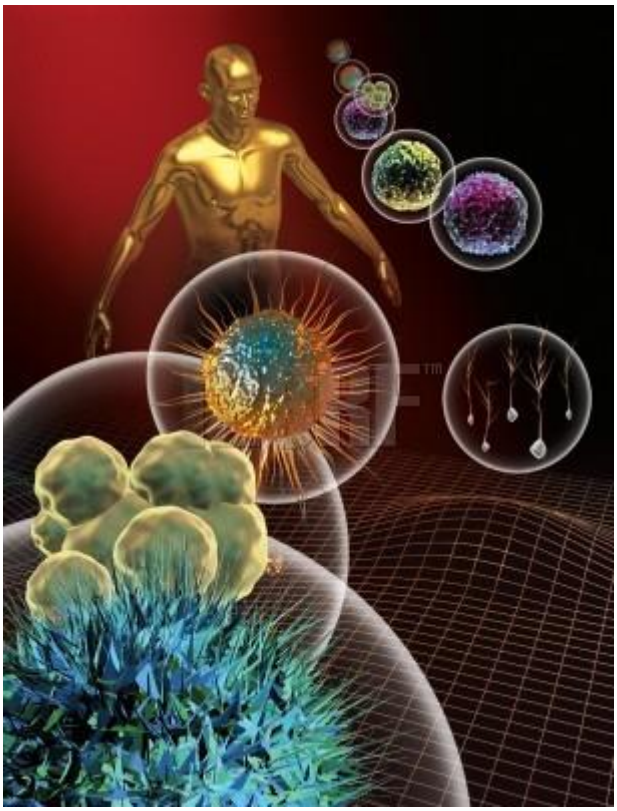
MICR 4154

LABORATORY OF IMMUNOLOGY



Dr. Rosa Maldonado

Laboratory Syllabus – MICR4154 Fall 2018

Instructor of Record: Dr. Rosa Maldonado, ramaldonado@utep.edu
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Lab attendance is required. Absence from the lab will be excused **ONLY** if notice is given to the instructor 24 hours in advance. Otherwise, you will receive 1 point off your grade for each unexcused lab absence. Make every effort to arrive on time.

		Points
LABORATORY GRADING	Lab reports (6):	20
	Notebook:	20
	Quizzes:	20
	Exams 1 and 2:	40
	Total:	**100**

LABORATORY REPORT

The laboratory reports will be prepared in groups. It should contain title, objectives, methodology, results, discussion, and references.

Basics

- It should be double spaced
- 1 inch margins
- Arial or Tahoma or Verdana fonts
- 12 point font (no less)
- Each Subsection should be clearly delineated (clearly highlight the Research Objective, Hypothesis, and Significance)
- You must have at least (a minimum of) two journalistic sources (not websites)
- **Websites CAN NOT be used as sources (unless it is data relating to health statistics)**

LABORATORY NOTEBOOK

Each student should maintain a detailed lab notebook, which will count 20 points towards the laboratory grade. This lab book is intended to serve as a detailed diary of the protocols, discussions, experiments, and results pertaining to each of the lab exercises performed during the semester. Legibility, neatness, and organization are important. Handouts do not need to be recopied into the lab book but may be cut and pasted or taped into the appropriate section. Work with lab partners to put together findings and other materials ***but write up each exercise in your own words*****. Plagiarism will**

not be tolerated. Do not loan out your finished product for others to copy. Lab books will be checked at randomly. Completed lab books are due in class on the last day.

Read this section carefully. *For each lab exercise your notebook should contain the following:*

I. Title

Brief and descriptive. Example: Lab Exercise # 5 - Handling of Animals, Immunizations

II. Introduction

It describes the purpose of the lab exercise/experiment and includes background information presented by the instructor or included in the handout. It should have a minimum of 2 paragraphs in length.

III. Materials and Methods

It should describe the supplies, reagents and equipment used and how the experiment or technique was performed. It should be sufficiently detailed to permit another person to reproduce the work.

IV. Results

A written account of observations, findings, and raw data. The Results section should also include data in the form of drawings, graphs or tables, when appropriate. Drawings, tables, and graphs should be numbered and have their own brief titles.

V. Discussion

A summary of what was learned from both methodological and principle standpoints. Also, you should describe problems encountered and possible solutions.

QUIZZES

Before each lab there will be a quiz about the exercise/experiment to be carried out.

LAB # 1- Safety Orientation

Biosafety: prevention of large-scale loss of biological integrity, focusing both on ecology and human health.

Biosafety is related to several fields:

- in ecology (referring to imported life forms from beyond ecoregion borders),
- in agriculture (reducing the risk of alien viral or transgenic genes, or prions such as BSE/"MadCow", reducing the risk of food bacterial contamination)
- in medicine (referring to organs or tissues from biological origin, or genetic therapy products, virus; levels of lab containment protocols measured as 1, 2, 3, 4 in rising order of danger),
- in chemistry (i.e., nitrates in water, PCB levels affecting fertility) and
- in exobiology (i.e., NASA's policy for containing alien microbes that may exist on space samples - sometimes called "biosafety level 5").

When biological warfare or new, currently hypothetical, threats (i.e., robots, new artificial bacteria) are considered, biosafety precautions are generally not sufficient. The new field of biosecurity addresses these complex threats.
Biosafety level refers to the stringency of biocontainment precautions deemed necessary by the Centers for Disease Control and Prevention (CDC) for laboratory work with infectious materials.

Principles of Biosafety

Lab Practices and Techniques

- Personnel
    - Aware of potential hazards
    - Training in practices/techniques
- Biosafety manual specific to lab

Safety Equipment

- Personal protective clothing
    - Gloves
    - Gowns (Lab coat)
- Pipetting Devices
- Safety centrifuge cups and rotors
-

Students will watch the safety videos, and sign the form

LAB # 2- Pipetting

HOW TO PIPETTE

- **choose the appropriate device**
- **dial the appropriate volume**
- **put a tip on the pipettor from a sterile tip box, banging the tip securely onto the device, do not touch the tip or allow it to touch anything, do not put the pipettor down on the bench**
- **depress the plunger to the first stop**
- **insert the tip into the fluid in the bottle to the depth that you expect the tip to fill, do not touch the side of the bottle with the device, the plastic tip should touch the fluid only (do not do step 5 before step 4!)**
- **release the plunger slowly to fill the tip**
- **remove the device from the bottle and place the tip onto the inside of the receptacle tube, touching the bottom or the side of the tube.**
- **while carefully watching, slowly depress the plunger**
- **depress the plunger all the way, using the expulsion volume to clear the tip**
- **always visually assess the transfer of the fluid, don't just poke and hope**

*table courtesy of http://71a32.lehman.cuny.edu/molbio_course/Basic_techniques.htm

Ranges of micropipettes (in microliters):

P1000: 200-1000

P200: 20-200

P20: 2-20

P2: 0.02-2

MICROPIPETTING EXERCISE

Today we will be ensuring that we are pipetting accurately. Your pipetting skills need to be perfected in order to generate accurate results. Pipetting will be a skill that we will be using in every single exercise. Your group depends on you to pull your weight in this area. Each person in the group must achieve proficiency.

Instructions: Place a weigh boat on the analytical balance. Close all the chamber doors of the analytical balance. Press the tare button (zero). Using the appropriate micropipette, transfer the following amounts of water from the beaker to the weigh boat. Follow the directions as they appear on the top of this form.

