334 MBIO
Biochemical Instrumentation Techniques

- Lab 6 -

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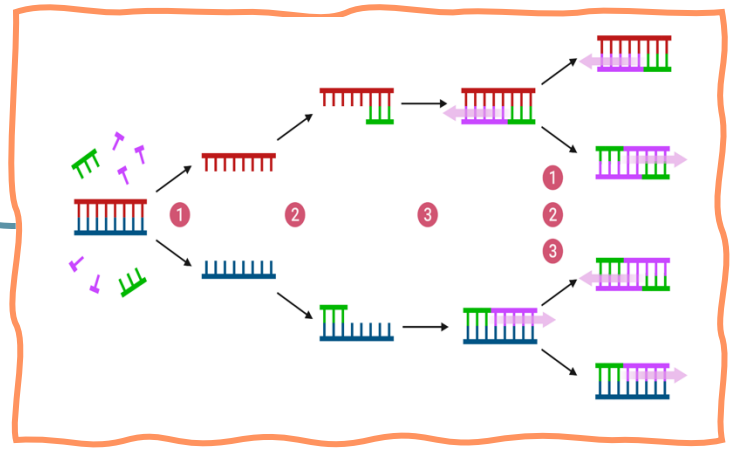
2025

Cloning Recombinant DNA

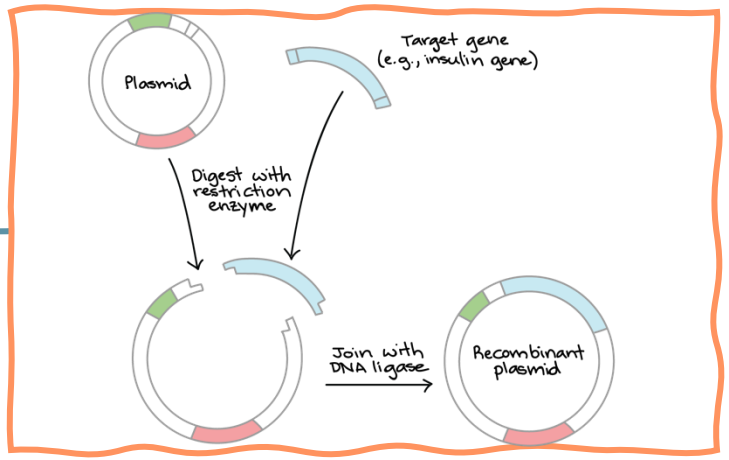
- The solution is to do **amplification of parts of DNA**.

Mainly there are two methods:

Polymerase chain reaction

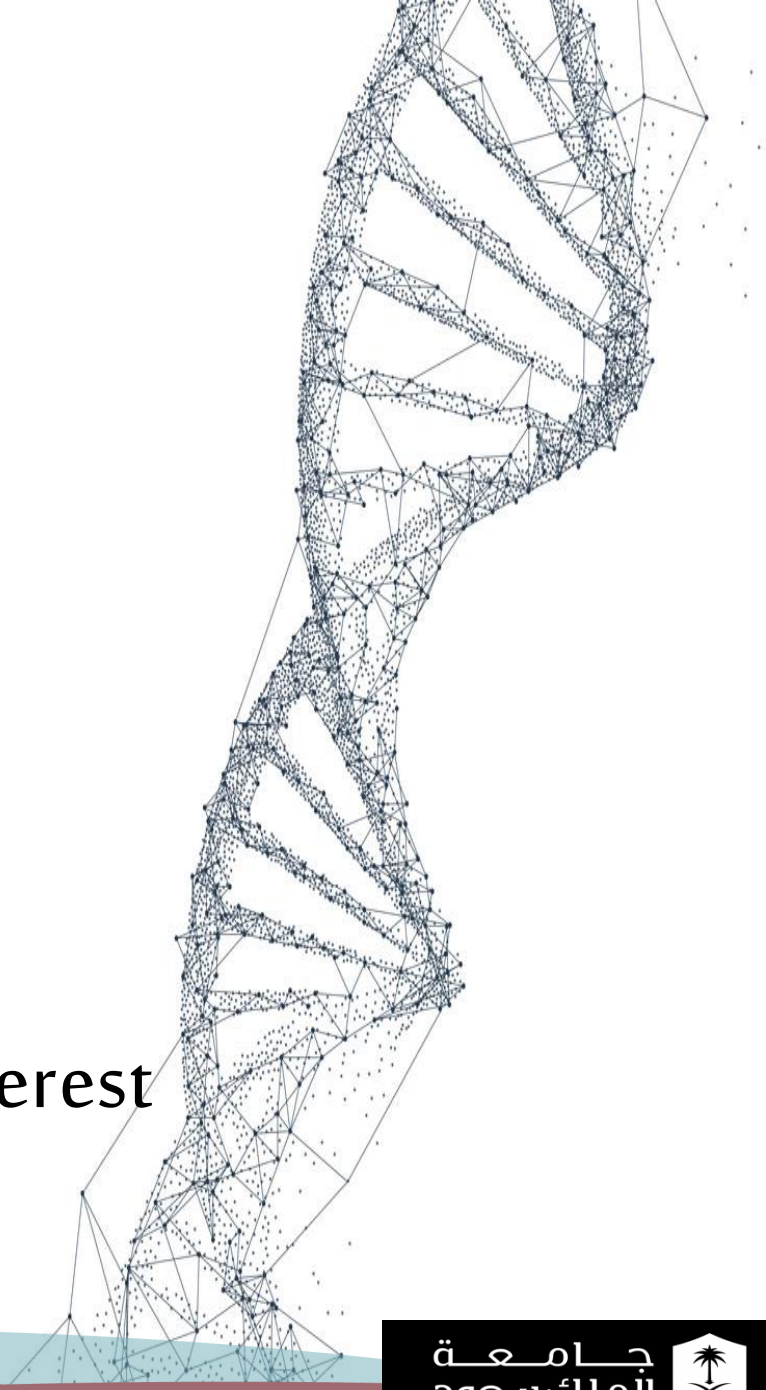


DNA Cloning



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.



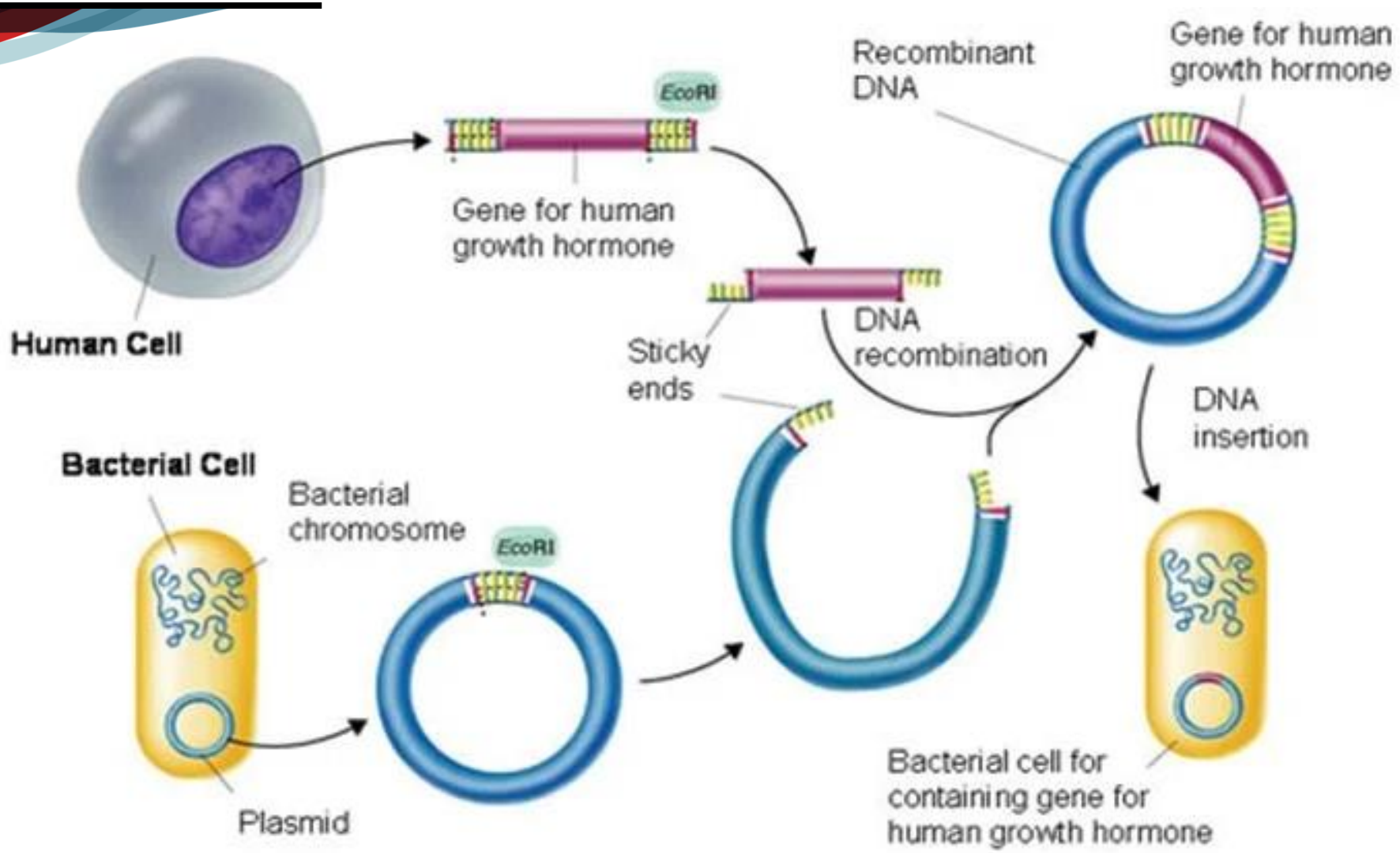
DNA Cloning

- 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.
- **It is the first stage of most of the genetic engineering experiments:**
  - Production of DNA libraries (essential step for genome mapping).
  - DNA sequencing .

