CORE IDEA 2: GENETICS AND INHERITANCE
TOPIC 2.9: DNA MOLECULAR TECHNIQUES

H2

Learning Outcomes:

(k) Describe the principles and procedures of these molecular techniques:
   i. polymerase chain reaction (including advantages and limitations)
   ii. gel electrophoresis
   iii. Southern blotting and nucleic acid hybridisation

References:
Reece, J. B., et al. (2011). *Campbell Biology (9th Ed)*, Chapter 20: Biotechnology, pp449 – 453

1 INTRODUCTION

- Gene expression can be studied using fundamental techniques of molecular biology such as the polymerase chain reaction (PCR), gel electrophoresis, Southern blotting and nucleic acid hybridisation.

- Such molecular techniques can separate and visualise specific fragments of DNA that are of interest e.g. disease-causing alleles in sickle cell anemia.

2 POLYMERASE CHAIN REACTION

Learning Outcome 2(k)(i):
Describe the principles and procedure of polymerase chain reaction (including advantages and limitations).

The Polymerase Chain Reaction (PCR) can **rapidly amplify a specific segment of DNA** *in vitro* (outside the cell) for the purpose of cloning and analysis.

The method is designed to permit **selective amplification** of a specific target DNA sequence within a heterogenous collection of DNA sequence.

Possible applications of PCR:

- Amplify specific gene or DNA fragment
- DNA fingerprinting
- Studying human ancestry
- Paternity testing

2.1 Reagents

(i) Target DNA Sequence

Target sequence refers to DNA to be copied, usually a gene of interest which is to be studied. The target sequence to be amplified is usually ~1 to 2 kb in length.

(ii) Primers

Primers are synthetic short DNA sequences (single-stranded oligonucleotides) approximately 20 – 30 bp in length.

Primers (**forward** and **reverse** primers) are **complementary** to DNA sequences at the end of the DNA target sequence.

- The forward primer will hybridise to the DNA coding stranding, 3' to 5' direction
- The reverse primer will hybridise to the DNA non coding strand, 5' to 3' direction
- The primer provides a **free 3' OH** end for DNA polymerase to replicate the target sequence by elongating the DNA strand in the 5' to 3' direction. This allows the replication of the double-stranded DNA molecule.

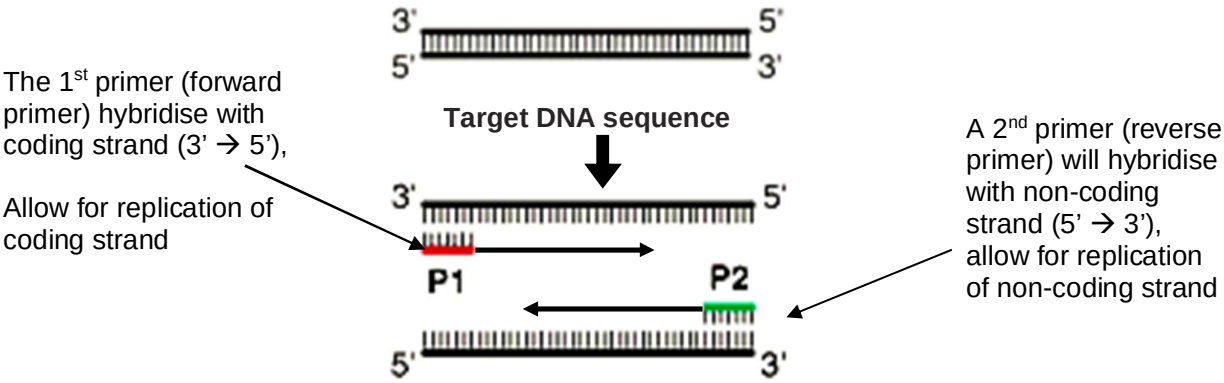


Fig. 2.1.1: Use of primer to initiate PCR process

CHECKPOINT 1

Deduce the sequence of two 5-nucleotide long primers that can be used to amplify the complete target DNA segment. Specify the polarity of your primers.

target DNA sequence

5'	**T**	**T**	**C**	**G**	**A**	**T**	**G**	**C**	**C**	**T**	**A**	**T**	**G**	**C**	**T**	**A**	**T**	**G**	**C**	**C**	**3'**
3'	**A**	**A**	**G**	**C**	**T**	**A**	**C**	**G**	**G**	**A**	**T**	**A**	**C**	**G**	**A**	**T**	**A**	**C**	**G**	**G**	**5'**

primer **1** ***5' G G C A T 3'***

primer **2** ***5' T T C G A 3'***

*Recall, DNA can only be synthesized in the 5' to 3' direction
*Common misconception by students: The primers are complementary to each other. The example clearly shows that the primers are not complementary.

(iii) DNA Polymerase

A DNA polymerase derived from a thermophilic bacterium, ***T**hermus **aq**uaticus* (***Taq* polymerase**) is used in PCR.

Taq polymerase is **thermally stable** (i.e. capable of withstanding high temperatures), with an optimum temperature of ~75°C. This is important as the PCR process requires repeated cycles of heating and cooling.

Taq polymerase binds to the 3' OH end of the primer, adding deoxyribonucleotides complementary to the target sequence in a 5' → 3' direction, to elongate the DNA strand.

(iv) Free Deoxyribonucleotides (dNTP)

The four types of deoxyribonucleotides, dATP, dTTP, dGTP and dGTP, are added in approximately equal proportions in **excess**.

2.2 Process

- The process involves a 3-stage cycle of denaturation, annealing and elongation which results in an exponential increase in the number of copies of the target DNA molecule of identical sequences.

- PCR reagents are added in a reaction tube and PCR is carried out in a thermal reactor, known as a **thermal cycler**, which automates the temperature and timing of each step in the amplification cycle.

Stage 1: DNA denaturation

The target DNA sample and required reagents are heated to **~ 95°C** for 1 min, to denature and separate the double-stranded (ds) DNA strands into single-stranded (ss) DNA.

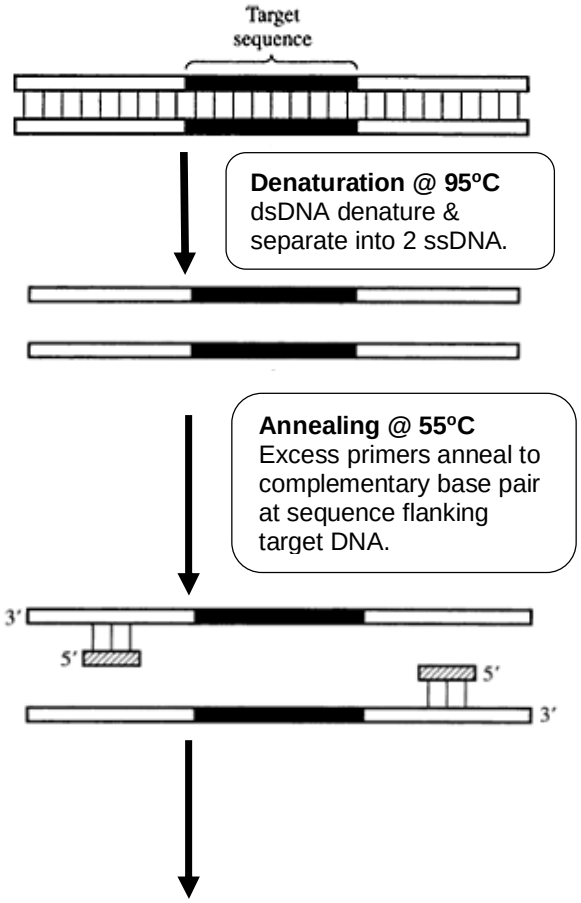
Heating increases kinetic energy of DNA molecules, resulting in breaking of hydrogen bonds between complementary base pairs.

