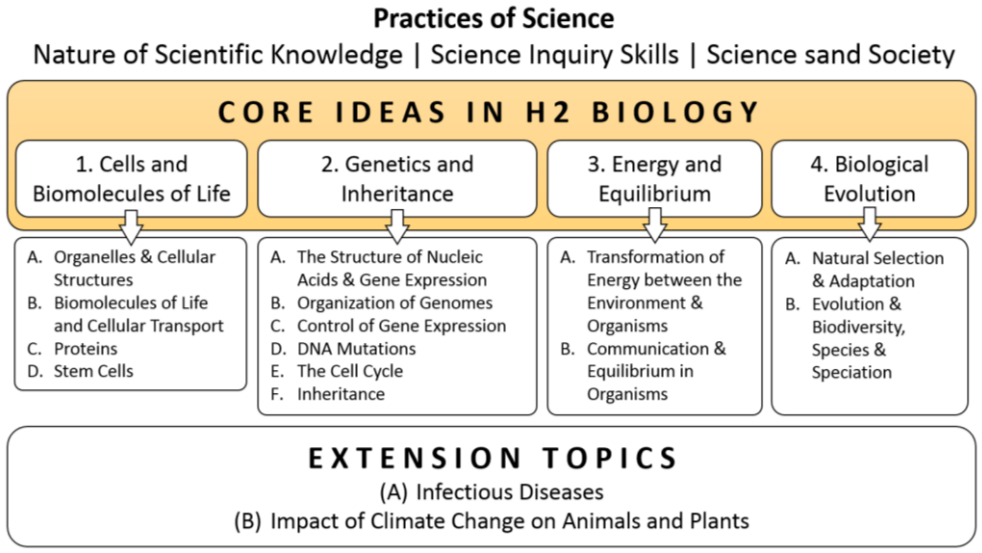
Core Idea 2C

7. Genetics & Inheritance (IV) – Molecular Techniques in DNA Analysis



SYLLABUS OVERVIEW		
No.	**Overarching Idea**	**Topics**
1	**Core Idea 1** The Cell and Biomolecules of Life	Cell – The Basic Unit of Life
2		Biomolecules of Life and Cellular Transport
3	**Core Idea 3** Energy and Equilibrium	Transformation of Energy – Photosynthesis and Cellular Respiration
4	**Core Idea 2** Genetics and Inheritance	Genetics and Inheritance (I) – The Cell Cycle
5		Genetics and Inheritance (II) – DNA Replication and Gene Expression
6		Genetics and Inheritance (III) – DNA Mutations and their Consequences
7		Genetics and Inheritance (IV) – Molecular Techniques in DNA Analysis
8		Genetics and Inheritance (V) – Organization of Genome & Control of Gene Expression in Eukaryotes *[Includes Core Idea 1D: Stem Cells]*
9		Genetics and Inheritance (VI) – Organization and Inheritance of Viral Genomes
10		Genetics and Inheritance (VII) – Organization of Genome & Control of Gene Expression in Prokaryotes
11		Genetics and Inheritance (VIII) - Inheritance
12	**Core Idea 3** Energy and Equilibrium	Communication and Equilibrium in Multicellular Organisms
13	**Core Idea 4** Biological Evolution	Biological Evolution
14	**Extension Topic A** Infectious Diseases	Immunity and Infectious Diseases
15	**Extension Topic B** Impact of Climate Change on Animals & Plants	Climate Change – Causes and Impacts on Animals and Plants

NARRATIVES

An understanding of *Genetics and Inheritance* that would help make sense of the transition from molecular to organismal level. *Genetics and Inheritance* provides the molecular basis to the understanding of how variations in populations arise and this is important in the study of biological evolution. At the cellular level, expression of genes involves cellular structures such as the nucleus, endoplasmic reticulum and ribosome. Many essential products of gene expression are enzymes involved in biochemical pathways which control physiological functions. As such, mutation of genes may give rise to dysfunctional proteins which in turn could result in diseases. Sickle cell anemia and cancer are raised as examples of a monogenic and a multi-genic disease respectively.

The following questions should help students frame their learning:

- How does the genetic make-up of an organism influence its appearance, behavior and survival?
- How can we ensure continuity of human as a species?

Heritable information, in the form of DNA (and in some cases RNA), provides for continuity of life

Genetic information is stored in an organism's DNA; expression of genes results in the synthesis of functional products, such as rRNA, tRNA and proteins. These products play a role in intra- and extra-cellular biochemical pathways and influence the physiological processes in organisms.

Genomes contain heritable information necessary for continuity of life at all levels: cell, organism and system. This information is stored and passed on to subsequent generations via DNA. Reproduction can occur at the cellular or organismal level; each progeny needs to receive heritable genetic information from its parents. 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 hybridization.

LEARNING OUTCOMES

Core Idea 2C: Control of Gene Expression
Basic molecular techniques allow scientists to study gene expression.

Candidates should be able to:

c) Describe the principles and procedures of these molecular techniques:
- **i)** polymerase chain reaction (including its advantages and limitations);
- **ii)** gel electrophoresis; and
- **iii)** Southern blotting and nucleic acid hybridization

LECTURE OUTLINE

0. Overview of molecular techniques used in DNA analysis

1. Polymerase chain reaction
    - 1.1 Introduction
    - 1.2 Principles and procedures
    - 1.3 Advantages and limitations of PCR
    - 1.4 Applications (FYI)

2. Gel electrophoresis
    - 2.1 Introduction
    - 2.2 Principles and procedures
    - 2.3 Applications (FYI)

3. Southern blotting and nucleic acid hybridization
    - 3.1 Introduction
    - 3.2 Principles and procedures
    - 3.3 Applications (FYI)

TEXTBOOK REFERENCES

1. Biology, Campbell and Reece, 9th Edition, *pgs 442 - 469*
2. Biology of Microorganisms, Brocks, Chapter 8 Genetic Engineering and Biotechnology
3. Genetics, Analysis & Principles, Robert J.Brooker, 2nd Edition, Chapter 20 Structural Genomics

INTERNET ANIMATIONS

Polymerase Chain Reaction

http://www.dnalc.org/view/15475-The-cycles- of-the-polymerase-chain-reaction-PCR-3D- animation-with-no-audio.html
http://highered.mcgraw-hill.com/sites/0072437316/student_view0/chapter16/animations.html#
http://www.sumanasinc.com/webcontent/animations/content/pcr.html
http://learn.genetics.utah.edu/content/labs/pcr/

