iClicker ? BLAST assignment

A. What is BLAST – I have not started yet.

B. I tried to my first BLAST run but was not successful.

C. I have done my 1st BLAST run and identified a protein that contains my 20 a.a. sequence.

D. I have my identified my protein and found its gene sequence.

E. I have finished my BLAST assignment.

Question to be repeated – answers can vary!

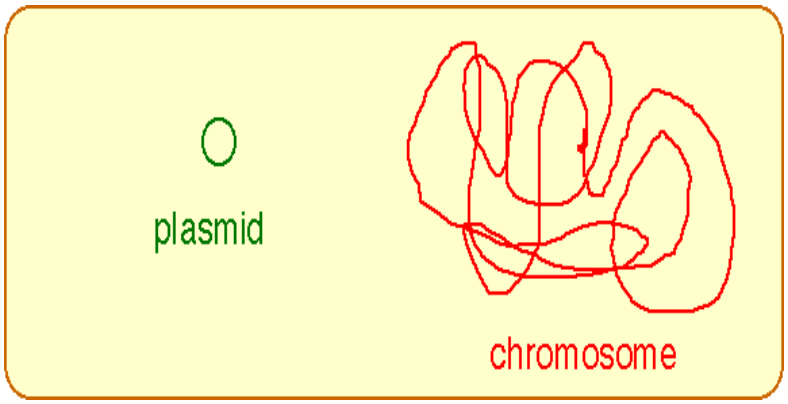
Protein Expression and Purification

Goals for this unit:

1. **Review steps in cloning and expression**
   - **Vectors / Restriction sites / Induction / Tags**

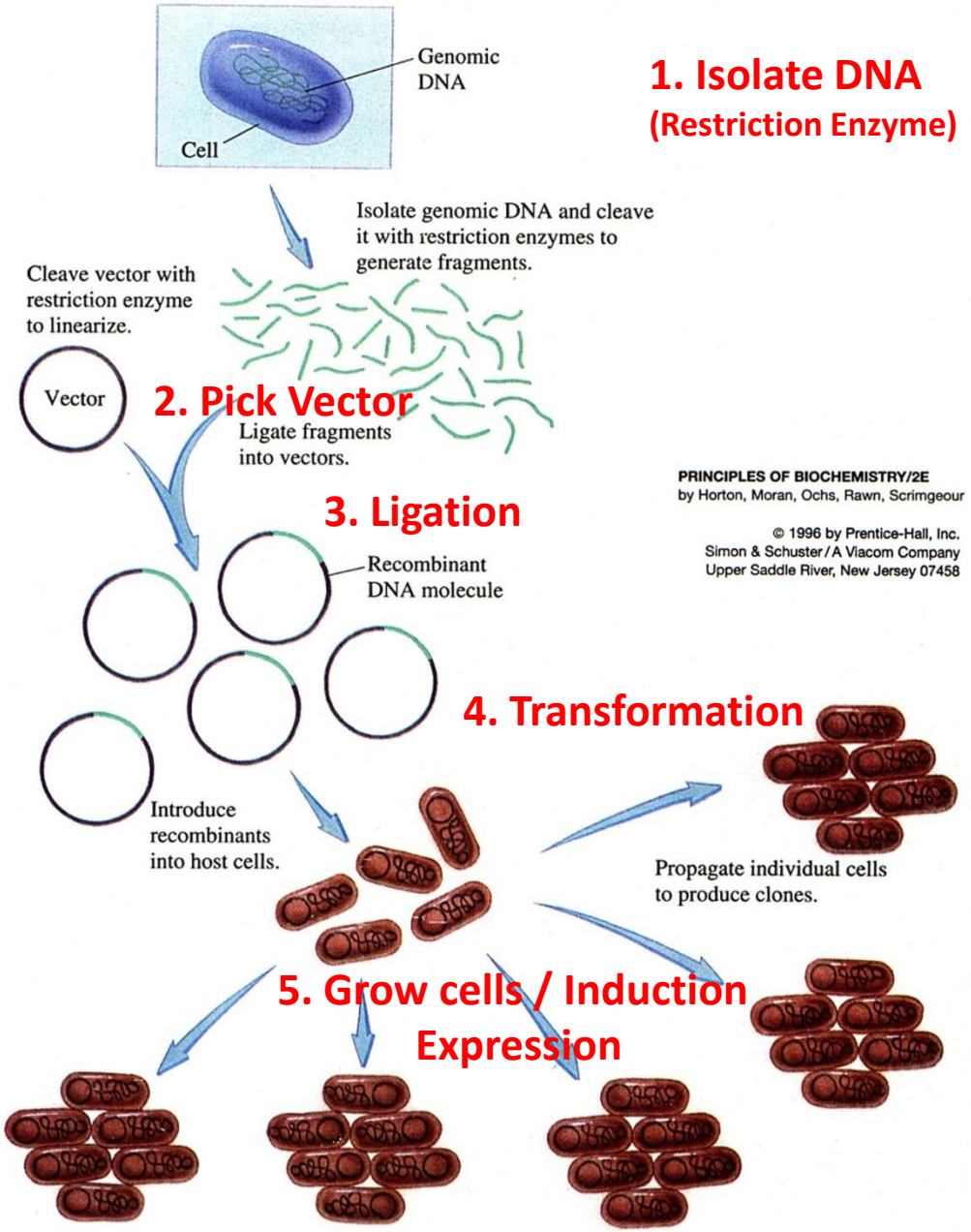
Why do we do cloning?

