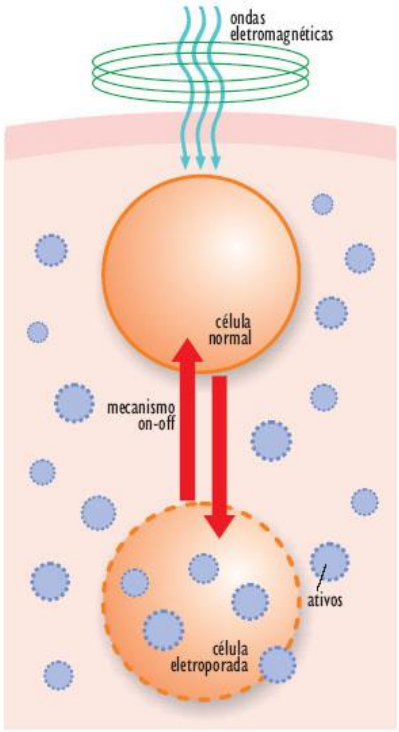
TECNOLOGIA DO DNA RECOMBINANTE: TRANSFORMAÇÃO BACTERIANA E PCR

Aula 4

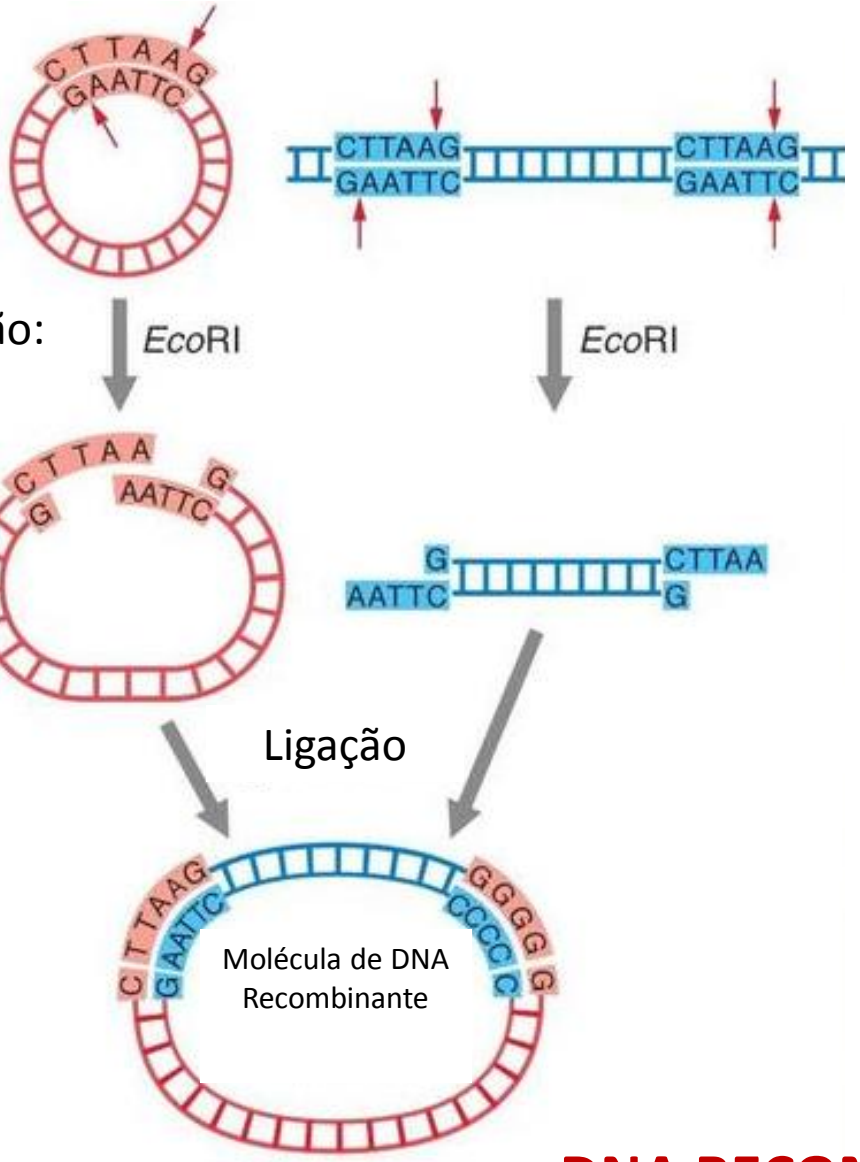
LGN0232 – Genética molecular



Maria Carolina Quecine
Departamento de Genética
mquecine@usp.br

AULA PASSADA:

Enzimas de Restrição:



DNA RECOMBINANTE!!!!

CLONAGEM CELULAR











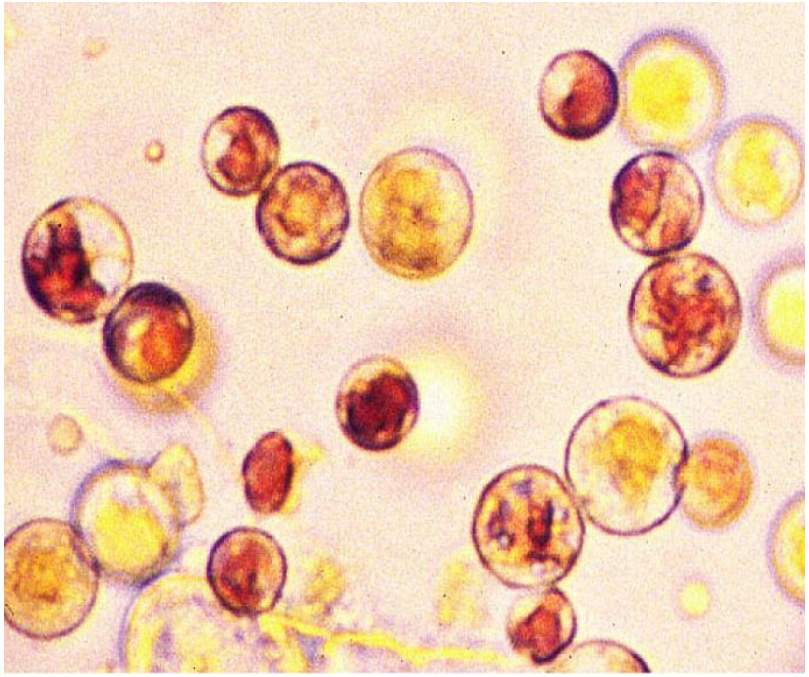
CÓPIAS IDENTICAS!!

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



CLONAGEM MOLECULAR

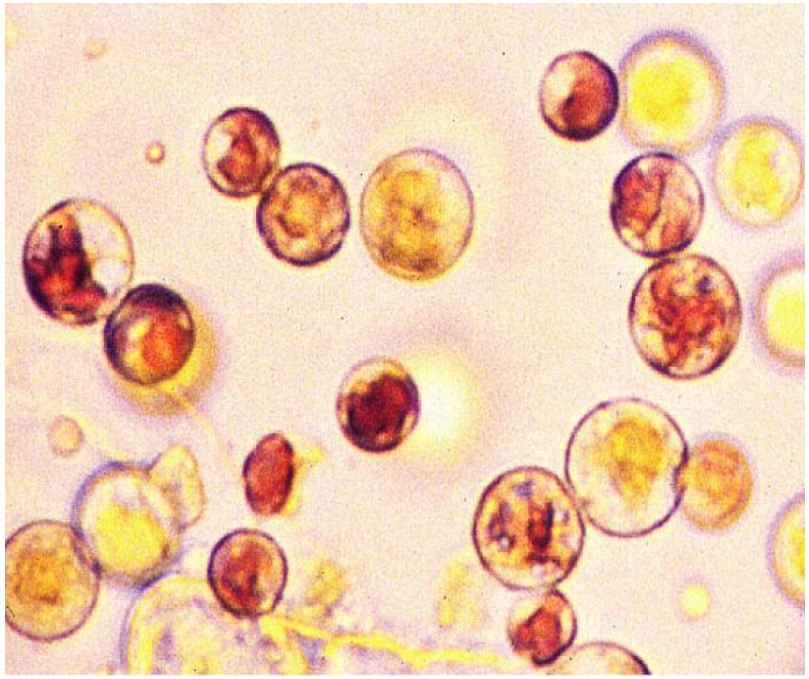
Dependente de células vivas



Independente de células vivas (PCR)



CLONAGEM DEPENDENTE DE CÉLULAS VIVAS



CLONAGEM DE FRAGMENTOS DE DNA

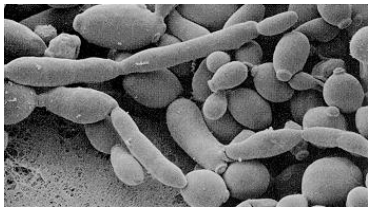
Ferramentas de clonagem:

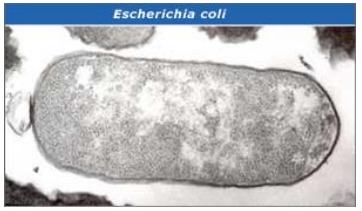
Enzimas: enzimas de restrição
DNA ligase

