Unit V
Genetics, Biotechnology and Microbiology

Module 2. Biotechnology

History and concept of biotechnology

Humans have used biotechnology since the dawn of civilization. Egyptians used yeasts to bake leavened bread, the Chinese developed fermentation techniques for brewing and cheese making, and the Aztecs used Spirulina algae to make cakes. Today, when most people think of biotechnology, they probably think of recombinant DNA. Although much of modern biotechnology does deal with manipulating DNA, classical biotechnology began long before we even knew about genes or chromosomes. What began as recipes for production of food now includes technology to enhance everything from farming to pharmaceuticals.

What we think of as modern biotechnology began around the end of the nineteenth century. By then, Mendel's work on genetics was completed and institutes for investigating fermentation along with other microbial processes had been founded by Koch, Pasteur, and Lister.

At the beginning of the twentieth century, industry and agriculture started to incorporate biotechnology. The growing automobile industry, for example, used fermentation processes to produce acetone and paint solvents. By mid-century, major advances in genetics dominated biotechnology research. The discovery that DNA carries the genetic code and the structure of the "double helix" were described by Watson and Crick. Soon, new techniques were developed to allow manipulation of DNA. Genetically engineered plants,

microbes, animals, and products like insulin, highlight the future of biotechnology.

The importance of the new genetics culminated in The Human Genome Project, an international project instituted by the Department of Energy and the National Institutes of Health to "map the human genome." The goal of this project is to identify the structure of the entire human genome, including its three billion base pairs and approximately 22,000 genes. The hope is that this knowledge will help scientists identify, prevent and treat many of the illnesses resulting from genetic malfunction.

Discussion of the ethical implications of recombinant experiments began several decades ago. James Watson was the elected as the first director of the Office of Human Genome Research at the NIH. With great insight, he suggested a portion of the budget of the human genome project be set aside to study the ethical, legal and social implications of the project. As genetic discoveries have progressed, the importance of regulating how the knowledge will be used has become of primary importance. Philosophers and ethicists now work side by side with research scientists and lawyers to help determine not only what we can do, but also what we should do with genetic knowledge.

Today, biotechnology is being used in countless areas including agriculture, clincial applications, bioremediation and forensics, where DNA sequencing is a common practice. Industry and medicine alike use the techniques of PCR, immunoassays and recombinant DNA. Genetic manipulation has been the primary reason that biology is now seen as the science of the future and biotechnology as one of the leading industries.

What Is The Definition of Biotechnology?

The basic concept of biotechnology involves using plants and living systems for the development of products. This process can use a variety of different techniques and is often associated with genetic engineering.

Basics of Biotechnology

The word 'biotechnology' is derived from the Greek words bios (life) and techne (art). Biotechnology refers to the application of living organisms or their products in developing new solutions for human needs. The term was coined by British scholar Samuel Butler who used it as a synonym for science applied to life-related enterprises.

Biotechnology basics have been around for a long time. The first known use of biotechnology was the cultivation of yeast by ancient Egyptians to create beer and wine about 12,000 years ago.

Recognized as one of the oldest branches of engineering, Biotech is defined as "the application of biological organisms or their products in developing new solutions to human needs, especially when those solutions are not found in nature."

Biotech is a 300 billion dollar industry with more than 300 biotech companies and 17 countries ranked among the world's top 20 biotechnology research centers. Biotechnological innovations have had an enormous impact on agriculture, healthcare, food production, and environmental protection. It is important to understand the basic concept of biotechnology in order to understand its principles and processes.

Principles of Biotechnology

According to the definition of Biotechnology, it is a field of applied biology and natural sciences. It uses the principles of genetics, molecular biology, biochemistry, and other life sciences to develop products or processes that improve our lives. It is essential to understand biotechnology principles and processes. Hence, below is a detailed explanation regarding principles of biotechnology:

1) Genetic Engineering

Genetic engineering is a technique that alters the DNA of an organism to create new genes and/or traits in living organisms. It is one of the core principles of biotechnology that you need to be well-versed in. This can be done by polymerase chain reaction (PCR) or using recombinant DNA methods. It may also refer to conventional breeding techniques as well as newer genetic manipulation technologies such as gene edits, RNA interference, and genome editing.

Uses Of Genetic Engineering

- With this technology, we can create high-yielding crop plants resistant to herbicides or insects, for instance.
- This technique is used in the production of foods such as genetically modified corn (maize).
- Genetic engineering has also been applied in medical research to produce new medicines, e.g., human insulin.
- Genetic engineering is also used to produce biofuels; for example, genetically modified yeast can be used to turn sugars into ethanol fuel.
- This technique could help in the future by providing food and fuels as well as medicines for a growing world population.

The Principles of Genetic Engineering

- The biotechnology process of gene splicing involves extracting the desired gene from one cell and inserting it into another.
- It is also called recombinant DNA technology.
- Genetic engineering can be used to insert desirable genes in order to improve the quality or quantity of produce, eliminate allergens, create disease-resistant plants, etc.

Process of Genetic Engineering:

- The process begins by isolating the gene of interest.
- It is inserted into a host organism such as bacteria, viruses, or animal cells.
- It can also be done with plant and animal genes in vitro (outside living organisms).
- A vector(s) is used to transfer this DNA molecule from one cell to another.
- The vector can be a simple molecule that tricks the cell into absorbing it or an intricate virus with parts to inject its own DNA inside cells.
- Once transferred, the gene is inserted in the place of existing genes and replicated as part of the normal cellular replication process. This results in daughter cells where new copies are made containing the added gene.
- These daughter cells can then be used to create a new organism with the desired gene or inserted in another cell from which other organisms are derived.

The methods of genetic engineering:

- Recombinant DNA technology, also known as recombination cloning; where genes and pieces of DNA are cut out of one organism's genome and inserted into the genome of another organism.
- Genome editing is a technique that can be used to modify and rewrite DNA sequences with precision by cutting out, adding, or altering sections of the gene sequence.
- This technology has led to dramatic advances in our understanding of how genes work together and could ultimately lead to treatments for certain diseases such as HIV.
- Transgenesis is the process of inserting a gene from one species into another, sometimes to make it resistant to certain diseases or environmental conditions.

2) Bioprocess Engineering

One of the most noted and integral principles of biotechnology is Bioprocess engineering. Bioprocess engineering is the process of designing and managing biological systems. This typically includes production, control, optimization, and qualification of bioproducts and processes through life sciences.

It involves fundamental understanding as well as application of physical science such as thermodynamics or chemical kinetics for modeling biochemical reactions.

Bioprocess Engineering is also responsible for process control and plant design, as well as developing monitoring systems, production methods, and quality assurance.

Principles of Bioprocess Engineering:

- This biotechnology Process design requires considering bioreactor parameters such as volume capacity, residence time, retention of materials, etc., to maximize production while minimizing costs.
- This is done by integrating the biochemical and mechanical design of a process, which both have to be optimized for maximum production.
- The Bioprocess Engineering principles also include optimization at multiple levels, such as cellular replication or protein expression, to achieve desired product qualities.
- Another key principle involves maximizing the use of available nutrients within bioreactors in order to minimize input costs.
- The Bioprocess Engineering principles also include understanding the biochemical reaction process, a systems engineering approach for optimizing bioreactor design, and integration between different aspects of the biological production system such as biomass growth or protein expression.

Process of Bioprocess Engineering:

- The Bioprocess Engineering process begins with understanding the needs of customers and what they want to produce.
- It then involves designing a manufacturing system that can deliver quality products for those specified requirements at minimum cost.
- This includes selecting optimum bioreactor types, best growth conditions, and engineering control systems for maintaining desired production levels.
- Finally, the process ends with designing and implementing a Quality Management system to ensure product quality is maintained.

Vectors

A vector is a DNA molecule that has the abil-ity to replicate autonomously in an appropri-ate host cell and into which the gene of inter-est (a foreign genetic sequence) is integrated. When we insert a foreign genetic sequence into the vector the aim is either to obtain numer-ous copies of the gene of interest or to obtain the product of that.

Accordingly the design and features of the vectors used for the assigned activity vary. Due to this there are two types of vectors - the expression vectors and clon-ing vectors.

Characteristic Features of an Ideal Vec-tor:

1. It should be able to replicate autono-mously. When the objective of cloning is to obtain a large number of copies of the DNA insert, the vector replication must be under relaxed control so that it can gener-ate multiple copies of itself in a single host cell.
2. It should be easy to isolate and purify.
3. It should be easily introduced into the host cells, i.e., transformation of the host with the vector should be easy.

4. The vector should have suitable marker genes that allow easy detection and/or se-lection of the transformed host cells.
5. When the objective is gene transfer, it should have the ability to integrate either itself or the DNA insert it carries into the genome of the host cell.
6. The cells transformed with the vector con-taining the DNA insert (recombinant DNA) should be identifiable and selectable from those transformed by the unaltered vector.
7. A vector should contain unique target sites for as many restriction enzymes as pos-sible into which the DNA insert can be in-tegrated.
8. When expression of the DNA insert is de-sired, the vector should contain at least suitable control elements, e.g., promoter, operator and ribosome binding sites.

Types of vector

Vectors are of two types:

- Cloning vector
- Expression vector

Cloning vectors

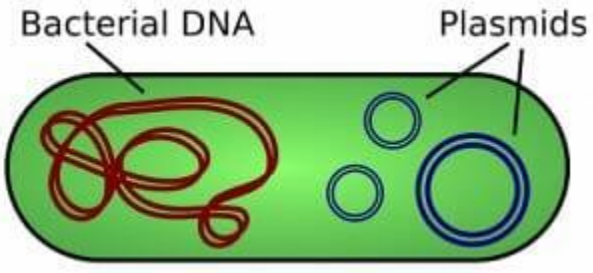
- Cloning vectors are small piece of DNA which have the ability and used to introduce foreign gene of interest into the host cell.
- They can be stably maintained insides the host cell.
- Cloning vector are generally used to obtain multiple copies of desired foreign gene.
- **Example-** Plasmid, Cosmid and Phages, BACs, YACs.
- These type of vectors generally contains selectable marker, origin of replication and a restriction site.

Expression vector

- Expression vector is a type of vector which not only introduces a gene of interest into the host cell but also aids in the analysis of the foreign gene via relevant protein product expression.
- It is type of vector which is used to obtain or analyses the gene product, which may be RNA or protein of the inserted desired gene.
- **Example-** Only plasmid vector.
- Expression vector contains enhancer, promoter region, start/stop codon, transcription initiation, selectable marker, ori sites, and restriction site.

Plasmids

- A plasmid is a small, circular piece of DNA that is different than the chromosomal DNA, which is all the genetic material found in an organism's chromosomes.
- It replicates independently of chromosomal DNA.
- Plasmids are mainly found in bacteria, but they can also be found in archaea and multicellular organisms.
- Plasmids usually carry at least one gene, and many of the genes that plasmids carry are beneficial to their host organisms.
- Although they have separate genes from their hosts, they are not considered to be independent life.



Functions of Plasmids

- Plasmids have many different functions.

- They may contain genes that enhance the survival of an organism, either by killing other organisms or by defending the host cell by producing toxins.
- Some plasmids facilitate the process of replication in bacteria.
- Since plasmids are so small, they usually only contain a few genes with a specific function (as opposed to a large amount of noncoding DNA).
- Multiple plasmids can coexist in the same cell, each with different functions.

General Types of Plasmids

- **Conjugative and Non-Conjugative**
  - There are many ways to classify plasmids from general to specific.
  - One way is by grouping them as either conjugative or non-conjugative.
  - Bacteria reproduce by sexual conjugation, which is the transfer of genetic material from one bacterial cell to another, either through direct contact or a bridge between the two cells.
  - Some plasmids contain genes called transfer genes that facilitate the beginning of conjugation.
  - Non-conjugative plasmids cannot start the conjugation process, and they can only be transferred through sexual conjugation with the help of conjugative plasmids.

Incompatibility

- Another plasmid classification is by incompatibility group.
- In a bacterium, different plasmids can only co-occur if they are compatible with each other.
- An incompatible plasmid will be expelled from the bacterial cell.

- Plasmids are incompatible if they have the same reproduction strategy in the cell; this allows the plasmids to inhabit a certain territory within it without other plasmids interfering.

Specific Types of Plasmids

There are five main types of plasmids:

- **Fertility F-plasmids**
- **Resistance plasmids**
- **Virulence plasmids**
- **Degradative plasmids**
- **Col plasmids**

- **Fertility F-plasmids**
  - Fertility plasmids, also known as F-plasmids, contain transfer genes that allow genes to be transferred from one bacteria to another through conjugation.
  - These make up the broad category of conjugative plasmids.
  - F-plasmids are episomes, which are plasmids that can be inserted into chromosomal DNA.
  - Bacteria that have the F-plasmid are known as F positive (F+), and bacteria without it are F negative (F–).
  - When an F+ bacterium conjugates with an F– bacterium, two F+ bacterium result.
  - There can only be one F-plasmid in each bacterium.

- **Resistance Plasmids**
  - Resistance or R plasmids contain genes that help a bacterial cell defend against environmental factors such as poisons or antibiotics.
  - Some resistance plasmids can transfer themselves through conjugation.

- When this happens, a strain of bacteria can become resistant to antibiotics.
- Recently, the type bacterium that causes the sexually transmitted infection gonorrhea has become so resistant to a class of antibiotics called quinolones that a new class of antibiotics, called **cephalosporins**, has started to be recommended by the World Health Organization instead.
- The bacteria may even become resistant to these antibiotics within five years.
- According to NPR, overuse of antibiotics to treat other infections, like urinary tract infections, may lead to the proliferation of drug-resistant strains.

- **Virulence Plasmids**
  - When a virulence plasmid is inside a bacterium, it turns that bacterium into a pathogen, which is an agent of disease.
  - Bacteria that cause disease can be easily spread and replicated among affected individuals.
  - The bacterium Escherichia coli (E. coli) has several virulence plasmids. E. coli is found naturally in the human gut and in other animals, but certain strains of E. coli can cause severe diarrhea and vomiting.
  - Salmonella enterica is another bacterium that contains virulence plasmids.

- **Degradative Plasmids**
  - Degradative plasmids help the host bacterium to digest compounds that are not commonly found in nature, such as camphor, xylene, toluene, and salicylic acid.
  - These plasmids contain genes for special enzymes that break down specific compounds.

    - Degradative plasmids are conjugative.

- **Col Plasmids**
    - Col plasmids contain genes that make bacteriocins (also known as colicins), which are proteins that kill other bacteria and thus defend the host bacterium.
    - Bacteriocins are found in many types of bacteria including E. coli, which gets them from the plasmid ColE1.

Applications of Plasmids

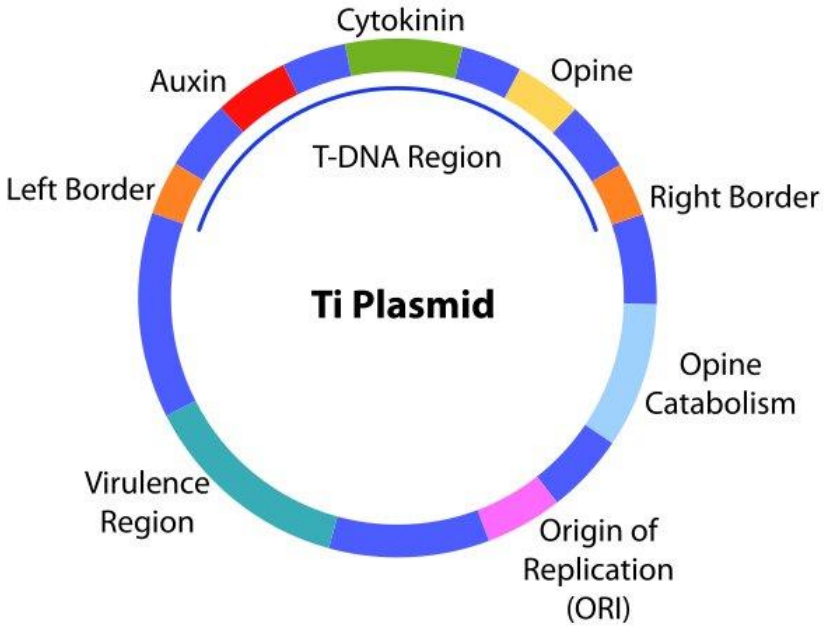
- Humans have developed many uses for plasmids and have created software to record the DNA sequences of plasmids for use in many different techniques.
- Plasmids are used in genetic engineering to amplify, or produce many copies of, certain genes.
- In molecular cloning, a plasmid is a type of vector.
- A vector is a DNA sequence that can transport foreign genetic material from one cell to another cell, where the genes can be further expressed and replicated.
- Plasmids are useful in cloning short segments of DNA. Also, plasmids can be used to replicate proteins, such as the protein that codes for insulin, in large amounts.
- Additionally, plasmids are being investigated as a way to transfer genes into human cells as part of gene therapy.
- Cells may lack a specific protein if the patient has a hereditary disorder involving a gene mutation.
- Inserting a plasmid into DNA would allow cells to express a protein that they are lacking.

Ti plasmid

- The **Ti-plasmid** in the bacteria is known to induce crown gall disease in plants by transferring crucial regions from the plasmid.
- These crucial regions were seen to modify the plant cells into a tumour to produce synthetic plant hormones and cause crown gall.
- This led the scientists to believe that there is a scope for bioengineering techniques to modify the plants using Ti-plasmid for our own use.

Features of Ti Plasmid

- **Virulence Region:** The virulence region codes for virulence genes that are responsible for the transfer of T-DNA to the plant cells and also recruiting various effector proteins for infecting the plant cells.



- **T-DNA:** The T-DNA region is the crucial region that gets transferred to the plant cell for infection. It is approximately 15-20 kbp in length and is transferred to the plant cell via means of genetic recombination.
- **Opine Catabolism:** The opine catabolism region is the region from where the bacteria sources its nutrients for the whole process. Opines are derivatives of amino acid or sugar phosphates that can be catabolized

to use in the form of nutrients. The types of opines found in Ti-plasmid are nopaline and octopine types.

- **Origin of Replication:** The origin of replication is the region where replication of the plasmid is initiated.

Uses in Bioengineering

- The ability of the Ti-plasmid to modify the plant cells has been taken advantage of for the production of transgenic plants.
- The plasmids have been modified into a cloning vector now which are no more pathogenic to plants.
- They are being used to transfer genes of our interest into the plant and produce plants with better quality and quantity.
- It is therefore known as 'nature's genetic engineer'.

Bacteriophage

- A bacteriophage is a virus that infects a bacterial cell and reproduces inside it.
- They vary a lot in their shape and genetic material.
- A bacteriophage may contain DNA or RNA.
- The genes range from four to several thousand.
- Their capsid can be isohedral, filamentous, or head-tail in shape.

Bacteriophage Structure

The bacteriophage consists of a polyhedral head, a short collar and a helical tail.

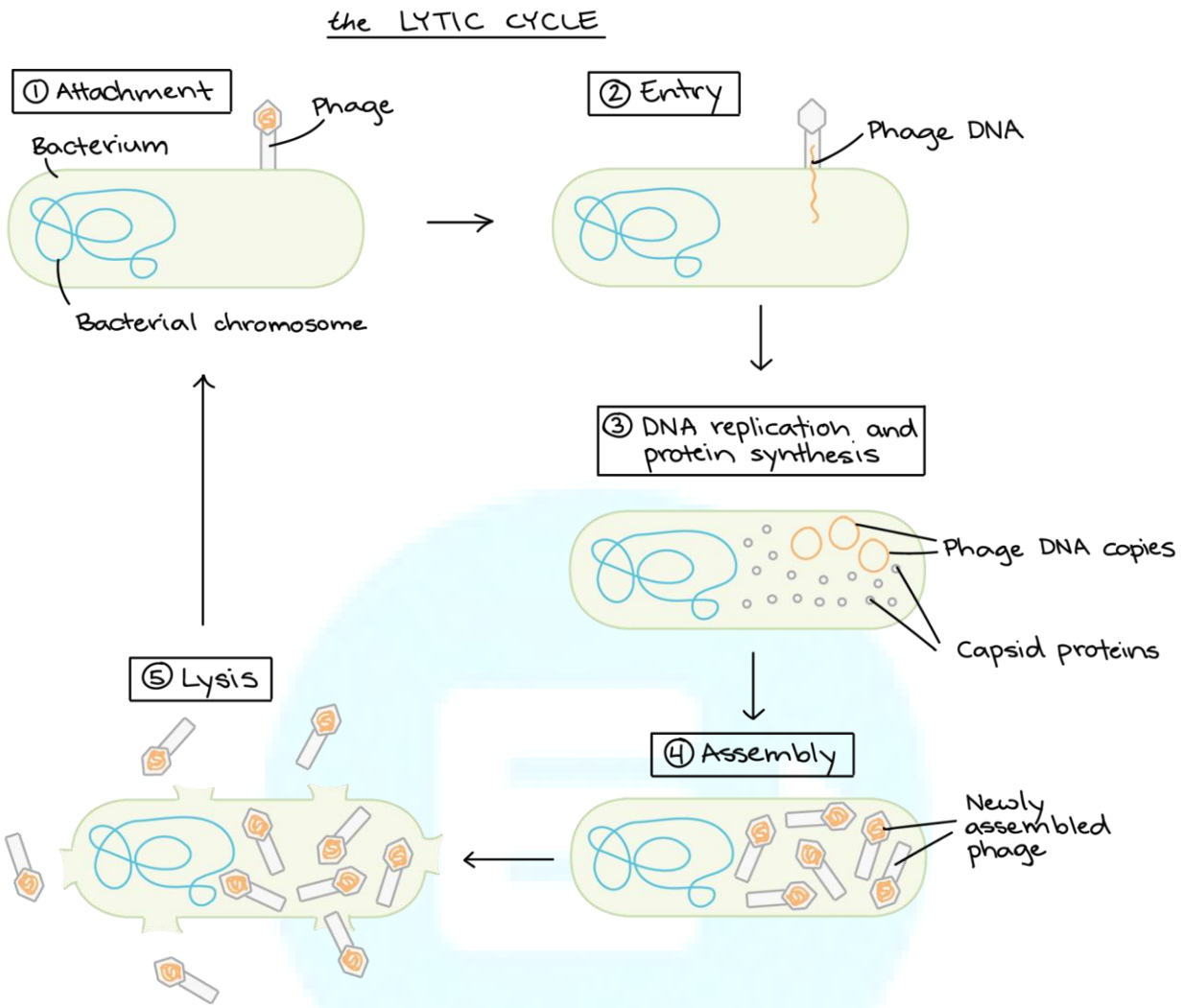
- **Head-** The head consists of 2000 capsomeres with double-stranded DNA enclosed within.
- **Tail-** The tail consists of an inner hollow tube which is surrounded by a contractile sheath with 24 annular rings. The distal end consists of a basal plate with tail fibres at each corner. The bacteriophage attaches to the bacteria with the help of these tail fibres.

Bacteriophage Life Cycle

Bacteriophage exhibits two major types of life cycles:

- **Lytic Cycle or Virulent Cycle**
- **Lysogenic Cycle or Temperate Cycle**

- **Lytic cycle**
  - In the lytic cycle, a phage acts like a typical virus: it hijacks its host cell and uses the cell's resources to make lots of new phages, causing the cell to lyse (burst) and die in the process.