DNA Cloning

- A single DNA molecule can be amplified allowing it to be:
  - Studied - Sequenced
  - Manipulated - Mutagenised or Engineered
  - Expressed - Generation of Protein

DNA Cloning



Essential Concepts for DNA Cloning

1. Cloning Vectors
2. Restriction sites (Digestion)
3. Ligation
4. Transformation/Transfection
5. Competent Cells

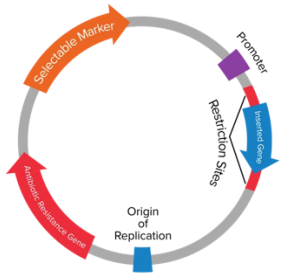
1. Cloning Vectors

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

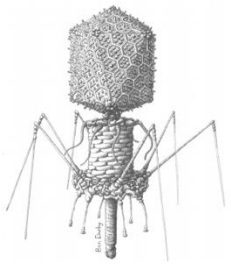
- **Cloning vectors share three common properties:**
  - Ability to promote autonomous replication.
  - Contain a genetic marker (usually dominant) for selection.
  - Unique restriction sites to facilitate cloning of insert DNA.

Types of Vectors

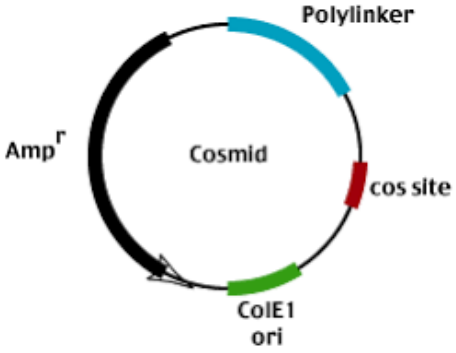
Plasmids



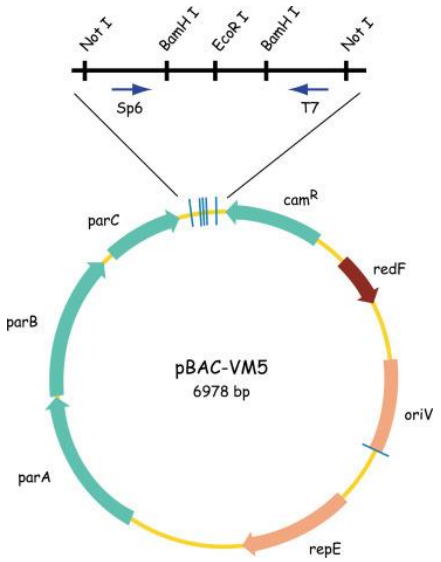
Phage



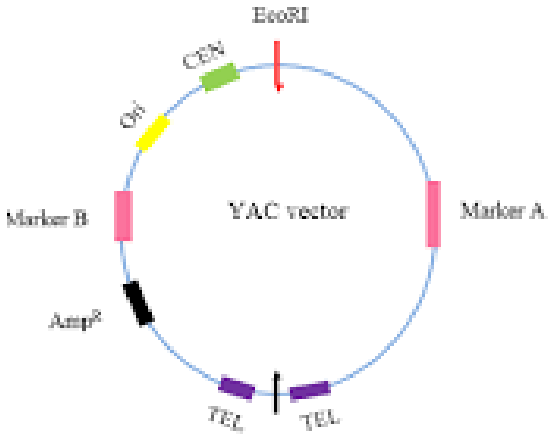
Cos site of λ phage + plasmid (Cosmid)



Bacterial Artificial Chromosome (BAC)

