PCR and Mutation detection strategies

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Mutation

- Change in the genetic material which may involve only a few bases or the large-scale chromosome abnormality
  - germline mutations are passed on to one's offspring; somatic mutations are not heritable.
- It may be caused through the action of damaging chemicals, or radiation, or through the **errors inherent in the DNA replication and repair mechanisms.**

Polymorphism

- A polymorphism must have a frequency of at least 1% in the population.

Types of mutation

1. Substitution
2. Deletion
3. Insertion
4. Exon skipping

Why mutation detection is essential?

- Determine the cause of the disease
- Acts as a diagnostic tool for various genetic diseases
- Helps to offer prenatal diagnosis

How to go about it?

- **Source of DNA**
  - Any nucleated cells (**WBC**, buccal cells, hair, finger nails)
- **DNA Extraction**
  - by **lysing** the cells using lysing solution containing Na_2EDTA and NaCl
  - **deproteinizing** (Dissociation of proteins using phenol or saturated NaCl)
  - **removal of lipids** from nucleic acid (chloroform)
- **PCR methods**
  - DNA used as a template

POLYMERASE CHAIN REACTION

- **Kary Mullis** – awarded the Nobel Prize in 1993
- By the mid1980s the technique was first time used to diagnose a disease sickle cell anemia.
- What has led to the success of amplifying DNA in vitro by PCR technique?
  - ability of double stranded DNA to separate or **denature** without disrupting covalent bonds and reforming very rapidly (renaturation)
  - Discovery of **thermostable enzyme**

POLYMERASE CHAIN REACTION

Basic components:

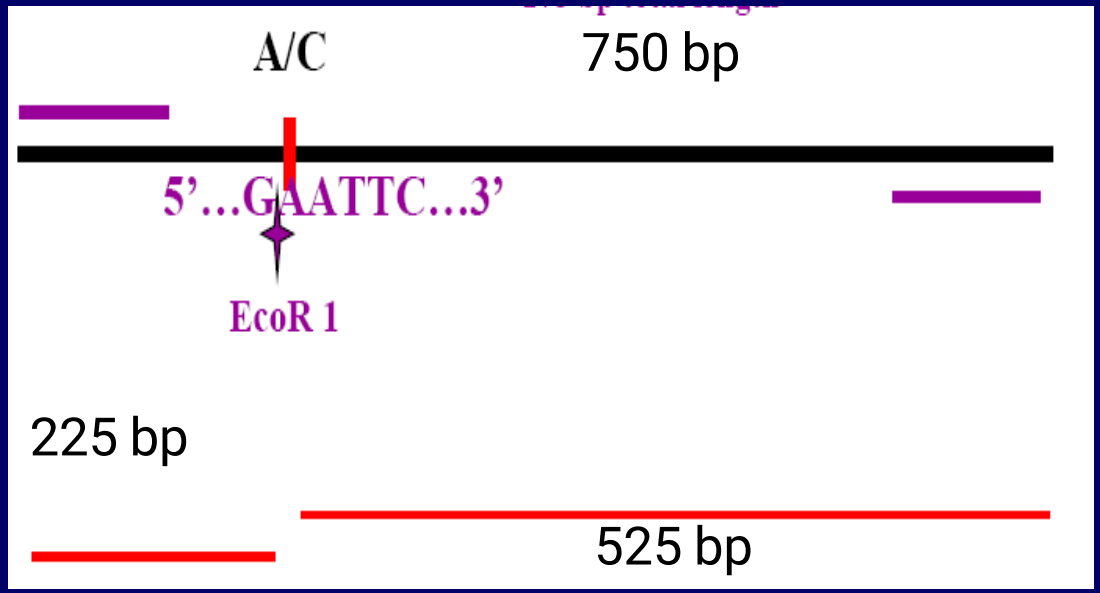
- ***DNA template***, which contains the region of the DNA fragment to be amplified
- Two **primers**, which flanks the region of interest
- ***dNTPs*** *(dATP, dGTP, dCTP, dTTP) f*rom which the DNA Polymerase builds the new DNA
- ***Taq polymerase*** (catalyzes the addition of mononucleotide units to the 3'-OH end of a primer chain; bases are added complementary to the template)
- ***Buffer***, which provides a suitable chemical environment for the DNA Polymerase

