Cloning Vectors

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DNA Cloning with Cloning Vectors

- **The genomes of even the simplest cells are much too large to directly analyze in detail at the molecular level and the problem is compounded for complex organisms.**
- **Human genome, contains about 6×10^9 base pairs (bp) in the 23 pairs of chromosomes.**

DNA Cloning with Cloning Vectors

- **Cleavage of human DNA with restriction enzymes produce about one cut for every 3000 base pairs yields (2million fragments).**
- **This obstacle to obtaining pure DNA samples from large genomes has been overcome by recombinant DNA technology.**
- **With this method (Cloning) any gene can be purified.**

Recombinant DNA technology step by step

The recombinant DNA technology is the preparation of large numbers of identical DNA molecules (fragment)

1. DNA fragment of interest is linked through standard $3' \rightarrow 5'$ phosphodiester bonds to a vector DNA molecule, which can replicate when introduced into a host cell.

Recombinant DNA technology step by step

2. When a single recombinant DNA molecule, composed of a vector plus an inserted DNA fragment, is introduced into a host cell (competent cell).

3. The inserted DNA is reproduced along with the vector, producing large numbers of recombinant DNA molecules that include the fragment of DNA originally linked to the vector.

Function of the Cloning Vectors

The two molecules that are required for cloning are DNA to be cloned and a cloning vector.

Function of the Cloning vector:

1. Carries foreign DNA into a host cell.

2. Replicates inside a bacterial or yeast cell.

3. Produces many copies of itself and the foreign DNA.

Cloning Vectors

The vector therefore should contains features that allow the convenient insertion or removal of DNA fragment in or out of the vector.

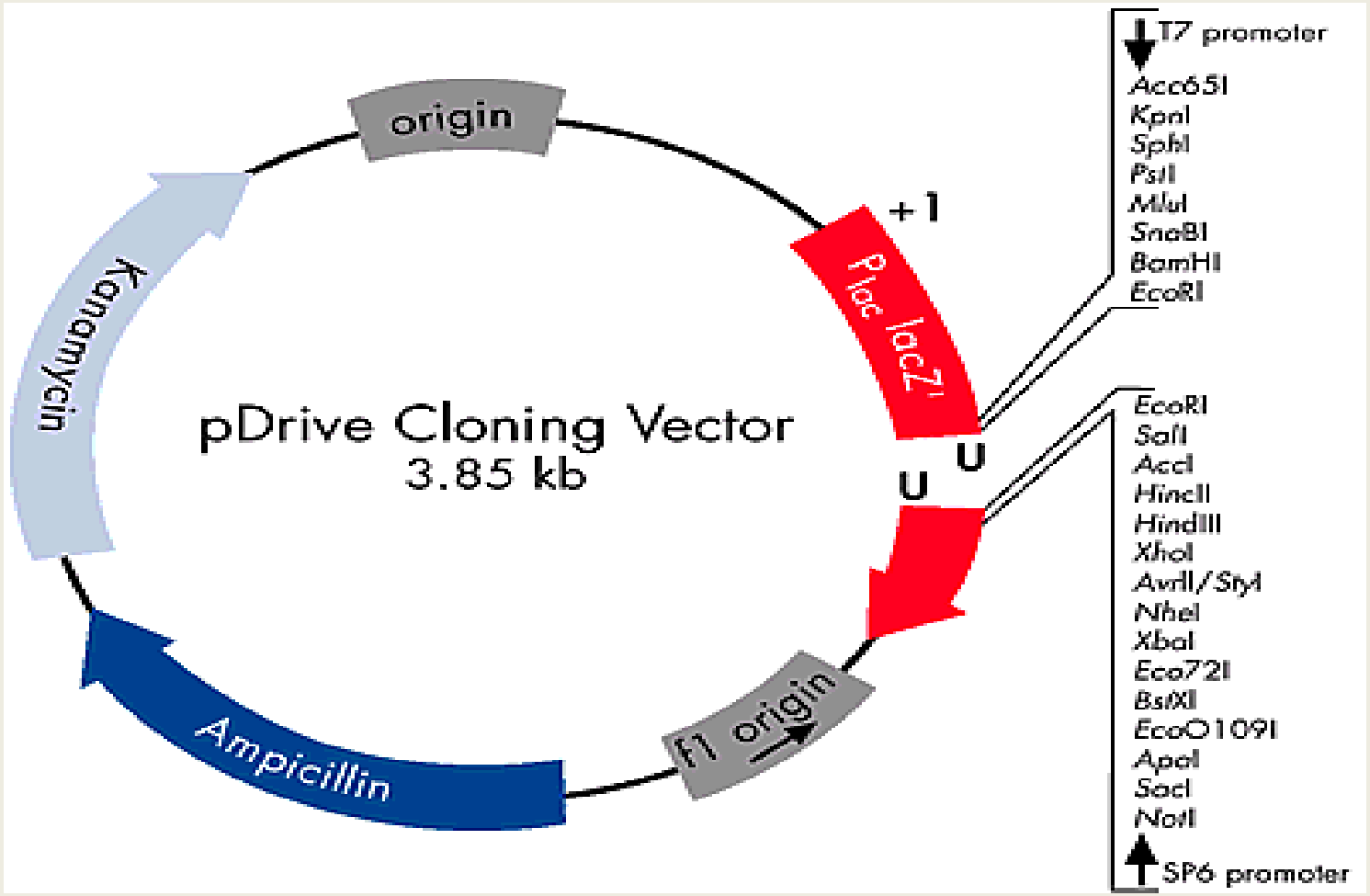
The features of all cloning vectors

1. **Small in size.**
2. **Sequences that permit the propagation of itself (The replication origin) in said the host cell (bacteria).**
3. **A cloning site to insert foreign DNA, good vectors contain a site that can be cut by many restriction enzymes.**

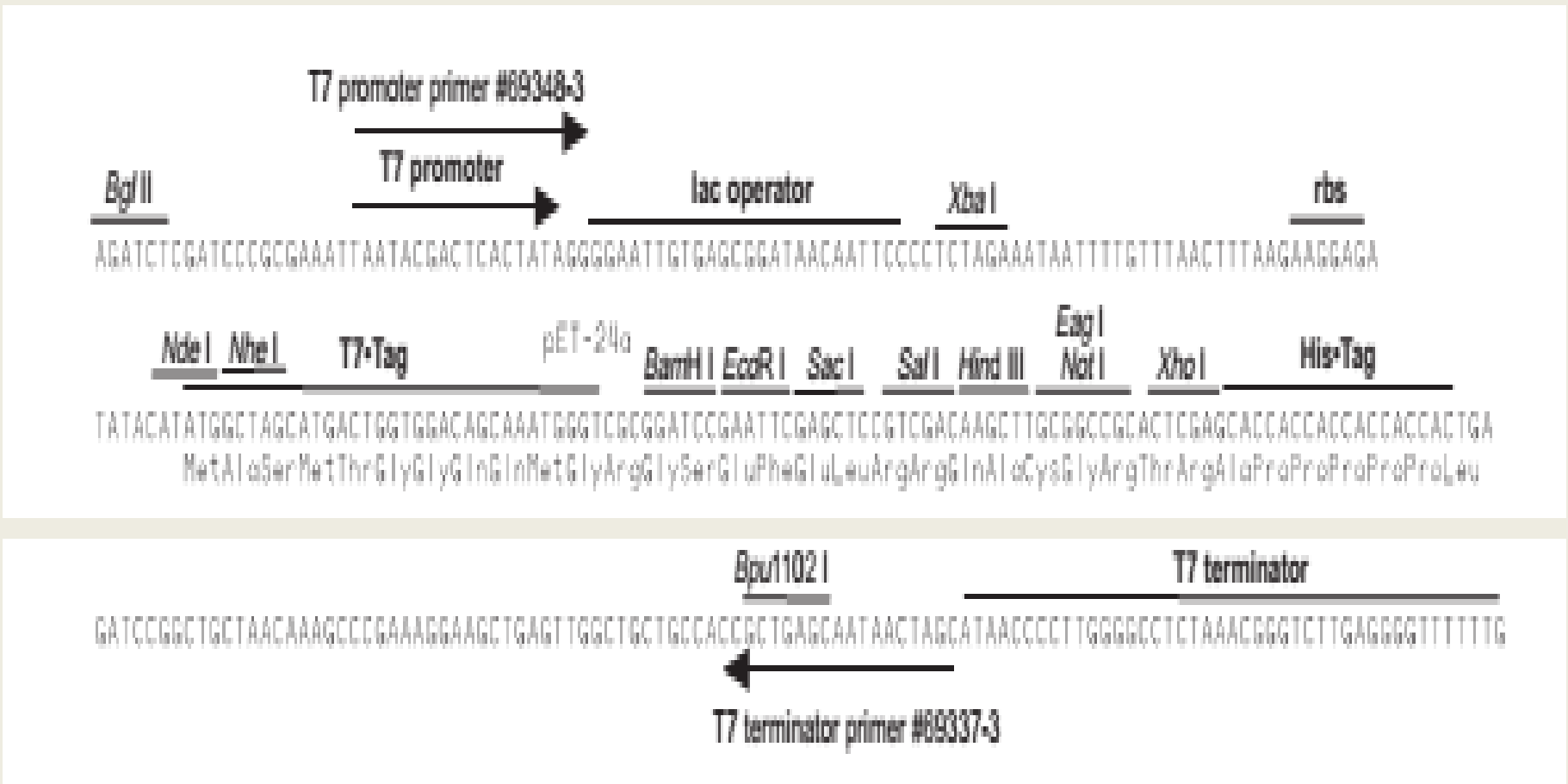
Multiple cloning site (MCS) site

- **A multiple cloning site (MCS) contains many restriction sites.**
- **These restriction sites are first cleaved by restriction enzymes and the target gene also, digested with the same enzymes → then ligated into the vectors using DNA ligase.**

Multiple Cloning Site (MCS)