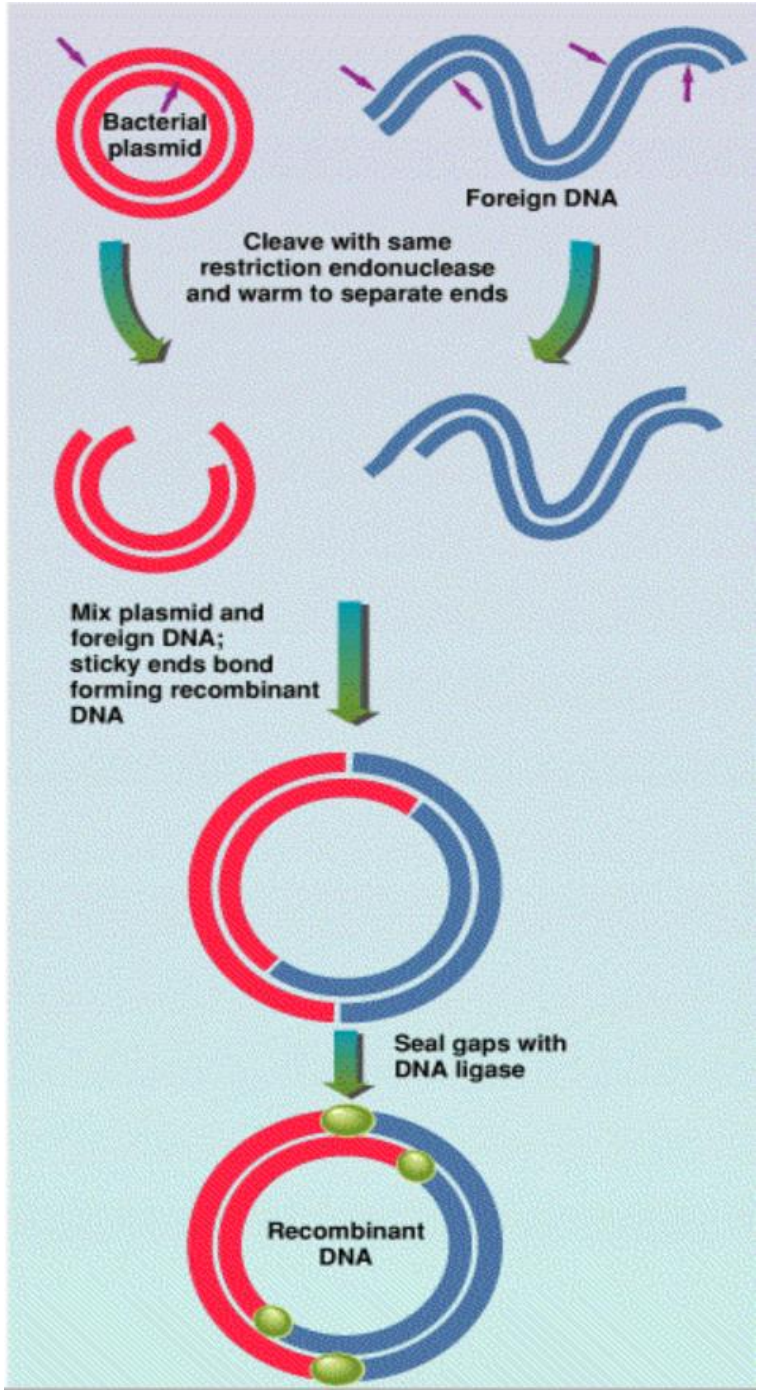
- **A single DNA molecule can be amplified allowing it to be:**
  - Studied - Sequenced
  - Manipulated - Mutagenesis or Engineered
  - Expressed - Generation of Protein

