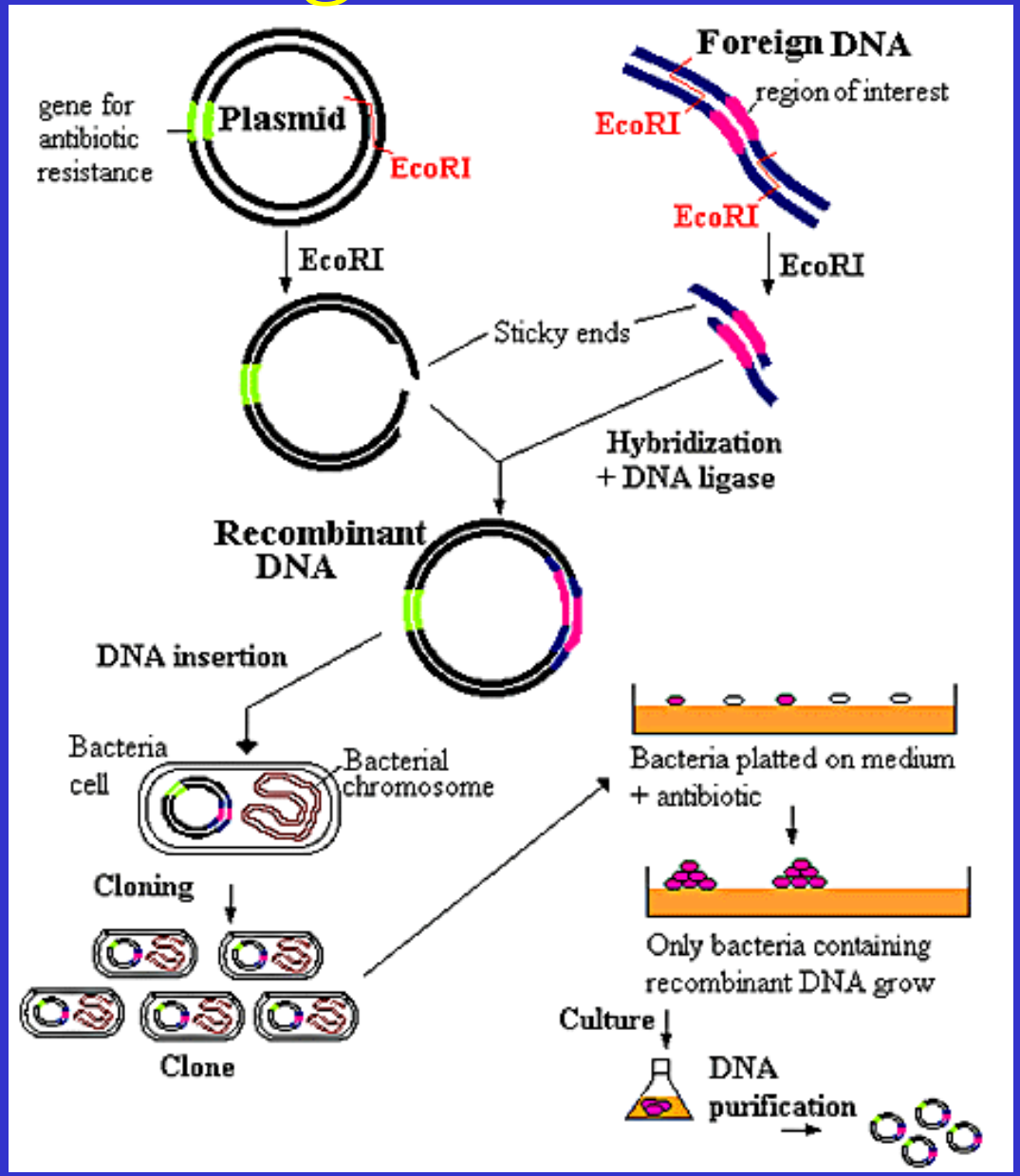
Recombinant DNA Technology

PLASMID VECTORS

Cloning into a Plasmid



Bacteria are useful hosts.

1. They are easily grown
2. They are cheap to grow
3. They grow fast
4. They are easily manipulated in the laboratory
    1. DNA can be inserted - transformation
    2. DNA can be easily isolated
5. Bacteria contain natural plasmids and viruses which are useful vectors for recombinant DNA

types

Plasmids are classified

1. by their ability to be transferred to other bacteria

1. Conjugative
   The **sexual transfer** of plasmids to another bacterium through a pilus. those plasmids possess the 25 genes required for transfert
2. Non-conjugative
   Non-conjugative plasmids don't initiate conjugaison. They can only be transferred with the help of conjugative plasmids.

2. by function

1. Fertility-(F) plasmids,
   They are capable of conjugation (they contains the genes for the pili).
2. Resistance-(R) plasmids,
   contain gene (s) that can build resistance against one or several antibiotics or poisons.
3. Col-plasmids,
   contain genes coding for colicines, proteins that can kill other bacteria.
4. Degradative plasmids,
   able to digest unusual substances, e.g., toluene or salicylic acid.
5. Virulence plasmids,
   turn a bacterium into a pathogen.

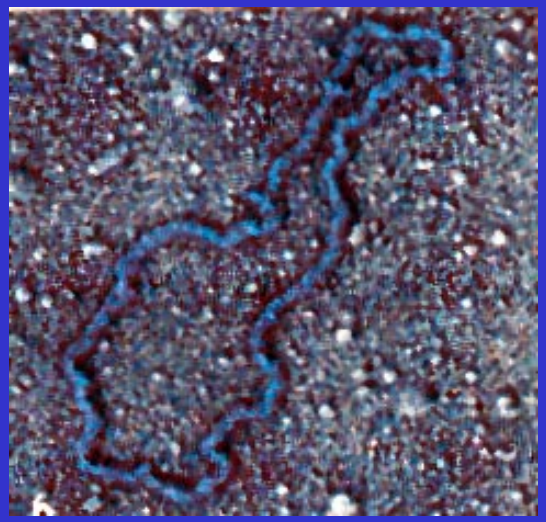
3. Copy number

High copy number = 10-100 copies / cell → generally Non conjugative

Low copy number = 1-4 copies / cell → generally conjugative

Plasmid Cloning Vectors

- Small circular piece of extrachromosomal DNA
- Must be a self-replicating genetic unit
- Plasmid DNA must replicate every time host cell divides or it will be lost
    a. DNA replication
    b. partitioning
- replication requires host cell functions



Plasmid replication

1. All self replication plasmids have a *ori*: origin of replication → it determines host and copy number
2. Plasmid segregation is maintained by a *par* locus-a partition locus that ensures each daughter cells gets on plasmid. Not all plasmids have such sequences. Essential for low copy number plasmids.

incompatibility groups:

Several types of plasmids could coexist in a single cell.
On the other hand, related plasmids are often 'incompatible', resulting in the loss of one of them from the cell line. Due to same *ori* or same *par*

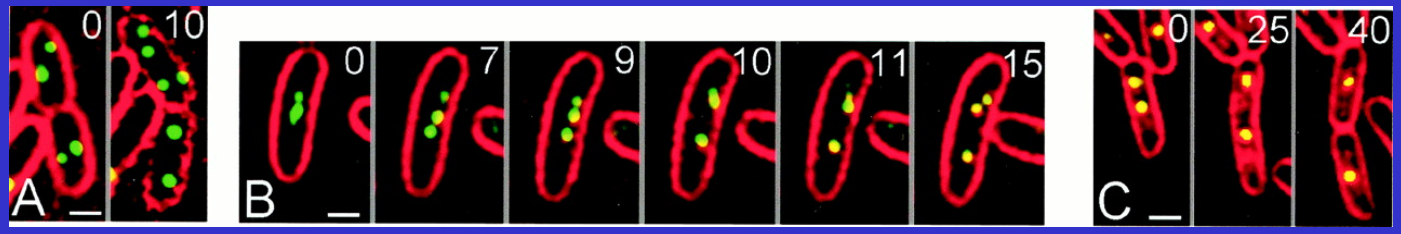
Copy Number:

- Antisense RNA
- Protein mediated

Incompatibility Groups

1. Not all plasmids can live together.
2. Plasmids that are able to coexist in the same cell do not interfere with each other's replication
3. A single cell can have as many Inc group plasmids as it can tolerate and replicate!

Partion Locus: a region on broad host range plasmids that binds to a structure on the inner membrane of the cell to ensure proper segregation. Plasmids labeled with fluorescent protein
-move to each daughter cell during division.



Pogliano, Joe et al. (2001) Proc. Natl. Acad. Sci. USA 98, 4486-4491

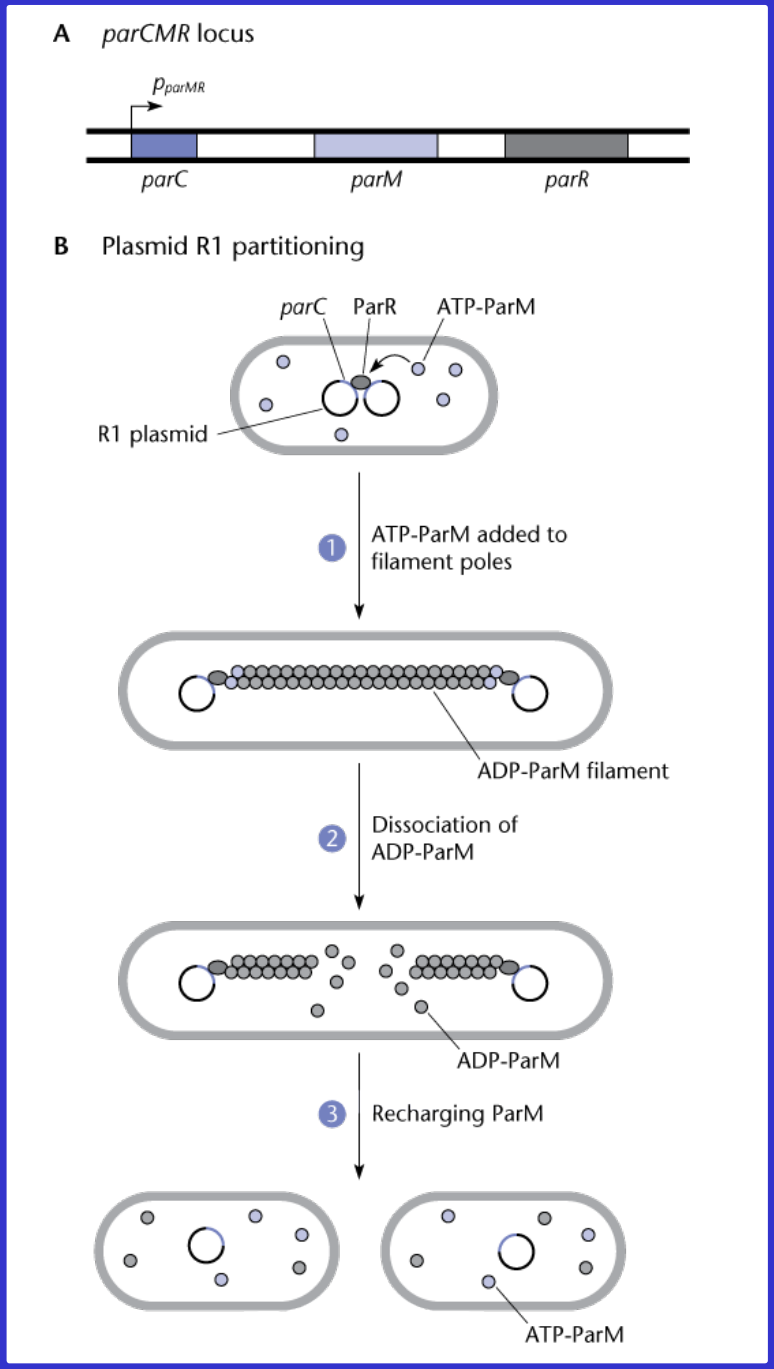


Figure 4.18

Par locus

-think of this as a primitive centromere

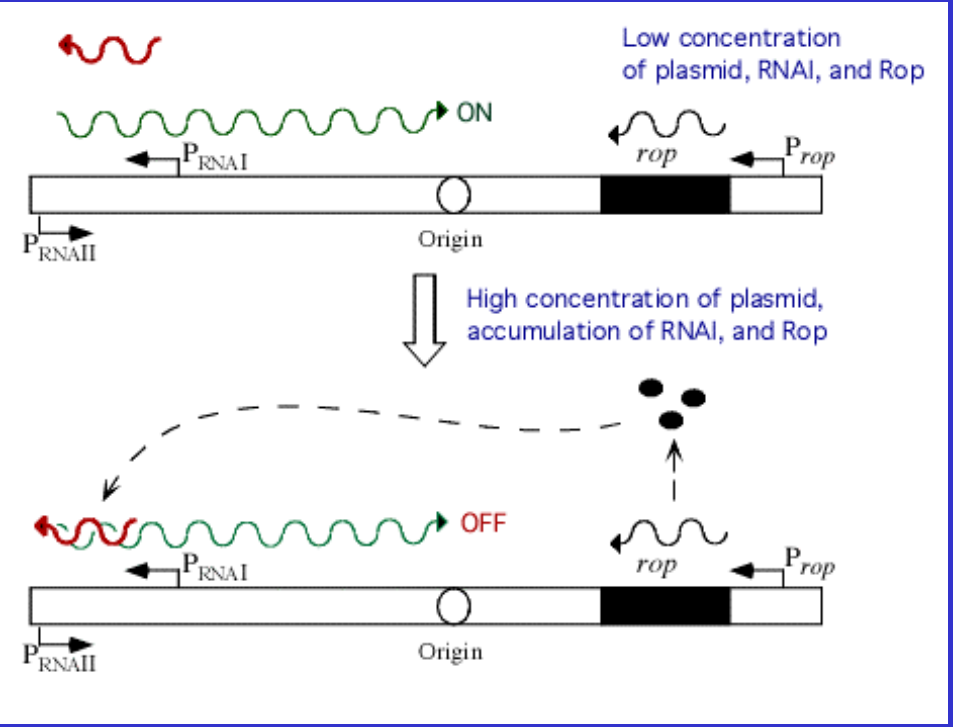
-the growing filaments push the plasmids to the opposite poles of the cells

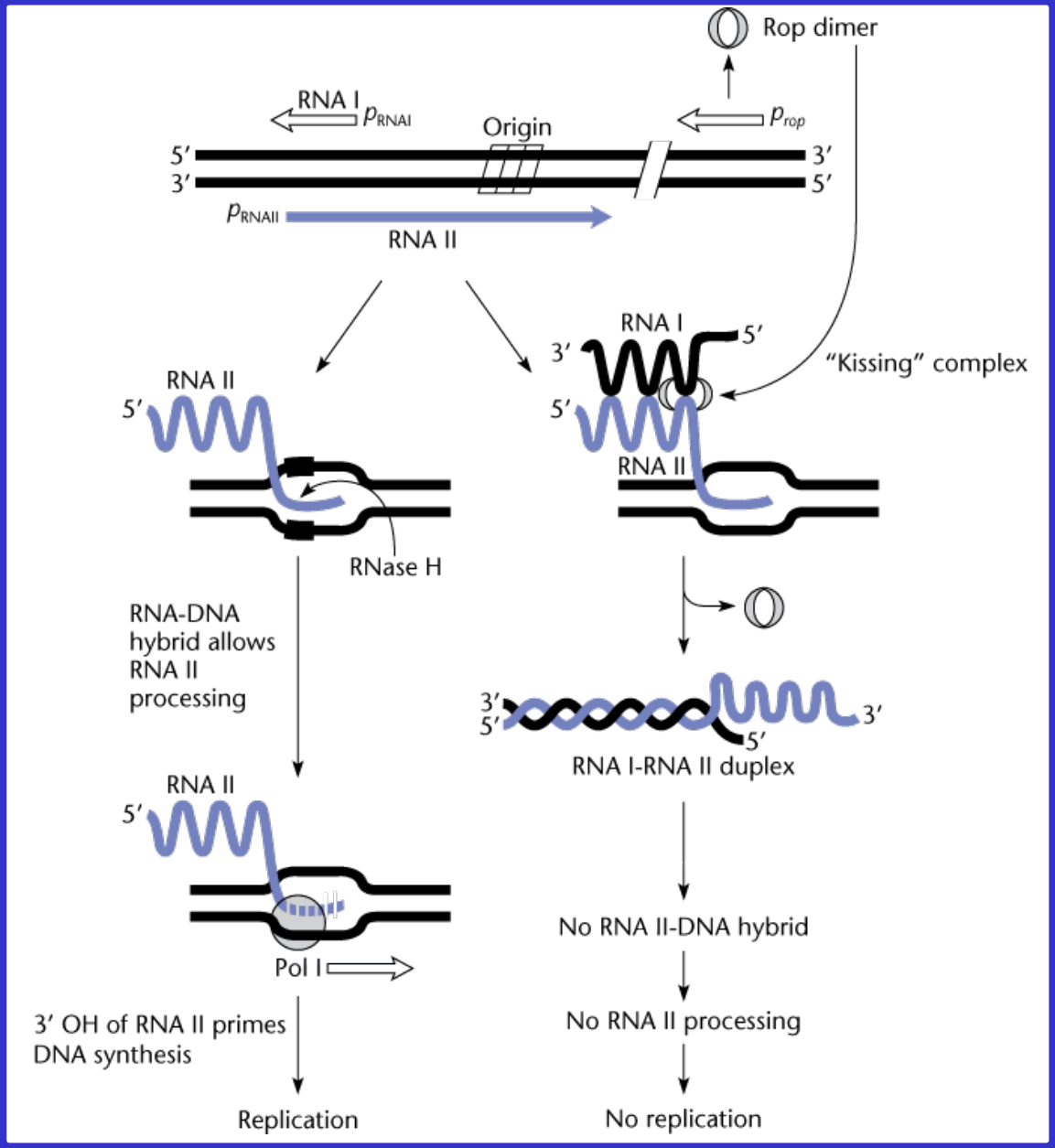
Col E1 replication: Anti-sense RNA control

RNAII will act as a primer for DNA replication

RNA I-small inhibitory RNA that binds to RNAII. Its amount is proportional to plasmid copy number

Rop: plasmid encoded proteins which stabilizes the RNAI-RNAII complex





ColE1 Replication Control- an example of primer control of replication

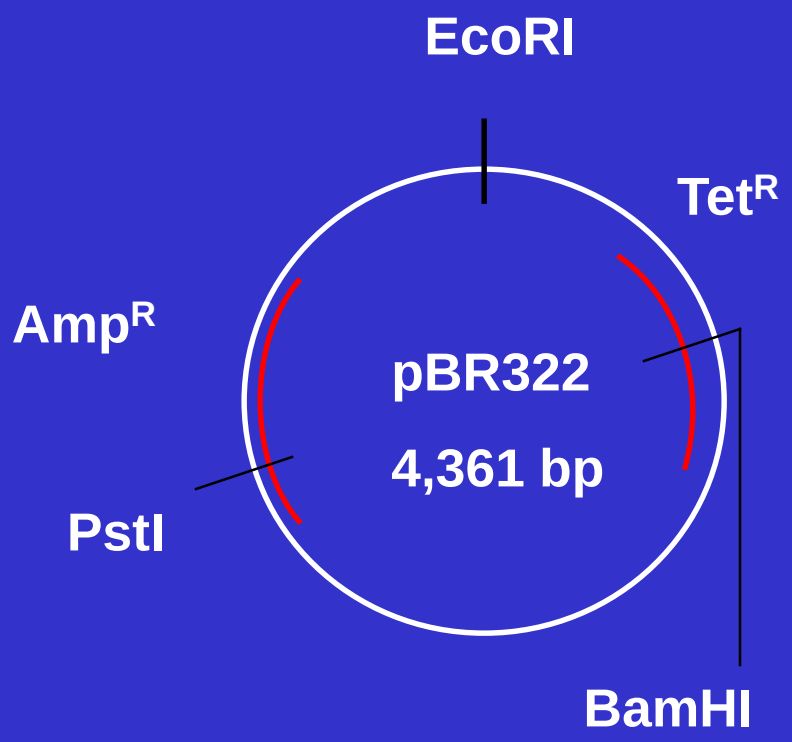
1. RNAII will serve as a primer for the replication fork.
2. The 3' end is processed by host RnaseH to allow efficient RNA-DNA hybrid to form
3. The hybrid acts as a primer for host Pol1
4. As the concentration of plasmid increases, Rop does also
5. Rop stabilizes the RNA1-II complex
6. No RNA for replication priming.

