MBT 2001P; Molecular Biology and Genetics (Practical)

Molecular Cloning; Basics of Plasmid, Isolation and Purification

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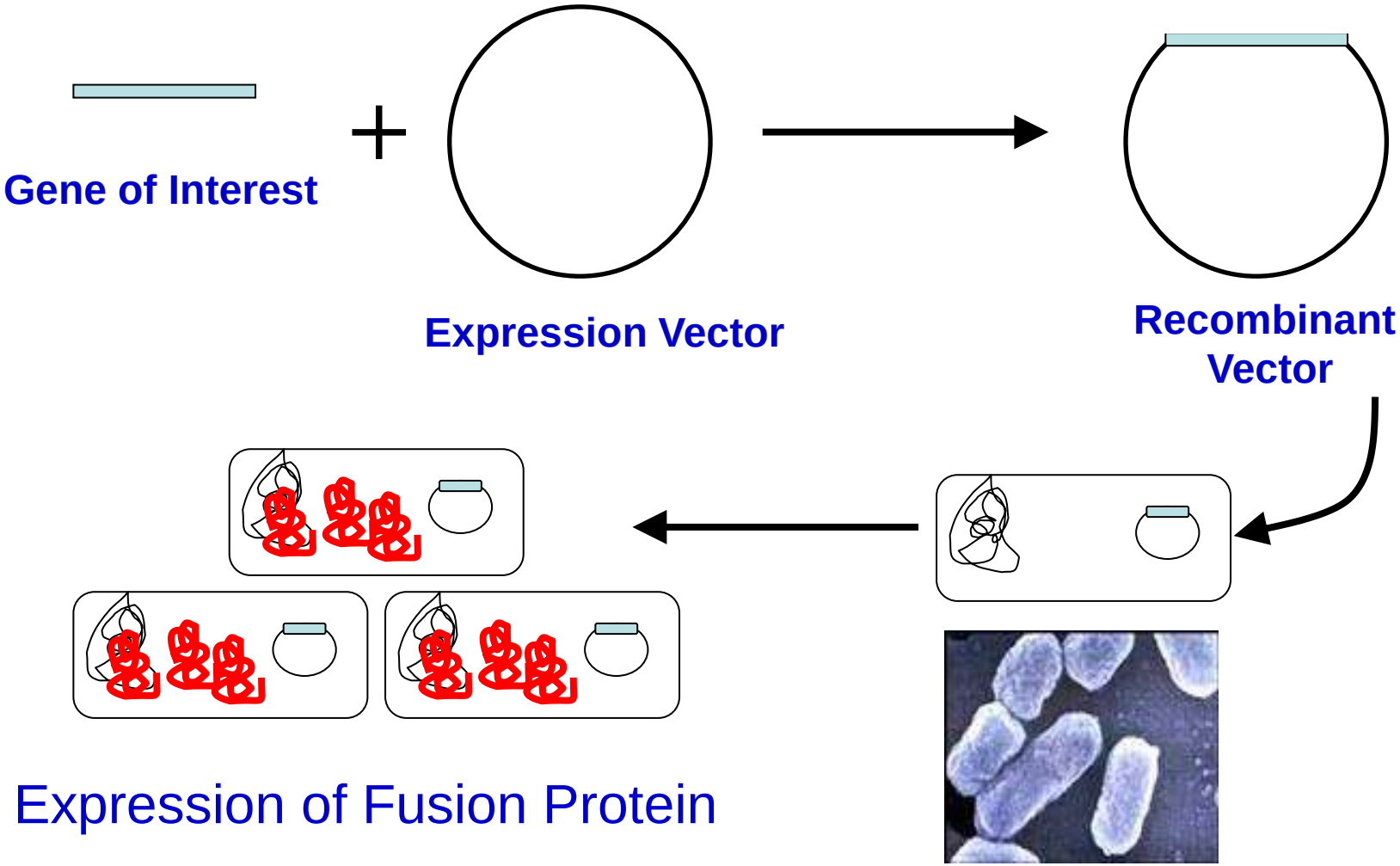
Overview

