UNIT-2

Genetic Engineering

Genetic engineering is the direct manipulation of an organism's genes using biotechnology. It is the process by which pieces of DNA are transferred from one organism to another. For example, from human to bacterium, or from bacterium to plant.

Recombinant DNA → is artificially made DNA strand that is formed by the combination of two or more gene sequences.

Genetically Modified Organism (GMO) → organism with the recombinant DNA.

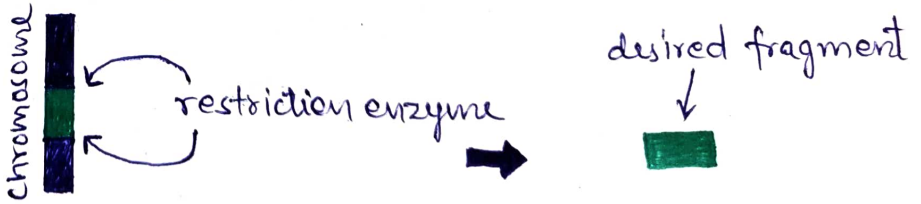
Recombinant DNA technology or Gene cloning is the insertion of a specific piece of desired DNA into a host cell in such a way that the inserted DNA is replicated and passed onto daughter cells during cell division.

→ The steps involved in gene cloning are:

(i) Isolation of gene to be cloned.
(ii) Insertion of gene into another piece of DNA called vector which will allow it to be taken by bacteria and replicated within them as the cells grow and divide.
(iii) Transfer of recombinant vector into bacterial cells.
(iv) Selection of those cells which contain the desired recombinant vectors.
(v) Growth of bacteria, that can be continued indefinitely to give as much cloned DNA as needed.
(vi) Expression of the gene to obtain the desired product.

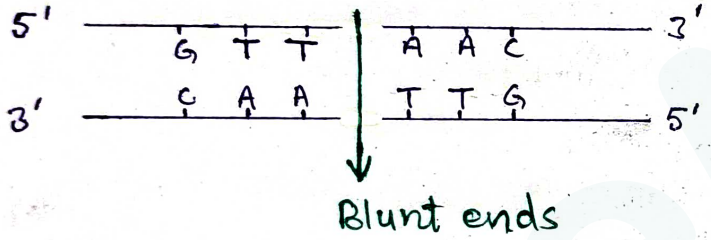
Isolation of DNA fragments

Restriction endonucleases are a group of enzymes that recognises specific nucleotide sequences and cut them at the desired region of DNA. They cut both strands of DNA, often 4 or 6 base pairs long. They are site specific.

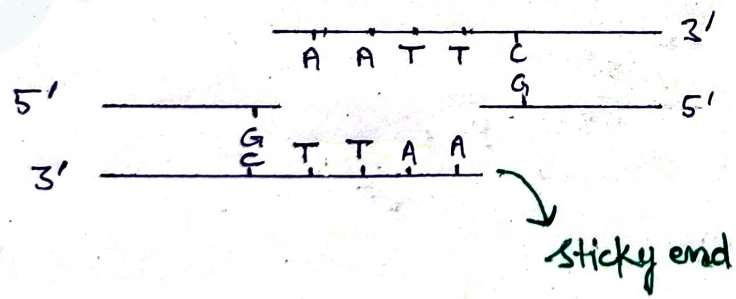


Two types of cuts are made by these enzymes:

(i) Blunt ends → If it cleaves both DNA strands at precisely opposite points on the two strands, it leads to blunt ends fragments which are difficult to ligate or join to the vector.



(ii) Cohesive or sticky ends → The two DNA strands are not cut directly at opposite ends, instead they are staggered. sticky ends are best suited for cloning purposes as they facilitate binding of another piece of DNA.



Insertion of Desired Gene into a Vector

Once the desired DNA fragment is obtained, it has to be transferred to the host cell. cloning vehicles or vectors may be small plasmids, phage or animal virus DNA molecules, used to transfer a DNA fragment into a living cell. cloning vehicles should have following properties:

(i) Origin of replication to enable independent replication.

(ii) presence of recognition sites for restriction enzymes for insertion of DNA fragment.

(iii) Must be able to replicate in host cell after transfer.

(iv) presence of several markers for selection

Introduction into the host cell

Once the vector and the desired DNA molecule is ligated, it has to be transferred to a host cell where it would replicate and produce copies of the desired gene.

Selection or Screening

After the recombinant DNA is transferred to host via the vector, it is integrated into the host cell DNA and starts replicating alongwith the host. The host cell becomes factory where the desired gene is replicated and expressed.

