Genetics and Inheritance – Molecular Techniques

1. Introduction

Molecular biology techniques are common methods used in molecular biology, biochemistry, genetics and biophysics which generally involve manipulation and analysis of DNA, RNA, proteins, and lipids. Since around 1960, molecular biologists have developed methods to identify, isolate, and manipulate molecular components in cells including DNA, RNA, and proteins. One important tool used in molecular biology techniques especially in molecular cloning is the restriction enzyme. These enzymes are found in bacteria and provide a defense mechanism against invading viruses. It is an enzyme that cuts DNA at or near specific recognition nucleotide sequences known as restriction sites.

Several techniques used in the field of molecular biology include:

- Polymerase chain reaction (PCR)
- Gel electrophoresis
- Macromolecule blotting and probing e.g. Southern blotting, Northern blotting, Western blotting and Eastern blotting
- Expression cloning

2. Learning Outcomes

(k) describe the principles and procedures of these molecular techniques:

i. polymerase chain reaction (including its advantages and limitations)
ii. gel electrophoresis
iii. Southern blotting and nucleic acid hybridisation

Use the knowledge gained in this section in new situations or to solve related problems.

3. References

i. T.A.Brown (2010) Gene cloning & DNA Analysis, 6th Edition, Wiley-Blackwell. Chapter 1 to 7, 9.
ii. Watson, Mayers, Caudy, Witkowski (2007), Recombinant DNA, Genes and Genomes – A short Course. Cold Spring, Harbor Lab Press. Chapter 4
iii. Campbell and Reece (2011) Biology, 9th Edition, Pearson Education. Chapter 20.
iv. Turner, McLennan, Bates and White (2001) Molecular biology, 2nd Edition, BIOS. Sections G, H, I and J.
v. Green, Stout and Taylor (1997), Biological Science Volumes 1 and 2, 3rd Edition.
vi. Hoh Yin Kiong (2003), Longman A-Level Course in Biology, Core Syllabus Volume 1 (for AS and A-Level)
vii. Weaver (2008), Molecular Biology(4th ed.). McGraw-Hill.
viii. http://www.dnalc.org/resources/spotlight/index.html (Cold Spring Harbor Laboratory DNA Learning Centre. Look for Polymerase Chain Reaction).

Contents

4. Molecular Biology Techniques

Notes to self

A. Polymerase Chain Reaction

What is PCR?

Polymerase Chain Reaction (PCR) is a technique that allows **amplification** (making many copies) of a specified segment of DNA *in vitro* (in a test tube).

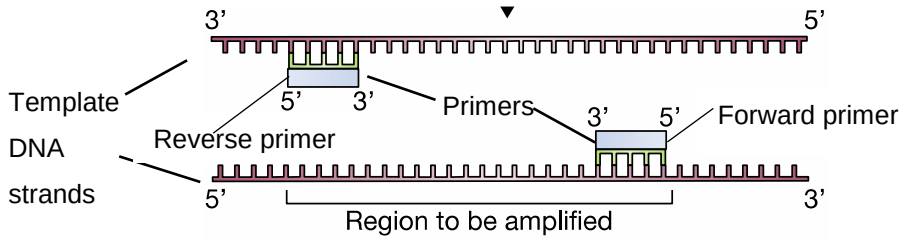
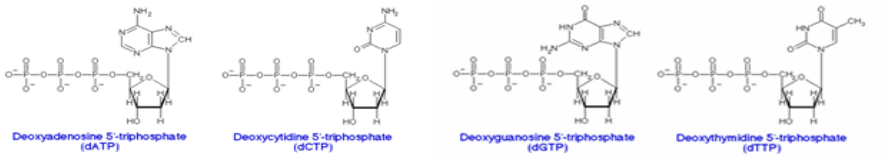
- Amplifying DNA through PCR has extensive applications such as in basic research, human genetics testing and forensics.

Until the mid-1980s, the only way to make many copies of DNA was to insert the DNA pieces into bacteria and select the desired one from many different colonies growing on a plate (this takes several weeks!). In 1985, **Kary Mullis** (shown right) invented a precise and radical new method of selecting and amplifying a section of DNA – the PCR which takes less than a few hours to complete. The revelation came to this eccentric character and avid surfer on a drive in northern California with his girlfriend. Kary Mullis was a recipient of the 1993 Nobel Prize in Chemistry. His award has drawn controversy in that he was not the first to describe nor 'invent' the processes used in PCR but others have defended that it was Mullis who first put the processes together into a system for molecular biology.	

Prior Knowledge Required

1. Structure of DNA:
   - DNA strands unzip and separate at high temperature as **hydrogen bonds** between the complementary base pairs are broken (**denature**).
   - DNA strands **anneal** through formation of hydrogen bonds between **complementary base-pairs** upon cooling.
2. DNA replication:
   - **Primers** are needed during DNA replication *in vitro* as DNA polymerase needs an existing **free 3' hydroxyl group** for chain extension to take place.

<u>PCR reaction mixture</u> contains the following reagents/components:

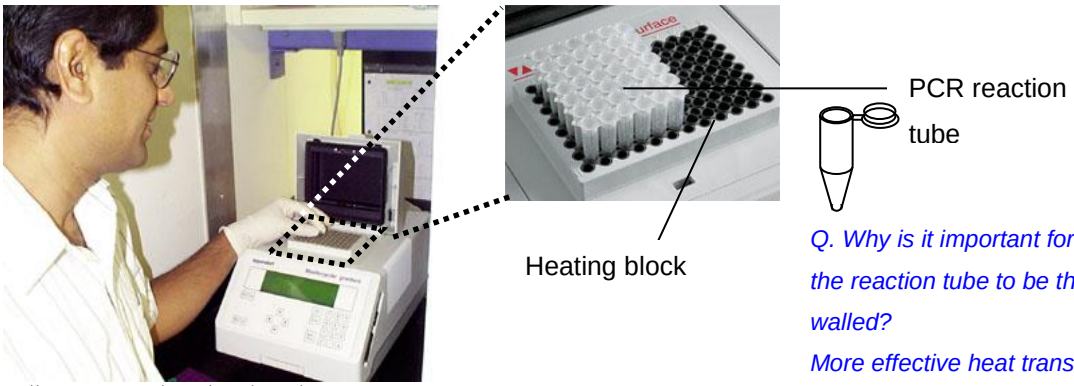
PCR component	Details
Template DNA	▪ DNA containing the segment or sequence of bases to be amplified. This can usually be genomic DNA, plasmid, or fragmented DNA.
Oligonucleotide primers **(oligo = short)**	▪ Synthetic **single-stranded DNA** typically 20-30 nucleotides long. ▪ Needed to **initiate DNA synthesis.** ▪ The primers (**forward** and **reverse** primers) are complementary to sequences at the 3' ends of the target DNA sequence to be amplified. ▪ Primers serve to: ➢ mark out the section of DNA to be amplified by attaching to complementary bases on the 3' ends of the target DNA sequence; • provide a free 3'-OH end for DNA polymerase to add DNA nucleotides onto to elongate a new strand of DNA. ▪  ▪ Knowledge of the **sequences flanking / at the ends of** the DNA segment of interest is thus crucial to artificially synthesise the primers. ▪ Primers are present in **large excess** in reaction mixture to increase likelihood of primers binding to target DNA (decrease likelihood of template DNA strands reannealing to each other again). ▪ The primers become part of the amplified segments.
Taq polymerase	▪ A thermostable (resistant to denaturation at high temperatures) **DNA polymerase** isolated from a thermophilic prokaryote living in hot springs, *Thermus aquaticus* (*Taq*). ▪ Enzyme is stable at 95°C and works optimally at 72°C. ▪ It binds to the primer at the 3' end. ▪ Using the DNA template, *Taq* polymerase catalyses the formation of a phosphodiester bond between the adjacent nucleotides, synthesising new DNA strands via complementary base-pairing in the 5' to 3' direction.
Deoxyribonucleoside triphosphates (dNTPs)	▪ dATP, dTTP, dCTP and dGTP are substrates for DNA replication. 

Buffer containing Mg^{2+}	▪ Mg^{2+} is a cofactor[1] for proper polymerase function.

PCR Procedure

Protocol:

1. Place all the components into a PCR tube~~,~~ and place the tube into the thermocycler (Fig. 1). Run a standard programme that involves heating the tube to different temperatures for different periods of time. Entire process is now fully automated.



http://lamar.colostate.edu/~reddy/assets/labtour/pcrvaka.JPG

Figure 1: The thermocycler has a heating block (inset) whose temperature and duration of heating can be programmed precisely. The many wells in the heating block enable several reactions to run simultaneously.

2. Steps during PCR:

The following three steps constitute **1 cycle**. The cycle is then **repeated** (Figs. 2, 3).

Step	Details
1. Denaturation	• A brief heat treatment (up to **95°C**) to **denature**/unzip and separate the two strands of DNA double helix. This exposes the bases for complementary base pairing required in steps 2 and 3.
2. Primer annealing	• Cooling of the DNA (e.g. **65°C**) in presence of a **large excess of DNA primers** allows their specific **annealing** to complementary sequences at the 3' ends of each of the template DNA strand. • Without an excess of primers, it is likely that the separated DNA strands will re-anneal back to a double-stranded form.
3. Extension	• *Taq* polymerase **synthesises** the complementary DNA strand by catalysing the formation of phosphodiester bonds between dNTPs at an optimum **72°C**. • Chain extension occurs from 3' end of DNA primer which provides free 3' OH group required by *Taq* polymerase. When the above elongation is completed, the primer has been lengthened into a new complementary strand of the single-stranded DNA fragment. • Because both separated DNA strands are used as templates, there are now two copies of the original fragment (one molecule of DNA becomes two).

