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Transformação em *Cryptococcus neoformans*

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1933

Recombinação

- **Homóloga**
- **Não-Homóloga**

Recombinação Homóloga

Reconhecimento do dano por um complexo proteico MRX (Mre11, Rad50 e Xrs2).

Sae2, Dna2 e Exo1são recrutadas que atua sinergicamente com o complexo MRX - *trimming*

Helicase Sgs1 – desenrola as fitas expondo DNA fita simples.

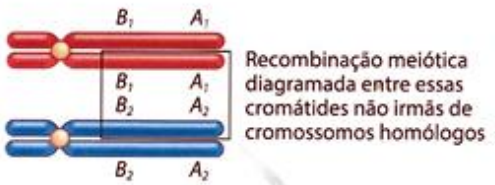
A proteína mediadora Rad52 faz a interação entre as sequências homólogas e um nucleofilamento

A proteína Rad51 se liga ao DNA de fita simples e invade o DNA homólogo

Regiões homologas alinhadas, a DNA polimerase faz síntese de DNA para reparar a molécula

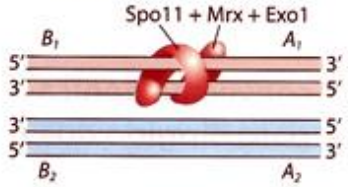
FIGURA BÁSICA 12.25

Modelo molecular de recombinação meiótica

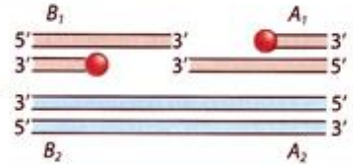


Recombinação meiótica diagramada entre essas cromátides não irmãs de cromossomos homólogos

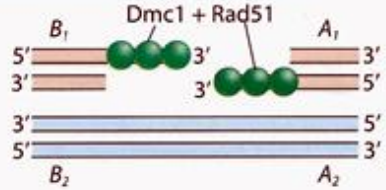
1 A Spo11 cria quebra de fita dupla em um dúplex de DNA.



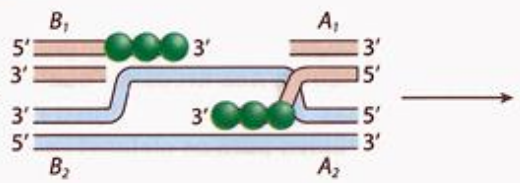
2 A digestão enzimática 5′ → 3′ pela Mrx cria segmentos de fita simples.



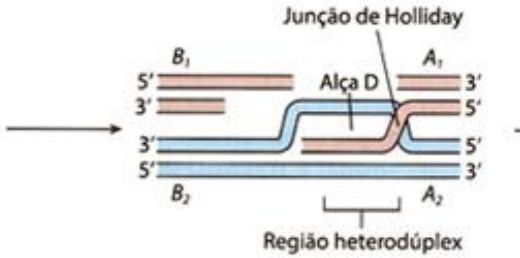
3 A Dmc1 e a Rad51 montam filamentos nucleoproteicos de intercâmbio de fitas.



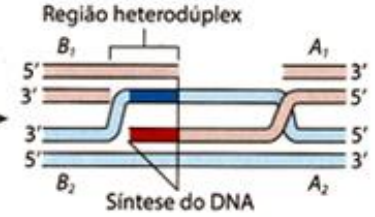
4 Os filamentos de intercâmbio de fitas promovem a invasão da fita.



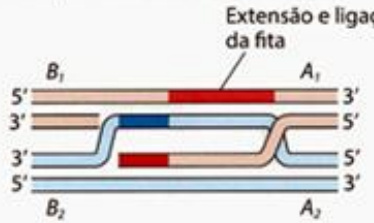
5 A invasão da fita cria uma alça D e a primeira região heterodúplex.



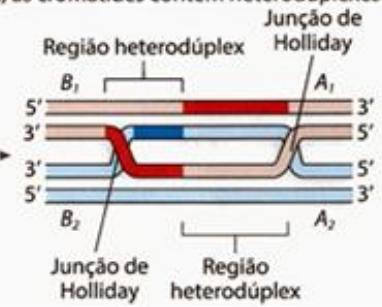
6 A extensão da fita pela DNA polimerase desloca o DNA da alça D, que pareia com o DNA de fita simples complementar e forma a segunda região heterodúplex.



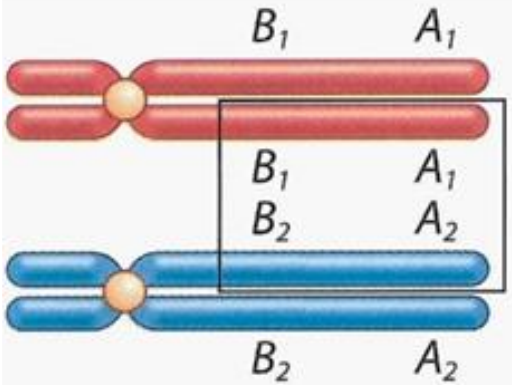
7 A extensão e a ligação da fita preenchem a lacuna de fita simples na fita pareada com o DNA da alça D.



8 As junções duplas de Holliday se formam após a incisão estar vedada; as cromátides contêm heteroduplexes deslocados.

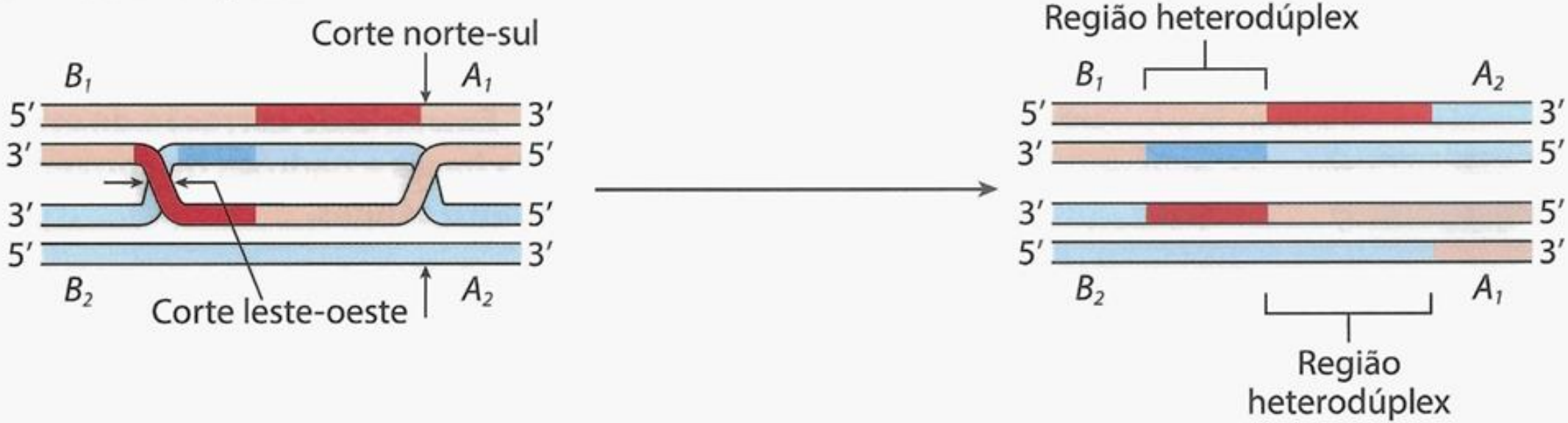


Sanders & Bowman, eds, 2014. Person, SP. 847p



Recombinação meiótica diagramada entre essas cromátides não irmãs dos cromossomos homólogos.

2 Resolução em sentido oposto



A resolução em sentido oposto é muito comum, gerando recombinação dos genes laterais e criando regiões heterodúplex deslocadas.

Sanders & Bowman, eds, 2014. Person, SP. 847p

Recombinação Não-Homóloga

Recombinação não homóloga em fungos filamentosos

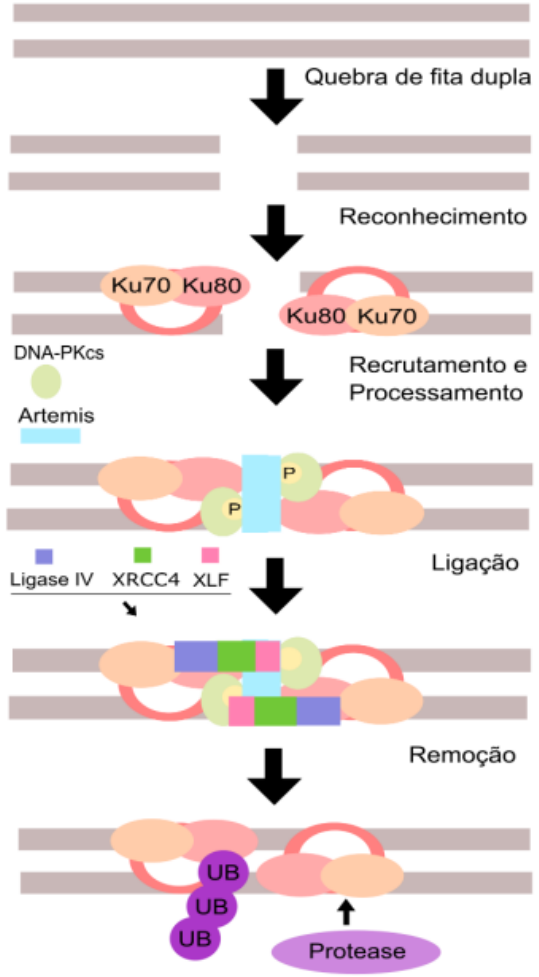


Figura 5. Esquema da via de recombinação não homóloga no reparo de quebras de fita dupla do DNA. O reconhecimento da quebra de fita dupla do DNA ocorre pelo heterodímero Ku, que recruta outras proteínas responsáveis pelo processamento das extremidades danificadas, produzindo extremidades compatíveis, as quais são unidas por atividade do complexo de ligação LigaseIV-XRRC4-XLF. Após o reparo, Ku é removido por protease e via de ubiquitinação. Figura baseada em (Fell & Schild-Poulter, 2015).

Alexia de Matos Czeczot, TCC, 2016.
Instituto de Biociências da UFRS

Recombinação não homóloga em fungos filamentosos

Tabela 1. Lista de fungos filamentosos que apresentaram aumento na taxa de recombinação homóloga pela metodologia de inativação de genes da via de recombinação não homóloga.

