Genetics Engineering (Zoo-455)

Principle and steps involved in CDS cloning of a gene

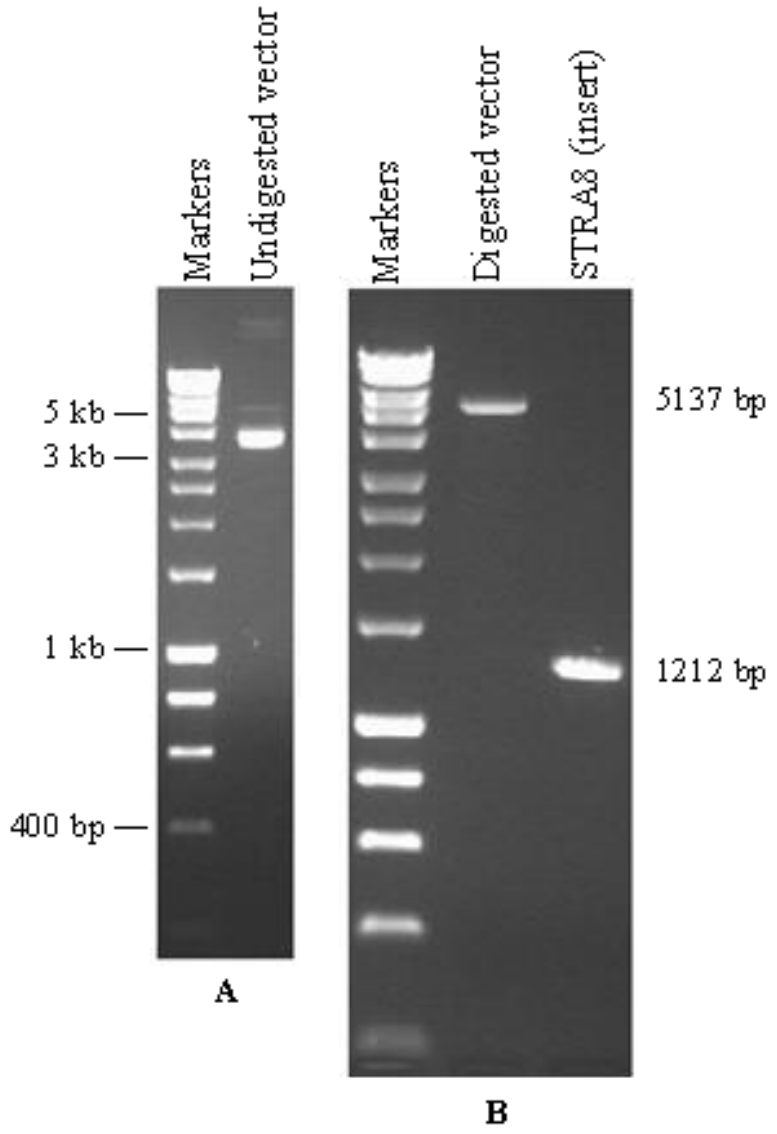
Lecture-6

Principle of CDS cloning:

1) Primers design for cloning (Refer to Lecture 2).
2) Isolation of mRNA (Refer to Lecture 5)
3) Synthesis of cDNA (Refer to Lecture 5).
4) PCR Amplification (Refer to Lecture 5).
5) Loading of PCR product onto agarose gel electrophoresis (Refer to Lecture 5).
6) Digestion of PCR insert by restriction enzyme (Refer to Lecture 3).
7) Digestion of vector with the same restriction enzyme used in PCR insert.
8) Calculation the insert and vector molar ratio for ligation.
9) Ligation of digested PCR insert and vector
10) Transformation of recombination vector into competent *E. coli* cells.
11) PCR colony screening.
12) Purification and digestion of recombinant vector from E. coli
13) Confirming the orientation and DNA sequencing of insert.

Digestion of PCR insert and vector with *Xho*I restriction enzyme

Figure. Amplification of *STRA8 CDS* from the testis cDNA. Agarose gel picture showing undigested (panel **(A)**). Panel **(B)** (right) shows the vector after digestion by *Xho*I restriction enzyme and purification. The enzyme linearizes the 5137 bp plasmid into one single fragment. The right side of gel B displays the amplification of the *STAR8* CDS sequence from the testis cDNA. This band was digested with the same restriction enzyme, *Xho*I, and then underwent purification.