*because of the density of water (1g/mL), the mass reading is equivalent to the volume dispensed. You need to obtain 3 consistent readings (precision). Try to be as accurate as possible (within 1% error). You do not need to continue the trials to the sixth trial if you were able to achieve 3 replicate readings.

Using the p1000:

Micropipettor	**Set Value (uL)**	**Actual wt. 1**	**Actual wt. 2**	**Actual wt. 3**	**Actual wt. 4**	**Actual wt. 5**	**Actual wt. 6**
p1000	900						
p1000	450						
p200	100						
p200	50						
p20	10						
p20	5						
p2	1						
p2	0.5						

Micropippetors

These are delicate (and expensive) instruments that require care and maintenance. They should be checked for accuracy frequently. Common manufacturers are Eppendorf and Gilson (Pipetman). Only the use of Pipetman is described here; other brands are similar. Pipetman come in different sizes with different volume ranges and are used with disposable plastic tips that come in various sizes to match. Accuracy of measurement depends on selection of the appropriate pipettor for your desired measurement. Three such sizes are commonly in use are:

NAME	RANGE	DISPOSABLE TIP COLOR
P20	1-20 µL	yellow
P200	20-200 µL	yellow
P1000	200-1000 µL	blue

On a **P20**, the following settings indicate the volumes shown:

1 µL	10 µL	15 µL	20.5µL
0 1 0	1 0 0	1 5 0	2 0 5

On a **P200**, the following settings indicate the volumes shown:

20 µL	45 µL	120 µL	200 µL
0 2 0	0 4 5	1 2 0	2 0 0

On a **P1000**, the following settings indicate the volumes shown:

220 µL	0.5 ml	720 µL	1 ml
0 2 2	0 5 0	0 7 2	1 0 0

Accuracy and precision of measurement also depend on pipetting skill. Repeatability of experiments is heavily dependent on precision (repeatability of measurements) and success is dependent on accuracy. Both accuracy and precision in micropipetting require much practice. Please practice with patience: it is necessary for success in this molecular laboratory and many other types of labs.

LAB # 3- Haematocytometer & cell counter

Materials:

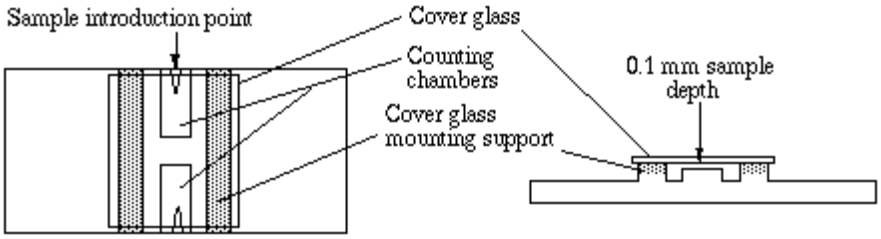
1. Mice blood collected with an anticoagulant
2. Neubauer Hemocytometers
3. Microscopes
4. Kim Wipes

Background

Using a Counting Chamber

For microbiology, cell culture, and many applications that require use of suspensions of cells it is necessary to determine cell concentration. One can often determine cell density of a suspension spectrophotometrically, however that form of determination does not allow an assessment of cell viability, nor can one distinguish cell types.

A device used for determining the number of cells per unit volume of a suspension is called a counting chamber. The most widely used type of chamber is called a hemocytometer, since it was originally designed for performing blood cell counts.

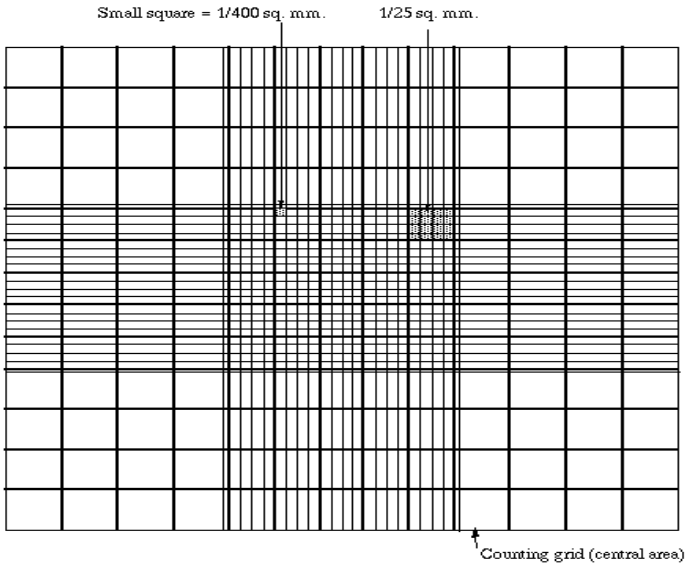


To prepare the counting chamber the mirror-like polished surface is carefully cleaned with lens paper. The coverslip is also cleaned. Coverslips for counting chambers are specially made and are thicker than those for conventional microscopy, since they must be heavy enough to overcome the surface tension of a drop of liquid. The coverslip is placed over the counting surface prior to putting on the cell suspension. The suspension is introduced into one of the V-shaped wells with a pasteur or other type of pipet. The area under the coverslip fills by capillary action. Enough liquid should be introduced so that the mirrored surface is just covered. The charged counting chamber is then placed on the microscope stage and the counting grid is brought into focus at low power.

It is essential to be extremely careful with higher power objectives, since the counting chamber is much thicker than a conventional slide. The chamber or an objective lens may be damaged if the user is not careful. One entire grid on standard hemacytometers with Neubauer rulings can be seen at 40x (4x objective). The main divisions separate the grid into 9 large squares (like a tic-tac-toe grid). Each square has a surface area of one square mm, and the depth of the chamber is 0.1 mm. Thus the entire counting grid lies under a volume of 0.9 mm-cubed.

Cell suspensions should be diluted enough so that the cells do not overlap each other on the grid, and should be

uniformly distributed. To perform the count, determine the magnification needed to recognize the desired cell type. Now systematically count the cells in selected squares so that the total count is 100 cells or so (number of cells needed for a statistically significant count). For large cells this may mean counting the four large corner squares and the middle one. For a dense suspension of small cells you may wish to count the cells in the four 1/25 sq. mm corners plus the middle square in the central square. Always decide on a specific counting pattern to avoid bias. For cells that overlap a ruling, count a cell as "in" if it overlaps the top or right ruling, and "out" if it overlaps the bottom or left ruling.



http://www.ruf.rice.edu/~bioslabs/methods/microscopy/cellcounting.html

The hemocytometer chamber represents a volume of 0.1 mm^3, or 1 $x10^{-4}$ cm^3 . Because 1 cm^3 is equivalent to 1 ml, the cell concentration per ml can be calculated using a chamber volume conversion of 10^{-4} in the equation below

cells/ml = average count per square x dilution factor x chamber conversion factor

e.g. If 200 cell were counted in one chamber from a 1:2 dilution of cells, the number of cells per ml in the original suspension is calculated as:

200 cells counted x 2 x 10^4 = 1x 10^6 cell/ml

The total number in the original sample is then:

cells/ml x total volume of sample (ml)

Which, for a 10 ml suspension would be:

1 x 10^6 cells/ml x 10 ml = 1x 10^7 total cells

Manual WBC counting

The manual procedure, using a Unopette system and a hemocytometer, is no longer used except for white cell counts in body fluids with extremely few cells (< 1000 cells/μL), e.g. cerebrospinal fluid. It involves diluting blood with a Unopette system that contains a diluent that lyses the red cells to remove them from view.

