Lecture 8

Bacteriophage Cloning vectors

Bacteriophage

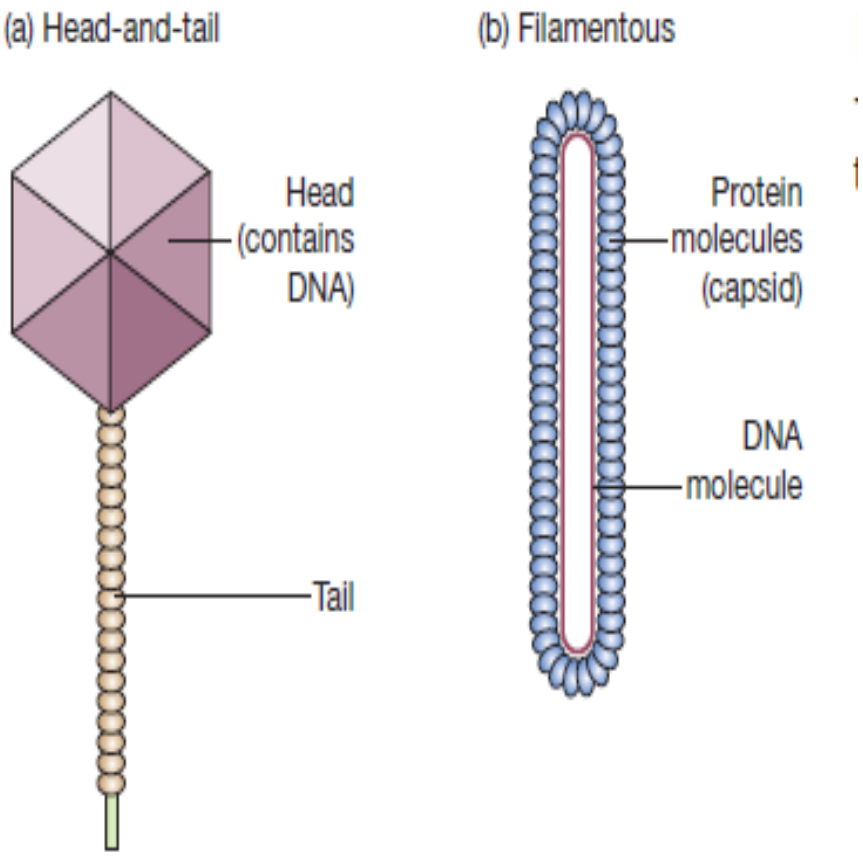


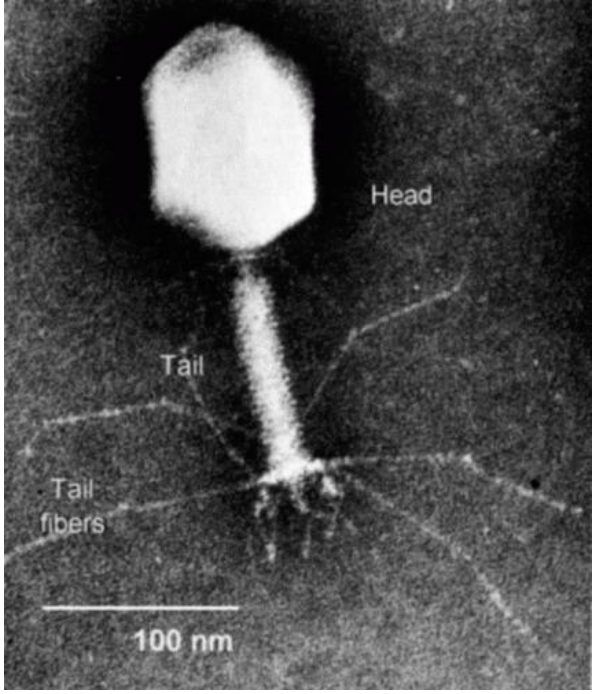
Figure 2.5

The two main types of phage structure: (a) head-and-tail (e.g. λ); (b) filamentous (e.g. M13).

- Plasmid Vectors
- Lambda Vectors
- Selection of Recombinants
- Cosmid Vectors
- M13 Vectors- (ssDNA prep)
- Phagemid Vectors
- BAC vectors
- YAC vectors

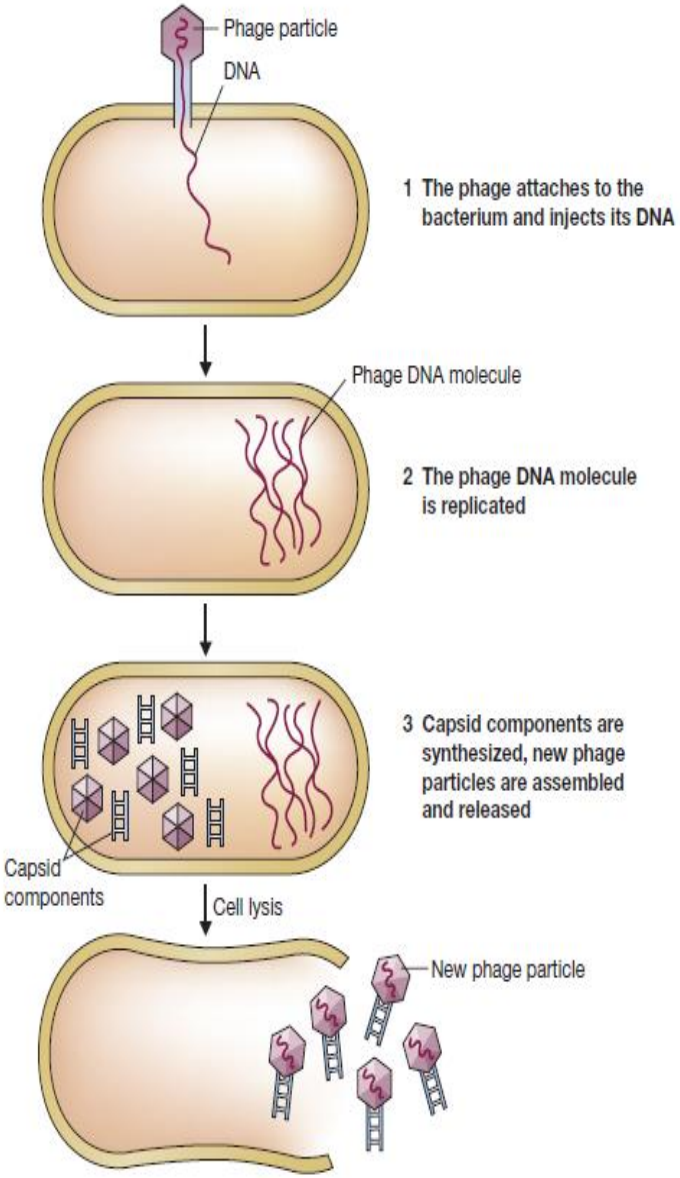
Viral vectors

1. Bacteriophage Lambda

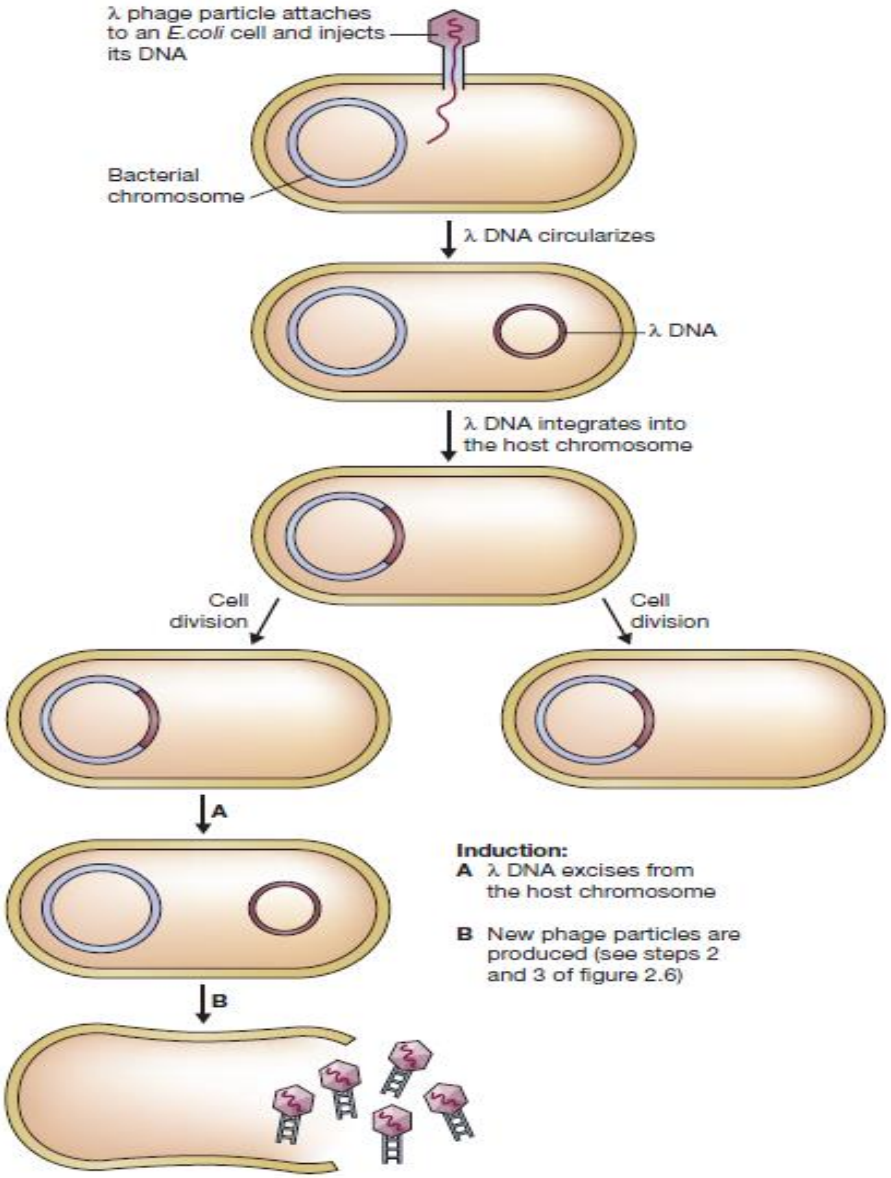


- Virus of *E.coli*
- Lytic or Lysogenic life style
- Virulent phage
  - do not integrate their DNA into the host bacterium genome and they usually kill the host (lytic)
- Temperate phage may integrate into the host genome causing lysogeny

Lytic Life Cycle



Lysogenic Life Cycle

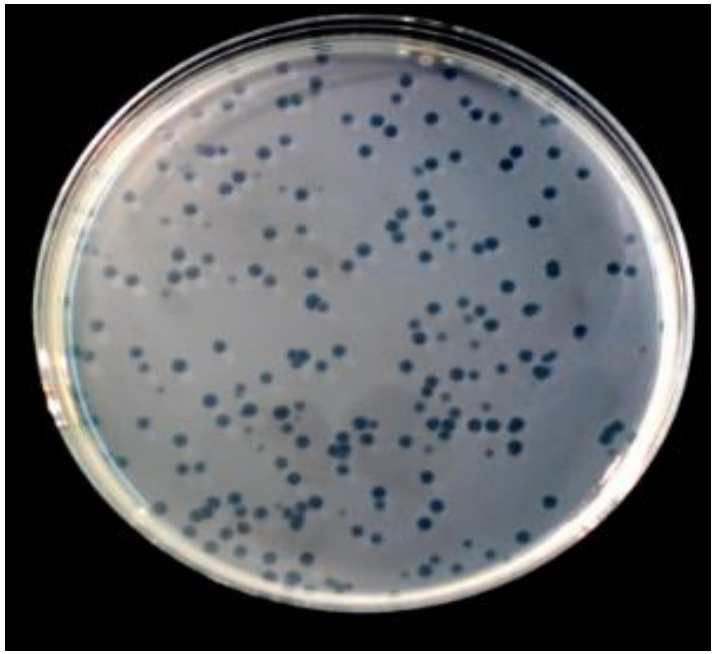


The infection process is about thousand times more efficienct than transformation with plasmid vectors.

10^6 tansformed colonies per microgram of plasmid vector

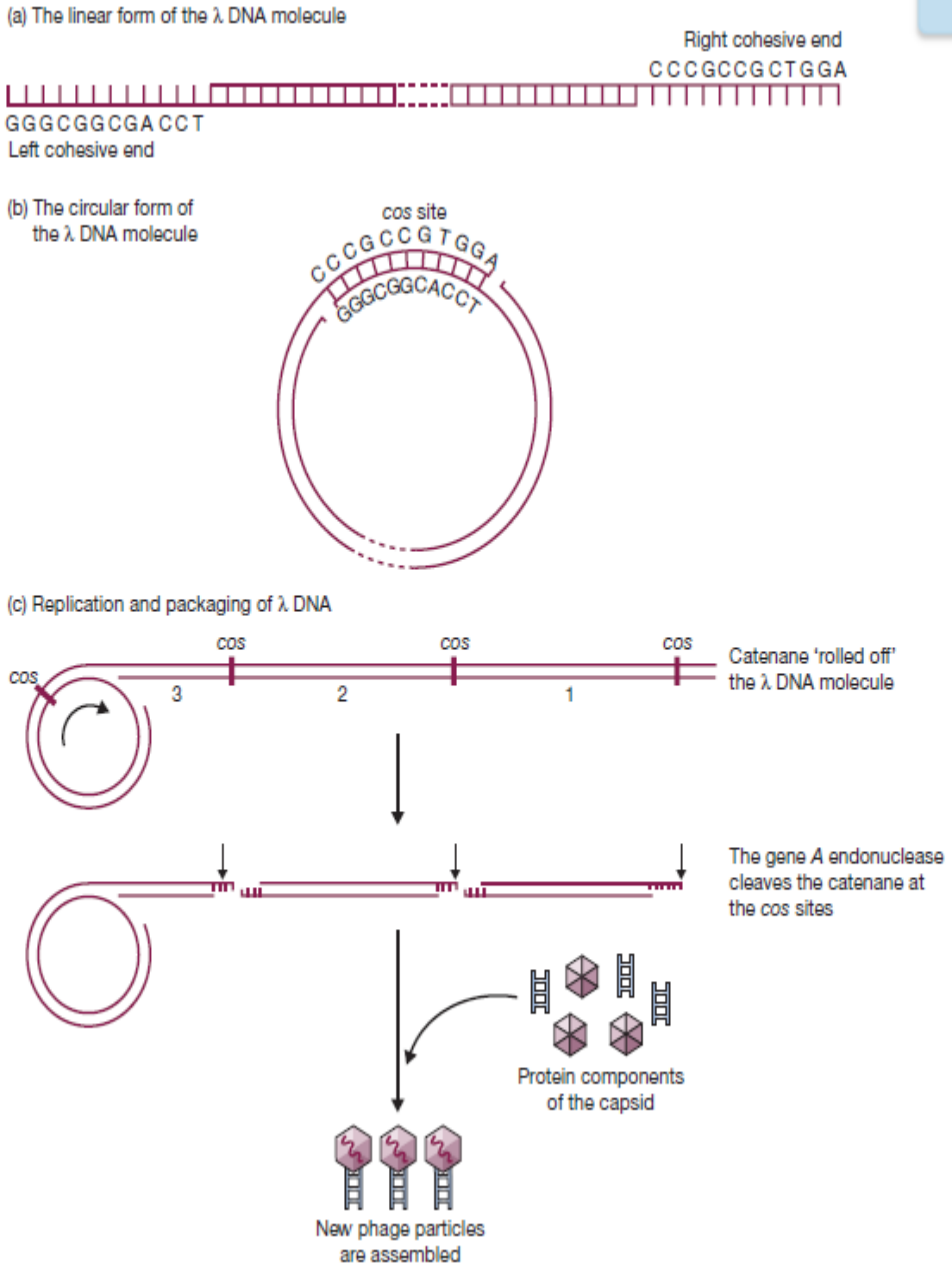
10^9 plaques per microgram of recombinant Lambda vector

