cases, tighten acceptance criteria, based on manufacturing experience as clinical development proceeds. Acceptance criteria should also be established, based on clinical lots shown to be safe and effective, when appropriate. We also recommend that sponsors develop testing to assess product potency and have this assay in place prior to initiating studies used to support product efficacy for licensure. For licensure, a complete set of specifications to ensure the safety and effectiveness of the product must include the general biological products standards, as outlined in 21 CFR Part 610.

EMA for the IMPD – Viral Vectors and Genetically Modified Patient Cells [3]

S.4.1. Specification

Additional information for confirmatory clinical trials

As knowledge and experience increase, the addition or removal of parameters and modification of analytical methods may be necessary. Parameters, analytical methods and acceptance criteria set for previous trials should be reviewed and, where appropriate, adjusted to the current stage of development. For confirmatory trials, the active substance specifications should be in place to allow sufficient and accurate evaluation of quality that is linked to the clinical outcome.

S.4.5. Justification of Specification

Further refinement is expected as knowledge increases and data become available. Changes to a previously applied specification (e.g. addition or removal of parameters, widening of acceptance criteria) should be indicated and justified.

P.5.1. Specification

As knowledge and experience increases the addition or removal of parameters and modification of analytical methods may be necessary. Specification and acceptance criteria set for previous trials should be reviewed for confirmatory clinical trials and, where appropriate, adjusted to the current knowledge and stage of development.

14.5.1.3 Seeking Market Approval Stage

At the time of submission of the market approval dossier for the biopharmaceutical, it is expected by the regulatory authorities that (1) the control strategy incorporating the specifications for the release of the drug substance and drug product is defined, and (2) the assigned ranges or limits can be justified.

For release specifications, at BLA/MAA submission stage, the following regulatory guidance is provided:

ICH Q6B – Recombinant Proteins and Monoclonal Antibodies [17]

SPECIFICATIONS

Selection of tests to be included in the specifications is product specific. The rationale used to establish the acceptable range of acceptance criteria should be described. Acceptance criteria should be established and justified based on data obtained from lots used in preclinical and/or clinical studies data from lots used for demonstration of manufacturing consistency, and data from stability studies, and relevant development data.

FDA BLA – Recombinant Proteins and Monoclonal Antibodies [18]

Drug Substance Specifications and Tests.

(a) *Specifications and analytical methods used for release testing, shelf life and distribution should be described.*

Specifications and tests for the drug substance sufficient to assure its identity, purity, strength and/or potency, as well as lot-to-lot consistency should be submit-

ted. Validation of the analytical systems and the data should be provided for non-compendial methods to demonstrate the system suitability.

(b) *Certificates of Analysis and Analytical Results*

Certificates of analysis and analytical results for at least three consecutive qualification lots of the drug substance should be submitted.

Impurities Profile.

A discussion of the impurities profiles, with supporting analytical data, should be provided. Profiles of variants of the protein drug substance (e.g., cleaved, aggregated, deamidated, oxidized forms, etc.), as well as non-product related impurities (e.g., process reagents and cell culture components), should be included.

Drug Product Specifications & Methods.

A description of all test methods selected to assure the identity, purity, strength and/or potency, as well as the lot-to-lot consistency of the finished product and the specifications used for the drug product should be submitted. Certificates of analysis and analytical results for at least three consecutive batches should be provided. The validation data for system stability for all non-compendial tests should be provided. If compendial methods have to be validated to ensure non-interference of special inactive ingredients, the results of those validation studies should be submitted.

EMA MAA – Viral Vectors [11]

Specifications for the drug substance

The criteria for acceptance or rejection of a production batch must be provided. DS specifications should be given and justified according to principles outlined in *ICH guideline Q6B*. A specification table (including parameters, methods and specifications or criteria for acceptance) should be provided. The specifications for the drug substance should normally encompass tests for identity, purity, content, activity, sterility, endotoxin level and mycoplasma. Tests indicated in relevant sections of *Ph.Eur. 5.14* should be considered in the specifications or any departure or omission justified. The analytical methods should be relevant and techniques validated. The following sections provide an indication of the tests expected to be included in the set of specifications but do not provide an exhaustive list as the tests required will be essentially product- and production process-specific (see *ICH guideline Q6B*, *Ph.Eur. 5.14 and Ph. Eur. 2.6.16*).

- Identity and integrity. The genetic identity and integrity of the drug substance should be assured using tests that identify both the therapeutic sequence and the vector. Such tests might include DNA sequencing or restriction enzyme mapping and immunological assays. The identity of the drug substance may also be confirmed through infection/transduction assays and detection of expression/activity of the therapeutic sequence(s) (see potency assay section). This identity test is especially important for complexed nucleic acid sequences.
- Content. The quantity of the drug substance should be established. For viral vectors, infectious titre should be quantified; the number of particles (infectious/non-infectious, empty/genome containing) should also be determined. Particle to infectivity ratio should be included to define the content of the drug substance. For plasmids and other forms of nucleic acids, the quantity or concentration of nucleic acid should be established.
- Potency Assay. A suitable measure of the potency of the DS should be established. At least one biological potency specification should be established, the attribute(s) reflecting the physiological mode of action and/or the pharmacological effects of the GTMP. The potency assay should normally encompass an evaluation of the efficiency of gene transfer (infectivity /transduction/delivery) and the level of expression of the therapeutic sequence or its direct activity. Where possible the potency assay should include a measure of the functional activity of the therapeutic sequence or the product of it. This functional test may be supplemented with immunochemical methods to determine the integrity and quantity of an expressed protein product if

appropriate. For release testing simpler surrogate assays (e.g. based on nucleic acid amplification) may be acceptable, provided a correlation to the more functional test or the clinical outcome has been established in bridging studies. *In vitro* biological potency tests should be developed. If not feasible, biological potency tests in animal tissues maintained *ex vivo* or in whole animals can be considered. Transgenic animals or animals with transplanted human tissues or systems, e.g. a suitable xenograft model, may be suitable for this purpose. In order to reduce the use of animals in accordance with the 3R principles a validated *in vitro* method is generally preferred over animal testing wherever possible (Directive 2010/63/EU). Suitable ways for expressing potency of DS (vectors) in reference to an appropriately qualified reference material should be established (including a range and specifications) whenever possible.

- Product-Related Impurities. The presence of product-related impurities such as non-functional forms of the vector, or the presence of co-packaged unwanted genetic sequences should be included in the specification and acceptance limits set to exclude or limit these impurities as appropriate and justified. For viral vectors, empty particle number, aggregates and replication competent vectors should be controlled. For plasmid DNA limits for different forms of plasmid should be included. Other impurities may need to be considered. Impurity limits should be justified with respect to clinical safety.
- Process-Related Impurities. Specifications should be set for materials used in vector production, unless process validation data have been provided to demonstrate that such residues are consistently reduced to acceptable levels. For the release specifications, tests should be developed and relevant (upper) limits set to monitor the residual levels of contaminants of cellular origin, e.g. host cell protein (including helper virus protein) or DNA from the bacterial or packaging cell line, as well as raw materials that may have been used during the production process such as benzonase or resins. Other process-related impurities may include: nucleic acids derived from bacteria used for the production of plasmid DNA, extraneous nucleic acids in vector preparations, helper viruses or other impurities such as residual animal serum proteins (e.g. BSA) used in production. If tumourigenic/immortalised cell lines are used during production the total residual DNA level should be strictly controlled and kept at a minimum unless otherwise justified. Reference is made to the *CPMP Position Statement on DNA and Host Cell Proteins (HCP) Impurities, Routine Testing versus Validation Studies* and the *Ph.Eur. 5.2.3*. Impurity limits should be justified with respect to clinical safety and efficacy.
- Extraneous agents. Tests for extraneous agents should be included to ensure the safety of the vector. For replication-deficient or conditionally-replicating viral vectors, a test for replication competent virus should be included. In the case of vectors which are potentially hazardous to patients' health in their replication competent forms, such as members of the *Retroviridae*, absence of replication competence should be demonstrated using a validated assay. In other justified cases, it may be acceptable to release vector lots with an upper limit for replication competent vector. In these cases the justification for the limit should include qualification on the basis of non-clinical and/or clinical data for batches with similar levels.
- Physicochemical properties. Limits should be applied to measurement of pH and any other relative physicochemical properties such as opalescence, refractive index. Particle number, molecular size average and size distribution should be controlled, as appropriate.
- Pharmacopoeial tests. Depending on the nature of the drug substance, other pharmacopoeial tests will apply for release; this includes sterility testing and bioburden which should be done in accordance with Ph.Eur. requirements.

Drug Product specification

Quality control tests should be performed at the DP level, unless appropriate justification can be provided based on release testing at the drug substance level. Tests on attributes which are specific to the formulated product in its final container and quality attributes which may have been impacted by the formulation steps should be included in the release testing. Unless otherwise justified, the release specifications for each batch of DP are expected to embrace the following:

- The range of quality attributes listed under "Drug substance" above, including identity and potency. Tests for impurities and process-related impurities from the DS steps could be omitted based on relevant justification and validation data.
- Infectivity or transduction efficiency: *in vitro* infectivity or transduction efficiency of the DP in its final formulation should be included.
- Specification should be applied for appearance and physicochemical properties (e.g. pH and any other relative physicochemical properties such as opalescence, refractive index and osmolality, visible and subvisible particles) specific to the drug product.
- Sterility, endotoxin, particulate matter and other pharmacopoeial tests such as extractable volume or residual moisture should be included as appropriate.
- Where appropriate, and subject to a risk-based approach, replication competent virus acceptance criteria should be applied to ensure the safety of the DP.
- Assays for critical excipients, such as albumin or complexing materials used in the formulation (of either DS or DP) should be included, particularly where these ensure the expected bioactivity and/or maintain the stability of the final formulated vector.
- Specifications should also be set for materials used in the DP formulation and filling unless process validation data have been provided to demonstrate that such residues are consistently reduced to acceptable levels.
- Where the DP contains a device, specific release testing, including functional release tests (e.g. for syringes) may be required.

14.5.1.4 Interim Regulatory Specification – Post-market Commitment

All too often today, for biopharmaceuticals, there is very limited manufacturing batch experience with which to set scientifically sound and appropriate regulatory specifications at market approval. Manufacturers are in a hurry to complete clinical development and seek market approval, at times with ten or less manufactured batches being produced to drive the entire clinical development program. None of the statistical analysis tools provide much confidence in their calculations with such few batch data points to use. As early as the 1990's, this issue of limited data availability at the time of filing of the market application dossier in setting meaningful specifications was identified by ICH [19]:

> It is recognized that only a limited amount of data may be available at the time of filing, which can influence the process of setting acceptance criteria. As a result it may be necessary to propose revised acceptance criteria as additional experience is gained with the manufacture of a particular drug substance or drug product (example: acceptance limits for a specific impurity). The basis for the acceptance criteria at the time of filing should necessarily focus on safety and efficacy. When only limited data are available, the initially approved tests and acceptance criteria should be reviewed as more information is collected, with a view towards possible modification. This could involve loosening, as well as tightening, acceptance criteria as appropriate.

But today, with the various clinical expedited programs in place, more and more manufacturers are struggling with assigning meaningful specifications at the time of seeking market approval with fewer and fewer batches of product.

'*If you don't ask, you won't receive*.' This is an issue that should be discussed with the regulatory authority before filing the market application dossier. Lay out the concerns, and the proposed strategy to address them. Seek their guidance, and hopefully, an allowance to be dealt with during the regulatory review. During the formal regulatory review of the market application dossier, the reviewers will decide if they want to proceed with an interim regulatory specification approach.

If the interim regulatory specification approach is accepted, as part of the market approval, a post-marketing CMC commitment will be required by the regulatory authority. Table 14.4 presents some case examples of these post-marketing CMC commitments for several market-approved biopharmaceuticals [8, 20].

It should not be surprising that these post-marketing CMC commitments require an additional set of 30 manufactured batches (at 30 batches the range calculated using a typical tolerance interval – 99% incorporation with 95.5% confidence – equals about the range calculated using mean ± 3 standard deviations). Having such a large data set reduces the uncertainty associated in the statistical calculation used;

Table 14.4 Interim regulatory specification post-market approval commitments

Post-Marketing CMC Commitment Interim Regulatory Specification	
Biopharmaceutical Type	**Commitment in Market Approval Letter**
Monoclonal Antibody Margenza (margetuximab-cmkb)	Reassess release and stability specifications for margetuximab drug substance and/or drug product, as appropriate, by December 30, 2022 or following manufacture of 30 lots (if earlier) for the following assays: N-glycosylation (OS), residual host cell protein levels (OS), potency by CGI bioassay, HER2 binding ELISA, and FcγRIIIa binding ELISA (OS and DP), visible particles (DP), and protein content (DP). Submit the final report as a Changes Being Effected-30 Supplement (CBE-30). Study Completion By: December 2022 (Market Approval: December 2020)
Viral Vector (rAAV) Zolgensma (onasemnogene abeparvovec)	Control of vector genome integrity: The applicant should develop and implement a release test and define scientifically justified acceptance criteria. When results of the first 30 batches tested with the in-house method are available, the continued need for a release test for vector genome integrity may be re-evaluated.

thus, the revised specification ranges/limits become more reflective of the true manufacturing process capability. Depending upon the frequency of manufacturing, completion of this post-marketing CMC commitment can take years. The expectation is that the specification ranges/limits will tighten after the analysis.

14.5.2 Minimum … Continuum Applied to Shelf-Life Specifications

As discussed in Chap. 4, Sect. 4.3.1, the regulatory authorities accept a minimum CMC regulatory compliance continuum risk-based approach for biopharmaceutical manufacturing. For shelf-life specifications, the regulatory requirements and expectations can be divided into three stages: (1) initiation of clinical development stage (FIH), (2) later clinical development stage (initiation of pivotal study) and (3) seeking market approval stage.

14.5.2.1 Initiation of Clinical Development (FIH)

For shelf-life specifications, at the initiation of clinical development stage (e.g., First-in-Human, FIH), regulatory authority expectations are, in summary, as follows:

- A stability program is to be established for both the drug substance and the drug product to obtain shelf-life data
- CQAs across all seven groups (see Chap. 13) are to be included in the evaluation.
- The selection of tests in the stability program is driven by the characterization studies on the biopharmaceutical and the release test methods
- Recommended that at least one test method be stability-indicating
- At this early stage in clinical development, the FDA does not require an assigned shelf-life; but EMA does require an assigned shelf-life, and stability data to support it.

For shelf-life specifications, at the initiation of clinical development stage, three major regulatory guidance documents, covering the different biopharmaceutical types, have been provided:

> ***EMA for the IMPD – Recombinant Proteins and Monoclonal Antibod***ies [2]
> *S.7. Stability*
> *Stability summary and conclusions (protocol/material and method)*
> A stability protocol covering the proposed storage period of the active substance should be provided, including specification, analytical methods and test intervals. The testing interval should normally follow the guidance given in ICH Q5C. The quality of the batches of the active substance placed into the stability program should be representative of the quality of the material to be used in the planned clinical trial. The active substance entered into the stability program should be stored in a container closure system of the same type and made from the same materials as that used to store active substance batches to be used in the clinical trial. Containers of reduced size are usually

acceptable for the active substance stability testing. Studies should evaluate the active substance stability under the proposed storage conditions. Accelerated and stress condition studies are recommended as they may help understanding the degradation profile of the product and support an extension of the shelf-life. The methods used for analysing the stability-indicating properties of the active substance should be discussed, or cross-reference to S.4.3 made to provide assurance that changes in the purity/impurity profile and potency of the active substance would be detected. A potency assay should be included in the protocol, unless otherwise justified. A re-test period (as defined in ICH Q1A guideline) is not applicable to biological/biotechnology derived active substances.

Stability data/results

Stability data should be presented for at least one batch made by a process representative of that used to manufacture material for use in the clinical trial. In addition, supportive stability data on relevant development batches or batches manufactured using previous manufacturing processes should be provided, if available. Such batch data may be used in the assignment of shelf life for the active substance provided an appropriate justification of the representative quality for the clinical trial material is given. The relevant stability data should be summarised in tabular format, specifying the batches tested, date of manufacture, process version, composition, storage conditions, time-points, test methods, acceptance criteria and results. For quantitative parameters, actual numerical values should be presented. Any observed data trends should be discussed.

Shelf-life determination

The claimed shelf-life of the active substance under the proposed storage conditions should be stated and accompanied by an evaluation of the available data. Any observed trends should be discussed. The requested storage period should be based on long term, real time and real temperature stability studies, as described in ICH Q5C. However, extension of the shelf-life beyond the period covered by real-time stability data may be acceptable, if supported by relevant data, including accelerated stability studies and/or relevant stability data generated with representative material. The maximum shelf-life after the extension should not be more than double, or more than twelve months longer than the period covered by real time stability data obtained with representative batch(es). However, extension of the shelf life beyond the intended duration of the long term stability studies is not acceptable. Where extensions of the shelf-life are planned, the applicant should commit to perform the proposed stability program according to the presented protocol, and, in the event of unexpected issues, to inform Competent Authorities of the situation, and propose corrective actions. Prior knowledge including platform technologies could be taken into consideration when designing a stability protocol. However, on its own this data is not considered sufficient to justify the shelf-life of the actual active substance.

P.8. Stability

The same requirements as for the active substance are applied to the medicinal product, including the stability protocol, stability results, shelf-life determination, including extension of shelf-life beyond the period covered by real-time stability data, stability commitment and post-approval extension. Stability studies should provide sufficient assurance that the IMP will be stable during its intended storage period. The presented data should justify the proposed shelf life of the product from its release to its administration to patients. The stability protocol for the IMP should take into account the knowledge acquired on the stability profile of the active substance. Bracketing and matrixing approaches may be acceptable, where justified. In-use stability data should be presented for preparations intended for use after reconstitution, dilution, mixing or for multidose presentations. These studies are not required if the preparation is to be used immediately after opening or reconstitution.

FDA for the IND – Viral Vectors and Genetically Modified Patient Cells [16]

Stability (3.2.S.7)

a. Stability Summary and Conclusions (3.2.S.7.1)

We recommend that you describe in your original IND submission the types of stability studies (either conducted or planned) to demonstrate that the DS is within acceptable limits. The protocol should describe the storage container, formulation, storage conditions, testing frequency, and specifications (i.e., test methodologies and acceptance criteria). Please note that stability studies may evolve with product development, and if the DS is immediately processed into a DP, long term DS stability data may not be needed. Your stability analysis may include measures of product sterility (or container integrity), identity, purity, quality, and activity or potency. We recommend that you provide justification for the test methods and acceptance criteria used in the stability analysis. It is often helpful to demonstrate that at least one or more of the test methods in your stability analysis are stability-indicating. You may demonstrate a test is stability-indicating using forced degradation studies, accelerated stability studies, or another type of experimental system that demonstrates product deterioration. Information to help you design your stability studies may be found in the following guidance documents (references ICH Q5C, Q1A(R2) and Q1E).

c. Stability Data (3.2.S.7.3)

We recommend that you provide the results of your stability studies in your IND and update this information on a regular basis (e.g., annual reports). Information on the qualification of analytical procedures used to generate stability data should be included in your original IND submission.

Stability (3.2.P.8)

a. Stability Summary and Conclusion (3.2.P.8.1)

You should summarize the types of studies conducted, protocols used, and the results of the studies. Your summary should include, for example, conclusions regarding storage conditions and shelf life as well as in-use and in-device storage conditions. If a short-term clinical investigation is proposed, or if a DP manufacturing process has limited product hold times, stability data submitted may be correspondingly limited. Early in development, stability data for the gene therapy may not be available to support the entire duration of the proposed clinical investigation. Therefore, we recommend that you submit a prospective plan to collect stability information and update this information to the IND in a timely manner (e.g., in an annual IND update).

c. Stability Data (3.2.P.8.3)

You should provide results of the stability studies in your IND in an appropriate format (e.g., tabular, graphic, narrative). Information on the analytical procedures used to generate the data should also be included, and this may be referenced to other sections of your submission (e.g., "Analytical Procedures (3.2.P.5.2)" section of the CTD).

EMA for the IMPD – Viral Vectors and Genetically Modified Patient Cells [3]

S.7. Stability

Stability summary and conclusions (protocol/material and method)

A stability protocol covering the proposed storage period and storage conditions of the active substance should be provided, including specification, analytical methods and test intervals. Unless justified, the testing interval should follow ICH Q5C. The re-test period (as defined in ICH Q1A guideline) is, however, not applicable to ATMPs. The quality of the batches of the active substance placed into the stability program should be representative of the quality of the material to be used in the planned clinical trial. The stability samples of active substance entered into the stability program should be stored in containers that use the same materials and container closure system as the active substance used to manufacture the clinical trial batch. Containers of reduced size are usually acceptable for the active substance stability testing. Studies should evaluate the active substance stability under the proposed storage conditions. Accelerated and stress condition studies may help understanding the degradation profile of the product and support extension of shelf-life and comparability studies. Stability-indicating methods

should be included in this stability protocol to provide assurance that changes in the purity/impurity profile and potency of the active substance would be detected. A potency assay should be included in the stability protocol, unless otherwise justified.

CBIMP:

For CBIMPs, particularly in the autologous setting, stability studies can pose a challenge, due to ethical considerations of using patient material. In these cases, it is acceptable to base early stability evaluations on results with cells from healthy donors. The representativeness of this approach for patient material, however, needs to be justified and investigated as development proceeds.

GTIMP:

For GTIMP, vector integrity, biological activity (including transduction capacity) and strength are critical product attributes which should always be included in stability studies. It is appreciated, however, that during early development the potency assay may not be fully developed. Where feasible forced degradation studies may also provide important information on degradation products and identify stability indicating parameters to be tested. In the case of products formulated with carrier or support materials, the stability of the complex formed with the drug substance should be studied.

Stability data/results

Stability data should be presented for at least one batch representative of the manufacturing process of the clinical trial material. In addition, stability data of relevant development batches or batches manufactured using previous manufacturing processes could be provided. Such batch data may be used in the assignment of shelf life for the active substance provided appropriate justification of representative quality for the clinical trial material is given.The relevant stability data available should be summarised in tabular format, specifying the batches tested, date of manufacture, process version, composition, storage conditions, time-points, test methods, acceptance criteria and results.

For quantitative parameters, actual numerical values should be presented. Any observed data trends should be discussed. The increase of available data and improved knowledge about the stability of the active substance will need to be demonstrated during the different phases of clinical development. For confirmatory clinical trials the applicant should have a comprehensive understanding of the stability profile of the active substance.

Shelf-life determination

The claimed shelf-life of the active substance under the proposed storage conditions should be provided and accompanied by an evaluation of the available data. Any observed trends should be discussed. The foreseen storage period should be based on long term, real time and real temperature stability studies, as described in ICH Q5C. Extension of the shelf-life beyond the period covered by real-time stability data may be acceptable, if supported by relevant data, including accelerated stability studies and/or relevant stability data generated with representative material.The maximum shelf-life after the extension should not be more than double, or twelve months longer, whichever is the longest, than the period covered by real time stability data obtained with representative batch(es). Where extensions of the shelf-life are planned, the applicant should commit to perform the proposed stability program according to the presented protocol, and, in the event of unexpected issues, to inform Competent Authorities of the situation, and propose corrective actions.