Plasmid Cloning Vectors

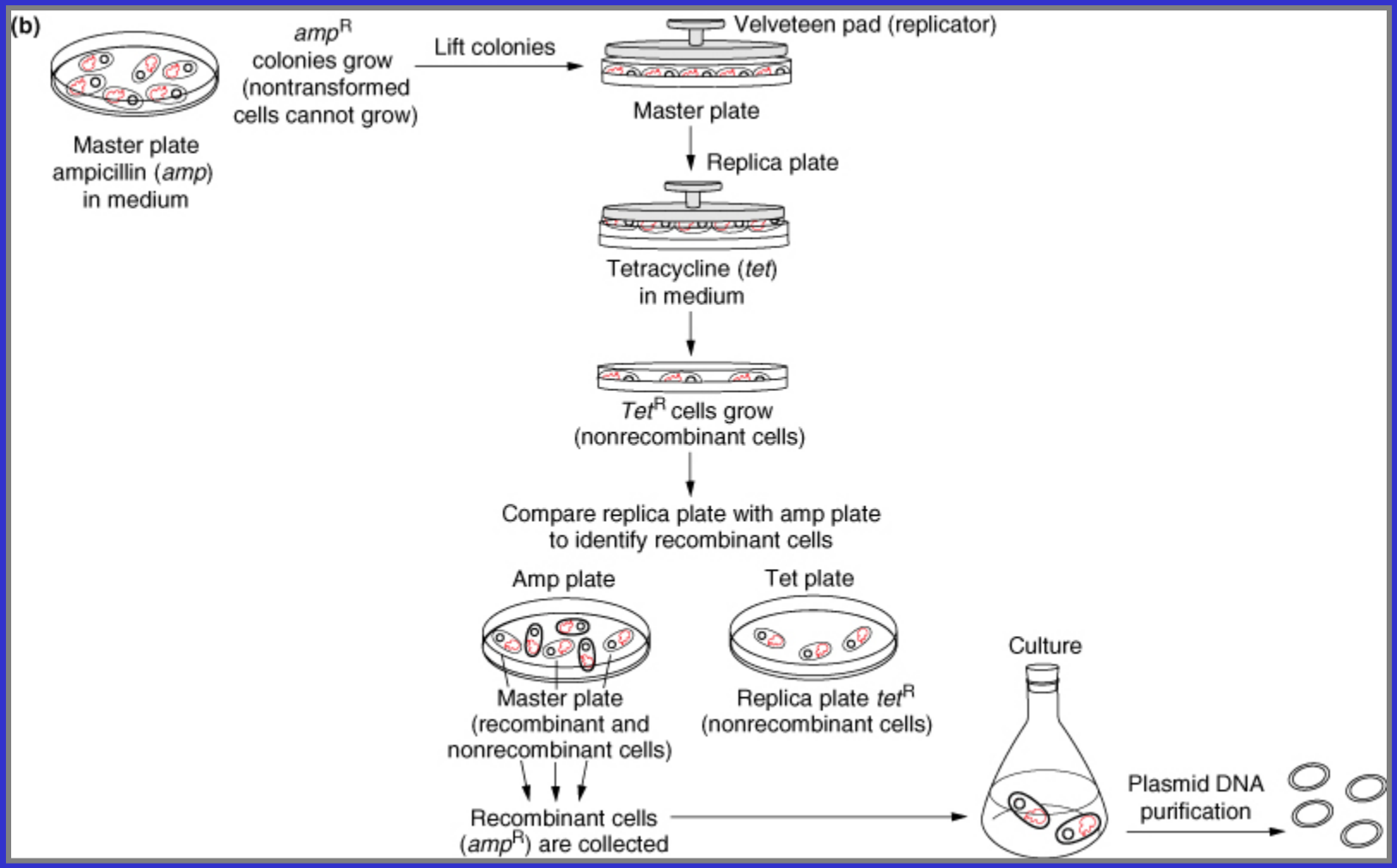
- Derived from naturally occurring plasmids
- Altered features
  - small size (removal of non-essential DNA) →higher transformation efficiency, manipulation and purification easier
  - unique restriction enzyme sites
  - one or more selectable markers
  - other features: promoters, etc.

The Cadillac of Cloning Vectors

- pBR322
  - Clone fragment in one antibiotic gene
  - Select for other antibiotic resistance
  - Screen for presence of one resistance gene (selects against untransformed bacteria) and loss of resistance to interrupted antibiotic resistance gene (selects for recombinant molecule)

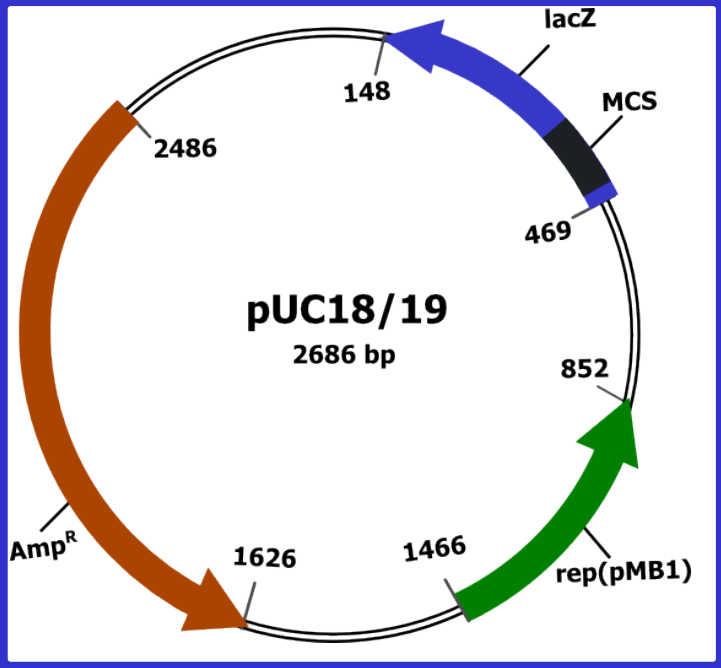


Screening bacteria by replica plating



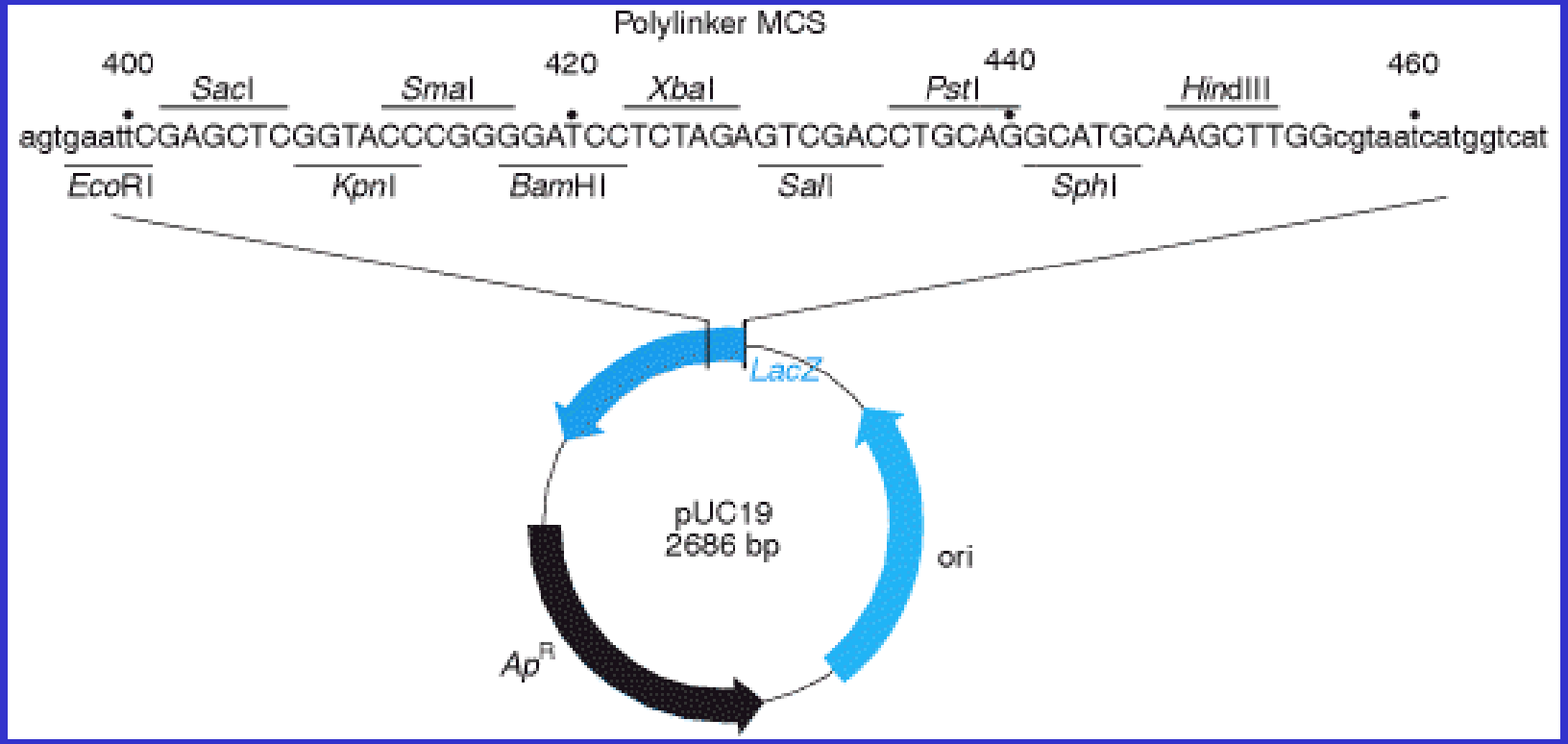
Next Major Advance in Plasmid(ology)

- The inclusion of polylinkers into plasmid vectors
- Polylinker is a tandem array of restriction endonuclease sites in a very short expanse of DNA
- For example, pUC18's polylinker
  - Sites for 13 RE's
  - Region spans the equivalent of 20 amino acids or 60 nucleotides



The Polylinker Advantage

- Unique sites (usually)
- Insert excision facilitated
- Restriction endonuclease mapping and Subcloning made easier
- Directional cloning



Another Major Advance: Blue-White Screening

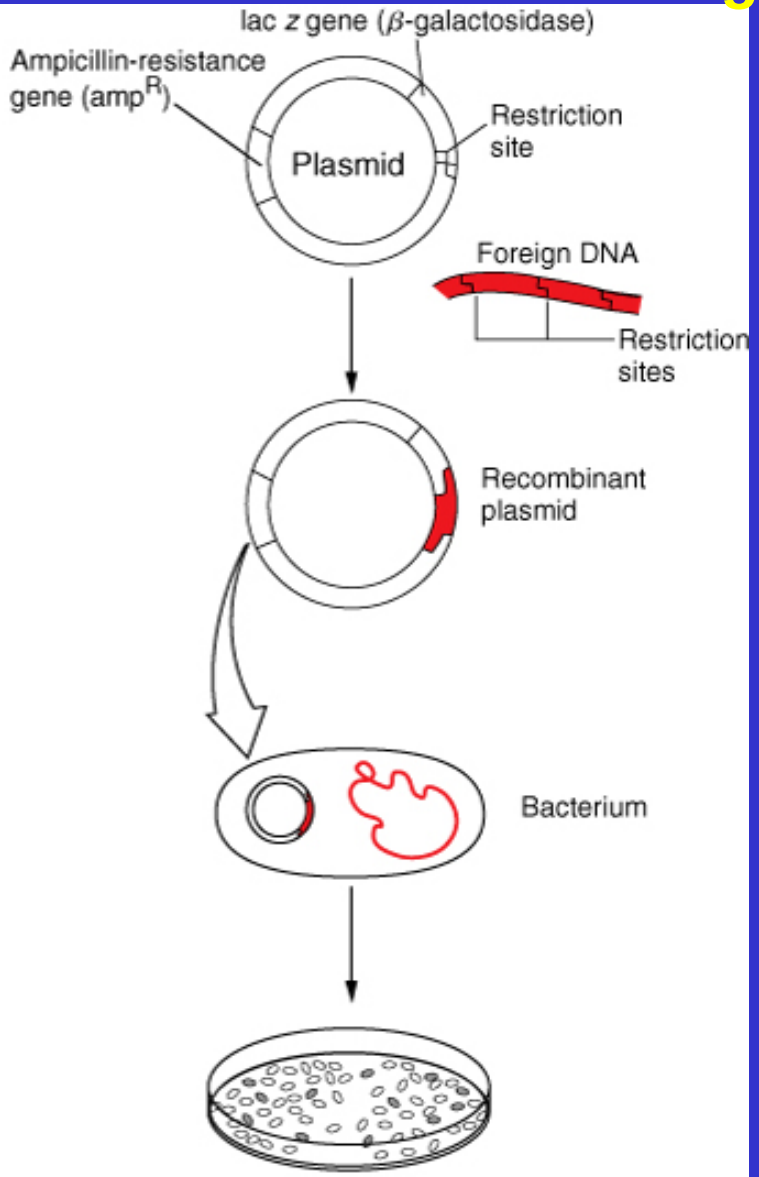
1) Plasmid DNA and foreign DNA are both cut with the same restriction enzyme.

2) Foreign DNA is inserted into the plasmid, where it inactivates the lac z gene.

3) The recombinant plasmid is introduced into a bacterium, which becomes ampicillin-resistant.

4) All treated bacteria are spread on a nutrient agar plate containing ampicillin and β-galactosidase substrate and incubated.

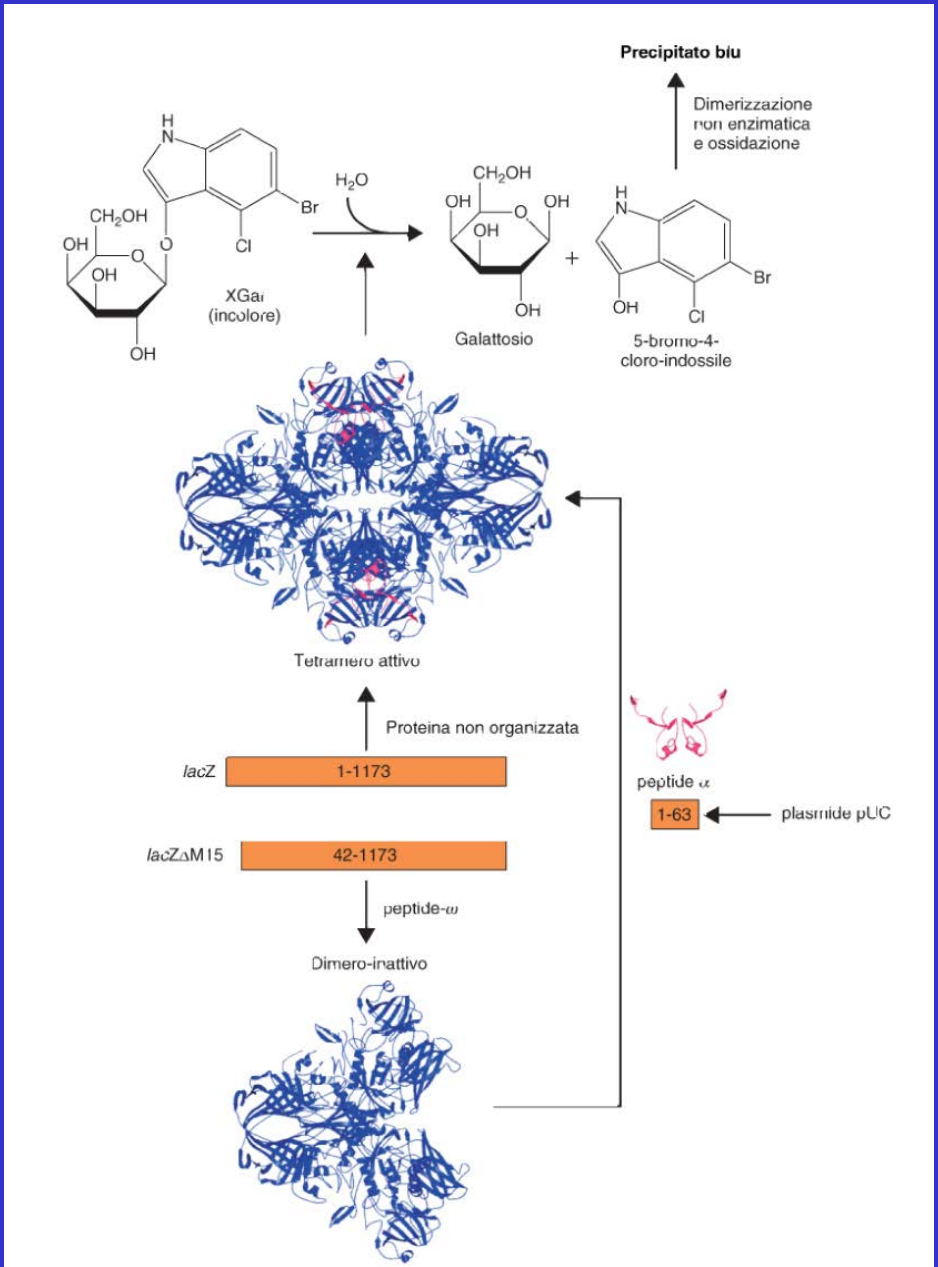
5) White colonies that appear must contain foreign DNA. Blue (gray in this illustration) colonies do not contain foreign DNA.



Alpha complementation:

LacZ→ Beta galactosidase
(Homotetramer)
1021aa →3,1kbp

- Bacteria carry mutant allele (LacZΔM15) lacking N-terminal domain → inactive protein
- Alpha peptide carried by plasmid
- Exploits X-Gal (5-bromo-4-cloro-3-indolil-Betagalattoside), a chromogenic substrate analog to galactose
- MCS inserted into LacZ alpha peptide →
  - With insert = white colonies
  - Without insert = blue colonies