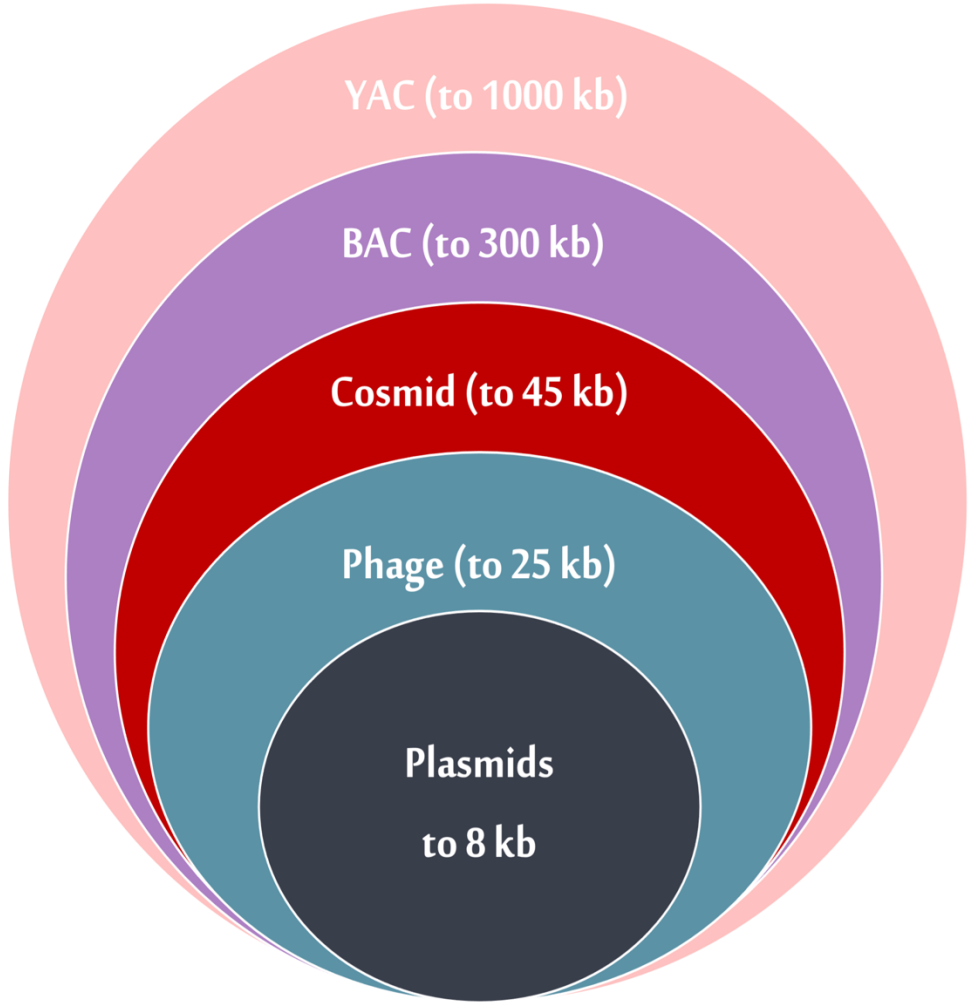


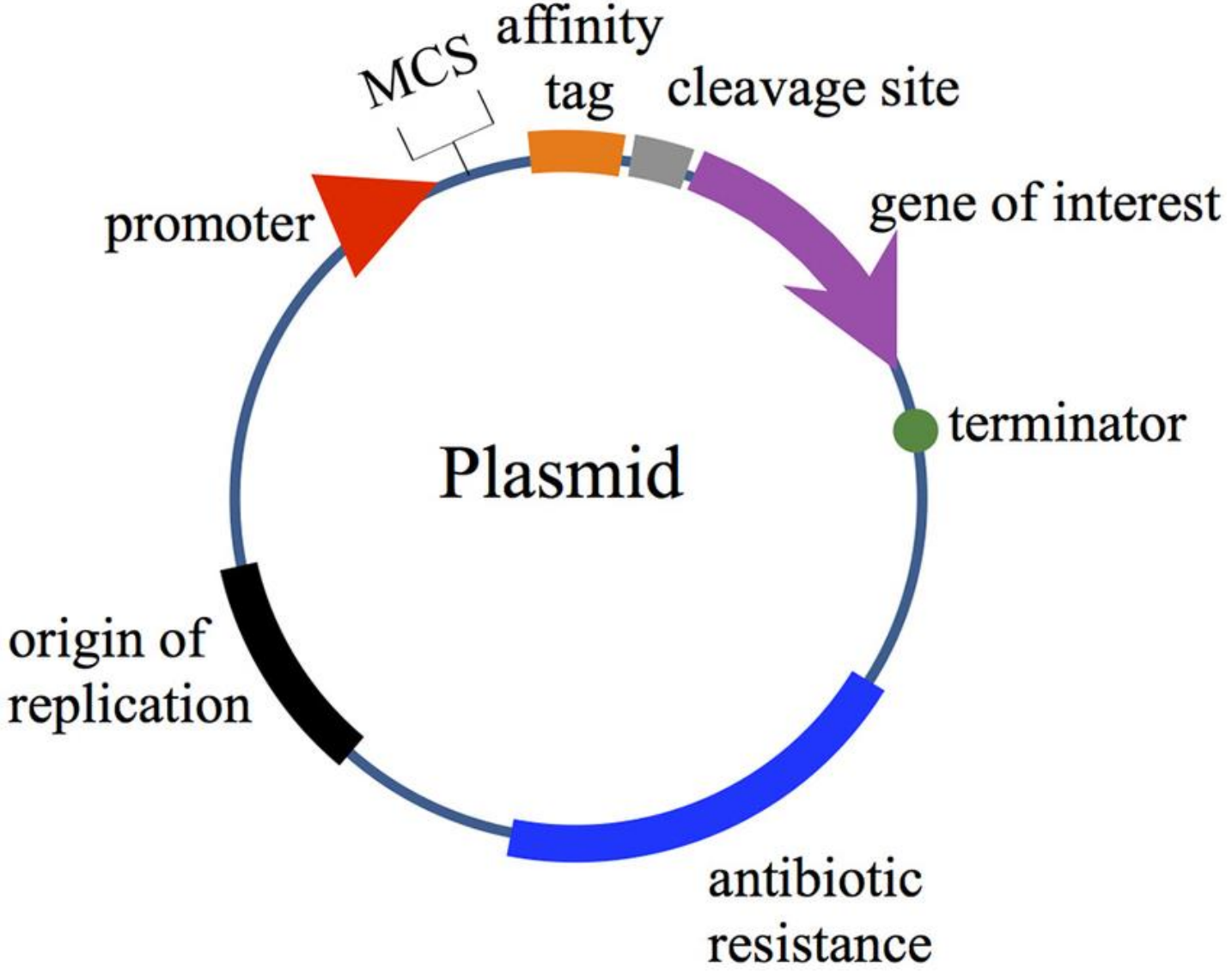
Yeast Artificial Chromosome (YAC)



Features of Vectors

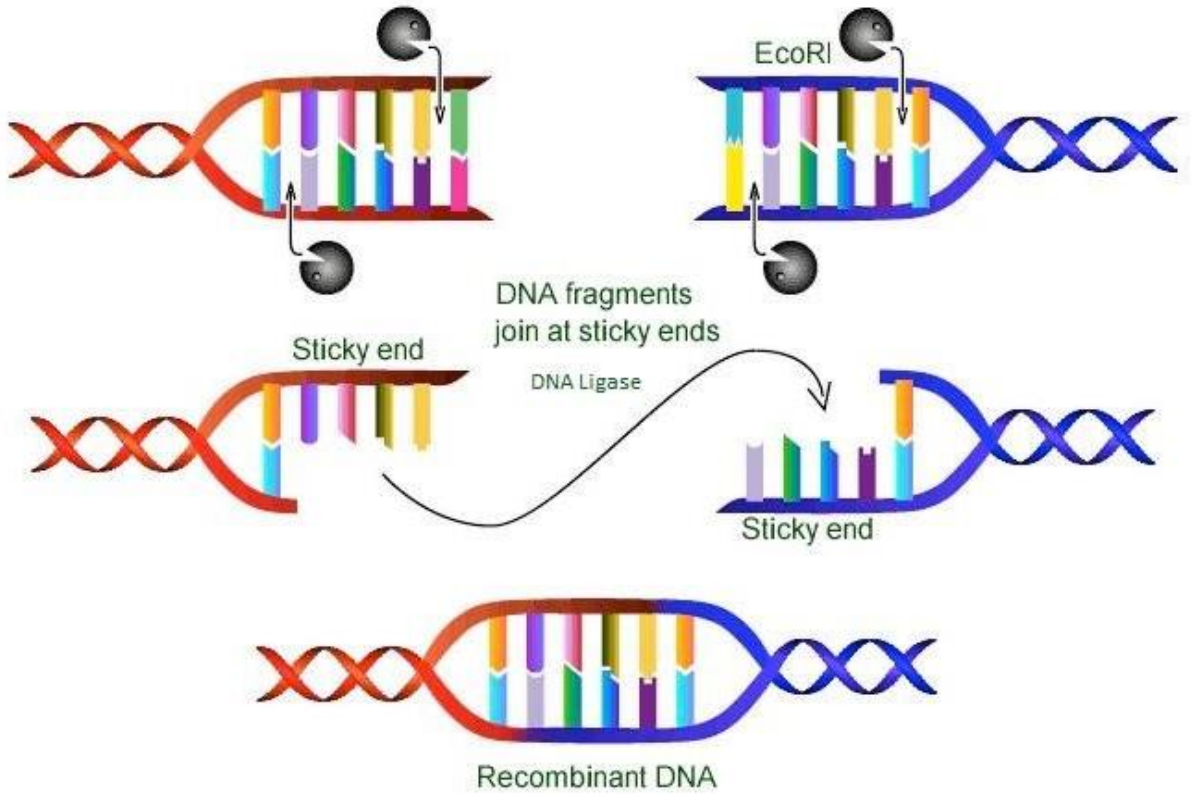
- An Ori (origin of replication)
- Selective markers gene (amp^R, kan^R, tet^R, etc.).
- Multiple cloning sites (MCS).
- Some have special promoters to induce expressions.
- Expression vectors contain **affinity tags**.





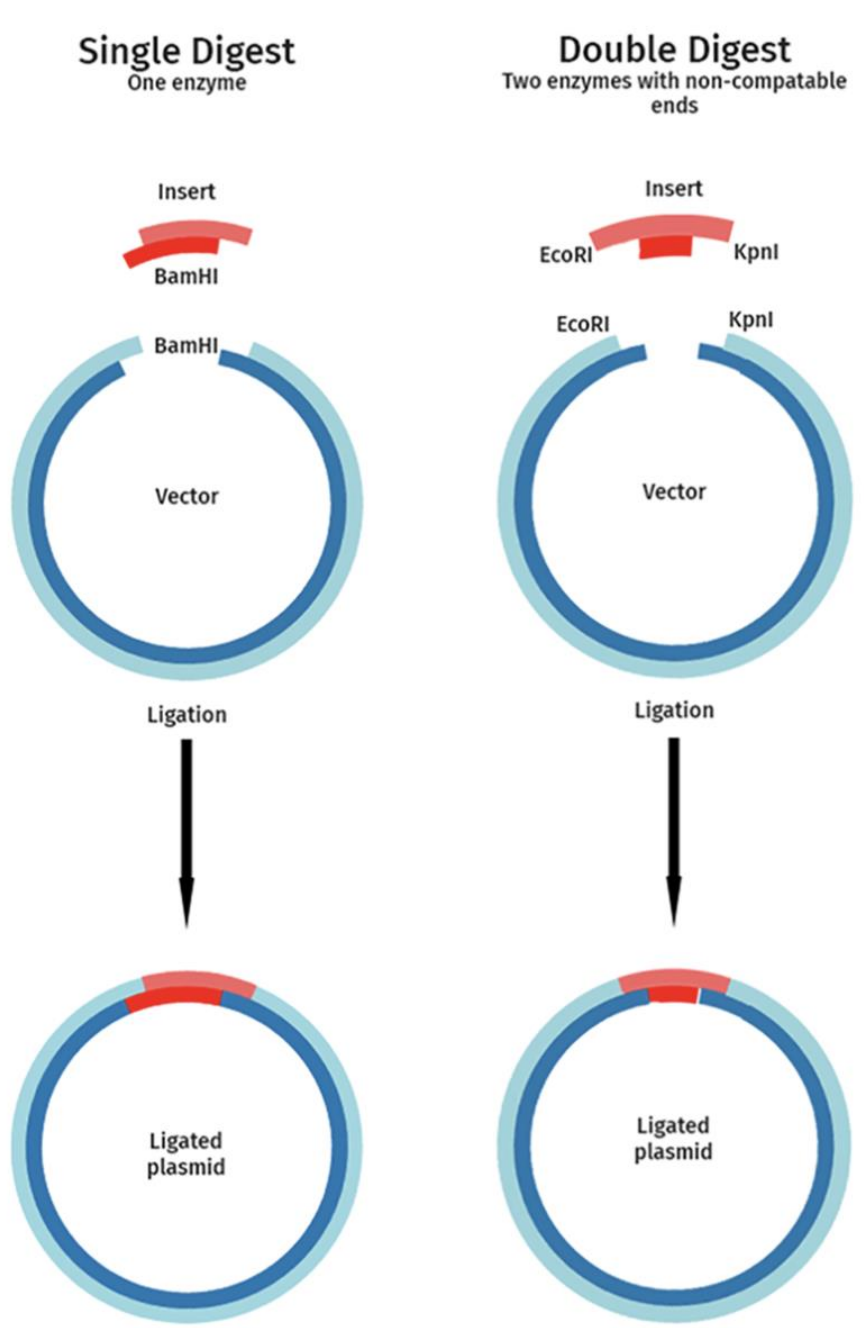
2. Restriction sites (Enzymes)

