Biotecnologia

ACH5545 Engenharia Genética

Atividades de Laboratório

2º Semestre 2024

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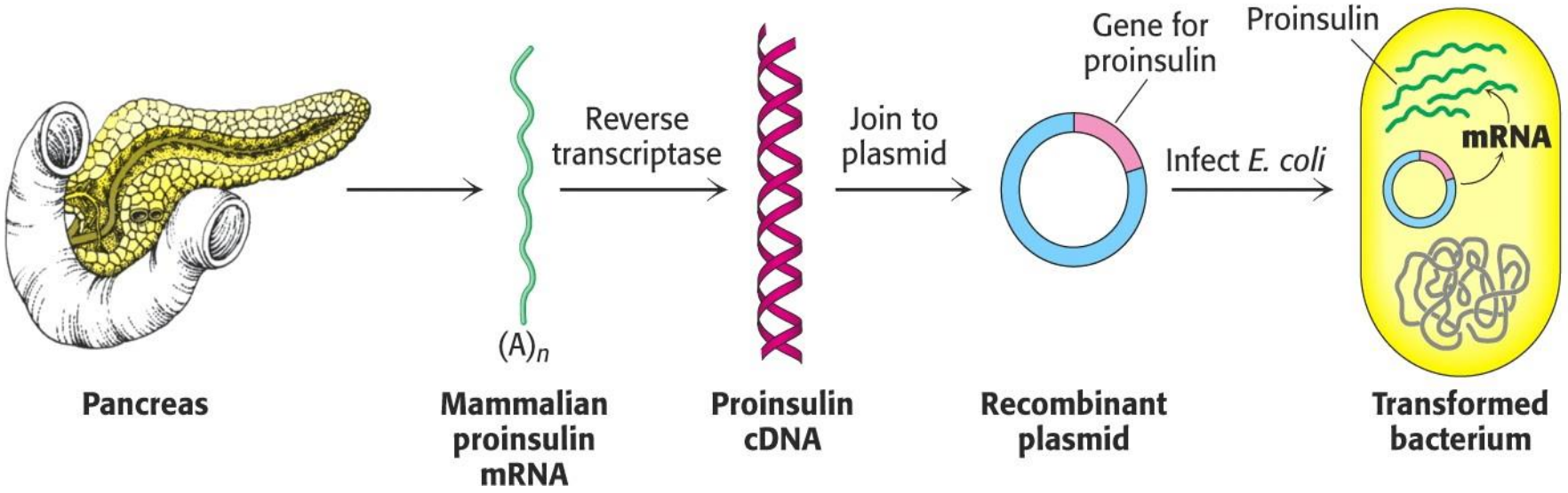
Período: Quinta-feira (14h00 -18h00), Laboratório de Biotecnologia – Edifício A2, 1º andar

USP - 2024

Atividades

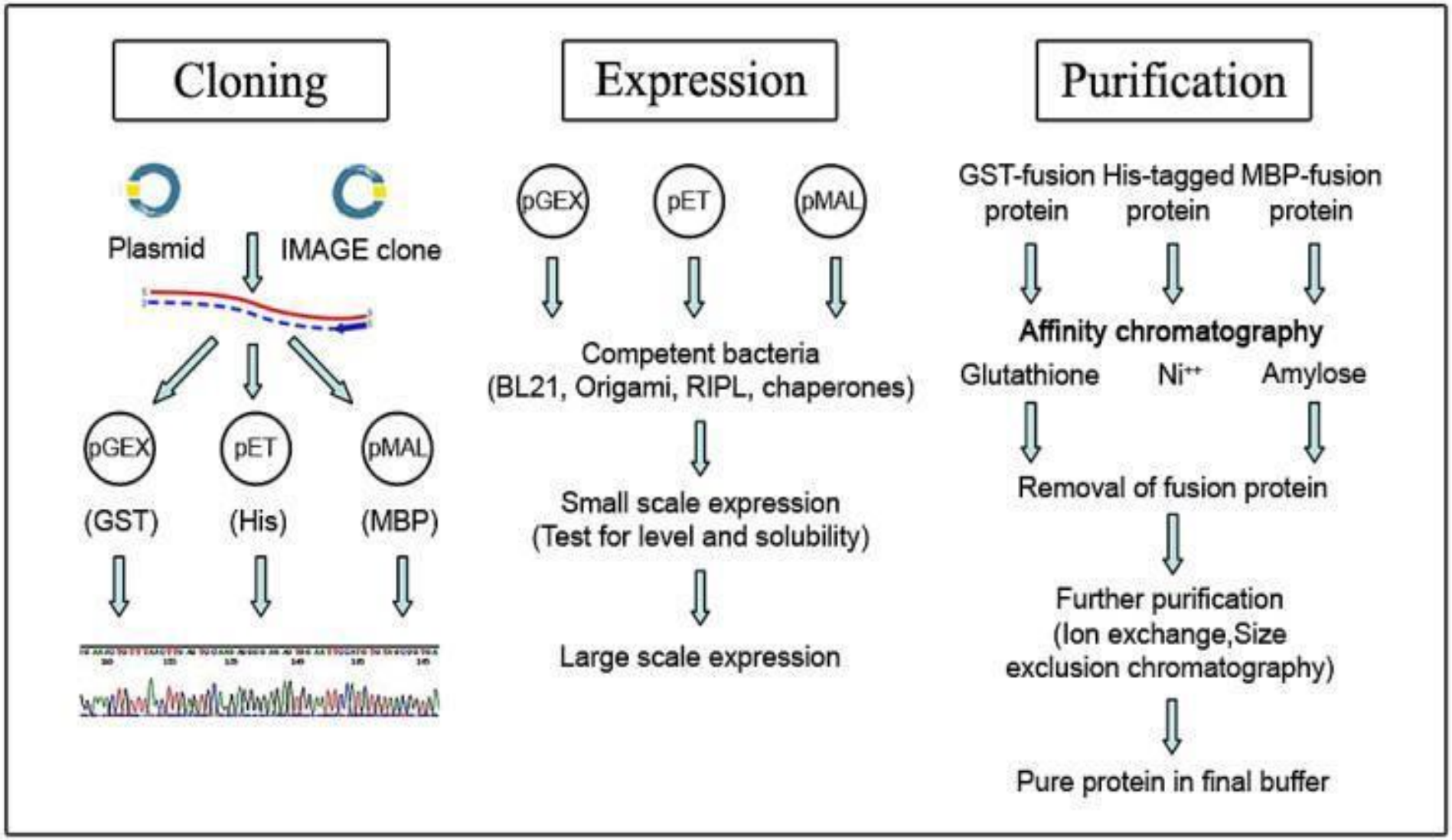
1. DNA recombinante:
- Sistemas Procarióticos - Vetores de Clonagem e Expressão.
2. Enzimas utilizadas em Clonagem molecular:
- Digestão de DNA - enzimas de restrição
- Ligação
3. Transformação de células procarióticas

Como produzir uma proteína recombinante?