Esempio di selezione bianco-blu in pUC

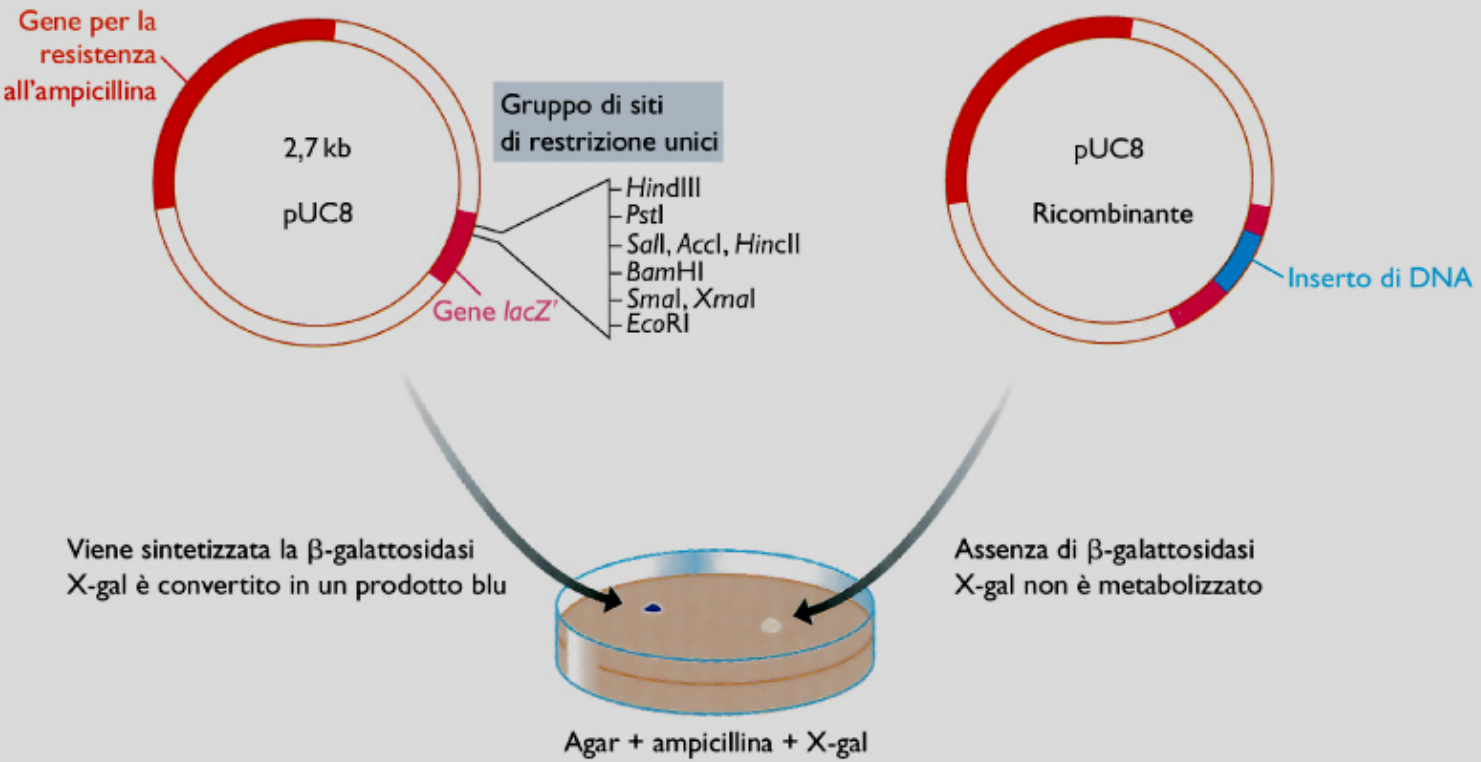
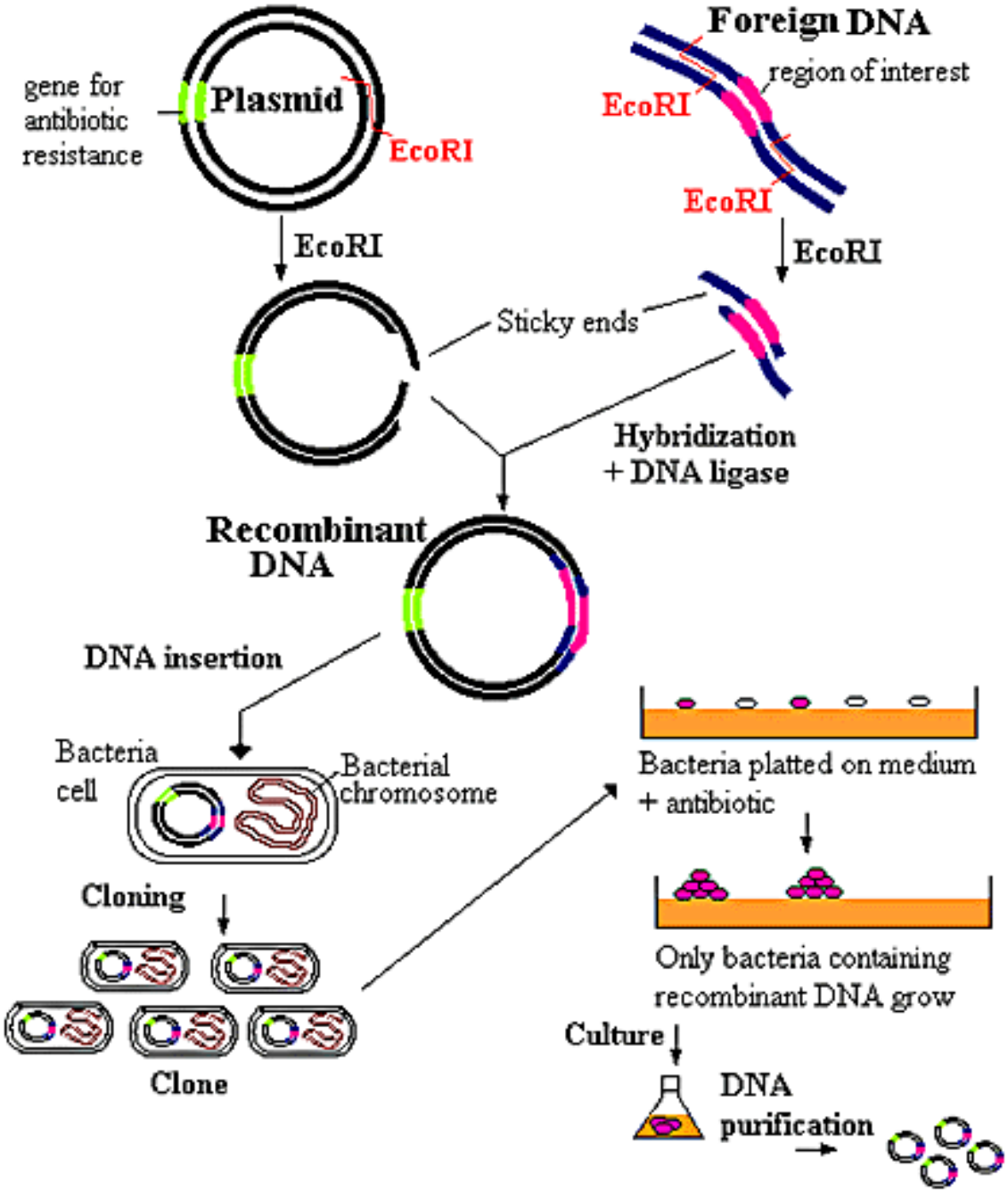


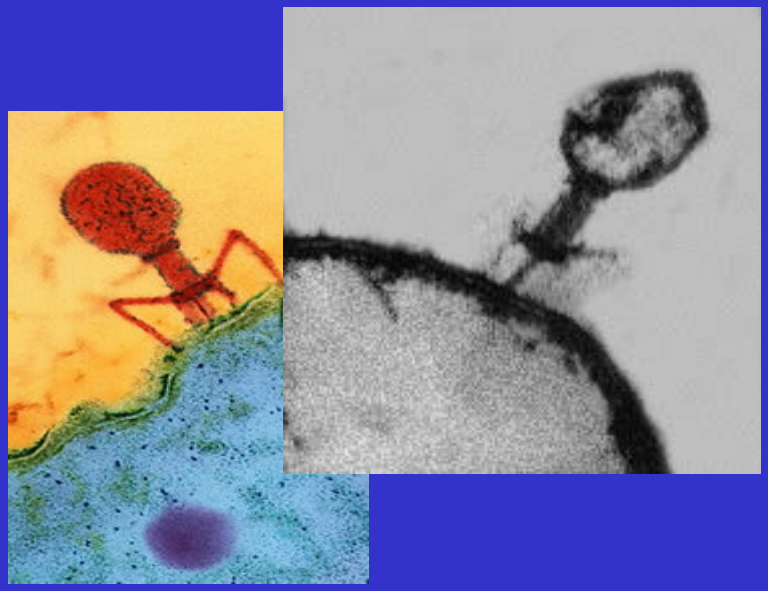
Figura 4.19 Selezione dei ricombinanti con pUC8.

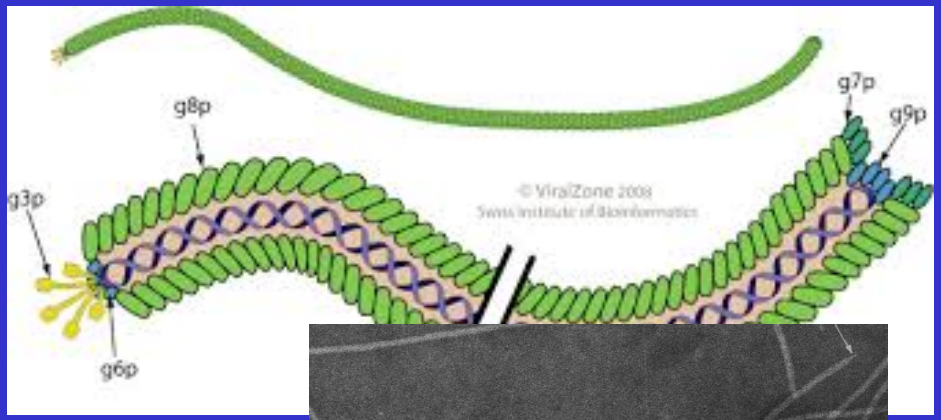
T.A. Brown
Genomi
EdiSES



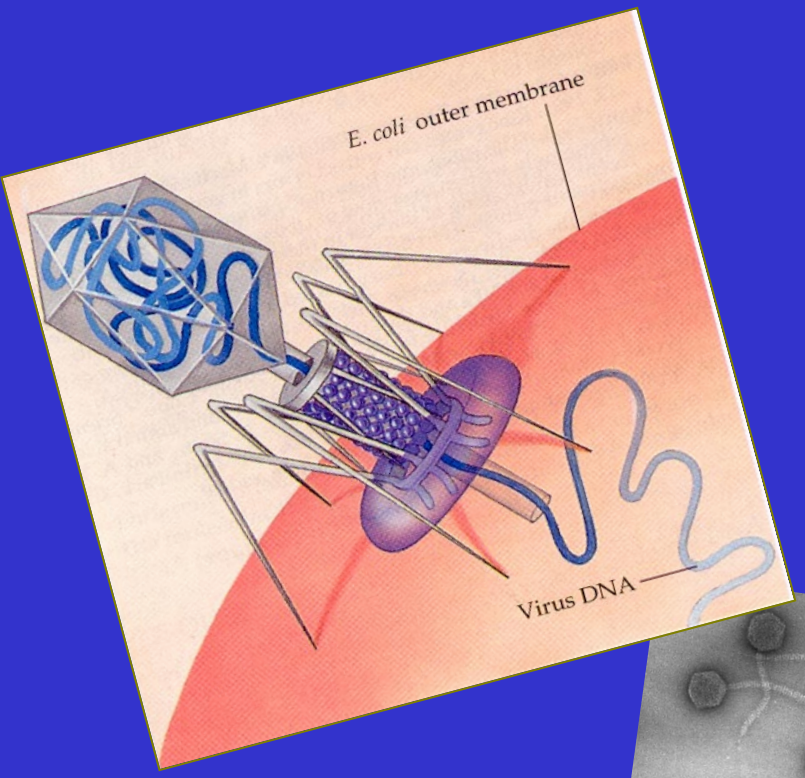
Cloning into a plasmid

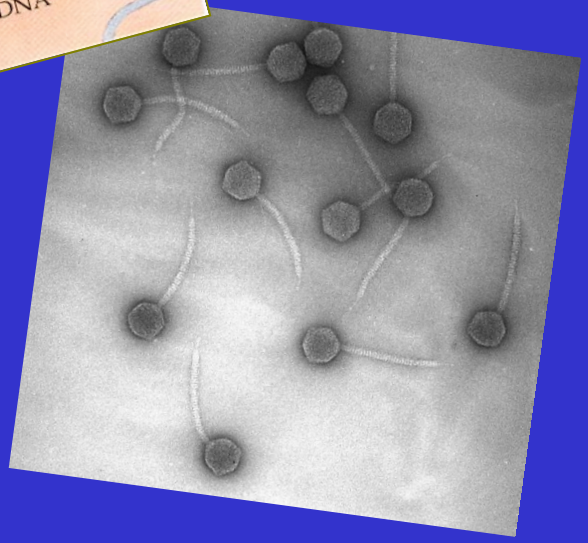
λ and M13 phage-based plasmid



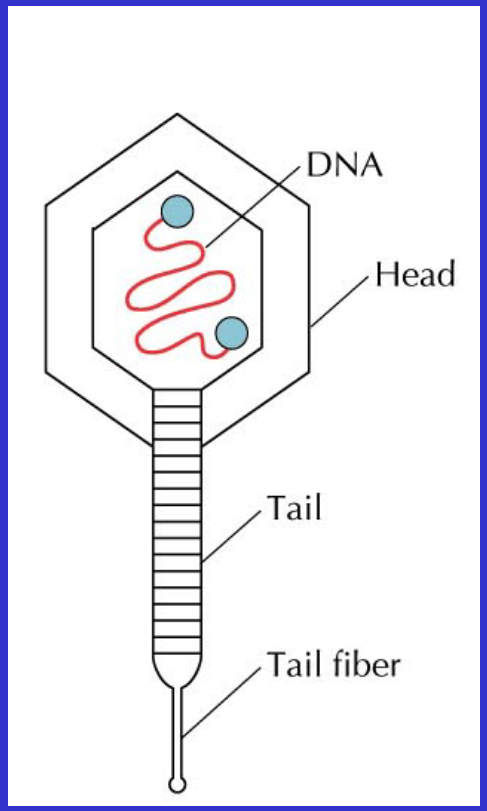


Bacteriophage lambda (λ)





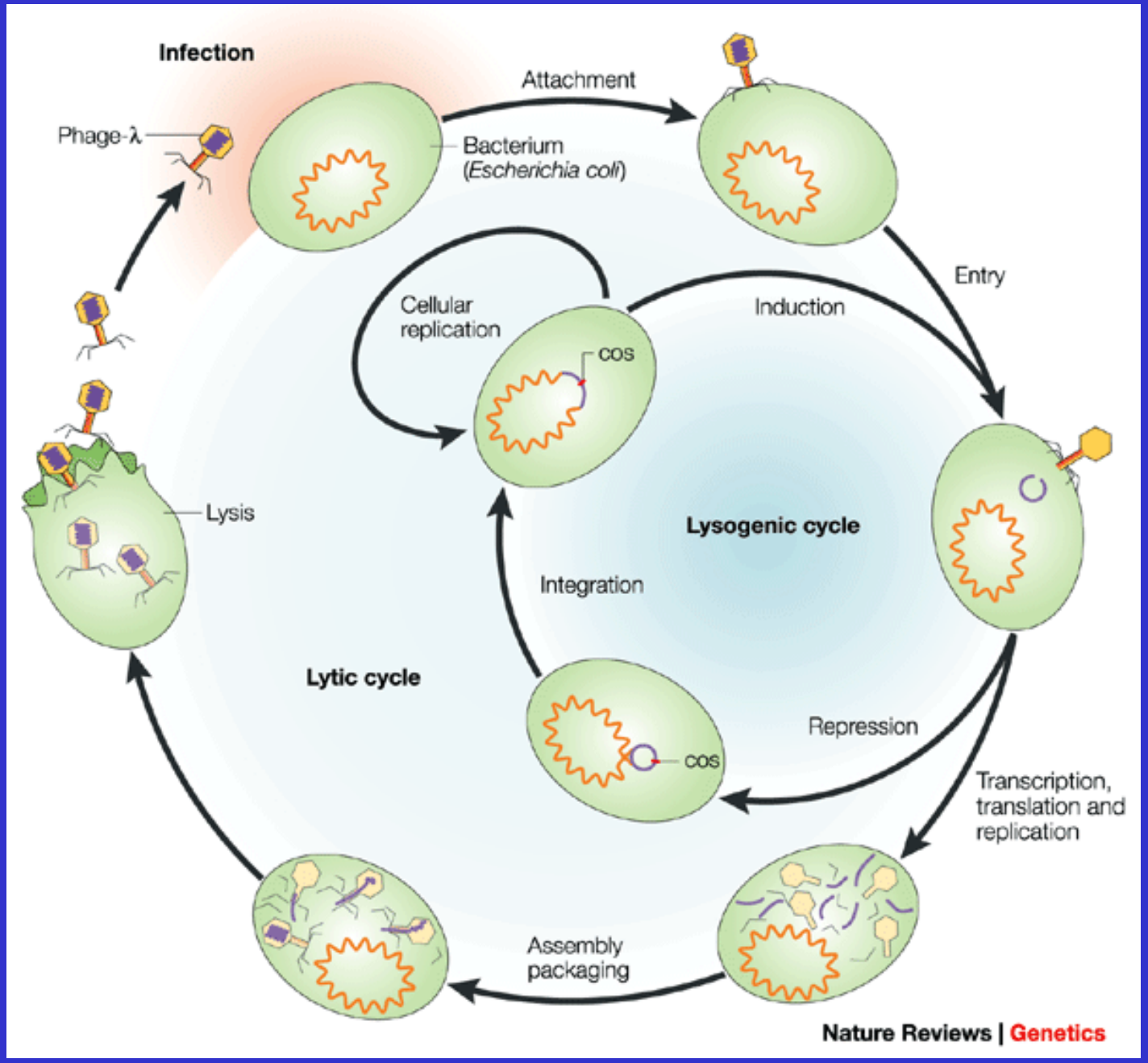
- A virus that infects bacteria

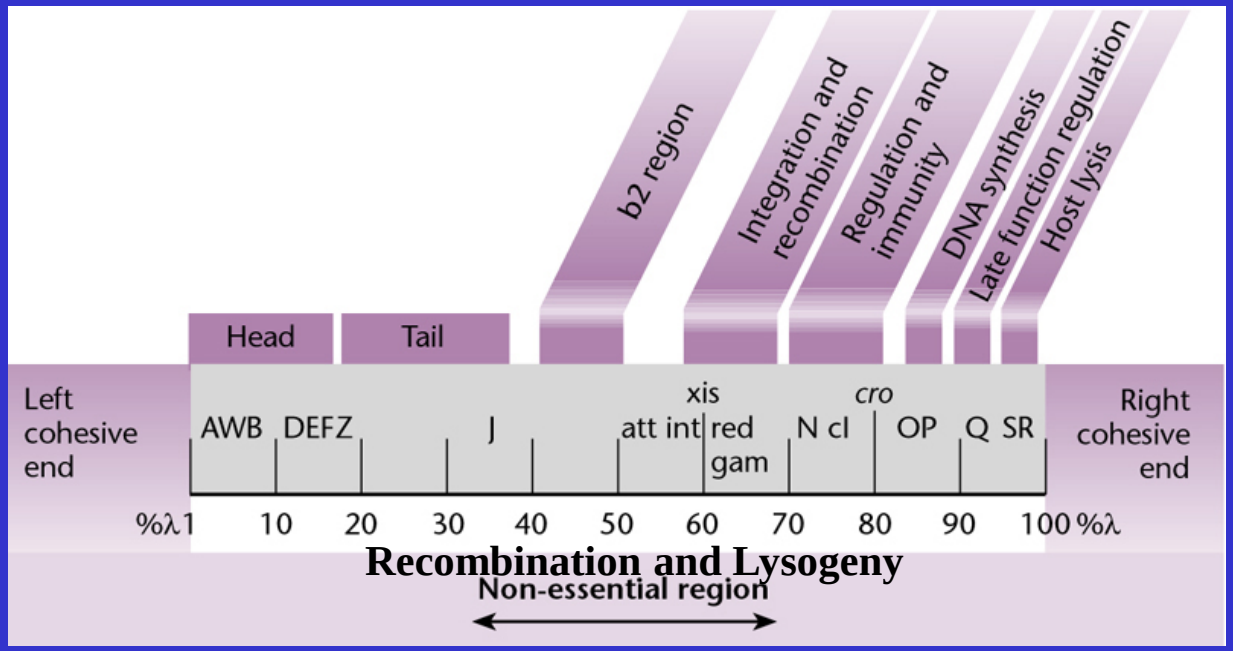


Bacteriophage lambda

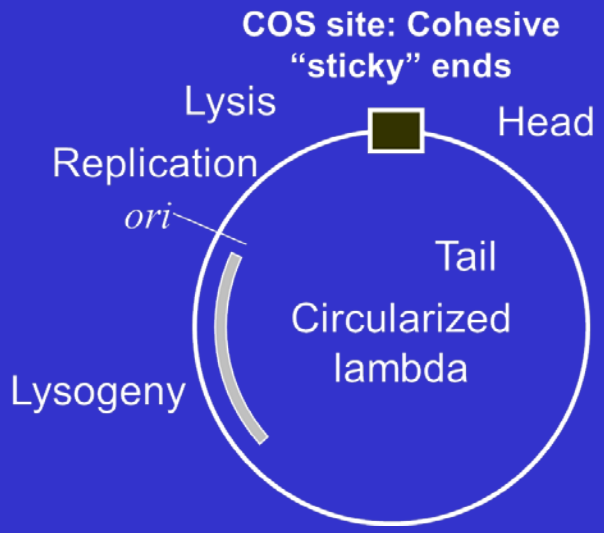
- “head and tail” phage, very well-studied
- Large, linear genome--48.5 kb
- Two lifestyle modes
  - Lytic: replicative mode
  - Lysogenic: latent mode
- Useful for cloning 5-25 kb DNA fragments

Lytic and Lysogenic cycle





Cos site :at the ends short (12bp) ss-complementary region "cohesive or sticky" ends--- circulation after infection



Only 30 kb is required for lytic growth.