Calculation the insert and vector molar ratio for ligation

- The amount of insert and vector needed was calculated using the following formula:
- The amount of insert = Amount of vector in ng × Length of insert in bp × molar ratio / length of vector in bp
  - **For example:**
    - If the concentration of vector = 20 ng/µL, and volume = 50 µL. Calculate the amount of vector !!.
    - For typical cloning, 50 ng of the vector is recommended.
    - If the concentration of insert = 10 ng/µL
    - Length of insert (STRA8) = 1212 bp
    - Length of vector (pcDNA5/FRT/TO) = 5137 bp
    - For insert of similar size to the vector, a 1:1 molar ratio of insert to vector is recommended.
    - If the insert is smaller than the vector, a 3:1 molar ratio of insert to vector is recommended

- The amount of insert = 50 × 1212 × 3/5137 = 35 ng
- 35 ng of insert = 35 × 1/10 = 3.5 µL

Ligation of digested PCR insert and vector

- **Ligation reaction:**
  - Add 3.5 µL of insert
  - Add 2.5 µL of vector
  - Add 4 µL of nuclease-free water
  - Add 10 µL of 2x quick ligation buffer and mix
  - Add 1 µL of quick T4 DNA ligase and mix thoroughly
  - Centrifuge briefly and incubate at RT (25°C) for 5 min
  - Chill on ice for 1 min and then transform or store at -20°C
- **T4 DNA Ligase:**
  - Derived from the T4 bacteriophage representing the most commonly used ligases in cloning protocol.
  - Formation of a phosphodiester bond between 5'-phosphate and 3'-hydroxyl.

Features and types of the host cells for cloning vectors:

- Ideal host cells should be:
  - Capable of rapid growth in inexpensive medium
  - Nonpathogenic
  - Capable of incorporating DNA
  - Genetically stable in culture
  - Equipped with appropriate enzymes to allow replication of the vector
- *Escherichia coli, Bacillus subtilis, Saccharomyces cerevisiae*
- *E. coli* is one of the most commonly used hosts in gene cloning.

Transformation of recombination vector into competent *E. coli* cells

- Inserting a gene into a vector is an important first step in the gene cloning process.
- If the ultimate goal is to produce a large amount of a particular protein, the vector must replicate to make sure that there are many copies of the gene to produce the encoded protein.
- A process of inserting recombinant vector into *E. coli* bacteria is called transformation.
- *E. coli* bacteria have complex plasma membranes that separate the external environment from the internal environment of the cell and carefully regulate which substances can enter and exit the cell.
- In addition, the cell wall is negatively charged and repels negatively charged DNA molecules.
- Competent ***E. coli*** cells can be made by treating the bacteria with a calcium solution.
- Calcium ions are positively charged, and will neutralize the negatively charged outer membrane on the *E. coli* bacteria.
- With the positive charge now coating the membrane, the negatively charged DNA molecules will move through the plasma membranes and into the cell.

Methods of Transformations:

1) **Electroporation**: The use of high-voltage electric shock to create tiny holes in the bacteria cell wall that allow recombinant DNA to eneter.

2) **Chemical transformation:**

- Treat bacterial cells with calcium chloride
- The use of heating shock to introduce recombinant DNA into *E. coli* cells (Put the cells on ice, follow by heating shock at 42°C and then place the cells on ice.

