الجامعة الألمانية الأردنية
German Jordanian University

School of Medical Sciences

Pharmaceutical and Chemical Engineering Department

Laboratory Manual

for

Biochemistry

BM229

Edition: 2022

Contents

Preface

This practical course is a 3rd year level that is co-requisite to Biochemistry course. This lab is a 1 credit hour course but scheduled to be given for 3 hours once a week.

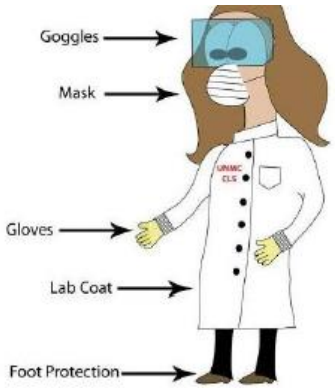
This lab covers the following:

> Preparation of buffers, methods for the isolation, purification, and characterization of amino acids, proteins, nucleic acids, carbohydrates and lipids. In more details, the experiments performed in this lab are: Qualitative tests for carbohydrates, Qualitative and Quantitative analysis of lipids, Paper chromatography of amino acids, Spectrophotometric analysis of protein-containing samples, Extraction of proteins from blood, Gel electrophoresis for proteins (SDS-PAGE), Isolation of DNA from whole blood followed by PCR and Analysis of DNA by Agarose gel electrophoresis.

It is not the role of your instructor or TA to do the lab for you. You are responsible for coming to lab prepared and ready to work during each session. During the lab sessions you must complete an experimental procedure and solve the questions related to the experiment. It is your responsibility to ensure you have all the necessary results and to have completed any calculations before leaving the lab.

During the first lab session, you will be introduced to some of the safety equipment in the lab as well as the rules and regulations for your class. Pay close attention to this discussion so that you can react properly in case of an emergency!

What Will You Need to Bring With You to Lab?



It is important to come prepared for your laboratory session. You will be required to bring:

Your manual, a lab-coat and protective eyewear (goggles or glasses with side-shields) as well as a calculator.

Please note you will not be allowed to enter the lab if you do not come prepared with a lab-coat, goggles and solved Pre-lab.

Lab Reports

Each lab session is divided into three main parts:

the **Pre-laboratory section** which should be completed before attendance. Sometimes these pre-laboratory questions will include numerical problems similar to those you will encounter in processing the data you will be collecting in the experiment. Obviously, if you review a calculation before lab, things will be much easier when you are actually in the lab!

Pre-lab assignments MUST be graded AT THE BEGINNING of the lab session. If you do not complete the pre-lab assignment and turn it in at the beginning of the lab, you will NOT be allowed to participate in the lab that day.

the **Experimental results** which are to be recorded while following the experimental methodology.

the **Post-laboratory questions**.

Your final lab report including all three sections must be submitted to your TA or instructor before leaving the lab.

Assessment Policy		
Reports	The least graded report will not be counted	**40%**
Evaluation	Participation and following lab rules	**10%**
Quizzes	The least graded quiz will not be counted	**20%**
Final Exam	Including all experiments	**40%**

Regulations	
Signing the Consent Form	During your first lab session you have to read and sign the consent form given in the next page of this manual and hand it to your TA (or instructor).
Attendance	Lab session attendance is mandatory. The student is allowed maximally 15% absentia of the total module hours. According to the rules you are allowed to be absent maximum for 2 lab session (even with excuses). More than this percentage, a student with an excuse will be drawn from the module. Otherwise, the student will be deprived from the module with zero mark assigned.
Online-sessions	Regarding the online-given lab sessions: If you don't submit your report on the assigned time, you will be considered 'absent' on that session.
Reports	You have to depend on yourself to prepare your report. Copying from each other is NOT allowed. **Similar copies of reports will be graded ZERO.** You may discuss together, search for the solution or ask your instructor or TA; but at the end you should write the answers in your own language.

School of Applied Medial Sciences

Bioorganic Chemistry Lab. – BM229

Consent Form

Student Full Name:	Student ID Number:
Student Emergency Contact Telephone Number:	

I hereby acknowledge receipt of safety codes and procedures that are required in the current edition of SAMS/PCE department safety regulations, all of which I have read, instructed, viewed, understood and agree to observe. Moreover, I will be aware of my special risks with the work I am doing and that should use protective equipment without any exemption. My own health and that of safety and that of others depend on this. In addition, it is my responsibility to cooperate with safety measures and seek consultation from lab supervisor about any unclear procedure/statement. I understand that I have registered in this practical course with the number of students decided by the PCE administrative department and I understand that the instructors in this course applied their best to keep people apart to help reduce the spread of coronavirus (COVID-19). On that aspect I will be committed to 1) have face covering to minimize the time of sharing breathing zone; 2) to be apart from my colleagues or staff by 2 meters through following floor tapes or signage to remember this social distancing; 3) to work side by side or facing away rather than face to face; 4) to limit the movement and follow the instructor regulations to rotate between equipment/tools; 5) to limit touching of surfaces or any stuff I will not use and to keep myself belongings without sharing ; 6) to report your concern of developing any of COVID 19 symptoms*; 7) to safely discard disposable items and clean reusable ones thoroughly; and 8) to ensure washing my hand thoroughly with soap and water (20 seconds) or an alcohol-based hand sanitizer as soon as possible before and after entering the laboratory assigned hall. Otherwise of all the above I understand that my activity will be stopped, and the staff will end my class.

Signed…… Date……

Instructor:

TA:

*The main symptoms of coronavirus are:

- a high temperature – this means you feel hot to touch on your chest or back (you do not need to measure your temperature)
- a new, continuous cough – this means coughing a lot for more than an hour, or 3 or more coughing episodes in 24 hours (if you usually have a cough, it may be worse than usual)
- a loss or change to your sense of smell or taste – this means you've noticed you cannot smell or taste anything, or things smell or taste different to normal.

Most people with coronavirus have at least one of these symptoms. Once you report any of these symptoms you are recommended to kindly leave the class and visit the **university clinic** and we will give you all the support needed, so please keep us posted so we can assist you and trace your case.

Safety in Chemistry Labs

Chemistry is an experimental science. You cannot learn it without getting your hands dirty. All new chemistry students face the prospect of lab work with some apprehension and fear, and it would be untruthful to say that this is completely unwarranted. *Chemicals can be dangerous!* The more you study chemistry, the more danger you will face, but also the more knowledge you will have to protect yourself. If you approach your lab work calmly and studiously, you will minimize any risk.

During your first lab session, you will be given a brief tour of your laboratory. You will be shown the locations of various pieces of emergency equipment that are there for your safety. If you have other specific questions about your safety in the lab consult with your laboratory instructor.

Protection for Your Eyes

It is a requirement for the labs (and it is just common sense!!) that you must wear **protective eyewear (safety goggles)** while you are in the laboratory. Such eyewear must be worn even if you are personally not working on an experiment.

In addition to protective goggles, an **eyewash fountain** provides eye protection in the laboratory. Should a chemical splash near your eyes, you should use the eyewash fountain before the material has a chance to run in behind your safety glasses. A typical eyewash fountain is shown in the accompanying Figure. If you ever need to use the eyewash fountain, don't be afraid or modest – *use it immediately!!*

Protection from Fire

The danger of fire in a chemistry laboratory is real, since the lab usually has a large number of flammable liquids in it, and open-flame gas burners are sometimes used for heating. With careful attention, though, the danger of fire can be reduced considerably, and even avoided completely.

Always check the lab before lighting a gas burner (Bunsen burner) to be sure than no one is pouring or using flammable chemicals near you. Since the vapors of most flammable liquids are heavier than air, be especially careful around sinks as they tend to concentrate there.

In spite of all the precautions you take, fires may still occur. The method you use to fight them depends on their size and on the substance that is burning. If only a small amount of flammable material has caught fire and there is no chance it can spread, depriving it of oxygen is the best and safest method to extinguish the flames. To do this, put a beaker over the fire and it will quickly go out. Leave the beaker in place for several minutes to ensure that the flammable material has cooled and will not flare up again.

In the unlikely event that a larger chemical fire occurs, carbon dioxide fire extinguishers are available in front of the lab door. When using a **CO_2 fire extinguisher**, direct the spray at the base of the fire. This not only deprives it of oxygen, but also cools the flammable material quickly. If this does not quickly work to extinguish the fire, immediately evacuate the laboratory and call the fire department. Be aware that the CO_2 fire extinguishers should not be used on fires in which magnesium (or other reactive metals) are burning – this may only make the fire worse. Consult your instructor before using a fire extinguisher to be certain you have chosen the correct method.

One of the most frightening and potentially dangerous accidents in the chemistry laboratory is the igniting of a person's clothing or hair. For this reason, certain types of clothing should never be worn in the lab, including long flowing sleeves; clothing made of silk or other flammable fabrics; and long hair should be tied back. In the unlikely event that a student's hair or clothing catches fire, his or her lab-mates must act quickly to extinguish the flames and prevent serious burns. The Figure on the side shows the kind of **emergency shower** you will find in our labs. This type of equipment provides a very large amount of water very quickly to put out most types of fires.

Protection from Chemical Burns

Most acids, alkalis (bases) and oxidizing and reducing agent are corrosive to the skin. Since it is impossible to avoid using these substances completely, you must learn how to handle them properly and safely. As a general rule, a material's corrosiveness is related to its concentration – the higher the concentration, the more dangerous it will be. Even though the concentrations of the chemicals you will be dealing with in this lab are generally small, this does not completely remove the danger. You should wash your hands frequently during lab, especially if any chemical substance is spilled on your skin.

After working with a substance that you know to be particularly dangerous or corrosive, wash your hands, even if you did not spill the substance – you don't know if the student using the bottle before you spilled any on the outside of the container. Whenever you spill a corrosive substance on your skin you should tell your instructor immediately. If there is any sign whatsoever of damage to your skin, you should see a doctor as soon as possible.

In the event of a major chemical spill, in which substantial portions of your body or clothing are affected, use the emergency shower – this is not the time for modesty, your health and safety are much more important!!!

Protection from Toxic Fumes

Many chemical substances are volatile (easily become a gas) and have toxic vapors. As a rule, in the chemistry lab, be careful that "if you can smell it, it can hurt you!" Some toxic fumes can overpower you immediately (like ammonia), whereas some fumes are even more dangerous and can cause harm without you even knowing it. There is no need to expose yourself to these toxic fumes in the lab. Our chemistry labs are equipped with **fume hoods** (figure on the side) that have exhaust fans to pull the vapors into the hood and away from you. Flammable solvents should also be stored in the hood to reduce the risk of fire.

Protection from Cuts and Burns

The most common injuries to students in the general chemistry lab are simple cuts and burns. You will use thermometers and glassware for nearly every experiment, and they are often not used properly.

Broken glassware should not be touched with your bare hands. Use a broom and dustpan to clean up the spills and shards of glass. Each lab has a designated place to put broken glass – do not throw it in the garbage, someone else may get injured if you do!

Simple burns occur in the laboratory when students forget that an apparatus is hot and touch it. Never touch a piece of equipment that has been heated until it has cooled for at least five minutes. Use tongs if you are unsure! Perhaps the

most common accident in the lab is the tipping over of a flask or beaker while it is being heated or handled.

Another common mistake occurs while heating liquids in test tubes. If the contents of the tube are heated too quickly, they can super-heat and blow out of the test tube like a bullet from a gun!! Ideally, heat a test tube in a beaker of boiling water, or if you must heat it directly over a heat source (like a Bunsen burner) hold it at a 45º angle, pointed away from yourself and anyone else in the lab. Gently move the tube through the flame, only briefly should it come in contact with the fire.

Report any cuts or burns to your instructor immediately, no matter how minor they seem. If there is a damage to your skin, you will likely be sent to see a doctor. What may seem like a scratch could become infected from the chemicals you were using and should be treated by a professional.

Safety Rules and Regulations

General Lab Rules

- Do not enter the laboratory before your instructor or TA arrives.
- Wear safety goggles and lab coat at all time when you are in the laboratory.
- Do not wear short skirts, shorts, or open-toed shoes in the laboratory.
- Do not wear scarves or neckties in the lab, because they may accidentally be ignited in the flame of a Bunsen burner.
- Jewelry should be removed. Chemicals can cause a severe irritation if concentrated, under a ring, wristwatch, or bracelet; chemicals on fingers or gloves can cause irritation around earrings, necklaces, and so on. It is just a good practice of laboratory safety to remove jewelry.
- Girls with long hair should tie it back before entering the lab, it, too, may accidentally catch fire.
- Never chew gum, eat, drink, or smoke in the laboratory.
- Contact lenses should not be worn in the lab, even if goggles are worn. Lenses can absorb vapors and are difficult to remove in an emergency.
- Prepare your work area Before you begin a lab, clear the lab bench or work area of all your personal items, such as backpacks, books, sweaters, and coats. Find a storage place in the lab for them. All you will need is your laboratory manual, a calculator, a pen or pencil, and equipment from your lab drawer.

Heating Safety Tips

- Never leave a lighted Bunsen burner unattended.
- Never heat solutions to dryness, this can sometimes cause an explosion.
- Never heat a "closed system" such as a stoppered flask.

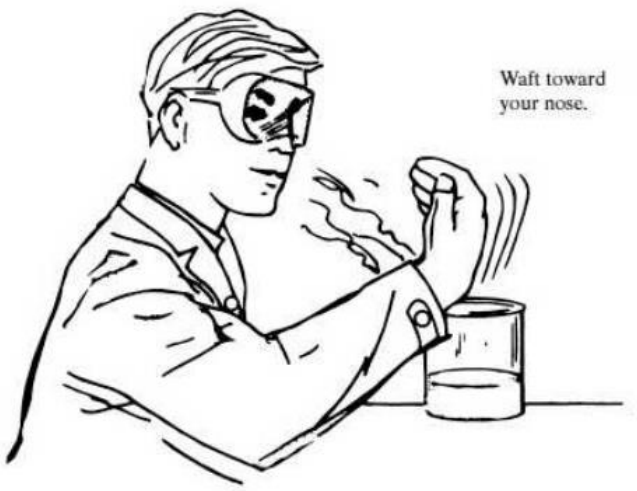
- To heat liquids, add 2-3 boiling stones to help it heat evenly and boil smoother.

Waste Disposal

- Always use the smallest amount of substance required for an experiment; more is never better in chemistry.
- Never return unused portions of chemicals to their original bottle – use a waste container.
- Dispose of all reaction products as directed by your instructor. In particular, observe the special disposal techniques necessary for flammable or toxic substances.
- Dispose of all glass products in the special container provided.

Other Rules

- Do not leave your experiment unattended during the laboratory period: This is often a time when accidents occur.
- Never remove any chemical substance from the laboratory. This is grounds for expulsion from our class and from the university.
- Handle chemicals carefully. Check the labels of all bottles before removing the contents. Read the labels three times: before you pick up the container, when the container is in your hand, and when you put the bottle back.
- Do not insert droppers into reagent bottles. Pour a small amount of the chemical into a beaker.
-
- Keep your work area clean and help keep the common areas of the laboratory clean. If you spill something in a common are, remember that this substance may injure someone else.
- Never fully inhale vapors of any substance. Waft a tiny amount of the vapor toward your nose if you need to smell it.



- Never add water to a concentrated reagent (like an acid) when diluting the reagent. Always add the reagent to water. The reverse may cause it to splash out on you.
- Never perform any experiment that is not specifically authorized by your instructor. DO NOT play games with chemicals!
- Don't use any glassware that has any cracks, chips, star fractures, or any other deformity.

Lab Safety Rules

Science labs offer great opportunities for learning, teaching, and research. They also pose hazards that require proper safety precautions.

Stay safe when conducting your labs by following these guidelines.

Dress appropriately

Tie back long hair, and wear suitable gloves, goggles, and other protective equipment.

Proper supervision

Don't perform lab experiments without instructor supervision (unless given permission to do so).

Know location of emergency numbers & safety equipment

Know the location of safety equipment and emergency phone numbers (such as poison control) so you can access them quickly if necessary.

No food

Don't eat or drink in the lab—and never taste chemicals.

ID hazards

Identify hazardous materials before beginning labs.

Be attentive

Be attentive while in the lab. Don't leave lit Bunsen burners unattended or leave an experiment in progress.

Be careful when handling hot glassware

Turn off all heating appliances when not in use. Keep flammable objects away from your workspace.

Keep a clean workspace

Don't obstruct work areas, floors, or exits. Keep coats, bags, and other personal items stored in designated areas away from the lab. Don't block sink drains with debris.

Handle glassware carefully

Properly dispose of anything that breaks. Report cuts, spills, and broken glass to your instructor immediately.

Clean up

After completing the lab, carefully clean your workspace and the equipment, and wash your hands.

Student Name:	**Student ID no.:**
Date:	**Section:**
Instructor Name:	**TA name:**

Safety Quiz

This quiz will test you on the preceding safety discussion. Circle the correct answer(s) in each of the following questions:

1. Approved eye protection is to be worn

   a) for certain experiments
   b) only for hazardous experiments
   c) all the time

2. Eating in the laboratory is

   a) not permitted
   b) allowed at lunch time
   c) all right if you are careful

3. If you need to smell a chemical, you should

   a) inhale deeply over the test tube
   b) take a breath of air and fan the vapors toward you
   c) put some of the chemical in your hand, and smell it

4. When heating liquids in a test tube, you should

   a) move the tube back and forth through the flame
   b) look directly into the open end of the test tube to see what is happening
   c) direct the open end of the tube away from other students

5. Unauthorized experiments are

a) all right as long as they don't seem hazardous
b) all right as long as no one finds out
c) not allowed

6. If a chemical is spilled on your skin, you should

a) wait to see if it stings
b) flood the area with water for 10 minutes
c) add another chemical to absorb it

7. When taking liquids from a reagent bottle,

a) insert a dropper
b) pour the reagent into a small container
c) put back what you don't use

8. In the laboratory, open-toed shoes and shorts are

a) okay if the weather is hot
b) all right if you wear a lab apron
c) dangerous and should not be worn

9. When is it all right to taste a chemical used in the lab?

a) never
b) when the chemical is not hazardous
c) when you use a clean beaker

10. After you use a reagent bottle,

a) keep it at your desk in case you need more
b) return it to its proper location
c) play a joke on your friends and hide it

11. Before starting an experiment,

a) read the entire procedure
b) ask your lab partner how to do the experiment
c) skip to the laboratory report and try to figure out what to do

12. Working alone in the laboratory without supervision is

a) all right if the experiment is not too hazardous
b) not allowed
c) allowed if you are sure you can complete the experiment without help

13. You should wash your hands

a) only if they are dirty
b) before eating lunch in the lab
c) before you leave the lab

14. Personal items (books, clothes, etc.) should be

a) kept on your lab bench
b) left outside
c) stored out of the way, not on the lab bench

15. When you have taken too much of a chemical, you should

a) return the excess to the reagent bottle
b) store it in your lab locker for future use
c) discard it using proper disposal procedures

16. In the lab, you should wear

a) practical, protective clothing
b) something fashionable
c) shorts and loose-sleeved shirts

17. If a chemical is spilled on the table,

a) clean it up right away
b) let the stockroom help clean it up
c) use appropriate adsorbent if necessary

18. If mercury is spilled,

a) pick it up with a dropper
b) call your instructor
c) push it under the table where no one can see it

19. If a student's hair or shirt catches on fire,

a) use the safety shower to extinguish the flames
b) get the student to the floor and roll
c) roll the student in a fire blanket

20. Hazardous waste should be

a) placed in a special waste container
b) washed down the drain
c) placed in the wastebasket

Experiment 1

Basic Techniques

Objectives

- Understand the concepts related to the concentration of a solution.
- Prepare a series of diluted solutions starting with a concentrated one.

Background

The living cell is composed of 80-90% water by weight, in addition, all of the chemical reactions in living systems occur in aqueous solution. The most important parameters of the solution that must be accurately known and strictly maintained are the concentrations of hydrogen ions and the critical salts.

Solution:

Homogenous mixtures of solutes and solvent.

Concentration:

Concentration of a solution is the amount of solute dissolved in a given amount of solvent. If a small amount of solute dissolved in a large amount of solvent, the solution is said to be diluted. While, if the amount of solute is increased until no more solute could be dissolved at a given temperature, the solution will be saturated.

Several methods have been developed for the preparation of solutions, but only two of these methods are used in Biochemistry:

1. Percent solution.

2. Molar solution (Morality).

- **Percent solution**:

The term percent means parts of hundred. It is easy to prepare, and there are three procedures for preparing this percent solution:

1. Weight per weight method (w/w).

2. Weight per volume method (w/v).

3. Volume per volume method (v/v). (Used when both the solute and solvent are liquids).

Examples:

1. Prepare 10% NaCl (w/w), weigh 10 g of NaCl and 90 g water.

2. Prepare 10% NaCl (w/v), weigh 10 g of NaCl and dissolve in sufficient distilled water to give final volume of 100 mL.

3. Prepare 25% ethanol (v/v), dissolve 25 mL of ethanol in sufficient distilled water to give final volume of 100 mL.

- **Molar solution**:

Prepared by dissolving gram/ molecular weight (mole) of a substance in a liter (1000 mL) of a solution.

*mole= $6x10^{23}$ molecules/liter.

Consequently, a molar solution of any substance contains $6x10^{23}$ molecules/liter.

Example:

Preparation of 1M NaOH:

You need 40 g of NaOH (Mwt= 40) and complete with sufficient quantity of distilled water to have a final volume of 1000 mL (1 L).

Usually, we express the concentration of substances in our body in units of molarity, however, these concentrations are extremely low.

What is Molarity?

For a solution, it is the number of moles of that species found in one liter of the solution.

Example:

To prepare: 0.5 M of H_2SO_4 (M. wt.= 98.08 g/mol)

0.5(moles) x 98.08(g/mol) = 49.04 g H_2SO_4 per liter of solution.

What is Molality?

For a solution, it is 1 mole of solute in 1 Kg of solvent (moles of solute/Kg solvent)

*In comparison with molar solution, the final volume here can exceed 1000 mL.

What is Normality?

Normality is the number of equivalent weight per liter of solution. N=M*F

F:

1. Is the number of electrons or protons transferred in the reaction.
2. The number of hydrogen ions that a molecule transfers
3. The valence state of an element or compound.

Equivalent weights are determined by the valence

The equivalent weight of an element or compound is equal to the molecular weight divided by the valence (F).

Equivalent weight= Mw/F

Dilution:

Dilution can be defined as expression of concentration. Dilution expresses the amount of volume or weight of a substance in a specific total final volume.

A 1:5 dilution can be stated as the common fraction 1/5. This fraction enables one to calculate the actual concentration of a diluted solution. (dilution factor).

The most commonly used equation for preparing dilution is:

$$V_1 \times C_1 = V_2 \times C_2$$

Where

V_1= the volume taken from stock solution

C_1= concentration of stock solution

C_2= concentration of diluted solution.

V_2= volume of diluted solution

Example:

How do you prepare 25 mL of 0.1 M HCl from a stock of 1 M HCl?

$V_1 \times C_1 = V_2 \times C_2$

$V_1 \times 1 = 25 \times 0.1 \rightarrow V_1 = 2.5\text{mL}$

Take 2.5 mL from the original solution and complete it (dilute it) with sufficient distilled water to a final volume of 25 mL using a 25 mL-volumetric flask.

Apparatus/Reagents Needed

- Pipette
- Burette
- Analytical balance
- Beakers
- Volumetric flasks (100 mL, 1L, 500 mL)
- flasks (50 mL and 100 mL)
- Pasteur pipette

Procedure

General steps in solution preparation:

1. Refer to laboratory instructions on preparation of the particular solution and the bottle label for any specific precautions in handling the chemical.
2. Weigh out the desired amount of chemical(s) (using an analytical balance) or take the volume accurately in case of liquids (burette or pipette).

Special considerations:

3. Place chemical(s) into appropriate size beaker with a stir bar.
4. Add less than required amount of water; prepare all solutions with distilled water.

5. When the chemical is dissolved, transfer to a graduated cylinder or volumetric flask and add the required amount of distilled water to achieve the final volume.
6. If the solution needs to be at a specific pH, check the pH meter with fresh buffer solutions and follow instructions for using pH meter.

