INDEPENDENT RESEARCH II

Laboratory Manual

Dr Hongxing Lu
Hongxing.lu@utrgv.edu

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Date	Laboratory Experience		Points	
Week 1	Introduction and Laboratory Safety		Note	Report
Week 2	Introduction to the biomedical science research field Practice : centrifuge and spectrophotometer	Lecture Lab	Quiz for lab safety 5 Lab note and results 5	
Week 3	Research methods in the cell biological and the human study--lecture paper guild line			
Week 4	Research methods in the biomedical and molecular biological research field and protein concentration determination: ice-cold isolation medium prepare (lab)	Lecture Lab		
Week 5	Cellular fraction part 1	Lab	5	5
Week 6	Cellular fraction part 2 Protein concentration analysis	Lab	5	5
Week 7	Elisa Western blot IRA	Lecture		
Week 8	Western-Protein extraction	Lab		
Week 9	western	Lab		
Week 10	Western	Lab		
Week 11	Western	Lab	15	15
Week 12	Methods to measured lipids Lipid Extraction Kit	Lab	5	5
Week 13	Research in animal research Visit UTRGV animal center	Lab	(TB, tetanus)	

Week 14	Paper writing		15
Week 15	Paper 1 due presentation		15
	Total		

LAB SAFETY

- Follow all instructions given by your teacher.
- **No eating or drinking in the lab**
- Report all accidents, injuries, and breakage of glass or equipment to instructor immediately.
- Long hair (chin-length or longer) must be tied back to avoid catching fire.
- Wear sensible clothing including footwear.
- Wear **safety goggles** to protect your eyes
- Do not taste or smell chemicals.
- **Never** point a test tube being heated at another student or yourself. Never look into a test tube while you are heating it.
- Unauthorized experiments or procedures **must not** be attempted.
- Leave your work station clean and in good order before leaving the laboratory.
- Do not lean, hang over or sit on the laboratory tables.
- Do not lift any solutions, glassware or other types of apparatus above eye level.
- Wash hands thoroughly with soap and water before leaving the lab.

CELL AND MOLECULAR BIOLOGY LAB NOTEBOOK

a. The notebook must be either spiral bound or hardcover.

b. The first page should contain the Table of Contents.
All pages should be numbered.

c. The notebook should be kept in continuous chronological order. The date should be written on the upper left hand side of each page.

d. No pages or lines are to be skipped (left blank). If you want to leave a space between entries, you must "X" out the page or line.

e. All data must be placed in the notebook in tables that are clearly titled. Your lab instructor must be able to tell by looking at the data where the numbers came from, i.e. OD400 readings or Apparent Molecular Mass. **Numbers without units are meaningless**. Tables from the lab protocol handouts may be taped or pasted into the lab notebook.

f. In general, materials and methods need not be written out extensively in the notebook unless we deviate from the methods described herein. In addition to providing the place for your data, the purpose of the notebook is to document what you actually did. Therefore, using the lab manual and the notebook, another scientist should be able to reproduce your work.

g. In addition to quantitative data, any observations of interest should be noted.

h. At the end of each experiment you should discuss the data with respect to relevant aspects of Cell Biology theory as covered in the lecture and the lab. In addition, you should note any errors or deviations in the outcome of your experiment from the expected and discuss the potential sources for the error or deviation.

i. Entries in the notebook should be done in lab or immediately thereafter. **Notebooks must be legible.** However, excessive neatness often indicates a notebook done well after the fact! You need to write things down as they occur because important facts or events are quickly forgotten or become unclear in memory. The purpose of your notebook is to faithfully describe what you actually did. It does not need to be a work of beauty ready for display on a museum shelf!

LAB REPORT

Lab Title

Student' Name

Instructor' Name

Date Exp Completed

Introduction

Purpose

- Descriptions of experiment purpose or/and research questions.

Procedure

- List what materials and equipment used in this experiment
- Explain the steps in this experiment

Data and Results

The data that collected from this experiment should be presented and summarized.

- All data and results should be listed, including those that do not support the purpose of this experiment.
- The results may be explained in text, figures and tables.

Discussion

The discussion section should explain the results based on purpose of the experiment. The discussion should contain:

- Summarize the important findings of observations during experiment.
- Explain how the results relate to purpose of this experiment.
- If the results contained errors, analyze the possible reasons for the errors.

LABORATORY EQUIPMENT

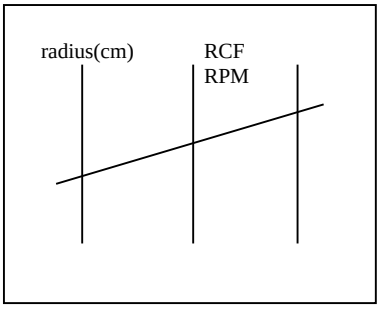
Centrifuges

Centrifuges are used to spin down cells, organelles. Different types, microcentrifuge (eppendorf tubes), centrifuge (15ml and 50 mL tubes) and ultra centrifuges (high speed for separating organelles). For tissue culture purposes regular centrifuge is used. Typical RCFs for cells 200-400g. Anything higher exerts too much force of cells. Actually if isolating mRNA where you want cells densely packed spin down at higher g.

Adjustable Parameters:
Temperature: Primarily 4□C to minimize metabolism and maximize recovery of viable cells. If working with blood R.T
Acceleration/Deceleration: 0-9, Use 0 when when you want to minimize disruption of pellet/buffy coat
Speed: Expressed as RPM (rotations per minute) or RCF (i.e 200g). Better to report speed as RCF because it is universal. RPM are rotor dependent.
RCF (g)= (1.118×10^{-5}) R (rotor radius) x $(S)^2$ (RPM) Ex. 1000 RPM, R=17 cm
RCF=190g

To determine RPM when given RCFs: $RPM=((RCF)/(1.118 \times 10\text{-}5) \times R)^{1/2}$ Ex. Determine what speed (RPM) to use to spin down cells at 400g
$RPM= ((400)/(1.118 \times 10^{-5}) \times 17)^{1/2}$
$=$1450 RRM

Alternatively if scale is provided use a ruler to match your RCF with RPM. You need to know the radius of the rotor.



Centrifuges must always be balanced!!! If not they shut off by themselves

Rotors have limited lifespan Log must be kept to record usage

Incubators

Incubators serve 3 main purposes:
Provide an environment with a constant temperature (most common 37□C)
Provide an environment with 5% CO_2
Provide a sterile environment (air is constantly being filtered)

Modern incubators have alarms that get activated when temperature and CO_2 levels fall below certain values
You can tell if CO_2 levels are not normal by the color of the culture. If CO_2 is too high then medium becomes yellow
If low CO_2 medium becomes purple
Phenol red is responsible for this color changes

Incubators should be cleaned with alcohol and water bath replaced with fresh MilliQ water regularly to avoid contamination.
Water bath ensures high humidity in incubator Frequent heating (weekly) minimizes contamination
Check CO_2 cylinder regularly if no back is available
Modern incubators do not make use of water jackets, easier to handle

Biological Hoods Laminar Flow Hood Vertical and Horizontal
Horizontal takes air from top filters end ejects air from the side facing you Vertical takes air from side, recirculates and ejects portion of air to the top Demonstrate use
Leave hood to run for a few minutes before starting work
UV light should be used during night with closed glass window If window is not a proper level alarm sounds
Show where not to put objects to avoid interference with air flow

1.5 Microscopes
Inverted microscopes are used in tissue culture so flasks can be mounted on stage Adjust ocular lenses according to your interpupilary length
Demonstrate use of microscope

Hemocytometer
Explain why we multiply by 10^4 to convert to cells/mL(0.1 mm x1mm x 1mm)= 0.1 mm^3 1 mL=10mm x 10mm x 10mm=1000 mm^3
Explain the formula again DF x # x 10^4 Demonstrate use of hemocytometer

Pipetman
Come in 3 sizes
2 uL, 200 uL, 1000 uL
They use different tips
Demonstrate proper use of pipetman. Show how to handle small volumes Avoid fast pipeting.
Validate pipetman regularly if high accuracy is needed Keep sterile pipetman in hood permanently to minimize contamination

Let students practice with pipetmen and water for 10 minutes

Ethanol and bleach
Explain why 70% ethanol and not 100%
10% Bleach. In essence it is a dilute solution of Na-Cl-O (sodium hypochlorite)Very effective disinfectant. It is made by mixing chlorine (gas) and liquid NaOH. Resulting sodium hypochlorite

Centrifuge Use

There are a few important guidelines for operating a centrifuge, even a small one. Following them can prevent damage to the centrifuge and possible serious injury to you and others. Use

The work surface must be level and firm. Do not use the centrifuge on an uneven or slanted work surface.

Balance the tubes in the rotor! If you want to run a tube with 10 mL of liquid, put another tube with 10 mL of water in the opposing hole on the rotor. If the liquid has a higher or lower density than water, you must balance the tubes by mass, not volume

. 3. Do not open the lid while the rotor is moving. Even though many centrifuges have a "safety shutoff" if the lid is opened, the only thing this does is stop powering the rotor. The rotor will still spin due to its own inertia for a while until friction slows and eventually stops it.

If you see it wobbling or shaking, turn it off or pull the plug. A little vibration is normal, but excessive amounts can mean danger. FIRST, double check that you correctly balanced the tubes. If the answer is yes and the wobbling still happens, contact the manufacturer or dealer and get the unit serviced. Do NOT continue to run a centrifuge that wobbles visibly when the rotor is spinning.

Wear a face shield and / or safety goggles if you have to work anywhere near a centrifuge that's in use.

Do not bump, jar, or move the centrifuge while the rotor is spinning. Make sure you don't have the cord dangling from a table edge where someone could catch their foot in it and pull down the centrifuge.

The following procedures for centrifugation shall be used when working with biohazardous materials:
.

Examine tubes and bottles for cracks or stress marks before using them. Discard any centrifuge tubes that have cracks in them.

When working with biohazardous materials, wipe outside of tubes with disinfectant prior to removal from the biological safety cabinet and before placing in safety cups or rotors

Place all tubes in safety buckets or sealed rotors when centrifuging infectious materials. Inspect the "O" ring seal of the safety bucket and the inside of safety buckets or rotors.

Spectrophotometer Basics

Introduction

The spectrophotometer is an instrument commonly used by biologists. The spectrophotometer enables biologists to investigate many chemical and physiological processes that characterize the living cells. The purpose of the first exercise is to describe in simple terms how this instrument works and to provide an exercise directly utilizing the spectrophotometer to demonstrate the *Beer-Lambert Law* and prepare a standard concentration curve for a colored solution used in future laboratory exercises (2,6- dichlorophenol inophenol, or DCPIP). Your ability to understand the concepts introduced in this exercise will be essential for the remaining laboratory exercises in this course. In addition, you are given instructions for the use of automatic micropipetters, which are used throughout this course.

The spectroscopic measurement of protein concentration uses a spectrophotometer. A cuvette of sample cell length (thickness) 1 cm containing a solution of the protein is placed in a beam of monochromatic radiation of intensity I_o. The intensity of the emerging beam will be decreased to a value I because the solution absorbs some of the radiation.