[1] Cofactors are additional non-protein substances required by some enzymes for catalytic activity. Cofactors can be 1) inorganic ions that are metal ions such as Ca^{2+}, Mg^{2+}; 2) Organic coenzymes such as NAD or 3) prosthetic groups such as haem in cytochrome oxidase or haemoglobin.

3. Each cycle results in a **doubling in number of DNA** molecules being replicated. *n* cycles will yield **2^n molecules** of target DNA. The amount of desired sequence hence **increases exponentially** after multiple cycles.
   Usually 25-30 cycles are run, resulting in eventual synthesis of millions of copies of the desired fragment.

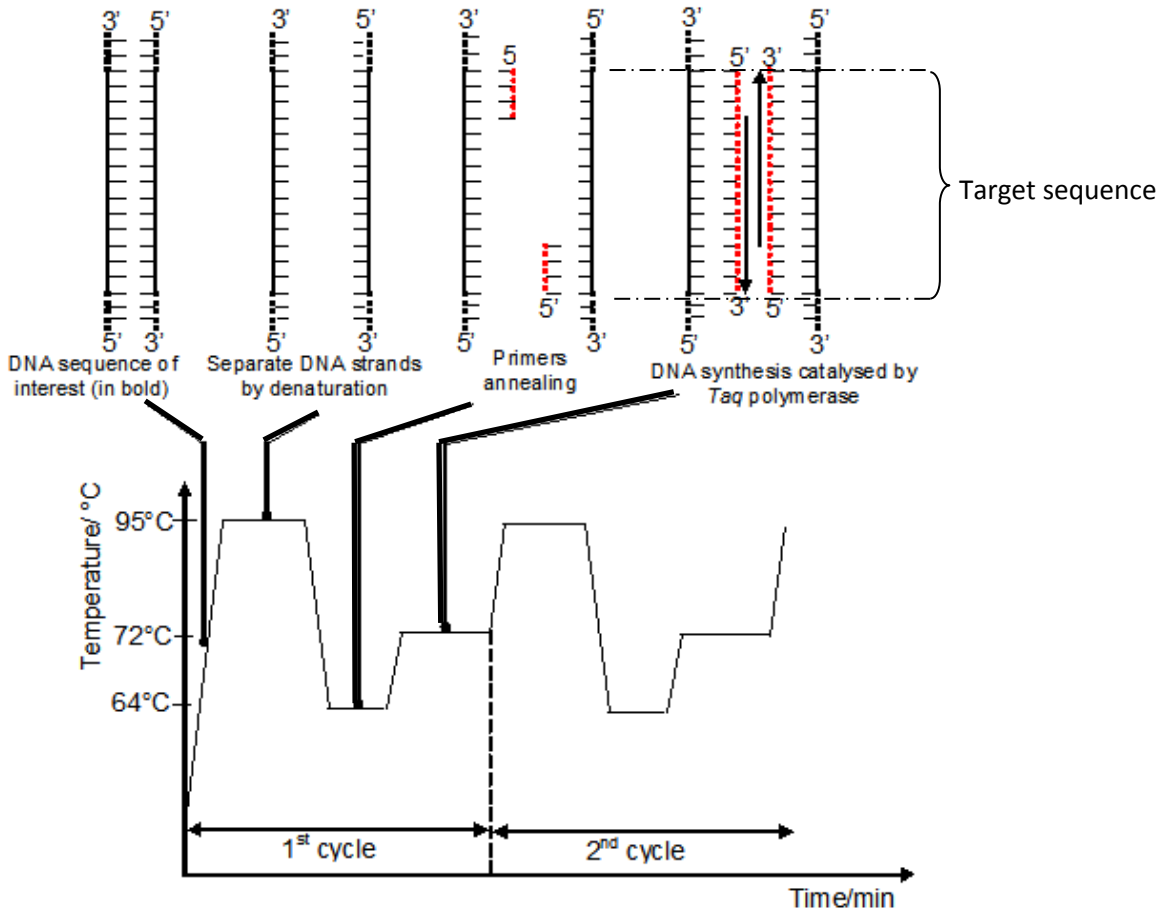


Figure 2: Principle behind Polymerase Chain Reaction (PCR)

Q: Why is it usually recommended to run about 25 to 30 cycles for PCR?

Fewer cycles – not enough DNA for analysis

Too many cycles – too many mistakes in replication, due to lack of proof-reading capability in this reaction

Q: Why is the discovery of Taq polymerase a breakthrough? What would you do if you have only normal DNA polymerase?

When first developed, PCR was slow and laborious because a crucial enzyme for the process, DNA polymerase, was denatured at high temperature and had to be replaced after each cycle. However, the introduction of the thermostable enzyme, Taq DNA polymerase revolutionized PCR so that the technique could be fully automated.

Other questions you might want to ask:

Q: Does PCR only target genes?

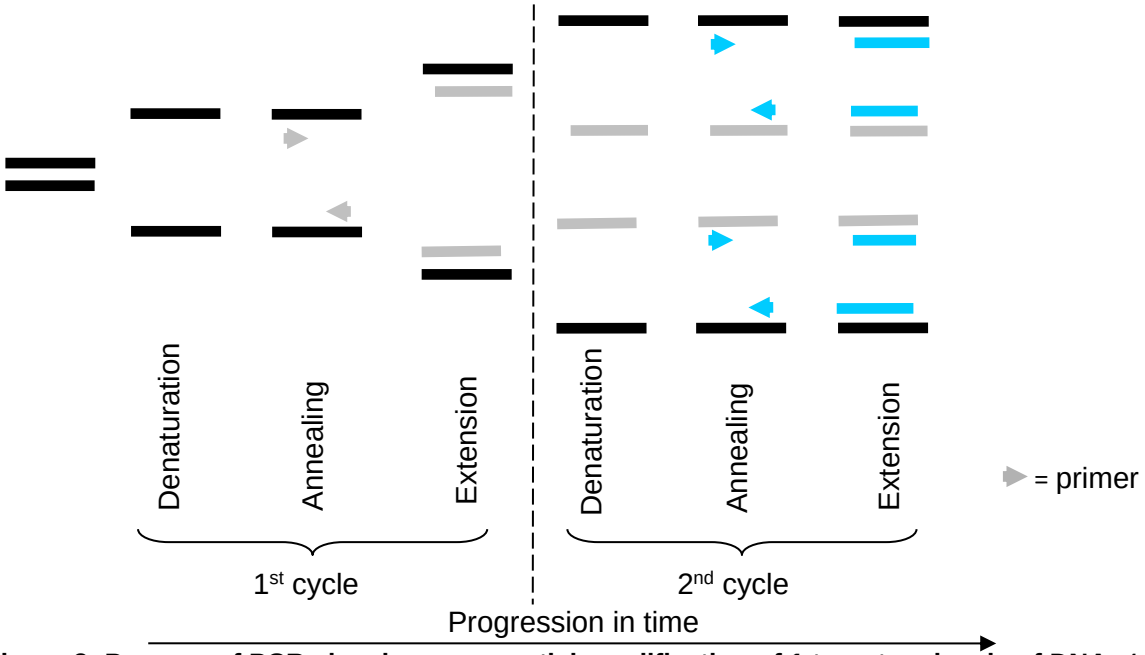


Figure 3: Process of PCR showing exponential amplification of 1 target molecule of DNA. 4 copies of target DNA molecules are produced at the end of 2 PCR cycles.

Number of copies of target DNA $= 2^n$

(n = number of cycles)

VIDEO LINK on polymerase chain reaction: http://tinyurl.com/pg98dat

VIDEO LINK on PCR (amplicons that match target sequences): http://tinyurl.com/y87dlmp

Concept check

1. Write down the nucleotide sequence of the primers you could use to amplify the target DNA sequence. Place them at the appropriate end of the two strands.

|----------TARGET DNA SEQUENCE----------|

5' CACGGC**TAAGATCTGAATTTTCCGAG……TTGGGCAATAATGTAGCGCC**TTAAT 3'

3' GTGCCG**ATTCTAGACTTAAAAGGCTC……AACCCGTTATTACATCGCGG**AATTA 5'

2. How many DNA molecules will have been produced from one molecule of DNA after 8 complete cycles?

3. How is the ability of *Taq* DNA polymerase functioning at high temperature an advantage in the polymerase chain reaction (PCR)?