Vetores: plasmídios
fagos (vírus de bactérias)
cosmídeos
cromossomos artificiais

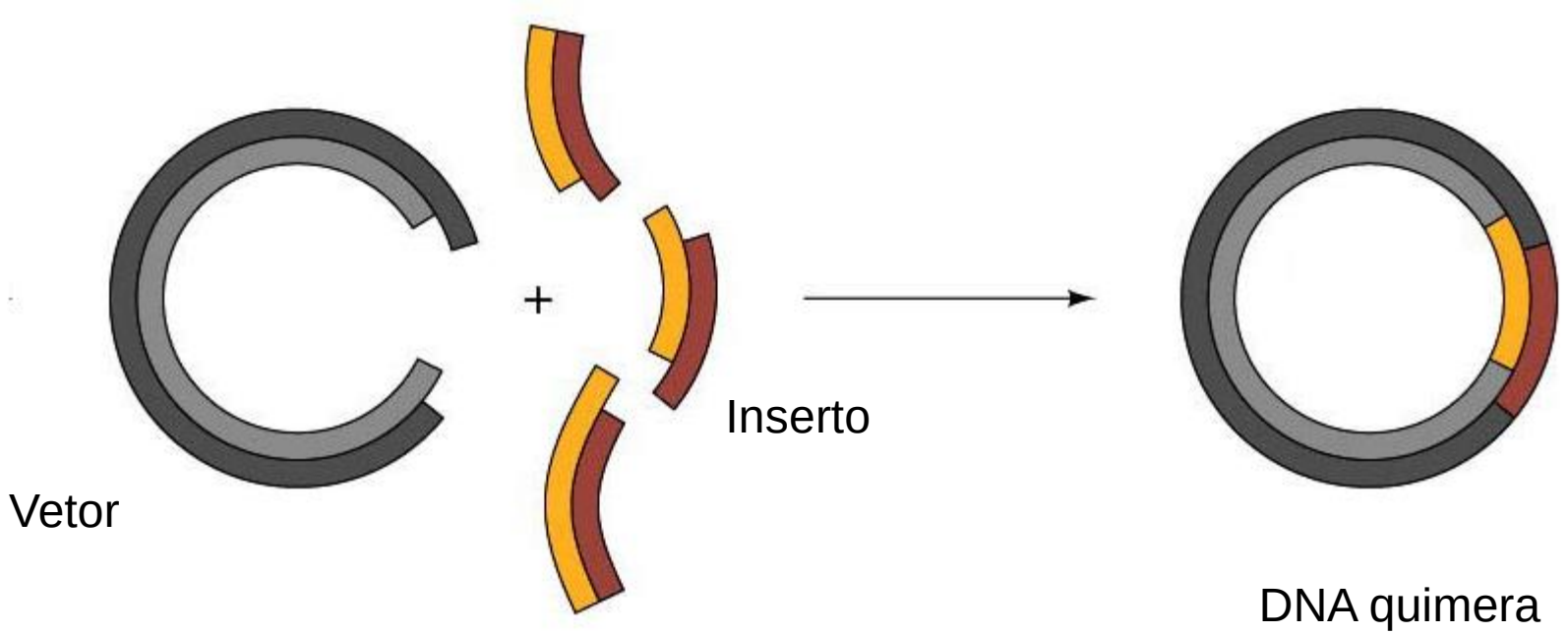


Hospedeiros: *E. coli*
levedura
células animais
células vegetais

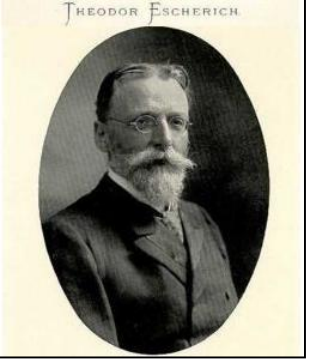




Lembrando: vetores de clonagem - multiplicam moléculas de DNA recombinante (clones) em organismos vivos, gerando quantidades abundantes de clones



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



Escherichia coli



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



Uma geração: 20 minutos

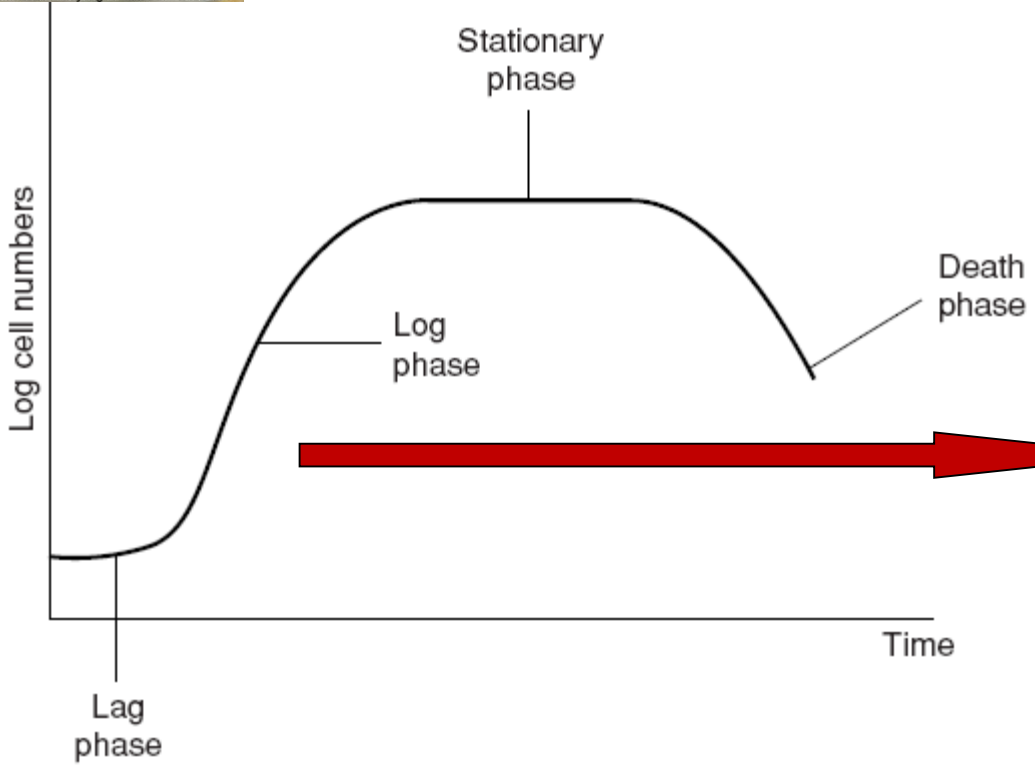
- Pouco exigente quanto a nutrição;
- Meio mínimo ou rico:
  - Glicose - melhor fonte de C
  - Extrato de levedura - vitaminas
  - Triptona e Peptona: fonte aminoácidos

