Biotecnologia

ACH5545 Engenharia Genética

Atividades de Laboratório

2º Semestre 2024

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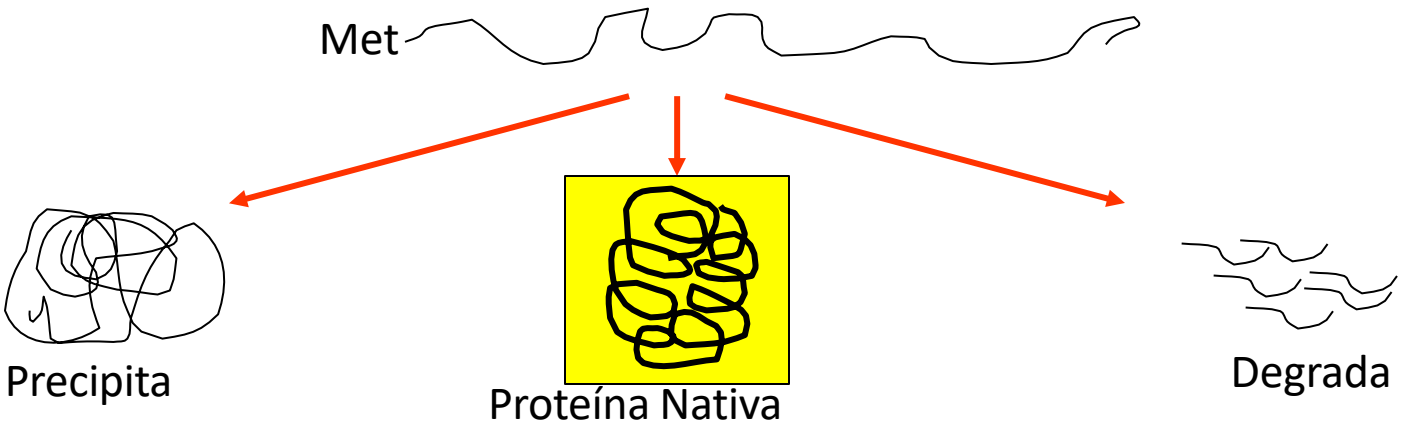
Créditos: 4
Período: Quinta-feira (14h00 -18h00), Laboratório de Biotecnologia – Edifício A2, 1º andar

USP - 2024

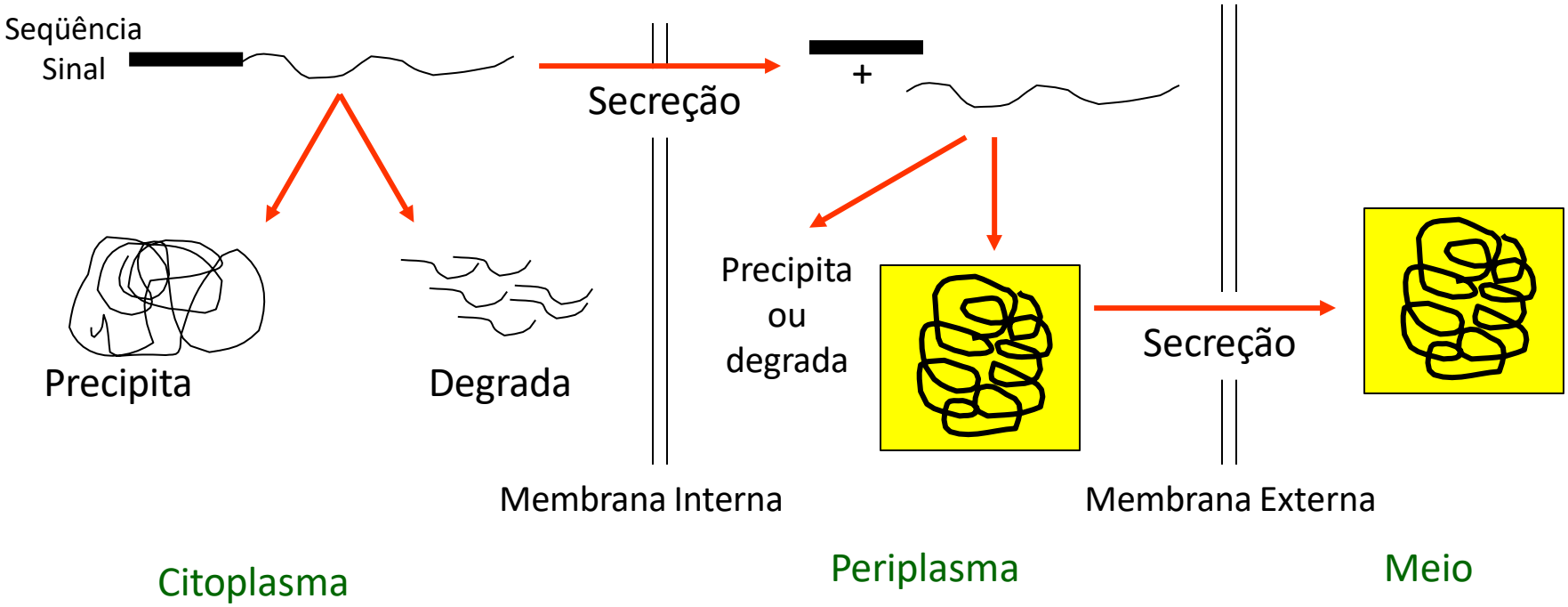
Atividade

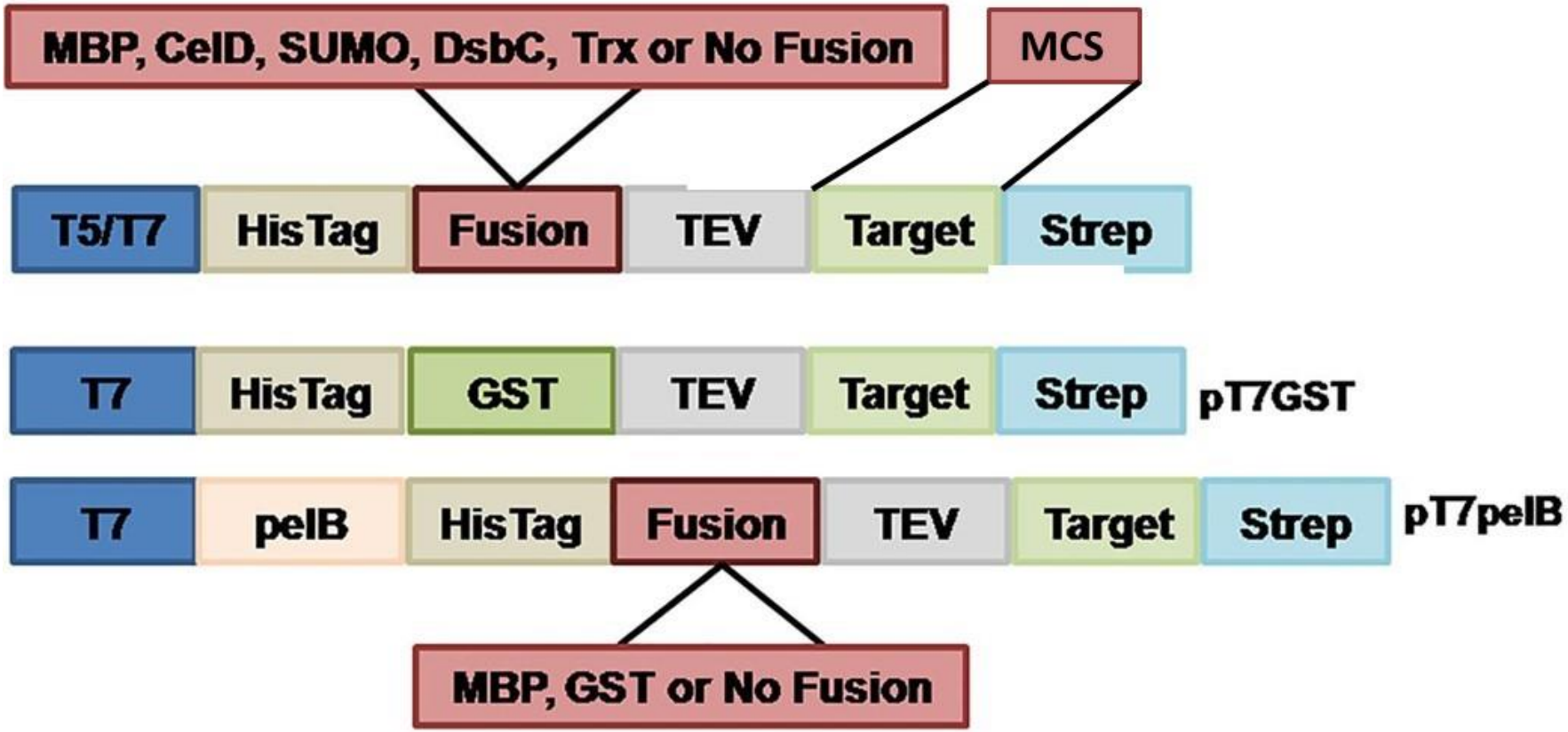
- Indução com IPTG a partir de pré-inóculo.
- Curva de crescimento bacteriano
- Recuperação da massa bacteriana por centrifugação