Gel Electrophoresis

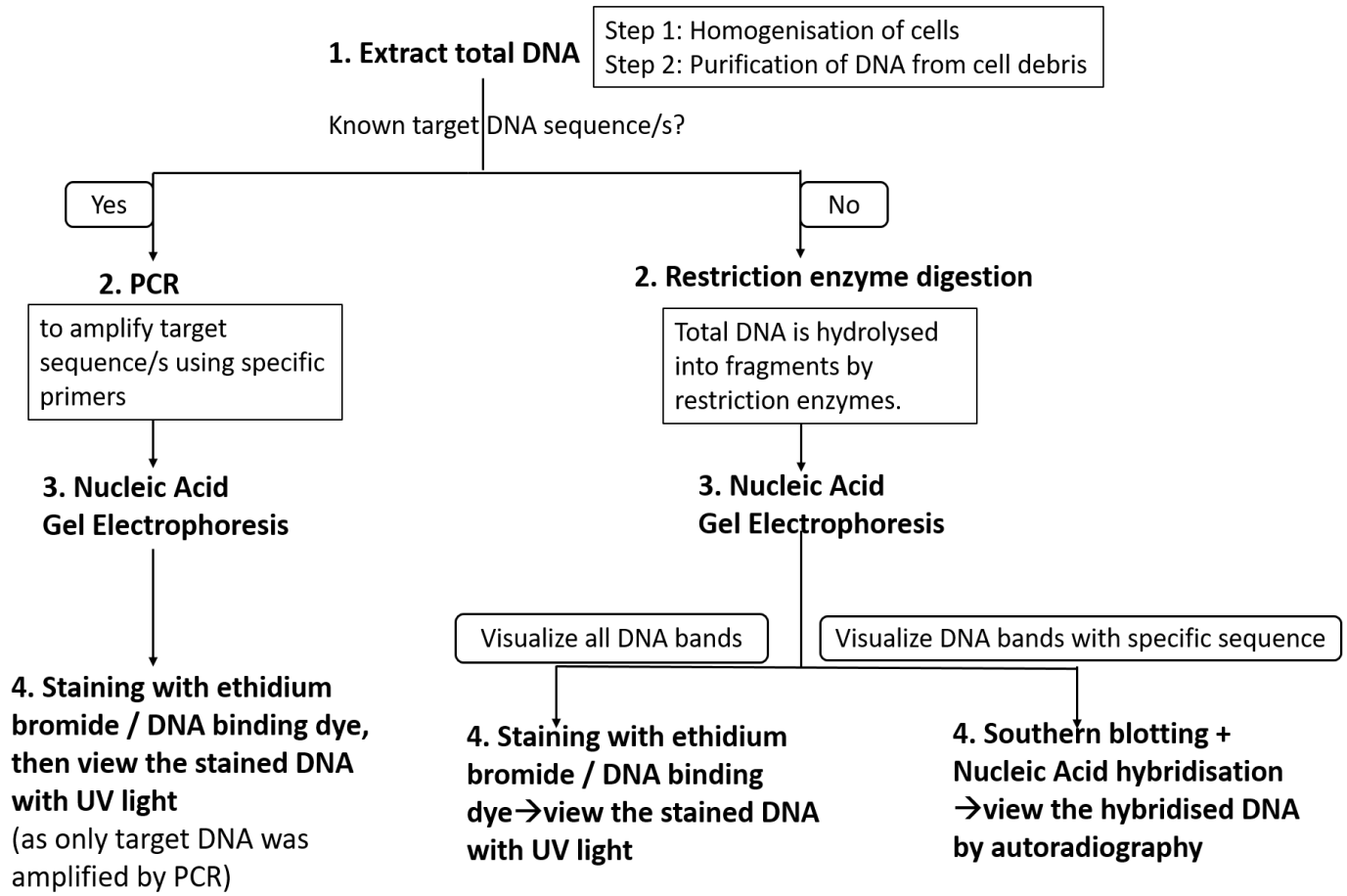
http://www.dnalc.org/resources/animations/gel electrophoresis.html
http://learn.genetics.utah.edu/content/labs/gel/
http://www.dnalc.org/resources/animations/gelelectrophoresis.html

Southern Blotting and Nucleic acid hybridisation

http://highered.mcgraw-hill.com/sites/0072437316/student_view0/chapter16/animations.html#

0. Overview of Molecular Techniques for DNA Analysis

A target DNA sequence can refer to either a gene of interest or non-coding sequences.

What are you trying to do?	Possible Methods
1. **Extract total DNA** (Breaking cells apart to remove DNA from the other cellular content) 2. **Isolate** target DNA sequence(s) 3. **Separation** of target DNA sequence/s from other DNA fragments (according to molecular size) 4. **Visualization** of DNA	

1. Polymerase chain reaction (PCR)

1.1 Introduction

The molecular biologist studying a particular gene faces a challenge. Naturally occurring DNA molecules are very long and a single molecule carries many genes. Moreover, in many eukaryotic genomes, genes only occupy a small portion of the chromosomal DNA, the rest being noncoding nucleotide sequences. Hence to work directly with specific genes or other DNA segments, scientists have developed laboratory techniques to obtain **multiple identical copies of a specific DNA sequence**, a process known as ***DNA cloning***. The production of multiple copies of a single gene is called *gene cloning.*

DNA cloning can be achieved using cells (e.g. *E.coli* bacteria) or through **polymerase chain reaction (PCR)** using a thermal cycler. DNA cloning using cells remains the best method for preparing large quantities of a particular gene or DNA sequence. However, when DNA sample for cloning is in small quantities or impure, PCR is a quicker and more selective method by which large quantities of a specific DNA sequence can be obtained in a short time.

Devised in 1985 by Dr. Kary Mullis, PCR revolutionized DNA research. For inventing this method, Dr. Mullis was award the 1993 Nobel Prize in Chemistry.

Polymerase chain reaction (PCR)

- **Polymerase chain reaction (PCR)** is a technique in which a sequence of DNA is **amplified** (copied many times) *in vitro* (outside of living organism) using a thermal cycler.

- In this method, any specific DNA target segment within one or many DNA molecules can be quickly amplified in a test tube. With automation, PCR can make billions of copies of a target segment of DNA in only a few hours (e.g. 2-3 hours).

1.2 Principles and procedures

(a) Reagents and equipment for PCR

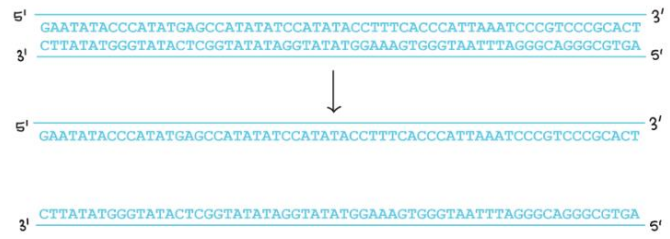
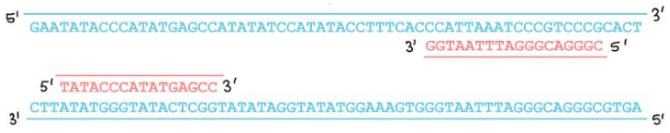
What is needed?	Description
1. **DNA sample** (source DNA) to be amplified	• The DNA to be amplified can be obtained from a **small original sample** from any source. (eg. DNA extracted from blood, sperm, or any other tissue, from older forensic specimens, bacterial colonies or purified DNA). • The **target** DNA sequence to be amplified by PCR must be **known to some extent** so that **DNA primers can be designed.**
2. **Excess** supply of **2 different single-stranded DNA primers** flanking (on each side of) the DNA to be amplified	• Primers are **short** (20 - 30 nucleotides), **synthetic, single-stranded DNA fragments** that are **COMPLEMENTARY to the 3' regions of the target DNA sequence**. • **2 different primers** (forward and reverse primers) are required, one for each strand of DNA, to provide a **free 3'OH group** for ***Taq*** **DNA**

What is needed?	Description

	polymerase to **elongate** the **new DNA strand from 5' to 3' direction.**
	• Primers should **flank the target DNA sequence** / gene of interest and may extend into the target sequence to **define the ends of the DNA segment to be amplified**.
	• Primers cannot be too short or too long.
	  ○ A shorter primer increases the chance of it annealing to non-target sequences.
	  ○ A primer that is too long will decrease the rate at which it anneals to the target DNA, hence decreasing the efficiency of the reaction.
	• Primers are added in excess to **prevent the DNA strands from reannealing** and to ensure that **no replenishment is required** during PCR.