Stage 2: Annealing

The mixture is then cooled to **~ 55°C** for 2 min, in the presence of excess primers.

Forward and **reverse primers** would bind to complementary sequences flanking the target DNA sequence via hydrogen bonds.

Due to presence of excess primers, the probability of primer annealing is higher than the DNA target sequence renaturing.



Stage 3: Elongation
The temperature is raised to **72°C** for 3 min.

Taq polymerase synthesizes the complementary DNA strand in the 5' to 3' direction by adding free dNTPs to the free 3' OH end of the primers using the target DNA sequence acts as template.

Elongation @ 72°C
Taq polymerase extends forward & reverse primers at free 3' OH ends.

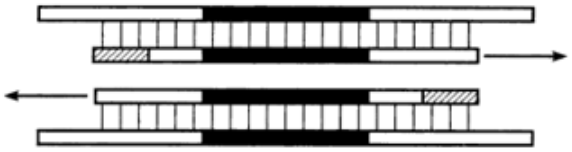


Fig. 2.2.1: One PCR cycle

- After the first cycle, the same process (denaturation, annealing, elongation) is **repeated 20 to 30** times in an automated thermal cycler.

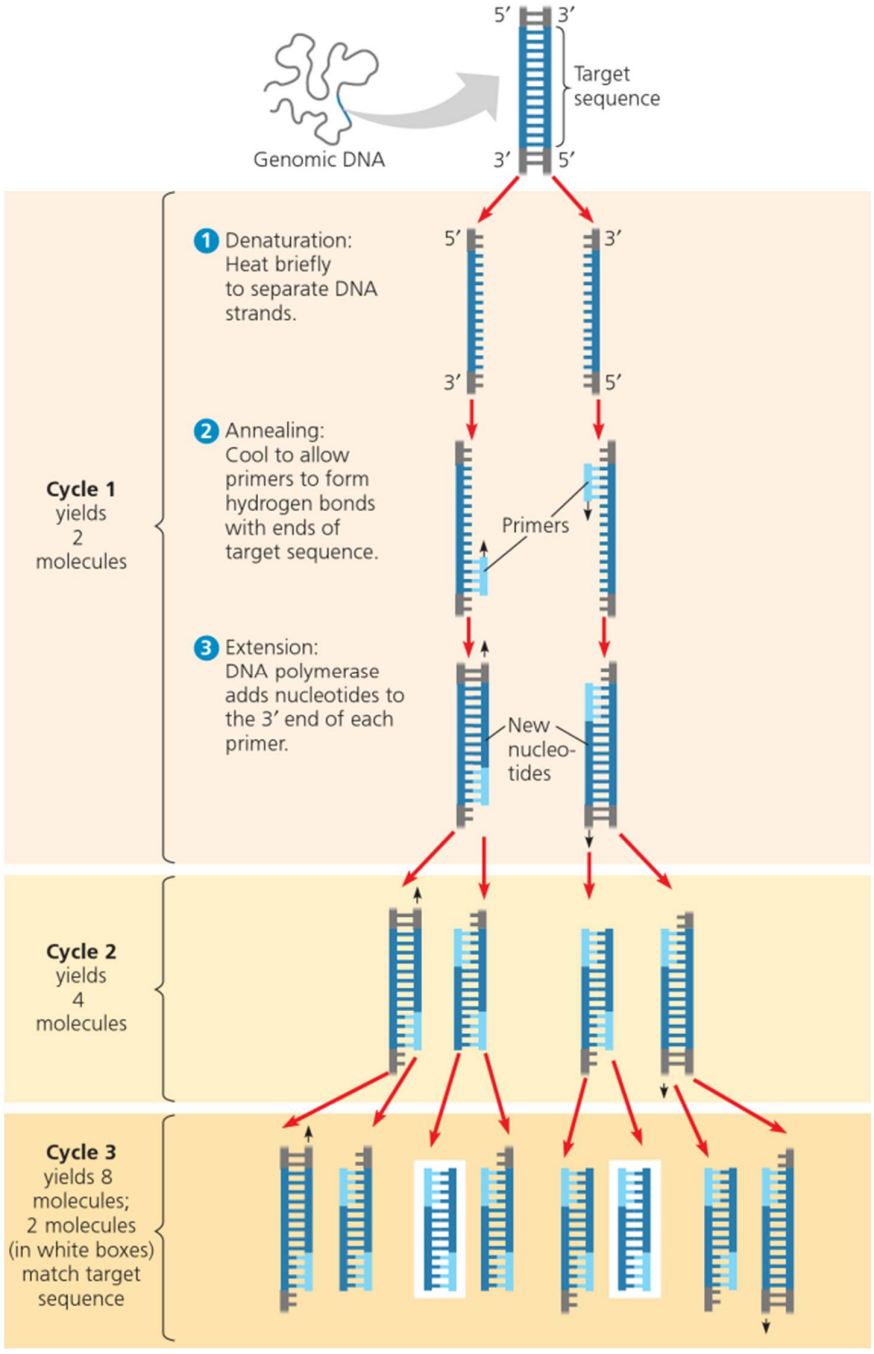


Fig. 2.2.2: PCR process

- Each cycle results in the doubling of the target DNA sequence. Thus, number of copies of dsDNA is $\mathbf{2^n}$ where, n is the number of cycles.

