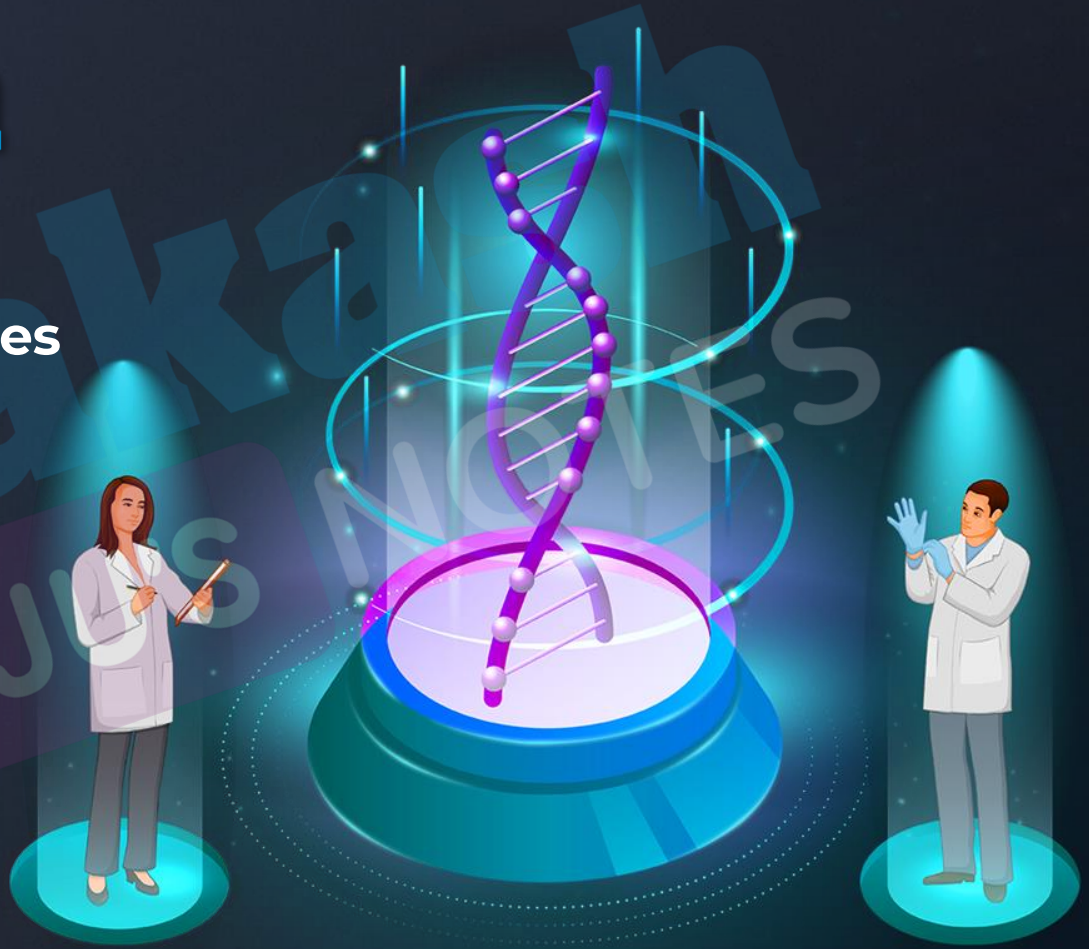
Aakash + BYJU'S NOTES

Biotechnology Principles and Processes



Key Takeaways

1. **Biotechnology**
   - Principles
   - Gene cloning
   - rDNA technology
2. **Tools of rDNA technology**
   - Restriction enzyme
   - DNA Ligase and DNA polymerase
   - Cloning vectors
   - Role of vector

3 **Selection using antibiotic resistance and insertional resistance**

4 **Process of rDNA technology**

- Isolation of the DNA
- Fragmentations of DNA by RE
- Separation and isolation of a desired DNA fragment
- Amplification of gene interest using PCR
- Ligation of DNA fragment into a vector
- Insertion of recombinant DNA into the host cell/organism
- Culturing the host cells in a nutrient medium for obtaining foreign gene product
- Extraction of the desired product through downstream processing

Summary

Biotechnology

- Biotechnology refers to the **use of living organisms** or **their products,** to **modify human health** and **human environment.**
- Examples of **microbe-mediated processes** are making curd, bread or wine and producing **genetically modified organisms.**
- Other processes are in vitro fertilisation (test-tube baby), genetic modification, developing a DNA vaccine.

Wine


Curd


Bread

European Federation of Biotechnology (EFB):

The integration of natural science and organisms, cells, parts thereof, and molecular analogues for products and services.

Principles of Biotechnology

Two core techniques that enabled birth of **modern biotechnology** are:

Genetic engineering

Techniques to alter the **chemistry** of **genetic material** (**DNA** and **RNA**) and change the phenotype of the organism

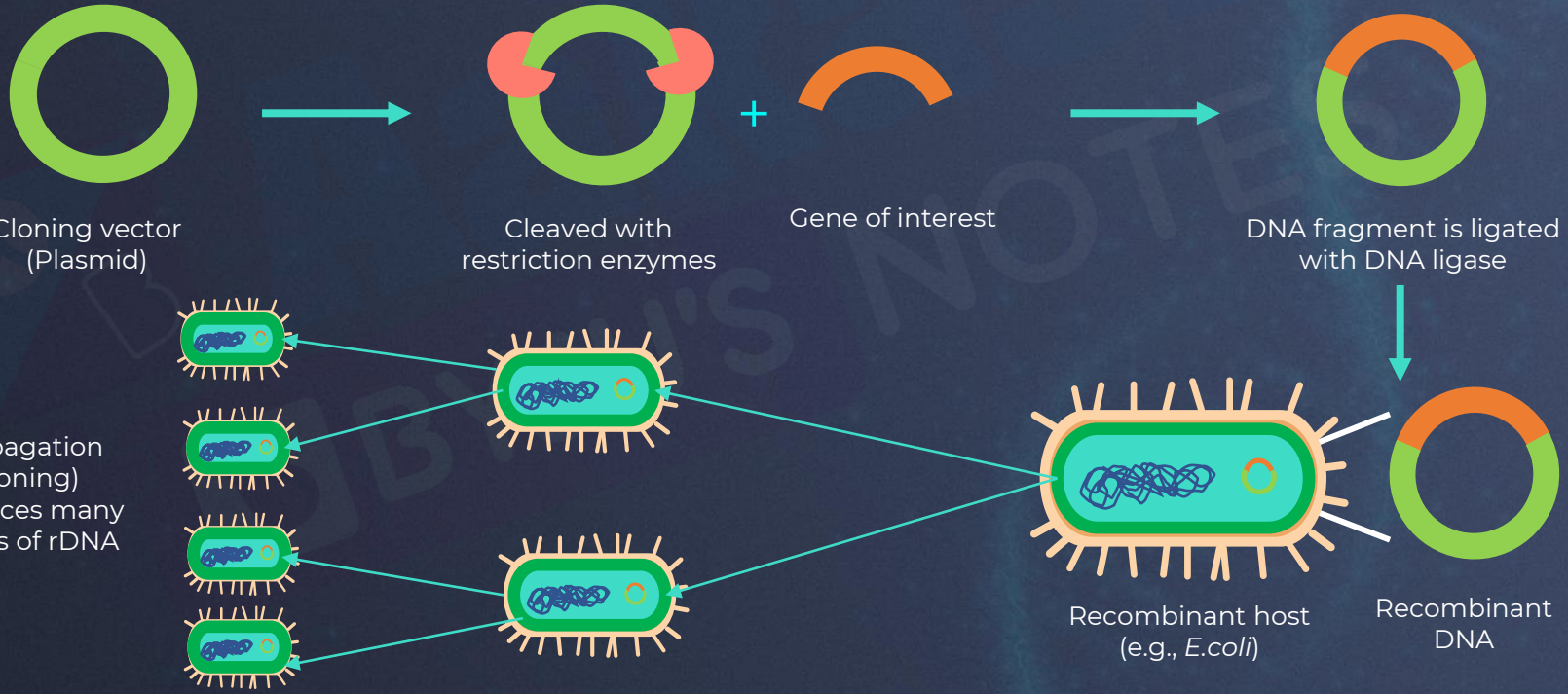
- **Gene cloning**
- **Gene transfer**
- **Recombinant DNA**

Bioprocess engineering

Maintenance of **sterile ambience** in chemical engineering processes to **enable growth** of only the desired microbial cells

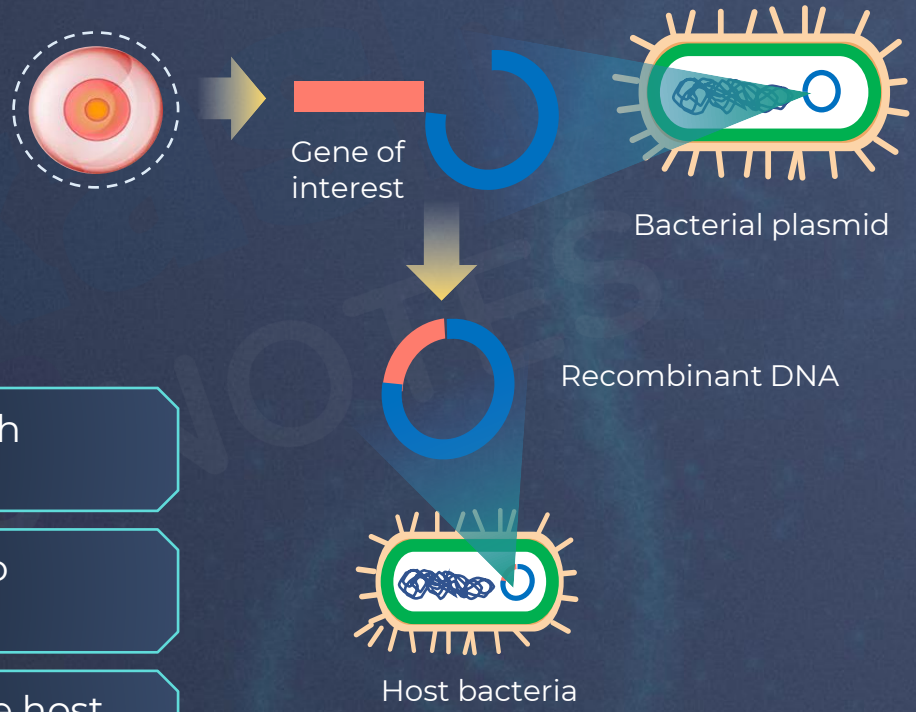
Gene Cloning

Cloning is making multiple identical copies of any template DNA



Recombinant DNA Technology

- Used for **introducing desired DNA sequence** in an organism
- First demonstrated by **Stanley Cohen and Herbert Boyer in 1972**



