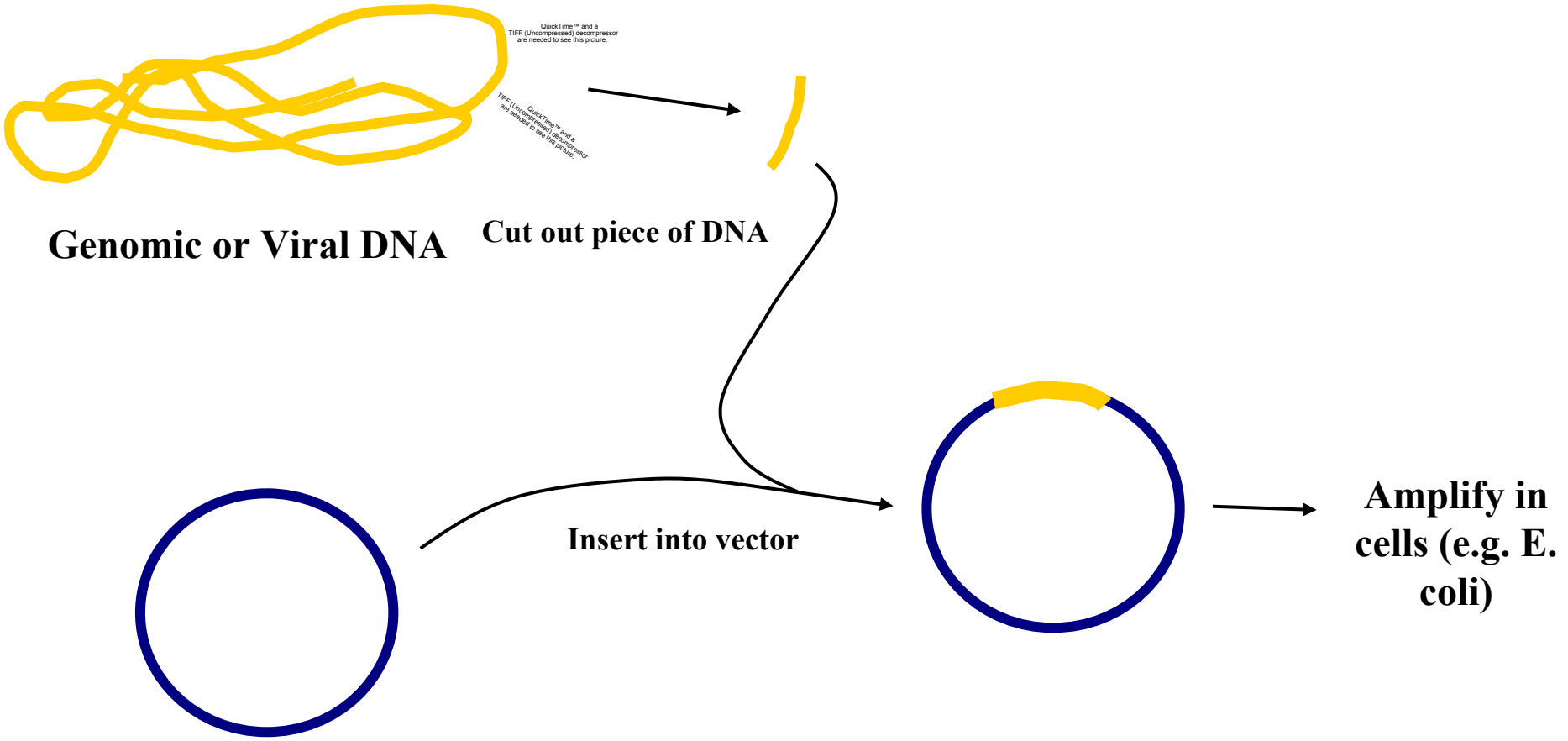
How do you isolate and propagate a piece of DNA (for example a gene)?



Vector (= plasmid or viral DNA that can replicate in a desired organism - often *E. coli*)

Restriction enzymes cleave DNA at specific (usually palindromic) sequences

Table 4.1 Recognition Sequences and Cutting Sites of Selected Restriction Endonucleases

Enzyme	Recognition Sequence*
*Alu*I	A G ↓ C T
*Bam*HI	G ↓ G A T C C
*Bgl*II	A ↓ G A T C T
*Cla*I	A T ↓ C G A T
*Eco*RI	G ↓ A A T T C
*Hae*III	G G ↓ C C
*Hin*dII	G T Py ↓ Pu A C
*Hin*dIII	A ↓ A G C T T
*Hpa*II	C ↓ C G G
*Kpn*I	G G T A C ↓ C
*Mbo*I	↓ G A T C
*Pst*I	C T G C A ↓ G
*Pvu*I	C G A T ↓ C G
*Sal*I	G ↓ T C G A C
*Sma*I	C C C ↓ G G G
*Xma*I	C ↓ C C G G G
*Not*I	G C ↓ G G C C G C

*Only one DNA strand, written 5′ → 3′ left to right is presented, but restriction endonucleases actually cut double-stranded DNA as illustrated in the text for *Eco*RI. The cutting site for each enzyme is represented by an arrow.

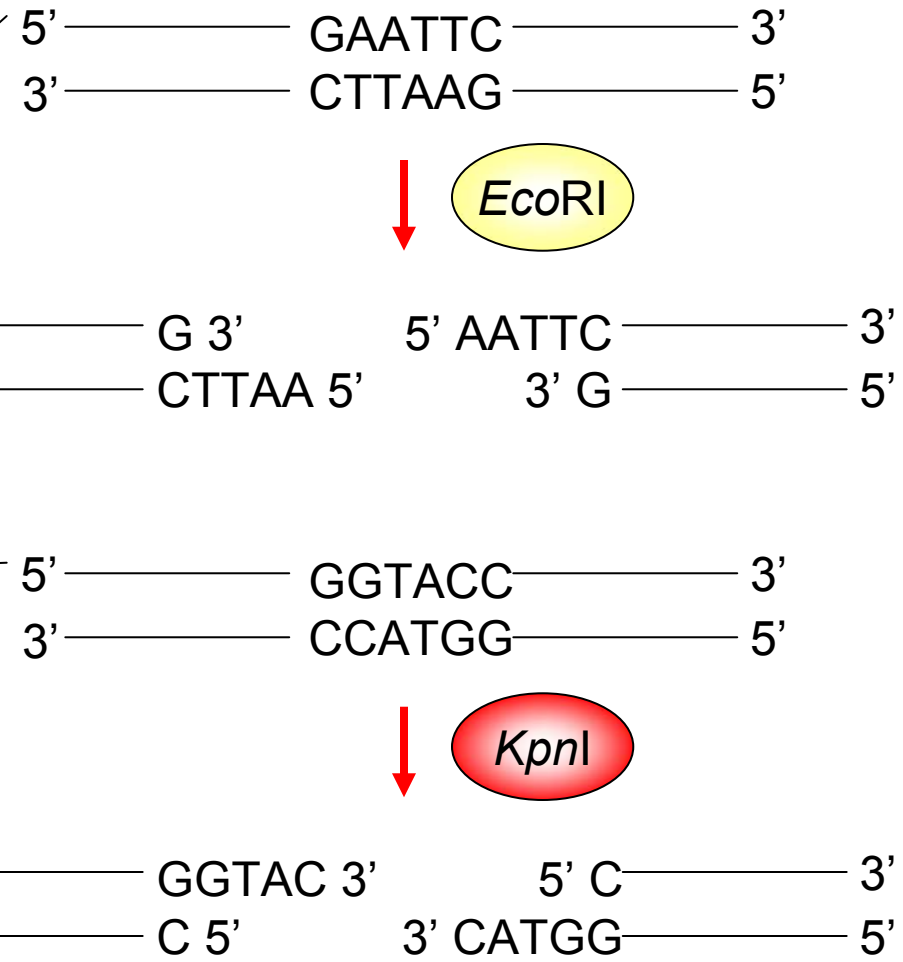
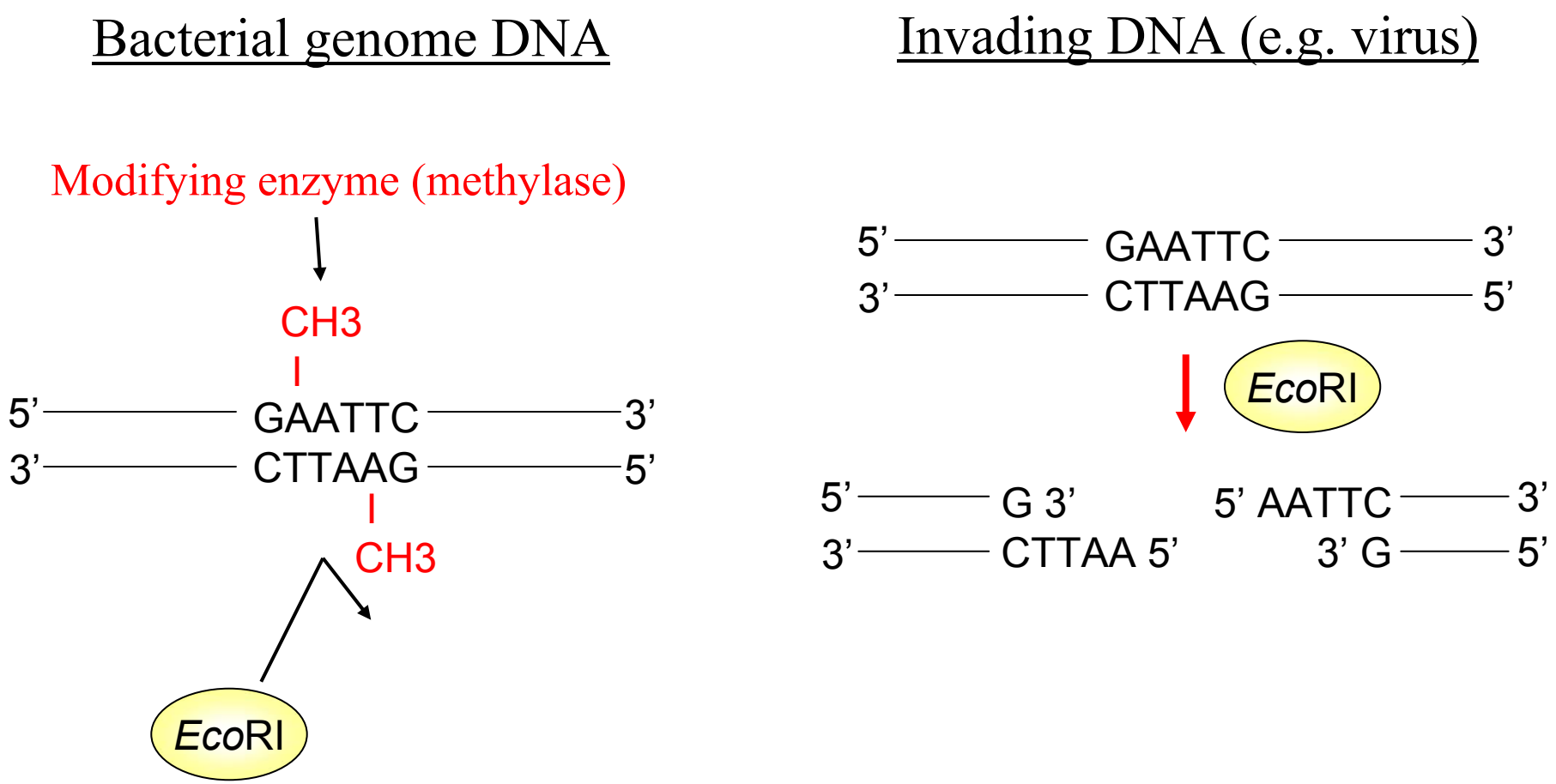


