Bharathidasan University

Tiruchirappalli- 620024,
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Programme: M.Sc., Botany

Course Title: Plant Biotechnology
Course Code: 22PGBOTCC204

Unit Iv - MOLECULAR TECHNIQUES

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In the seminar will be described

1.What is the genetic testing?

2.Aims of molecular medical genetics laboratory?

3.Molecular techniques(DNA and RNA extraction, Different types of PCR,ARMS,RFLP,MLPA and hybridization technique.

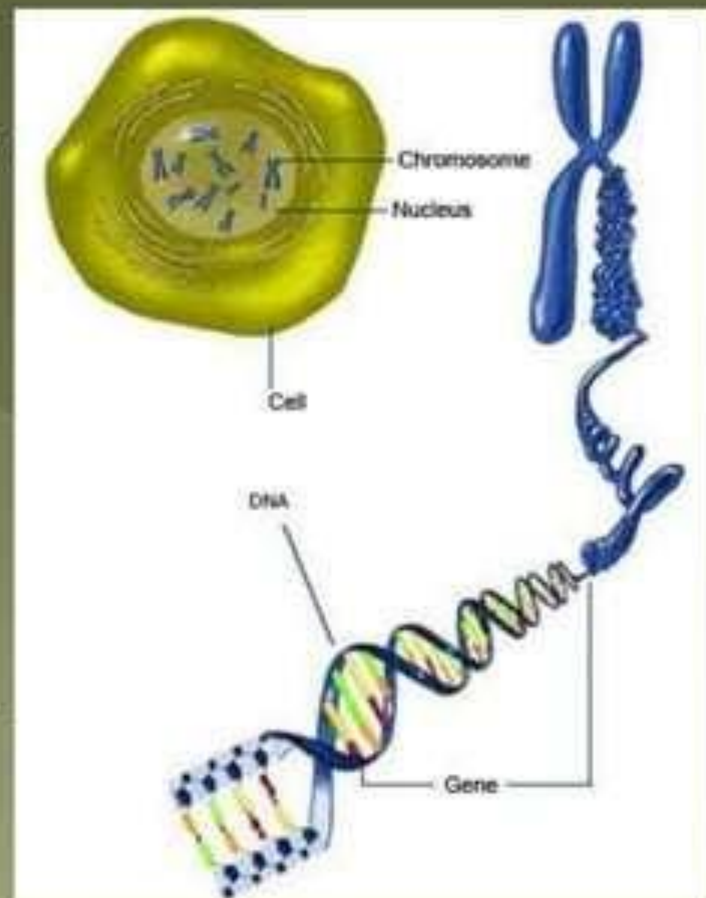
4.Applying molecular technique in forensic medicine (DNA fingerprinting)

5. Sex determination of fetus in 7th week of pregnancy by molecular techniques

6.Prenatal Genetic Diagnosis(PND)

What is Genetic testing?

- called DNA-based test
- type of medical test that identifies changes(mutation) in chromosomes, genes, or proteins
- newest and most sophisticated of techniques used
- these testes are done in molecular medical genetic laboratory



Aims in molecular genetics laboratory

determining mutations and detecting genotypes in molecular level which causes to

1. Single gene inherited disorders (thalasemia.FMF)
2. Multifacturial gene inherited disorders (cancer)
3. Mitochondrial inherited disorders (LHON)

4. Infertilitiy disabilities (Azo spermi and recurrent abortion)

5. Infection diseases (CMV.HIV)

6. Forensic medicine (DNA fingerprinting)

7. Sex determination of fetus in 7th week of pregnancy

8. PND

Molecular techniques

1.DNA and RNA extraction

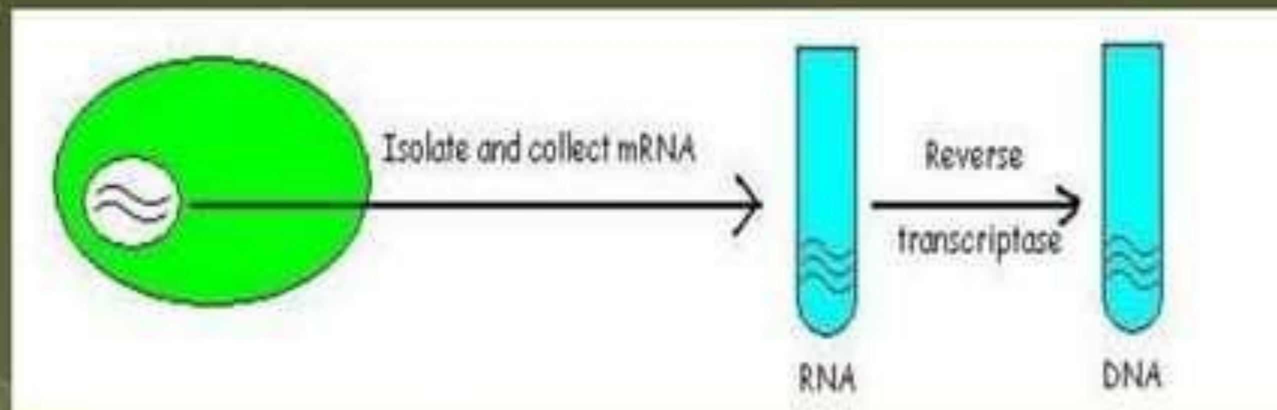
- In medical molecular genetics lab DNA and RNA are extracted from
- Blood
- Tissue
- DNA and RNA isolation is a routine procedure to collect DNA or RNA for subsequent molecular or forensic analysis
- There are lots of kits and methods for extraction DNA/RNA

Protocol

- The function of the lysis buffer is to aid in the breaking of the cell . The lysis buffer contains protease enzymes and essential salts to bring about this process.
- The purpose of TE buffer is to solubilize DNA or RNA, while protecting it from degradation concentrated on a filter
- The DNA/RNA on the filter is washed to remove inhibitors
- An elution buffer removes the DNA from channel walls, and the DNA is collected at the end of the channel

- **RNA extraction** is the purification of RNA from biological samples.
- Protocols of RNA extraction and DNA extraction are same but lysis buffer is different.it contains deoxyribonuclease enzyme for degradation of DNA
- This procedure is complicated by the ubiquitous presence of [illegible] enzymes in cells and tissues, which can rapidly degrade RNA

So :



complementary DNA (**cDNA**) is DNA synthesized from a messenger RNA (mRNA) template in a reaction catalyzed by the enzyme reverse transcriptase and the enzyme DNA polymerase

2.PCR (Polymerase Chain Reaction)

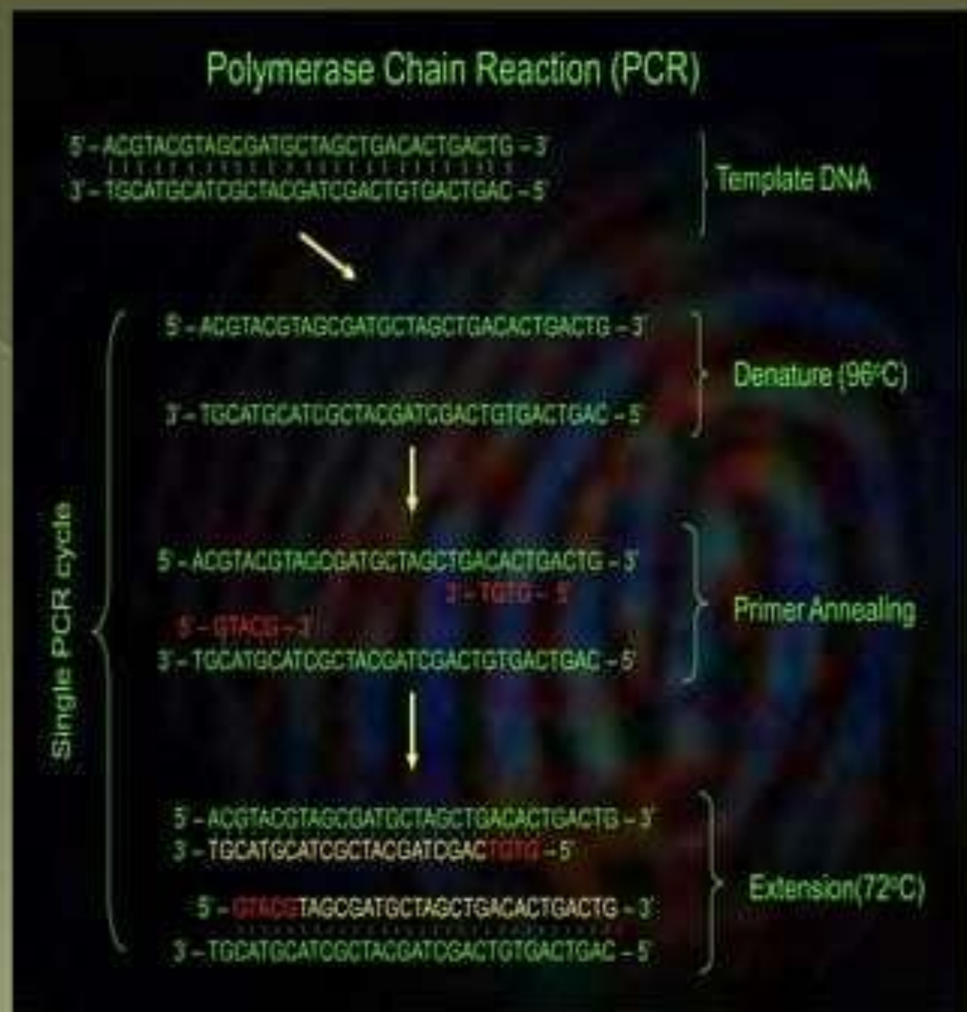
- Main method in molecular laboratory
- The purpose of a PCR (Polymerase Chain Reaction) is to make a huge number of copies of a gene.
- PCR allows isolation of DNA fragments from genomic DNA by selective amplification of a specific region of DNA

PCR Reaction Mix

- **Template DNA**
- **PCR Buffer**
- **dNTP (A,T,C,G)**
- **Forward Primer**
- **Reverse Primer**
- **Polymerase Enzyme (Taq)**