The step thereafter includes screening of the host cells to check for the successful integration and replication of the desired gene.

This can be achieved by the following methods:

(i) Genetic method → This involves the expression of certain traits.

(ii) Nucleic acid hybridisation → It uses a defined nucleic acid probe which will identify the presence of desired gene sequence.

(iii) Immunological screening → Here, instead of a nucleic acid probe, a specific antibody is used. Detection may be by radioactive or non-radioactive method.

Analysis of cloned genes

This method involves the identification of the protein product by two methods based on translation of mRNA in vitro. These methods are known as Hybrid Release Translation (HRT) and Hybrid Arrest Translation (HART).

HRT is the preferred method.

Blotting Techniques

Blotting is like a detective tool used to study and identify big molecules like DNA, RNA or proteins in a complex mixture of related molecules.

South blotting → It is used for running DNA samples. Gel agarose is used.

Northern blotting → It is used for running RNA samples. Here also agarose gel used.

Western blot → It is used to find proteins.

Cloning Vectors

Cloning vectors are used as vehicles for transporting foreign genetic material into another cell (host cell). This foreign segment of DNA is replicated and expressed using the machinery of host organism.

"A cloning vector is a small piece of DNA, taken from any organism into which a foreign DNA fragment can be inserted for cloning purposes."

→ It can be extracted from a virus, plasmid or cells of higher organism.

Ideality for cloning vectors

→ should be small in size

→ must have an origin of replication (ori)

ori → is the specific sequence of nucleotide in a DNA from where the replication starts.

→ must be compatible with host organism.

→ must possess a restriction site

→ Multiple cloning sites should be present.

→ The introduction of donor DNA fragment must not intervene with the self-replicating property of the cloning vector.

→ A selectable marker must be present to identify screen the recombinant cells. The cloning vector must possess a selectable marker gene as it allows the selection of the host cells which carry the recombinant DNA and separate them from those that do not.

→ It should be capable of of working under the prokaryotic as well as eukaryotic system.

Different types of cloning vectors

- Plasmids
- Phagemids
- Bacteriophase
- Cosmids
- Retroviral vectors
- Bacterial Artificial Chromosome (BAC)
- Yeast Artificial Chromosome (YAC)
- Human Artificial Chromosome (HAC)

1) Plasmids → Plasmids were the first vectors to be used in gene cloning. They are naturally occuring and autonomously replicating extra-chromosomal double-stranded DNA molecules. They are present in bacteria, archaea and eukaryotes. The size of plasmids ranges from 1 Kb to 250 Kb. DNA insert of upto 10 Kb can be cloned in the plasmids.
examples, pBR322, pUC 18, F plasmid, Col plasmid

pBR322 cloning vector has the following elements:
p = plasmid
B = Bolivar (name of the scientist)
R = Rodriguez (name of scientist)
322 = number of plasmids discovered in the same lab.

2) Bacteriophase → Bacteriophases or phases are viruses which infect bacterial cells. The most common bacteriophases utilised in gene cloning are Phase λ and M13 Phase. A maximum of 53 Kb DNA can be packaged into the phase. They are more efficient than plasmids for cloning large inserts.

3) Phagemids → They are prepared artificially. They are generally used as a cloning vector in combination with M13 phage. They contain multiple cloning sites.

4) Cosmids → Cosmids are plasmids. They are capable of incorporating the phage λ DNA segment.

5) BACs → It is derived from naturally occuring F plasmid. It is a modification of F plasmid and is artificially synthesized. DNA insert size varies between 150 – 350 Kb.
Example– pUvBBAC.

6) Yeast Artificial Chromosome → They are used for cloning inside eukaryotic cells. A yeast centromere sequence (CEN) is present which allows proper segregation during meiosis. A large DNA insert of upto 200 Kb can be cloned.

7) Human Artificial Chromosome → They are utilised for gene transfer into human cells. HAC avoids the possibility of insertional mutagenesis.

8) Retroviral vectors → These are viruses with RNA as genetic material. Any gene of interest can be introduced into the retroviral genome. The viral RNA can be converted into DNA with the help of reverse transcriptase and hence, efficiently integrated into the host cell.

The following factors should be considered in choosing a vector according to the interest:

(i) DNA insert size
(ii) Size of the vector
(iii) Restriction size
(iv) Efficiency of cloning

Restriction Endonucleases

→ These are the enzymes that cuts the sugar-phosphate backbone of DNA strands at the restriction sites.