Strategies for mutation detection

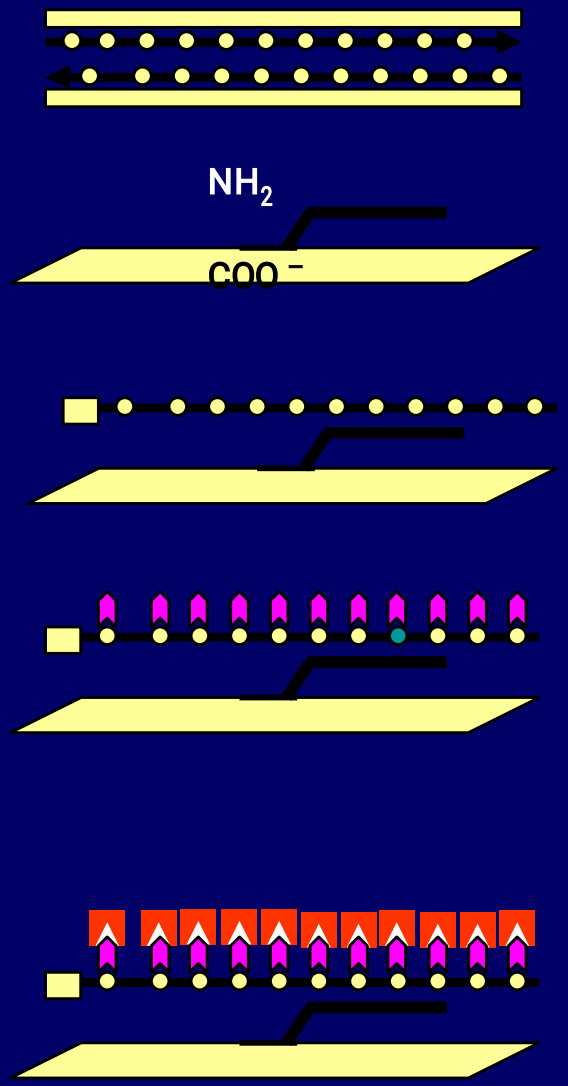
- **Diagnostic mode, involves detection of known mutations**
  - PCR-RFLP (Restriction Fragment Length Polymorphism)
  - RDB (Reverse Dot Blot)
  - ARMS (Amplification Refractory Mutation System)
- **Scanning mode, involves screening of unknown mutations**
  - SSCP (Single Strand Conformation Polymorphism)
  - DGGE (Denaturing Gradient Gel Electrophoresis)
  - TTGE (Temporal Temperature Gradient Gel Electrophoresis)
  - CSGE (Conformation Sensitive Gel Electrophoresis)
  - SEQUENCING

PCR-RFLP

- RE - recognize short sequences on double stranded DNA as target for cleavage.
- recognizes unique sequences ranging from 4-8 bp.
- A nucleotide change can either create or abolishes the restriction enzyme site.
- detect the presence of wild or mutant allele
- precise region of DNA, mapping the target amplified by PCR and subjected to digestion using a specific restriction enzyme



