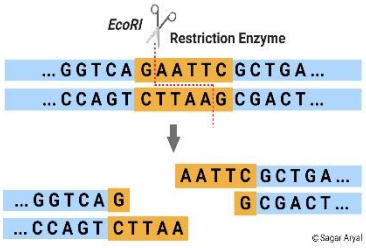
Paper VI - rDNA Technology

Unit 1 – Restriction and Modifications

Restriction Enzyme (Restriction Endonuclease) Definition

- Restriction enzyme, also called restriction endonuclease, is a protein produced by bacteria that cleaves **DNA** at specific sites along the molecule.
- Restriction endonucleases cut the DNA double helix in very precise ways. It cleaves DNA into fragments at or near specific recognition sites within the molecule known as restriction sites.
- They have the capacity to recognize specific base sequences on DNA and then to cut each strand at a given place. Hence, they are also called as 'molecular scissors'.



Source of Restriction Enzymes

- The natural source of restriction endonucleases are bacterial cells.
- These enzymes are called restriction enzymes because they restrict infection of bacteria by certain viruses (i.e., bacteriophages), by degrading the viral DNA without affecting the bacterial DNA. Thus, their function in the bacterial cell is to destroy foreign DNA that might enter the cell.
- The restriction enzyme recognizes the foreign DNA and cuts it at several sites along the molecule.
- Each bacterium has its own unique restriction enzymes and each enzyme recognizes only one type of sequence.

Recognition Sites

- The DNA sequences recognized by restriction enzymes are called palindromes. Palindromes are the base sequences that read the same on the two strands but in opposite directions.

For example, if the sequence on one strand is GAATTC read in 5'→3′ direction, the sequence on the opposite strand is CTTAAG read in the 3'→5′ direction, but when both strands are read in the 5'→ 3′ direction the sequence is the same. The palindrome appears accordingly —

5′ GAATTC 3′
3′ CTTAAG 5′

In addition, there is a point of symmetry within the palindrome. In the example, this point is in the center between the AT/AT.

- The value of restriction enzymes is that they make cuts in the DNA molecule around this point of symmetry.
- Some enzymes cut straight across the molecule at the symmetrical axis producing blunt ends.

- Of more value, however, are the restriction enzymes that cut between the same two bases away from the point of symmetry on two strands, thus, producing a staggering break.

Mechanism of Cleavage of Restriction Enzymes

When a restriction endonuclease recognizes a particular sequence, it snips through the DNA molecule by catalyzing the hydrolysis (splitting of a chemical bond by addition of a water molecule) of the bond between adjacent nucleotides. To cut DNA, all restriction enzymes make two incisions, once through each sugar-phosphate backbone (i.e. each strand) of the DNA double helix.

Types of Restriction Enzymes

Traditionally, four types of restriction enzymes are recognized, designated I, II, III, and IV, which differ primarily in structure, cleavage site, specificity, and cofactors.

1. **Type I enzymes** cleave at sites remote from a recognition site; require both ATP and S-adenosyl-L-methionine to function; multifunctional protein with both restriction and methylase activities.
2. **Type II enzymes** cleave within or at short specific distances from a recognition site; most require magnesium; single function (restriction) enzymes independent of methylase.
3. **Type III enzymes** cleave at sites a short distance from a recognition site; require ATP (but do not hydrolyze it); S-adenosyl-L-methionine stimulates the reaction but is not required; it exists as part of a complex with a modification methylase.
4. **Type IV enzymes** target modified DNA, e.g. methylated, hydroxymethylated and glucosyl-hydroxymethylated DNA.

Nomenclature of Restriction Enzymes

Since their discovery in the 1970s, many restriction enzymes have been identified while Type II restriction enzymes have been characterized.

Each enzyme is named after the bacterium from which it was isolated, using a naming system based on bacterial genus, species and strain. For example, the name of the EcoRI restriction enzyme was derived as:

E – *Escherichia*: Genus

co- *coli:* specific species

R- RY13: strain

I- First identified: order of identification in the bacterium

Applications of Restriction Enzymes

- Restriction enzymes can be isolated from bacterial cells and used in the laboratory to manipulate fragments of DNA, such as those that contain genes; for this reason, they are indispensable tools of recombinant DNA technology (genetic engineering).
- The most useful aspect of restriction enzymes is that each enzyme recognizes the same unique base sequence regardless of the source of the DNA. It means that these enzymes establish fixed landmarks along an otherwise very regular DNA molecule. This allows dividing a long DNA molecule into fragments that can be separated from each other by size with the technique of gel electrophoresis.
- Each fragment, thus generated, are also available for further analysis, including the sequencing.

- One value of cutting DNA molecule up into discrete fragments is being able to locate a particular gene on the fragment where it resides which is done by the general technique of Southern blotting.

Some examples of Restriction Enzymes

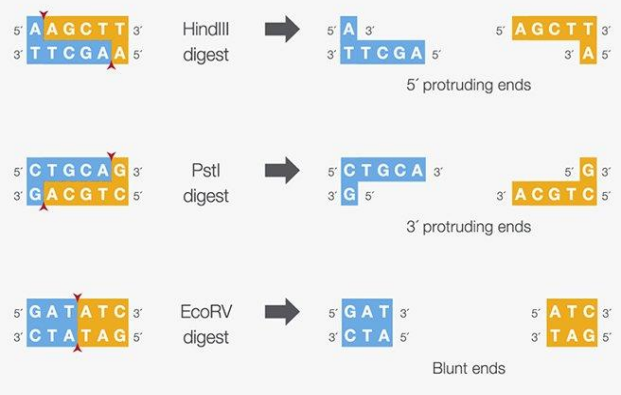


Figure: Sticky or protruding ends (5′ or 3′) or blunt ends produced by specific restriction enzymes.

One of the most popular restriction enzymes is called EcoRI from *E. coli* (bacterium).

- Hundreds of other restriction enzymes with different sequence specificities have been isolated from several bacteria and are commercially available.

Enzyme	**Obtained from**	**Recognition Sequence**
EcoRI	*Escherichia coli*	5'GAATTC 3'CTTAAG
EcoRII	*Escherichia coli*	5'CCWGG 3'GGWCC
BamHI	*Bacillus amyloliquefaciens*	5'GGATCC 3'CCTAGG
HindIII	*Haemophilus influenzae*	5'AAGCTT 3'TTCGAA

Enzymes used in molecular cloning

Prokaryotic DNA polymerase types

DNA Polymerase I

- This is a type A or Family A polymerase enzyme that was initially isolated from *E. coli* and most abundantly found in *E. coli.*
- Its main function is excision repair of DNA strands from the 3′-5′ direction to the 5′-3 direction, as an exonuclease.
- It also helps with the maturation of Okazaki fragments, which are short DNA strands that make up the lagging strand during DNA replication.
- Its role during replication is the addition of nucleotide at the RNA primer and it moves along the 5′-3′ direction.
- The binding site for DNA polymerase I is known as octylglucoside.

DNA Polymerase II

- It belongs to Type B or Family B of the polymerases.
- Its major function is the 3′ – 5′ exonuclease activity and to also restart replication after replication stops due to DNA strand damages.

- The DNA polymerase II is found in the replication fork, to help in directing the activities of other polymerases.

DNA Polymerase III

- This is the primary enzyme that is used in DNA replication, belonging to the Family C or Type C.
- It is responsible for the synthesis of new strands by adding nucleotides to the 3′- OH group of the primer.
- It has a 3′-5′ exonuclease activity hence it can also proofread the errors that may arise during DNA strand replication.

DNA Polymerase IV

- It belongs to the Family Y and it is involved in the non-targeted mutagenesis.
- Its activation is based on the stalling activity of the replication fork.
- When it is activated, it creates a checkpoint, stops replication, and gives time to properly repair lesions in the new DNA strand.
- It is also involved in the repair mechanism of translesion synthesis.
- It does not have nuclease activity, therefore it is prone to errors in DNA replication.

DNA Polymerase V

- It belongs to Family Y, with high regulatory activity.
- It is produced only when DNA is damaged and it requires translesion synthesis.
- It also lacks exonuclease functions and hence it can not proofread the synthesis of DNA replicas making it less efficient.

Taq DNA polymerase

- ***Taq* polymerase** is a thermostable type of DNA polymerase 1 that was initially isolated from a thermophilic eubacterial known as *Thermus aquaticus*.
- It is abbreviated as Taq or Taq pol.
- It is commonly used in Polymerase Chain Reaction to amplify short strands of DNA.
- Due to its thermophilic nature, it is able to withstand denaturation that is required during PCR, hence it replaced DNA polymerase from *E. coli.*

DNA polymerase vs RNA polymerase

S.N.	Characteristics	DNA polymerase	RNA polymerase
1.	**Definition**	It is an enzyme that synthesizes the DNA	It is an enzyme synthesizes the RNA
2.	**Mechanism**	DNA polymerase's mechanism is during replication whereby it synthesizes new DNA strands	RNA polymerase functions during transcription, which is the synthesis of RNA
3.	**Strands**	It synthesizes a double-stranded DNA molecule	It synthesizes a single-stranded RNA molecule

4.	**Presence or absence of Primer**	Its replication mechanism is initiated by a short-RNA primer	It does not need a primer to initiate transcription
5.	**Nucleotide insertion**	It inserts nucleotides after finding the free 3' OH end by the assistance of the primer-synthesizer, primase enzyme	It adds nucleotides directly.
6.	**Amino acid bases**	It adds dATP (Adenine-Thymine), dGTP, dCTP and dTTP to the growing new DNA strand	It inserts dATP, dGTP, dCTP and dUTP to the growing RNA strand.
7.	**Functionality**	It has polymerization and proofreading activity	RNA polymerase only has a polymerization activity.
8.	**Polymerization rate**	The rate of polymerization by DNA polymerase is about 1000 nucleotides per second in prokaryotes	The rate of RNA polymerase is 40 to 80 nucleotides per second.
9.	**Efficiency**	DNA polymerase enzyme is faster, efficient, and more accurate considering its proofreading activity.	RNA polymerase is slower, inefficient, and inaccurate.
10.	**Subtypes**	DNA polymerase has three different subtypes: Type 1, 2, and 3.	RNA polymerase has five different subtypes in eukaryotes
11.	**Termination**	The DNA synthesis continues until the end when the strand ends, that is when polymerization stops, thus the entire chromosomal DNA is synthesized.	The polymerization is terminated when RNA polymerase finds the stop codon or termination codon on the nucleic acid strand.

DNA Ligase

DNA ligases indicate the basic class of enzymes necessary for all entities to sustain structural integrity of the genome. This enzyme connects two strands of DNA together as a result of association between phosphate group of one strand and deoxyribose group on the other strand. The DNA ligase is functional in joining the Okazaki fragments that take shape on the lagging strand while the DNA replicates. The DNA ligase is able to join two of the DNA fragments as a

result of formation of a phosphodiester bond between them with the help of a molecule of energy.

DNA Ligase – Function

The importance of DNA ligases to maintain genomic integrity is immense. It does so by joining the breaks in the DNA's phosphodiester backbone occurring while recombination and replication takes place in addition to the result of DNA damage and its repairing.

The primary role of DNA ligase is to ligate two strands of DNA, which could be single or double strands despite the fact that ligases are differently used for various purposes in vitro and vivo processes.

Role of DNA ligase in replication

4 different daughter single-stranded DNA molecules are produced in the process of replication from a single DNA duplex. To bring about complete replication, various enzymes perform a range of activities. Replication of DNA is initiated with the introduction of the RNA primer via primase enzyme. Primer's 3′ end is used as the initial point to add nucleotides by the DNA polymerase at the leading strand. The process terminates at the lagging strand through the synthesis of the Okazaki fragments. When the process is about to complete, the primer is extracted out and loaded with nucleotides in the gaps between the Okazaki fragments by the DNA polymerase. However, the strands so produced are yet to be joined.

The role of DNA ligase here is to fill gaps by producing phosphodiester links between the gaps once the primer between the Okazaki fragments is removed. Though ligation 5′ end of a strand and 3′ end of another end are joined through the elimination of the pyrophosphate from the triphosphate. On the contrary, the DNA replication uses ligation, the same is not applicable on blunt ended ds DNA or double stranded DNA.

Role of DNA ligase in recombination DNA technology

DNA ligase I, II or IV are used in cloning experiments, but eukaryotic DNA ligase is not used. The phage T4 DNA ligase instead, is used to perform various ligation methods. Two types of DNA ends are generated by restriction digestion, they are blunt or sticky ends. For various molecular biological techniques, different ends are generated.

DNA Ligase – Types

The following are the different types of DNA Ligase:

DNA Ligase I – It ligates the nascent DNA on the lagging strand particularly the gaps between the Okazaki fragments.

DNA Ligase II – Primarily, it participates in the DNA repair pathway.

DNA Ligase III – Participates in the DNA repair, particularly the nucleotide excision repair. This ligase is the one found in the mitochondrial DNA also.

DNA Ligase IV – It joins double-stranded DNA and does take part in double-strand break repair pathway, specially, the non-homologous end-joining.

Alkaline Phosphatase:

Alkaline phosphatase removes 5′ phosphate groups from DNA and RNA (Fig. 13.4). It will also remove phosphates from nucleotides and proteins. These enzymes are most active at alkaline pH therefore known as alkaline phosphatase.

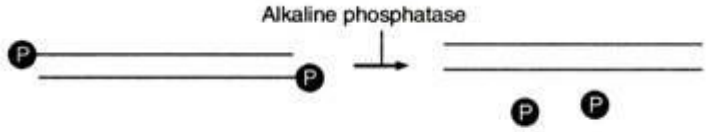


Fig. 13.4. Removal of phosphate by alkaline phosphatase.