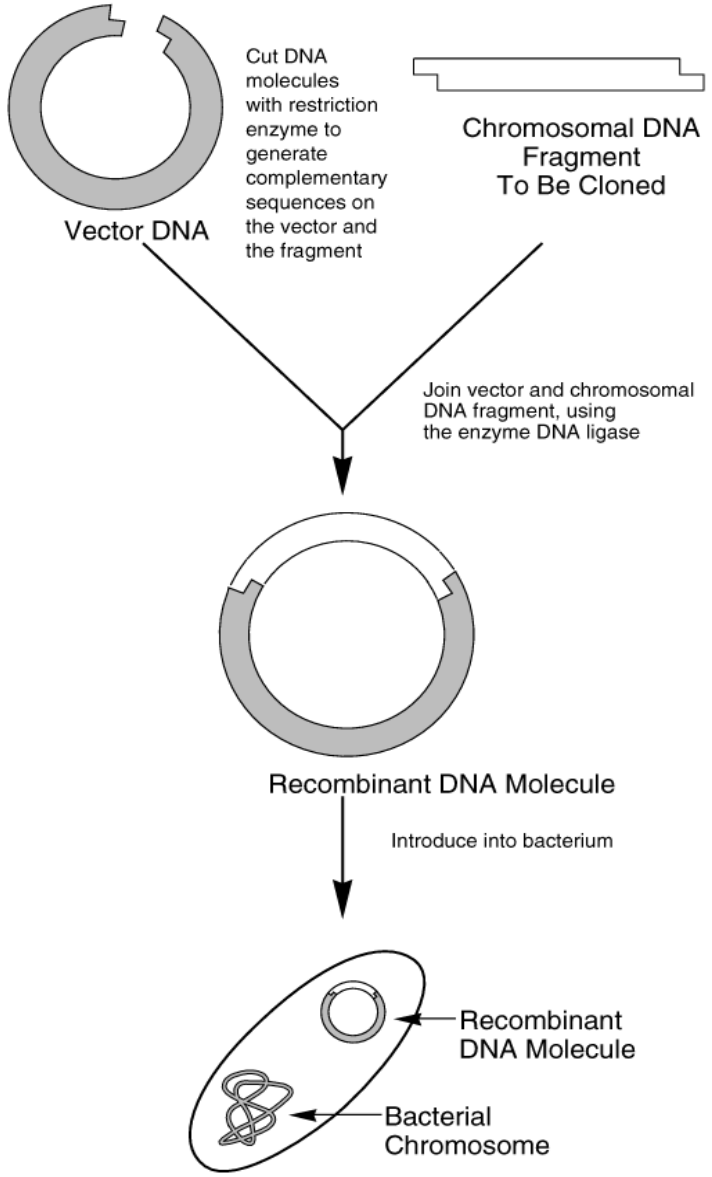
- Introduction
- Molecular Cloning and Plasmid Vectors
- Plasmid Isolation and Purification

