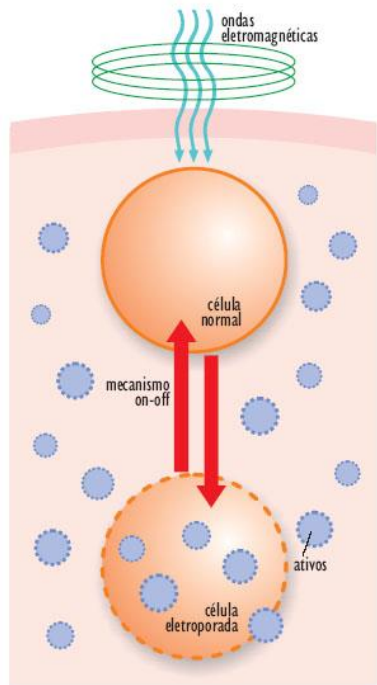


LGN0232 - Genética Molecular

Tecnologia do DNA recombinante: Transformação bacteriana e PCR

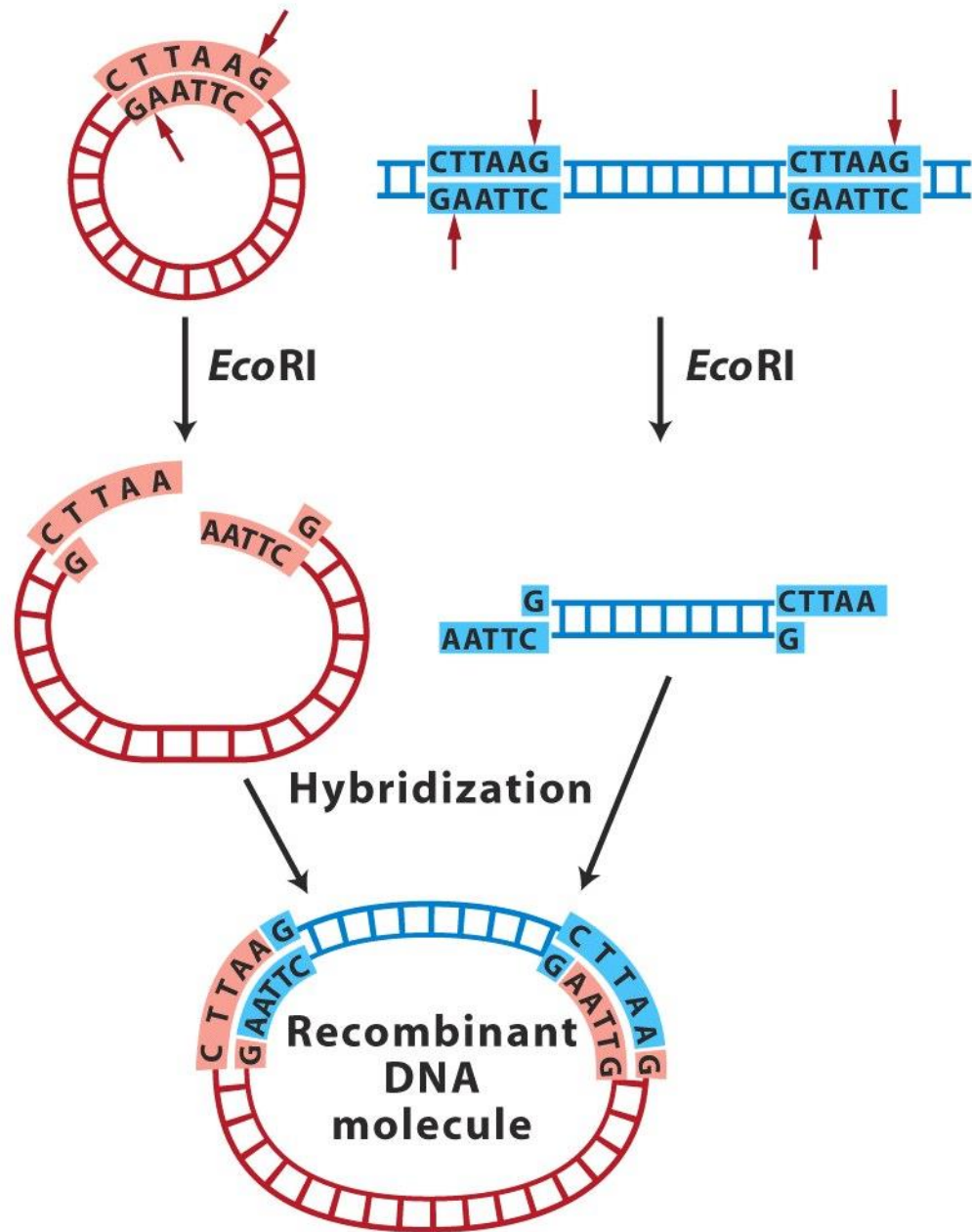


4^a aula

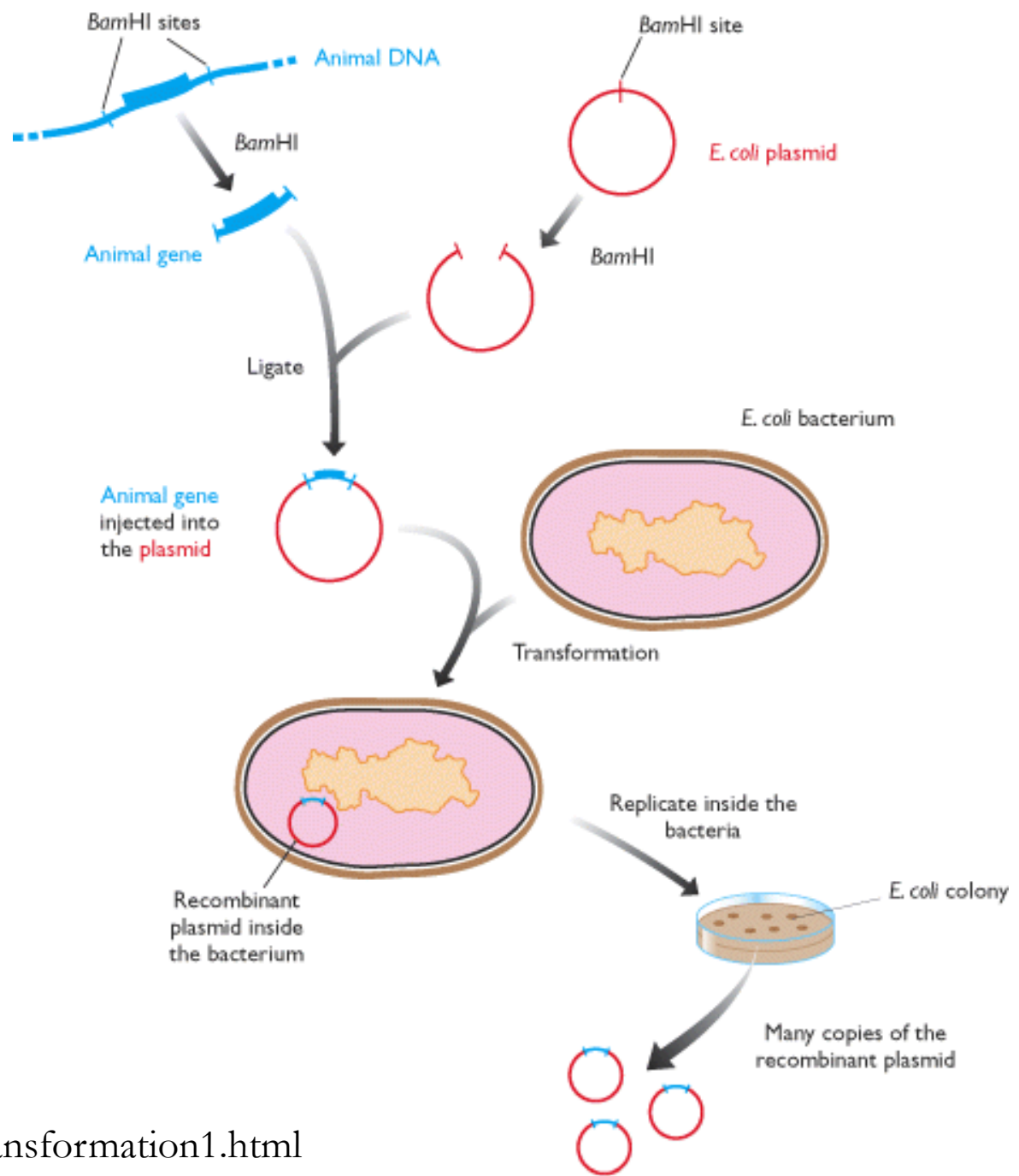
Antonio Figueira

CENA

figueira@cena.usp.br



Formação de uma
molécula de
DNA recombinante



Clonagem Celular



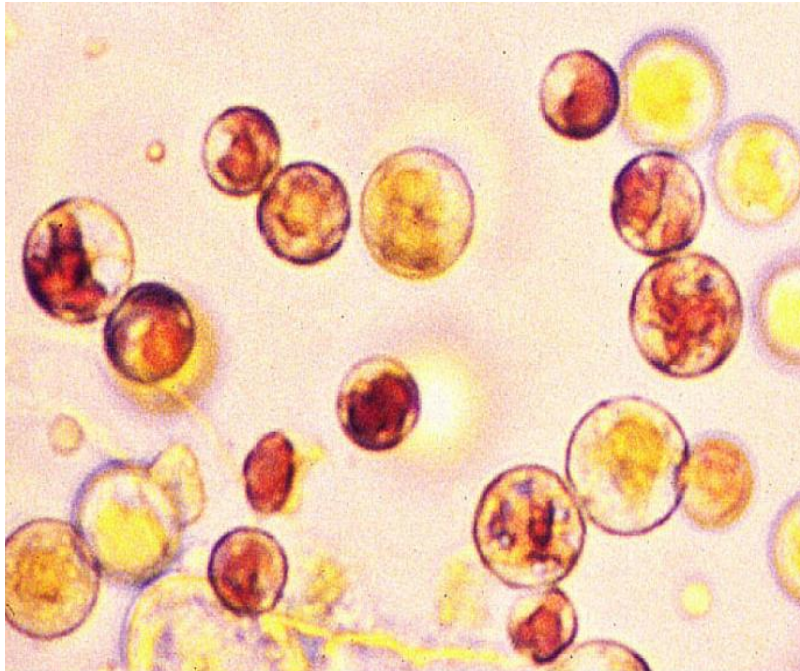
Cópias Idênticas!!

