Introduction to Animal Biotechnology & Recombinant Technology

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Biotechnology Principles and Processes

What is Biotechnology

Biotechnology deals with techniques of **using live organisms or enzymes from organisms to produce products and processes useful to humans**.

e.g., making curd, bread or wine

Further, many other processes/techniques are also included under biotechnology. For example, *in vitro* fertilisation leading to a 'test-tube' baby, **synthesising a gene** and using it, developing a **DNA vaccine** or **correcting a defective gene**, are all part of biotechnology.

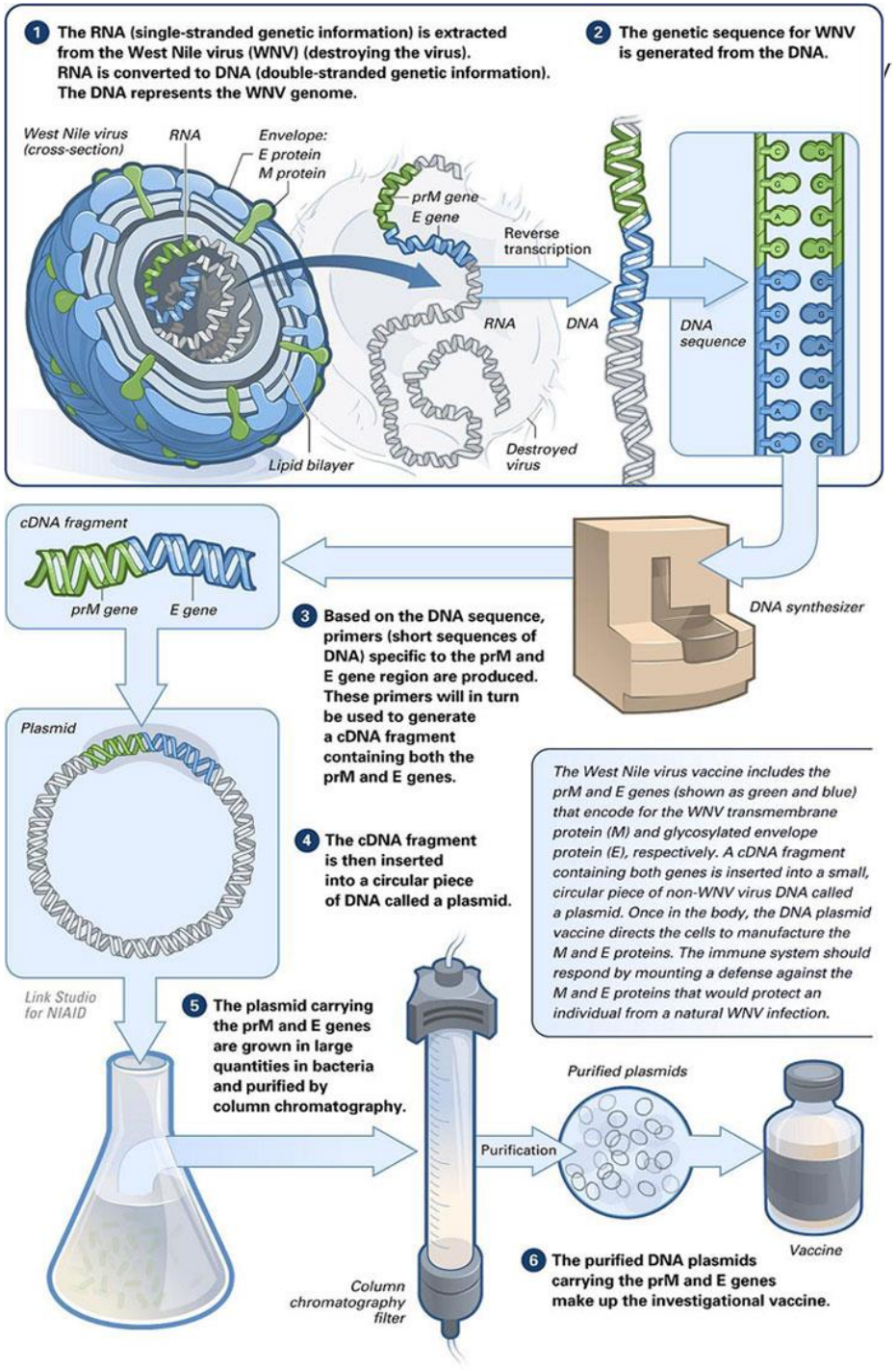
Definition as per European Federation of Biotechnology (EFB) :

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

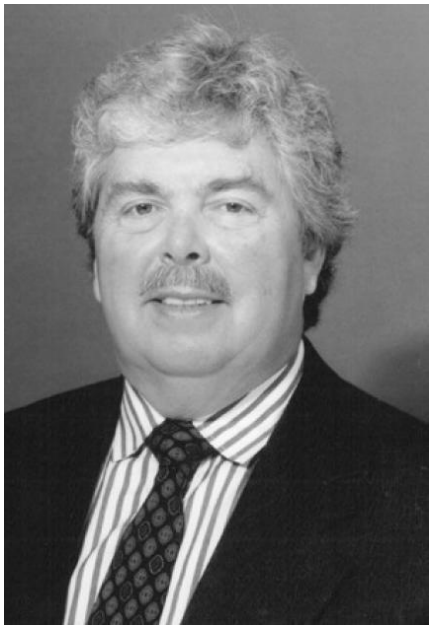
Biotechnology Principles and Processes

mRNA / cDNA Vaccines

cDNA vaccines are third generation vaccines, made up of small, circular pieces of cDNA, plasmids. These plasmids are genetically engineered to produce specific antigens from a pathogen.

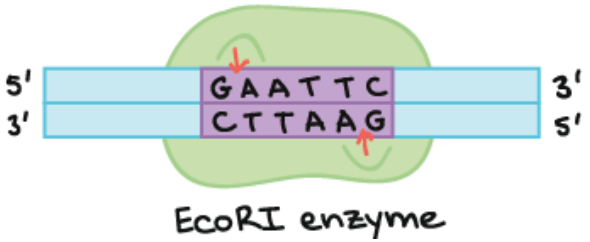


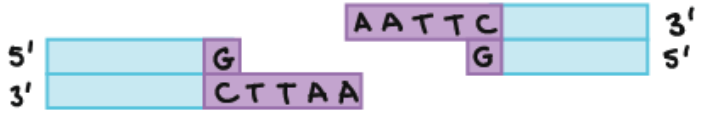
Biotechnology Principles and Processes



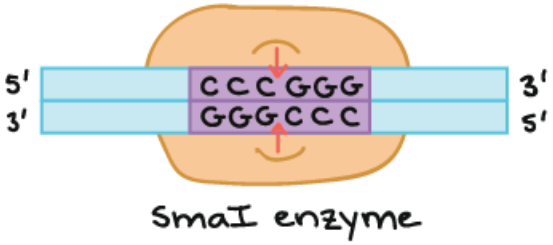
Herbert W. Boyer

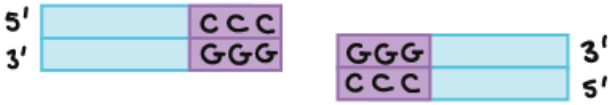
Herbert Boyer: **R**estriction enzymes of the *E. coli* bacterium have the capability of cutting DNA strands in a particular fashion to give 'sticky ends' on the strands. These clipped ends made pasting together pieces of DNA a precise exercise.





'Sticky ends' - single-stranded DNA "overhangs"





'Blunt-ends'

Blunt-ended fragments are harder to ligate together (the ligation reaction is less efficient and more likely to fail) because there are no single-stranded overhangs to hold the DNA molecules in position.

Biotechnology Principles and Processes

Restriction enzymes **R**ecognition Sequence

EcoR I

5′. . . G|AATTC . . . 3′

3′. . . CTTAA|G . . . 5′

Escherichia coli

*Eco*RI is a restriction endonuclease that is used in molecular biology applications to cleave DNA at the recognition site 5′-G/AATTC-3′, generating fragments with 5′-cohesive termini.

Sma I

5′. . . CCC|GGG . . . 3′

3′. . . GGG|CCC . . . 5′

Serratia marcescens

*Sma*I is a restriction endonuclease used to cut DNA at the recognition sequence 5′-CCC/GGG-3′, generating DNA fragments with blunt termini.

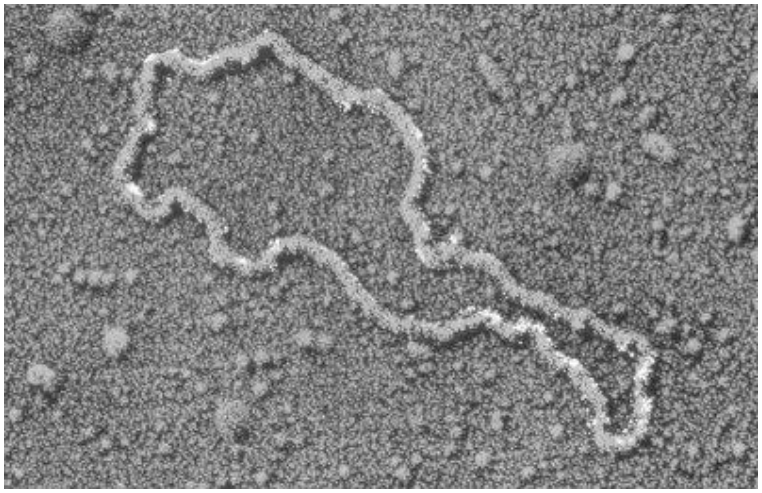
Restriction enzymes Recognition Sequence

Source and recognition sequences (indicated by arrow) of various restriction enzymes:

Restriction Enzyme	Source (Organism and strain)	Recognition sequence	Product	End products produced
Alu I	*Arthobacter luteus*	5' ---A- G-▼-C -T--- 3' 3' ---T- C-▲-G-A--- 5'	5'---A-G 3'---T-C C-T---3' G-A---5'	Blunt ends
Bam HI	*Bacillus amyloliquefaciens H*	5'---G-▼-G-A-T-C-C---3' 3'---C-C-T-A-G-▲-G---5'	5'---G 3'---C-G-T-A-G G-A-T-C-C---3' G---5'	Sticky ends
EcoRI	*Escherichia coli* Ry13	5' ---G-▼-A-A-T-T-C---3' 3' ---C-T-T-A-A-▲-G--- 5'	5'---G 3'---C-T-T-A-A A-A-T-T-C---3' G---5'	Sticky ends
Hind II	*H. influenzae* Rd	5'--G-T-C-▼-G-A-C--3' 3'--C-A-G-▲-C-T-G--5'	5'--G-T-C 3'--C-A-G G-A-C--3' C-T-G--5'	Blunt ends

Biotechnology Principles and Processes

Stanley Cohen



Electron micrograph of plasmid DNA

Cohen had been studying **plasmids**.
Cohen had developed a method of removing these plasmids from the cell and then reinserting them in other cells.