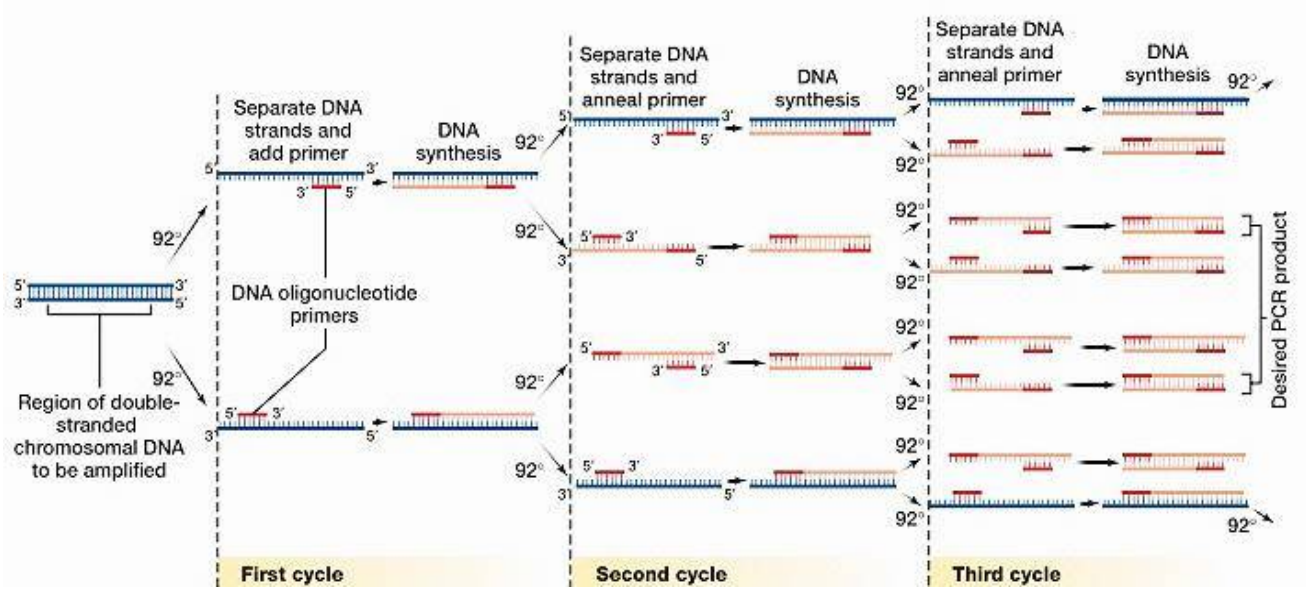


Fig. 2.2.3: Exponential increase in DNA copies

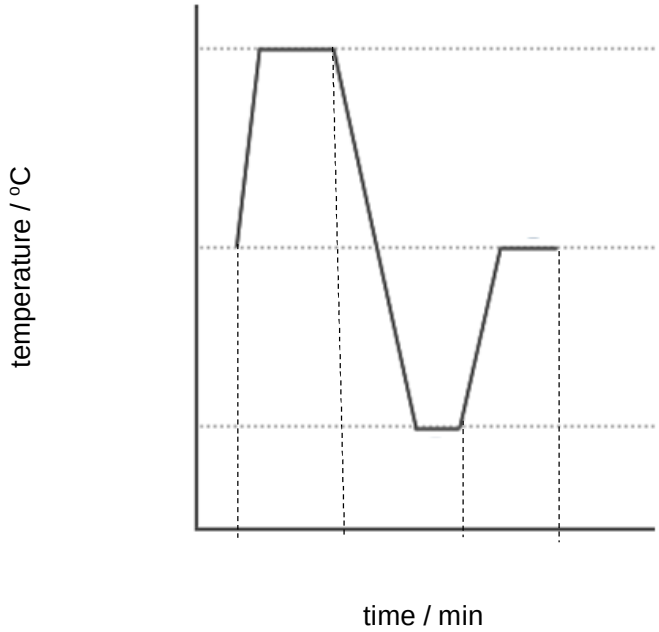
CHECKPOINT 2

Calculate the number of DNA molecules (double-stranded) and DNA strands (single-stranded) after each PCR cycle.

Cycle	Number of target DNA molecules (ds)	Number of DNA strands (ss)
0	1	2
1		
2		
3		
30		
n		

CHECKPOINT 3

(a) Label the graph to show the stages of polymerase chain reaction.
(b) Label the appropriate temperature on the y-axis and time on the x-axis.



2.3 Advantages

- **Sensitive**
  The process is sensitive and can amplify minute samples of target DNA.

  In criminal investigations, DNA fingerprints can thus be prepared from cells in a tiny speck of blood or from the base of a single human hair. In pediatric medicine, physicians can detect genetic defects in very early embryos by collecting a few cells and amplifying their DNA.

- **Robust**
  PCR is a robust process which can amplify DNA sequences from badly degraded material or even DNA embedded in material that is difficult to extract, as long as a few molecules contain the complete target sequence.

- **Rapid and relatively easy to use**
  Each cycle takes only 3 - 5 min. Thus, a PCR reaction involving 30 cycles of denaturation, annealing and elongation can be completed in a few hours, yielding a large number of DNA molecules and amplifying the target sequence.

  PCR can be performed using relatively simple equipment, a thermal cycler. Reagents are added to reaction tubes in appropriate amounts and conditions (e.g temperature, duration, number of cycles) are set. The reaction process is then fully automated and the cycles can run unattended.

- **Relatively high fidelity**
  The amplification is relatively accurate with error rates ranging between 1 in 10,000 bases to 1 in 100,000 bases. Error rates vary with the choice of polymerase.

2.4 Limitations

- **Too sensitive**
  Even a tiny amount of contaminant DNA in a sample may become amplified if it includes a DNA sequence complementary to the primers, potentially leading to a erroneous conclusion.

- **Need for target DNA sequence information for primer design**
  The specificity of the amplification process is dependent on specificity of the primers. Thus, the base sequence flanking the target sequence is required in order to synthesize specific primers.

  Short primers are easier to synthesize but tend to bind to multiple sites along the DNA molecule, which may lead to inaccurate amplification. Long primers are more specific to but their synthesis is more difficult and costly.

- **Limited length of target sequence**
  The length of target DNA restricted to 0.1 to 5 kb with an optimum length of 2 to 3 kb. A further increase in length of the target sequence decreases efficiency of amplification because polymerase tends to detach before chain extension is complete.

- **Error in replication**
  Taq polymerase lacks proofreading activity. This results in an error rate of approximately 1 in 10,000 bases.If the error occurs early in the PCR cycle, the erroneous sequence will be amplified together with the target sequence.

3 GEL ELECTROPHORESIS

Learning Outcome 2(k)(ii):
Describe the principles and procedure of gel electrophoresis.

Agarose gel electrophoresis allows the separation of DNA on the basis of molecular weight using differential migration rates across a gel in an electrical field. Gel electrophoresis can also be used to separate molecules like RNA and proteins.

3.1 Agarose Gel Electrophoresis

Agarose gel electrophoresis is used in the separation of DNA fragments.

Steps:

1. Agarose powder is dissolved in Tris-Borate-EDTA (TBE) buffer and poured into the gel casting tray. A comb is inserted to form wells.

2. Agarose is left to cool and harden forming an **agarose gel** of 0.8% to 2%.

3. The gel is placed into a gel chamber and submerged in TBE buffer. The buffer **maintains appropriate pH** and **contains ions that conduct a direct electric current** across the gel.