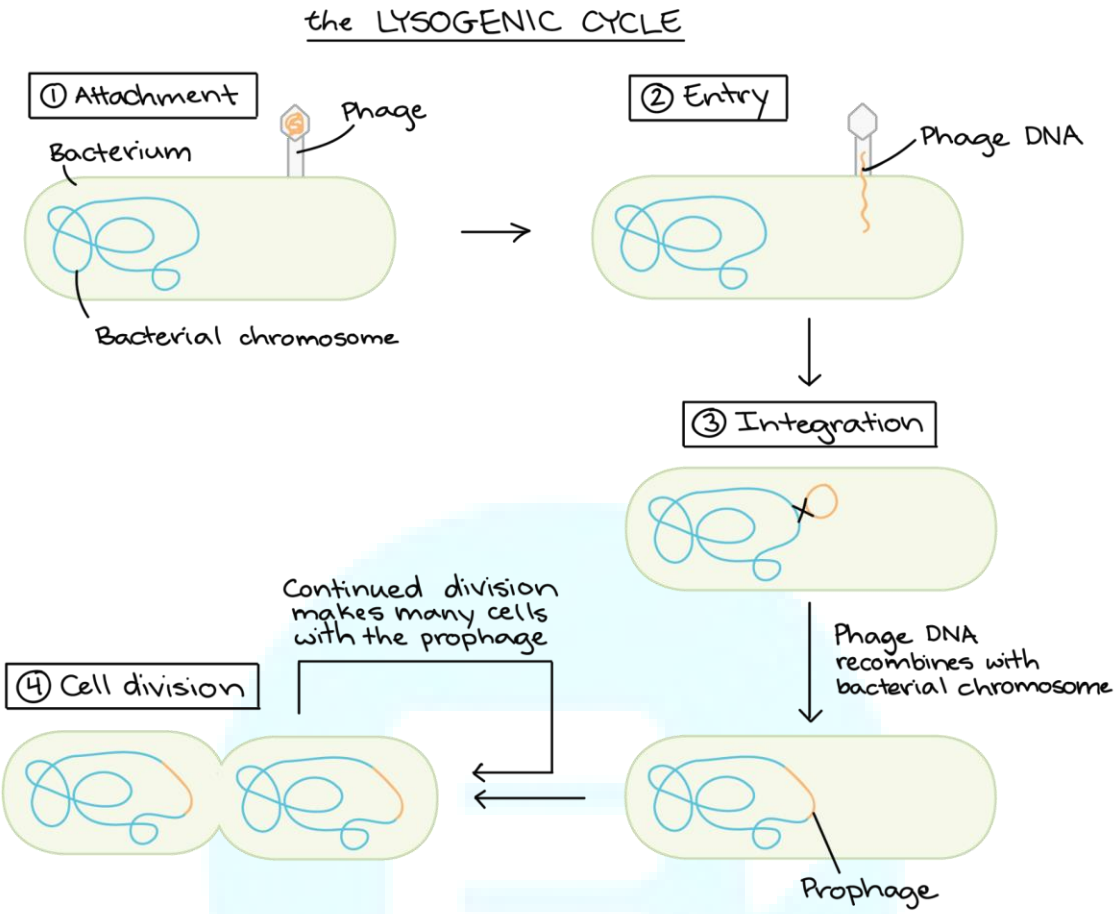


- <u>The stages of the lytic cycle are:</u>
  - **Attachment:** Proteins in the "tail" of the phage bind to a specific receptor (in this case, a sugar transporter) on the surface of the bacterial cell.
  - **Entry:** The phage injects its double-stranded DNA genome into the cytoplasm of the bacterium.
  - **DNA copying and protein synthesis:** Phage DNA is copied, and phage genes are expressed to make proteins, such as capsid proteins.

    - **Assembly of new phage:** Capsids assemble from the capsid proteins and are stuffed with DNA to make lots of new phage particles.
    - **Lysis:** Late in the lytic cycle, the phage expresses genes for proteins that poke holes in the plasma membrane and cell wall. The holes let water flow in, making the cell expand and burst like an overfilled water balloon.

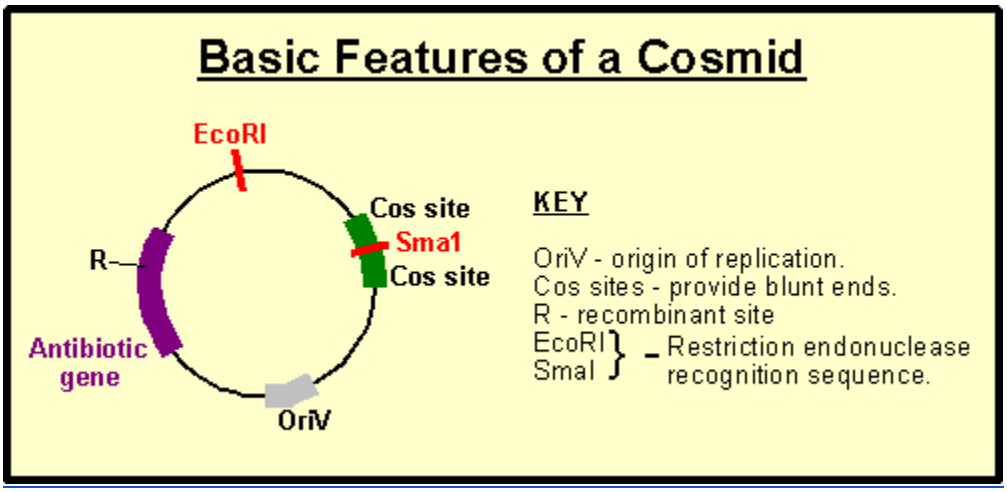
Cell bursting, or lysis, releases hundreds of new phages, which can find and infect other host cells nearby. In this way, a few cycles of lytic infection can let the phage spread like wildfire through a bacterial population.

- **Lysogenic cycle**
    - The lysogenic cycle allows a phage to reproduce without killing its host.
    - Some phages can only use the lytic cycle, but the phage we are following, lambda (\lambdaλlambda), can switch between the two cycles.
    - In the lysogenic cycle, the first two steps (attachment and DNA injection) occur just as they do for the lytic cycle.
    - However, once the phage DNA is inside the cell, it is not immediately copied or expressed to make proteins.
    - Instead, it recombines with a particular region of the bacterial chromosome.
    - This causes the phage DNA to be integrated into the chromosome.



- The integrated phage DNA, called a **prophage**, is not active: its genes aren't expressed, and it doesn't drive production of new phages.
- However, each time a host cell divides, the prophage is copied along with the host DNA, getting a free ride.
- The lysogenic cycle is less flashy (and less gory) than the lytic cycle, but at the end of the day, it's just another way for the phage to reproduce.
- Under the right conditions, the prophage can become active and come back out of the bacterial chromosome, triggering the remaining steps of the lytic cycle (DNA copying and protein synthesis, phage assembly, and lysis).

Cosmids



- A cosmid, first described by Collins and Hohn in 1978, is a type of hybrid plasmid with a bacterial "ori" sequence and a "cos" sequences derived from the lambda phage.
- It is formed by joining ends of a linearized plasmid DNA with cos-site of lambda DNA.
- It is a derived vector.
- The cosmid DNA can be packed in a capsid of lambda phage in vitro to form recombinant phage particles.
- It is linear inside the phage capsid.
- The cosmid gets circularized and behaves like a plasmid.
- Cosmid has an origin of replication, selectable markers, and gene cloning sites of plasmid DNA.
- They lack structural and regulatory genes of lambda DNA.
- Hence there is no lysis and integration of cosmid DNA in the host cell.
- Examples: **Col EI cosmid, pHC 79, pJB8, pWE cosmid, etc.**

Salient features of Cosmids

- Cosmid is a circular ds DNA
- It has two complementary single-stranded regions at both ends of a plasmid DNA. The two cos-ends form a duplex by base pairing.
- The cosmid DNA does not code for phage proteins and host cell lysis.
- It does not involve in ,multiplication of phage particles.

- It has an origin of replication from plasmid DNA for independent replication.
- It has selectable marker genes and gene cloning sites of plasmid DNA
- The cosmid DNA is packed within protein coat of bacteriophage to form inactive phage particles. Cos-site is a prerequsites for invitro packaging of cosmid in phage protein coat.
- After infection, the cosmid DNA does not integrate into host chromosomal DNA. It exits as a definite extra chromosomal DNA and replicates independently.

Cosmid pLFR5

- pLFR5 is the commonly used cloning vector suitable for cloning large DNA fragments upto 45 kbp.
- It is 6 kbp in size.
- It is constructed from E.coli plasmid pBR322 and two cos-ends of lambda DNA.
- The plasmid derived portion contributes an origin of replication (Ori) and tetracycline resistance gene.
- There is a MCS between the orogin of replication and cos-site.
- A foreign DNA of upto 45 kbp is inserted into the MCS of pLR5 and the rDNA is packed into bacteriophage head in vitro.
- The phage thus formed delivers the DNA into E.coli while infecting the cell.

Cosmid pJB8

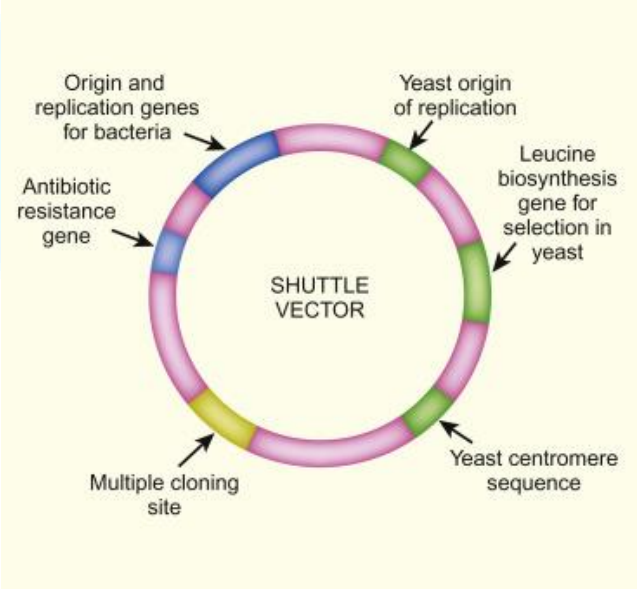
- pJB8 is constructed from the plasmid pBR322 and cos sites of lambda DNA.
- It is 5.4 kbp in size.
- It has an origin of replication and ampicillin resistance gene derived from pBR322 and two cos-ends from lambda DNA.
- A foreign DNA of about 45 kbp is inserted into BamHI or RcoRI or HindIII restriction site of the cosmid.

- The recombinant cosmid is packaged into lambda phage head to form an infective phage particle.
- The phage delivers its rDNA into E.coli while infecting the cell.

Cosmid pHC79

- pHC79 is constructed from pBR322 and cos-sites of lambda DNA.
- It is 6.5 kbp in size.
- It can carry DNA fragments upto 40 kbp.
- The derivative of pBR322 has an origin of replication and two marker genes- ampicillin resistance gene and tetracycline resistance gene.
- The derivative of the cosmid into lambda phage head.

Shutter Vector



- The plasmid cloning vector that can exist and replicate in two different organisms is called shuttle vectors.
- It has two origins of replication, each of which is specific to a host.
- It is also called as **Bi-functional vectors.**
- It can also exist in 2 different host.
- **Ex: pHV14,pEB10,pHP3,** etc. replicate both in bacillus subtilis & E.coli
- pJDB219 is another, can replicate E.coli and yeast.

Yeast Vectors

- Yeast are eukaryotes and thus contain complex internal cell structures similar to those of plants and animals.
- Unlike bacteria, yeast can post-translationally modify proteins yet they still share many of the same technical advantages that come with working with prokaryotes.
- This includes but is not limited to: rapid growth, ease of replica plating and mutant isolation, a well-defined genetic system, and a highly versatile DNA transformation system.Unlike most other microorganisms, yeast have both a stable haploid and diploid state which is useful for genetic analysis, as well as an efficient mechanism of homologous recombination to facilitate simple gene replacement/mutation.
- Yeast expression plasmids used in the lab typically contain all the necessary components to allow shuttling between E. coli and yeast cells.
- To be useful in the lab, the vectors must contain a yeast-specific origin of replication (ORI) and a means of selection in yeast cells, in addition to the bacterial ORI and antibiotic selection markers.

The four main types of yeast plasmids are defined below:

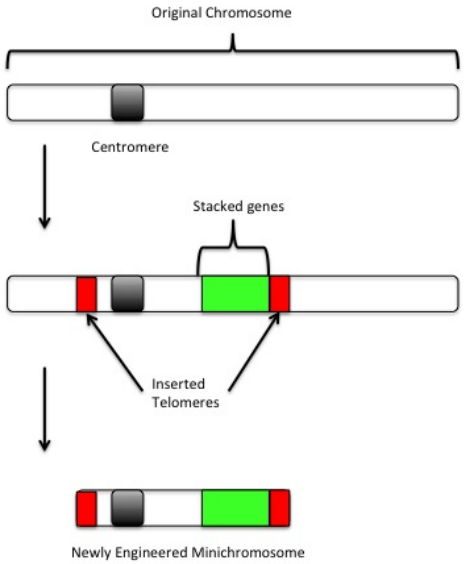
- **Yeast Integrating plasmids (YIp):** These plasmids lack an ORI and must be integrated directly into the host chromosome via homologous recombination.
- **Yeast Replicating plasmids (YRp):** These vectors contain an Autonomously Replicating Sequence (ARS) derived from the yeast chromosome. As the name suggests, these vectors can replicate independently of the yeast chromosome; however, they tend to be unstable and may be lost during budding.
- **Yeast Centromere plasmids (YCp):** These are considered low copy vectors and incorporate part of an ARS along with part of a centromere sequence (CEN). These vectors replicate as though they are small

independent chromosomes and are thus typically found as a single copy. Unlike the ARS vectors, CEN vectors are stable without integration.

- **Yeast Episomal plasmids (YEp):** These are most similar to bacterial plasmids and are considered “high copy”. A fragment from the 2 micron circle (a natural yeast plasmid) allows for 50+ copies to stably propogate per cell. The copy number of these vectors can also be controlled if specific regulatable elements are included

Minichromosomes

A minichromosome is a small chromatin-like structure resembling a chromosome and consisting of centromeres, telomeres and replication origins but little additional genetic material. They replicate autonomously in the cell during cellular division. Minichromosomes may be created by natural processes as chromosomal aberrations or by genetic engineering.



Structure

Minichromosomes can be either linear or circular pieces of DNA. By minimizing the amount of unnecessary genetic information on the

chromosome and including the basic components necessary for DNA replication (centromere, telomeres, and replication sequences), molecular biologists aim to construct a chromosomal platform which can be utilized to insert or present new genes into a host cell.

Artificial chromosomes

- **Artificial chromosome is a type of cloning vector** that has some features of true chromosomes and is used to clone relatively large fragments of DNA.
- **Bacterial artificial chromosomes (BACs)** are based on the F (fertility) plasmid found naturally in E. coli bacteria (see sex factor).
- They can accommodate inserts of foreign DNA up to about 300 kilobase (kb) in length.
- Also included are several bacterial genes necessary for replication of the plasmid by the host cell and a gene (usually for resistance to an antibiotic) that allows selection of BAC-containing cells.
- Larger DNA fragments are cloned using yeast artificial chromosomes (YACs).
- These are linear vectors derived from a circular plasmid found naturally in baker's yeast (Saccharomyces cerevisiae) and capable of accommodating DNA inserts of up to 1000 kb.
- YACs have a centromere, enabling them to attach to the mitotic spindle of their yeast host and undergo normal segregation during cell division.
- They are also engineered with telomeres, the DNA sequences that cap either end of a chromosome.
- Thus YACs behave like **mini-chromosomes.**
- They are used for cloning eukaryotic genes or gene segments, for making DNA libraries of organisms with large genomes (e.g. mammals), and for studying gene function.

Probes

The following points highlight the three types of nucleic acid probes. The probes are:

1. **Oligonucleotide Probes**
2. **DNA Probes**
3. **RNA Probes**

1. **Oligonucleotide Probes:**
    - These are synthesized chemically as oligonucleotides based on the information available on the amino acid sequence of the protein of interest.
    - These oligo nucleotides can be used as a probe the identification of gene which encode for that particular protein.
    - However, due to degeneracy of the genetic code, construction of oligonucleotide is car-ried out with those that are rich in methionine or tryptophan residue or with only two codons.
    - Generally oligonucleotide probe is used to screen cDNA libraries.

2. **DNA Probes:**
    - These are longer than the oligonucleotides.
    - Thus, clones of longer DNA sequence is used as a DNA probes.
    - The sequence obtained from cDNA library (cDNA clones) can be used to probe genomic library to identify genomic clones.
    - The same probe can also be used to reprobe the same cDNA library to identify more cDNA clones.
    - The genomic DNA clones are used to screen cDNA library or a genomic library.

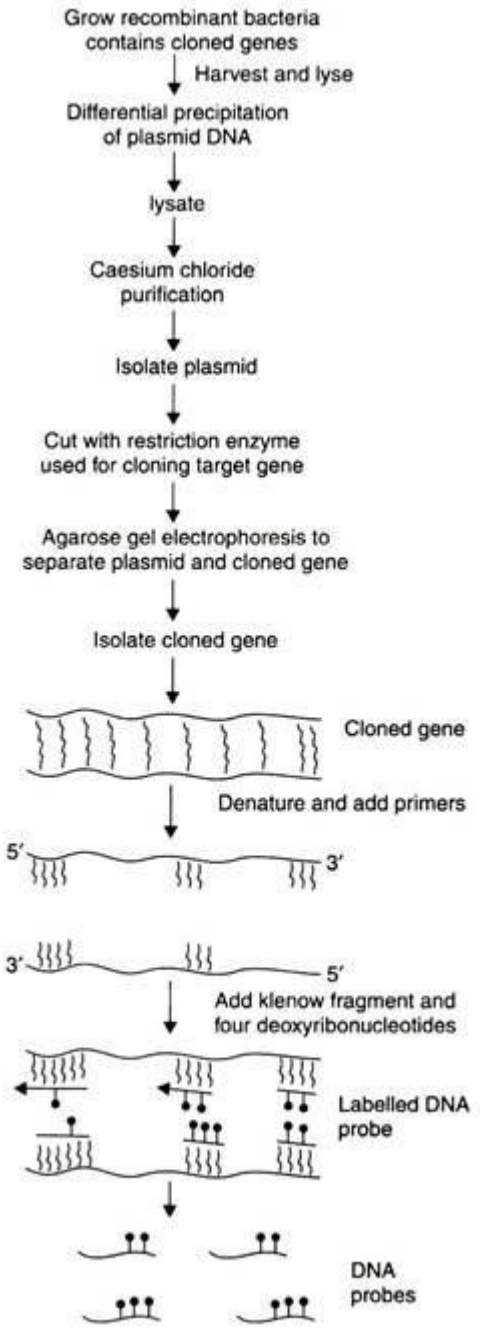


Fig. 13.8 Preparation of labelled probes

Preparation of DNA Probe:

The DNA probe is prepared by random primer method as follows:

- In double stranded DNA containing the sequence that is to act as the probe is dena-tured and an oligonucleotide sample containing all possible sequences of six nucleotides

is added (it is statistical certainty that some of the molecules of the oligonucleotide mixture will hybridize to the unlabelled, denatured probe DNA).

- In the presence of klenow fragment and four deoxyribunucleotides, one of the four deoxyribonucleotides is labelled.
- The bound oligonucleotides act as primers for DNA synthesis.
- The synthesised DNA is labelled and can be used as a probe to detect the presence of a complementary DNA sequence in a source DNA sample.

- There are at least **two possible sources of probes**: one is from cloned DNA, second is the nucleotide sequence of a synthetic probe, based on the probable nucleotide sequence that is deduced from the known aminoacid sequence of the protein encoded by the target gene.

3. **RNA Probes:**
   - RNA probes used only under certain circumstances.
   - Purification of particular RNA is generally used as a specific probe for the corresponding DNA specifies.
   - By employing positive- negative screening or differential hybridized approach, often possible to identify clones for RNA in one population but not so in other population.

   - The colony is first probed with labelled RNA from one population.
   - After the location of hybridized colonies, labelled RNA is then washed off from the membrane and the membrane is then probed with labelled RNA from the second population and hybridized colonies are identified.

- The first RNA probes were mRNAs of a gene that are abundantly expressed in a cell, labelled with 32po4.
- One of the efficient ways of preparing RNA probes involves transcription from a target gene cloned in a plasmid.
- Transcription of this gene is initiated from a promoter that is specifically recognised by an RNA polymerase.
- Due to unspecificity of RNA pol recogni-tion, specific promoter can be selected.
- The promoter of a bacteriophage is very specific for the phage polymerase.
- Some researchers have utilized phage (SP6) T7 promoter and the corre-sponding enzyme to transcribe a DNA (target gene) cloned in a plasmid vector.

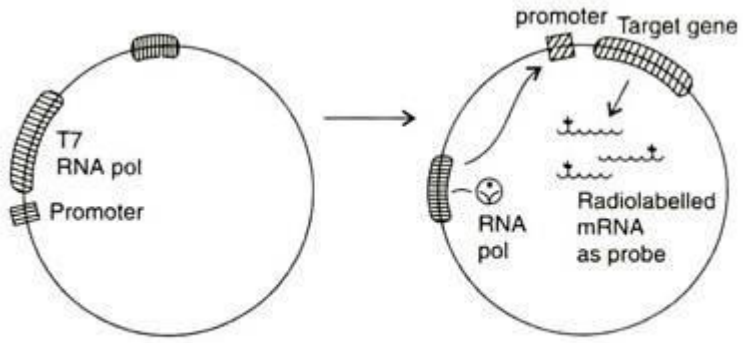


Fig 13.9 Preparation of RNA probe

- The gene for the phage polymerase and the phage promoter were both cloned in a vector, and a target gene sequence for transcription inserted downstream of the phage promoter.
- Cells transformed with recombinant DNA are supplied with RNA precursor nucleotides, of which one nucleotide is radio labelled.
- Transcription of the inserted gene is therefore labelled and may be used as RNA probes.

ENTRI

Molecular marker

- Molecular marker is identified as genetic marker.
- Molecular marker is a DNA or gene sequence within a recognized location on a chromosome which is used as identification tool.
- In the pool of unknown DNA or in a whole chromosome, these molecular markers helps in identification of particular sequence of DNA at particular location.

- **Applications:**
    - It plays a crucial role in gene mapping by identifying the position of linked genes in the chromosome which inherited together.
    - It also detect any alteration in a sequence of DNA or any genetic oddity. It ascertains genes involved in genetic disorders.
    - It is used to determine different characters in a DNA sequence which is used to distinguish between individuals, populations or species.
- Different types of genetic polymorphism can be used as Genetic markers. On the basis of polymorphisms detected in the genetic makeup of individuals that may vary in the length of a DNA sequence or in the identity of nucleotides located at specific position in chromosome, some of the common genetic markers are- RFLP, SSLP, etc
- Genetic markers can be classified as PCR based and hybridization based.
    - PCR based genetic markers: RAPD, ISSR, EST-SSR, microsatellite, CAPS etc.
    - Hybridization based genetic markers: RFLP, VNTRs, in which targeted gene is digested with restriction enzymes and then hybridized with RFLP probe.

Quality for a good genetic marker:

- Genetic markers should be largely polymorphic in nature

- They should be selectively neutral
- Assay for detecting markers should be simple and rapid
- Genetic markers should occur frequently within genome
- The genetic marker (gene) should show codominant inheritance pattern.
- They should be highly reproducible
- They should not interact with other markers while using multiple markers at a same time

Properties and type of Isozymes

Isozymes (also known as isoenzymes) are enzymes that differ in amino acid sequence but catalyze the same chemical reaction. These enzymes usually display different kinetic parameters (i.e. different KM values), or different regulatory properties. The existence of isozymes permits the fine-tuning of metabolism to meet the particular needs of a given tissue or developmental stage (for example lactate dehydrogenase (LDH)). In biochemistry, isozymes (or isoenzymes) are isoforms (closely related variants) of enzymes. In many cases, they are coded for by homologous genes that have diverged over time. Although, strictly speaking, allozymes represent different alleles of the same gene, and isozymes represent different genes whose products catalyse the same reaction, the two words are usually used interchangeably.

Isozymes were first described by hunter and Markert (1957) who defined them as different variants of the same enzyme having identical functions and present in the same individual. This definition encompasses (1) enzyme variants that are the product of different genes and thus represent different loci (described as isozymes) and (2) enzymes that are the product of different alleles of the same gene (described as allozymes).

Isozymes are usually the result of gene duplication, but can also arise from polyploidisation or hybridization. Over evolutionary time, if the function of the new variant remains identical to the original, then it is likely that one or the other will be lost as mutations accumulate, resulting in a pseudogene.

However, if the mutations do not immediately prevent the enzyme from functioning, but instead modify either its function, or its pattern of gene Expression, then the two variants may both be favoured by natural selection and become specialised to different functions. For example, they may be expressed at different stages of development or in different tissues.

Allozymes may result from point mutations or from insertion-deletion (indel) events that affect the dna coding sequence of the gene. As with any other new mutation, there are three things that may happen to a new allozyme:

It is most likely that the new allele will be non-functional — in which case it will probably result in low fitness and be removed from the population by natural selection.

Alternatively, if the amino acid residue that is changed is in a relatively unimportant part of the enzyme, for example a long way from the active site then the mutation may be selectively neutral and subject to genetic drift.

In rare cases the mutation may result in an enzyme that is more efficient, or one that can catalyse a slightly different chemical reaction, in which case the mutation may cause an increase in fitness, and be favoured by natural selection.