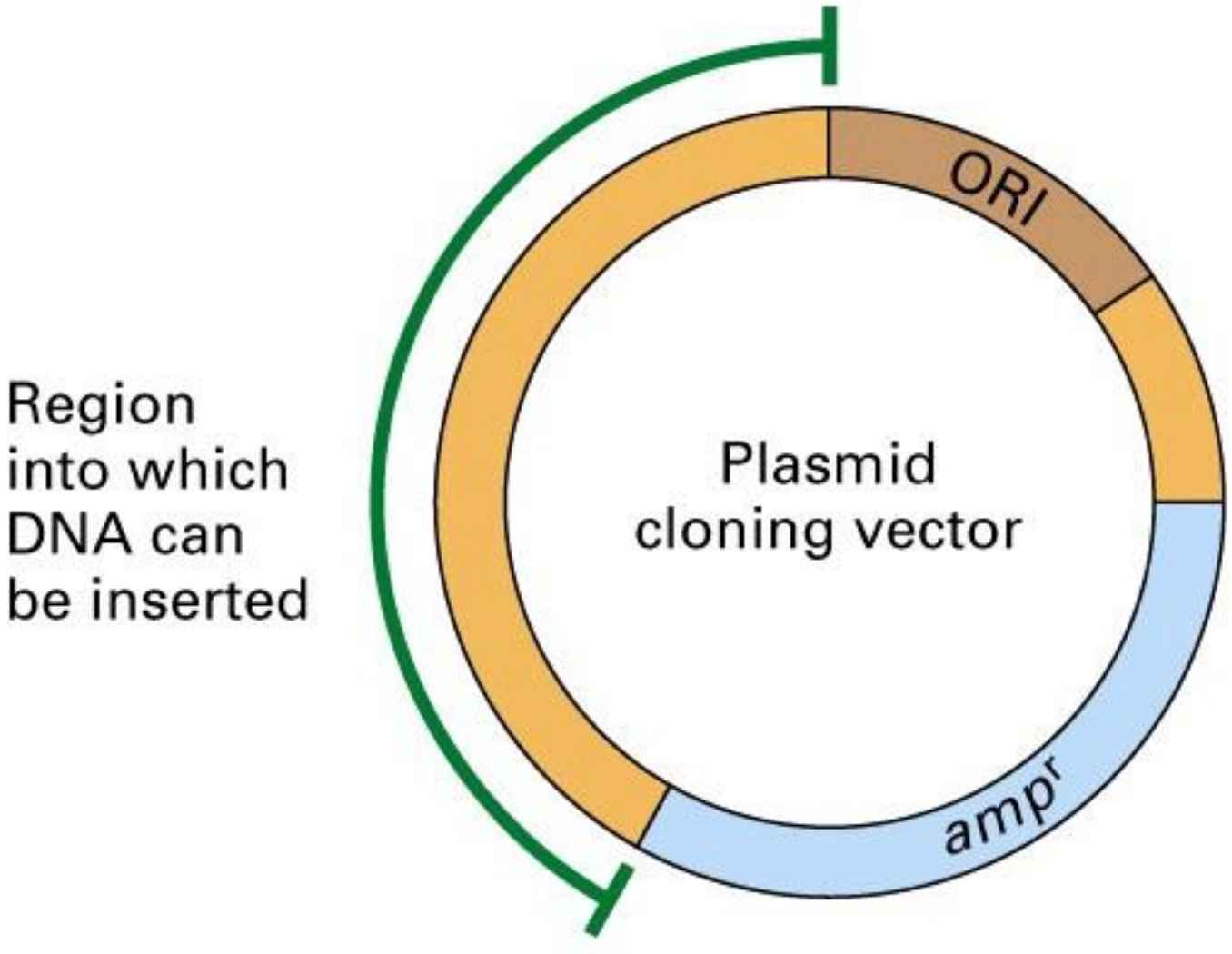
0.1 - 1% da proteína celular

10 - 70% da proteína celular

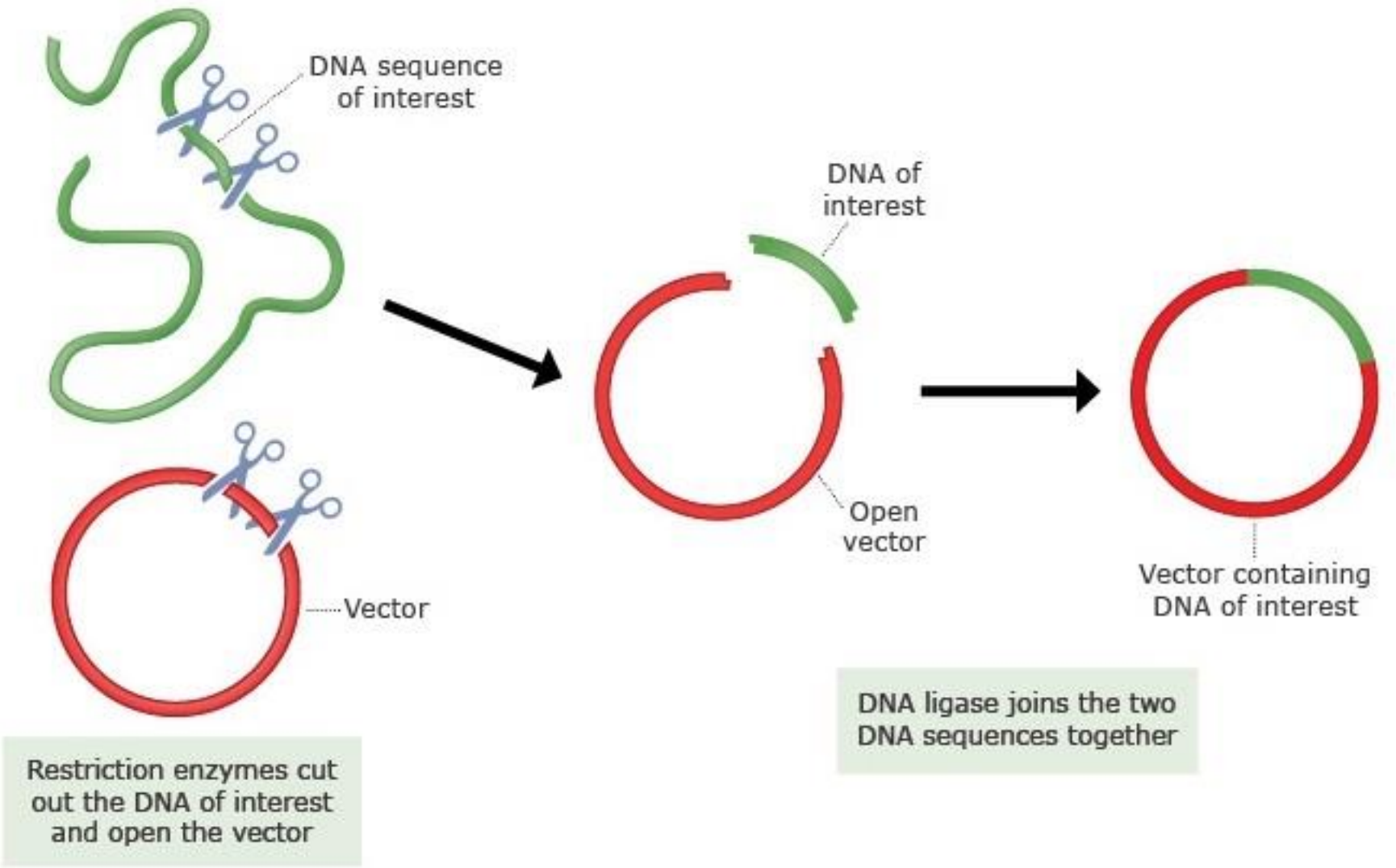


https://www.southampton.ac.uk/

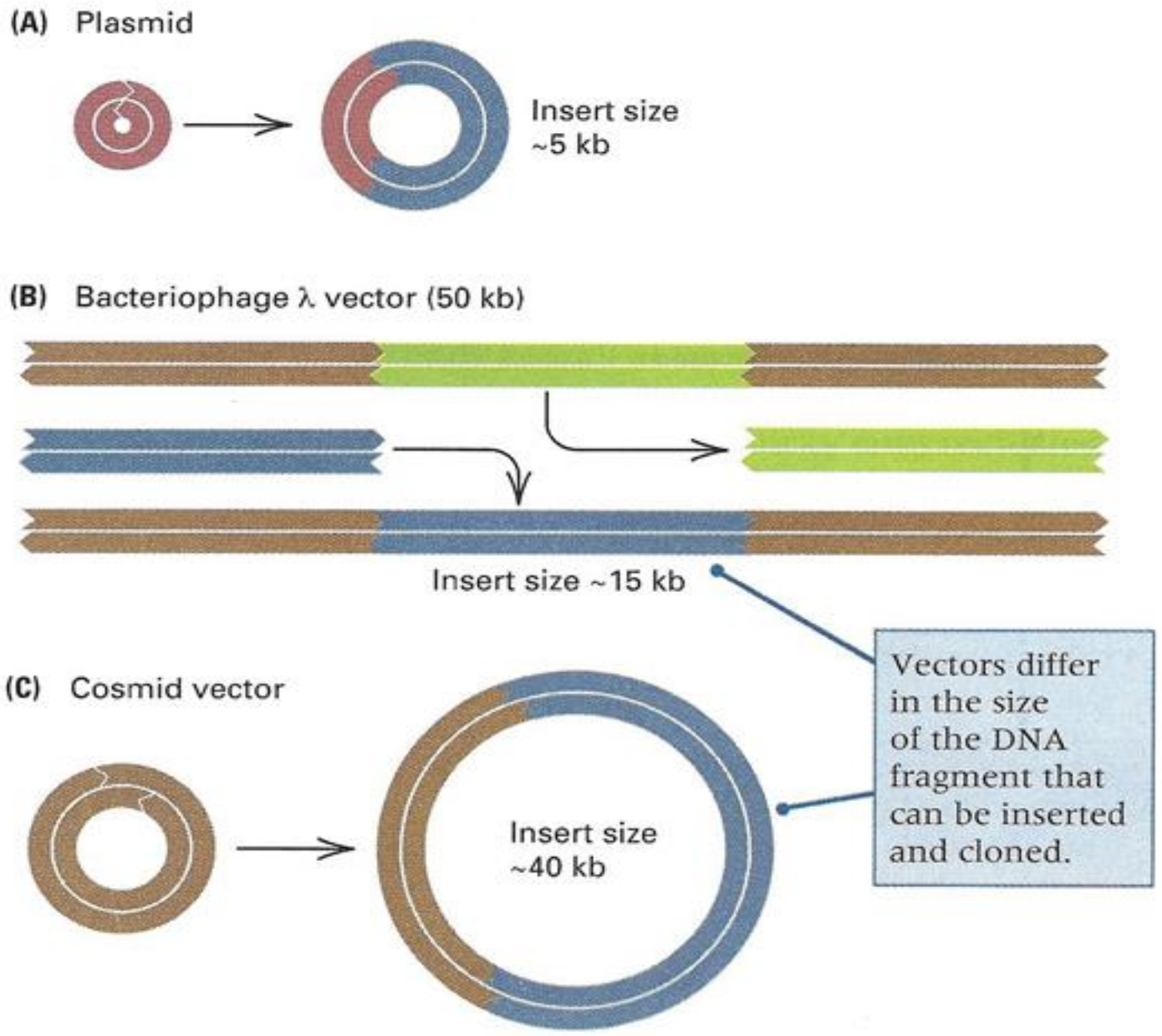
Diagram of a simple cloning vector derived from a plasmid, a circular, double-stranded DNA molecule that can replicate within an *E. coli* cell.



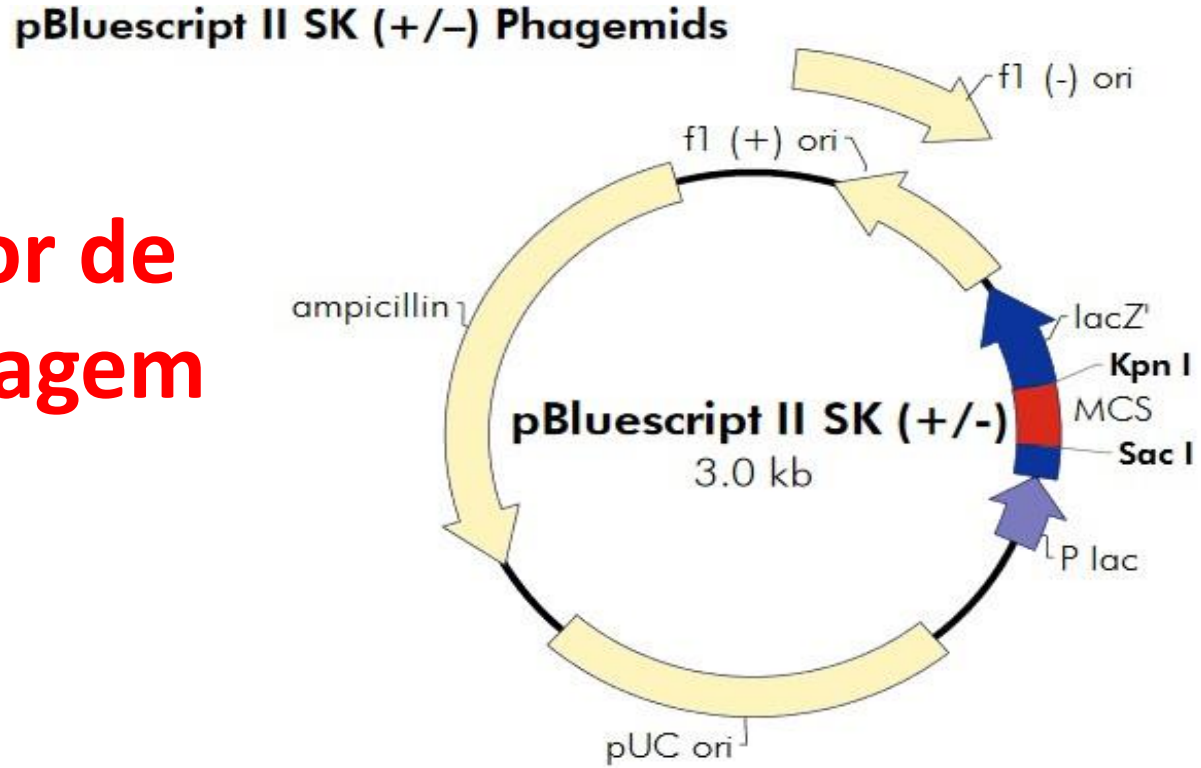
DNA Recombinante



Vetor de Clonagem



Vetor de Clonagem



pBluescript II SK (+/–) Multiple Cloning Site Region (sequence shown 598–826)

BssH II — T7 Promoter — Kpn I — Apa I / EcoO109 I / Dra II — Xho I — Hinc II / Acc I / Sal I

TTGTAAAACGACGGCCAGTGAGCGCGCGTAATACGACTCACTATAGGGCGAATTGGGTACCGGGCCCCCCCTCGAGGTCGAC...

M13 –20 primer binding site | T7 primer binding site | KS primer binding site...