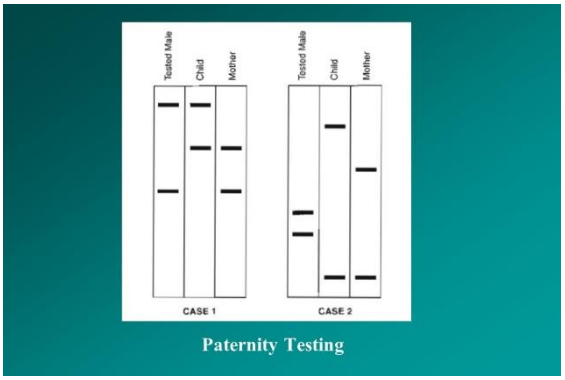
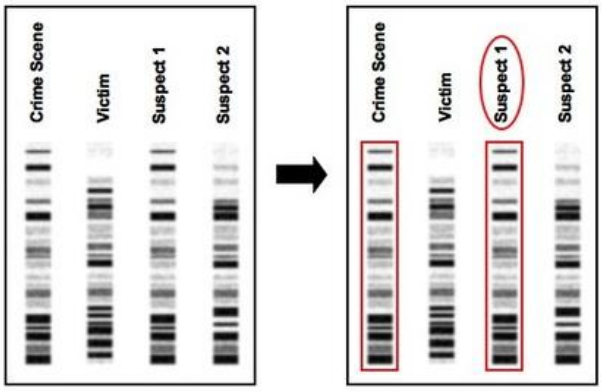
Advantages of PCR

Notes to self

As an analytical tool, PCR has several advantages.

Feature	Advantages
High sensitivity	PCR is capable of amplifying sequences from minute amounts of target DNA. This is especially useful in forensic work where DNA may not be present in high quantity.
Speed	Millions of copies of target DNA can be obtained in a relatively short amount of time i.e. a few hours. Target DNA is doubled after every round of PCR (i.e. No. of DNA molecules obtained = $\mathbf{2^n}$ where n = number of cycles), leading to exponential increase of target DNA after 25-30 cycles of PCR.
Specificity	As primers complementary base pairs via hydrogen bonds only to complementary sequences, PCR is a specific process which amplifies only target sequences.
Robustness	PCR can permit amplification of specific sequences from material in which the DNA is badly degraded or embedded in a medium (e.g. DNA from formalin-fixed tissue samples or from archaeological remains).
Automation	The use of *Taq* polymerase which is able to withstand high temperatures without being denatured allows the technique to be fully automated. There is no need to replace the enzyme after every cycle.
Ease of use	It is easy to set up and use a thermal cycler to carry out PCR. Computer software for primer design are easily available.

Applications of PCR

Field	Use	Details
Clinical diagnosis	Pre-natal screening for certain genetic diseases	Fetal cells can be removed from amniotic fluid and DNA obtained from the cells is subjected to PCR even before the phenotype is expressed. A gene of interest can be amplified through PCR and subsequently sequenced to detect disease-causing mutation. Such genetic screening enables couples to assess risks of their unborn children, e.g. cystic fibrosis.
	Early detection of infection	PCR can detect presence of HIV genome within a patient at very early stages of infection, before symptoms appear, when only a few thousand blood cells in a patient are infected with the virus. This provides physicians a head-start in treatment of the disease.
Paternity Testing	Determines biological father of a child	DNA samples will be painlessly collected through buccal swabs from the mother, child, and alleged father. Then, the samples will be replicated through PCR and compared for similarities. Because half of the child's DNA is inherited from the mother and the other half from the father, the child's DNA should match portions of both biological parents. 
Forensic analysis	Identification of suspects	Minute traces of DNA in organic matter e.g. hair, semen, blood, can be amplified to sufficiently large amounts to be analysed. This helps to identify suspects by matching their DNA with DNA found at crime scenes. 
Evolution	Study evolutionary relatedness	Fragments of DNA found in prehistoric specimens can be amplified for further study. DNA sequences from both living (extant) and extinct organims can be compared to determine evolutionary relationships.

Limitations of PCR

Feature	Limitations
Taq polymerase **lacks 3' to 5' proofreading ability**	*Taq* polymerase cannot check and/or remove an incorrectly incorporated base. **Errors** occurring early in the PCR reaction will get compounded with each replication cycle and all daughter molecules resulting from this early error will be exponentially affected.
Synthesis of PCR primers depends on **sequence information** from target region	Success of PCR **requires knowledge of sequences** flanking target region to be amplified. If the flanking sequences of a gene of interest are unknown, no proper primers can be synthesised to amplify the target DNA sequence. If primers are designed incorrectly, no amplification occurs / wrong DNA fragment(s) may be amplified.
Limit to size of DNA fragment to be amplified	DNA fragments to be amplified are **limited to about 3 kb[2].** Further increase in length of target sequence decreases efficiency of amplification. This is because the polymerase tends to 'fall off' DNA template before chain extension is complete.
Exponential amplification of **contaminant DNA**	It is possible to contaminate a fresh PCR reaction with **minute amounts** of contaminant DNA due to poor laboratory skills. Such unwanted DNA sequences may be amplified to significant amounts, alongside the target DNA sequences.

Q: Why is proof-reading activity from 3' to 5' and not 5' to 3'?

It takes place only after the nucleotide has been added in the 3' end of the extending chain. Correction (removal of wrongly-incorporated nucleotide) can take place in the reverse orientation.

Q: Which is worse? An error occurring earlier or later during PCR?

Early. Errors occurring early will get compounded with each replication cycle and all daughter molecules resulting from this early error will be affected.

Q: Is it possible to amplify an entire eukaryote or even prokaryotic genome using PCR?

No. Genomic DNA is too large to be amplified entirely by PCR.

[2] Kb or **kilobase** is a measurement of the length of a DNA/RNA molecule. 1kb is the length of 1,000 nucleotides.

B. Gel Electrophoresis

What is gel electrophoresis?

Gel electrophoresis is a technique that separates DNA, RNA or proteins according to molecular size.

Agarose gel electrophoresis

Agarose gel electrophoresis separates DNA based on size.

Principle (Fig. 4)**:**

1. Electrophoresis is the movement of charged molecules in an electric field.
2. Biological molecules such as DNA, RNA and proteins exist in solution as electrically-charged particles at a given pH. DNA, for example, is **negatively-charged** due to phosphate groups of sugar-phosphate backbone.
3. When placed in an electric field, DNA molecules which are **negatively-charged** will move away from the negative electrode (cathode) and **towards the positive electrode (anode)**.
4. **Agarose** is a polysaccharide derived from seaweed that forms a gel when heated and dissolved in aqueous solution.
5. Meshwork of polymer fibers that makes up agarose gel impedes movement of the DNA fragments, affecting the longer fragments more than shorter ones. Therefore, **shorter DNA fragments will move towards the positive electrode at a higher rate** than longer fragments.
6. DNA sample separates out into fragments of different sizes, based on their different rates of migration.

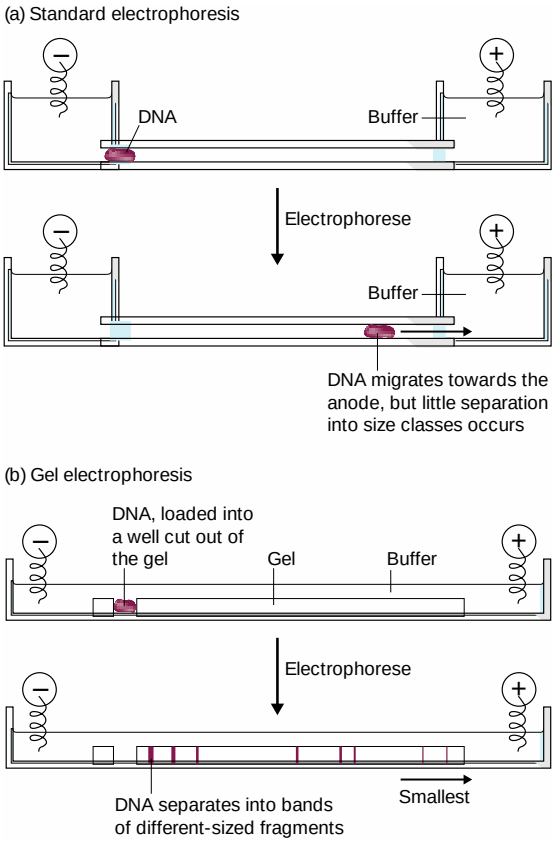


Figure 4: Principles of agarose gel electophoresis. (a) When placed in a electric field, negatively-charged DNA molecules move towards the anode. (b) The meshwork of polymer fibers of the agarose gel impedes this movement, affecting larger fragments more than shorter ones. This results in the separation of DNA fragments based on size.

Protocol:

1. A slab of agarose gel is placed in a buffer solution which allows conduction of electricity to generate an electric field (Figs. 4, 5). The gel has been pre-cast with little indentations called **wells** at one end of the gel slab, using an apparatus called a comb. DNA samples are loaded into these wells. Each well corresponds to one lane.
2. Prior to loading, the DNA sample is first mixed with a **dense loading buffer** which helps the DNA sink to the bottom of the well. The loading buffer also contains a loading dye which will travel along the gel with DNA but doesn't bind to it. (*The loading dye DOES NOT make DNA visible to the naked eye)*.
3. As DNA is invisible to the naked eye, loading dyes help to monitor progress of electrophoresis (Fig. 6).

Q: If I was running a gel to separate DNA fragments of different s, when do I stop electrophoresis?
Stop it before the dark blue band runs out of the gel.