3. ***Taq*** **DNA polymerase** – a **thermostable** enzyme purified from the hot-spring bacteria *Thermus aquaticus*	• Works at an **optimum temperature of 72°C.** • The enzyme is stable enough to withstand changes in temperatures between 50 to 95°C for the PCR cycles to be repeated many times in the thermal cycler. i.e. can be **reused after every cycle**. • Has similar function to DNA polymerase except that it **does not have 3' to 5' exonuclease proofreading function.** This leads to its **low replication fidelity** and an error rate of about one in 9,000 nucleotides.
4. Excess supply of **deoxyribonucleotides** (A, T, C, G)	• The raw materials for the DNA synthesis. • They are added in **excess** so that **no replenishment is required during PCR.**
5. Buffer solution - contains Mg^{2+}	• Provides a suitable chemical environment for optimum activity and stability of *Taq* DNA polymerase.
6. The above reagents is mixed together in a microfuge tube and placed in an **automated thermal cycler** (Fig. 1.2a)**.**	• Equipment programmed to carry out the PCR cycles.  **Fig. 1.2a:** Thermal cycler

(b) Three major steps for each PCR cycle

Steps and essential conditions: One PCR cycle consists of 3 steps **(Fig. 1.2b)**	**Rationale**
1. Denaturation of DNA molecules (95˚C – temperature of 94 to 96˚C acceptable) 	• DNA mixture is **heated to <u>95˚C</u>** • T**he double-stranded DNA is separated into 2 complementary single-stranded DNA**. • This is done by **breaking the hydrogen bonds between the complementary base pairs** holding the two strands together.
2. Annealing of DNA Primers (55˚C – temperature of 50 to 65 ˚C acceptable) 	• The DNA mixture is subsequently **cooled to <u>55˚C</u>** • This allows the **2 DNA primers** to **hybridize with/anneal to 3' regions of the single-stranded target DNA** via **complementary base pairing**, and thus **flank** the target DNA. • **Excess DNA primers** present would also **prevent the DNA strands from reannealing.**
3. Extension of DNA Primers (72˚C) 	• The DNA mixture is subsequently **heated to <u>72˚C</u>**. • ***Taq* polymerase adds deoxyribonucleotides to the free 3' OH ends of the DNA primers.** • Both strands of DNA are replicated in **5' to 3' direction** and thus, there are now 2 copies of the original DNA molecule.
• **Each cycle** of the above steps is **repeated 30-40 times**. Each time the cycle is repeated, the amount of DNA is **doubled.** Hence**,** there is **exponential increase of target DNA sequences** as products. • **Note:** The target sequence is actually not obtained until the 3rd cycle (Fig. 1.2b).	

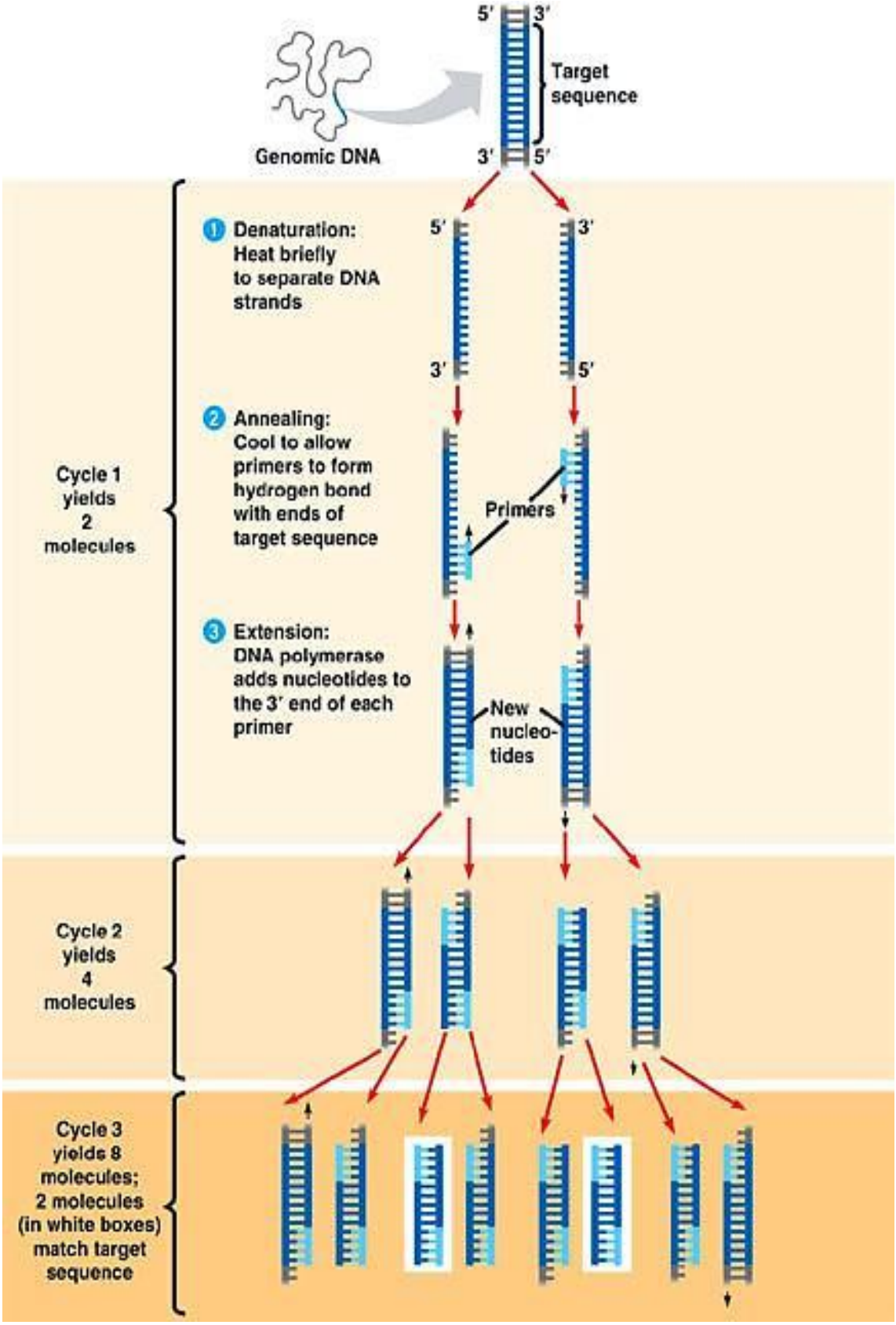


Fig. 1.2b: The Polymerase Chain Reaction (PCR)

Question 1:
State how many **molecules** of DNA will be present after 10 cycles?

Question 2:
State how many **strands** of DNA will be present after 10 cycles?

1.3 Advantages and Limitations of PCR

Advantages:

1. Only **minute amounts of DNA** needs be present in the starting material.

2. A particular gene or DNA sequence of interest can be **amplified to large quantities without using cells** (eg. bacteria).

3. Hence, this is a **faster and more efficient** method since DNA can be **cloned and amplified exponentially within a few hours rather than in days.**

4. PCR is a very **specific** process that **targets only the desired DNA sequence to be copied**. This is because each DNA primer is designed to **anneal to only the 3' regions** that **flank the target DNA sequence** / gene of interest and **not to any other regions** of the genomic DNA.

Limitations:

1. PCR is **sensitive**, so **contamination** of sample with extraneous (foreign) DNA can result in unintended sequences being amplified.

2. **The sequence flanking the target DNA needs to be known** for **suitable DNA primers** to be synthesized. Hence, PCR cannot serve as the starting point for the analysis of genes or genomic regions that have not yet been cloned and sequenced.

3. *Taq* DNA polymerase has **low replication fidelity** since it **lacks a 3' to 5' exonuclease proofreading activity**. There is an error rate of about one in 9,000 nucleotides. Hence, the amplified DNA contains errors which are different from the original sequence, imposing limits on the number of good copies that can be made.

    - However other thermostable DNA polymerases such as *Pfu* polymerase, has been discovered in recent years. Unlike *Taq* DNA polymerase, *Pfu* DNA polymerase has 3' to 5' exonuclease proofreading activity. Hence, use of *Pfu* DNA polymerase overcomes this limitation as it gives rise to a lower error rate.

4. Standard PCR **only copies DNA fragments up to 25 kb (25000 bases) in length**; it **cannot amplify longer regions** of interest.