Table 4.1

Many bacteria contain restriction-modification systems to "restrict" invasion by foreign DNA



Based on Fig. 4.1

Ligating a DNA fragment to a vector

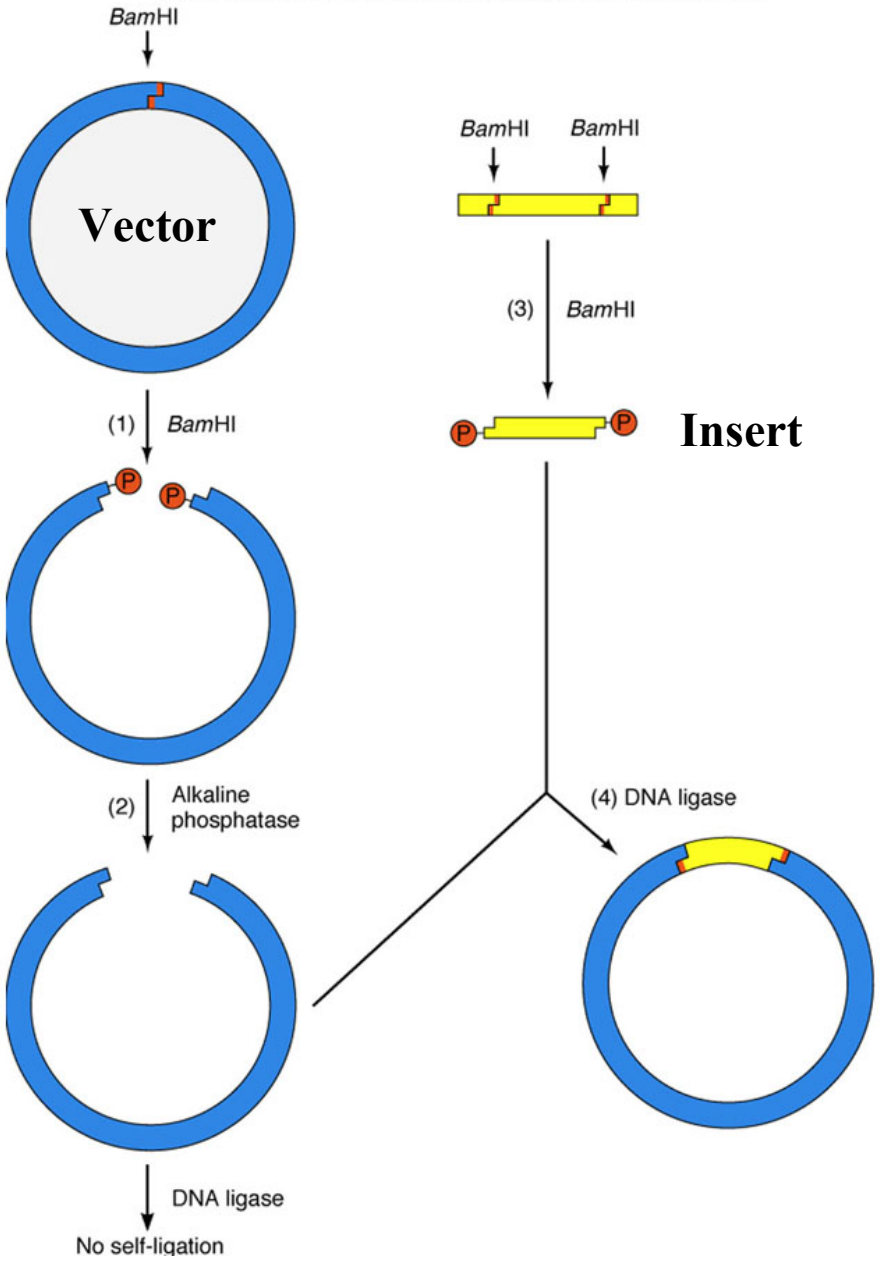


Fig. 4.7

Obtaining bacterial clones with your recombinant plasmid

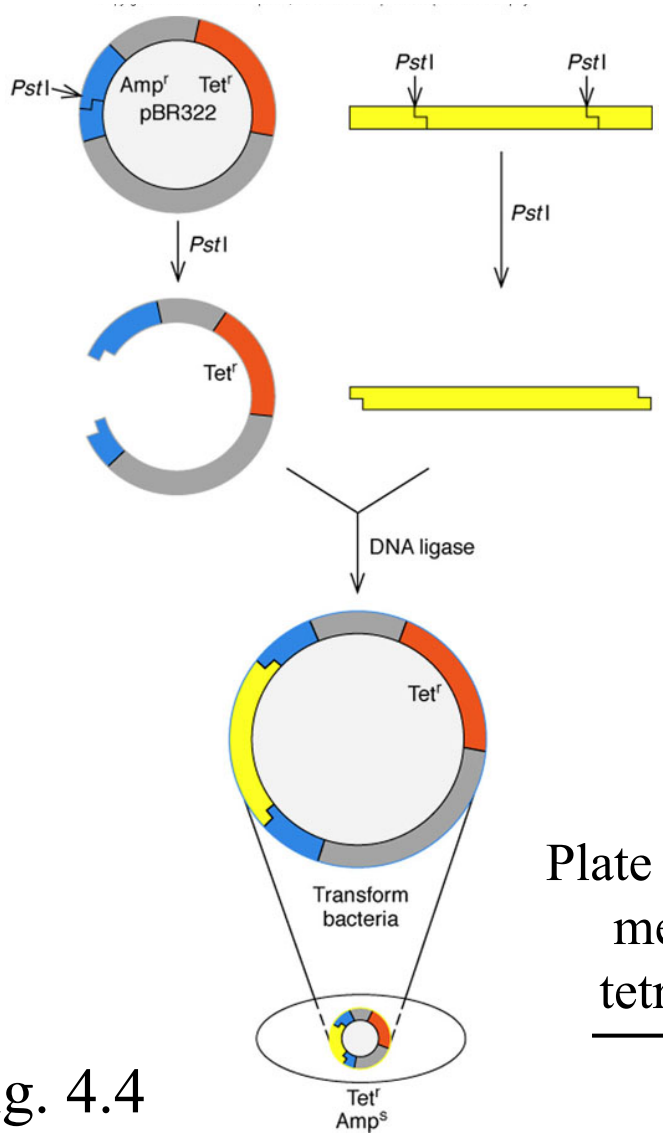
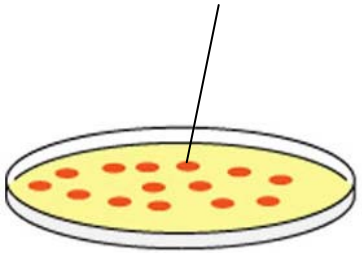


Fig. 4.4

Plate on selective media (e.g. tetracycline)

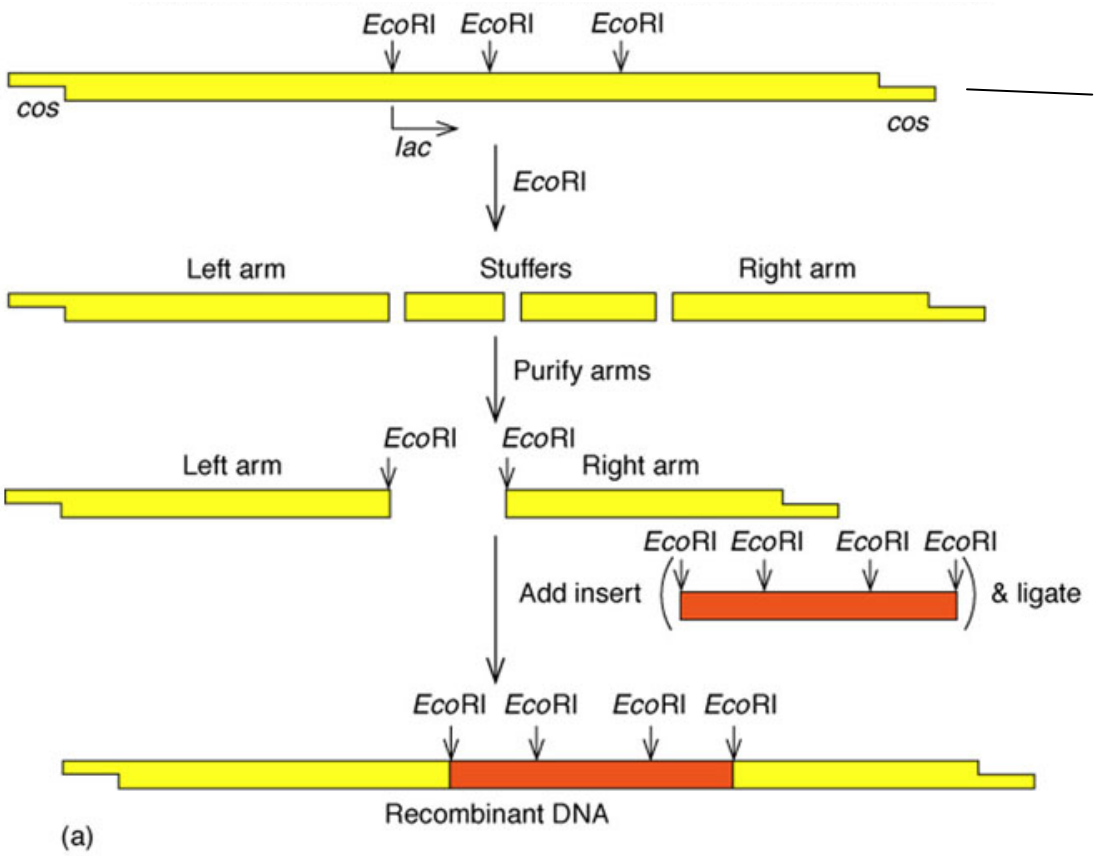


Bacterial colonies containing your plasmid (only plasmid-containing bacteria will survive on tetracycline)

Pick a colony - you have your clone!

- Clicker Question -

Bacteriophages can be used instead of plasmids as cloning vectors (can take larger inserts)



(Bacterio)phage lambda

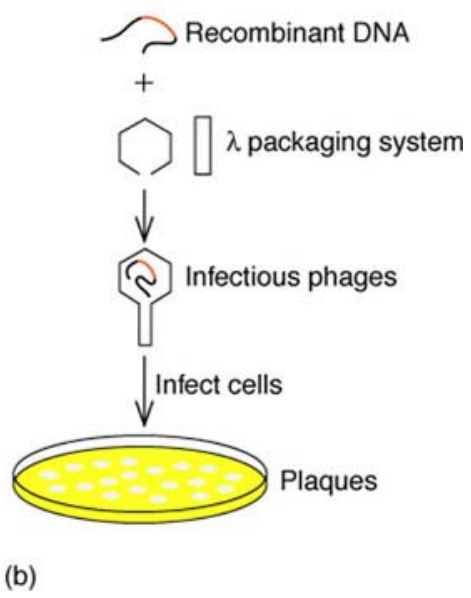
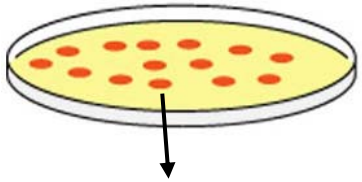


Fig. 4.8

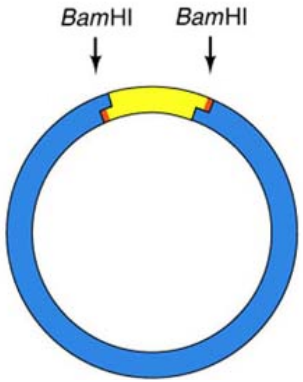
How do you know your bacterial clone contains your plasmid (and not e.g. self-ligated plasmid)?