A)Proteínas Citoplasmáticas



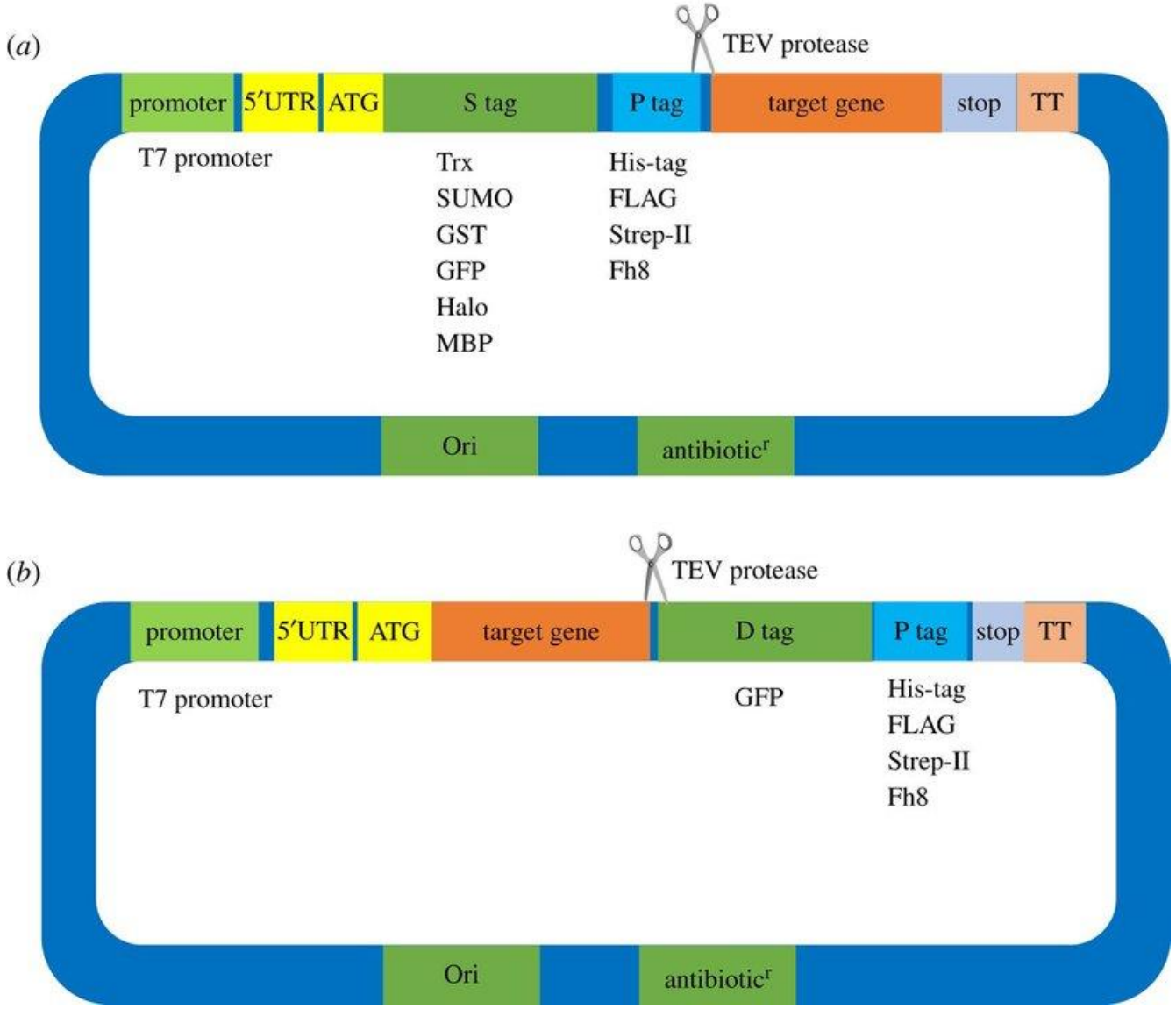
B)Secreção de Proteínas





- **T5/T7, T5, or T7** – Sequencia do Promotor
- **pelB** - Sequencia sinal para o espaço periplásmico
- **TEV** - sitio de reconhecimento para a protease do Virus Etch do Tabacco:
- **Strep-Tag** - Cauda de streptavidina (8 aa) que liga resina de biotina:
- **His-Tag** – Cauda de Histidina (6 - 11 aa) que liga ions Ni+2 imobilizados na resina
- **MBP** (maltose-binding protein) – cauda de proteína para purificação em coluna de amilose e eluição com maltosa. Aumenta a solubilidade da proteína de interesse.
- **GST** (glutathione-S-transferase) - cauda de proteína para purificação em coluna com glutationa e eluição com glutationa. Aumenta a solubilidade da proteína de interesse.
- **SUMO** (Small Ubiquitin-like Modifier), **Trx** (Thioredoxin), DsbC (Disulfide bond C), **MBP**, ou **CelD** (cellulase D) – Proteínas que aumentam a solubilidade da proteína de interesse.

