DNA-Technology

Part II

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Introduction

Advances in human genetics are one of the most rapidly occurring developments in medicine. There is no doubt that every physician who practices in the 21st century must have an in-depth knowledge of the principles of human genetics and their applications which span a wide variety of clinical problems.

The aim of this core-course is to acquaint the students to versatile tools and techniques employed in genetic engineering and recombinant DNA technology. A sound knowledge on methodological repertoire allows students to innovatively apply these in basic and applied fields of biological research.

This course provides theoretical bases to properties and applications of versatile DNA modifying enzymes, cloning strategies, vector types, host genotype specificities for selection and screening of recombinants and/or recombinant transformants. Students will also be introduced to prominent nucleic acid labeling techniques. Introduction to various types of vectors viz. cloning, transformation, expression; and also vectors for genomic and cDNA library and whole genome sequencing will be provided.

At the end of this course, you should be able to:

- To illustrate creative use of modern tools and techniques for manipulation and analysis of genomic sequences.
- To expose students to application of recombinant DNA technology in biotechnological research.
- To train students in strategizing research methodologies employing genetic engineering techniques.

Restriction Nucleases

A **restriction enzyme** is a nuclease enzyme that cleaves DNA sequence at a random or specific recognition sites known as restriction sites. In bacteria, restriction enzymes form a combined system (restriction+ modification system) with modification enzymes that methylate the bacterial DNA. Methylation of bacterial DNA at the recognition sequence typically protects the own DNA of the bacteria from being cleaved by restriction enzyme.

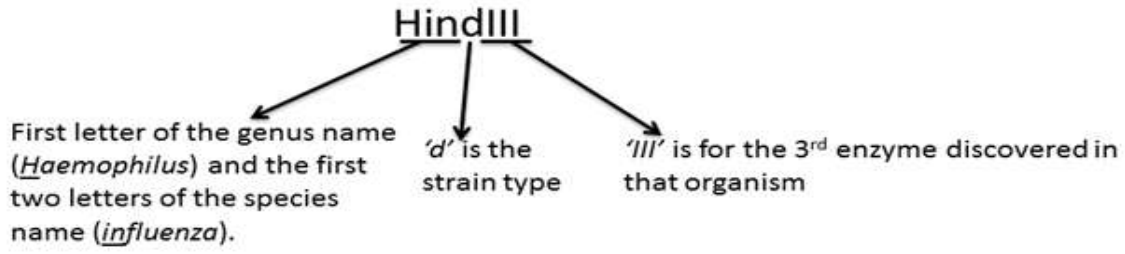
There are two different kinds of restriction enzymes:

(1) Exonucleases catalyses hydrolysis of terminal nucleotides from the end of DNA or RNA molecule either 5'to 3' direction or 3' to 5' direction. Example: exonuclease I, exonuclease II etc.

(2) Endonucleases can recognize specific base sequence (restriction site) within DNA or RNA molecule and cleave internal phosphodiester bonds within a DNA molecule. Example: EcoRI, Hind III, BamHI etc.

Restriction Endonuclease Nomenclature:

Restriction endonucleases are named according to the organism in which they were discovered, using a system of letters and numbers. For example, *HindIII* (pronounced *"hindee-three")* was discovered in *Haemophilus influenza* (strain d). The Roman numerals are used to identify specific enzymes from bacteria that contain multiple restriction enzymes indicating the order in which restriction enzymes were discovered in a particular strain.



Classification of Restriction Endonucleases

There are three major classes of restriction endonucleases based on the types of sequences recognized, the nature of the cut made in the DNA, and the enzyme structure:

• Type I restriction enzymes

• Type II restriction enzymes

• Type III restriction enzymes

Type I restriction enzymes

• These enzymes have both restriction and modification activities. Restriction depends upon the methylation status of the target DNA.

• Cleavage occurs approximately 1000 bp away from the recognition site.

• The recognition site is asymmetrical and is composed of two specific portions in which one portion contain 3–4 nucleotides while another portion contain 4–5 nucleotides and both the parts are separated by a non-specific spacer of about 6–8 nucleotides.

• They require S-adenosylmethionine (SAM), ATP, and magnesium ions (Mg^{2+}) for activity.

• These enzymes are composed of mainly three subunits, a specificity subunit that determines the DNA recognition site, a restriction subunit, and a modification subunit.

Type II restriction enzymes

• Restriction and modification are mediated by separate enzymes so it is possible to cleave DNA in the absence of modification. Although the two enzymes recognize the same target sequence, they can be purified separately from each other.

• Cleavage of nucleotide sequence occurs at the restriction site.

• These enzymes are used to recognize rotationally symmetrical sequence which is often referred as palindromic sequence.

• These palindromic binding site may either be interrupted (e.g. BstEII recognizes the sequence 5´-GGTNACC-3´, where N can be any nucleotide) or continuous (e.g. KpnI recognizes the sequence 5´-GGTACC-3´).

• They require only Mg^{2+} as a cofactor and ATP is not needed for their activity.

• Type II endonucleases are widely used for mapping and reconstructing DNA *in vitro* because they recognize specific sites and cleave just at these sites.

The steps involved in DNA binding and cleavage by a type II restriction endonuclease:

- These enzymes have nonspecific contact with DNA and initially bind to DNA as dimmers.
- The target site is then located by a combination of linear diffusion or "sliding" of the enzyme along the DNA over short distances, and hopping/jumping over longer distances.
- Once the target restriction site is located, the recognition process (coupling) triggers large conformational changes of the enzyme and the DNA, which leads to activation of the catalytic center.

- Catalysis results in hydrolysis of phosphodiester bond and product release.

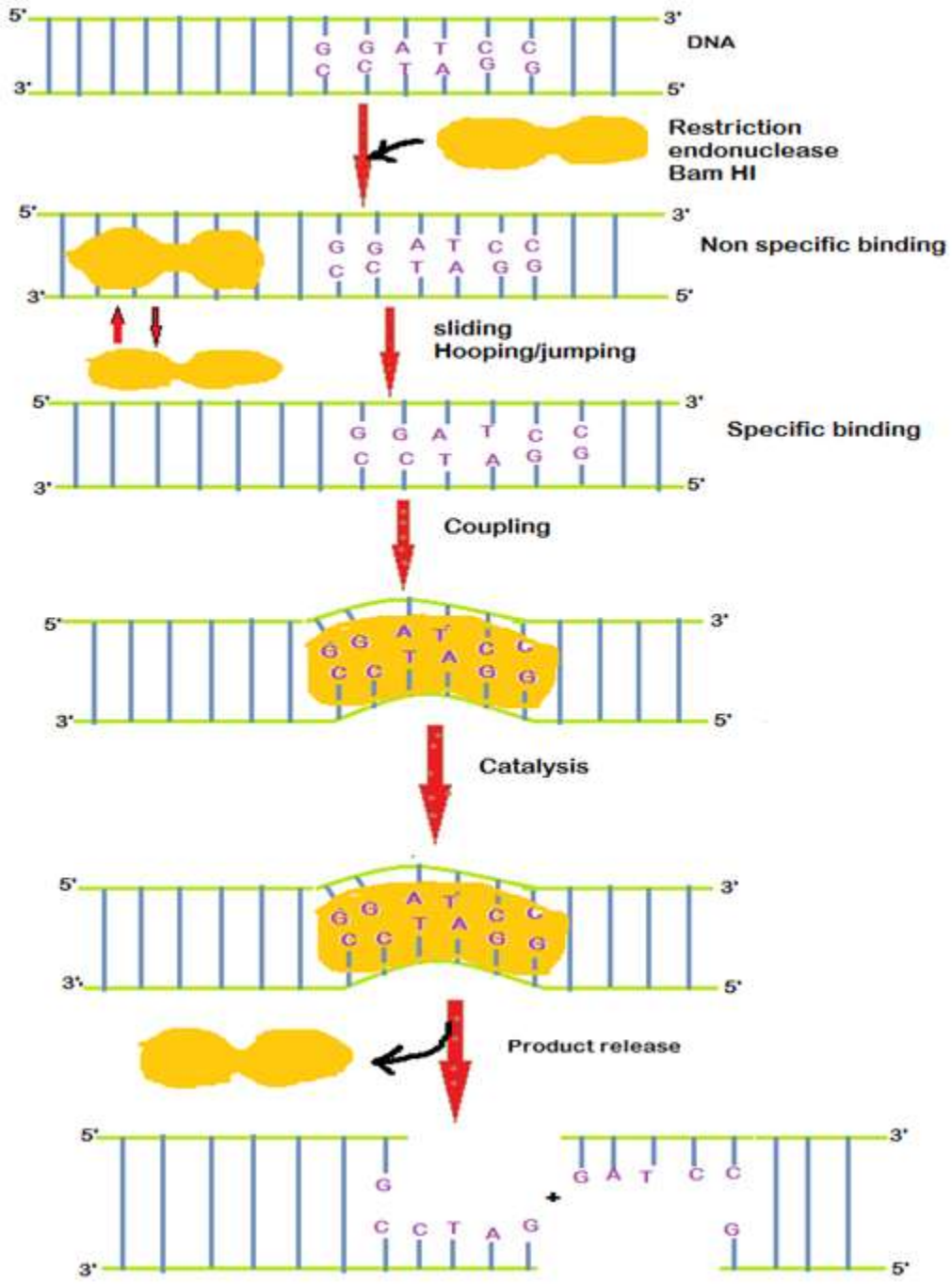


Fig (1): Structures of free, nonspecific, and specific DNA-bound forms of BamHI. The two dimers are shown in brown, the DNA backbone is in green and the bases

in gray. *BamH* I become progressively more closed around the DNA as it goes from the nonspecific to specific DNA binding mode.

Type III restriction enzymes:

• These enzymes recognize and methylate the same DNA sequence but cleave 24–26 bp away.

• They have two different subunits, in which one subunit (M) is responsible for recognition and modification of DNA sequence and other subunit (R) has nuclease action.

• Mg^{+2} ions, ATP are needed for DNA cleavage and process of cleavage is stimulated by SAM.

• Cleave only one strand. Two recognition sites in opposite orientation are necessary to break the DNA duplex.

Applications:

In various applications related to genetic engineering DNA is cleaved by using these restriction enzymes.

• They are used in the process of insertion of genes into plasmid vectors during gene cloning and protein expression experiments.

• Restriction enzymes can also be used to distinguish gene alleles by specifically recognizing single base changes in DNA known as single nucleotide polymorphisms (SNPs). This is only possible if a mutation alters the restriction site present in the allele.

• Restriction enzymes are used for Restriction Fragment Length Polymorphism (RFLP) analysis for identifying individuals or strains of a particular species.

Comparative properties of restriction enzymes:

Property	Type I RE	Type II RE	Type III RE
Abundance	Less common than Type II	Most common	Rare
Recognition site	Cut both strands at a non- specific location > 1000 bp away from recognition site	Cut both strands at a specific, usually palindromic recognition site (4-8 bp)	Cleavage of one strand, only 24-26 bp downstream of the 3´ recognition site
Restriction and modification	Single multifunctional enzyme	Separate nuclease and methylase	Separate enzymes sharing a common subunit
Nuclease subunit structure	Heterotrimer	Homodimer	Heterodimer
Cofactors	ATP, Mg^{2+} , SAM	Mg^{2+}	Mg^{2+} (SAM)
DNA cleavage requirements	Two recognition sites in any orientation	Single recognition Site	Two recognition sites in a head-to-head orientation
Enzymatic turnover	No	Yes	Yes
DNA translocation	Yes	No	No
Site of methylation	At recognition site	At recognition site	At recognition site

DNA cloning

DNA cloning is the process of making multiple, identical copies of a particular piece of DNA. In a typical DNA cloning procedure, the gene or other DNA fragment of interest (perhaps a gene for a medically important human protein) is first inserted into a circular piece of DNA called a **plasmid**. The insertion is done using enzymes that "cut and paste" DNA, and it produces a molecule of **recombinant DNA**, or DNA assembled out of fragments from multiple sources.

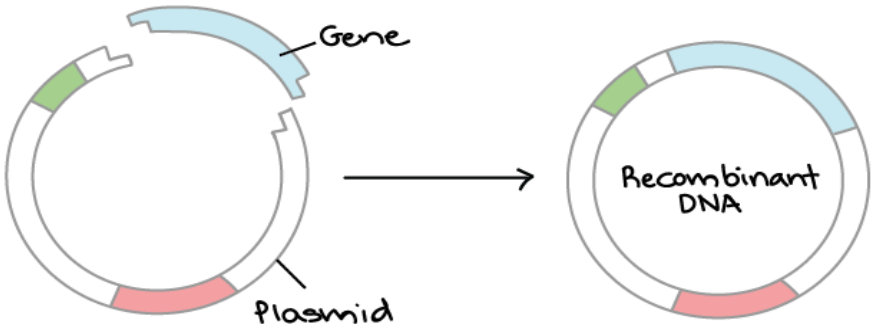


Fig (2): Diagram showing the construction of a recombinant DNA molecule.

A circular piece of plasmid DNA has overhangs on its ends that match those of a gene fragment. The plasmid and gene fragment are joined together to produce a gene-containing plasmid. This gene-containing plasmid is an example of recombinant DNA, or a DNA molecule assembled from DNA from multiple sources.

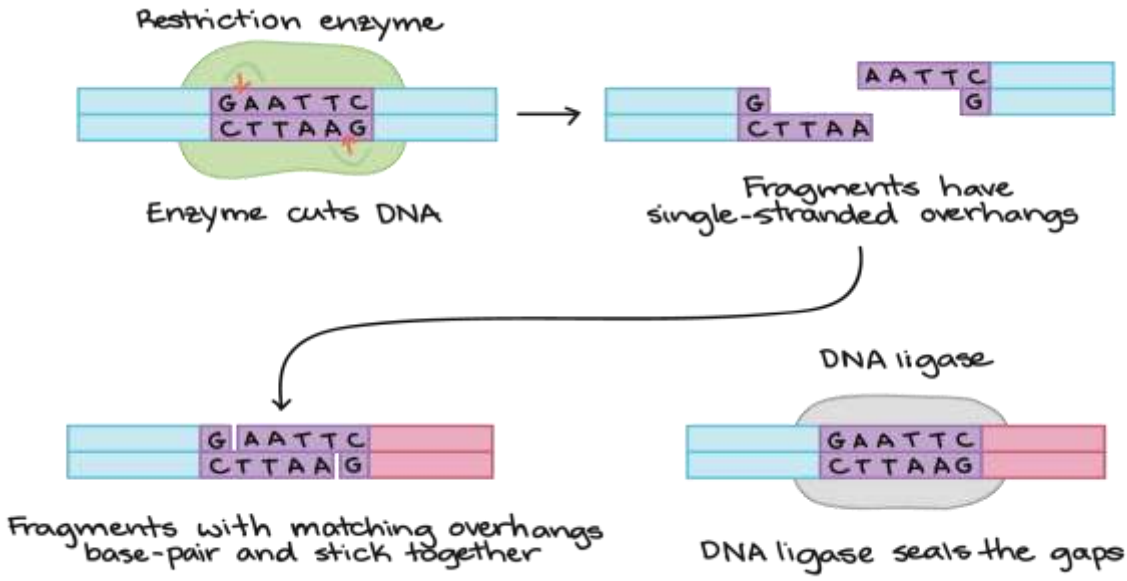
Next, the recombinant plasmid is introduced into bacteria. Bacteria carrying the plasmid are selected and grown up. As they reproduce, they replicate the plasmid and pass it on to their offspring, making copies of the DNA it contains.

The basic steps are:

Step (1) Cutting and pasting DNA:

Cut open the plasmid and "paste" in the gene. This process relies on restriction enzymes (which cut DNA) and DNA ligase (which joins DNA).

A **restriction enzyme** is a DNA-cutting enzyme that recognizes a specific target sequence and cuts DNA into two pieces at or near that site. Many restriction enzymes produce cut ends with short, single-stranded overhangs. If two molecules have matching overhangs, they can base-pair and stick together. However, they won't combine to form an unbroken DNA molecule until they are joined by **DNA ligase**, which seals gaps in the DNA backbone.