For volumes >5mL

Exact volume	**Approximate volume**
Pipette, burette	Graduated cylinder

For volumes <5mL

Exact volume	**Approximate volume**
Graduated pipette	Pasteur pipette

Useful links

Diluting a Volumetric Flask

https://www.youtube.com/watch?v=SeOyYK-sW2E

Dilution of a Solution

https://www.youtube.com/watch?v=tAgmKC18dRI

Student Name:	**Student ID no.:**
Date:	**Section:**
Instructor Name:	**TA name:**

Experiment 1: Basic Techniques

Pre-lab Questions

1. Define each of the following: Molarity:

Normality:

Dilution:

2. How do you prepare 100 mL of 95% solution of alcohol from pure 100% solution?

3. How do you prepare 100 mL of 10% solution of alcohol from 95% solution?

Post-lab Questions

1. How many grams of NaOH are required to prepare 600 mL of a 0.05 M solution? (Mwt = 40 g/mol)

2. Express the concentration of solution in Q1 in terms of normality, and in g/L (w/v).

3. How do you prepare 2 liter of 0.5 M solution of HCL that has specific gravity of 1.19 and is assayed as being 38% HCl (w/w)?

4. How much H_2SO_4 (Mw=98.08g/mol) is needed to make 100 mL of 0.25 M?

Experiment 2

Carbohydrates

Objectives

• predict the reactivity of various carbohydrates when reacted with Fehling's reagent.

• predict the outcome of selected carbohydrates that are reacted with the Barfoed's reagent.

• predict reaction results when various carbohydrate compounds are reacted with Seliwanoff's reagent.

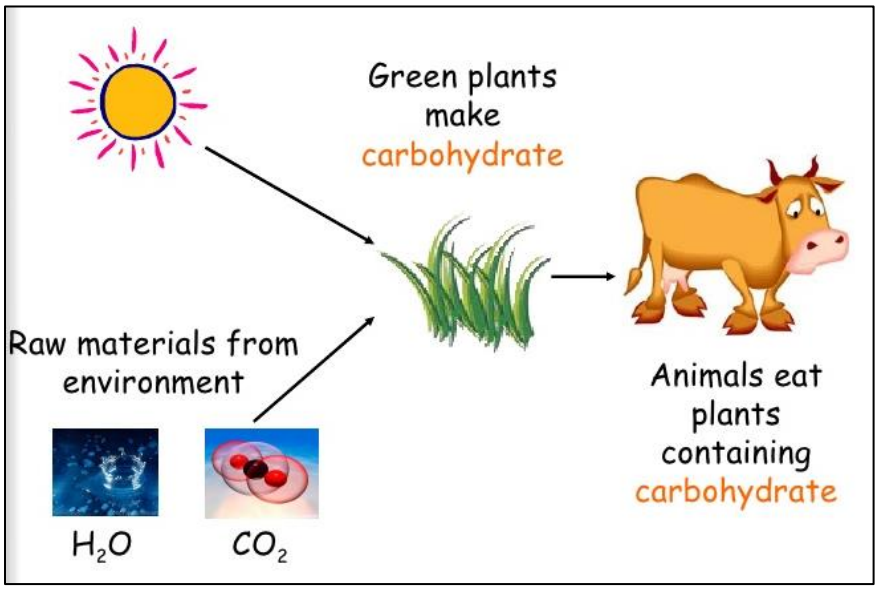
• predict the reactivity of various carbohydrates when reacted with an iodine solution.

• hydrolyze sucrose and describe the change in its behavior when it is reacted with Fehling's reagent.

Background

The word carbohydrate conjures up thoughts of foods, specifically sugar or starch-based foods. Sugars and starches constitute only a few examples of carbohydrate compounds. Cellulose, substances that make up part of the DNA structure, and substances that are part of the coating of living cells are also classified as carbohydrates.

Carbohydrates are formed by the process of photosynthesis. Energy from the sun converts H_2O and CO_2 into carbohydrates via photosynthetic plants; the carbohydrates then serve as a potential source of energy for animals. The idea of photosynthesis is shown in the following figure.



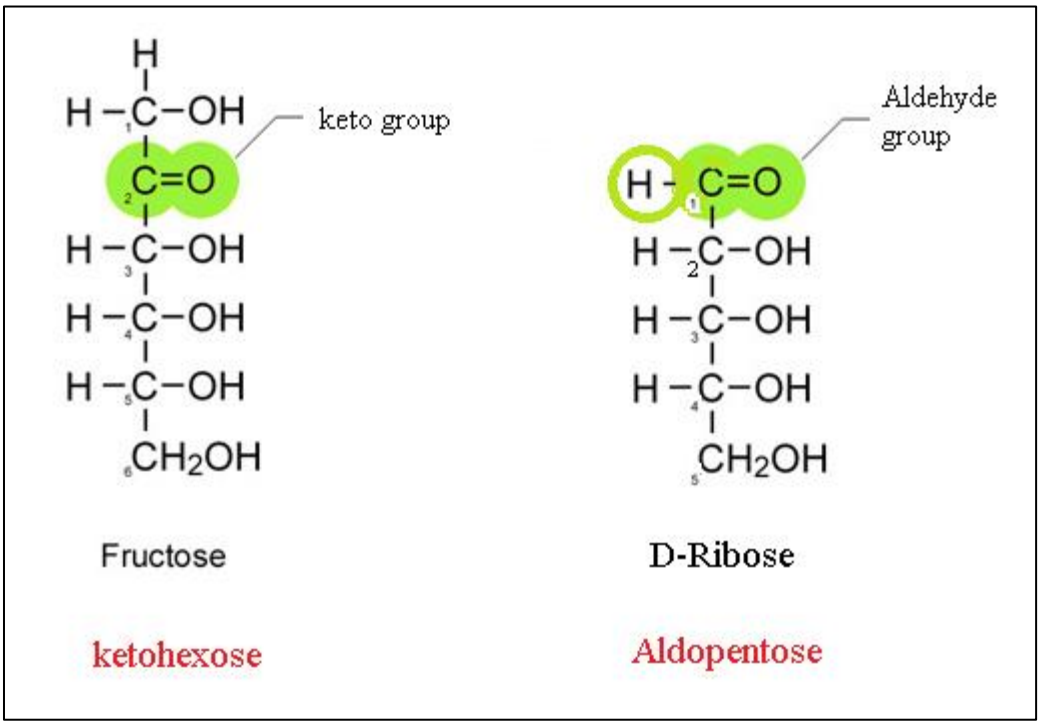
photosynthesis

Carbohydrates were initially defined as compounds having the empirical formula $\mathbf{C_n(H_2O)_n}$. This definition does not encompass all the molecules that are now known to belong to this group. For example, starch molecules contain fewer parts of water than their corresponding monosaccharide parts.

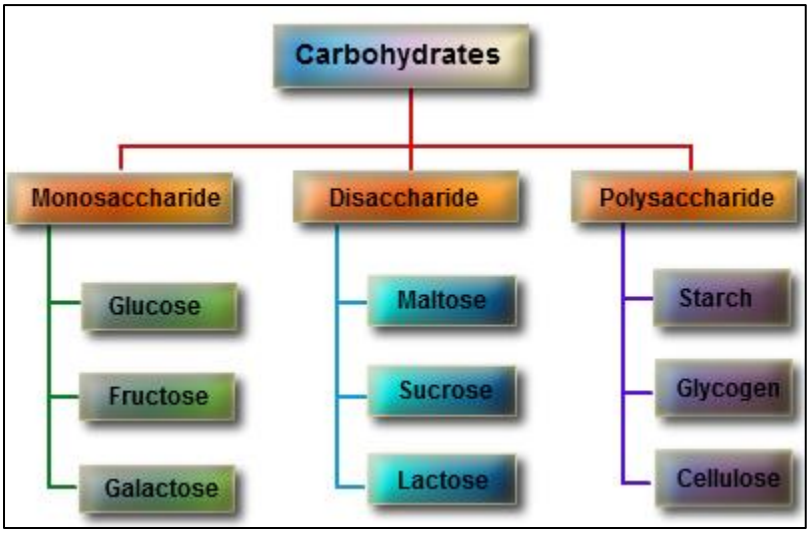
Carbohydrates are now more correctly defined as *polyhydroxy aldehydes or polyhydroxy ketones, or compounds that break down into these substances*. By examining the Fisher projection of D-ribose (see figure below), a 5-carbon carbohydrate, a better understanding of this definition is attained. Carbon 1 is the aldehyde carbon. Carbons 2 to 5 contain the hydroxyl group (—OH), hence polyhydroxy.

Carbohydrates can be categorized according to the number of carbons in the structure and whether a ketone or an aldehyde group is present. For example, ribose is classified as an aldopentose (aldo = aldehyde; pent = 5 [carbons]; ose =

carbohydrate ending). Fructose is a 6-carbon carbohydrate containing the ketone functional group and is therefore classified as a **ketohexose**.



A more general classification exists where carbohydrates are broken into the groups: **monosaccharides, disaccharides, and polysaccharides**. The following figure illustrates this classification with some examples on each class.



- Monosaccharides are often termed simple carbohydrates or simple sugars. These compounds cannot be broken down into smaller sugars by acid hydrolysis. Glucose, ribose, and fructose are examples of monosaccharides.

- Disaccharides are formed by the linkage of two monosaccharides. Sucrose, better known to us as table sugar, contains two carbohydrate units. It is synthesized when a glycosidic bond forms between glucose and fructose. Lactose and maltose are two other examples of disaccharides.

- Polysaccharides are formed by the joining of monosaccharide units. These compounds have molecular masses. Starch and cellulose are polysaccharides. In relation to food, starch is often termed the complex carbohydrate.

PHYSICAL PROPERTIES

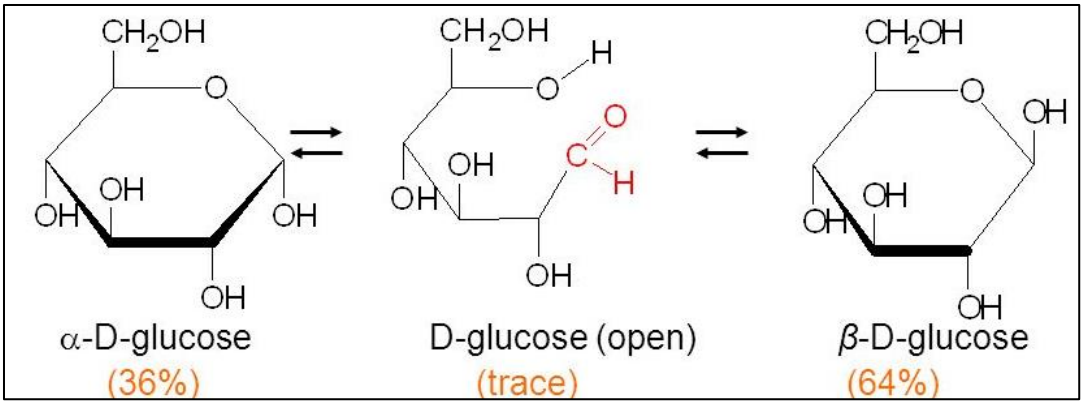
Since carbohydrates contain so many hydroxy groups, they tend to be very water-soluble. All mono- and disaccharides are extremely water-soluble, while some of the polysaccharides are not. The extensive amount of hydrogen bonding available in the lower molecular mass carbohydrates allows for water solubility.

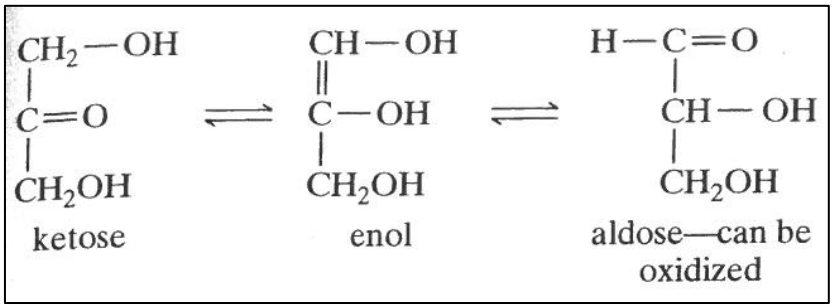
Other physical properties of many carbohydrates include high melting points, sweet taste, and white crystalline textures.

REACTIVITY

Oxidation:

The open-chain form of glucose, which accounts for less than 1% of a glucose solution at equilibrium, possesses an aldehyde functional group. This is susceptible to attack by oxidizing agents as shown below.

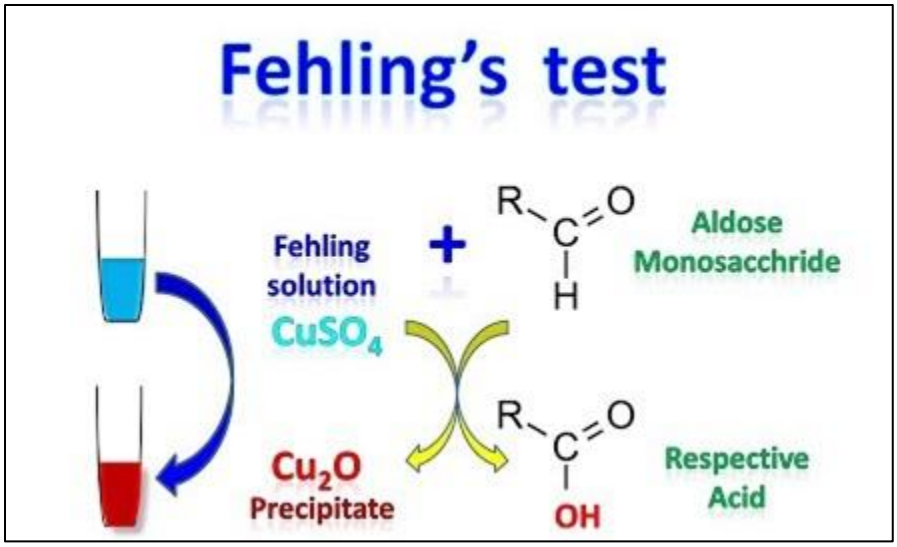




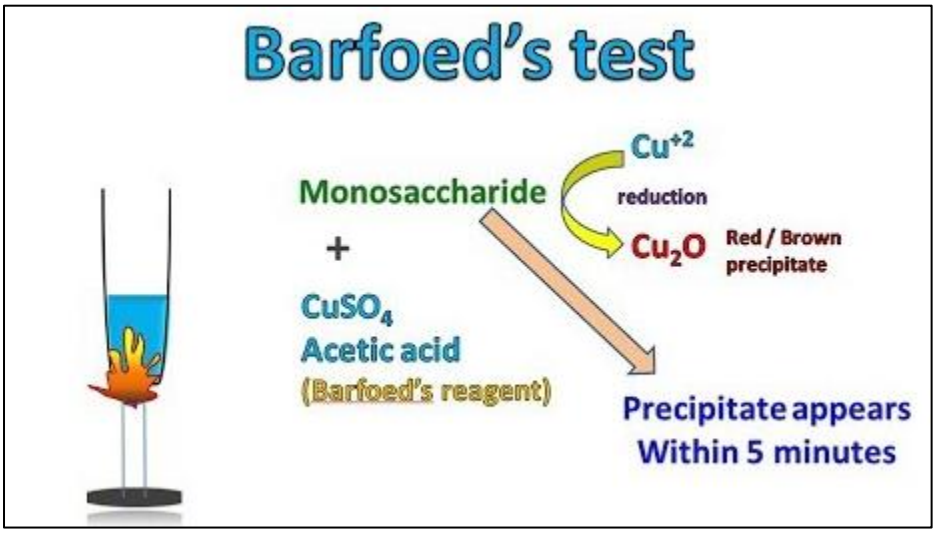
Carbohydrates that can undergo oxidation (i.e., glucose) are known as **reducing sugars**. All monosaccharides are reducing sugars since they have either the aldehyde functional group or an alpha-hydroxy ketone group that can undergo oxidation due to the equilibrium that exists as shown above.

Many disaccharides are also reducing sugars. Lactose and maltose both undergo oxidation reduction reaction. On the other hand, sucrose is not a reducing sugar (Why Not?)

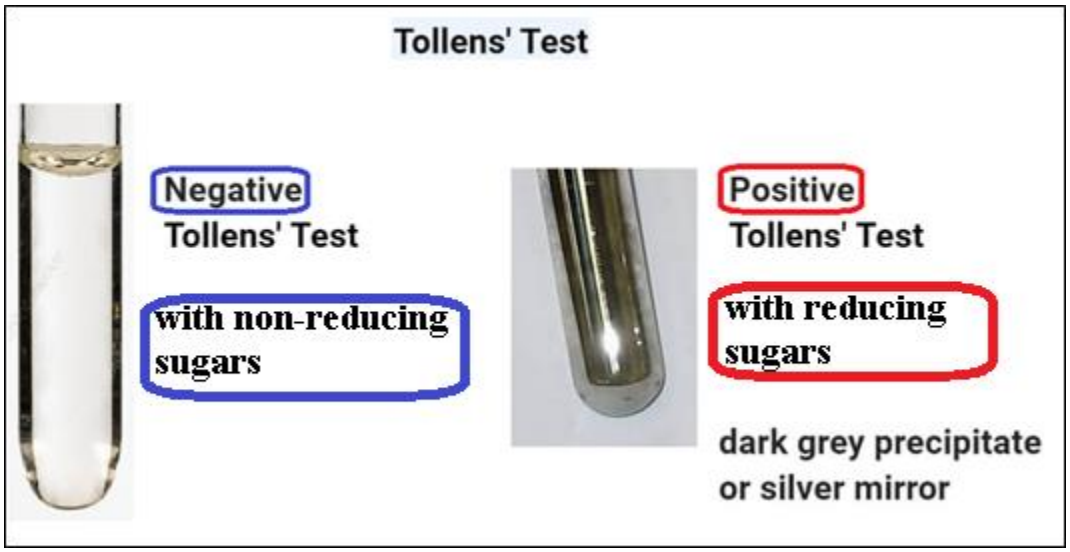
Fehling's reagent will be used to test the ability of carbohydrates as reducing sugars. (Remember that a substance acts as a reducing agent undergoes oxidation itself). A red precipitate signals a positive reaction.



One reagent that can distinguish between reducing monosaccharides and reducing disaccharides is called **Barfoed's reagent**. Reducing disaccharides undergo slow reaction or no reaction at all, while reducing monosaccharides react quickly with Barfoed's reagent. Again, the presence of a red precipitate indicates a positive reaction. Barfoed's reagent work as a mild oxidizing agent.

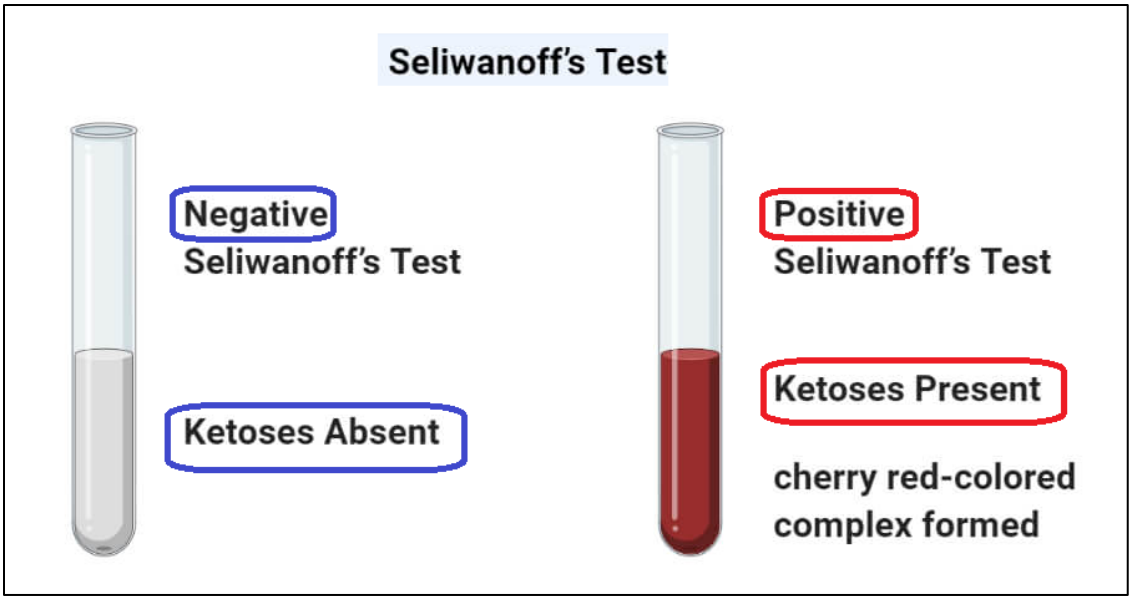


Tollen's test: This test is given by reducing sugars. Carbohydrates react with Tollen's reagent and forms a silver mirror on the inner walls of the test tube. This confirms the presence of reducing sugars. Silver ions are reduced to metallic silver.

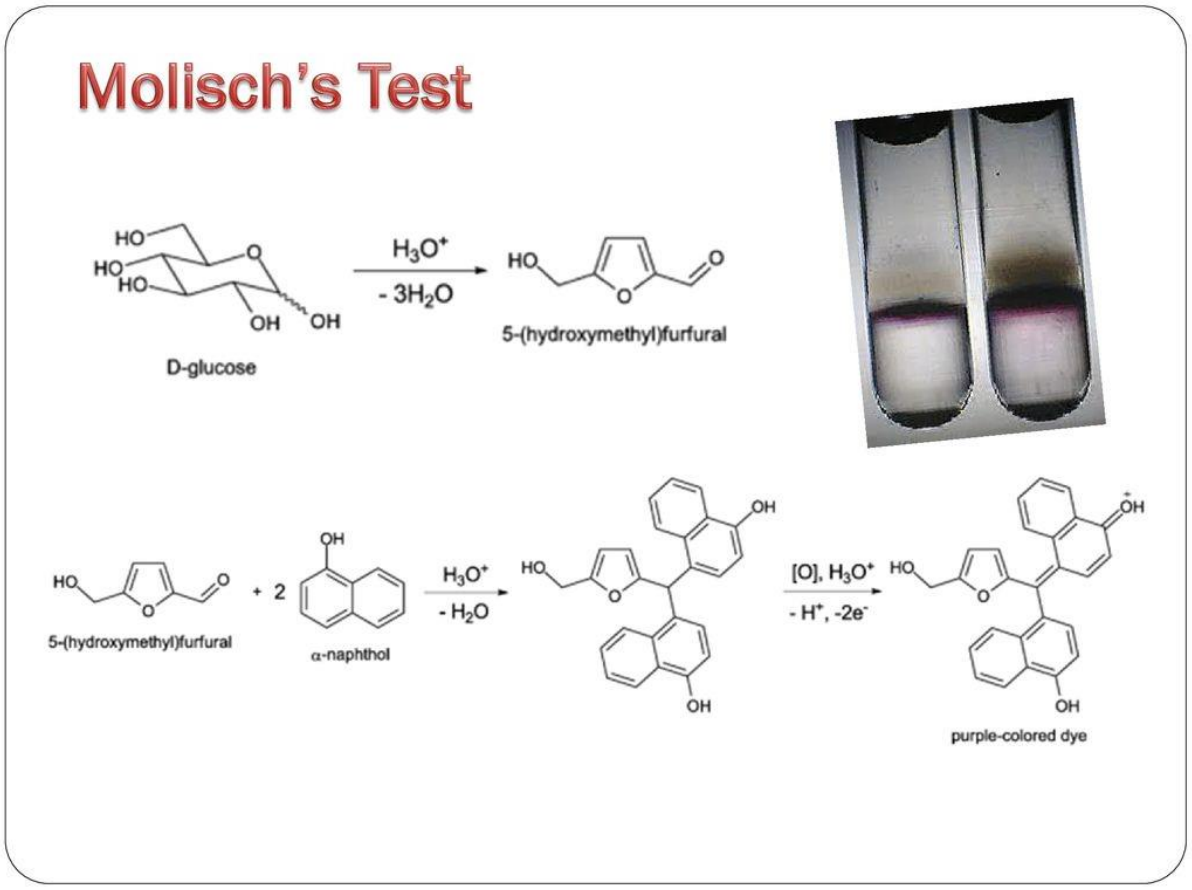


Dehydration:

Carbohydrates can undergo dehydration reactions when mixed with nonoxidizing aldoses and ketoses. To differentiate between aldoses and ketoses, **Seliwanoff's reagent** will be employed. When a ketose reacts with this reagent (resorcinol in 6 M HCI), a cherry-red-colored complex forms. On the other hand, if an aldose reacts with this reagent, the reaction takes place at a much slower pace. Disaccharides and polysaccharides eventually hydrolyze into monosaccharides, which, in time, also form red-colored solutions.



