Recombinant DNA Technology

Manipulation of Gene Expression in Prokaryotes

Advantages of Genetically Engineering Products

- More efficient
- Safer products
- More economical
- Commercially valuable traits

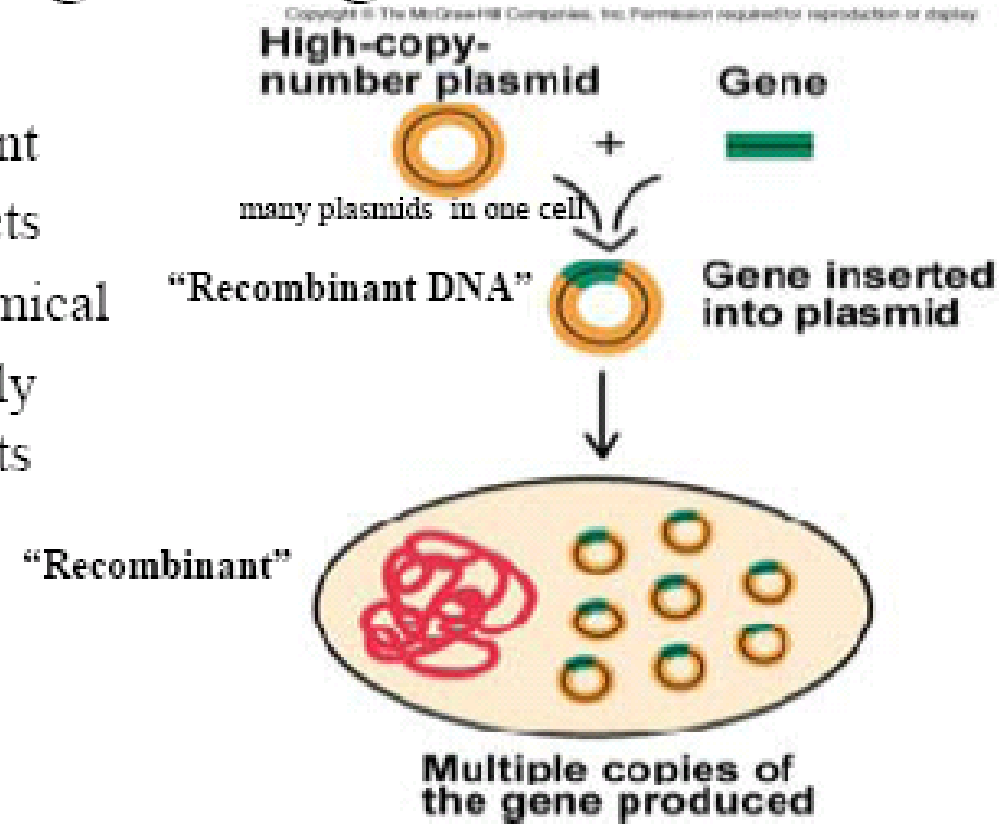
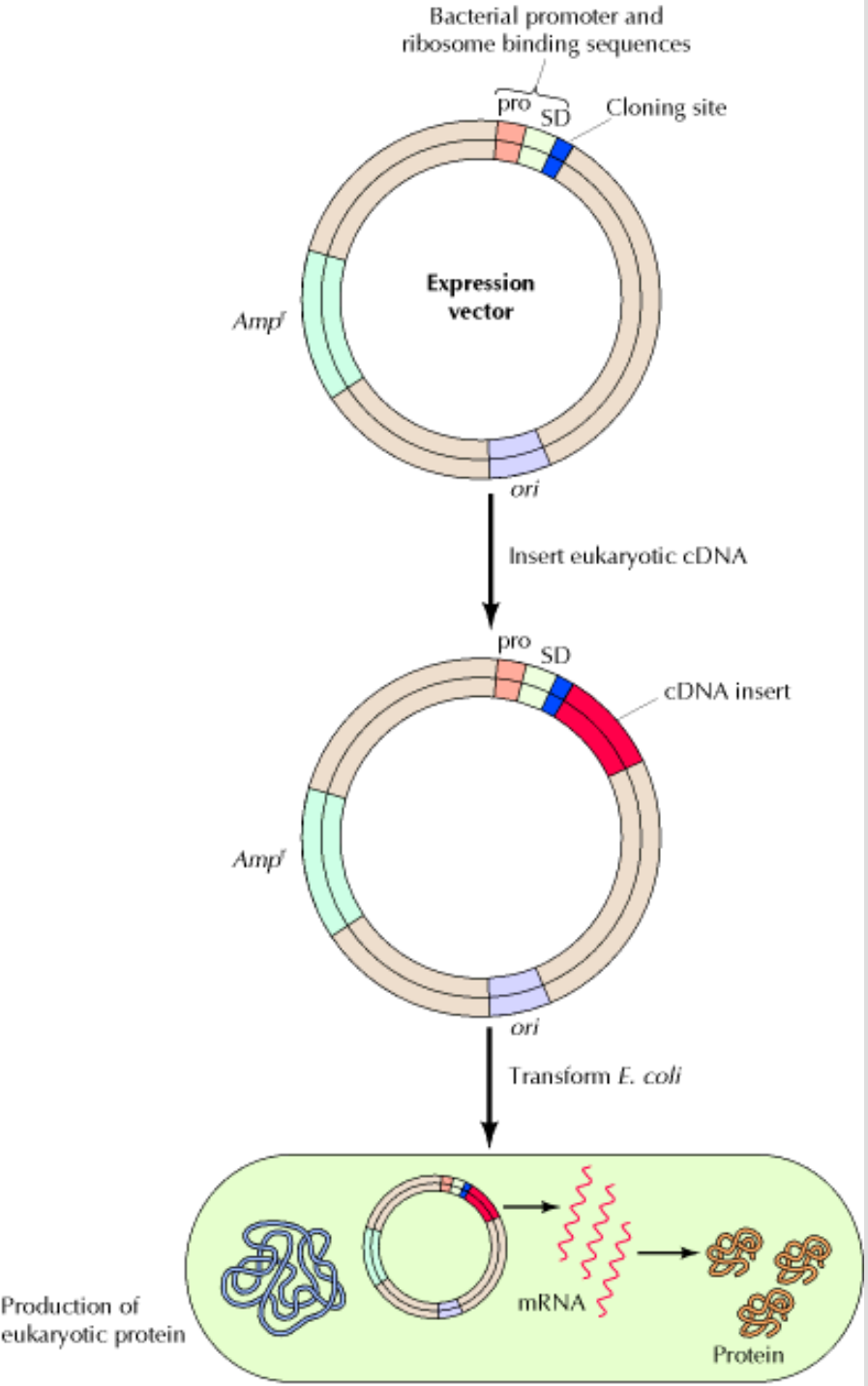


Table 9.2 Some Applications of Genetic Engineering

Medically Important Substances

Genetically Engineered Bacteria	Application
Protein Production	
Pharmaceutical proteins	
Alpha interferon	Treating cancer and viral infections
Erythropoietin	Treating some types of anemia
Beta interferon	Treating multiple sclerosis
Deoxyribonuclease	Treating cystic fibrosis
Factor VIII	Treating hemophilia
Gamma interferon	Treating cancer
Glucocerebrosidase	Treating Gaucher disease
Growth hormone	Treating dwarfism
Insulin	Treating diabetes
Platelet derived growth factor	Treating foot ulcers in diabetics
Streptokinase	Dissolving blood clots
Tissue plasminogen activator	Dissolving blood clots
Vaccines	
Hepatitis B	Preventing hepatitis
Lyme disease	Preventing Lyme disease
Foot-and-mouth disease	Preventing foot-and-mouth disease in animals



Expression of cloned genes in bacteria Expression vectors contain promoter sequences (pro) that direct transcription of inserted DNA in bacteria and sequences required for binding of mRNA to bacterial ribosomes (Shine-Delgarno [SD] sequences). A eukaryotic cDNA inserted adjacent to these sequences can be efficiently expressed in *E. coli*, resulting in production of eukaryotic proteins in transformed bacteria.

Plasmid and / or gene can be altered to accomplish high-level expression

1) transcriptional promoter & terminator
2) Shine Dalgarno Sequence (ribosome binding site)
3) efficiency of translation
4) Stability of the protein
5) Final cellular location (secreted?)
6) Number of copies of cloned gene

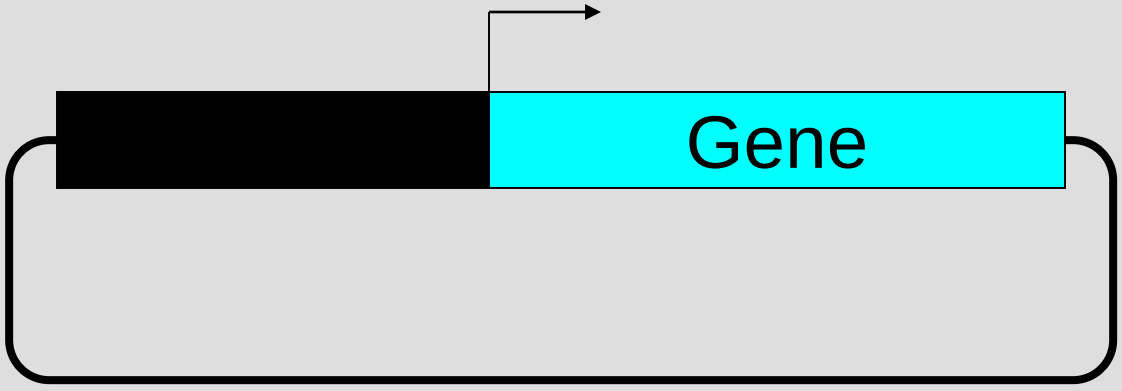
Plasmid vs. chromosomal

many vs. 1

7) Host organism

Gene Expression from a Strong, Regulatable Promoter

Some plasmids (Expression Plasmids) have promoters upstream of cloning sites for expression of genetic info encoded by DNA fragment



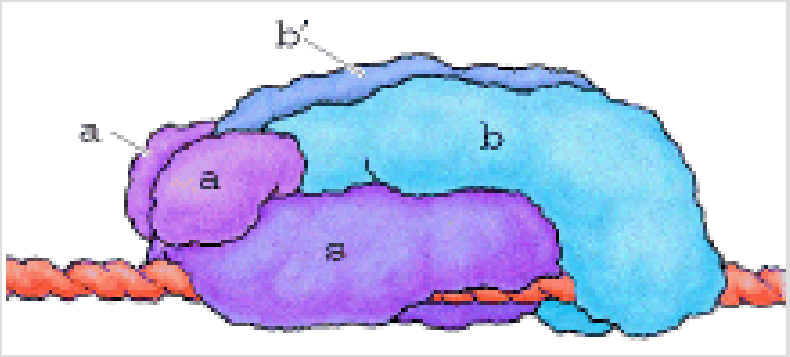
Gene Expression from a Strong, Regulatable Promoter

- **Strong**
  - **high affinity for RNA polymerase**
  - **tight binding - frequently transcribed**
  - **weak binding - RNA Pol falls off, no txn**
- **Regulatable**
  - **researcher can control when gene is expressed**
  - **use inducers / co-repressors**

Promoters of Importance in Biotech

- **lac**
- **trp**
- **tac (or trc)**
- **phage λ p^L**
- **phage T7 gene 10**

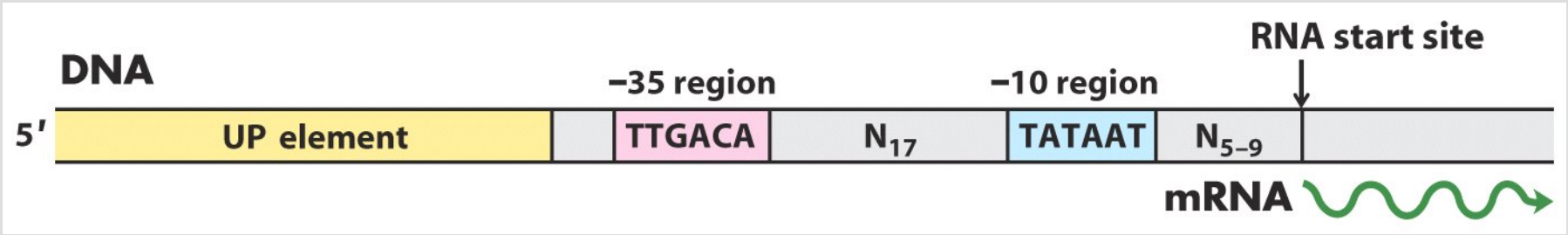
Initiation

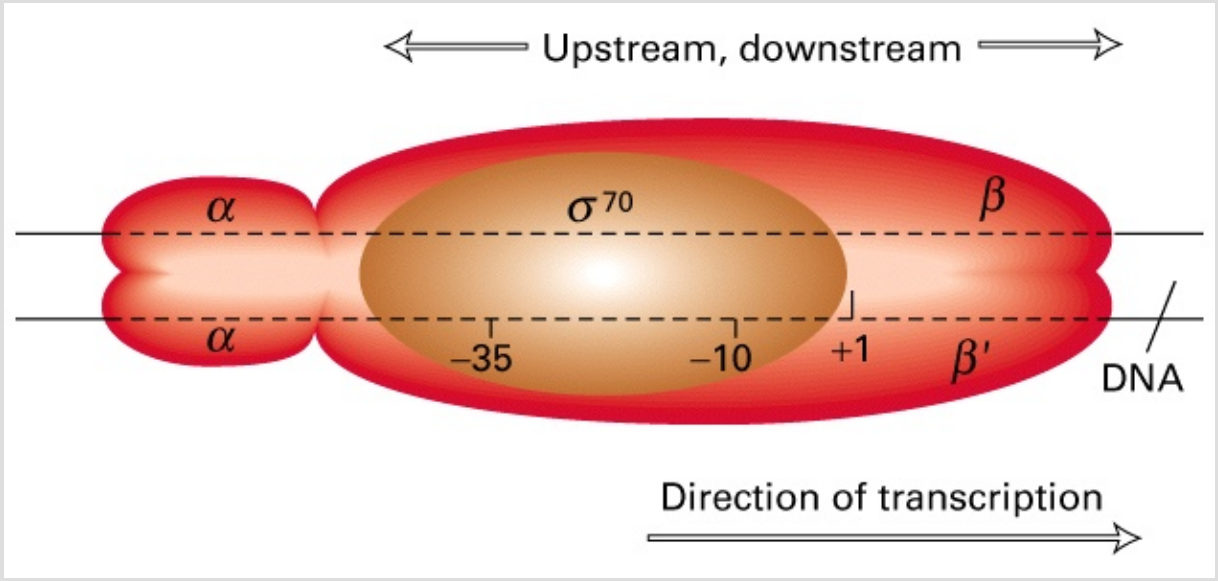


- RNA polymerase α α β β′σ
- Transcription factors
- Sigma factor (σ)– determines promoter specificity

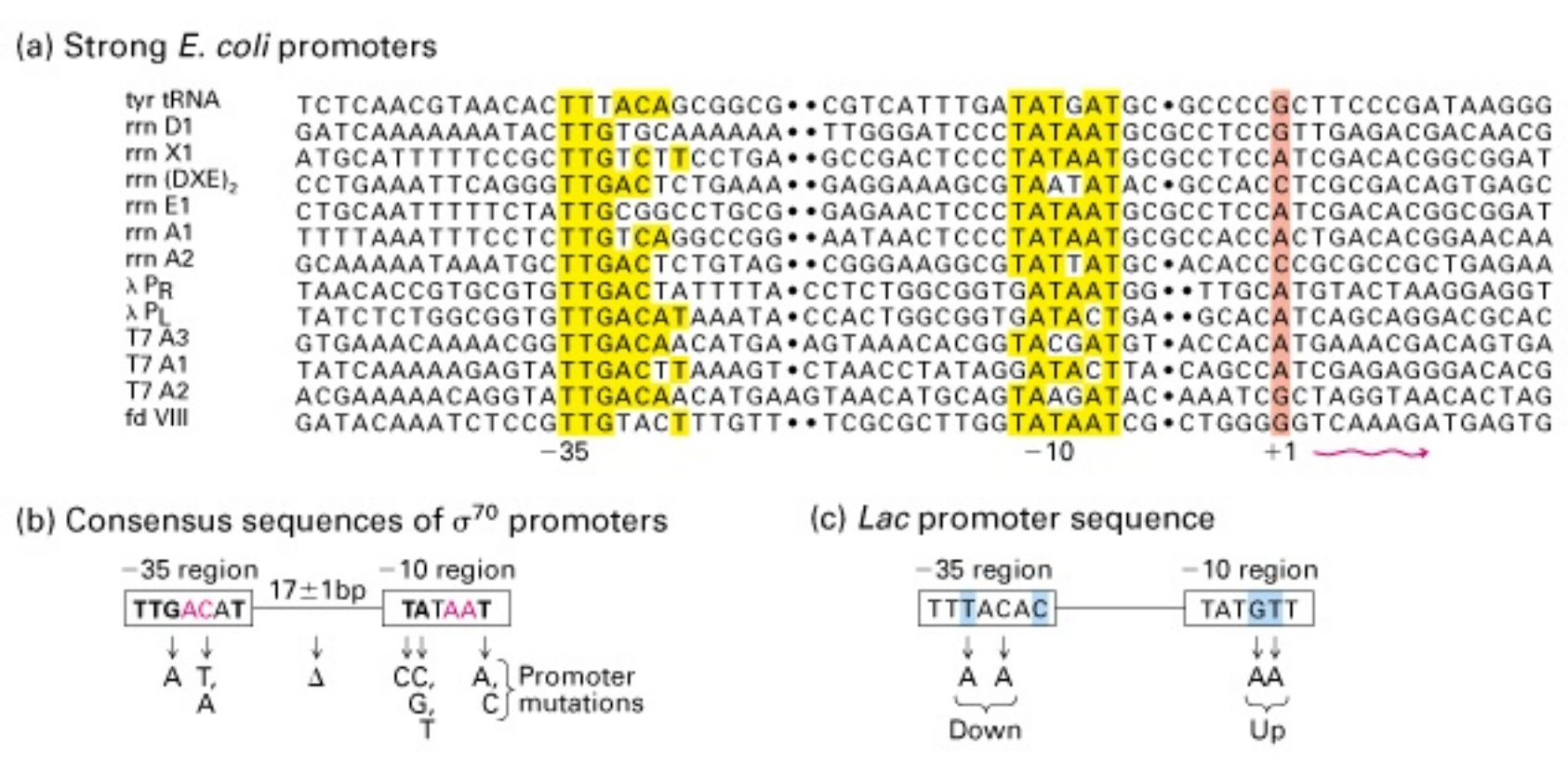
Start site of txn is +1

E. coli Promoter Sites





E. coli Promoter Sites



Deviation from consensus -10 , -35 sequence leads to weaker gene expression

lac promoter

- *lac operon*

- *negative regulation*
  - *lac repressor binds to operator sequence*
  - *inducer binds to repressor - makes it non-functional*
  - *Inducer = allolactose, IPTG*

Operons

- An operon is a group of genes that are **transcribed at the same time**.
- They usually control an important biochemical process.
- They are **only found in prokaryotes**.

Jacob, Monod & Lwoff

The *lac* Operon

- The *lac* operon consists of **three genes** each involved in processing the sugar lactose
- One of them is the gene for the enzyme **β-galactosidase**
- This enzyme hydrolyses lactose into glucose and galactose

Adapting to the environment

- *E. coli* can use either glucose, which is a monosaccharide, or lactose, which is a disaccharide
- However, lactose needs to be hydrolysed (digested) first
- So the bacterium prefers to use glucose when it can

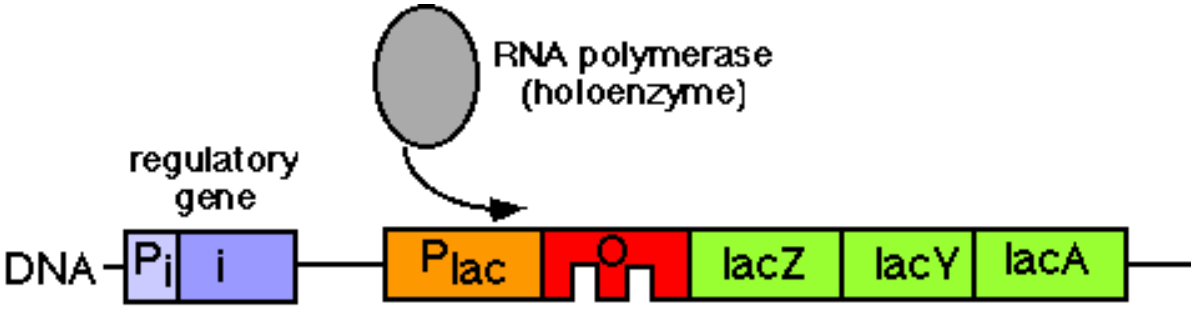
Four situations are possible

1. When glucose is **present** and lactose is **absent** the E. coli does **not** produce β-galactosidase.

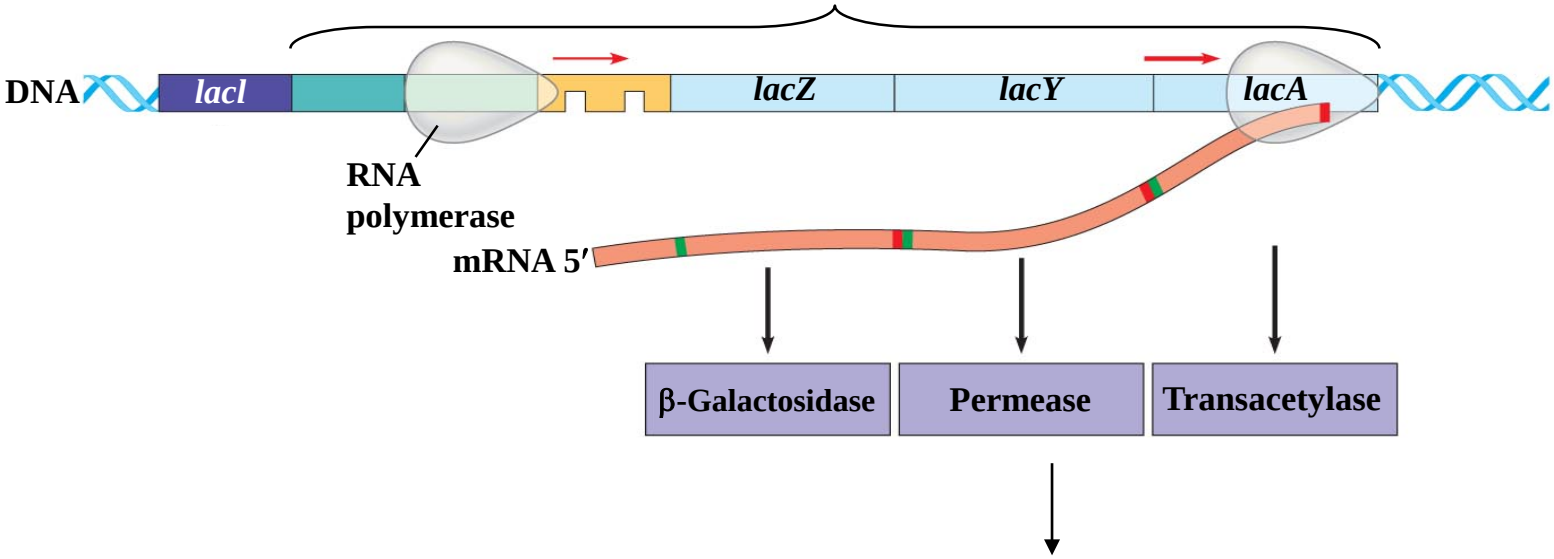
2. When glucose is **present** and lactose is **present** the E. coli does **not** produce β-galactosidase.