1.4 Applications (FYI)

1. To amplify DNA for cloning or for analysis from small quantities of DNA. Example –

    - from extinct organisms (eg. fragments of ancient DNA from a 40,000-year-old frozen wooly mammoth)

    - for forensic investigations. From tiny amount of blood, semen or tissues found at the scenes of violent crimes, the tiniest amount of DNA can be amplified for analysis. This helps in the identification of suspects.

2. To test for/detect the presence of specific disease-causing genes. Example –

    - Using PCR to amplify the DNA, scientists developed tests to pick up the genetic changes which take place in cancerous cells very early in the development of the disease. PCR has already made it possible to detect bowel cancer from the DNA of cells extracted from the faeces – an easy, quick and non-intrusive way of making a diagnosis which gives the treatment a much better chance of success.

3. To detect infections.

    - Amplifying the DNA from a single bacterium or virus using PCR can provide a speedy and accurate diagnosis for serious infections, where getting the right treatment quickly can mean the difference between life and death. PCR is already used in the diagnosis of AIDS, viral meningitis, TB, etc.

    - To test for the presence of a particular virus in a tissue sample, the virus genome in the sample is tested by using PCR primers that will hybridize to it. If the PCR reaction generates a product, the virus is present. If the virus is not present, the PCR primers will not hybridize and no product will be generated.

Checkpoint 1:

1. Is PCR a naturally occurring process?
2. Differentiate between PCR and DNA Replication:

Features	PCR	DNA Replication (naturally occurring process)
Location		
Temperature		
Primer type How primer is synthesized		
Enzyme involved in elongation		
Proofreading		
Replication		

Checkpoint 2: DNA Primer Design Exercise

Similar to DNA replication, PCR requires primers to initiate DNA synthesis by *Taq* DNA polymerase. PCR primers are short, synthetic DNA fragments usually having a minimum length of 20 -30 nucleotides. Two primers are required to replicate a target sequence.

The average IQ score of humans range between 90 to109. Less than 0.5% of the human population possesses an IQ of above 140. In 2006, a study of a Dutch family indicated a significant correlation between the *CHRM2* gene on chromosome 7 and intelligence. You are part of a team of scientists attempting to clone this gene for further, large scale investigation. Since only a very small amount of DNA is available, you decided to use PCR to obtain many copies of this gene.

The figure below shows the DNA nucleotide sequence of the *CHRM2* gene (region shaded in grey). The unboxed regions represent sequences flanking the gene.

Target DNA =

5'-ATTTTGGGCCAGTCCTAAGGGCTC…..GAGTACATTGCCATTCTTGAGTATCTACCGAGT-3'

3'-TAAAACCCGGTCAGGATTCCCGAG…..CTCATGTAACGGTAAGAACTCATAGATGGCTCA-5'

(a) Based on your knowledge of DNA replication, design primers which are 20 nucleotides long to amplify the *CHRM2* gene. Write your answer in the figure and state clearly the direction of the primers.

Points to consider:

1. **Why should there be 2 primers?**

2. **Where should each primer anneal to?**

3. **In which direction are the primers extended by *Taq* polymerase?**

4. **On which side of strand 1 should primer 1 anneal to? Why?**

5. **On which side of strand 2 should primer 2 anneal to? Why?**

2. Gel electrophoresis

2.1 Introduction

Once DNA cloning or PCR provides us with large amounts of a specific DNA/gene sequence, we can tackle interesting questions about genes and their functions. For example, how does a particular human gene differ from person from person? Are the different alleles of that gene related to any genetic disorders?

To do so, it is essential to learn about other common techniques applied in the **analysis of DNA** – **Gel electrophoresis**, **Southern Blotting and Nucleic Acid Hybridization** *(details in Section 3).*

Gel electrophoresis is a technique developed by Sharp, Sambrook and Sugden.

There are two types of Gel Electrophoresis –

(1) **Nucleic Acid Gel Electrophoresis** (separation of DNA & RNA)
(2) SDS-PAGE (separation of proteins) *(not required in syllabus)*.

Nucleic Acid Gel Electrophoresis (Fig. 2.1)

- **Nucleic acid gel electrophoresis** is a technique that **separates nucleic acids** on the basis of their **rate of movement through a gel matrix in an electrical field**.

- Rate of movement depends on **molecular size/weight,** electrical charge, and other physical properties (e.g. shape) of the nucleic acids.

- The gel is made of agarose (hence the name agarose gel), a type of polysaccharide derived from seaweed. The agarose gel is a **cross-linked matrix** and functions as a 'molecular sieve' where the **matrix forms little pores (holes) through which DNA / RNA can travel through.**

Think:
DNA molecules are negatively-charged. In order for DNA to move from one end of the gel to the other and be separated as they move, at which end of the electrical field should the DNA be placed - positive end or negative end?

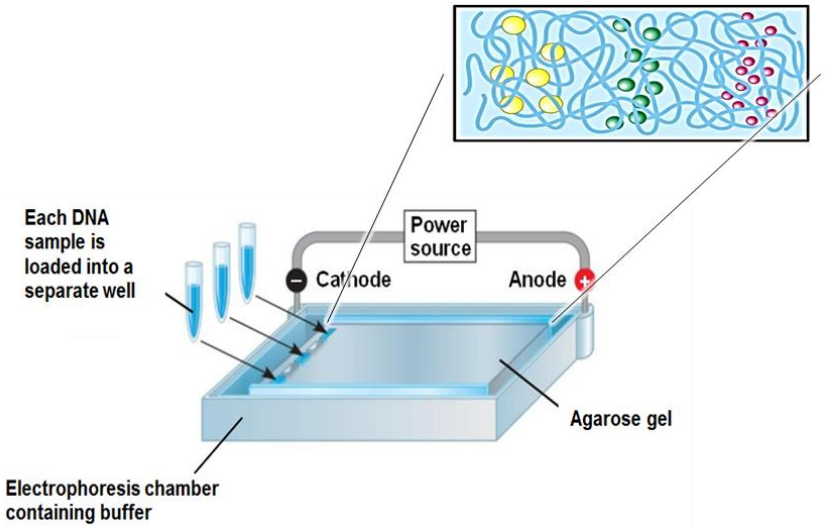
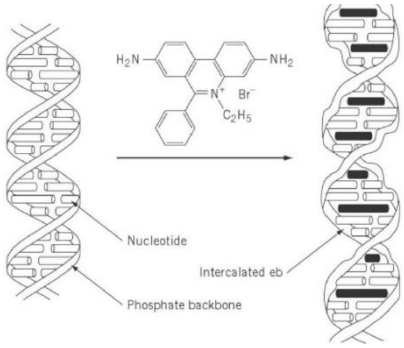
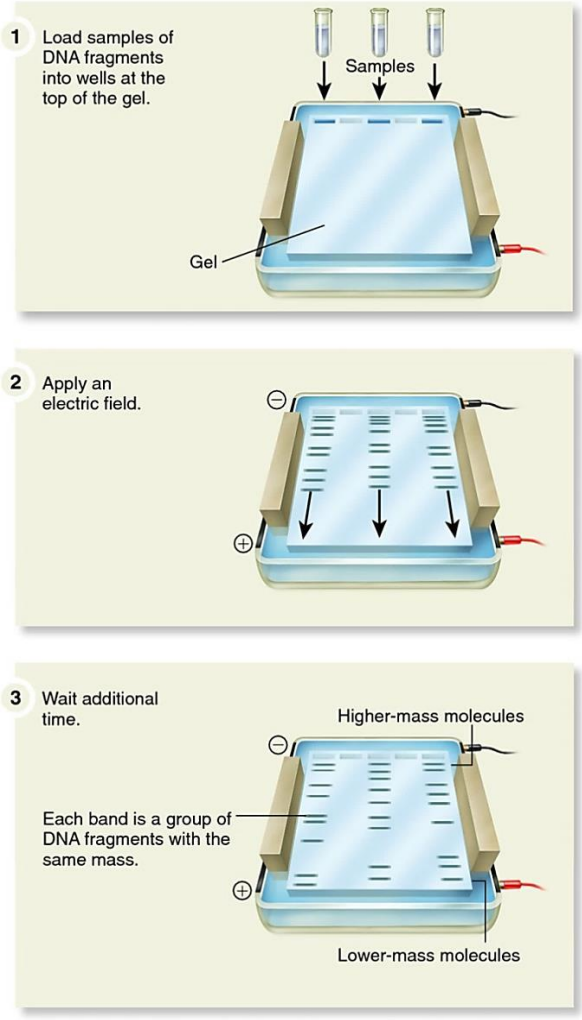


Fig. 2.1: Agarose gel electrophoresis setup.

