

Molecular Biology

-Practical-

(BCH 361)- Handout

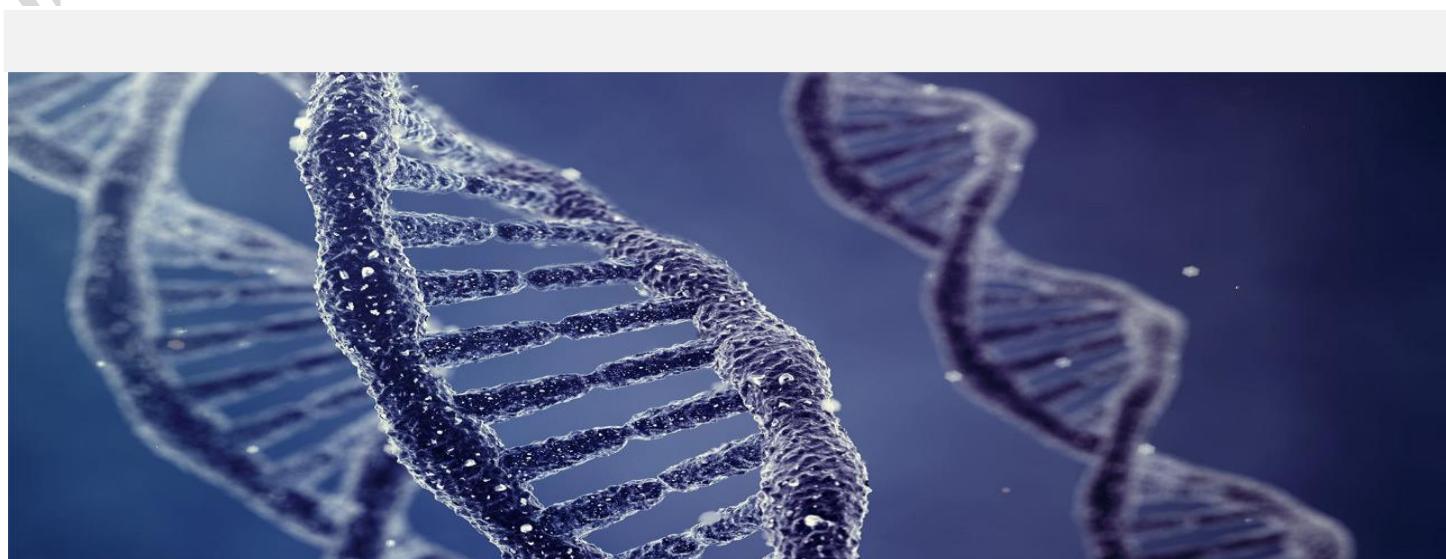




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Introduction to Molecular Biology

❖ What is Molecular Biology?

Molecular Biology is the study of biology at the molecular level. It is the study of essential cellular macromolecules, including **DNA, RNA, and proteins**, and the biological pathways between them (replication, transcription, translation). Researchers in Molecular Biology field, design and perform experiments to gain insight into how these components operate, organization and communicate.

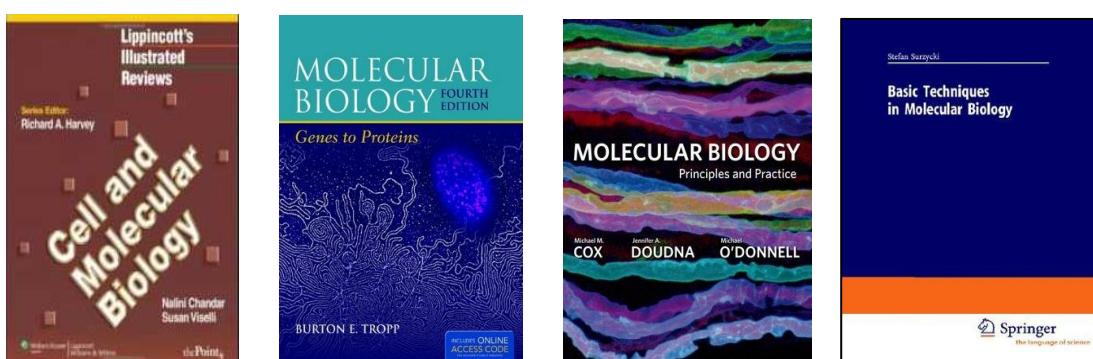
The techniques used for these studies are referred to as: “**Techniques of Molecular Biology**”.⁽¹⁾

❖ Why Understanding of Molecular Biology is Important?

Molecular biology may have a relatively short history, but its impact on the human experience is already considerable. Medicine, modern agriculture, forensic science, and many other efforts rely on **technologies developed by molecular biologists**. Our current understanding of information pathways has given rise to diagnostic tests for genetic diseases, forensic DNA analysis, crops with improved yields and resistance to disease, new cancer therapies, track pandemics, new treatment methods, new approaches to the generation of energy, and much more.⁽¹⁾

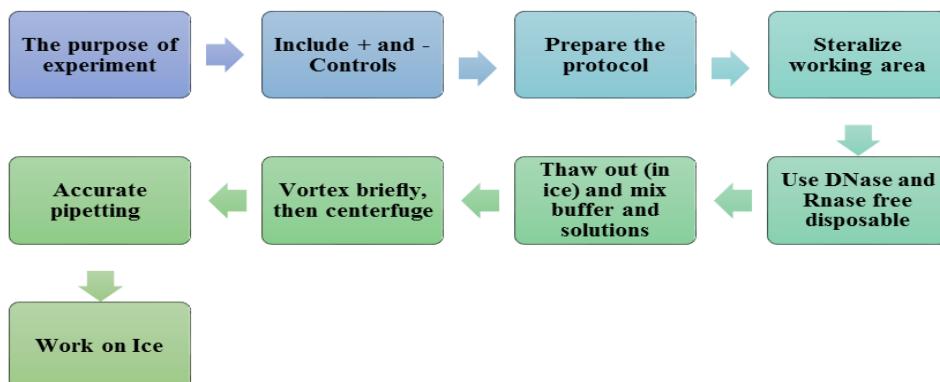
❖ Things you must to know as Molecular Biologist:

- Basic Molecular Biology background:



➤ Molecular Biology techniques.

For conducting a successful molecular biology experiment:



➤ Lab safety:

1. Before Start Working:

- Wearing of laboratory coats.
- Wear gloves and goggles when working with toxic chemicals or UV light.
- Disinfect your lab bench at the beginning using 70% ethanol.

2. During Working:

- Clean up as you proceed through experiments and keep your work area organized.
- Do not work with UV light on.
- Read the labels on the chemical that you are using carefully, some chemical are mutagens like ethidium bromide.

3. After Working:

- Wash your hands.
- Disinfect your lab bench at the beginning using 70% ethanol.
- Wash your glassware and organize your working area.

➤ Types of hazards in molecular biology lab:

1. Biological hazards:

Include human body fluids that may carry infections. All experiments with tissue and cell cultures should be conducted in microbiological cabinets that are provided with a sterile airflow away from the operator.

2. Chemical hazards:

All chemicals are, to varying extents, capable of causing damage to the body.

***Ethidium bromide (EtBr):** is a mutagen and a potential carcinogen and must be treated with respect. EtBr solutions can be handled safely as long as gloves are worn.

3. Physical, Electrical and Mechanical hazards:

- **Ultraviolet (UV) light.**
- **Electricity:** Electrophoresis experiments present a potential shock hazard. It is advisable not to touch any part of the apparatus while the unit is on.
- **Centrifugation:** certain that appropriate tubes or bottles are used containers not designed for centrifugation may shatter or collapse under the forces generated in centrifugation. Be certain that tubes and rotors are balanced.

➤ Website Sources of information:

- The most fundamental skill in bioinformatics is the ability to carry out an **efficient** and **comprehensive search** of the scientific literature to find out what is known about a specific subject.
- **Source of information:** Books, Articles, Websites.
- **Some academic research tools:**



- **Types of scientific articles:**

1. Primary research article:

It's a peer-reviewed report of new research on a specific question (or questions).

2. Review article:

Review articles are also peer-reviewed, and don't present new information, but summarize multiple primary research articles, to give a sense of the consensus, debates, and unanswered questions within a field. **It is better to start with it for reading new topic.**

➤ Writing a lab report.

References:

1. Cox M, Doudna J, O'Donnell M. Molecular Biology genes to proteins. p.3. (2012)

Experiment (1): Extraction of Genomic DNA from Rat Blood

Aim:

- To isolate pure genomic DNA from rat blood sample.

Introduction:

Genomic DNA constitutes the total genetic information of an organism. The genomes of almost all organisms are DNA, the only exceptions being some viruses that have RNA genomes. Genomic DNA molecules are generally large, and in most organisms are organized into DNA–protein complexes called chromosomes. The size, number of chromosomes, and nature of genomic DNA varies between different organisms. Genomic DNA contains genes, discrete regions that encode a protein or RNA. A gene comprises the coding DNA sequence, as well as the associated regulatory elements that control gene expression. Nuclear eukaryotic genes also contain noncoding regions called introns. The number of genes varies widely between different organisms.

DNA isolation is an essential technique in molecular biology; it is the first step in the study of specific DNA sequences, genomic structure, DNA fingerprinting, restriction fragment length polymorphism (RFLP), and PCR analysis. The quantity, quality and integrity of the isolated DNA will directly affect these results.

Sources for DNA extraction are very diverse, practically DNA can be isolated from any part of human body such as semen, saliva, hair roots, mouth swabs and even from several skin cells left on the surface after it has been touched. However, the most common sources are soft tissue or blood samples. There are many different methods which can be used to perform DNA extraction on such samples such as organic extraction, salting out, magnetic separation and silica based technology. The choice of a method depends on many factors: the tissue type, the concentration of DNA, sample number, safety of the experiment and cost. Regardless of the used methods, they happen to follow some common procedures aimed to achieve effective **cell lysis, proteins and RNA removal, and lastly DNA precipitation**. Resulting in a homogeneous DNA preparation that represent the entire genetic information contained within the cell.

 PAUSE AND THINK ➔ Can we obtain DNA from mature RBC? Why?