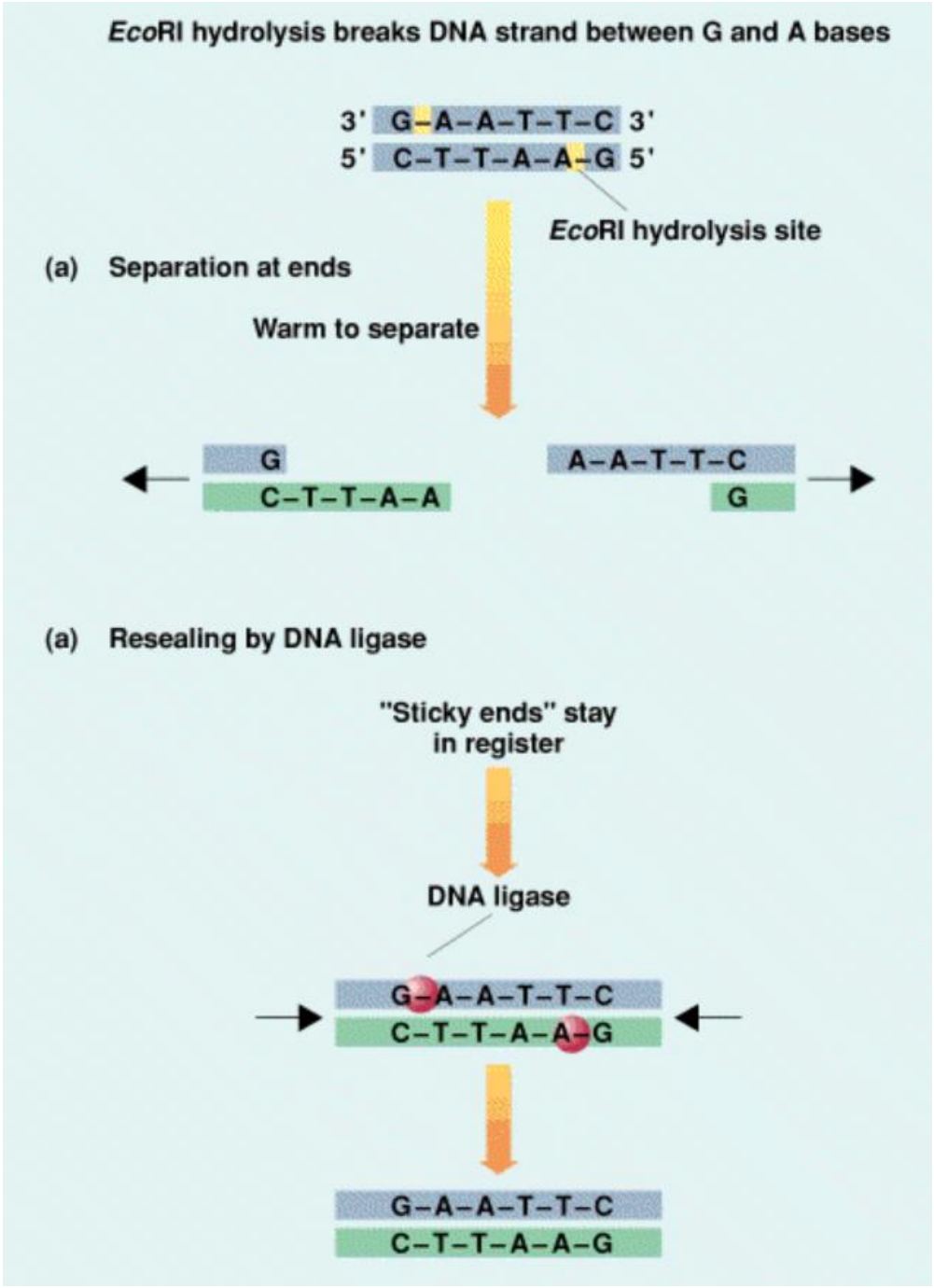


- Bacterial cells may contain extra-chromosomal DNA called plasmids.
- Plasmids are usually represented by small, circular DNA.
- Some plasmids are present in multiple copies in the cell

T–100 Fig. 24.1 General steps in the generation of a recombinant DNA molecule




Bacterial plasmid
Foreign DNA
Cleave with same restriction endonuclease and warm to separate ends
Mix plasmid and foreign DNA; sticky ends bond forming recombinant DNA
Seal gaps with DNA ligase
Recombinant DNA

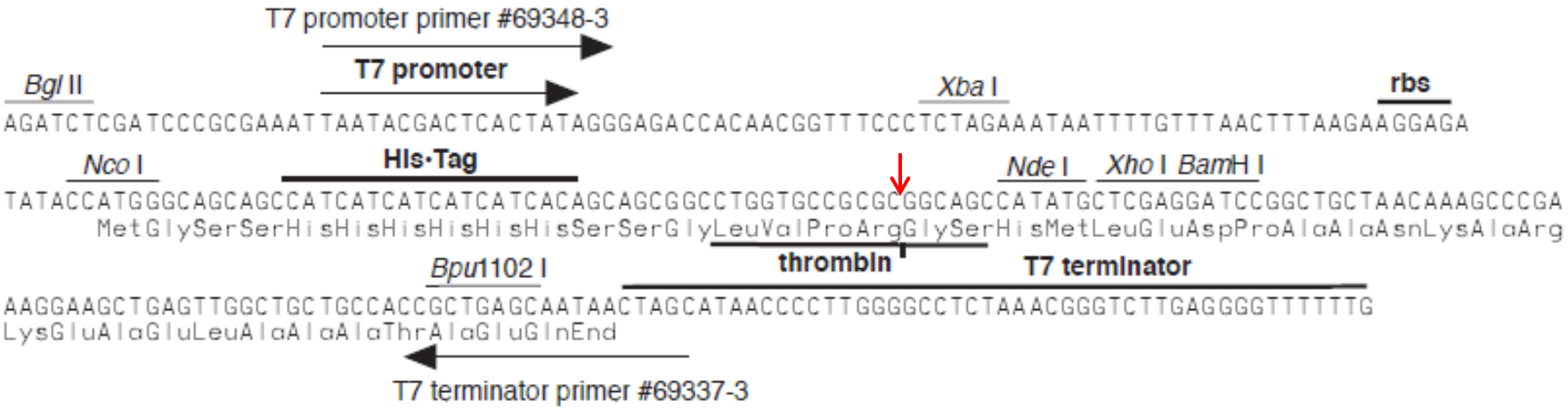

*Eco*RI hydrolysis breaks DNA strand between G and A bases
3' G–A–A–T–T–C 3'
5' C–T–T–A–A–G 5'
*Eco*RI hydrolysis site
(a) Separation at ends
Warm to separate
G
C–T–T–A–A
A–A–T–T–C
G
(a) Resealing by DNA ligase
"Sticky ends" stay in register
DNA ligase
G–A–A–T–T–C
C–T–T–A–A–G
G–A–A–T–T–C
C–T–T–A–A–G

1. Isolate DNA (Restriction Enzyme)

What restriction enzyme to choose?

First check the vector you want to insert your gene into.

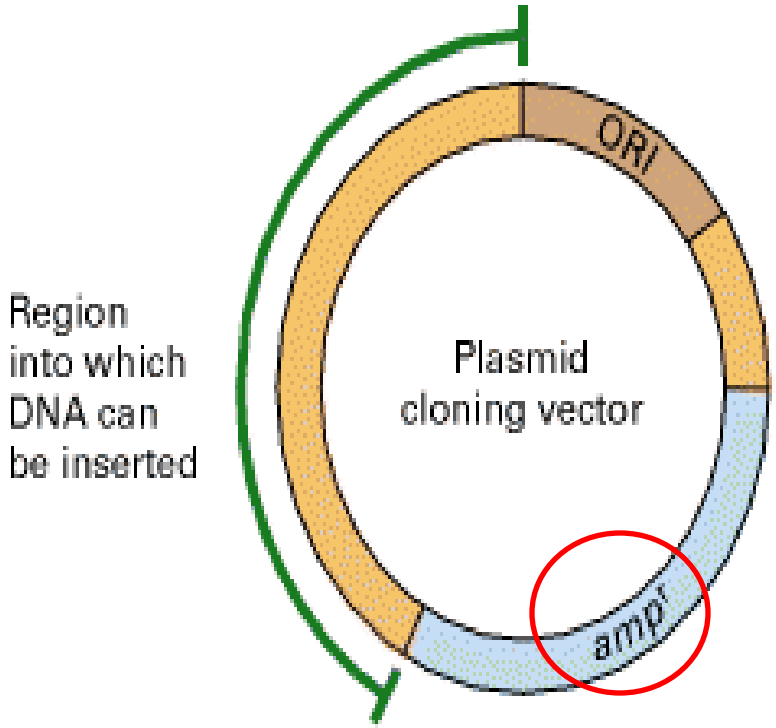
[PDF] **pET14b**
biochem.web.utah.edu/hill/links/**pET14b**.pdf
Novagen • ORDERING 800-526-7319 • TECHNICAL SUPPORT 800-207-0144 ori (2845). A p (3606-4463). Cla I(24). Hind III(29). Nhe I(229). Bpu1102 I(458).