Clone: uma coleção de moléculas ou células, todas idênticas a uma molécula ou célula original.



Clonagem Molecular

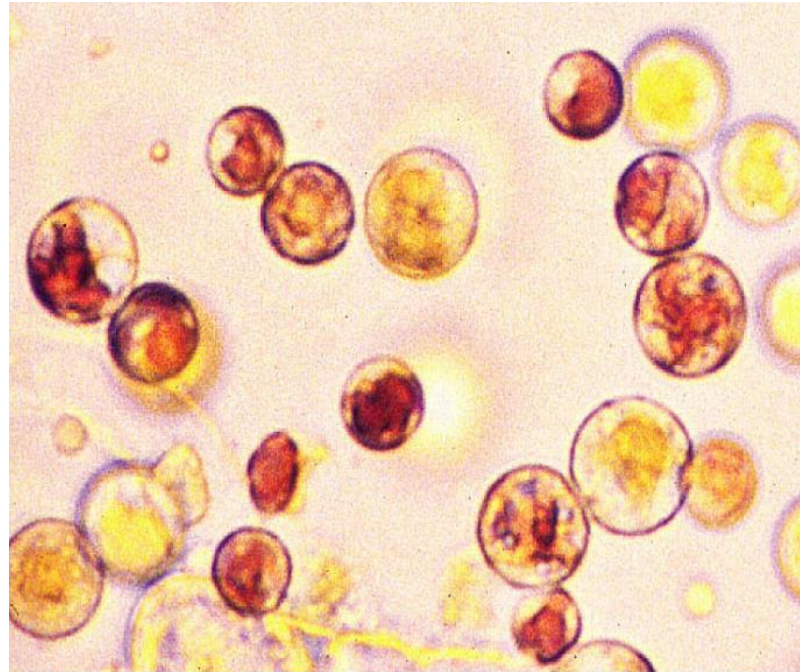
Dependente de células
vivas



Independente de células vivas
(PCR)



Clonagem dependente de células vivas



Atores no DNA Recombinante

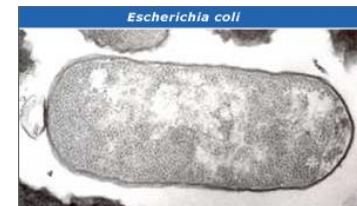
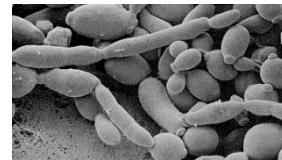
Ferramentas de clonagem

Enzimas: enzimas de restrição
DNA ligase

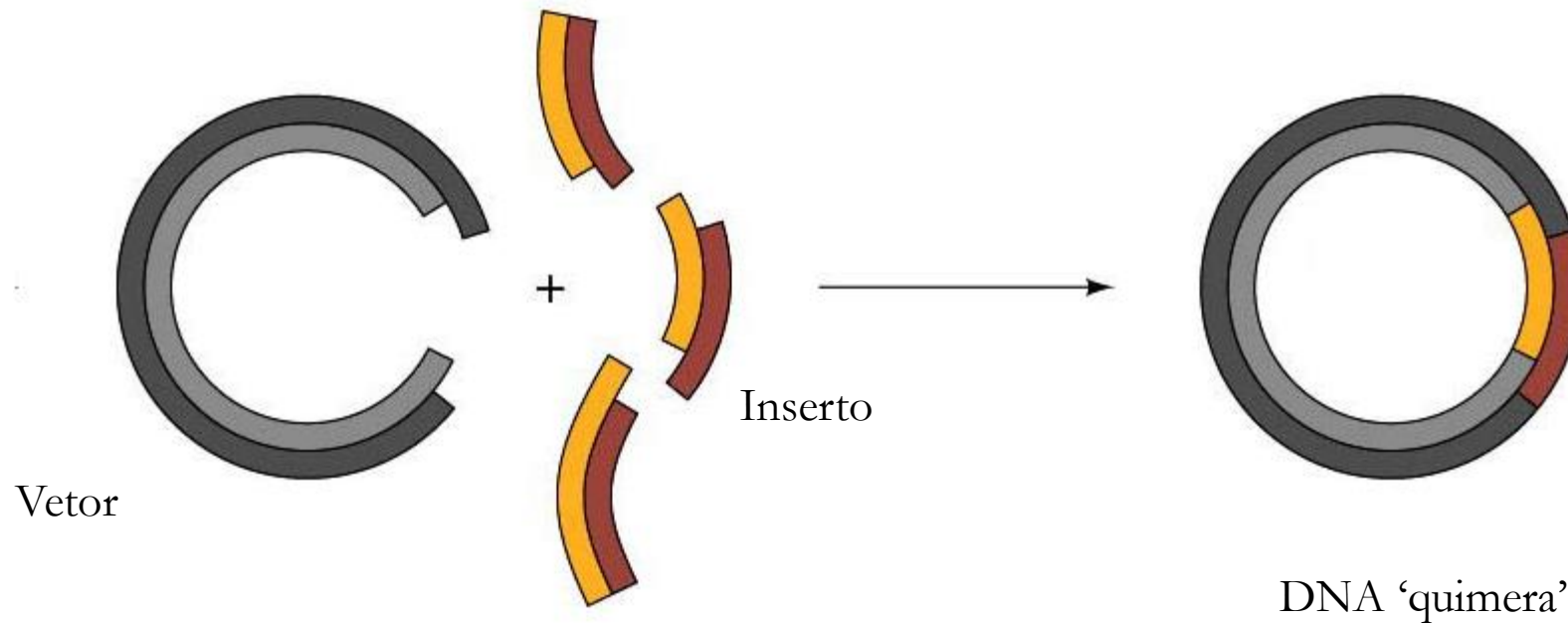
Vetores: plasmídeos
fagos (vírus de bactérias)
cosmídeos
cromossomos artificiais (YAC, BAC)

Hospedeiros: *Escherichia coli*

leveduras
células animais
células vegetais
células de insetos,..

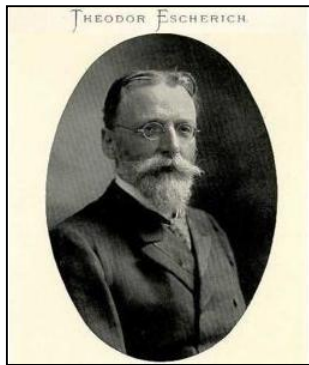


Lembrando: vetores de clonagem –
multiplicam moléculas de DNA recombinante
(clones) em organismos vivos, gerando
quantidades abundantes de clones



Fragmentos de DNA ligados a um **vetor de clonagem**

DNA recombinante



Escherichia coli



- Unicelular, na forma de bastão;
- Cresce rapidamente a 37°C ;
 - ciclo de 20 min

Uma geração a cada 20 minutos



- Pouco exigente quanto a nutrição;
- Meio mínimo ou rico:
 - Glicose - melhor fonte de C
 - Extrato de levedura - vitaminas
 - Triptona e Peptona: fonte aminoácidos

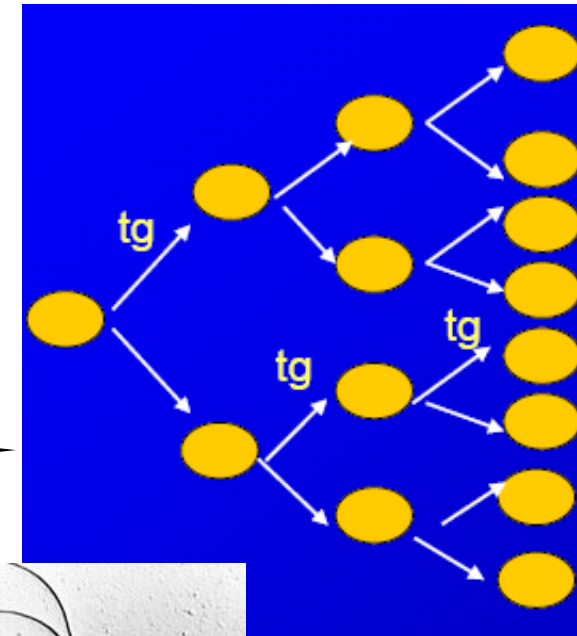
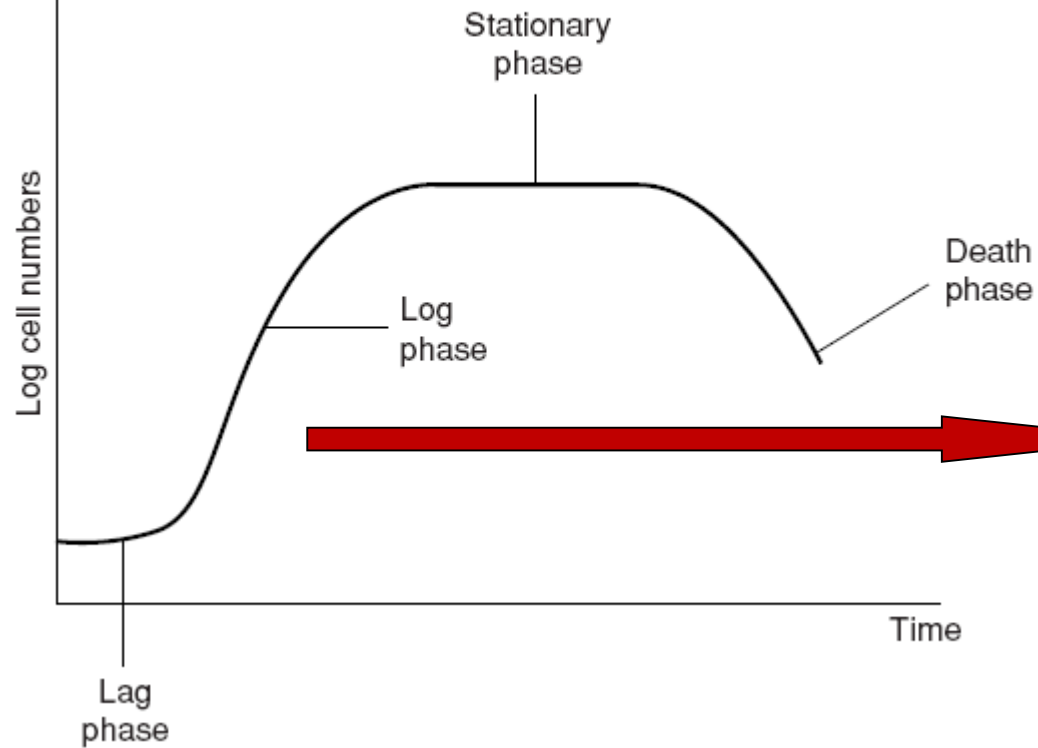