The greater the number of molecules capable of absorbing light of a given wavelength (□), the greater the extent of absorption. Furthermore, the more effectively a molecule absorbs light of a given wavelength (□), the greater the extent of light absorption. From these ideas the following equation, known as the **Beer-Lambert's Law** may be formulated:

A = $\log (I_o/I) = w \cdot c \cdot l$ A = absorbance

I_o = intensity of light incident upon the same cell I = intensity of light leaving the sample cell

c = molar concentration of the solute l = length of the sample cell (cm)

w = molar absorptivity (extinction coefficient)

The dimensions of the extinction coefficient depend on the concentration units employed. If protein concentration is measured in molarity, the unit for □ has to be $M^{-1} cm^{-1}$ since the absorption is a dimensionless quantity.

A plot of the absorbance of a substance in solution at a given wavelength, as a function of the molar amount of the substance present, is called a calibration plot or standard curve. If the length of the sample cell is 1cm, the slope of a standard curve is the extinction coefficient. An absorbance spectrum of a given compound is a graphical representation of the absorbance at each wavelength over a given wavelength range (usually UV-visible), that is, A vs. □. Chemically different compounds have their own distinctive absorbance spectrum. You will be determining the absorbance spectrum for the electron acceptor 2,6-dichlorophenol inophenol (DCPIP) and will also plot absorbance spectra in a future laboratory exercise on plant pigments.

SPECTROSCOPY: The Instrument

The typical UV-visible spectrophotometer consists of a **light source**, a **monochromator**, and a **detector**. The light source is usually a deuterium lamp, which emits electromagnetic radiation in the UV region of the spectrum. A second light source, a tungsten lamp, is used for the wavelengths in the visible region of the spectrum. The monochromator is a diffraction grating; its role is to spread the beam of light into its component wavelengths. A system of slits focuses the desired wavelength on the sample cell. The light, which passes through the sample cell, reaches the multiplier tube. In a typical double beam instrument, the light emanating from the light source is split into two beams, the sample beam and the reference beam. When there is no sample cell in the reference beam, the detected light is taken to be equal to the intensity of light entering the sample.

The sample cell must be constructed of a material that is transparent to the electromagnetic radiation being used in the experiment. For measurements in the visible range of the spectrum, cells composed of glass or plastic are generally suitable. For measurements in the ultraviolet region of the spectrum, however, glass and plastic cannot be used because they absorb ultraviolet radiation. Instead, cells made of quartz must be used, since quartz does not absorb radiation in this region.

The instrument design just described is quite suitable for measurement at only one wavelength. If a complete spectrum is desired, this type of instrument has some deficiencies. A mechanical system is required to rotate the monochromator and provide a scan of all desired wavelengths.

Using the spectrophotometer

The machine needs to warm up prior to use. Turn on the *power switch* and allow the machine to warm up for at least 15 minutes.

Using the A/T/C option on the spectrophotometer, select the absorbance mode.

Using the nm button, select the appropriate wavelength.

Insert your cuvette by positioning it firmly in the opening provided.

Insert your blank and press OABS/100%T to calibrate the machine with your blank.

Remove the blank and insert your samples one by one and record the absorbance. You do not need to blank between measurements.

EXPERIMENT PROTOCOL

LAB EQUIPMENT PRACTISE

Spectrophotometric and centrifuge practice

Materials needed (per group):

Food color

Liquid Broth

4 -1.5ml micro centrifuge tubes of LB (Liquid Broth)

Micropipette 1000uL

Pipettes tips

Centrifuge

15mL Conical tubes

Cuvettes

Spectrophotometer

Distilled water

Waste container

Procedure:

1. Label 4 tubes “1/2”, “1/4”, and “1/8” and blank respectively. These values represent the dilutions that you will be making. Label the 4th tube “Blank”.

2. Transfer 5mL of food color solution into Conical tube (15 ml) add

3. Centrifuge food color for 5 min at 3000 g. *pay attention to the tube balance

4. Pipette 1mL of food color and 1 ml water into the “1/2” tube and. Mix by gently flicking the bottom of the tube with one finger, or by rolling the tube in your hand. Be careful not to invert the tube or it will leak.

5. Pipette 1mL from the “1/2” tube and 1 ml water to the tube marked “1/4” and mix. Put the “1/2” tube in your rack—you will need it in a minute.

6. Pipette 1mL from the “1/4” tube and 1ml water to the tube marked “1/8” and mix. Put both tubes in your rack.

7. Fill a clean cuvette with broth (1mL) from the “Blank” tube and insert into the spectrophotometer. Zero the spectrophotometer.

8. Remove the cuvette and pour the broth into the waste container provided. Do not rinse cuvette.

9. Fill the cuvette with sample from the “1/8” tube. Place in the spectrophotometer and record the absorption value in the table below.

10. Important: DO NOT zero the spectrophotometer at this point, or you will have to start all over (think about what you would be doing here).

11. Remove the cuvette and pour the broth into the waste container provided. Rinse with distilled water.

12. Repeat steps 7 and 8 for the “1/4” and “1/2” tubes.

Food color 1/8 dilution (0.125uL) ¼ dilution (0.25uL) ½ dilution (0.5uL)

Absorption

If you did your dilutions accurately, the 1/4 dilution should have approximately half the absorption of the 1/2 dilution, and about twice the absorption of the 1/8 dilution.

In general, the linear relationship between absorption and cell numbers holds up until absorption reaches about 1.0. Beyond this point, the cell numbers are so high that some of the light initially scattered by one cell may be scattered forward to the detector by another cell, thus causing the amount of light hitting the detector to increase. This is why dilutions are made.

CELLULAR FRACTIONATION

AND

PROTEIN QUANTIFICATION OF CELLULAR FRACTIONS

WEEK 1 PREPARE SOLUTION

To prepare a 1M Phosphate Buffer Solution:

1M HCl and dilute it to 0.1M HCl
Use 900ml of ddH20 and add 100mL of 1M HCl
1M NaOH and dilute to 0.1M NaOH
For 1M NaOH = 40g of NaOH Pellets to 1000mL of ddH20
For 0.1M NaOH = 4g NaOH Pellets to 1000mL of ddH20
1M disodium hydrogen phosphate
For 1M disodium hydrogen phosphate
Combine 14.2g Na2HPO4 + 1000mL ddH20

- Adjust pH by adding HCl or NaOH

Phosphate Buffer Combine:
955.1mL of disodium phosphate solution and add 44.9mL of HCl and adjust PH to 7.8 by using HCl and NaOH

To Prepare Isolation Medium:

0.1M EDTA –
To Prepare 3M of NaOH
120g of NaOH Pellets + 1000ml of ddH2O
Then add 2.9224g of EDTA

$$1000\text{ml} \times \frac{0.01\text{ mol}}{1000\text{mL}} \times \frac{292.24\text{g}}{1\text{mol}} = 2.9224\text{g EDTA}$$

0.1M Phosphate Buffer Solution

$$1000\text{ml} \times 0.01\text{ mol} \times 141.96\text{g Na2HPO4} = 14.2\text{g Na2HPO4}$$

---------- ----------
1000mL 1mol

0.4M Sucrose

1000ml x 0.01 mol x 342.3g Na2HPO4 = 136.92g Sucrose
---------- ----------
1000mL 1mol

For isolation Medium Combine:

- Prepare the 0.01M EDTA first put 900 mL in a 1L media bottle
- ***The disodium phosphate solution and sucrose will not dissolve if the EDTA isn't prepared first.***

1.
2. If a 1M phosphate buffer solution is already made add 100mL of 1M phosphate Buffer Solution to 900mL of 0.01M EDTA.
- If the phosphate buffer solution isn't made then add 14.2g of disodium hydroxide phosphate
3. Add 0.4M of Sucrose 136.92g

WEEK 2 CELLULAR FRACTIONATION

INTRODUCTION

All eukaryotic cells contain organelles that perform specific functions. **Cellular fractionation** is a technique that enables the researcher to separate and isolate bulk quantities of organelles and other cellular components. Once isolated, the functioning of the cellular constituents can be studied more easily than within the intact cell.

Fractionation begins with **homogenization**, which involves the disruption of the cells by mechanical shearing, ultrasound, or osmotic shock. This process releases the cellular organelles, which can then be separated from each other by **centrifugation**.
Centrifugation at low speeds causes the sedimentation of only the largest and densest of components. These components can be collected and the "unpelleted" portion, or supernatant, can be centrifuged again at higher speeds and for longer durations to "pellet" successively smaller components. Separation of organelles and other particles in solution can be described mathematically using Stokes' Law.

$\mu = 2cr^2 (d\text{-}d / 9\eta)$

Where;
μ = velocity of sedimentation of the particle
c = centrifugal acceleration and is proportional to revolutions per minute of the centrifuge
r = radius of the organelle
d and d_0 = the density of the organelle and medium respectively
η = viscosity of the medium

Therefore, the rate at which an organelle settles is proportional to its radius and density.

Once isolated, the components can be assessed for purity by light or electron microscopy and their biochemical activities assayed to enable structure/function relationships to be determined.

In the following exercise, you will fractionate tissue from Mung Bean (*Phaseolus mungo aureus*) seedlings to isolate cellular functions enriched in nuclei, mitochondria, and cytoplasm. An overview of the procedures is provided in figure 1.1.

Materials
Bean seedling
Cheese cloth
Blender
500 ml glass beaker
Ice-cold isolation medium
50 ml cold, polypropylene centrifuge tubes
2 ml Microcentrifuge tubes
Refrigerated Centrifuge
Ice
Marker pen
Scalpels

PRODEDURE FOR CELLULAR FRACTIONATION

PART 1: HOMOGENIZATION OF SEEDLINGS

Steps 1 through 3, part 1 should be performed per lab groups. All solutions and glassware should be maintained on ice.

1. Chop 20 g of bean seedlings and add to 80 ml of ice-cold isolation medium. Remember to keep the tissue cold.

2. Pour the suspended tissue into a blender and homogenize the tissue with several short bursts (10 seconds) of the blender. The tissue suspensions from different labs benches may be pooled to save time. This blended solution will be referred to as the homogenate.

3. Place 2-4 layers of cheesecloth, pre-wetted with isolation medium, over a clean 400 ml beaker. Pour the homogenate though the cheesecloth into the beaker maintained on ice. Rinse the blender with a further 10 ml of ice-cold isolation medium and pass the wash through the cheesecloth into the beaker. Gently squeeze the cloth to remove the remaining fluid from the tissue fragments. You should have approximately 90 ml of tissue homogenate with a tissue: buffer ratio of 1:4.5 (weight to volume). At this point, we will split the homogenate between the two groups.

4. Split the homogenate in half by pouring equal amounts into each of two 50 ml cold, polypropylene centrifuge tubes. You must balance the tubes at this point. Each group will now work with one of the tubes for the remainder of the procedure. Keep the tubes on ice.

5. Aliquot two 1.5 ml samples of the homogenate into clean, labeled microcentrifuge tubes. Label the tubes with your group name, the volume and sample type (in this case homogenate) and place on ice. These samples will be used for later assays.