There are several sources of alkaline phosphatase that differ in how easily they can in-activated:

1. Bacterial alkaline phosphatase (BAP) is the most active of the enzymes, but also the most difficult to destroy at the end of the dephosphorylation reaction.

2. Calf intestinal alkaline phosphatase (CIP) is purified from bovine intestine. This is phosphatase most widely used in molecular biology labs because, although less active than BAP, it can be effectively destroyed by protease digestion or heat (75 °C for 10 minutes in the presence of 5 mM EDTA).

3. Shrimp alkaline phosphatase is derived from a cold-water shrimp and is promoted for being readily destroyed by heat (65°C for 15 minutes).

There are two primary uses for alkaline phosphatase in DNA manipulations:

1. Removing 5′ phosphates from plasmid and bacteriophage vectors that have been cut with a restriction enzyme. In subsequent ligation reactions, this treatment prevents self-ligation of the vector and thereby greatly facilitates ligation of other DNA fragments into the vector (e.g. sub-cloning).

2. Removing 5′ phosphates from fragments of DNA prior to labeling with radioactive phosphate. Polynucleotide kinase is much more effective in phosphorylating DNA if the 5′ phosphate has previously been removed.

It is usually recommended that dephosphorylation of DNAs with blunt or 5′-recessed ends be conducted using a higher concentration alkaline phosphatase or at higher temperatures than for DNAs with 5′ overhangs.

Nucleases: DNase and RNase:

Most of the time nucleases are the enemy of the molecular biologist who is trying to preserve the integrity of RNA or DNA samples. However, deoxyribonucleases (DNases) and ribonucleases (RNases) have certain indispensible roles in molecular biology laboratories.

Numerous types of DNase and RNase have been isolated and characterized. They differ among other things in substrate specificity, cofactor requirements, and whether they cleave nucleic acids internally (endonucleases), chew in from the ends (exonucleases) or attack in both of these modes.

In many cases, the substrate specificity of a nuclease depends upon the concentration of enzyme used in the reaction, with high concentrations promoting less specific cleavages. The most widely used nucleases are DNase I and RNase A, both of which are purified from bovine pancreas: Deoxyribonuclease I cleaves double-stranded or single stranded DNA. Cleavage preferentially occurs adjacent to pyrimidine (C or T) residues, and the enzyme is therefore an endonuclease. Major products are 5′-phosphorylated di, tri and tetra nucleotides.

In the presence of magnesium ions, DNase I hydrolyzes each strand of duplex DNA independently, generating random cleavages. In the presence of manganese ions, the enzyme cleaves both strands of DNA at approximately the same site, producing blunt ends or fragments with 1-2 base overhangs. DNase I does not cleave RNA, but crude preparations of the enzyme are contaminated with RNase A; RNase-free DNase I is readily available.

Applications:

1. Eliminating DNA (e.g. plasmid) from preparations of RNA.

2. Analyzing DNA-protein interactions via DNase foot printing.

3. Nicking DNA prior to radio-labeling by nick translation.

Ribonuclease A is an endoribonuclease that cleaves single-stranded RNA at the 3′ end of pyrimidine residues. It degrades the RNA into 3′-phosphorylated mononucleotides and oligonucleotides. The major use of RNase A is eliminating or reducing RNA contamination in preparations of plasmid DNA.

a. Exonucleases are enzymes (found as individual enzymes, or as parts of larger enzyme complexes) that cleave nucleotides one at a time from an end of a polynucleotide chain. These

enzymes hydrolyze phosphodiester bonds from either the 3′ or 5′ terminus of a polynucleotide molecule (Fig. 13.9).

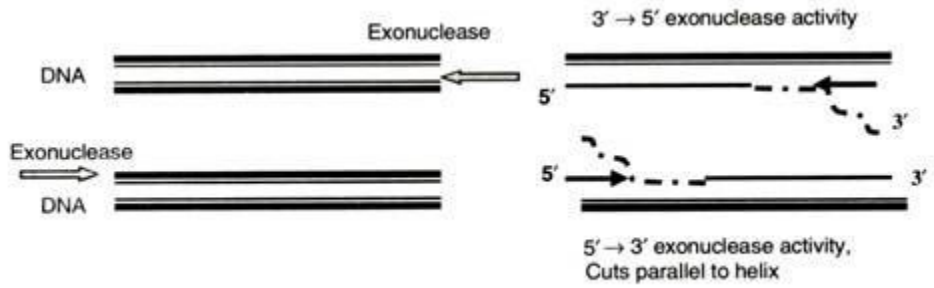


Fig. 13.9. Action of exonuclease.

b. Endonucleases are enzymes that cleave the phosphodiester bond within a polynucleotide chain, in contrast to exonucleases, which cleave phosphodiester bonds at the end of a polynucleotide chain. Restriction endonucleases (Restriction Enzymes) cleave DNA at specific sites, and are divided into three categories, Type I, Type II, and Type III, according to their mechanism of action. These enzymes are often used in genetic engineering to make recombinant DNA for introduction into bacterial, plant, or animal cells (Fig. 13.10).

Fig. 13.10. Action of endonuclease.

Polynucleotide Kinase:

Polynucleotide kinase (PNK) is an enzyme that catalyzes the transfer of a phosphate from ATP to the 5′ end of either DNA or RNA. It is a product of the T4 bacteriophage, and commercial preparations are usually products of the cloned phage gene expressed in E. coli.

The enzymatic activity of PNK is utilized in two types of reactions:

1, In the “forward reaction”, PNK transfers the gamma phosphate from ATP to the 5′ end of a polynucleotide (DNA or RNA). The target nucleotide is lacking a 5′ phosphate either because it has been dephosphorylated or has been synthesized chemically.

2. In the “exchange reaction”, target DNA or RNA that has a 5′ phosphate is incubated with an excess of ADP, PNK will first transfer the phosphate from the nucleic acid onto an ADP,

forming ATP and leaving a dephosphorylated target. PNK will then perform a forward reaction and transfer a phosphate from ATP onto the target nucleic acid (Fig. 13.11).

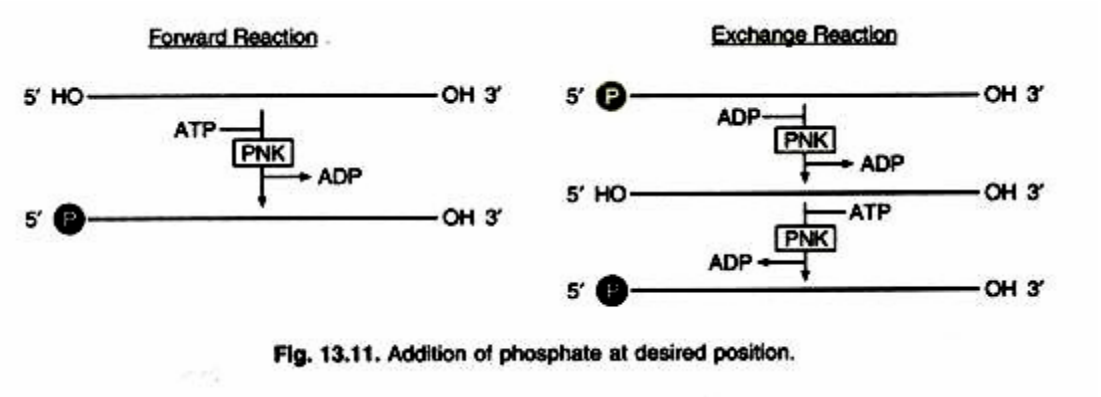


Fig. 13.11. Addition of phosphate at desired position.

As you might expect, the efficiency of phosphorylating via the exchange reaction is considerably less than for the forward reaction. In addition to its phosphorylating activity, PNK also has 3′ phosphatase activity, although this has little significance to molecular technologists.

There are two major indications for phosphorylating nucleic acids and hence uses of PNK are:

1. Phosphorylating linkers and adaptors (fragments of DNA ready for ligation) which require a 5′ phosphate. This includes products of polymerase chain reaction, which are typically generated using non-phosphorylated primers.

2. Radiolabelling oligonucleotides, usually with 32P, for use as hybridization probes. PNK is inhibited by small amounts of ammonium ions, so ammonium acetate should not be used to precipitate nucleic acids prior to phosphorylation. Low concentrations of phosphate ions, or NaCl concentrations greater than about 50 mM, also inhibit this enzyme.

Blotting techniques: Blots are techniques for transferring DNA, RNA and proteins onto a carrier so they can be separated, and often follows the use of a gel electrophoresis. **The Southern blot is used for transferring DNA, the Northern blot for RNA and the western blot for PROTEIN.**

Southern Blotting: Southern blot named after Sir Edwin Southern Developed in 1975

- This method Involves separation, transfer and hybridization.
- The Southern blot is used to detect the presence of a particular piece of DNA in a sample.
- The DNA detected can be a single gene, or it can be part of a larger piece of DNA such as a viral genome.
- The key to this method is Hybridization.

- Hybridization - Process of forming a double-stranded DNA molecule between a single-stranded DNA probe and a single-stranded target patient DNA.

Steps:

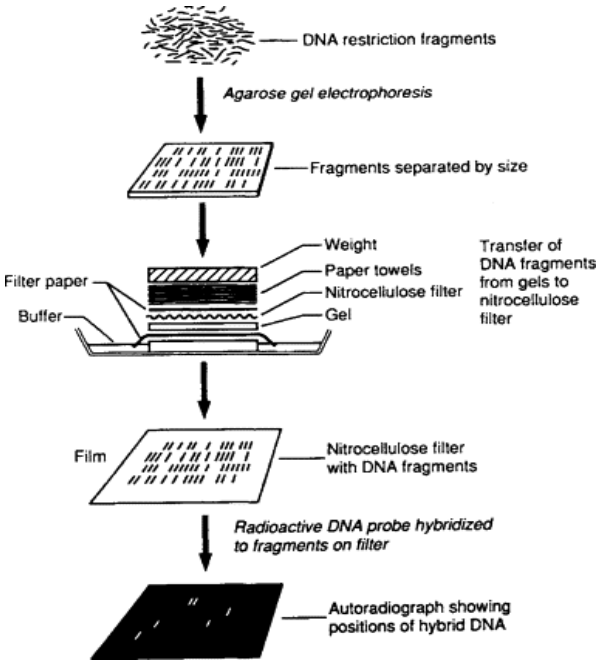
1. The DNA to be analyzed, such as the total DNA of an organism, is digested to completion with a restriction enzyme.

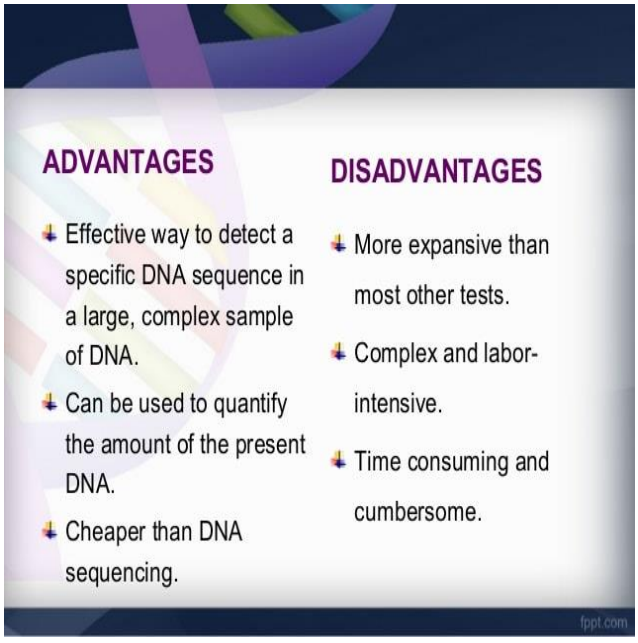
2. The complex mixture of fragments is subjected to gel electrophoresis to separate the fragments according to size.

3. The restriction fragments present in the gel are denatured with alkali and transferred onto a nitrocellulose filter or nylon membrane by blotting. • This procedure preserves the distribution of the fragments in the gel, creating a replica of the gel on the filter.

4. The filter is incubated under hybridization conditions with a specific radiolabeled DNA probe. The probe hybridizes to the complementary DNA restriction fragment.

5. Excess probe is washed away and the probe bound to the filter is detected by autoradiography, which reveals the DNA fragment to which the probe hybridized.





Applications:

• Southern blots are used in several main areas including gene discovery and mapping, evolution and development studies, diagnostics and forensics.

• Southern blots allow investigators to determine the molecular weight of a restriction fragment and to measure relative amounts in different samples.

• Southern blot is used to detect the presence of a particular bit of DNA in a sample .

• Analyze the genetic patterns which appear in a person's DNA.

• Used in DNA fingerprinting, genetic engineering, & forensic science for tests such as: – Paternity testing – Personal identification – Sex determination.

• In regards to genetically modified organisms, Southern blotting is used as a definitive test to ensure that a particular section of DNA of known genetic sequence has been successfully incorporated into the genome of the host organism.

• Diagnosis of congenital inherited diseases such as congenital adrenal hyperplasia (CAH).

• Identify mutations , deletions , and gene rearrangements

• Used in prognosis of cancer and in prenatal diagnosis of genetic diseases

• Leukemias • Diagnosis of HIV-1 and infectious disease.

Northern blotting:

Northern blotting is a technique for detection of specific RNA sequences. Northern blotting was developed in 1977 by James Alwine, David Kemp and George Stark at Stanford University and was named such by analogy to Southern blotting .