4. The comb is removed.

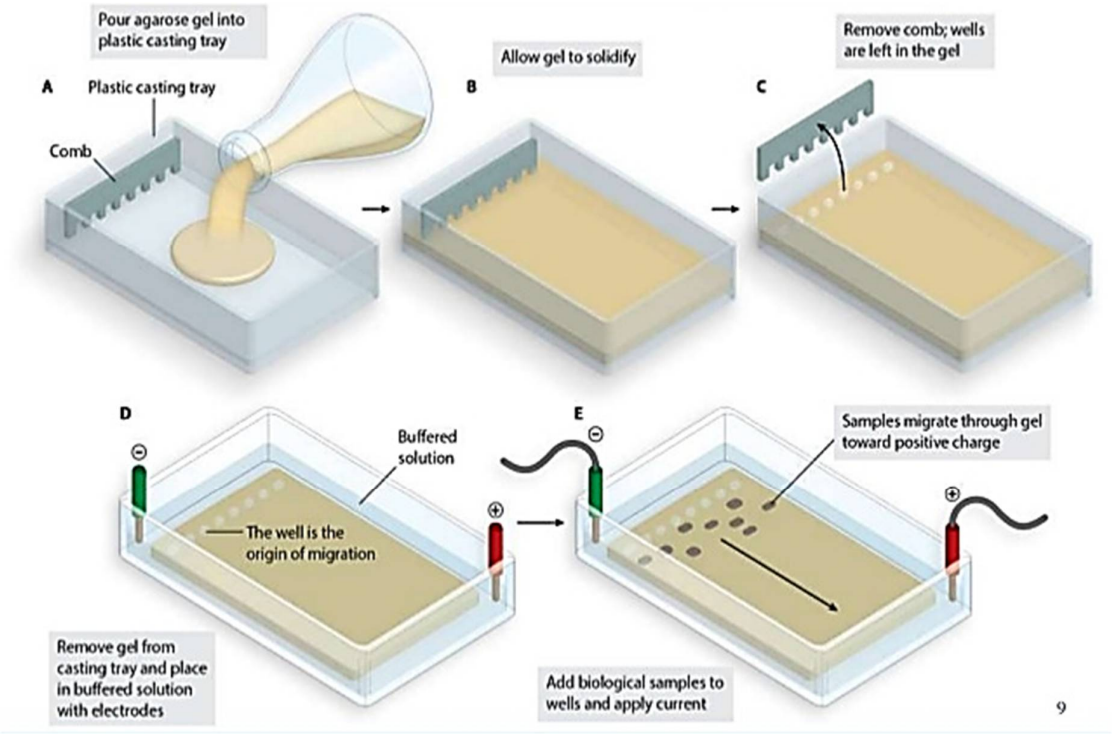


Fig. 3.1.1: Preparation of gel

5. DNA molecules are mixed with loading dye and tracking dye, and loaded in wells of the agarose gel near the **cathode** (negative electrode) using a micropipette.

   The **loading dye** contains **glycerol** that helps **weigh the DNA fragments** into the wells. DNA fragments are light.
   - Without the loading dye, they would float out of the well and disperse in the buffer. Migration would not be able to take place.

   The **tracking dye** which serves as a visual marker for migration during electrophoresis and indicates when to turn off the power.
   - The tracking dye move towards the anode, similar to DNA molecules, when placed in an electric field.
   - It is usually of lower molecular weight than DNA, so once it reaches the end of the gel, it signals for the current to be turned off.
   - This prevents the DNA from migrating out of the gel.

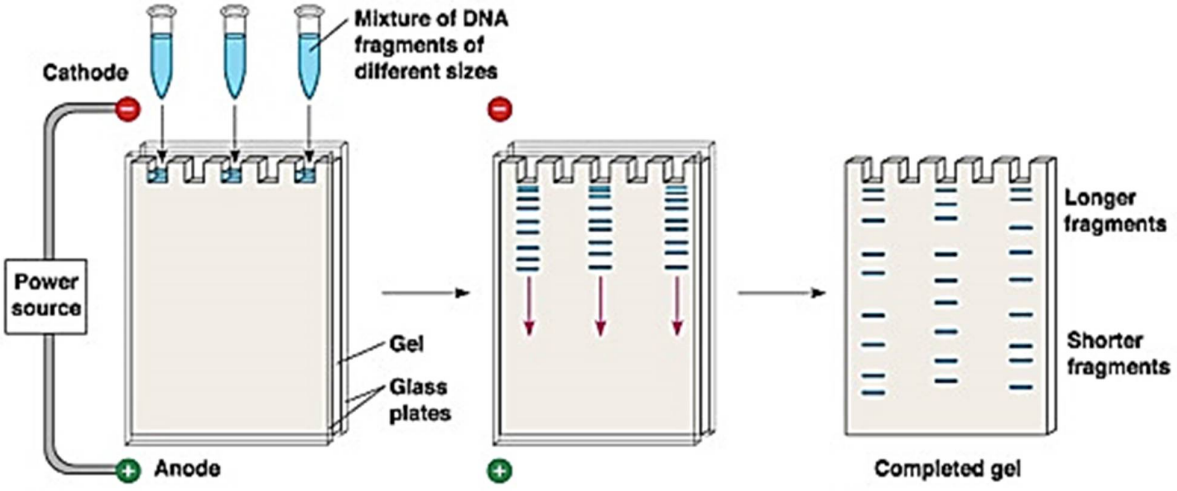


Fig. 3.1.2: Gel electrophoresis

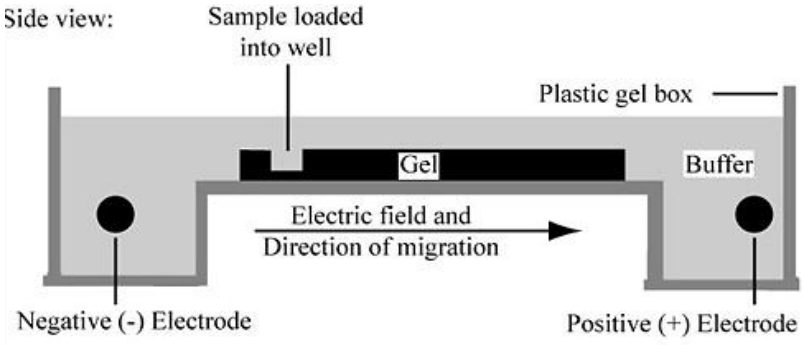


Fig. 3.1.3: Side view of gel chamber

6. A **DNA ladder**, consisting of molecules migrating at speeds of **known-sized fragments**, can be loaded (usually in the first well) for comparison to indicate size (fragment length) of the separated DNA.

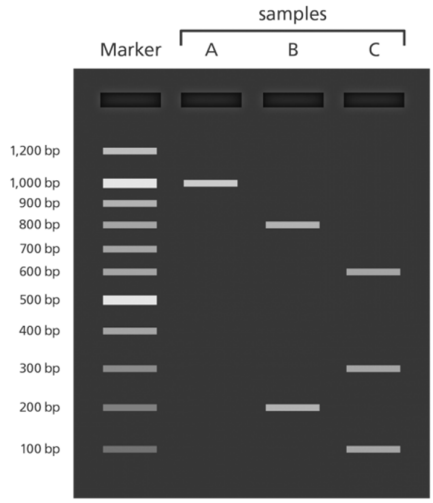
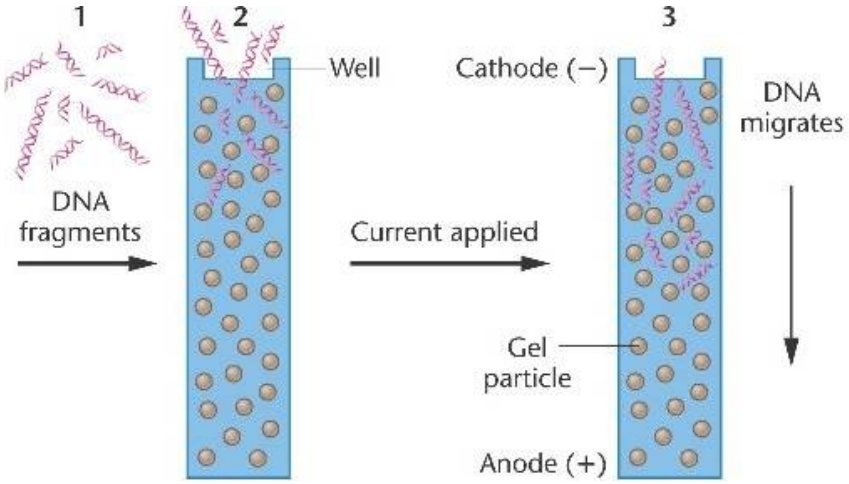