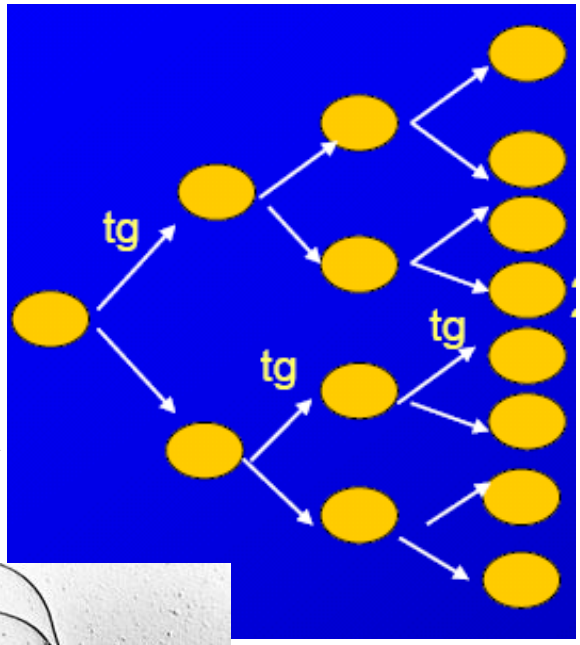


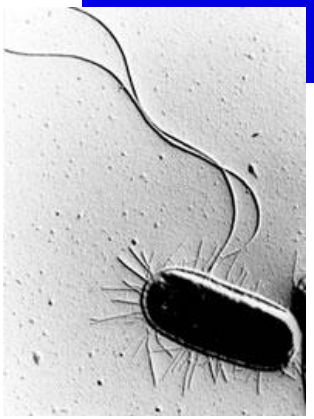
PROGRESSÃO GEOMÉTRICA DO CRESCIMENTO POPULACIONAL BACTERIANO



Uma geração: 20 minutos







Curva de crescimento

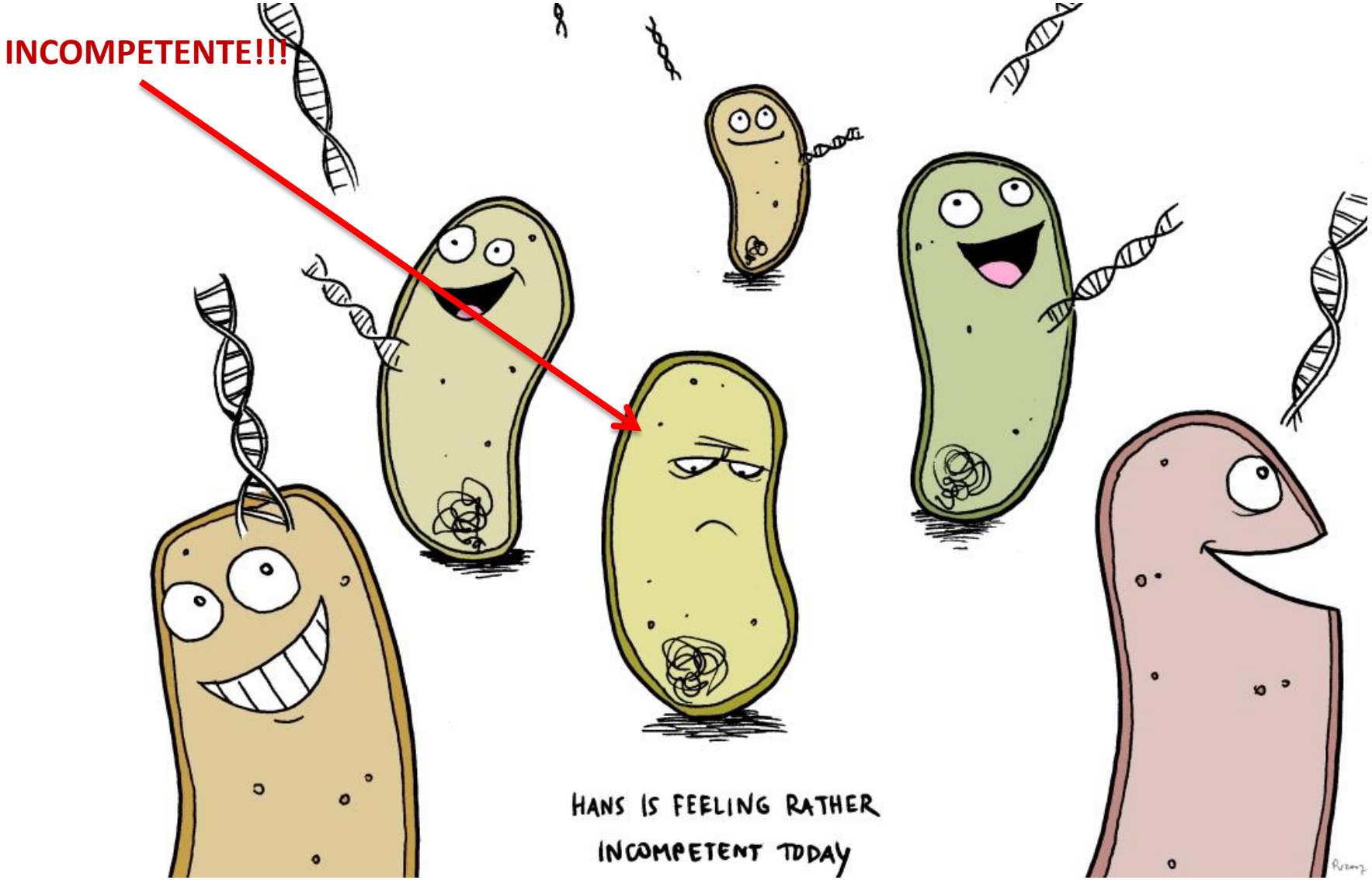
BACTÉRIAS PARA TRANSFORMAÇÃO

- Não apresentam DNA plasmidial



- Precisam ser preparadas
  - Tornar-se competentes = aptas a receberem a molécula de DNA recombinante

CÉLULAS COMPETENTES

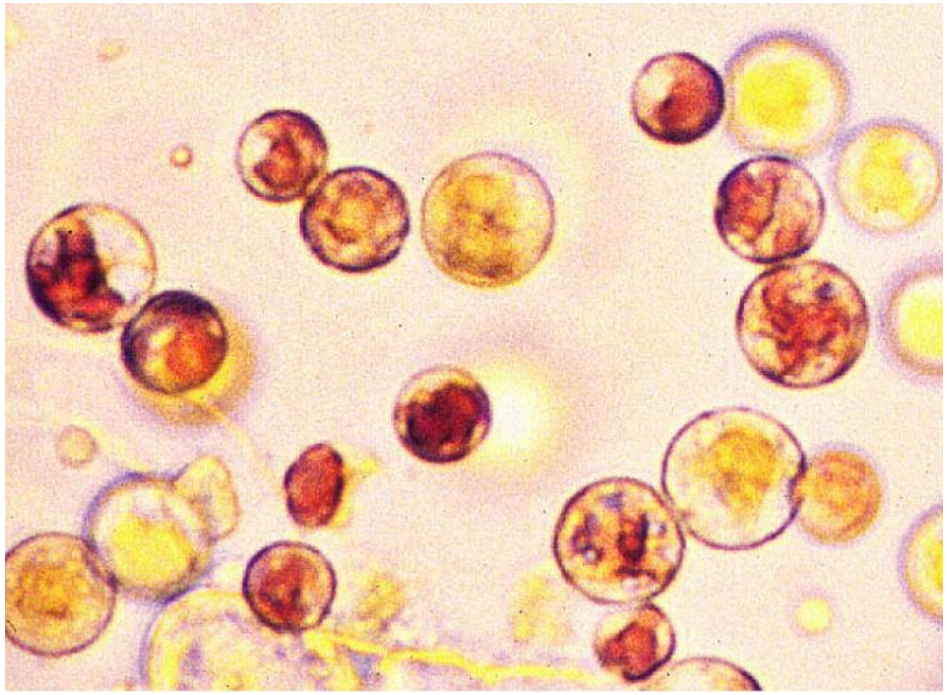


Uma bactéria é **competente** quando tem a **capacidade de receber e multiplicar DNA estranho**.

