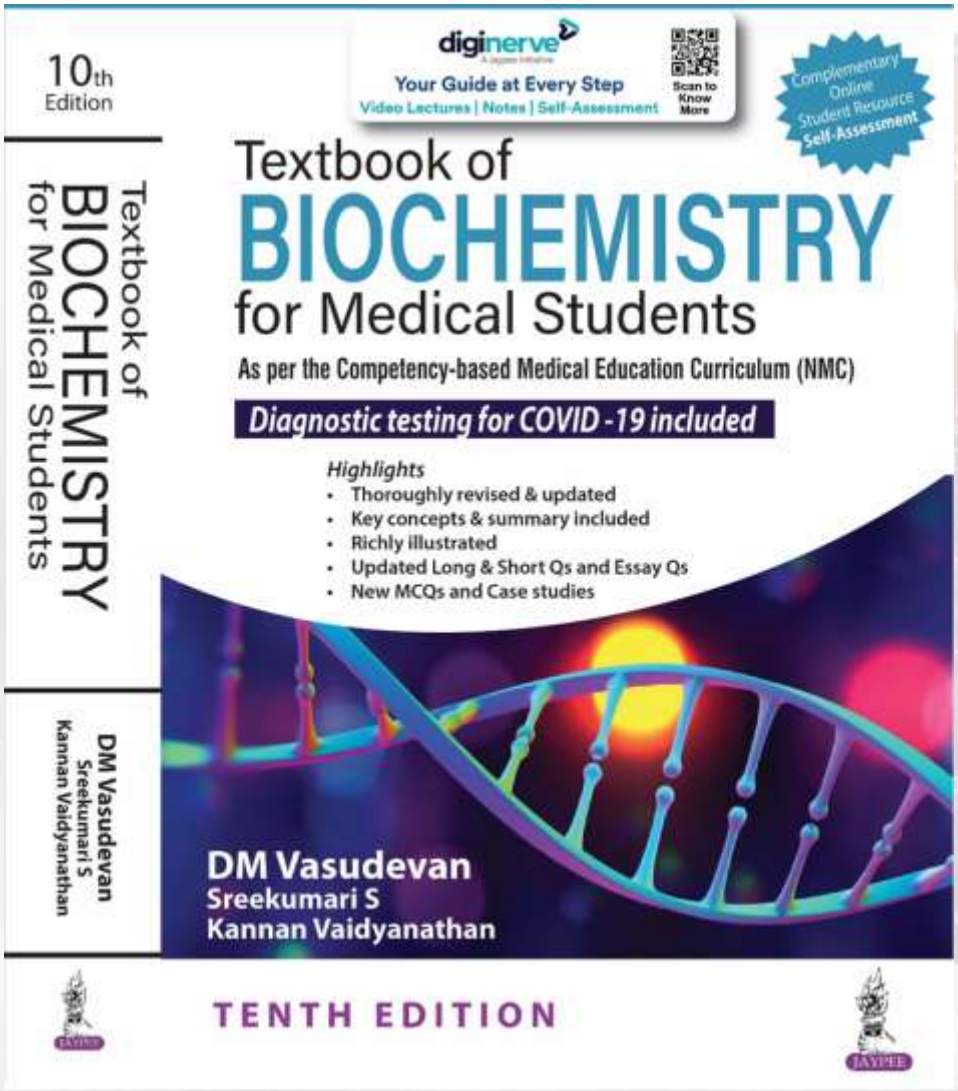
Chapter 38:

Molecular Diagnostics

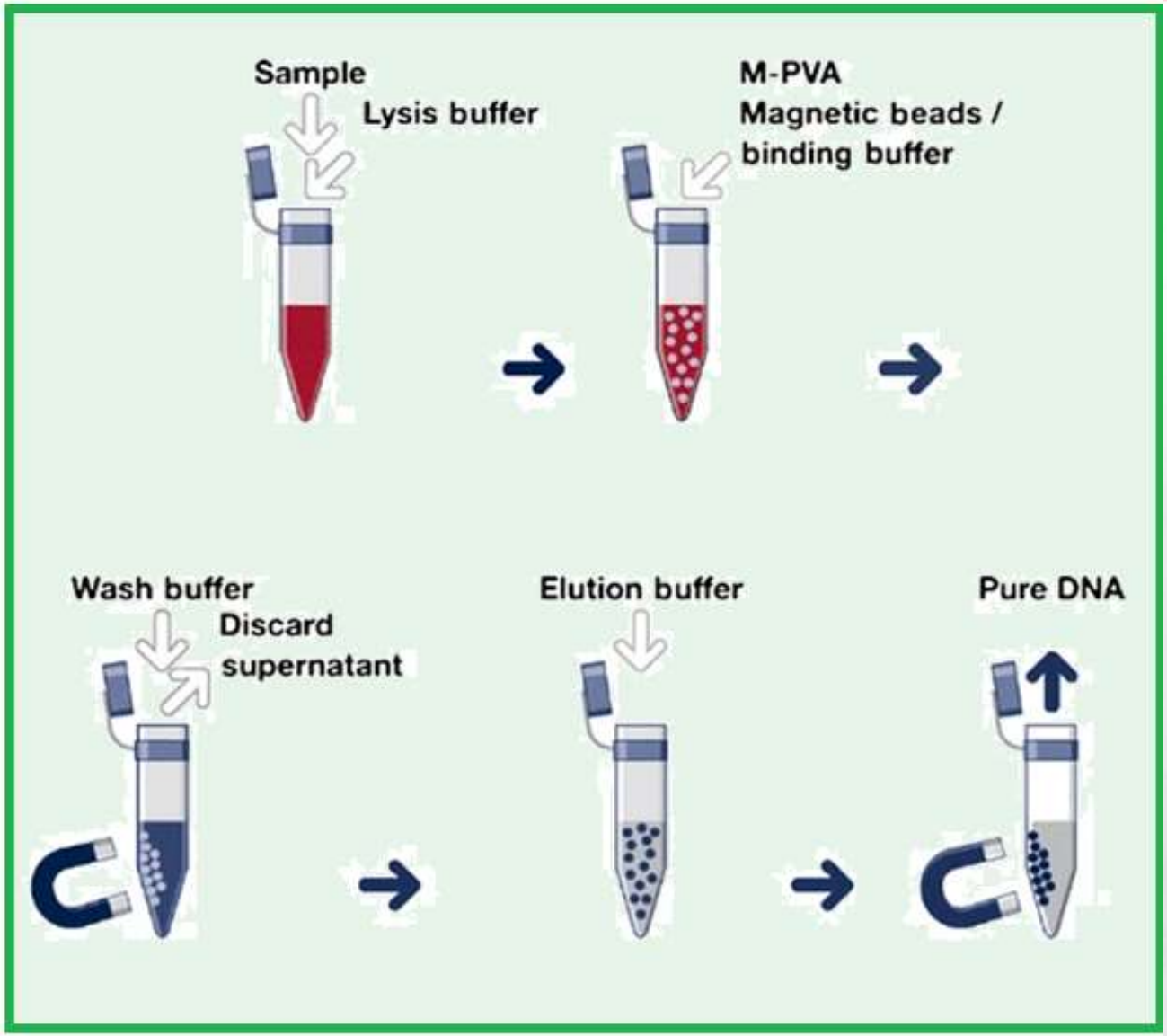


Textbook of
BIOCHEMISTRY
for Medical Students

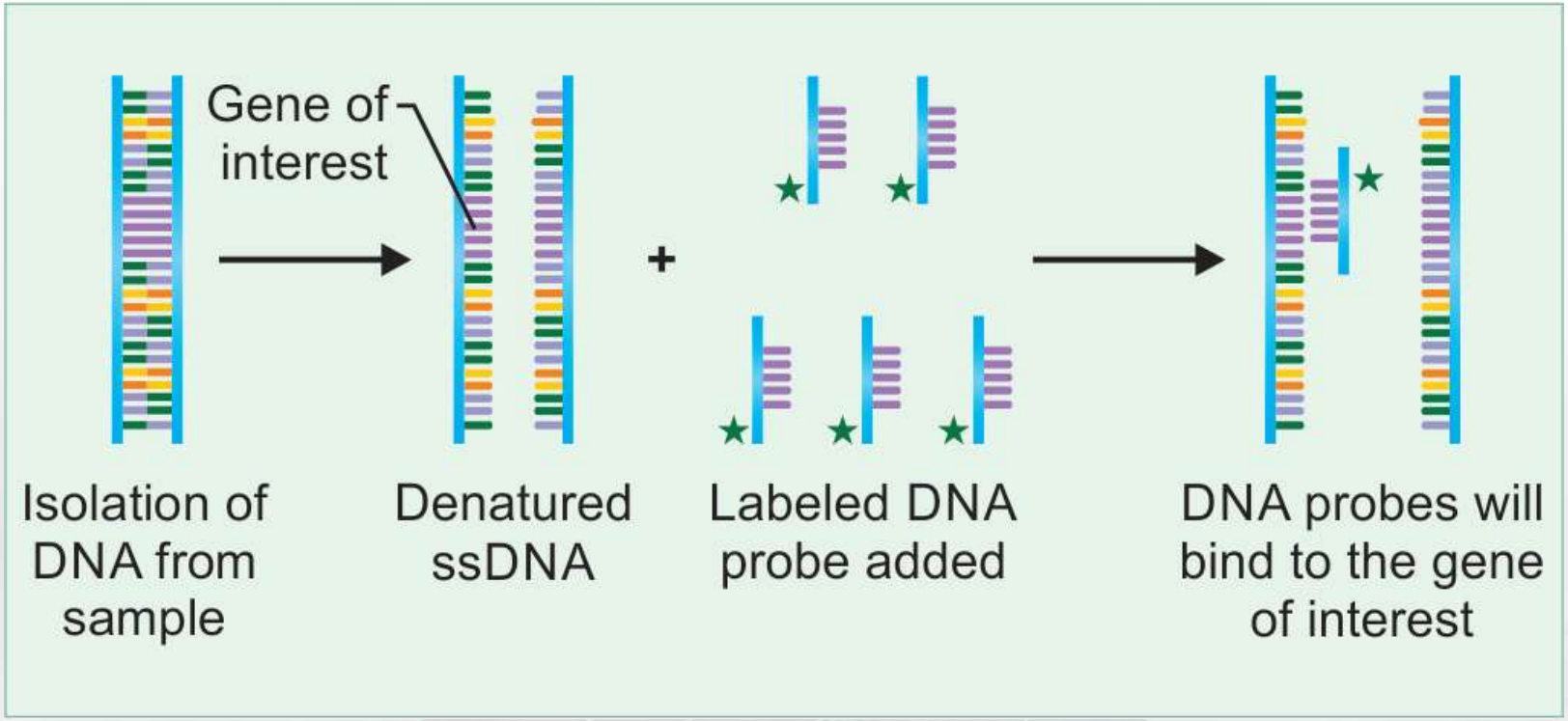
By DM Vasudevan, *et al.*

TENTH EDITION

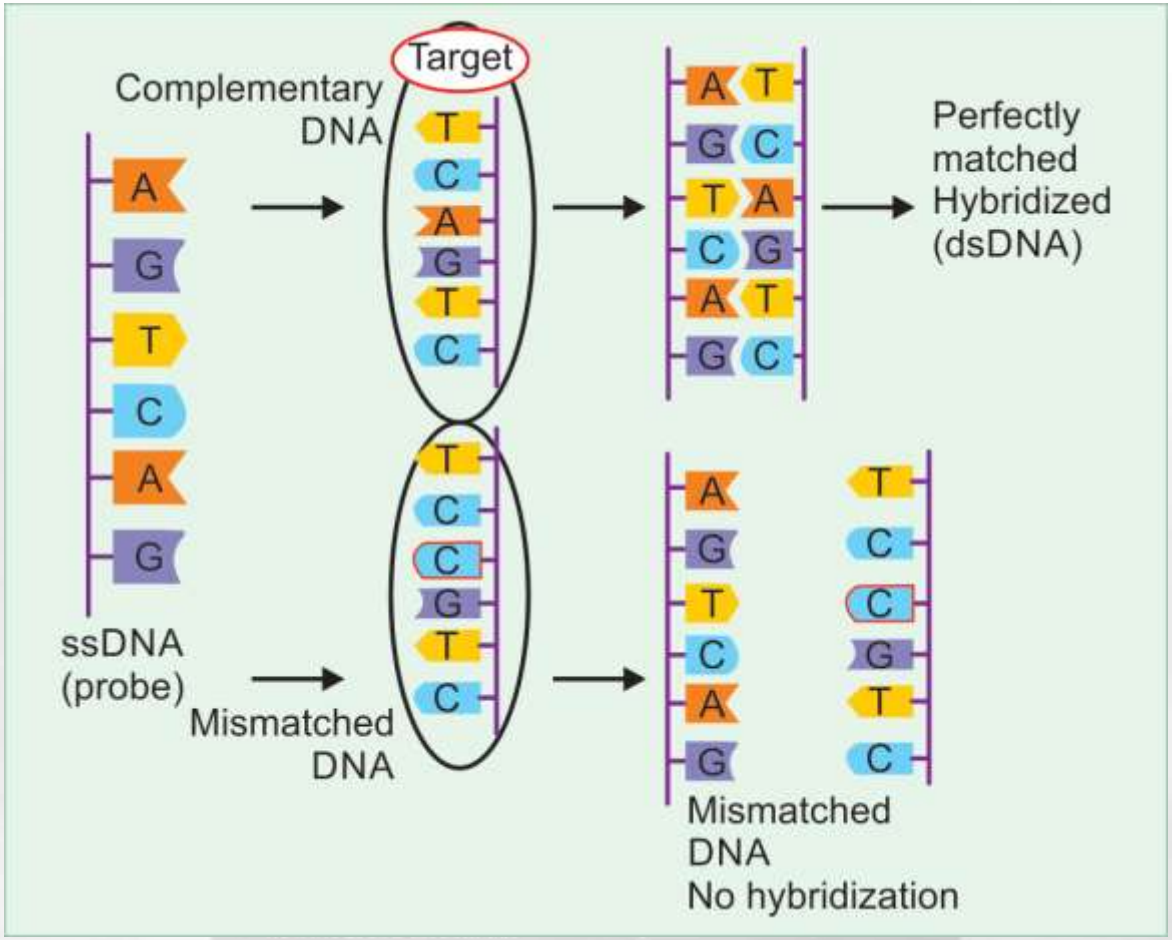
Magnetic Beads Based Extraction of DNA



DNA-DNA Hybridisation



DNA probe is used for hybridization technique.