Another dehydrating reagent is the **Molisch reagent**. This simply detects the presence of a carbohydrate, since all carbohydrates form purple-colored products when reacted with this reagent.



Reaction with iodine:

Starch reacts with a solution containing iodine to reveal a deep blue color. This color may vary according to the structure of the polysaccharide and the concentration of the iodine solution. Simpler carbohydrates will not change the color of the iodine solution.

Hydrolysis:

Polysaccharides and disaccharides can be broken down in the presence of acid; this process is an **acid hydrolysis**. A carbohydrate such as sucrose that has only one glycosidic bond becomes fully hydrolyzed in an acid solution. The products are the two monosaccharides that make up sucrose: glucose and fructose. On the other hand, a very large starch molecule undergoes partial hydrolysis, producing many shortened starch molecules, known as dextrins. We will test the reducing properties of hydrolyzed sucrose and the ability of hydrolyzed starch to complex with iodine.

Apparatus/Reagents Needed

- Several small test tubes, 1 medium test tube, 1 large test tube, beaker for water bath, hot plate, litmus paper, glass stirring rod

- 1% glucose solution, 1% sucrose solution, 1% lactose solution, 1% fructose solution, 1% starch solution, Fehling's A and B solutions, Barfoed's reagent, Seliwanoff's reagent, Tollen’s reagent, iodine solution, 3 M HCI, 3 M NaOH.

Procedure

Fehling's test:

(Test: glucose, fructose, lactose, sucrose, starch, and your unknown).

To each of 6 test tubes, add 6 drops of the substances to be tested. In a large test tube, mix 6 mL of Fehling's solution A with 6 mL of Fehling's solution B. Add 2 mL of this mixture to each test tube. Stir each solution thoroughly. Place the test tubes in a boiling-water bath for 5 minutes and record your observations. The formation of a red precipitate indicates a positive reaction.

Barfoed's test:

(Test: glucose, fructose, lactose, sucrose, starch, and your unknown)

To each of 6 test tubes, add 1 mL of each of the solutions to be tested. Then add 3 mL of Barfoed's reagent to each test tube and mix thoroughly. Place the test tubes in a boiling water bath for 5 min. Let the test tubes cool, and then run cold water over each test tube. Record your observations. A dark red precipitate will form when a reaction occurs.

Tollen’s test:

(Test: glucose, fructose, lactose, sucrose, starch, and your unknown)

To each of 6 test tubes, add 1 mL of each of the solutions to be tested. Then add 3 mL of Tollen's reagent to each test tube and mix thoroughly. Place the test tubes in

a boiling water bath for 10 min. The appearance of shiny silver mirror confirms the presence of reducing sugars.

Seliwanoff's test:

(Test: glucose, fructose, lactose, water, and your unknown)

Avoid contact with this solution since it contains hydrochloric acid. To each of 4 test tubes, add 10 drops of the solutions to be tested. A fifth test tube containing 10 drops of distilled water should then be prepared. Add 4 mL of Seliwanoff's reagent to each test tube and mix. Place the test tubes in a boiling-water bath and note the time needed for any color change to occur. Discontinue heating after 10 minutes. A color change indicates a positive reaction. Record your observations.

Iodine test:

(Test: glucose, fructose, lactose, sucrose, starch, water, and your unknown)

Add 1 mL of the solutions to be tested to separate, labeled test tubes and then add 3 drops of iodine solution to each one. Compare the colors and record your observations.

*** In the following tests, avoid contact with the NaOH and HCI solutions.*

Hydrolysis of sucrose (use 1% sucrose solution):

Add 0.5 mL of 3 M HCl to 5 mL of a 1% sucrose solution. Heat and stir the mixture in a boiling-water bath for 20 minutes. Cool the solution. Add 3 M NaOH dropwise until the solution tests neutral on litmus paper.

Transfer 8 drops of this solution to a small test tube. Mix together 1 mL of Fehling's solution A with 1 mL of Fehling's solution B and then add to the test tube. Heat a few minutes and record your observations.

Hydrolysis of starch (use 1% starch solution):

Add 0.5 ml of 3M HCl to 3 ml of a 1% starch solution. Heat for 10 minutes in a boiling- water bath. Cool and neutralize with 3M NaOH.

Transfer 1 ml of this hydrolyzed starch solution to a small test tube and add 3 drops of iodine reagent. Record the color on your data sheet.

** DECANT THE LIQUID DOWN THE SINK WITH PLENTY OF RUNNING WATER AND DISPOSE OF THE SOLID IN THE GARBAGE.

Useful links

- Introduction about Carbohydrates:

https://www.youtube.com/watch?v=kIIS59cjzjI

- Fehling's Test:

https://www.youtube.com/watch?v=_C1llXoimns

- Barfoed's Test:

https://www.youtube.com/watch?v=WLt0LRXS7FU

-Tollen's Test:

https://www.youtube.com/watch?v=7U69JXm0rPM

- Qualitative Tests for Carbohydrates:

https://www.youtube.com/watch?v=ojhdTFmkY1c

Student Name:	Student ID no.:
Date:	Section:
Instructor Name:	TA name:

Experiment 2: Carbohydrates

Pre-lab Questions

1. Fill in the blanks in the following table:

Sugar	glucose	fructose	lactose	sucrose	starch
Structure (open form)			--	--	--
Mono, di, or polysaccharide?					
Aldose or ketose?			--	--	--
Pentose or hexose?			--	--	--

2. What test could be used to differentiate between the compounds in each set? Explain your answer.

- Sucrose & Maltose

- Fructose & Glucose

- Lactose & Glucose

3. What are the products of the sucrose hydrolysis?

4. Explain what is meant by the term "reducing sugar."

Results and Observations

Fehling's Test (Oxidation):

	Glucose	Lactose	Sucrose	Starch
Observations				
Positive/negative result				

What is the aim of Fehling's test?

Barfoed's test (Mild oxidation):

	Glucose	Lactose	Sucrose	Starch
Observations				
Positive/negative result				

What is Barfoed's test for?

Explain the negative results.

Tollen's Test (Oxidation):

	Glucose	Lactose	Sucrose	Starch
Observations				
Positive/negative result				

What is the aim of Tollen's test?

Explain the negative results.

Seliwanoff's test (Dehydration):

	Glucose	Lactose	Sucrose	Starch
Observations				
Positive/negative result				

What is the aim of Seliwanoff's test?

Iodine Test:

	Glucose	Lactose	Sucrose	Starch
Observations				
Positive/negative result				

What is iodine test is used for?

Hydrolysis of sucrose and starch:

Substance	Observations
Sucrose + Fehling's	
Hydrolyzed sucrose + Fehling's	
Starch + iodine	
Hydrolyzed starch + iodine	

Compare the result of reaction of Sucrose with Fehling's reagent after and before hydrolysis. Explain these results.

Compare the result of reaction of Starch with iodine solution after and before hydrolysis. Explain these results.

Post-lab Questions

1. An unknown carbohydrate showed a positive test reaction when tested with Fehling's reagent, turned red when reacted with Seliwanoff's reagent, and reacted quickly with Barfoed's reagent. What conclusion can be made about this compound?

2. Sucrose differs from honey in that honey is a mixture of glucose and fructose. Sucrose is a disaccharide made up of them. Explain why honey is a quicker source of energy than sucrose.

3. Predict whether or not a reaction will occur. Write the predicted results (time, color, etc.) for a positive result or NR for no reaction.

Sugar	**+ Reagent**	**Result**
sucrose	Barfoed's	
fructose	Fehling's	
fructose	Seliwanoff's	
lactose	Barfoed's	
sucrose	I_2 solution	
starch	Hydrolysis followed by I_2 solution	

Experiment 3

Amino Acids

Objectives

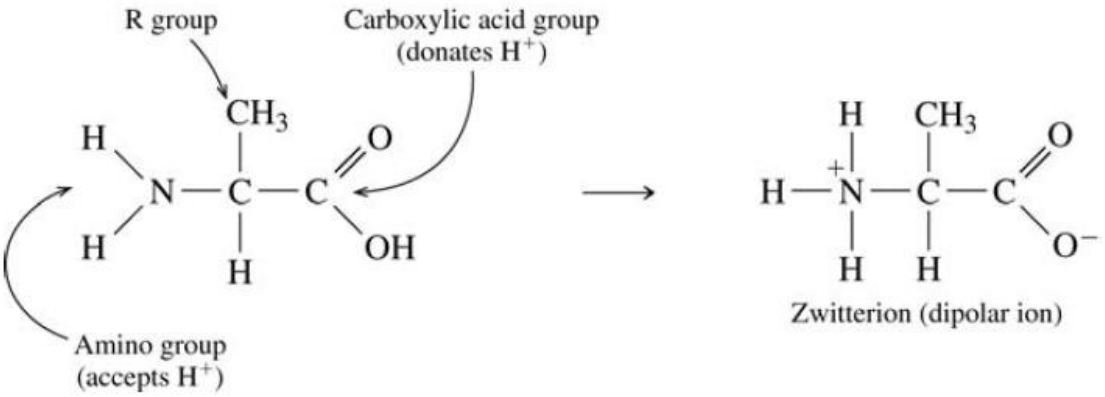
• Use R groups to determine if an amino acid will be nonpolar (hydrophobic), polar (hydrophilic), acidic, or basic.

• Use paper chromatography to separate and identify amino acids.

• Calculate R_f values for amino acids.

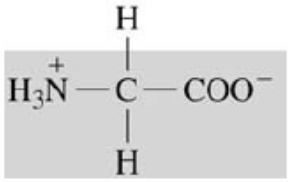
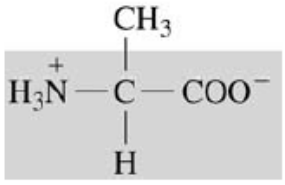
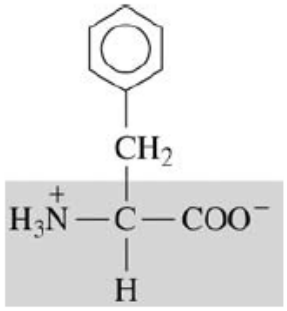
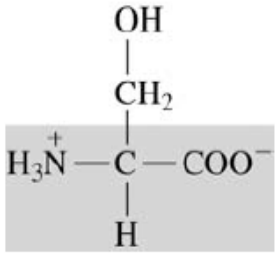
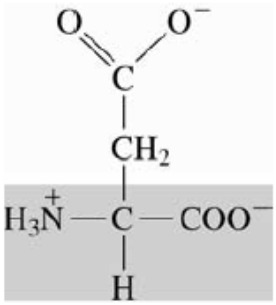
Background

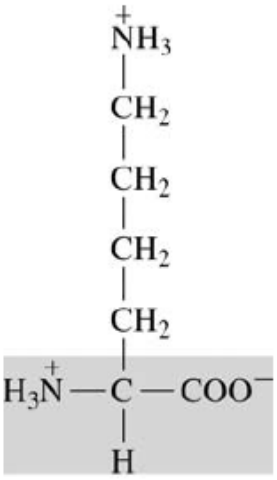
In our bodies, amino acids are used to build tissues, enzymes, skin, and hair. **Essential amino acids**, about half of the naturally occurring amino acids, must be obtained from the proteins in the diet because the body cannot synthesize them. Amino acids are similar in structure because each has an ionized amino group ($-NH_3^+$) and an ionized carboxylate group ($-COO^-$). Individual amino acids have different organic groups (R groups) attached to the alpha (α) carbon atom. Variations in the R groups determine whether an amino acid is nonpolar (hydrophobic), polar (hydrophilic), acidic, or basic.



Some R groups contain carbon and hydrogen atoms only, which makes the amino acids nonpolar and hydrophobic ("water-fearing"). Other R groups contain -OH or -SH atoms and provide a polar area that makes the amino acids soluble in water; they are hydrophilic ("water-loving"). Other hydrophilic amino acids contain R groups that are carboxylic acids (acidic) or amino groups (basic). The R groups of some amino acids used in this experiment are given in the following Table (next page).

Table: **Amino Acids Found in Nature**

	Amino Acid	Symbol	Polarity	Reaction to Water
	Glycine	Gly	Nonpolar	Hydrophobic
	Alanine	Ala	Nonpolar	Hydrophobic
	Phenylalanine	Phe	Nonpolar	Hydrophobic
	Serine	Ser	Polar	Hydrophilic
	Aspartic acid	Asp	Acidic	Hydrophilic

	Lysine	Lys	Basic	Hydrophilic

Ionization of Amino Acids

In acidic solutions (low pH), the ionized amino acid *accepts* a proton (H^+) to form an ion with a positive charge. When placed in a basic solution (high pH), the ionized amino acid *donates* a proton (H^+) to form an ion with a negative charge (see Table in the next page). This is illustrated using alanine.

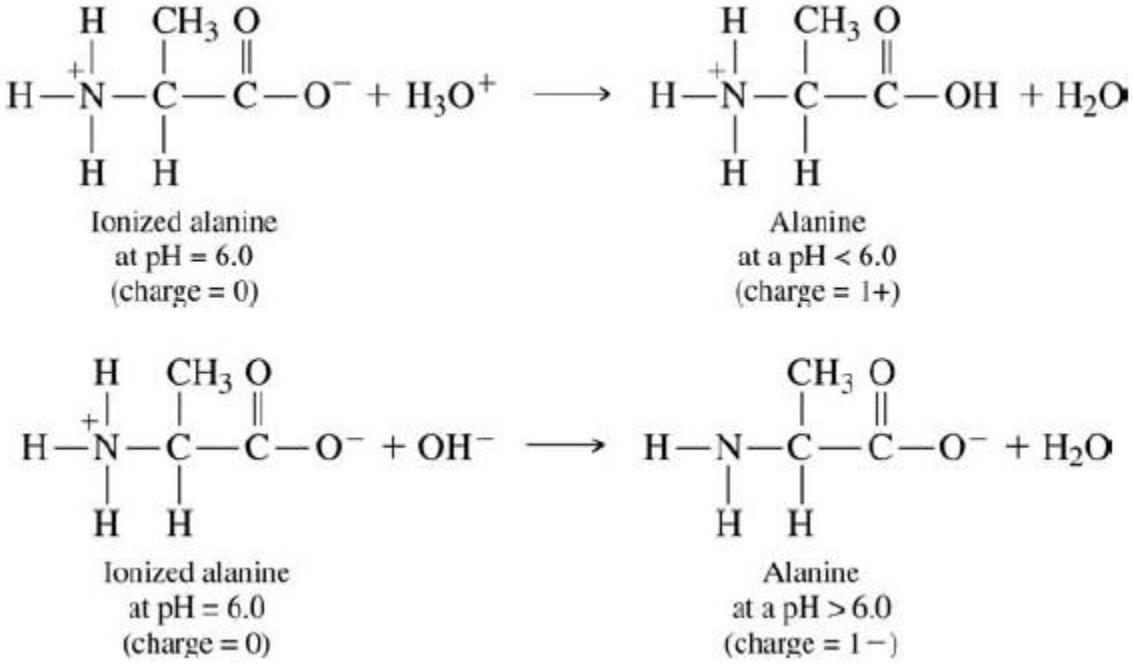


TABLE **Ionized Forms of Nonpolar and Polar Neutral Amino Acids**

Solution	pH < pI (acidic)	pH = pI	pH > pI (basic)
Change in H^+	$[H_3O^+]$ increases	No change	$[H_3O^+]$ decreases
Carboxylic Acid/Carboxylate	—COOH	$—COO^-$	$—COO^-$
Ammonium/Amino	$—NH_3^+$	$—NH_3^+$	$—NH_2$
Overall Charge	1+	0	1−

Chromatography of Amino Acids

Chromatography is used to separate and identify the amino acids in a mixture. In **paper chromatography**, the stationary phase is a very uniform absorbent paper. The mobile phase is a suitable liquid solvent or mixture of solvents. Small amounts of amino acids and unknowns are placed along one edge of Whatman #1 paper, which makes the chromatogram. The chromatogram is then placed in a container with solvent. With the paper acting like a wick, the solvent flows up the chromatogram, carrying amino acids with it. Amino acids that are more soluble in the solvent will move higher on the paper. Those amino acids that are more attracted to the paper will remain closer to the origin line. After removing and drying the chromatogram, the amino acids can be visualized by spraying the dried chromatogram with ninhydrin. The distance each amino acid travels from the starting line is measured and the R_f values calculated (see the following Figure).

$$R_f = \frac{\text{distance traveled by an amino acid}}{\text{distance traveled by the solvent}}$$

To identify an unknown amino acid, its R_f value and color with ninhydrin are compared to the R_f values and colors of known amino acids. In this way, the amino acids present in an unknown mixture of amino acids can be separated and identified.

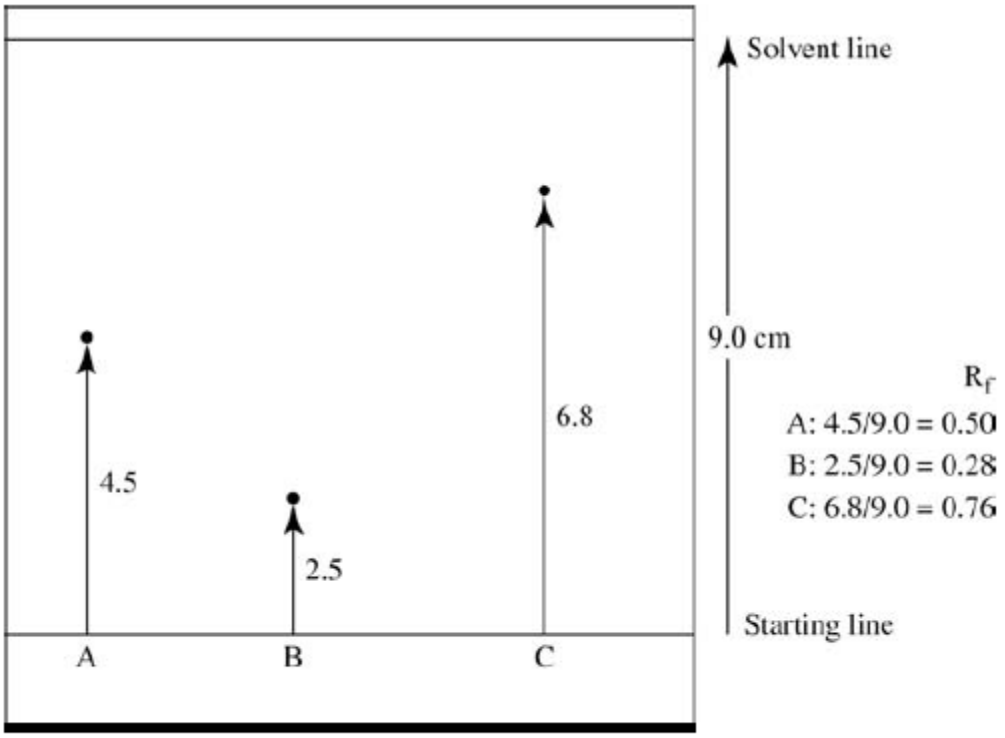


FIGURE A developed chromatogram (R_f values calculated for A, B, and C).

Apparatus/Reagents Needed

600-mL beaker and watch glass or chromatography chamber with cover; thread; plastic gloves; Whatman chromatography paper #1 (12 cm × 24 cm); capillary tubing; drying oven (80 °C) or hair dryer; metric ruler; stapler; amino acids (1% solutions): phenylalanine, alanine, glycine, serine, lysine, aspartic acid, and unknown amino acid; chromatography solvent: isopropyl alcohol, 0.5M NH_4OH; 0.2% ninhydrin spray.

Procedure

** Keep your fingers off the chromatography paper because amino acids can be transferred from the skin.

** Do not use pen, the ink will dissolve in the solvent and ruin your results.

1- Using forceps, plastic gloves, or a paper towel, pick up a piece of Whatman #1 chromatography paper that has been cut to a size of 12 cm × 24 cm.

2- Draw a pencil line about 2 cm from the long edge of the paper or plate. This will be the starting line (origin). With a pencil, mark off 7 equally spaced points about 3 cm apart along the line as you can see in the following Figure.

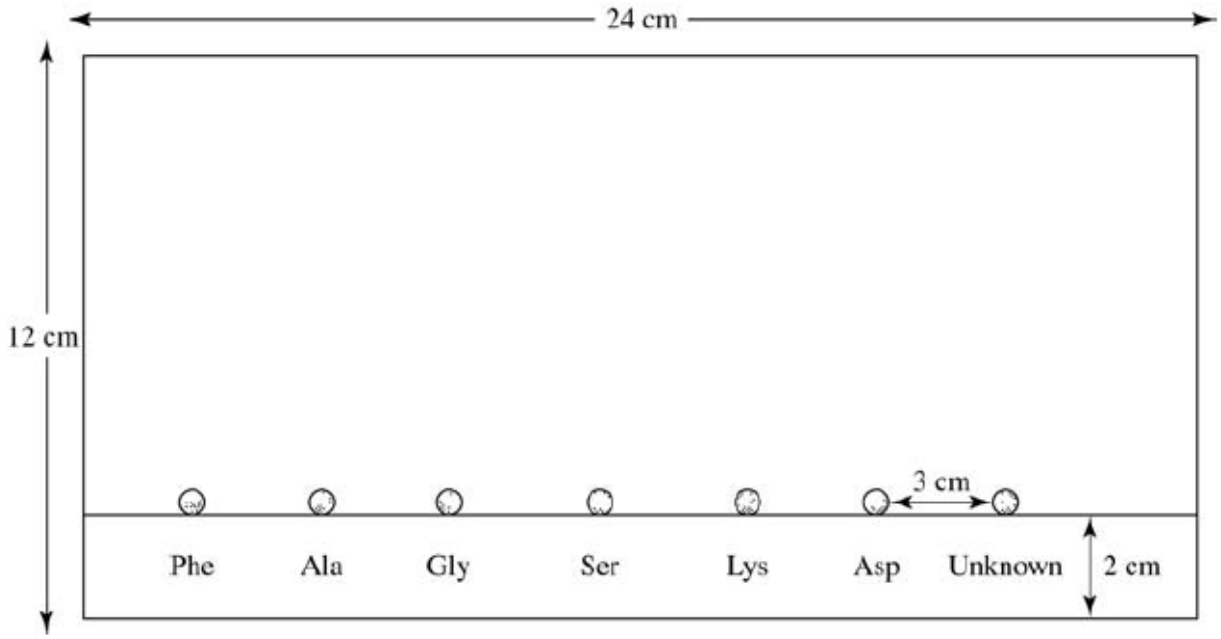
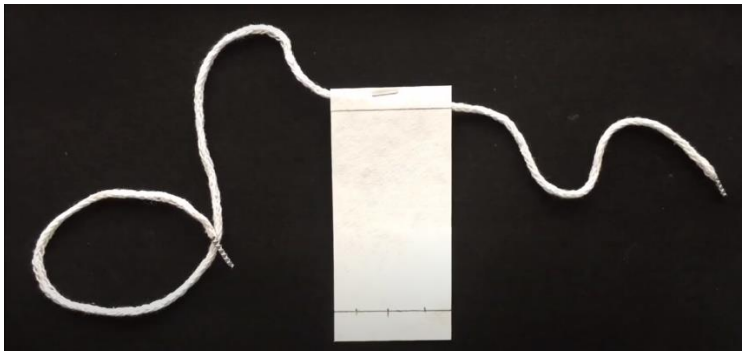


FIGURE Preparation of a chromatogram.

