



DNA CLONING

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DNA CLONING

- **DNA cloning is a technique for reproducing DNA fragments.**
- **It can be achieved by two different approaches:**
 - **cell based**
 - **using polymerase chain reaction (PCR).**
- **a vector is required to carry the DNA fragment of interest into the host cell.**



DNA CLONING

- DNA cloning allows a copy of any specific part of a DNA (or RNA) sequence to be selected among many others and produced in an unlimited amount.
- This technique is the first stage of most of the genetic engineering experiments:
 - production of DNA libraries
 - PCR
 - DNA sequencing

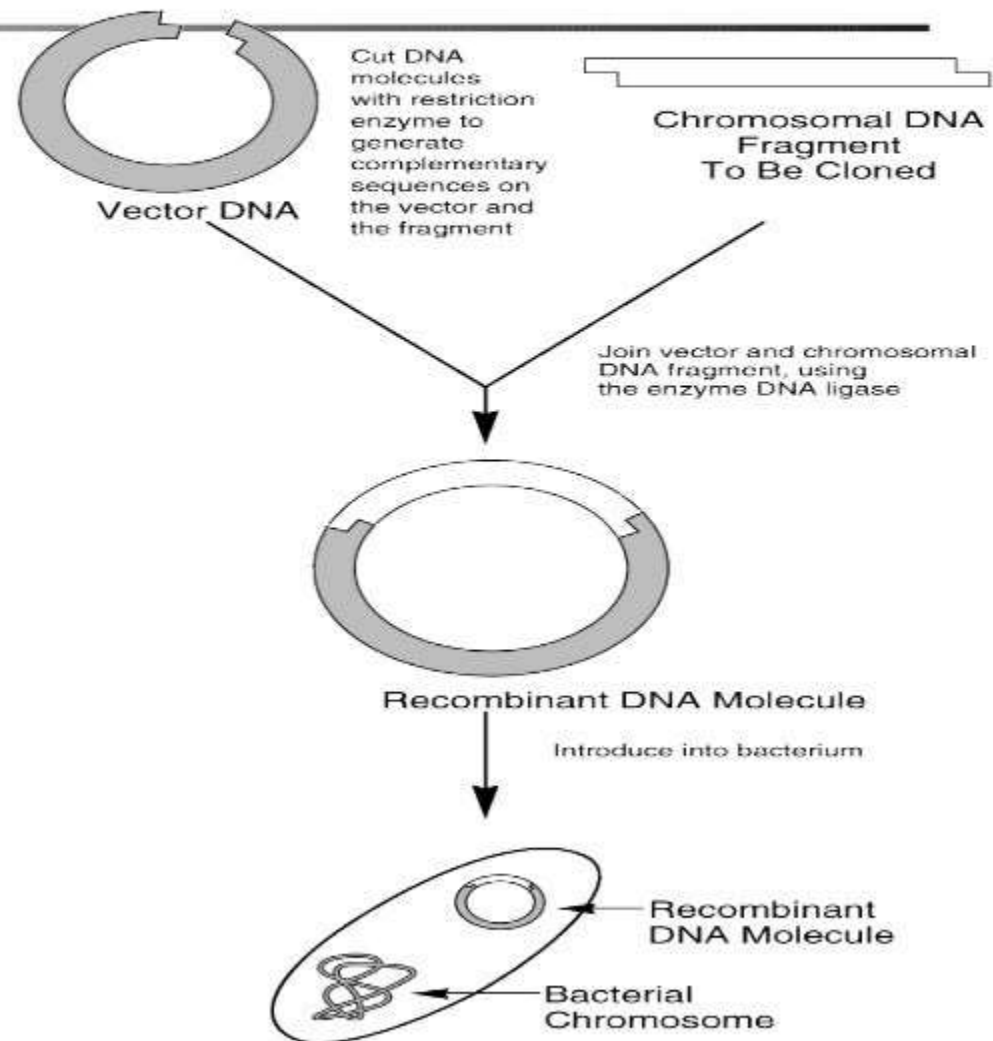


DNA CLONING

- **Massive amplification of DNA sequences**
- **Stable propagation of DNA sequences**
- **A single DNA molecule can be amplified allowing it to be:**
 - **Studied - Sequenced**
 - **Manipulated - Mutagenised**
 - **Expressed - Generation of Protein**

CLONING PROCESS

- Gene of interest is cut out with RE
- Host plasmid is cut with same RE
- Gene is inserted into plasmid and ligated with ligase
- New plasmid inserted into bacterium (transform)





PLASMID CLONING STRATEGY

Involves five steps:

Enzyme restriction digest of DNA sample.

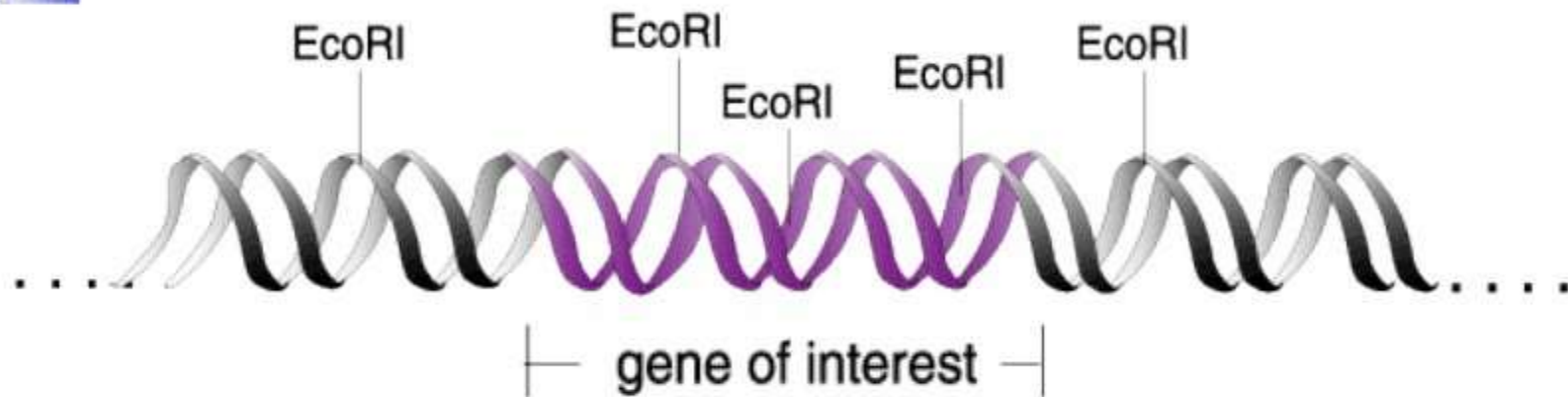
Enzyme restriction digest of DNA plasmid vector.

Ligation of DNA sample products and plasmid vector.

Transformation with the ligation products.

Growth on agar plates with selection for antibiotic resistance.