REVERSE DOT BLOT



Amplification of target DNA with incorporated biotin

Binding of amino modified ASO probe to negatively charged nylon membrane

Hybridization of membrane bound ASO probe with biotin labeled PCR fragments

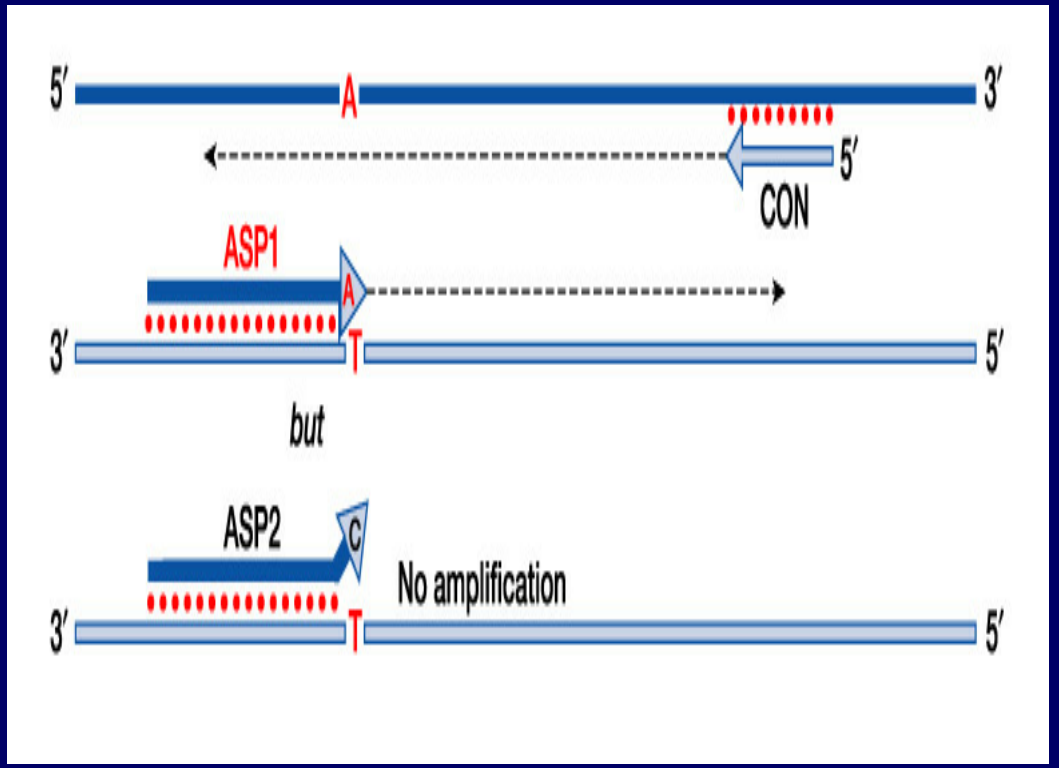
Binding of avidin-alkaline phosphatase

detection with NBT and X-phosphate

Reverse dot blot is a widely used technique for identifying point mutations

AMPLIFICATION REFRACTORY MUTATION SYSTEM

- PCR that allows the detection of known point mutations by allele specific amplification of genomic DNA.
- One of the primers contains / may not contain a mismatch at the 3' end.
- Presence of mismatch does not allow extension during PCR.
- A primer having a complementary base to the known substitution in the genomic DNA will amplify