3. When glucose is **absent** and lactose is **absent** the E. coli does **not** produce β-galactosidase.

4. When glucose is **absent** and lactose is **present** the E. coli **does** produce β-galactosidase



Lac operon



Enzymes facilitate lactose import and breakdown for cellular energy

The control of the *lac* operon

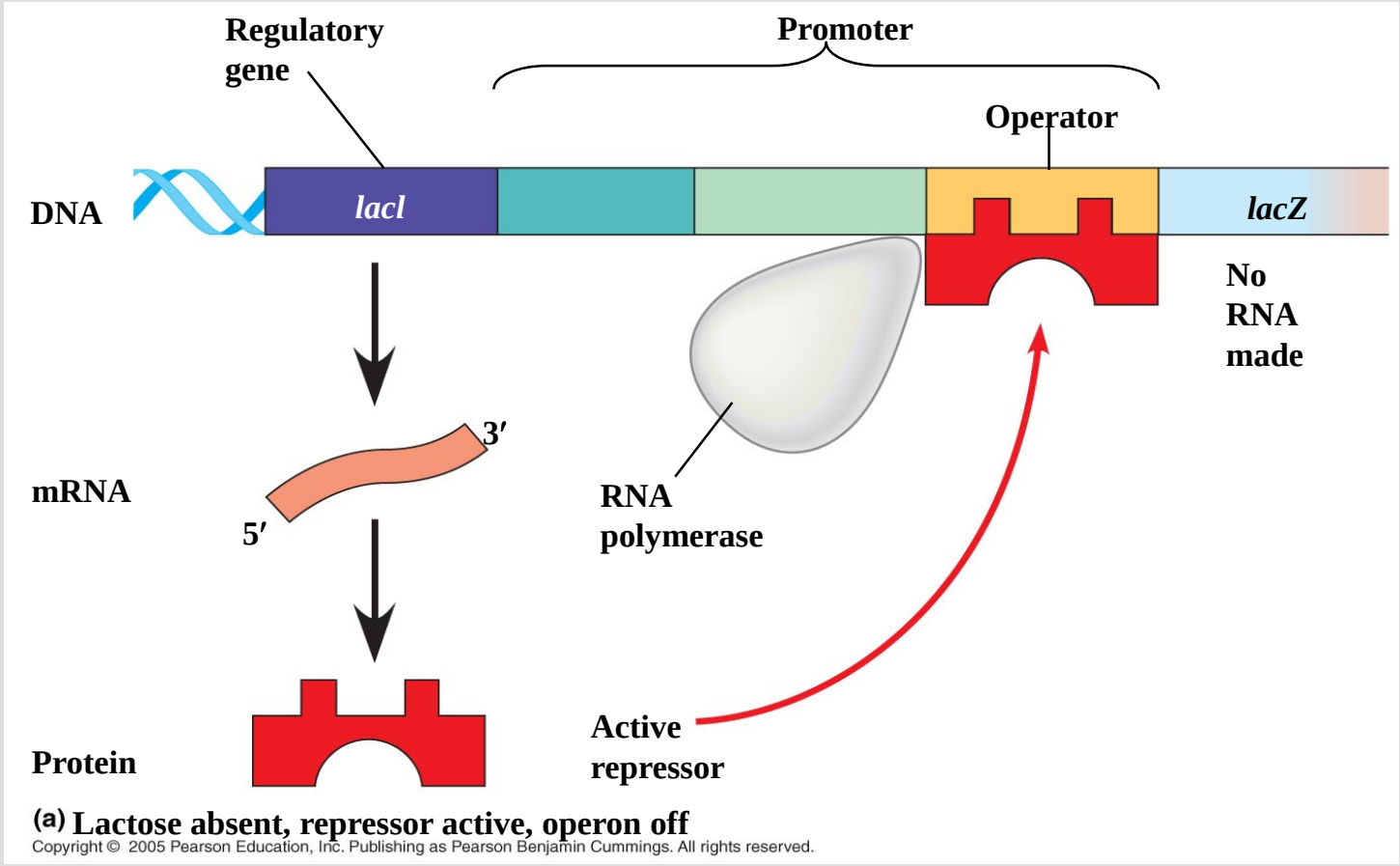
Let’s assume bacteria prefer glucose to lactose as a carbon source.

If glucose is available in the surroundings, does it make sense for the lac operon to be ON?

If it’s a waste of energy then how do bacteria repress (turn OFF) the Lac operon?

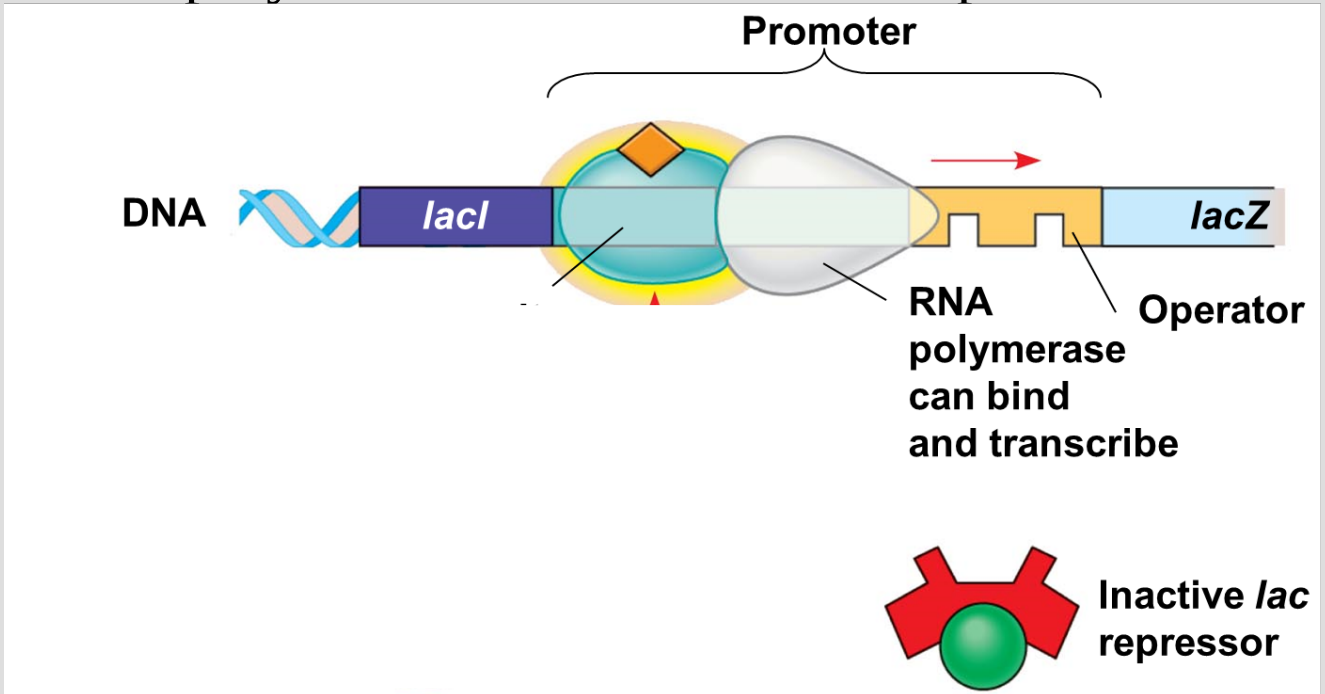
1. When lactose is absent, glucose present

- A repressor protein is continuously synthesised. It sits on a sequence of DNA just in front of the *lac* operon, the **Operator site**
- The **repressor protein** blocks the **Promoter site** where the RNA polymerase settles before it starts transcribing



2. When lactose is present

- A small amount of a sugar allolactose is formed within the bacterial cell. This fits onto the repressor protein at another active site (**allosteric site**)
- This causes the repressor protein to change its shape (a **conformational change**). It can no longer sit on the operator site. RNA polymerase can now reach its promoter site



3. When both glucose and lactose are present

- This explains how the *lac* operon is transcribed only when lactose is present.
- BUT….. this does not explain why the operon is not transcribed when both glucose and lactose are present.

Positive Gene Regulation

- Activator protein turns on Lac operon
  - catabolite activator protein (CAP)

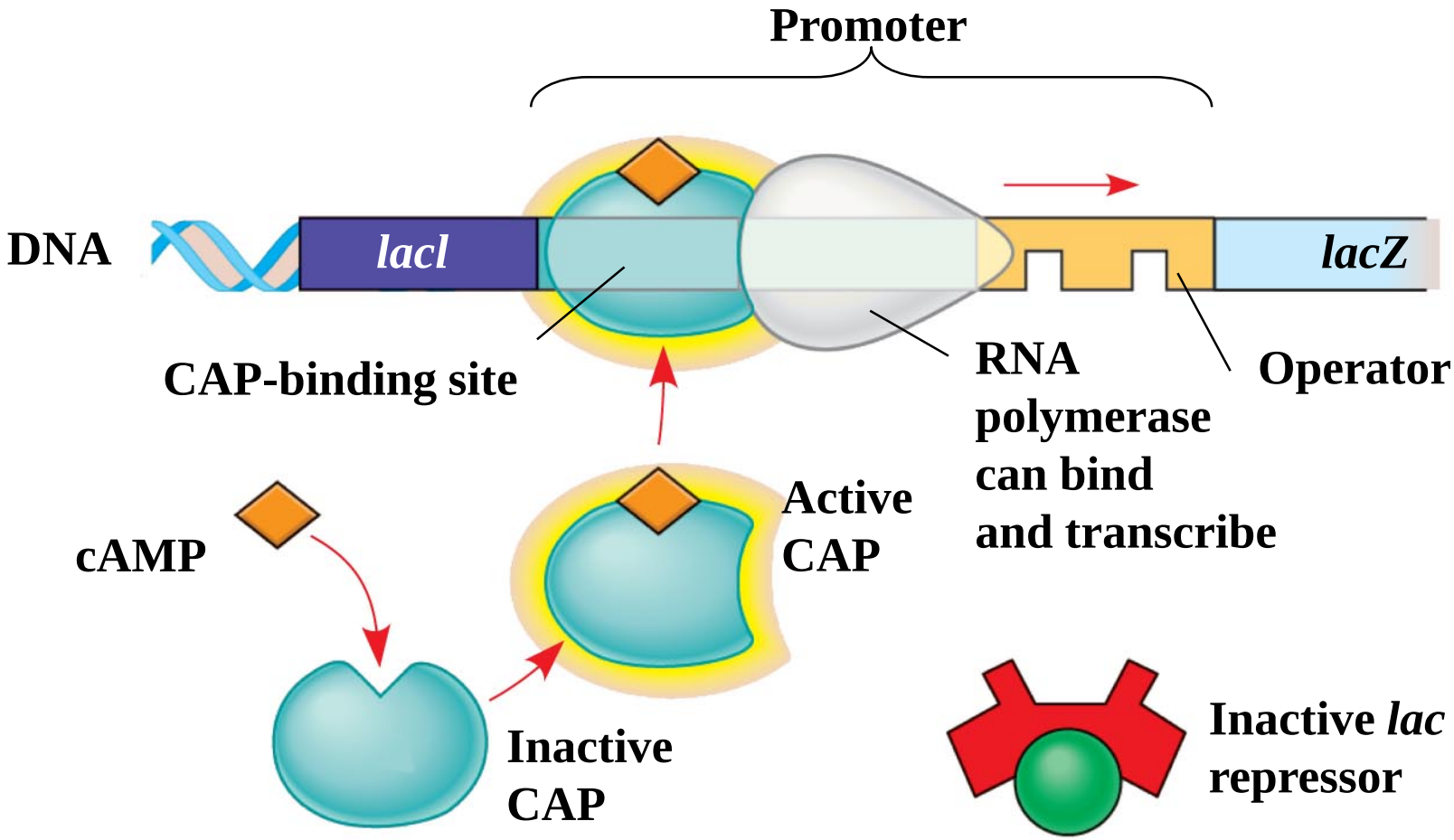
Glucose high cAMP low

Glucose low cAMP high

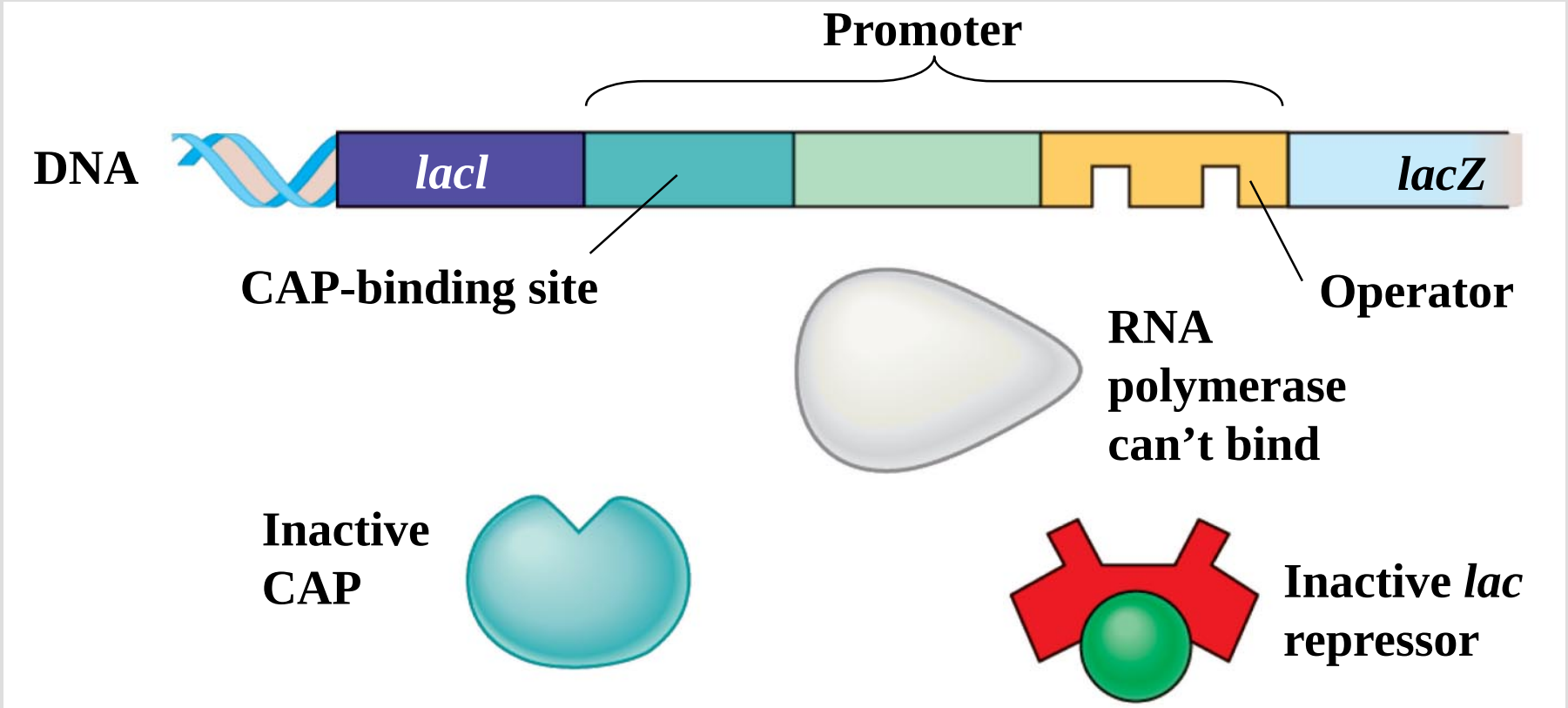
CAP-cAMP binds Lac promoter and induces transcription

When would this occur, when glucose is high or low?

Low



(a) Lactose present, glucose scarce (cAMP level high): abundant *lac* mRNA synthesized

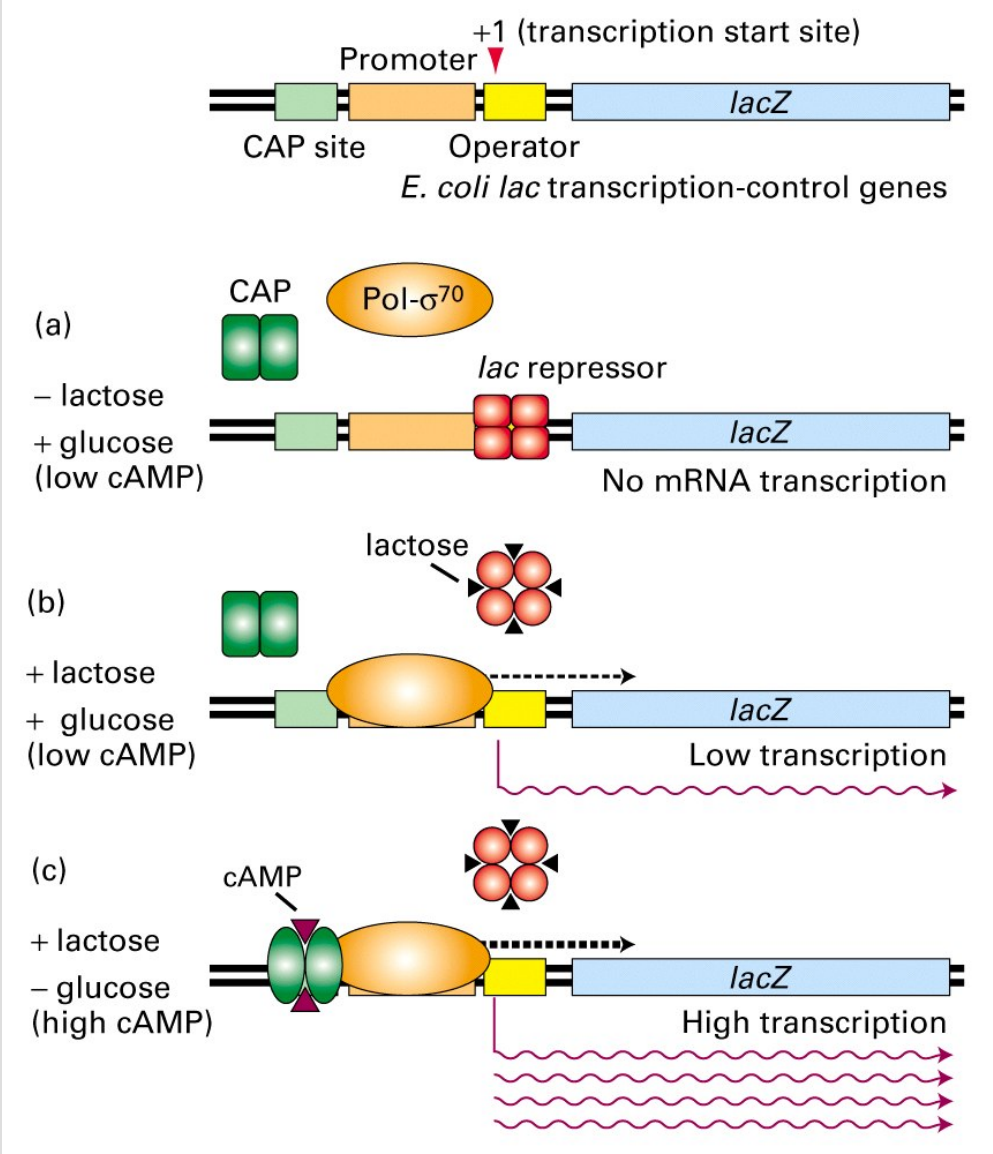


(b) Lactose present, glucose present (cAMP level low): little *lac* mRNA synthesized

Summary

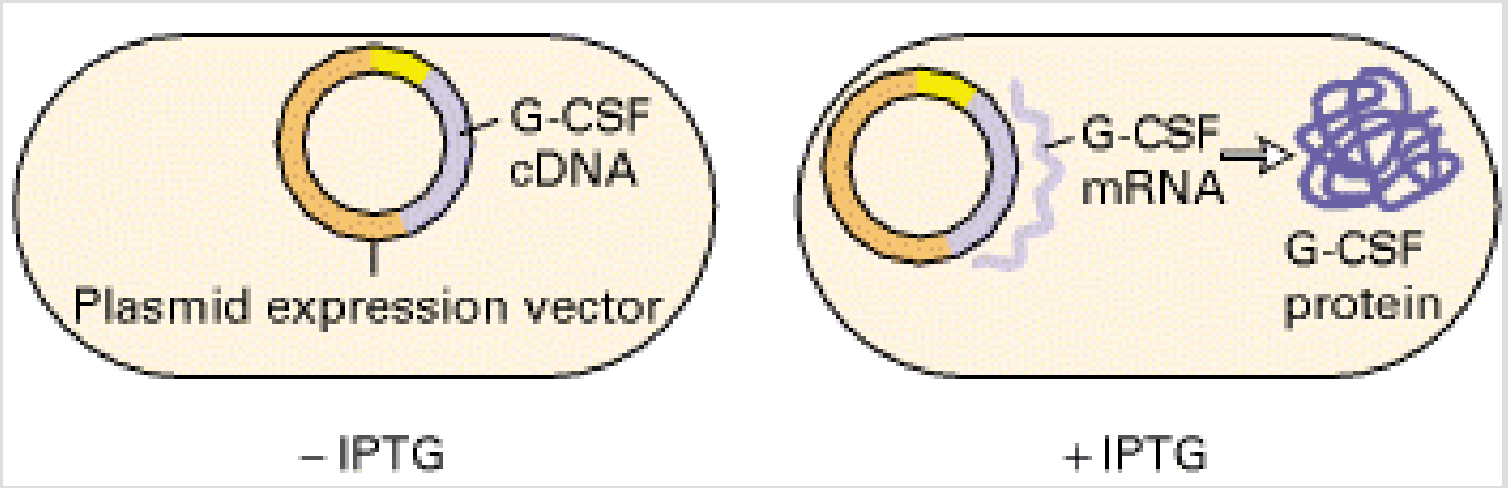
Carbohydrates	Activator protein	Repressor protein	RNA polymerase	*lac* Operon
+ GLUCOSE + LACTOSE	Not bound to DNA	**Lifted off operator site**	Keeps falling off promoter site	**No transcription**
+ GLUCOSE - LACTOSE	Not bound to DNA	Bound to operator site	Blocked by the repressor	**No transcription**
- GLUCOSE - LACTOSE	**Bound to DNA**	Bound to operator site	Blocked by the repressor	**No transcription**
- GLUCOSE + LACTOSE	**Bound to DNA**	**Lifted off operator site**	**Sits on the promoter site**	**Transcription**

The *Lac* promoter System



A simple *E. coli* expression vector utilizing the *lac* promoter.

In the presence of the lactose analog IPTG, RNA polymerase normally transcribes the *lacZ* gene, producing *lacZ* mRNA, which is translated into the encoded protein, G-CSF

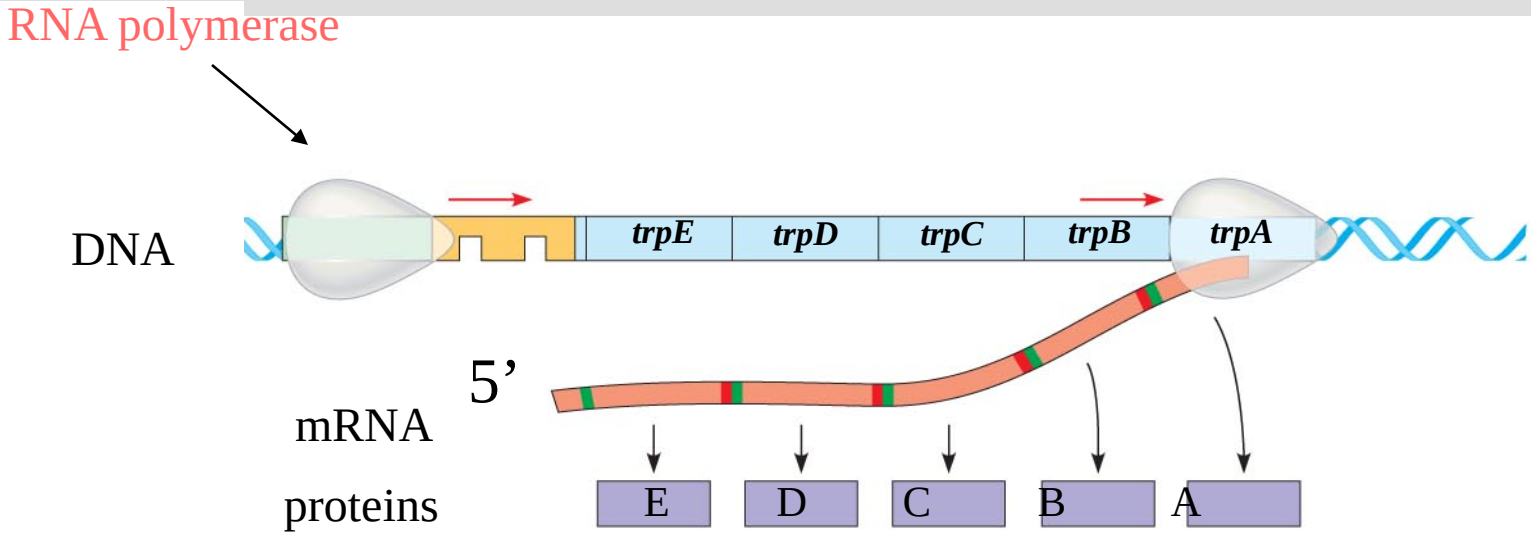


Trp promoter

- from E. coli tryptophan operon
- controlled by
  - trp repressor - co-repressor (tryptophan)
  - repressor synthesized in non-functional form
  - repressor-co-repressor complex is functional

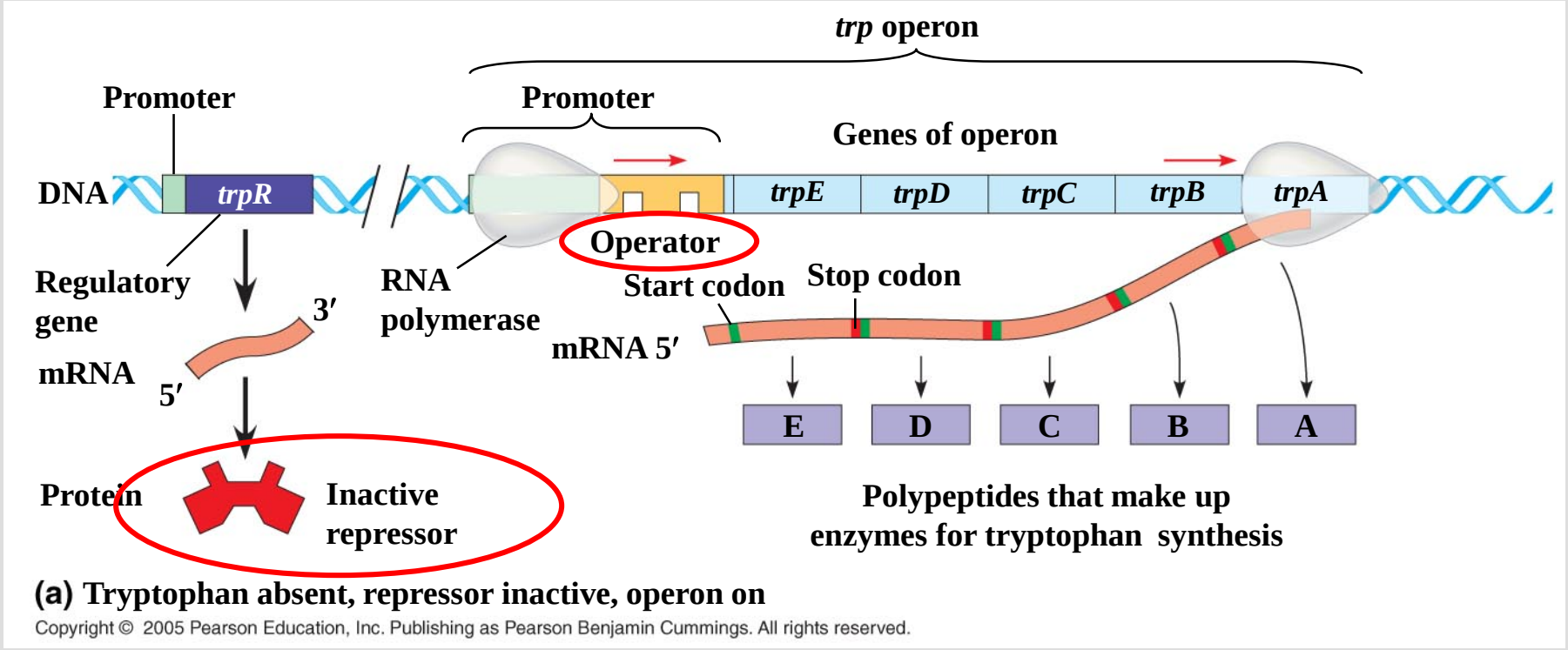
Trp Operon

Group of genes that encode enzymes
for tryptophan synthesis (an amino acid)