Fig. 3.1.4: Use of DNA ladder for estimation of DNA fragment length

7. A **voltage** between 90V to 150V is applied across the gel.

   As DNA fragments are **negatively charged** due to presence of **phosphate groups**, DNA will migrate across the gel from the cathode (negative electrode) towards the anode (positive electrode).

   The gel acts as a **molecular sieve** that separates the DNA fragments by size (molecular weight) and shape. **Smaller** fragments are less impeded by the gel and migrate **faster** than larger fragments. Supercoiled fragments which are more compact migrate faster than relaxed ones.



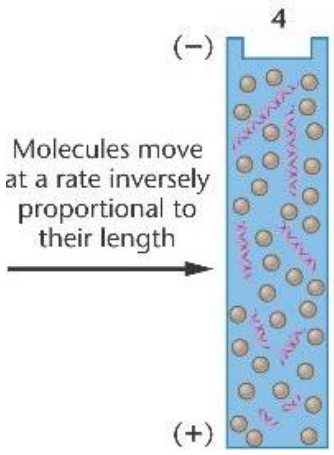


Fig. 3.1.5: Agarose gel acts as molecular sieve to separate DNA fragments based on size

8. After electrophoresis, DNA fragments of the same size and shape are localised in the same region of the gel forming a **band**. However, DNA molecules are not visible to the naked eye. Thus, the gels need to be **stained** for the DNA bands to be seen.

   The electrophoresed gel is submerged in stains such as:
   - **methlyene blue**, a safe to use but low sensitivity dye (cannot detect small amounts of DNA)
   - **ethidium bromide** (EtBr), a higher sensitivity dye but is a carcinogenic chemical which binds to DNA and fluorescence under UV light.

   However, stains such methylene blue and EtBr, stains all DNA present in the gel and may result in a smear if many DNA fragments of similiar length are present in the lane. If only specific DNA fragments are to be visualised, Southern blot and nucleic acid hybridisation can be used instead.

CHECKPOINT 4

What are the principles of Gel Electrophoresis?

What are the different dyes invovled in Gel Electrophoresis and their respective functions?

Dye	**Function**

CHECKPOINT 5

Fig. 5.1 shows the result of a gel electrophoresis.

(a) On Fig. 5.1, label
(i) the cathode and anode.
(ii) the direction of movement of DNA fragments during gel electrophoresis.
(iii) the longest and shortest DNA fragments in lane 1.

Different individuals have different DNA base sequences in our chromosomes thus different number of restriction sites for specific REs. Thus restriction digestion results in different number of DNA fragments of different sizes. DNA banding patterns from restriction digestion from individuals can be unique, like fingerprints, thus known as DNA fingerprint.

(b) Fig. 5.1 shows the results of gel electrophoresis of DNA involved in forensic investigation. Lane 1 contains DNA found at a crime scene. Lanes 2 – 4 contained DNA from Suspect 2, 3 and 4 of the crime investigated.

With reference to Fig. 5.1 state the suspect was at the crime scene. Explain your answer.

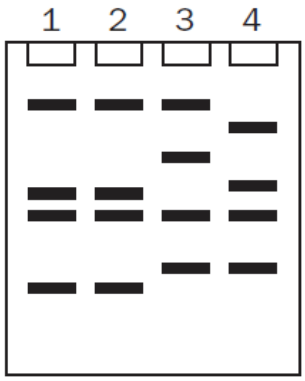


Fig. 5.1

3.2 Polyacrylamide Gel Electrophoresis (PAGE)

- Agarose is frequently used in gel electrophoresis as it is easy to use, relatively cheap, and non-toxic. However, it has relatively **low resolving power** i.e. DNA fragments of very similar sizes cannot be separated in an agarose gel.

- Polyacrylamide, on the other hand, has higher resolution. It is capable of separating DNA fragments differing by **one base pair**. However, polyacrylamide is more difficult to use, relatively more expensive than agarose and polyacrylamide solution is a neurotoxin. Thus, PAGE is usually used to separate DNA molecules for purposes such as DNA sequencing which requires resolution up to 1 base pair while agarose gel electrophoresis is used for most other applications.

- PAGE is also commonly used for separating proteins of different molecular weight.

3.3 Applications of Gel Electrophoresis : Identification of normal / mutant alleles

Gel electrophoresis is used to compare two alleles of the same gene.

- A **restriction enzyme** recognises and cuts DNA molecules at a specific base sequence (restriction site).

- A change in even 1 base pair will prevent the restriction enzymes from cutting that particular restriction site.

- Hence if the base sequence difference between two alleles occurs within a restriction site, cutting with the restriction enzyme that recognises the site will produce a different picture of fragments from each allele. Each mixture will give its own band pattern in gel electrophoresis.

Analysis of the normal and sickle-cell allele of the beta globin gene

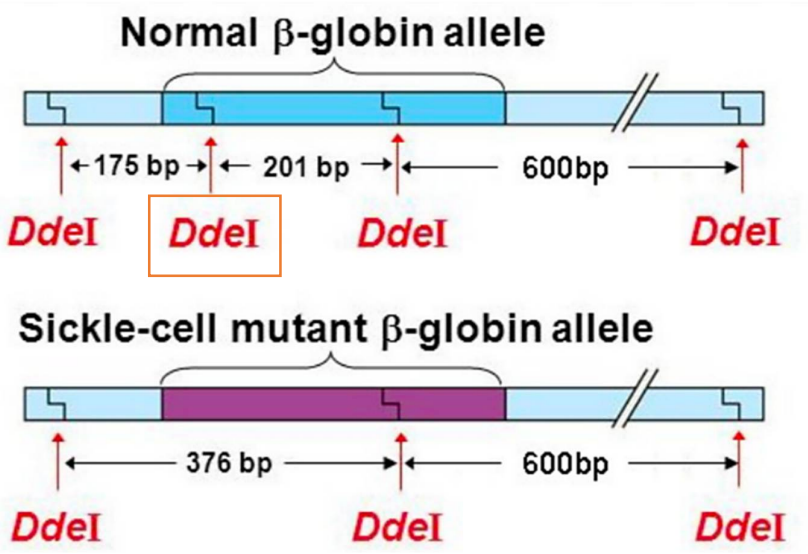


Figure 3.3.1 Gel Electrophoresis and Southern Hybridisation of Nomal and Sickle-Cell DNA

- Sickle cell anaemia is caused by a point mutation (base substitution) within a restriction site in the human β-globin gene. (Recall Topic 2.6 DNA mutations)
- The point mutation destroys one of the Ddel restriction sites at the 5' end of the β-globin gene.
- Gel electrophoresis may be used to distinguish the normal and sickle-cell alleles of the β-globin gene.