Organismo	Gene inativado	Recombinação Homóloga (%)	Referência
Neurospora crassa	*ku70/ku80*	100	Ninomiya *et al.* (2004)
Aspergillus fumigatus	*ku70*	75 - 95	Krappmann *et al.* (2006)
	ku80	80	Da Silva Ferreira *et al.* (2006)
Aspergillus nidulans	*ku70*	90	Nayak *et al.* (2006)
Aspergillus oryzae	*ku70/ku80*	64.2 - 75	Takahashi *et al.* (2006)
Aspergillus sojae	*ku70/ku80*	63.4	Takahashi *et al.* (2006)
Cryptococcus neoformans	*ku70/ku80*	90 - 100	Goins *et al.* (2006)
Sordaria macrospora	*ku70*	85.7 - 100	Pöggeler & Kück (2006)
Aspergillus niger	*ku70*	>80	Meyer *et al.* (2007)
Podospora anserina	*ku70*	100	El-Khoury *et al.* (2008)
Botrytis cinerea	*ku70/ku80*	37 - 100	Choquer *et al.* (2008)
Claviceps purpurea	*ku70*	50 - 60	Haarmann *et al.* (2008)
Magnaporthe grisea	*ku80*	>80	Villalba *et al.* (2008)
Hypocrea jecorina	*ku70*	>95	Guangtao *et al.* (2009)
Penicillium chrysogenum	*ku70*	60 - 69	Boer *et al.* (2010)
Trichoderma virens	*ku70*	88	Catalano *et al.* (2011)
Yarrowia lipolytica	*ku70*	43	Verbeke *et al.* (2013)
Monascos ruber	*ku70/ku80*	22.2/31.5	He *et al.* (2013)
Rhodosporidium toruloides	*ku70*	75.3	Koh *et al.* (2014)
Metarhizium robertsii	*ku70*	86	Xu *et al.* (2014)
Verticillium dahliae	*ku70*	22.8 - 34.7	Qi *et al.* (2015)
Penicillium digitatum	*ku70*	11.4	Gandía *et al.* (2016)

Alexia de Matos Czeczot, TCC, 2016.
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TCC

Alexia de Matos Czeczot, 2016.
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Sugestão de leitura:
Páginas 15 a 21.

Link: https://bit.ly/4kQIRpq
Acesso: 03/06/2025

Transformação em *Cryptococcus neoformans*

- Eletroporação
- Biolísitica

Eletroporação

MOLECULAR AND CELLULAR BIOLOGY, Sept. 1990, p. 4538–4544
0270-7306/90/094538-07$02.00/0

Vol. 10, No. 9

Isolation of the *URA5* Gene from *Cryptococcus neoformans* var. *neoformans* and Its Use as a Selective Marker for Transformation

JEFFREY C. EDMAN[1]* AND K. J. KWON-CHUNG[2]

https://pmc.ncbi.nlm.nih.gov/articles/PMC361041/pdf/molcellb00045-0110.pdf
Acesso: 03/06/2025

Biolística

JOURNAL OF BACTERIOLOGY, Mar. 1993, p. 1405–1411 Vol. 175, No. 5
0021-9193/93/051405-07$02.00/0

Gene Transfer in *Cryptococcus neoformans* by Use of Biolistic Delivery of DNA

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DAVID T. DURACK,[1] AND JOHN R. PERFECT[1]*

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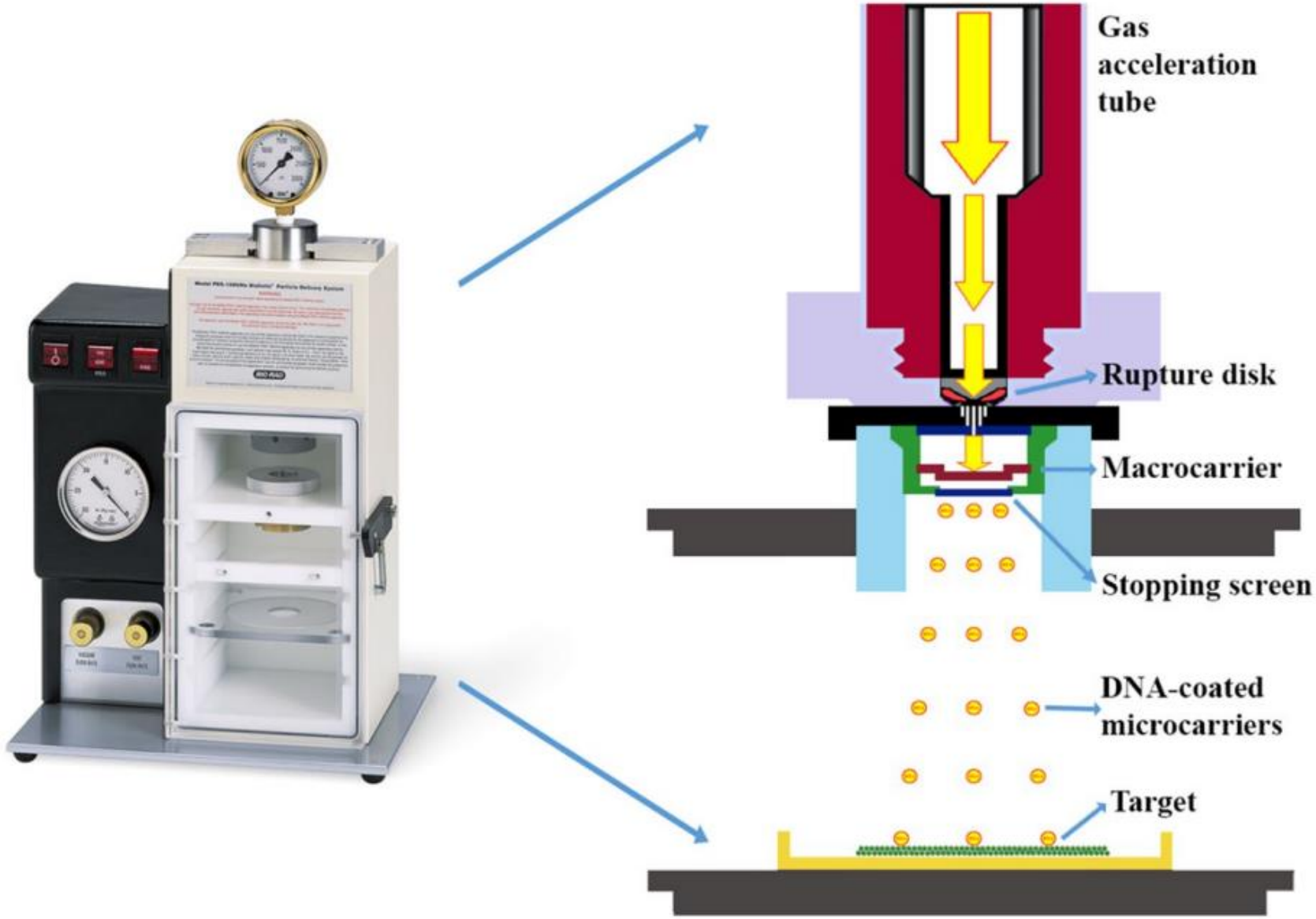
Received 8 October 1992/Accepted 7 January 1993

A transformation scheme for *Cryptococcus neoformans* to yield high-frequency, integrative events was developed. Adenine auxotrophs from a clinical isolate of *C. neoformans* serotype A were complemented by the cryptococcal phosphoribosylaminoimidazole carboxylase gene (*ade2*) with a biolistic DNA delivery system. Comparison of two DNA delivery systems (electroporation versus a biolistic system) showed notable differences. The biolistic system did not require linear vectors and transformed each auxotrophic strain at similar frequencies. Examination of randomly selected transformants by biolistics showed that 15 to 40% were stable, depending on the recipient auxotroph, with integrative events identified in all stable transformants by DNA analysis. Although the *ade2* cDNA copy transformed at a low frequency, DNA analysis found homologous recombination in each of these transformants. DNA analysis of stable transformants receiving genomic *ade2* revealed ectopic integration in a majority of cases, but approximately a quarter of the transformants showed homologous recombination with vector integration or gene replacement. This system has the potential for targeted gene disruption, and its efficiency will also allow for screening of DNA libraries within *C. neoformans*. Further molecular strategies to study the pathobiology of this pathogenic yeast are now possible with this transformation system.

https://pmc.ncbi.nlm.nih.gov/articles/PMC193227/pdf/jbacter00047-0205.pdf
Acesso: 03/06/2025



Ozyigit & Kurtoglu 2020. Molecular Biology Reports https://doi.org/10.1007/s11033-020-06001-5

Overlapping PCR

Mutação sítio dirigida – overlapping PCR

Gene, 77 (1989) 51–59
Elsevier

GEN 02940

Site-directed mutagenesis by overlap extension using the polymerase chain reaction

(Genetic engineering; sequencing; recombinant DNA; *Taq* polymerase; oligodeoxyribonucleotide primers; major histocompatibility complex mutants)

Steffan N. Ho[a], Henry D. Hunt[a], Robert M. Horton[b], Jeffrey K. Pullen[a] and Larry R. Pease[a]

[a] Department of Immunology and [b] Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN 55905 (U.S.A.)

Received by M.R. Culbertson: 15 December 1988
Accepted: 19 December 1988

SUMMARY

Overlap extension represents a new approach to genetic engineering. Complementary oligodeoxyribonucleotide (oligo) primers and the polymerase chain reaction are used to generate two DNA fragments having overlapping ends. These fragments are combined in a subsequent 'fusion' reaction in which the overlapping ends anneal, allowing the 3′ overlap of each strand to serve as a primer for the 3′ extension of the complementary strand. The resulting fusion product is amplified further by PCR. Specific alterations in the nucleotide (nt) sequence can be introduced by incorporating nucleotide changes into the overlapping oligo primers. Using this technique of site-directed mutagenesis, three variants of a mouse major histocompatibility complex class-I gene have been generated, cloned and analyzed. Screening of mutant clones revealed at least a 98% efficiency of mutagenesis. All clones sequenced contained the desired mutations, and a low frequency of random substitution estimated to occur at approx. 1 in 4000 nt was detected. This method represents a significant improvement over standard methods of site-directed mutagenesis because it is much faster, simpler and approaches 100% efficiency in the generation of mutant product.

Gene, 77 (1989) 61–68
Elsevier

GEN 02941

Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension

(Genetic engineering; polymerase chain reaction; recombinant DNA; *Taq* polymerase; complementary sequences; frequency of errors; exon; intron; mosaic fusion protein; mouse histocompatibility genes)

Robert M. Horton*, Henry D. Hunt, Steffan N. Ho, Jeffrey K. Pullen and Larry R. Pease

*Department of Immunology and * Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN 55905 (U.S.A.)*

Received by M.R. Culbertson: 15 December 1988
Accepted: 19 December 1988

SUMMARY

Gene splicing by overlap extension is a new approach for recombining DNA molecules at precise junctions irrespective of nucleotide sequences at the recombination site and without the use of restriction endonucleases or ligase. Fragments from the genes that are to be recombined are generated in separate polymerase chain reactions (PCRs). The primers are designed so that the ends of the products contain complementary sequences. When these PCR products are mixed, denatured, and reannealed, the strands having the matching sequences at their 3′ ends overlap and act as primers for each other. Extension of this overlap by DNA polymerase produces a molecule in which the original sequences are 'spliced' together. This technique is used to construct a gene encoding a mosaic fusion protein comprised of parts of two different mouse class-I major histocompatibility genes. This simple and widely applicable approach has significant advantages over standard recombinant DNA techniques.