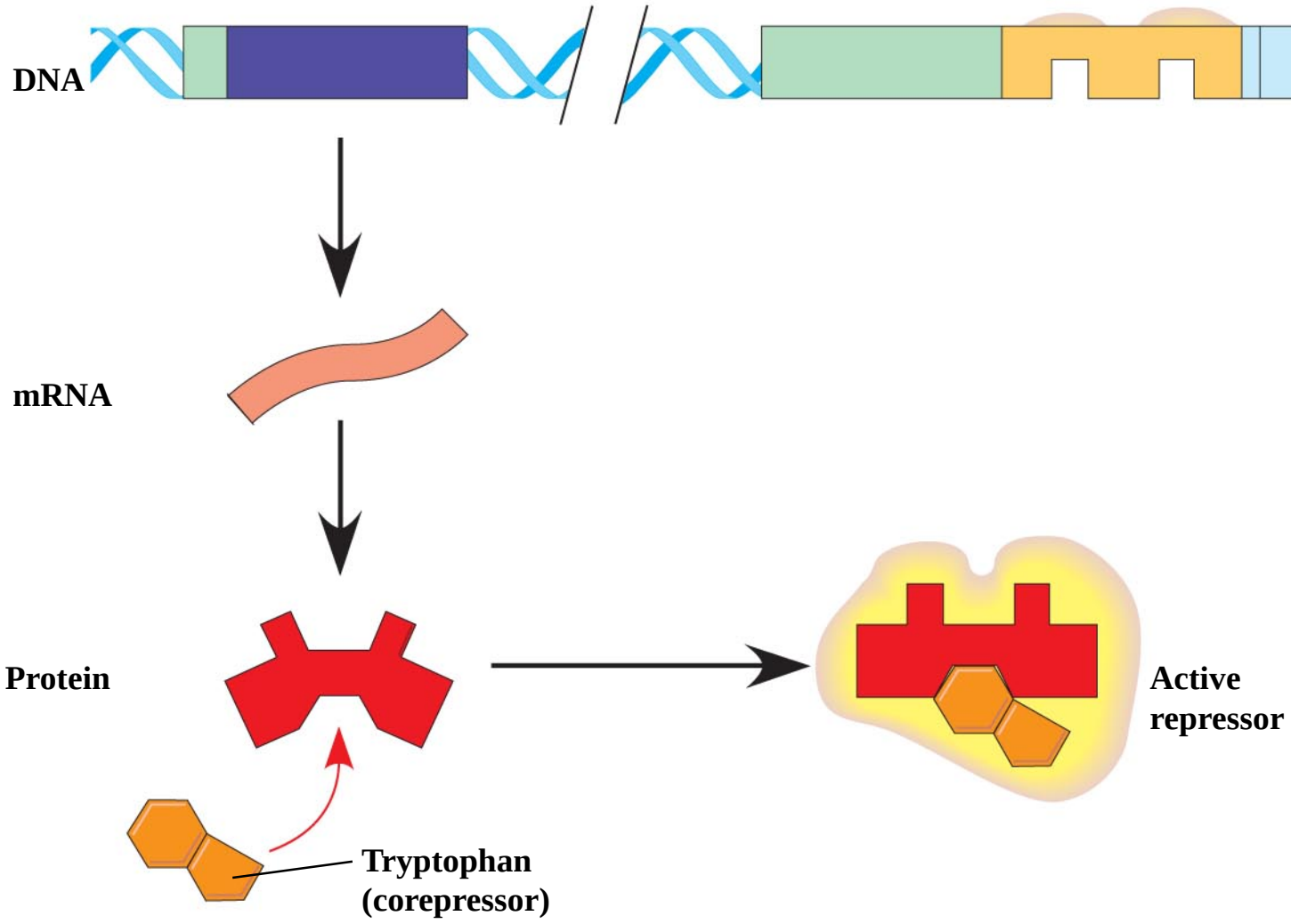
Trp Operon ON most of the time

TrpR gene also ON: makes inactive repressor protein

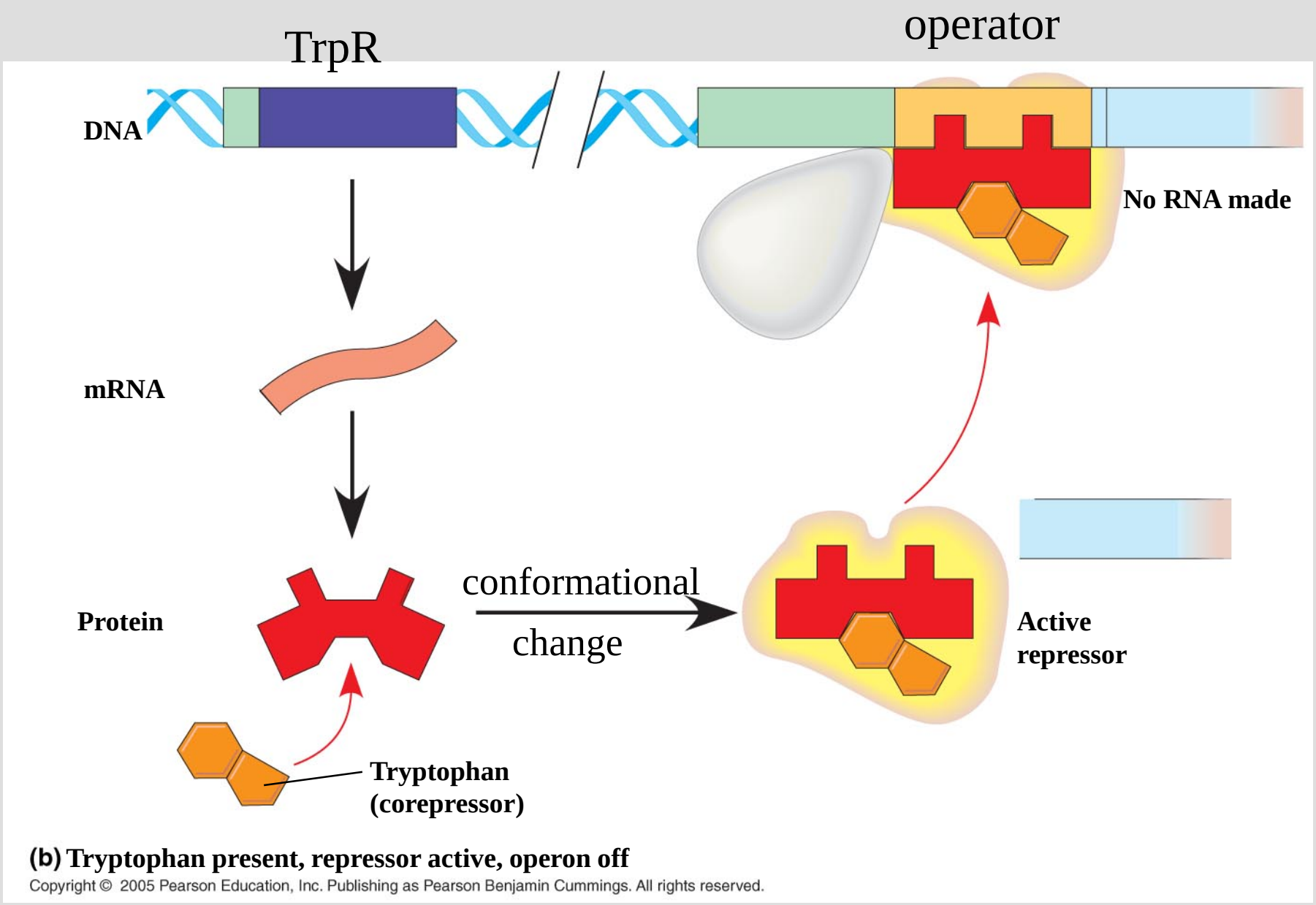


If the cell is not synthesizing much protein (e.g. low nutrients), will it need to continue to make trp?

How to shut off the trp operon?



(b) Tryptophan present, repressor active, operon off



(b) Tryptophan present, repressor active, operon off

Is the trp operon repressible or inducible?

ON unless excess trp binds and activates repressor protein->

Active TrpR binds operator

Blocks transcription

Trp operon OFF

Trp promoter

Repressor only ⟶ Gene Expressed

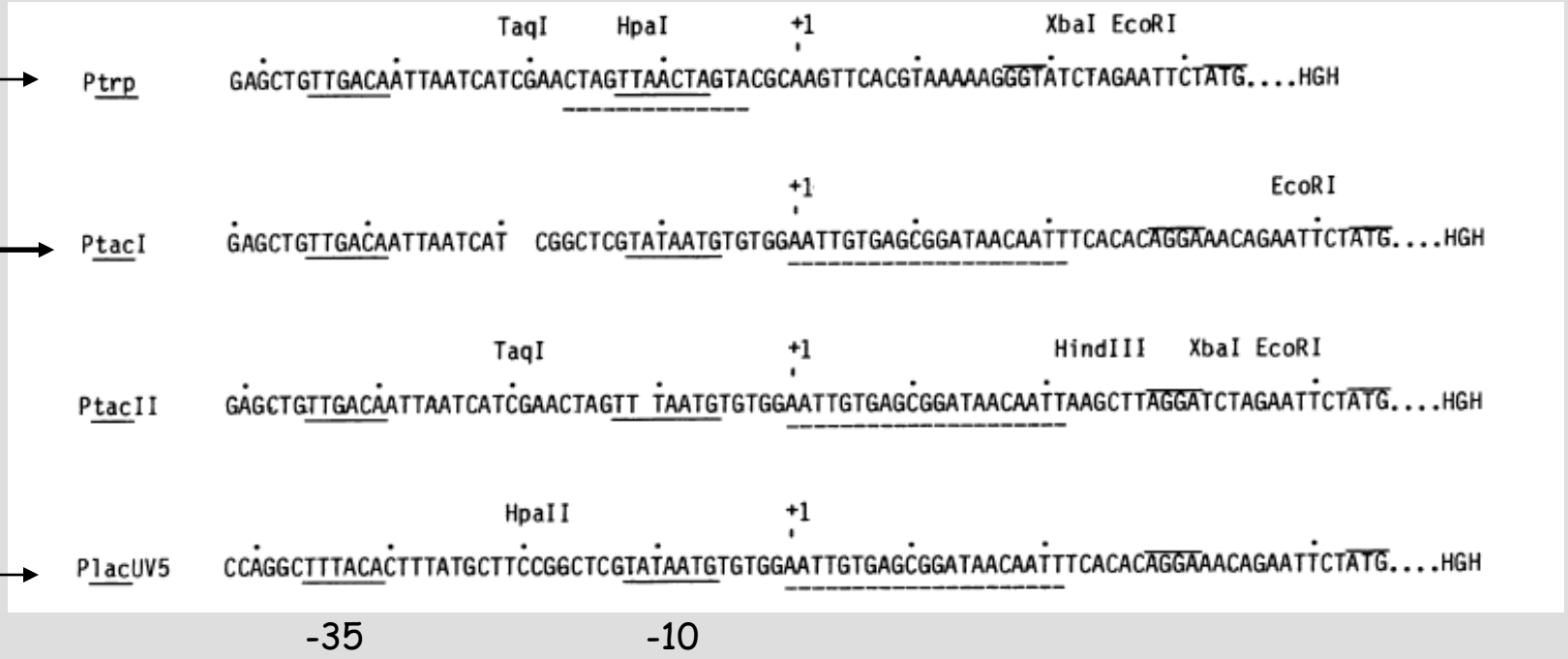
Repressor + co-repressor (Trp) ⟶ No Expression

tac (trc) promoter

- **Hybrid of lac and trp promoters**
  - **-35 region from trp**
  - **-10 region from lac**
  - **– separated by 16bp = tac promoter**
  - **– separated by 17bp = trc promoter**

- **3x stronger than trp**
- **5-10x stronger than lac**

Synthetic *E. coli* promoters



Hybrid Promoters:

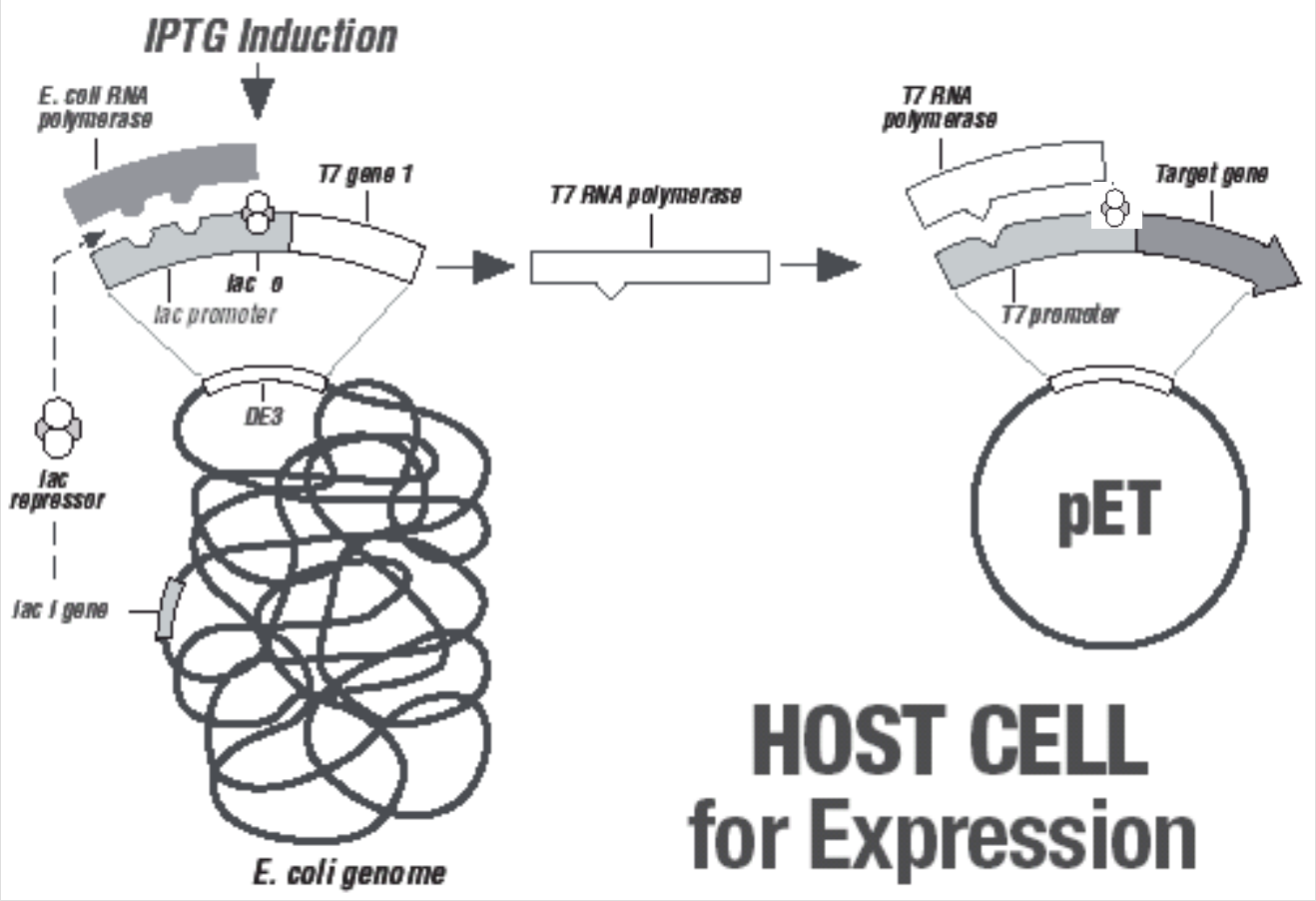
ptac Promoter (P_{trp} –35 +16pb + P_{lac} –10; IPTG)

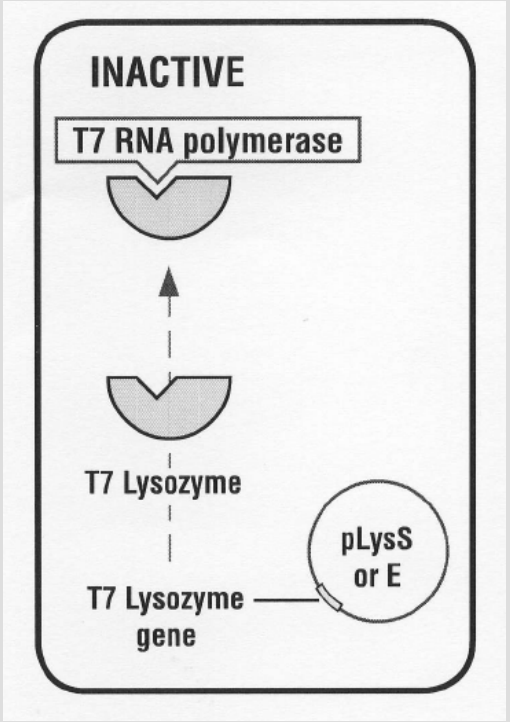
ptrc Promoter (P_{trp} –35 +17pb + P_{lac} –10; IPTG)-> invitro most powerful -> invivo 90% of *ptac*

ptic Promoter (P_{trp} –35 +18pb + P_{lac} –10; IPTG) -> 65 % of *ptac*

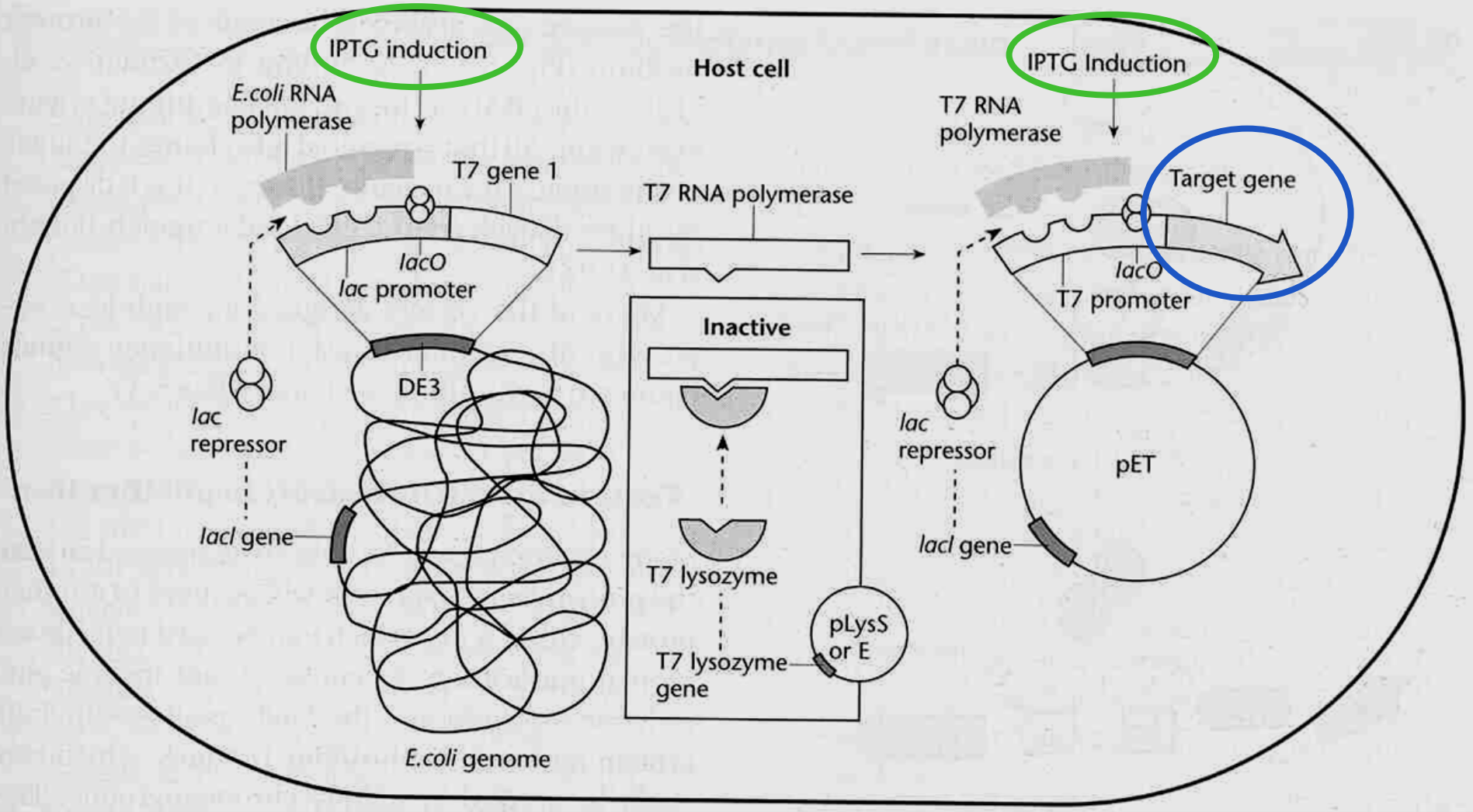
T7 promoter

- From T7 phage
- Use T7 polymerase – need it





pET vectors: protein expression



Enhancing Translation

- Strong promoter will produce lots of mRNA
- Also need effective translation to obtain lots of protein

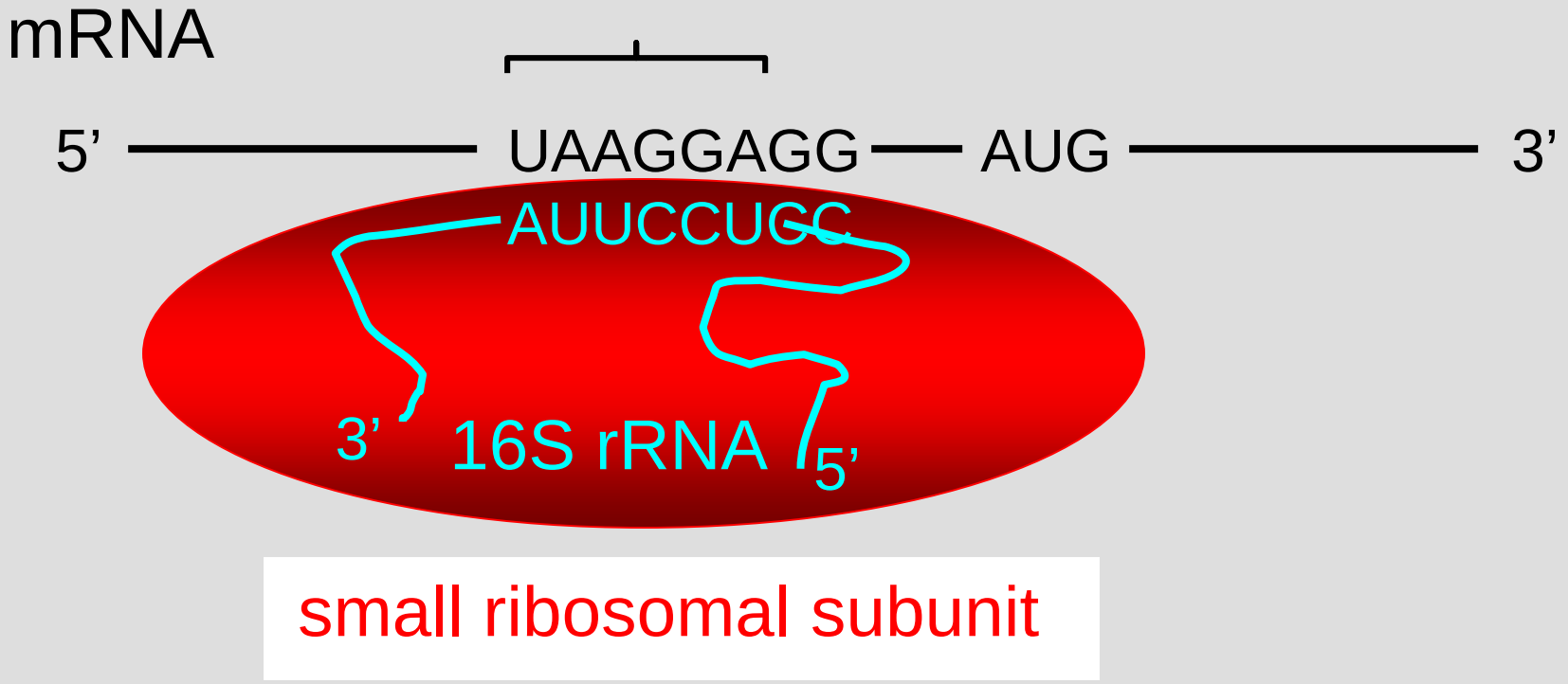
- DNA ⟶ RNA ⟶ Protein

Enhancing Translation

- In Prokaryotes
  - translation signal is RBS
  - ribosome binding site
  - Shine-Dalgarno sequence
  - 6-8 nt in length
  - located short distance (~10nt) upstream of AUG translation start codon

