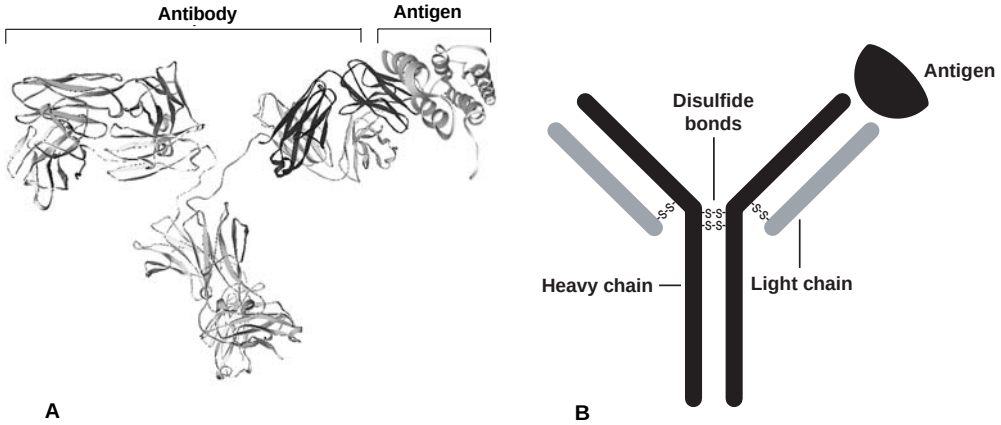
Student Manual

Introduction

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". The ELISA uses antibodies to detect the presence of a disease agent, (for example, viruses, bacteria, or parasites) in your blood or other body fluid. You will then track the disease back to its source.

When you are exposed to a disease agent, your body mounts an immune response. Molecules that cause your body to mount an immune response are called antigens, and may include components of infectious agents like bacteria, viruses, and fungi. Within days, millions of antibodies — proteins that recognize the antigen and bind very tightly to it — are circulating in your bloodstream. Like magic bullets, antibodies seek out and attach themselves to their target antigens, flagging the invaders for destruction by other cells of the immune system.

Over 100 years ago, biologists found that animals' immune systems respond to invasion by "foreign entities", or antigens. Today, antibodies have become vital scientific tools, used in biotechnology research and to diagnose and treat disease. The number of different antibodies circulating in the blood has been estimated to be between 10^6 and 10^{11}, so there is usually an antibody ready to deal with any antigen. In fact, antibodies make up to 15% of your total blood serum protein. Antibodies are very specific; each antibody recognizes only a single antigen.



A) Structure of IgG bound to the HIV capsid protein p24 as determined by X-ray crystallography (Harris et al. 1998, Momany et al. 1996). These structures can be downloaded from the Protein Data Bank (www.pdb.ufmg.br, (Berman et al. 2000) using the PDB identification codes 1IGY and 1AFV and manipulated using free online software such as Rasmol and Protein Explorer. **B)** A commonly used representation of an antibody bound to an antigen.

How Are Antibodies Made?

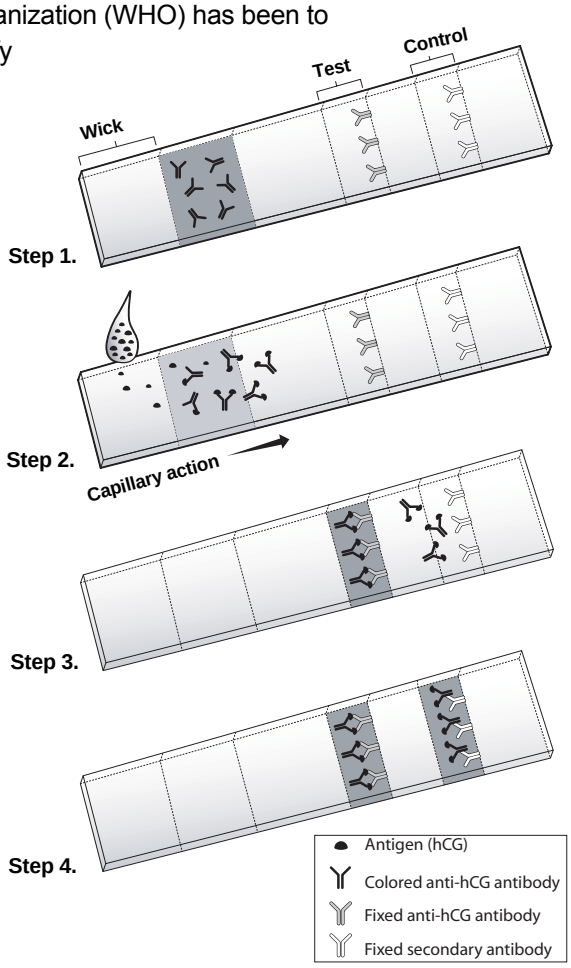
Scientists have learned to use the immune response of animals to make antibodies that can be used as tools to detect and diagnose diseases. The study of the immune system is called "immunology". Animals such as chickens, goats, rabbits, and sheep can be injected with an antigen and, after a period of time, their serum will contain antibodies that specifically recognize that antigen. If the antigen was a disease agent, the antibodies can be used to develop diagnostic tests for the disease. In an immunoassay, the antibodies used to recognize antigens like disease agents are called primary antibodies; primary antibodies confer specificity to the assay.

