

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.

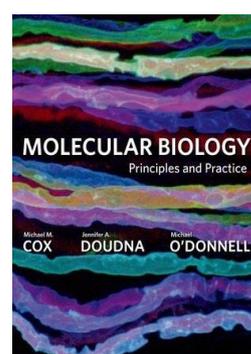
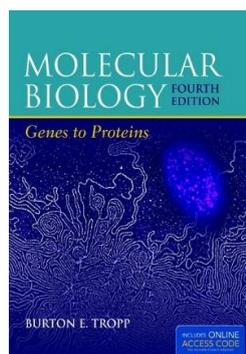
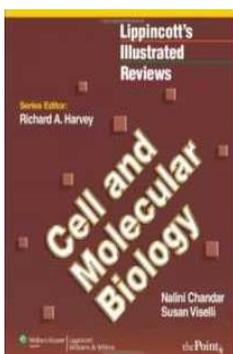
The techniques used for these studies are **referred to as: “Techniques of Molecular Biology”**.⁽¹⁾

🧬 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⁾

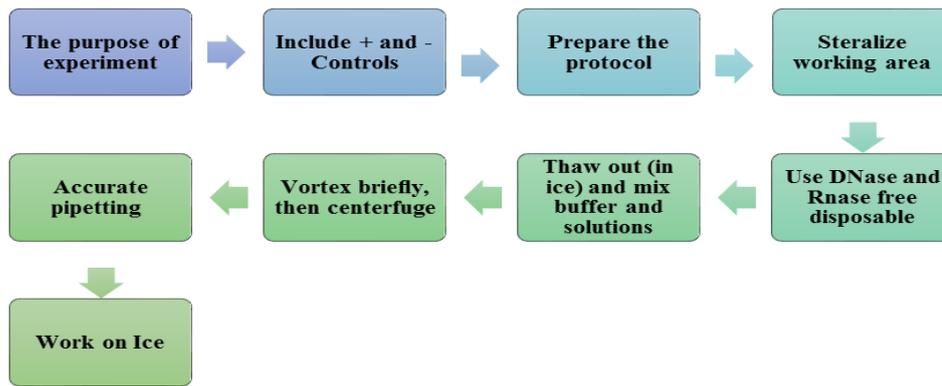
🧬 Things you must to know as Molecular Biologist:

- 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): Preparation of Genomic DNA from Rat Blood (Manual).

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 $^{\circ}$ 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).

Experiment (2): Preparation of Total RNA from Rat Blood (Kit).

Aim:

- To isolate pure and intact total RNA from Rat blood.

Introduction

Obtaining pure RNA is an essential step in the analysis of patterns of gene expression and understanding the mechanism of gene expression. Thus, isolation of pure, intact RNA is one of the central techniques in molecular biology and represents an important step in Northern analysis, RNA mapping, RT-PCR, cDNA library construction and in vitro translation experiments. There are three main types of RNA: messenger RNA (mRNA), ribosomal RNA (rRNA), and transfer RNA (tRNA).

Two strategies of RNA isolation are usually employed: isolation of total RNA and isolation of mRNA. Isolation of total RNA is most frequently used when pure RNA is required for experiments. Depending on the nature of RNA required, various techniques for RNA preparation are widely available that can be classified into: organic extraction methods, filter-based spin basket formats, and magnetic particle methods.

The physical and chemical properties of RNA and DNA are very similar, thus, the basic procedures used in RNA purification are similar to those of DNA: disruption of cells or tissue, effective denaturation of nucleoprotein complexes and removal of proteins, concentration of RNA molecules. However, RNA isolation is much more difficult than DNA purification largely due to the sensitivity of RNA to degradation by internal and external ribonucleases. These enzymes are very stable and active enzymes that require no cofactors to function.

 **PAUSE AND THINK** → What feature is used to separate mRNA from the other RNA in cells

Principle:

In general, most procedures for isolating RNA from eukaryotic cells involve lysing and denaturing cells to liberate total nucleic acids in the presence of RNase inhibitory agents (typically strong denaturants like guanidine salts or SDS). Additional steps are then required to remove DNA.

In Organic extraction methods: the sample is homogenized in a phenol-containing solution and then centrifuged. During centrifugation, the sample separates into three phases: a lower organic phase, a middle phase that contains denatured proteins and gDNA, and an upper aqueous phase that contains RNA.