A hemocytometer is charged with the diluted blood, and nuclei are counted in the appropriate areas of the grid using a light microscope. The manual WBC is a reasonable test to do in-office since it is not terribly time- and labor-intensive and gives acceptably accurate results. Most veterinary practices have a microscope and the cost of the hemocytometer and the Unopettes is not prohibitive.

(http://www.diaglab.vet.cornell.edu/clinpath/modules/hemogram/wbc.htm)

Experimental Procedure:

Erythrocyte Counting

1. **Charge the Hemocytometer -** If the sample has been allowed to stand, mix the diluted blood thoroughly to resuspend the cells. a) Using PBS dilute the blood 1:1000 and 1:10000; b) With a micropipette take 10 □L of the diluted blood and carefully put it in the hemocytometer, by inclining the micropipette in an angle of aprox. 30 ° just next to the chamber cover slip.
2. **Count and Calculate -** An erythrocyte count is done with a Neubauer hemocytometer as follows: a) Using 40X magnification, count the erythrocytes in the four corner squares and the one center square within the large center square of the chamber.
3. **Limitation of the Procedure -** A highly elevated erythrocyte count may make accurate counting difficult. In this instance, a secondary dilution should be made. When calculating the total count, adjust the formula to allow for the secondary dilution.
4. **Technical Note -** Cells and diluent(s) must be adequately mixed and counting chambers should be properly filled if errors in manual counting procedures are to be avoided.

White Blood Cell Counting

1. Follow the procedure as above for erythrocyte counting, but use the Unopette for white blood cells.
2. **Count and Calculate -** A white blood cell count is done with a Neubauer hemocytometer

LAB # 4- White cell staining

Materials:

1. SureStain Wright (Fisher Diagnostics CS432)
2. Hematoxylin and Eosin Stain
3. Methanol
4. Distilled water
5. Microscope Slides
6. Blood

Background

White Blood Cells (leukocytes)

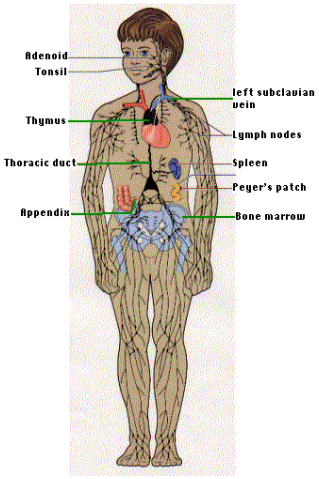
White blood cells

- are much less numerous than red (the ratio between the two is around 1:700),
- have nuclei,
- participate in protecting the body from infection,
- consist of **lymphocytes** and **monocytes** with relatively clear cytoplasm, and three types of **granulocytes**, whose cytoplasm is filled with granules.

Lymphocytes

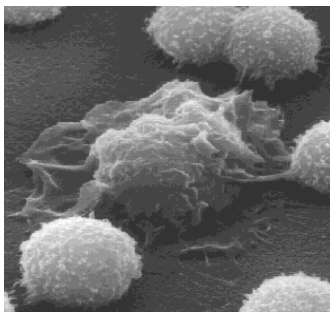
There are several kinds of lymphocytes (although they all look alike under the microscope), each with different functions to perform. The most common types of lymphocytes are

- **B lymphocytes** ("B cells"). These are responsible for making antibodies.
- **T lymphocytes** ("T cells"). There are several subsets of these:
    1. **inflammatory T cells** that recruit macrophages and neutrophils to the site of infection or other tissue damage
    2. **cytotoxic T lymphocytes** (CTLs) that kill virus-infected and, perhaps, tumor cells
    3. **helper T cells** that enhance the production of antibodies by B cells



Although bone marrow is the ultimate source of lymphocytes, the lymphocytes that will become T cells migrate from the bone marrow to the **thymus**, where they mature. Both B cells and T cells also take up residence in lymph nodes, the spleen and other tissues where they encounter antigens, continue to divide by mitosis, AND mature into fully functional cells.

Monocytes



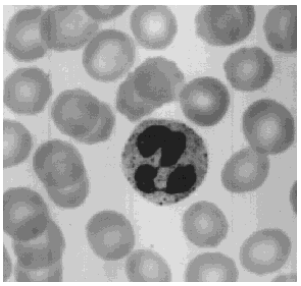
Monocytes leave the blood and become **macrophages**.

This scanning electron micrograph (courtesy of Drs. Jan M. Orenstein and Emma Shelton) shows a single macrophage surrounded by several lymphocytes.

Macrophages are large, phagocytic cells that engulf

- foreign material (antigens) that enter the body
- dead and dying cells of the body.

Neutrophils



The most abundant of the WBCs. This photomicrograph shows a single neutrophil surrounded by red blood cells.

Neutrophils squeeze through the capillary walls and into infected tissue where they kill the invaders (e.g., bacteria) and then engulf the remnants by phagocytosis.

This is a never-ending task, even in healthy people: Our throat, nasal passages, and colon harbor vast numbers of bacteria. Most of these are commensals, and do us no harm. But that is because neutrophils keep them in check.

However, heavy doses of radiation, chemotherapy and many other forms of stress can reduce the numbers of neutrophils so that formerly harmless bacteria begin to proliferate. The resulting **opportunistic infection** can be life-threatening.

Eosinophils

The number of eosinophils in the blood is normally quite low (0–450/µl). However, their numbers increase sharply in certain diseases, especially infections by parasitic worms. Eosinophils are cytotoxic, releasing the contents of their granules on the invader.

Basophils

The number of basophils also increases during infection. Basophils leave the blood and accumulate at the site of infection or other inflammation. There they discharge the contents of their granules, releasing a variety of mediators such as:

- histamine
- serotonin
- prostaglandins and leukotrienes

which increase the blood flow to the area and in other ways add to the inflammatory process. The mediators released by basophils also play an important part in some allergic responses such as

- hay fever and
- an anaphylactic response to insect stings.

Experimental Procedure

Procedure A

1. Prepare a thin blood smear and allow to air dry.
2. Dip the slide in SureStain Wright for 5 to 15 seconds.
3. Dip the slide in distilled water for 10 to 20 seconds.
4. Rinse the slide by dipping in fresh distilled water for a few seconds and air dry.
5. Observe the cells by using oil immersion.
6. Perform a differential cell count by counting 100 cells and keeping track of the number of neutrophils, basophils, eosinophils, monocytes, and lymphocytes.

Procedure B: Hematoxylin and Eosin Stain

1) Prepare smear
2) Fixed the cell with methanol
3) Poured the Hematoxylin directly on the slide, allow 15 minutes to stain
4) Rinse 3 times with water (5 minutes each rinse)
5) Stain for 3 seconds with 1% eosin.
6) Rinse with water
7) Dry
8) Observe in the microscope at 100X with immersion oil
9) Count 100 white cells and identify the different types.
10) Calculate a percentage of white cells that found

LAB # 5 – ELISA

Objectives

- Learn about antigen-antibody interactions
- Understand how HIV is detected in the laboratory
- Learn how disease agents are transmitted, diagnosed, and tracked
- Understand how antibodies are produced in the lab for use in diagnostic tests
- Study enzyme-substrate mechanics

Materials

Antigen (chicken gamma-globulin), 1 vial

Primary antibody (rabbit anti-chicken polyclonal antibody), 1 vial

Secondary antibody (goat anti-rabbit antibody conjugated to horseradish peroxidase, or HRP), 1 vial

HRP enzyme substrate (TMB), 30 ml

10x PBS, 100 ml

10% Tween 20, 5 ml

Disposable plastic transfer pipets, 80

Empty 30 ml bottles and caps, 3 of each

Microplate with 12-well strips (8 rows of 12 wells), 3 plates

Yellow micro test tubes, 2.0 ml, 60

Colored micro test tubes, 2.0 ml, 75

microplate reader

Background

What is an ELISA?