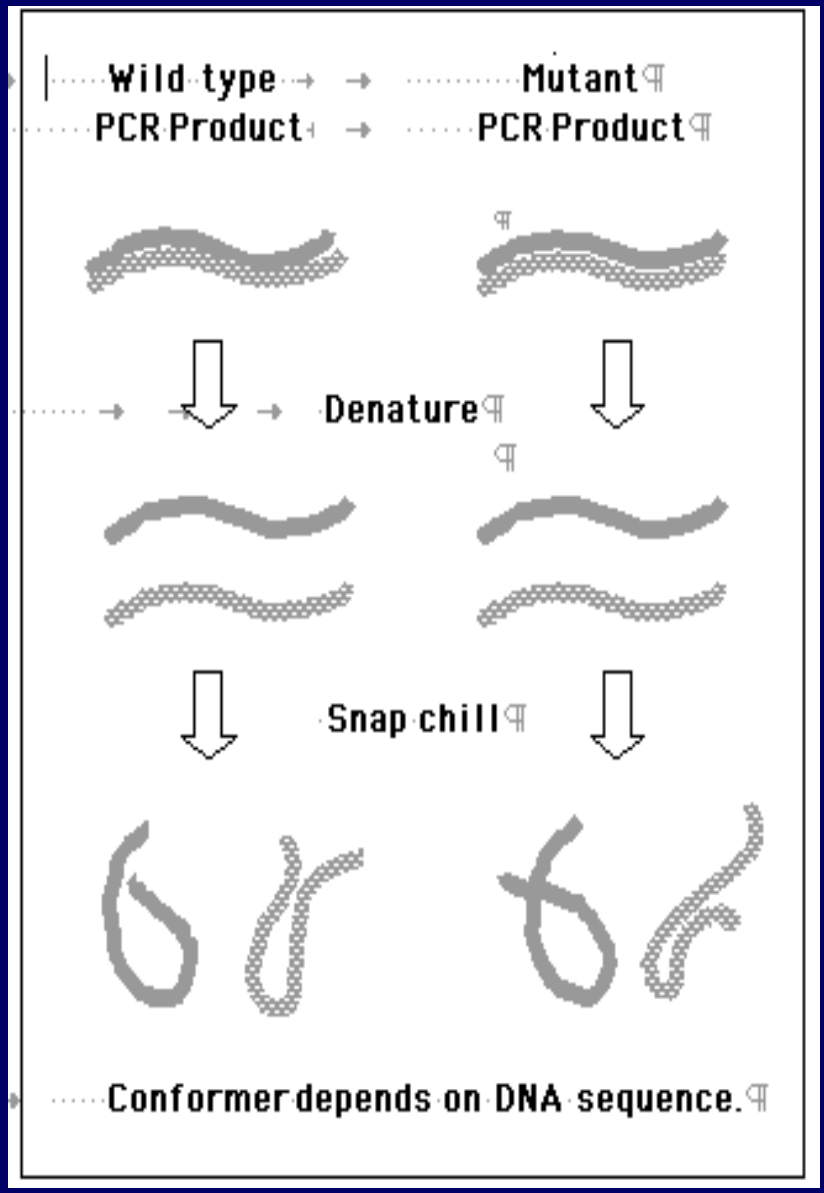
Rapid non radioactive method and genotyping is possible simply by inspection of PCR reaction mixtures after agarose gel electrophoresis

DGGE/TTGE

- Mutation screening is based on the melting behavior of the DNA molecules under denaturing environment
  - Denaturant can be temperature or chemical like urea or formamide
- ds DNA molecule may have several melting domains with several characteristic melting temperature
- determined by the nucleotide sequences
- Any alteration in the sequence will tend to modify its melting point

Single-stranded conformational polymorphism (SSCP)

- based on the electrophoretic behavior of a ss DNA molecule through a non-denaturing acrylamide gel
- A ssDNA molecule has the property of forming secondary structures through internal base pairings
- Formation of secondary structures are
  - sequence dependent and result in particular conformation for each single-stranded molecul
  - may vary depending on physical conditions, e.g. temperature and ionic environment.
- Conformational differences causes the DNA strands to migrate