Analysis by restriction digestion



Pick a colony (and expand in selective medium)

Isolate plasmid



Restriction digest (e.g. BamHI)

Analyze by agarose gel electrophoresis

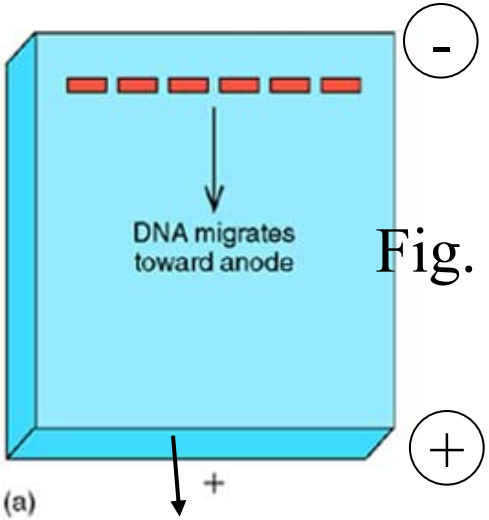
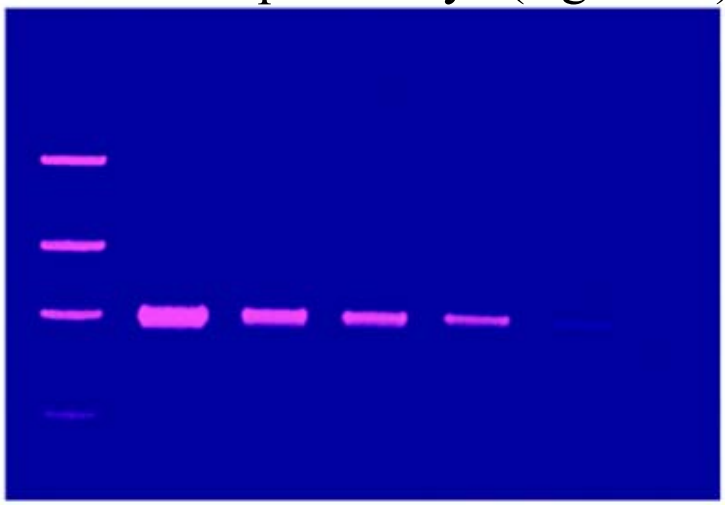


Fig. 5.1

Stain with DNA-specific dye (e.g. EtBr)

Large DNA

Small DNA

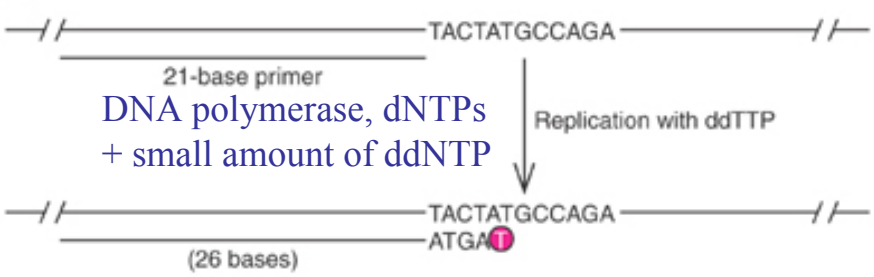


- Clicker Question -

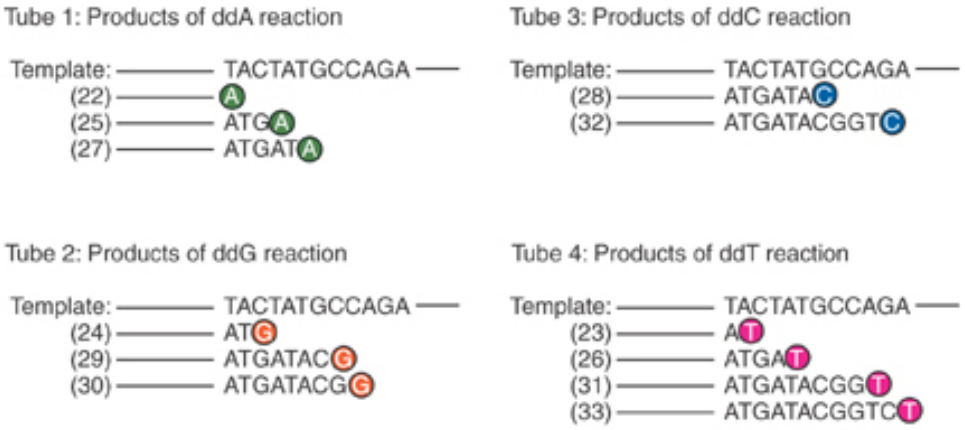
How do you know your bacterial clone contains your plasmid ?

Sanger dideoxy DNA sequencing

(a) Primer extension reaction



(b) Products of the four reactions



(c) Electrophoresis of the products

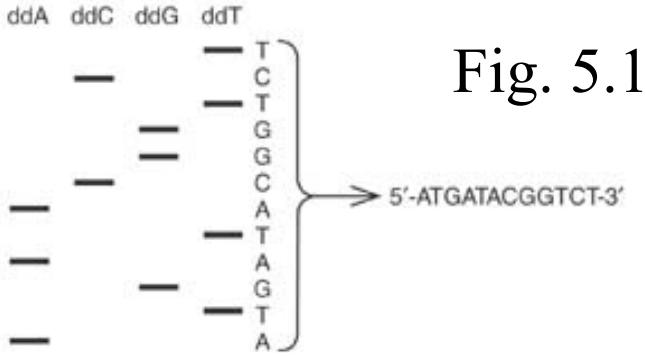


Fig. 5.18

Detection by ^{32}P-labeling (autorad)

or

By fluorophor labeling (fluorescence)

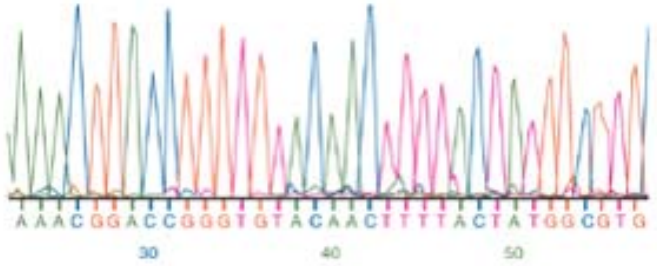
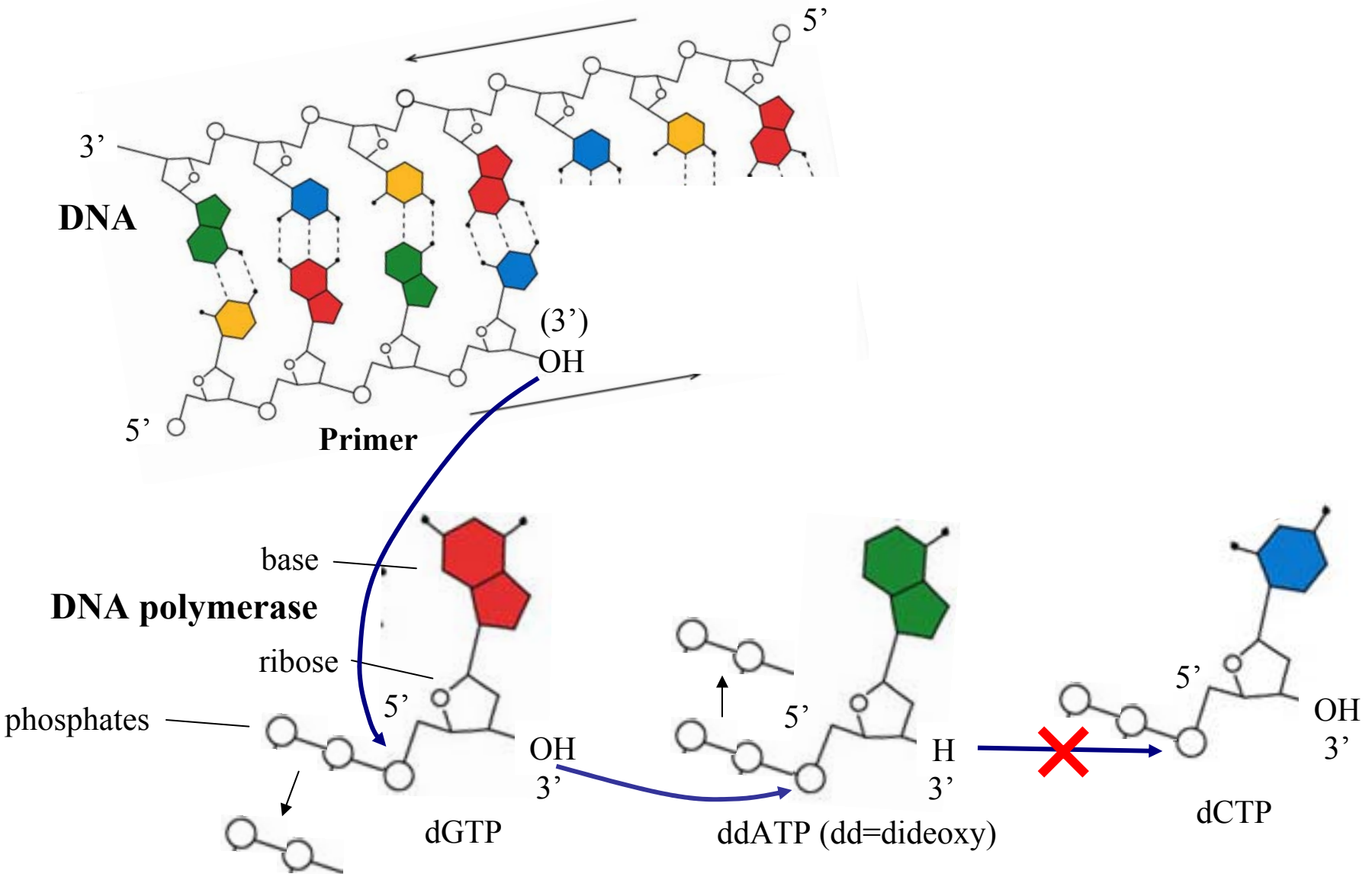


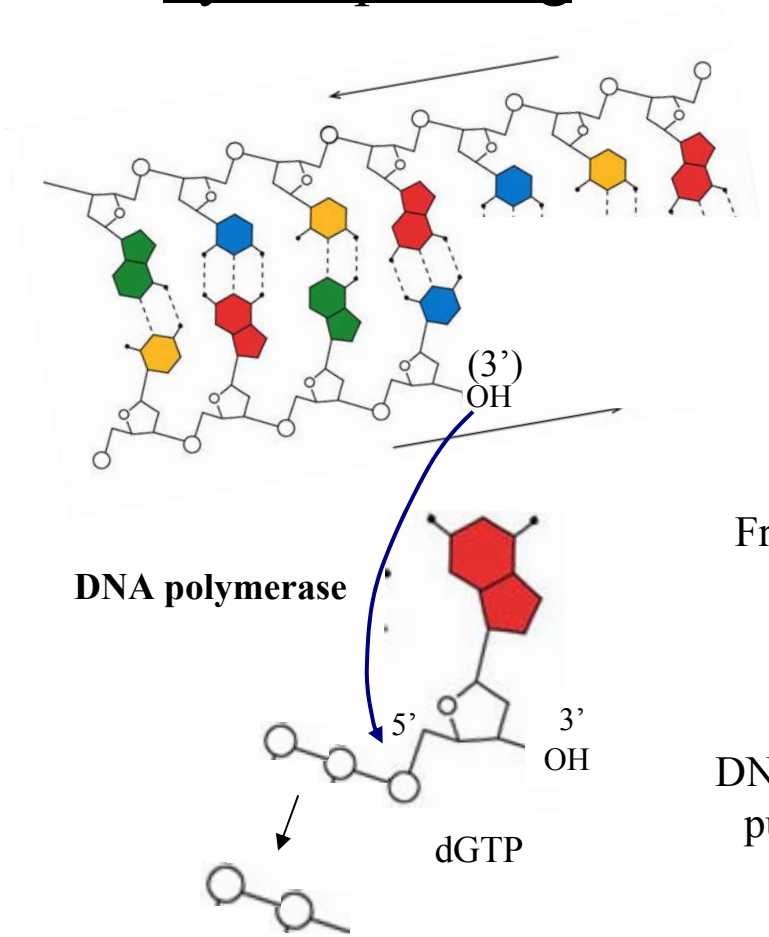
Fig. 5.20

How do dideoxynucleotides terminate DNA polymerization?