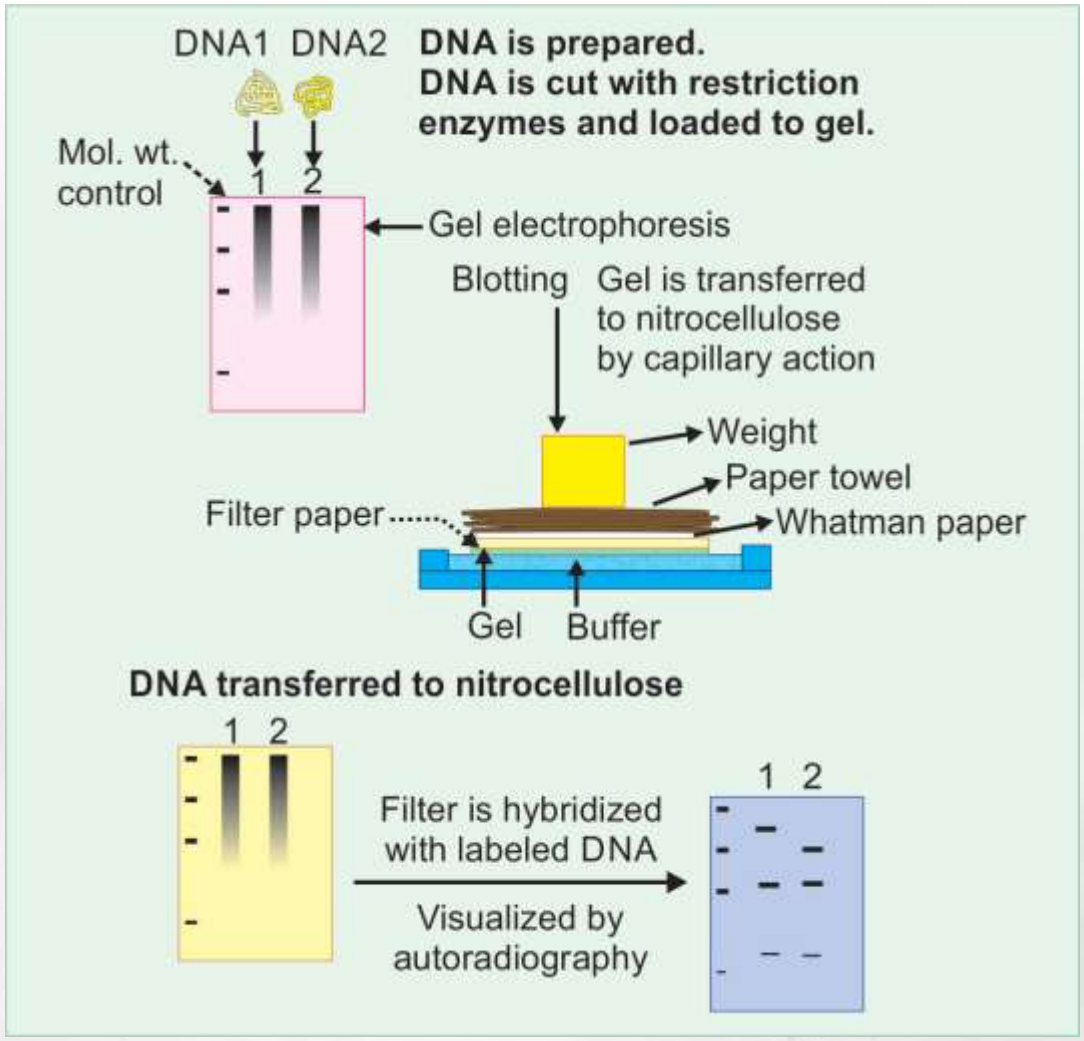
Explanation of hybridization. Only when there is a complementary strand in the DNA, the probe could hybridize.

Hybridisation and Blot Techniques

Southern Blot Technique

This is used to detect a specific segment of DNA in the whole genome. It is based on the specific base pairing properties of omplementary nucleic acid strands.

A probe is defined as a single stranded piece of DNA, labelled (either with radio-isotope or with non-radioactive label), the nucleotide sequence of which is complementary to the target DNA.



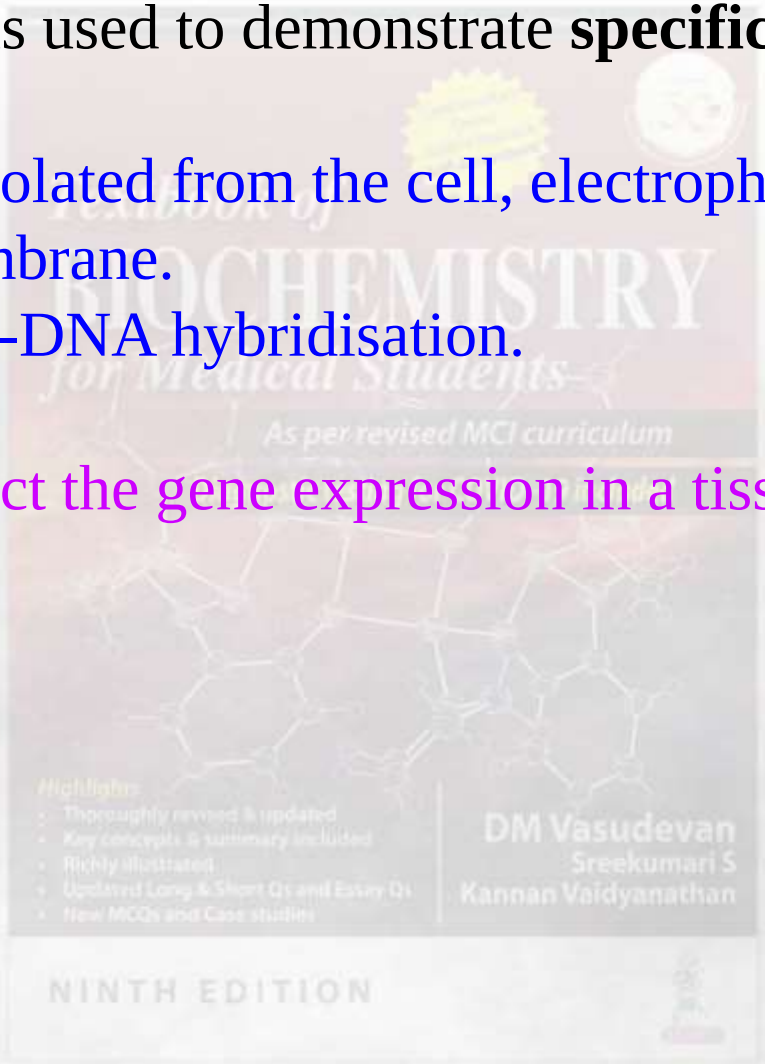
Southern blot technique.

Northern Blotting

The Northern blot is used to demonstrate **specific RNA**.

The total RNA is isolated from the cell, electrophoresed and then blotted on to a membrane.
There will be RNA-DNA hybridisation.

This is used to detect the gene expression in a tissue.

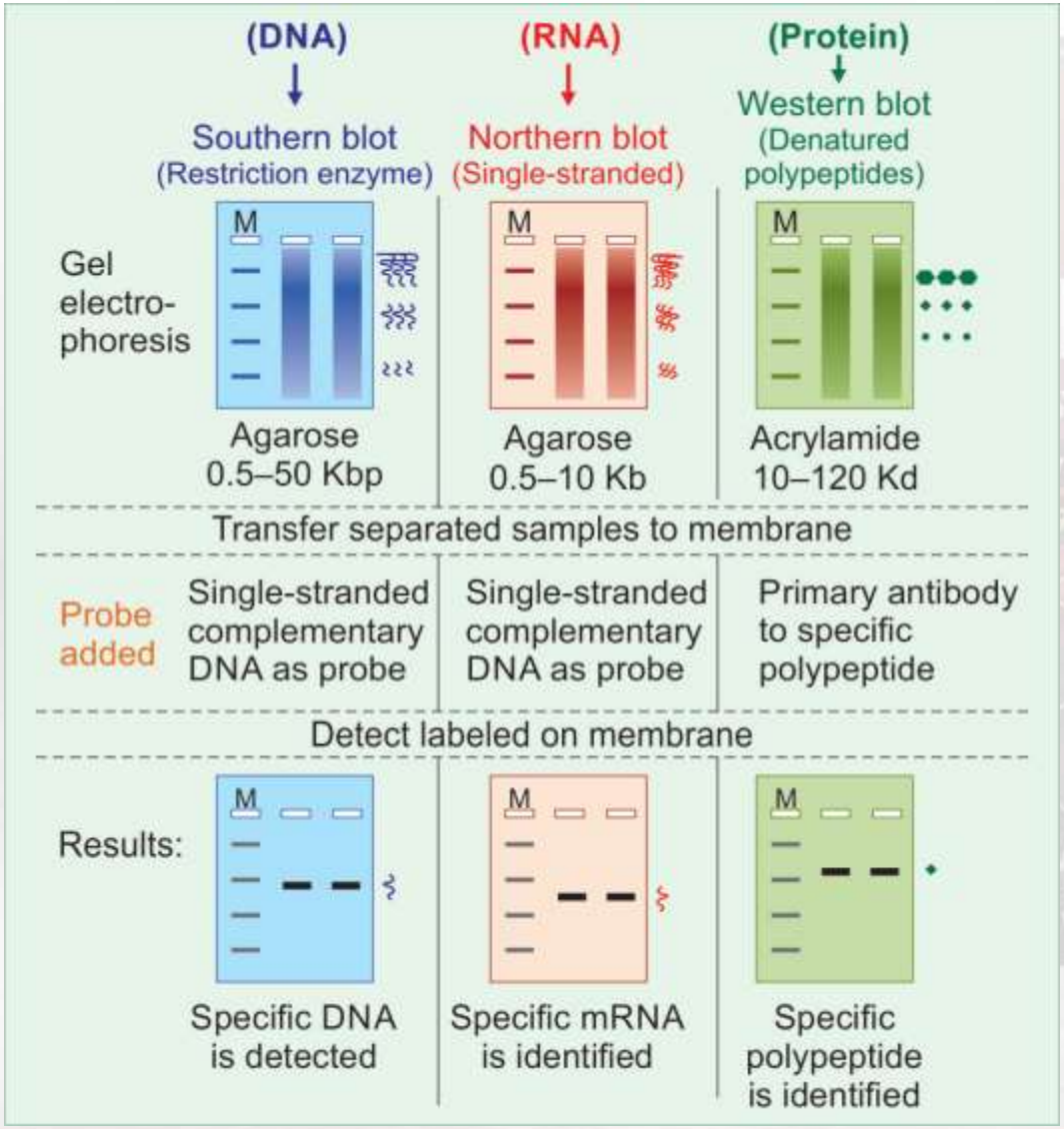


Western Blot Analysis

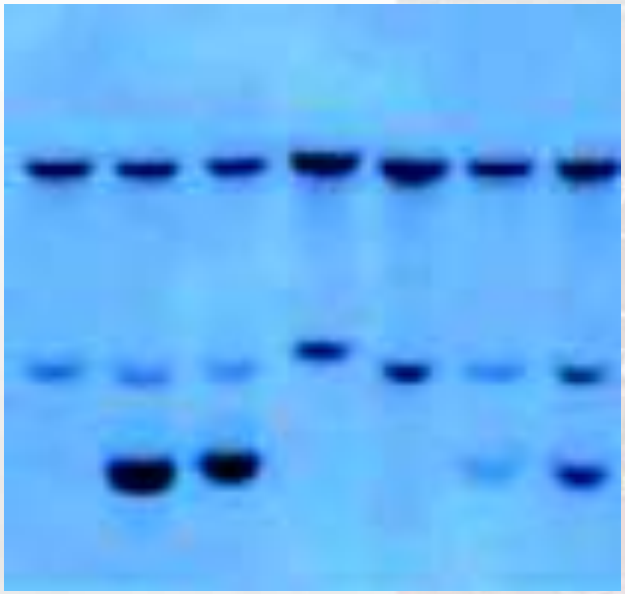
In this technique, **proteins** (not nucleic acids) are identified.

The proteins are isolated from the tissue and electrophoresis is done; transferred on to a **nitrocellulose** membrane, it is probed with radioactive **antibody** and auto-radiographed.

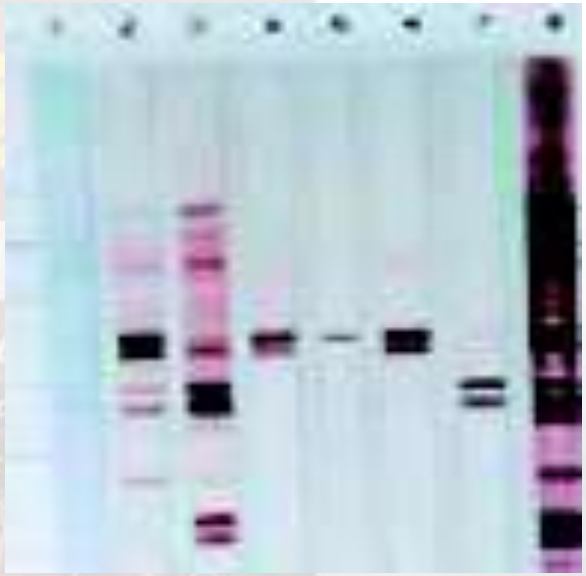
This technique is very useful to identify the specific protein in a tissue, thereby showing the **expression of a particular gene**.



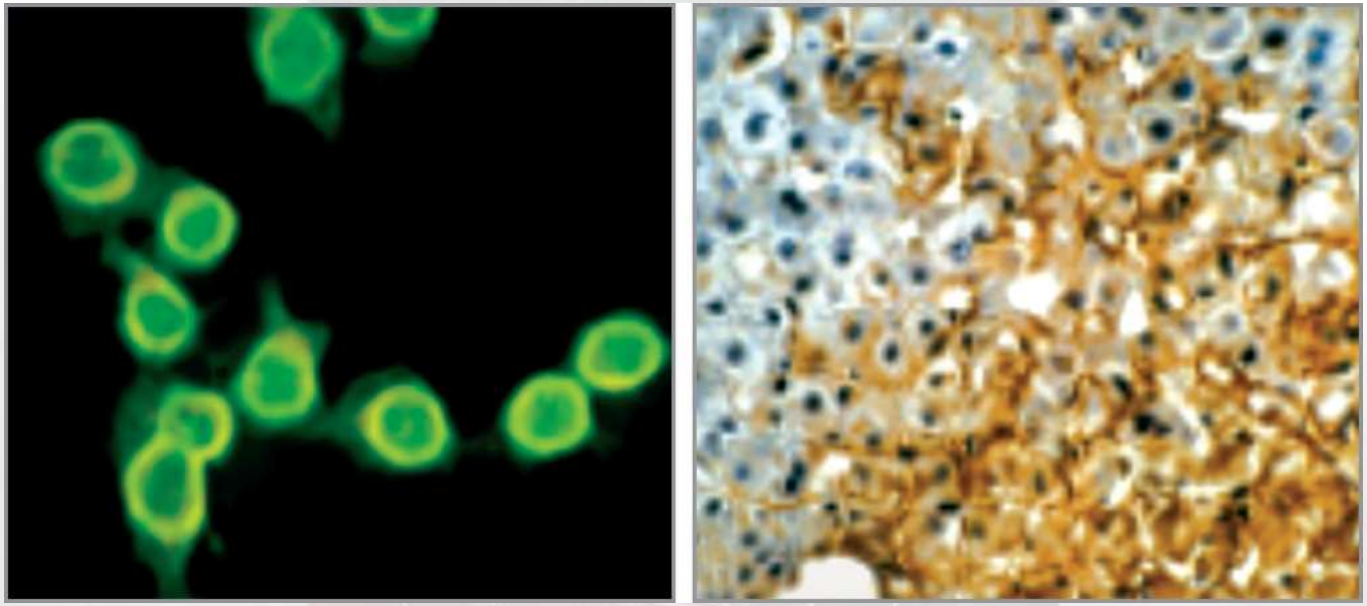
Comparison of blot transfer techniques.