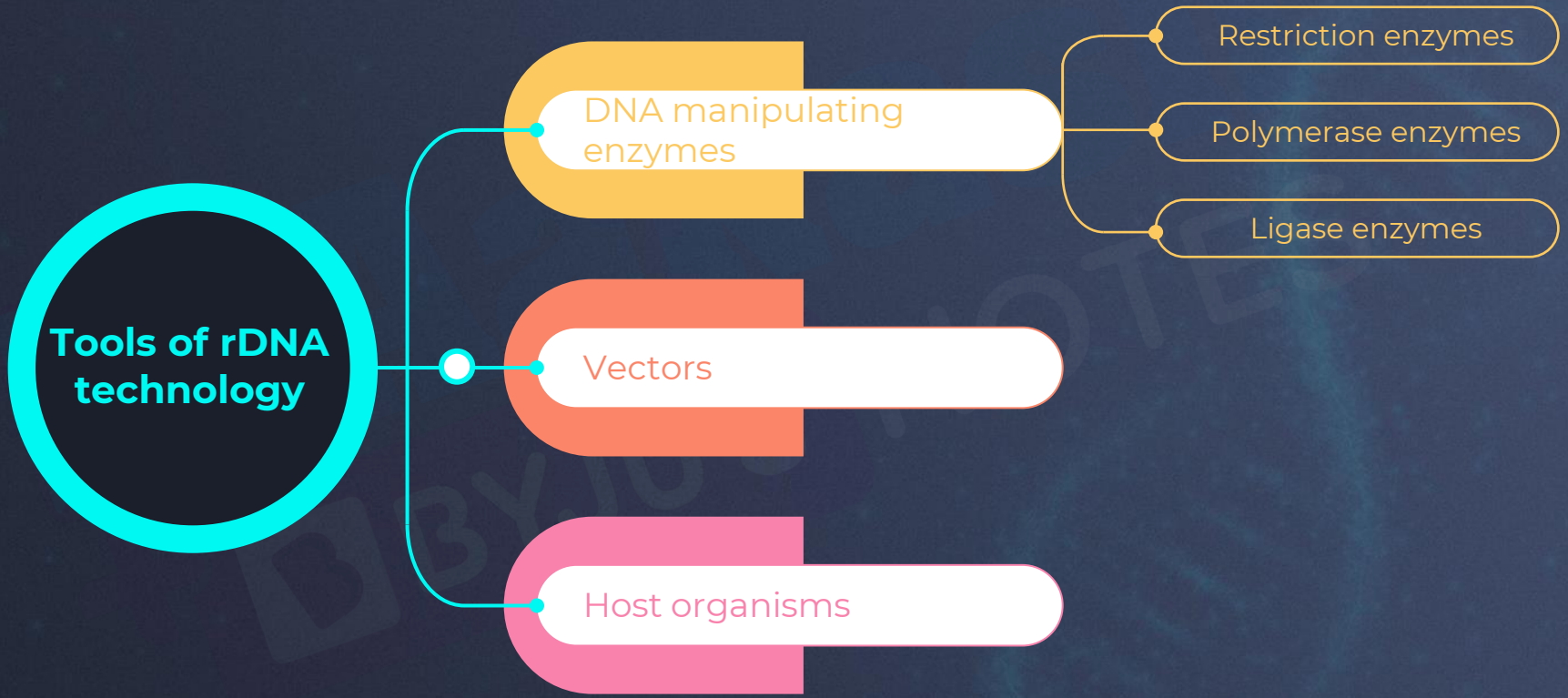
Steps of rDNA technology

01 Identification and isolation of DNA with desirable genes

02 Introduction of the identified DNA into the host

03 Maintenance of introduced DNA in the host and transfer of the DNA to its progeny

Tools of rDNA Technology



DNA Manipulating Enzymes

Restriction enzymes

- **Restriction enzymes** serve as knives to **cut genes** (DNA) into defined fragments
- Used :
  - to **determine** the order of genes
  - to **analyze** the chemical structure of genes
  - to **create** new combinations of genes
- **First restriction endonuclease** - *HIND II*
- **Recognition sequence** : specific base sequence at which DNA is cut by a particular restriction enzyme
- Number of isolated restriction enzymes - **900**

DNA Manipulating Enzymes

- Restriction enzymes belong to a larger class of enzymes called **nucleases**

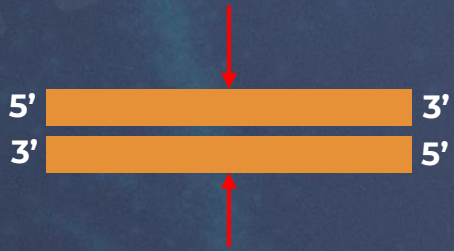
Restriction enzymes

Exonucleases

- Makes cut **at the end** of DNA molecule

Endonucleases

- Makes cut **within** the DNA molecule
- Recognizes a specific **palindromic nucleotide sequence** in the DNA
  - Palindrome is a group of letters that form the **same words, when read either forward** or **backward.**



DNA Manipulating Enzymes

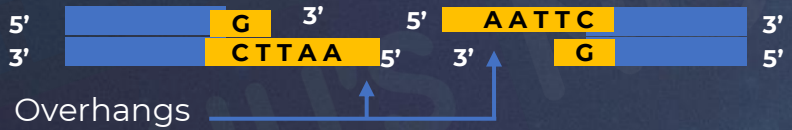
DNA Ligase

- DNA cut by the same restriction enzyme results in DNA fragments with same kind of **'sticky-ends'**
- Sticky ends can be joined together (end-to-end) using **DNA ligases**

DNA Polymerase

- **Synthesize new strand of DNA** complementary to an existing DNA template in 5' to 3' direction
- Commonly used - **DNA Polymerase I**

EcoR I (Restriction endonuclease)



DNA Ligase - joins

DNA Polymerase - synthesize new strands

Cloning Vectors

- Vectors act as the carriers or vehicle for the **DNA fragment** while **transferring** it into the **host cell.**

Features required to facilitate cloning into a vector

1. Origin of replication (ori)

- Sequence from where **replication starts**
- Any piece of DNA when linked to this sequence can be made to **replicate** within the **host cells**
- Responsible for **controlling** the **copy number** of the **linked DNA**

2. Selectable marker

- Helps in **identifying** and **eliminating non-transformants** and **selectively** permitting the growth of the **transformants**

Cloning Vectors

3. Cloning sites

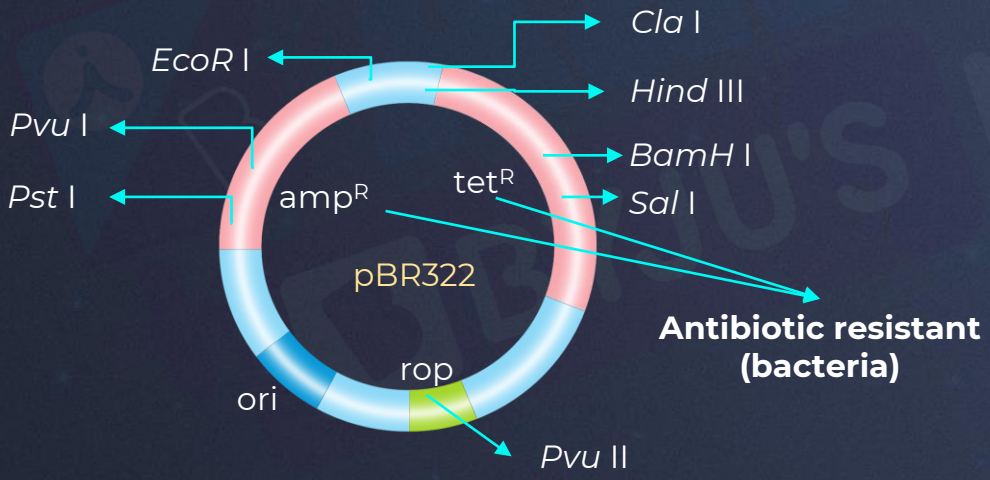
- Cloning site is the site where **DNA is inserted into the vector**.
- Restriction enzyme recognises the recognition site and cuts the DNA for insertion of foreign DNA.
- **Multiple cloning site**: is the region with a cluster of unique restriction enzyme sites in proximity.

4. Size of vectors

- Should be small, as large molecules have tendency to breakdown during purification.
- **Examples of vectors** commonly used in RDT are plasmids, bacteriophage, cosmid, YAC vector, BAC vector, phagemid, transposons etc.

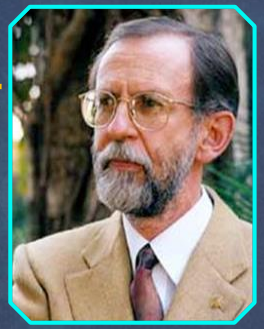
Plasmid - pBR322

- **Extrachromosomal** and **circular**
- **Non-essential** and **double-stranded**
- Autonomous and **self-replicating pieces of DNA** in bacteria and yeast



