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.
- Cleavage of human DNA with restriction enzymes that produce about one cut for every 3000 base pairs yields some 2 million fragments, far too many to separate from each other directly.

DNA Cloning with Cloning Vectors

- This obstacle to obtaining pure DNA samples from large genomes has been overcome by recombinant DNA technology.
- With this method any gene can be purified.
- Its sequence determined, the functional regions of the sequence explored by altering it in planned ways and reintroducing the DNA into cells and into whole organisms.

DNA Cloning with Cloning Vectors

- The recombinant DNA technology is the preparation of large numbers of identical DNA molecules.
- A 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.
- When a single recombinant DNA molecule, composed of a vector plus an inserted DNA fragment, is introduced into a host cell, 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.

Cloning Vectors

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

- **Cloning vector** is a DNA molecule that carries foreign DNA into a host cell, replicates inside a bacterial or yeast cell and produces many copies of itself and the foreign DNA.

Cloning Vectors

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

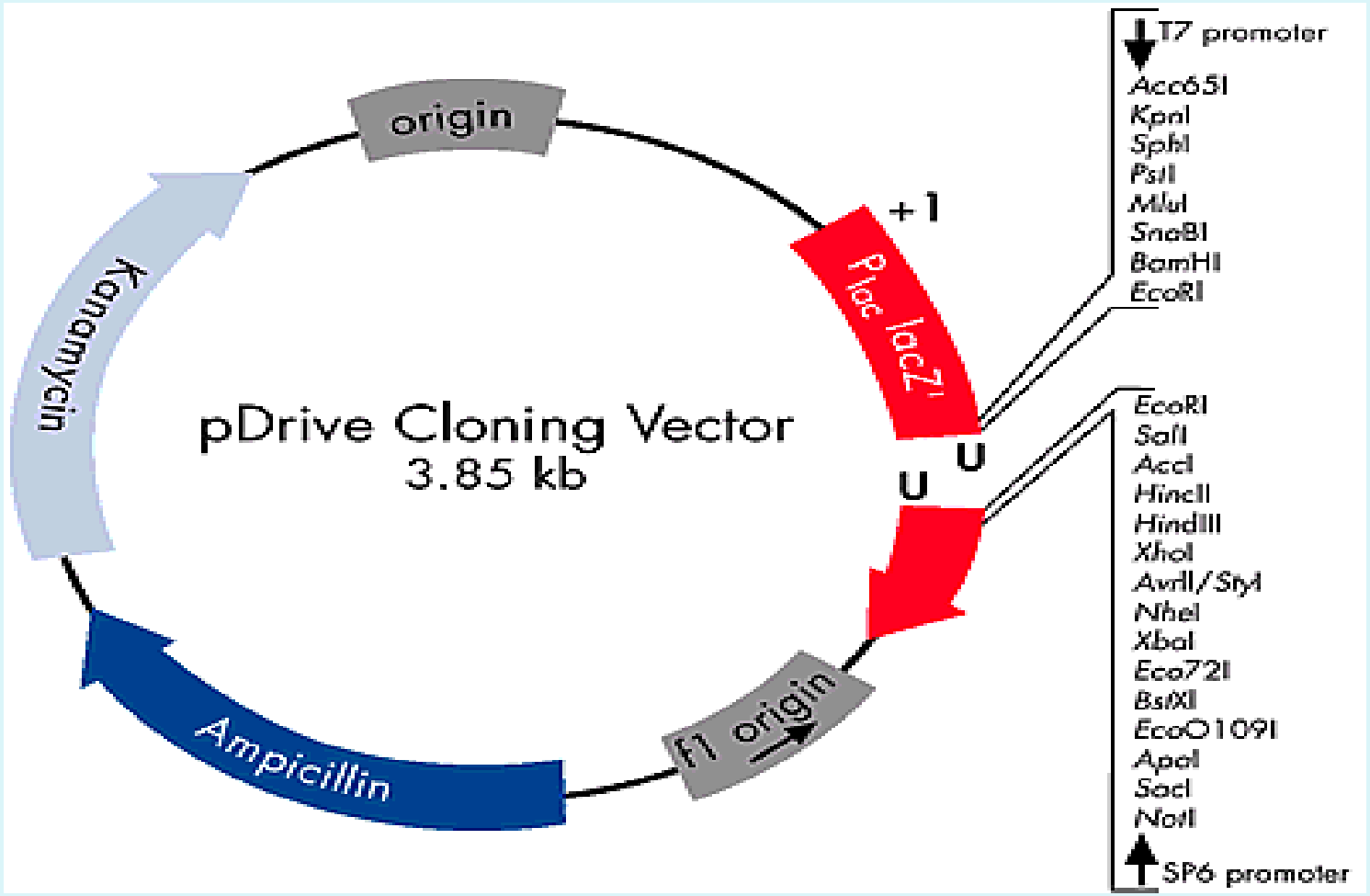
Three features of all cloning vectors

1. Small in size.
2. Sequences that permit the propagation of itself in bacteria or in yeast(The replication origin).
3. A cloning site to insert foreign DNA; the most versatile vectors contain a site that can be cut by many restriction enzymes.