Other kinds of antibody tools, called secondary antibodies, are made in the same way. In an immunoassay, secondary antibodies recognize and bind to the primary antibodies, which are antibodies from another species. Secondary antibodies are prepared by injecting antibodies made in one species into another species. It turns out that antibodies from different species are different enough from each other that they will be recognized as foreign proteins and provoke an immune response. For example, to make a secondary antibody that will recognize a human primary antibody, human antibodies can be injected into an animal like a rabbit. After the rabbit mounts an immune response, the rabbit serum will contain antibodies that recognize and bind to human antibodies. The secondary antibodies used in this experiment are conjugated to the enzyme horseradish peroxidase (HRP) which produces a blue color in the presence of its substrate, TMB. These antibody and enzyme tools are the basis for the ELISA.

Where Is ELISA Used in the Real World?

With its rapid test results, the ELISA has had a major impact on many aspects of medicine and agriculture. ELISA is used for such diverse purposes as pregnancy tests, disease detection in people, animals, and plants, detecting illegal drug use, testing indoor air quality, and determining if food is labeled accurately. For new and emerging diseases like severe acute respiratory syndrome (SARS), one of the highest priorities of the US Centers for Disease Control (CDC) and the World Health Organization (WHO) has been to develop an ELISA that can quickly and easily verify whether patients have been exposed to the virus.

Some tests give positive or negative results in a matter of minutes. For example, home pregnancy dipstick tests are based on very similar principles to ELISA. They detect levels of human chorionic gonadotropin (hCG), a hormone that appears in the blood and urine of pregnant women within days of fertilization. The wick area of the dipstick is coated with anti-hCG antibody labeled with a pink compound (step 1). When the strip is dipped in urine, if hCG is present it will bind to the pink antibody, and the pink hCG-antibody complex will migrate up the strip via capillary action (step 2). When the pink complex reaches the first test zone, a narrow strip containing an unlabeled fixed anti-hCG antibody, the complex will bind and concentrate there, making a pink stripe (step 3). The dipsticks have a built-in control zone containing an unlabeled fixed secondary antibody that binds unbound pink complex (present in both positive and negative results) in the second stripe (step 4). Thus, every valid test will give a second pink stripe, but only a positive pregnancy test will give two pink stripes.



Why Do We Need Controls?

Positive and negative controls are critical to any diagnostic test. Control samples are necessary to be sure your ELISA is working correctly. A positive control is a sample known to be positive for the disease agent, and a negative control is a sample that does not contain the disease agent.

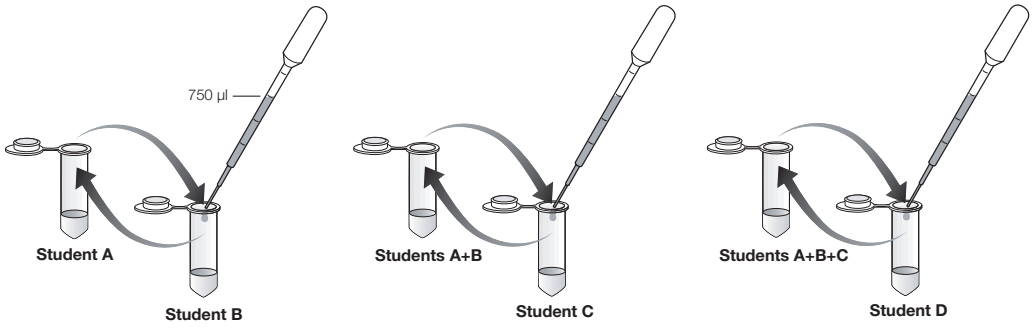
Your Task Today

You will be provided the tools and an experimental protocol to perform an ELISA. You will be given a simulated “body fluid” sample that you will share with your classmates. One or two of the samples in the class have been “infected”. You will also be provided with positive and negative control samples. Then you and your fellow students will assay your samples for the presence of the “disease agent” to track the spread of the disease through your class population.