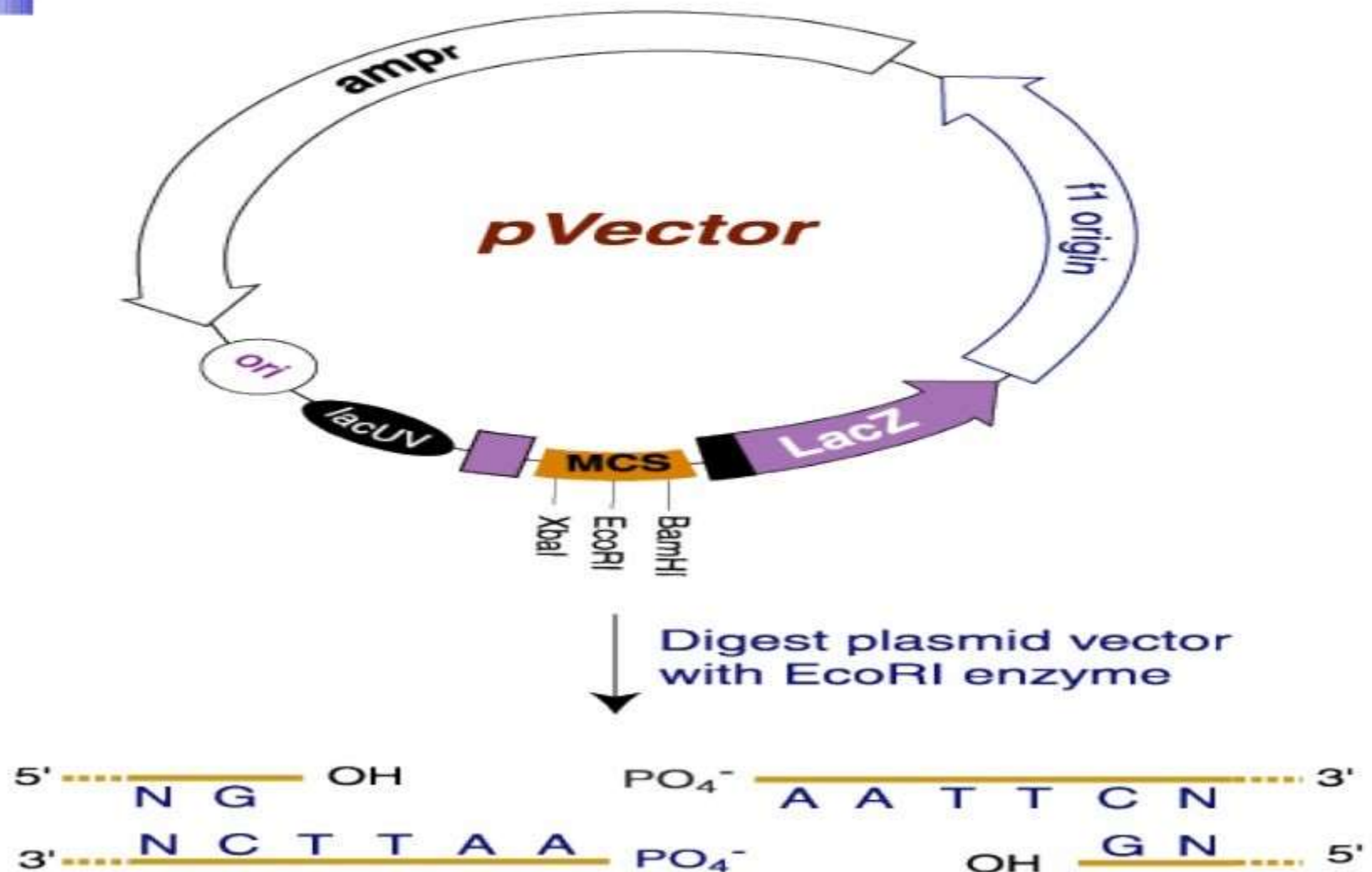
STEP 1. RE DIGESTION OF DNA SAMPLE



Digest DNA sample
with EcoRI enzyme

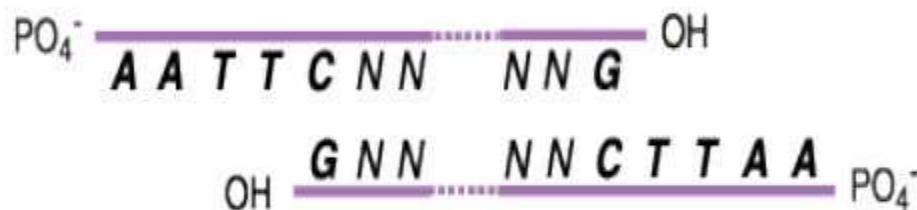


STEP 2. RE DIGESTION OF PLASMID DNA

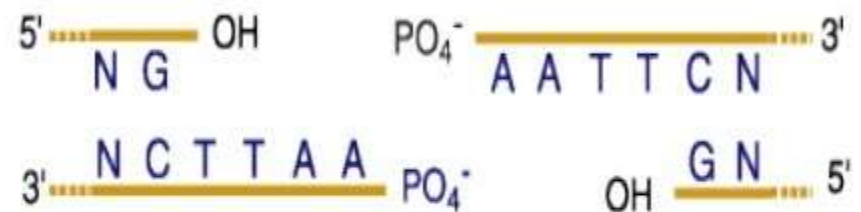


STEP 3. LIGATION OF DNA SAMPLE AND PLASMID DNA

EcoRI digested DNA sample



EcoRI digested plasmid vector



combine and add
DNA ligase + ATP

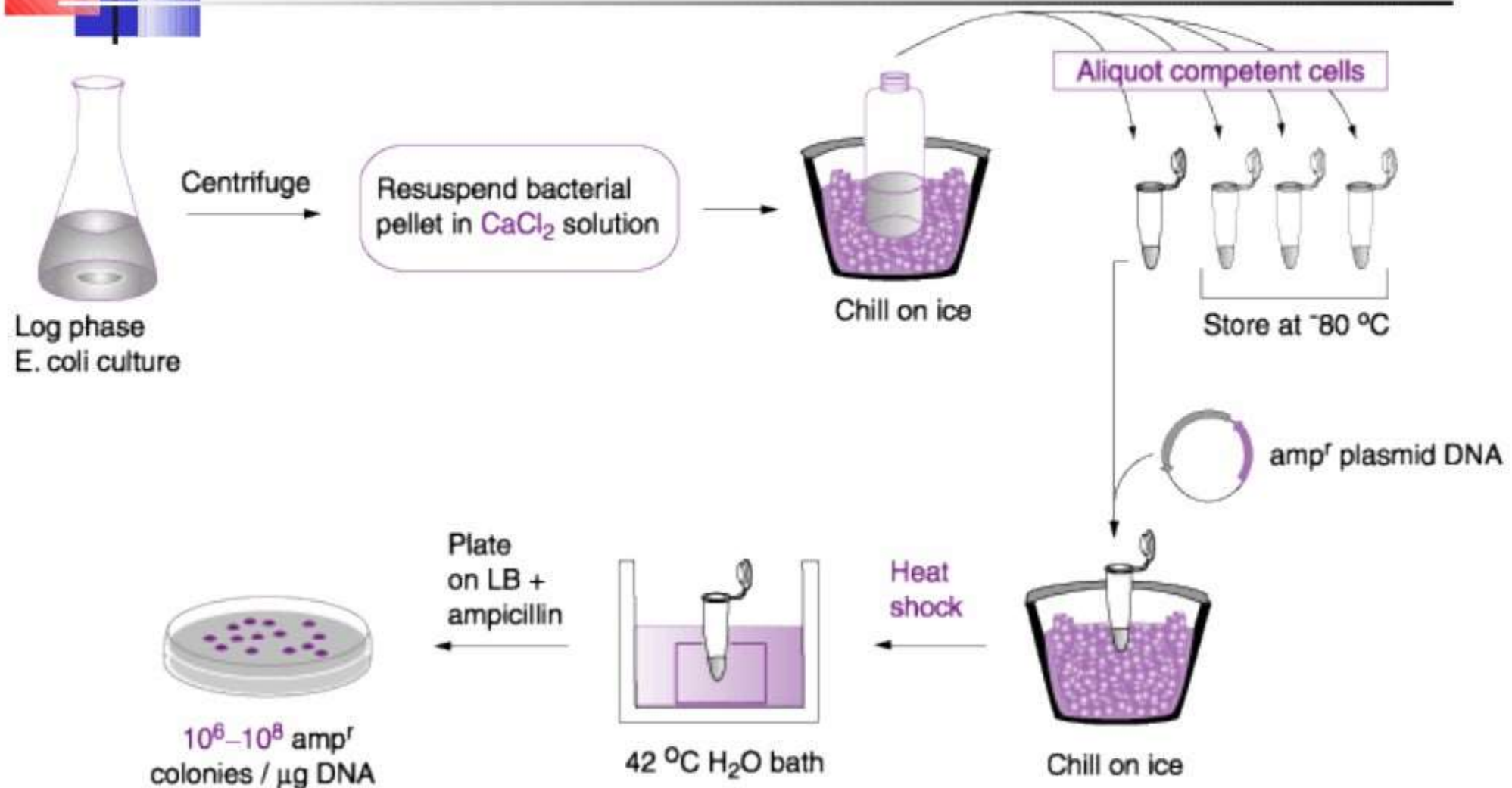
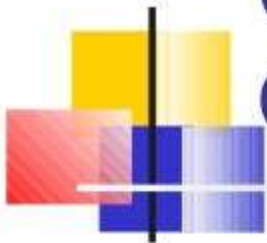