PART 2: DIFFERENTIAL CENTRIFUGATION

6. Take balanced pairs of centrifuge tubes from step A4 and centrifuge in a pre- cooled centrifuge (4^{o}C) for 5 min. at 500 g. Pour or pipette the supernatant (taking care not to resuspend the pellet) into a cold, clean 50 ml centrifuge tube. Discard the pellet, which consists mainly of whole cells and cell wall debris.

7. Centrifuge the supernatant from step B1 at 600 g for 10 min. Make sure tubes are balanced. Pour or pipette the supernatant (taking care not to re-suspend the pellet) into a cold, clean 50 ml centrifuge tube and maintain on ice.

8. Re-suspend the pellet in the centrifuge tube using 20 ml of isolation medium and gently agitating with a paint brush or Pasteur pipette. Re-centrifuge at 800 g for 10min. Discard the supernatant and resuspend the pellet in 5 ml of ice-cold isolation medium. This fraction is the **nuclear fraction.**

9. Centrifuge the supernatant obtained during step B2 for 15 min. at 15,000 g. Transfer the supernatant into a cold, clean centrifuge tube. This is the **cytosolic fraction.**

10. Resuspend the pellet obtained from Step B4 in 5 ml of ice-cold isolation medium. This sample is the **mitochondrial fraction.**

You should produce 4 samples: the crude homogenate (two x 1.5 ml), nuclear (approx. 5 ml), mitochondrial (5 ml) and cytosolic fractions (approx. 40 ml.). Keep these samples on ice.

STORAGE OF CELLULAR FRACTIONS

Divide the remainder of each of your four samples as shown below in table 1.1.
Use microcentrifuge tubes and label them carefully using indelible markers. Include the fraction name, volume, and group name on the label. Place samples in the -80^{o}C freezer. These samples will be used in the later exercises. The crude homogenate samples were prepared in step A5.

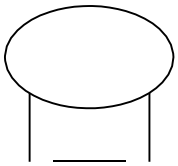
Table 1.1

Sample	Aliquot Size	
	Tube 1	Tube 2
Crude Homogenate	1.5 ml	1.5 ml
Nuclear Fraction	1.5 ml	1.5 ml
Mitochondrial	1.5 ml	1.5 ml
Cytosolic	1.5 ml	1.5 ml

METHODS OVERVIEW (for quick reference):

Preparation of Bean Sprouts

1. Weigh out 20 grams (2 groups share)
2. Chop (crudely) with Scalpel
3. Add ice cold isolation medium (80 ml)
4. Blender (short bursts!)
5. Filter through cheesecloth

Add extra 10ml of isolation medium to "wash" blender (total  volume = 90 ml)

↓

Homogenate ⟶ Reserve 2x 1.5ml samples*

6. Split in half for the 2 groups (2 x 50 ml tubes)

Centrifugation

1. Balance the centrifuge tubes. If they are off balanced you could severely damage the centrifuge.
2. Centrifuge @ 500xg "Junk Step" - Discard pellet
3. Centrifuge supernatant from step 2 @ 600xg for 10 min

↘

resuspend pellet in 20ml isolation medium, re-spin (centrifuge) @ 800xg for 10 min, resuspend pellet in 5ml isolation medium (nuclear)

* reserve 2 x 1.5ml

4. Centrifuge supernatant from step 3 @ 15,000xg for 15 min

↘

Resuspend pellet in 12ml isolation medium (mitochondrial)
*reserve 2x 1.5ml

Supernatant = cytosol
reserve 2 x 1.5ml

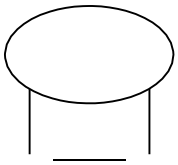
Table 1.1

Sample	Aliquot Size	
	Tube 1	Tube 2
Crude Homogenate	1.5 ml	1.5 ml
Nuclear Fraction	1.5 ml	1.5 ml
Mitochondrial	1.5 ml	1.5 ml
Cytosolic	1.5 ml	1.5 ml

METHODS OVERVIEW (for quick reference):

Preparation of Bean Sprouts

1. Weigh out 20 grams (2 groups share)
2. Chop (crudely) with razor
3. Add ice cold isolation medium (80 ml)
4. Blender (short bursts!)
5. Filter through cheesecloth



Add extra 10ml of isolation medium to "wash" blender (total volume = 90 ml)

Homogenate → Reserve 2x 1.5ml samples*

6. Split in half for the 2 groups (2 x 50 ml tubes) Centrifugation

1. Balance tubes
2. Centrifuge @ 500xg "Junk Step" - Discard pellet
3. Centrifuge supernatant from step 2 @ 600xg for 10 min

resuspend pellet in 20ml isolation medium, re-spin (centrifuge) @ 800xg for 10 min, resuspend pellet in 5ml isolation medium (nuclear)

* reserve 2 x 1.5ml

4. Centrifuge supernatant from step 3 @ 15,000xg for 15 min

Supernatant = cytosol
* reserve 2 x 1.5ml

Resuspend pellet in 12ml isolation medium (mitochondrial)
* reserve 2x 1.5ml

WEEK 3 PROTEIN QUANTIFICATION OF CELLULAR FRACTIONS

INTRODUCTION

The biochemical activity of a cellular protein is often expressed in the form of activity mg^{-1}. This expression requires that the protein content of the sample be quantified. In this exercise, you will determine the protein content of the cellular fractions obtained in the previous exercise using the Coomassie Blue method. You will be required to prepare a standard curve by making serial dilutions of bovine serum albumen (BSA) from a concentrated solution (2.0 mg ml^{-1}). The standard curve can be used to calculate the protein content of each cellular fraction.

3 Materials
BSA.
Coomassie Brilliant Blue G-250
spectrophotometer
Cuvette

PART 3 PROCEDURE FOR PREPARATION OF A PROTEIN STANDARDIZATION CURVE

1. You are provided with an ampule containing 2.0 mg ml^{-1} BSA. Prepare 4 ml of BSA solution at a concentration of 333.3 µg ml^{-1} by diluting the BSA solution in the ampule with the appropriate amount of distilled water.

2. Label 7 cuvettes and prepare a dilution series of BSA at the concentrations listed in table 2.1. Start by adding 4ml of Coomassie Blue to each cuvette (the final total volume will be 5 ml --see Table 2.1). The reagent contains the dye Coomassie Brilliant Blue G-250, which binds to proteins to form a blue complex whose absorbance is directly proportional to protein concentration.

3. Remember to calculate the volume first (see tables), then prepare all of the solutions for the standard curve together (add appropriate volumes of BSA to the Coomassie Blue). Mix the tubes thoroughly immediately after the dye has been added. Wait five minutes, then read absorbance for each sample on the spectrophotometer.

4. Set the spectrophotometer wavelength to 595nm. Use tube#1 as a blank and set the spectrophotometer to zero. Using this wavelength, measure the remaining tubes.

5. Plot the results on graph paper, plotting the absorbance of each tube on the y-axis (vertical), and the protein concentration (µg/ml) on the x-axis (horizontal).

Table 2.1

Tube #	BSA added (Solution 1) (ml)	Distilled water added (ml)	Coomassie Reagent added (ml)	Protein content (µg)	Protein concentration (µg/ml)	Absorbance at λmax
1	0	1	4	0	0	
2			4	33.3	6.66	
3			4	66.7	13.32	
4			4	133.3	26.64	
5			4	200.0	39.96	
6			4	266.6	53.28	
7	1.0	0.0	4	333.3	66.66	

PART 4: CALCULATION OF PROTEIN CONTENT OF CELLULAR FRACTIONS COLLECTED EARLIER

Materials:

Glass Test Tubes
Spectrophotometer
Hot Plate
0.1% SDS in 1mM EDTA
Test Tube Tongs
Beaker
ddH20
Cuvettes
Coomassie Blue

1. Defrost one tube of each of your refrigerated sample types prepared during cellular fractionation and pipette 500 µl from each into four separate glass test tubes. Lyse the samples by the addition of 2000 µl of 0.1% SDS in 1mM EDTA (prepared for you by the technician) and immerse the tubes in boiling water for two minutes. Remove the tubes with the tongs provided – take care not to get burned.

2. Dilute 200 µl of each of our boiled samples with 1.8ml of distilled water in a clean cuvette. Mix the contents thoroughly using a new pipette tip. You should have two tubes for each fraction type with concentrations of protein differing by an order of magnitude. This is referred to as a serial dilution. Transfer 1ml of each sample to a new tube and add 4 ml of the Coomassie reagent. Once again, gently, mix the contents thoroughly by inverting a number of times. Measure the absorbance at the 595nm and record the data in Table 2.2. This assay is temperature dependent. Make sure your samples are at room temperature before reading.

3. Determine the concentration of protein in the original sample held in the freezer from the BSA protein standard curve. Make sure to account for the dilutions. Which fraction contains the most

protein? These values will be used in the following exercise.

Outline of Experiment

A. PREPARE PROTEIN STANDARD CURVE
Ampule contains 1ml of 2mg/ml solution (BSA is a protein standard)

1. 2mg/ml (1ml) = 2mg
0.333mg/ml (xml) = 2 mg x = 2mg/0.333mg/ml = 6ml
(1ml ampule & 5 ml H_2O) ⟶ this is solution (1)

solution (1) will be used to make most concentrated BSA solution for standard curve

will be: 1ml solution (1)
4ml coomassie blue reagent
final conc. = 333.3/5 = 66.6 µg/ml

2. Calculate volumes required to make all solutions of the standard curve

3. Assemble all blue solutions at once, wait 5 mins, then read at 595nm (abs. Max)

B. PREPARE SAMPLES (from previous lab)

1. Remove one each of your 1.5ml aliquots from freezer, thaw

2. Prepare boiling water bath (600ml beaker on hotplate)

3. Take 500 µl from each sample (above), add 2000 µl SDS solution (prepared by technician)
Boil x 2 min
Take care (use tongs)! This is the 1:5 dilution

4. Take 200 µl from step 3 above, add 1.8ml water. This is the 1:50 dilution

5. Take <u>1ml</u> from each of these diluted, boiled samples, add 4.0ml Coomassie Blue, wait 5 min., read absorbance.

6. Will extrapolate from standard curve to get unknown concentration.

*Remember to multiply by 5 or 50 to get actual protein concentration Take the average of your 2 answers to get the protein concentration.
Alt use the reading obtained from 1:5 dilution
<u>only</u> (if @ 600 Abs. Range, this is most accurate)

TABLE 2.2

Sample	Absorbance	Protein Concentration (µg/ml) Corrected for Dilution
Homogenate		
1/5 Dilution*		
1/50 Dilution		
Cytosol Fraction		
1/5 Dilution		
1/50 Dilution		
Mitochondrial Fraction		
1/5 Dilution		
1/50 Dilution		
Nuclear Fraction		
1/5 Dilution		
1/50 Dilution		

