Describe the principles and procedures of the polymerase chain reaction (PCR) (including its advantages and limitations)

Polymerase Chain Reaction

➔ **Amplifies DNA** from a **limited source** of DNA so that there is **sufficient amount** for **analysis**.

*** Process: (3 steps)**

1. **Denaturation: Double stranded DNA denatures** into **single stranded DNA** by heating to **95°C** as weak **hydrogen bonds** between **complementary bases** of each strand is **broken** due to increased molecular vibrations

2. **Primer annealing:** Each **primer anneals specifically** to the **3'end** of each **single stranded target DNA sequence** via **complementary base pairing** when the temperature is lowered to **64°C**

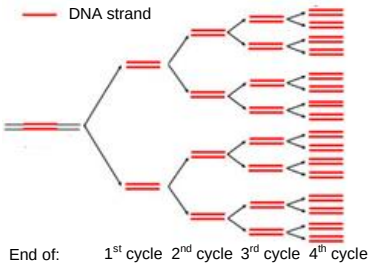
3. **Extension: Taq polymerase** synthesizes the **complementary DNA strand** from the **free 3OH' end** of the **DNA primer** by catalyzing the formation of **phosphodiester bonds** between dNTPS when the temperature is increased to **72°C**

*** Advantages:**

1. Only a **minute amount of DNA** is required to carry out PCR as with **each round of PCR**, the number of copies of **target DNA is doubled**. Thus the number of desired sequence **increases exponentially** and there will be **sufficient DNA for analysis**.
2. Use of **thermostable** (i.e. **resistant to denaturation at high temperatures)** Taq polymerase allows PCR to be **automated** so DNA can be amplified very **quickly**.

*** Limitations:**

1. **Taq polymerase lacks 3' to 5' proofreading ability**. Hence **errors** occurring early in the PCR reaction will get **compounded** with each subsequent replication cycle.
2. **Knowledge of sequences flanking (i.e. at the 3' ends of) the target sequence** is required in order to **design** appropriate **primers**.
3. **Taq polymerase tends to 'fall off' the DNA template** before chain extension is complete if the strand is too long. Hence there is a **limit to the size** of DNA fragment (~3kb) to be amplified.
4. **Minute amounts of contaminant DNA** can be exponentially **amplified along with target DNA** and affect the **reliability** of the results.



* Number of copies of double stranded DNA = 2^n
(where n is the number of cycles)
* Number of (single) strands of DNA = 2^{n+1}
(where n is the number of cycles)

End of n cycle where n is	1	2	3	4
Copies of ds DNA	2	4	8	16
Strands of ss DNA	4	8	16	32

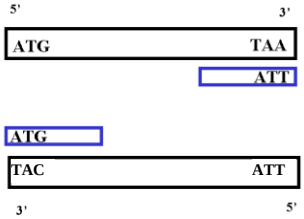
PCR Components

*** Template DNA**

➔DNA containing the target sequence to be amplified

*** Primers**

➔synthetic single-stranded DNA fragment (20-30 nucleotides long)
➔needed to **initiate DNA synthesis** by providing a free 3'OH group for Taq polymerase to bind to and extend
➔ **2 different primers** are required. Each is complementary to the sequence at **3'end of each single stranded target DNA sequence.**



➔ are required in **large excess** to increase the likelihood of them binding to target DNA sequences (relative to template strands binding each other)

*** Taq polymerase**

➔ **thermostable** DNA polymerase which is resistant to denaturation at high temperature