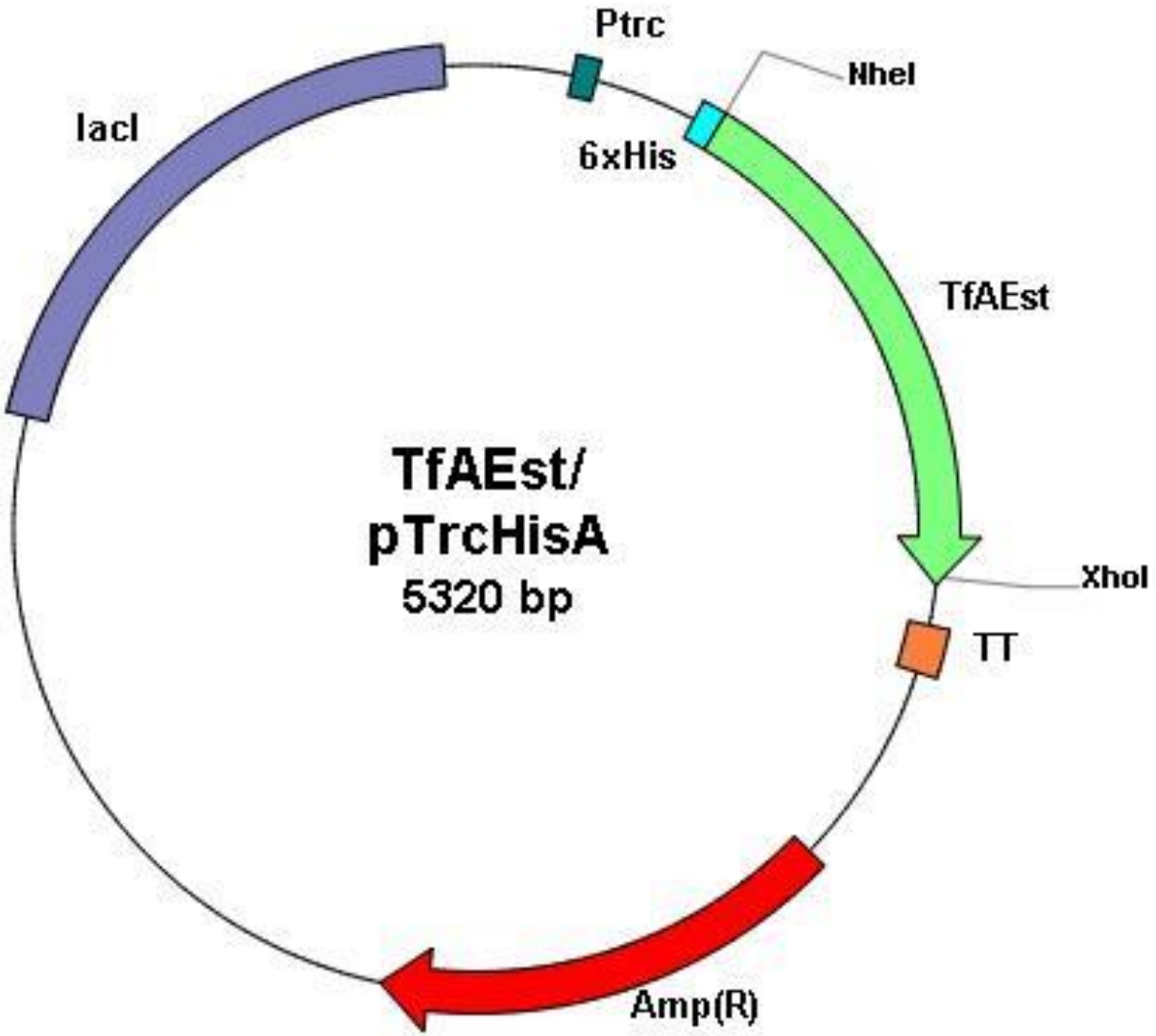
https://doi.org/10.3389/fmicb.2018.01384



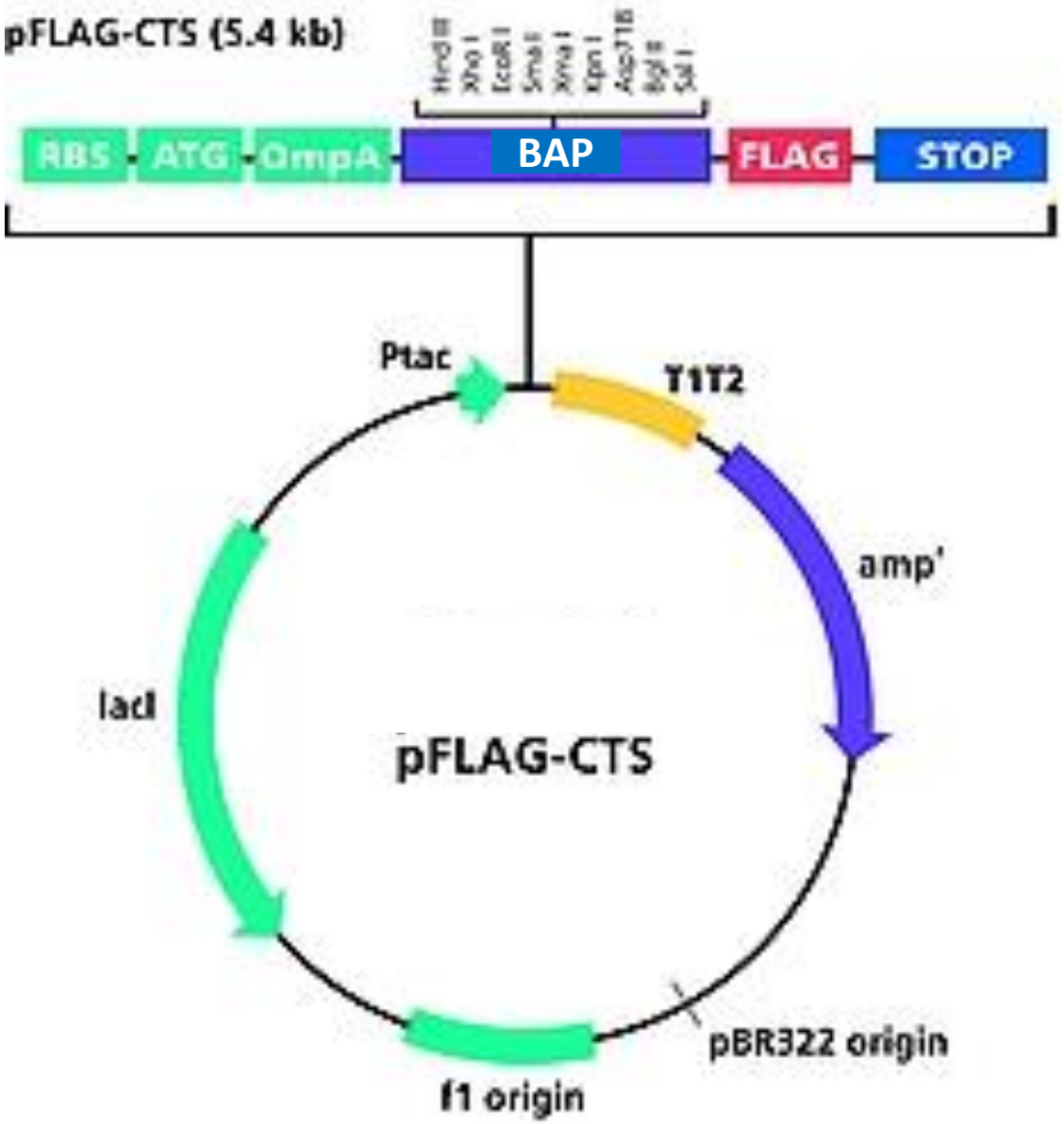
Basic expression vectors for high-throughput expression in *E. coli* of (a) cytoplasmic proteins and (b) membrane proteins. The T7 promoter is used to control expression of the protein in E. coli. The vectors requires tandem affinity tags, larger tag for protein expression initiation, protein solubility and soluble detection, and smaller tag for purification. TEV protease can be used to remove the tags. The tags for membrane proteins are located at the C-terminus for protein targeting, and GFP is a favourabel choice for use as an indicator of protein folding. D tag, detection tag; P tag, purification tag; S tag, solubility and translation initiation tag; TT, transcriptional terminator; 5′UTR, 5′ untranslated region.