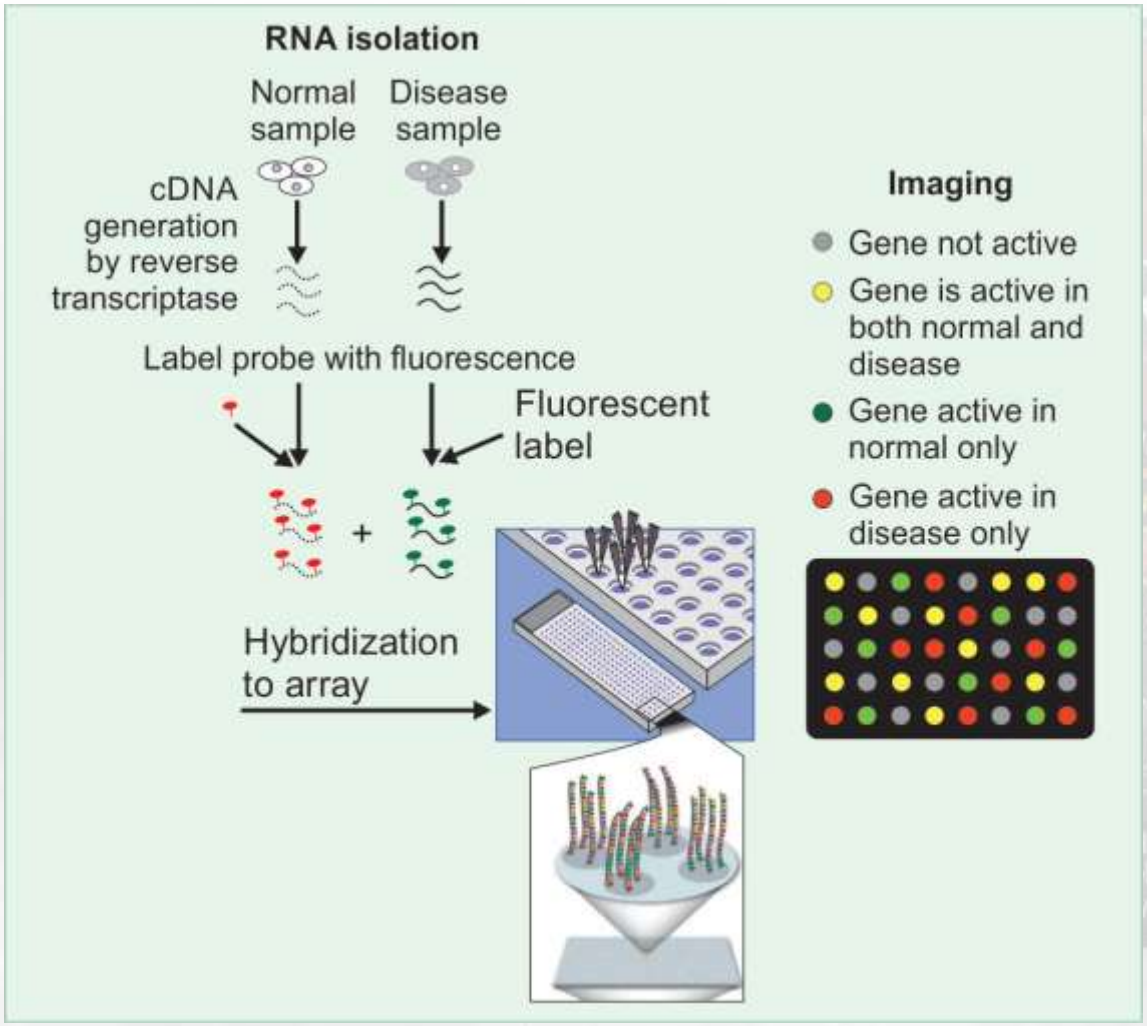
Southern blot



Western blot



(A) Fluorescent in situ hybridization (FISH); the cells are incubated with specific antibody showing surface immunofluorescence.
(B) immunoperoxidase technique



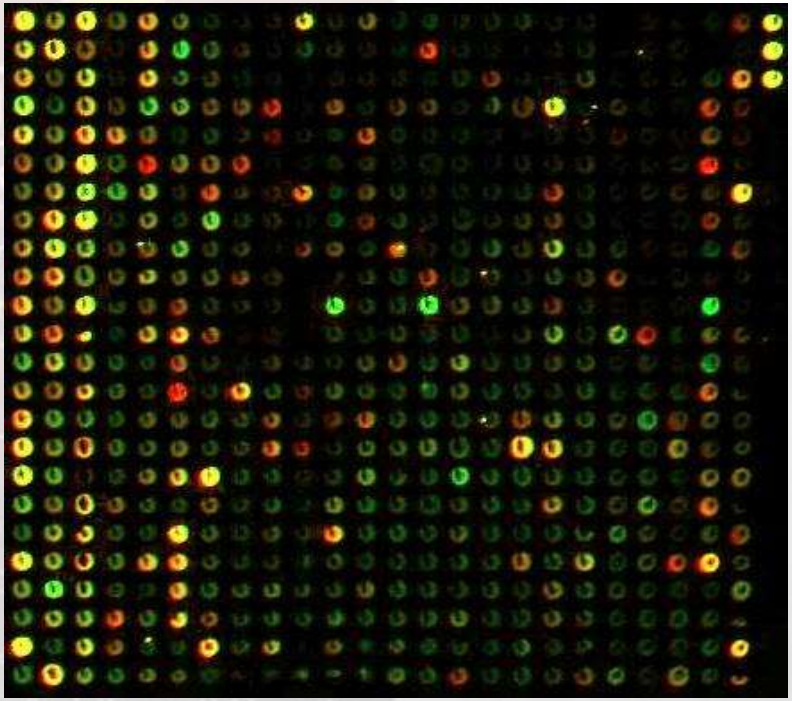
Microarray technology. Red mark means diminished gene activity. Green light indicates increased gene activity.

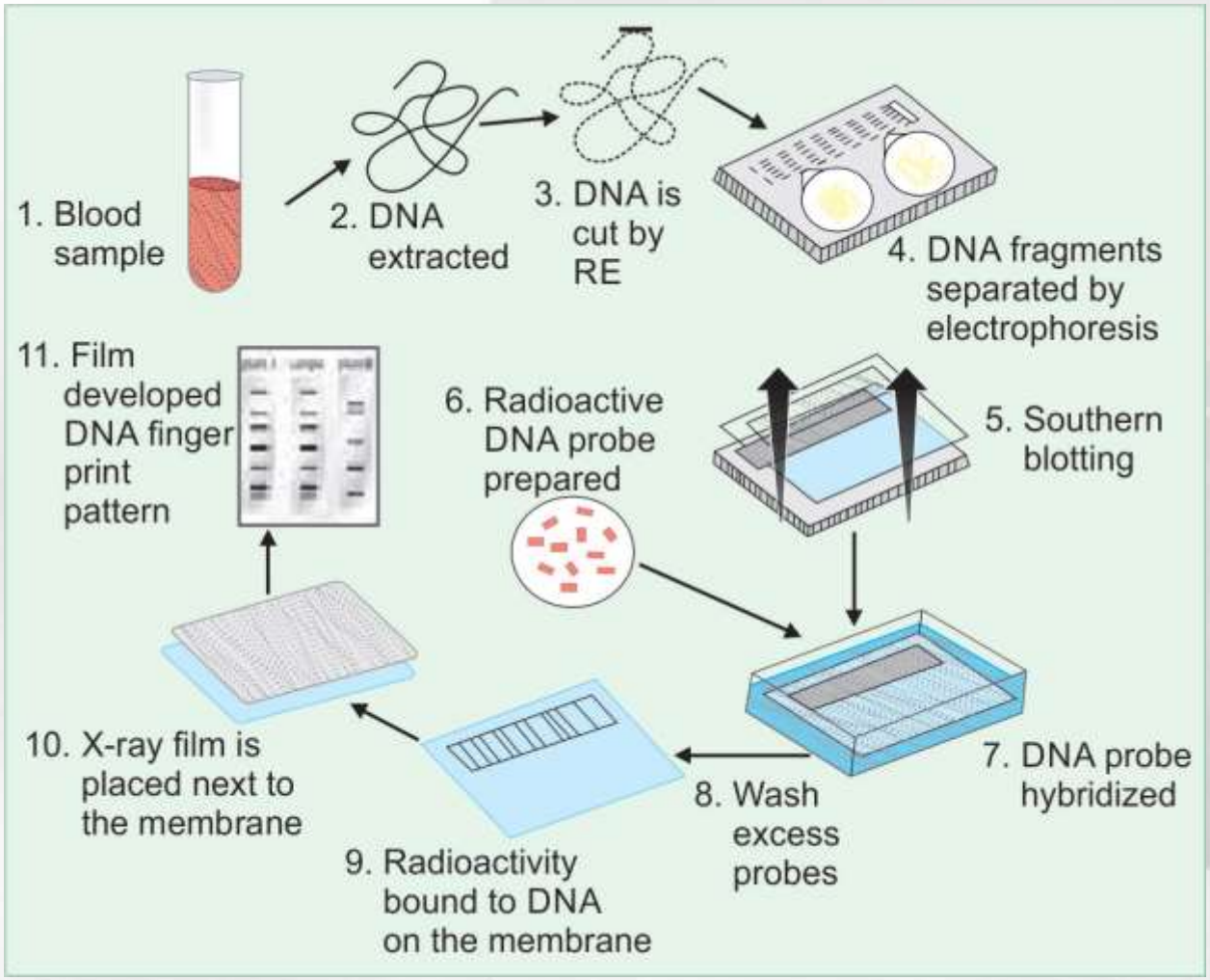
Micro-Array Technique

DNA probes of about 500 to 5000 genes are prepared and added to very small wells in polystyrene plates.

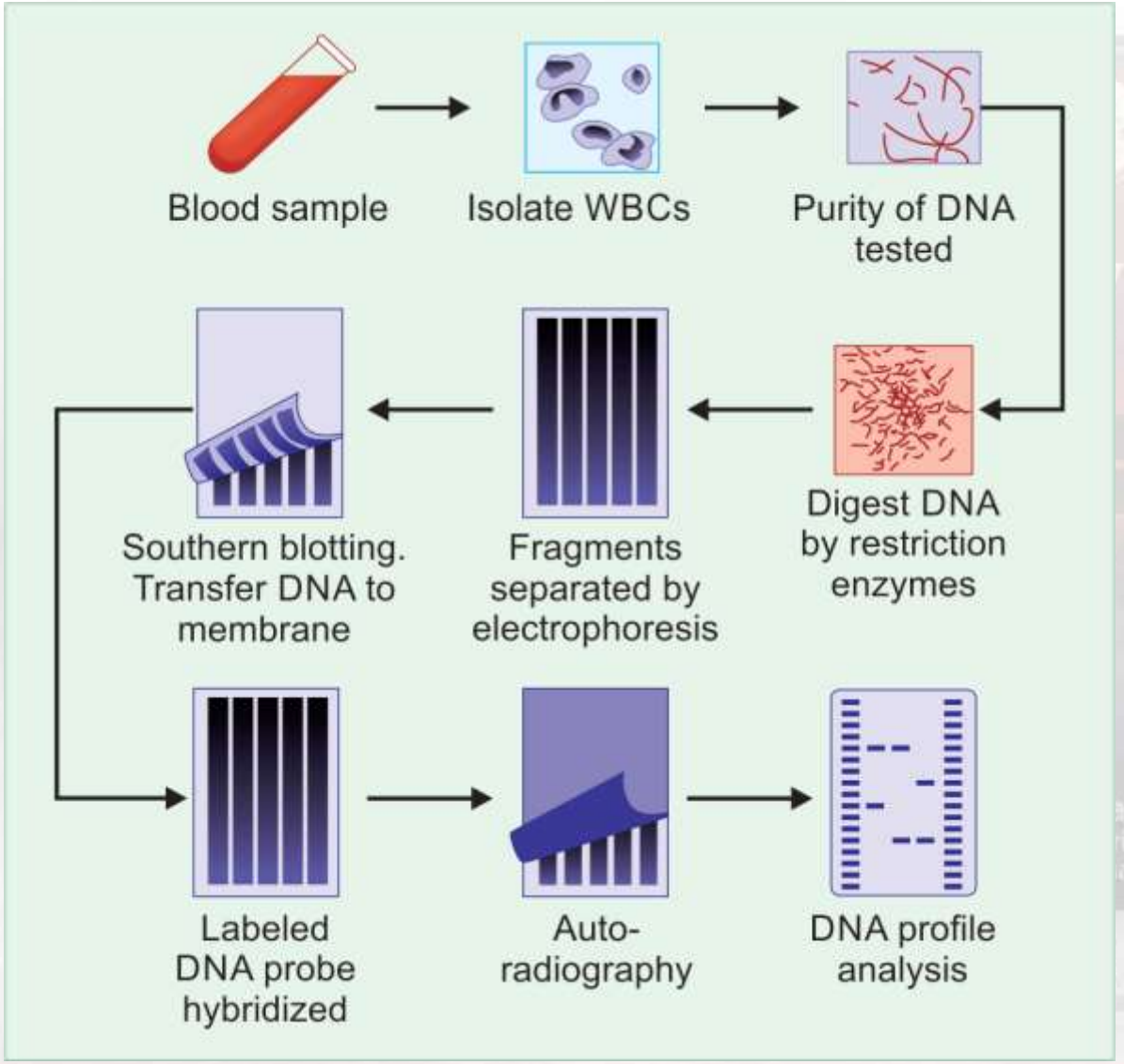
DNA prepared from patient's tissue is added to each well, hybridised.

Fluorescence is assessed. **So, the activity of various genes may be identified quickly.**





DNA ingerprinting. The technique is used to pinpoint the culprit of the crime, and also to settle the disputes of parenthood. DNA can be isolated from blood or semen stains on clothing, even several years after the crime.



Restriction fragment length polymorphism (RFLP).

Restriction Fragment Length Polymorphism

The human genome contains hundreds of variations in base sequences that do not affect the phenotype. The *property of the molecules to exist in more than one form is known as* **polymorphism**.

Difference in mutation and DNA polymorphism:

If more than 1% of the population has a particular alteration in the sequence, it is polymorphism. If only a few individuals have it, then it is a **mutation**. Polymorphism is the normal variation, and generally having no deleterious effect. Mutation is abnormal, and sometimes will have defective function, e.g. phenylketonuria.

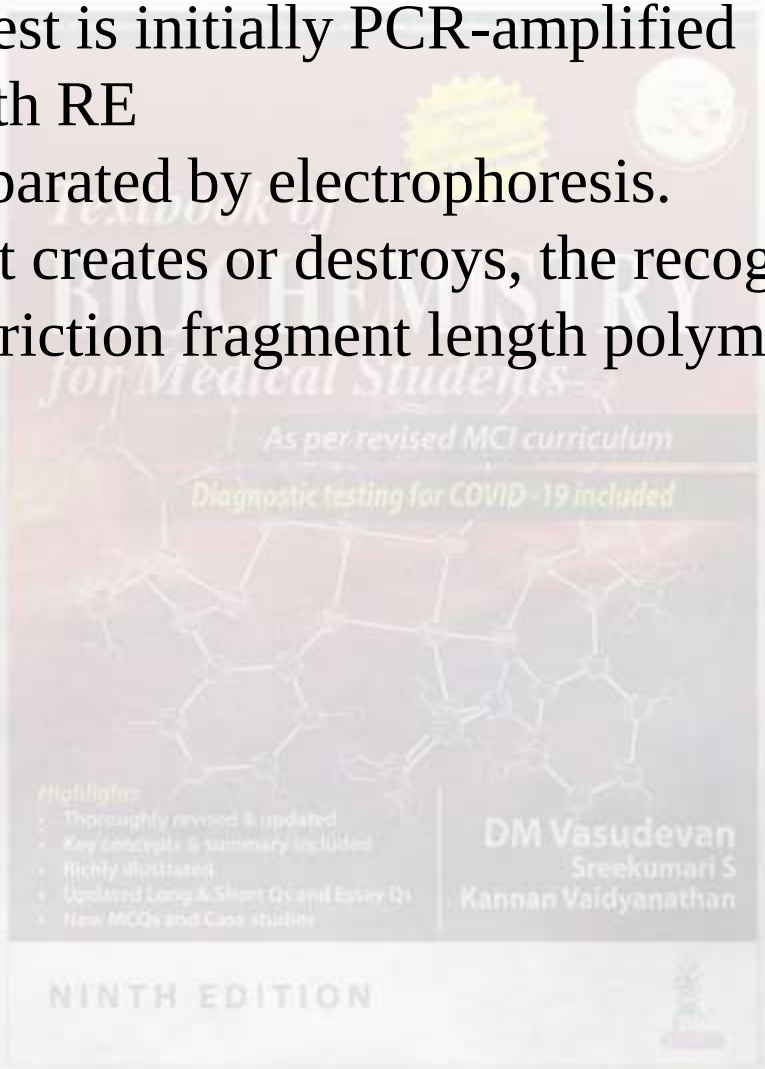
The existence of two or more types of **restriction fragment patterns** is called restriction fragment length polymorphism (RFLP) and can be used as a **genetic marker**. DNA is treated with restriction enzymes, then electrophoresis is done in agarose gels, then fragments are transferred on to nitrocellulose paper (Southern blotting) and hybridized with labeled probe sequences.