*** Deoxyribonucleotides (dNTPs)**

➔ **substrates** for DNA replication made up of dATP, dTTP, dCTP and dGTP

*** Buffer**

➔ contains cofactor, **Mg^{2+}**, for proper polymerase function

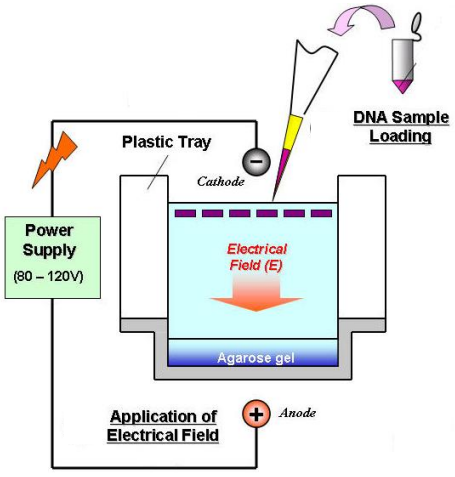
Describe the principles and procedures of gel electrophoresis

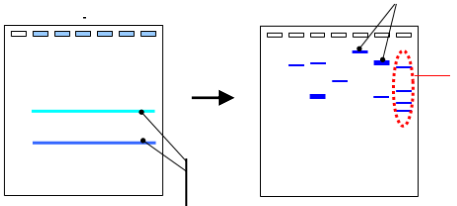
Agarose gel electrophoresis

➔ separates DNA based on **fragment size**

*** Steps:**

1. A slab of agarose gel is placed in a **buffer solution** contains **ions** which **allows the conduction of electricity** when the current is turned on.
2. The DNA sample is mixed with a **dense loading dye** containing **glycerol** & **2 coloured dyes**. Glycerol makes the **DNA sample denser** than the buffer solution so that the DNA sample can **sink to the bottom of the well.**
3. Since **DNA is invisible**, the dyes **colour the DNA sample** and will indicate if the DNA has been **loaded correctly into the well**. (NB: Loading dyes do not bind to the DNA.)
4. One dye (corresponds to a 100bp DNA fragment) and often **runs ahead of the DNA sample** and gives an indication of **when electrophoresis must be stopped so that the samples do not run out of the gel.** The other dye (corresponds to a 1100bp DNA fragment) and gives an indication of the **position of the larger fragments** on the gel.
5. The **2 coloured dyes** thus act as **visual markers** which help to **monitor** the **progress of the migration** of the invisible **DNA fragments** in the gel during electrophoresis.
6. DNA samples are pipetted into the **wells** in the gel near the **negative electrode**.
7. A **DNA ladder** (i.e. DNA molecular weight markers) which contains **DNA fragments of known sizes**, is run in one of the lanes and acts as a standard for which to compare fragments of unknown size in the sample.
8. **Negatively charged DNA** is attracted towards the **positive electrode (anode)** when subjected to an **electric current.**
9. The **agarose gel** matrix made of a **meshwork of polymer fibres** which **impedes movement** of longer fragments more than shorter fragments. The **longer fragments** thus migrate **more slowly** compared to **shorter fragments**, leading to a banding pattern observed on the gel.
10. Before the loading dye reaches the end of the gel, the current is turned off.
11. To **visualize the bands**, the gel can be treated with a staining **dye that binds DNA** (e.g. **ethidium bromide, a carcinogen**) and **fluoresces under uv light**.
12. Thus a) the **fragment size** can be estimated (based on **position** of the band relative to bands in the molecular weight marker) and
    b) the **amount of DNA** can possibly be estimated (based on **intensity** & **thickness** of the band).





DNA molecular weight marker acts as standard to compare to fragments of unknown sizes

The top line corresponds to fragments that are 1100bp and the bottom line corresponds to 100bp fragments.

Note: Bands are only visible after treating with staining dye such as ethidium bromide (visible under UV light).

NB: The higher the concentration of agarose, the finer the pores in the meshwork.

➔Thus smaller fragments can be effectively separated.

