# Biology 196 Laboratory
# Pipetting and Enzyme-Linked Immunosorbent Assay

## Laboratory Objectives

After completing this lab you should be able to:

- Use the three different sizes of micropipettes to accurately pipet liquid volumes.
- Define the basics of antibodies and antigens and how they are related.
- Describe the uses and functions of an ELISA.
- Perform an ELISA to determine the presence of a serum antigen.

## Micropipettes

Micropipettes dispense very small volumes of liquid very accurately. The unit of volume that they dispense is the microliter (abbreviated "$\mu$l"), which is 1 x $10^{-6}$ liters or 1 x $10^{-3}$ milliliters.

### Precautions When Using Micropipettes:

- Set pipette volume only within the range specified for that micropipette. Do not attempt to set a volume beyond the pipet's minimum or maximum values.
- When using a micropipette, first apply a tip. Forgetting to do this would cause liquid leakage into the nose cone. Since a micropipette works by air displacement, its internal mechanism must remain dry.
- Always keep a micropipette in a vertical position when there is fluid in the tip. Do not allow liquid to accidentally run back into the nose cone.
- Use your thumb to control the speed at which the plunger rises after taking up or ejecting fluid. Releasing the plunger too abruptly will cause liquid leakage.

### Setting and Preparing the Micropipette:

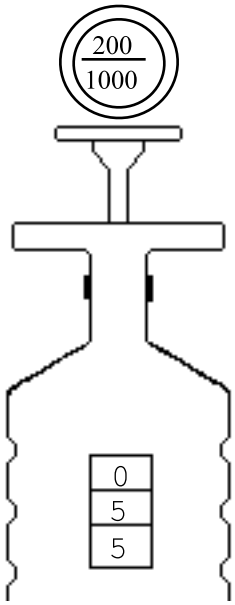
- Check that you have the right micropipette. There may be three sizes in the lab -- a "P-20" (for 2 to 20 µl), a "P-200" (for 20-200 µl), and a "P-1000" (for 200-1000 µl).
- Dial the desired volume. Do you understand how to read the scale? If not -- ASK!
- Push the end of the pipette into the proper-size tip. The yellow tips are for P-20's and P-200's; the larger blue tips are for P-1000's. Use sufficient force to create an air-tight seal.
- When pushing down on the plunger to expel atmospheric air be sure to stop at the first resistance point you feel. If you push further you are going to pipet the wrong volume.
- Always hold the tubes and pipet tips at *eye level*.

Before moving on to the ELISA experiment be sure you are comfortable with the pipet devices and that you can answer the questions in the example exercises below.

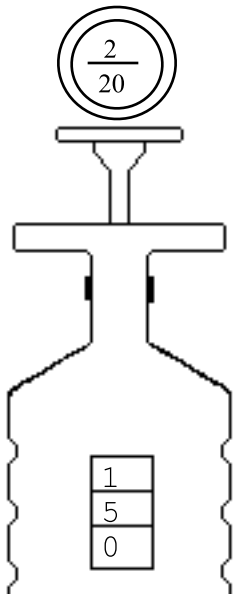
In addition, your instructor may assign a few sample volumes for you to pipet as practice.

## Examples:

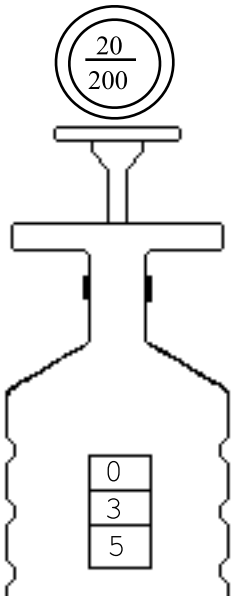
Observe the volume of liquid that is measured by micropipettes a, b, and c.