An ELISA is a type of immuno assay. Immuno assays are a group of powerful techniques that have been developed recently. Using the bodies own defence system, scientists can determine the quantity of very small amounts of specific molecules. The tests are quick and yield information that would be hard to obtain using other methods.

ELISA is an **E**nzyme **L**inked **I**mmuno **S**orbant **A**ssay. It is used to determine the level of antibodies in a sample. These tests are useful because they are so specific and are relatively simple to perform.

Using an enzyme link on the antibody, scientists can attach a fluorescent dye to an antibody which means they can accurately measure the quantity of antibody, and therefore antigen, present.

What can it be Used For?

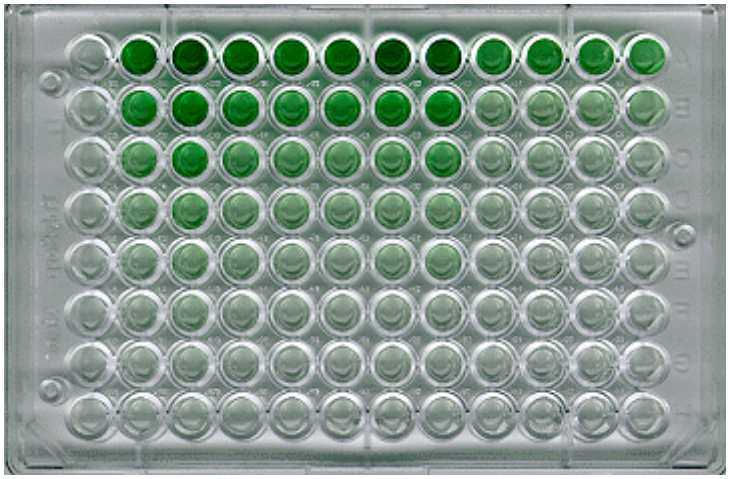
It is used to identify the type of antigen and the quantity that is present in a sample.

Antibodies can be used to detect the presence of drugs, viral and bacterial products and other unusual or abnormal substances in the blood.This is important because of the role antibodies play in disease. For example, antibodies can be used to develop a vaccine for a specific disease and to diagnose that disease. Mammals make antibodies to almost anything that is not normally part of the body such as viruses and bacteria. You can get a mammal to make an antibody to almost anything by injecting it into the bloodstream.

Antibodies are really useful because they are so specific. They latch on to only one type of antigen and ignore all the others. Hence it is easy to identify any antigens that are present in the sample.

An HIV ELISA, sometimes called an HIV enzyme immunoassay (EIA) is the first and most basic test to determine if an individual is positive for a selected pathogen, such as HIV. The test is performed in a 8 cm x 12 cm plastic plate which contains an 8 x 12 matrix of 96 wells, each of which are about 1 cm high and 0.7 cm in diameter. The next page illustrates how an HIV ELISA is performed.

An ELISA plate



The ELISA Method

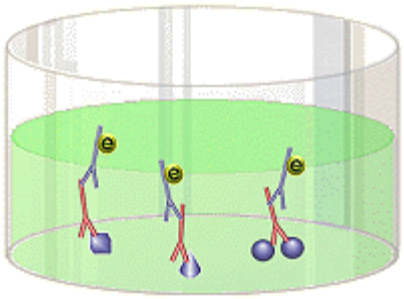
Partially purified, inactivated HIV antigens pre-coated onto an ELISA plate

Patient serum which contains antibodies. If the patient is HIV+, then this serum will contain antibodies to HIV, and those antibodies will bind to the HIV antigens on the plate.

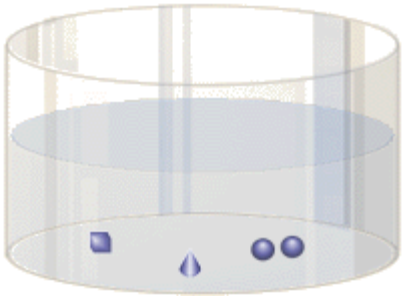
Anti-human immunoglobulin coupled to an enzyme. This is the second antibody, and it binds to human antibodies.

Chromogen or substrate which changes color when cleaved by the enzyme attached to the second antibody.