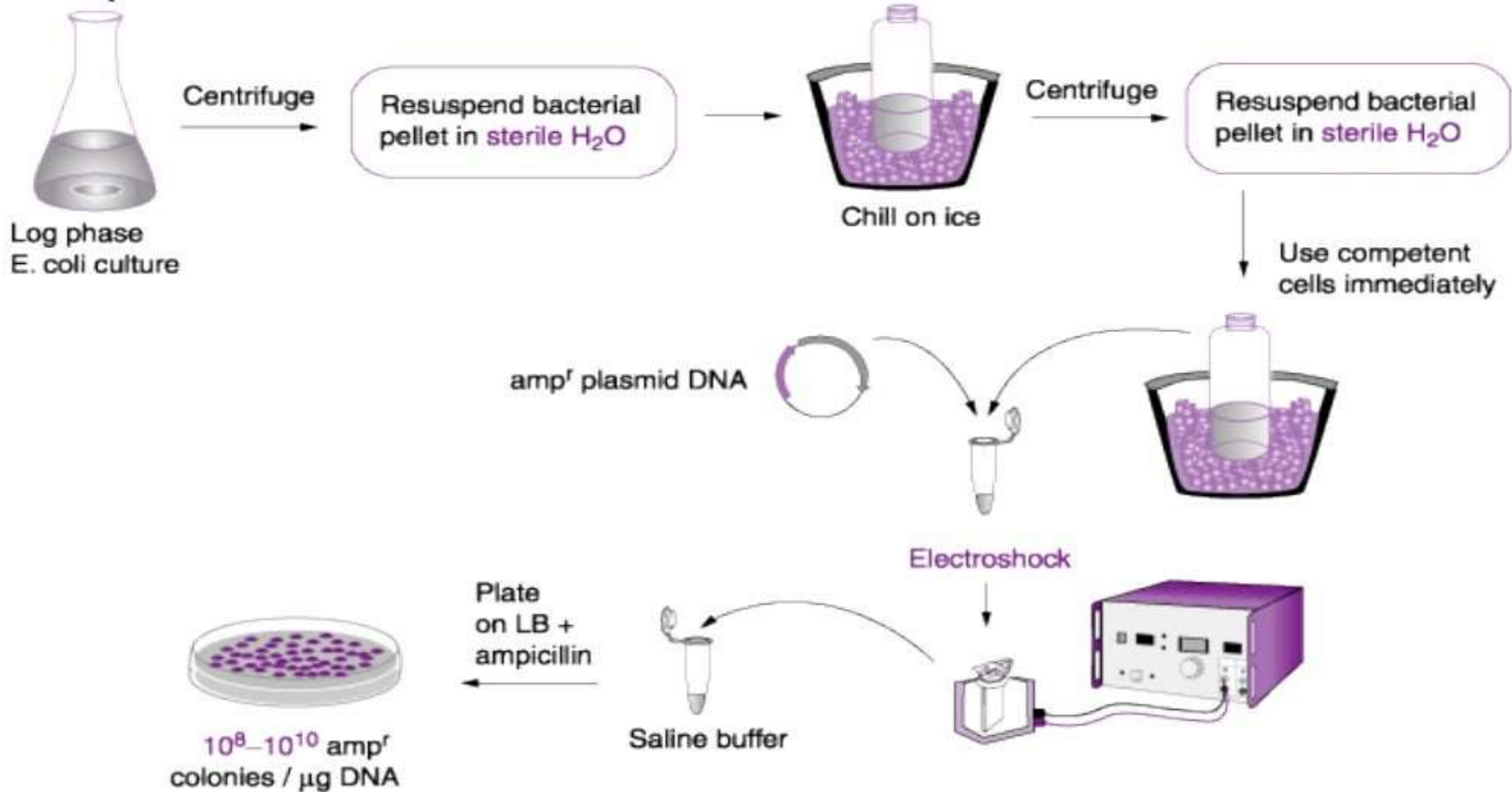
STEP 4. TRANSFORMATION OF LIGATION PRODUCTS

- The process of transferring exogenous DNA into cells is call **“transformation”**
- There are basically two general methods for transforming bacteria. The first is a **chemical method utilizing CaCl_2** and heat shock to promote DNA entry into cells.
- A second method is called **electroporation** based on a short pulse of electric charge to facilitate DNA uptake.