Figure 5: Principles of gel electrophoresis

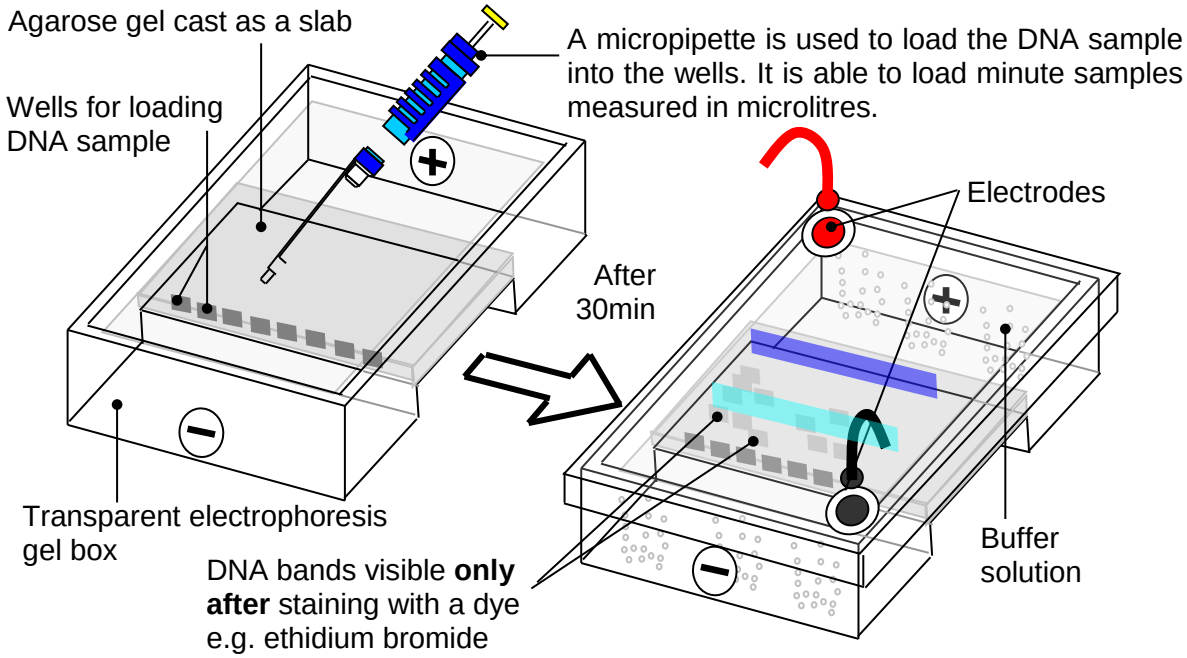
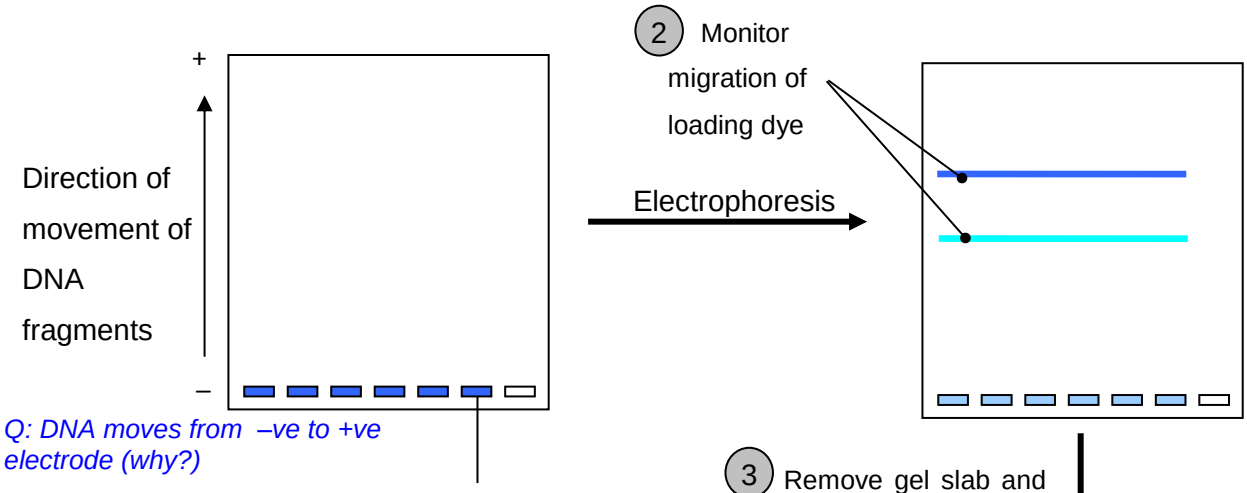


Figure 6: Patterns on gel slab during and after electrophoresis



4. Usually, **markers/ DNA ladder** are run in one or two lanes. These are prepared mixtures of DNA fragments of **known sizes**, which will form the basis of comparison for the unknown fragments in your sample. These are normally loaded into the wells at either extreme lanes.
5. When the current is turned on, the negatively-charged DNA fragments migrate out of the well into the agarose. DNA is **attracted towards the positive electrode (anode)** due to negative charges on its sugar phosphate backbone.
6. Meshwork of polymer fibers that makes up agarose gel impedes movement of the DNA fragments, affecting the longer fragments more than shorter ones. Therefore, **shorter DNA fragments will move towards the positive electrode at a higher rate** than longer fragments.
7. DNA sample separates out into fragments of different sizes, based on their different rates of migration. If the amount of the fragments is high enough, these are seen as discrete **bands** on the gel after staining. Each band consists of DNA molecules of the same length.
8. Before loading dye reaches end of the gel, the current is turned off.
9. To visualise the bands, gel slab is next stained with a DNA-binding dye, usually **ethidium bromide** (a carcinogen). When the gel is placed under UV light afterwards, DNA bands will be revealed as the dye bound to the DNA fluoresces.
10. It is possible to alter resolution of the gel used by varying concentration of agarose used. The higher the concentration, the better the resolution i.e. even very small differences in fragment size can be effectively separated.

Q: Can you account for the higher resolution?
Higher concentration → tighter meshwork → finer pores → can separate fragments with small differences in size.

11. Electrophoresis help estimate:
    (a) **fragment size** (by comparing position of the band relative to bands of the DNA ladder)
    (b) **amount** of DNA (Note: Amount of DNA may be reflected through the intensity in which the band fluoresces, since more DNA allows more staining dye to bind, this increases fluorescence. This may be translated to thickness of band on 2D gel diagrams. E.g. on Fig. 7, the 1857 bp band on the ladder fluoresces more in comparison to the band on lane 1. Thus, one can infer that there is a greater amount of DNA on the ladder than on lane 1.). This is however not an accurate measure.

Applications of gel electrophoresis

- Results from gel electrophoresis can be used to analyse and verify DNA fragments (Fig. 7).

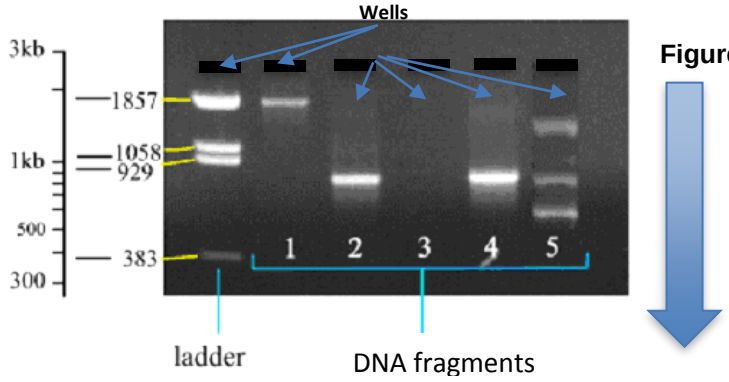


Figure 7: Verification of DNA fragments (in this case, PCR products) on agarose gel. DNA fragments always move away from the wells with the larger fragments moving the slowest and hence, found nearest the well. The smallest fragments move fastest and are hence found furthest from the well.

VIDEO LINK on gel electrophoresis and southern blot: http://tinyurl.com/yb8xh5t

C. Southern Blotting – Nucleic acid hybridisation technique for RFLP analysis

Southern blot is typically carried out after gel electrophoresis which was used to separate DNA fragments according to size. It is a technique used to **detect specific nucleotide sequences** within a sample of DNA (a better method to visualise separated DNA fragments). In particular, it is useful for comparing sizes of restriction fragments produced from different samples of genomic DNA in RFLP (pg 19). It is named after its inventor Sir Edwin Mellor Southern.

Protocol:

1. After the DNA sample is cut by restriction enzymes and the fragments separated by electrophoresis as shown in Fig. 7,
2. Gel slab containing DNA fragments is placed under a nitrocellulose membrane and a stack of paper towels (Fig. 8). These are placed on top of an absorbent sponge in a tray of alkaline solution. A heavy weight is stacked right on top of the paper towels.
3. The absorbent paper towels draw the solution towards themselves. This capillary action draws the **alkaline** solution upwards through the gel, **denaturing** the double-stranded DNA fragments in there. The **single-stranded DNA** on the gel is then drawn upwards **onto nitrocellulose membrane and binds to the membrane**. The DNA binds to the membrane in exactly the same position as they were in the gel.