Steps:

1. RNA is isolated from several biological samples (e.g. various tissues, various developmental stages of same tissue etc.) * RNA is more susceptible to degradation than DNA.
2. Sample’s are loaded on gel and the RNA samples are separated according to their size on an agarose gel . • The resulting gel following after the electrophoresis run.
3. The gel is then blotted on a nylon membrane or a nitrocellulose filter paper by creating the sandwich arrangement.
4. The membrane is placed in a dish containing hybridization buffer with a labeled probe. • Thus, it will hybridize to the RNA on the blot that corresponds to the sequence of interest.
5. The membrane is washed to remove unbound probe.
6. The labeled probe is detected via autoradiography or via a chemiluminescence reaction (if a chemically labeled probe is used). In both cases this results in the formation of a dark band on an X-ray film. • Now the expression patterns of the sequence of interest in the different samples can be compared.

Applications:

• A standard for the direct study of gene expression at the level of mRNA (messenger RNA transcripts). • Detection of mRNA transcript size • Study RNA degradation • Study RNA

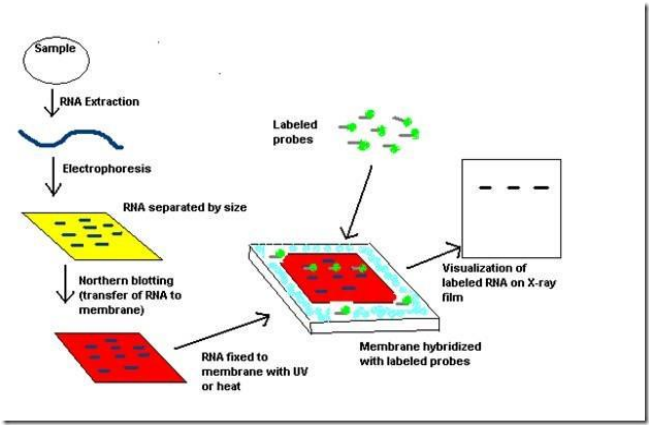
splicing - can detect alternatively spliced transcripts • Study RNA half-life • Study IRES (internal ribosomal entry site) - to remove possibility of RNA digestion vs. 2nd cistron translation. • Often used to confirm and check.

Advantages:

• It is a widely accepted and well regarded method • northern blotting is a straight-forward method • Often it is used as a confirmation or check • Often a gold-standard • it is a versatile protocol as it can allow the usage of many types of probes (vs Real time PCR) including: radiolabeled and non-radiolabeled, in vitro transcribed RNA and even oligonucleotides such as primers.

Disadvantages:

• Often radioactivity is used. This prevents ease of performing it, use and disposal. New methods of non - radioactive detection have been generated allowing non-radioactive detection. • The whole process of northern blotting takes a long time usually, from sample preparation through to detection. • If RNA samples are even slightly degraded by RNases, the quality of the data and quantitation of expression is quite negatively affected.



Western blotting: • Western blotting is an Immunoblotting technique which rely on the specificity of binding between a molecule of interest and a probe to allow detection of the molecule of interest in a mixture of many other similar molecules. • introduced by Towbin, et al. in 1979 • In Western blotting, the molecule of interest is a protein and the probe is typically an antibody raised against that particular protein. • The SDS PAGE technique is a prerequisite for Western blotting .

Steps: 1. A protein sample is subjected to electrophoresis on an SDS polyacrylamide gel.

2. Electroblotting transfers the separated proteins from the gel to the surface of a nitrocellulose membrane.

3. The blot is incubated with a generic protein (such as milk proteins or BSA) which binds to any remaining sticky places on the nitrocellulose.

4. An antibody that is specific for the protein of interest (the primary antibody - Ab1) is added to the nitrocellulose sheet and reacts with the antigen. Only the band containing the protein of interest binds the antibody, forming a layer of antibody molecules.

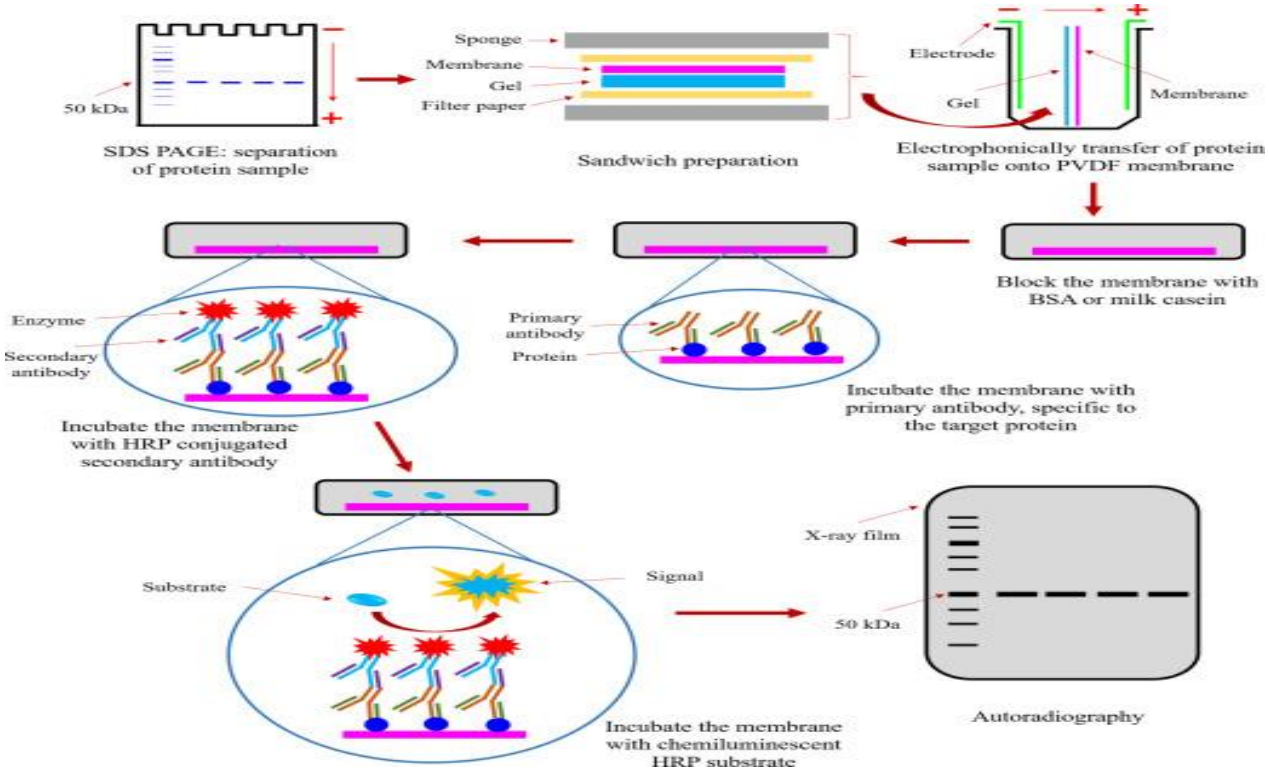
5. Following several rinses for removal of non-specifically bound Ab1, the Ab1-antigen complex on the nitrocellulose sheet is incubated with a second antibody (Ab2), which specifically recognizes the Fc domain of the primary antibody and binds it. Ab2 is radioactively labeled, or is covalently linked to a reporter enzyme, which allows to visualize the protein-Ab1-Ab2 complex.

Applications:

• The confirmatory HIV test employs a Western blot to detect anti-HIV antibody in a human serum sample. Proteins from known HIV-infected cells are separated and blotted on a membrane then, the serum to be tested is applied in the primary antibody incubation step; free antibody is washed away, and a secondary anti-human antibody linked to an enzyme signal is added. The stained bands then indicate the proteins to which the patient's serum contains antibody . • A Western blot is also used as the definitive test for Bovine spongiform encephalopathy (BSE, commonly referred to as 'mad cow disease(• Some forms of Lyme disease testing employ Western blotting .

Advantages: • Whereas ELISA measures antibody to whole virus and gives a "positive," "negative" or indeterminate test result, Western blotting is a more specific test. Western blot analysis can detect one protein in a mixture of any number of proteins while giving you information about the size of the protein. This provides more specific results than the ELISA protocol by providing additional information regarding protein size and multiple bands. • Western blot test is referred to as the 'Gold Standard', meaning it trumps any other positive tests . . • Western blotting tells you how much protein has accumulated in cells.

Disadvantages: • It is time-consuming (compared to ELISA) • If a protein is degraded quickly, Western blotting won't detect it well . • It might also be more costly. • Additionally, it requires optimizing of the experimental conditions (i.e. in terms of protein-isolation, buffers, type of separation, gel-concentration, etc.).



Unit 3 – Cloning Vectors

Vector Definition

A vector is a substance, usually a piece of DNA that carries a sequence of DNA or other genetic material and introduces it into a new cell.

- Vectors act as vehicles to transfer genetic material from one cell to the other for different purposes like multiplying, expressing, or isolation.
- Vectors are used as a tool in molecular cloning procedures so as to introduce the desired DNA insert into a host cell.
- The DNA insert that is transmitted by a vector is termed recombinant DNA, and the process is also known as recombinant DNA technology.
- Usually, the vectors are DNA sequences that carry different parts involved in different functions. Vectors usually have an insert, also known as a transgene, that carries the recombinant DNA and a larger sequence called the backbone of the vector responsible for the structure of the vector.
- Vectors can be classified into different types depending on different characteristics. The selection of vectors thus depends on the purpose of the process.

Characteristics or Features of vectors

The following are some of the characteristic features of vectors;

1. Vectors should be capable of replicating autonomously, which, in turn, depends on the presence of particular sequences in the vector that enables them to initiate replication and propagation within the host cell. Some vectors might even have sequences that allow the production of proteins essential for the inserted DNA, regulation of the process, and further transfer of the insert between different vectors.

2. The size of an ideal vector should also be small enough for it to be incorporated into the host genome. The small size of the vector also enables it to incorporate a large-sized insert for transfer.
3. Vectors should be easy to isolate and purify as these need to be recovered and reused for multiple processes.
4. For a vector to be effective, these should also have certain components that facilitate the process of determining whether the host cell has received the vector. Most vectors used in this process have a gene that either provides resistance to an antibiotic or produces a particular type of protein. These components are called marker genes.
5. Many vectors also require unique restriction enzyme recognition sites that enable the insertion of the vector DNA in the presence of specific restriction enzymes. However, many vectors have been designed with a series of restriction sites close to multiple cloning sites that increases the possible restriction enzymes that can be used to digest the sequence.
6. The introduction of vectors into the host cell should be easy, which depends on a number of factors.
7. In the case of gene transfer processes, it is important that the vector is capable of integrating itself or the recombinant DNA into the genome of the host cell.
8. It is important that the introduction of recombinant DNA into the vector doesn't affect the replication cycle of the vector.

Types of vectors

Vectors can be classified into different groups depending on the purpose of the process and the type of particles used in the process. The following are the commonly studied group of vectors that are used for different purposes:

1. Cloning vectors – Plasmids, Cosmids, Bacteriophages, Bacterial artificial chromosome, Yeast artificial chromosome, Human artificial chromosome
2. Viral Vectors
3. Expression Vectors
4. Shuttle Vectors
5. Secretion Vectors

Definition of Plasmids:

In addition to bacterial chromosome (nucleoid), bacterial cells normally contain genetic elements in their cytoplasm. These genetic elements exist and replicate separately from the chromosome and are called plasmids. The very existence of plasmids in bacterial cytoplasm was revealed by Lederberg in 1952 while working on conjugation process in bacteria.

Lederberg coined the term 'plasmid' to refer to the transmissible genetic elements that were transferred from one bacterial cell to another and determined the maleness in bacteria.

Physical Nature and Copy Number of Plasmids:

The physical nature of plasmids is quite simple. They are small double-stranded DNA molecules. Majority of the plasmids are circular, but many linear plasmids are also known.

Naturally occurring plasmids vary in size from approximately 1 kilobase to more than 1 megabase, and a typical plasmid DNA is considered to be less than 5% the size of the bacterial chromosome. Most of the plasmid DNA isolated from bacterial cells exist in the supercoil configuration, which is the most compact form for DNA to exist within the cell.

The copy number refers to the fact that different plasmids occur in cells in different numbers. Some plasmids are present in the cell in only 1-3 copies, whereas others may be present in over 100 copies. Copy number is controlled by genes on the plasmid and by interactions between the host and the plasmid.

Properties of Plasmids:

(i) They are specific to one or a few particular bacteria.

(ii) They replicate independently of the bacterial chromosome.

(iii) They code for their own transfer.

(iv) They act as episomes and reversibly integrate into bacterial chromosome.

(v) They may pick-up and transfer certain genes of bacterial chromosome,

(vi) They may affect certain characteristics of the bacterial cell,

(vii) Plasmids differ from viruses in following two ways.

(viii) They do not cause damage to cells and generally are beneficial.

(ix) They do not have extracellular forms and exist inside cells simply as free and typically circular DNA.

Plasmid classification: The plasmids are divided into 6 major classes as described below depending on the phenotype:

i) **Resistance or R plasmids** carry genes which give resistance to the bacteria from one or more chemical agents, such as antibacterial agents. R plasmids are very important in clinical microbiology as they can have profound consequences in the treatment of bacterial infections.