2.2 Principles and Procedures

Steps (Fig. 2.2a)	Rationale
1. Obtain **DNA samples** to be analysed. Each sample is a solution containing DNA fragments of various lengths. The DNA fragments of different lengths are obtained either via PCR or restriction digestion *(Section 3)*.	• Gel electrophoresis will separate the **DNA fragments** by their **molecular size/weight** (i.e. length of the DNA fragment). Hence, each sample forms a characteristic pattern of bands on the gel after gel electrophoresis.
2. The DNA samples are mixed with **loading dye**, which contains **bromophenol blue** and **glycerol.** The mixture is then loaded in the well at one end of a slab of agarose gel.	• **Bromophenol blue –** a dye that **aids in visualizing the loading of DNA sample into the wells**. It also moves ahead of DNA fragments, allowing us to **track the progress of electrophoresis** to ensure that DNA molecules will not migrate out of gel. • **Glycerol** – **increases the density of the mixture,** to allow the sample to **sink** to the bottom of the well and not float away into the buffer solution.
3. For some experiments, a solution containing **DNA ladder** is loaded into a separate well on the gel (Fig. 2.2c).	• **DNA ladder** – DNA fragments of **known size/lengths** which serve as **reference** to **estimate the fragment sizes** of the sample.
4. The gel is submerged in aqueous **buffer** solution (e.g. TAE buffer) in the gel electrophoresis chamber.	• **Buffer** – Contains **ions** which **allow electricity to pass through** the solution. It also **maintains a relatively constant pH** so that **DNA retains its negative charge**, which allows it to **migrate to the positive electrode.**
5. A **negatively charged electrode (cathode)** is then attached to the chamber at the **DNA-containing end** of the gel and a positive electrode (anode) to the other end. The **DNA molecules** which are **negatively charged** will **migrate towards the positive electrode (anode)** and are **separated according to molecular size.** **Shorter / smaller fragments** (molecules of lower molecular weight) **migrate faster and further** than larger fragments**.**	• DNA molecules have a high **negative charge** due to the presence of **phosphate groups**. • The DNA molecules are pulled towards the positive end by the current and through the agarose gel which acts as a **molecular sieve** to separate the DNA fragments by molecular weight. • The **smaller molecules** are able to move through the pores of the agarose gel **faster** than the larger molecules, so they will travel **further** in the gel, and vice versa.

Steps (Fig. 2.2a)	Rationale
6. After electrophoresis, depending on the goal of the experiment, the **DNA bands** in the agarose gel can be **visualized** by two different methods.	• Visualisation is required as **DNA is colourless** (not visible to the naked eye).
(i) If the goal is **to visualize <u>ALL</u>** the DNA fragments and their band patterns:	
• **Stain the DNA with <u>ethidium bromide</u> or DNA-binding dyes** and **view under <u>UV light</u>**.	• Ethidium bromide **intercalates** (inserts itself) **between stacked nitrogenous bases** and fluoresces under UV light (Fig. 2.2d).
• The gel can be pre-stained (ie. DNA-binding dye mixed together with agarose gel) or stained after gel electrophoresis. Examples of DNA –binding dyes ▪ SYBR Green emits green light of wavelength 522nm; ▪ Gel Red emits red light of wavelength 300nm	 **Fig. 2.2d:** Intercalation of ethidium bromide between stacked DNA bases
• **<u>All</u>** the separated DNA fragments appear as bands in the agarose gel (Fig. 2.2c)**.**	
(ii) If the goal is **to view and identify a <u>SPECIFIC</u>** DNA fragment among many fragments:	
• Carry out **Southern Blot, followed by nucleic acid hybridization** *(details in Section 3).*	• These probes are **short, specific sequences of nucleotides** that are **complementary to the target DNA sequence**.
• **Single-stranded radioactive or fluorescent <u>probes</u>** are used. ○ If **radioactive probes** are used, view DNA bands with **autoradiography.** ○ If **fluorescent probes** are used, view under **UV light.**	
• Only the DNA bands bound by the probes are observed (Fig. 3.2a).	



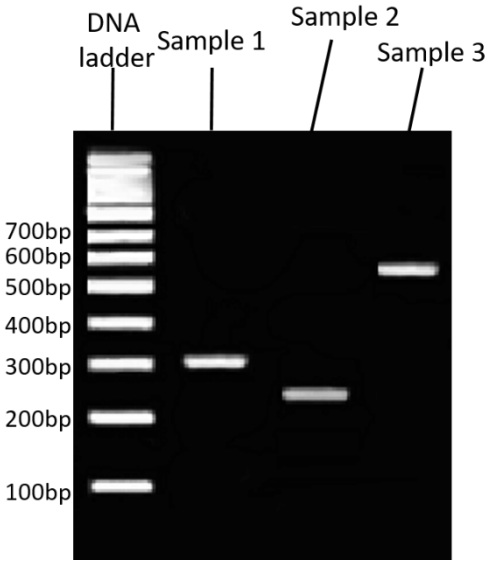


Fig. 2.2c: A photo of DNA samples separated by Gel Electrophoresis and viewed under UV light

[Qn: Can you estimate the molecular size of each DNA sample?]

2.3 Applications (FYI)

1. Estimate size of DNA molecules

    - By comparing the bands in a DNA sample to the bands from the DNA ladder, the size of the DNA fragments can be estimated.

2. Analysis of PCR products

    - Electrophoresis can be carried out on products of PCR on an agarose gel, to determine the results of the amplification (e.g. presence or absence of PCR products).
    - The amount of DNA in a sample can be estimated. The thicker and more distinct the DNA band, the larger the amount of DNA present.

3. Isolation and Purification of a DNA Sample

    - After gel electrophoresis, DNA can be recovered undamaged from gels. Hence this procedure provides a way to prepare pure samples of individual fragments. DNA fragment purified from the gel can be subjected to further DNA analysis. (e.g. DNA sequencing to determine nucleotide sequence).

3. Southern Blotting and Nucleic acid hybridisation

3.1 Introduction

Nucleic acid gel electrophoresis is widely used to separate DNA/RNA molecules. After gel electrophoresis, there is often a need to **detect one or more DNA/RNA fragments** containing a **specific nucleotide sequence** (e.g. a gene sequence) among many other fragments.

This is often done through **Southern blotting** followed by **nucleic acid hybridization.**

Southern blotting and nucleic acid hybridization

- Southern blotting was invented by E.M. Southern in 1975.
- **Southern blotting** is a procedure in which **DNA fragments,** after separation by gel electrophoresis, are **transferred from the agarose gel to a nitrocellulose membrane**.
  (FYI: Northern Blotting - for RNA fragments).
- **Nucleic acid hybridization** is then carried out, where the **DNA** is **denatured and then hybridized** to a **short, single-stranded, radioactively-labelled / fluorescent-labelled DNA/RNA probe**. The probe has a specific sequence that is **complementary to the target DNA** sequence.
- Southern blotting and nucleic acid hybridization are often used when total / genomic DNA undergoes restriction digestion before nucleic acid gel electrophoresis. This is because **too many DNA fragments would be produced to be visualized distinctly using ethidium bromide or DNA-binding dyes**.