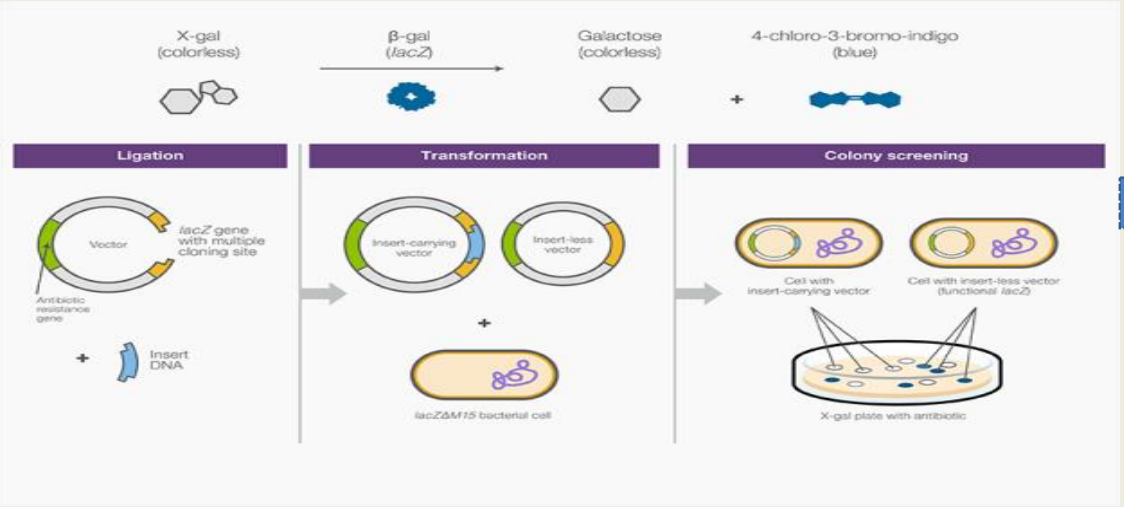
Multiple Cloning Site (MCS)



The features of all cloning vectors

4. A method of selecting for bacteria or yeast containing a vector with foreign DNA.

Usually accomplished by selectable markers for Reporter genes or/and drug resistance .



Reporter gene

- **Reporter genes are used in some cloning vectors to facilitate the screening of successful clones, the features of these genes allows successful clone to be easily identified.**
- **Such features present in cloning vectors may be the *lacZα* fragment for α complementation in blue-white selection.**

Reporter gene

- If the ligation was successful, the bacterial colony will be white; if not, the colony will be blue.
- The principle of this screening is based on the ability of β-galactosidase to produce a blue cleavage product from an artificial chromogenic substrate, The presence of an active β-galactosidase can be detected by X-gal, a colourless analog of lactose that may be cleaved by β-galactosidase.
- This results in a characteristic blue color in cells containing a functional β-galactosidase. Blue colonies therefore show that they may contain a vector with an uninterrupted *lacZα* (therefore no insert), while white colonies, where X-gal is not hydrolyzed, indicate the presence of an insert in *lacZα* which disrupts the formation of an active β-galactosidase.
- Introduction of a cloned DNA fragment into the multicloning site of the vector, which is embedded in the coding region of the *LacZ*α gene, disrupts the amino-terminal fragment of *β*-galactosidase, which is no longer capable of producing active β- galactosidase

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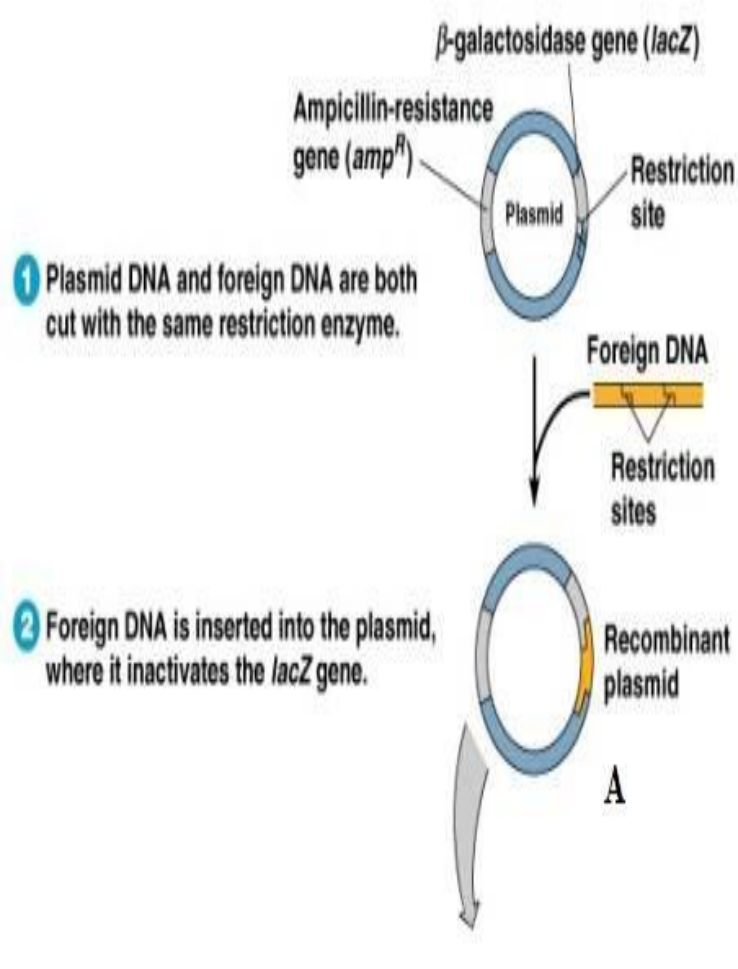


Selectable marker

A selectable marker is carried by the vector to allow the selection of positively transformed cells (cell with cloned vector).

1. Antibiotic resistance: For example: The beta-lactamase gene which confers resistance to the penicillin group of beta-lactam antibiotics like ampicillin.

The features of all cloning vectors

5. The replication origin (ORI) is a specific DNA sequence of 50 – 100 base pairs that must be present in a plasmid for its replication.

- **Host-cell enzymes bind to ORI, initiating replication of the circular plasmid.**
- **Once DNA replication is initiated at ORI, it continues around the circular plasmid regardless of its nucleotide sequence.**
- **Thus any DNA sequence inserted into such a plasmid is replicated along with the rest of the plasmid DNA.**

How can you choose the cloning Vector?

A large number of cloning vectors are available, and choosing the vector may depend a number of factors:

1. **The size of the insert.**
2. **Number of Copy needed.**
3. **Cloning method**

How can you choose the cloning Vector?

Large insert may not be stably maintained in a general cloning vector, especially for those with a high copy number, therefore cloning large fragments may require more specialized cloning vector.

Types of Cloning Vectors

1. Plasmid: An extra-chromosomal circular DNA molecule that autonomously replicates inside the bacterial cell; cloning limit to 100 to 10,000 base pairs. or 0.1-10 kilobases (kb).

(bp↔kb 1 kb = 1000 bp)

2. Phage: Derivatives of bacteriophage lambda; linear DNA molecules, this region can be replaced with foreign DNA without disrupting its life cycle; cloning limit to 8-24 kb.

Types of Cloning Vectors

3. Cosmids: An extra-chromosomal circular DNA molecule that combines features of plasmids and phage; cloning limit to 35-50 kb.

4. Bacterial Artificial Chromosomes (BAC).

5. Yeast Artificial Chromosomes (YAC).

6. Human Artificial Chromosomes (HAC).

7. Mouse Artificial Chromosomes (MACs)

Plasmids

- **Plasmids are circular, double-stranded DNA (dsDNA) molecules that are separate from a cell's chromosomal DNA.**
- **Plasmid is an autonomously replicating circular extra-chromosomal DNA.**

Plasmids

- **It occur naturally in bacteria, yeast, and some higher eukaryotic cells, exist in a parasitic or symbiotic relationship with their host cell.**
- **Plasmids range in size from a few thousand base pairs to more than 100 kilobases (kb).**
- **Like the host-cell chromosomal DNA, plasmid DNA is duplicated before every cell division.**

Plasmids

- **During cell division, at least one copy of the plasmid DNA is segregated to each daughter cell for assuring continued propagation of the plasmid through successive generations of the host cell.**
- **Many naturally occurring plasmids contain genes that provide some benefit to the host cell.**

Plasmids

- **For example, some bacterial plasmids encode enzymes that inactivate antibiotics.**
- **Such drug-resistance plasmids have become a major problem in the treatment of a number of common bacterial pathogens.**

Plasmids

- **Many of these plasmids also contain "transfer genes" encoding proteins that can form a macromolecular tube, or *pilus*, through which a copy of the plasmid can be transferred to other host cells of the same or related bacterial species. Such transfer can result in the rapid spread of drug-resistance plasmids.**

Plasmids As A cloning vector

They are the standard cloning vectors and the most commonly used

- **The plasmids most commonly used in recombinant DNA technology replicate in *E. coli*.**
- **Generally, these plasmids have been engineered (Not wild) to optimize their use as vectors in DNA cloning.**

Plasmids As A cloning vector

- **To simplify working with plasmids, <u>their length is reduced</u>; many plasmid vectors are only ≈3kb in length, which is much shorter than in naturally occurring *E. coli* plasmids.**