→ Named after the organism from which derived:
(i) EcoRI from Escherichia coli
(ii) BamHI from Bacillus amyloliquefaciens

Restriction site → Each restriction enzyme identifies a specific sequence of nucleotides (between four and eight bases) and makes cut in both strands of the double-stranded DNA. Here, this sequence of nucleotides is called restriction site.

→ Different restriction enzymes that recognise the same restriction sequences but cut at different location, called neoschizomers. On the other hand, when recognise and cut in the same site or location are called isoschizomers.

→ When restriction enzymes cut, they can generate either sticky or blunt ends. Example of restriction enzyme that make sticky ends is EcoRI and that for blunt ends is SmaI.

Types of Restriction Enzymes

1) Type-I → cuts DNA on both strands but at a non-specific location at far distances from the sequence that is recognised by the restriction enzyme.

2) Type-II → Cuts the strands of DNA within the particular sequence recognised by the restriction enzyme.

DNA Ligase

DNA ligase is a DNA-joining enzyme. If two pieces of DNA have matching ends, ligase can link them to form a single, unbroken molecule of DNA.

DNA ligase catalyses the formation of phosphodiester bond between the 5'-phosphate of one strand of DNA or RNA and 3'-hydroxyl of another.

Types of DNA ligases

(i) Bacteriophase T_4 DNA ligase → derived from T_4 bacteriophase.

(ii) E.coli DNA ligase → derived from E.coli

(iii) Taq DNA ligase → Thermostable ligase

(iv) T_4 RNA ligase

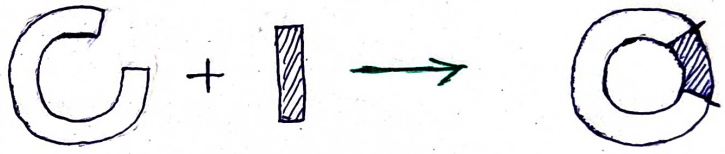
Recombinant DNA Technology rDNA

The Technology used for producing artificial DNA by the combination of different DNAs from different sources.

Basic principles of rDNA Technology

(i) Selection and generation of desired piece of DNA

(ii) Insertion of selected DNA into a cloning vector to create rDNA.



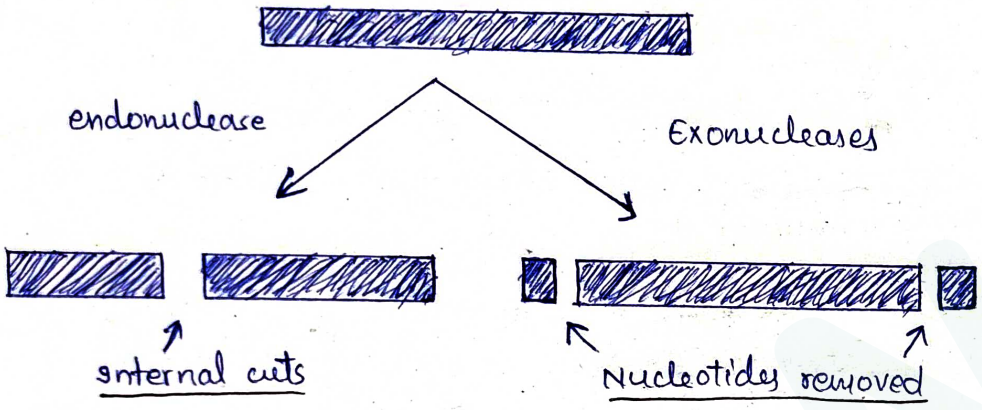
vector DNA fragment rDNA molecule

(iii) Introduction of recombinant vectors into host cells.

(iv) Mutiplication of rDNA

(v) Expression of gene to produce the desired product.

Activity of nucleases



endonucleases cut DNA internally by cutting the phosphodiester backbone, exonucleases act by removing the nucleotides from the ends of the strands.

Applications of Genetic Engineering in medicine

(i) Production of Transgenic Medicinal Plants
(ii) Production of transgenic Animals
(iii) Production of Hormones
(iv) Vaccine production, antibiotic production
(v) Commercially important chemicals
(vi) Prevention and diagnosis of diseases, forensics
(vii) Gene therapy

Genetic Engineering in Products

Interferons

(IFNs)

Interferons are proteins, made and released by host cells in response to the presence of pathogens such as viruses, bacteria, parasites or tumor cells.

IFNs are named after their ability to interfere with viral replication within host cells.