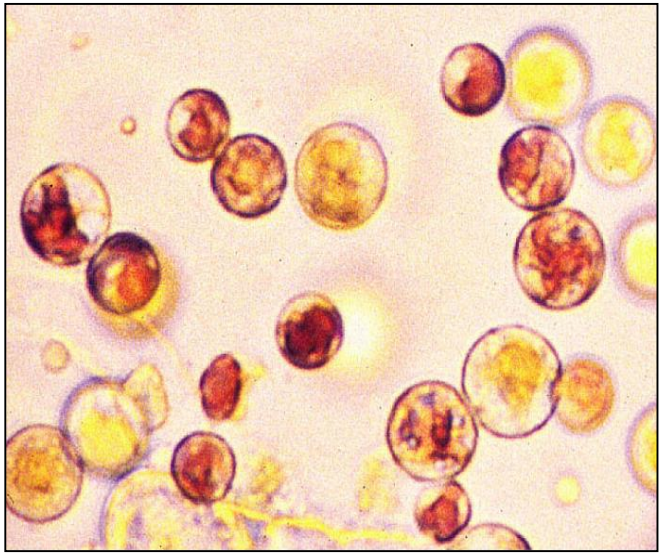
INSERÇÃO DO DNA DENTRO DA CÉLULA BACTERIANA – O HOSPEDEIRO

- Transformação química
- Eletroporação



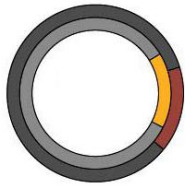


TRANSFORMAÇÃO QUÍMICA



Suspensão de células competentes

+

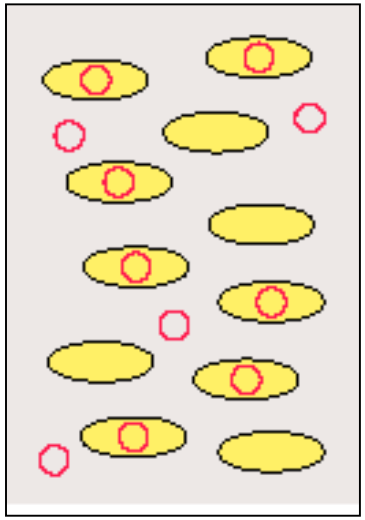


Moléculas de DNA recombinante

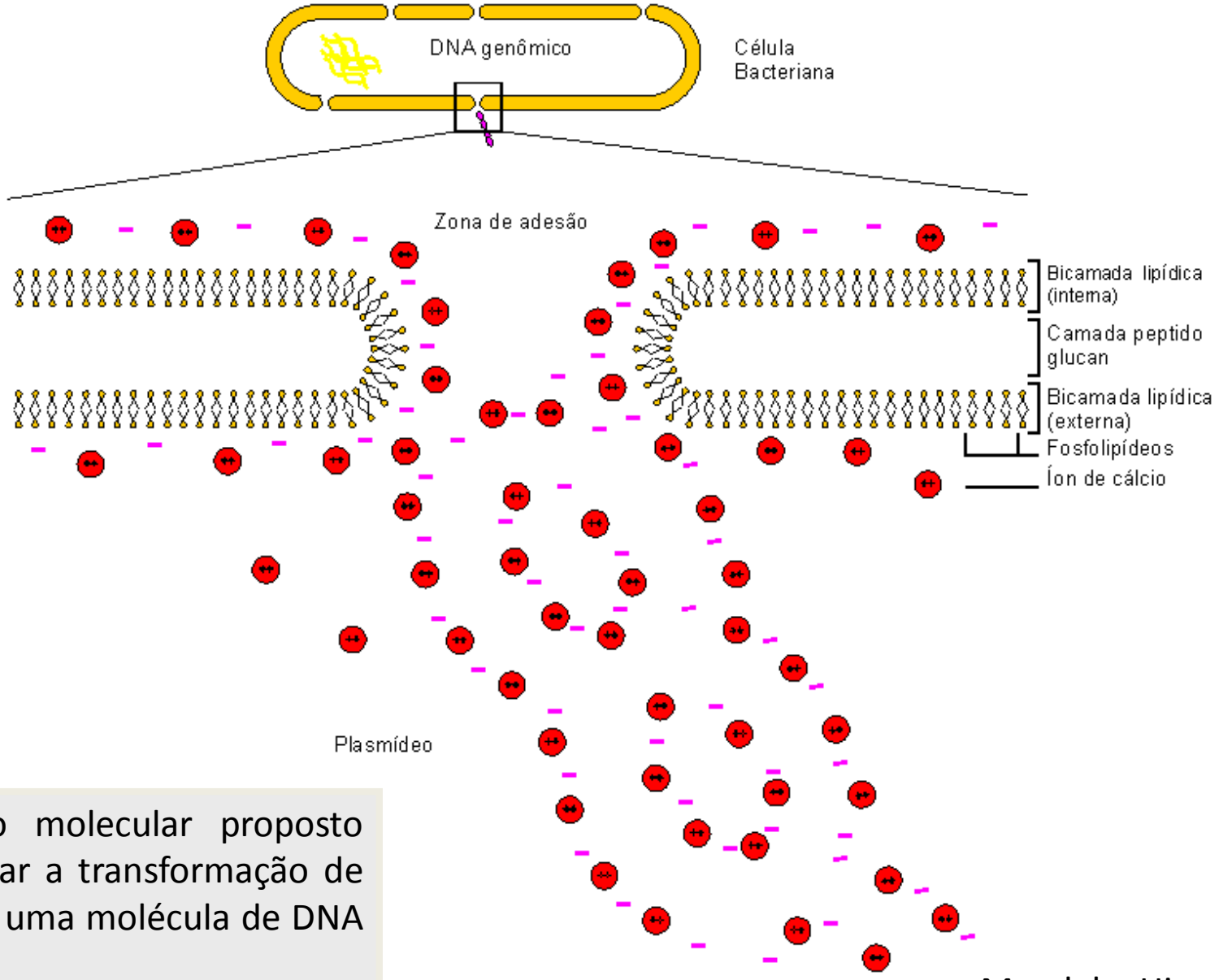
=

Incubar 30 min no gelo

Choque térmico (42°C/30 s)



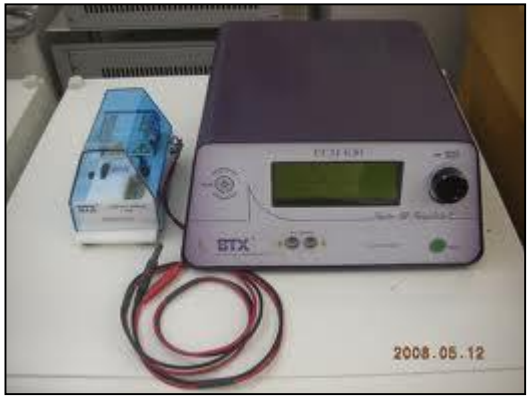
TRANSFORMAÇÃO QUÍMICA



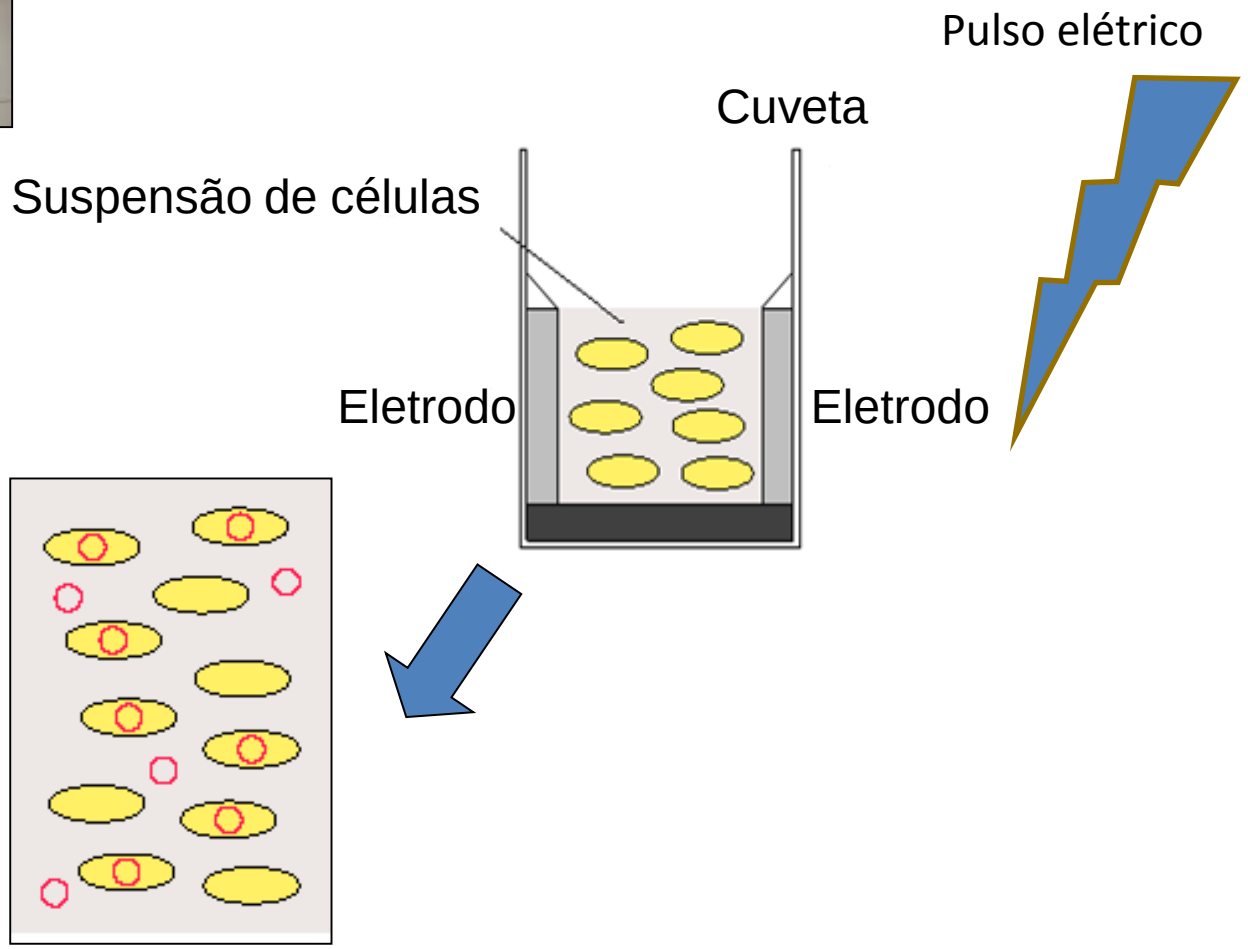
Mecanismo molecular proposto para explicar a transformação de *E. coli* com uma molécula de DNA exógeno.