INTRODUCTION UOLPHYSIOLOGY

ELISA Simulation Kit

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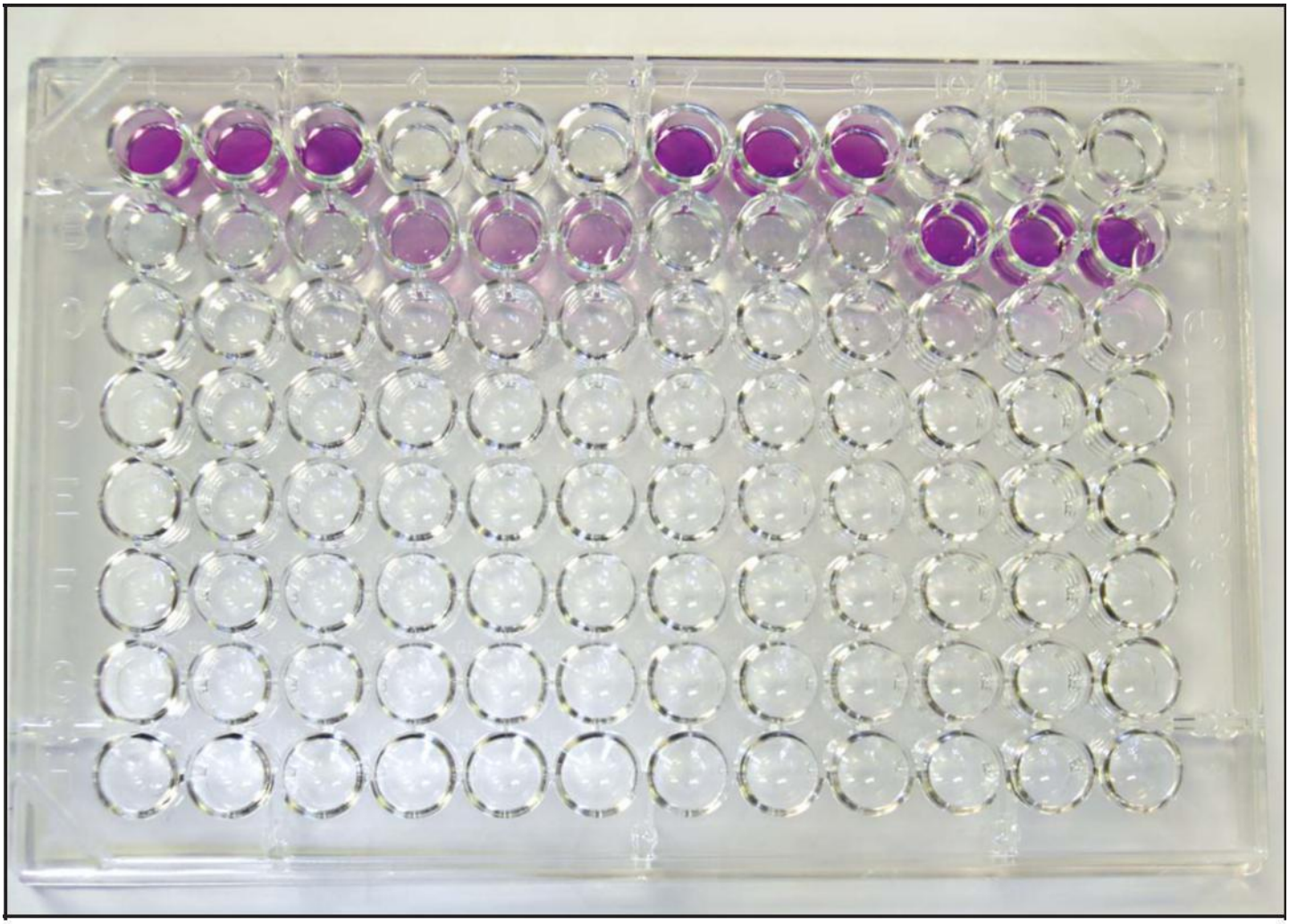

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ELISA Simulation Kit

Overview

Using the ELISA Simulation Kit, students explore how the principles of antibody- based human immunity apply to a common laboratory test called ELISA (enzyme- linked immunoabsorbant assay). ELISA is commonly used to test blood serum for the presence of antibodies against disease-causing pathogens such as viruses and bacteria.In this way, the assay indirectly detects infection by particular disease-causing agents. Students work in pairs to perform a hands-on simulation of an indirect ELISA using simulated reagents. Students take an investigative approach to diagnose six fictitious patients for either HIV, Lyme Disease, Avian Influenza (Bird Flu), or West Nile virus.

Hypothetical scenarios are provided for each patient being tested for each disease. This kit is a simulated ELISA and does not include actual serum samples, antibodies, antigens, or disease agents. As such, there is no risk of infection from the materials included in this kit.

Objectives

•To understand the basic principles of antibody-mediated immunity

To understand the steps involved in performing an ELISA and how it is used as a diagnostic tool •To understand the disease-causing agent and transmission patterns of certain infectious diseases

Background

The Body's Defense System

The body possesses several lines of defense against infection by pathogenic organisms. Pathogens are tiny, disease-causing agents including viruses, bacteria, protozoa, molds, and other microorganisms. Pathogens invade the body and multiply; they can cause sickness or even the death of the invaded individual. The body employs three lines of defense to prevent and fight off such dangerous intrusions. The first two defense modes are nonspecific. They include the body's physical barriers and the nonspecific immune system. These defenses function without regard to the type of pathogenic intruder. The third layer of defense is the body's specific immune system.

Specific immune responses are tailored to the type of invading pathogen.

Nonspecific Barriers

The primary defense against intruding pathogens is the external protective covering of the body, the skin, and the mucous membranes that line the mouth, nostrils, and other potential gateways. This armor presents a nonspecific physical barrier to foreign invaders. Secretions from the skin and mucous membranes, such as sweat, tears, and saliva, also inhibit entry into the body.

Nonspecific Immune Response

If the body's exterior barriers are breached, several internal, nonspecific immune defenses are launched. One such response is a fever, a rise in body temperature. Many pathogens cannot function well at body temperatures even slightly higher than normal. A fever, therefore, can slow the spread of an infection.

The inflammatory response is another nonspecific immune defense. This response increases blood flow to an infected area, resulting in localized redness and swelling. White blood cells called neutrophils rush to the area of infection as part of the blood flow. They engulf and destroy invading organisms by phagocytosis. Other white blood cells called monocytes develop into

macrophages, which are large phagocytic cells that also ingest intruding pathogens.

Another nonspecific internal defense includes antimicrobial proteins of the complement system and antiviral interferon proteins. These proteins act to directly destroy some pathogens and enhance other mechanisms of immunity.

Although the nonspecific immune system indiscriminately attacks invading pathogens, it can distinguish its own cells (self) from foreign cells (non-self). It can also recognize and destroy abnormal body cells—cells that could lead to cancer. Natural killer cells play a role in nonspecific cell-mediated immunity by attacking abnormal body cells and infected cells.

Specific Immune Response

Invading microbes also encounter the force of the specific immune system. The specific immune system is complex and involves several organs and tissues, including the thymus, spleen, lymph nodes, bone marrow, and white blood cells. This section focuses on the aspects of specific immunity that apply to the ELISA test discussed in the next section.

Specific immune responses are triggered by antigen molecules. Antigens include proteins and other molecules produced by pathogens. The key players in the specific immune defense are dendritic cells, macrophages, and small white blood cells called B lymphocytes (B cells) and T lymphocytes (T cells).

Phagocytic macrophages and dendritic cells break down pathogens and display antigenic fragments from the pathogens on the surface of their cell membranes. B and T lymphocytes circulate through the body in the blood and lymph. When
T cells see displayed antigenic fragments, they stimulate specific B cells to reproduce and generate antibodies designed against the specific structure of the antigen encountered. Thus, the word *antigen* is derived from the term "antibody generator."

Antibodies are a group of serum proteins (also referred to as immunoglobulins) that are found in the bloodstream or bound to cell membranes. These proteins all have the same basic Y-shaped structure, but have different antigen binding sites at their ends. Antigen binding sites are designed to fit the shape of specific antigens. Antibodies bind to antigens like a lock and key, forming antigen-antibody complexes (see Figure 1).

Figure 1. Antigen-antibody complex
When an antibody forms an antigen-antibody complex, generally it marks the invading organism/antigen for destruction or for clearance from the bloodstream by phagocytic cells. This removal is designed to prevent the organism/antigen from infecting the cell. Antigen-antibody complexes also stimulate additional immune responses to aid the body in clearing an infection.

The specific immune system has an incredible ability to learn and remember. The first time the body is exposed to an antigen, a primary immune response is launched and antibody-producing B cells and T cells work steadily over time to eliminate the infection. If the same antigen is encountered a second time, the body is primed and remembers how to respond. It launches a more potent secondary
immune response that can rid the body of the invading antigen more quickly. This is known as

immunological memory.

ELISA

Scientists have applied the basic principles of antibody-mediated immunity to an assay for detecting infection by specific organisms. This assay is called an ELISA (enzyme-linked immunoabsorbant assay) and is based on the principle that antibodies produced in response to pathogens attach to their antigen targets with great specificity to form antigen-antibody complexes. ELISAs can be used to test for infection by HIV, influenza virus, the bacterium that causes Lyme Disease, smallpox virus, SARS coronavirus, West Nile virus, and other disease agents.

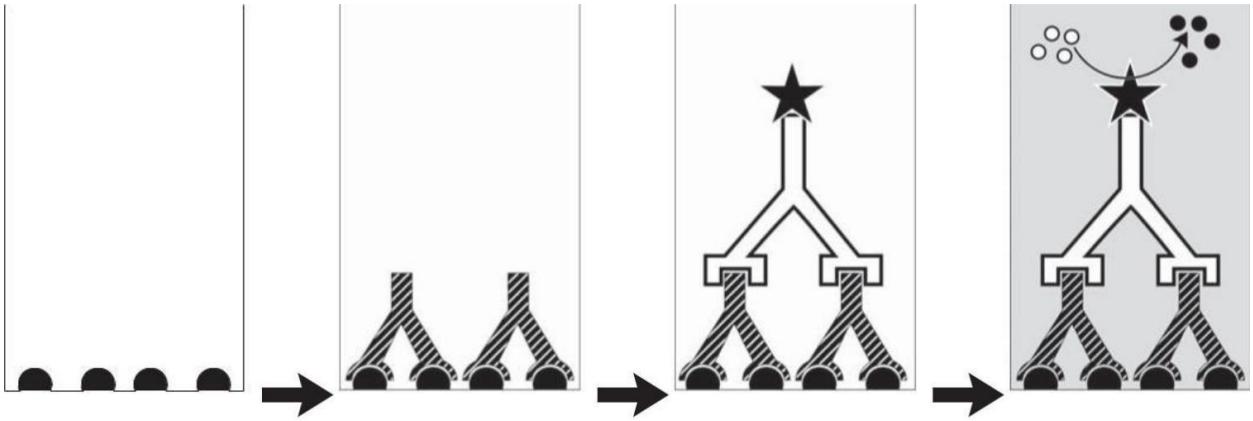
There are two types of ELISA tests—direct ELISA and indirect ELISA. Indirect ELISA is used to detect infection by testing patients' blood for the presence or absence of antibodies against a particular pathogen. The presence of such antibodies indicates that the individual has been infected and that their body has launched an immune response against the disease-causing agent. Direct ELISA assays for the presence or absence of certain antigens in patients' blood. The lab performed in this kit simulates the indirect ELISA test.