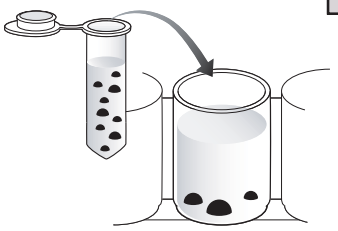
Now let’s put this all together.

Your task will be to:

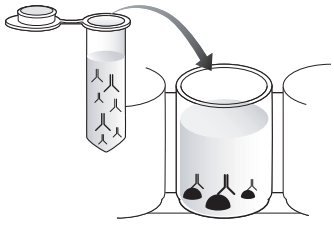
1. Share your (simulated) body fluids randomly with your classmates.



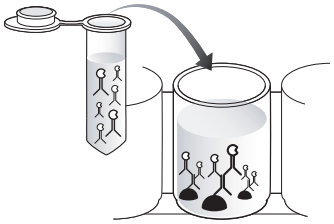
2. Add your shared sample plus control samples to the wells in a microplate strip. Your samples contain many proteins and may or may not contain the disease agent (antigen). Incubate for 5 minutes to allow all the proteins in the samples to bind to the plastic wells via hydrophobic interaction. This is an immunosorbent assay because proteins adsorb (bind) to the plastic wells.



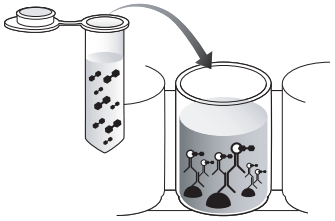
3. Add anti-disease antibody (primary antibody) to the wells and incubate. The primary antibody will seek out the antigen from the many proteins bound to the well. If your sample was "infected", the antibodies will bind tightly to the disease agent (antigen) in the wells.



4. Detect the bound antibodies with HRP-labeled secondary antibody. If the primary antibodies have bound to the antigen, the secondary antibodies will bind tightly to the primary antibodies.



5. Add enzyme substrate to the wells, wait 5 minutes, and evaluate the assay results. If the disease antigen was present in your sample, the wells will turn blue. This is a positive diagnosis. If the wells remain colorless, the disease antigen was not present in your sample and the diagnosis is negative.



Pre-Lab Focus Questions

1. How does the immune system protect us from disease?

2. How do doctors use the immune response to protect you from disease?

3. What are some ways that diseases spread?

4. What is an example of a disease that attacks the human immune system?

5. What problems can prevent the immune system from working properly?

6. Why are immunosuppressant drugs necessary when someone has an organ transplant?

7. Why is rapid detection of disease exposure important?

8. What does ELISA stand for?

9. Why are enzymes used in this immunoassay?

10. Why do you need to assay positive and negative control samples as well as your experimental samples?

Laboratory Guide

Student Workstation Checklist

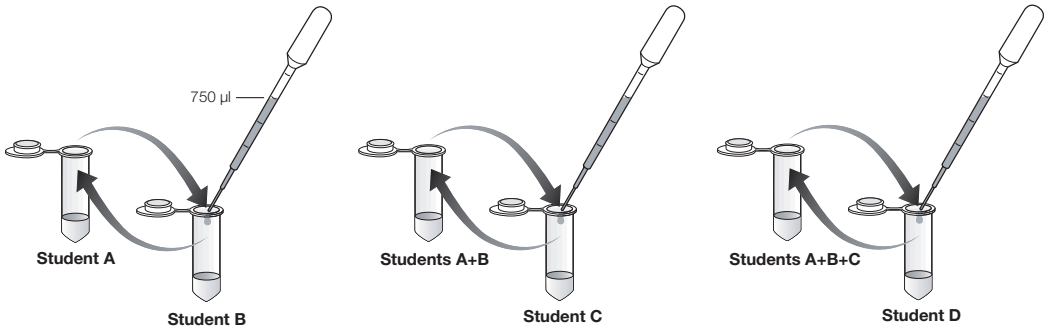
One workstation serves 4 students

Items	Contents	Number	(✔)
Yellow tubes	Student samples (0.75 ml)	4 (1 per student)	❑
Violet tube (+)	Positive control (0.5 ml)	1	❑
Blue tube (–)	Negative control (0.5 ml)	1	❑
Green tube (PA)	Primary antibody (1.5 ml)	1	❑
Orange tube (SA)	Secondary antibody (1.5 ml)	1	❑
Brown tube (SUB)	Enzyme 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 pipets		5	❑
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