P.8. Stability

The same requirements as for the active substance are applied to the medicinal product, including the stability protocol, stability results, shelf-life determination, including extension of shelf-life beyond the period covered by real-time stability data and stability commitment. The storage conditions including temperature range should be defined and stability studies should provide sufficient assurance that the IMP will be stable during the intended storage period. The stability protocol for the ATIMP should take into

account the knowledge acquired on the stability profile of the active substance. Transportation and storage conditions should be supported by experimental data with regard to the maintenance of cell integrity and product stability during the defined period of validity. Where applicable, product-specific methods for freezing and thawing should be documented and justified. For preparations intended for use after reconstitution, dilution or mixing, a maximum shelf life needs to be defined and supported by in-use stability data. The stability of the non-cellular components should be assessed in the presence and absence of cellular components in order to determine whether the non-cellular component undergoes degradation, or physico-chemical alterations (e.g. aggregation, oxidation) that may impact on the quality of the product by affecting cellular behaviour and survival. The effect of the cellular component or of the surrounding tissues on the degradation (rate and, if appropriate, products) or stability of the structural component should be assessed, considering also the effect of the non-cellular components throughout the expected lifetime of the product. Bracketing and matrixing approaches may be acceptable, where justified.

14.5.2.2 Later Clinical Development Stage (Pivotal Clinical Studies)

For shelf-life specifications, at the later clinical development stages (e.g., pivotal clinical studies), regulatory authority expectations are increased compared to the earlier clinical development stage, in summary, as follows:

- Shelf-life test methods will be modified – some added, some deleted – based on changes to the release program
- Desire to have more stability-indicated test methods identified
- Expectation that the manufacturer has a comprehensive understanding of the stability of the biopharmaceutical
- Especially for the viral vectors and genetically modified patient cells, the FDA recommends a discussion to discuss how the shelf-life date will be determined

For shelf-life specifications, at this later clinical development stage, the following regulatory guidance is provided:

EMA for the IMPD – Recombinant Proteins and Monoclonal Antibodies [2]

Progressive requirements will need to be applied to reflect the amount of available data and emerging knowledge about the stability of the active substance during the different phases of clinical development. By phase III the applicant should have a comprehensive understanding of the stability profile of the active substance.

FDA for the IND – Viral Vectors and Genetically Modified Patient Cells [16]

b. Post-Approval Stability Protocol and Stability Commitment (3.2.S.7.2)

We do not recommend that you provide a post-approval stability protocol and stability commitment in the IND. However, as you progress with product development, you may want to consider which stability studies would be required to determine an expiry date. We recommend the discussion of these items at your late phase IND meetings.

b. Post-Approval Stability Protocol and Stability Commitment (3.2.P.8.2)

We do not recommend that you provide a post-approval stability protocol and stability commitment in your IND submission. However, as product development continues, we recommend that you consult with your Quality Reviewer to determine the type of studies that will be necessary to support product expiration dates for commercial manufacturing.

EMA for the IMPD – Viral Vectors and Genetically Modified Patient Cells [3]

The increase of available data and improved knowledge about the stability of the active substance will need to be demonstrated during the different phases of clinical development. For confirmatory clinical trials the applicant should have a comprehensive understanding of the stability profile of the active substance.

14.5.2.3 Seeking Market Approval Stage

At the time of submission of the market approval dossier for the biopharmaceutical, it is expected by the regulatory authorities that (1) an expiration date is proposed for both the drug substance and the drug product that can be justified by the acquired stability data, and (2) the assigned ranges or limits to the shelf-life specifications can be justified. In the submitted BLA/MAA, all aspects of the stability program will be evaluated by the regulatory authority – the number and representativeness of the batches on study, the stability-indicating test methods used, the time periods and conditions of study, the analysis of the stability profile (typically a regression line fit of the data) and the establishment of shelf-life specifications, and whether they are the same or different from the release specifications.

For shelf-life specifications, at BLA/MAA submission stage, the following regulatory guidance is provided:

ICH Q5C – Recombinant Proteins and Monoclonal Antibodies [10]
SELECTION OF BATCHES
Drug Substance (Bulk Material)
Where bulk material is to be stored after manufacture but prior to formulation and final manufacturing, stability data should be provided on at least 3 batches for which manufacture and storage are representative of the manufacturing scale of production. A minimum of 6 months stability data at the time of submission should be submitted in cases where storage periods greater than 6 months are requested. For drug substances with storage periods of less than 6 months, the minimum amount of stability data in the initial submission should be determined on a case-by-case basis. Data from pilot-plant scale batches of drug substance produced at a reduced scale of fermentation and purification may be provided at the time the dossier is submitted to the regulatory agencies with a commitment to place the first 3 manufacturing scale batches into the long-term stability program after approval. The quality of the batches of drug substance placed into the stability program should be representative of the quality of the material used in preclinical and clinical studies and of the quality of the material to be made at manufacturing scale. In addition, the drug substance (bulk material) made at pilot-plant scale should be produced by a process and stored under conditions representative of that used for the manufacturing scale. The drug substance entered into the stability program should be stored in containers which properly represent the actual holding containers used during manufacture. Containers of reduced size may be acceptable for drug substance stability testing provided that they are constructed of the same material and use the same type of container/closure system that is intended to be used during manufacture.
Drug Product (Final Container Product)
Stability information should be provided on at least 3 batches of final container product representative of that which will be used at manufacturing scale. Where possible, batches of final container product included in stability testing should be derived from different batches of bulk material. A minimum of 6 months data at the time of submission should be submitted in cases where storage periods greater than 6 months are requested. For drug products with storage periods of less than 6 months, the minimum

amount of stability data in the initial submission should be determined on a case-by-case basis. Product expiration dating will be based upon the actual data submitted in support of the application. Since dating is based upon the real-time/real-temperature data submitted for review, continuing updates of initial stability data should occur during the review and evaluation process. The quality of the final container product placed on stability studies should be representative of the quality of the material used in the preclinical and clinical studies. Data from pilot-plant scale batches of drug product may be provided at the time the dossier is submitted to the regulatory agencies with a commitment to place the first 3 manufacturing scale batches into the long term stability program after approval. Where pilot-plant scale batches were submitted to establish the dating for a product and, in the event that product produced at manufacturing scale does not meet those long-term stability specifications throughout the dating period or is not representative of the material used in preclinical and clinical studies, the applicant should notify the appropriate regulatory authorities to determine a suitable course of action.

STABILITY-INDICATING PROFILE

On the whole, there is no single stability-indicating assay or parameter that profiles the stability characteristics of a biotechnological/biological product. Consequently, the manufacturer should propose a stability-indicating profile that provides assurance that changes in the identity, purity and potency of the product will be detected. At the time of submission, applicants should have validated the methods that comprise the stability-indicating profile and the data should be available for review. The determination of which tests should be included will be product-specific. The items emphasised in the following subsections are not intended to be all-inclusive, but represent product characteristics that should typically be documented to adequately demonstrate product stability.

SPECIFICATIONS

Although biotechnological/biological products may be subject to significant losses of activity, physicochemical changes, or degradation during storage, international and national regulations have provided little guidance with respect to distinct release and end of shelf-life specifications. Recommendations for maximum acceptable losses of activity, limits for physicochemical changes, or degradation during the proposed shelf-life have not been developed for individual types or groups of biotechnological/biological products but are considered on a case-by-case basis. Each product should retain its specifications within established limits for safety, purity, and potency throughout its proposed shelf-life. These specifications and limits should be derived from all available information using the appropriate statistical methods. The use of different specifications for release and expiration should be supported by sufficient data to demonstrate that clinical performance is not affected as discussed in the tripartite guideline on stability.

FDA BLA – Recombinant Proteins and Monoclonal Antibodies [18]

Drug Substance Stability

A description of the storage conditions, study protocols and results supporting the stability of the drug substance should be submitted in this section. Data from tests to monitor the biological activity

and degradation products such as aggregated, deamidated, oxidized, and cleaved forms should be included, as appropriate. Data supporting any proposed storage of intermediate(s) should also be provided.

Drug Product Stability

A description of the storage conditions, study protocols and results supporting the stability of the drug product should be provided. This should include information on the stability of intermediate fluids or formulated bulk under specified holding or shipping conditions, as appropriate. For products administered through pumps or other such delivery devices, data on the stability of the drug product in the delivery system should be pro-

vided. Stability data supporting the proposed shelf-life of the reconstituted drug product and for all labeled dilutions should be included. The results of all tests used to monitor biological activity and the presence of degradation products such as aggregated, deamidated, oxidized, cleaved, etc. forms of the drug substance should also be included.

EMA MAA – Viral Vectors [11]

Stability for drug substance and drug product

Stability protocols, stability data, justifications for the container-closure system used, and proposed shelf-lives and storage conditions, should be presented for the drug substance, drug product and any intermediate product stored during production (i.e. intermediates for which a holding time is scheduled on the production process scheme). The principles outlined in ICH stability guidelines (and particularly *ICH guideline Q5C*) should be followed. Real time stability studies should be undertaken, in particular for the DS and DP intended for marketing. However, it is acknowledged that accelerated stability studies (e.g. at elevated temperatures or under other stress conditions relevant for the product of interest) may provide complementary supporting evidence for the stability of the product and help to establish the stability profile. Forced degradation studies provide important information on degradation products and identify stability indicating tests. In general, the shelf-life specifications should be derived from the release specifications, with additional emphasis on the stability-indicating features of tests used and tests/limits for degradation products. Vector integrity, biological potency (including transduction capacities) and strength are critical product attributes which should always be included in stability studies. In the case of products formulated with carrier or support materials, the stability of the complex formed with the drug substance should be studied. Where relevant, the in-use stability of the drug product (after reconstitution or after thawing) should be properly investigated including its compatibility with any diluents used in reconstitution and if appropriate, devices used for administration. The recommended in-use time period should be justified. The impact of the transport conditions on the stability of DS or DP with a short term shelf life should be considered.

14.6 Release Specification Limits – Required Versus Recommended

While it is fairly straightforward to list the critical quality attribute (CQA) categories, as well as the list of tests in each category, for a biopharmaceutical (see Chap. 13), it is challenging to fill in the release specification limits that might go with those tests. There are two groups of specification limits – those that are regulatory required and those that are recommended.

14.6.1 Required Regulatory Release Specification Limits

Within the seven categories of CQAs (see Chap. 13), the regulatory required release specification limits are in two categories – Safety (sterility, adventitious mycoplasma, replication competent virus (RCV)), and Purity (endotoxin, residual host cellular DNA).

Sterility

Limit applies to all biopharmaceutical types: Pharmacopeia requirement is that there be 'no growth' detected in the compendial test method, at a detection level of NMT 100 CFU.

Mycoplasma

Limit applies to all biopharmaceutical types: Pharmacopeia requirement is that there be 'no growth' detected in the compendial test method at a detection level of NMT 100 CFU. For recombinant proteins, monoclonal antibodies and viral vectors, the testing for adventitious mycoplasma contamination is at the unprocessed bulk stage (i.e., during the cell culture stage of the production process, immediately prior to harvest); the most sensitive location for detection). For genetically modified patient cells, the testing for adventitious mycoplasma is at the cellular final drug product stage.

Replication Competent Virus (RCV)

Limit applies to viral vectors and genetically modified patient cells [21].

> Current manufacturing experience indicates that <1 RCR/dose equivalent is an achievable level for retroviral vector preparations intended for clinical use. Therefore, we now recommend that sufficient supernatant be tested to ensure a 95% probability of detection of RCR if present at a concentration of 1 RCR/dose equivalent.

Notice that the residual RCV limit is expressed as 'dose equivalent', not the typical unit of measurement that Quality Control would use in the release test method. Therefore, it is important to know the maximum potential dose of the biopharmaceutical that will be administered to a patient in the clinic, so that either the correct volume, viral particles or cells can be tested. It also is important that if changes are made in the clinical dosing amount, that Quality is notified in order to doublecheck the safety level.

Endotoxin

Limit applies to all biopharmaceutical types [16]:

> Although the rabbit pyrogen test method is the current required method for testing certain licensed biological products for pyrogenic substances (21 CFR 610.13(b)), we generally accept alternative test methods, such as the Limulus Amebocyte Lysate (LAL).). For any parenteral drug, except those administered intrathecally or intraocularly, we recommend that the upper limit of acceptance criterion for endotoxin be 5 Endotoxin Unit (EU)/kg body weight/hour. For intrathecally-administered drugs, we recommend an upper limit of acceptance be set at 0.2 EU/kg body weight/hour. For intraocularly-administered drugs, we recommend acceptance criterion of not more than (NMT) 2.0 EU/dose/eye for injected or implanted DPs or NMT 0.5 EU/mL for ophthalmic irrigation products.

Notice that the residual endotoxin limit is expressed as 'kg body weight/hour', not the typical 'mg' that Manufacturing and Quality staff use for the release specification. Therefore, it is important to know how the biopharmaceutical is administered in the clinic: (1) who the patients are (kg weight differences between adults and

children), and (2) the dose amount that is delivered within an hour time period), so that the correct conversion to biopharmaceutical weight (i.e., EU/mg) can be made. It also is important that if changes are made in the clinical dosing (either amount/hour or patient population), that Quality is notified in order to doublecheck the safety level.

Residual Host Cellular DNA

Limit applies to recombinant proteins, monoclonal antibodies, and viral vectors [22]:

> Since some cell substrates also harbor tumorigenic genetic sequences or retroviral sequences that may be capable of transmitting infection, we recommend that you take steps to minimize the biological activity of any residual DNA associated with your viral preparation. This can be accomplished by reducing the size of the DNA to below the size of a functional gene and by decreasing the amount of residual DNA. We recommend that you limit the amount of residual DNA for continuous non-tumorigenic cells to less than 10 ng/dose and the DNA size to below approximately 200 base pairs.

Notice that the residual host cellular DNA limit is expressed as 'dose', not the typical 'mg' that Manufacturing and Quality staff use for the release specification. Therefore, it is important to know the maximum potential dose of the biopharmaceutical that will be administer to a patient in the clinic, so that the correct conversion to biopharmaceutical weight (i.e., ng/mg) can be made. It also is important that if changes are made in the clinical dosing amount, that Quality is notified in order to doublecheck the safety level.

14.6.2 Recommended Release Specification Limits

Recommended release specification limits can come from the regulatory authorities or from best practices in the biopharmaceutical industry. Within the seven categories of CQAs (see Chap. 13), the recommendations on release specification limits are in one category – Purity: cell viability, protein aggregation, residual host cell proteins, protein 'purity'.

Cell Viability

The FDA recommended limit for % cell viability applies to genetically modified patient cells: [22].

> You should establish minimum release criteria for viability, where appropriate. For ex vivo genetically modified cells, we recommend a minimum acceptable viability of at least 70 percent. If this level cannot be achieved, we recommend that you submit data in support of a lower viability specification, demonstrating, for example, that dead cells and cell debris do not affect the safe administration of the product and/or the therapeutic effect.

Dead genetically modified patient cells are a product-related impurity. The transgene is present in the cells, and in the case of CAR T cells the binding protein

receptors are assembled on the cells, but the cells are dead, non-functioning, not able to complete the purpose for the genetic modification.

Protein Aggregation (% Monomer)

Proteins like recombinant proteins and monoclonal antibodies can aggregate, and when administered to patients can initiate an immunogenic response. Size exclusion HPLC (SEC-HPLC) is the standard test method for separating and measuring protein monomer and protein aggregates (higher molecular weight proteins). During clinical development, ≥95% monomer level (as measured by SEC-HPLC) has been a biopharmaceutical industry target for controlling the protein aggregation level [23]. For market approval, the % monomer level across the manufactured batches is calculated to set the release specification limit; and in general, results in a higher % monomer level (i.e., lower aggregation level). However, if there are any clinical concerns of significant immunogenicity arising in patients, or if the aggregation significantly increases over the shelf-life, then a lower % aggregation level is warranted.

Residual Host Cell Proteins (HCPs)

Residual host cell proteins, which can be immunogenic, are present in those biopharmaceutical types that involve production in cell culture – recombinant proteins, monoclonal antibodies, and viral vectors. There is no regulatory upper limit requirement. During clinical development, NMT 100 ppm (100 ng/mg) has been a biopharmaceutical industry target for controlling HCPs [23]. For market approval, the NMT level across the manufactured batches is calculated to set the release specification limit; and in general, results in an or lower HCP level. However, if there are any clinical concerns of significant immunogenicity arising in patients, then a lower NMT HCP level is warranted.

Protein 'Purity'

Protein purity of recombinant proteins, monoclonal antibodies, and viral vectors (i.e., capsid protein) is typically determined using an electrophoretic (e.g., SDS-PAGE or CGE) or chromatographic (HPLC) test method. There is no regulatory % protein purity lower limit requirement. During clinical development, ≥ 95% purity has been a biopharmaceutical industry target. This perception arises due to regulatory guidances issued in the 1980's, such as the 1983 guidance for recombinant interferons which states, *"High Performance Liquid Chromatography. This method can be used to assess protein purity. Greater than or equal to 95% of the protein should elute as a single peak using reverse phase chromatography in each of two different solvent systems"* [24]. However, with today's sophisticated analytical test methods (e.g., capillary electrophoresis, improved resolution HPLC resins), single protein peaks can readily become multiple protein peaks. Therefore, today, multiple product-related protein peaks are summed together to be reported as % protein purity. For market approval, the % protein purity value across the manufactured batches is calculated to set the release specification limit; and in general, results in

a higher % level. And, based upon the characterization of the protein peaks present, the peaks that have been summed may change.

14.7 Need to Get This Right the First Time

A hard lesson to learn is that incorrectly setting a release or shelf-like specification, or not having adequate stability data to confirm a shelf-life assignment, can cause frustration and market approval delays when the market application dossier is under review. The regulatory authority will closely examine all of your support provided in the CMC submission, and if they are not convinced of your proposals, then a major setback can occur. Such was the case for FKD Therapies, Oy.

After 6 months of review and discussion with the FDA concerning their BLA submission for the rAAV viral vector, Adstiladrin (nadofaragene firadenovec), the manufacturer received a Complete Response Letter (CRL) in April 2020 [25], which stopped the BLA review. One of the main issues that concerned the FDA dealt with how the manufacturing facility was not being operated under appropriate cGMP control, especially concerning aseptic processing control for the biopharmaceutical. But there were other major concerns from the FDA. A request to recalculate a proposed release specification ('*the sensitivity of your test method does not support this acceptance limit*'). And a request to redo the drug product stability program:

> Redo the stability program. The stability information for the DP is incomplete. You proposed a shelf life of 12 months for the DP; however, the stability data included in the BLA for the proposed commercial product (RTU formulation) is only for 9 months. Please provide updated stability information. Please note that the stability information should also include stability data to address the following outstanding issues:
>
> (a) Supporting data to show that the DP is stable when stored for 12 months. Please note that the stability evaluation should take into consideration the storage period for the DS to show that quality attributes of the DP are not compromised when the DS is stored initially for the proposed period of month prior to being formulated into the DP and stored for an additional 12 months.
> (b) You provided updated stability information to show that the novel excipient Syn3NODA is stable in the final formulation for a period of 12 months when stored at -60°C. However you have not provided data to show that the Syn3NODA is also stable when the drug product is stored at -20°C, the proposed storage temperature at the clinical site.
> (c) You have not yet validated the analytical method used to detect the presence of the functional excipient, Syn3NODA, in the DP. Analytical method validation is required to support the stability of Syn3NODA in the DP. Please provide the assay validation for the detection of the functional excipient, Syn3NODA, in the formulated DP.

Then, there was a request to provide adequate *in use* instructions for the correct handling of the viral vector with the urinary catheter device in the clinic:

> In your draft labeling in Module 1.14.1.3, submitted in Amendment 25 dated January 10, 2020, you state in Sections 2.2 Preparation and Handling and 2.3 Administration, that the drug product should be withdrawn from four (4) vials into a syringe(s) and instilled into the bladder using a urinary catheter. However, you did not include critical parameters for these delivery devices. Please propose critical device parameters (e.g., volume,

material(s) of construction, French gauge, length, coatings, colorants, connector style, tip style, etc.) to include in the labeling in order to guide the clinician in selecting a syringe and urinary catheter that are compatible with your DP. While it is possible these parameters may include a range of selections/values (e.g., different materials of construction, different lengths, etc.), all proposed parameters and selections/values should be supported by compatibility testing and suitable for clinical delivery of the product. If there are any catheter types that should not be used with your product (e.g., in-dwelling catheters, catheters with antimicrobial coatings, etc.), please also include this information in the labeling. To support your proposed parameters and selections/values, please provide:

(i) a discussion of how each proposed parameter and selection/value is supported by your compatibility data.
(ii) information regarding the catheters that were selected for use during your clinical studies, along with a summary of your clinical experience using these urethral catheters to deliver the DP (including any delivery-related adverse events) and how the catheters used in the clinical study compare to the catheters used in the compatibility testing and the proposed critical device parameters.

A tremendous amount of work had to be done or redone: completing release assay validations, reassessing release specifications, redoing a drug product stability program, and confirming correct in use directions for the clinic setting. It took the manufacturer 2 months to do all the work and resubmit the revised BLA, the FDA took another 6 months to complete its review; an 8-month delay in receiving market approval.

In conclusion, it cannot be emphasized strongly enough, that the justification of the release and shelf-life specifications, and the *in use* handling instructions for the clinic, will be held to a sound and appropriate scientific standard by the regulatory authorities when seek market approval.

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Chapter 15
The Challenge of Demonstrating Biopharmaceutical Product Comparability

Abstract Manufacturers need the freedom to make changes for improving their biopharmaceutical processes, not only during clinical development, but also for continuous process improvement once in the marketplace. Along with this freedom to make manufacturing process changes, comes the responsibility of carefully assessing the potential safety and efficacy impact on the biopharmaceutical due to those changes. Demonstrating comparability for a biopharmaceutical after a manufacturing process change is no easy task, whether it be for a recombinant protein, monoclonal antibody, viral vector, or genetically modified patient cells. In this chapter, the three risk-based concerns that need to be addressed by an effective comparability study will be examined: (1) the stage of clinical development when the change is planned, (2) the nature (type, extent, process location) of the change that is planned, and (3) the residual uncertainty remaining after the analytical/functional testing has been evaluated. Also, a firmer understanding of how to obtain a post-approval change management plan (PACMP) with a regulatory authority, will be discussed.

Keywords Highly similar · Comparability · Value-added · Contract · Analytical · Functional · Limitations · PACMP · Exercise

Change is inevitable, but demonstrating biopharmaceutical comparability after a manufacturing process change is no easy task. Biopharmaceuticals are biomolecules ranging in size and complexity from the large/complex recombinant proteins and monoclonal antibodies, to the much larger/more complex viral vectors, and finally to the very large/very complex genetically modified patient cells. Manufacturers need the freedom to make changes for improving their biopharmaceutical processes, not only during clinical development, but also for continuous process improvement once in the marketplace. Along with this freedom to make manufacturing process changes, comes the responsibility of carefully assessing the potential safety and efficacy impact on the biopharmaceutical due to those changes. But, demonstrating comparability for a biopharmaceutical after a manufacturing

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process change is no easy task. In this chapter, the three risk-based concerns that need to be addressed by an effective comparability study will be examined: (1) the stage of clinical development when the change is planned, (2) the nature (type, extent, process location) of the change that is planned, and (3) the residual uncertainty remaining after the analytical/functional testing has been evaluated. Also, in this chapter, a firmer understanding of how to obtain a post-approval change management plan (PACMP) with a regulatory authority, will be discussed.