CHEMICAL TRANSFORMATION WITH CALCIUM CHLORIDE



TRANSFORMATION BY ELECTROPORATION

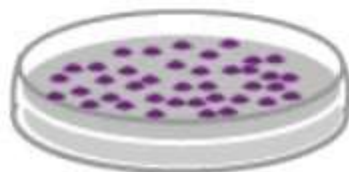
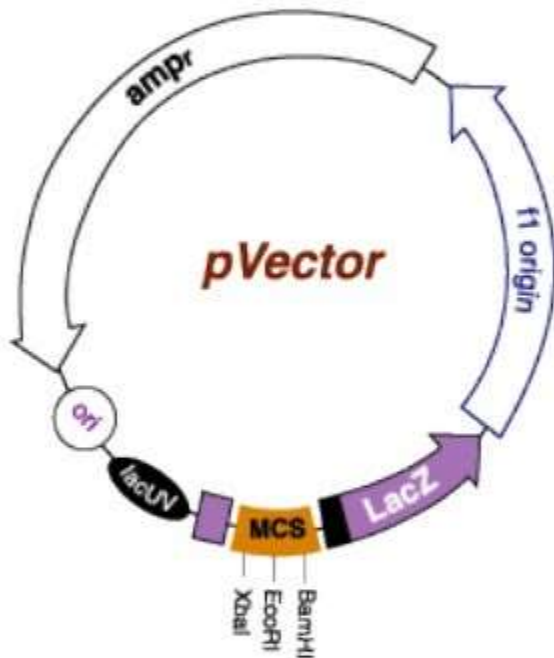


STEP 5. GROWTH ON AGAR PLATES

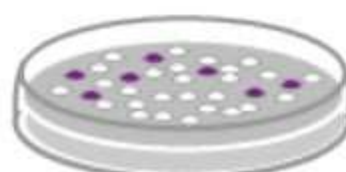
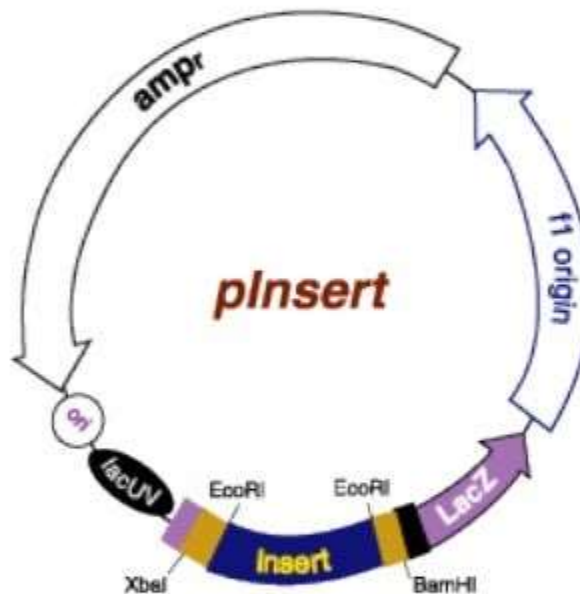
Vector religation

Vector + insert ligation

Insert self-ligation



Amp+X-Gal plate



Amp+X-Gal plate



Amp+X-Gal plate



STEP 5

- **Blue colonies** represent Ampicillin-resistant bacteria that contain pVector and express a functional **alpha fragment** from an intact **LacZ alpha coding sequence**.

White colonies represent Ampicillin-resistant bacteria that contain pInsert and do **not** produce **LacZ alpha fragment**



TERMS USED IN CLONING

- **DNA recombination.**
The DNA fragment to be cloned is inserted into a vector.
- **Transformation.**
The recombinant DNA enters into the host cell and proliferates.
- **Selective amplification.**
A specific antibiotic is added to kill *E. coli* without any protection. The transformed *E. coli* is protected by the antibiotic-resistance gene
- **Isolation of desired DNA clones**



CLONING VECTORS

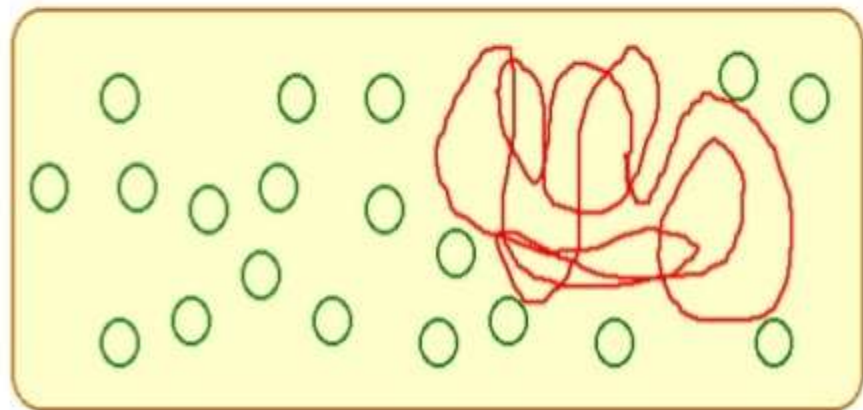
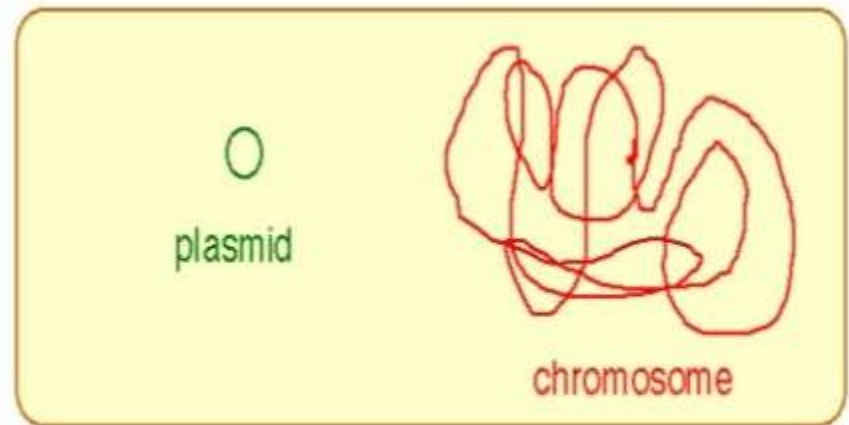
- **Cloning vectors** are DNA molecules that are used to "transport" cloned sequences between biological hosts and the test tube.

Cloning vectors share four common properties:

- 1. Ability to promote autonomous replication.**
- 2. Contain a genetic marker (usually dominant) for selection.**
- 3. Unique restriction sites to facilitate cloning of insert DNA.**
- 4. Minimum amount of nonessential DNA to optimize cloning.**