In Filter-based, spin basket method: Samples are lysed in a buffer that contains RNase inhibitors and nucleic acids are bound to the membrane by passing the lysate through the membrane using centrifugal force. Wash solutions are subsequently passed through the membrane and discarded. An appropriate elution solution is applied and the sample is collected into a tube by centrifugation.

Protocol & materials:

According to the manufacturer's instruction of **QIAamp® RNA Blood Mini Kit (QIAGEN)**.

Results:

The isolated RNA can be confirmed by measuring the O.D at 260nm.

References:

1. Surzycki S. Basic techniques in molecular biology. Springer. (2000).
2. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (eds.) Current Protocols in Molecular Biology. John Wiley & Sons, Inc. New York. 2003.

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

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 (concentration, 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 $0.02 (\mu\text{g/ml})^{-1}\text{cm}^{-1}$. 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 complementary 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 T_m 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 (T_m) 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:

DNA concentration and purity from proteins and carbohydrates, peptides, ethanol or any organic compounds can be determined by measuring the absorption of ultraviolet light. This achieved by using Beer-Lambert law and calculating the A_{260}/A_{280} and A_{260}/A_{230} ratios. Meanwhile, 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 T_m 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 800 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 → $\mu\text{g/ml}$ → 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 microcentrifuge tube, prepare 1 ml of the sample by diluting your extracted DNA (your stock) to 10 $\mu\text{g/ml}$ with 0.1 X SSC buffer.
2. In another microcentrifuge tube, pipette 1 ml of distilled water.
3. Place the tubes in a thermomixer at 25°C and allow temperature to equilibrate (5 min).
4. Immediately read the absorbance at 260 nm.
5. Raise the temperature to 50 °C, 60 °C, 70 °C, and boiling, then repeat step 4.

Results:

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

Wavelength (nm)	Absorbance of DNA	
	Rat	
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 (°C)	DNA Absorbance at 260 nm	
	Rat	
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:

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

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.

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 linear polymer composed of alternative residues of D-galactose and 3,6-anhydro-L-galactopyranose joined by α (1 \rightarrow 3) and β (1 \rightarrow 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 \rightarrow 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 **CAREFULLY** 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).

Experiment (5): cDNA Synthesis

Aim:

- Reverse transcript RNA to cDNA.

Introduction:

The synthesis of DNA from an RNA template, via reverse transcription, produces complementary DNA (cDNA). cDNA synthesis is the first step for many protocols in molecular biology. For the cDNA synthesis to be successful there are critical considerations related to the **template**, **reagents/components**, and **reaction conditions**. Regarding template preparation, it is critical to maintain RNA integrity and isolate and purify RNA.

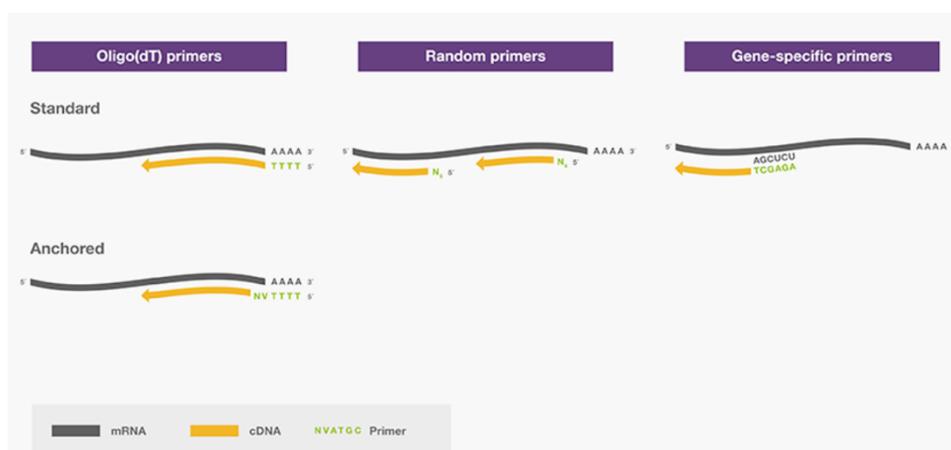
 PAUSE AND THINK → How can the RNA integrity be maintained?