3- Label each with the abbreviation of one of the amino acids.

4- Place your name or initials in the upper corner with the pencil.

5- staple a thread to the top of the paper as shown below:



5- Use the capillary tubes provided in each 1% amino acid solution to make a small spot (the size of the letter o) by lightly touching the tip to the paper or plate. Always return the tube to the same amino acid solution.

6- Dry the spot either with a hair dryer or by allowing it to air dry.

7- After the spot dries, repeat the application of the amino acids twice more, for a total of three applications.

8- Prepare the solvent by mixing 10 mL of 0.5 M NH_4OH and 20 mL of isopropyl alcohol.

9- Pour the solvent into a chamber to a depth of about 1 cm but not over 1.5 cm. ** The height of the solvent must not exceed the height of the starting line on your chromatography paper.

10- Cover the chamber.

11- Slowly lower the paper into the solvent of the chromatography chamber with the row of amino acids at the bottom. Make sure that the paper does not touch the sides of the chamber (see Figure 33.3).

12- Cover the beaker with the plastic wrap and leave the tank and paper undisturbed.

13- Let the solvent flow up the paper until the solvent front is 2–3 cm from the top edge of the paper. It may take 45–60 min. *Do not let the solvent run over the top of the paper*.

14- Carefully remove the paper from the tank and spread the chromatogram out on a paper towel. *Immediately*, use a ruler and pencil to mark the solvent line.

15- Allow the chromatogram to dry completely. A hair dryer or an oven at about 80°C may be used to speed up the drying process.

16- ***Working in the hood***, spray the paper lightly, but evenly, with a ninhydrin solution. Distinct, colored spots will appear as the ninhydrin reacts with the amino acids.

***Caution: Do not breathe the fumes or get spray on your skin.**

17- Draw the chromatogram on the report sheet or staple the original to the report sheet.

18- Outline each spot with a pencil. Place a dot at the center of each spot. Record the color of each spot on the drawing or original.

19- Calculate and record the R_f values for the known amino acid samples and the unknown.

20- Compare the color and R_f values produced by the unknown amino acids to those of the known samples. Identical amino acids will give similar R_f values and form the same color with ninhydrin. Identify the amino acid(s) in the unknown

Useful links

Introduction about Paper Chromatography:

https://www.youtube.com/watch?v=TdJ57SQ6GAQ

Paper Chromatography of amino acids:

https://www.youtube.com/watch?v=8wmQ_xWqZbo

Student Name:	Student ID no.:
Date:	Section:
Instructor Name:	TA name:

Experiment 3: Amino Acids

Pre-lab Questions

1. Which two functional groups are in all amino acids?

2. How does an R group determine if an amino acid is acidic, basic, or nonpolar? Explain with examples.

3. In a chromatography experiment, a student calculated an R_f value for alanine of 0.70 and 0.91 for leucine. Which amino acid traveled higher on the chromatography paper? Explain your reasoning.

4. In a chromatography experiment, a student calculated that the solvent front was 6.8 cm above the starting line. Arginine traveled a distance of 4.9 cm and glycine traveled 3.4 cm. What R_f values would the student calculate for arginine and glycine?

Results and Observations

1. Structural formulas (ionized) of amino acids		
Asparagine	Leucine	Lysine
2. Polar or nonpolar?		
3. Structural formulas of amino acids in an *acidic* solution		
4. Structural formulas of amino acids in a *basic* solution		

1. Structural formulas (ionized) of amino acids	
Proline	Tryptophan
2. Polar or nonpolar?	
3. Structural formulas of amino acids in an *acidic* solution	
4. Structural formulas of amino acids in a *basic* solution	

Draw your paper after spraying with Ninhydrin then fill in the following table.

Amino acid	Color	Distance (cm) solvent traveled	Distance (cm) amino acid traveled	R_f value
Asp				
Leu				
Lys				
Pro				
Try				

- The mixture has 3 spots. What are the amino acids present in it?

- The R_f value of Leu is greater than that of Lys. Explain.

Post-lab Questions

1- What do fingerprint specialists use ninhydrin for? Why?

2- Give the general equation for the reaction of an amino acid with ninhydrin to produce a colored compound.

Experiment 4

Spectrophotometric Determination of Protein Concentration

Objectives

• Learn how to use a spectrophotometer to calculate concentrations using Beer's law.

• Calculate absorbances, concentrations, and extinction coefficients, using Beer's law.

• Do a simple protein assay and learn how to create standard curves for the determination of the protein concentration in an unknown.

Background

Almost all biochemical experiments eventually use spectrophotometry to measure the amount of a substance in solution. **Spectrophotometry** is the study of the interaction of electromagnetic radiation with molecules, atoms, or ions.

Light, or electromagnetic radiation, has a wave and particle nature. The **wavelength** λ of light is the distance between adjacent peaks in the wave. The **frequency** ν is the number of waves passing a fixed point per unit of time (see Figure 5.1).

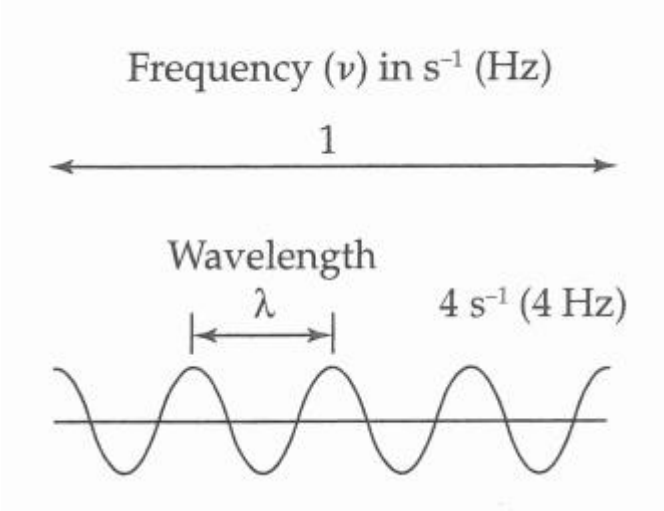


Figure 5.1: Wave nature of light.

These parameters can be further defined by the equation:

$$\lambda = \frac{c}{\nu}$$ where ***c*** is the speed of light.

Photons of different wavelength have different energies. These energies can be calculated by the equation:

$$E = hc/\lambda = h\nu$$ where ***h*** is Planck's constant.

Therefore, the longer the wavelength, the less energy the light has and vice versa.

Figure 5.2 shows the relationship between the wavelength of light and the common types of electromagnetic radiation. As you can see, those regions where the wavelength is very short correspond to the types of radiation that you know are powerful and often harmful, such as X-rays, gamma-rays, and ultraviolet radiation.

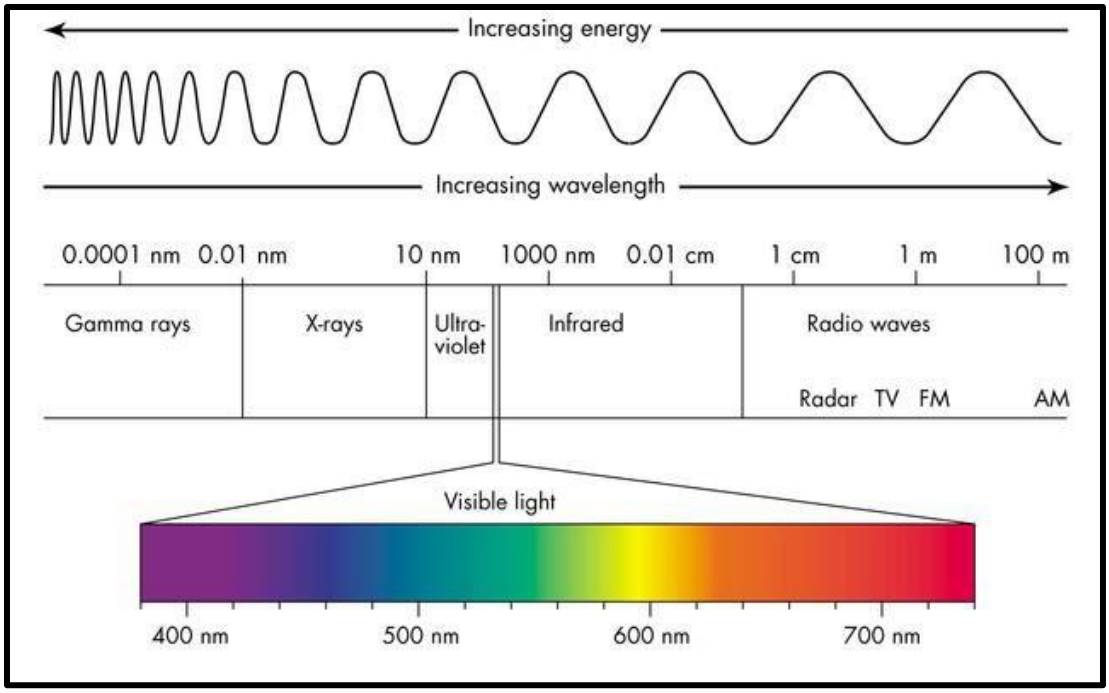


Figure 5.2: Wavelength regions of light.

Most compounds have a certain characteristic wavelength or wavelengths of light that they absorb. Figure 5.3 diagrams this process. Thus, the solution looks green to us because green light (blue and yellow) is transmitted while the red light is absorbed.

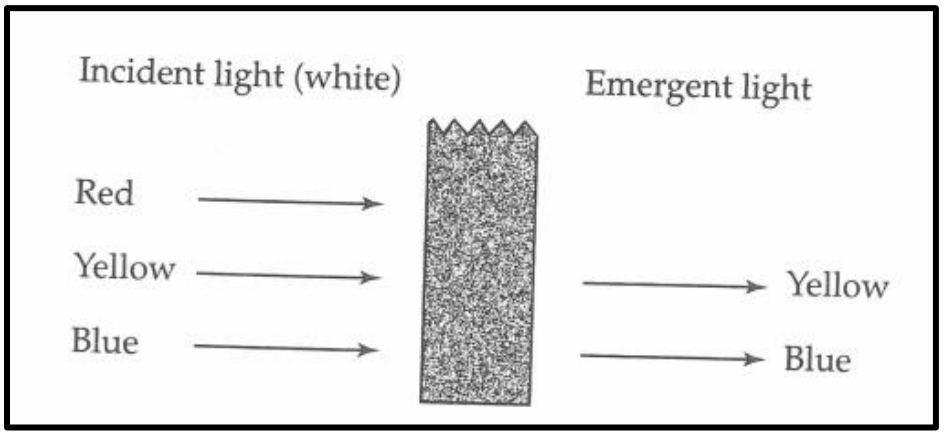


Figure 5.3: Absorption of light by a solution.

A solution may contain many compounds that absorb at many different wavelengths. However, if a compound that we are interested in absorbs at a unique wavelength, we can determine its concentration even in a solution of other compounds.

The Beers-Lambert Law

If a ray of monochromatic light (one wavelength) of initial intensity 1_0 passes through a solution, some of the light may be absorbed so that the transmitted light I is less than 1_0 (Figure 5.4). The ratio of intensities $1/1_0$ is called the **transmittance** and is dependent on several factors:

1. If the concentration c of the absorbing solution increases, then the transmittance will decrease.
2. If the pathlength l that the light must travel through increases, then the transmittance will decrease.
3. If the nature of the substance changes or another substance that absorbs more strongly is used, then the transmittance will change. The nature of the substance is reflected in ε, the **extinction coefficient**, also called the **absorptivity constant**.

An equation can be written that incorporates these ideas:

$$\log \frac{I_0}{I} = \varepsilon l c$$

where I_0 = intensity of incident light
I = intensity of transmitted light
ε = extinction coefficient
l = pathlength through solution
c = concentration of absorbing solution

Log I_0/I is usually called the **absorbance** and is abbreviated **A**.

This law $A = \varepsilon l c$

is called the **Beer-Lambert law** (**Beer's law**).

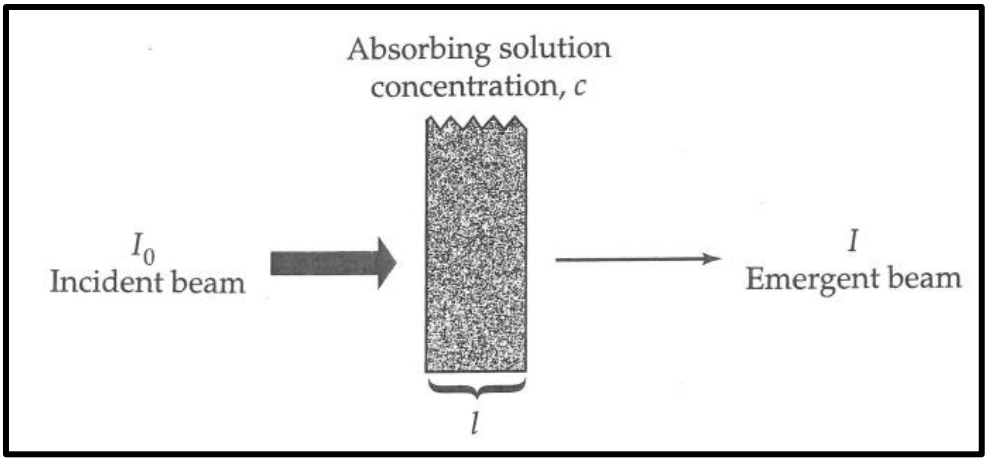


Figure 5.4: Relationship between I, I_0, l, and c for a solution absorbing monochromatic light.

Some Points to Consider

1. Absorbance A has no units. It is just a number that can be read off of the spectrophotometer. The wavelength is often specified along with the absorbance, such as $A_{540} = 0.3$.

2. The extinction coefficient ε is the absorbance of a unit solution and has units of reciprocal concentration and pathlength. The most common ε values recorded are for a pathlength of 1 cm and a 1 M solution. Therefore, the expression $\varepsilon_{600} = 4000\ M^{-1}cm^{-1}$ means that a 1 M solution has an absorbance at 600 nm of 4000 if a 1-cm diameter cuvette is used.

3. Remember that the pathlength l is usually in centimeters and, if not specified, is assumed to be 1 cm.

4. The concentration c has units that are the reciprocal of the units for ε.

5. At least 2 mL of solution is needed in a cuvette in order to read it with standard spectrophotometers.

6. Many things can interfere with your use of a spectrophotometer. If the cuvette is smudged or scratched, light will be scattered by the tube rather than absorbed by the solution. If you do not have sufficient volume (see point 5), the light may pass

over the solution instead of going through it. The spectrophotometer must be well calibrated before use.

If a substance obeys the Beer—Lambert law, then a plot of *A* versus *c* is straight, as in the "ideal" line shown in Figure 5.5. More often, however, the line is curved, shown as "reality" in Figure 5.5.

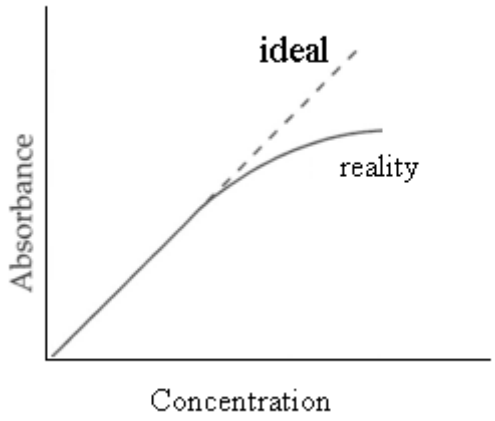


Figure 5.5: Absorbance versus concentration.

Reagent Blanks

A **reagent blank** is *a control in which everything is included except the substance for which we are testing*. One problem often encountered in spectrophotometry is that an absorbance is present at a given wavelength not due to the substance of interest. We handle that by mixing up all solutions in a tube except that substance and then read the absorbance. The absorbance of the reagent blank is then subtracted from the other readings.

Example: We want to read the absorbance at 595 nm of a protein solution mixed with the colorizing solution called Bradford reagent. What is a suitable reagent blank?

We want everything that might contribute to an absorbance at 595 nm except the protein we are trying to measure. Therefore, the best reagent blank is a tube of

Bradford reagent without any added protein. By zeroing the machine on this tube, the absorbance due to the Bradford reagent is subtracted out automatically.

Standard Curves

Determining the concentration of a substance works well if you know the extinction coefficient and if you know that the system obeys Beer's law at that concentration (as in question 1, Pre-lab). When these things are not known, which is most of the time, a standard curve is prepared. A **standard curve** is a plot of A versus a varying amount of a substance. Then, an unknown concentration can be determined from the graph.

Example: We have a compound X of varying concentrations in a phosphate buffer, pH 7.0, and the following absorbencies:

Tube no.	Concentration (mM)	Absorbance	Corrected Absorbance= (Sample Abs -blank Abs)
1	0	0.05	0.00
2	1	0.15	0.10
3	2	0.25	0.20
4	3	0.35	0.30
5	4	0.45	0.40
6	5	0.55	0.50

What is the concentration of a sample of X if the absorbance equals 0.30?

First, you must understand corrected absorbencies. Tube 1 has an absorbance of 0.05, but it does not contain any of the compound that we are measuring. This tube is our reagent blank, and it has an absorbance. The graph must have a curve that goes through zero. There are two ways of dealing with this. The first way is to subtract the absorbance (0.05) from all the absorbencies. This gives the data shown in the last column. The second way is to zero the spectrophotometer with tube 1. That way, the subtraction is done automatically. We will always use corrected

curves even though the difference between a corrected curve and an uncorrected one is largely cosmetic.

Continuing with the example, first subtract the reagent blank (tube 1) from the rest to give the corrected absorbance; then plot A versus c (corrected) (Figure 5.6). On the graph, look for the A that corresponds to the unknown, 0.30 - 0.05 (blank) = 0.25. From the graph, A = 0.25 corresponds to the concentration of 2.5 mM, which is the answer.

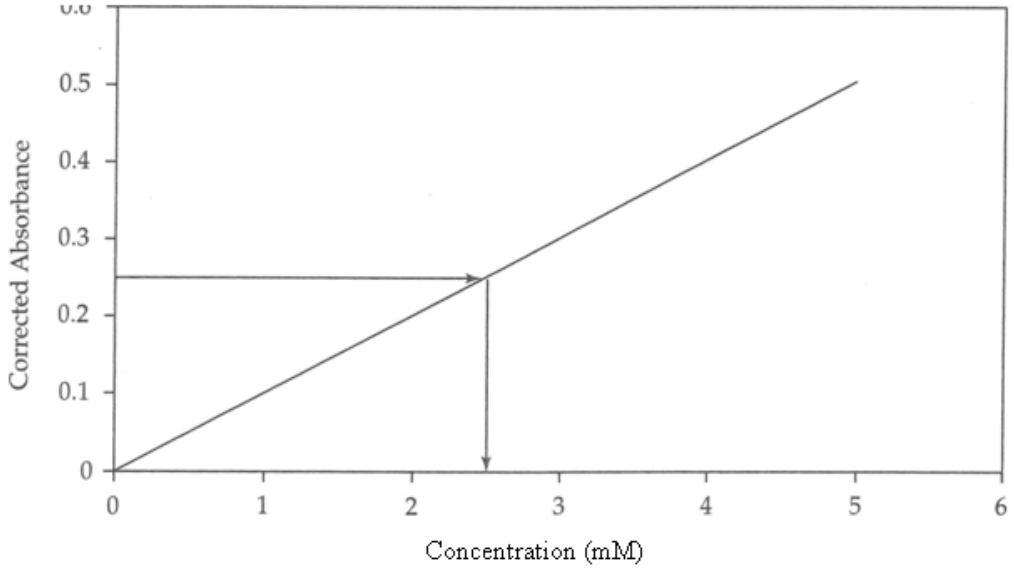


Figure 5.6: Corrected absorbance versus concentration for the Example.

Protein Assays

One of the most common uses for spectrophotometry, which also happens to use standard curves, is the **protein assay**. Many biochemical studies at some point require the knowledge of the amount of protein that you have in a sample.

Proteins can be assayed easily if you have a spectrophotometer that can measure light in the ultraviolet (UV) region. The amino acids tryptophan and tyrosine absorb strongly at 280 nm, which enables the scientist to scan for proteins at this wavelength. This is often done as a protein is purified with some chromatographic technique. However, to get a quantitative answer, you have to know the exact ε_{280}

for the protein. If the protein contains few aromatic residues or the extinction coefficient is low, the UV method would not be suitable. Also, many spectrophotometers found in teaching labs do not have UV capability.

Many assays can compensate for the inability to use UV absorption. Most of them depend upon certain dye molecules that react with parts of the protein to give a colored complex that can then be measured. Once you have a colored complex, you can use visible light spectrophotometry.

One of the most common and easiest to use is the **Bradford method**. This method uses a dye called Coomassie Brilliant Blue G-250, which has a negative charge on it. The dye normally exists in a red form that absorbs light maximally at 465 nm. When the dye binds to the positive charges on a protein, it shifts to the blue form, which absorbs maximally at 595 nm (see Figure 5.7). Many proteins have the same response curve to this dye, making the Bradford method reproducible among many experimenters. It is also very rapid. The reaction occurs in a couple of minutes, and the colored product is stable for over an hour. In addition, the protocol calls for a protein sample of up to 100-µL to be added to 3-5 mL of Bradford reagent. With such a large difference in volumes between the sample and the protein reagent, bringing all samples to the same 100-µL volume is not necessary before reagent addition. This saves time in setting up the assays.

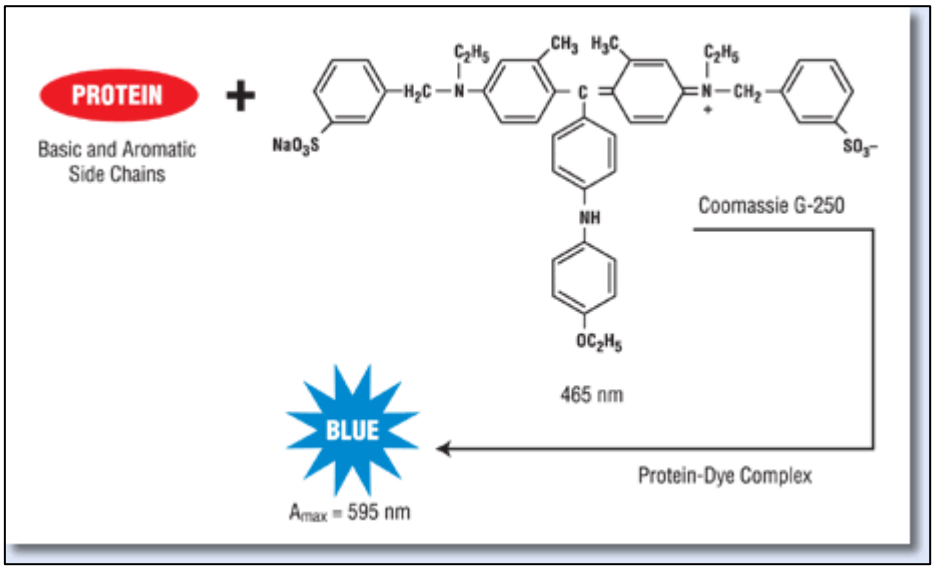


Figure 5.7: the principle of Bradford assay.

No single piece of equipment is used more in biochemistry or any life science than the **spectrophotometer**. We measure almost everything using one. Granted, most research labs have very sophisticated spectrophotometers that do more work, but basically it has a simple light source, a prism or grating for controlling the wavelength, and a sample holder. Getting caught up in the fun of pushing buttons without understanding how the machine works is far too easy. A spectrophotometer with its general principle is shown in Figure 5.8.