Bacterial Colonies

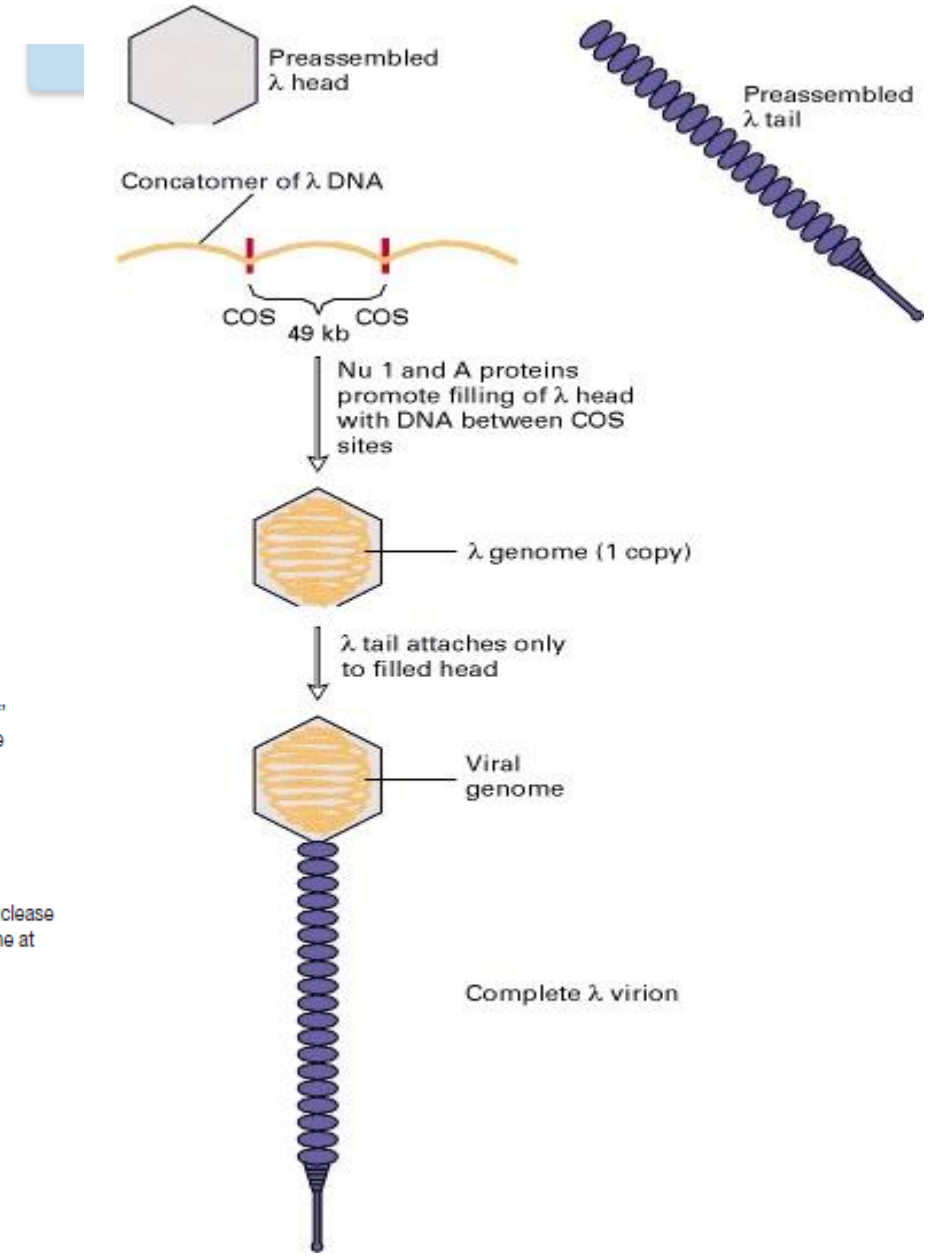


Viral Plaque

Lambda Phage
49kb, linear, dsDNA



Assembly of bacteriophage λ virions



Cloning Vectors based on Lambda Phage

Problem-Packaging efficiency 78%-105% of the lambda genome.

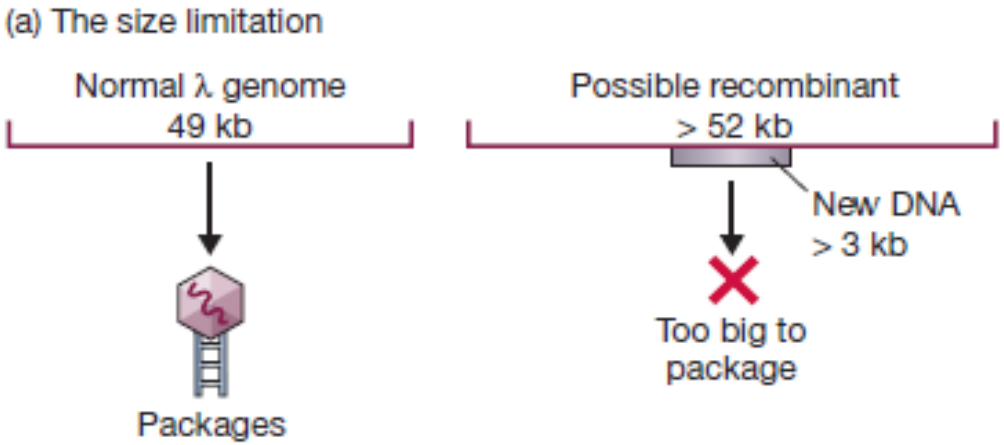
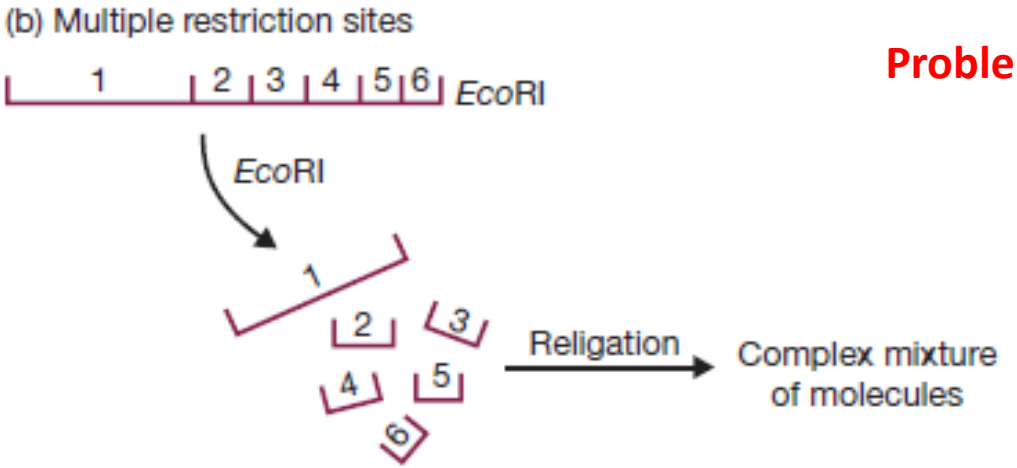


Figure 6.9

The two problems that had to be solved before λ cloning vectors could be developed. (a) The size limitation placed on the λ genome by the need to package it into the phage head. (b) λ DNA has multiple recognition sites for almost all restriction endonucleases.



Problem- Multiple Restriction Sites

Segments of the L genome can be deleted without impairing viability

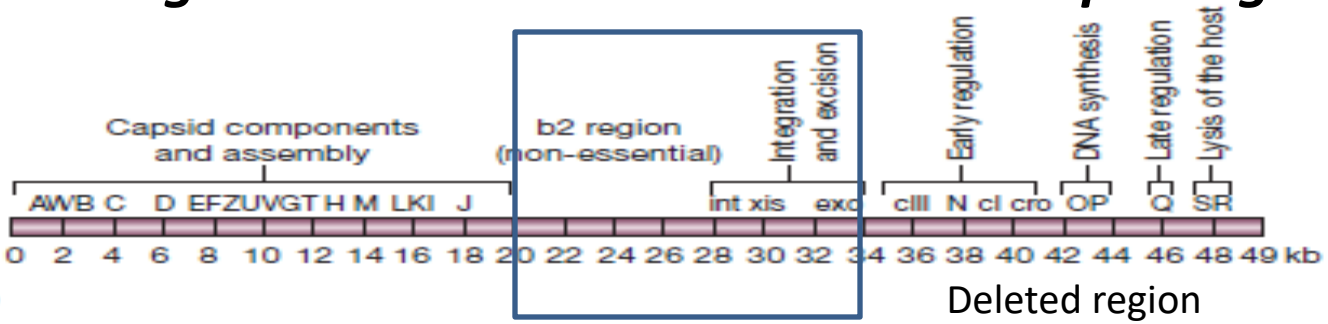


Figure 2.9

The λ genetic map, showing the positions of the important genes and the functions of the gene clusters.

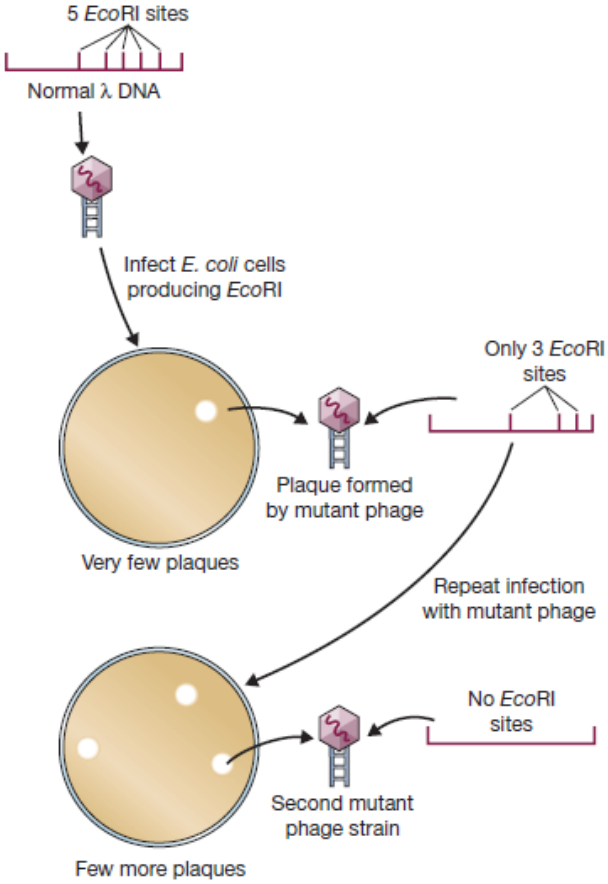
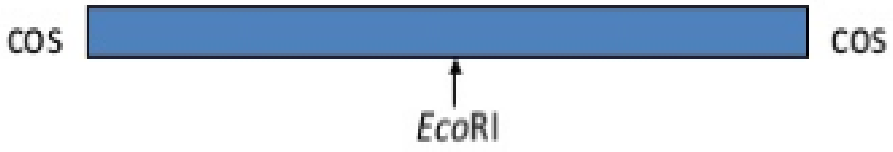


Figure 6.11

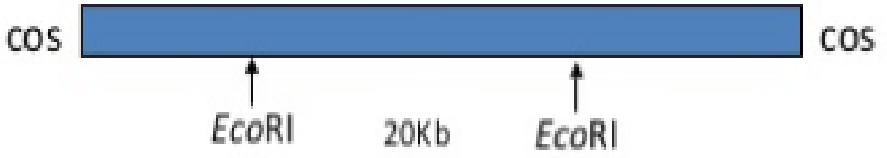
Using natural selection to isolate λ phage lacking *Eco*RI restriction sites.

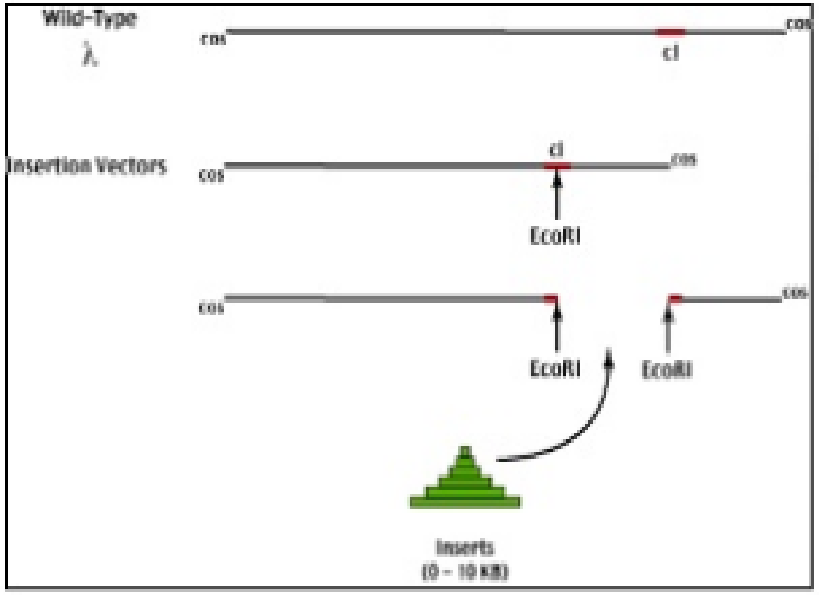
Lambda vectors

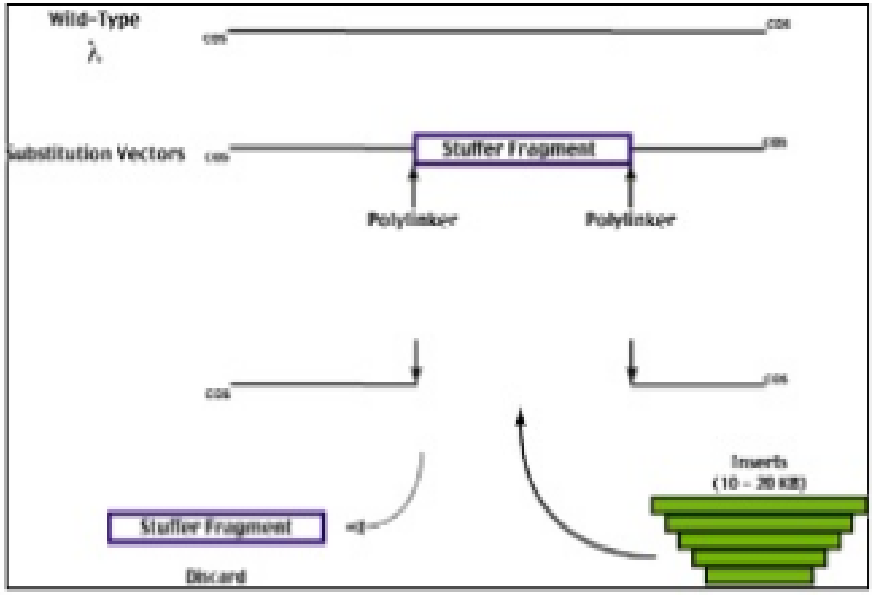
- Insertion vectors:



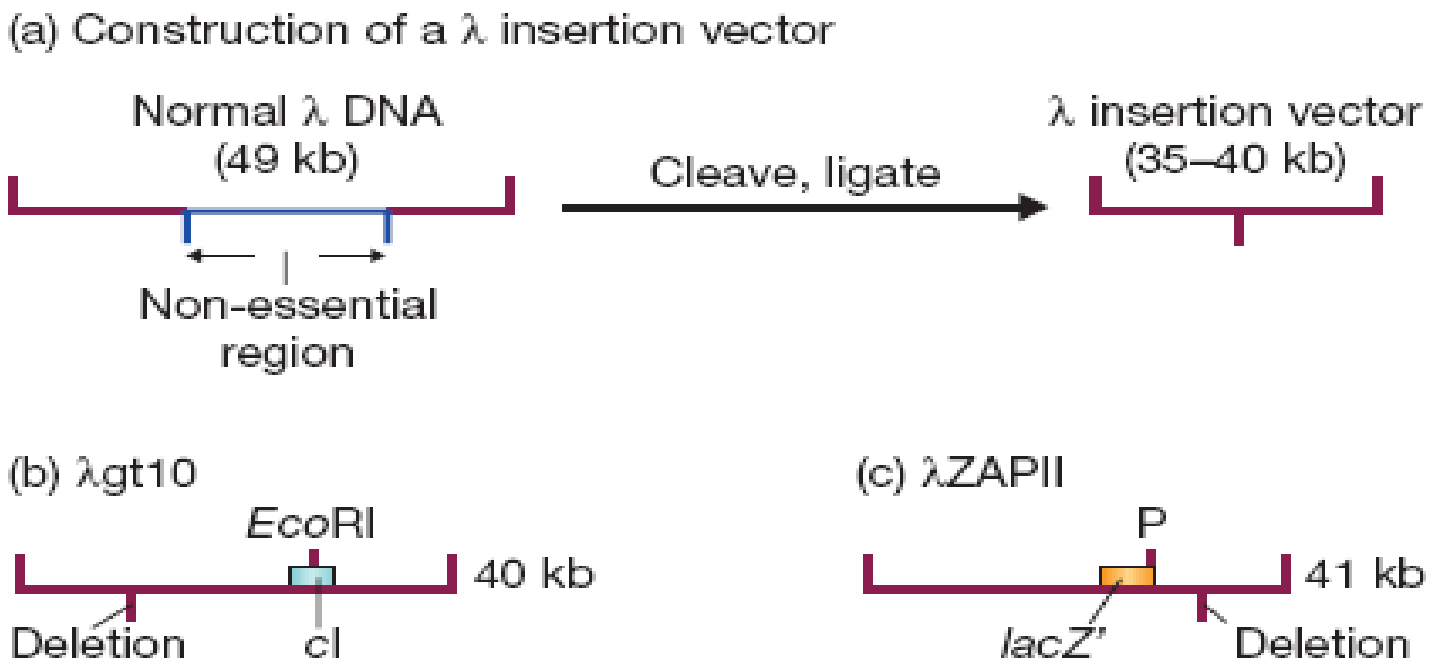
- Replacement vectors:







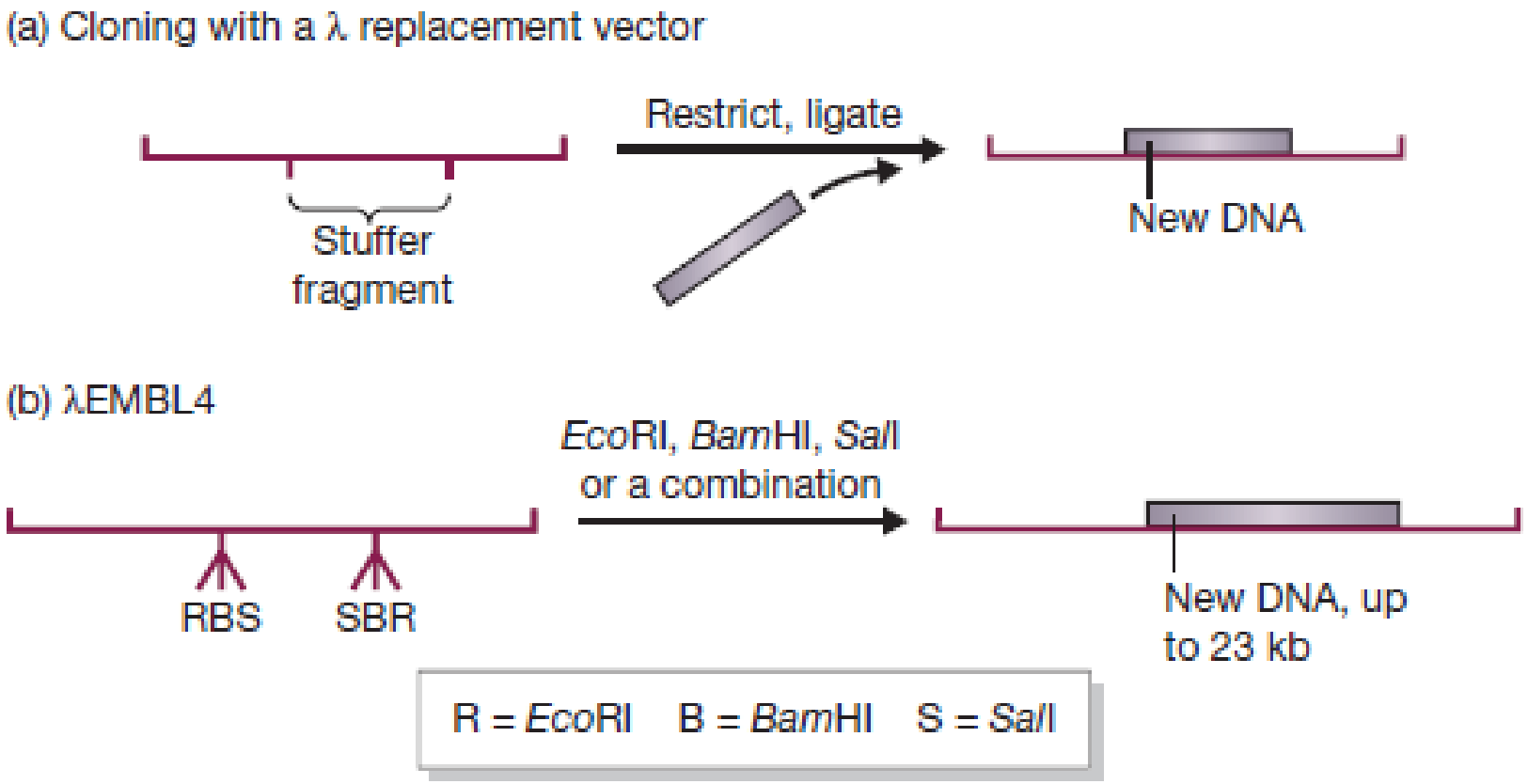
Lambda Insertion Vectors



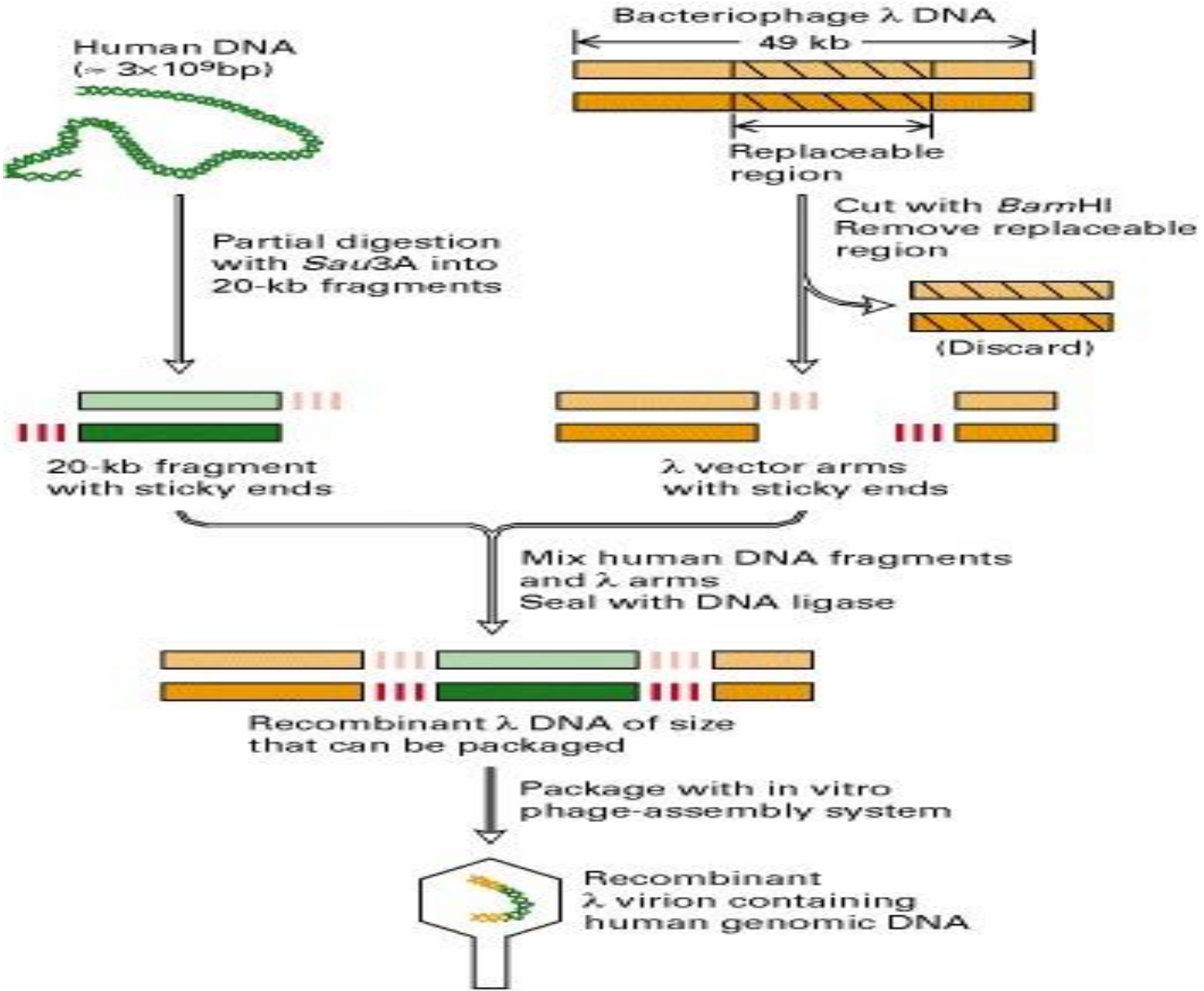
Disruption of cI gene- cI promotes lysogeny, thus, recombinants are distinguished as clear rather than turbid plaques

Disruption of lacZ'- Recombinants give clear rather than blue plaques on X-gal agar.

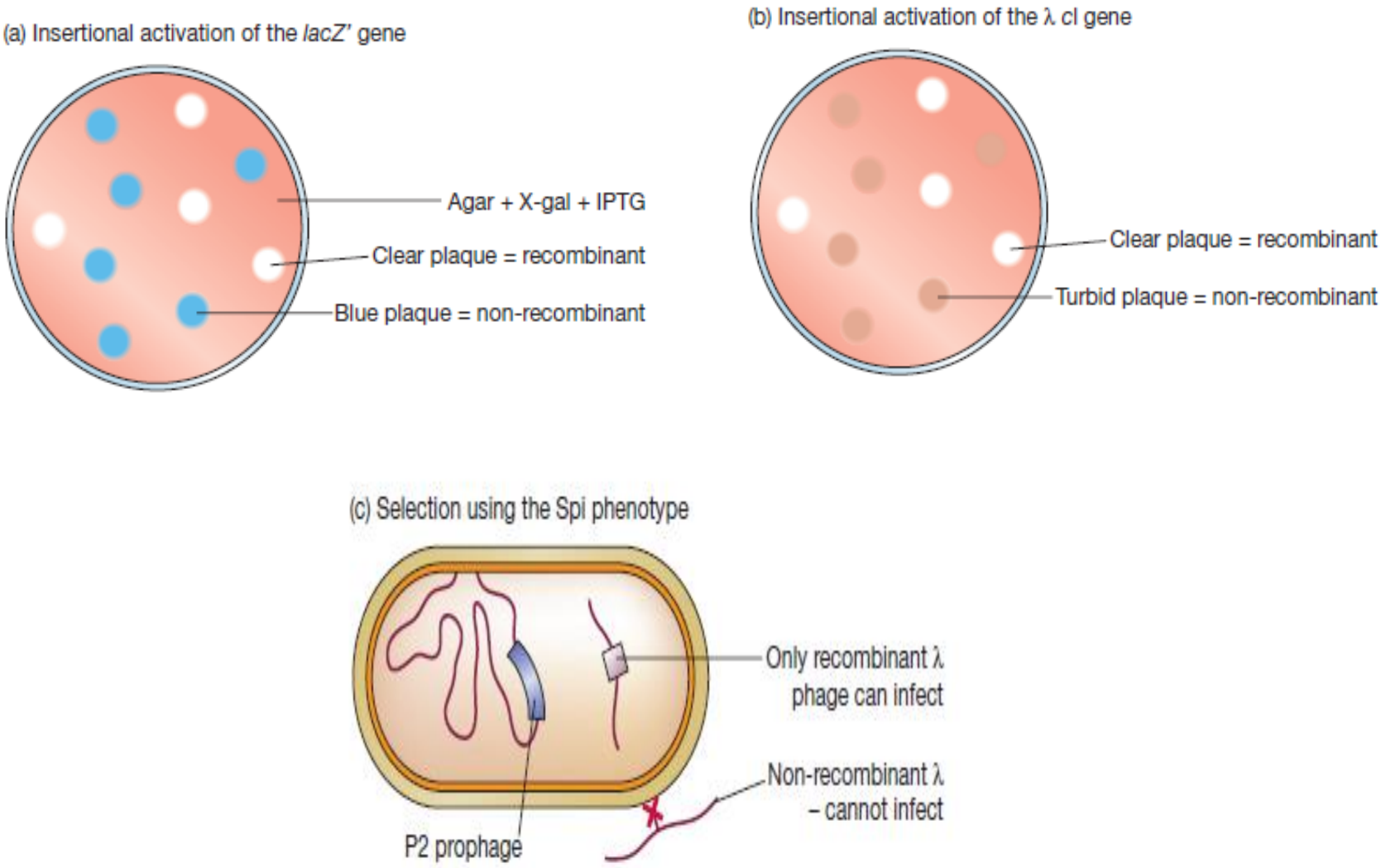
Lambda Replacement Vectors



Construction of a genomic library of human DNA in a bacteriophage λ vector.