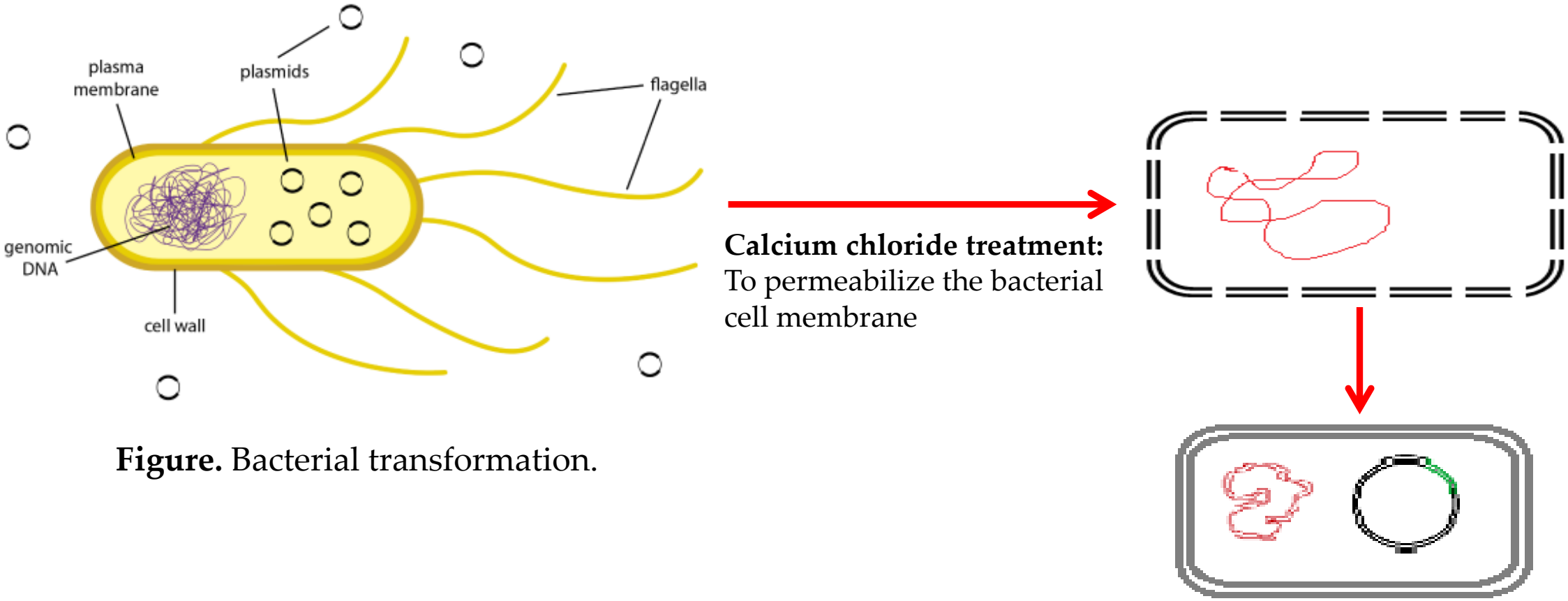
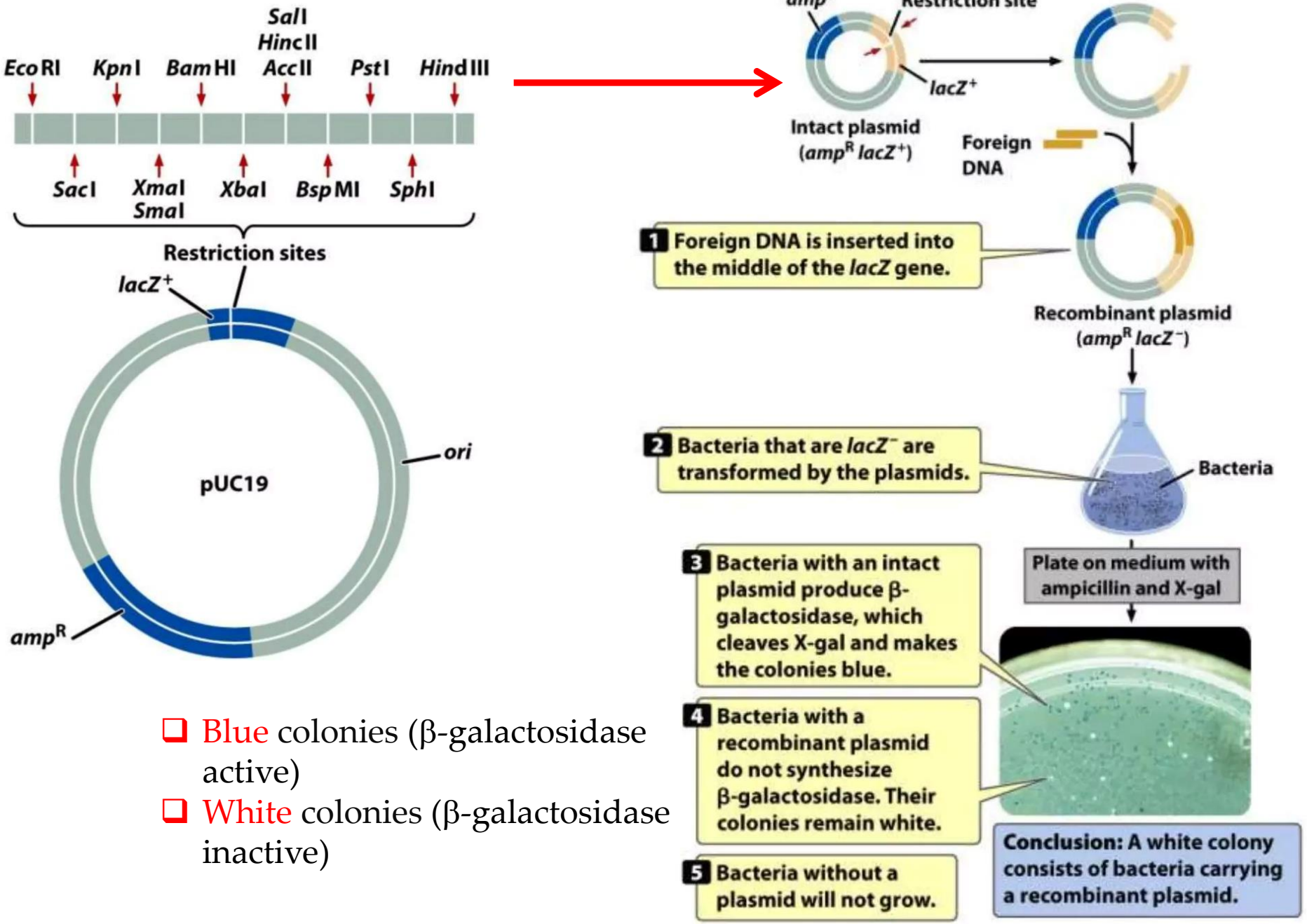