Our goal in cloning is to insert a target gene (e.g., for human insulin) into a plasmid. Using a carefully chosen restriction enzyme, we digest:

- The plasmid, which has a single cut site
- The target gene fragment, which has a cut site near each end

Then, we combine the fragments with DNA ligase, which links them to make a recombinant plasmid containing the gene.

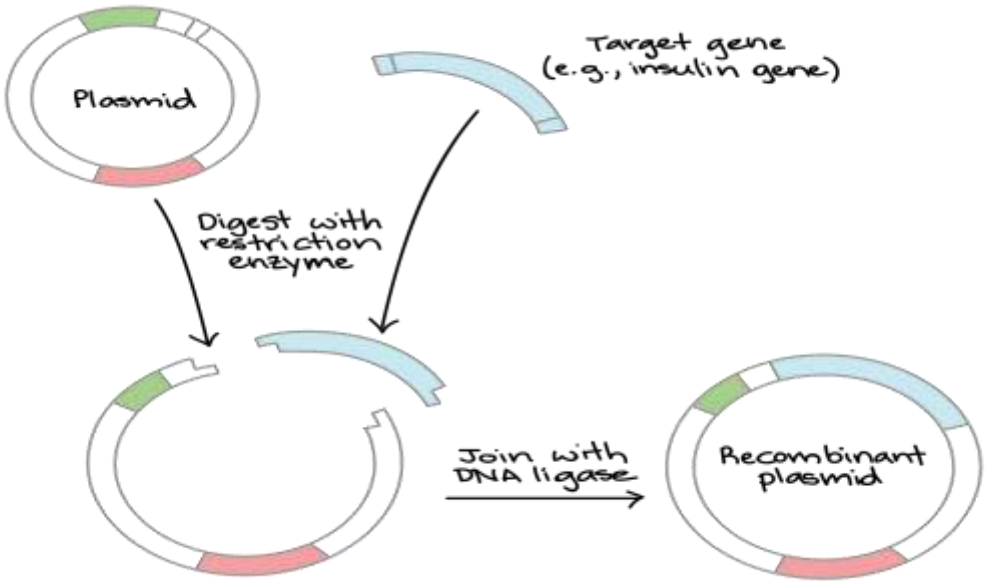


Fig (3): Diagram depicting restriction digestion and ligation in a simplified schematic.

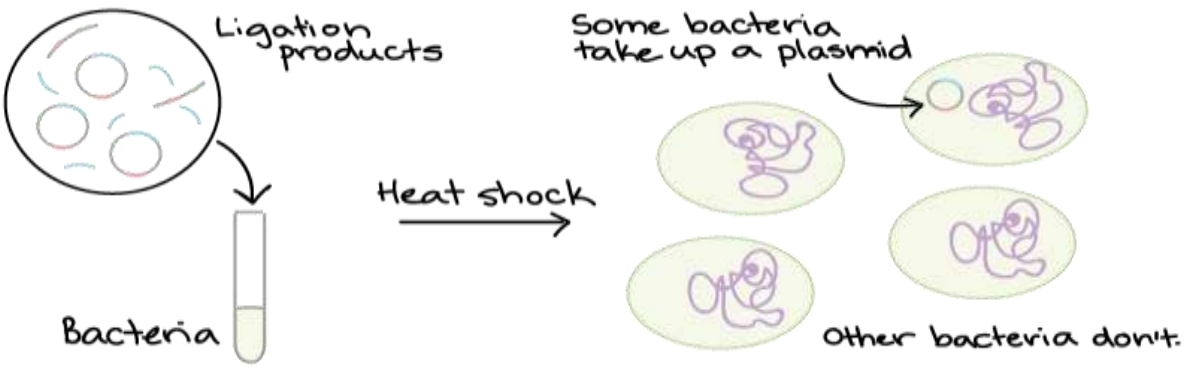
We start with a circular bacterial plasmid and a target gene. On the two ends of the target gene are restriction sites, or DNA sequences recognized by a particular restriction enzyme. In the plasmid, there is also a restriction site recognized by that same enzyme, right after a promoter that will drive expression in bacteria.

Both the plasmid and the target gene are (separately) digested with the restriction enzyme. The fragments are purified and combined. They have matching "sticky ends," or single-stranded DNA overhangs, so they can stick together.

The enzyme DNA ligase joins the fragments with matching ends together to form a single, unbroken molecule of DNA. This produces a recombinant plasmid that contains the target gene.

Step (2) Bacterial transformation and selection:

Plasmids and other DNA can be introduced into bacteria, such as the harmless *E. coli* used in labs, in a process called **transformation**. During **transformation**, specially prepared bacterial cells are given a shock (such as high temperature) that encourages them to take up foreign DNA.



The DNA produced by ligation (which may be a mix of desired plasmids, side-product plasmids, and linear DNA pieces) is added to bacteria. The bacteria are given a heat shock, which makes them more apt to take up DNA by transformation. However, only a tiny minority of the bacteria will successfully take up a plasmid.

A plasmid typically contains an **antibiotic resistance gene**, which allows bacteria to survive in the presence of a specific antibiotic. Thus, bacteria that took up the plasmid can be selected on nutrient plates containing the antibiotic. Bacteria without a plasmid will die, while bacteria carrying a plasmid can live and reproduce. Each surviving bacterium will give rise to a small, dot-like group, or **colony**, of identical bacteria that all carry the same plasmid.

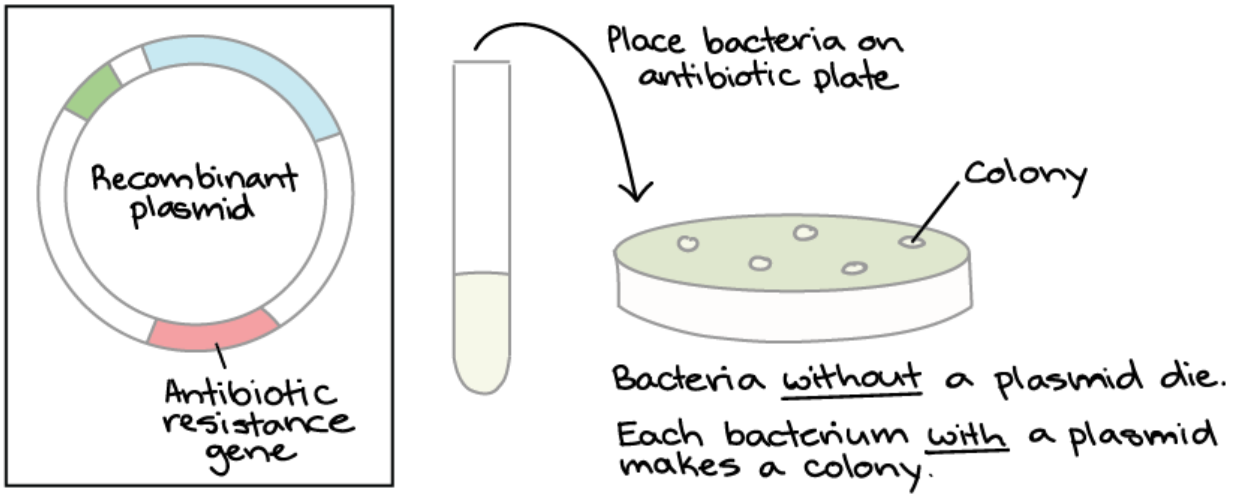


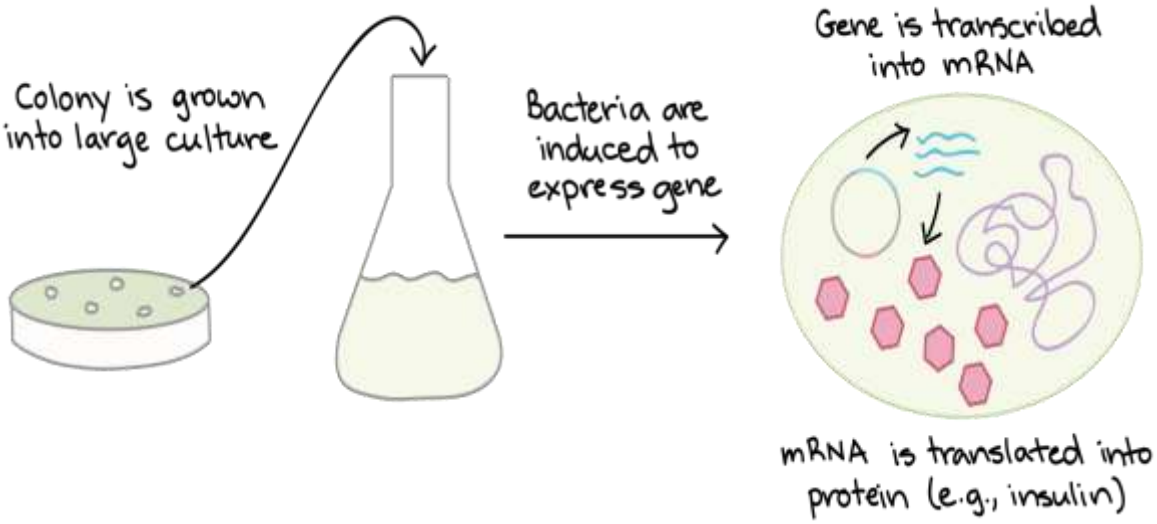
Fig (4): Left panel: Diagram of plasmid, showing that it contains an antibiotic resistance gene. **Right panel:** all the bacteria from the transformation are placed on an antibiotic plate. Bacteria without a plasmid will die due to the antibiotic. Each

bacterium with a plasmid makes a colony, or a group of clonal bacteria that all contain the same plasmid. A typical colony looks like a small, whitish dot the size of a pinhead.

Step (3) Protein production:

Grow up lots of plasmid-carrying bacteria and use them as "factories" to make the protein. Harvest the protein from the bacteria and purify it.

The bacteria serve as miniature “factories," churning out large amounts of protein. For instance, if our plasmid contained the human insulin gene, the bacteria would start transcribing the gene and translating the mRNA to produce many molecules of human insulin protein.



A selected colony is grown up in a large culture (e.g., a 1-liter flask). The bacteria in the large culture are induced to express the gene contained in the plasmid, causing the gene to be transcribed into mRNA, and the mRNA to be translated into protein. The protein encoded by the gene accumulates inside of the bacteria.

Once the protein has been produced, the bacterial cells can be split open to release it. There are many other proteins and macromolecules floating around in bacteria besides the target protein (e.g., insulin). Because of this, the target protein must be **purified**, or separated from the other contents of the cells by biochemical techniques. The purified protein can be used for experiments or, in the case of insulin, administered to patients.

Uses of DNA cloning:

DNA molecules built through cloning techniques are used for many purposes in molecular biology. A short list of examples includes:

- **Biopharmaceuticals.** DNA cloning can be used to make human proteins with biomedical applications, such as the insulin mentioned above. Other examples of recombinant proteins include human growth hormone, which is given to patients who are unable to synthesize the hormone, and tissue plasminogen activator (tPA), which is used to treat strokes and prevent blood clots. Recombinant proteins like these are often made in bacteria.
- **Gene therapy.** In some genetic disorders, patients lack the functional form of a particular gene. Gene therapy attempts to provide a normal copy of the gene to the cells of a patient’s body. For example, DNA cloning was used to build plasmids containing a normal version of the gene that's nonfunctional in cystic fibrosis. When the plasmids were delivered to the lungs of cystic fibrosis patients, lung function deteriorated less quickly^22start superscript, 2, end superscript.
- **Gene analysis.** In basic research labs, biologists often use DNA cloning to build artificial, recombinant versions of genes that help them understand how normal genes in an organism function.

DNA Library

A DNA library is a collection of DNA fragments that have been cloned into vectors so that researchers can identify and isolate the DNA fragments that interest them for further study.

There are basically two kinds of libraries:

A. Genomic DNA libraries

Genomic library refers to a large collection of bacterial cells, each containing a random piece of human genomic DNA. For constructing a gene library, the entire DNA of cell is cleaved into small pieces by using different REs. All these cut fragments are then introduced into appropriate vectors.

This forms a large collection of different recombinant clones, which are then introduced into the host bacterial cells to form the gene library. All the genes of an organism are represented in the gene library. In order to produce a complete gene library for E. coli , about 1500 fragments are required, whereas about 10 lakhs fragments are required for human gene library

B. cDNA Library

cDNA library is a collection of all the expressed DNA of a particular cell type or tissue . For example, a cDNA from pancreatic β-cell contains clones with cDNA for proinsulin.

On the other hand, a cDNA library from bone marrow cell contains many clones with cDNA for α- and β-chains of haemoglobin. Thus, for a cDNA library the tissue of origin is important.

For a genomic library, the tissue of origin is unimportant because the genomic material is same in all cell types of an organism.

Building of cDNA library: The mRNA is extracted from a specific tissue. It is used as a template for synthesis of complementary DNA strand; the enzyme catalyzing this synthesis is reverse transcriptase, which yields a single-stranded cDNA. Double-stranded cDNA is then obtained from it by adding the DNA polymerase. The latter is incorporated in plasmid, λ phage or cosmid and introduced into host bacterial cell.

DNA Repair

DNA, like any other molecule, can undergo a variety of chemical reactions. Because DNA uniquely serves as a permanent copy of the cell genome, however, changes in its structure are of much greater consequence than are alterations in other cell components, such as RNAs or proteins.

Mutations can result from the incorporation of incorrect bases during DNA replication. In addition, various chemical changes occur in DNA either spontaneously or as a result of exposure to chemicals or radiation. Such damage to DNA can block replication or transcription, and can result in a high frequency of mutations—consequences that are unacceptable from the standpoint of cell reproduction. To maintain the integrity of their genomes, cells have therefore had to evolve mechanisms to repair damaged DNA.

These mechanisms of DNA repair can be divided into three general classes:

Direct reversal: Some DNA-damaging chemical reactions can be directly "undone" by enzymes in the cell.

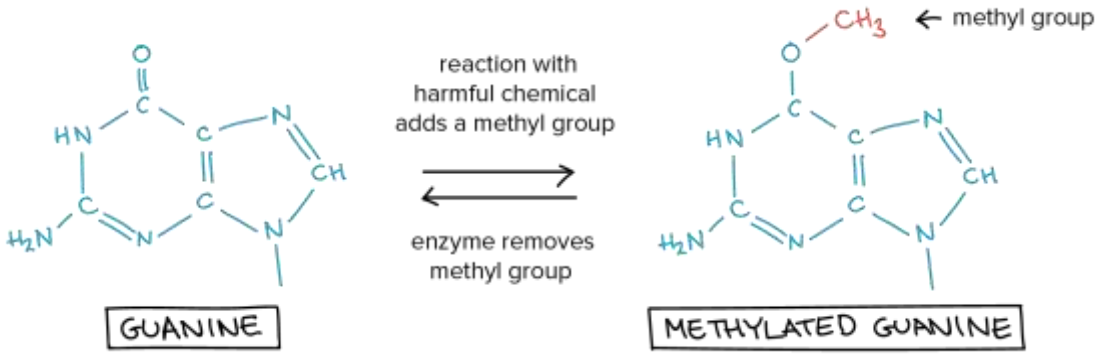
Excision repair: Damage to one or a few bases of DNA is often fixed by removal (excision) and replacement of the damaged region. In base excision repair, just the damaged base is removed. In nucleotide excision repair a patch of nucleotides is removed.

Double-stranded break repair: Two major pathways, non-homologous end joining and homologous recombination, are used to repair double-stranded breaks in DNA (that is, when an entire chromosome splits into two pieces).

Reversal of damage

In some cases, a cell can fix DNA damage simply by reversing the chemical reaction that caused it.