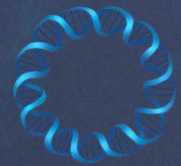
pBR322

Francisco Bolívar Zapata

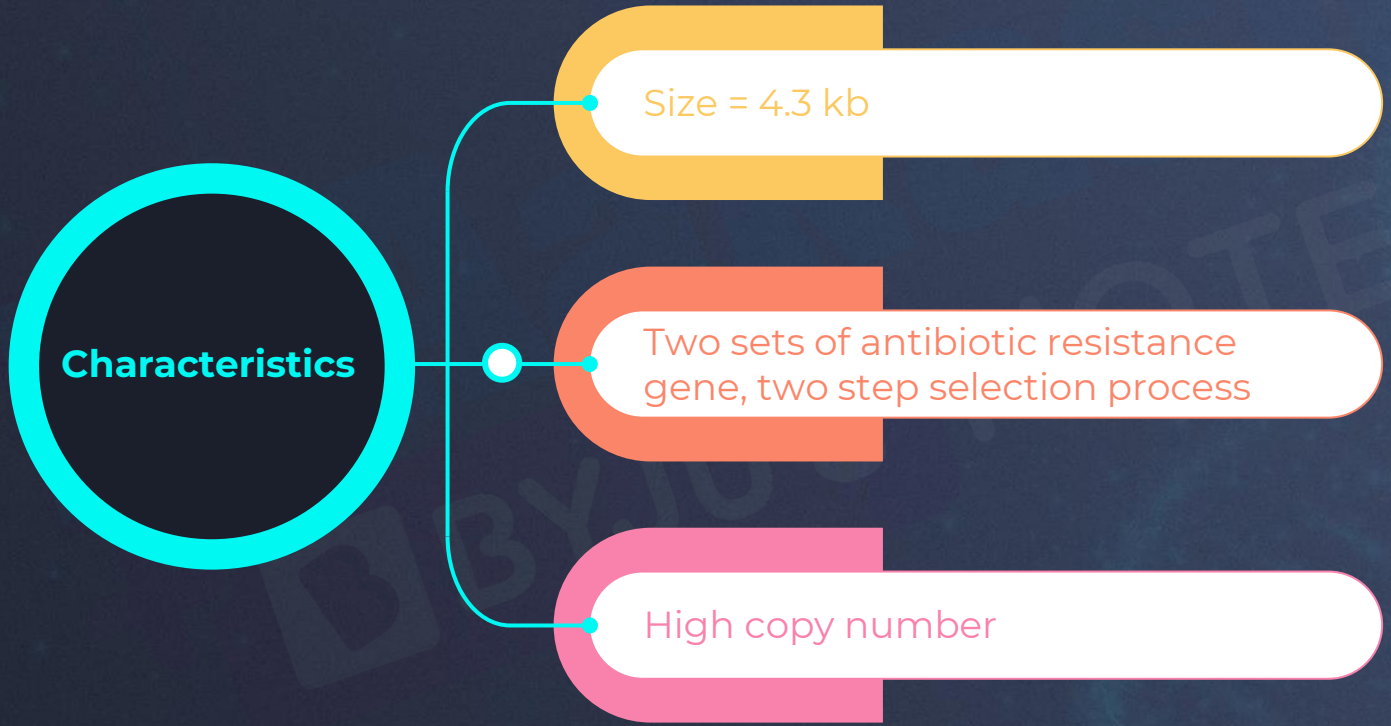


Raymond L. Rodriguez

Plasmid



Plasmid - pBR322



Difference between Plasmid DNA and Chromosomal DNA

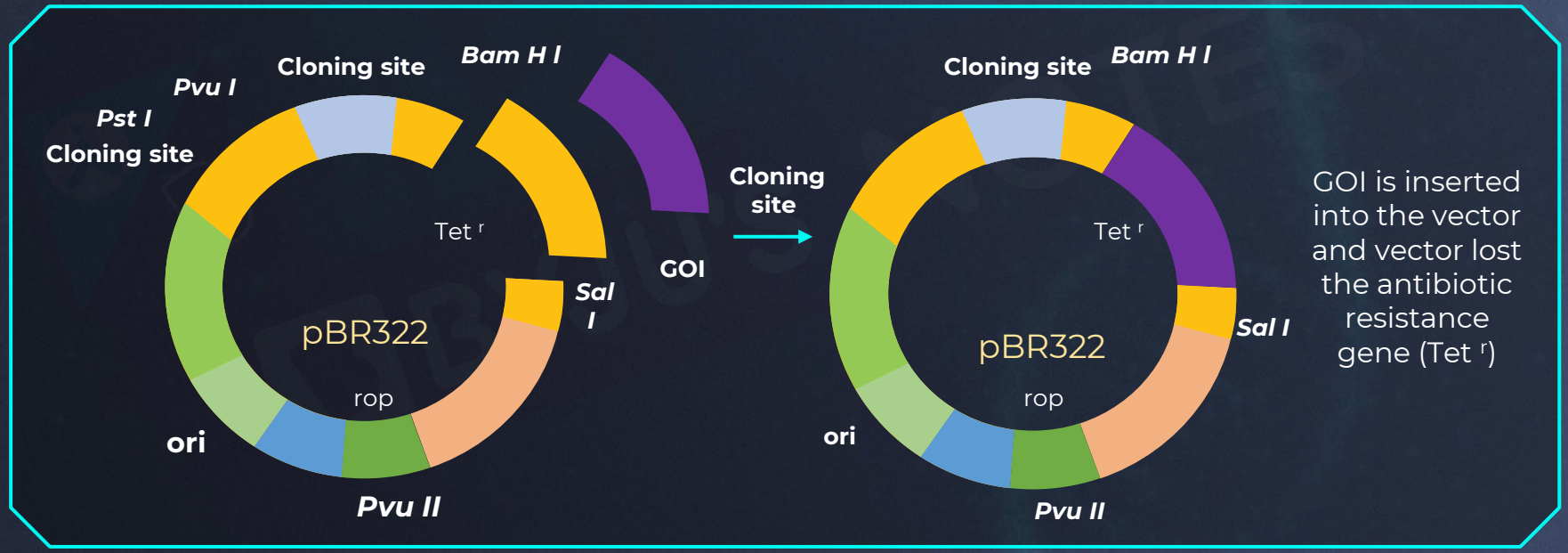
Plasmid DNA	Chromosomal DNA
• Always double stranded	• Single or double stranded
• Circular	• Linear or circular
• Naked without histone protein	• Coated with histone protein
• Does not carry any vital gene necessary for cell	• Carries vital gene necessary for cell
• Replicates independently	• Replicates with genome
• Introns are absent	• Exons and introns are present

Role of Vector

- Vector DNA helps in **carrying the gene of interest (GOI)** into the host cell.
- The host organism can be prokaryote **(*E.coli*)** or eukaryote (yeast, plants).
- *E.coli,* thus, ends up having its own DNA, and the inserted gene of interest
- The resultant host cell is called **recombinant host**
- The **vector DNA plays two major roles**:
  - It helps in **carrying the gene of interest** into the host cell
  - It **replicates** when the host cell divides, thus **producing copies of GOI**

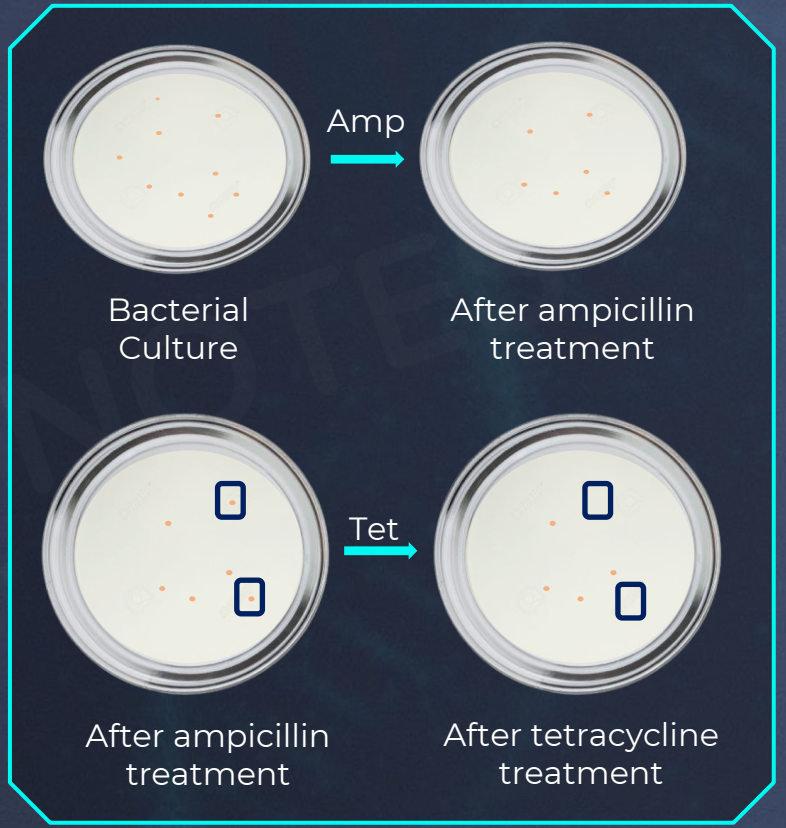
Selection Using Antibiotic Resistance

- In *E. coli*, the **ligation of foreign DNA** is carried out at *BamH I* site of tetracycline resistance gene in the vector pBR322.
- Recombinant plasmids **lose resistance** due to insertion of foreign DNA but can still be **selected** out from **non-recombinants.**



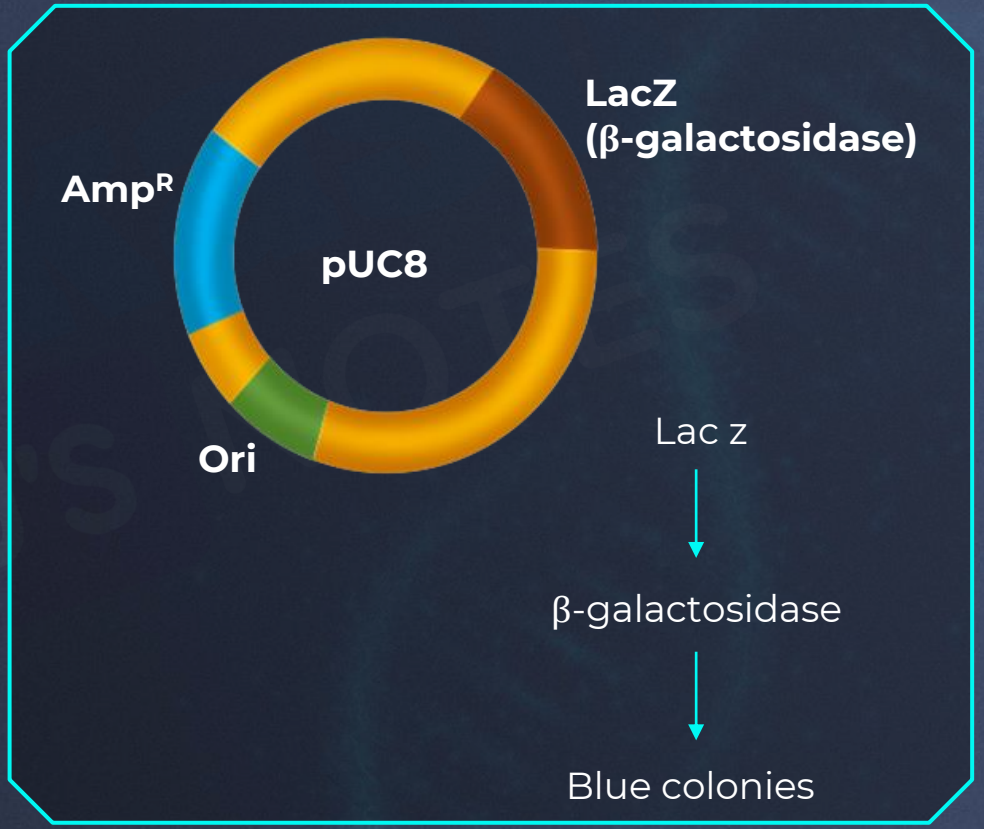
Selection Using Antibiotic Resistance

- The normal bacteria dies by the action of the antibiotic, ampicillin.
- Bacteria which are alive after treatment of ampicillin are again treated with tetracycline
- After tetracycline treatment,
  - Colonies with rDNA do not grow in tetracycline medium, they are **recombinant colonies**
- Recombinant bacteria which have ampicillin resistant gene intact are **resistant to ampicillin**
- Recombinant bacteria which do not have tetracycline resistant gene are **sensitive to tetracycline**



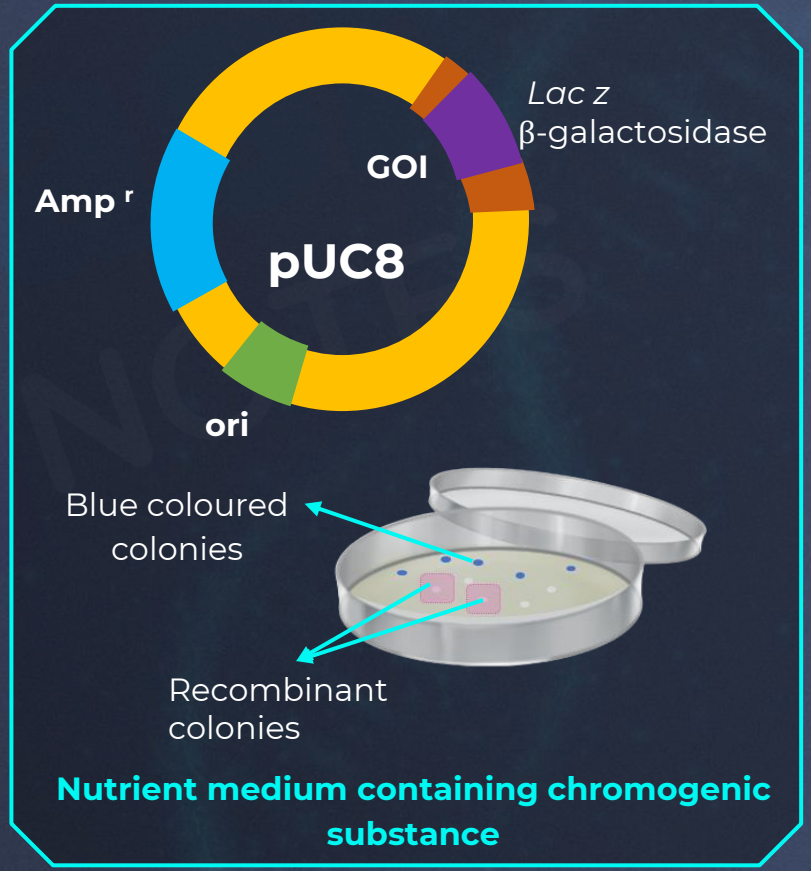
Selection Using Insertional Inactivation

- **pUC8** is one of the **ideal vectors**
- **Overcomes** the **disadvantage of pBR322**
- Has lac z gene that codes for **β-galactosidase**
- Bacteria with pUC8 give **blue color colonies** in presence of a chromogenic substance in media.
- However, when lac z gene is inactivated due to insert the colonies obtained are colorless.