DOI:10.1098/rsob.160196

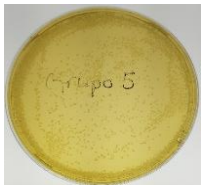
Enzima Acetil Esterase (969 pb; 35 kDa)



Enzima Fosfatase Alcalina Bacteriana (1338 pb; 49,5 kDa)



Procedimento:



***E. coli* recombinante**

10 mL de meio
LB + amp +
37 °C, *overnight*,
150 rpm

2,5 ml/250 mL de
meio LB + amp +
37 °C, 150 rpm
DO_{600}=0,4-0,6

①

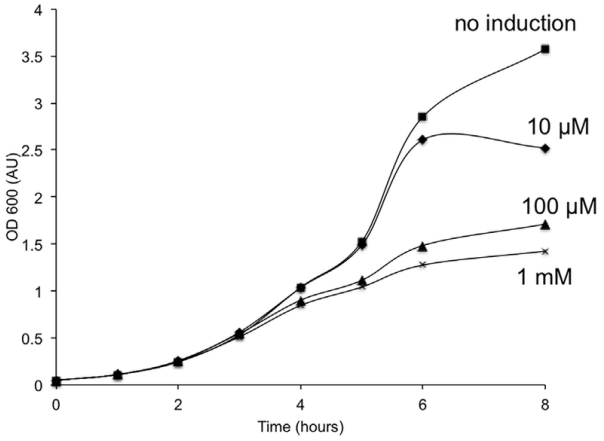
***E. coli* recombinante**
INDUZIDO
+ IPTG 0,5 mM

+

②

***E. coli* recombinante**
NÃO INDUZIDO
Sem IPTG

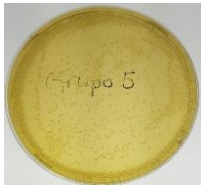
Cultura 37 °C,
150 rpm
Leitura DO_{600} no
tempo indicado



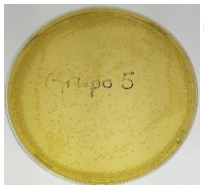
Curva de crescimento bacteriano

8h	**10h**	12h	14h	16h	18h
Inóculo	**Indução**	duas	quatro	seis	oito

Coletar a massa bacteriana de 1 ml, após cada leitura de DO_{600}



TfAEST



BAP

***E. coli* recombinante**

10 mL de meio
LB + amp +
37 °C, *overnight*,
150 rpm

2,5 ml/250 mL de
meio LB + amp +
37 °C, 150 rpm
DO_{600}=0,4-0,6

1

***E. coli* recombinante**
INDUZIDO
+ IPTG 0,5 mM

2

***E. coli* recombinante**
NÃO INDUZIDO
Sem IPTG

Cultura 37 °C,
150 rpm
Leitura DO_{600} no
tempo indicado

8h	10h	12h	14h	16h	18h
Inóculo	Indução	duas	quatro	seis	oito

Tabela. Crescimento bacteriano $DO600_{nm}$.

Cultura/Leitura OD_{600}	Indução (0 h)	Indução –IPTG após - 2h		Indução 4 h		Indução 6 h		Indução 8 h	
		NIndz*	Indz**	NIndz	Indz	NIndz	Indz	NIndz	Indz
TfAEST									
BAP									

* Cultura bacteriana não induzida com IPTG
** Cultura bacteriana induzida com IPTG 0,5 mM

Procedimento:

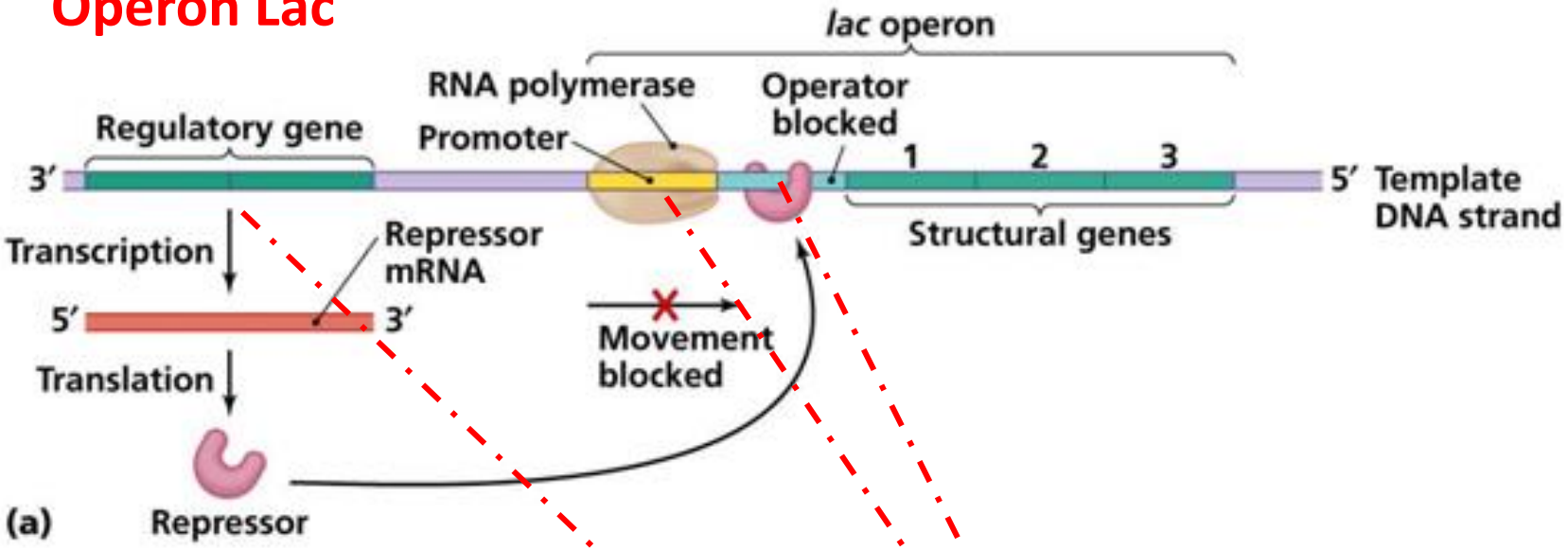
Recuperar 1 ml de cultura em condição estéril.

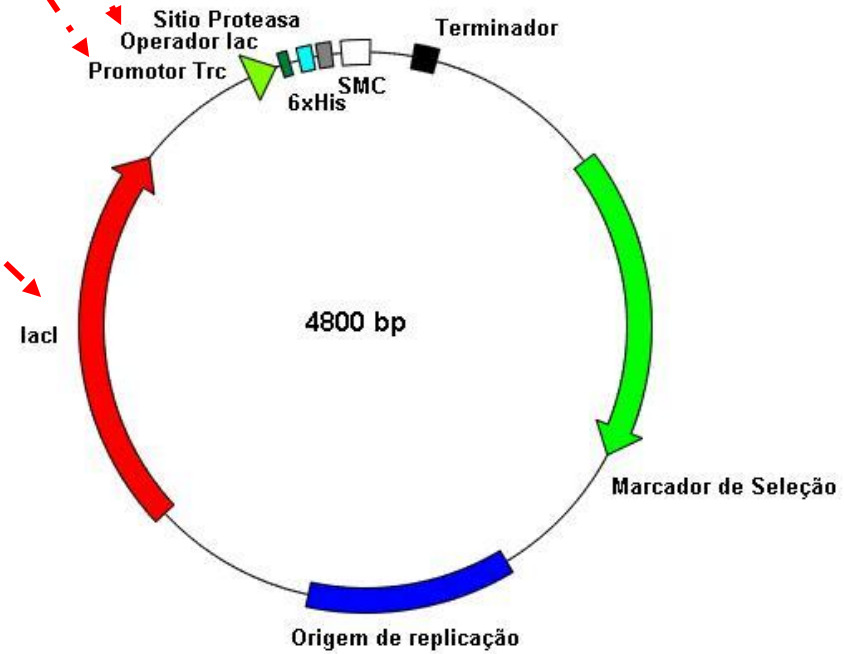
1- Transferir 100 ul da cultura a tubo limpo contendo 900 ul de água destilada, misturar, transferir a cuveta, realizar a leitura DO_{600} nm e anotar na tabela.