Bsp106 I / Cla I — Hind III — EcoR V — EcoR I — Pst I — Sma I — BamH I — Spe I — Xba I — Not I / Eag I — BstX I — Sac II — Sac I

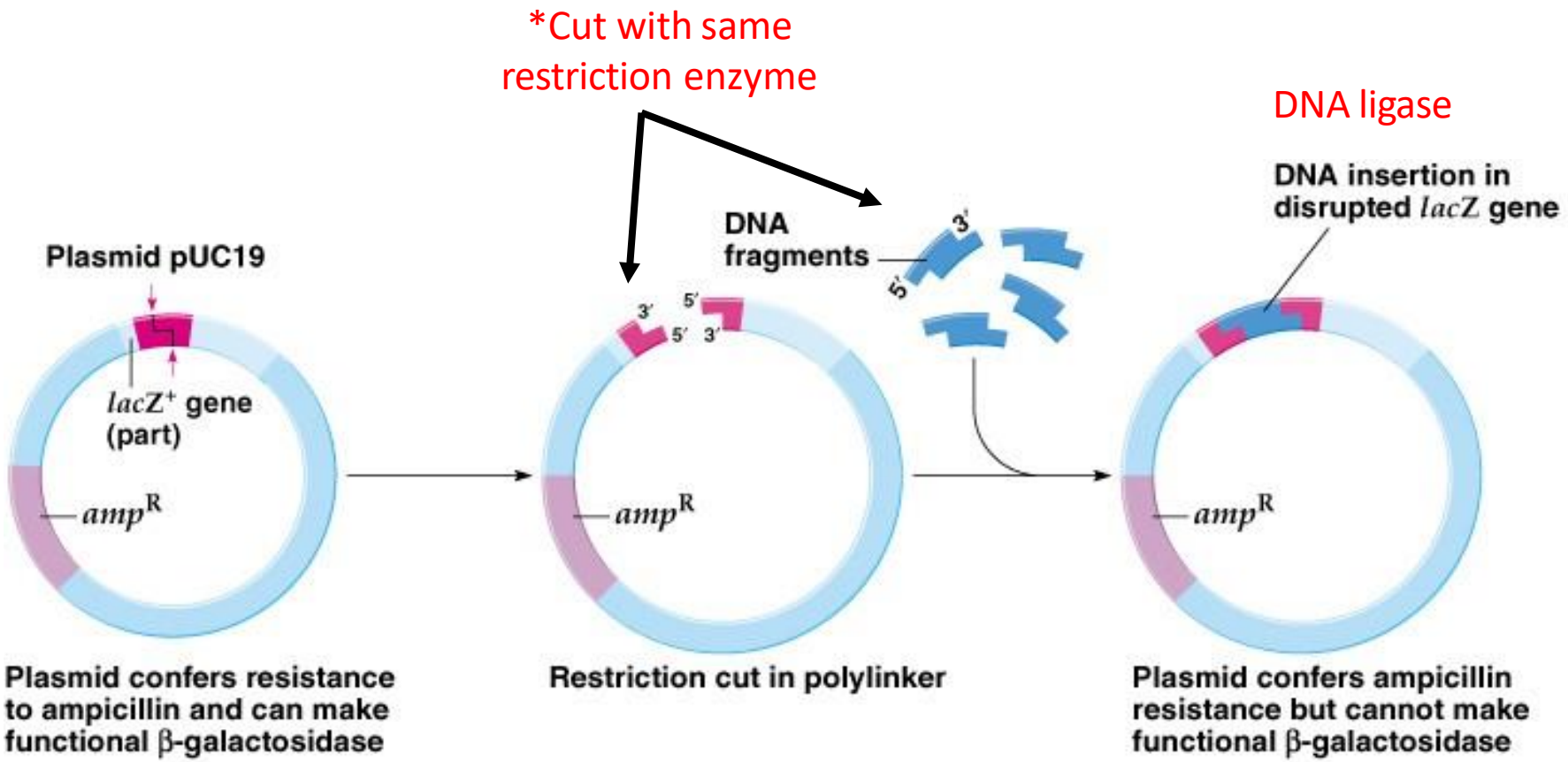
...GGTATCGATAAGCTTGATATCGAATTCCTGCAGCCCGGGGGATCCACTAGTTCTAGAGCGGCCGCCACCGCGGTGGAGCTC...

...KS primer binding site | SK primer binding site

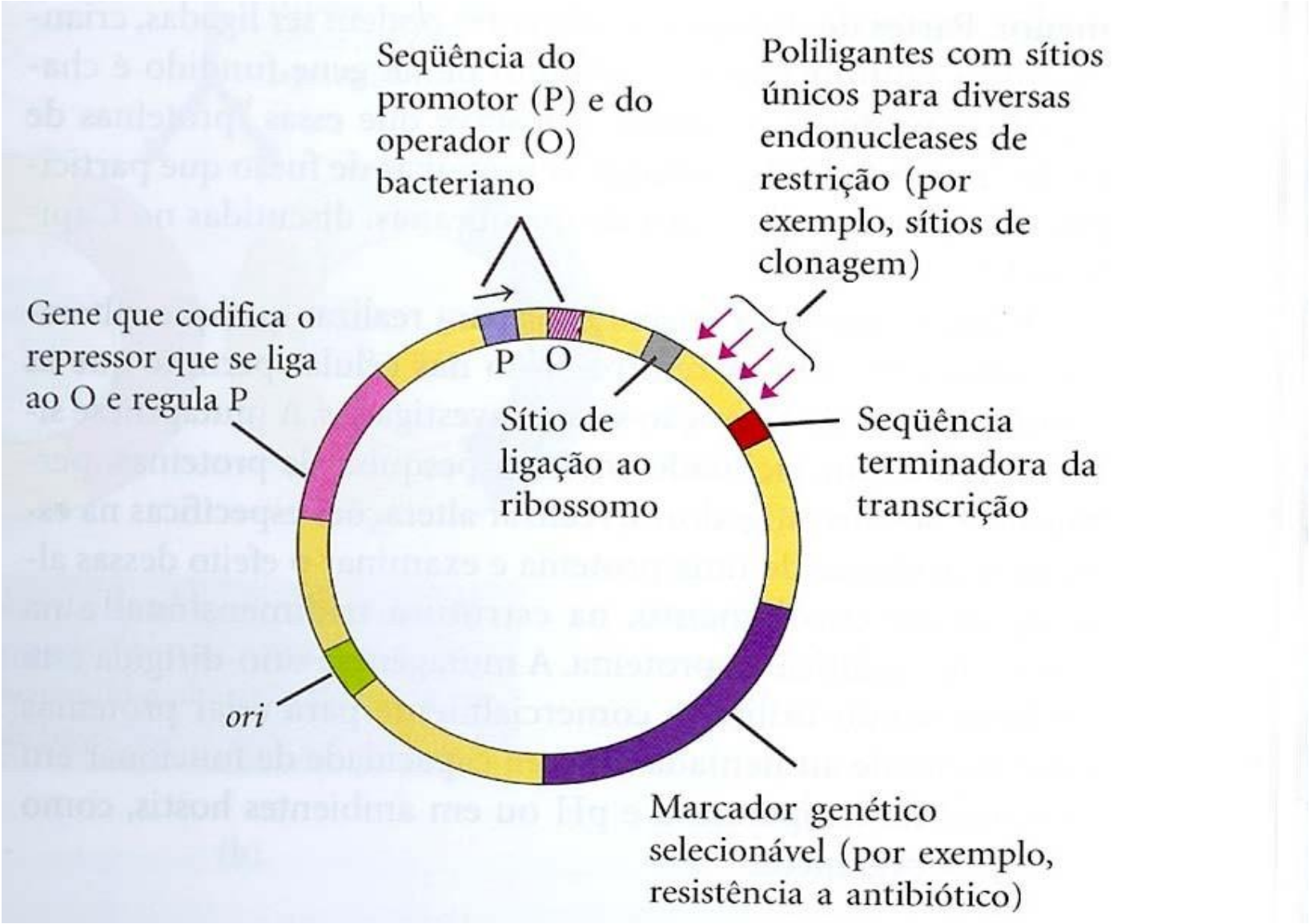
T3 Promoter — BssH II — β-gal α-fragment

...CAGCTTTTGTTCCCTTTAGTGAGGGTTAATTGCGCGCTTGGCGTAATCATGGTCATAGCTGTTTCC

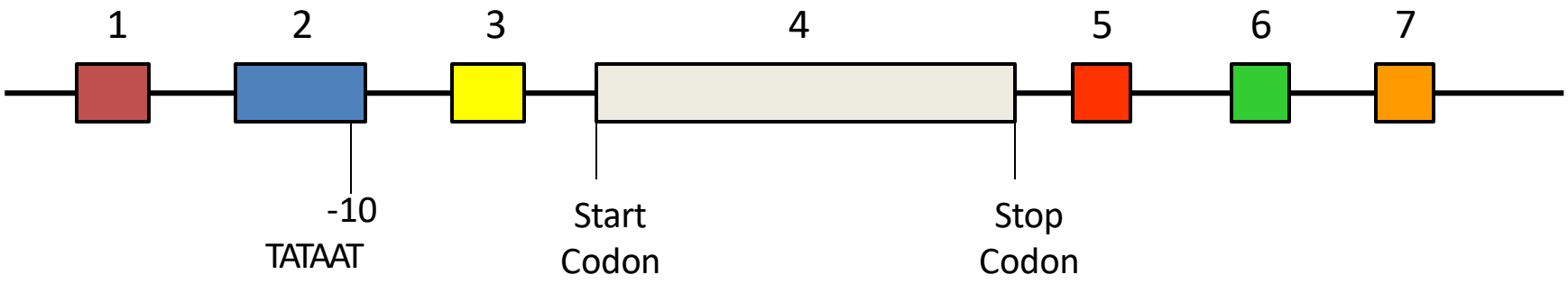
T3 primer binding site | M13 Reverse primer binding site


*Cut with same restriction enzyme
DNA ligase
Plasmid pUC19
lacZ+ gene (part)
ampR
DNA fragments
3′
5′
5′
3′
3′
5′
ampR
DNA insertion in disrupted lacZ gene
ampR
Plasmid confers resistance to ampicillin and can make functional β-galactosidase
Restriction cut in polylinker
Plasmid confers ampicillin resistance but cannot make functional β-galactosidase

Vetor de Expressão



Elementos de um vetor de expressão procariótico



1- Regulador do promotor: Proteína que modula o promotor

2- Promotor: Deve ser forte (lac, trp, tac, λp^L, gene 10 do fago T7)

3- Seqüência Shine-dalgarno: Sitio de ligação do ribossomo, (RBS).

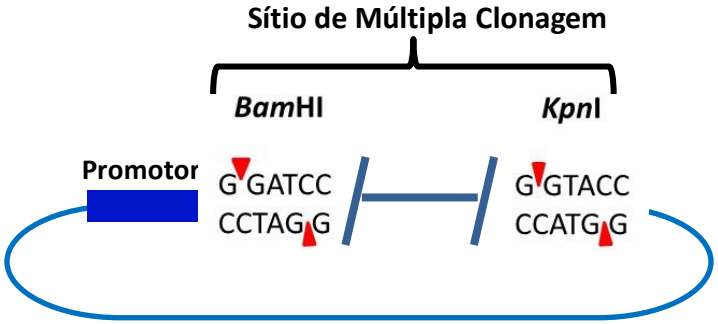
4- Região codificadora: sítios de múltipla clonagem

5- Terminador de transcrição: Estabiliza o mRNA

6- Marcador genético (antibiótico de seleção)

7- Ori: Origem de replicação.