Microbiology (2002), **148**, 2607–2615 Printed in Great Britain

A PCR-based strategy to generate integrative targeting alleles with large regions of homology

Robert C. Davidson, Jill R. Blankenship, Peter R. Kraus, Marisol de Jesus Berrios, Christina M. Hull, Cletus D'Souza, Ping Wang and Joseph Heitman

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Departments of Molecular Genetics and Microbiology, Pharmacology and Cancer Biology, and Medicine, and the Howard Hughes Medical Institute, Duke University Medical Center, Research Drive, Durham, NC 27710, USA

***Cryptococcus neoformans* is an opportunistic fungal pathogen with a defined sexual cycle for which genetic and molecular techniques are well developed. The entire genome sequence of one *C. neoformans* strain is nearing completion. The efficient use of this sequence is dependent upon the development of methods to perform more rapid genetic analysis including gene-disruption techniques. A modified PCR overlap technique to generate targeting constructs for gene disruption that contain large regions of gene homology is described. This technique was used to disrupt or delete more than a dozen genes with efficiencies comparable to those previously reported using cloning technology to generate targeting constructs. Moreover, it is shown that disruptions can be made using this technique in a variety of strain backgrounds, including the pathogenic serotype A isolate H99 and recently characterized stable diploid strains. In combination with the availability of the complete genomic sequence, this gene-disruption technique should pave the way for higher throughput genetic analysis of this important pathogenic fungus.**

Davidson et al, 2002. Microbiology, 148, 2607–2615
https://bit.ly/4kpnTyb
Acesso: 03/06/2025

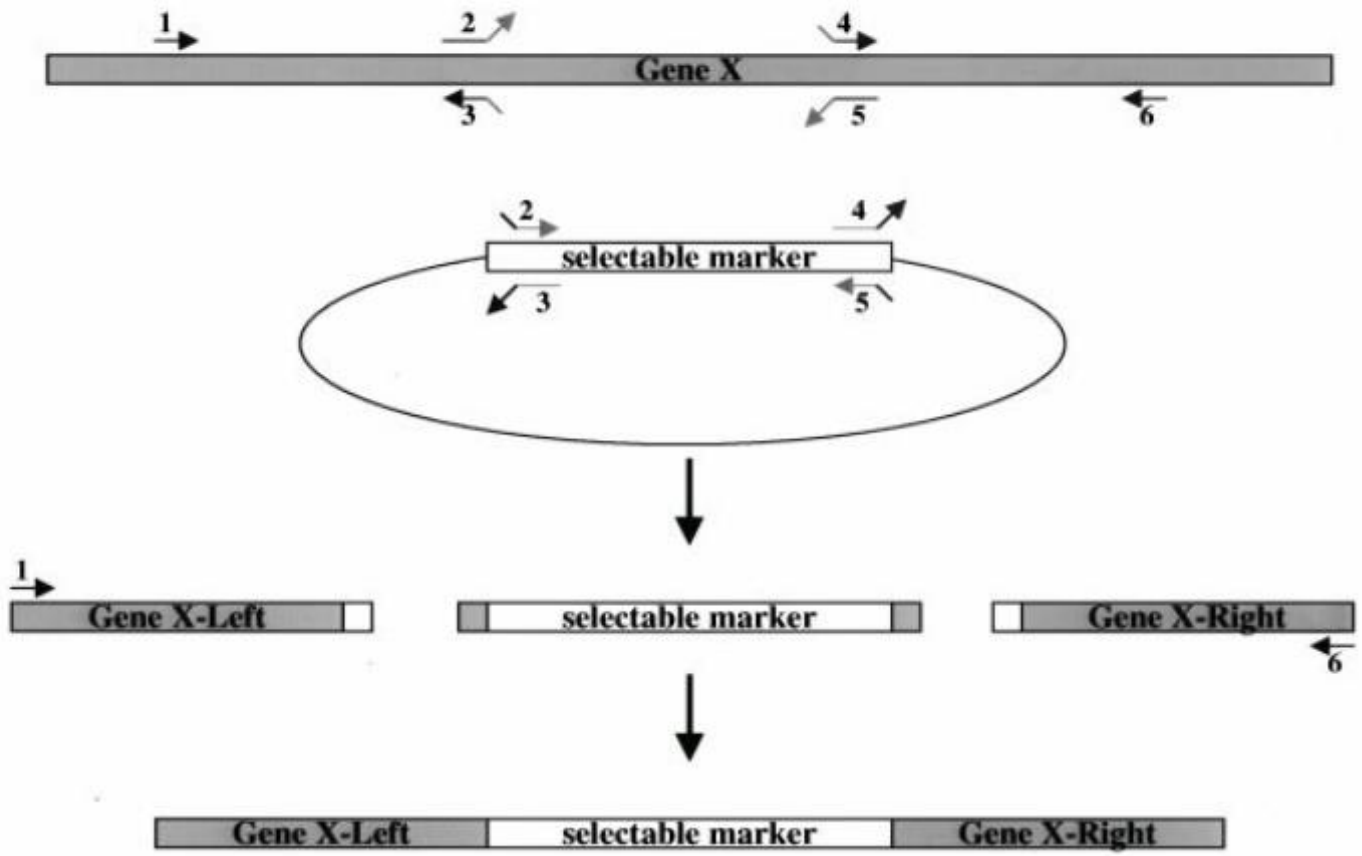
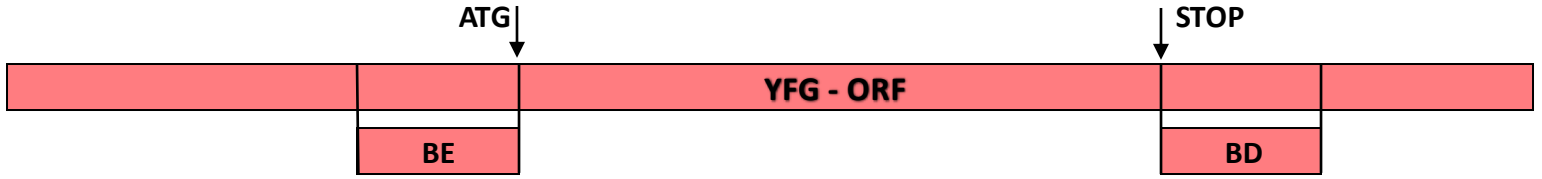


Fig. 1. The PCR overlap procedure. A gene deletion allele is constructed by PCR as follows: the 5′ end of the gene to be deleted (Gene X) is amplified with primers 1 and 3 to generate fragment Gene X-Left, the 3′ end of Gene X is amplified with primers 4 and 6 to generate fragment Gene X-Right, and the selectable marker is amplified with primers 2 and 5. The amplified products are then used as templates for an overlap reaction using primers 1 and 6. Primers 2 and 3 and primers 4 and 5, respectively, are complementary to one another and will therefore allow primers 1 and 6 to overlap the three first-round products into a linear PCR-amplified GeneX::selectable marker deletion allele.