Reaction components:

For the cDNA to be synthesised the following components are needed: reverse transcriptase, Primers, dNTPs, water, buffer, and the RNA template. Two important considerations for cDNA synthesis are the **reverse transcriptase** and the **type of primers**.

The reverse transcriptases which synthesizes cDNA using RNA as template can be differ in functional activities and properties. Their properties impact their ability to reverse-transcribe long RNA transcripts, GC-rich RNA, RNA with significant secondary structures. Most reverse transcriptases used in molecular biology are derived from the pol gene of avian myeloblastosis virus (AMV) or Moloney murine leukemia virus (MMLV).

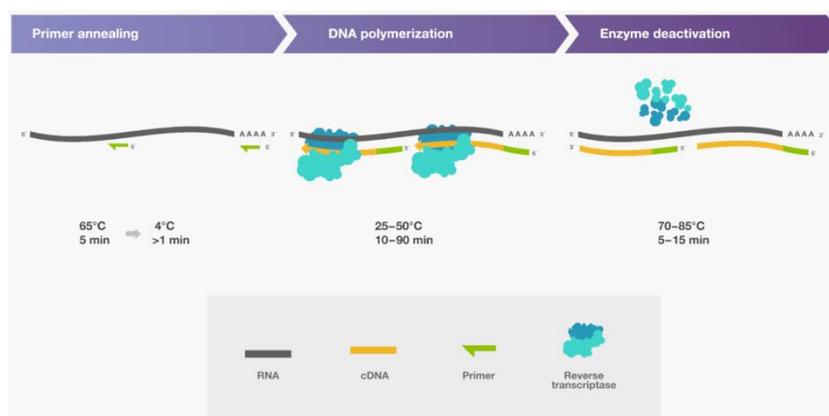
To initiate reverse transcription, reverse transcriptases require a short DNA oligonucleotide called a primer to bind to its complementary sequences on the RNA template and serve as a starting point for synthesis of a new strand. Depending on the RNA template and the downstream applications, primers of three basic types are available as shown in the figure below: oligo(dT) primers, random primers, and gene-specific primers.



Oligo(dT) primers consist of a stretch of 12–18 deoxythymidines that anneal to poly(A) tails of eukaryotic mRNAs, and they are not suitable for degraded RNA (Why?). **Random primers** are oligonucleotides (often six) with random base sequences, and are usually referred to as random hexamers. **Gene-specific primers** offer the most specific priming in reverse transcription. These primers are designed based on known sequences of the target RNA.

Reaction conditions:

As shown in the figure below, reverse transcription reactions involve three main steps: primer annealing, DNA polymerization, and enzyme deactivation.



Primer annealing: The primer is mixed with the RNA template, heated to 65°C for 5 min, then incubated on ice for at least 1 min. This helps ensure that the RNA is single-stranded and that the primer anneals to the target efficiently.

DNA polymerization: In this step, reaction temperature and duration may vary according to the primer choice and reverse transcriptase used.

Enzyme deactivation: The final step in reverse transcription reactions is to deactivate the reverse transcriptase. The deactivation temperature may be in the range of 70–85°C, depending upon the thermostability of the enzyme.

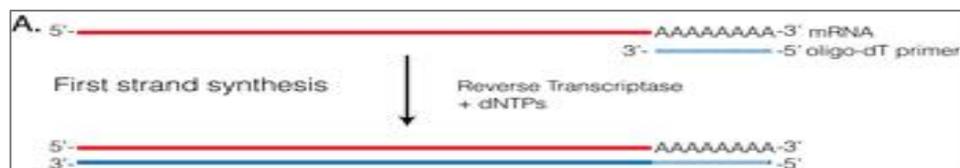
Step	Temperature	Duration
Primer annealing	65 °C	5 min
DNA polymerization*	Oligo(dT): 35–50 °C	>60 min
	Random hexamers: 10–15 °C	
	Primer mixture: 25 °C	
Enzyme deactivation	70–85 °C	5–15 min

* Reaction temperature and duration may vary according to the primer choice and reverse transcriptase used.