Mandel e Higa, 1970

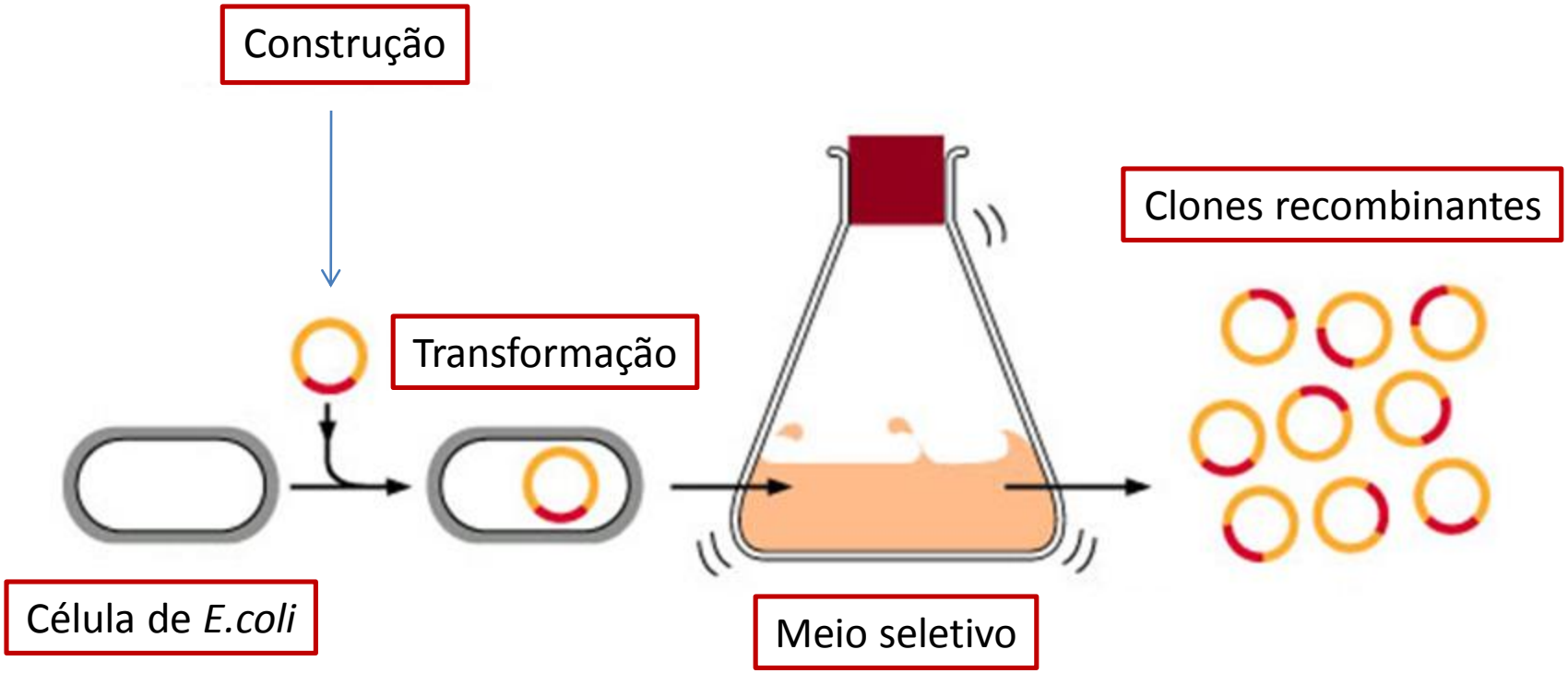
ELETROPORAÇÃO



Eletroporador

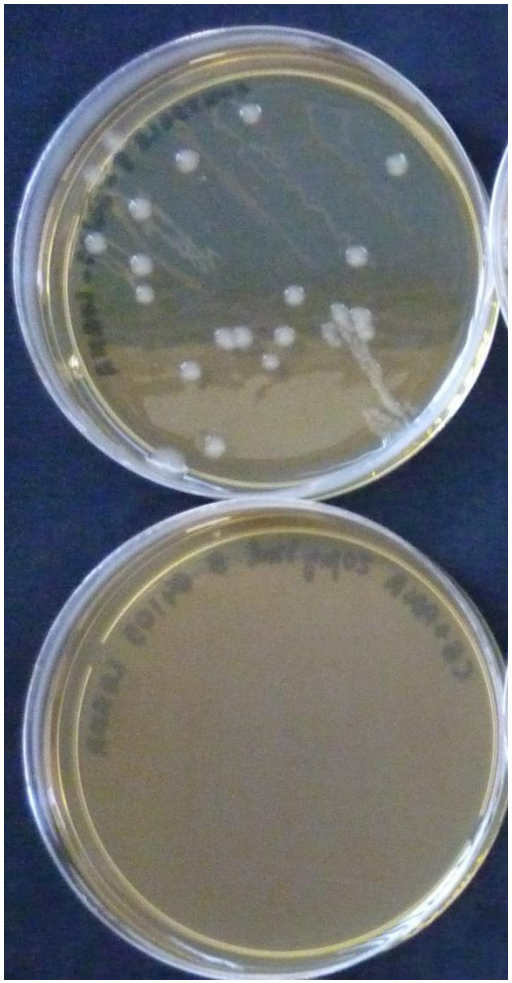


SELEÇÃO DE CLONES RECOMBINANTES



Ex: Meio com antibiótico (ampicilina)

MC + ampicilina



Células transformadas

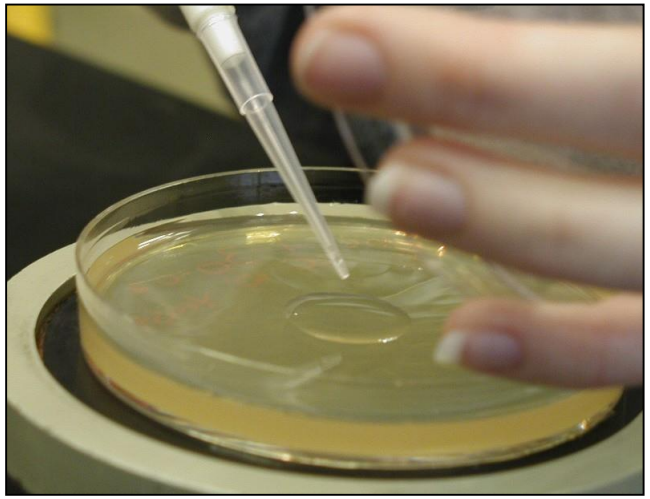
Clones resistentes a ampicilina

Células controle (não transformadas)

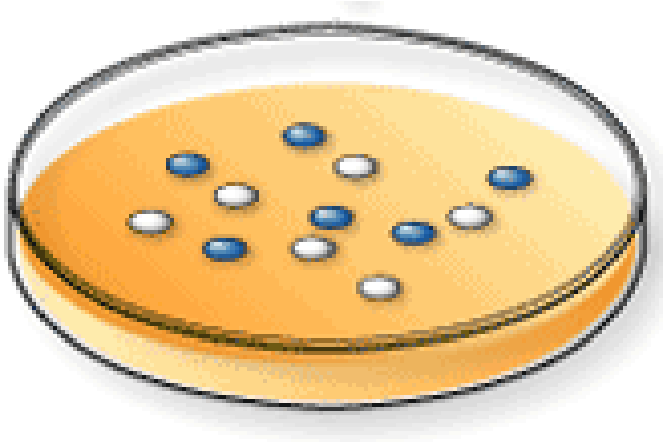
Clones sensíveis a ampicilina

SELEÇÃO DE CLONES RECOMBINANTES





MC+X-gal+ampicilina



Período de incubação
12-16h / 37°C)

*MC = Meio de cultura

SELEÇÃO DE CLONES RECOMBINANTES

➢Podem ser também utilizados marcadores que são inativados com a inserção do DNA estranho no plasmídio.

Exemplo: sistema de diferenciação branco-azul

• Plasmídios da série pUC possuem um gene (gene *lac*Z) que codifica a produção da enzima β-galactosidase. A inserção do DNA estranho no sítio de clonagem do plasmídeo causa a interrupção do gene, levando à perda da função da β-galactosidase.

Como resultado:

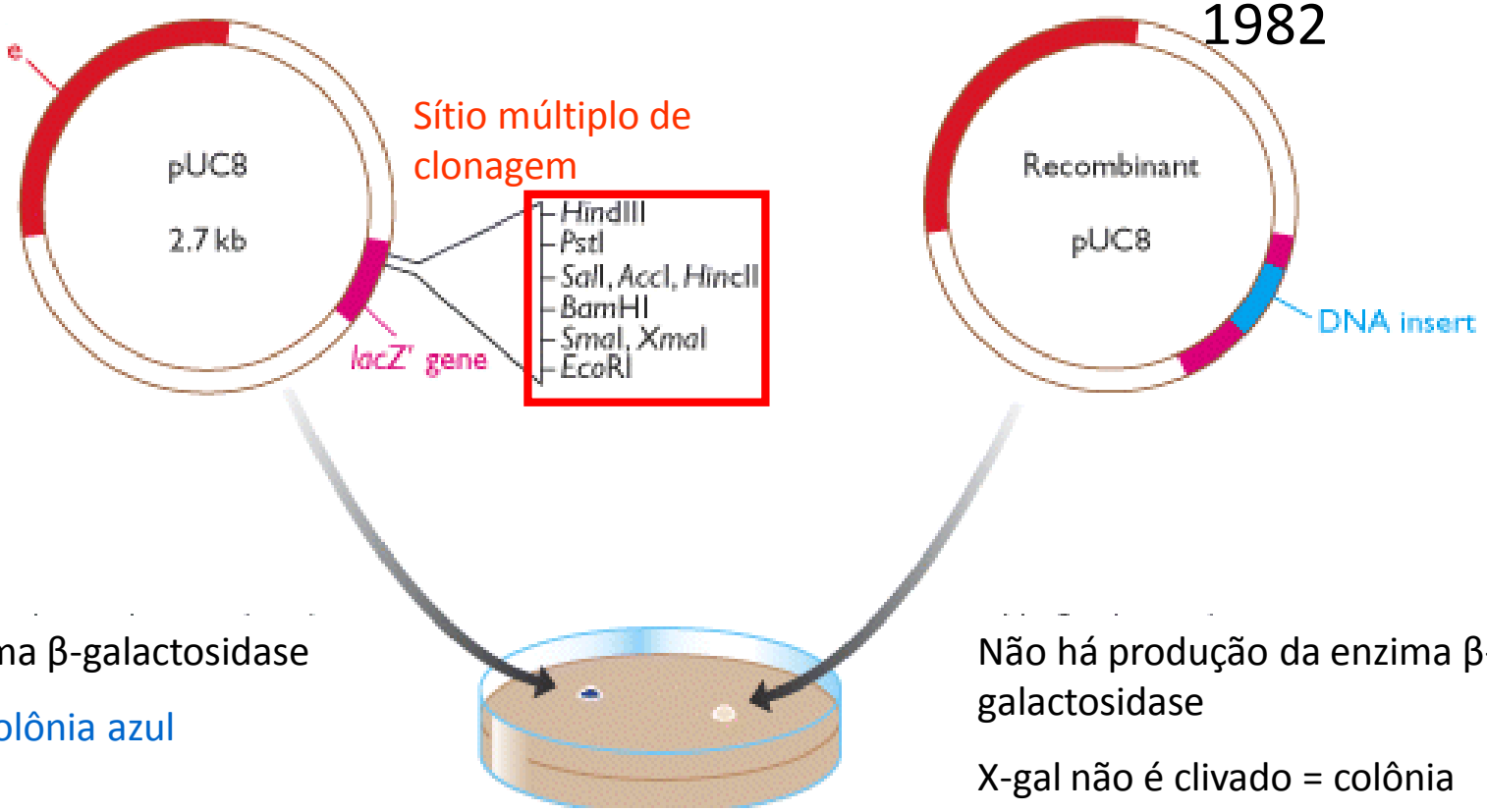
• colônias brancas (β-gal inativa) - positivas (com inserto)
• colônias azuis (β-gal ativa) - negativas (sem inserto)