Davidson et al, 2002. Microbiology, 148, 2607–2615

Overlapping PCR

1º Passo



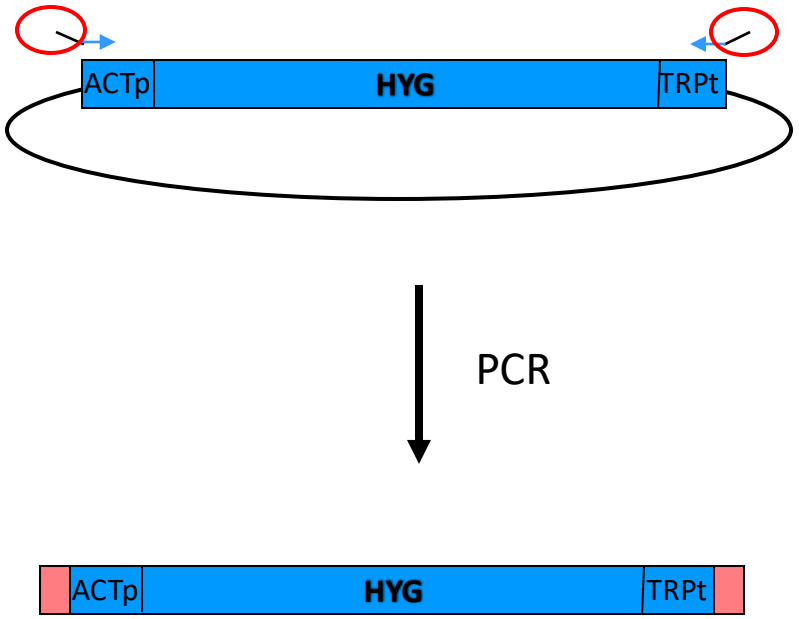
2º Passo

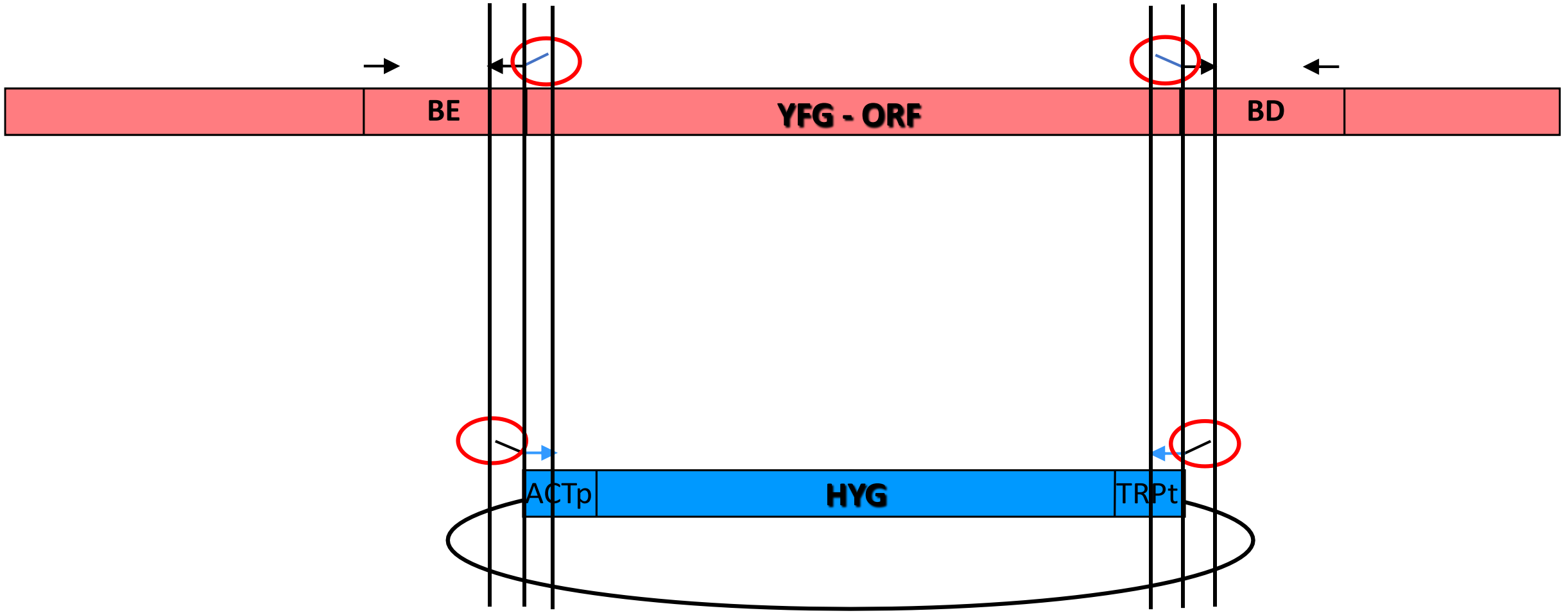
PCR

3º Passo

Overlapping PCR

Marcador
4º Passo

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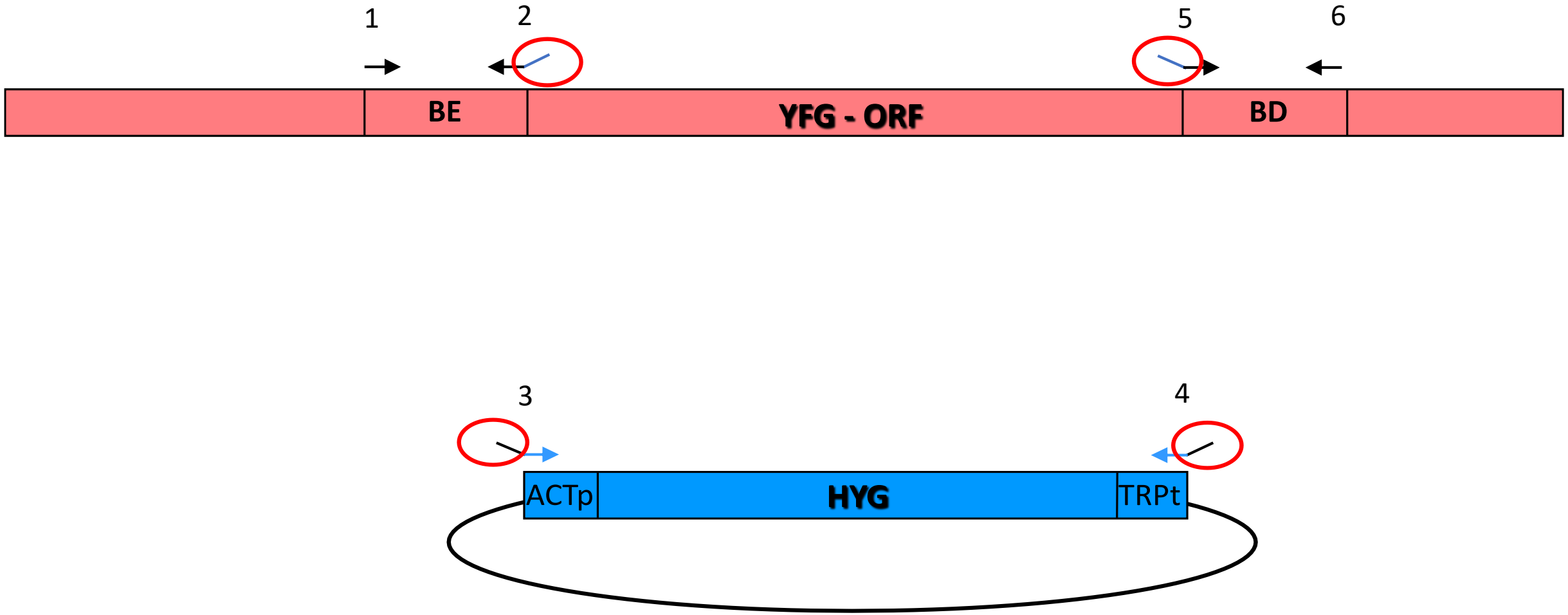
YFG - ORF
BD
ACTp
HYG
TRPt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2
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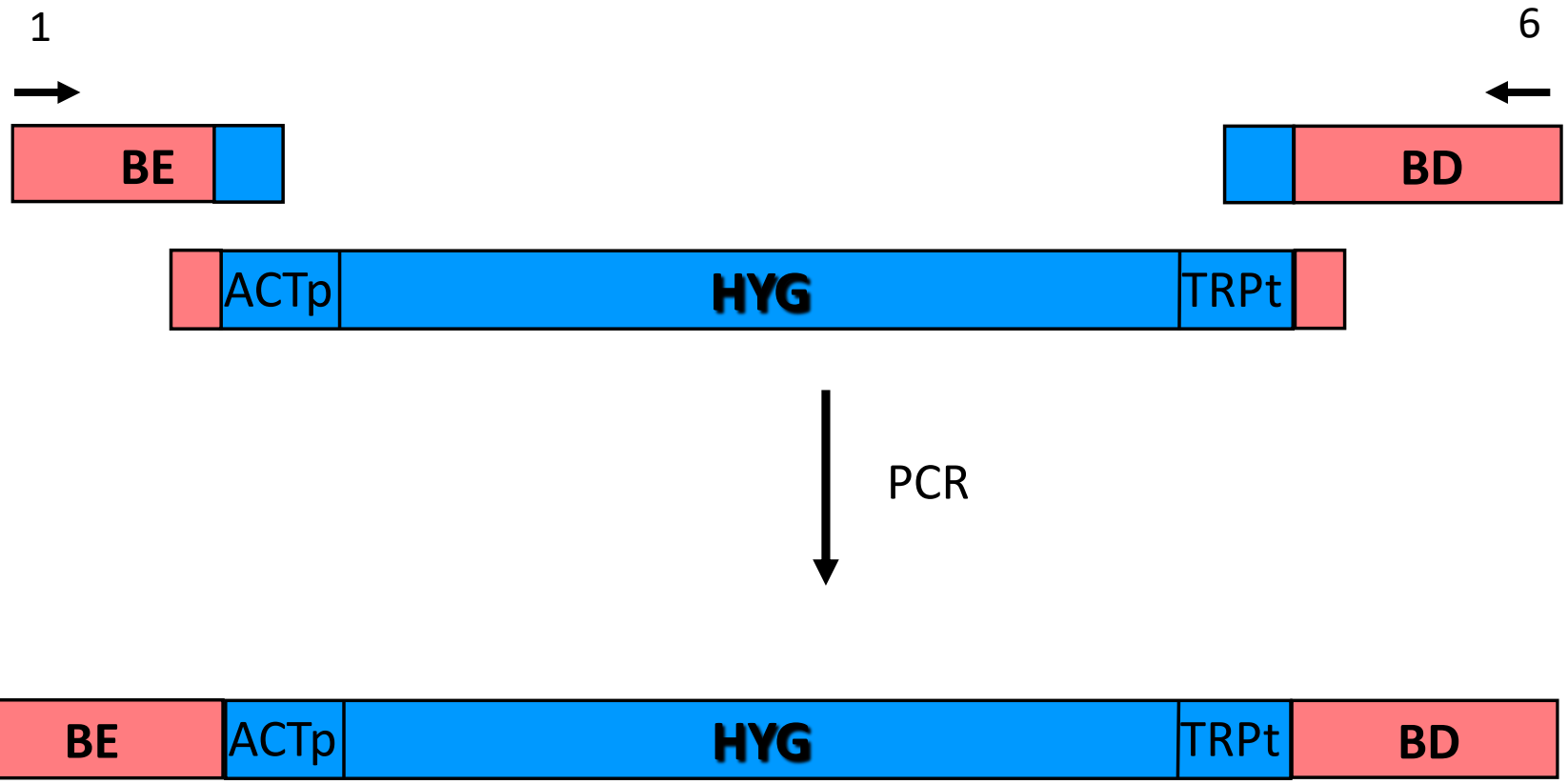
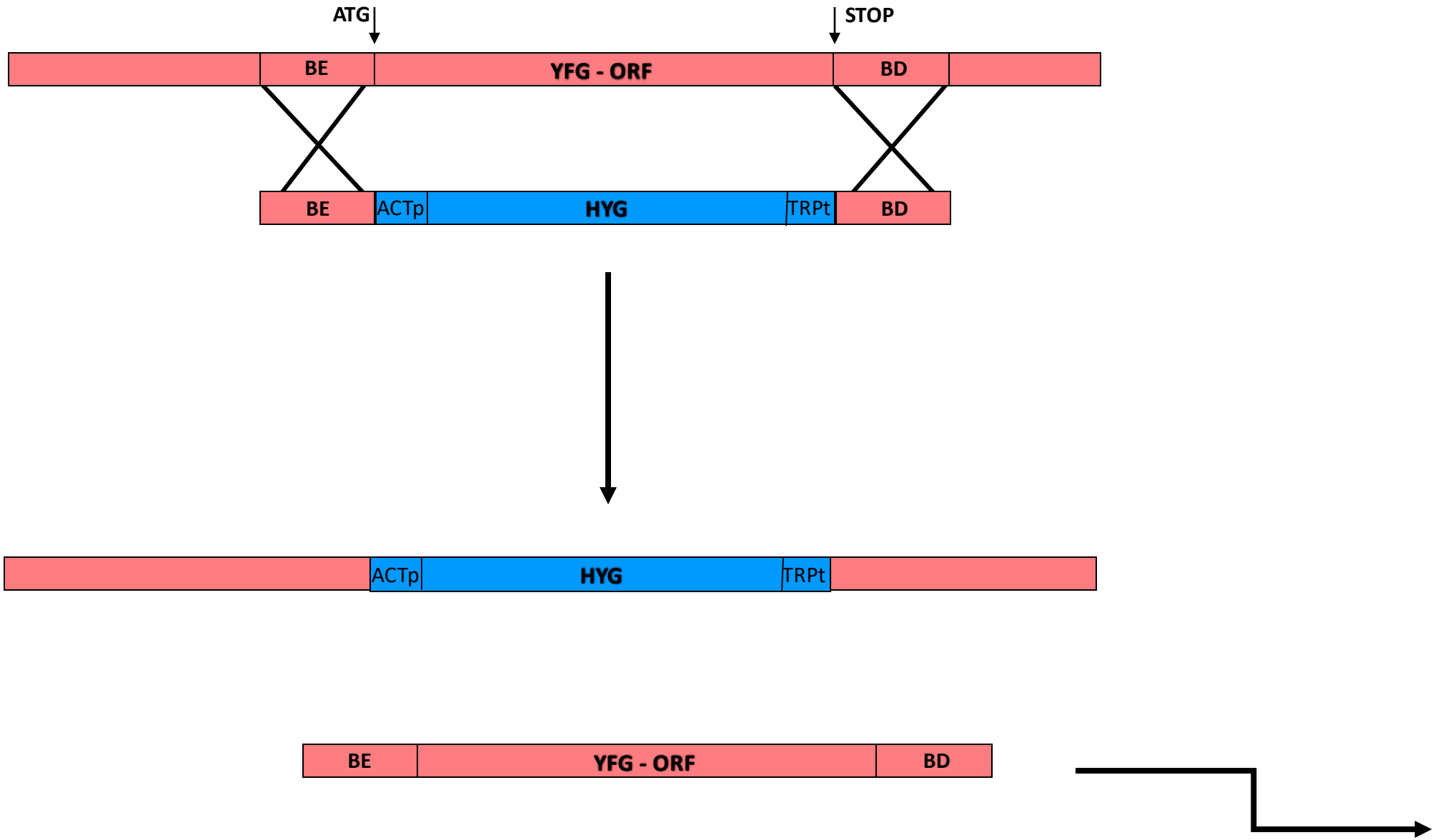
BE
YFG - ORF
BD
3
4
ACTp
HYG
TRPt