Selection Using Insertional Inactivation

- Hence, the **gene of interest** is **inserted** into the **vector pUC8** where the gene *LacZ* is present, to inactivate the synthesis of gene.
- This results into **insertional inactivation** of the gene.
- Consequently, in the presence of chromogenic substrate
  - **If blue coloured colonies** obtained, then the bacteria does not have an insert.
  - **If colorless colonies** are obtained, then they are identified as recombinant colonies i.e., successfully transformed colonies.



Plasmids

pBR322	pUC8
Disadvantages	**Advantages**
• Inactivation of antibiotics makes the process **cumbersome** for selecting recombinants • As it requires **simultaneous plating** on two plates having different antibiotics	• **High copy number** • **Identification** of recombinant cells achieved by a **single step** i.e., plating cells onto agar medium containing ampicillin and X-gal (chromogenic substrate)

Examples of Vectors Used in rDNA Technology

Bacteriophage

- Virus that **infects bacteria**
- Ability to **replicate within bacterial cells**
- **Independent** of the control of chromosomal DNA
- Have high number per cell and **high copy number**

Commonly used bacteriophages are:

Lambda phage vector

- Clone DNA fragments upto 23 kb length

M13 phage vector

- Filamentous phage
- Infects *E.coli*
- Foreign DNA can be inserted into it without disrupting any of essential gene

Examples of Vectors Used in rDNA Technology

Cosmid

- **Cos site of lambda phage** + **plasmid DNA**

YAC vector
(Yeast artificial chromosome)

- **Telomeric sequence** + **centromere** + **autonomously replicating sequence**
- Used to clone DNA fragments of size 100kbp

BAC vector
(Bacterial artificial chromosome)

- Based on F plasmid (fertility) of *E.coli*
- Contains **genes for replication and maintenance of F factor** + **selectable marker** + **cloning sites**
- Used to clone DNA fragments of the size 300-350 kbp

Examples of Vectors Used in rDNA Technology

Phagemid

- **Bacteriophage** + **plasmid**
- Used for carrying larger DNA sequence

Transposons

- Unit of DNA which **moves from one DNA molecule to another**

Shuttle vector

- **Replicate in both** eukaryotic cell and *E coli*
- Contains two types of ori and selectable marker

Retroviruses

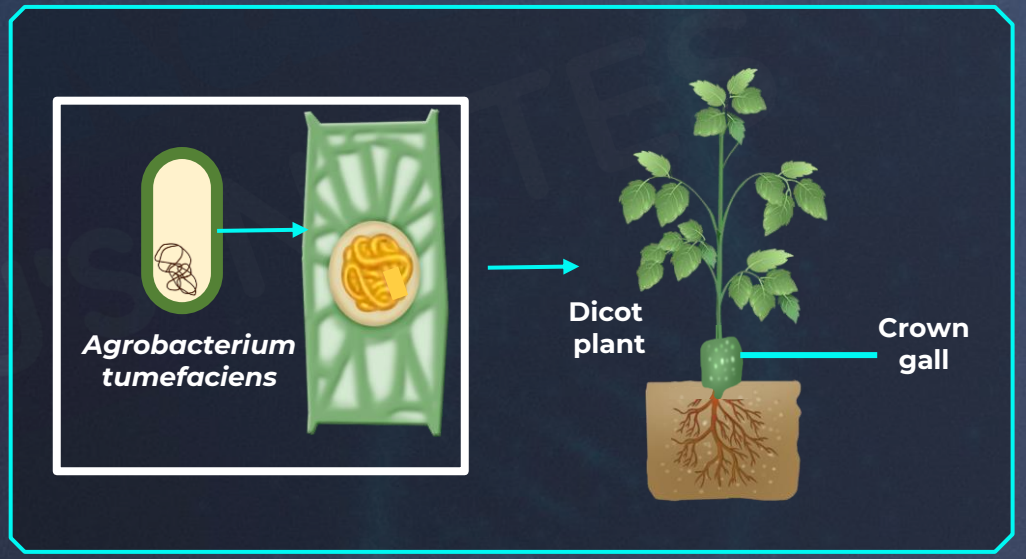
- Viruses used to **clone genes in animals**
- Ability to transform normal cells to cancerous cell
  - Hence, they are disarmed before being used as vectors

Examples of Vectors Used in rDNA Technology

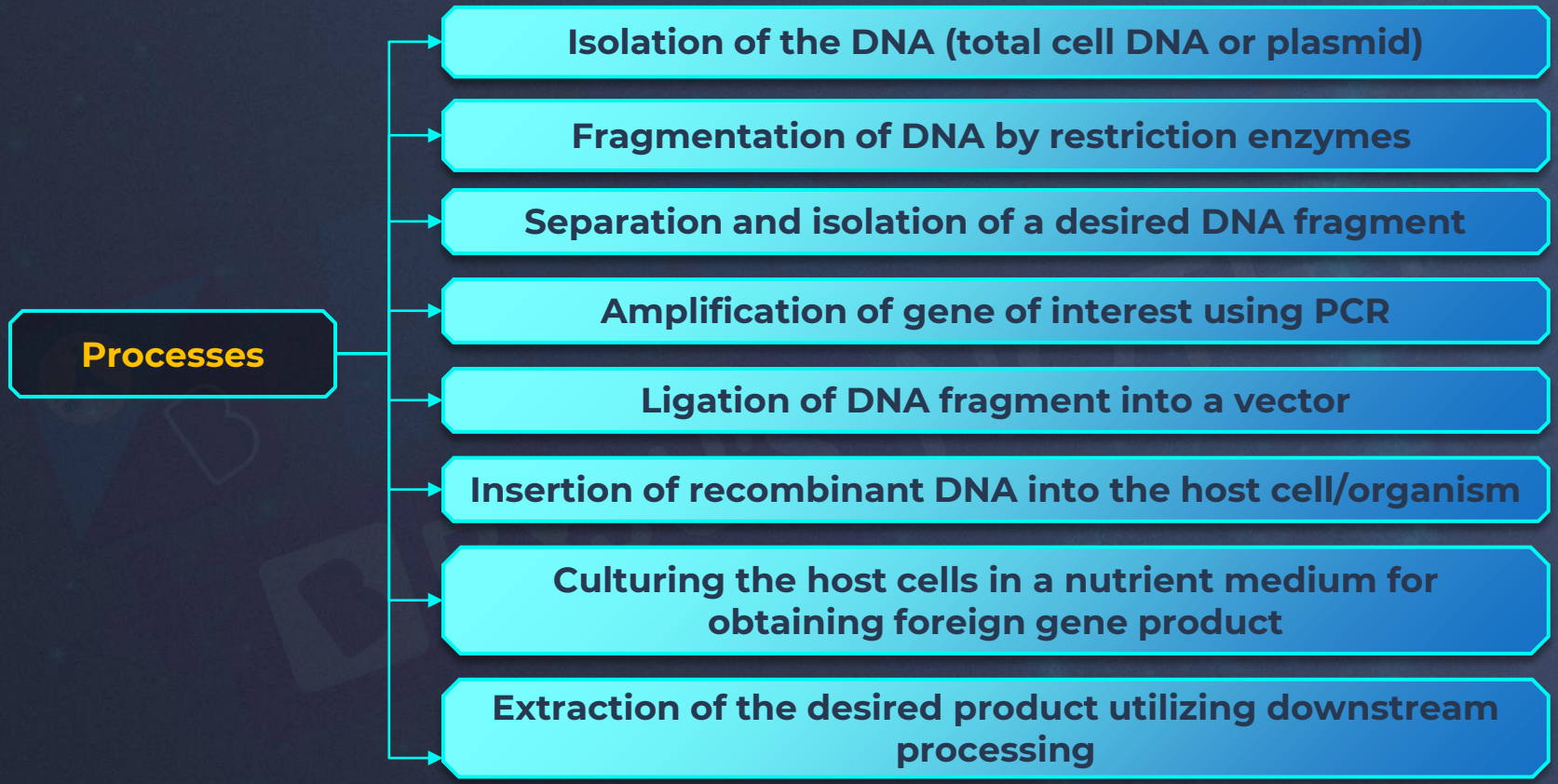
Ti plasmid of *Agrobacterium tumefaciens*

Agrobacterium tumefaciens, a pathogen of several dicot plants is able to deliver T-DNA to transform normal plant cells into tumor cells.

- This transformation causes them to produce chemicals required by the bacterial pathogen to cause **crown gall disease.**
- Tumour inducing plasmid (**Ti plasmid**) is present in *Agrobacterium tumefaciens*.
- Ti plasmid is **modified into a cloning vector** which is no more pathogenic to the plants but **able to deliver genes of our interest** into a variety of plants.



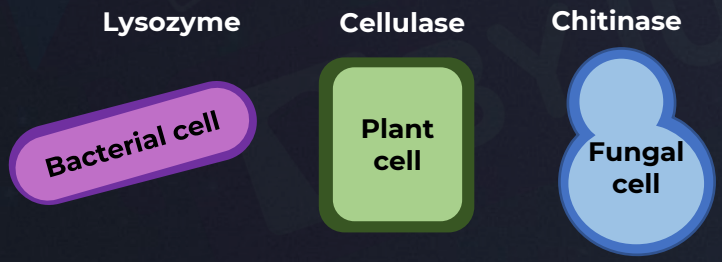
Process of rDNA Technology



Isolation of the DNA

- In animal cell, DNA is **enclosed** within the **cell membrane,** as they lack cell wall.
- There are **specific enzymes** used to **breakdown the cell wall** of different organisms based on the constituents of the cell wall.
- **Detergents** destroy the cell membrane and all lipids inside the cell, thus letting the cytoplasm leak.