In the first step of an indirect ELISA, antigen proteins purified from the infectious agent, or genetically engineered versions of the antigens, are added to the wells of plastic microtiter plates. These antigen proteins bind to the bottom of the well by forming hydrophobic associations with the plastic surface (see Figure 2a). The wells of the plate are then washed with a buffer to remove any unbound material.

Next, blood serum from the patient(s) being tested is added to the treated wells. If these serum samples contain antibodies against the bound antigen, the antibodies will attach to the antigens, forming tight complexes (see Figure 2b). Such antigen-antibody complexes are not visible by eye, so detection steps (described in the next paragraph) must be employed to visualize them. The wells are again washed to remove any unbound proteins.

Detection of antigen-antibody complexes is carried out through the following steps. A secondary antibody that recognizes antibodies produced by humans (anti-human antibody) is added to the wells. If antigen-antibody complexes formed in the wells, this secondary antibody recognizes and binds to the primary antibodies from the patients' serum (see Figure 2c). The secondary antibody is attached to an enzyme that will facilitate the final detection. (This antibody/enzyme combination is called a conjugate.) The wells are rinsed one last time to remove unbound molecules.

In the final step, a chromogen substrate is added to the wells of the plate. If present, the enzyme that is linked to the secondary antibody facilitates a chemical reaction that changes the color of the chromogen (see Figure 2d). A color change indicates that the patient possesses antibodies to the antigen and has been infected. No change in color indicates that the patient has not been infected, or that their body has not yet launched an immune response to produce antibodies against the invading antigen.



nd antigen | gen-serum y complex | bound secondary antibody | (d) enzyme-induced color change

Key:

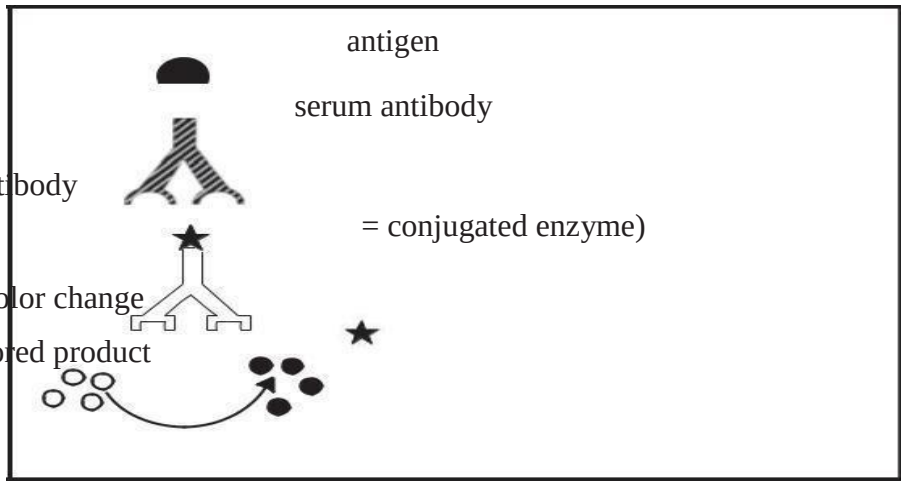


Figure 2. ELISA schematic of a positive result

In this simulated assay, each sample will be tested in triplicate to ensure reproducibility. Known positive and negative samples are included as controls. For ease of performance, the well washing steps of a true ELISA have been eliminated in this simulation. It is important to note that the washes are a necessary step of an actual assay. Likewise, it is critical to use a clean pipet for each new sample or reagent to prevent cross-contamination of the wells.

Patient Profiles for HIV Testing

HIV stands for Human Immunodeficiency Virus. It is the virus responsible for causing the Acquired Immune Deficiency Syndrome, or AIDS. HIV attacks and destroys a type of T lymphocyte of the immune system called $CD4^+$ cells, and thereby severely weakens immune defense. Shortly after infection by HIV, the body begins producing antibodies against the virus, and the infected person may

feel flu-like symptoms. The virus then enters a latent phase where the infected person does not experience symptoms but the virus and the immune cells continue to attack each other. Eventually, the HIV virus gains the upper hand. The immune system loses its ability to fight off the opportunistic infections it could normally combat, and advanced AIDS results. HIV is communicable and can be transmitted from person to person through blood, pregnancy, breast milk, and other bodily fluids.

Laboratory Guide

Student Workstation Checklist

One workstation serves 4 students.

Items	Contents	Number	(✓)
Yellow tubes	Student samples (0.25 ml)	4	❑
Green tube (AG)	Purified antigen (1.5 ml)	1	❑
Violet tube (+)	Positive control (0.5 ml)	1	❑
Blue tube (–)	Negative control (0.5 ml)	1	❑
Orange tube (SA)	Secondary antibody (1.5 ml)	1	❑
Brown tube (SUB)	Substrate (1.5 ml)	1	❑
12-well microplate strips		2	❑
50 µl fixed-volume micropipet or 20–200 µl adjustable micropipet		1	❑
Yellow tips		10–20	❑
Disposable plastic transfer pipet		1	❑
70–80 ml wash buffer in beaker	Phosphate buffered saline with 0.05% Tween 20	1	❑
Large stack of paper towels		2	❑
Black marking pen		1	❑

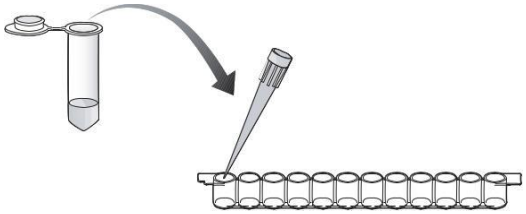
Laboratory Procedure

1. The yellow tubes contain the serum samples that will be tested for the presence of antibodies. Label each yellow tube to identify the sample being tested.

2. Label the outside wall of each well of your 12-well strip. Two students may share a strip of 12 wells. On each strip label the first three wells with a "+" for the positive controls and the next three wells with a "–" for the negative controls. Label the remaining wells to identify the samples being tested. For example, Florence Nightingale and Alexander Fleming would label their shared strip like this:

FN

3. Use a pipet to transfer 50 µl of the purified antigen (AG) from the green tube into all 12 wells.



Purified antigen

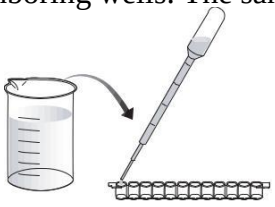
4. Wait 5 minutes while the antigen binds to the plastic wells.

5. Wash unbound antigen out of the wells:

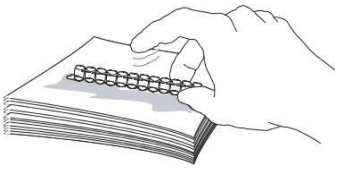
a. Tip the microplate strip upside down onto the paper towels so that the samples drain out, then gently tap the strip a few times upside down on the paper towels. Make sure to avoid splashing sample back into wells.

b. Discard the top paper towel.

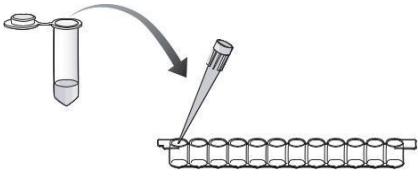
c. Use a transfer pipet filled with wash buffer from the beaker to fill each well with wash buffer, taking care not to spill over into neighboring wells. The same transfer pipet will be used for all washing steps.



d. Tip the microplate strip upside down onto the paper towels so that the wash buffer drains out, then gently tap the strip a few times upside down on the paper towels. scard the top 2–3 paper towels.
e. Discard the top 2–3 paper towels.

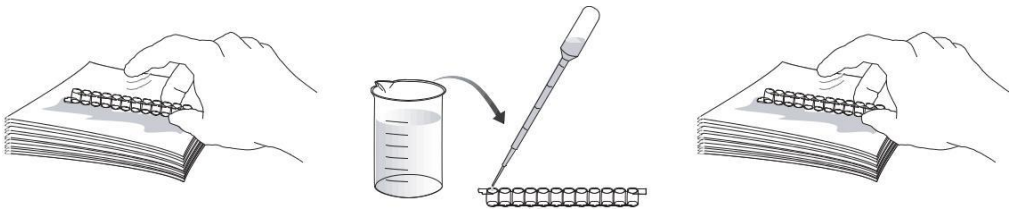


6. Use a <u>fresh</u> pipet tip to transfer 50 µl of the positive control (+) from the violet tube into the three "+" wells.

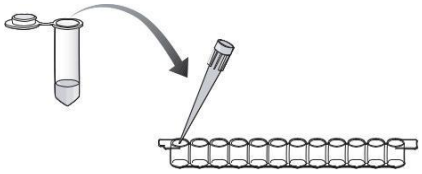


Control or serum

7. Use a fresh pipet tip to transfer 50 µl of the negative control (–) from the blue tube into the three "–" wells.
8. Use a fresh pipet tip to transfer 50 µl of each student's serum sample into the appropriately initialed three wells.
9. Wait 5 minutes to allow the serum antibodies in the samples to bind to the antigen.
10. Wash the samples out of the wells by repeating wash step 5.



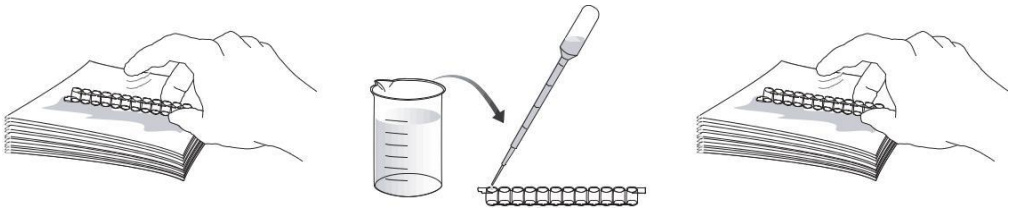
11. Use a fresh pipet tip to transfer 50 µl of the positive control (+) from the violet tube into the three "+" wells.



Secondary antibody

12. Wait 5 minutes for the secondary antibody to bind to the primary antibody.

13. Wash the unbound secondary antibody out of the wells by repeating wash step 5 **two times**.



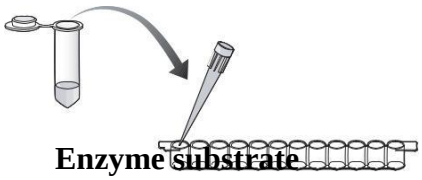
The secondary antibody is attached to an enzyme (HRP) that chemically changes the enzyme substrate, turning it from a colorless solution to a blue solution. Predict which wells of your experiment should turn blue, which should remain colorless, and which wells you are not sure about.

_____

_____

_____

14. Use a fresh pipet tip to transfer 50 µl of enzyme substrate (SUB) from the brown tube into all 12 wells of the microplate strip.



