PLNT2530
2024

Unit 6a

# Gene Cloning

## Vectors

Molecular Biotechnology (Ch 4)

Principles of Gene Manipulation (Ch 3 & 4)

Analysis of Genes and Genomes (Ch 5)

# Cloning terminology

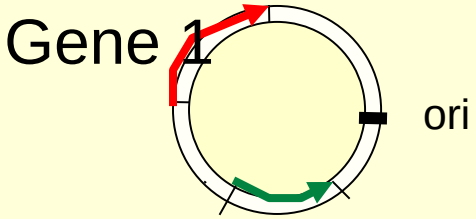
**Plasmid** - autonomously replicating mini-chromosome, typically circular

**Bacteriophage** - virus that infects bacteria

**Vector** - plasmid or bacteriophage genetically engineered for cloning foreign insert DNA

**Construct** - Recombinant DNA molecule in which a foreign DNA fragment has been inserted into a vector.

# Plasmids




## Naturally occurring plasmids

-occur widely in bacteria

-are covalently closed circular dsDNA

-are replicons, stably inherited as extra-chromosomal DNA

-can be 1 kbp to 500 kbp in size  (compared to 4000 kbp chromosome)

-bacteria can contain several different types of plasmid simultaneously

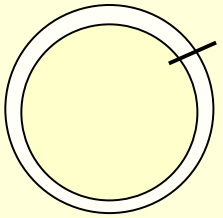
-many naturally occurring plasmids carry genes for restriction enzymes, antibiotic resistance, or other genes

# Bacterial Vectors

All vectors :

1. -must replicate autonomously in their specific host even when joined to foreign DNA

ori - origin of replication
sequence at which DNA polymerase initiates replication



2. - should be easily separated from host chromosomal DNA

- *E. coli* chromosomal DNA: ~ 4 million bp
- typical plasmid vector: ~ 3 to 10 kb

Most modern cloning vectors in E. coli are derived from naturally-ocurring plasmid col E1.
Most of col E1 was deleted except for an origin of replication and an antibiotic resistance gene.

# Vectors

Types

plasmids- can occur naturally in as circular dsDNA in bacteria (up to 15 kb)

cloning small fragments eg. single genes

bacteriophage -viruses of bacteria (~10-50 kb) used in the construction of cDNA and genomic libraries

cDNA cloning, high-efficiency cloning

BAC-bacterial artificial chromosome (130-150 kb

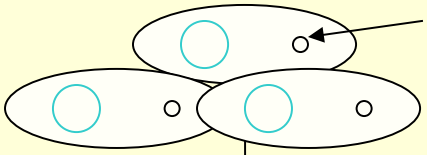
YAC-Yeast artificial chromosome (1000-2000 kb)

genomic libraries with large inserts

Each type of vector has specific applications but primary function is to carry foreign DNA (foreign to bacteria) and have it replicated by the bacteria

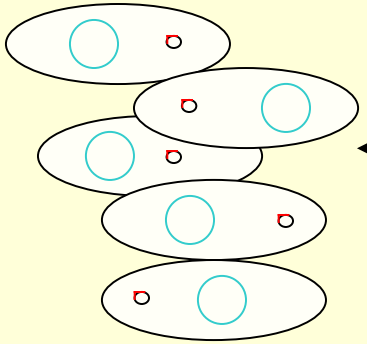
# Introduction of foreign DNA into E. coli

Bacterial cells with plasmid

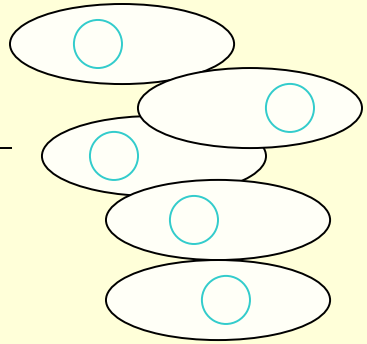


Plasmid vector

Lyse cells and recover plasmids

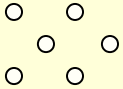


transform

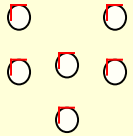


"Competent" bacterial cells

Plasmid transformed bacteria

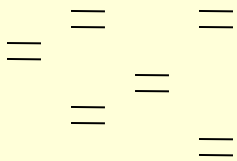


Isolate plasmid vectors

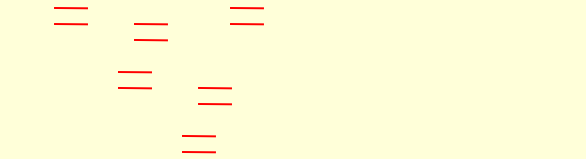


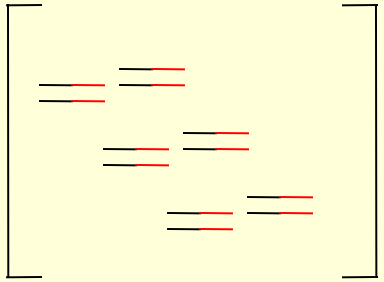
Recombinant plasmid vectors

Cut with a restriction enzyme to linearize without fragmenting

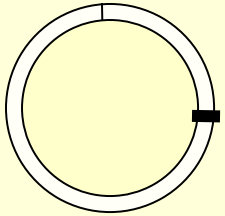


Add foreign DNA with compatible ends and ligate





# Plasmids




Plasmid vectors (engineered) should

1. have naturally-ocurring copy number control sequences deleted

   Result: higher plasmid copy number in the bacteria

   (eg. 20-200 copies/cell)

   eg. pUC19

2. be small in size (3-5 kbp)
   - to facilitate separation from chromosomal DNA
   - transfer efficiency declines for plasmids >15 kbp

3. carry selectable markers to allow selection of:

   i) plasmid transformed bacteria

   ii) transformed bacteria that carry the recombinant vector

## 3. Selectable markers -explanation

i) selection of plasmid-transformed bacteria

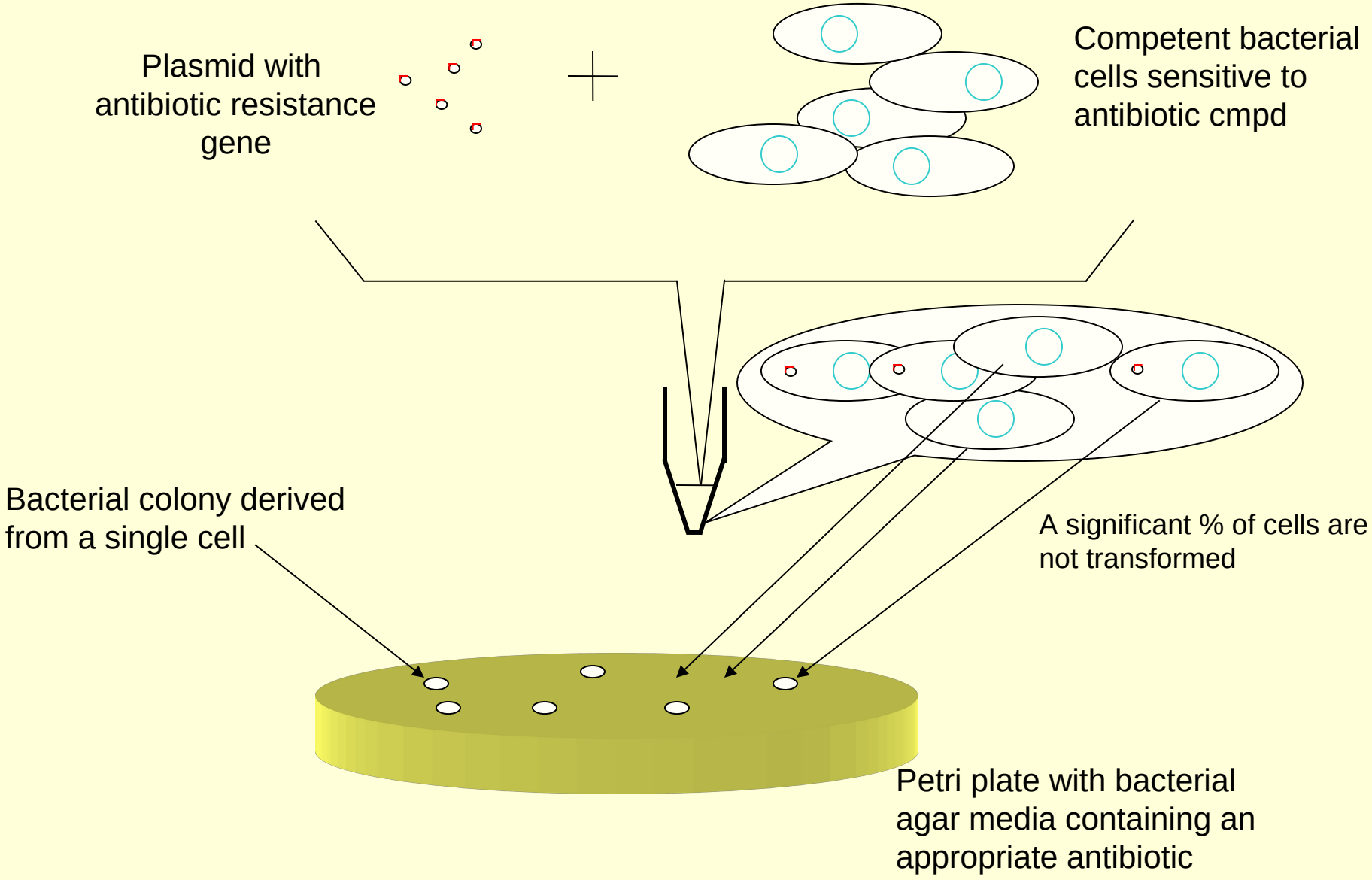
Achieved by engineering a plasmid to carry and express a gene for antibiotic detoxification which will allow any bacterium carrying that plasmid (and gene) to be resistant to the toxic effects of the antibiotic.

Following transformation of bacterial cells (antibiotic sensitive without this plasmid), cells are plated on an antibiotic containing media.

Commonly used antibiotics: Ampicillin, streptomycin, kanamycin, hygromycin

Eliminates non-transformed bacteria

# Bacterial transformation



## 3. Markers for Screening and Selection

Antibiotic selection - kills all cells that did not get the vector

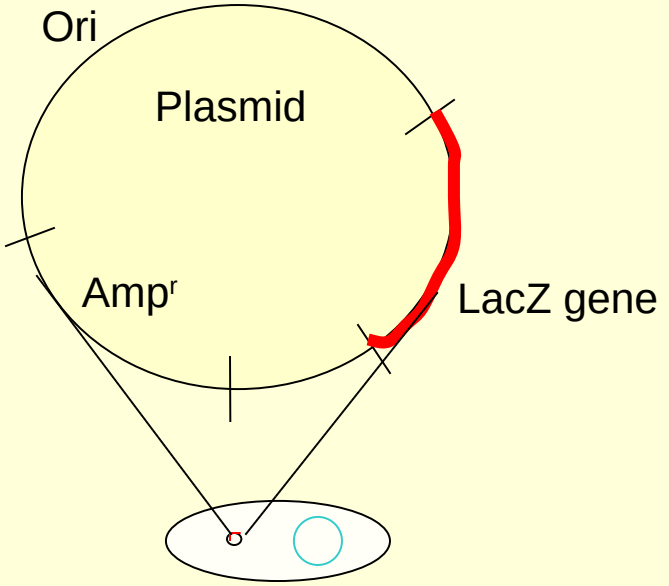
However - there is no guarantee that all vectors got an insert.

Therefore, many cloning vectors let us screen for those with inserts based on insertional inactivation of a gene.

Example: X-gal assay for the LacZ gene

# Expression of LacZ gene

*this is what happens if we don't insert a fragment*




Expression of gene

in bacterial cell

(5-bromo 4-chloro 3-indolyl β-galactoside)

**X-gal**

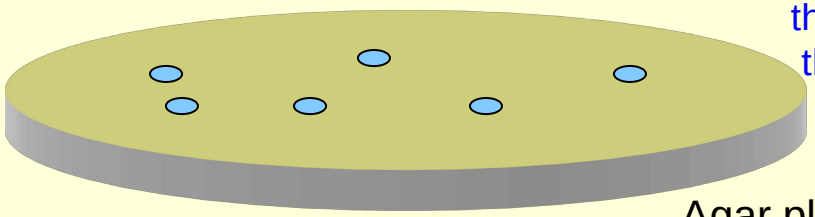
(colourless substrate)

β-galactosidase

galactose

Released indolyl cmpd dimerizes after oxidation to yield an insoluble indigo dye (blue color)