3.2 Principles and Procedure

Steps (Fig. 3.2a and Fig. 3.2b)	Rationale
1. Obtain the **DNA samples** to be analysed. ○ E.g. **extract** the **total DNA** from different organisms or from different cell types. ○ Digest the DNA samples with the **appropriate restriction enzyme(s)** (also called endonuclease). This produces restriction fragments of various lengths (Fig 2.2b)	• Each restriction enzyme (Eg. *EcoRI*) recognizes a **specific** sequence of 4 to 8 nucleotide bases on the DNA molecule, known as a **restriction site.** *(Note: Even a point mutation within one of its restriction sites will prevent the restriction enzyme from recognising it)* • The restriction enzyme acts on the restriction site by **breaking the phosphodiester bond** between 2 specific nucleotides. It acts like a molecular scissors, cutting up DNA molecules into smaller fragments to produce **restriction fragments of different lengths**.

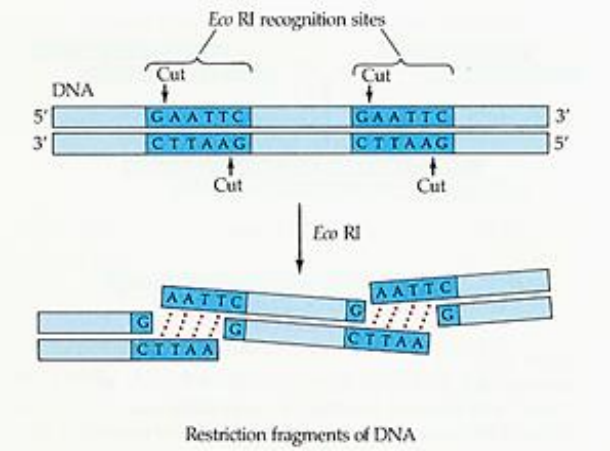


Fig. 2.2b: *Eco*RI (a restriction enzyme) detects a specific sequence GAATTC (*Eco*RI restriction site) on the DNA and cuts it, generating restriction fragments.

- Restriction enzymes are **synthesized naturally in bacteria**.

2. Each sample is loaded into separate wells in an agarose gel, and then separated by **nucleic acid <u>gel electrophoresis</u>**.	• The DNA fragments **are separated by <u>molecular weight/size</u>**. Hence, each sample containing DNA fragments of varying lengths would form a characteristic pattern of bands.
3. Carry out **<u>Southern blotting</u>**: • **DNA fragments** from the gel are **transferred** to a sheet of **nitrocellulose membrane** by capillary action. This means that the nitrocellulose membrane will contain **DNA fragments in a pattern** that is a **<u>replica</u> of** / identical to the **agarose gel.** • The DNA fragments on the nitrocellulose membrane are then **denatured** into **single-stranded DNA** by adding **sodium hydroxide (NaOH) solution.**	• Transfer/blotting is done as it is difficult to denature the DNA in the gel directly without accidentally breaking the gel apart. Detecting a specific DNA fragment on the nitrocellulose membrane also allows the DNA fragments on the agarose gel that are not transferred to be further used in other analysis / experiments. • Denaturation is required as the **probe cannot hybridize with the double-stranded DNA** fragments in the nitrocellulose membrane. • NaOH causes the **hydrogen bonds between the complementary base pairs** of the double-stranded DNA to **break**.
4. Carry out **<u>nucleic acid hybridization:</u>** • Incubate the nitrocellulose membrane with **many copies** of **short, single-stranded, radioactively/florescent-labelled DNA/RNA probe** • The probe undergoes **complementary base pairing** by forming **hydrogen bonds** with the target DNA sequence.	• Probes are **labelled to detect the presence and/or location** of specific DNA fragment that it hybridizes to. • Complementary base pairing ensures **specificity** of the probe **to the target DNA** and not to other DNA sequences.

• Wash away excess / unhybridised probes.	• This prevents unhydridised probes from interfering with the visualization later.

5. **<u>Visualise</u>** only the **specific** DNA fragments that hybridize with the probe.
 	
• Only target DNA fragments bound by the probes would be visible as bands. The rest of the DNA bands would remain invisible.
 	
• If the probe is **<u>radioactively-labeled</u>**, carry out **<u>autoradiography</u>**.
  ○ A sheet of X-ray film is placed over the nitrocellulose filter. The radioactive probes that hybridize with the DNA bands exposes the X-ray film, showing up as dark bands.
 	
• If the probe <u>is **fluorescently-labelled**</u>, the DNA bands that hybridize with the probe can be visualized under **<u>UV light</u>**.
  ○ The florescent probes absorb specific wavelengths of UV light and emit light of different colours.
	• Visualisation is required as **DNA is colourless** (not visible to the naked eye).

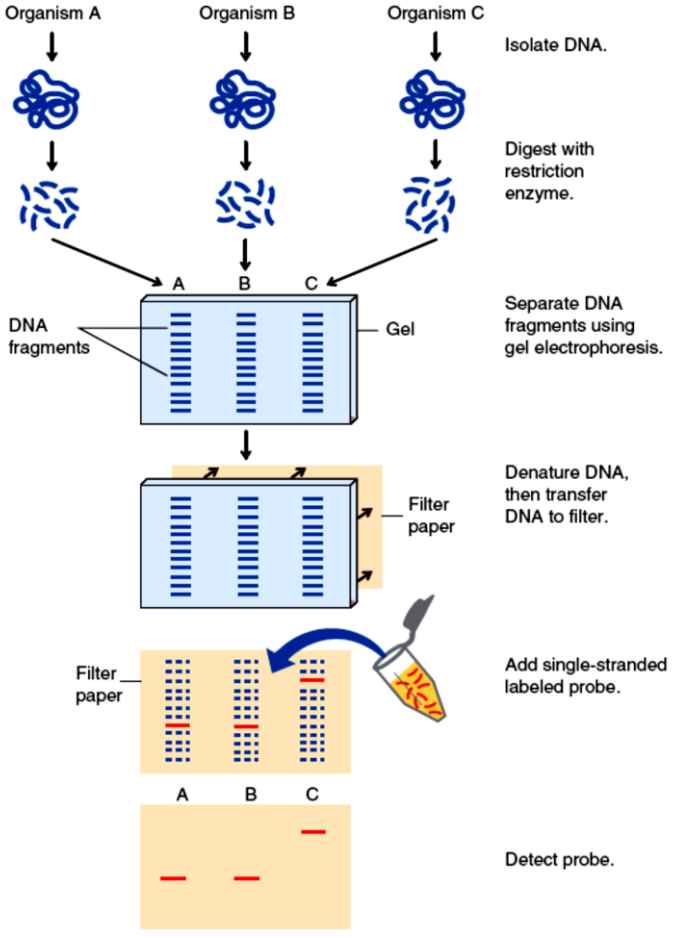


Fig. 3.2a: Overview of Southern Blotting and Nucleic acid hybridization.