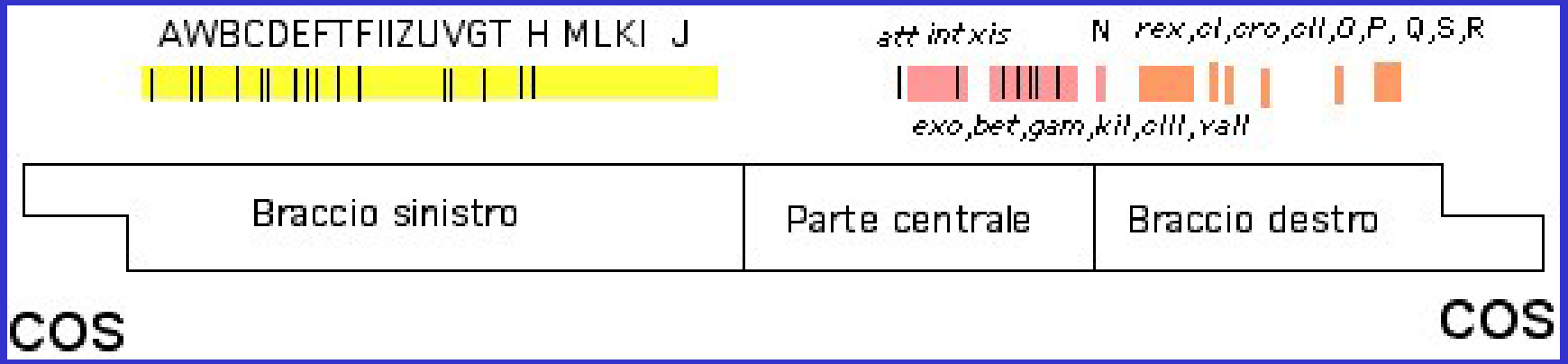
Thus, one could clone 19 kb of "foreign" DNA.

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

Left Arm: structural genes for head and tail

Central Region: genes for lysogenic growth and ricombination/insertion of genome into baterial genome

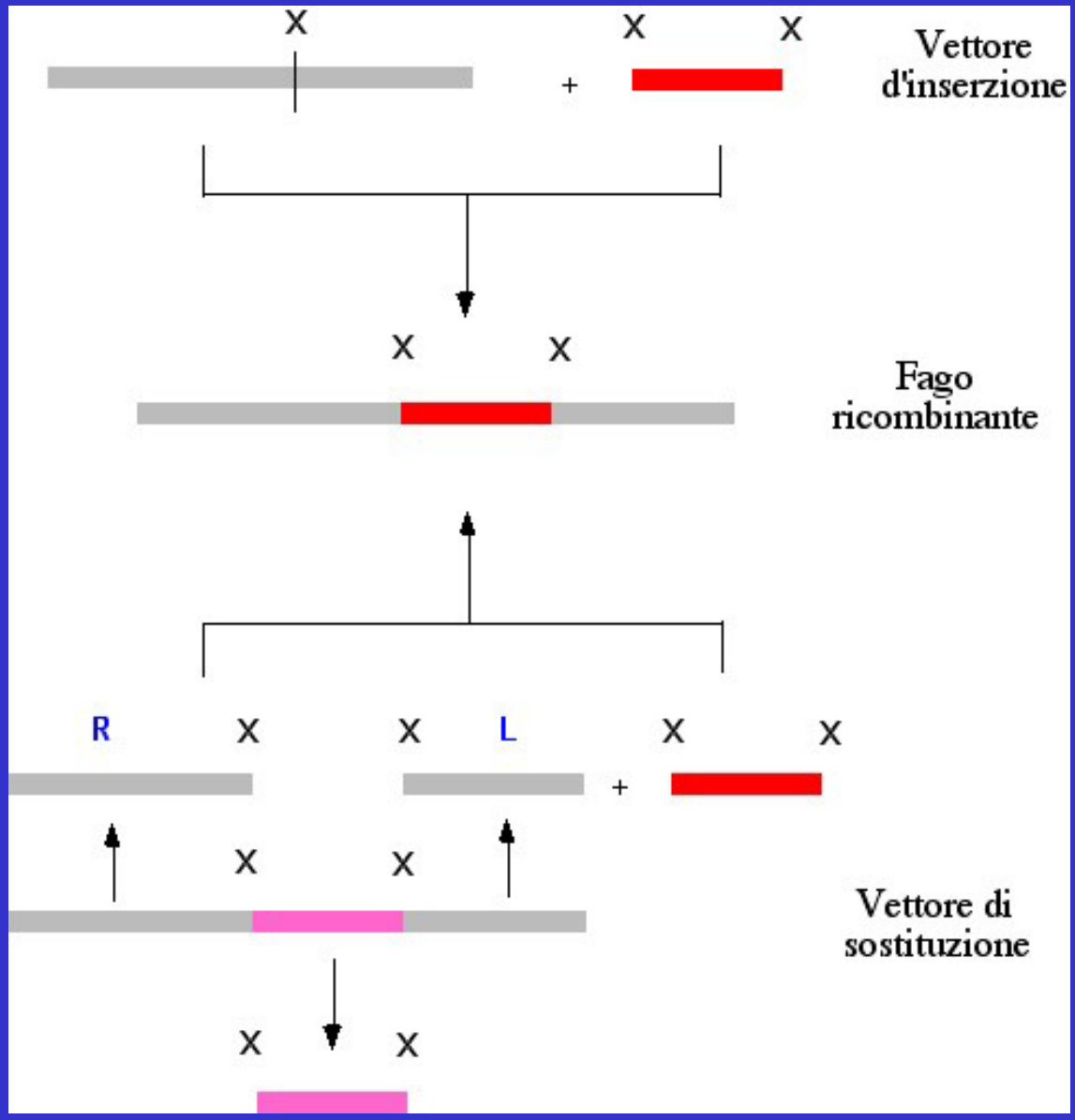
Right Arm: genes involved in DNA replication and lytic growth



Lambda as a cloning vector

- Insertional vectors (clone into one or multiple restriction sites, can only increase genome size by 5% (size of foreign DNA insert depends on the original size of the phage vector, about 5 to 11 kb)

- Replacement vectors (removing “stuffer”), can clone larger pieces of DNA, 8 to 24 kb (sufficient for many eukaryotic genes)


X
X
X
+
Vettore
d'inserzione
X
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Fago
ricombinante
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X
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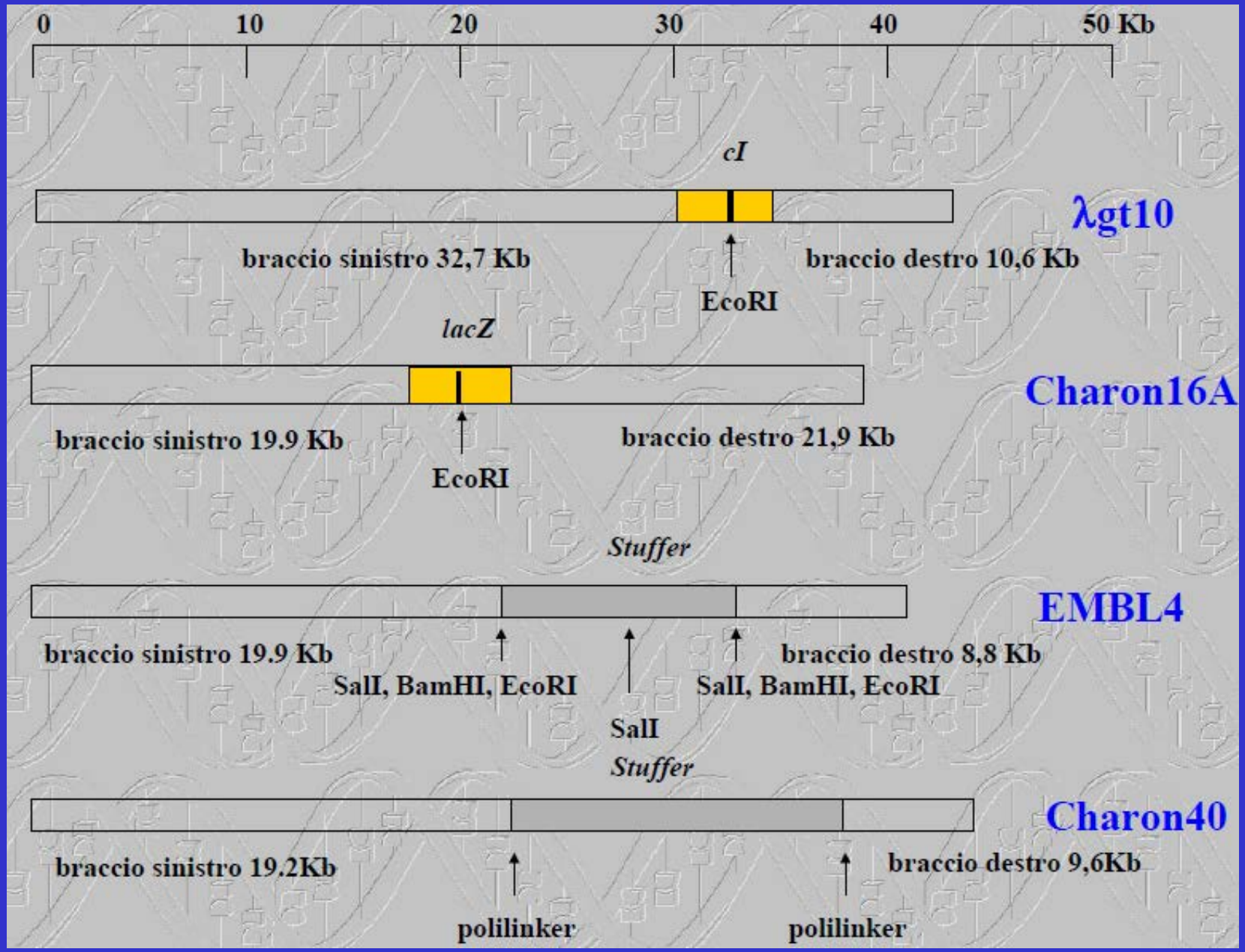
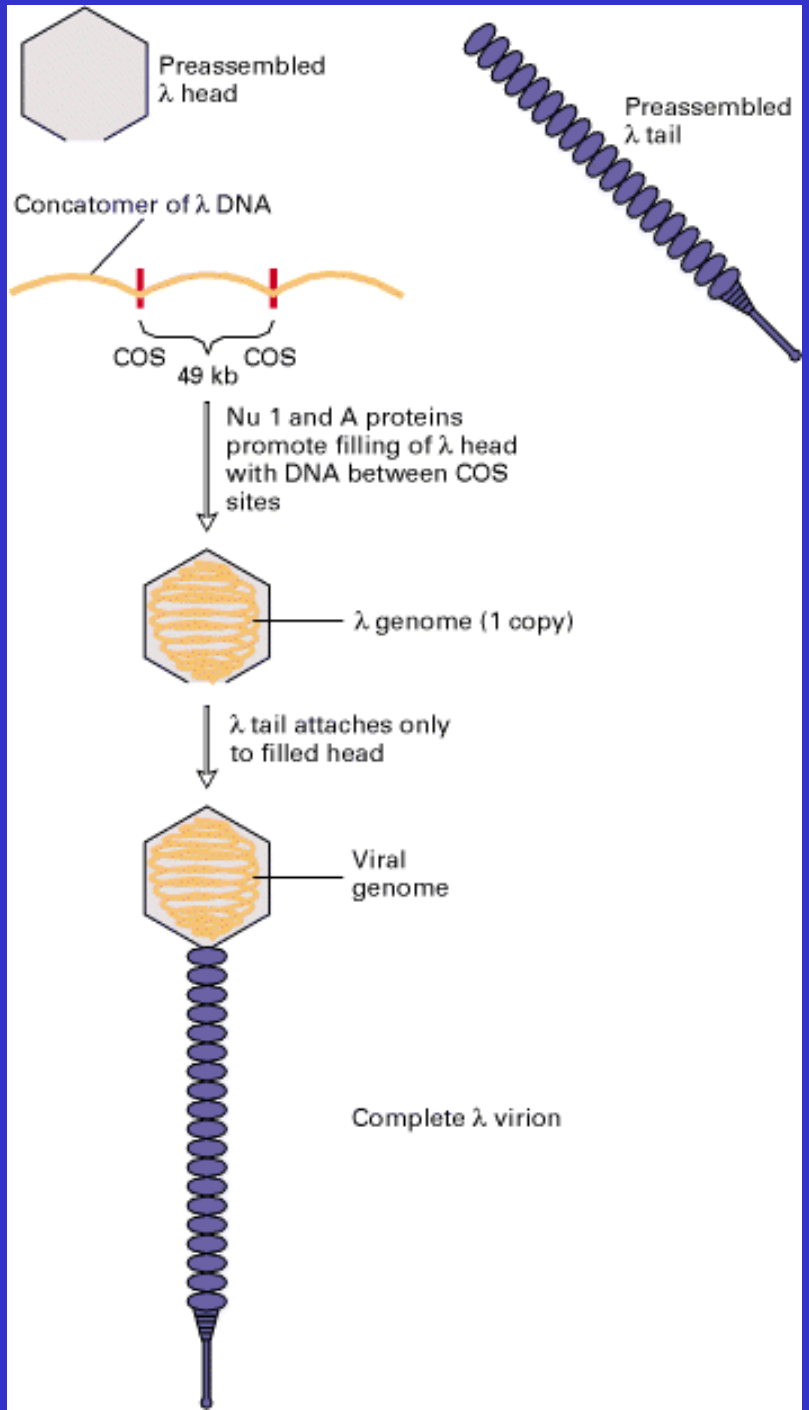
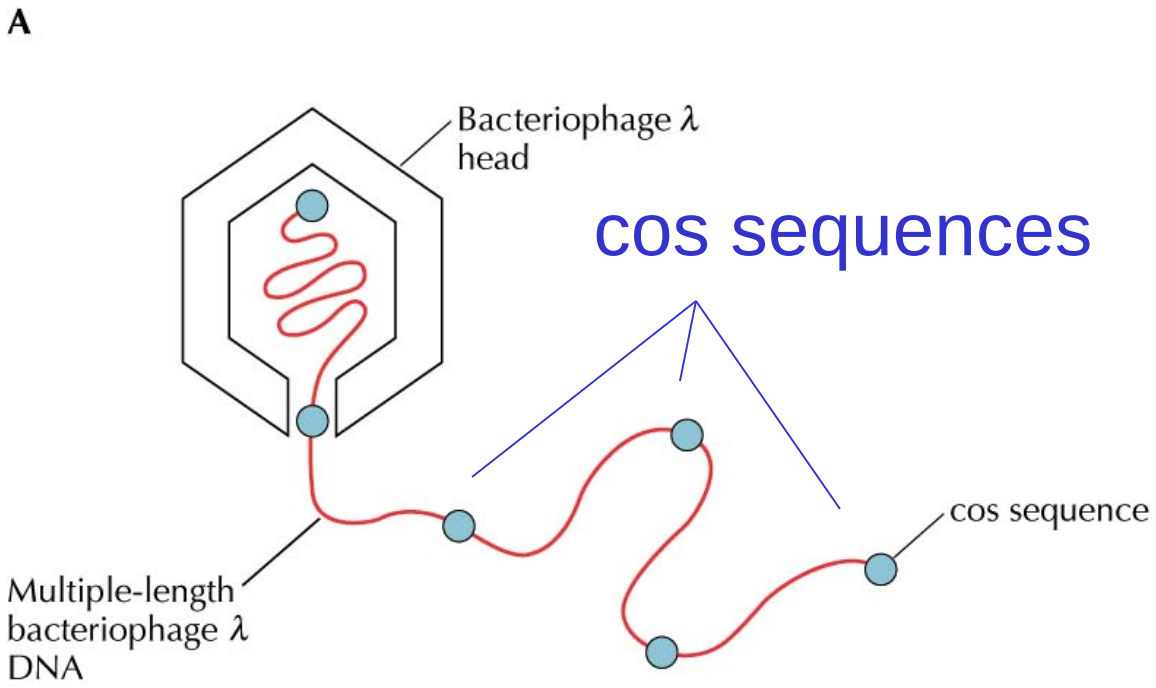
Vettore di
sostituzione
X
X

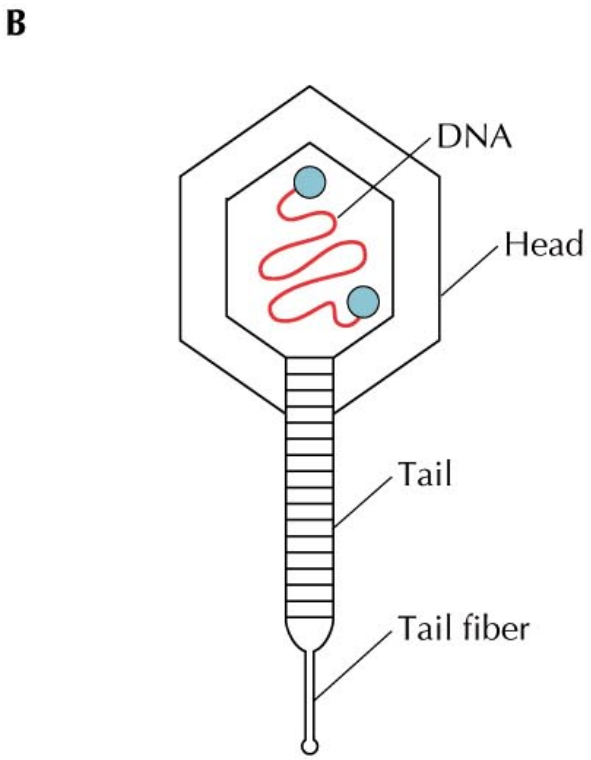

0
10
20
30
40
50 Kb
cI
λgt10
braccio sinistro 32,7 Kb
braccio destro 10,6 Kb
EcoRI
lacZ
Charon16A
braccio sinistro 19.9 Kb
braccio destro 21,9 Kb
EcoRI
Stuffer
EMBL4
braccio sinistro 19.9 Kb
braccio destro 8,8 Kb
SalI, BamHI, EcoRI
SalI, BamHI, EcoRI
SalI
Stuffer
Charon40
braccio sinistro 19.2Kb
braccio destro 9,6Kb
polilinker
polilinker

But bacterial transformation with recombiant lambda phages is very ineffective

In Vitro Packaging

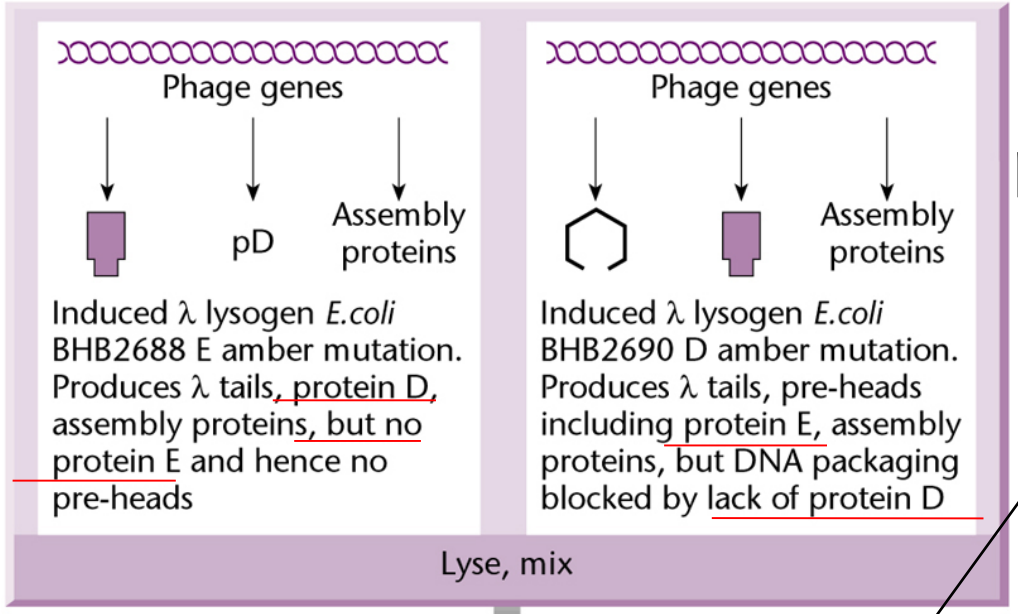

Preassembled
λ head
Preassembled
λ tail
Concatomer of λ DNA
COS
49 kb
COS
Nu 1 and A proteins
promote filling of λ head
with DNA between COS
sites
λ genome (1 copy)
λ tail attaches only
to filled head
Viral
genome
Complete λ virion



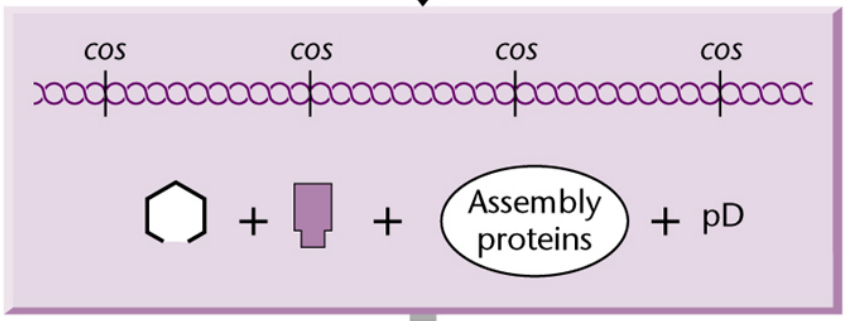


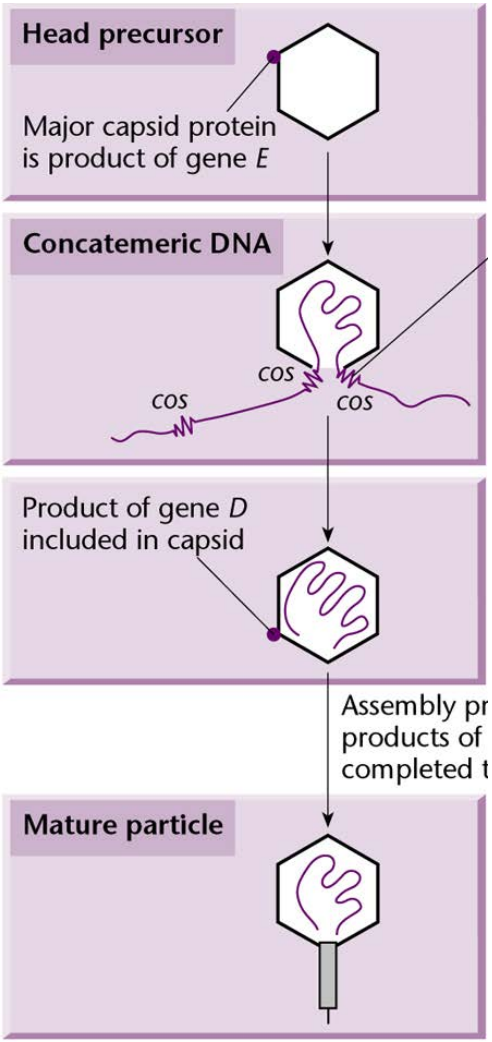
DNA can be packaged into phage particle in vitro

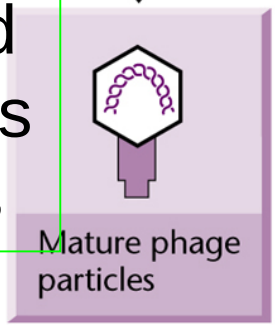
How to transfer recombinant lambda into cells?