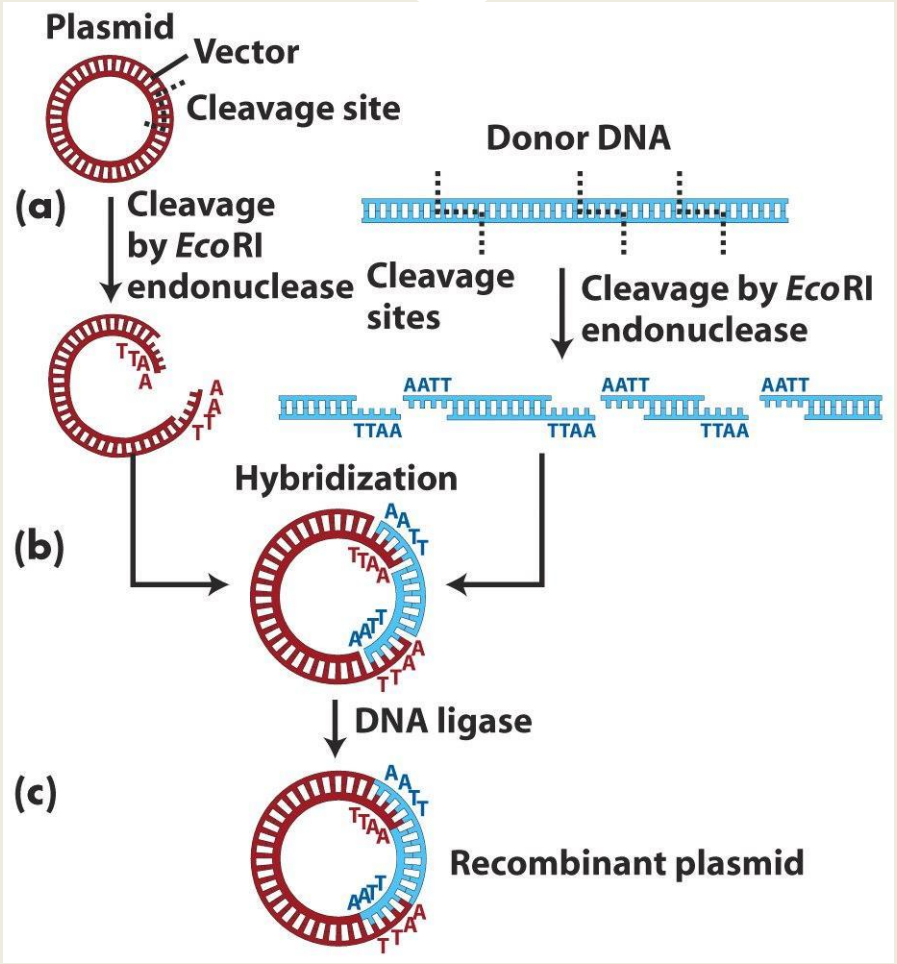
The circumference of plasmids usually is referred to as their "length," even though plasmids are almost always circular DNA molecules.

Plasmids As A cloning vector

- **Many plasmids have high copy number, for example pUC19 which has a copy number of 500-700 copies per cell and high copy number is useful as it produces greater yield of recombinant plasmid for subsequent manipulation.**

However low-copy-number plasmids may be preferably used in certain circumstances, for example, when the protein from the cloned gene is toxic to the cells.

Plasmids As A cloning vector



Bacteriophages As A cloning vector

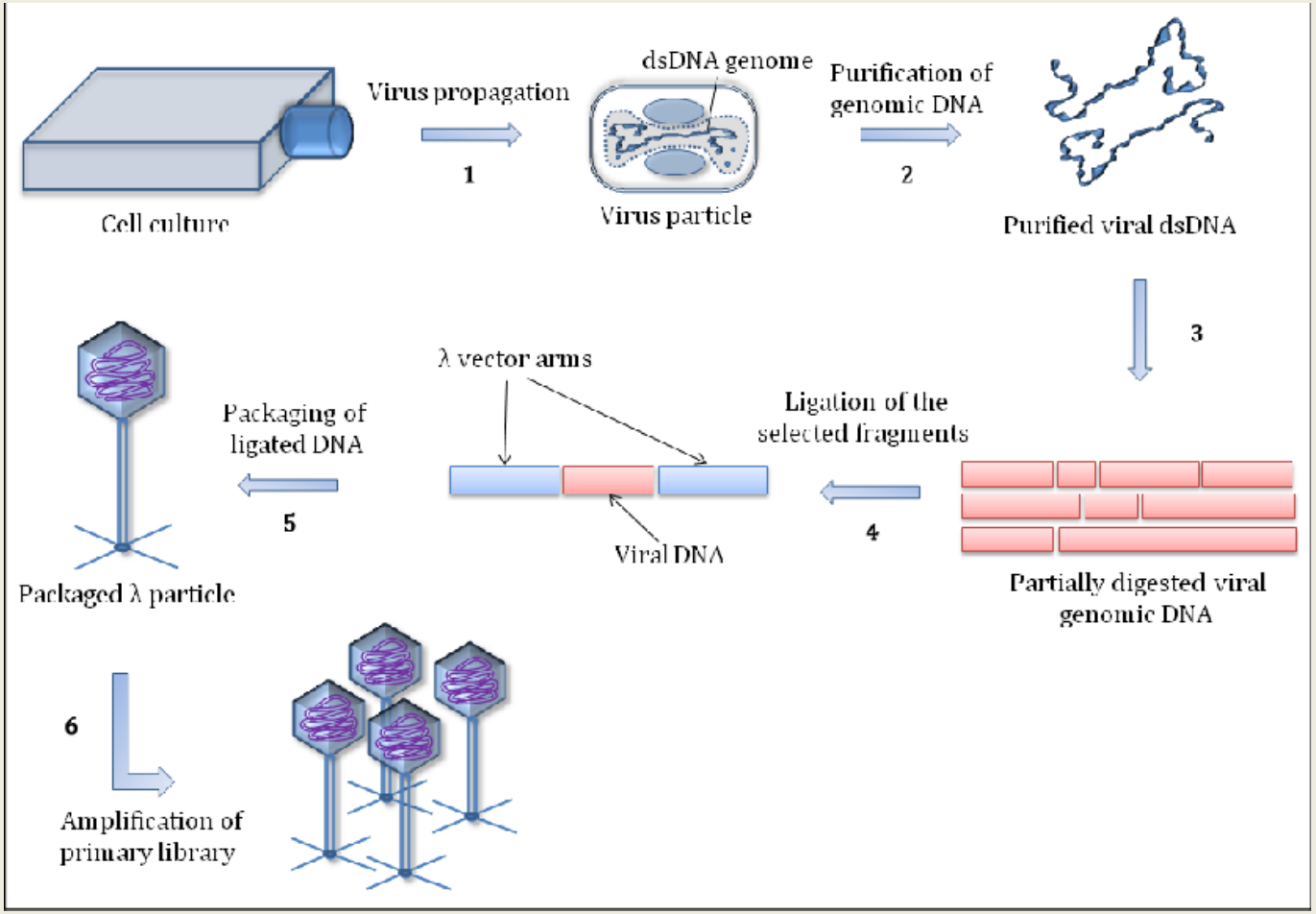
- **Bacteriophages used for cloning are the phage λ and M13 phage.**
- **There is an upper limit on the amount of DNA that can be packed into a phage (a maximum of 25: 53 kb).**
- **To allow foreign DNA to be inserted into phage, phage cloning vectors need to have some non-essential genes deleted.**

Bacteriophages As A cloning vector

- **There are two kinds of λ phage vectors → insertion vector and replacement vector.**
- Insertion vectors: contain a unique cleavage site where foreign DNA with size of 5–11 kb may be inserted.
- In replacement vectors: the cleavage sites flank a region containing genes not essential for the lytic cycle, and this region may be deleted and replaced by the DNA insert in the cloning process, and a larger sized DNA of 8–24 kb may be inserted.