   Eg: RP4 plasmid, which is commonly found in Pseudomonas and in many other bacteria.

ii) **Fertility or F plasmids** are conjugative plasmid found in F+ bacterium with higher frequency of conjugation. F plasmid carries transfer gene (tra) and has the ability to form Conjugation Bridge (F pilus) with F- bacterium. Eg: F plasmid of E. coli.

iii) **Col plasmids** have genes that code for colicins, proteins that kill other bacteria. Eg: ColE1 of E. coli.

iv) **Degradative plasmids** allow the host bacterium to metabolize unusual molecules such as toluene and salicylic acid. Eg TOL of Pseudomonas putida.ca 12/10/2019 DS/RKMV/MB 8

v) **Virulence plasmids** confer pathogenicity on the host bacterium. Eg: Ti plasmids of Agrobacterium tumefaciens, which induce crown gall disease on dicotyledonous plants.

vi) **Cryptic Plasmids** do not have any apparent effect on the phenotype of the cell harboring them. They just code for enzymes required for their replication and maintenance in the host cell.

Based on the origin or source of plasmids, they have been divided into two major classes: such as natural and artificial.

i) **Natural plasmids**: They occur naturally in prokaryotes or eukaryotes. Example: ColE1.

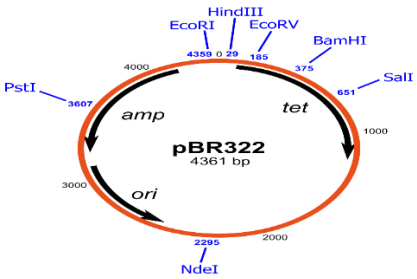
ii) **Artificial plasmids**: They are constructed in-vitro by re-combining selected segments of two or more other plasmids (natural or artificial). Example: pBR322.

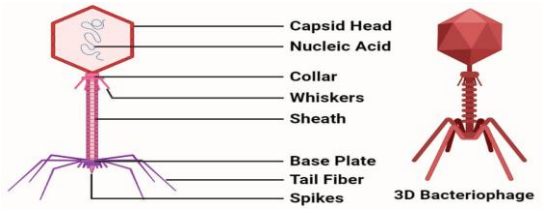
pBR322:

1. pBR322 is a widely-used E. coli cloning vector.
2. It was created in 1977 in the laboratory of Herbert Boyer at the University of California San Francisco.
3. The p stands for "plasmid" and BR for "Bolivar" and "Rodriguez", researchers who constructed it. '322' distinguishes this plasmid from others developed in the same laboratory (there are also plasmids called pBR325, pBR327, pBR328, etc.).
    - pBR322 is 4363 base pairs in length.
    - pBR322 plasmid has the following elements: ¬ "rep" replicon from plasmid pMB1 which is responsible for replication of the plasmid. ¬ "rop" gene encoding Rop protein, are associated with stability of plasmid and also controls copy number (increase number).
4. The source of "rop" gene is pMB1plasmid. ¬ "tet" gene encoding tetracycline resistance derived from pSC101 plasmid. ¬ "bla" gene encoding β lactamase which provide ampicillin resistance (source: transposon Tn3).
5. The first useful feature of pBR322 is its size. pBR322 is 4363 bp, which means that not only can the vector itself be purified with ease, but also any recombinant DNA molecules constructed with it. Even with 6 kb of additional DNA, a recombinant pBR322 molecule is still of a manageable size.
6. The second feature of pBR322 is that, it carries two sets of antibiotic resistance genes. Either ampicillin or tetracycline resistance can be used as a selectable marker for cells containing the plasmid, or each marker gene contains unique restriction sites that can be used in cloning experiments.
7. A third advantage of pBR322 is that it has a reasonably high copy number. Generally, there are about 15 molecules present in a transformed E. coli cell, but this number can be increased up to between 1000 and 3000 by plasmid amplification in the presence of a

protein synthesis inhibitor such as chloramphenicol. An E. coli culture therefore provides a good yield of recombinant pBR322 molecules.

8. It has 528 restriction sites for 66 restriction enzymes. Among them 20 restriction enzymes cut it at unique restriction sites. Tetracycline has 6 unique sites for 6 restriction enzymes. Ampicillin gene has 3 unique restriction site.
9. The sequences other than Tet and Amp genes, have unique sites for 1 restriction enzymes. There is no restriction inactivation when gene is inserted into any one of these sites.





Bacteriophage vector

- Bacteriophage vectors are viruses that only infect bacteria and transform them efficiently while carrying large inserts.
- Bacteriophages or phages have higher transformation efficiencies which increase the chances of recovering a clone containing the recombinant DNA segments.
- The most important feature of a phage is the packaging system which enables the incorporation of large eukaryotic genes and their regulatory elements.
- The use of phages also facilitates the isolation of larger quantities of DNA that can be used for the analysis of the insert.
- Even though there are a number of phages that can and have been used as vectors, phage λ is the most convenient cloning vector.
- It can selectively package a chromosome about 50 kb in length, and the size of the phage can be adjusted by removing the central part of the genome as it is not necessary for replication or the packaging of the donor DNA.
- The use of a bacteriophage vector that can incorporate larger DNA segments decreases the number of clones required to obtain a particular DNA library with the entire genome of the organism.
- Phage vectors are also effective as cloning vectors as the recombinant molecules formed after the cloning process are packaged into infective particles that can then be stored or handle efficiently.
- Some of the common phages used as vectors include M13 phages, λ phages, and P1 phages.

Applications of Vectors

The application of vectors in molecular biology and genetic engineering has increased with time due to the simplicity, cost-effectiveness, and rapidity of the process. The following are some of the major applications of vectors in molecular biology;

1. Cloning vectors are the most important group of vectors that are used for the transfer of foreign DNA into host cells for different purposes.

2. One of the most important applications of vectors is to generate engineered organisms for a particular function, like engineering *E. coli* bacteria for insulin production.
3. Vectors can be used to isolate a particular gene sequence within a genome and to determine its nucleotide sequence through DNA sequencing.
4. It also helps determine control sequences and regulatory sequences in genomes for their study and analysis.
5. Cloning vectors can be used for studying the structure, function, and production of protein in different organisms.
6. Phage therapy is a form of therapy that uses bacteriophage vectors to treat different bacterial infections in humans and other animals.
7. Vectors can also be used to identify mutations in different regions of DNA sequences as well as to diagnose gene defects related to certain diseases.
8. Recombinant DNA technology has been used in clinical microbiology in different approaches like recombinant antigens, recombinant vaccines, and diagnostic probes.
9. Recombinant antigens prepared by cloning techniques by using cloning vectors have been used for the screening of diseases like HIV, HCV, and CMV.
10. Vectors are one of the components in molecular biology which enable numerous studies related to cell structure, nucleic acid composition, and genetic engineering techniques.

Limitations of Vectors

The following are some of the limitations of vectors;

1. Vectors are not very stable due to changes in metabolic energy and changing pH and temperature in different hosts. The stability of vectors depends largely on the type of vector and host genotypes.
2. Overexpression of a particular type of genes in the host cell is a common problem associated with the use of vectors.
3. The use of a single type of vector might not be sufficient for a particular purpose. The use of multiple vectors is complex and results in difficulties during the process.
4. Even though a large number of studies are done in the field of molecular biology for the production of more efficient vectors, it is a time-consuming and expensive process.

DNA library or the gene library:

DNA library or the gene library is the collection of DNA sequences from different organisms. These DNA sequences have been extracted, purified, sequenced and associated with a vector so as to use them for the purpose of analysis, comparative detection etc.

Depending on the source of DNA, the DNA library can be of the following two types:

A) Genomic Library: In these libraries, the whole genome of an organism is used. The information of the whole genome is made available. These can be further of two types:

1. Nuclear Genomic Library: Nuclear DNA is purified and stored.

2. Organelle Genomic Library: Organellar DNA (from mitochondria or chloroplast) Is purified and stored

B) **cDNA Library:** When the mRNAs from a cell are extracted and DNA is made from mRNA through reverse transcription, such a DNA is called cDNA (complementary DNA). In cDNA library, only the cDNA (that are functional in a cell) are made and stored. In other words, cDNA library has a collection of mRNAs of an organism, in the form of cDNA.

Advantages of DNA libraries:

1. Ease of studying the genome of different organisms
2. Identification of genes in an organism.
3. Creation of transgenic organisms.
4. Gene therapy.
5. Pharmaceutical applications through the study of genetic defects.

Disadvantages of DNA libraries:

1. Extraction and sequencing of larger genome is a difficult task.
2. cDNA libraries give a picture of exons only, the alternative splicing os not considered.
3. Contamination of DNA

Genomic library

A genomic library contains all the sequences present in the genome of an organism. In the construction of genomic libraries it is feasible to use vectors that could accommodate large size of inserts.

The first step in the construction of genomic library is the isolation of genomic DNA, and entire DNA is subjected to restriction digestion. The fragmented DNA of suitable size is ligated in the appropriate cloning vectors. It is necessary to use partially digested DNA with partially used restriction enzyme to generate a random collection of fragments with a suitable size distribution.

The recombination vectors are transferred and maintained in organisms such as bacteria, virus or yeast. A target DNA sequence present in particular cell clones are identified, sub-cultured and maintained as cell lines, widely known as gene bank or a clone bank.

Steps in Genomic Library Construction:

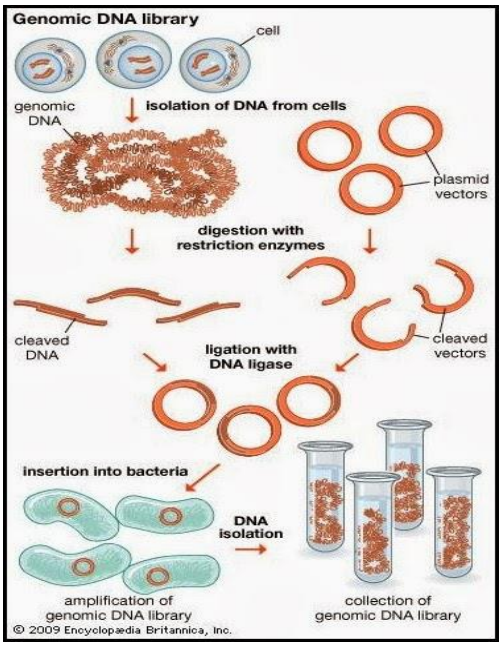
Construction of genomic library involves following steps:

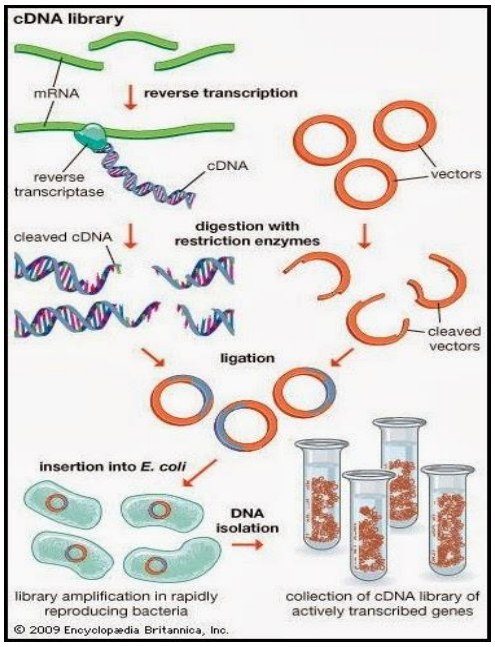
(a) Isolation of target DNA: Genomic libraries can be constructed by isolation of complete DNA from bacteria, virus, plants and animals. In eukaryotes, high molecular weight DNA is isolated by CTAB or SDS methods. The isolated DNA is then purified by caesium chloride and other methods.

(b) Restriction Fragments: Fragmentation can be done by mechanical shearing or using suitable restriction enzymes. Partial digestion is essential to procure proper size DNA fragments. Therefore, treatment times and concentration of enzyme is very important for desirable result.

(c) Cloning the fragments in vector: The restricted digested DNA sample is electrophoresed and subjected to. Target DNA fragments are identified by hybridization with probes and then cloned in suitable vectors like lambda or cosmid vectors and maintained as library.

(d) Screening of Genomic library: Genomic library can be screened for clones by hybridization with probe, western blotting to detect protein product and also screening of protein activity.





Advantages of genomic libraries

1. Identification of a clone encoding a particular gene of interest.
2. It is useful for prokaryotic organisms having relatively small genomes.
3. Genomic libraries from eukaryotic organisms are very important to study the genome sequence of a particular gene, including its regulatory sequences and its pattern of introns and exons.

Disadvantages of genomic library

1. Genome libraries from eukaryotes having very large genomes contain a lot of DNA which does not code for proteins and also contain non-coding DNA such as repetitive DNA and regulatory regions which makes them less than ideal.
2. Genomic library from a eukaryotic organism will not work if the screening method requires the expression of a gene.

Applications

1. To determine the complete genome sequence of a given organism.
2. To study and generate transgenic animals through genetic engineering, serving as a source of genomic sequence.
3. To study the function of regulatory sequences in vitro.
4. To study the genetic mutations.
5. Used for genome mapping, sequencing and the assembly of clone contigs.

cDNA Library:

Steps of cDNA cloning:

1. **Isolation of mRNA**
2. **Synthesis of first strand of cDNA**
3. **Synthesis of second strand of cDNA**
4. **Cloning of cDNA**
5. **Introduction to host cells**
6. **Clone selection**

1. Isolation of mRNA:

- A crude extract of the tissue with the gene of interest is prepared.
- The extract must be free from proteins, polysaccharides and all other contaminants.
- The technique of oligo-deoxythymine (oligo-dT) cellulose chromatography is used for the further purification of many eukaryotic mRNAs from the total or polysomal fraction.
- mRNAs consist of poly A (adenosine residues) tail at their 3′ end.
- Under favourable conditions, this tail will bind to a string of thymidine residues immobilized on cellulose and then poly (A)+ fraction can be eluted.
- Two or three passages of the poly (A)+ fraction through such a column produces a fraction highly enriched for mRNA.
- This fraction includes different mRNA sequences, however certain techniques can be employed for extracting a particular mRNA species.
- After the preparation of the fraction, it is essential to confirm if the extracted mRNA consists of the sequence of interest.
- It is performed by translation of mRNA in vitro and identification of suitable polypeptides in the products obtained.