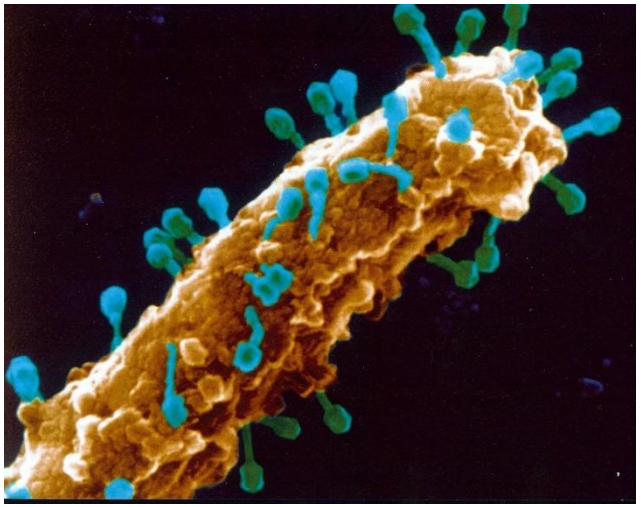
Combining this process with that of **DNA splicing enabled Boyer** and **Cohen to recombine segments of DNA in desired configurations and insert the DNA in bacterial cells**, which could then act as manufacturing plants for specific proteins.

Biotechnology Principles and Processes

TOOLS OF RECOMBINANT DNA TECHNOLOGY

1. Restriction Enzymes

In the year 1963, the two enzymes responsible for restricting the growth of bacteriophage in *Escherichia coli* were isolated. One of these added methyl groups to DNA, while the other cut DNA. The later was called **restriction endonuclease**.



The first restriction endonuclease–*Hind II*

900 restriction enzymes that have been isolated from over 230 strains of bacteria each of which recognise different recognition sequences

EcoRI comes from *Escherichia coli* RY 13.

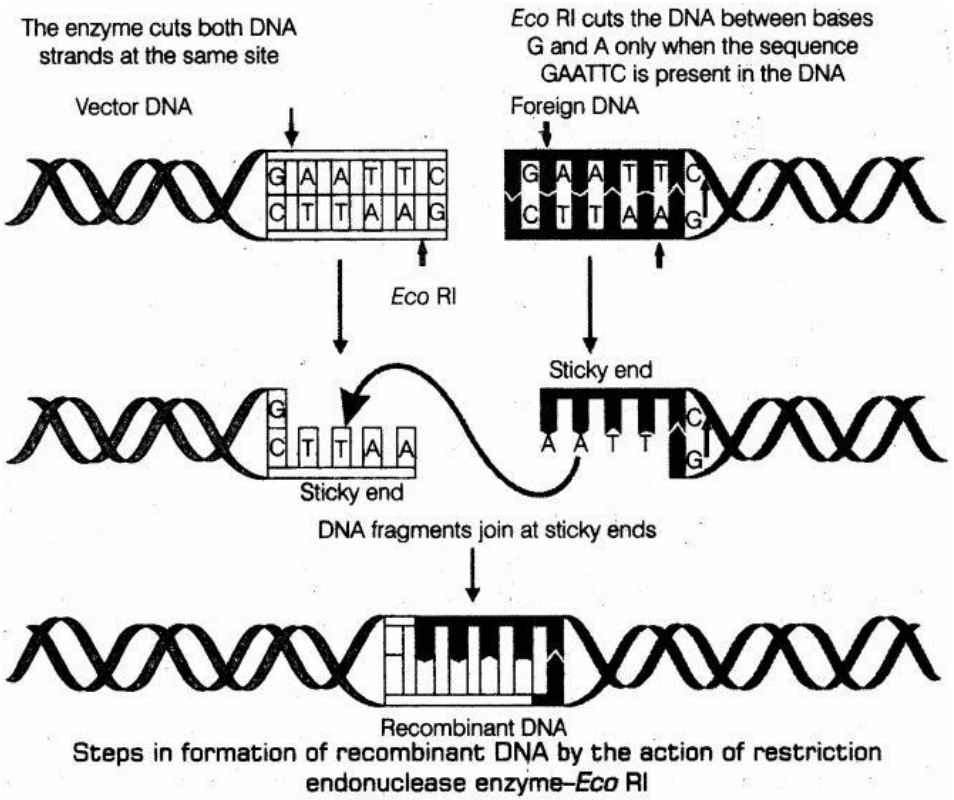
In EcoRI, the letter 'R' is derived from the name of strain. Roman numbers following the names indicate the order in Which the enzymes were isolated from that strain of bacteria.

Restriction enzymes

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

These are of two kinds; **exonucleases** and **endonucleases**.
Exonucleases remove nucleotides from the ends of the DNA whereas, **endonucleases** make cuts at specific positions within the DNA.

Each restriction endonuclease functions by 'inspecting' the length of a DNA sequence. Once it finds its specific recognition sequence, it will bind to the DNA and **cut each of the two strands of the double helix at specific points in their sugar -phosphate backbones** (Figure 11.1). Each restriction endonuclease recognises a specific palindromic nucleotide sequences in the DNA.



Steps in formation of recombinant DNA by the action of restriction endonuclease enzyme–*Eco* RI

Biotechnology Principles and Processes

The construction of the first recombinant DNA
emerged from the possibility of linking a **gene encoding antibiotic resistance** with a **native plasmid (autonomously replicating circular extra-chromosomal DNA) of *Salmonella typhimurium***.

Stanley Cohen and Herbert Boyer accomplished this in 1972 by isolating the antibiotic resistance gene by cutting out a piece of DNA from a plasmid which was responsible for conferring antibiotic resistance. The cut piece of DNA was then linked with the plasmid DNA.

- These plasmid DNA act as **vectors** to transfer the piece of DNA attached to it.
- Inserted in Escherichia coli.
- Replicated using the new host's DNA polymerase enzyme and make multiple copies. **The ability to multiply copies of antibiotic resistance gene in *E. coli* was called cloning** of antibiotic resistance gene in *E. coli*.

Recombinant DNA

- *Recombinant DNA* Technology (rDNA) is the process of joining two different DNA molecules and inserting it into a host organism to produce desired product.

- Recombinant DNA is the general name for a piece of DNA that has been created by combining two or more fragments from different sources.

Recombinant Technology

- Restriction endonucleases are used in genetic engineering to form 'recombinant' molecules of DNA, which are composed of DNA from different sources/genomes.

- When cut by the same restriction enzyme, the resultant DNA fragments have the same kind of 'sticky-ends' and, these can be joined together (end-to-end) using DNA ligases (Figure 11.2).

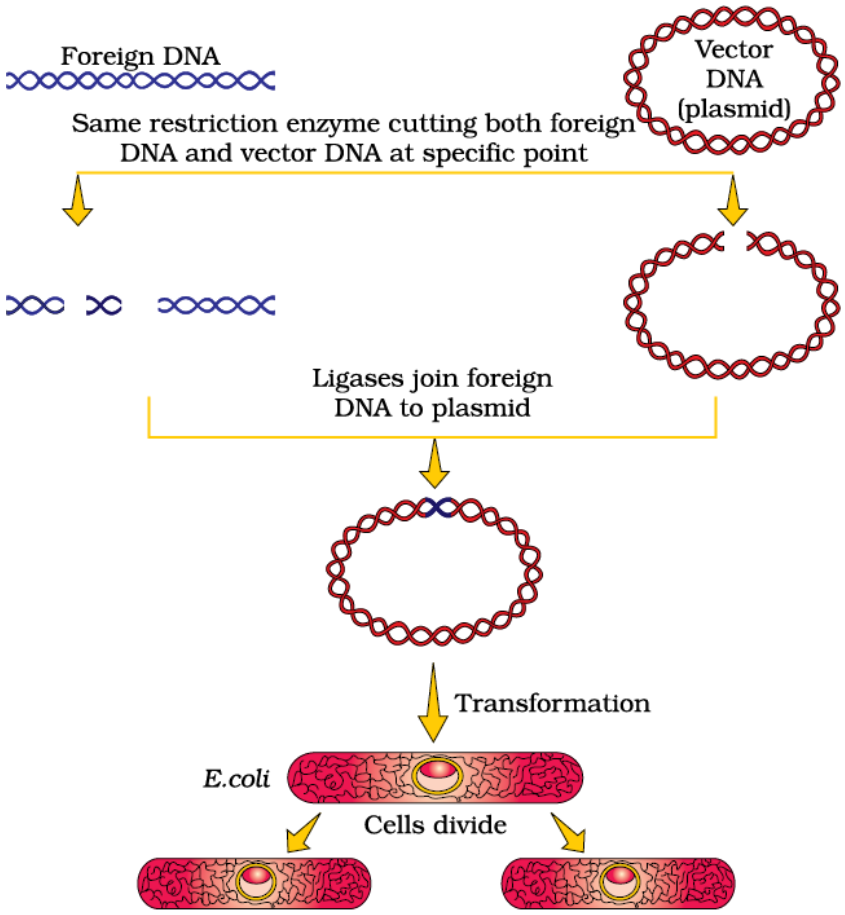
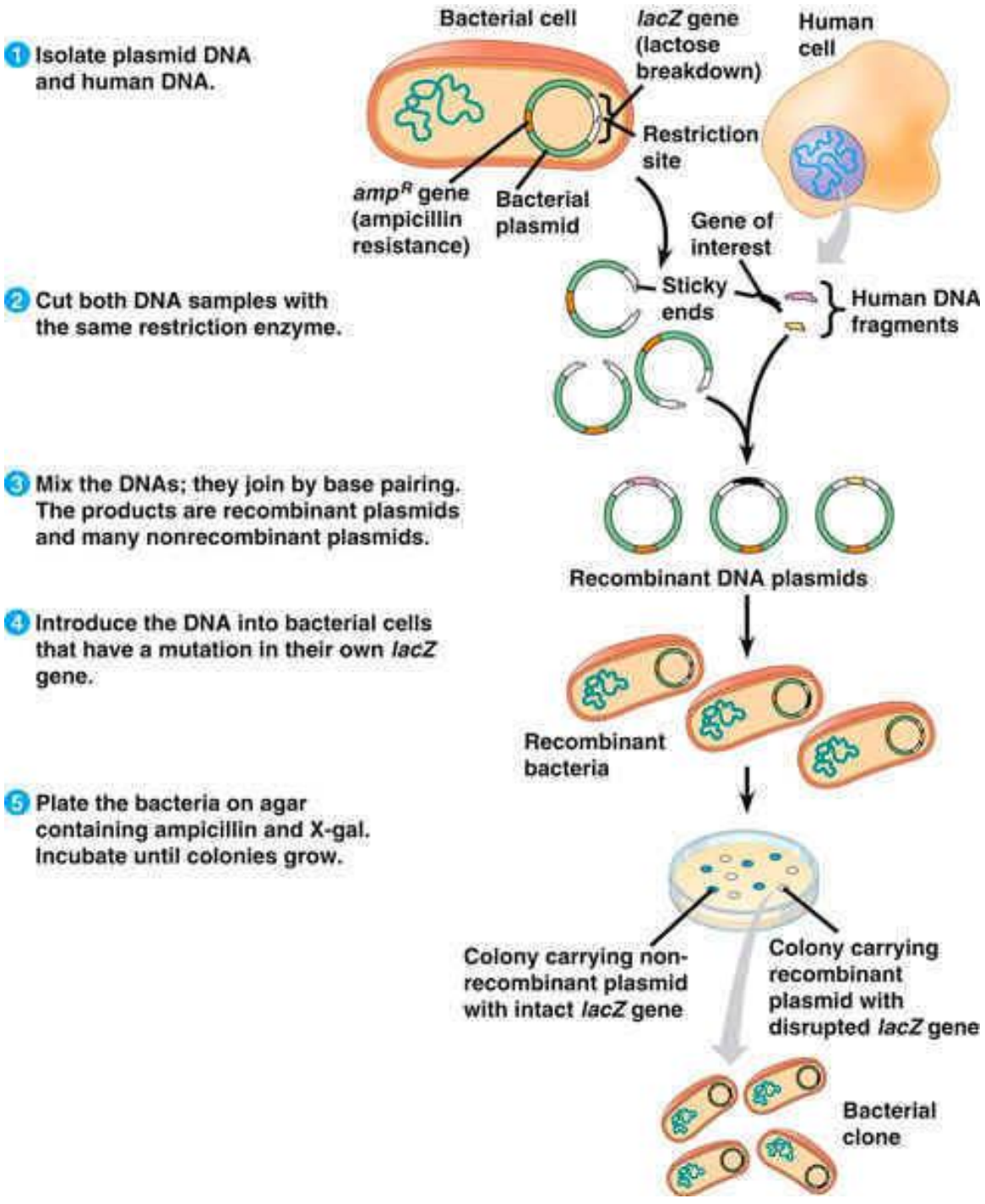