Enzyme substrate

15. Wait 5 minutes. Observe and record your results.
16. **Results Section** Label the figure below with the same labels you wrote on the wells in step 1. In each of the wells, put a "+" if the well turned blue and a "–" if there is no color change.

Is your sample positive? Explain your results.

Post-Lab Focus Questions

1. Did your serum have antibodies to the disease?

2. If you tested positive for antibodies, does this mean that you have been exposed to the disease?

3. What reasons could there be for a positive test when you actually do not have the disease?

4. Why did you assay your samples in triplicate?

5. When you added serum samples to the wells, what happened to the serum antibodies if the sample was positive? What if it was negative?

6. Why did you need to wash the wells after every step?

7. When you added secondary antibody, what happened if your serum sample was positive? What if it was negative?

8. What antibody-based tests can you buy at your local pharmacy?

WESTERN BLOT

Western blot is often used in research to separate and identify proteins. In this technique a mixture of proteins is separated based on molecular weight, and thus by type, through gel electrophoresis. These results are then transferred to a membrane producing a band for each protein. The membrane is then incubated with labels antibodies specific to the protein of interest.

The unbound antibody is washed off leaving only the bound antibody to the protein of interest. The bound antibodies are then detected by developing the film. As the antibodies only bind to the protein of interest, only one band should be visible. The thickness of the band corresponds to the amount of protein present; thus doing a standard can indicate the amount of protein present. The paper will first describe the protocol for western blot, accompanied by pictures to help the reader and theory to rationalize the protocol. This will be followed by the theoretical explanation of the procedure, and in the later section, troubleshooting tips for common problems.

Theory

Sample preparation

Cell lysates are the most common form of sample used for western blot. Protein extraction attempts to collect all the proteins in the cell cytosol. This should be done in a cold temperature with protease inhibitors to prevent denaturing of the proteins. Since tissue sample display a higher degree of structure, mechanical invention, such as homogenization, or sonication is needed to extract the proteins.

After extracting the protein, it is very important to have a good idea of the extract's concentration. This eventually allows the researcher to ensure that the samples are being compared on an equivalent basis. Protein concentration is often measured using a spectrophotometer. Using this concentration allows to measure the mass of the protein that is being loaded into each well by the relationship between concentration, mass, and volume.

After determining the appropriate volume of the sample, it is diluted into a loading buffer, which contains glycerol so that the samples sink easily into the wells of the gel. A tracking dye (bromophenol blue) is also present in the buffer allowing the researcher to see how far the separation has progressed. The sample is heated after being diluted into a loading buffer, in order to denature the higher order structure, while retaining sulfide bridges. Denaturing the high structure ensures that the negative charge of amino acids is not neutralized, enabling the protein to move in an electric field (applied during electrotransfer).

It is also very important to have positive and negative controls for the sample. For a positive control a known source of target protein, such as purified protein or a control lysate is used. This helps to confirm the identity of the protein, and the activity of the antibody. A negative control is a null cell line, such as β-actin, is used as well to confirm that the staining is not nonspecific.

Gel electrophoresis

Western blot uses two different types of agarose gel: stacking and separating gel. The higher, stacking gel is slightly acidic (pH 6.8) and has a lower acrylamide concentration making a porous gel, which separates protein poorly but allows them to form thin, sharply defined bands. The lower gel, called the separating, or resolving gel, is basic (pH 8.8), and has a higher polyacrylamide content, making the gel's pores narrower. Protein is thus separated by their size more so in this gel, as the smaller proteins to travel more easily, and hence rapidly, than larger proteins.

The proteins when loaded on the gel have a negative charge, as they have been denatured by heating, and will travel toward the positive electrode when a voltage is applied. Gels are usually made by pouring them between two glass or plastic plates, using the solution described in the protocol section. The samples and a marker are loaded into the wells, and the empty wells are loaded with sample buffer. The gel is then connected to the power supply and allowed to run. The voltage is very important, as a high voltage can overheat and distort the bands.

Blotting

After separating the protein mixture, it is transferred to a membrane. The transfer is done using an electric field oriented perpendicular to the surface of the gel, causing proteins to move out of the gel and onto the membrane. The membrane is placed between the gel surface and the positive electrode in a sandwich. The sandwich includes a fiber pad (sponge) at each end, and filter papers to protect the gel and blotting membrane [Figure 12]. Here two things are very important: (1) the close contact of gel and membrane to ensure a clear image and (2) the placement of the membrane between the gel and the positive electrode. The membrane must be placed as such, so that the negatively charged proteins can migrate from the gel to the membrane. This type of transfer is called electrophoretic transfer, and can be done in semi-dry or wet conditions. Wet conditions are usually more reliable as it is less likely to dry out the gel, and is preferred for larger proteins.

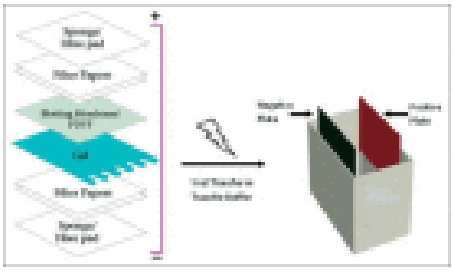


Figure 12
Assembly of a sandwich in western Blot

The membrane, the solid support, is an essential part of this process. There are two types of membrane: nitrocellulose and PVDF. Nitrocellulose is used for its high affinity for protein and its retention abilities. However, it is brittle, and does not allow the membrane to be used for reprobing. In this regard, PVDF membranes provide better mechanical support and allow the blot to be reprobed and stored. However, the background is higher in the PVDF membranes and therefore, washing carefully is very important.

Washing, blocking and antibody incubation

Blocking is a very important step of western blotting, as it prevents antibodies from binding to the membrane nonspecifically. Blocking is often made with 5% BSA or nonfat dried milk diluted in TBST to reduce the background.

Nonfat dried milk is often preferred as it is inexpensive and widely available. However, milk proteins are not compatible with all detection labels, so care must be taken to choose the appropriate blocking solution. For example, BSA blocking solutions are preferred with biotin and AP antibody labels, and antiphosphoprotein antibodies, since milk contains casein, which is itself a phosphoprotein and biotin, thus interfering with the assay results. It is often a good strategy to incubate the primary antibody with BSA since it is usually needed in higher amounts than the secondary antibody. Putting it in BSA solution allows the antibody to be reused, if the blot does not give good result.

The concentration of the antibody depends on the instruction by the manufacturer. The antibody can be diluted in a wash buffer, such as PBS or TBST. Washing is very important as it minimized background and removes unbound antibody. However, the membrane should not be left to wash for a really long time, as it can also reduce the signal.

The membrane is then detected using the label antibody, usually with an enzyme such as horseradish peroxidase (HRP), which is detected by the signal it produces corresponding to the position of the target protein. This signal is captured on a film which is usually developed in a dark room.

Quantification

It is very important to be aware that the data produced with a western blot is typically considered to be semi-quantitative. This is because it provides a relative comparison of protein levels, but not an absolute measure of quantity. There are two reasons for this; first, there are variations in loading and transfer rates between the samples in separate lanes which are different on separate blots. These differences will need to be standardized before a more precise comparison can be made. Second, the signal generated by detection is not linear across the concentration range of samples. Thus, since the signal produced is not linear, it should not be used to model the concentration

Troubleshooting

Even though the procedure for western blot is simple, many problems can arise, leading to unexpected results. The problem can be grouped into five categories: (1) unusual or unexpected bands, (2) no bands, (3) faint bands or weak signal, (4) high background on the blot, and (5) patchy or uneven spots on the blot.

Unusual or unexpected bands can be due to protease degradation, which produces bands at unexpected positions. In this case it is advisable to use a fresh sample which had been kept on ice or alter the antibody. If the protein seems to be in too high of a position, then reheating the sample can help to break the quaternary protein structure. Similarly, blurry bands are often caused by high voltage or air bubbles present during transfer. In this case, it should be ensured that the gel is run at a lower voltage, and that the transfer sandwich is prepared properly. In addition, changing the running buffer can also help the problem. Nonflat bands can be the result of too fast of a travel through the gel, due to low resistance. To fix this the gel should be optimized to fit the sample. Finally, white (negative) bands on the film are due to too much protein or antibody.

Another problem: no bands can also arise due to many reasons related to antibody, antigen, or buffer used. If an improper antibody is used, either primary or secondary, the band will not show. In addition, the concentration of the antibody should be appropriate as well; if the concentration is too low, the signal may not be visible. It is important to remember that some antibodies are not to be

used for western blot. Another reason for no visible bands is the lowest concentration or absence of the antigen. In this case, antigen from another source can be used to confirm whether the problem lies with the sample or with other elements, such as the antibody. Moreover, prolonged washing can also decrease the signal. Buffers can also contribute to the problem. It should be ensured that buffers like the transfer buffer, TBST, running buffer and ECL are all new and noncontaminated. If the buffers are contaminated with sodium azide, it can inactivate HRP.

Similarly, weak signals can be caused by low concentration of antibody or antigen. Increasing exposure time can also help to make the band clearer. Another reason could be nonfat dry milk masking the antigen. In this case use BSA or decrease the amount of milk used.

High background is often caused by too high concentration of the antibody, which can bind to PVDF membranes. Another problem could be the buffers, which may be too old. Increasing the washing time can also help to decrease the background. Additionally, too high of an exposure can also lead to this problem. Therefore, it is advisable to check different exposure times to achieve an optimum time.

Patchy and uneven spots on the blot are usually caused by improper transfer. If there are air bubbles trapped between the gel and the membrane, it will appear darker on the film. It is also important to use a shaker for all incubation, so that there is no uneven agitation during the incubation. Once again, washing is of utmost importance as well to wash the background. This problem can also be caused by antibodies binding to the blocking agents; in this case another blocking agent should be tried. Filtering the blocking agent can also help to remove some contaminants. Finally, this problem can also be caused by aggregation of the secondary antibody; in this case, the secondary antibody should be centrifuged and filtered to remove the aggregated.

https://www.youtube.com/watch?v=VgAuZ6dBOfs

Immunoblotting (Western Blotting) Protocol

Purpose

The purpose of this lab is to introduce students to the utility and basic methodology of Western blotting (including fundamentals of polyacrylamide gel electrophoresis and immunology).

During the course of this laboratory assignment the successful student will:

1. Isolate and quantify protein from animal tissue (week 1).
2. Prepare a polyacrylamide gel (week 2).
3. Separate protein by polyacrylamide gel electrophoresis (week 2).
3. Use a monoclonal antibody to characterize the tissue distribution of a protein of interest by Western blot (week 3).

Introduction

Polyacrylamide gel electrophoresis (PAGE)

Western blotting relies on the separation of proteins by gel electrophoresis. Unlike DNA which is normally linear in conformation and intrinsically negative in charge, proteins are folded into a wide range of shapes and may possess a net negative, positive or even neutral charge. Fortunately each of these challenges to uniform separation by gel electrophoresis may be overcome through the addition of sodium dodecyl sulfate (SDS) to a protein mixture. SDS denatures proteins by coating them and inhibiting protein folding (this is especially effective when used in combination with a reducing agent such as mercaptoethanol or DDT to break apart disulfide linkages). SDS also confers a uniform negative charge on the protein, so that the when placed in the presence of an electrical field (see Figure 1) the proteins will migrate toward the positive based on size alone. The smaller proteins will migrate through the gel faster than the larger proteins.