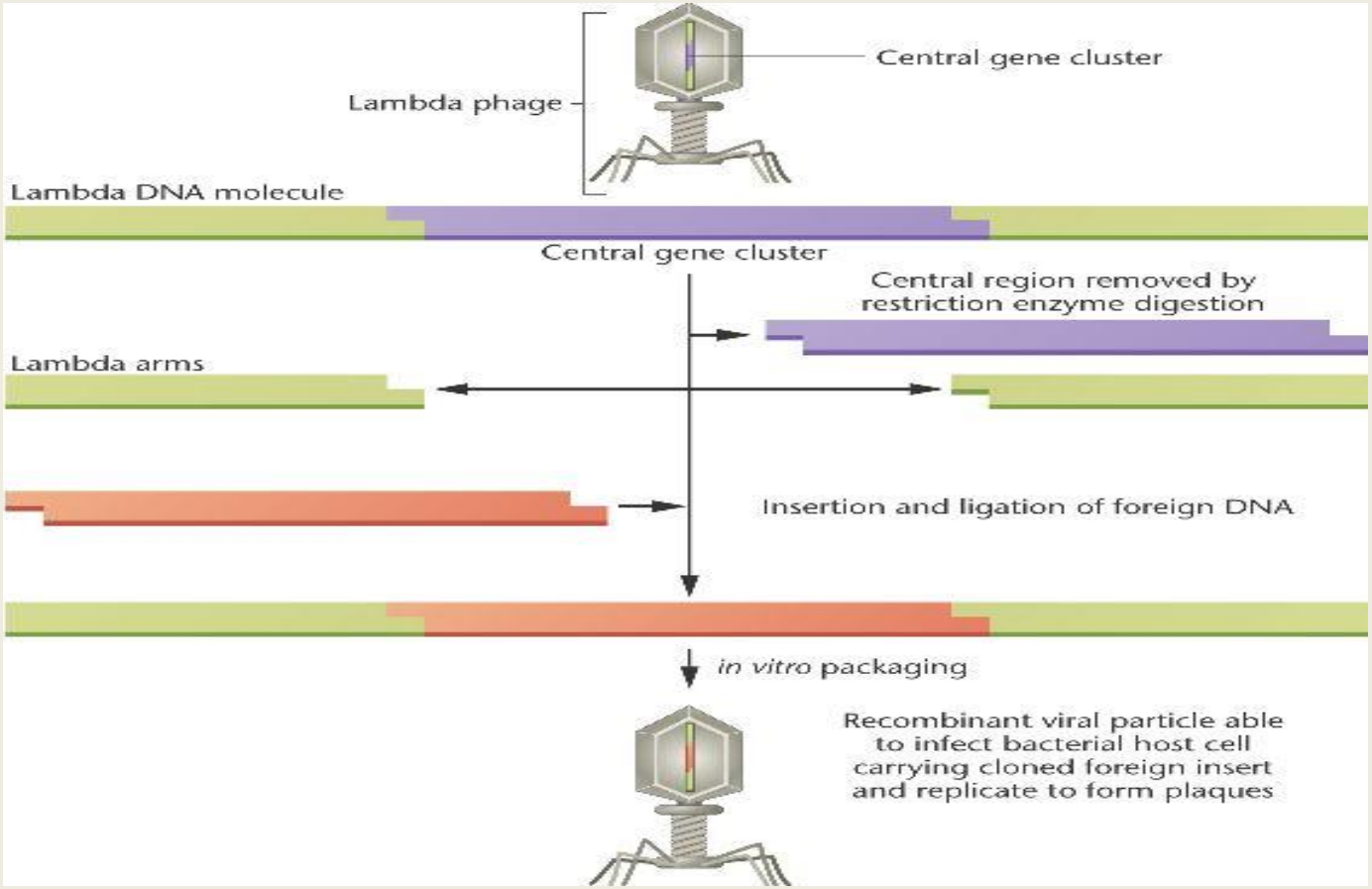
Bacteriophages As A cloning vector

Insertion vectors



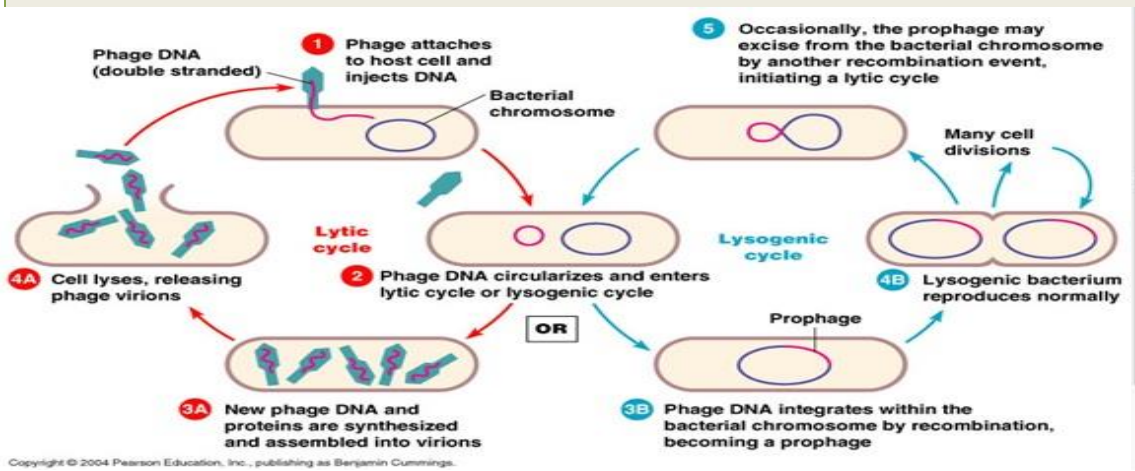
Bacteriophages As A cloning vector

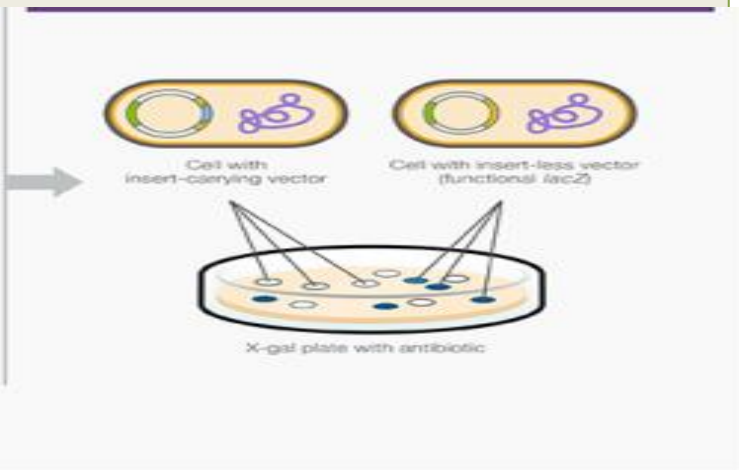
Replacement vectors



Difference between plasmid an bacteriophage

- **Plasmid vectors replicate along with their host cells.**
- **λ vectors replicate as lytic viruses, killing the host cell and packaging the DNA into virions.**





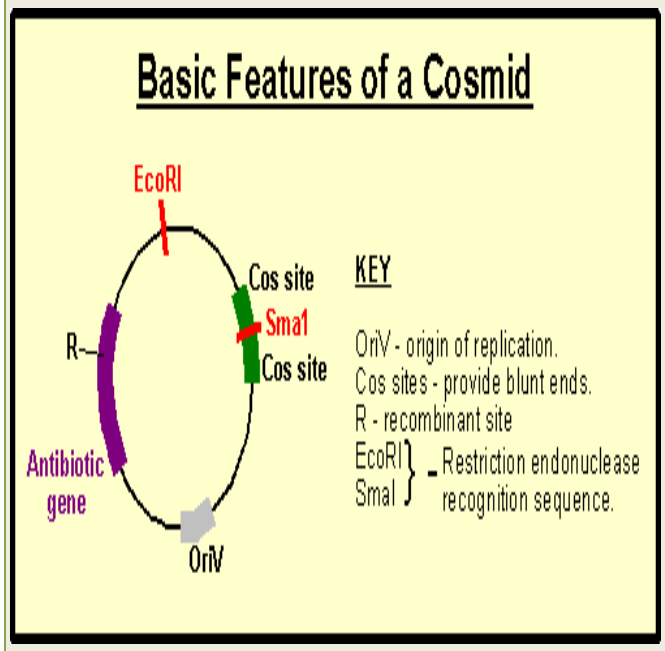
Cosmids as cloning vectors

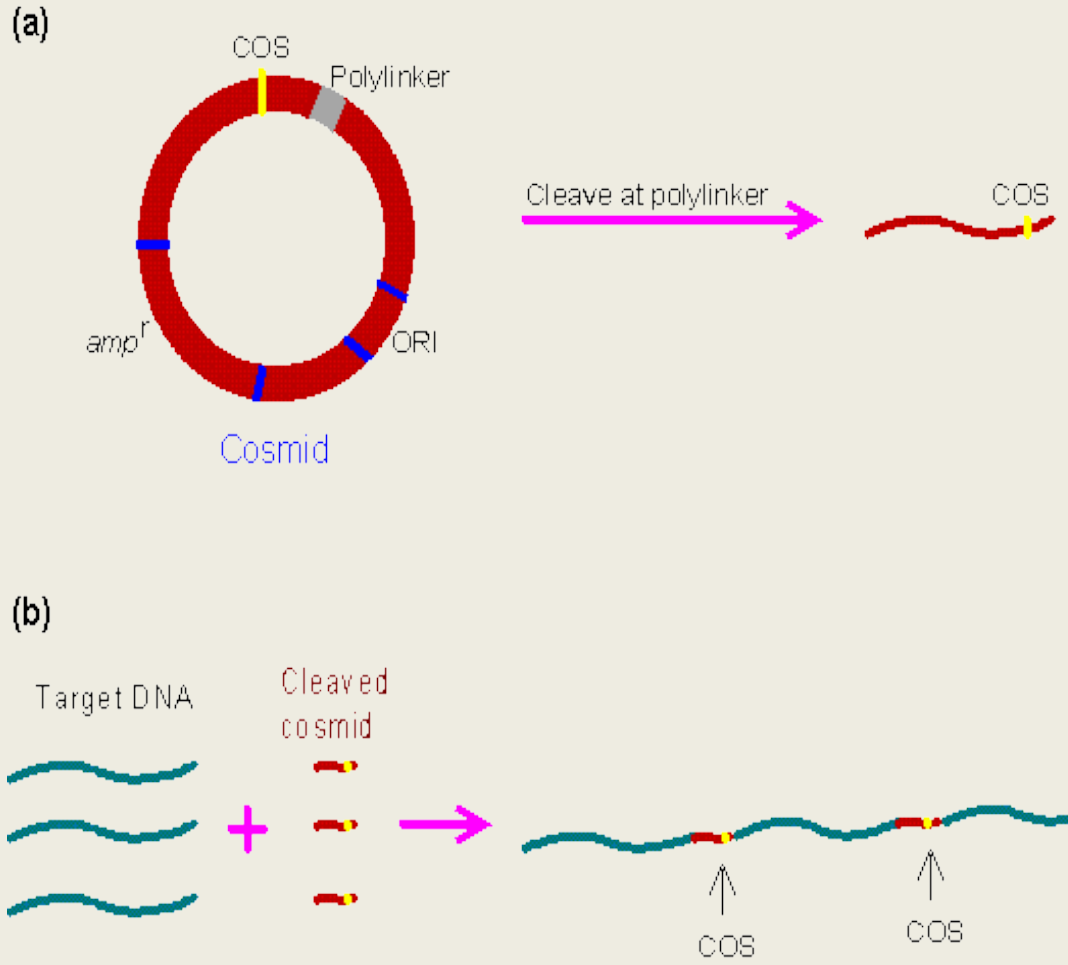
- **Cosmids are plasmids that incorporate a segment of bacteriophage λ DNA that has the cohesive end site (COS) which contains elements required for packaging DNA into λ head particles.**
- **It is normally used to clone large DNA fragments between 28 to 45 Kb.**