Strategies for selection of recombinant phages



The need for vectors with larger inserts

Genomic Libraries

How to select a vector [edit]

Vector selection requires one to ensure the library made is representative of the entire genome. Any insert of the genome derived from a restriction enzyme should have an equal chance of being in the library compared to any other insert. Furthermore, recombinant molecules should contain large enough inserts ensuring the library size is able to be handled conveniently.[14] This is particularly determined by the amount of clones needed to have in a library. The amount of clones to get a sampling of all the genes is determined by the size of the organism's genome as well as the average insert size. This is represented by the formula (also known as the Carbon and Clarke formula):[15]

$$N = \frac{ln(1-P)}{ln(1-f)}$$

where,

N is the necessary number of recombinants[16]

P is the desired probability that any fragment in the genome will occur at least once in the library created

f is the fractional proportion of the genome in a single recombinant

f can be further shown to be:

$$f = \frac{i}{g}$$

where,

i is the insert size

g is the genome size

Thus, increasing the insert size (by choice of vector) would allow for fewer clones needed to represent a genome. The proportion of the insert size versus the genome size represents the proportion of the respective genome in a single clone.[14] Here is the equation with all parts considered:

$$N = \frac{ln(1-P)}{ln(1-\frac{i}{g})}$$

Vector selection example [edit]

The above formula can be used to determine the 99% confidence level that all sequences in a genome are represented by using a vector with an insert size of twenty thousand basepairs (such as the phage lambda vector). The genome size of the organism is three billion basepairs in this example.

$$N = \frac{ln(1 - 0.99)}{ln[1 - \frac{2.0 \times 10^4 basepairs}{3.0 \times 10^9 basepairs}]}$$

$$N = \frac{-4.61}{-6.7 \times 10^{-6}}$$

$$N = 688,060 \text{ clones}$$

Thus, approximately 688,060 clones are required to ensure a 99% probability that a given DNA sequence from this three billion basepair genome will be present in a library using a vector with an insert size of twenty thousand basepairs.

Table 4.2. Sizes of inserted DNA commonly obtained with different cloning vectors

Cloning vector	Size of insert
Standard high copy number plasmid vectors	0–10 kb
Bacteriophage λ insertion vectors	0–10 kb
Bacteriophage λ replacement vectors	9–23 kb
Cosmid vectors	30–44 kb
Bacteriophage P1	70–100 kb
PAC (P1 artificial chromosome) vectors	130–150 kb
BAC (bacterial artificial chromosome) vectors	up to 300 kb
YAC (yeast artificial chromosome) vectors	0.2–2.0 Mb

Cosmids-Long DNA fragments can be cloned using a cosmid

Refer:

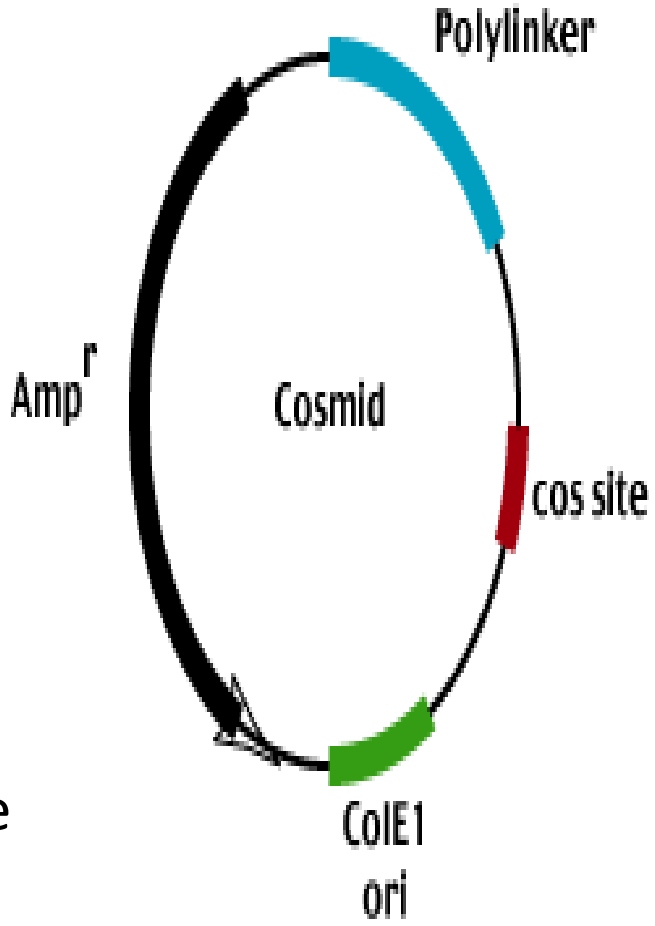
https://www.ncbi.nlm.nih.gov/books/NBK21696/

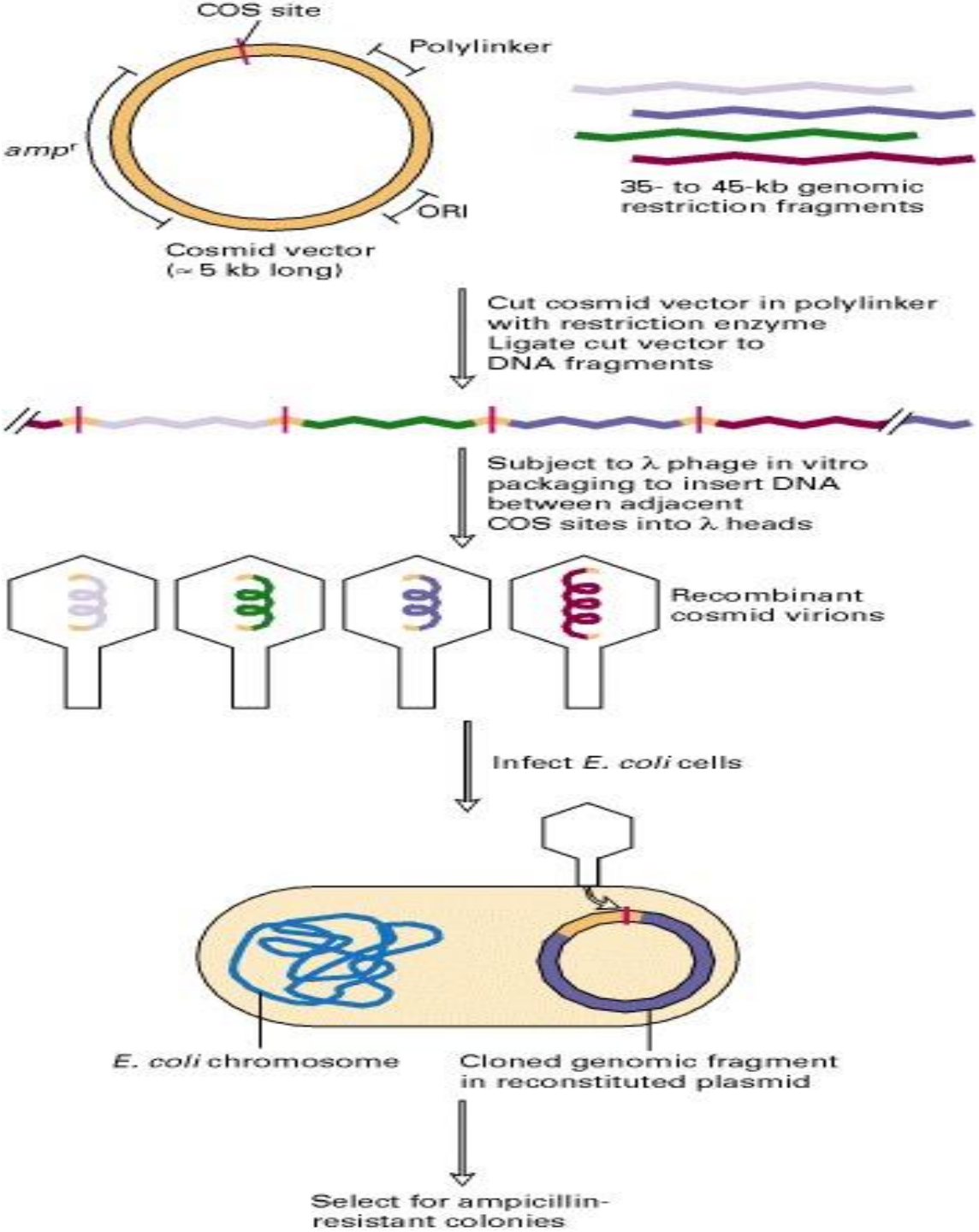
Cosmids

- Cross between phage and plasmid
- Circular ds DNA
- Contain cos sites and can be packaged into phage heads
- Carry more DNA than plasmid and can be maintained and manipulated as plasmids

Cloning using cosmids

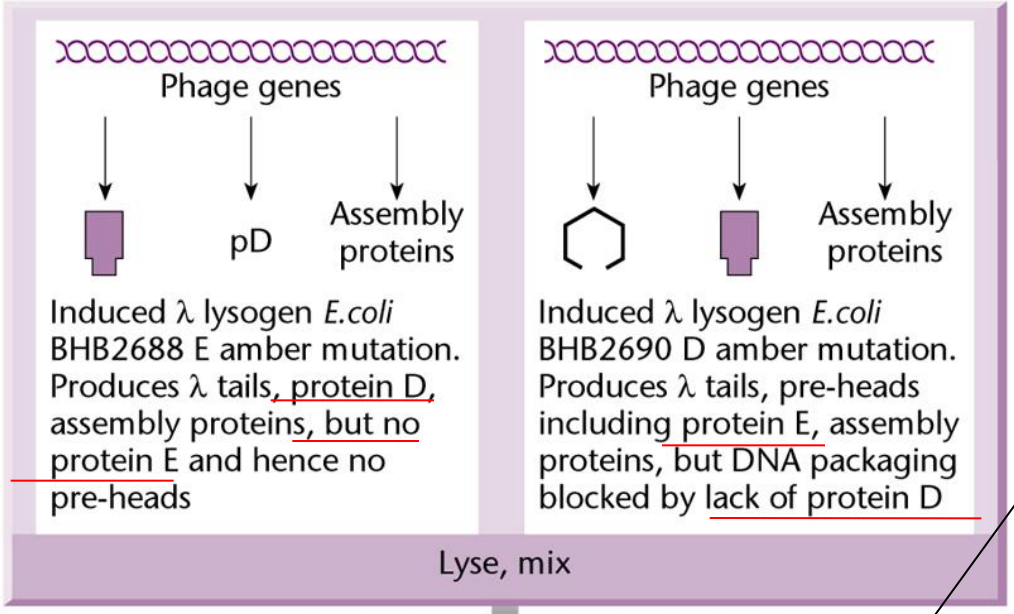
- Clone into vector as would with plasmid
- Introduce DNA to cell as you would with phage
- Propagate as you would a plasmid
- Cosmids have about 5kb DNA and therefore can get ~ 33-48kb foreign DNA into a phage head.



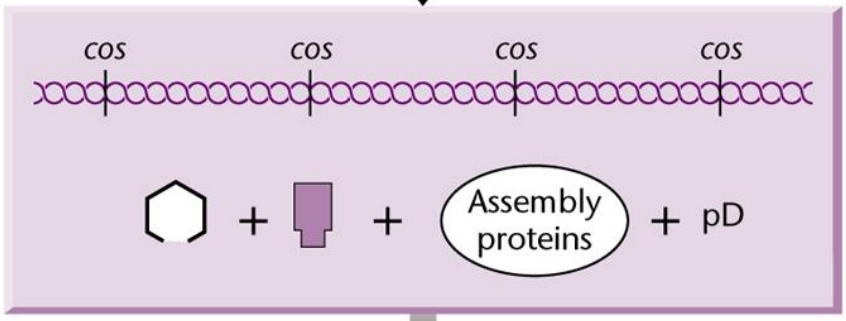

COS site
Polylinker
amp^r
ORI
Cosmid vector
(≈ 5 kb long)
35- to 45-kb genomic
restriction fragments
Cut cosmid vector in polylinker
with restriction enzyme
Ligate cut vector to
DNA fragments
Subject to λ phage in vitro
packaging to insert DNA
between adjacent
COS sites into λ heads
Recombinant
cosmid virions
Infect E. coli cells
E. coli chromosome
Cloned genomic fragment
in reconstituted plasmid
Select for ampicillin-
resistant colonies

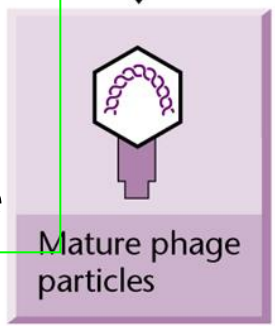
In-vitro packaging

How to transfer recombinant lambda into cells?

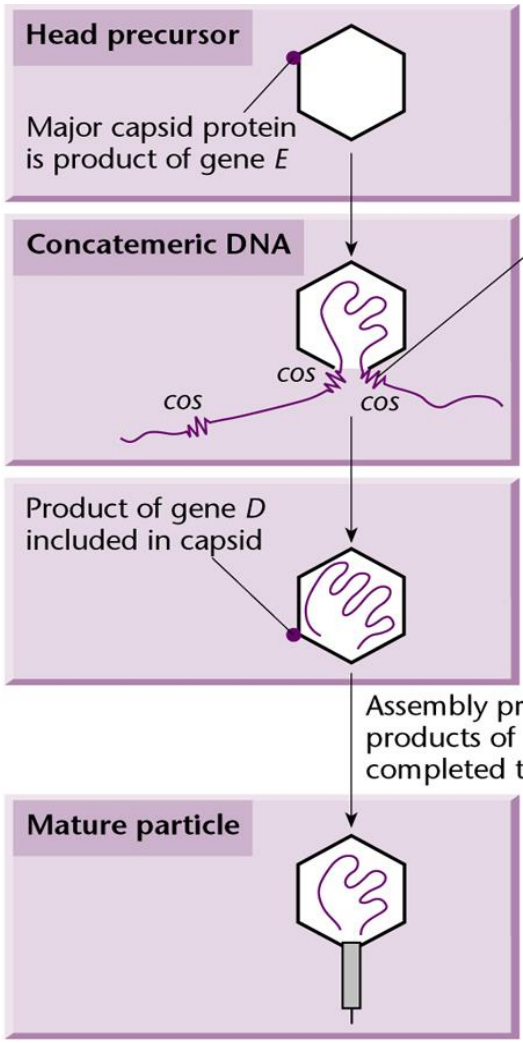


Add ATP and concatemerized λDNA





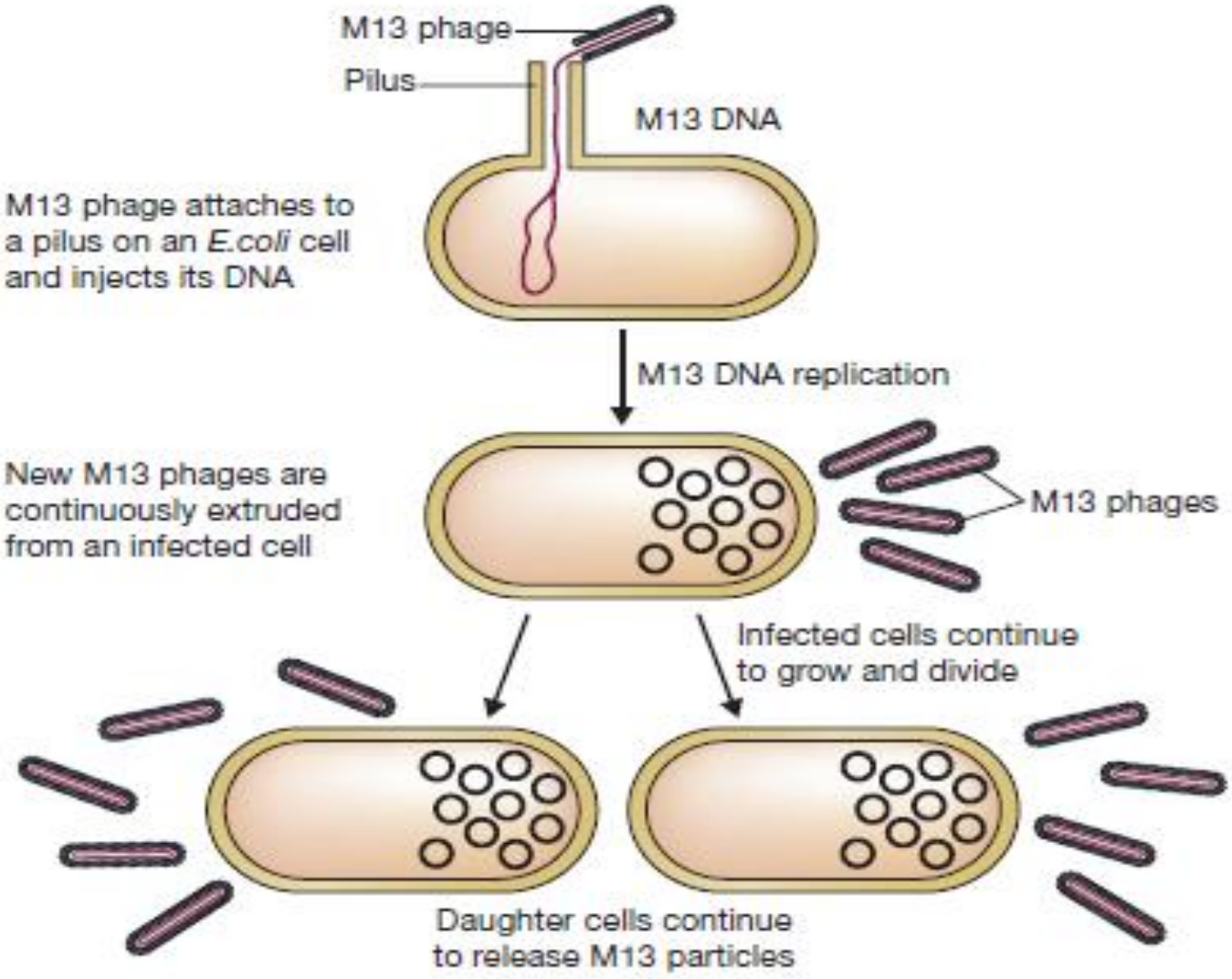
The packaged phage particles are infectious



Other vectors

- Other high-capacity vectors have been constructed from bacteriophage
- **P1, which has the advantage over lambda of being able to squeeze 110 kb of DNA into its** capsid structure.
- Cosmid-type vectors based on P1 have been designed and used to clone DNA fragments ranging in size from 75 to 100 kb.