Latest technology: Deep sequencing

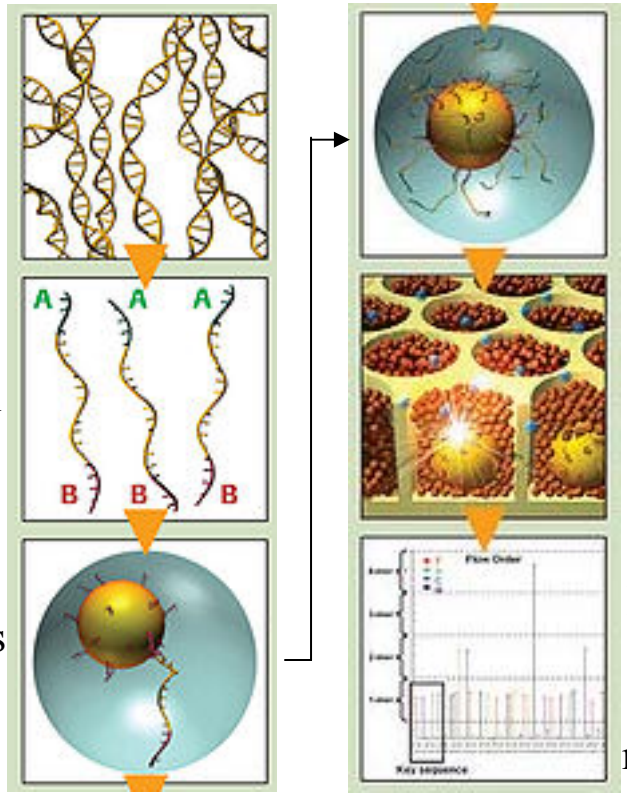
Pyrosequencing



Diphosphate (pyrophosphate) release is measured

dNTPs added one at a time - only correct dNTP causes release of pyrophosphate

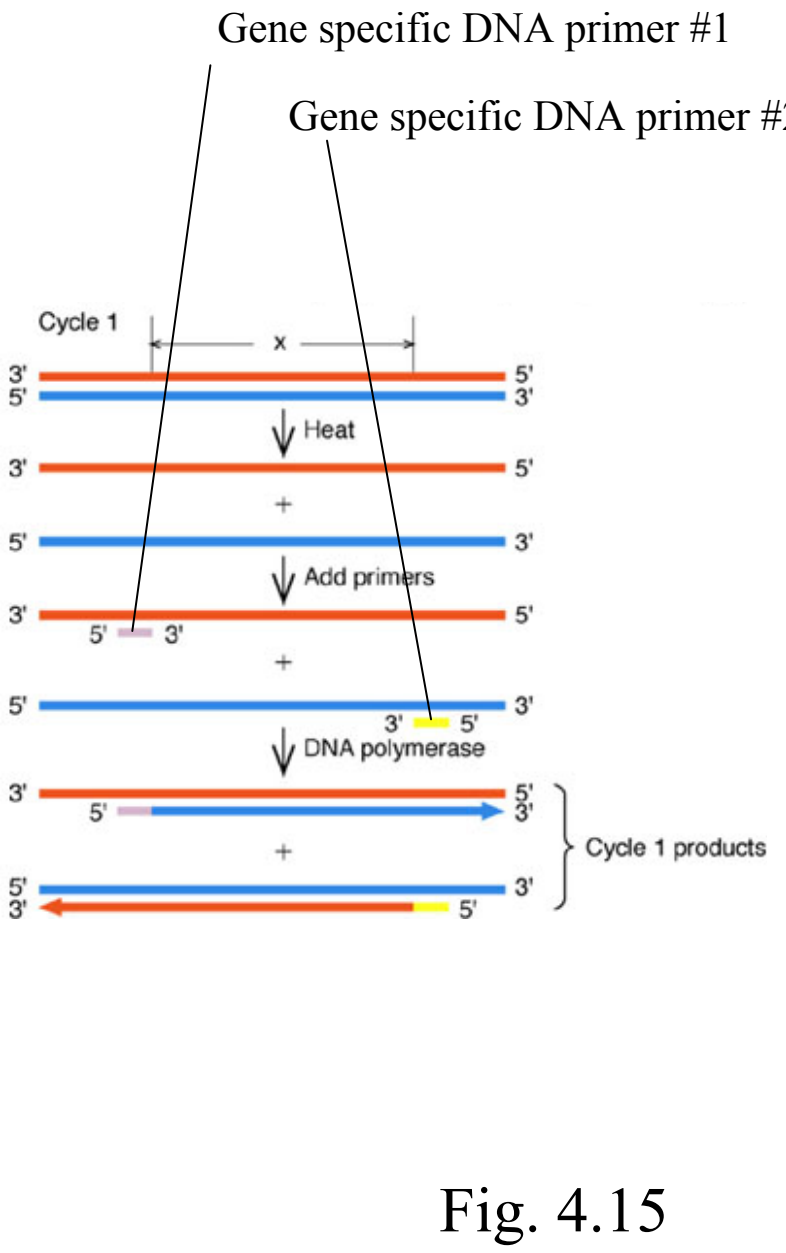
Deep sequencing



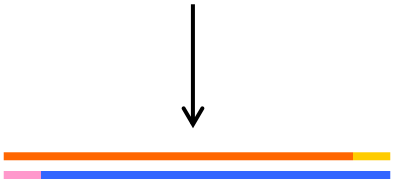
Currently sequences ≈500 million base pairs in 10 hours

How do you obtain your insert DNA (e.g. a gene)?

The polymerase chain reaction (PCR) for amplifying DNA



Cycles 3,4 etc…



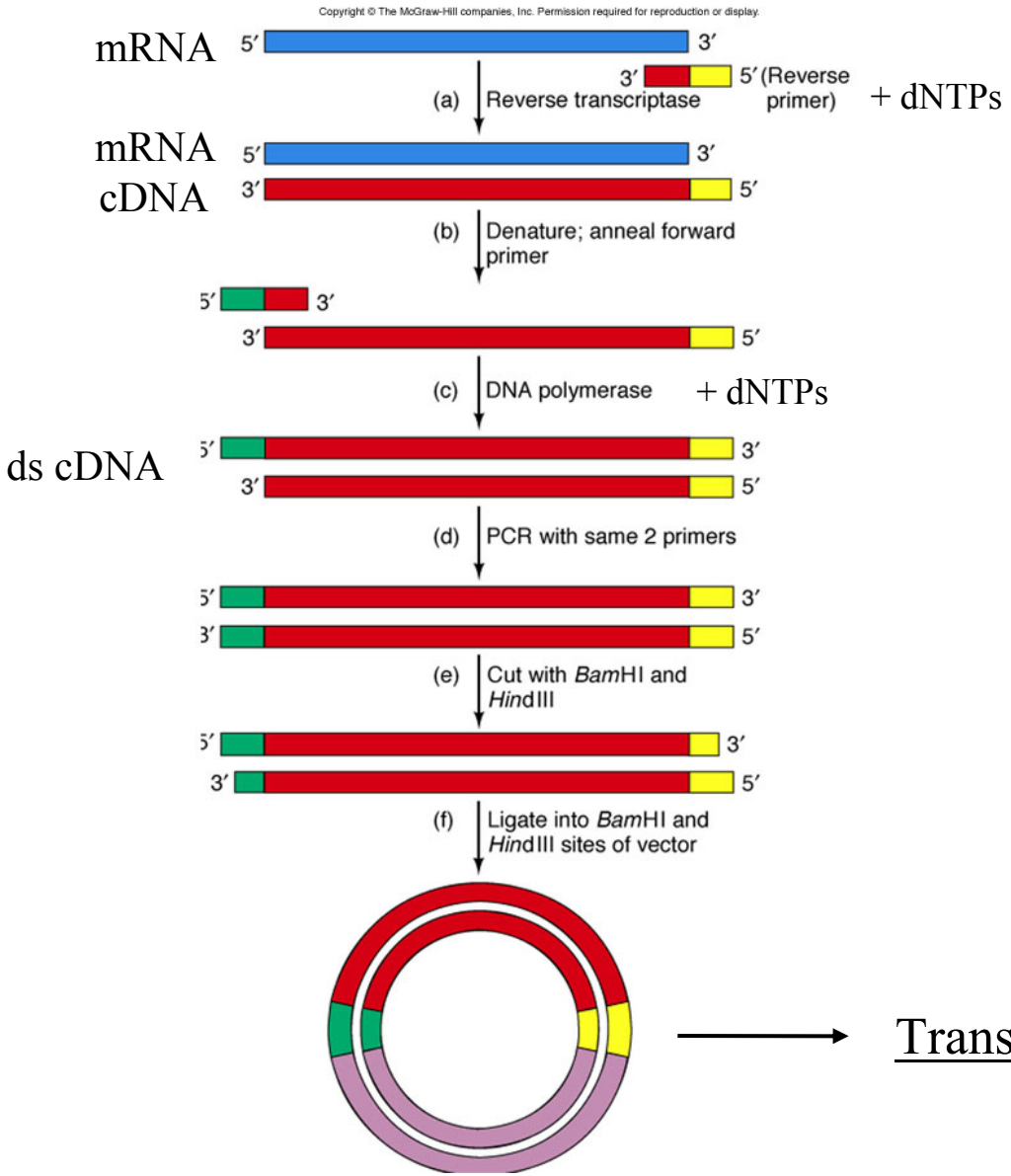
Final product

Max yield:
[start conc] * $2^{\#cycles}$
e.g: 30 cycles starting with one molecule of DNA => $1 * 2^{30}$ molecules ≈ 10^9 (one billion).

Fig. 4.15

How many cycles of PCR would (minimally) be required to amplify a piece of DNA one million times?