Figure 11.2 Diagrammatic representation of recombinant DNA technology

Recombinant DNA



TOOLS OF RECOMBINANT DNA TECHNOLOGY

1. Restriction Enzymes
2. Cloning Vectors
3. Competent Host (For Transformation with Recombinant DNA)

TOOLS OF RECOMBINANT DNA TECHNOLOGY

1. Restriction Enzymes

Separation and isolation of DNA fragments

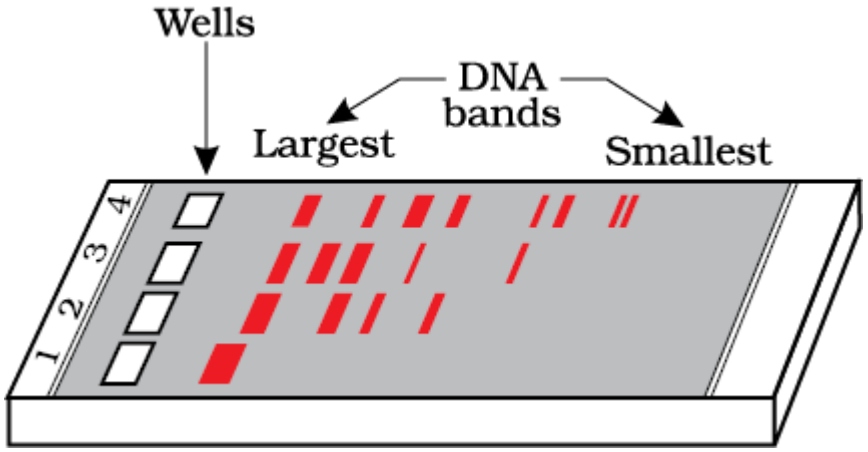


Figure. A typical agarose gel electrophoresis showing migration of undigested (lane 1) and digested set of DNA fragments (lane 2 to 4)

Ethidium bromide

Elution

Vectors

- ***Vector* is a DNA molecule that acts as a vehicle in transporting foreign DNA into the host**.
- Furthermore, they replicate and transfer information inside the host cell.
- A vector containing foreign DNA is termed recombinant DNA.
- Examples of vectors – Plasmids, Bacteriophages, Cosmids, etc. Sometimes. Artificial chromosomes also act as vectors.

Cloning Vectors

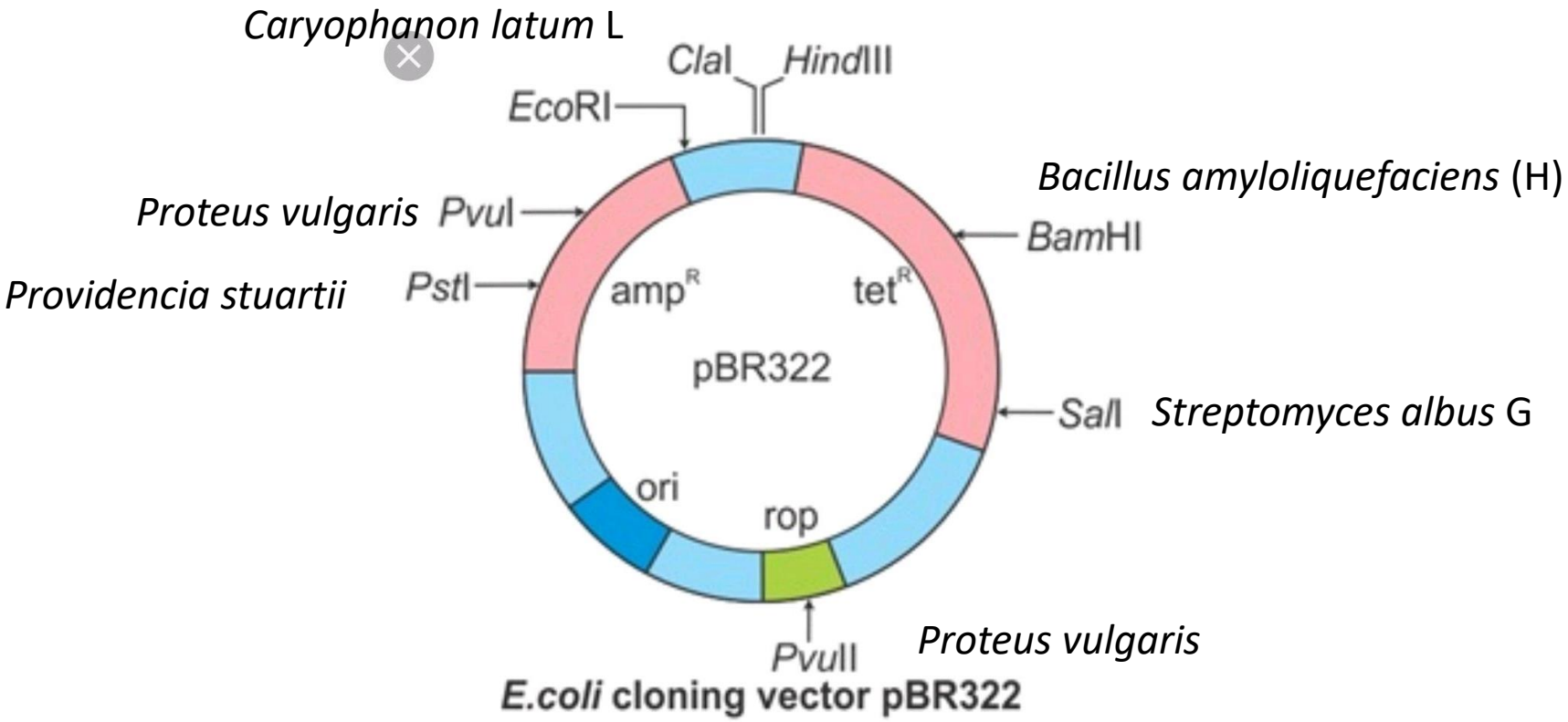


Figure *E. coli* cloning vector pBR322 showing restriction sites (*Hind* III, *Eco*R I, *Bam*H I, *Sa*l I, *Pvu* II, *Pst* I, *Cla* I), **ori** and **antibiotic resistance genes** (ampR and tetR).

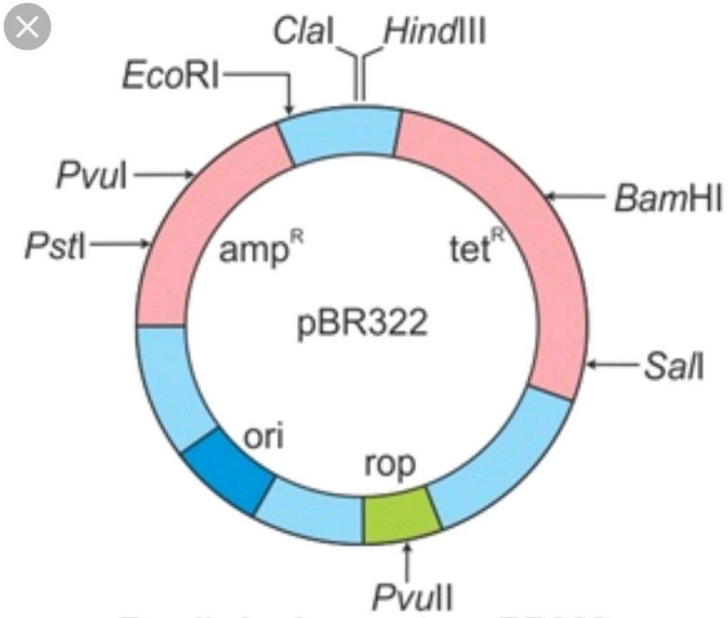
rop codes for the proteins involved in the replication of the plasmid.

Features that are required to facilitate cloning into a vector

(i) ***Origin of replication (ori)*** *:* This is a sequence from where replication starts and any piece of DNA when linked to this sequence can be made to replicate within the host cells. This sequence is also responsible for controlling the copy number of the linked DNA.