2. Synthesis of first strand of cDNA:

- Reverse transcriptase is a RNA dependent DNA polymerase which is used to copy the mRNA fraction into the first strand of DNA.

- This enzyme, like all other DNA polymerases, can only add residues at the 3′-OH group of an existing primer, which is base paired with the template.
- The most commonly used primer is oligo-dT for cloning of cDNAs.
- Oligo-dT primer is 12-18 nucleotides in length, that binds to the poly (A) tract at the 3′ end of mRNA molecules.
- The RNA strand of the resulting RNA-DNA hybrid is destroyed prior to second strand synthesis through alkaline hydrolysis.

3. Synthesis of second strand of cDNA:

- The second strand of cDNA can be synthesized by two techniques. They are:
- **i. Self-priming cDNA:**
  - In Self-priming, the mRNA hybrid obtained is denaturated for the synthesis of second strand on the single strand of cDNA by the klenow fragment of DNA polymerase I.
  - The transitory hairpin structure at the 3′ end of single-stranded DNA can be used to prime the synthesis of second strand of cDNA by the klenow fragment of *Escherichia coli* DNA polymerase I.
  - Single-strand specific S1 nuclease digests the hairpin loop and any single-stranded overhung at the other end.
  - The ultimate product is a population of double-stranded, blunt-ended DNA molecules complementray to the original mRNA fraction.
- **ii. Replacement synthesis:**
  - In this method, the cDNA:mRNA hybrid works as a template for a nick translation reaction.
  - In the mRNA strand of the hybrid, RNase H produces nicks and gaps, creating a series of RNA primers.
  - These RNA primers are used by *E. coli* DNA polymerase I during the synthesis of second strand of cDNA.
  - The advantages of this technique are:
    - – very efficient
    - – can be performed directly using the products of the first strand reaction
    - – eliminates the need to use nuclease S1 to cleave the single-stranded hairpin loop in the double stranded cDNA.

4. Cloning of cDNA:

- The most frequently used technique for cloning cDNAs involves the addition of complementary homopolymeric tracts to double stranded cDNA and to the plasmid vector.
- To the cDNA, strings of cytosine residues are added using the enzyme terminal transferase to form oligo-dC tails on the 3′ ends.
- Likewise, a plasmid is cut open at a unique restriction endonuclease site and tailed with oligo-dG.
- Now, the vector and the double stranded cDNA are joined by hydrogen bonding between the complementary homopolymers.

- It results in the formation of open circular hybrid molecules capable of transforming *E. coli.*

5. Introduction to host cells:

- For the transforming of bacteria, the recombinant plasmids are used, usually the *E. coli* K-12 strain.
- The uptake of plasmid molecules from the surrounding medium is performed by E. coli cells treated with calcium chloride.
- Any gaps in the recombinant plasmid will be repaired by the host cells.
- The transformed bacteria can be isolated from non-transformed ones on the basis of antibiotic resistance.
- Majority of cloning plasmids contain two antibiotic resistance genes, one of which is destroyed during cloning.
- For instance, in the case of pBR322, cloning into unique PstI site destroys ampicillin resistance but leaves tetracycline resistance intact.
- Bacteria transformed with a recombinant plasmid will be sensitive to ampicillin but resistant to tetracycline.

6. Clone selection:

- The antibiotic resistance selection already performed has recognized which clones carry a recombinant plasmid, however there will be thousands of various inserts.
- The cloning process generally commences with a whole population of mRNA sequences.
- Selection of clones carrying the sequence of interest is the tough job.
- If the gene is expressed, then the simplest selection is to screen for the presence of the protein.
- It can be screened either by bacterial phenotype it produces or by the protein detection methods usually based on immunological or enzymological techniques.
- If the protein is not expressed, then other methods such as nucleic acid hybridization are used.
- Identification of the gene is discussed after the genomic DNA cloning.

Advantages of cDNA Library:

A cDNA library has two advantages. First, it is enriched with fragments from actively transcribed genes. Second, introns do not interrupt the cloned sequences; introns would pose a problem when the goal is to produce a eukaryotic protein in bacteria, because most bacteria have no means of removing the introns.

Disadvantages of cDNA Library:

The disadvantage of a cDNA library is that it contains only sequences that are present in mature mRNA. Introns and any other sequences that are altered after transcription are not present; sequences, such as promoters and enhancers that are not transcribed into RNA also are not present in a cDNA library.

It is also important to note that the cDNA library represents only those gene sequences expressed in the tissue from which the RNA was isolated. Furthermore, the frequency of a particular DNA sequence in a

cDNA library depends on the abundance of the corresponding mRNA in the given tissue. In contrast, almost all genes are present at the same frequency in a genomic DNA library.

Applications of cDNA Library:

1. Discovery of novel genes.
2. Cloning of full-length cDNA molecules for in vitro study of gene function.
3. Study of the repertoire of mRNAs expressed in different cells or tissues.
4. Study of alternative splicing in different cells or tissues.

Unit 4 – Methods of gene sequencing and gene transfer

DNA sequencing: A laboratory technique employed to known the correct DNA sequence by the sequential chemical reaction is known as DNA sequencing.

Various methods of DNA sequencing are explained here.

- Maxam and Gilbert method
- Chain termination method
- semiautomated method
- automated method
- Pyrosequencing
- The whole-genome shotgun sequencing method
- Clone by the clone sequencing method
- Next-generation sequencing method

Maxam-Gilbert sequencing:

The *Maxam* and *Gilbert* method was developed in 1977. It is also referred to as a chemical cleavage method. By using this method, they had sequenced 24 nucleotides only. However, their method was published after two years of sangers method.

The brief principle of the present method is as stated,

"The single-stranded DNA is cleaved at the specific location with the help of the chemicals at specific base location and the fragments of DNA is then run on polyacrylamide gel."
Obviously, DNA extraction is the very first step. After that, the DNA is denatured using the heat denaturation method and single-stranded DNA is generated.

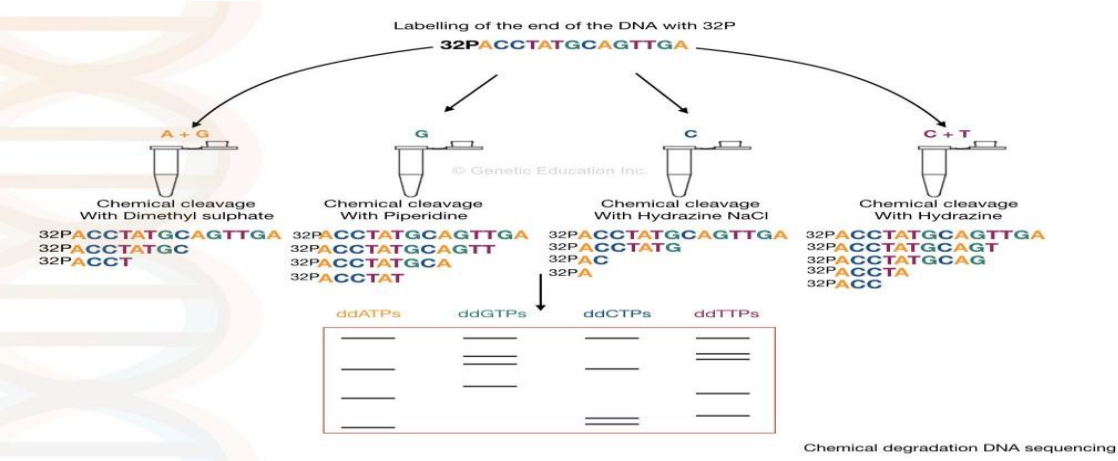
The phosphate (5' P) end of the DNA is removed and labeled by the radiolabeled P32. The enzyme named phosphatase removes the phosphate from the DNA and simultaneously, the kinase adds the 32P to the 5' end of it.

4 different chemicals are used to cleave DNA at four different positions; hydrazine and hydrazine NaCl are selectively attack pyrimidine nucleotides while dimethyl sulfate and piperidine attack purine nucleotides.

- Hydrazine: T + C
- Hydrazine NaCl: C
- Dimethyl sulfate: A + G
- Piperidine: G

An equal volume of 4 different ssDNA samples is taken into 4 different tubes each containing 4 different chemicals. The samples are incubated for some time and electrophoresed in polyacrylamide gel electrophoresis. The results of the chemicals cleavage of four different tubes are shown in the figure below.

Autobiography is used to visualize the separation of DNA fragments. Due to the radiolabelled 32P end of the DNA, the DNA bands are visualized through autoradiography.



In the present automation time, the present method is used in DNA fingerprinting and genetic engineering studies.

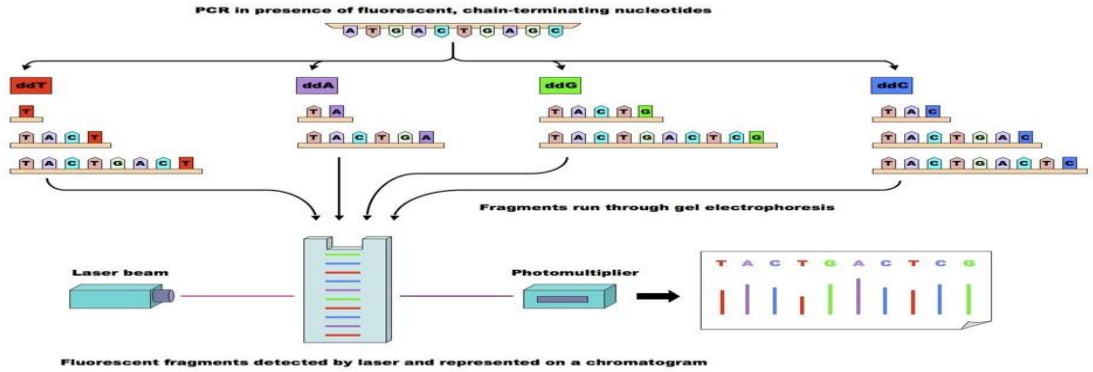
Advantages

- Purified DNA can be read directly
- Homopolymeric DNA runs are sequenced as efficiently as heterogeneous DNA sequences
- Can be used to analyze DNA protein interactions (i.e. footprinting)
- Can be used to analyze nucleic acid structure and epigenetic modifications to DNA

Disadvantages

- It requires extensive use of hazardous chemicals.
- It has a relatively complex set up / technical complexity.
- It is difficult to “scale up” and cannot be used to analyze more than 500 base pairs.
- The read length decreases from incomplete cleavage reactions.
- It is difficult to make Maxam-Gilbert sequencing based DNA kits.

Chain Termination Method (Sanger Dideoxy Method)



- The chain terminator method is more efficient and uses fewer toxic chemicals and lower amount of radioactivity than the method of Maxam and Gilbert.
- The key principle of the Sanger method was the use of dideoxynucleotide triphosphates (ddNTPs) as DNA chain terminators.
- The chain termination method requires a single-stranded DNA template, a DNA primer,a DNA polymerase, radioactively or fluorescently labelled nucleotides,and modified nucleotides that terminate DNA strand elongation.
- The DNA sample is divided into four separate sequencing reactions, containing all four of the standard deoxynucleotides (dATP, dGTP, dCTP, dTTP) and the DNA polymerase.
- To each reaction is added only one of the four dideoxynucleotide (ddATP, ddGTP, ddCTP, ddTTP) which are the chain terminating nucleotides, lacking a 3'-OH group required for the formation of a phosphodiester bond between two nucleotides, thus terminating DNA strand extension and resulting in DNA fragments of varying length.
- The newly synthesized and labelled DNA fragments are heat denatured, and separated by size by gel electrophoresis on a denaturing polyacrylamide-urea gel with each of the four reactions run in one of the four individual lanes (lanes A, T, G, C).
- The DNA bands are then visualized by autoradiography or UV light, and the DNA sequence can be directly read off the X-ray film or gel image.
- A dark band in a lane indicates a DNA fragment that is result of chain termination after incorporation of a dideoxynucleotide (ddATP, ddGTP, ddCTP, or ddTTP).
- The relative position of the different bands among the four lanes are then used to read (from bottom to top) the DNA sequence.
- The technical variations of chain termination sequencing include tagging with nucleotides containing radioactive phosphorus for labelling, or using a primer labelled at the 5' end with a fluorescent dye.
- Dye- primer sequencing facilitates reading in an optical system for faster and more economical analysis and automation.

Key Features

- Uses dideoxy nucleotides to terminate DNA synthesis.
- DNA synthesis reactions in four separate tubes
- Radioactive dATP is also included in all the tubes so the DNA products will be radioactive.
- Yielding a series of DNA fragments whose sizes can be measured by electrophoresis.
- The last base in each of these fragments is known.

Advantage

Chain termination methods have greatly simplified DNA sequencing.

Limitations

- Non-specific binding of the primer to the DNA, affecting accurate read-out of the DNA sequence.
- DNA secondary structures affecting the fidelity of the sequence.