Describe the principles and procedures of Southern blotting and nucleic acid hybridisation

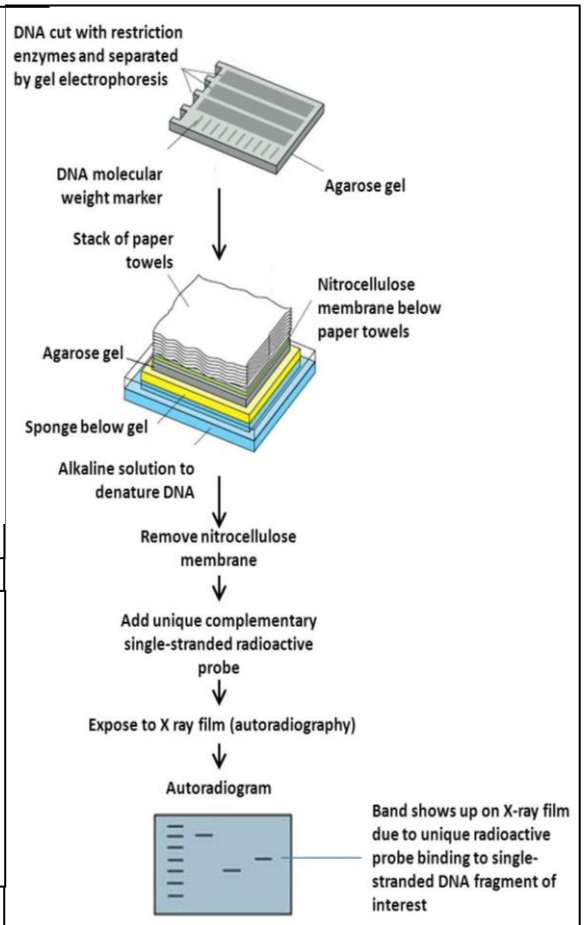
Southern blotting: a nucleic acid hybridisation technique used in RFLP analysis

Tool to **detect specific nucleotide sequences** within a sample of DNA

Steps:
(Continued from Gel electrophoresis)

1. Gel slab is placed on top of the sponge and under a **nitrocellulose membrane**. A stack of paper towels placed on top of nitrocellulose membrane. These are placed in a tray of **alkaline solution**. A heavy weight is placed above the paper towels.
2. Absorbent paper towels draw the solution towards themselves and the **alkaline solution denatures double-stranded DNA** into **single-stranded DNA**.
3. Single stranded DNA on the gel is then **drawn upwards onto the nitrocellulose membrane** and **binds to the membrane** (in exactly the same position as they were in the gel).
4. Nitrocellulose membrane is removed and incubated with single-stranded **radioactive DNA probe** which **hybridises** via **complementary base pairing** to the target sequence.
5. The excess unhybridised probes are washed off.
6. **Autoradiography** is performed placing **X-ray film** over membrane. Radioactive regions **exposes the film** forming an image that correspond to the bands that have base-paired with probe.

[Note: RFLP analysis is a principle/ way of analyzing data. PCR, gel electrophoresis and nucleic acid



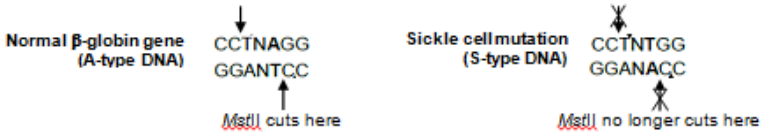
What are RFLPs?

- RFLP = Restriction Fragment Length Polymorphisms
- RFLPs are restriction patterns that arise from restriction digestion using a specific restriction enzyme.
- RFLPs arise due to **DNA polymorphisms** which are **small nucleotide differences** in **different individuals** at **specific locus**.
- Thus due to the **polymorphic** nature of DNA in different individuals, there will be **variations** in the
  1. **number/location of restriction sites** or
  2. **number of tandemly repeated nucleotide sequences**. This will result in a **unique banding pattern** amongst individuals.