SELEÇÃO DE CLONES RECOMBINANTES

pUC8

Vieira & Messing, 1982

Gene de resistência à ampicilina



Produção da enzima β-galactosidase

X-gal é clivado = colônia azul

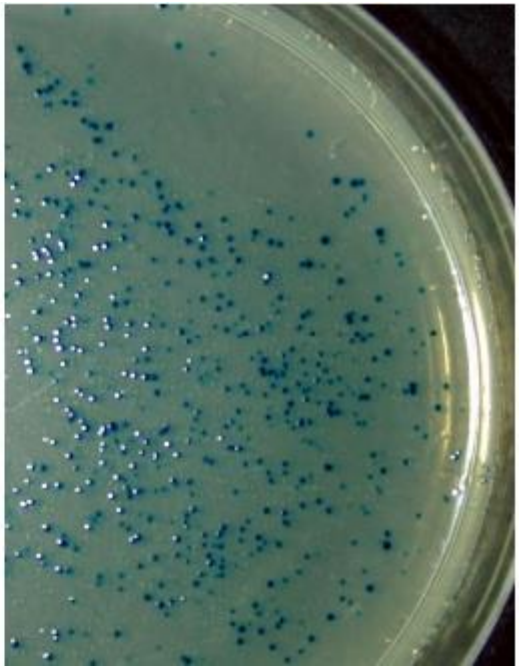
Não há produção da enzima β-galactosidase

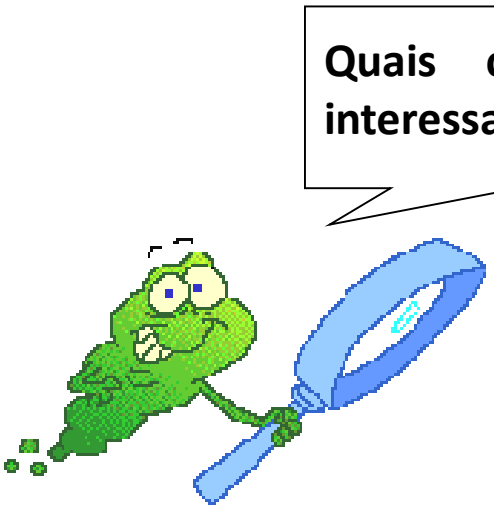
X-gal não é clivado = colônia branca

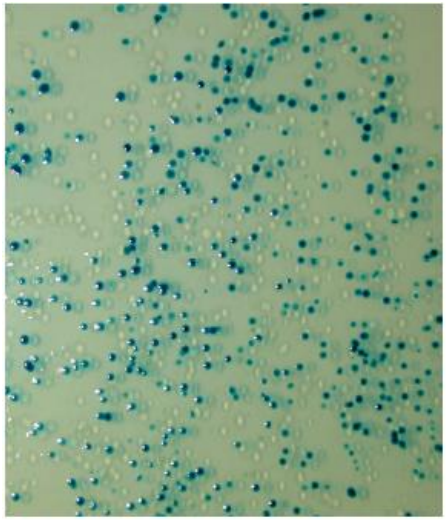
Ágar + ampicilina + **X-gal**

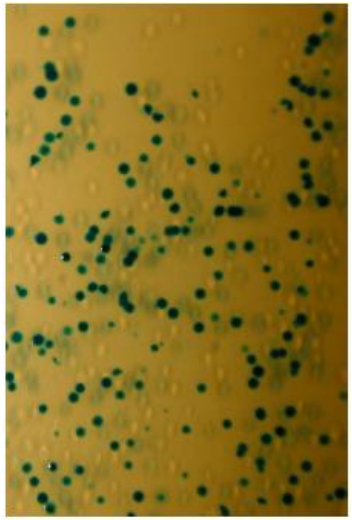
Gene *lacZ* = codifica a enzima β-galactosidase





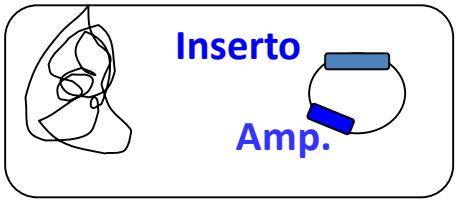

Quais colônias nos interessam e por quê?





POSSÍVEIS RESULTADOS APÓS A ETAPA DE TRANSFORMAÇÃO

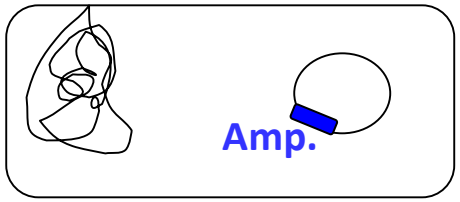
Célula bacteriana



MC+X-gal+amp

Plasmídeo com inserto

- ✓Resistência à ampicilina
- ✓*Lac*Z não funcional
- ✓**Colônias brancas**



MC+X-gal+amp

Plasmídeo sem inserto

- ✓Resistência à ampicilina
- ✓*Lac*Z funcional
- ✓**Colônias azuis**

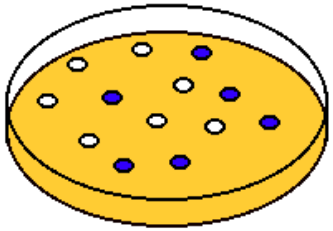
DNA cromossomal



MC+X-gal+amp

Sem plasmídeo

- ✓**Sem** resistência à ampicilina
- ✓**Sem** gene *Lac*Z
- ✓**Sem** crescimento



*MC = Meio de cultura

CLONAGEM MOLECULAR

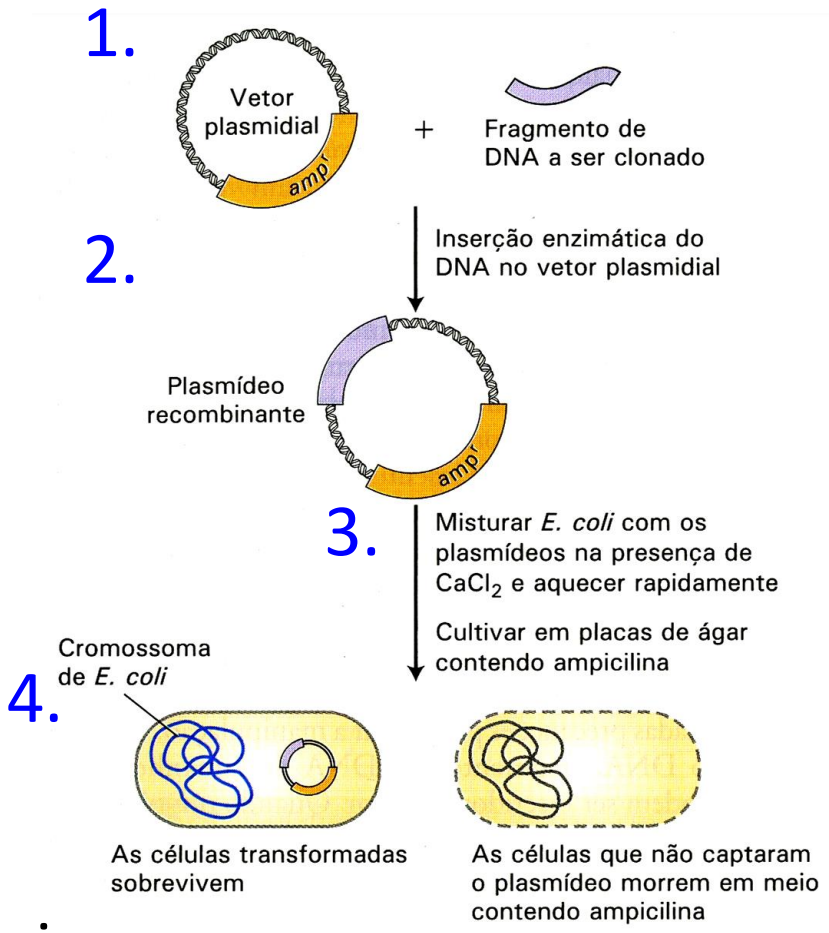
RESUMO DAS ETAPAS:

1. Preparação do vetor e do inserto

2. Ligação do vetor e inserto

3. Transformação em célula hospedeira

4. Seleção de clones



CLONAGEM MOLECULAR INDEPENDENTE DE CÉLULAS VIVAS PCR



PCR: *Polymerase Chain Reaction*
(Reação em cadeia da polimerase)



- O processo de PCR foi **inventado** por Kary Mullis no início da década de 80 →atribuído o Premio Nobel da Química pelo seu trabalho.

- É um método de amplificação (de criação de múltiplas cópias) de DNA **sem** o uso de um organismo vivo, por exemplo, *Escherichia coli* (bactéria) ou leveduras.

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 $^{87}Sr/^{86}Sr$ 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 $^{87}Sr/^{86}Sr$ 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 $^{87}Sr/^{86}Sr$ 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." Science 239 (1988): 487-491.

PCR: *Polymerase Chain Reaction*

- **1. Desnaturação** (94-96ºC, 30-600 segundos).

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

A DNA polimerase é 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**) (45-80º 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.

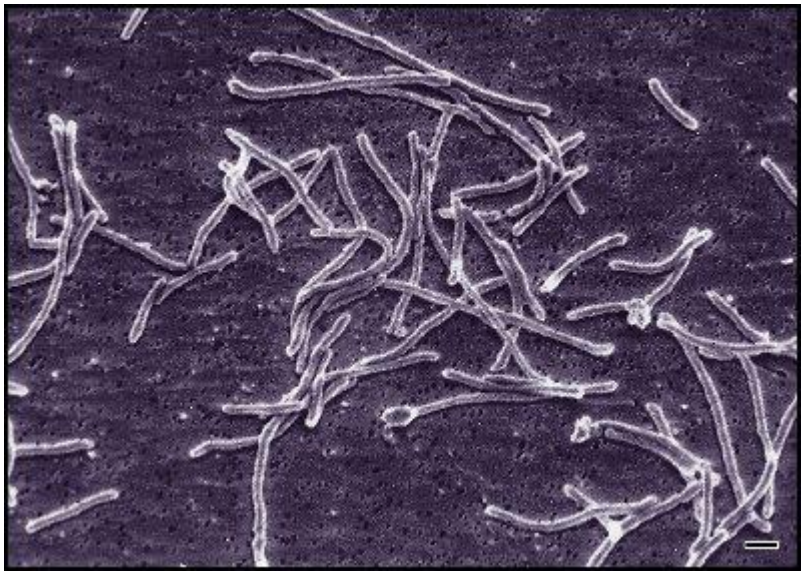
- **3. Alongamento (polimerização)** (65-80º C, 30-120 segundos).