(ii) ***Selectable marker :***
*G*enes encoding resistance to antibiotics such as ampicillin, chloramphenicol, tetracycline or kanamycin, etc., are considered useful selectable markers for *E. coli. Or* ***LacZ*** *gene*

(iii) ***Cloning sites:*** Preferably single, recognition sites for the commonly used restriction enzymes

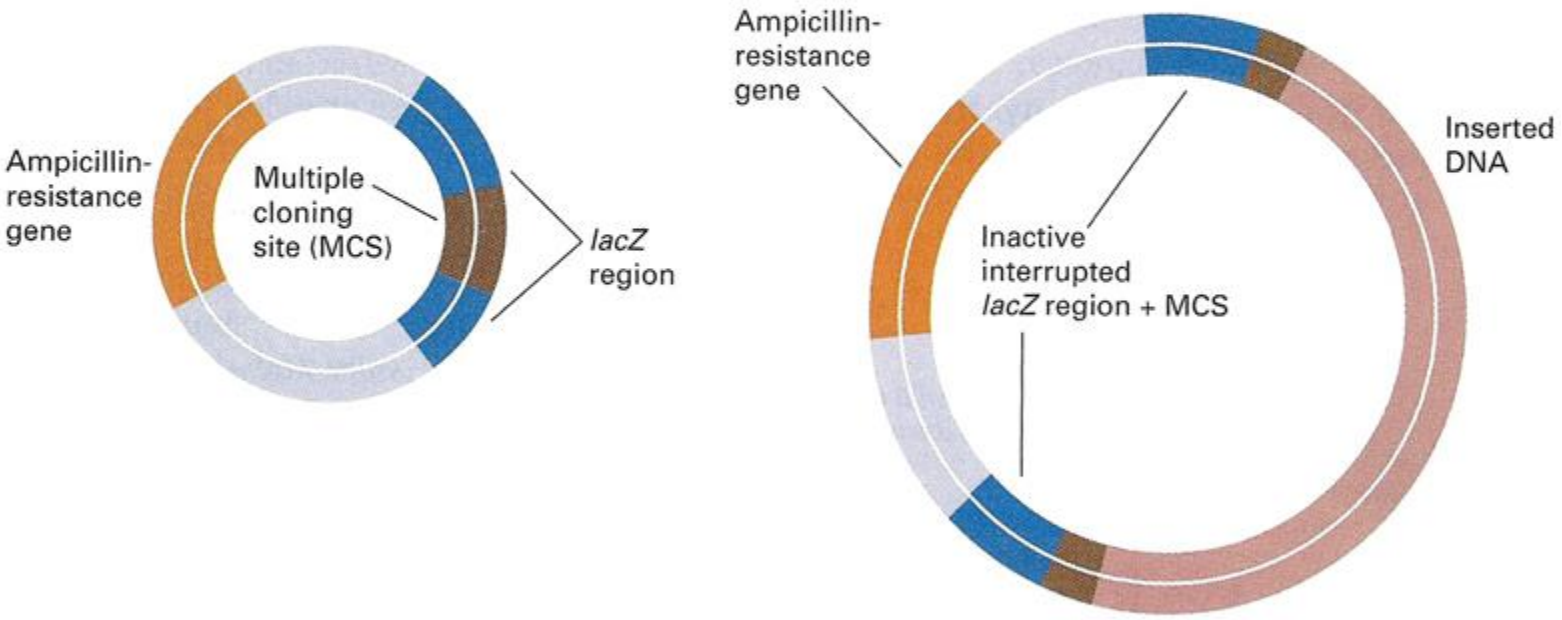


E.coli **cloning vector pBR322**

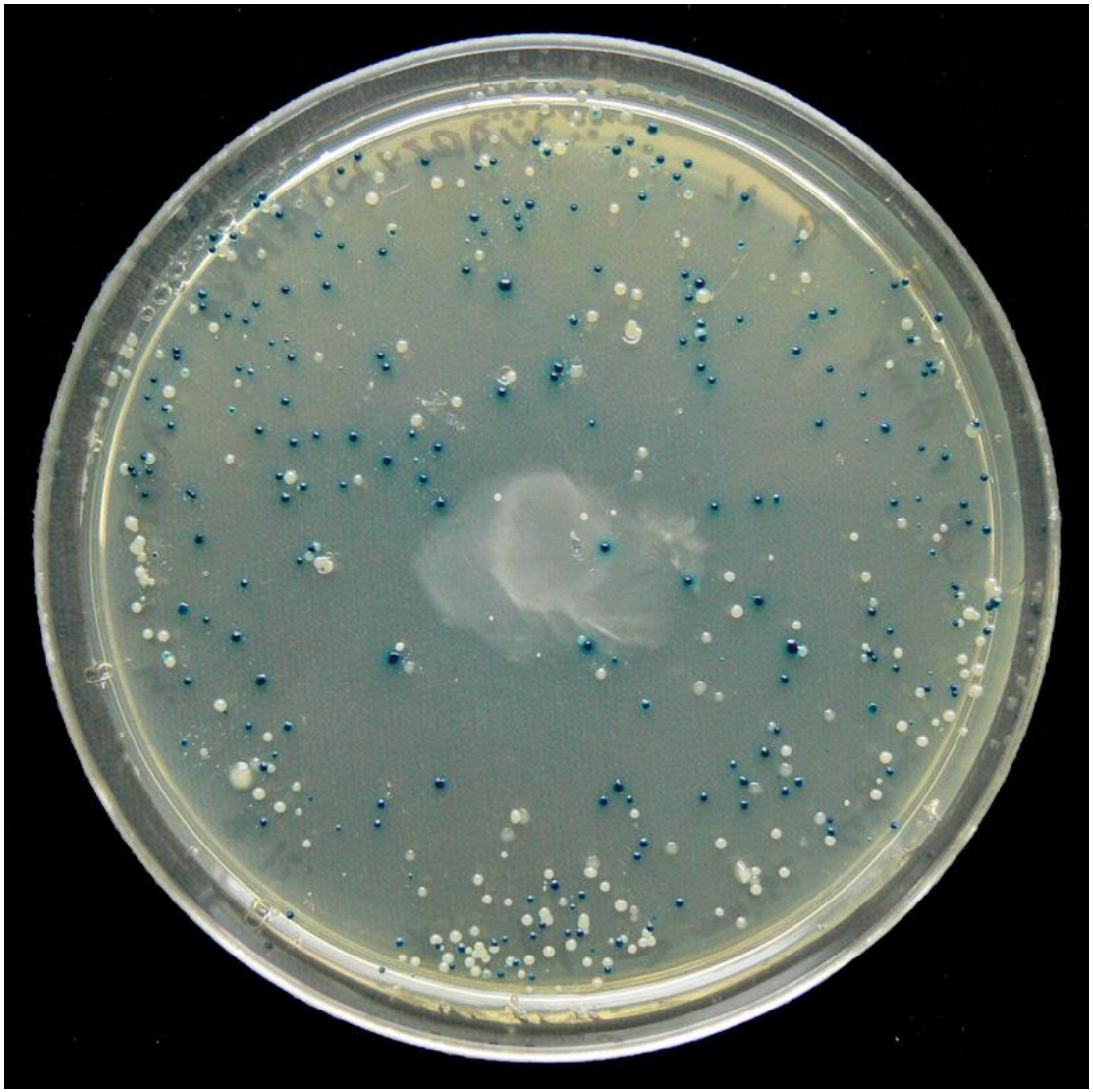
LacZ *gene*

Genes in the lac operon specify proteins that help the cell utilize lactose. lacZ encodes an enzyme that splits lactose into monosaccharides (single-unit sugars) that can be fed into glycolysis

Plasmid optimized for cloning



Blue White Colonies Screening



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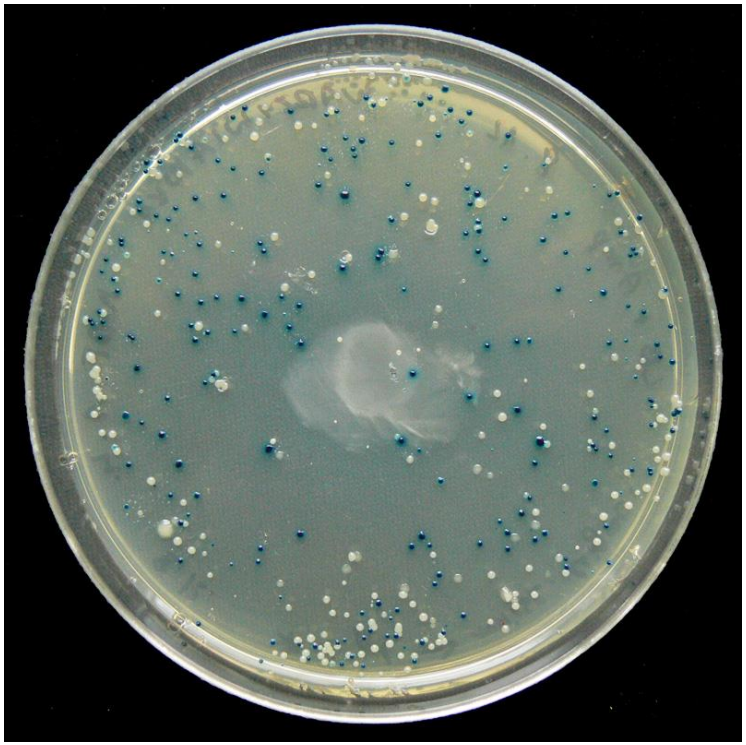
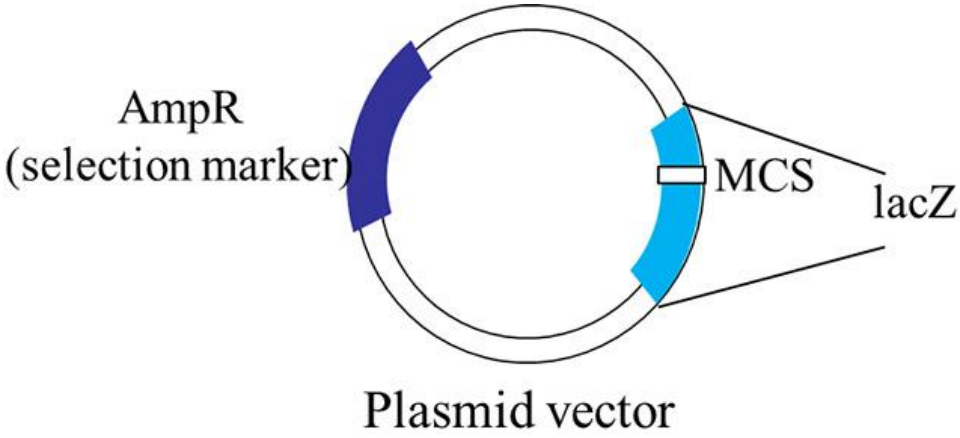
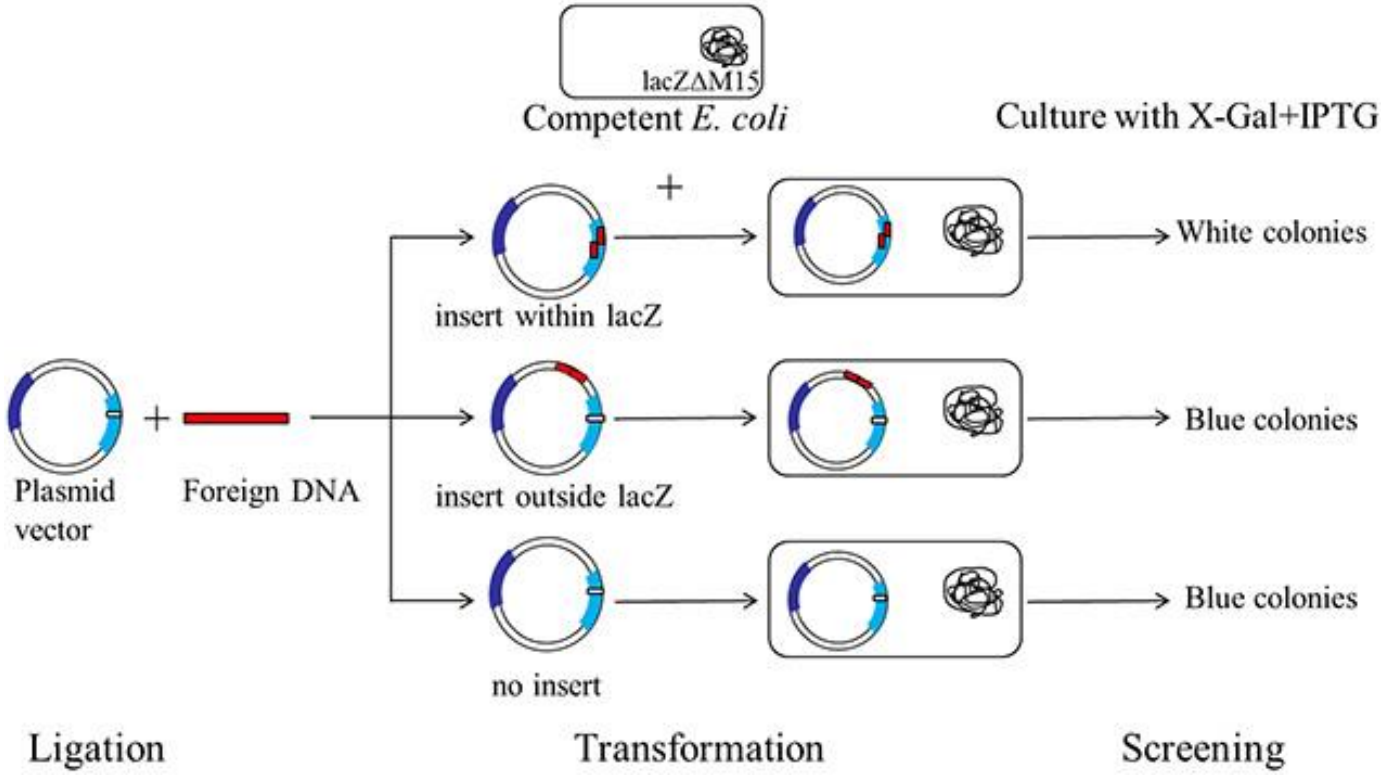