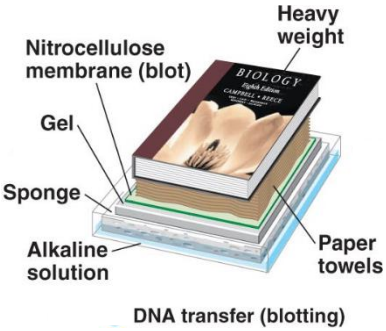


Figure 8: Transfer to DNA fragments from gel slab to nitrocellulose membrane (protocol, step 2).

4. The nitrocellulose membrane is removed and incubated with a **radioactive single-stranded DNA probe**. The probe is a single-stranded DNA synthesised to be complementary to part of the target sequence to be detected. DNA fragments containing this part of the target sequence will **hybridise to the probe by complementary base-pairing** (Fig. 9).
5. After hybridisation, membrane is washed to remove any unhybridised probes.
6. **Autoradiography** is performed by placing an X-ray film over the membrane. The radioactivity of the bound probes exposes the film to form an image corresponding to the bands that have base-paired to the probe.

Notes to self

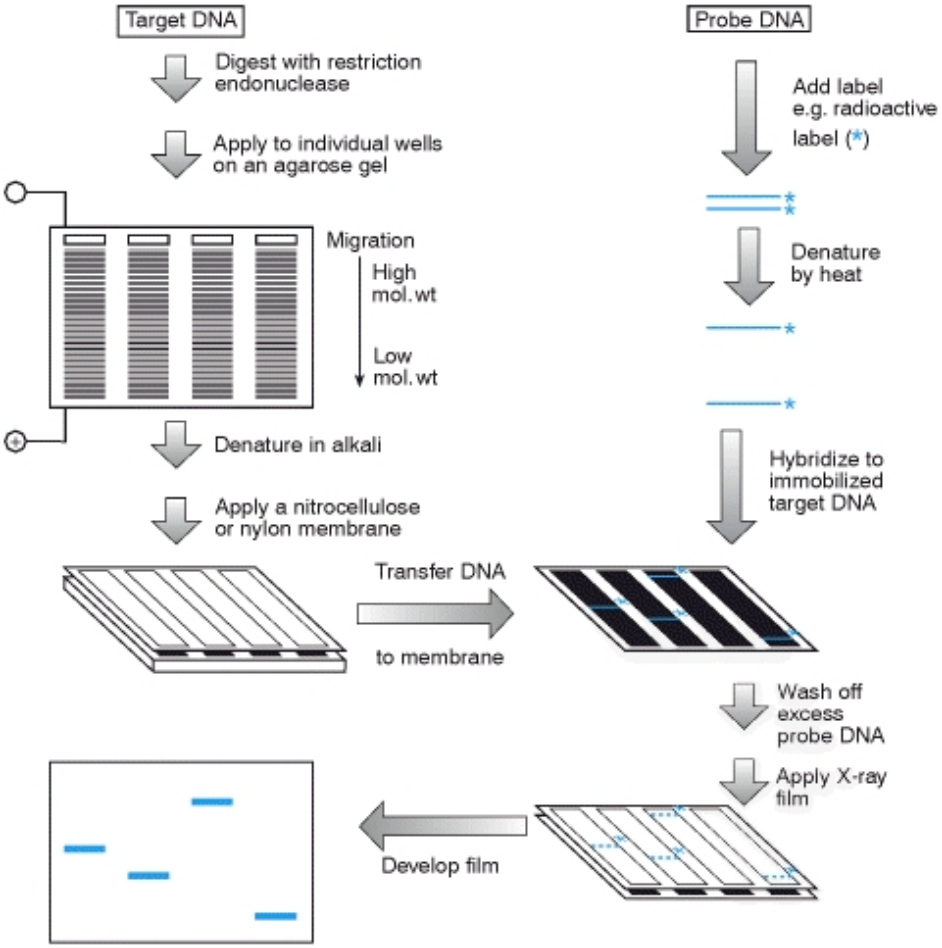


Figure 9: Southern blotting used to detect DNA fragments that have been separated according to size by gel electrophoresis.

VIDEO LINK on southern blot: http://tinyurl.com/ko4oqa2

5. Restriction Enzymes

Notes to self

a. *Origin of restriction enzymes*

- In nature, **restriction enzymes** are produced by **bacteria**.
- Each enzyme is named after the bacterium from which it originates. E.g. The enzyme *Eco*RI comes from *E. coli.*

b. *Restriction enzymes as tools for cutting DNA*

- Restriction enzymes / restriction endonucleases belong to a group of enzymes called nucleases which **break phosphodiester bonds** that link adjacent nucleotides in DNA.
- Restriction enzymes recognise and bind to specific nucleotide sequences known as **restriction sites** in double-stranded DNA, and cleave a **phosphodiester bond on both strands** of the DNA.
- Most restriction sites are about 4 or 6 bases long and are **palindromic**, i.e. the sequences when read from 5' to 3' on both strands are the same.

Example of a restriction site (fill in the blanks):

5' –	G	A	T				– 3'
3' –				T	A	G	– 5'

- Some restriction enzymes produce **sticky ends** while others produce **blunt ends** (Fig. 10).
  - Sticky ends:
    - Produced when restriction enzymes leave a **staggered cut** resulting in single stranded overhangs / sticky ends**.**
    - These short overhangs will form **hydrogen bonds** and **anneal** by **complementary base pairing** with complementary single-stranded stretches on other DNA molecules cleaved with the **same** restriction enzyme.
  - Blunt ends:
    - Produced when restriction enzymes make a simple **cut across both strands** at a single point**.**

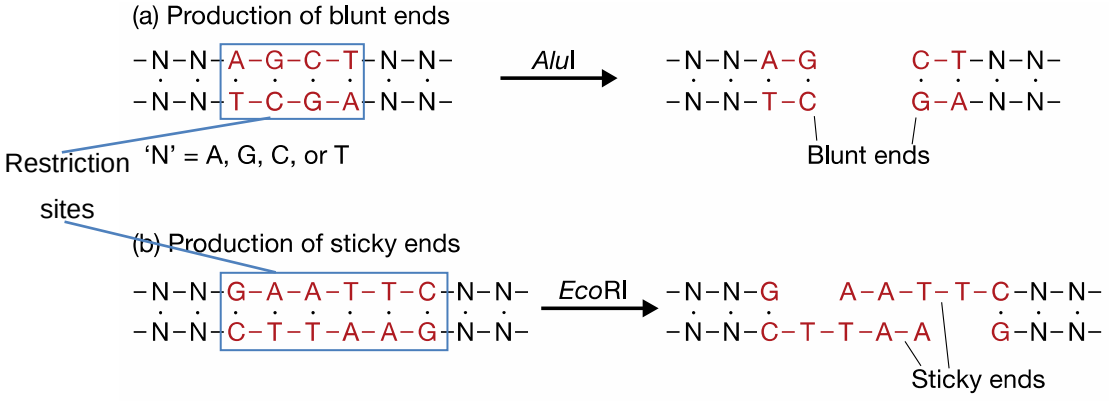


Figure 10: Ends produced by cuts with different restriction enzyme

(a) *Alu*I producing blunt ends (b) *Eco*RI producting sticky ends

c. Natural function of restriction enzymes

- Bacteria produce restriction enzymes as a **defense mechanism** – they **cleave foreign DNA** molecules (from sources such as bacteriophage) into **non-infective** fragments.
- When a bacteriophage infects a bacterium, it first injects its DNA into the bacterium. Cleaving the phage DNA prevents the phage from replicating, conferring the bacterium with resistance to bacteriophage infection.
- The bacterium's own DNA is not cleaved because it is **methylated** at the restriction sites that are recognised by its own restriction enzyme. (Fig. 11)
  - Methylation:

    Methylase **adds methyl groups** ($-CH_3$) to adenine or cytosine residues in the DNA sequence that constitutes the **restriction site**. This changes the conformation of the DNA at the restriction site such that it is **no longer complementary in shape and charge to** the enzyme's **active site**. Hence, the restriction enzyme will be unable to recognise and to cleave the DNA.

    Therefore, methylation protects bacterial DNA from cleavage by its own restriction enzymes.

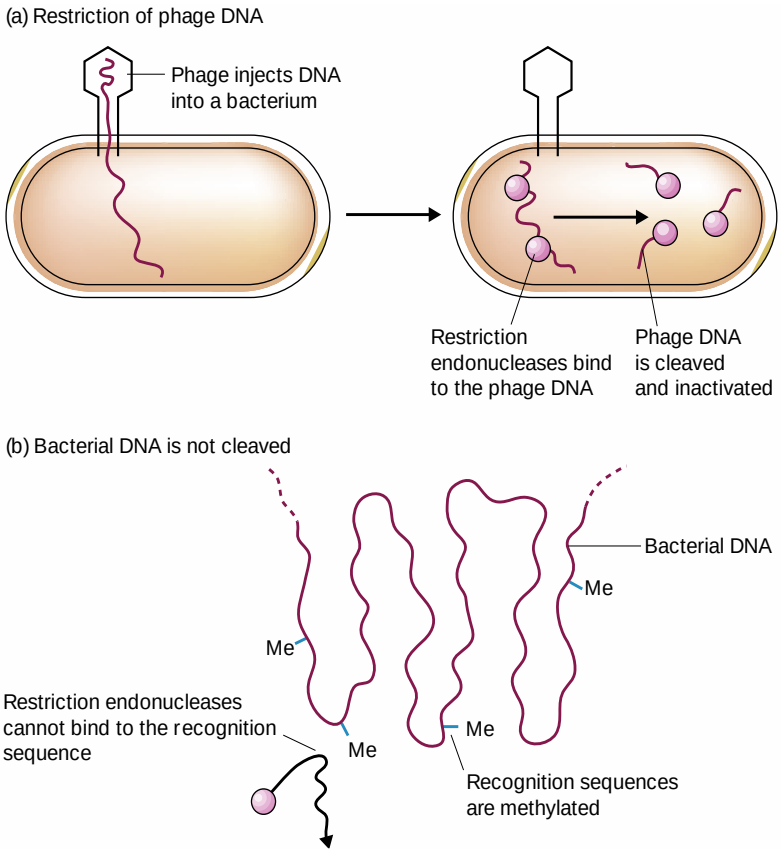


Figure. 11: Function of restriction enzymes in a bacterium

(a) Phage DNA is cleaved (b) Bacterial DNA protected due to methylation of restriction sites.