Progressão Geométrica do Crescimento Populacional Bacteriano

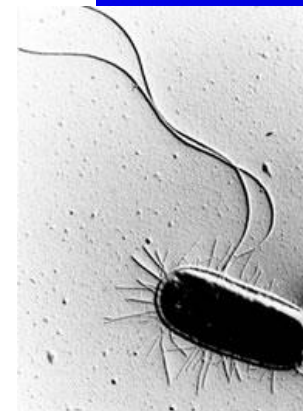


MICROBIAL GROWTH

Uma geração: 20 minutos



Curva de crescimento



Bactérias para Transformação

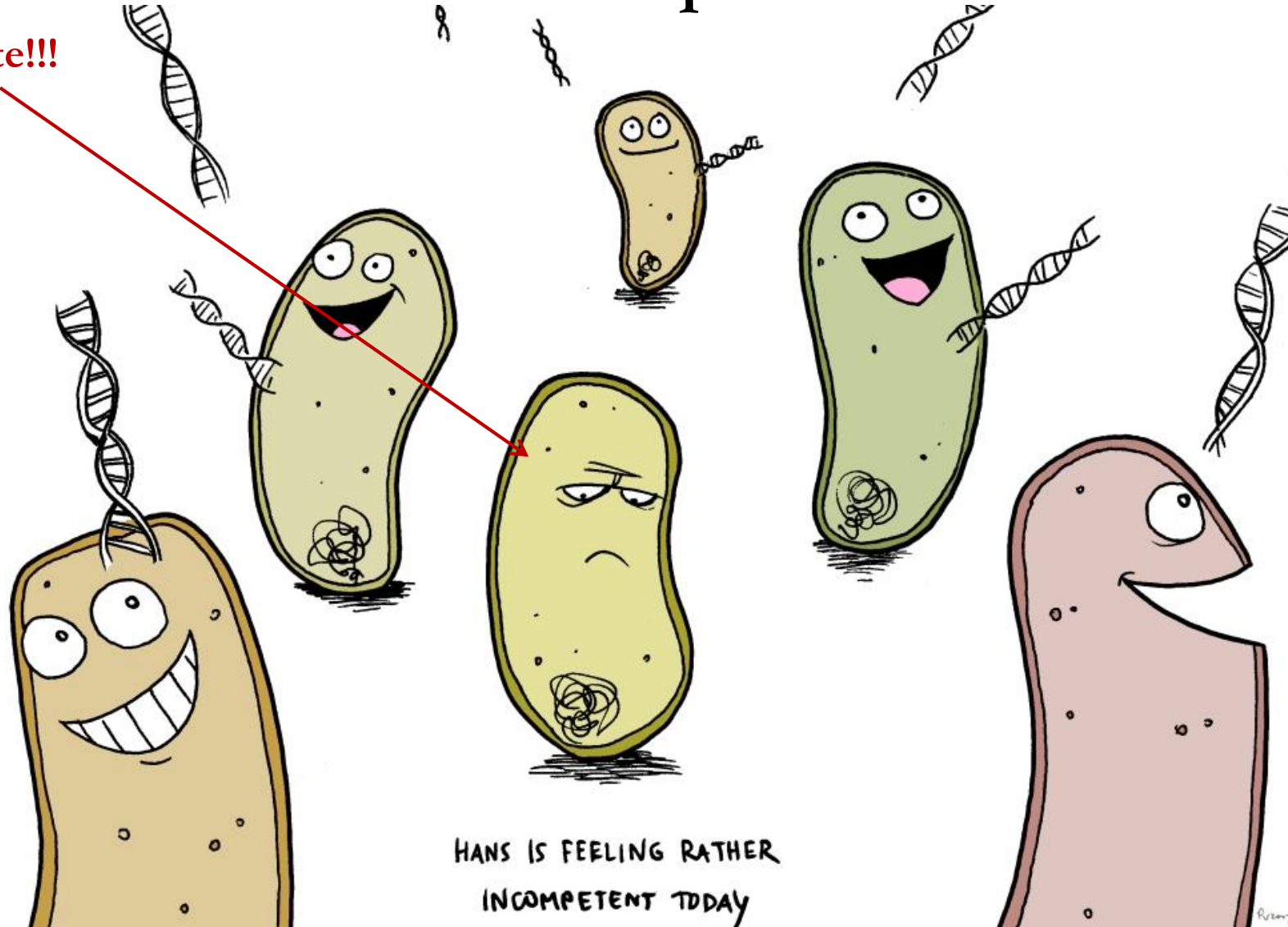
- Não apresentam DNA plasmidial



- Células das bactérias precisam ser preparadas
 - Tornar-se competentes = aptas a receberem a molécula de DNA recombinante
 - Na fase de crescimento exponencial!

Células Competentes

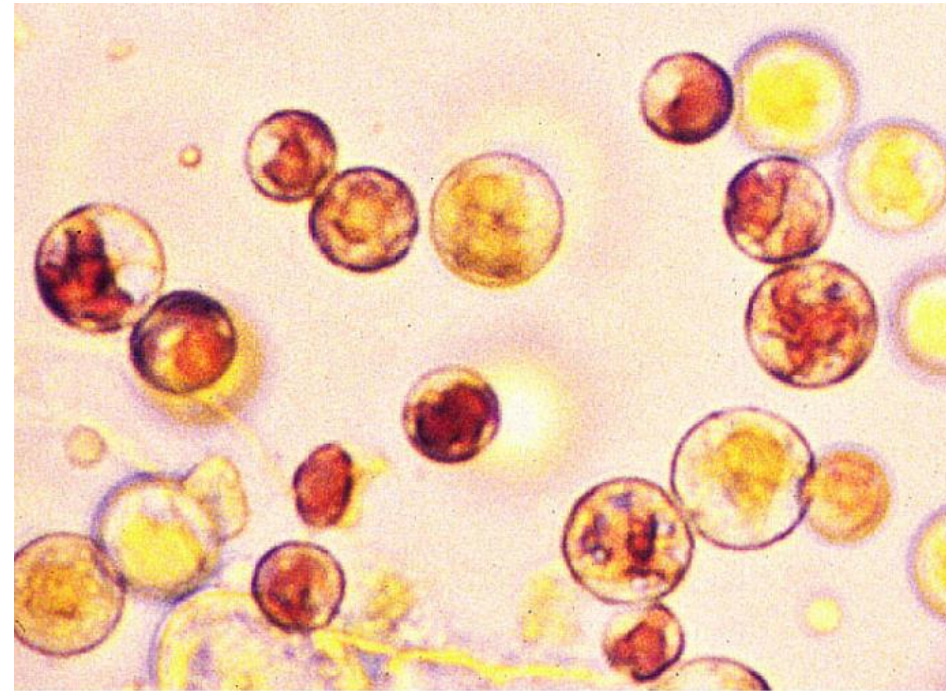
Incompetente!!!



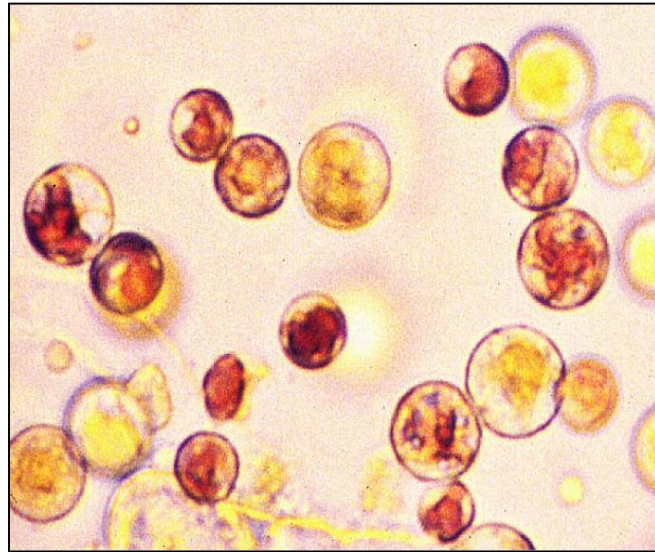
Uma bactéria é **competente** quando tem a **capacidade de receber e multiplicar DNA exogeno.**

Inserção do Plasmídeo (DNA) dentro da Célula Bacteriana – Hospedeiro

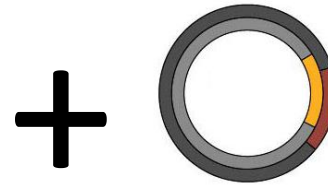
- Transformação química (choque térmico)
- Eletroporação



Transformação Química

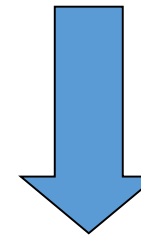


Suspensão de células competentes

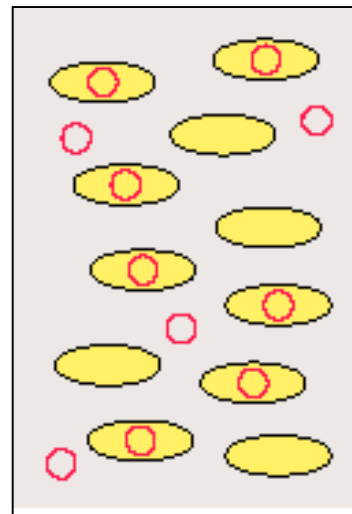
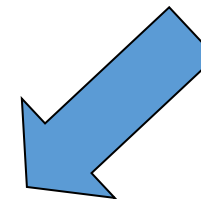