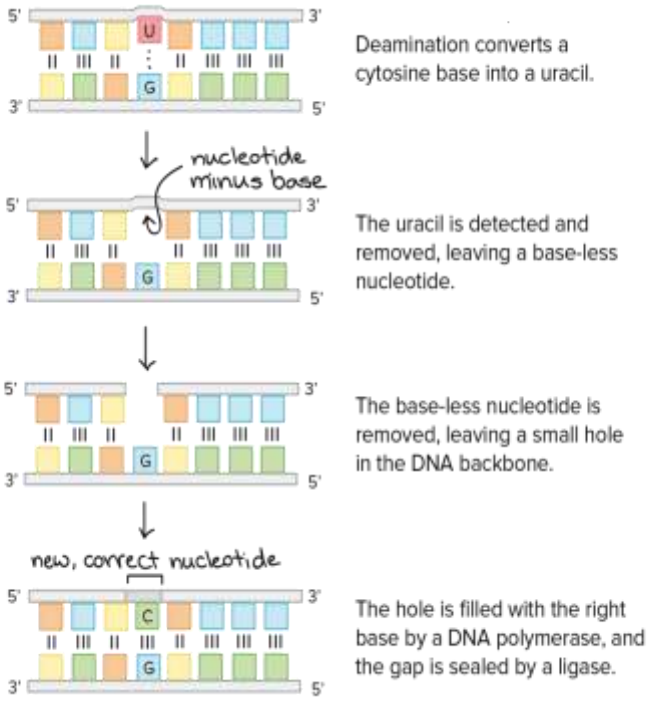
For example, guanine (G) can undergo a reaction that attaches a methyl {-CH_3} group to an oxygen atom in the base. The methyl-bearing guanine, if not fixed, will pair with thymine (T) rather than cytosine (C) during DNA replication. Luckily, humans and many other organisms have an enzyme that can remove the methyl group, reversing the reaction and returning the base to normal.



Base excision repair

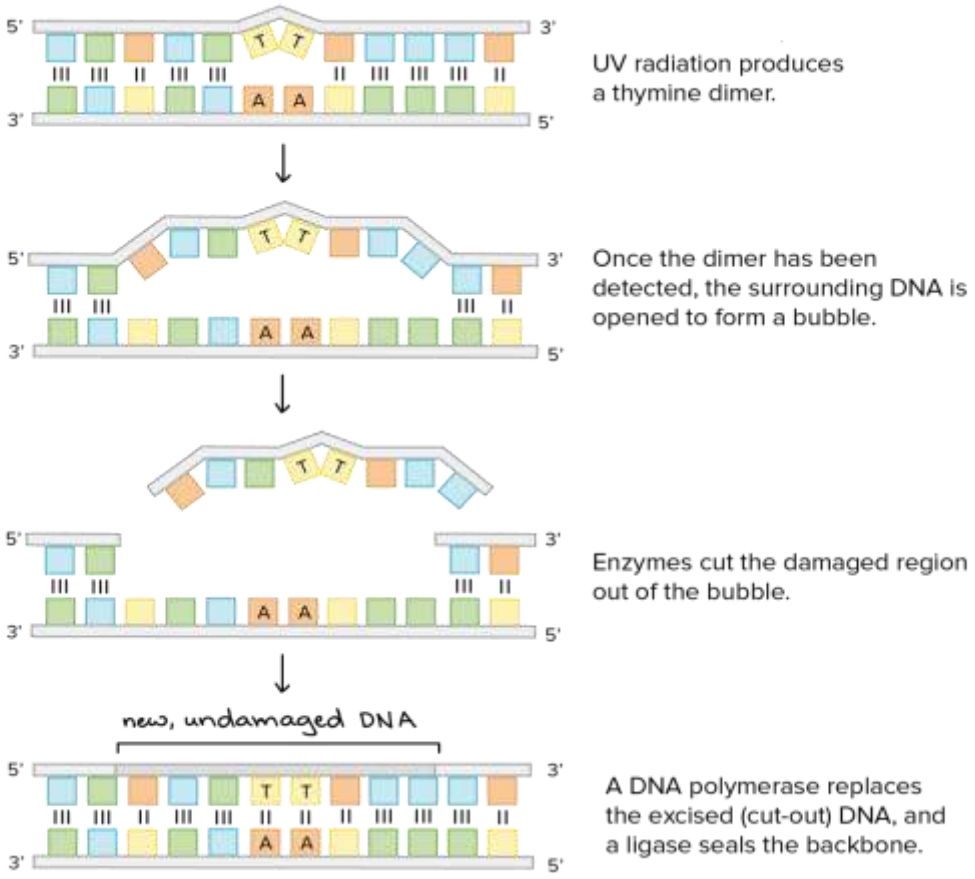
Base excision repair is a mechanism used to detect and remove certain types of damaged bases. A group of enzymes called glycosylases play a key role in base excision repair. Each glycosylase detects and removes a specific kind of damaged base.

For example, a chemical reaction called deamination can convert a cytosine base into uracil, a base typically found only in RNA. During DNA replication, uracil will pair with adenine rather than guanine (as it would if the base was still cytosine), so an uncorrected cytosine-to-uracil change can lead to a mutation. To prevent such mutations, a glycosylase from the base excision repair pathway detects and removes deaminated cytosines. Once the base has been removed, the "empty" piece of DNA backbone is also removed, and the gap is filled and sealed by other enzymes.



Nucleotide excision repair

In nucleotide excision repair, the damaged nucleotide(s) are removed along with a surrounding patch of DNA. In this process, a helicase (DNA-opening enzyme) cranks open the DNA to form a bubble, and DNA-cutting enzymes chop out the damaged part of the bubble. A DNA polymerase replaces the missing DNA, and a DNA ligase seals the gap in the backbone of the strand.



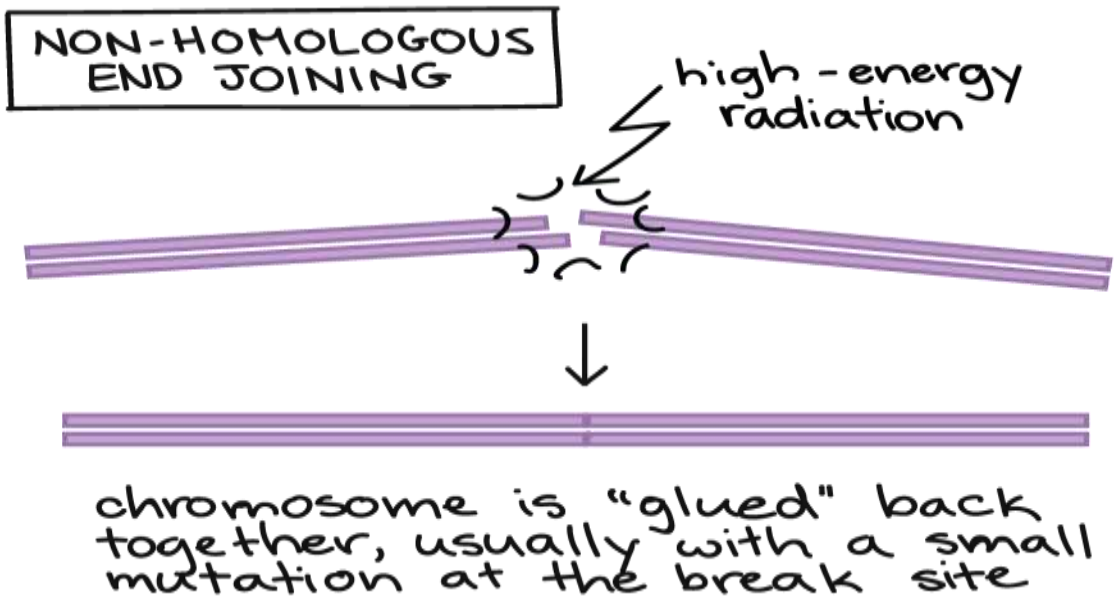
Double-stranded break repair

Some types of environmental factors, such as high-energy radiation, can cause double-stranded breaks in DNA (splitting a chromosome in two). This is the kind of DNA damage linked with superhero origin stories in comic books, and with disasters like Chernobyl in real life.

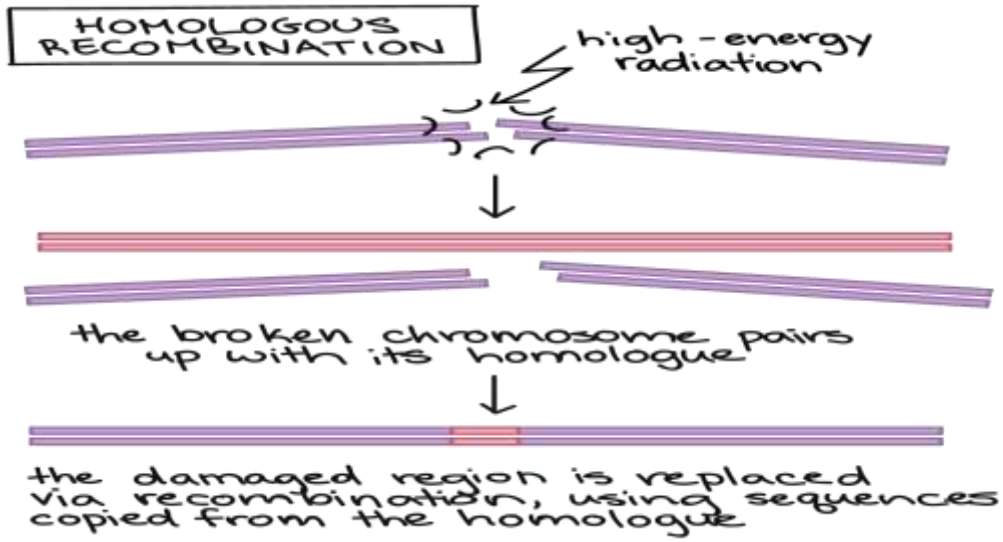
Double-stranded breaks are dangerous because large segments of chromosomes, and the hundreds of genes they contain, may be lost if the break is not repaired. Two pathways involved in the repair of double-stranded DNA breaks are the non-homologous end joining and homologous recombination pathways.

In **non-homologous end joining**, the two broken ends of the chromosome are simply glued back together. This repair mechanism is "messy" and typically involves the loss, or sometimes addition, of a few nucleotides at the cut site. So,

non-homologous end joining tends to produce a mutation, but this is better than the alternative (loss of an entire chromosome arm)

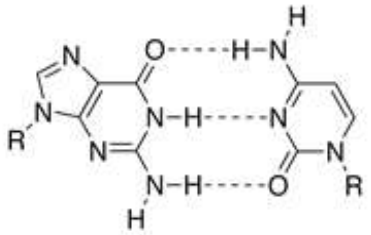


In **homologous recombination**, information from the homologous chromosome that matches the damaged one (or from a sister chromatid, if the DNA has been copied) is used to repair the break. In this process, the two homologous chromosomes come together, and the undamaged region of the homologue or chromatid is used as a template to replace the damaged region of the broken chromosome. Homologous recombination is "cleaner" than non-homologous end joining and does not usually cause mutations.

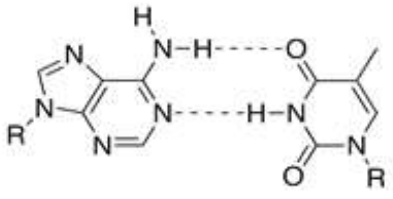


Nucleic acid hybridization

Nucleic acid hybridization is a basic technique in molecular biology which takes advantage of the ability of individual single-stranded nucleic acid molecules to form double-stranded molecules. According to Watson-Crick base pairing, adenine binds with thymine and guanine binds with cytosine by hydrogen bonding.



A GC base pair demonstrating three intermolecular hydrogen bonds



An AT base pair demonstrating two intermolecular hydrogen bonds

Nucleotide Probes

To search a desired DNA sequence of interest from a vast array of DNA fragments present in a genomic library, a reagent is needed which would react only with the correct fragment and ignore the rest. This type of reagent is called a nucleotide-based probe. It is a single-stranded piece of DNA (sometimes RNA), which can range in size from as little as 15 bp to several hundred kilobases.

It can identify, through base pairing, a specific DNA fragment of the library, which contains complementary sequence. A probe DNA, for example, will form complementary base pairing with another DNA strand (termed template) if the two strands are complementary and a sufficient number of hydrogen bonds are formed.

An effective molecular hybridization between the probe and the template requires that both must be single stranded. Likewise, a mRNA can be used as a probe: it will bind to the DNA fragment that contains exon sequences of its gene. RNA probe, termed riboprobe , can be produced by in vitro transcription of cloned DNA inserted into a plasmid vector. Synthetic oligonucleotide probes , constructed by chemical methods, are most commonly used. The probe has to be at least 15–18 nucleotides long because shorter sequences may be present, by chance,

at multiple sites in the genome (e.g. a trinucleotide every 4^3 base pairs). Oligonucleotides of this size can be easily synthesized.

A. Probes must have a Label to be Identified

To render them recognizable, probes are labelled with the radioisotopes, such as

^{32}P or tritium. These probes can be detected by autoradiography, which involves placing the sample in direct contact with the photographic material, usually an X-ray film. Alternatively, end-labelling probes with fluorescent tags can be used. The latter are visible under the UV lamp.

B. Techniques for Labelling Probes

There are two general ways in which a labelled nucleotide can be incorporated into the structure of the probe:

1. End-labelling: Addition of a labelled group to one terminal of the probe is done, for example, by exchanging a labeled γ-phosphate from ATP with a phosphate from the 5'-terminal on (single or double-stranded) DNA.

2. Polymerase-based labelling: Using a DNA polymerase , multiple-labelled-nucleotides are incorporated into the probe during DNA synthesis. Such a reaction

requires dNTPs, and it is customary to have one of them to be labelled, e.g. dGTP. Because on an average 25% of the nucleotides incorporated are labelled, this type has a higher specific activity than the end-labelling where only terminal nucleotide is labelled.

C. Uses of Nucleotide Probes

1. To search specific DNA sequences of DNA library.

2. In Southern and Northern blot techniques, probes are used to identify DNA or RNA fragments respectively.

3. In diagnosis of genetic disorders, such as sickle cell anaemia, thalassaemia, cystic fibrosis , etc.

Blotting Techniques

These are standard techniques for the identification of a specific DNA, an RNA or a protein from a vast expanse of others. The technique for DNA identification is termed Southern blot, whereas Northern blot is for RNA and Western blot for protein identification.

Southern hybridization:

The basic principle behind the southern hybridization is the nucleic acid hybridization. Southern hybridization commonly known as southern blot is a technique employed for detection of a specific DNA sequence in DNA samples that are complementary to a given RNA or DNA sequence. It was the first blotting technique to be devised, named after its pioneer E.M Southern, a British biologist. Southern blotting involves separation of restricted DNA fragments by electrophoresis and then transferred to a nitrocellulose or a nylon membrane, followed by detection of the fragment using probe hybridization.

Separated by electrophoresis is transferred from gel to a membrane which in turn is used as a substrate for hybridization analysis employing labeled DNA or RNA probes specific to target fragments in the blotted DNA. Southern hybridization helps to detect specific fragment against a background of many other restriction fragments. Southern blotting is a technique which is used to confirm the identity of a cloned fragment or for recognition of a sub-fragment of interest from within the cloned DNA, or a genomic DNA. Southern blotting is a prerequisite to techniques such as restriction fragment length polymorphism (RFLP) analysis.

Procedure:

1. The high-molecular-weight DNA strands are fractioned using restriction enzymes.

2. The DNA fragments are separated based on size by agarose gel electrophoresis.

3. The gel with the restricted fragments is then laid on a filter paper wick which serves as a connection between the membrane and the high salt buffer.

4. The nitrocellulose membrane is placed on top of the gel and a tower of filter papers is used to cover it and these are kept in place with a weight. The capillary

action drives the buffer soaking through the filter paper wick, through the gel and the membrane and into the paper towels. Along with the buffer passing through the gel the DNA fragments are also carried with it into the membrane and they bind to the membrane. This causes an effective transfer of fragments (up to 15 kb in length taking around 18hours or overnight.

For DNA fragments larger than 15 kb, before blotting an acid such as diluted HCl is used to treat the gel that depurinates the DNA fragments causing breakage of DNA into smaller pieces, resulting in more efficient transfer from the gel to membrane.