6. Restriction Fragment Length Polymorphism (RFLP)

1. Introduction

1. The completion of the Human Genome Project revealed that only 1-2% of the total DNA in the human cell represents protein-coding genes. The rest of the non-coding DNA include the following regions, among others:
   - **introns** (intervening sequences between exons)
   - **control elements** that help regulate transcription, including promoter region, enhancers and silencers
   - **tandem repeats** (nucleotide sequence that are repeated and the repeats are adjacent to one another)
2. DNA analyses showed that a non-coding DNA sequence at a particular locus on a chromosome often exhibits small nucleotide sequence differences in different individuals. These differences are termed **DNA polymorphisms**. In general, DNA polymorphisms at a particular chromosomal locus differ either
   (a) in **nucleotide sequence** or
   (b) in **numbers** of tandemly repeated nucleotide units.

Definition of RFLP

- Due to the presence of DNA polymorphisms in homologous regions of different individuals
  1. **Single nucleotide polymorphisms** (SNPs, pronounced 'snips') - differences at a single base-pair,
  2. Varying **number** of **tandem repeats**
     - produce fragments of **different length** among individuals.
- When genomic DNA are **digested by restriction enzymes, different-sized restriction fragments** called **restriction fragment length polymorphisms** (RFLPs) are generated.
- After separation by **gel electrophoresis** and upon visualization and analysis, a **unique banding pattern** is produced for each individual.

Q: Why do non-coding DNA sequences tend to have nucleotide sequences differences between individuals?

Since these sequences do not code for any protein or RNA product, mutations occurring here are tolerated and are allowed to accumulate over time.

VIDEO LINK on restriction fragment length polymorphisms (RFLPs):
http://tinyurl.com/mbj36b6

2. Applications of RFLP Analysis

1. *RFLP analysis in* ***disease detection – sickle-cell anaemia***

Detecting ***Single Nucleotide Polymorphisms (SNPs)***

1. The vast majority of SNPs occurs in non-coding regions of the genome, but some **occur in coding regions** as well, which can lead to genetic diseases in humans.
   e.g. sickle-cell anaemia is due to point mutation
2. These single nucleotide mutations **alter the DNA sequence leading to formation or elimination of restriction site(s).**
   Hence single nucleotide polymorphisms (SNPs) can lead to different number of restriction fragments in different individuals.

3. RFLP analysis can thus be used to detect diseases directly.
   - The single nucleotide substitution is the disease-causing mutation and occurs within the coding region.
     e.g. in **sickle-cell anaemia**, the disease-causing mutation occurs at restriction site for *Mst*II within the β-globin gene. (Fig. 12)

Notes to self

     - In the disease allele, this *Mst*II restriction site is eliminated.
     - In the normal allele, this *Mst*II restriction site is retained.

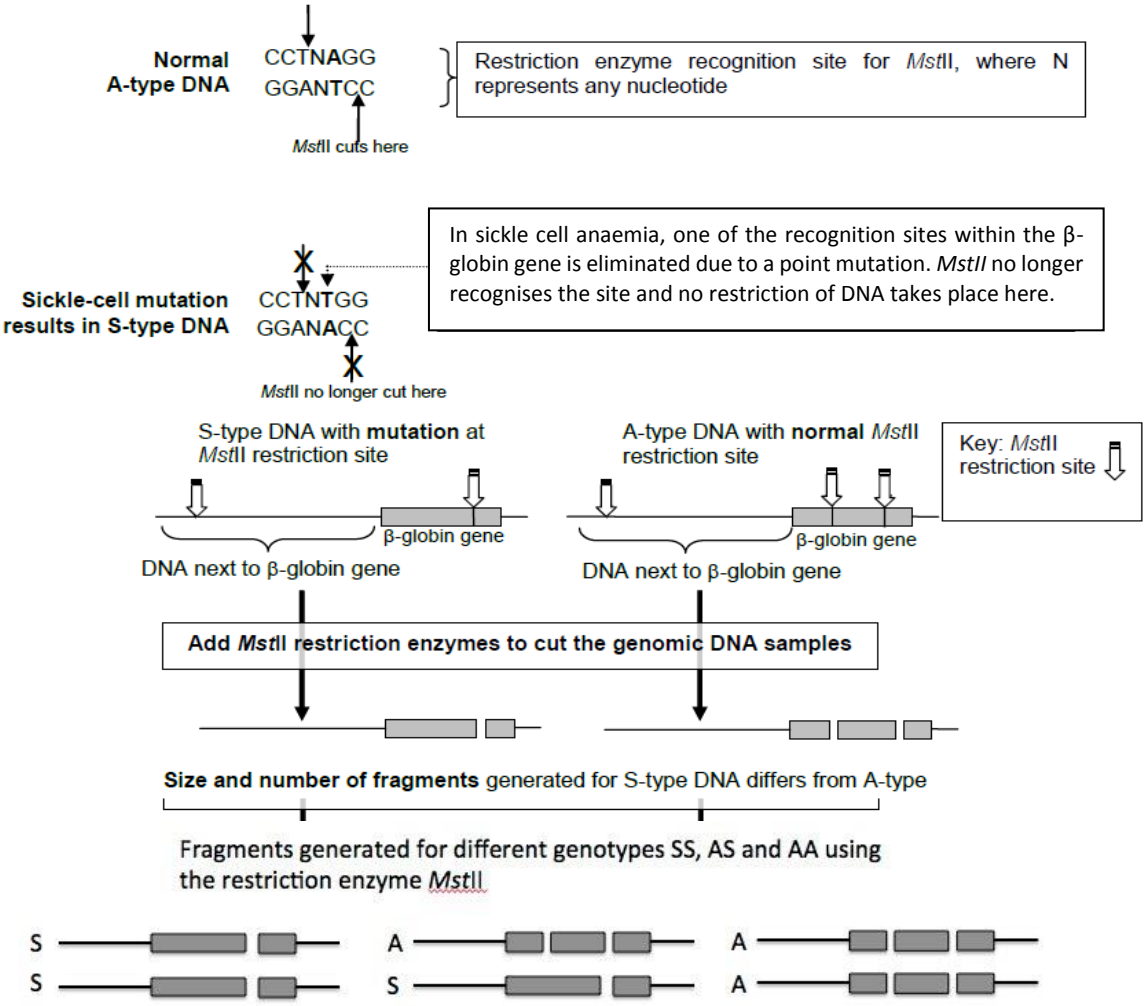


Figure 12: The basis of using RFLP to detect the sickle-cell anemia allele.

Protocol:

Method 1 (Fig. 13a)	Method 2 (Fig. 13b)
Genomic DNA from an individual with sickle cell anaemia and a normal individual are isolated.	
1. Digest with **restriction enzyme**, *Mst*II Different-sized restriction fragments are produced	1. Conduct **PCR** By designing primers that flank the boundaries of the β-globin gene
2. Run **gel electrophoresis**	2. Digest with **restriction enzyme**, *Mst*II Different-sized restriction fragments are produced
3. Southern blot and nucleic acid hybridisation using a specific radioactive **probe** that complementary to part of the β-globin gene. Visualise bands via autoradiography.	3. Run **gel electrophoresis** Visualise bands by adding ethidium bromide, and view under UV light.
4. Analysis of results	

Note: Both methods are similar, the 1st method uses specific radioactive probe (complementary to β-globin gene) to detect the relevant bands, whereas in the 2nd method, probes are not needed, as PCR primer allows only for the target sequence (β-globin gene) to be amplified.