Polyacrylamide gels are made using acrylamide and N,N-methylenebis-acrylamide (Bis). The polymerization of these molecules is catalyzed by the addition of ammonium persulfate and TEMED. Higher acrylamide concentrations will result in smaller pore size within the gel. Gels with smaller pore size will resolve smaller proteins (see the table below for a guide in selecting acrylamide percentage).

[1] Burnette, W.N. (1981). Western blotting: electrophoretic transfer of proteins from sodium dodecyl sulfate — polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Analytical Biochemistry **112**:195–203.

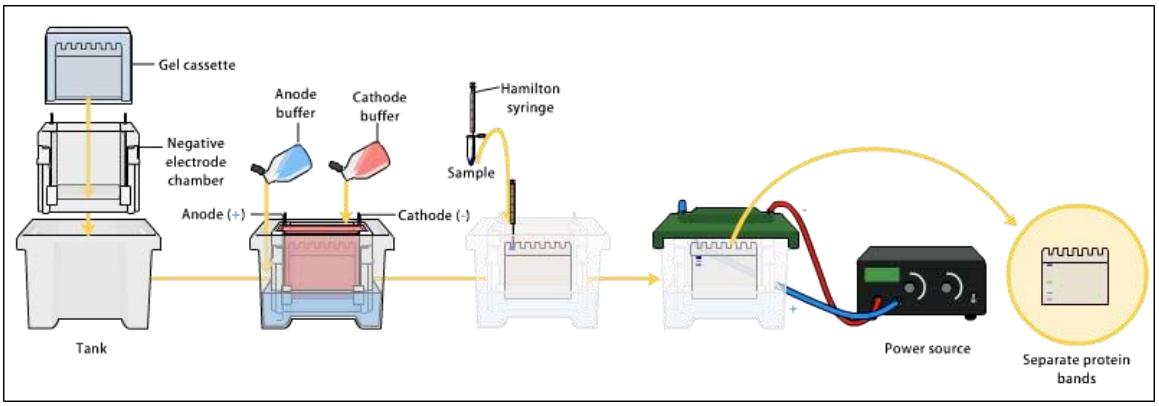


Figure 1. Proteins coated with SDS may be separated by size using electrophoresis through a polyacrylamide gel.

Electroblotting

Individual proteins may be identified using an antibody that binds specifically to the protein of interest. To make the proteins seperated by PAGE accessible to antibody detection, they must first be moved from the gel onto a thin membrane made of nitrocellulose. Electric current may again be used to move or "electroblot" the seperated protein onto the nitrocellulose membrane (see Figure 2).

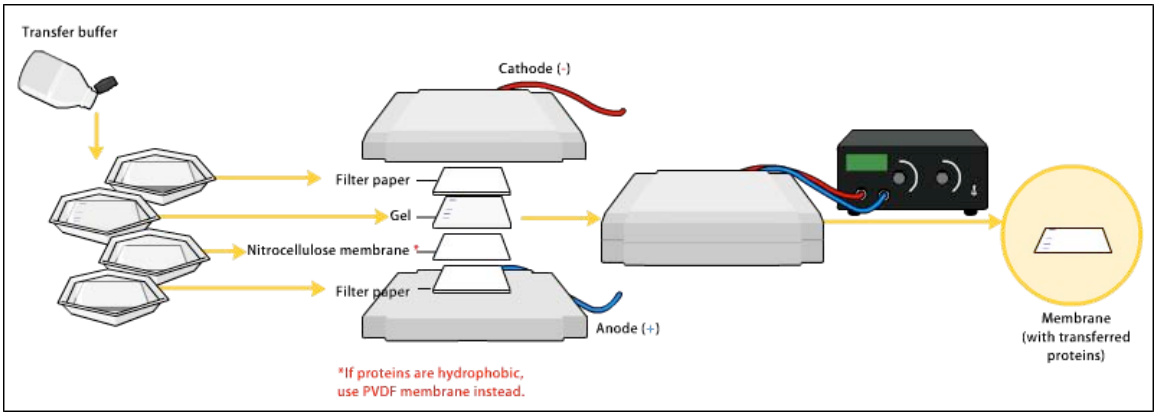


Figure 2. Proteins seperated according to size using electrophoresis may be transferred to a nitrocellulose membrane to facilitate antibody detection.

Immunodetection

Once the proteins have been blotted onto a membrane the membrane may be cut into strips for parellel experiments. One strip may be stained using a universal protein stain such as coomassie blue or India ink, while the other strip may be probed using a specific antibody.

The antigen-antibody complexes on the membrane cannot be visualized without any treatment since they are not colored. Enzymes that catalyze the reaction of soluble, colorless substances into insoluble colored products at the site of the antigen (protein of interest) can be used to allow visualization of these complexes.

In this lab, you will be using a primary antibody which has the enzyme horseradish peroxidase (HRP) bound to it. The enzyme HRP oxidizes the substrate 4-Chloro-1-naphthol in the presence of hydrogen

peroxide (oxidizing agent) and converts it to a purple-colored precipitate on the membrane making it readily visible to the eye (colorimetric detection).

In this lab, we are using a primary antibody with the HRP enzyme bound to it to save time (omitting the secondary antibody step). Usually, the enzyme is bound to the secondary antibody. We will first have to bind the secondary antibody to the primary antibody and then add 4-Chloro-1-naphthol in the presence of hydrogen peroxide (oxidizing agent) to detect the bands

PRE-LAB QUESTINNAIRE

For Week 1

1. Why do we lyse the cells?
2. What is a Bradford assay used for?
3. What is usually used as a standard for protein quantification?
4. Why do we have to denature the protein?
5. What is the reason for using SDS, BME and bromophenol blue in the loading dye?
6. What is the reason for using BME the loading dye?
7. What is the reason for using bromophenol blue in the loading dye?
8. What is the reason for using glycerol in the loading dye?

For Week 2

1. When making gels, do you face the short or large plate outside?
2. When making gels, why is TEMED added last?
3. When making gels, do you put the stacking or resolving/separating gel solution first?
4. Why is a MW ladder used?
5. Which protein will appear lower down the gel and why?
6. What is the order of the transfer components (the two sponges, the membrane, the two filter papers, the gel)
7. What is the charge of the protein? Will it flow to the positive or negative end? Should the gel or membrane be nearer to the positive end?

For Week 3

1. Why is blocking performed?
2. What is the blocking solution usually made from?
3. What is Ponceau Red used for?
4. Draw the interaction of the target protein, primary, secondary, enzyme and enzyme substrate for the detection of a specific protein in immunoblotting. In words, describe the process.

REMEMBER TO KEEP ALL YOUR SAMPLES ON ICE AT ALL TIMES

WEEK 1: SAMPLE PREPARATION

Lysing the Cells

Materials

- Lysis buffer

Lysis buffer recipes:

NP-40 buffer
150 mM sodium chloride
1.0% NP-40 (Triton X-100 can be substituted for NP-40)
50 mM Tris pH 8.0
This is a popular buffer for studying proteins that are cytoplasmic, or membrane-bound, or for whole cell extracts. If there is concern that the protein of interest is not being completely extracted from incoluble material or aggregates, RIPA buffer may be more suitable, as it contains ionic detergents that may more readily bring the proteins into solution.

- Eppendorf tubes
- Refrigerated microcentrifuge
- Fast spin microcentrifuge

Method

1. Add 100 µL lysis buffer to the cell pellet
2. Break pellet by pipetting up and down
3. Place on rotator in refrigerator for 30 min
4. Label tubes while waiting for samples to be lysed with your name – 1 for storage (Labelled "Storage"), 2 for denaturing (Labelled "Denatured")
5. Spin down for 10 min, 10,000 rcf, 4°C
6. Collect the supernatant (liquid on top of the pellet) in the "Storage" tube and discard the pellet

Bradford Protein Quantification Assay

Materials

- BSA
- Bradford Protein Quantification Dye

Method

1. Prepare 1x Bradford Protein Buffer
2. Make the standard solutions of BSA with the calculation
3. Pipet 10 µL of each standard and sample solution into a separate well, add 10 µL of lysis buffer for the blank/control
4. Add 200 µL of dye reagent to each well
5. Swirl the 96 well plate carefully to mix the solution

6. Leave at room temperature for at least 5 minutes
7. Read the 96-well plate in the microplate reader using the Gen5 software at 595 nm

Denaturing Samples

Denaturing is performed to unfold the protein to allow access and detection of the protein by antibodies.
Antibodies usually recognize a small portion of the protein of interest (epitope) which may be located within the 3D conformation of the protein.

Materials

- Protein Loading Dye (including BME)
- Lysis buffer
- Hot plate

Method

1. Calculate the amount of protein that is in 30 µL of the least concentrated sample:
Amount of protein in the least concentrated sample = 30 µL x concentration of sample

2. Based on that amount, calculate the volume of lysate from the other samples that will equal to the amount of protein in the least concentrated sample:
Volume of sample = Amount of protein in the least concentrated sample/Concentration of sample

3. Calculate the volume of lysis buffer needed to result in a total volume of 30 µL:
Volume of lysis buffer = 30 µL – volume of sample

4. Prepare 2 of these samples in the tubes labelled “Denatured”
5. Add 30 µL loading buffer to each tube (make sure the loading dye has 10% v/v BME)
6. Vortex the tube to mix the sample
7. Fast spin down to collect solution
8. Boil for 8 min in a 100°C hotplate
9. Allow to cool to room temperature on a rack
1) Fast spin down to collect solution
2) Place samples in the freezer for next week

WEEK 2: SDS-PAGE GEL ELECTROPHORESIS

VERY IMPORTANT TO WEAR GLOVES WHEN WORKING WITH THE GEL. ACRYLAMIDE, POTENT CUMULATIVE NEUROTOXIN

Gel Preparation
Loading dye components
BME or DTT is used to reduce disulfide bridges

Glycerol is used to increase the density of the sample to make sure it remains in the bottom of the well

Bromophenol blue is used to enable visualization of the lysate and migration. When the dye molecule reaches the bottom of the gel, the run can be stopped

The anionic detergent **sodium dodecyl sulfate (SDS)** in the loading buffer denatures the protein as well as make them negative charge

Materials
- ddH2O
- 1.0 M Tris-HCl, pH 8.0
- 1.0 M Tris-HCl, pH 6.8
- 30% acrylamide/bis (stored at 4C)
- 10% (w/v) SDS
- 10% ammonium persulfate (w/v) (APS) (stored at -20C)
- Tetramethylethylenediamine (TEMED)
- Gel preparation aparatus

Prepare the following separating and stacking gel solutions in 15 mL tubes

10% separating/resolving gel (10 mL)
- 2.71 mL ddH2O
- 3.75 mL 1.0 M Tris-HCl, pH 8.0
- 3.33 mL 30% acrylamide/bis
- 100 µL 10% (w/v) SDS
- 100 µL 10% (w/v) APS
- 10 µL TEMED (add last, when you are ready to pour the solution)

4% stacking gel (5 mL)
- 3.6 mL ddH2O
- 625 µL 1.0 M Tris-HCl, pH 6.8
- 670 µL 30% acrylamide/bis
- 50 µL 10% (w/v) SDS
- 50 µL 10% (w/v) APS
- 5 µL TEMED (add last, when you are ready to pour the solution)

Method
1. Place plates into gel holder
2. Transfer separating gel between plates with a pipette
3. Use transfer pipette to add some H_2O on top to help even out the gel

4. Wait 15-20 min for gel to set (look at remaining gel in tube)
5. Decant H_2O by lifting/tilting the apparatus to pour the H_2O out
6. Transfer stacking gel on top with a pipette
7. Insert comb at an angel (to ensure no bubbles)
8. Wait 15-20 min for gel to set (look at remaining gel in tube)

Running the Gel

Materials

- Crystalgen MW ladder
- WB blot systems
- Denatured samples
- Running buffer

Running buffer: Tris/Glycine/SDS
25 mM Tris
190 glycine

1.1% SDS
Check the pH and adjust to pH 8.3 if necessary.