Overlapping PCR

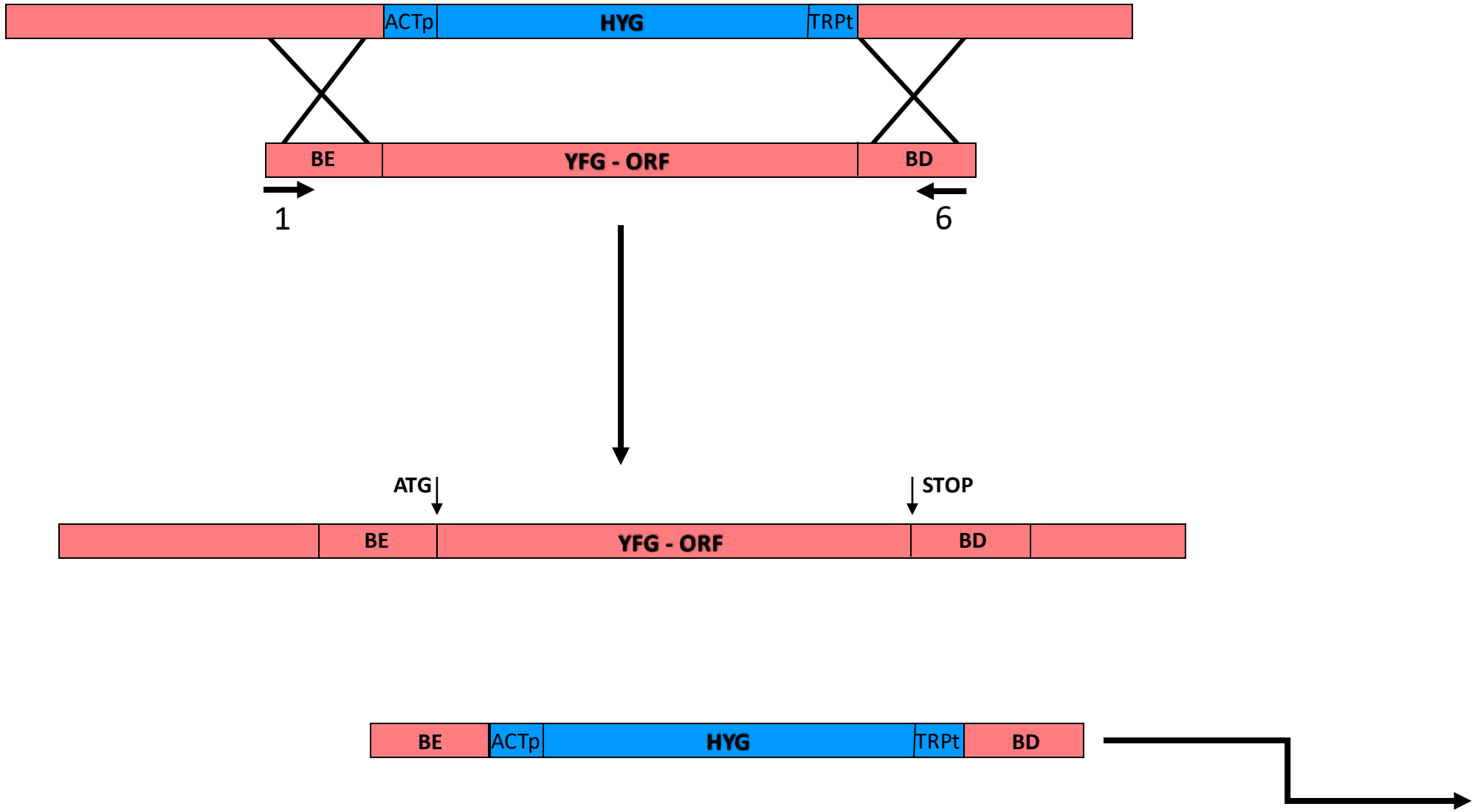


Overlapping PCR



Overlapping PCR

Reconstituição



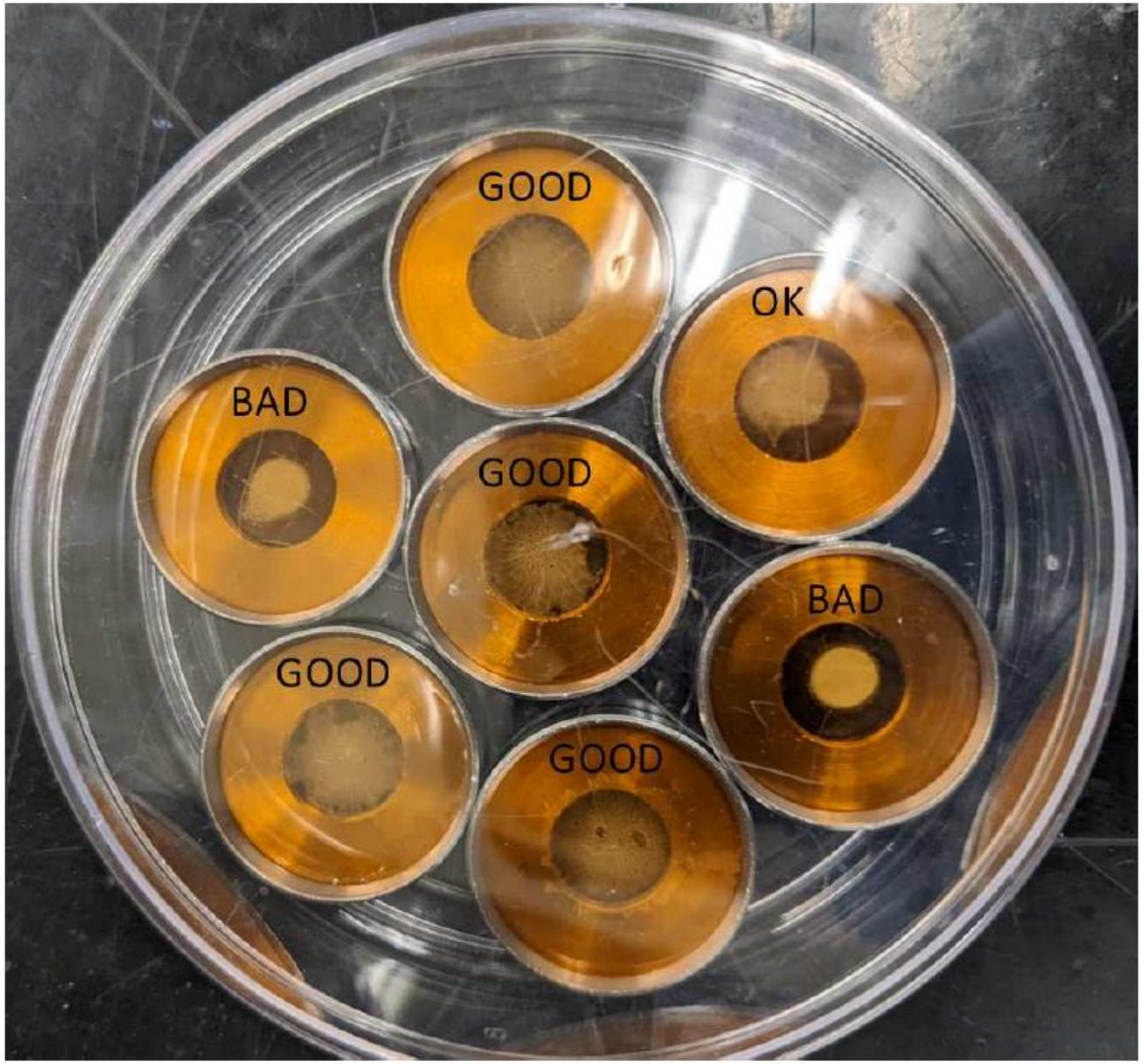
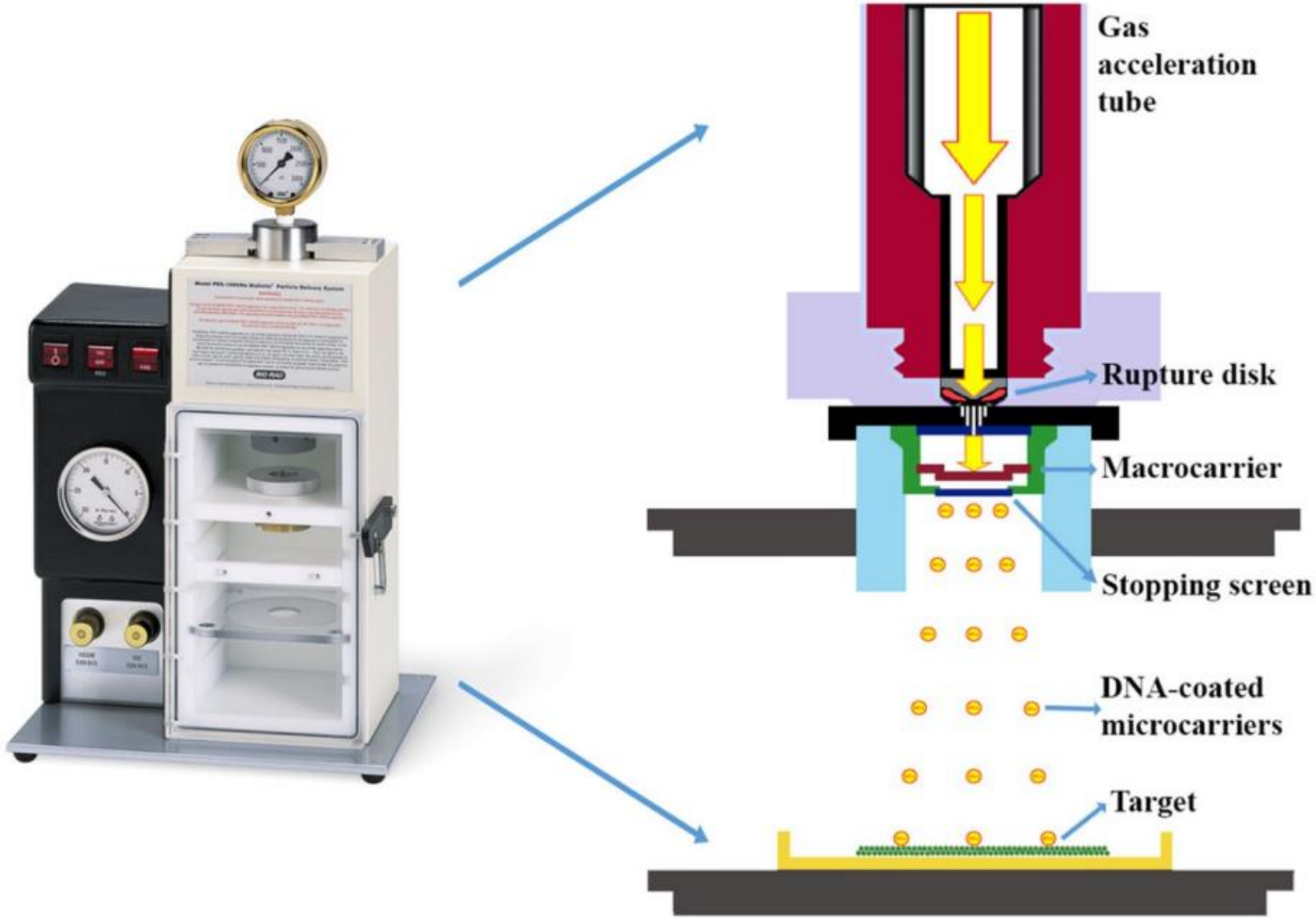