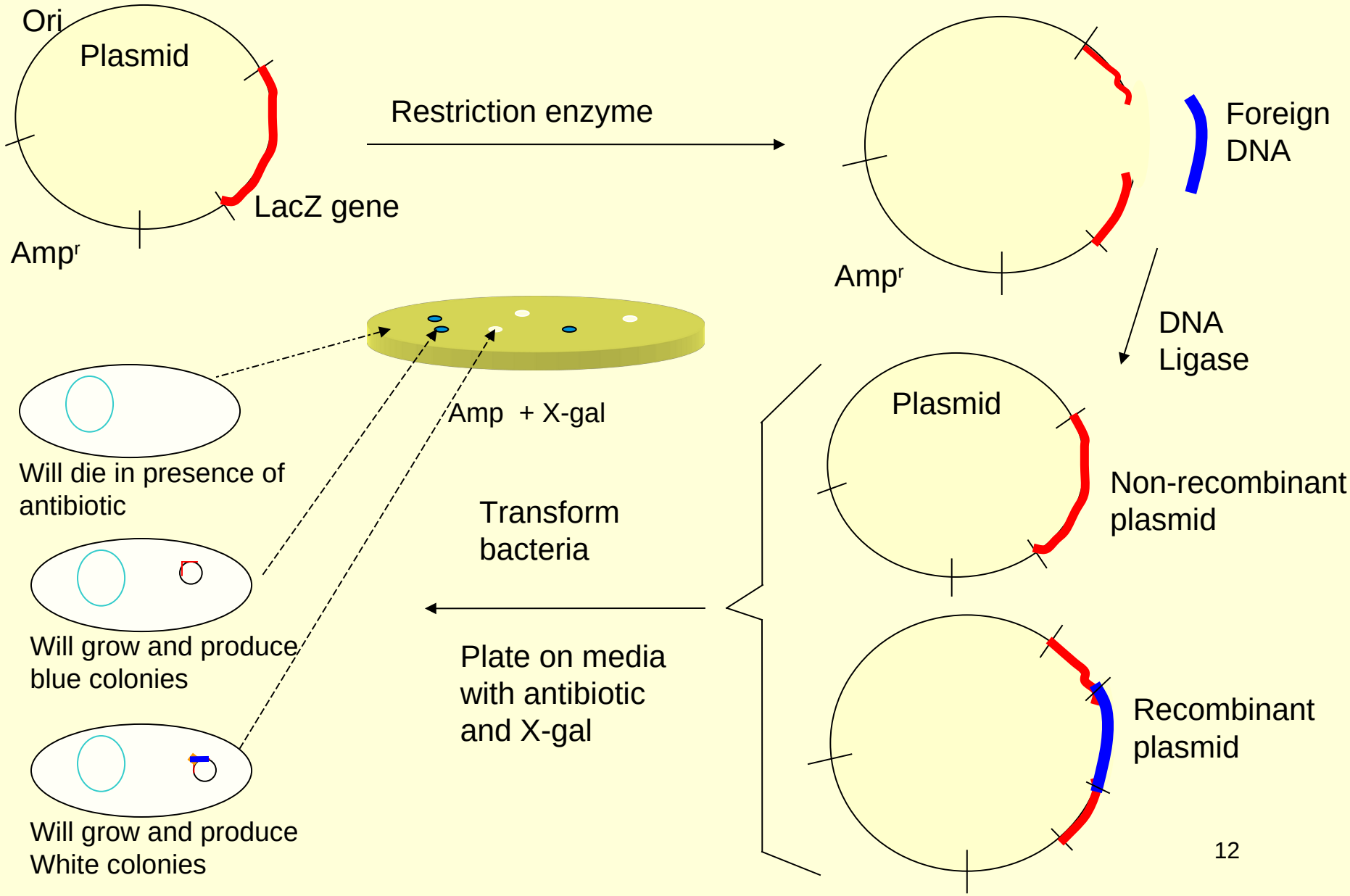
Colonies expressing LacZ gene

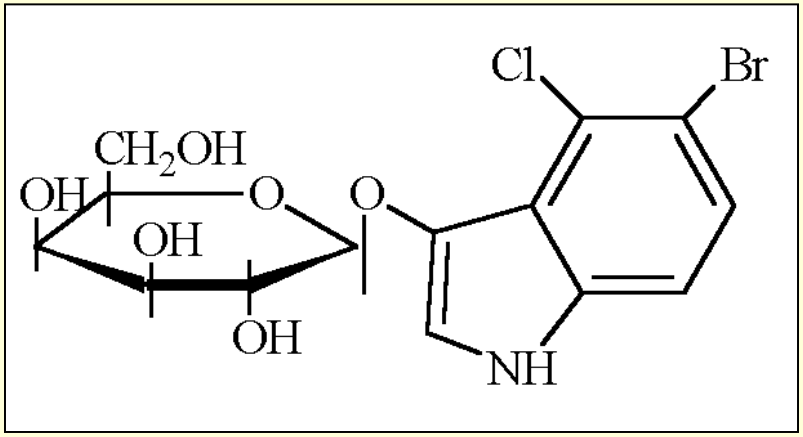


Agar plate
Amp
X-gal

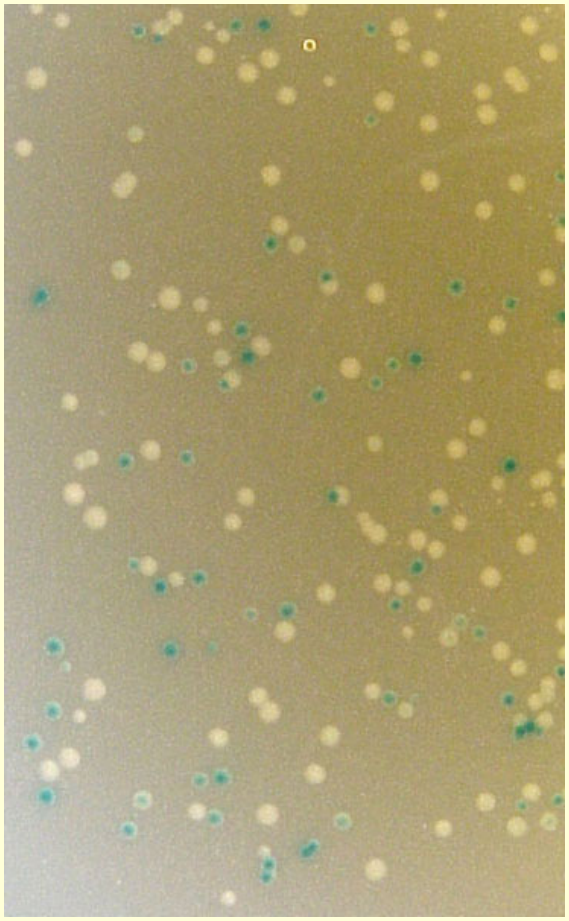
NOTE: X-gal is taken up by cells but the product becomes insoluble inside the cells

# Screening for bacteria carrying recombinant plasmids






- X-Gal structure



# Plasmids

Plasmid vectors (engineered) should: (cont'd)

4. Have a multiple cloning site (MCS) region A constructed ds sequence containing a series of consecutive, unique (ie only one site in the plasmid) restriction enzyme recognition sites. The sequence is a multiple of 3 bp long and is inserted into the coding region of the selectable marker (i.e. LacZ gene) gene.

Sequence is relatively short (54 bp in pUC19)

As multiple of 3, the insertion does not change the reading frame of coding region of the gene, adds 18 amino acids to protein - but doesn't affect product activity.

Value: Allows different restriction enzymes to be used to open plasmid to match the restriction enzyme used to create the DNA being inserted.