Cosmids as cloning vectors

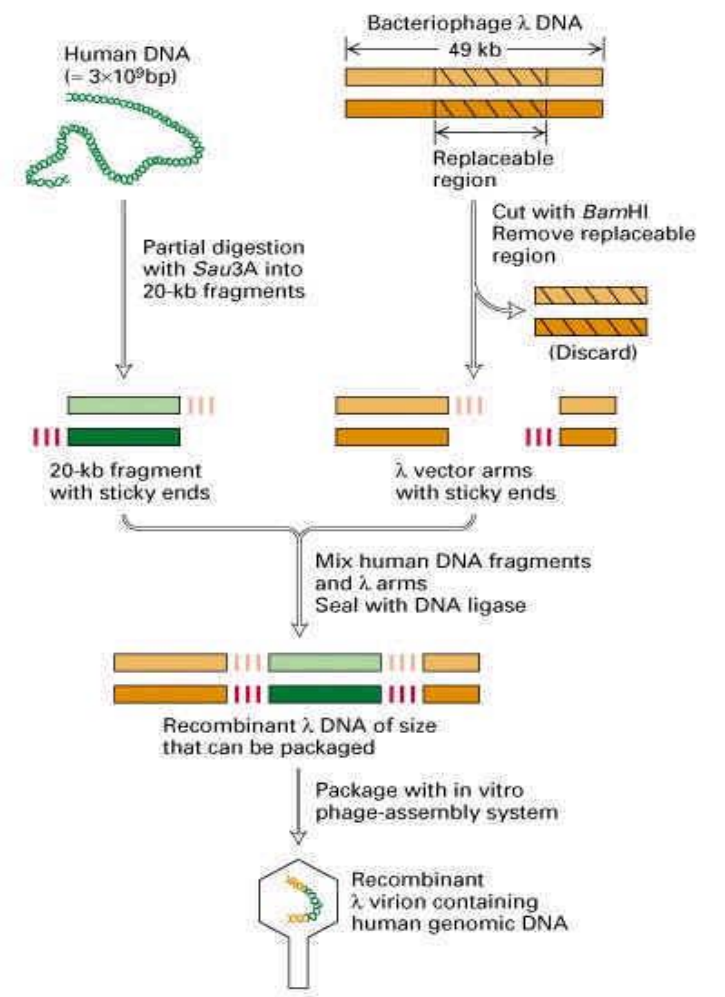
- **It is a cloning vector consisting of the phage cos site inserted into a plasmid i.e. simply plasmid and cos sites.**
- **cos site is one of the cohesive, single stranded extensions present at the ends of the DNA molecules of certain strains of lamda phage.**

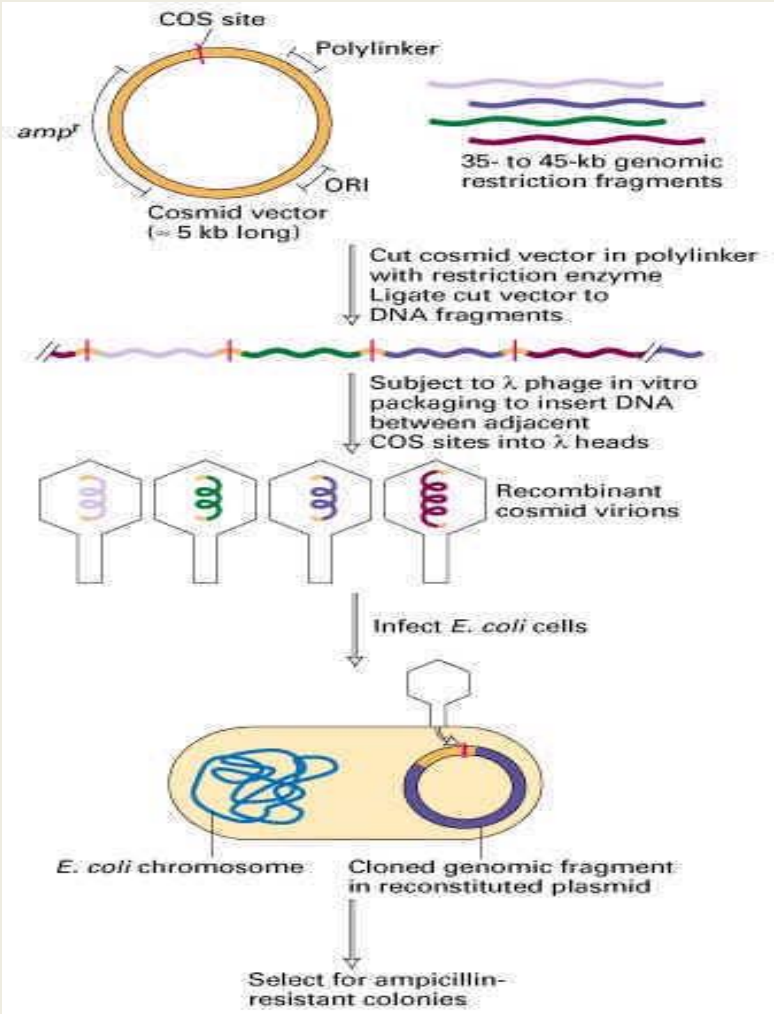
Cosmid


Basic Features of a Cosmid
EcoRI
Cos site
SmaI
Cos site
R
Antibiotic gene
OriV
KEY
OriV - origin of replication.
Cos sites - provide blunt ends.
R - recombinant site
EcoRI
SmaI
Restriction endonuclease recognition sequence.


(a)
COS
Polylinker
ampr
ORI
Cosmid
Cleave at polylinker
COS
(b)
Target DNA
Cleaved cosmid
COS
COS

Cosmid Vs. Bacteriophage





Cosmids as cloning vectors

Development of the cosmid vector is to overcome the disadvantages of plasmids and phage vectors:-

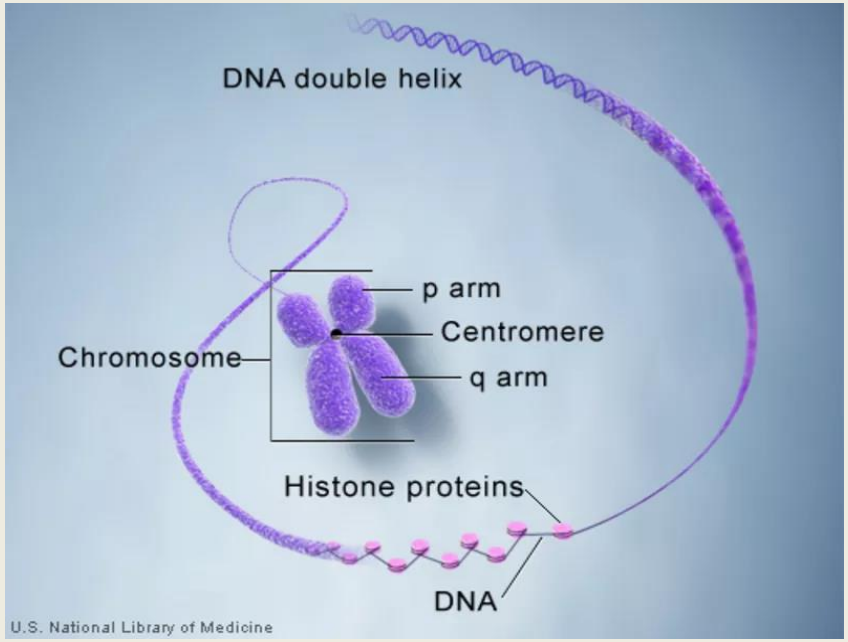
1. **The low copy number property of plasmids overcome because of cos sites.**
2. **The lysis of culture by the phage virus overcome because the vector does not have sequence for the functional phage production.**

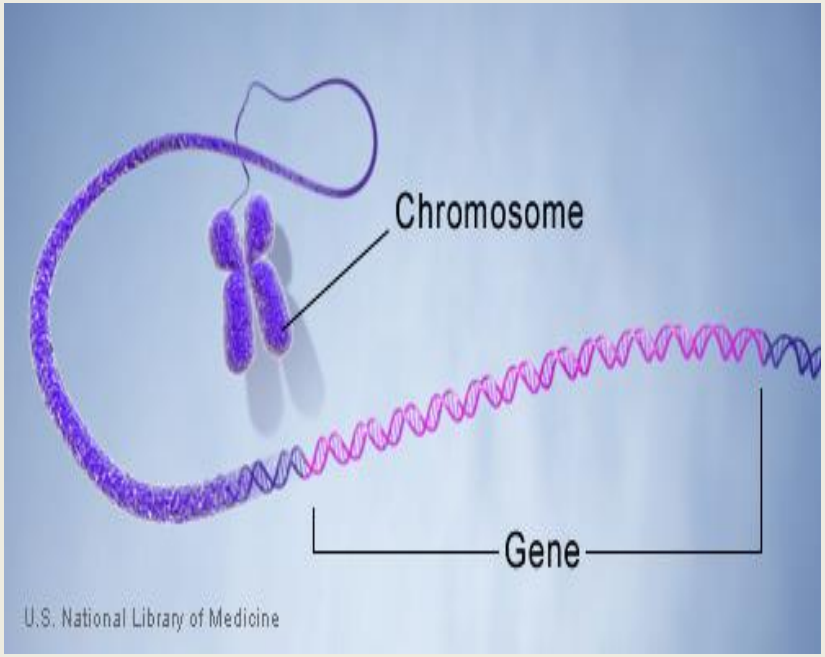
Artificial chromosomes

Artificial chromosomes were first assembled in budding yeast and have since been useful in many aspects of yeast genetics.

Several attempts have been made at building artificial chromosomes in mammals, although these have been met with limited success. Consequently, mini-chromosomes of defined structure have been developed to address questions regarding mammalian chromosome function and for biotechnological applications.

Artificial chromosome





Bacterial artificial chromosome

- **Bacterial artificial chromosome:**

Insert size of up to 350 kb can be cloned in bacterial artificial chromosome (BAC).

BACs are maintained in *E. coli* with a copy number of only one per cell.