An example of an isozyme:

An example of an isozyme is **glucokinase**, a variant of hexokinase which is not inhibited by glucose 6-phosphate. Its different regulatory features and lower affinity for glucose (compared to other hexokinases), allows it to serve different functions in cells of specific organs, such as control of insulin release by the beta cells of the pancreas, or initiation of glycogen synthesis by liver cells. Both of these processes must only occur when glucose is abundant, or problems occur.

Characteristics of Isozymes:

- They catalyze the same reaction but they can be distinguished by physical meth-ods such as electrophoresis or by immu-nological methods.
- The difference between some isozymes are due to differences in the quarternary structure of the enzymes, e.g., **lactate dehydro-genase** exists in five isozymic forms.
- The isozymic forms of lactate dehydroge-nase are tetramers, each is made up from two types of units H and M. The molecu-lar weight of active lactate dehydrogenase is 1,30,000. Only the tetrameric molecule possesses catalytic activity.

- The **subunits** are expressed in the following **5 ways:**

H H H H
H H H M
H H M M
H M M M
M M M M

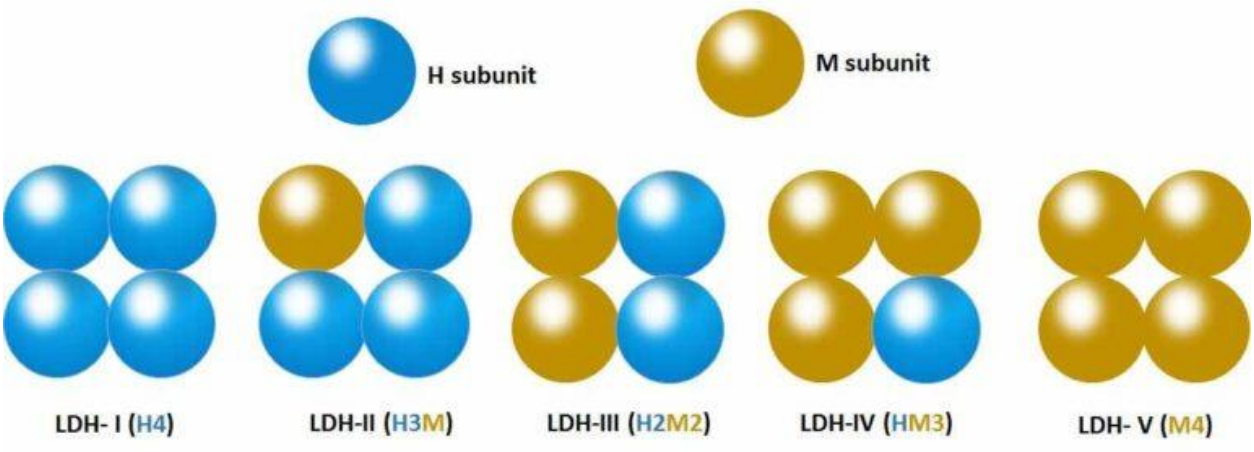
- Splitting and reconstitution of lactate de-hydrogenase –I1 or lactate dehydrogenase- 15 produces on new isozymes. Therefore, each consists of a single subunit.

But when a mixture of purified lactate de-hydrogenase – I1 and lactate dehydroge-nase – I5 is subjected to splitting and re- constitution, lactate dehydrogenase – I2, – I3 and – I4 are also produced.

<u>Examples of Isozymes</u>

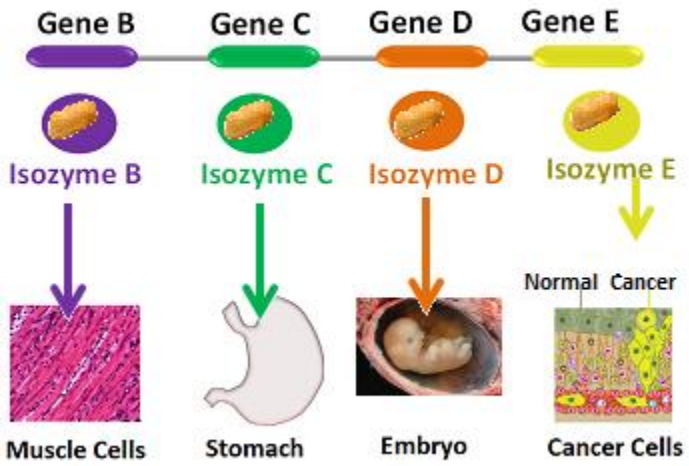
- The well-studied isozymes are those of lactate dehydrogenase (LDH), the enzymes that are responsible for the reduction of pyruvate to lactate.
- LDH is a tetrameric enzyme that can be found in homotetrameric or heterotetrameric forms.
- There are five possible isozymes of LDH that have been studied.

a. **LDH- I (H4)** is a homotetramer of 'H' subunits; present in heart cells.
b. **LDH-II (H3M), LDH-III (H2M2), and LDH-IV (HM3)** are tetramers distributed in different tissues.
c. **LDH- V (M4)** is a homotetramer of 'M' subunits present in skeletal muscle cells.



The heart form of LDH has very high Km for substrate and very low affinity and Vmax. These properties are very common for heart form since pyruvate accumulation rarely occurs due to the aerobic nature of heart muscle cells.

The skeletal muscle form has very low Km, high Vmax, and a very high affinity for the substrate. Skeletal muscle is well studied for rapid conversion of pyruvate to lactate. This conversion is very crucial as the skeletal muscle cells are bound to anaerobic respiration. More activity of LDH in skeletal muscle cells leads to an accumulation of lactate, resulting in muscle fatigue.



Isozymes of LDH are also used in clinical biochemistry as diagnostic markers. LDH-I is a marker for myocardial infection, while LDH-V is a marker enzyme for muscular dystrophy.

Types of genetic markers:

1. Random Amplified Polymorphic DNA (RAPD):

- RAPD was developed by Welsh and McClelland along with Williams in 1990.
- It is pronounced as 'rapid'.
- It is based on PCR assay and it doesn't need require any prior sequencing of DNA.
- This procedure uses short arbitrary primer of 8-12 bp that randomly amplifies the region of DNA.
- This primer serves as both forward and reverse primer.
- This reaction proceeds when a single primer anneals to the genomic DNA at two distinct sites on the complementary strand of DNA template.
- The amplification of segment of DNA depends on the positions complimentary to the primers' sequence.

- The fragments obtained from RAPD are between 0.2 to 5.0kb and can be viewed by using agarose gel electrophoresis stained by ethidium bromide or with the help of polyacrylamide gel electrophoresis.
- If any mutation occurs in the primer binding region then no any PCR product will be produced, yielding a distinct pattern of amplified DNA segments on the gel.
- **Application:**
    - Distinct pattern of amplification is seen in different samples. This is why RAPD can be used for studying polymorphism.
    - RAPD is applicable for the mapping of genome, analyzing linkage, and individual specific genotyping.
    - RAPD markers are dominant in nature so it has restrictions for mapping purpose.
    - RAPD is strictly laboratory dependent so it requires sensitivity.
- **Demerits:**
    - It has demerits as poor reproducibility, yields faint products, problems occur in band scoring.

2. Restriction Fragment Length Polymorphism (RFLP):

- It was one of the first methods used for the analysis of DNA in various fields such as forensic science.
- It is a hybridization based technique.
- It was invented by Alec Jeffreys, an English scientist in 1984 during his research in genetic diseases.
- RFLP uses particular restriction endonuclease enzymes that cuts at its specific site yielding fragments of various lengths along with the fragment of interest.
- The length of the distinct fragments is determined by using blotting, now replaced with sequencing.
- RFLP markers are largely locus-specific and are co-dominant in nature due to the nature of restriction endonuclease used.

- **Steps for RFLP are as follows:**
    - DNA extraction is done from saliva, blood or other samples and is purified.
    - Restriction endonucleases digests the purified DNA resulting restriction fragments.
    - Now the restriction fragments are examined using gel electrophoresis.
    - The gel is now treated with luminescent dyes for the visibility of DNA bands.
- **Applications:**
    - RFLP was one of the first techniques applied for genetic fingerprinting/profiling.
    - It is used for identification of inherited diseases, carrier of that diseases, genetic mapping, and heterozygous detection.
    - The molecular basis of the RFLP is that any point mutations as such deletions, substitutions and insertions or alterations like duplications, inversions within the genome can eliminate or form new restriction sites. These alterations in genome can be detected by analyzing fragments of variable length, digested with restriction endonuclease enzyme

- **Demerits:**
    - requires relatively large DNA sample
    - laborious and tedious process
    - sensitivity and more precautions for contamination required

3. Amplified Fragment Length Polymorphism (AFLP):

- Zabeau and Vos invented the AFLP technique in 1993.
- AFLP was originally developed by the KeyGene in 1990.
- It is a PCR based technique for fingerprinting. It includes both PCR and RFLP.

- The basis of AFLP is the amplification of selected fragments followed by restriction digestion of whole genomic DNA of specific organism.
- **The steps for the AFLP are as follows:**
    - DNA extraction and its restriction digestion followed by ligation with the short adaptor sequences.
    - Amplification of restricted fragments by PCR
    - Analysis of results in gel electrophoresis or PAGE followed by autoradiography.
- **Applications:**
    - AFLP has its ability for rapid generation of marker fragments for any organism without prior sequencing of DNA is required.
    - Also, it needs only small fragments of starting template DNA relative to RAPD and ISSR (inter-simple sequence repeats) and has much higher reproducibility.
    - AFLP is largely used for crop improvement programs, parentage and genomic interpretation of various crop species.
- **Demerits:**
    - AFLP require large DNA samples and require purification

VNTR

- **VNTR or the Variable Number of Tandem Repeats** are the repeated DNA sequences at a defined locus.
- The repeats are clustered together and oriented in the same direction.
- Individual repeats can be added or removed through replication and recombination errors.
- This forms alleles with different number of repeats.
- The DNA segments vary in different individuals and are hence beneficial in identifying individuals in case of a crime scene or a paternity dispute. This is known as **DNA fingerprinting.**

- The tandem repeat sequences of DNA are also termed as “satellite DNA”. These are of three main types:
    - **Satellite**
    - **Minisatellite**
    - **Microsatellite**

Types Of VNTR

1. **Satellite DNA**
    - These are highly repetitive DNA sequences and each DNA sequence consists of several thousand base pairs.
    - A satellite can measure up to 100 million base pairs.
    - These are found occurring in the regions of heterochromatin.
    - The Y chromosome has abundant satellites.
    - This makes it convenient for the researchers studying paternal genetic transmission in mammals.
    - The density of the DNA is the function of its base and sequence. The satellite DNA with its highly repetitive DNA has a reduced density compared to the rest of the genome. Therefore, the name “satellite DNA” is coined.

    - The satellite DNA has several **biological functions:**
        - Satellite DNA is present in the centromeric and pericentromeric regions. It regulates the functions of the centromere.
        - They help in the formation of heterochromatin.
        - Satellite RNA transcripts are found in plants, vertebrates, and invertebrates
2. **Minisatellite**
    - In a minisatellite, each repeat ranges from 9 to 100 base pairs. It is an array of tandem repeats 500 to 300,000 base pairs long.

- Minisatellites have been found in association with important features of the human genome such as gene regulation, imprinting, and chromosomal fragile sites. They provided the first highly polymorphic, multiallelic markers for linkage studies.
- Most of the minisatellites are GC rich. They possess a strong strand symmetry.

3. **Microsatellite**
    - The repeats are very short, 2-6 base pairs each. The whole array ranges from 10,000 to 100,000 base pairs long. They are therefore called short tandem repeats or simple sequence repeats.
    - They are usually found in insect and plant chromosomes, and euchromatin regions of vertebrates.
    - Microsatellites are important to the population geneticists because of the variable number of repeats among the individuals of a population.
    - Microsatellite markers are inherited from both the parents. Therefore, they are useful for paternity tests. Highly polymorphic loci increase our ability for parental analysis.
    - Microsatellites are not affected by natural selection. But they are influenced by gene flow, genetic drift, and mutation.

Importance of VNTR

- VNTRs are found on many chromosomes and vary in length among different individuals. Each variant helps in personal or parental identification.
- VNTRs are an important source of genetic marker RFLP which is used in the linkage analysis of genomes. A banding pattern unique to each individual is produced by the VNTRs.
- VNTR has its applications in forensic science, DNA fingerprinting, and other genetics and biology researches.

ENTRI

Techniques in Genetic Engineering: Selection and isolation of desired genes

Gene cloning involves separation of specific gene or DNA fragments from a donor cell, attaching it to small carrier molecule called vector and then replicating this recombinant vector into a host cell.

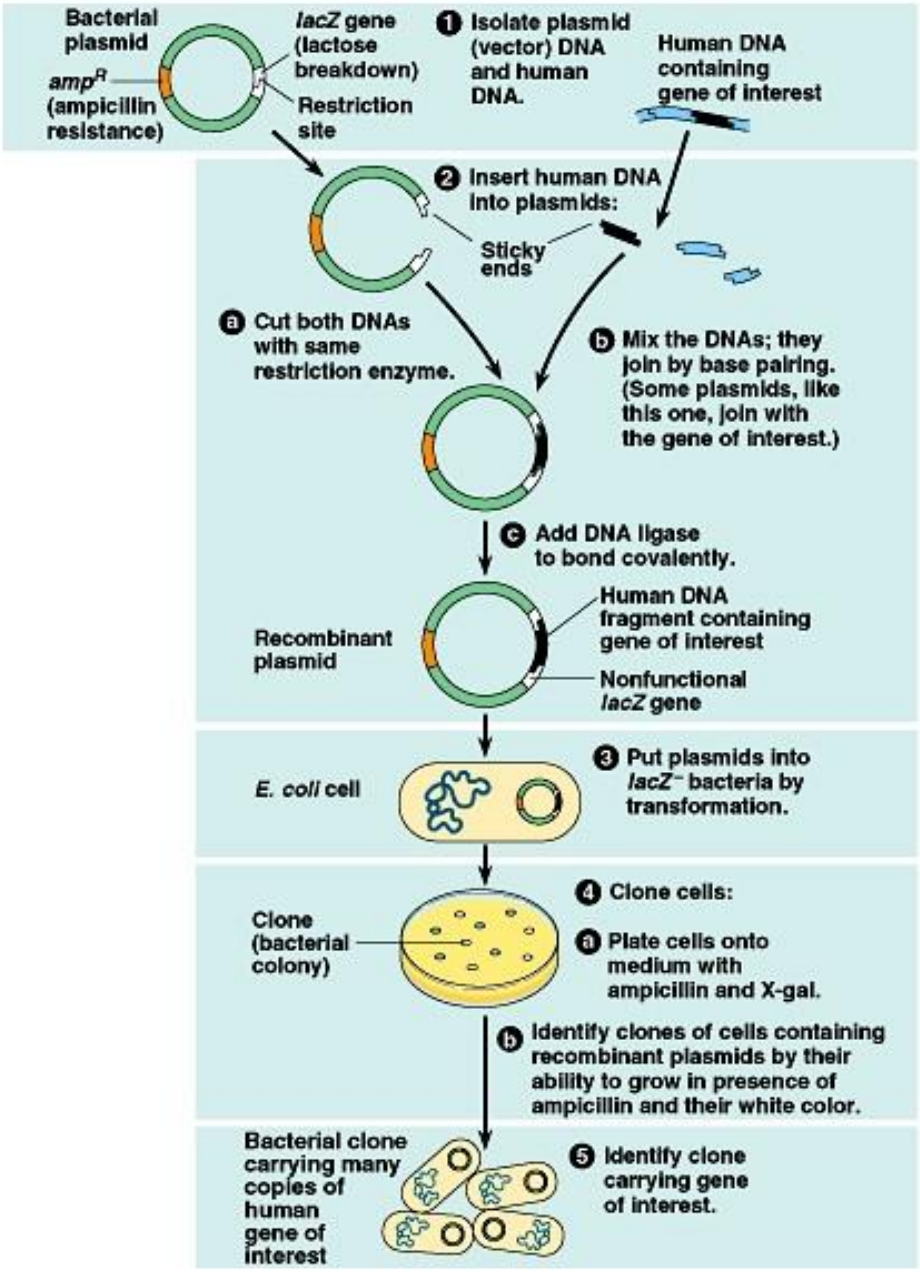
Major steps in cloning

Steps involved in gene cloning:

1. **Isolation of donor DNA fragment or gene**
2. **Selection of suitable vector**
3. **Incorporation of donor DNA fragment into the vector**
4. **Transformation of recombinant vector into a suitable host cell**
5. **Isolation of recombinant host cell**

1. Isolation of donor DNA fragment or gene

- At first a donor DNA fragment should be isolated. There are two method for isolation of desired gene or DNA fragment.
- Using restriction endonuclease enzyme: the enzyme restriction endonuclease is a key enzyme in molecular gene cloning. It has specific restriction site for its action. The enzyme RE generates a DNA fragment either with blunt end or with sticky end
- Using reverse transcriptase enzyme: reverse transcriptase enzyme Synthesizes complementary DNA strand of the desired gene using its mRNA.



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2. Selection of suitable cloning vector:

- When donor DNA fragment is incorporated into a host cell, it will not replicates because the isolated gene do not have the capacity to replicated itself. So before introduction of donor fragment into host, a suitable vector should be selected.

- Cloning vector is the DNA molecule capable of self-replication inside the host cell. the main function of cloning vector is to replicates the inserted DNA fragment inside the host cell.
- Examples of cloning vectors: Plasmid, BAC, YAC, Λ-bacteriophase, expression vectors etc.
- Characteristics of a cloning vectors
    - It must be self-replicating inside host cell
    - It must possess restriction site for RE enzymes
    - Introduction of donor DNA fragment must not interfere with replication property of the vector
    - It must possess some marker gene such that it can be used for later identification of recombinant cell.

3. Incorporation of donor DNA fragment with Plasmid vector:

- The plasmid vector is cut open by the same RE enzyme used for isolation of donor DNA fragment
- The mixture of donor DNA fragment and plasmid vector are mixed together.
- In the presence of DNA ligase, base pairing of donor DNA fragment and plasmid vector occurs forming recombinant vector in the mixture.

4. Transformation of recombinant vector into suitable host:

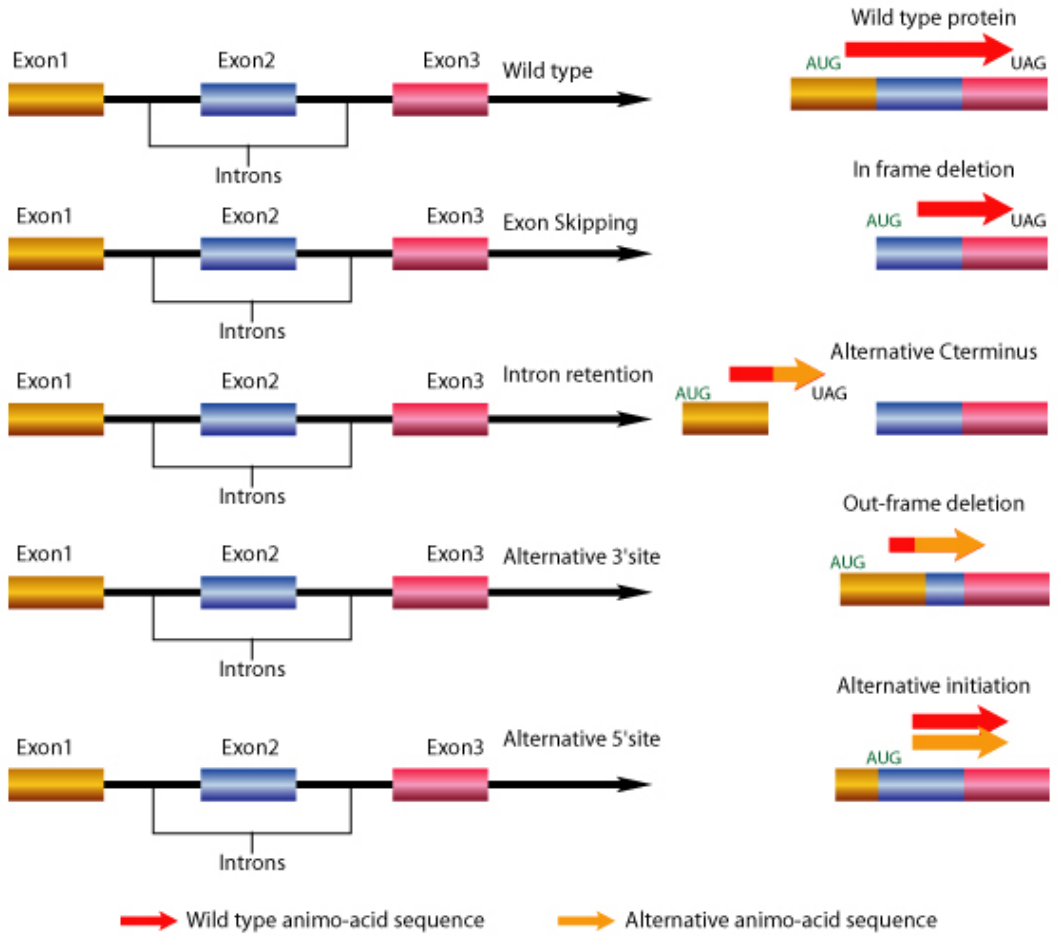
- The recombinant vector is transformed into suitable host cell. ie bacterial cell
- Some bacteria are naturally transformable, they take up the recombinant vector automatically. For examples: Bacillus, Haemophillus, Helicobacter pylori, are naturally competent
- Some other bacteria are not naturally competent, in those bacteria recombinant vector are incorporated by artificial method such as Ca++ ion treatment, electroporation etc.

5. Isolation of recombinant cell:

- The recombinant host cell is then grown in culture media but the culture may contains colonies both recombinant cell and non-recombinant cell.
- For isolation of recombinant cell from non-recombinant cell, marker gene of plasmid vector is employed.
- For examples, PBR322 plasmid vector contains different marker gene (Ampicillin resistant gene and Tetracycline resistant gene. When pst1 RE is used it knock out Ampicillin resistant gene from the plasmid, so that the recombinant cell become sensitive to Ampicillin.

Gene splicing

Gene splicing is a post-transcriptional modification in which a single gene can code for multiple proteins. Gene Splicing is done in eukaryotes, prior to mRNA translation, by the differential inclusion or exclusion of regions of pre-mRNA. Gene splicing is an important source of protein diversity. During a typical gene splicing event, the pre-mRNA transcribed from one gene can lead to different mature mRNA molecules that generate multiple functional proteins. Thus, gene splicing enables a single gene to increase its coding capacity, allowing the synthesis of protein isoforms that are structurally and functionally distinct. Gene splicing is observed in high proportion of genes. In human cells, about 40-60% of the genes are known to exhibit alternative splicing.



Gene Splicing Mechanism

There are several types of common gene splicing events. These are the events that can simultaneously occur in the genes after the mRNA is formed from the transcription step of the central dogma of molecular biology.