Principle:

Successful nucleic acid isolation protocols have been published for nearly all biological materials. They involve the physical and chemical processes of tissue homogenisation (to increase the number of cells or the surface area available for lysis), cell permeabilization, cell lysis (using hypotonic buffers), removal of nucleases, protein degradation, protein precipitation, solubilisation of nucleic acids and finally various washing steps. Cell permeabilization may be achieved with the help of non-ionic (non DNA-binding) detergents such as Triton.

Materials:

Chemical

Ethylene diamine tetra acetate (EDTA), NaOH, Tris-HCl, sucrose, MgCl₂, Triton X100, Sodium dodecyl sulphate (SDS), NaCl, Sodium perchlorate, TE buffer or double distilled water, cold chloroform, cold ethanol.

Preparation of solutions

1) 0.5 M EDTA, pH 8.0

Add 146.1 g of anhydrous EDTA to 800 ml of distilled water. Adjust pH to 8.0 with NaOH (about 20 g). Make up the volume to 1 L with distilled water.

2) 1 M Tris-HCl, pH 7.6

Dissolve 121.1 g of Tris base in 800 ml of distilled water. Adjust pH with concentrated HCl (about 60 ml). Make up the volume to 1 L with distilled water.

3) Reagent A (Red Blood Cell Lysis Solution)

Containing: 0.01M Tris-HCl (pH 7.4), 320 mM Sucrose, 5 mM MgCl₂, and 1% Triton X100.

Add 10 ml of 1 M Tris to 109.54 g of sucrose, 0.47 g MgCl₂ and 10 ml Triton X100 to 800 of distilled water. Adjust pH to 8.0; make up the volume to 1 L with distilled water.

4) Reagent B (White blood Cell Lysis Solution)

Containing: 0.4 M Tris-HCl, 150 mM NaCl, 0.06 M EDTA, 1% SDS, pH 8.0.

Take 400 ml of 1 M Tris (pH 7.6), 120 ml of 0.5 M EDTA (pH 8.0), 8.75 g of NaCl, adjust pH to 8.0 with NaOH. Make up the volume to 1 L with distilled water. Autoclave at 15 p.s.i. for 15 min. After autoclaving the mixture, add 10 g of SDS.

Protocol:

1. Place 3 mL of whole blood in a 15-mL falcon tube (centrifuge tube).
2. Add 12 mL of reagent A.
3. Mix on a rolling or rotating blood mixer for 4 min at room temperature (to prevent leakage, close the lid tightly).
4. Centrifuge at 3000g for 5 min at room temperature.
5. Discard supernatant without disturbing cell pellet. Remove remaining moisture by inverting the tube and blotting onto tissue paper.
6. Add 1 mL of reagent B and vortex briefly to re-suspend the cell pellet.
7. Add 250 μ L of 5 M NaCl and mix by inverting tube several times.
8. Place tube in water bath for 15 to 20 min at 65 °C.
9. Add 2 mL of ice-cold chloroform.
10. Mix on shaker for 20 min.
11. Centrifuge at 2400g for 2 min.
12. Transfer upper phase into a clean falcon tube using a sterile pipette.
13. Add 2 to 3 ml of ice-cold ethanol and invert gently to allow DNA to precipitate (if a cloudy did not form, add more ethanol).
14. Using a clean Pasteur pipette spool the DNA onto the hooked end.
15. Immediately transfer to a 1.5-mL microcentrifuge tube.
16. Spin the microcentrifuge tube at 6000 rpm for about 5 minutes.
17. Gently remove the supernatant (ethanol layer) without disrupting the DNA pellets, and leave it to dry.
18. Re-suspend in 200 μ L of TE buffer or doubled distilled water and label the tube.
19. As a final step in nucleic acid isolation, the yield and purity of the extracted nucleic acid may need to be determined (Lab No. 3).

Results:

Cloudy precipitation can be seen by the naked eye, and it represents the isolated genomic DNA.

References:

1. Surzycki S. Basic techniques in molecular biology. Springer. (2000).

Analysis Sheet #1

Name: _____

ID: _____

❖ Write the principle of genomic DNA isolation from blood as steps:

1. _____
2. _____
3. _____
4. _____

❖ Write the methodology:

Problem.1:

What is the purpose of the following? How it performs its role?

❖ EDTA: _____

❖ The cell lysis solution: _____

❖ 70% Ethanol: _____

Problem.2:

Isolated DNA should be free from contaminating proteins, heme and other cellular macromolecule, what precautions did you take to solve this situation?

Problem.3:

In this procedure, what other salts can be used instead of 5M sodium chloride?

Experiment (2): Genomic DNA Extraction from Plant Tissue

Aim:

- To isolate pure genomic DNA from plant tissue.

Introduction

Studying plant genome allows us to characterize and modify plant genes and metabolic pathways, as well as understanding the genetic variation in species. Transgenic (GM) plants are those that have been genetically modified using recombinant DNA technology. This may be to express a gene that is not native to the plant or to modify endogenous genes. The protein encoded by the gene will confer a particular trait or characteristic (eg: growth or survival) to that plant. The technology can also be used to improve the nutritional content of the plant, as well as production of some industrial products, such as monoclonal antibodies, vaccines, plastics and biofuels.

Method used for extracting DNA from plants is different from extracting DNA from animal sources as the plant contains hard cellulose cell wall and large DNA molecule. Essentially, breaking the cell wall and cellular membranes (lysis) using mechanical or non-mechanical methods to allow access to nuclear material, without its degradation. For this, usually an initial grinding step is employed to break down cell wall material and allow access to DNA while cellular enzymes are inactivated.

The main goal of developing number of protocol for isolating DNA from plant, is to extract DNA with pure and high quality. DNA must be purified from cellular material in a manner that prevents degradation.

 PAUSE AND THINK → How extraction of plant DNA differ from animal DNA?

Principle:

Breaking the cell wall and cellular membranes (lysis) by using mechanical or non-mechanical methods. Mechanical methods is to force the **cell wall** to open and spilling the contents. On the other hand, non-mechanical method is the addition of enzymes or chemicals that specifically break down cell wall components in combination with mechanical force. The advantage of mechanical disruption over the non-mechanical is that no chemicals are introduced that might interfere with the extracted substance, and these chemicals need to be removed from the sample afterwards.

After lysis, small cracks were formed in the **cell membrane** for accessibility of detergents. Detergents will break down the cell membranes due to the amphipathic (having both hydrophilic and hydrophobic regions) nature of both cellular membranes and detergent molecules.

The DNA is then precipitated from the protein in a subsequent step with isopropanol or ethanol. The clean DNA is now suspended in a 1XTE buffer or dd.H₂O.

Materials:

Chemical

Strawberry, Extraction solution, 96% Cold ethanol or isopropanol, and TE buffer or double distilled water.

Preparation of extraction solution

Add 100 ml detergent to 750 ml of distilled water and then add 11 g NaCl. Make up the volume to 1 L with distilled water.

Equipment and Glassware

Microfuge centrifuge, electronic balance, Razor blade, Mortar and pestle, cheesecloth, Funnel, Gradual cylinder 25 ml, Beaker 50 ml, Test tube, centrifuge tube, and Pasteur pipette, micropipette, tips.

Protocol:

1. After removing the green leaf of the strawberry, weight the plant using sensitive balance.
2. Place the plant onto a mortal. Chop it into small pieces using a clean razor blade.
3. Add the DNA extraction buffer on a 1:1 ratio (e.g. if the plant weight 20 g, we will add 20 ml of the solution).
4. Then mix the chopped strawberry pieces using a pestle for 5 minutes.
5. Pour the mixture through cheesecloth into clean beaker.
6. Pipette 2 ml of the mixture into a clean test tube.
7. On the same tube, slowly add 2 ml of cold ethanol (DON'T MIX).
8. DNA will appear as a clear white thread.
9. Using a clean Pasteur pipette, spool the DNA onto the hooked end.
10. Immediately transfer to centrifuge tube.
11. Spin the centrifuge tube at 6000 rpm for about 5 minutes.
12. Gently remove the supernatant (ethanol layer) without disrupting the DNA pellets, and leave it to dry.
13. Suspend the pellet in 0.5 - 1.5 ml TE buffer or double distilled water.

Results:

Cloudy precipitation can be seen by the naked eye, and it represent the isolated DNA.

References:

1. Surzycki S. Basic techniques in molecular biology. Springer. (2000).

Analysis Sheet #2

Name: _____

ID: _____

❖ Write the principle briefly as steps:

1. _____
2. _____
3. _____
4. _____

❖ Write the methodology:

Problem.1:

DNA from other sources like mitochondrion and chloroplast can precipitate out with your nuclear DNA; discuss how you can overcome this problem?

Problem.2:

What are possible sources that can contaminate your plant DNA sample?

Problem.3:

In general, what precautions you should keep in mind while isolating DNA?

Problem.4:

In general, why it is important to remove the ethanol completely before re-suspending DNA?

Experiment (3): Characterization of DNA by Spectrophotometric Assay and Melting Temperature (Tm)

Aim:

- Determination of the concentration and purity of extracted DNA using UV spectrophotometer.
- Determination of DNA melting temperature and GC content percentage.

Introduction:

DNA extracts must meet downstream applications requirements. For that after each extraction approach, DNA undergo characterization process, where quantity and quality (purity and intactness) must be measured. The characterization of DNA could be performed with a number of different techniques. In this experiment, spectrophotometric and melting temperature will be used to determine DNA concentration, purity, and GC content.

Characterization of extracted DNA by spectrophotometric assay: DNA concentration and purity can be determined by measuring the absorption of ultraviolet light. The DNA has a maximum and minimum absorbance at 260 nm and 234nm, respectively and the purines and pyrimidine in nucleic acid are responsible for these absorptions. At 260 nm double-stranded DNA has specific absorption coefficient of $0.02 \text{ } (\mu\text{g/ml})^{-1}\text{cm}^{-1}$. Moreover, the A_{260}/A_{280} ratio allow to detect nucleic acid purity from proteins contamination since proteins have maximum absorption at 280 nm. Highly purified DNA samples have a 260/280 nm ratio of (1.8-1.9), thus below (1.8) a significant amount of protein impurity may present within the sample. The A_{260}/A_{230} ratio determined to confirm that the sample is pure from carbohydrates, peptides, ethanol or any organic compounds, and it is usually between 2 and 2.2.