A. 550 µl




B. 15 µl

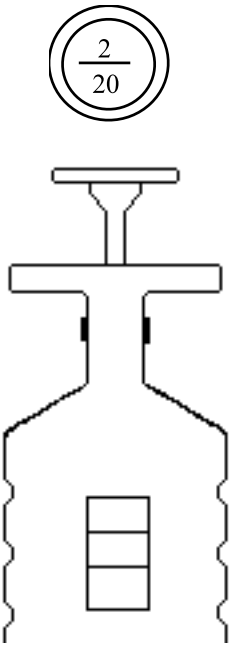



C. 35 µl

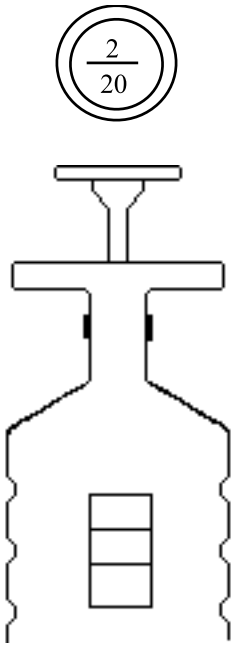
Which micropipette (a, b, or c) is the P-20? _____ What is its range? _____
Which micropipette (a, b, or c) is the P-200? _____ What is its range? _____
Which micropipette (a, b, or c) is the P-1000? _____ What is its range? _____

Select the appropriate micropipette and show what the dial should read to measure each of the following amounts of liquid. Write the amount on the line beneath each drawing.

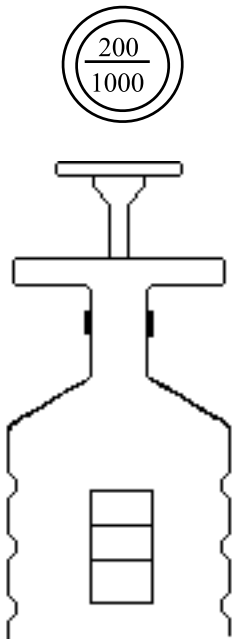
a. 150 µl b. 2.5 µl c. 300 µl d. 7 µl



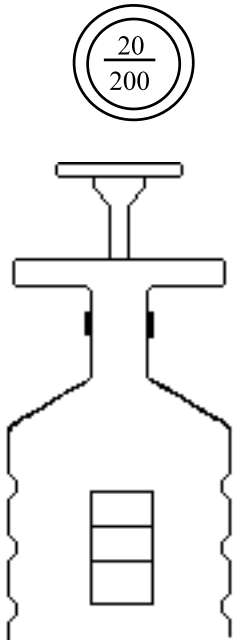

i.____




ii._____




iii._____




iv._____

# Enzyme-Linked Immunonosorbent Assay (ELISA)[1]

## Immunology

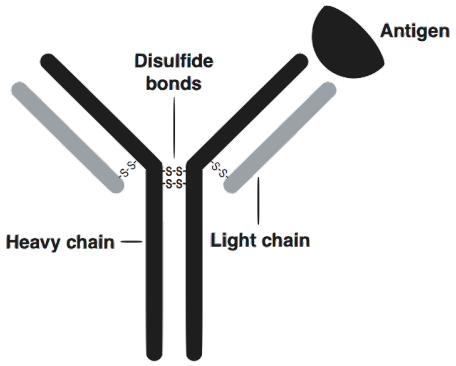
**Immunology** is the study of the immune system and how the body protects itself against foreign, potentially disease-causing microorganisms and molecules. The immune system has three fundamental functions. They are:

- To recognize intruders
- To respond appropriately to intruders in a way that protects the body
- To respond the next time the intruders are encountered

Mammalian immune systems produce molecules called **antibodies** that recognize intruder molecules with incredible specificity. Like magic bullets, antibodies locate and attach themselves to their targets, called **antigens** ("antibody generators").

Antibodies do not bind the whole antigen, but specifically bind to antigenic determinants or **epitopes** of the antigen. Epitopes are those regions of an antigen that are recognized and bound by the antibody

By attaching to the invading foreign entities, antibodies make the invaders recognizable to other cells of the immune system so that they can be destroyed. Antibodies have become vital high-tech tools, used in biotechnology research and disease diagnosis and treatment.




*A commonly used representation of an antibody bound to an antigen*

Only the antibody with the correct 3D conformation will respond to a particular epitope. As stated in class many times during the semester, what a protein does and what it interacts with depends upon its overall 3D conformation. Today's lab is an excellent example of this process in action.

Characteristics of antibodies include:

- a Y-shaped structure
- two short, light chains and two long, heavy chains
- the chains are held together with di-sulfide bonds
- a variable region exists at the top ends of the Y, representing about 25% of the entire antibody structure
- the variable region is different in all antibodies and it is this region that interacts with the epitopes of an antigen through ionic bonds, H bonds, and hydrophobic interactions
- a constant region occurs in the bottom 75% of the antibody and is used for recognition by other components of our immune system.