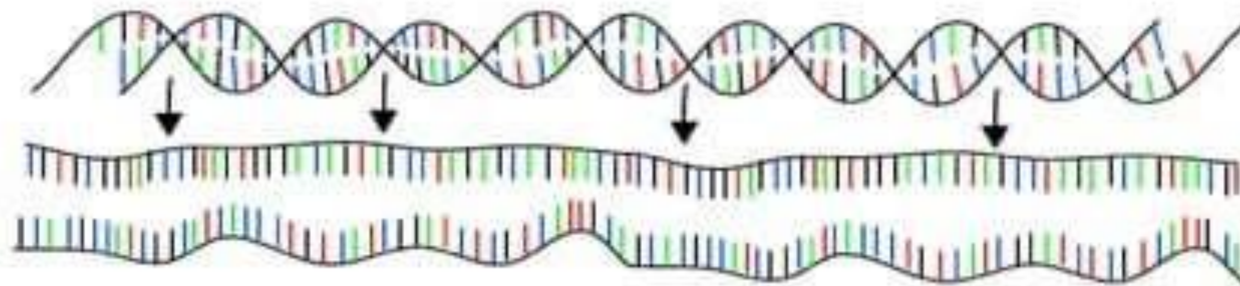
The cycling reactions :

- There are three major steps in a PCR, which are repeated for 30 or 40 cycles. This is done on an automated cycler, which can heat and cool the tubes with the reaction mixture in a very short time



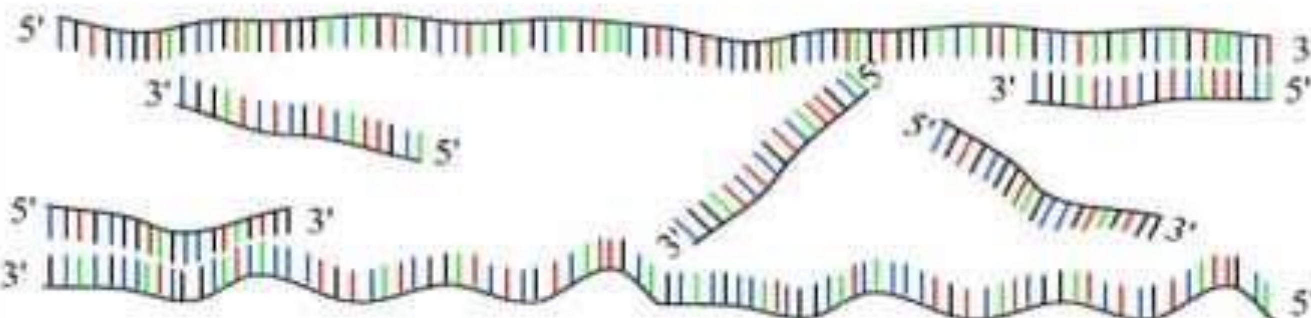
PCR : Polymerase Chain Reaction

30 - 40 cycles of 3 steps :



Step 1 : denaturation

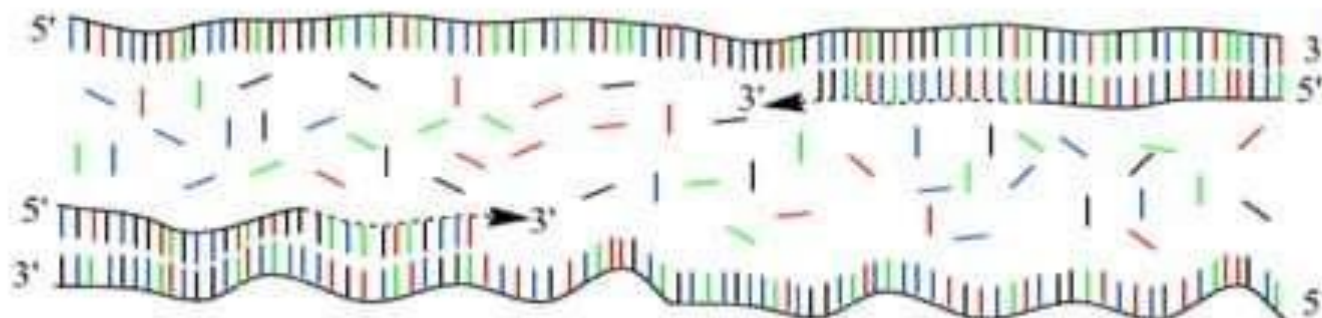
1 minut 94 °C



Step 2 : annealing

45 seconds 54 °C

forward and reverse primers !!!

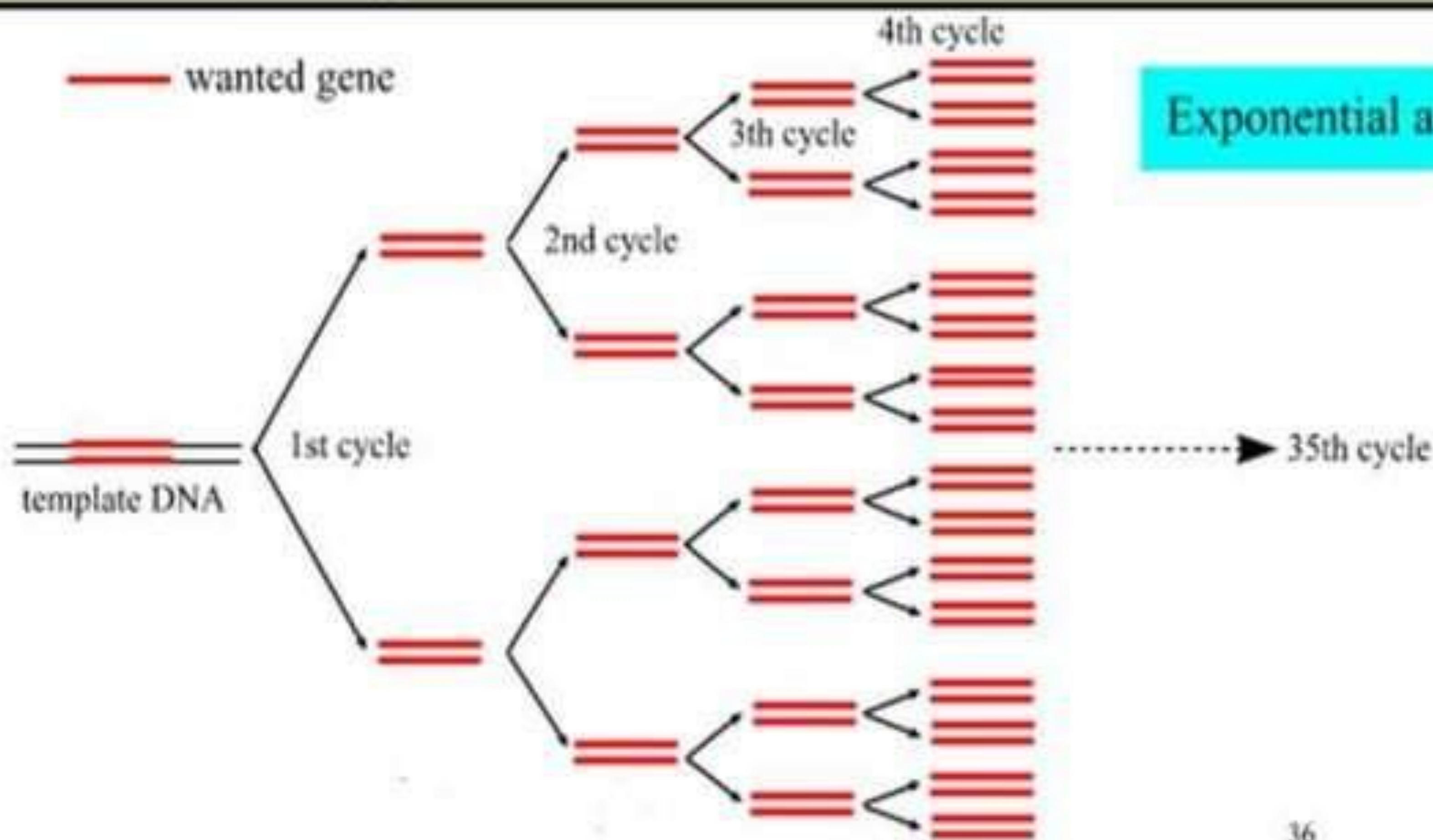


Step 3 : extension

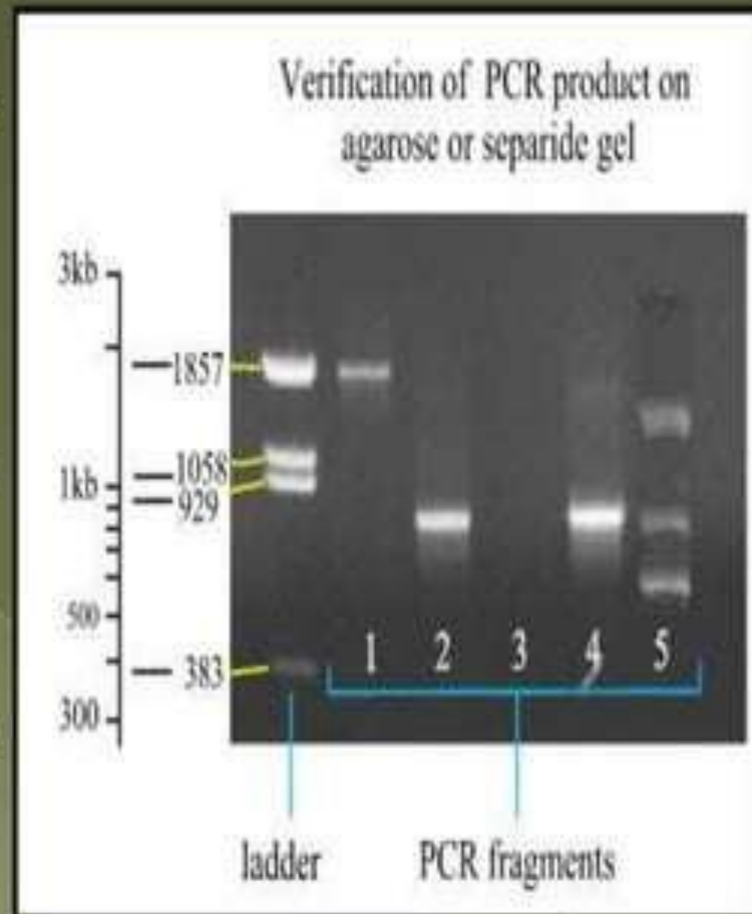
2 minutes 72 °C
only dNTP's

(Andy Vierstraete 1999)

there is an exponential increase of the number of copies of the gene



- Verification of the PCR product on gel.
- The ladder is a mixture of fragments with known size to compare with the PCR fragments. Notice that the distance between the different fragments of the ladder is logarithmic. Lane 1 : PCR fragment is approximately 1850 bases long. Lane 2 and 4 : the fragments are approximately 800 bases long. Lane 3 : no product is formed, so the PCR failed. Lane 5 : multiple bands are formed because one of the primers fits on different places.