Using reverse transcriptase (RT)-PCR for amplifying a specific copy DNA (cDNA)



:Restriction site (e.g. BamHI)

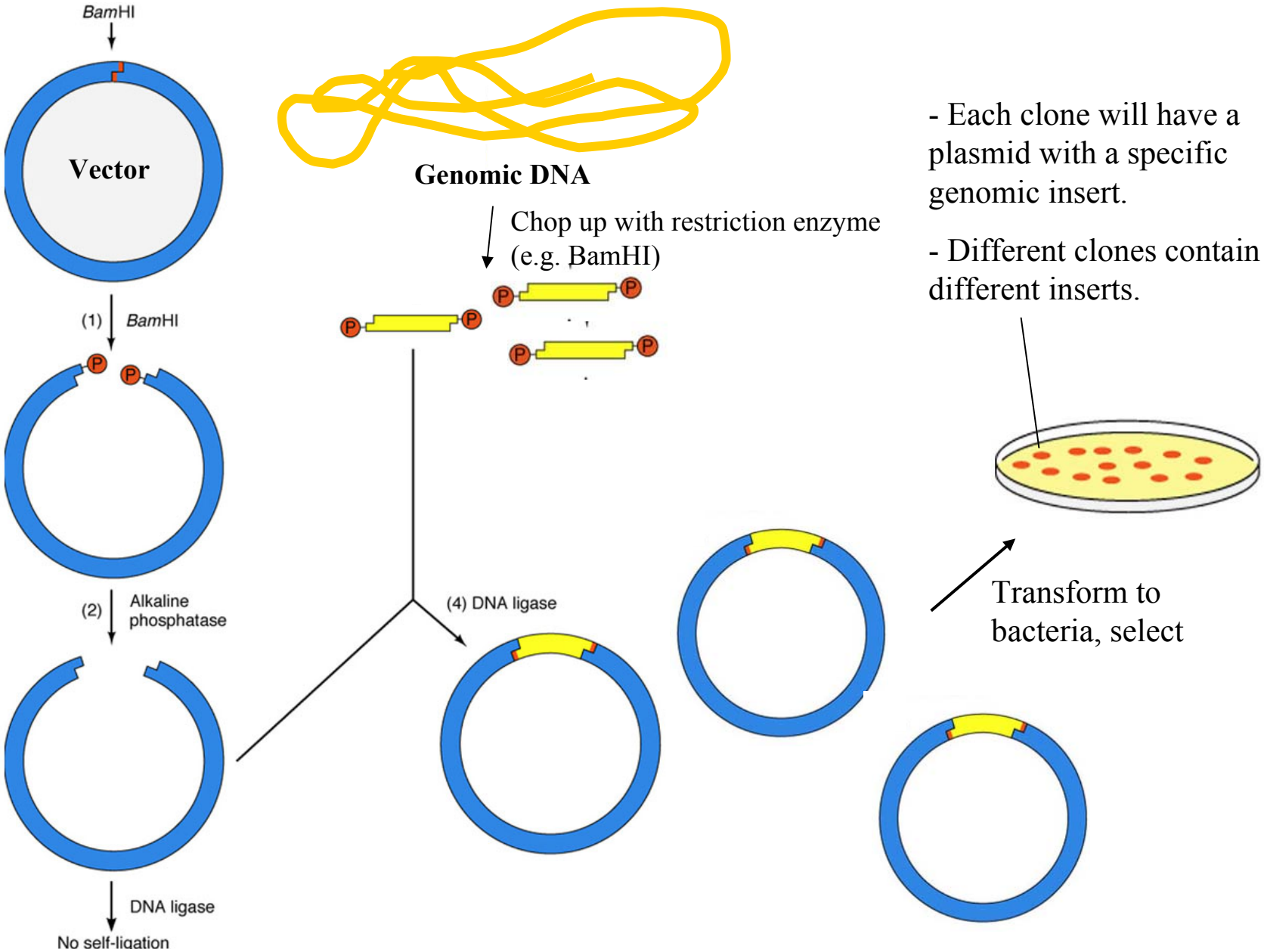
:Restriction site (e.g. HindIII)

Transform to bacteria, select

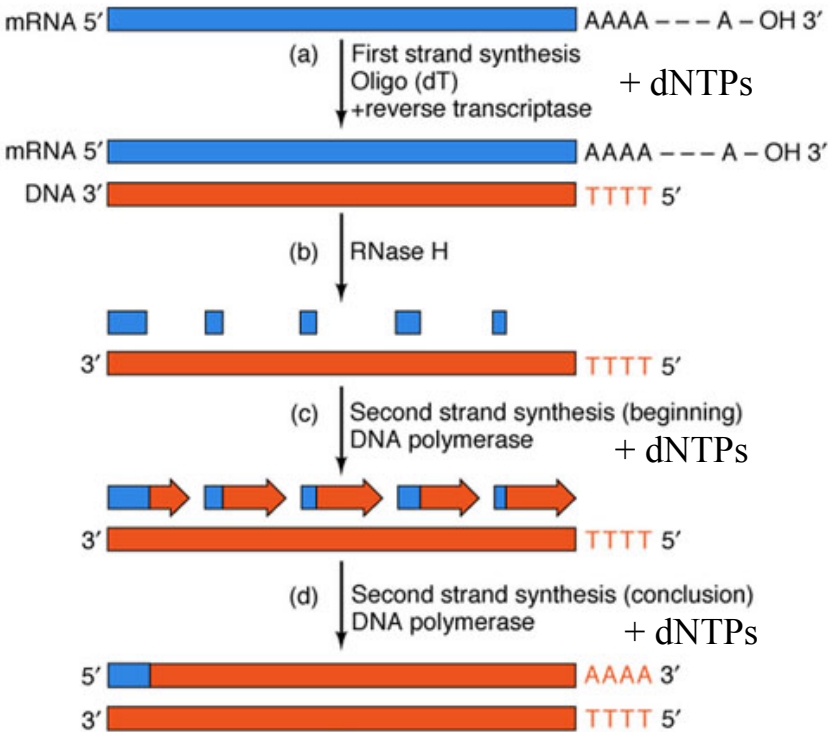
Fig. 4.16

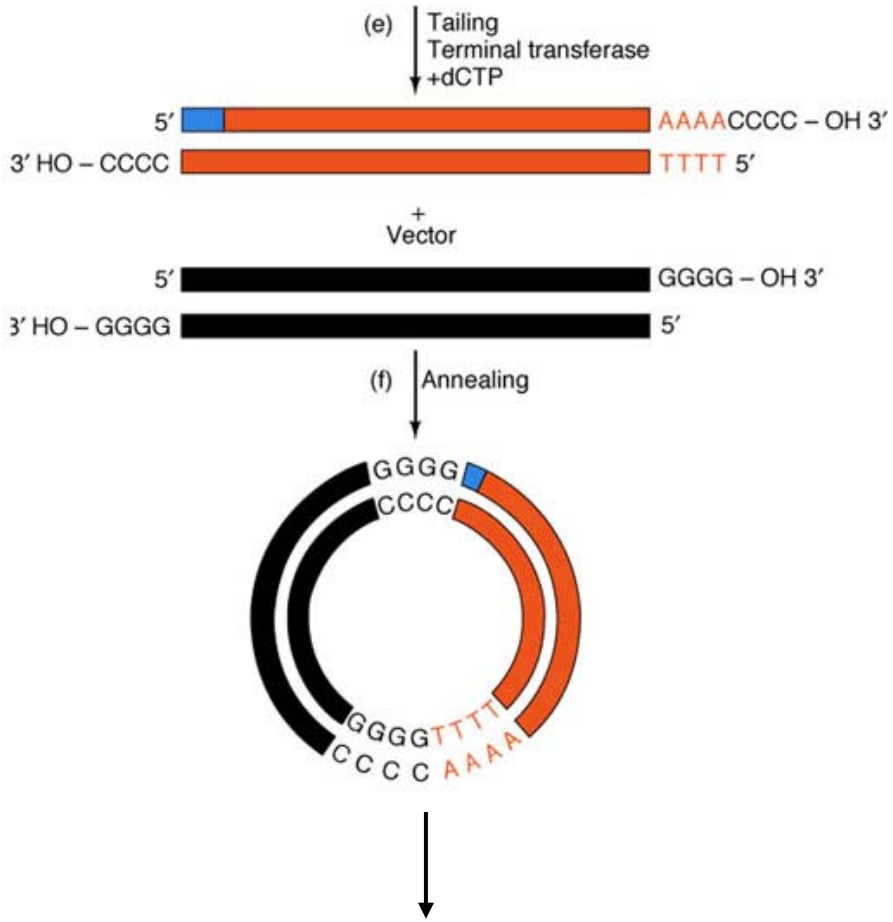
- Clicker Question -

Creating a genomic library



Creating a cDNA library

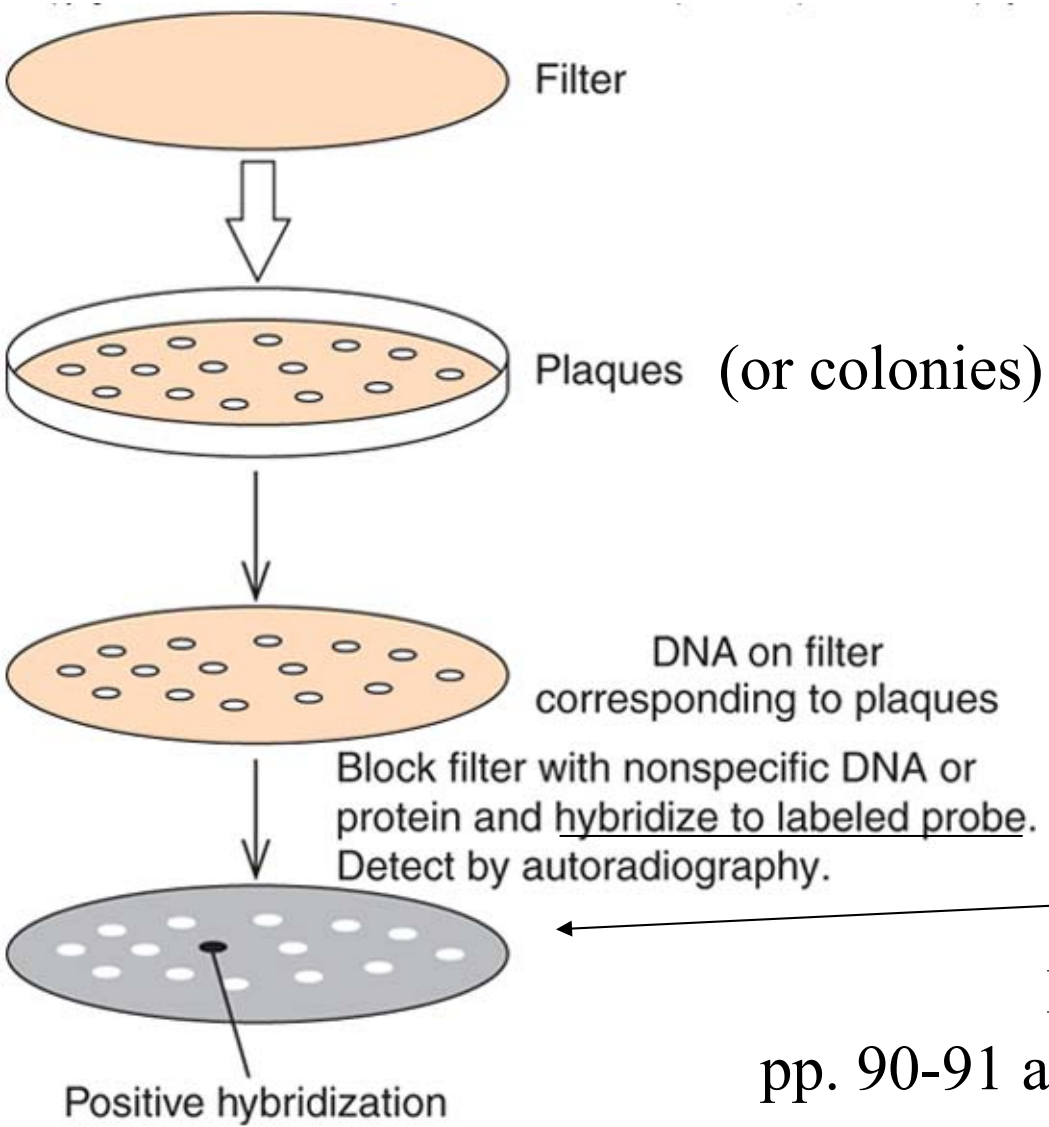




Transform to bacteria, select

Fig. 4.12

Finding the colony that contains a plasmid with your gene of interest in a library