## What is an ELISA?

**ELISA** stands for enzyme-linked immunosorbent assay. This powerful antibody-based test is used to diagnose diseases such as HIV/AIDS and malaria and to track pathogenic agents in water, food, and the air, whether these emerge naturally or through acts of aggression. ELISA is also used to identify genetically modified organisms (GMOs) and to trace food allergens and molecular markers of pregnancy and drug use.

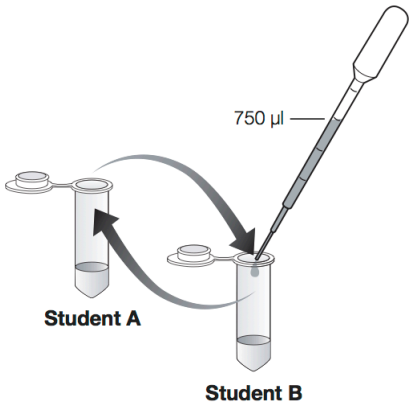
## Procedural Background

You are about to perform an experiment in which you will share simulated "body fluids" with your classmates. After sharing, you will perform an enzyme-linked immunosorbent assay or ELISA to determine if you have been exposed to a contagious "disease". While the antigen in the simulated bodily fluid is actually a completely benign

[1] Reproduced for class use only from BioRad ELISA Immuno Explorer™ Kit - catalog# 166-2400EDU

protein, for the sake of the experiment we will pretend it is the **Hepatitis A** virus, a contagious human disease that can be transmitted via the fecal-oral route. Using the ELISA you will then track the disease back to its source.

## Procedure:




Part 1- Spread the epidemic

To mimic the spread of this disease, students will share bodily fluids. Obtain a yellow tube with 750ul of bodily fluids and a plastic transfer pipet. One of the yellow tubes will be contaminated with hepatitis A[2].

A. Your tube number: _____

B. Share your (simulated) body fluids randomly with your classmates. Use the pipet to transfer all of your bodily fluids into the tube of another student. Gently mix the tube and then take back 750ul of the now mixed sample to put into your original tube.

C. Write down the name and tube number of the student that you shared with

Student #1 name: _____ Number: _____

D. Repeat the sharing protocol a second time with a different person.

Student #2 name: _____ Number: _____

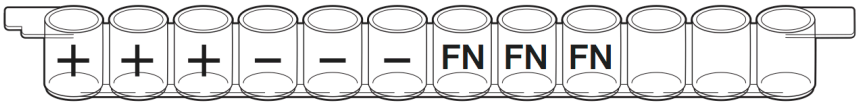
E. If you have more than 12 students, repeat the sharing protocol for a third and final time. If you have fewer than 12 students skip this third sharing step and move on to Part 2.

Student #3 name: _____ Number: _____

Part 2- Bind the antigen to the wells

The reactions will be performed in microplate wells made of polystyrene which adsorbs proteins by hydrophobic interactions. Label your 12 well strip as follows: the first three wells will be labeled as + for a positive control, the second set of three will be labeled – for a negative control, the third set of three will be labeled with your initials.

For example:




A. Using a micropipette with a clean pipet tip, transfer 50 µl of the + control into the first three + wells. The + control will be in the violet tubes. This represents a sample containing hepatitis A.

B. Using a micropipette with a clean pipet tip, transfer 50 µl of the (-) control into the three (-) wells. The (-) control will be in the blue tubes.

C. Using a micropipette with a clean pipet tip, transfer 50 µl of your bodily fluids into the wells with your initials.

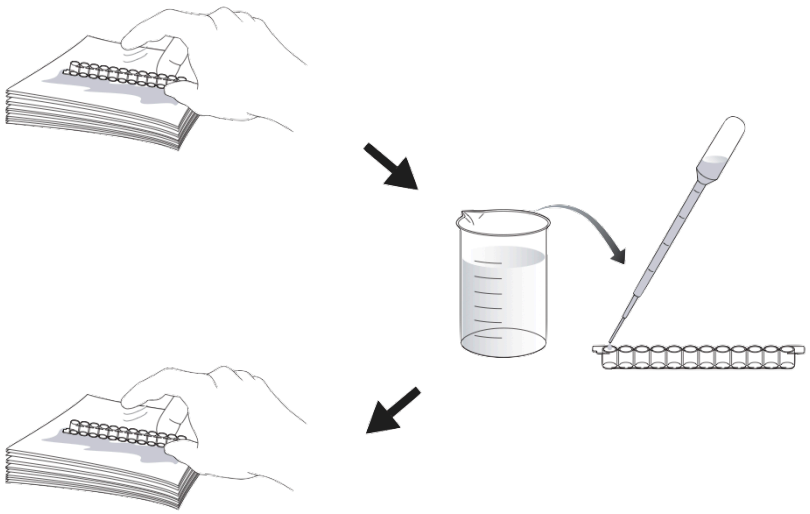
D. Wait five minutes for the proteins in the samples to bind to the plastic.