Significance of DNA Sequencing

- Information obtained by DNA sequencing makes it possible to understand or alter the function of genes.
- DNA sequence analysis demonstrates regulatory regions that control gene expression and genetic "hot spots" particularly susceptible to mutation.
- Comparison of DNA sequences shows evolutionary relationships that provide a framework for definite classification of microorganisms including viruses.
- Comparison of DNA sequences facilitates identification of conserved regions, which are useful for development of specific hybridization probes to detect microorganisms including viruses in clinical samples.
- DNA sequencing has become sufficiently fast and inexpensive to allow laboratory determination of microbial sequences for identification of microbes. Sequencing of the 16S ribosomal subunit can be used to identify specific bacteria. Sequencing of viruses can be used to identify the virus and distinguish different strains.
- DNA sequencing shows gene structure that helps research workers to find out the structure of gene products.

Polymerase Chain Reaction (PCR)- Principle, Steps, Applications

- PCR is an enzymatic process in which a specific region of DNA is replicated over and over again to yield many copies of a particular sequence.
- The most widely used target nucleic acid amplification method is the polymerase chain reaction (PCR).
- This method combines the principles of complementary nucleic acid hybridization with those of nucleic acid replication applied repeatedly through numerous cycles.
- This method is able to amplify a single copy of a **nucleic acid** target, often undetectable by standard hybridization methods, and multiply to 10^7 or more copies in a relatively short period.
- This thus provides ample target that can be readily detected by numerous methods.

Principle of PCR

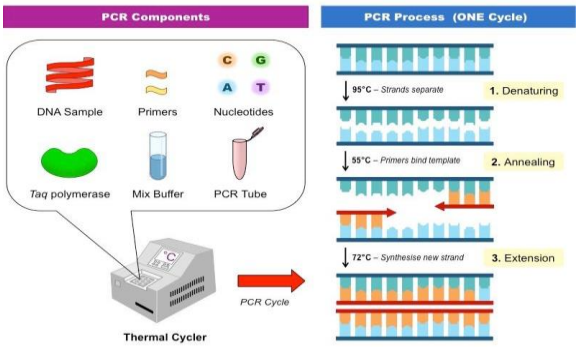
The target sequence of nucleic acid is denatured to single strands, primers specific for each target strand sequence are added, and **DNA** polymerase catalyzes the addition of deoxynucleotides to extend and produce new strands complementary to each of the target sequence strands (cycle 1). In cycle 2, both double-stranded products of cycle 1 are denatured and subsequently serve as targets for more primer annealing and extension by DNA polymerase. After 25 to 30 cycles, at least 10^7 copies of target DNA may be produced by means of this thermal cycling

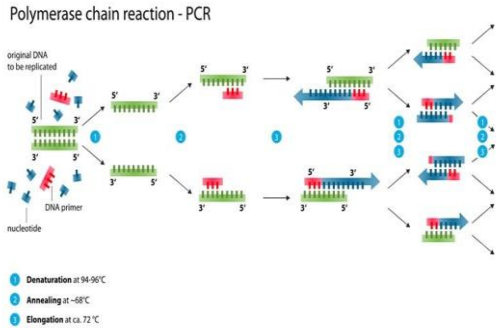
Requirements for PCR

- A PCR reaction contains the target double-stranded DNA, two primers that hybridize to flanking sequences on opposing strands of the target, all four deoxyribonucleoside triphosphates and a DNA polymerase along with buffer, co-factors of **enzyme** and water.

- Since the reaction periodically becomes heated to high temperature, PCR depends upon using a heat-stable DNA polymerase.
- Many such heat-stable enzymes from thermophilic bacteria (bacteria that live in high temperature surroundings) are now available commercially.
- The first one and the most commonly used is the Taq polymerase from the thermophilic bacterium *Thermus aquaticus*.

Steps Involved





A. Extraction and Denaturation of Target Nucleic Acid

- For PCR, nucleic acid is first extracted (released) from the organism or a clinical sample potentially containing the target organism by heat, chemical, or enzymatic methods.
- Once extracted, target nucleic acid is added to the reaction mix containing all the necessary components for PCR (primers, nucleotides, covalent ions, buffer, and enzyme) and placed into a thermal cycler to undergo amplification.

B. Steps in Amplification

- Conventional PCR involves 25 to 50 repetitive cycles, with each cycle comprising three sequential reactions:

1. **Denaturation** of target nucleic acid
2. **Primer annealing** to single-strand target nucleic acid extension of primer target duplex.
3. **Extension** of the primer-target duplex.

Denaturation

- The reaction mixture is heated to 95°C for a short time period (about 15–30 sec) to denature the target DNA into single strands that can act as templates for DNA synthesis.

Primer annealing

- The mixture is rapidly cooled to a defined temperature which allows the two primers to bind to the sequences on each of the two strands flanking the target DNA.
- Primers are short, single-stranded sequences of nucleic acid (i.e., oligonucleotides usually 20 to 30 nucleotides long) selected to specifically hybridize (anneal) to a particular nucleic acid target, essentially functioning like probes.
- This annealing temperature is calculated carefully to ensure that the primers bind only to the desired DNA sequences (usually around 55^{o}C).
- One primer binds to each strand. The two parental strands do not re-anneal with each other because the primers are in large excess over parental DNA.

Extension

- The temperature of the mixture is raised to 72°C (usually) and kept at this temperature for a pre-set period of time to allow DNA polymerase to elongate each primer by copying the single-stranded templates.
- Annealing of primers to target sequences provides the necessary template format that allows the DNA polymerase to add nucleotides to the 3' terminus (end) of each primer and extend sequence complementary to the target template
- Taq polymerase is the enzyme commonly used for primer extension, which occurs at 72°C. This enzyme is used because of its ability to function efficiently at elevated temperatures and to withstand the denaturing temperature of 94°C through several cycles.
- The ability to allow primer annealing and extension to occur at elevated temperatures without detriment to the polymerase increases the stringency of the reaction, thus decreasing the chance for amplification of non-target nucleic acid (i.e., nonspecific amplification).

The three steps of the PCR cycle are repeated.

- Thus in the second cycle, the four strands denature, bind primers and are extended. No other reactants need to be added. The three steps are repeated for a third cycle and so on for a set of additional cycles.
- By the third cycle, some of the PCR products represent DNA sequence only between the two primer sites and the sequence does not extend beyond these sites.
- As more and more reaction cycles are carried out, the double-stranded DNA are synthesized more in number. After 20 cycles, the original DNA has been amplified a million-fold and this rises to a billion fold (1000) million after 30 cycles.

C. Product Analysis

- Gel electrophoresis of the amplified product is commonly employed after amplification.
- The amplified DNA is electrophoretically migrated according to their molecular size by performing agarose gel electrophoresis.
- The amplified DNA forms clear bands which can be visualized under ultra-raviolet (UV) light.

Advantages of PCR

- PCR (polymerase chain reaction) is an extremely simple yet immensely powerful technique.
- It allows enormous amplification of any specific sequence of DNA provided that short sequences either side of it are known.
- Allow faster diagnosis and identification while enhancing sensitivity and maintaining specificity.

Applications of PCR

PCR already has very widespread applications, and new uses are being devised on a regular basis.

- PCR can amplify a single DNA molecule from a complex mixture, largely avoiding the need to use DNA cloning to prepare that molecule. Variants of the technique can similarly amplify a specific single RNA molecule from a complex mixture.
- DNA sequencing has been greatly simplified using PCR, and this application is now common.
- By using suitable primers, it is possible to use PCR to create point mutations, deletions and insertions of target DNA which greatly facilitates the analysis of gene expression and function.

- PCR is exquisitely sensitive and can amplify vanishingly small amounts of DNA. Thus, using appropriate primers, very small amounts of specified bacteria and viruses can be detected in tissues, making PCR invaluable for medical diagnosis.
- PCR is now invaluable for characterizing medically important DNA samples. For example, in screening for human genetic diseases, it is rapidly replacing the use of RFLPs.
- Because of its extreme sensitivity, PCR is now fundamentally important to forensic medicine. It is even possible to use PCR to amplify the DNA from a single human hair or a microscopic drop of blood left at the scene of a crime to allow detailed characterization.

Gene transfer techniques:

1. Transformation:

Transformation is the method of introducing foreign DNA into bacterial cells (e.g. E.coli). The uptake of plasmid DNA by E.coli is carried out in ice-cold $CaCl_2$ (0-5°C), and a subsequent heat shock (37-45°C for about 90 sec). By this technique, the transformation frequency, which refers to the fraction of cell population that can be transferred, is reasonably good e.g. approximately one cell for 1000 (10^{-3}) cells.

Transformation efficiency:

It refers to the number of trans-formants per microgram of added DNA. For E.coli, transformation by plasmid, the transformation efficiency is about 10^7 to 10^8 cells per microgram of intact plasmid DNA. The bacterial cells that can take up DNA are considered as competent. The competence can be enhanced by altering growth conditions.

The mechanism of the transformation process is not fully understood. It is believed that the $CaCI_2$ affects the cell wall, breaks at localized regions, and is also responsible for binding of DNA to cell surface. A brief heat shock (i.e. the sudden increase in temperature from 5°C to 40°C) stimulates DNA uptake. In general, large-sized DNAs are less efficient in transforming.

Other chemical methods for transformation:

Calcium phosphate (in place of $CaCI_2$) is preferred for the transfer of DNA into cultured cells. Sometimes, calcium phosphate may result in precipitate and toxicity to the cells. Some workers use diethyl amino ethyl dextran (DEAE -dextran) for DNA transfer.

2. Calcium phosphate Transfection

It was the first chemical transfection method to be used with animal cells. Calcium phosphate is probably the most widely used transfection method. This is a simple, reliable method applicable to many cultured cell lines, and the reagents are inexpensive. It can be used both for transient and stable transformation. The principle of the technique is that DNA in a buffered phosphate solution is mixed gently with calcium chloride, which causes the formation of a fine DNA-calcium phosphate coprecipitate. The precipitate settles onto the cells and some of the particles are taken up by endocytosis. The most efficient transfection occurs in cells growing as a monolayer, because these cells become evenly coated with the precipitate. The procedure was developed in 1973 by Graham and van der Erb for the introduction of adenovirus DNA into rat cells. In a report by Szybalska and Szybalski published in 1962 the presence of calcium was

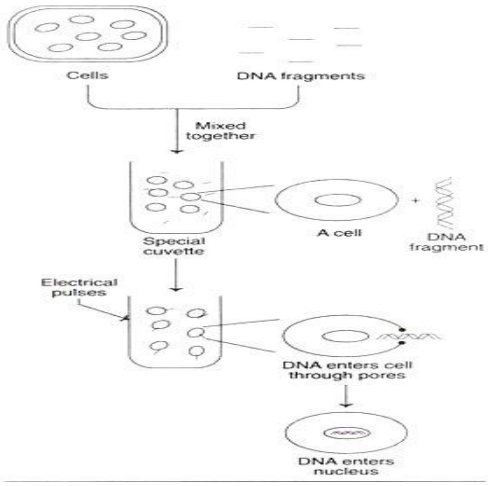
shown to be responsible for the successful transformation of human cells with genomic DNA. The first mammalian cell lines stably transfected with plasmid DNA were also produced by calcium phosphate transfection, in 1978.

3. Electroporation:

Electroporation is based on the principle that high voltage electric pulses can induce cell plasma membranes to fuse. Thus, electroporation is a technique involving electric field-mediated membrane permeabilization. Electric shocks can also induce cellular uptake of exogenous DNA (believed to be via the pores formed by electric pulses) from the suspending solution.

Electroporation is a simple and rapid technique for introducing genes into the cells from various organisms (microorganisms, plants and animals).

The cells are placed in a solution containing DNA and subjected to electrical shocks to cause holes in the membranes. The foreign DNA fragments enter through the holes into the cytoplasm and then to nucleus.



Electroporation is an effective way to transform E.coli cells containing plasmids with insert DNAs longer than 100 kb. The transformation efficiency is around 10^9 transformants per microgram of DNA for small plasmids (about 3kb) and about 10^6 for large plasmids (about 130 kb).

Advantages of electroporation:

i. This technique is simple, convenient and rapid, besides being cost-effective.

ii. The transformed cells are at the same physiological state after electroporation.

iii. Efficiency of transformation can be improved by optimising the electrical field strength, and addition of spermidine.

Limitations of electroporation:

i. Under normal conditions, the amount of DNA delivered into plant cells is very low.

ii. Efficiency of electroporation is highly variable depending on the plant material and the treatment conditions.

iii. Regeneration of plants is not very easy, particularly when protoplasts are used.

4. **Microinjection:**

Microinjection is a direct physical method involving the mechanical insertion of the desirable DNA into a target cell. The target cell may be the one identified from intact cells, protoplasts, callus, embryos, meristems etc. Microinjection is used for the transfer of cellular organelles and for the manipulation of chromosomes.

The technique of microinjection involves the transfer of the gene through a micropipette (0.5-10.0 pm tip) into the cytoplasm/nucleus of a plant cell or protoplast. While the gene transfer is done, the recipient cells are kept immobilized in agarose embedding, and held by a suction holding pipette (Fig. 49.11).

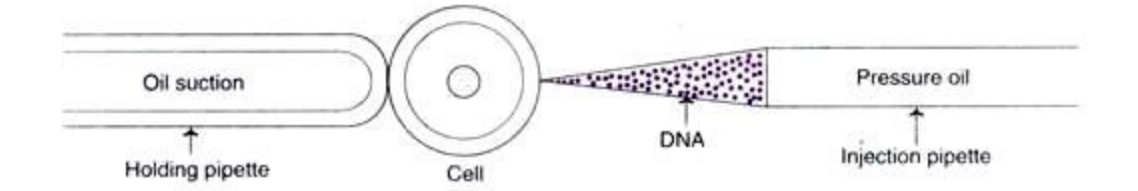


Fig. 49.11 : Microinjection of DNA by holding pipette method.