Methods

1. Put gel into holder and add running buffer until the lower portion of the gel is submerge in buffer
2. Add running buffer between the gel plates
3. Wash wells by pipeting up and down with a 200 µL pipette (set at ~150 µL)
4. Connect the gel box to the power source and run machine to check that everything is set correctly
5. Load 3 µL of the MW ladder into the gel
6. Load 20 µL of your denatured sample
7. Run gel for 30 min at 200 V

Proteins are separated by **Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE)** are based on **molecular weight** as the protein travels in the pores of the gels

Lower molecular weight proteins travel **faste**r and will appear towards the **bottom** of the gel

A **molecular weight marker** allows determination of protein size and progress of the run

The proteins can travel through the gel in an **electric field** due to having a **charge** (negative charge due to the **SDS** bound on them)

Transferring the Gel

Materials

- Polyvinylidene difluoride (PVDF) Transfer membrane
- Western blotting filter paper
- Transfer buffer

25 mM Tris

190 mM glycine

20% methanol

Check the pH and adjust to pH 8.3 if necessary

Transferring

1. Pour chilled transfer buffer into a plastic bucket
2. Soak membrane in methanol
3. Prepare transferring in the following order: Sponge, Filter Paper, Membrane, Gel, Filter Paper, Sponge
4. Put in machine: Membrane by the positive end and Gel by the negative end
5. Pour transfer buffer into machine
6. Transfer for 1 h at 100 V (You can leave once you start the transfer)

The proteins can be **transferred** from a gel to a membrane in an electric field due to being charged

The **negatively-charged proteins** travel towards the **positively-charged electrode** but the membrane stops them which cause them to remain on the membrane

WEEK 3: DETECTION OF PROTEIN BANDS

Materials

- Hydrogen peroxide
- B-actin HRP
- 4-chloro-1-naphthol (4CN)
- Nonfat Milk for Blocking
- TBST for washing

Ponceau Stain, Blocking, Primary and Secondary Antibodies

1. Immerse the membrane into Ponceau Stain and leave for 5 minutes on a shaker
2. Discard Ponceau into container and wash with 1X TBST(1st and 2nd time just rinse and pour out, 3rd time allow to shake for 5 min at room temperature) with TBST
3. Observe if the protein is on the membrane (red lines in wells next to markers)
4. Soak in ~30 mL blocking solution (5% milk, 2.5 g milk in 50 mL TBST) for 15 min, shaking at room temperature
5. Discard blocking solution and add actin primary (10 µL of primary antibody in 20 mL of blocking solution)
6. Leave for 1 hr shaking at room temperature
7. Discard primary antibody and wash with 1xTBST (1st and 2nd time just rinse and pour out, 3rd time allow to shake for 15 min at room temperature, 4th time just rinse with TBS (without Tween) and pour out) – WHILE WAITING FOR THE 15 MIN, PREPARE THE DETECTION SOLUTION BELOW

Detection

1. Prepare Solution A: Dissolve 30 mg of 4CN into 10 mL methanol and protect from light (cover with Aluminum foil)
2. Prepare Solution B: Mix 30 µL of ice cold 30% hydrogen peroxide to 50 mL of TBS (without tween)
3. Put solution (A) and (B) **immediately** after adding hydrogen peroxide in the membrane box
4. Wait and observe the purple bands (5-30 minutes)

Lipid Extraction Kit Catalog Number MAK174

Product Description Lipids are structural components of cell membranes that play a critical role in gene transcription, signaling, and metabolism. Several lipid species exist in biological systems, including phospholipids, triglycerides, and fatty acids. The Lipids Extraction kit enables the extraction of these lipids in one step. The extracted lipids can then be transesterified and quantified using gas chromatography (GC) with flame-ionization detection (FID).

Lipids are typically extracted using a dual solvent partition system containing a lipophilic solvent and an aqueous solvent. The Folch method has been conventionally used to extract lipids from biological samples, using chloroform, methanol, and water to separate lipids from aqueous-soluble compounds.1 In this procedure, lipids are retained in the lower chloroform layer; whereas, aqueous-soluble compounds are retained in the upper methanol-water layer. The sample is then centrifuged to achieve uniform separation and the bottom chloroform layer is transferred with a pipette to another test tube. An aliquot of the transferred layer is then transesterified with 14% boron trifluoride in methanol or 1% sulphuric acid in methanol. This transesterification reaction results in fatty acid methyl esters: R-COOH ® R-COCH3

The methyl esters can be separated from the transesterification medium with water, and heptane or hexane, and injected directly into a GC-FID system for quantitation.

The Lipid Extraction Kit shortens the extraction process by eliminating the need to prepare solvents and standards, centrifugation, and pipetting. Once the sample is homogenized and dissolved in the Extraction Solvent containing the internal standard, it is inverted twice and poured into the syringe containing a filter, which preferentially elutes the chloroform layer containing total lipids. The user then has to squeeze the plunger to ensure the lipids are eluted

Reagents and Equipment Required but Not Provided.
· Homogenizer to homogenize solid samples · Capped PyrexÒ glass tubes to collect the total lipid extract
· Gas chromatography system (GC), preferably with a flame-ionization detector (FID)
· Polar gas chromatography column
· Sulfuric acid (Catalog Number 258105 or equivalent) in methanol (Catalog Number 414719 or equivalent)
OR
Boron trifluoride-methanol solution (Catalog Number B1252 or equivalent) · Hexane (Catalog Number 414700 or equivalent)

Procedure

Sample Preparation
1. Weigh the sample. Add 3 mL of Extraction Solvent to each sample. Lipids can be extracted from

up to
0.15 g of sample containing <10% lipids, 0.01 g of adipose tissue, or <5 mg of oil.
2. Homogenize if the sample is a solid and vortex. Note: The Extraction Solvent can also be added after homogenizing the sample. 3. Add 0.5 mL of Aqueous Buffer. Invert twice or vortex.
4. Place the syringe containing the filter on top of a collecting tube that can hold at least 2 mL of liquid.
5. Pour the solution into the syringe, attach plunger, and push the plunger to elute lipids into the collecting tube. The eluted solvent contains the total lipid extract.
Note: Avoid excessive plunging. Although the filter selectively traps water, excessive plunging may inadvertently force water though the filter.
6. The total lipid extract may now be transesterified and analyzed by GC-FID.

Transesterification
1. Aliquot 100 mL of the total lipid extract from Sample Preparation, step 5 and dry under nitrogen for transesterification.
2. Two suggested reactions for transesterification:
a. After drying, add 1 mL of 1% H_2SO4 in methanol and 0.5 mL of hexane, cap, and heat at 70 °C for 3 hours. Add 1 mL of hexane and 1 mL of 5% NaCl.

OR
b. Add 1 mL of Boron trifluoride-methanol solution (Catalog Number B1252) and 0.3 mL of hexane, cap, and heat at 95 °C for 1 hour. Add 1 mL of hexane and 1 mL of distilled water.

3. Vortex and centrifuge at 500 ´ g for 5 minutes.

4. Transfer the top hexane layer and dry under nitrogen. Reconstitute the transesterified lipids with 65–100 mL of hexane and add to a GC vial. Inject into a GC-FID system with appropriate column. GC/MS can also be used for quantitation, following determination of the response factor for each fatty acid.

RNA purification using the RNeasy Lipid Tissue Mini Kit

Notes before starting
Add 4 volumes of ethanol (96–100%) to Buffer RPE for a working solution.

1. Disrupt and homogenize ≤100 mg fatty tissue (≤50 mg other tissue) in 1 ml QIAzol Lysis Reagent using the TissueRuptor®, TissueLyser LT, or TissueLyser II (see Table 1).
2. Incubate the homogenate at room temperature (15–25°C) for 5 min.
3. Add 200 µl chloroform, and shake vigorously for 15 s.
4. Incubate sample at room temperature for 2–3 min.
5. Centrifuge at 12,000 x g for 15 min at 4°C.
6. Transfer upper, aqueous phase to a new tube. Be careful to avoid the interphase. Add 1 volume of 70% ethanol, and vortex. Do not centrifuge. Proceed at once to step 7.
7. Transfer up to 700 µl of the sample to RNeasy Mini spin column in 2 ml collection tube (supplied). Close the lid, centrifuge at room temperature for 15 s at ≥8000 x g, and discard flow-through.
8. Using the same collection tube, repeat step 7 using the remainder of the sample. Discard the flow-through. Optional DNase digest: Follow steps in "Optional on-column DNase digestion with the RNase-Free DNase Set" in Appendix C of the RNeasy Lipid Tissue Handbook.
9. Add 700 µl Buffer RW1 to RNeasy column. Close lid, centrifuge for 15 s at ≥8000 x g, and discard flow-through. (Skip this step if performing optional DNase digestion.)
10. Add 500 µl Buffer RPE to RNeasy column. Close lid, centrifuge for 15 s at ≥8000 x g, and discard flow-through.
11. Add 500 µl Buffer RPE to RNeasy column. Close lid and centrifuge for 2 min at ≥8000 x g. Optional: To further dry membrane, place RNeasy column in new 2 ml tube, close lid, and centrifuge at full speed for 1 min.)
12. Place RNeasy column in a new 1.5 ml tube. Add 30–50 µl RNase-free water, close lid, and centrifuge for 1 min at ≥8000 x g

Table 1. Recommended methods for sample homogenization Sample Amount Dish Disruption and homogenization

Simple	Amount	Dish	Disruption and homogenization
Animal cells	≤1 x 107	< 6cm	Add Buffer RLT, vortex or use QIAshredder, TissueRuptor, or needle and syringe
	≤1 x 105	6–10 cm	
Animal tissues	<20 mg		TissueLyser LT; TissueLyser II; TissueRuptor, or mortar and pestle followed by QIAshredder or needle and syringe
	≤30 mg		