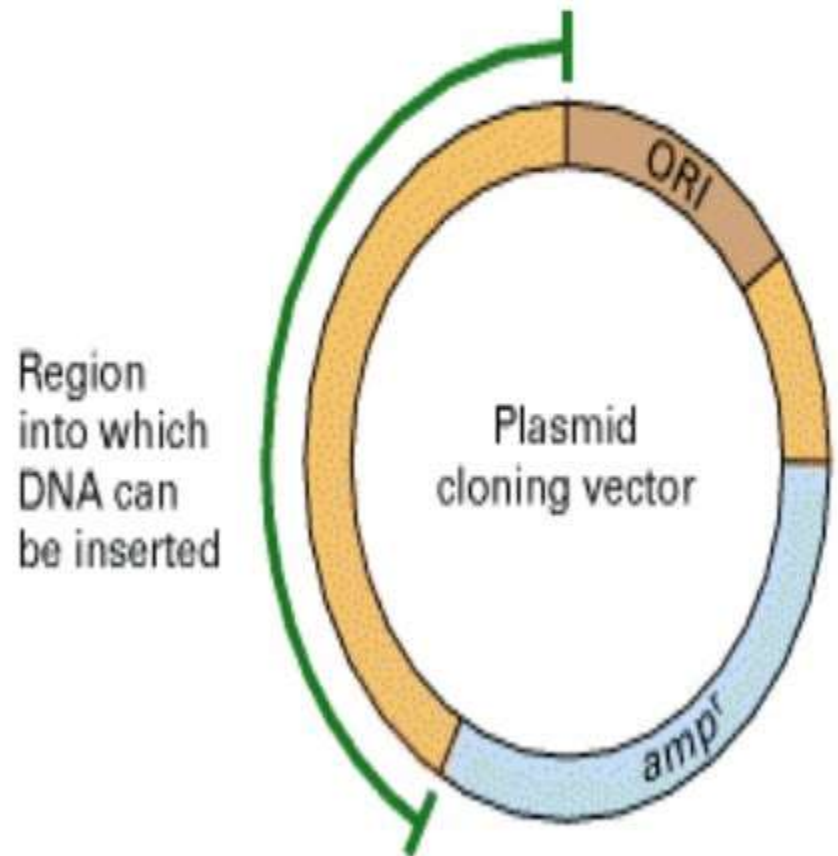
PLASMIDS

- Bacterial cells may contain extra-chromosomal DNA called plasmids.
- Plasmids are usually represented by small, circular DNA.
- Some plasmids are present in multiple copies in the cell



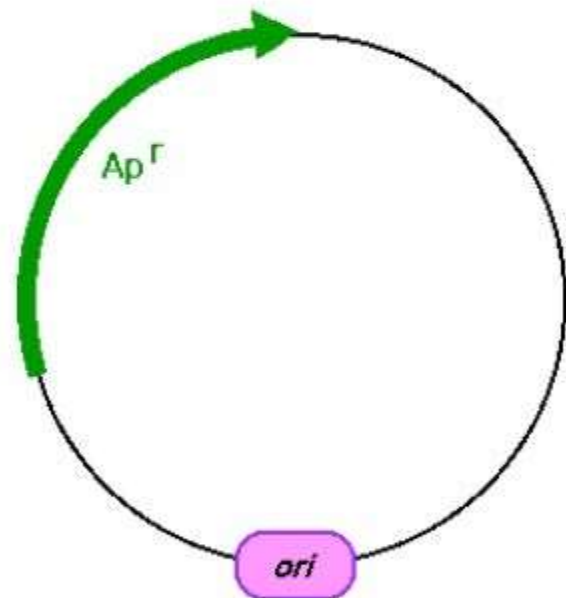
PLASMID VECTORS

- Plasmid vectors are ≈ 1.2 – 3 kb and contain:
- replication origin (ORI) sequence
- a gene that permits selection,
- Here the selective gene is *amp^r*; it encodes the enzyme b-lactamase, which inactivates ampicillin.
- Exogenous DNA can be inserted into the bracketed region .



SELECTIVE MARKER

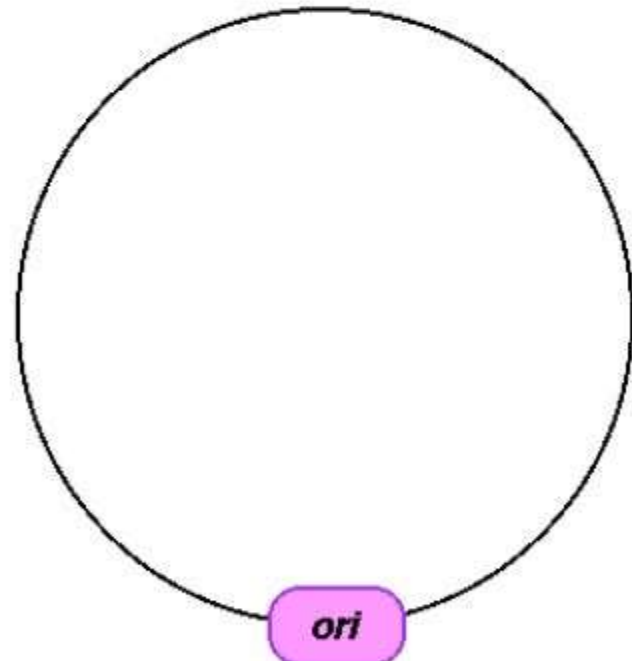
- **Selective marker** is required for maintenance of plasmid in the cell.
- Because of the presence of the selective marker the plasmid becomes useful for the cell.
- Under the selective conditions, only cells that contain plasmids with selectable marker can survive
- Genes that confer resistance to various antibiotics are used.
- Genes that make cells resistant to ampicillin, neomycin, or chloramphenicol are used





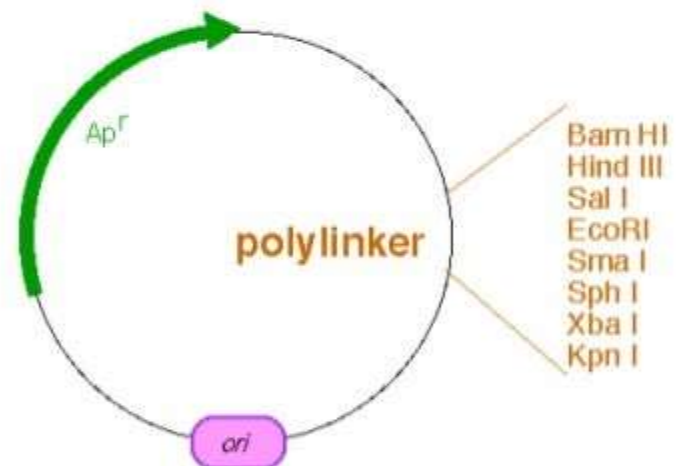
ORIGIN OF REPLICATION

- **Origin of replication** is a DNA segment recognized by the cellular DNA-replication enzymes.
- Without replication origin, DNA cannot be replicated in the cell.