Moléculas de DNA recombinante

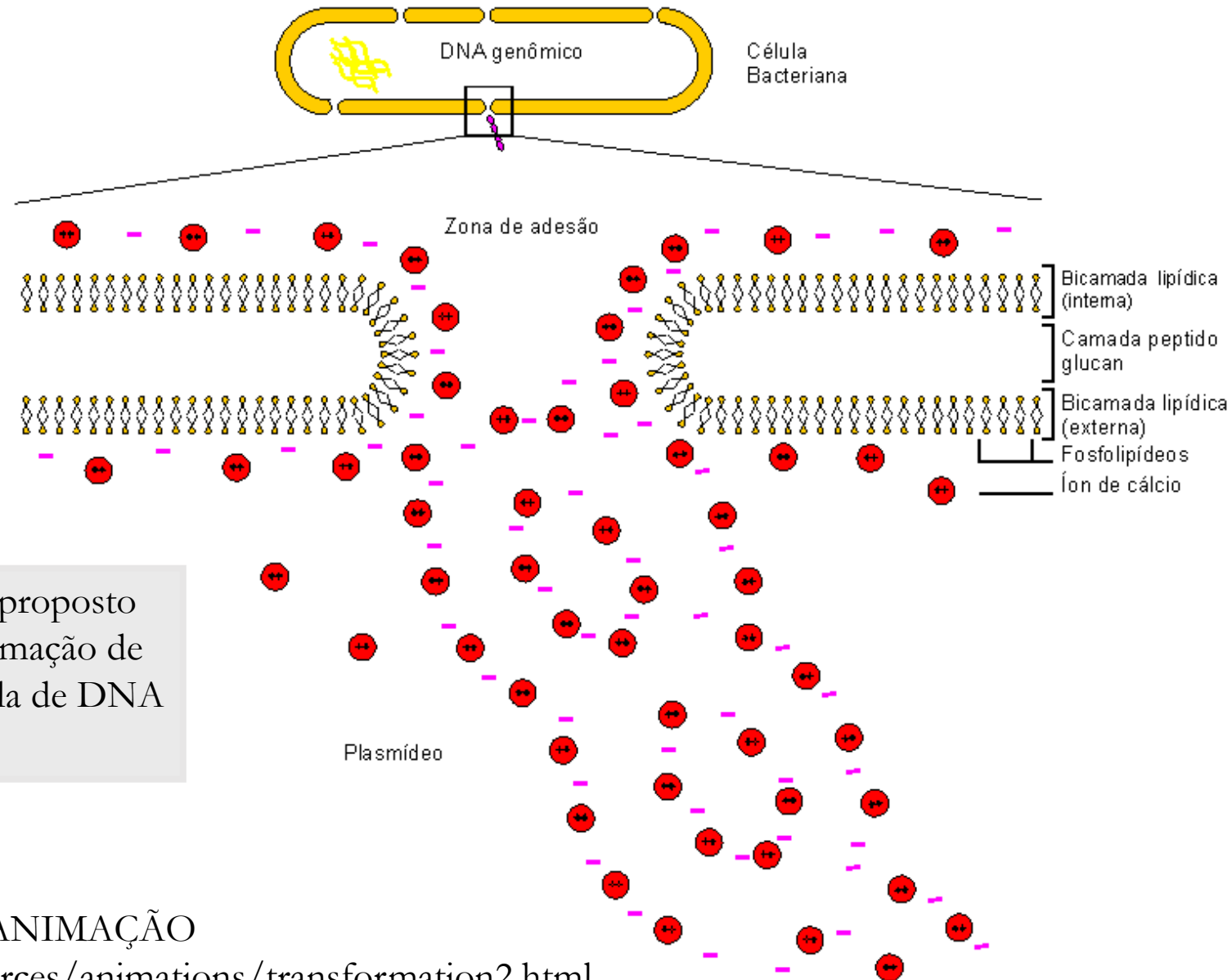
= Incubar por 30 min no gelo



Choque térmico
(42°C/30 s)



Transformação Química



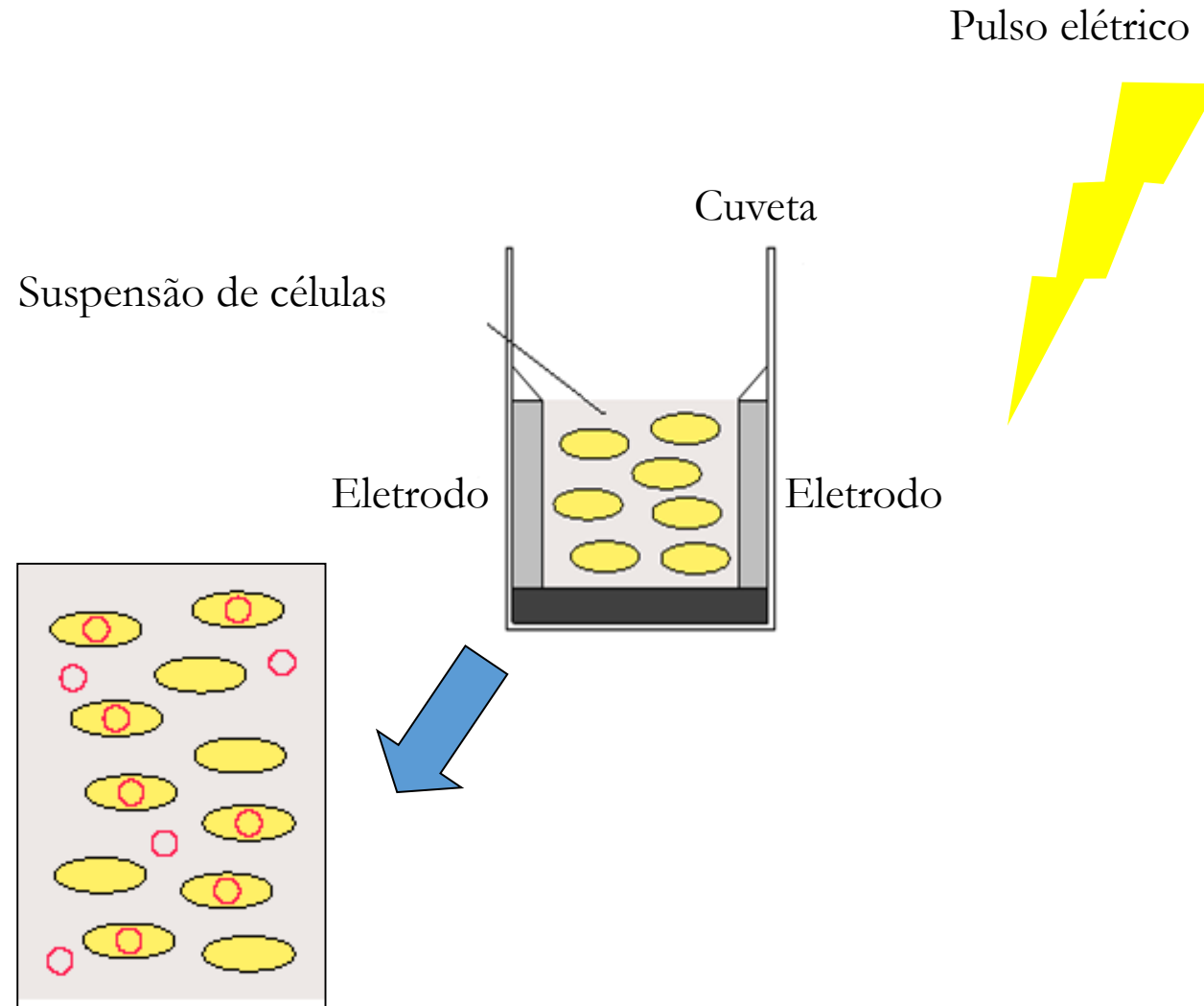
Mecanismo molecular proposto para explicar a transformação de *E. coli* com uma molécula de DNA exógeno.