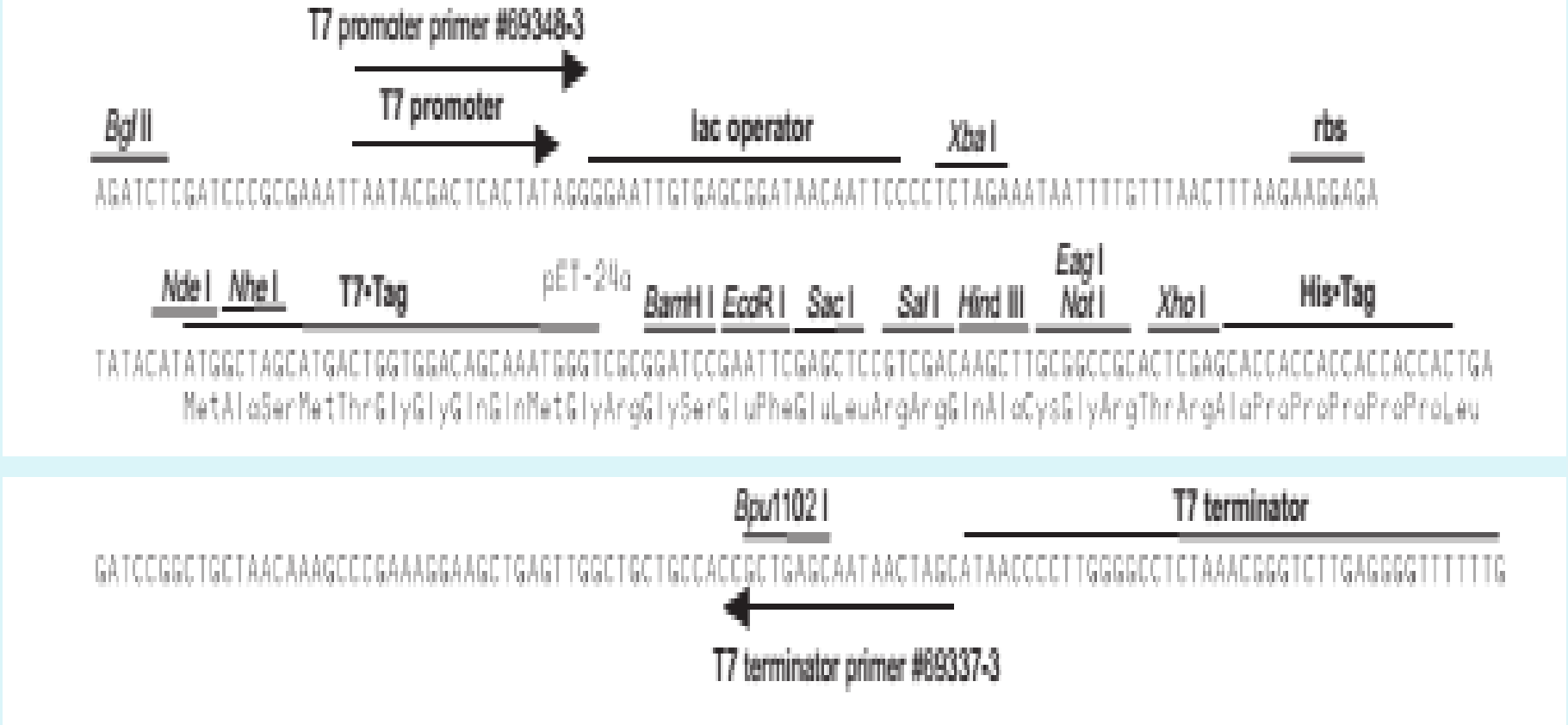
Multiple Cloning Site (MCS)

3. A method of selecting for bacteria or yeast containing a vector with foreign DNA; usually accomplished by selectable markers for drug resistance or/and Reporter genes.

Cloning vector



Cloning vector



The replication origin (ORI)

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

Cloning multiple cloning site (MCS) site

- A multiple cloning site (MCS) which contains many unique restriction sites.
- The restriction sites in the MCS are first cleaved by restriction enzymes, and a PCR-amplified target gene, also digested with the same enzymes, is then ligated into the vectors using DNA ligase.

Selectable marker

- A selectable marker is carried by the vector to allow the selection of positively transformed cells.
- Antibiotic resistance is often used as marker, an example is the beta-lactamase gene which confers resistance to the penicillin group of beta-lactam antibiotics like ampicillin.