Cloning and expression of target gene

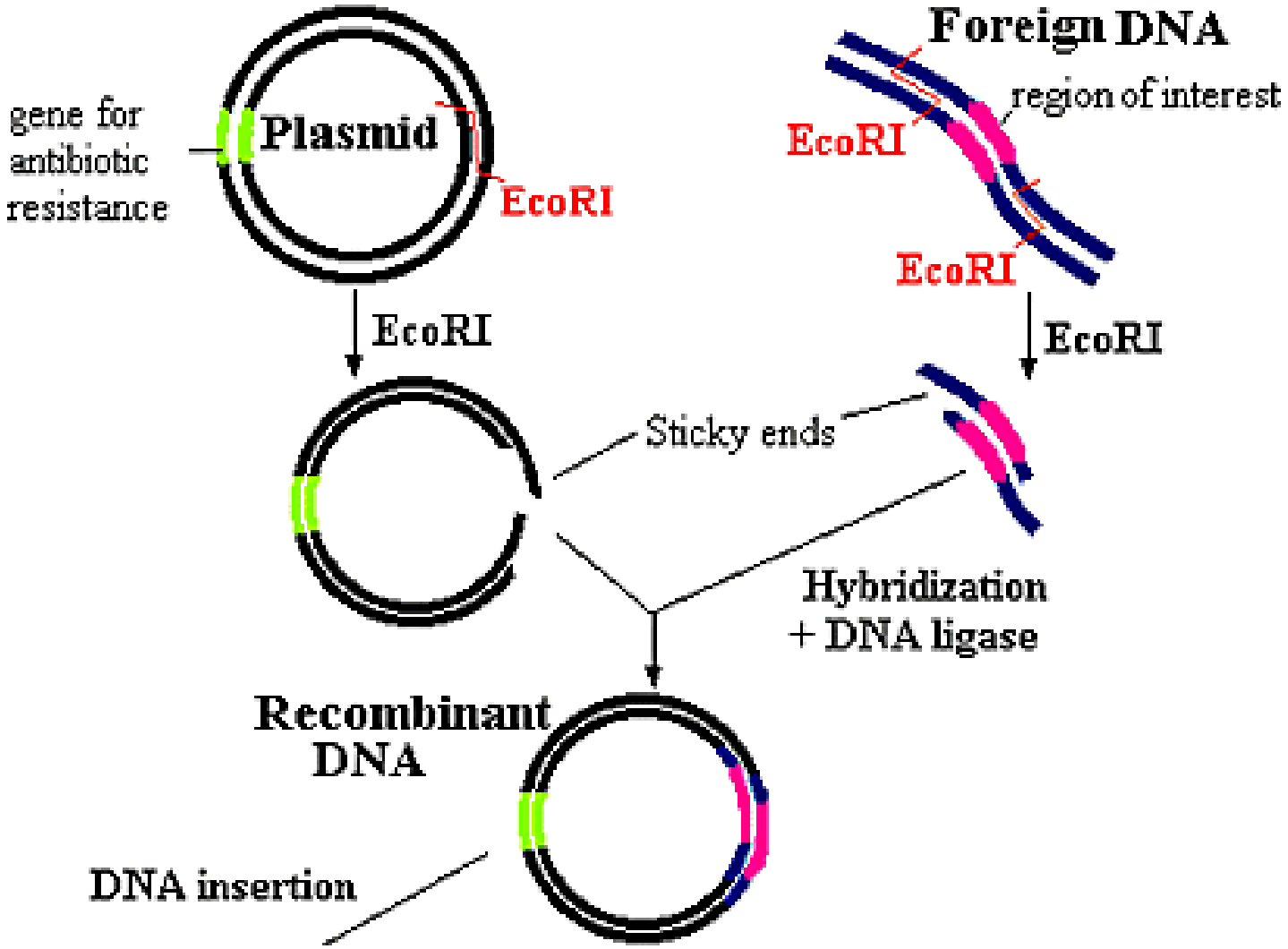


Cloning Process

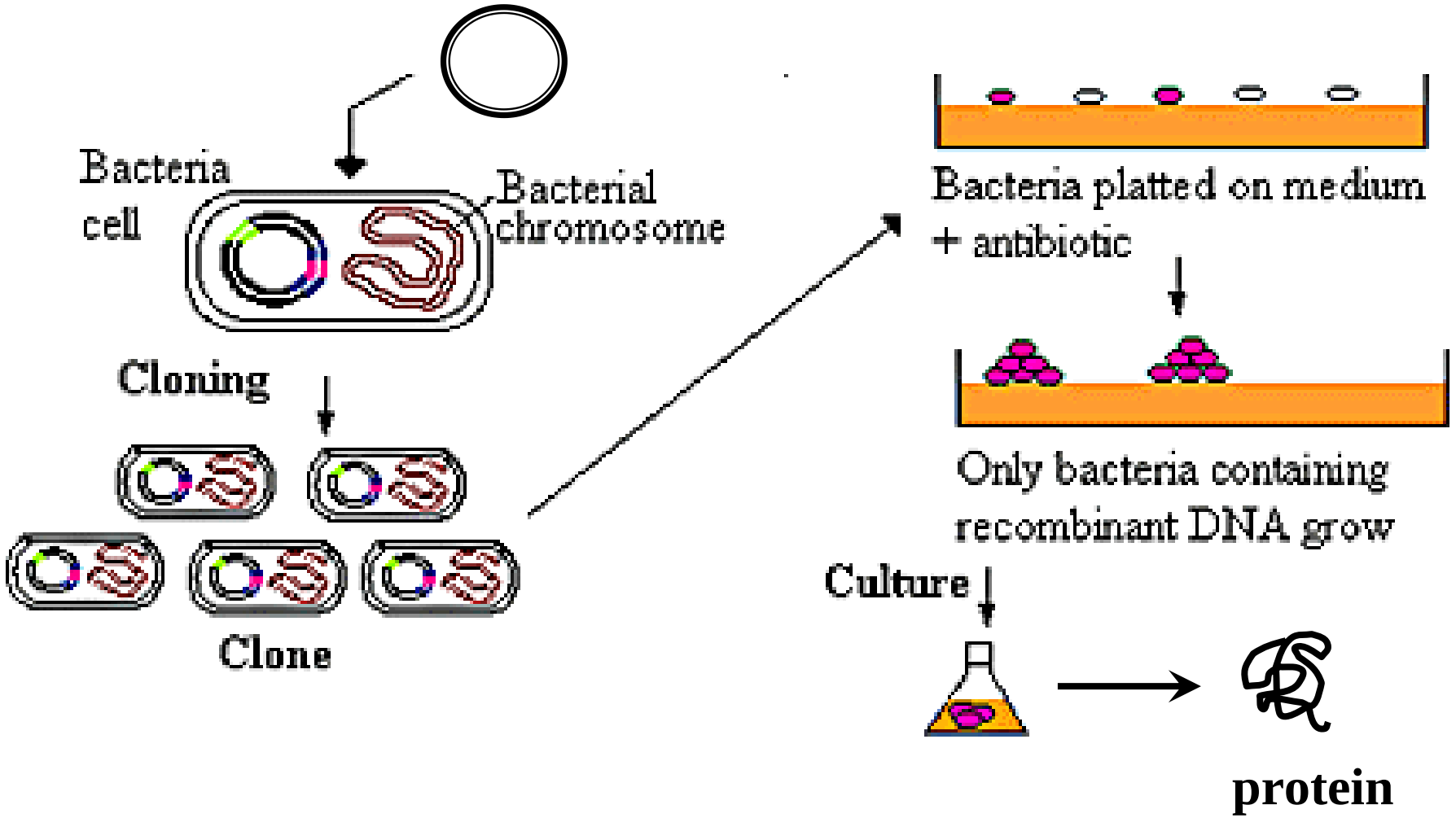
- ✓ Gene of interest is cut out with restriction enzymes (RE)
- ✓ Host plasmid (circular chromosome) is cut with same REs
- ✓ Gene is inserted into plasmid and ligated with ligase
- ✓ New (engineered) plasmid inserted into bacterium (transform)