ANIMAÇÃO

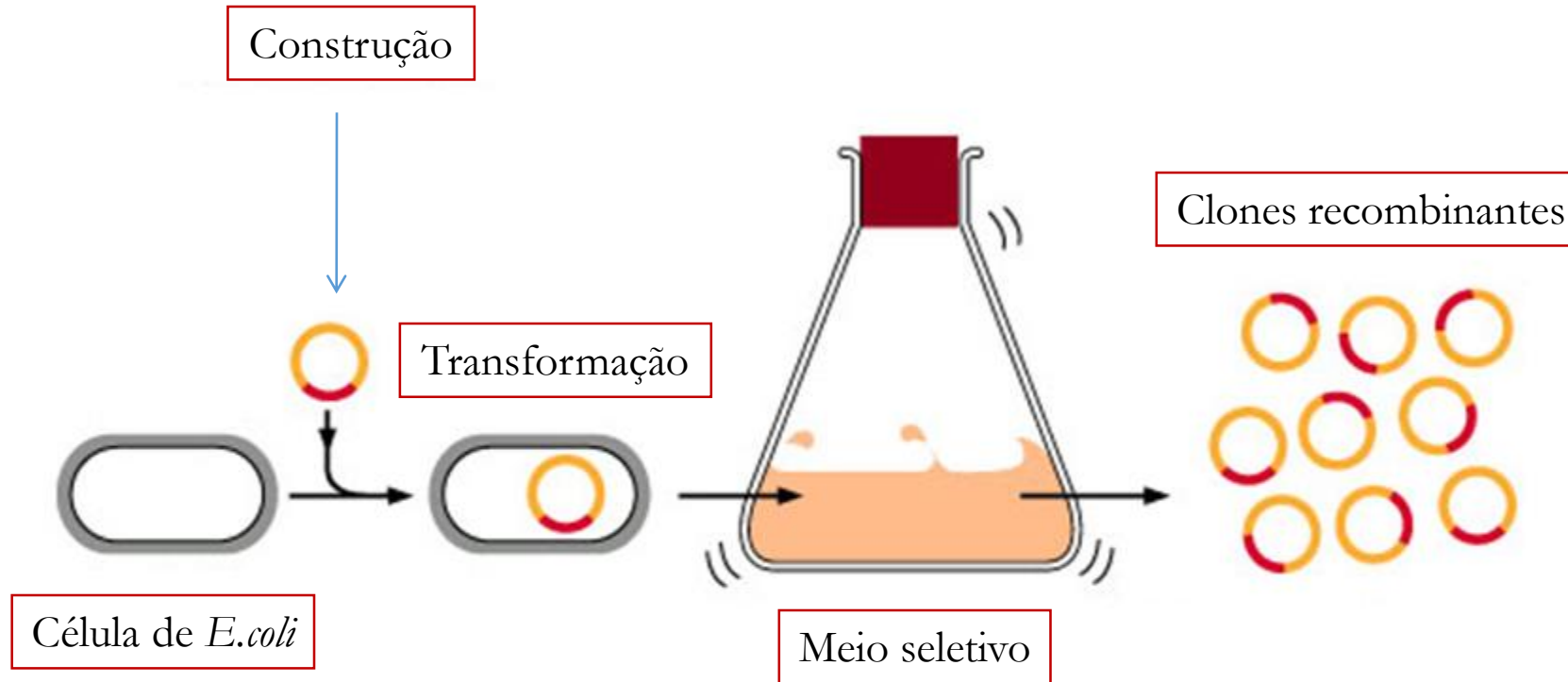
Eletroporação



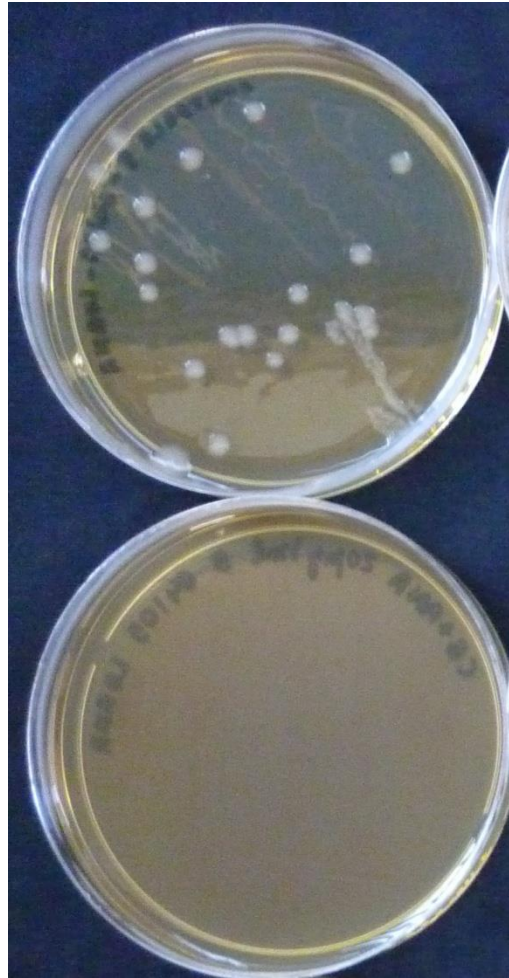
Eletroporador



Seleção de Clones Recombinantes



Meio de Cultura + ampicilina



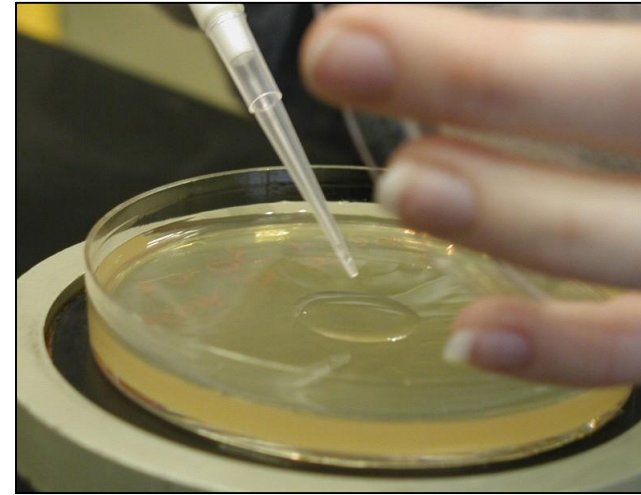
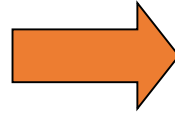
Células transformadas

Clones resistentes a ampicilina

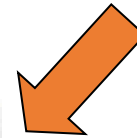
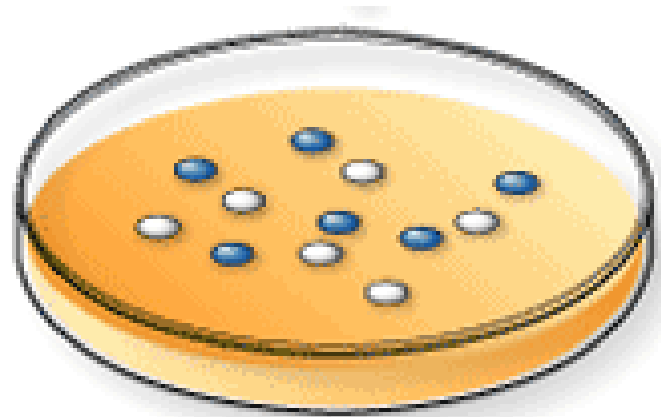
Células controle (não transformadas)

Clones sensíveis a ampicilina

Seleção de Clones Recombinantes



MC+X-gal+ampicilina



Período de incubação = 12-16 h a 37°C

*MC = Meio de cultura

Seleção de Clones Recombinantes

➤ Utilizados marcadores que são **inativados** com a inserção do DNA exógeno no plasmídeo

Exemplo: sistema de diferenciação **branco-azul**

- Plasmídeos da série pUC possuem um gene (gene *lacZ*) que codifica a produção da enzima β -galactosidase.
A inserção do DNA exógeno (inserto) no sítio de clonagem do plasmídeo causa a interrupção do gene, levando à perda da função da β -galactosidase.

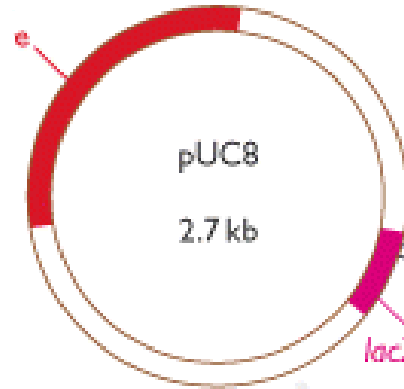
Como resultado:

- colônias brancas (β -gal inativa) - positivas (**com inserto**)
- colônias **azuis** (β -gal ativa) - negativas (**sem inserto**)

Seleção de Clones Recombinantes

pUC8
Vieira & Messing, 1982

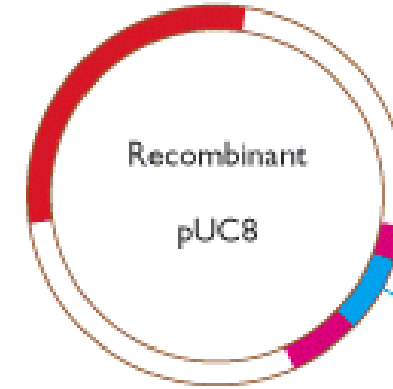
Gene de resistência à
ampicilina



Sítio múltiplo de
clonagem

- HindIII
- PstI
- SalI, AclI, HincII
- BamHI
- SmaI, XmaI
- EcoRI

lacZ' gene



DNA insert

Produção da enzima β -galactosidase

X-gal é clivado = **colônia azul**

Não há produção da enzima

β -galactosidase

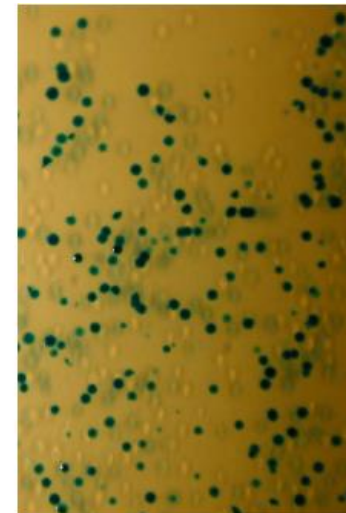
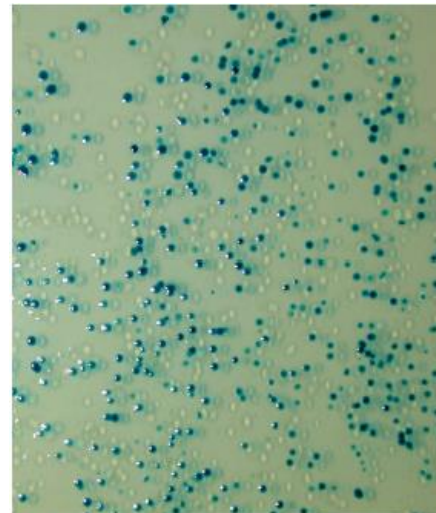
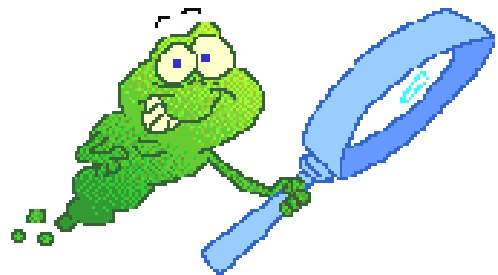
X-gal não é clivado = colônia branca

Ágar + ampicilina + X-gal

Gene *lacZ* = codifica a enzima β -galactosidase

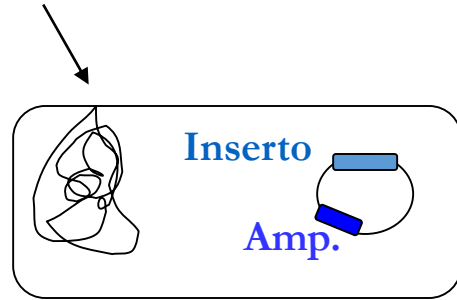


Quais colônias nos interessam e por quê?