DNA melting temperature and GC content: The two strands of the DNA double helix separate when hydrogen bonds between the paired bases are disrupted and this can occur in vitro if the pH of the DNA solution is altered, or if the solution is heated. When DNA is heated, the double-stranded DNA (dsDNA) unwinds and separates into single-stranded (ssDNA) by breaking the hydrogen bonds between the bases (A=T and G=C). This process called DNA denaturation and it can be monitored by measuring its absorbance at 260 nm. The absorbance of DNA at 260 nm increases as the DNA becomes denatured, a phenomenon known as the hyperchromic effect. The opposite, a decrease of absorbance is called hypochromic effect. The Tm is the temperature at which 50% of the DNA is unpaired (denatured), and it is depending on both length and GC content of the DNA. The GC content of the DNA that is critical for its stability, and it can be provided by melting temperature (Tm) profile. This profile can be achieved by gradual denaturation of dsDNA into ssDNA.

 PAUSE AND THINK ➔ What is the principle behind hyper/hypochromic effect?

❖ Principle:

When a dilute aqueous DNA solution is heated slowly, the two strands of the double helix gradually separate, leading to the formation of a single stranded DNA (denaturation). It results in an increase in absorbance at 260 nm. Temperature for midpoint of denaturation gives Tm by increasing the temperature slowly and measuring absorbance at 260 nm as melting profile can be generated. The DNA of each species has a specific denaturation curve which is dependent on the % GC content and length. In double stranded DNA, G and C base pairing is more stable and requires more heat energy to break the three hydrogen bonds to separate the strands.

❖ Materials:

The extracted blood and plant DNA from previous experiments, 0.1 X SSC buffer.

Preparation of 20X SSC buffer

Dissolve 175.3 g of NaCl, 88.2g of sodium citrate dehydrate in 8000 ml distilled water. Adjusts pH to 7.0 with diluted HCl. Make up the final volume to 1 L by distilled water.

❖ Protocol:

A. Characterization of DNA by Spectrophotometric Assay (concentration and purity):

1. Prepare 1 ml of the sample by diluting your extracted DNA (your stock) in 0.1 X SSC buffer with 1:10 ratio.
2. Place the DNA sample in a quartz cuvette (why?) along with a second cuvette contains distal water as a blank, then set the spectrophotometer as follows:
Nucleic acid → DNA → 10 mm → µg/ml → yes → Enter the dilution factor.

OR

2. Traditionally, measure the absorbance at 230, 260 and 280 nm.

B. Melting Temperature of DNA:

1. In a test tube, prepare 1 ml of the sample by diluting your extracted DNA (your stock) to 10 µg/ml with 0.1 X SSC buffer.
2. In another test tube, pipette 1 ml of distilled water.
3. Cover the tubes by aluminium foil. Then place them into a water bath at 25°C and allow temperature to equilibrate (4 min).
4. Immediately, transfer the sample and the blank into quartz cuvette, re-place them in the water bath, and allow temperature to equilibrate for 1 min.
5. Immediately read the absorbance at 260 nm.
6. Raise the temperature of the water bath to 50 °C, 60 °C, 70 °C, and boiling, then repeat steps 3-5.

Results:

A. Characterization of DNA by Spectrophotometric Assay (concentration and purity):

Wavelength (nm)	Absorbance of DNA	
	Rat	Plant
230		
260		
280		

➤ Find out the concentration of the DNA samples using the following equation:

$$\text{Concentration of DNA } (\mu\text{g/ml}) = (A_{260} / \epsilon L) \times \text{Dilution factor (DF)}$$

➤ Determine the purity of the DNA samples by calculating A_{260}/A_{280} and A_{260}/A_{230} ratios.

B. Melting Temperature:

Temperature ($^{\circ}\text{C}$)	DNA Absorbance at 260 nm	
	Rat	Plant
25		
50		
60		
70		
Boiling		

➤ Plot the value of absorbance vs. temperature and calculate the T_m for sample DNA.

➤ Find out the GC content of your sample using the following formula:

$$(G + C)\% = (T_m - 69.3) \times 2.44$$

References:

- Surzycki S. Basic techniques in molecular biology. Springer. (2000).

Analysis Sheet #3

Name: _____

ID: _____

❖ Write the principle as diagram:

Problem.1:

How pure is your DNA samples? Reflect on the possible sources of contaminations of your samples? What your sample should have?

Problem.2:

Discuss the curve of your melting temperature, is it the same pattern that it supposes to be or not and why?

Problem.3:

Compare between the melting temperatures of DNA obtained from the two different sources.

Problem.4:

Answer the following:

- ❖ What does the ratio of AT/CG tells you?

- ❖ What is the relationship between the DNA melting temperature and GC content?
Explain the reason behind this relationship?

Experiment (4): Agarose Gel Electrophoresis

Aim:

- Evaluating the intactness of the extracted DNA by agarose gel electrophoresis.
- To separate and calculate the molecular size of DNA fragment by comparing the separated bands with known standard molecular weight marker.
- To quantify DNA fragment by comparing the separated band with known quantity of DNA.

Introduction:

Agarose gel electrophoresis is method for separation (by size), quantifying, purification of nucleic acids fragments mixture, and analysis of DNA restriction fragments. It is one of the most widely-used techniques in biochemistry and molecular biology. Agarose is a liner polymer composed of alternative residues of D-galactose and 3,6-anhydro-L-galactopyranose joined by α (1→3) and β (1→4) glycosidic linkages. Agarose and acrylamide matrices are used to separate DNA by gel electrophoresis. The choice of gel matrices and gel concentration depends on the size of nuclear acid molecules, as the concentration of the agarose or acrylamide determine the pores size:

w/v % Gel type	Size of DNA fragments (Kb = 1000 bp)
0.5 %	1 kb to 30 kb
0.7 %	800 bp to 12 kb
1.0 %	500 bp to 10 kb
1.2 %	400 bp to 7 kb
1.5 %	200 bp to 3 kb
2.0 %	50 bp to 2 kb

 PAUSE AND THINK ➔ What is the relation between the concentration of the gel and the pores size?

Under physiological conditions, DNA is a negatively charged molecule due to the presence of phosphate groups in the backbone. Therefore, in aqueous media, under the influence of an electrical field, DNA molecules will move through an agarose matrix towards the positively charged anode, at a rate that is inversely proportional to the molecular weight. The electrophoretic migration rate of DNA through agarose gel depends on the following: size of DNA molecules, concentration of agarose gel, voltage applied, conformation of DNA, and the buffer used for electrophoresis.

Several buffers are used for agarose gel electrophoresis, but the most common are: Tris-acetate EDTA buffer (TAE) and Tris-borate EDTA buffer (TBE). The DNA mobility in TBE buffer is approximately two times slower than in TAE buffer. This is due to the lower porosity of agarose gel when agarose polymerizes in the presence of borate.

Since DNA is colourless, the loaded sample need to be tracked. This is achieved by using a loading dye solution. Finally, to visualize DNA (results), agarose gels are usually stained with ethidium bromide and illuminated with UV light.

PAUSE AND THINK ➔ How the DNA is visualized by ethidium bromide?

Identifying the size of a DNA sample is one of the common AGE uses and this accomplished through what called: DNA marker (Ladder). A DNA and RNA size markers contain a mixture of DNA (or RNA) fragments of known length, making them suitable for estimating the fragment length of concurrently run samples.

Principle:

Nucleic acids are separated by applying an electric field, so these negatively charged molecules will move through an agarose matrix towards the anode, and the biomolecules are separated by size in the agarose gel matrix, where the distance travelled by a DNA molecule is inversely correlated with its size.

Materials:

Agarose powder, 1X TBE buffer (89 mM Tris-base, 89 mM boric acid and 2 mM EDTA) prepared from 10X TBE, Ethidium Bromide (5 mg/ml), Gel loading dye (Glycerol and orange dye), 1 kb and 100 bp DNA ladder, horizontal electrophoresis apparatus and power supply.

Protocol:

1. Measure the desired grams of agarose to make 1% agarose gel.
2. Heat the solution to boiling in the microwave to dissolve the agarose to produce a homogeneous mixture.
3. Add 4 µl of ethidium bromide CARFULLY to the dissolved agarose and mix .
4. Get a gel plate and a comb. Put the two dams into the slots on each side of the gel plate. Make sure that they fit tight. Pour the melted agarose onto the gel plate in the electrophoresis tray.
5. Place the comb in its place. Let the gel cool to room temperature.
6. Place the gel in the electrophoresis chamber.
7. Pour enough electrophoresis buffer (1X TBE) to cover the gel to prevent overheating of the gel.

8. Carefully remove the comb.
9. Prepare the DNA sample by mixing around 300 ng of DNA sample with 3-4 μ l of loading dye.
10. Add 3 μ l DNA ladder into the first well by using a micropipette.
11. Carefully place the prepared samples into adjacent wells
12. Electrophorese the samples at 95 V for 45 minutes. (Check the gel while it is running).
13. Carefully remove the gel, place it onto the UV light box and take a picture for the gel.

Results:

Picture of the gel.

References:

1. Surzycki S. Basic techniques in molecular biology. Springer. (2000).

Analysis Sheet #4

Name: _____

ID: _____

❖ Write the principle as steps:

1. _____
2. _____
3. _____
4. _____

❖ Write the methodology:

Problem.1:

What is the role of the following chemicals in the loading dye:

❖ Bromophenol blue / orange dye:

❖ Glycerol/Sucrose:_____

Problem.2:

1. Discuss the mobility of different sizes and shape of the DNA on the agarose gel, and which band is closest to the positive electrode and which is the closest to the negative electrode?

2. Why do you think selection of the right percentage of agarose gel is crucial in separation of the DNA sample?

3. Why should we use low agarose concentration gels to separate large DNA fragments?

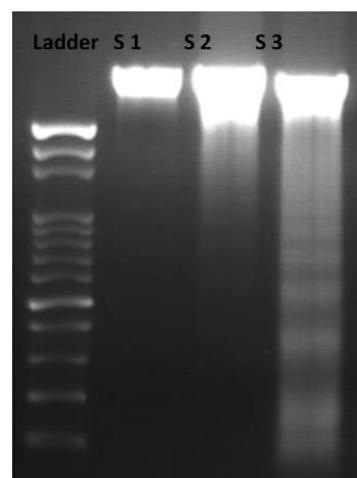
Problem.3:

Below is an agarose gel electrophoresis result of genomic DNA. Comment on each sample.