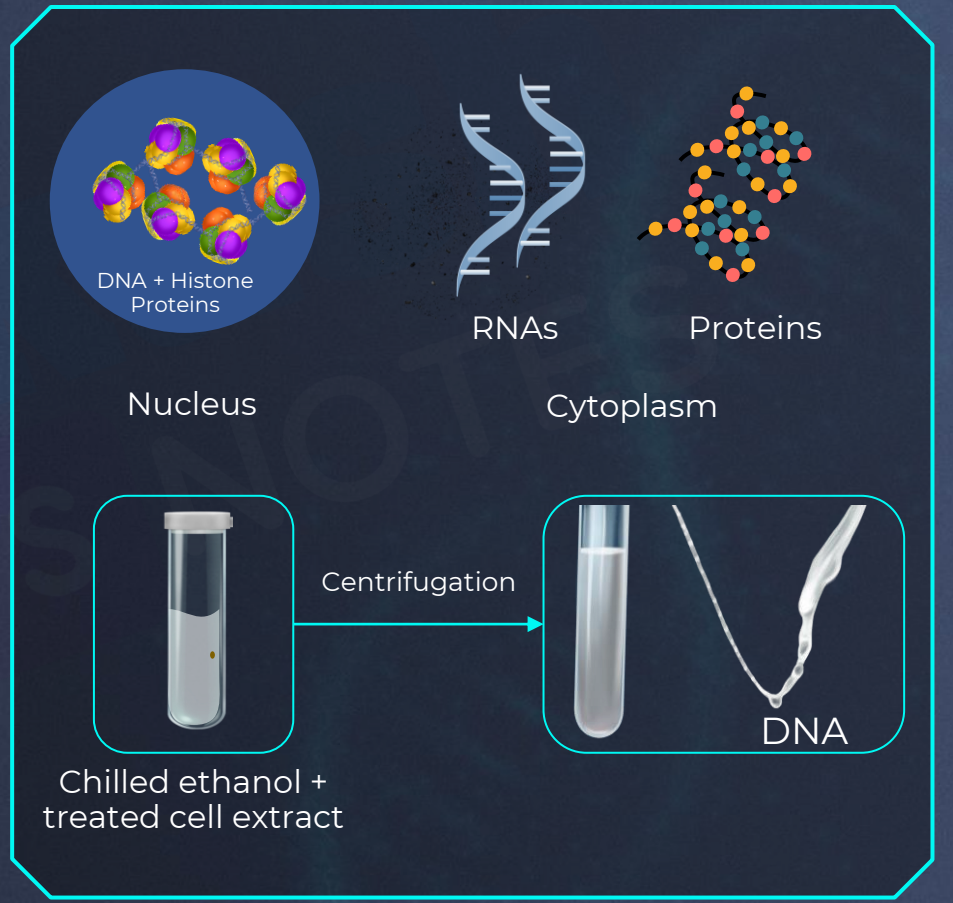
Enzymes to breakdown the cell wall



Cell membrane and lipids disintegrated

Isolation of the DNA

- The cell has several other components.
  - DNA + Histones in the nucleus
  - Debris from lipids destroyed
  - Proteins
  - RNA in the cytoplasm
- To obtain pure DNA following treatments are done:
  - **Treatment with protease**: destroys the histone proteins and the proteins in the cytoplasm
  - **Treatment with ribonuclease (RNase)** : destroys the RNA in the cytoplasm
- Once cell is treated, cell extract obtained is **mixed with chilled ethanol** and **centrifuged**. This gives us the **pure DNA.**



Fragmentations of DNA by Restriction Enzymes

- **Restriction enzyme digestions** are performed by incubating purified DNA molecules with the **restriction enzyme**, at the optimal conditions for that specific enzyme.

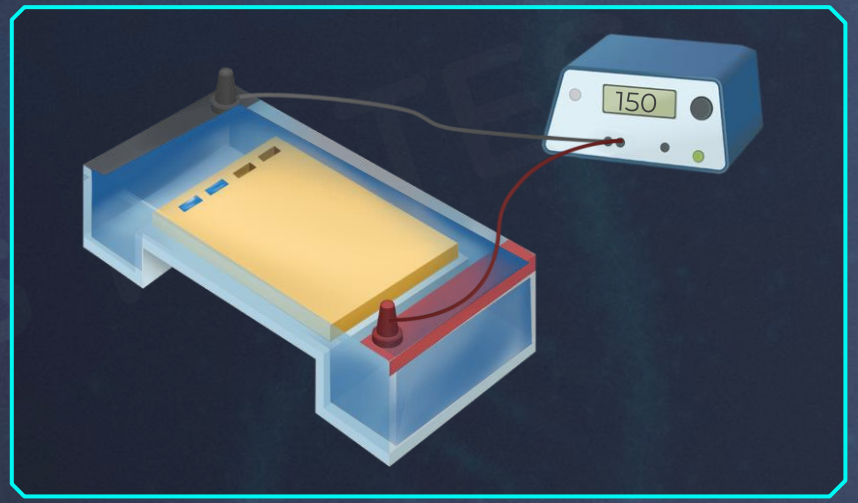
- This is done to **identify the location** wherein a **designated gene is introduced into a vector genome**.

- **Agarose gel electrophoresis** is employed to check the **progression of a restriction enzyme digestion.**
- The process is repeated with the vector DNA, with the same restriction enzyme.

Separation and Isolation of DNA Fragments

Separation

- The fragments of DNA can be separated by **gel electrophoresis.**
- **DNA - negatively charged molecules** (move towards anode) under an electric field through a matrix.
- Matrix used - **Agarose**, a natural polymer extracted from sea weeds.

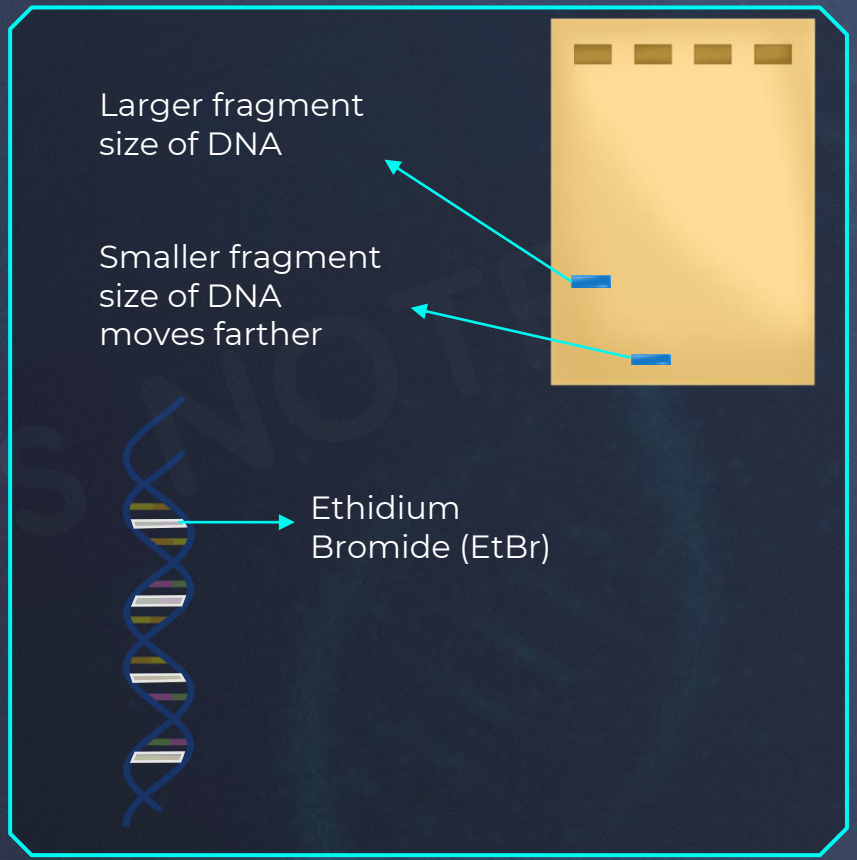


Separation and Isolation of DNA Fragments

Separation

- DNA molecules move and **separate based on size** due to the sieving effect
- **Smaller the fragment size, the farther it moves**

- **Ethidium bromide (EtBr)** - a chemical added during the preparation of agarose gel
- Ethidium bromide **binds to DNA** by inserting itself between the stacked bases in double-stranded DNA



Separation and isolation of DNA Fragments

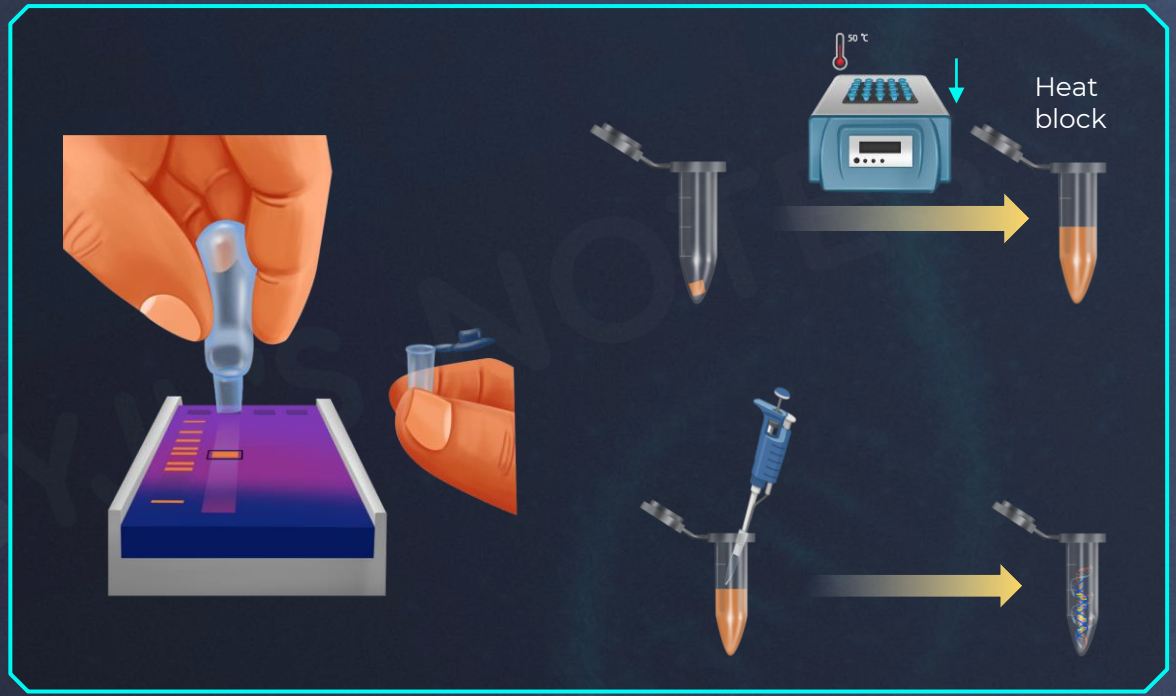
Separation

- DNA is **visualized under UV light as bright orange bands**

Separation and isolation of DNA Fragments

Isolation

- **Elution**: Separated bands with gene of interest are cut out from the agarose gel and extracted from the gel piece.
- DNA fragments are **purified**, for constructing recombinant DNA by joining them with cloning vectors.