As the process of microinjection is complete, the transformed cell is cultured and grown to develop into a transgenic plant. In fact, transgenic tobacco and Brassica napus have been developed by this approach. The major limitations of microinjection are that it is slow, expensive, and has to be performed by trained and skilled personnel.

5. **Particle Bombardment (Biolistics):**

Particle (or micro projectile) bombardment is the most effective method for gene transfer, and creation of transgenic plants. This method is versatile due to the fact that it can be successfully used for the DNA transfer in mammalian cells and microorganisms.

The micro projectile bombardment method was initially named as biolistics by its inventor Sanford (1988). Biolistics is a combination of biological and ballistics. There are other names for this technique- particle gun, gene gun, bio blaster. A diagrammatic representation of micro

projectile bombardment system for the transfer of genes in plants is depicted in Fig. 49.10, and briefly described below.

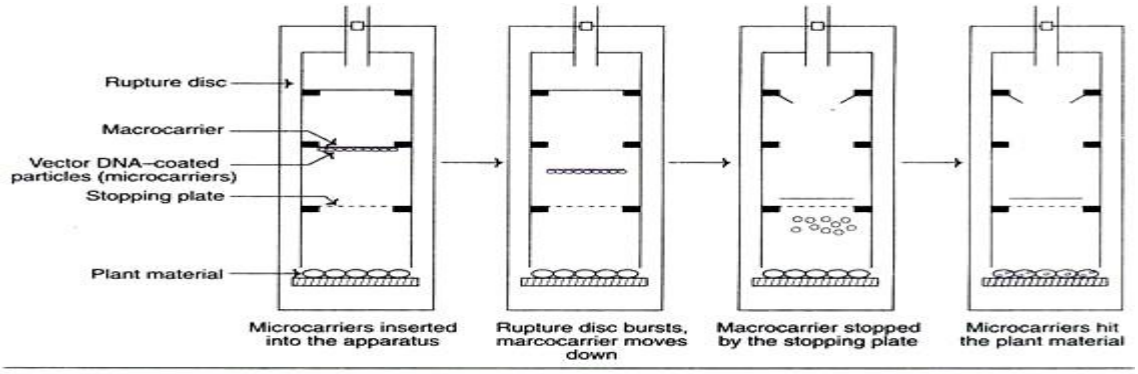


Fig. 49.10 : A diagrammatic representation of particle bombardment (biolistics) system for gene transfer in plants.

Micro carriers (micro projectiles), the tungsten or gold particles coated with DNA, are carried by macro carriers (macro projectiles). These macro-carriers are inserted into the apparatus and pushed downward by rupturing the disc.

The stopping plate does not permit the movement of macro carrier while the micro carriers (with DNA) are propelled at a high speed into the plant material. Here the DNA segments are released which enter the plant cells and integrate with the genome.

Plant material used in bombardment:

Two types of plant tissue are commonly used for particle bombardment:

1. Primary explants which can be subjected to bombardment that are subsequently induced to become embryo genic and regenerate.

2. Proliferating embryonic tissues that can be bombarded in cultures and then allowed to proliferate and regenerate.

In order to protect plant tissues from being damaged by bombardment, cultures are maintained on high osmoticum media or subjected to limited plasmolysis.

Advantages of particle bombardment:

i. Gene transfer can be efficiently done in organized tissues.

ii. Different species of plants can be used to develop transgenic plants.

Limitations of particle bombardment:

i. The major complication is the production of high transgene copy number. This may result in instability of transgene expression due to gene silencing.

ii. The target tissue may often get damaged due to lack of control of bombardment velocity.

iii. Sometimes, undesirable chimeric plants may be regenerated.

6. Agrobacterium-Mediated Gene Transfer (Transformation) in Plants

What is Agrobacterium?

Agrobacterium is a phytopathogen that infects plants through wound sites, causing crown gall disease, and is one of the most popular plant transformation tools used in agriculture to date.

- Agrobacterium tumefaciens is the soil pathogen that utilizes its bacterial type IV secretion system for the transfer of its transferred (T)-DNA into the host cells.
- The genus Agrobacterium consists of different species depending on their disease symptomology and host range. Some of the species of Agrobacterium include A. radiobacter, A. vitis, A. rhizogenes, A. rubi and A. tumefaciens.
- The organisms of this genus are most notably known as plant transformation tools used in a wide range of host cells.

Factors affecting Agrobacterium-mediated Gene Transfer

- There are a number of methods that are employed to obtain transgenic plants, some of which include Agrobacterium-mediated transformation, particle bombardment, protoplasts mediated by polyethene glycol, and liposome-mediated transformation.
- Among all these methods, Agrobacterium-mediated transformation results in single-copy transgenes, which are comparatively more stably expressed than multiple gene copies.
- The process is, however, influenced by different factors like bacterial strains and cell density, plant species, plant growth regulators, and environmental factors.
- In order to develop an efficient transformation protocol, it is necessary to find the right combination of these factors.

The following are some of the factors that influence Agrobacterium-mediated transformation:

a. Explants

b. Explants Wounding c. Plant species and Genotype

d. Antibiotics

e. Plant Growth Regulators (PGR)

f. Light g. Temperature

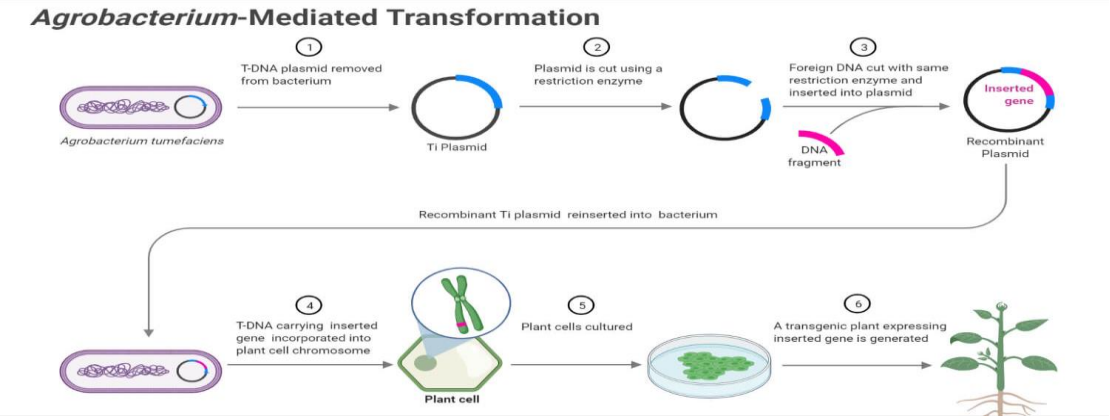
h. Agrobacterium Strains

Procedure of Agrobacterium-mediated Gene Transfer

The protocol or procedure for the Agrobacterium-mediated transformation might differ depending on the type of explants selected for the process. The following is the protocol for Agrobacterium-mediated transformation in the case of an embryo;

a. **Sterilization and Germination of seeds**

- The seeds are sterilized with Cl2 for 1-2 hours, and the seeds are then soaked in water for 2 hours at room temperature in a Petri dish.
- The seed coats are removed with forceps from the seed and are further sterilized with 75% ethanol for 30 seconds. These are then rinsed with 20 ml of the 3% sodium hypochlorite.
- The sterilized seeds are placed on Petri plates containing seed germination medium and are incubated at 28°C for 2 days in the dark. Each plate can contain about 15-25 seeds.



b. **Inoculum Preparation**

- 2 ml of the LB medium containing rifampicin and kanamycin is inoculated with a single Agrobacterium colony. The culture is then incubated in the shaker incubator at 28°C.
- The Agrobacterium culture prepared is centrifuged at 4000g for 10 minutes. The supernatant is removed, and the pellets are cleaned with a 1 ml MS liquid medium.

c. **Preparation of the explants**

- The seeds are pulled out of the germination medium and are placed on empty sterile Petri plates with a stack of filter papers.
- The radicle is removed, and the seed is cut to remove ½ of the cotyledons and endopleura.
- The cotyledons are separated with a sterile scalpel and are wiped off. The detached cotyledons are collected into the MS liquid medium in a sterile glass beaker.
- The MS liquid medium containing the cotyledons are poured into MS liquid medium containing Agrobacterium and shaken gently.
- The glass beaker is covered with a container and sealed with film, and ut into the desiccators attached to the vacuum pump.
- The explants are left on the medium for 5 minutes after two sessions of vacuum infiltration.
- A sterile filter paper is packed on the cocultivation medium, and the infected explants are placed on the filter paper with forceps. The adaxial surface of the cotyledon is to be kept upwards.
- The Petri plates are then sealed and incubated in the dark for 2 days at 28°C.

d. **Shoot Initiation**

- The explants are then transferred to the shooting medium containing kanamycin and carbenicillin in order to inhibit the growth of Agrobacterium.
- Every plate can contain 5-6 explants that are then incubated under light for 2-3 weeks at 25°C.

e. **Regeneration**

- Once the shoots begin to appear on the explants, these are pulled out and placed on sterile Petri plates with a stack of filter paper.
- The shoots are cut off with a sterile scalpel, and the embryoid part of the shoots are removed.
- The shoots are then transferred to a 100 ml glass flask or tissue culture vessels with a rooting medium.
- About 3-4 shoots can be added per vessel. The vessels are incubated under light for 1-2 weeks at 25°C.
- After 2 weeks, if no roots are observed, the unfolded leaves and end parts of the shoots are cut off, and the shoots are transferred to a new rooting medium.

f. **Plant acclimation**

- After the roots begin to appear, the over on the flasks are loosened and further incubated at 25°C for 3 days.
- The plants are then pulled out from the medium and washed off with running water.
- These are then transferred to pots filled with wet compost and watered.
- The pots are covered with zip bags to keep the moisture. These are incubated in a good condition chamber under light at 25°C for 1-2 weeks.
- Once the plants grow in good condition, the zip bags are removed, and the plants are watered.

Applications of Agrobacterium-mediated Gene Transfer

The following are some of the important applications of Agrobacterium-mediated transformation;

1. The Agrobacterium-mediated transformation has been used as a method of genetic modification of plants for the production of various substances like proteins, antibodies, and even vaccines.
2. Different plants have also been modified to produce life-saving pharmaceutical products like anticoagulants, human epidermal growth factors, and interferons.
3. Transgenic plants prepared with Agrobacterium serve as biomonitors to detect the presence of toxic compounds in the environment as well as to detoxify the contaminated soil and water.
4. Agrobacterium-mediated transformation has also remarkably increased crop yields by modifying the shelf-life and biosynthesis of the plants.
5. Plants can be modified to enhance tolerance against biotic and abiotic factors, nutrient capture with increased pest resistance.
6. Agrobacterium-mediated transformation has been used to produce insect resistance crops by the incorporation of various toxic genes like the Bt toxin genes.
7. The increase in pest resistance results in a reduction in the use of harmful agrochemicals and herbicides.
8. Agrobacterium-mediated transformation is one of the less complicated genetic engineering techniques which has the possibility of being upgraded to use with other organisms as well.

Limitations of Agrobacterium-mediated Gene Transfer

Even though Agrobacterium-mediated transformation has been advancing over the years with much success, there are some problems and limitations associated with this technique. Some of the commonly encountered limitations and problems with the technique are;

1. The most important limitation associated with this technique is the narrow host range, as it is still limited to particular plant species.
2. Even though a lot is known about the mechanism of T-DNA transfer in the bacteria, not much is known about the plant-encoded factors that affect the efficiency of this process.
3. The technique is labor-intensive as it requires the development of plant regeneration protocols and detailed time-consuming processes. Many of these processes are prone to in vitro variations, resulting in unfavorable results.
4. The success of transformation in the case of monocots depends on the use of embryos as the explants; however, these are only available for a short period of time during the year.
5. Agrobacterium-mediated transformation cannot transfer large DNA molecules into more economically important plants, which indicates a possible introduction of a powerful vector system.

Unit 5 - Applications of rDNA Technology

Applications of rDNA Technology in Agriculture (Transgenic plants): The term transgenic plants refers to the plants whose DNA is modified through genetic engineering. This means that one or several genes from a different species are introduced and combined with the genetic material of the plant which changes the traits of the original genome.

While horizontal gene transfer has been shown to occur naturally in nature (between plants that grow close to each other), various artificial techniques are used to insert gene sequences to some plants with the aim of increasing yields, making them more tolerant of various environmental conditions, or making them more resistant to given biotic stresses, etc.

For this reason, transgenic plants are particularly valuable in agriculture as well as in various industries (e.g. in the pharmaceutical industry).

The first transgenic plant was produced in 1982. It was a tobacco plant that exhibited resistance to antibiotics.

The new genetic material inserted into the genome of a plant can be derived from a different plant or from a different species.

Applications of transgenic plants

With regards to transgenic plants, there are two main areas of applications.

These include:

1. **In Agriculture**

As these techniques involve changing the genomic information of plants, the agricultural sector is one of the main areas of agriculture.

Since 1982, the technology has been used to transform the characteristics of different types of plants ranging from corn and barley to onion and tomato.

Here, gene transfer is used for various functions ranging from increasing yields (crops and animals feeds etc) as well as increasing plant/crop tolerance to various biological and environmental factors.

2. Biopharming/Pharmaceutical Industries

Apart from the agricultural sector, gene transfer in plants has also found many applications in the pharmaceutical industry. Gene transfer has been used for the large scale production of various proteins and chemicals that plants would not naturally produce.

As compared to some of the other cells used in biopharmaceuticals, transgenic plants are suggested to be more cost-effective. Moreover, they have in place the machinery that can transform molecules into given structures that can serve the required biological activities.