Share Body Fluids

1. Label each yellow tube with your initials. These are your "body fluid" samples that will be shared randomly with your classmates.
2. Label a plastic transfer pipet with your initials; you will use this to mix your sample with your fellow students.
3. When you are told to do so, find another student and use a pipet to transfer all 750 µl of your sample into the tube of the other student. (It doesn't matter whose tube is used to mix both samples.) Gently mix the samples by pipetting the mixture up and down. Then take back half of the shared sample (about 750 µl) to your own tube. Write down the name of that student next to "Sharing Partner #1" below.



Sharing Partner #1 _____

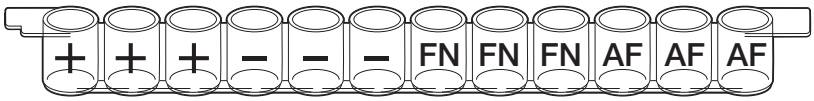
Sharing Partner #2 _____

Sharing Partner #3 _____

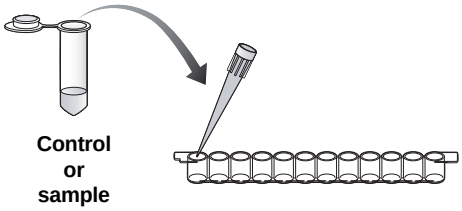
4. When told to do so, repeat the sharing protocol two more times with 2 other students so that you have shared your sample with 3 other students total. Make sure that you record their names in the order in which you shared. Discard your transfer pipets after this step. You may proceed directly to the next step or store your samples overnight at 4°C.

Perform ELISA

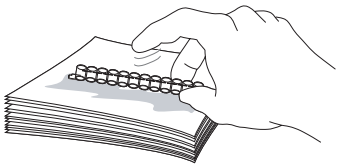
5. Label the outside wall of each well of your 12-well strip. Two students may share a strip of 12 wells. On each strip, label the first three wells with a "+" for the positive controls and the next three wells with a "–" for the negative controls. On the remaining wells write your and your partner's initials. For example, Florence Nightingale and Alexander Fleming would label their shared strip like this:



6. Bind the antigen to the wells:
    a. Use a pipet to transfer 50 µl of the positive control (+) from the violet tube into the three "+" wells.
    b. Use a <u>fresh</u> pipet tip to transfer 50 µl of the negative control (–) from the blue tube into the three "–" wells.
    c. Use a <u>fresh</u> pipet tip for each sample and transfer 50 µl of each of your team's samples into the appropriately initialed three wells.

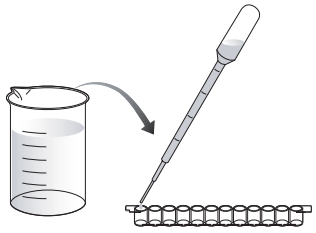


7. Wait 5 minutes while all the proteins in the samples bind to the plastic wells.
8. Wash the unbound sample out of the wells:
    a. Tip the microplate strip upside down onto the paper towels so that the samples drain out, then gently tap the strip a few times upside down on the paper towels. Make sure to avoid samples splashing back into wells.

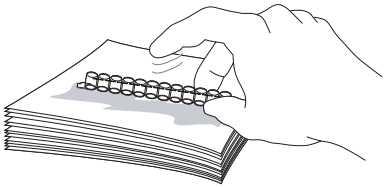


    b. Discard the top paper towel.

c. Use a fresh 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.

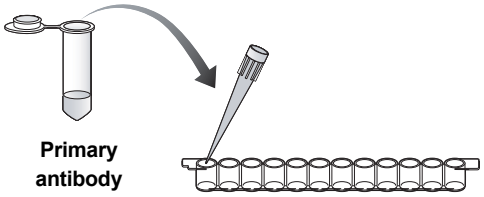


d. Tip the microplate strip upside down onto the paper towels so that the wash buffer drains out, then gently tap the strip a few times upside down on the paper towels.