Enhancing Translation-Shine Dalgarno

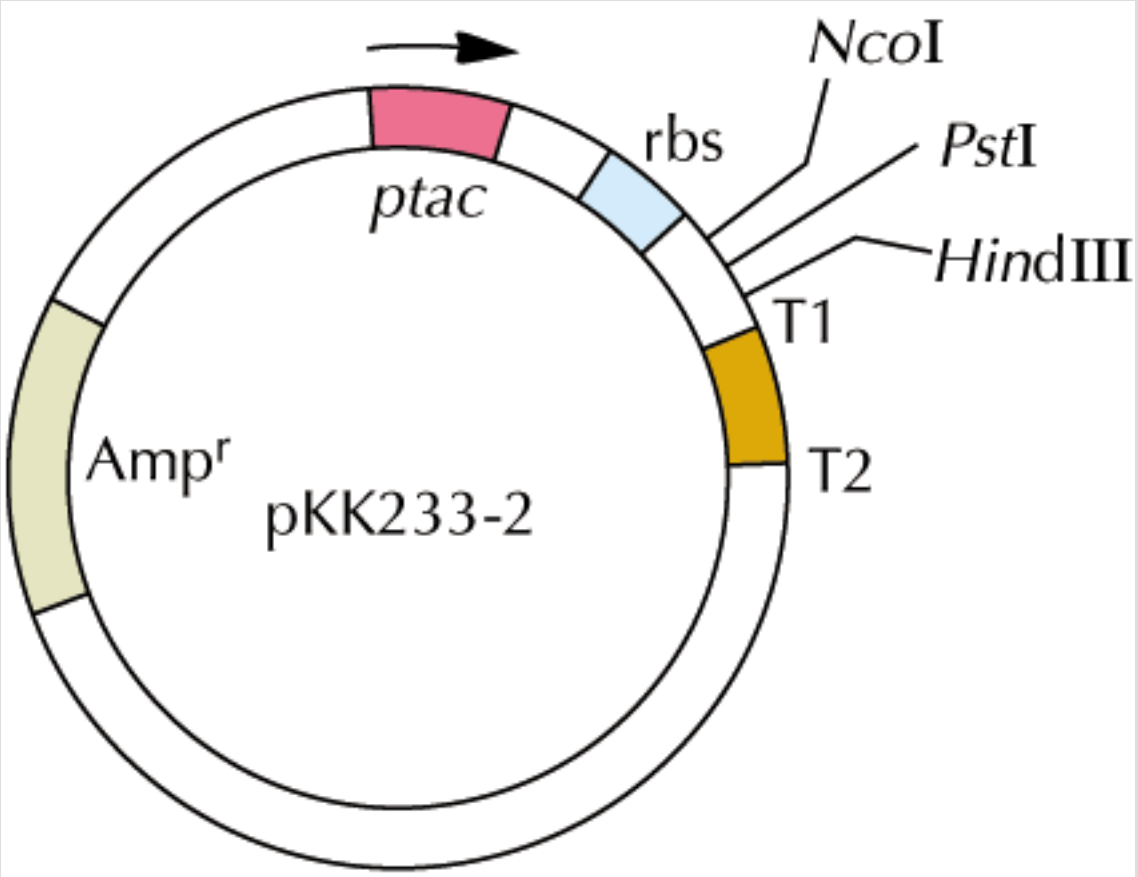
- sequence complementary to region on 16S rRNA of small subunit of ribosome



Expression Vector pKK233-2

selectable marker
tac promoter
RBS
unique REase sites
Txn terminators

not shown:
ori of replication



Codon Optimization

UUU F 0.57	UCU S 0.11	UAU Y 0.53	UGU C 0.42
UUC F 0.43	UCC S 0.11	UAC Y 0.47	UGC C 0.58
UUA L 0.15	UCA S 0.15	UAA * 0.64	UGA * 0.36
UUG L 0.12	UCG S 0.16	UAG * 0.00	UGG W 1.00
CUU L 0.12	CCU P 0.17	CAU H 0.55	CGU R 0.36
CUC L 0.10	CCC P 0.13	CAC H 0.45	CGC R 0.44
CUA L 0.05	CCA P 0.14	CAA Q 0.30	CGA R 0.07
CUG L 0.46	CCG P 0.55	CAG Q 0.70	CGG R 0.07
AUU I 0.58	ACU T 0.16	AAU N 0.47	AGU S 0.14
AUC I 0.35	ACC T 0.47	AAC N 0.53	AGC S 0.33
AUA I 0.07	ACA T 0.13	AAA K 0.73	AGA R 0.02
AUG M 1.00	ACG T 0.24	AAG K 0.27	AGG R 0.03
GUU V 0.25	GCU A 0.11	GAU D 0.65	GGU G 0.29
GUC V 0.18	GCC A 0.31	GAC D 0.35	GGC G 0.46
GUA V 0.17	GCA A 0.21	GAA E 0.70	GGA G 0.13
GUG V 0.40	GCG A 0.38	GAG E 0.30	GGG G 0.12

[Codon/a.a./fraction per codon per a.a.]
E. coli K12 data from the Codon Usage Database

GENOSPHERE BIOTECHNOLOGIES

GENE BUILDER: Optimise your sequence

www.genosphere-biotech.com

Gene	AAT	AGT	ATT	ATT	TTA	AGT	AGT	GTA	TTG	AGC	TTA	CAA	AAC	AAT	AAA	AAT	GCT
Protein	N	S	I	I	L	S	S	V	L	S	L	Q	N	N	K	N	A
Build1	AAT	AGC	ATT	ATT	CTG	TCT	TCT	GTA	CTG	AGC	CTG	CAA	AAC	AAT	AAA	AAT	GCT
Build2	AAT	AGC	ATC	ATT	CTG	TCT	TCT	GTC	CTG	TCT	CTG	CAA	AAC	AAC	AAA	AAC	GCC
Build3																	

TTA	ACA	TCA	GGA	AAT	AAT	GCA	GAA	GTG	AGG	TTT	GGA	GAT	ATA	GTA	AAT	CTT	AAT
L	T	S	G	N	N	A	E	V	R	F	G	D	I	V	N	L	N
CTG	ACT	TCA	GGC	AAT	AAT	GCA	GAA	GTG	CGT	TTT	GGT	GAT	ATC	GTA	AAT	CTG	AAT
CTG	ACT	TCT	GGC	AAC	AAC	GCA	GAA	GTA	CGT	TTC	GGT	GAC	ATC	GTG	AAC	CTG	AAT

ACG	ACA	AAT	GAC	TAT	AAA	GAT	TCA	AAA	TCA	GGA	GAT	AAA	AAT	GTT	CAA	AAT	TTA
T	T	N	D	Y	K	D	S	K	S	G	D	K	N	V	Q	N	L
ACG	ACC	AAT	GAC	TAT	AAA	GAT	TCA	AAA	TCT	GGC	GAT	AAA	AAT	GTT	CAA	AAT	CTG
ACC	ACC	AAC	GAT	TAC	AAA	GAT	TCT	AAA	TCT	GGC	GAC	AAA	AAC	GTT	CAG	AAC	CTG

Choice of Host Organism

- ***E.coli is organism of choice***
  - well characterized growth
  - lots of experience
  - NOT always the best organism

- **Sometimes overexpression can reduce amount of recovered protein**
  - Protein forms insoluble aggregates
  - Proteins is found in Inclusion bodies
  - Degradation
  - results in low level of expression
  - difficulty in purifying desired protein

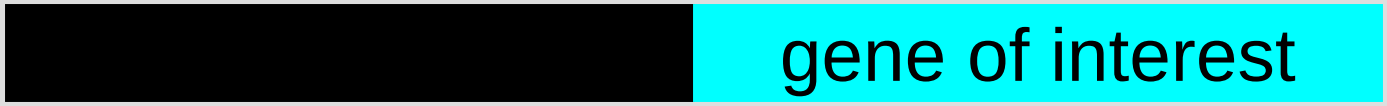
HOW OVERCOME THESE DRAWBACKS:

- Inducible promoters
- Decrease T e growth rate
- Little changes into aa sequences (i.e introducing Cys)
- **Fusion protein** with Tyreodoxin

Fusion Proteins

- Protein fusion done at level of DNA (genes)
  - coding region of cellular protein fused in frame to coding region of target protein gene
  - when transcribed and translated generates a ***fusion protein***

Fusion Proteins



MET ... LEU ARG THR MET VAL ILE ... End

ATG ... GTG CGA ACC ATG GTG ATC ... TAG

Nco I

Note: translation stop of cellular gene must be removed

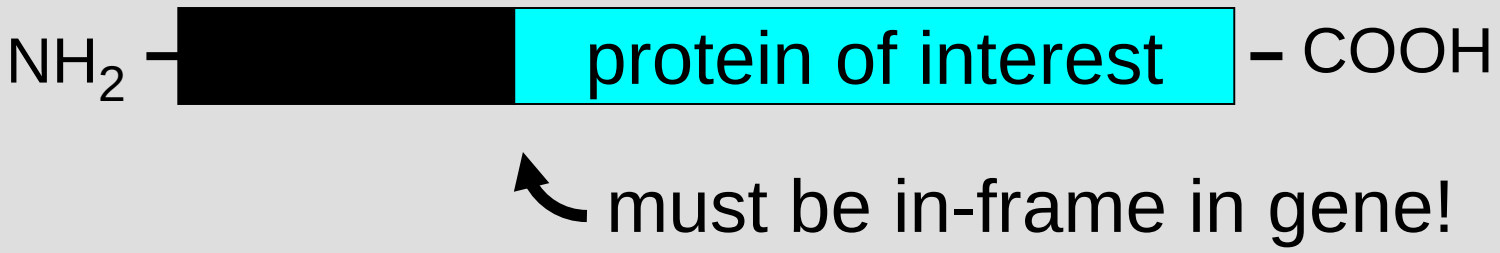
Note: reading frame of fusion protein must be contiguous

Secretion of Protein of Interest

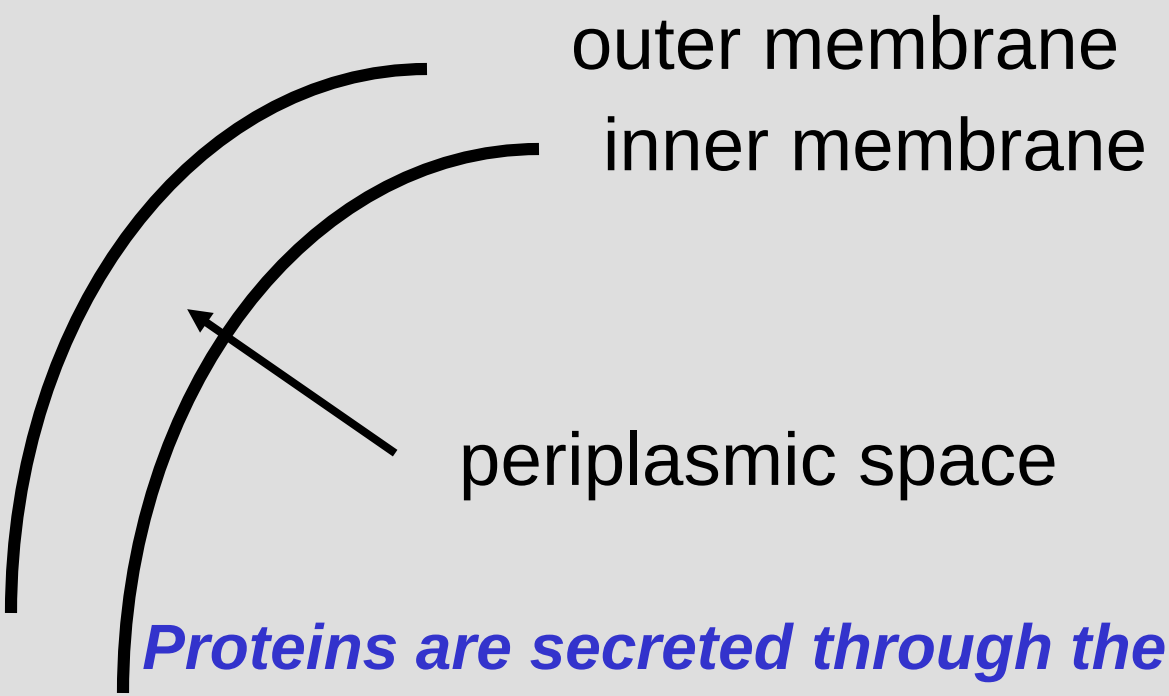
- ***Why secrete Protein of Interest?***
  - stability of the protein may be increased
  - protein may be easier to purify
- ***Stability of the protein may be increased***
  - remove from cytoplasm and its proteases
  - recombinant human proinsulin (rh-proinsulin) stability is increased 10X if secreted

Secretion of Protein of Interest

- ***What is required for secretion?***
  - **Signal peptide sequence** required for passage through cell membrane
  - added at NH_2 end of protein (5' end of gene)



Secretion in Gram - Bacteria

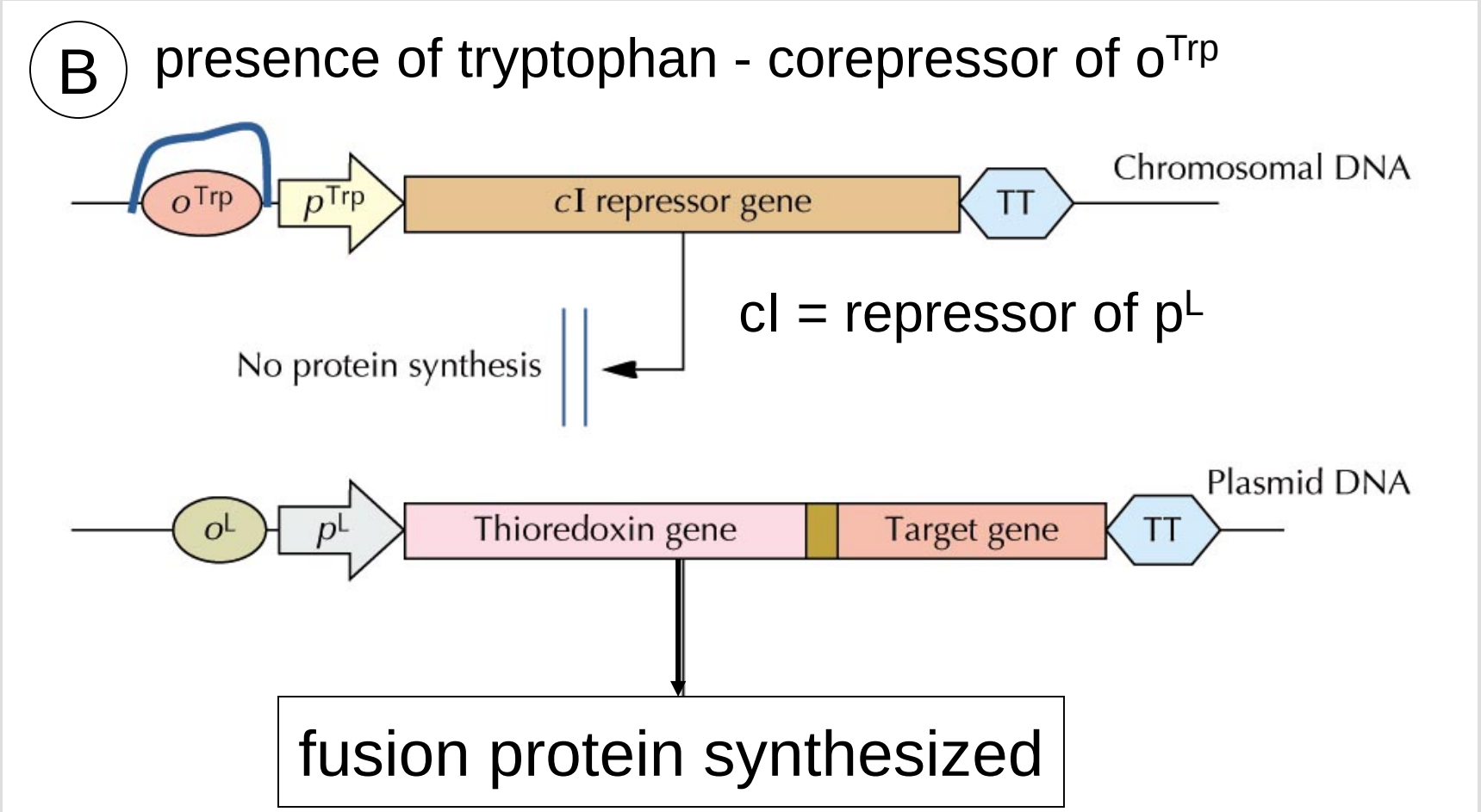


Proteins are secreted through the inner (cell) membrane, but in general cannot pass through the outer membrane.
Proteins remain trapped in the periplasmic space.

Increase stability

- Insolubility may be due to aggregation caused by incorrect folding
  - Solution:
    - ***make a fusion protein with thioredoxin***
    - 11.7 kdal protein
    - can keep the fusion protein soluble even when it makes up 40% of total protein

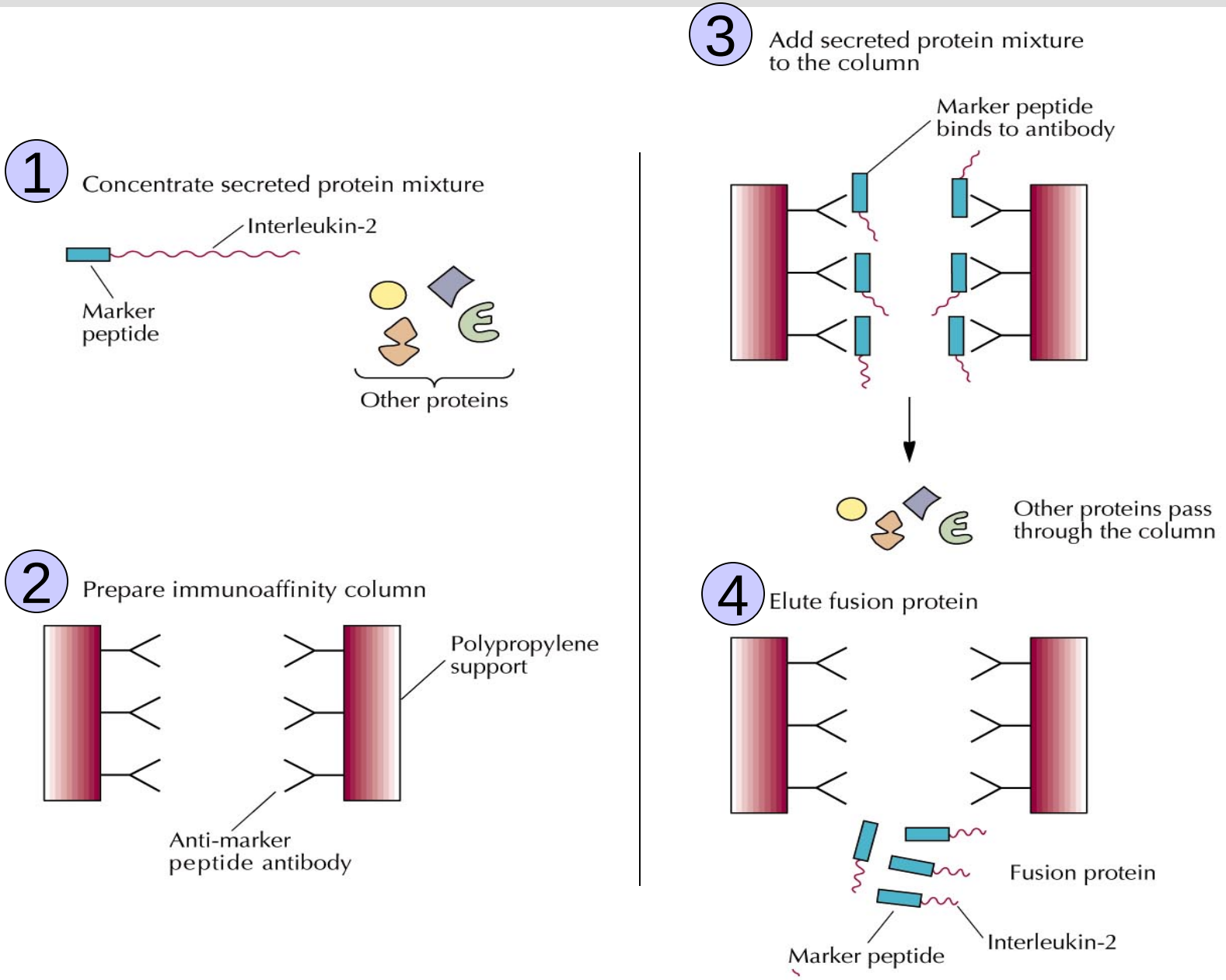
Thioredoxin-target fusion protein



Construction of Fusion Proteins might help purification

- Fusion partner (“tag”) binds to small molecule or Antibody (Ab)
- Small molecule or Ab can be linked to inert matrix
- Fusion protein will bind, other proteins won’t
- Elute purified protein from column

Affinity Chromatography Purification



Helper tags for protein production and purification

- 6/7 histidine tag: interacts very specifically with Ni2+ ions, which can be immobilized on columns or beads

- Biotin carboxylase: covalently attaches to biotin, biotin binds to streptavidin which can be immobilized on columns or beads

- Epitopes (e.g. c-myc) for specific antibodies can be included as tags--purify on antibody column

- Tags can be engineered to be removable

Fusion partner	Size	Ligand	Elution condition
ZZ	14 kDa	IgG	Low pH
His tail	6–10 aa	Ni^{2+}	Imidazole
Strep-tag	10 aa	Streptavidin	Iminobiotin
PinPoint	13 kDa	Streptavidin	Biotin
MBP	40 kDa	Amylose	Maltose
β-Lactamase	27 kDa	Phenyl-boronate	Borate
GST	25 kDa	Glutathione	Reducing agent
Flag	8 aa	Specific MAb	Low calcium

Adapted from Nygren et al., 1994, *Trends Biotechnol.* **12:**184–188.

Abbreviations: aa, amino acids; kDa, kilodaltons; ZZ, a fragment of *Staphylococcus aureus* protein A; His, histidine; Strep-tag, a peptide with affinity for streptavidin; PinPoint, a protein fragment which is biotinylated in vivo in *E. coli*; MBP, maltose binding protein; GST, glutathione *S*-transferase; Flag, a peptide recognized by enterokinase.