Reporter gene

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

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

TABLE 4.2 Copy numbers of some plasmids

Plasmid	Approximate copy number
F	1
P1 prophage	1
RK2	4–7 (in *E. coli*)
pBR322	16
pUC18	~30–50
pIJ101	40–300

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

- **Plasmid** - an extrachromosomal circular DNA molecule that autonomously replicates inside the bacterial cell; cloning limit: 100 to 10,000 base pairs or 0.1-10 kilobases (kb)
- **Phage** - derivatives of bacteriophage lambda; linear DNA molecules, whose region can be replaced with foreign DNA without disrupting its life cycle; cloning limit: 8-20 kb
- **Cosmids** - an extrachromosomal circular DNA molecule that combines features of plasmids and phage; cloning limit - 35-50 kb

Types of Cloning Vectors

- **Bacterial Artificial Chromosomes (BAC).**
- **Yeast Artificial Chromosomes (YAC)**

an artificial chromosome that contains telomeres, origin of replication, a yeast centromere, and a selectable marker for identification in yeast cells; cloning limit: 100-1000 kb.

- **Human Artificial Chromosomes (HAC).**

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.
- 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).

Plasmids

- Like the host-cell chromosomal DNA, plasmid DNA is duplicated before every cell division.
- During cell division, at least one copy of the plasmid DNA is segregated to each daughter cell, 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, fulfilling the plasmid's portion of the symbiotic relationship.

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.
- As antibiotic use became widespread, plasmids containing several drug-resistance genes evolved, making their host cells resistant to a variety of different antibiotics simultaneously.

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, expanding the number of antibiotic-resistant bacteria in an environment such as a hospital.

Plasmids

- 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 to optimize their use as vectors in DNA cloning.
- To simplify working with plasmids, their length is reduced; 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

- 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.

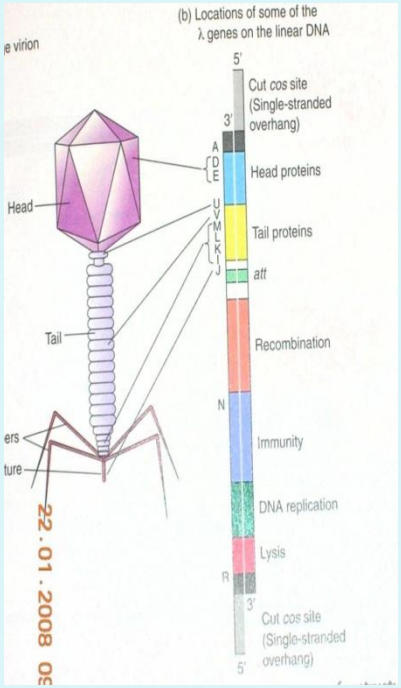
Bacteriophages

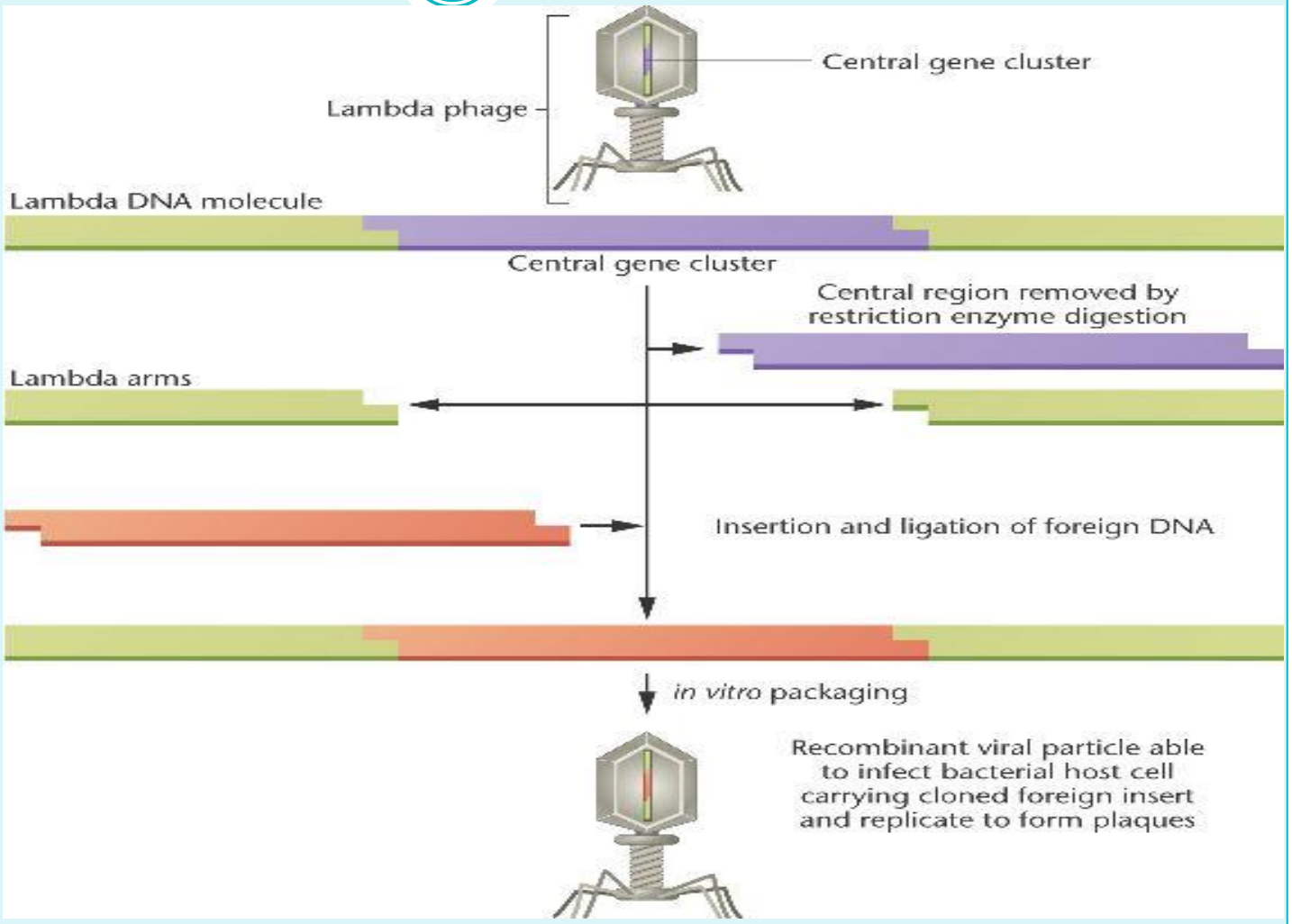
- The bacteriophages used for cloning are the phage λ and M13 phage.
- There is an upper limit on the amount of DNA that can be packed into a phage (a maximum of 53 kb).
- To allow foreign DNA to be inserted into phage, phage cloning vectors need to have some non-essential genes deleted.
- There are two kinds of λ phage vectors → insertion vector and replacement vector.