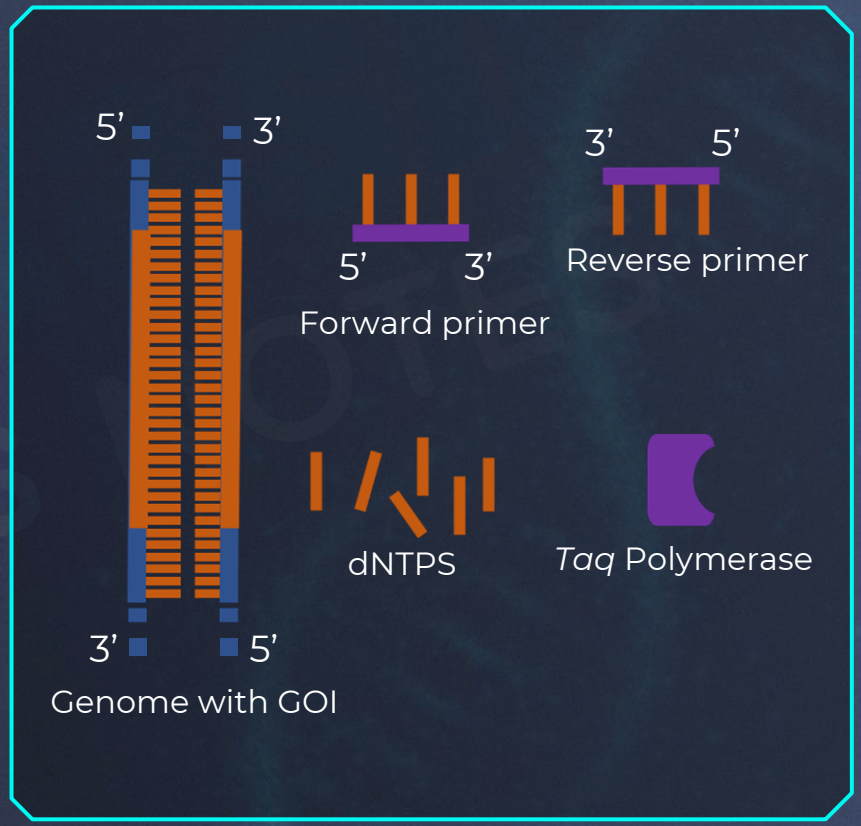
Amplification of Gene of Interest Using PCR

- PCR (**Polymerase Chain Reaction**): process of selective amplification of a specific region of DNA in vitro is called PCR (polymerase chain reaction).
- It was discovered by **Kary Mullis**.

Amplification of Gene of Interest Using PCR

PCR ingredients :

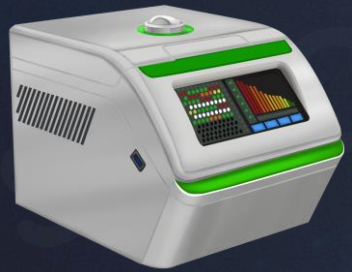
- Initial template - **small amount of DNA**
- Substrate - **dNTPs**
- **Ions** and **salts**
- **Pair of primers** - exposed 3'-OH (binds with the particular sequence of interest in the DNA template) **one primer (5' to 3')** and **other (3' to 5') each to initiate replication of each of the two DNA strands**
- Thermostable DNA polymerase (**Taq polymerase**), which remains active during the high temperature
  - isolated from ***Thermus aquaticus***



Amplification of Gene of Interest Using PCR

Vial with reaction ingredients is put into the thermal cycler

Thermal cycler



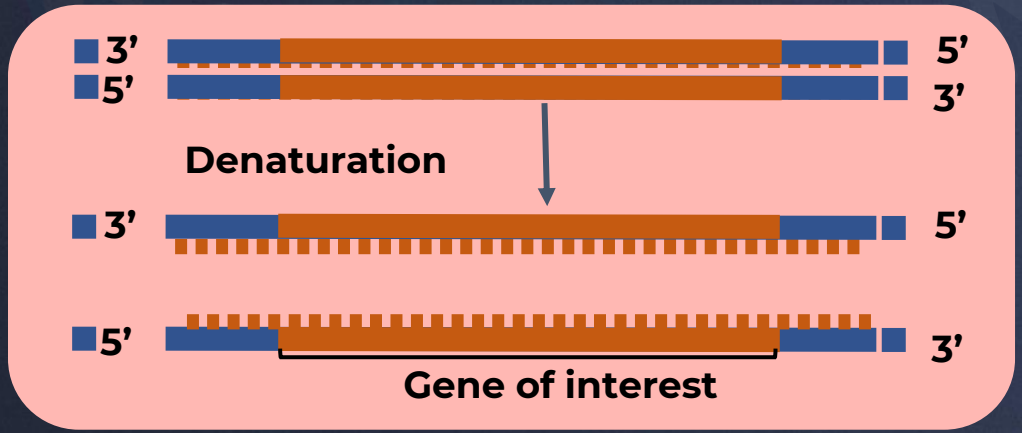
Loaded thermal cycler

Controls temperature and time duration

Amplification of Gene of Interest Using PCR

PCR cycle consists of three steps:

1. **Denaturation**:
   - PCR mixture is heated at **94 - 96℃**
   - DNA strands are **separated into single strands** as the **hydrogen bonds** between them **breaks**
   - Therefore, **single strand acts as a template** for the new strands



Denaturation

↓

Annealing

↓

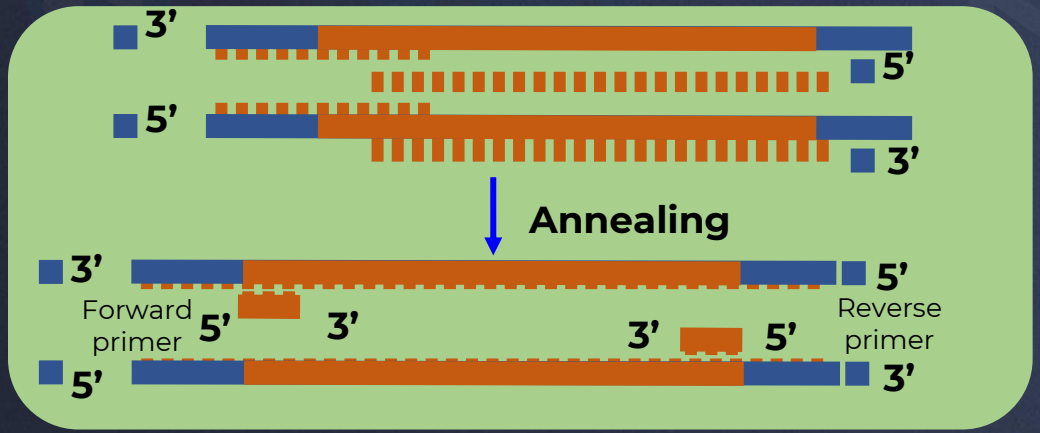
Extension

Amplification of Gene of Interest Using PCR

PCR cycle consists of three steps:

2. Annealing:

- The temperature is reduced to **50-60℃**
- At this temperature, the **primers bind** to the respective complementary sequences on single stranded templates



Denaturation

↓

Annealing

↓

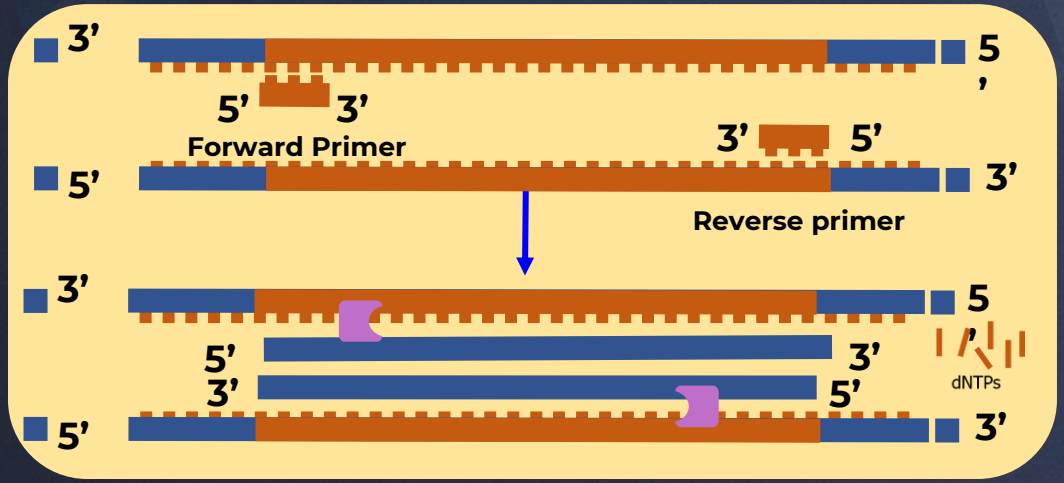
Extension

The two separated strands run in the opposite directions and consequently there are two primers - a **forward primer** and a **reverse primer.**

Amplification of Gene of Interest Using PCR

PCR cycle consists of three steps:

3. **Primer extension (polymerisation):**
   - The temperature is raised to **72 - 80°C**
   - **DNA polymerase adds nucleotides to the primer**



Denaturation

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Annealing

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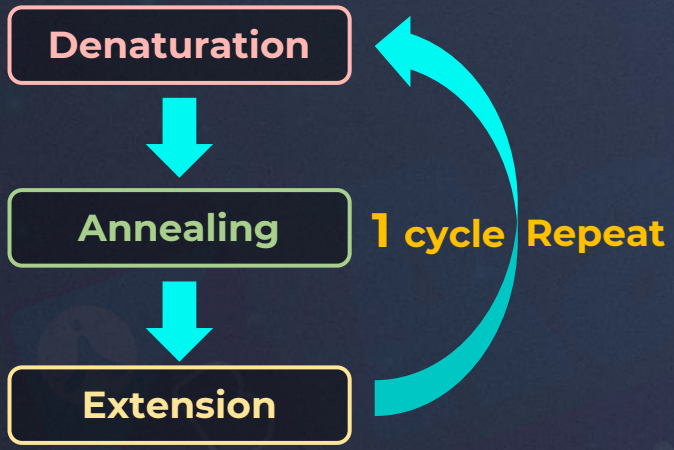
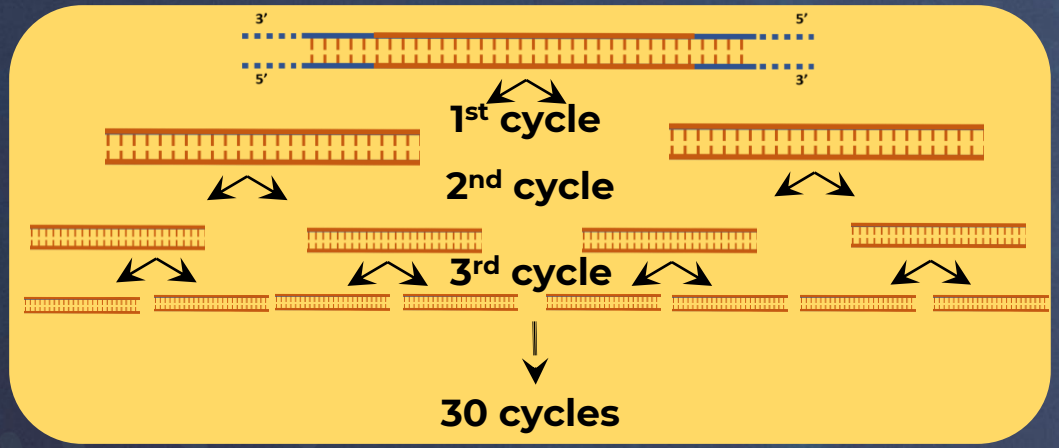
Extension