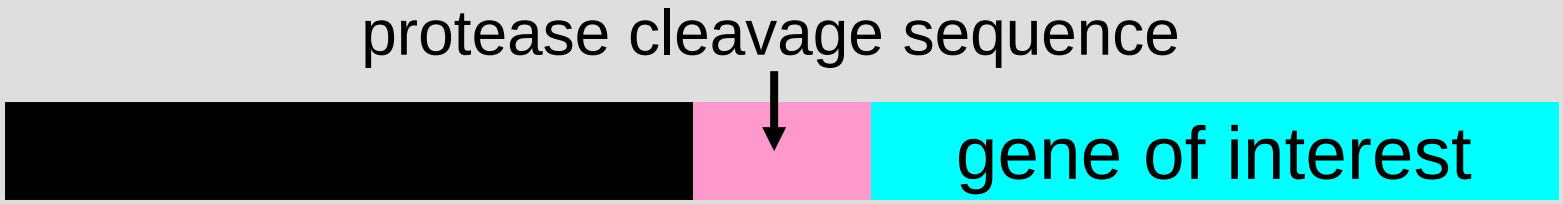
Fusion Proteins

- Produce fusion protein
  - prevent degradation
  - increase ease of purification

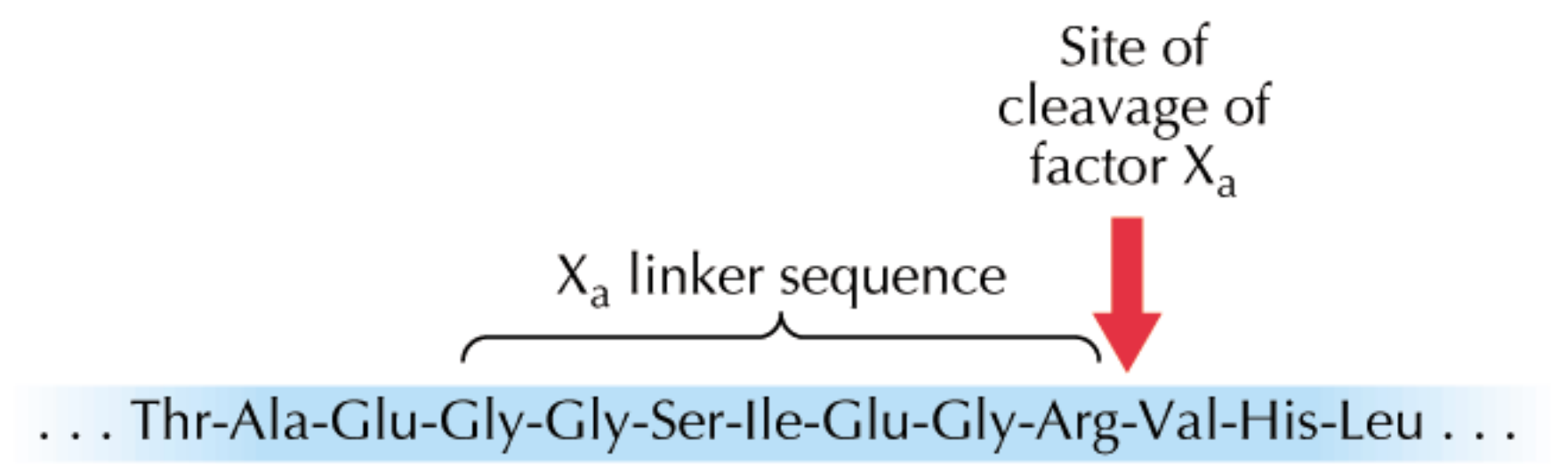
- ***May need to remove fusion "tag"***
  - may effect biological functioning
  - may make it unsuitable for clinical use
    - FDA might not grant approval

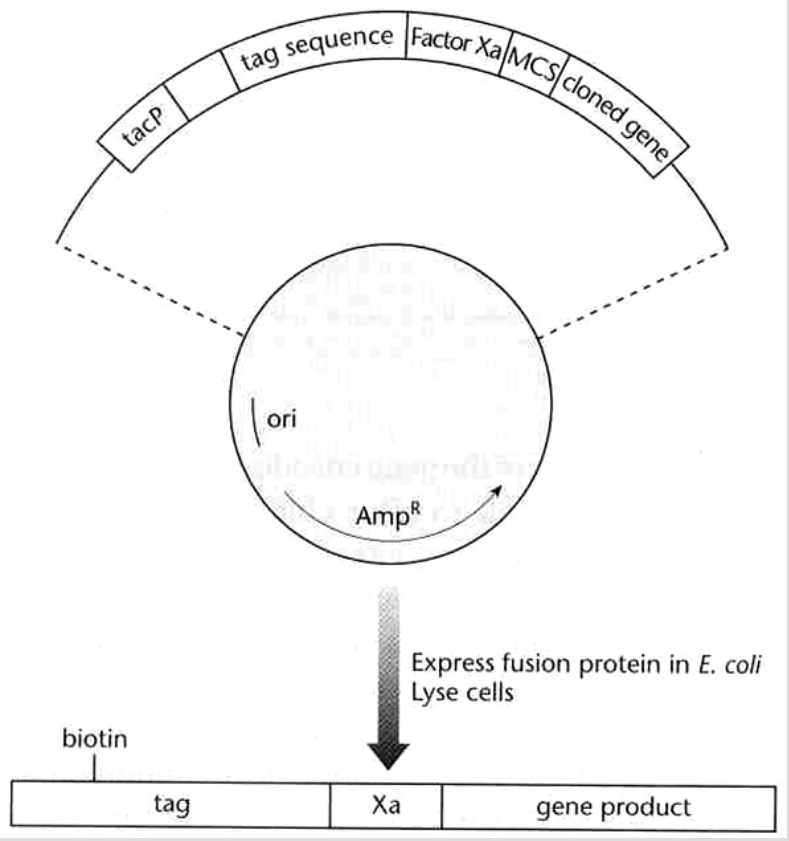
Fusion Proteins

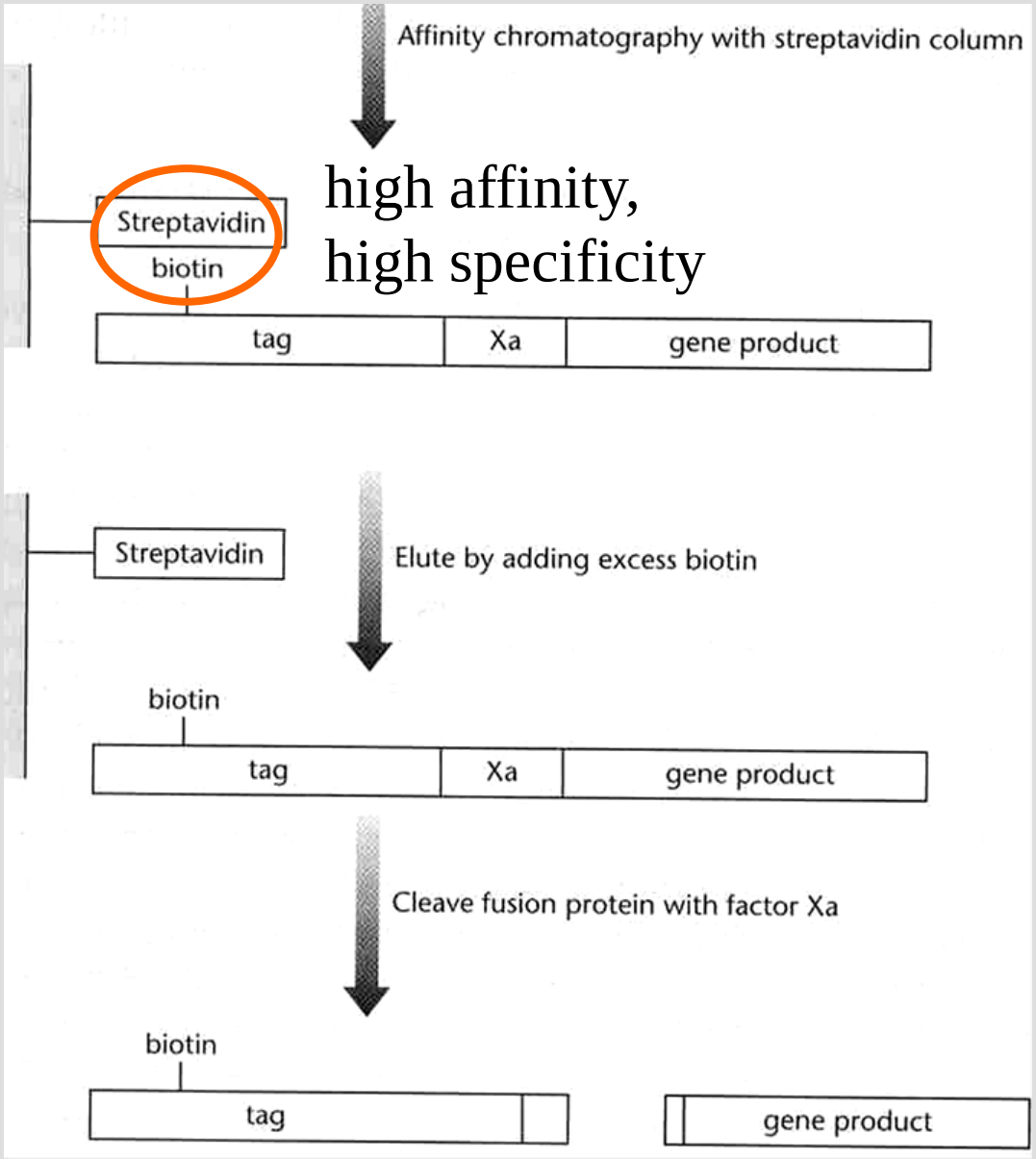
- Need Proteases equivalent to REases
  - Several have been developed
  - cleave a short defined aa sequence
  - insert protease cleavage sequence between fusion partner gene and G of I



Cleavage by Factor Xa



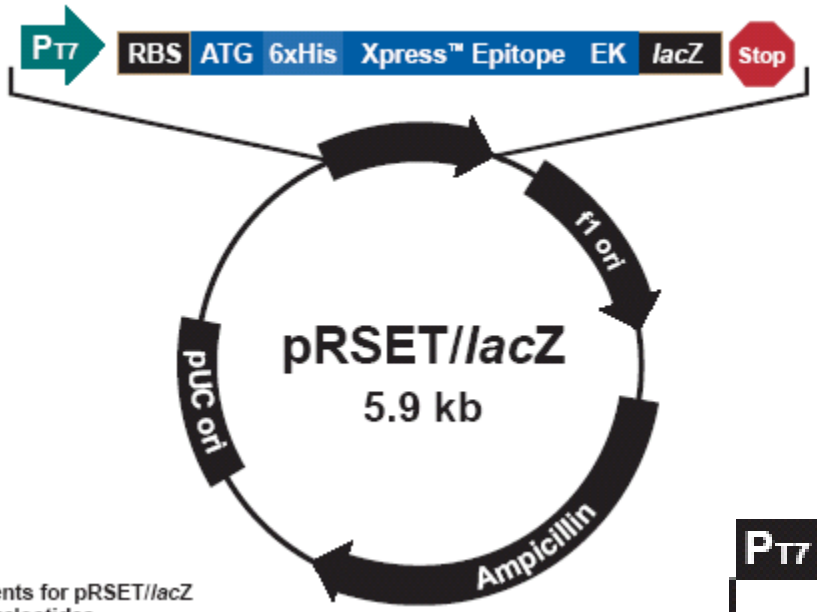




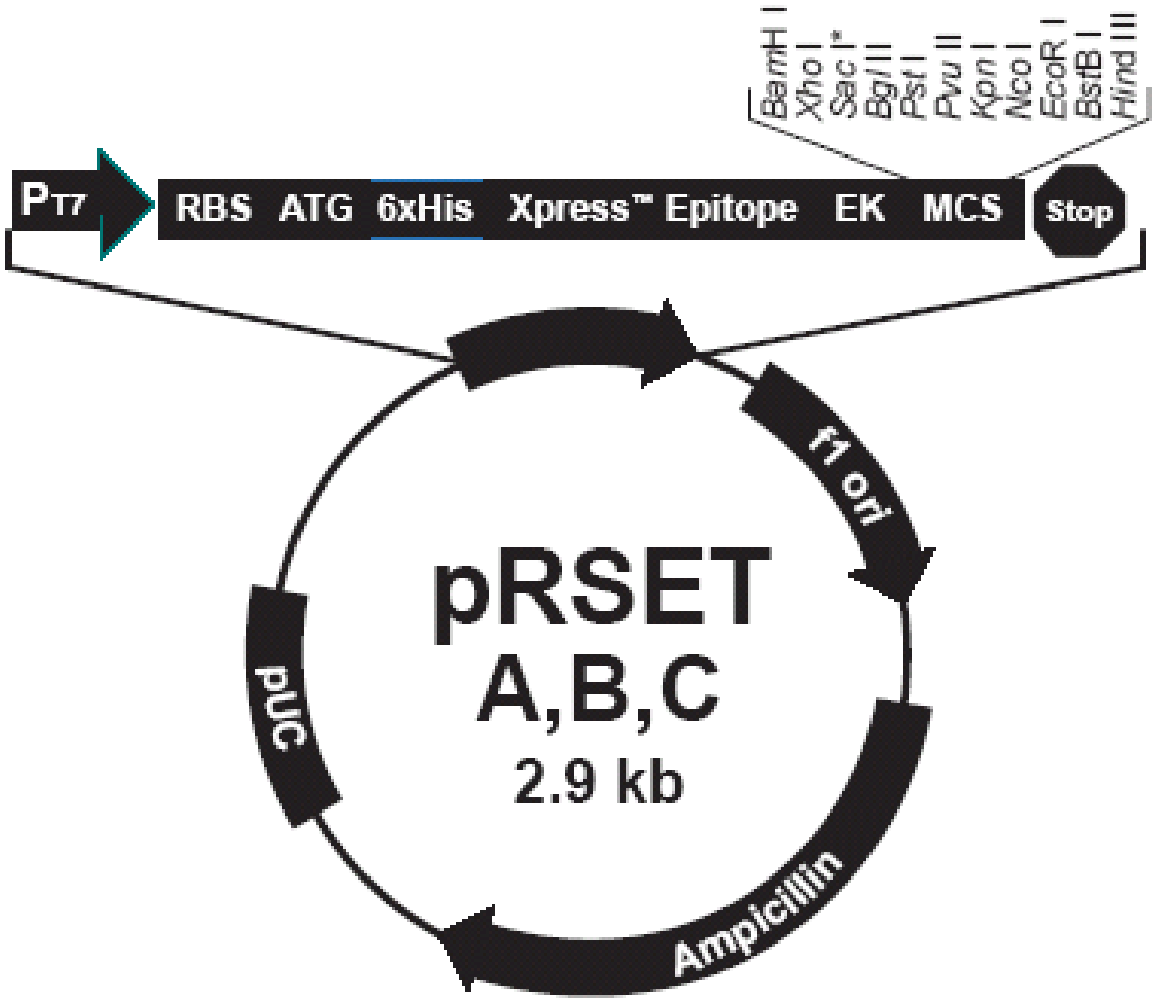
Using tags in protein purification

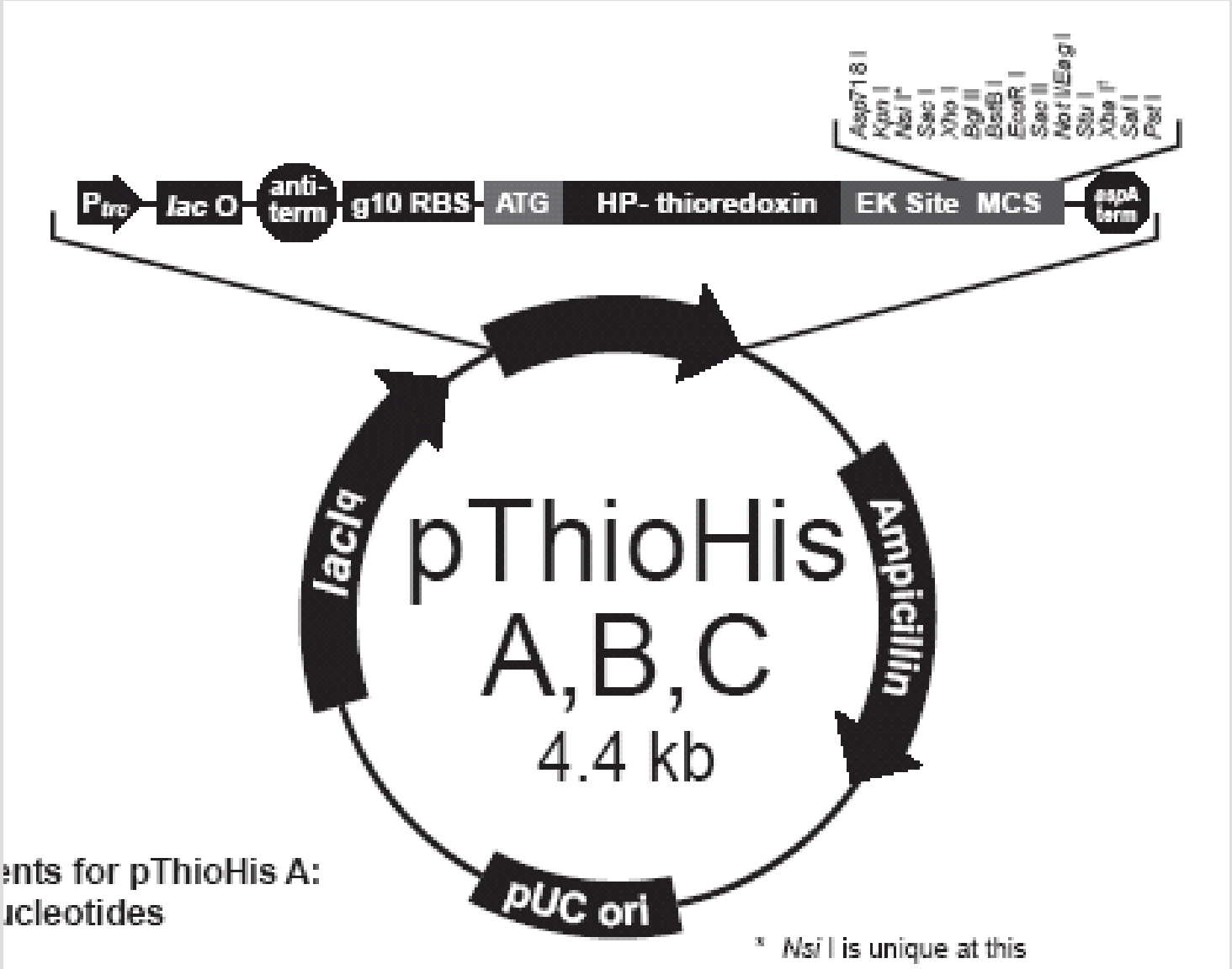
Fusion Protein Cleavage

Protease	Source
Factor Xa	blood clotting factors
Thrombin	
Enterokinase	



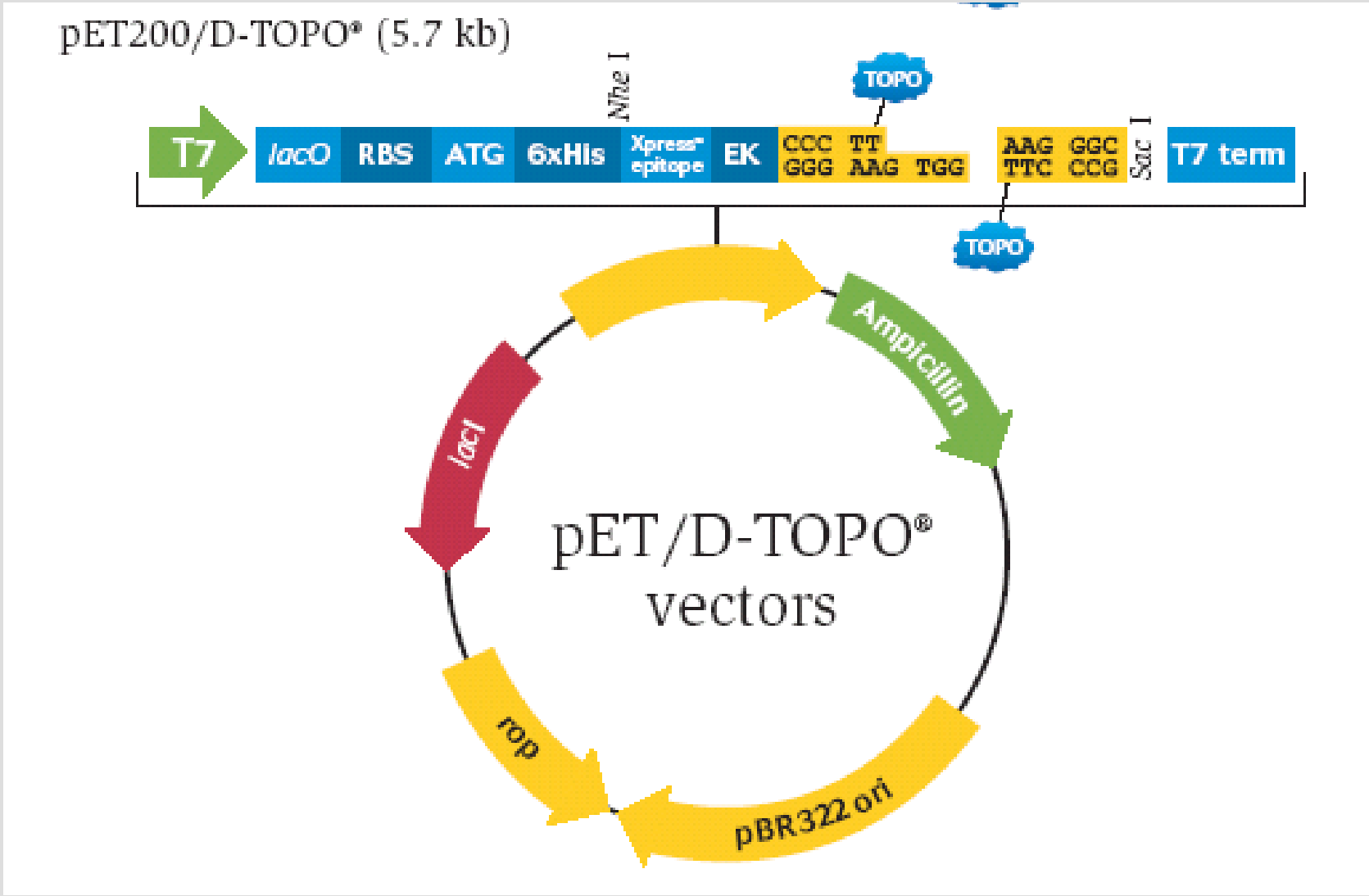
ents for pRSET/*lacZ*



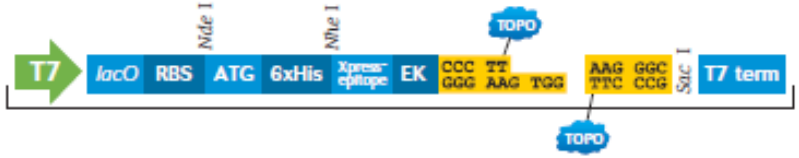


ents for pThioHis A:
ucleotides

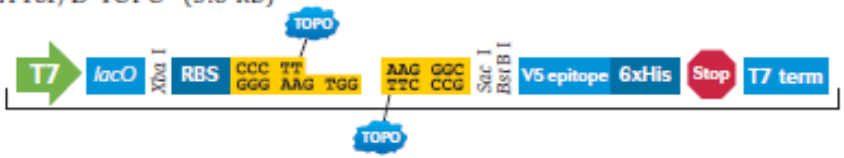
* *Nsi* I is unique at this


pET200/D-TOPO® (5.7 kb)
Nhe I
TOPO
T7
lacO
RBS
ATG
6xHis
Xpress™ epitope
EK
CCC TT
GGG AAG TGG
AAG GGC
TTC CCG
Sac I
T7 term
TOPO
Ampicillin
lacI
pET/D-TOPO®
vectors
rop
pBR322 ori

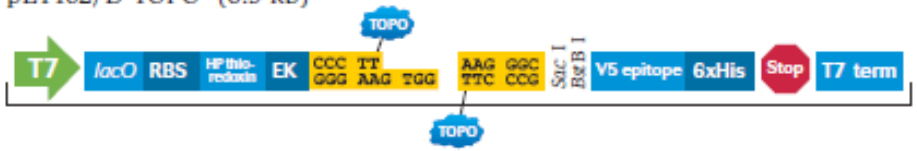
pET100/D-TOPO® (5.8 kb)



pET101/D-TOPO® (5.8 kb)

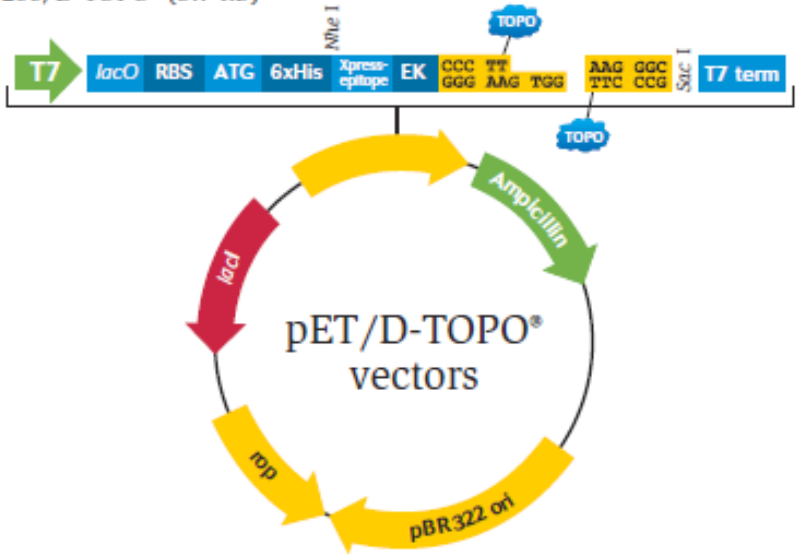


pET102/D-TOPO® (6.3 kb)



pET151/D-TOPO® (5.8 kb)

pET200/D-TOPO® (5.7 kb)



Recombinant DNA Technology

Manipulation of Gene Expression in mammalian cells

Recombinant Protein Production in Eukaryotic Cells

- **Occassionally problems will arise when eukaryotic proteins are expressed in prokaryotic cells**
  - **unstable**
  - **no biological activity**
  - **prokaryotic contaminants**

Eukaryotic Expression Systems

- **To eliminate prokaryotic expression problems, eukaryotic expression systems were developed**
  - **Esp. important for therapeutic proteins**
  - **Need to have identical: biochemical**
    biophysical
    functional properties
    as the natural protein

Euk. Expression Systems

- **What's different?**
  - **Post-translational modification of most eukaryotic proteins**

  Such as:
  - **Correct disulfide bond formation**
  - **Proteolytic cleavage of inactive precursor**
  - **Glycosylation - addition of sugar residues**
  - **Alteration of amino acids in protein**
    - **phosphorylation**
    - **acetylation**
    - **sulfation**
    - **fatty acid addition**

Eukaryotic Cell Exp. Systems

- Therapeutic proteins may need to be expressed in eukaryotic cells to be effective
  - correct post-translational modifications

Protein Drugs Produced by Eukaryotic Cell Culture

Protein	Condition
Factor IX & VIIIc	hemophiliacs
CD4 receptor	AIDS
erythropoetin	cancer
β & γ interferons	cancer
Interleukin-2	cancer
growth hormone	dwarfism
tissue plasminogen activator	heart attack/stroke
Hepatitis B surface antigen	vaccine
monoclonal antibodies	various