# Multiple cloning site (MCS) from pUC 18/19 plasmids

EcoRI SacI KpnI BamHI XbaI

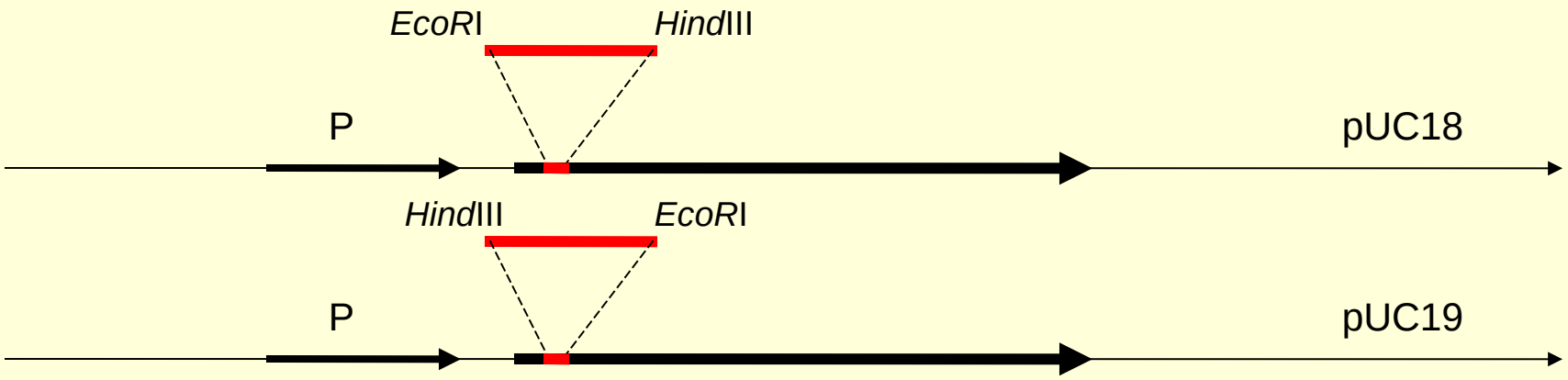
gaattCGAGCTCGGTACCCGGGGATCCTCTAGA

SmaI

SalI PstI SphI HindIII

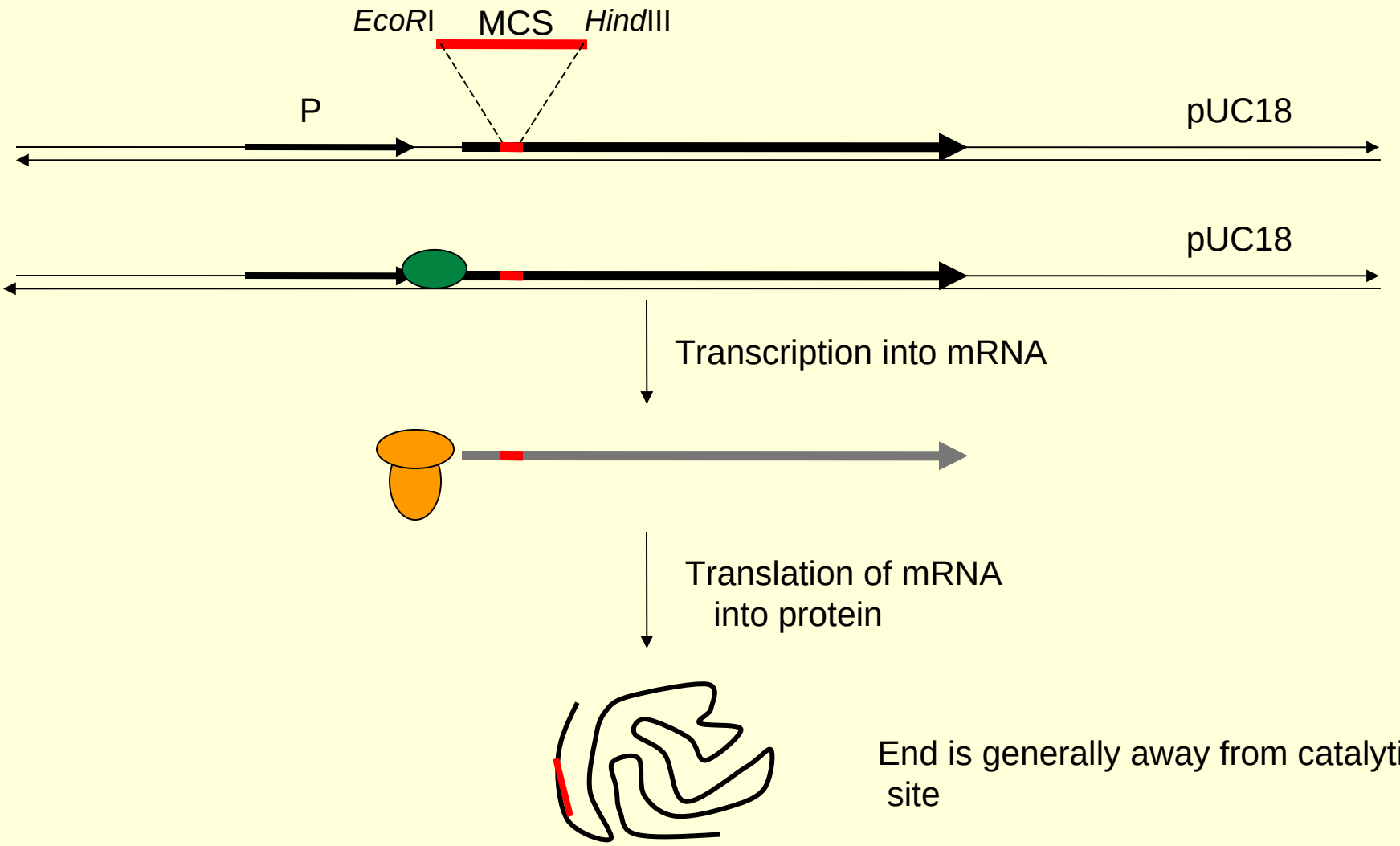
GTCGACCTGCAGGCATGCAAGCTTGGctg

10 unique restriction enzyme sites

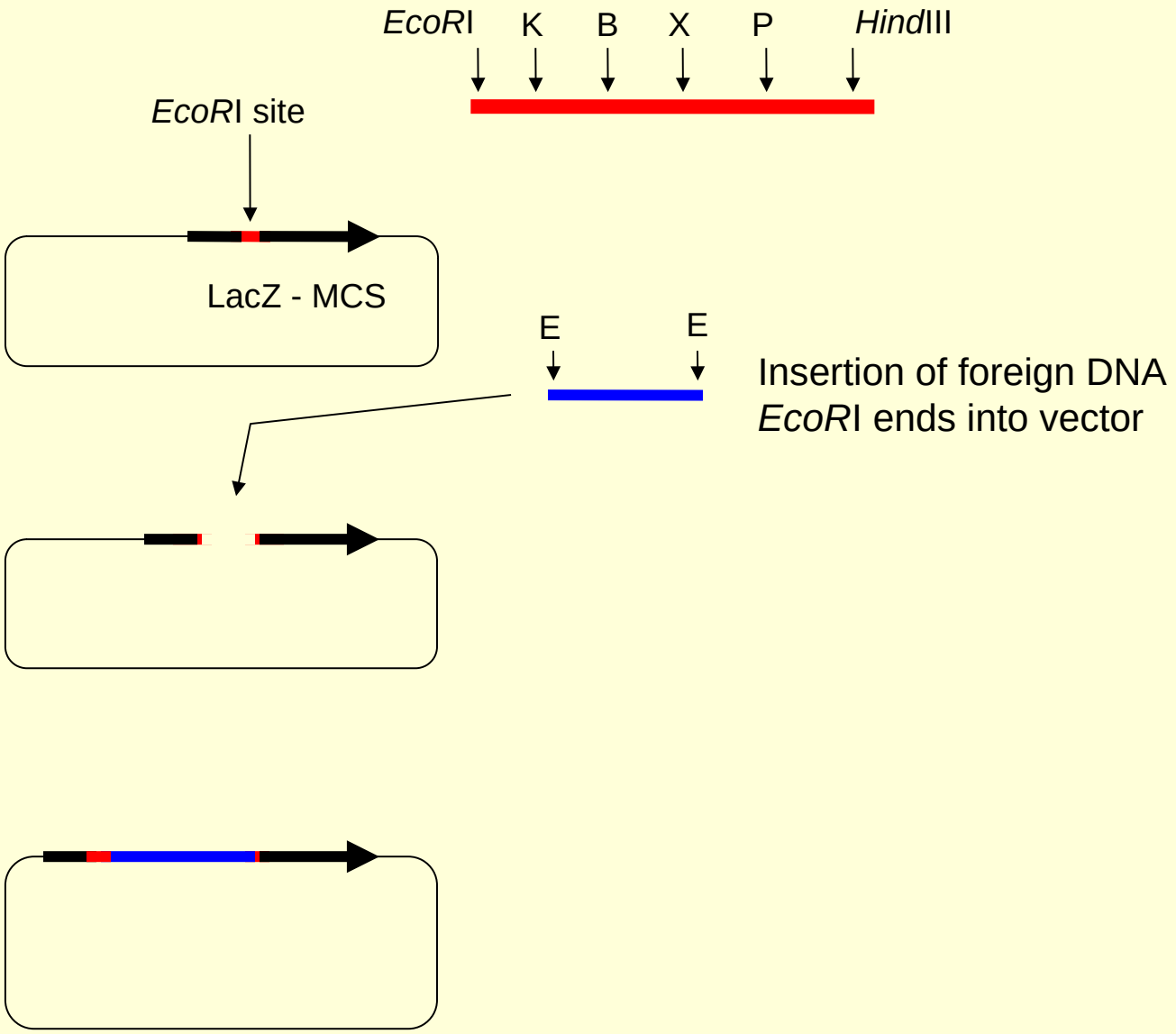



Paired vectors have oppositely oriented MCS

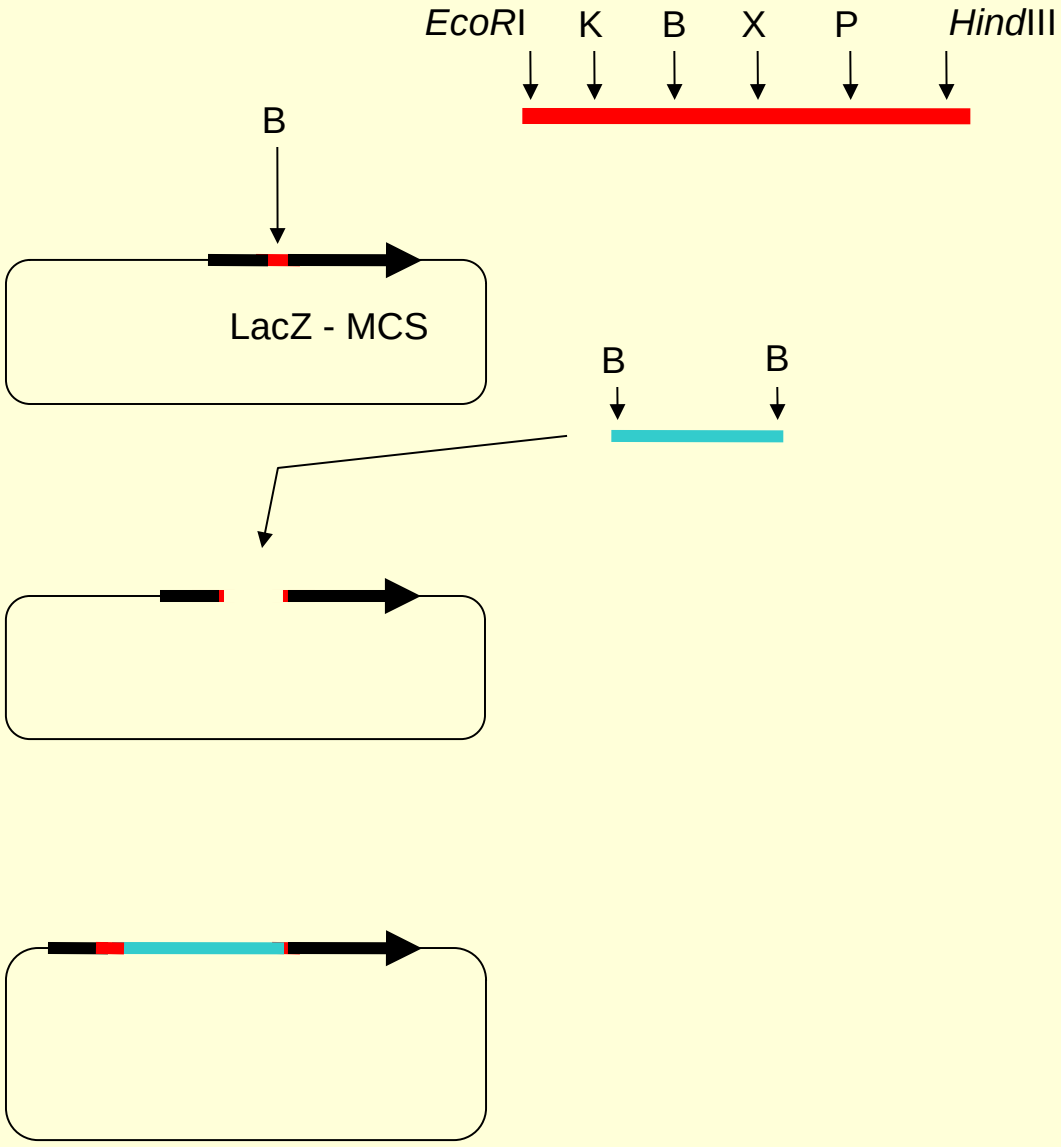
# In-frame insertion of MCS adds 12-18 amino acids



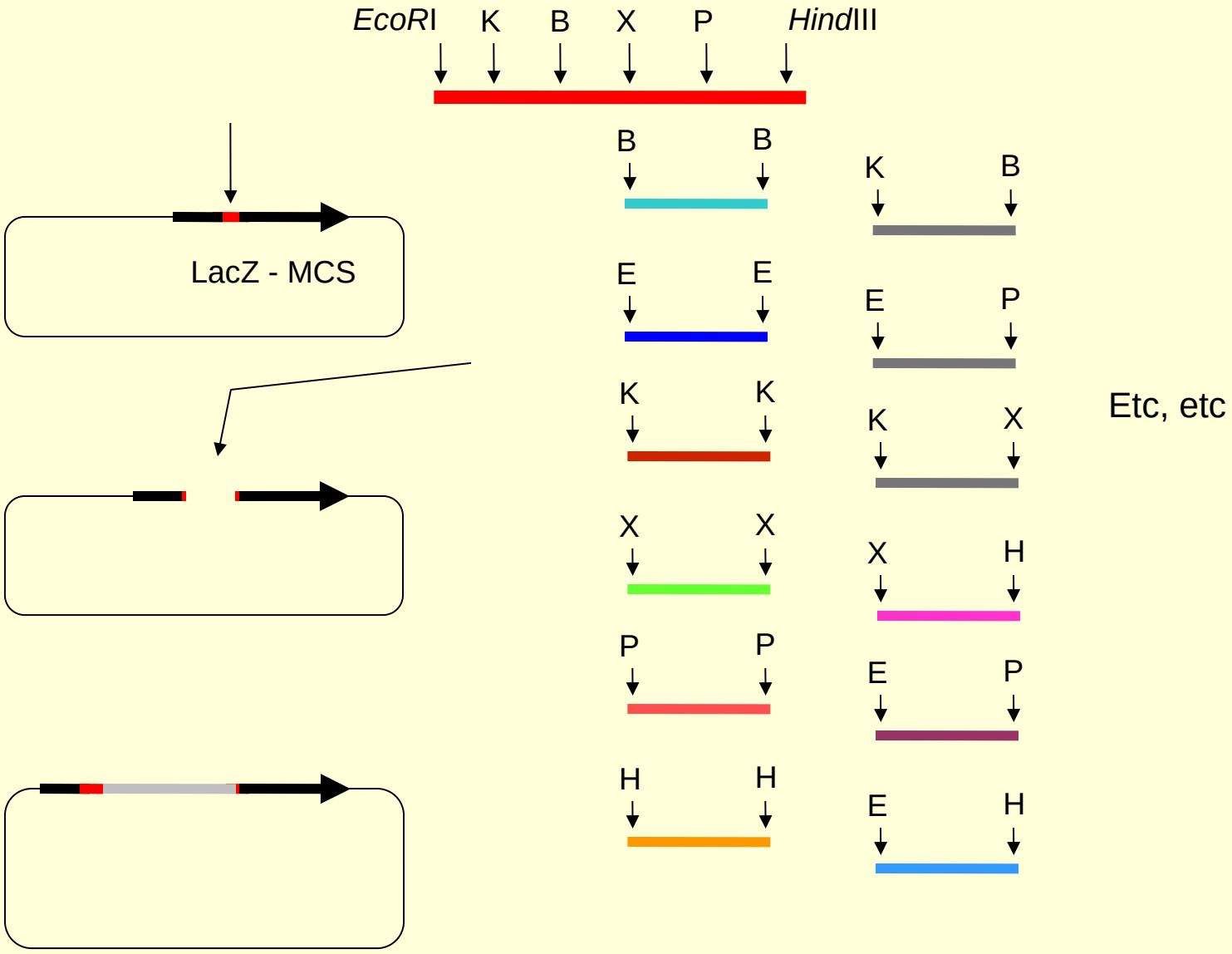
# Example: Insertion at an Eco RI site



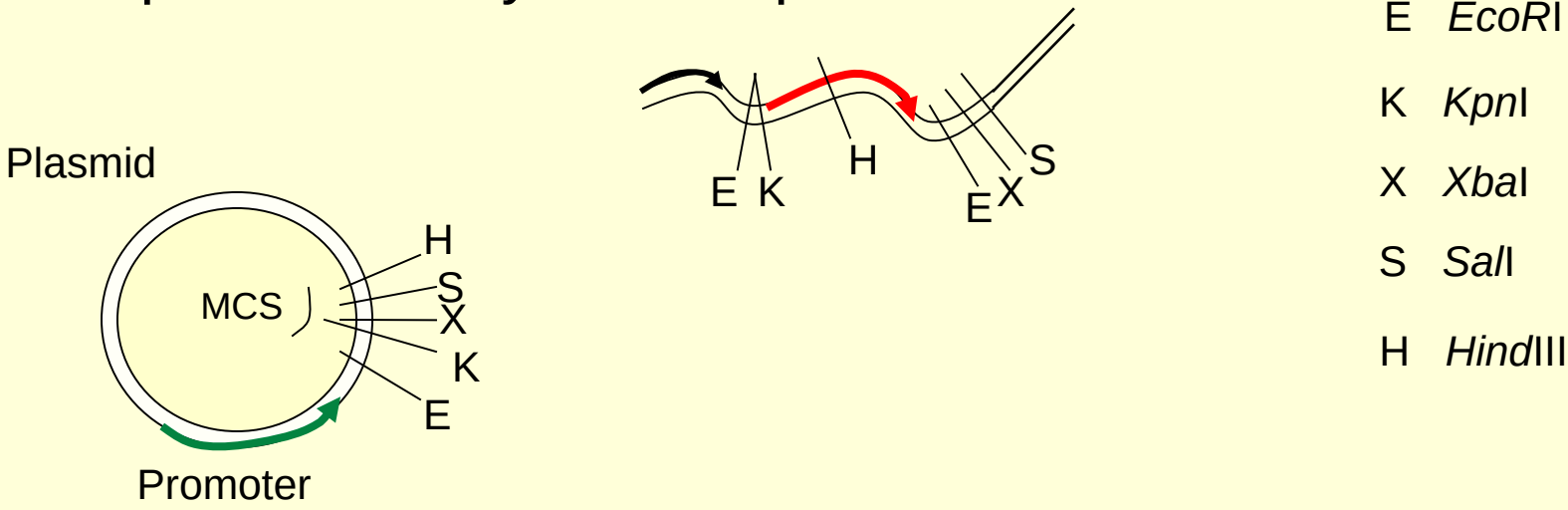
# Example: Insertion at a Bam HI site



# Fragments can be inserted using either 1 or 2 restriction sites



# Example: One enzyme, two possible orientations




E *EcoR*I

K *Kpn*I

X *Xba*I

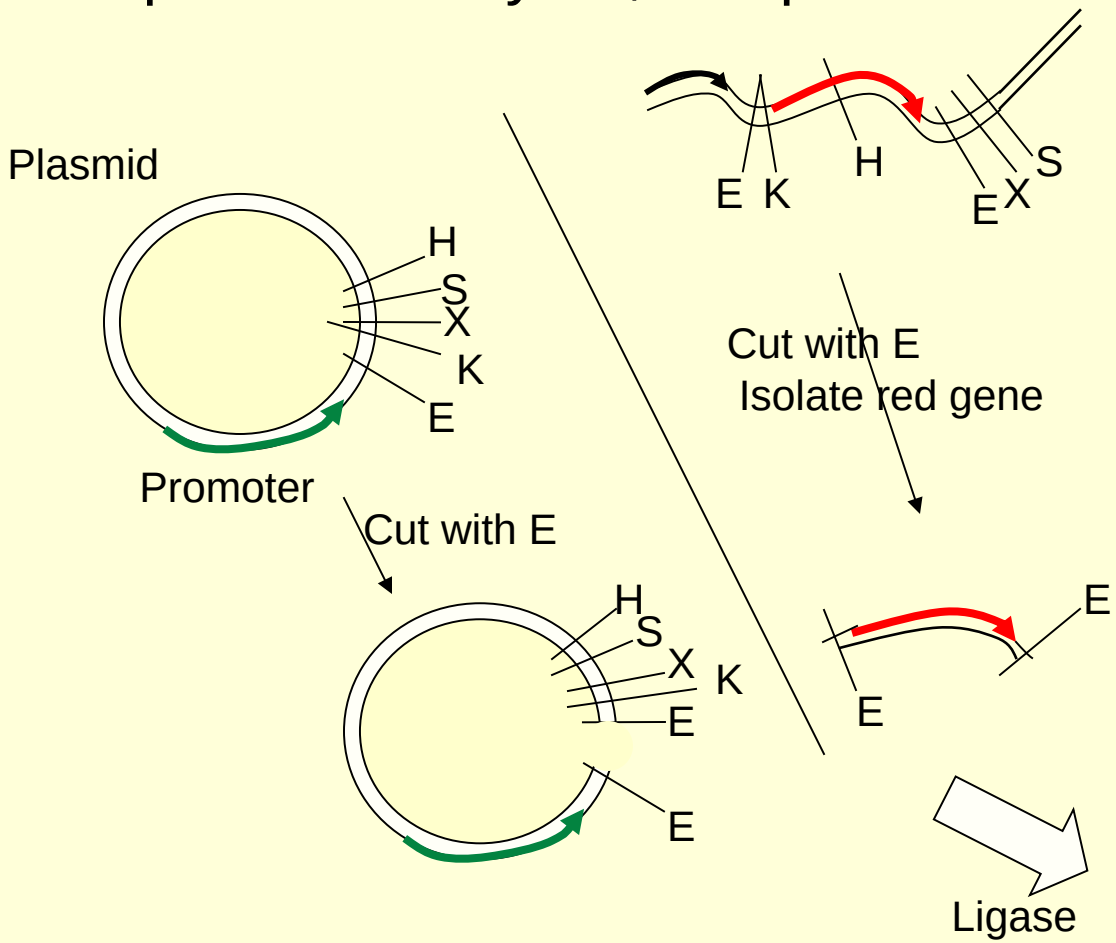
S *Sal*I

H *Hind*III

Possibilities?