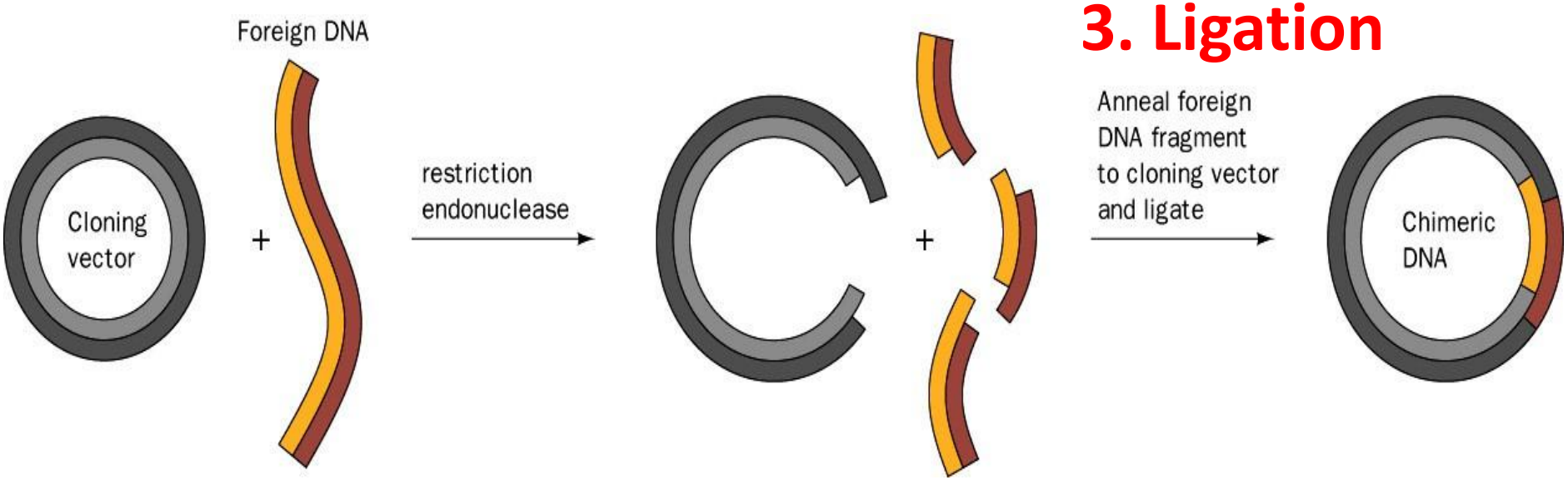
pET-14b cloning/expression region

2. Pick Vector

- replication origin (ORI) sequence
- a gene that permits selection,
- Here the selective gene is *ampr*; it encodes the enzyme b-lactamase, which inactivates ampicillin.
- Exogenous DNA can be inserted into the bracketed region .
- Under the selective conditions, only cells that contain plasmids with selectable marker can survive