3.Reverse Transcription Polymerase Chain Reaction (RT-PCR)

- is a variant of polymerase chain reaction (PCR). RT PCR contains 3steps a)RNA extraction b)cynthesize of CDNA c)the resulting cDNA is amplified using PCR
- highly sensitive technique in which a very low copy number of RNA molecules can be detected
- RT PCR is commonly used in studying the genomes of viruses whose genomes are composed of RNA, such as [illegible] and [illegible] like [illegible]

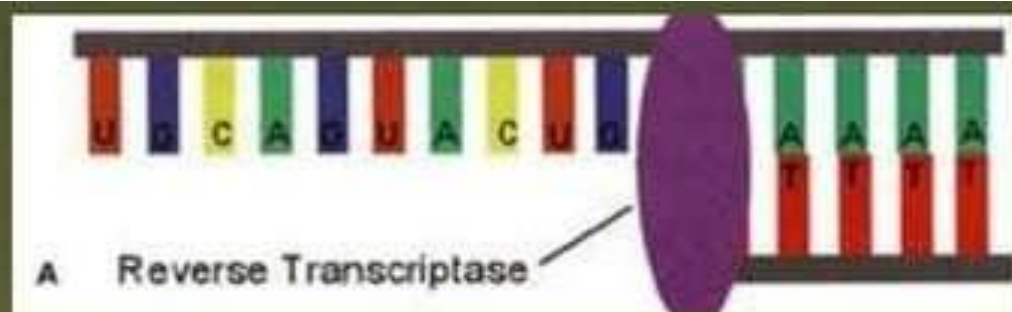
Protocol

- 1.RNA is extracted

2.Cynthsis of CDNA

A.poly-T oligonucleotide primer is hybridized onto the poly-A tail of the mature mRNA template, (Reverse transcriptase requires this double-stranded segment as a primer to start its operation)

- **B.** Reverse transcriptase is added, along with deoxynucleotide triphosphates (A, T, G, C)

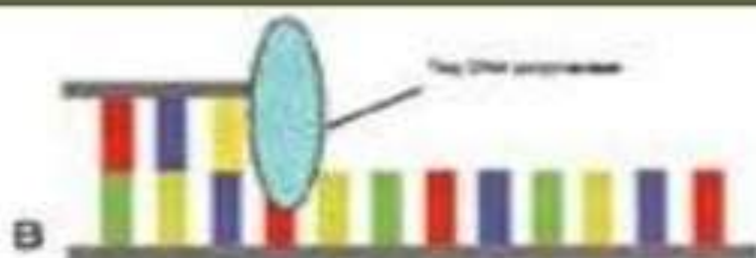


This synthesizes one complementary strand of DNA hybridized to the original mRNA strand

C. To synthesize an additional DNA strand, you need to digest the RNA of the hybrid strand, using an enzyme like RNase H

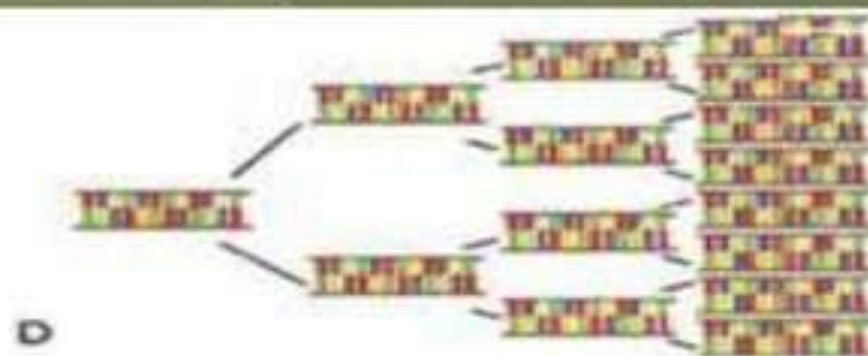
- D.The oligonucleotide primer is allowed to anneal CDNA template

E.Taq polymerase adds complimentary nucleutides beginning at the primer anealing site



- F.The resultant product is double stranded CDNA

- 8.Three steps of PCR (denaturation,anealing and extention)are repeated

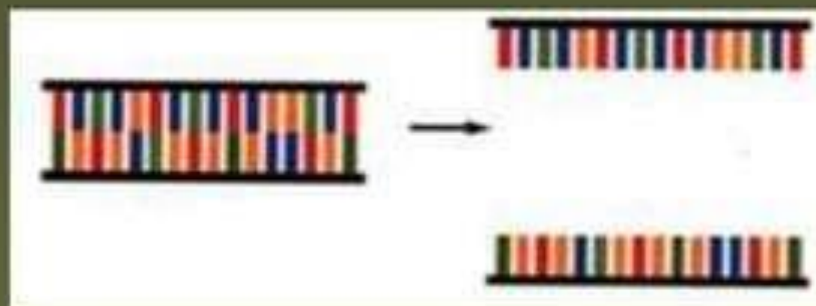


4. Real Time PCR

- is a laboratory technique based on the PCR
- enables both detection and quantification
- DNA,CDNA /RNA can be detected
- Real-Time chemistry provides fast, precise and accurate results
- Real-Time PCR is designed to collect data as the reaction is proceeding, which is more accurate for DNA and RNA quantitation and does not require laborious post PCR methods
- its key feature is that the amplified DNA is detected as the reaction progresses in real time

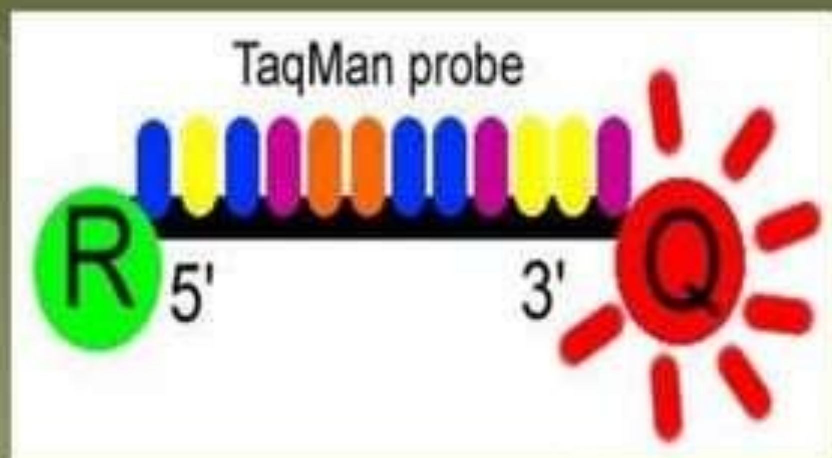
Protocol

- 1.DNA is extracted .The template DNA is denatured

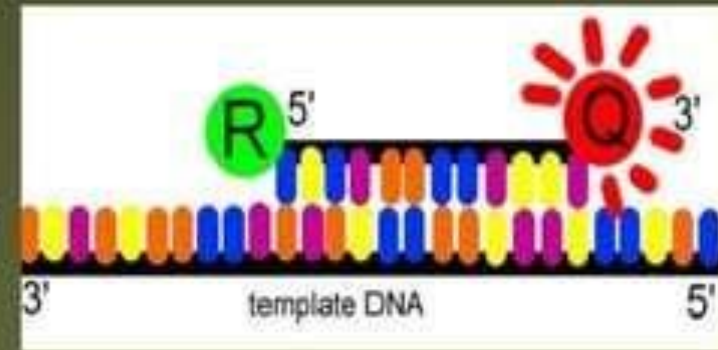


- 2.Hybridization probe and annealing

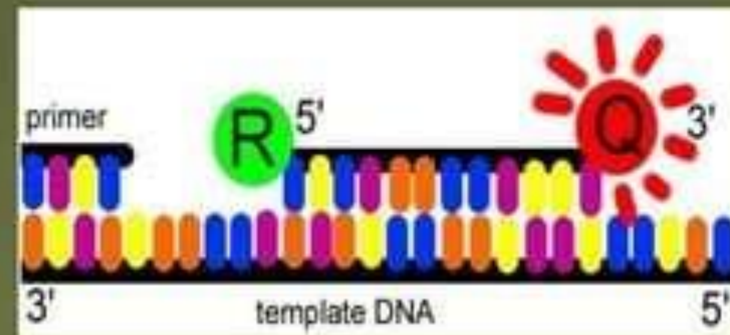
A non extendable hybridization probe is designed to bind the single stranded DNA internal to the PCR product.the probe contains a reporter fluorescent dye on the 5' end R and quencher dye on 3' end Q Thereby preventing detection of fluorescent probe



- During annealing phase of PCR the hybridization probe bind to template DNA. the annealing temperature for annealing the probe is generally 5 to 10 C greater than primer annealing temperature