Bacterial artificial chromosome (BAC)

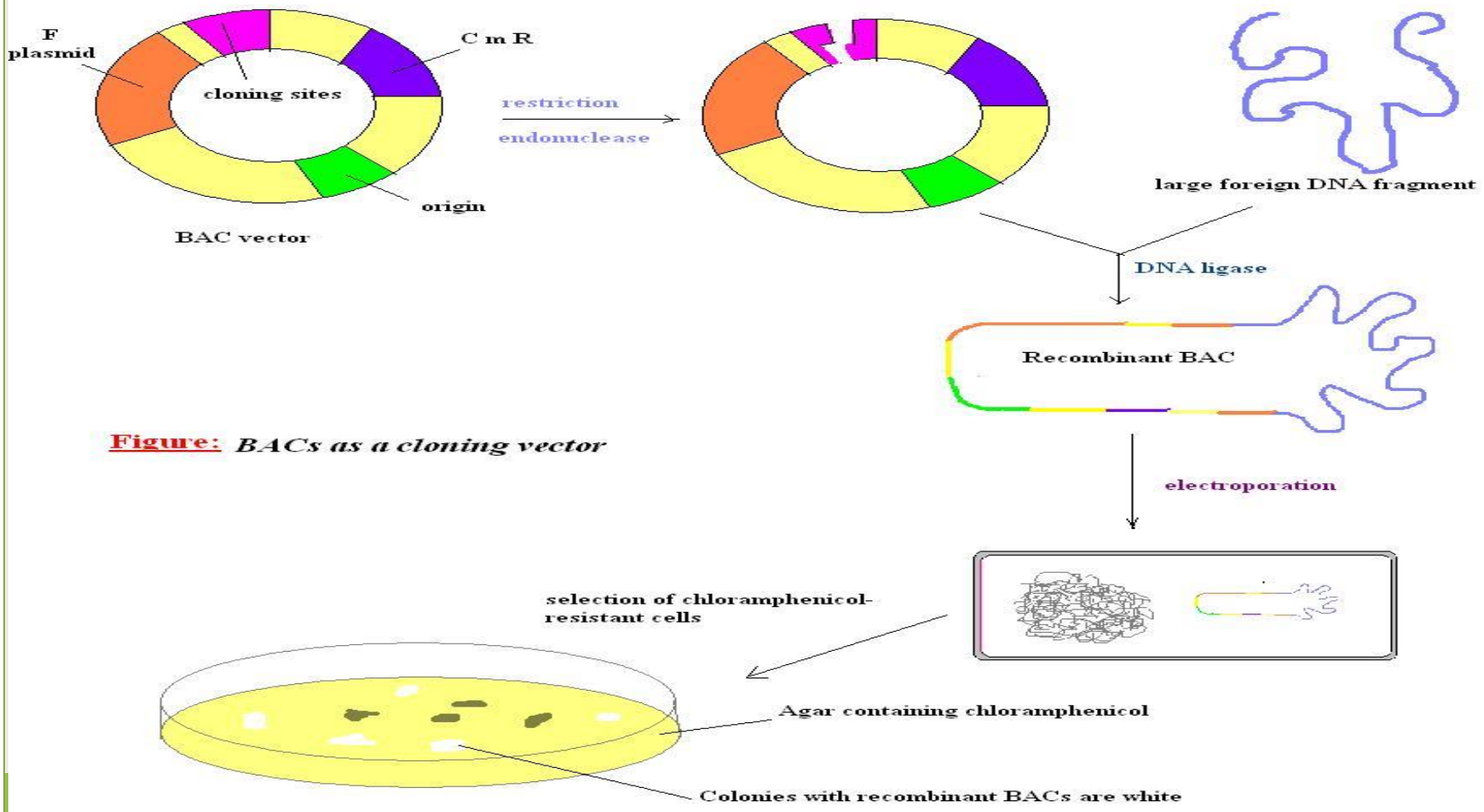
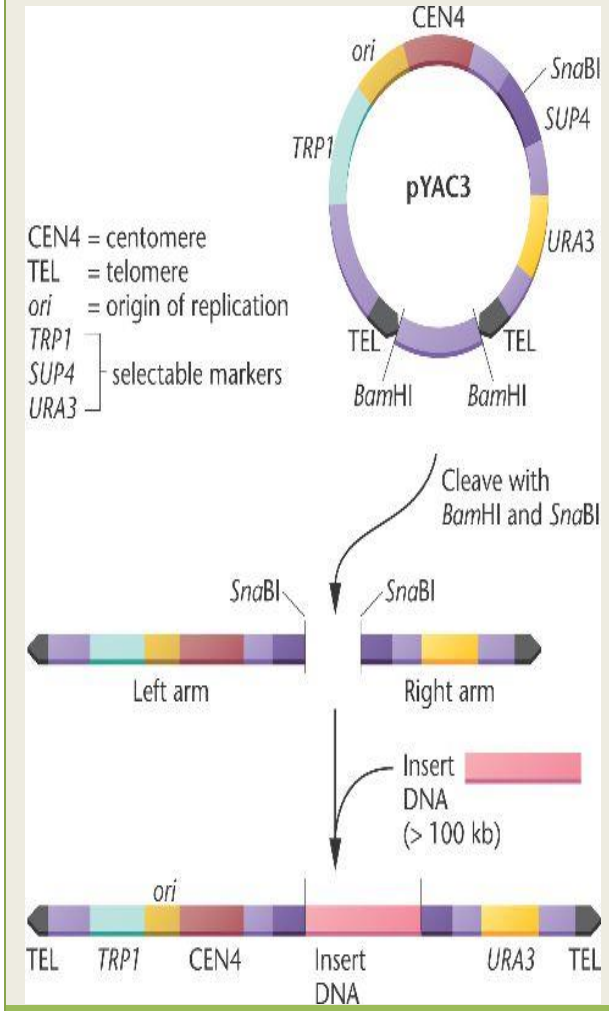


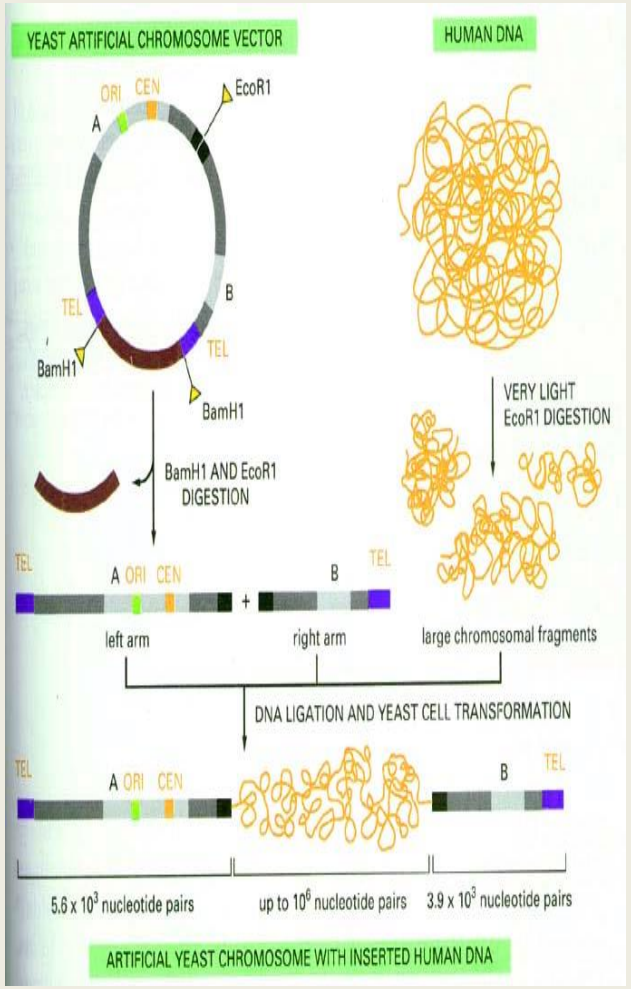
Figure: *BACs as a cloning vector*

Yeast artificial chromosome

- **Yeast artificial chromosome (YAC): It is genetically engineered chromosomes derived from the DNA of the yeast.**
- **Insert of up to 3,000 kb may be carried by yeast artificial chromosome.**
- **This is the process that was initially used for the Human Genome Project, however due to stability issues, YACs were abandoned for the use of Bacterial artificial chromosomes (BAC).**

Yeast artificial chromosome





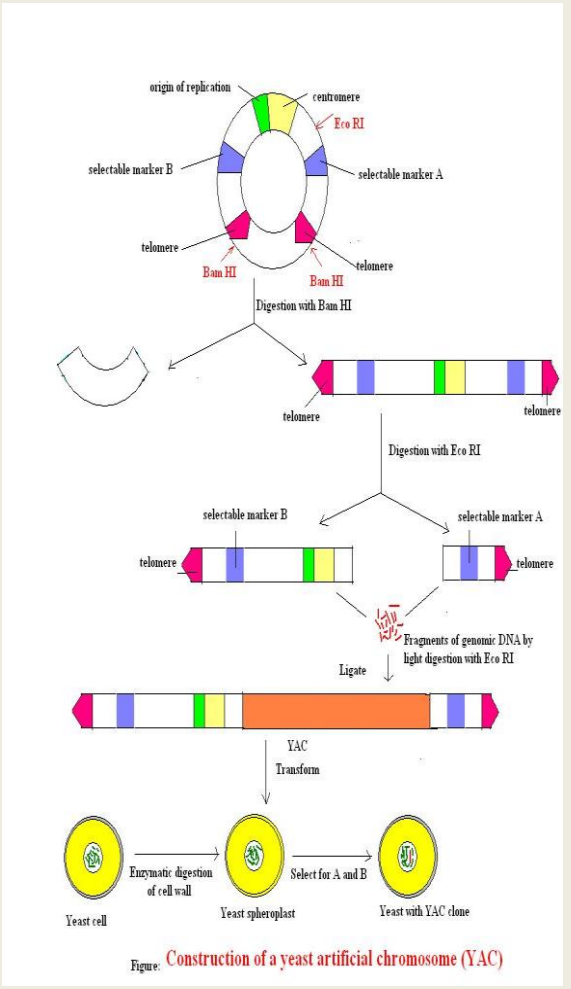
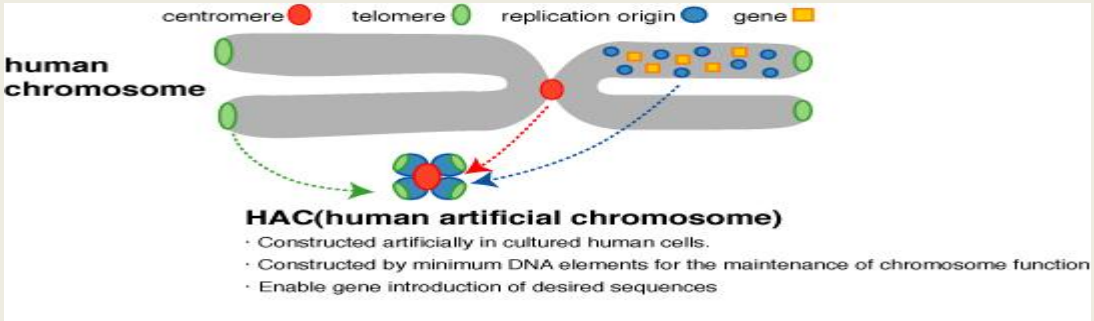