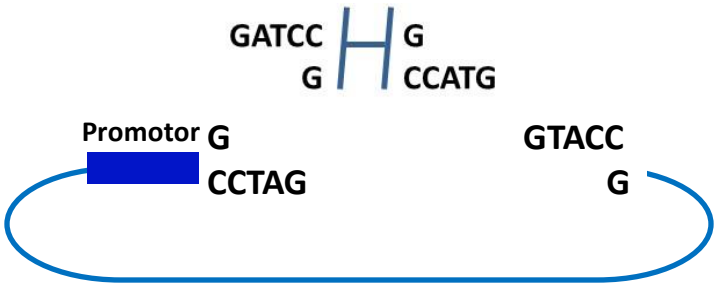
Estratégia de Clonagem no Vetor de Expressão



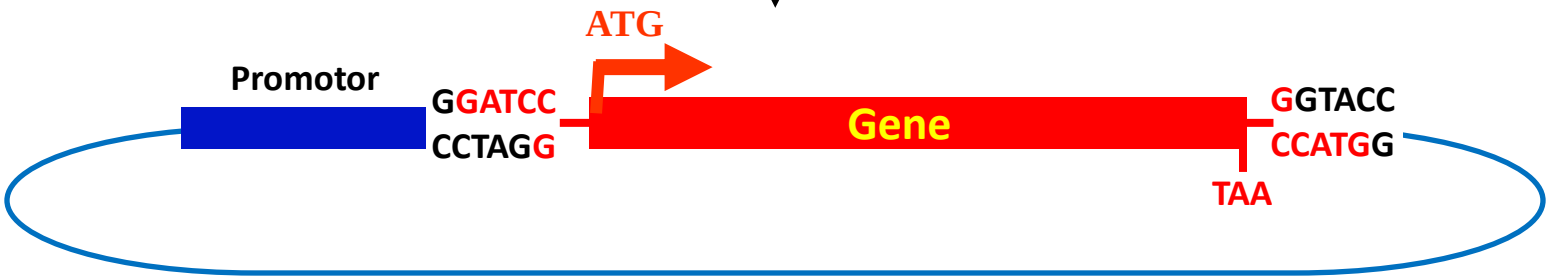
Vetor de Expressão

Gene de Interesse

Clivagem com enzimas de restrição *Bam*HI e *Kpn*I



Ligação com enzima DNA Ligase



Vetor de Expressão/Gene de Interesse

ThermoFisher SCIENTIFIC

Vector Selection Tool

We've made finding the right vector for your research easier. Simply search by keyword or filter your results by vector type, host system, or cloning system. Once the initial results are displayed, they can be further refined by selecting among characteristics such as promoter, inducer, and antibiotic selection. In addition we also have a wide selection of products related to vectors, including competent cells, media, plasmid purification kits and more.

Search by Keywords: Find

Vector Type
Expression (212)
Cloning (84)
Cloning for Sequencing (13)
RNAi (7)
DNA Assembly (5)
Two Hybrid (1)

Host System
Mammalian (129)
Bacteria (35)
Yeast (28)
Insect (21)
In vitro (7)

Cloning System
Restriction & Ligation (91)
Gateway (89)
TOPO (39)
TOPO TA (38)
TA (14)
TOPO adapted Gateway (13)
All Cloning System

Promoter
CMV (92)
lac (52)
T7 (29)
lac operator (15)
EF1alpha (13)
AOX1 (12)
All Promoter

Inducer
IPTG (27)
methanol (13)
Tetracycline (10)
Arabinose (7)
galactose (7)
copper sulfate (3)
All Inducer

Selection for plasmid
Ampicillin (255)
Kanamycin (94)
Zeocin (49)
Blasticidin (20)
Spectinomycin (10)
Gentamicin (9)
All Selection for plasmid

N-term tag
6xHis (30)
Xpress (17)
alpha factor (11)
V5 (9)
lacZ (5)
HP–Thioredoxin (5)
All N-term tag

C-term tag
6xHis (72)
V5 (72)
Myc (26)
None (5)
Lumio (5)
EmGFP (3)
All C-term tag

N-term Protease cleavage
EK (26)
TEV (16)
None (5)
SUMO (1)

Select the best expression host for your work!

Host System	Advantages	Challenges
Cell-Free Expression (*in vitro*)	• Scalable • Simple format • Rapid expression directly from plasmid • Open system—easily add components to enhance solubility or functionality	• Large-scale expression > 3 mg
Bacterial Expression	• Scalable • Low cost • Simple culture conditions	• Protein solubility • Minimal posttranslational modifications • May be difficult to express functional mammalian proteins
Yeast Expression	• Eukaryotic protein processing • Scalable up to fermentation (grams per liter) • Simple media requirements	• Fermentation required for very high yields • Growth conditions may require optimization
Algae Expression	• Genetic modification and expression systems for photosynthetic microalgae • Superb experimental control for biofuel, nutraceuticals and specialty chemical production • Optimized system for robust selection and expression	
Insect Expression	• Postranslational modifications similar to mammalian systems • Greater yield than mammalian systems	• More demanding culture conditions
Mammalian Expression	• Highest level of correct post-translational modifications • Highest probability of obtaining fully functional human proteins	• Multimilligram per liter yields only possible in suspension cultures • More demanding culture conditions

https://www.thermofisher.com

Atividade 2: Extração de DNA plasmidial de bactérias em pequena escala: "Miniprep" (Plasmid DNA Purification using the QIAprep Spin Miniprep Kit, www.qiagen.com)

Procedimento:

1. Realizar uma cultura de *E.coli,* contendo o plasmídeo, em 5 mL de meio LB com antibiótico, incubar por 12-18 h sob agitação à 37°C.

2. Posteriormente, colocar 1,5 mL da cultura em microtubo de 1,5 mL.

3. Centrifugar à 10.000 rpm em temperatura ambiente por 5 minutos e remover o sobrenadante.

4. Acrescentar 1,5 mL da cultura nos respectivos tubos e repetir os passos 2 e 3.

5. Suspender o sedimento bacteriano em 250 µL da Solução P1.

6. Acrescentar 250 µL da Solução P2. Misturar por inversão, **NÃO VORTEXAR OS TUBOS**. Uma solução viscosa deverá ser formada imediatamente devido à lise das células.

7. Acrescentar 350 µL da Solução N3. Misturar por inversão, **NÃO VORTEXAR OS TUBOS**. Um precipitado branco deverá ser formado imediatamente. Este precipitado contém DNA genômico e restos celulares. O DNA plasmidial permanecerá em solução.

8. Centrifugar os tubos à 12.000 rpm, temperatura ambiente, por 10 minutos.

9. Remover 750 µL do sobrenadante cuidadosamente e transferi-lo para coluna QIAprep Spin, com tubo reservatório.

10. Centrifugar os tubos à 10.000 rpm, temperatura ambiente, por 1 minuto, descartar o líquido do tubo reservatório.

11. Lavar a coluna QIAprep Spin, adicionando 0.75 mL de solução PE

12. Em seguida, centrifugar à 10.000 rpm, temperatura ambiente, por 1 minuto.

13. Descartar o líquido do tubo reservatório, centrifugar à 10.000 rpm, temperatura ambiente, por 1 minuto.

14. Transferir a coluna QIAprep Spin a tubo limpo, eluir o DNA por adição de 60 uL de solução EB (10 mM Tris·Cl, pH 8.5) ou água.

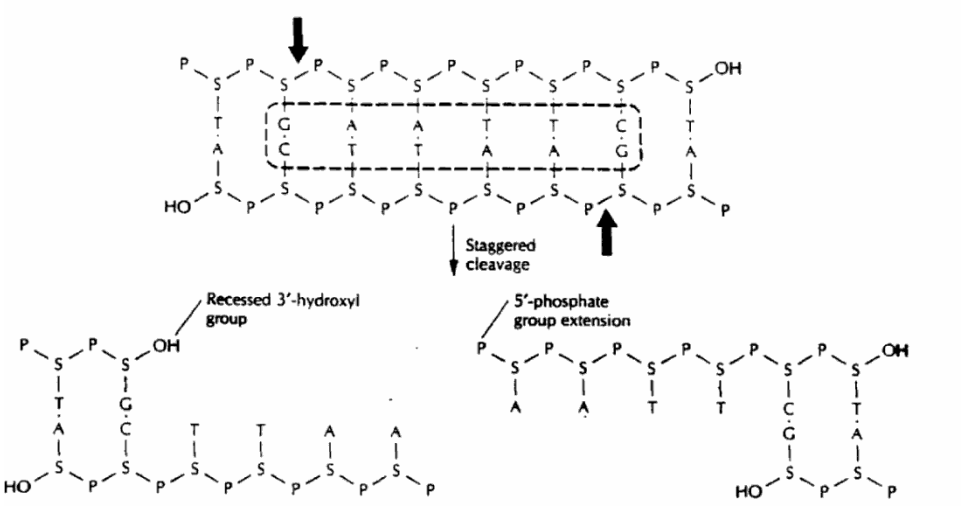
15. Centrifugar os tubos à 10.000 rpm, temperatura ambiente, por 1 minuto, descartar a coluna QIAprep Spin, o DNA está em solução.

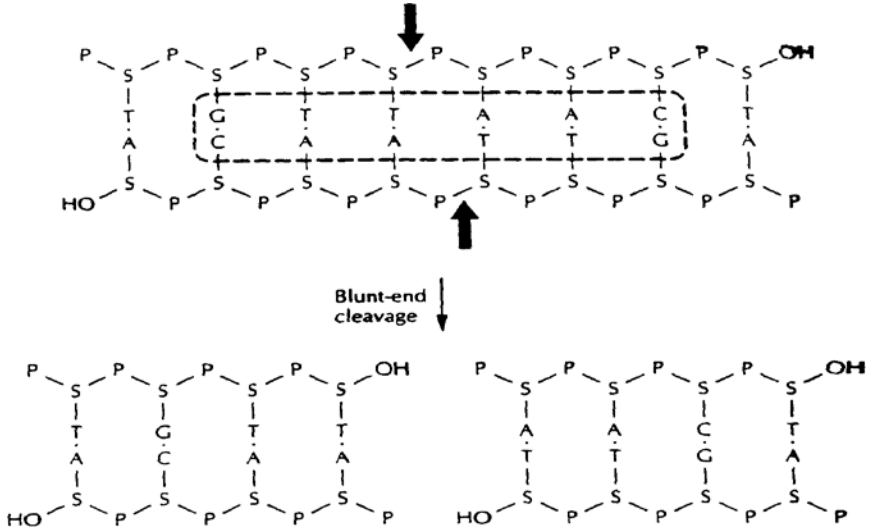
16. Realizar a Eletroforeses e quantificar.

Enzimas de Modificação de DNA e Mapas de restrição

1. Enzimas de Restrição

Restriction-recognition sites are short DNA sequences recognized and cleaved by various restriction endonucleases.