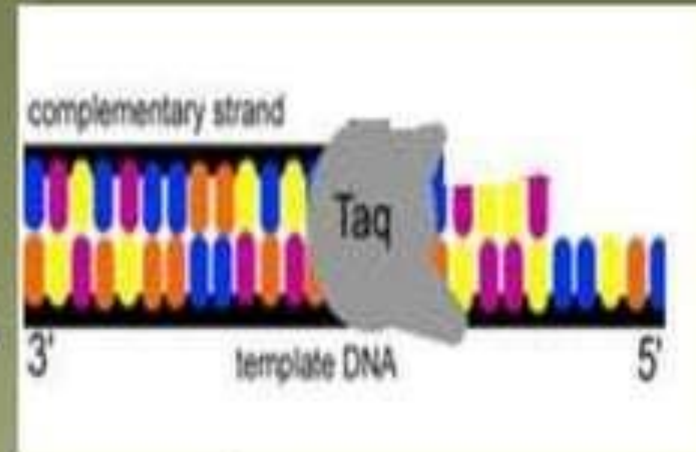


- 3.anealing of oligonucletide primer

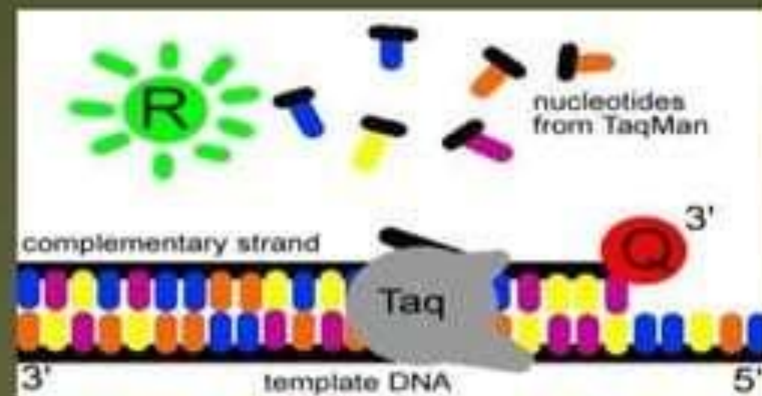


- 4.Nuclease activity and synthesis of new DNA

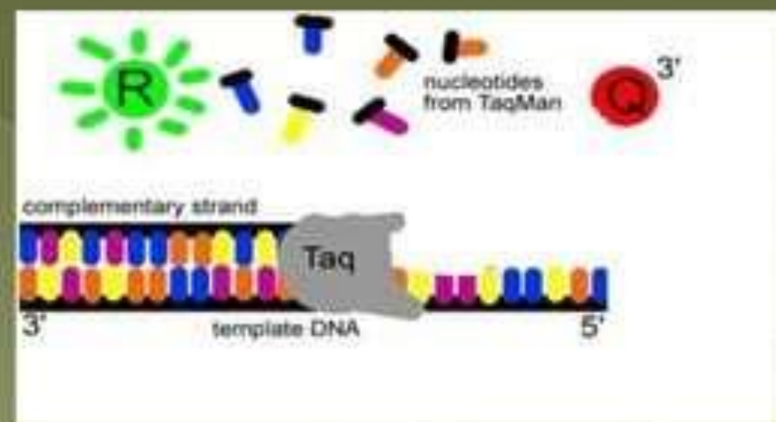
A)Taq polymerase synthesis new DNA



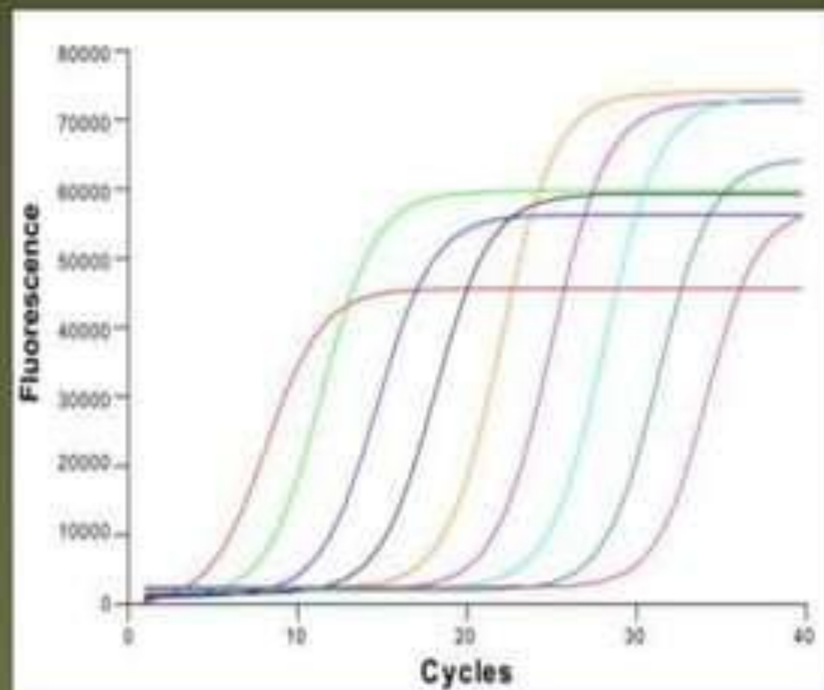
B)Taq plolymerase has 5' to 3' exonuclease activity that allows it cleave terminal nucleotides. this activity removes these nucleotides from template DNA



C) as the hybridization probe is degraded Q is separated from R and allowing detection fluorescent dye



- 5.Product analysis



the best technique for detection of infections like HIV ,leishmania, helicobacter pylori,mycobacterium.

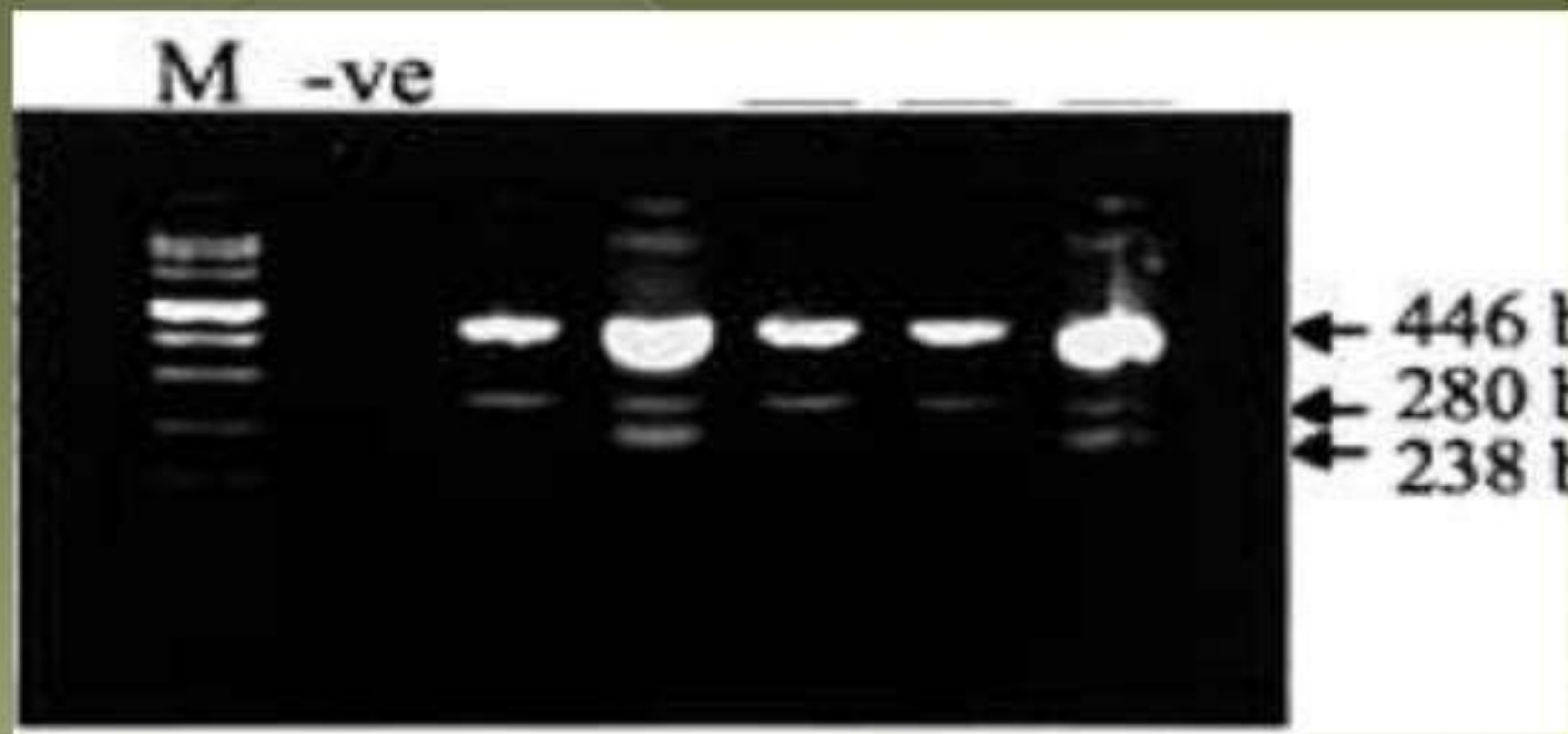
5.ARMS (Amplification Refractory Mutation System)

- detecting known point mutations were first described
- it has been developed for the diagnosis of all the common b-thalassaemia mutations found in all the main ethnic groups
- 3 primers are utilized (One primer is constant and complementary to the template in both reactions. the other primers are specific for Wild and mutant type of interested gene

- PROTOCOL
- 1.DNA is extracted
- 2.Two tubes are used.at first tube Common primer+wild type primer is added .at the next one Common primer +mutant type primer is added
- 3.dntps.taq polymerase and pcr buffer added and PCR is done
- 4.Product s of two tubes are loaded to agars gel
- 5.analysis of productions

- if the sample is homozygous mutant or homozygous wild type amplification will only occur in only one of the tubes, if the sample is heterozygous amplification will be seen in both tubes

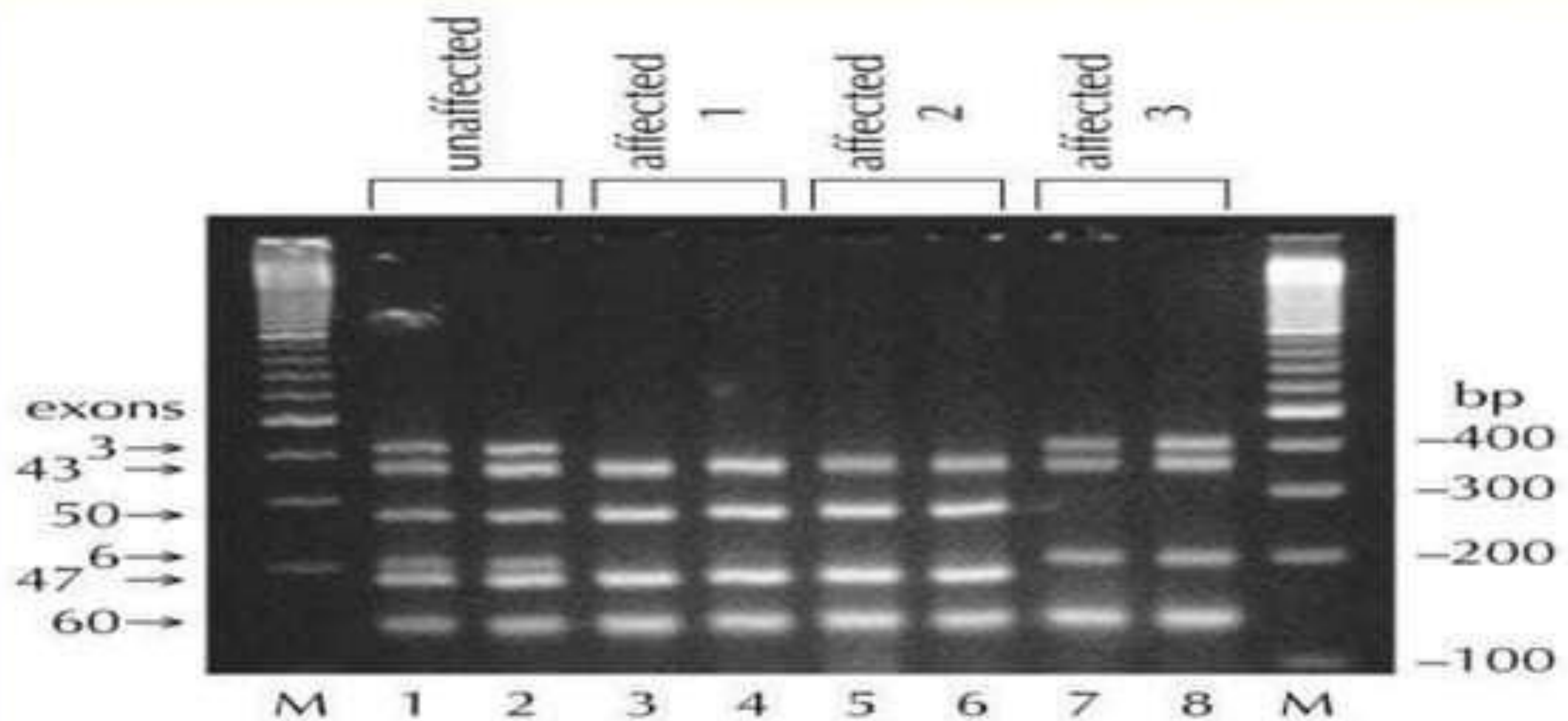
- ARMS-PCR shows a 446 bpcontrol band, the 280 bp band indicates the presence of the wild type allele and the 238 bp band indicates the presence of the E237G variant.