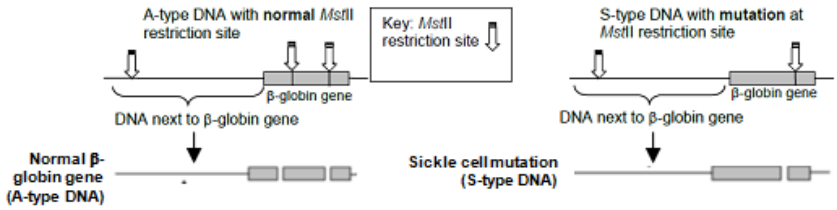
- The DNA polymorphism in sickle cell anaemia is a **single nucleotide polymorphism** (**SNP**). There is a difference in a **single base pair** due to a **point mutation**.
- In **sickle cell anaemia** this SNP is within the **coding region**. However the **majority of SNPs** used for RFLP analysis are found in **non-coding regions**.

i) Use of RFLP analysis in disease detection e.g. sickle cell anaemia

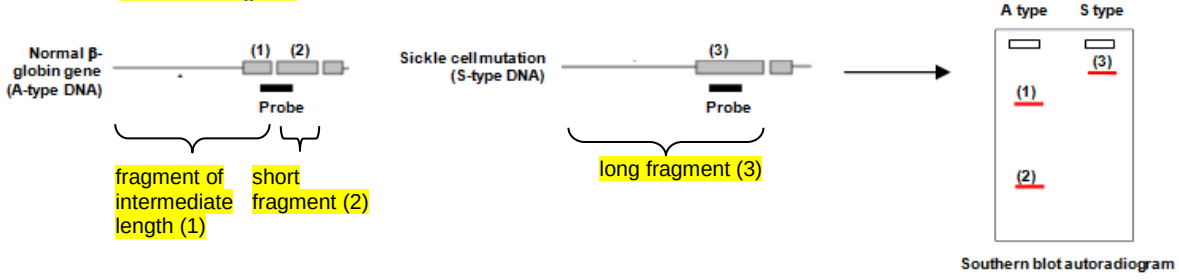
- A **difference in single nucleotide** can result in a **gain OR loss of a restriction site** of an enzyme. Thus when a particular section of DNA in individuals with the mutation and without the mutation is digested with the same restriction enzyme, **DNA fragments of different lengths** will result. **Analysis of the banding pattern** that arises, will allow determination of the **presence of disease-causing allele** or the **normal allele**.
- e.g. in **sickle-cell anaemia**, the disease-causing mutation occurs at restriction site for ***Mst* II** within the **β-globin** gene.
  1. In the **disease causing** allele, the *Mst* II restriction site is **eliminated**.
  2. In the **normal** allele, *Mst* II restriction site is **retained**.



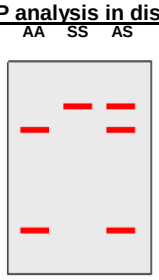
- How to determine the genotype of a person for **β-globin** gene?
  1. Digest **genomic** DNA from both samples with *Mst*II to obtain different restriction fragments will arise. (i.e. cut both DNA samples with the SAME restriction enzyme, Mst II)



  2. Perform **gel electrophoresis** following by **southern blotting** (use of nitrocellulose membrane, radioactive probes and autoradiography). The **same single-stranded radioactive probe** (which is complementary to part of the target sequence) will be used to detect both the presence of A-type and S-type DNA. Hence the site that the probe binds on the DNA fragments should give different banding patterns on the autoradiogram.



i) Use of RFLP analysis in disease detection e.g. sickle cell anaemia (continued)