To determine if a person is heterozygote for the mutant sickle-cell allele

- Compare the genomic DNA from
    - A person who is homozygous for the normal allele
    - A person who is homozygous for mutant allele

- Note that electrophoresis of genomic DNA cut with a restriction enzyme result in too many bands upon staining with a DNA binding dye.

- Hence there is a need to perform a Southern blot and nucleic acid hybridisation. This involves the use of a radioactive single-stranded DNA probe that is complementary to the β-globin gene.

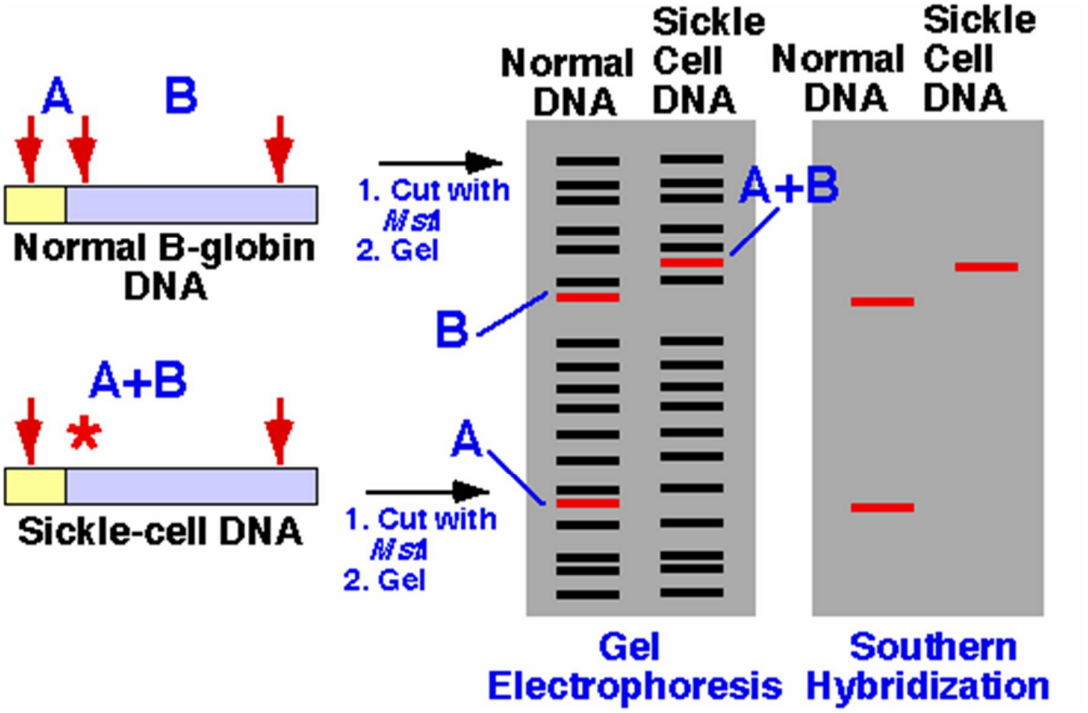


Figure 3.3.2 Gel Electrophoresis and Southern Hybridisation of Nomal and Sickle-Cell DNA

4 VISUALISATION OF DNA

Learning Outcome 2(k)(iii):
Describe the principles and procedure of Southern blotting and nucleic acid hybridisation.

- Staining the electrophoresed gel with methylene blue or EtBr allows us to visualize **all** DNA bands in the gel (as shown in Figure 3.3.2) , which may result in a **smear**, making it difficult to **locate a particular DNA fragment**.

- Southern blotting and nucleic acid hybridization will identify within the smear the size of the particular fragment containing the gene of interest.

  (i) **Southern blotting** is a method of transferring DNA from a gel after electrophoresis to a membrane.

  (ii) After immobilising the DNA onto the membrane, **nucleic acid hybridisation** is performed to detect specific sequences, using a radioactively-labelled probe that binds to the target sequence via hydrogen bonding between complementary base pairs.

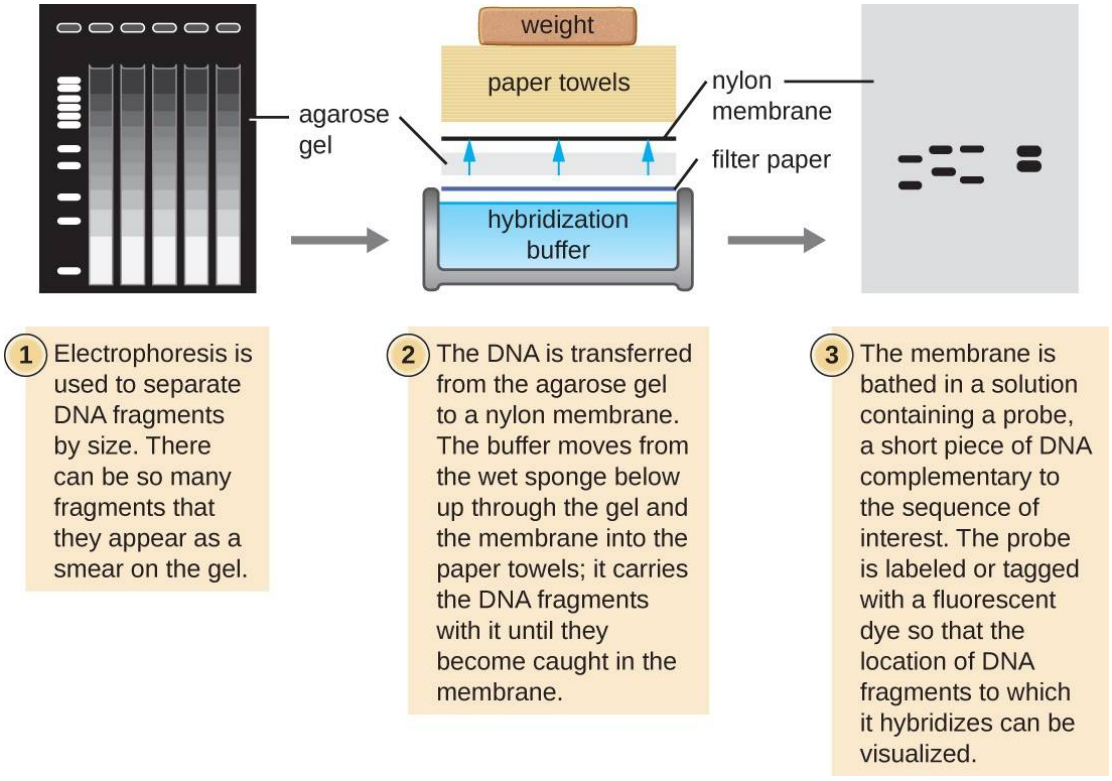


Fig. 4.1: Nucleic acid hybridisation and Southern blotting

4.1 Southern Blotting

Steps:

1. A **nitrocellulose** or nylon membrane / paper is laid over the electrophoresed gel containing separated DNA fragments.