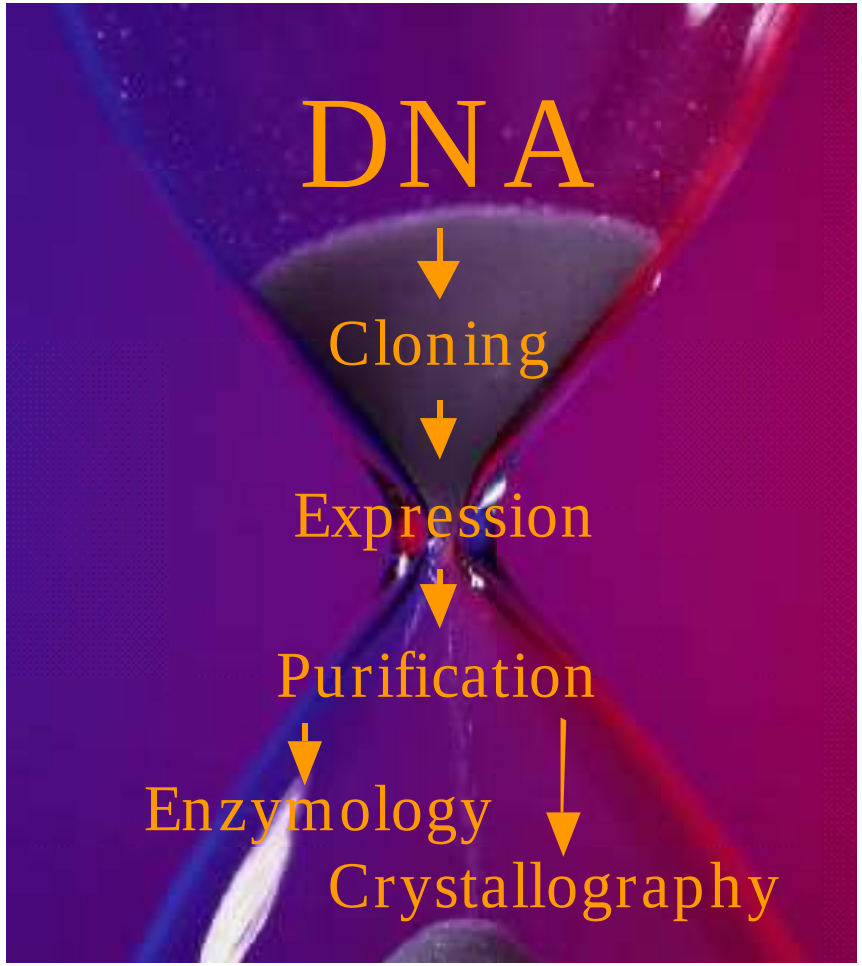
Cloning (Details)



Cloning (Details)



Protein Expression Bottleneck



- Protein Biochemistry
  - soluble, purifiable protein
- Enzymology
  - soluble, active protein
  - 0.1-10 mg of protein
- Crystallography
  - soluble, crystallizable protein
  - 5-100 mg of protein

Which Vector?

- ✓Must be compatible with host cell system (prokaryotic vectors for prokaryotic cells, eukaryotic vectors for eukaryotic cells)
- ✓Needs a good combination of
  - strong promoters
  - ribosome binding sites
  - termination sequences
  - affinity tag or solubilization sequences
  - multi-enzyme restriction site

Plasmids and Vectors

- ✓Circular pieces of DNA ranging in size from 1000 to 10,000 bases
- ✓Able to independently replicate and typically code for 1-10 genes
- ✓Often derived from bacterial “mini” chromosomes (used in bacterial sex)
- ✓May exist as single copies or dozens of copies (often used to transfer antibiotic resistance)

Key Parts to a Vector

- ✓Origin of replication (ORI) – DNA sequence for DNA polymerase to replicate the plasmid
- ✓Selectable marker (Amp or Tet) – a gene, when expressed on plasmid will allow host cells to survive
- ✓Inducible promoter – Short DNA sequence which enhances expression of adjacent gene
- ✓Multi-cloning site (MCS) – Short DNA sequence that contains many restriction enzyme sites