cDNA synthesis

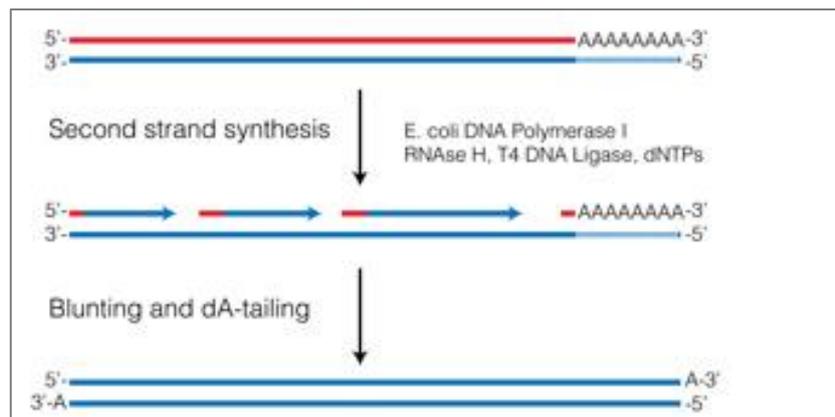
First-strand cDNA synthesis

Is the generation of cDNA:RNA hybrid. The RNA of the cDNA:RNA hybrid is cleaved during first-strand synthesis via RNase H activity (presence in reverse transcriptases). The first-strand cDNA (with or without the RNA annealed to it) may be used directly in some applications such as RT-PCR.



Second-strand cDNA synthesis.

When the first-strand cDNA is used as a template to generate double-stranded cDNA representing the RNA targets via DNA polymerase. This type is used in cDNA library construction and sequencing.



References:

1. <https://www.thermofisher.com/sa/en/home/life-science/cloning/cloning-learning-center/invitrogen-school-of-molecular-biology/rt-education/reverse-transcription-setup.html>
2. <https://www.bio-rad.com/featured/en/cdna-synthesis.html>
- 3.

Experiment (6): 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⁾

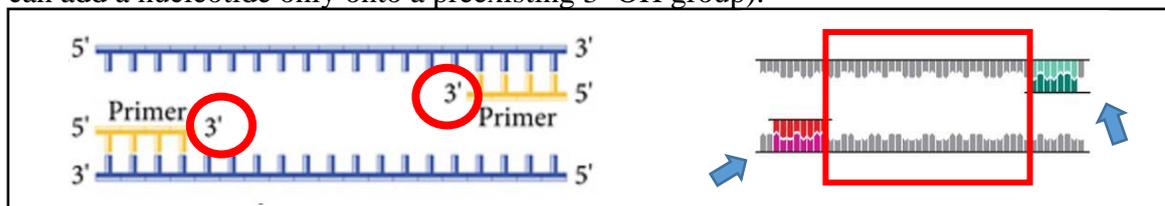


Figure.1. Two primers are prepared, complementary to sequences on opposite strands of the target DNA.

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.

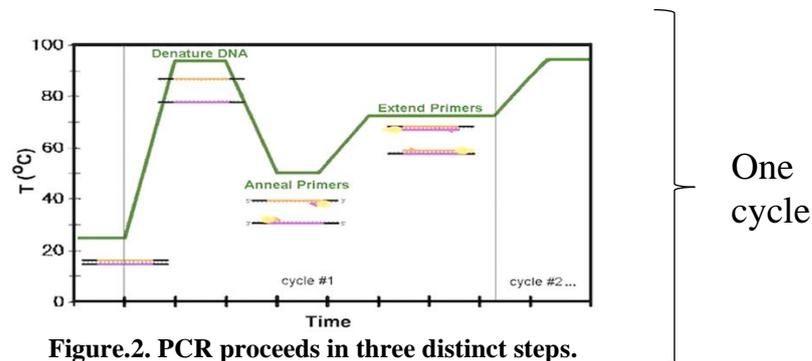
Components	Function
Template DNA	The template carries the DNA segment or (target) you wish to amplify.
Forward and reverse primers	A primer is a short, single-stranded piece of DNA that anneals (attaches) to its complementary sequence on the template. A pair of primers will bind to either side of the 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**:



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.