15.1 Manufacturing Process Change Is Inevitable

Manufacturers need the freedom to make changes for improving their biopharmaceutical processes, not only during clinical development but also for continuous process improvement once in the marketplace. But along with this freedom to make manufacturing process changes comes the responsibility of carefully assessing the potential safety and efficacy impact on the biopharmaceutical due to the changes being implemented. All too often, the words of Robert Burns, Scottish poet, come true *"the best laid schemes of mice and men go often askew."* For biopharmaceuticals, great care needs to be exercised to effectively minimize the impact of a manufacturing process change on the recombinant protein, monoclonal antibody, viral vector, or genetically modified patient cells.

15.1.1 Process Change – Anytime and Anywhere

Ideally, manufacturing process changes should occur as early as possible during clinical development. Ideally, low-risk manufacturing process changes are preferred. Realistically, however, manufacturing process changes, both low-risk and high-risk, will occur throughout the clinical development and the post-market approval life cycle of the biopharmaceutical.

The following are several case examples of successfully implemented manufacturing process changes that occurred during clinical development of biopharmaceuticals that are now market-approved:

Monoclonal Antibody, Ilumetri (Tildrakizumab) [1]

The commercial active substance manufacturing process was developed in parallel with the clinical development program. Process I active substance was used for non-clinical studies. Process I was up-scaled and transferred to another site. This produced material was used for Phase 1 and 2 clinical trials. Process I underwent further refinement resulting in process II. The main changes were usage of a WCB instead of a MCB, column changes and a change in the active substance formulation among other smaller changes. The material of process II was used for phase 3 clinical trials. Finally, process II was transferred to the commercial site and up-scaled. The main change beside facility and scale fits was the introduction of a new WCB, which was cultured and is stored without using FBS.

Monoclonal Antibody, Idacio (Adalimumab-aacf) Biosimilar [2]

During development, per advice from the Agency, the Applicant made changes to the manufacturing process to better align the glycan profile of the proposed commercial material with that of US-Humira.

One of the major changes was change from a citrate formulation, the same formulation as US-Humira, to an acetate formulation to address patent issues.

Viral Vector, Zolgensma (Onasemnogene Abeparvovec, rAAV) [3]

Three finished product manufacturing processes are described: Process A (manufactured at a research institute), Process B-initial, and Process B-commercial (both manufactured at Avexis). Only one clinical batch manufactured according to Process A is listed, which was used in the Phase I clinical study that supports the present application; the batches manufactured according to Process B-initial were used in development and in the ongoing pivotal clinical trial. Batches manufactured according to Process B-commercial were used for process validation and used in clinical studies. The finished product presentation is different for each process with regard to container closure system, fill volume, and vector concentration. These changes were implemented to support long-term storage and achieve the desired dose and dose volumes. With regard to the finished product manufacturing process, the main difference is the presence of an active substance thaw step in Process A and Process B-commercial, which is not present in Process B-initial. Manual filling in Process A was replaced by an automated filler in Process B-initial and -commercial.

Genetically Modified Patient Cells, Libmeldi (Atidarsagene Autotemcel) [4]

Several different active substance/finished product manufacturing processes are identified. Differences include the starting material (BM or mPB), the CD34+ enrichment procedure, the presence or absence of an additional cryopreservation step for the CD34+ enriched cells, the container closure system, and the final formulation (fresh or cryopreserved). The active substance manufacturing process downstream of the CD34+ enrichment was the same for all clinical batches. It is noted that apart from these changes there were also the changes in the manufacturing of the LVV vector. Upon request, the applicant has performed a comparability assessment for clinical batches manufactured at different sites. Some minor differences were observed that could be attributed to the enrichment step or the starting material (BM vs mPB), none of which is expected to impact efficacy or safety. Overall, the data from the clinical batches sufficiently support comparability between manufacturing sites.

Process changes continue into market approval. A survey published in 2016 of twenty-nine (29) EMA market-approved monoclonal antibodies revealed that significant post-marketing manufacturing process changes averaged about 1–2 per year per product [5].

15.1.2 Process Change – Should Be Value-Added

Manufacturing process changes should not be viewed as threatening. During clinical drug development, there is the important need to mature the development of the manufacturing process to ensure that it will be adequately robust to yield a consistent quality product when it reaches commercialization. And, after commercialization, there is the continuing need to ensure that the manufacturing process remains under validated control with appropriate process improvements, manufacturing site changes, and scale-ups (or scale-downs), as needed.

Every manufacturing process change carries the risk of potentially impacting the biopharmaceutical. Therefore, every manufacturing process change should provide added value to offset this potential risk. The following are some examples of value-added manufacturing process changes:

1. ***Improving manufacturing process robustness and control***
   - Tightening of cell culture or purification in-process controls
   - Replacing a chromatography resin type to improve process-related impurity removal
   - Manufacturing site change to enhance cGMP compliance
2. ***Improving biopharmaceutical purity, quality, or safety***
   - Addition of a new chromatography polishing step
   - Tightening of biopharmaceutical release and/or shelf-life specifications
   - Changing the acceptance criteria of a critical raw material to a higher quality standard
3. ***Increasing manufacturing capacity***
   - Exchanging a recombinant cell line to one with higher biopharmaceutical productivity
   - Scale-up (or scale-out) to increase production capacity
   - Addition of additional biopharmaceutical manufacturing sites
4. ***Business reasons***
   - Reduction in cost of goods (COGs)
   - Acquisitions/mergers requiring manufacturing site changes
   - Responding to a regulatory authority or compendial required change

Thus, from a risk-benefit perspective, the benefit of a manufacturing process change should always exceed the risk of negatively impacting the biopharmaceutical.

15.1.3 Process Change – ICH Q5E Adapted for all Biopharmaceutical Types

The core regulatory guidance for assessing potential biopharmaceutical impact after a manufacturing process change is ICH Q5E *Comparability of Biotechnological/Biological Products Subject to Changes in Their Manufacturing Process* [6]. This regulatory consensus guidance document, which was issued in 2004, addresses comparability testing for manufacturing process changes specifically for recombinant proteins and monoclonal antibodies:

Scope

The principles adopted and explained in this document apply to:

- Proteins and polypeptides, their derivatives, and products of which they are components, e.g., conjugates. These proteins and polypeptides are produced from recombinant or non-recombinant cell-culture expression systems and can be highly purified and characterised using an appropriate set of analytical procedures;
- Products where manufacturing process changes are made by a single manufacturer, including those made by a contract manufacturer, who can directly compare results from the analysis of pre-change and post-change product; and
- Products where manufacturing process changes are made in development or for which a marketing authorisation has been granted.

But ICH Q5E has now been adapted and applied to all the other types of biopharmaceuticals:

Viral Vectors [7]

Where, during development, changes to the design of the vector are made to obtain new improved product characteristics, principles outlined in the *Reflection paper on changes during development of gene therapy medicinal products* and *ICH guideline Q5E* should be taken into consideration.

Genetically Modified Patient Cells [8]

Analytical comparability of CAR T cells pre- and post-change may be assessed following the general principles described in ICH Q5E. Note that the "comparability" does not necessarily mean that pre- and post-change products are identical, but that they are highly similar and that any differences in product CQAs have no adverse impact on CAR T cell quality, safety, or efficacy.

15.1.4 The Comparability Study Must Address Three Risk-Based Concerns

Prior to proceeding with the planning for a comparability study, is the initiation of the process change review within the manufacturer's Pharmaceutical Quality System, and the activation of the cGMP change control system [9]:

CHANGE CONTROL

11.1. The control of change is an important part of knowledge management and should be handled within the pharmaceutical quality system.

11.2. Written procedures should be in place to describe the actions to be taken if a planned change is proposed to a starting material, product component, process, equipment, premises, product range, method of production or testing, batch size, design space or any other change during the lifecycle that may affect product quality or reproducibility.

11.3. Where design space is used, the impact on changes to the design space should be considered against the registered design space within the marketing authorization and the need for any regulatory actions assessed.

11.4. Quality risk management should be used to evaluate planned changes to determine the potential impact on product quality, pharmaceutical quality systems, documentation, validation, regulatory status, calibration, maintenance and on any other system to avoid unintended consequences and to plan for any necessary process validation, verification or requalification efforts.

11.5. Changes should be authorised and approved by the responsible persons or relevant functional personnel in accordance with the pharmaceutical quality system.

11.6. Supporting data, e.g. copies of documents, should be reviewed to confirm that the impact of the change has been demonstrated prior to final approval.
11.7. Following implementation, and, where appropriate, an evaluation of the effectiveness of change should be carried out to confirm that the change has been successful.

Part 11.4 in the above guidance is where the evaluation of the planned process change takes place, and the determination of what all is needed for the comparability study to support that the biopharmaceutical remains highly similar before and after the proposed process change. The manufacturer's comparability study program is then activated to prepare the necessary study protocol and required department review and approval signatures, usually a four-step process (very similar to any other type of validation):

***Step 1*.** Prepare the proposed comparability study protocol (also referred to as the comparability exercise). Describe the details of the proposed process change – the 'who, what, when, where, how and why'. The clarity of what is required to be carried out is essential to guide the work that will be necessary. Identify how the comparability study experimental results will be documented, and the required approval signatures needed.
***Step 2*.** Obtain the required signoffs before proceeding with the planned comparability study (depending upon the type of change this could be Development, Manufacturing, Quality Control; always will be Quality Assurance, and in some companies also Regulatory Affairs).
Step 3. Execute the comparability study workplan and complete all of the required comparison testing and documentation. Prepare the comparability study report.
Step 4. Obtain the required signoffs on the comparability study report (signoffs by the same departments as on the comparability protocol in Step 2). Regulatory Affairs to communicate the change, under the required pathway, to regulatory authorities. Implementation of the manufacturing process change may have to wait until regulatory authority approval,

The expected standard of measurement needed to establish 'comparable' differs between chemical drugs and biopharmaceuticals. For a chemical drug, a comparable product is one that is considered equivalent to the product before the manufacturing process change, that is, it remains 'unchanged' [10]:

> For chemical drug substances, you should include appropriate structural characterization, analytical procedures to be used, and acceptance criteria to be achieved to ensure that the chemical structure remains unchanged in a CP for any manufacturing process change that could affect the chemical structure (e.g., stereochemical configuration) of the drug substance (e.g., change in route of synthesis or manufacturing process).

But, for a biopharmaceutical, which is a large and complex biomolecule, a comparable product is one that is considered 'highly similar' to the product before the manufacturing process change [6]:

> The demonstration of comparability does not necessarily mean that the quality attributes of the pre-change and post-change product are identical, but that they are highly similar and that the existing knowledge is sufficiently predictive to ensure that any differences in quality attributes have no adverse impact upon safety or efficacy of the drug product.

Differences are allowed, and expected with today's sophisticated analytical methodology, but the differences cannot have an adverse impact on the safety or efficacy of the biopharmaceutical. Thus, the definition of 'highly similar' is

experimentally-based on a case-by-case basis. As ICH Q5E states, "*a careful evaluation of all foreseeable consequences for the product should be performed*." But different manufacturers and different regulatory authority reviewers will subjectively interpret whether such a careful evaluation has been accomplished, which can sometimes lead to frustration for the manufacturer. However, since the regulatory authority has the final say on what is considered highly similar and what is not, it is most important to ensure that the regulatory reviewers have a clear and comprehensive understanding of what was changed in the manufacturing process and the available data to support that the biopharmaceutical has not been adversely impacted by the change. There is no excuse for mislabeling a manufacturing process change as yielding a comparable biopharmaceutical if a careful evaluation of all foreseeable consequences has not been carried out or if the experimental data generated does not justify that conclusion.

An effective biopharmaceutical comparability study addresses three risk-based concerns: (1) the level of risk due to the stage of clinical development when the change is planned, (2) the level of risk due to the nature (type, extent, process location) of the planned process change, and (3) the level of risk due to residual uncertainty remaining after all required testing is completed.

15.2 Level of Risk Due to Stage of Clinical Development When Change Is Planned

As discussed in Chap. 4, Section 4.3.1, the regulatory authorities accept a minimum CMC regulatory compliance continuum risk-based approach for biopharmaceutical manufacturing. For a biopharmaceutical comparability study, the regulatory requirements and expectations to support a manufacturing process change can be divided into three stages: (1) early stage clinical (including the initiation of first-in-human clinical development stage (FIH)), (2) middle stages clinical (refining medical target and dose optimization) and (3) late stage clinical (pivotal study and seeking market approval), as illustrated in Fig. 15.1.

As clinical development advances, the ongoing gathering of safety and efficacy data increases. Issues with product comparability after a process change could impact the safety and efficacy data captured up to that point, even to the point of having already acquired patient data disqualified. Clinical measure of safety and efficacy success is based on the product not changing in the clinical program:

> ***Recombinant Proteins and Monoclonal Antibodies*** [6]
> Comparability studies conducted for products in development are influenced by factors such as the stage of product development, the availability of validated analytical procedures, and the extent of product and process knowledge, which are limited at times due to the available experience that the manufacturer has with the process. Where changes are introduced in development before nonclinical studies, the issue of assessing comparability is not generally raised because the manufacturer subsequently conducts nonclinical and clinical studies using the post-change product as part of the development

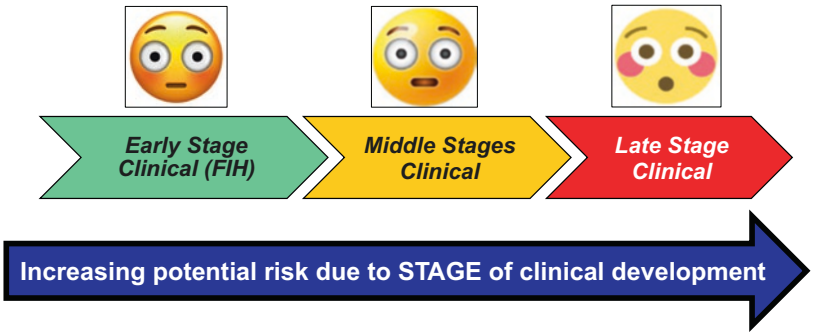

Fig. 15.1 Risk concern increases as the stage of clinical development advances

process. During early phases of nonclinical and clinical studies, comparability testing is generally not as extensive as for an approved product. As knowledge and information accumulate, and the analytical tools develop, the comparability exercise should utilise available information and will generally become more comprehensive. Where process changes are introduced in late stages of development and no additional clinical studies are planned to support the marketing authorisation, the comparability exercise should be as comprehensive and thorough as one conducted for an approved product.

Viral Vectors [11]

For first-in-human studies, any differences between toxicology lots and clinical lots should be assessed for their impact on product safety. For later phase studies, especially those designed to measure product efficacy, differences in clinical lots should be assessed for their impact on product safety and activity.

Genetically Modified Patient Cells [8]

Additionally, the stage of product development may impact whether an analytical comparability study is warranted. For changes to be implemented during early-stage development, the major consideration should be avoiding a negative impact on product safety. However, when considering changes to be made at later stages of product development, the sponsor should evaluate the impact of the change on both safety and efficacy. Regardless of the product development stage, the IND must be updated to reflect the change in manufacturing process (a change in manufacturing process would be considered new chemistry information requiring an information amendment; 21 CFR 312.31(a)). When changes are introduced during late stages of development, and there are no plans for additional clinical studies to support a BLA, the analytical comparability studies should be as comprehensive and thorough as those conducted for a licensed product. Differences in CQAs may warrant new nonclinical or clinical studies. For a licensed product, manufacturing changes must take place within the context of existing change control procedures (21 CFR Parts 210 and 211. Such procedures should be designed to ensure that manufacturing changes do not affect CAR T cell quality. If changes to product release criteria are proposed, clinical data generated under an IND may be requested to support the safety and efficacy of the post-change product.

In summary, the concern level of a regulatory authority for a biopharmaceutical manufacturing process change increases as the stage of clinical development advances. Prior to the start of the first-in-human (FIH) clinical study, the manufacturer has complete freedom to make manufacturing process changes, as long as the changes do not impact the validity of any existing pre-clinical toxicology study. At the early stage of clinical studies (including FIH), regulatory authorities expect that

a comparability study will be performed to ensure that patient safety is not impacted, with the study level at this point in clinical development being referred to as 'adequate'. By the time of the late stage of clinical studies (pivotal clinical stage onwards into market approval), regulatory authorities expect that the comparability study will ensure that both patient safety and product efficacy is not impacted, with the study level at this point in clinical development being is referred to as 'comprehensive and thorough'.

Due to the increasing risk associated with process changes later in clinical development, the preference of regulatory authorities is that manufacturing process changes should occur 'earlier than later', if at all possible, to reduce the risk of impacting the validity of efficacy data being gathered, especially for the viral vectors and genetically modified patient cells:

Viral Vectors [11]

We further acknowledge that understanding and defining product characteristics that are relevant to the clinical performance of the gene therapy may be challenging during early stages of product development, when product safety and quality may not be sufficiently understood. Therefore, we recommend that you evaluate a number of product characteristics during early clinical development to help you identify and understand CQAs. This will also help ensure your ability to assess manufacturing process controls, manufacturing consistency, and stability as development advances. This is especially important for sponsors of gene therapy products who are pursuing expedited development programs.

We recommend that you develop detailed SOPs for how your analytical procedures are conducted at early stages of product development as a part of your quality system. We acknowledge that, during product development, analytical methods may be modified to improve control and suitability. However, assay control is critical during all phases of clinical development to ensure product quality and safety and to allow for comparability studies, following manufacturing changes.

Genetically Modified Patient Cells [8]

Analytical testing for CAR T cells often requires complex assays and development of product-specific biological assays. Thus, we recommend that sponsors begin assay development in early stages of CAR T cell development and use a variety of assays to characterize their product.

In some cases, a change might alter CQAs that cannot be adequately measured in analytical assays. In such a case, analytical comparability studies will be inadequate to evaluate comparability. Therefore, we recommend sponsors anticipate changes needed to establish a scalable and robust manufacturing process and make those changes prior to initiating clinical studies that are intended to provide primary evidence of effectiveness to support a marketing application.

Genetically Modified Patient Cells [12]

The introduction of substantial changes to the manufacturing process and the final product during pivotal clinical studies are not recommended due the complexity of the comparability exercise and the possible impact of its results on the acceptability of the clinical data. In cases where late stage changes in the manufacturing process are unavoidable, it is recommended to seek for EMA scientific advice.

On the other hand, the preference of manufacturers is to delay costly manufacturing process changes (e.g., funding costly validation activities; transferring manufacturing to a more cGMP compliant site) until later in clinical development to ensure that the expense of those changes is justified. Waiting makes financial sense, since some biopharmaceuticals that enter a pivotal clinical program may not meet the

risk-benefit ratio needed for market approval. The downside to this approach is what happens if the clinical program is expedited. No one wins if a biopharmaceutical market approval is delayed because of a manufacturing process change that should have occurred. For this reason, the regulatory authorities encourage discussion with them of pending manufacturing process changes (see Chap. 16).

15.3 Level of Risk Due to Nature (Type, Extent, Location) of Planned Process Change

All manufacturing process changes do not carry the same level of risk of potentially impacting the product. It is most important to consider the nature of that change – location within the process and importance of that process step under change and type of change. For example, changing a filter vendor is a low risk change if the product contact composition and the filtering pore size are equivalent. But the level of potential risk is much higher for this change if the filter is a sterilizing filter for the drug product rather than a microbial reduction filter used in the upstream drug substance process. For a biopharmaceutical comparability study, the nature of the manufacturing process change carries different levels of risk that must be addressed, as illustrated in Fig. 15.2.

The regulatory authorities have commented on the different levels of risk associated, and the corresponding amount of effort, due to the nature of the manufacturing process change:

> ***Recombinant Proteins and Monoclonal Antibodies*** [6]
> The process assessment should consider such factors as the criticality of the process step and proposed change, the location of the change and potential for effects on other process steps, and the type and extent of change. Information that can aid this assessment is generally available from several sources. The sources can include knowledge from process development studies, small scale evaluation/validation studies, experience with earlier process changes, experience with equipment in similar operations, changes in similar manufacturing processes with similar products, and literature. Although infor-

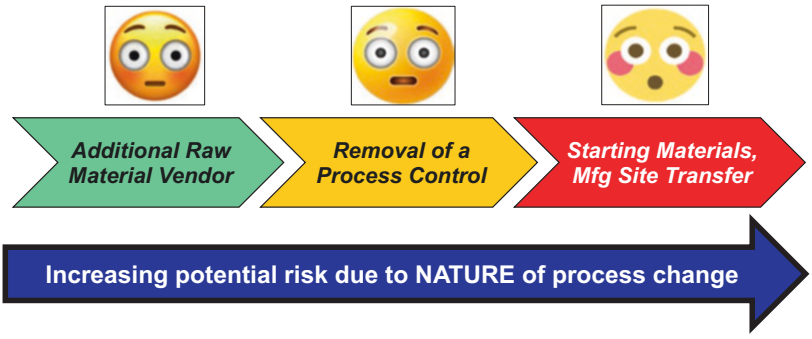

Fig. 15.2 Risk concern increases as the perceived risk of the nature of the change increases

mation from external sources is useful to some extent, it is within the context of the specific manufacturing process and specific product that the change should be assessed.

Genetically Modified Patient Cells [8]

Early product characterization to establish CQAs facilitates the design of studies. Using a variety of characterization assays throughout CAR T cell development provides a greater understanding of the product and supports the evaluation of quality attributes that may be affected by proposed manufacturing changes. For example, you may propose to change the cytokines used for CAR T cell culturing to alter the cell expansion rate. However, this change may also affect the cellular subpopulations and activation state. Therefore, a variety of product attributes, including cellular surface markers, should be monitored using reliable analytical methods, in addition to those attributes typically tested for lot release.

We recognize there may be changes to the CAR T cell design, manufacturing process, or manufacturing facility during product development or post-approval. Changes during the CAR T cell product lifecycle, including changes to the final container, cytokines used during culture, or duration of cell expansion, may impact product quality, safety, efficacy, or stability. There are some changes (e.g., changes to the CAR construct or changing from an autologous to allogeneic product) which would generally result in a new product that should be submitted in a new IND.

- Substantial changes to the vector manufacturing process (e.g., changing from adherent to suspension culture) should be supported by comparability studies. Due to the essential role of the vector in CAR T cell activity, the impact of such changes should be assessed on both the vector and the CAR T cells. Studies should include side-by-side analyses of the pre- and post-change vector. Additionally, CAR T cells manufactured with pre- and post-change vector should be assessed using side-by-side analysis by using the same cellular starting material (e.g., splitting the leukapheresis starting material from the same donor).
- The complexity of comparability assessments may differ depending on the extent of the change to the CAR T cell manufacturing process. For example, a small change in the volume of culture media to manufacture CAR T cells may generally be supported by cell viability and expansion data. In contrast, a more robust comparability study should be conducted for a change to the concentration or type of growth factors or supplements in the culture media.
- When the CAR T cells or vector manufacturing facility is changed, comparability between manufacturing facilities should be established to ensure that the properties of the investigational product are not altered in a manner that would prohibit using preclinical data to support the clinical study or combining the clinical data resulting from the product produced at each manufacturing facility.