Goal: Express Red gene by Green promoter in plasmid

# Example: One enzyme, two possible orientations

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Goal: Express Red gene by Green promoter in plasmid

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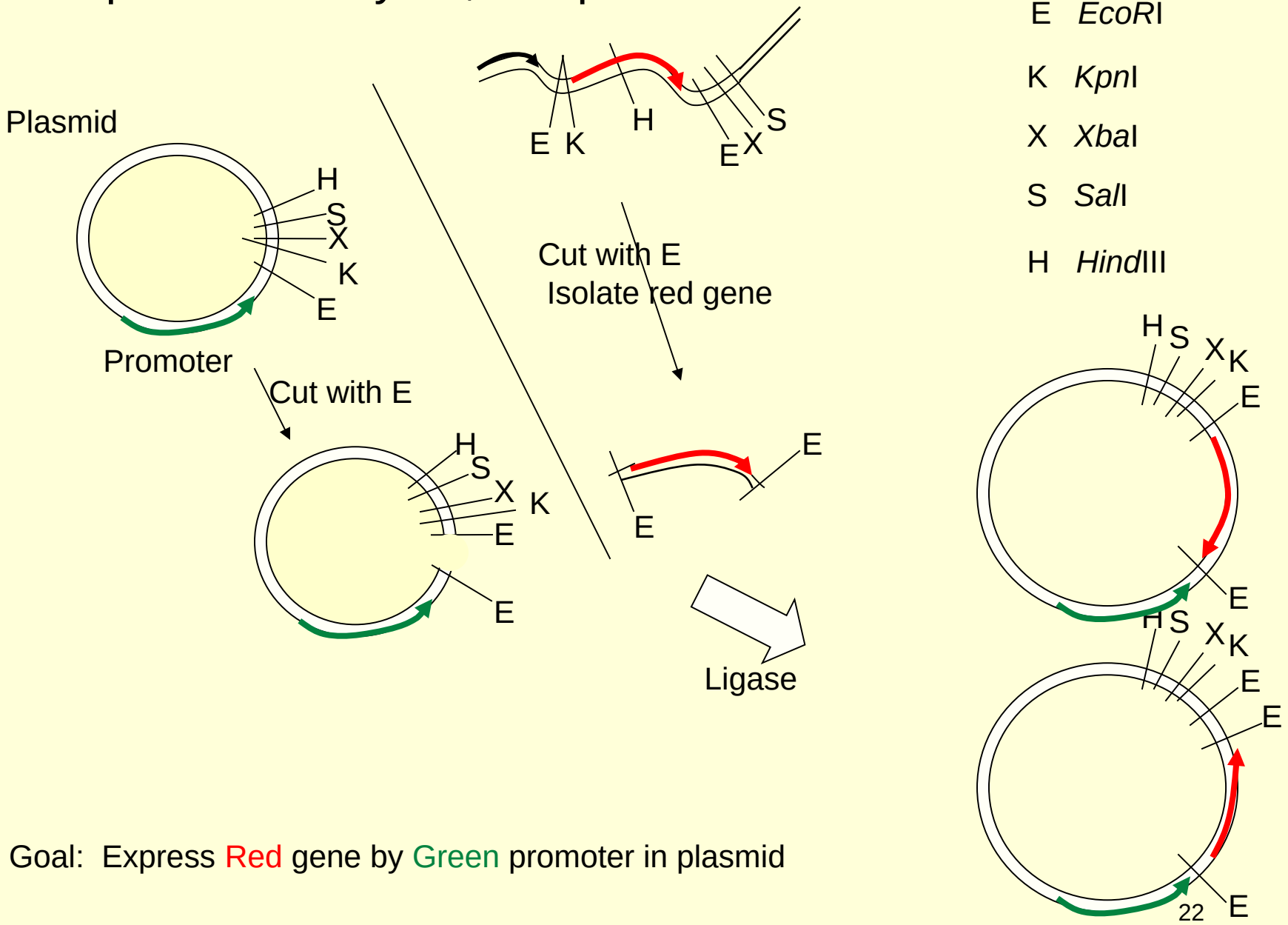
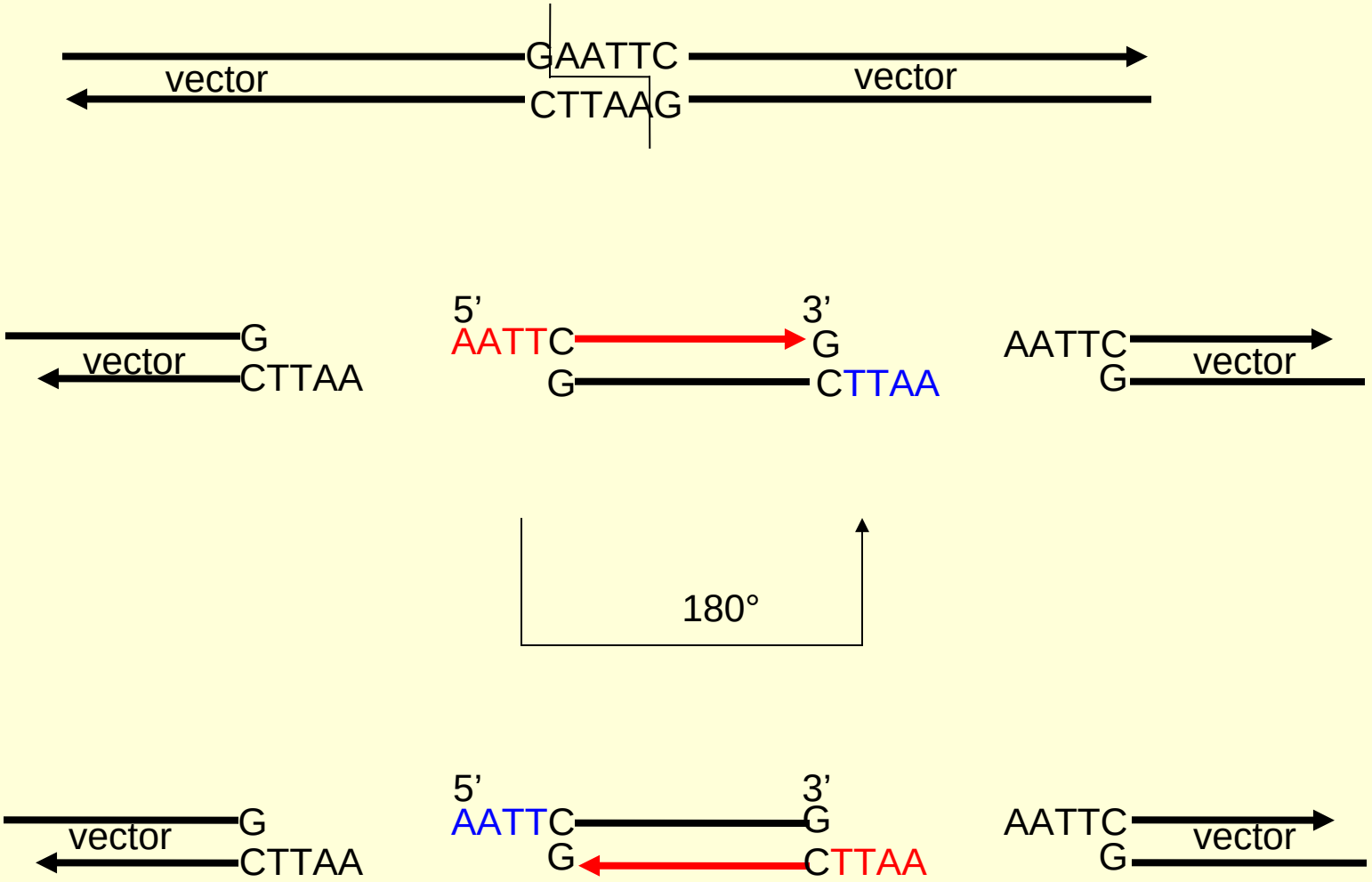


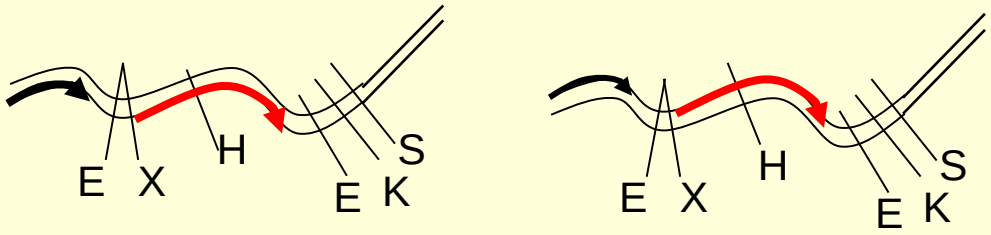

Goal: Express Red gene by Green promoter in plasmid

When the same restriction enzyme cuts on both ends, the insert can be ligated to the vector in either of two possible orientations.



Example: Two enzymes, one possible orientation
This is called directional cloning

Orientation is defined with reference to the direction of transcription →



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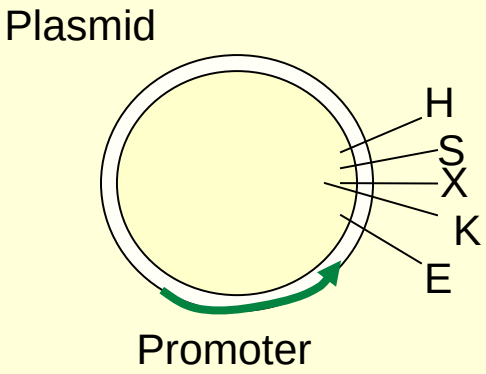


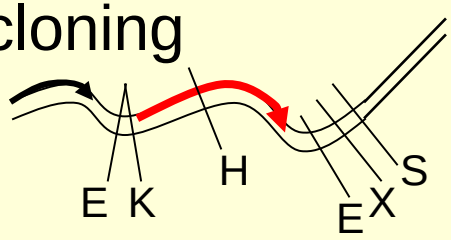


E *EcoR*I

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Goal: Express Red gene by Green promoter in plasmid

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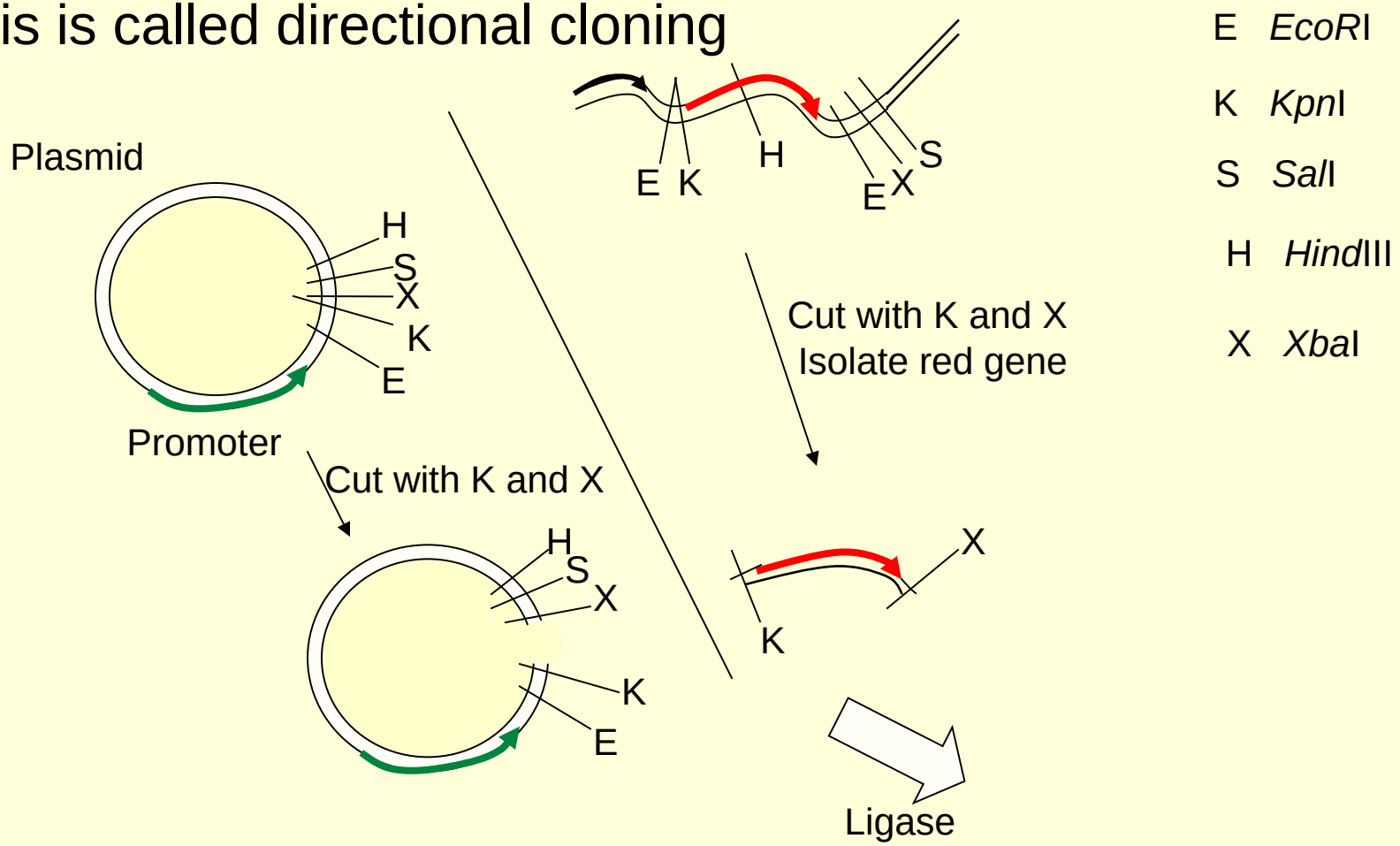