Positive ELISA Test



Negative ELISA Test

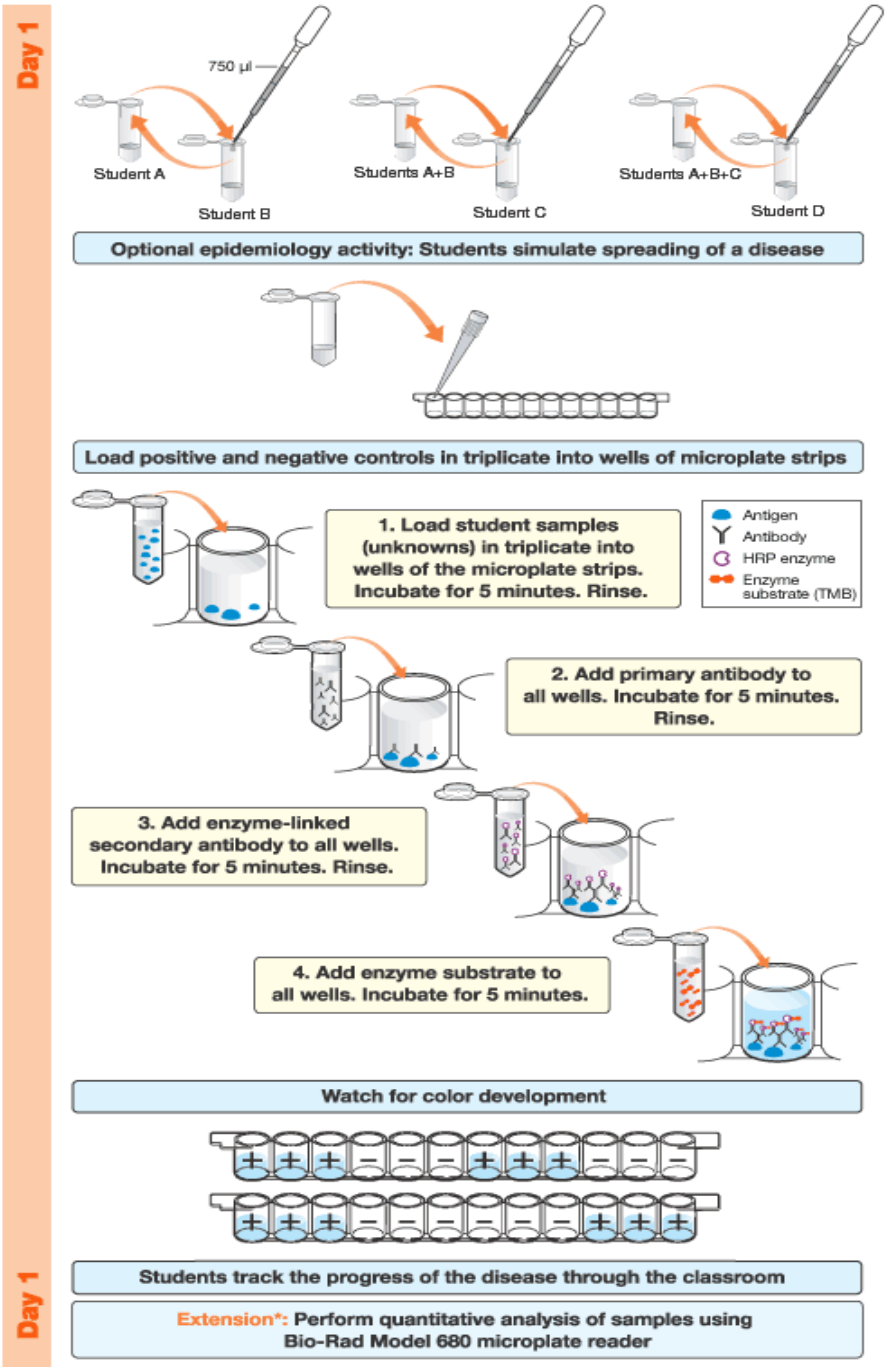


False positives. It is entirely possible that an individual not infected with HIV has antibodies which may give a positive result in the HIV ELISA. This is called a false positive. One reason for this is that people (especially women who have had multiple pregnancies) may possess antibodies directed against human leukocyte antigens (HLA) which are present on the host cells used to propagate HIV. As HIV buds from the surface of the host cell, it incorporates some of the host cell HLA into its envelope. False negatives can occur during the window between infection and an antibody response to the virus (seroconversion).

Use these problems to test your understanding of this topic.

1. What does ELISA measure?
2. What if serum were left out?
3. Omission of the wash step
4. Which patient is HIV positive?

Experimental Procedure



***Analysis of results:** An ELISA can give qualitative (yes or no) or quantitative (how much?) information. Qualitative results can be determined visually by eye. For quantitative determination of precise concentrations, a microplate reader is required. A detailed lesson extension for quantitative ELISA is included in the kit curriculum (BioRad).

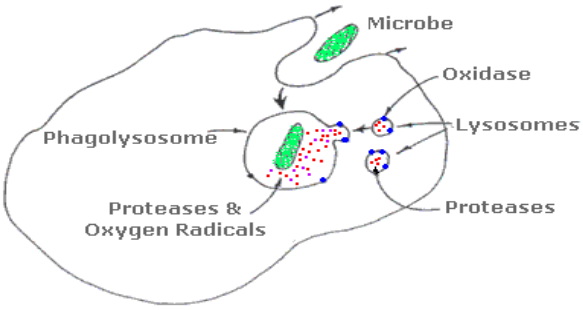
LAB # 6 – Phagocytosis

Objetive:

Observe and understand the phagocytosis process and the type of defense cells involved

Background

Several types of cells in the immune system engulf microorganisms via **phagocytosis**.



Phagocytosis is mediated by macrophages and polymorphonuclear leucocytes.

Phagocytosis involves the ingestion and digestion of the following:

- microorganisms
- insoluble particles
- damaged or dead host cells
- cell debris
- activated clotting factors

- Neutrophils. Neutrophils are abundant in the blood, quickly enter tissues, and phagocyte pathogens in acute inflammation.

- Macrophages. Macrophages are closely related to monocytes in the blood. These longer-lived cells predominate in chronic inflammation. They also release some important inflammatory paracrines.

- Dendritic Cells and B Lymphocytes. Phagocytosis in these cells is important for the elaboration of a specific immune response rather than for directly destroying the pathogens.

Phagocytosis begins with the neutrophil or macrophage flowing around the pathogen and engulfing it so that it winds up enclosed in a **phagosome** (phagocytic vesicle). But this is only the first step, because the more challenging task of destroying the microorganisms remains. Indeed, some pathogens have special, effective mechanisms for frustrating this destruction step.

The next step is the fusion of **lysosomes** with the phagosome. The result is called a **phagolysosome**. Lysosome are derived from the Golgi apparatus, much like secretion vesicles, but their proteins are focused on destroying microorganisms. The proteins are of two sorts:

- Anti-microbial Proteins. Especially important here are **proteases** which break down protein.

- NADPH Oxidase. This complex is found in the membrane of a lysosome and generates **oxygen radicals** in the phagosome. These highly reactive molecules react with proteins, lipids and other biological molecules.

In addition to destroying the microorganism, macrophages also release **paracrines** that alert other parts of the immune system that an infection is present. (Two important examples are IL-1 and TNF-alpha.) Among other things, these paracrines promote inflammation.

Identification of Pathogen

Neutrophils and macrophages have some ability on their own to recognize microorganisms and begin phagocytosis. This is because such organisms have molecules much different than those found in a human. But phagocytosis is far more effective if microorganisms are labelled as such by special molecules that bind to their surface. (Any molecule that binds to a microorganism and thereby speeds phagocytosis is called an **opsonin**). Most important here are **antibodies** (such as IgG), which specifically identify molecules at the surface of specific microorganisms. With this attached to the surface of the microorganisms, phagocytosis is much more effective and rapid.

Difficult Pathogens

But, as mentioned above, sometimes phagocytes have a difficult time with certain pathogens. **Tuberculosis** is an important example. A macrophage can usually engulf the tuberculosis bacterium, but then apparently the bacterium has a means for preventing the lysosomes from fusing with the phagosome. If the macrophage is not "activated" by paracrines from a specific immune response, the bacteria may remain alive for long periods within the macrophage. In this circumstance, other macrophages surround and wall off the infected macrophages, forming a type of chronic inflammation called a **granuloma**. Leprosy is another bacterium that is difficult for macrophages to destroy.

Anthrax is an example of a bacterium surrounded by a **capsule** that make phagocytosis difficult. Anthrax spores from the lungs or a cut in the skin make their way first to lymph nodes, where they change to their "vegetative form" and begin dividing. But because they are difficult to destroy, they quickly become quite numerous and accumulate in the blood, causing **septicemia**. Not only do the bacteria release toxins, but also macrophages respond to the crisis by releasing enough IL-1 and TNF-alpha to cause inflammation throughout the body. Indeed, this hyperinflammation by itself can quickly be fatal.

There are several stages of phagocytosis:

1. Chemotaxis

This is the movement of cells up a gradient of chemotactic factors. It may be directly induced by a substance such as C5a, produced as a result of complement activation. It can also be indirectly induced as a consequence of release of preformed mediators within mast cells by the action of C3a or C5a e.g. eosinophil chemotactic factor, or neutrophil chemotactic factor. Leukotrienes, produced by the metabolism of mast cell arachidonic acid, are also chemotactic.

2. Adherence

This works reasonably well for whole bacteria or viruses, but less so for proteins or encapsulated bacteria. In order to deal more effectively with encapsulateed bacteria, antibodies directed against the capsule enable the phagocytic cells to ingest the organisms, using their Fc receptors (see below).

3. Pseudopodium formation

This is the protrusion of membranes to flow round the "prey".

4. Phagosome formation

Fusion of the **pseudopodium** with a membrane enclosing the "prey" leads to the formation of a structure termed a **phagosome**.

5. Phago-lysosome formation

The phagosome moves deeper into the cell, and fuses with a **lysosome**, forming a phago-lysosome. These contain hydrogen peroxide, active oxygen species (free radicals), peroxidase, lysozyme and hydrolytic enzymes. This is known as the **oxidative burst**, and leads to digestion of the phagolysosomal contents, after which they are eliminated by exocytosis. Some peptides however, undergo a very important separate process at this stage. Instead of being eliminated, they attach to a host molecule called MHC class II and end up being expressed on the surface of the cell within a groove on the MHC molecule (antigen presentation).