2. The gel is supported on a layer of sponge in a bath of **alkaline** solution.

3. Paper towels are stacked on top of the nitrocellulose membrane to **wick** buffer through the gel and nitrocellulose membrane by **capillary action**.

4. As the buffer passes through the gel, it **denatures** the DNA fragments from dsDNA to ssDNA. It also transfers the ssDNA fragments from the gel to the nitrocellulose membrane.

5. The membrane is then baked in an oven at 80°C for 2 hours or exposed to UV to fix the DNA fragments firmly onto nitrocellulose membrane.

4.2 Nucleic Acid Hybridisation

Steps:

1. The nitrocellulose membrane is peeled off the gel and placed in a sealed plastic bag of solution containing **radioactive probes**.

   Probes are short fragments of nucleic acid with base sequence complementary to the target DNA fragment. One element (e.g. carbon) used to synthesized the probe is radioactive (e.g. ^{13}C), making the probe radioactive.

2. The mixture is incubated to allow radioactive probes to **hybridise** with target DNA sequence via **complementary base pairing** by forming hydrogen bonds.

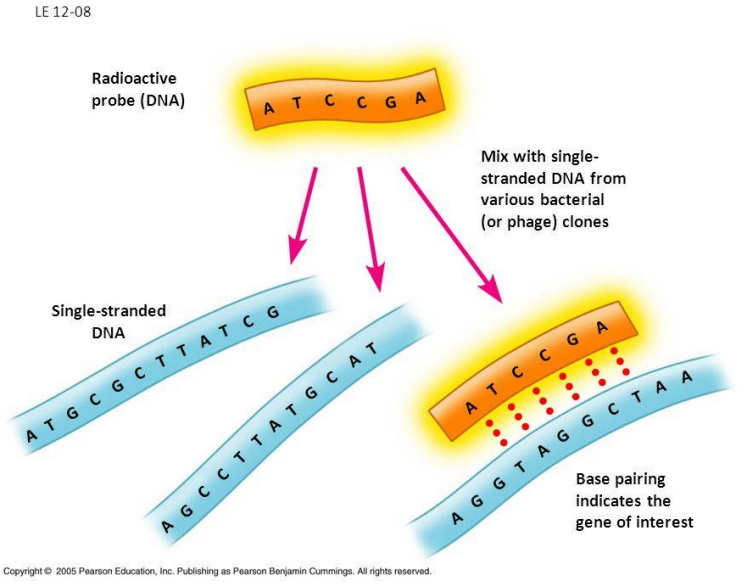


Fig. 4.2.1: Nucleic acid hybridisation

3. The nitrocellulose membrane is removed from the bag. Excess, unbound probes are washed away.

4. The nitrocellulose membrane is subjected to **autoradiography** by exposing it to an X-ray film or **photographic film**. The DNA band that has hybridized to the radioactive probe will show up as **dark bands** on the autoradiograph.

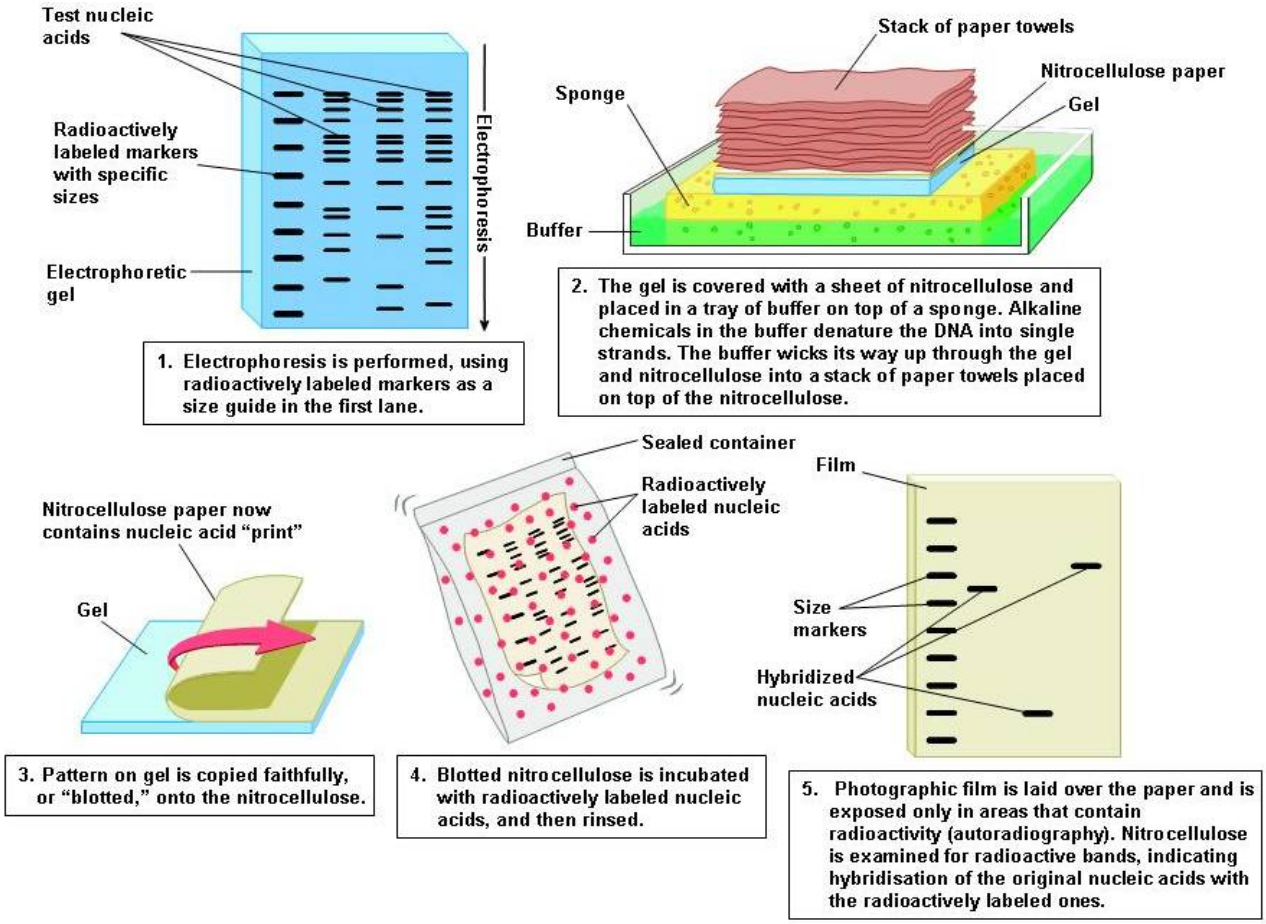


Fig. 4.2.2: Southern blotting and nucleic acid hybridisation

CHECKPOINT 6: Detecting Sickle-Cell Anaemia

- Sickle cell disease is a recessively inherited genetic disorder. Glu is replaced by Val at the 6th position of the β-globin chain of the haemoglobin molecule due to a T → A substitution in the template strand. The mRNA codon GAG, coding for glu, is changed to GUG, coding for val.