For screening the clones containing recombinant DNA, a chromogenic substrate known as X-gal is added to the agar plate. If β-galactosidase is produced, X-gal is hydrolyzed to form 5-bromo-4-chloro-indoxyl, which spontaneously dimerizes to produce an insoluble blue pigment called 5,5'-dibromo-4,4'-dichloro-indigo. The colonies formed by non-recombinant cells, therefore appear blue in color while the recombinant ones appear white. The desired recombinant colonies can be easily picked and cultured.

Blue White Colonies Screening



Cloning Vectors

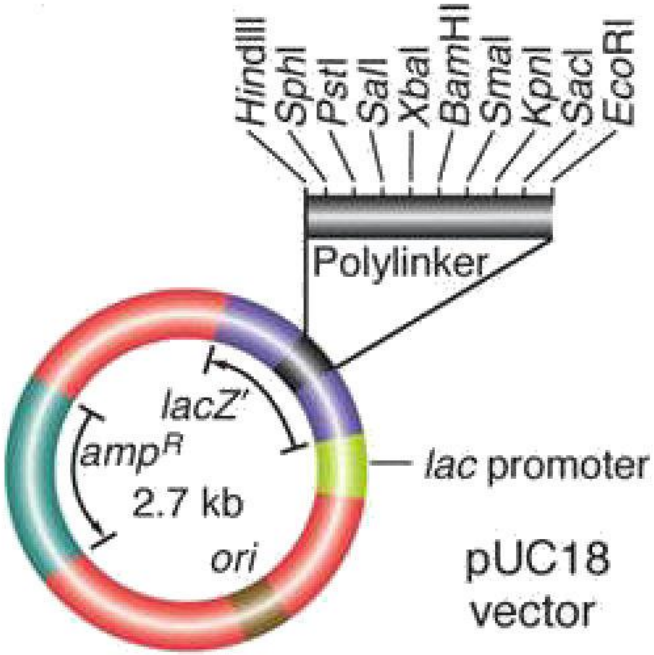
Selection of recombinants due to inactivation of antibiotics is a cumbersome procedure

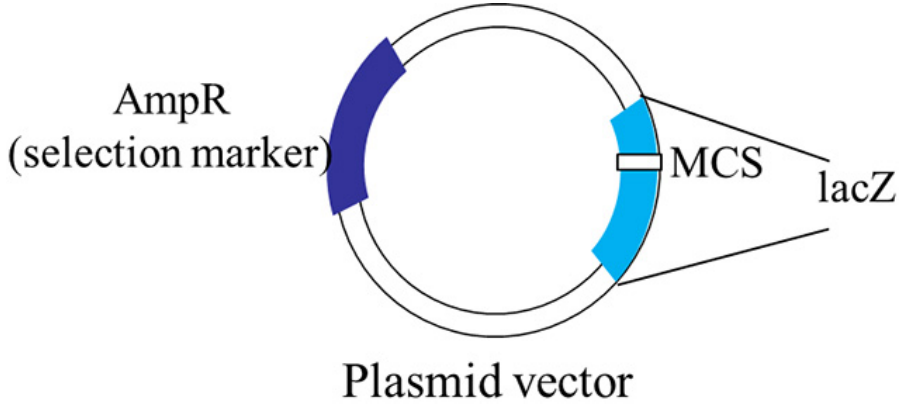
Alternative selectable markers have been developed - on the basis of their ability to produce colour in the presence of a chromogenic substrate.

In this, a recombinant DNA is inserted within the coding sequence of an **enzyme, β-galactosidase**. This results into inactivation of the enzyme, which is referred to as **insertional inactivation**.

The presence of a chromogenic substrate gives **blue coloured colonies if the plasmid in the bacteria does not have an insert**.

Presence of insert results into insertional inactivation of the â-galactosidase and the colonies do **not produce any colour**, these are identified as recombinant colonies.





Cloning Vectors

(iv) ***Vectors for cloning genes in animals :***

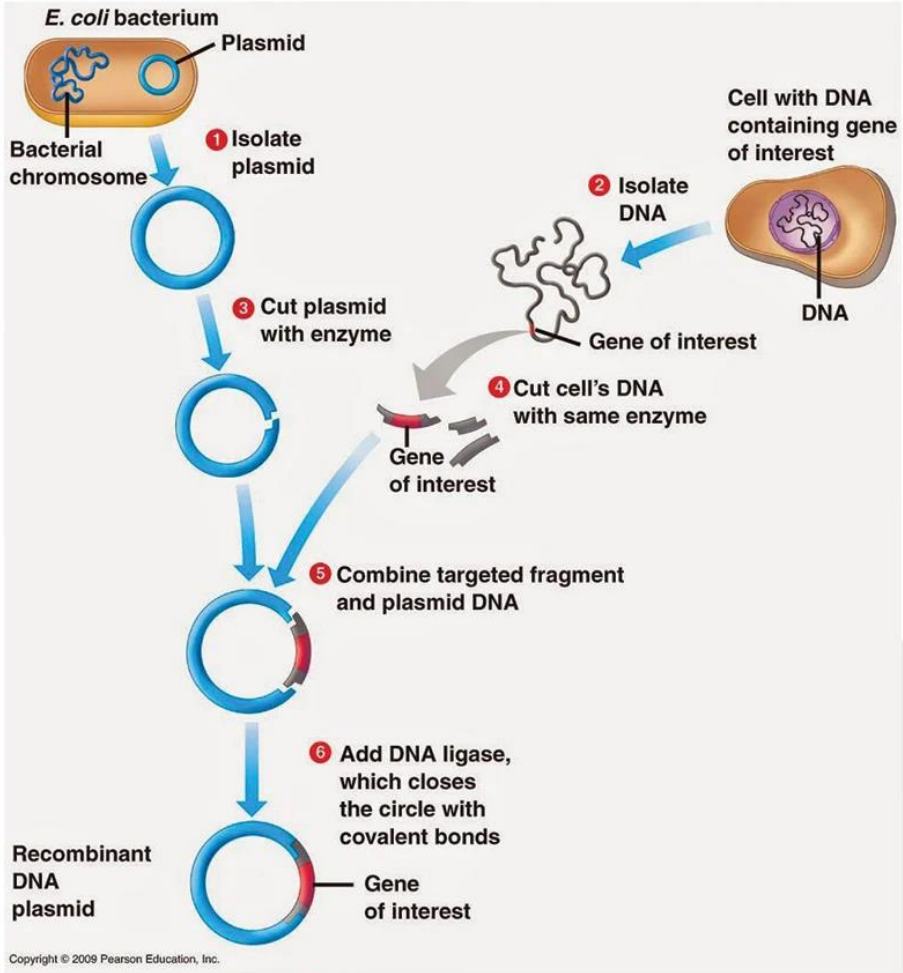
How to deliver genes to transform eukaryotic cells and force them to do what the bacteria or viruses want.

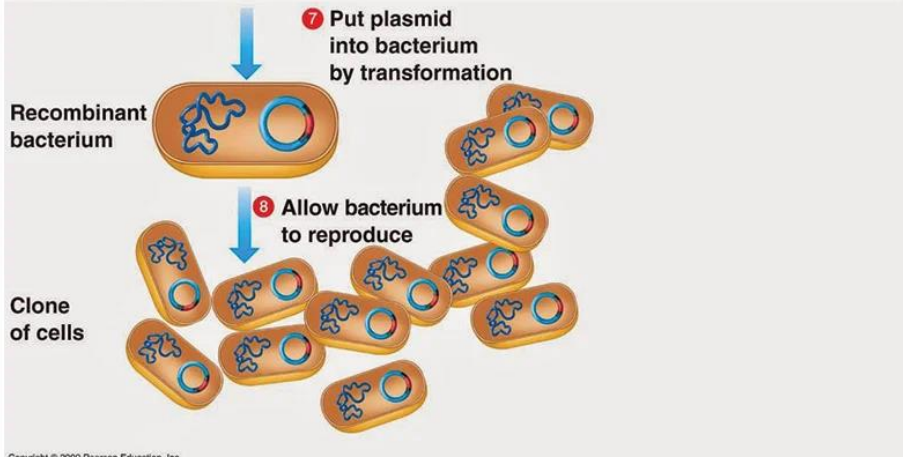
For example, Retroviruses in animals have the ability to transform normal cells into cancerous cells. A better understanding of the art of delivering genes by pathogens in their eukaryotic hosts has generated knowledge to transform these tools of pathogens into useful vectors for delivering genes of interest to humans.