Basic elements of a plasmid/vector

pET developed by WF Studier & BA Moffatt in 1986

1) Ap = ampicillin resistance

2) ori = ColE1/pBR322 origin of replication

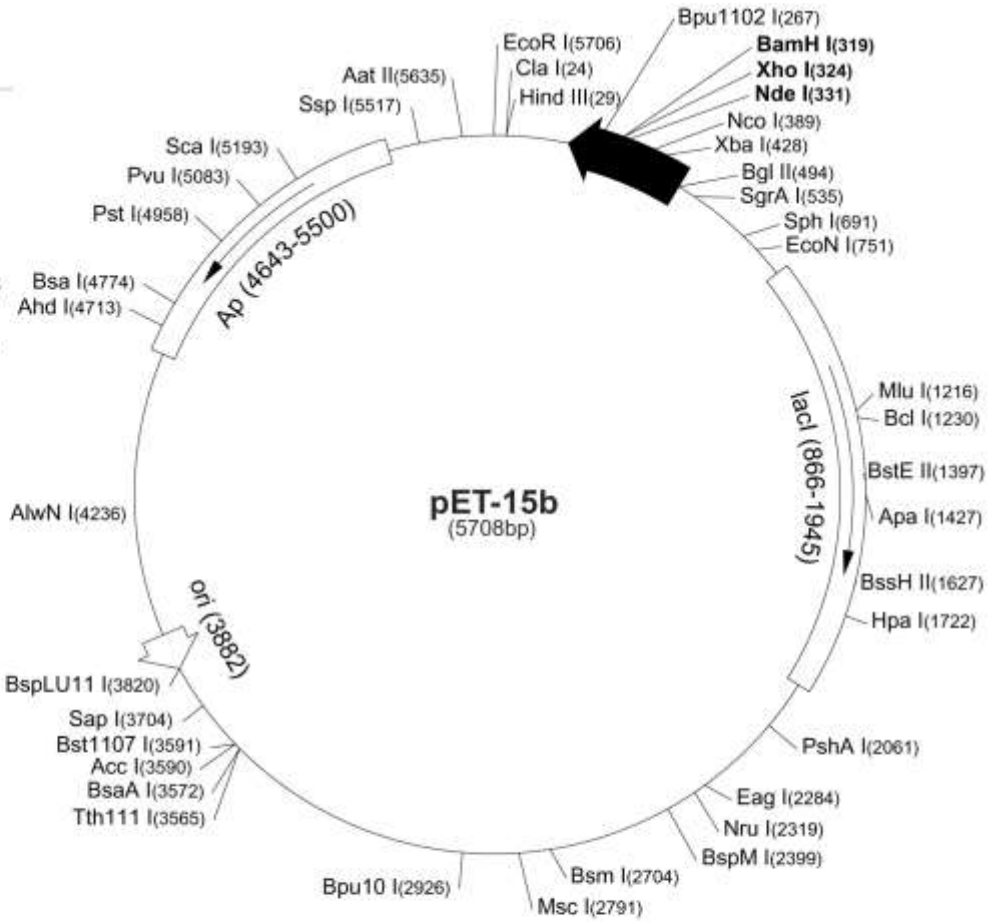
3) lacI = lac repressor; bind lacO until IPTG induction