Durante o alongamento, a DNA polimerase cria a cadeia de DNA complementar à medida que percorre o DNA de cadeia simples, incorporando desoxirribonucleótideos 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.

Thermus aquaticus

Thermus aquaticus é uma espécie de bactéria que pode suportar temperaturas elevadas, uma de várias bactérias termófilas. Esta é a 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.



Thermus aquaticus



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

REAÇÃO DE PCR



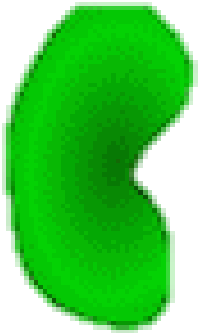
DNA molde

H_2O + tampão



primers



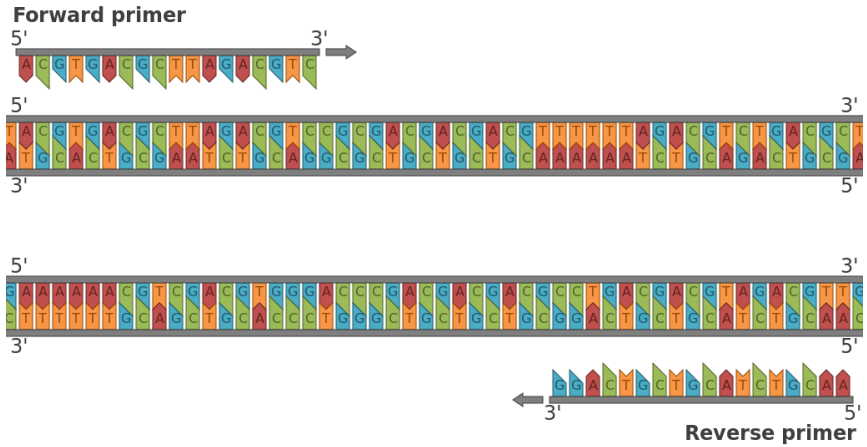


DNA polimerase

A T C G

nucleotídeos

DESENHO DE *PRIMERS*:



molbiol-tools.ca/PCR.htm

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 Tm 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).

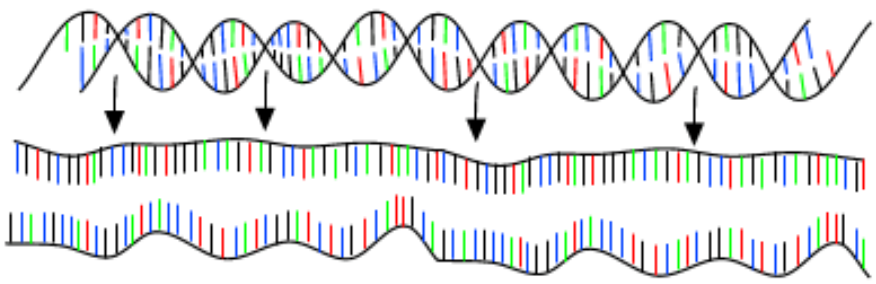
- Primer-BLAST was developed at NCBI to help users make primers that are specific to the input PCR template. It uses Primer3 to design PCR primers and then submits them to BLAST search against user-selected database. The blast results are then automatically analyzed to avoid primer pairs that can cause 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)

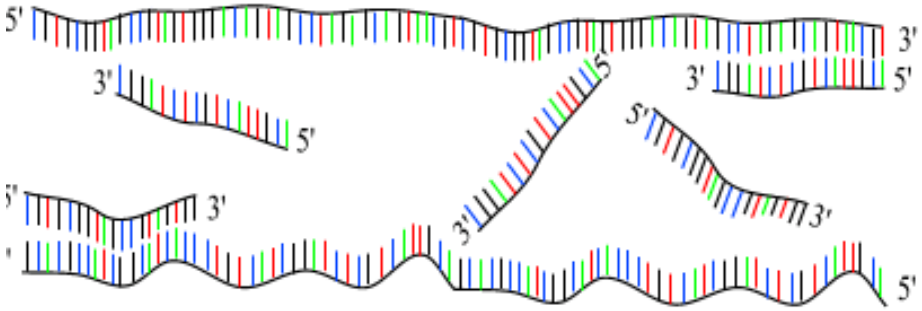
- Primer Design and Search Tool

- 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).

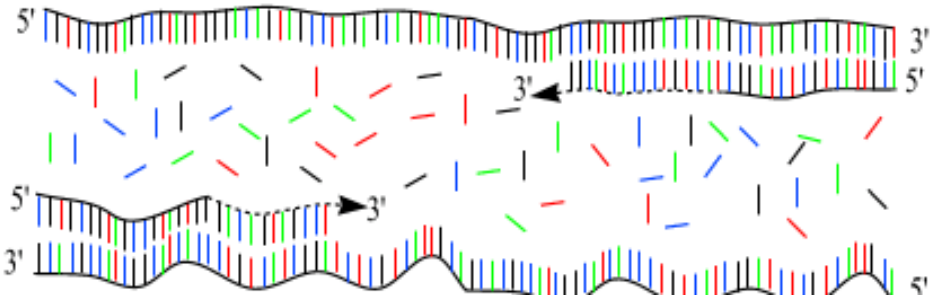
http://molbiol-tools.ca/PCR.htm



Step 1 – desnaturação
1 minuto – 94ºC

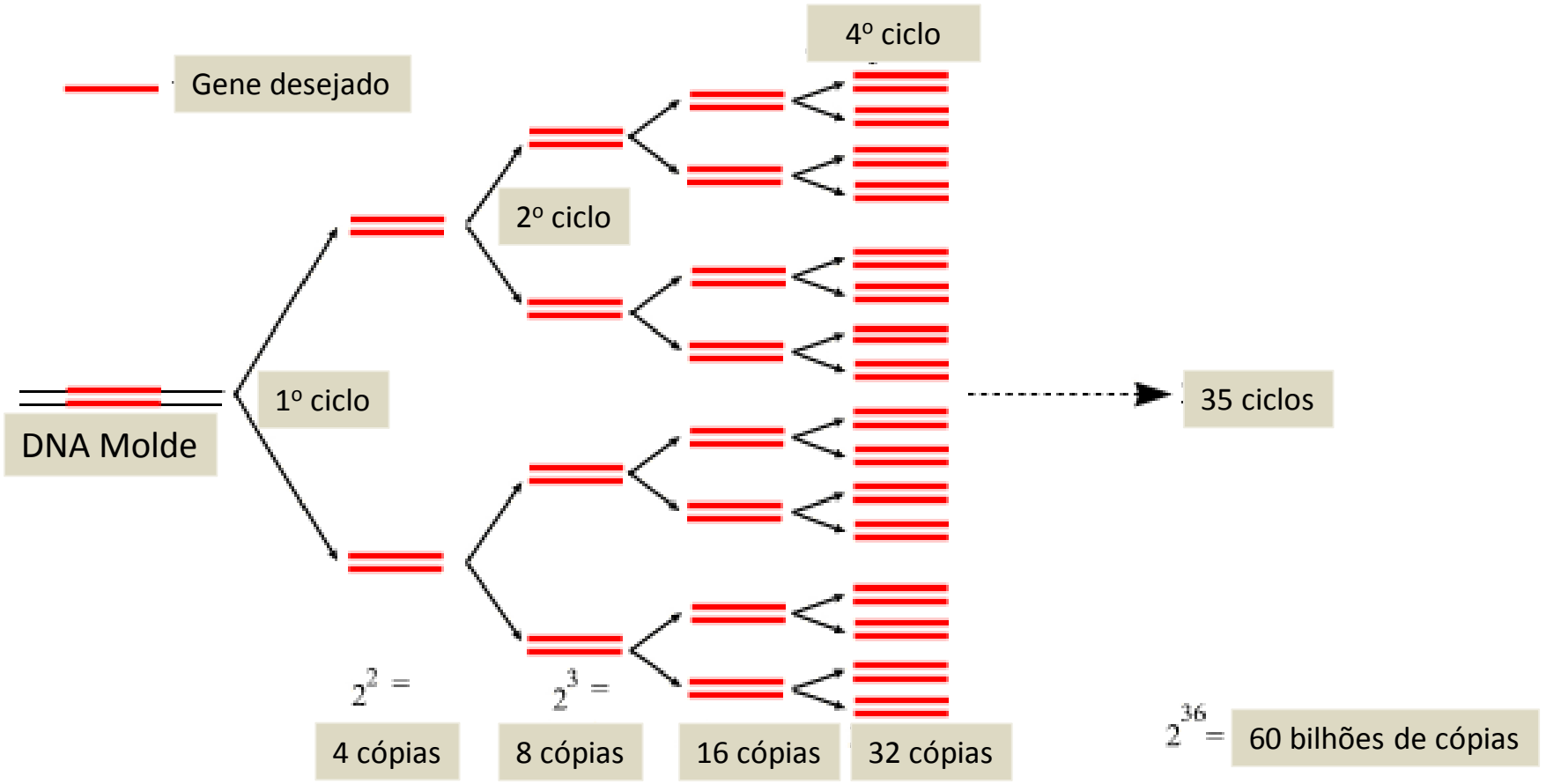


Step 2 – anelamento
45 segundos– 55ºC



Step 3 – extensão
45 segundos– 72ºC

A AMPLIFICAÇÃO É EXPONENCIAL!!

