ICS 2

Polymerase Chain Reaction - Amplifies DNA from a limited source of DNA so that there is sufficient for analysis
Protocol

1. Design primers that are complementary to the 3' end of the regions flanking the target DNA sequence
2. (**Denaturation**) Heat to 95°C to denature the dsDNA double helix into ssDNA by breaking hydrogen bonds between complementary bases due to increased molecular vibrations
3. (**Primer Annealing**) Cool to 64°C for **DNA primers** to anneal specifically to the 3' ends of the single stranded target DNA via complementary base pairing
4. (**Extension**) Heat to 72°C, which is the optimum temperature for **Taq polymerase** to extend the free 3' OH end of DNA primer to synthesise complementary DNA strand
5. Repeat the cycle 20 times

- Each cycle results in the doubling of the number of DNA molecules being replicated ⇒ n cycles yields 2^n molecules of target DNA
- Usually 20-25 cycles are run

Components of PCR mixture

- 2 different synthetic single stranded primers that are complementary to the 3' end of the target DNA sequence on both DNA strands are required in large excess
- Template DNA
- Taq polymerase
- dNTPs
- Buffer containing cofactor Mg^{2+} for proper polymerase function

Advantages:

- Only a small amount of source DNA is required as amount of desired sequence increases exponentially with each round of PCR
- DNA can be amplified very quickly as Taq polymerase is **thermostable** ⇒ Can be automated

Disadvantages:

- Taq polymerase lacks 3' to 5' proofreading ability ⇒ Errors that occur early in the PCR

reaction will get compounded with each replication cycle
- Requires knowledge of the sequences flanking the target region in order to design the DNA primers
- Length of DNA fragments to be amplified is limited to about 3kb as Taq polymerase tends to fall off the DNA template before the chain extension is complete if the strand is too long
- Small amounts of contaminant DNA may be amplified to significant amounts alongside the target DNA sequences

How is ability of Taq polymerase to function at high temperatures an advantage in PCR?

1. dsDNA is heated to high temperatures of about 95°C in the first step of PCR to break hydrogen bonds and denature the DNA
2. Taq polymerase is not denatured at this high temperature as it is thermostable ⇒ First step can proceed
3. Can be reused after every cycle of PCR is completed ⇒ PCR can be automated
4. Ability of Taq polymerase to withstand high temperatures also allows primer extension to occur rapidly and so DNA amplification can occur within a relatively shorter time

Agarose Gel Electrophoresis - Separates DNA based on size
Protocol

1. Dense loading buffer mixed with DNA sample to help it sink to the bottom of the well
    - Loading buffer = Glycerol (denser than buffer) + 2 coloured dyes
2. Loading dyes indicates that the DNA has been loaded correctly into the well + acts as visual markers to allow visualisation of separation process
    - 1 dye corresponds to smaller fragment to show when electrophoresis should be stopped so that sample does not run out of the gel
    - Other dye corresponds to larger fragment to give indication of position of larger fragments on the gel
    - Dyes do NOT bind to DNA fragments
3. Fragments of DNA pipetted into wells at the top of the agarose gel furthest from the positive electrode
4. DNA ladder containing DNA fragments of known sizes is added to act as a standard to compare to
5. Negatively charged DNA migrates out of the well towards direction of positive electrode when subjected to electric field
6. Fragments migrate through the agarose gel matrix made up of a meshwork of polymer fibres
7. Meshwork impedes the movement of longer fragments more than shorter fragments ⇒ Longer fragments migrate slower than shorter fragments
8. Stain the gel with DNA binding dye ethidium bromide which will fluoresce when viewed under UV light, hence allowing the banding pattern to be visualised

Role of buffer solution

- Contains ions which allows for conduction of electric current
- Thus allowing negatively charged DNA molecules to move from the -ve electrode to the +ve electrode

Southern Blotting - Detect specific nucleotide sequences
Protocol

1. After gel electrophoresis, DNA on agarose gel is pressed against a nitrocellulose membrane to transfer the DNA to the membrane
2. Stack of paper towels placed on top of the membrane
3. Placed in a tray with **NaOH** to denature double stranded DNA to single stranded DNA using alkaline solution
4. Paper towels draw solution towards themselves, drawing the ssDNA upwards onto the nitrocellulose membrane causing them to bind to membrane
5. Incubate membrane with a single stranded radioactive DNA probe which will **hybridise** with the DNA fragment through **complementary base pairing**
6. Wash off excess probe
7. Using **autoradiography** where an X-Ray film is placed over the membrane and radioactive probes cause X-ray film to blacken, the banding pattern can be visualised