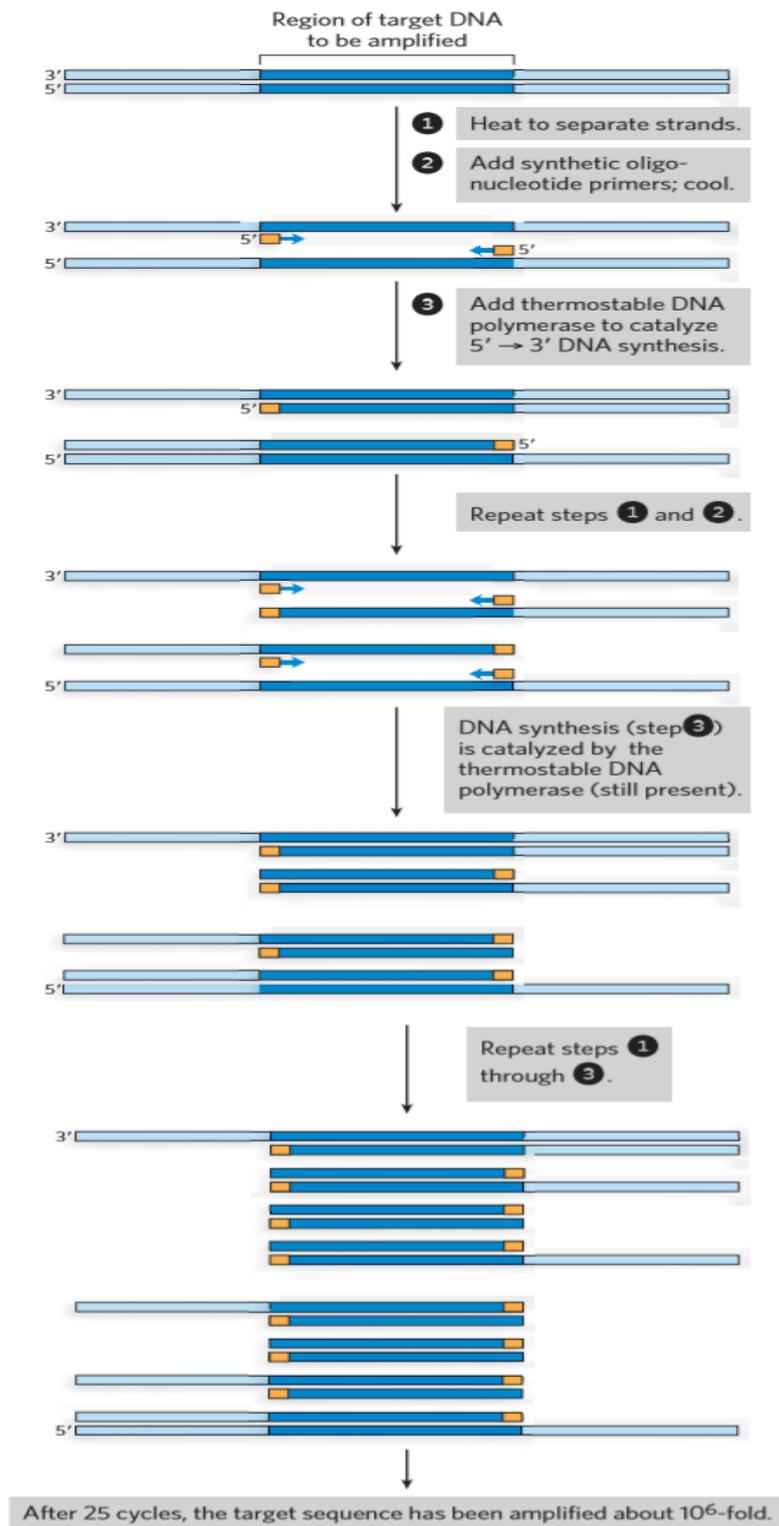


Figure.3. Amplification of DNA segment by PCR.⁽⁵⁾

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 (T_m):

The melting temperature (T_m) 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 T_m for both primers should differ by no more than 5 °C (difference between forward and reverse primer). The T_m of the primer can be calculated by the following formula:

$$\text{T}_m = [(G + C) \times 4] + [(A + T) \times 2]$$

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

5. Annealing temperature (T_a):

The primer melting temperature is the estimate of the DNA-DNA hybrid stability and critical in determining the annealing temperature. Too high T_a will produce insufficient primer-template hybridization resulting in low PCR product yield. Too low T_a 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

5'-CAACATAATAGCGACAACA **CTAGA**-3'

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 you 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>
<https://www.youtube.com/watch?v=2KoLnIwoZKU&t=13s>
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.
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3. Mullis KB. The unusual origin of the polymerase chain reaction. Sci Am.1990;4: 56-61.
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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.
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Experiment (7): Optimization of Annealing Temperature