Clinical Applications of RFLP

- The analysis of parental origin of each allele of a genetic locus helps to establish or eliminate biological parentage in cases of *disputed parenthood.*
- RFLP is also useful in human *population genetics*, geographical distribution and genetic makeup of people belonging to different races and ethnicities can be studied.
- *Genetic diseases* will produce alteration in size distribution of RE fragments, and show RFLP.

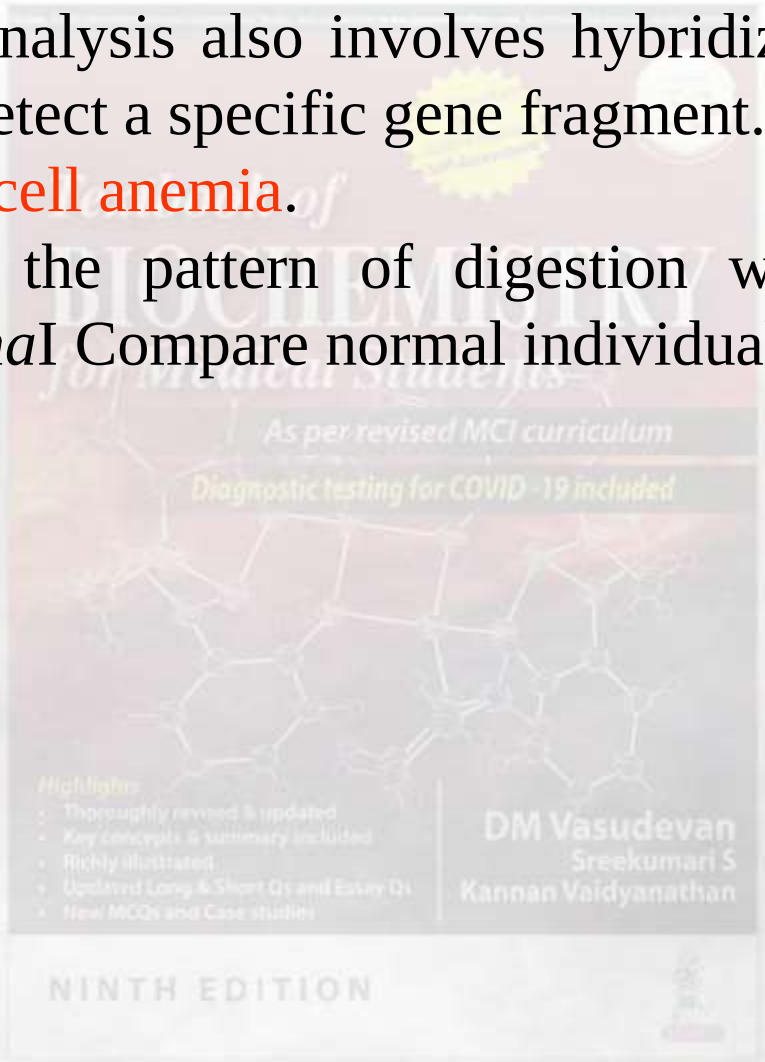
Restriction Fragment Length Polymorphism (RFLP)

- The gene of interest is initially PCR-amplified
- Then digested with RE
- Fragments are separated by electrophoresis.
- Any mutation that creates or destroys, the recognition sequence for RE leads to a restriction fragment length polymorphism (RFLP).

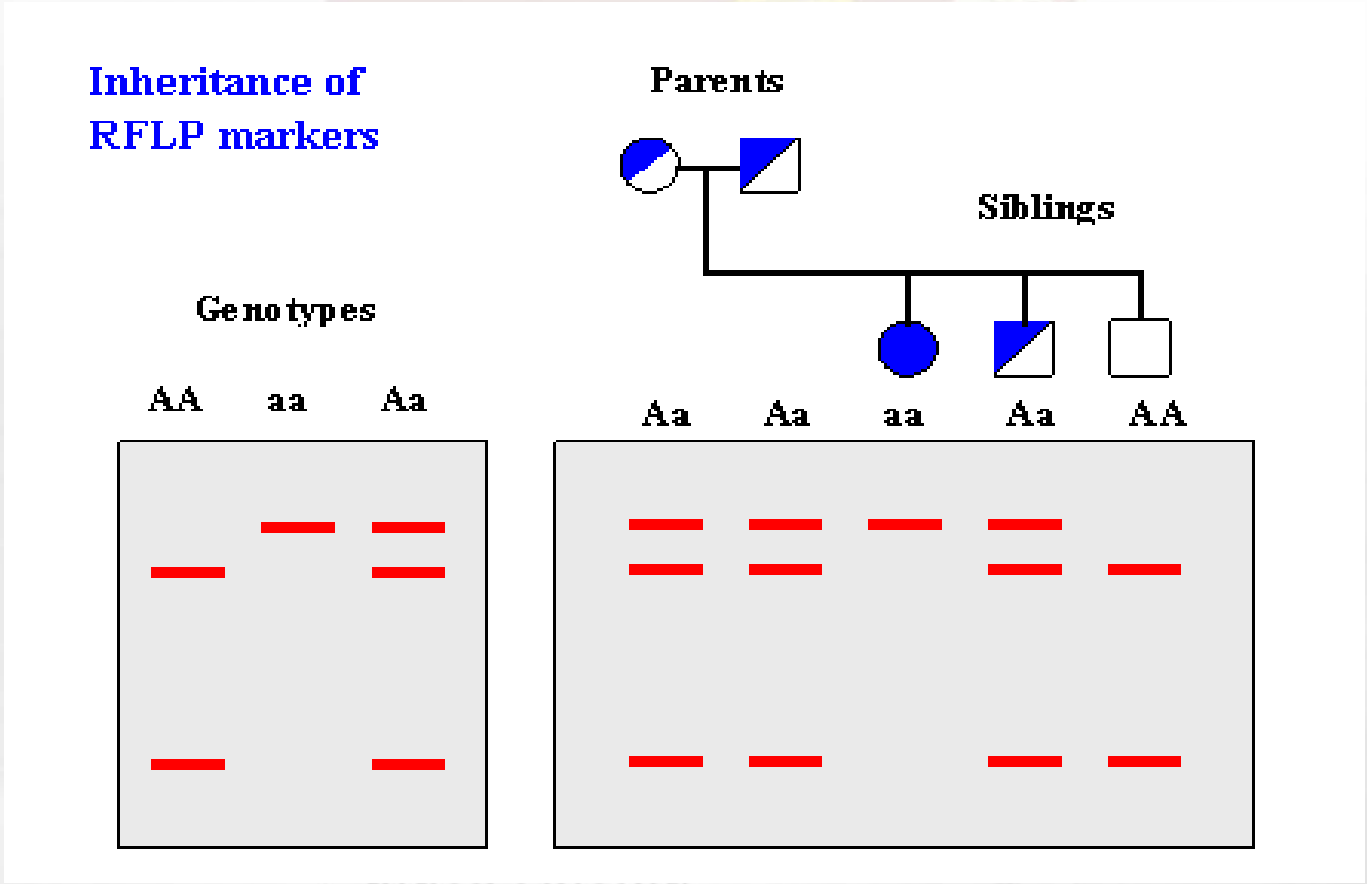


Restriction Fragment Length Polymorphism (RFLP)

- Routine RFLP analysis also involves hybridization with labeled gene probes to detect a specific gene fragment.
- Example, sickle cell anemia.
- a difference in the pattern of digestion with the restriction endonuclease *Hha*I Compare normal individuals and patients with the disease.



Restriction fragment length polymorphism (RFLP)



Polymerase Chain Reaction

PCR is an in vitro DNA amplification procedure.

Step 1: Separation (Denaturation)

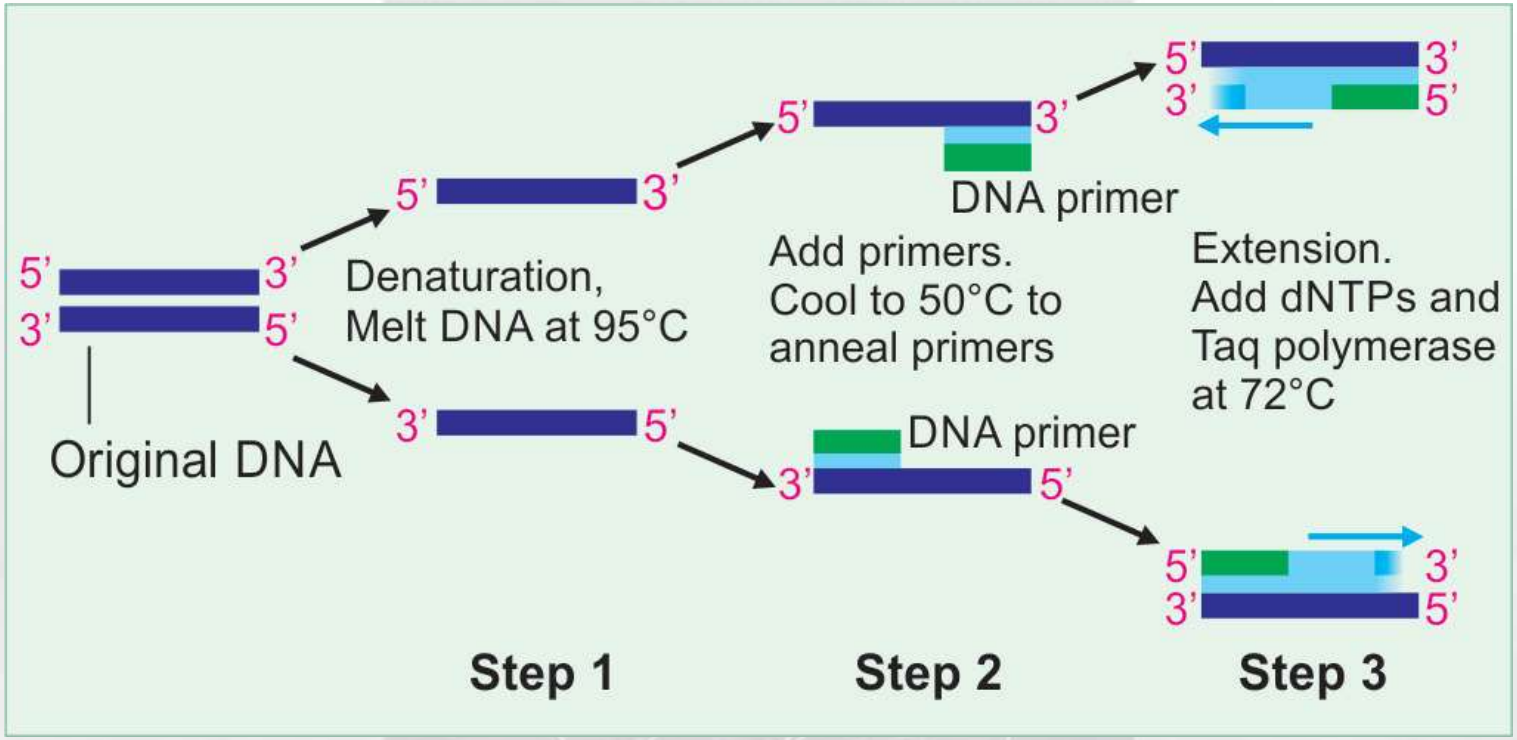
The DNA strands are separated (**melted**) by heating at 95°C for 15 sec to 2 min.

Step 2: Priming (Annealing)

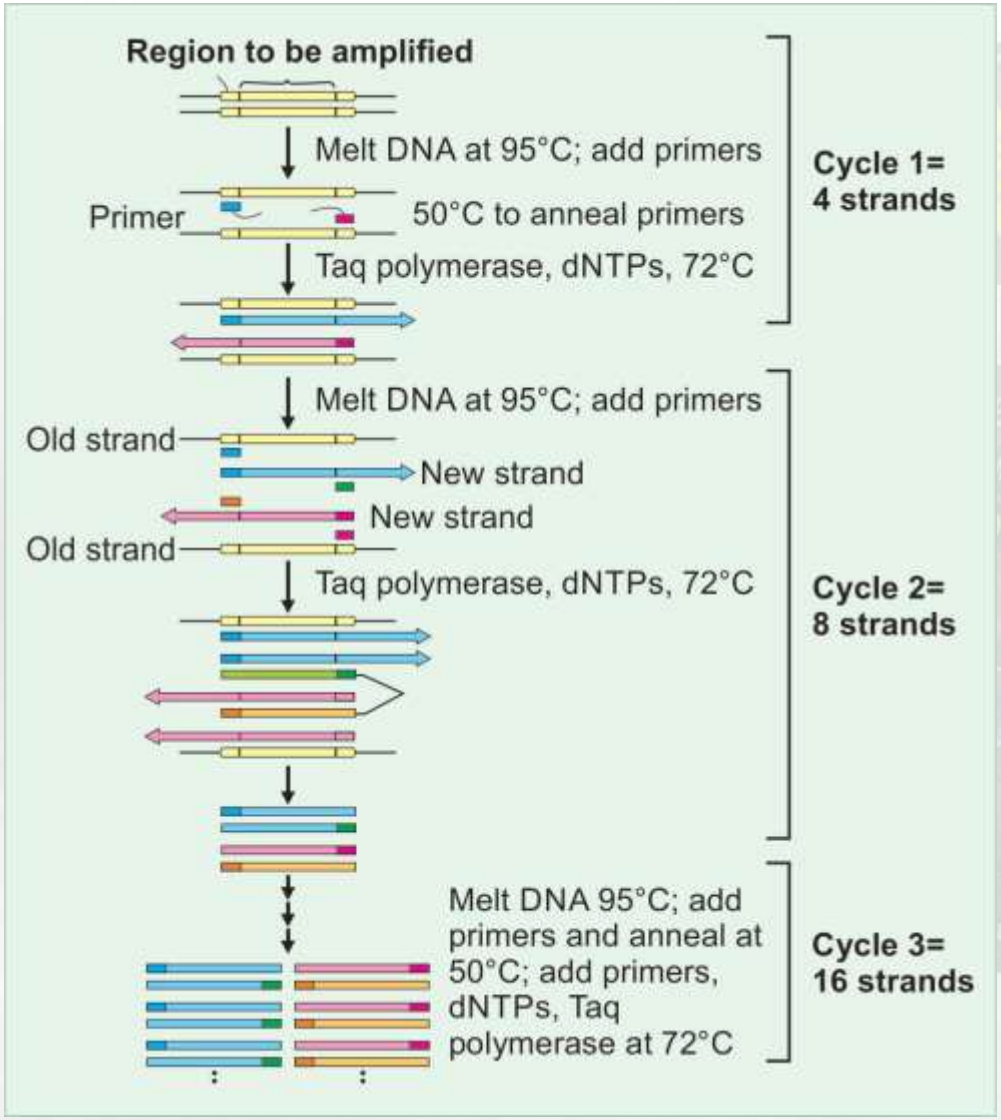
The primers are **annealed** by cooling to 50°C for 0.5 to 2 minutes. The primers hybridize with their complementary single-stranded DNA.