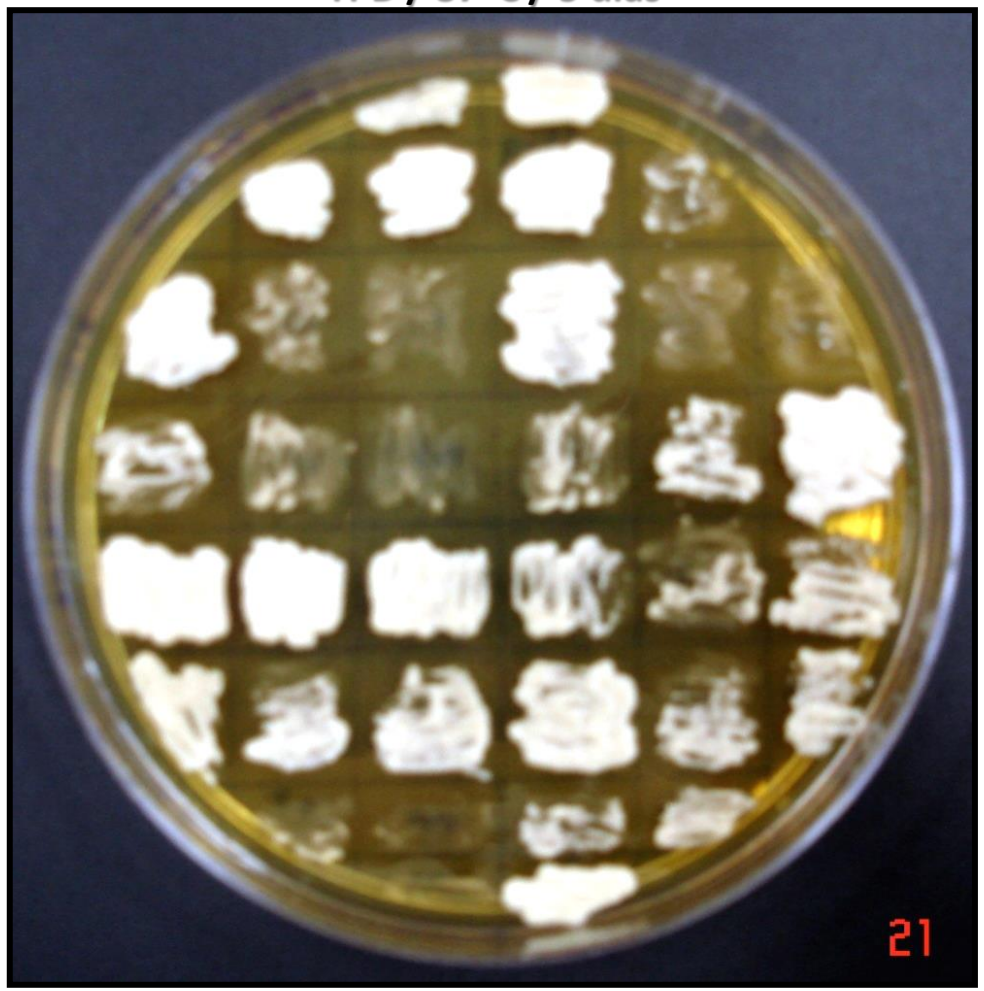
Figure 3. Macrocarriers with a range of gold spread. Gold spread evenly over the macrocarrier will have better transformation efficiency.

Lafferty & Li 2024. https://dx.doi.org/10.17504/protocols.io.3byl49792go5/v1



Ozyigit & Kurtoglu 2020. Molecular Biology Reports https://doi.org/10.1007/s11033-020-06001-5

200µg/mL Higromicina B
YPD / 37ºC / 5 dias



306 hyg+

22 35 36 43

55 61 81 82 136 138

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190 191 192 194 197 210

214 218 219 231 237 238

257 266 274 296

K99α (+)

Double-joint Overlapping PCR

Biochemical and Biophysical Research Communications 390 (2009) 983–988

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journal homepage: www.elsevier.com/locate/ybbrc

An efficient gene-disruption method in *Cryptococcus neoformans* by double-joint PCR with *NAT*-split markers

Min Su Kim [a,1], Seo-Young Kim [a,1], Ja Kyung Yoon [a], Yin-Won Lee [b], Yong-Sun Bahn [a,*]

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[b] Department of Agricultural Biotechnology, Center for Fungal Pathogenesis, Seoul National University, Seoul 151-921, Republic of Korea

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NAT
Overlap-PCR
Recombination
Split marker

ABSTRACT

Targeted gene disruption via biolistic transformation and homologous recombination is a method widely used to identify and investigate the function of genes in *Cryptococcus neoformans* that causes fatal fungal meningitis if not timely treated. Currently, most laboratories employ the overlap-PCR method to generate a gene-disruption cassette with dominant selectable markers, such as nourseothricin acetyltransferase (*NAT*). However, the conventional overlap-PCR method is often found to be inefficient because of the presence of multiple templates and of the long length of the final overlap-PCR products. In this report, we suggested an efficient gene-disruption method for *C. neoformans*, termed a double-joint PCR with *NAT*-split markers. Here we demonstrated that the gene-disruption cassette generated using double-joint PCR with *NAT*-split markers can be used successfully for targeted *C. neoformans* gene disruption with the advantages of providing a more convenient construction of gene-disruption cassettes and high targeted-integration frequency.