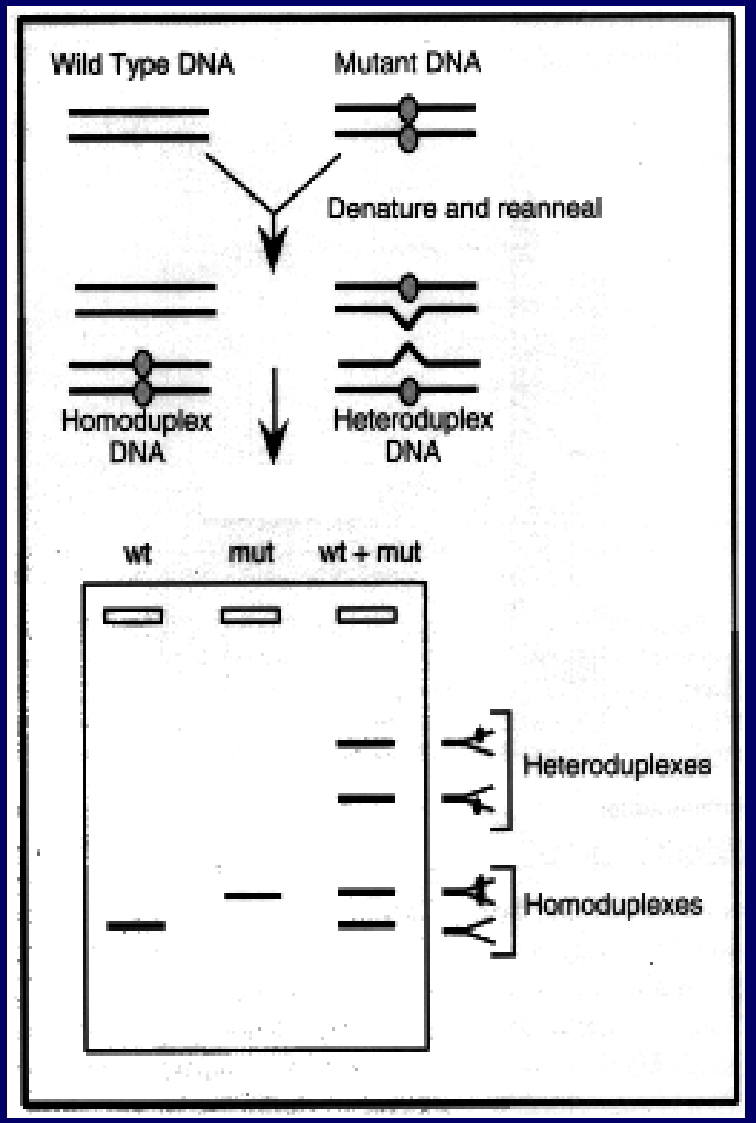


CONFORMATION SENSITIVE GEL ELECTROPHORESIS

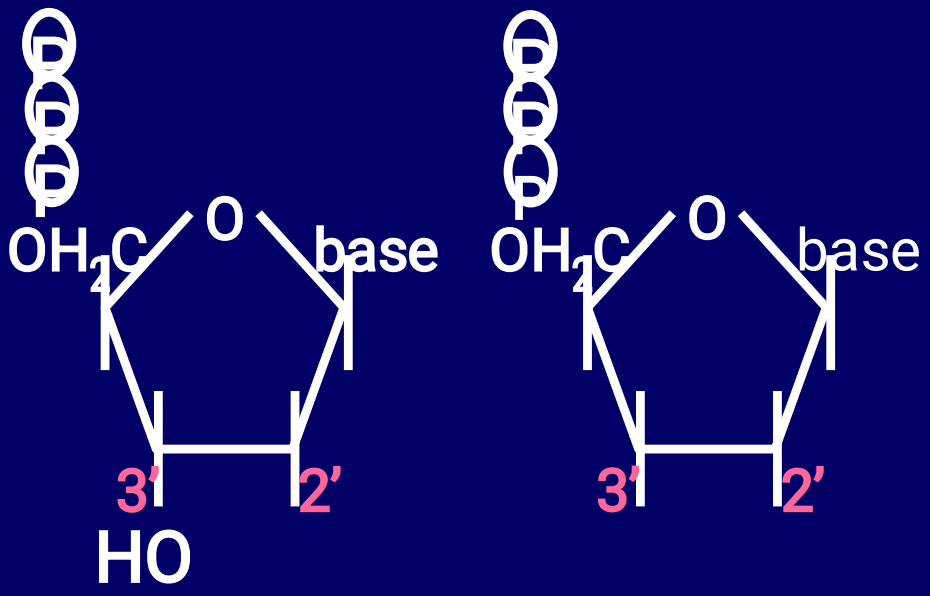
- Rapid, non radioactive heteroduplex based detection method for mutation screening.