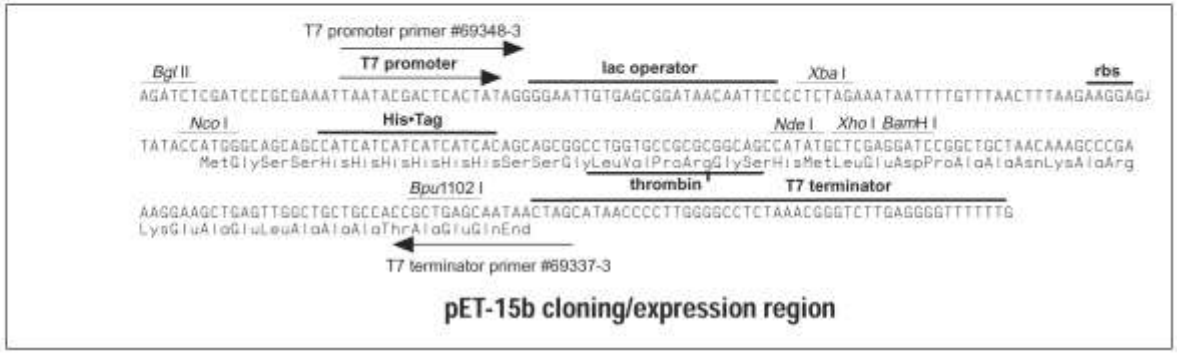
4) T7P = T7 Polymerase promoter

5) lacO = lac operator where lac repressor binds

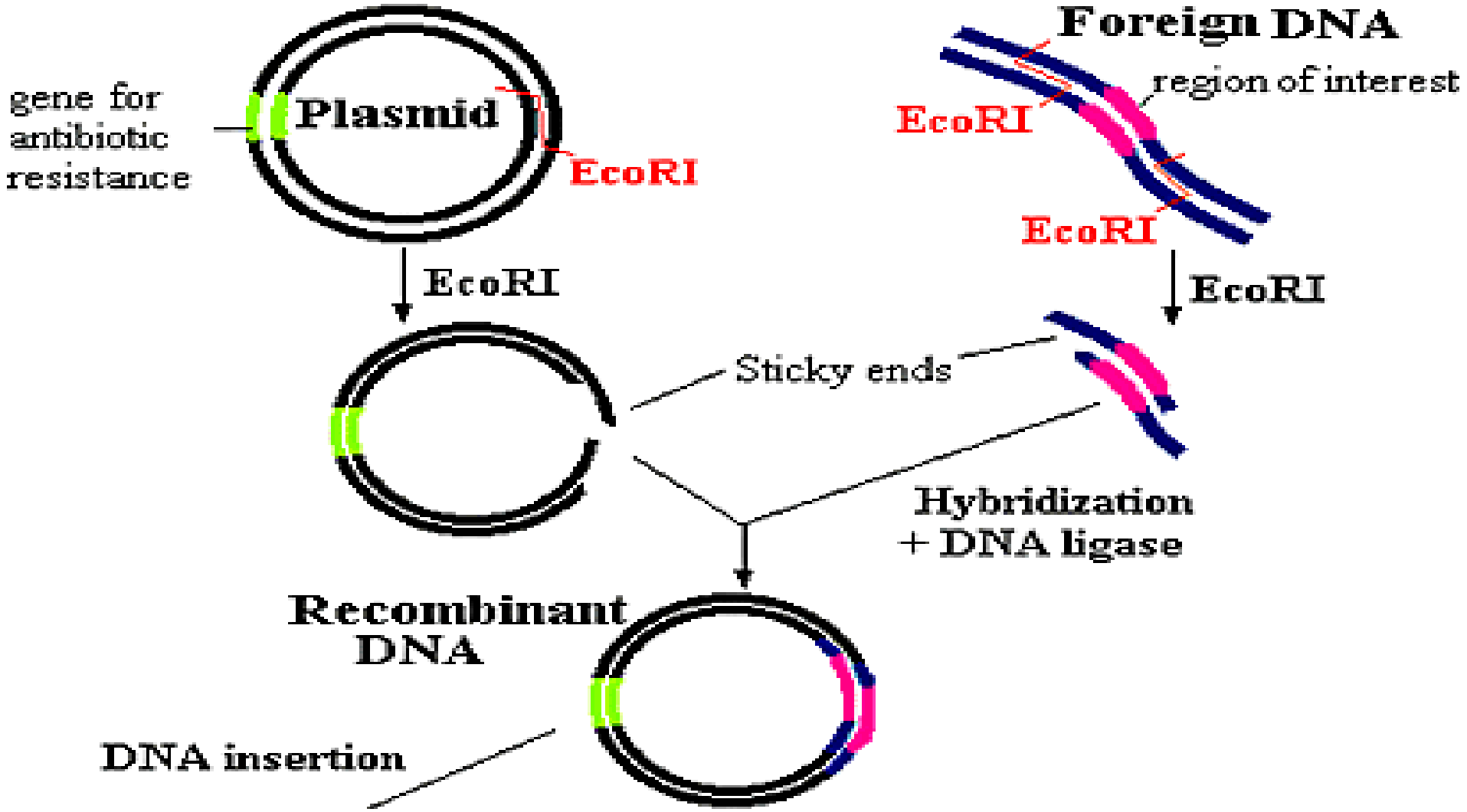
6) ➡ = multiple cloning site

pET-15b sequence landmarks	
T7 promoter	453-469
T7 transcription start	452
His•Tag coding sequence	362-380
Multiple cloning sites (*Nde* I - *Bam*H I)	319-335
T7 terminator	213-259
lacI coding sequence	(866-1945)
pBR322 origin	3882
bla coding sequence	4643-5500

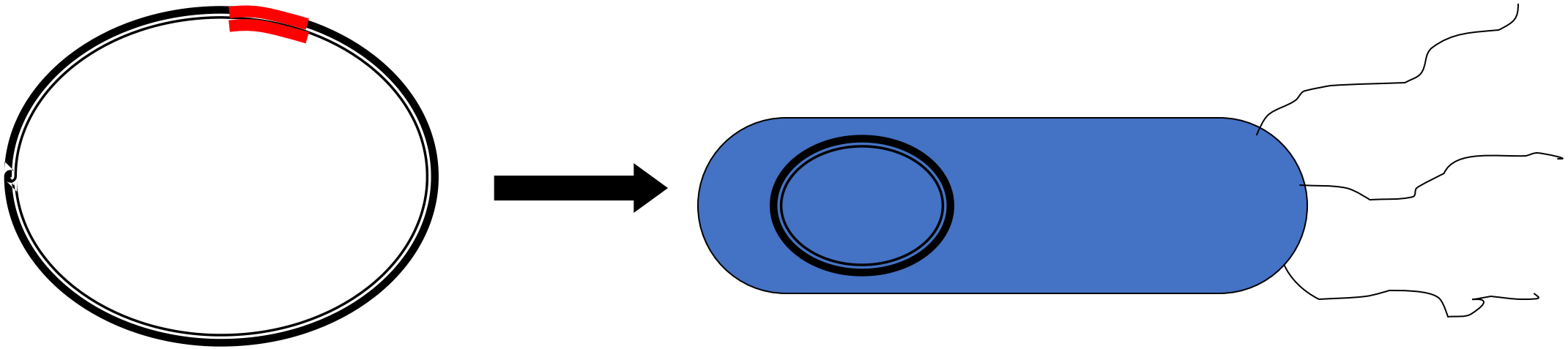




Gene Introduction (Bacteria)



