RECOMBINANT DNA TECHNOLOGY

Recombinant DNA technology involves several steps in specific sequence such as isolation of DNA, fragmentation of DNA by restriction endonucleases, isolation of a desired DNA fragment, ligation of the DNA fragment into a vector, transferring the recombinant DNA into the host, culturing the host cells in a medium at large scale and extraction of the desired product.

TOOLS OF RECOMBINANT DNA TECHNOLOGY

Recombinant DNA technology can be accomplished only if we have the key tools, i.e., **restriction enzymes**, **polymerase enzymes**, **ligases**, **vectors** and the **host organism** .

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. Later was called **restriction endonuclease**.

The first restriction endonuclease–Hind II, whose functioning depended on a specific DNA nucleotide sequence was isolated and characterized five years later. It was found that Hind II always cut DNA molecules at a particular point by recognizing a specific sequence of six base pairs. This specific base sequence is known as the recognition sequence for Hind II.

Besides Hind II, today we know more than 900 restriction enzymes that have been isolated from over 230 strains of bacteria each of which recognize different recognition sequences.

The convention for naming these enzymes is the first letter of the name comes from the genus and the second two letters come from the species of the prokaryotic cell from which they were isolated, e.g., 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 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 1). Each restriction endonuclease recognizes a specific **palindromic nucleotide sequences** in the DNA**.**

Do you know what **palindromes** are? These are groups of letters that form the same words when read both forward and backward, e.g., "MALAYALAM". As against a word-palindrome where the same word is read in both directions, the palindrome in DNA is a sequence of base pairs that reads same on the two strands when orientation of reading is kept the same. For example, the following sequences read the same on the two strands in 5′ ⟶ 3′ direction. This is also true if read in the 3′ ⟶ 5′ direction.

5′ —— GAATTC —— 3′

3′ —— CTTAAG —— 5′

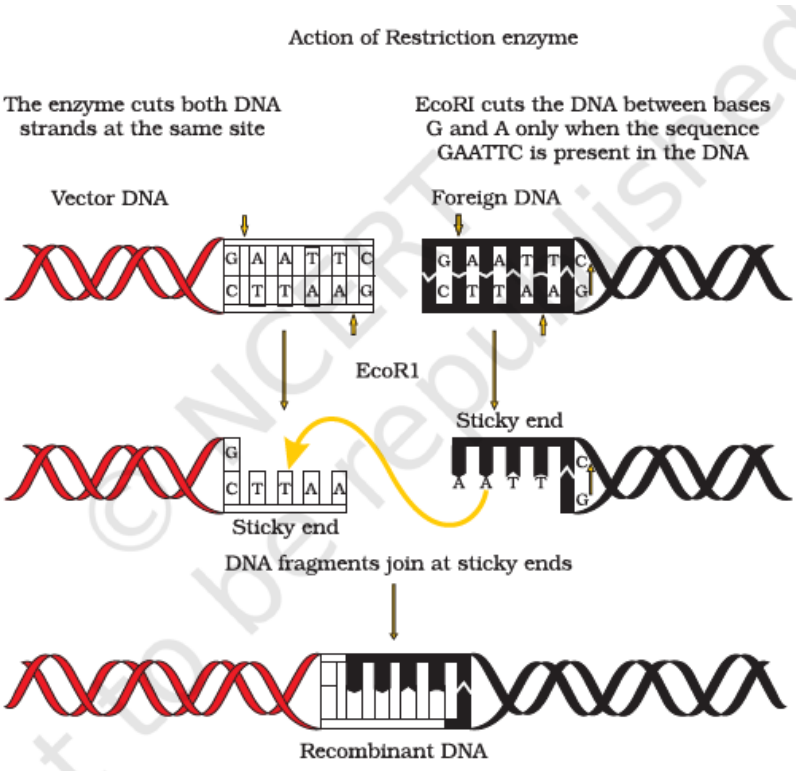


Figure 1: Steps in formation of recombinant DNA by action of restriction endonuclease enzyme – EcoRI

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 2).