- Enzymes that **recognise a specific base sequence in DNA and cleave at that site**.
- **"Molecular scissors"**



❖ Digestion

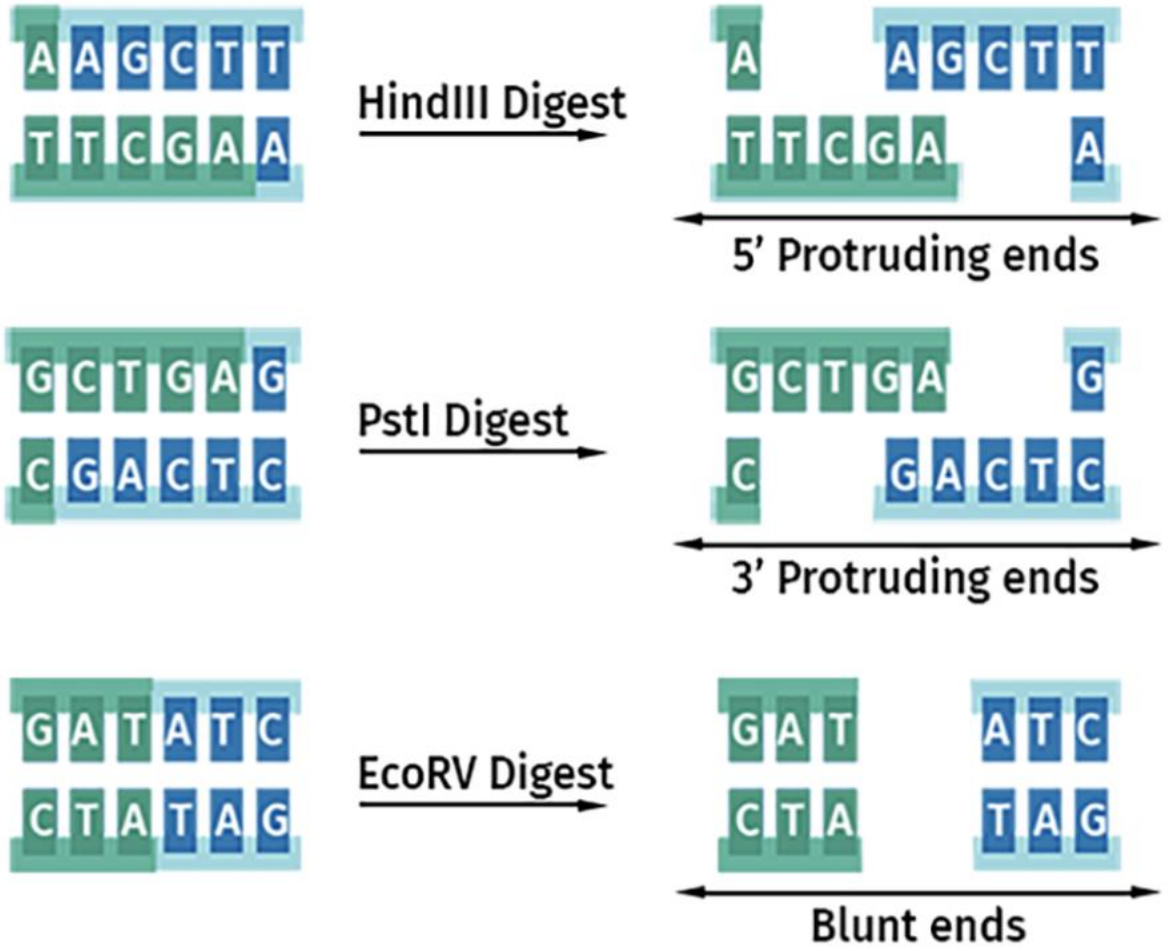
- **Digestion is the process of cutting DNA** using **restriction enzymes**. There are two types of digestion:
  - **Single Digestion** ⟶ Uses **one restriction** enzyme to cut the DNA at a specific site.
  - **Double Digestion** ⟶ Uses **two different restriction enzymes** simultaneously to cut at **two distinct sites**, which helps in precise cloning and directional insertion of DNA fragments.



Single vs. double digest

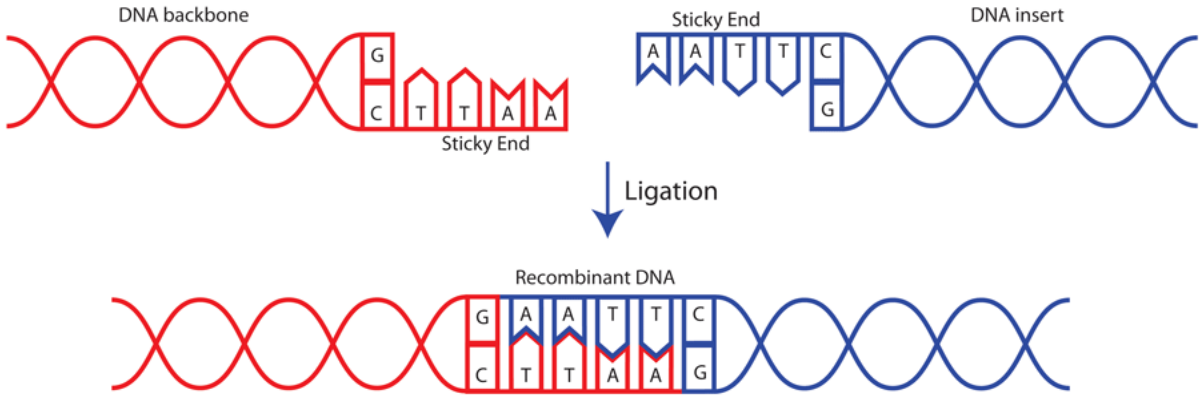
❖ Recognition sequence

- **Palindrome** - sequence is **read the same on either strand**, when read from 5′ to 3′.
- Creates either **sticky ends or blunt ends.**



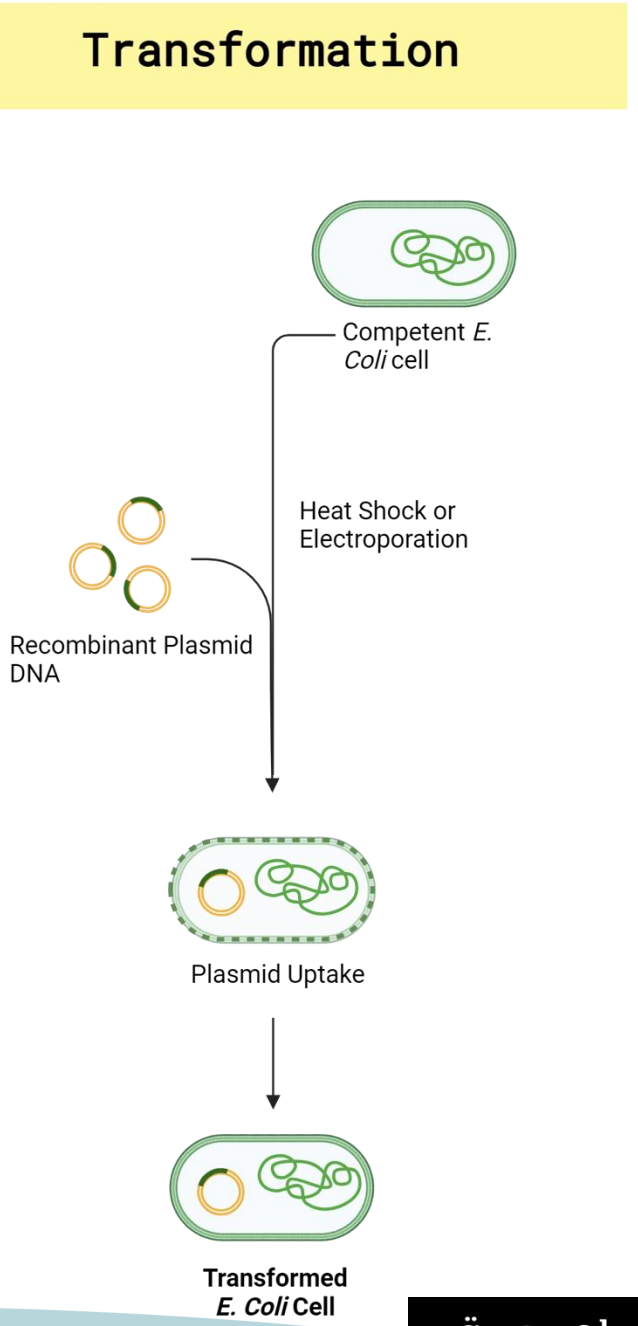
3. Ligation

- **Ligation is the process of joining DNA fragments** together using the enzyme **DNA ligase**.
- When DNA fragments have **complementary overhangs (sticky ends)** after digestion, ligase easily joins them through base pairing and covalent bonding.