Similarly, **retroviruses** have also been disarmed and are now used to deliver desirable genes into animal cells. So, once a gene or a DNA fragment has been ligated into a suitable vector it is transferred into a bacterial, plant or animal host (where it multiplies).

DNA cloning

- *DNA cloning* is the molecular biology technique used for creating copies of *DNA* fragments, cells, or organisms using restriction enzymes and *DNA* ligase.





TOOLS OF RECOMBINANT DNA TECHNOLOGY

1. Restriction Enzymes
2. Cloning Vectors
3. Competent Host (For Transformation with Recombinant DNA)

Competent Host (For Transformation with Recombinant DNA)

Transformation, a bacterium takes up a piece of DNA floating in its environment.

- DNA is a **hydrophilic** molecule, it cannot pass through cell membranes.
- In order to force bacteria to take up the plasmid, the bacterial cells must first be made **'competent'** to take up DNA.
- This is done by **treating them with a specific concentration of a divalent cation**, such as **calcium**, which **increases the efficiency with which DNA enters** the bacterium through pores in its cell wall.

- Recombinant DNA can then be forced into such cells by incubating the cells with recombinant DNA on ice, followed by placing them briefly at 42^0C (heat shock), and then putting them back on ice. This enables the bacteria to take up the recombinant DNA.

Comparison of Transformation and Transfection

Transformation	Transfection
1. Applicable to bacteria	1. Applicable to eukaryotic cells*
2. Exogenous genetic material is taken up by competent bacteria	2. Exogenous genetic material is introduced into the eukaryotic cells
3.Bacteria can be made competent either chemically or by electroporation	3. Introduction of exogenous genetic material may be liposome-mediated, by electroporation or by using viral vector
4.The exogenous genetic material may integrate into the bacterial genome or exist as a plasmid	4. The exogenous genetic material is either integrated into the genome or is degraded
5.Transformation enables the expression of multiple copies of DNA resulting in large amounts of protein or enzyme that are not normally expressed by bacteria	5. Genetic material of transformed bacteria may be used to transfect eukaryotic cells for DNA or protein expression studies

Other ways to introduce alien DNA into host cells

- Animal cell :

  Micro-injection, recombinant DNA is directly injected into the nucleus of an animal cell.

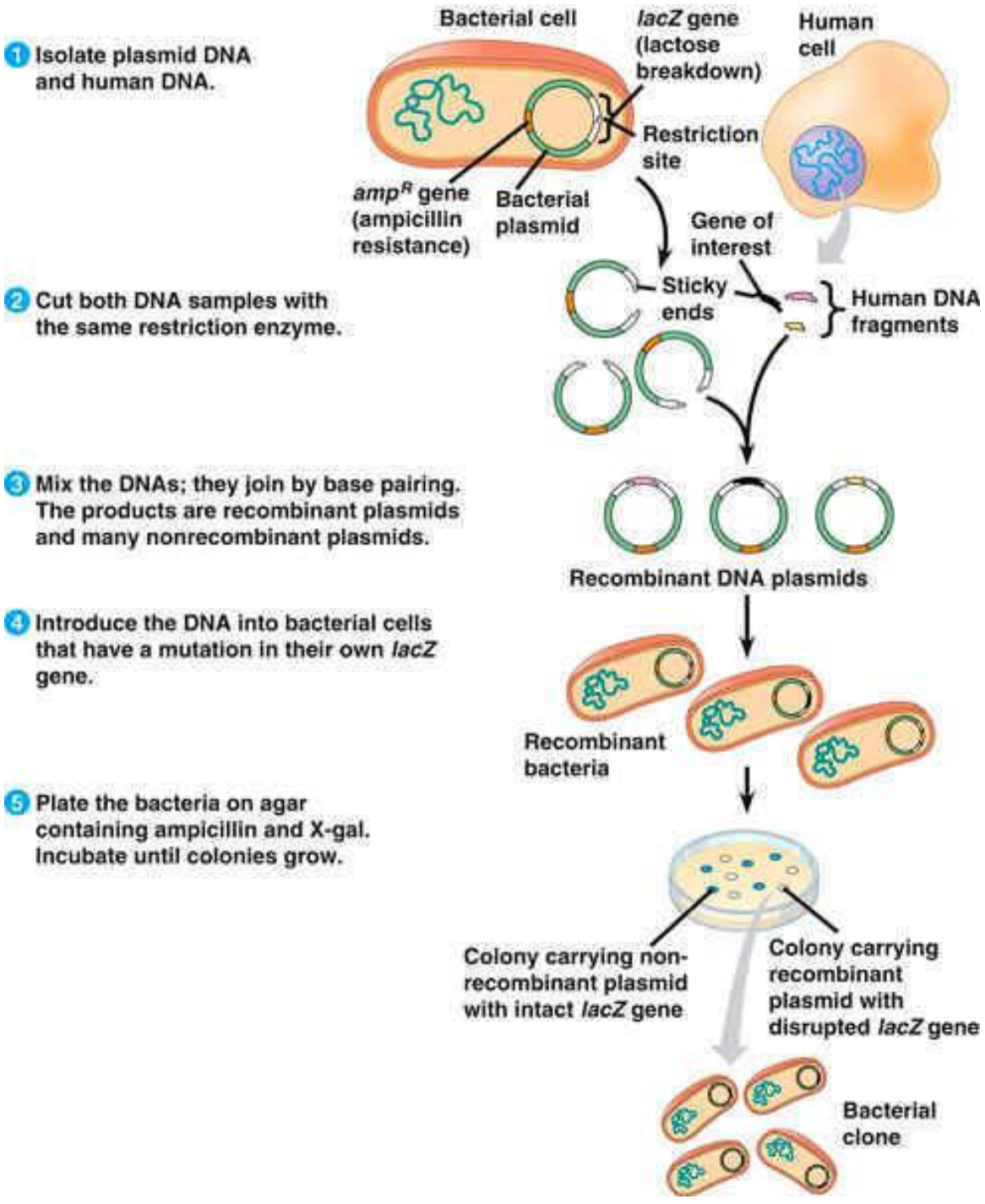
- **'Disarmed pathogen'** vectors, which when allowed to infect the cell, transfer the recombinant DNA into the host.

Recombinant DNA

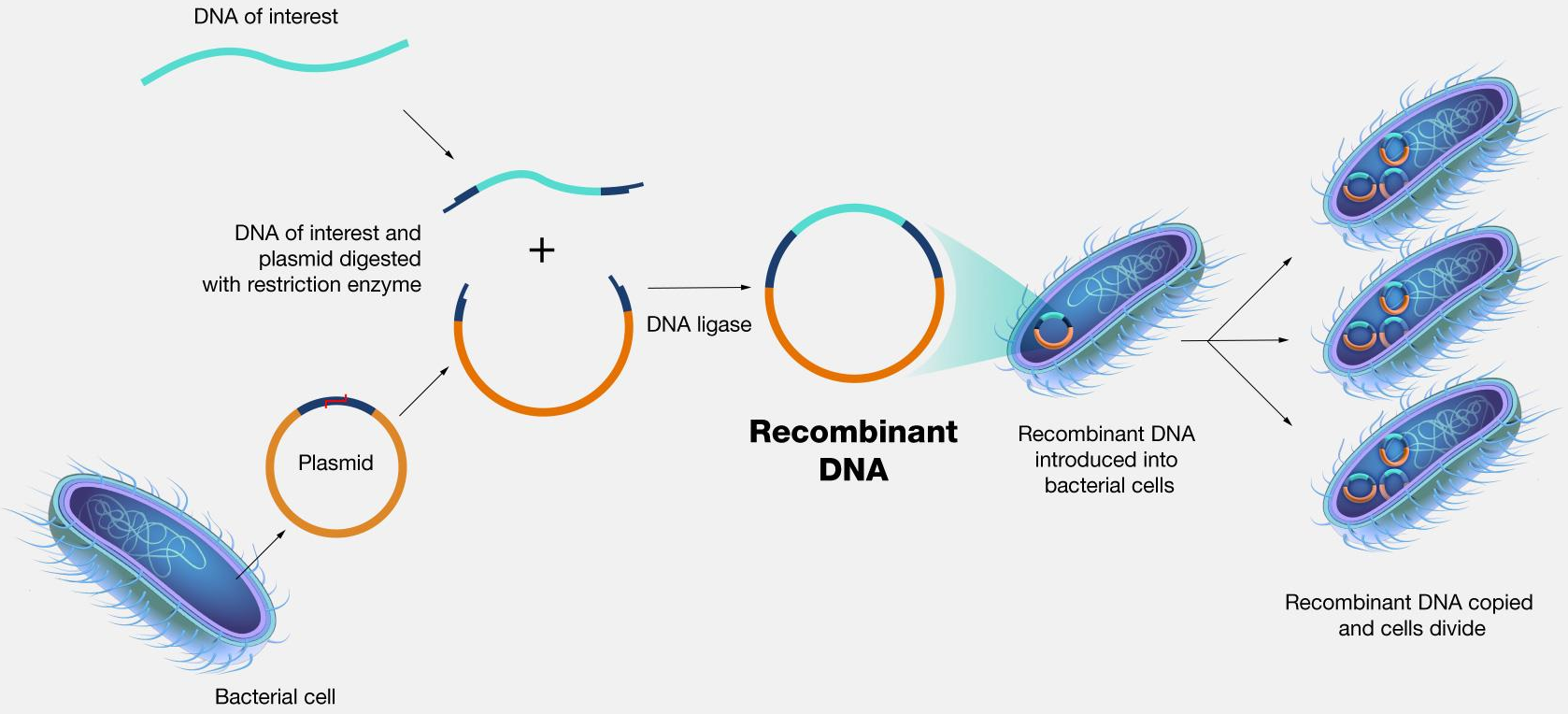
Recombinant DNA technology involves using enzymes and various laboratory techniques to manipulate and isolate DNA segments of interest. This method can be used **to combine (or splice) DNA from different species or to create genes with new functions.** The resulting copies are often referred to as **recombinant DNA**.

Such work typically involves propagating the recombinant DNA in a bacterial or yeast cell, whose cellular machinery copies the engineered DNA along with its own.

Recombinant DNA



Recombinant DNA



PROCESSES OF RECOMBINANT DNA TECHNOLOGY

Recombinant DNA technology involves several steps in specific sequence such as

1. Isolation of DNA,
2. Cutting of DNA at specific locations by RE,
3. Isolation of a desired DNA fragment,
4. Ligation of the DNA fragment into a vector, transferring the
5. Recombinant DNA into the host,
6. Culturing the host cells in a medium at large scale and
7. Extraction of the desired product.

1. Isolation of Genetic material DNA

- Nucleic acid is the genetic material of all organisms
- In majority of organisms this is DNA.
- In order to cut the DNA with restriction enzymes, it needs to be in pure form, free from other macro-molecules.
- DNA is enclosed within the membranes, we have to break the cell open to release DNA along with other macromolecules such as RNA, proteins, polysaccharides and also lipids.