- relies on the differential migration of DNA heteroduplexes in comparison with homoduplexes during polyacrylamide gel electrophoresis under mildly denaturing conditions.

Heteroduplex formation



Sequencing (Sanger method)



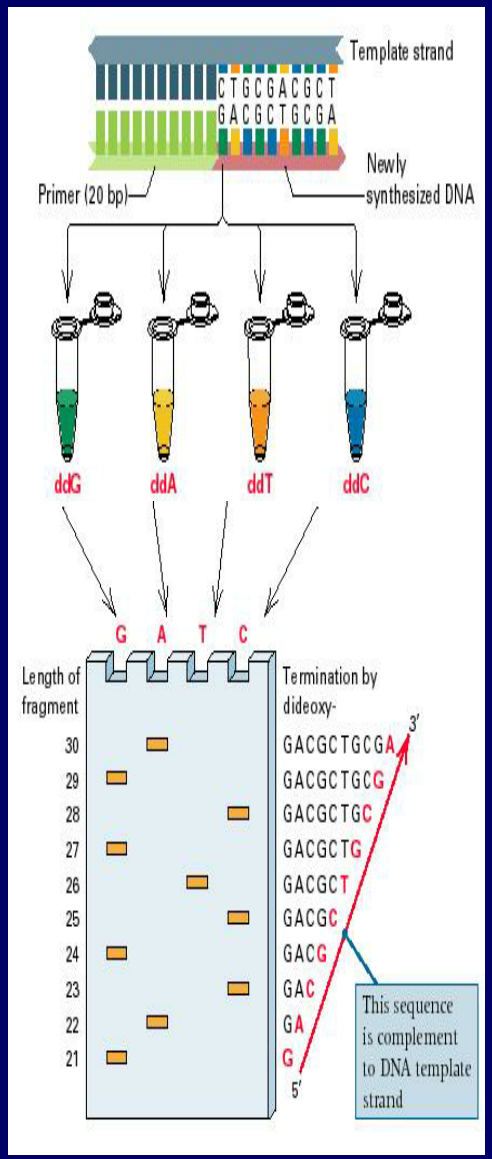
dNTP ddNTP

Frederick Sanger

If dNTP is incorporated, synthesis proceeds; if ddNTP is incorporated, synthesis is terminated.

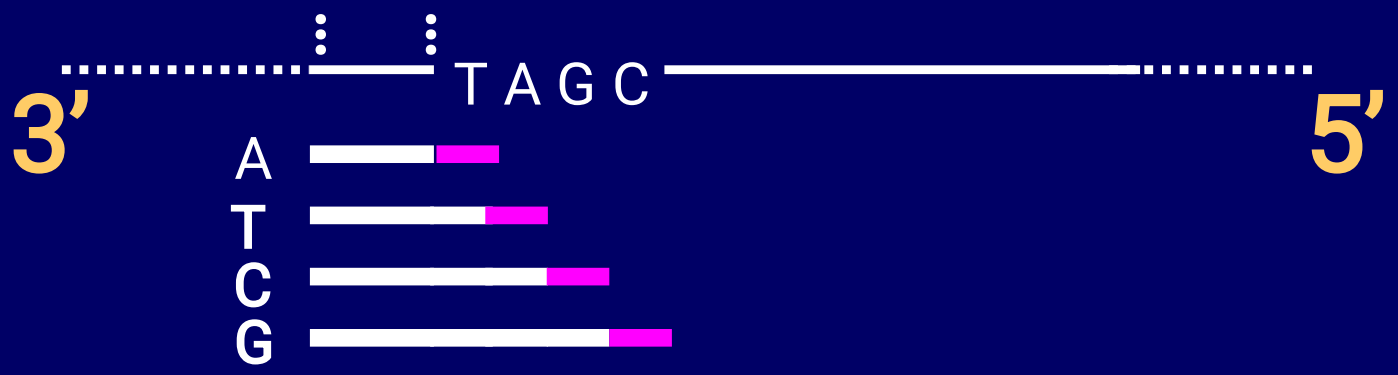
(Nobel prize 1980 with Paul Berg and Walter Gilbert)