Step 3: Extension

The new DNA strands are synthesized by **Taq polymerase. T**he enzyme is not denatured at 72°C for 30 seconds in presence of dNTPs. Both strands of DNA are now duplicated. The steps 1, 2 and 3 are repeated. In each cycle, the DNA strands are doubled. Thus, 20 cycles provide for 1 million times amplifications. These cycles are generally repeated by automated instrument, called **TempCycler**.



Polymerase chain reaction (PCR).



Polymerase chain reaction, three cycles are shown.

Polymerase Chain Reaction

Cycle 4 = 32 strands

Cycle 5 = 64 strands

Cycle 6 = 128 strands

Cycle 7 = 256 strands

Polymerase Chain Reaction

1. Diagnosis of bacterial and viral diseases

In early phases of tuberculosis, the sputum may contain only very few tubercle bacilli, so that usual acid fast staining may be negative.

But PCR could detect even one bacillus present in the specimen. Any other bacterial infection could also be detected.

The specific nucleotide sequences of the bacilli are amplified by PCR and then detected by Southern blot analysis.

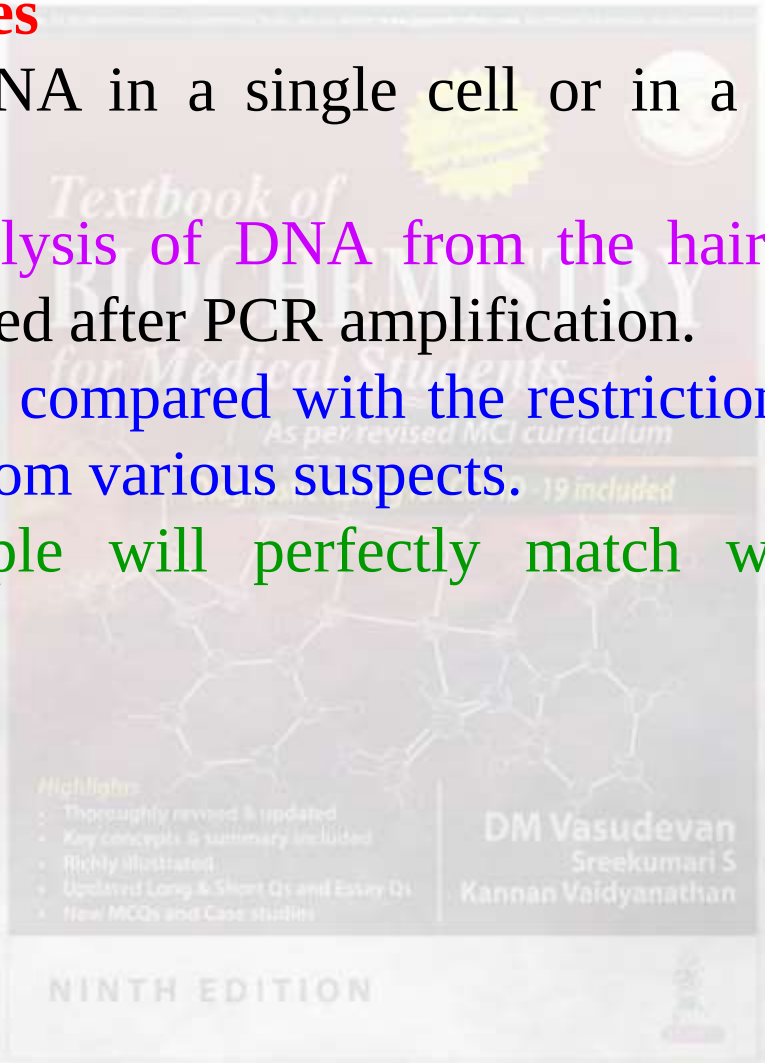
2. Medicolegal cases

PCR allows the DNA in a single cell or in a hair follicle to be analysed.

The restriction analysis of DNA from the hair follicle from the crime scene is studied after PCR amplification.

This pattern is then compared with the restriction analysis of DNA samples obtained from various suspects.

The culprit's sample will perfectly match with that of PCR amplified sample.



3. Diagnosis of genetic disorders

The PCR technology has been widely used to amplify the gene segments that contain known mutations for diagnosis of inherited diseases such as sickle cell anemia, thalassemia, cystic fibrosis, etc.

4. Prenatal diagnosis of inherited diseases

5. Cancer detection: PCR is widely used to monitor residual abnormal cells present in treated patients. Similarly, identification of mutations in oncosuppressor genes such as p53, or retinoblastoma gene can help to identify individuals at high risk of cancer.

Reverse Transcriptase PCR (RTPCR)

Instead of Taq polymerase, Tth polymerase from *Thermus thermophilus* may be used.

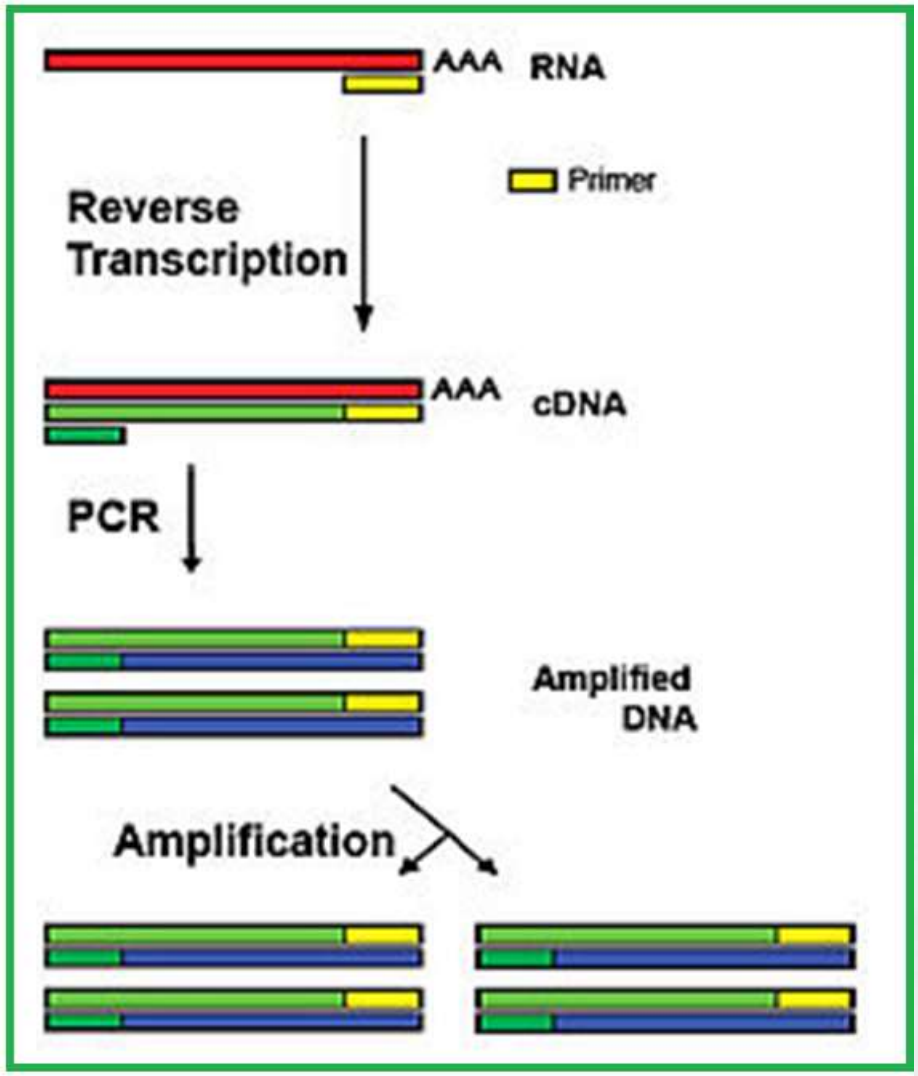
This enzyme has both DNA polymerase and reverse transcriptase activities at 95°C.

So mRNA is copied to cDNA synthesis followed by PCR amplification.

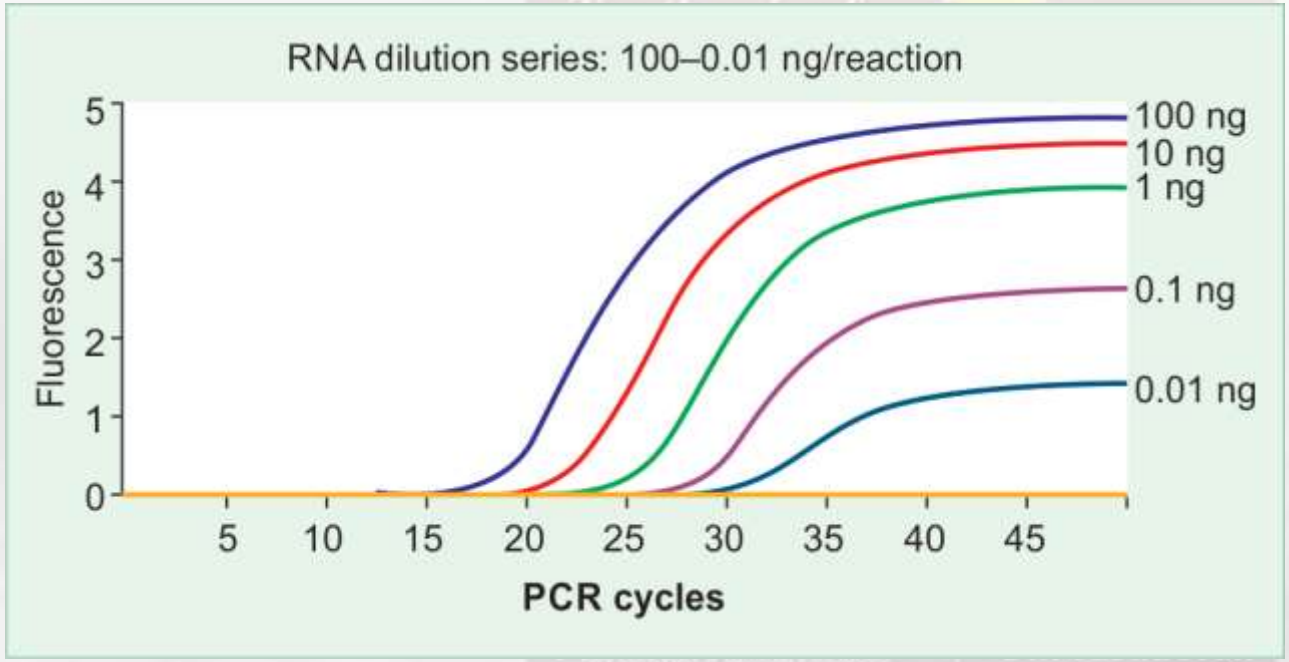
In ordinary PCR, DNA is detected; that DNA could be from a living or non-living organism.

But in reverse PCR, mRNA is detected; that means, it is derived from a living organism.

Reverse Transcriptase PCR (RTPCR)



Real Time Quantitative (QPCR)



By this method, quantitation of the number of virus present in a sample can be calculated., e.g.,viral load in HIV (human Immuno deficiency virus or HBV (hepatitis B virus).

Nested PCR

Nested PCR is intended to reduce the contamination in products due to the amplification of unexpected primer binding sites.

Two sets of primers are used in two successive PCR runs; the second set intended to amplify a secondary target within the product of the first round of amplification.

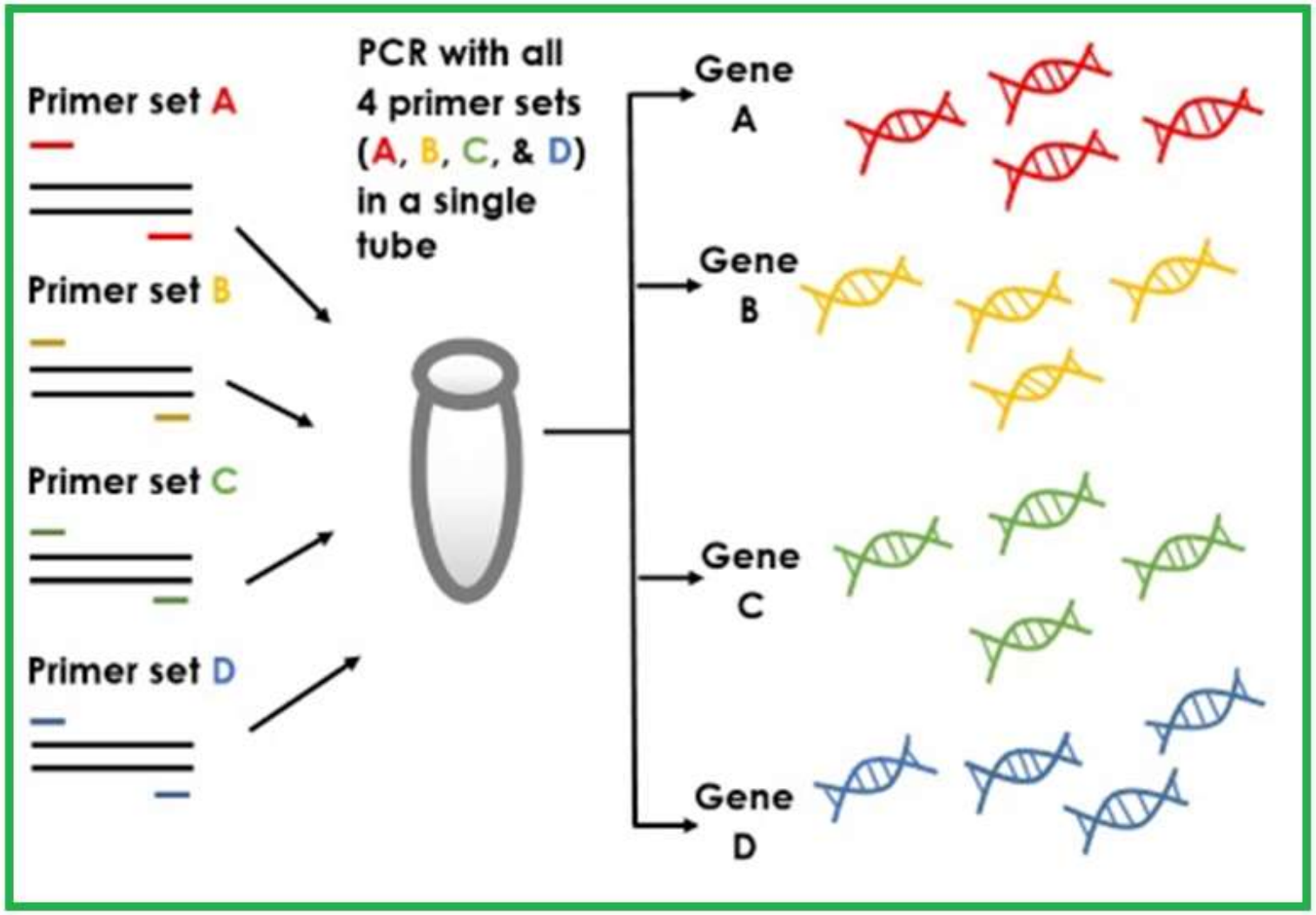
Nested PCR is intended to reduce the contamination in products due to the amplification of unexpected primer binding sites.