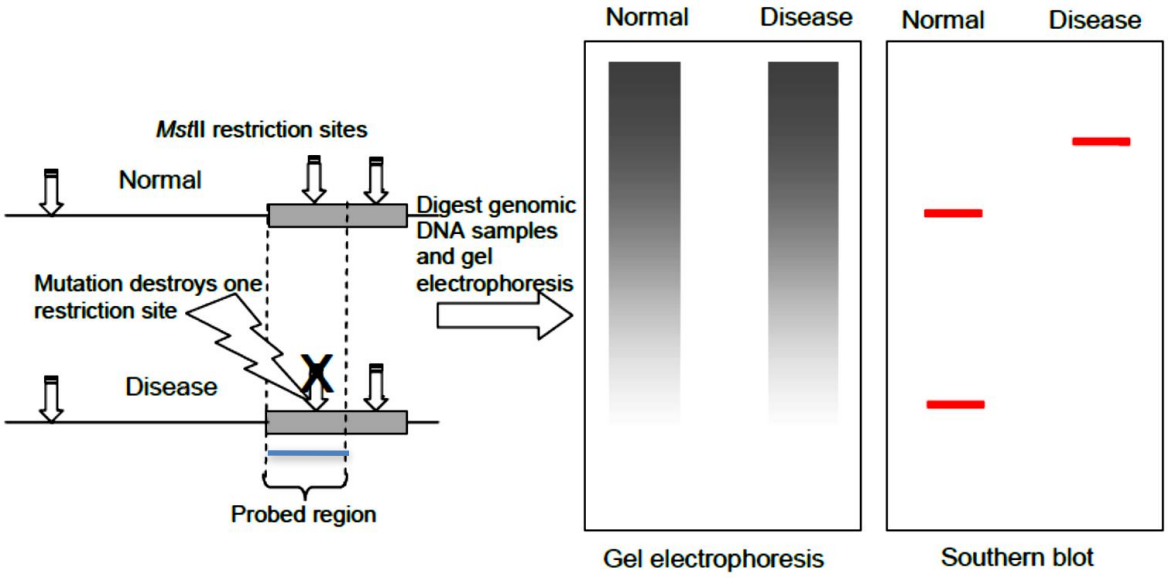


Figure 13a) Method 1 - RFLP can be used for detection of sickle-cell anaemia. In order to detect discrete bands in the smear, Southern blotting must be carried out.

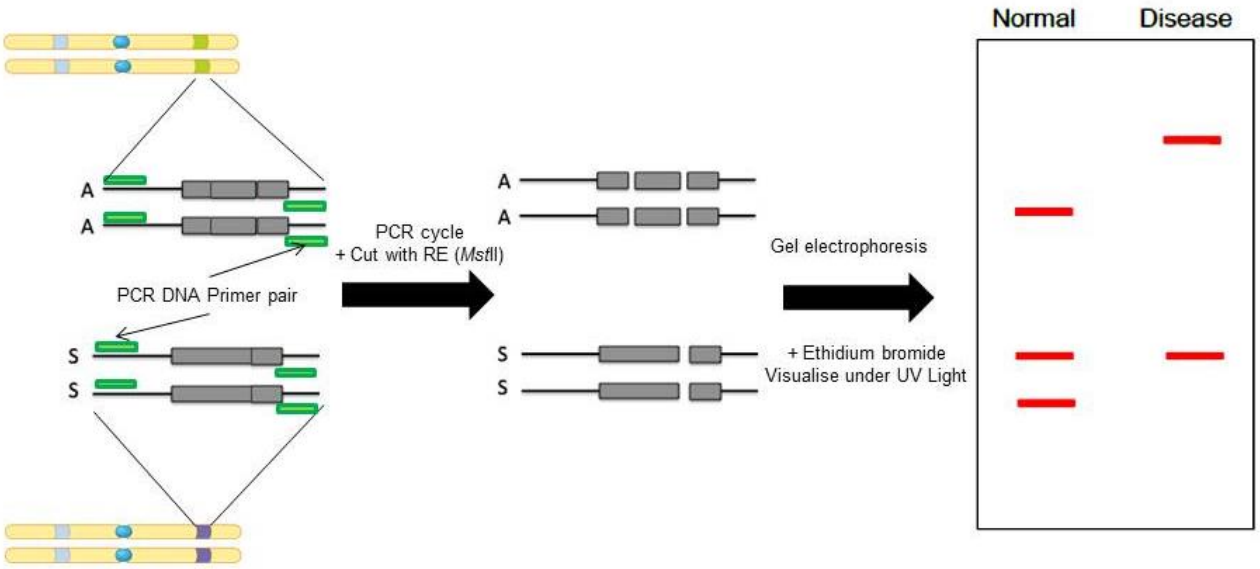


Figure 13b) Method 2 - RFLP can be used for detection of sickle-cell anaemia. Using PCR primers to amplify on the desired locus (β-globin gene).

2. *RFLP in **DNA fingerprinting***

*Detecting varying **number** of **tandem repeats***

1. No two humans (except identical twins) have exactly the same genome.
2. Discovery of DNA polymorphisms has led to the development of **DNA fingerprinting** (a.k.a. DNA typing / profiling) techniques which have major applications in:
   - **forensic science** and
   - **paternity testing**.
3. These tandem repeats of a particular DNA sequence occur in **varying numbers** in different individuals – discriminating enough to generate a **unique DNA fingerprint** for each individual (Fig 14, Fig 15).

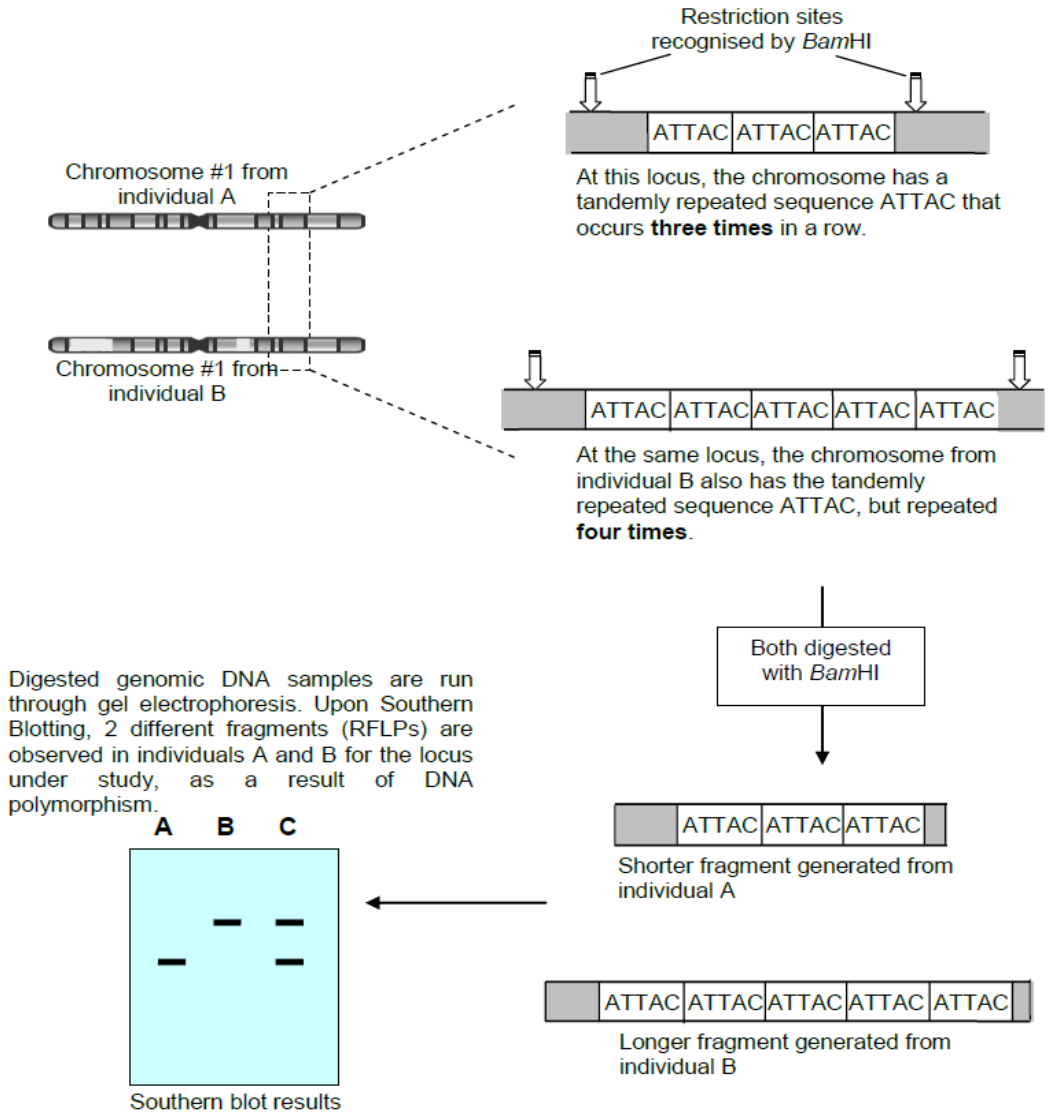


Figure 14: DNA polymorphism in terms of tandem repeats can result in different restriction fragments. Genomes from 2 individuals A and B, are cut using *Bam*HI. The 2 individuals differ in the number of tandem repeats (of the sequence ATTAC) at a particular locus on chromosome #1. As a result of the larger number of tandem repeats present, individual B generates a longer restriction fragment which travels a shorter distance down the gel during electrophoresis. The 2 different-sized fragments are detected using the same probe which has a sequence complementary to segments of both fragments e.g. ATTAC.

4. Since the number of repeats of a particular sequence is heritable, if the banding patterns of two DNA samples are very similar, it means that they are from two very closely-related individuals.
   - The probability of 2 individuals having the exact same banding pattern is infinitesimally small; there is a calculated probability of 1/ 575 trillion that two persons will have the same DNA fingerprint.