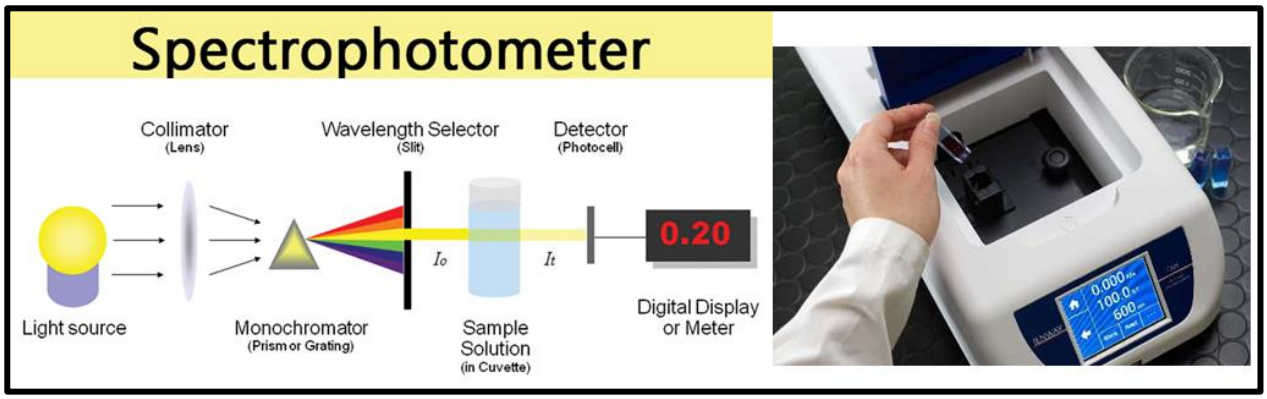


Figure 5.8: A spectrophotometer and its general principle.

Apparatus/Reagents Needed

Spectrophotometer
Cuvettes
Micropipettes
Vortex
NADH unknowns
Bradford protein reagent
BSA (Bovine serum albumin), mg/mL
BSA of unknown concentration


Cuvettes


Micropipette

Vortex

Procedure

Part A: Using Beer's Law to Determine Concentration.

In this part of the experiment, you use Beer's law to determine the concentration of a solution of NADH. NADH has an extinction coefficient of 6220 M^{-l} cm^{-1} at 340 nm. The pathlength for the cuvette is 1 cm.

1. Obtain a solution of NADH of unknown concentration.

2. Warm up and zero the spectrophotometer at 340 nm or at the wavelength as close to 340 nm that you can. Sometimes older machines cannot be zeroed at 340 nm but can be zeroed somewhere between 340 and 360.

3. Make a minimal dilution of the NADH to provide enough solution to measure in the cuvette.

4. Measure the absorbance of the NADH. If the absorbance is greater than 0.8, dilute it with water and remeasure. Record these dilutions. You need to know how much NADH you added to how much water.

5. Use the absorbance and any dilutions you made to determine the concentration of the NADH millimolar (mM).

Part B: Setting Up a Standard Curve

In this part, you set up a standard curve for a protein determination, called the Bradford method. The protein standard is bovine serum albumin (BSA), a generic protein generally used for protein assays. Usually, a series of tubes is set up with varying amounts of BSA and a constant amount of Bradford reagent. By plotting the milligrams or micrograms of BSA on the x axis and the corrected absorbance on the y axis, we can determine the concentration of unknown proteins from the graph.

1. Warm up the spectrophotometer at 595 nm.

2. Set up 10 large, clean test tubes to use for the assays. As a general rule, it is better to use large tubes for the reactions and then pour a couple milliliters of the solution into a cuvette-sized tube to read it, rather than setting up the reaction in the

cuvettes. Cuvettes are too small to mix most reaction solutions, and you also risk permanently discoloring the cuvettes.

3. Set up a protocol as in Table below. Using the most accurate pipet available, pipet the BSA standard into the tubes. The protein concentration is very high, and the volume is low, so any pipetting error will lead to poor standard curves.

	Reagent /Tube/mL								
	1 (Blank)	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9 (Unk)**
BSA standard, 1 mg/mL (μL)	0	10	20	30	40	50	75	100	-
Unknown	0	-	-	-	-	-	-	-	?
Bradford reagent	5 mL	5 mL	5 mL	5 mL	5 mL	5 mL	5 mL	5 mL	5 mL

4. Obtain an unknown BSA solution. Choose a volume of the unknown to assay and pipet into tube 9. This volume must be 100 μL or less.

5. Add 5 mL of Bradford reagent to each tube. Vortex immediately after adding the reagent to each tube. Do not wait until you have added it to all tubes.

6. Let the tubes sit about 10 min before reading the absorbencies. Once the color develops, it is stable for over an hour.

7. Make a plot to determine how much BSA you can add without the curve straying from linear.

Useful links

- Introduction to Protein Structure

https://www.youtube.com/watch?v=HSCUAjZQhXI

- Spectrophotometry and Beer's Law

https://www.youtube.com/watch?v=zuUvQN8KXOk

- Spectrophotometry- Standard curve

https://www.youtube.com/watch?v=b-mlIWb1lDQ

- Bradford Assay

https://www.youtube.com/watch?v=7o65va089S4

https://www.youtube.com/watch?v=TAMrj0Z9FOk

Student Name:	Student ID no.:
Date:	Section:
Instructor Name:	TA name:

Experiment 4: Spectrophotometric Determination of Protein Concentration

Pre-lab Questions

1. We measure the absorbance of a solution of compound X, which absorbs at 540 nm. The cuvette has a width of l cm, the extinction coefficient at 540 nm is 10,000 M^{-1} cm^{-1}, and the absorbance is 0.4. What is the concentration of compound X?

2. If your spectrophotometer can measure an absorbance up to 1.5, what is the maximum concentration of NADH that you can measure without diluting?

3. What is Vortex? When do use it?

4. When do we use micropipettes not normal graduated pipettes?

5. Convert:

5 mL= μL

2 μL= mL

2 mL= L

3 μL= L

Results and Observations

Part A: Using Beer's Law to Determine Concentration.

- Use the absorbance and any dilutions you made to determine the concentration of the NADH millimolar (mM).

- If you add 3 mL of water to 1 mL of NADH, mix and get an absorbance of 0.2, what is the concentration of the original NADH solution? (*Show calculations*)

(*Remember*: NADH extinction coefficient = 6220 M^{-1} cm^{-1} at 340 nm.

$M_{conc}V_{conc} = M_{dil}V_{dil}$)

Part B: Setting Up a Standard Curve

Suppose you got the following absorbances.

	Reagent /Tube/mL								
	1 (Blank)	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9 (Unk)**
BSA standard, 1 mg/mL (μL)	0	10	20	30	40	50	75	100	-
Unknown (μL)	0	-	-	-	-	-	-	-	80
Final Conc. (**mg/mL**)	0	0.002							
Absorbance									

** One can zero the instrument on the blank or take the blank absorbance and subtract it from all other readings. Here the instrument was zeroed on the blank.

1- Find all final concentrations in Tubes (3-8).

Example: Tube 2: 10 μL (= $10*10^{-3}$ mL) were taken from (1 mg/mL) solution and 5-mL Barford reagent was added.

$$M_{conc}V_{conc} = M_{dil}V_{dil}$$

1 mg/mL* $10*10^{-3}$ mL = M_{dil} * 5.01 mL

➔ M_{dil} = 0.002 mg/mL

2- Using Excel draw Absorbance vs. concentration (mg/mL) for Tubes 1-8.

Paste your graph here showing the equation of the Trendline.

(* Don't forget to set the intercept to zero)

3- From the equation of the graph, find the concentration of the unknown (Tube 9).

4- Remember that the conc. Found above is the final conc. Of the unknown. Find the original concentration of the unknown.

Post-lab Questions

1. What is the theoretical absorbance at 340 nm of a 0.01 M solution of NADH, assuming a 1-cm pathlength?

2. What dilution would be necessary to get the absorbance from Question 1 down to 3.1? (consider the final volume to be 2 mL) (*Show calculations*).

(----------µL of 0.01 M NADH to ---------- µL water)

3. Absorbance at 340 nm of a 0.02 mM NADH solution is 0.124 with a 1-cm pathlength. What is the absorbance with a 1.2-cm pathlength?

4. Five µL of an unknown BSA sample were added to 5 mL of Bradford reagent. The absorbance at 595 nm was 0.78 and, according to a standard curve, corresponds to 0.015 mg/mL of protein on the x axis. What is the protein concentration of the original solution?

5. Why did we not use Beer's law in Part B?

6. Why is the absorbance versus concentration curve for a substance rarely straight for all concentrations? (*i.e*. deviation from Beer's law).

Experiment 5

Isolating proteins from white blood cells

Objectives

• Extract total proteins from white blood cells.

Background

The study of proteins in living organisms is an integral part of life science research. Proteins are the most diverse group of biologically important molecules and are essential for cellular structure and function. The first step in protein analysis is cellular extraction. Because proteins are so heterogeneous, there is no one method or reagent that is optimal for general protein isolation. In addition, protein extraction techniques vary depending on the source of the starting material, the location within the cell of the protein of interest and the downstream application. Many techniques have been developed to obtain the best protein yield and purity for different types of cells and tissues, taking into account where appropriate, the subcellular location of the protein and the compatibility of the protein extract with the next step in the experiment.

In life science research, proteins are typically extracted from cultured mammalian cells, mammalian tissues or primary cells. When extracting proteins from mammalian tissues, mechanical disruption is required to isolate the cells from their tissue matrix. For cultured mammalian and primary cells, which have only a plasma

membrane separating the cell contents from the environment, reagents containing detergents and other components can easily disrupt the protein-lipid membrane bilayer, making total protein extraction relatively straightforward.

Because certain proteins are localized in specific organelles, protein yield and enrichment are greatly improved if the protein is extracted directly from its subcellular compartment or organelle. Some types of mechanical lysis alone can disrupt all cellular compartments, making it difficult to achieve subcellular fractionation. However, by the careful optimization of physical disruption and detergent-buffer formulations, procedures have been developed that enable the separation of subcellular structures. For example, with the appropriate detergents, hydrophobic membrane proteins can be solubilized and separated from hydrophilic proteins.

Cell lysis disrupts cell membranes and organelles resulting in unregulated proteolytic activity that can reduce protein yield and function. To prevent degradation of extracted proteins and obtain the best possible protein yield and activity following cell lysis, protease and phosphatase inhibitors can be added to the lysis reagents. Numerous compounds have been identified and used to inactivate or block the activities of proteases and phosphatases by reversibly or irreversibly binding to them. Because some detergents used in protein extraction formulations may inactivate the function of enzymes of interest or affect their long-term stability, it may be important to remove the detergents following cell lysis. In addition, high concentrations of detergents or salts can interfere with common research methods such as protein assays, protein purification, immunoprecipitation, gel electrophoresis and mass spectrometry (MS).

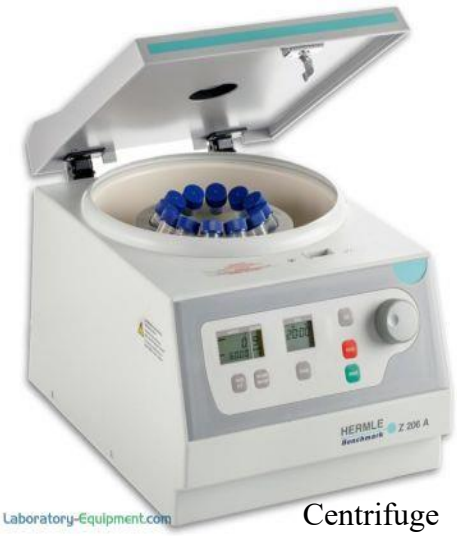
Apparatus/Reagents Needed

Sheep blood, EDTA, heparin or citrate anticoagulant tubes, RBC lysis solution (0.84% NH_4Cl), sterile 15-mL centrifuge tubes, Centrifuge, micropipette, PBS solution, 1.5-mL microcentrifuge tubes, extraction buffer, microcentrifuge tubes heater, ice bath.

Procedure

** Caution: When handling blood samples, follow recommended procedures for biohazardous material.

In this experiment, you will extract proteins from a sheep blood sample.



Centrifuge

- White blood cells (WBC) proteins:

1. Collect 3 mL blood in EDTA, heparin or citrate anticoagulant tubes to prevent clotting.

2. Gently rock the tube of blood until thoroughly mixed.

3. Add 9.0 mL of RBC lysis solution (0.84% NH_4Cl) to a sterile 15-mL centrifuge tube → in this step, you separate the component of the blood according to their densities, leading to have heavier component on the bottom and the lighter ones above.

** *Lysis solution contains NH_4Cl which is a salt that cause bursting of the cell releasing its content due to osmosis effect.*

4. Transfer blood to the tube containing the lysis solution. Invert the tube 5-6 times to mix.

5. Incubate the mixture for 10 minutes at room temperature (invert 2-3 times during the incubation) to lyse the red blood cells.

6. Centrifuge at 4000 RPM (round-per-minute) for 10 minutes at room temperature.

7. Remove and discard as much supernatant as possible without disturbing the visible white pellet. Approximately 50-100 µL of residual liquid will remain in the 15-mL tube.

Note: Some red blood cells or cell debris may be visible along with the white blood cells. If the pellet appears to contain only red blood cells, add an additional aliquot of the lysis solution after removing the supernatant above the cell pellet, and then repeat Steps 3-7.

The pellet here consists of intact WBC that is heavier in density, while the supernatant is composed of lighter substances, such as lysed RBC, NH_4Cl and serum.

** From now on, everything should be done on ice! (***Because in the later steps you will extract protein from its cell to the surrounding that is full of proteases leading to destruction and denaturation of them, in ice they will be inhibited).***

8. Add 500 µL cold PBS (Phosphate Buffer Saline) and pipette the cells vigorously until the white blood cells are resuspended (10-15 seconds).

9. Move your cell suspension to a 1.5 mL microcentrifuge tube and centrifuge at maximum speed for 1 minute at 4°C.

10. Remove and discard as much supernatant as possible without disturbing the visible white pellet.

11. Add 100-150 µL of the **extraction buffer**. Vortex or pipette thoroughly until all clumps disappear and your sample becomes viscous.

12. Boil the sample for 3-5 minutes then keep on ice. **Why boiling?**

To completely denature the proteins to homogenate the sample and to make it less gummy by melting the DNA in the sample.

***** Extraction buffer (1.5 ml per group):**

2 mL of 10% SDS

0.1 mL of 1M Tris buffer pH 7.5

0.1 mL of 1M NaF

0.05 mL of 1M DTT

0.2 mL of 0.1M EGTA

7.55 mL distilled water

- **10% SDS**: anionic detergent , denatures proteins by disturbing the non covalent forces, also gives a net negative charge for the proteins.
- **1M Tris buffer** pH 7.5: maintain the pH constant and protect the proteins.
- **1M NaF**: inhibitor for protein phosphoseryl and phosphothreonyl phosphatases (PSPs), to preserve the proteins phosphorylation state in the cells.
- **1M DTT:** reduces the disulfide bonds of proteins, to prevent intramolecular disulfide bonds from forming cysteine residues of proteins.
- **0.1M EGTA:** chelating agent and inhibitor

Useful Links

What is a micropipette and when we use it:

https://www.youtube.com/watch?v=QGX490kuKjg&feature=share&fbclid=IwAR1QIXPBY1N-nha7Rao7hr5s9PW57xTI9Qk5Qv8PT7ltZYmdAgk1zbljMi0

Centrifuge and its purpose:

https://www.youtube.com/watch?v=F3788O7jy2I&feature=share&fbclid=IwAR071i5bGU1BIwHAQ2wCUBDKBJqfdtj52-HvOS5-RbeclLhZEKjww5ewn6E

Vortex mixer that is used for vigorous mixing:

https://www.youtube.com/watch?v=aGbogxCyTq0

Protein purification:

https://www.youtube.com/watch?v=rom85WMAm08

Experiment 6

SDS PAGE

Objectives

• Prepare polyacrylamide gels and assemble the gel chamber.

• Prepare and load protein samples into the sample wells.

• Stain and destain the gels with Coomassie Blue.

• Determine the molecular weight of your protein samples.

Background

Electrophoresis is the movement of charged particles in an electric field. A negatively charged particles will move toward positive pole and vice versa. For most biological molecules, their net charge depends on the medium in which you put them. If the pH of the buffer in the system is changed, the net charges will change on some of the molecules.

Although electrophoresis could be used to separate charged molecules from any class of biomolecule, the two most common are proteins and nucleic acids.

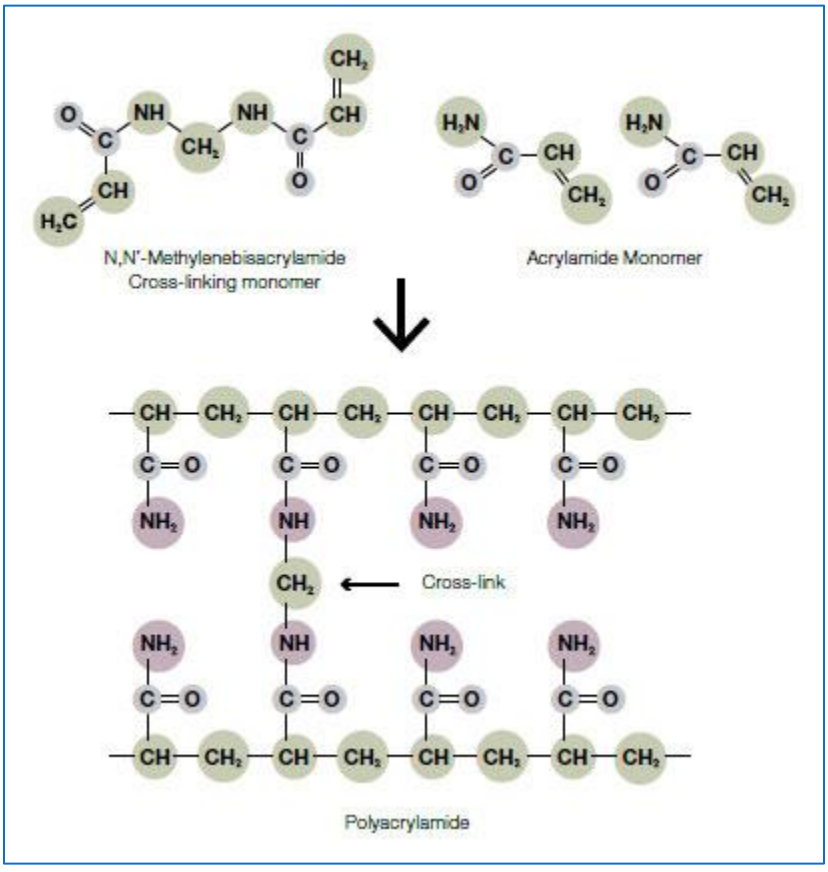
Proteins are different from one another based on their amino acid sequence. The amino acid sequence gives each protein a unique charge character as well as size and

shape. All these factors act together to affect how the proteins migrate with electrophoresis.

Many different media can be used for electrophoresis, such as liquid, paper, or gel. Most electrophoresis done today uses a gel-based medium.

Polyacrylamide Gels

Polyacrylamide gels are long polymers of acrylamide cross-linked with N,N'-methylenebisacrylamide as shown below.



A polyacrylamide (PA) gel has many components, and the nature of the gel is controlled by the amounts chosen.

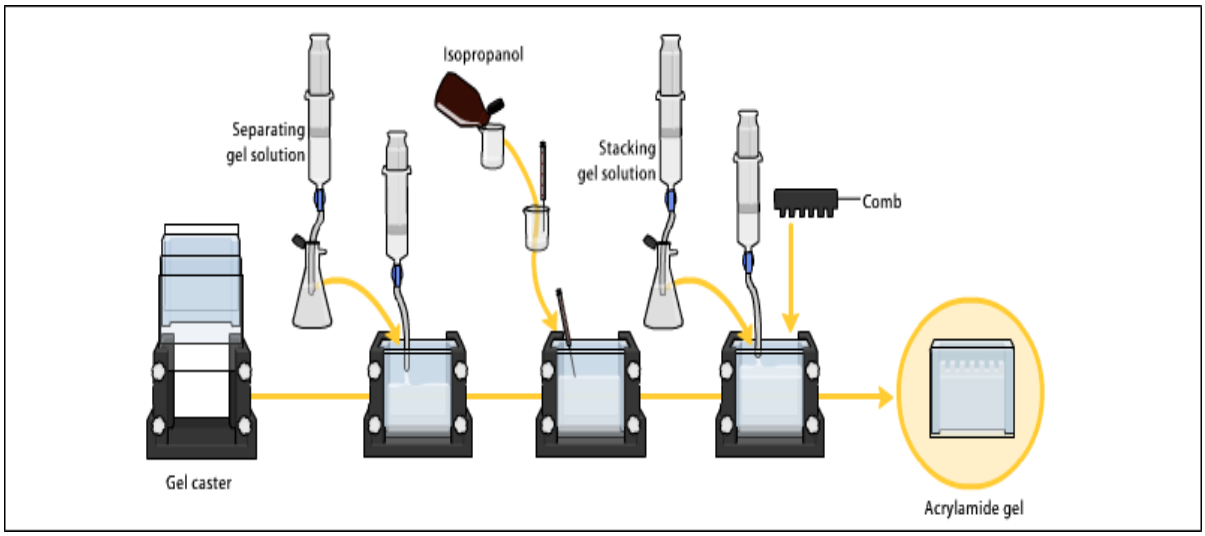
- Acrylamide
- Bisacryalmide crosee-linker, the best gels has 3-5% cross liner of the total.

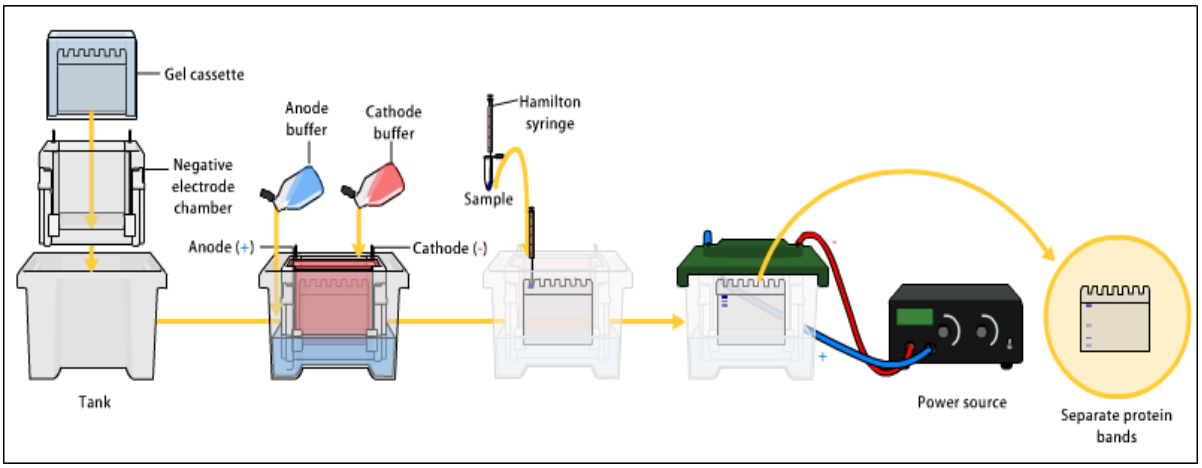
- TEMED: is a catalyst that stimulates the formation of free radicals during the reaction that links acrylamide molecules. The amount of TEMED controls the speed at which the gel will harden.
- Ammonium persulfate: is the initiator of the reaction. It creates free radicals and begins a chain reaction that links all the acrylamide molecules together. Like TEMED, the amount of ammonium persulfate controls the speed. Small volumes of ammonium of highly concentrated ammonium persulfate are usually used. Weigh and pipet it carefully. A small error can be the difference between the gel not solidifying during this geological epoch and it solidifying in the flask before you can pour it between the plates.

Many times a discontinuous gel is made where the bulk of the gel is high percentage at pH 8.5, but a couple of centimeters of gel on top is low percentage (3%) at pH 6.5. This upper gel is the **stacking gel** because it tends to compress all of the proteins into a thin band. The lower gel is called the **running, resolving, separating gel** because the proteins in it separate from each other with the small proteins running fast. The stacking gel works in two ways: First, because it is a low-concentration gel, protein move quickly in it. When the proteins encounter the higher-density separating gel, they slow down. Therefore, proteins that enter the separating gel first are going slower than the ones still in the stacking gel. This causes the protein sample to become much thinner as enters the separating gel. Second, the pH difference plays upon the charge nature of the compounds being used. Glycine, which is in the buffer, has only a partial negative charge at pH 6.5. this causes zones to be set up in the lane where proteins are sandwiched between chloride ions and lots of negative charge and glycine ions with less charge. This causes voltage differences between the zones that tend to push all proteins together. Once proteins and glycine enter the separating gel, the pH increases to 8.6 puts more negative charge on glycine, relieving this effect. Proteins will then move at different rates based on their size.