Amplification of Gene of Interest Using PCR





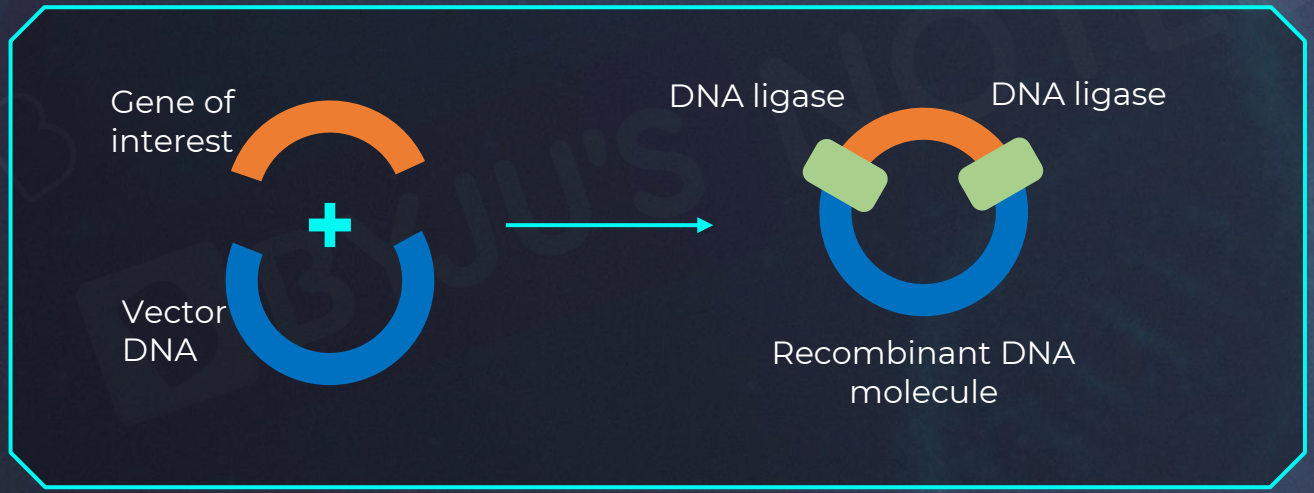
This process is **repeated 30 times.** Each new **DNA piece** can act as a **new template for each cycle**. So, after 30 cycles, **1 million copies of a single fragment of DNA** is produced

- **30 PCR cycles** = **1 billion number of copies**
- **n (no. of cycles)** yields **2^n molecules**

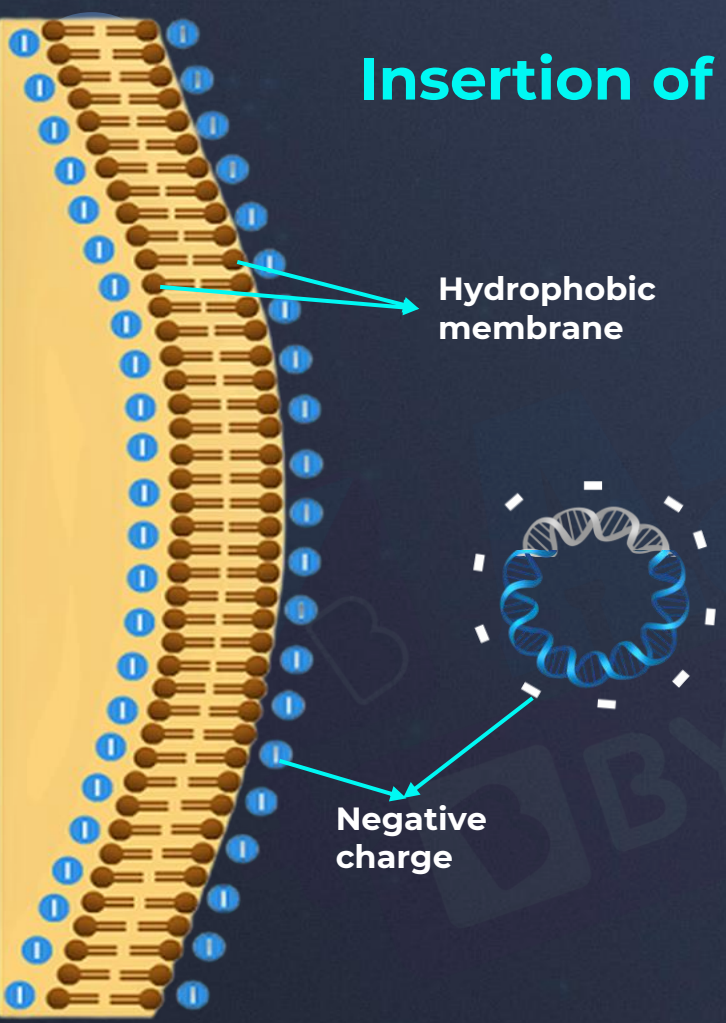
Applications of PCR

Ligation of the Fragment into a Vector

- Desired amplified fragment can be used to ligate with the vector for further cloning.
- Vector and GOI are mixed and **ligase** is added. This results in the preparation of **recombinant DNA**.



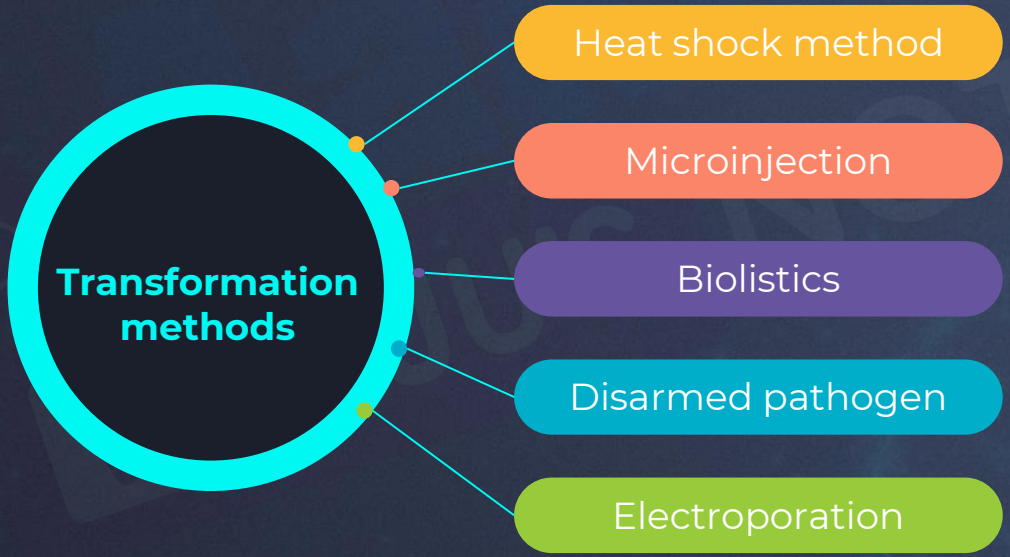
Insertion of Recombinant DNA into the Host



- **Bacterial cells** have **hydrophobic membrane** and **rDNA** is **hydrophilic**, so the naked DNA **can not pass** into the bacteria **directly**.
- Charges on both the cells and DNA are negative hence they **repel** each other.
- In order to counter negative-negative charge repulsion, **divalent cation** treatment is given to rDNA.
- **Calcium** is one such divalent cation that is used to force the host cell to take up the **naked exogenous DNA** molecule.

Insertion of Recombinant DNA into the Host

- Cells that can take up the DNA are known as **competent cells.**
  - Ability to readily take up the rDNA is called **competence.**
- The process of introducing the DNA into competent cells is known as **transformation**.



Insertion of Recombinant DNA into the Host

Heat shock method

- Recombinant DNA can be forced into host cells by incubating the cells with recombinant DNA on ice, followed by placing them briefly at **42℃ (heat shock),** and then putting them **back on ice.**
- This enables the bacteria to take up the **recombinant DNA.**

- Competent bacterial cells are **incubated on ice** along with rDNA.

- Competent bacterial cells with rDNA are briefly placed in water bath at **42℃**

- The cells are again introduced to the **cold temperature.**

Insertion of Recombinant DNA into the Host

Micro-injection

- Recombinant DNA is **directly injected** into the nucleus of an animal cell.

Biolistics/Gene gun

- Cells are bombarded with **high velocity micro-particles** of gold or tungsten coated with DNA.

Disarmed pathogen

- 'Disarmed pathogen' vectors, which when **allowed to infect the cell**, transfer the recombinant DNA into the host.

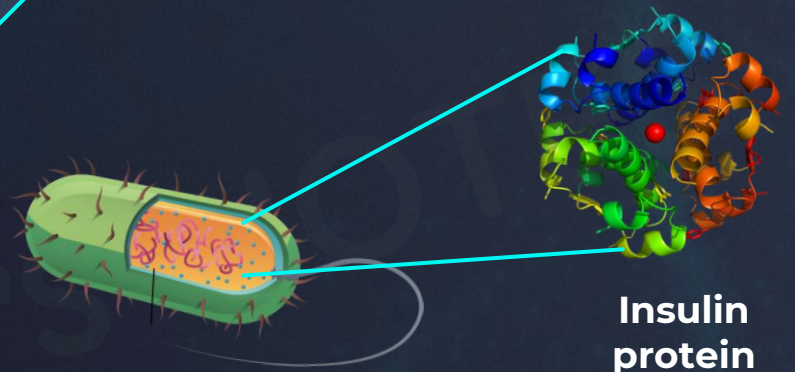
Electroporation

- **Short electrical impulses** of high field strength are given.
- **Increases the permeability of protoplast** membrane by **creating microscopic pores.**
- This opportunity is used to introduce DNA into the cell.

Foreign Gene Product

Culturing the host cells in a nutrient medium at a large scale for obtaining the foreign gene product

- The aim of all recombinant technologies is to **produce a desirable protein** by the expression of recombinant DNA.
- **Recombinant protein** is any protein that gets expressed in a heterologous host.



Culturing of the Host Cells

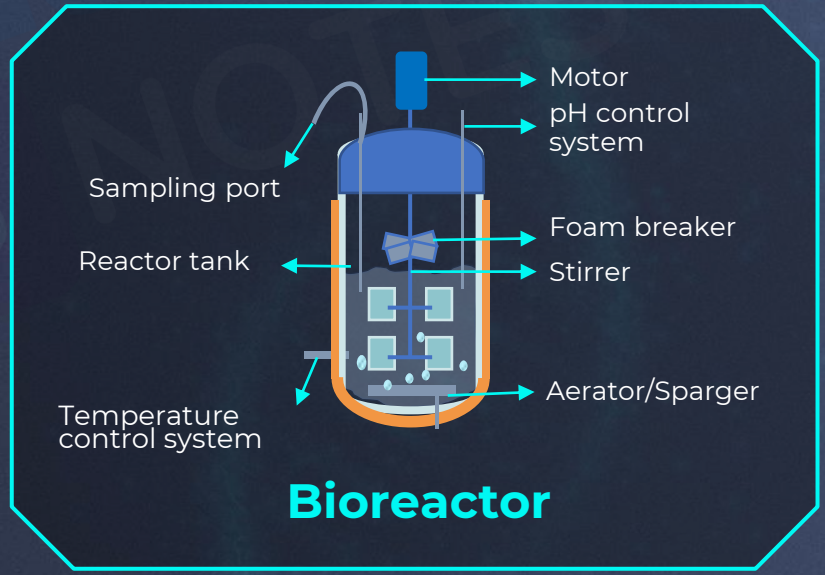
- The cells harboring cloned genes of interest may be grown on a **small scale in laboratories.**
- Desired protein can be **extracted** and then **purified.**
- The initial process starts with the **pilot plant**, which involves the use of small fermenter (a tank or vessel in which the process will be carried out).