❖ S1: _____

❖ S2: _____

❖ S3: _____



Experiment (5): Polymerase Chain Reaction (PCR)

Aim:

- Amplification of a specific region on DNA.
- Primer design.
- Determine the parameters that may affect the specificity, fidelity and efficiency of PCR.

Introduction:

Nucleic acid **amplification** is an important process in biotechnology and molecular biology and has been widely used in research, medicine, agriculture and forensics.⁽¹⁾ In order to study individual genes or specific DNA regions of interest, it is often necessary to obtain a large quantity of nucleic acid for study, rather than isolate a single copy of the target DNA from a large number of cells. It is often more useful to generate multiple copies of a target from a single molecule of DNA or mRNA, via an in vitro amplification method.⁽²⁾

What is PCR (DNA photocopier)?

Polymerase chain reaction (PCR), a process conceived by Kary Mullis in 1983.⁽³⁾ It is a laboratory version of DNA replication in cell where **particular piece of DNA** can be **amplified** in billions of copies in a short time. The PCR amplify a precise fragment of DNA from a complex mixture of starting material **termed the template DNA** which controlled by heating and cooling. It does require the knowledge of some DNA sequence information which flanks the fragment of DNA to be amplified (**target DNA**).⁽⁴⁾

 PAUSE AND THINK ➔ How you will determine your target sequence?

To amplify a specific piece of DNA (target DNA), **two synthetic oligonucleotides are synthesised** called **primers** each complementary to a stretch of DNA to the 3' side of the target DNA, one oligonucleotide for each of the two DNA strands (DNA polymerase can add a nucleotide only onto a preexisting 3'-OH group).⁽⁵⁾

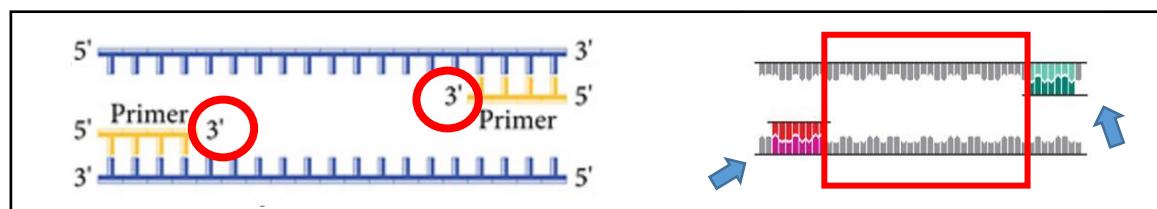


Figure 1. Two primers are prepared, complementary to sequences on opposite strands of the target DNA.

So, PCR **does not copy all of the DNA** in the sample. It copies only a very specific sequence, targeted by the PCR primers.

PCR components:

Basic PCR reaction requires four components:

- (1) DNA template.
- (2) Primers.
- (3) Deoxynucleotide triphosphates (dNTPs).
- (4) Thermostable DNA polymerase.

The below table illustrate the function of each components and other needed PCR components.

Table.1. Basic PCR components and the function of each one.

Components	Function
Template DNA	The template carries the DNA segment or (target) you wish to amplify.
Forward and Reverse Primers	A primer is a short, single-stranded piece of DNA that anneals (attaches) to its complementary sequence on the template. A pair of primers will bind to either side of the target DNA segment providing initiation sites for DNA synthesis. → e.i. Providing initiation site + specify the amplification to the target DNA segment.
Thermostable DNA polymerase	This is the enzyme used to synthesize new strands of DNA. The DNA polymerase adds nucleotides onto the end of an annealed primer. → PCR uses a heat-stable DNA polymerase, such as the <u>Taq polymerase</u> , which remains active after every heating step and does not have to be replenished. It is named after the thermophilic bacterium <i>Thermus aquaticus</i> from which it was originally isolated. ⁽⁷⁾
dNTPs	These are the four nucleotides used by DNA polymerase to extend an annealed primer (building blocks).
Magnesium	DNA polymerase requires magnesium for activity (co-factor). Magnesium is usually supplied to a PCR amplification in the form of magnesium chloride.
PCR buffer	PCR buffer is necessary to create optimal conditions for activity of Taq DNA polymerase.

* Additional reagents may include like, DMSO, BSA, potassium salt K⁺ or glycerol.

In addition PCR require a special PCR tubes and a device called **thermal cycler** to perform the heating and cooling cycles (see below).

PCR cycle:

PCR proceeds in THREE distinct steps Governed by Temperature:

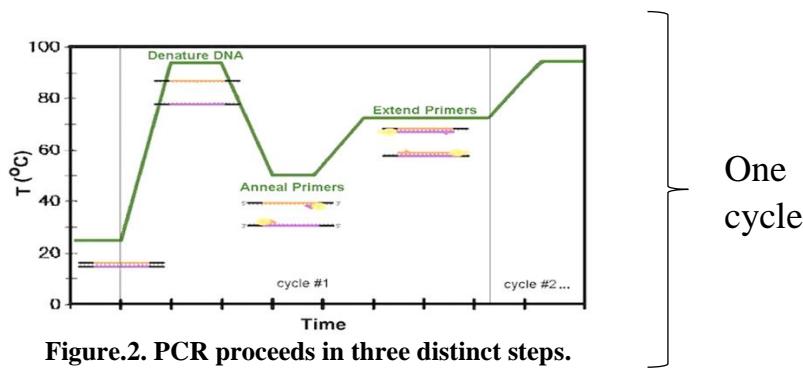


Figure 2. PCR proceeds in three distinct steps.

1. Denaturation:

This is the first step in cycling event and consists of heating the reaction to 94–97 °C for 20–30 seconds. This causes DNA melting, or denaturation, of the double-stranded DNA template by breaking the hydrogen bonds between complementary bases, yielding two single-stranded DNA molecules.



2. Annealing:

The reaction temperature is cooled to (50-65°C)* for 15-60 seconds allowing annealing of the primers to the single-stranded DNA template. The single strands of the template are too long and complex to be able to reanneal during this rapid cooling phase. Stable hydrogen bonds are only formed when the primers anneal to sequences that are complementary to them. During this annealing step the thermostable DNA polymerase will be active to some extent and will begin to extend the primers as soon as they anneal to the template.

PAUSE AND THINK ➔ What determine the annealing temperature? See PCR optimization



3. Extension/elongation:

The reaction is heated to a temperature depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 72-80°C and commonly a temperature of 72°C is used with this enzyme (optimum temperature). At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTP's that are complementary to the template in 5' to 3' direction. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified.



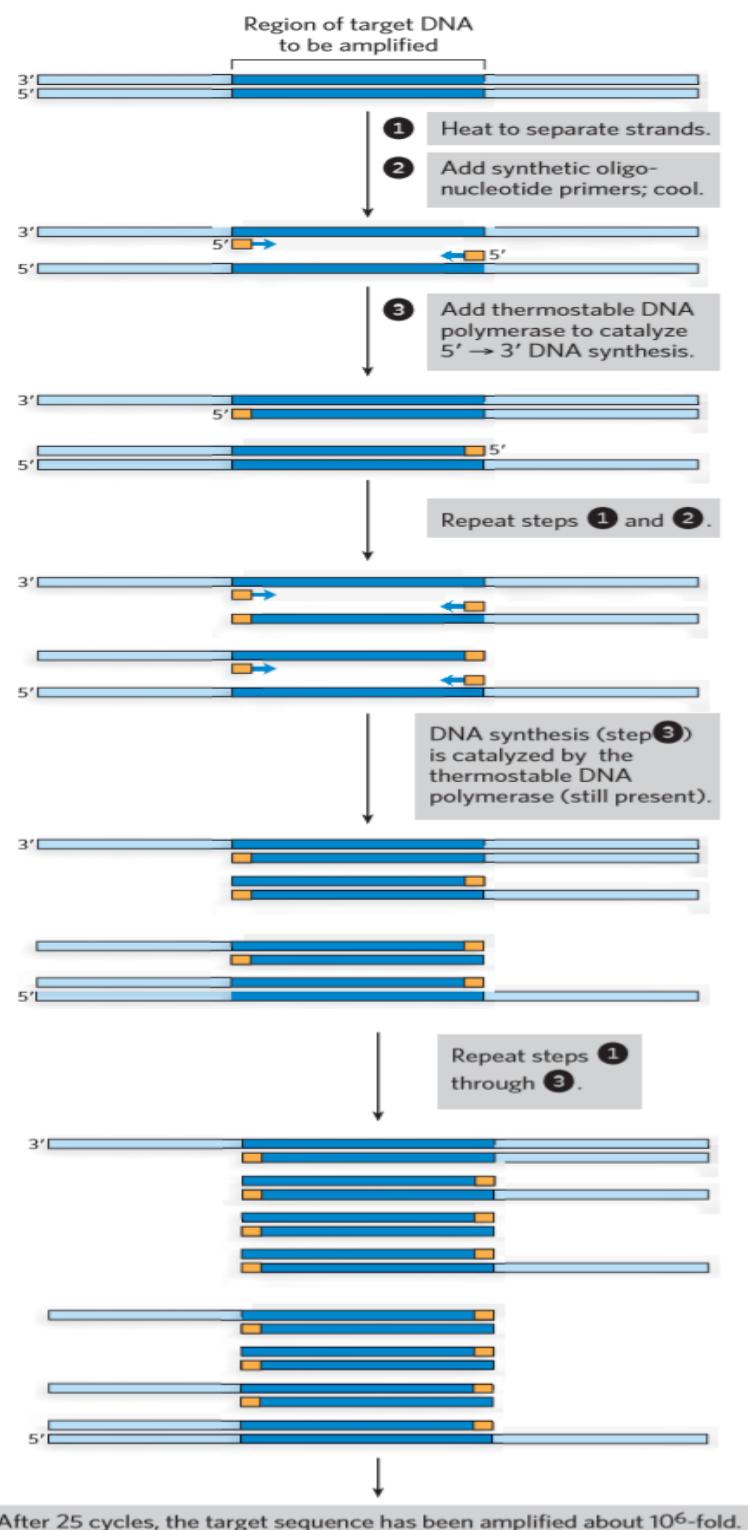


Figure 3. Amplification of DNA segment by PCR.⁽⁵⁾

At the end of the PCR reaction, the specific sequence will be accumulated in billions of copies called **amplicons**. In only 20 cycles, PCR can produce about a million (2^{20}) copies of the target. [Number of copies -amplicons- = $2^{\text{number of cycles}}$]

❖ Performing PCR steps:

1. Identification the location of the target sequence in the DNA template.
2. Primer design and primer specificity.
3. PCR optimization.
4. Post-PCR analysis results using agarose gel electrophoresis (AGE).
5. PCR troubleshooting.
6. Start your PCR and visualize the results by AGE.