6.Multiplex PCR

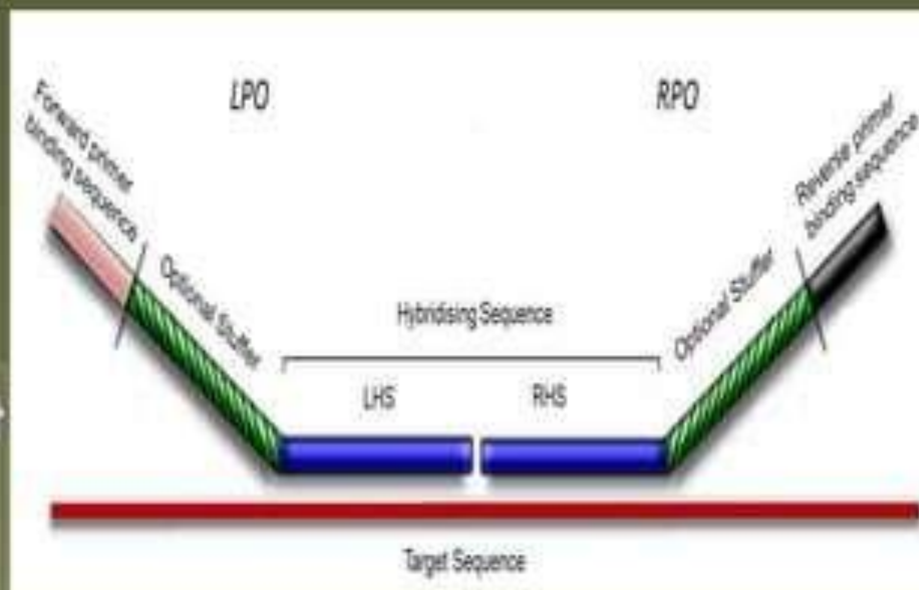
- is a modification of polymerase chain reaction in order to rapidly detect deletions or duplications in a large gene
- amplifies genomic DNA samples using multiple primers and a temperature-mediated DNA polymerase in a thermal cycler
- consists of multiple primer sets within a single PCR mixture to produce [illegible] of varying sizes that are specific to different DNA sequences

- Annealing temperatures for each of the primer sets must be optimized to work correctly within a single reaction, and amplicon sizes
- their base pair length, should be different enough to form distinct bands when visualized by gel electrophoresis
- different infection causes can be detected
- its useful for detection of genetic diseases which have large gene like Duchenne Muscular Dystrophy has 79 exons

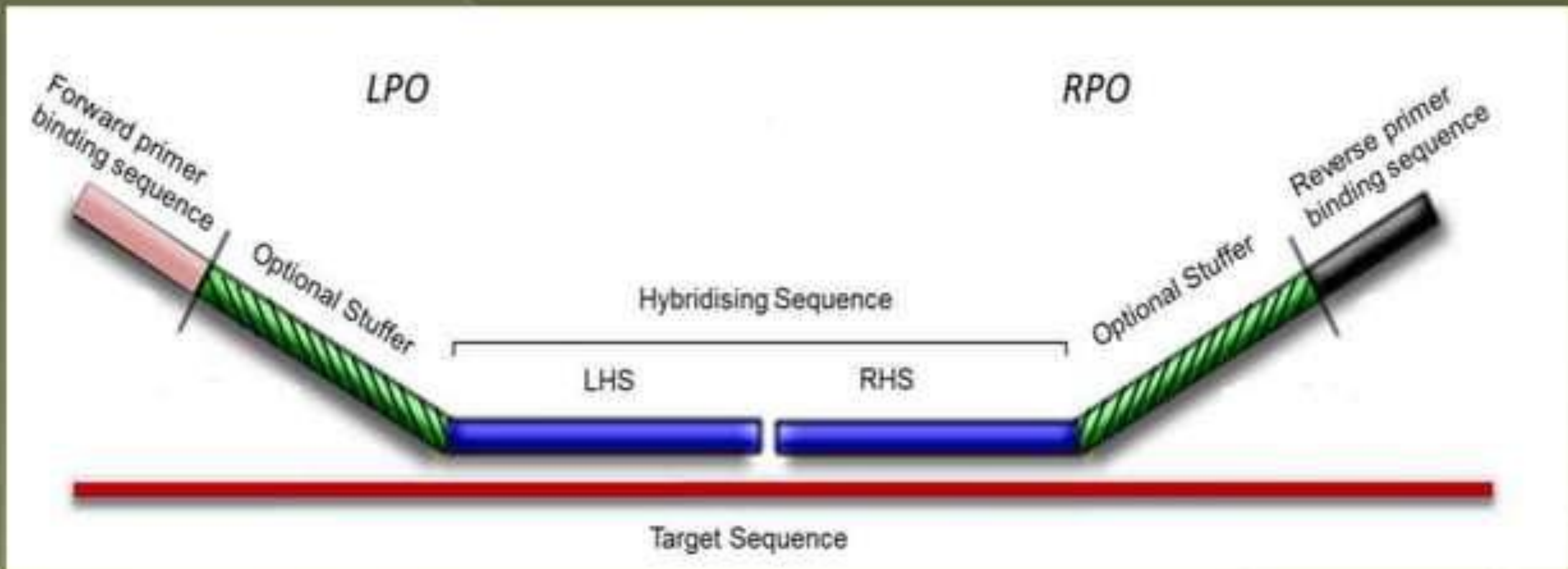


7.Multiplex ligation-dependent probe amplification (MLPA)

- is a variation of the multiplex polymerase chain reaction that permits multiple targets to be amplified with only a single primer pair
- Each probe consists of a two oligonucleotides which recognise adjacent target sites on the DNA
- One probe oligonucleotide contains the sequence recognised by the forward primer, the other the sequence recognised by the reverse primer

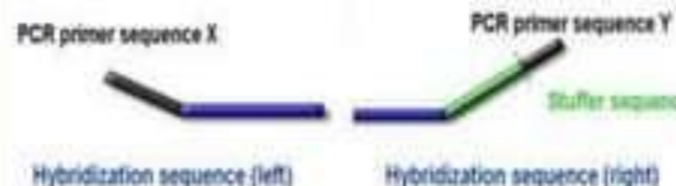


- LPO : Left Probe Oligonucleotide,LHS left hybridization sequence
- RPO : Right Probe Oligonucleoitde,RHS right hybridization sequence



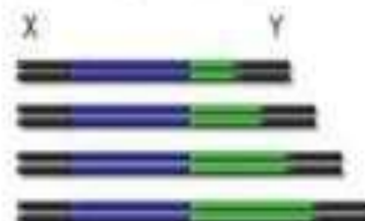
- Consist of 3 steps:
- 1.denaturation and hybridization
- 2.Ligation:Only when both probe oligonucleotides are hybridised to their respective targets, they can be ligated into a complete probe
- 3.Amplification:Each complete probe has a unique length, so that its resulting amplicons can be separated and identified by (capillary) electrophoresis
- the forward primer used for probe amplification is fluorescently labeled, each amplicon generates a fluorescent peak which can be detected by a capillary sequencer