** Glycine has been used as the source of trailing ion or slow ion because its pK_a is 9.69 and mobility of glycinate are such that the effective mobility can be set at a value below that of the slowest known proteins of net negative charge in the pH range. The minimum pH of this range is approximately 8.0.

Gels are polymerized in a gel caster. First the separating gel is poured and allowed to polymerize. Next a thin layer of isopropanol is added. Next the loading gel is poured, and a comb is placed to create the wells. After the loading gel is polymerized the comb can be removed and the gel is ready for electrophoresis.





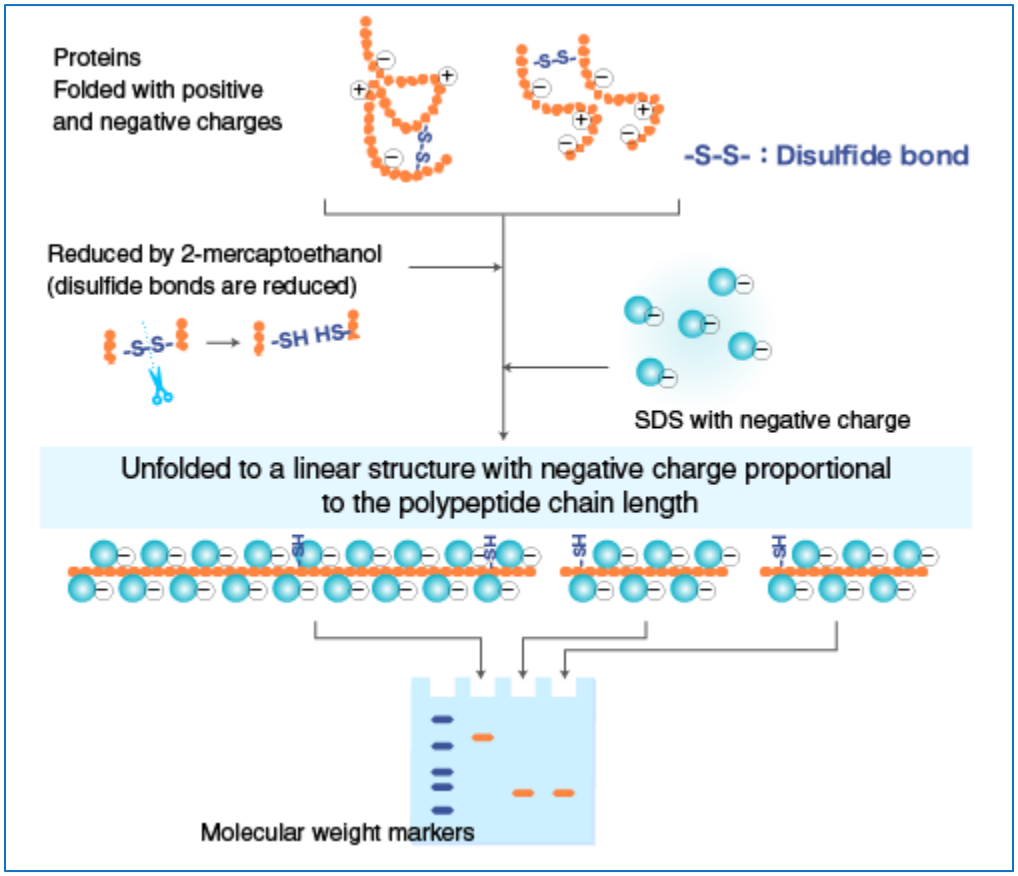
SDS-PAGE

When using electrophoresis to determine the molecular weight of a protein, electrophoresis is usually done in the presence of the detergent sodium dodecyl sulfate (**SDS**), which has the structure:

$$CH_3\text{-}(CH_2)_{11}\text{-}SO_4^-$$

SDS binds to proteins in a constant ration of 1.4 g of SDS /gram of protein and covers the protein with negative charge. SDS and some **β-mercaptoethanol**

included in the sample buffer also denature the proteins and break up any polymers into their subunits. The β-mercaptoethanol reduces any disulfide bridges present. The effect is that all proteins attain the same shape (random coil) and have the same charge-to-mass ratio. The only variable left is mass. Proteins therefore separate on the gels solely based on their molecular weight.



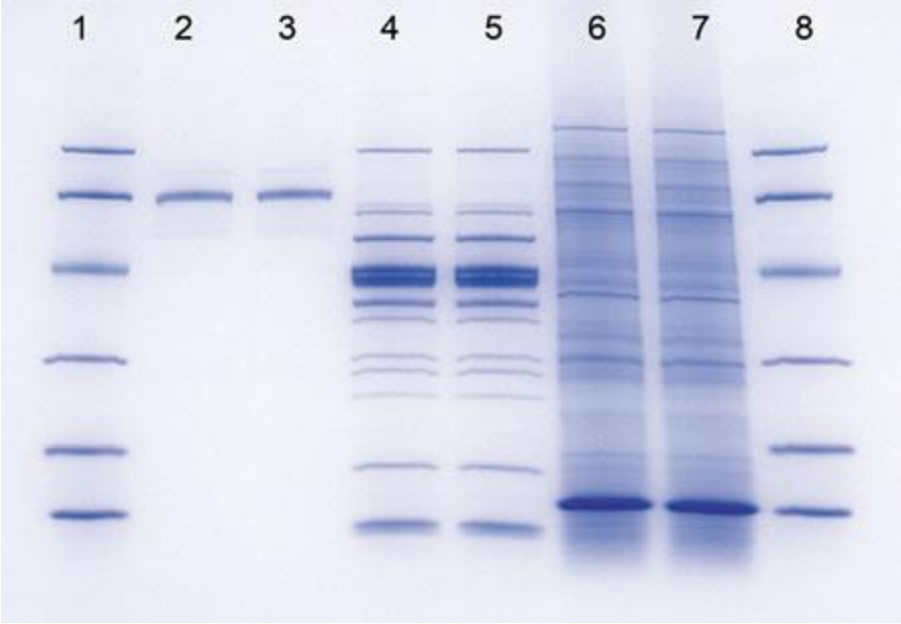
One of the main uses of SDS-PAGE (Polyacrylamide gel electrophoresis) is the determination of molecular weight. When gels are stained and destained, blue bands show up at the locations on the gel based on molecular weight.

Staining Gels

Many different dyes are used during electrophoresis, and it is important to keep their uses straight. The first type is **bromophenol blue**, which is included in the sample buffer (also called **tracking dye** or **loading dye**). This acts as a marker so that you can see how the separation is proceeding. During the run, it is the only band that you will see. Bromophenol blue is negatively charged and small, so it migrates more

quickly than the proteins that you are trying to separate. By using bromophenol blue, you can be confident that if the dye has not run off the gel, then neither have your proteins.

The second is **Coomassie Blue**, similar to the dye used in the Bradford protein assay. Used after an electrophoresis is over, it stains all proteins blue and shows where the proteins are on the gel. The following Figure shows a typical result of the Coomassie gel.



Apparatus/Reagents Needed

- 30% acrylamide/bisacrylamide (Teacher's bench)
- 1.5M Tris/HCl buffer, pH 8.8 (50 mL per group)
- 1.0M Tris/HCl buffer, pH 6.8 (50 mL per group)
- Ammonium persulfate (1 g per group)
- TEMED (teacher's bench)
- 10% SDS (20 mL per group)
- Distilled water (500 mL per group)
- 10X Tris Glycine (2 liters per lab):

60.5 g Tris + 288 g Glycine in 2 Liters distilled water. Do not pH this solution!

- 1X running buffer: dilute 200 mL of 10X Tris Glycine to 1980 mL and add 20 mL 10% SDS.

- 4X Sample Prep Buffer (1 mL per group):
12.5 mL 1 M Tris pH 6.8
5 mL glycerol
2.5 mL 20% SDS
5 mL 2-mercaptoethanol
Tiny bit of bromophenol blue

- Coomassie Blue for staining gels (100 mL per group):
0.125 g Coomassie Blue R-250
50 mL methanol
10 mL acetic acid
H_2O to 100 mL

Gravity filter before use!

- Destaining solution (100 mL per group): 5-10% methanol, 10% acetic acid
- RBC lysis solution (0.84% NH_4Cl): 10 mL
- PBS (10 ml per group)

- Extraction buffer (1.5 mL per group).

Supplies and equipment (per group):
- Vertical electrophoresis set up and power supply
- 10 mL, 25 mL, 50 mL graduated cylinders
- Ice bucket
- 1.5 mL microcentrifuge tubes
- Five 50 mL conical tubes
- Five 15 mL conical tubes
- Racks for tubes
- Yellow and Blue tips
- P100 or P200 micropipette, P1000 micropipette
- 5 of each 5 mL, 10 mL pipettes

- Sterile 1 mL syringe and needles
- Containers for staining and destaining
- 2 Blood tubes with anticoagulant
- Centrifuge
- Microcentrifuge

Procedure

**Tris (tris (hydroxy methyl) aminomethane) Buffer has been used as a buffer because it is an innocuous substance to most proteins. Its pK_a is 8.3 at 20 °C, making it a very satisfactory buffer in the pH range from roughly 7 to 9.

1. We will prepare two 10% SDS-PA gels.

2. Prepare the two gels according to the following recipes:

% resolving gel	**7.5%**	**10%**	**12.5%**	**15%**
30% Acryl:bis (29:1)	2.5 mL	3.3 mL	4.2 mL	5.0 mL
1.5 M Tris (pH=8.8)	2.5 mL	2.5 mL	2.5 mL	2.5 mL
H_2O	4.8 mL	4.0 mL	3.1 mL	2.3 mL
10% SDS	100 µL	100 µL	100 µL	100 µL
10% Ammonium persulfate (APS)	100 µL	100 µL	100 µL	100 µL
TEMED	10 µL	10 µL	10 µL	10 µL
Total volume	10 mL	10 mL	10 mL	10 mL

Stacking gel 4%:

30% Acryl:bis (29:1)	1.33 mL
1.0M Tris (pH=6.8)	1.25 mL
H_2O	7.2 mL
10% SDS	100 µL
10% Ammonium persulfate	100 µL
TEMED	10 µL
Total volume	10 mL

* The ammonium persulfate should be made fresh.

* The 10 mL-gel ingredients should be enough for one gel

3. After pouring the separating gel portion, layer with water saturated with butanol or water.

4. Wait for 20-30 min, and then drain the water.

NOW GO TO ISOLATION OF TOTAL PROTEINS PROCEDURE

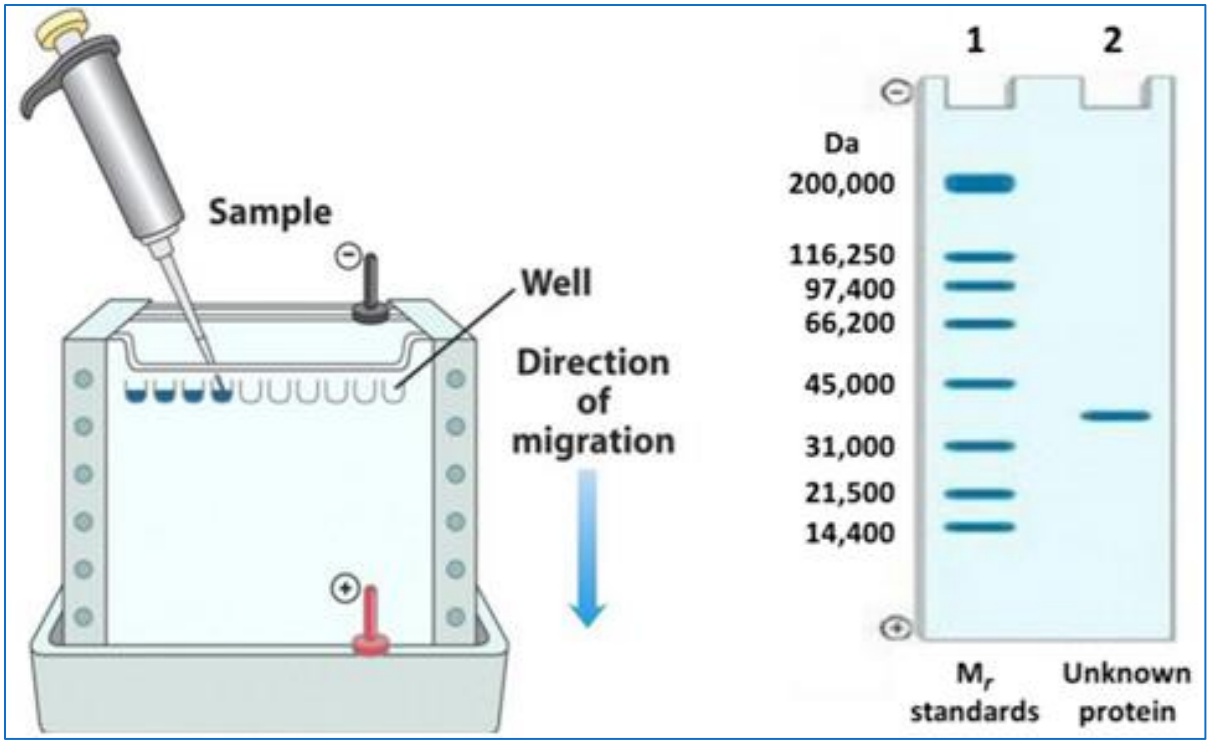
5. Place the comb on top of the resolving gel and then pour the stacking gel and wait 20-30 min.

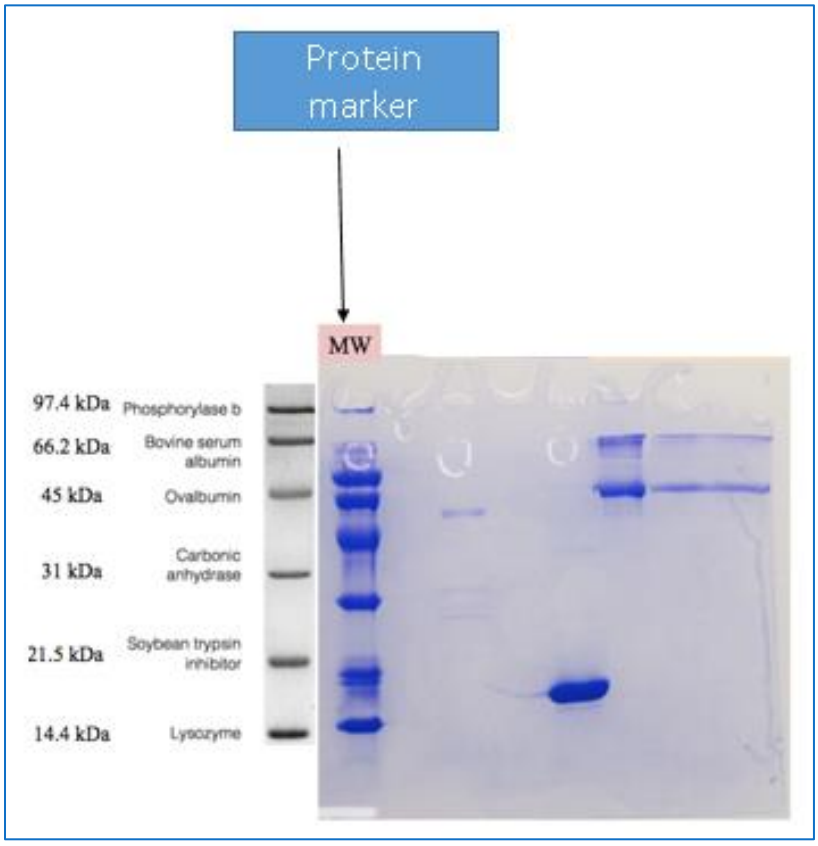
6. Fill the inner chamber with the running buffer and make sure your chamber is not leaky!

7. In the first lane, add 5 µl of the **protein marker**, then in the third and fifth lane, add 15 µg of your protein sample which was mixed previously with 1:1 volume of 4X sample preparation buffer.

Note: keep your protein sample on ice all the time!

8. Fill the outer chambers with the running buffer, and then hook your set up with the power supply and perform SDS-PAGE at 100 mV constant voltage for an hour or till your proteins reach the end of the run.


Sample
Well
Direction of migration
1
2
Da
200,000
116,250
97,400
66,200
45,000
31,000
21,500
14,400
M_r standards
Unknown protein


Protein marker
MW
97.4 kDa
Phosphorylase b
66.2 kDa
Bovine serum albumin
45 kDa
Ovalbumin
31 kDa
Carbonic anhydrase
21.5 kDa
Soybean trypsin inhibitor
14.4 kDa
Lysozyme

Useful Links

The principle of SDS-PAGE

https://www.youtube.com/watch?v=On_ZotdZexI

Separating Proteins using SDS Polyacrylamide Gel Electrophoresis

https://www.youtube.com/watch?v=eaETFKXtNRA&feature=share&fbclid=IwAR1C-PxHFbtC12JgqD1I49LIbIdfjeNe7axpTofJf4IaxJvnAUewQx4JsZw

Student Name:	**Student ID no.:**
Date:	**Section:**
Instructor Name:	**TA name:**

Experiments 5+6

Pre-lab Questions

1- What is meant by:

- Cell lysis

- Protein denaturation

- Primary structure of a protein

2- What is the principle of centrifugation?

Results and Observations

1- put the following steps of SDS-PAGE in the correct order.

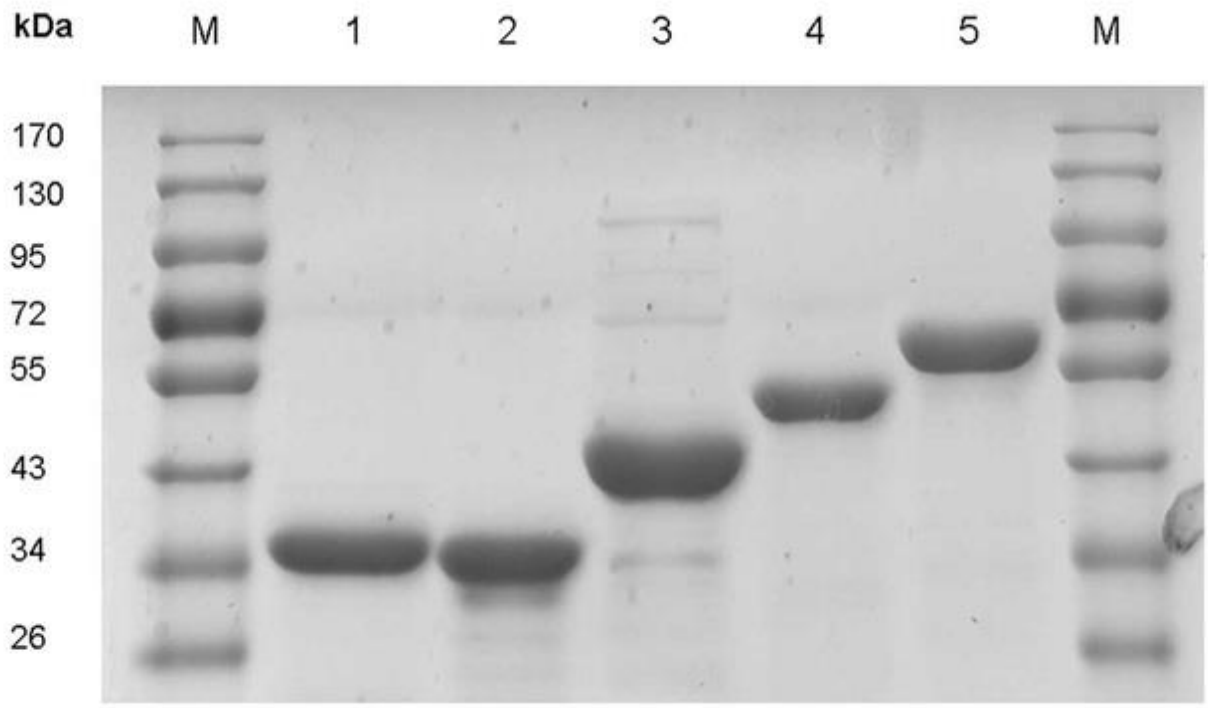
A. Staining

B. Preparing the gels

C. Applying the electric current

D. Loading the samples

2- Suppose you did SDS-PAGE for 5 unknown proteins and got the following results:



- What is the molecular weight of protein in sample 2? Explain.

- Which protein has higher molecular weight, that is sample 5 or sample 3? Explain.

Post-lab Questions

1. You have an enzyme that is composed of three subunits. Two subunits are 28 kD, and the other is 14 kD. How many bands do you see on SDS-PAGE?

2. You forget to include SDS in your samples for SDS-PAGE. What happens when you run the gel?

3. What is the importance of NH_4Cl in the lysis solution (Exp. 6)?

4. What is the purpose of each of the following in SDS-PAGE:

- SDS:

- β-mercaptoethanol:

- TEMED:

- Ammonium persulfate:

- bromophenol blue:

- Coomassie blue:

- protein marker:

Experiment 7

Lipids

Objectives

• Observe the solubility of lipids in polar and nonpolar solvents and to compare saturated and unsaturated fats in their chemical reaction with bromine.

• Determine the %fat in a food by extraction of the fat and weighing it.

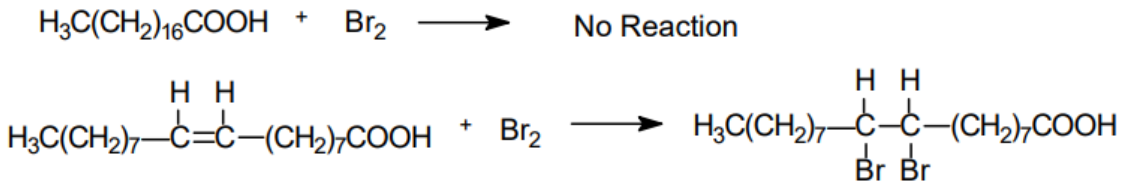
• Know the principle of Soxhlet extraction of fats.

Background

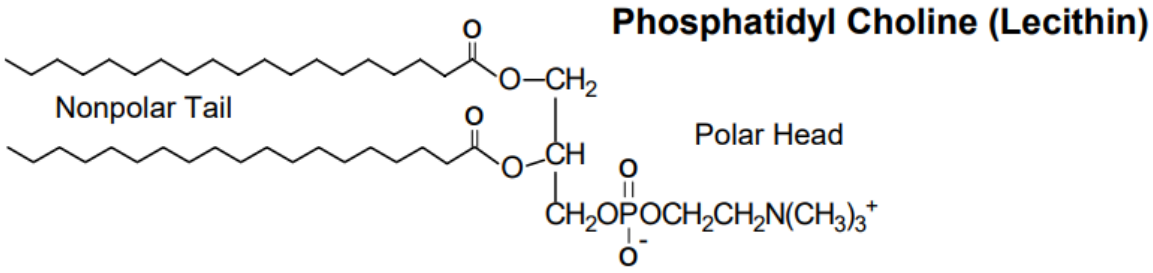
Lipids by definition, are natural substances that do not mix with water but dissolve in organic solvents. There are several classes of lipids, including: fatty acids, waxes, triacylglycerols (fats and oils), phospholipids and steroids. The fatty acids are usually not free in nature, but are components of triacylglycerols, waxes and phospholipids. Animal fats or vegetable oils (especially palm oil) are used to make soap.

Fatty acids are classified as saturated or unsaturated, according to whether they have C=C bonds or not. The C=C bonds are sited where the molecules are not saturated with hydrogen and are susceptible to chemical attack. One substance that readily attacks theses double bonds is the element bromine (Br_2). The product of his reaction is a brominated fatty acid (see the reaction below). One measure of the degree of

unsaturation of fats and oils used by food chemists is known as the iodine number, where iodine is used in place of bromine for this reaction.