doi:10.1016/j.bbrc.2009.10.089

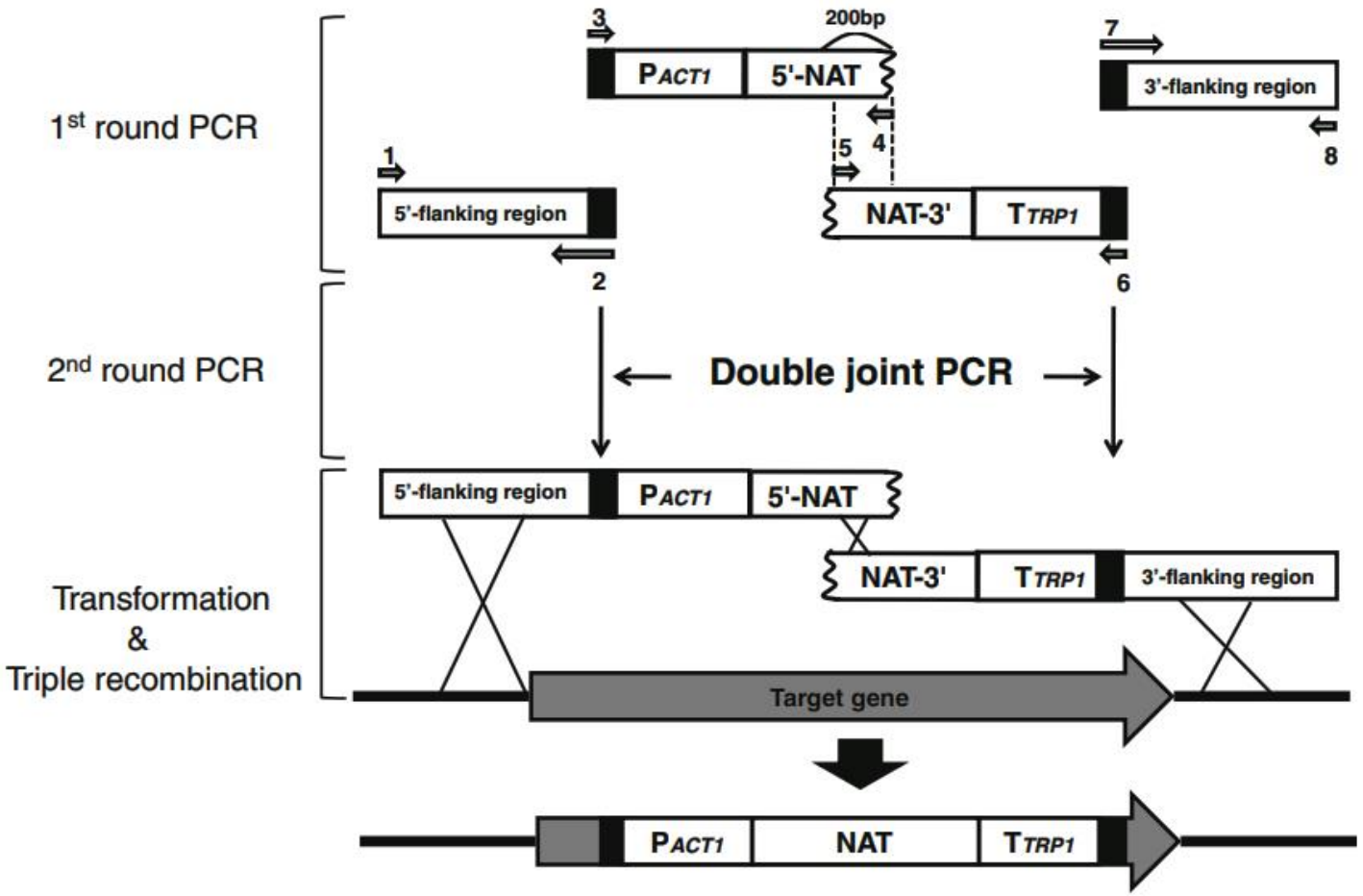
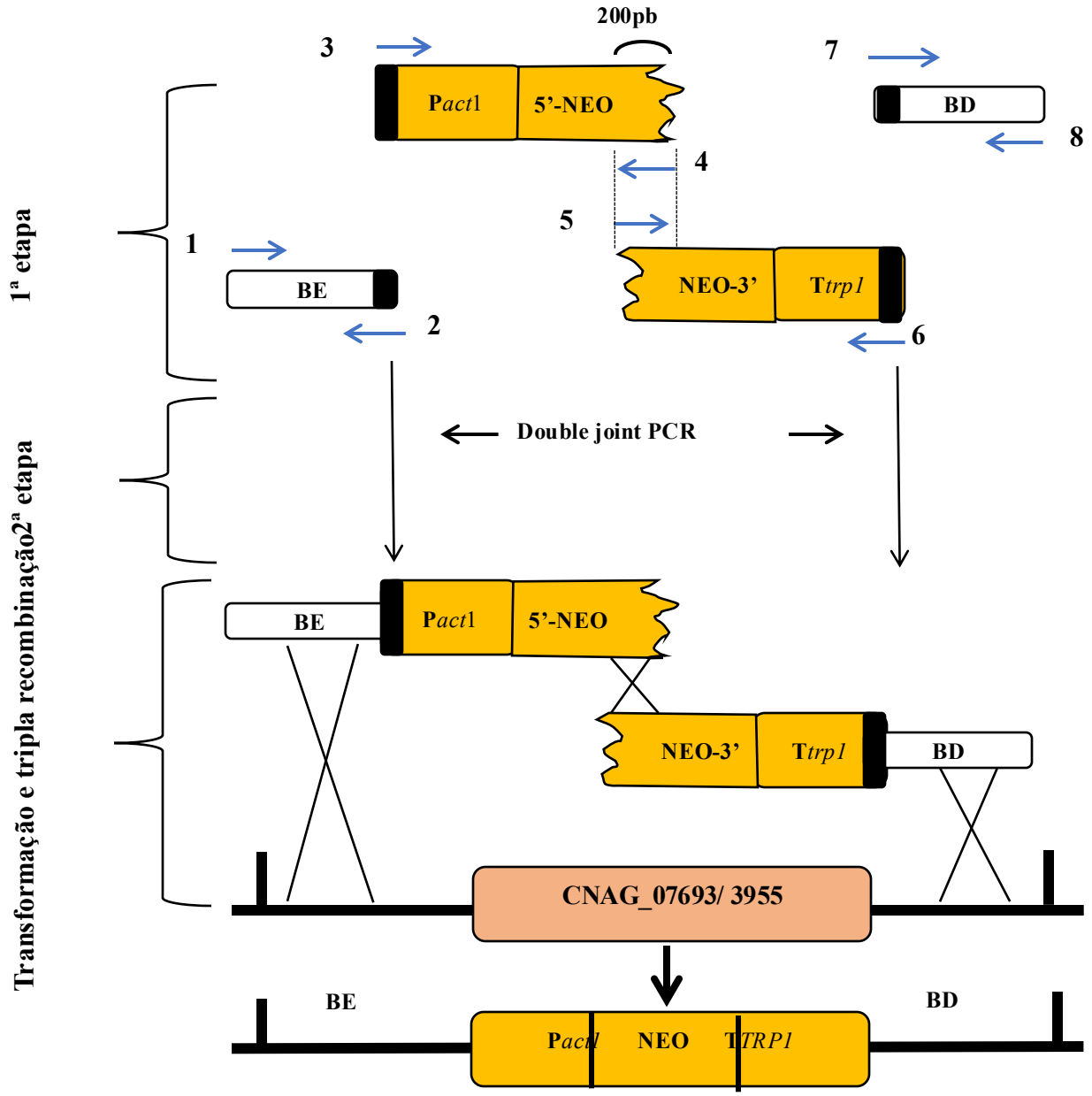
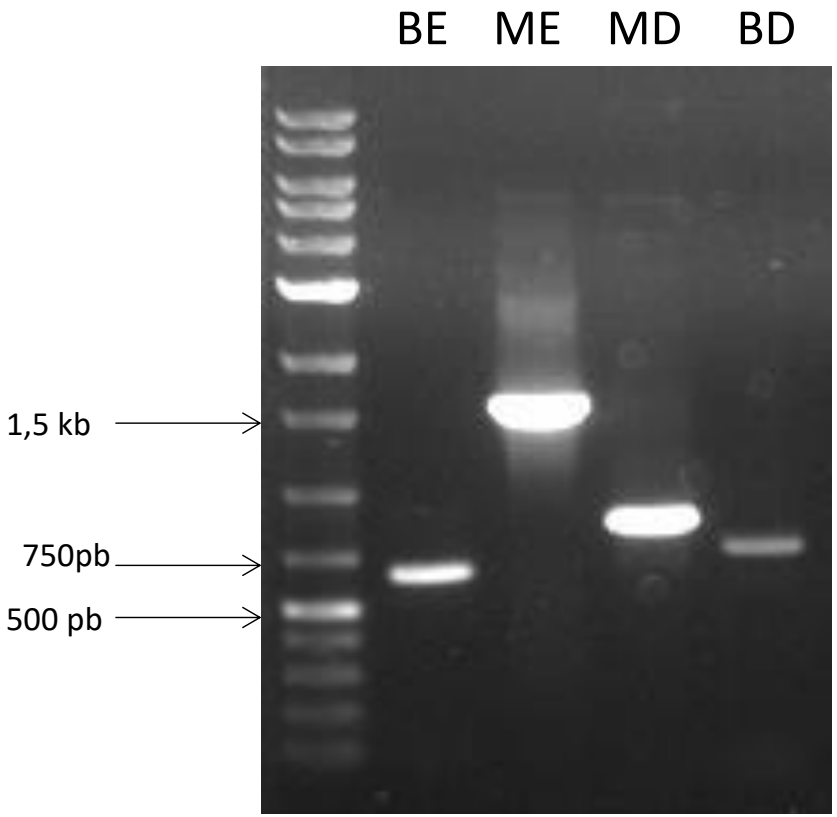


Fig. 1. The procedure for the double-joint PCR using *NAT*-split markers. At the first round of PCR, 5′- and 3′-flanking regions of a gene to be deleted were amplified using primers 1 and 2, and primers 7 and 8, respectively. The 5′- and 3′-*NAT*-split markers were amplified using primers 3 and 4, and primers 5 and 6, respectively. At the second round of PCR, the 5′-flanking region of the target gene and 5′-*NAT*-split marker were combined and amplified using primers 1 and 4 (double-joint PCR). Similarly, the 3′-flanking region of the target gene and 3′-*NAT*-split marker were combined and amplified using primers 5 and 8. The two DJ-PCR fragments were combined and introduced using biolistic transformation into the cell, where triple recombination occurs between the native locus of the target gene and split disruption cassette. P_{ACT1}, the *ACT1* promoter; T_{TRP1}, the *TRP1* terminator; *NAT*, nourseothricin acetyltransferase.

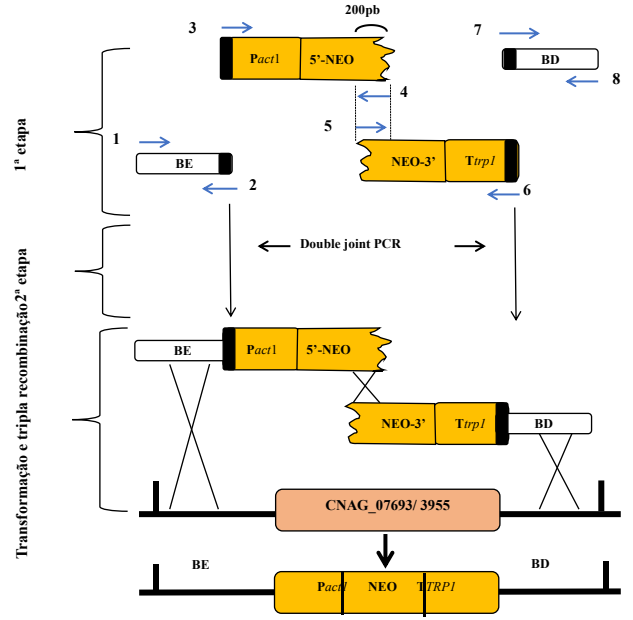
doi:10.1016/j.bbrc.2009.10.089

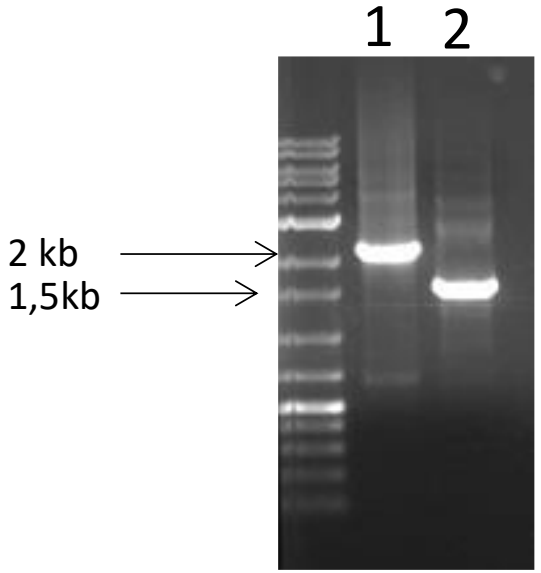


Possari, et al 2025.