- **Exon Skipping:** This is the most common known gene splicing mechanism in which exon(s) are included or excluded from the final gene transcript leading to extended or shortened mRNA variants. The exons are the coding regions of a gene and are responsible for producing proteins that are utilized in various cell types for a number of functions.
- **Intron Retention:** An event in which an intron is retained in the final transcript. In humans 2-5 % of the genes have been reported to retain introns. The gene splicing mechanism retains the non-coding (junk)

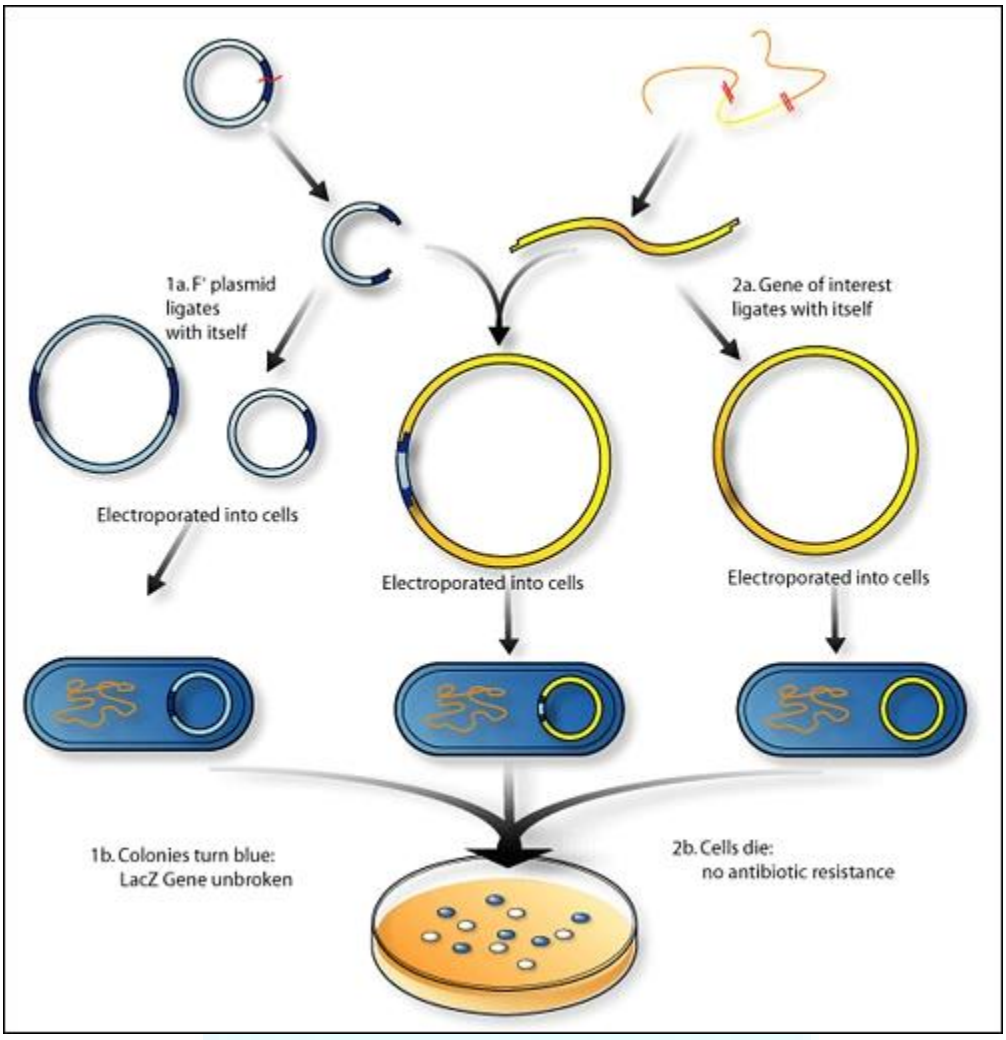
portions of the gene and leads to a demornity in the protein structure and functionality.

- **Alternative 3' Splice Site and 5' Splice Site:** Alternative gene splicing includes joining of different 5' and 3' splice site. In this kind of gene splicing, two or more alternative 5' splice site compete for joining to two or more alternate 3' splice site.

Introduction of rDNA into host cells

There are many methods to introduce recombinant vectors and these are dependant on several factors such as the vector type and host cell. Some commonly used procedures are discussed below.

- **Transformation:** In rDNA technology, the most common method to introduce rDNA into living cells is called transformation. In this procedure, bacterial cells take up DNA from the surrounding environment. Many host cell organisms such as E. coli, yeast and mammalian cells do not readily take up foreign DNA and have to be chemically treated to become competent to do so. In 1970, Mandel and Higa found that E. coli cells become markedly competent to take up external DNA when suspended briefly in cold calcium chloride solution.



- **Transfection:** Another method to transfer rDNA into host cells involves mixing the foreign DNA with charged substances like calcium phosphate, cationic liposomes or DEAE dextran and overlaying on recipient host cells. Host cells take up the DNA in a process called transfection.
- **Electroporation:** An electric current is used to create transient microscopic pores in the recipient host cell membrane allowing rDNA to enter.
- **Microinjection:** Exogenous DNA can also be introduced directly into animal and plant cells without the use of eukaryotic vectors. In the procedure of microinjection, foreign DNA is directly injected into recipient cells using a fine microsyringe under a phase contrast microscope to aid vision.

- **Biolistics:** A remarkable method that has been developed to introduce foreign DNA into mainly plant cells is by using a gene or particle gun. Microscopic particles of gold or tungsten are coated with the DNA of interest and bombarded onto cells with a device much like a particle gun. Hence the term biolistics is used.

Selection of clone containing DNA insert

After the introduction of r-DNA into a suitable host cell, it is essential to identify those cells which have received the r-DNA molecule. This process is called screening. The vector or foreign DNA present in recombinant cells expresses the characters, while the non-recombinants do not express the characters or traits. For this some of the methods are used and one such method is Blue-White Selection method.

1. **Insertional Inactivation - Blue-White Colony Selection Method**
    - It is a powerful method used for screening of recombinant plasmid. In this method, a reporter gene lacZ is inserted in the vector. The lacZ encodes the enzyme β-galactosidase and contains several recognition sites for restriction enzyme.
    - β-galactosidase breaks a synthetic substrates called **X-gal (5-bromo-4-chloro-indolyl-β -D-galacto-pyranoside)** into an insoluble blue coloured product. If a foreign gene is inserted into lacZ, this gene will be inactivated. Therefore, no- blue colour will develop (white) because β-galactosidase is not synthesized due to inactivation of lacZ.

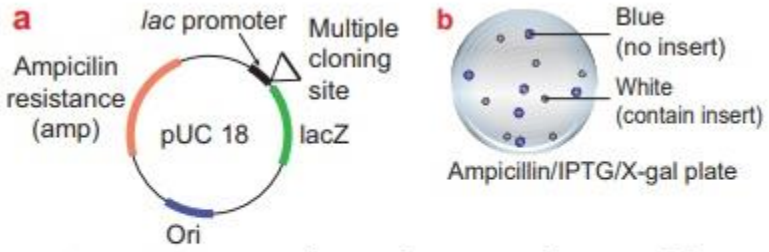


Figure 4.18: a. Plasmid vector designed for blue-white screening b. Blue-white colony selection method

Therefore, the host cell containing r-DNA form white coloured colonies on the medium contain X-gal, whereas the other cells containing non-recombinant DNA will develop the blue coloured colonies. On the basis of colony colour, the recombinants can be selected.

2. **Antibiotic resistant markers**
    - An antibiotic resistance marker is a gene that produces a protein that provides cells with resistance to an antibiotic.
    - Bacteria with transformed DNA can be identified by growing on a medium containing an antibiotic.
    - Recombinants will grow on these medium as they contain genes encoding resistance to antibiotics such as ampicillin, chloro amphenicol, tetracycline or kanamycin, etc., while others may not be able to grow in these media, hence it is considered useful selectable marker.

3. **Replica plating technique**
    - A technique in which the pattern of colonies growing on a culture plate is copied.
    - A sterile filter plate is pressed against the culture plate and then lifted. Then the filter is pressed against a second sterile culture plate. This results in the new plate being infected with cell in the same relative positions as the colonies in the original plate. Usually,

the medium used in the second plate will differ from that used in the first.

- It may include an antibiotic or without a growth factor. In this way, transformed cells can be selected.

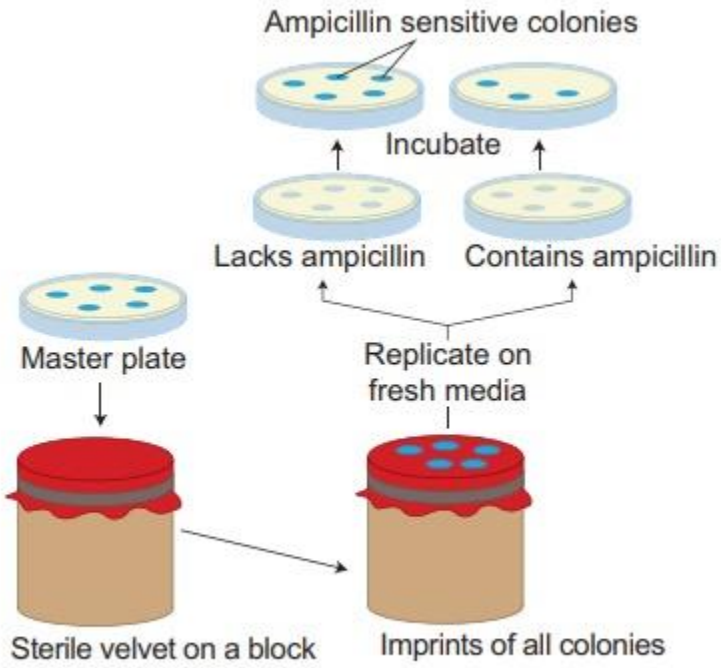


Figure 4.19: Replica plating technique

4. **Molecular Techniques - Isolation of Genetic Material and Gel Electrophoresis**
    - Electrophoresis is a separating technique used to separate different biomolecules with positive and negative charges.

    Principle
    - By applying electricity (DC) the molecules migrate according to the type of charges they have. The electrical charges on different molecules are variable.

+ve	charged	Cations	will move towards	−ve	Cathode
−ve	charged	Anions	will move towards	+ve	Anode

Agarose GEL Electrophoresis

- It is used mainly for the purification of specific DNA fragments.
- Agarose is convenient for separating DNA fragments ranging in size from a few hundred to about 20000 base pairs.
- Polyacrylamide is preferred for the purification of smaller DNA fragments.
- The gel is complex network of polymeric molecules. DNA molecule is negatively charged molecule under an electric field DNA molecule migrates through the gel.
- The electrophoresis is frequently performed with marker DNA fragments of known size which allow accurate size determination of an unknown DNA molecule by interpolation.
- The advantages of agarose gel electrophoresis are that the DNA bands can be readily detected at high sensitivity.
- The bands of DNA in the gel are stained with the dye Ethidium Bromide and DNA can be detected as visible fluorescence illuminated in UV light will give orange fluorescence, which can be photographed.

DNA fingerprinting

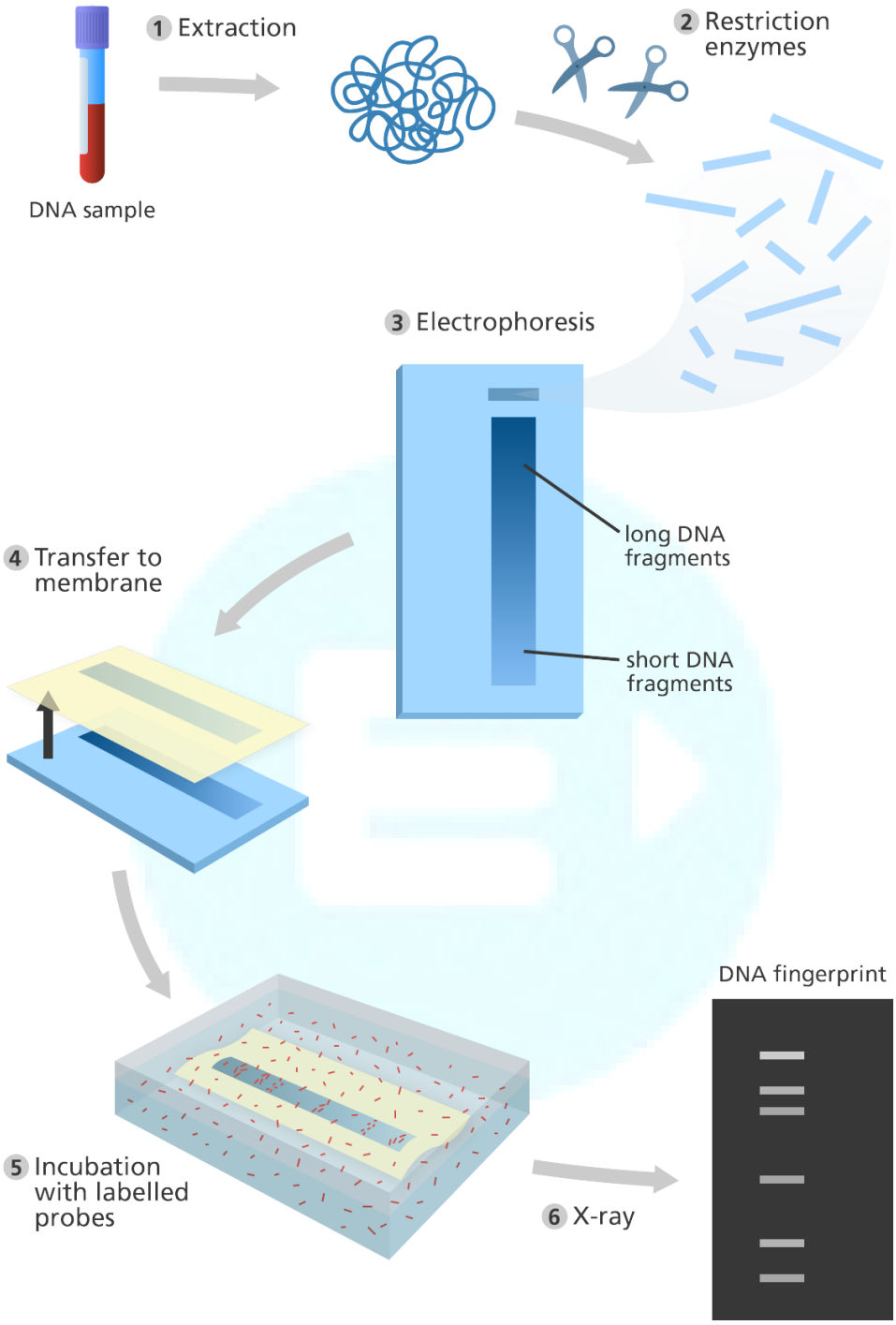
- "DNA fingerprinting is a procedure that shows the hereditary cosmetics of living things. It is a strategy for finding the distinction between the satellite DNA areas in the genome."
- DNA profiling, DNA testing, DNA examination, Genetic profile, DNA distinguishing proof, genetic fingerprinting, and genetic investigation are

a portion of the mainstream names utilized for DNA fingerprinting. This technique was invented by Alec Jeffreys in 1984.

Principle of DNA fingerprinting

- The human genome consists of innumerable small noncoding sequences which are inheritable and repeatedly present.
- They can be separated from the bulk DNA as satellite upon performing density gradient centrifugation and thus known as satellite DNA.
- They can be categorized into either microsatellites or microsatellites depending on the length, base composition and tandemly repetitive units.
- These satellite DNAs show polymorphism and this polymorphism is the basis of DNA fingerprinting.
- The repeat regions can be divided into two groups based on the size of the repeat - variable number tandem repeats (VNTRs) and short tandem repeats.
- These repeats act as genetic markers and every individual inherits these repeats from their parents.
- Thus, every individual has a particular composition of VNTRs and this is the main principle of the DNA fingerprinting technique.

DNA Fingerprinting Steps



1. Collection of organic example blood, spit, buccal swab, semen, or solid tissue.

2. DNA extraction.

3. Restriction absorption or PCR intensification.
4. Agarose gel electrophoresis, slim electrophoresis or DNA sequencing.
5. Interpreting outcomes.

The Process of DNA Fingerprinting

Sample collection, DNA extraction, absorption or intensification and investigation results are significant advances.

- **Stage 1: Sample Collection**
  - DNA can be acquired from any bodily sample or liquid. Buccal smear, salivation, blood, amniotic liquid, chorionic villi, skin, hair, body liquid, and different tissues are significant kinds of samples utilized.

- **Stage 2: DNA Extraction**
  - We need to initially get DNA. To play out any genetic applications, DNA extraction is one of the most significant advances. Great quality and amount of DNA expands the conceivable outcomes of getting better outcomes.
  - Following strategies can utilize:
    - Phenol-chloroform DNA extraction strategy
    - CTAB DNA extraction strategy
    - Proteinase K DNA extraction strategy

- **Stage 3: Restriction Absorption, Enhancement or DNA Sequencing**
  - Three regular strategies are utilized:
    - RFLP based STR investigation
    - PCR based investigation
    - Real-time PCR investigation
- **Stage 4: Analysis of Results**

- Utilizing the southern blotting, agarose gel electrophoresis, narrow electrophoresis, ongoing intensification, and DNA sequencing, the outcomes for different DNA profiling can be gotten in which rt-PCR and sequencing are much of the use in forensic science.

- **Stage 5: Interpreting Results**
  - By looking at DNA profiles of different examples, varieties and likenesses between people can be distinguished.
  - Outstandingly, the whole procedure is presently nearly automatic. We don't need to do anything, the computer gives us conclusive outcomes.

Applications of DNA Fingerprinting

- Utilizing the DNA fingerprinting strategy, the natural personality of an individual can be uncovered. For approving one's character, there is no other preferable alternative over DNA fingerprinting.
- Gravely harmed dead bodies can be distinguished.
- It is utilized to detect maternal cell contamination.
- One of the significant downsides of pre-birth determination is maternal cell tainting. The amniotic liquid or CVS test contains the maternal DNA or maternal tissue, once in a while. Contamination expands the opportunity of false-positive outcomes, particularly on account of carrier recognition. Utilizing VNTRs and STRs markers with PCR-gel electrophoresis, maternal cell tainting can be recognized during pregnancy hereditary testing.
- One of the most significant uses of the current strategy is in the crime scene examination and criminal check. The example is gathered from the crime site which could be salivation, blood, hair follicle, or semen. DNA is removed and investigated against the suspect, utilizing the two

markers we clarified previously. By coordinating DNA band designs criminal's connected to wrongdoing can be built up.

Utilizing Blood-Typing in Paternity Tests

- The procedure of DNA fingerprinting was discovered by Alec Jeffreys in 1984, and it originally opened up for paternity testing in 1988.
- Before this kind of DNA investigation was accessible, blood classifications were the most widely recognized calculation considered human paternity testing. Blood bunches are a mainstream case of Mendelian hereditary qualities at work.
- All things considered, there are various human blood bunches with numerous alleles, and these alleles display a scope of predominance designs.

DNA Fingerprinting and Farming

- A few DNA minisatellite tests have yielded piece profiles that show up valuable for plant reproducing work.
- These part profiles show no variety when vegetative spread material is broken down.
- So also, examples obtained through self-inbreeding species show indistinguishable profiles.
- Interestingly, hereditary recombination in cross-pollinating species brings about exceptionally factor, normally singular, explicit piece profiles.
- Along these lines various cultivars can be recognized, as additionally can genotypes of wild species in characteristic populaces.
- These piece profiles can likewise be used in parentage examination, as has just been led in rice and apples, in this way empowering us to explain the source of deficiently recorded cultivars.

- Also, evaluations of hereditary variety dependent on similitude lists determined from section profiles show a nearby relationship with known degrees of hereditary relatedness.

DNA sequencing

- DNA sequencing refers to methods for determining the order of the nucleotides bases adenine,guanine,cytosine and thymine in a molecule of DNA.
- The first DNA sequence was obtained by academic researchers, using laboratories methods based on 2- dimensional chromatography in the early 1970s.
- By the development of dye based sequencing method with automated analysis, DNA sequencing has become easier and faster.

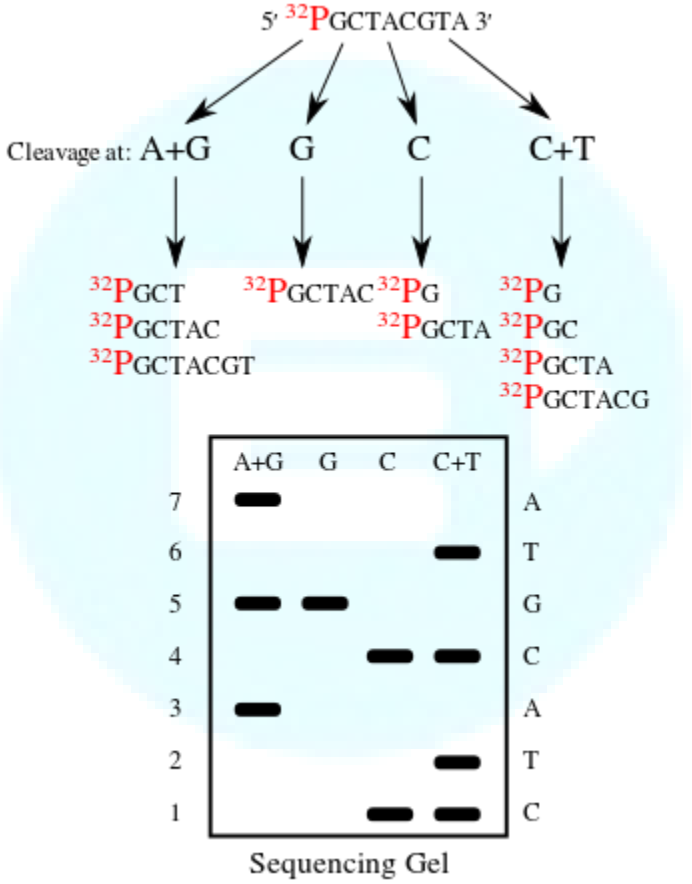
Two main methods are widely known to be used to sequence DNA:

  - **The Chemical Method** (also called the Maxam–Gilbert method after its inventors).
  - **The Chain Termination** Method (also known as the Sanger dideoxy method after its inventor).
- Maxam–Gilbert technique depends on the relative chemical liability of different nucleotide bonds, whereas the Sanger method interrupts elongation of DNA sequences by incorporating dideoxynucleotides into the sequences.
- The chain termination method is the method more usually used because of its speed and simplicity.

Maxam-Gilbert method

- In 1976-1977, Allan Maxam and Walter Gilbert developed a DNA sequencing method based on chemical modification of DNA and subsequent cleavage at specific bases.

- The method requires radioactive labelling at one end and purification of the DNA fragment to be sequenced.
- Chemical treatment generates breaks at a small proportions of one or two of the four nucleotide based in each of four reactions (G,A+G, C, C+T).
- Thus a series of labelled fragments is generated, from the radiolabelled end to the first 'cut' site in each molecule.
- The fragments in the four reactions are arranged side by side in gel electrophoresis for size separation.



- To visualize the fragments, the gel is exposed to X-ray film for autoradiography, yielding a series of dark bands each corresponding to a radiolabelled DNA fragment, from which the sequence may be inferred.

Key Features

- Base-specific cleavage of DNA by certain chemicals
- Four different chemicals, one for each base

- A set of DNA fragments of different sizes
- DNA fragments contain up to 500 nucleotides

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.

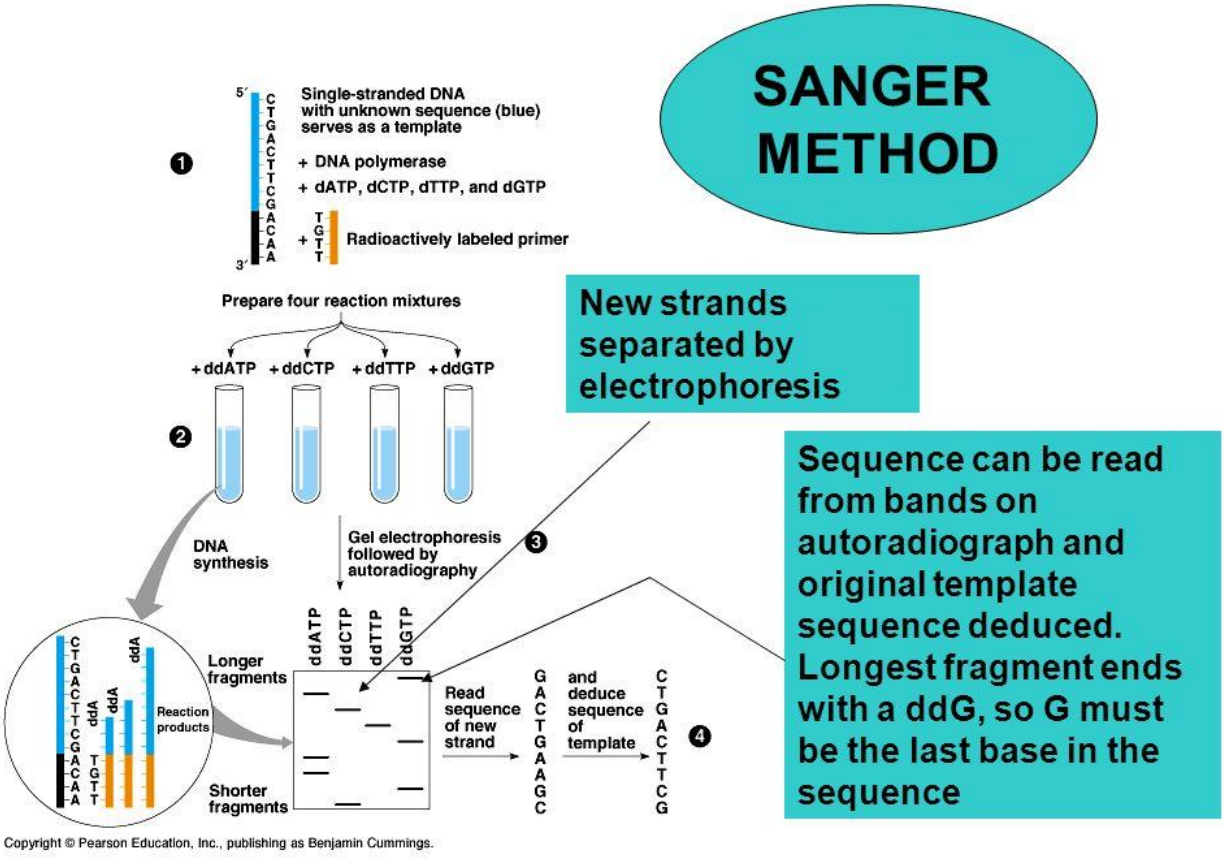
Sanger-Coulson method

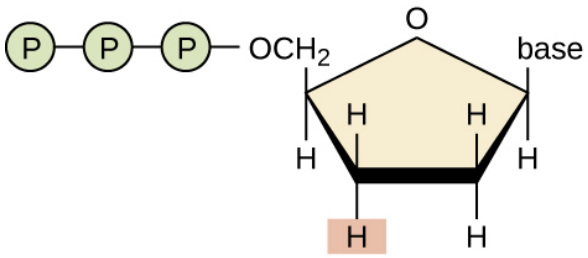
- Sanger's method of gene sequencing is also known as dideoxy chain termination method. It generates nested set of labelled fragments from a template strand of DNA to be sequenced by replicating that template strand and interrupting the replication process at one of the four bases.
- Four different reaction mixtures are produced that terminates in A. T. G or C respectively.