Bacteriophages

- Insertion vectors contain a unique cleavage site whereby 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





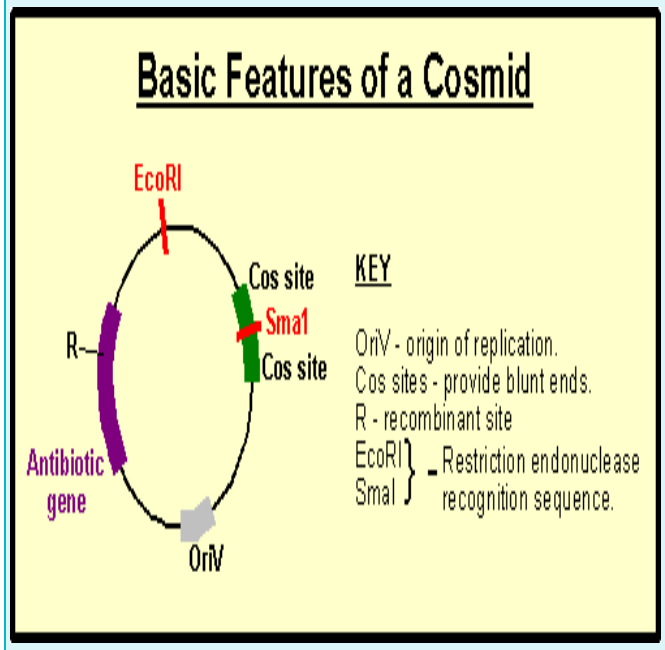
Difference between plasmid and bacteriophage