Now a days blotting is also done by applying electric field. This **electro blotting** technique depends upon current and transfer buffer solution to nucleic acids onto a membrane. Following electrophoresis, a standard tank or semi-dry blotting transfer system is set up. A stack is put together in the following order from cathode to anode: sponge, three sheets of filter paper soaked in transfer buffer gel, PVDF or nitrocellulose membrane, three sheets of filter paper soaked in transfer buffer and then again sponge. Importantly the membrane should be located between the gel and the positively-charged anode, as the current and sample will be moving in that direction. Once the stack is prepared, it is placed in the transfer system, and suitable current is applied for a specific period of time according to the materials being used.

5. For using alkaline transfer methods, the DNA gel is placed into an alkaline solution (like that of sodium hydroxide) causing denaturation of the double-stranded DNA. Denaturation in an alkaline environment enhances the binding between the negatively charged DNA and the positively charged membrane, causing separation to single DNA strands for further hybridization to the probe, alongside destroying any residual RNA that may persist in DNA. The membrane is washed with buffer to remove unbound DNA fragments.

6. The membrane which contains the transferred fragments is heated in presence or absence of vacuum at 80°C for 2 hours or exposed to ultraviolet radiation (nylon membrane) for permanent attachment of the transferred DNA to the membrane.

7. The obtained membrane is then hybridized with a probe (a DNA fragment with a specific sequence whose presence in the target DNA is to be determined).

8. Labeling of the probe DNA is done for easy detection, usually radioactivity is incorporated or the molecule is tagged with a fluorescent or chromogenic dye. The hybridization probe may be made of RNA, instead of DNA in some cases where the target is RNA specific.

9. Washing of the excess probe from the membrane is done by using saline sodium citrate(SSC buffer) after the hybridization step and the hybridization pattern is studied on an X-ray film by autoradiography (for a radioactive or fluorescent probe), or via color development on membrane if a chromogenic detection method is employed.

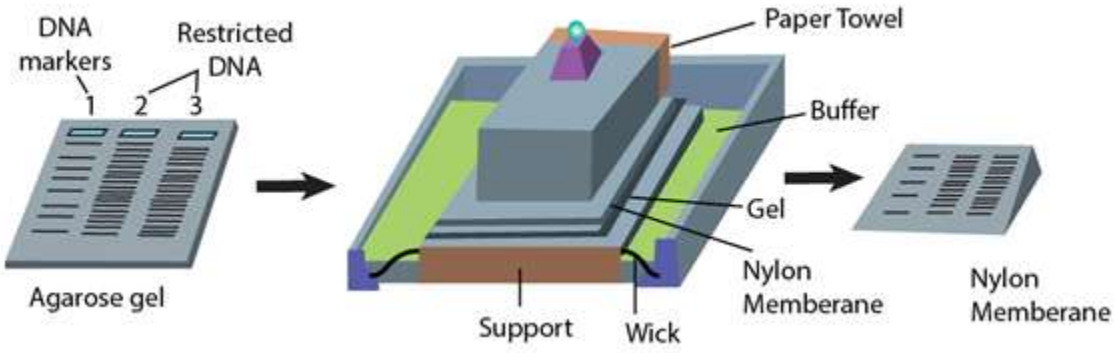


Fig (5): Steps of Southern Hybridization

Analysis of Southern Blot:

Hybridization of the probe to a specific DNA fragment on the membrane indicates the presence of a complementary fragment in the DNA sequence. Southern hybridization performed by digestion of genomic DNA using a restriction enzyme digestion, helps in determining the number of sequences (or gene copies) in the genome. For a probe hybridizing to a single DNA segment that has not been cut by the restriction enzyme, a single band is observed and on the other hand multiple bands will likely be observed when the hybridization occurs between the probe and several highly similar target sequence (Due to sequence duplication). Alterations in the hybridization conditions like enhancing the hybridization temperature or decreasing salt concentration, helps in altering specificity and hybridization of the probe to sequences that are less than 100% similar.

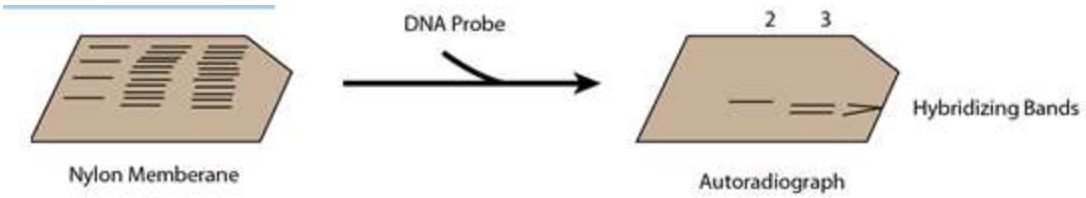


Fig (6): Southern hybridization analysis

Applications:

Southern blotting has been exploited for various applications which include:

a) Clone identification: One of the most common applications of Southern blotting is identification and cloning of a specific gene of interest. Southern blotting is carried out for identification of one or more restriction fragments that contain the gene of interest in genomic DNA. After cloning and tentative identification of the desired recombinant by employing colony or plaque hybridization, southern blotting is further is used to confirm the clone identification and possibly to locate a shorter restriction fragment, containing the sequence of interest.

b) Restriction fragment length polymorphism Analysis: Another major application of Southern hybridization is restriction fragment length polymorphism (RFLP) mapping, which is crucial in construction of genome maps.

Northern hybridization:

Northern blotting was developed by James Alwine, George Stark and David Kemp (1977). Northern blotting drives its name because of its similarity to the first blotting technique, which is Southern blotting, named after the biologist Edwin Southern. The major difference is that RNA being analyzed rather than DNA in the northern blot.

Expression of a particular gene can be detected by estimating the corresponding mRNA by Northern blotting. Northern blotting is a technique where RNA fragments are separated by electrophoresis and immobilized on a paper sheet.Identification of a specific RNA is then done by hybridization using a labeled nucleic acid probe. It helps to study gene expression by detection of RNA (or isolated mRNA) in a sample.

In Northern blotting, probes formed of nucleic acids with a sequence which is complementary to the sequence or to a part of the RNA of interest. The probe can be DNA, RNA or chemically synthesized oligonucleotides of minimum 25 complementary bases to the target sequence.

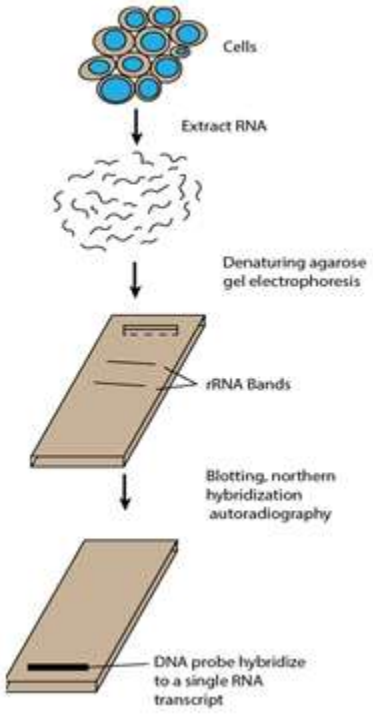


Fig (7): Steps of Northern Hybridization

Procedure:

The northern blotting involves the following steps:

1. Total RNA is extracted from a homogenized tissue sample or cells. Further eukaryotic mRNA can then be isolated by using of oligo (dT) cellulose chromatography to isolate only those RNAs by making use of a poly A tail.

2. The isolated RNA is then separated by gel electrophoresis.

3. The RNA samples separated on the basis of size are transferred to a nylon membrane employing a capillary or vacuum based system for blotting.

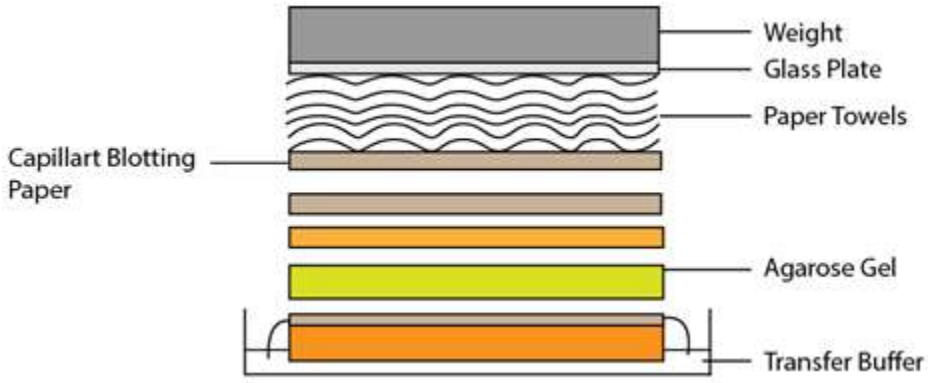


Fig (8): Setup for Northern blotting

4. Similar to Southern blotting, the membrane filter is revealed to a labeled DNA probe that is complementary to the gene of interest and binds.

5. The labeled filter is then subjected to autoradiography for detection.

The net amount of a specific RNA in a sample can be estimated by using Northern blot. This technique is widely used for comparing the amounts of a particular mRNA in cells under different conditions. The separation of RNA samples is often done on agarose gels containing formaldehyde as a denaturing agent as it limits the RNA to form secondary structure.

Analysis of Northern Blot:

RNA extract is electrophoresed in an agarose gel, using a denaturing electrophoresis buffer (containing formaldehyde) to ensure that the RNAs do not form inter- or intra-molecular base pairs, as base pairing would affect the rate at which the molecules migrate through the gel. After electrophoresis, the gel is blotted onto a nylon or nitrocellulose membrane, and hybridized with a labeled probe. If the probe is a cloned gene, the band that appears in the autoradiograph is the transcript of that gene. The size of the transcript can be determined from its position within the gel, and if RNA from different tissues is run in different lanes of the gel, then the possibility of differentially expressed gene can be examined.

Applications:

Northern blotting helps in studying gene expression pattern of various tissues, organs, developmental stages, pathogen infection, and also over the course of treatment. It has been employed to study overexpression of oncogenes and down-regulation of tumor-suppressor genes in cancerous cells on comparison with healthy tissue, and also for gene expression of immune-rejection of transplanted organ.

The examination of the patterns of gene expressions obtained under given conditions can help determine the function of that gene.

Northern blotting is also used for the analysis of alternate spliced products of same gene or repetitive sequence motif by investigating the various sized RNA of the gene. This is done when only probe type with variation in one location is used to bind to the target RNA molecule.

Variations in size of a gene product may also help to identify deletions or errors in transcript processing, by altering the probe target that can be used along the known sequence and make it possible to determine the missing region of the RNA.

Colony Hybridization:

It is a rapid method of isolating a colony containing a plasmid harboring a particular sequence or a gene from a mixed population. The colonies to be screened are first replica-plated on to a nitrocellulose filter disc that has been placed on the surface of an agar plate prior to inoculation. Master plate is retained for reference set of colonies. The filter bearing the colonies is removed and treated with alkali so that the bacterial colonies are lysed and the DNA they contain is denatured. The filter is then treated with proteinase K to remove protein and leave denatured DNA bound to the nitrocellulose. The DNA is fixed firmly by baking the filter at 80°C. A labeled probe is hybridized to this DNA which is monitored by autoradiography. A colony whose DNA print gives a positive auto radiographic result on X-ray film can then be picked from the reference plate.

Colony hybridization can be used to screen plasmid or cosmid based libraries.

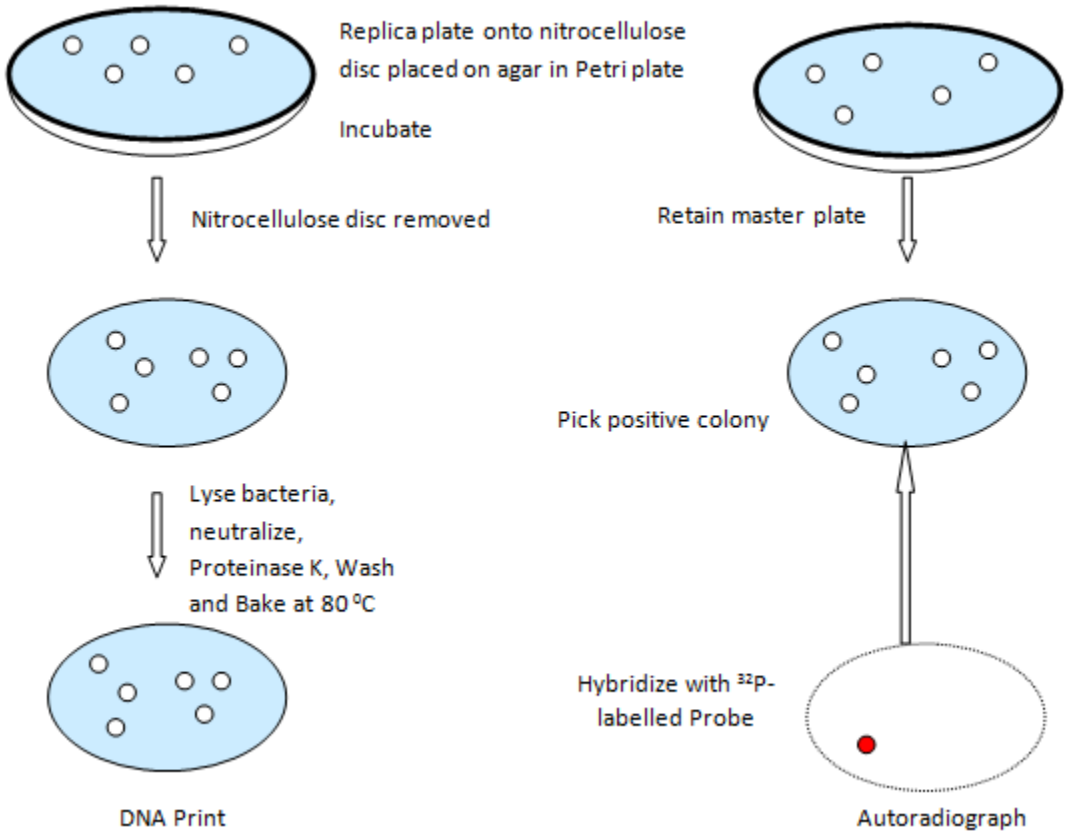


Fig (9): Colony hybridization

In Situ Hybridization (ISH)

It is a technique that employs a labeled complementary nucleotide strand (i.e. probe) for localizing specific DNA or RNA sequence targets within fixed tissues and cells (i.e *in situ*). Probes used for hybridization can be double-stranded DNA probes, single-stranded DNA probes, RNA probes, synthetic oligonucleotides. There are two ways available to detect DNA or RNA targets:

- Chromogenic (**CISH**) *in situ* hybridization
- Fluorescence *in situ* hybridization (**FISH**).

Chromogenic *in situ* hybridization (CISH)

It uses the labeling reactions involving alkaline phosphatase or peroxidase reactions to visualize the sample using bright-field microscopy. It is primarily used in molecular pathology diagnostics. CISH can also be employed for samples like fixed cells or tissues, blood or bone marrow smears and metaphase chromosome spreads.