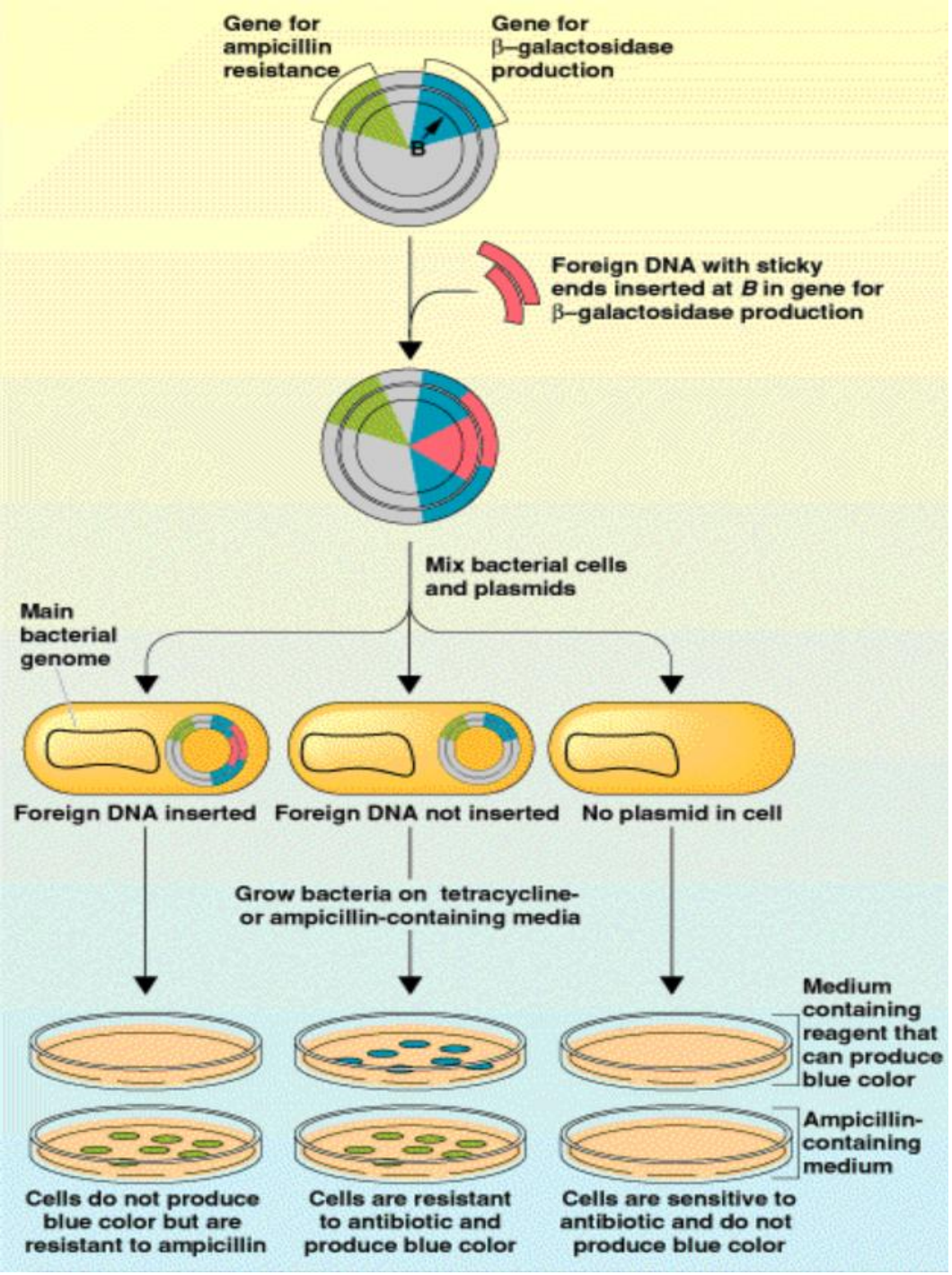
3. Ligation



Transformation

- Upon heat shock or electric pulses, bacterial cell walls can temporarily become permeable and allow DNA to get in.
- The mechanism for such take-up is still unknown.
- Overall take-up efficiency is low. But once the plasmid enters, the ligated ones will be replicated in bacteria along with bacteria growth.
- *Use antibiotics to select those bacterial cells that take-up the foreign DNA.*
- So the specific DNA you want are duplicated many times in bacterial cells.
- When you spread them on agar plate and put it to selection, the ones that have plasmid will grow as colonies.