Phospholipids, such as lecithin, have a polar or charged portion and a nonpolar portion consisting of long chain fatty acids within the same molecule. Consequently, the polar or charged portion of these molecules will mix with water and the nonpolar portion repels the water but mixes with lipids.



These phospholipids will form small globules (more or less spherical) in water that make the water look opaque or milky. When lipids mix with water, this is known as emulsification and the mixture is known as an emulsion. This is what gives ilk its opaque appearance. This is similar to the process by which cells form membranes, where the phospholipid membrane acts as a barrier between the watery areas inside and outside the cell.

In this lab you will be testing the solubility of some lipids. You will also determine the degree of unsaturation of some lipids by determining how much bromine they will absorb (or react with). You will determine the lipid content of foods by extracting the fats and oils into a nonpolar organic solvent evaporating the solvent and measuring the amount of lipid extracted. At the end you will be introduced to extraction of fats by soxhlet extraction.

Procedure

Part A: Solubility of some lipids

You will need test tubes and different fats and oils. Test the solubility of each fat/oil firstly in water the in dichloromethane. For oils take about 3 drops and for fats take a small amount using a spatula. Try o dissolve them in 1-2 mL of each solvent.

Part B: Measuring Unsaturation of some lipids with bromine/water solution

Take 2 types of oils. Put 5 drops of each oil in a separate test tube then add bromine water to each oil dropwise and count how may drops you needed until the color of bromine persists. If your sample contains no double bonds, it will not react with bromine and the orange color of bromine will not disappear, even if only one drop of bromine is added. If your sample contains double bonds, it will react with the added bromine, and the orange color will disappear. The more double bonds present in the sample, the more bromine will be needed to react with the sample. The orange color of bromine remains in the solution when all of the double bonds have reacted.

** don't pour the resulting mixtures in the sink!

Part C: Saponification of fats and oils

Saponification is the alkaline hydrolysis of lipids

Hydrolysis of fat or oil is carried out either by alkali or acid or even appropriate enzyme as pancreatic lipase.

In alkaline hydrolysis, soap will be formed:

Triglyceride (triacylglycerol) + NaOH → Sodium soap + glycerol

Soaps are cleansing agent because of their emulsification properties.

1. Add 5 mL of 20% alcoholic solution of KOH to 1 mL olive oil in a clean test tube.

2. Mix well by shaking.

3. Heat in a boiling water bath for 5 minutes.

4. While mixing, add 5 mL of water, and heat for additional 5 minutes.

Write your observation and explain it.

Part D: Extraction of lipids from food

1. Accurately weigh a small amount (10 to 20 g) of solid food, such as potato chips, bran muffin, donut or croissant. You should note and record any nutrition facts on the bag or container of food, if it is given.

2. Record the weight of your sample on the Report Sheet.

3. Crush or crumble the food sample into small pieces and place it in a large Erlenmeyer flask. (See diagram on next page).

4. Add 25 mL of petroleum ether to the flask containing your food sample and swirl the flask for several minutes to get the lipids to dissolve in the petroleum ether.

5. While you are waiting for the lipids to dissolve, weigh a clean 100 mL beaker as accurately as possible. Be sure the balance is set at zero before placing the beaker on the pan to weigh it.

6. Record the mass of the beaker on the Report Sheet.

7. Carefully decant the petroleum ether from the flask containing your food sample to the weighed beaker. (Pour only the clear liquid, not the solid portion of the food).

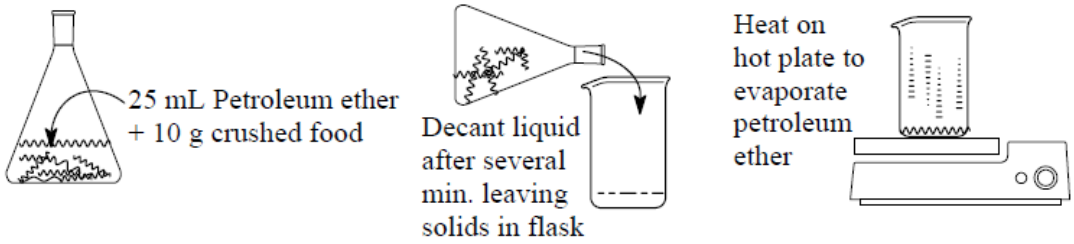
8. Place the beaker on a hot plate or water bath in the hood to evaporate all of the petroleum ether.

9. When you think it has all evaporated, check to see that there is no longer a strong smell DO NOT ATTEMPT TO EVAPORATE THE PETROLEUM ETHER WITH A FLAME!!!

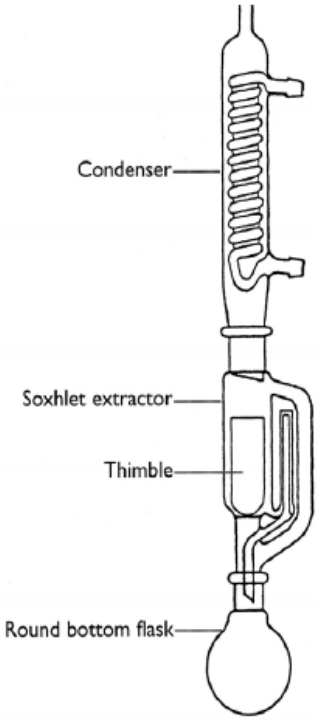
10. After the beaker has cooled to room temperature, make sure it is dry on the outside and weigh it again.

11. Record the weight on the Report Sheet.

12. Calculate the mass of lipid extracted from the food sample and determine the weight percent of fat in that food.



Part D: Soxhlet Extraction of lipids



The "Soxhlet" method described here is recognized by the Association of Official Analytical Chemists (AOAC) as the standard method for crude fat analysis.

Crude fat content is determined by extracting the fat from the sample using a solvent, then determining the weight of the fat recovered. The sample is contained in a porous thimble that allows the solvent to completely cover the sample. The thimble is contained in an extraction apparatus that enables the solvent to be recycled over and over again. This extends the contact time between the solvent and the sample and allows it time to dissolve all of the fat contained in the sample. In order for the solvent to thoroughly penetrate the sample it is necessary for the sample to be as finely comminuted as possible.

The porous thimble loaded with a solid sample is placed inside the main chamber of the Soxhlet extractor. By refluxing the solvent through the thimble using a condenser and a siphon side arm, the extraction cycle is typically repeated many times. Soxhlet extraction is a rugged, well-established technique and permits unattended extraction. However, it requires a long extraction time and the consumption of a large amount of solvent.

Student Name:	**Student ID no.:**
Date:	**Section:**
Instructor Name:	**TA name:**

Experiment 7: Lipids

Pre-lab Questions

1- What functional group is present in a triglyceride?

2. What functional group is present in a fatty acid?

3. Draw the structure of oleic acid.

5. What do lipids have in common?

6. What type of solvent would be needed to remove an oil spot? Why?

7. The melting point of stearic acid is 70°C, and the melting point of oleic acid is 4°C. Explain in detail why their melting points are so different.

8. Describe the differences between saturated fatty acids and unsaturated fatty acids in terms of their chemical structures.

9. What are trans-fatty acids? In what kinds of foods are these trans-fatty acids found? Are trans- fatty acids saturated or unsaturated fatty acids?

Results and Discussion

Part A: Solubility of some lipids

Fat/ oil	Solubility in water	Solubility in DCM

Part B: Measuring Unsaturation of some lipids with bromine/water solution

Oil name	No. of Bromine/water drops till color persists

Part C: Saponification of fats and oils

Write your observation and explain it.

Part D: Extraction of lipids from food

Mass of food sample	
Mass of empty dry beaker	
Mass of (beaker + extracted lipids)	
Mass% of lipids in the food	

How does your value compare with the value on the food wrapper (if it was available)?

Part E: Soxhlet Extraction of lipids

Have a look on the animated figure in this link:
https://en.wikipedia.org/wiki/Soxhlet_extractor#/media/File:Soxhlet_mechanism.gif

what are the advantages of this method?

Experiment 9

DNA Extraction from Whole Blood

Objectives

• Isolate the DNA from blood using a kit (see below). The DNA that you will isolate from this technique will be used in PCR experiment afterwards.

Background

Deoxyribonucleic acid (DNA) extraction has considerably evolved since it was initially performed back in 1869. It is the first step required for many of the available downstream applications used in the field of molecular biology. Whole blood samples are one of the main sources used to obtain DNA, and there are many different protocols available to perform nucleic acid extraction on such samples. These methods vary from very basic manual protocols to more sophisticated methods included in automated DNA extraction protocols.

Human blood like other mammals; has nonnucleated erythrocytes (RBCs), so when extracting DNA from blood we extract it from leukocytes (WBCs). In order to extract DNA from these cells we first break open (lysis) the cells while protecting our DA from the nucleic acid degrading action of nucleases by inhibition of such nucleases (in our kit we are destroying them) at this stage a homogenous cell lysate composed mainly of degraded proteins, phospholipids ad intact nucleic acid (DNA). This lysate is the passed through silica filter o which the nucleic acid (DNA) ind (adsorption) specifically and tightly; which enables effective washing of impurities out of the filter. Then the pure DNA is eluted sing elution buffers or simply distilled water which interrupt the adsorption in presence of centrifugation force which aid and accelerate the whole process of elution.

A brief and simplifies explanation of the kit

In this experiment you will use QIAamp DNA Blood Mini Kit depending on spin column-based DNA purification technique.

Cell lysis buffer (Buffers ATL and AL in our kit)

Lysis solutions that break open tissue, cell, and nuclear membranes. Generally, the aim of any lysis buffer is to mimic the internal conditions of the cell (or cell compartment) in order to preserve the structure and/or function of the contents of the lysate. Most lysis buffers contain salts (e.g. Tris-HCl or EDTA) to regulate the acidity and osmolarity of the lysate, while detergents (such as Triton X-100 or SDS) are added to break up membrane structures.

Proteases (Proteinase K in our Kit)

Proteinase K (endopeptidase K): Proteinase K is used for the destruction of proteins in cell lysates and for the release of nucleic acids, since it very effectively inactivates DNases and RNases. It is commonly used for its broad specificity and stability over a wide pH range (4-12). It is proteolytic residual activity is calcium independent (i.e. not inactivated by EDTA).

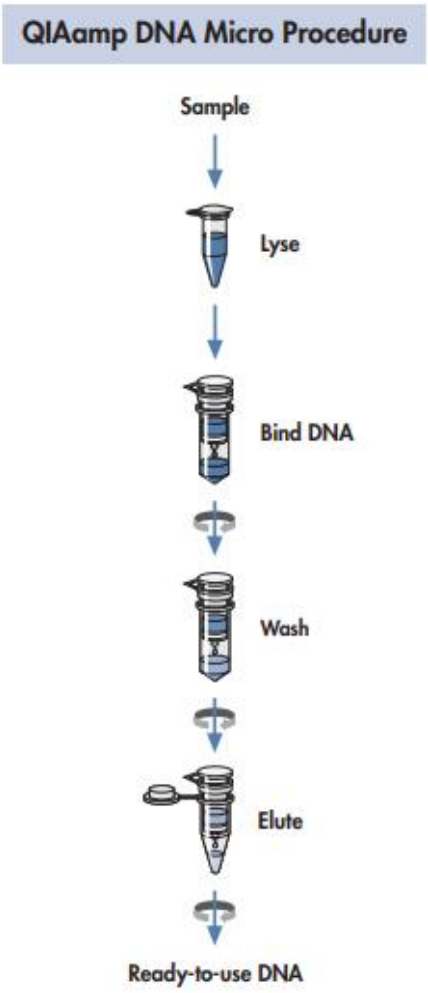
DNA separation by silica adsorption

A proposed explanation of how DNA binds to silica is that the positively charged ions form a salt bridge between the negatively charged silica and the negatively charged DNA backbone in high salt concentrations. The DNA can then be washed with high salt and ethanol, and ultimately eluted with low salt strength solution or simply water which would disrupt the salt bridges and ends in DNA elution out of the column.

Caution: When handling blood samples, follow recommended procedures for biohazardous material.

Procedure

**Note: always refer to instructions given with the kit being used.
* All mixing, vortexing or centrifugation are done with closed tubes lids.
* Avoid both touching silica filters and wetting tube's rims while adding or transferring any reagent.



Step 1: Cell lysis and protein degradation

1. Pipet 1-100 µL (EDTA anticoagulated blood) into a 1.5 mL microcentrifuge tube.
2. Add buffer **ATL** to a final volume of 100 µL.
3. Add 10 µL **proteinase K** to degrade nucleases and other proteins.
4. Add 100 µL buffer **AL**; then mix by pulse-vortexing for 15 s.
5. Incubate at 56°C for 10 min while shaking every 2 minutes.
   This incubation period provides sufficient time for the reactions.
6. Briefly centrifuge the 1.5 mL tube to remove drops from inside the lid.
7. Add 50 µL ethanol (96-100%), close the lid and mix thoroughly by pulse-vortexing for 15 s. Incubate for 3 min at room temperature.
8. Briefly centrifuge the 1.5 mL tube to remove drops from inside the lid.

Step 2: DNA binding to the silica membrane

9. Carefully transfer the entire lysate from step 8 to the MinElute column (in a 2 mL collection tube) then centrifuge at 8000 rpm for 1 min. Place the QIAamp MinElute column in a clean 2 mL collection tube and discard the collection tube containing the flow-through.

This step (centrifugation) ensures that most DNA have passed into (i.e. bound to the column) while the lysate has been eluted.

Step 3: Bound DNA wash

10. Add 500 L buffer **AW1** (washing buffer) then centrifuge at 8000 rpm for 1 min. Place the MiElute column in a clean 2 mL collection tube, and discard the collection tube containing the flow-through.
11. Add 500 L buffer **AW2** (washing buffer) then centrifuge at 8000 rpm for 1 min. Place the MiElute column in a clean 2 mL collection tube, and discard the collection tube containing the flow-through.
12. Centrifuge at full speed (14,000 rpm) for 3 min to dry the membrane completely.

This step is necessary, since ethanol carryover into the eluate may interfere with some downstream applications.

Step 4: DNA elution and recovery

13. Place the MinElute column in a clean 1.5 mL microcentrifuge tube and discard the collection tube containing the flow-through. Add about 40 µL buffer **AE** or distilled water to the center of the membrane of the column.
14. Close the lid and incubate at room temperature (15-25 ℃) for 1.5 min. Centrifuge at full speed (14,000 rpm) for 1 min. (add another 30 µL buffer AE then incubate and centrifuge again for more DNA elution in the same microcentrifuge tube).
15. Store your labeled sample in the refrigerator for the next lab session.

Useful links

DNA extraction from blood
https://www.youtube.com/watch?v=gmNw6CWtN5k&t=545s&fbclid=IwAR0UOvfTMIH1uAw7GbnGXtiowV5Qq6PogjQDdwQLwylkI-O8gWmPc2AOnow\

Student Name:	Student ID no.:
Date:	Section:
Instructor Name:	TA name:

Experiment 9: DNA Extraction

Questions

1- Answer the following with True /False:

( ) a- Any tissue that has cells with nuclei can be used to extract DNA.
( ) b- DNA is soluble in ethanol.
( ) c- Cell membrane can be broken down by salt.
( ) d- AW1, AW2. AE Buffers came with known composition.

2- What was the purpose of changing the tips when sampling?

3- In the experiment we worked in a sterile environment, mention two of the precautions taken to prevent contamination.

4- Mention the function of each of the following:

A- Elution buffer:

B- Proteinase K:

C- AL buffer:

D- Silica membrane in the filtering tube:

Experiment 10

Polymerase Chain Reaction (PCR)

Objectives

• Amplify the DNA you isolated from blood last experiment by PCR technique.

Background

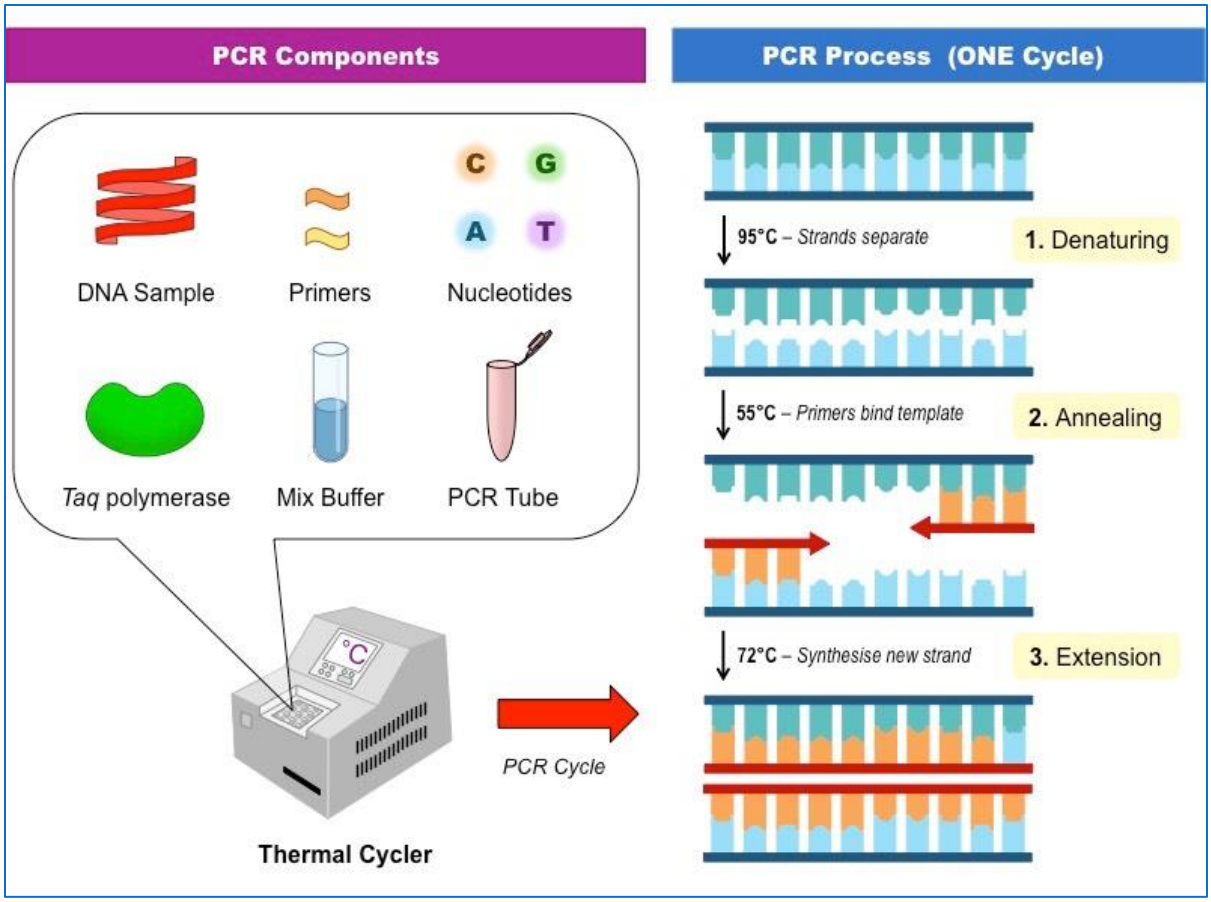
Polymerase chain reaction (PCR) technique is a method used to amplify DNA. It is so sensitive that it can produce over 1 µg of specific target DNA from les than 50 ng in a few hours.

PCR is an automated procedure carried out in a machine called a **thermocycler** that controls the time and temperature of the amplification reactions. The key o the process was the discovery f a heat-stabile DNA polymerase (Taq) from the bacterium *Thermus aquaticus*. This enzyme allows the reactions to proceed at high temperatures without denaturating the key enzyme the replicates the DNA. Other components of the reaction include the target DNA that you want to amplify, the full set of deoxynucleoside triphosphates (dNTPs), and specific primers that are complementary to the DNA in or near the target DNA.

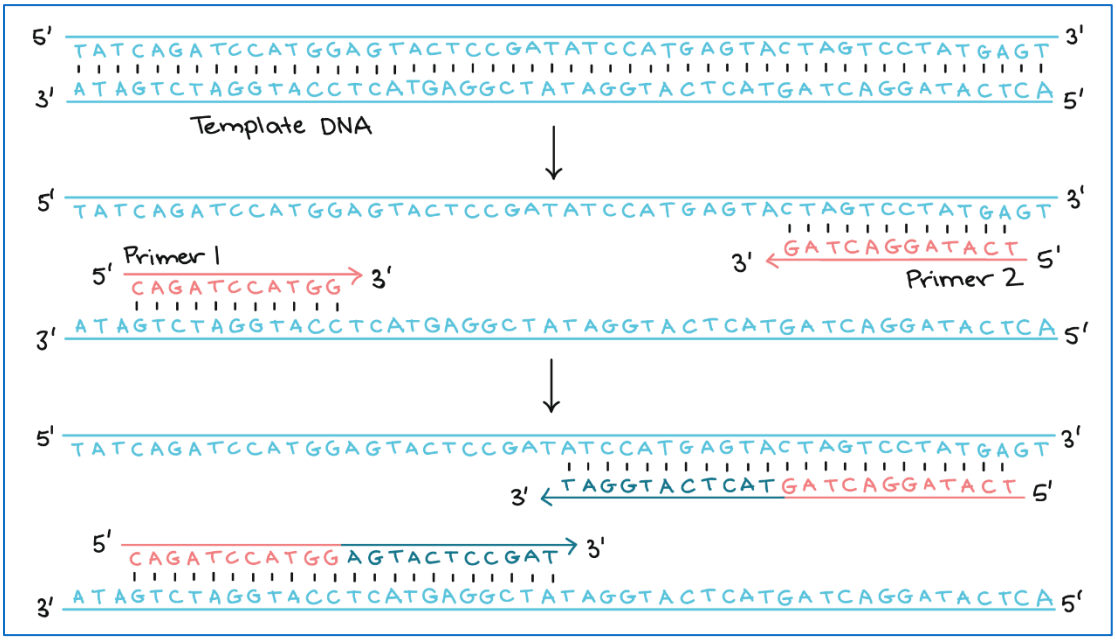
Many enzymes are involved when an organism replicates its DNA, and the reaction occurs at biological temperatures (20-40°C). Some enzymes are involved in the synthesis and subsequent proofreading and repair of the new DNA. Other enzymes are involved in the unwinding of the DNA double helix so that the replication can proceed. The complexity of this process, and the requirements for ambient

temperatures so that enzymes can work, make it impossible to automate a DNA replication outside the organism. If we want to produce DNA chemically, we have to find a way to separate DNA strands first. This requires a temperature that inactivates the average DNA polymerase used to replicate the single strand.

In 1993, Dr. Kary Mullis won the Nobel Prize in science for his PCR procedure. He used the DNA polymerase from *T. aquaticus*, known now as Taq polymerase. The bacteria were found living in undersea vents at temperatures near 100℃. Any enzymes from these bacteria have to work at high temperatures. Using Taq polymerase allowed the automation of PCR. A thermocycler can raise the temperature high enough to separate strands of DNA without killing the enzyme. Next the thermocycler reduces the temperature, allowing primers to anneal. The temperature is then raised again to the one optimal for the polymerase to work. The polymerase copies the single strands, starting at the primer and going from 5' to 3' as all DNA synthesis does. Once enough time has elapsed to replicate the required DNA sequence, the thermocycler raises the temperature again to separate DNA strands. Each round of replication doubles the amount of DNA. It takes 1 min. to replicate 1 kilobase (kb) of DNA. So, with this process, if the target DNA is 1 kb, the sequence can be doubled every couple of minutes. At that rate, a huge amplification occurs in a few hours. The following figure demonstrates the PCR components and PCR process (one cycle). Noten that the amount of both primer and dNTPs actually controls the maximum possible PCR product. Once either runs out, the reaction stops.



The next figure illustrates the template DNA and the three major steps of PCR which are: DNA strands separation, annealing then extension.



PCR principles and procedure

PCR is used to amplify specific regions of a DNA strand (the DNA target). This can be a single gene, a part of a gene, or a non-coding sequence. Most PCR methods typically amplify DNA fragments of up to 10 kilobase pairs (kb), although some techniques allow for amplification of fragments up to 40 kb in size.