The speed of phagocytosis can be increased markedly by bringing into action two attachment devices present on the surface of phagocytic cells:

- **Fc receptor:** which binds the Fc portion of antibody molecules, chiefly IgG. The IgG will be attached the organism via its Fab site.
- **Complement receptor:** the third component of complement (C3) also binds to organisms and then attaches to the complement receptor.

This coating of the organisms by molecules that speed up phagocytosis, is termed **'opsonization'**, and the Fc portion of antibody, and C3 are termed **'opsonins'**.

Ref.: http://courses.washington.edu/conj/bloodcells/phagocytosis.htm
http://www-micro.msb.le.ac.uk/MBChB/bloodmap/Phagocytosis.html

Experimental Procedure

Fluorescent labeling of yeast (perform by TA)

1) Resuspend 5 grams of yeast in 50 ml PBS in 100 ml flask
2) Put flask for 30 minutes in boiling water bath while stirring.
3) Wash 5 x with PBS and 2 x in PB.
4) Adjust the concentration to of particles to 10^9 particles/ml. Can now be frozen at - 20 °C.
5) For labeling, resuspend the pellet of 2 x 10^{10} particles in 20 ml Na_2HPO_4 (50 mM, pH 9.2)
6) Add 2 mg TRITC; incubate 30 minutes at 37°C on a rotary shaker.
7) Wash 2 x in Na_2HPO_4 (50 mM, pH 9.2) and 4 x in PB.
8) Freeze aliquots of 10^9 particles/ml at -20°C.

Phagocytosis assay (perform by students)

1) Grow macrophages, the density not exceeding 5 x 10^6 cells/ml
2) Harvest 2 x 10^7 cells, wash 1 x in PBS and resuspend in 1 ml PBS.
3) Mix 100 □l cells (2 x 10^6 with 12□□l labeled yeast 1.2 x 10^7) in glass tubes.
4) Incubate for 0, 5, 15, 30, 40 minutes.
5) Stop with 1 ml cold PBS.
6) Add 100 □l Trypan blue (2 mg/ml in 20 mM citrate, 150 mM NaCl, pH 4.5).
7) Mix and shake for 5 minutes.
8) Spin 3 minutes 500 x g.
9) Remove supernatant. Resuspend in 1 ml PBS.
10) Read in fluorescence spectrofotometer at 544 nm excitation, 574 nm emission.
11) Include the following blanks:
    a) **For autofluorescence**: 100□□l cells and 12 □l unlabeled yeast in 1 ml PBS + Trypan blue.
    b) **For background**, unquenched fluorescence, 12 □l labeled yeast in 1 ml PBS + Trypan blue.
12) Prepare 2 slides of each incubation time

Standard curve:

0, 5, 10 □l labeled yeast in 1 ml PB.

LAB # 7 Western Blot

Material

Pre-cast page gel (15%)
Running buffer
Loading buffer
Molecular marker
Electrophoresis system
Power supply
Tips
Tubes
Heat blot
Microcentrifugue
Ice
Blot
Anti-myosin antibody
Anti-rabit IgG peroxidase-conjugate
PBS
Blotto
Developer solution

Background

SDS-PAGE (polyacrylamide gel electrophoresis)

The purpose of this method is to separate proteins according to their size, and no other physical feature. In order to understand how this works, we have to understand the two halves of the name: SDS and PAGE.

SDS: since we are trying to separate many different protein molecules of a variety of shapes and sizes, we first want to get them to be linear so that the proteins no longer have any secondary, tertiary or quaternary structure (i.e. we want them to have the same linear shape). Consider two proteins that are each 500 amino acids long but one is shaped like a closed umbrella while the other one looks like an open umbrella. If you tried to run down the street with both of these molecules under your arms, which one would be more likely to slow you down, even though they weigh exactly the same?

This analogy helps point out that not only the mass but also the shape of an object will determine how well it can move through and environment. So we need a way to convert all proteins to the same shape – then we use SDS.

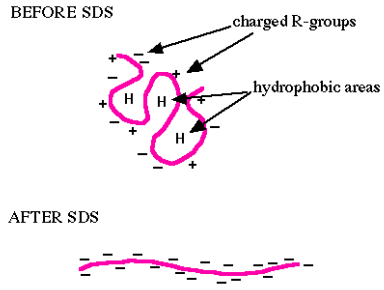


Figure 1. This cartoon depicts what happens to a protein (pink line) when it is incubated with the denaturing detergent SDS. The top portion of the figure shows a protein with negative and positive charges due to the charged r-groups of the particular amino acids in the protein. The large h represents hydrophobic domains where nonpolar R-groups have collected in an attempt to get away from the polar water that surrounds the protein. The bottom portion shows that SDS can break up hydrophobic areas and coat proteins with many negative charges which overwhelms- any positive charge in the protein due to positively charged r-groups. The resulting protein has been denatured by SDS (reduced to its primary structure) and as a result has been linearized

SDS (sodium dodecyl sulfate) is a detergent (soap) that can dissolve hydrophobic molecules but also has a negative charge (sulfate) attached to it. Therefore, if a cell is incubated with SDS, the membranes will be dissolved and the proteins will be solubIlized by the detergent, plus all the proteins will be covered with many negative charges. So a protein that started out like the one shown in the top part of Figure 1 will be converted into the one shown in the bottom part of Figure 1. The end result has two important features: 1) all proteins contain only primary structure and 2) all proteins have a large negative charge which means they will all migrate towards the positive pole when placed in an electric field.

PAGE: if the proteins are denatured and put into an electric field, they will all move towards the positive pole at the same rate, with no separation by size. So we need to put the proteins into an environment that will allow different sized proteins to move at different rates. The environment of choice is polyacrylamide, which is a polymer of acrylamide monomers. When this polymer is formed, it turns into a gel and we will use electricity to pull the proteins through the gel so the entire process is called polyacrylamide gel electrophoresis (PAGE). A polyacrylamide gel is not solid but is made of a labyrinth of tunnels through a meshwork of fibers (Figure 2).

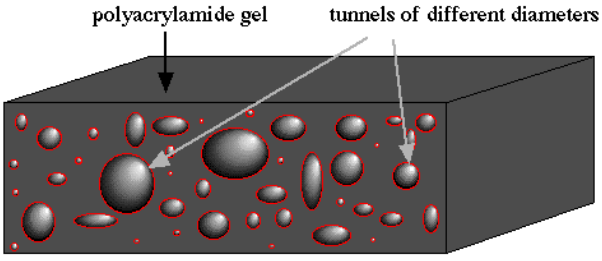


Figure 2. This cartoon shows a slab of polyacrylamide (dark gray) with tunnels (different sized red rings with shading to depict depth) exposed on the edge. Notice that there are many different sizes of tunnels scattered randomly throughout the gel.

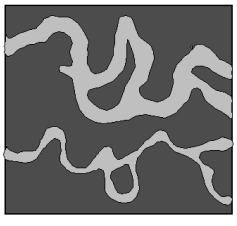
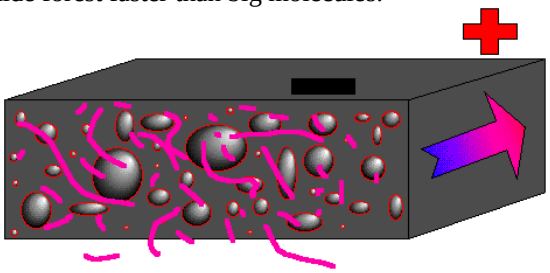


Figure 3. This is a top view of two selected tunnels (only two are shown for clarity of the diagram). These tunnels extend all the way through the gel, but they meander through the gel and do not go in straight lines. Notice the difference in diameter of the two tunnels.