Figure. Bacterial transformation.

Types of selection methods of recombinant bacteria after transformation:

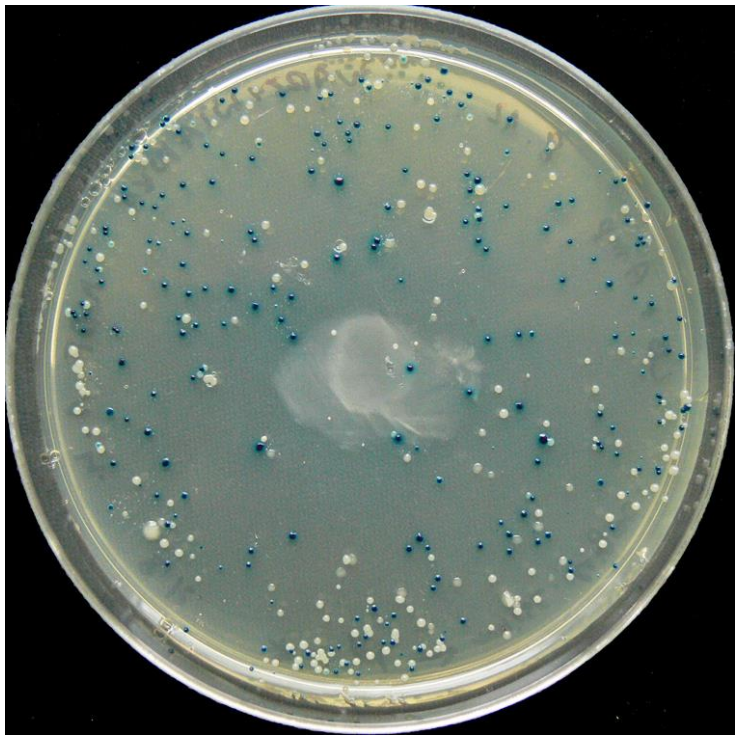
- Selection is a process designed to facilitate the identification of recombinant bacteria while preventing the growth of non-transformed bacteria and bacteria that contain plasmid without foreign DNA.