[2] There are no pathogens present in this exercise. The “hepatitis A” is actually a chicken gamma globulin.

E. Wash the unbound sample out of each well by tipping the strip of wells upside down onto a few paper towels. Gently tap the strip to get as much liquid out as possible. Discard the used paper towels.

F. Using a fresh plastic transfer pipet (NOT the micropipette), fill each well about ¾ full with wash buffer. Do not let the wells overflow and *do not let the pipet touch the sides of the wells* (especially the +++ wells)! If you do it will contaminate the other wells and ruin the experiment.

G. Remove the wash buffer from each well by tipping the strip of wells upside down onto a few paper towels. Gently tap the strip to get as much liquid out as possible. Discard the used paper towels.



*washing the wells with wash buffer*

H. Repeat parts F and G once more.

## Part 3- Adding the primary antibody

A. Using a micropipette with a clean pipet tip, transfer 50 ul of the primary antibody into each well of the strip. The primary antibody will be in the green tubes.

B. Wait five minutes for the primary antibody to bind to the antigen.

C. Discard the unbound sample out of each well by tipping the strip of wells upside down onto a few paper towels. Gently tap the strip to get as much liquid out as possible. Discard the used paper towels.

D. Using a fresh plastic transfer pipet (NOT the micropipette), fill each well about ¾ full with wash buffer. Do not let the wells overflow!

E. Remove the wash buffer from each well by tipping the strip of wells upside down onto a few paper towels. Gently tap the strip to get as much liquid out as possible. Discard the used paper towels.

F. Repeat parts D and E once more.

## Part 4- Adding the secondary antibody

A. Using a micropipette with a clean pipet tip, transfer 50 ul of the secondary antibody into each well of the strip. The secondary antibody will be in the orange tubes. Note that the secondary antibody has the enzyme HRP (horse-radish peroxidase) attached.

B. Wait five minutes for the secondary antibody to bind to the primary antibody.

C. Wash the unbound sample out of each well by tipping the strip of wells upside down onto a few paper towels. Gently tap the strip to get as much liquid out as possible. Discard the used paper towels.

D. Using a fresh plastic transfer pipet (NOT the micropipette), fill each well about ¾ full with wash buffer. Do not let the wells overflow!

E. Remove the wash buffer from each well by tipping the strip of wells upside down onto a few paper towels. Gently tap the strip to get as much liquid out as possible. Discard the used paper towels.

F. Repeat parts D and E **two more** times.

Part 5- The enzymatic color reaction

Using a micropipette with a clean pipet tip, transfer 50 $\mu$l of the enzyme substrate into all twelve wells of the strip. The enzyme substrate will be in the brown tubes. Wait 5 minutes and record any color changes.

HRP will reduce hydrogen peroxide into water while at the same time oxidizing 3, 3', 5, 5'-tetramethyl benzidine (TMB) into cationic form. TMB is colorless, but its cationic form has a blue color. Thus the presence of a blue color will denote a + result and signify the presence of hepatitis A.

Record your data in the table below

| Sample wells | Color |
|---|---|
| 1-3 | |
| 4-6 | |
| 7-9 | |

Your instructor should be recording all students' data on the whiteboard.
Can you determine who started the epidemic?

## Study Checklist

To perform well on the quiz, you need to have a thorough knowledge of the following:

- You should know and understand all of the terms which appear in **boldface** type.
- Determine the proper pipet and dial it to the proper setting for any given liquid volume.
- Describe the structure and function of the components of an antibody
- What are the uses for the ELISA, and what are the advantages/disadvantages it has over other procedures.
- Why is it important to be able to detect antibodies in people who don't appear sick?
- Why do you need to assay positive and negative control samples as well as your experimental samples?
- What was happening inside the wells for each step of the ELISA procedure