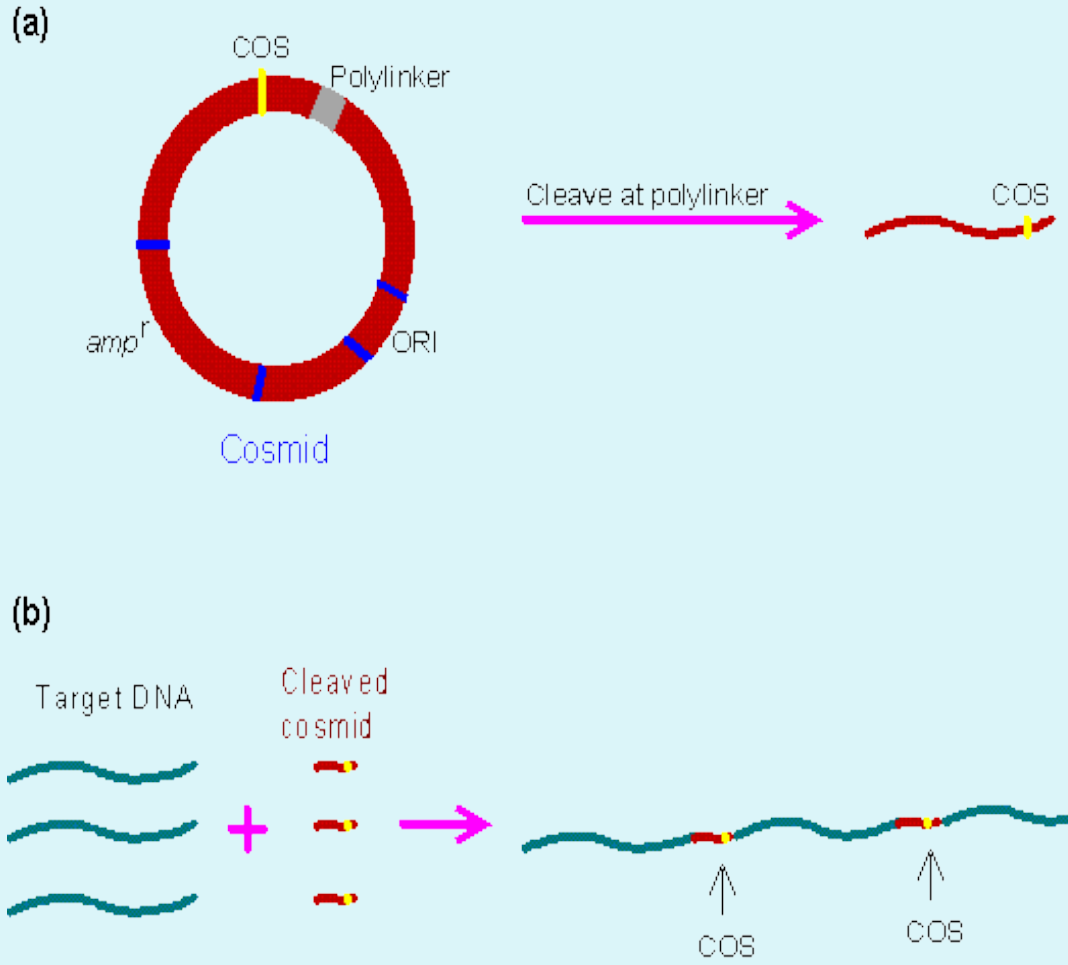
- Plasmid vectors replicate along with their host cells.
- λ vectors replicate as lytic viruses, killing the host cell and packaging the DNA into virions.

Cosmid, Bacterial artificial chromosome

- **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. (?)
- **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 1 per cell.

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

Bacterial artificial chromosome (BAC)