15.3.1 Assigning Risk Levels to Proposed Manufacturing Process Changes

Both the stage of clinical development and the nature of the proposed manufacturing process change, impact the risk level associated with that change. The risk level determines the amount of experimental data necessary to evaluate the potential impact of the manufacturing process change adequately and appropriately. Lower risk levels might mean acquiring some process data before and after the change, or maybe even simply a written justification of why no testing is necessary; while

higher risk levels typically mean extensive product characterization data, stability data and even possibly clinical data. Using a risk-based approach (RBA), the risk level for the change is to be determined [13]:

> The potential impact of the proposed change should always be evaluated for its risks to the quality of the final product and the impact on the efficacy and safety profile of the product. The overall extent of the comparability exercise for ATMPs should therefore be driven by a risk-based approach (RBA). Namely, the RBA should be used to determine an appropriate amount of comparability data and to select a suitable set of relevant critical quality attributes (CQAs) to be compared, taking into account the stage of product development and the number of batches available. Changes that are considered to have a high risk/impact will require an extensive exercise of comparison at the in-process control level, characterization and release. Whenever relevant, the generation of additional/new validation data has to be taken into account. On the other hand, low risk/impact changes may entail a more limited amount of comparability data. A more comprehensive data package is required to support manufacturing changes in pivotal clinical trials or to the marketing authorisation.

Sounds easy enough. But risk levels are challenging, and definitely not easy to assign, for a biopharmaceutical manufacturing process change. In addition, the level assigned to a manufacturing process change can be somewhat subjective, frequently based on one's own experience in the biopharmaceutical industry (i.e., lessons learned of what can possibly happen with that type of change), coupled with the risk tolerance culture of the manufacturer (i.e., different manufacturers may want difference levels of assurance that a proposed change will not impact the biopharmaceutical).

Lessons can be learned by viewing how regulatory authorities assign the level of risk to the different biopharmaceutical manufacturing process changes that they have to review and approve. Insight can also be obtained on regulatory authorities' view of the minimum CMC regulatory compliance continuum applied to the level of risk for similar process changes between clinical development and market-approval. In the next two sections, risk levels according to the regulatory authorities for the different types of manufacturing process changes by biopharmaceutical type will be closely examined:

Section 15.3.2 Recombinant Proteins and Monoclonal Antibodies
Section 15.3.3 Viral Vector and Genetically Modified Patient Cells

15.3.2 Risk Levels for Recombinant Proteins and Monoclonal Antibodies

The regulatory authorities have published a few guidances to help determine the level of risk that they associate with a number of manufacturing process changes for recombinant proteins and monoclonal antibodies.

15.3.2.1 Manufacturing Process Changes During Clinical Development

EMA has published a list of manufacturing process changes that can occur during clinical development. The level of risk is divided into two main CMC risk level categories [14]:

- Substantial Modification (process change must be submitted to IMPD, and approved by national competent authority (NCA) prior to implementation)

  Substantial modification means any change which is likely to have a substantial impact on the safety and rights of the subjects or on the reliability and robustness of the data generated in the clinical trial. Assessment of an IMPD should be focused on patient safety. Therefore, any modification involving a potential new risk has to be considered a substantial modification. This may be especially the case for changes in impurities profile, microbial contamination, viral safety, or the risk of TSE contamination or in some particular cases to stability when degradation products of concern may be generated.

- Non-Substantial Modification (no prior approval required, but submitted to IMPD at next update)

  For non-substantial modifications, documentation should not be proactively submitted, but the relevant internal and study documentation supporting the change should be recorded within the company and if appropriate, at investigator site. At the time of an overall IMPD update or submission of a substantial modification the non-substantial changes should be incorporated into the updated documentation.

The list of manufacturing process changes is non-exhaustive in this regulatory guidance, but it does give an indication of the risk level that EMA applies to some changes; and it provides an insight into how they might apply the risk level also for other types of changes. By definition, if it is not listed as a substantial, it is a non-substantial change. Table 15.1 presents some recombinant protein and monoclonal manufacturing process change examples for the two risk levels.

15.3.2.2 Manufacturing Process Changes Post-Market Approval

Both FDA and EMA have published lists of recombinant protein and monoclonal antibody manufacturing process changes that can occur post-market approval. The FDA guidance consists of two stand-alone documents [15, 16], while the EMA guidance is under the Variation Guidelines applicable to both chemical drugs and biologics [17]. The level of risk is divided into three main CMC categories, that determine how the process change can be implemented into the BLA/MAA, see Table 15.2.

The list of manufacturing process changes is non-exhaustive in these two regulatory guidances, but it does give an indication of the risk level that FDA and EMA apply to some changes; and it provides an insight into how they might apply the risk level also for other types of changes. Table 15.3 presents some recombinant protein and monoclonal manufacturing process change examples for two of the FDA risk levels (the highest risk and the lowest risk categories).

Table 15.1 Process change risk levels: protein-based biopharmaceuticals in clinical development

RECOMBINANT PROTEINS AND MONOCLONAL ANTIBODIES		
Changes to IMPD	**SUBSTANTIAL Risk Level**	**NON-SUBSTANTIAL Risk Level**
Drug Substance Manufacturing Process	Changes such as: • new master cell bank • introduction of a working cell bank if prepared from an approved MCB • change in scale of the production bioreactor (upstream process), • changes in the purification process (downstream): addition or removal of a purification step • changes in the process conditions of any steps that have been identified as contributing to virus removal/inactivation, • changes leading to the occurrence of new impurities and product related substances	Changes such as: • Addition or tightening of IPC if not due to safety reasons • Modification of the process parameters (same process, analogous raw materials) where no effect on product quality is demonstrated. • reprocessing if adequately described and accepted in the initial submission • minor changes in the process which do not require a comparability exercise • changes to the controls of non-critical raw materials
Specifications Release & Shelf Life	• Change in the specification, if acceptance criteria are widened or deleted • Addition of specification or acceptance criteria for safety/quality reasons	• Tightening acceptance criteria or adding acceptance criteria for no safety/quality reasons • Addition, deletion or replacement of a specification due to compendial change

Table 15.2 Regulatory authority guidances on process change risk levels post-market approval

Risk-Level for Manufacturing Process Change			
Regulatory Authority	**Major Risk**	**Moderate Risk**	**Minor Risk**
	Prior Approval Required	30 Day Wait After Submission	No Waiting
FDA	Prior Approval Supplement (PAS)	Change Being Effective (CBE-30)	Annual Report
EMA	Type II Variation	Type 1B Variation	Type 1A Variation

A word of caution, FDA has issued several other guidance documents addressing the risk of manufacturing process changes. It is important to check out the scope of coverage for any FDA guidance document, as many do not apply to biopharmaceuticals, or specifically to certain types of biopharmaceuticals. For example, the FDA

Table 15.3 Process change risk levels: protein-based biopharmaceuticals post-market approval

RECOMBINANT PROTEINS AND MONOCLONAL ANTIBODIES Changes to BLA	
MAJOR (PAS) Risk Level	**MINOR (ANNUAL REPORT) Risk Level**
• extension of culture growth time leading to significant increase in number of cell doublings beyond validated parameters; • new or revised purification process, including a change in a column; • a change in the chemistry or formulation of solutions used in processing; • a change in the sequence of processing steps or addition, deletion, or substitution of a process step; • reprocessing of a product without a previously approved protocol. • scale-up requiring a larger fermenter, bioreactor, and/or purification equipment • change of the site(s) at which manufacturing, other than testing, is performed, addition of a new location, or contracting of a manufacturing step in the approved application, to be performed at a separate facility. • conversion of production and related area(s) from single to multiple product manufacturing area(s).	• changes in batch sizes that do not involve use of different equipment (e.g., minor changes in roller bottle number, fermenter volume or load volumes for chromatography columns) • manufacturing batch size or scale change caused by minor changes in the size of pooled or separated batches to perform the next step in the process if all batches meet the approved in-process control limits and the CPP ranges for the next step remain unaffected • replacement of a nonspecific identity test with a discriminating identity test that includes a change in acceptance value (e.g., replacing SDS-PAGE with peptide mapping) • tightening of an existing acceptance criterion • for sterile drug products, changes to the ranges of filtration process parameters (such as flow rate, time, pressure) that are within previously validated parameters

guidance above that deals with the Annual Report minor process changes listed above, is specifically for the protein-based biopharmaceuticals. The guidance states clearly: "*The guidance does not apply to .. cellular and gene therapy products …*".

A word about the EMA guidance is under the Variation Guidelines listed above. Sometimes the determination of the risk level associated with a process change is not so clearly delineated. For example, in the EMA Variation Guideline, introducing a new Working Cell Bank is not specifically listed, so manufacturers have to decide if the change is considered a minor change (which means the change is annual reportable) or a significant change (which requires prior-approval). Fortunately, EMA also issues post-authorisation procedural advice, and in that guidance [18], they make it very clear that if a post-approval change management plan (PACMP) has been pre-approved for the MAA, then the change is a Type IB variation (annual reportable), but if no PACMP has been approved, then the change is a Type II (prior-approval supplement).

15.3.3 Risk Levels for Viral Vectors and Genetically Modified Patient Cells

The regulatory authorities have published a few guidances to help determine the level of risk associated with a number of manufacturing process changes for the gene therapy-based biopharmaceuticals (viral vectors and genetically modified patient cells).

15.3.3.1 Manufacturing Process Changes During Clinical Development

Unfortunately, neither FDA nor EMA have published to date their thoughts on a list of manufacturing process change that can occur during clinical development with an assigned risk level.

15.3.3.2 Manufacturing Process Changes Post-Market Approval

Both FDA and EMA have published lists of viral vector and genetically modified patient cells manufacturing process changes that can occur post-market approval. The FDA guidance is a stand-alone document [19], while the EMA guidance is under the Variation Guidelines applicable to both chemical drugs and biologics [17]. The level of risk is divided into three main CMC categories (see Table 15.2), that determine how the process change can be implemented into the BLA/MAA.

The list of manufacturing process changes is non-exhaustive in these two regulatory guidances, but it does give an indication of the risk level that FDA and EMA apply to some changes; and it provides an insight into how they might apply the risk level also for other types of changes. Table 15.4 presents some viral vectors and genetically modified patient cells manufacturing process changes examples for two of the FDA risk levels (the highest risk and the lowest risk categories).

At first glance, the FDA guidance for changing the biopharmaceutical manufacturing scale in the BLA and the EMA guidance for changing the biopharmaceutical manufacturing scale in the MAA, appear to have different risk level assignments. The EMA Variance Guidance appears to indicate that scaling-up or scaling-down the manufacturing process up to ten-fold, compared to the approved size in the MAA is a Type IA variation (annual-reportable). But looking closer at the footnotes in the table, it states clearly that if this is a biological process, then the 10X rule doesn't apply, and a Type II variation (prior-approval) is necessary. Thus, making the two guidances in agreement on this level of risk.

The FDA guidance also refers to 'with an approved protocol' and 'without and approved protocol'. This is referring to PACMPs (discussed in a following section).

A word of caution, FDA has issued several other guidance documents addressing the risk of manufacturing process changes. It is important to check out the scope of coverage for any FDA guidance document, as many do not apply to

Table 15.4 Process change risk levels: gene therapy-based biopharmaceuticals post-market approval

GENE THERAPY BIOPHARMACEUTICALS Changes to BLA	
MAJOR (PAS) Risk Level	**MINOR (ANNUAL REPORT) Risk Level**
Addition of new equipment (e.g., bioreactor/fermenter, purification/ inactivation) that results in a change in a batch size (increase or decrease) or operating parameters.	**Addition or replacement of equipment of the same size and material of construction used in harvesting and pooling with no change in the process parameters specified in the BLA.**
Change in a batch size (increase or decrease), using different equipment or involving change in process parameters, controls and specifications.	**Change in fermentation step batch size (e.g., minor adjustments in volume) using the same equipment with no change in process parameters, controls and specifications.**
Increase in the number of cycles of resin and membrane re-use without an approved protocol.	**Increase in the number of cycles of resin and membrane re-use according to an approved protocol.**
Generation of a working cell bank or working virus seed from an approved master cell bank or master virus seed without an approved protocol.	**Generation of a working cell bank or virus seed from an approved master cell bank or master virus seed according to an approved protocol. [but requires a CBE 30 day wait]**
Widening of acceptance criteria for in-process control.	**Tightening of acceptance criteria for in-process control.**
Addition or replacement of an existing building for production of Drug Substance, Drug Product or intermediates within an approved manufacturing location.	**Relocation of manufacturing equipment within an approved manufacturing location to improve product/personnel/raw material flow and segregation of materials.**

biopharmaceuticals, or specifically to certain types of biopharmaceuticals. For example, the FDA guidance above, is specifically for the gene therapy-based biopharmaceuticals. The guidance states clearly: "*The guidance does not apply to .. recombinant proteins, monoclonal antibodies, biosimilars …*"

With the newness of the gene therapy-based biopharmaceuticals, biopharmaceutical manufacturers have recently joined together to publish consensus documents, to illustrate from their perspective, how to best manage the CMC regulatory compliance risk concerns for these gene therapy-based biopharmaceuticals, including what comparability studies might be needed and how to appropriately carry them out:

A-Gene (2021): A case study-based approach to integrating QbD principles in gene therapy (viral vectors) CMC programs – over 60 individuals volunteered their time [20]

A-Cell (2022): A case study-based approach to integrating QbD principles in cell-based therapy (CAR T cells) CMC programs – over 40 individuals volunteered their time [21]

15.3.4 Established Conditions (ECs)

As noted above, one of the challenges that a biopharmaceutical manufacturer faces is knowing which process changes can be carried out within their pharmaceutical quality system (PQS) and which process changes must go through a regulatory authority review and approval process. There is no way that any regulatory authority can issue enough guidances to address all of the potential manufacturing process changes that are possible. Therefore, if the manufacturer can reach agreement with a regulatory authority ahead of time on a significant portion of likely process changes across their entire manufacturing process, then not only is the risk of incorrectly managing a process change reduced but also a faster implementation of some low-risk process changes can occur.

An established condition (EC) is an attempt to reach such a broad agreement on process changes. EC is binding information considered necessary to assure product quality. As a consequence, any change to an EC necessitates a submission to the regulatory authority. And, as a consequence, any change to a non-EC (also referred to as 'supportive information') does not necessitate a submission to the regulatory authority, but is managed within the PQS.

ICH Q12 [22] lays out the proposal for ECs. The manufacturer of the market-approved biopharmaceutical initiates the request with the regulatory authority, and the regulatory authority has to decide if they want to agree with the EC proposal. ICH Q12 provides a template to serve as a discussion point for some ECs to be considered, see Table 15.5 for an example of a drug substance proposed template. EC proposals are submitted in Module 3.2.R.

ICH Q12 Annex [23] drills down into the manufacturing process with two examples of applying ECs to a monoclonal antibody manufacturing process – specifically an upstream cell culture production step and an anion exchange chromatographic step. The document shows how three different approaches, to controlling the manufacturing process, impact the list of ECs: minimal parameter-based approach, enhanced parameter-based approach, and performance-based approach [22]. Basically, the more investment in scientifically understanding the manufacturing process and the characterization of the biopharmaceutical, (i.e., investment in the QbD approach), the more freedom of operation is offered (i.e., less EC controls).

The greatest challenge in reaching agreement on ECs is the level of confidence (or lack thereof) that the regulatory authority has in the manufacturer's comprehensive scientific understanding of controlling their challenging manufacturing process along with confidence in the strength of the manufacturer's PQS.

Table 15.5 Example of Established Conditions (ECs) template

Established Conditions (ECs) for the Drug Substance	
3.2.S CTD Outline	EC in blue Non-EC in yellow
3.2.S.1	General Information
3.2.S.1.1	Nomenclature
3.2.S.1.2	Structure
3.2.S.1.3	General Properties
3.2.S.2	Manufacture
3.2.S.2.1	Manufacturer(s)
3.2.S.2.2	Description of Manufacturing Process
3.2.S.2.3	Control of Materials
3.2.S.2.4	Control of Critical Steps
3.2.S.2.5	Process Validation/Evaluation
3.2.S.2.6	Manufacturing Process Control
3.2.S.3	Characterization
3.2.S.3.1	Elucidation of Structure
3.2.S.3.2	Impurities
3.2.S.4	Control of Drug Substance
3.2.S.4.1	Specifications
3.2.S.4.2	Analytical Procedures

15.4 Level of Risk Due to Residual Uncertainty Still Remaining

The overall goal of a comparability study is not to eliminate all potential patient safety and efficacy risk. Biopharmaceuticals are too large and complex, and the analytical tools too limited to investigate all possible change that could lead to harm and then eliminate them all. According to ICH Q5E – '*Demonstration of comparability does not necessarily imply that the quality attributes of the pre- vs post-change product are identical, but that they are highly comparable and that the existing knowledge is sufficiently predictive to ensure that any differences in quality attributes have no adverse impact on the safety and efficacy of the drug product*' [6, 24]. Therefore, the overall goal of a comparability study is to reduce the risk of harm to the patient to an acceptable level (i.e., to be 'sufficiently predictive' of no adverse impact on quality, safety, efficacy) – this is referred to as reduction in 'residual uncertainty'.

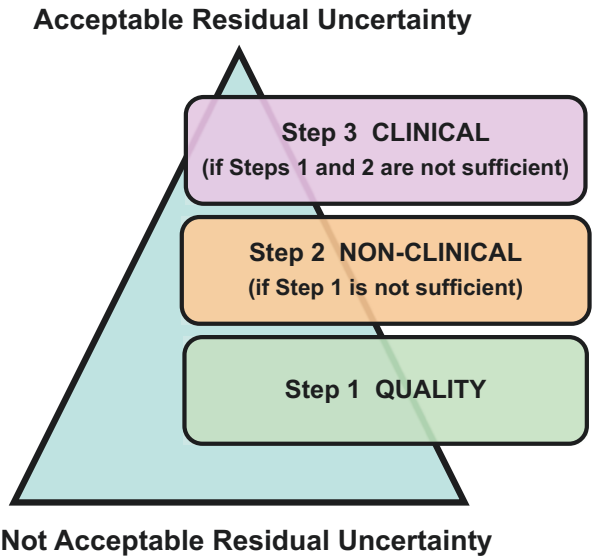

Fig. 15.3 Stepwise approach to reducing residual uncertainty for a process change

To reduce residual uncertainty, the regulatory authorities recommend a stepwise approach to the design of the comparability study, as shown in Fig. 15.3. Step 1 is to first determine if the Quality (analytical/functional) comparison testing can provide sufficient confirmation for the biopharmaceutical of no adverse impact due to the process change. If there is an unacceptable residual uncertainty about the sufficiency of Step 1, then additional Nonclinical (Step 2, studies in animals, typically pharmacokinetics) comparison testing is to be carried out. If there is still an unacceptable residual uncertainty about the sufficiency of Steps 1 and 2, then additional Clinical (Step 3, bridging human studies) comparison testing is necessary. Regulatory guidances clearly state the recommended step-wise approach:

> ***Recombinant Proteins and Monoclonal Antibodies*** [14]
> This comparability exercise should normally follow a stepwise approach, including comparison of quality attributes of the active substance and relevant intermediates, using suitable analytical methods. Analytical methods usually include routine tests, and may be supplemented by additional characterisation tests (including orthogonal methods), as appropriate. Where the manufacturers' accumulated experience and other relevant information are not sufficient to assess the risk introduced by the change, or if a potential risk to the patients is anticipated, a comparability exercise based only on quality considerations may not be sufficient.
>
> ***Viral Vectors and Genetically Modified Patient Cells*** [13]
> Overall, the general principles of ICH Q5E can be applied to ATMPs:
>
> - The comparability exercise should be conducted stepwise, starting with the physicochemical and biological properties of the product. This will be based on analytical testing e.*g.*, routine batch analysis, in-process controls, process validation/evaluation data, characterization and stability studies, as applicable.

- The investigation should focus on the manufacturing process steps most appropriate to detect a change. This may require an evaluation on all critical steps/in-process controls/materials of the manufacturing process downstream of the change.
- Analytical methods should be suitable for purpose and sufficiently sensitive to ensure the detection of differences/modifications. Any observed analytical difference should be evaluated in relation to its impact on the product quality, safety and efficacy.
- If required due to non-comparable results that can have impact on the relevance of the safety and/or efficacy data gathered so far, the comparability exercise should proceed with the generation and evaluation of comparability non-clinical and/or clinical data as necessary to contribute to the conclusion of comparability of the product.

For most manufacturing process changes, the Step 1 Quality (analytical/functional) comparability study can be sufficient to reaching the acceptable residual uncertainty threshold. But it is important to stress that the analytical/functional comparison is more than just comparing release specifications for the biopharmaceutical before and after a process change:

Recombinant Proteins and Monoclonal Antibodies [6]

When considering the comparability of products, the manufacturer should evaluate, for example:

- Relevant physicochemical and biological characterization data regarding quality attributes;
- Results from analysis of relevant samples from the appropriate stages of the manufacturing process (e.g., intermediate, drug substance, and drug product);
- The need for stability data, including those generated from accelerated or stress conditions, to provide insight into potential product differences in the degradation pathways of the product and, hence, potential differences in product-related substances and product-related impurities;
- Batches used for demonstration of manufacturing consistency;
- Historical data that provide insight into potential "drift" of quality attributes with respect to safety and efficacy, following either a single or a series of manufacturing process changes. That is, the manufacturer should consider the impact of changes over time to confirm that an unacceptable impact on safety and efficacy profiles has not occurred.

In addition to evaluating the data, manufacturers should also consider:

- Critical control points in the manufacturing process that affect product characteristics, e.g., the impact of the process change on the quality of in-process materials, as well as the ability of downstream steps to accommodate material from a changed cell culture process;
- Adequacy of the in-process controls including critical control points and in-process testing: In-process controls for the postchange process should be confirmed, modified, or created, as appropriate, to maintain the quality of the product
- Nonclinical or clinical characteristics of the drug product and its therapeutic indications

Gene Therapy-Based Biopharmaceuticals [13]

Overall, the general principles of ICH Q5E can be applied to ATMPs:

- The comparability exercise should be conducted stepwise, starting with the physicochemical and biological properties of the product. This will be based on analytical testing e.g., routine batch analysis, in-process controls, process validation/evaluation data, characterization and stability studies, as applicable.

- The investigation should focus on the manufacturing process steps most appropriate to detect a change. This may require an evaluation on all critical steps/in-process controls/materials of the manufacturing process downstream of the change.
- Analytical methods should be suitable for purpose and sufficiently sensitive to ensure the detection of differences/modifications. Any observed analytical difference should be evaluated in relation to its impact on the product quality, safety and efficacy.
- If required due to non-comparable results that can have impact on the relevance of the safety and/or efficacy data gathered so far, the comparability exercise should proceed with the generation and evaluation of comparability non-clinical and/or clinical data as necessary to contribute to the conclusion of comparability of the product.

Genetically Modified Patient Cells [24]

Any change in the manufacturing process should be assessed for its risk to affect the quality of the final product. The results of this assessment will determine the extent of the comparability study. For changes concluded to have a high risk, such as a manufacturing site change, comparability between pre- and post-change products should include release tests, relevant stability studies, extended characterisation and in-process controls as well as any other relevant process parameter.

Interpretation of what is an 'acceptable' residual uncertainty will be subjective; and, will lead to disagreements within the manufacturer (Quality Assurance is typically more conservative in their interpretation than senior management) as well as between the manufacturer and the regulatory authority reviewers. Bottom line, the group that has the final say in whether the manufacturing process change is acceptable or not makes the final decision – and that is the regulatory authority.