❖ Primer design:

To design a primer, many parameters should be considered including: primer sequence, length, GC content, melting temperature, annealing temperature and GC clamp.⁽⁸⁾

1. Primer sequence:

Primers must be complementary to flanking sequences of target region. Avoid repeat (ex: ATATATAT) and run (ex: AGCGGGGGAT) sequence which leads to misprime, maximum number is 4 di/nucleotides respectively. It is important to be sure that primer is match to the target sequence at the 3' end. In addition, complementary sequences between primers should be avoided to prevent primer dimer (self and cross dimers). Avoid cross homology sequences that lead to nonspecific amplification.

2. Primer length:

It is generally accepted that the optimal length of primers is 18-25 bp. Length should be long enough for adequate specificity and short enough for primers to bind easily to the template at the annealing temperature. It prefers that the two primers have a similar length.

3. GC content:

GC% = the number of G's and C's in the primer as a percentage of the total bases. Optimal GC content should be ranged 40-60%. GC percentage can be calculated by using the below formula:

$$\text{GC\%} = [(G + C) / (G + C + A + T)] \times 100$$

4. Melting temperature (Tm):

The melting temperature (Tm) is defined as the temperature at which half of the DNA strands are in the single-stranded (ssDNA) state. Melting temperatures in the range of 50-60 °C generally produce the best results. The Tm for both primers should differ by no more than 5 °C (difference between forward and reverse primer). The Tm of the primer can be calculated by the following formula:

$$T_m = [(G + C) \times 4] + [(A + T) \times 2]$$

As shown, the GC content of the sequence gives a fair indication of the primer Tm. In addition there are some online tools that calculate the Tm.

5. Annealing temperature (Ta):

The primer melting temperature is the estimate of the DNA-DNA hybrid stability and critical in determining the annealing temperature. Too high Ta will produce insufficient primer-template hybridization resulting in low PCR product yield. Too low Ta may possibly lead to non-specific products caused by a high number of base pair mismatches.

6. GC clamp:

Presence of G or C bases within the last five bases from the 3' end of primers. It promote a specific binding at the 3' end. GC clamp should be not more than 2 G's or C's .



There are different tools for primer design, the following are two of the most used tools:

1. NCBI Primer design tool: <https://www.ncbi.nlm.nih.gov/tools/primer-blast/>
2. Primer3 or primer3Plus: <http://bioinfo.ut.ee/primer3-0.4.0/primer3/>

After designing the primer, the **specificity of the primer** should be checked to avoid amplification of related pseudogenes or homologs. It could be useful to run a BLAST on NCBI to check for the target specificity of the primers.

❖ PCR Optimization :

There is no single set of conditions that is optimal for all PCR reactions. PCR optimization means to find the most effective condition. This part will be discussed in the next lab.

❖ Post-PCR analysis:

Once the PCR has finished, you need to analyze the products (amplicons). The usual way of doing this is to size fractionate the DNA through an agarose gel. Examining the gel provides evidence for success or failure. The concentration of agarose depends on the product size.⁽⁹⁾

 PAUSE AND THINK ➔ How you will make sure that your target sequence is amplified?

By knowing the target sequence size (product size).

❖ Advantages of PCR:

Simplicity, easier methodology, sensitive, extensively validated standard operating procedure and availability of reagents and equipment.⁽¹⁾

❖ PCR application:

The polymerase chain reaction has been elaborated in many ways since its introduction and is now commonly used for a wide variety of applications including: genotyping, cloning, mutation detection, sequencing, microarrays, RT-PCR, forensics, and paternity testing. ⁽⁶⁾

❖ Supporting materials:

1. Polymerase Chain Reaction: Basic Protocol Plus Troubleshooting and Optimization Strategies: <http://www.bio-rad.com/en-sa/applications-technologies/pcr-troubleshooting>
2. PCR animation: <https://www.youtube.com/watch?v=DkT6XHWne6E>
3. PCR troubleshooting: <http://www.bio-rad.com/en-sa/applications-technologies/pcr-troubleshooting>
4. History of PCR: http://siarchives.si.edu/research/videohistory_catalog9577.html

❖ References:

1. Fakruddin M, Mannan KS, Chowdhury A, Mazumdar RM, Hossain MN, Islam S, et al. Nucleic acid amplification: Alternative methods of polymerase chain reaction. J PHarm Bioallied Sci. 2013;5:245-52.
2. <https://www.neb.com/applications/dna-amplification-pcr-and-qpcr>
3. Mullis KB. The unusual origin of the polymerase chain reaction. Sci Am. 1990;4: 56-61.
4. <http://himedialabs.com/TD/HTBM016.pdf>
5. Cox M, Doudna J, O'Donnell M. Molecular Biology genes to proteins. p.226. (2012).
6. <http://www.bio-rad.com/en-gu/applications-technologies/pcr-polymerase-chain-reaction>
7. Chien A, Edgar DB, Trela JM. Deoxyribonucleic acid polymerase from the extreme thermophile Thermus aquaticus. J Bacteriol. 1976;3: 1550-7.
8. <https://www.neb.com/tools-and-resources/usage-guidelines/guidelines-for-pcr-optimization-with-onetaq-and-onetaq-hot-start-dna-polymerases>
9. Mcpherson M, Møller S. PCR. Garland Science. (2007).

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- ❖ Write the principle as one paragraph:

Problem.1:

What is the purpose of the polymerase chain reaction?

Problem.2:

What is the role of each of the following in the PCR reaction:

- ❖ Heating around 95°C: _____
- ❖ Primers: _____
- ❖ Thermostable DNA Polymerase: _____
- ❖ Heating around 72°C: _____

Problem.3:

Regarding the following primers:

Forward: 5'-CTG AAC CCC ATG TGG AAC GA-3' Tm: _____ GC%: _____

Reverse: 5'- GGC ATC CAT CAC CTA GCT ACA-3' Tm: _____ GC%: _____

1. Calculate the Tm and GC% for each one. Is the Tm of the two primers are similar? Why it is important to be similar?

Problem.4:

How you will choose the concentration of the agarose to see your PCR product?

Problem.5:

Identify the error/s and the outcome/s of it in each primer pair of the following:

Primer pair.1:

Forward: 5'- GATACGTCATCTGGCGGA – 3' Tm: 57.9 °C

Reverse: 5'- TGCCTGTTTTCTGCCAAG – 3' Tm: 59 °C

➔ Error/s: _____

➔ Outcome/s: _____

Primer pair.2:

Forward: 5'- GATTAAGGATCGCTGGCGGA – 3' Tm: 61.6 °C

Reverse: 5'- TGCCTGTCATCTCTGCCAAG – 3' Tm: 61.8 °C

➔ Error/s: _____

➔ Outcome/s: _____

Primer pair.3:

Forward: 5'- GATTAAGGATCGCTTAATC – 3' Tm: 50.6 °C

Reverse: 5'- TGCCTGTCATCACTGTCAAG – 3' Tm: 59.4 °C

➔ Error/s: _____

➔ Outcome/s: _____

Experiment (6): Optimization of Annealing Temperature

❖ Aim:

- To optimize different parameters that effects PCR results/performance.
- Optimization of PCR annealing temperature.
- Be familiar with PCR technique and thermal cycler device.

❖ Introduction:

PCR optimization means to find the most effective and optimum conditions. Failure to amplify under optimum conditions can lead to the generation of multiple undefined and unwanted products, even to the exclusion of the desired product.⁽¹⁾ When developing a protocol for PCR amplification of a new target, it may be important to optimize all parameters including reagent concentrations, cycling temperatures, and cycle number.

 PAUSE AND THINK → There is no single set of conditions that is optimal for all PCR reactions. Why?

❖ PCR Optimization :

In PCR optimization, you need to optimize:

- (1) PCR components (reagents) concentration.
- (2) Thermal cycling condition.

In this lab, we will briefly review the conditions that should optimized and the focus will be on the annealing temperature optimization.

1. PCR components concentration:

Optimization of PCR reagents aim to find out the most optimum concentration of all PCR component including, primer concentration, MgCl₂, DNA template...etc. It is important to note that while optimization of one parameter, other parameters should be fixed and not changed (one factor at a time). Generally, the concentration of PCR components should be as shown in the **table.1**.

 PAUSE AND THINK → How you will know that you reached to the optimum conditions?

Table.1. Standard concentrations of PCR components.

Component	Concentration
Taq polymerase	0.5–2.0 units, ideally 1.25 units.
dNTPs	Typical concentration is 200 μM of each dNTP.
Magnesium	1.5-2.0 mM is optimal for Taq DNA Polymerase.
Forward and reverse primers	Typically 0.1-0.5 μM of each primer.
DNA Template	1ng–1μg of genomic templates.

2. Thermal cycling condition:

Optimization of thermal cycling condition aims to reach optimum cycling temperatures, duration of each step in PCR and number of cycles. Setting up the thermal cycling conditions is divided to three stages. **First stages** is initial denaturation, a typical reaction will start with a three minutes denaturation at 94-97°C, this stage aim to denature the template and activate “hot-start” DNA polymerase. **Next stage** is the three PCR steps (denaturation, annealing and elongation), which will be repeated from 25 to 35 cycles. **Last stage** is the final elongation phase; a period of 5 minutes or longer to allow synthesis of many uncompleted amplicons to finish. **Table.2** shows the general PCR thermal cycling condition.

Table.2. General PCR thermal cycling condition.

Step	Temperature	Duration	Cycle
Initial denaturation	94–97 °C	3 min	x1
Denaturation	94–97 °C	30 sec	x (25-35)
Annealing	50-65 °C*	30 sec	
Elongation	72-80 °C◊	30-60 sec	
Final elongation	75-80 °C	5-7 min	x1

* Depend on the primer annealing temperature.

◊ Depend on DNA polymerase optimum temperature.

From all, optimizing the annealing temperature of your PCR assay is one of the most critical parameters for reaction specificity.