1. Denaturation and Hybridization



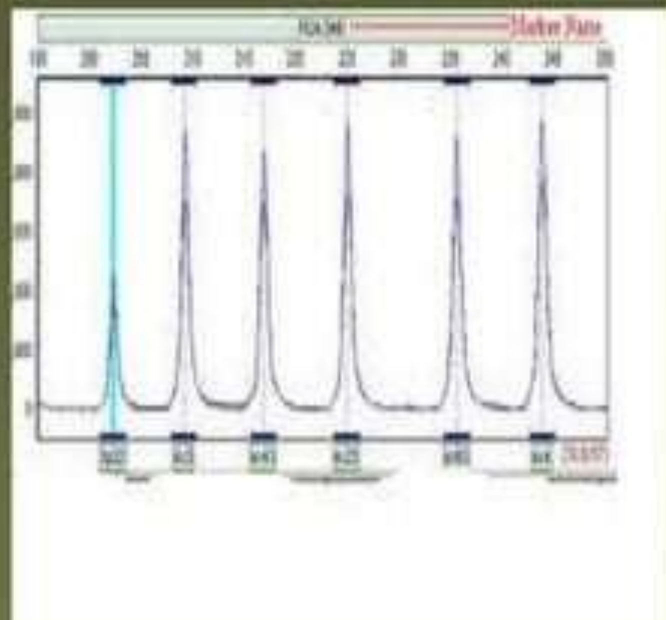
2. Ligation

3. PCR with universal primers X and Y
exponential amplification of ligated probes only



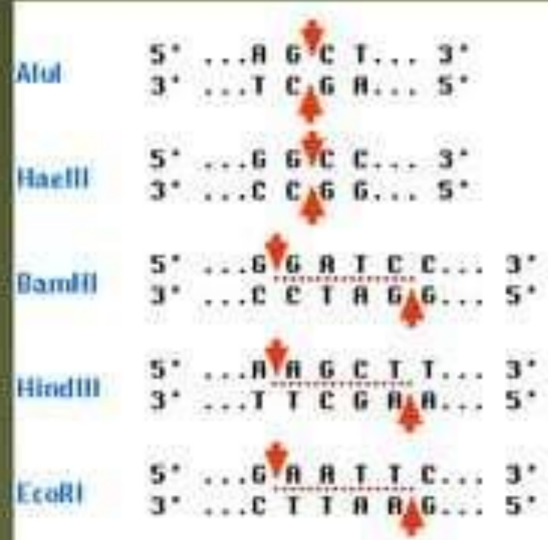
4. Fragment analysis

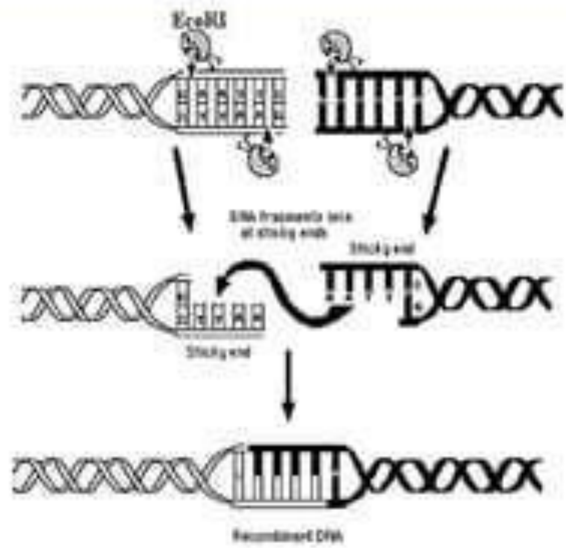
- its used for detection of an abnormal number of chromosomes, gene deletions, gene duplications, and gene expansions
- limitation of this technique : MLPA requires the creation of labor-intensive probes for each new gene or chromosome



what are restriction enzymes?

- found in bacteria and archaea, are thought to have evolved to provide a defence mechanism against invading viruses. Inside a bacterial host, the restriction enzymes selectively cut up *foreign* DNA in a process called *restriction*; host DNA is methylated by a modification enzyme (a methylase) to protect it from the restriction enzyme's activity
- DNA-cutting enzymes
- cut DNA at specific recognition nucleotide sequences thus there are many restriction enzymes





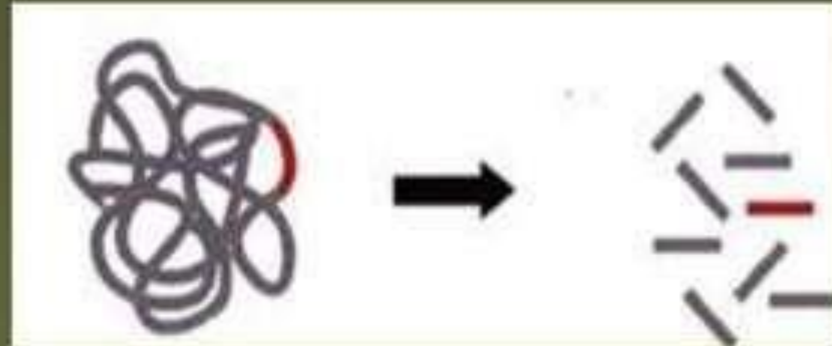
Restriction Enzyme Action of EcoRI

8.Southern hybridization

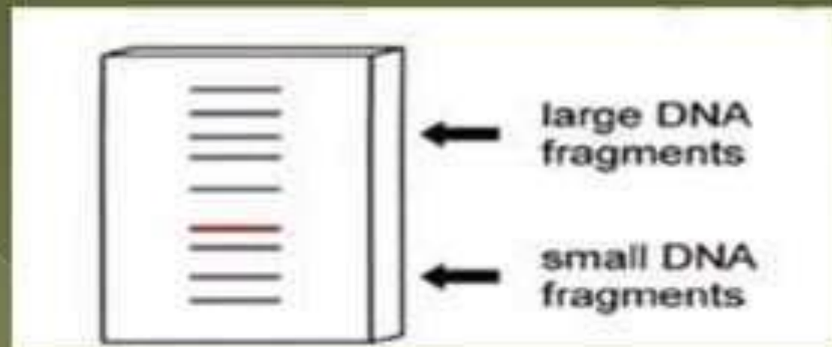
- Restriction enzymes are used
- there are 3types of hybridization (southern for DNA Western for Protein and Northern for RNA detection)
- Southern blotting combines transfer of electrophoresis-separated DNA fragments to a filter membrane and subsequent fragment detection by probe hybridization
- its used for diagnosis some disorders like Fragile x and widely used in fingerprinting

Protocol

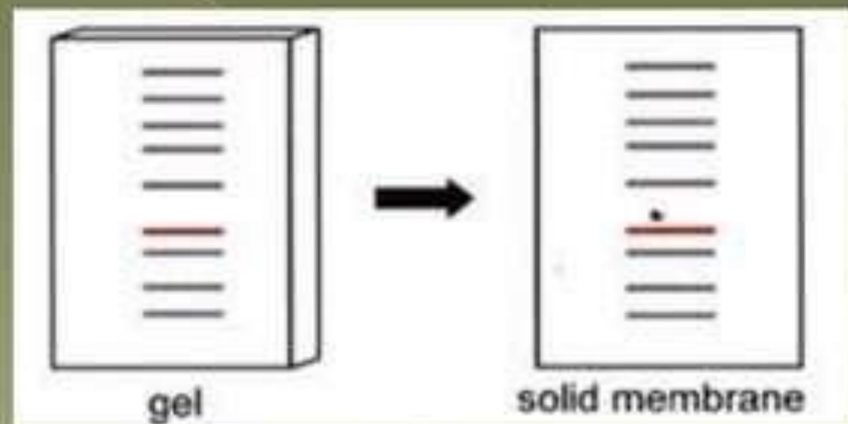
- 1 Restriction endonucleases are used to cut high-molecular-weight DNA strands into smaller fragments



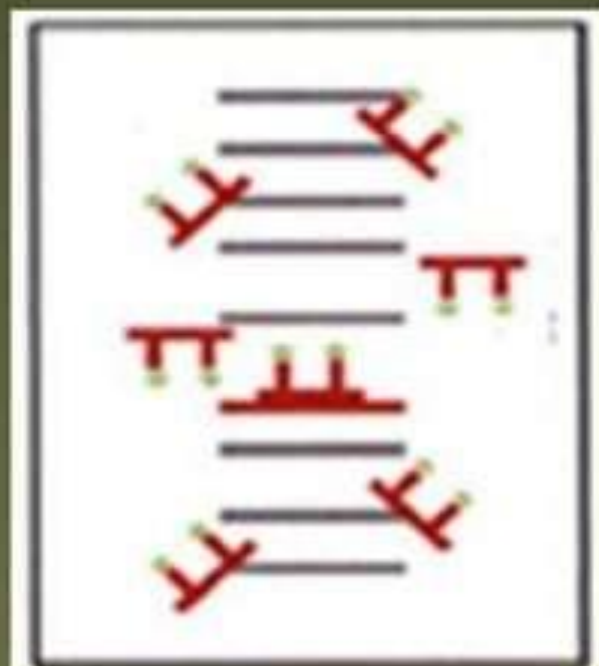
- 2 The DNA fragments are then electrophoresed on an agarose gel to separate them by size



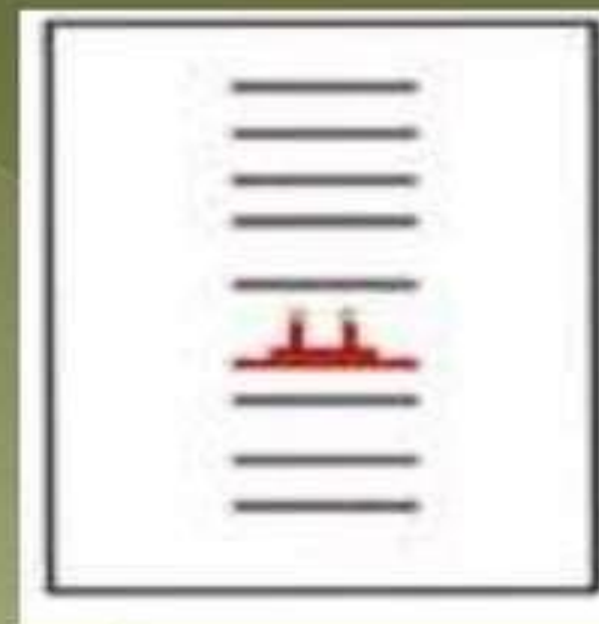
- 3 Double stranded DNA is denatured within the gel and then transferred to solid membrane



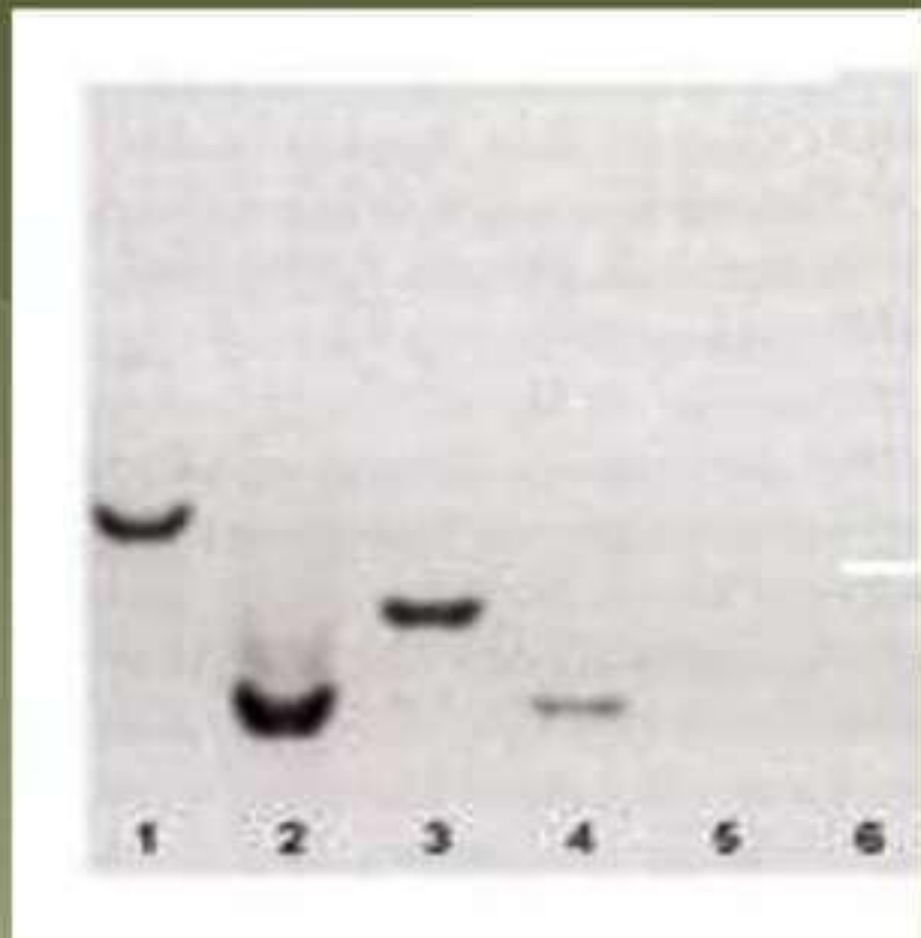
- 4. Small sequence of DNA containing gene of interest are conjugated with Radioactive labeled and added to solid membarne.the probe recognize and hybridize to the spesific sequence of complementary DNA within the gene of interest