Bacterial Transformation

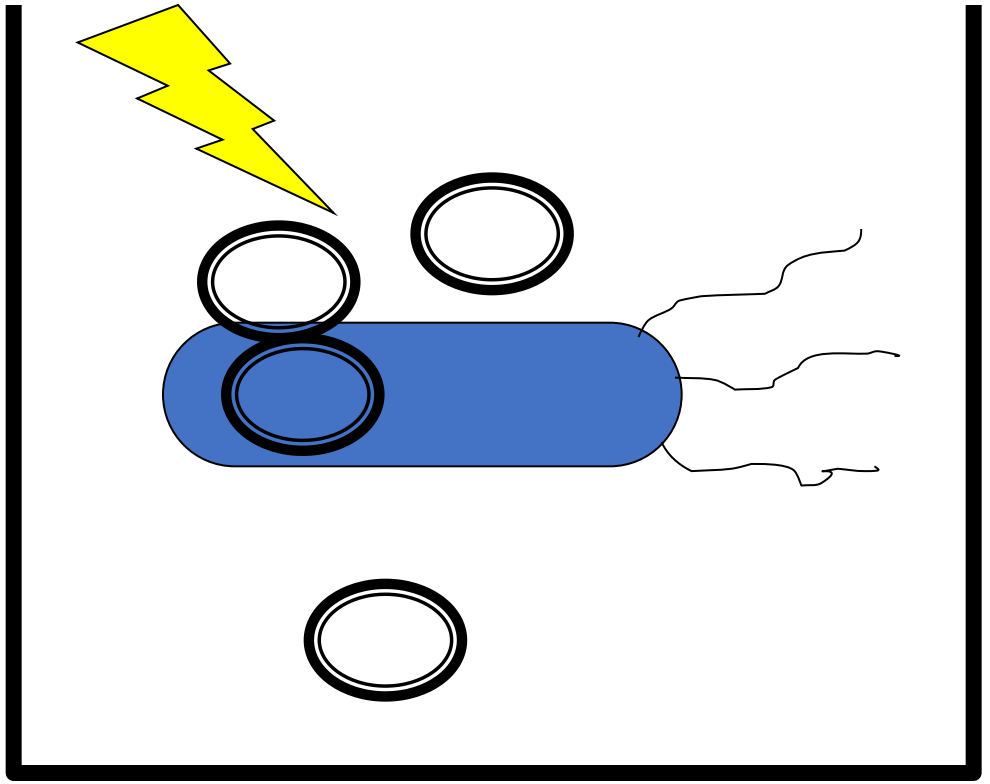


Bacterial Transformation

- ✓Moves the plasmid into bacterial host
- ✓Essential to making the gene “actively” express the protein inside the cell
- ✓2 routes of transformation
  - $CaCl_2$ + Temperature shock
  - Electroporation
- ✓Typical transformation rate is 1 in 10,000 cells (not very efficient) for $CaCl_2$, but 1 in 100 for electroporation