❖ Optimization of annealing temperature (Ta):

The purity yield of the reaction products depend on several parameters, one of which is the annealing temperature (Ta). Reaching the optimum Ta is critical for reaction specificity, as non-specific products may be formed as a result of non-optimal Ta.⁽²⁾ Optimization done by applying **temperature gradient PCR**, where PCR carried with different Ta starting at 5 °C below the lowest calculated melting temperature (Tm) of the primer pair. For example if your primer Tm is 58 °C, you will start from 53 °C and you will increase the temperature for 8 degree, so the Ta is often fall in the range of 53 - 60 °C.

 PAUSE AND THINK ➔ When optimizing Ta what you should do with other PCR component?

❖ Materials:

PCR buffer, DNA Taq polymerase, dNTPs, MgCl₂, primers, DNA template, Nuclease free water.

❖ Protocol:

- Start by applying the standard concentration of PCR component that work with majority of PCR reaction. Use the below table to calculate the needed volume of each PCR component.

Components	Stock concentration	Final concentration	Volume per reaction (μl)
PCR buffer	10X	1X	
Taq polymerase	5 U/μl	0.05 U/μl	
dNTPs	10 mM	200 μM	
MgCl ₂	25 mM	1.5 mM	
Forward primer	10 μM	0.4 μM	
Reverse primer	10 μM	0.4 μM	
DNA Template	45 ng/ μl	90 ng	
Water			
Total volume			50 μl

- Prepare a master mix that contains everything except the DNA template by multiplying the volume per reaction of each component by (number of desired reaction +1 for pipetting error).

Volume per reaction (μ l)	Master mix (Volume per reaction x)
50 μ l μ l

3. Using special PCR tubes, distribute the master mix by pipetting μ l to each tube.
4. Add the DNA template for each template.
5. Centrifuge the tubes briefly.
6. Set the thermal cycling condition as following:

Step	Temperature	Duration	Cycle
Initial denaturation	94 °C	3 min	x1
Denaturation	94 °C	30 sec	
Annealing	____ - ____ °C	30 sec	x 25
Elongation	72 °C	30 sec	
Final elongation	72 °C	5 min	x1
Storage	4 °C	∞	

7. Try different 8 annealing temperatures depending on your primer pair Tm.
8. Set the final volume in the thermal cycler to be 50 μ l.
9. Start PCR.

❖ Results:

Analyse the results using 2% agarose gel, and determine the optimum Ta.

❖ References:

1. Roux KH. Optimization and troubleshooting in PCR. PCR Methods Appl, 1995;5:185-94.
2. Rychlik W, Spencer WJ, Rhoads R. Optimization of the annealing temperature for DNA amplification in vitro. Nucleic Acids Res, 1990;21: 6409-12.

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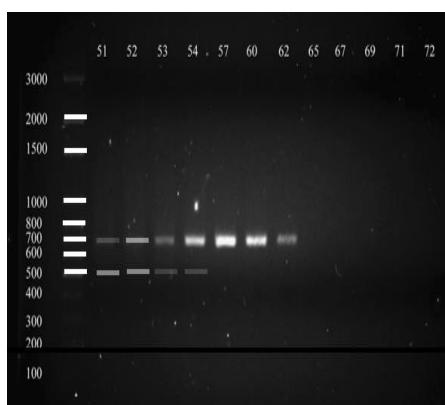
- ❖ Write the methodology:

Problem.1:

What does a PCR optimization means?

Problem.2:

You are working on a gradient PCR to determine the optimum annealing temperature for your primer, and you get the following result.



- ❖ What is the optimum annealing temperature if you know that the expected amplicon size is 700 bp? Why?

-
-
- ❖ Can 52°C or 62 °C be an optimum annealing temperature? Why?

Problem.3:

What is the objective of gradient PCR?

Problem.4:

What does a PCR master mix contain?

Problem.5:

Your target is to amplify a promoter region of *TRF2* gene. You design the following primer pair:

Forward: 5'- CAGCGCTGCCTGAAACTC-3' Tm: 58.6 °C, length: 18 bp , GC%: 61.11%

Reverse: 5'- GTCCTGCCCTTCACCTT-3' Tm: 59 °C, length: 18 bp, GC%: 61.11%

Product size: 200 bp

-You get the following result:



1. What is the best annealing temperature? Why?
-

2. Comment on each temperature.
-
-
-

3. What do you think the agarose concentration will be in this experiment?
-

4. What is the next step after you know the optimum temperature and concentrations of the PCR component?
-

Experiment (7): PCR Troubleshooting

Aim:

- Be familiar with common PCR difficulties.
- PCR troubleshooting.

Introduction:

PCR troubleshooting is a collection of techniques that alter PCR reactions in order to achieve optimum PCR results. Even with the simplest PCR reaction things can go wrong, so you need to have a good checklist of ideas for PCR troubleshooting and rectifying the problem. Fixing the error is done by changing the parameters (PCR components and thermal cycling condition) that discussed in the previous lab.

Common Issues in PCR:

Common issues of PCR is usually fall in the three following categories:

1. No or low amplification (no band or faint band).
2. Non-specific band or primer dimer.
3. Incorrect product size.
4. Smeared Bands.

The possible causes of each error are listed below, depending on the error, PCR troubleshooting could performed.

1. No or low amplification (no band or faint band):

Causes related to cycling condition	Causes related to PCR components
Too Few cycles were used.	No enough template was in the reaction.
Extension time was too short.	Primer concentration was too low.
Incorrect annealing temperature.	Impure primers, dNTPs, or water.
Denaturation temperature was too low	PCR product has high GC content.
	Primers were designed or synthesized incorrectly.
	Not enough Mg ²⁺ .

2. Non-specific band or primer dimer:

Causes related to cycling condition	Causes related to PCR components
Annealing temperature was too low.	Too much primer was added.
Too many cycles were used.	Too much Mg ²⁺ was added.
Extension time was too long.	Primers were designed or synthesized incorrectly.
	Impure primers, dNTPs, or water.

To identify Primer dimers, it always has a very low molecular weight (less than 100 bp usually).

3. Incorrect product size:

To have a single band, however it is not the same size of your target.

Causes related to cycling condition	Causes related to PCR components
Incorrect annealing temperature	Mispriming.
	Improper Mg ²⁺ concentration.
	Impure primers, dNTPs, or water.
	Primers were designed or synthesized incorrectly.

4. Smeared Bands:

Causes related to cycling condition	Causes related to PCR components
Too many cycles were used.	Too much template was added.
	Impure primers, dNTPs, or water.
	Template contained an exonuclease or was degraded.

❖ Common PCR additive reagents:

Additive reagents may yield results when all else fails. Understanding the reagents and what they are used for is critical in determining which reagents may be most effective in the acquisition of the desired PCR product. The following is a list of some of the common additives and the purpose of them.⁽²⁾

1. Additives that benefit GC Rich templates:

1. 1-10% DMSO (Dimethylsulfoxid):

In PCR experiments in which the template DNA is particularly GC rich (GC content >60%), adding DMSO may enhance the reaction by disrupting base pairing and effectively lowering the Tm.⁽²⁾

2. Q solution:

Q-Solution will often enable or improve PCR systems that have a high degree of secondary structure or that are GC-rich. In addition, Q-Solution increases PCR specificity in certain primer-template systems.⁽³⁾

3. PCRx Enhancer:

For problematic and/or GC-rich templates, the PCRx Enhancer System offers higher primer specificity, broader magnesium concentration optima, broader annealing temperature optima and improved thermostabilization of Taq DNA polymerase.⁽⁴⁾

2. Additives That Help PCR in the Presence of Inhibitors:

1. 400 ng/µl BSA (Bovine serum albumin).

2. Non-ionic detergents: Ex: 0.1 to 1% Triton X.⁽²⁾

❖ References:

1. <http://www.bio-rad.com/en-sa/applications-technologies/pcr-troubleshooting>
2. Lorenz TC. Polymerase chain reaction: basic protocol plus troubleshooting and optimization strategies. J Vis Exp,2012;63:e3998.
3. Taq PCR Handbook from qiaGen.
4. PCRx Enhancer System handbook from invitrogen.

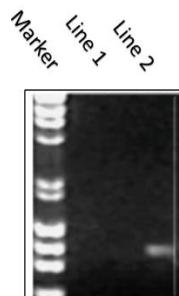
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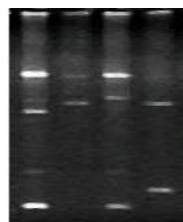
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Problem.1:

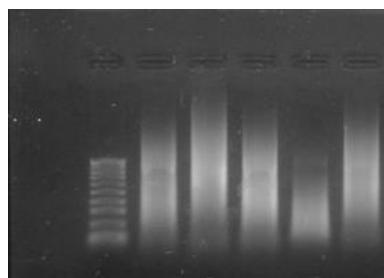
Identify the problem in the following PCR results. Think in some causes. How to solve it?





Problem.2:

A student was working on PCR but she was not careful and cautious enough and she get the following result. What do you think happened?



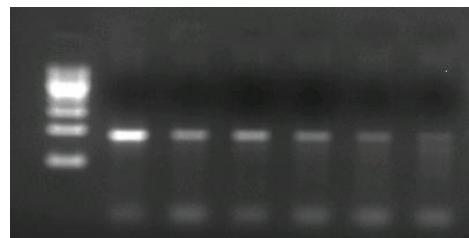
Problem.3:

A student was working on PCR and she performs the additives shown in (A) and get the result in (B):

A.

Component	Volume per reaction (μ l)
10X PCR buffer	2.5
MgSO ₄	1
dNTPs (10mM)	0.5
Enzyme	0.2
Forward Primer (10 pmole)	1
Reverse Primer (10 pmole)	1
RNase/DNase – free water	14.8
DNA	4 μ l
Total volume	25

B.



❖ What is the problem in her result?

❖ What do you think she should change in her protocol to avoid the problem?

Problem.4:

If you have a GC rich template, what PCR additives you may use? What is their function? Can manipulating the thermal cycling condition help? How?

Experiment (8): Digestion of DNA with Restriction Enzymes

Aim:

- Restriction of genomic DNA.