All interferons share common effects: they are anti-viral agents and can fight tumors.

Types of interferons:

(i) alpha (leukocyte interferon): produced by virus infected leukocytes.
(ii) beta (fibroblast interferon): produced by virus infected fibroblasts or epithelial cells
(iii) gamma (immune interferon): produced by certain activated T cells and natural Killer cells. They can also activate other immune cells such as macrophages.

Production

(i) A DNA sequence coding for the product was synthesized and inserted into E.coli.

(ii) Large scale manufacture needs an initial fermentation step.

(iii) The recombinant product accumulates intracellularly as inclusion bodies.

(iv) After harvesting, the E. coli. cells are homogenized and the inclusion bodies recovered via centrifugation.

(v) After solubilization, the interferon is purified to homogeneity by a combination of chromatographic steps.

(vi) The final product is formulated in the presence of a phosphate buffer and sodium chloride.

(vii) It is resented as a 30 mg/ml solution in glass vials and displays a shelf-life of 24 months when stored at 2-8°C.

Vaccines

Vaccine is a substance which is used to stimulate the production of anti-bodies, providing immunity against diseases.

Types

(i) Live-attenuated vaccines → Live vaccines use a weakened form of the germ that causes a disease.

Live vaccines are used to protect against:

- Measles, mumps, rubella (MMR vaccine)
- Rotavirus

(ii) Inactivated vaccines → These vaccines use the killed version of the germ that causes a disease. These are milder than live vaccines. These are used against:

- Hepatitis A
- Polio

(iii) Toxoid vaccines → These vaccines use a toxin made by the germ that causes a disease.

used against: diphtheria, Tetanus.

Recombinant Vaccines

DNA vaccines → DNA vaccination is a technique for protecting an organism against disease by injecting it with genetically engineered DNA to produce an immunological response. Here, the gene encoding for immunogenic protein is isolated from the donor DNA and used to produce recombinant DNA which acts as vaccine which is injected into an individual.

Hepatitis B vaccine → The vaccine gives protection against the hepatitis B virus, which is a major cause of serious liver disease, including liver cancer and cirrhosis.

Recombinant Hepatitis B vaccine production:

(i) HBs antigen producing gene is isolated from the HB virus by normal isolation process.
(ii) A plasmid DNA is extracted from a bacterium, E. coli.
(iii) The HBs antigen producing gene is incorporated into the plasmid vector to form rDNA.
(iv) This recombinant DNA, is itroduced into a yeast cell (Saccharomyces cerevisiae) forming the recombinant yeast cell.
(v) The recombinant yeast cell multiplies in the fermentation tank and produces HBs antigens.
(vi) After 48 hrs. yeast cells are ruptured to free HBs antigens.
(vii) The HB antigens are purified.

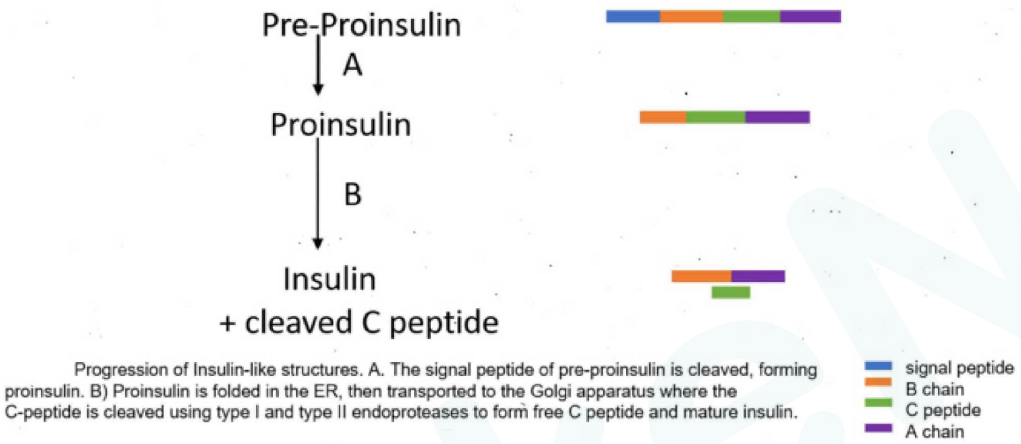
Insulin

→ Insulin is a hormone that regulates the amount of glucose in the blood.
→ Insulin is produced by cells in the pancreas called islets of Langerhans.
→ Human insulin is available in two forms:
(i) Regular (short-acting) : Humulin S, Actrapid
(ii) NPH (intermediate-acting) : Humulin I

Method of production

(i) The sequence that codes for proinsulin is inserted into the non-pathogenic E. coli bacteria.
(ii) The bacteria go through the fermentation process where it reproduces and produces proinsulin.