M13 Phage
6.4 kb, circular, ss DNA



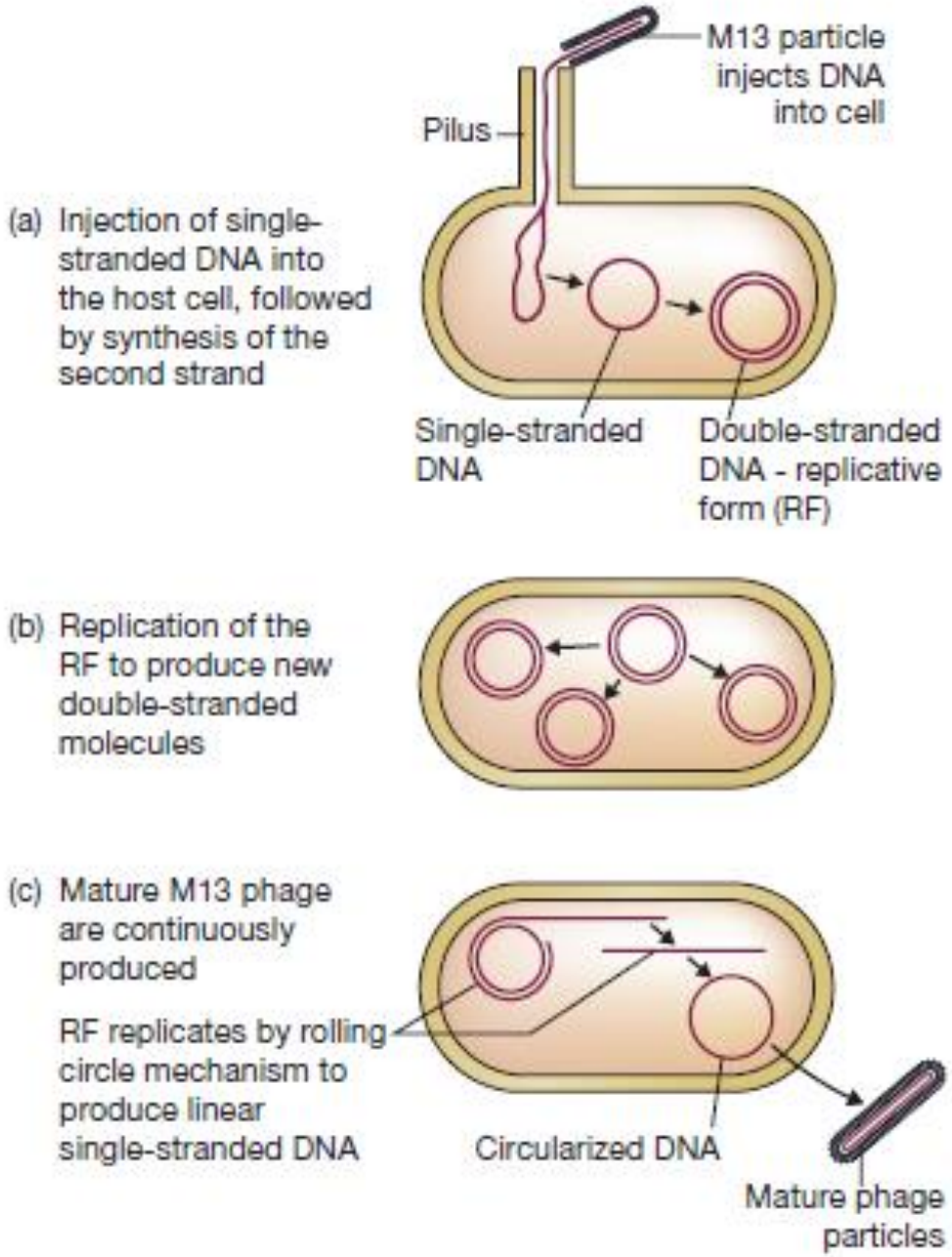


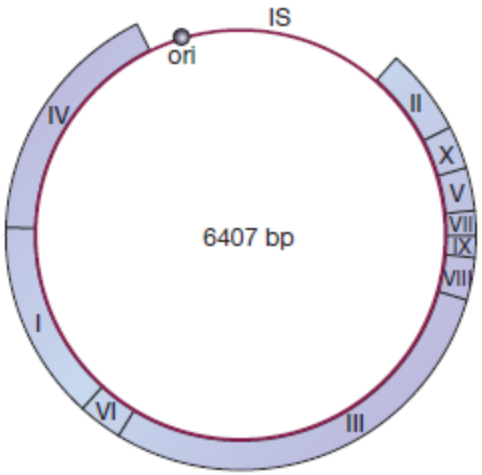
Figure 2.11

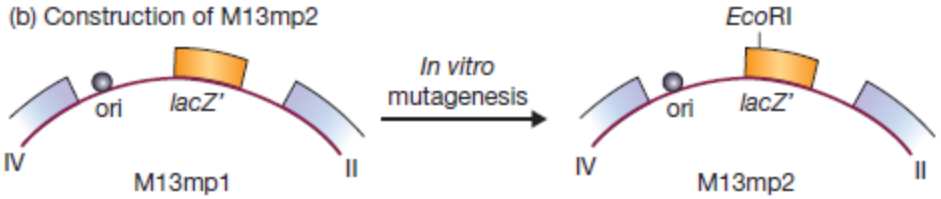
The M13 infection cycle, showing the different types of DNA replication that occur. (a) After infection the single-stranded M13 DNA molecule is converted into the double-stranded replicative form (RF). (b) The RF replicates to produce multiple copies of itself. (c) Single-stranded molecules are synthesized by rolling circle replication and used in the assembly of new M13 particles.

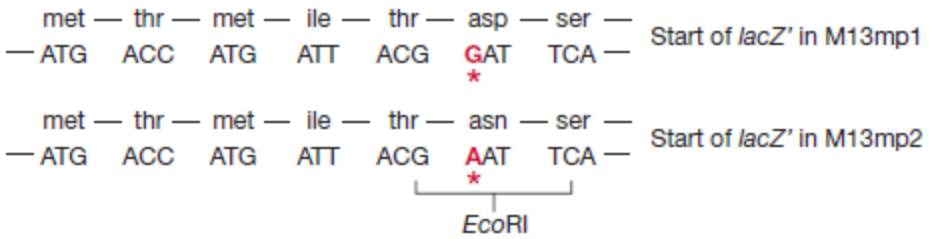
M13 as cloning vector-Advantages

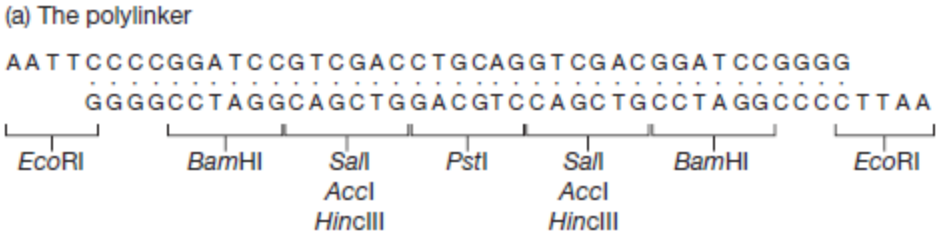
- The genome is less than 10 kb in size(~6.4 KB), well within the range desirable for a potential vector. In addition,
- The double-stranded **replicative form (RF) of the M13 genome behaves very much** like a plasmid, and can be treated as such for experimental purposes (It is easily prepared from a culture of infected *E. coli cells)*.
- Most importantly, genes cloned with an M13-based vector can be obtained in the form of single-stranded DNA. Single-stranded versions of cloned genes are useful for several techniques, notably DNA sequencing and *in vitro mutagenesis.*
- M13 vectors are also used in **phage display, a technique for**
- identifying pairs of genes whose protein products interact with one another.

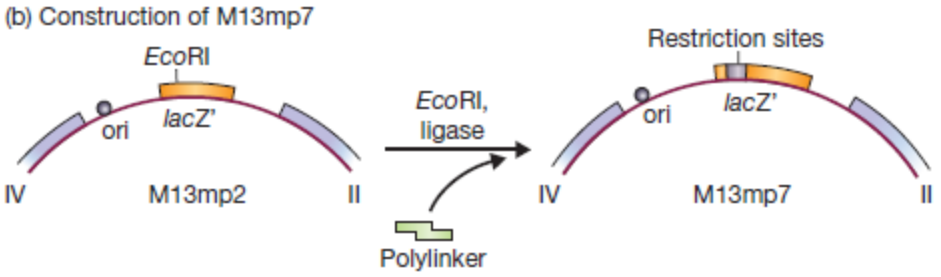
M13 cloning vectors



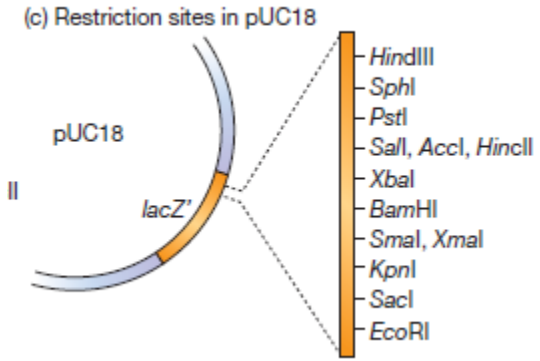








M13mp8 with same MCS as PUC8



Phagemids: plasmid/M13 hybrids

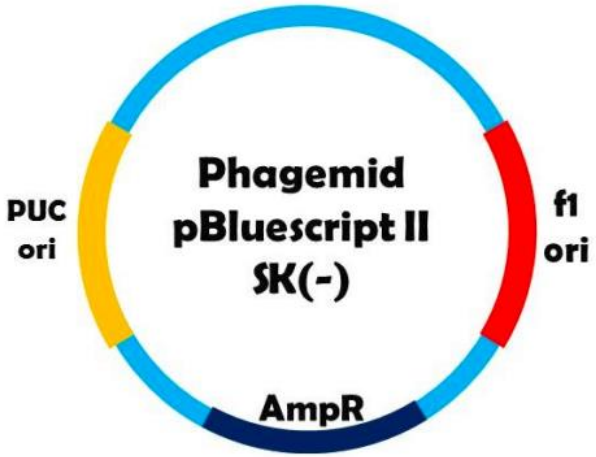
• Plasmids containing both plasmid (colE1) origin and bacteriophage M13 origin of replication

• To recover single-stranded version of the plasmid (for sequencing, e.g.), infect transformed strain with a helper phage (M13KO7)

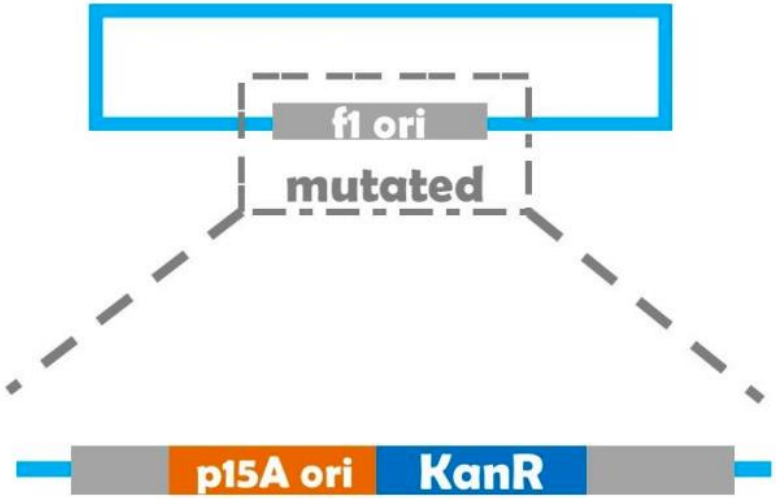
• Helper phage cannot produce single stranded copies of itself (bcoz ori is mutated), but provides replication machinery for single-stranded copies of the phagemid DNA and packaging of the phagemid.