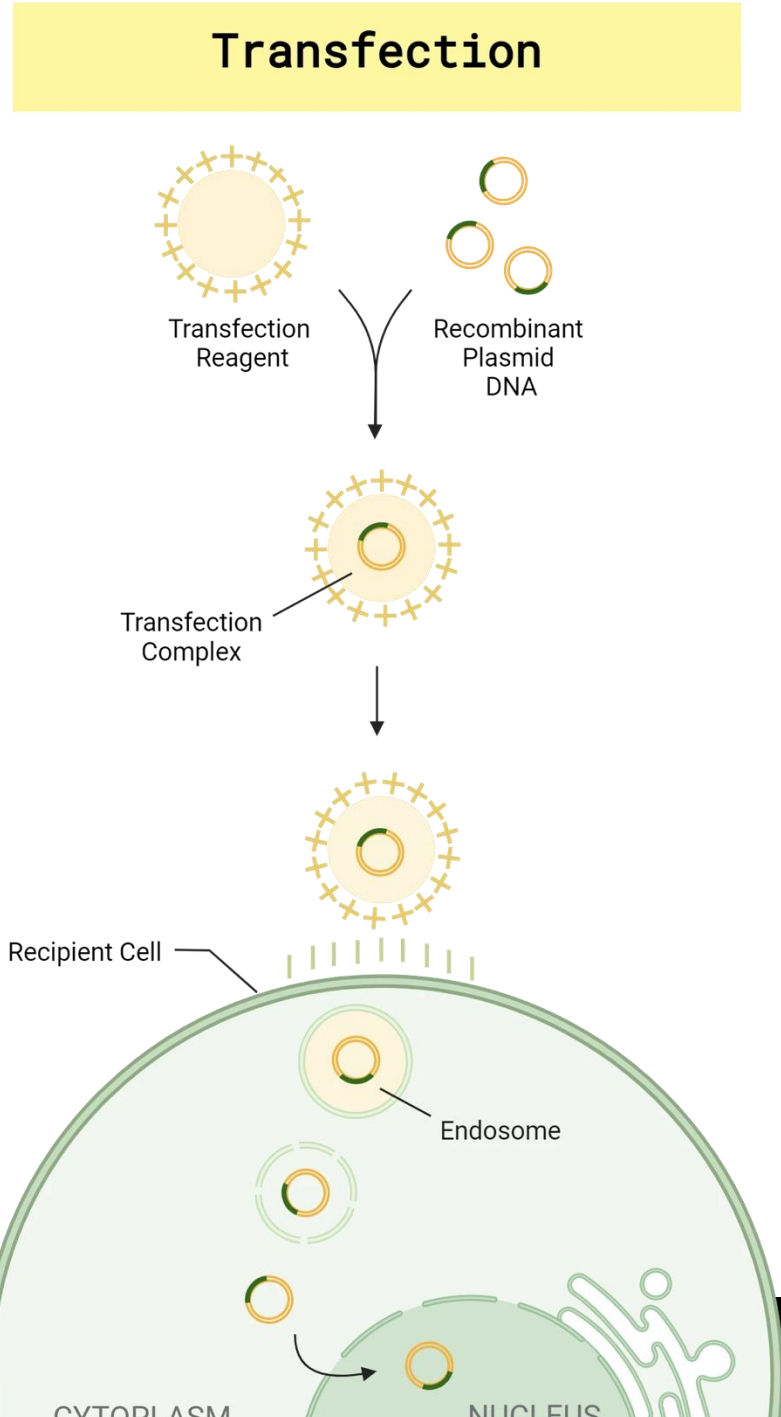
4. Transformation and Transfection

- **Transformation** is the uptake of foreign DNA from the environment, such as plasmid DNA, typically by bacterial cells
- ***E. coli* competent cells** transformations are regularly used to propagate recombinant plasmid in fast-growing bacteria for purification and further analysis.

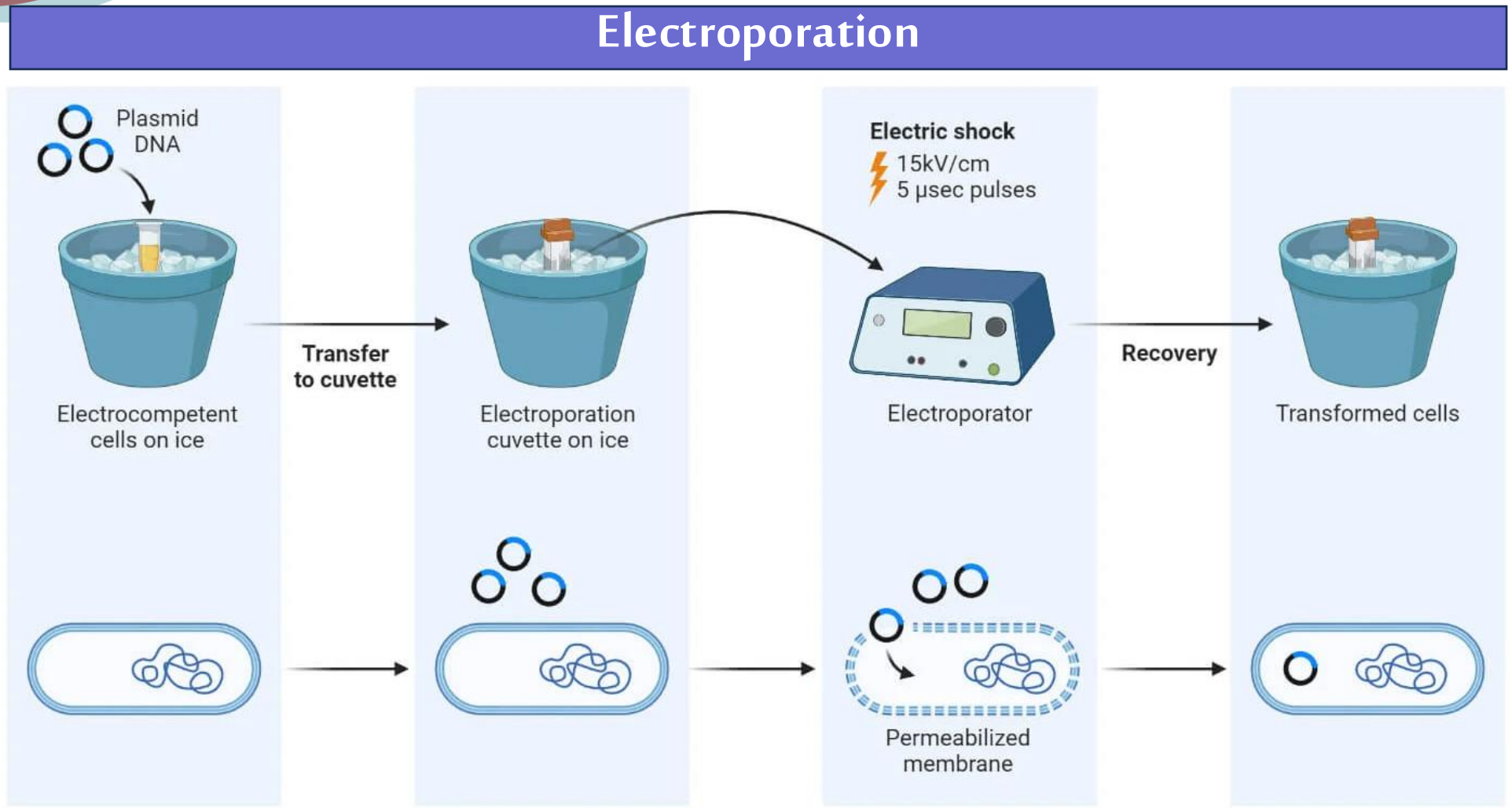


4. Transformation and Transfection

- **Transfection** is s when eukaryotic cells take up foreign DNA or RNA by non-viral means.
- **Chemical transfection reagents (Lipid-based)**, form positively charged nucleic acid containing liposomes that can integrate with the host membrane, enabling the entry of foreign nucleic acids, such as plasmid DNA.