Possíveis Resultados após a Etapa de Transformação

Célula bacteriana



MC+X-gal+amp

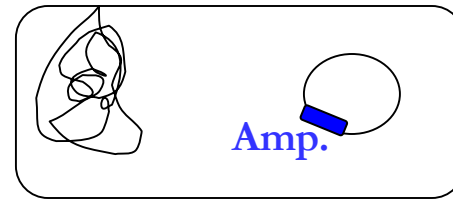


Plasmídeo com inserto

✓ Resistência à ampicilina

✓ *LacZ* não funcional

✓ **Colônias brancas**



MC+X-gal+amp



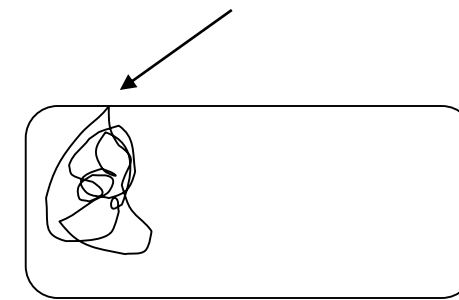
Plasmídeo sem inserto

✓ Resistência à ampicilina

✓ *LacZ* funcional

✓ **Colônias azuis**

DNA cromossomal



MC+X-gal+amp

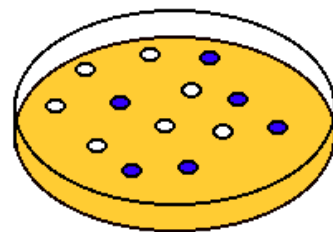


Sem plasmídeo

✓ **Sem** resistência à ampicilina

✓ **Sem** gene *LacZ*

✓ **Sem** crescimento

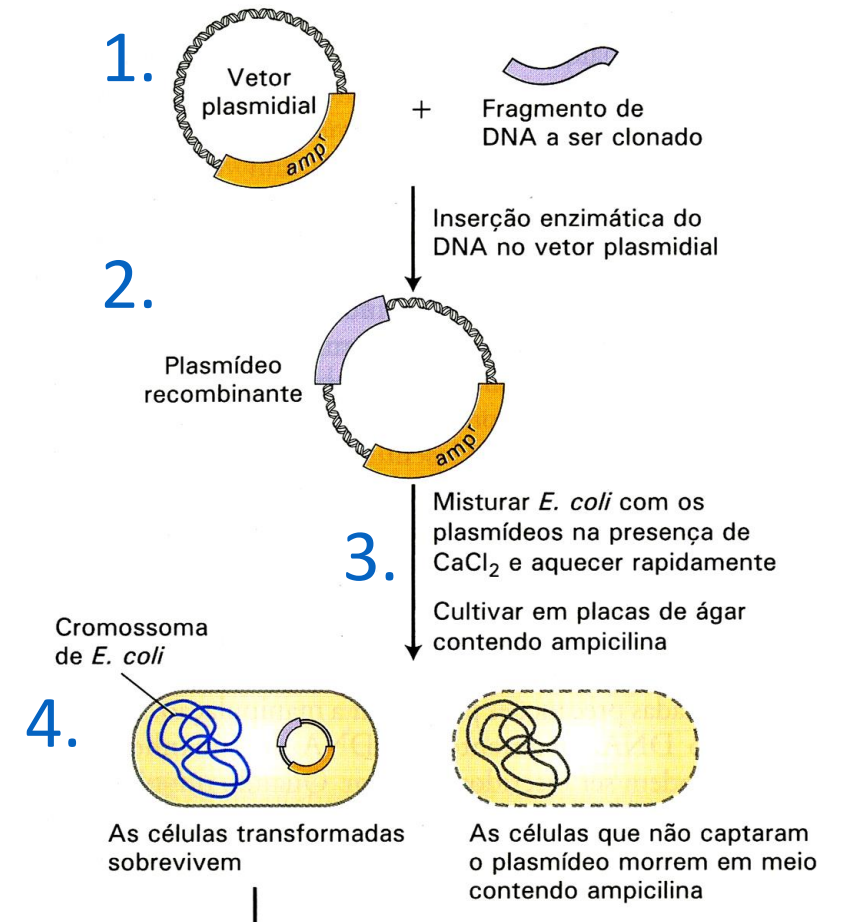


*MC = Meio de cultura

Clonagem Molecular

RESUMO DAS ETAPAS:

1. Preparação do vetor e do inserto
2. Ligação do vetor e inserto
3. Transformação em célula hospedeira
4. Seleção de clones



Clonagem Independente de Células Vivas

PCR



PCR: Polymerase Chain Reaction

(Reação em cadeia da polimerase)



- O processo de PCR foi **inventado** por Kary Mullis na década de 1980 → atribuído o Premio Nobel da Química 1993
- É um método de amplificação (criação de múltiplas cópias) enzimática de DNA **sem** o uso de um organismo vivo, como por exemplo, *Escherichia coli* (bactéria) ou leveduras.

<http://www.karymullis.com/pcr.shtml>

If the K-T boundary isotopic spike is indeed the result of impact-related acid rain, the oceanic strontium isotope record may reveal other large impacts. The seawater strontium curve of Burke *et al.* (9), which spans the past 500 million years, shows at least two other prominent high spikes in the $^{87}\text{Sr}/^{86}\text{Sr}$ ratio, one in the mid-Cretaceous, at ~ 100 million years, and the other in the Pennsylvanian, at ~ 290 million years. The first appears to precede by a few million years the mass extinction event at the Cenomanian-Turonian boundary. There is also a large increase in $^{87}\text{Sr}/^{86}\text{Sr}$ across the Permian-Triassic boundary (9), the time of the most extreme mass extinction in the Phanerozoic record (17). However, the increase appears to be rather gradual, extending over 20 million to 25 million years, and is thus quite different in character from the K-T spike. Nevertheless, data are sparse for this interval, and more work will be required to determine the exact nature of the increase.

The occurrence of a spike toward higher values in the seawater $^{87}\text{Sr}/^{86}\text{Sr}$ record at the K-T boundary is tantalizing evidence for

18. I thank many colleagues at Scripps for comments on the ideas expressed in this report, in particular G. Arrhenius, S. Galer, J. Gieskes, M. Kastner, D. Lal, G. Lugmair, and H.-G. Stosch. Comments from two anonymous reviewers also improved the original manuscript. I thank P. Hey for preparation of

the manuscript. This work was supported in part by grants from the National Science Foundation and the National Aeronautics and Space Administration.

28 September 1987; accepted 7 December 1987

Primer-Directed Enzymatic Amplification of DNA with a Thermostable DNA Polymerase

RANDALL K. SAIKI, DAVID H. GELFAND, SUSANNE STOFFEL, STEPHEN J. SCHARF, RUSSELL HIGUCHI, GLENN T. HORN, KARY B. MULLIS,* HENRY A. ERLICH

A thermostable DNA polymerase was used in an *in vitro* DNA amplification procedure, the polymerase chain reaction. The enzyme, isolated from *Thermus aquaticus*, greatly simplifies the procedure and, by enabling the amplification reaction to be performed at higher temperatures, significantly improves the specificity, yield, sensitivity, and length of products that can be amplified. Single-copy genomic sequences were amplified by a factor of more than 10 million with very high specificity, and DNA segments up to 2000 base pairs were readily amplified. In addition, the method was used to amplify and detect a target DNA molecule present only once in a sample of 10^5 cells.

Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich. Primer-Directed Enzymatic Amplification of DNA with a Thermostable DNA Polymerase. 1988. *Science* 239: 487-491.

PCR: *Polymerase Chain Reaction*

- **1. Desnaturalização** (94-96°C, 30-600 segundos).

Durante a desnaturalização, a cadeia dupla do DNA é separada em duas cadeias simples.

A DNA polimerase deve ser estável a altas temperaturas pois é obtida de organismos que vivem em ambientes extremos (extremófilos). A DNA polimerase mais usada é a *Taq* polimerase (obtida de *Thermus aquaticus*).

- **2. Annealing** (emparelhamento, pareamento, hibridização) (45-80°C, 30-120 segundos).

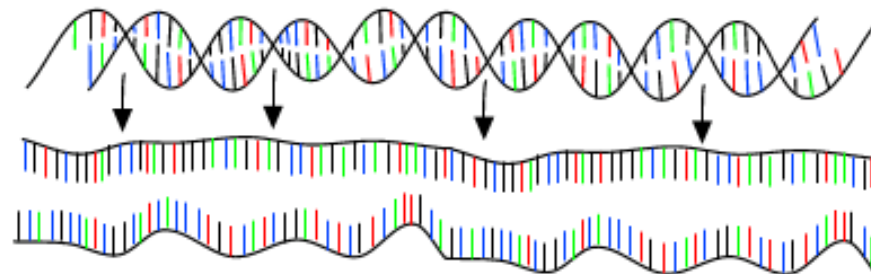
Durante o emparelhamento, os iniciadores (*primers*) ligam-se ao DNA de cadeia simples e a DNA polimerase liga-se aos iniciadores emparelhados.

- **3. Alongamento (polimerização)** (65-80° C, 30-120 segundos).

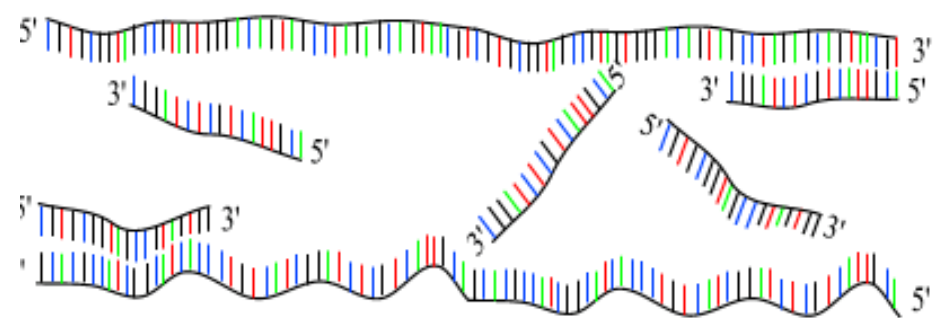
Durante o alongamento, a DNA polimerase cria a cadeia de DNA complementar à medida que percorre o DNA de cadeia simples, incorporando desoxirribonucleótidos presentes na reação.