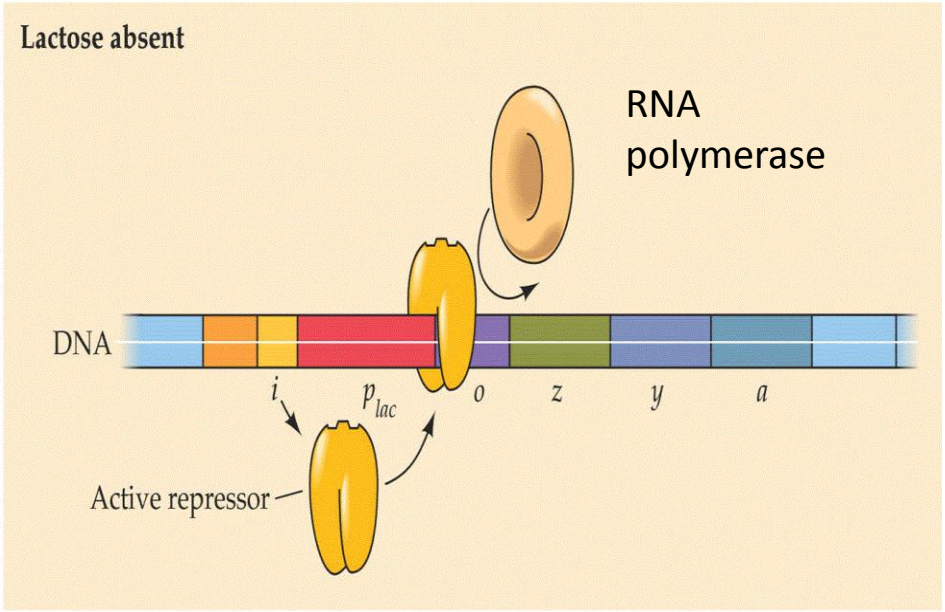
4. Transformation

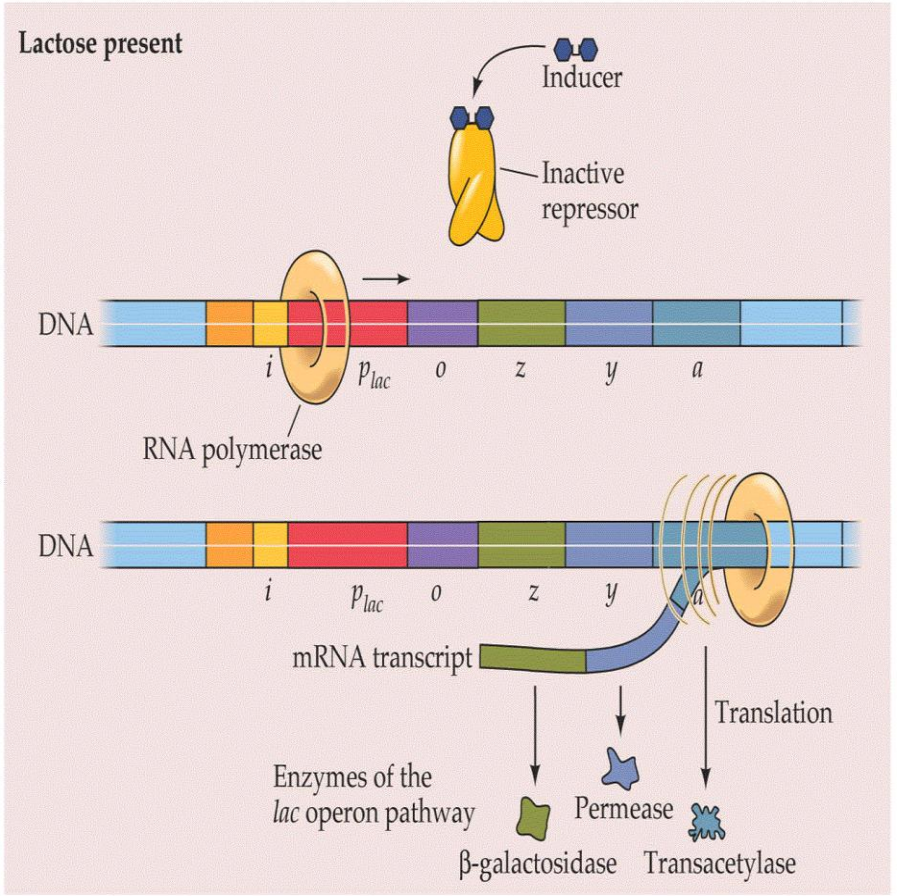


IPTG-inducible protein expression

Isopropyl β-D-1-thiogalactopyranoside

5. Grow cells / Induction Expression





LIFE: THE SCIENCE OF BIOLOGY, Seventh Edition, Figure 13.17 The *lac* Operon: An Inducible System (Part 2)
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LIFE: THE SCIENCE OF BIOLOGY, Seventh Edition, Figure 13.17 The *lac* Operon: An Inducible System (Part 1)
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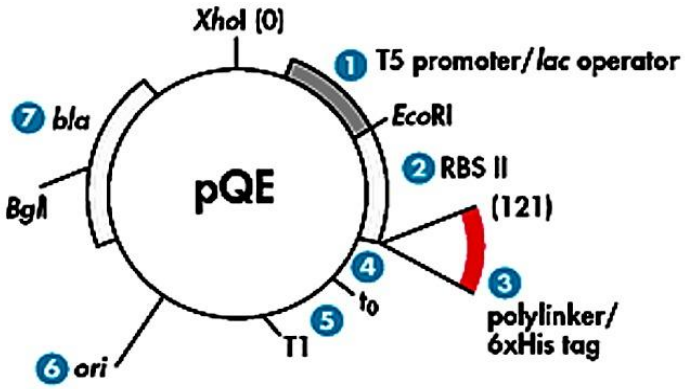
Elements present in QIAexpress pQE Vectors

pQE Vectors



	Element	Description
1	Optimized promoter/operator element	Consists of the phage T5 promoter and two lac operator sequences, which increase the probability of lac repressor binding and ensure efficient repression of the powerful T5 promoter
2	Synthetic ribosomal binding site - RBSII	For efficient translation
3	6xHis-tag coding sequence	Either 5' or 3' to the polylinker cloning region
4	Translational stop codons	In all reading frames for convenient preparation of expression constructs
5	Two strong transcriptional terminators	t0 from phage lambda, and T1 from the rrnB operon of E. coli, to prevent read-through transcription and ensure stability of the expression construct
6	ColE1 origin of replication	From pBR3227
7.	beta-lactamase gene (bla)	Confers ampicillin resistance

Most used organisms for protein overexpression

- **Bacterial culture**
  - Easy to manipulate.
  - Don’t get contaminated easily.
  - Grow fast. (one day turn-around).
  - Cheap!
- **Eukaryotic systems**:
  - Yeast
    - Cheap and fast turn-around
    - Sometimes good yield.
    - Translational modification still very different from mammalian system.
  - Baculovirus and insect cells.
    - Stable expression.
    - Great yield.
    - Overcome problem like folding or glycosylation.
    - Less non-specific band during purification.
    - But long turn-around.
    - $$$
  - Mammalian
    - Very pricey.
    - Yield low.
    - Expression unstable
    - Only used when small amount is required.

So, why not use a bacterial expression system every single time?

- Codon usage.
- Toxicity
- Sugar linkage.
- Folding issue and disulfide bond formation.

Companies have developed vectors and bacterial strains to improve the chances of success!

QIAgenes:* Easy access to high-yield expression of human proteins — in *E. coli*, insect, and mammalian cells

Lack of sufficient amounts of protein often slows down or makes structural and functional analyses of proteins impossible. Expression of optimized genes helps to overcome this obstacle by improving codon usage and avoiding mRNA secondary structure or motifs that interfere with the transcription/translation process (internal ribosomal binding sites, sequence repeats etc.).