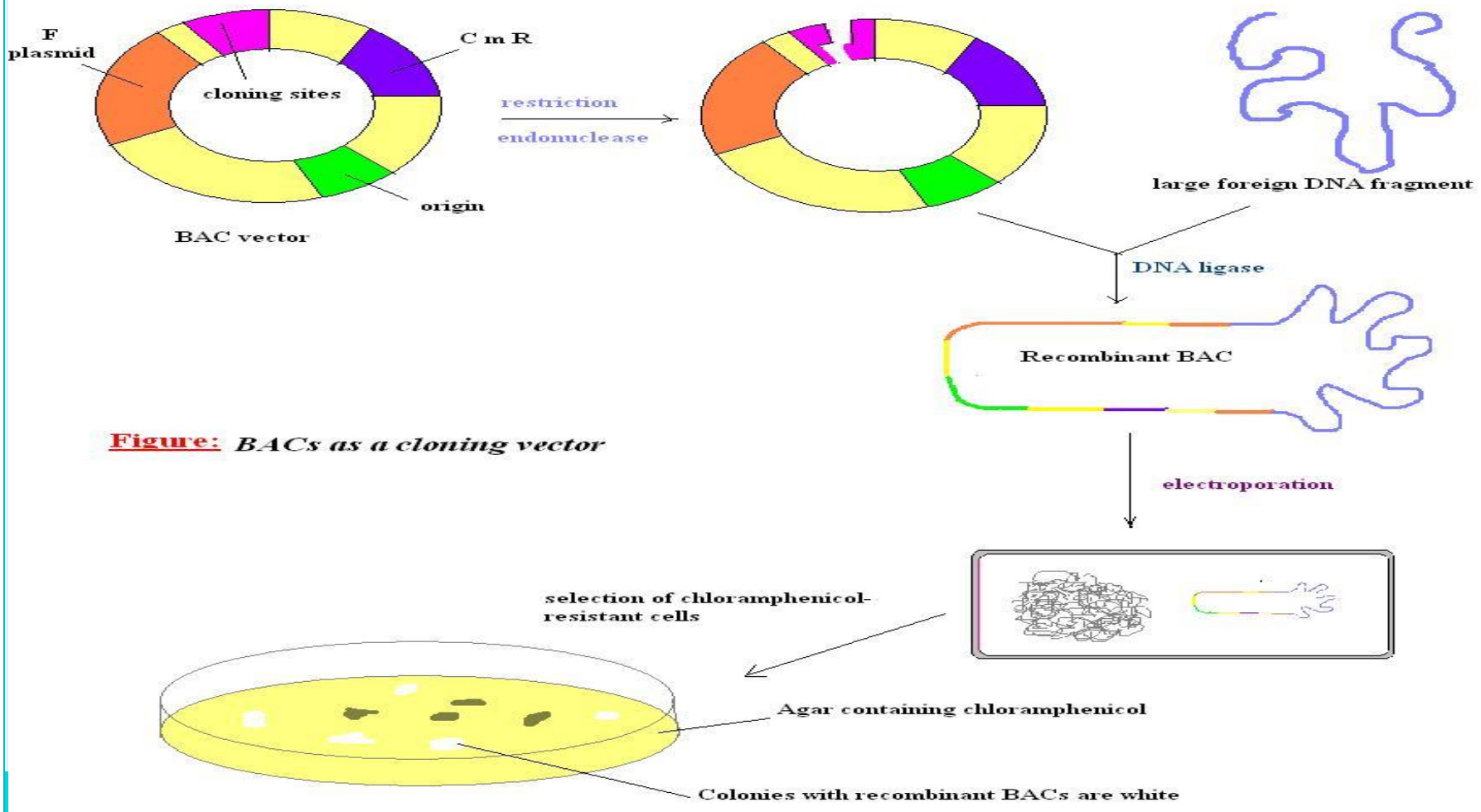
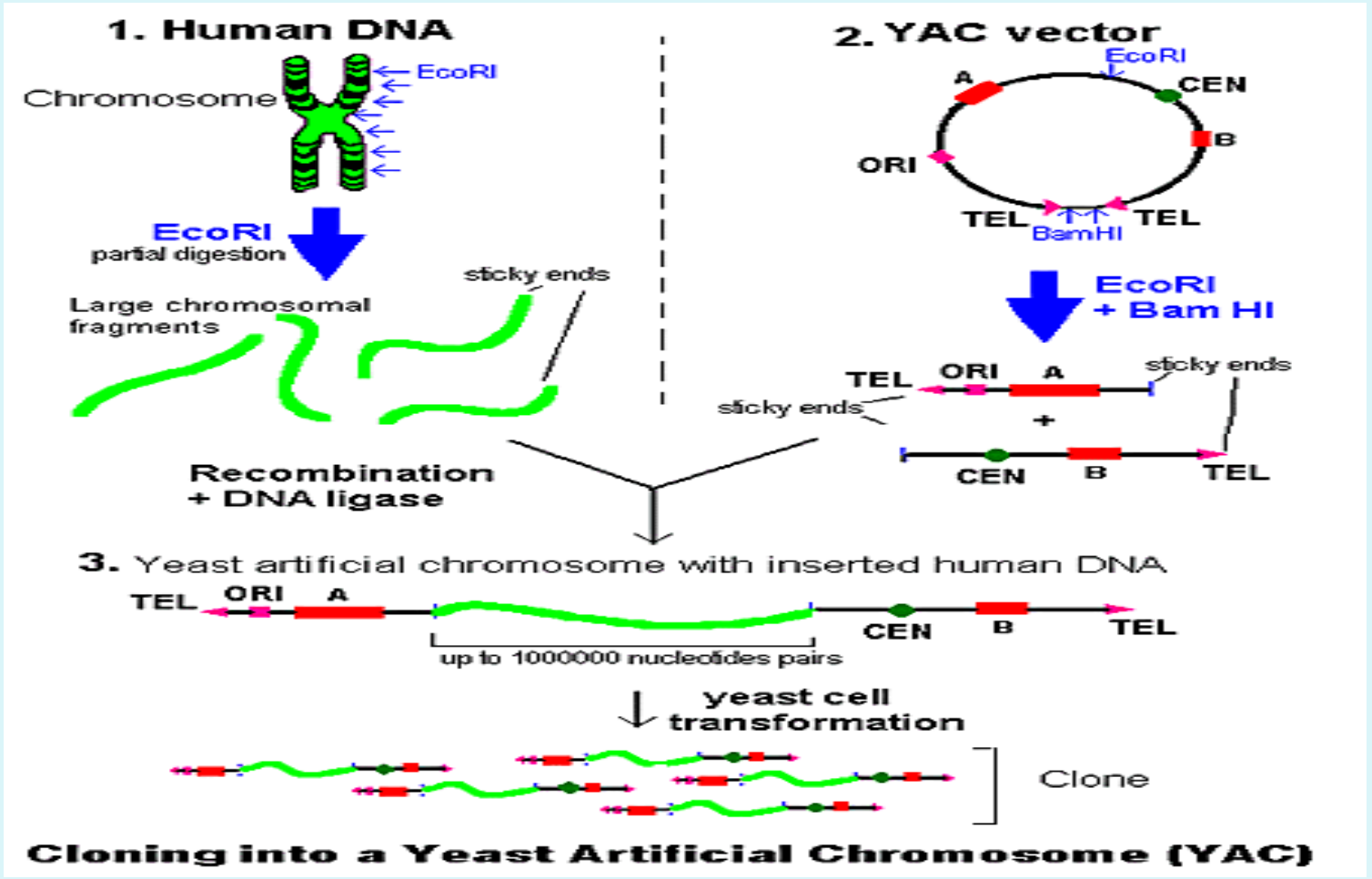