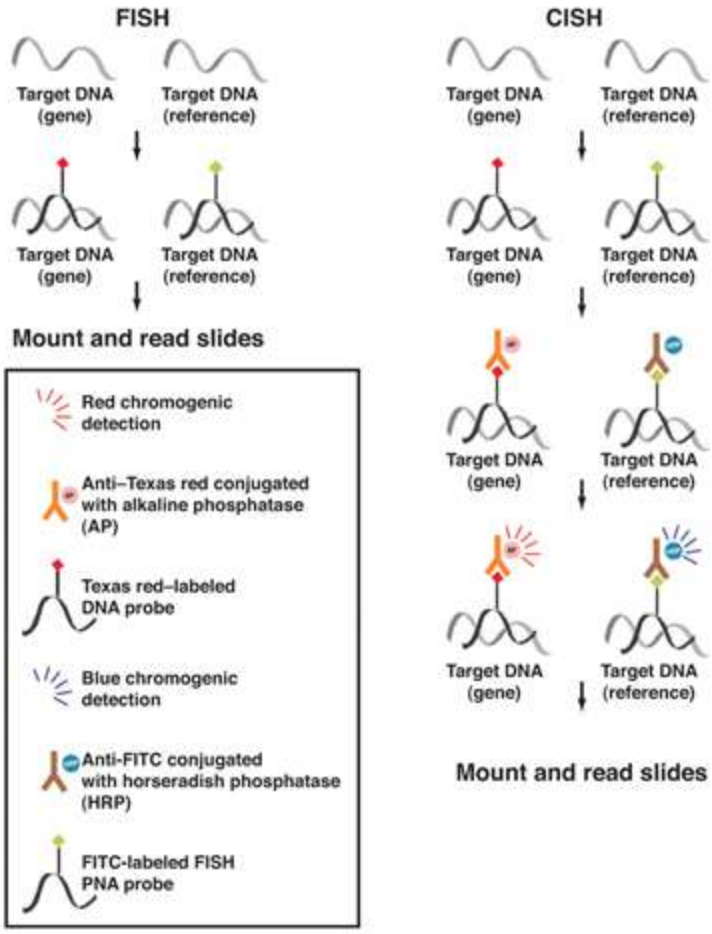


Fig (10): Use of dual-color Chromogenic in situ hybridization (CISH) in combination with fluorescence in situ hybridization (FISH) probes. FITC, fluorescein isothiocyanate; PNA, peptide nucleic acid.(Obtained from http://ajcp.ascpjournals.org/content/133/2/205/F1.expansion.html)

Fluorescence *in situ* hybridization (FISH)

FISH is a cytogenetic technique that uses fluorescent probes that bind to complementary targets and sample is visualized using epi-fluorescence or confocal microscopy. Using differently labeled probes, we can visualize several targets in a single sample. It is used for spatial-temporal patterns of gene expression and resolving genetic elements in chromosomal preparations.

Cells or tissues to be analyzed are fixed and permeabilized with Proteinase K to allow target accessibility. Probe is constructed and tagged using non-radioactive labels like biotin, digoxigenin or fluorescent dye (FISH). Probe must be large enough to hybridize specifically with its target. Probe is applied to fixed sample and incubated to several hours to allow hybridization. Washing is done to remove non-specific or unbound probe removal. Results are then visualized using either bright-field or confocal microscopy.

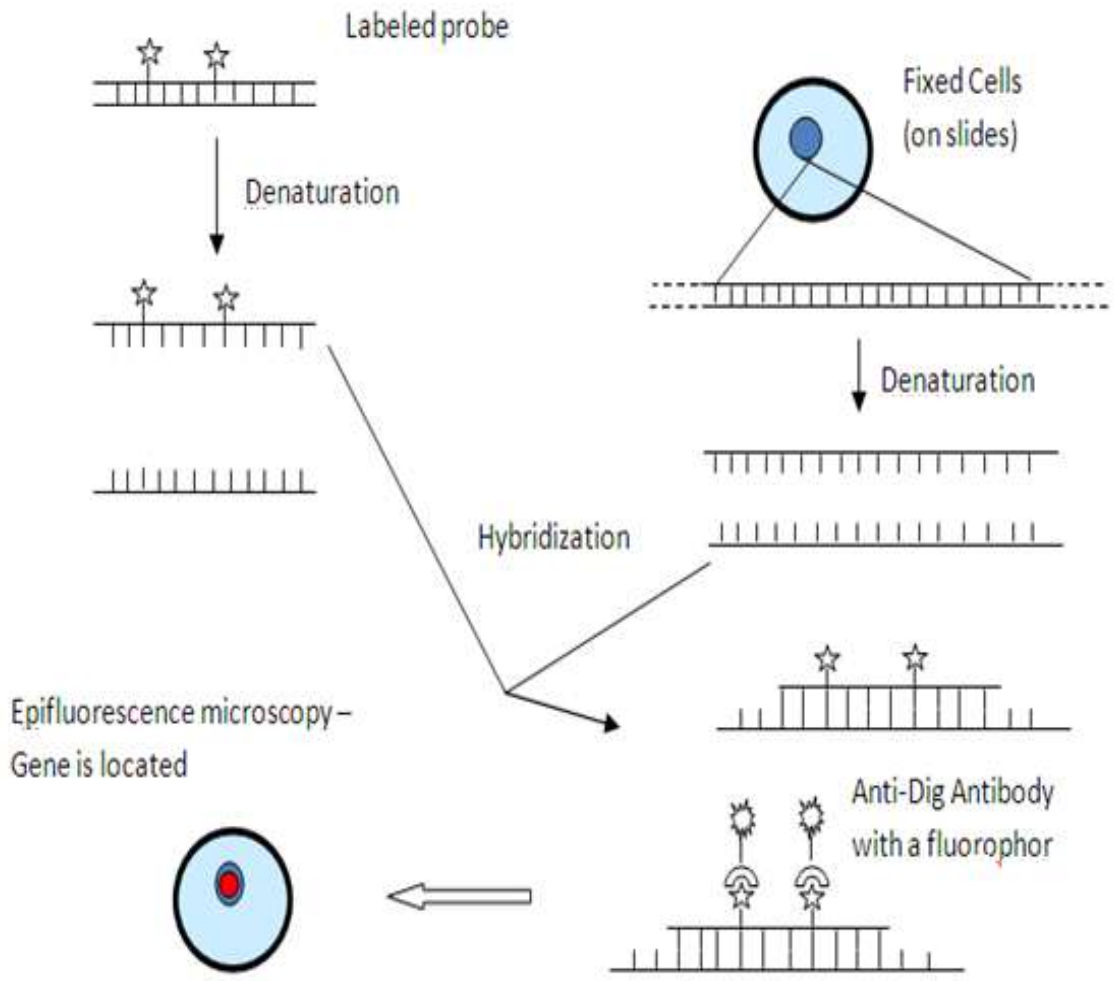


Fig (11): Fluorescent *in-situ* hybridization

Dot Blot and Slot Blot Hybridization

These two techniques represents the simplification of Southern and Western blots saving the time involved in procedures of chromatography, electrophoresis, restriction digestion and blotting of DNA or proteins from the gel to membrane. Here nucleic acid mixture is directly applied (blotted) on to the nylon or nitrocellulose membrane where hybridization between probe and target takes place, denatured to single-stranded form and baked at 80°C to bind DNA target to membrane. In dot-blot, target is blotted as circular blots whereas in slot-blots, it is in the form of rectangular blots. Due to this, slot-blot offers greater precision in observing different hybridization signals. After blotting, membrane is allowed to dry and non-specific sites are blocked by soaking in blocking buffer containing BSA. It is then followed by hybridization of labeled probe for detection of specific sequences or gene.

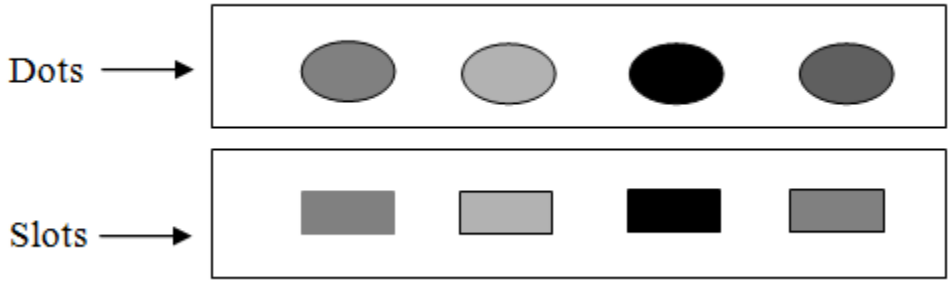


Fig (12): Dots and slots in dot and slot blot hybridization

These procedures can only detect presence and absence of particular sequence or gene. It cannot distinguish between two molecules of different sizes as they appear as single dot on membrane. It also has application in detecting alleles that differ in single nucleotide with the help of allele-specific oligonucleotides.

DNA microarray

DNA microarrays are solid supports, usually of glass or silicon, upon which DNA is attached in an organized pre-determined grid fashion. Each spot of DNA, called a probe, represents a single gene. DNA microarrays can analyze the expression of tens of thousands of genes simultaneously. There are several synonyms of DNA microarrays such as DNA chips, gene chips, DNA arrays, gene arrays, and biochips.

Principle of DNA Microarray Technique

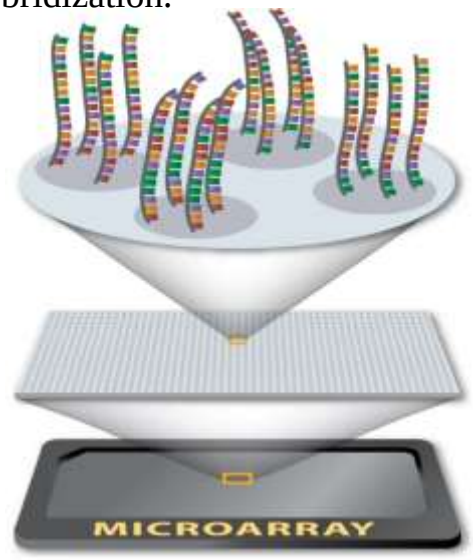
The principle of DNA microarrays lies on the hybridization between the nucleic acid strands. The property of complementary nucleic acid sequences is to specifically pair with each other by forming hydrogen bonds between complementary nucleotide base pairs. For this, samples are labeled using fluorescent dyes.

At least two samples are hybridized to chip. Complementary nucleic acid sequences between the sample and the probe attached on the chip get paired via hydrogen bonds. The non-specific bonding sequences while remain unattached and washed out during the washing step of the process. Fluorescently labeled target sequences that bind to a probe sequence generate a signal. The signal depends on the hybridization conditions (ex: temperature), washing after hybridization etc while the total strength of the signal, depends upon the amount of target sample present. Using this technology the presence of one genomic or cDNA sequence in 1,00,000 or more sequences can be screened in a single hybridization.

Types of DNA Microarrays

There are 2 types of DNA Chips/Microarrays:

1. cDNA based microarray
2. Oligonucleotide based microarray



Requirements of DNA Microarray Technique

- DNA Chip
- Target sample (Fluorescently labelled)
- Fluorescent dyes

- Probes
- Scanner

Steps Involved in cDNA based Microarray

The reaction procedure of DNA microarray takes places in several steps:

1. **Collection of samples**

   The sample may be a cell/tissue of the organism that we wish to conduct the study on. Two types of samples are collected: healthy cells and infected cells, for comparison and to obtain the results.

2. **Isolation of mRNA**

   RNA is extracted from the sample using a column or solvent like phenol-chloroform. From the extracted RNA, mRNA is separated leaving behind rRNA and tRNA. As mRNA has a poly-A tail, column beads with poly-T-tails are used to bind mRNA. After the extraction, the column is rinsed with buffer to isolate mRNA from the beads.

3. **Creation of labeled cDNA**

   To create cDNA (complementary DNA strand), reverse transcription of the mRNA is done. Both the samples are then incorporated with different fluorescent dyes for producing fluorescent cDNA strands. This helps in distinguishing the sample category of the cDNAs.

4. **Hybridization**

   The labeled cDNAs from both the samples are placed in the DNA microarray so that each cDNA gets hybridized to its complementary strand; they are also thoroughly washed to remove unbounded sequences.

5. **Collection and analysis**

   The collection of data is done by using a microarray scanner.This scanner consists of a laser, a computer, and a camera. The laser excites fluorescence of the cDNA, generating signals. When the laser scans the array, the camera records the images produced. Then the computer stores the data and provides the results immediately. The data thus produced are then analyzed. The difference in the intensity of the colors for each spot determines the character of the gene in that particular spot.

Applications of DNA Microarray

In humans, they can be used to determine how particular diseases affect the pattern of gene expression (the expression profile) in various tissues, or the identity (from the expression profile) of the infecting organism.

Besides, it has applications in many fields such as:

- Diagnostics and genetic engineering
- Alternative splicing detection
- Proteomics, functional genomics, DNA sequencing and gene expression profiling
- Toxicological research (Toxicogenomics)

Advantages of DNA Microarray

- Provides data for thousands of genes in real time.
- Single experiment generates many results easily.
- Fast and easy to obtain results.
- Different parts of DNA can be used to study gene expression.

Disadvantages of DNA Microarray

- Expensive to create.
- The production of too many results at a time requires long time for analysis.
- The DNA chips do not have very long shelf life.

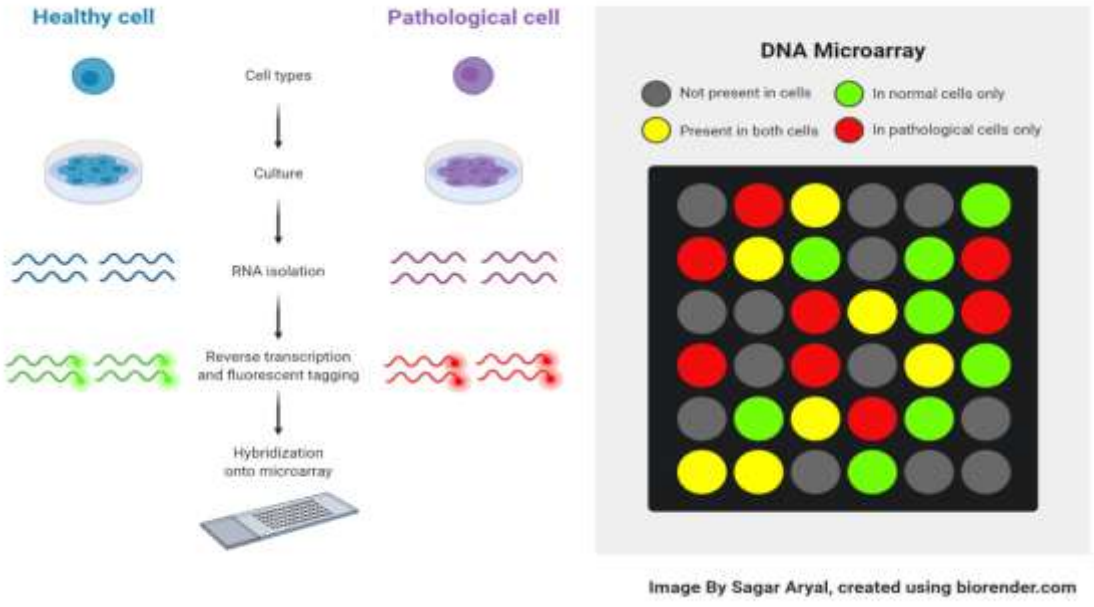


Image By Sagar Aryal, created using biorender.com

Poymerase chain reaction (PCR) and its Applications

Polymerase chain reaction (PCR) is a widely employed technique in molecular biology to amplify single or a few copies of DNA, generating millions of copies of a particular DNA sequence. The polymerase chain reaction results in the selective amplification of a target region of a DNA or RNA molecule. PCR has been extensively exploited in cloning, target detection, sequencing etc. The method consists of thermal cycles of repeated heating followed by cooling of the reaction mixture to achieve melting and primer hybridization to enable enzymatic replication of the DNA.

In 1985, Kary Mullis invented a process Polymerase Chain Reaction (PCR) using the thermo-stable *Taq* polymerase for which he was awarded Nobel Prize in 1993.

Fig (13): PCR Thermo cycler (*Adapted from http://products.invitrogen.com/ivgn/product/4452300*)

Basic Protocol for Polymerase Chain Reaction:

Components and reagents:

A basic PCR set up requires the following essential components and reagents:

1. Template DNA containing the DNA region (target) to be amplified.

2. Primers that are complementary to the 5' ends of each of the sense (Forward primer) and anti-sense strand of the DNA target (Reverse primer).

3. *Taq* polymerase or other thermostable, high fidelity DNA polymerase (Pfu polymerase isolated from *Pyrococcus furiosus*).