A basic PCR set up requires several components and reagents. These components include:

1. DNA template that contains the DNA region (target) to be amplified.

2. Two primers (Forward and Reverse), which are complementary to the DNA regions at the 5' or 3' ends of the DNA region.

3. Taq polymerase or another DNA polymerase with a temperature optimum at around 70°C.

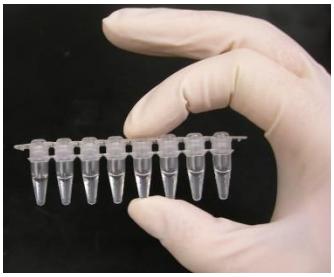
4. Deoxynucleoside triphosphates (dNTPs; also very commonly and erroneously called deoxynucleotide triphosphates), the building blocks from which the DNA polymerases synthesizes a new DNA strand.

5. Buffer solution, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase.

6. Divalent cations, magnesium or manganese ions; generally, Mg^{2+} is used, but Mn^{2+} can be utilized for PCR-mediated DNA mutagenesis, as higher Mn^{2+} concentration increases the error rate during DNA synthesis

7. Monovalent cation potassium ions.

The components from 3-7 are all premixed in one single concentrated solution optimized for PCR, named as **Master Mix**.



The PCR is commonly carried out in a reaction volume of 10-200 µL in small reaction tubes (0.2-0.5 mL volumes) in a thermocycler. The thermocycler heats and cools the reaction tubes to achieve the temperatures required at each step of the

reaction. Thin-walled reaction tubes permit favorable thermal conductivity to allow for rapid thermal equilibration. Most thermal cyclers have heated lids to prevent condensation at the top of the reaction tube.

PCR optimization

In practice, PCR can fail for various reasons, in part due to its sensitivity to contamination causing amplification of spurious DNA products. Because of this, a number of techniques and procedures have been developed for optimizing PCR conditions. Contamination with extraneous DNA is addressed with lab protocols and procedures that separate pre-PCR mixtures from potential DNA contaminants.

Before running the PRC, we will add 2 controls to the reaction tubes (in addition to our samples) to ensure the success of the PCR process:

1- **Negative control**: it is the one which should not give you amplicons, typically the negative control will contain NO template but the rest of the mixture is present. Setting up negative controls, each containing only the forward or reverse primer, should not provide visible amplicons. Therefore, any visible bands might be a result of contamination.
2- **Positive control**: it is the one that you expect to work under the conditions given. It contains the template gene you are testing besides other PCR components. The positive control will test your master mix, $MgCl_2$ amounts, primer annealing temperature, and extension times. If your positive control does not work, those results indicate that something is wrong with your annealing or extension times or temperatures, or something is wrong with your $MgCl_2$ or master mix set up.

General Procedure

The PCR usually consists of a series of 20 to 40 repeated temperature changes called cycles; each cycle typically consists of 2-3 discrete temperature steps. Most commonly PCR is carried out with cycles that have three temperature steps. The cycling is often preceded by a single temperature step (called hold) at a high temperature (>90°C) and followed by one hold at the end for final product extension or brief storage. The temperatures used and the length of time they are applied in each cycle depend on a variety of parameters. These include the enzyme used for

DNA synthesis, the concentration of divalent ions and dNTPs in the reaction, and the melting temperature (Tm) of the primers.

*** Initialization step:** This step consists of heating the reaction to a temperature of 94-96°C (or 98°C if extremely thermostable polymerases are used), which is held for 1-9 minutes. It is only required for DNA polymerases that require heat activation by hot-start PCR.

*** Denaturation step:** This step is the first regular cycling event and consists of heating the reaction to 94-98°C for 20-30 seconds. It causes melting of DNA template and primers by disrupting the hydrogen bonds between complementary bases of the DNA strands, yielding single strands of DNA.

* **Annealing step:** The reaction temperature is lowered to 50-65°C for 20-40 seconds allowing annealing of the primers to the single-stranded DNA template. Typically, the annealing temperature is about 3-5 degrees Celsius below the Tm of the primers used. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA synthesis.

*** Extension/elongation step:** The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75-80°C, and commonly a temperature of 72°C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. As a rule-of-thumb, at its optimum temperature, the DNA polymerase will polymerize a thousand bases per minute. Under optimum conditions, i.e., if there are no limitations due to limiting substrates or reagents, at each extension step, the amount of DNA target is doubled, leading to exponential (geometric) amplification of the specific DNA fragment.

*** Final elongation:** This single step is occasionally performed at a temperature of 70-74°C for 5-15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

*** Final hold:** This step at 4-15°C for an indefinite time may be employed for short-term storage of the reaction.

Procedure

There are many PCR techniques. Today we will follow the PCR technique called **Allele-specific PCR technique**. This diagnostic or cloning technique is used to identify or utilize single-nucleotide polymorphisms (SNPs) (single base differences in DNA). It requires prior knowledge of a DNA sequence and uses primers whose 3' ends encompass the SNP. PCR amplification under stringent conditions is much less efficient in the presence of a mismatch between template and primer, so successful amplification with an SNP-specific primer signals presence of the specific SNP in a sequence.

You will investigate one of the most common allelic variants (CYP2C19*3) of CYP2C19 gene using PCR (Tammingaet al., 2001). For CYP2C19*3 detection, a segment of exon 4 which contains the base pair substitution 636G>A will be amplified using the primer set presented in (CYP2C19*3 (F) AAATTGTTTCCAATCATTTAGCT/ CYP2C19*3 (R) ACTTCAGGGCTTGGTCAATA). The PCR product should yield one band (271 bp). The PCR reaction of CYP2C19*3 will be prepared using the PCR mixture given in the following Table. A negative control (does not contain a DNA template) will be included with the PCR run.

****Note**: always refer to instructions given with the master mix being used.

Table: Concentrations and volumes of the PCR mixture for 50 µL reaction volume

PCR Mixture	**Control Volume (µL)/ Final concentration**	**Experimental Volume (µL)/ Final concentration**
Master mix (2x)	25 µL/1x	25 µL/1x
Forward primer	2 µL/0.4 µM	2 µL/0.4 µM
Reverse primer	2 µL/0.4 µM	2 µL/0.4 µM
DNA template	-	-
Nuclease free water	?	?
Total	**50 µL**	**50 µL**

Reaction setup:

1. Thaw the master mix. Vortex for 5-10 seconds, and store on ice. Thaw the nuclease-free water.
2. Label the required number of reaction tubes (Control, Experimental). Place the reaction tubes on ice.
3. Vortex the Multiplex Master Mixes for 5-10 seconds. Place 20 µL of each Multiplex Master Mix in the appropriately labeled reaction tubes on ice.
4. For each DNA sample, prepare a genomic DNA mixture on ice following the table given above.
5. Centrifuge the tubes briefly to bring the contents to the bottom of the tubes. Place reaction tubes on ice until ready for thermal cycling.

Thermal Cycling:

Place the tubes in the thermocycler and run the recommended program.

Cycling Profile:

Preheating at 40 ℃ for 2 min.

Initial denaturation at 94°C for 5 minutes,

then:

94° for 1 minute

54°C for 1 minute

72°C for 1 minute

For **35 cycles**,

then:

72°C for 5 minutes

4°C overnight

Useful links

PCR

https://www.youtube.com/watch?v=iQsu3Kz9NYo

PCR

https://www.youtube.com/watch?v=matsiHSuoOw&t=137s&fbclid=IwAR1UK_S-Zi-2-I_lOCMaFPGt7jVsnwDTCnRn1kDwmoTqEhahW5Eb2Apf5dk

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Date:	Section:
Instructor Name:	TA name:

Experiment 10: PCR

Questions

1. What is the main principle of PCR? Explain.

2. What are the component of the Master Mix in PCR reaction?

3. You want to use PCR to amplify **X gene** from a cDNA library. Using the provided cDNA sequence, choose the best pair of primers from the following:

5' ..atgtggccagtggccaccatccttaacatgcat…aacctagctcctaaccgctacccaccttga..3'

a) 5'-atggtggccacatggccacat-3'

b) 5'-taccaccggtgtaccggtgta-3'

c) 5'-tcaaggtgggtagcggttagg-3'

d) 5'-agttccacccatcgccaatcc-3'

e) 5'-ggattggcgatgggtggaact-3'

4. What is the name of the enzyme used for PCR?

5. Did you run a negative or a positive control in your PCR? Why do you need to include a positive and negative control? Explain the components of each of them?

6. Name the 3 steps in each PCR cycle?

7. What is the major factor that defines the selectivity of the PCR results? Explain.

Experiment 11

Analysis of DNA

Objectives

• Do gel electrophoresis for the DNA you produced from PCR experiment.

Background

After amplifying the DNA sample in PCR, you can use it now to detect the presence or absence of specific gene(s) using **Mini gel Electrophoresis Method**.

Agarose gel electrophoresis is the easiest and most common way of separating and analyzing DNA. This technique has lots of applications in forensics, molecular biology, genetics, microbiology and biochemistry.

Electrophoresis is the movement of charged particles in an electric field. A negatively charged particles will move toward positive pole and vice versa.

Agarose is the matrix of choice for separation of DNA fragments larger than 1000 base pairs. All DNA is of uniform shape and charge-mass ratio, so the fragments separate by molecular weight or size which are measured as base pairs or kilobase pairs.

Agarose is a natural polysaccharide of galactose and 3,6-anhydrogalactose derived from agar, which is itself obtained from certain red algae. Agarose chains tend to make left-handed helices that intertwine with each other. This rise to a gel that is quite dense for its concentration. A solution of only 1.0% w/v agarose will solidify into a fairly dense gel than can be used to separate proteins or nucleic acids. Agarose gels are prepared by boiling a defined quantity of the dry polymer in buffer until it

melts. The melted agarose suspension is then poured into a casting tray with a well-formed comb and allowed to cool and solidify.

It is important to remember that the ions in the buffer carry most of the current during electrophoresis. Because this buffer is also in the gel, some current goes through the gel so that the samples can move. If you accidently make up your gel in water, no current will flow through the gel and your sample will not move.

Agarose gels are usually run horizontally where the gel is completely immersed in buffer. This aids in heat reduction. It's very fragile, that's why we use it horizontally not vertically as in SDS-PAGE.

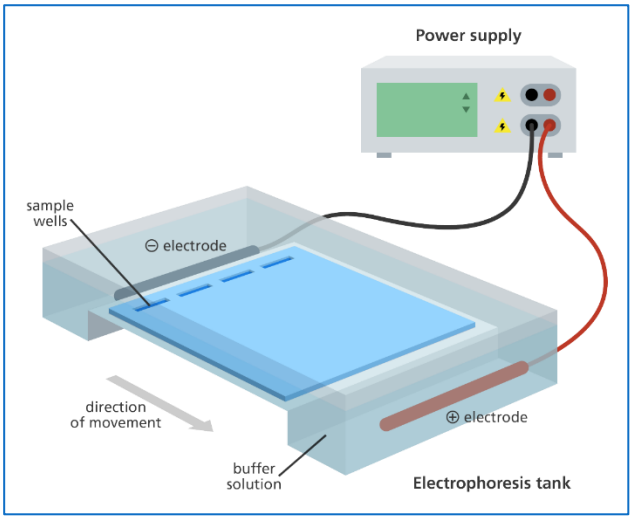
Equipment:

1) Power pack: supplies direct current between the electrodes in electrophoresis unit.

2) Electrophoresis unit: which are available for running either vertical-usually for separating proteins in acrylamide gel -or horizontal units.

Horizontal unit

In its simplest form, a horizontal gel apparatus consists of a box which is divided into two compartments by a platform in the middle. The gel is placed on this platform, and buffer is added until the gel is fully submerged. Electrodes in each compartment supply the electric field. The resulting current flows through both the gel and the buffer over the gel, so the thickness of these must be controlled for fully reproducible results.



Principle of DNA gel electrophoresis:

Double stranded DNA molecules are negatively charged rods (due to phosphate groups) under an electrical current all DNA molecules move toward the anode through the agarose gel. The agarose gel is a cross-linked matrix that is somewhat like a three-dimensional mesh or screen. The DNA molecules are pulled to the positive end by the current, but they encounter resistance from this agarose mesh. The smaller molecules are able to navigate the mesh faster than the larger one, so they make it further down the gel than the larger molecules. This is how agarose electrophoresis separates different DNA molecules according to their size. Consequently, the mobility of DNA molecules during gel electrophoresis will depend on size (length of the rod).

Single-stranded DNA or RNA tend to fold up into molecules with complex shapes and migrate through the gel in a complicated manner based on their tertiary structure. Therefore, agents that disrupt the hydrogen bonds, such as sodium hydroxide or formamide, are used to denature the nucleic acids and cause them to behave as long rods again.

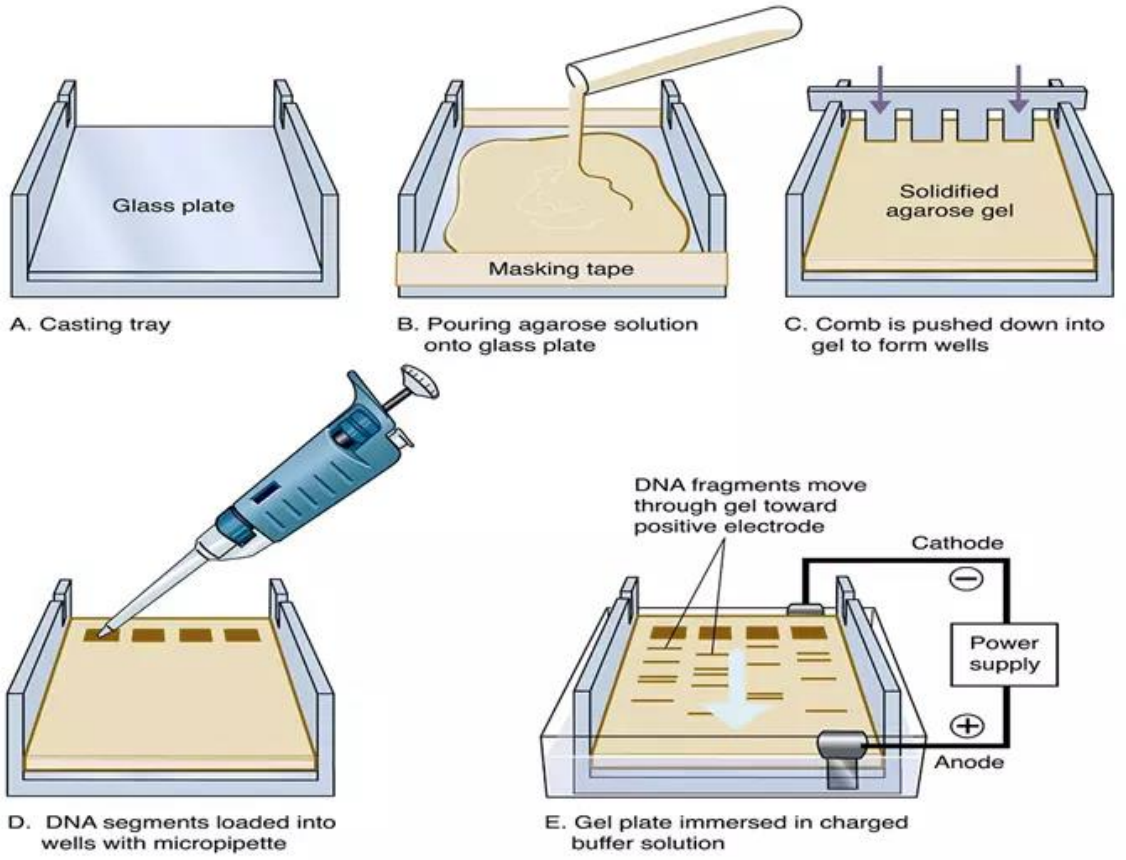
Why is a buffer important?

To maintain constant pH since the electrical current used in electrophoresis can separate water molecules, the liberated hydrogen ions that accumulate near the negative electrode would cause the pH there to be lower, and the hydroxide ions accumulating near the positive electrode would raise the pH. Either of these pH changes could denature the DNA in the sample. The gel buffer prevents these pH changes, thus protecting the sample. Additionally, a buffer is essential to maintain constant state of ionization of the molecules being separated. Any change in pH will alter the charge and hence the rate of migration and separation.

CASTING A GEL

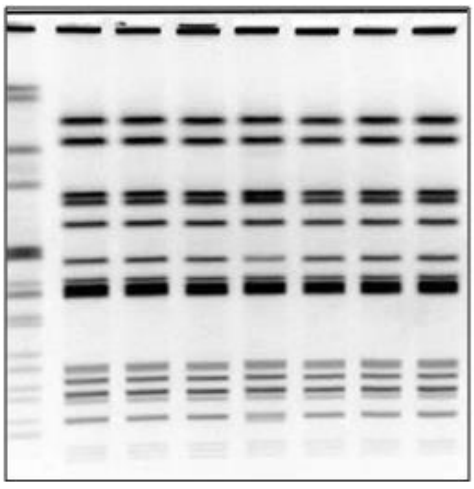
Agarose must be in a semi-solid state to be used in electrophoresis. The hot liquid agarose can be formed into any shape. Since you will be using gel electrophoresis, the agarose must be cast into a flat sheet. This sheet is called a gel, and the gel must be sized appropriately for the electrophoresis apparatus being used. In addition,

special containment areas, called wells, must be formed in the gel to accept the DNA samples.



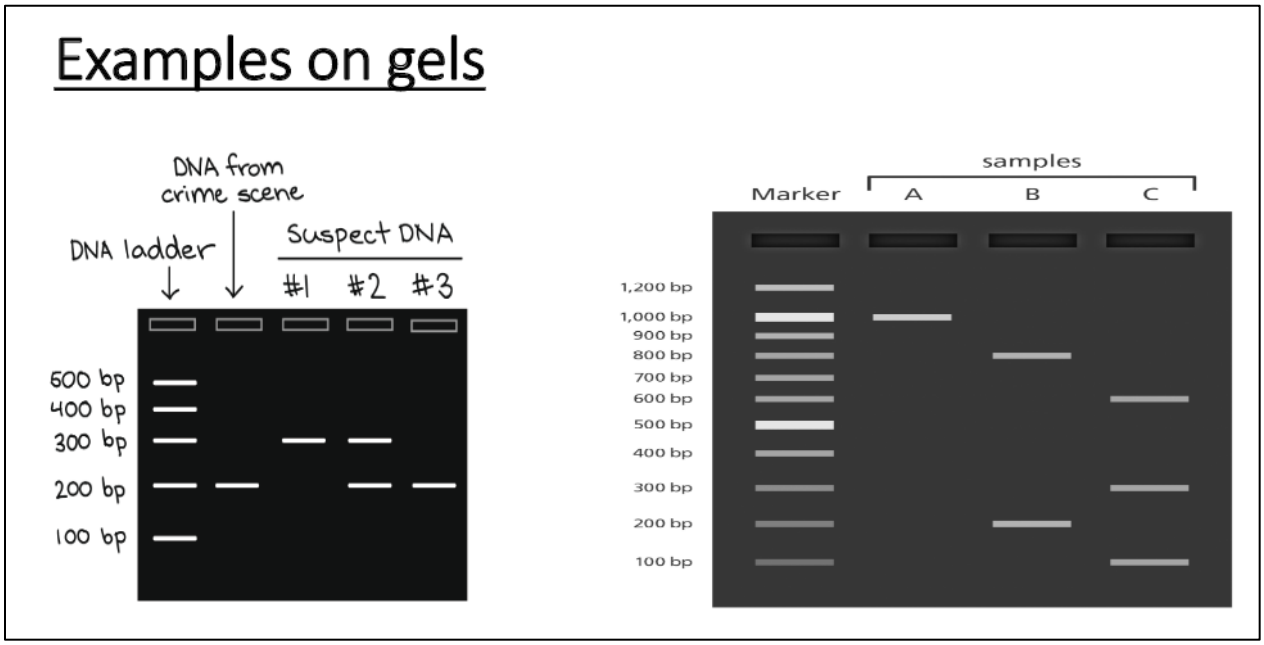
Staining DNA

The most common way to visualize DNA is by putting the chemical ethidium bromide (EtBr) into the gel or reservoir buffer. Ethidium bromide intercatales into DNA and fluoresces bright orange when exposed to UV light. If EtBr is in the buffer during the electrophoresis, then the progress can be monitored, and one can actually watch the bands separate. The disadvantage to this is that EtBr is a strong carcinogen and must be handled with extreme care. Hence other stains like **Red safe** is used.



An ethidium-stained gel photographed under UV light. Each band in is a collection of millions of DNA molecules, all of the same length.

The extent of the run can be monitored as usual by using tracking dyes. On a 1% agarose gel, bromophenol blue migrates with fragments of around 400 base pairs.



Procedure

I. Casting an Agarose Gel

1. Level the gel caster.

2. Insert gel casting tray into gel caster. Make sure the cam lever is pointing towards the gel tray. Test the casting for leaks by pouring water into the tray.

3. Weigh 1.5 g Agarose in a 125 mL Erlenmeyer flask. Add 10 mL of TBE (90 mM Tris-Borate, 1 mM EDTA, pH 8.3) or TAE buffer. What is the concentration of your agarose gel?

4. Heat the mixture on a hot plate with stirring. Heat till you get a clear solution then stop. Make sure it doesn't boil.

5. Add 3-5 µL of Red Safe to your agarose mixture.

6. Pour the dissolved agarose solution into the gel tray.

7. Insert comb immediately at the top of the gel.

8. Let the gel solidify. This may take 20-40 minutes.

9. Carefully remove the comb.

10. Fill the electrophoresis chamber and cover the gel with TBE/TAE buffer (about 275 mL of buffer).

II. Agarose Gel Electrophoresis

1. Obtain DNA marker/ladder / standard (M). Be sure to mix well before taking you sample. Take only 6 µL.

2. Check that the walls of the agarose gels are near the black (-) electrode and the bottom edge of the gel is near the red (+) electrode.

4. Mix 16 µL of your sample with 2 µL of the loading dye (contains Formamide and NaOH to disrupt Hydrogen bond that could form between the nucleotides, also it contains a bromophenol blue dye to help tracking the sample) .

5. Mix an equal volume of a series of DNA standards (0.5-50 µg/mL, approximately the same size as your sample DNA) and gel-loading buffer.

6. Load each sample into separate wells in the gel chamber in the following order:

Lane	Sample
1	DNA ladder
2	Sample 1 (-ve control)
3	Sample 2
4	Sample 3
5	Sample 4 (+ve control)

7. Carefully place the lid on the electrophoresis chamber. Connect the electrical leads into the power supply, red to red and black to black.

8. Turn on the power and run the gel at 120 V for 1 hour or until the dye is a little over halfway down the gel.

** you may notice that there is bubbling near the wire of the machine, this result from the hydrolysis of H2O at the cathode and the anode. The same will happen in SDS-PAGE and that means that the machine is functioning.

III. Visualization of DNA

1. When the electrophoresis run is complete, turn off the power and remove the top of the chamber. Carefully remove the gel and tray from the gel box. Be careful – the gel is very slippery. Slide the gel into a tray.

2. Ask the professor or a TA to help you take a picture of the gel using gel documentation system.

3. Place the gel in the waste bag (in the hood).

4. Determine the DNA concentration by comparison to the standard DNA as above or to the marker band with an equivalent fluorescence.

Useful links

Agarose gel electrophoresis/practical

https://www.youtube.com/watch?v=vq759wKCCUQ

Gel electrophoresis/theory

https://www.youtube.com/watch?v=lU8gHrA5QCQ

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Experiment 11: Analysis of DNA

Questions

1. Why do scientists load DNA of known sizes into the agarose gel?

2. What is ethidium bromide? Why is it used in Agarose gel electrophoresis?

3. Why does Agarose gel electrophoresis is done horizontally not vertically?

4. Fill the following table showing the difference between SDS-PAGE of proteins and DNA gel electrophoresis.

	SDS-PAGE	DNA gel electrophoresis
The used gel		
Vertical or horizontal		
The used stain		