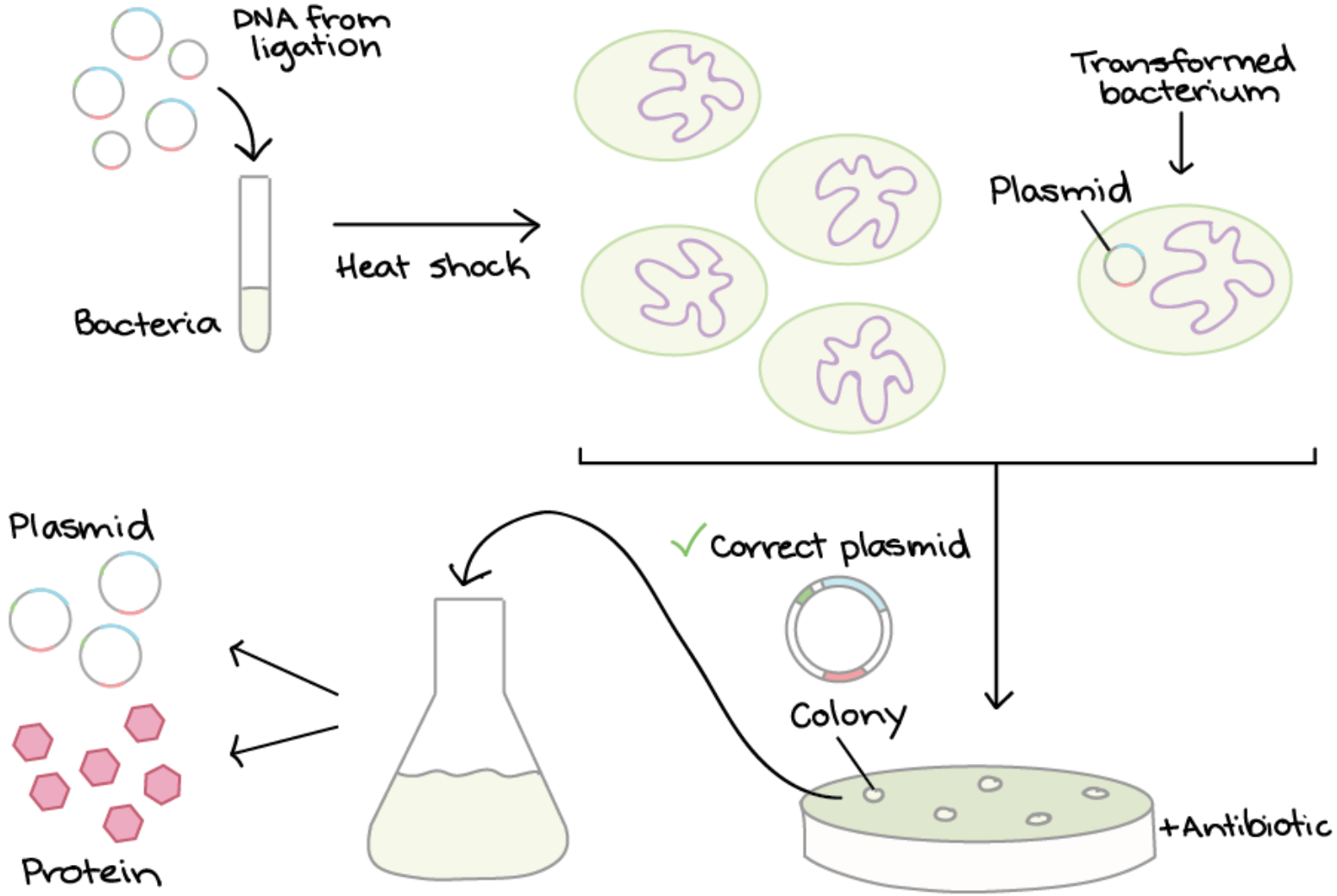
1) Antibiotic selection: plate transformed cells on Petri dishes plates containing a specific antibiotic to identify recombinant bacteria and non-transformed bacteria.
2) Blue-white selection:
   - The *lacZ* gene activity results in the production of β-galactoisdase enzyme that metabolizes the lactose.
   - DNA insert is cloned into the restriction site in the *lacZ* gene of the vector.
   - The *lacZ* gene is disrupted by the insertion of foreign DNA and cannot produce β-galactosidase.
   - When X-gal (artificial lactose) is added to the plate, if functional *lacZ* is present = blue colony
   - The β-galactosidase enzyme can break down the X-gal into galactose and an insoluble blue pigment.
   - Non-functional lacZ= white colony = clone = genetically identical bacterial cells each containing copies of recombinant plasmid.
   - The presence of lactose in the surrounding environment triggers the *lacZ* gene in *E. coli*.