(iii) Then the connecting sequence between the A and B chains is spliced away with an enzyme and the resulting insulin is purified.



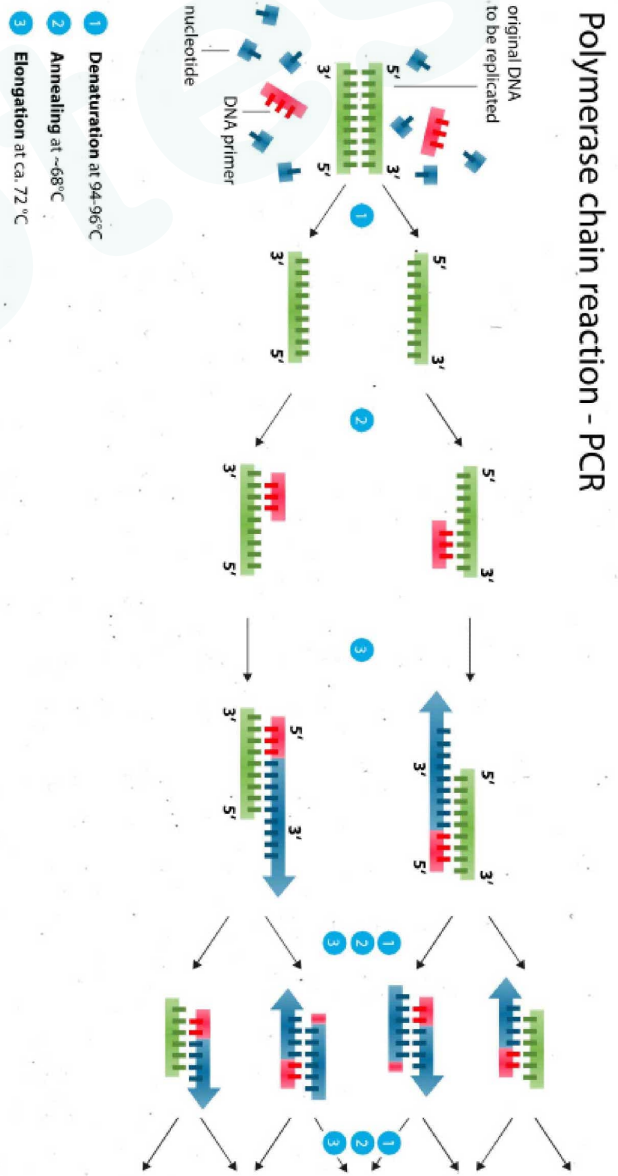
Progression of Insulin-like structures. A. The signal peptide of pre-proinsulin is cleaved, forming proinsulin. B) Proinsulin is folded in the ER, then transported to the Golgi apparatus where the C-peptide is cleaved using type I and type II endoproteases to form free C peptide and mature insulin.

Polymerase chain Reaction or PCR

PCR is a technique to make many copies (millions or billions) of a specific DNA region in vitro (in a test tube).

PCR was originated and developed by Kary Mullis.

Principle of PCR



(i) The target sequence of nucleic acid is denatured to single strands,

(ii) Primers (nucleic acid sequence) specific for each target strand sequence are added.

(iii) DNA polymerase catalyses the addition of deoxynucleotides to extend and produce new strands complementary to each of target strand. (Cycle-1)

(iv) In cycle-2, both double stranded products of cycle-1 are denatured and subsequently serve as targets for more primer anealing and extension by DNA polymerase.

(v) After 25-30 cycles, at least 10^7 copies of target DNA may be produced by means of this thermal cycling.

Stages of PCR

1) **Denaturation** → On heating the DNA to a temperature of 96°C for a minute, it gets denatured and two strands separate.

2) **Anealing or Renaturation** → As the temperature of mixture is slowly cooled to about 55-65°C, the primers bind to their complementary sequences on the single-stranded DNA template.

3) **Extension** → Raise the reaction temperature to 72°C, so Taq polymerase extends the primers, synthesizing new strands of DNA.

Results of PCR

The results of a PCR reaction are usually visualised using gel electrophoresis.

Gel electrophoresis is a technique in which DNA fragments are pulled through a gel matrix by an electric current and it separates DNA fragments according to size.