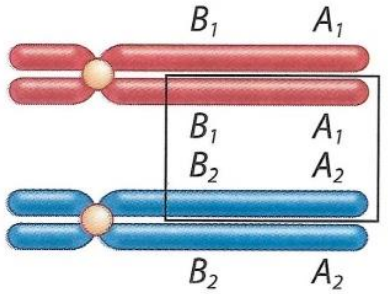
BE – Braço Esquerdo
ME – Marcador Esquerdo
MD - Marcador Direito
BD – Braço Direito





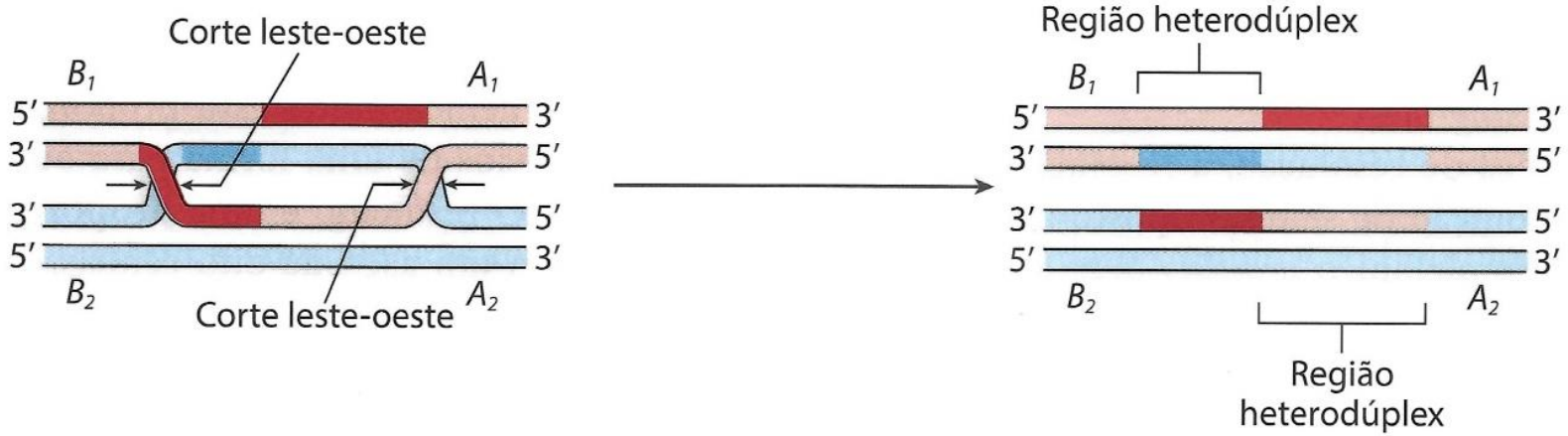
1- Fusão fragmentos esquerdos (E) purificado
2- Fusão fragmentos direitos (D) purificado

Obrigado



Recombinação meiótica diagramada entre essas cromátides não irmãs dos cromossomos homólogos

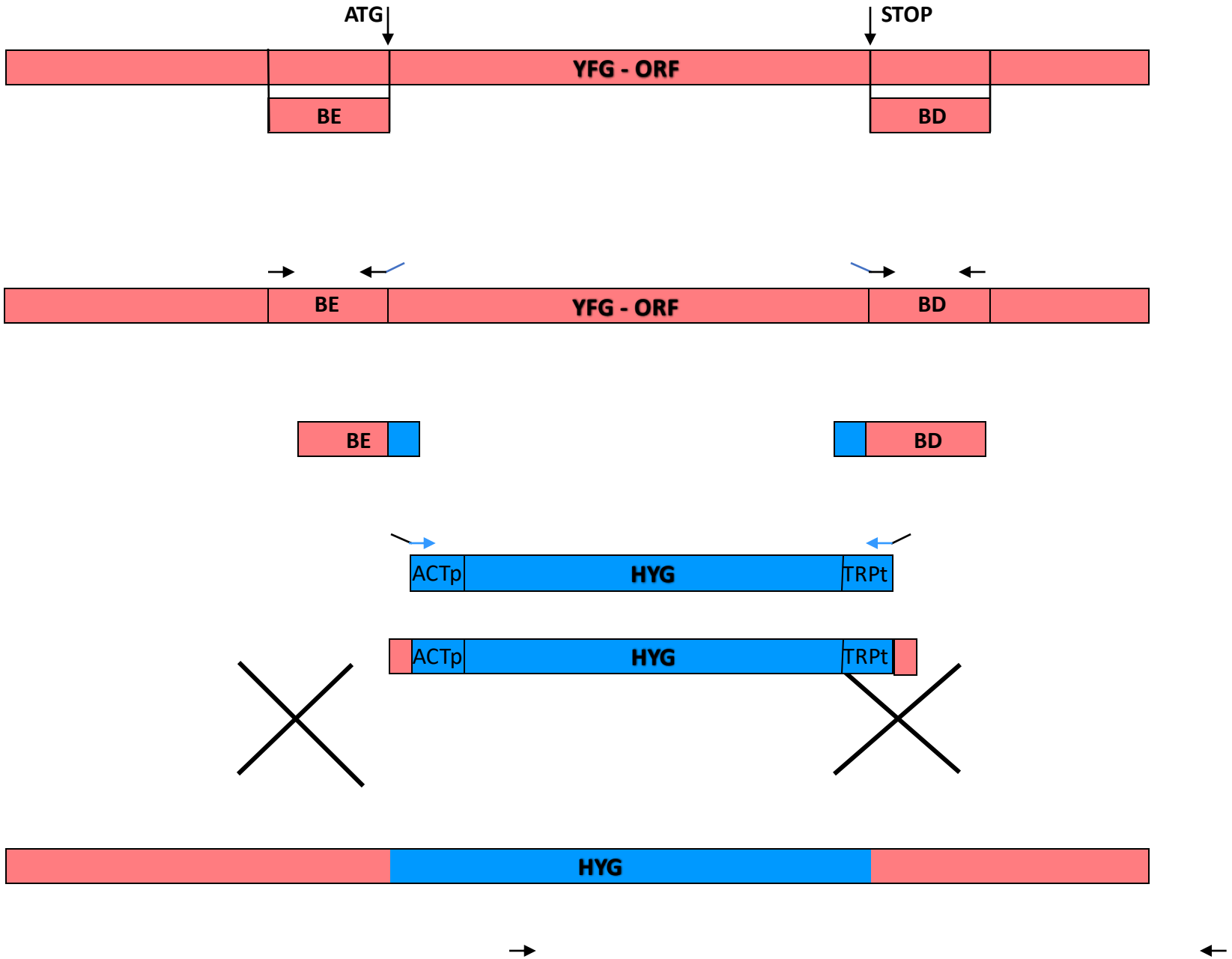
1 Resolução de mesmo sentido



A resolução de mesmo sentido produz regiões heterodúplex deslocadas, mas nenhuma recombinação dos genes laterais. Essa forma de resolução raramente ocorre.

Sanders & Bowman, eds, 2014. Person, SP. 847p

Overlapping PCR



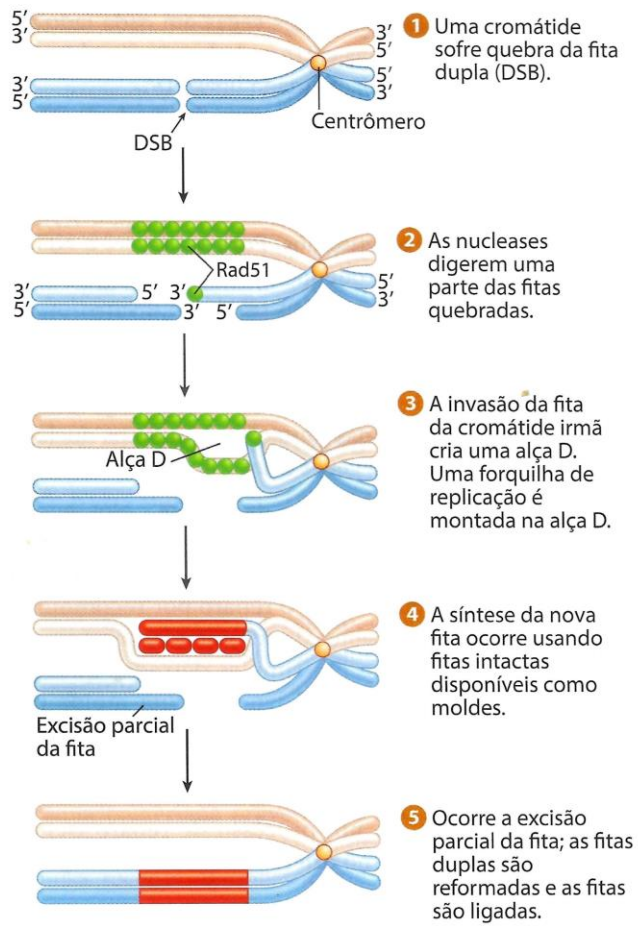


Figura 12.24 Anelamento da fita dependente da síntese (SDSA).

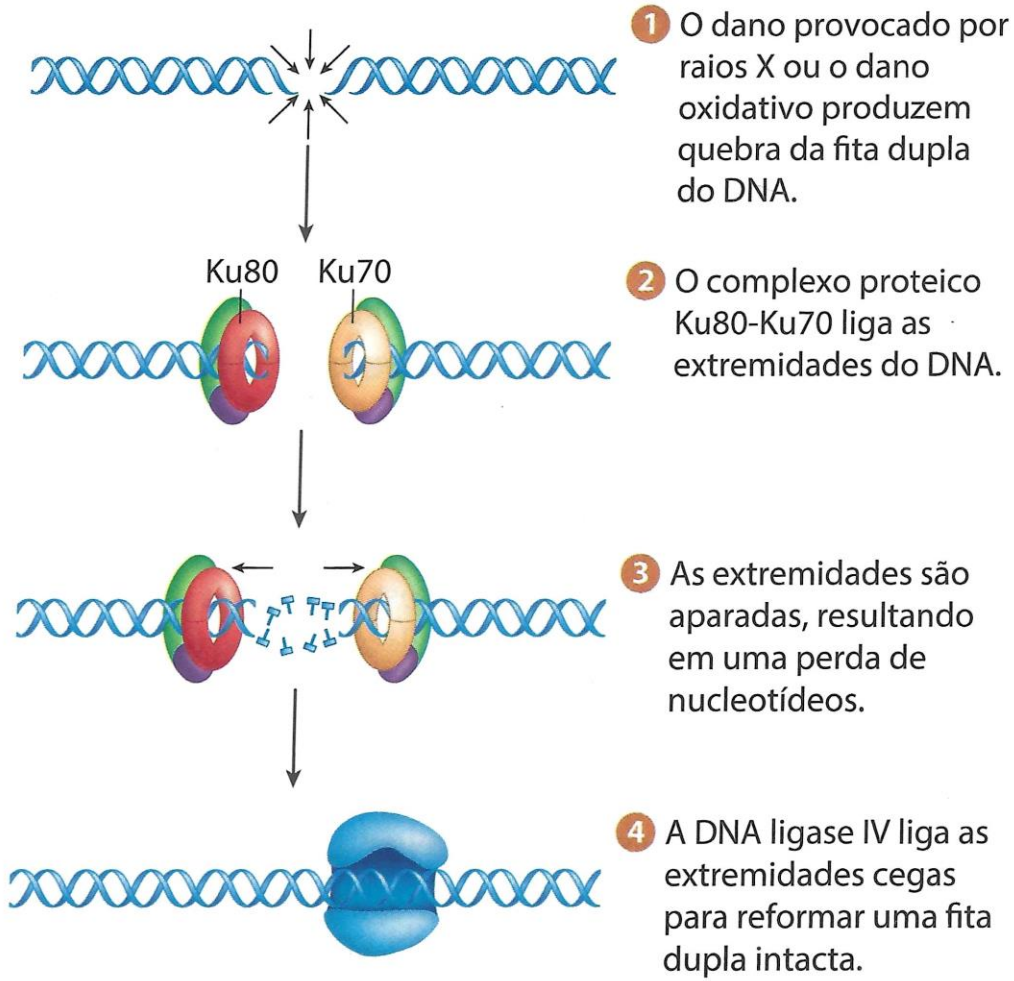


Figura 12.23 Ligação das extremidades não homólogas. A NHEJ é um sistema propenso a erros que junta novamente as fitas do DNA após uma quebra da fita dupla.

Sanders & Bowman, eds, 2014. Person, SP. 847p