• Phagemid single stranded DNA is packaged and extruded into supernatant--can then be isolated for sequencing, etc.

phagemid



Helper phage genome



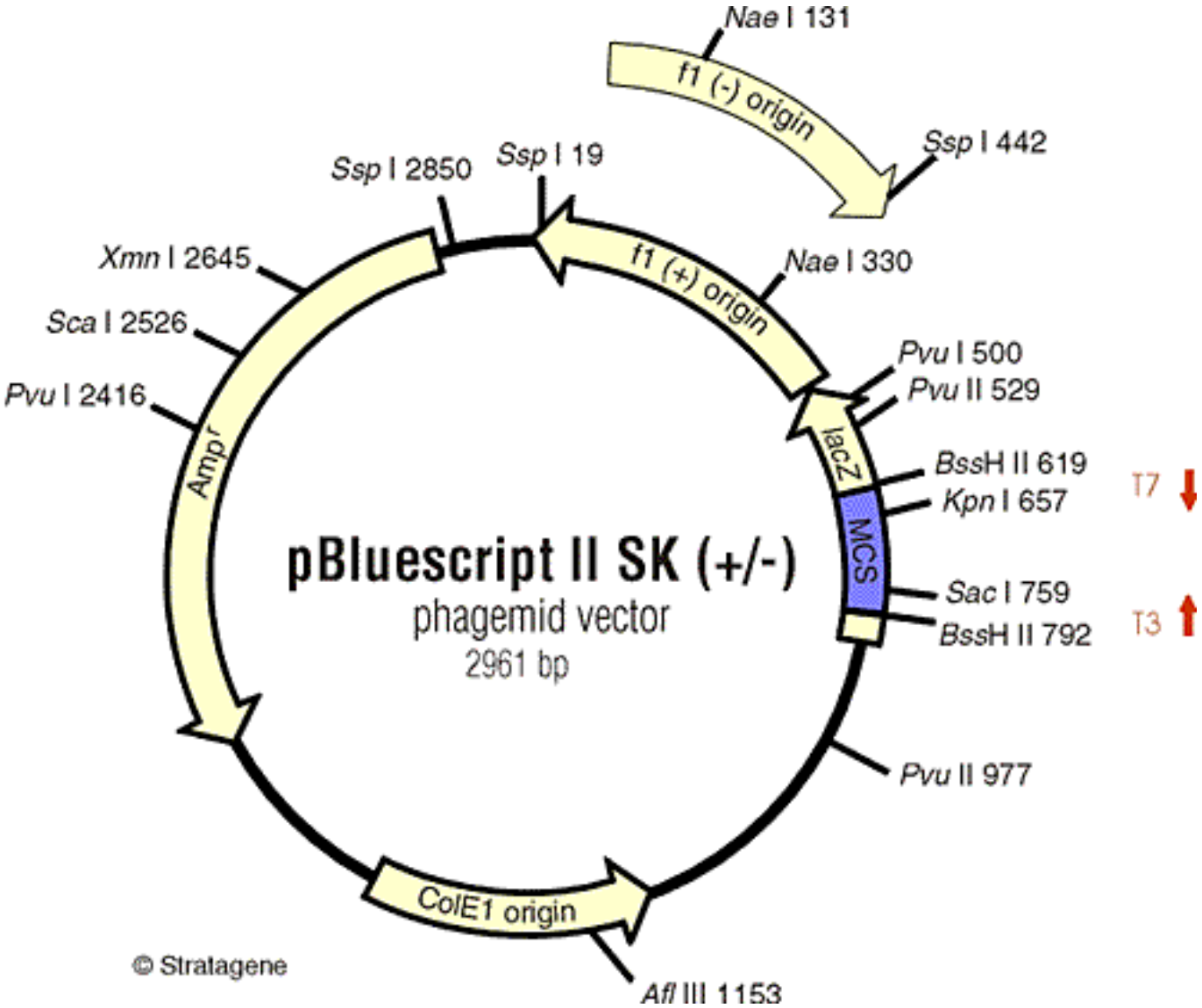

Nae I 131
f1 (-) origin
Ssp I 442
Ssp I 2850
Ssp I 19
Xmn I 2645
f1 (+) origin
Nae I 330
Sca I 2526
Pvu I 500
Pvu I 2416
Pvu II 529
lacZ
BssH II 619
Ampr
Kpn I 657
T7
MCS
pBluescript II SK (+/-)
phagemid vector
2961 bp
Sac I 759
T3
BssH II 792
Pvu II 977
ColE1 origin
© Stratagene
Afl III 1153

Table 4.2. Sizes of inserted DNA commonly obtained with different cloning vectors

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BAC (bacterial artificial chromosome) vectors	up to 300 kb
YAC (yeast artificial chromosome) vectors	0.2–2.0 Mb

BACs: Bacterial Artificial Chromosomes

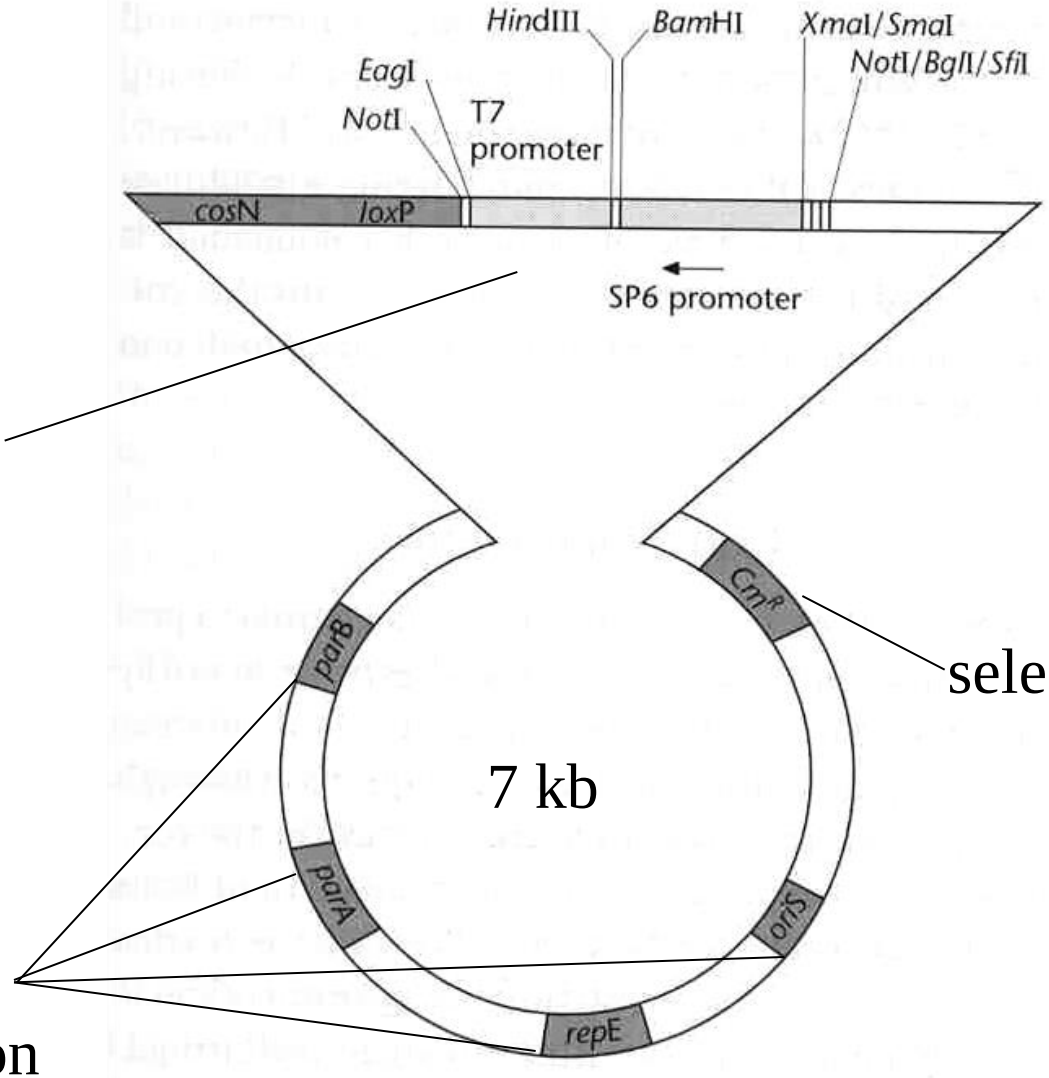
- Based on the F factor of E. coli:
  --100 kb plasmid, propagates through conjugation
  --low copy number (1-2 copies per cell)
  --2 genes (parA and parB): accurate partitioning during cell division

- BACs: just have par genes, replication ori, cloning sites, selectable marker

- Can propagate very large pieces of DNA:
up to 300 kb

- Move into cells through electroporation.

General BAC vector

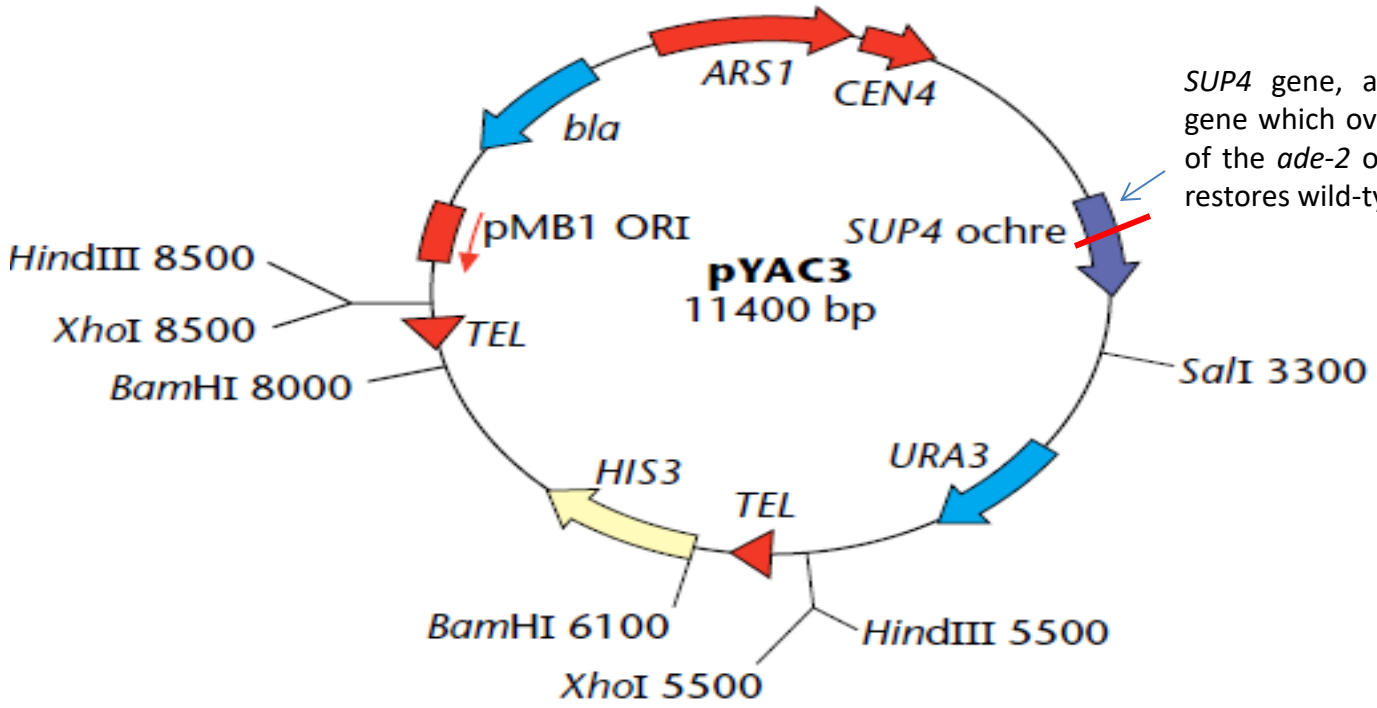


Cloning, etc

selection

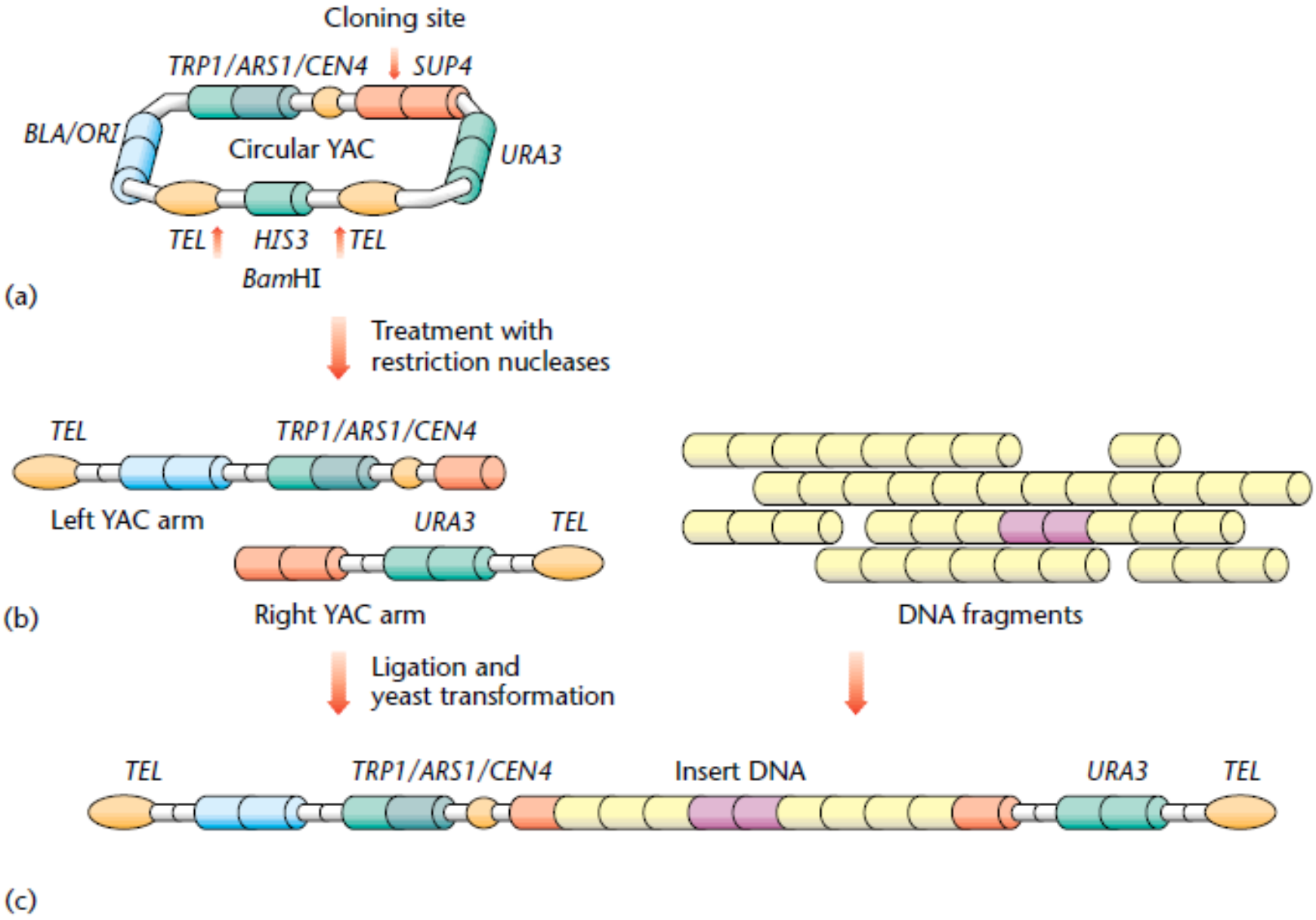
replication

YACs: Yeast Artificial Chromosomes



SUP4 gene, a suppressor tRNA gene which overcomes the effect of the *ade-2* ochre mutation and restores wild-type activity.

- Based on the chromosome of Yeast
- Features:
- CEN1, centromere sequence→segregation
- TEL, telomere sequences→extremity protection
- ARS1, autonomous replicating sequence→replication
- *Amp*
- ori, origin of replication for propagation in an *E. coli* host.


Cloning site
TRP1/ARS1/CEN4
SUP4
BLA/ORI
Circular YAC
URA3
TEL
HIS3
TEL
BamHI
(a)
Treatment with
restriction nucleases
TEL
TRP1/ARS1/CEN4
Left YAC arm
URA3
TEL
Right YAC arm
(b)
DNA fragments
Ligation and
yeast transformation
TEL
TRP1/ARS1/CEN4
Insert DNA
URA3
TEL
(c)

SUP4, *which is the marker* into which new DNA is inserted during the cloning experiment.
(SUP4 gene, a suppressor tRNA gene which overcomes the effect of the *ade-2* ochre mutation and restores wild-type activity, resulting in colorless colonies.
SUP4 gene compensates for a mutation in the yeast host cell that causes the accumulation of red pigment. The host cells are normally red, and those transformed with YAC only, will form colorless colonies. Cloning of a foreign DNA fragment into the YAC causes insertional inactivation of the gene, restoring the red color. Therefore, the colonies that contain the foreign DNA fragment are red.)