2- Centrifugar 4 tubos com 50 ul de cultura, 5 min/10000 rpm, descartar sobrenadante e reservar para eletroforeses SDS-PAGE (02 tubos) e para análise de atividade enzimática (02 tubos), congelar a massa celular, devidamente identificada

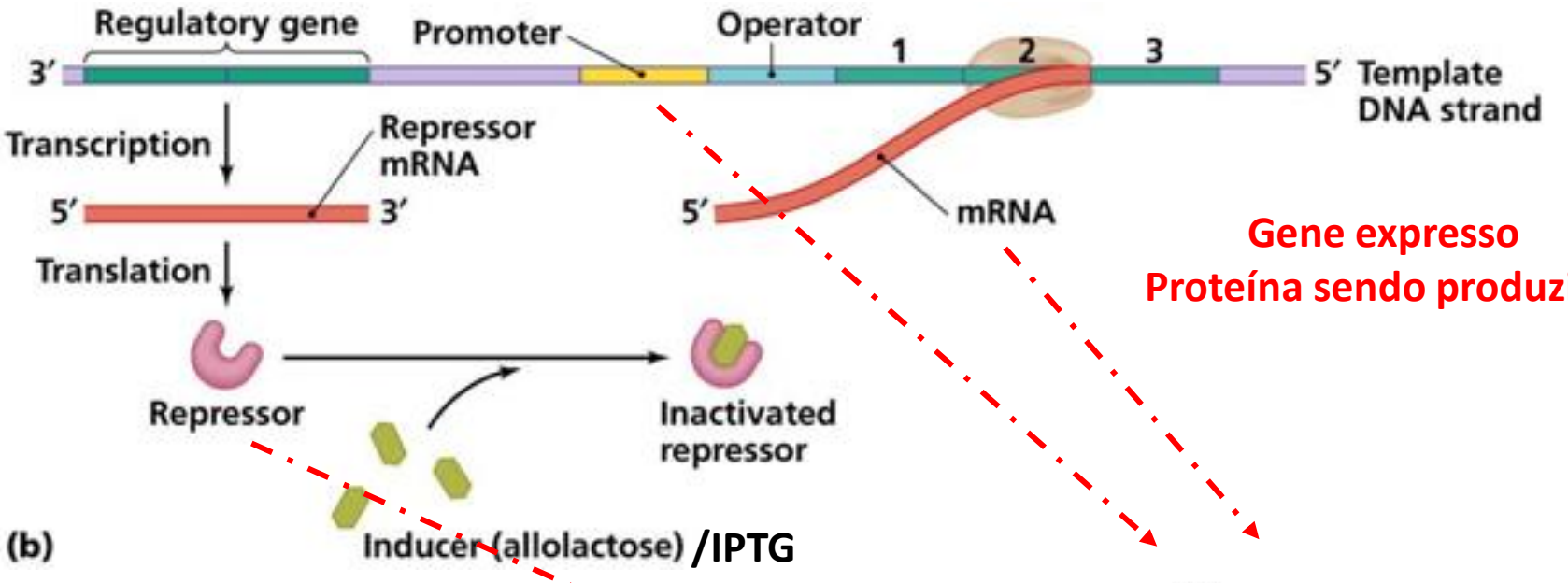
3- O restante da cultura (700 ul), centrifugar 5 min/10000 rpm, descartar o sobrenadante e congelar a massa celular, devidamente identificada.

Operon Lac

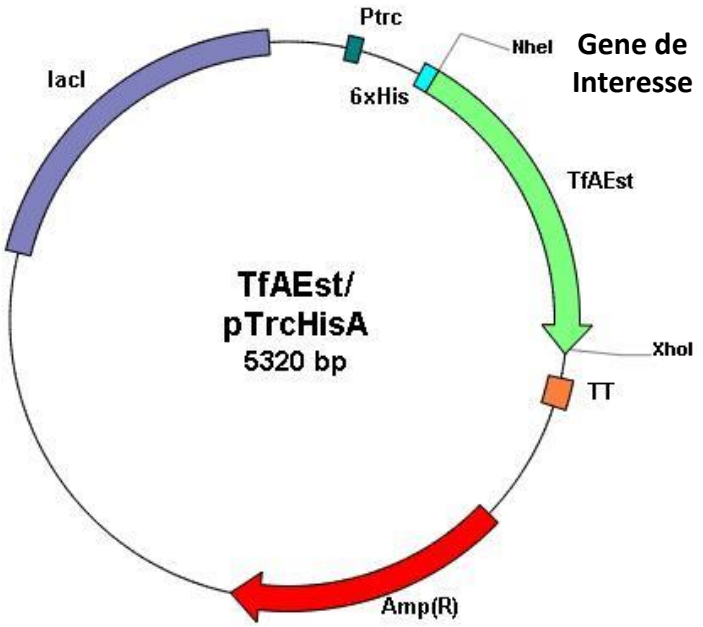

lac operon
RNA polymerase
Operator
blocked
Regulatory gene
Promoter
1
2
3
3′
5′ Template
DNA strand
Structural genes
Transcription
Repressor
mRNA
5′
3′
Movement
blocked
Translation
(a)
Repressor