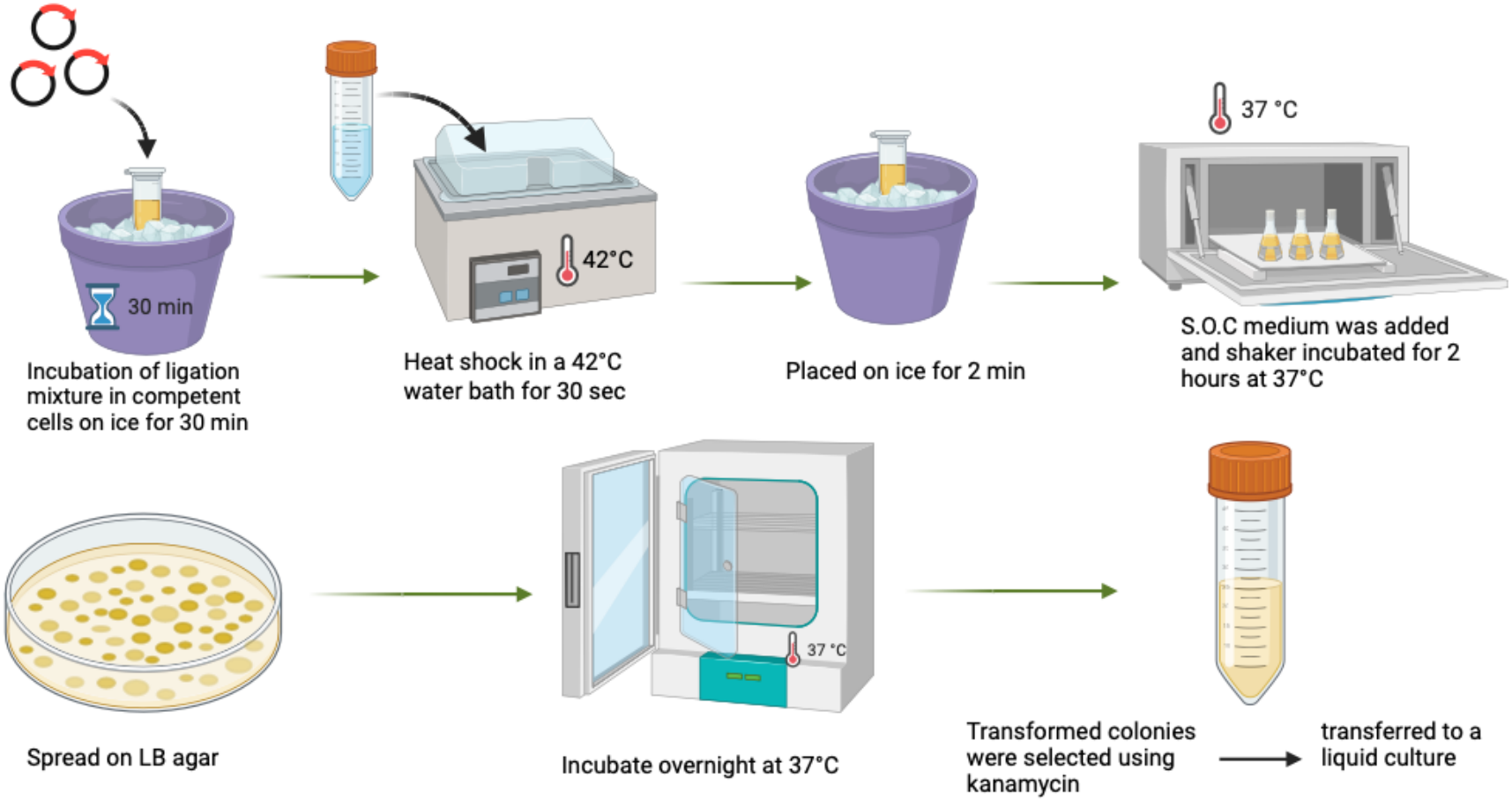


4. Transformation Protocol



4. Transformation Protocol

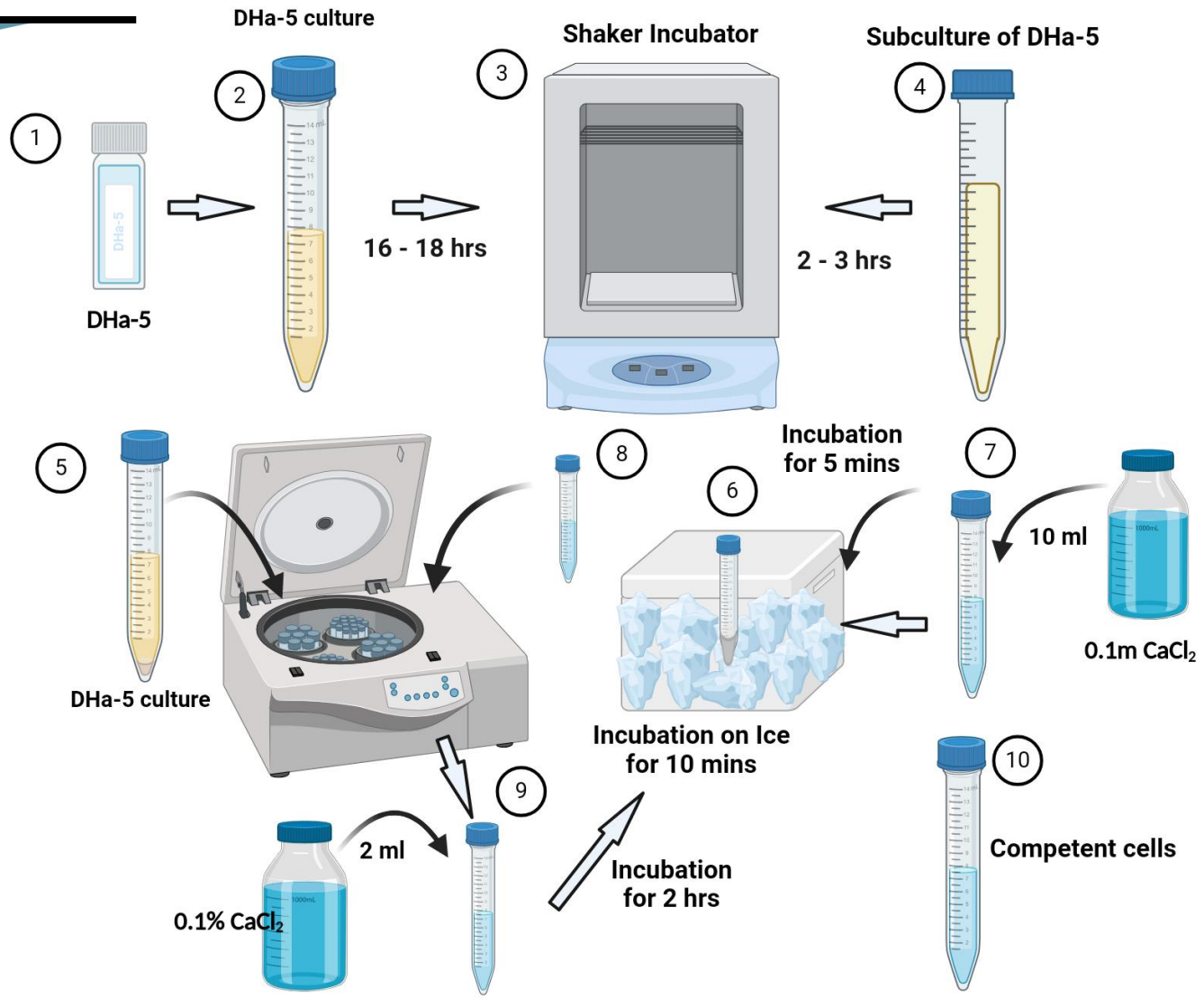
Heat Shock



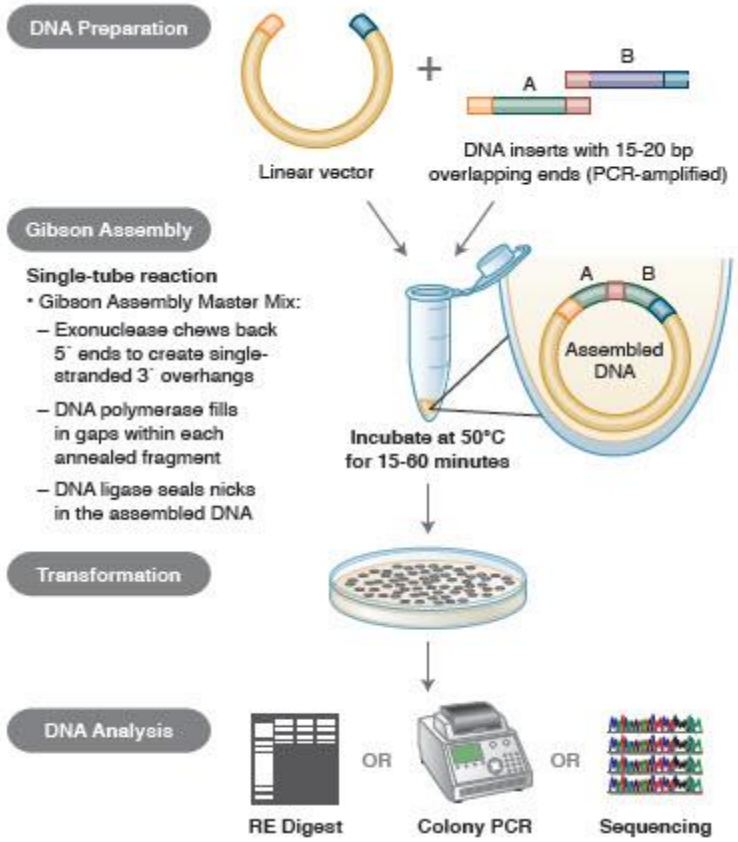
5. Competent Cells

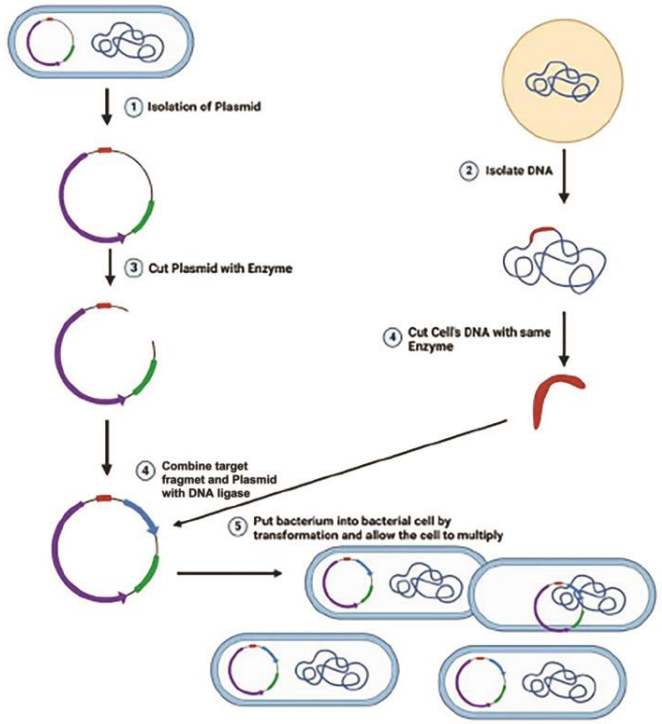
- **Competent cells** are bacterial cells that have an enhanced ability to uptake foreign DNA from their surroundings compared to normal cells.
- **Horizontal gene transfer (HGT)** is more difficult in eukaryotic cells than prokaryotic cells because genetic material must get through both the cell membrane and the nuclear membrane, which is absent in bacterial cells.
- Competent cells can be **prepared chemically** using calcium chloride ($CaCl_2$) or **obtained commercially, such as ClearColi™.**