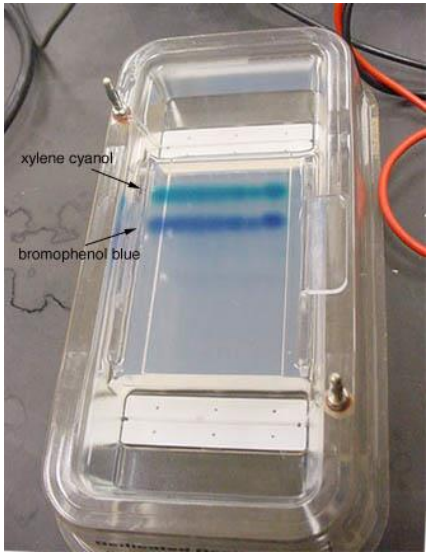


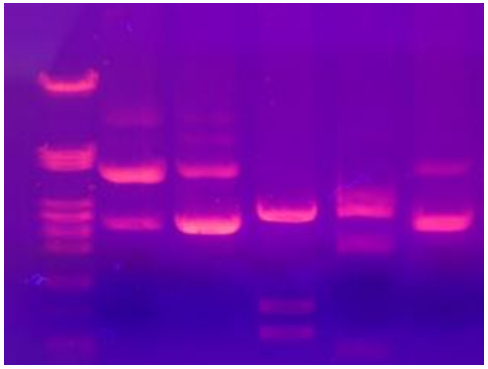
TERMOCICLADOR

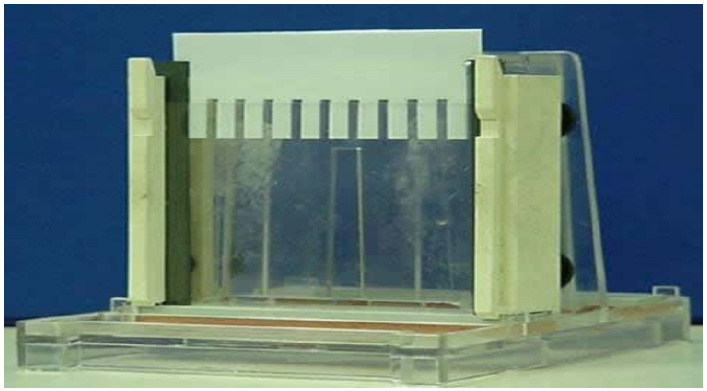


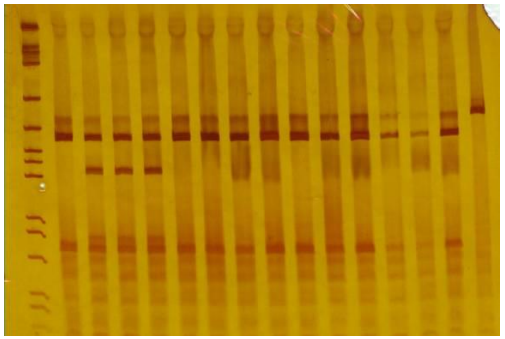


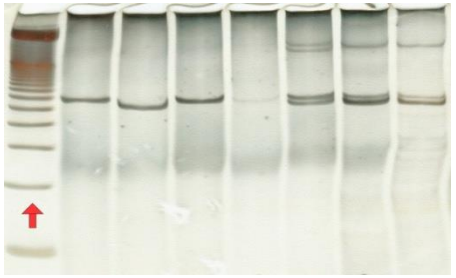
VISUALIZANDO O MATERIAL AMPLIFICADO













ANIMAÇÕES

https://www.youtube.com/watch?v=rn40R5w5Fkw

https://www.youtube.com/watch?v=iQsu3Kz9NYo

"The emergence of recombinant DNA technology occurred via the appropriation of known tools and procedures in novel ways that had broad applications for analyzing and modifying gene structure and organization of complex genomes. Although revolutionary in their impact, the tools and procedures per se were not revolutionary. Rather, the novel ways in which they were applied was what transformed biology."

Paul Berg and Janet E. Mertz (Personal Reflections on the Origins and Emergence of Recombinant DNA Technology, DOI: 10.1534/genetics.109.112144)

Um exemplo de aplicação...

Evidência molecular da ocorrência de um fitoplasma associado ao lenho mole da macieira

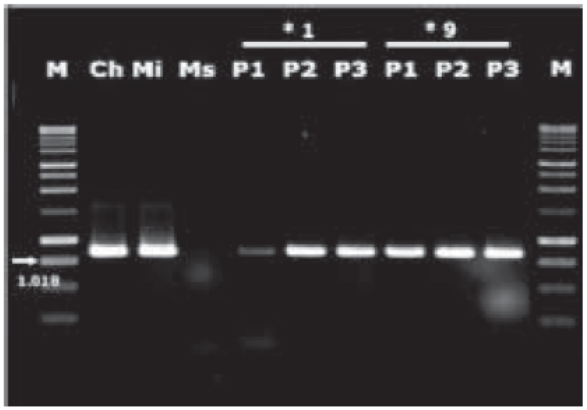
Luiz Fernando Caldeira Ribeiro[1], Ivan Paulo Bedendo[1], Rosa Maria Valdebenito Sanhueza[2]

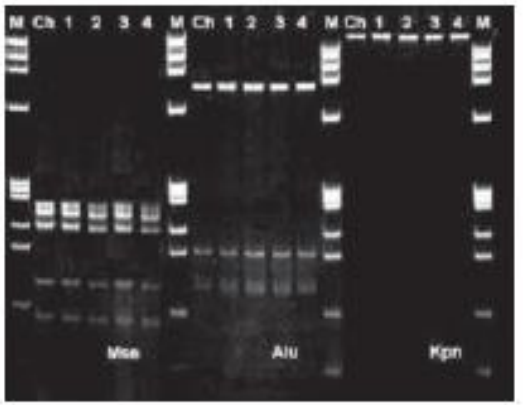
[1]Setor Fitopatologia / ESALQ–USP, Av. Pádua Dias, 11 CEP 13418-900 Piracicaba /SP, e-mail: ipbedend@esalq.usp.br, [2]Embrapa Uva e Vinho, rua Livramento, 515, CEP 95700-000 Bento Gonçalves, RS.
Autor p/ correspondência: Ivan Paulo Bedendo
Data de chegada: 15/08/2005. Aceito para publicação em: 04/05/2006.

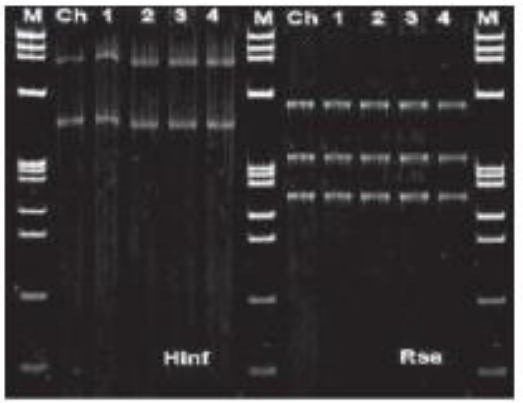
RESUMO

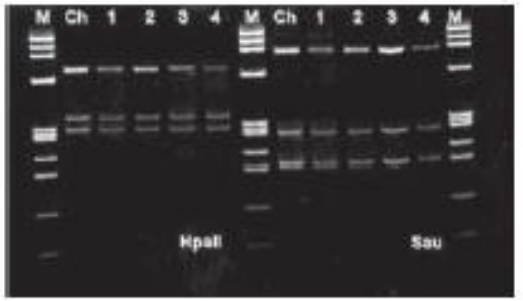
Ribeiro, L.F.C.; Bedendo, I.P.; Sanhueza, R.M.V. Evidência molecular da ocorrência de um fitoplasma associado ao lenho mole da macieira. ***Summa Phytopathologica***, v.33, n.1, p.30-33, 2007.

O lenho mole da macieira é uma doença relevante em diversas partes do mundo. Sintomas típicos desta doença têm sido observados em pomares instalados em estados do sul do território brasileiro desde a década de oitenta. Enxertia tem revelado a natureza infecciosa da doença e a observação de corpúsculos filamentosos no floema tem evidenciado possível associação com fitoplasma. No presente trabalho plantas com sintomas de lenho mole foram coletadas em pomar comercial, visando demonstrar a presença de fitoplasma em tecido doente, bem como identificar molecularmente este fitoplasma. Através do emprego de duplo PCR com iniciadores universais R16mF2/R1 e R16F2n/R2, fitoplasma foi consistentemente detectado em plantas sintomáticas. A identificação conduzida com duplo PCR usando-se iniciadores específicos R16(III)F2/R demonstrou que o fitoplasma detectado pertencia ao grupo 16SrIII. Análises de RFLP conduzidas com as endonucleases *Alu*I, *Kpn*I, *Hinf*I, *Hpa*II, *Mse*I, *Rsa*I e *Sau*IIIA confirmaram que o fitoplasma era um representante típico do grupo 16SrIII. A detecção e identificação molecular se constitui numa forte evidência que um fitoplasma está associado ao lenho mole da macieira no Brasil, complementando os trabalhos realizados anteriormente com transmissão por enxertia e observação por microscopia eletrônica .









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

Capitulo 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.