Now we are ready to apply the mixture of denatured proteins to the gel and turn on the current (Figure 4). If all the proteins enter the gel at the same time and have the same force pulling them towards the other end, which ones will be able to move through the gel faster? Think of the gel as a tiny forest with many branches and twigs throughout the forest but they form tunnels of different sizes. If we let children and adults run through this forest at the same time, who will be able to get through faster? The children of course. Why? Because of their small size, they are more easily able to move through the forest. Likewise, small molecules can maneuver through the polyacrylamide forest faster than big molecules.

Figure 4. Cartoon showing a mixture of denatured proteins (pink lines of different lengths) beginning their journey through a polyacrylamide gel (gray slab with tunnels). An electric filed is established with the positive pole (red plus) at the far end and the negative pole (black minus) at the closer end. Since all the proteins have strong negative charges, they will all move in the direction the arrow is pointing (run to red).



You have to remember that when we work with proteins, we work with many copies of each kind of protein. As a result, the collection of proteins of any given size tends to move through the gel at the same rate, even if they do not take exactly the same tunnels to get through. Proteins tend to move through a gel in bunches, or bands, since there are so many copies of each protein. Figure 5 shows a cartoon gel and Figre 6 shows a real gel. Notice that the actual bands are equal in size, but the proteins within each band are of different sizes.

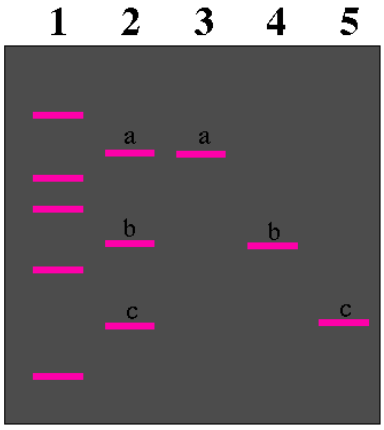


Figure 5. This shows a top view of an SDS page after the current has been on for a while (positive pole at the bottom) and then turned off. The gel (gray box) has five numbered lanes where five different samples of proteins (many copies of each kind of protein) were applied to the gel. (lane 1, molecular weight standards of known sizes; lane 2, a mixture of three proteins of different sizes with a being the biggest and c being the smallest protein; lane 3, protein a by itself; lane 4, protein b by itself; lane 5 protein c by itself.) Notice that each group of the three proteins migrated the same distance in the gel whether they were with other proteins (lane 2) or not (lanes 3-5). The molecular weight standards are used to measure the relative sizes of the unknown proteins (a, b, and c).

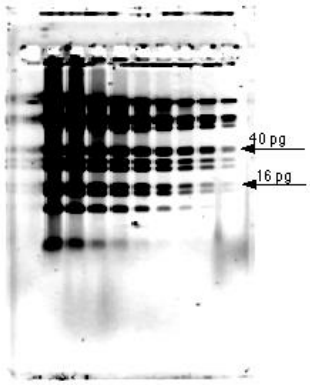


Figure 6. This photo shows a variety of different proteins being separated on a gel. This particular image is showing a serial dilution of the same protein sample to indicate how little protein is needed in order to be detected

This image was taken from a home page operated by Hitachi software (http://www.hitachi-soft.com/hitsoft/gs/fmbio/feb.htm)

Western blotting

Western blot analysis can detect **one** protein in a mixture of any number of proteins while giving you information about the size of the protein. It does not matter whether the protein has been synthesized in vivo or in vitro. This method is, however, dependent on the use of a high-quality antibody directed against a desired protein. So you must be able to produce at least a small portion of the protein from a cloned DNA fragment. You will use this antibody as a probe to detect the protein of interest.

Blotting procedures combine the resolution of gel electrophoresis with the specificity of antibody detection. Blotting can be used to ascertain a number of important characteristics of protein antigens, including the presence and quantity of an antigen, the molecular weight of the antigen, and the efficiency of antigen extraction. This method is especially helpful when dealing with antigens that are insoluble, difficult to label, or easily degraded, and thus not amenable to procedures such as immunoprecipitation.

Steps in a Western blot

- The first step is **gel electrophoresis**. The proteins of the sample are separated according to size on a gel, usually using SDS-PAGE. Usually the gel has several lanes so that several samples can be tested simultaneously. However, it is also possible to use a 2-D gel which spreads the proteins from a single sample out in two dimensions.
- **Nitrocellulose transfer** - The proteins in the gel are then transferred onto a membrane made of nitrocellulose or PVDF, by pressure or by applying a current. This is the actual blotting process and is necessary in order to expose the proteins to antibody (see below). The membrane is "sticky" and binds proteins non-specifically (i.e. binds all proteins equally well).

PVDF is often used because it is sturdier and can be "stripped" of antibodies and reused. Unlike nitrocellulose, PVDF must be soaked in 100% methanol before using.

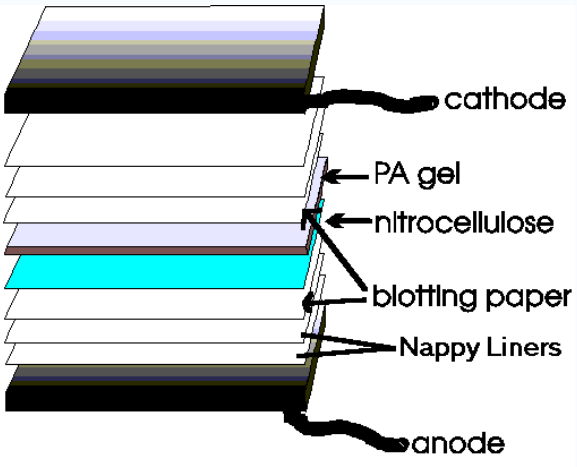


Figure 7. Ensemble of the western blot sandwich

- **Blocking** - The membrane is then *blocked*, in order to prevent non-specific protein interactions between the membrane and the antibody protein (next step, below). This is done by a solution of Bovine serum albumin (BSA) or dry milk. (Without the blocking, the antibody to be applied in the next step would bind to the nitrocellulose.)
- The first antibody (often called the *primary antibody*) is incubated with the membrane. This antibody recognizes only the protein of interest, and will not bind any of the other proteins on the membrane. It is obtained by immunizing an animal (usually a rabbit or goat) with the protein of interest (i.e., injecting the protein into the animal's body) and collecting the antibodies the animal produces against that protein. Some high affinity monoclonal antibodies can also be used for Western blots. An alternative to using an enzyme that is coupled to the secondary antibody is to use a radioactive label. An antibody-binding protein such as *Staphylococcus* Protein A can be used and labeled with a radioactive isotope of iodine.
- After rinsing to remove unbound primary antibody a *secondary antibody* is incubated with the membrane. It binds to the first antibody, and is usually produced by a different animal. For example, goat anti-rabbit antibody might be used if the first antibody was produced by rabbits. This secondary antibody is usually linked to an enzyme that can allow for visual identification of where on the membrane it has bound. As with the ELISPOT and ELISA procedures, the enzyme can be provided with a substrate molecule that will be converted by the enzyme to a colored reaction product that will be visible on the membrane (see the figure below with blue bands). Alternately, the reaction product may produce enough fluorescence to expose a sensitive sheet of film when it is placed against the membrane.