Add ATP and concatemerized λDNA







The packaged phage particles are infectious

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

Filamentous phages: M13

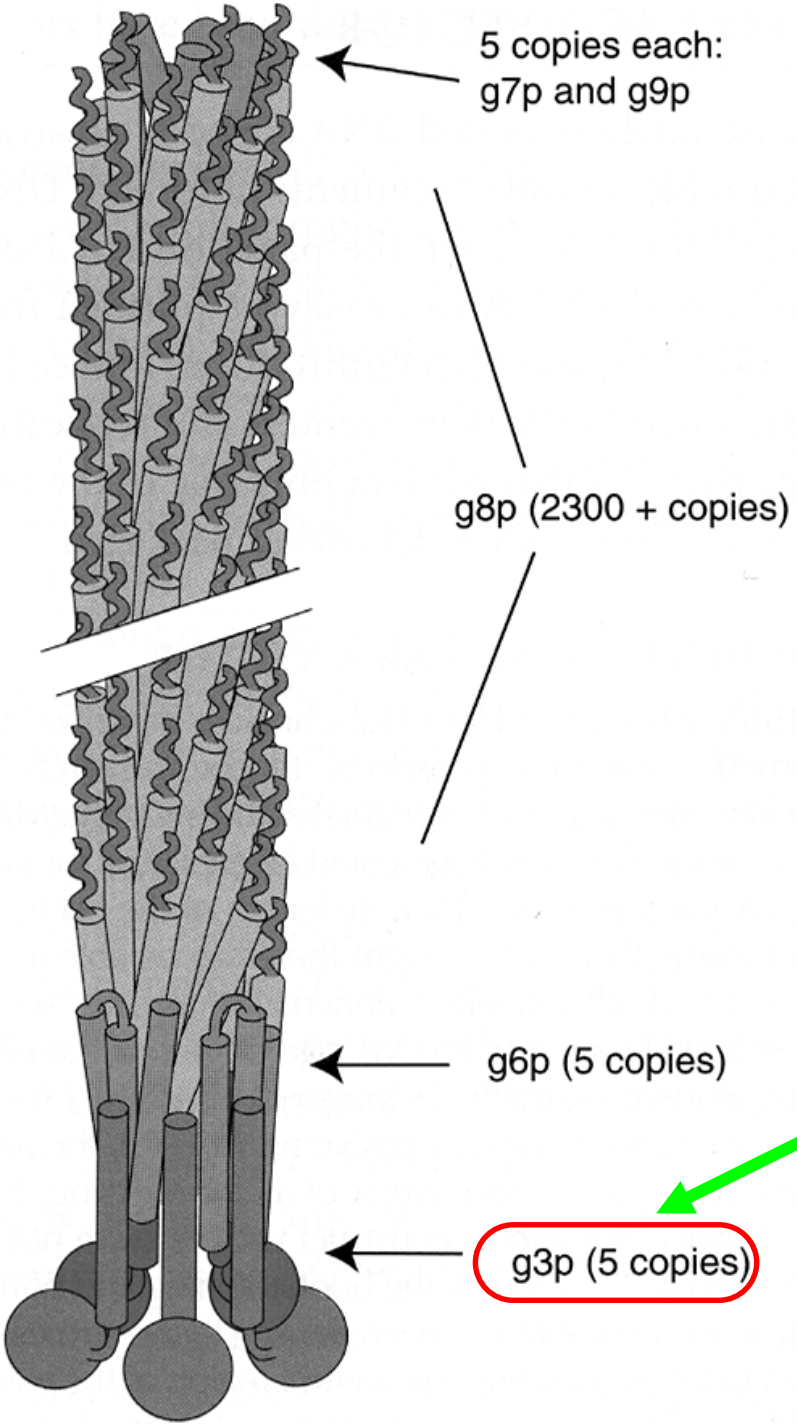
- Single-stranded, circular genome, 6.4 kb
- Infect only F+ bacteria, using pilus F- coded

- Can clone pieces of DNA up to 6X the M13 genome size (36 kb) -- but the larger the DNA, the less stable the clone is…..

- Useful for
  - Sequencing
  - Site-directed mutagenesis (later)
  - Any other technique that requires single stranded DNA

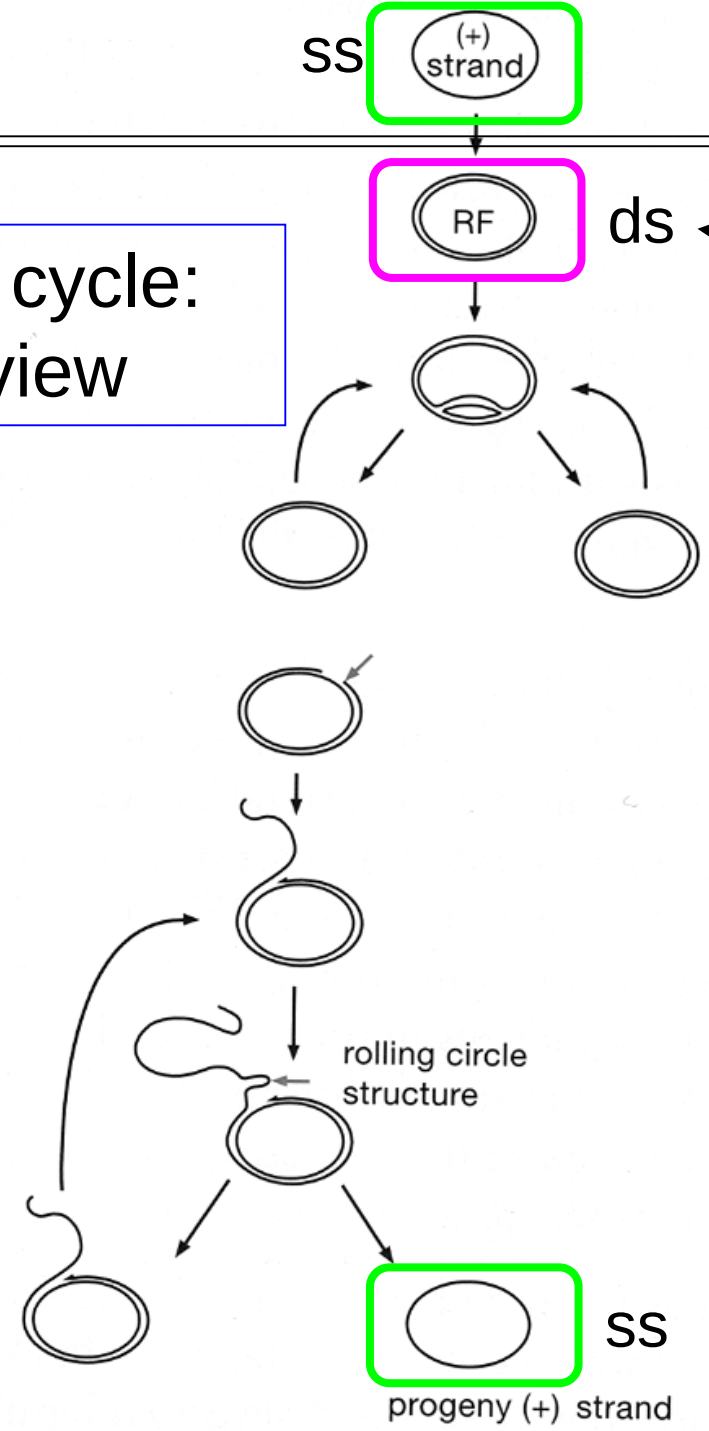
- Drawback: foreign DNA can be unstable (slows down host cell growth, so deletions confer a selective advantage)

M13 structure



Used in ‘phage display’ techniques

M13 life cycle: an overview



Infecting single-stranded circular viral DNA is converted to double-stranded replicating form (RF) DNA by host-encoded enzymes.

Isolate for cloning

Several rounds of replication occur through θ structures.

The (–) strand of the RF DNA is transcribed into viral mRNAs.

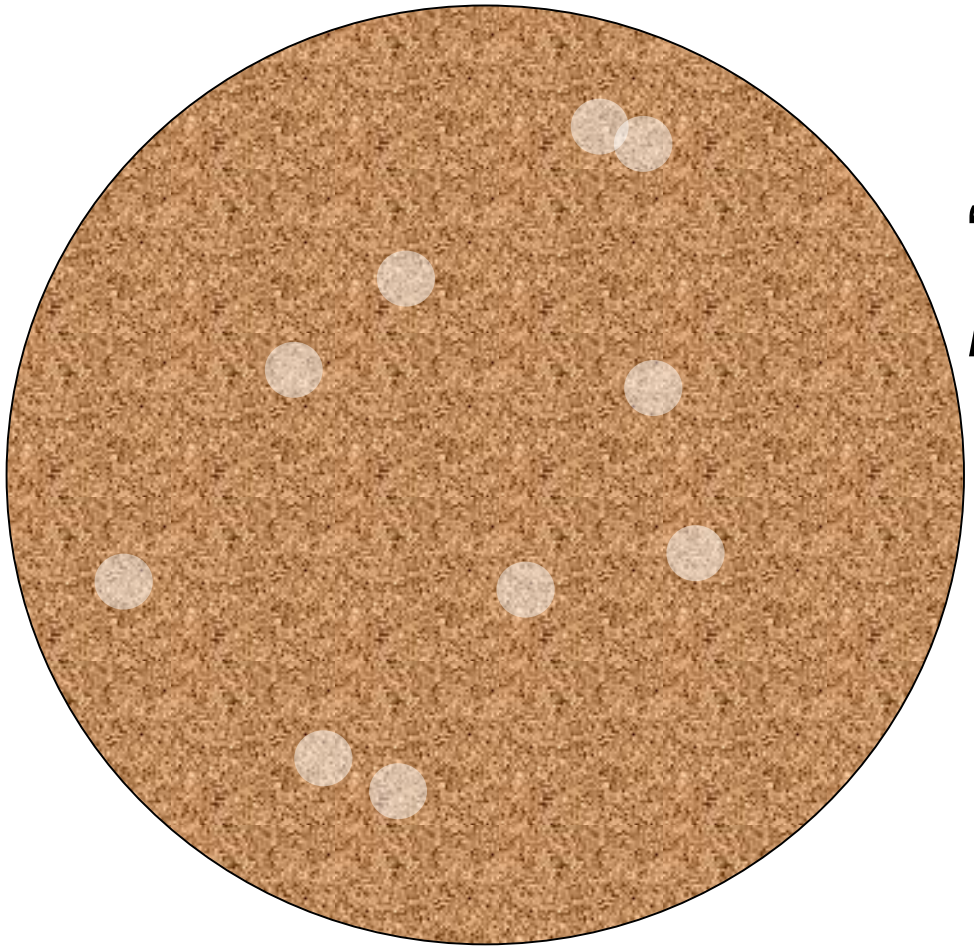
The viral gene II product introduces a nick at a specific site (red arrow) in the (+) strand of the RF DNA.

Progeny (+) strand is synthesized continuously by movement of the replication machinery around the (–) strand template (rolling circle replication).

The completed progeny (+) strand is cleaved from the rolling circular structure by the viral gene II product (red arrow). The progeny strand then circularizes.

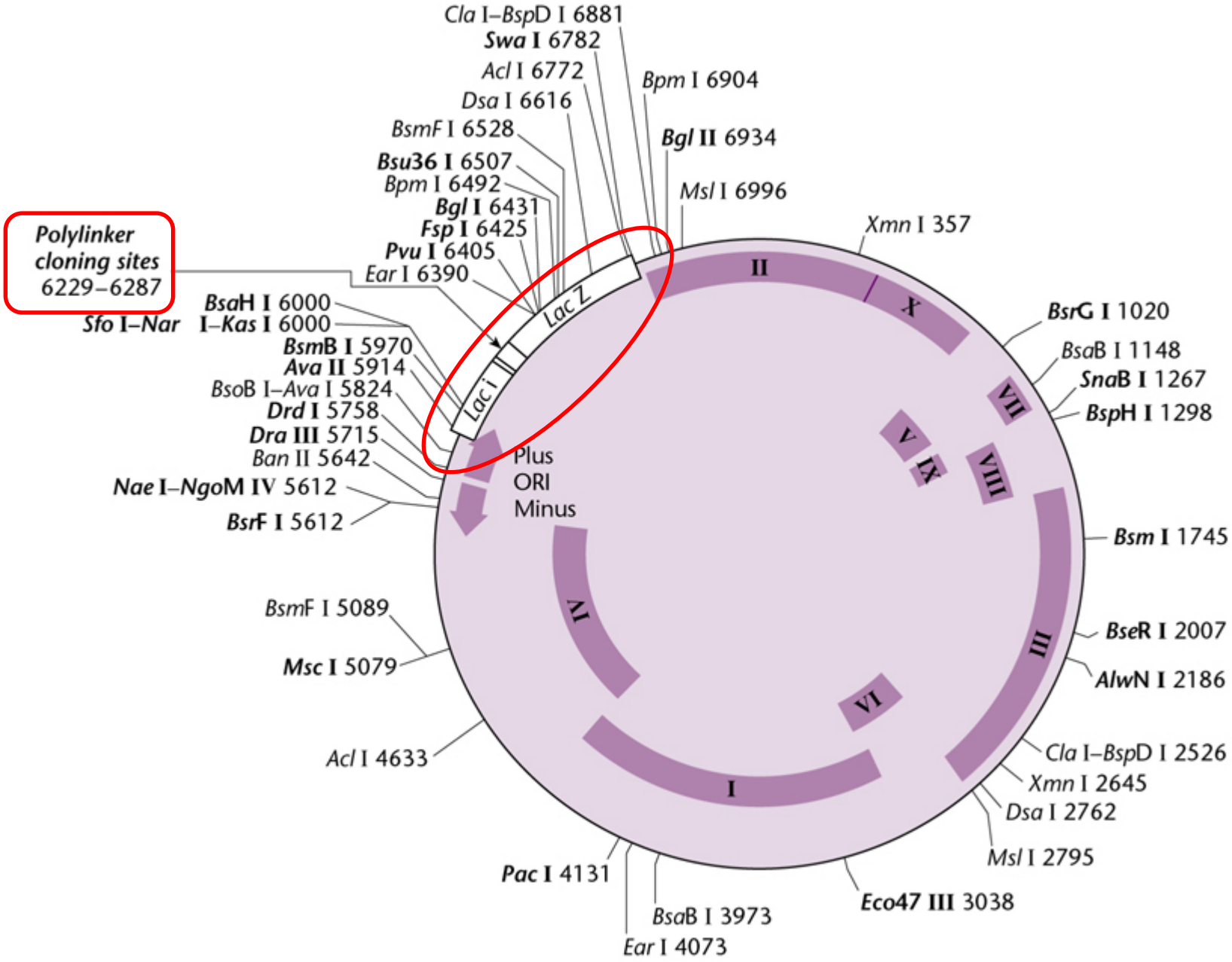
Synthesis of progeny (+) strand continues.

M13 doesn’t lyse cells, but it does slow them down



“lawn” of
E. coli

M13 infections form plaques, but they are “turbid”



M13 mp18: engineered for alpha complementation

Uses of Bacteriophages:

Lambda -- large-ish DNA fragments
- for gene cloning (large eukaryotic genes)
- Excellent selection capability (stuffer stuff)
- Clone lots of precisely-sized DNA fragments for library construction

M13 -- single-stranded DNA
- Sequencing
- Site-directed mutagenesis
- Etc.

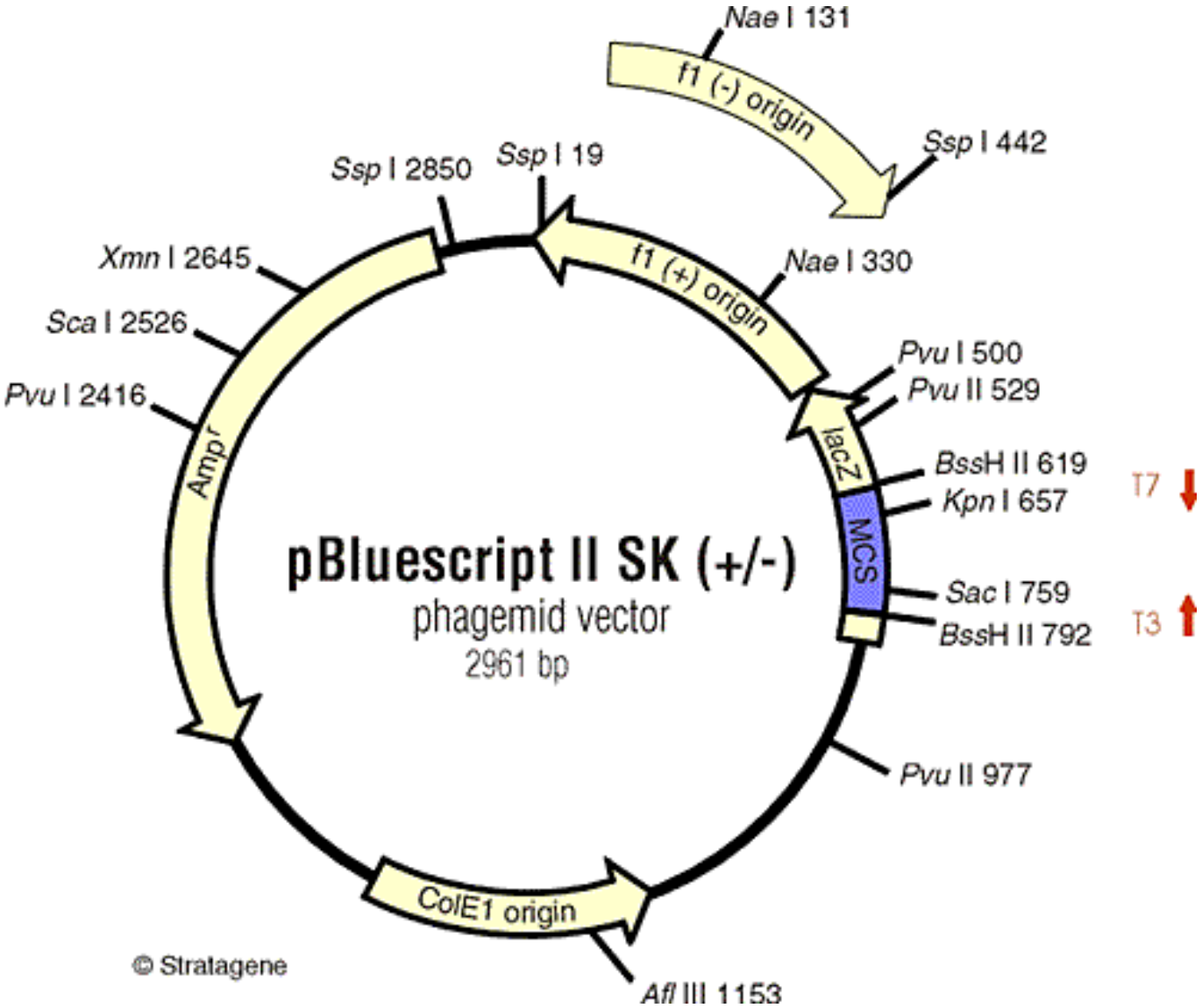
Phagemids: plasmid/M13 hybrids

• 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 (male) strain with a helper phage (M13KO7)