Large Scale Production

1. Maintaining aseptic conditions
2. Physical factors
3. Adequate oxygen
4. Anti-foaming agents
5. Circulation of cooling water
6. Monitoring devices and control processes

Fermenter / Bioreactors

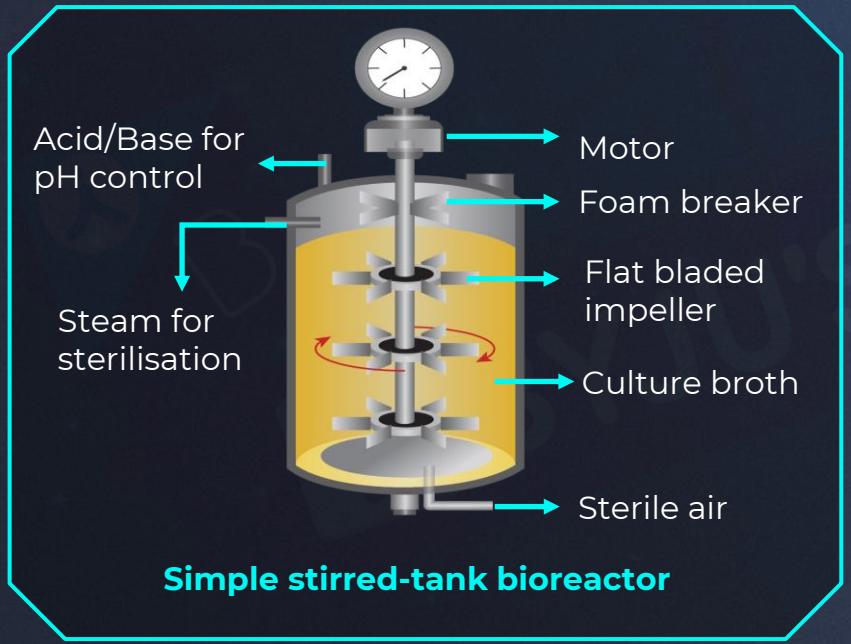
- **Bioreactors** can produce desirable product in large quantities.
  - **large volumes (100-1000 litres)** of culture can be processed
- **Bioreactor** is a vessel in which **raw materials are biologically converted into specific products**, individual enzymes, etc., using microbial, plant, animal or human cells.
- Bioreactor has -
  - **agitator** system
  - **oxygen delivery** system
  - **foam control** system
  - **temperature control** system
  - **pH control** system
  - **sampling ports** - to periodically withdraw small volumes of the culture



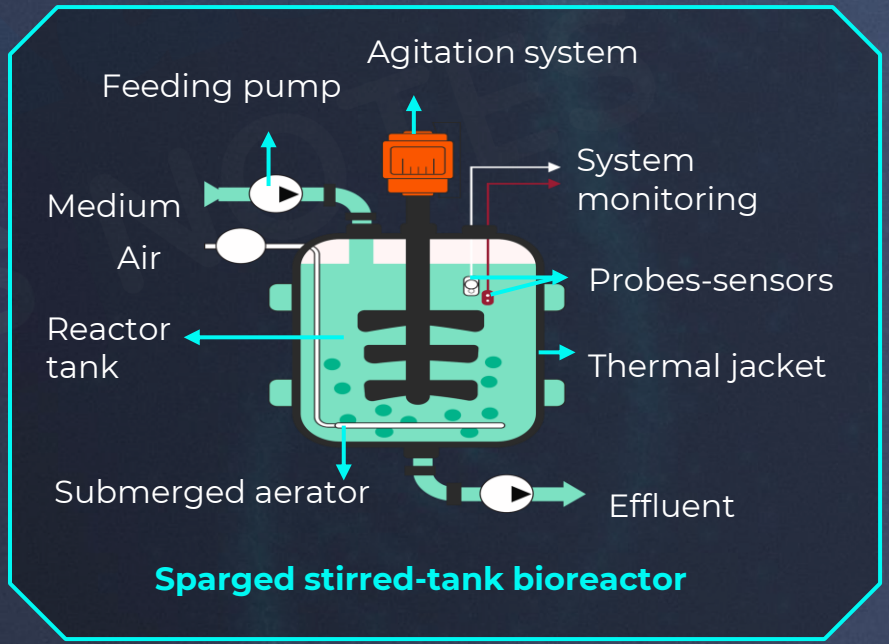
Bioreactor

Fermenter Design and Use

- The most commonly used **bioreactors** are of **stirring type** -
  - Stirrer facilitates even **mixing** and **oxygen availability** throughout the **bioreactor**
  - Alternatively, **air** can be **bubbled** through the **reactor**.



Simple stirred-tank bioreactor



Sparged stirred-tank bioreactor

Fermenter

Types of fermentation

Batch fermentation (closed system)

- **Conditions are set up** and not changed from outside once the fermentation starts
- **No more nutrients are added**
- Process is stopped once sufficient product has been formed

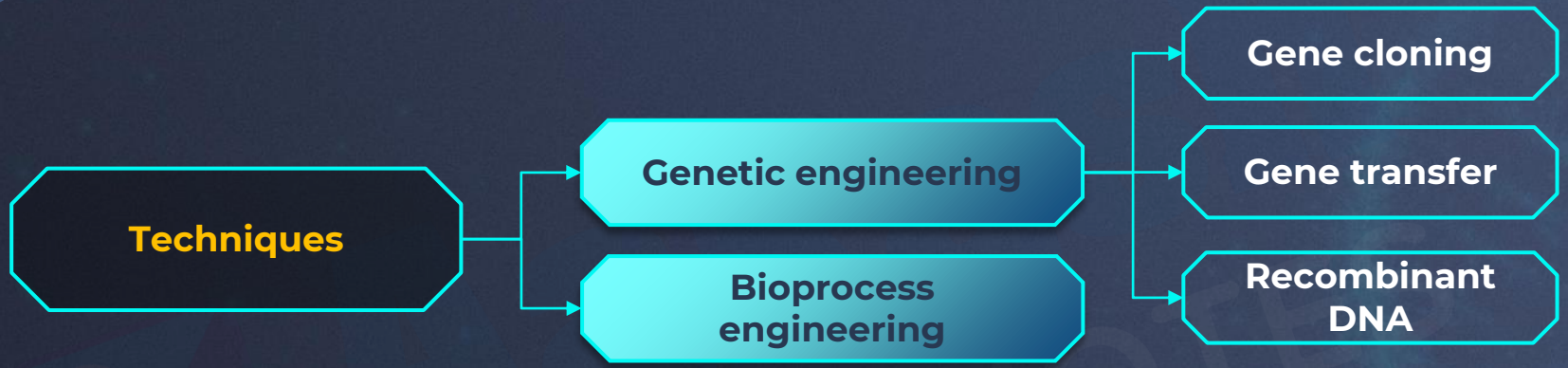
Continuous culture (open system)

- **Used medium** is **drained** out from **one side** , while **fresh medium** is **added**
- This maintains the physiological activity
- **Produces larger biomass** i.e., higher yields of desired protein

Extraction of the Desired Product

- The stage **after fermentation** when the desired product is **recovered** and **purified** through a **series** of **processes** before **marketing** as a **finished product.**
- Processes include
  - **separation**
  - **purification**
- The product has to be formulated with **suitable preservatives.**
- Such formulation has to undergo thorough clinical trials and quality control testing.

Summary



Steps of rDNA technology

01 Identification and isolation of DNA with desirable genes

02 Introduction of the identified DNA into the host

03 Maintenance of introduced DNA in the host and transfer of the DNA to its progeny

Summary

Processes

- Isolation of the DNA (total cell DNA or plasmid)
- Fragmentations of DNA by restriction enzymes
- Separation and isolation of a desired DNA fragment
- Amplification of gene of interest using PCR
- Ligation of DNA fragment into a vector
- Insertion of recombinant DNA into the host cell/organism
- Culturing the host cells in a nutrient medium for obtaining foreign gene product
- Extraction of the desired product utilizes downstream processing

Summary

PCR cycle consists of three steps:

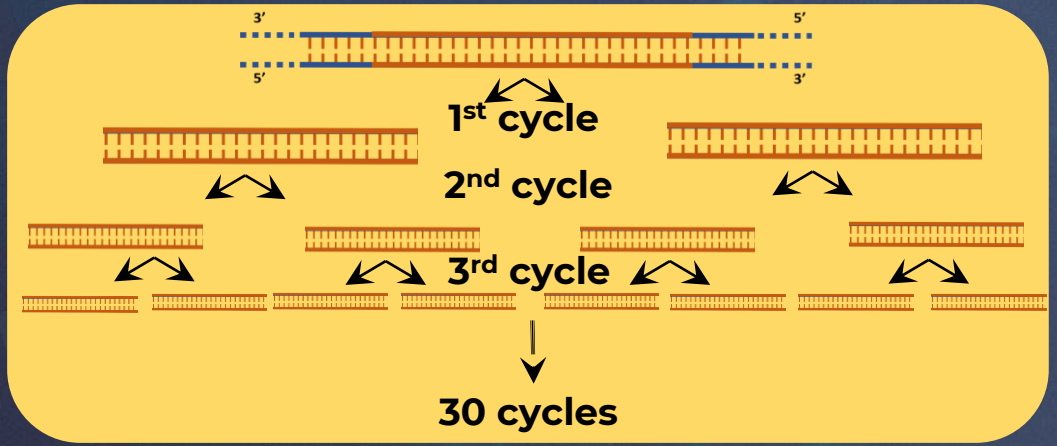
Denaturation
(dsDNA to ssDNA

Annealing
(Primers bind to the ssDNA)

Extension
(Synthesis of new DNA strand)

1 cycle

Repeat



Summary

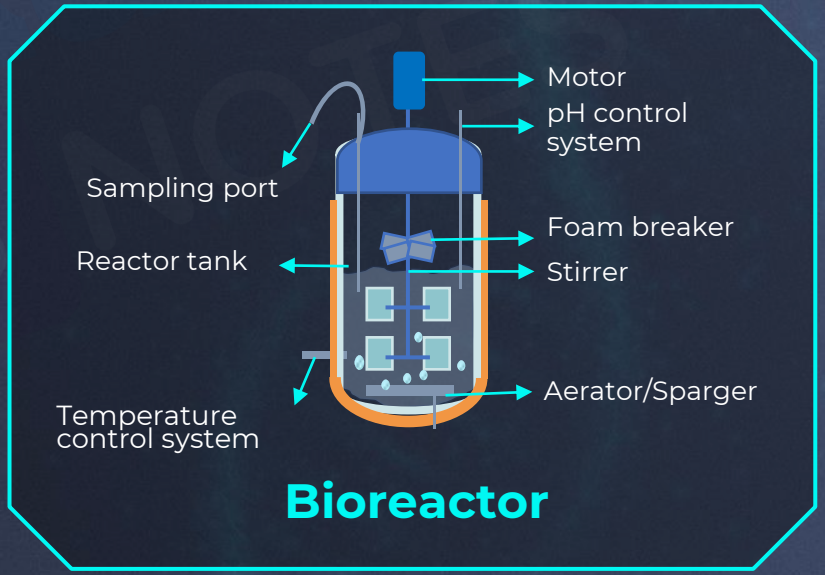
Applications of PCR

Summary

Transformation methods

- Heat shock method
- Microinjection
- Biolistics
- Disarmed pathogen
- Electroporation

Bioreactor is a vessel in which raw materials are biologically converted into specific products, individual enzymes, etc., using microbial plant, animal or human cells.



Bioreactor