15.5 PACMPs – Comparability 'Contracts'

With so much riding on the need to have regulatory authority prior-approval for many biopharmaceutical manufacturing process changes, having to wait for regulatory approval not only is expensive (batches cannot be released until approval of the change is accepted), but it also can tie up a manufacturing facility with the change in place but yet not able to be used. Discussions about product comparability plans with the regulatory authorities (as discussed in Chap. 16) to reach agreement on the comparability work that needs to be done helps reduce the uncertainty of getting a surprise reaction from the regulatory reviewers after the work for the change has been completed. But it doesn't change the time waiting for approval. But, a regulatory tool now available – a 'contract' with the regulatory authority – not only reduces the surprise reaction when the comparability report is delivered to them, but also reduces the time for their acceptance of the change. This contract is called the 'comparability protocol' or 'CP' by the FDA [10], or more internationally now called the post-approval change management protocol or 'PACMP' [22]. If the PACMP is agreed to by the regulatory authority, it can reduce the time between submitting the comparability report to them and the timing for completion of their review. The goal

being to reduce the review risk level from a prior-approval submission to a change being effective – 30 day wait, or even down to an annual report filing.

Seeking agreement ahead of time with a regulatory authority is no easy task. Don't underestimate the amount of descriptive detail that has to be submitted. The regulatory authority's expectation is that the proposal is a 'comprehensive, prospectively written plan'. The following key elements, as stated by ICH Q12, are required to be included [22]:

Elements of a PACMP

The development of the PACMP is informed by the application of process and product understanding gained from product development and/or manufacturing experience. A PACMP would typically include the following, e.g.:

- A detailed description of the proposed change(s), including a rationale. The differences before and after the proposed change(s) should be clearly highlighted (e.g., in a tabular format).
- Based on an initial risk assessment, a list of specific tests and studies to be performed to evaluate the potential impact of the proposed change(s), such as: characterisation, batch release, stability (as appropriate), in-process controls. The PACMP should include an appropriate description of the analytical procedures and proposed acceptance criteria for each test or study.
- Discussion regarding the suitability of the approved control strategy or any changes needed to the control strategy associated with the planned change(s).
- Any other conditions to be met, such as confirmation that certain process qualification steps will be completed before implementation.
- Where applicable, supportive data from previous experience with the same or similar products related to: development, manufacturing, characterisation, batch release, and stability to allow for risk mitigation.
- Proposed reporting category for step 2 of the PACMP.
- Confirmation, as appropriate, that ongoing verification will be performed under the PQS to continue to evaluate and ensure that there is no adverse effect of the change(s) on product quality. In cases where monitoring of the impact on product quality following implementation of the change(s) is required, a summary of the quality risk management activities should be provided to support the proposed PACMP. If multiple changes are to be implemented, these activities should address the potential risk from the cumulative effect of multiple changes and how they are linked.

The MAH should demonstrate in the PACMP suitable scientific knowledge and understanding of aspects impacted by the proposed change in order to conduct an appropriate risk assessment of the proposed change(s). Typically, more complex changes would require enhanced product/process understanding.

The manufacturer needs to convince the regulatory authority that (a) they have the scientific knowledge to know how to carry out the proposed process change, (b) they have a comprehensive understanding of the biomolecule to be able to predict the impact of the process change on the biopharmaceutical, and (3) the regulatory authority can trust them to complete what they proposed in the detailed plan.

The more complicated the intended manufacturing process change, the more time it typically takes to negotiate the final agreement with the regulatory authority. Keep in mind that the total elapsed time – from the preparation of the comparability plan to the regulatory authority to its final approval for implement – is not shortened by the PACMP procedure. A PACMP primarily does two things: (1) shifts the time

interval, between submitting the final comparability report and obtaining regulatory approval, to the upfront time period needed to negotiate the agreement, and (2) reduces the risk of rejection by the regulatory authority when the final report is sent to them by reaching an agreement ahead of work initiation. Keep in mind that if the negotiated acceptance criteria are not met, the protocol reverts to a normal prior-approval supplement, with no savings in time.

Use of these change management plans are quite common today for biopharmaceutical manufacturers for advance planning of future changes typically expected for a market-approved product. Frequently, the following future process changes are submitted as a PACMP for advanced approval in a BLA/MAA (typically in the CTD Module 3.2.R section):

- Qualifying a new Working Cell Bank
- Qualifying a new Reference Material or Standard
- Concurrent validation to complete the column resin lifetime study
- Reprocessing (especially for sterile filtration)
- Shelf-life extension using the ongoing stability protocols in the submitted dossier

The following is a case example of an approved PACMP for the market-approved monoclonal antibody, Imjudo (tremelimumab), submitted in the MAA dossier, to use alternate single-use disposable filters across the drug substance manufacturing process [25]:

> The applicant introduced a Post-Approval Change Management Protocol (PACMP) to support the use of alternative single-use disposable filters across a number of steps in the active substance manufacturing process. Details regarding the planned technical assessment, assessment of extractables and leachables, small-scale studies and at-scale verification studies for the purpose of demonstration of comparability were provided. The upcoming changes will not have an impact on the composition, active substance and finished product specifications, active substance manufacturing process, critical steps, in-process controls or hold times and at-scale active substance batches will be placed on stability. Overall, the proposed PACMP is considered acceptable.

The following is a case example of several approved PACMPs for the market-approved bispecific monoclonal antibody, Lunsumio (mosunetuzumab-axgb), submitted in the BLA dossier [26]:

> *Drug Substance protocols approved*
>
> - Master cell bank and working cell bank stability protocol
> - Primary and secondary reference standard re-qualification protocol
> - Future secondary reference standard qualification protocol
> - Post-approval annual stability protocol
> - Multi-use drug substance facility expanded protocol for Genentech, Inc. South San Francisco, Building 3 (FEI: 2917293)

The following is a case example of an approved PACMP for the market-approved monoclonal antibody engineered fragment, Ximluci (ranibizumab), submitted in the MAA dossier, to increase manufacturing scale ten-fold [27]

> *Post-approval change management protocol(s)*

> A post-approval change management protocol (PACMP) is proposed for the scale-up of active substance manufacture by a factor of 10. Three consecutive validation batches are planned to support the scale-up. All of the final CPPs/PPs will be verified and reported for the process validation batches. In addition, the FMEA performed to verify that no change to manufacturing process controls is required was provided and supports the proposed approach. The proposal to verify the proposed in-process hold times and temperatures as part of the process validation is acceptable. Active substance batches will be included in a long-term and accelerated conditions stability program. An updated risk assessment for extractables and leachables will be performed which is agreed. Comparability of the current and proposed process will be evaluated by in-process data and release testing of active substance from the validation batches. The testing panel for comparability includes release tests and additional extended characterisation tests. Results from historical process batches will be included in the comparability exercise and used to generate comparability acceptance criteria. The testing panel and acceptance criteria for the comparability exercise have been registered to the PACMP and are acceptable. Historical process batches will be statistically evaluated for quantitative quality attributes and the acceptance criteria will be based on mean ±3SD for all CQAs except when this is not feasible for the method. In the latter case, the Min-Max range will be used. The PACMP proposed for the scale-up of the active substance manufacturing process is considered acceptable.

It is interesting to note that the manufacturer in the same MAA dossier submission attempted to seek approval for a second PACMP, a future pre-filled syringe presentation, but in this case the request was denied, and the manufacturer had to remove the PACMP out of the MAA dossier [27]:

> Following the withdrawal of the PFS presentation during the procedure, the Applicant proposed a PACMP for the future registration of the PFS presentation. However, numerous deficiencies were noted in the PACMP. While some aspects could be addressed by significant revision of the PACMP, there were numerous issues that did not appear to be resolvable. As a consequence, the Applicant decided to remove the PACMP for the PFS from the dossier.

15.6 Clear Communication – Comparability Missteps Not Allowed

It is most important that the manufacturer provide adequate and appropriate information to the regulatory authority so that they may understand the type of manufacturing process change made, the location in the manufacturing process at which the change was made, and the studies carried out that confirm the highly similar properties of the biopharmaceutical after the process change. Nothing will speak stronger to the regulatory reviewers than good science, solid data, and an honest critical evaluation, in interpreting the conclusions of the comparability results. A case example of a thorough scientifically executed comparability study is reported in the EMA market-approval for the bispecific monoclonal antibody, Lunsumio (mosunetuzumab) [28]:

> *Comparability*

> A comparability exercise considered the types of changes introduced in the mosunetuzumab manufacturing process with v0.2 and the potential impact of observed product quality differences on safety and efficacy. The comparability evaluation included active substance release analysis, extended characterisation comparison and in addition a pharmacokinetic study in cynomolgus monkeys, to compare the pharmacokinetic characteristics of mosunetuzumab from the v0.1 and v0.2 processes. Overall, the results of the comparability exercise showed that the manufacturing process changes did not have an adverse impact on the quality, safety, or efficacy of mosunetuzumab, and determined that v0.1 material and toxicology material were comparable to v0.2 material. In addition to comparability evaluation, process impact assessment was performed, see details in sections above. The data presented in the process impact assessment support the conclusion that the manufacturing process produces active substance of consistent quality. Comparability is considered sufficiently addressed.

Then there are other case examples where the market application dossier probably should not have been filed in the first place. The case example of the monoclonal antibody biosimilar Hervelous (trastuzumab) at day 120 of EMA review received 20 major objections and135 other objections concerning CMC regulatory compliance aspects. Needless to say, the biopharmaceutical never achieved market approval because the comparability data could not prove that (1) the commercial manufactured product was comparable to their own clinical manufactured product used in the clinical development program, and (2) the biosimilar was not highly similar to the innovator's marketed biopharmaceutical [29]:

> Through multiple rounds of responses several of the major objections raised at day 120 and 180 could be satisfactorily resolved, as discussed in the above sections of this report. However, at the time of opinion three major objections remained unresolved. These relate to the following deficiencies:
>
> - The clinical trial material is not considered representative of the proposed commercial material: Multiple quality attributes with high criticality directly impacting the mode of action or which can have an effect on efficacy, safety, pharmacokinetic and immunogenicity, demonstrate significant variation between the manufacturing processes used during clinical development and the proposed commercial manufacturing process. Therefore, the batches from the different clinical manufacturing processes cannot be considered comparable to the commercial process material.
> - Significant concerns identified in the presented biosimilarity exercise preclude a conclusion of biosimilarity between HD201 and EU-sourced Herceptin. The approach taken by the applicant to address the identified concerns by post hoc reanalysis of data, including arbitrary exclusion or inclusion of certain data, is not considered acceptable. It is rather creating uncertainty around the credibility of the results presented and the integrity of the data.
> - Data provided on the quality profile for the reference product are not in line with the known quality profile of the reference product (including ADCC activity, HER2 binding, anti-proliferation, afucosylation), creating further uncertainty around the credibility of the results presented and the integrity of the data. Therefore, it cannot be concluded whether the currently presented data ranges fully represent the underlying variability for the reference product.

Don't try to hide a problematical comparability concern in the back of the submitted comparability study report. Consider introducing the comparability concern early in the report and provide the justification for why the manufacturer believes the biopharmaceutical is still considered comparable. Do this before the regulatory

reviewer forms a different opinion if surprised in finding it buried in the submitted report.

All too often the conclusion of the comparability report is “comparable”, even when the data speaks otherwise. And at times, even the conclusion of ‘comparable’ has been sometimes written down in the draft comparability report even before the comparability studies are carried out. This is not good science.

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Chapter 16
Strategic CMC-Focused Interactions with Regulatory Authorities

Abstract The development, design and control of a biopharmaceutical manufacturing process to obtain product of required quality, safety and efficacy, has never been more challenging, as the biomolecules have increased in size and complexity – from monoclonal antibodies to viral particles to genetically modified patient cells. In this chapter, the critical importance of the biopharmaceutical manufacturer communicating with the regulatory authority, to ensure that their CMC regulatory compliance strategy is sufficient to carry the product all the way through to the end of clinical development and into the marketplace, will be stressed. And the importance of the biopharmaceutical manufacturer to listen to what the regulatory authority reviewers have to say, and either follow their CMC guidance or develop an alternative CMC regulatory compliant strategy that will address their identified concerns. Taking advantage of the established regulatory review process, involving milestone submissions and meeting opportunities throughout the clinical development program, to discuss CMC regulatory compliance strategy, reduces the risk of surprises or delays when later seeking market approval. In this chapter, the opportunities available to interact with the regulatory authority reviewers will be examined, as well as how to maximize the benefit to the biopharmaceutical manufacturer from these interactions. But then comes the time for submitting the market application dossier (BLA or MAA). This is the time when the developed CMC regulatory compliance strategy is evaluated to see if the biopharmaceutical meets the regulatory authority standard for market approval. Intense interactions with the reviewers occur. In this chapter, responding accurately, thoroughly, and timely to reviewer's requests for more CMC explanation or additional data will be examined. Avoiding a BLA Complete Response Letter (CRL) or a MAA Withdrawal will be discussed.

Keywords Communicating · Listening · Encouragement · EOP2 · Pre-Submission · Teamwork · Milestones · Withdrawal · Advice · Refusal · Meetings

Senior management readily agree that deficiencies in the Clinical regulatory strategy can seriously delay, and even terminate, advancing a clinical development

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program to achieve market approval for a biopharmaceutical. However, not all senior management would necessarily agree on the level to the seriousness that deficiencies in the CMC regulatory strategy can have on advancing the clinical development program. A comment that this consultant still hears from senior management is "*We know how to do CMC strategy better than the regulatory authorities, so why involve them and muddy the waters?"* In this chapter, the critical importance of communicating with the regulatory authorities to ensure that one's CMC regulatory compliance strategy is appropriate and adequate, will be stressed. Communicating is important, but even more important, is listening to what the regulatory authority reviewers have to say, and either follow their CMC guidance or develop an alternative CMC regulatory compliant strategy that will address the identified concerns. Taking advantage of the established regulatory review process, involving milestone submissions and meeting opportunities throughout the clinical development program, to discuss CMC regulatory compliance strategy, reduces the risk of surprises or delays when later seeking market approval. In this chapter, the opportunities available to interact with the regulatory authority reviewers will be examined, as well as how to maximize the benefit to the biopharmaceutical manufacturer from these interactions. But then comes the time for submitting the market application dossier (BLA or MAA). This is the time when the developed CMC regulatory compliance strategy is evaluated to see if the biopharmaceutical meets the regulatory authority standard for market approval. Intense interactions with the reviewers occur in the defense of the applied CMC regulatory compliance strategy. In this chapter, the need to respond accurately, thoroughly, and timely to reviewer's requests for more CMC explanation or additional data will be examined. Avoiding a BLA Complete Response Letter (CRL) or a MAA Withdrawal will be discussed. Finally, in this chapter, an encouragement is given to senior management to take advantage of the interaction opportunities available to discuss their CMC strategy with the regulatory authorities.

16.1 CMC Regulatory Compliance Strategy – Teamwork Required

CMC regulatory compliance strategy requires a team effort to be effective. Within the biopharmaceutical manufacturer, teamwork among Development, Manufacturing, Quality Control, Quality Assurance and Regulatory Affairs is critical for CMC success. But teams can become dysfunctional, and drift apart into different priorities. Hopefully, the Project Manager can use the QTPP (discussed in Chap. 4) to keep the CMC team on focus. Teamwork is also necessary between the biopharmaceutical manufacturer and the regulatory authority. This team can also drift apart, by setting a different critical concern level on various activities to be carried out. This is where ongoing communication and discussion of CMC regulatory

strategic issues between the manufacturer and the regulatory authority is essential to try to prevent this drift, either intentionally or unknowingly.

The bottom-line for the biopharmaceutical manufacturer is: 'When do you want to find out that you haves a serious problem with your current CMC regulatory compliance strategy?' Is it when the advancement of the clinical development program is placed on clinical hold? Is it when a submitted market application dossier (BLA or MAA) either is refused-to-file (RTF) or needs to be withdrawn? Sound business practice dictates that knowing of a pending serious problem sooner is preferred to discovering the problem later. Throughout the previous 15 chapters in this book, I have tried to identify the many CMC regulatory compliance strategic issues and challenges that need to be addressed by the biopharmaceutical manufacturer. Earlier discovery of problems with any of those CMC regulatory compliance strategic issues allows the biopharmaceutical manufacturer to either shift their CMC strategy or develop a new CMC strategy that might prevent eventual derailing of clinical development.

"Communication" is more than talking to one another. The definition of communication that I like best is "*a two-way process of reaching mutual understanding, in which participants not only exchange (encode-decode) information, news, ideas and feelings but also create and share meaning*."

Three primary benefits for communicating with the regulatory authorities on CMC regulatory compliance strategic issues are:

1. Facilitates evaluation of the CMC program by the regulatory authority, in advance of a significant submission to be sent to them. This takes the form of pre-submission discussions/meetings (e.g., pre-IND, pre-BLA). This introduction to the CMC program can open the door for further detailed discussion with the regulatory authority reviewer of the manufacturer's overall CMC regulatory compliance strategy for the biopharmaceutical.
2. Identifies strategic CMC issues during the course of clinical development. At times there will be disagreement between a manufacturer and the regulatory authority reviewers on the significance of some CMC issues, but at least those disagreements will be out in the open – knowing that resolution needs to be obtained.
3. Seeks resolution of significant CMC issues that have been identified, in a timely manner, during the course of clinical development; trying to prevent any delay that the regulatory authority might place on the advancing program. Keep in mind that the reviewer probably is aware of how other manufacturers have resolved similar problems; and they have on occasion, shared a direction of resolution for the manufacturer to pursue.

16.2 Clinical Development Milestones – Opportunities for CMC Strategy Discussions

The pathway for regulatory approval for all biopharmaceuticals (recombinant proteins, monoclonal antibodies, viral vectors, genetically modified human patient cells, mRNA non-viral vectors), both within the United States (regulated by the U.S. FDA) and the European Union (regulated by National Competent Authorities (NCAs) during clinical and EMA for market approval), was examined in Chap. 2. The clinical development pathway milestones for regulatory approval are shown in Fig. 16.1.

Each of the clinical development pathway milestones, require submissions to the regulatory authority to be allowed to proceed forward. But, along with each milestone regulatory submission comes an opportunity to discuss CMC regulatory compliance strategy with the regulatory authority. Both FDA and EMA are strong proponents of providing direction to the biopharmaceutical industry, including providing opportunities for meetings.

16.2.1 FDA's Encouragement for Milestone Meetings

FDA's meetings are governed by two separate systems: Prescription Drug User Fee Amendments (PDUFA) and Biosimilar User Fee Amendments (BsUFA). PDUFA applies to all biopharmaceutical types: recombinant proteins, monoclonal antibodies, viral vectors, genetically modified patient cells, mRNA non-viral vectors. BsUFA applies only to the recombinant protein and monoclonal antibody biosimilars.

FDA believes that the clinical development milestone meeting opportunities provide appropriate feedback to manufacturers that can result in greater efficiency of the biopharmaceutical development process. Bottomline: Take advantage of them!

Fig. 16.1 Clinical development pathway milestones for regulatory approval

16.2.1.1 PDUFA Meeting Opportunities

PDUFA sets out three types of meetings with the FDA:

- Type A: Critical Path/Urgent Meeting (when the clinical development program is stopped – Clinical Hold, Refusal to File, Complete Response – and advice is needed to unstick)
- Type B: Advancing Clinical Development (associated with milestones)
- Type C: if not A or B

Type B clinical development milestone meetings include the following:

- Pre-IND Meeting (prior to initiation of the first-in-human clinical study)
- End-of-Phase 2 Meeting (prior to initiation of the pivotal clinical program)
- Pre-BLA Submission Meeting (prior to submission of the marketing application dossier)

The FDA is a strong proponent of holding Type B milestone meetings with manufacturers [1]:

> FDA believes that scientific and regulatory recommendations provided during drug development meetings with sponsors may result in more efficient and robust development programs. This philosophy is articulated in 21 CFR 312.47, 21 CFR 312.82, FDA's meetings guidances CDER's Manuals of Policies and Procedures (MAPPs), and CBER's Standard Operating Policy and Procedures (SOPPs). Sponsors can request meetings with FDA at any time during drug development, and FDA strongly encourages sponsors to request the critical milestone meetings, and BIA or BPD meetings identified in the references cited above.
>
> Meetings are useful in resolving questions and issues raised during the life cycle of drug development. There are important reasons for sponsors to discuss development plans with FDA. FDA can provide valuable scientific and regulatory advice, resulting in more efficient and robust development programs. FDA can also help sponsors understand the evidence that will be necessary to demonstrate effectiveness, safety, highly similar or no clinically meaningful differences, and product quality. It is critical for sponsors to ascertain FDA's views on the applicable statutory and evidentiary requirements well in advance of submission of a marketing application to ensure an efficient development program.
>
> Meetings between FDA and a sponsor at critical junctures in drug development can be especially helpful in minimizing wasteful expenditures of time and resources and thus in speeding the drug development and evaluation process.

FDA has issued a guidance identifying specific CMC strategic topics that would be appropriate for discussion with the manufacturer during the Type B clinical development milestone meetings [2]:

> ***Pre-IND Meeting***
>
> With respect to CMC information, the purpose of pre-IND meetings for phase l/phase 2 is to discuss safety issues related to the proper identification, strength, quality, purity, or potency of the investigational drug, as well as to identify potential clinical hold issues. Meetings at the pre-IND stage regarding CMC information are often unnecessary when the project is straightforward. For certain types of drugs, such as biotechnological drugs, biological drugs, natural products, complex dosage forms, and drug-device combinations, it may be appropriate to discuss the CMC information in more detail. Examples where detailed discussion may be appropriate include, but are not limited to:

Biotechnology drugs, particularly rDNA proteins from cell line sources (e.g., adequacy of characterization of cell banks, potential contamination of cell lines, removal or inactivation of adventitious agents, potential antigenicity of the product)
Novel excipients
Drug-device delivery systems (e.g., demonstration of device and its characteristics, potential for overly rapid release of dose, particle size distribution considerations, where applicable)

End-of-Phase 2 (EOP2) Meeting

The purpose of the EOP2 meeting, with respect to CMC information, is to provide an opportunity for the sponsor and reviewing division to (1) evaluate the results of the drug development program to date; (2) discuss the sponsor's plans and protocols relative to regulations, guidances, and Agency policy; (3) identify safety issues, scientific issues, and/or potential problems and resolve these, if possible, prior to initiation of phase 3 studies; and (4) identify additional information important to support a marketing application. The CMC portion of the EOP2 meeting is a critical interaction between the sponsor and the chemistry review team to ensure that meaningful data will be generated during phase 3 studies. The goal is to identify potential impediments to further progress at an early stage, thus reducing the number of review cycles for the proposed marketing application. Although the EOP2 meeting is important for all drugs, it is particularly important for new molecular entities, biotechnology drugs, biological drugs, natural products, complex dosage forms, and/or drug-device delivery systems. CMC issues that can be addressed in EOP2 meetings for rDNA protein biotechnology drugs include, but are not limited to:

Adequacy of physicochemical and biological characterization (e.g., peptide map, amino acid sequence, disulfide linkages, higher order structure, glycosylation sites and structures, other post-translational modifications, and plans for completion, if still incomplete)
Bioassay (e.g., appropriateness of method, specificity, precision)
Adequacy of cell bank characterization (e.g., update from phase l/phase 2, plans for completion, if still incomplete)
Removal of product- and process-related impurities (e.g., misfolded proteins, aggregates, host cell proteins, nucleic acid)
Bioactivity of product-related substances and product-related impurities relative to desired product

Pre-BLA Submission Meeting

The CMC portion of the pre-NDA or pre-BLA meeting is a critical interaction between the CMC review team and the sponsor to ensure the submission of a well-organized and complete NDA or BLA. The pre-NDA or pre-BLA meeting should focus on addressing the specific questions related to filing and format issues. Typically the meeting also includes a discussion to identify problems that can cause a refuse-to-file recommendation or hinder the review process. Examples of CMC issues that could be addressed in pre-NDA or pre-BLA meetings include, but are not limited to:

Discussion of the format of the proposed NDA or BLA submission, including whether an electronic submission will be provided
Confirmation that all outstanding issues discussed at the EOP2 meeting or raised subsequently will be adequately addressed in the proposed NDA or BLA
Assurance that all activities in support of the proposed NDA or BLA have been coordinated, including the ml1 and timely cooperation of DMF holders or other contractors and suppliers
Discussion of the relationship between the manufacturing, formulation, and packaging of the drug product used in the phase 3 studies and the final drug product

> intended for marketing, and assurance that any comparability or bridging studies agreed upon at the EOP2 meeting have been appropriately completed
> Assurance that the submission will contain adequate stability data in accordance with stability protocols agreed upon at the EOP2 meeting
> Confirmation that all facilities (e.g., manufacturing, testing, packaging) will be ready for inspection by the time of the NDA or BLA submission
> Identification of any other issues, potential problems, or regulatory issues that should be brought to the attention of the Agency or sponsor

There are two important points to notice in this CMC-focus meeting guidance. First, FDA's extra importance placed on the EOP2 CMC strategic meeting. If CMC regulatory compliance strategic issues are surfaced during this meeting, there is potentially adequate time for the manufacturer to correct the problem to avoid a delay in the future filing of the BLA. Second, FDA places strong emphasis on whose responsibility it is to bring up CMC regulatory strategy concerns – it is the biopharmaceutical manufacturer's responsibility, not the FDA's.