Introduction:

Restriction enzymes (RE) are enzymes that have the ability **to recognizes a specific**, short nucleotide sequence and cleave the sugar phosphate backbones in double stranded DNA at that specific site, which is known as **RESTRICTION SITE** or target sequence. RE naturally found in a wide variety of prokaryotes. In live bacteria, restriction enzymes function to defend the cell against invading viral bacteriophages by cleaving its DNA at specific sites and so prevent replication. Over 300 restriction enzyme have been isolated and the nomenclature depends on the organism from which they are derived e.g. *EcoRI*: is isolated from ***E. coli*** strain RY13, **I** (Roman numeral) indicates it was the first enzyme of that type isolated from *E. coli* RY13. **Table.1** present few examples of restriction enzymes, their origin and restriction site.

 PAUSE AND THINK → Bacterium is immune to its own restriction enzymes, even if it has the target sequences ordinarily targeted by them. Why?

How Restriction Enzyme cut the DNA?

A fragment of DNA produced by a pair of adjacent cuts is called a RESTRICTION FRAGMENT. Restriction enzymes can generate two different types of cuts (**Figure.1**) depending on whether they cut both strands at the center (Blunt end) of the recognition sequence or each strand closer to one end of the recognition sequence (Sticky end). The latter (sticky ends) are more cohesive compared to blunt ends, which have no nucleotide overhangs. Both are useful in molecular genetics and can be used for join DNA fragments.



Figure.1. Generation of blunt and sticky ends fragments my different RE.

Table.1. Examples of RE and their restriction site.

RE name	Origin	Restriction site
EcoRI	Escherichia coli	5' ... G A A T T C ... 3' 3' ... C T T A G ... 5'
BamHI	Bacillus amyloliquefaciens H	5' ... G G A T C C C ... 3' 3' ... C C T A G G ... 5'
HindIII	Haemophilus influenza RD	5' ... A A G C T T T ... 3' 3' ... T T C G A A ... 5'
HaeIII	Haemophilus aegyptius	5' ... G G C C C ... 3' 3' ... C C G G G ... 5'
AluI	Arthrobacter luteus	5' ... A G C T ... 3' 3' ... T C G A ... 5'

*Arrows denote phosphodiester bonds cleaved by each restriction endonuclease.

Mechanism of Action:

Restriction Endonuclease scan the length of the DNA, binds to the DNA molecule when it recognizes a specific sequence and makes one cut in each of the sugar phosphate backbones of the double helix by hydrolyzing the phosphodiester bond. Specifically, the bond between the 3' O atom and the P atom is broken.

Uses in Biotechnology:

The ability of restriction enzymes to cut DNA at specific sequences has led to the widespread use of these tools in many molecular genetics techniques. Restriction enzymes can be used to map DNA fragments or genomes. Mapping means determining the order of the restriction enzyme sites in the genome. Perhaps the most important use of restriction enzymes has been in the generation of recombinant DNA molecules, which are DNAs that consist of genes or DNA fragments from two different organisms (gene cloning). In addition, Restriction enzymes also have applications in several methods for identifying individuals or strains of a particular species. Restriction Fragment Length Polymorphism (RFLP) is a tool to study variations among individuals (humans and other species). This technique able to differentiate minor nucleotide sequence variations in homologous fragments of DNA. It relies on the specificity of restriction endonucleases, which are highly sequence-specific and cut the double-stranded DNA only at their recognition sites.

Principle:

Cleavage by RE is accomplished by the incubating of genomic DNA or DNA fragments obtained following amplification using PCR with the RE under appropriate experimental conditions of temperature, pH and ionic strength. The RE restricts the DNA at sites where the specific sequence recognized by the RE are presents, resulting in the production of different size fragments. These fragments can be separated on agarose gel electrophoresis.

In this experiment restriction of genomic DNA will be done using *Mst*II, which cut the DNA at ‘5-CCTNAGG-3’

Materials:

DNA solution (0.5 µg/µl), *Mst*II (3U/ µl), 10X restriction buffer, NaCl solution, nuclease free water, 0.5 M EDTA.

Protocol:

1. Label a clean micro-centrifuge tube, and add the following:

Component	Volume (µl)
DNA solution (1 µg/µl)	1
10X restriction buffer	2
NaCl solution	1
Water	15

2. Add *Mst*II (3 U for each one µg DNA) and incubate the reaction mixture for 20 min at 37 °C in an incubator.
3. Stop the reaction by adding 0.5 µl of 0.5 M EDTA.
4. Prepare it for agarose gel electrophoresis by adding 5 µl of gel loading buffer.

Results:

Analyse the results using agarose gel electrophoresis.

References:

1. <https://www.thebalance.com/what-are-restriction-enzymes-375674>
2. <http://medicine.jrank.org/pages/2779/Restriction-Enzymes-Use-Restriction-Enzymes-in-Biotechnology.html>
3. <https://www.thermofisher.com/sa/en/home/life-science/cloning/cloning-learning-center/invitrogen-school-of-molecular-biology/molecular-cloning/restriction-enzymes/restriction-enzymes-genome-mapping.html>

Analysis Sheet #8

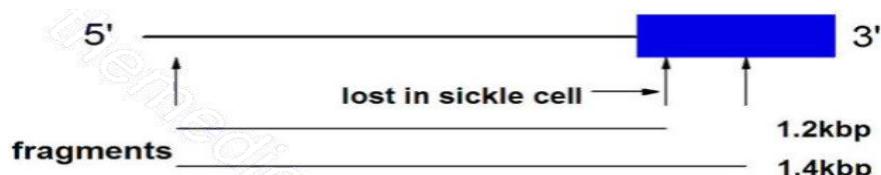
Name: _____

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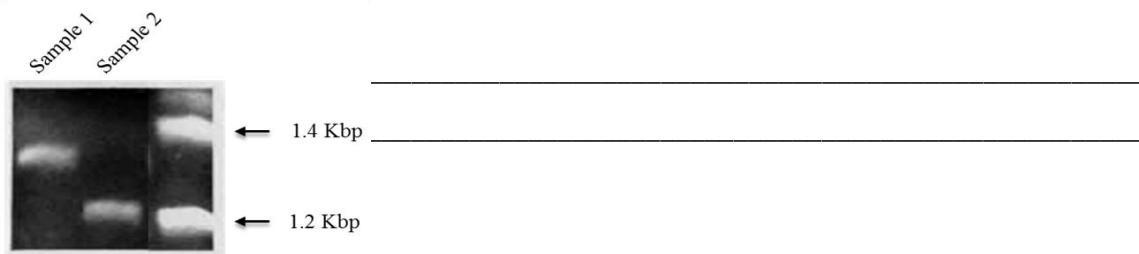
❖ Write the Principle as steps:

1. _____
2. _____
3. _____

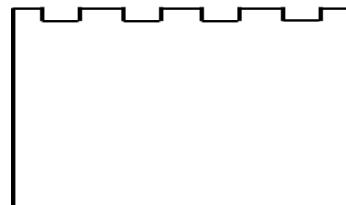
❖ Write the methodology:

Problem.1:If you that *Mst*II cuts the DNA around the β -globin gene in three sites as shown:

1. Determine whether the results below belong to normal or sickle cell anaemia patient? Why?



2. Draw the expected gel result for a carrier patient.



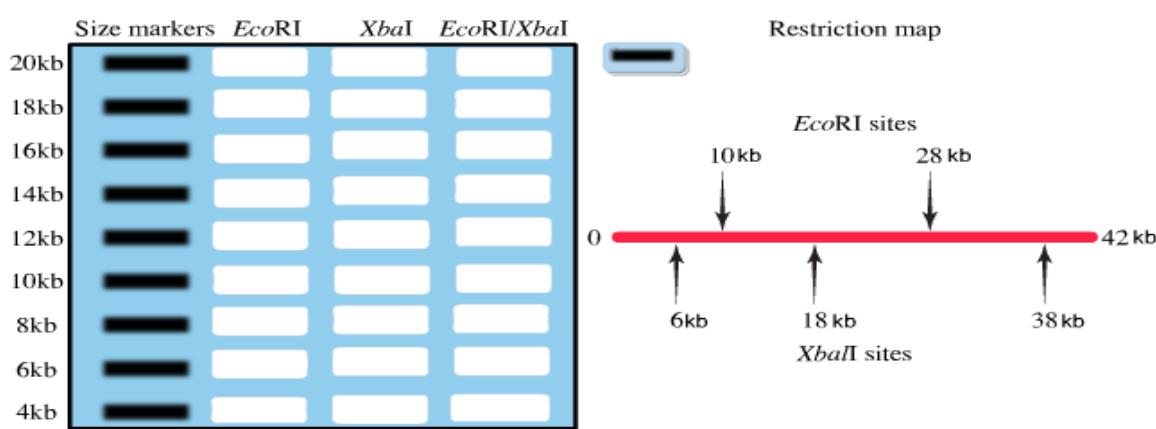
Problem.2:

By drawing, show how *Eco*RI will digest the following sequence, and the resulted fragments.

'5-CCATTACGAGCTCAGTCGGAATTCAACGGATTACCGCATGCCAATACCA-3'
 3'-GGTAATGCTCGAGTCAGCCTAACGTTGCCTAATGGCGTACCGGTTATGGT-5'

Problem.3:

Use the linear restriction map below to predict where you would expect to see bands on a gel if a digest is performed using the specified restriction enzymes.



Experiment (9): Sanger Sequencing

Aim:

- To determine the order of the nucleotides in a given DNA sample.

Introduction:

The term DNA sequencing refers to methods for determining the precise order of nucleotide bases (As, Ts, Cs, and Gs) in a molecule of DNA. Knowledge of DNA sequences has become necessary in numerous applied fields such as medical diagnosis, biotechnology and forensic biology. A sequencing can be done by different methods including: Maxam – Gilbert sequencing (chemical degradation method), **Sanger sequencing** (dideoxy chain-termination method) and high-throughput sequencing technologies. The most commonly used method is the dideoxy chain termination method developed by Sanger and co-workers in 1975 owing to its relative ease and reliability (**Figure.1**).