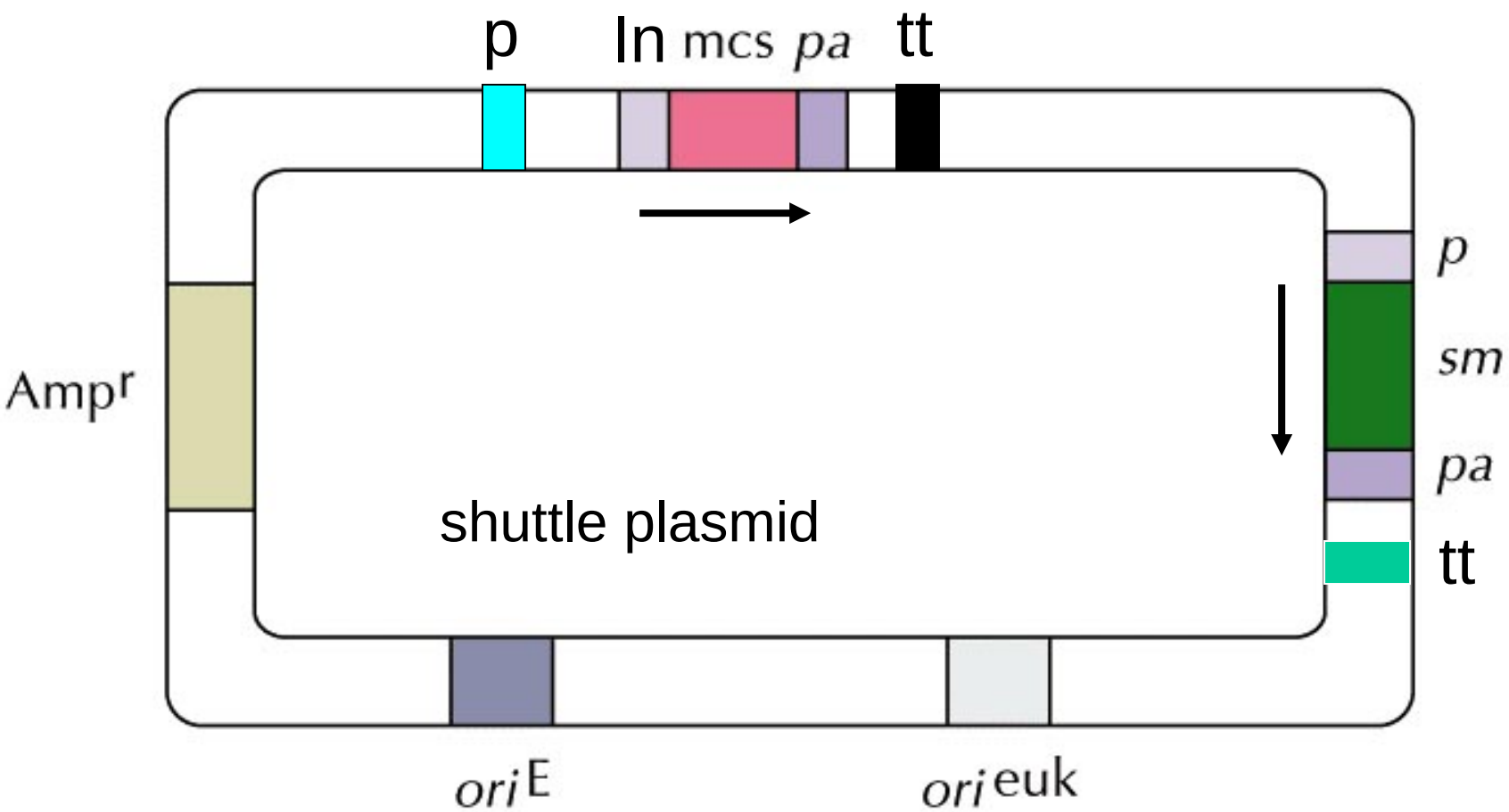
Eukaryotic Expression Vectors

- Same sorts of genetic features
  - selectable marker (for eukaryotic cell)
  - eukaryotic promoter
  - mRNA polyadenylation signal
  - ori of replication (if plasmid based)
  - chromosomal DNA segment for homologous recombination into host Chr

Eukaryotic Expression Vectors

- They have genetic features for selection and maintenance in E.coli cells
    - ori of replication
    - selectable marker

Generalized Mammalian Expression Vector



Eukaryotic Expression Vector

- **Ori of replication**
  - generally derived from animal virus (SV40)
- **Promoters**
  - generally derived from animal viruses or from highly expressed mammalian genes
  - SV40, cytomegalovirus (CMV), herpes simplex virus (HSV), etc,

Translation control elements

Transcribed Region of Gene

1 AUG (Kozak sequence = CCRCCAUGG)
2 Signal sequence for secretion
3 Affinity tag for purification
4 Proteolytic cleavage site

Euk. Selectable Markers

Agent	Action	Marker Gene
Xyl-A	DNA damage	Adenine deaminase
Blasticidin S	Inhibits protein syn.	BlasticidinS deaminase
G-418	**Inhibits protein syn.**	**Neomycin phosphotransferase**
MSX	Inhibits glutamine syn.	Glutamine synthetase
MTX	**Inhibits DNA syn.**	**Dihydrofolate reductase**

and others

Eukaryotic Selectable Markers

- **G418 (Geneticin)**
  - blocks translation
  - neomycin phosphotransferase confers resistance (Neo^r)

Eukaryotic Selectable Markers

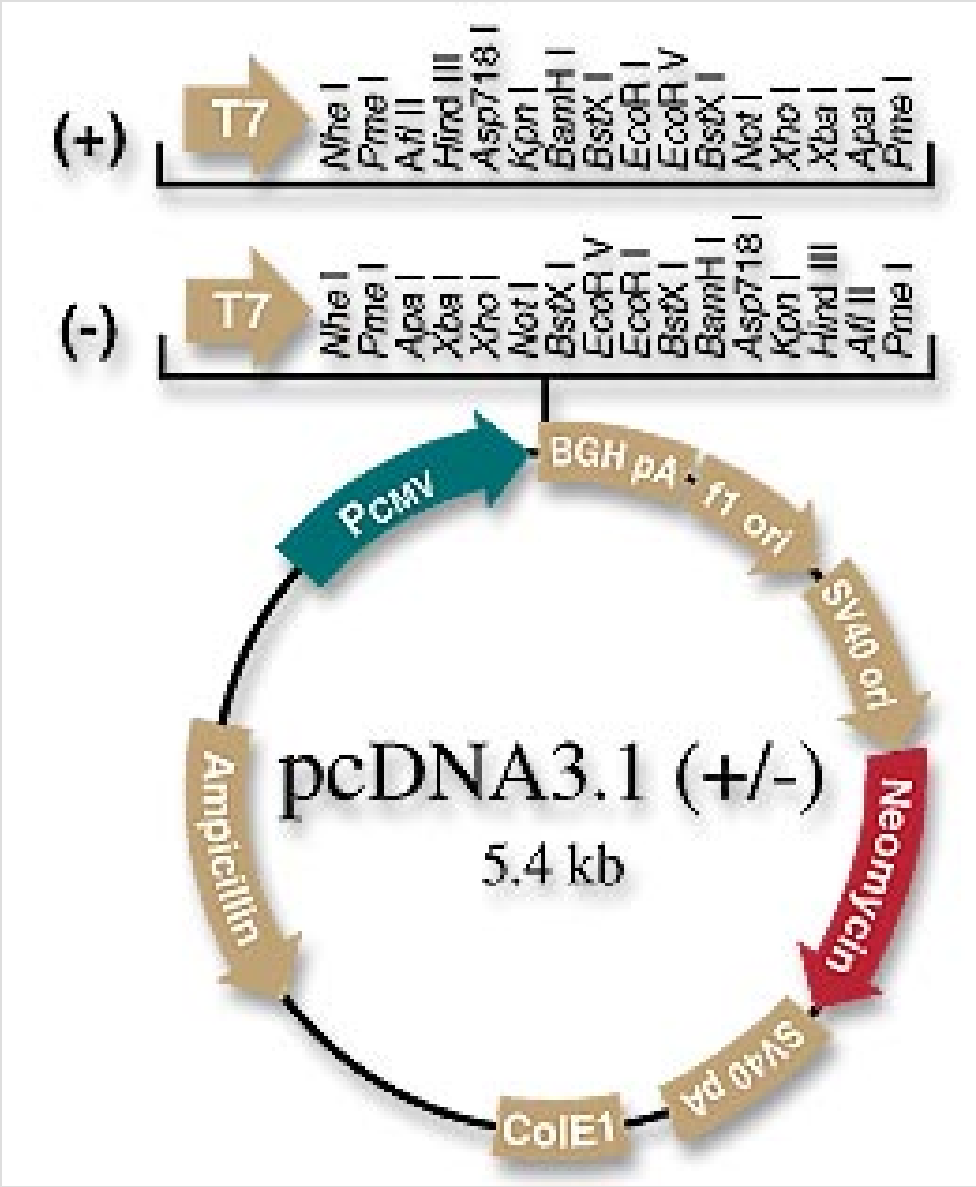
- **Methotrexate (MTX)**
  - kills cells which lack dihydrofolate reductase
  - host cell line made $DHFR^-$
  - vector carries a functional DHFR gene

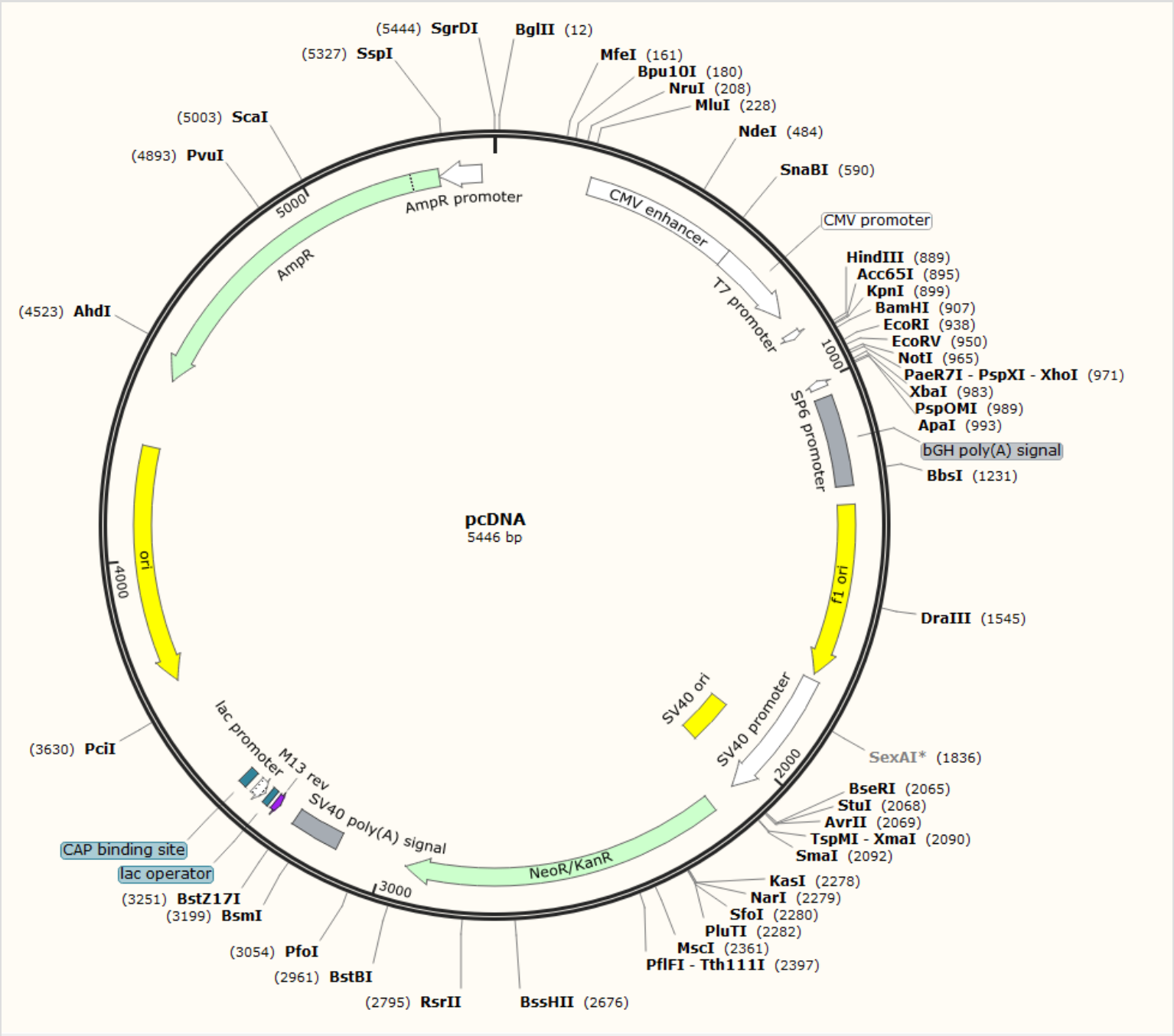
Proteins can be also expressed as fusion protein as described before

Fusion partner	Size	Ligand	Elution condition
ZZ	14 kDa	IgG	Low pH
His tail	6–10 aa	Ni^{2+}	Imidazole
Strep-tag	10 aa	Streptavidin	Iminobiotin
PinPoint	13 kDa	Streptavidin	Biotin
MBP	40 kDa	Amylose	Maltose
β-Lactamase	27 kDa	Phenyl-boronate	Borate
GST	25 kDa	Glutathione	Reducing agent
Flag	8 aa	Specific MAb	Low calcium

Adapted from Nygren et al., 1994, *Trends Biotechnol.* **12:**184–188.

Abbreviations: aa, amino acids; kDa, kilodaltons; ZZ, a fragment of *Staphylococcus aureus* protein A; His, histidine; Strep-tag, a peptide with affinity for streptavidin; PinPoint, a protein fragment which is biotinylated in vivo in *E. coli*; MBP, maltose binding protein; GST, glutathione *S*-transferase; Flag, a peptide recognized by enterokinase.


(+)
T7
Nhe I
Pme I
Afl II
Hind III
Asp718 I
Kpn I
BamH I
BstX I
EcoR I
EcoR V
BstX I
Not I
Xho I
Xba I
Apa I
Pme I
(-)
T7
Nhe I
Pme I
Apa I
Xba I
Xho I
Not I
BstX I
EcoR V
EcoR I
BstX I
BamH I
Asp718 I
Kpn I
Hind III
Afl II
Pme I
PCMV
BGH pA
f1 ori
SV40 ori
Neomycin
SV40 pA
ColE1
Ampicillin
pcDNA3.1 (+/-)
5.4 kb


pcDNA
5446 bp
(5444) SgrDI
BglII (12)
(5327) SspI
MfeI (161)
Bpu10I (180)
NruI (208)
MluI (228)
(5003) ScaI
NdeI (484)
(4893) PvuI
SnaBI (590)
5000
AmpR promoter
CMV enhancer
CMV promoter
AmpR
T7 promoter
HindIII (889)
Acc65I (895)
KpnI (899)
BamHI (907)
(4523) AhdI
EcoRI (938)
EcoRV (950)
1000
NotI (965)
PaeR7I - PspXI - XhoI (971)
XbaI (983)
SP6 promoter
PspOMI (989)
ApaI (993)
bGH poly(A) signal
BbsI (1231)
ori
4000
f1 ori
DraIII (1545)
SV40 ori
SV40 promoter
(3630) PciI
lac promoter
M13 rev
SexAI* (1836)
2000
BseRI (2065)
StuI (2068)
AvrII (2069)
SV40 poly(A) signal
TspMI - XmaI (2090)
SmaI (2092)
CAP binding site
NeoR/KanR
KasI (2278)
lac operator
NarI (2279)
(3251) BstZ17I
SfoI (2280)
(3199) BsmI
3000
PluTI (2282)
MscI (2361)
(3054) PfoI
PflFI - Tth111I (2397)
(2961) BstBI
(2795) RsrII
BssHII (2676)

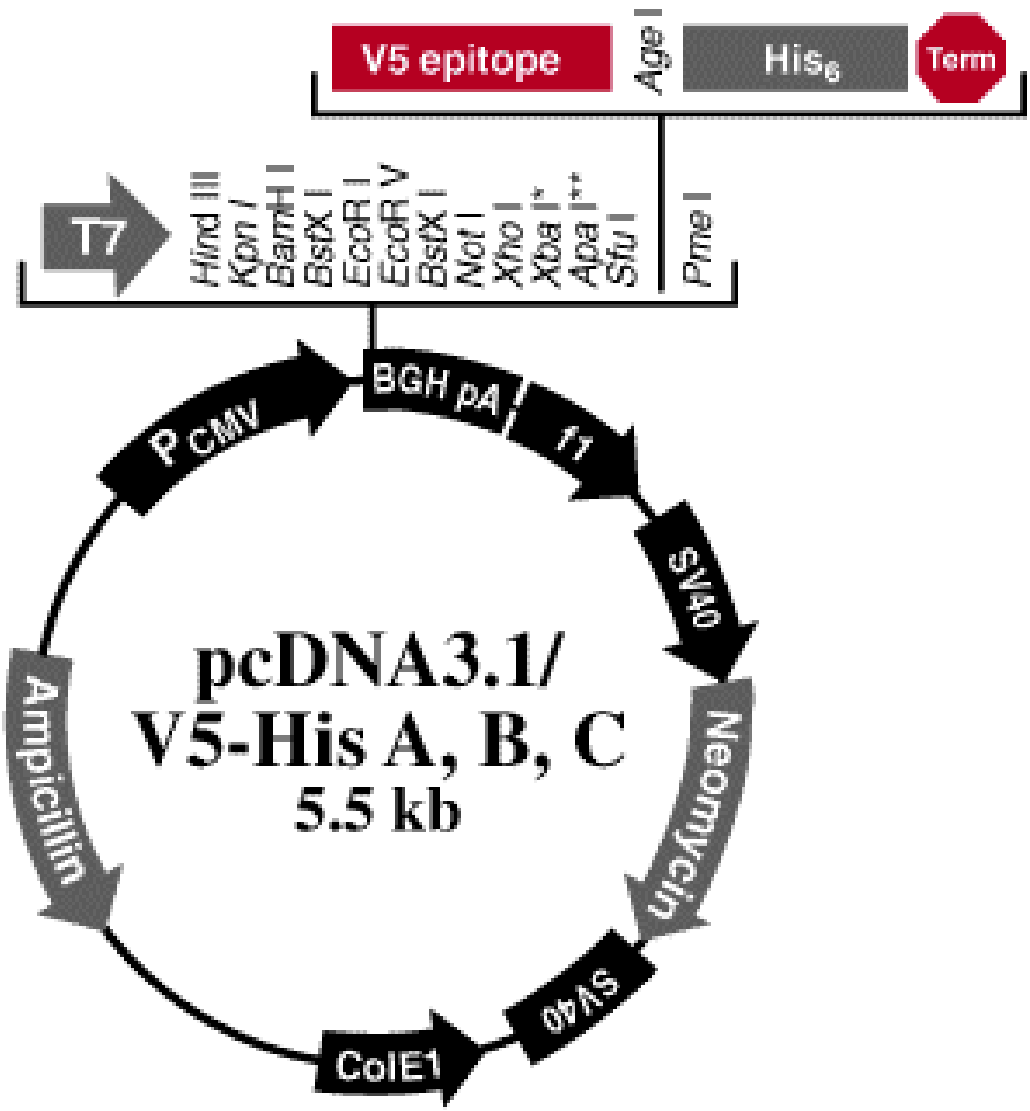
Comments for pcDNA3.1/V5-His A

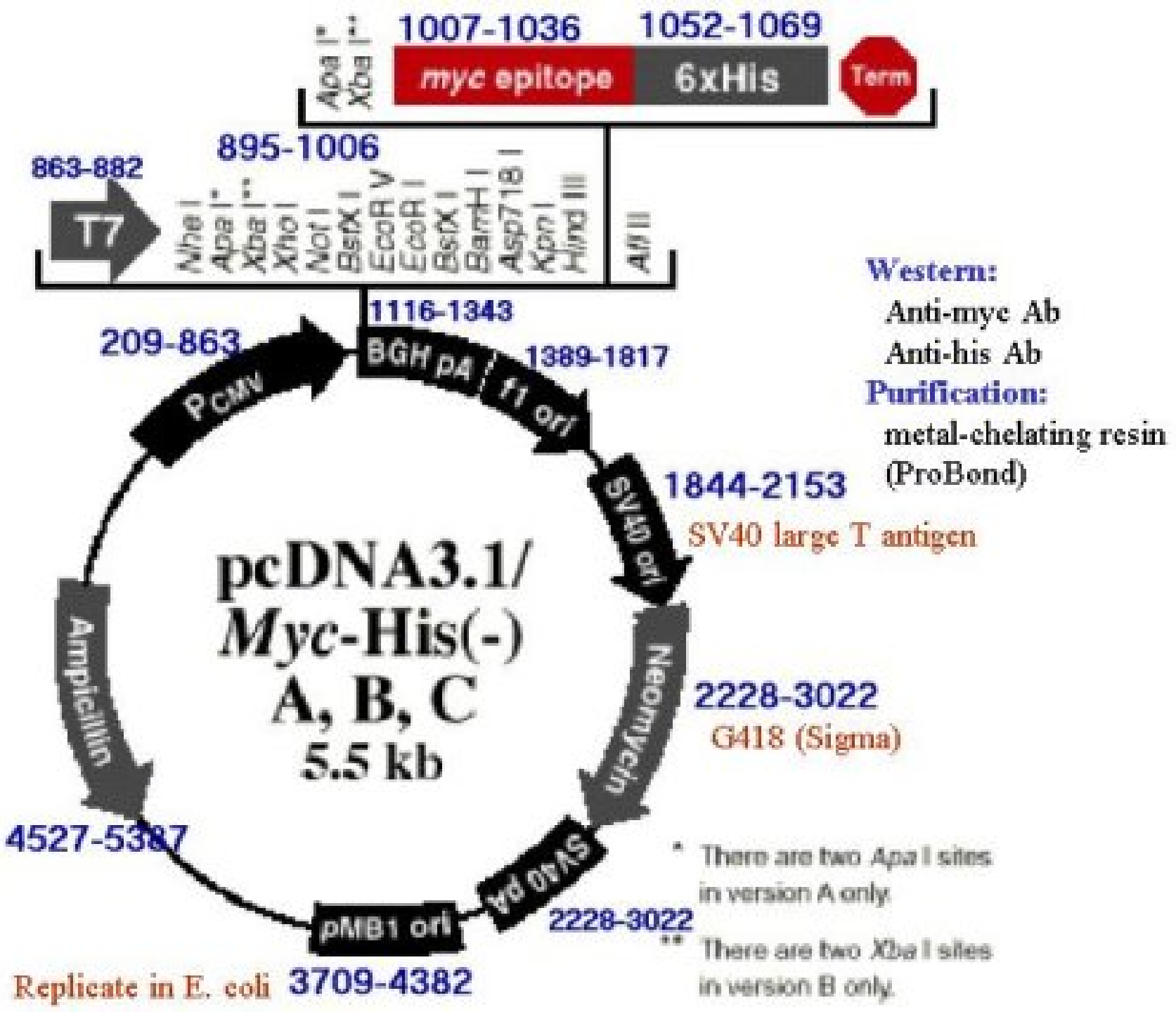
5503 nucleotides

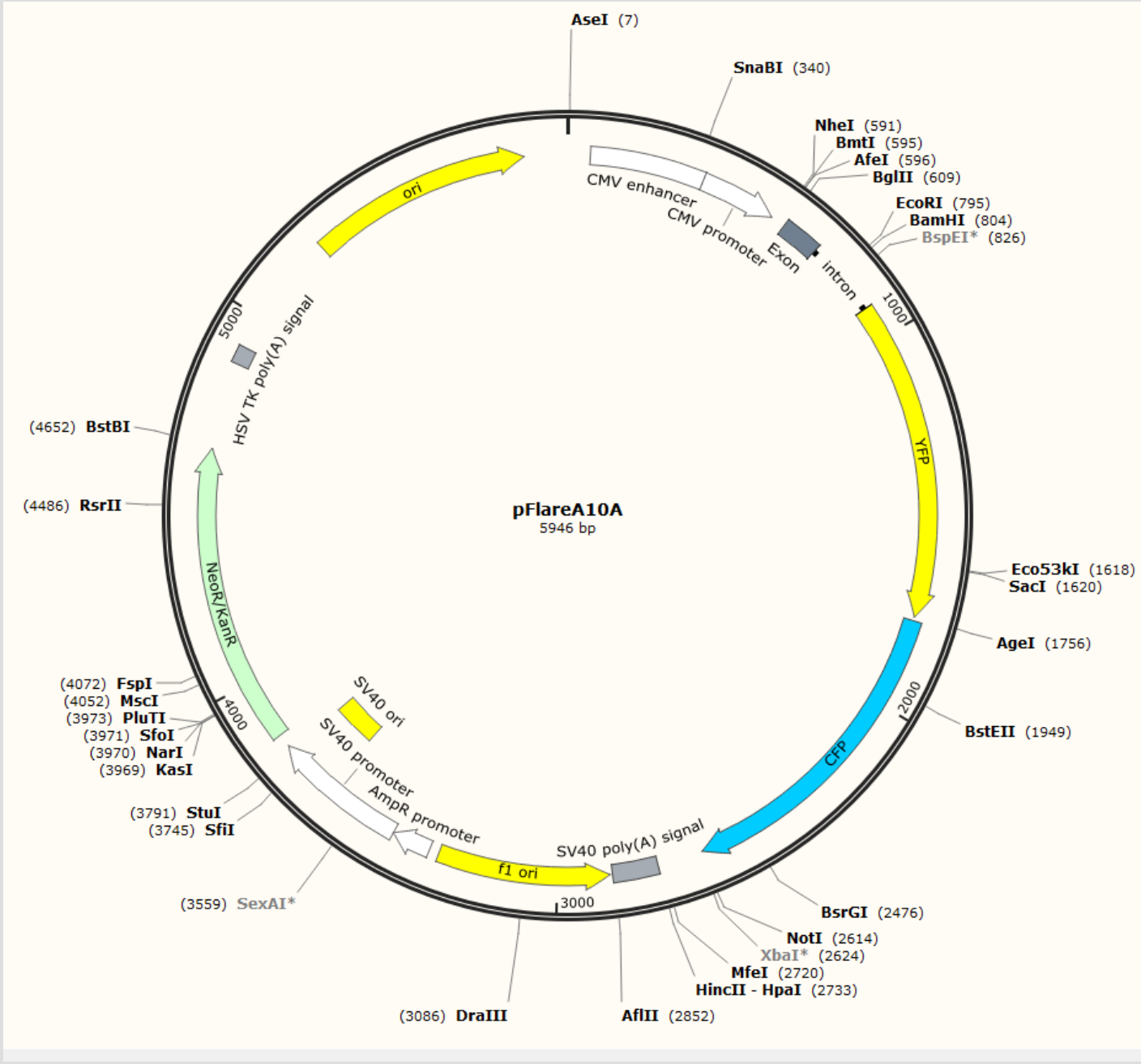
CMV promoter: bases 209-863
T7 promoter/priming site: bases 863-882
Multiple cloning site: bases 902-999
V5 epitope: bases 1000-1041
Polyhistidine tag: bases 1051-1068
pcDNA3.1/BGH reverse priming site: bases 1091-1108
BGH polyadenylation signal: bases 1090-1304
f1 origin of replication: bases 1357-1780
SV40 promoter and origin: bases 1845-2170
Neomycin resistance gene: bases 2206-3000
SV40 polyadenylation signal: bases 3019-3257
ColE1 origin: bases 3689-4362
Ampicillin resistance gene: bases 4507-5367

* After the *Xho* I site, there is a unique *BstE* II site, but no *Xba* I or *Apa* I sites in version C.