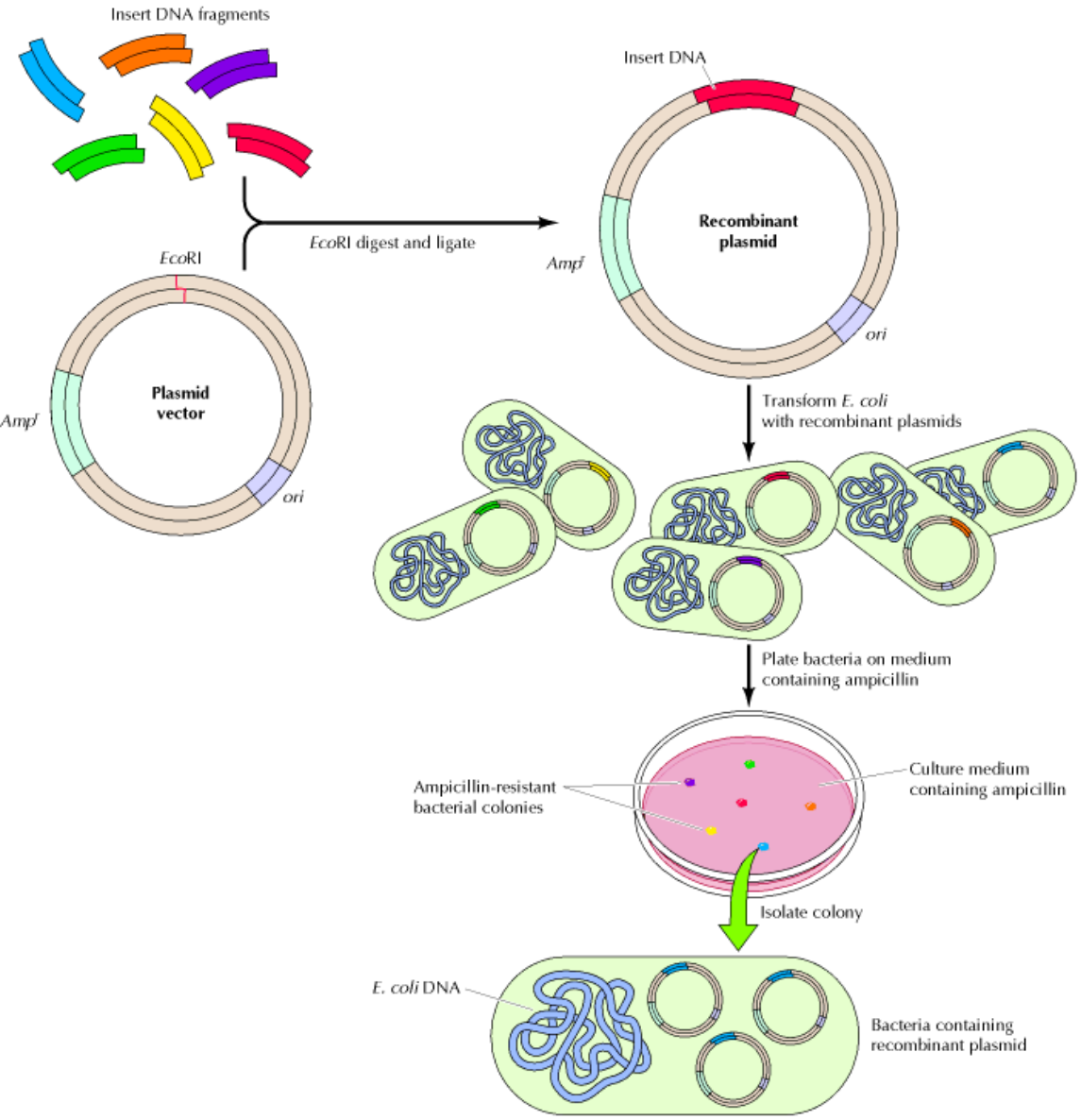
The host cells are also designed to have recessive *trp1* and *ura3* alleles which can be complemented by the corresponding *TRP1* and *URA3* alleles in the vector, providing a selection system for identifying cells containing the YAC vector.

URA3- This gene codes for orotidine-5'-phosphate decarboxylase (an enzyme that catalyzes one of the steps in the biosynthesis pathway for pyrimidine nucleotides) and is used as a selectable marker.

TRP1*. This gene, which is involved in tryptophan biosynthesis, is* located adjacent to a chromosomal origin of replication.

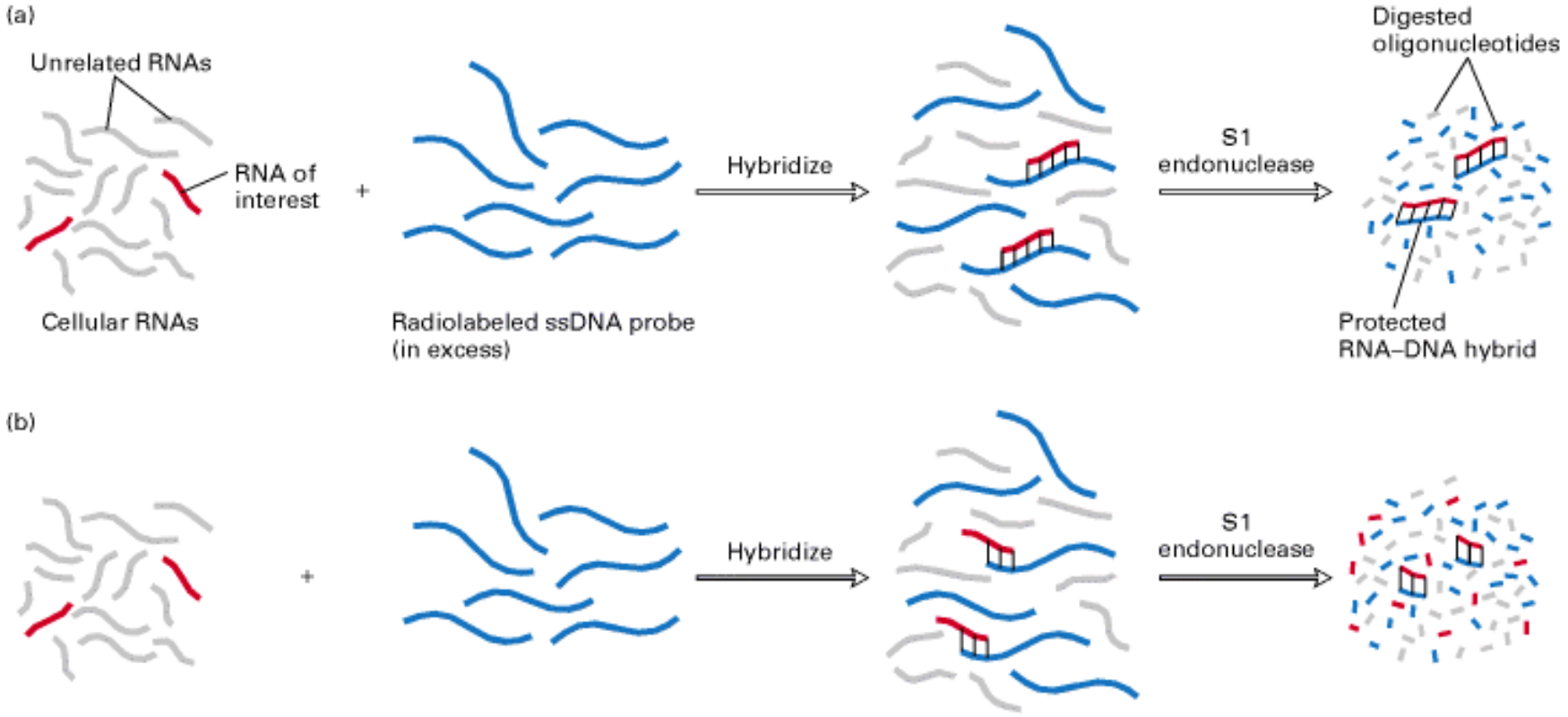
Table 4.2. Sizes of inserted DNA commonly obtained with different cloning vectors

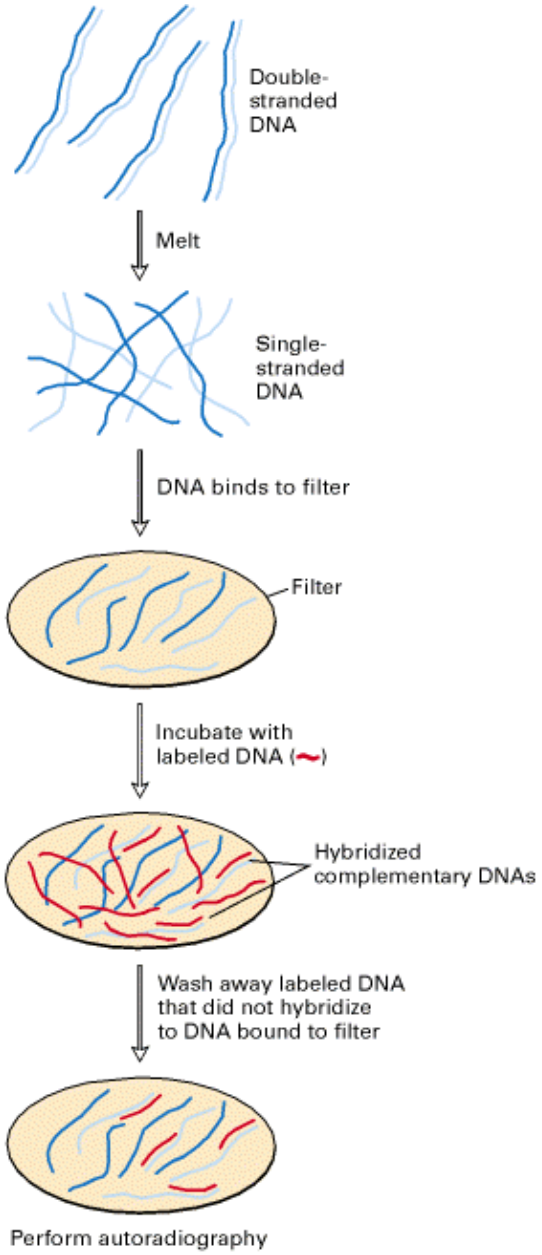
Cloning vector	Size of insert
Standard high copy number plasmid vectors	0–10 kb
Bacteriophage λ insertion vectors	0–10 kb
Bacteriophage λ replacement vectors	9–23 kb
Cosmid vectors	30–44 kb
Bacteriophage P1	70–100 kb
PAC (P1 artificial chromosome) vectors	130–150 kb
BAC (bacterial artificial chromosome) vectors	up to 300 kb
YAC (yeast artificial chromosome) vectors	0.2–2.0 Mb

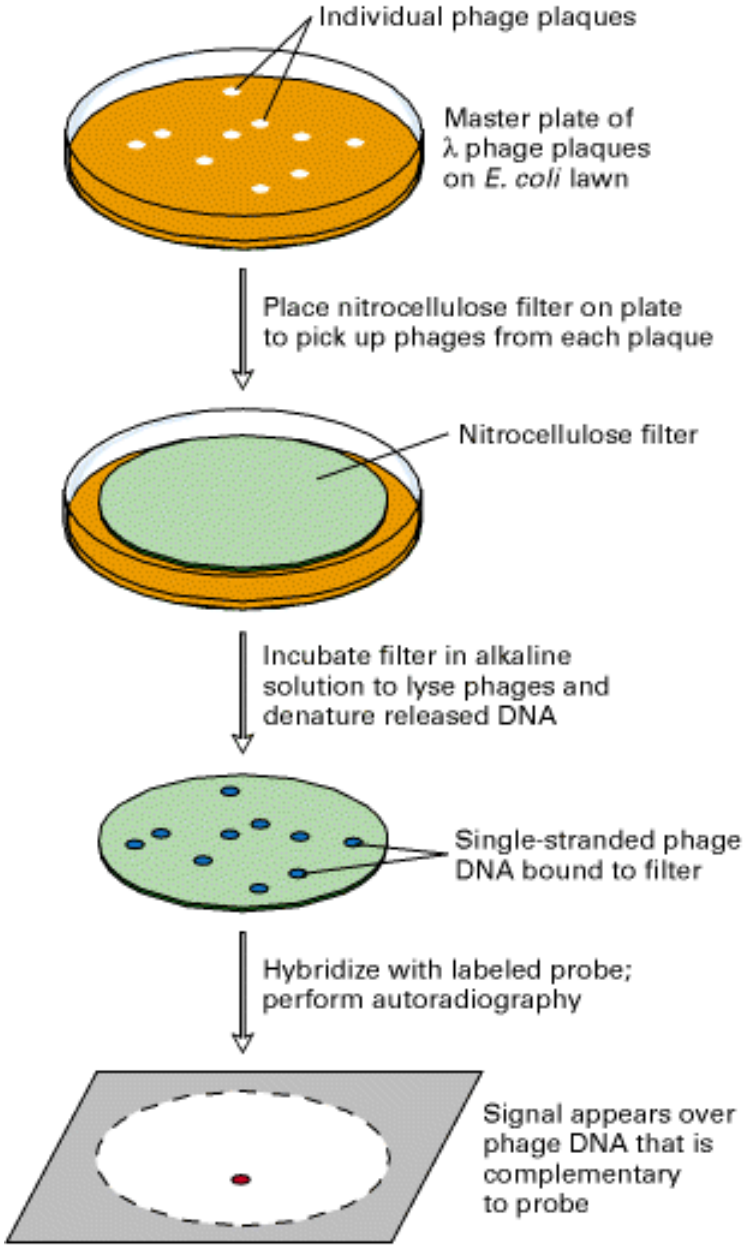


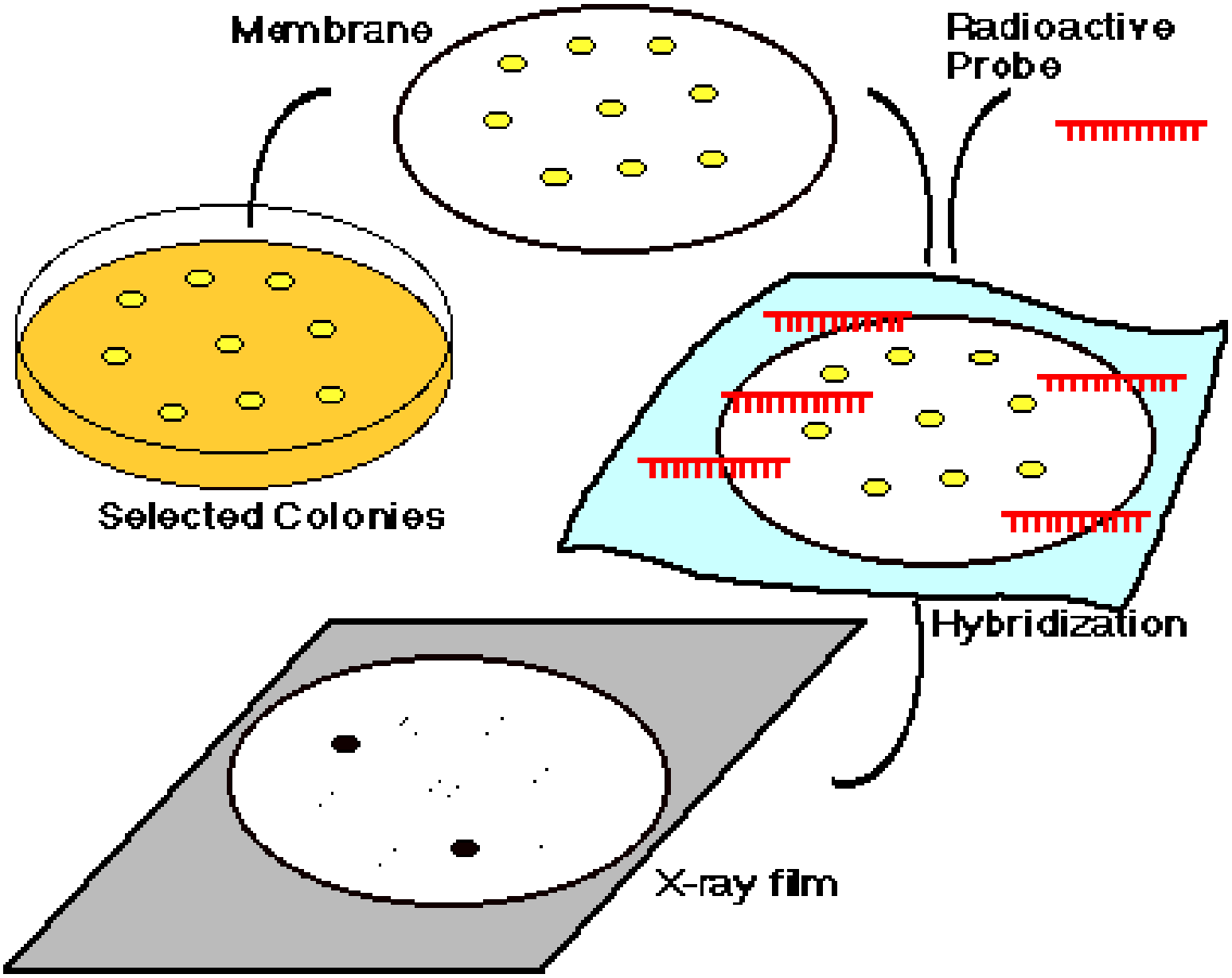
Not all vectors permit the identification of the desired clones by simple selection or color based strategies.