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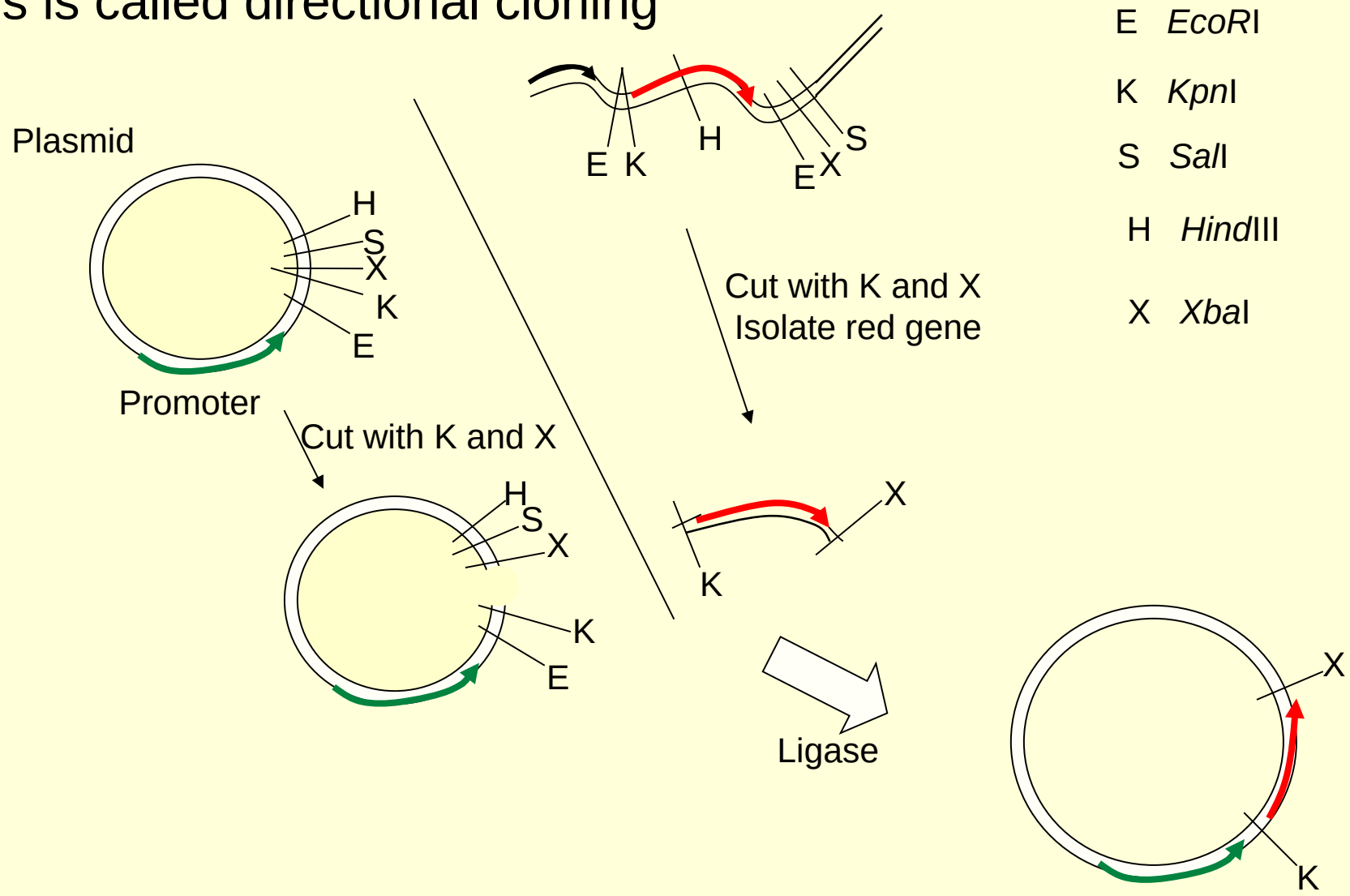



Goal: Express Red gene by Green promoter in plasmid

# Bacteriophage Derived Vectors – 2nd type of vector

Common phage (virus) of E. coli is lambda ($\lambda$)

Viruses typically consist of a DNA (RNA) based genome and a protein coat

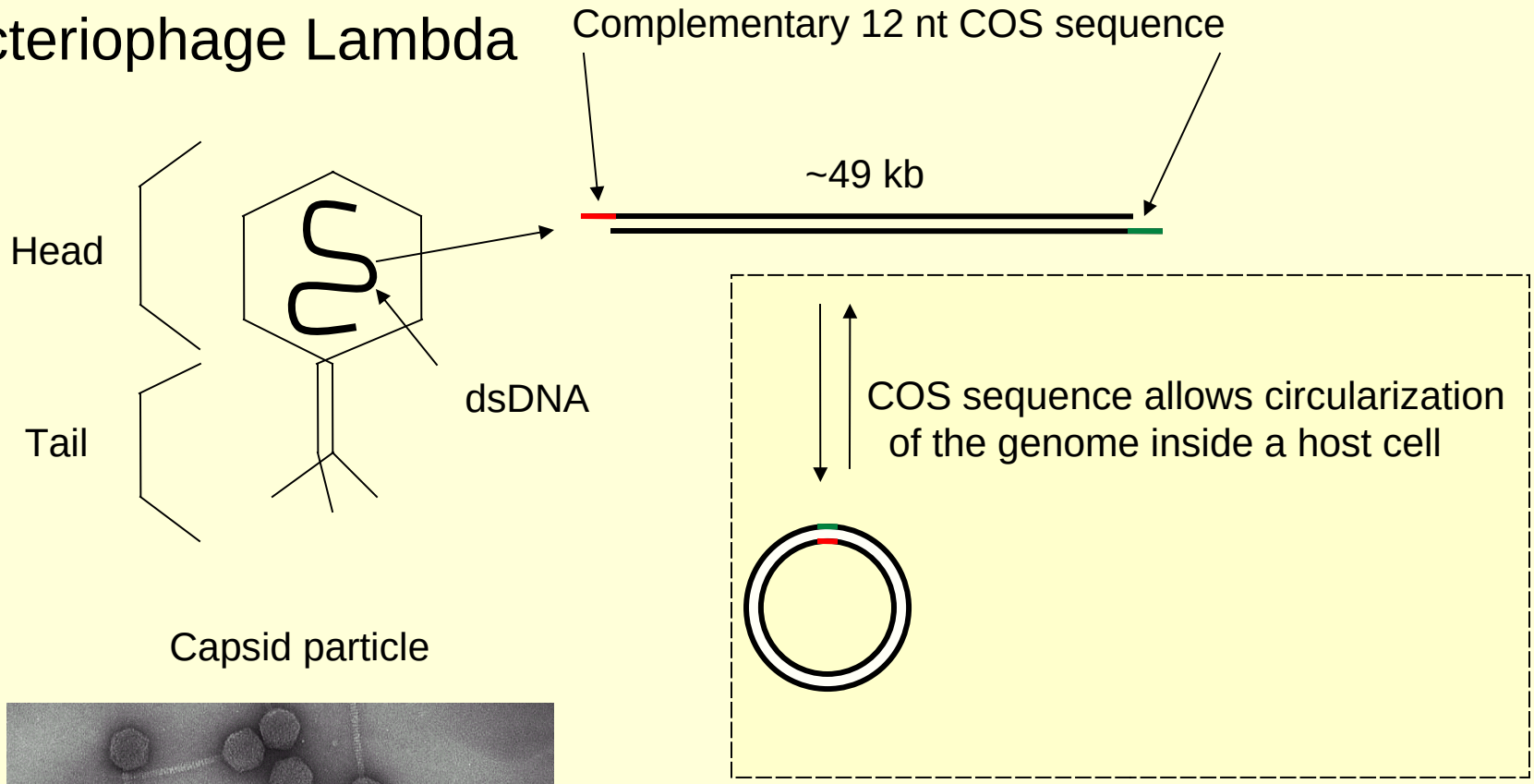
A virus uses host cells to replicate and express their genome

<u>Features of natural lambda</u>

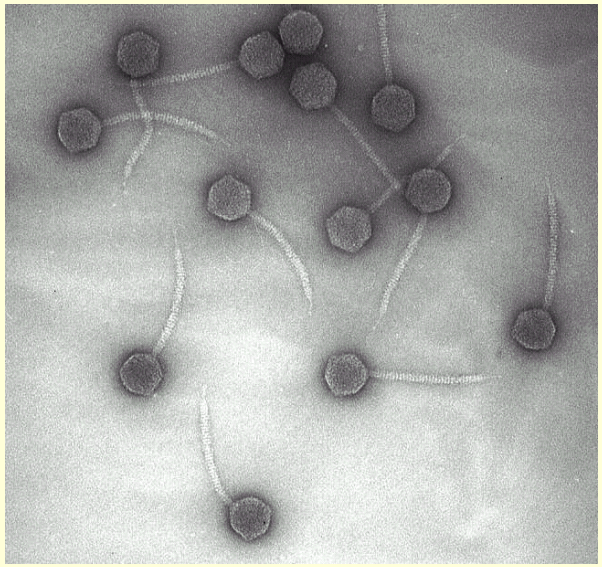
ds DNA , ~49 kbp,  encodes 67 genes
outside its host, phage exists as a phage particle (capsid particle)

linear dsDNA wrapped in a protein coat
ds except for both 5' termini which are 12 nt extensions
the extensions are complementary allowing circularization
The 12 nt extension is called a COS site

# Bacteriophage Lambda




Capsid particle



# Bacteriophage Lambda

the **cos site** in (multimer) Lambda DNA looks like this:

```
---GGGGCGGCGACCT---
---CCCCGCCGCTGGA---
```

terminase cuts like this:

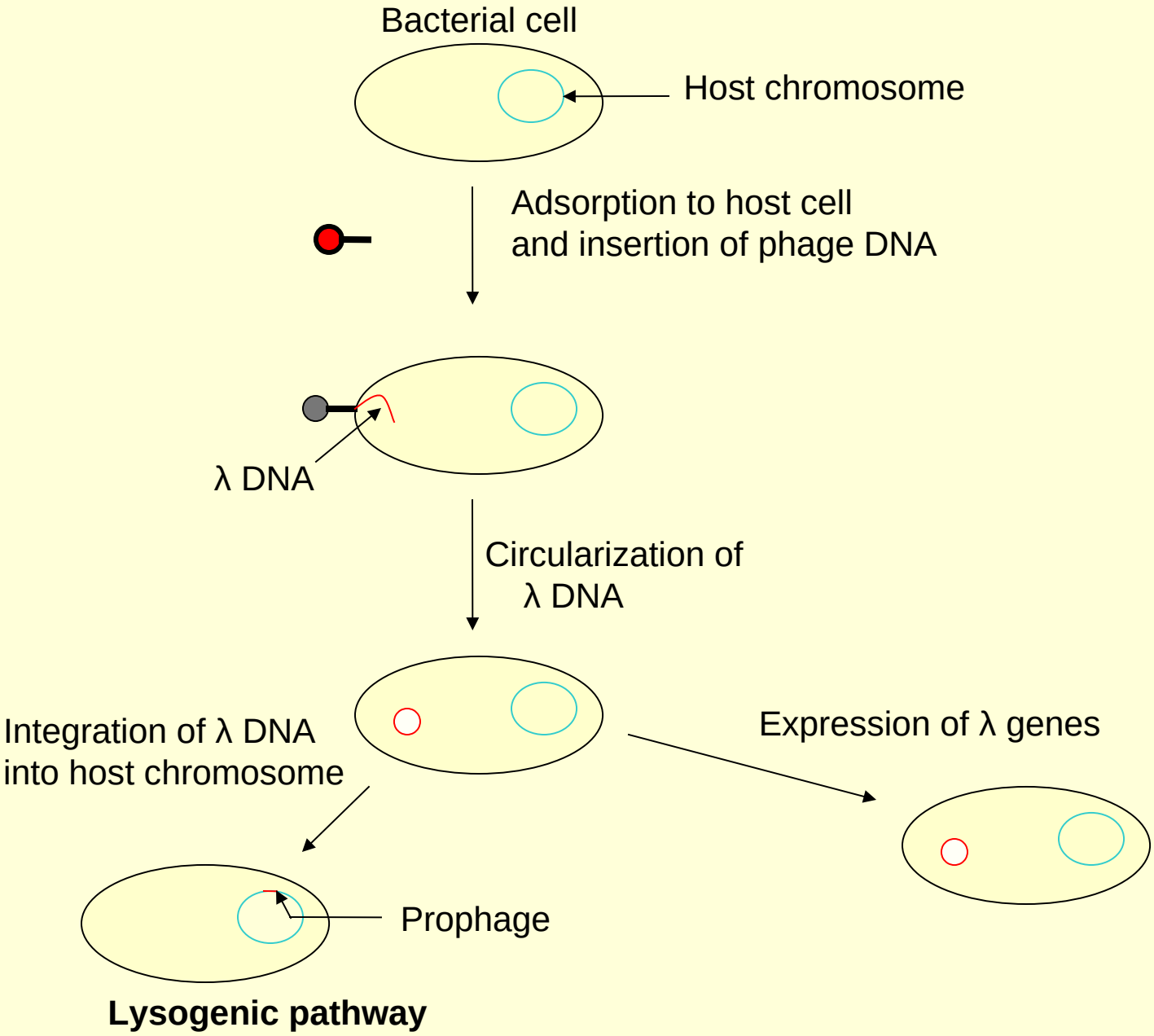
```
---G/GGGCGGCGACCTC---
---CCCCGCCGCTGGA/G---
```

resulting in these **cos ends** in the linear Lambda DNA:

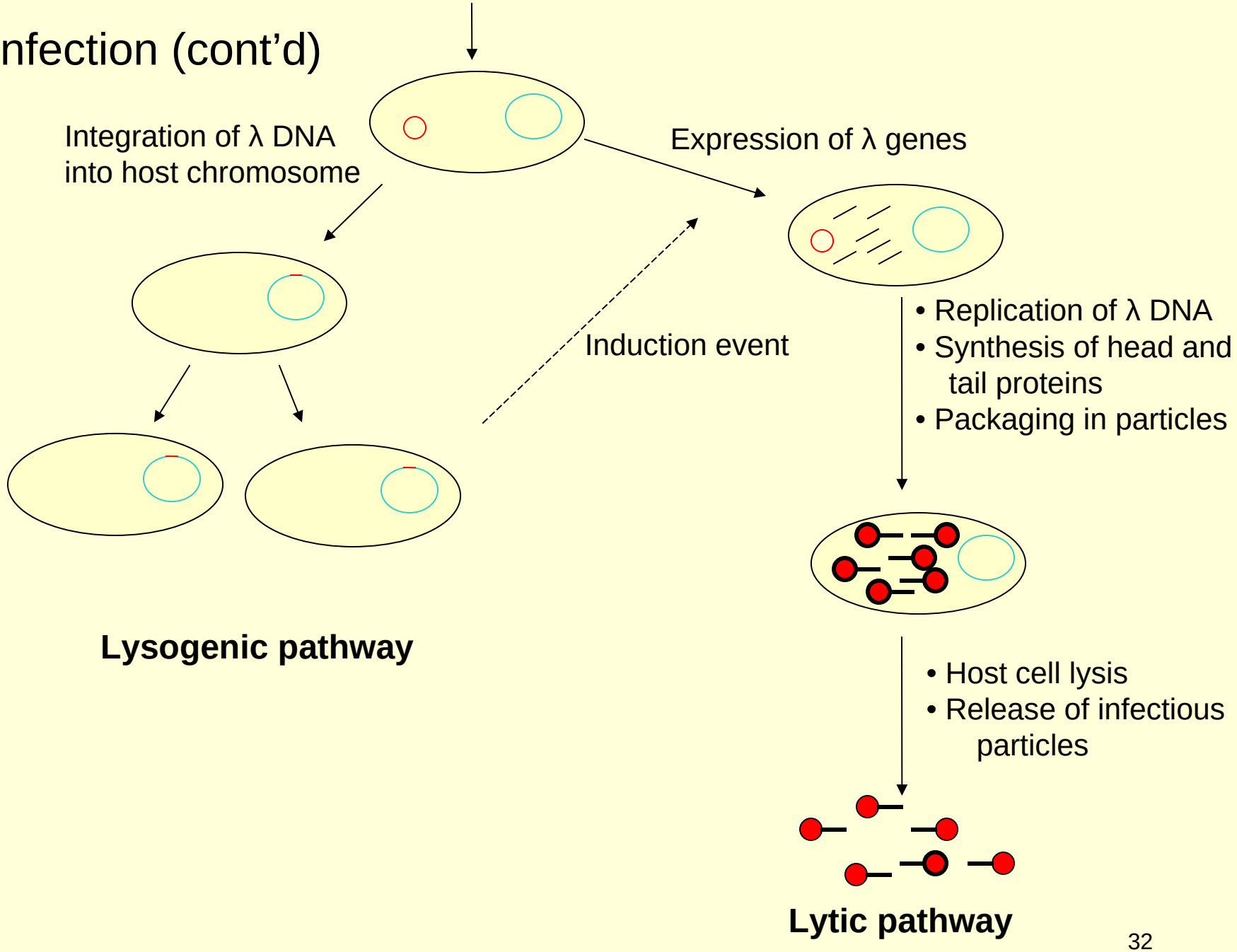
```
---G                          GGGCGGCGACCTC---
---CCCCGCCGCTGGA                          G---
```

from http://www.bioinformatics.nl/molbi/SCLResources/lambda.htm

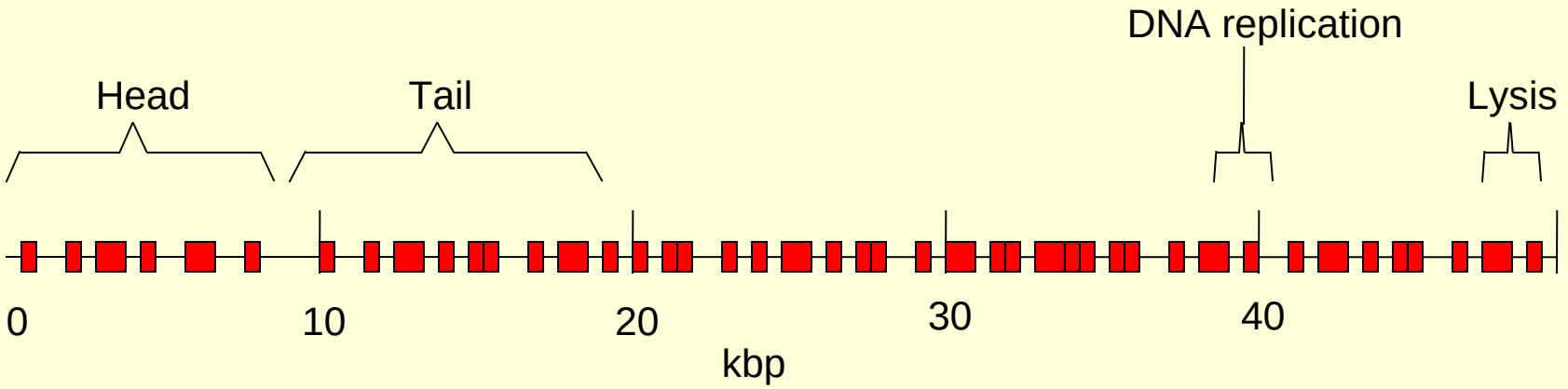
# Bacteriophage lambda (λ) infection of *E. coli*



# Infection (cont'd)



# Lambda genome




Only one gene is expressed in the lysogenic pathway - cI repressor protein which prevent the first stage in the sequential expression of the lambda genome genes.

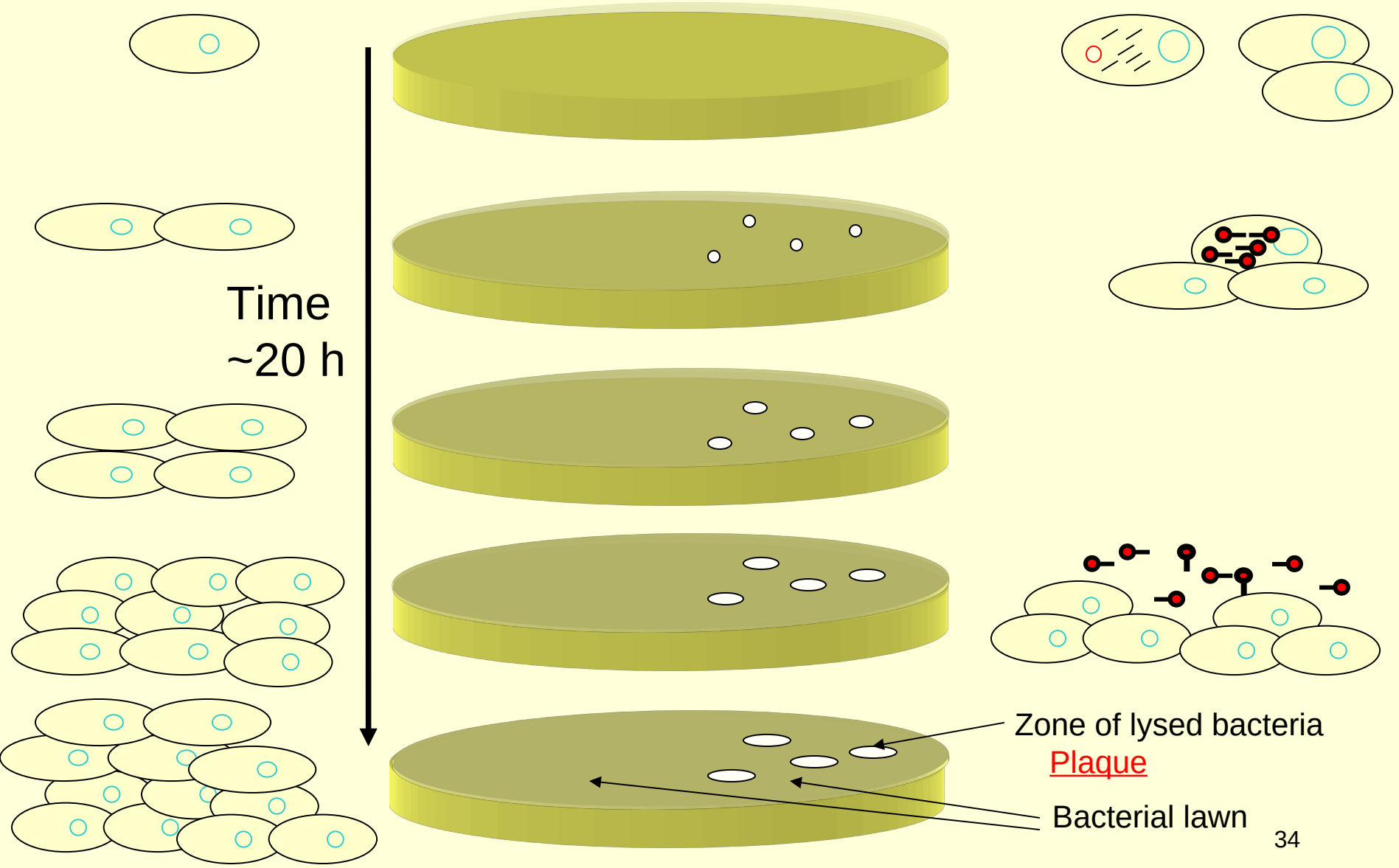
During lytic pathway, different sets of genes are expressed early (DNA replication) mid (synthesis of head and tail structures and assemble of capsid particle) and late (for lysis of host cell) in the pathway

The capsid coat (head and tail structures) are very important to the phage as it allows the phage to get its DNA into healthy *E. coli* cells.

# Lytic Infectious process

Bacteria spread uniformly over surface

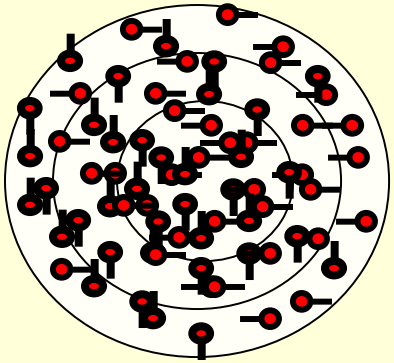
Bacteria with very low level of lambda-infected cells spread over plate (1/10,000)

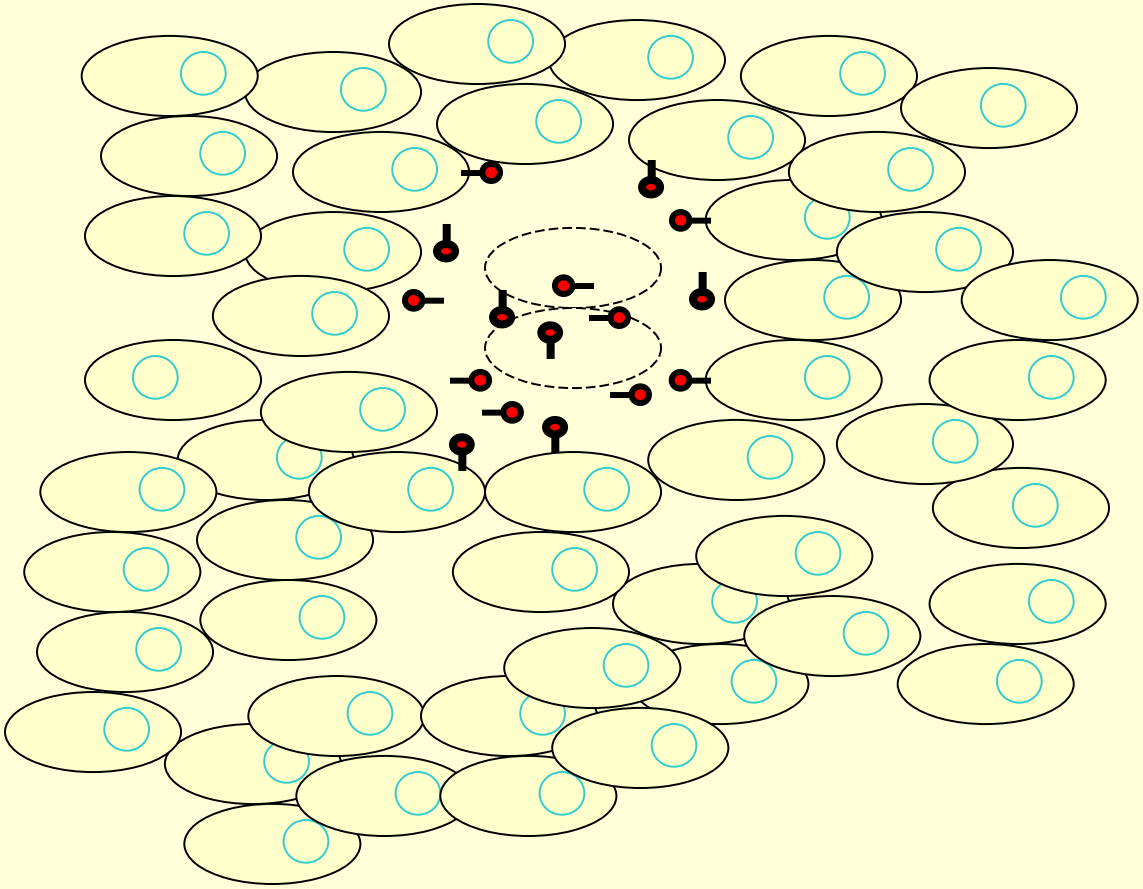


# Plaque

-results from a repeated infection, phage replication and lysis with release of phage particles

Lysed cell zone contains free phage particles and proteins expressed in the *E. coli* prior to lysis





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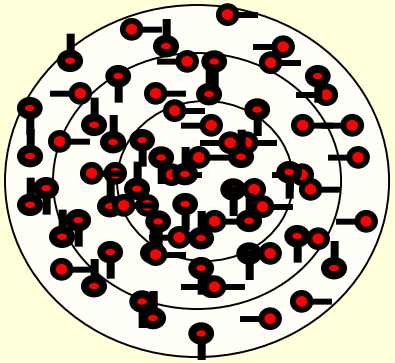
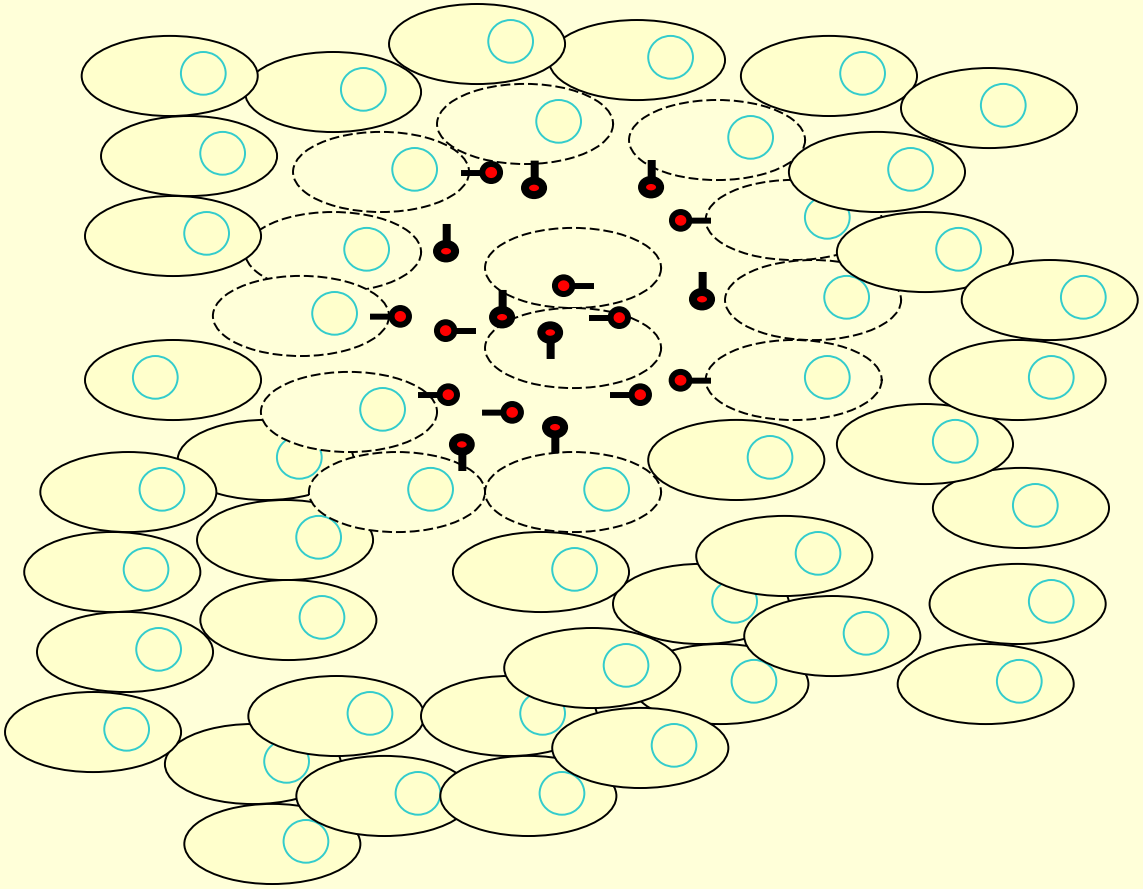