Restriction Fragment Length Polymorphism - Unique banding pattern among individuals when genomic DNA is digested by restriction enzymes and separated by gel electrophoresis

- Different alleles produce different bands in the agarose gel resulting in unique banding pattern in individuals
- Arise due to DNA polymorphisms (small nucleotide differences) in different individuals at specific locus ⇒ Variation in number/location of restriction sites or variation in number of tandemly repeated nucleotide sequences ⇒ Unique banding pattern

Function:

- Detection of diseases
- DNA fingerprinting in forensics/paternity testing
- Facilitate linkage mapping

Detection of genetic disease through SNPs

- **Single nucleotide polymorphism** = Difference in a single base pair due to point mutation
- Usually found in non-coding region although for sickle cell anaemia, found in coding region

- Results in **loss/gain of restriction site** ⇒ DNA fragments of different lengths produced when digested with same restriction enzyme ⇒ Analyse banding pattern to determine presence of disease-causing allele/normal allele

Procedure:

1. Digest genomic DNA using same restriction enzyme
2. Gel electrophoresis
3. Southern blotting
4. Analyse banding pattern

e.g. Sickle Cell Anaemia

- Sickle cell anaemia is an autosomal recessive genetic disease caused by a substitution mutation in DNA coding for beta-globin chain of haemoglobin where T is substituted for A
- Mutation is located within a restriction site for **MstII**
    - Disease-causing allele → Loss of MstII restriction site
    - Normal allele → Retained MstII restriction site
- Disease-causing HbS allele → 1 large fragment ⇒ Move a shorter difference
- Normal HbA allele → 2 smaller fragments
- 1 large band ⇒ 2 HbS alleles ⇒ sufferer;
- 1 intermediate band + 1 small band ⇒ 2 HbA alleles ⇒ Normal individual
- 1 large + 1 intermediate + 1 small band ⇒ Heterozygote ⇒ Normal individual but carrier

Detection of genetic disease through STRs

- Short tandem repeats = Repeating sequences of DNA → No. of repeats can differ + May be tightly linked with a disease causing gene locus
- Results in different length of DNA fragments when cleaved before and after the tandem repeats

Procedure:

1. Digest genomic DNA using same restriction enzyme
2. Separate restriction fragments using gel electrophoresis
3. Southern blotting where dsDNA is made single stranded by denaturing using NaOH and transferring to nitrocellulose membrane for nucleic acid hybridisation using a radioactively labelled ssDNA probe that is complementary to beta-globin gene at the restriction site
4. Analyse banding pattern

- 1 large band ⇒ Homozygous for gene linked to longer STR
- 1 large band, 1 short band ⇒ Heterozygous
- 1 short band ⇒ Homozygous for gene linked to shorter STR

DNA fingerprinting protocol

- DNA profile = Restriction banding pattern that identifies individuals
- Use probes for STRs
- At a particular RFLP locus, different individuals will have different number of STR repeats ⇒ Different fragment lengths → 1 allele inherited from each parents
- The more RFLP loci used, the more unique the fingerprint

Explain why RFLP allele must be tightly linked to the gene locus of the disease allele

- So that RFLP allele and disease allele will have a higher chance of being inherited together
- OR Minimise chance of crossing over between disease allele and marker allele so that linkage will not be disrupted

How genetic fingerprint can be used to determine how closely related 2 individuals are:

1. Different alleles produce different bands in the agarose gel resulting in unique banding pattern in individuals
2. Different bands arise due to **polymorphic** nature of DNA in different individuals, there will be variations in number and location of restriction sites and number of tandemly repeated nucleotide sequences among individuals
3. Genetic fingerprint of one individual can be compared against that of another to see how closely related they are
4. The more similar the fingerprint patterns, the more closely related the 2 individuals

Recombination frequency = no. of recombinant progeny/total no. of progeny x 100% = Distance between the 2 loci in centiMorgans
Chromosome map = Diagram showing position where genes occur along a chromosome and their relative distance apart

How RFLP can be used to construct a chromosome map

- Identify genes that can be detected as RFLPs
- Parents with known genotypes crossed to find recombination frequency in offspring
- The greater the percentage of recombinants, the greater the distance between genes
- 1% = 1cM
- Recombination frequency = no. of recombinant progeny/total no. of progeny x 100%

Limitations of RFLP in disease detection

- More than one mutation may cause a disease → Negative test for 1 point mutation does not necessarily rule out presence of mutations at other loci which may also cause the disease
- Crossing over between adjacent RFLP marker and disease causing allele may occur → ↓ reliability in the case of indirect detection
- Disease could be caused by multiple gene interactions
- Gene causing the disease and its nucleotide sequence must be known for molecular tests to be available