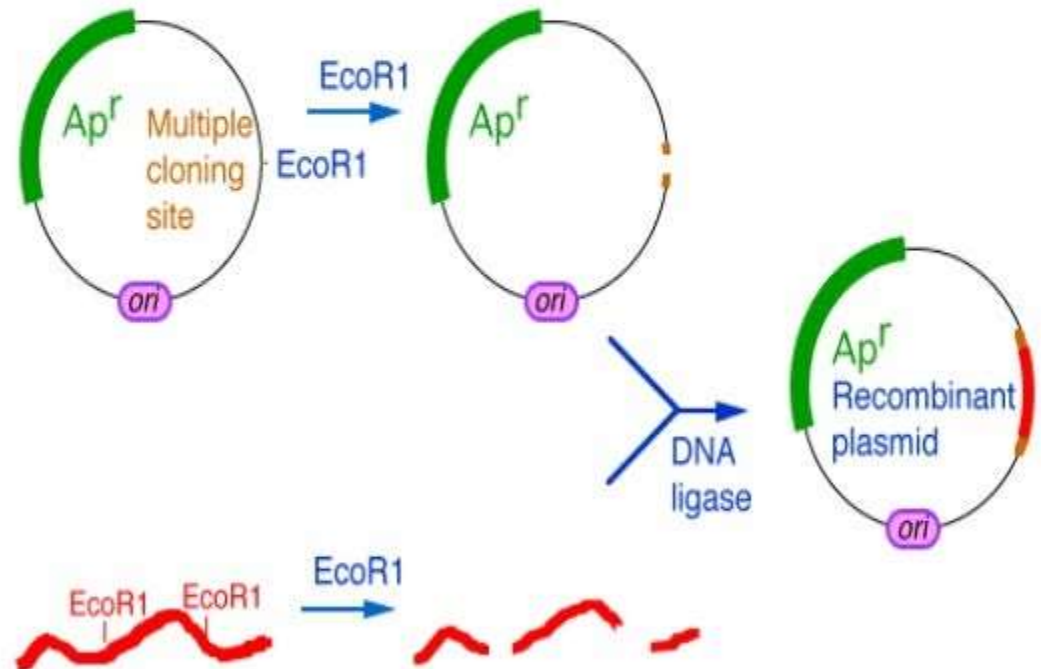
MULTIPLE CLONING SITE

- Many cloning vectors contain a **multiple cloning site** or **polylinker**: a DNA segment with several unique sites for restriction endo- nucleases located next to each other
- Restriction sites of the polylinker are not present anywhere else in the plasmid.
- Cutting plasmids with one of the restriction enzymes that recognize a site in the polylinker does not disrupt any of the essential features of the vector



MULTIPLE CLONING SITE

- Gene to be cloned can be introduced into the cloning vector at one of the restriction sites present in the polylinker



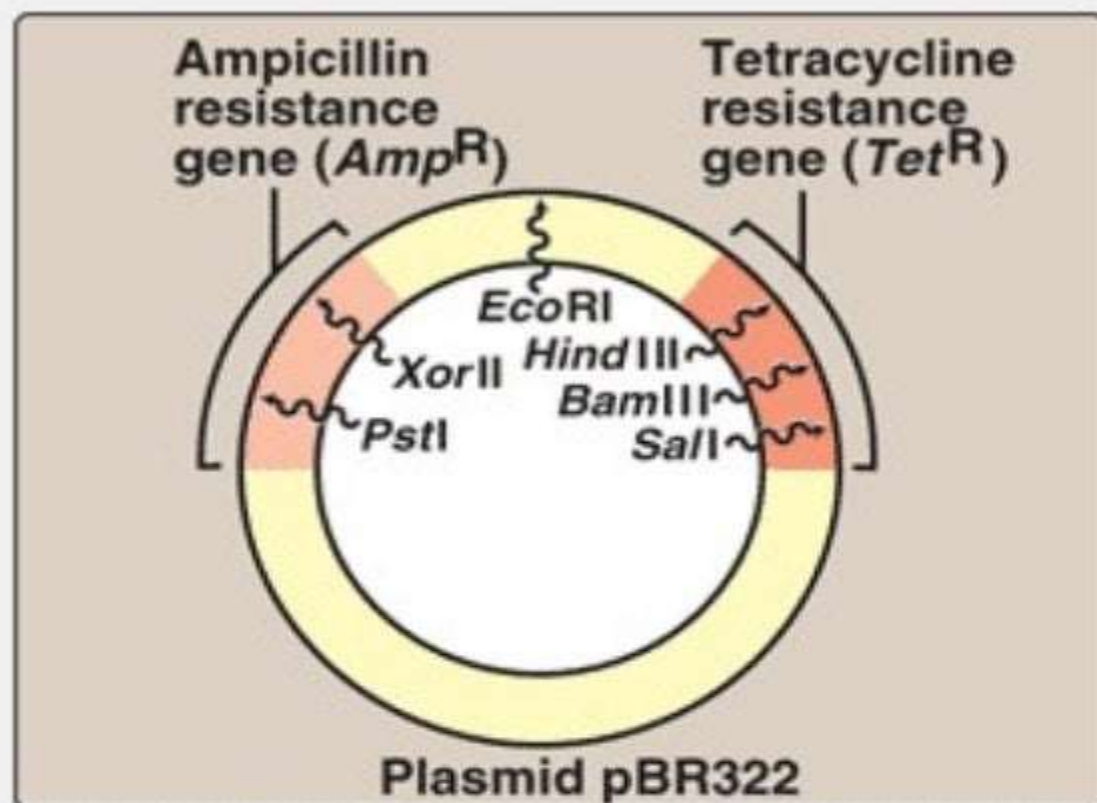
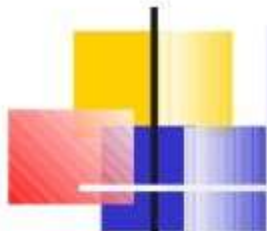


Figure 33.5 A restriction map of plasmid pBR322 indicating the positions of its antibiotic resistance genes and the sites of nucleotide sequences recognized by specific restriction endonucleases.

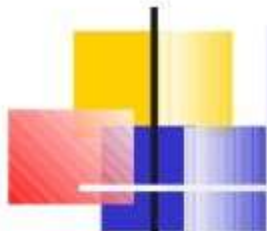
Types of expression systems

- Bacterial: plasmids, phages
- Yeast: expression vectors: plasmids, yeast artificial chromosomes (YACs)
- Insect cells: baculovirus, plasmids
- Mammalian:
 - viral expression vectors (gene therapy):
 - vaccinia virus
 - adenovirus
 - retrovirus
 - Stable cell lines (CHO, HEK293)



EXPRESSION VECTORS

- **Allows a cloned segment of DNA to be translated into protein inside a bacterial or eukaryotic cell.**
- **Vectors will contain the :**
 - (a) *in vivo* promoter**
 - (b) Ampicillin selection**
 - (c) Sequencing primers**



EXPRESSION VECTORS

- Produces large amounts of a specific protein
- Permits studies of the structure and function of proteins
- Can be useful when proteins are rare cellular components or difficult to isolate