Aim:

- To know how 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 μ M of each dNTP.
Magnesium	1.5-2.0 mM is optimal for Taq DNA Polymerase.
Forward and reverse primers	Typically 0.1-0.5 μ M of each primer.
DNA Template	1ng–1 μ g 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 stages aim to denature the template and activate “hot-start” DNA polymerase. **Next stage** is the three PCR steps (denaturation, annealing and elongation), which will repeated from 25 to 35 cycles. **Last stage** is the final elongation phase; a period of 5 minutes or longer to allows 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 \diamond	30-60 sec	
Final elongation	75-80 °C	5-7 min	x1

* Depend on the primer annealing temperature.

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

 **PAUSE AND THINK** → When optimizing T_a what you should do with other PCR component?

Materials:

PCR buffer, DNA Taq polymerase, dNTPs, $MgCl_2$, primers, DNA template, Nuclease free water.

Protocol:

1. 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 (μ l)
PCR buffer	10X	1X	
Taq polymerase	5 U/ μ l	0.05 U/ μ l	
dNTPs	10 mM	200 μ M	
$MgCl_2$	25 mM	1.5 mM	
Forward primer	10 μ M	0.4 μ M	
Reverse primer	10 μ M	0.4 μ M	
DNA Template	45 ng/ μ l	90 ng	
Water			
Total volume			50 μl

2. 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).

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

PCR buffer		
Taq polymerase		
dNTPs		
MgCl ₂		
Forward primer		
Reverse primer		
Water		
Total volume	50 µl µl

- Using special PCR tubes, distribute the master mix by pipetting µl to each tube.
- Add the DNA template for each template.
- Centrifuge the tubes briefly.
- Set the thermal cycling condition as following:

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

- Try different 8 annealing temperatures depending on your primer pair T_m.
- Set the final volume in the thermal cycler to be 50 µl.
- Start PCR.

Results:

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

References:

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

Experiment (8): 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.
	No 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 T_m. ⁽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 by changing the melting behaviour of the DNA. 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.

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 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 easy and reliability (**Figure.1**).

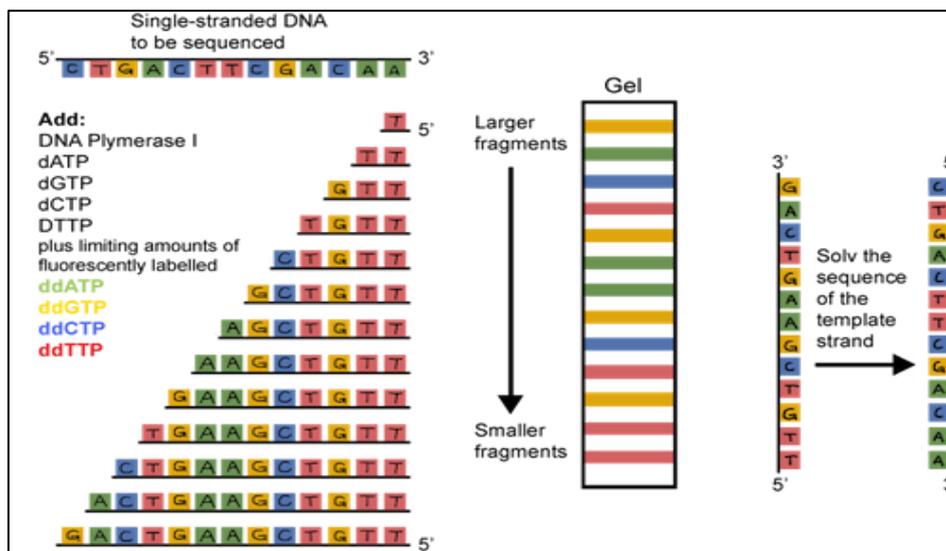
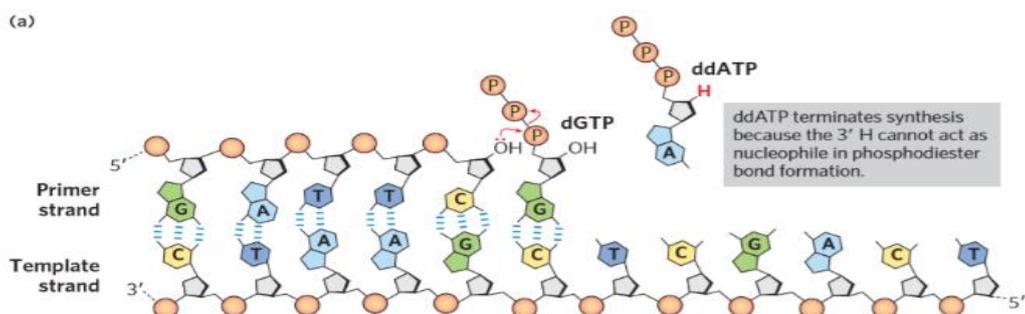