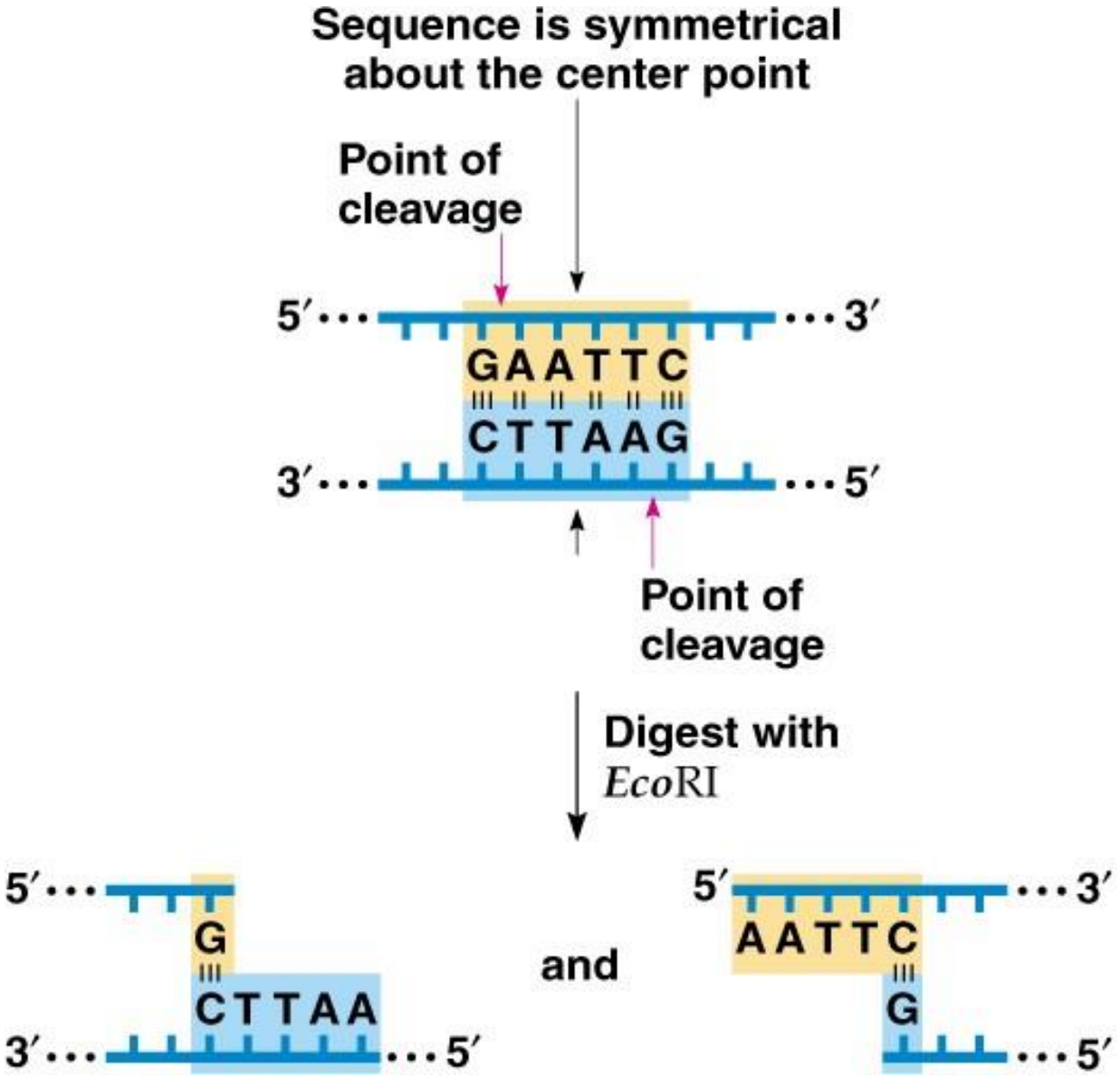
Extremos coesivos

*Eco*RI

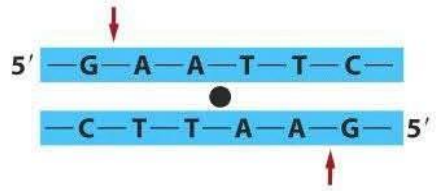
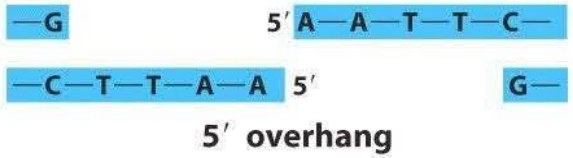
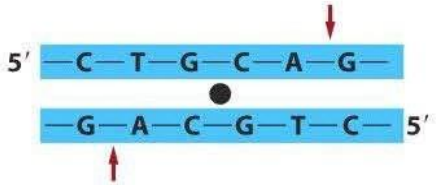
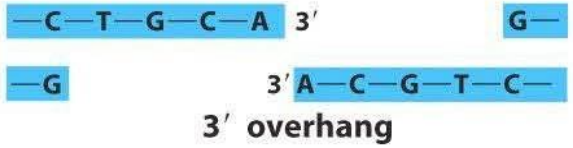
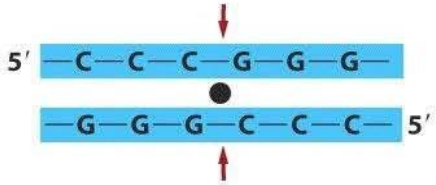
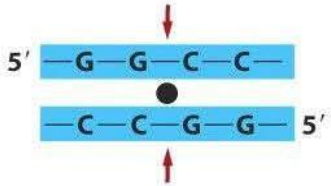
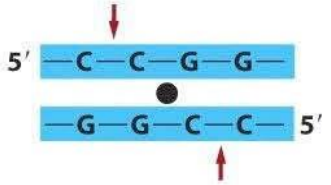
Extremos cegos

*Hind*II

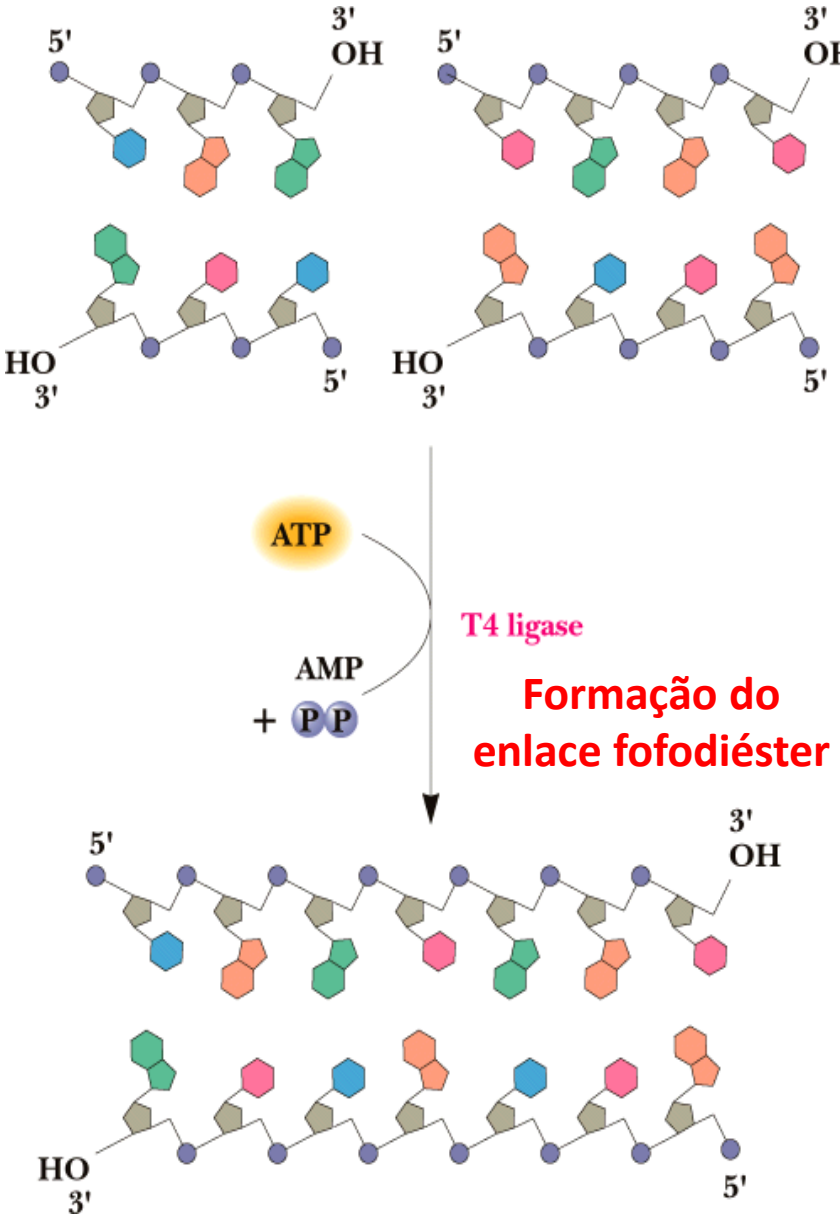
RADAR


Sequence is symmetrical about the center point
Point of cleavage
5′
3′
GAATTC
CTTAAG
3′
5′
Point of cleavage
Digest with *Eco*RI
5′
3′
G
CTTAA
3′
5′
and
5′
3′
AATTC
G
5′

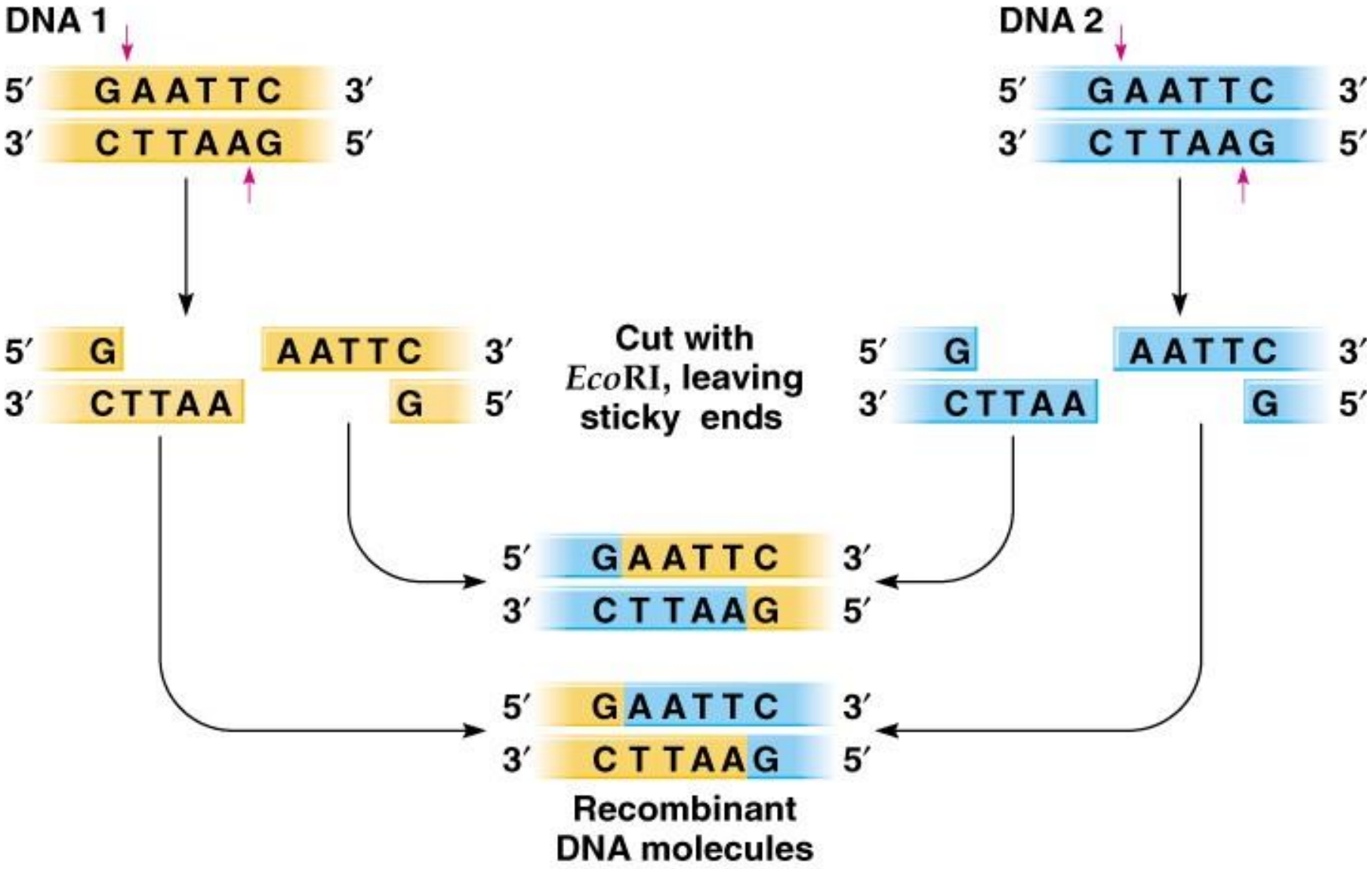
Some restriction enzymes

Enzyme	Source organism	Restriction recognition site in double-stranded DNA	Structure of the cleaved products
(a)			
*Eco*RI	*Escherichia coli*		
			5′ overhang
*Pst*I	*Providencia stuartii*		
			3′ overhang
*Sma*I	*Serratia marcescens*		
			Blunt ends
(b)			
*Hae*III	*Haemophilus aegyptius*		
			Blunt ends
*Hpa*II	*Haemophilus parainfluenzae*		
			5′ overhang