Figure: Construction of a yeast artificial chromosome (YAC)

Human artificial chromosome

- **A human artificial chromosome (HAC) is a microchromosome that can act as a new chromosome in human cells.**
- **That is, instead of 46 chromosomes, the cell could have 47 with the 47th being very small.**



Human artificial chromosome

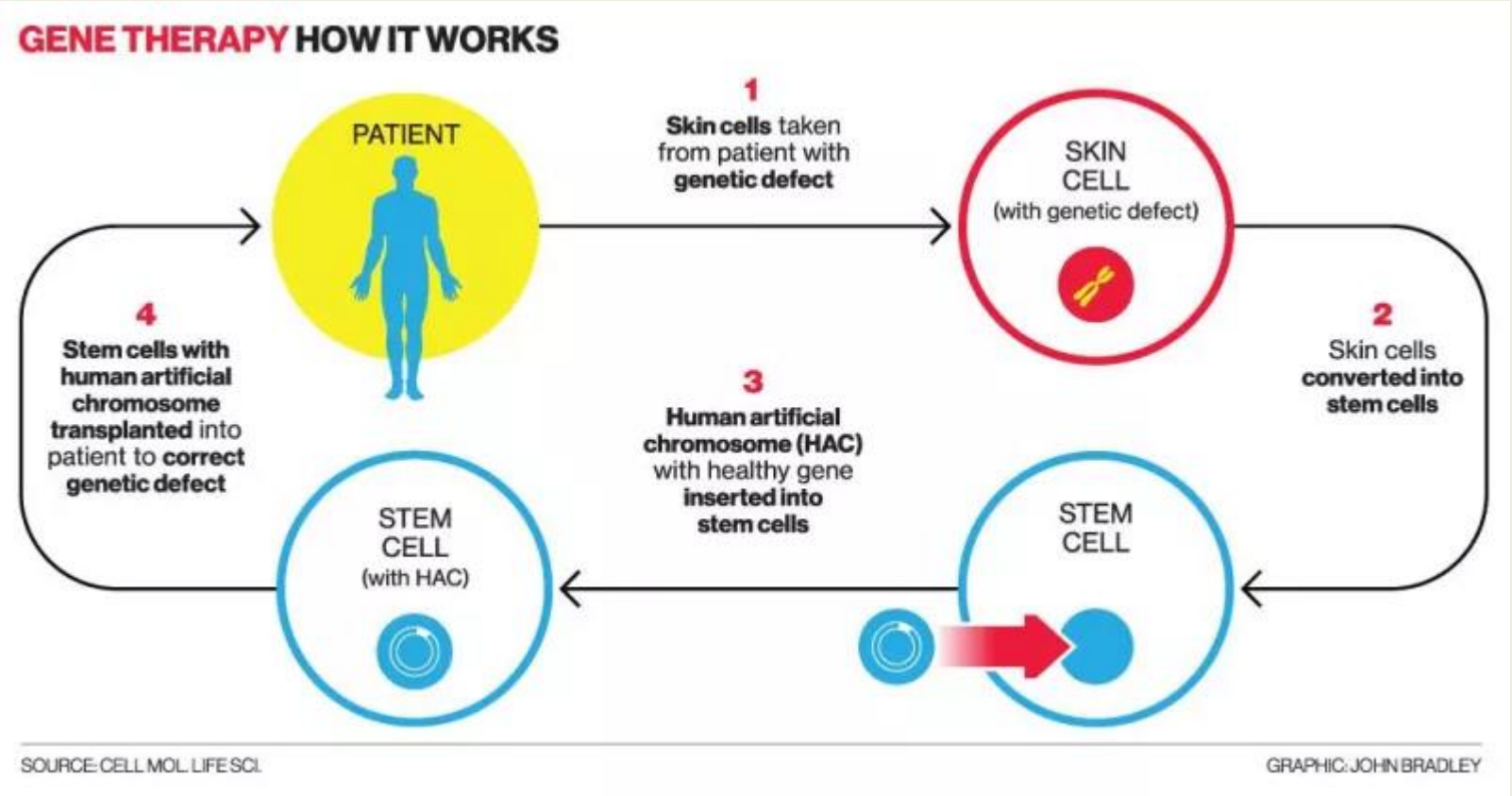
- **Human artificial chromosome (HAC):** is potentially useful as a gene transfer vectors for gene delivery into human cells, a tool for expression studies and determining human chromosome function.
- It can carry very large DNA fragment (there is no upper limit on size for practical purposes).
- It also avoids possible insertional mutagenesis caused by integration into host chromosomes by viral vector.

Human artificial chromosome

Alternative methods of creating transgenes, to over come the problems caused by yeast artificial chromosomes and bacterial artificial chromosomes that lead to unpredictable problems.

The genetic material introduced by these vectors not only leads to different expression levels, but the inserts also disrupt the original genome

Human artificial chromosome



Advantages of AHCs

1. **All HACs by definition contain a functional centromere that enables the long-term stable maintenance of HACs as single copy episomes without integration into the host chromosomes.**
2. **<u>Secondly,</u> there is no upper size limit to DNA cloned in a HAC, Indeed, not only single genes but groups of genes encoding complex pathways can be carried on a single HAC.**

Advantages of AHCs

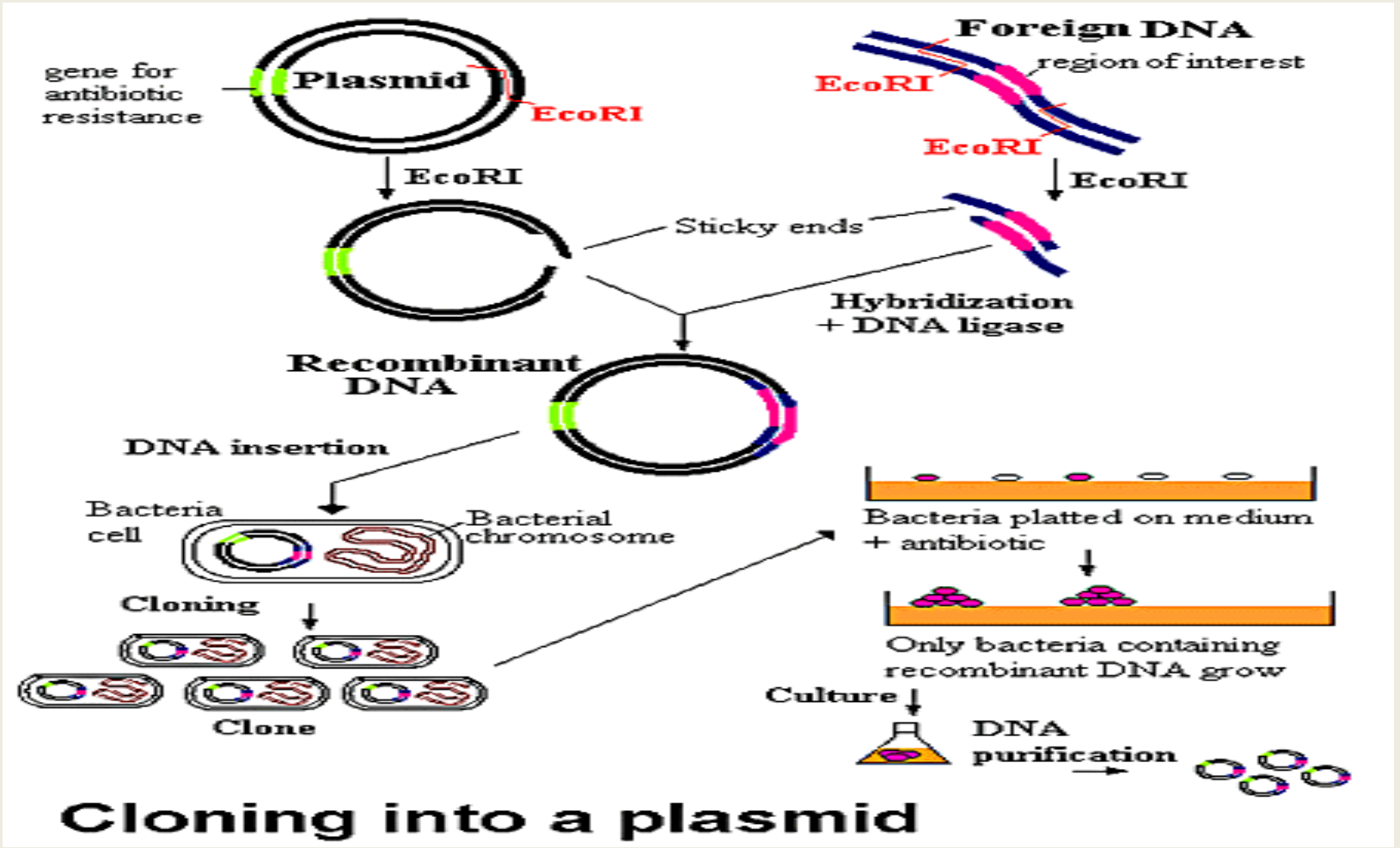
3. Thirdly, the HACs can be transferred from one cell to another.

4. Finally, because of the lack of viral sequences, HAC vectors minimize adverse host immunogenic responses and the risk of cellular transformation.