# Capsid head structure

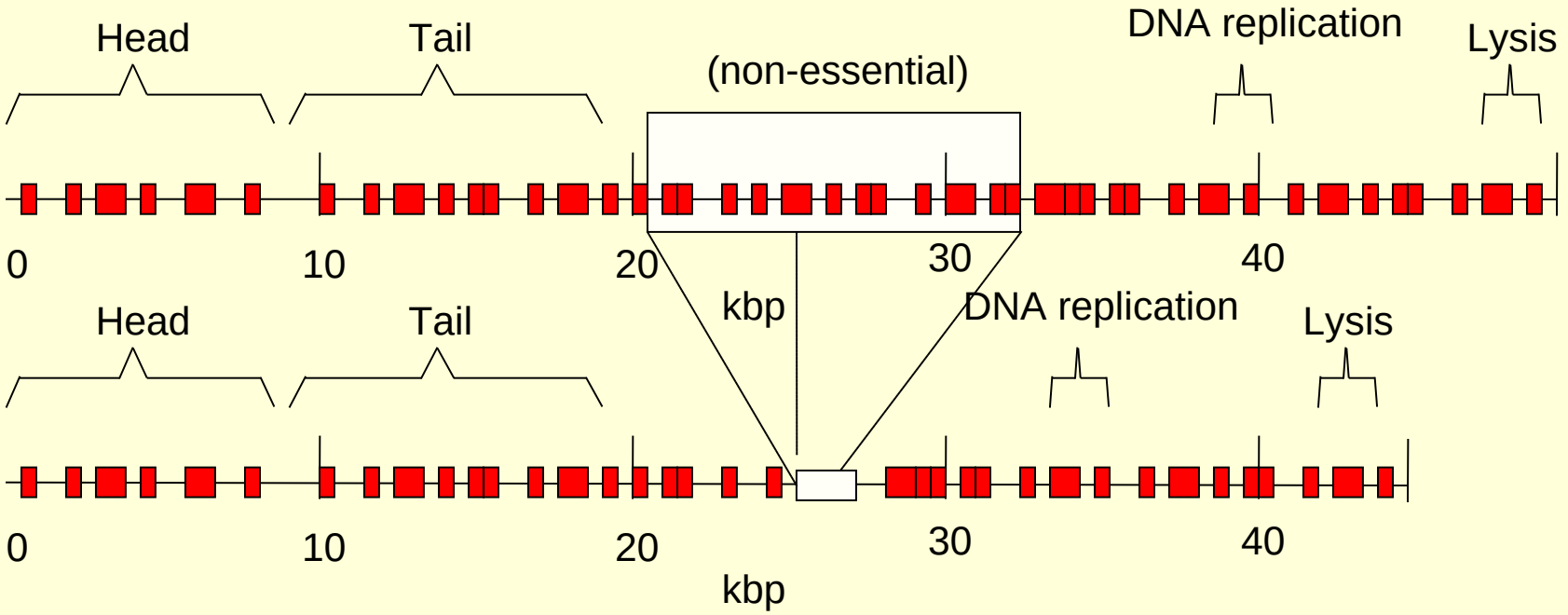
The head structure is a defined size

To be a viable phage particle the head structure must contain between 38-51 kbp of linear dsDNA which is terminated by 5’ COS sequences

# Lambda vectors - features

Must create space in the lambda genome for insertion of extra DNA

1. Lambda vectors have had part of the genome deleted to allow 1-8 kb of foreign DNA to be inserted. Deleted part is non-essential to function as a vector. Eg the vectors are 41 – 47 kbp long

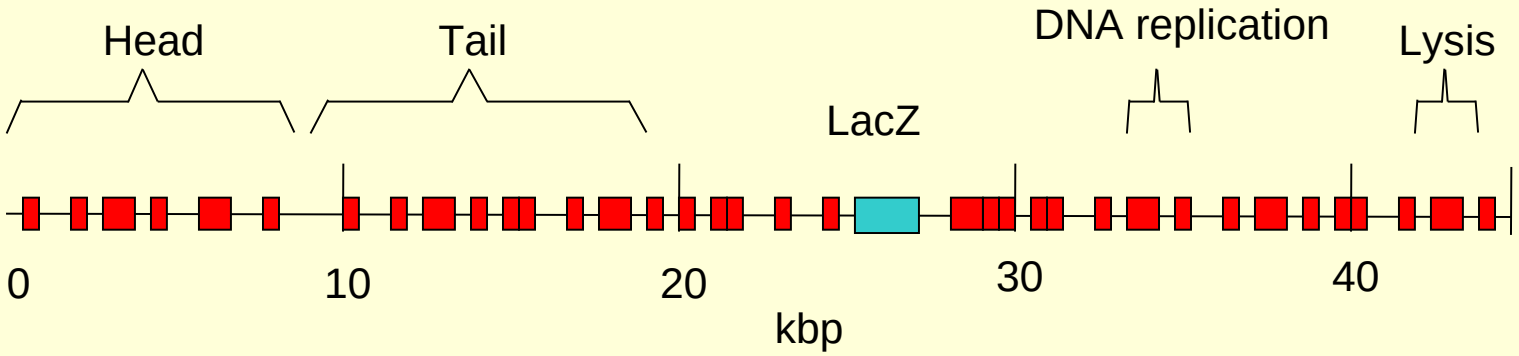


# Lambda vectors - features

2. Reporter gene to distinguish recombinant from non-recombinant vectors.
(No antibiotics are required as uninfected cells are required.)

If LacZ gene is used as a cloning site:

- no insert : blue plaque
- insert in LacZ: clear plaque



# Lambda vectors - features

3. One or more unique restriction sites within the selectable marker to allow for insertion of foreign DNA.

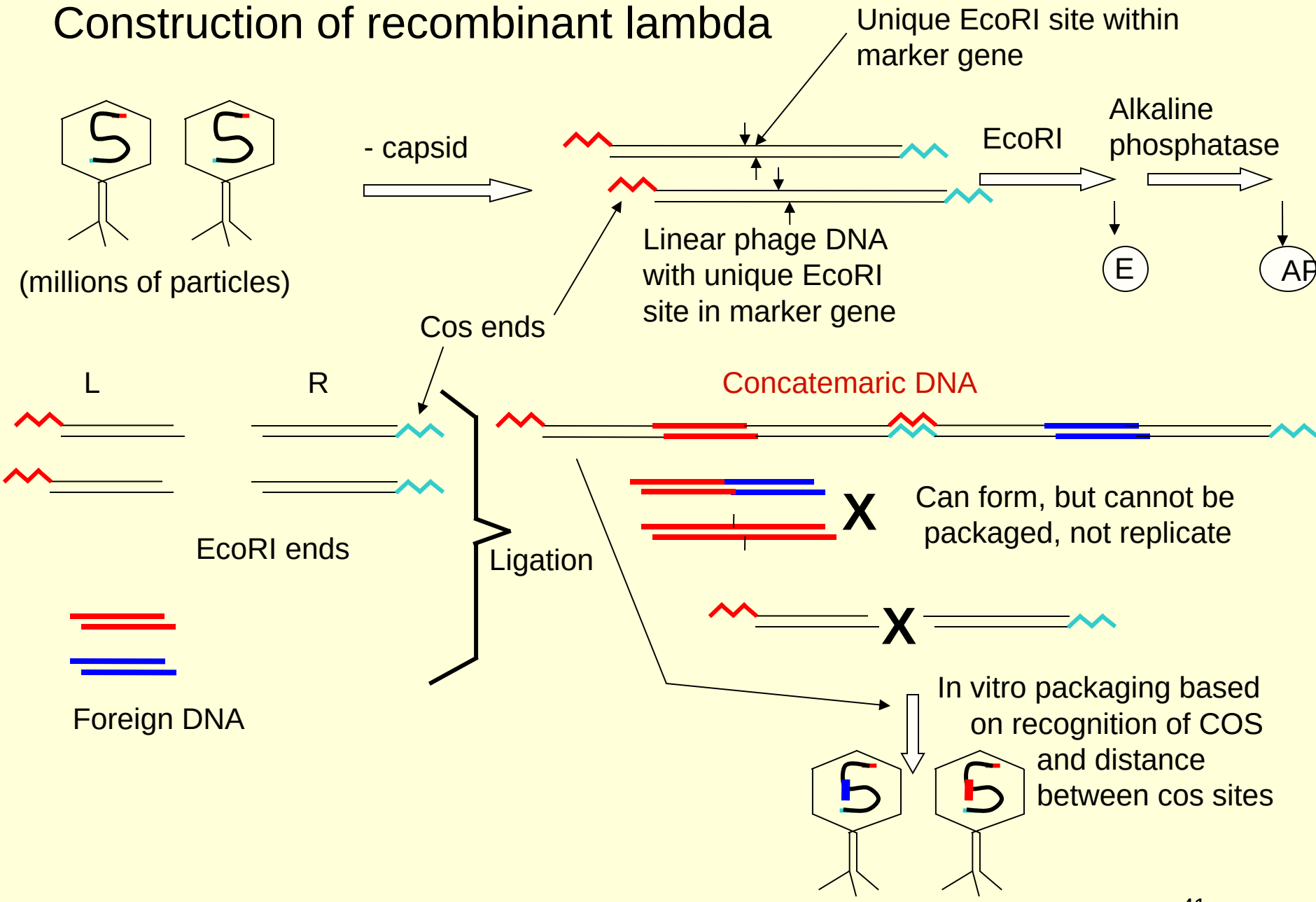
Because the vector is large (~43 kbp) getting multiple restriction sites which are unique (occur only once in the vector) is more difficult but has been achieved with specific vectors.

4. The ability to repackage recombinant phage DNA into a capsid particle.

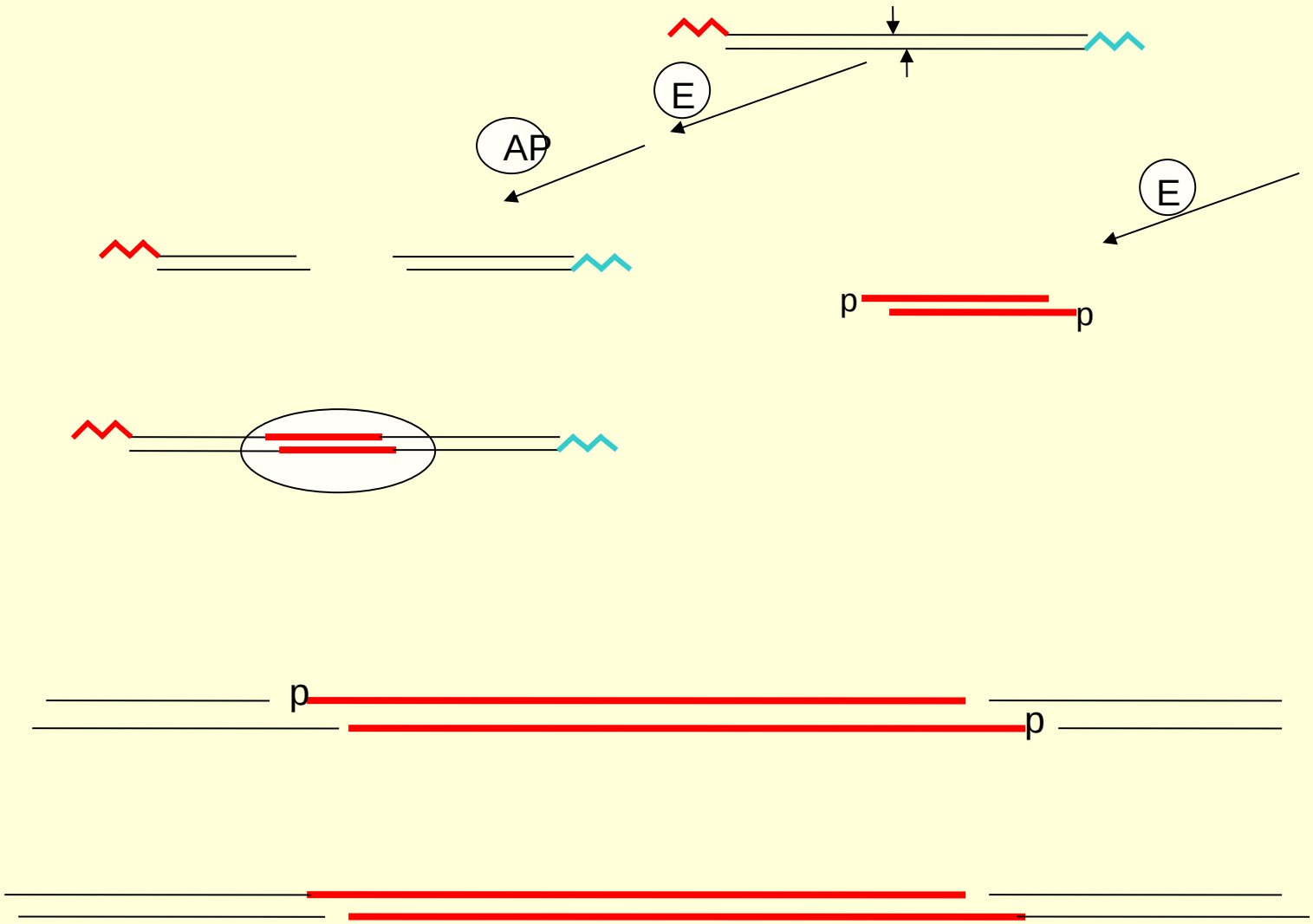
For phage this process is normally done inside the bacterial cell but for recombinant DNA work it must be done *in vitro* if this is to be useful vector.