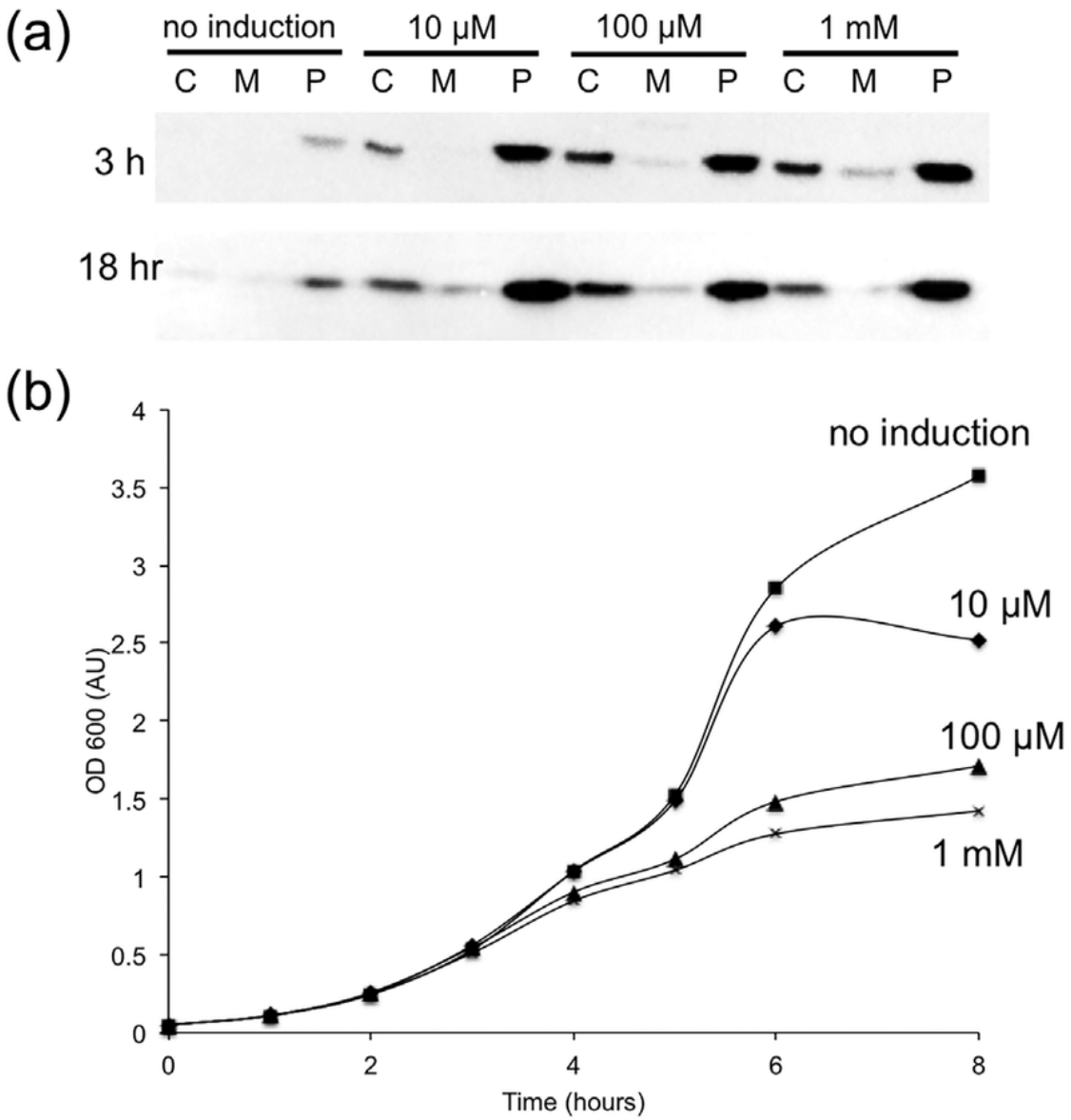

Sitio Proteasa
Operador lac
Promotor Trc
6xHis
SMC
Terminador
lacI
4800 bp
Marcador de Seleção
Origem de replicação

Operon Lac / IPTG



Gene expresso
Proteína sendo produzida





Excessive IPTG concentrations lead to inhibition of growth and reduced export in TatExpress cells. (a) expression of TorA-hGH in W3110 TatExpress cells was induced by the addition of IPTG at the concentrations shown (after 3 hr growth at approximately 0.5 AU). Cells were fractionated to generate cytoplasm/membrane/periplasm samples and the fractions were immunoblotted as shown in Figure 3; (b) effects of IPTG concentration on growth curves of the cultures; uninduced cells are represented by squares and the growth characteristics of cells induced using 10 µm IPTG (diamonds), 100 µm IPTG (triangles), and 1 mm IPTG (crosses) are illustrated

August 2017. Biotechnology and Bioengineering 114(12). DOI:10.1002/bit.26434

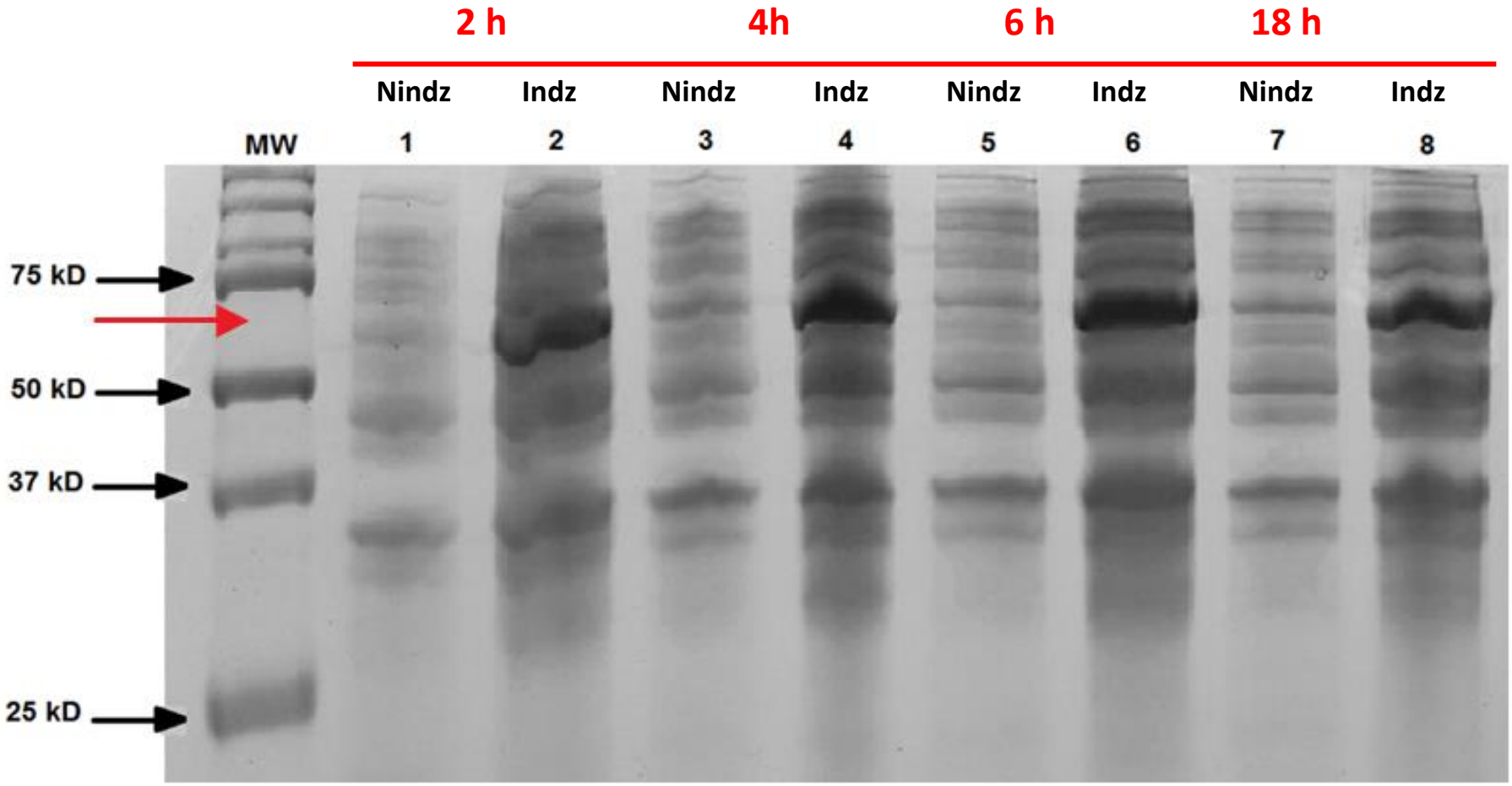


Figura 10. Eletroforese em SDS-PAGE (12,5%) da expressão da proteína recombinante TrCat utilizando o vetor pTrc-HisA, variando-se o tempo de indução. (MW) *Precision Plus Protein Standards* (*BIO-RAD*). A seta vermelha indica, aproximadamente, a posição da proteína recombinante.