- Blue colonies (β-galactosidase active)
- White colonies (β-galactosidase inactive)

Steps of bacterial transformation and selection:





PCR colony screening:

- One colony was picked from the overnight plates by using a micropipette tip and rinsing it into a new Eppendorf tube containing medium with antibiotic (ampicillin).
- PCR screening is directly carried out.

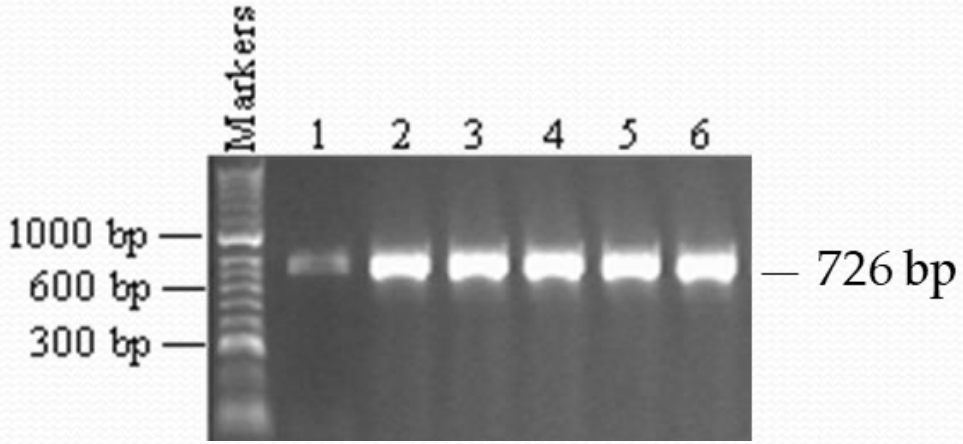


Figure. PCR screening of colonies for the cloning of *STRA8*. Agarose gel picture showing PCR screening of *E. coli* colonies for the cloning of *STRA8* into vector using an internal primer for the *STAR8* gene. The results show that all the 6 samples contain a *STRA8* insert of the expected size, 726 bp.

Purification and digestion of recombinant vector from *E. coli*:

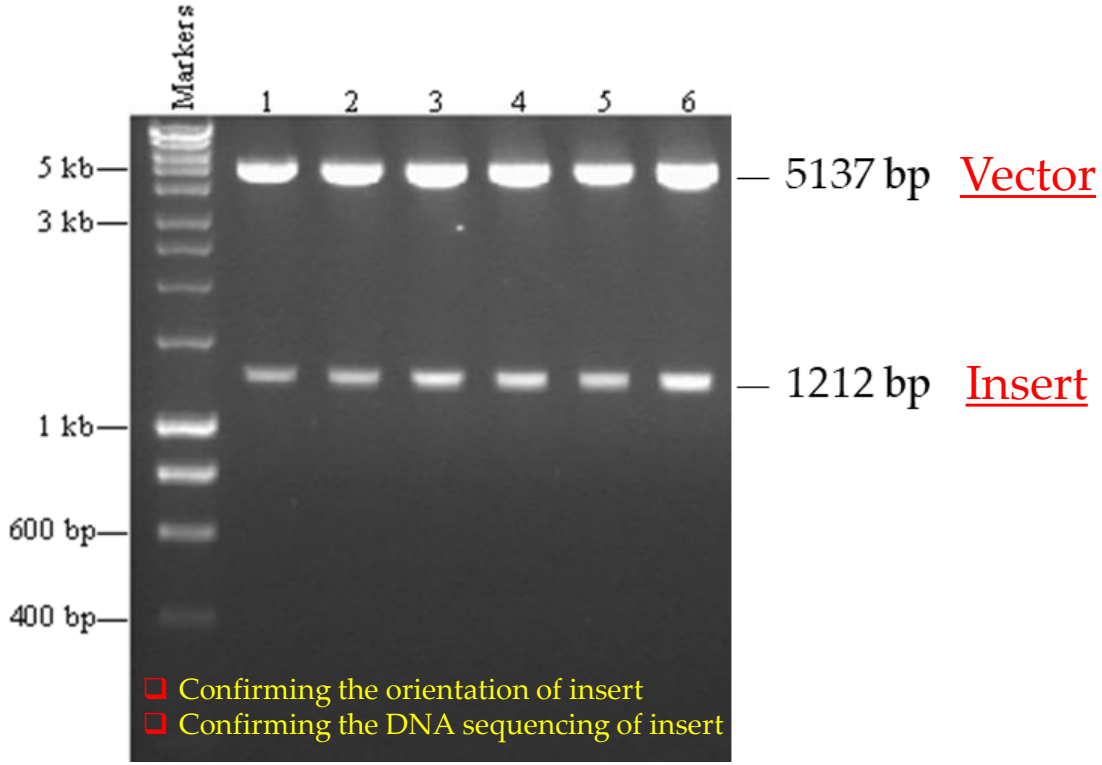
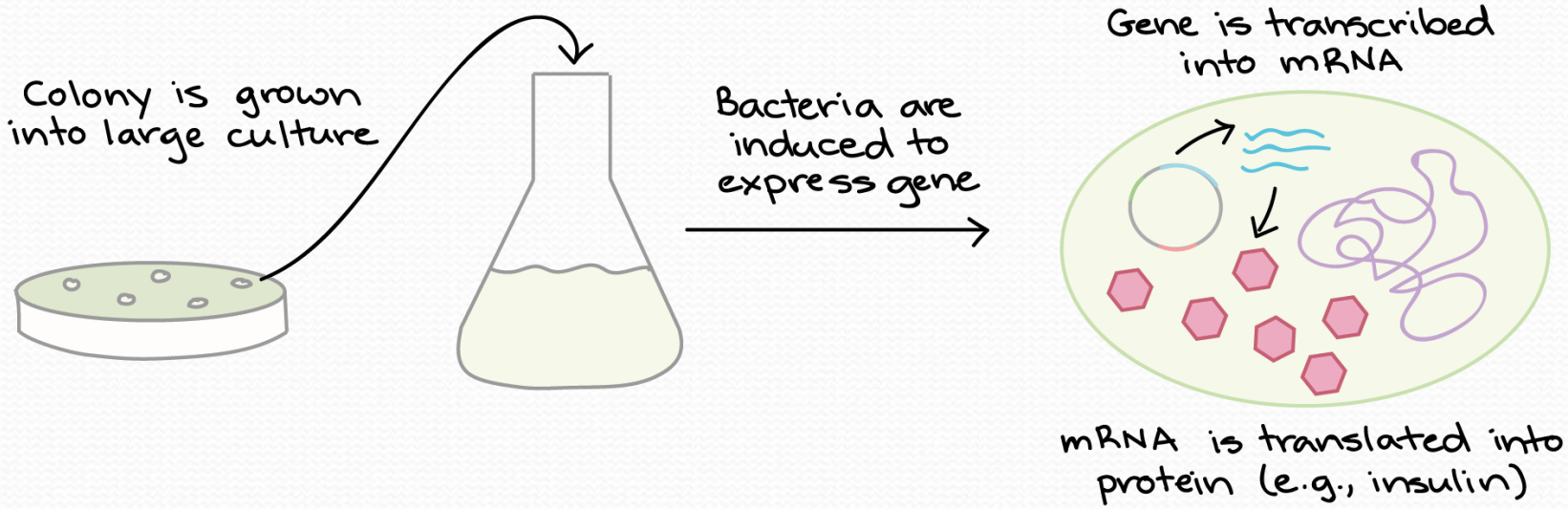


Figure. Digestion of recombinant vectors. Agarose gel picture showing the digestion of the recombinant purified vectors by the *Xho*I restriction enzyme. Two bands are formed for all the recombinants. The upper bands show a vector of 5137 bp, while the lower bands show the full open reading frame of the *STRA8* gene of 1209 bp in size. All the samples in lanes 1-6 show successful cloning of *STRA8* CDS.

Protein production:

- Once we have found a bacterial colony with the right vector, we can grow a large culture of bacteria. Then, we give the bacteria a chemical signal that instructs them to make the target protein.
- The bacteria serve as factories to produce large amounts of protein. For instance, if our vector contained the human insulin gene, the bacteria would start transcribing the gene and translating the mRNA to produce many molecules of human insulin protein.



- The target protein must be purified, or separated from the other contents of the cells by biochemical techniques.

Questions?