Figure: *BACs as a cloning vector*

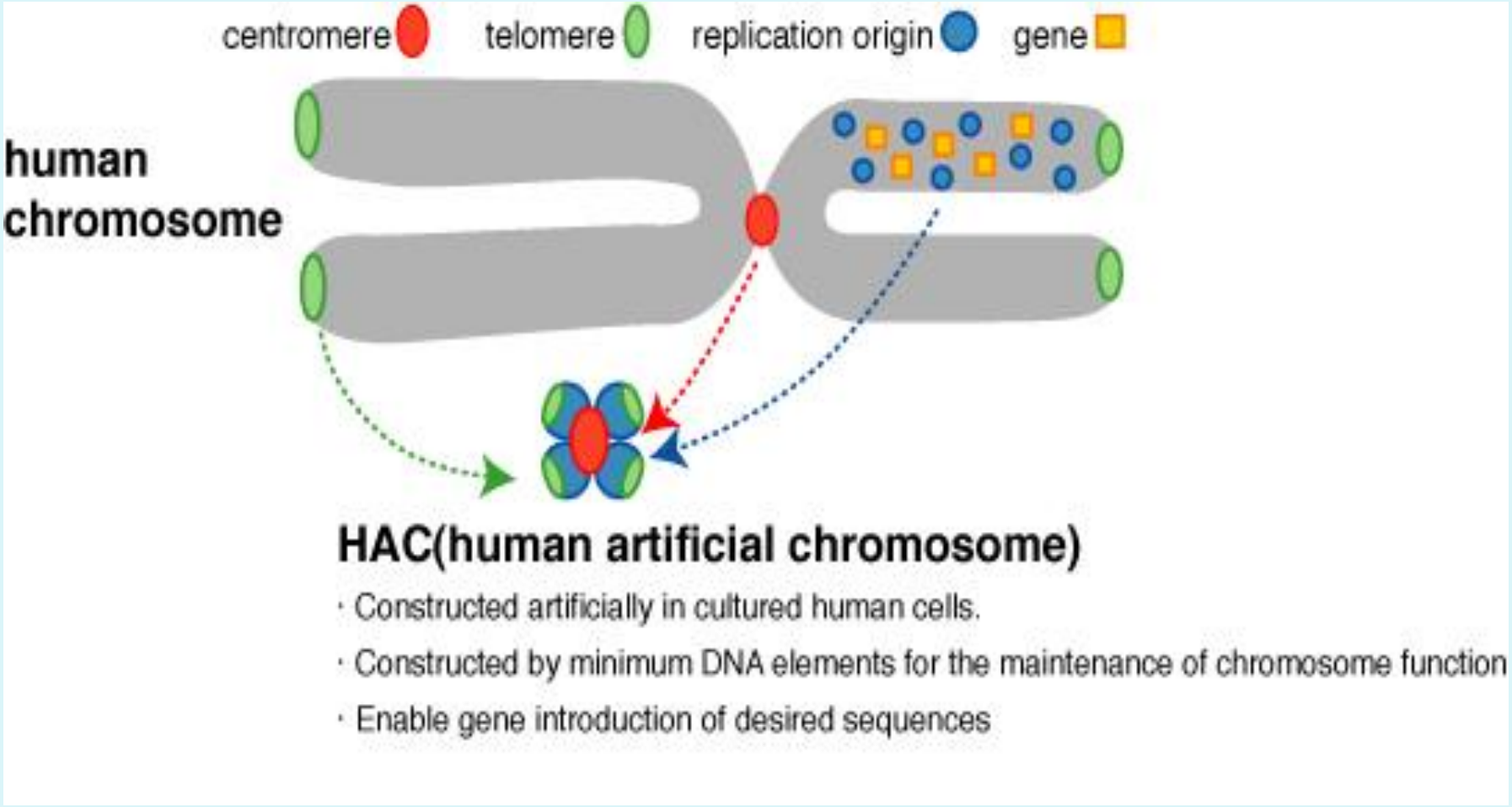
Yeast artificial chromosome Human artificial chromosome

- **Yeast artificial chromosome (YAC):** Insert of up to 3,000 kb may be carried by yeast artificial chromosome.
- **Human artificial chromosome (HAC):** may be potentially useful as a gene transfer vectors for gene delivery into human cells, and 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.