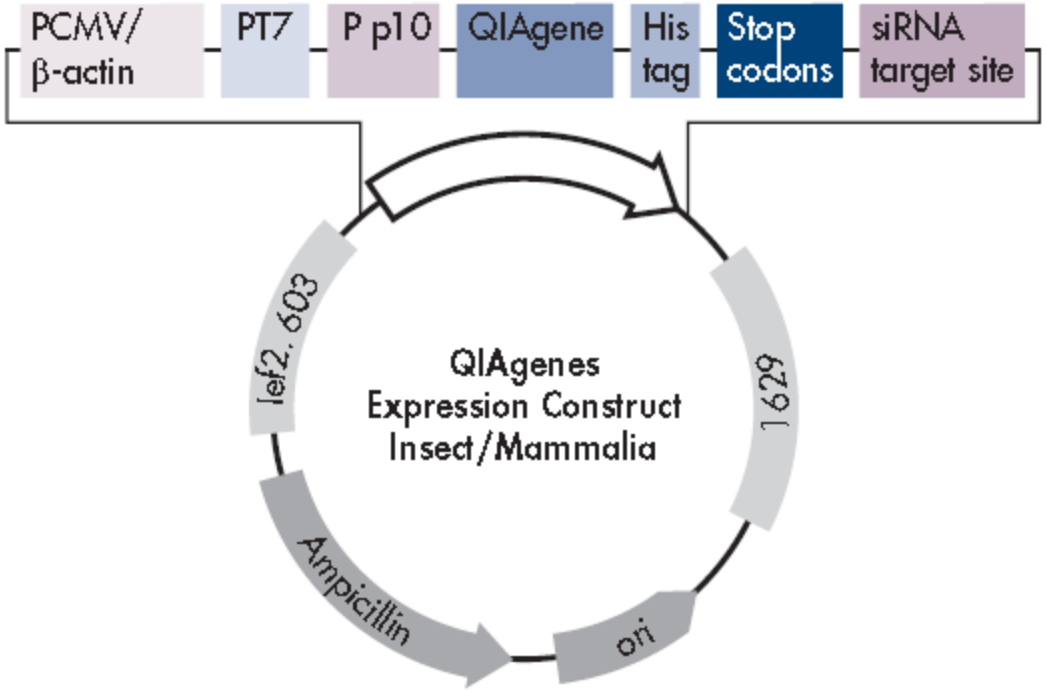


Figure 1. Elements within QIAgenes Expression Constructs Insect/Mammalia.

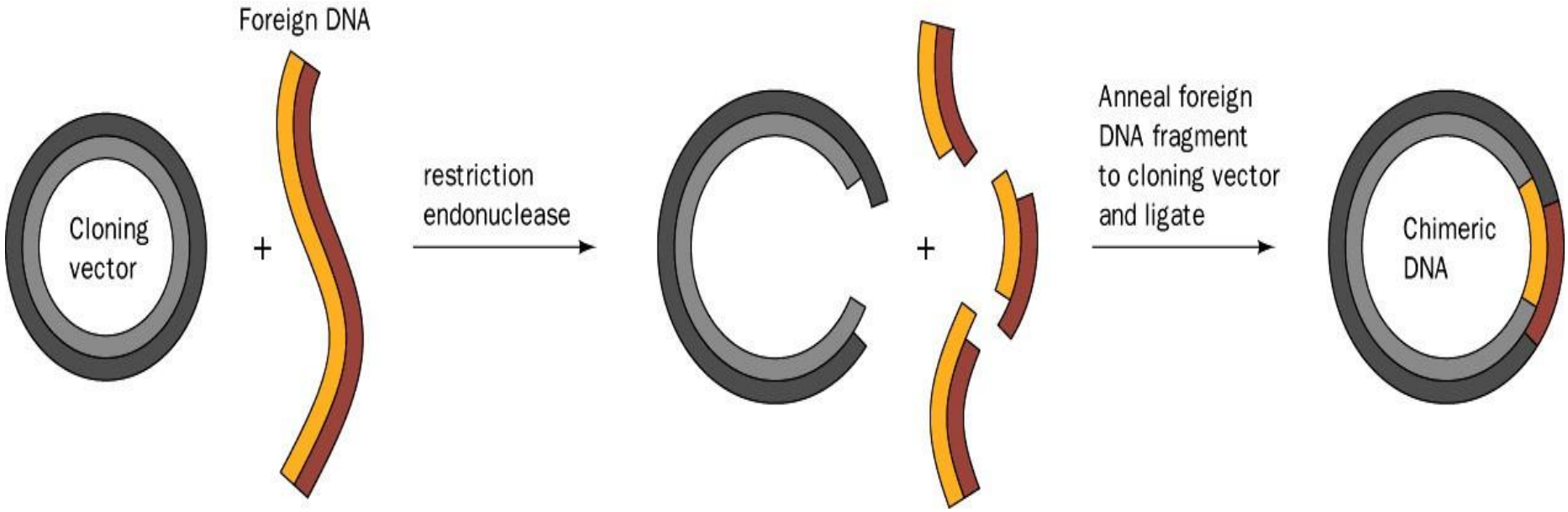
Strategy to overcome the problems

- If the protein is toxic, use (DE3) strain to prevent expression before induction. Additionally, pLysS or pLysE strain can make sure no protein expression until addition of **IPTG**.

- If the protein is from eukaryotes, use codon plus strain if there are a lot of rare codons.

- If the protein has problem in folding, you can either refold from inclusion body or use **GroEL** or DnaK supplemented strain. Sometimes using special vector with thioredoxin can help.