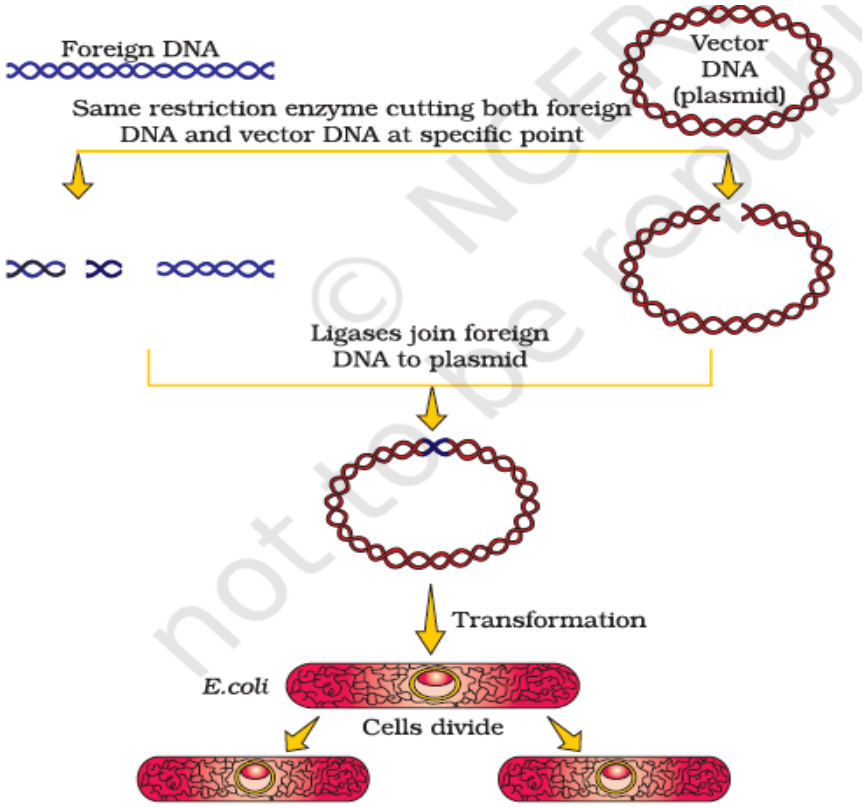


TABLE The Specificity of Some Restriction Enzymes

Name	Origin	Recognition Sequence
*Bam*HI	*Bacillus amyloliquefaciens* H	5′ G//GATCC 3′
		3′ CCTAG//G 5′
*Eco*RI	*Escherichia coli* RY13	5′ G//AATTC 3′
		3″CTTAA//G 5′
*Hind*III	*Haemophilus influenzae* Rd	5′ A//AGCTT 3′
		3′ TTCGA//A 5′
*Hae*III	*Haemophilus aegyptius*	5′ GG//CC 3′
		3′ CC//GG 5′
*Kpn*I	*Klebsiella pneumoniae*	5′ GGTAC//C 3′
		3′ C//CATGG 5′

Double bars indicate the cleavage site in the DNA strand.

Cloning Vectors

Plasmids and bacteriophages have the ability to replicate within bacterial cells independent of the control of chromosomal DNA.

Bacteriophages because of their high number per cell have very high copy numbers of their genome within the bacterial cells. Some plasmids may have only one or two copies per cell whereas others may have 15-100 copies per cell. Their numbers can go even higher. If we are able to link an alien piece of DNA with bacteriophage or plasmid DNA, we can multiply its numbers equal to the copy number of the plasmid or bacteriophage. Vectors used at present, are engineered in such a way that they help easy linking of foreign DNA and selection of recombinants from non-recombinants.

The following are the features that are required to facilitate cloning into a vector.

1- **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. So, if one wants to recover many copies of the target DNA it should be cloned in a vector whose origin support high copy number.