Principle

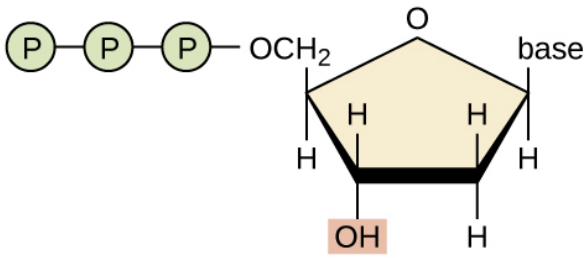
- A DNA primer is attached by hybridization to the template strand and deoxynucleosides triphosphates (dNTPPs) are sequentially added to the primer strand by DNA polymerase.
- The primer is designed for the known sequences at 3′ end of the template strand.

- M13 sequences is generally attached to 3′ end and the primer of this M13 is made.
- The reaction mixture also contains dideoxynucleoside triphosphate (ddNTPs) along with usual dNTPs.
- If during replication ddNTPs is incorporated instead of usual dNTPs in the growing DNA strand then the replication stops at that nucleotide.





dideoxynucleotide (ddNTP)

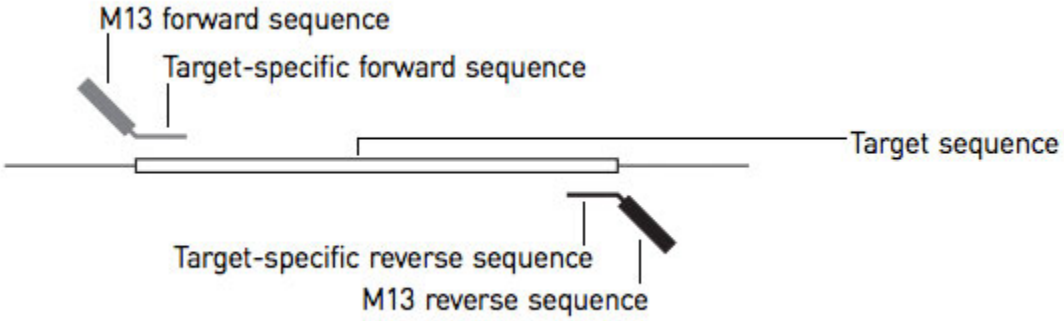


deoxynucleotide (dNTP)

- The ddNTPs are analogue of dNTPs.
- ddNTPs lacks hydroxyl group (-OH) at c3 of ribose sugar, so it cannot make phosphodiester bond with nest nucleotide, thus terminates the nucleotide chain.
- Respective ddNTPs of dNTPs terminates chain at their respective site. For example ddATP terminates at A site. Similarly ddCTP, ddGTP and ddTTP terminates at C, G and T site respectively.

Procedure

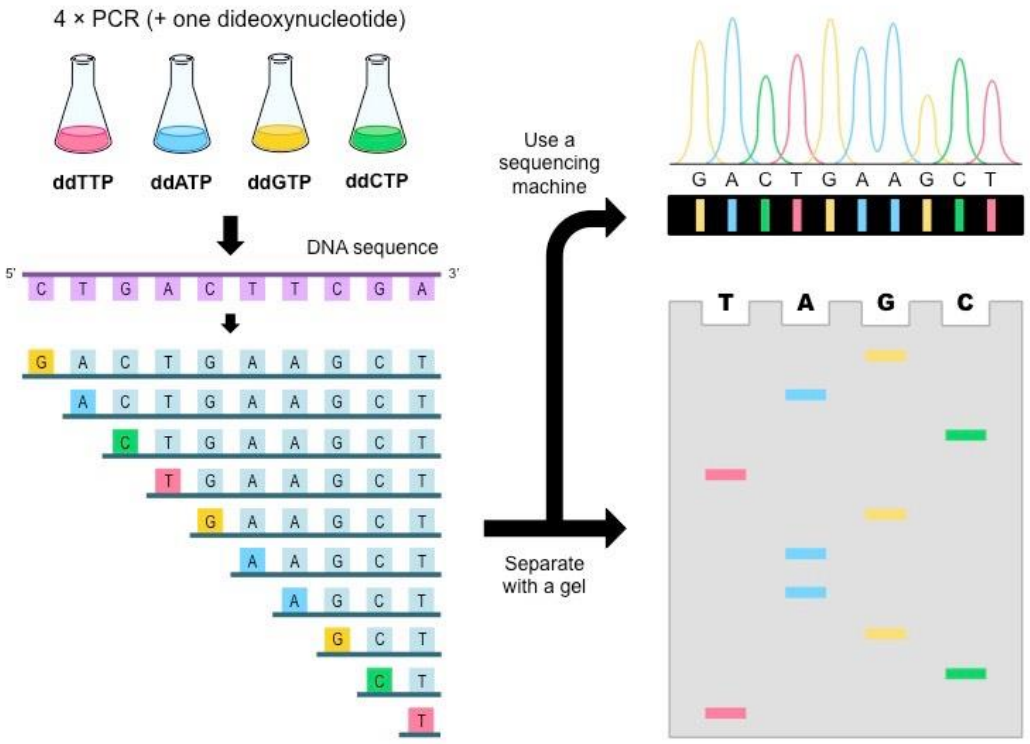
1. **Template preparation:**



- Copies of template strand to be sequenced must be prepared with short known sequences at 3' end of the template strand.
- A DNA primere is essential to initiate replication of template , so primer preparation of known sequences at 3'end is always required.
- For this purpose a single stranded cloning vector M13 is flanked with template strand at 3'end which serves as binding site for primer.

2. **Generation of nested set of labelled fragments:**

- Copies of each template is divided into four batches and each batch is used for different replication reaction.
- Copies of standard primer and DNA polymerase I are used in all four batches.
- To synthesize fragments that terminates at A, ddATP is added to the reaction mixture on batch I along with dATP, dTTP,dCTP and dGTP, standard primer and DNA polymerase I.
- Similarly, to generate, all fragments that terminates at C, G and T, the respective ddNTPs ie ddCTP,ddGTP and ddTTP are added respectively to different reaction mixture on different batch along with usual dNTPs.



3. **Electrophoresis and gel reading:**

- The reaction mixture from four batches are loaded into four different well on polyacrylamide gel and electrophoresed.

- The autoradiogramof the gel is read to determine the order of bases of complementary strand to that of template strand.
- The band of shortest fragments are at the bottom of autoradiogram so that the sequences of complementary strand is read from bottom to top.

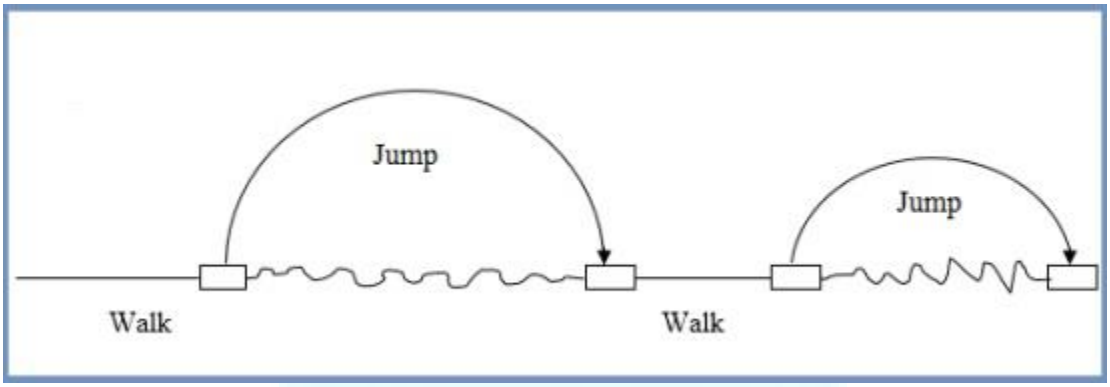
Significance of DNA Sequencing

- 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.

Chromosome jumping

- Chromosomal jumping is a technique used in molecular biology for physical mapping of genomes of the organisms.
- This technique was introduced to overcome a barrier of the chromosomal walking which arose upon finding the repetitive DNA regions during the cloning process. Therefore, chromosome jumping

technique can be considered as a special version of chromosomal walking. It is a rapid method compared to chromosomal walking and enables bypassing of the repetitive DNA sequences which are not prone to be cloned during chromosomal walking.



- Chromosomal jumping narrows the gap between the target gene and the available known markers for genome mapping.
- Chromosome jumping tool starts with the cutting of a specific DNA with special restriction endonucleases and ligation of the fragments into circularized loops.
- Then a primer designed from a known sequence is used to sequence the circularized loops.
- This primer enables jumping and sequencing in an alternative manner.
- Hence, it can bypass the repetitive DNA sequences and rapidly walk through the chromosome for the search of the target gene.
- The discovery of the gene encodes for cystic fibrosis disease was done using the chromosomal jumping tool.
- Combined together, chromosomal jumping and walking can enhance the genome mapping process.

Genomic library

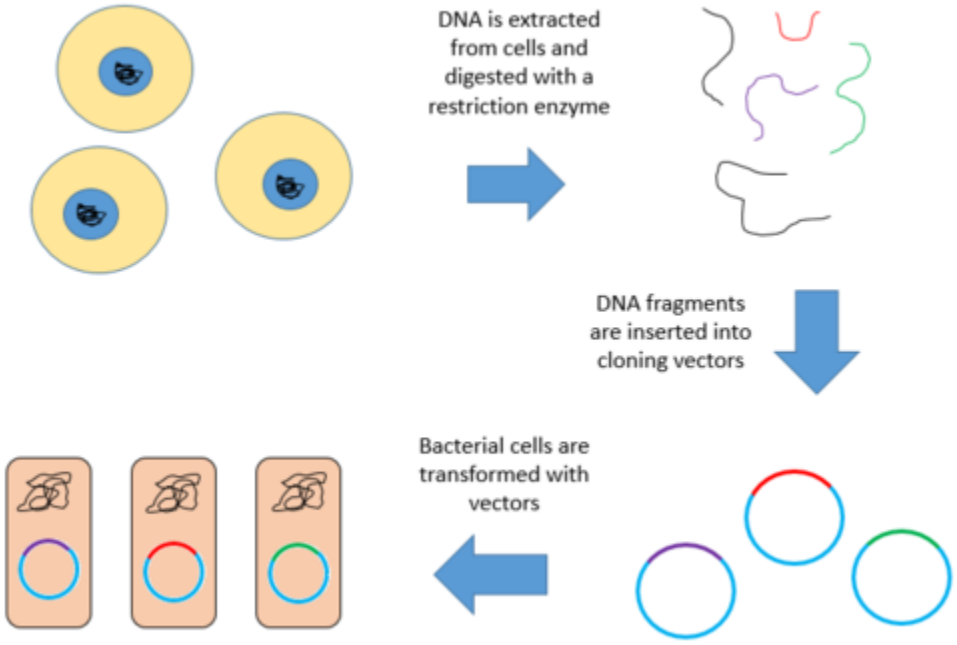
- A genomic library is a collection of the total genomic DNA from a single organism.

- The DNA is stored in a population of identical vectors, each containing a different insert of DNA.
- In order to construct a genomic library, the organism's DNA is extracted from cells and then digested with a restriction enzyme to cut the DNA into fragments of a specific size.
- The fragments are then inserted into the vector using DNA ligase.
- Next, the vector DNA can be taken up by a host organism - commonly a population of Escherichia coli or yeast - with each cell containing only one vector molecule. Using a host cell to carry the vector allows for easy amplification and retrieval of specific clones from the library for analysis.
- There are several kinds of vectors available with various insert capacities.
- Generally, libraries made from organisms with larger genomes require vectors featuring larger inserts, thereby fewer vector molecules are needed to make the library.
- Researchers can choose a vector also considering the ideal insert size to find the desired number of clones necessary for full genome coverage.
- Genomic libraries are commonly used for sequencing applications.
- They have played an important role in the whole genome sequencing of several organisms, including the human genome and several model organisms

Genomic library construction

- Construction of a genomic library involves creating many recombinant DNA molecules.
- An organism's genomic DNA is extracted and then digested with a restriction enzyme.
- For organisms with very small genomes (~10 kb), the digested fragments can be separated by gel electrophoresis.
- The separated fragments can then be excised and cloned into the vector separately.

- However, when a large genome is digested with a restriction enzyme, there are far too many fragments to excise individually.
- The entire set of fragments must be cloned together with the vector, and separation of clones can occur after.
- In either case, the fragments are ligated into a vector that has been digested with the same restriction enzyme.
- The vector containing the inserted fragments of genomic DNA can then be introduced into a host organism.



Below are the steps for creating a genomic library from a large genome.

- Extract and purify DNA.
- Digest the DNA with a restriction enzyme. This creates fragments that are similar in size, each containing one or more genes.
- Insert the fragments of DNA into vectors that were cut with the same restriction enzyme. Use the enzyme DNA ligase to seal the DNA fragments into the vector. This creates a large pool of recombinant molecules.

- These recombinant molecules are taken up by a host bacterium by transformation, creating a DNA library.

cDNA library

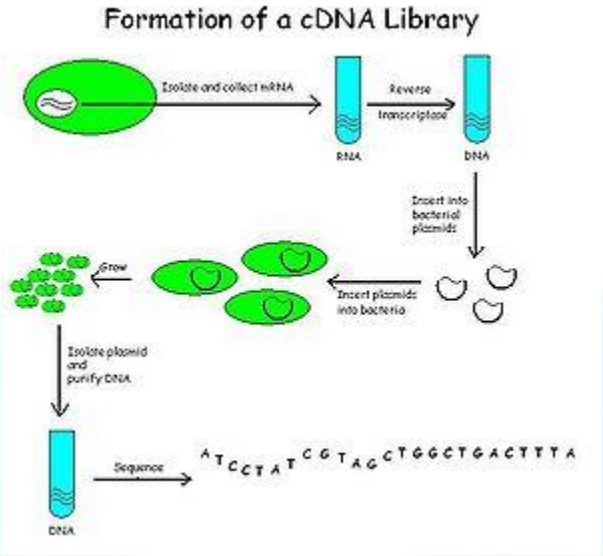
- A cDNA library is a combination of cloned cDNA (complementary DNA) fragments inserted into a collection of host cells, which constitute some portion of the transcriptome of the organism and are stored as a "library".
- cDNA is produced from fully transcribed mRNA found in the nucleus and therefore contains only the expressed genes of an organism.
- Similarly, tissue-specific cDNA libraries can be produced.
- In eukaryotic cells the mature mRNA is already spliced, hence the cDNA produced lacks introns and can be readily expressed in a bacterial cell.
- While information in cDNA libraries is a powerful and useful tool since gene products are easily identified, the libraries lack information about enhancers, introns, and other regulatory elements found in a genomic DNA library.

cDNA Library Construction

- cDNA is created from a mature mRNA from a eukaryotic cell with the use of reverse transcriptase. In eukaryotes, a poly-(A) tail (consisting of a long sequence of adenine nucleotides) distinguishes mRNA from tRNA and rRNA and can therefore be used as a primer site for reverse transcription.
- This has the problem that not all transcripts, such as those for the histone, encode a poly-A tail.

    - **mRNA extraction**
        - Firstly, the mRNA is obtained and purified from the rest of the RNAs.

- Several methods exist for purifying RNA such as trizol extraction and column purification.
- Column purification is done by using oligomeric dT nucleotide coated resins where only the mRNA having the poly-A tail will bind. The rest of the RNAs are eluted out.
- The mRNA is eluted by using eluting buffer and some heat to separate the mRNA strands from oligo-dT.



- **cDNA construction**
  - Once mRNA is purified, oligo-dT (a short sequence of deoxy-thymidine nucleotides) is tagged as a complementary primer which binds to the poly-A tail providing a free 3'-OH end that can be extended by reverse transcriptase to create the complementary DNA strand.
  - The mRNA is removed by using an RNAse enzyme, leaving single-stranded cDNA (sscDNA).
  - The sscDNA is converted into double-stranded DNA with the help of DNA polymerase.
  - However, for DNA polymerase to synthesize a complementary strand, a free 3'-OH end is needed.

- This is provided by the sscDNA itself by coiling on itself at the 3' end, generating a hairpin loop.
- The polymerase extends the 3'-OH end, and later the loop at 3' end is opened by the scissoring action of S1 nuclease.
- Restriction endonucleases and DNA ligase are then used to clone the sequences into bacterial plasmids.

The cloned bacteria are then selected, commonly through the use of antibiotic selection. Once selected, stocks of the bacteria are created which can later be grown and sequenced to compile the cDNA library.

cDNA Library uses

- cDNA libraries are commonly used when reproducing eukaryotic genomes, as the amount of information is reduced to remove the large numbers of non-coding regions from the library.
- cDNA libraries are used to express eukaryotic genes in prokaryotes.
- Prokaryotes do not have introns in their DNA and therefore do not possess any enzymes that can cut it out during transcription process.
- cDNA does not have introns and therefore can be expressed in prokaryotic cells.
- cDNA libraries are most useful in reverse genetics where the additional genomic information is of less use.
- Additionally, cDNA libraries are frequently used in functional cloning to identify genes based on the encoded protein's function.
- When studying eukaryotic DNA, expression libraries are constructed using complementary DNA (cDNA) to help ensure the insert is truly a gene.

Gene cloning

ENTRI

Cloning techniques in animals

Clones of adult animals are created by the processes of artificial twinning and somatic cell nuclear transfer. There are two variations of the somatic cell nuclear transfer method. They are the Roslin Technique and the Honolulu Technique. It is important to note that in all of these techniques the resulting offspring will be genetically identical to the donor and not the surrogate unless the donated nucleus is taken from a somatic cell of the surrogate.

Cloning Techniques

1. **Somatic Cell Nuclear Transfer**
    - The term somatic cell nuclear transfer refers to the transfer of the nucleus from a somatic cell to an egg cell. A somatic cell is any cell of the body other than a germ cell (sex cell). An example of a somatic cell would be a blood cell, heart cell, skin cell, etc.

    - In this process, the nucleus of a somatic cell is removed and inserted into an unfertilized egg that has had its nucleus removed. The egg with its donated nucleus is then nurtured and divides until it becomes an embryo. The embryo is then placed inside a surrogate mother and develops inside the surrogate.

2. **The Roslin Technique**
    - The Roslin Technique is a variation of somatic cell nuclear transfer that was developed by researchers at the Roslin Institute. The researchers used this method to create Dolly. In this process, somatic cells (with nuclei intact) are allowed to grow and divide and are then deprived of nutrients to induce the cells into a suspended or dormant stage. An egg cell that has had its nucleus removed is then placed in close proximity to a somatic cell and

both cells are shocked with an electrical pulse. The cells fuse and the egg is allowed to develop into an embryo. The embryo is then implanted into a surrogate.

3. **The Honolulu Technique**
    - The Honolulu Technique was developed by Dr. Teruhiko Wakayama at the University of Hawaii. In this method, the nucleus from a somatic cell is removed and injected into an egg that has had its nucleus removed. The egg is bathed in a chemical solution and cultured. The developing embryo is then implanted into a surrogate and allowed to develop.

4. **Artificial Twinning**
    - While the previously mentioned techniques involve somatic cell nuclear transfer, artificial twinning does not. Artificial twinning involves fertilization of a female gamete (egg) and separation of resulting embryonic cells in the early stages of development. Each separated cell continues to grow and can be implanted into a surrogate. These developing embryos mature, eventually forming separate individuals. All of these individuals are genetically identical, as they were originally separated from a single embryo. This process is similar to what happens in the development of natural identical twins.

Amplification of DNA by PCR

- **Polymerase chain reaction (PCR)** is a common laboratory technique used to make many copies (millions or billions!) of a particular region of DNA.
- This DNA region can be anything the experimenter is interested in.

- For example, it might be a gene whose function a researcher wants to understand, or a genetic marker used by forensic scientists to match crime scene DNA with suspects.
- Typically, the goal of PCR is to make enough of the target DNA region that it can be analyzed or used in some other way. For instance, DNA amplified by PCR may be sent for sequencing, visualized by gel electrophoresis, or cloned into a plasmid for further experiments.
- PCR is used in many areas of biology and medicine, including molecular biology research, medical diagnostics, and even some branches of ecology.

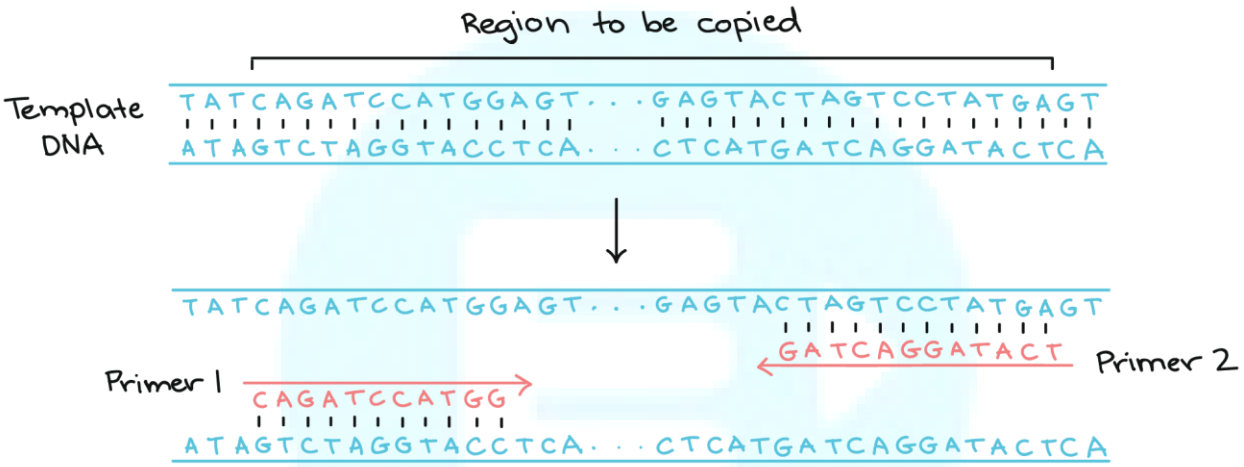
Taq polymerase

- Like DNA replication in an organism, PCR requires a DNA polymerase enzyme that makes new strands of DNA, using existing strands as templates.
- The DNA polymerase typically used in PCR is called Taq polymerase, after the heat-tolerant bacterium from which it was isolated (Thermus aquaticus).
- T. aquaticus lives in hot springs and hydrothermal vents.
- Its DNA polymerase is very heat-stable and is most active around 70 °\text C70°C70, °, start text, C, end text (a temperature at which a human or E. coli DNA polymerase would be nonfunctional).
- This heat-stability makes Taq polymerase ideal for PCR. As we'll see, high temperature is used repeatedly in PCR to denature the template DNA, or separate its strands.