- **This can be achieved by treating the bacterial cells/plant or animal tissue with enzymes such as lysozyme (bacteria), cellulase (plant cells), chitinase (fungus).**
- You know that genes are located on long molecules of DNA interwined with proteins such as histones. **The RNA can be removed by treatment with ribonuclease** whereas **proteins can be removed by treatment with protease.**
- Other molecules can be removed by appropriate treatments and purified **DNA ultimately precipitates out after the addition of chilled ethanol**. This can be seen as collection of **fine threads in the suspension** (Figure).

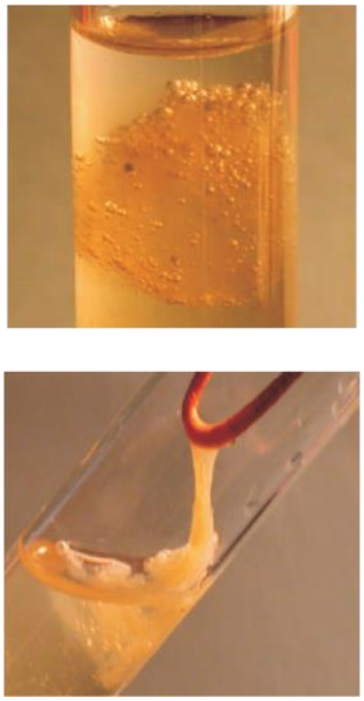


Figure : DNA that separates out can be removed by spooling

2. Cutting of DNA at Specific Locations

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

Agarose gel electrophoresis is employed to check the progression of a restriction enzyme digestion. DNA is a negatively charged molecule, hence it moves towards the positive electrode (anode). The process is repeated with the vector DNA also.

Joining of DNA involves several processes. After having cut the source DNA as well as the vector DNA with a specific restriction enzyme, the cut out 'gene of interest' from the source DNA and the cut vector with space are mixed and ligase is added. This results in the preparation of **recombinant DNA**.

3. Amplification of Gene of Interest using PCR

In this reaction, multiple copies of the gene (or DNA) of interest is synthesised *in vitro* using two sets of primers (small chemically synthesised oligonucleotides that are complementary to the regions of DNA) and the enzyme DNA polymerase.

The enzyme extends the primers using the nucleotides provided in the reaction and the genomic DNA as template.

If the process of replication of DNA is repeated many times, the segment of DNA can be amplified to approximately billion times, i.e., 1 billion copies are made. Such repeated amplification is achieved by the use of a thermostable DNA polymerase (isolated from a bacterium, ***Thermus aquaticus***), which remain active during the high temperature induced denaturation of double stranded DNA.

The amplified fragment if desired can now be used to ligate with a vector for further cloning (Figure).

3. Amplification of Gene of Interest using PCR

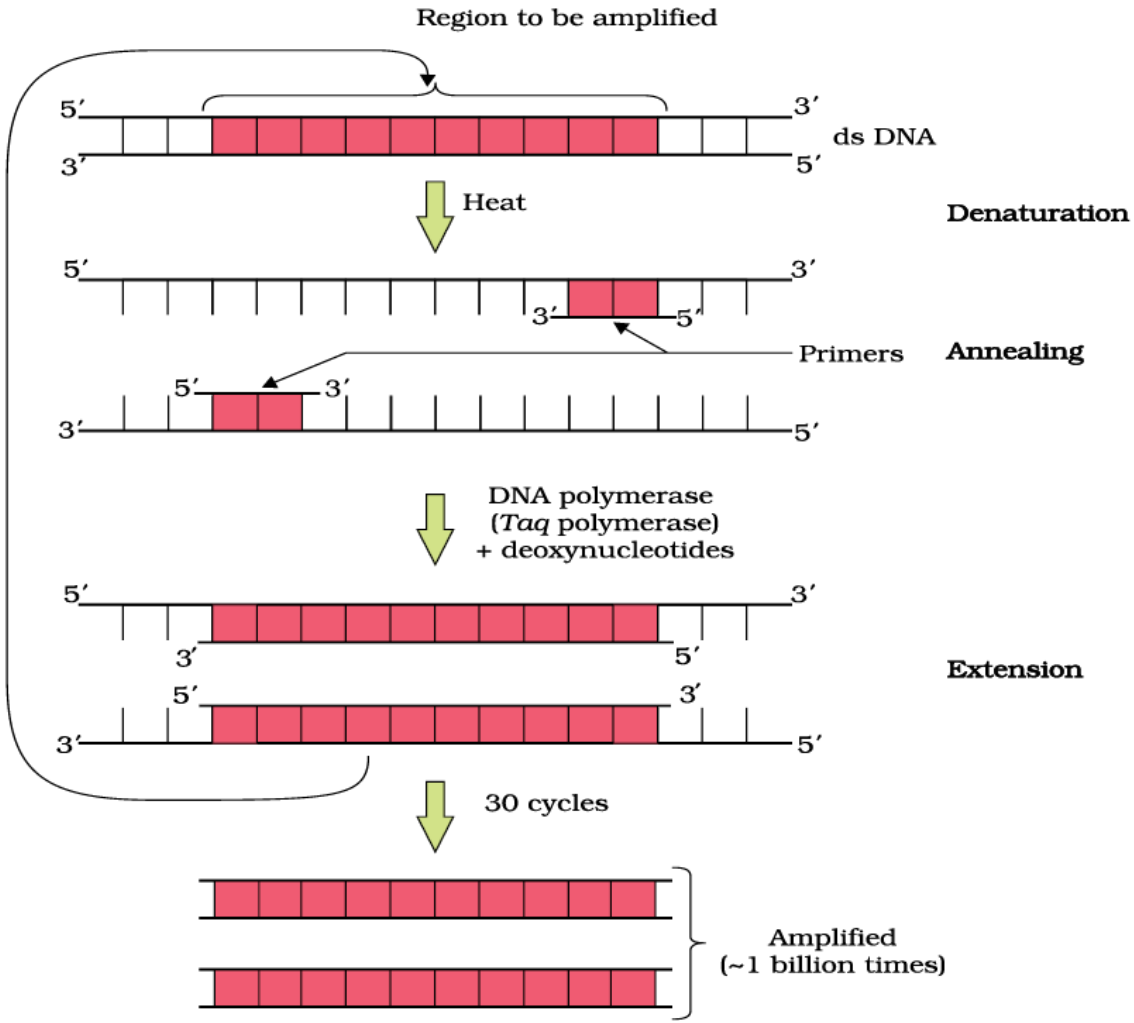


Figure. Polymerase chain reaction (PCR) :
Each cycle has three steps: (i) Denaturation; (ii) Primer annealing; and (iii) Extension of primers

4. Insertion of Recombinant DNA into the Host Cell/Organism

There are several methods of introducing the ligated DNA into recipient cells. Recipient cells after making them '**competent**' to receive, take up DNA present in its surrounding. So, if a recombinant DNA bearing gene for resistance to an antibiotic (e.g., ampicillin) is transferred into *E. coli* cells, the host cells become transformed into ampicillin-resistant cells. If we spread the transformed cells on agar plates containing ampicillin, only **transformants** will grow, untransformed recipient cells will die. Since, due to ampicillin resistance gene, one is able to select a transformed cell in the presence of ampicillin. **The ampicillin resistance gene in this case is called a selectable marker**.

5. Obtaining the Foreign Gene Product

Having cloned the gene of interest

Having optimised the conditions to induce the expression of the target protein

If any protein encoding gene is expressed in a heterologous host, it is called a **recombinant protein**.

5. Obtaining the Foreign Gene Product

The cells can also be multiplied in a **continuous culture system** wherein the used medium is drained out from one side while fresh medium is added from the other to maintain the cells in their physiologically most active log/exponential phase. produces a larger biomass leading to higher yields of desired protein.

To produce in large quantities, the development of **bioreactors**, where large volumes (100-1000 litres) of culture can be processed

A **bioreactor** provides the optimal conditions for achieving the desired product by providing optimum growth conditions (temperature, pH, substrate, salts, vitamins, oxygen).

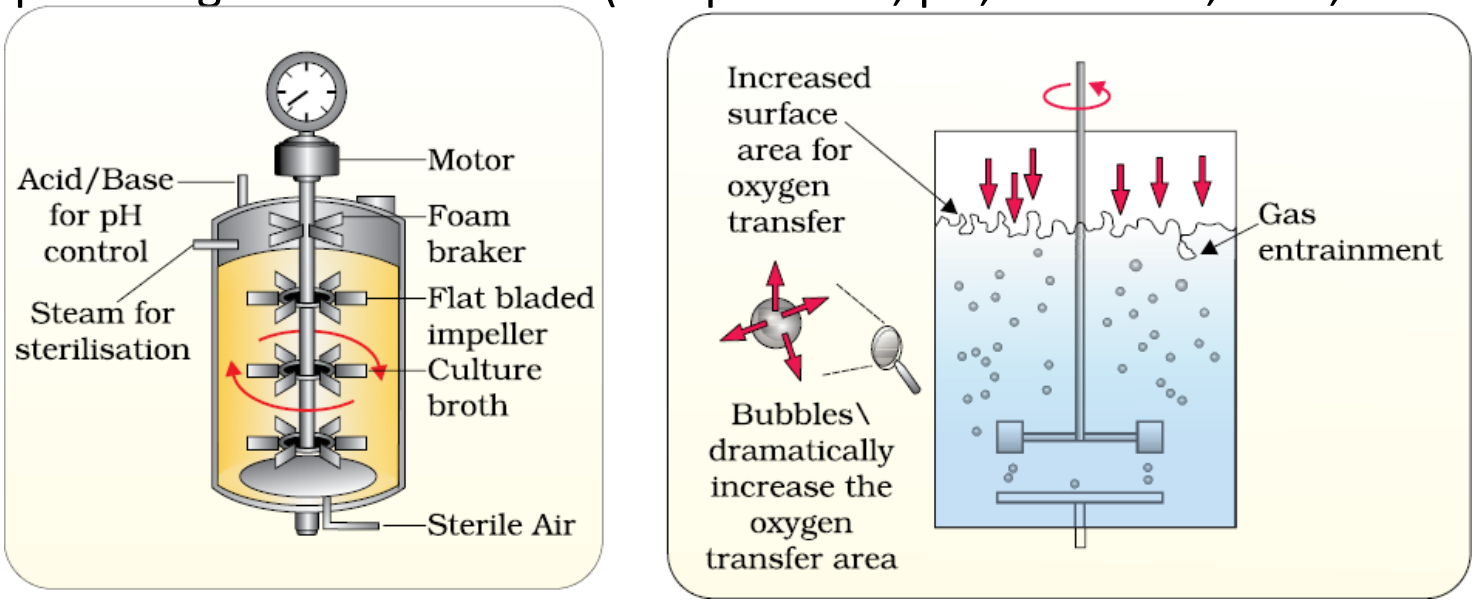


Figure 11.7 (a) Simple stirred-tank bioreactor; (b) Sparged stirred-tank bioreactor through which sterile air bubbles are sparged

Downstream Processing

After completion of the **biosynthetic stage**, the product has to be subjected through a series of processes before it is ready for marketing as a finished product.