2- **Selectable marker**

In addition to 'ori', the vector requires a selectable marker, which helps in identifying and eliminating non-transformants and selectively permitting the growth of the transformants. Transformation is a procedure through which a piece of DNA is introduced in a host bacterium. Normally, the genes encoding resistance to antibiotics such as ampicillin, chloramphenicol, tetracycline or kanamycin, etc., are considered useful selectable markers for *E. coli*. The normal *E. coli* cells do not carry resistance against any of these antibiotics.

3- **Cloning sites**

In order to link the alien DNA, the vector needs to have very few, preferably single, recognition sites for the commonly used restriction enzymes. Presence of more than one recognition sites within the vector will generate several fragments, which will complicate the gene cloning. The ligation of alien DNA is carried out at a restriction site present in one of the two antibiotic resistance genes. For example, you can ligate a foreign DNA at the BamH I site of tetracycline resistance gene in the vector pBR322.

The recombinant plasmids will lose tetracycline resistance due to insertion of foreign DNA but can still be selected out from non-recombinant ones by plating the transformants on ampicillin containing medium. The transformants growing on ampicillin containing medium are then transferred on a medium containing tetracycline. The recombinants will grow in ampicillin containing medium but not on that containing tetracycline. But, nonrecombinants will grow on the medium containing both the antibiotics. In this case, one antibiotic resistance gene helps in selecting the transformants, whereas the other antibiotic resistance gene gets 'inactivated due to insertion' of alien DNA, and helps in selection of recombinants.

4- Vectors for cloning genes in plants and animals

How to deliver genes to transform eukaryotic cells and force them to do what the bacteria or viruses want. For example, *Agrobacterium tumifaciens*, a pathogen of several dicot plants is able to deliver a piece of DNA known as 'T-DNA' to transform normal plant cells into a tumor and direct these tumor cells to produce the chemicals required by the pathogen. Similarly, 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. The tumor inducing (Ti) plasmid of *Agrobacterium tumifaciens* has now been modified into a cloning vector which is no more pathogenic to the plants but is still able to use the mechanisms to deliver genes of our interest into a variety of

plants. 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).

PROCESSES OF RECOMBINANT DNA TECHNOLOGY

1- Isolation of the Genetic Material (DNA)

In order to cut the DNA with restriction enzymes, it needs to be in pure form, free from other macro-molecules. Since the 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).

Genes are located on long molecules of DNA intertwined 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.

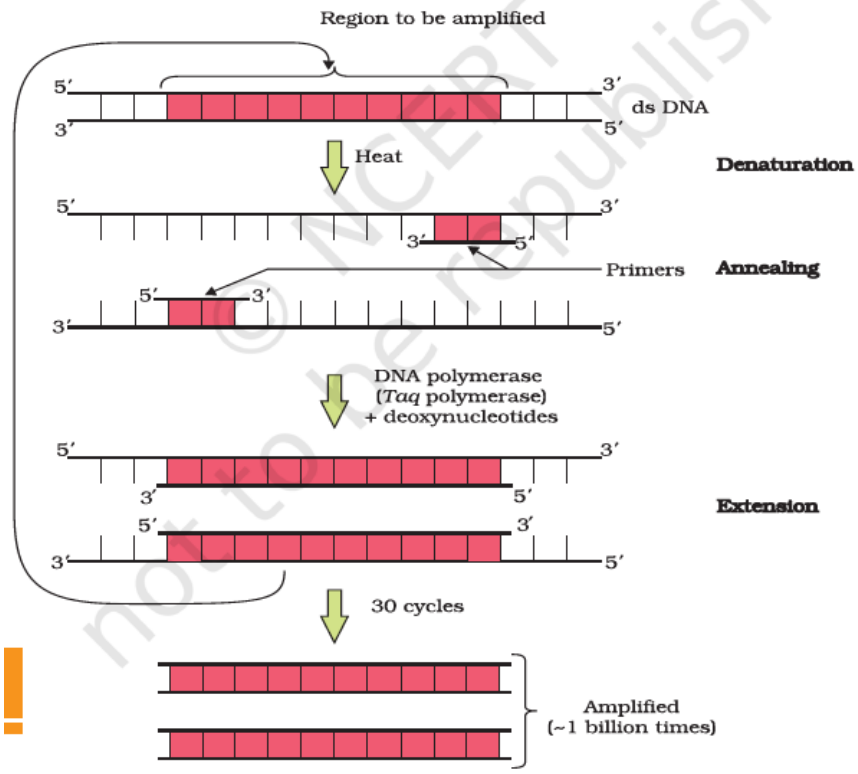
2- Cutting of DNA at Specific Locations

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. The 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

PCR stands for Polymerase Chain Reaction. In this reaction, multiple copies of the gene (or DNA) of interest is synthesised *in vitro* using **two sets** of primers (**small chemically synthesized 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.