The rate of ligation is not high



To optimize ligation, different ratio of vector and insert should be tested

1 vector : 3 insert

1 vector : 10 insert

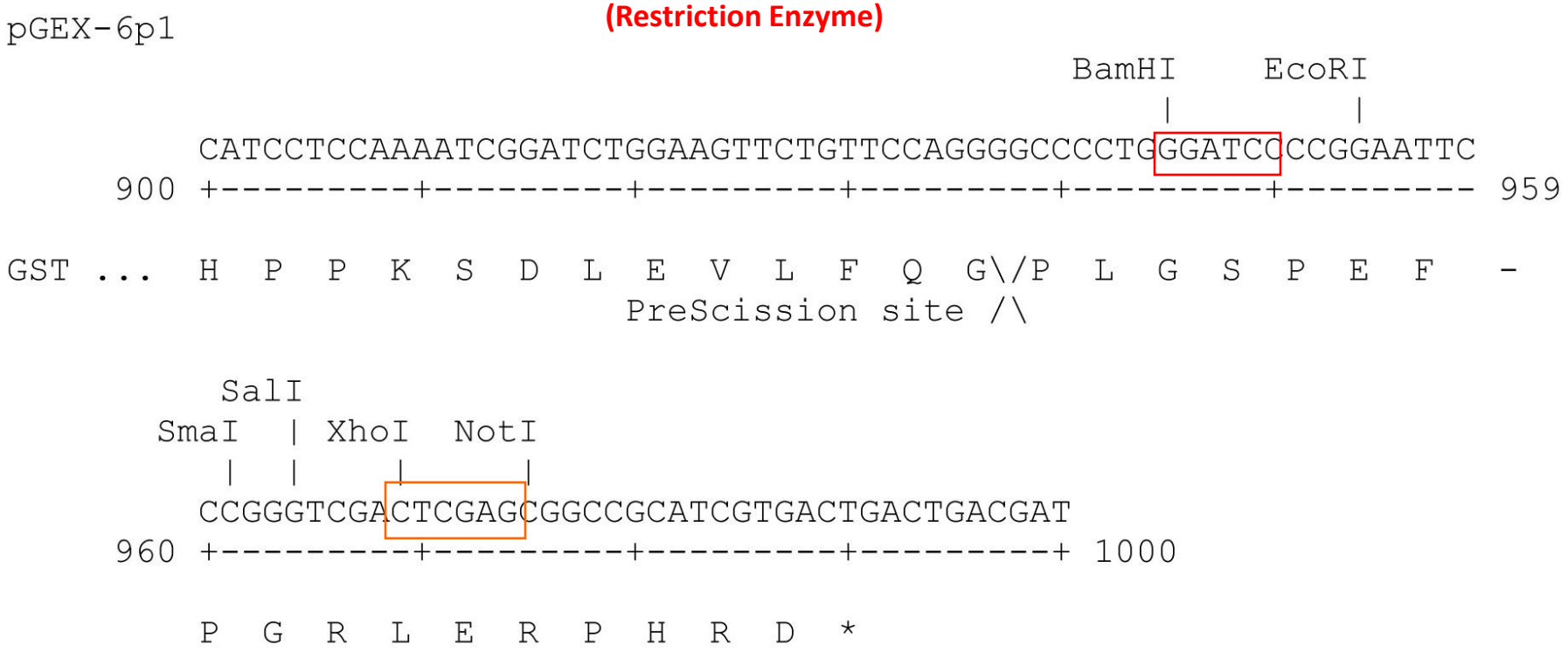
1 vector: 50 insert

Assumption: all cuts are 100%

What restriction enzyme to choose?

- First check the vector you want to insert your gene into.

1. Isolate DNA
(Restriction Enzyme)



Bacterial expression vectors

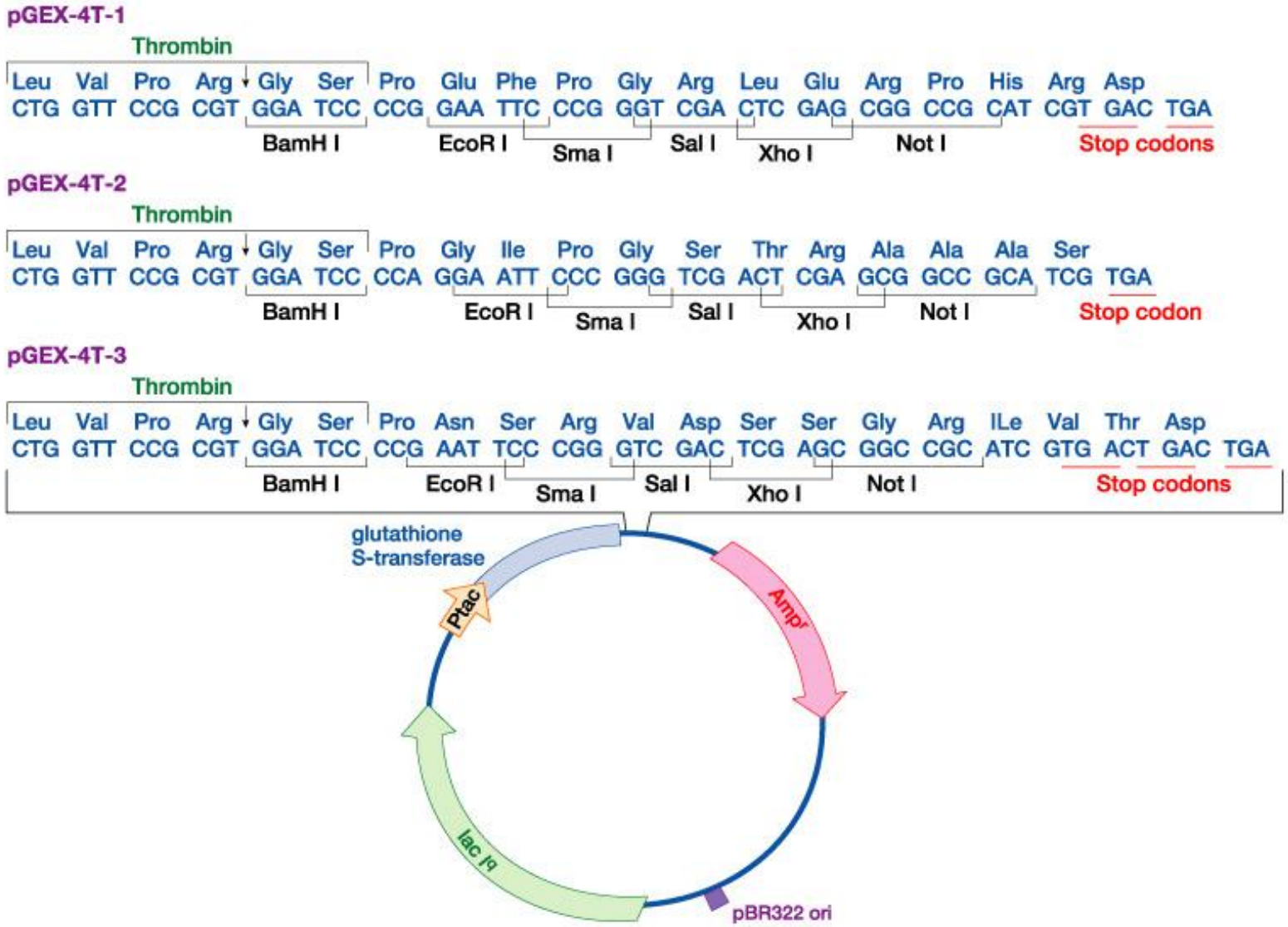


Figure 5-21 part 2 of 2 Human Molecular Genetics, 3/e. (© Garland Science 2004)