Yeast artificial chromosome (YAC)



Human artificial chromosome (HAC)

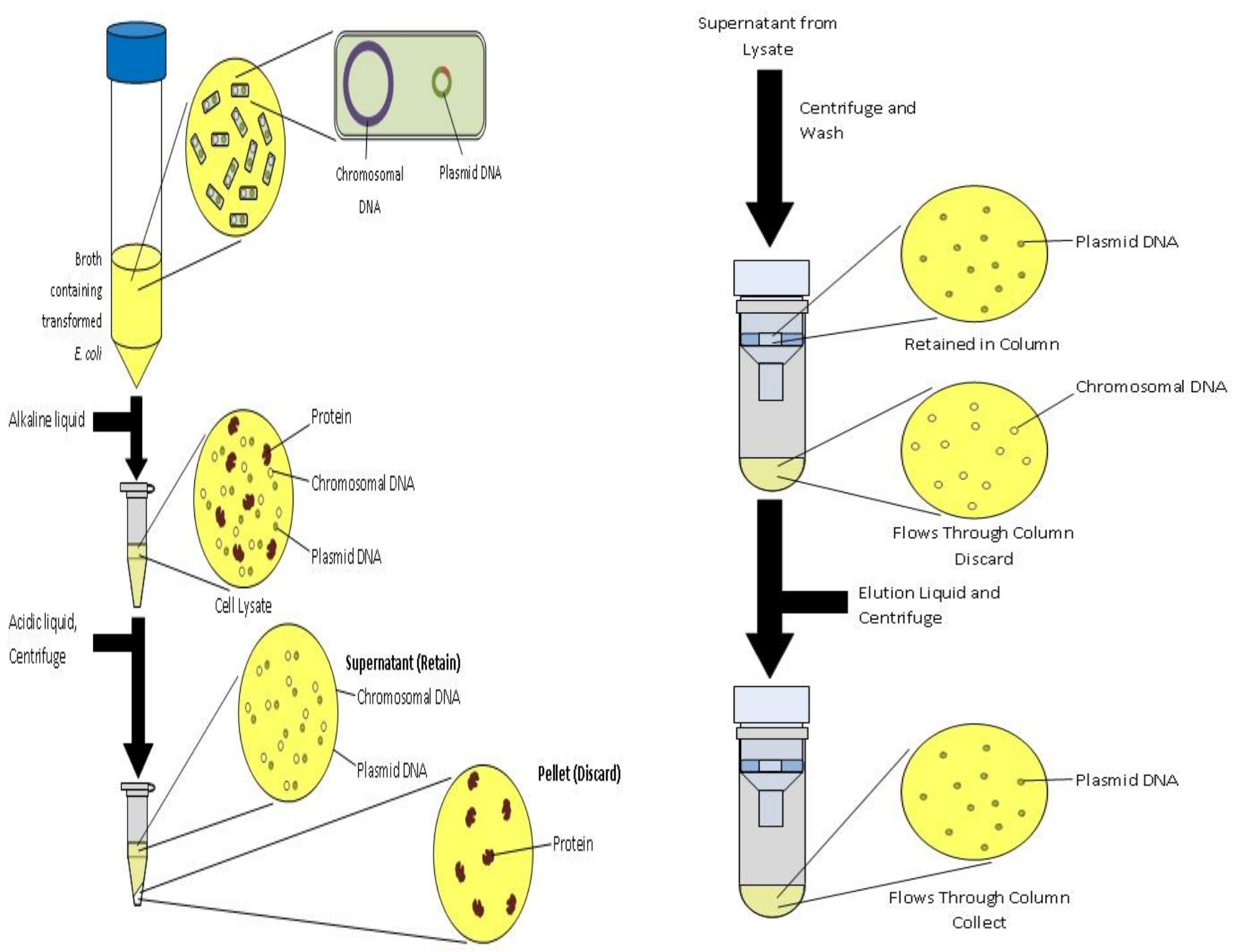


General Steps of Cloning with Any Vector

- Prepare the vector and DNA to be cloned by digestion with restriction enzymes to generate complementary ends.
- Ligate the foreign DNA into the vector with the enzyme DNA ligase.
- Introduce the DNA into bacterial cells (or yeast cells for YACs) by transformation.
- Select cells containing foreign DNA by screening for selectable markers (usually drug resistance).

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

Expression vector

- An expression vector has features that any vector may have.
- Origin of replication, selectable marker, multiple cloning site.
- 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.

Expression vector

- The cloning process is normally performed in *Escherichia coli*, and vectors used for protein expression in organisms other than *E.coli* may have, in addition to a suitable origin of replication for its propagation in *E. coli*, elements that allow them to be maintained in another organism, and these vectors are called **shuttle vectors**.

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.

Elements for expression

- Where the promoter is present, the expression of the gene is preferably tightly controlled and **inducible** so that proteins are only produced when required.
- Protein expression may also be also constitutive (i.e. protein is constantly expressed) in some expression vectors. Low level of constitutive protein synthesis may occur even in expression vectors with tightly controlled promoters.

Elements for expression

- The promoter initiates the transcription and is therefore the point of control for the expression of the cloned gene.
- After the expression of the gene product, it is usually necessary to purify the expressed protein.
- To make this purification process easier, a purification tag may be added to the cloned gene.

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. These peptide and protein pharmaceuticals may be 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.
- Used to produce pharmaceuticals and other proteins.
- They may also be engineered to have advantageous or useful traits.
- Green fluorescent protein is sometimes used as tags which results in animal that can fluoresce, and this have been exploited commercially to produce the fluorescentGloFish.

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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