Recombinant Lambda DNA can be packaged in-vitro using bacterial cell extracts prepared from cells expressing the mid stage of the lytic infection genes. The extract contains the enzymes and precursors for the head and tail structure and for packaging of the recombinant DNA into capsid particles. Addition of the extract to ligated phage/foreign DNA will result in packaging any DNA which is 38-51 kbp long and terminated by cos sequences.

# Construction of recombinant lambda



# Alkaline phosphatase - prevents ligation of lambda arms to each other



Foreign DNA alone cannot be packaged as an infectious particle as packaging requires recognition of a cos site at each end of the sequence (and 38-51 kb of DNA between the cos sites).

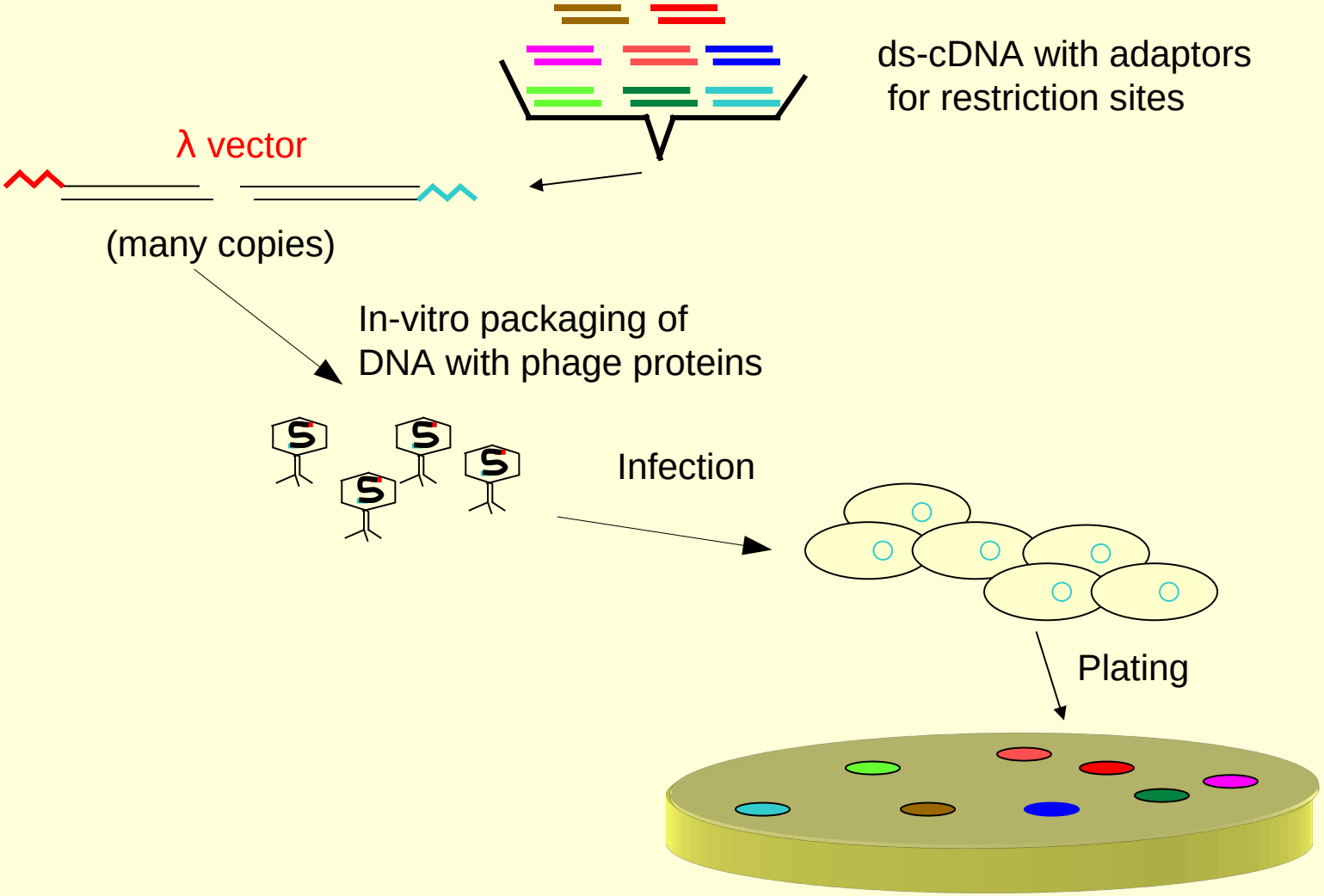
Molar ratio of vector to foreign DNA arranged to favor foreign DNA insertion vs self-ligation of foreign DNA (higher molar amount of the vector)

The resulting phage particles are used to infect *E. coli* cells and plated as described previously.

If insertion site is in the λ repressor (cI gene), then all plaques will be due to recombinant phage.

If insertion site is in LacZ marker gene, then all clear plaques will indicate recombinant phage and blue plaques will be due to non-recombinant phage.

# Lambda phage is most commonly used for cDNA cloning




Each plaque from phage with different insert

# BAC (Bacterial Artificial Chromosome) vector

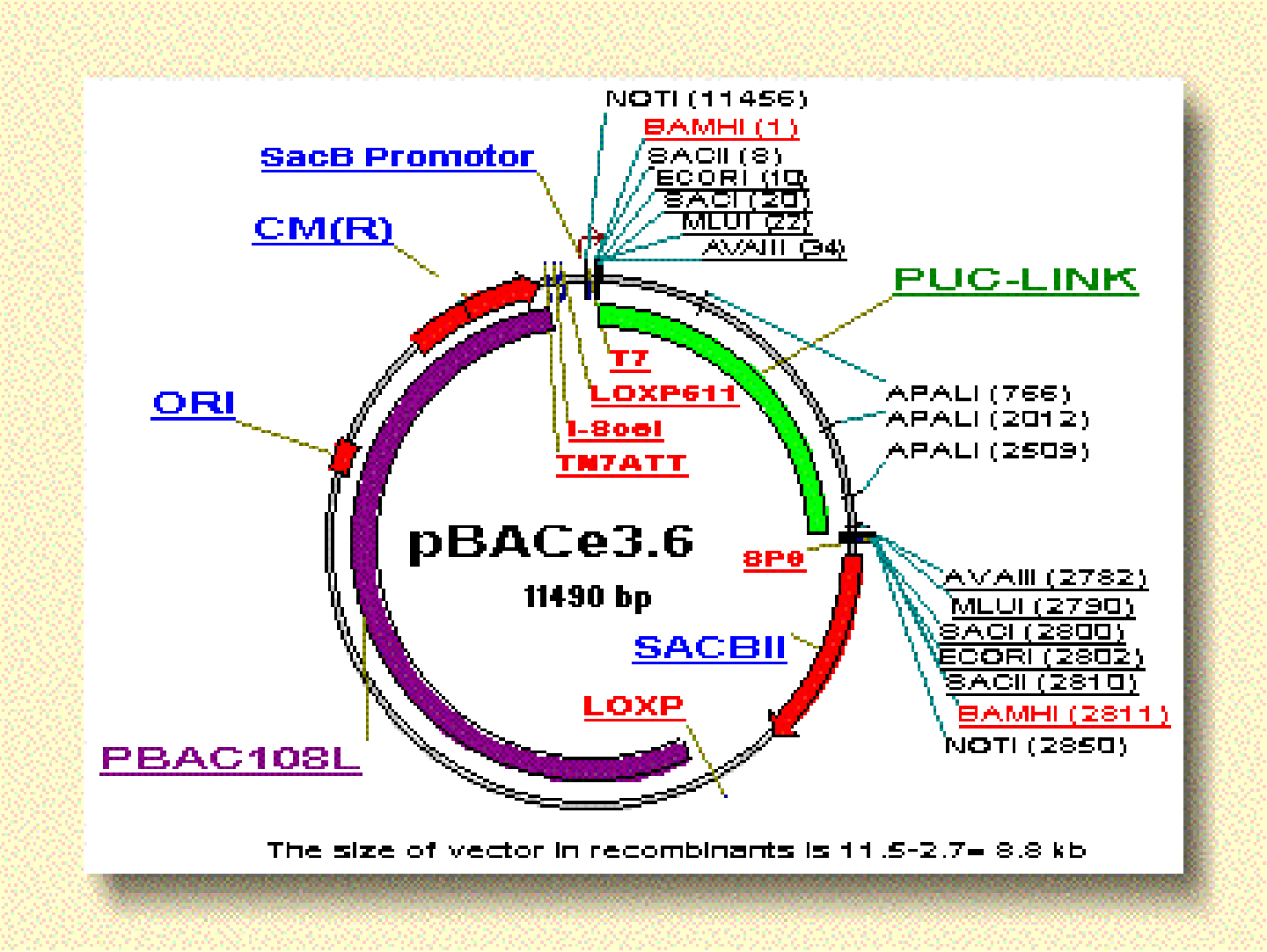
Designed for **cloning of large pieces of genomic DNA** (50-250 kbp)

BAC vectors are used to find contiguous pieces of genomic DNA so that the arrangement of the sequences in a chromosome can be determined

BAC vectors are ds circular DNA which contains an ORI with tight replication control so that ideally only a single copy of the BAC with inserted DNA is retained in any cell. (Ori is derived from F' plasmid of E coli)

Multiple copies of large DNA inserts leads to a low (but unacceptable) frequency of recombination

# BAC vector




from
http://escience.ws/b572/L18/L18.htm

# BAC vectors

**Sequences for maintanence in E.coli**

- ori - E.coli rep. derived from high-copy plasmid pUC9
- CM(R) - chloramphenicol resistance gene; typical plasmid vectors use ampicillin resistance as a selectible marker, so it's better to have a different gene for BACs
- cloning site - site for insert

**Selection against clones with no inserts**

- PUCLINK stuffer fragment interrupts the sacB gene
- sacB encodes levanosucrase, which converts sucrose to levan, which is toxic to E. coli.
- PUCLINK stuffer fragment can be excised with restriction enzymes NotI (5'GC^GGCCGC3') , BamHI (5'G^GATCC3') or EcoRI (5'G^AATTC3').
- If the plasmid is recircularized with itself, the sacB promoter will now be directly upstream from the sacB coding sequence, and sacB will be expressed
- If the plasmid ligates with an insert, then sacB will not be expressed, and cells will survive.

**Working with large inserts**

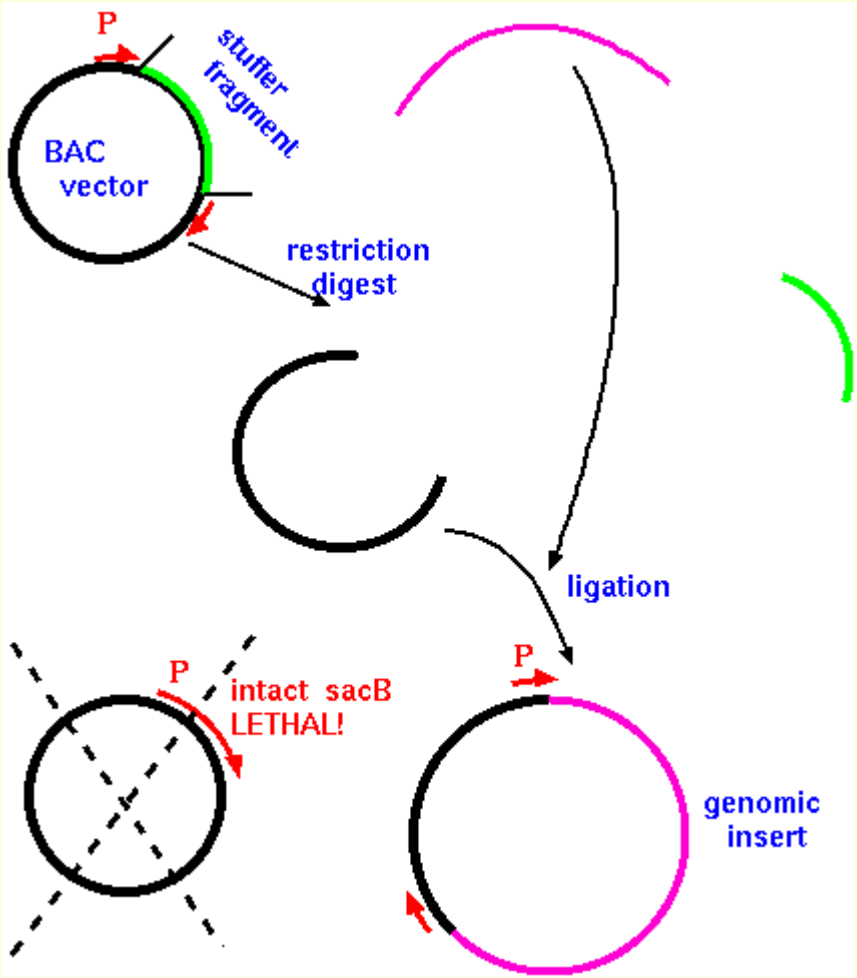
- NotI site is best, because the recognition is 8 bp (5'GC^GGCCGC3'), rather than 6. (Remember, $4^6$=4096; $4^8$=65536). Because NotI cuts, on the average, once every 65536 bp, most inserts can be excised as a single fragment, or perhaps several large fragments. 6-cutter enzymes would produce many smaller fragments.
- For physical reasons, circular BACs are much less susceptible to shearing than linear YACs

# BAC - Selection for clones with inserts

Transformants are grown on levanosucrose.

If the vector recircularizes with itself, sacB is expressed, and cells are killed.

If the vector contains an insert, sacB is not expressed. Thus, only clones with inserts will be selected.



# Summary of Vectors

- Plasmids -designed for cloning small pieces (0-8 kb) for foreign DNA
  -high copy number, high amplification
  -chemically treated cells (competent cells)
  allows plasmids to leak through
  weaken plasma membrane of bacteria
  (size limitations) Transformation ~ $10^8$ transformants/µg DNA

- Bacteriophage lambda -primarily used for cloning small cDNAs (0-8 kb) (single genes)
  - » - Valuable because of high transfection efficiency ~$10^9$/µg DNA and ability to segregate large numbers of plaques/plate
- BAC vectors -designed for cloning very large pieces of genomic DNA
  (50-250 kb) requires special transformation system to get large DNA into *E.coli* cells ~$10^7$/µg DNA