- This results in the loss of a *Mst*II restriction site in the sickle cell allele.

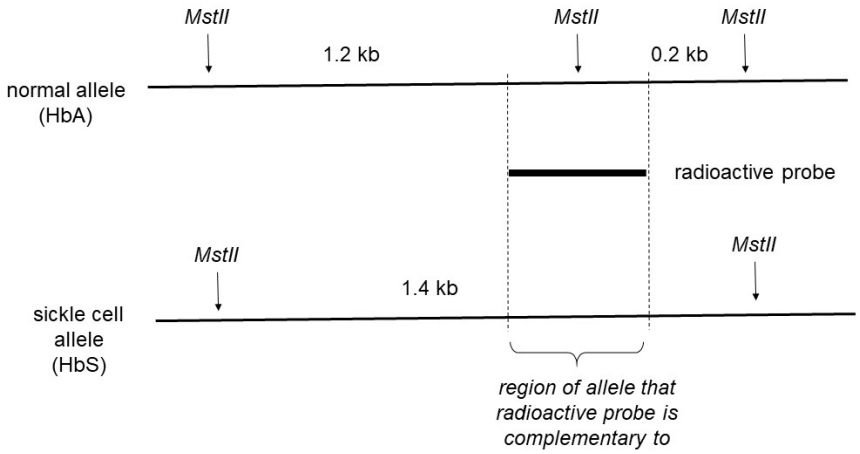
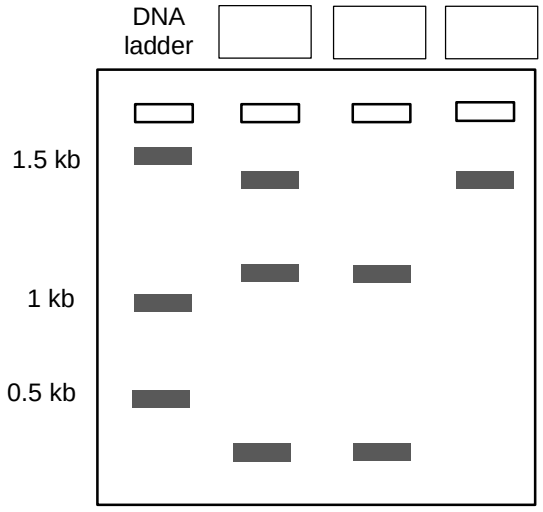


Fig. 6: MstII restriction sites in normal Hb allele and sickle cell allele

- Restriction digestion of the normal (Hb^A) and sickle cell (Hb^S) allele results in restriction fragments of different lengths.

- Separation of DNA fragments suing gel electrpohoresis and subsequent visualisation using nucleic acid hybrdisation following Southern Blotting results in an autoradiograh with different banding patterns that allows identifcation of the individual's genotype.

  Identify the genotypes shown by the Southern Blotting results below



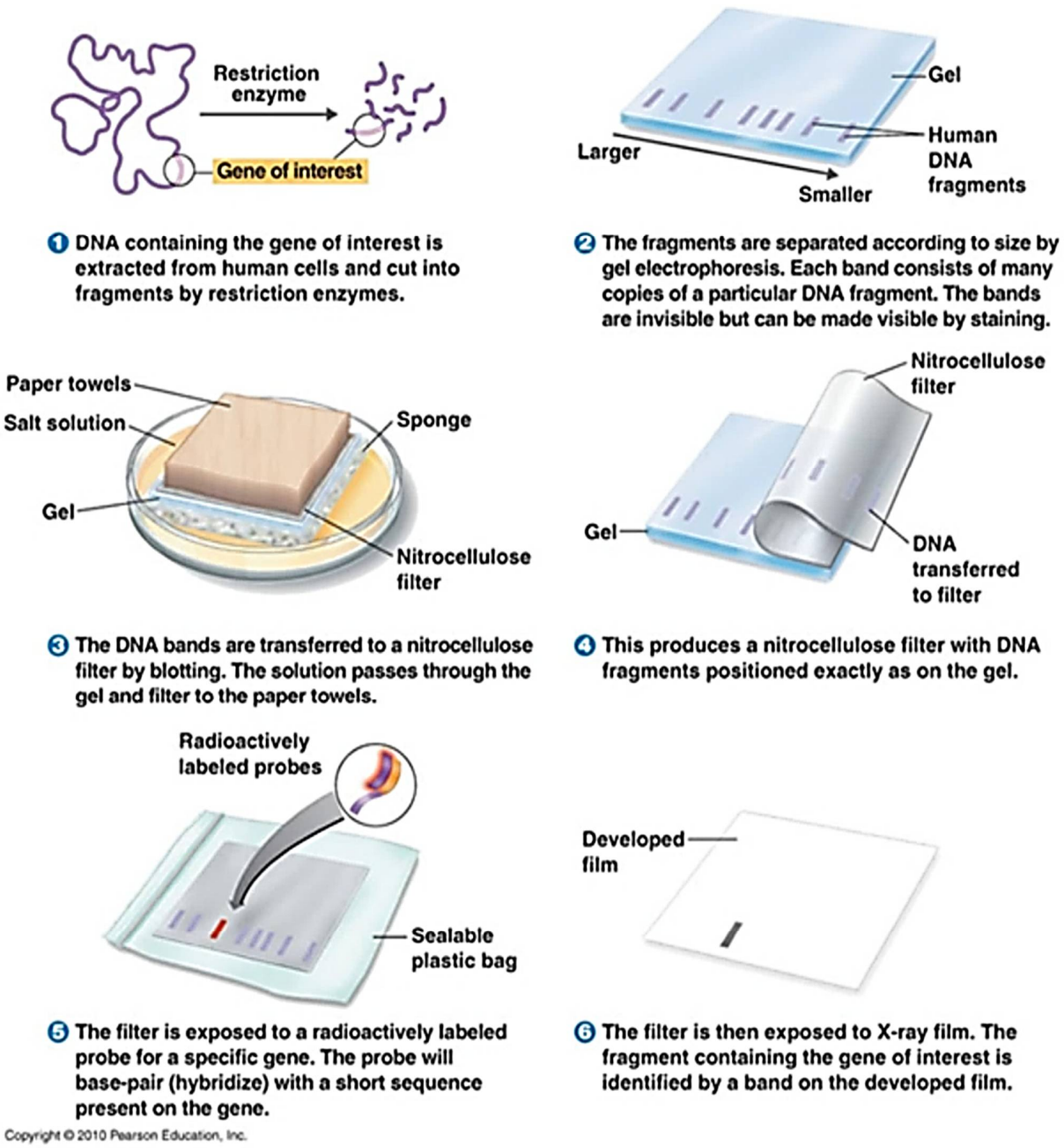


Fig. 4.2.3: Detection of sickle cell allele