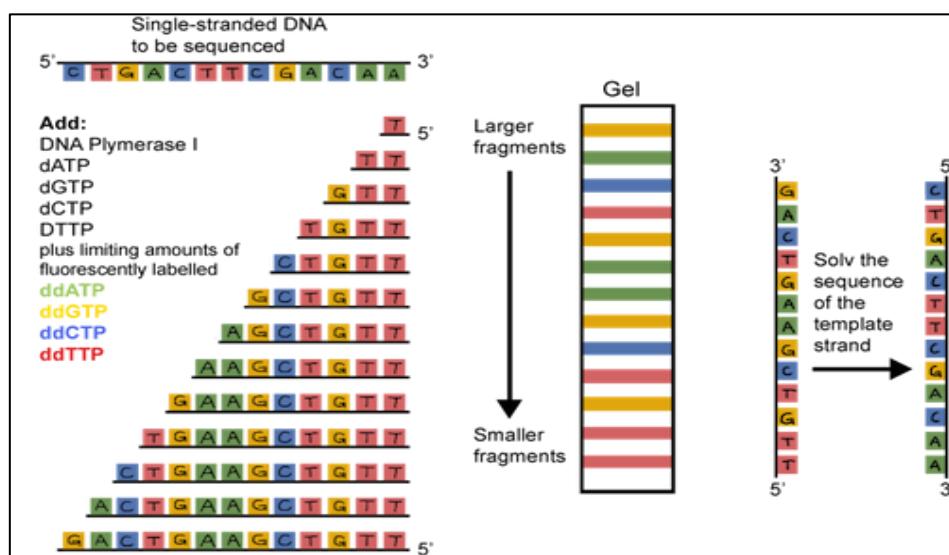
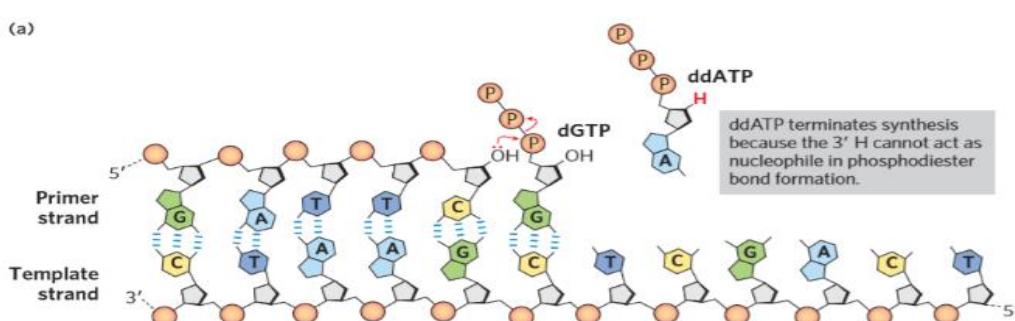


Figure.1. Chain Termination DNA Sequencing.

PAUSE AND THINK ➔ Why the reaction terminated by the ddNTPs (dideoxynucleosides) and cannot be continued?



Principle of automated Sanger method:

In this method, **cycle sequencing**, the **dideoxynucleosides** -not the primers- are tagged with different colored fluorescent dyes, thus all four reactions occur in the same tube and are separated in the same lane on the gel. As each labelled DNA fragment passes through the bottom of the gel, a laser reader detect the fluorescence of each fragment (blue, green, red or yellow) and compiles the data into an image (**Figure.2**).

This method makes use of the mechanism of DNA synthesis by DNA polymerases. It requires the enzymatic synthesis of a DNA strand complementary to the strand under analysis, using **ddNTPs** tagged with fluorescence dye (different color for each nucleotide). In the reaction catalyzed by DNA polymerase, the 3'-hydroxyl group of the primer reacts with an incoming deoxynucleoside triphosphate (dNTP) to form a new phosphodiester bond. The identity of the added deoxynucleotide is determined by its complementarity, through base pairing, to a base in the template strand. In the Sanger sequencing reaction, nucleotide analogs called dideoxynucleoside triphosphates (ddNTPs) interrupt DNA synthesis because they lack the 3'-hydroxyl group needed for the next step. For instance, the addition of ddCTP to an otherwise normal reaction system causes some of the synthesized strands to be prematurely terminated at the position where dC would normally be added, opposite a template dG, and the same for the others nucleotides. This results in different colored DNA fragments, which can be separated by size in an electrophoretic gel in a capillary tube. All fragments of a given length migrate through the capillary gel together in a single band, and the color associated with each band is detected with a laser beam. The DNA sequence is read by identifying the color sequences in the bands as they pass the detector. The amount of fluorescence in each band is represented as a peak in the computer output.

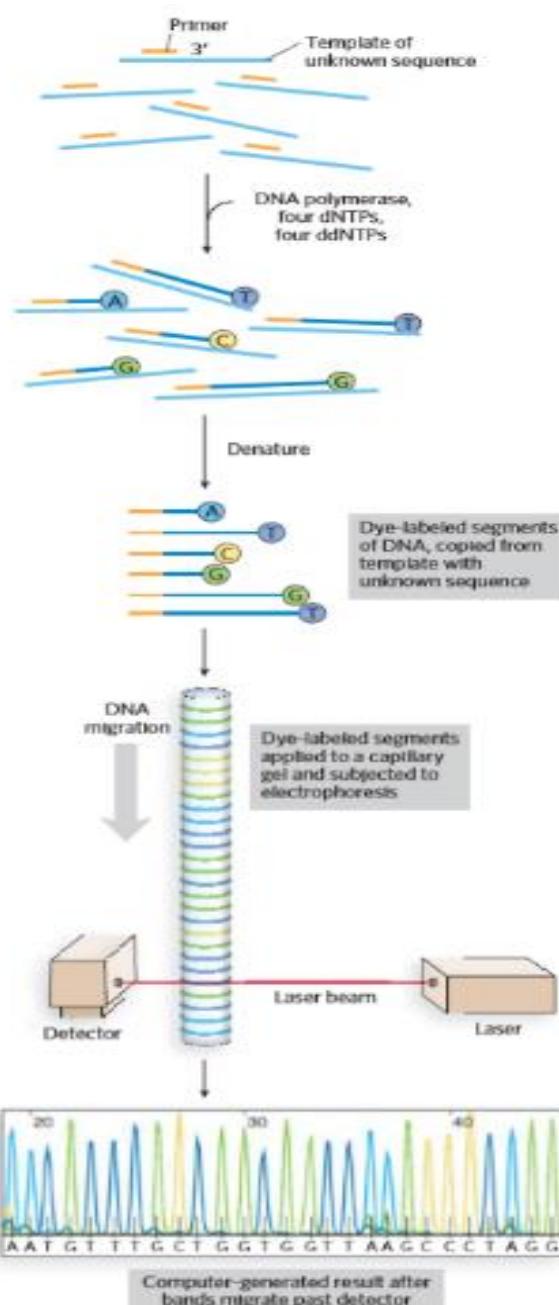


Figure.2. Automation of DNA-sequencing reactions.

❖ Sanger sequencing performing steps:

1. PCR amplification.
2. Purification of PCR product: removal of unwanted primers and dNTPs from PCR product mixture.
3. Sequencing reaction.
4. Post reaction clean-up: The post sequencing reaction product needs to be purified for removal of excess dye terminators and unused primer by using ethanol precipitation protocol.
5. Capillary electrophoresis.
6. Data analysis

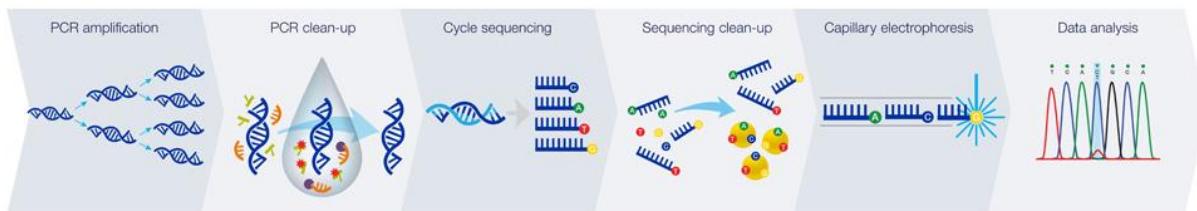


Figure.3. Sanger sequencing workflow.

❖ Sanger sequencing application:

Sanger sequencing supports a wide range of DNA sequencing applications including:

1. Single nucleotide polymorphism (SNP) detection.
2. Single-strand conformation polymorphism (SSCP).
3. Mutations detections.

❖ References:

1. Cox M, Doudna J, O'Donnell M. Molecular Biology genes to proteins. p.226. (2012).
2. <http://www.thermofisher.com/sa/en/home/life-science/sequencing/sanger-sequencing/sanger-sequencing-workflow.html>
3. Munshi A. DNA sequencing –methods and applications. InTech, 2012.

Analysis Sheet #9

Name: _____

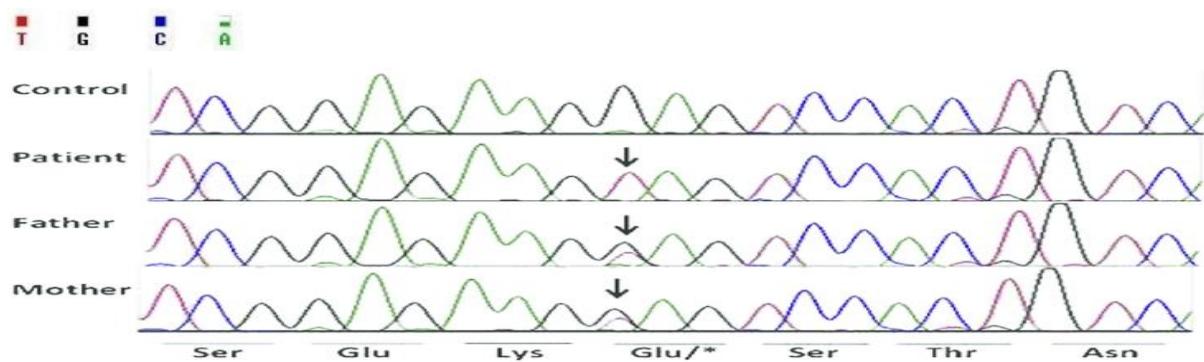
ID: _____

- ❖ Write the principle as steps:

1. _____
2. _____
3. _____
4. _____

Problem.1:

Below is electropherogram for exon 7 of the PLCE1 gene (phospholipase C, epsilon1) in a patient and his parents.



1. What is the nucleotide sequence of the patient?

2. What is the difference in the sequence between the patient and his parents in the position indicated by arrows?

3. Does the patient has an identical alleles for the gene? Why?

4. What is the zygosity of the parents genotype?

5. What type of mutation does the patient has?

Problem.2:

Compare between manual and automated Sanger sequencing.

Problem.3:

The following is a gel resulted from manual Sanger sequencing. Determine the order of the nucleotide in that DNA molecule.