Multiplex-PCR

The use of multiple, unique primer sets within a single PCR reaction to produce amplicons of varying sizes specific to different DNA sequences. By targeting multiple genes at once, additional information may be elicited from a single test run that otherwise would require several times the reagents and technician time to perform.

The FilmArray is a multiplex PCR system that integrates sample preparation, amplification, detection and analysis. This technique helps in getting quick results in cases of infection and sepsis.

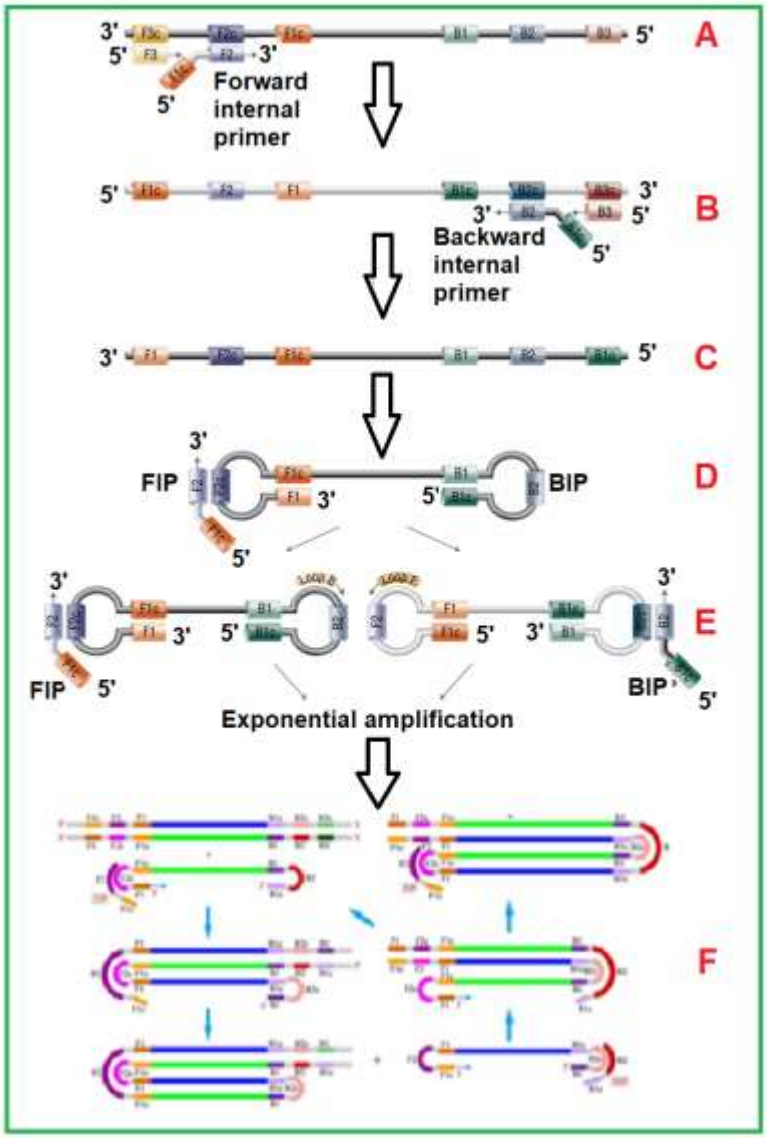
Multiplex-PCR



LAMP Test

Loop-mediated Isothermal amplification (LAMP), allows DNA amplification at a constant temperature (60°C), thus avoiding the thermal cycing. Here, the initiates the melting of strands, then proceeds to synthesize the new DNA strand. Typically, 4 different primers are used, which adds specificity. Using these primers, the new DNA is synthesized at both ends. As there are complementary sequences available in the end regions, loops are formed in the 3' and 5- ends. Thus, small stem-loop ends are formed on both sides of the ssDNA strand. the stem-loop will act as an automatic primer for the target region. New copies would automatically have a primer bound to it (via stem-loop). In this way, there will be exponential amplification, so that within a few minutes, millions of copies of DNA are produced (Fig.38.18F).

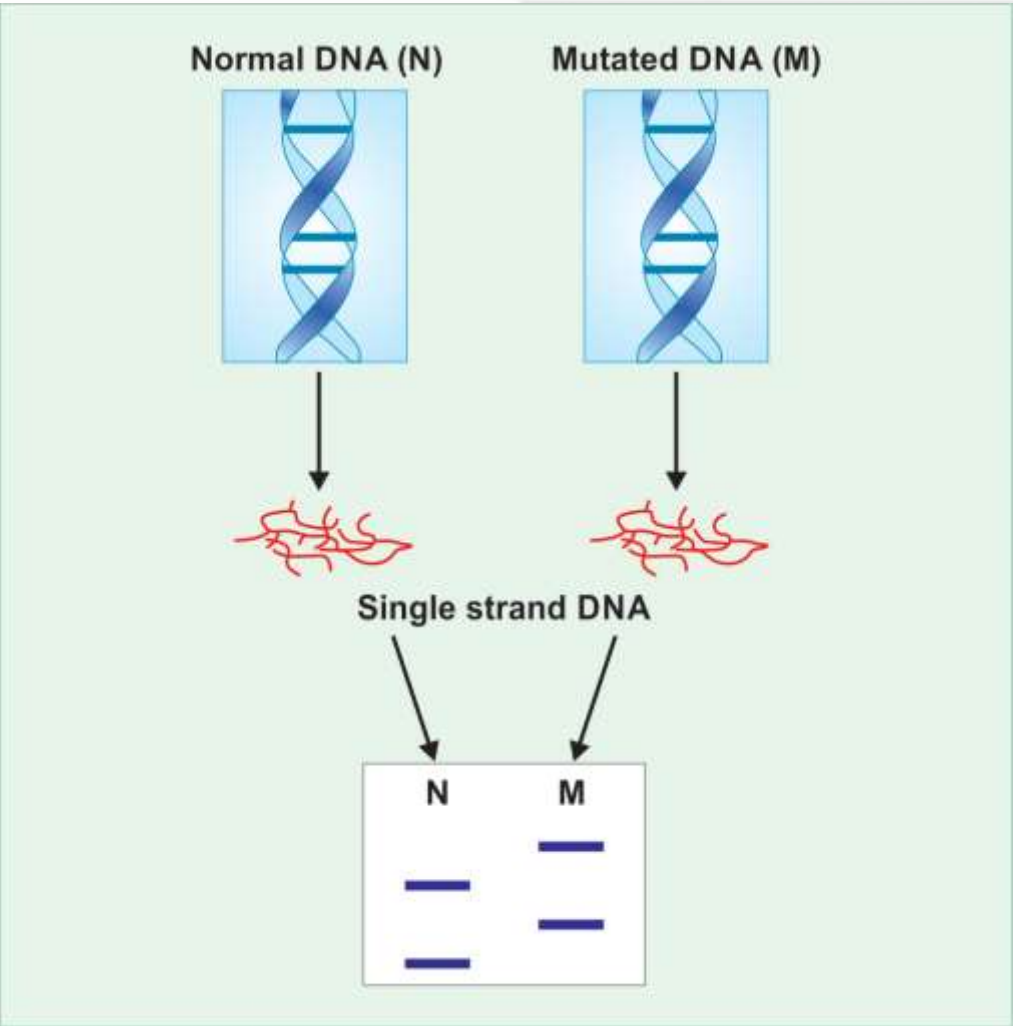
LAMP Test



Advantages of LAMP technology over standard PCR technique are

1. Lamp reactions are isothermal, and therefore there is no need for a thermal cycler.
2. PCR reaction generally requires a few hours, while LAMP needs only a few minutes.
3. LAMP yield is 100 to 1000 higher than PCR.
4. The initial laboratory set up cost for LAMP is only one-fourth of the cost of PCR set up.

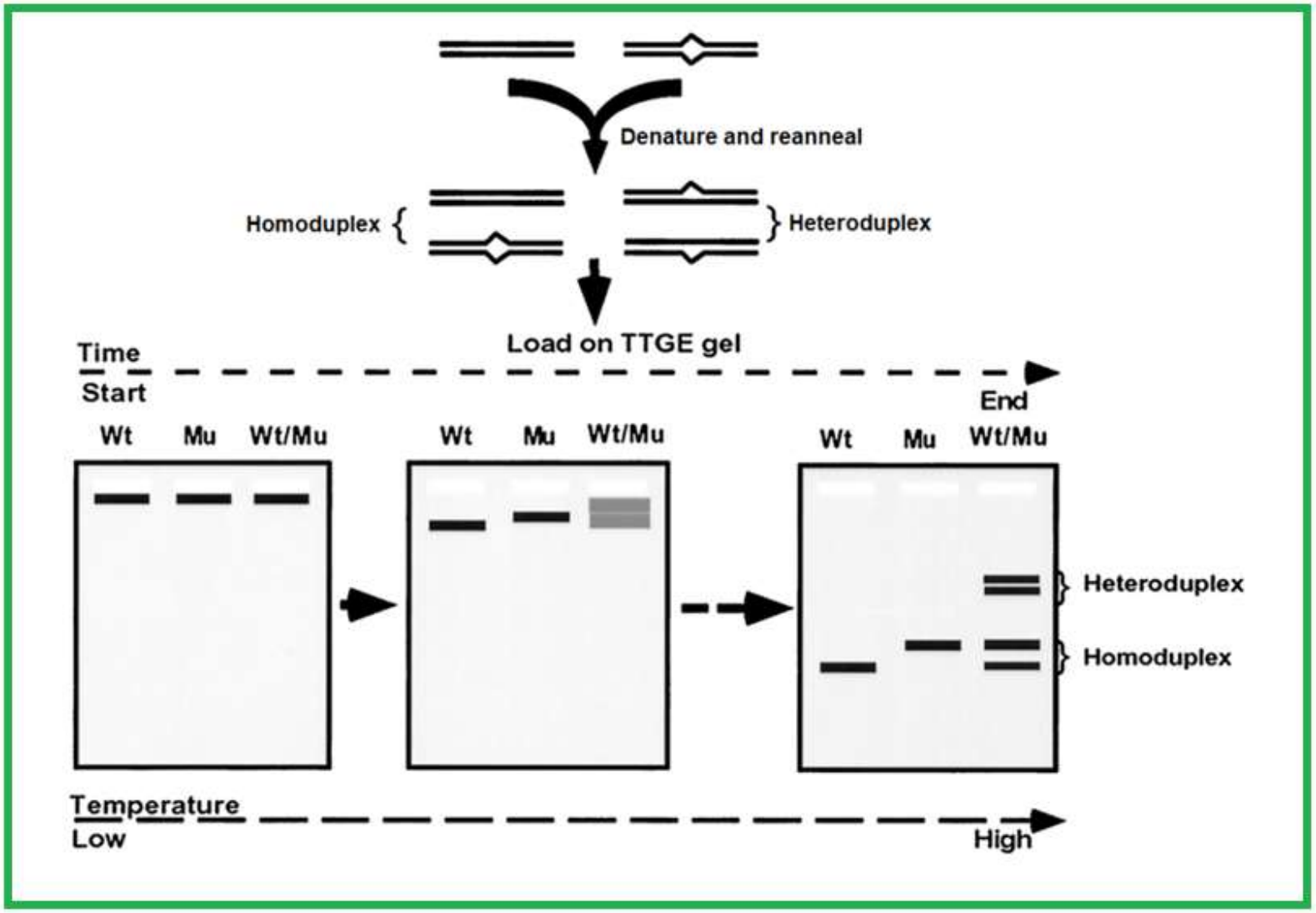
SSCP Analysis



Single–Strand conformation polymorphism (SSCP) analysis.

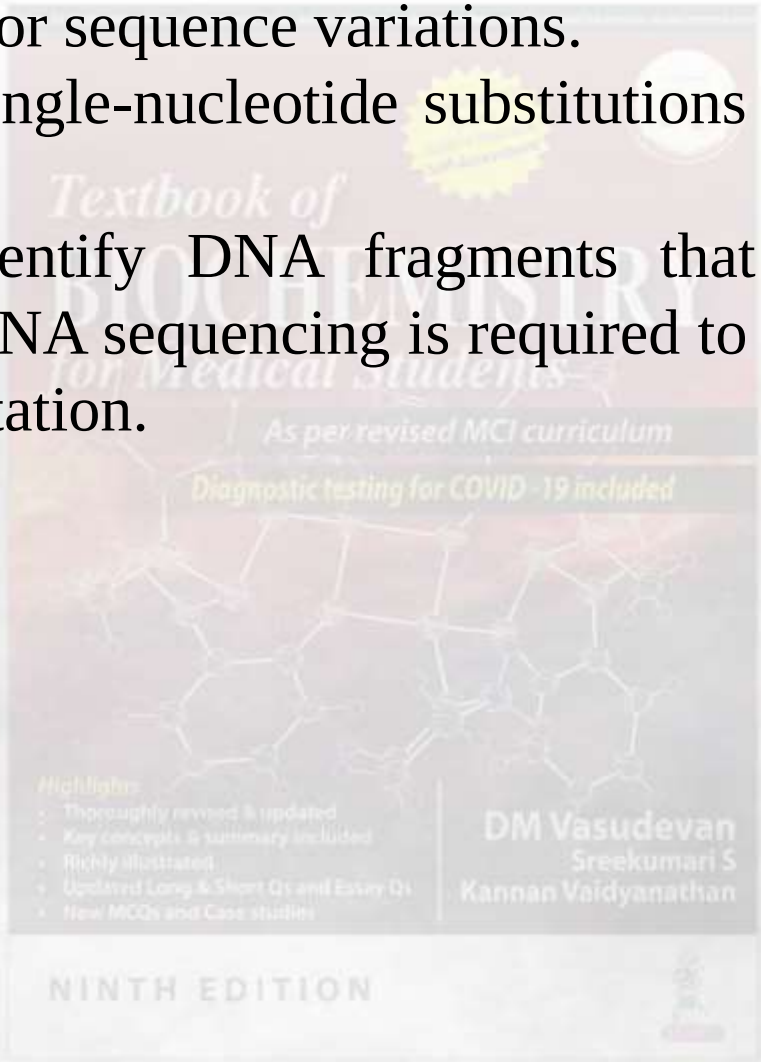
A single nucleotide polymorphism of either thymine (T) or cytosine (C) leads to different single-stranded confirmations of DNA, which results in different mobilities in electrophoresis.