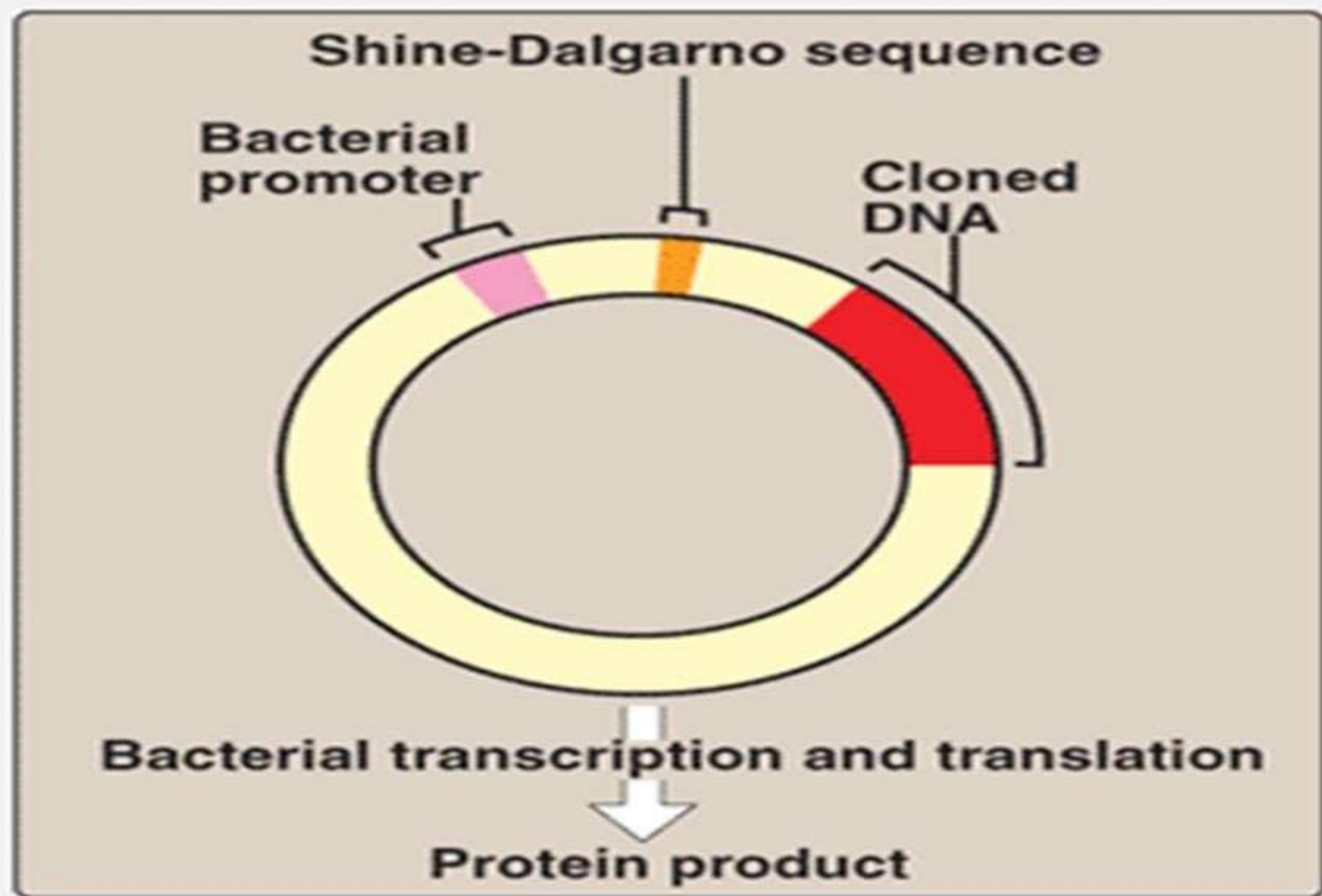
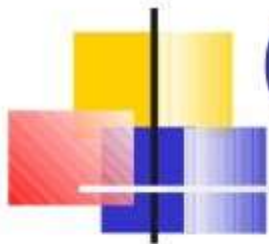


Figure 33.8 An expression vector.



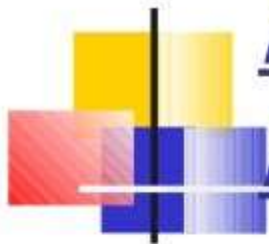
Common problems with bacterial expression systems

- Low expression levels:
 - change promoter
 - change plasmid
 - change cell type
 - add rare tRNAs for rare codons on second plasmid
- Severe protein degradation:
 - use proteasome inhibitors and other protease inhibitors
 - try induction at lower temperature
- Missing post-translational modification: co-express with kinases etc.
- Glycosylation will not be carried out:
 - use yeast or mammalian expression system
- Misfolded protein (inclusion bodies):
 - co-express with GroEL, a chaperone
 - try refolding buffers



CLONING STRATEGY

- **Strategy** depends on the starting information and desired endpoint.
- **Starting Information or Resources:**
 - Protein sequence
 - Positional cloning information
 - mRNA species / sequence
 - cDNA libraries
 - DNA sequence known or unknown
 - genomic DNA libraries
 - PCR product



How Are Genes Cloned Using Plasmids?

- To understand how genes are cloned, we need introduce three terms.
- Recombinant DNA- is mixed DNA
- Vector -it carries recombinant DNA into cells.
- Plasmids - are tiny circular pieces of DNA that are commonly found in bacteria.



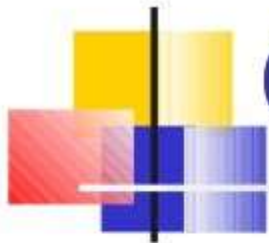
Why Plasmids are Good Cloning Vectors

- small size (easy to manipulate and isolate)
- circular (more stable)
- replication independent of host cell
- several copies may be present (facilitates replication)
- frequently have antibiotic resistance (detection easy)



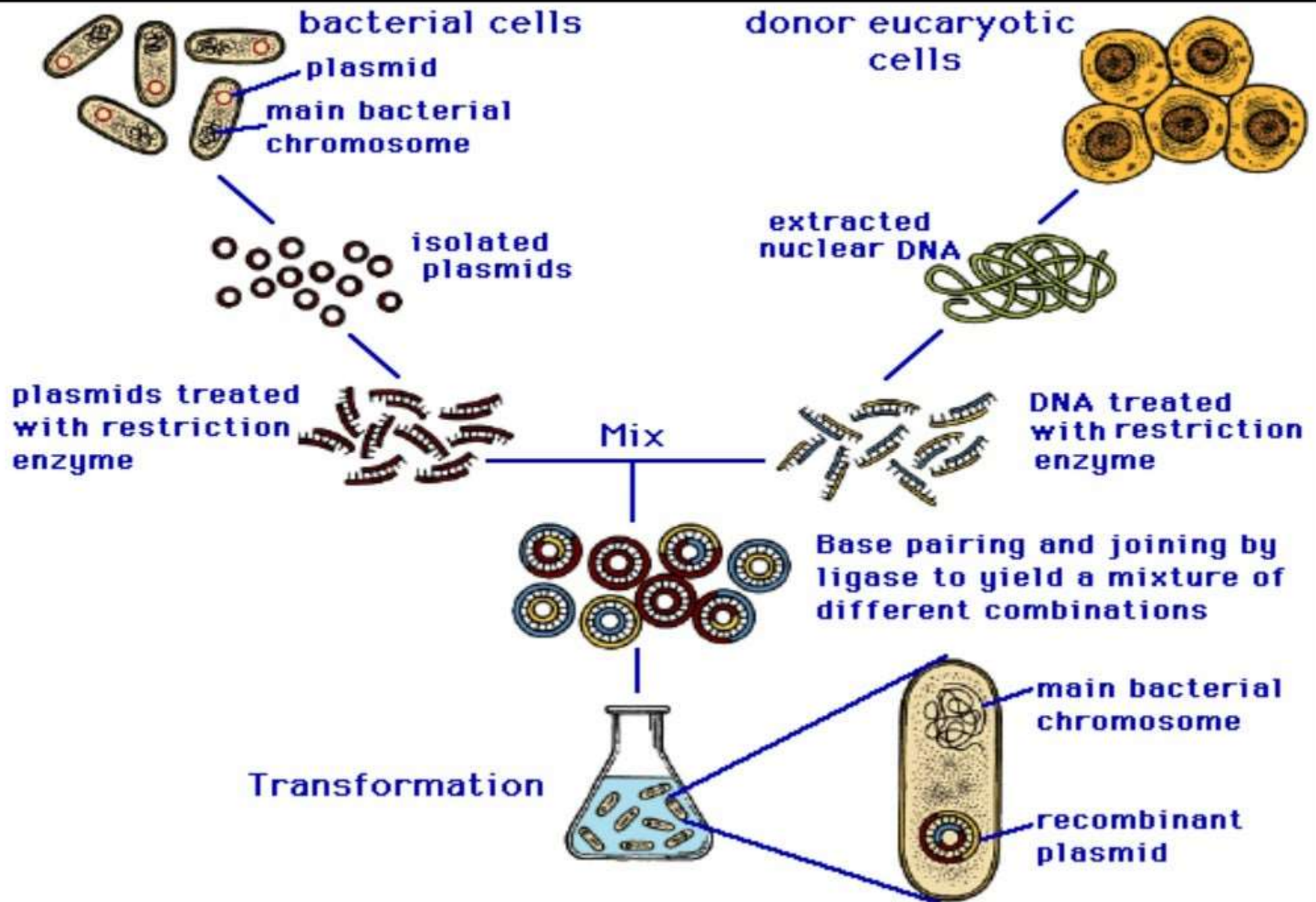
How is foreign DNA Inserted into a Plasmid?