4. Deoxyribonucleotide triphosphates (dNTPs), which are the building-blocks for a newly synthesized DNA strand.

5. Buffer solutions to provide a suitable chemical condition for optimum activity and stability of the DNA polymerases.

6. Divalent cations (eg. magnesium or manganese ions). They act as a co-factor for Taq polymerase which increases its polymerase activity. Generally Mg^{2+} is used, but Mn^{2+} can be applied to achieve PCR-mediated DNA mutagenesis. This is because higher Mn^{2+} concentration leads to higher error rate during DNA synthesis.

Procedure:

Typically, PCR is designed of 20-40 repeated thermal cycles, with each cycle consisting of 3 discrete temperature steps: denaturation, annealing and extension. The thermal cycles are often proceeded by a temperature at a high range (>90°C), and followed by final product extension or brief storage at 4 degree celsius. In PCR cycles, the temperatures and the duration of each cycle is determined based on various parameters like the type of DNA polymerase used, the melting temperature (Tm) of the primers, concentration of divalent ions and dNTPs in the reaction etc. The various steps involved are:-

a) Initial Denaturation

b) Denaturation

c) Annealing

d) Extension

e) Final extension

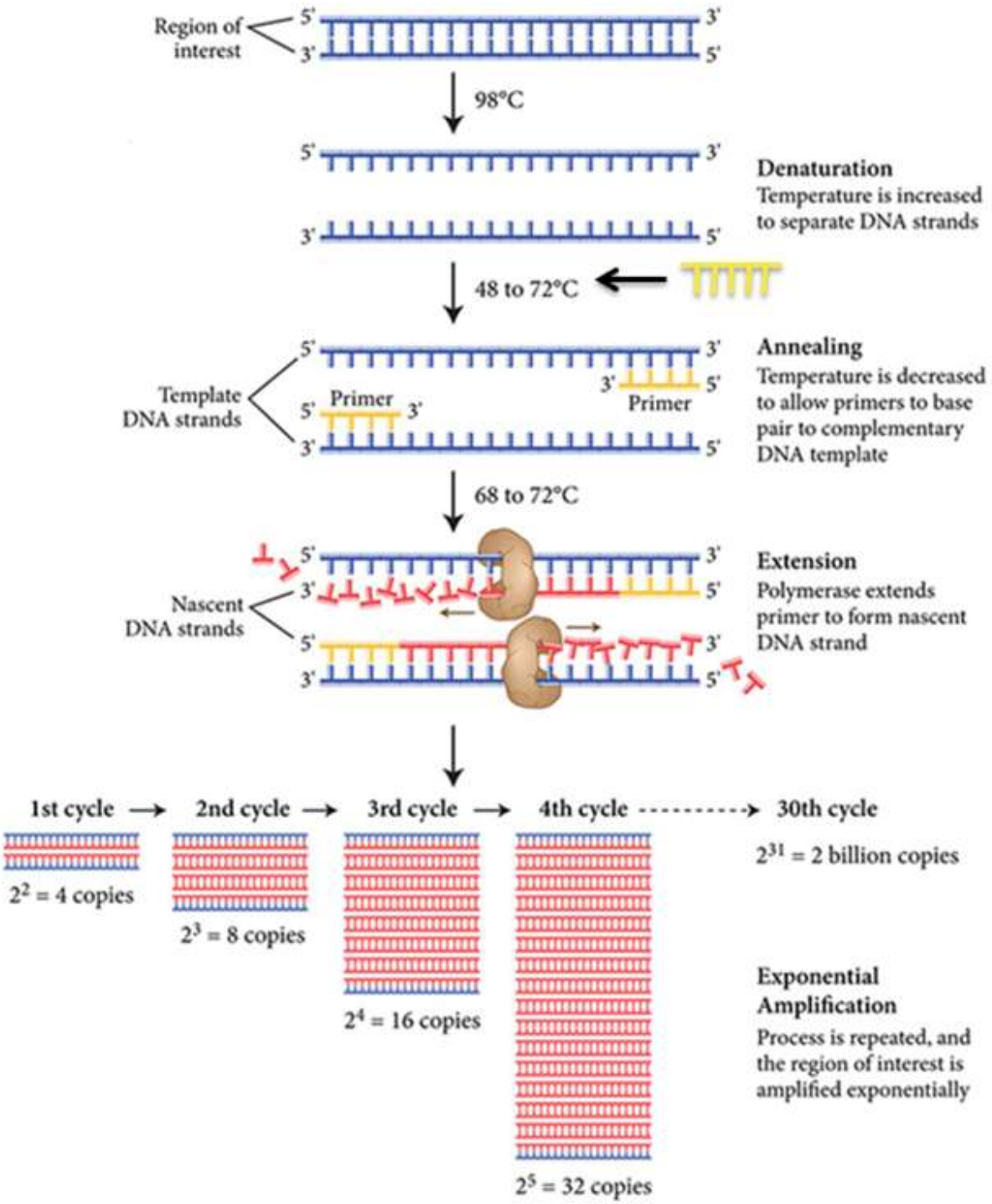


Fig: The sequential steps of PCR

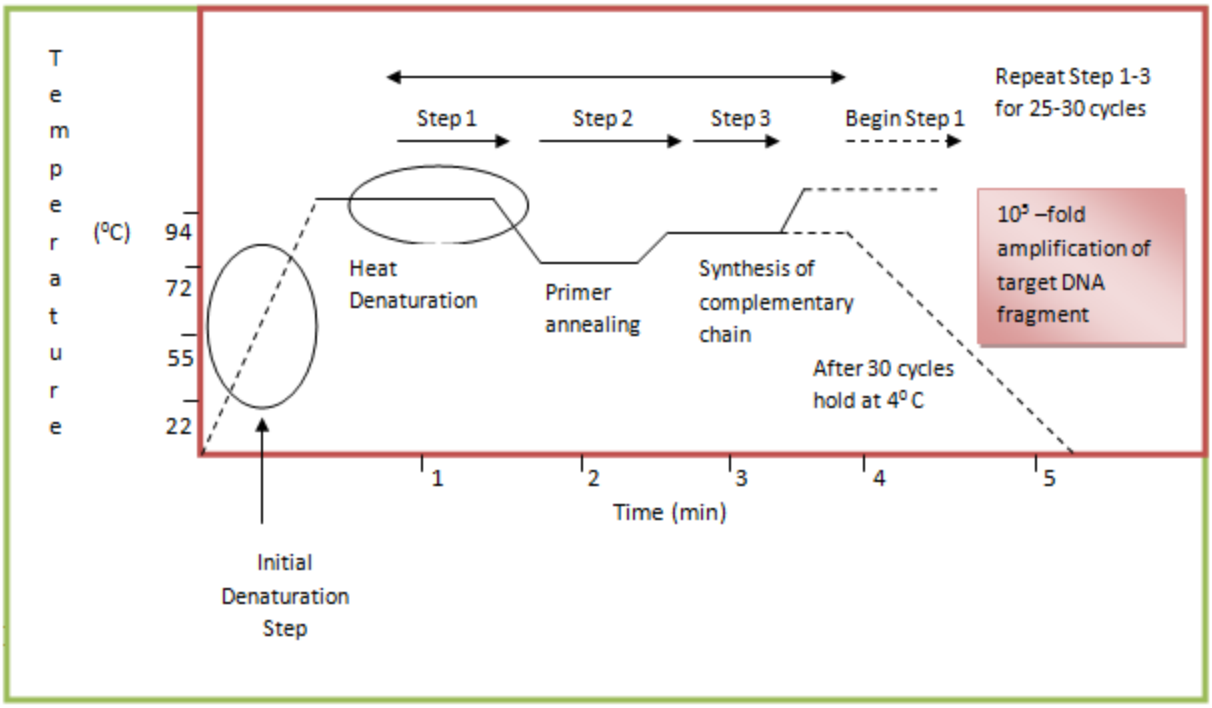


Fig (14): Basic Thermal Profile of PCR

Initial denaturation:

Initial denaturation involves heating of the reaction to a temperature of 94–96°C for 7-10 minutes (or 98°C if extremely thermostable polymerases are used). For specifically engineered DNA polymerases (Hot start Taq polymerases) activity requires higher range of temperature. The initial heating for such a long duration also helps in gradual and proper unfolding of the genomic DNA and subsequent denaturation, and thus exposing target DNA sequence to the corresponding primers.

Denaturation:

Denaturation requires heating the reaction mixture to 94–98°C for 20–30 seconds. It results in melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.

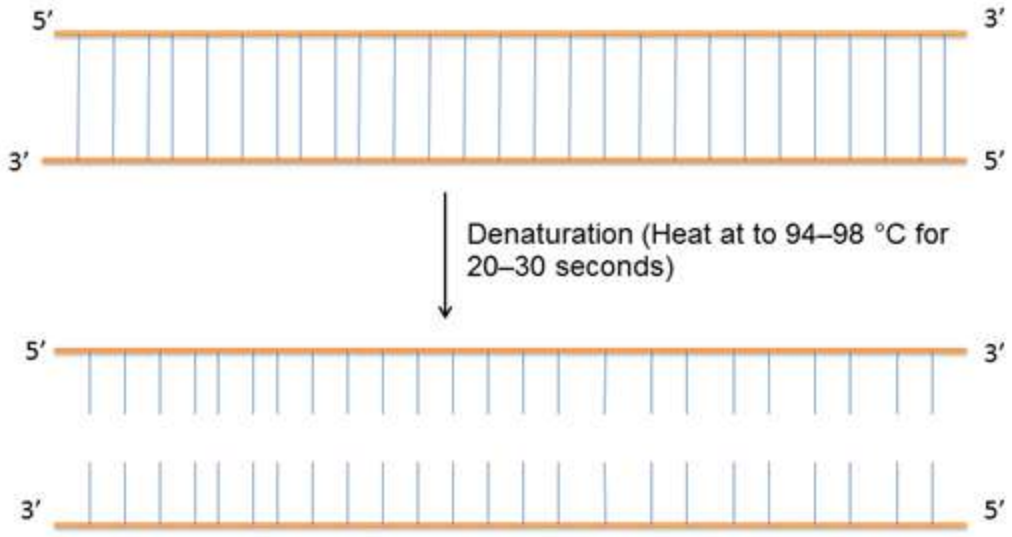


Fig (15): Denaturation of double stranded DNA to single stranded DNA

Annealing:

Following the separation of the two strands of DNA during denaturation, the temperature of the reaction mix is lowered to 50–65°C for 20–50 seconds to allow annealing of the primers to the single-stranded DNA templates. Typically the annealing temperature should be about 3-5°C below the T_m of the primers. Stable complimentary binding are only formed between the primer sequence and the template when there is a high sequence complimentarity between them. The polymerase enzymes initiate the replication from 3' end of the primer towards the 5'end of it.

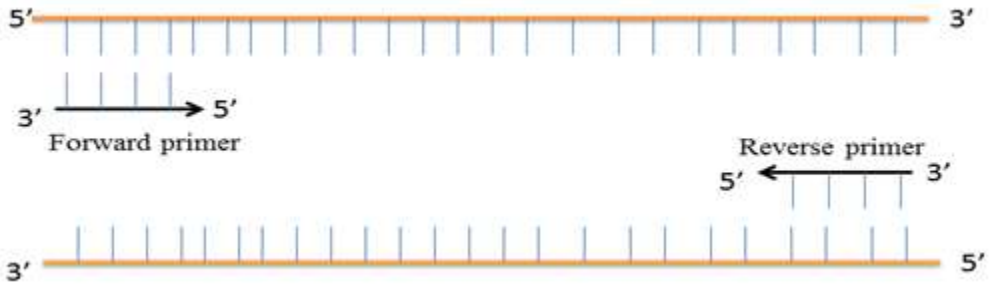


Fig (16): Annealing of primer

Extension /elongation:

Extension/elongation step includes addition of dNTPs to the 3' end of primer with the help of DNA polymerase enzyme. The type of DNA polymerase applied in the

reaction determines the optimum extension temperature at this step. DNA polymerase synthesizes a new DNA strand complementary to its template strand by addition of dNTPs, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand. Conventionally, at its optimum temperature, DNA polymerase can add up to a thousand bases per minute. The amount of DNA target is exponentially amplified under the optimum condition of elongation step. The drawback of Taq polymerase is its relatively low replication fidelity. It lacks a 3' to 5' exonuclease proofreading activity, and has an error rate measured at about 1 in 9,000 nucleotides.

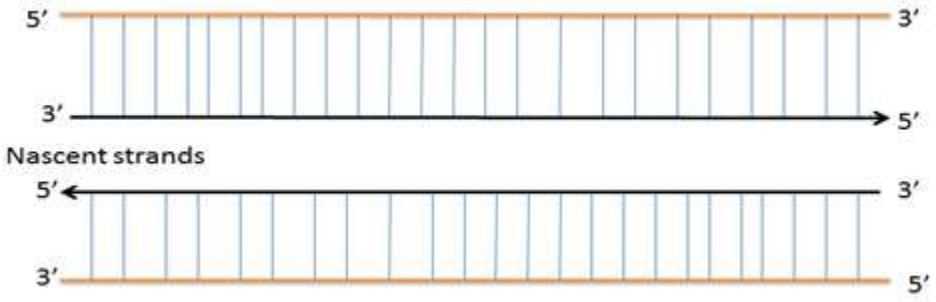


Fig (17): Extension/ Elongation

Final elongation & Hold:

Final elongation step is occasionally performed for 5–15 minutes at a temperature of 70–74°C after the last PCR cycle to ensure amplification of any remaining single-stranded DNA.

Final hold step at 4°C may be done for short-term storage of the reaction mixture.

After around 30 cycles of denaturation, annealing and extension, there will be over a billion fragments that contain only your target sequence. This will yield a solution of nearly pure target sequence. To check the desired PCR amplification of the target DNA fragment (also sometimes referred to as the amplicon or amplimer), agarose gel electrophoresis is employed for separation of the PCR products based on size. The determination of size(s) of PCR products is performed by comparing with a DNA ladder, which contains DNA fragments of known size, run on the gel alongside the PCR products.

Key factor affecting the polymerase chain reaction (Designing of Primers):

The specificity of the PCR depends crucially upon the primers. The following factors are important in choosing effective primers.

a) Primers should be 17 to 30 nucleotides in length. However for certain studies the RAPD primer length might be of 10-12 nucleotides.

b) Ideally GC content of the primers is 50% .For primers with a low GC content, it is desirable to choose a long primer so as to avoid a low melting temperature.

c) The basic formula to calculate melting temperature is

T_m = 4°C x (number of G's and C's in the primer) + 2°C x (number of A's and T's in the primer)

Two primers must have a similar Tm value. In case of several primer candidates, we have to choose primers which have the higher Tm value among them.

d) Sequences with long runs (i.e. more than three or four) of a single nucleotide should be avoided.

e) Primers with significant secondary structures (self –hair pin, loop formation) are undesirable.

f) There should be no base complementarities between the two primers.

Applications

Infectious disease diagnosis, progression and response to therapy

PCR technology facilitates the detection of DNA or RNA of pathogenic organisms and, as such, helps in clinical diagnostic tests for a range of infectious agents like viruses, bacteria, protozoa etc. These PCR-based tests have numerous advantages over conventional antibody-based diagnostic methods that determine the body's immune response to a pathogen.

PCR-based diagnostics tests are available for detecting and/or quantifying a number of pathogens, such as:

1. HIV-1, which causes AIDS

2. Hepatitis B and C viruses, might lead to liver cancer

3. Human Papillomavirus, might cause cervical cancer

Genetic counseling

Genetic counseling is done for the parents to check the account of genetic disease beforehand to make a decision on having children. This is of course governed by national laws and guidelines. Detection of genetic disease before implantation of an embryo in IVF (*In vitro* fertilization) also known as pre-implantation diagnosis can also be done exploiting PCR based method. Further to diagnose inherited or a spontaneous disease, either symptomatic or asymptomatic (because of family history like Duchene muscular dystrophy) PCR based method is very useful.

Forensic sciences

Genetic fingerprint is one of the most exploited application of PCR (also known as DNA profiling).Profiles of specific stretches of DNA are used in genetic fingerprinting (generally 13 loci are compared) which is differ from person to person. PCR also plays a role in analysis of genomic or mitochondrial DNA, in which investigators used samples from hair shafts and bones when other samples are not accessible.