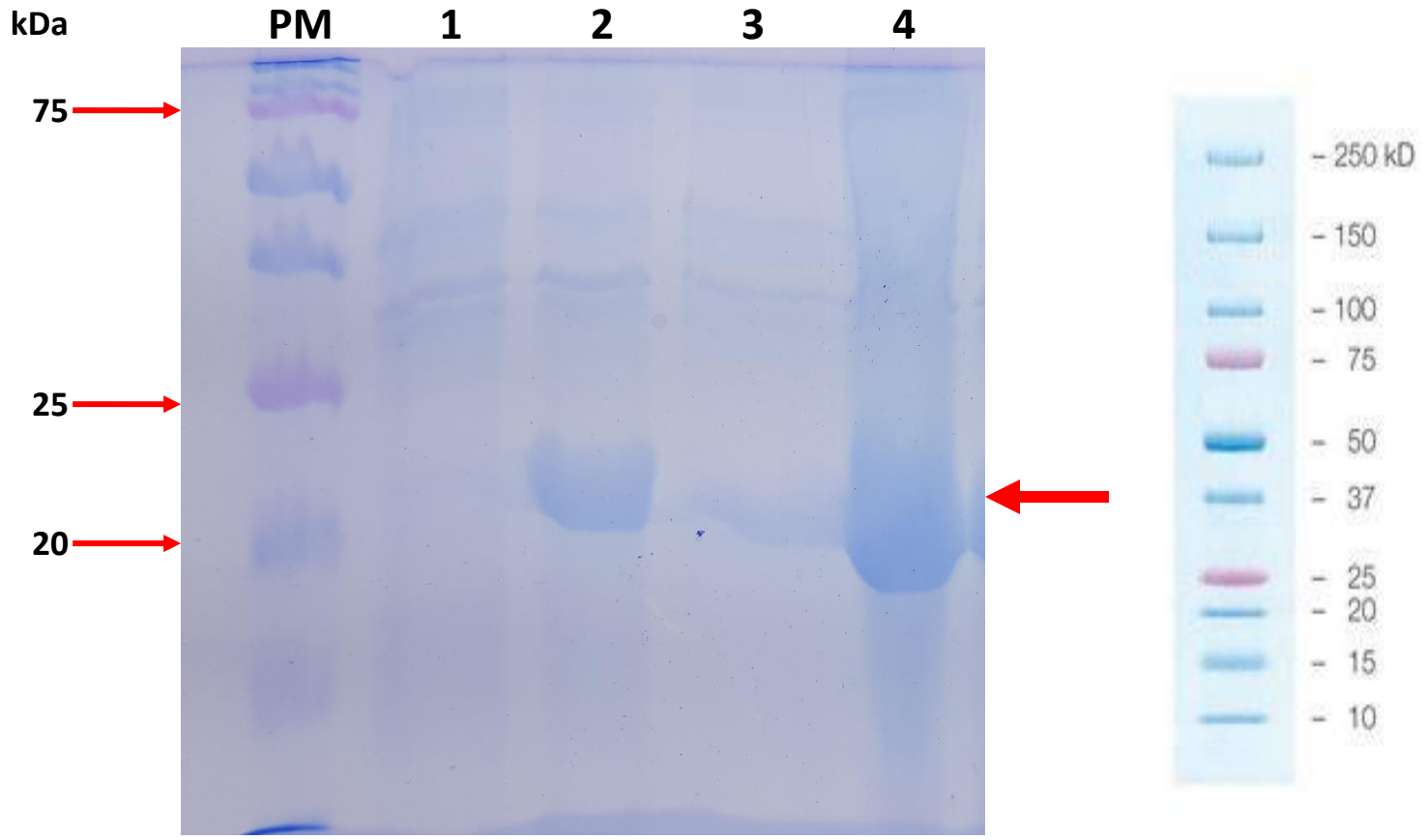


Figura – Análise por eletroforeses SDS-PAGE (12,5%) da expressão da proteína recombinante, em *E. coli* BL21. (1) Amostra de 20 µL da bactéria não induzida; (2) Amostra de 20 µL da bactéria induzida; (3) Amostra de 20 µL do sobrenadante bacteriano (solúvel); (4) Amostra de 20 µL do precipitado bacteriano (não solúvel). (PM) peso molecular Precision Plus Protein™ Dual Color Standards (Bio-Rad Laboratories).

Problem	Possible explanation	Solutions
No or low expression	Protein may be toxic before induction	Control basal induction: • add glucose when using expression vectors containing *lac*-based promoters • use defined media with glucose as source of carbon • use pLysS/pLysE bearing strains in T7-based systems • use promoters with tighter regulation
		Lower plasmid copy number
	Protein may be toxic after induction	Control level of induction: • Tuneable promoters • Use strains that allow control of induction [Lemo21(DE3) strain] or *lacY*$^{-}$ strains (Tuner^{TM})
		Lower plasmid copy number
		Use strains that are better for the expression of toxic proteins (C41 or C43)
		Direct protein to the periplasm
	Codon bias	Optimize codon frequency in cDNA to better reflect the codon usage of the host
		Use codon bias-adjusted strains
		Increase biomass: • Try new media formulations • Provide good aeration and avoid foaming
Inclusion body formation	Incorrect disulfide bond formation	Direct protein to the periplasm
		Use *E. coli* strains with oxidative cytoplasmic environment
	Incorrect folding	Co-express molecular chaperones
		Supplement media with chemical chaperones and cofactors
		Remove inducer and add fresh media
		Lower production rate: • Lower temperature. If possible, use strains with cold-adapted chaperones • Tune inducer concentration
	Low solubility of the protein	Fuse desired protein to a solubility enhancer (fusion partners)
	An essential post translational modification is needed	Change microorganism
Protein inactivity	Incomplete folding	Lower temperature
		Monitor disulfide bond formation and allow further folding *in vitro*
	Mutations in cDNA	Sequence plasmid before and after induction. If mutations are detected, the protein may be toxic.
		Use a *recA*$^{-}$ strain to ensure plasmid stability
		Transform *E. coli* before each expression round

Proteína recombinante em Corpo de Inclusão

O nível de expressão e solubilidade da proteína recombinante é um pré-requisito para estudos estruturais, funcionais e bioquímicos de uma proteína.

(1)alterando o vetor,
(2)alterando o hospedeiro,
(3)alterando os parâmetros de cultura da cepa hospedeira recombinante,
(4)coexpressão de outros genes e
(5)alterando as sequências genéticas, o que pode ajudar a aumentar a expressão e o dobramento adequado da proteína desejada.

Table 1. Common E.coli strains used as expression host.

Bacterial Strain	Features	Benefit	Growth Condition	Company
BL21(DE3)	*Has DE3 lysogen that expresses T7 RNA polymerase *Deficient in lon and ompT proteases *Induced by IPTG	Suitable for the expression of nontoxic genes	1 % glucose in the medium	Novagen
BL21(DE3) pLysS	*Has DE3 lysogen that expresses T7 RNA polymerase *Has T7 lysozyme to decompose the enzyme T7 polymerases before induction	*Prevents leaky expression *Suitable for the expression of toxic genes	Chloramphenicol 34 µg/m	Novagen
BL21 Star	Mutation in the gene rne131, so mRNA has more stability			Novagen
Lemo21(DE3)	*Contains features of BL21(DE3) *Tunable expression of difficult clones by varying the level of lysozyme (lysY)	Suitable for the expression of challenging protein including: toxic proteins, membrane proteins, and low soluble ones	L-Rhamnose 0–2,000 µM	NEB
Tuner (DE3)	Has lac permease (lacY) mutation	*Allows uniform entry of IPTG into all cells in the population. *Suitable for toxic and insoluble proteins	None	Novagen
Origami	Has mutation in trxB and gor genes	Enhances disulfide bond formation in the cytoplasm	Kanamycin 15 µg/mL Tetracycline 12.5 µg/mL	(Novagen, 2006–2007)
SHuffle	*Expresses disulfide bond isomerase DsbC *Deficient in proteases Lon and Omp	*Promotes the correct folding of mis-oxidized proteins *resistance to phage T1 (fhuA2)		NEB
Rosetta	*BL21 lacZY (Tuner) derivatives *Has additional copies of genes encoding the tRNAs for rare codons AUA, AGG, AGA, CUA, CCC, GGA	Suitable for the expression heterogeneous proteins	Chloramphenicol 34 µg/mL	Novagen
Rosettagami (DE3)	Derived from Origami and also has tRNAs for rare codons			Novagen
C41(DE3) and C43(DE3)	Mutant strains of BL21(DE3) that prevents cell death associated with the expression of toxic recombinant proteins	suitable for the expression of toxic and/or membrane proteins from all classes of organisms		Lucigen