- To open up the DNA a **restriction enzyme** is used.
- Cut the DNA at a specific place called a restriction site.
- The result is a set of double-stranded DNA pieces with single-stranded ends
- These ends that jut out are not only "sticky" but they have gaps that can be now be filled with a piece of foreign DNA
- For DNA from an outside source to bond with an original fragment, one more enzyme is needed
- **DNA ligase** seals any breaks in the DNA molecule



CLONING METHODOLOGY

- **Cut** the cloning vector with R.E. of choice, eg *Eco* RI
- **Cut** DNA of interest with same R.E. or R.E. yielding same sticky ends, e.g. *Bam* HI and *Sau* 3A
- **Mix** the restricted cloning vector and DNA of interest together.
- **Ligate** fragments together using **DNA ligase**
- Insert ligated DNA into host of choice - **transformation** of *E. coli*
- Grow host cells under **restrictive conditions**, grow on plates containing an antibiotic





DIRECTIONAL CLONING

- Often one desires to insert foreign DNA in a particular orientation
- This can be done by making two cleavages with two different **restriction** enzymes
- Construct foreign DNA with same two **restriction** enzymes
- Foreign DNA can only be inserted in one direction



Contd...

- Good efficiency of ligation of foreign DNA into a vector can be achieved if both the vector and the insert DNA are cut with 2 different restriction enzymes which leave single stranded ends (**cohesive ends**).
- The DNA is ligated in only one direction, and there is only a low background of non-recombinant plasmids.



Contd...

- If only one restriction enzyme is used to cut the vector and insert, then efficiency of ligation is lower, DNA can be inserted in two directions and tandem copies of inserts may be found.
- To avoid high background of non-recombinants, **alkaline phosphatase** is used to remove 5' phosphate groups from the cut vector to prevent self-ligation.

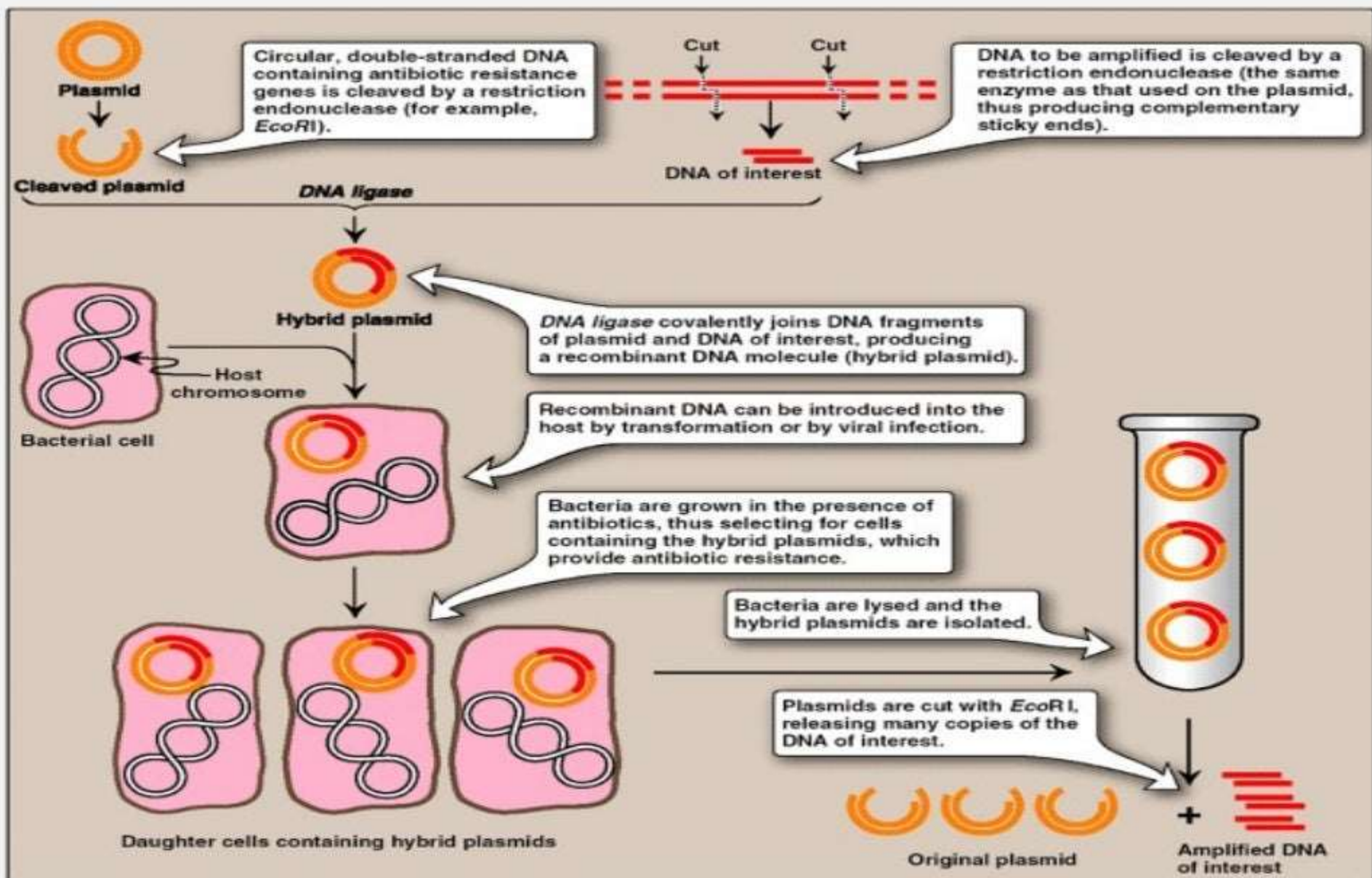


Figure 33.6 Summary of gene cloning.