Heteroduplex Analysis



Denaturing High Performance Liquid Chromatography (DHPLC)

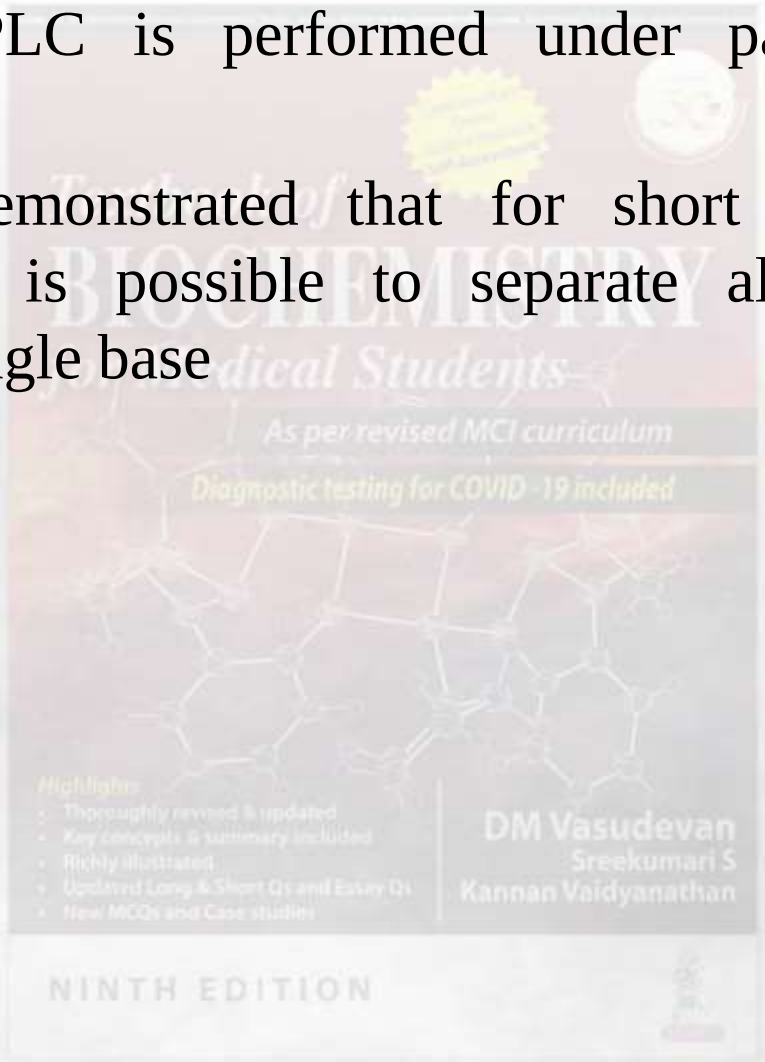
- to screen genes for sequence variations.
- used to detect single-nucleotide substitutions or small insertions or deletions.
- DHPLC can identify DNA fragments that contain sequence variations, but DNA sequencing is required to confirm the precise nature of the mutation.



Denaturing High Performance Liquid Chromatography (DHPLC)

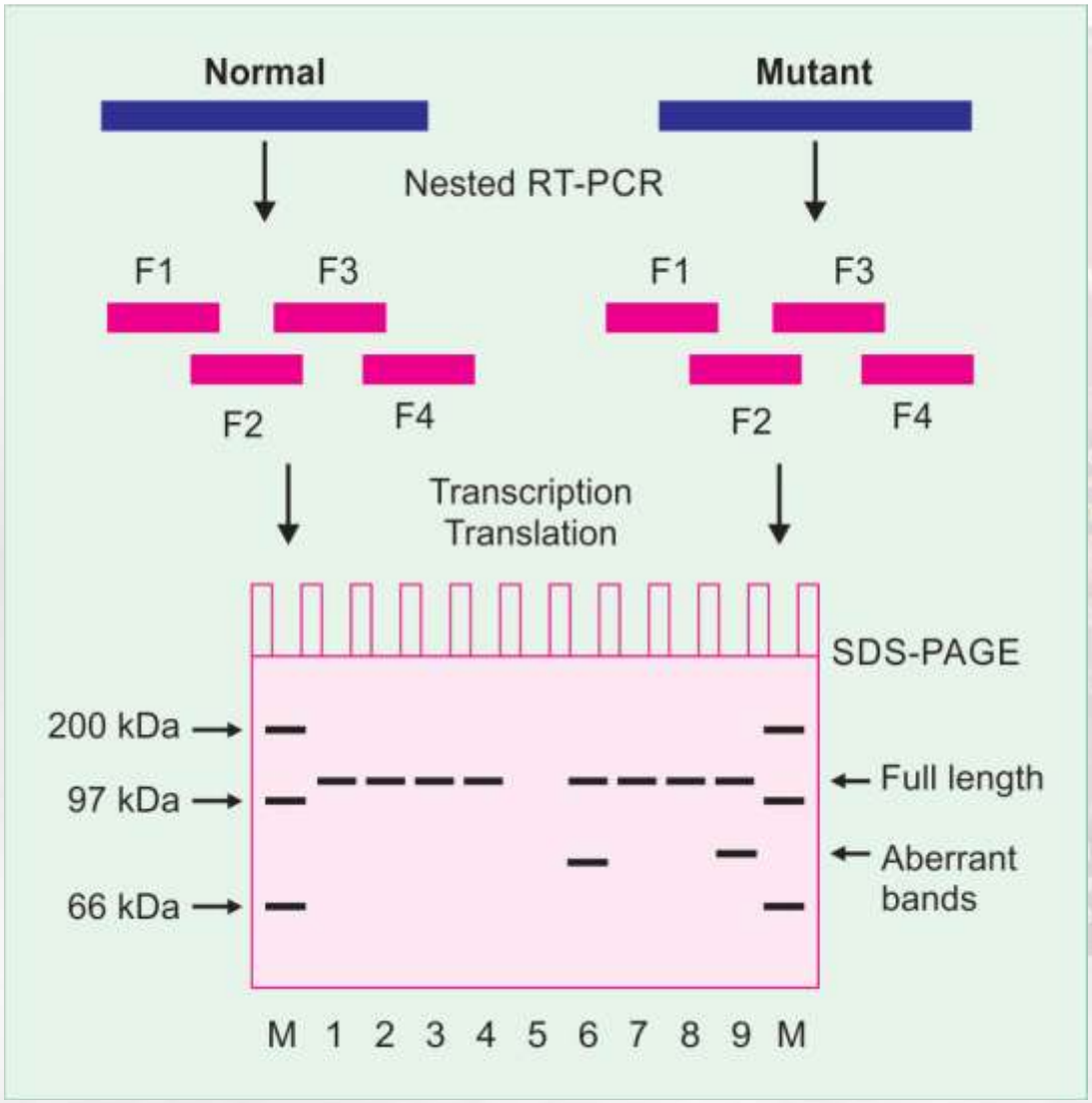
- Denaturing HPLC is a form of ion-pair reversed-phased chromato-graphy in which nucleic acids can be bound to a hydrophobic column [usually poly- styrene- di vinyl benzene particles]
- The affinity of this interaction is dependent on size, nucleotide composition, and column temperature.
- Under nondenaturing conditions (i.e., column temperatures at which double-stranded DNA remains fully paired), the interaction is almost completely dependent on fragment size.
- It is possible to accurately quantify the size of PCR fragments.
- At high column temperatures, double-stranded DNA is completely denatured into single strands and elution from the column is dependent on both size and sequence.

- Generally, DHPLC is performed under partially denaturing conditions.
- It has been demonstrated that for short fragments (< 30 nucleotides), it is possible to separate all oligonucleotides differing by a single base



Protein Truncation Test

Protein truncation test (PTT) is also referred to as the **in vitro synthesized protein assay (IVSPA)**. This is a method for screening the coding region of a gene for mutations that result in the premature termination of mRNA translation. The PCR is used to amplify a DNA template. Truncated proteins are identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The complete coding sequence of a large gene, can be amplified in several overlapping fragments by reverse transcription PCR (RT-PCR). The size of the truncated protein indicates the position of the premature stop codon, and DNA sequencing of genomic DNA is performed to confirm the presence of a mutation. Several kilobase segments of a gene can be rapidly screened in a single reaction.

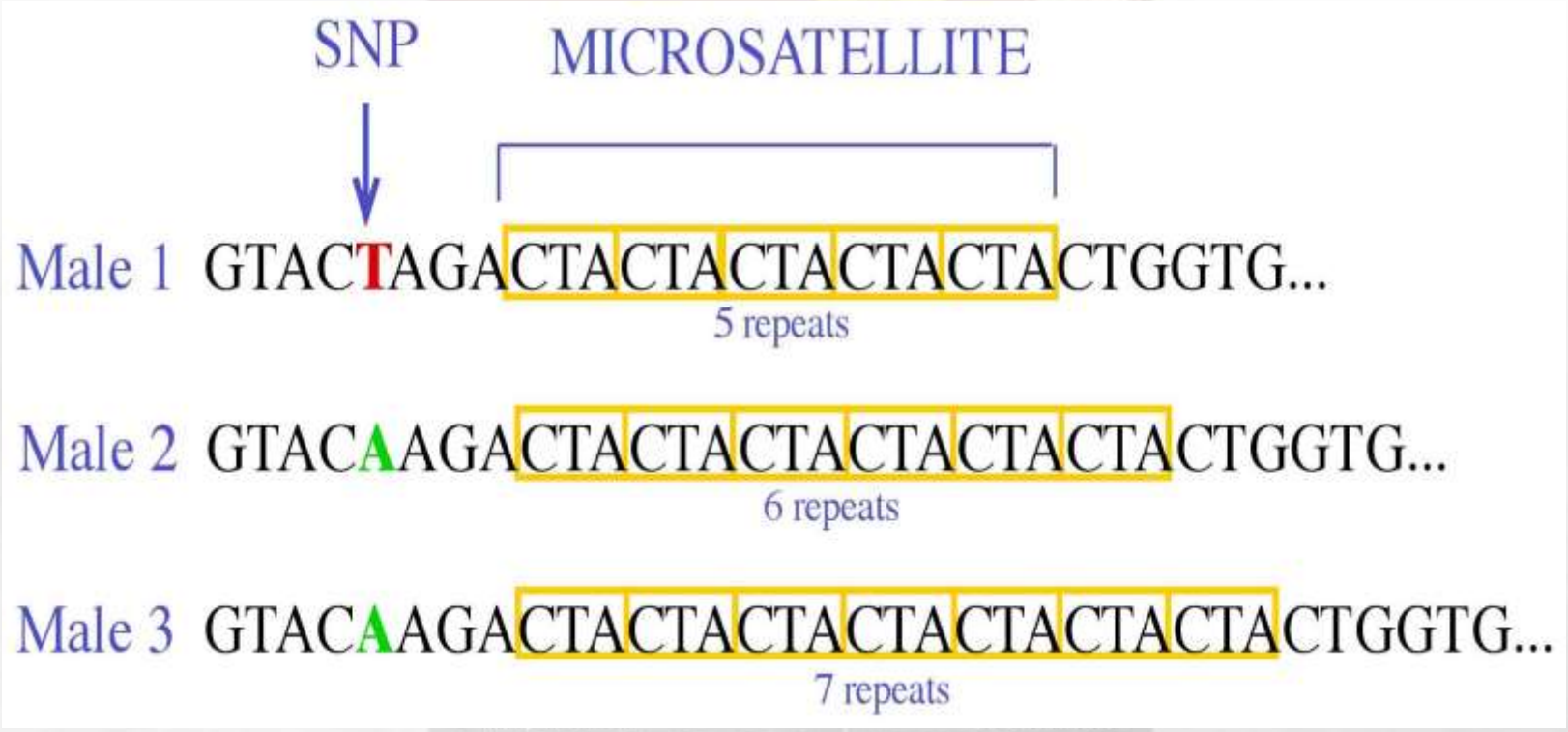


Protein truncation test (PTT)

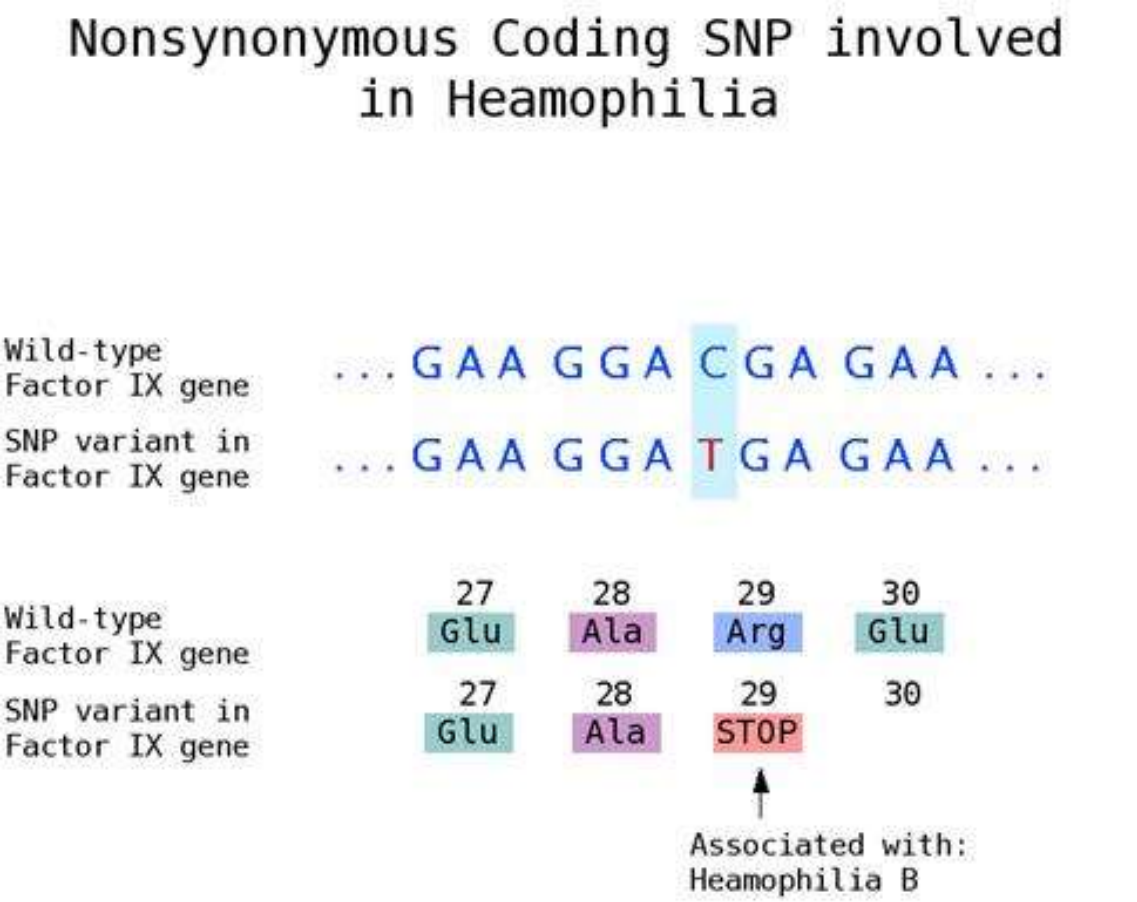
Single Nucleotide Polymorphism (SNP)

- Pronounced as "snip".
- SNPs are substitutions of one base at a precise location within the genome.
- Those that occur in coding regions are termed cSNPs.
- An SNP occurs once in every 300 bases; So approx 10 million in the human genome.
- These differences can account for the differences in disease susceptibility, drug metabolism, and response to environmental factors between individuals.