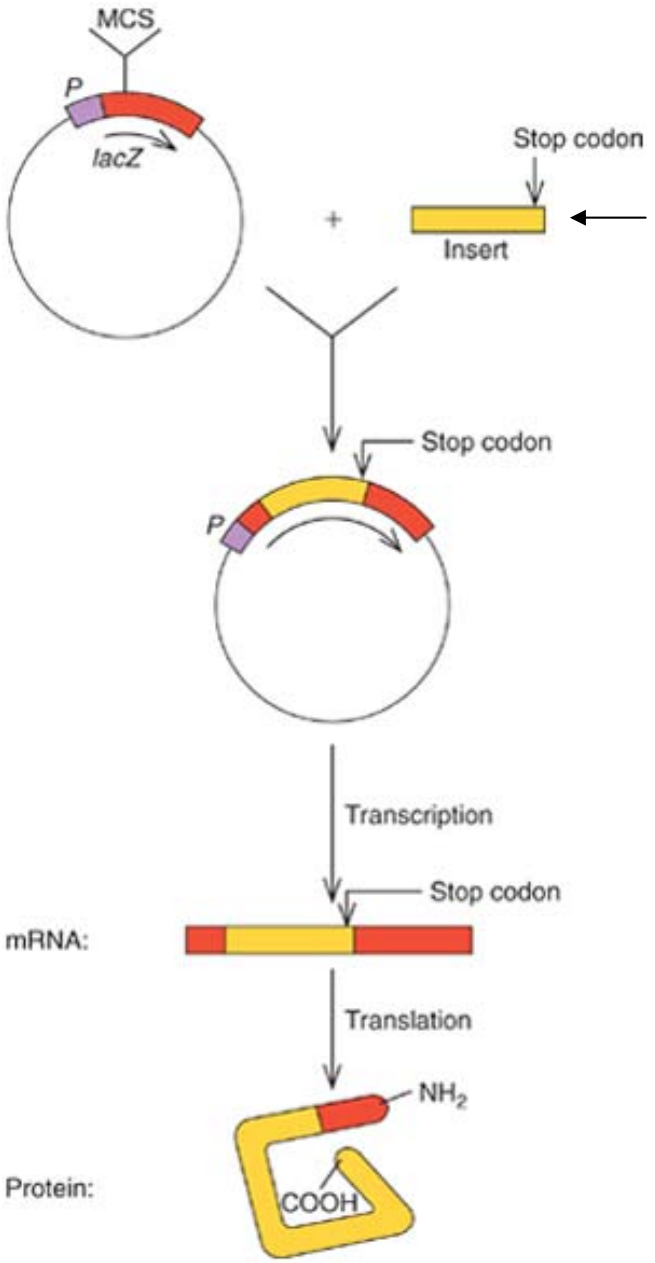


^{32}P-labeled probe (DNA or RNA) corresponding to your gene of interest

Fig. 4.9 (see also Chapter 5 pp. 90-91 and 93-94 for hybridization)

- Clicker Question -

Expression of recombinant protein in bacteria



Protein coding region
(Eukaryotic cDNA or bacterial DNA)

Fig. 4.18

How do you know your protein is expressed?

SDS-PAGE
(PolyAcrylamide Gel Electrophoresis)

Protein + SDS

Stain for proteins using a protein-binding dye (e.g. coomassie blue)

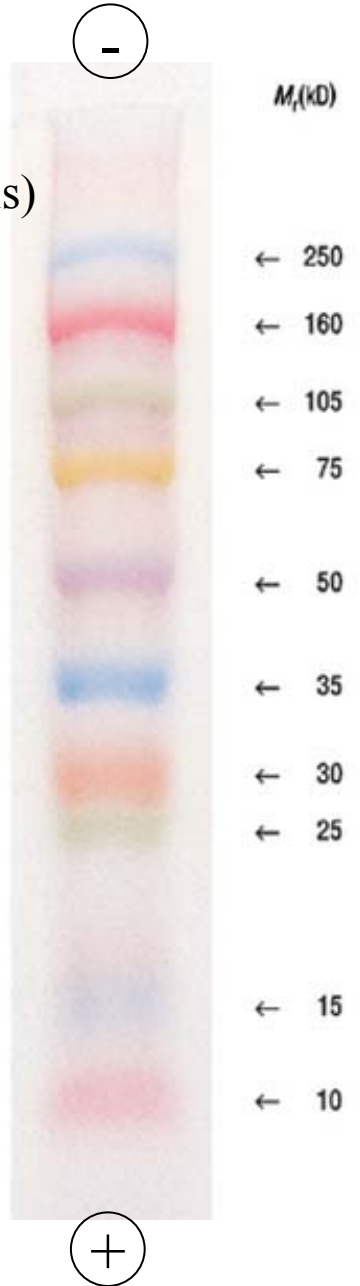
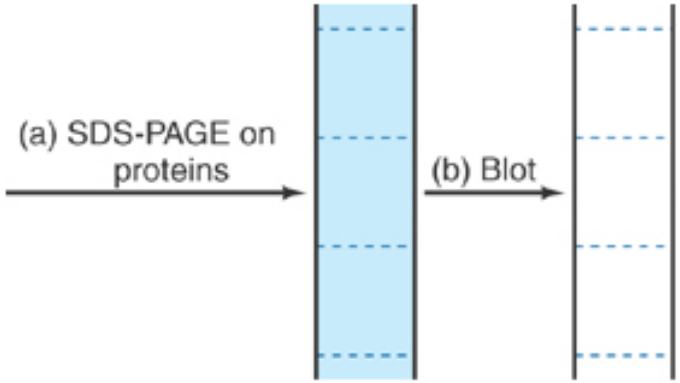


Fig. 5.4

Immunoblotting
(Western blotting)



blot: transfer protein from gel to a membrane

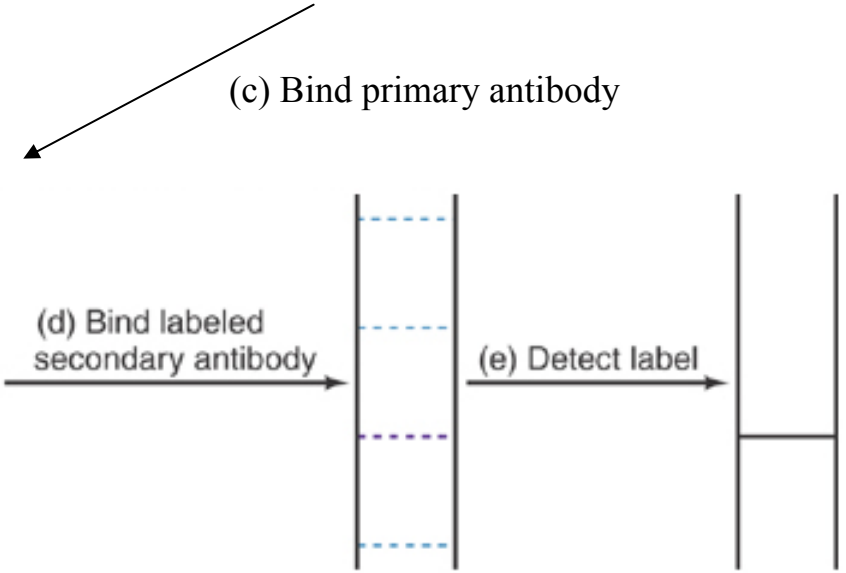
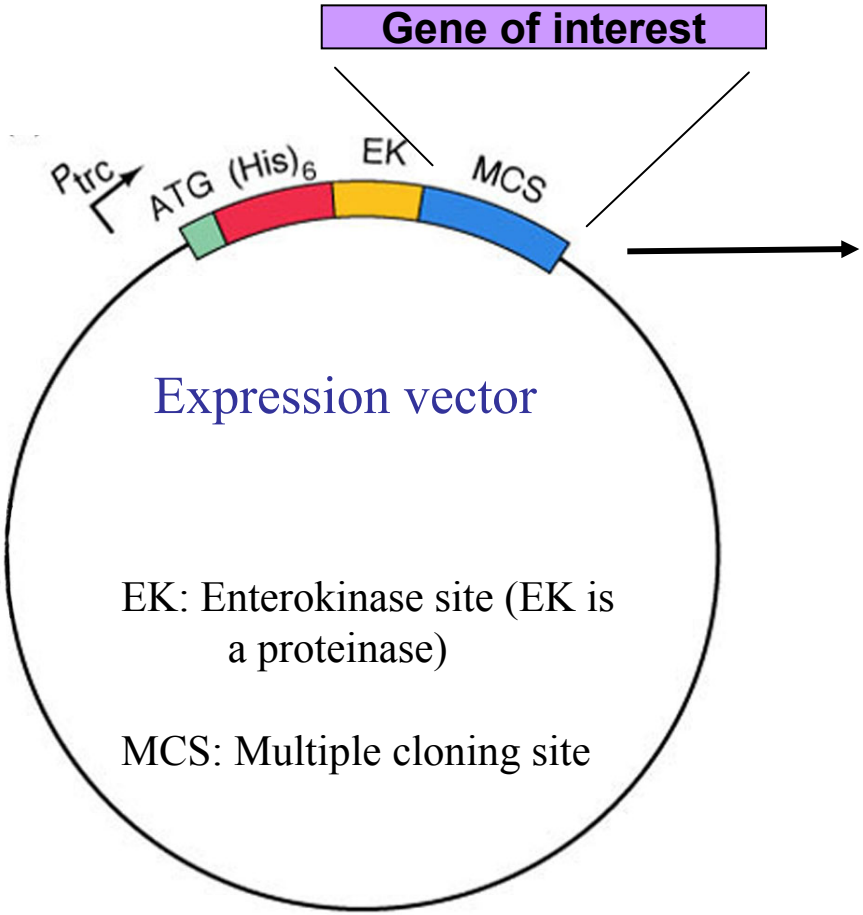
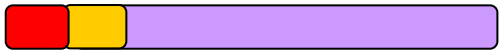


Fig. 5.17

How do you purify your protein?
The use of purification tags



Fusion protein:



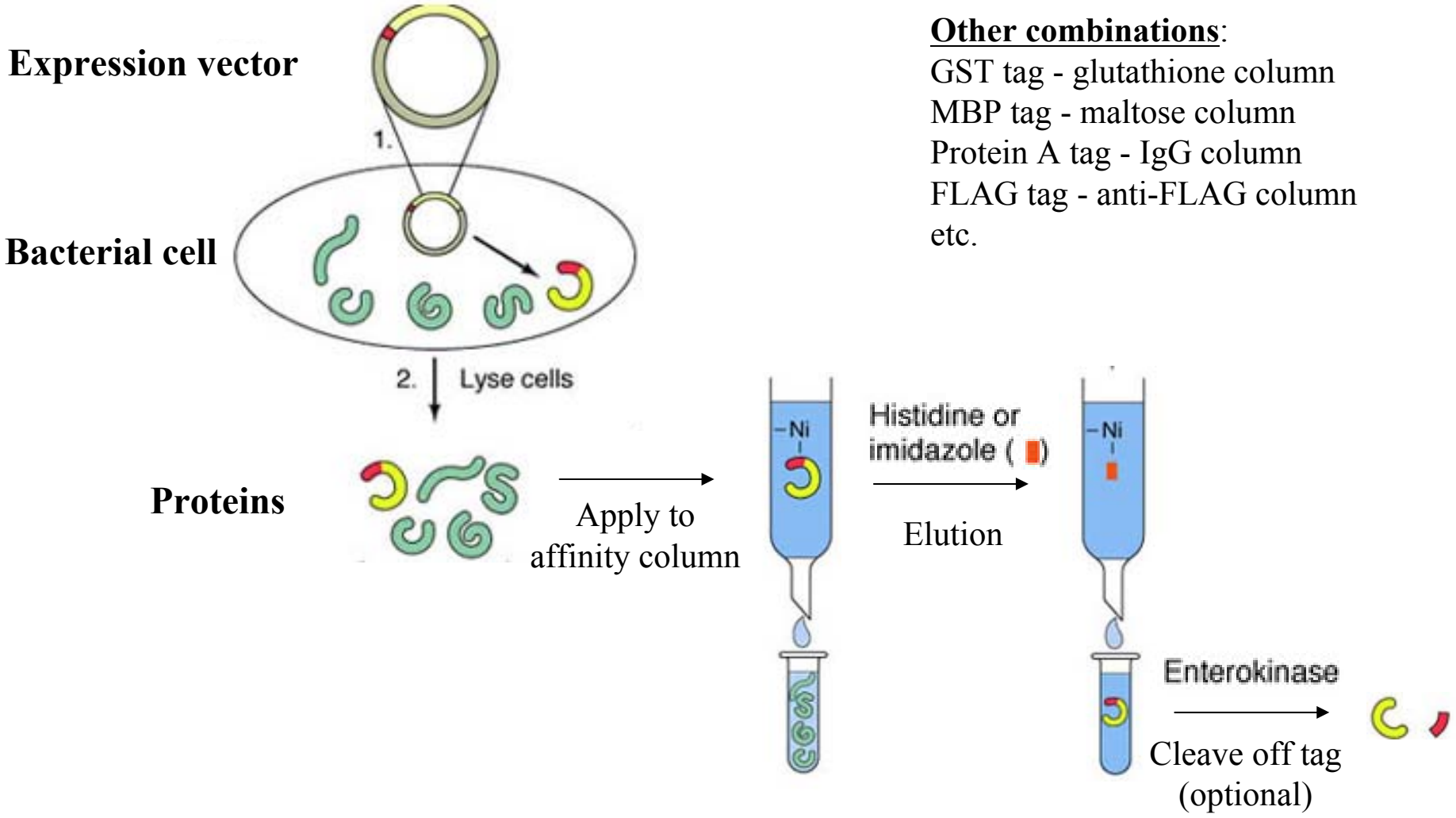
His_6 Protein of interest

His_6: Six histidines - purification tag (has high affinity for Ni^{2+})

Many other tags can be used, e.g. GST, MBP, Protein A etc....

Fig. 4.20

Using affinity chromatography to purify your protein (e.g. His_6 tag and Ni^{2+} column)



Other combinations:
GST tag - glutathione column
MBP tag - maltose column
Protein A tag - IgG column
FLAG tag - anti-FLAG column
etc.

Modified Fig. 4.20 (see also Chapter 5, pp. 88-90)

The usage of inducible promoters (e.g. arabinose-regulated)

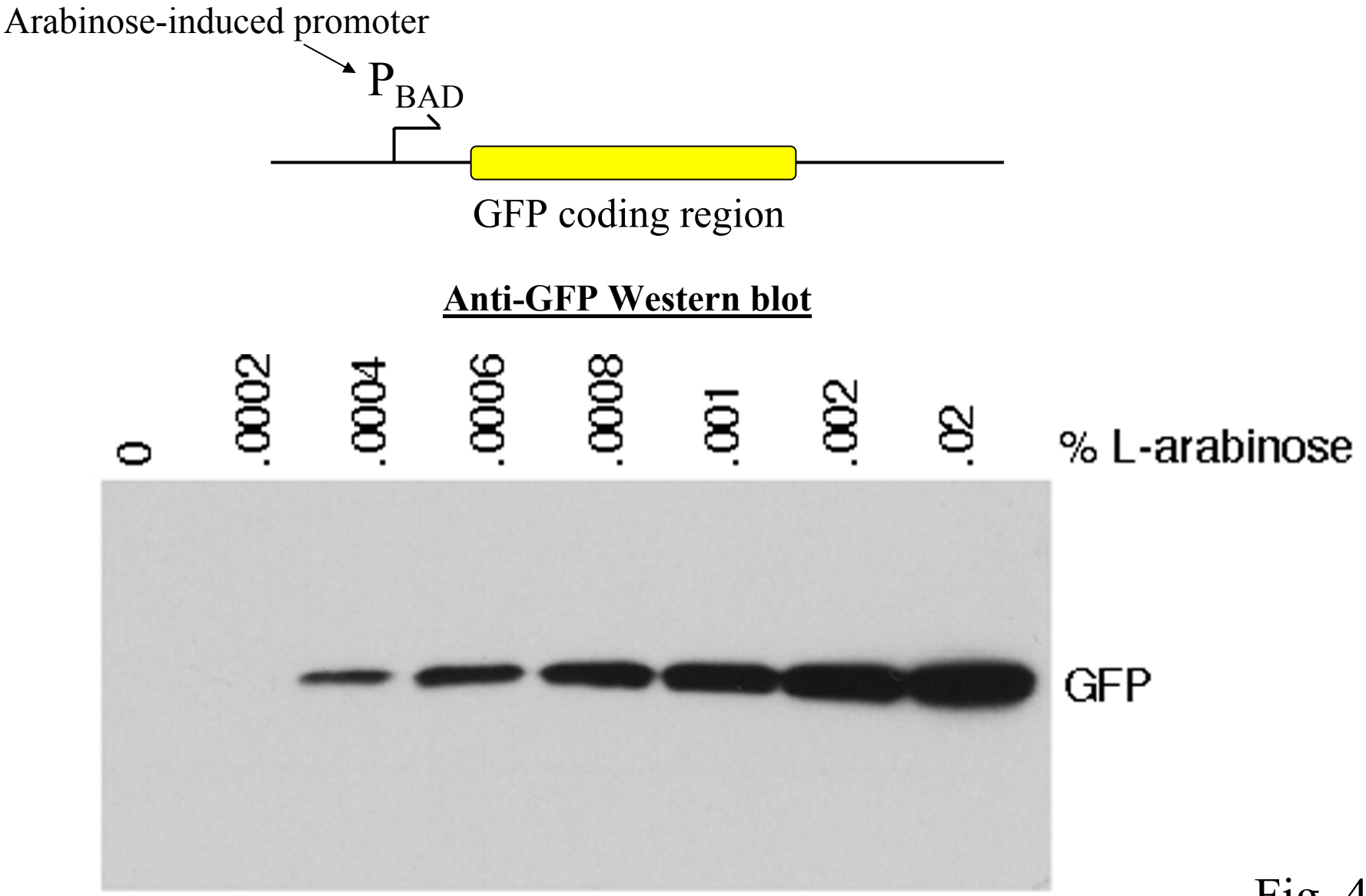
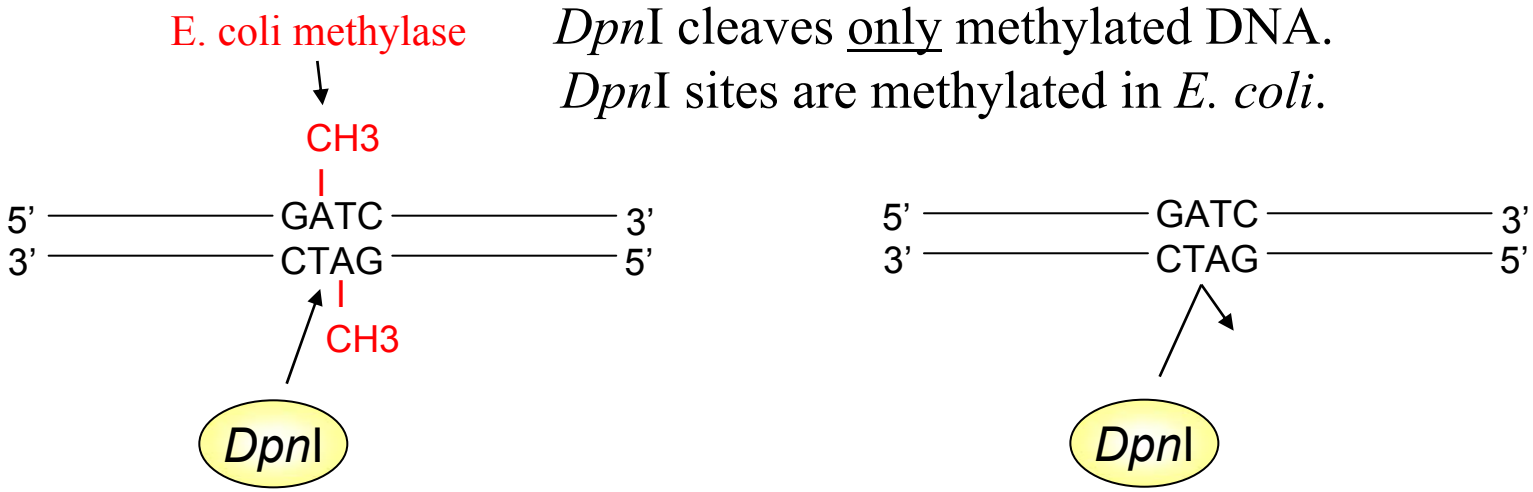