** There is a unique *Sac* II site between the *Apa* I site and the *Sfu* I site in version B only.




1007-1036
1052-1069
Apa I *
Xba I **
myc epitope
6xHis
Term
895-1006
863-882
T7
Nhe I
Apa I *
Xba I **
Xho I
Not I
BstX I
EcoR V
EcoR I
BstX I
BamH I
Asp718 I
Kpn I
Hind III
Afl II
1116-1343
209-863
P CMV
BGH pA
f1 ori
1389-1817
SV40 ori
1844-2153
SV40 large T antigen
pcDNA3.1/
Myc-His(-)
A, B, C
5.5 kb
Ampicillin
Neomycin
2228-3022
G418 (Sigma)
4527-5387
SV40 pA
2228-3022
pMB1 ori
Replicate in E. coli
3709-4382
* There are two Apa I sites in version A only.
** There are two Xba I sites in version B only.
Western:
Anti-myc Ab
Anti-his Ab
Purification:
metal-chelating resin
(ProBond)


AseI (7)
SnaBI (340)
NheI (591)
BmtI (595)
AfeI (596)
BglII (609)
EcoRI (795)
BamHI (804)
BspEI* (826)
1000
YFP
Eco53kI (1618)
SacI (1620)
AgeI (1756)
2000
CFP
BstEII (1949)
BsrGI (2476)
NotI (2614)
XbaI* (2624)
MfeI (2720)
HincII - HpaI (2733)
AflII (2852)
3000
SV40 poly(A) signal
f1 ori
SV40 promoter
AmpR promoter
SV40 ori
(3559) SexAI*
(3791) StuI
(3745) SfiI
4000
NeoR/KanR
(4072) FspI
(4052) MscI
(3973) PluTI
(3971) SfoI
(3970) NarI
(3969) KasI
(4486) RsrII
(4652) BstBI
5000
HSV TK poly(A) signal
ori
CMV enhancer
CMV promoter
Exon
intron
(3086) DraIII
pFlareA10A
5946 bp

Transfection of Euk. cells

- **Introduction of DNA into prokaryotic (and yeast) is cells termed Transformation**
- **Transformation of animal cells refers to changes in growth characteristics of cells in culture**
- **Transfection is term used for uptake of foreign DNA into eukaryotic cells resulting in inherited change**

METODI DI TRASFEZIONE

- CARATTERISTICHE:
  - Elevata efficienza
  - Bassa tossicità
  - Riproducibilità *in vitro* e *in vivo*
- PROCESSO:
  - Introduzione del DNA nella cellula
  - Ottenimento dell'espressione del gene d'interesse
  - (selezione delle cellule che si sono trasfettate stabilmente)
  - Caratterizzazione del gene/ proteina prodotta
- PROBLEMATICHE:
  - Come superare le barriere naturali???
    - DNA: fortemente POLARE (carica negativa)
    - MEMBRANA CELLULARE LIPOFILA

METODI DI TRASFEZIONE

1) METODI CHIMICI

2) METODI FISICI

2

Factors Affecting Transfection

Host Cell

Cell health

Cell culture

Genetic Material

DNA quality and quantity

Cell Health

- Cells should be grown in appropriate medium with all necessary factors
- Cultures must be free of contamination
- Fresh medium must be used if it contains chemically unstable components, such as thiamine
- Cells should be incubated at 37°C with CO_2 supplied at the correct percentage (5–10%) and 100% relative humidity
- Cells should be maintained in log phase growth

Cell Culture

Confluency and Growth Phase

- Cells should be transfected at 40–80% confluency (cell type dependent)
  - Too few cells cause cell cultures to grow poorly without cell-to-cell contact
  - Too many cells result in contact inhibition, making cells resistant to uptake of DNA and other macromolecules
- Actively dividing cells take up DNA better than quiescent cells (breakdown and perforation of the nuclear membrane during mitosis enable nuclear delivery)

Number of Passages for Primary Cells

- The number of passages should be low (<50)
- The number of passages for cells used in a variety of experiments should be consistent
- Cell characteristics can change over time with immortalized cell lines and cells may not respond to the same transfection conditions
- Cells may not respond to the same transfection conditions after repeated passages

DNA Quality and Quantity

- Use high-quality plasmid DNA that is free of proteins, RNA, and chemicals for transfections; endotoxin removal should be part of the preparation procedure
- Typically, DNA is suspended in sterile water or TE buffer to a final concentration of 0.2–1 mg/ml
- The optimal amount of DNA to use in the transfection will vary widely depending upon the type of DNA, transfection reagent/method, target cell line, and number of cells

4

Common Transfection Methods

Reagent-Based Methods

Lipids

Calcium phosphate

Cationic polymers

DEAE-dextran

Activated dendrimers

Magnetic beads

Instrument-Based Methods

Electroporation

Biolistic technology

Microinjection

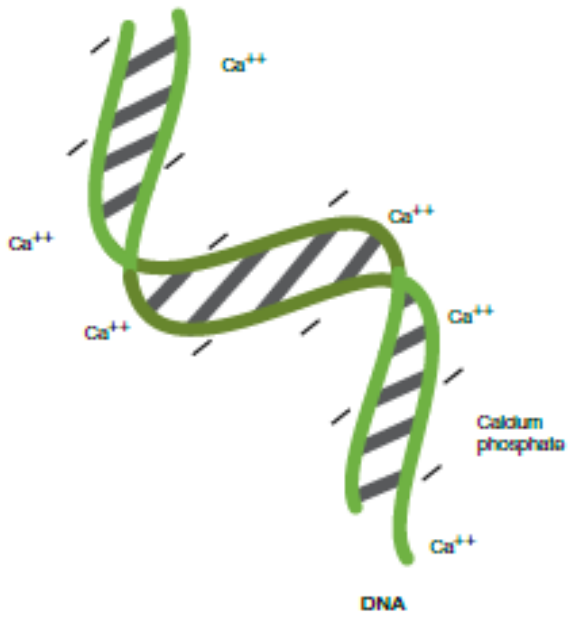
Laserfection/optoinjection

Virus-Based Methods

Calcium Phosphate

The protocol involves mixing DNA with calcium chloride, adding this in a controlled manner to a buffered saline/ phosphate solution, and allowing the mixture to incubate at room temperature.

This step generates a precipitate that is dispersed onto the cultured cells. The precipitate is taken up by the cells via endocytosis or phagocytosis.



Method Overview

Solution A: DNA in calcium solution

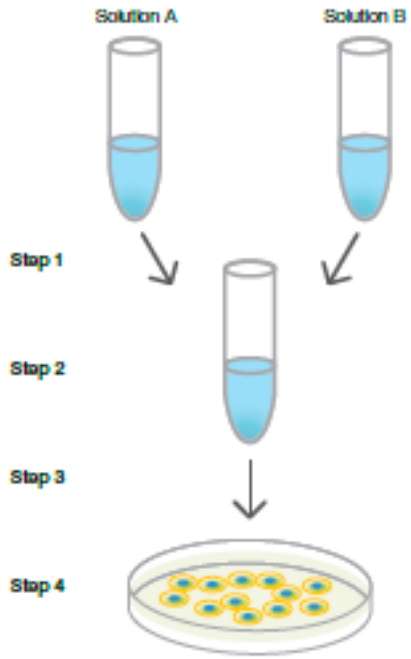
Solution B: 2x Hanks buffered saline solution

1 Add solution A to solution B while vortexing.

2 Incubate 20–30 min. Apply the solution to a subconfluent cell culture.

3 Incubate 2–12 hr. Replace the solution with complete growth medium.

4 Assay for transient gene expression or begin selection for stable transformation line.



Pros and Cons

Advantages of Calcium Phosphate

- Inexpensive
- High efficiency (cell type dependent)
- Can be applied to a wide range of cell types
- Can be used for transient and stable transfection

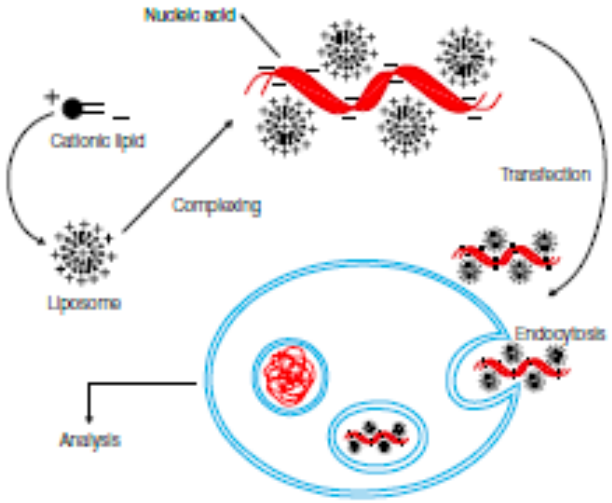
Disadvantages of Calcium Phosphate

- Reagent consistency is critical for reproducibility
- Small pH changes (±0.1) can compromise transformation efficiency
- Size and quality of the precipitate are crucial to the success of transfection
- Calcium phosphate precipitation does not work in RPMI, due to the high concentration of phosphate within the medium

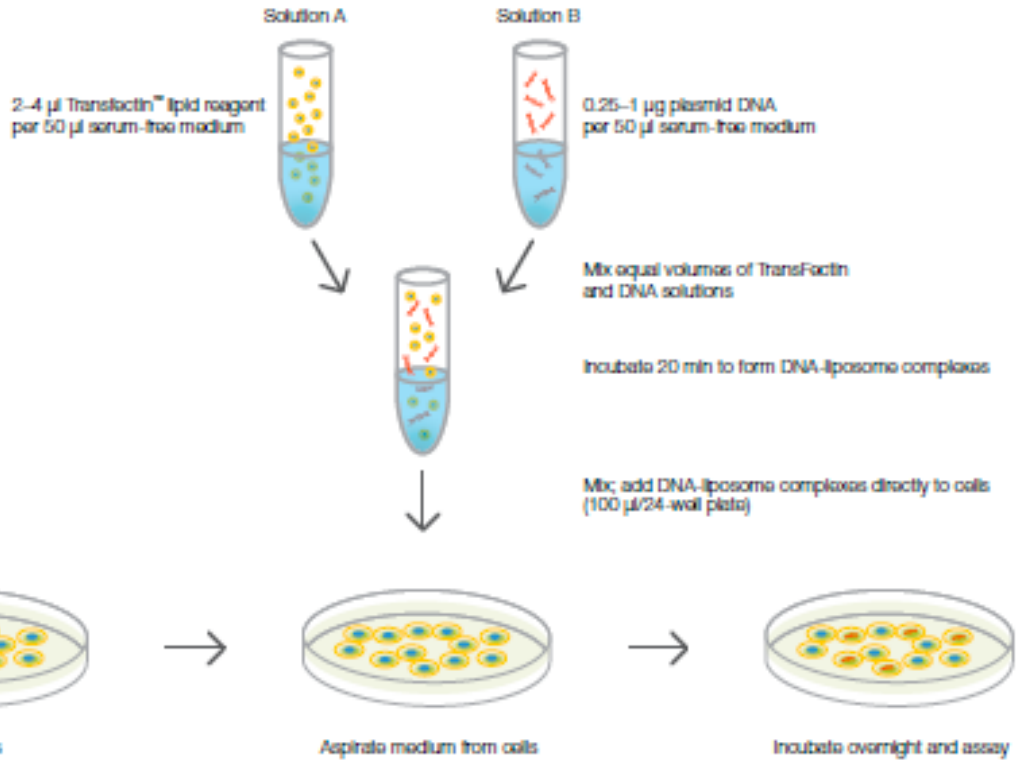
Lipid-Mediated Gene Delivery

Lipid-mediated gene delivery is also referred to as lipofection, or liposome-based gene transfection. It uses lipids to cause a cell to absorb exogenous DNA.

Transfer of genetic material into the cell takes place via liposomes, which are vesicles that can merge with the cell membrane since they are both made of a phospholipid bilayer.



Method Overview



Pros and Cons

Advantages of Lipids

- Deliver nucleic acids to cells in a culture dish with high efficiency
- Easy to use, minimal steps required; adaptable to high-throughput systems
- Using a highly active lipid will reduce the cost of lipid and nucleic acid, and achieve effective results

Disadvantage of Lipids

- Not applicable to all cell types

Cationic Polymers

Cationic polymers differ from cationic lipids in that they do not contain a hydrophobic moiety and are completely soluble in water. Given their polymeric nature, cationic polymers can be synthesized in different lengths, with different geometry (linear versus branched). The most striking difference between cationic lipids and cationic polymers is the ability of the cationic polymers to more efficiently condense DNA.

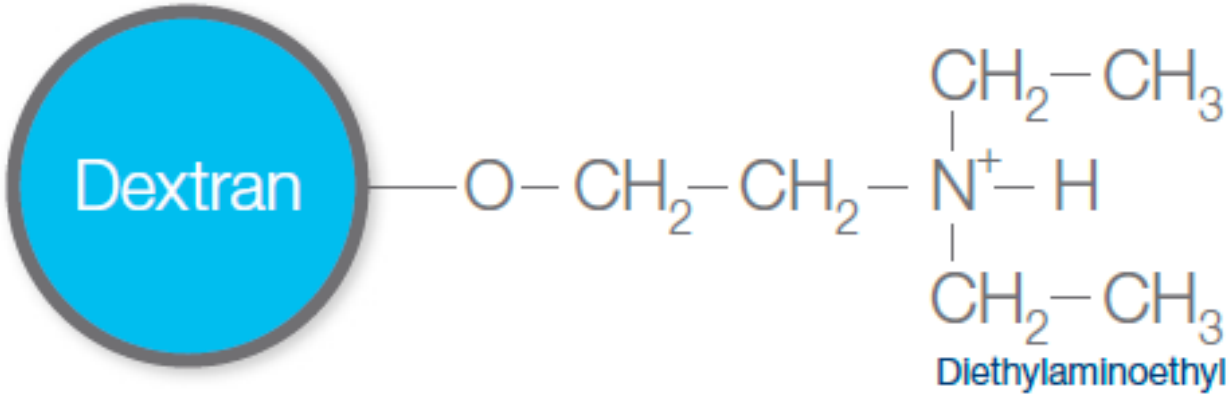
There are three general types of cationic polymers used in transfections:

- Linear (histone, spermine, and polylysine)
- Branched
- Spherical

Cationic polymers include polyethyleneimine (PEI) and dendrimers.

DEAE-Dextran

DEAE-dextran is a cationic polymer that tightly associates with negatively charged nucleic acids. The positively charged DNA:polymer complex comes into close association with the negatively charged cell membrane. DNA:polymer complex uptake into the cell is presumed to occur via endocytosis or macropinocytosis.



Method Overview

Solution A: DNA (~1–5 µg/ml) diluted into 2 ml of growth medium with serum containing chloroquine

Solution B: DEAE-dextran solution (~50–500 µg/ml)

Solution C: ~5 ml of DMSO

Solution D: Complete growth medium

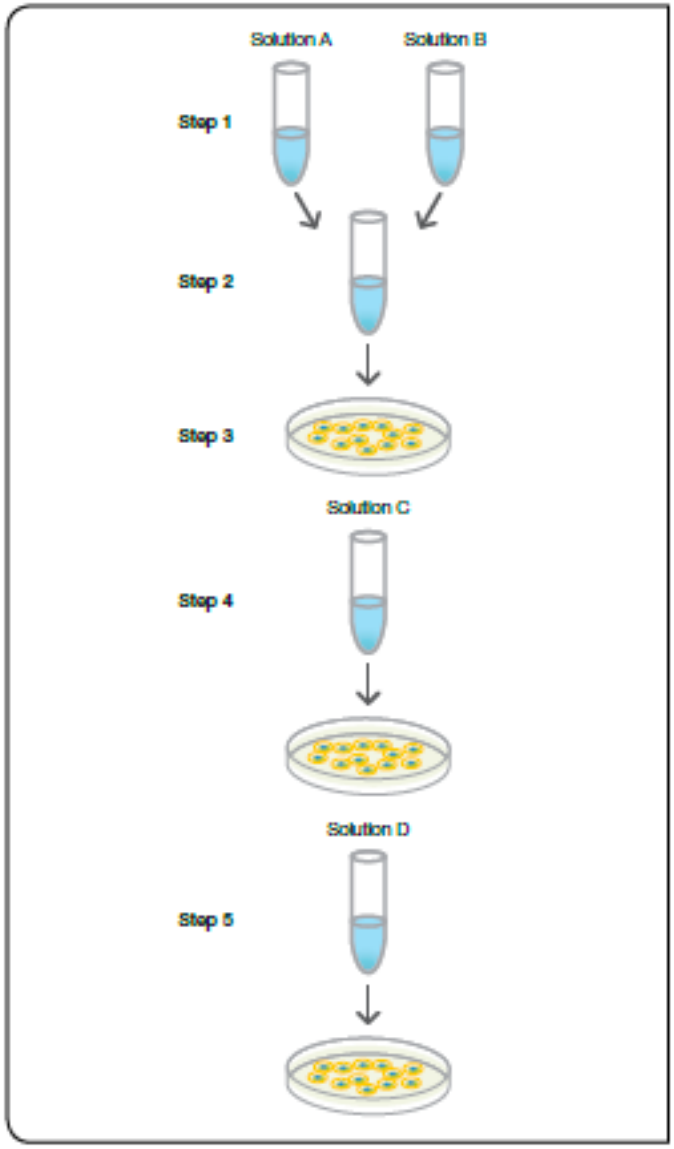
1 Add solution A to solution B, then mix gently.

2 Aspirate cell medium and apply the mixed A and B solutions to the subconfluent cell culture. Incubate the DNA mixture for ~4 hr.

3 Aspirate supernatant.

4 Add solution C to induce DNA uptake.

5 Remove DMSO and replace with complete growth medium; assay for transient gene expression.



Pros and Cons

Advantages of DEAE-Dextran

- Inexpensive
- Easy to perform and quick
- Can be applied to a wide range of cell types

Disadvantages of DEAE-Dextran

- High concentrations of DEAE-dextran can be toxic to cells
- Transfection efficiencies will vary with cell type
- Can be used only for transient transfection
- Typically produces less than 10% delivery in primary cells

Magnet-Mediated Transfection

Magnet-mediated transfection uses magnetic force to deliver DNA into target cells.

Nucleic acids are first associated with magnetic nanoparticles. Then, application of magnetic force drives the nucleic acid–particle complexes toward and into the target cells, where the cargo is released.

Method Overview

1 Dilute nucleic acid in medium.

2 Add magnetic nanoparticle.

3 Incubate 10–20 min.

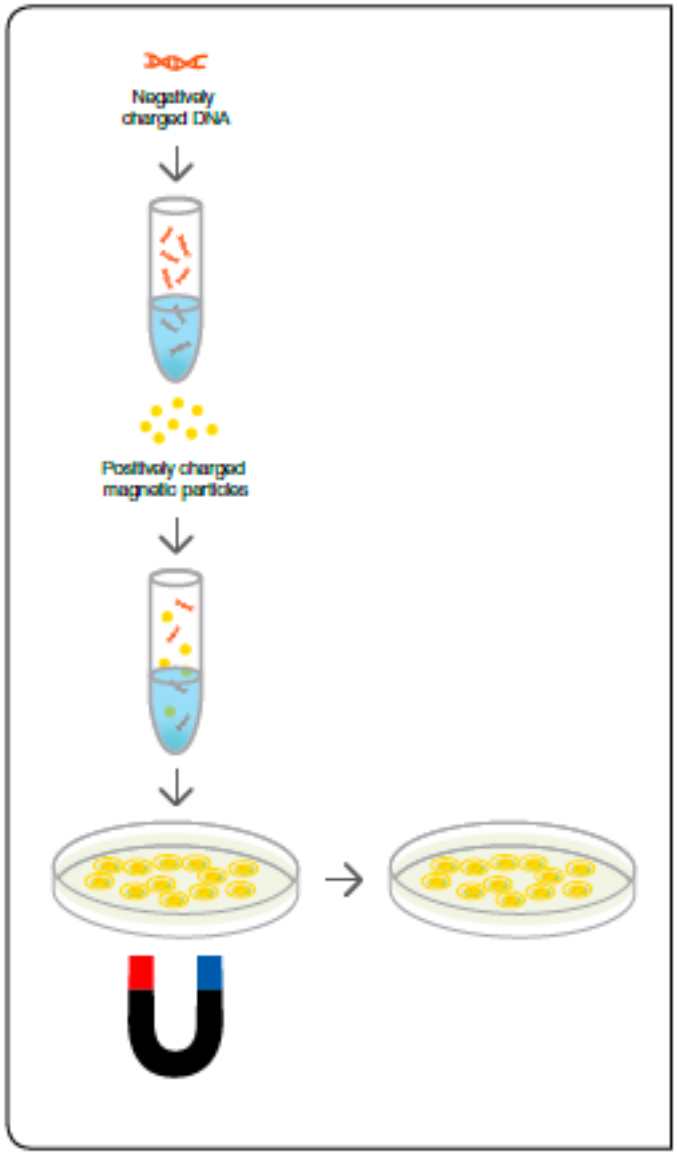
4 Add medium to adherent cells (2–4 x 10^5 cells).

5 Add nucleic acid/nanoparticle solution.

6 Place culture plate on magnet plate.

7 Incubate 15 min.

8 Remove magnet plate.



Pros and Cons

Advantages of Magnetic Beads

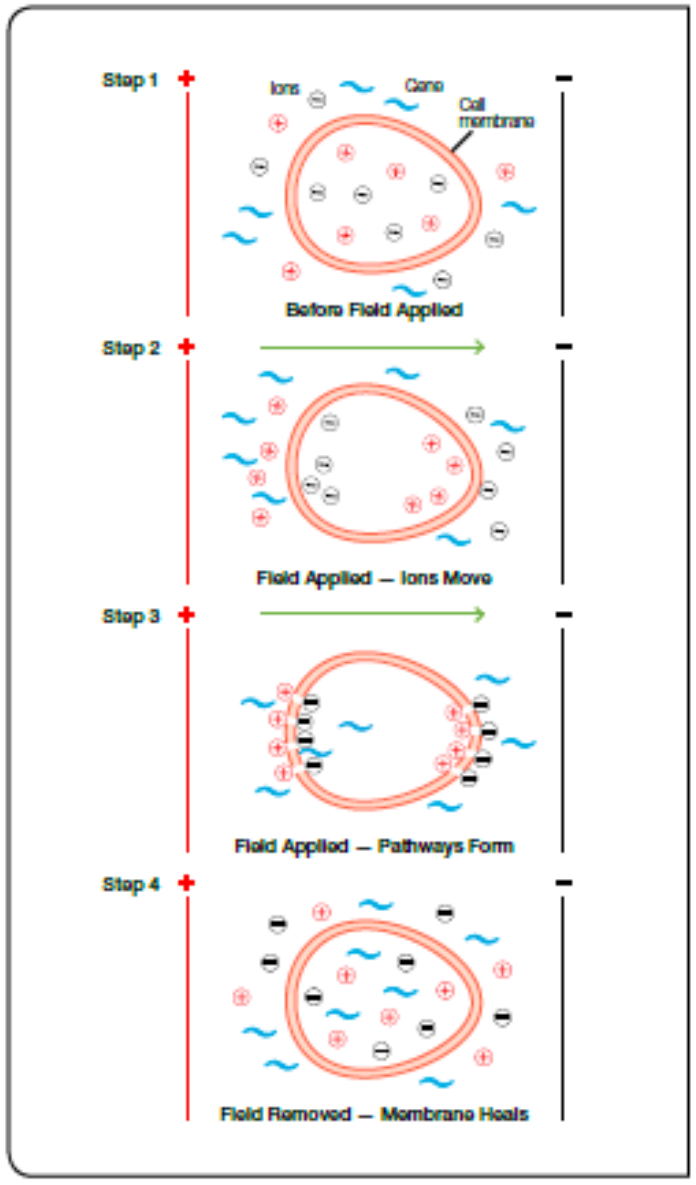
- Rapid
- Increased transfection efficiency by the directed transport, especially for low amounts of nucleic acids
- High transfection rates for adherent mammalian cell lines and primary cell cultures (suspension cells and cells from other organisms also successfully transfected but need to be immobilized)
- Mild treatment of cells
- Can also be performed in the presence of serum

Disadvantages of Magnetic Beads

- Relatively new method
- Requires adherent cells; suspension cells need to be immobilized or centrifuged

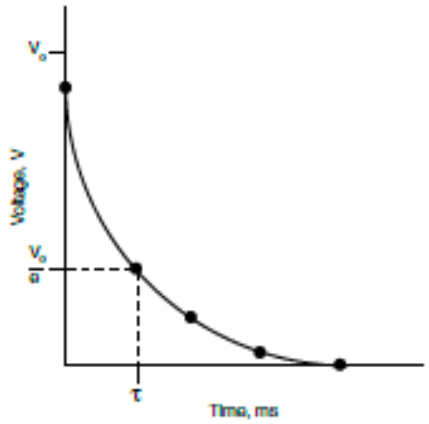
How Electroporation Works

1. Electroporation exposes a cell to a high-intensity electric field that temporarily destabilizes the membrane.
2. During this time the membrane is highly permeable to exogenous molecules present in the surrounding media.
3. DNA then moves into the cell through these holes.
4. When the field is turned off, the pores in the membrane reseal, enclosing the DNA inside.