- 5. washing steps remove the unbounded probes



- 6 Autoradiography or colorimetric detection demonstarte the peresens of hybridization probe to the sequence of DNA and indicate the size and number of DNA fragments that containe sequence of interest



9.Restriction Fragment Length Polymorphism(RFLP)

- is a technique that exploits variations in homologous DNA sequences
- It refers to a difference between samples of homologous DNA molecules that come from differing locations of restriction enzyme sites
- DNA sample is broken into pieces (digested) by restriction enzymes and the resulting *restriction fragments* are separated according to their lengths by gel electrophoresis
- Its technique based on Southern hybridization
- its used for some tests like paternity test , and localization of genes for genetic disorders and gene mapping

Restriction Fragment Length Polymorphism (RFLP)

DNA Extracted from blood cells

Restriction eznyme cleavage of DNA

Bloodstain

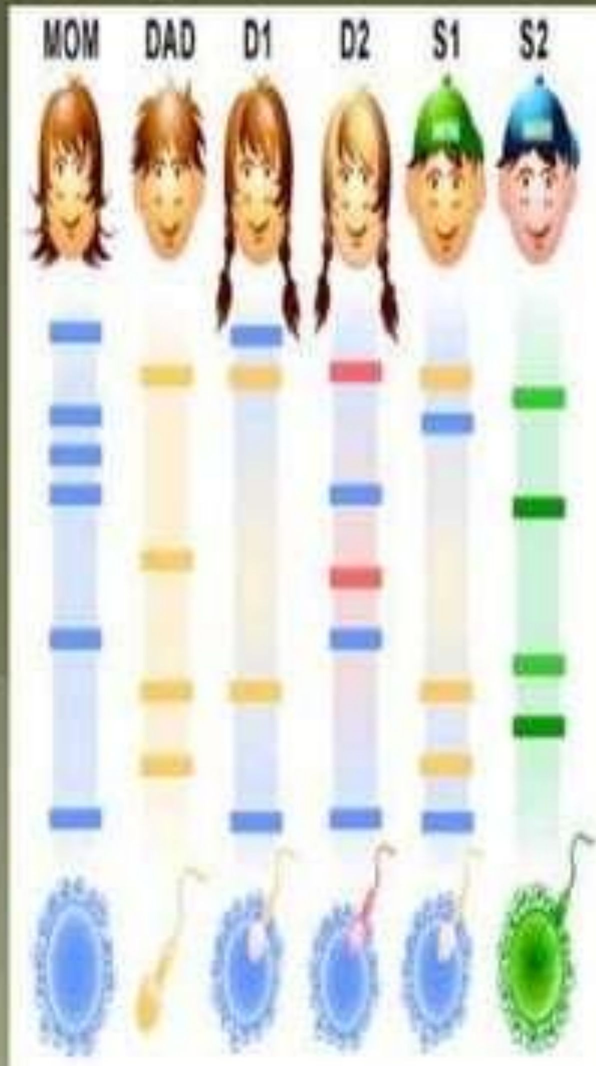
Radioactive DNA probe binds to specific DNA fragments

Transfer of DNA fragments to a membrane (Southern blott)

Fragments of DNA are separated by electrophoresis

Membrane is washed free of excess probe

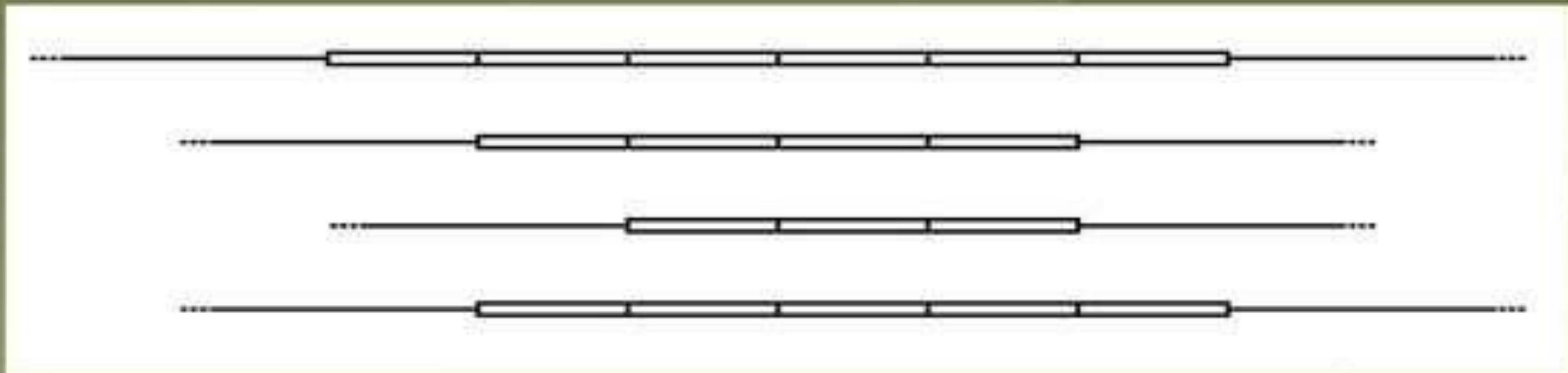
X-ray film, sandwiched to the membrane to detect radioactive pattern



10. DNA fingerprinting

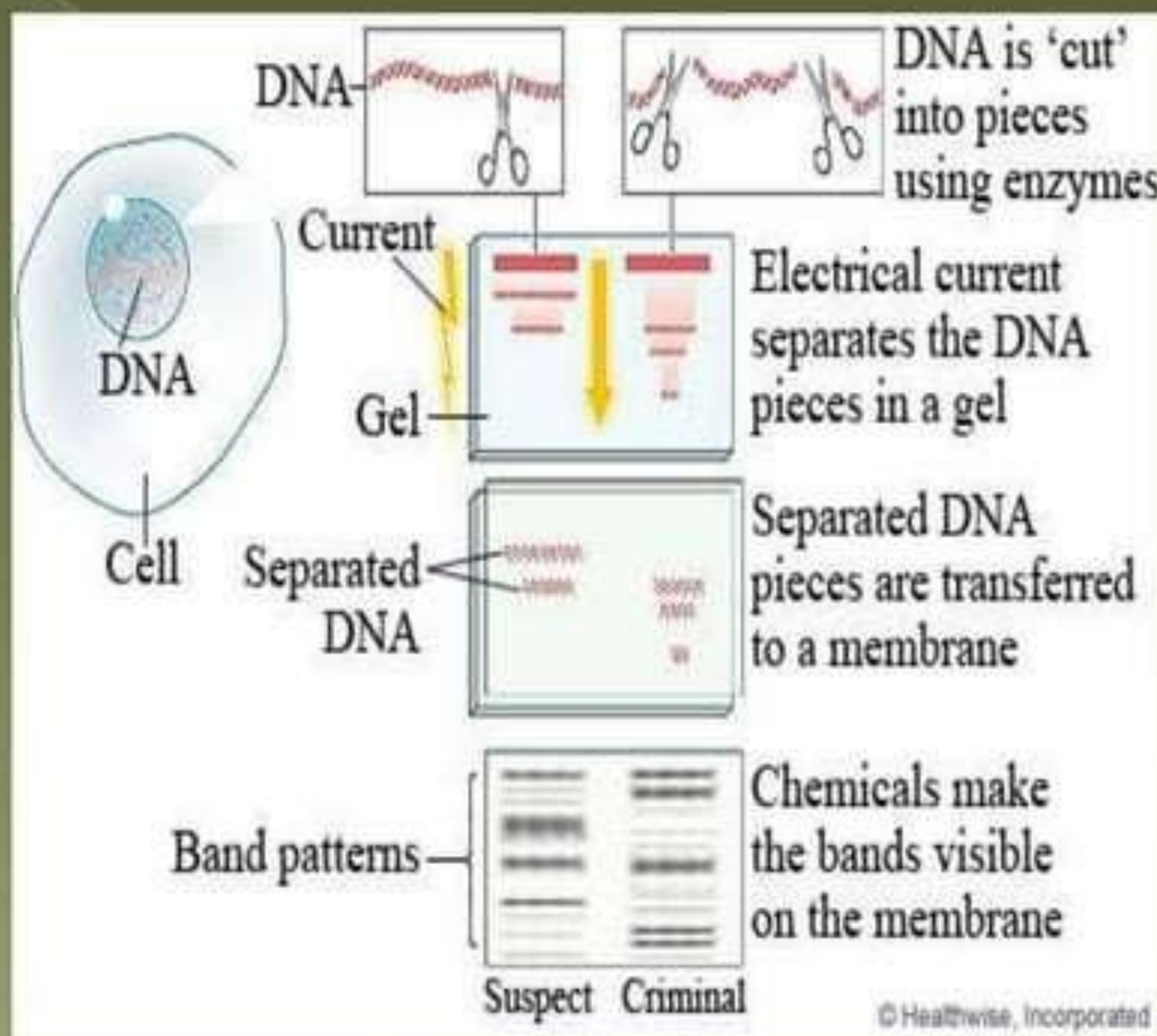
- 99.9% of human DNA sequences are the same in every person, enough of the DNA is different to distinguish one individual from another, unless they are monozygotic twins

- DNA profiling uses repetitive ("repeat") sequences that are highly variable,called variable number tandem repeats (VNTRs)