Single nucleotide polymorphism (SNP)

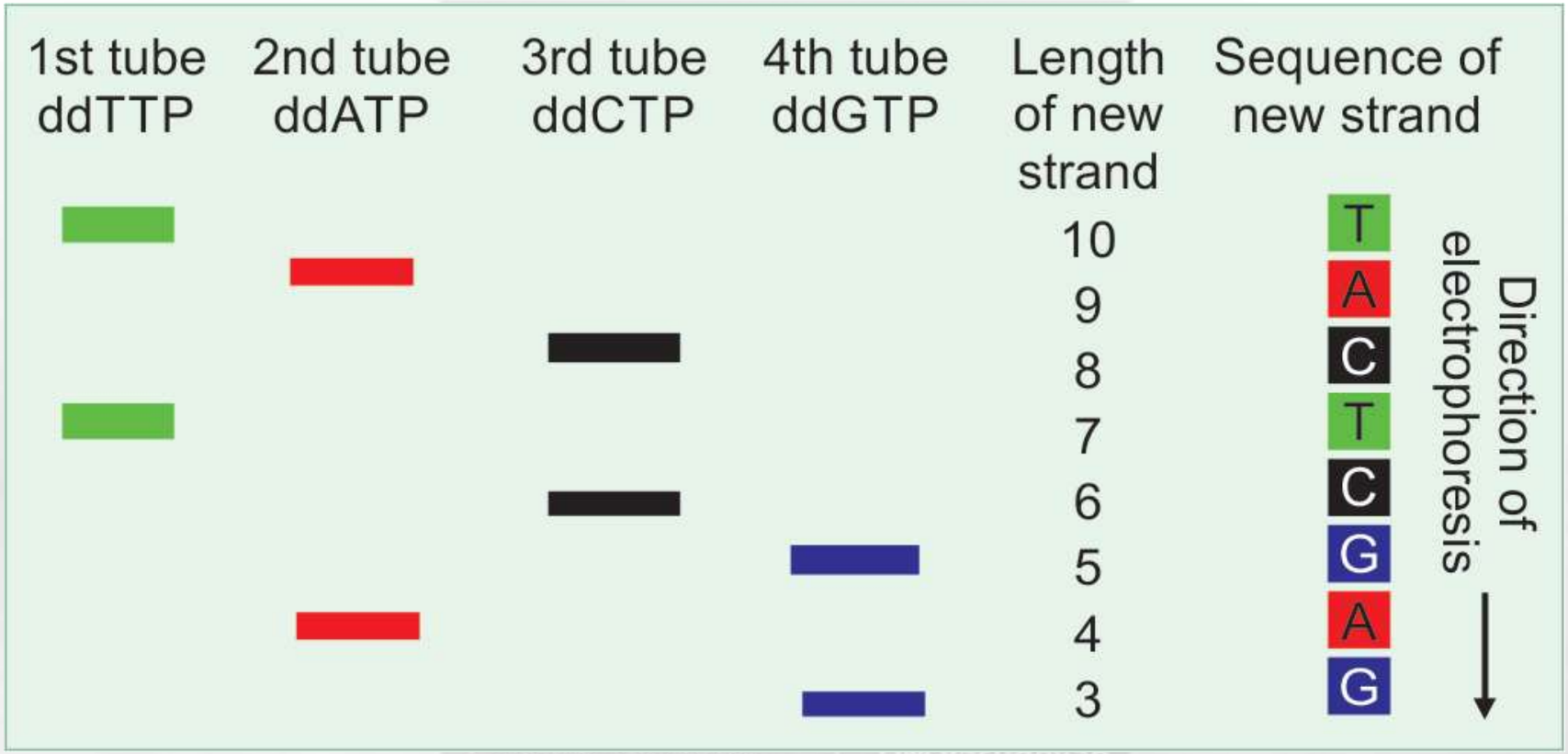


SNP may or may not be associated with disease condition. In this example it has led to hemophilia.

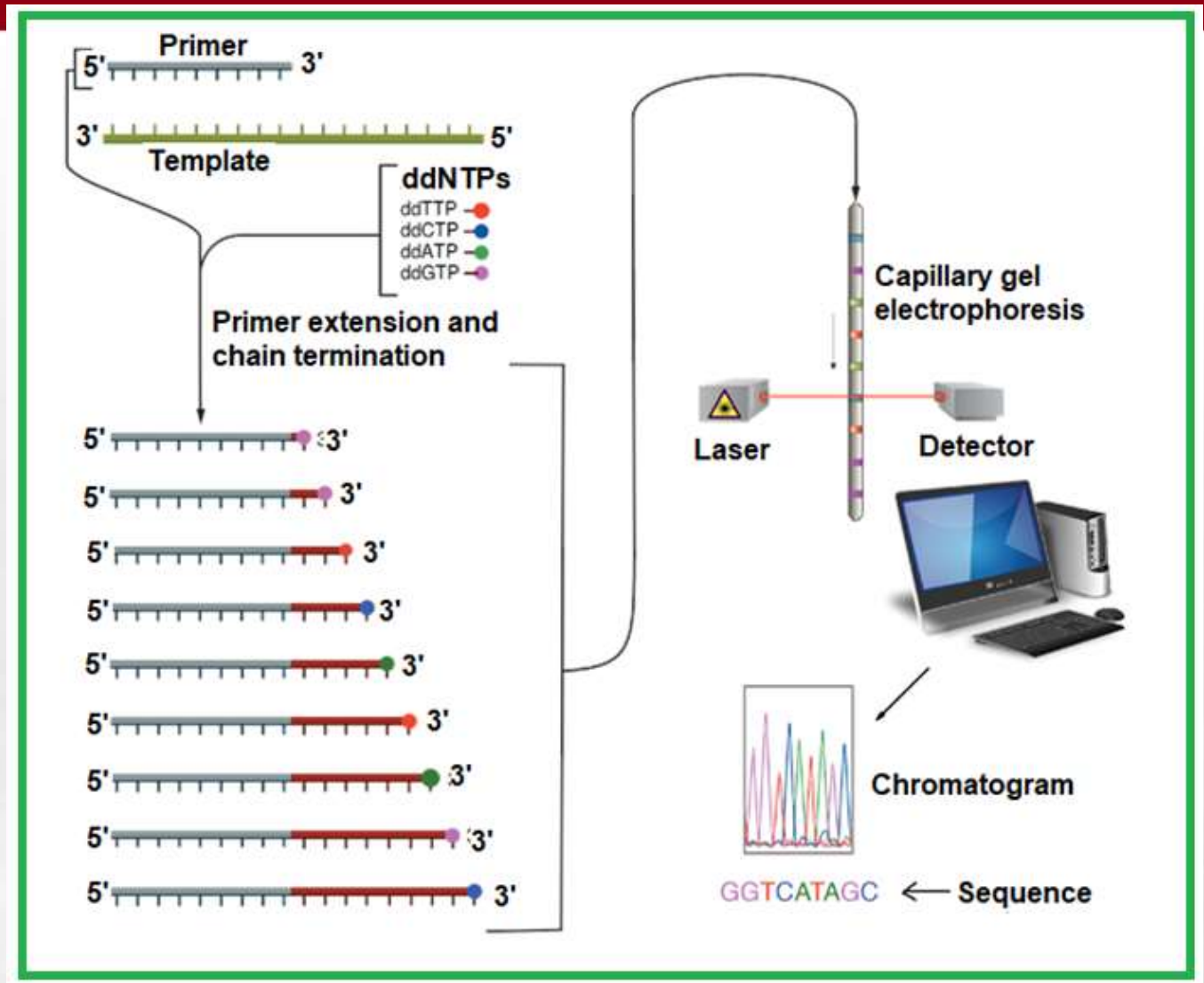


DNA Sequencing

Sanger's technique is called "controlled termination of synthesis"; as it uses chain terminating agents. DNA sample is taken in 4 different test tubes. In all tubes, the **Klenow enzyme** (DNA polymerase without exonuclease activity) and radiolabeled TT as the primer are added. In all tubes, radioactive dNTPs (all the 4 nucleotides which are with 32P) are added. Synthesis of a new strand of DNA is started. But in the 1st test tube, ddTTP (2',3'-di deoxy TTP) is also added. The ddTTP will add the T, but ddTTP will stop chains at T. Then the contents of each tube are simultaneously examined on polyacrylamide electrophoresis. The gel is then autoradiographed. Thus, the sequence of the newly synthesized strand is known. The complementary sequence will be present in the original unknown DNA. At present, the automated sequencers can quickly analyze fragments of DNA and provide the full sequence of the gene as a graphic representation.



DNA sequencing by Sanger's method.



Sanger's sequencing with fluorescent label.

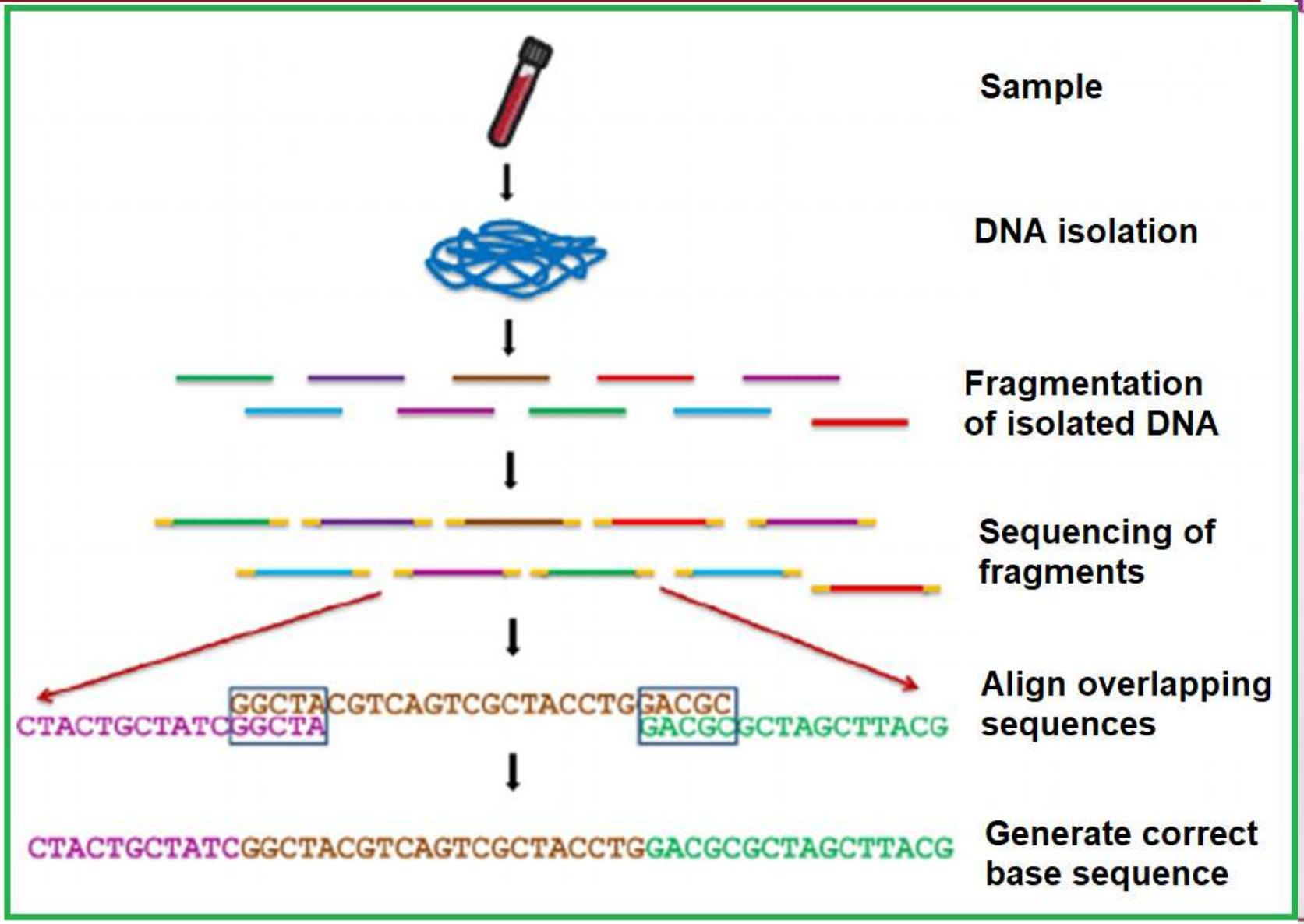
Next Generation Sequencing

Next generation sequencing (NGS) is a form of high throughput sequencing technology and has revolutionized the sequencing. Using the NGS, the whole human genome can be sequenced within a single day.

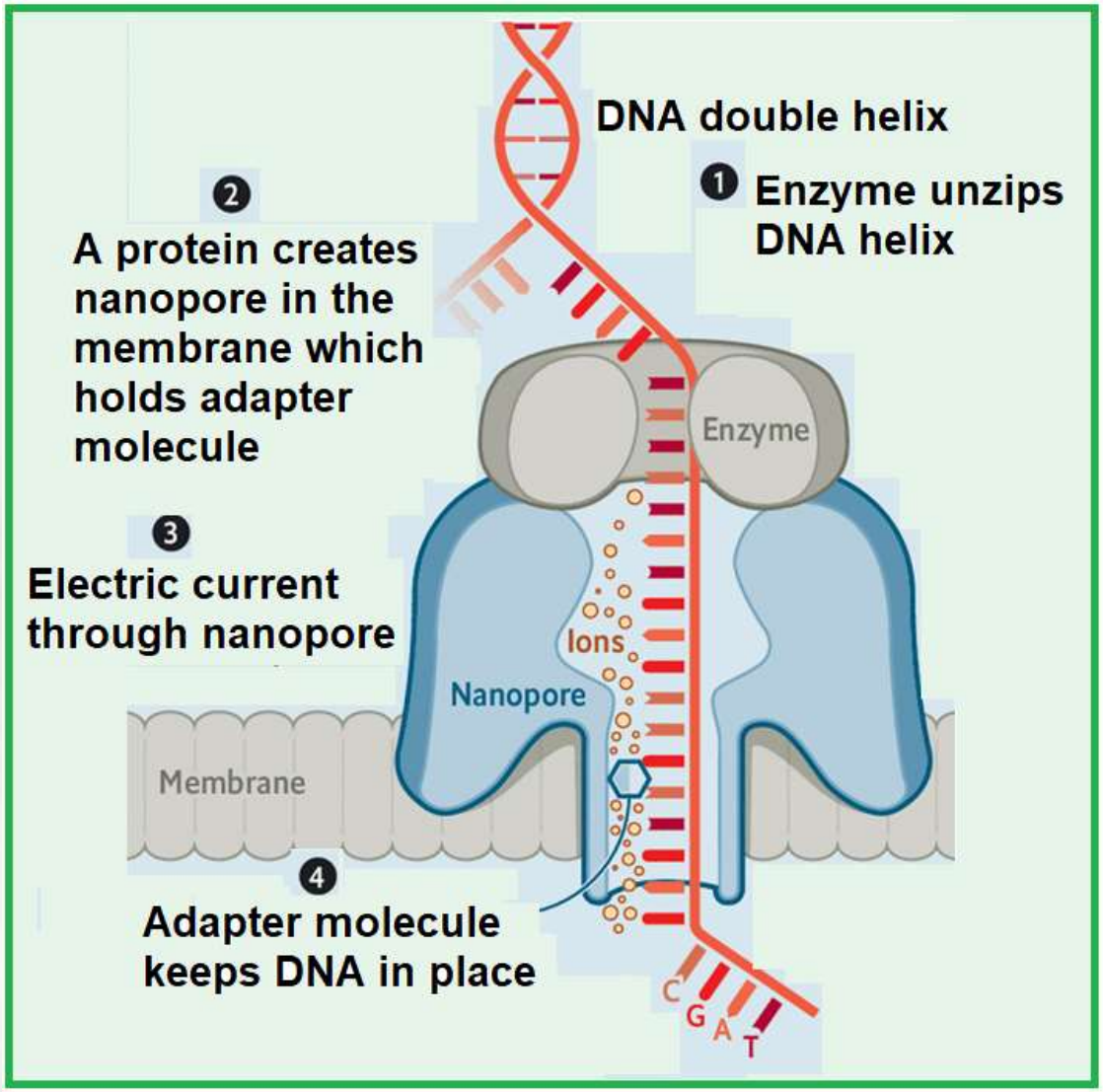
Millions of DNA nsegments are sequenced in parallel. The major limitation is the infrastructure requirement, including the interpretation of the huge volumes of data generated. With the advent of personalized medicine, it is now applied in actual clinical scenario to detect specific mutations in genes causing cancer and other non-communicable diseases.

In general, NGS has three steps: (1) library preparation, (2) amplification and (3) sequencing.

Next Generation Sequencing



Nanopore Sequencing



In Nanopore sequencing, the analysis of DNA strand is done directly as the molecule is drawn through a tiny pore suspended in a membrane. Changes in electrical current, or tunnelling currents, are measured to detect the individual bases.. The nanopore is a naturally occurring protein.