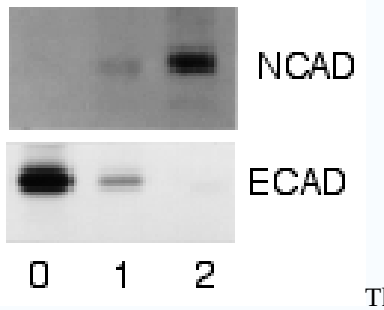


Figure 8. Western blot using radioactive detection system

The unbound secondary antibodies are washed away, and the enzyme substrate is incubated with the membrane so that the positions of membrane-bound secondary antibodies will become visible. If a radioactive label is used, the radioactive membrane can be placed against a sheet of medical X-ray film. Bands corresponding to the detected protein of interest will appear as dark regions on the developed film (see figure to right).

Since the first antibody only recognizes the protein of interest, and the second antibody only recognizes the first antibody, if there is stain present on the membrane then the protein of interest must also be present on the membrane. Thus, the protein bands on the membrane that are stained contain the protein that was to be detected, the other locations on the membrane do not. Size approximations can be done by comparing the stained bands to that of a pre-stained protein size marker.

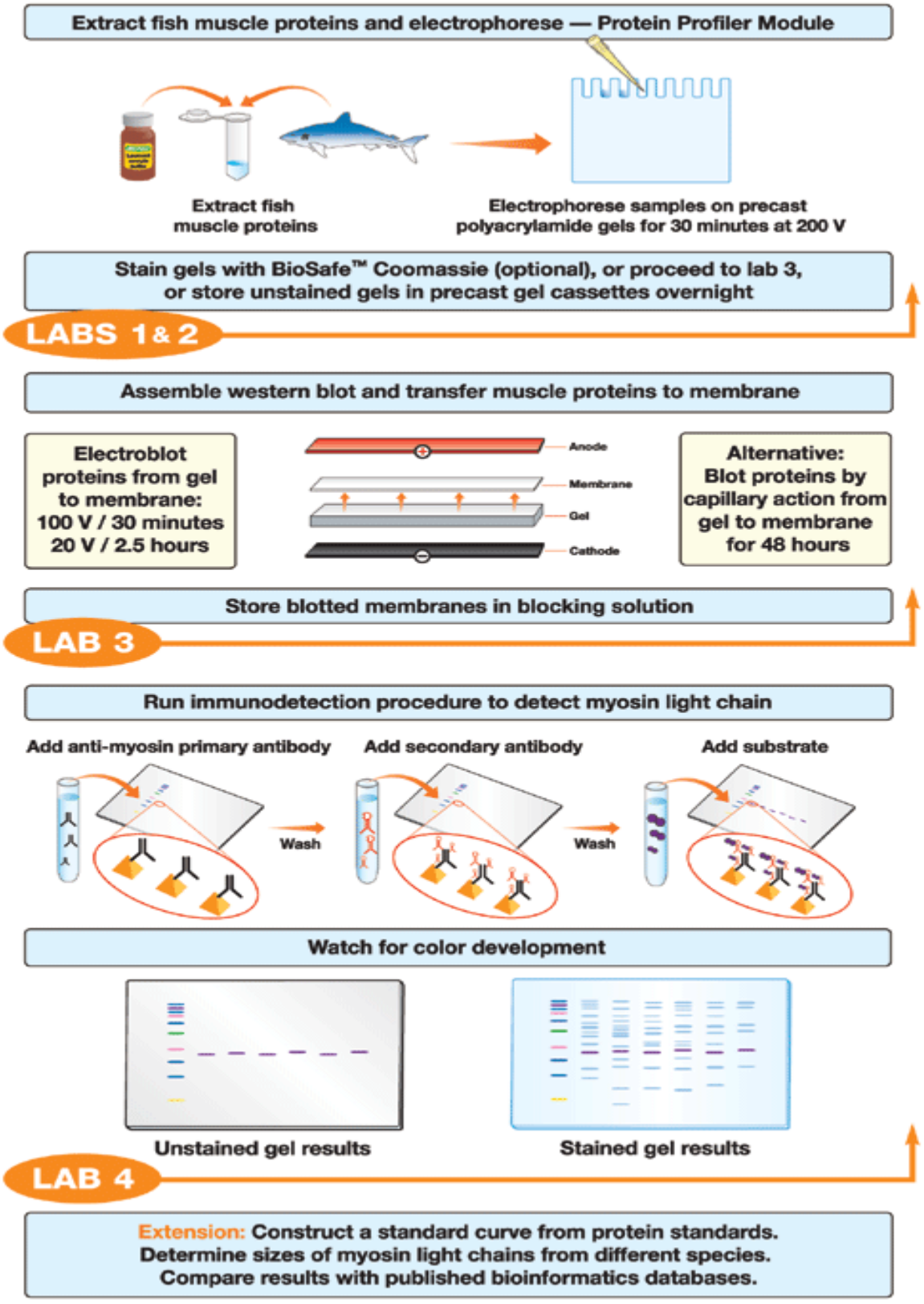
Usually, the gel is not completely devoid of proteins after blotting. Protein staining solution will show all protein bands on the gel. The stained gel can then be compared with the stained membrane to identify which bands contain the wanted protein and which do not.

In principle, one could bind the chemical signal directly to the first antibody, but production of the antibodies is easier if the two functions *recognition* and *signalling* are separated

http://en.wikipedia.org/wiki/Western_blot

http://lsvl.la.asu.edu/resources/mamajis/western/western.html

Experimental Procedure (BioRad kit)



LAB # 8 - Bioinformatics tools for vaccines development

Materials:

Protein sequence

Computer

Background

Development of effective vaccines for some of the major infectious diseases contributed to dramatic improvements in public health worldwide over the past 100 years. In the post-genomics and information era, the application of computational tools to vaccine research and development (R&D) has contributed to progress in the development of new vaccines. Computational vaccinology is a branch of vaccinology that is focused on solving scientific questions in vaccinology using computer-driven algorithms. This interdisciplinary field of research spans computer science, mathematics, statistics, molecular biology, microbiology, immunology, and vaccinology. Computational vaccinology has also been called vaccine informatics [1]. The two terms are used interchangeably to represent the same concept at this point in the history of vaccinology.

Role of immunoinformatics

Computational vaccinology or vaccine informatics is closely related to immunoinformatics. Many immunoinformatics methods have been developed since 1980s to predict T-cell immune epitopes and B-cell functional neutralizing or cross-reactive epitopes [2]. These epitopes are useful for the development of diagnostic tests, for the development and design of vaccines, and for characterizing targets of immune responses to vaccines and infections. A large number of computational algorithms and software programs have been developed for immune epitope prediction. In general, T-cell immune epitope prediction can be successful. However, functional B-cell antibody epitopes prediction remains a challenge, especially conformational epitopes [3].

Reverse vaccinology

Computational vaccinology has also contributed to the "reverse vaccinology" approach to vaccine development. Reverse vaccinology starts with bioinformatics analysis of genome or proteome sequences of pathogens [4] and proceeds "backwards" to identifying critical antigens, rather than beginning with selection of a single antigen and moving forward with iterative testing. This comprehensive initial screening of genome sequences enables the selection of antigen candidates that are highly likely to be relevant for vaccine development. An example of successful reverse vaccinology is the Meningitis B vaccine developed by Rino Rappuoli's group (Novartis). This vaccine is under review by the European Medicines Agency. Approval of this vaccine would signal the maturation of computational biology field. A number of other vaccines are in development, using reverse vaccinology have also been reported [1,5].

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