Figure.1. Chain Termination DNA Sequencing.

-  **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.

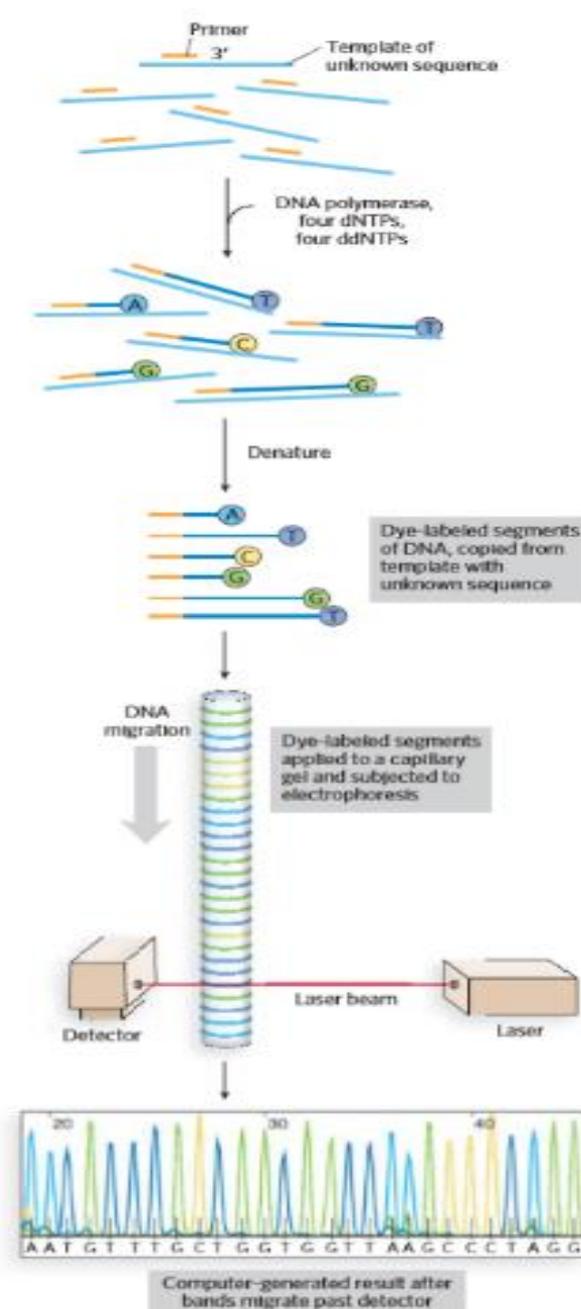


Figure.2. Automation of DNA-sequencing reactions.

🧬 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

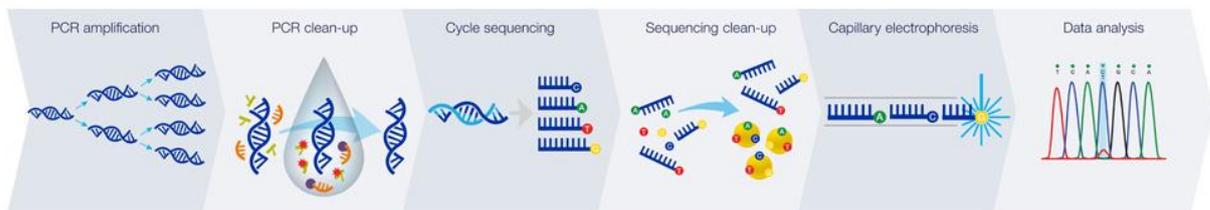


Figure.3. Sanger sequencing workflow.

🧬 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).
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