In these PDUFA clinical development milestone meetings, the FDA holds two types: (1) a multi-disciplined meeting (where multiple scientific disciplines are represented – Clinical is usually present, with others such as CMC, Toxicology, etc.), and (2) a discipline-focused meeting (if CMC-focused, where only CMC subject matter experts would be present). The FDA encourages discipline-focused meetings for CMC strategy discussions over multi-disciplined meetings (a recognition that if Clinical is present, the clinical questions get priority over the CMC questions). CMC-focused meetings provide adequate discussion time for the CMC regulatory compliance strategy issues and allows for an adequate number of CMC subject matter experts from the FDA review teams to participate.

CDER's medical application Offices review all therapeutic recombinant proteins and monoclonal antibodies. They are assisted by the CDER Office of Biotechnology Products (OBP).

CBER's Office of Gene Therapy CMC (within the Office of Therapeutic Products (OTP)) reviews all gene therapy-based biopharmaceuticals (viral vectors, genetically modified patient cells).

16.2.1.2 BsUFA Meeting Opportunities

BsUFA sets out five types of meeting with the FDA for biosimilars:

- BIA Meeting (advice meeting prior to major commitment into biosimilar studies)
- BPD Type 1 Meeting (Urgent, same as under PDUFA Type A)
- BPD Type 2 Meetings (specific questions during the course of studies)
- BPD Type 3 Meetings (advice/review on a completed comparability section)
- BPD Type 4 Meeting (BLA pre-submission meeting, same as under PDUFA)

FDA has issued a guidance identifying specific CMC strategic topics that would be appropriate for discussion with the manufacturer during the three BsUFA meetings associated with clinical development milestones [3]:

Biosimilar Initial Advisory (BIA) Meeting

A Biosimilar Initial Advisory meeting is an initial assessment limited to a general discussion regarding whether licensure under section 351(k) of the PHS Act may be feasible for a particular product, and, if so, general advice on the expected content of the development program. This meeting type does not include any meeting that involves substantive review of summary data or full study reports. However, preliminary comparative analytical similarity data from at least one lot of the proposed biosimilar biological product compared to the U.S.-licensed reference product should be provided in the meeting package. The analytical similarity data should be sufficient to enable the FDA to make a preliminary determination as to whether licensure under section 351(k) of the PHS Act may be feasible for a particular product, and to provide meaningful advice. A general overview of the development program, including synopses of results and findings from all completed studies and information about planned studies, also should be provided.

Biosimilar Biological Product Development (BPD) Type 3 Meeting

Examples of a BPD Type 3 meeting submission include: Comprehensive analytical similarity data that permit the FDA to make a preliminary evaluation of analytical similarity during development. The level of analytical data provided should be similar to what the sponsor or applicant intends to submit in a 351(k) BLA (e.g., full study reports and/or datasets that support the full study reports).

Biosimilar Biological Product Development (BPD) Type 4 Meeting

A BPD Type 4 meeting is a meeting to discuss the format and content of a biosimilar biological product application or supplement to be submitted under section 351(k) of the PHS Act.

CDER's medical application Offices review all biosimilars. They are assisted by the CDER Office of Therapeutic Biologics and Biosimilars (OTBB).

16.2.2 EMA's Encouragement for Scientific Meetings

Unlike the FDA which reviews and approves both clinical development and market approval activities, the National Competent Authorities (NCAs) in each of the countries within the European Union reviews and approves the clinical development activities, while the EMA reviews and approves the market approval and post-market approval activities. But the EMA provides meeting opportunities both during clinical development and post-market approval. While input from the NCAs is sought during the clinical development stages, input via scientific advice is also sought concurrently from EMA. Since the final market approval decision comes from EMA, it is most important to seek their advice sooner than later.

EMA's meetings are referred to as 'scientific advice' meetings between the Scientific Advice Working Party (SAWP) and the manufacturer. EMA strongly encourages scientific advice meetings with manufacturers [4]:

> Scientific advice is one of the Agency's key instruments for supporting the development of high-quality, effective and safe medicines, for the benefit of patients. Early dialogue and scientific advice lead to better development plans, promote the collection of high-quality data and, most importantly, help to ensure that patients only take part in those clinical trials that are likely to be robust enough to generate data that are relevant to

support the evaluation of a marketing authorization application or extension of indication.

2021 Summary

71% of applicants who were granted a positive opinion for their medicine had received scientific advice or protocol assistance from EMA during their product's development phase. This early engagement with developers allows EMA to clarify what kind of evidence is required to later evaluate a medicine for authorisation. This encourages generation of more robust data for regulatory assessment, and thus protects patients from taking part in unnecessary or poorly designed clinical trials.

As in previous years, 81% of the requests for scientific advice included questions related to clinical issues, 39% to preclinical issues and 37% to quality issues. In terms of development stage, 63% of requests related to medicines in phase III, 23% to medicines in phase II, 12% to medicines in phase I and 2% to medicines in phase IV of their clinical development.

EMA has not issued a guidance document, like the FDA, identifying potential CMC strategic topics that would be appropriate for discussion between the SAWP and the manufacturer. In fact, the scope of SAWP discussion topics related to CMC issues is simply stated as: *"Scientific advice will be given by the SAWP/CHMP on questions concerning quality (manufacturing, chemical, pharmaceutical and biological testing)…"*. Instead EMA uses another pathway to inform manufacturers: European Public Assessment Reports (EPARs) [5]:

During the development and assessment phases, the detailed advice given to a medicine developer is not made public. This is because disclosing information at this stage may undermine research and development efforts and discourage research in new medicines. However, information is made available after a medicine obtains marketing authorisations. All medicines whose assessment report was finalized since 2019 include a summary of the developer's questions and key elements of EMA's advice and whether or not the developer complied with this advice with the assessment report.

The following two case examples from market-approved biopharmaceutical EPARs illustrate some CMC regulatory compliance strategic issues that were discussed under EMA scientific advice:

Antibody Drug Conjugate (ADC) Blenrap (Belantamab Mafodotin) [6]

- Cell line control strategy.
- Demonstration of analytical comparability and, provided comparability can be demonstrated at quality level, that no additional non-clinical or clinical tests will be needed to qualify the proposed process changes, comparability acceptance criteria, list of potentially critical quality attributes.
- Acceptability of the validation approach for mAb, ADC DS and DP as well as stability.
- Proposed activity and potency assays and their categorization as release and stability or characterization assays for commercialisation.
- API starting materials, agreement that the drug-linker component of the product is a non-biologically derived drug substance. Advice was also sought regarding the tests and acceptance criteria to control the quality of the linker component.

CAR T Cell Genetically Modified Patient Cells, Abecma (Idecabtagene Vicleucel) [7]

- Proposed release and stability specifications, including the proposed tests for potency, sterility, mycoplasma and replication-competent lentivirus, and the comparability strategy to support manufacturing changes during development;

- The new commercial and clinical facility that is being constructed for bb2121; the process qualification of process validation for bb2121 drug product; the viral risk management strategy for human AB serum raw material;
- The strategy to demonstrate comparability between bb2121 pivotal trial lots and commercial bb2121 lots for MAA (analytical comparability assessment for the lentiviral vector and for the bb2121 drug product); the adequacy of the functional potency assay (IFNγ) to support release of the commercial LVV; the need to test for replication competent lentivirus (RCL) at bb2121 commercial drug product release.

EMA scientific advice is open to all types of biopharmaceuticals – recombinant proteins, monoclonal antibodies, viral vectors, and genetically modified patient cells. When a gene therapy-based biopharmaceutical (e.g., viral vector, genetically modified patient cells) is under discussion, the SAWP utilizes the expertise of the Committee for Advanced Therapies (CAT).

Complying with scientific advice does not guarantee market approval, but it does increase the chances. Bottomline: Take advantage of the scientific advice meetings!

16.3 Expedited Clinical Development – Opportunities for Additional Discussions

'Expedited clinical pathways' are a tremendous advantage for the clinical development program. These 'clinical speedways' allow for move rapid clinical development programs, such as clinical hybrids (e.g., instead of Phase 2 followed by Phase 3, expediting can allow a seamless Phase 2/3) and even the possibility of completing the pivotal clinical program after market approval (rather than prior to filing of the BLA/MAA).

FDA has three major expedited clinical programs that are open for accelerating the clinical development of biopharmaceuticals:

- Accelerated Approval (AA) – open to all biopharmaceuticals, but not biosimilars
- Breakthrough Therapy Designation (BTD) – open to all biopharmaceuticals, but not biosimilars
- Regenerative Medicine Advanced Therapy (RMAT) – open only to gene therapy biopharmaceuticals

EMA also has three major expedited clinical programs that are open for accelerating clinical development of all biopharmaceuticals, but not biosimilars:

- Conditional Marketing Authorisation (CMA)
- Exceptional Circumstances (EC)
- Priority Medicine (PRIority MEdicine, PRIME)

In addition, both FDA and EMA have accelerated review procedures that are initiated upon submission of the BLA/MAA – Priority Review for FDA and Accelerated Assessment for EMA. But these accelerated reviews only shorten the regulatory

review time of a BLA/MAA, they do not shorten the clinical development time to get the BLA/MAA ready for submission.

Expediting clinical development is exciting for the Clinical team to have the biopharmaceutical move faster through the clinical program. But expediting clinical development has a mixed review from the CMC team. On the one hand, shortening the clinical development time period, can create a nightmare for those managing the CMC regulatory compliance strategy due to the loss of significant time to complete some of the planned more time-intensive activities (e.g., site transfers, completion of shelf-life studies, validations, etc.). But on the other hand, receiving an expedited clinical pathway designation, is a blessing to the CMC team because it opens up the door for more intensive interaction with the regulatory authority – including CMC regulatory compliance strategy discussions:

FDA Expedited Programs [8]

Manufacturing and Product Quality Considerations

The sponsor of a product that receives an expedited drug development designation may need to pursue a more rapid manufacturing development program to accommodate the accelerated pace of the clinical program. The sponsor's product quality and CMC teams should initiate early communication with FDA to ensure that the manufacturing development programs and timing of submissions meet the Agency's expectations for licensure or marketing approval. When sponsors receive an expedited drug development designation, they should be prepared to propose a commercial manufacturing program that will ensure availability of quality product at the time of approval. The proposal should consider estimated market demand and the commercial manufacturing development plan. The proposal should also consider manufacturing facilities and a lifecycle approach to process validation. Additionally, the proposal should include a timeline for development of the manufacturing capabilities with goals aligned with the clinical development program. After the initial discussion following designation, frequent communication during development will generally facilitate meeting manufacturing development goals and product quality goals.

Sponsors of such products should allow for an earlier submission of the CMC section (including product quality information) for timely review, and, critically, for inspection activities. Coordination with the sponsor and contract manufacturers may be necessary to ensure that manufacturing facilities and equipment are ready for inspection during review of the clinical section of the application. A comprehensive meeting with FDA's product quality review groups in advance of submission may facilitate the quality assessment of products designated for expedited programs. Although sponsors must ensure the availability of quality product at the time of approval, FDA may exercise some flexibility on the type and extent of manufacturing information that is expected at the time of submission and approval for certain components (e.g., stability updates, validation strategies, inspection planning, manufacturing scale-up). The level of flexibility will be determined on a case-by-case basis after consideration of factors such as the following: (1) product characteristics, (2) seriousness of the condition and medical need, (3) manufacturing processes, (4) the robustness of the sponsor's quality system, and (5) the strength of the sponsor's risk-based quality assessment. FDA's consideration of the sponsor's proposal for an integrated postmarketing plan will also take into account whether elements of the plan may be appropriately executed as a postmarketing commitment or requirement. For example, FDA will consider impacts on clinical performance, such as safety and immunogenicity. Sponsors should meet with the Agency to discuss their proposed plan as soon as possible and no later than the pre-NDA or pre-BLA meeting.

EMA PRIME [9]

The Priority Medicines (PRIME) scheme was launched to enhance EMA support to the development of medicines that target an unmet medical need with the aim to help patients to benefit from these therapies as early as possible. This is achieved by optimising the medicines' development plans and speeding up their evaluation.

Once a candidate medicine has been selected for PRIME, EMA will:

- appoint a rapporteur from the Committee for Medicinal Products for Human Use (CHMP) or from the Committee on Advanced Therapies (CAT) in the case of an advanced therapy to provide continuous support and help to build knowledge ahead of a marketing-authorisation application;
- assign a dedicated contact point from EMA and a dedicated EMA Quality specialist. Other team support will be involved as needed (e.g. Inspections Office);
- organise a kick-off meeting with the CHMP/CAT rapporteur and a multidisciplinary group of experts, so that they provide guidance on the overall development plan and regulatory strategy;
- provide scientific advice at key development milestones, involving additional stakeholders such as health-technology-assessment bodies, to facilitate quicker access for patients to the new medicine

Experience to date has shown that applicants face challenges to complete quality and manufacturing development data requirements during development of products in early access approaches. In order to address and overcome these challenges, EMA wishes to support applicants with guidance regarding their pharmaceutical development programme and flexibility on the provision and type of data packages in the context of a MAA taking into consideration the overall benefit-risk of the product. Specific guidance covers prior knowledge, risk assessment, process validation, specification setting, GMP compliance, stability testing, and comparability, as well as early identification of quality issues/attributes that are critical to the clinical use of the medicinal product. This toolbox guidance summarises scientific and regulatory approaches which can be considered and applied by applicants, tailored to their product development in question, to facilitate the development and preparation of robust quality data packages. A well-prepared and robust Module 3 will support timely access to the medicine for patients whilst providing assurance that product quality and efficacy and patient safety are not compromised. Similarly, applicants should ensure that manufacturers are compliant with EU GMP and are inspection ready at the time of submission. The scientific and regulatory approaches described in this document can offer flexibility in terms of the time point for full completion of certain quality data packages when there is an unmet medical need and should always be considered in the context of the specific benefit-risk of the product.

A case example of additional CMC regulatory compliance strategy discussions with a regulatory authority due to clinical expediting is found in the case example of the market-approved genetically modified patient cells biopharmaceutical, Carvykyi (ciltacabtagene autoleucel). According to the EPAR, the following occurred immediately after receiving the EMA's designation of PRIME [10]:

Carvykti was granted eligibility to PRIME on 28 March 2019. Upon granting of eligibility to PRIME, Jan Mueller-Berghaus was appointed by the CHMP as rapporteur. A kick-off meeting was held on 19 June 2019. The objective of the meeting was to discuss the development programme and regulatory strategy for the product. The applicant was recommended to address the following key issues through relevant regulatory procedures:

CMC: The EMA recommended that the lentiviral comparability plan should be included in a Scientific Advice request. EMA also recommended that the characterisation testing for both lentiviral vector and drug product should be included in a Scientific Advice request

to ensure appropriate release specifications. Furthermore, a Scientific Advice request should be considered in order to clarify the exemption from batch release testing in the EU due to e,g., limited amount of material available or the short shelf life (GMP for ATMPs 11.27) of JNJ-68284528.

Scientific Advice (CMC)

- the definition of the plasmids used for lentiviral vector manufacturing, the lentiviral vector, the apheresis material, the CAR T cell pellet as well as on the final cryopreserved medicinal product; drug substance and drug product specification review strategy;
- adherence of the apheresis material to Directive 2002/98/EC and Directive 2004/23/EC;
- comparability study for the clinical manufacturing process to be used in the clinical trials and to be the commercial process;
- the approach to demonstrate analytical comparability between the manufacturing process and the commercial process.
- process performance qualification of the lentiviral vector (LV) and drug product manufacturing process as well as the accompanying analytical testing strategies and specification setting plan

Both FDA and EMA recognize the pressures on the CMC regulatory compliance strategy due to the shortened time period of the expedited clinical development programs. They believe the increased opportunities for discussions and meetings can achieve the balance of moving faster yet meeting the necessary manufacturing and quality requirements of obtaining market approval. Bottomline: Take advantage of the increased opportunities for CMC strategy interaction if clinical expediting is granted!

16.4 Securing a CMC Strategy-Focused Meeting

If you don't ask, you won't get one! But the manufacturer must ask according to the procedures established by the regulatory authorities. And the manufacturer must convince the regulatory authority that there is potential utility of a meeting, rather than a written response only (WRO). The regulatory authorities are just as busy as we are, so they have limited time to use for the meetings [1]:

> FDA's decision to grant or deny meeting requests is resource-dependent and is based on the maturity of the drug's development at the time of the meeting request, taking into consideration the potential utility of the meeting. The procedures for requesting and conducting effective meetings between sponsors and FDA are fully described in the meetings guidances.

As questions are formulated, remember each party's role. The manufacturer is the 'initiator' of the questions. The manufacturer is the CMC expert for their biopharmaceutical, having developed and operated the manufacturing process, and characterized the biomolecule. The regulatory reviewing body is the 'responder' to the questions raised by the manufacturer. The response to a question can only be as valid as the clarity of the specific question being raised.

16.4.1 PDUFA Meetings with FDA

The FDA clearly spells out the three-step process of trying to secure a Type A, B or C meeting with the FDA: (1) request the meeting, (2) wait to see if the meeting request is accepted, (3) if accepted, provide the required briefing package in the required time period before the meeting [11].

16.4.1.1 Request the Meeting

The meeting request letter is the introduction of the manufacturer's need for the meeting:

> When a meeting is needed, a written request must be submitted to the FDA via the respective center's document room (paper submissions) or via the electronic gateway, as appropriate. Requests should be addressed to the appropriate review division or office and, if previously assigned, submitted to the application (e.g., investigational new drug application (IND), new drug application (NDA), biologics license application (BLA)). Meeting requests sent by fax or email are considered courtesy copies only and are not a substitute for a formal submission. The meeting request should include adequate information for the FDA to assess the potential utility of the meeting and to identify FDA staff necessary to discuss proposed agenda items.

The guidance document is very clear on what elements are to be included in the request letter, and there are really no surprises of what they ask for: (1) product name and proposed regulatory pathway (BLA since this is for a biopharmaceutical), (2) meeting type being requested (Type A, Type B, or Type C), (3) suggested dates and times for the meeting that are consistent with the appropriate scheduling time frame for the meeting type being requested, (4) proposed meeting format (face-to-face, teleconference, written response only), (5) a brief statement of the purpose of the meeting, (6) etc.

But what determines the FDA's reception to the meeting request letter is the list of questions:

> The objectives and agenda provide overall context for the meeting topics, but it is the list of questions that is most critical to understanding the kind of information or input needed by the requester and to focus the discussion should the meeting be granted. Each question should be precise and include a brief explanation of the context and purpose of the question. The questions submitted within a single meeting request should be limited to those that can be reasonably answered within the allotted meeting time, taking into consideration the complexity of the questions submitted. Similar considerations regarding the complexity of questions submitted within a WRO should be applied.

When you are writing down the list of questions, ask yourself, if I was the regulatory reviewer, 'is this a meeting that I would want to attend, that I feel I could make a contribution'. Everyone is busy and resourced constrained, not only in the biopharmaceutical company but also at the regulatory authorities. With over 4500 meeting requests being sent into the FDA each year, why would they choose your

meeting versus just sending back a written response only (WRO). Hopefully, this puts in perspective the importance of the list of questions.

16.4.1.2 Timely Prepare the Meeting Package

If the FDA accepts the meeting request, then the manufacturer must meet the required time period for when the meeting package must be submitted. Figure 16.2 illustrates the tight timelines that are necessary to meet. The narrow window of time requires the Type A meeting packages to be submitted along with the meeting request letter. The narrow window of time also puts a lot of pressure on the CMC team for the Type B meeting package (to be submitted within 2 weeks of received confirmation of FDA meeting). Unfortunately what happens, the meeting package is rushed and ends up not being clear or thorough in the message that is trying to be delivered.

Manufacturers need to understand that the meeting package is their 'voice' at the FDA, when prior to the scheduled meeting, the FDA internal team reviews the meeting package and provides a written response to every question, as shown in Fig. 16.2. The only representative from the manufacturer at that internal FDA team meeting is the meeting package. The clarity and content of the meeting package explains why manufacturers sometimes get a FDA written response such as 'not enough data was provided to give a response' or get a written response that was not the intent of what the manufacturer thought was the basis for the question.

Even though the manufacturer will receive a copy of the written FDA team response prior to the scheduled meeting, the damage is done and there will not be enough time to correct an FDA misunderstanding during the fixed, limited time allowed for the meeting.