• Helper phage cannot produce single stranded copies of itself, but provides replication machinery for single-stranded copies of the phagemid DNA

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


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
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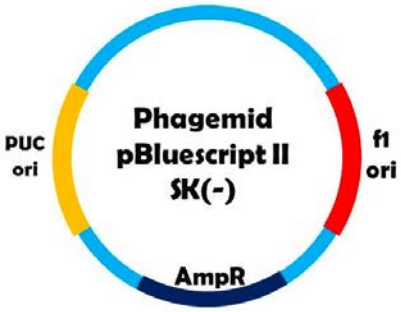
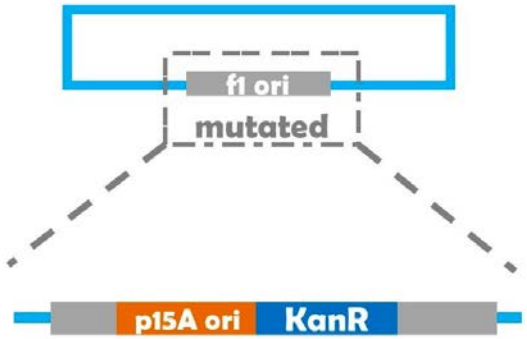
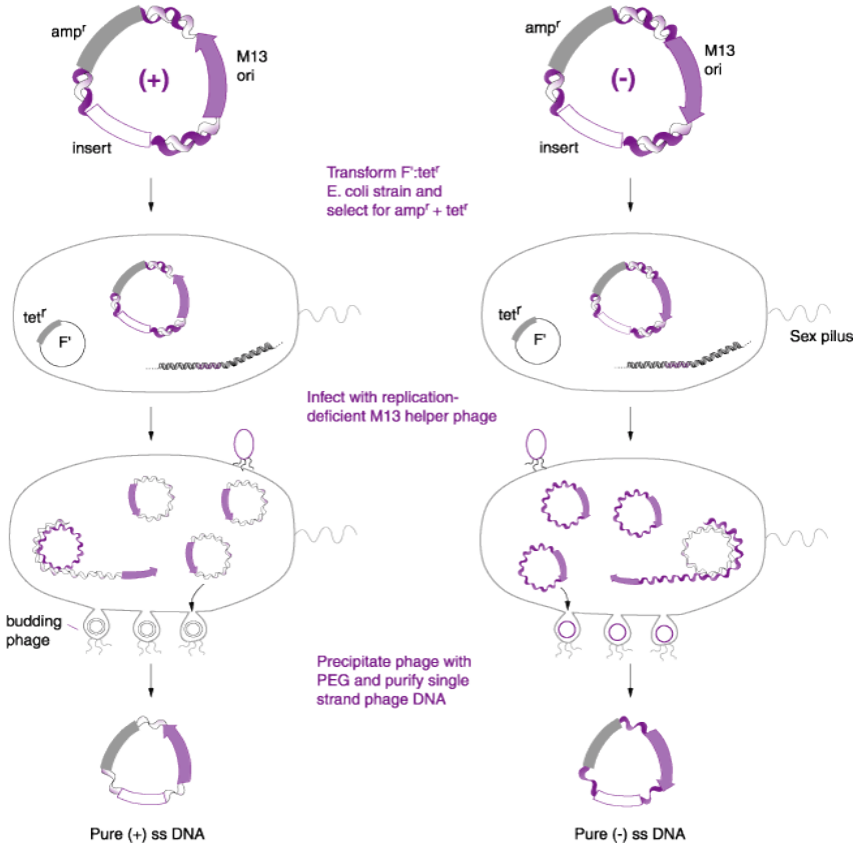
T3
BssH II 792
Pvu II 977
ColE1 origin
© Stratagene
Afl III 1153

phagemid



Helper phage genome

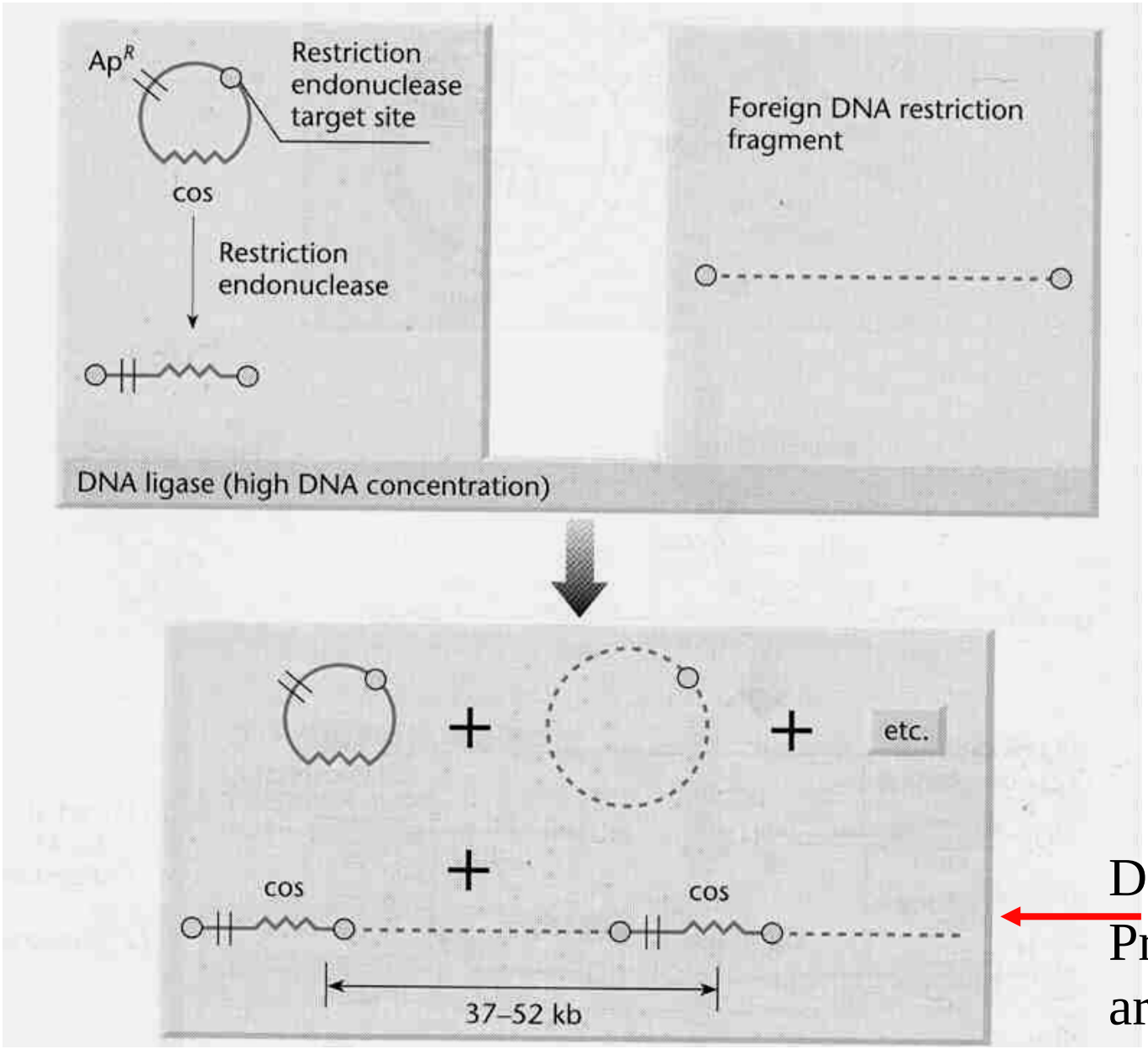




Cosmids:

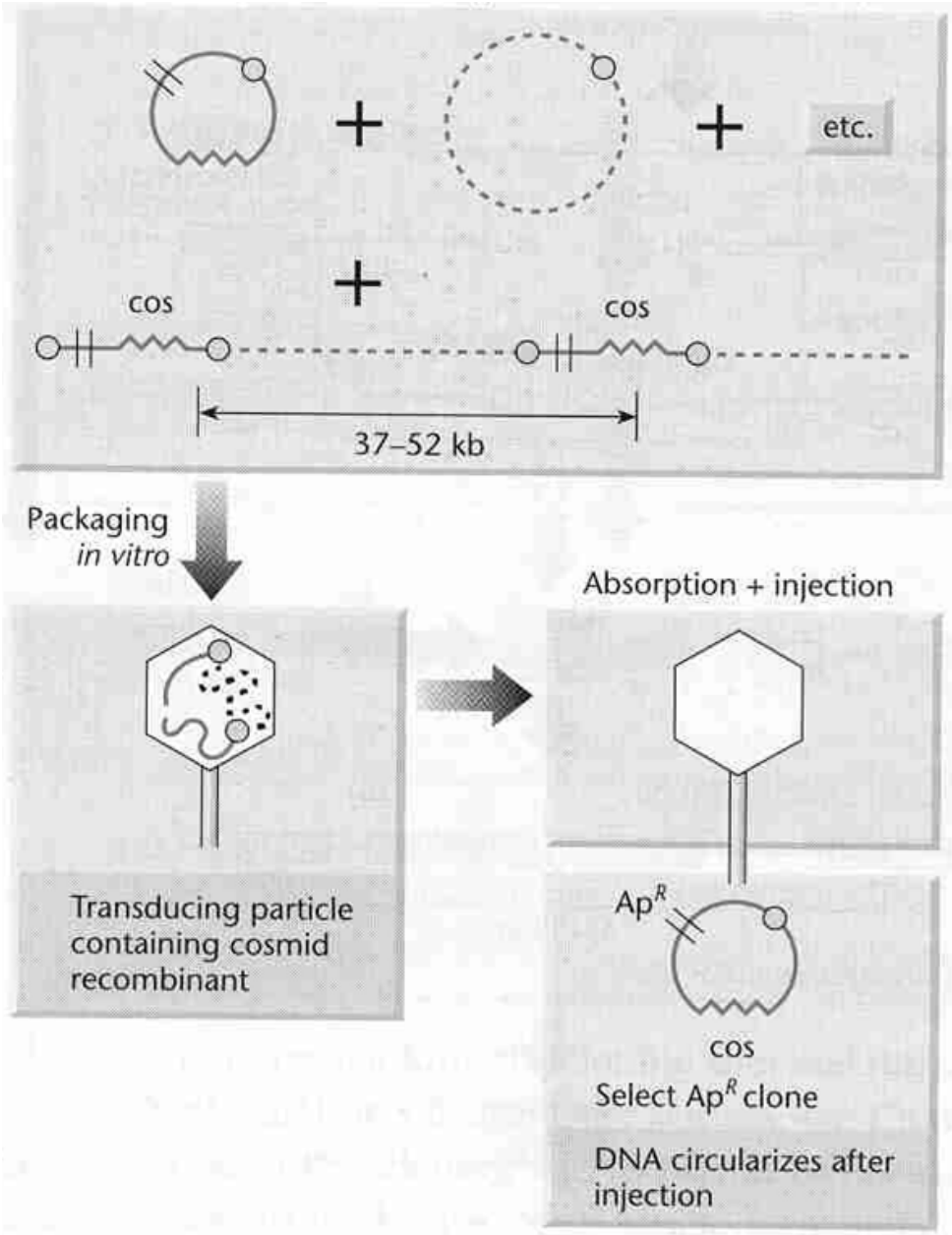
- 5 kb plasmids, antibiotic resistance, plasmid origin of replication
- Contain lambda cos sites required for packaging into lambda phage heads
- Packaging only occurs with 37-52 kb fragments--selection for large fragments
- Packaged DNA is inserted into cells and then replicates as a very large plasmid

Cloning in a cosmid



Desired ligation Products--these are packaged

Cloning in a cosmid



Instead of transformation, desired ligation products are packaged and then transfected into cells

Selection for colonies, not screening of plaques (not infectious)

BACs: Bacterial Artificial Chromosomes

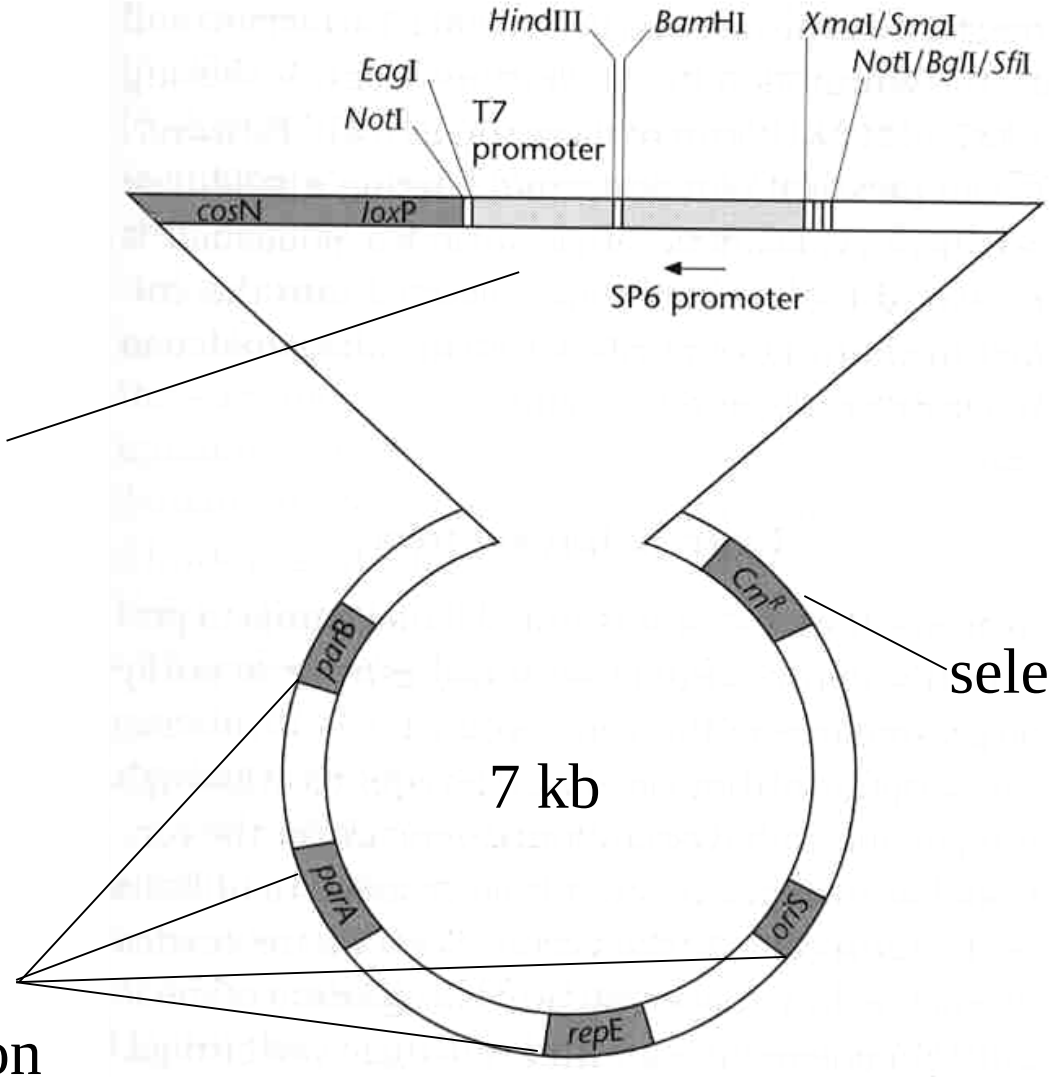
- Based on the F factor of E. coli:
  --100 kb plasmid, propagates through conjugation
  --low copy number (1-2 copies per cell)
  --2 genes (parA and parB): accurate partitioning during cell division

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

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

- Relatively easy to manipulate: move into cells by transformation (electroporation)

General BAC vector

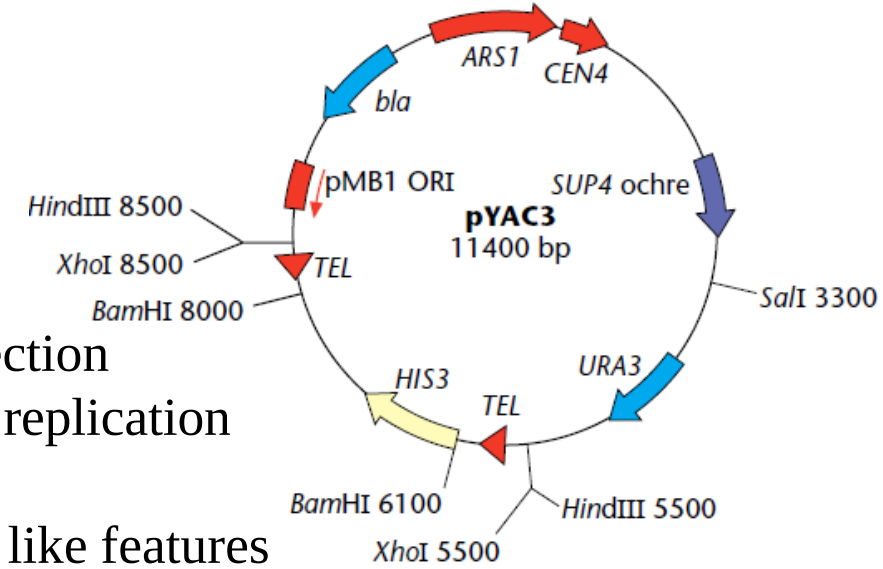


Cloning, etc

selection

replication

YACs: Yeast Artificial Chromosomes



- Based on the chromosome of Yeast
- Features:
- CEN1, centromere sequence→segregation
- TEL, telomere sequences→extremity protection
- ARS1, autonomous replicating sequence→replication
- *Amp*
- Acquiring 150kbp it acquires chromosome like features
- ori, origin of replication for propagation in an *E. coli* host.
- *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.
- 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.