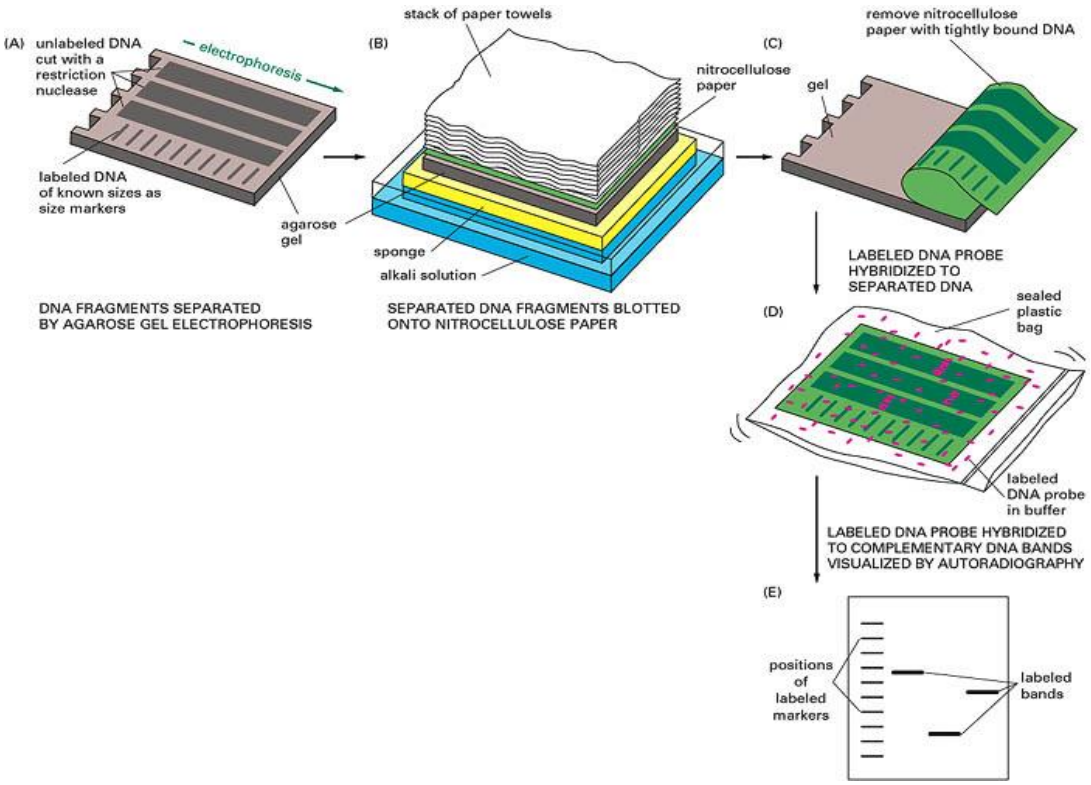


Fig. 3.2b: Various steps in Southern Blotting and Nucleic acid hybridization.

3.3 Applications (FYI)

1. To determine the presence or absence of a target DNA sequence (e.g. a gene) in a DNA sample.

2. To isolate DNA fragment containing the target sequence for further analysis: Example –

    - Carry out sequencing to determine the full length sequence of a specific gene, or

    - Discover functionally related genes with similar sequence.

3. To test genetically modified organisms (GMOs) to ensure that the specific gene inserted has been successfully incorporated into the genome of the host organism.

4. To allow for DNA fingerprinting through analysis of restriction fragment patterns.

    - When a sample of DNA is digested by a certain restriction enzyme and the fragments are separated by electrophoresis, a band pattern characteristic of that DNA molecule and the restriction enzyme used is obtained. This unique pattern of DNA fragments which differ from individual to individual is known as DNA fingerprint.

    - DNA fingerprinting is used in –
        1) Crime Scene Investigation--In solving criminal cases, this method can be used to compare DNA samples from the crime scene, suspect and victim.

        2) Paternity Testing-- In paternity testing, comparison between the DNA of mother, child and supposed father can determine parentage. Each DNA band from the child must coincide with either the father or the mother. (Fig. 3.3)

        3) Phylogenetic relationship for evolutionary study-- In determining phylogenetic relationship / molecular homology among different species of organisms. The more closely related two species are, the more similar the band patterns (Refer to lecture on Biological Evolution).

Genetic fingerprinting

The DNA coding for every individual is unique, and each of our cells carries an identical set of this unique DNA. These are the assumptions on which DNA fingerprinting is used in forensic science.

We believe our individuality is controlled by our genes (the combined 'exons' of the DNA strands of our chromosomes, Figure 4.16, page 46). The bulk of our DNA, however, does not code for proteins as far as we know (this includes the 'introns'). Surprisingly, it is parts of the non-coding regions as a whole that are exploited in 'fingerprinting', that is, in uniquely identifying individuals (Figure 5.12). This is because huge lengths of the non-coding DNA consist of unusual sequences of bases, repeated over and over again. We inherit a distinctive combination of these apparently non-functional 'repeat regions', half from our mother and half from our father.

5 Explain why the composition of the DNA of identical twins challenges an underlying assumption of DNA fingerprinting, but that of non-identical twins does not.

Figure 5.12 Steps to genetic fingerprinting.

DNA fingerprints are used to eliminate (or incriminate) suspects in crimes in which blood, tissue or body fluid samples are available, or to establish relationships, typically parentage. The process in outline is as follows:

1 Sample of DNA is taken from blood, semen, hair root, or body tissues, etc. and the **DNA extracted.**

2 DNA is cut into small, double-stranded fragments using **restriction enzyme** (HinfI in UK, HaeIII in USA), since these cut close to, but not within, the highly repetitive, 'nonsense' regions of DNA.

3 Resulting DNA fragments are of varying lengths, and are separated by **gel electrophoresis** into (invisible) bands.

4 Gel is treated with alkali to **split double-stranded DNA** into single strands.

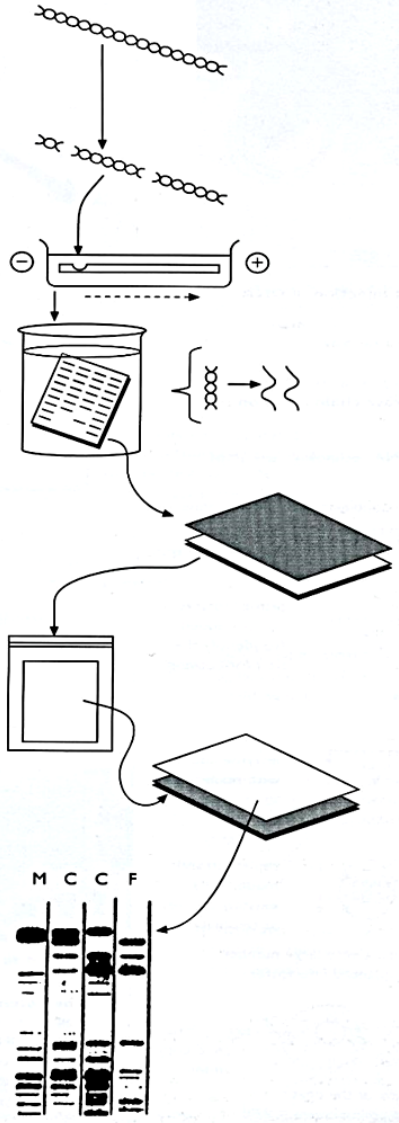
5 **Southern blotting** of gel copies the strands to a nylon membrane (see Figure 5.14).

6 **Heat treatment** of the nylon membrane binds the DNA copies to it.

7 Selected, **radioactively labelled DNA probes** are added to bind to particular bands of DNA – then excess probes are washed away.

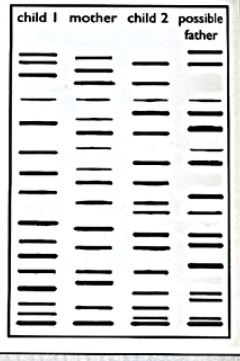
8 Nylon membrane is now **overlayed with X-ray film** which is selectively 'fogged' by emission from the retained labelled probes.

9 **X-ray film is developed**, showing up the positions of the bands (fragments) to which probes are attached.



6 In the 'genetic fingerprint' shown, is child 1 and/or child 2 the offspring of the parents shown here?

A genetic fingerprint of four individuals
In DNA samples from unrelated people only one in four 'fingerprint' bands match.