Após cada ciclo, a quantidade de DNA duplica. Assim, após múltiplas ciclos, o aumento da quantidade de DNA é exponencial de base 2.

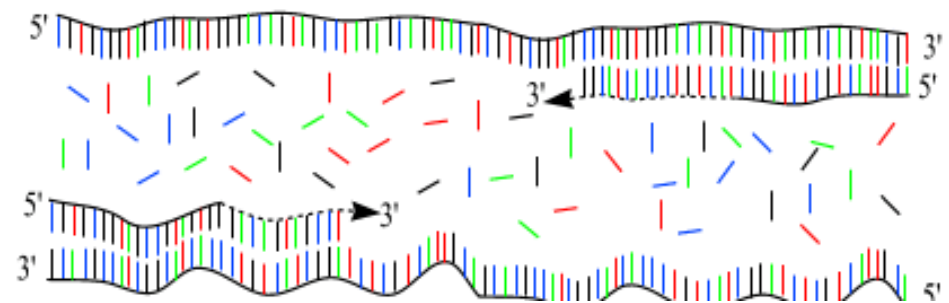
<https://www.youtube.com/watch?v=2KoLnIwoZKU>



Step 1 – desnaturação
1 minuto – 94°C

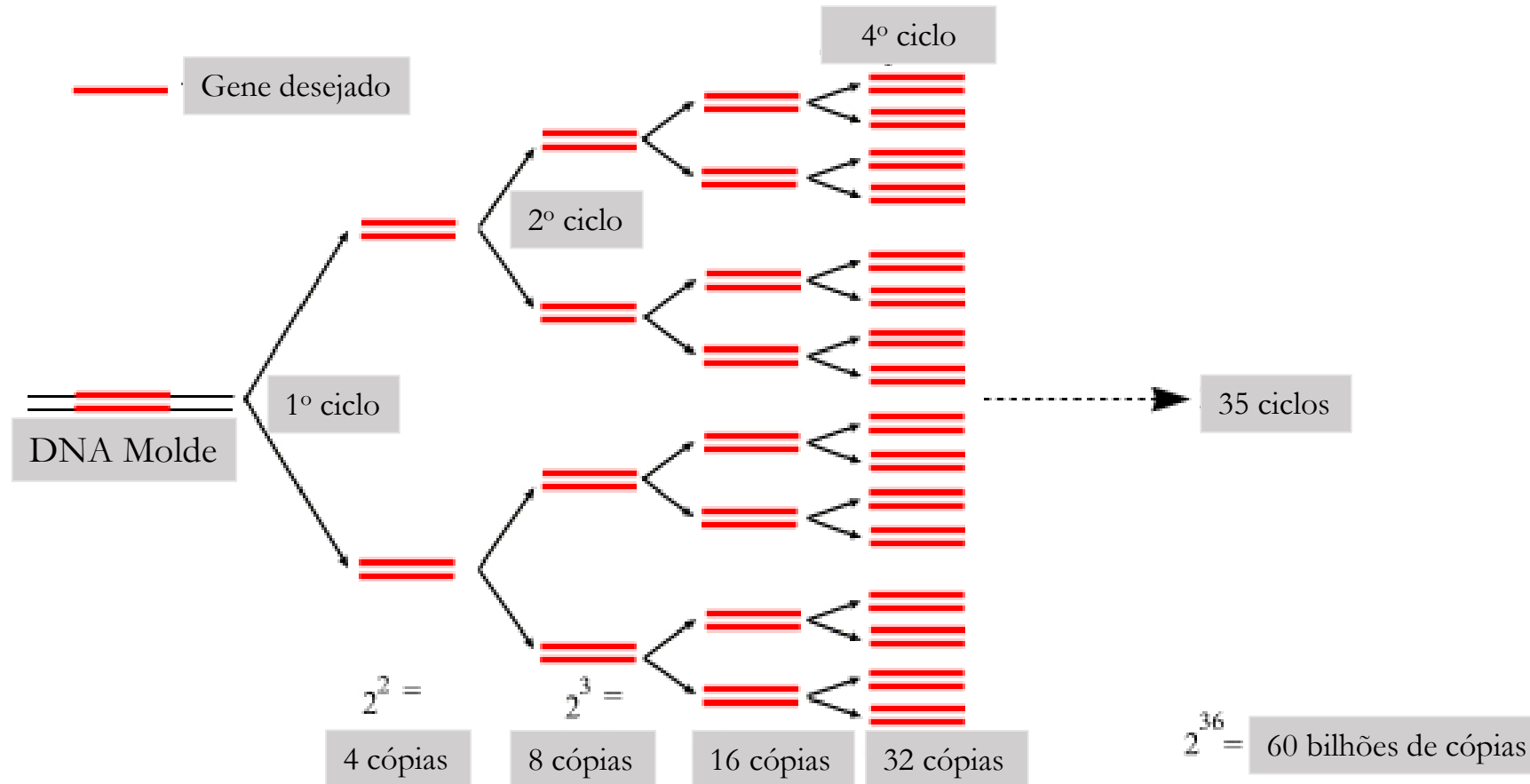


Step 2 – anelamento
45 segundos – 55°C



Step 3 – extensão
45 segundos – 72°C

A amplificação é exponencial

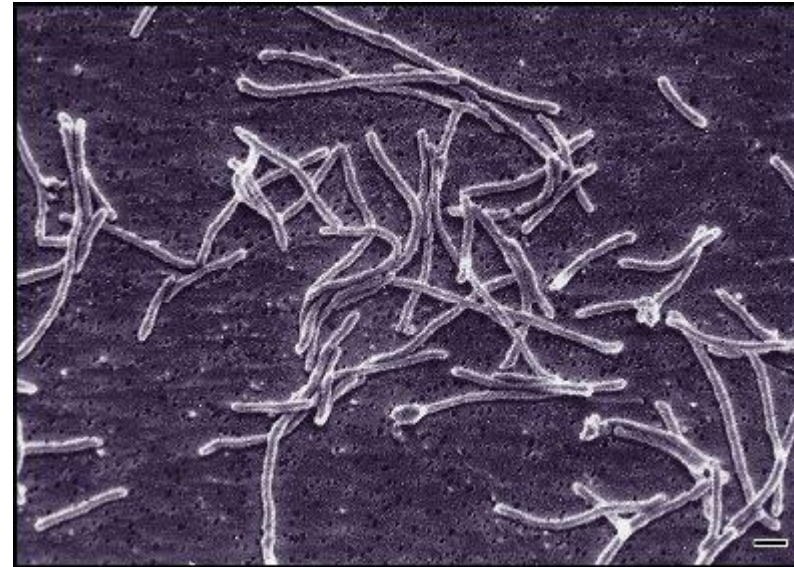


Thermus aquaticus

Thermus aquaticus: bactéria que pode suportar temperaturas elevadas, uma de várias bactérias termófilas.

Fonte da enzimas resistente ao calor como a *Taq* polimerase de DNA , um dos mais importantes enzimas na biologia molecular devido à sua utilização na reação em cadeia da polimerase (PCR), técnica de amplificação de DNA

A bactéria foi descoberta pela primeira vez no Lower Geyser Basin do Parque Nacional de Yellowstone



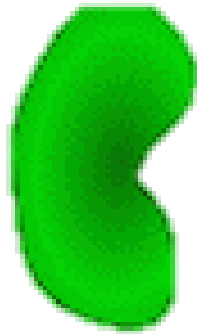
Thermus aquaticus



Reação de PCR



DNA molde



DNA polimerase

H₂O + tampão

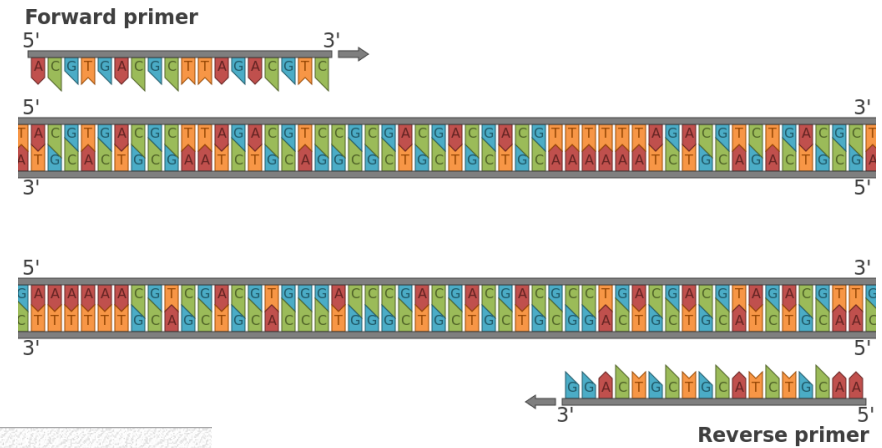


primers

A T C G

nucleotídeos

Desenho de *Primers* Oligonucleotídeos



DESIGN PCR PRIMERS

BACKGROUND INFORMATION: For sites describing PCR theory, as well as companies marketing PCR products you might want to begin by visiting [Highveld](#). For PCR techniques see [PCRlink.com](#).