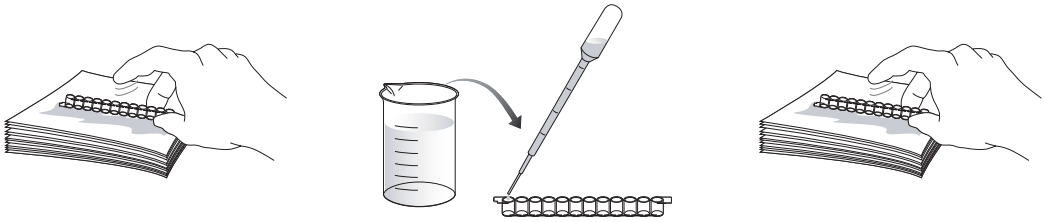


e. Discard the top 2–3 paper towels.

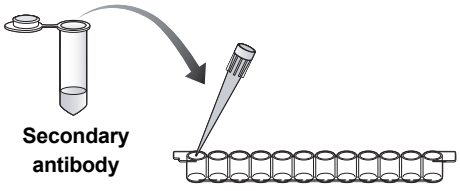
9. Repeat wash step 8.
10. Use a fresh pipet tip to transfer 50 µl of primary antibody (PA) from the green tube into all 12 wells of the microplate strip.



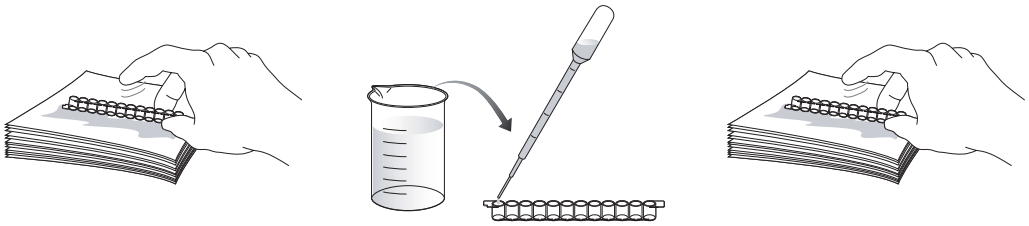
11. Wait 5 minutes for the primary antibody to bind.
12. Wash the unbound primary antibody out of the wells by repeating wash step 8 **two** times.



13. Use a fresh pipet tip to transfer 50 µl of secondary antibody (SA) from the orange tube into all 12 wells of the microplate strip.



14. Wait 5 minutes for the secondary antibody to bind.
15. Wash the unbound secondary antibody out of the wells by repeating wash step 8 **three** 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 and which should remain colorless and which wells you are not sure about.

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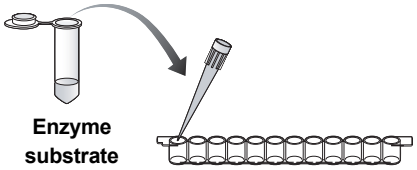
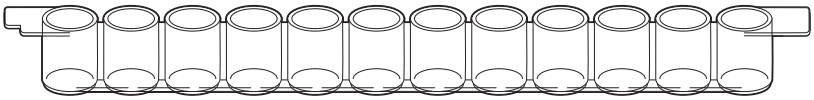


16. Use a fresh pipet tip to transfer 50 µl of enzyme substrate (SUB) from the brown tube into all 12 wells of the microplate strip.
17. Wait 5 minutes. Observe and record your results.

Results Section

Label the figure below with the same labels you wrote on the wells in step 5. In each of the wells, put a "+" if the well turned blue and a "–" if there is no color change.



Are you "infected" with the disease? YES/NO (circle one)

You can track the progress of the disease through the class by having each student record whether they tested positive or negative next to the names of the people with whom they shared "body fluids".

Class Results For Tracking Disease Transmission

Student's name	+ /–	Sharing partners (+ or –)	Student's name	+ /–	Sharing partners (+ or –)

Post-Lab Focus Questions

1. The samples that you added to the microplate strip contain many proteins and may or may not contain the disease antigen. What happened to the proteins in the plastic well if the sample contained the antigen? What if it did not contain the antigen?

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

3. When you added primary antibody to the wells, what happened if your sample contained the antigen? What if it did not contain the antigen?

4. When you added secondary antibody to the wells, what happened if your sample contained the antigen? What if it did not contain the antigen?

5. If the sample gave a negative result for the disease-causing agent, does this mean that you do not have the disease? What reasons could there be for a negative result when you actually do have the disease?

6. Why did you assay your samples in triplicate?

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

8. If you tested positive for disease exposure, did you have direct contact with one of the original infected students? If not, what conclusions can you reach about transmissibility of disease in a population?