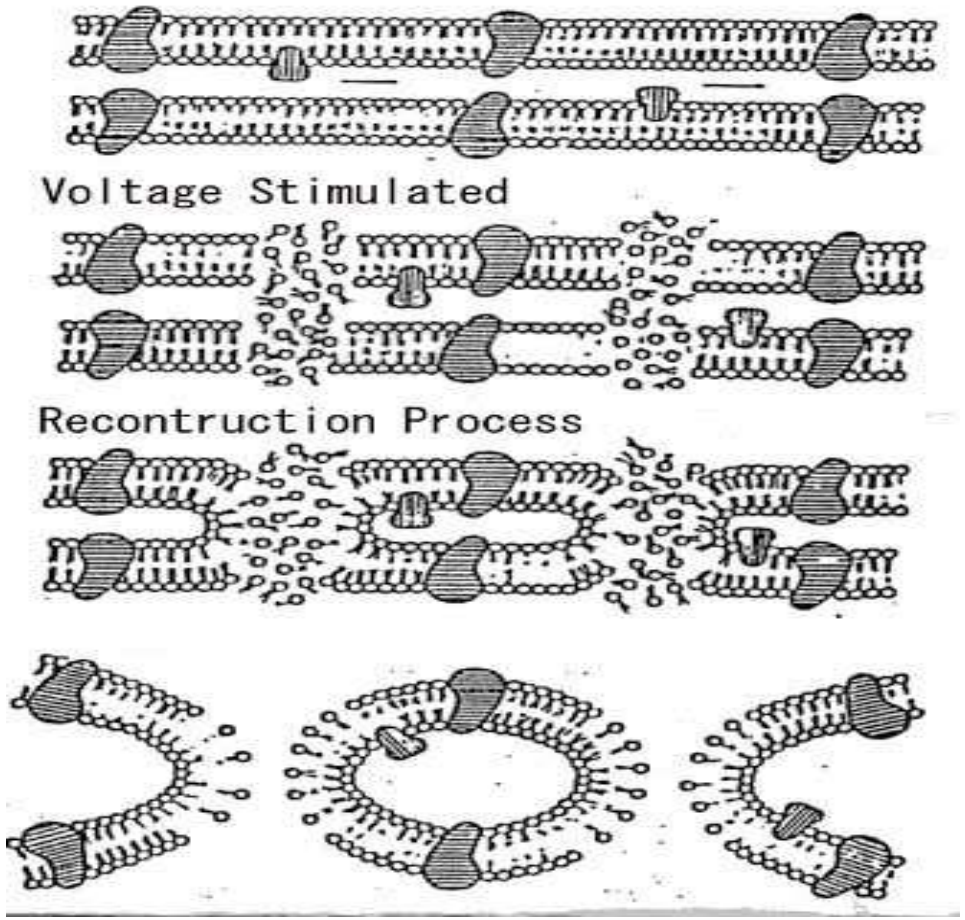
Electroporator

25 microfarads = 2500 V
@ 200 ohms for 5 ms



Electroporation

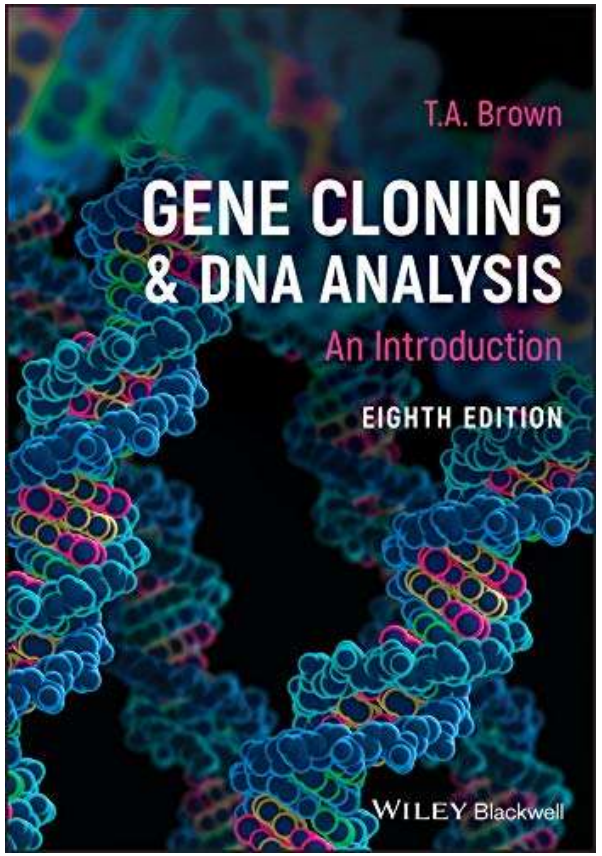
- Seems to cause disruption in cell membrane
- Reconstitution of membrane leads to large pores which allow DNA molecules to enter
- Works for bacteria, yeast and animal cells

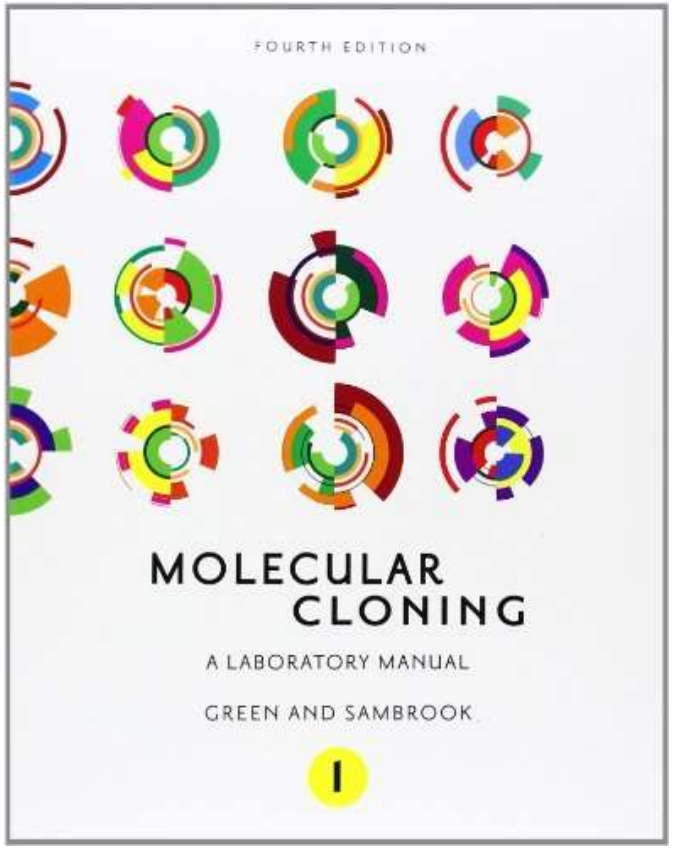


References

- ✓Protocol: https://sci-hub.se/10.1101/pdb.prot093344
- ✓Youtube: Lecture 41 : Isolation of Plasmid DNA – YouTube
  https://www.youtube.com/watch?v=Jyk2RzkxUXw
  https://www.youtube.com/watch?v=O4oLyd2mZv8

Acknowledgement





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