2. DNA Ligase: Junção de moléculas

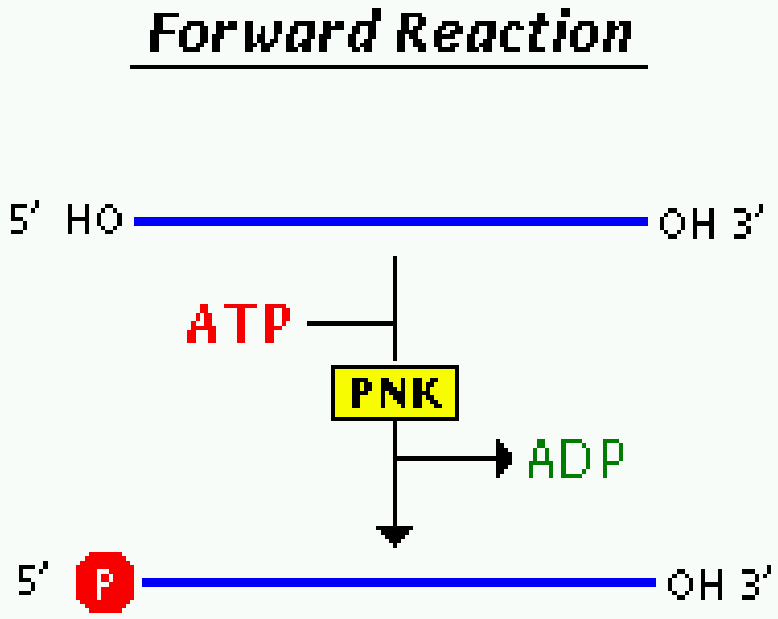


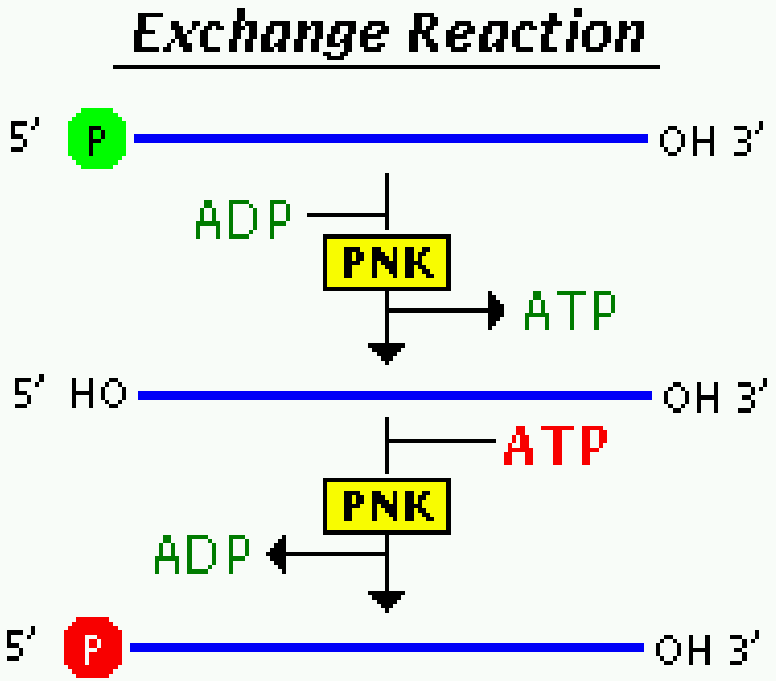
Clivagem e ligação de duas moléculas de DNA com *Eco*RI ---> DNA Recombinante



3. T4 polynucleotide kinase

- Transfers gamma phosphate of ATP to the 5’ end of polynucleotides
- Useful for preparing DNA fragments for ligation (if they lack 5’ phosphates)
- Useful for radiolabelling DNA fragments using gamma ^{32}P ATP as a phosphate donor





4. Fosfatasa alcalina

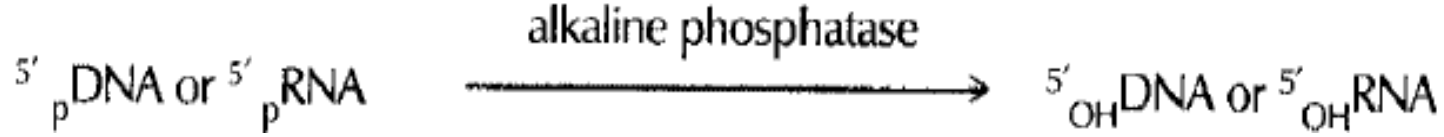
- Remoção do 5’ (e 3’) fosfato dos polinucleotídeos
- Useful for treating restricted vector DNA sequences prior to ligation reactions, prevents religation of vector in the absence of insert DNA

ALKALINE PHOSPHATASES

Activity: Phosphatase

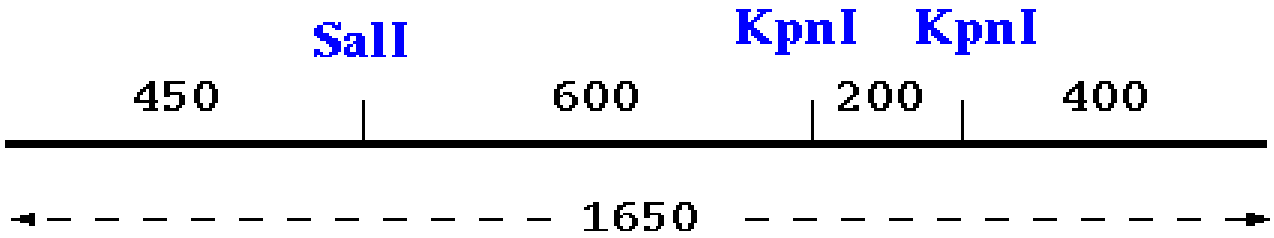
Substrate: Single- or double-stranded DNA and RNA; rNTPs and dNTPs.

Reaction:

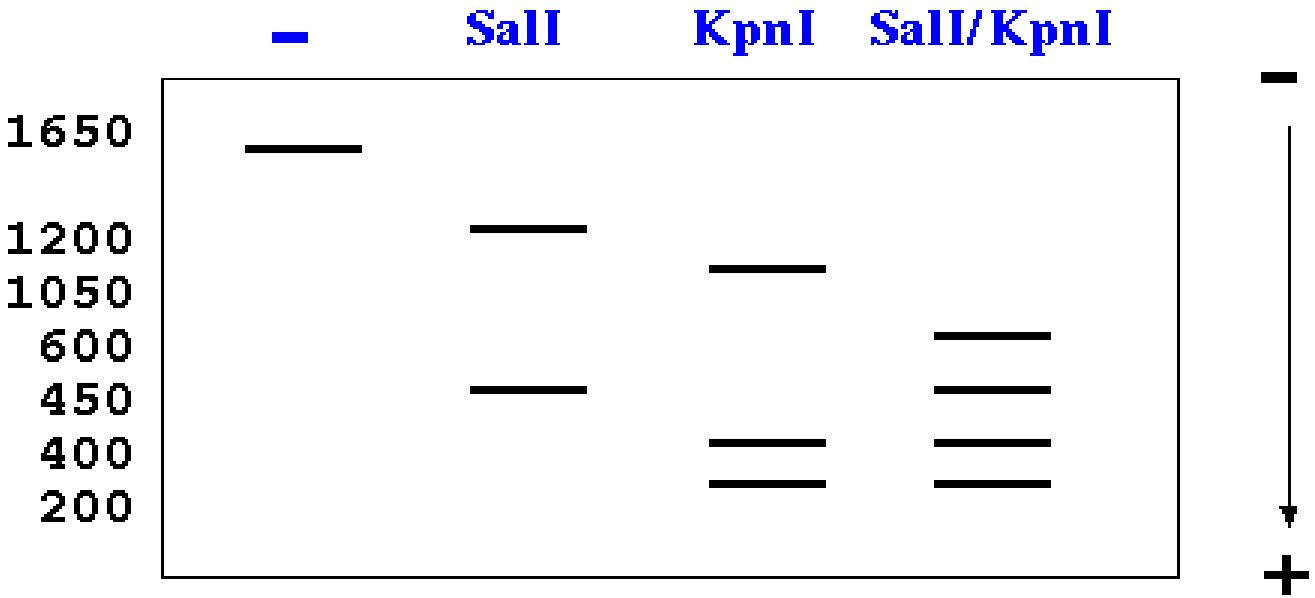


Mapa de Restrição

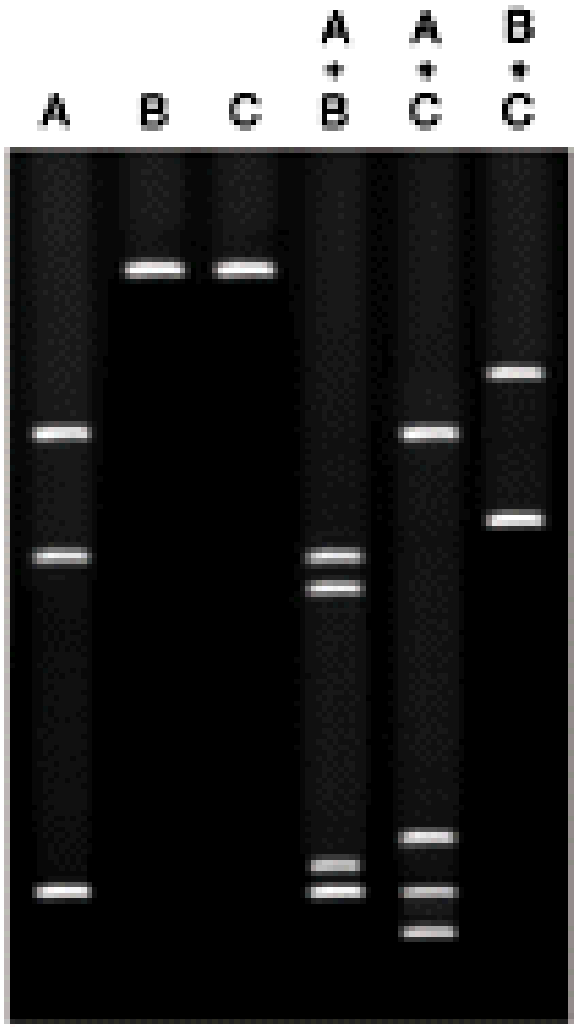
Restriction map

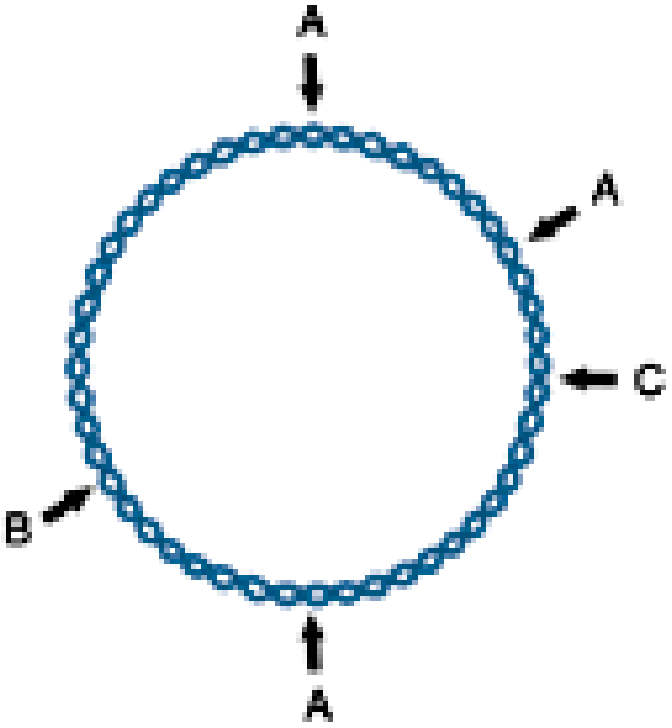


Predicted digest fragments

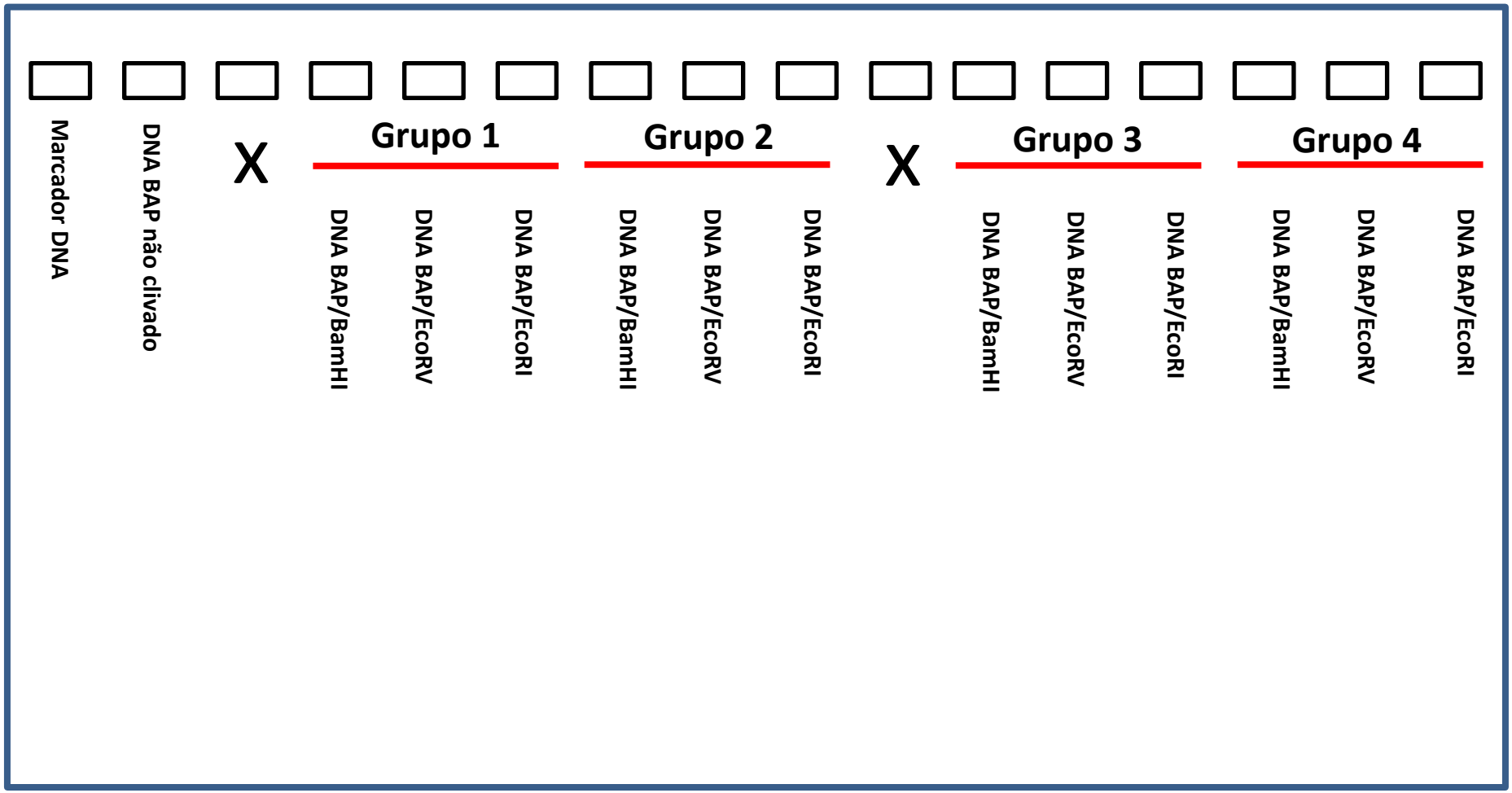


Mapa de Restrição

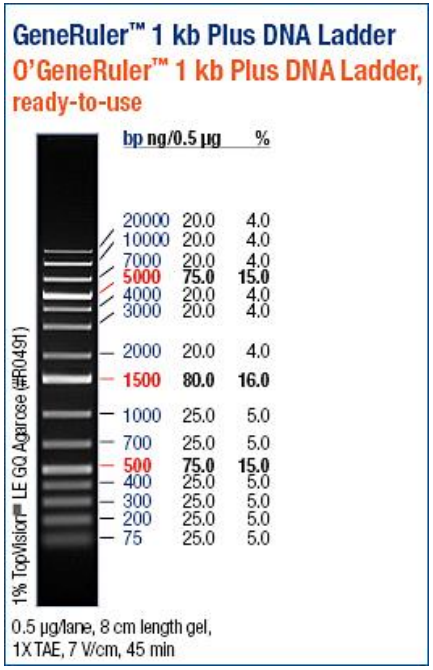


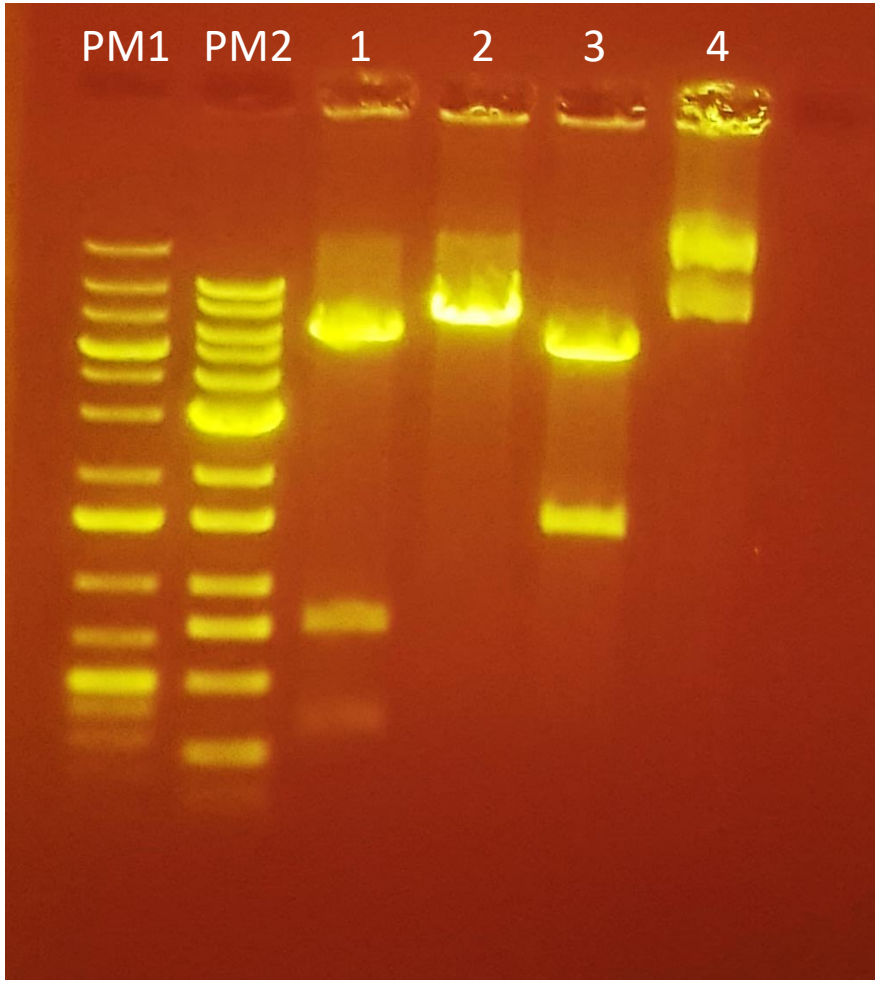


Eletroforeses de DNA



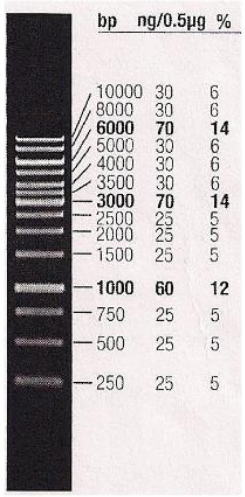
PM1





LGC Biotecnologia

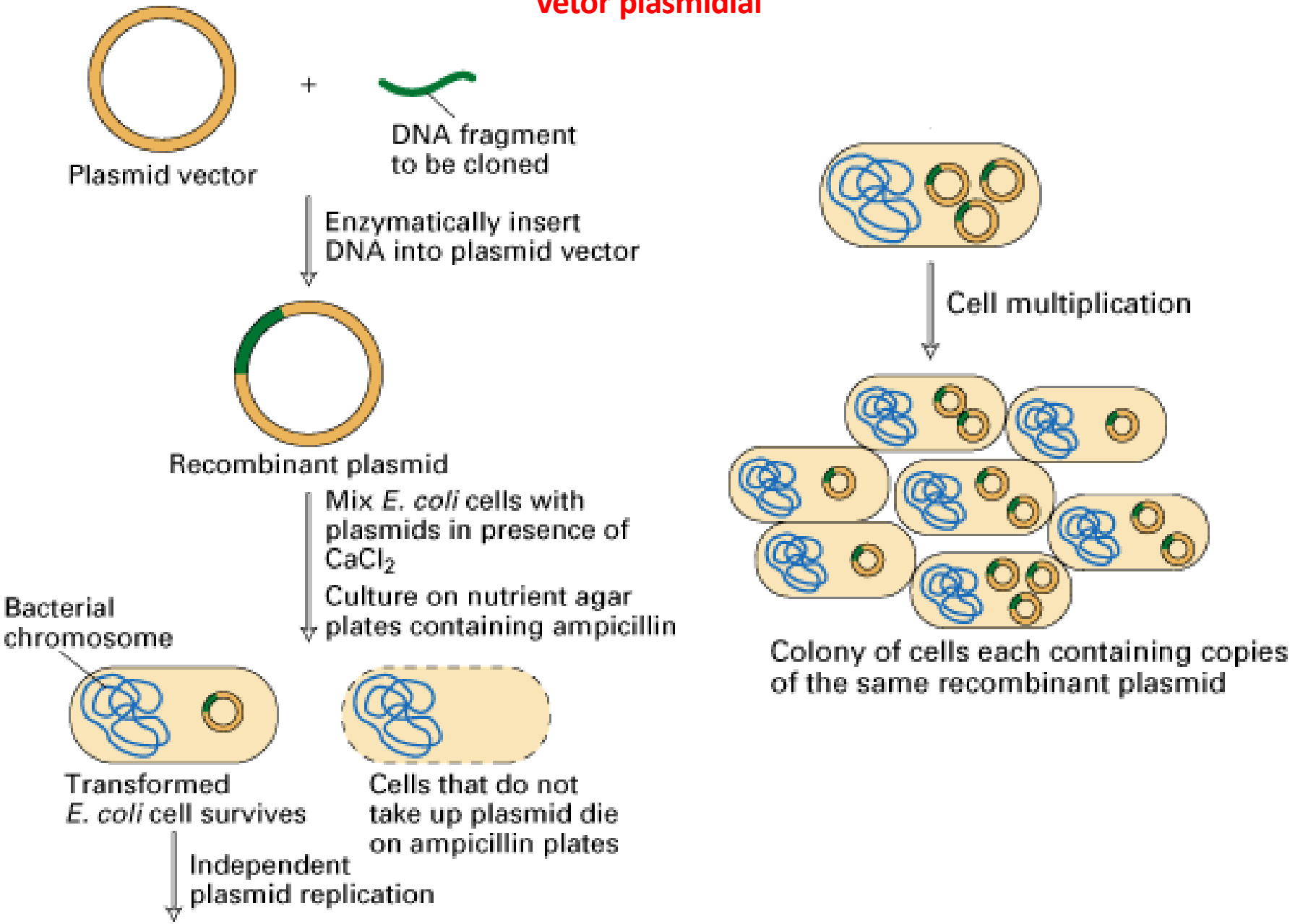
Marcador de Peso molecular
1 Kb DNA Ladder
Código Nº:
13-4004-01: 100 ng/µL
Volume: 500 µL
Rendimento: 100 aplicações
Armazenamento: -15° a - 30°C