Protocol:

(Note: Method is similar to the 2 methods mentioned in pg 21)

1. Digest with restriction enzyme(s) that **cut on either side of a tandem repeat loci**.

   Separation of restriction fragments by gel electrophoresis.
   Southern blotting and incubating with **radioactive probes** for **tandem repeats** (Fig. 15), visualise via autoradiography.

   OR

2. Design **multiple PCR primer** pairs which anneal to the **flanking** sequences of **different tandem repeats** loci.

   Amplification of multiple tandem repeat loci.
   Separation of products by gel electrophoresis.
   Stain with ethidium bromide and visualise under UV light.

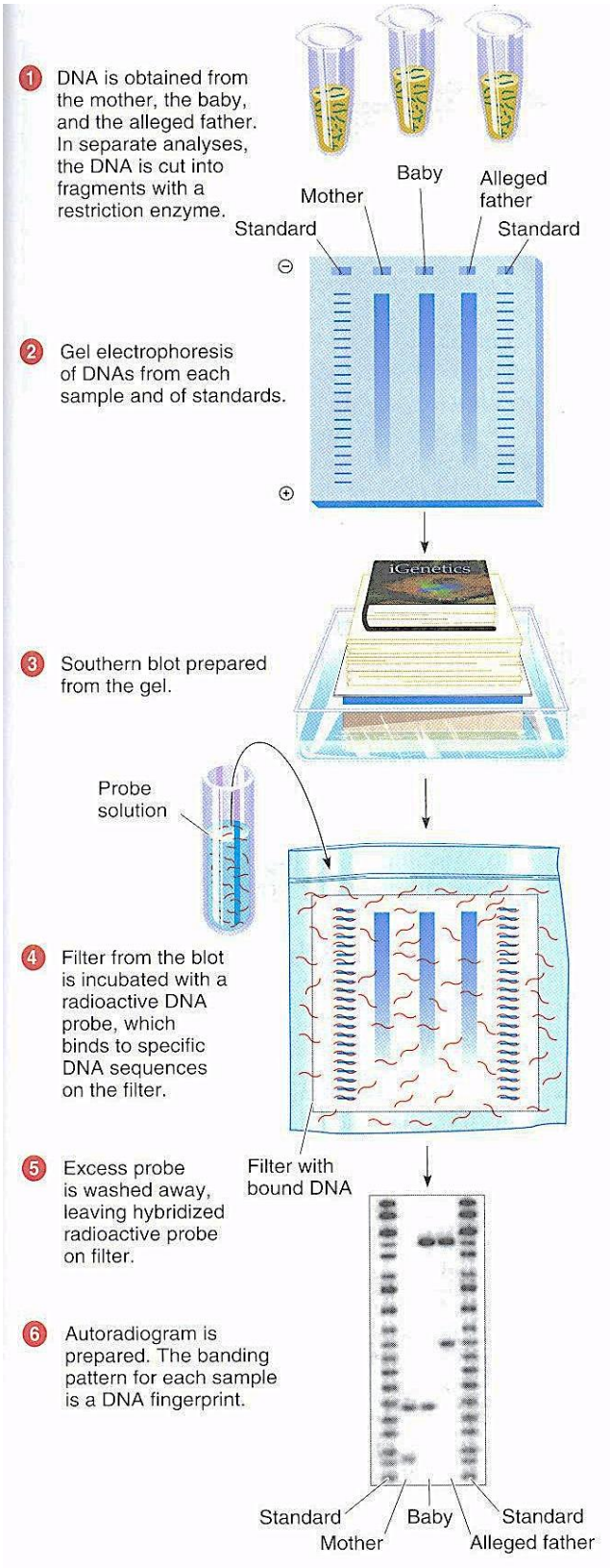
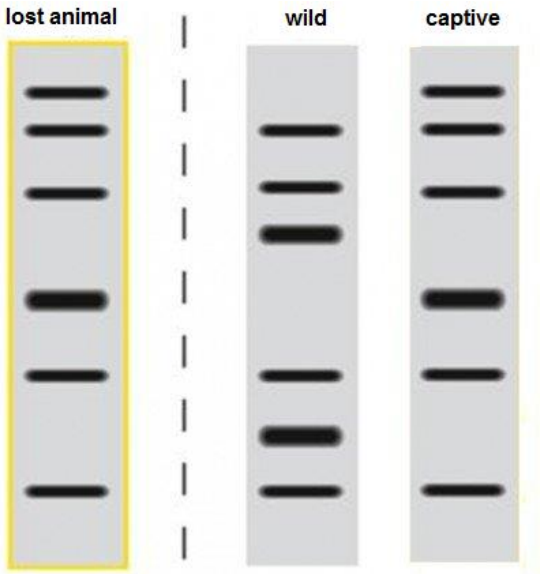


Figure 15: RFLP used in paternity testing. A particular locus on the chromosome is targeted for RFLP analysis.

Concept check

Explain the process of Southern blotting and suggest how the genetic fingerprint shown in the figure below can be used to verify if a lost animal belonged to the wild or was bred in captivity. [4]



Concept check

The maps of the sites for restriction enzyme R in the wild type and the mutated genes are shown schematically in Figure 1 below:

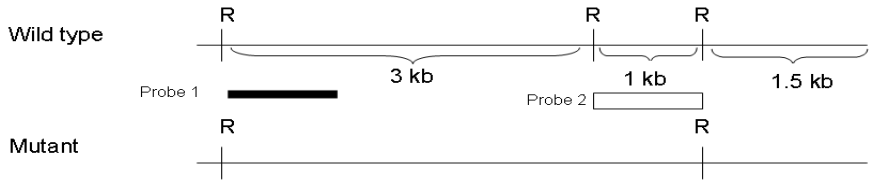
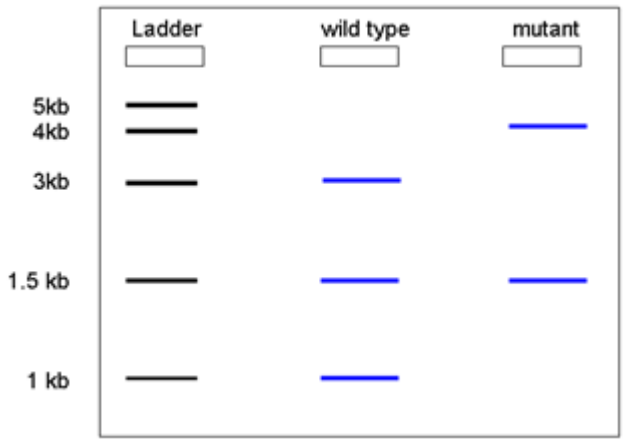


Fig.1

(a) Draw the location of the bands in the gel below when the gene was digested with restriction enzyme R and separated by gel electrophoresis. [2]



Note: "Ladder" is equivalent to DNA marker".

(b) Circle the band(s) to which the probe 1 will hybridise to. [1]

(c) Circle (using a different colour) the band(s) to which the probe 2 will hybridise to. [1]

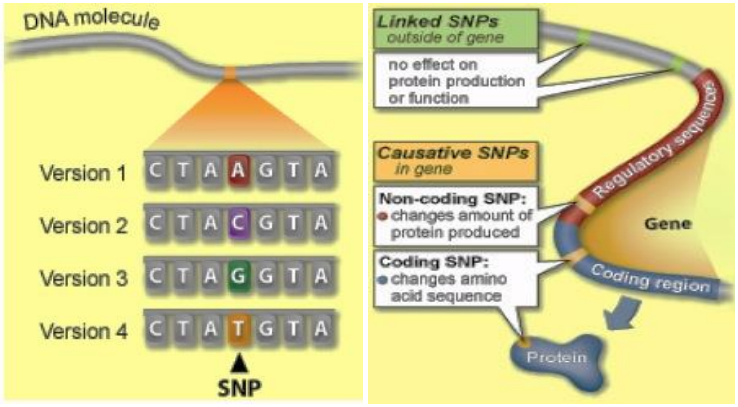
(d) On Fig. 1, draw the location of a third probe ('Probe 3') which would give the same banding pattern for both wild type and mutant samples and hence is not able to differentiate between both samples. [1]

(e) Explain why the DNA fragments move in the gel and why do they separate out on the gel? [3]

Appendix

Additional Information:

More about SNPs (not in syllabus)



SNP (pronounced "snip") stands for Single Nucleotide Polymorphism. SNPs are single-nucleotide substitutions of one base for another. Each SNP location in the genome can have up to four versions: one for each nucleotide, A, C, G and T. Not all single-nucleotide changes are SNPs, though. To be classified as a SNP, two or more versions of a sequence must each be present in at least one percent (1%) of the general population.

SNPs occur throughout the human genome - about one in every 300 nucleotide base pairs. This translates to about 10 million SNPs within the 3-billion-nucleotide human genome.

SNPs and disease-causing mutations: Not exactly the same!

If you know what a point mutation is, then the description of a SNP might sound similar. True, both are single-nucleotide differences in a DNA sequence, but SNPs should not be confused with disease-causing mutations. There are some differences:

First, to be classified as a SNP, the change must be present in at least one percent (1%) of the general population. No known disease-causing mutation is this common.

Second, most disease-causing mutations occur within a gene's coding or regulatory regions and affect the function of the protein encoded by the gene. SNPs are not necessarily located within genes, and they do not always affect the way a protein functions.