Others

PCR has numerous applications in various fields. The Human Genome Project (HGP) for determining the sequence of the 3 billion base pairs in the human genome relied heavily on PCR. The genes associated with a variety of diseases have been identified using PCR. For example, Duchene muscular dystrophy, which is caused by the mutation of a gene, identified by a PCR technique called Multiplex PCR. PCR can help to study for DNA from various organisms such as viruses or bacteria.

DNA SEQUENCING

Sequencing is the method of resolving the order of the nucleotide bases in a DNA molecule (genomic DNA, cDNA, or organellar DNA). It is a primary step in assessing regulatory sequences, coding and non-coding regions. For past few years, DNA sequencing has been a solid foundation for various research fields such as taxonomy, phylogeny, ecology and genetic studies.

There are two classical methods of sequencing:

• Sanger dideoxy sequencing

• Maxam-Gilbert sequencing

Sanger dideoxy sequencing

The Sanger or chain termination method was first developed by Fred Sanger and colleagues in the mid-1970s. It involves *in vitro* synthesis of DNA on a single-stranded template by using a primer, a set of labeled deoxynucleotide triphosphates (dNTPs), and dideoxynucleotide triphosphates (ddNTPs). Each ddNTP is fluorescently-labeled and impedes chain elongation. It lacks hydroxyl group at the third position of the sugar component which is required for attachment of the next nucleotide.

The template DNA to be sequenced can be obtained by *in vivo* or *in vitro* cloning. It can be elongated by incorporation of deoxynucleotides at random positions. The ratio of dNTP to dideoxy NTP should be such that an individual strand can be polymerized for a significant distance before addition of dideoxyNTP molecule. The reaction terminates at the position where the ddNTP rather than dNTP incorporates into the growing DNA chain. This process results in the generation of amplicons of different sizes each ending in dideoxyNTP.

The next step involves separation of these fragments which can be achieved by acrylamide gel electrophoresis to obtain a sequence of up to a few hundred bases. Four parallel reactions can be carried out in parallel, one for each base. The base sequence can then be read by autoradiography of the banding patterns.

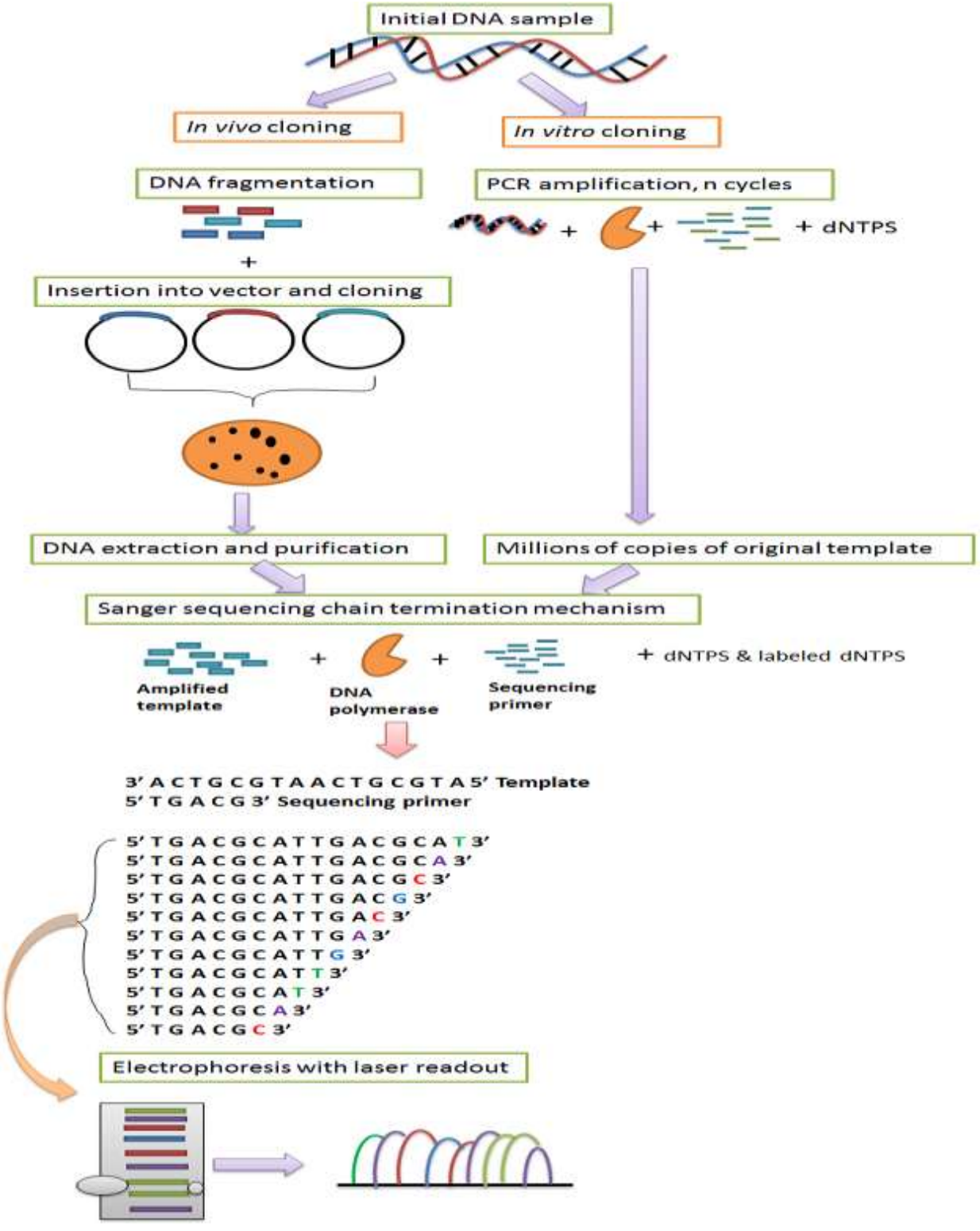


Fig (18): Schematic representation of Sanger sequencing workflow. *(Adapted and modified from Hoy MA. 2013. DNA Sequencing and the Evolution of the "-Omics". Insect Molecular Genetics.3 rd Edn. Academic Press, San Diego, 251-305.)*

Maxam-Gilbert Sequencing

This method was developed by Maxam and Gilbert in 1977 and is also known as chemical degradation method. In this method, double-stranded DNA to be sequenced is radioactively labeled at the 5′ end and undergoes selective fragmentation for the breakdown of specific base-pairing and dissociation of DNA. The resulting fragments are then loaded onto a polyacrylamide gel. Depending on the sizes of the fragments, the radioactively labeled fragments can be separated by electrophoresis, and the sequences are identified by autoradiography.

In contrast to Sanger sequencing, the samples to be sequenced need less complex preparation, but shorter reads (maximum 100 bp). This method itself is technically complex and utilizes hazardous chemicals.

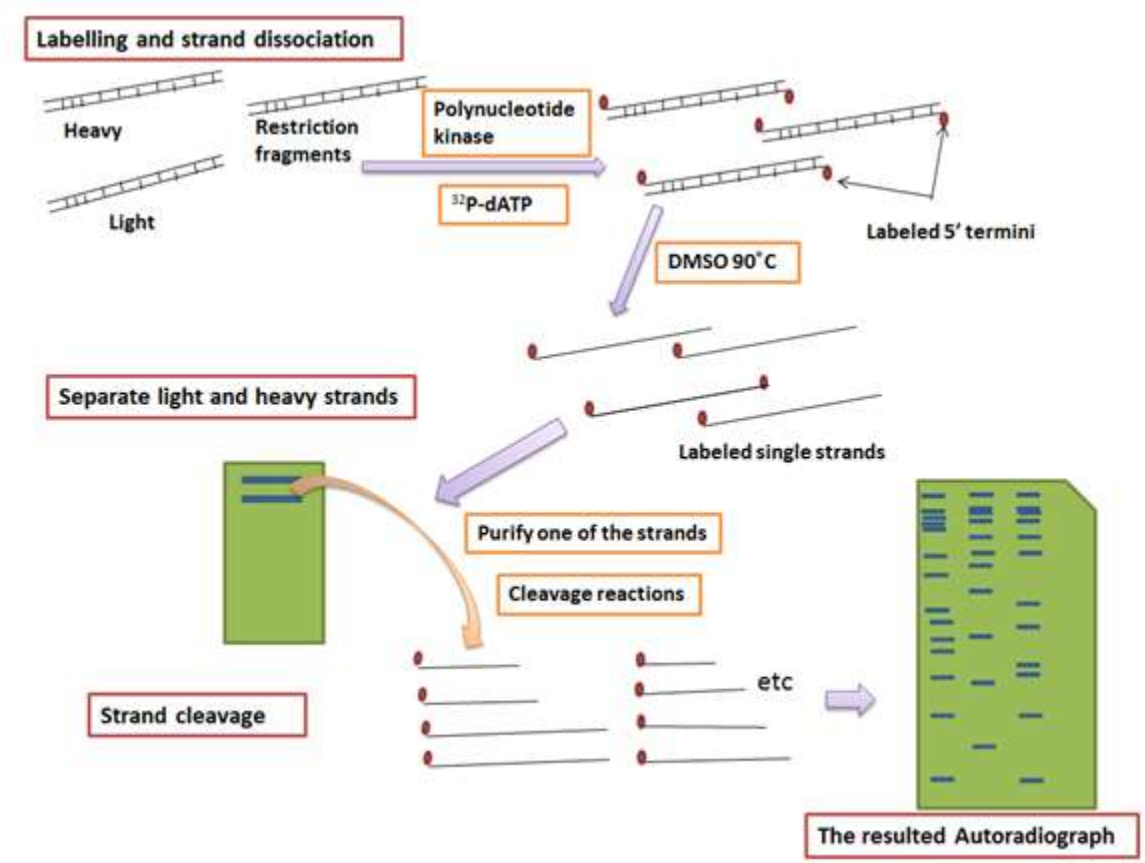


Fig (19): Schematic representation of Maxam-Gilbert sequencing workflow. *(Adapted and modified from Brown TA. 2006. Gene cloning and DNA analysis: an introduction. 5 th ed. Blackwell Scientific.)*

Karyotyping

Scientists have developed several different tools and techniques for studying chromosomes, genes and base pairs in humans and other organisms. One of the most useful techniques is karyotyping.

Karyotyping is the process by which cytogeneticists take photographs of chromosomes in order to determine the chromosome complement of an individual, including the number of chromosomes and any abnormalities.

White blood cells, cells obtained from amniotic fluid, or cells from cancerous tumors are cultured (incubated) in media containing essential nutrients, at a temperature that resembles the body, allowing the cells to continue to undergo mitosis. Mitosis is stopped at metaphase using chemicals, and the cells are 'harvested' and then placed onto a microscope slide, spread out, and stained so they can be seen. The most commonly used stain, Giemsa, stains densely packed DNA more darkly than less- densely packed DNA, creating a characteristic banding pattern for each chromosome. The cells are viewed under a microscope that is specially adapted with a camera to take a picture of the chromosomes from one or more of the cells.

Once the picture is taken and enlarged, the chromosomes are cut out and arranged in pairs according to size, banding pattern, and location of the centromere. There are 22 pairs of chromosomes called **autosomes**, which should match up exactly. In females the XX **sex chromosomes** match, while in males, the **XY sex chromosomes** do not match.

Karyotyping is used by all cytogenetic technologists, medical geneticists, or genetic counselors to determine whether a diagnosed birth defect is due to a chromosomal abnormality or to predict whether a fetus may be at risk for a genetic disorder.

The chromosomes in the karyotype are arranged in homologous pairs according to size (largest to smallest). Homologous pairs can be determined by centromere placement, equal length of top and bottom arms as well as similar band placement on each arm.

Karyotyping helps geneticists to diagnose and treat genetic disorders. They use a normal human karyotype and compare it to the karyotype of a patient to determine if there are abnormalities. Some of the characteristics used for comparison are:

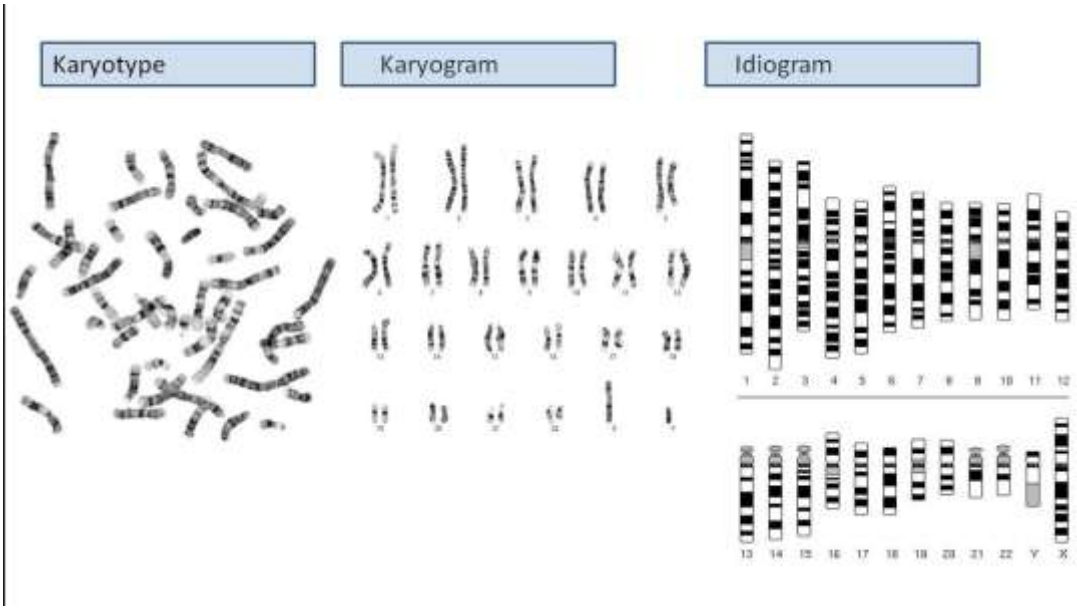
a) Total number of chromosomes - normal humans has 46 chromosomes (23 pairs), so if the number is higher or lower then an abnormality exists;

b) Homologous pairs for the first 22 pairs of chromosomes (autosomes) – once centromeres are aligned, top and bottom arms are of equal length and if not then an abnormality exists;

c) Sex Chromosomes (23rd pair) - if female, then 2 homologous X chromosomes (XX) will be present and if male, an X chromosome and a Y chromosome (XY) will be present, so if there are additional or fewer sex chromosomes then an abnormality exists.

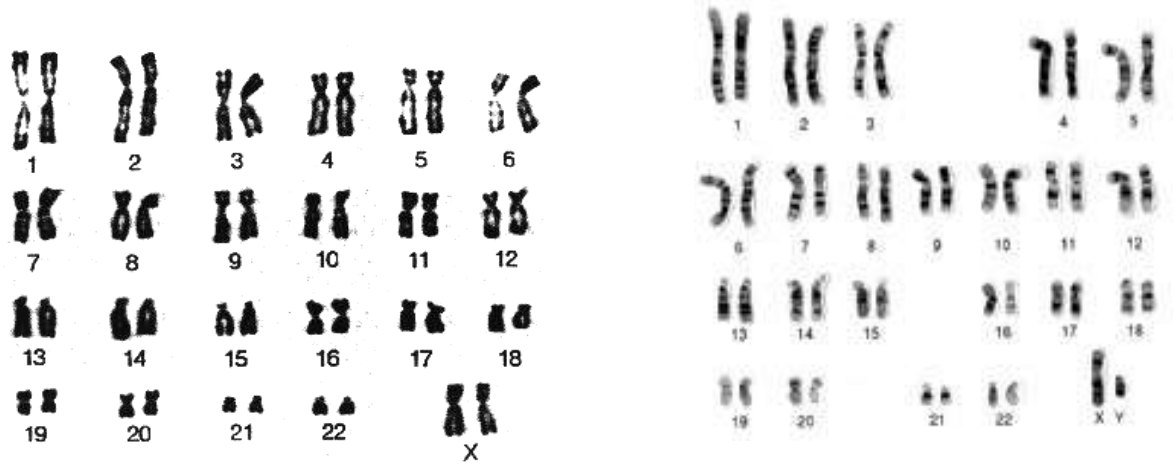
Karyogram: a graphic representation of a karyotype (Graphic representation of chromosomal set where chromosomes are arranged by their size).