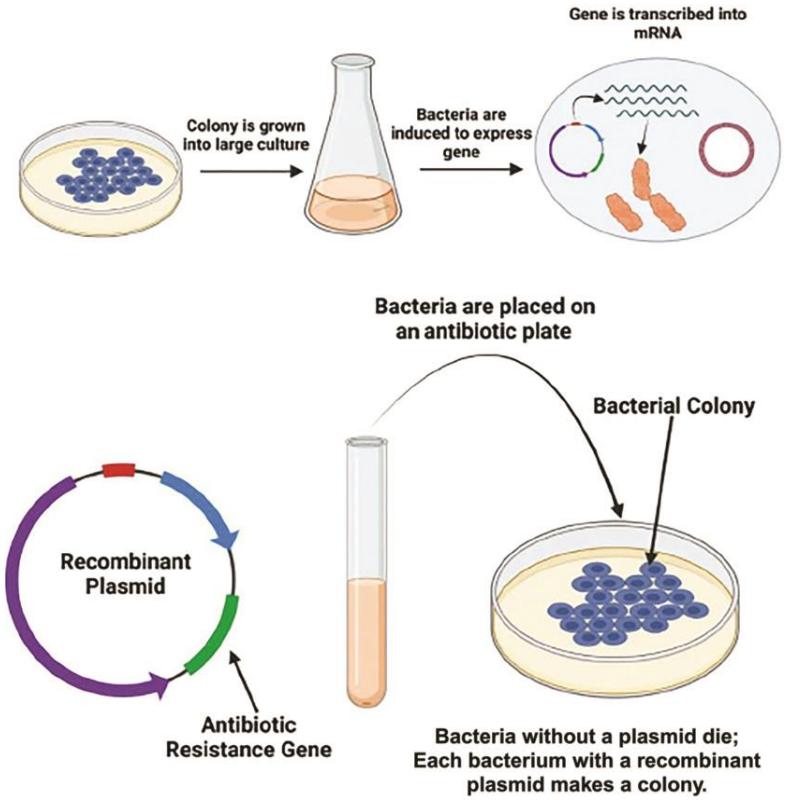
5. Competent Cells



DNA Cloning Protocol







DNA Cloning Protocol

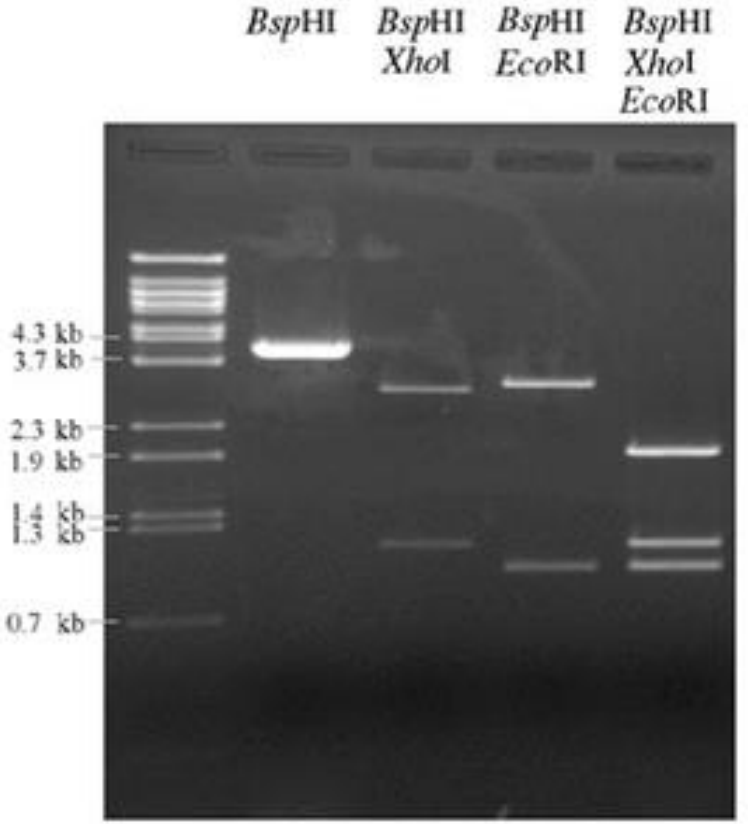
- Often **one desires** to insert foreign DNA in a **particular orientation**.
- This can be done by **making two cleavages with two different restriction enzymes.**
- Construct foreign DNA with **same two restriction enzymes**.
- Foreign DNA can **only be inserted in one direction**.
- To avoid high background of non-recombinants, **alkaline phosphatase** is used to remove 5' phosphate groups from the **cut vector to prevent self-ligation.**

Identification of Positive Clones

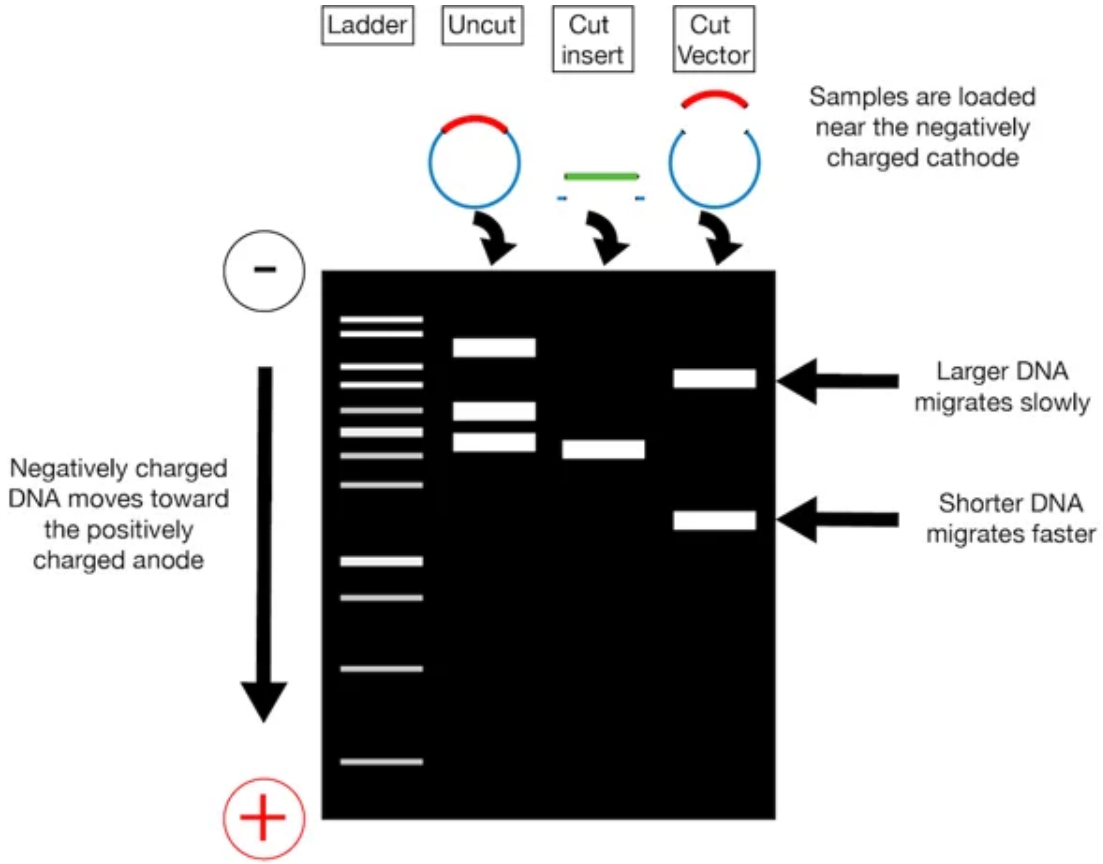
- One of the first steps is to **identify clones carrying the recombinant plasmid**, with the **desired DNA insert**.
- This can be done by 'picking' clones - choosing individual bacterial colonies to isolate the plasmid DNA from each of them.
- Single bacterial colonies are grown in culture broth containing **the selection antibiotic** to maintain the plasmid.
- The plasmid DNA is extracted by the standard **minipreparation technique** and then analysed by restriction digest.