DOI: 10.2174/138161282466618013112194O

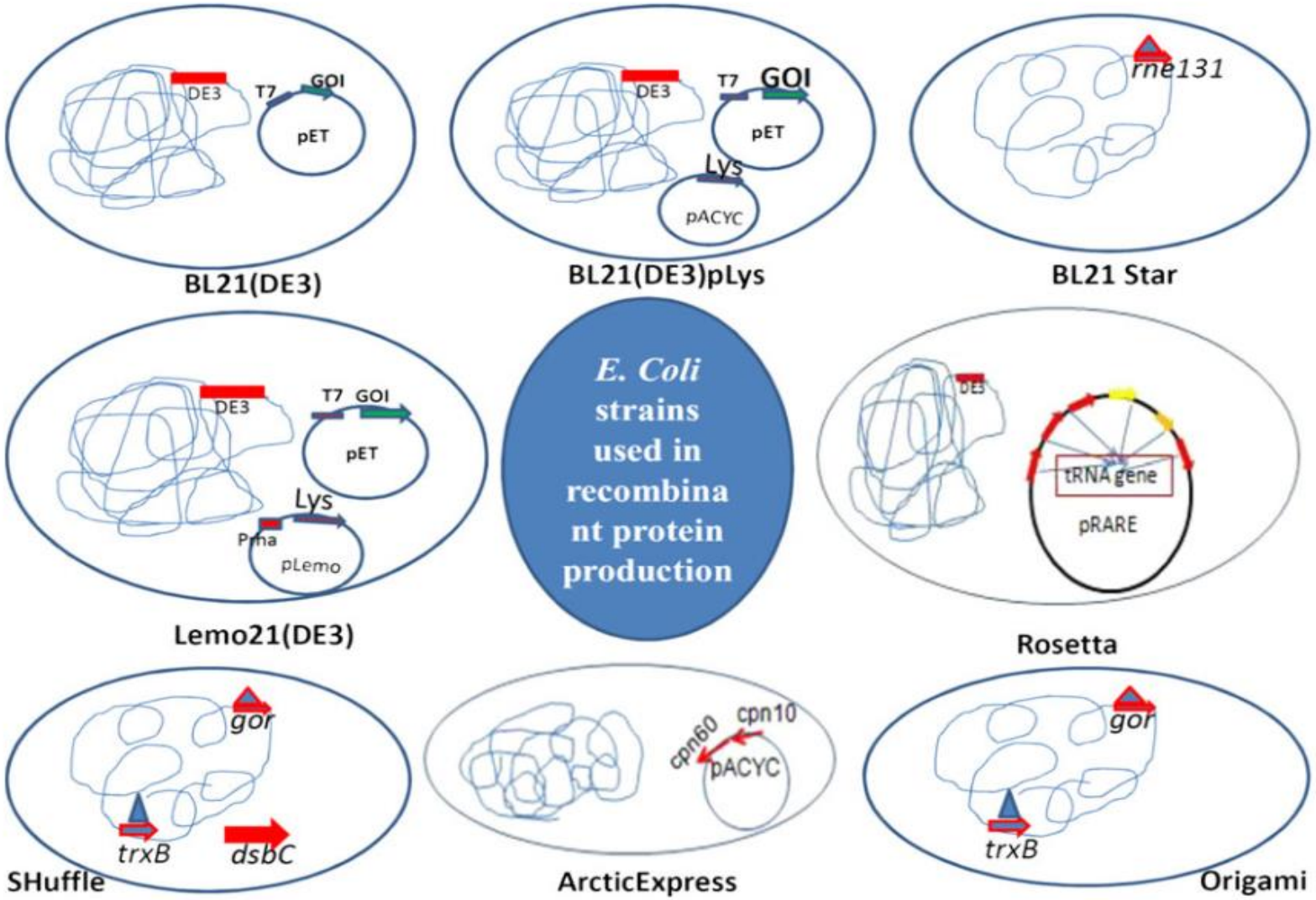


Fig. 1 *E. coli* strains frequently used in recombinant protein production having different vector, promoter, multiple cloning sites, tRNA and other genes

Protein J. DOI 10.1007/s10930-013-9502-5

Table 2 Characteristics of *E. coli* strains used in recombinant protein expression

E. coli strain	Used for	Characteristics at genetic level
ArcticExpress	Grow at low temperature	Low temperature adapted chaperone encoded gene
BL21	Less protease degradation of recombinant protein	*Lon* and *OmpT* protease deficient
BL21-Codonplus (RIL)	Overcome the effect of codon biasness (AT rich gene)	tRNA encoding gene (*argU, ileY* and *leuW*)
BL21(DE3)	Expression under T7 promoter	T7 pol encoded
BL21(DE3)pLys S/E	Controlled expression	Lysozyme encoded plasmid
BL21 Star	Increase stability of mRNA	*rne31* gene mutated
C41(DE3)	Overexpressing toxic proteins	Carry the lambda DE3 lysogen which expresses T7 RNA polymerase from the lacUV5 promoter by IPTG induction
C43(DE3)	Membrane and globular protein	-Same-
Codon plus (RP)	Overcome the effect of codon biasness (GC rich gene)	tRNA encoding gene (*argU* and *proL*)
Lemo21(DE3)	Membrane, globular and toxic protein expression	Lysozyme encoded under rhamnose inducible promoter
M15	Gene under T5 promoter	Constitutively expresses *lac* repressor at high levels
Origami	Protein required disulphide bond formation	*gor* and *trxB* genes mutated
Rossetta	Both the AT and GC rich gene	All the rare tRNA coding gene
SG13009	Enabling *trans* repression of protein expression prior to IPTG induction	Carry the repressor plasmid pREP4, which constitutively expresses *lac* repressor at high levels
Shuffle	Proper disulphide bond formation	*DsbC* gene encoded

Table 1.1. Constitutive and inducible promoters commonly used in recombinant protein expression systems.

Host	Expression System	Constitutive Promoters	Inducible Promoters	Inducers
Mammalian	*In vivo*	CMV (cytomegalovirus); EF-1 alpha (human elongation factor alpha) 1; UbC (human ubiquitin C); SV40 (simian virus 40)	Promoter with TetO2 (tetracycline operator); promoter with GAL4-UAS (yeast GAL4 upstream activating sequence)	Tetracycline or doxycycline; mifepristone
	Cell free (rabbit reticulocyte)	None	NA	NA
	Cell free (HeLa or CHO)	Requires T7 promoter and T7 RNA polymerase for transcription	NA	NA
Insect	*In vivo*	Ac5 (actin); OpIE1 & 2; PH (polyhedrin); p10	MT (metallothionein)	Copper
Yeast	*In vivo*	GAP (glyceraldehyde-3-phosphate dehydrogenase)	AOX1 (aldehyde oxidase); GAL1 (galactokinase)	Methanol; galactose
E. coli	*In vivo*	Not commonly available	Lac (lactose operon); araBAD (L-arabinose operon)	IPTG; L-arabinose
	Cell free	Requires T7 promoter and T7 RNA polymerase for transcription	NA	NA

Book-Protein Expression ThermoFisher

Obrigado

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