General Steps of Cloning with Any Vector

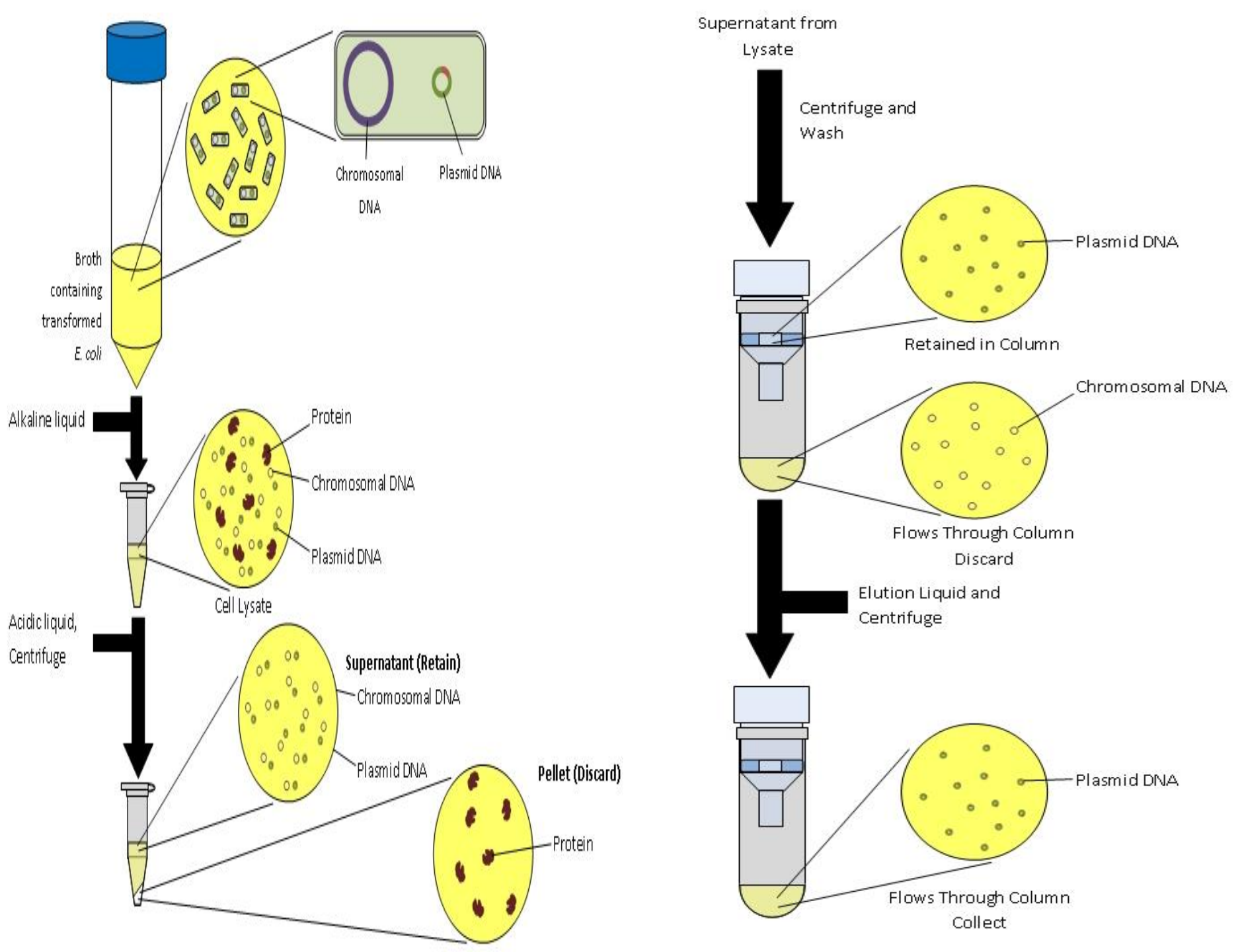
1. **Prepare the vector and DNA to be cloned by the same restriction enzymes to generate complementary ends.**
2. **Ligate the foreign DNA into the vector with the enzyme DNA ligase.**
3. **Introduce the cloned vector into bacterial cells by transformation.**
4. **Select cells containing foreign DNA by screening for selectable markers (usually drug resistance).**

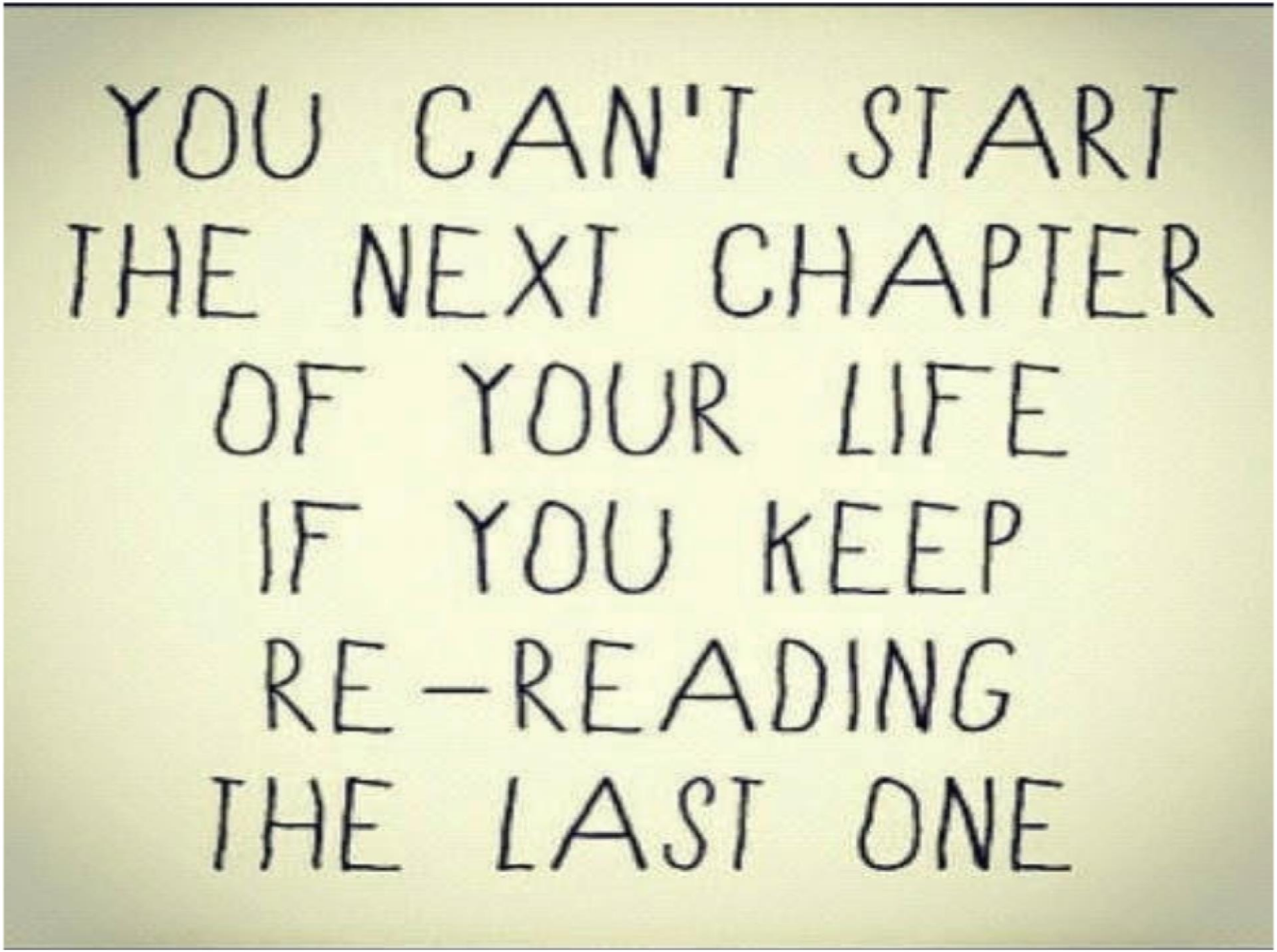
General Steps of Cloning with Any Vector



Plasmid isolation

Plasmids may be easily isolated by cell lysis followed by precipitation of proteins, which traps chromosomal DNA in insoluble fraction and after centrifugation, plasmid DNA can be purified from soluble fraction.


Chromosomal DNA
Plasmid DNA
Broth containing transformed E. coli
Alkaline liquid
Protein
Chromosomal DNA
Plasmid DNA
Cell Lysate
Acidic liquid, Centrifuge
Supernatant (Retain)
Chromosomal DNA
Plasmid DNA
Pellet (Discard)
Protein
Supernatant from Lysate
Centrifuge and Wash
Plasmid DNA
Retained in Column
Chromosomal DNA
Flows Through Column Discard
Elution Liquid and Centrifuge
Plasmid DNA
Flows Through Column Collect


YOU CAN'T START
THE NEXT CHAPTER
OF YOUR LIFE
IF YOU KEEP
RE-READING
THE LAST ONE

Expression vector

- **Expression vector has similar features like any vector.**
- **The cloned gene may be transferred from a specialized cloning vector to an expression vector, although it is possible to clone directly into an expression vector.**

Elements for expression

- **An expression vector must have elements necessary for protein expression.**
- **These may include a strong promoter, the correct translation initiation sequence such as a ribosomal binding site and start codon, a strong termination codon, and a transcription termination sequence.**

Applications

1. Laboratory use:

Expression vector in an expression host is now the usual method used in laboratories to produce proteins for research.

2. Production of peptide and protein pharmaceuticals:

Most protein pharmaceuticals are now produced through recombinant DNA technology using expression vectors (hormones, vaccines, antibiotics, antibodies, and enzymes).

Applications

3. Transgenic plant and animals:

Expression vectors have been used to introduce specific genes in organisms,

For example in agriculture it is used to produce transgenic plants, introduce vitamin A precursor, beta-carotene, into rice plants, this product is called golden rice.

Introduce a gene into plants that produces an insecticide, which reduces the need for farmers to apply insecticides.

Applications

4. Transgenic animals:

- Produced to study animal biochemical processes and human diseases.
- Green fluorescent protein is sometimes used as tags which results in animal that can fluoresce, and this have been exploited commercially to produce the fluorescent GloFish.

5. Gene therapy is a promising treatment for a number of diseases where a “Normal" gene carried by the vector is inserted into the genome, to replace an “Abnormal" gene or supplement the expression of particular gene.

Thanks a lot

with my Best Regards and My Best wishes

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