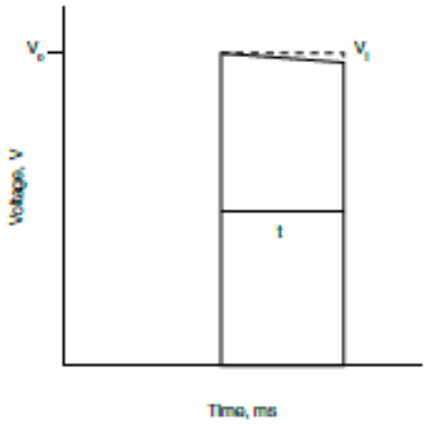
Type of Electrical Pulse — the Waveform

The most common electrical fields are exponential and square waveforms. The waveform has a significant effect on the transfection efficiency for different cell types. Both exponential-decay and square-wave pulses have been used very effectively for electroporation.



Exponential Waveform

- Capacitors charged to a set voltage
- Set voltage is released from the selected capacitor and decays rapidly (exponentially) over time



Square Waveform

- Determined by pulse duration and/or number of pulses
- Several short pulses may be more beneficial than one long pulse

Pros and Cons

Advantages of Electroporation

- Nonchemical method that doesn't seem to alter the biological structure or function of the target cells
- Easy to perform
- High efficiency
- Can be applied to a wide range of cell types

Disadvantage of Electroporation

- Cell mortality (if using suboptimal conditions)

Biolistic Particle Delivery

Biolistic transformation is the delivery of nucleic acids into cells via high velocity nucleic acid–coated microparticles.

Helios® Gene Gun

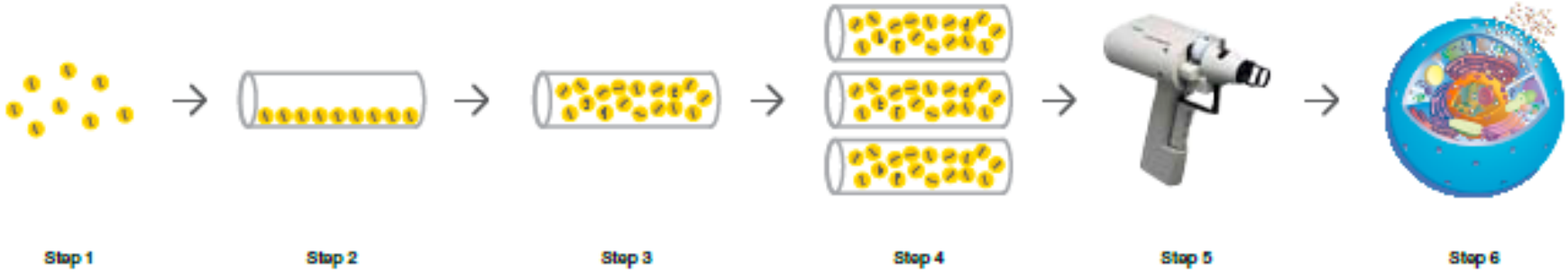
- For in situ, in vivo, and in vitro transformations
- Applications for animals, plants, cell culture, nematodes, yeast, and bacteria
- Pressure range 100–600 psi enables fine-tuning of penetration
- Highly portable — can be used in the field
- Small target area for accurate targeting

PDS-1000/He™ Biolistic Particle Delivery System

- For in vitro, ex vivo, and in vivo (for some plants and microbes) transformations
- Applications for animal cell and organ cultures, plant cell cultures and explants, pollen, insects, algae, fungi, and bacteria
- Pressure range 450–2,200 psi gives flexibility and penetration — ideal for plant applications
- Large target area — more cells can be transformed

Helios® Gene Gun — Process Overview

1. Precipitate DNA onto gold particles.
2. Load DNA/gold into tubing.
3. Rotate tubing to coat DNA gold over inside surface.
4. Cut tubing into cartridges.
5. Load cartridges into gene gun.
6. Deliver DNA into target cells.



PDS-1000/He™ System — Process Overview

1. DNA-coated gold particles (microcarrier) are spread over the central area of a thin plastic disk (macrocarrier).
2. Disk loaded with the DNA-gold particles is placed into a holder inside the PDS-1000/He system.
3. The system uses high-pressure helium, released by a rupture disk, and partial vacuum to propel the macrocarrier loaded with microcarrier toward the target cells.
4. Macrocarrier is stopped after a short distance by a stopping screen.
5. DNA-coated gold particles continue travelling toward the target to penetrate the cells.
6. Sample chamber is subjected to partial vacuum, from 15 to 29 inches of mercury, depending on the target cells.

Pros and Cons

Advantages of Biolistic Technology

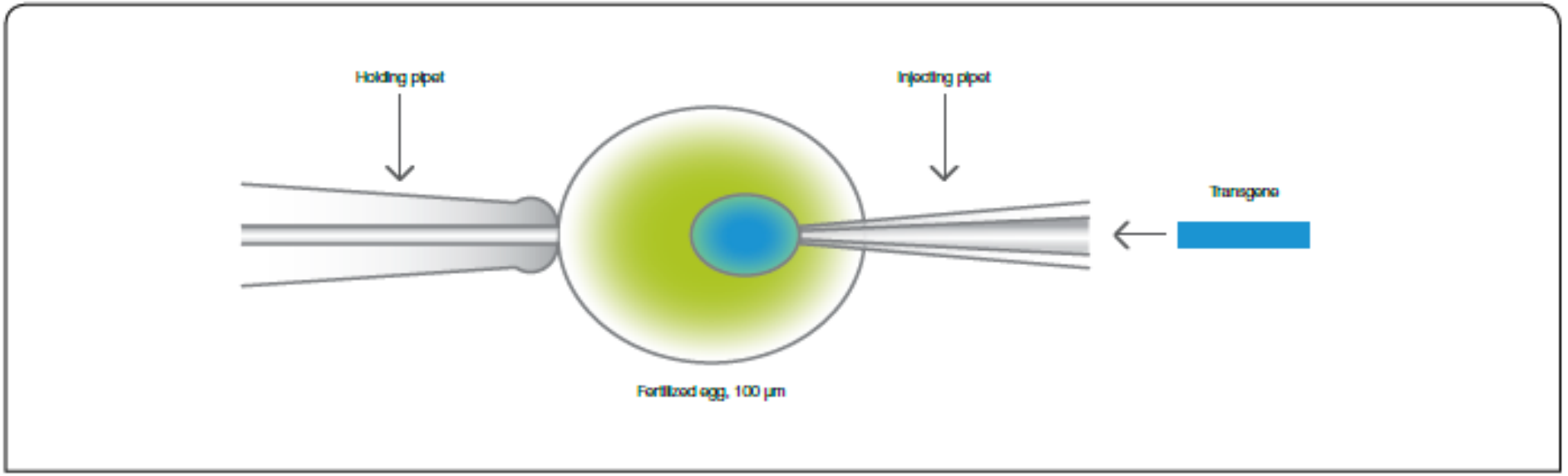
- Simple, rapid, versatile technique
- Targeted intracellular gene delivery
- Cell type independent
- Uses small amounts of DNA
- Delivers single or multiple genes
- No carrier DNA needed
- Can deliver large DNA fragments
- No extraneous genes or proteins delivered
- Requires little manipulation of cells
- High reproducibility

Disadvantages of Biolistic Technology

- Generally lower efficiency compared to electroporation or viral- or lipid-mediated transfection
- Limited bacterial transfection data
- Preparation of microparticles
- Instrument cost
- Requires purchase agreement

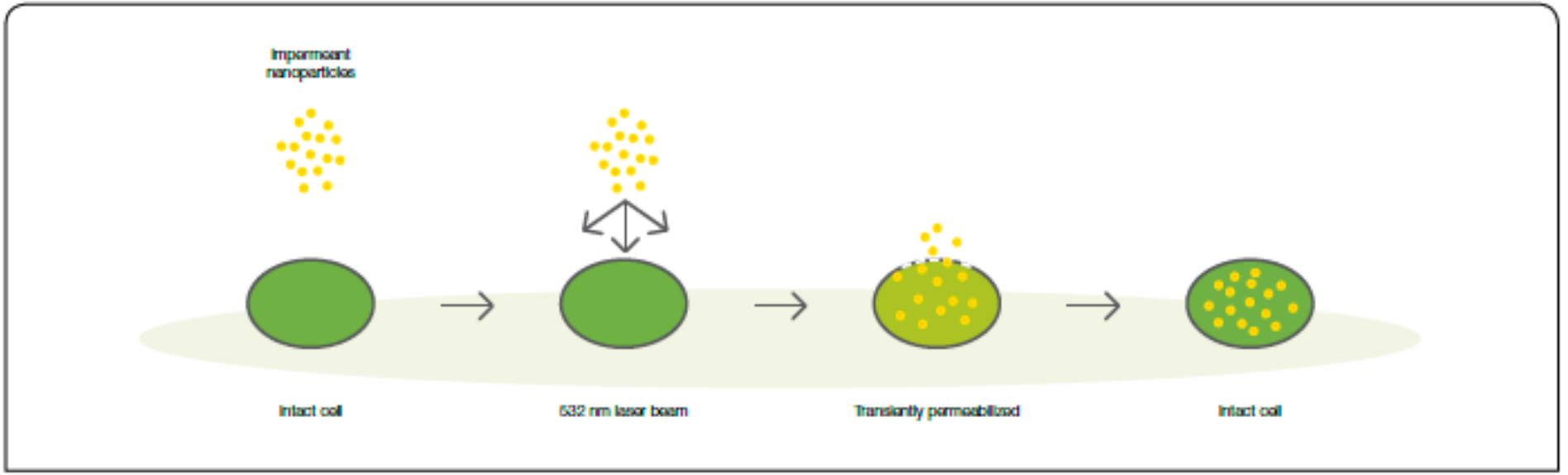
Microinjection

- Direct injection of naked DNA
- Laborious (one cell at a time)
- Technically demanding and costly
- Can be used for many animals



Laserfection/Optoinjection

- This procedure uses laser light to transiently permeabilize a large number of cells in a very short time
- Various substances can be efficiently optoinjected, including ions, small molecules, dextrans, short interfering RNAs (siRNAs), plasmids, proteins, and semiconductor nanocrystals, into numerous cell types
- Advantages: very efficient; works with many cell types; fewer cell manipulations needed
- Disadvantages: requires the cells to be attached; expensive laser-based equipment needed



Viral Vectors

Retroviruses

- Murine leukemia virus (MuLV)
- Human immunodeficiency virus (HIV)
- Human T-cell lymphotropic virus (HTLV)

DNA Viruses

- Adenovirus
- Adeno-associated virus (AAV)
- Herpes simplex virus (HSV)

Retroviruses — a class of viruses that can create double-stranded DNA copies of their RNA genomes; these copies can be integrated into the chromosomes of host cells. HIV is a retrovirus.

Adenoviruses — a class of viruses with double-stranded DNA genomes that cause respiratory, intestinal, and eye infections in humans. The virus that causes the common cold is an adenovirus.

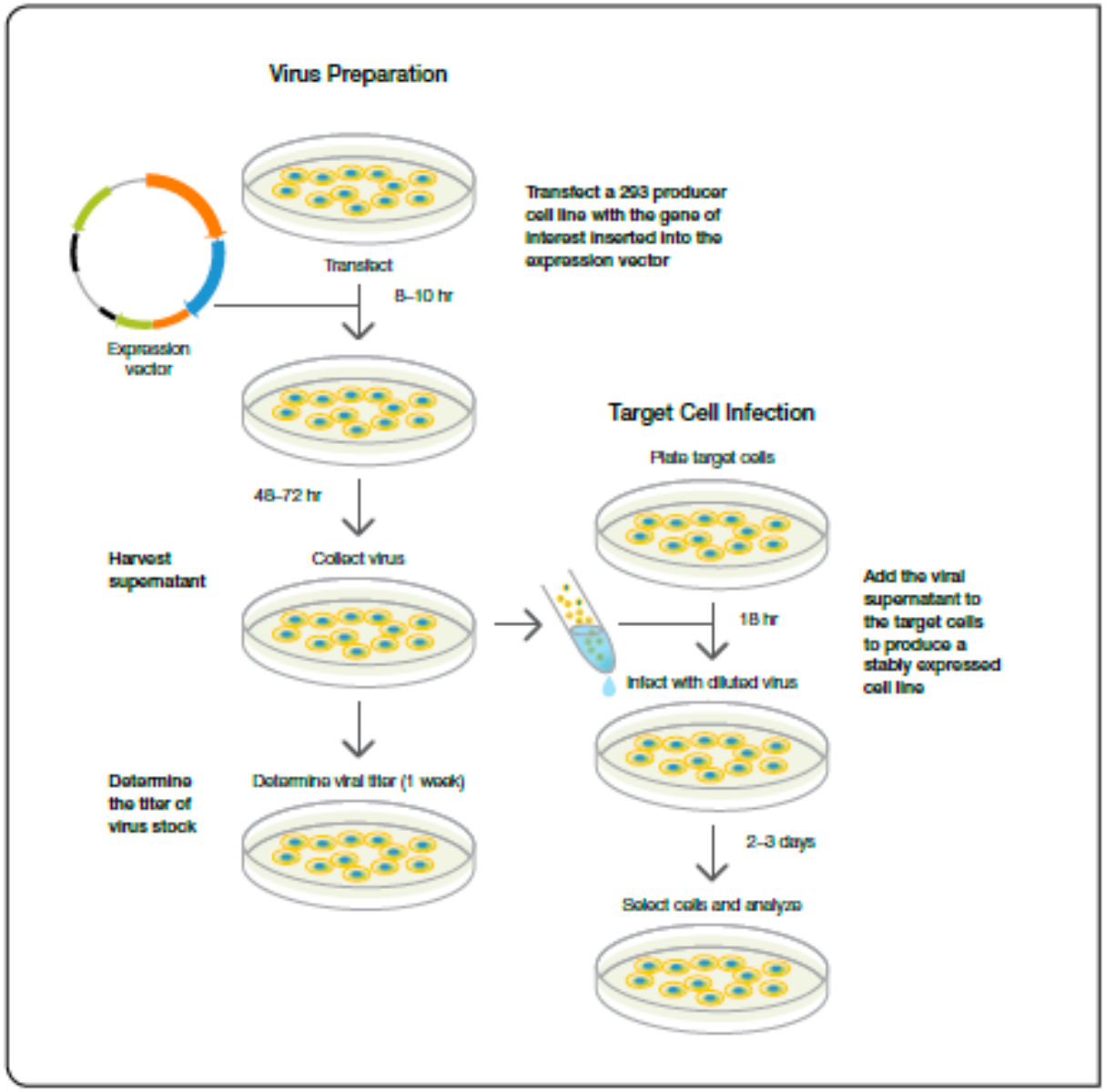
Adeno-associated viruses — a class of small, single-stranded DNA viruses that can insert their genetic material at a specific site on chromosome 19.

Herpes simplex viruses — a class of double-stranded DNA viruses that infect a particular cell type, neurons. Herpes simplex virus type 1 is a common human pathogen that causes cold sores.

Viral Attributes

Viral Vector	DNA Insert Size	Maximum Titer	Cell Type	Expression	Pitfalls
Retroviral	8 kb	1×10^9	Dividing cells	Stable	Random insertion site
Lentivirus	9 kb	1×10^9	Dividing cells Nondividing cells	Stable	Random insertion site
Adenovirus	8 kb	1×10^{13}	Dividing cells Nondividing cells	Transient	Highly immunogenic
Adeno-associated virus (AAV)	5 kb	1×10^{11}	Dividing cells Nondividing cells	Stable, site-specific location	Requires helper virus to grow; difficult to remove helper virus
Herpes simplex virus	30–40 kb	1×10^9	Dividing cells Nondividing cells	Transient	No gene expression during latent infection
Vaccinia virus	25 kb	3×10^9	Dividing cells	Transient	Potential cytopathic effects

Viral Workflow



Pros and Cons

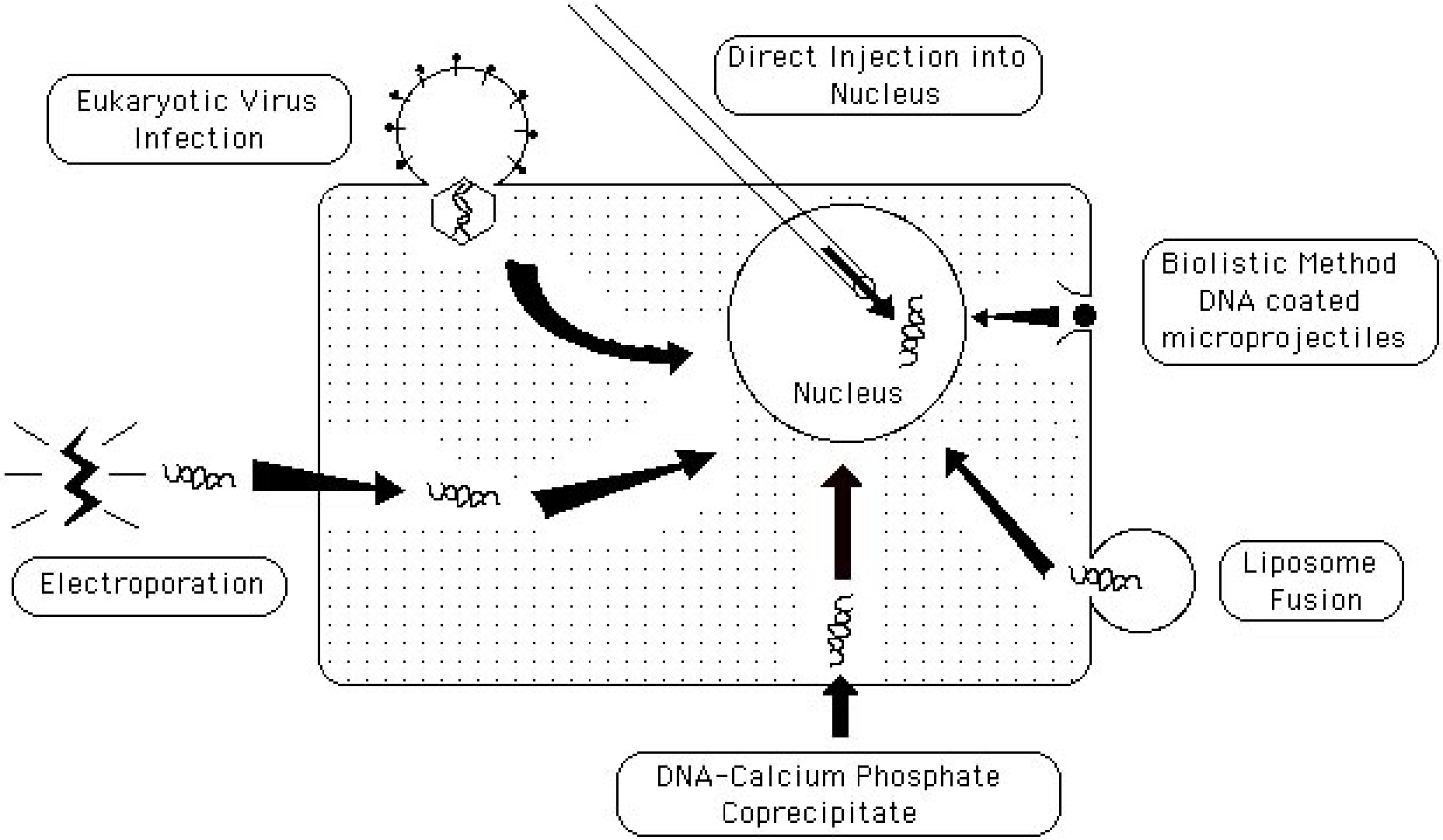
Advantages of Virus-Based Methods

- Very high gene delivery efficiency, 95–100%
- Simplicity of infection

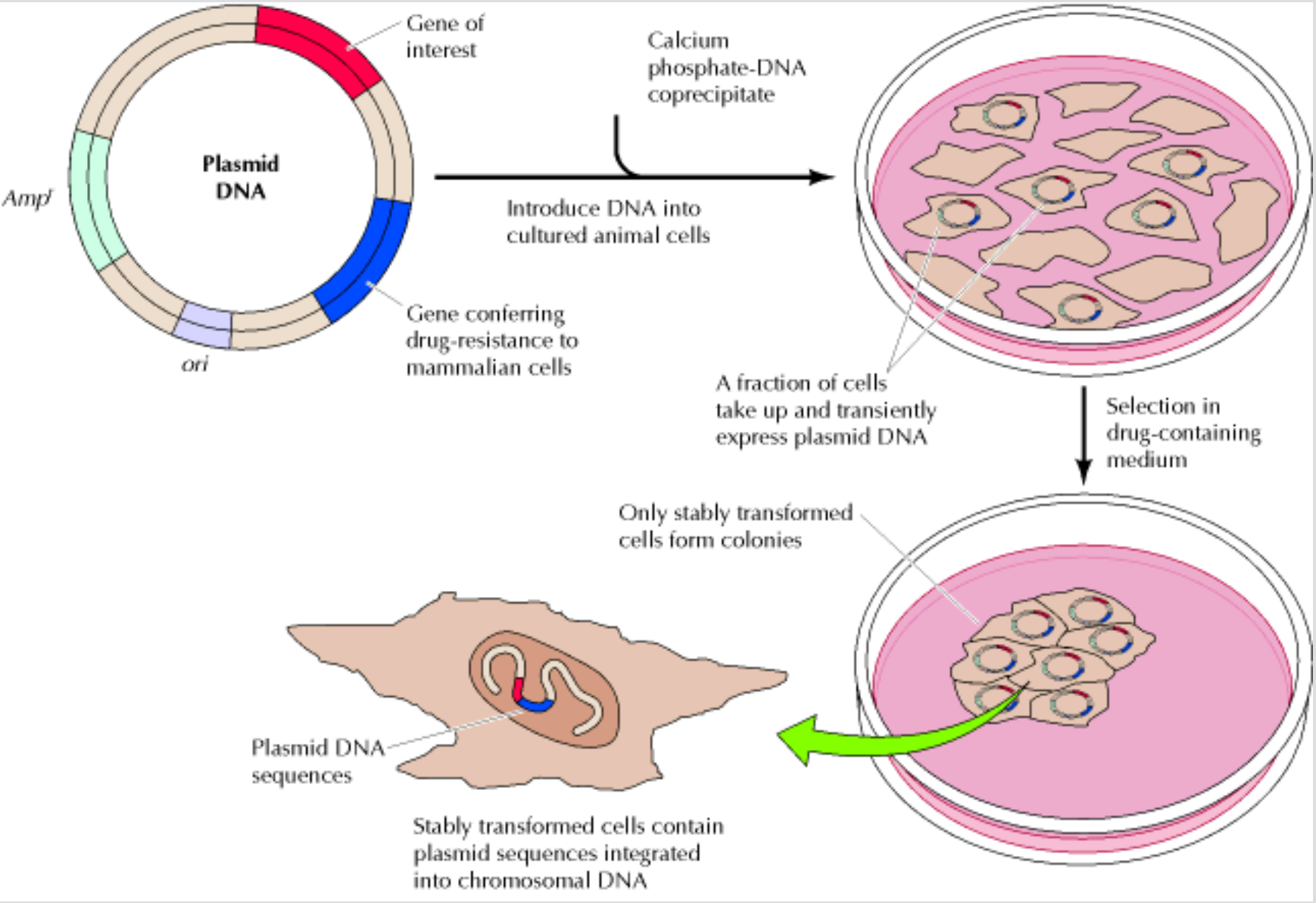
Disadvantages of Virus-Based Methods

- Labor intensive
- Best for introducing a single cloned gene that is to be highly expressed
- P2 containment required for most viruses
  - Institutional regulation and review boards required
  - Viral transfer of regulatory genes or oncogenes is inherently dangerous and should be carefully monitored
  - Host range specificity may not be adequate
- Many viruses are lytic
- Need for packaging cell lines

Transfection Methods



STABLE TRANSFECTION



Generalized Mammalian Expression Vector