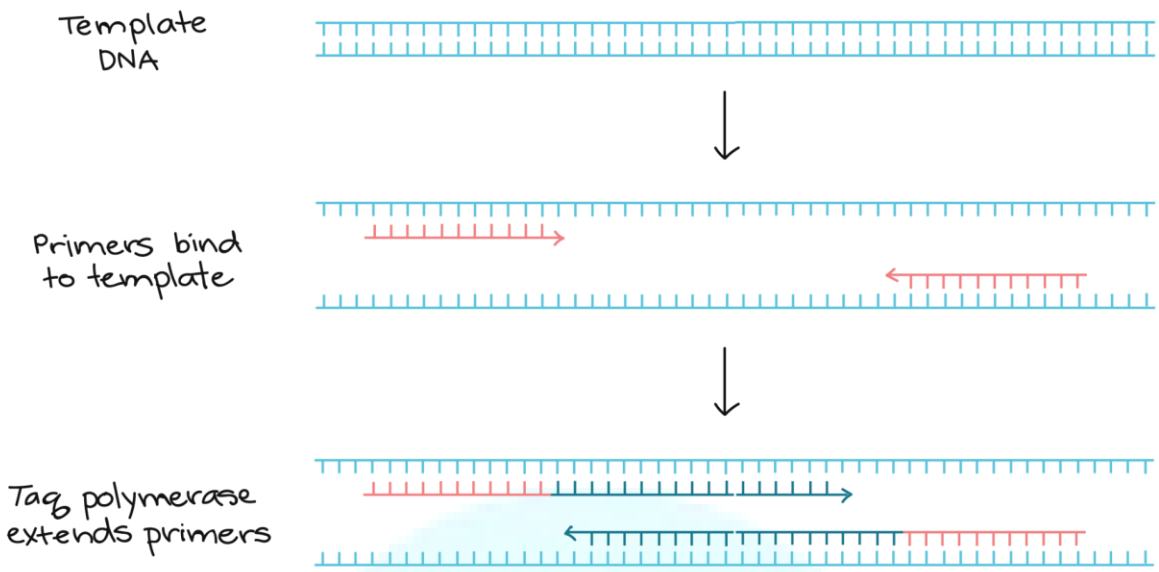
PCR primers

- Like other DNA polymerases, Taq polymerase can only make DNA if it's given a primer, a short sequence of nucleotides that provides a starting point for DNA synthesis.

- In a PCR reaction, the experimenter determines the region of DNA that will be copied, or amplified, by the primers she or he chooses.
- PCR primers are short pieces of single-stranded DNA, usually around 202020 nucleotides in length.
- Two primers are used in each PCR reaction, and they are designed so that they flank the target region (region that should be copied).
- That is, they are given sequences that will make them bind to opposite strands of the template DNA, just at the edges of the region to be copied.
- The primers bind to the template by complementary base pairing.



When the primers are bound to the template, they can be extended by the polymerase, and the region that lies between them will get copied

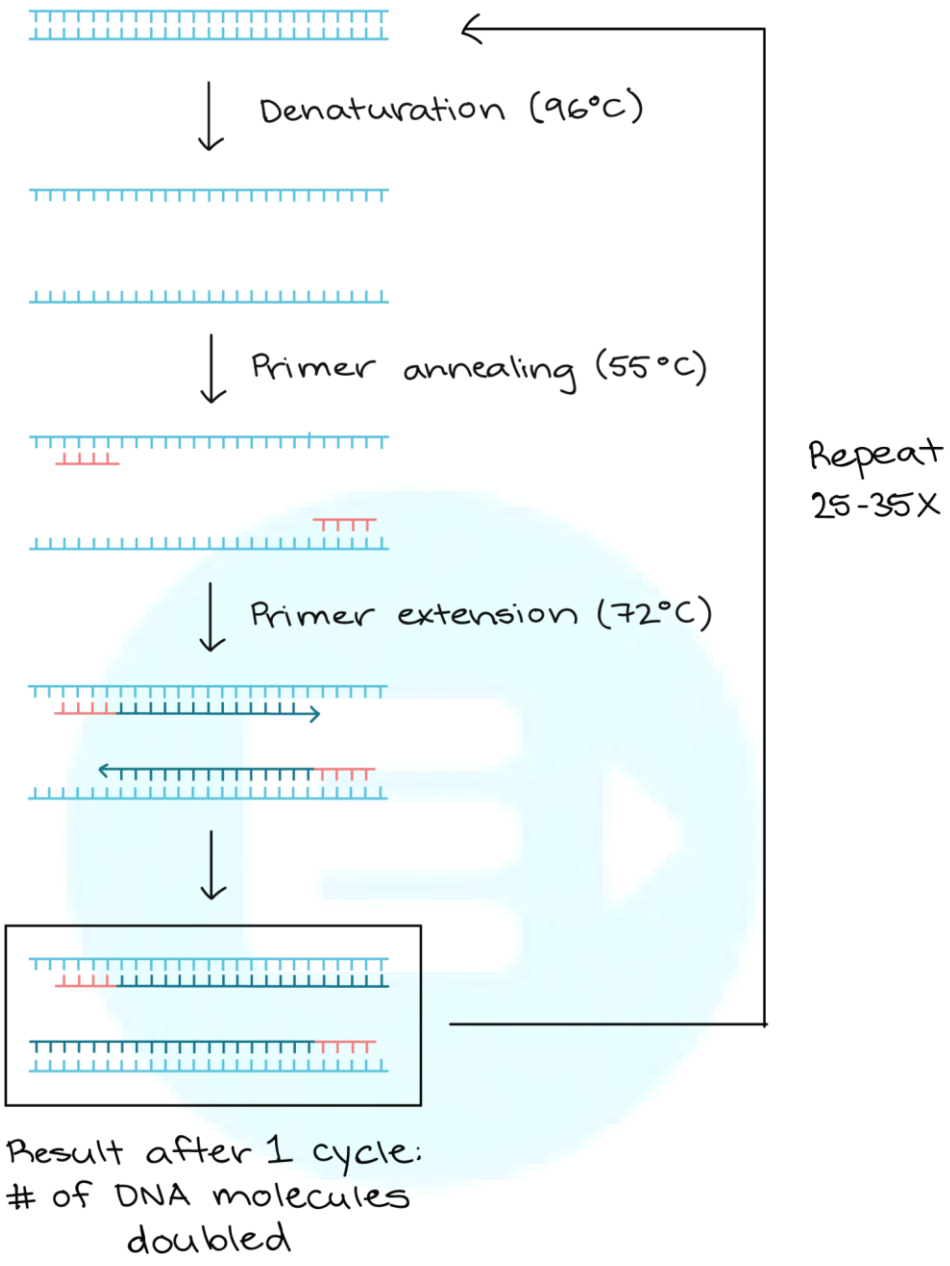


The steps of PCR

The key ingredients of a PCR reaction are Taq polymerase, primers, template DNA, and nucleotides (DNA building blocks). The ingredients are assembled in a tube, along with cofactors needed by the enzyme, and are put through repeated cycles of heating and cooling that allow DNA to be synthesized.

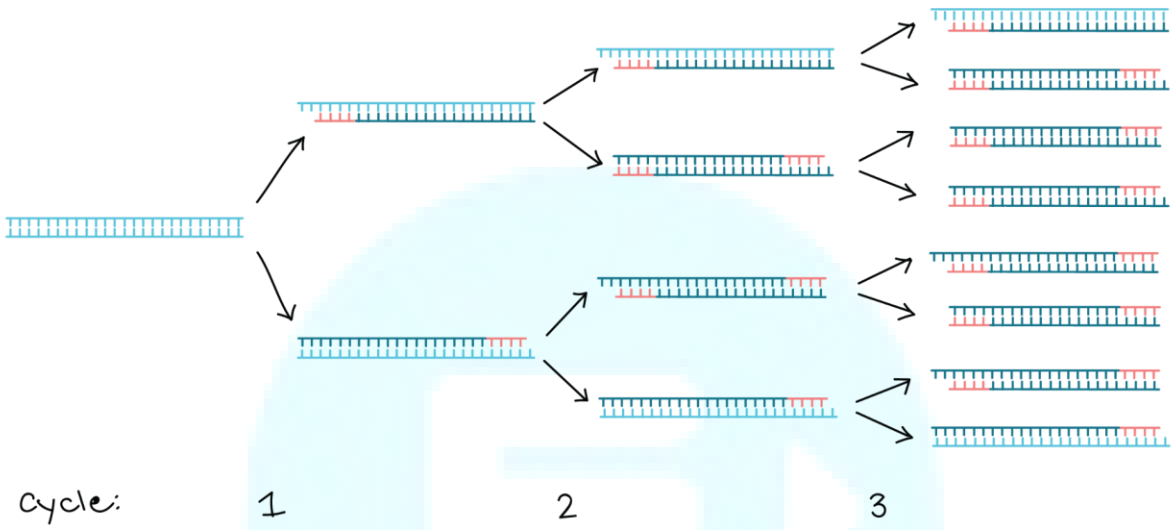
The basic steps are:

- **Denaturation (96°C):** Heat the reaction strongly to separate, or denature, the DNA strands. This provides single-stranded template for the next step.
- **Annealing (55-65°C):** Cool the reaction so the primers can bind to their complementary sequences on the single-stranded template DNA.
- **Extension (72°C):** Raise the reaction temperatures so Taq polymerase extends the primers, synthesizing new strands of DNA.



- This cycle repeats 25-35 times in a typical PCR reaction, which generally takes 2-4 hours, depending on the length of the DNA region being copied.
- If the reaction is efficient (works well), the target region can go from just one or a few copies to billions.
- That's because it's not just the original DNA that's used as a template each time.

- Instead, the new DNA that's made in one round can serve as a template in the next round of DNA synthesis.
- There are many copies of the primers and many molecules of Taq polymerase floating around in the reaction, so the number of DNA molecules can roughly double in each round of cycling.



Applications of PCR

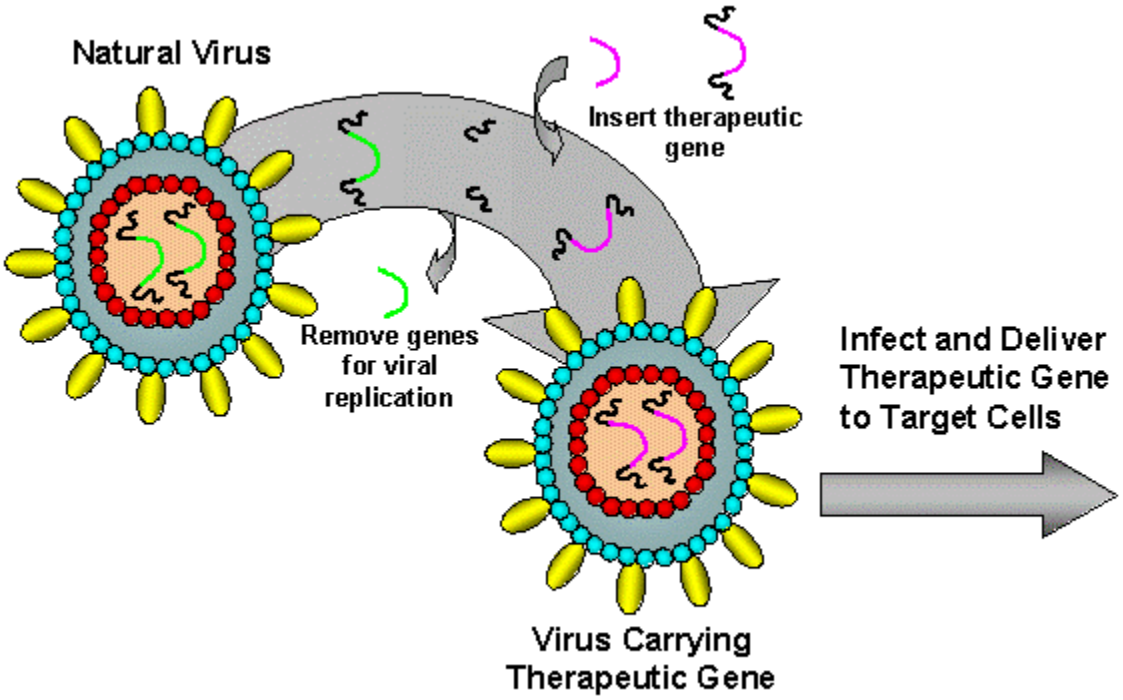
- Using PCR, a DNA sequence can be amplified millions or billions of times, producing enough DNA copies to be analyzed using other techniques.
- For instance, the DNA may be visualized by gel electrophoresis, sent for sequencing, or digested with restriction enzymes and cloned into a plasmid.
- PCR is used in many research labs, and it also has practical applications in forensics, genetic testing, and diagnostics.
- For instance, PCR is used to amplify genes associated with genetic disorders from the DNA of patients (or from fetal DNA, in the case of prenatal testing).
- PCR can also be used to test for a bacterium or DNA virus in a patient's body: if the pathogen is present, it may be possible to amplify regions of its DNA from a blood or tissue sample.

Gene transfer technology

Gene transfer is defined simply as a technique to efficiently and stably introduce foreign genes into the genome of target cells. Gene transfer technologies were originally developed as a research tool for investigating gene expression and function. However, as new gene transfer technologies are developed and old technologies refined, the potential applications have expanded dramatically. Currently, there are a number of gene transfer technologies available which vary greatly in their efficiency of gene transfer and the types of cells they are capable of delivering genes into. Genes can be delivered into cells using lipid-based vectors, naked DNA, electroporation (the application of electrical charge to cells), or viruses which are the most efficient gene transfer vectors.

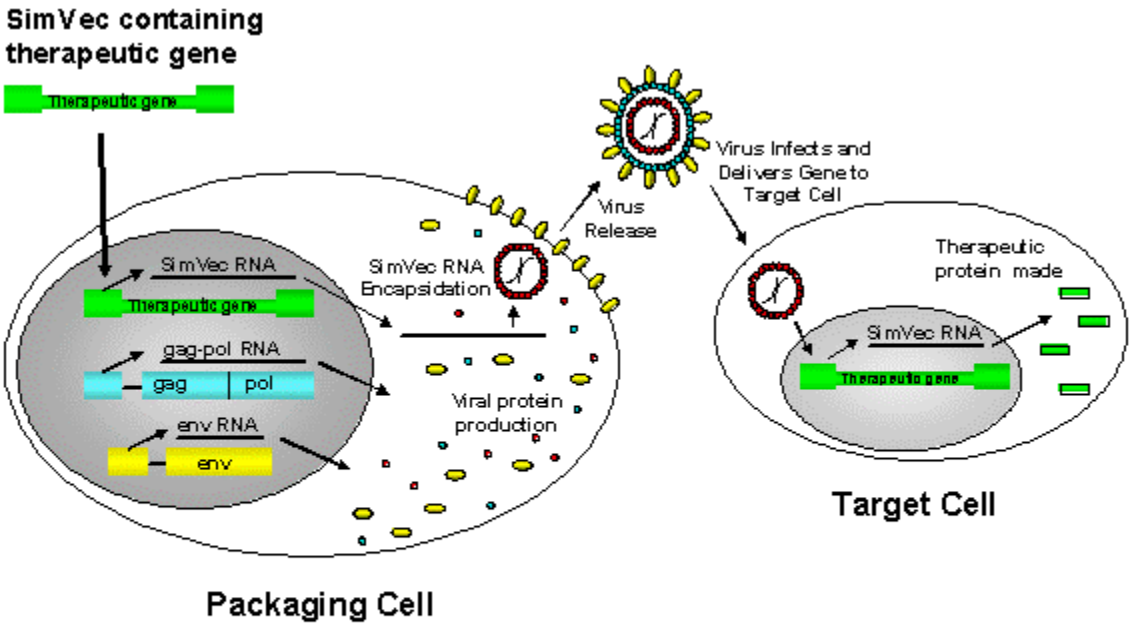
Recently, gene transfer technology has found its way into clinical applications designed to treat inherited diseases, cancer, and infectious diseases such as AIDS. When genes become altered or damaged so that the encoded protein is no longer functional, as is the case for inherited diseases and many cancers, disease development may occur. Disease development can also be the result of an acquired or infectious disease wherein a pathogenic protein or aberrant protein is produced. In either case, gene transfer technologies can be used to deliver a therapeutic gene into patients.

For human clinical applications, gene transfer technologies must be designed that are capable of efficiently delivering genes into primary human cells without harming the recipient. In general, a carrier molecule known as a "vector" is used to deliver the therapeutic gene into target cells. The most commonly used vector for clinical gene transfer applications is a virus which has been genetically engineered to carry the therapeutic gene, but does not have the ability to multiply or cause disease.



How our gene transfer technology, SimVec, works

GeneCure's patented gene transfer technology is based on a primate lentivirus, the simian immunodeficiency virus (SIV). The company's lentiviral gene transfer vector (SimVec) is a genetically engineered SIV genome that lacks the genes necessary for viral replication. A therapeutic gene can be introduced into the vector through standard molecular biology techniques. Virus particles encoding the therapeutic gene can be produced in a specialized cell line called a packaging cell line. Virus particles containing the therapeutic gene can then be collected and used to infect and deliver the gene to target cells. Importantly, the virus is capable of infecting and delivering the therapeutic gene to target cells such as non-dividing human cells but is unable to multiply and spread to other cells. Because lentiviruses stably integrate into the target cell's genome, the therapeutic gene can be expressed long-term and is replicated and passed on to all daughter cells during cell division.



Unique advantages of SimVec

1. **Efficient gene delivery into primary cells**

Current clinical gene transfer is hampered by the lack of effective means to deliver genes into primary human cells. The most commonly used gene transfer technologies in clinical studies are retroviral-based vectors derived from murine retroviruses. Unfortunately, these vectors have limited potential for clinical applications due to their inability to infect non-dividing cells. To address this concern, scientists have taken advantage of lentiviruses, which have the natural ability to infect non-dividing mammalian cells. Lentiviral-based vectors including those based on human immunodeficiency virus, HIV-1, have been developed to deliver genes to non-dividing human cells. However, as HIV-1 is a major human pathogen, the use of HIV-1-based gene transfer technology poses significant concerns for use in human gene transfer applications. GeneCure's unique technology is based on the simian immunodeficiency virus

(SIV), a lentivirus family member that does not cause disease in humans. Thus, GeneCure's gene transfer technology can effectively deliver genes into human cells including non-dividing and terminally differentiated cells without the risks associated with an HIV-1-based approach.

2. **Safety profile in pre-clinical and clinical trials**

In order for viral vectors to be worthy of clinical applications, they must be proven safe in animal models. The primary concern when using retroviruses for human gene transfer applications is the generation of replication-competent retrovirus (RCR). Studies performed in rhesus monkeys to monitor formation of replication-competent virus have validated GeneCure's gene transfer technology as a safe delivery system for future use in clinical studies.

Clinical studies of GeneCure's lead product, HIVAX, also demonstrated well-tolerated safety profiles in patients infected with HIV-1.

3. **Stable long-term expression of therapeutic genes**

Because lentiviruses permanently integrate into the target cell's genome, lentiviral vectors allow for stable long-term expression of the gene. Numerous reports demonstrate stable expression of reporter genes for greater than nine months. Additionally, unlike commonly used onco-retroviral vectors, where transcriptional silencing of the gene has been observed in numerous reports, no transcriptional silencing has been observed with lentiviral vectors. Thus, use of lentiviral vectors may overcome the challenges hindering current gene transfer technologies.

4. **No pre-existing immunity**

An important consideration when using viral vectors for clinical gene transfer applications is the presence of pre-existing immunity in the target population. Pre-existing immunity occurs when a patient has been previously exposed to the natural virus, as is common for vectors based on the adenovirus (cause of the common cold) and canarypox virus (a harmless relative of smallpox). Previous exposure the virus prepares the patient's body to quickly mount an immune response should it encounter the virus again. As a result, use of these viral vectors in patients with pre-existing immunity may dampen the effectiveness of the vector due to unwanted immune responses directed at the vector itself. Pre-existing immunity can limit both the ability of the vector to efficiently deliver the gene to target cells as well as limit the duration of gene expression due to immune-mediated destruction of infected cells. In addition, pre-existing immunity can reduce the efficiency of vector re-administration, limiting the usefulness of boosting or re-immunization with the same vector. GeneCure's gene transfer technology is based on the simian immunodeficiency virus (SIV), which is not a human pathogen. Accordingly, this technology can be used in clinical gene transfer applications without the problems associated with pre-existing immunity. This includes the ability to re-administer the viral vector to patients as necessary.

5. **Broad host range**

GeneCure's unique gene transfer technology allows production of lentiviral particles which have incorporated the vesicular stomatitis virus G protein (VSV-G) into the viral envelopes. Incorporation of VSV-G envelope protein permits the viral particles to infect a broad range of mammalian and non-mammalian host cells including human, primate, mouse, hamster, and fish cells. This technology greatly expands the applications of gene transfer technology to include studies using cell types which are resistant to infection with other vectors and to non-mammalian studies.

6. **Stable high titer production of viral particles**

Many viral-vector mediated gene transfer applications, particularly clinical applications, require a large quantity of viral vector. As a result, viral vectors must be produced at high titers and must be able to withstand further concentration. GeneCure's unique gene transfer technology allows production of lentiviral particles which have incorporated the vesicular stomatitis virus G protein (VSV-G) into the viral envelopes. Incorporation of VSV-G into viral particles greatly increases the amount and stability of vector produced. Viral vector can be consistently produced at a biological titer of 108 Transducting Units (TU)/ml and can be concentrated to 1011 TU/ml by ultracentrifugation.

Expression of induced genes

- Expression of target genes in response to extracellular stimuli is activated by means that include activation of **RNA polymerase II (Pol II)-dependent transcription.**
- Multiple processes during the early stages of Pol II-dependent transcription are subject to regulation and can therefore function as the rate-limiting step during activation of target genes.
- **Activator-dependent recruitment** of Pol II and the general transcription factors is essential for high levels of transcription at inducible genes. This process must occur at all induced genes before activation, whether or not recruitment of polymerase is rate-limiting with regards to activation.
- The kinetics of Pol II recruitment and transcription initiation can be stimulated by nucleosome-remodelling complexes that use the energy from ATP hydrolysis to move or displace histones from DNA.
- **Co-activators** are essential for activator-dependent recruitment of Pol II and the general transcription factors. Mediator and the histone acetyltransferase complex SAGA are well-characterized examples of co-activators.
- Transcription can be regulated at steps that occur after the recruitment of Pol II. These steps include the release of Pol II from a paused state close

to the promoter into active transcription elongation in the coding region of the gene.

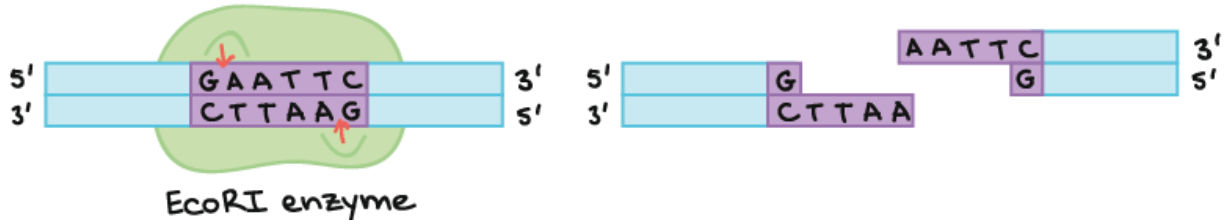
- The rate-limiting step of transcription can differ between various inducible genes.
- **Signalling kinases** such as MAPKs sometimes localize to the promoters of target genes at which they can function as transcriptional activators, rapidly facilitating the switch between activated and repressed states of gene expression.

Restriction enzymes

Restriction enzymes are found in bacteria (and other prokaryotes). They recognize and bind to specific sequences of DNA, called restriction sites. Each restriction enzyme recognizes just one or a few restriction sites. When it finds its target sequence, a restriction enzyme will make a double-stranded cut in the DNA molecule. Typically, the cut is at or near the restriction site and occurs in a tidy, predictable pattern.

As an example of how a restriction enzyme recognizes and cuts at a DNA sequence, let's consider EcoRI, a common restriction enzyme used in labs. EcoRI cuts at the following site:

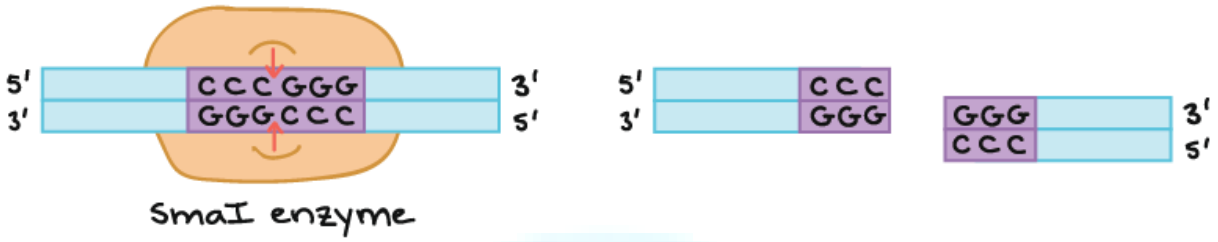
When EcoRI recognizes and cuts this site, it always does so in a very specific pattern that produces ends with single-stranded DNA “overhangs”:



If another piece of DNA has matching overhangs (for instance, because it has also been cut by EcoRI), the overhangs can stick together by complementary base pairing. For this reason, enzymes that leave single-stranded overhangs

are said to produce sticky ends. Sticky ends are helpful in cloning because they hold two pieces of DNA together so they can be linked by DNA ligase.