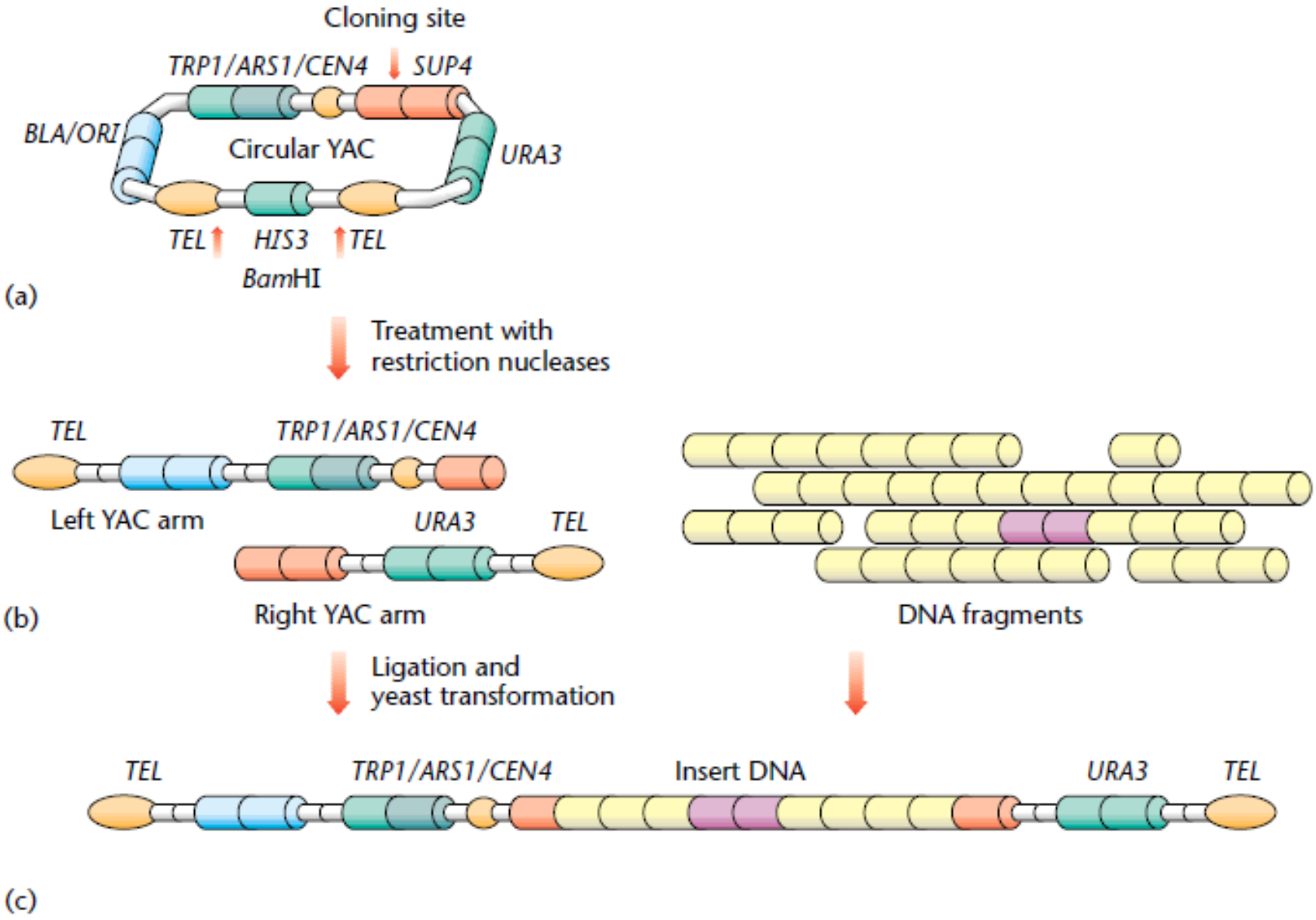
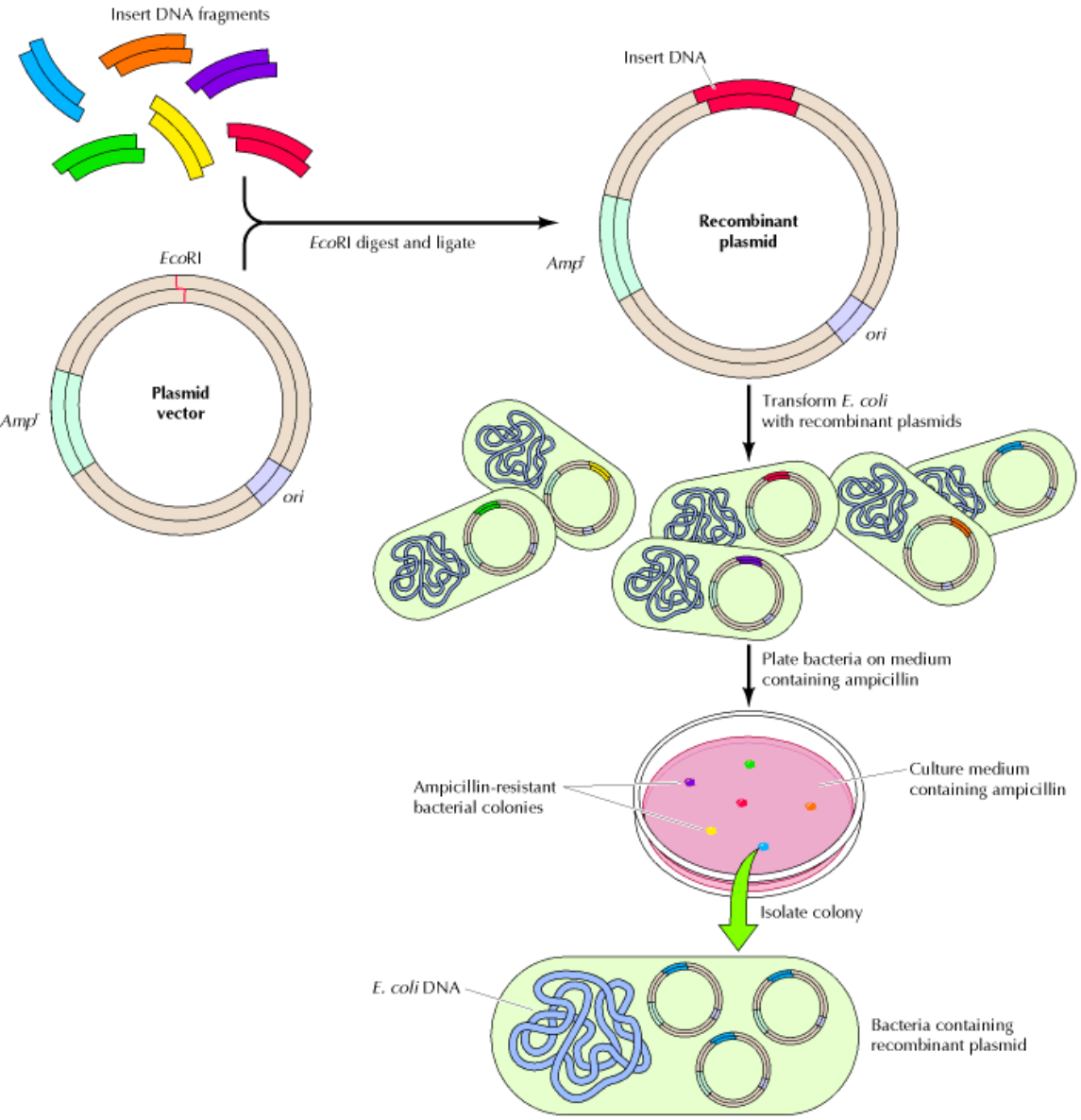

Cloning site
TRP1/ARS1/CEN4
SUP4
BLA/ORI
Circular YAC
URA3
TEL
HIS3
TEL
BamHI
(a)
Treatment with
restriction nucleases
TEL
TRP1/ARS1/CEN4
Left YAC arm
URA3
TEL
Right YAC arm
(b)
DNA fragments
Ligation and
yeast transformation
TEL
TRP1/ARS1/CEN4
Insert DNA
URA3
TEL
(c)

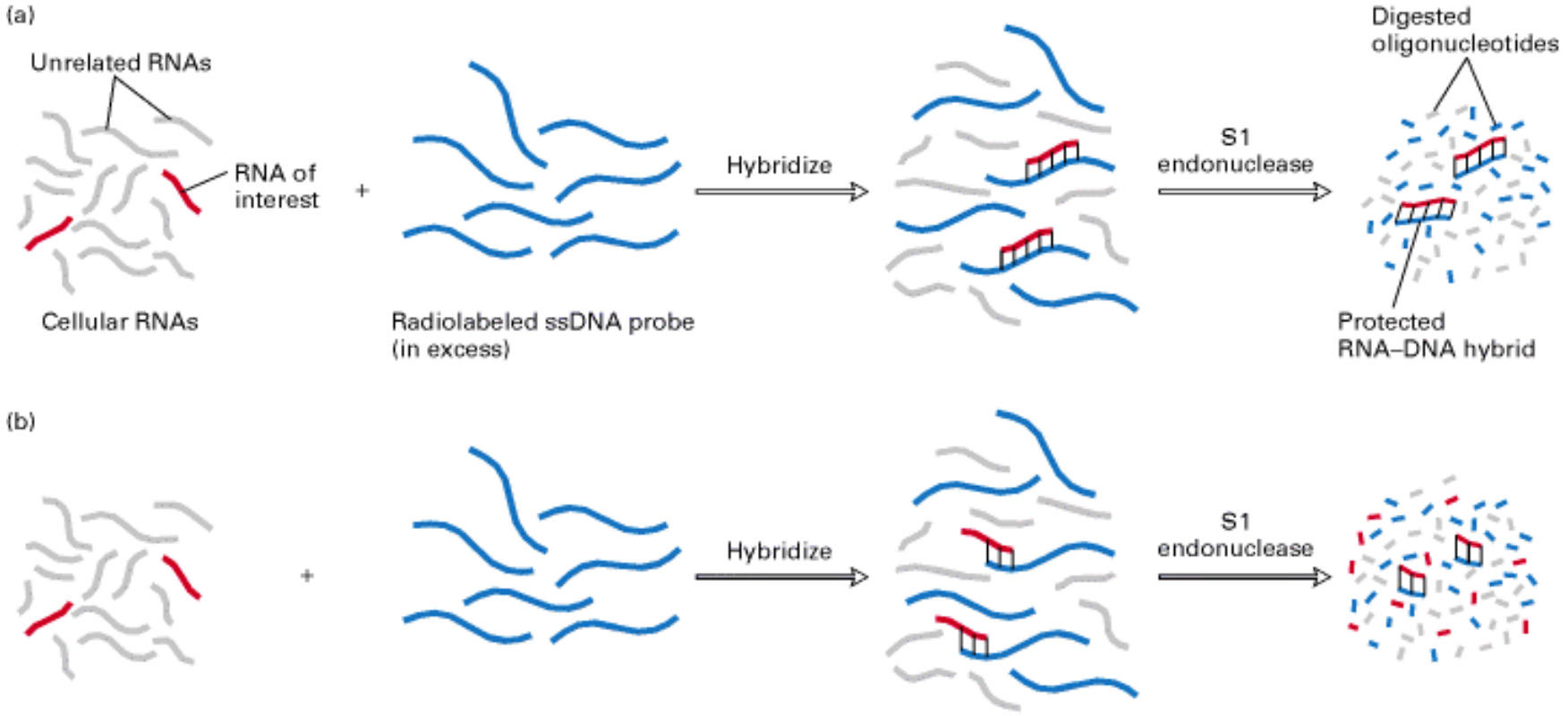
Table 4.2. Sizes of inserted DNA commonly obtained with different cloning vectors

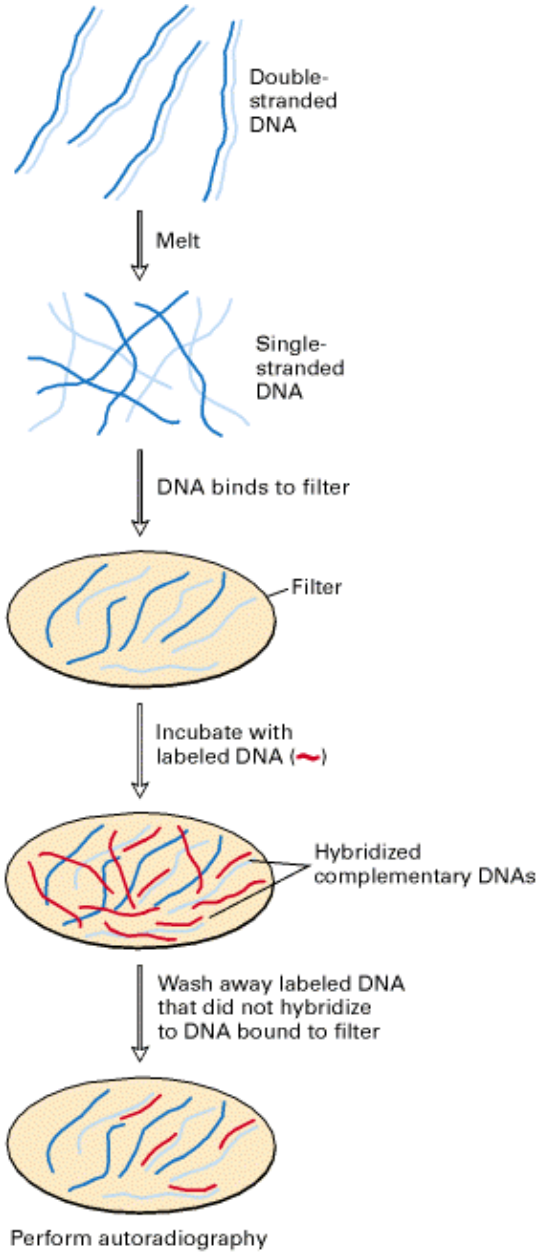
Cloning vector	Size of insert
Standard high copy number plasmid vectors	0–10 kb
Bacteriophage λ insertion vectors	0–10 kb
Bacteriophage λ replacement vectors	9–23 kb
Cosmid vectors	30–44 kb
Bacteriophage P1	70–100 kb
PAC (P1 artificial chromosome) vectors	130–150 kb
BAC (bacterial artificial chromosome)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

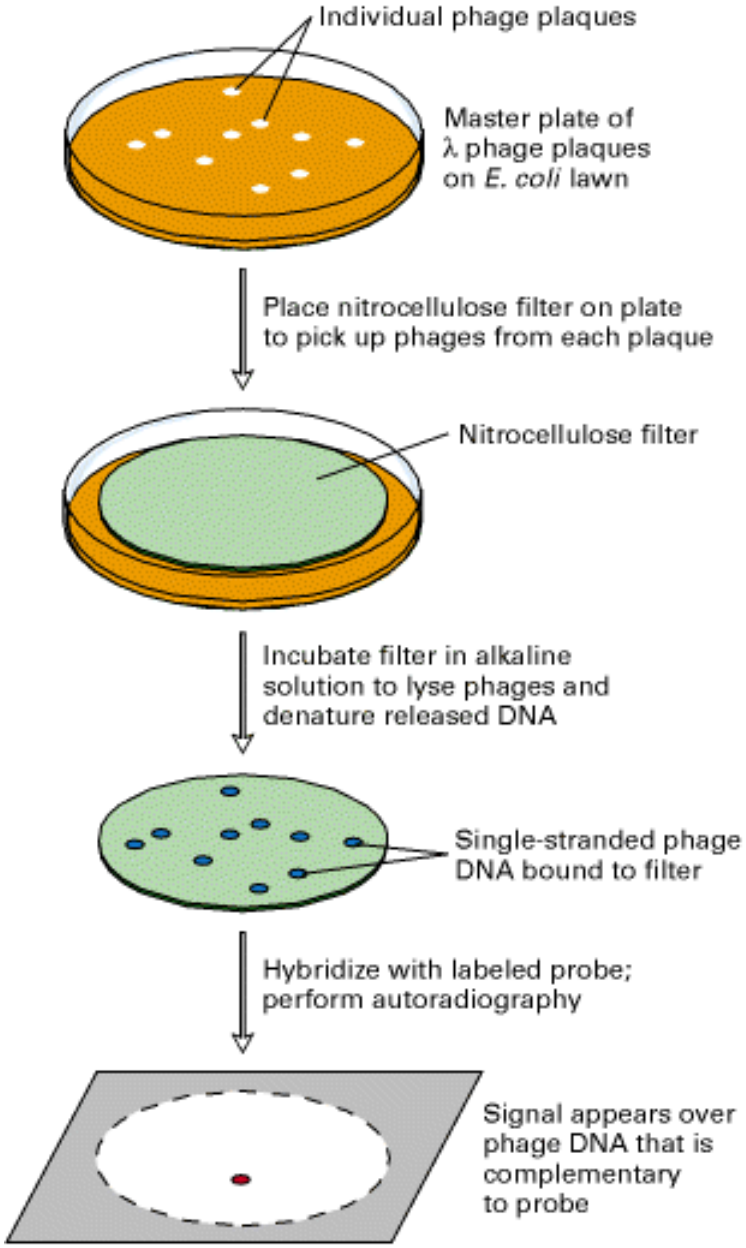


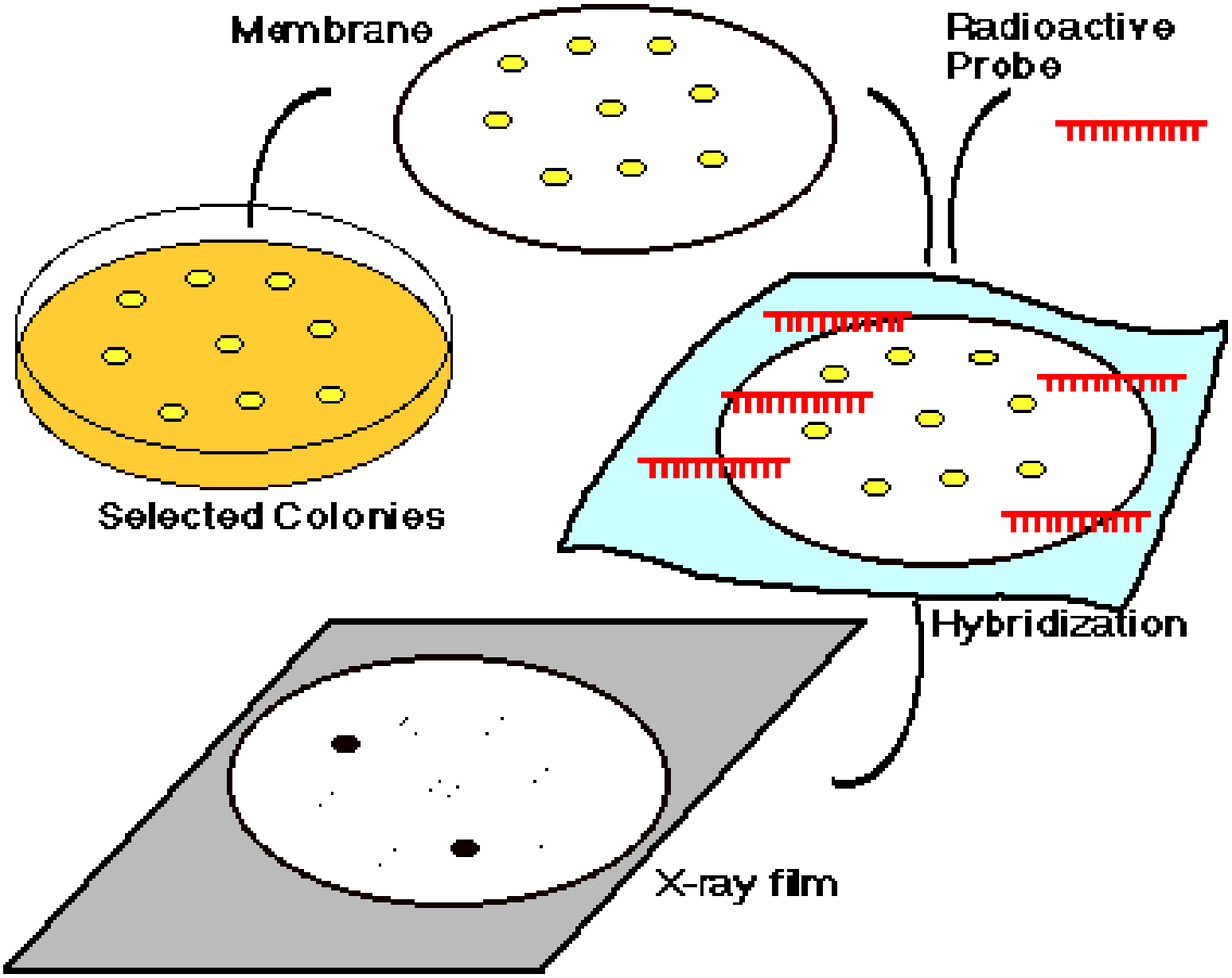
Not all vectors permit the identification of the desired clones by simple selection or color based strategies.

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

Source of the DNA Probe?