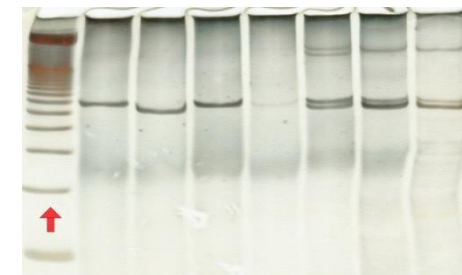
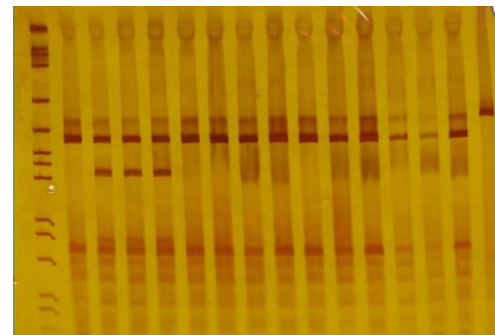
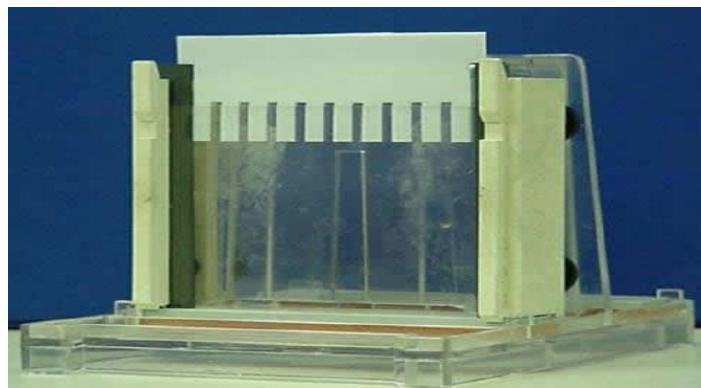
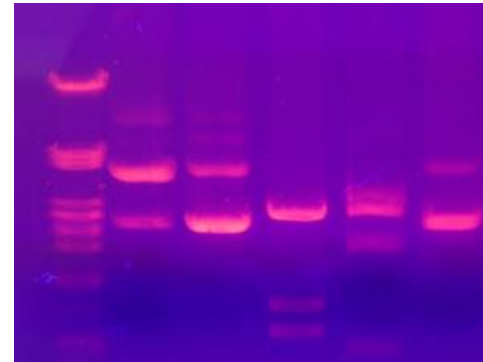
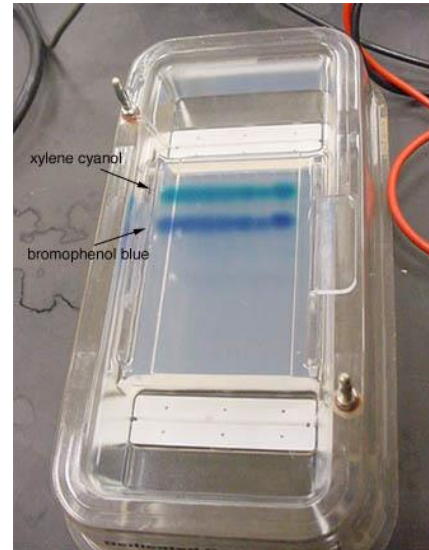
There are several excellent sites for designing PCR primers:

- [Primer3: WWW primer tool](#) (*University of Massachusetts Medical School, U.S.A.*) – This site has a very powerful PCR primer design program permitting one considerable control over the nature of the primers, including size of product desired, primer size and T_m range, and presence/absence of a 3'-GC clamp.
- [GeneFisher - Interactive PCR Primer Design](#) (*Universitat Bielefeld, Germany*) - a very good site allowing great control over primer design.
- [PCR Now](#) (*Computational Biology Group, PathoGene, Southwestern Medical Center, U.S.A.*) - created to design Real-Time Polymerase Chain Reaction (RT-PCR) primers for any number of user-defined coding sequences. Great control over primer properties. If you are interested in designing primers specific to published organismal or viral genes see the related site [PathoGene](#).
- [Primer3Plus](#) - a new improved web interface to the popular Primer3 primer design program (*Reference: A. Untergasser et al. 2007. Nucl. Acids Res. 35(Web Server issue):W71-W74*)
- [BiSearch](#) Primer Design and Search Tool - this is a useful tool for primer-design for any DNA template and especially for bisulfite-treated genomes. The ePCR tool provides fast detection of mispriming sites and alternative PCR products in cDNA libraries and native or bisulfite-treated genomes. (*Reference: Arányi T et al. 2006. BMC Bioinformatics 7: 431*).
- [Primer-BLAST](#) was developed at NCBI to help users make primers that are specific to the input PCR template. It uses [Primer3](#) to design PCR primers and then submits them to BLAST search against user-selected database. The blast results are then automatically analyzed to avoid primer pairs that can cause amplification of targets other than the input template.
- [MFEprimer-2.0](#) allows users to check primer specificity against genomic DNA and messenger RNA/complementary DNA sequence databases quickly and easily. This server uses a k-mer index algorithm to accelerate the search process for primer binding sites and uses thermodynamics to evaluate binding stability between each primer and its DNA template. Several important characteristics, such as the sequence, melting temperature and size of each amplicon, either specific or non-specific, are reported. (*Reference: Qu W et al. 2012. Nucl. Acids Res. 40 (Web Server issue): W205-W208*)
- [Primer Design and Search Tool](#)
- [PrimerDesign-M](#) - includes several options for multiple-primer design, allowing researchers to efficiently design walking primers that cover long DNA targets, such as entire HIV-1 genomes, and that optimizes primers simultaneously informed by genetic diversity in multiple alignments and experimental design constraints given by the user. PrimerDesign-M can also design primers that include DNA barcodes and minimize primer dimerization. PrimerDesign-M finds optimal primers for highly variable DNA targets and facilitates design flexibility by suggesting alternative designs to adapt to experimental conditions. (*Reference: Yoon H & Leitner T. 2015. Bioinformatics 31:1472-1474*).

Termociclador



Visualizando o Fragmento Amplificado



<https://www.dnalc.org/resources/animations/gelectrophoresis.html>

Aplicações de PCR

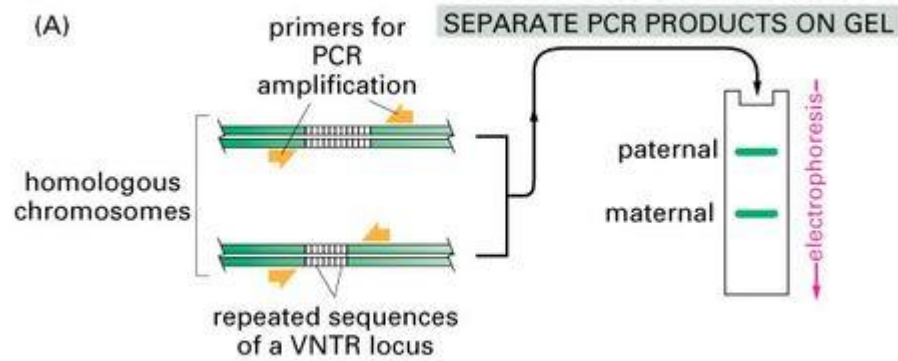
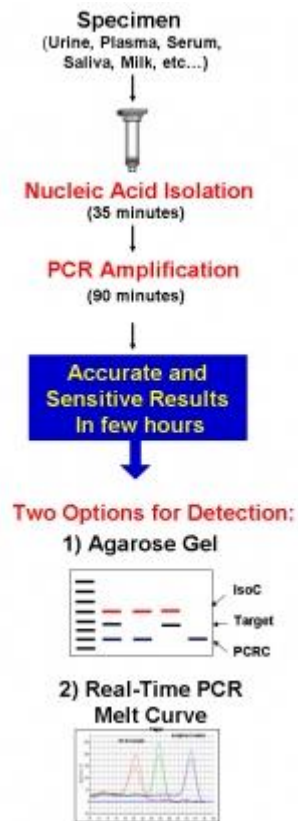
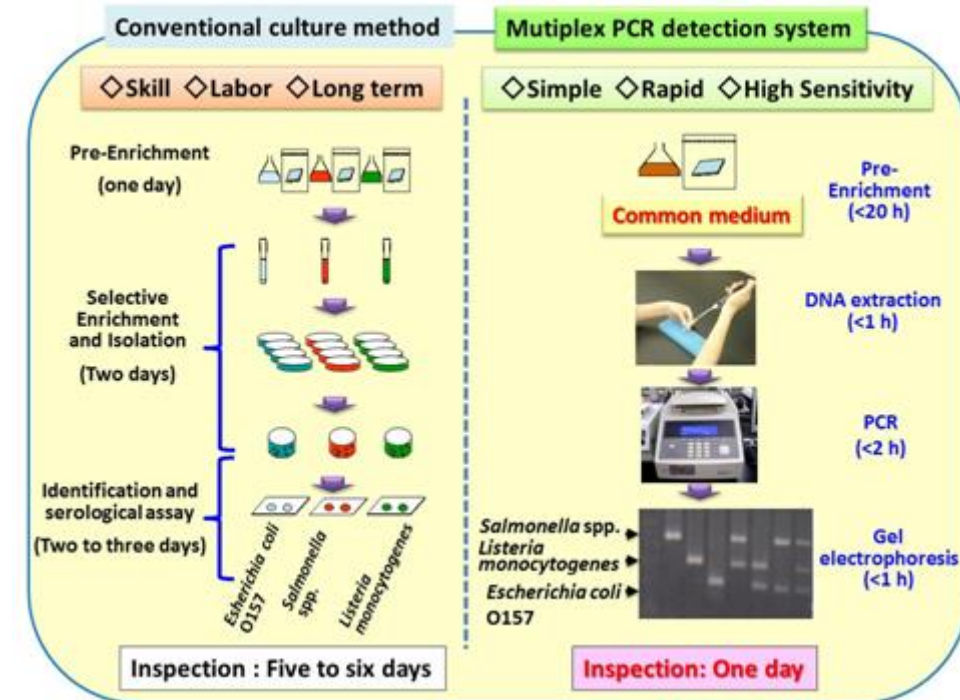


Figure 10-30 part 1 of 2 Essential Cell Biology, 2/e. (© 2004 Garland Science)

Teste de paternidade, forense



Detecção de vírus



Detecção de patógenos

Qual seria a aplicação na agricultura?

Estudo Dirigido

1. O que é transformação bacteriana? Qual o princípio? Quais as aplicações?
2. Como se faz a seleção de uma bactéria transformada?
3. O que é a PCR?
4. Qual aplicação dessa técnica?

Leitura

Capítulo 11 – Manipulando o gene /Técnicas de Biologia Molecular (páginas 197 a 241) . Menck, C.F.M.; Van Sluys, M.A. *Genética Molecular Básica: dos genes aos genomas*. Editora Guanabara Koogan, 2017.

Capítulo 5 - Técnicas de Genética Molecular (páginas 171-222). Lodish et al. *Biologia Celular e Molecular*. 7o Edição. Editora Artmed, 2014.