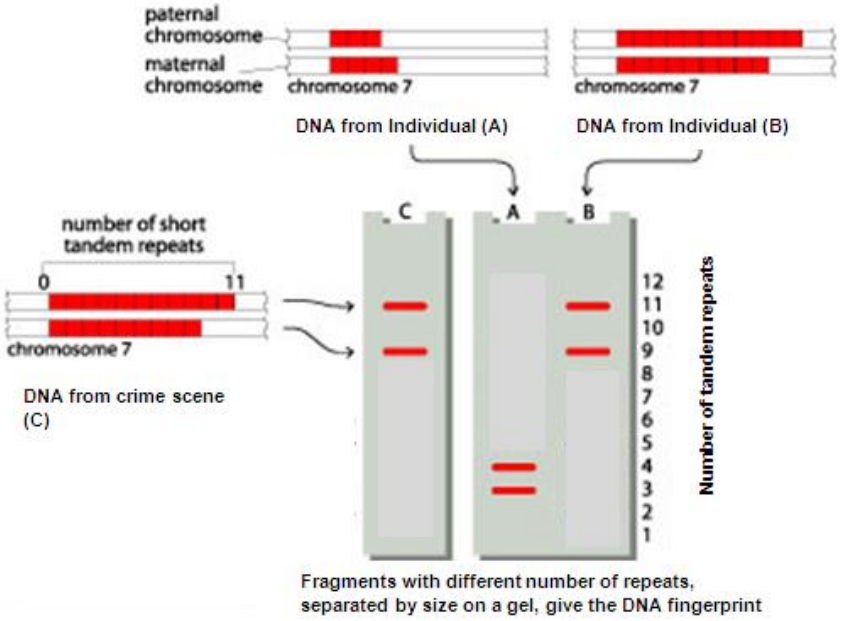


A type DNA will indicate presence of normal β globin.
S type DNA will indicate presence of abnormal β globin (sickle cell).
Note that normal β globin is dominant over abnormal β globin (sickle cell).

AA indicates that the individual is **homozygous dominant** and is **phenotypically normal**.
(The individual has 2 bands. One fragment of intermediate length and one short fragment.)
SS indicates that the individual **is homozygous recessive** and is **suffering from sickle cell anaemia**.
(The individual has 1 band corresponding to the long fragment.)
AS indicates that the individual is **heterozygous** and is **phenotypically normal.**
(The individual has 3 bands. One long fragment, one fragment of intermediate length and one short fragment.)

ii) Use of RFLP analysis in DNA fingerprinting

- As no individuals (exception of twins) have the same genome, therefore they will not have the same DNA profile.
- The DNA profile is the restriction banding pattern that identifies individuals.
- How to carry out DNA fingerprinting:
  1. **Restriction digestion** of **genomic DNA** by **restriction enzymes** followed by **gel electrophoresis** to **separate DNA fragments**
  2. **Southern blotting** using of **nitrocellulose membrane**.
  3. **Nucleic acid hybridisation** using **radioactive probes** complementary to the STRs
  4. **Visualisation of bands** via **autoradiography using X-ray films**
- At a particular RFLP locus, different individuals will have different number of STR repeats, hence different length of fragment.
- RFLP alleles of each locus is inherited from parents. Therefore individuals with similar banding patterns should be closely related.



- These RFLP alleles are all from the same RFLP locus.
  DNA from the crime scene (C) has 2 RFLP alleles, one with 11 repeats and the other with 9 repeats. Individual (A) has two RFLP alleles, one with 3 repeats and the other with 4 repeats. Hence Individual (A)'s DNA fingerprint is different from the DNA at the crime scene.

  (Note: Instead of using steps 1 and 2 (above) to carry out DNA fingerprinting, the different STR loci can be amplified using PCR and multiple PCR primers that anneal to flanking sequences of different STR loci. Then gel electrophoresis can be carried out to separate the DNA fragments. The DNA can then be stained by ethidium bromide and the fragments can be visualised under uv light. This alternative method will also give rise to the different banding patterns seen above.)

* **Restriction enzymes** (molecular scissors)
1) are isolated from **bacterial** cells
2) recognize & bind to specific **palindromic** base sequences (restriction sites) on DNA
3) create either **sticky ends/blunt ends** by cleaving of **phosphodiester bonds** between **nucleotides** in **both** strands of DNA

e.g. ***Alu* I**

```
NNAGCTNN
NNTCGANN

NNAG  CTNN
NNTC  GANN
```

blunt ends

e.g. ***Eco* RI**

```
G AATTC
CTTAA G

G      AATTC
CTTAA      G
```

sticky ends

4) **protect** bacterial cells from **invading viruses** by **degrading the foreign DNA** that enter them
5) do not cleave the bacterial DNA in the bacteria they are found in as the restriction sites in the bacterial DNA are methylated. **Methylation** protects bacterial DNA from degradation.