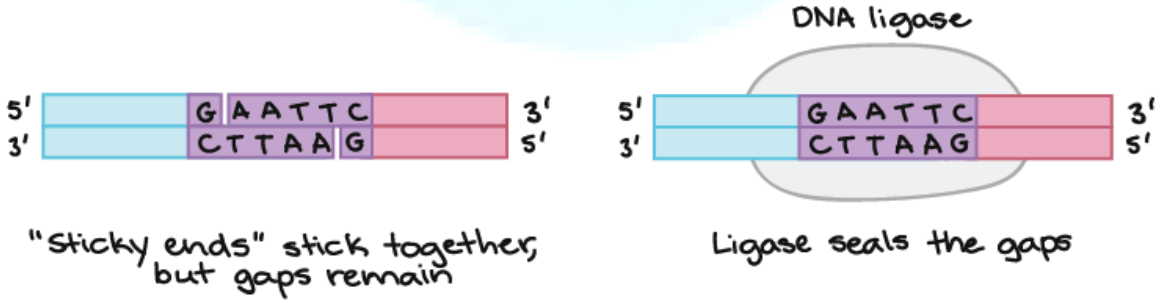
Not all restriction enzymes produce sticky ends. Some are "blunt cutters," which cut straight down the middle of a target sequence and leave no overhang. The restriction enzyme SmaI is an example of a blunt cutter:



Blunt-ended fragments can be joined to each other by DNA ligase. However, blunt-ended fragments are harder to ligate together (the ligation reaction is less efficient and more likely to fail) because there are no single-stranded overhangs to hold the DNA molecules in position.

DNA ligase

If you've learned about DNA replication, you may already have met DNA ligase. In DNA replication, ligase's job is to join together fragments of newly synthesized DNA to form a seamless strand. The ligases used in DNA cloning do basically the same thing. If two pieces of DNA have matching ends, DNA ligase can join them together to make an unbroken molecule.



Using ATP as an energy source, ligase catalyzes a reaction in which the phosphate group sticking off the 5′ end of one DNA strand is linked to the

hydroxyl group sticking off the 3′ end of the other. This reaction produces an intact sugar-phosphate backbone.

Applications of Biotechnology:

Blotting techniques (Southern, Northern, Western)

- Blotting is the technique in which nucleic acids or proteins are immobilized onto a solid support generally nylon or nitrocellulose membranes.
- Blotting of nucleic acid is the central technique for hybridization studies.
- Nucleic acid labeling and hybridization on membranes have formed the basis for a range of experimental techniques involving understanding of gene expression, organization, etc.
- Identifying and measuring specific proteins in complex biological mixtures, such as blood, have long been important goals in scientific and diagnostic practice.
- More recently the identification of abnormal genes in genomic DNA has become increasingly important in clinical research and genetic counseling.
- Blotting techniques are used to identify unique proteins and nucleic acid sequences.
- They have been developed to be highly specific and sensitive and have become important tools in both molecular biology and clinical research.

General principle

- The blotting methods are fairly simple and usually consist of four separate steps: electrophoretic separation of protein or of nucleic acid fragments in the sample; transfer to and immobilization on paper support; binding of analytical probe to target molecule on paper; and visualization of bound probe. Molecules in a sample are first separated

by electrophoresis and then transferred on to an easily handled support medium or membrane.

- This immobilizes the protein or DNA fragments, provides a faithful replica of the original separation, and facilitates subsequent biochemical analysis.
- After being transferred to the support medium the immobilized protein or nucleic acid fragment is localized by the use of probes, such as antibodies or DNA, that specifically bind to the molecule of interest.
- Finally, the position of the probe that is bound to the immobilized target molecule is visualized usually by autoradiography.
- Three main blotting techniques have been developed and are commonly called
    - **Southern blotting**
    - **Northern blotting**
    - **Western blotting**

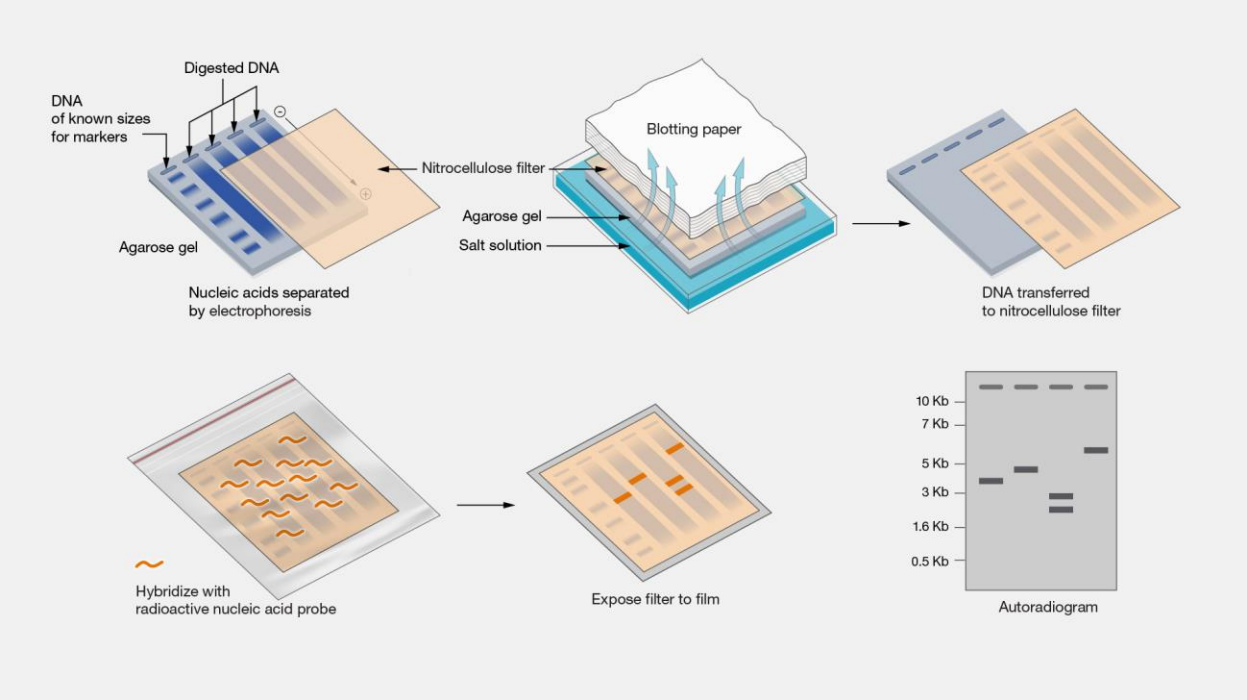
Southern blot

Southern blot is a method used to check for the presence of a DNA sequence in a DNA sample.

The method is named after its inventor, the British biologist Edwin Southern.

The procedure for Southern blot technique is as detailed below:

- Restriction endonucleases are used to cut high-molecular-weight DNA strands into smaller fragments, which are then electrophoresed on an agarose gel to separate them by size.
- If the DNA fragments are larger than 15 kb, then prior to blotting, the gel may be treated with an acid, such as dilute HCl, which depurinates the DNA fragments, breaking the DNA into smaller pieces, thus allowing more efficient transfer from the gel to membrane.
- If alkaline transfer methods are used, the DNA gel is placed into an alkaline solution (containing NaOH) to denature the double-stranded DNA.



- The denaturation in an alkaline environment may improve binding of the negatively charged DNA to a positively charged membrane, separating it into single DNA strands for later hybridization to the probe and destroys any residual RNA that may still be present in the DNA.
- A sheet of nitrocellulose (or nylon) membrane is placed on top of (or below, depending on the direction of the transfer) the gel. Pressure is applied evenly to the gel (either using suction, or by placing a stack of paper towels and a weight on top of the membrane and gel), to ensure good and even contact between gel and membrane.
- Buffer transfer by capillary action from a region of high water potential to a region of low water potential (usually filter paper and paper tissues) is used to move the DNA from the gel on to the membrane; ion exchange interactions bind the DNA to the membrane due to the negative charge of the DNA and positive charge of the membrane.
- The membrane is then baked in a vacuum or regular oven at 80 °C for 2 hours or exposed to ultraviolet radiation (nylon membrane) to permanently attach the transferred DNA to the membrane.

- The membrane is then exposed to a hybridization probe—a single DNA fragment with a specific sequence whose presence in the target DNA is to be determined.
- The probe DNA is labelled so that it can be detected, usually by incorporating radioactivity or tagging the molecule with a fluorescent or chromogenic dye.
- After hybridization, excess probe is washed from the membrane and the pattern of hybridization is visualized on X-ray film by autoradiography in the case of a radioactive or fluorescent probe, or by development of colour on the membrane if a chromogenic detection method is used.
- Hybridization of the probe to a specific DNA fragment on the filter membrane indicates that this fragment contains DNA sequence that is complementary to the probe.
- The transfer step of the DNA from the electrophoresis gel to a membrane permits easy binding of the labeled hybridization probe to the size-fractionated DNA. Southern blots performed with restriction enzyme-digested genomic DNA may be used to determine the number of sequences (e.g., gene copies) in a genome.
- A probe that hybridizes only to a single DNA segment that has not been cut by the restriction enzyme will produce a single band on a Southern blot, whereas multiple bands will likely be observed when the probe hybridizes to several highly similar sequences (e.g., those that may be the result of sequence duplication).
- Modification of the hybridization conditions (ie, increasing the hybridization temperature or decreasing salt concentration) may be used to increase specificity and decrease hybridization of the probe to sequences that are less than 100% similar.

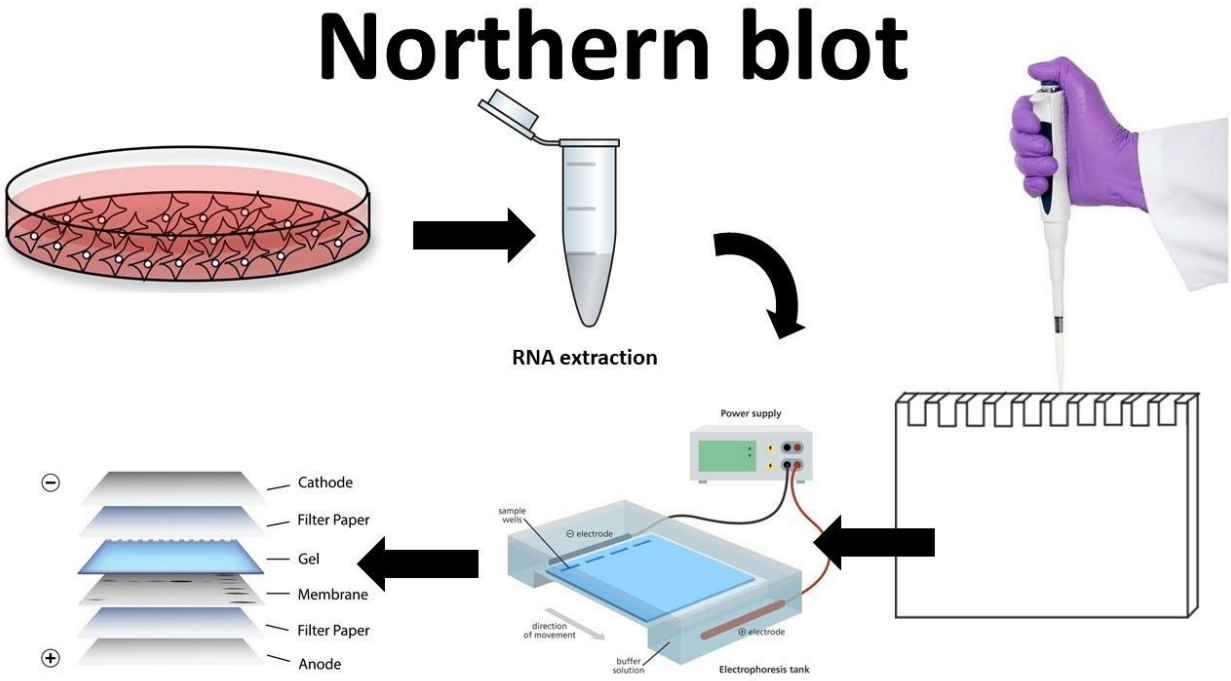
Northern blot

- The northern blot technique is used to study gene expression by detection of RNA (or isolated mRNA) in a sample.
- With northern blotting it is possible to observe cellular control over structure and function by determining the particular gene expression levels during differentiation, morphogenesis, as well as abnormal or diseased conditions.
- This technique was developed in 1977 by James Alwine, David Kemp and George Stark at Stanford University.
- Northern blotting takes its name from its similarity to the first blotting technique, the Southern blot.
- The major difference is that RNA, rather than DNA, is analyzed in the northern blot.

Procedure

- The blotting procedure starts with extraction of total RNA from a homogenized tissue sample.
- The mRNA can then be isolated through the use of oligo (dT) cellulose chromatography to maintain only those RNAs with a poly(A) tail.
- RNA samples are then separated by gel electrophoresis.
- A nylon membrane with a positive charge is the most effective for use in northern blotting since the negatively charged nucleic acids have a high affinity for them.
- The transfer buffer used for the blotting usually contains formamide because it lowers the annealing temperature of the probe-RNA interaction preventing RNA degradation by high temperatures.
- Once the RNA has been transferred to the membrane it is immobilized through covalent linkage to the membrane by UV light or heat.
- After a probe has been labeled, it is hybridized to the RNA on the membrane.
- The membrane is washed to ensure that the probe has bound specifically.

- The hybrid signals are then detected by X-ray film and can be quantified by densitometry.



Applications

- Northern blotting allows in observing a particular gene's expression pattern between tissues, organs, developmental stages, environmental stress levels, pathogen infection.
- The technique has been used to show over expression of oncogenes and down regulation of tumor-suppressor genes in cancerous cells when compared to 'normal' tissue, as well as the gene expression in the rejection of transplanted organs.
- If an up regulated gene is observed by an abundance of mRNA on the northern blot the sample can then be sequenced to determine if the gene is known to researchers or if it is a novel finding.
- The expression patterns obtained under given conditions can provide insight into the function of that gene.
- Since the RNA is first separated by size, if only one probe type is used variance in the level of each band on the membrane can provide insight

into the size of the product, suggesting alternative splice products of the same gene or repetitive sequence motifs.

- The variance in size of a gene product can also indicate deletions or errors in transcript processing, by altering the probe target used along the known sequence it is possible to determine which region of the RNA is missing.

Advantages & disadvantages

- Analysis of gene expression can be done by several different methods including RT-PCR, RNase protection assays, microarrays, serial analysis of gene expression (SAGE), as well as northern blotting. Microarrays are quite commonly used and are usually consistent with data obtained from northern blots, however at times northern blotting is able to detect small changes in gene expression that microarrays cannot.
- The advantage that microarrays have over northern blots is that thousands of genes can be visualized at a time while northern blotting is usually looking at one or a small number of genes.
- A problem in northern blotting is often sample degradation by RNases (both endogenous to the sample and through environmental contamination) which can be avoided by proper sterilization of glassware and the use of RNase inhibitors such as DEPC (diethylpyrocarbonate). The chemicals used in most northern blots can be a risk to the researcher, since formaldehyde, radioactive material; ethidium bromide, DEPC, and UV light are all harmful under certain exposures.
- Compared to RT-PCR northern blotting has a low sensitivity but it also has a high specificity which is important to reduce false positive results.
- The advantages of using northern blotting include the detection of RNA size, the observation of alternate splice products, the use of probes with partial homology, the quality and quantity of RNA can be measured on the gel prior to blotting, and the membranes can be stored and reprobed for years after blotting.

Western blot

- A western blot, sometimes called a protein immunoblot, is an antibody-based technique used to detect the presence, size and abundance of specific proteins within a sample.
- The technique was developed in 19791 by Harry Towbin and colleagues and later named the "western blot" due to the technique's similarity to Southern blotting.
- Briefly, proteins in an aqueous sample are separated by electrophoresis.
- Following transfer to an appropriate membrane, the samples are probed using target-specific antibodies.
- These antibodies can be detected, and the size and abundance of the bound proteins evaluated in comparison to known standards or controls.

Western blot protocol

- **Western blot gel**
    - Before a western blot can be performed, the proteins in the sample must be separated.
    - This is typically achieved by protein electrophoresis, such as sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) or native PAGE, which separates proteins based on their molecular weight or charge.
    - The specific separation method chosen will depend on the aim of the analysis.
    - For a clean image, samples are centrifuged to remove any solids, in order to load only the soluble fraction.
    - If your protein of interest is in the insoluble fraction (e.g., cell membrane-bound proteins) investigate pretreatment methods to liberate and solubilize it first.
    - Solids will impair the running of the gel and it is likely your protein of interest will remain in the stacking gel.

- It is also important to load appropriate control samples and size marker ladders to enable interpretation of the final blot.

- **Loading control for western blot**
  - It is essential, especially when trying to compare protein expression between different samples, to know how much sample has been loaded as this may not be apparent from the blot alone.
  - For example, when assessing a blot, the band from one sample may appear twice as bright as another sample.
  - This could mean that there is twice as much of the target protein in that sample, or it could mean that more sample or a more concentrated sample has been loaded in one lane than the other.
  - Running a duplicate protein gel and developing with Coomassie stain5 can help to remove this uncertainty as it will show the amount of total protein6 in each sample lane and can reveal any loading inconsistencies.
  - Detecting expression of a ubiquitous protein that should be even between all of your samples, such as actin in whole cell and cytoplasmic samples, can also be used as a loading control and helps to ensure consistent transfer of protein samples to the membrane.
  - However, this type of control can be problematic when comparing models in which “control” proteins are differently expressed, such as degeneration models.

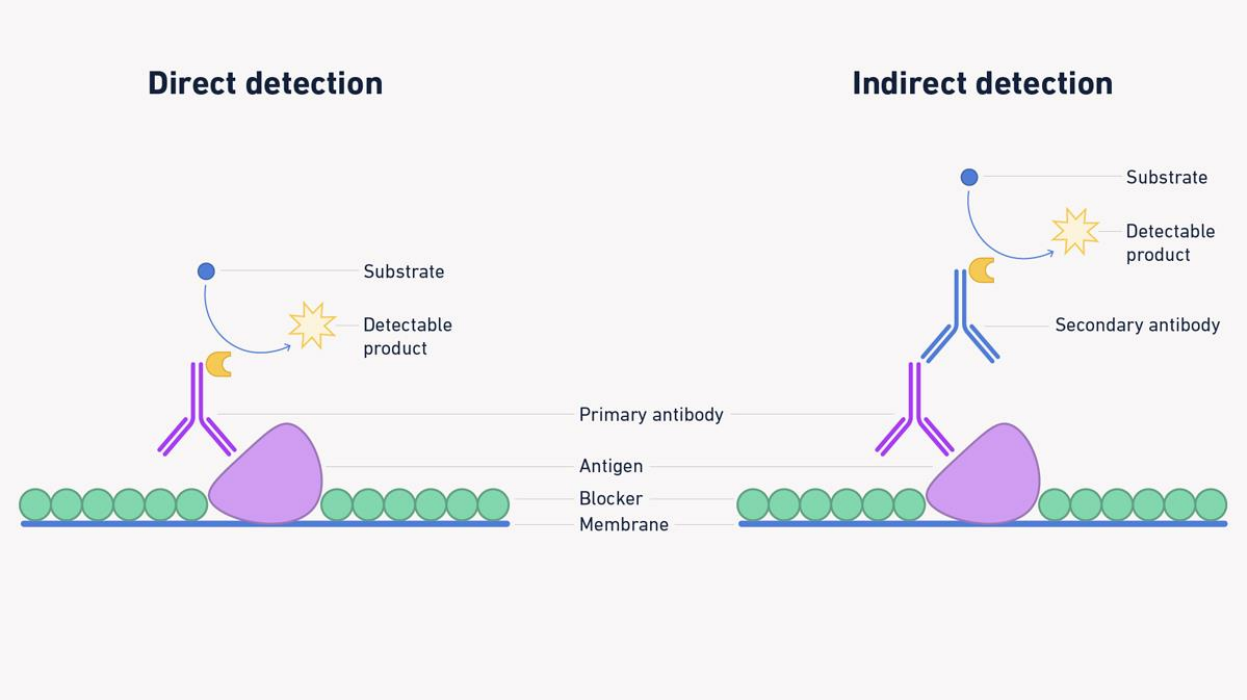
- **Protein transfer (blotting)**
  - Proteins must be transferred from the protein gel to an appropriate membrane (typically nitrocellulose or polyvinylidene difluoride (PVDF)) to facilitate antibody probing.

- A number of techniques can be used for transfer, including capillary transfer, diffusion transfer and **vacuum blotting**, but by far the most common due to its speed and efficiency is **electroblotting** (also called electroelution or electrophoretic transfer).
- Here, the protein gel is sandwiched against the transfer membrane and an electrical current is applied.
- Proteins from the gel are carried across and attach to the membrane tightly.
- Within electroblotting, there are also multiple strategies for transfer, known as wet, semi-dry and dry transfer.
- Wet transfer is efficient and offers flexible buffering but is time-consuming.
- Semi-dry transfer is quicker and still offers flexibility but is less efficient than wet transfer for large proteins.
- Dry transfer is efficient and quick but offers less flexibility than the other methods.
- Transfer efficiency can be assessed prior to probing using a removable stain such as Ponceau S.

- **Western blot blocking**
  - Due to the high affinity of blotting membranes for proteins, after transfer it is important to block any remaining binding sites to prevent subsequent non-specific binding of the assay detection antibodies.
  - This is achieved by incubating the membrane with a proteinaceous liquid such as milk or serum.
- **Western blot washing**
  - Following blocking, it is important to wash the membrane between each step to remove excess or unbound reagents.

- Insufficient or uneven washing can lead to poor quality/patchy blots and high background.
- However, over washing can diminish the target signal so it is important to optimize the number and duration of wash steps.
- Ensure the membrane is well covered with an appropriate buffer and apply gentle agitation to wash the membrane evenly without damaging it.
- Commonly used buffers include tris-buffered saline (TBS) and phosphate-buffered saline (PBS), often with the inclusion of Tween 20 (TBST and PBST).

- **Western blot antibody**

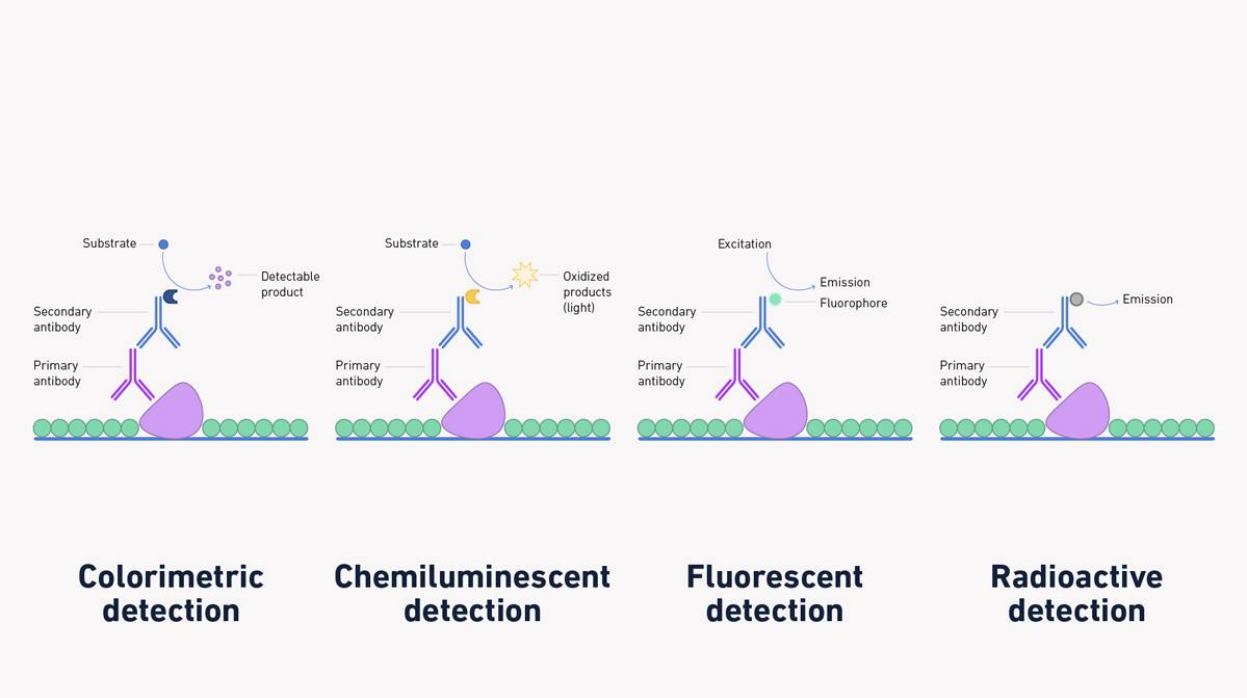


- While it is possible to use direct detection (a single antibody that recognizes the target and is detectable) for a western blot, more often an indirect method is applied.
- Here, a primary antibody is used to probe the membrane and bind any target protein present.