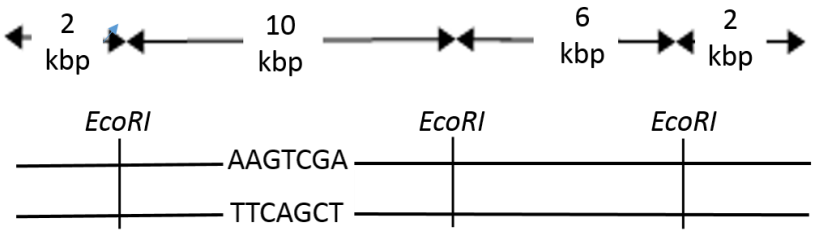


Note: DNA fingerprinting has been applied widely in biology. In ornithology, for example, a degree of 'promiscuity' has been established in nestlings reared by breeding pairs, the male of which was assumed to be father of the whole brood. In birds, the production of a clutch of eggs is extended over a period of days, with copulation and fertilisation preceding the laying of each egg. This provides the opportunity, in some species, for different males to fertilise the female.

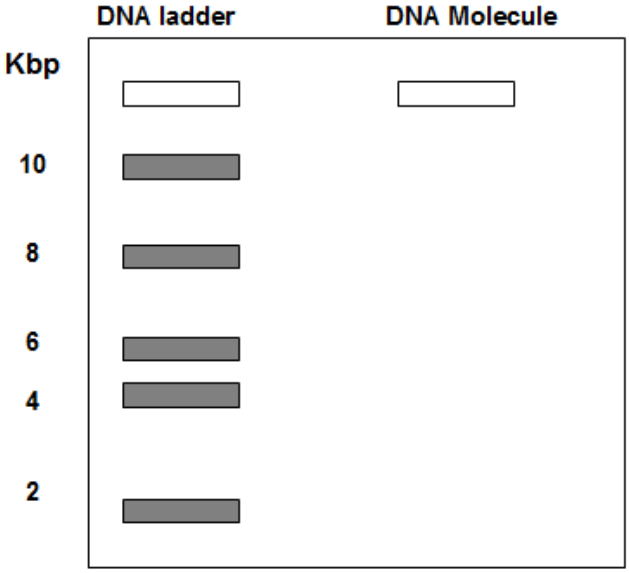
Fig 3.3: DNA fingerprints from a paternity testing case

Checkpoint 3: Detecting DNA fragments

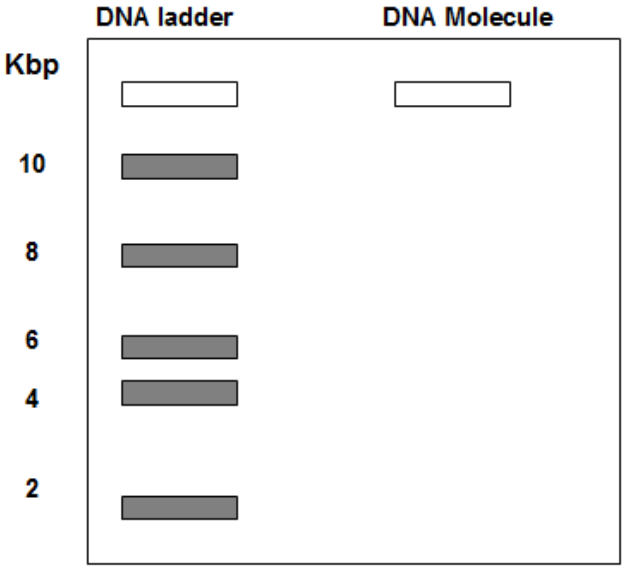
DNA molecule shown below contains **three restriction sites** for *EcoRI*.



Draw on the diagram below, the position of DNA molecule after restriction digestion with *EcoRI* and staining with ethidium bromide or DNA-binding dye.



Draw on the diagram below, the position of DNA molecule after restriction digestion with *EcoRI* followed by Southern blot and hybridization with a probe complementary to DNA sequence AAGTCGA .



End of lecture notes

Concept map

DNA analysis – PCR, gel electrophoresis, Southern blot and Nucleic Acid Hybridisation

A. PCR

- Function: To _____ a short sequence of DNA obtained from _____from a variety of sources, so that researchers can analyze the DNA.
- Procedure:
  - Occurs _____ (i.e. outside of cells), in a machine called the _____**.**
  - **One PCR cycle** consists of 3 stages. Describe the stages in the space below:

Stage 1: Denaturation of DNA strands (___ºC)

- Heat DNA mixture to ___**ºC** to _____ into **2** _____.
- This is done by _____ **between** _____ holding the strands together.

↓

Stage 2: Annealing of Primers (___ºC)

- ___ DNA mixture to ___**ºC.** This allows **complementary** _____ to _____to the ___ **ends of the single-stranded DNA template.**
- _____ **primers** present would **prevent the DNA strands from** _____**.**

↓

Stage 3: Primer Extension (___ºC)

- ____the temperature to ___**ºC**, elongation of the target DNA sequence occurs rapidly from _____ **direction**.
- Primers provide the _____ required by _____ to **add** _____ to the _____end of the primers. Both strands of DNA are replicated and thus, there are now **2 copies of the original** _____**.**
- Each cycle is **repeated** _____ **times**. Each time the cycle is repeated, the amount of DNA _____**.** Hence**,** there is **exponential increase of target DNA sequences** as products.

B. Agarose gel electrophoresis

- Function: To separate _____ according to their _____.
- Procedure:

1a. **Digestion:**
- Digest DNA samples using **specific** _____.

OR

1b. **PCR:**
- PCR DNA samples using specific _____ that anneal to ___ ends of regions that are _____ the target DNA sequence.

2. **Gel electrophoresis:**
- Mix digested DNA samples with _____ containing _____ (Function: _____ _____, and _____) and _____ (Function: _____ _____), and load samples in the well at one end of an agarose gel.
- Add _____ in a separate well (Function: serves as a _____ to _____ the fragment sizes).
- The DNA molecules which are _____-charged migrate towards the _____ **(**_____**)** electrode.
- **Longer** DNA fragments travel _____ and thus _____ through the _____ of the agarose gel.

3a. **Visualisation:**
- **Specific bands** that correspond to specific fragments can be visualized by _____ _____ (details in Section C).
- If radioactive probes are used, view DNA bands with _____.
- If Fluorescent probes are used, view under _____.

OR

3b. **Visualisation:**
- **ALL bands** of DNA can be visualized by adding _____ _____(non-selective intercalating agent/stain) to the gel.
- The bands are then viewed under _____.

C. Southern Blot and Nucleic Acid Hybrization

- Function of Southern Blot: To _____ **DNA fragments** from _____ to _____.
- Function of Nucleic Acid Hybridisation: To detect _____ through the use of a _____**- labelled** _____.
- Procedure:

1. Restriction Digestion:
- Digest DNA samples using _____ to produce _____ of various lengths.

↓

2. Gel electrophoresis (details in Section 2):
- Each sample is loaded into separate wells in an agarose gel, and the restriction fragments are _____ by molecular weight.

↓

3. Southern Blot:
- Transfer DNA fragments to a piece of **nitrocellulose membrane** by _____**.** It will contain DNA fragments in a pattern that is a _____ of the agarose gel.
- _____ the DNA on the _____ into _____ **DNA** by adding _____**.** This causes the _____ between the _____of a double helix to break.

↓

4. Nucleic acid hybridization:
- Incubate membrane with a *_____**-labelled** or ****_____**-labelled** ____ **or** ____ **probe** which is _____ to the _____ **DNA sequence.**
- Hence **complementary base pairing** *via* **hydrogen bonding** occurs between the _____ and the _____**.**

↓

5. Visualisation:
- The hybridized probe is detected by *_____ or **_____**.**