The processes include **separation and purification**, which are collectively referred to as downstream processing.

The product has to be **formulated with suitable preservatives**. Such formulation has to undergo thorough **clinical trials** as in case of drugs.

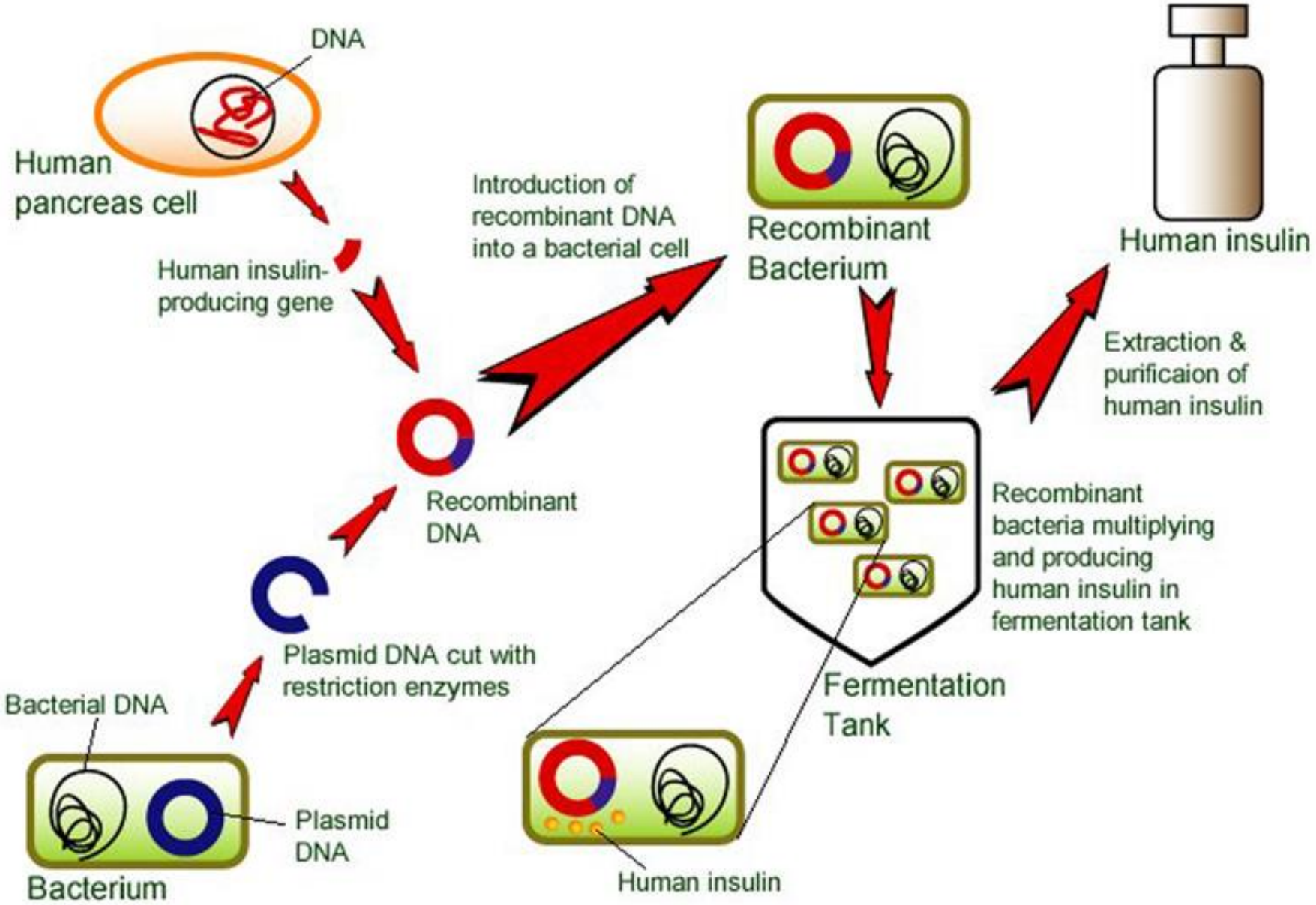
Strict quality control testing for each product is also required.

The **downstream processing and quality control testing vary from product to product**.

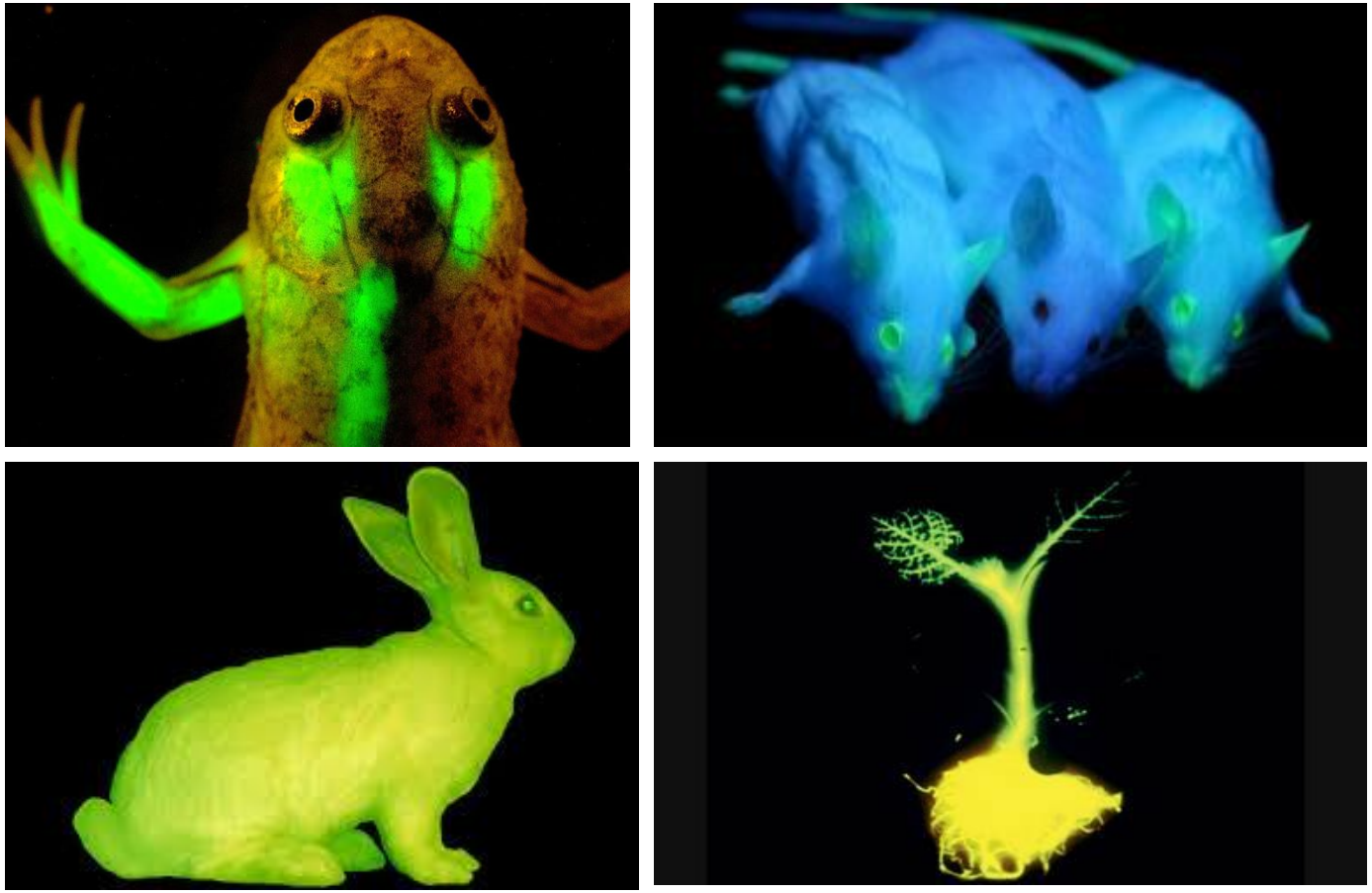
Application of Recombinant DNA Technology

- **Gene Therapy** – It is used as an attempt to correct the gene defects which give rise to heredity diseases.
- **Clinical diagnosis** – ELISA is an example where the application of recombinant
- Recombinant DNA technology is widely used in Agriculture to **produce genetically-modified organisms** such as Flavr Savr tomatoes, golden rice rich in proteins, and Bt-cotton to protect the plant against ball worms and a lot more.
- In the field of medicines, Recombinant DNA technology is used for the **production of Insulin**.
- **Development o Vaccine** in animals/human eg., mRNA vaccine SARS Corona virus-2
- **Production of transgenic plants or animals** with improved quality

Human Insulin Production



Genetically modified plants and animals



Modern biotechnology using genetically modified organisms was made possible only when man learnt to alter the chemistry of DNA and construct recombinant DNA. This key process is called recombinant DNA technology or genetic engineering.

THANKS

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Biotechnology Principles and Processes

Conceptual development of the principles of genetic engineering

Asexual reproduction preserves the genetic information, while **sexual reproduction** permits variation.

Traditional hybridisation procedures used in plant and animal breeding, very often lead to inclusion and multiplication of undesirable genes along with the desired genes.

The **techniques of genetic engineering** which include

-creation of **recombinant DNA**,

-use of **gene cloning** and **gene transfer**,

overcome this limitation and allows us to isolate and introduce only one or a set of desirable genes without introducing undesirable genes into the target organism.

Biotechnology Principles and Processes

Three basic steps in genetically modifying an organism —

(i) identification of DNA with desirable genes;

(ii) introduction of the identified DNA into the host;

(iii) maintenance of introduced DNA in the host and transfer of the DNA to its progeny.

Biotechnology Principles and Processes

GMO Salmon

Photo of a genetically engineered Salmon. Created so that it continuously produces growth hormones and can be sold as a full size fish after 18 months instead of 3 years.

Reference:
https://www.nationalgeographic.org/encyclopedia/genetically-modified-organisms/

Biotechnology Principles and Processes

PRINCIPLES OF BIOTECHNOLOGY

Among many, the two core techniques that enabled birth of modern biotechnology are :

(i) ***Genetic engineering*** *:* Techniques to alter the chemistry of genetic material (DNA and RNA), to introduce these into host organisms and thus change the phenotype of the host organism.

(ii) ***Bioprocess engineering****:* Maintenance of sterile (microbial contamination-free) ambience in chemical engineering processes to enable growth of only the desired microbe/eukaryotic cell in **large quantities for the manufacture of biotechnological products** like antibiotics, vaccines, enzymes, etc.