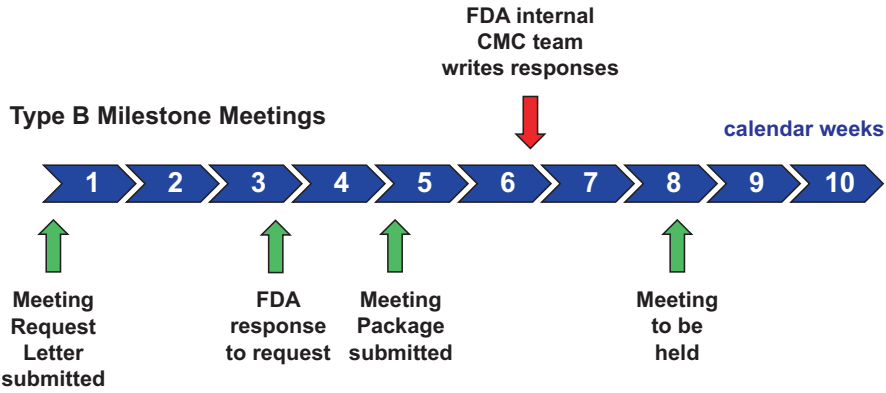

Fig. 16.2 Timeline commitments for PDUFA (Type B) meetings with the FDA

Do it right upfront – take time to ensure that the questions being asked the FDA to address are clear. The response to the question may not be what you wanted to hear from the FDA, but it will be the correct response from the FDA. A case example of hearing from the FDA in a milestone meeting is illustrated with Bluebird Bio's genetically modified patient cell biopharmaceutical for treating sickle cell disease [12]:

> *SICKLE CELL DISEASE (November 4, 2020)*
> *BIOLOGICS LICENSE APPLICATION (BLA) SUBMISSION* – Today, bluebird bio announces confirmation of its general agreement with the U.S. Food and Drug Administration (FDA) that the clinical data package required to support a BLA submission for LentiGlobin™ for sickle cell disease (bb1111) will be based on data from a portion of patients in the HGB-206 study Group C that have already been treated. bluebird bio is also announcing today that it has reached general agreement with FDA on its path to transition to commercial manufacturing using an analytical comparability strategy, including suspension-based lentiviral vector (sLVV). These developments meaningfully de-risk the bb1111 program and bring clarity on the path to approval. However, FDA requested the use of drug product manufactured from sickle cell disease (SCD) patient cells in addition to healthy donors as well as commercial lentiviral vector to demonstrate drug product comparability. Given this feedback, alongside COVID-19 related shifts and contract manufacturing organization COVID-19 impacts, bluebird is adjusting its submission timing to late 2022. The company looks forward to continuing to work with the Agency to find an innovative approach to reviewing the CMC portion of a BLA submission and address the high unmet need in sickle cell disease.

The manufacturer was not pleased that the BLA would be delayed an extra year due to the required comparability study required by the FDA – they had changed the manufacturing of the recombinant lentivirus (LV) starting material. But they stated clearly the value of the FDA's advice – "*These developments meaningfully de-risk the bb1111 program and bring clarity on the path to approval*."

16.4.2 BsUFA Meetings with FDA

The same principles mentioned above with the PDUFA meetings (e.g., request letter, meeting package) apply for BsUFA meetings [3]. The primary difference is the meeting scheduling time, which are more extended for the BsUFA meetings, see Table 16.1.

Note, the differences in timing for scheduling these meetings, and especially notice that the meeting package for all biosimilar meetings must be submitted with the request letter to the FDA.

Table 16.1 Timeline commitments for all BsUFA meetings with the FDA.

BsUFA Calendar Days				
Biosimilar Meeting Type	**Meeting Request Letter submitted**	**FDA response to request**	**Meeting Package submitted**	**Meeting to be held**
BIA	0	21	Must be submitted with the meeting request letter	75
BPD Type 1	0	14		30
BPD Type 2	0	21		90
BPD Type 3	0	21		120
BPD Type 4	0	21		60

16.4.3 Scientific Advice Meetings with EMA

For the one-size fits all EMA meetings, the scientific advice process follows the same eight-step pattern for all scientific advice discussions [13], as shown in Fig. 16.3.

As with any regulatory authority meeting, there is an established timing and set procedure for the meeting. For scientific advice, the first step is the submission of the letter of intent (LoI) and the briefing document. The briefing document has three parts: (1) summary, (2) list of questions and manufacturer's position, and (3) background information. EMA provides templates for these on its website. The closing timing is 40 days for the written response (70 days if the manufacturer had to make a presentation to the SAWG). EMA makes it clear the type of information they want to be sent to them, especially about the importance of the listed questions and the clarity of the support in the manufacturer's position [14]:

> The briefing document is the core of the SA request and consists of three main parts: I. summary, II. question(s) and applicant's position(s), and III background information on the product. The summary (part I), which should typically not be longer than three pages, contains background information on the disease to be treated and a brief description of the product including quality, non-clinical and clinical development, its regulatory status, and an explanation of the rationale for seeking SA. The questions (part II) are grouped according to the area of expertise and numbered sequentially. Questions should be phrased carefully, clearly, and unambiguously to obtain a clear and precise answer, and their scope neither too broad nor too narrow to obtain meaningful advice. questions are phrased starting with "Does the CHMP agree that/with" followed by the applicant's proposal, which is detailed and justified in the applicant's position following each question. The applicant's position includes a comprehensive justification of the chosen approach, including the context and consideration of alternative options, with a critical discussion of the relative advantages and disadvantages of each approach. With a recommended length of 1–3 pages, each applicant's position should contain sufficient detail to serve as a "stand-alone" argument,

Fig. 16.3 Eight (8) steps for EMA scientific advice

supported by cross-references to relevant parts of the briefing document or annexes supporting the argument, as needed. The background information (part III) provides a comprehensive overview of the medicine's development programme and presents detailed information on quality, non-clinical, and clinical aspects; though consideration should be given to the content and level of detail to keep the overall size of the briefing document reasonable.

As both EMA and FDA state clearly: "*Questions should be phrased carefully, clearly, and unambiguously to obtain a clear and precise answer, and their scope neither too broad nor too narrow to obtain meaningful advice.*" You only get what you ask for.

16.5 Defending the CMC Strategy During the BLA/MAA Review

The pre-submission meetings have now been held. For FDA, a pre-BLA submission meeting, Type B, is held typically about 6 months prior to the anticipated filing date. For EMA, a targeted MAA pre-submission meeting is required, typically 7 months before the anticipated filing date. That meeting is to evaluate the biopharmaceutical for eligibility under the centralized review procedure, and for assignment of the appropriate rapporteur and co-rapporteur. For the gene therapy-based biopharmaceuticals, members from the Committee for Advanced Therapy will be selected as rapporteurs.

After these pre-submission meetings, everything is now ready for the filing of the market application submission. But is it really? Depends upon the readiness of the market application dossiers, especially Module 3 for CMC. Unfortunately, there is an all too often difference in perspective of the readiness of these submissions, as illustrated in Fig. 16.4. This can help explain the detours and potholes that manufacturers encounter on the path to final market approval.

16.5.1 FDA BLA Review Process

FDA provides a diagram on their website of the BLA review process [15], see Fig. 16.5. This diagram includes standard and priority review timelines. This diagram references PDUFA timelines, but not BsUFA timelines. This diagram includes many of the Clinical review milestones as well as the CMC review milestones.

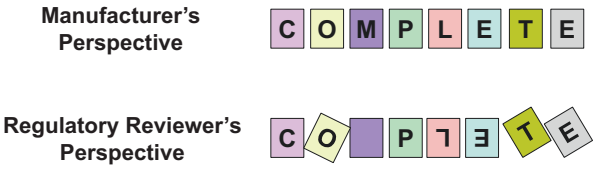

Fig. 16.4 Different perspectives on readiness of BLA/MAA filing

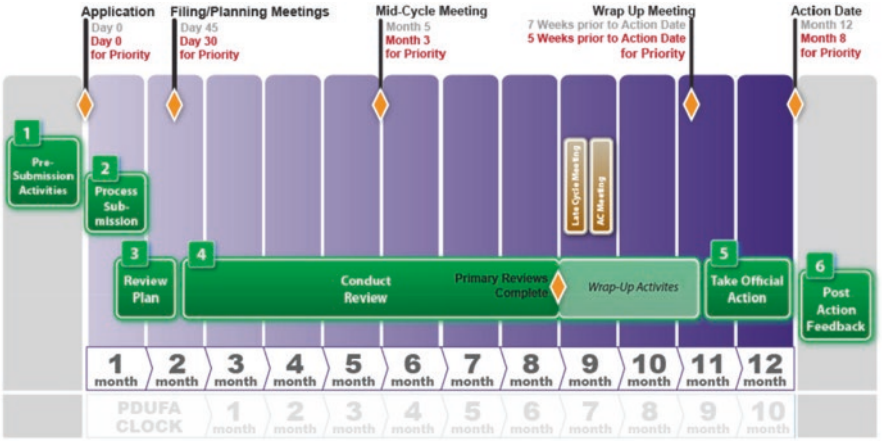

Fig. 16.5 BLA review process – Clinical and CMC milestones

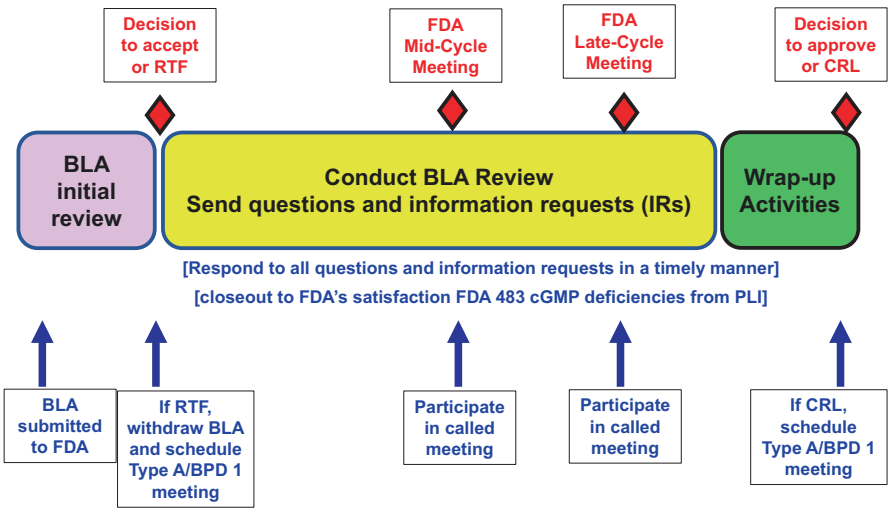

Fig. 16.6 BLA review process – CMC milestones only

To focus specifically on only CMC review milestones of the BLA, a more generalized diagram, Fig. 16.6, is provided. This diagram will be used to examine in more detail, the CMC review milestones on the pathway to market approval.

Upon receipt of the BLA, the regulatory authority review team has three general CMC milestones: (1) complete the BLA initial review, (2) complete the detailed BLA review to understand the content provided that supports market approval, and (3) wrap-up activities and make a decision on the approvability of the BLA.

16.5.1.1 BLA Initial Review

The BLA is received and processed by FDA's document control room staff and then distributed to the appropriate review division. The Regulatory Project Manager (RPM) conducts an initial assessment of the BLA to assure that certain regulatory requirements are met and that a user fee has either been paid, the fee waived, or the application exempted.

The FDA review team is selected and assignments are made at this time. The size of the CMC review team varies, but there could be more than ten CMC team members (especially for the gene therapy-based biopharmaceuticals – viral vectors, genetically modified patient cells).

The goal of the CMC review team at this first stage is to carry out an initial (but not detailed) assessment of the BLA to determine if it is 'fileable'. Not fileable means:

- Omission of a required section of the BLA
- Presentation of a section in so haphazard a manner as to render it incomplete on its face
- Inadequate content, presentation, or organization within the required technical sections and integrated summaries that would render a section incomplete on its face such as illegibility; data tabulations (line listings) or graphical displays that are not interpretable

By day 45 (day 30 if priority review was granted) the CMC team makes a recommendation to the broader BLA review team (including clinical and non-clinical):

> If fileable, internal discussions on the review timelines, and the timeline for the pre-license inspections are initiated, and a 'filing letter' is issued to the manufacturer.
>
> If non-fileable, all BLA review is stopped, and a 'Refusal to File' (RTF) letter is issued to the manufacturer.

Unfortunately, RTF letters are received for CMC deficiencies. When that happens the BLA submission is withdrawn and the manufacturer typically issues a press release spinning the significant 'road block' to market approval. Two case examples follow:

> ***Rolontis (Fc Fusion Protein), Spectrum Pharmaceuticals Press Release*** [16]
>
> Mar. 15, 2019 – Spectrum Pharmaceuticals, Inc. (NASDAQ-GS: SPPI) announced today that due to the U.S. Food and Drug Administration's (FDA) request for additional manufacturing-related information for ROLONTIS, the company has voluntarily withdrawn its Biologics License Application (BLA). Spectrum plans to resubmit a revised BLA as soon as possible. The FDA did not cite concerns related to the pre-clinical and clinical modules of the BLA or the need for additional clinical studies. Spectrum's decision to withdraw the BLA was the result of the company needing more time to provide certain additional manufacturing-related information, which was required before March 29, 2019, the day that the FDA's initial 60-day review period ends.
>
> ***Abecma (Genetically Modified Patient Cells), Bristol Myers Squibb, Bluebird Bio Press Release*** [17]
>
> 05/13/2020 – Bristol Myers Squibb (NYSE: BMY) and bluebird bio, Inc (Nasdaq: BLUE) today announced that the companies received a Refusal to File letter from the U.S. Food

and Drug Administration (FDA) regarding the Biologics License Application (BLA) for idecabtagene vicleucel (ide-cel; bb2121) for patients with heavily pre-treated relapsed and refractory multiple myeloma, which was submitted in March 2020. Upon preliminary review, the FDA determined that the Chemistry, Manufacturing and Control (CMC) module of the BLA requires further detail to complete the review. No additional clinical or non-clinical data have been requested or are required. Bristol Myers Squibb is planning to resubmit the BLA no later than the end of July 2020.

16.5.1.2 Conduct (and Complete) BLA Review

After the two-month BLA initial review, the official review timeclock is turned on, and it varies with the type of clinical expediting that was allowed and the type of biopharmaceutical under review:

- 10-month timeclock: biosimilars of recombinants and monoclonal antibodies
- 10-month timeclock: recombinant proteins, monoclonal antibodies viral vectors, genetically modified patient cells
- 6-month timeclock: 10 months reduced to 6 months if granted priority review
- 4-month timeclock: if granted breakthrough designation

Part of the total allocated timeclock period (about 1.5 weeks) is needed at the end for the Wrap-up Activities. So overall, there is not a lot of time for the complete review of the BLA CMC Module 3. This explains why it is most urgent that the manufacturer timely responds to the reviewer's questions, issues or clarifications as they arise.

For CMC, the following activities and milestones will be completed during the Conduct BLA Review period:

- FDA completing any required pre-license inspection(s) (PLIs), and the manufacturer responding back to FDA's satisfaction of any FDA 483 items that were issued
- Closeout of information requests (IRs) and any other information requested by the reviewers
- Advisory Committee meeting (only if necessary; primarily involves Clinical issues)
- Mid-Cycle Meeting to inform manufacturer of significant issues identified by FDA team
- Late-Cycle Meeting, with manufacturer invited, to discuss resolution of remaining issues

The FDA is responsible for convening the Mid-Cycle Meeting and the Late-Cycle Meeting. The primary purposes for these two FDA-called meetings is to keep the manufacturer's senior management current on any significant issues of concern to the FDA reviewers. The intent is that with these concerns made visible, senior management can be more proactive in supporting efforts to reach timely resolutions that will prevent delay in market approval. The Mid-Cycle Meeting is to inform the manufacturer of the review status and any significant issues that have been identified. For the Late-Cycle Meeting, the manufacturers is invited to attend.

When FDA publishes the content of these meetings (after a biopharmaceutical is market-approved), one can get a feel for the type of CMC issues that, if not timely corrected, could have negatively impact market approval. A case example of a Mid-Cycle Meeting and a Late-Cycle Meeting discussion, for market-approved viral vectors, follows:

Hemgenix (Viral Vector), Mid-Cycle Meeting with FDA [18]

Extractables and Leachables

FDA stated that the applicant's evaluation for E&L is inadequate and potential presence of toxic E&L-related impurities in the drug product (DP) is a safety concern. The applicant is primarily focusing on the drug product container closure system for the assessment of E&Ls, and FDA wants the applicant to broaden their assessment to storage container and manufacturing contact materials as well. FDA also stated that the applicant should perform/ complete formal E&L studies to detect/identify the compounds and their concentrations, and the theoretical risk assessment alone is not sufficient.

Stability

FDA clarified that the primary concern is the stability data to support the shelf-life of DP does not represent the worst-case scenario. FDA's expectation is the DP batches that are derived from aged DS batches should be used to support the shelf-life of the DP. The data provided in the BLA review utilizes relatively fresh DS batches to derive the DP. The applicant asked for further clarification regarding the allowable DS and DP shelf-life. FDA stated that in the absence of supporting cumulative stability data, the maximal allowable DS shelf-life will be based on the duration for which the DS was stored prior to formulating into DP batches used to determine DP shelf-life. The DP shelf-life will be determined based on real-time stability data submitted/to be submitted in the BLA.

Adventitious Viral Safety

Additional information regarding the performance and validation of the in vitro assay for adventitious viral agents (AVA) is needed to assess the safety risk from AVA.

Zolgensma (Viral Vector), Late-Cycle Meeting with FDA [19]

Stability

Only a few months of stability information have been submitted for the DS and for the DP commercial presentation. We acknowledge your plans to submit additional stability data by March 31, 2019. We may decide to approve a shorter shelf life than the that you have requested. **Update: FDA received the stability data on March 29, 2019. FDA has significant concerns that the stability data do not support the proposed shelf life for DS and DP.

Total Protein Assay

Total protein: In IR #23 (January 7, 2019) and our mid-cycle communication, we listed multiple concerns with the DP total protein concentration that have not been resolved. You informed us in submission number 40 (February 25, 2019) that the total protein assay is currently under investigation. Discussion: The applicant notified FDA that they are working on providing the requested information, but they are relying on a third-party contractor and unfortunately may not meet the April 10th deadline. The applicant indicated that they believe the data are explained by poor assay precision. The original assay validation was not performed correctly, and they are revalidating the assay to determine the precision. FDA acknowledged their response.

Labelling of Frozen DP Vials

The process for labeling of frozen DP vials has not been validated. Please validate the labeling process and submit the validation report to the BLA.

Reference Materials

Regarding control and qualification of reference materials used in assays, you agreed in submission number 38 (February 19, 2019) to implement an SOP by March 15, 2019 to

control inventory and lot-to-lot variability of reference materials. Please submit this SOP to the BLA, and list which reference materials this SOP will apply to.

Materials Not Physically Separated in Freezers

On February 7, 2019, FDA inspectors noted that frozen materials are not physically separated in freezers. You agreed to separate frozen materials by adding on freezer shelves. You agreed to implement these by March 31, 2019, and to submit confirmation to the BLA. When this change has been implemented, please provide confirmation to the BLA that frozen materials are physically separated in freezers.

16.5.1.3 Wrap-Up Activities

The timeclock is running out, and a decision has to be made: not approved, approved, Complete Response Letter.

If the decision is 'approved', then meetings take place to wrap up review activities. This includes discussions on labels, required post-market clinical studies, etc. For CMC, this includes discussions on the timelines for completing certain CMC activities after market-approval. These postmarketing commitments, contracted with the FDA, will appear in the FDA issued BLA approval letter. The following are some postmarketing commitments, along with the timeline for required completion, made by two biopharmaceutical manufacturers in order to receive BLA approval:

Tepezza (Teprotumumab-trbw) Monoclonal Antibody [20]
BLA approved: January 21, 2020

- Establish an in-house qualification program for the IGF-1R AlphaLISA commercial kit used to control the potency of teprotumumab drug substance and drug product at release and during storage. Submit the description of the qualification program, information and data to support the adequacy of the qualification program with respect to the assurance of consistent performance of the AlphaLISA commercial kit in final study report.

Final Report Submission: 03/2020 [by 2 months after market approval]

- Develop and validate a product-specific host cell protein (HCP) assay that has improved sensitivity and capability to detect a greater range of potential HCPs compared to the current assay and to implement this assay for teprotumumab drug substance release. The analytical procedure, validation report, proposed acceptance criterion, and data used to set the proposed acceptance criterion will be submitted as a CBE-30 to the BLA.

Final Report Submission: 06/2021 [by 17 months after market approval]

Briumvi (Ublituximab-xiiy) Monoclonal Antibody [21]
BLA approved December 28, 2022

- To optimize the antibody-dependent cellular cytotoxicity (ADCC) Potency Assay with the goal of reducing method variability as well as implement a comprehensive and robust control strategy to control ADCC activity of ublituximab-xiiy drug substance and drug product at release and stability, and to submit the proposed relevant specifications as a Prior Approval Supplement in accordance with 21 CFR 601.12 (b).

Final Report Submission: 12/2023 [by 12 months after market approval]

- To implement and validate an analytical method to control polysorbate 80 concentration for ublituximab-xiiy drug product at release and stability.

Final Report Submission: 03/2023 [by 3 months after market approval]

- To establish a ublituximab-xiiy working reference standard (WRS) and submit WRS qualification data for the first WRS as well as a WRS requalification protocol.

Final Report Submission: 01/2023 [by 1 month after market approval]

If the decision is 'Complete Response Letter', then the drafting of the significant issues not resolvable within the timeclock period allowance will be listed in the letter. The manufacturer has up to a year after receiving the letter to resubmit the BLA sections, and restart the review; if not, the BLA is considered withdrawn. The following are two FDA published Complete Response Letters that biopharmaceutical manufacturers have received (these were published by FDA after the BLA was resubmitted and subsequently received market approval):

Tzield (Teplizumab-mzwv) (Monoclonal Antibody) [22]

- Results of your ongoing real-time stability studies demonstrate unacceptable charge variation measured in PRV-031 drug substance manufactured at AGC Biologics and the resulting drug product under recommended storage conditions. Data preclude the ability to assign a shelf-life for either drug substance or drug product, not only because of the unacceptable degree of change, but also because stability behavior is not consistent between drug product lots manufactured using AGC material. The degree of change also prevents a determination as to whether there is a problem with product stability, the method, or both. Finally, the possibility that this variation arises from method variability also introduced uncertainty into the reliability of all results generated with this method, including analytical comparability assessment, highlighted by the difference in stability behavior between AGC lots and lots manufactured by Eli Lilly. To address these deficiencies:
  (a) Provide data and information regarding the source of the variability of the PRV-031 drug product and drug substance profile on stability as measured by the CEX-HPLC assay.
  (b) Address and remediate the source of the charge variation of PRV-031 manufactured at AGC.
  (c) Address the differences in PRV-031 stability behavior between clinical material manufactured at Eli Lilly and proposed commercial material manufactured at AGC Biologics.
- No information was provided in Section 3.2.S.2.3 regarding your plans to monitor Master Cell Bank (MCB) and Working Cell Bank (WCB) stability. To correct this deficiency, provide cell bank requalification protocols for the MCB and WCB to include, but not be limited to, the frequency of testing, a justification for this frequency, number of vials proposed to be tested at each testing timepoint, tests proposed/parameters to be evaluated, and appropriately justified acceptance criteria.
- Determine the extinction coefficient for PRV-031 experimentally to ensure that the concentration of PRV-031 is determined correctly in release and stability testing.

Adstiladrin (Nadofaragene Firadenovec-vncg) Viral Vector [23]

- CBER conducted a Pre-License Inspection (PLI) of the FinVector Oy facility from January 20-25 and January 27-28, 2020, and issued a Form FDA 483, List of Inspectional Observations. Your responses to the FDA 483 received through March 2, 2020, do not sufficiently address the concerns noted during the inspection as your corrective actions do not appear to be comprehensive enough to address the systemic issues.
- You state in Sections 2.2 Preparation and Handling and 2.3 Administration, that the drug product should be withdrawn from four (4) vials into a syringe(s) and instilled into the bladder using a urinary catheter. However, you did not include critical parameters for these delivery devices. Please propose critical device parameters (e.g., volume, material(s) of construction, French gauge, length, coatings, colorants,

connector style, tip style, etc.) to include in the labeling in order to guide the clinician in selecting a syringe and urinary catheter that are compatible with your DP. While it is possible these parameters may include a range of selections/values (e.g., different materials of construction, different lengths, etc.), all proposed parameters and selections/values should be supported by compatibility testing and suitable for clinical delivery of the product. If there are any catheter types that should not be used with your product (e.g., in-dwelling catheters, catheters with antimicrobial coatings, etc.), please also include this information in the labeling. To support your proposed parameters and selections/values, please provide:

(i) a discussion of how each proposed parameter and selection/value is supported by your compatibility data.
(ii) information regarding the catheters that were selected for use during your clinical studies, along with a summary of your clinical experience using these urethral catheters to deliver the DP (including any delivery-related adverse events) and how the catheters used in the clinical study compare to the catheters used in the compatibility testing and the proposed critical device parameters.