Sequencing-Manual



dATP
dTTP
dCTP
dGTP
+
ddATP
ddTTP
ddCTP
ddGTP

DNA polymerase + Buffer

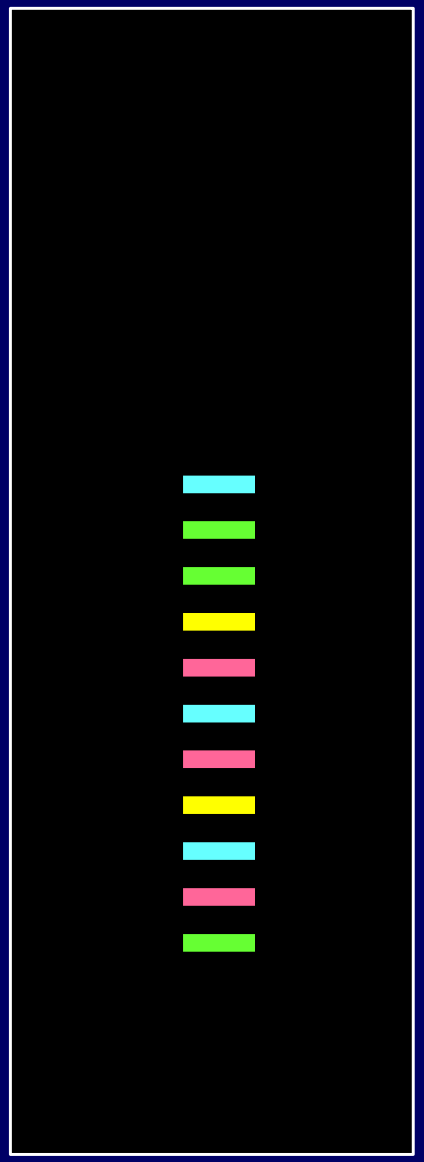


Lane number gives nucleotide

Product length gives nucleotide position

synthesis is 5' to 3', the gel reads 5' to 3' from the smallest to the largest chain terminated products

Sequencing –Automated method



dATP
dTTP
dCTP
dGTP
+
ddATP (JOE dye)
ddTTP (ROX dye)
ddCTP (FAM dye)
ddGTP (TAMRA dye)

Product colour gives nucleotide

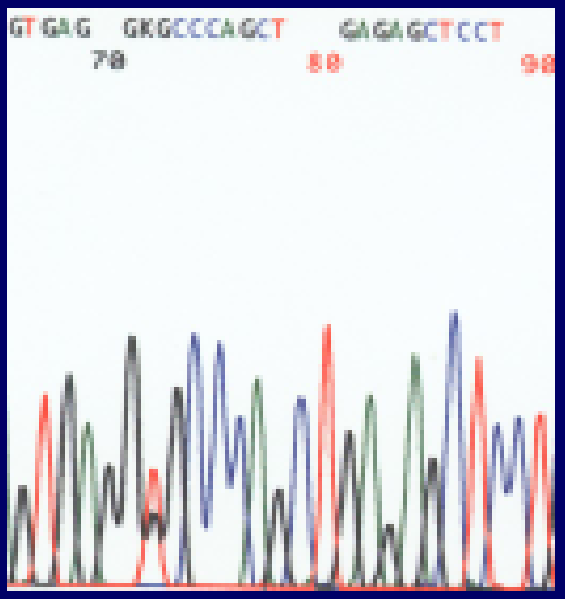
Product length gives nucleotide position

5’A T C G T C T G A A C 3’

Seq gel

Laser light

Electropherogram



detector

computer