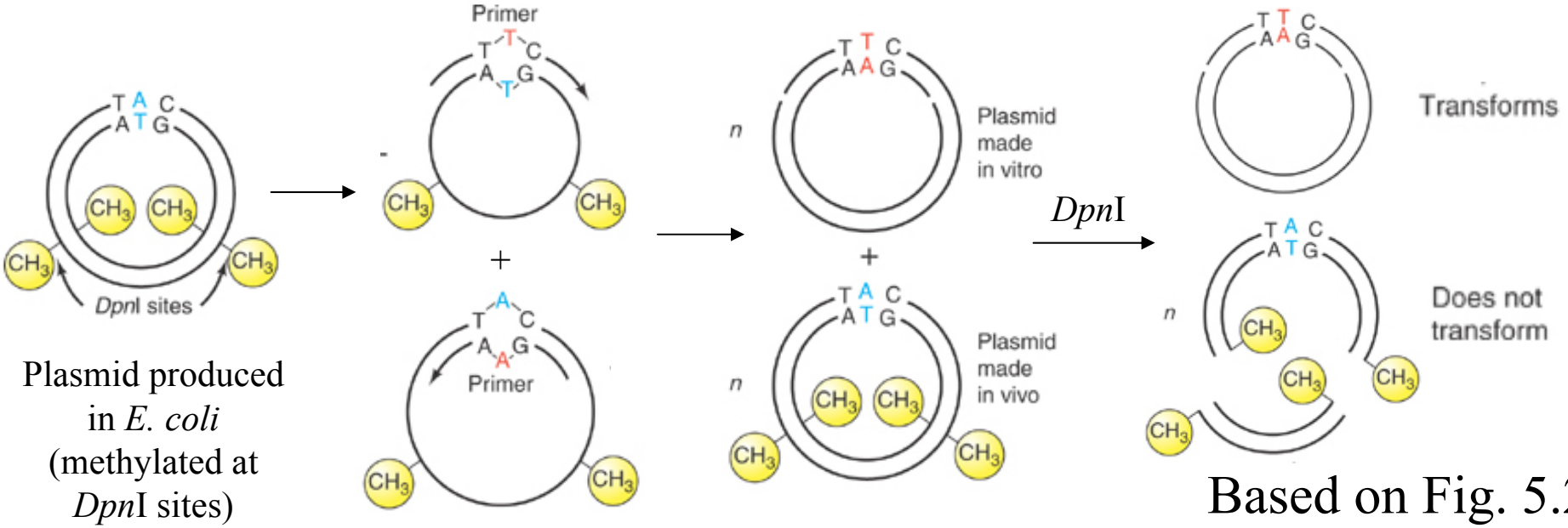
Fig. 4.19

- Clicker Question -

Commonly used procedure to create point mutations



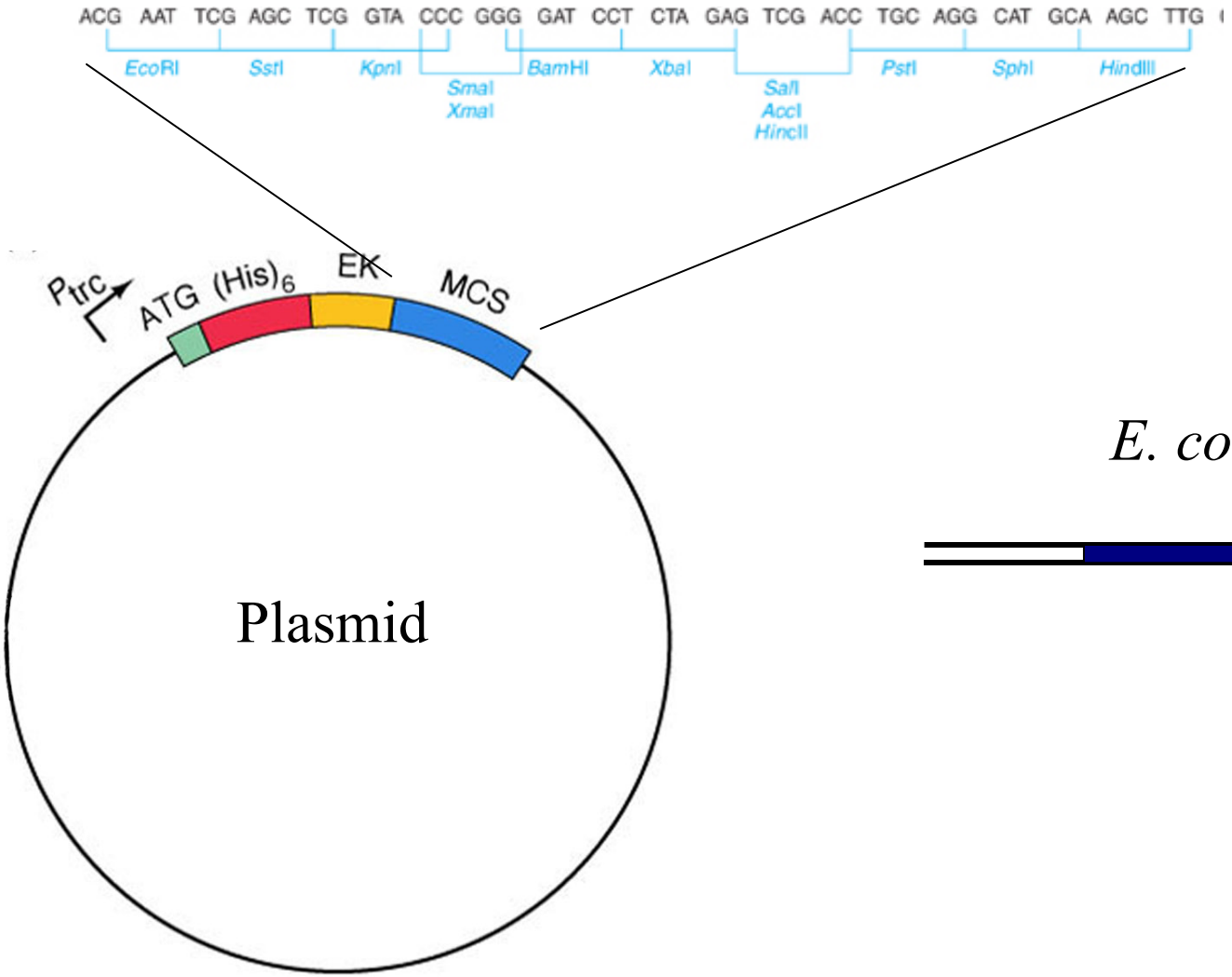
PCR with mutagenic primers



Plasmid produced in *E. coli* (methylated at *Dpn*I sites)

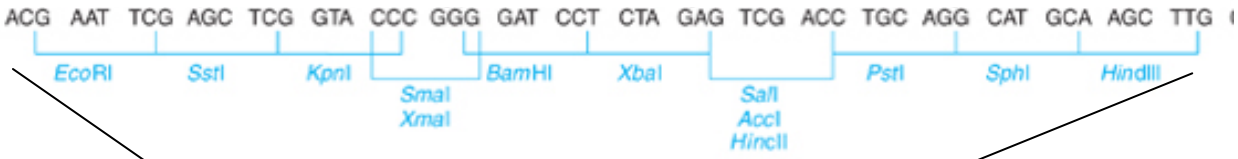
Based on Fig. 5.25

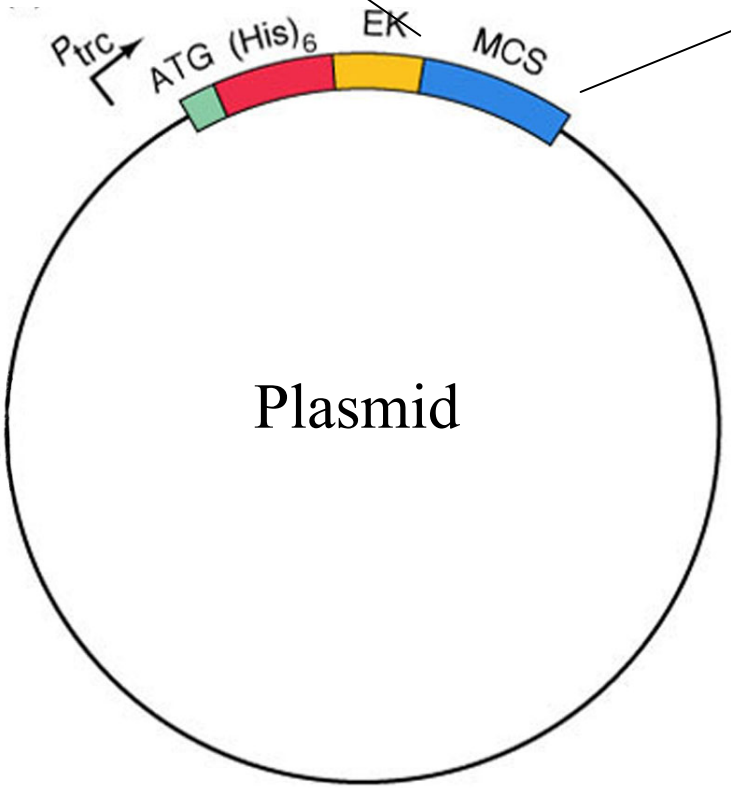
How would you make a plasmid for bacterial expression of an *E. coli* protein in fusion with His_6?



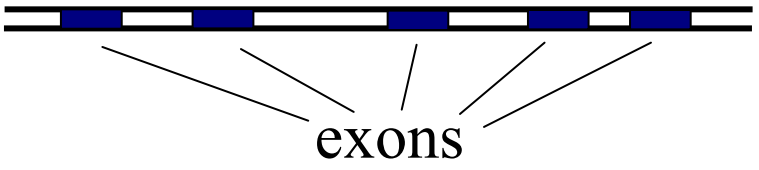
E. coli gene

How would you make a plasmid for bacterial expression of a human protein in fusion with His_6?





Human gene



Vectors exist for gene expression in eukaryotic cells

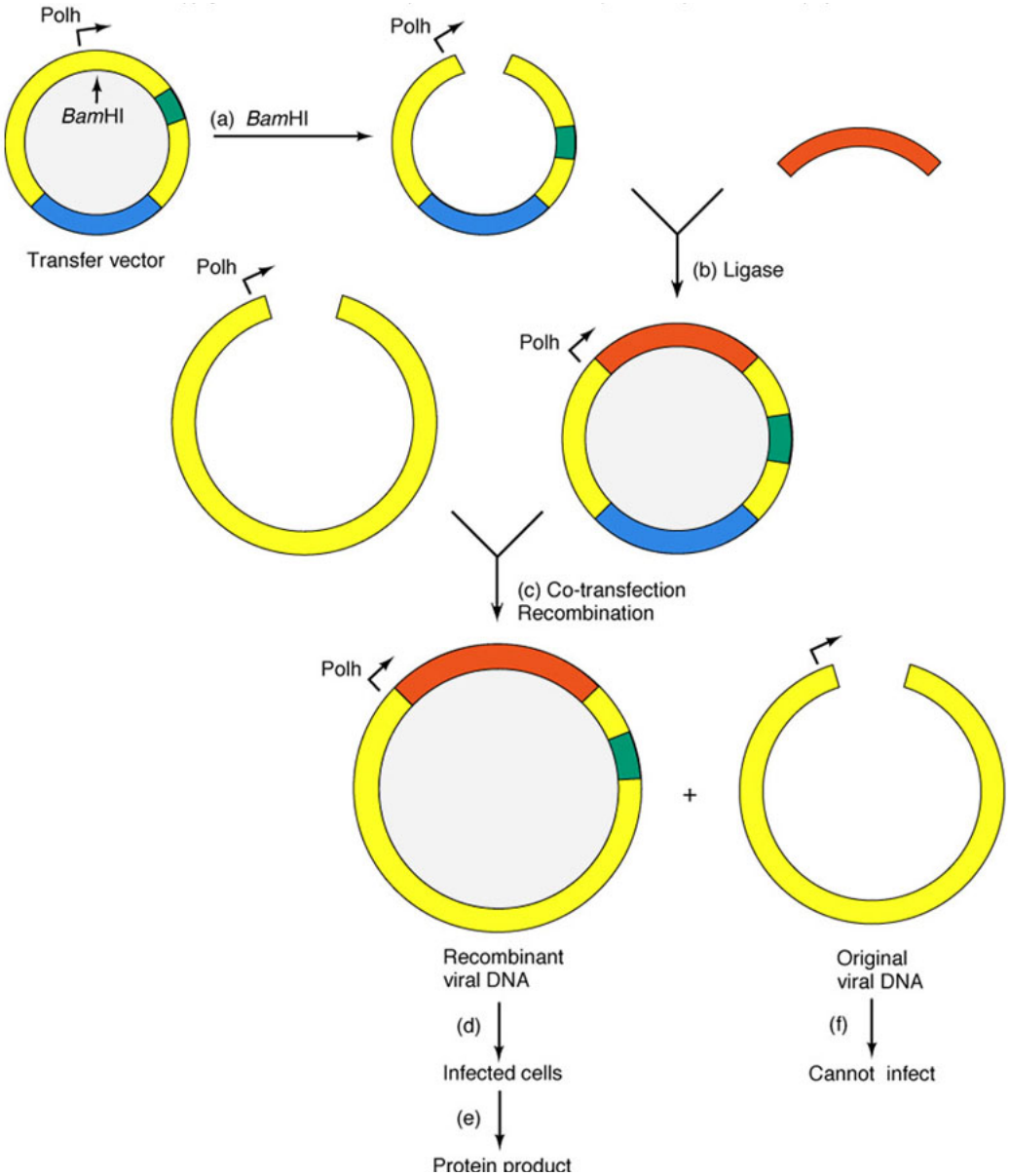


Fig. 4.23

- Clicker Question -