Idiogram: a diagram representing the characteristic features of the chromosome set, based on physical characteristics visible in the karyogram.



Normal human in nuclei of all human cells has 46 chromosomes, and that number is designated as **diploid chromosomal number (2n)**; while in germ cells number of chromosomes is reduced to half and designated as **haploid chromosomal number (n)**.

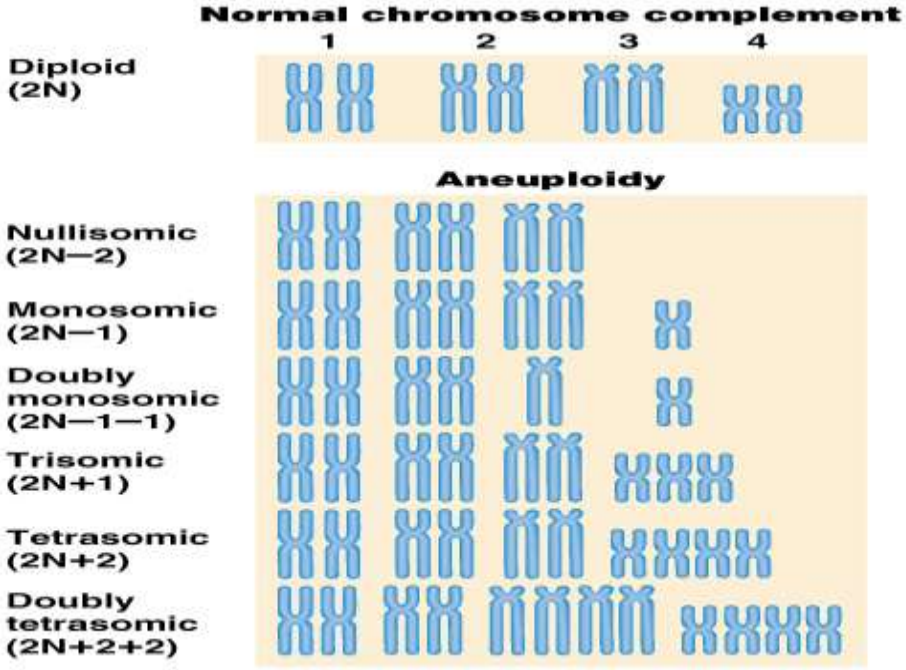
Normal karyotype formula of a male is 46, XY; and of a female: 46, XX.



Female karyotype **Male karyotype**

Fig (20) Human karyogram of a healthy female or male

<u>Aneuploidy</u>: variation in the number of individual chromosomes (but not the total number of sets of chromosomes).



- A **trisomic** cell has one extra chromosome (2n +1) = example: trisomy 21. (**Polyploidy** refers to the condition of having **three** homologous chromosomes rather than two)
- A **monosomic** cell has one missing chromosome (2n - 1) = usually lethal except for one known in humans: Turner's syndrome (monosomy XO).

Gene Therapy

Gene therapy is a novel treatment method which utilizes genes or short oligonucleotide sequences as therapeutic molecules, instead of conventional drug compounds. This technique is widely used to treat those defective genes which contribute to disease development. Gene therapy involves the introduction of one or more foreign genes into an organism to treat hereditary or acquired genetic defects. In gene therapy, DNA encoding a therapeutic protein is packaged within a "vector", which transports the DNA inside cells within the body. The disease is treated with minimal toxicity, by the expression of the inserted DNA by the cell machinery.

Types of gene therapy

There are several approaches for correcting faulty genes; the most common being the insertion of a normal gene into a specific location within the genome to replace a non-functional gene. Gene therapy is classified into the following two types:

1. Somatic gene therapy
2. Germ line gene therapy

Somatic Gene Therapy

In somatic gene therapy, the somatic cells of a patient are targeted for foreign gene transfer. In this case the effects caused by the foreign gene is restricted to the individual patient only, and not inherited by the patient's offspring or later generations.

Germ Line Gene Therapy

Here, the functional genes, which are to be integrated into the genomes, are inserted in the germ cells, i.e., sperm or eggs. Targeting of germ cells makes the therapy heritable.

Gene Therapy Strategies

Gene Augmentation Therapy (GAT)

In GAT, simple addition of functional alleles is used to treat inherited disorders caused by genetic deficiency of a gene product, e.g. GAT has been applied to autosomal recessive disorders. Dominantly inherited disorders are much less amenable to GAT.

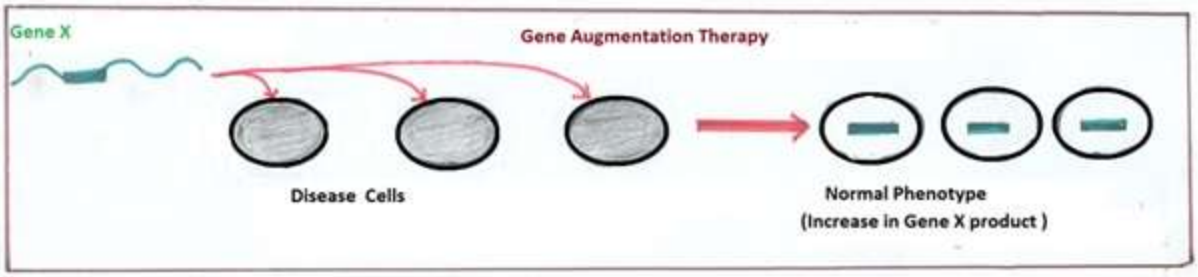


Fig (21): A gene therapy vector has been designed to treat the diseased cells with a gene X. This vector was introduced inside the diseased cells by various gene transfer methods. After a successful homologous recombination the treated cells will show the presence of gene X product as well as normal phenotype.

Targeted Killing of Specific Cells

It involves utilizing genes encoding toxic compounds (**suicide genes**), or **prodrugs** (reagents which confer sensitivity to subsequent treatment with a drug) to kill the transfected/ transformed cells. This general approach is popular in cancer gene therapies.

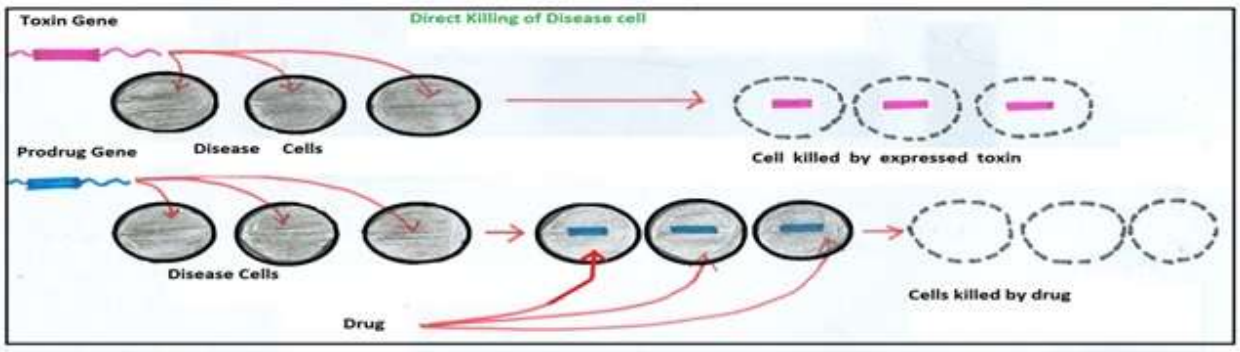


Fig (22): Direct killing of diseased cells by two methods. The first method is the introduction of toxin gene into the diseased cell which when expresses toxin protein the cells die. The second method involves incorporation of a certain gene (e.g. TK) in the gene therapy vector which shows a suicidal property on introducing certain drug (e.g. ganciclovir).

Thymidine kinase (TK) phosphorylates the introduced prodrug ganciclovir which is further phosphorylated by endokinases to form ganciclovir triphosphate, an

competitive inhibitor of deoxyguanosine triphosphate. Ganciclovir triphosphate causes chain termination when incorporated into DNA.

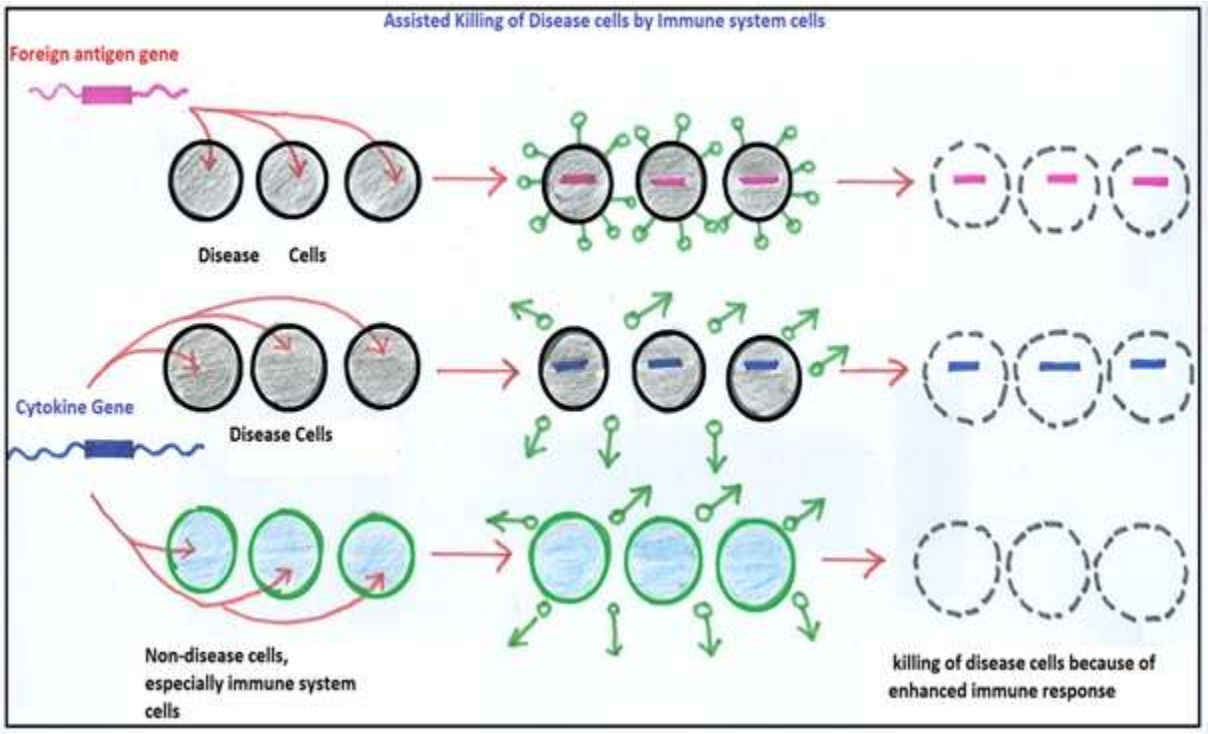


Fig (23): Assisted killing is another strategy of killing diseased cells. Here one method is to insert a well-known foreign antigen coding gene which induces immune cells for the killing of the diseased cells. Few more methods are based on immune cells activation in which a certain cytokine encoding gene incorporated into gene therapy vector and inserted into either diseased cells or non-diseased cells. This will lead to enhanced immune response followed by killing of diseased cells.

Targeted Inhibition of Gene Expression

This is to block the expression of any diseased gene or a new gene expressing a protein which is harmful for a cell. This is particularly suitable for treating infectious diseases and some cancers.

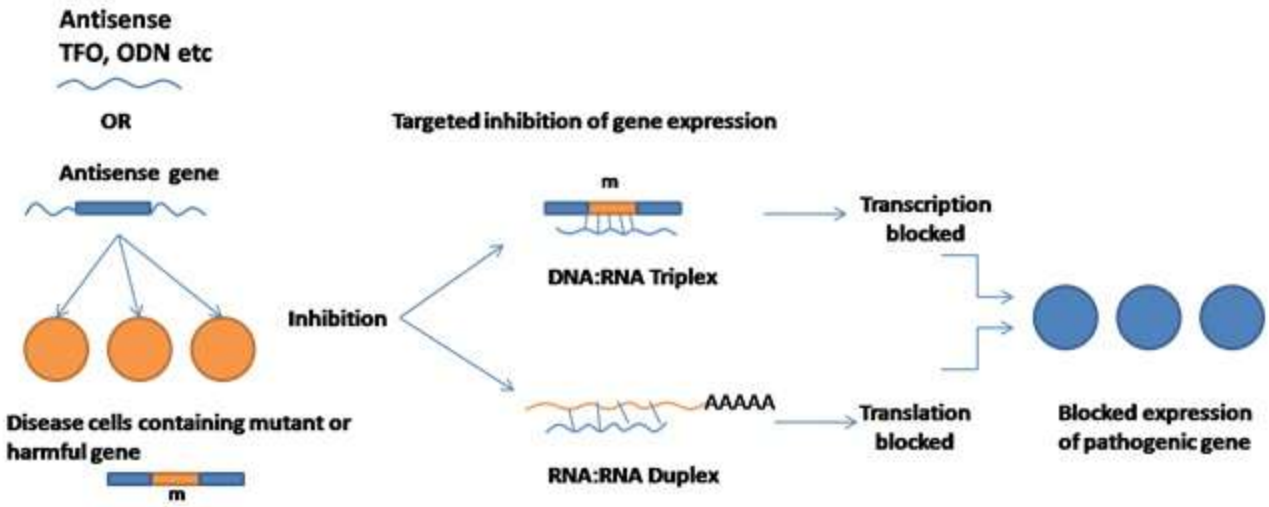


Fig (24): To inhibit the target gene expression in diseased cell the antisense mRNA coding gene inserted vector or triplex-forming oligonucleotides (TFO) or antisense oligonucleotide (ODN) can be introduced which will inhibit the gene expression either by forming DNA:RNA triplex inside the nucleus or forming RNA:RNA duplex by forming complementary mRNA strand of disease protein coding mRNA. This may lead to blocking of disease causing protein expression.

Targeted Gene Mutation Correction

It is used to correct a defective gene to restore its function which can be done at genetic level by homologous recombination or at mRNA level by using therapeutic ribozymes or therapeutic RNA editing.

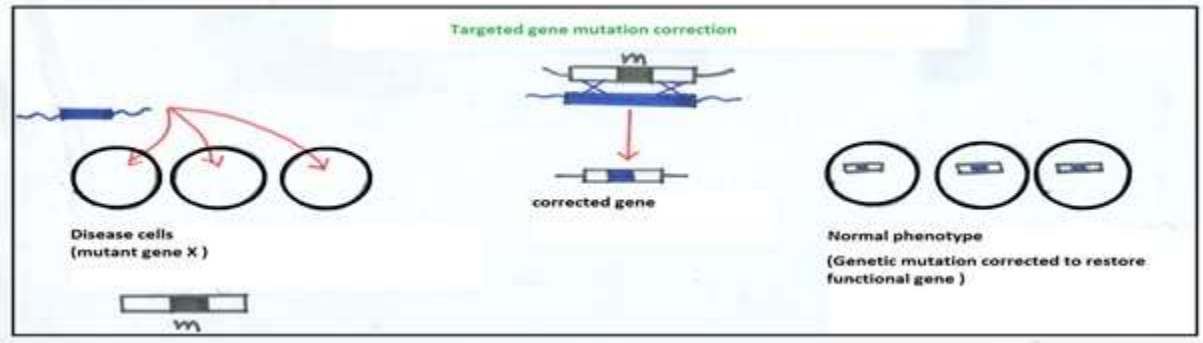


Fig (25): This is used for disease caused by mutation. The corrected gene will be swapped by the mutant gene X(m). Then diseased cells will become normal after the correction of mutation by gene therapy.

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