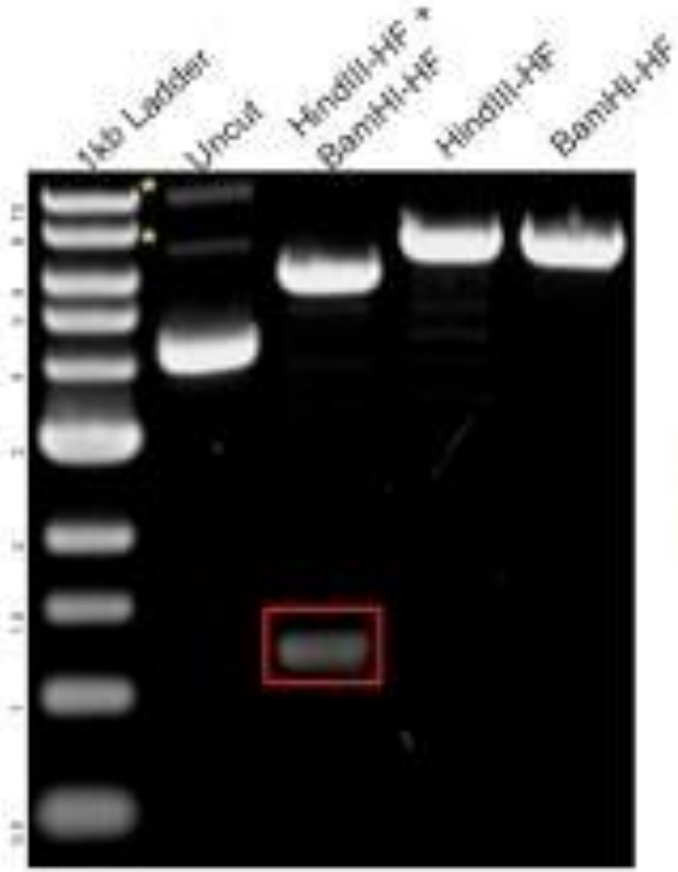
Identification of Positive Clones

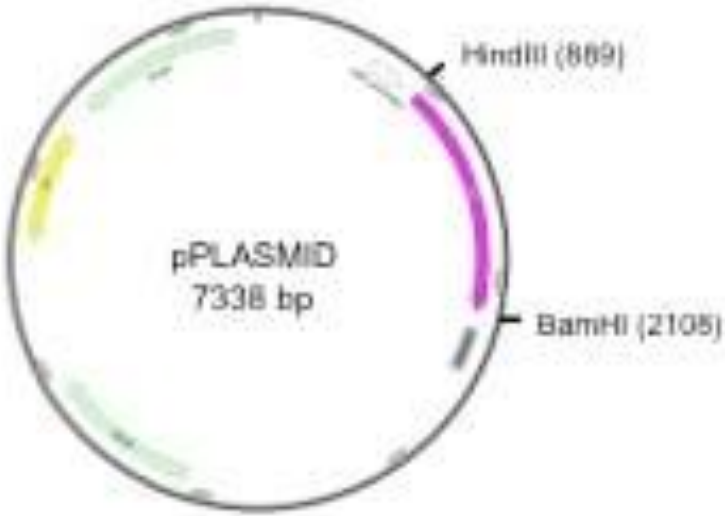
- After digesting the DNA, **different sized fragments are separated** by **agarose gel electrophoresis** and the sizes determined by comparison with known DNA molecular weight marker.
- **Gel extraction** can then be performed to remove the DNA from the gel, this will purify the DNA from any buffer components.



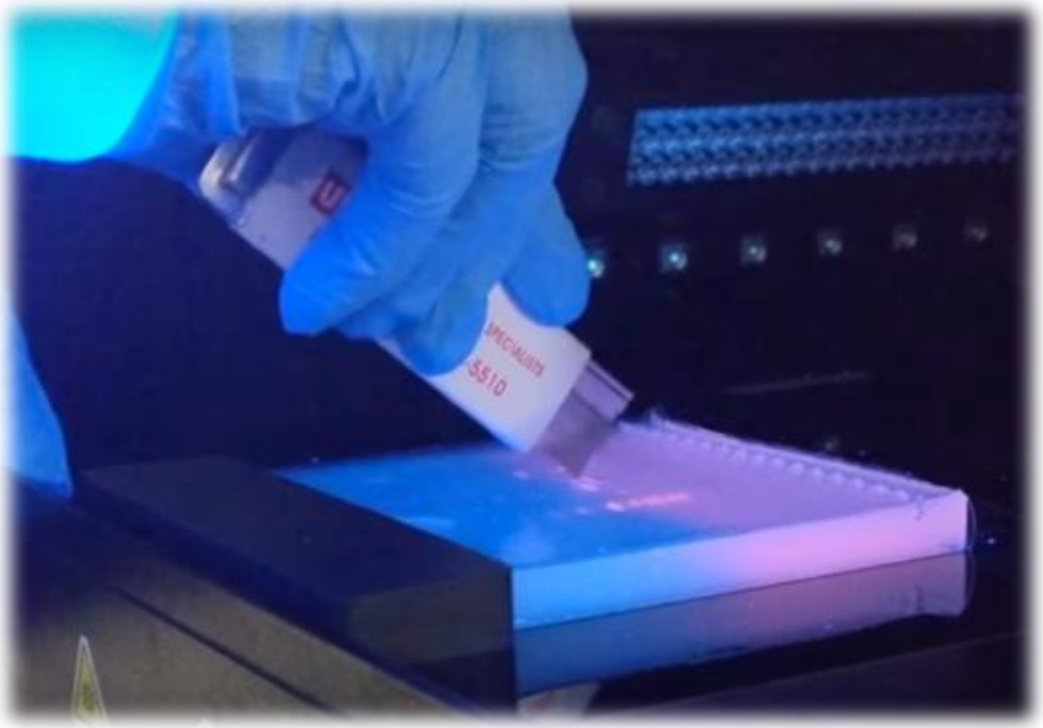
Identification of Positive Clones







Identification of Positive Clones



Recombinant DNA

- R.E. are a useful tool for **analysing Recombinant DNA** by:
  - Checking the size of the insert
  - Checking the orientation of the insert
- Sometimes it is important to determine **the orientation of the DNA insert** in relation to the vector sequence.

⟶ by using R.E that **cut the vector sequence near to the insert** and **cut within the insert sequence (asymmetrically).**

PCR Cloning Considerations

- **Nature of the Insert:** Not all PCR fragments will clone with the same efficiency into the same vector.
- **Insert Size:** The size of the fragment being cloned is a primary contributor to the overall cloning efficiency. **Large fragments** of DNA (≥ 5 kb) are amenable to cloning in high-copy number vectors, yet at a much **lower efficiency.**
- **Vector-to-Insert Ratio:** Optimisation of molar concentration ratios of the vector to insert is critical to ensure efficient cloning.

⟶ In most cases, when the vector's size is **larger** than the insert's size, **a ratio 3:1 insert to vector** should be suitable.

Analysis of Cloned DNA

1. **Restriction mapping** ⟶ determining the order of restriction sites in a cloned fragment.

2. **Gel electrophoresis** ⟶ separates DNA fragments by molecular weight.

3. **Southern Blot analysis** ⟶ DNA is transferred ("blotted") to filter paper. Filter is exposed to a DNA probe, binds specifically to target DNA immobilized on filter.

4. **DNA sequencing** ⟶ provides complete order of bases in a DNA fragment.

Exercise 2:
Performing Virtual DNA Cloning

- **Human Growth Hormone (hGH)**, encoded by the **GH1 gene**, is essential for growth, metabolism, and tissue regeneration. Recombinant hGH is widely used in treating growth disorders and metabolic conditions.
- In this assignment, you are going to **simulate the cloning of the GH1 gene into the pET22b (+) vector** using SnapGene. This process mimics real-world genetic engineering techniques used in biopharmaceutical production.

Instructions

1. Obtain the human GH1 gene coding sequence

- Download from PBD (Accession: 1HGU).

2. Download the pET22b Vector

- Download the pET22b(+) plasmid map from Addgene.
- This vector contains a T7 promoter for **high-level expression in *E. coli*** and an **Ampicillin** resistance gene.

3. Select Restriction Enzymes

- Identify suitable restriction sites on both the GH1 gene and pET22b.
- Suggested enzymes: **EcoRI and XhoI.**

4. Perform Virtual Cloning in SnapGene

- **Digest** both the GH1 gene and pET22b with the selected restriction enzymes.
- **Ligate** the GH1 gene into the vector using SnapGene's ligation tool.
- Verify correct orientation using SnapGene's sequence **analysis feature**.

5. Simulate Gel Electrophoresis

- **Run a virtual agarose gel** to confirm correct insert size and successful cloning.
- Compare fragment sizes before and after cloning.

6. Submit a Report (Include the Following)

- ✅ Final plasmid map (screenshot from SnapGene).
- ✅ List of restriction enzymes used and justification.
- ✅ Virtual gel electrophoresis image with an explanation.
- ✅ A brief explanation of the cloning process and how you verified success.

Best of Luck!

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