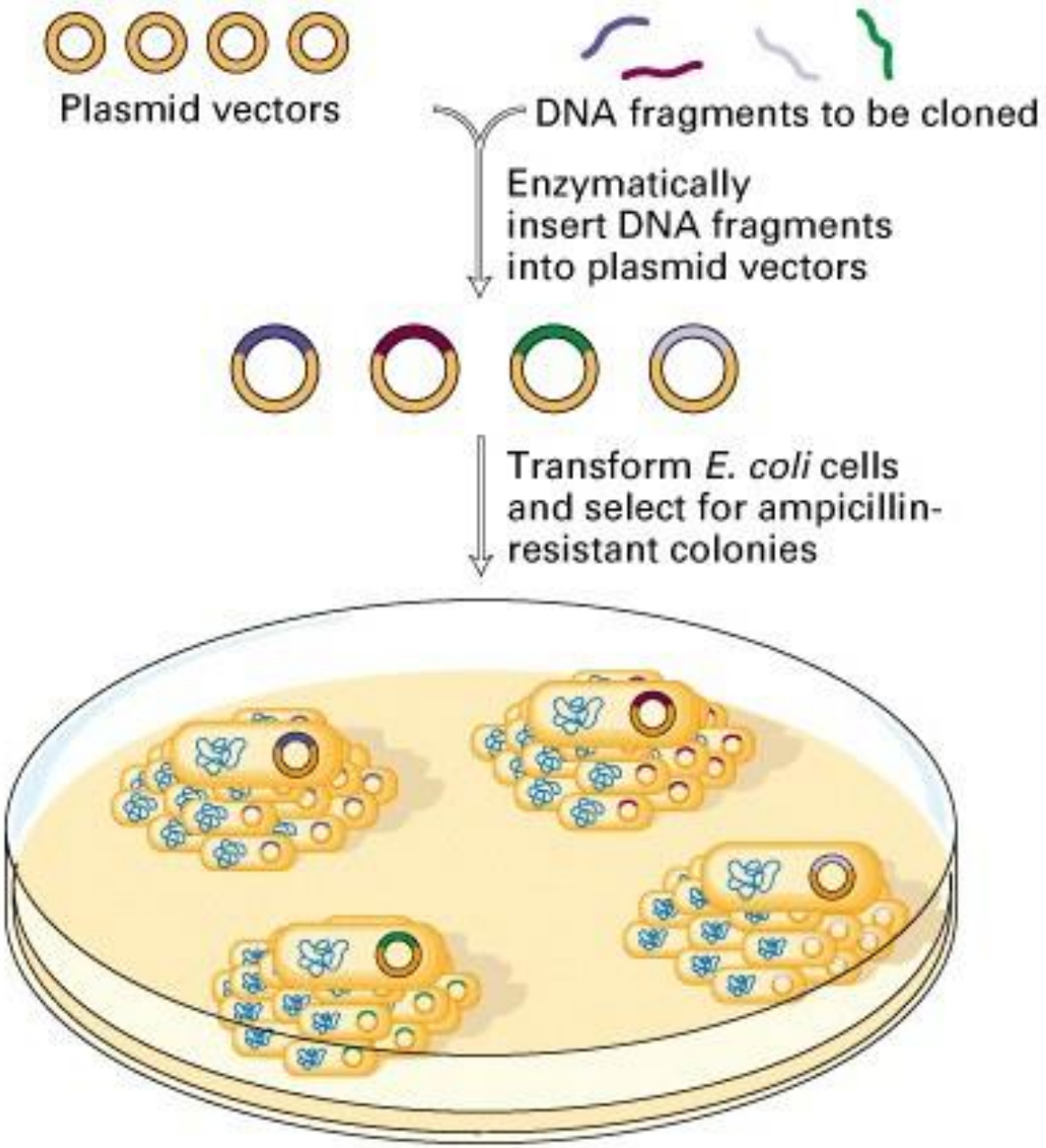


Marcador 1 Kb *Ladder LGC*. Eletroforese em gel de agarose 0,8%, mostrando os fragmentos gerados da aplicação de 5 µL do marcador.

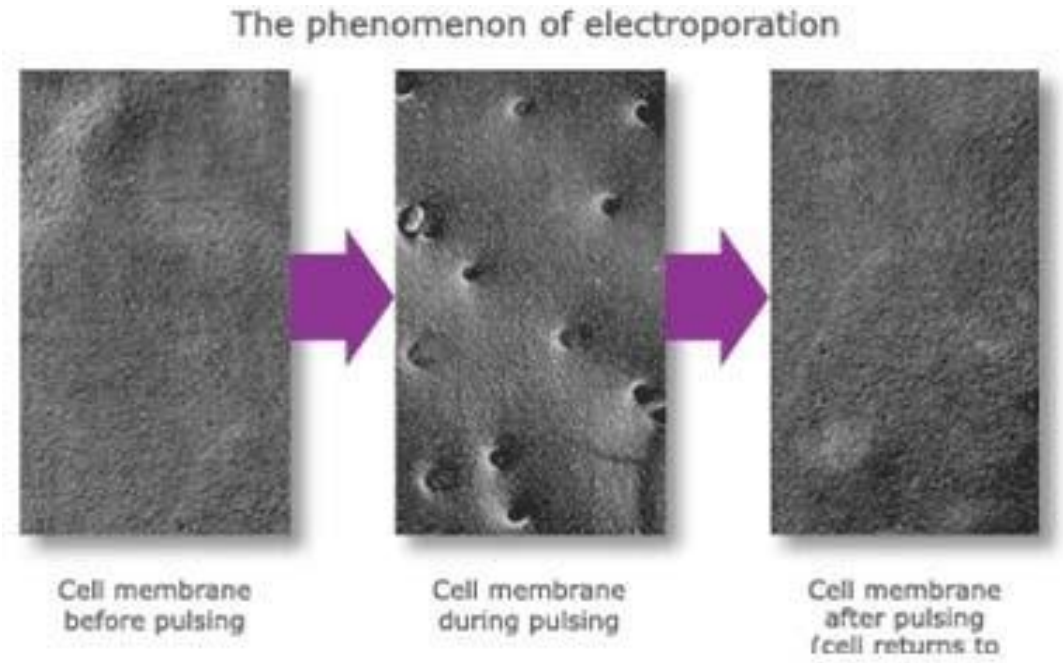
Figura 1. Eletroforeses em gel de agarose 1% da análise de restrição do DNA plasmidial contendo o gene Fosfatase alcalina bacteriana. Amostra de 17 uL de DNA foi clivada com 1 uL de enzima de restrição. Linha 1: EcoRI; linha 2: BamHI; linha 3: EcoRV; linha 4: Não clivado. PM1: Peso Molecular 1 kb DNA ladder. PM2: Peso Molecular 1 kb DNA ladder. Aplicados 20 uL da reação + 4 uL de tampão de corrida. Corante SYBR SAFE DNA gel stain (Lifetechnologies, USA).

Procedimento geral para clonagem de um fragmento de DNA em um vetor plasmidial




Plasmid vectors
DNA fragments to be cloned
Enzymatically
insert DNA fragments
into plasmid vectors
Transform *E. coli* cells
and select for ampicillin-
resistant colonies

Transformação de célula procariótica por Eletroporação



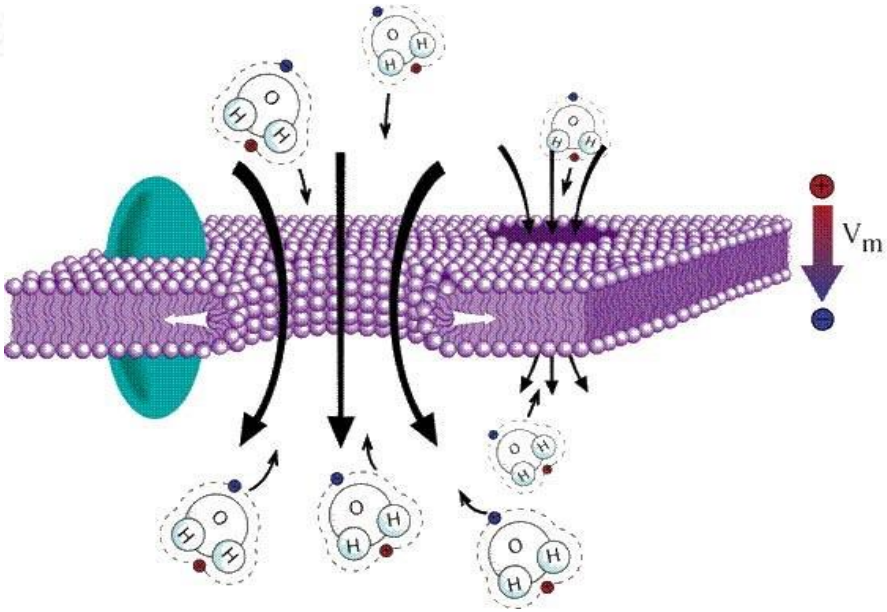
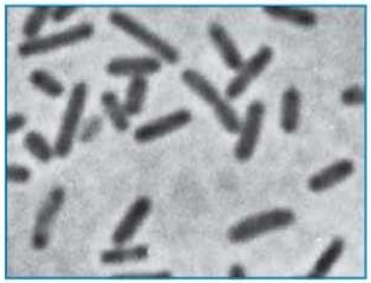


Figure 11.13

Bacteria

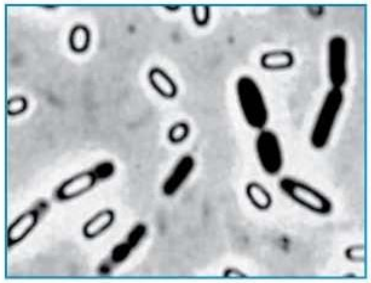
Escherichia coli



- Well-developed genetics
- Many strains available
- Best known bacterium

- Potentially pathogenic
- Periplasm traps proteins

Bacillus subtilis

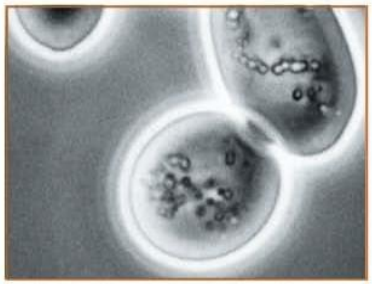


- Easily transformed
- Nonpathogenic
- Naturally secretes proteins
- Endospore formation simplifies culture

- Genetically unstable
- Genetics less developed than in *E. coli*

Eukaryote

Saccharomyces cerevisiae



- Well-developed genetics
- Nonpathogenic
- Can process mRNA and proteins
- Easy to grow

- Plasmids unstable
- Will not replicate most bacterial plasmids

Advantages | Disadvantages

Obrigado

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