- Then, a secondary antibody is used that binds the primary antibody and is detectable.
- As with all steps, optimization, in this case choosing the "right" antibody and determining the optimal concentration, is key to a good blot.

- **Western blot secondary antibody**
  - When an indirect detection assay is used, a secondary antibody will need to be applied after washing excess unbound primary antibody off the membrane.
  - The secondary antibody should be specific to the species of the primary antibody (e.g., mouse anti-rabbit if the primary antibody was derived from a rabbit) and possess the necessary conjugate for the chosen detection method.

- **Western blot analysis**



- There are multiple methods for detection and subsequent visualization of western blots including **colorimetric, chemiluminescent, fluorescent and radioactive detection**.
- Both **colorimetric and chemiluminescent detection** require conjugation of an enzyme to the detection antibody and are considered very sensitive techniques.
- Horseradish peroxidase (HRP) and alkaline phosphatase (AP) are the most commonly used enzymes, with HRP generally favored due to its stability, amenability to most conjugations and low cost.
- During detection, a substrate is added to the membrane, which is acted on by the conjugated enzyme, bringing about a chemical change.
- If performing colorimetric detection, a chromogenic substrate is chosen that will produce a change that can be visualized and imaged directly.
- However, prompt imaging of the blot is important as colors will fade as the blot dries.
- In chemiluminescent detection, the signal produced only lasts as long as the reaction between the enzyme and substrate is occurring (typically 1–24 hours).
- During this time, the signal can be recorded by exposing X-ray film or using digital imaging to make a permanent record.
- In fluorescent detection, the detection antibody is conjugated with a fluorophore rather than an enzyme.
- When light of a specific wavelength is shone on them, they become excited and emit light of a different specific wavelength.
- This can then be captured visually using digital imagers, such as an avalanche photodiode (APD), photomultiplier tube (PMT) or charge-coupled device (CCD) camera.

- While specialist equipment is required to undertake the excitation and detection steps, there is no substrate step in fluorescent detection, shortening the protocol.
- It is also possible to multiplex fluorophores11 within a western blot assay.
- Radioactive detection, where a radioisotope is conjugated to the detection antibody and the emitted radiation is detected on X-ray film, was used extensively in the past.
- However, the technique requires special handling to protect personnel from the radiation, is expensive and has a limited shelf life due to radioactive decay.
- Therefore, the technique has mostly been replaced in favor of other available detection methods.

Genetic engineering and its applications

Diagnosis of diseases

Genetic engineering techniques have solved the problem of conventional methods for diagnosis of many diseases. **DNA probe, monoclonal antibodies, and antenatal diagnosis** are some of the available methods used as a tool to diagnose a particular disease.

1. **Diagnosis of parasitic disease through DNA probe**

   Probes used for diagnosis of pathogens contain the most specific DNA sequences of genetic material of parasite. The specificity lies in such a way that the other related species or strain do not contain those sequences. These unrelated unspecific sequences of parasite are first recognized by using DNA hybridization technique. Then a DNA sequence, not present in any species, is identified, cleaved by using restriction enzymes, and inserted into a cloning vector (plasmid). The bacterial cells are transformed by the recombinant vector. The tranformants are

multiplied. Finally, the foreign DNA fragments are retrieved from the host cells. The DNA sequences of the parasite, thus obtained, are labeled with radioisotope and used as a probe. The probe can also be chemically synthesized.

Following are the **steps for diagnosis** of a particular disease :

- Isolate the parasite from the infected tissue of the patient. Extract the DNA from parasite and purify it
- Break the DNA by using restriction enzymes to get the DNA fragments of varying size
- Electrophorise the DNA solution of different length by using agarose gel just to get a smear of DNA
- Attach the DNA smear to more firmer support by Southern blotting techniques. Thus the filter paper carries the exact replica of the DNA adhered to it
- Hybridize the immobilized DNA on filter paper by incubating it with radiolabeled probe. Probe DNA complementary to certain DNA sequences of parasite DNA sticks to it and forms the hybrid
- Wash the filter paper to remove unhybridized probe. Keep the filter in contact of x-ray film. Dark bands appear where DNA probe had hybridized the specific target sequence. Thus, a parasitic disease is diagnosed positive. If dark bands on x-ray film do not appear, the absence of parasite is noted.

Specific DNA probes have been designed which have shown good results in diagnosing the infection caused by viruses, bacteria or protozoa. Tuberculosis (T.B.) caused by Mycobacterium tuberculosis is one of such diseases which can be diagnosed by this method. Moreover, a complete testing system for T.B. is marketed by Genprobe Inc, California. Similar efforts have also been made for diagnosis of leprosy, Kala azar (caused by Leishmania donovani), malaria, etc.

2. **Monoclonal antibodies**

- Antibodies are proteins synthesized in blood against specific antigens just to combat and give immunity in blood.
- They can be collected from the blood serum of an animal.
- Such antibodies are heterogeneous and contain a mixture of antibodies (i.e.polyclonal antibodies).
- Therefore, they do not have characteristics of sepcificity.
- If a specific lymphocyte, after isolation and culture in vitro, becomes capable of producing a single type of antibody which bears sepcificity against specific antigen, it is known as 'monoclonal antibody'.
- Due to the presence of desired immunity, monoclonal antibodies are used in the diagnosis of diseases.

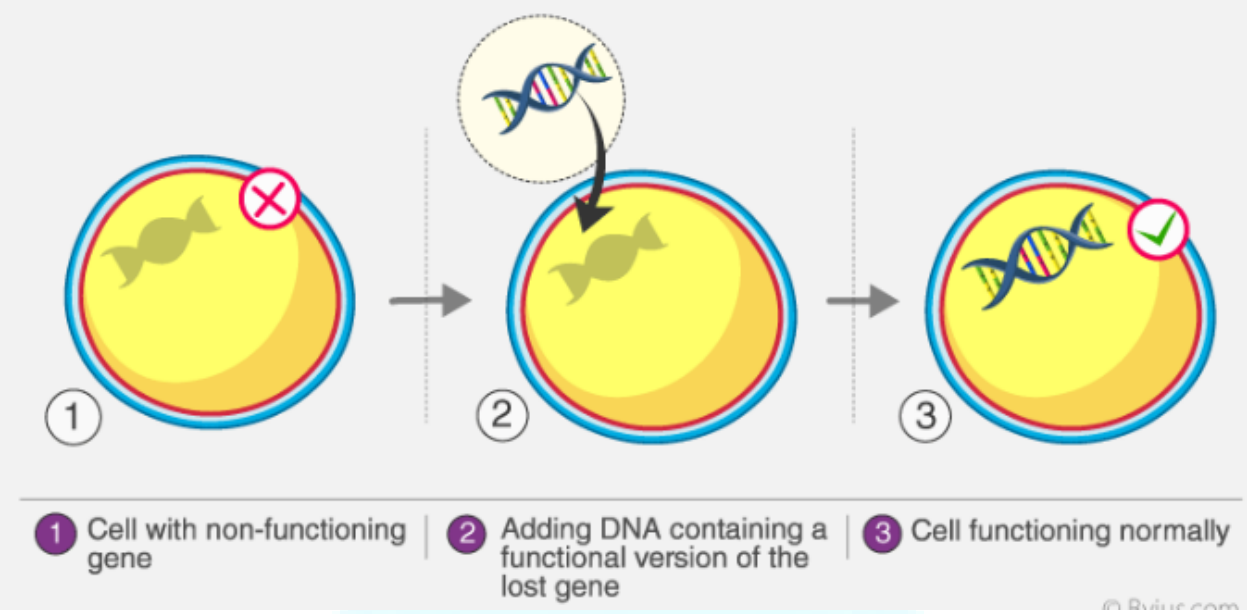
3. **Antenatal diagnosis of haemoglobinopathies**

- There are as many as 2,500 genetic defects known to occur in human beings. Some of them (e.g. phenylketonuria and haemophilia) have been cured but most of them are not understood.
- If a pregnant woman bears a child with a genetic defect, often she is advised to abort it but not to give birth to genetically defected child.
- Thus, the technique of diagnosis and thereby suggestion for abortion of a genetically defected child is known as antenatal diagnosis.
- The genetically determined defects of function or **synthesis of human haemoglobin is known as haemoglobinopathies.**

Gene Therapy

Gene therapy is a technique which involves the replacement of defective genes with healthy ones in order to treat genetic disorders. It is an artificial method that introduces DNA into the cells of the human body. The first gene therapy was successfully accomplished in the year 1989.

The simple process of gene therapy is shown in the figure below:



In the figure, the cell with the defective gene is injected with a normal gene which helps in the normal functioning of the cell. This technique is employed mainly to fight against the diseases in the human body and also to treat genetic disorders. The damaged proteins are replaced in the cell by the insertion of DNA into that cell. Generally, improper protein production in the cell leads to diseases. These diseases are treated using a gene therapy technique. For example, cancer cells contain faulty cells which are different from the normal cells and have defective proteins. Hence, if these proteins are not replaced, this disease would prove to be fatal.

Types of Gene Therapy

Basically, there are two types of gene therapy

1. **Somatic Gene Therapy**

   This type usually occurs in the somatic cells of human body. This is related to a single person and the only person who has the damaged cells will be replaced with healthy cells. In this method, therapeutic genes are transferred into the somatic cells or the stem cells of the human

body. This technique is considered as the best and safest method of gene therapy.

2. **Germline Gene Therapy**

   It occurs in the germline cells of the human body. Generally, this method is adopted to treat the genetic, disease causing-variations of genes which are passed from the parents to their children. The process involves introducing a healthy DNA into the cells responsible for producing reproductive cells, eggs or sperms. Germline gene therapy is not legal in many places as the risks outweigh the rewards.

Application of Gene Therapy

- It is used in the replacement of genes that cause medical ill-health
- The method generally destroys the problem causing genes
- It helps the body to fight against diseases by adding genes to the human body
- This method is employed to treat diseases such as cancer, ADA deficiency, cystic fibrosis, etc.

Metabolites production

Metabolites are the **intermediate products** produced during metabolism, catalyzed by various enzymes that occur naturally within cells. Eg., antibiotics, and pigments. The term metabolites are usually used for small molecules. The various functions of metabolites include; fuel, structure, signalling, catalytic activity, defence and interactions with other organisms.

The metabolites are produced by plants, humans and microbes.

Human Metabolites

- Humans have 2500 metabolites. Prostaglandin produces a metabolite Arachidonic acid. Both the molecules have the same physical properties,

functional groups and are linked by a series of enzyme-catalyzed reactions.

- Cholesterol produces steroid hormones, catecholamines arise from the amino acid tyrosine.
- All the information about the metabolites produced in the human body is freely accessible by an electronic database the Human Metabolome Database (HMDB).

Microbial Metabolites

- Different microbes use different strategies to produce metabolites. These are useful in differentiating between different species of microorganisms.
- Alcohol is one of the most common primary metabolites used for the fermentation- anaerobic respiration process for the production of wine and beer. Aspergillus niger produces citric acid widely used in food, pharmaceuticals and cosmetics industries.

Biocontrol agents

The biocontrol agents protect plants from their natural enemies like parasites from predation, etc. They help in controlling the infestation of plant pests such as weeds, nematodes, insects, and mites. The biological control agents are specific to harmful organisms and do not kill useful organisms present in the soil.

Types of Bio-Control Agents

Biological control can be categorized into **two types:**

1. **Classical biocontrol (Importation):** It uses the natural enemy of the pest as a biocontrol agent. This method adopts natural predators of the

invasive plant but sometimes the introduced species can become serious pests, if not chosen wisely.

2. **Inductive biocontrol (Augmentation):** A large number of natural enemies are released to kill the target weed. Nematodes are most commonly used in the inundative release.

List of Bio-Control Agents

- Insect Predators such as spiders, flies, ladybugs, wasps, beetles, and dragonflies.
- Pathogenic microorganisms such as viruses, bacteria, fungi, etc. Coccobacillus bacteria are more pathogenic to insects. They affect the digestive system of insects and are used against insects and aphids. The fungi Entomophaga is used against green peach aphids.
- Parasitoids lay eggs in the host body and kill it. It is later used as a source of food for the developing larva. It is commonly used as a biocontrol agent.

Merits and demerits of Bio-Control Agents

Merits

- Cost-effective.
- Reduces the use of chemicals and other pesticides.
- They are environment friendly and with no side effects.
- Effective in all the seasons, easy to use and easily available.

Demerits

- It affects product quality.
- Pest is not completely removed.
- Not very effective for short-scale applications.

ENTRI

Biofuels

- Any hydrocarbon fuel that is produced from an organic matter (living or once living material) in a short period of time (days, weeks, or even months) is considered a biofuel.
- Biofuels may be solid, liquid or gaseous in nature.
    - **Solid:** Wood, dried plant material, and manure
    - **Liquid:** Bioethanol and Biodiesel
    - **Gaseous:** Biogas
- These can be used to replace or can be used in addition to diesel, petrol or other fossil fuels for transport, stationary, portable and other applications. Also, they can be used to generate heat and electricity.
- Some of the main reasons for shifting to biofuels are the rising prices of oil, emission of the greenhouse gases from fossil fuels and the interest for obtaining fuel from agricultural crops for the benefit of farmers.

Categories of Biofuels

1. **First generation biofuels:**
    - These are made from food sources such as sugar, starch, vegetable oil, or animal fats using conventional technology.
    - Common first-generation biofuels include Bioalcohols, Biodiesel, Vegetable oil, Bioethers, Biogas.
    - Though the process of conversion is easy, but use of food sources in the production of biofuels creates an imbalance in food economy, leading to increased food prices and hunger.

2. **Second generation biofuels:**
    - These are produced from non-food crops or portions of food crops that are not edible and considered as wastes, e.g. stems, husks, wood chips, and fruit skins and peeling.
    - Thermochemical reactions or biochemical conversion process is used for producing such fuels.

- Examples include cellulose ethanol, biodiesel.
- Though these fuels do not affect food economy, their production is quite complicated.
- Also, it is reported that these biofuels emit less greenhouse gases when compared to first generation biofuels.

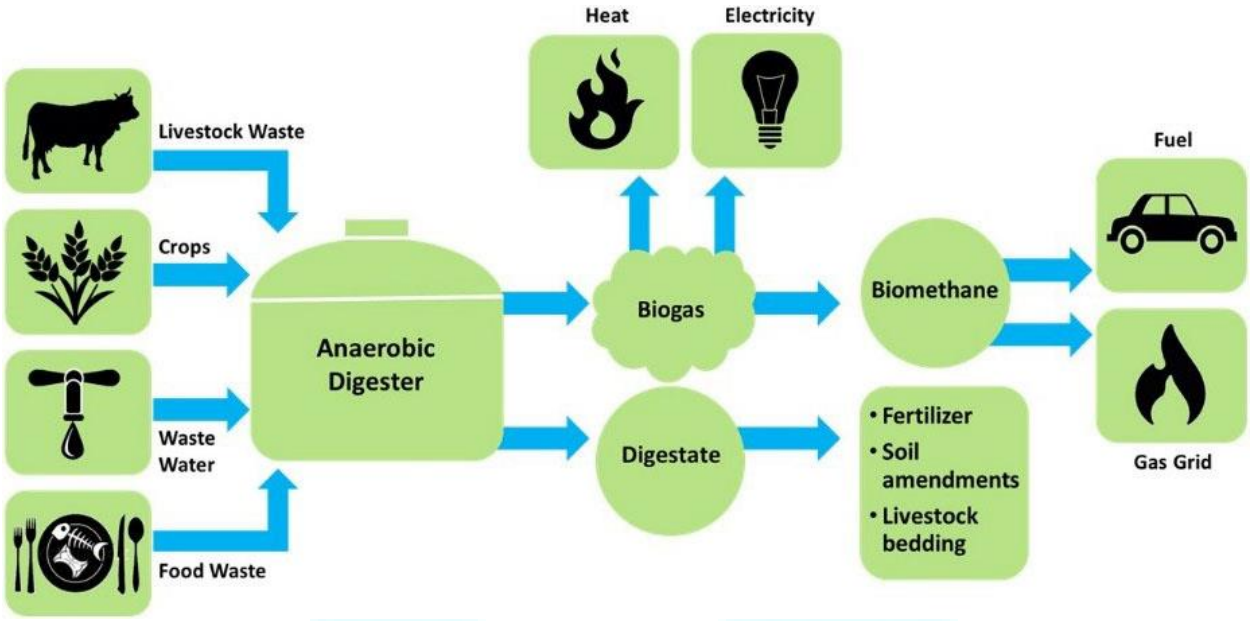
3. **Third generation biofuels:**
    - These are produced from micro-organisms like algae.
    - Example- Butanol
    - Micro-organisms like algae can be grown using land and water unsuitable for food production, therefore reducing the strain on already depleted water sources.
    - One disadvantage is that fertilizers used in the production of such crops lead to environment pollution.

4. **Fourth Generation Biofuels:**
    - In the production of these fuels, crops that are genetically engineered to take in high amounts of carbon are grown and harvested as biomass.
    - The crops are then converted into fuel using second generation techniques.
    - The fuel is pre-combusted and the carbon is captured. Then the carbon is geo-sequestered, meaning that the carbon is stored in depleted oil or gas fields or in unmineable coal seams.
    - Some of these fuels are considered as carbon negative as their production pulls out carbon from environment.

Biogas

- It mainly comprises hydro-carbon which is combustible and can produce heat and energy when burnt.

- Biogas is produced through a biochemical process in which certain types of bacteria convert the biological wastes into useful bio-gas.



- Since the useful gas originates from a biological process, it has been termed as bio-gas.
    - Methane gas is the main constituent of biogas.

Tansgenic Animals

- **Trangenic animals** are the animals with the modified genome.
- A foreign gene is inserted into the genome of the animal to alter its DNA.
- This method is done to improve the genetic traits of the target animal.
- Initially, the improvement of genetic traits was done by selective breeding methods.
- In this, the animals with desired genetic characteristics were mated to produce an individual with improved genetic characteristics.
- Since this technique was time-consuming and expensive, it was later replaced by recombinant DNA technology.
- **Transgenesis** is the phenomenon in which a foreign gene with desired characteristics is introduced into the genome of the target animal.

- The foreign gene that is introduced is known as the transgene, and the animal whose genome is altered is known as transgenic.
- These genes are passed on to the successive generations.
- The transgenic animals are genetically engineered and are also known as genetically modified organisms.
- The first genetically modified organism was engineered in the year 1980.
- Let us have a detailed look at the process, importance and applications of transgenic animals.

Methods for Creating Transgenic Animals

The transgenic animals are created by the following methods:

- **Physical Transfection**
  - In this method, the gene of interest is directly injected into the pronucleus of a fertilized ovum. It is the very first method that proved to be effective in mammals. This method was applicable to a wide variety of species. Other methods of physical transfection include particle bombardment, ultrasound and electroporation.

- **Chemical Transfection**
  - One of the chemical methods of gene transfection includes transformation. In this method, the target DNA is taken up in the presence of calcium phosphate. The DNA and calcium phosphate co-precipitates, which facilitates DNA uptake. The mammalian cells possess the ability to take up foreign DNA from the culture medium.

- **Retrovirus-Mediated Gene Transfer**
  - To increase the chances of expression, the gene is transferred by means of a vector. Since retroviruses have the ability to infect the

host cell, they are used as vectors to transfect the gene of interest into the target genome.

- **Viral Vectors**
  - Viruses are used to transfect rDNA into the animal cell. The viruses possess the ability to infect the host cell, express well and replicate efficiently.

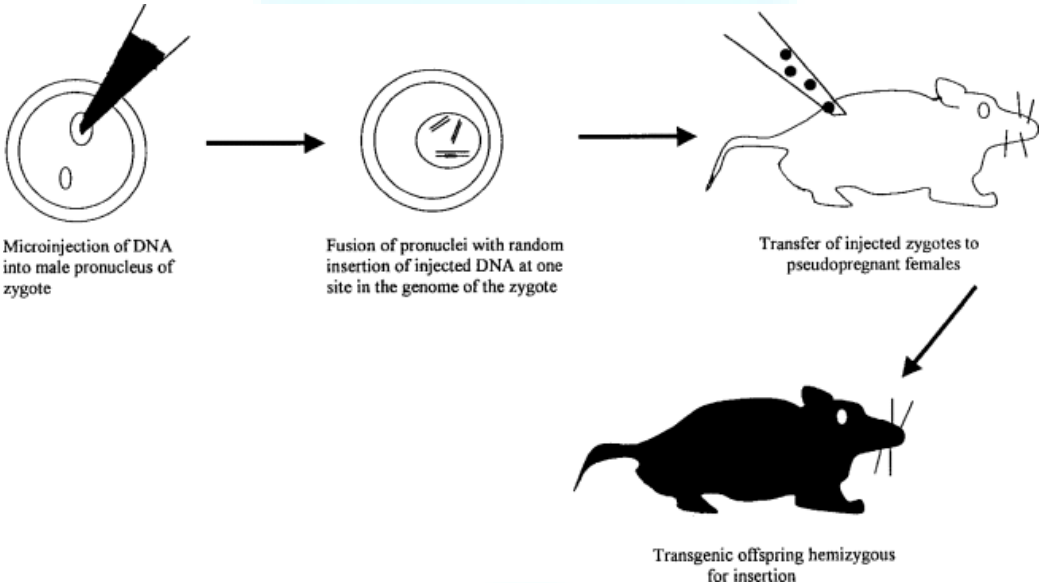
- **Bactofection**
  - It is the process by which the gene of interest is transferred into the target gene with the help of bacteria.

Examples of Transgenic Animals

Following are the examples of transgenic animals:

- **Dolly Sheep**
  - Dolly the sheep was the first mammal to be cloned from an adult cell. In this, the udder cells from a 6-year-old Finn Dorset white sheep were injected into an unfertilized egg from a Scottish Blackface ewe, which had its nucleus removed. The cell was made to fuse by electrical pulses. After the fusion of the nucleus of the cell with the egg, the resultant embryo was cultured for six to seven days. It was then implanted into another Scottish Blackface ewe which gave birth to the transgenic sheep, Dolly.

- **Transgenic Mice**
  - Transgenic mice are developed by injecting DNA into the oocytes or 1-2 celled embryos taken from female mice. After injecting the DNA, the embryo is implanted into the uterus of receptive females.



Applications Of Transgenic Animals

The transgenic animals are created because of the benefits they provide to the man. Let us discuss a few of them here.

- **Normal Physiology and Development**
  - In transgenic animals, a foreign gene is introduced due to which the growth factor is altered. Hence, these animals facilitate the

study of gene regulation and their effect on the everyday functions of the body.

- **Study of Diseases**
  - Transgenic animals are specially designed to study the role of genes in the development of certain diseases. Moreover, in order to devise a cure for these diseases, the transgenic animals are used as model organisms. These transgenic models are used in research for the development of medicines. For example, we have transgenic models for diseases such as Alzheimer's and cancer.

- **Biological Products**
  - A number of biological products such as medicines and nutritional supplements are obtained from transgenic animals. Research for the manufacture of medicines to treat diseases such as phenylketonuria (PKU) and hereditary emphysema is going on. The first transgenic cow, Rosie (1997), produced milk containing human protein (2.4 grams per litre). This milk contains the human gene alpha-lactalbumin and could be given to babies as an alternative to natural cow milk.

- **Vaccine Safety**
  - Transgenic animals are used as model organisms for testing the safety of vaccines before they are injected into humans. This was conventionally done on monkeys.