3. Role of Transgenic Plants as Bioreactors or Biofactories

As is the case with many other cells, plant cells have biological machinery involved in various processes. Here, the information (blueprint) required for these processes is contained in the genetic material.

By introducing new information in the form of new genes, it has become possible for researchers to take advantage of these processes to produce various proteins including vaccines and antibodies among others.

While bacteria and other types of cells have been used as bioreactors, transgenic plants have been gaining more attention in recent years because they are less expensive and are capable of post-translational modifications involved in the production of complex proteins.

Advantages of Transgenic Plants

As mentioned, the methods and technologies used to produce transgenic plants are applied in several industries/sectors. This is because transgenic plants present many advantages compared to natural ones.

Some of the main benefits of transgenic plants include:

They are resistant to various biotic and abiotic stress - Biotic stresses include those that result from other organisms in nature (bacteria, viruses, fungi, etc) while abiotic stresses are the type of stresses that result from the environmental conditions in which given plants are grown. By introducing specific genes, researchers have managed to make many types of plants/crops resistant to these stressors. In the process, farmers are able to avoid the heavy costs associated with plant/crop damage resulting from biotic and abiotic stressors.

Increased yields - The other advantage of transgenic plants is that they give high yields compared to natural plants. In general, there are two main reasons as to why these plants give

higher yields. The first reason is that it has become possible for researchers to introduce genes that can transform plants resulting in higher yields compared to natural plants/crops.

As well, by introducing genes that allow the plant to withstand biotic and abiotic stressors, the plant is able to survive various environmental conditions that would otherwise cause significant damage to the plants.

High quality of yields - One of the biggest advantages of transgenic plants is that they produce higher quality yields. As mentioned, there are many stressors in nature that can affect plant growth and yields. In addition, various chemicals used for the purposes of controlling insects, pests, and weeds, etc have also been shown to affect sensitive plants.

Through gene transfer, researchers have been able to introduce new characteristics into different types of plants allowing them to withstand these stressors. As a result, these plants provide high-quality yields because they are not significantly affected by factors that affect other plants and their yields.

Applications of rDNA Technology in Medicine:

1. Insulin

Human insulin production by genetic engineering

- **Insulin** is a hormone produced by β-cells of islets of Langerhans of pancreas. It was discovered by sir Edward Sharpey Schafer (1916) while studying Islets of Langerhans.
- Pancreas is a mixed gland situated transversely across the upper abdomen behind stomach and spleen.
- Insulin is a peptide hormone produced by pancreas and is a central regulator of carbohydrates and fat metabolism in the body.

Structure of Human Insulin:

- Chemically Human insulin is small, simple protein composed of 51 amino acids sequences and has a molecular weight of 5808 Da.
- Insulin hormone is a dimer of a A- chain and a B-chain which are linked together by a disulphide bond.
- Fredrick Sanger et al (1954) gave the first complete description of insulin. Insulin consists of two polypeptide chain,
  - o Chain A- 21 amino acids long
  - o Chain B-30 amino acids long
  - o Both chains are joined together by disulphide bond between two cysteine residue

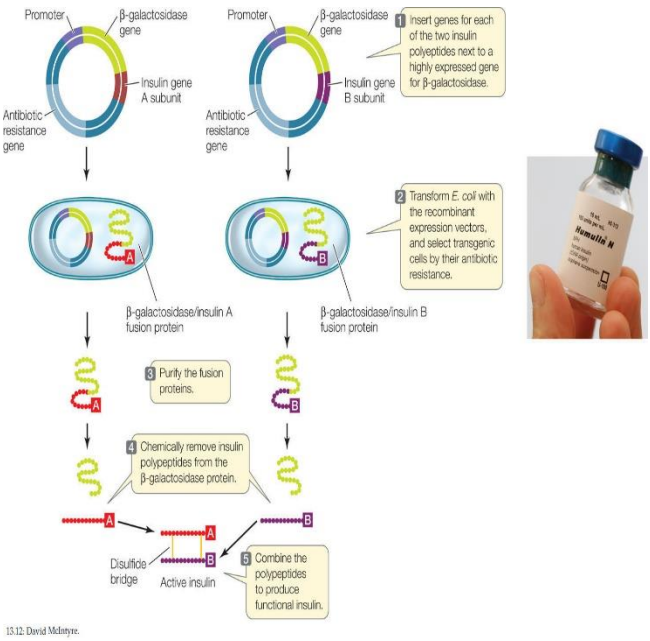
Insulin produced inside pancreas:

- At first Pancreatic β-cells synthesize pre-pro-insulin, which is a 109 amino acids long polypeptide
- Among 109 amino acids, 23 amino acids are signal molecules which allows the pre-pro-insulin to pass through cell membrane.
- Entering inside cell, it become 86 amino acids long pro-insulin. It is still inactive.

- Some Proteolytic enzymes cut and expose the active site of pro insulin converting it into active form of insulin of 51 amino acids long.

Insulin produced by recombinant DNA technology.

- The basic step in recombinant DNA technology is similar for insulin production also.
  - At first suitable vector (plasmid) is isolated from E. coli and then it is cut open by restriction endonuclease enzyme.
  - The gene of interest (ie. Insulin coding gene) is isolated from β-cell and inserted in opened plasmid.
  - Plasmid and gene of interest are recombined together by DNA ligase enzyme
  - This recombined plasmid is inserted into suitable host cell (ie E. coli) and now this recombined host cell starts producing insulin hormone.
- Hakura et al (1977) chemically synthesize DNA sequence of insulin for two chains A and B and separately inserted into two PBR322 plasmid vector.
  - These gene are inserted by the side of β-galactosidase gene of the plasmid.
  - The recombinant plasmid were then separately transformed into E. coli host.
  - The recombinant host produced pro-insulin chains ie. fused β-galactosidase-A chain and β-galactosidase-B-chain separately.
  - These pro-insulin chains A and B were separated from β-galactosidase by treatment with cyanogen bromide. The detachment of pro-insulin chains from β-galactosidase is possible because an extra codon form methionine was added at N-terminal of each gene for A and B-chain.
  - After detachment, A and B chains are joined invitro to reconstitute the naïve insulin by sulphonating the peptide chains with sodium disulphonate and sodium sulphite.



2. Human Growth Hormone:

Growth hormone is produced by the pituitary gland. It regulates the growth and development. Growth hormone stimulates overall body growth by increasing the cellular uptake of amino acids, and protein synthesis, and promoting the use of fat as body fuel.

Insufficient human growth hormone (hGH) in young children results in retarded growth, clinically referred to as pituitary dwarfism. The child usually is less than four feet in height, and has chubby face and abundant fat around the waist.

Traditional treatment for dwarfism:

The children of pituitary dwarfism were treated with regular injections of growth hormone extracted from the brains of deceased humans. It may be noted that only human growth hormone is effective for treatment of dwarfism. (This is in contrast to diabetes where animal insulin's are employed).

At least eight pituitary glands from cadavers must be extracted to get hGH adequate for treating a dwarf child just for one year! And such treatment has to be continued for 8-10 years!! Further, administration hGH isolated from human brains exposes the children to a great risk of transmitting the cadaver brain diseases (through virus or viral-like agents) e.g. Creuzfeldt- Jacob (CJ) syndrome characterized by convulsions, wasting of muscle etc.

Production of recombinant hGH:

Biotechnologists can now produce hGH by genetic engineering. The technique adopted is quite comparable with that of insulin production. The procedure essentially consists of inserting hGH gene into E. coli plasmid, culturing the cells and isolation of the hGH from the extracellular medium.

Limitation in hGH production:

The hGH is a protein comprised of 191 amino acids. During the course of its natural synthesis in the body, hGH is tagged with a single peptide (with 26 amino acids). The signal peptide is removed during secretion to release the active hGH for biological functions. The entire process of hGH synthesis goes on in an orderly fashion in the body.

However, signal peptide interrupts hGH production by recombinant technology. The complementary DNA (cDNA) synthesized from the mRNA encoding hGH is inserted into the plasmid. The plasmid containing E. coli when cultured, produces full length hGH along with signal peptide. But E. coli cannot remove the signal peptide.

Further, it is also quite difficult to get rid of signal peptide by various other means. Theoretically, cDNA encoding signal peptide can be cut to solve these problems. Unfortunately, there is no restriction endonuclease to do this job, hence this is not possible.

A novel approach for hGH production:

Biotechnologists have resolved the problem of signal peptide interruption by a novel approach. The base sequence in cDNA encoding signal peptide (26 amino acids) plus the neighbouring 24 amino acids (i.e a. total 50 amino acids) is cut by restriction endonuclease ECoRI.

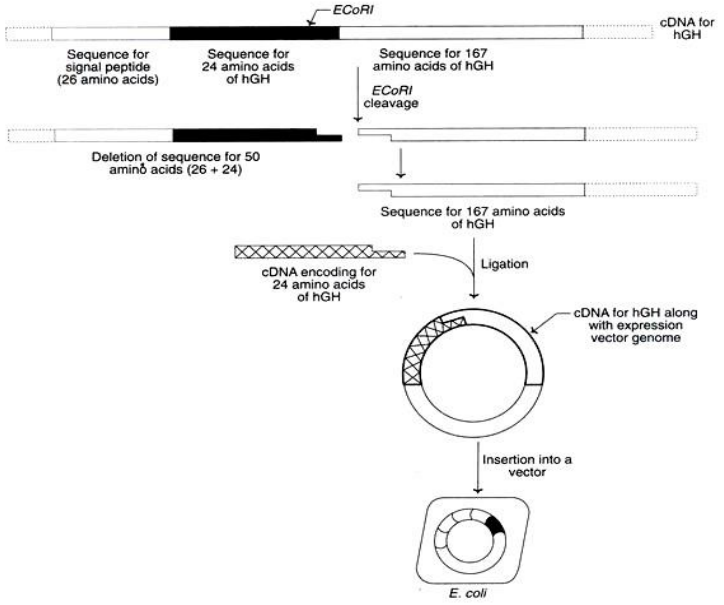


***Fig. 15.2** : The production of recombinant human growth hormone (cDNA–Complementary DNA, hGH–Human growth hormone)*

Now a gene (cDNA) for 24 amino acid sequence of hGH (that has been deleted) is freshly synthesized and ligated to the remaining hGH cDNA. The so constituted cDNA, attached to a vector, is inserted into a bacterium such as E. coli for culture and production of hGH. In this manner, the biologically functional hGH can be produced by DNA technology. Recombinant hGH was approved for human use in 1985. It is marketed as Protropin by Gene-tech Company and Humatrope by Eri Lilly Company.

3. Tissue Plasminogen Activator:

Tissue plasminogen activator (tPA) is a naturally occurring protease enzyme that helps to dissolve blood clots. tPA is a boon for patients suffering from thrombosis. The majority of natural deaths worldwide are due to a blockade of cerebral or coronary artery by a blood clot, technically called as thrombus. The phenomenon of thrombus blockage of blood vessels is referred to as thrombosis.

Chemically, thrombus consists of a network of fibrin, formed from the fibrinogen. In the normal circumstances, plasmin degrades fibrin and dissolves blood clots. This plasmin is actually produced by activation of plasminogen by tissue plasminogen activator.

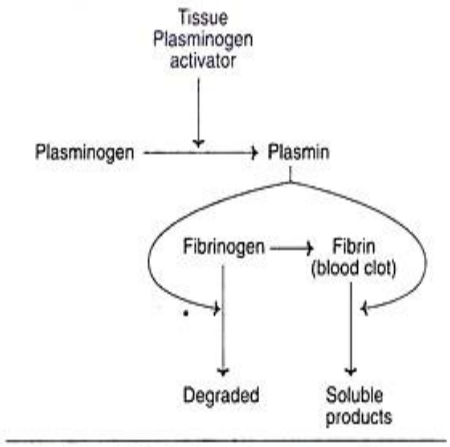


Fig. 15.3 : Role of tissue plasminogen activator in dissolving blood clots.

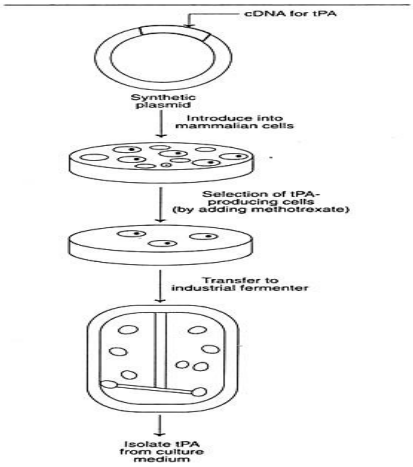


Fig. 15.4 : The production of recombinant tissue plasminogen activator (tPA).

The natural biological systems is however, not that efficient to remove the blood clots through this machinery. Tissue plasminogen activator is very useful as a therapeutic agent in dissolving blood clots (thrombi) by activating plasminogen. By removing the arterial, thrombi, the possible damage caused by them on heart and brain could be reduced.

Production of recombinant tPA:

DNA technologists synthesized the complementary DNA (cDNA) molecule for tissue plasminogen activator. This cDNA was then attached to a synthetic plasmid and introduced into mammalian cells (Fig. 15.4). They were cultured and tPA-producing cells were selected by using methotrexate to the medium.

tPA-producing cells were transferred to an industrial tank (fermenter). tPA, secreted into the culture medium, is isolated for therapeutic purpose. It may be noted here that tPA was the first pharmaceutical product to be produced by mammalian cell culture.

Recombinant tPA has been in use since 1987 for treatment of patients with acute myocardial infarction or stroke. Gene-tech was the first to market tPA with a trade name Activase.