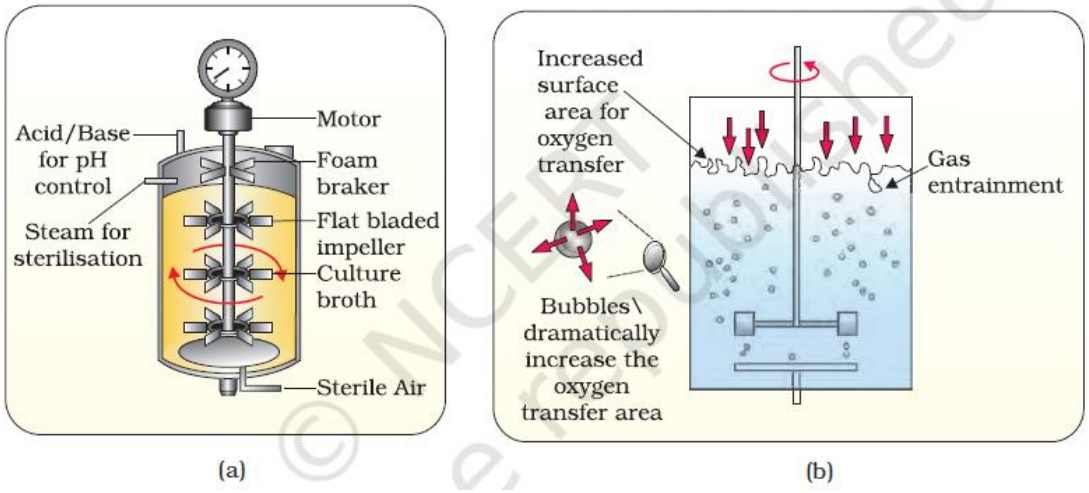
4- Insertion of Recombinant DNA into the Host Cell/Organism

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

When you insert a piece of alien DNA into a cloning vector and transfer it into a bacterial, plant or animal cell, the alien DNA gets multiplied. In almost all recombinant technologies, the ultimate aim is to produce a desirable protein. Hence, there is a need for the recombinant DNA to be expressed. The foreign gene gets expressed under appropriate conditions. After having cloned the gene of interest and having optimized the conditions to induce the expression of the target protein, one has to consider producing it on a large scale. If any protein encoding gene is expressed in a heterologous host, it is called a recombinant protein. The cells harbouring cloned genes of interest may be grown on a small scale in the laboratory. The cultures may be used for extracting the desired protein and then purifying it by using different separation techniques.

To produce in large quantities, the development of bioreactors, where large volumes (100-1000 liters) of culture can be processed, was required. Thus, bioreactors can be thought of as vessels in which raw materials are biologically converted into specific products, individual enzymes, etc., using microbial plant, animal or human cells. A bioreactor provides the optimal conditions for achieving the desired product by providing optimum growth conditions (temperature, pH, substrate, salts, vitamins, oxygen).



6- 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.

Recombinant DNA technologies allow the isolation, purification and selective amplification in specific host cells of discrete DNA fragments or genes from almost any organism .

Recombinant bacteria and fungi are used extensively in certain **industrial enzyme productions**, while **mammalian cell** lines are increasingly used for **recombinant protein production**. Gene manipulations are now widely used to **(a)** improve yield and quality of existing biomolecules (e.g. metabolites, proteins), **(b)** improve characteristics of existing products by protein engineering, and **(c)** alter pathways for synthesis of existing products.