- Probe DNA must have complementarity with target DNA
- Two sources
  - Related organism - heterologous probe
  - Reverse translate protein sequence

DNA Probe

- Probe from Related Organism
  - sequences are related evolutionarily
  - not identical but similar enough
  - Heterologous Probe

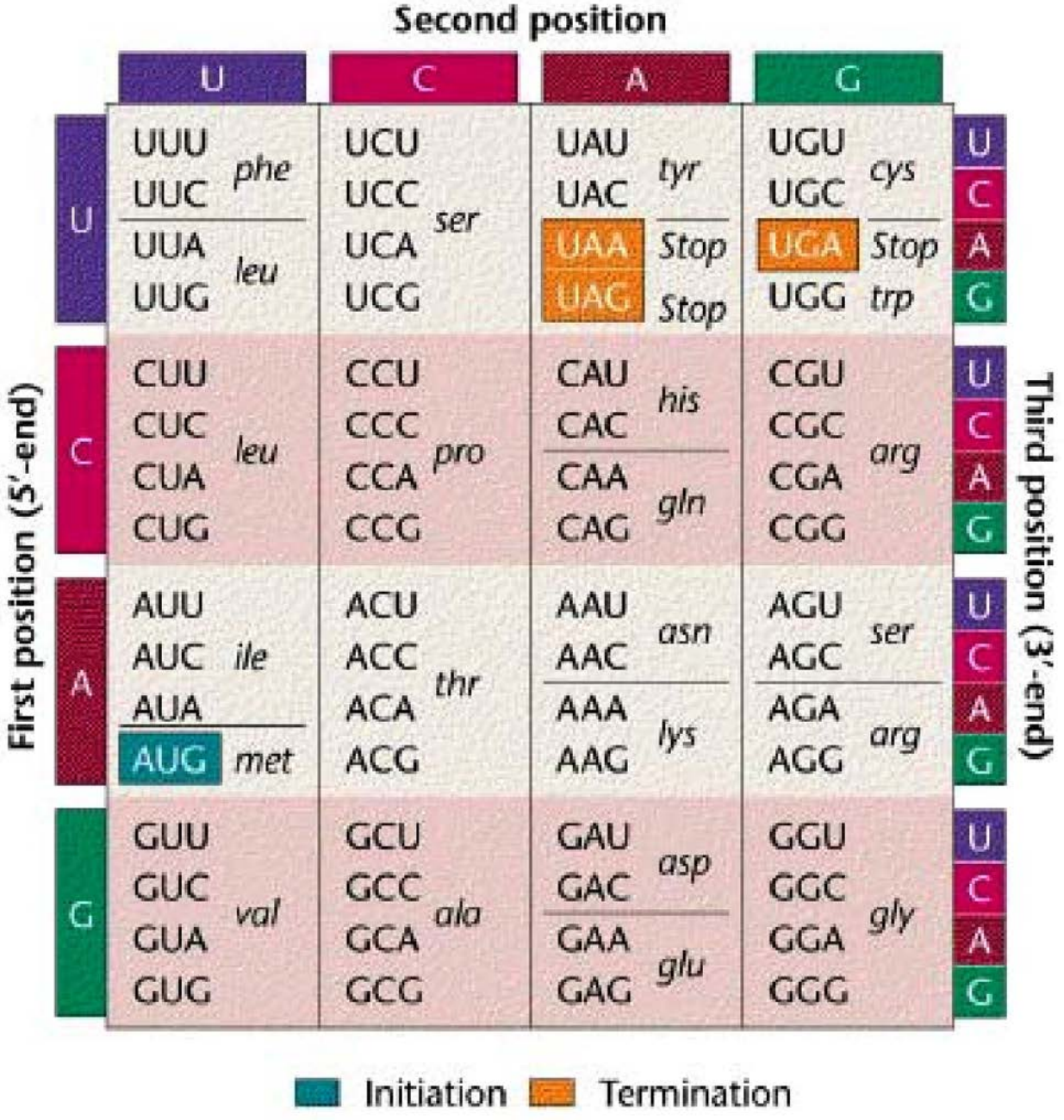
  - Use yeast gene to isolate Human gene

DNA Probe

- Reverse translate protein sequence
  - Use knowledge of Genetic Code to obtain DNA sequence(s) based on protein seq.
  - MET = AUG in RNA

    ATG in DNA (or 5’ CAT)

Genetic Code Table

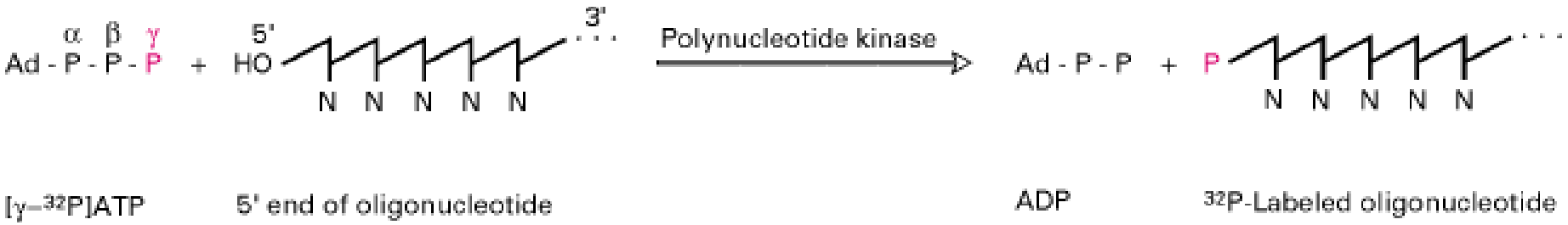


Reverse Translation of Protein Seq.

MET-TRP-TYR-GLN-PHE-CYS-LYS-PRO

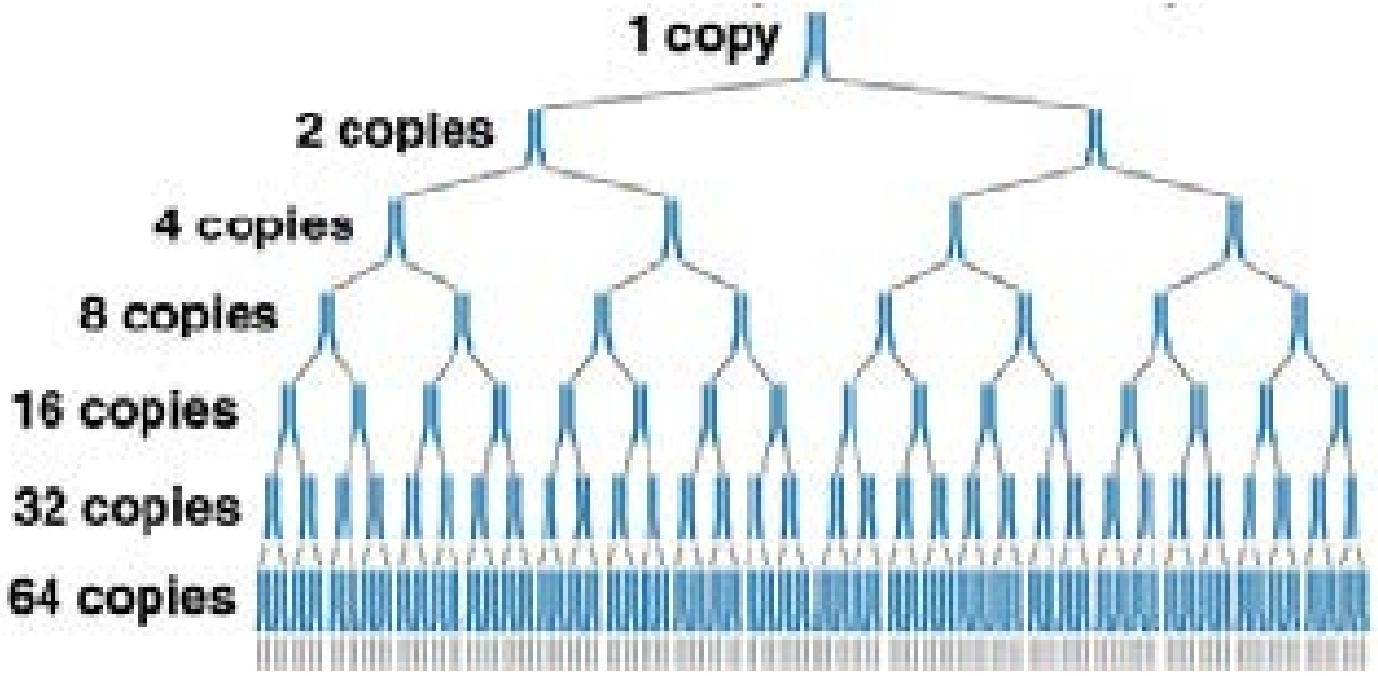
ATG-TGG-TAT-CAA-TTT-TGT-AGA-CCN
C G C C G

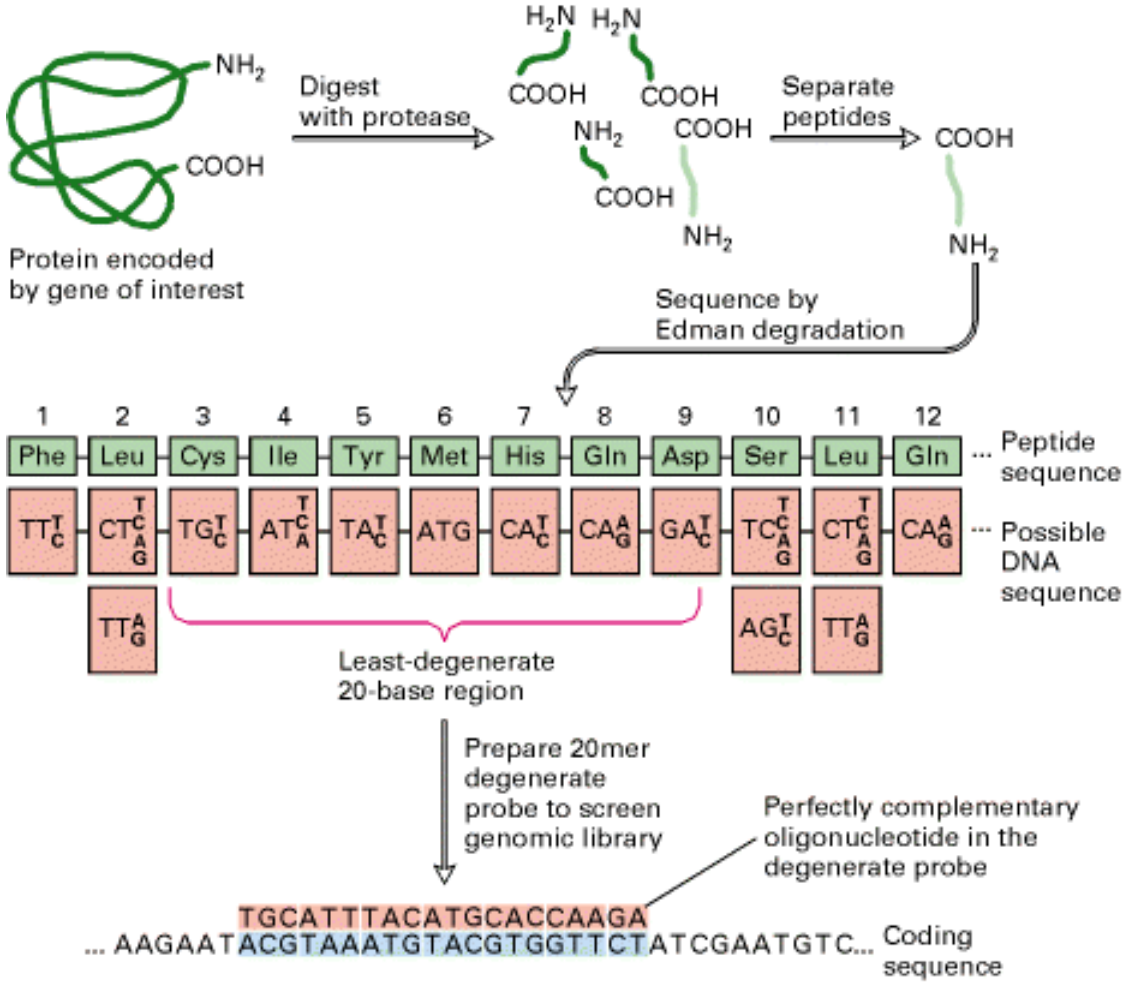
32 Different oligonucleotides for this peptide sequence (due to degeneracy of code)



Radiolabeling of an oligonucleotide at the 5 and with phosphorus-32. The three phosphate groups in ATP are designated the α, β, and γ phosphates in order of their position away from the ribose ring of adenosine (Ad). ATP containing the radioactive isotope ^{32}P in the γ-phosphate position is called *[γ-^{32}P]ATP*. Kinase is the general term for enzymes that transfer the γ-phosphate of ATP to specific substrates. Polynucleotide kinase can transfer the ^{32}P-labeled γ phosphate of [γ-^{32}P]ATP to the 5 end of a polynucleotide chain (either DNA or RNA). This reaction is commonly used to radiolabel synthetic oligonucleotides.

Labelling of PCR products using a radioactive dNTP!

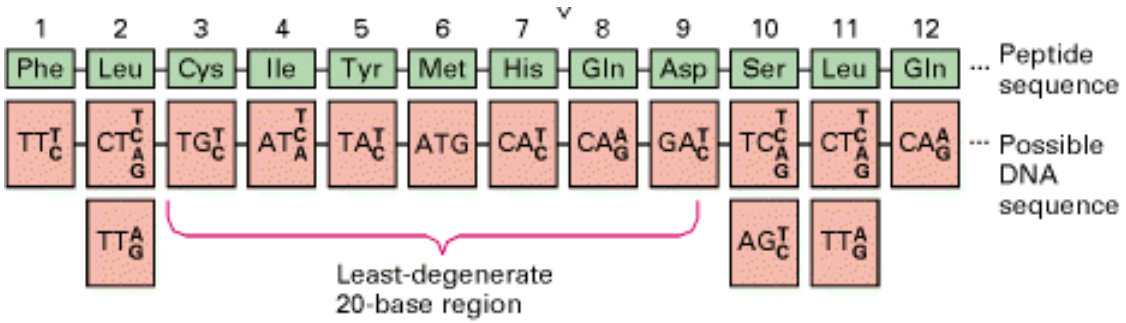




Designing oligonucleotide probes based on protein sequence. The determined sequences then are analyzed to identify the 6- or 7-aa region that can be encoded by the smallest number of possible DNA sequences. Because of the degeneracy of the genetic code, the 12-aa sequence (light green) shown here theoretically could be encoded by any of the DNA triplets below it, with the possible alternative bases at the same position indicated. For example, Phe-1 is encoded by TTT or TTC; Leu-2 is encoded by one of six possible triplets (CTT, CTC, CTA, CTG, TTA, or TTG). The region with the least degeneracy for a sequence of 20 bases (20-mer) is indicated by the red bracket. There are 48 possible DNA sequences in this 20-base region that could encode the peptide sequence 3 9. Since the actual sequence of the gene is unknown, a degenerate 20-mer probe consisting of a mixture of all the possible 20-base oligonucleotides is prepared. If a cDNA or genomic library is screened with this degenerate probe, the one oligonucleotide that is perfectly complementary to the actual coding sequence (blue) will hybridize to it.

CAC TGA AAG AAC AMT GAG TAT TT

AAA GAA CAG TGA HTA TTT CCA CAT A



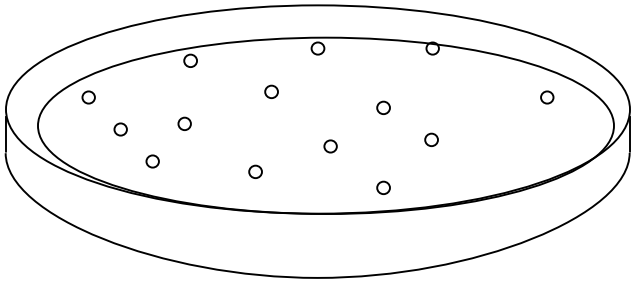
Nucleotide codes

A	Adenine
G	Guanine
C	Cytosine
T	Thymine
U	Uracil
R	Purine (A or G)
Y	Pyrimidine (C or T)
N	Any nucleotide
W	Weak (A or T)
S	Strong (G or C)
M	Amino (A or C)
K	Keto (G or T)
B	Not A (G or C or T)
H	Not G (A or C or T)
D	Not C (A or G or T)
V	Not T (A or G or C)

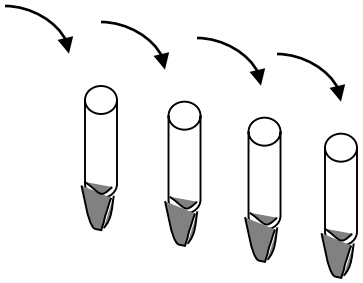
TGY ATH TAY ATG CAY CAR GAY

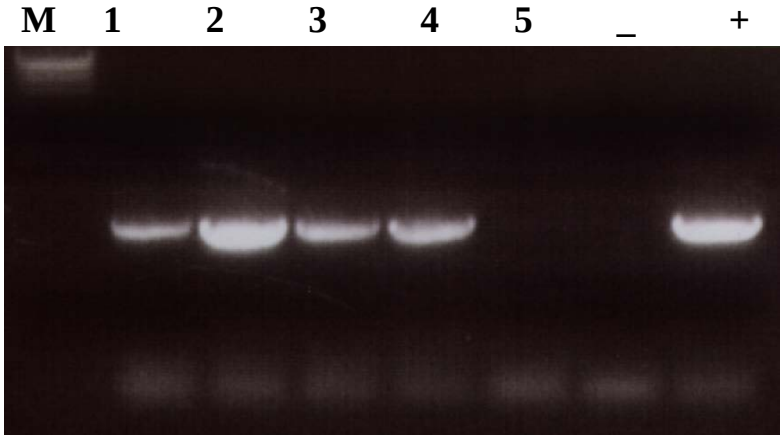
Colony PCR

1. Si piastra, come al solito, una trasformazione. I cloni trasformanti possono contenere il solo vettore o il vettore più l'inserto



2. Si mette su una reazione di PCR per ogni clone che si vuole analizzare, risospendendo nella mix di PCR una parte della colonia.Si utilizza una coppia di primers specifica per l'inserto clonato



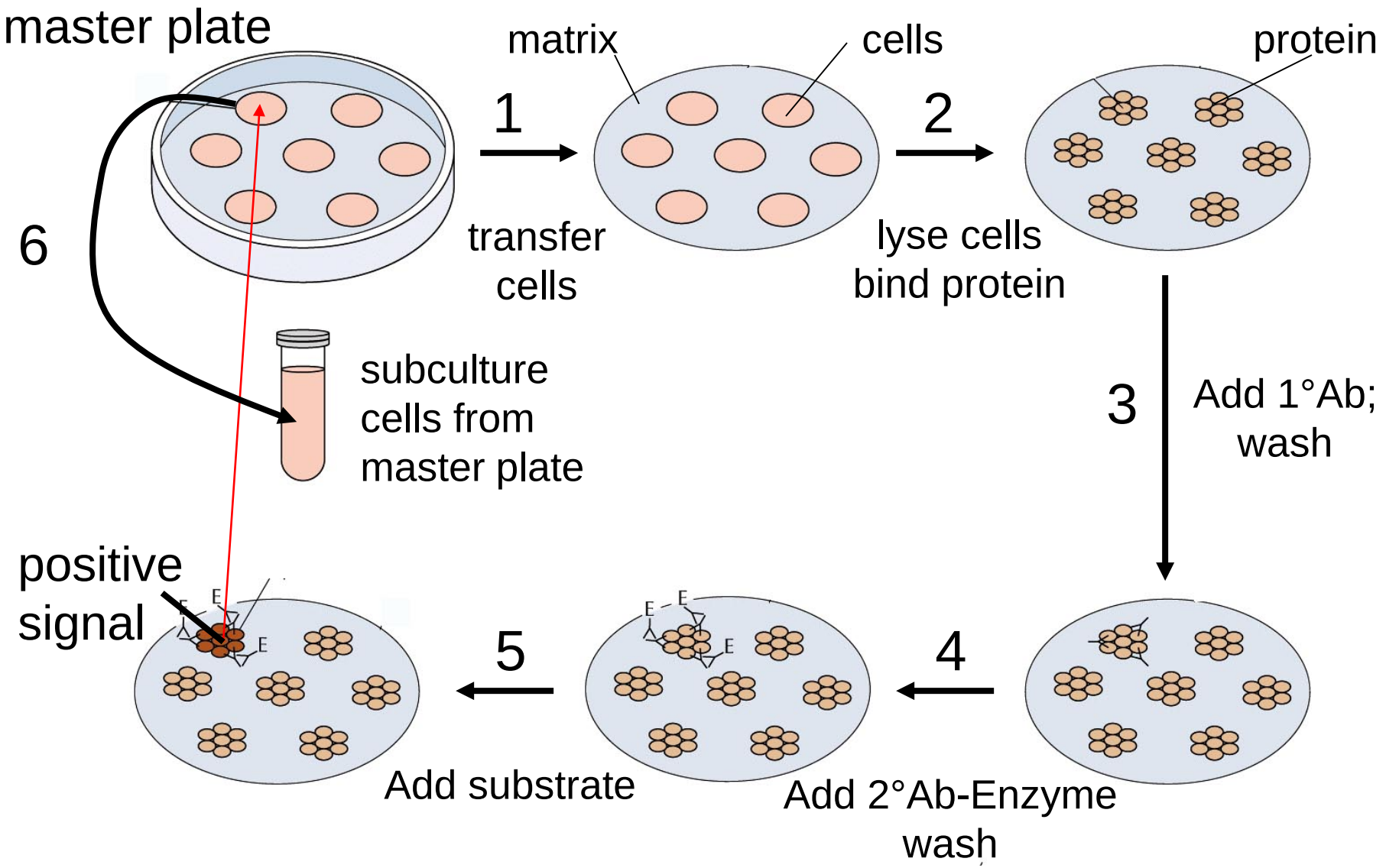


I cloni 1,2,3 e 4 contengono l'inserto. Il clone 4 contiene solo il vettore. "-" e "+" sono controlli negativo e positivo

Immunological Screening

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

Immunological Screen



Immunological Screen of Library