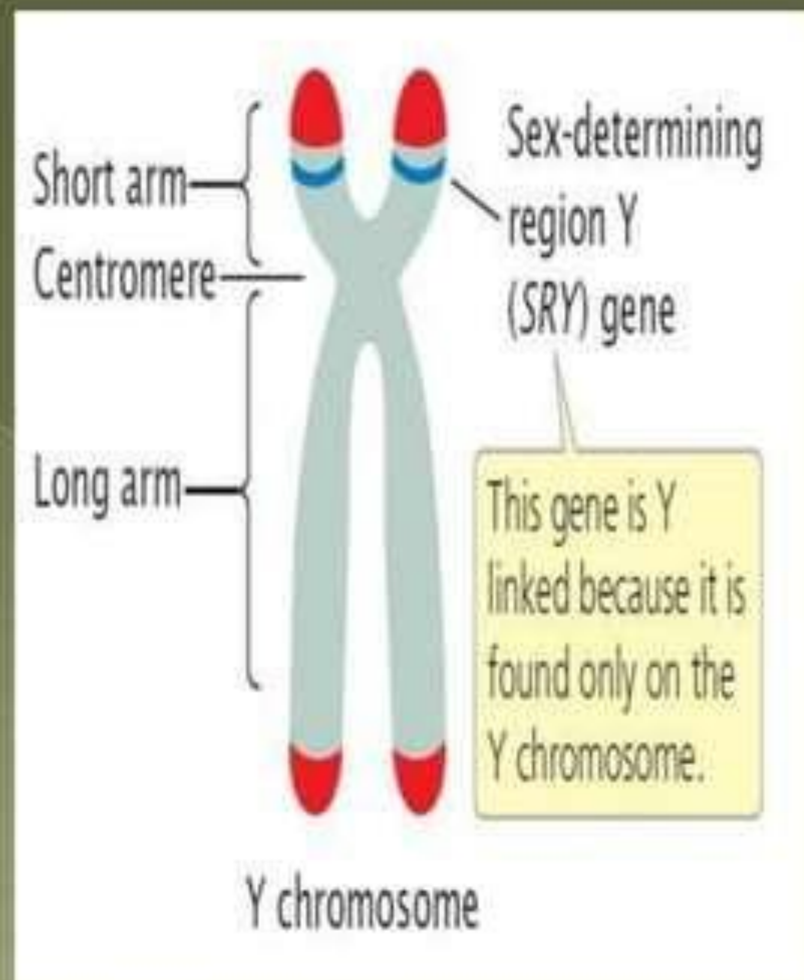
- Although the introns may seem useless, it has been found that they contain repeated sequences of base pairs. VNTR can contain anywhere from twenty to one hundred base pairs
- VNTR loci are very similar between closely related humans, but so variable that unrelated individuals are extremely unlikely to have the same VNTRs
- A given person's VNTRs come from the genetic information donated by his or her parents; he or she could have VNTRs inherited from his or her mother or father
- Because VNTR patterns are inherited genetically, a given person's VNTR pattern is more or less unique. The more VNTR probes used to analyze a person's VNTR pattern, the more distinctive and individualized that pattern, or DNA fingerprinting

- DNA fingerprinting protocol is same as RFLP just VNTR is detected instead interested genes in RFLP

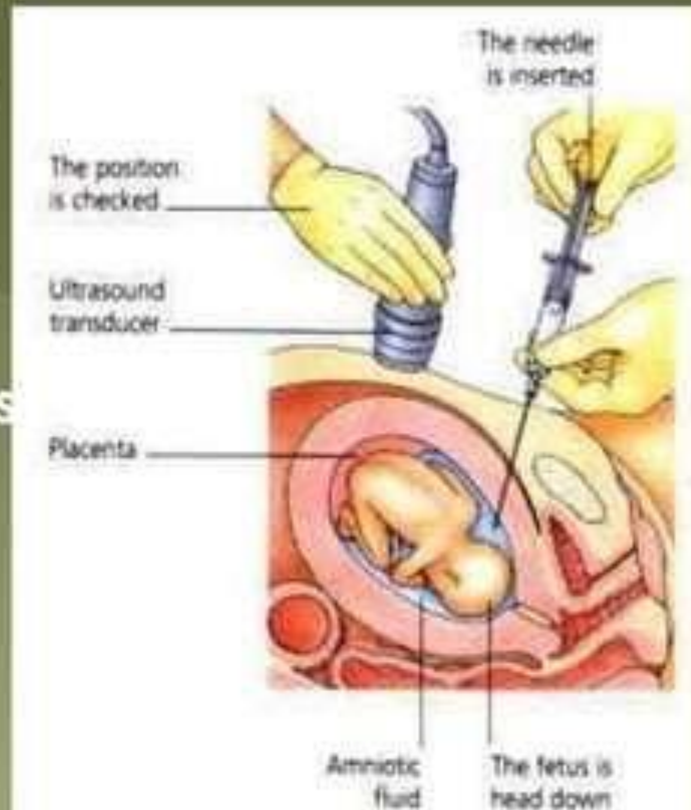


Sex determination of fetus

- Sex determination of fetus by mother's blood in 7th week of pregnancy with using real time technique
- SRY gene is specific gene which is located on Y chromosome
- If this gene is detected in mother's blood it means fetus is male
- fetus sex is determined in 7th

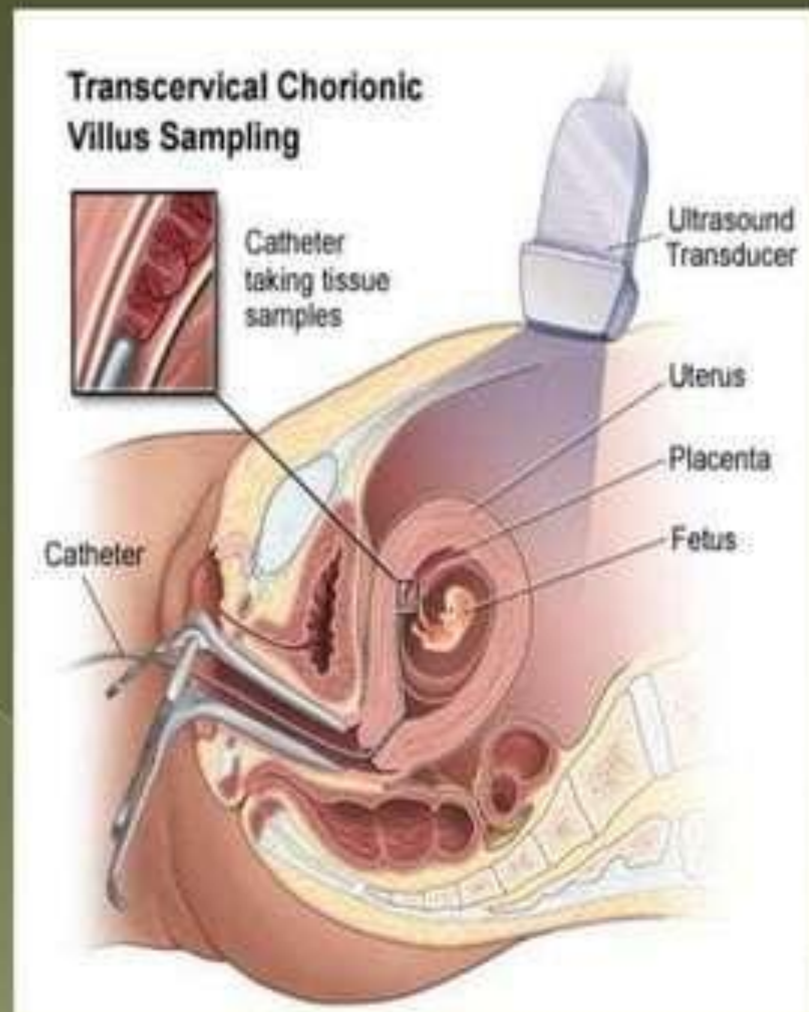


- 2. Prenatal diagnosis (PND)
- Prenatal diagnosis is performing chromosome and DNA analysis for diseases or conditions in a fetus or embryo before birth
- Prenatal diagnosis is performed to detect presence or absence of structural or numerical abnormalities in chromosomes and mutation in DNA
- Prenatal samples:
- Amniotic Fluid: It involves cells of fetal origin. Amniotic fluid consists of the cells of baby's skin, respiration system, digestive system and excretory system and mostly baby's urine



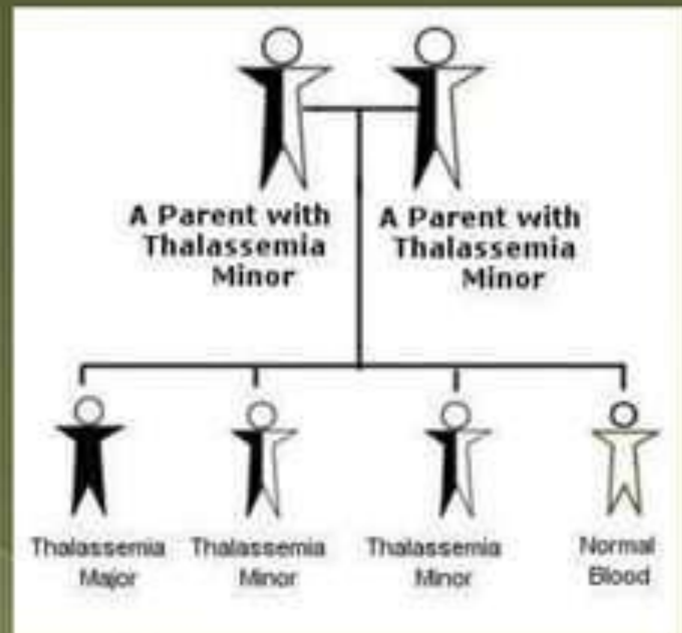
- **CVS (Chorionic Villus Sampling)**

  Chorionic villus sampling (CVS) is the removal of a small piece of placenta tissue (chorionic villi) from the uterus during early pregnancy to screen the baby for genetic defects. CVS usually takes place 10-12 weeks after the last period, earlier than amniocentesis



Prenatal diagnosis of β-*thalassemia*

- for example parents are carrier of β-*thalassemia* so
- 25% of children may have β-*thalassemia*
- Ultrasound can not diagnosis thalasemia but molecular technique can



- Steps
- DNA extraction from parents
- PCR is done then mutation or mutations are detected
- If parents haven't same mutation process is stoped because at the worst condition fetus will be carrier but if parents have same mutation
- DNA of fetus is extracted from CVS or Amniotic fluid
- PCR is done then mutation is detected
- If fetus has homozygote mutation it has major thalasemia so parent can abort it
- If fetus has heterozygote mutation it is carrier for thalassemia

Conclusion

- Molecular techniques help to prevention of morbidity of many inherited diseases
- diagnosis of bacteria , viruses and parasites are done at the earliest time and the most accurate result by molecular techniques