16.5.2 EMA MAA Review Process

The assessment of a MAA application for a new medicine takes up to 210 'active' days. This active evaluation time is the time spent by EMA experts to evaluate the evidence provided by the manufacturer in support of a marketing authorization application. The evaluation time is interrupted by one or two 'clock-stops' during which the manufacturer prepares the answers to any questions raised by the Committee Human Medicinal Products (CHMP). The maximum duration of a clock-stop depends on how long the manufacturer thinks it will take to respond, but must be agreed by the CHMP. The first clock-stop usually lasts 3 to 6 months and the second one 1 to 3 months. Therefore, overall, the assessment of a new medicine usually requires around 12 months to complete.

EMA has provided a diagram of the MAA review process, in 21 steps, covering the entire 210-day review process [13]. Three of the steps are milestones for the CMC regulatory review: Steps 2, 7 and 11. Each of these steps will be examined in more detail.

16.5.2.1 Step 2: Decision on Need for cGMP Compliance Inspection

Upon receipt of the MAA, the assigned rapporteur and co-rapporteur do an initial assessment of the submission. If the product is a gene therapy-based biopharmaceuticals, the rapporteur and co-rapporteur will have been appointed from the Committee for Advanced Therapies (CAT) members.

One of the first key decisions to be reached is whether there is the need of a pre-approval cGMP compliance inspection. Owing to the complexity of the biopharmaceutical manufacturing process and the need for a sterile injectable drug product, the decision is yes. The appropriate inspections are carried out by inspectors of the various national competent authorities (NCAs).

Fig. 16.7 MAA review process: Step 2 – Decision on need for cGMP inspection

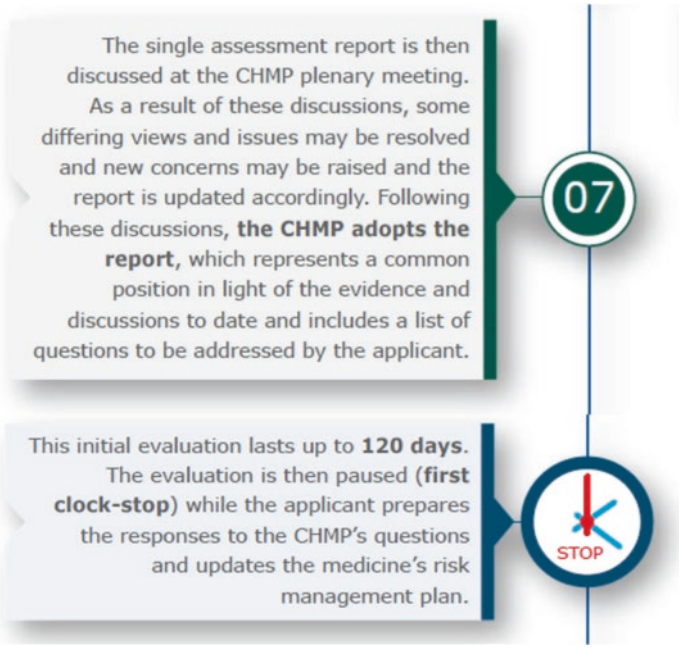

Fig. 16.8 MAA review process: Step 7 – Day 120 List of Questions (LoQ)

Satisfactory completion of any inspection issues will need to be resolved to the CHMP team's acceptance before the recommendation of market approval.

16.5.2.2 Step 7: Day 120 List of Questions (LoQ)

The submitted MAA was distributed at the beginning of the review process to all parties – the rapporteur and co-rapporteur, the other CHMP members, and the CHMP peer-reviewers – now by Day 115, a list of questions is now received from them. The

list of questions is reviewed and compiled, and on Day 120, the List of Questions (LoQ) is sent to the manufacturer. At this point, the official timeclock is paused.

Looking through the list of questions received for Clinical, Non-Clinical and CMC, the manufacturer has a critical decision to reach: (a) respond to all questions typically within 3-6 months, or (2) withdraw the MAA submission. Don't underestimate the amount of work it might take to respond to all of the questions in such a short time period, and typically there are plenty of CMC questions. When responding to the LoQ, all responses are expected to be complete and are submitted together.

The following case example presents the situation where an IgG4 humanized monoclonal antibody manufacturer chose to withdraw their MAA submission upon receiving the Day 120 LoQ, when realizing that it would not be possible to address all of the significant Clinical issues identified. But, in the published withdrawal report, major issues were also raised about CMC regulatory compliance, which gives an insight into the type of CMC regulatory compliance questions that are asked in a LoQ [24]:

- The process steps, process parameters, operation ranges and controls have been laid down in sufficient detail but critical process parameters (CPPs, defined as process parameters whose variability has an impact on a Critical Quality Attribute) are lacking for a number of important manufacturing steps such as protein A chromatography, depth filtration, CEX chromatography, anion exchange membrane chromatography and UF/DF. Define appropriate CPPs. Critical process parameters and in process controls have been specified. A question has been raised on the definition and number of CPPs for several process steps in the downstream process. Criticality is based on impact (severity), not on the residual risk after the implementation of the control strategy. Consequently, even if a critical parameter is adequately controlled, it will still be a critical parameter: the risk is lower but criticality is the same. As a result, the applicant is requested to revise their approach of CPPs based on the CQAs defined.
- Additional information is required for the process specific ELISA kit used for the determination of residual CHO cell protein. The applicant is requested to demonstrate that most of the representative HCP species of the intended manufacturing process are present (in line with PhEur 2.6.34). It also needs to be specified what is meant by "CHO HCP Antigen Standard Stock" and how, where and when this was produced. For system suitability incorporate dilutional linearity into the SST criteria.
- The validation document for PD-1 binding assay is missing and has been requested. A PD-1 binding ELISA and a cell-based blocking assay has been used to monitor biological activity in both release and stability of DS and DP. FcRn binding conditions were provided in the method description. The rationale for studying FcRn binding at these conditions should be justified.
- The absence of a test for major glycosylation forms in the release specification should be justified.
- The method description for determination of protein concentration is only briefly described. The applicant is asked to justify the use of the theoretical extinction coefficient and not the experimentally determined extinction coefficient when calculating the concentration.

- The applicant should strengthen the identification of retifanlimab in the DS by introducing identification criteria based on relevant structural properties. The applicant should implement a test which is able to unequivocally establish identity of retifanlimab, e.g. peptide map or other (combination of) highly specific methods.
- In general, the statistical approach for setting acceptance criteria is not endorsed and should be revised. The specification for potency in PD-1 binding assay is considered too wide. Similarly, the proposed specification for the PD-1 Blockade Bioassay is considered too wide. The applicant is requested to tighten these ranges in line with the statistical approach and/or batches used in clinical studies and assay variability.
- The proposed shelf life is 36 months for drug products stored at 5 ± 3 °C. This period is covered by just 2 batches from supportive lots but not by any of the primary stability batches. The applicant is requested to propose a new shelf life period that is supported by at least three representative batches.

16.5.2.3 Step 11: Day 180 List of Outstanding Questions (LoOQ)

The manufacture's responses to the LoQ are received by all parties for their review – the rapporteur and co-rapporteur, the other CHMP members, and the CHMP peer-reviewers. The official time clock is unpaused.

After review, comments are received from the review team members. Upon discussion and agreement, a new list of questions is compiled, and on Day 180, the List of Outstanding Questions (LoOQ) is sent to the manufacturer. At this point, the official time clock is paused again.

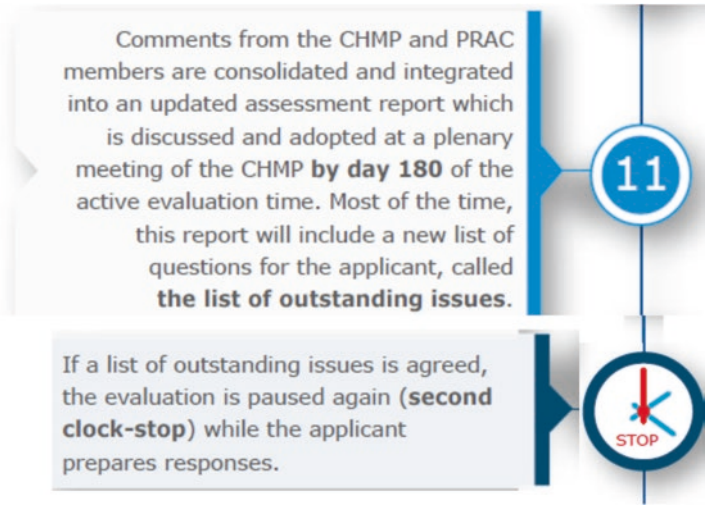

Fig. 16.9 MAA review process: Step 11 – Day 180 List of Questions (LoOQ)

Looking through all of the questions for each discipline on the LoOQ, the manufacturer again has a critical decision to reach: (a) respond to all questions typically within 1-2 months, or (2) withdraw the MAA submission. Don't under-estimate the amount of work it might take to respond to all of the new CMC questions in such a short time period. When responding to the LoOQ, all responses are expected to be complete and are submitted together.

The following case example presents the situation where an IgG chimeric monoclonal antibody (biosimilar to rituximab) manufacturer chose to withdraw their MAA submission upon receiving the Day 180 LoOQ, when realizing that it would not be possible to address all of the issues identified. CMC regulatory compliance issues were the major concern. While the CHMP review team acknowledged the manufacturer's response to the considerable CMC issues (specific items not identified) raised in the Day 120 LoQ, the new information provided raised even more serious CMC concerns. The ultimate assessment at Day 180 was 'the current MAA is not approvable from a quality point of view since major concerns are raised regarding biosimilarity to the reference product MabThera'[25]:

> After the assessment of the responses to the d180 LoQ, a major concern with regard to quality/biosimilarity remains to be solved. The manufacturing process reflects a standard process used for the manufacture of monoclonal antibodies. The individual steps are described in detail.
>
> After completion of the clinical trials the manufacturing process had been transferred to the commercial facility. Hence, a pre-requisite for biosimilarity is the comparability of MabionCD20 used in the phase III clinical trials vs. the proposed commercial MabionCD20. The applicant performed a retrospective approach to demonstrate comparability based on available data from the batches, supported by comparability and stability data. The approach taken by the company to demonstrate comparability is neither considered appropriate nor compliant with GMP principles and ICH Q5E. Moreover, based on the inconsistencies identified during review of the MAA, a reasonable uncertainty in respect to reliability of the data presented in the dossier remains. It can be concluded that the proposed manufacturing process is not considered finalised for commercial manufacturing.

16.5.2.4 Wrap-Up Activities

The timeclock is running out, and a decision has to be made: not approved, approved.

If the decision is 'approved', then for CMC, decisions for postmarketing commitments, contracted with the EMA, will appear in the EMA issued European Public Assessment Report (EPAR). The following are some postmarketing commitments, along with the timeline for required completion, required by two biopharmaceutical manufacturers in order to receive MAA approval:

> ***Vabysmo (Faricimab) Monoclonal Antibody*** [26]
> *MAA approved July 21, 2022*
> In the context of the obligation of the MAHs to take due account of technical and scientific progress, the CHMP recommends the following points for investigation:
>
> 1. To provide full leachable data for the active substance container closure system upon study completion (up to maximum 60 months). The study is foreseen to be completed in Q2 2025.

2. The long-term leachables study the finished product container closure system will be continued for at least up to 36 months covering the proposed finished product shelf life of 30 months, and any results that are above the toxicological thresholds or any new leachables not previously identified will be reported to EMA. Final results of the 36 months' time point will be available after study completion in October 2023.
3. To include defined additional tests when either a new primary reference standard or an updated protocol is submitted for post marketing approval.

Hemgenix (Etranacogene Dezaparvovec) Viral Vector [27]

1. The applicant commits to transfer the analytical methods and release testing to EU testing laboratories using a staggered approach. All finished product release testing should be conducted in the EU GMP-certified testing laboratories within defined timeframe following completion of analytical method transfer as per presented plan and successful Type II variation procedure outcome.

   Active substance
2. The applicant is recommended to include testing of porcine viruses in the testing panel for future MCB and MSV.
3. The applicant is recommended to submit the next time points of the stability data for WCB lot upon availability after testing.
4. The applicant is recommended, as committed as part of the ongoing CPV programme, to analyse data from all commercial batches at a regular frequency to ensure the process performance and the process control strategy are appropriate to ensure product quality. Special attention should be paid to data for biological activity.
5. The applicant is recommended to perform additional studies to improve performance of a downstream purification step in the active substance manufacture.
6. The applicant is recommended to perform additional studies to improve the performance of a downstream step in the active substance manufacture to reduce residual impurities.
7. The applicant is recommended to perform a post-hoc analysis of infectious titre ranges observed in the upstream step. The possibility of establishing an acceptance criterion for better control of this step should be explored.
8. The applicant is recommended, as committed, to perform a formal hold time validation study to collect additional data for extended hold times at full-scale at each relevant process intermediate. The applicant confirms that the hold time validation study results will be submitted in terms of a post-approval variation.
9. The applicant is recommended to complete, as committed, the experiments for an assay for the attribute purity.
10. The applicant is recommended to develop and incorporate, as committed, a release assay for the attribute purity, proposing to introduce the method as a release test as a post-approval variation. The introduction of this methodology as release testing is planned within a defined timeline.
11. The applicant is recommended, as committed, to provide the revised method validation report for assay to measure process related impurities.
12. The applicant is recommended to provide the additional results for active substance batches on stability post-approval.

    Finished product
13. The applicant is recommended, as committed, to revise the upper limit for finished product potency specification once additional data from finished product commercial batches manufactured are available.
14. The applicant is recommended, as committed, to reassess the finished product specification for the attribute biological activity once the data for finished product commercial batches tested using the newly validated method are available.

15. The applicant is recommended, as committed, to introduce a release method for the finished product, once the analytical method validation is accomplished and the release criteria is established.
16. The applicant is recommended, as committed, to provide the finished product leachable study for the timepoints until the study is completed.
17. The applicant is recommended to provide the GMP certificates for two testing sites once available.

16.6 Why So Many Problems with Biopharmaceutical CMC Strategy?

Working in the biopharmaceutical field for three decades you see some interesting patterns develop. Three thoughts come to my mind related to problems with biopharmaceutical CMC strategy.

My first thought is that the biopharmaceutical industry does not fully appreciate the level of support that a regulatory authority can provide to the CMC regulatory compliance strategy. On the one hand, we say that regulatory reviewers are team members, but on the other hand, we act like they might not have much to add to our CMC challenges. Knowing how important securing a CMC-focused meeting with the FDA can be, it was quite surprising to see the following discussion recorded during a multi-discipline pre-BLA meeting. The monoclonal antibody manufacturer actually turned down a strongly stated recommendation from the FDA themselves that they needed to meet with them and have a CMC-focused meeting [28]:

> **General FDA Comment**: We note that there are no questions or discussion focused on the Chemistry, Manufacturing and Controls or the Pharmacology/Toxicology portions of your proposed Biologics Licensing Application (BLA). The preBLA meeting is the time to reach agreement on the adequacy of the entire data package you intend to submit in support of your application. You do have an option for a specific CMC preBLA meeting. We strongly recommend that you seek a separate meeting with the Office of Biotechnology Products for discussion about the content of the CMC section of your BLA and the adequacy of the data you propose for that section.
>
> **Discussion at the Meeting**: The FDA reminded Ultragenyx that the purpose of the pre-BLA meeting is to reach agreement on the entire data package for the proposed BLA and explained that we would not be able to agree on the entire package because we have yet to discuss some aspects of the package, specifically the CMC, Nonclinical, and Clinical Pharmacology sections. The FDA reiterated the recommendation to have a CMC pre-BLA meeting.
>
> **Response**: Ultragenyx stated they did not think a pre-BLA CMC meeting was necessary based on previous detailed CMC comments received at the Initial Comprehensive Multidisciplinary Breakthrough Therapy Meeting in October 2016 and because Ultragenyx does not have any new data since that meeting. Ultragenyx stated they fully understand the expectations for the CMC section of the BLA.

Fortunately, in this case example, the manufacturer was able to meet the required CMC regulatory compliance strategy within the required time period, and did achieve market approval. But I believe it provides an example of how some manufacturers think that they can handle their CMC regulatory compliance strategy on

their own. The various case examples in the 15 chapters in this book indicate that that is not always the best pathway.

"I only wish I could find an institute that teaches people how to listen. Business people need to listen at least as much as they need to talk", commented Lee Iacocca, former CEO Chrysler Corporation. We can do, and must do, a better job of communicating with the regulatory authorities.

My second thought deals with submission surges. Every year, there seems to be an "end of the calendar year rush to file" for market dossier submissions. Regardless of whether the BLA/MAA is complete or not, some biopharmaceutical manufacturers push to get their market application submissions filed by a corporate-set target date, rather than ensuring that all the required CMC information is complete and properly and clearly presented in the submission. This has resulted in several embarrassing comments communicated by the regulatory authority reviewer to the manufacturer, that appeared in the public release review documents, after achieving market approval:

PEGylated Recombinant Protein (Udenyca, PEGfilgrstim-cbqv) [29]

Complete Response Letter

Overall, Module 3 of your BLA is not well prepared. Frequently, you refer to information/data in numerous reports, but do not provide an informative summary with your conclusions based on the information. While reports are important to verify some specific information or evaluate raw data your summaries with interpretations and conclusions form the basis for the Agency's review. Submitting a large number of reports with minimal data interpretation did not allow for an efficient review process. For example, reports related to process characterization and determination of in-process controls were difficult to interpret. Additionally, there are many inconsistencies, missing information, and typographical errors throughout your BLA. We expect that you will address these and other such issues in any resubmission.

Recombinant Protein (Oxervate, Cenegermin) [30]

EPAR

From the quality point of view the CHMP considered the quality dossier at submission, to be poorly presented and incomplete with respect to critical data to support a sufficient knowledge of active substance and an appropriate control strategy for both manufacturing process and active substance. This was reflected in the two major objections that were raised during the procedure namely (1) on the proposed manufacturing process control strategy which was considered insufficient to ensure consistent quality of the active substance and (2) on insufficient characterisation of the active substance and routine control of impurities for active substance/finished product. The major objections are interlinked as the insufficient characterisation of active substance impacts upon defining appropriate CQAs and upon the comparability studies carried out across the different historical versions of the manufacturing process. In addition, numerous inconsistencies and omissions were noted in the data presented which has been reflected in the number of other concerns raised throughout the procedure.

Viral Vector (Adstiladrin, Nadofaragene Firadenovec-vncg) [31]

A Complete Response Letter (CRL) was issued on April 24, 2020.

The BLA was then resubmitted on April 11, 2022. However, the submission was not complete, and an incomplete response letter was issued on May 10, 2022. In the April 11, 2022, submission, the files were not uploaded properly and eCTD format was not followed. Many of the relevant, originally submitted files were deleted or replaced with the newer versions that were incomplete or revised to contain substantially less information than the previous versions. The applicant was asked to update the BLA modules to

ensure a complete submission. The technical information submitted and the applicant's response to the CR was not reviewed.

The BLA was resubmitted again on June 30, 2022, and the submission was designated complete.

My third thought comes from years of CMC regulatory compliance consulting: let's stop 'spinning' the data. If the science is not there, admit it, and then work on a plan to get a clearer picture of what is happening. I enjoy the quote attributed to British economist and Nobel Prize winner in economics, Ronald H. Coase: "if you torture the data long enough, it will confess to anything."

Surely, we in the CMC regulatory compliance side of biopharmaceuticals can do a better job!

16.7 Biopharmaceutical CMC Regulatory Compliance Strategy Future

An interesting development is taking place in CMC regulatory compliance strategy: an attempt to shift from generating piles of CMC documents to a more facile processing of the abundance of data for more rapidly and thoroughly accomplish the necessary adequate and appropriate risk assessments.

From the biopharmaceutical industry's side, there is 'digitalization'. The use of computers and pre-defined formats to more efficiently process the large volumes of data generated [32]:

> Albert Einstein said that "the basis of all scientific work is the conviction that the world is an ordered and comprehensible entity." The current regulatory submission and review of drug applications is currently not considered to be an ordered process as it requires significant manual and repetitive labor by both sponsors and health authorities which delays the speed at which novel therapeutics become available to patients. Pharmaceutical companies generate abundant volumes of data, content, and, ultimately, electronic or paper documentation for regulatory submissions involving clinical trial applications, new drug approvals, and post-approval lifecycle management activities. In turn, each health authority must receive, review, and respond to these submissions, initiating further document generation between a health authority and sponsor throughout the lifecycle of the product.
>
> To show the importance of digitalization in pharmaceutical submissions, the eCTD initially includes approximately 45 independent granules (sections of the eCTD) that can be used for ICH and regionally specific sections in Module 3. Dissecting these granules, there are typically an average of 5 to10 documents per granule, each of which are typically created via 3 authoring events, 3 review events, and 3 data verification events, totaling approximately 5000 internal sponsor events in building the core Module 3. This immensely complex and time-consuming process only constitutes the first CMC module sent to a health authority, following which regional customization results in other required variants for a product's global approval which can include over 80 individual health authorities. All the previously described manual processing, manipulation, and verification of data, in addition to global variation in registered details, is for a product that is essentially the same for all global markets. These authoring and verification efforts continue throughout the product lifecycle, which for many products can extend to 20 years on the market, which further highlights the need for digitalization and global standardization.

> Structured Content and Data Management (SCDM) is an emerging field which is closely related to structured content management. Here, SCDM is defined as the integration of structured content with structured data and the management of those integrated components, currently specific to CMC activities that involve high volumes of data used to author CMC submissions. At the heart of SCDM is a core design principle which aims to shift a company's focus to managing data instead of managing documents.

From the regulatory authority's side (at least for the FDA) there is 'Knowledge-aided Assessment & Structured Application (KASA)'[33]:

> The KASA system is a data-based platform for structured quality assessments and applications that supports knowledge management. KASA is designed to:
>
> - Capture and manage knowledge during the lifecycle of a drug product;
> - Include established rules and algorithms to facilitate risk identification, mitigation, and communication for the drug product manufacturing process, and facilities;
> - Perform computer-aided analyses of applications for a comparison of regulatory standards and quality risks across the repository of approved drug products and facilities; and
> - Provide a structured assessment that radically eliminates text-based narratives and summarization of information from the applications.

The KASA system allows FDA to capture critical assessment information as highly specific structured data in a predefined format which improves the efficiency, consistency, and objectivity of regulatory actions. KASA represents a significant concept shift and revolutionizes FDA's ability to take sound comprehensive regulatory actions

It is the grand hope, that one day, we on the manufacturing side as well as those on the regulatory authority side, could move off of the intense focus of how to package (and re-package) all of the CMC information that is generated, and move more onto the scientific focus on what the data is actually telling us.

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