In the majority of cases we need alternative approaches!!!!


(a)
Unrelated RNAs
RNA of interest
Cellular RNAs
+
Radiolabeled ssDNA probe (in excess)
Hybridize
S1 endonuclease
Digested oligonucleotides
Protected RNA–DNA hybrid
(b)
+
Hybridize
S1 endonuclease

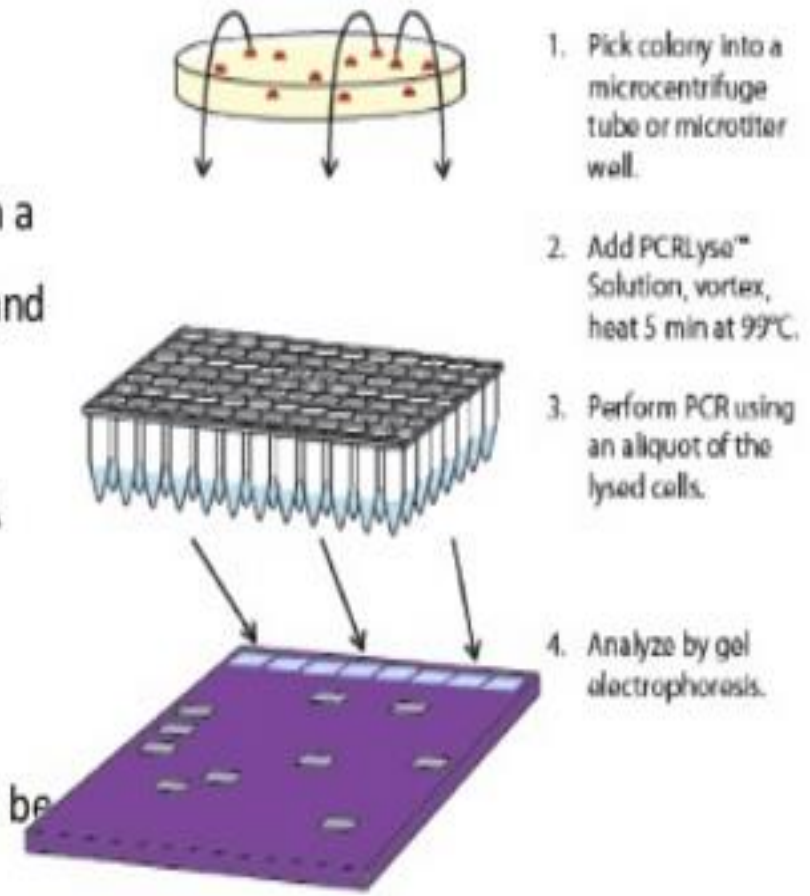

Double-stranded DNA
Melt
Single-stranded DNA
DNA binds to filter
Filter
Incubate with labeled DNA (~)
Hybridized complementary DNAs
Wash away labeled DNA that did not hybridize to DNA bound to filter
Perform autoradiography


Individual phage plaques
Master plate of λ phage plaques on E. coli lawn
Place nitrocellulose filter on plate to pick up phages from each plaque
Nitrocellulose filter
Incubate filter in alkaline solution to lyse phages and denature released DNA
Single-stranded phage DNA bound to filter
Hybridize with labeled probe; perform autoradiography
Signal appears over phage DNA that is complementary to probe


Membrane
Radioactive Probe
Selected Colonies
Hybridization
X-ray film

Colony PCR

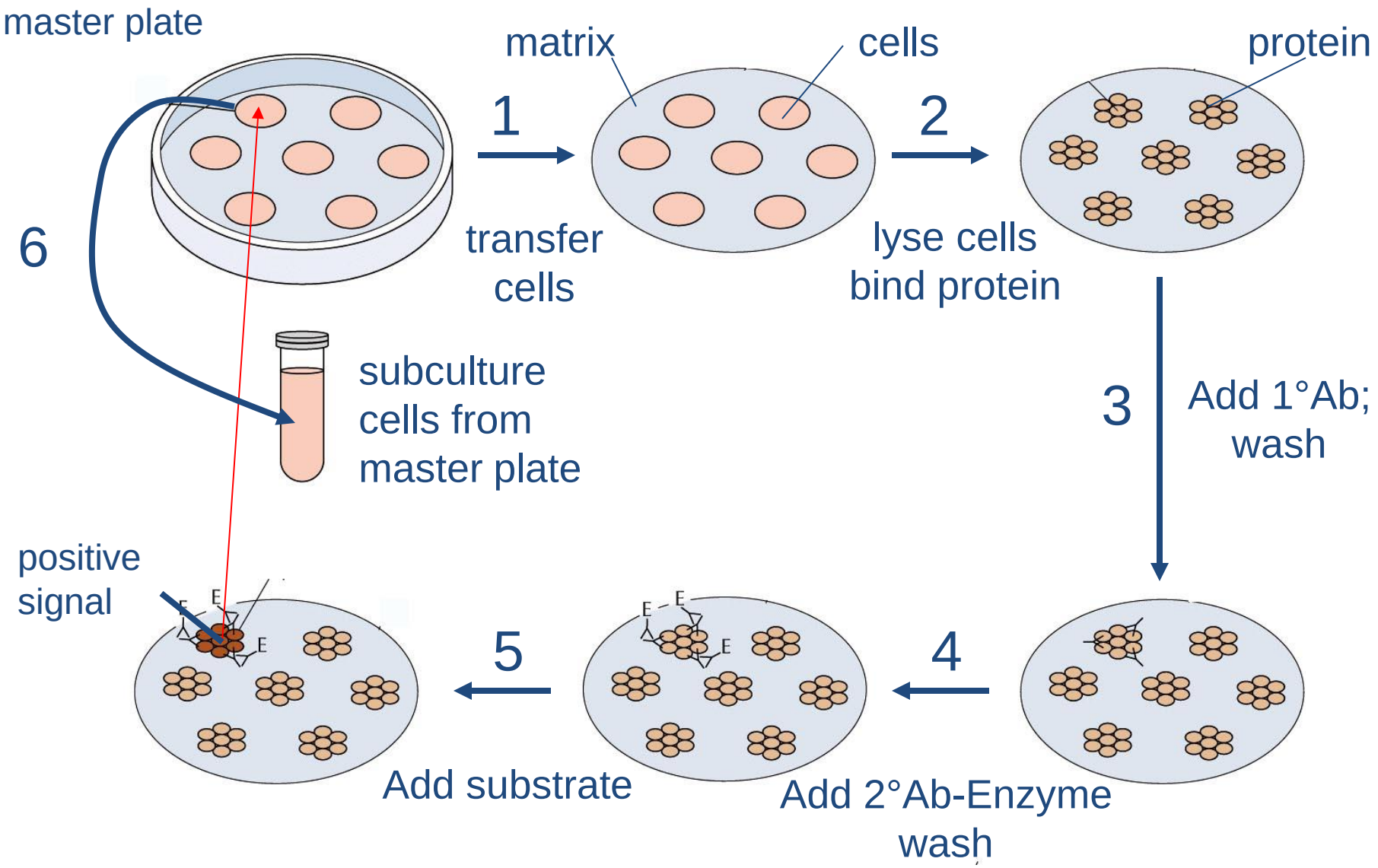
- Screening of bacteria (E.coli) or yeast clones for correct ligation or Plasmid products.
- Individual transformants can either be lysed in water with a short heating step or added directly to the PCR reaction and lysed during the initial heating step.
- Initial heating step causes the release of the plasmid DNA from the cell, so it can serve as template for the amplification reaction.
- Primers designed to specifically target the insert DNA can be used to determine if the construct contains the DNA fragment of interest and also insert orientation.



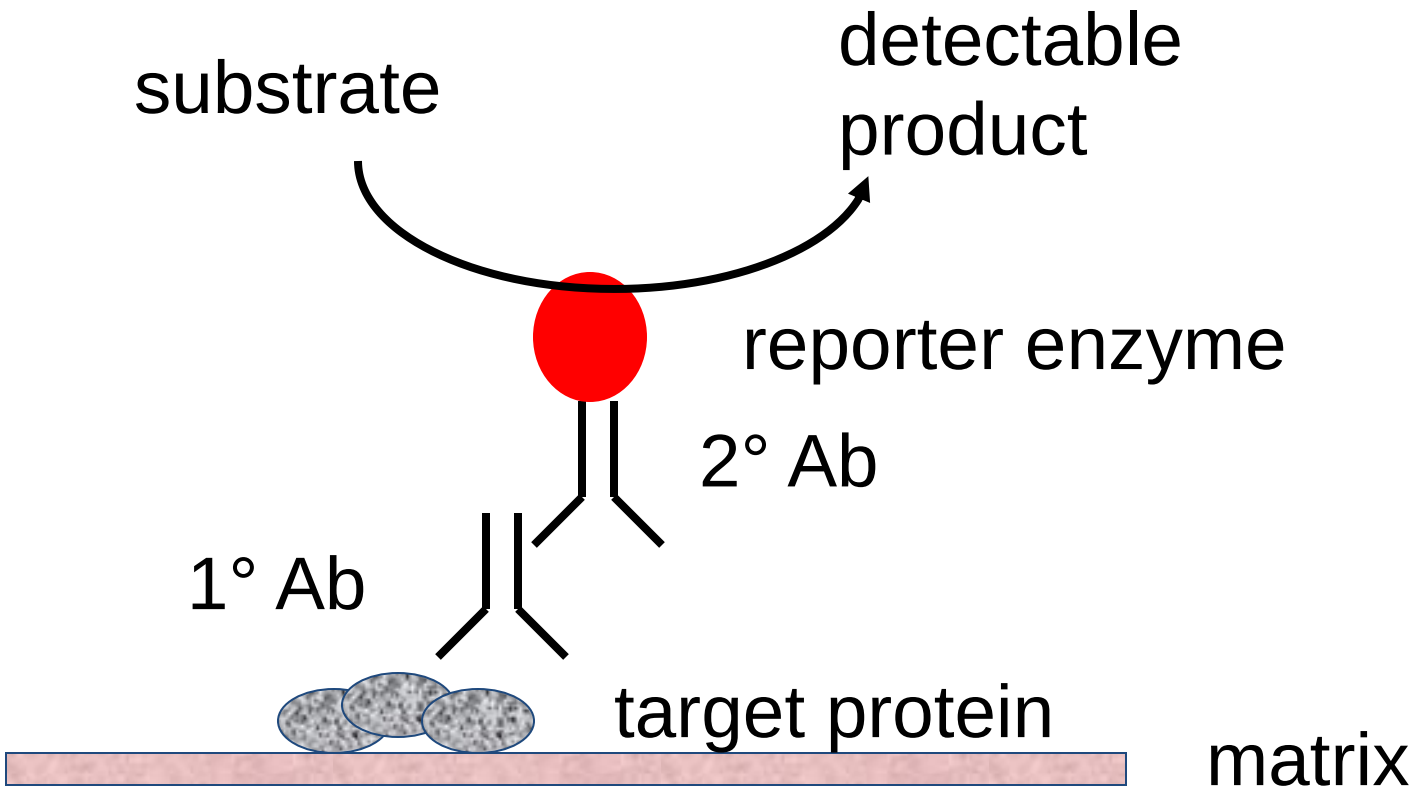
Immunological Screening

- *Antibodies to the protein encoded by the desired gene can be used to screen a library*

Immunological Screen



Immunological Screen of Library



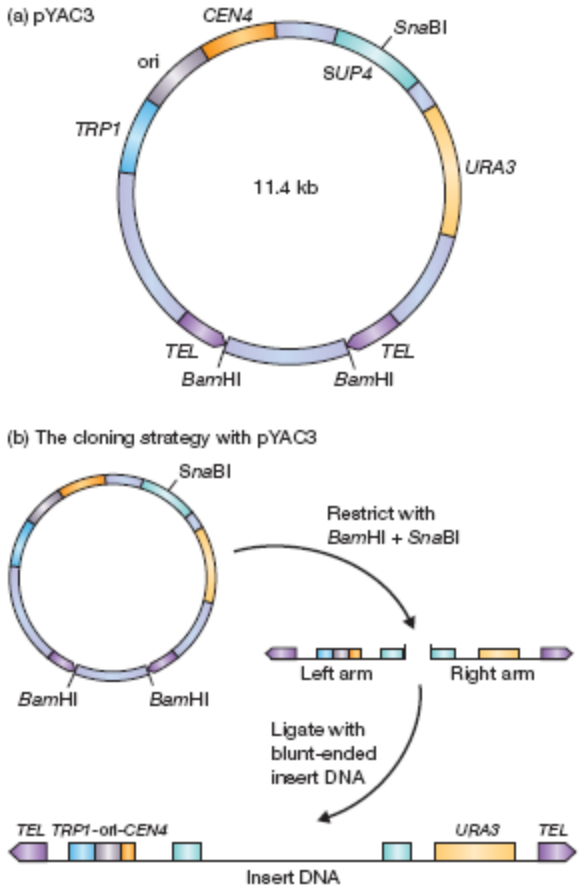


Figure 7.8
A YAC vector and the way it is used to clone large pieces of DNA.

The cloning strategy with pYAC3 is as follows (Figure 7.8b). The vector is first restricted with a combination of *Bam*HI and *Sna*BI, cutting the molecule into three