Protocol I: ELISA for Tracking Disease Outbreaks

Instructor's Guide

This interactive procedure provides a context for introducing a real-world, topical application of ELISA. Students first model the spread of disease in a population by the sharing of simulated "body fluids". Each student is given a sample to share, one or two of which are positive for the "disease agent". After students share their "body fluids", they assay their shared samples using ELISA.

Students' ELISA results reveal that a large portion of the class now tests positive for the disease! This leads to a guided, inquiry-based activity about how the disease has spread through the population. Because students have a personal connection to the results, this activity tends to captivate students' imaginations and is particularly relevant in light of the recently emerged contagious disease SARS.

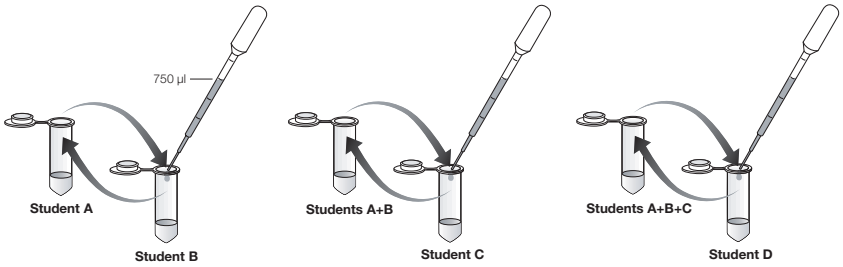
Many other diseases also work with this protocol, including West Nile virus, HIV, colds, influenza, and STDs, to name a few. A review of Appendix C will provide useful information about appropriate diseases and scenarios for implementation in the classroom. A simple approach may be to leave the actual disease unspecified for a generic lesson.

Implementation Timeline

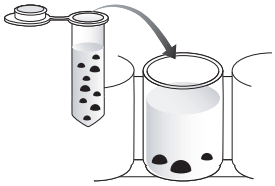
Lesson 1	Set the stage	Lecture and discussion
Lesson 2	Sharing of simulated body fluids	ELISA lab
Lesson 3	Analyze ELISA results	Tracking exercise

Instructor's Laboratory Overview

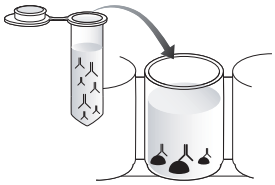
Step 1: Students share "body fluids" by mixing their sample with those of other students. Within each pair of students who share, each student takes back half of the combined sample. Each student repeats the sharing process with a different student either 1 or 2 more times (depending on class size) and records sharing partners. Note: To ensure dissemination of the "disease", the sharing must be performed in two or three separate rounds.



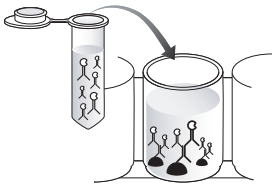
Step 2: Using a pipet, 50 µl of each student's sample (unknowns) along with positive and negative controls are added to the wells of the microplate strip and incubated for 5 minutes, allowing proteins in the sample to bind to the wells. The wells are rinsed with wash buffer (PBST: phosphate buffered saline containing 0.05% Tween 20) that also blocks the unoccupied protein binding sites in the wells.



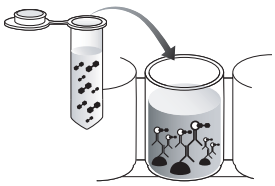
Step 3: Primary antibody (50 µl) is added to each well of the microplate strip and incubated for 5 minutes at room temperature. The primary antibody is an antibody that recognizes and binds to the "disease agent"/antigen. The wells are rinsed with wash buffer to remove unbound antibody.

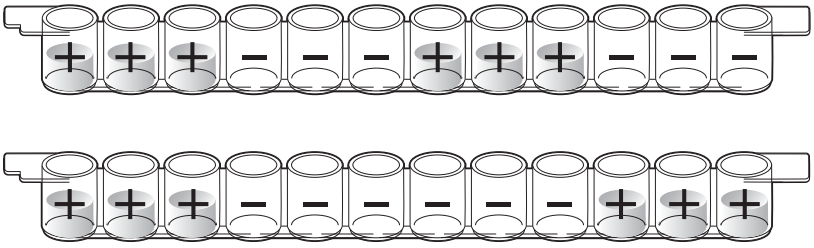


Step 4: Horseradish peroxidase (HRP)-labeled secondary antibody (50 µl) is added to each well and incubated for 5 minutes at room temperature. The secondary antibody is antibody that recognizes and binds to the primary antibody. HRP is an enzyme that will oxidize a color-producing substrate. Wells are rinsed with wash buffer to remove unbound secondary antibody.



Step 5: The enzyme substrate (50 µl) is added to each well and students watch color development. If HRP is present (meaning that the antigen was present in the sample), the solution in the wells will turn blue within 5 minutes. If the antigen was not present in the sample, the wells will remain colorless.





Typical ELISA results.

Using Students' ELISA Results to Track the "Disease"

The number of positive tests in the class results will depend on how many positive samples you released at the beginning. You can now track the progress of the disease through your class.

You may want the students to work out a method to track the disease to its source by themselves. For a more guided inquiry approach, we recommend using the class results table on page 32 in the student manual. Make a transparency from the page with the students' names listed and place it on an overhead projector. Ask each student to come up and write a plus (+) or a minus (-) in the second column to indicate if their ELISA tested positive or negative. Then, depending on whether their ELISA tested positive or negative, they should also write a plus (+) or a minus (-) respectively next to the names of the students with whom they shared their sample.

For example, if Kiko tested positive and shared with Alexander, Florence, and Mustafa, she would write a "+" by Alexander, Florence's, and Mustafa's names.

The students with all pluses against their names will be revealed as early sources of the infection.

Question: Why will the class not be able to track the infection to a single student?

Answer: When a single student who is the primary source of infection first shares his or her sample with a second student, the second student will also have all pluses. This is representative of the kind of problem that epidemiologists face in the real world. You may turn this occurrence to your advantage by discussing why epidemiologists investigate many factors when tracking diseases, such as patients' locations, histories, and behaviors, in addition to testing for the infection. You may also have your students perform a more detailed analysis involving tracking the order in which the samples were shared and deducing if some of the students can be eliminated from the pool of students suspected as being the original source.

Epidemiologists rarely have patient samples prior to the outbreak of infection, and rarely are they able to track an outbreak to a single source. However, you have the advantage of keeping a record of which students received the infected samples, which for the sake of this exercise may prove helpful. Alternatively, for a more anonymous approach, you may sequentially number all the student samples and record the numbers of tubes that are "infected". The source can be revealed at the end of the activity to see if it matches your students' data analysis.

Instructor's Advance Laboratory Preparation

This section is designed to help you prepare for the laboratory efficiently. We recommend that you read this section of the manual (Protocol I: Tracking Disease Outbreaks) in its entirety before beginning your preparation. In addition, if you are choosing to perform a scenario-based activity (for example, HIV testing), we recommend using the information given in Appendix C to help plan your lesson.

The most important thing for the students to do is to put the correct components in the assay wells in the correct order, so having the tubes clearly labeled and properly color-coded is crucial to a successful outcome.

Objectives

Step 1. Prepare buffers
Step 2. Rehydrate the freeze-dried antigen, primary antibody, and secondary antibody to make 50x stocks
Step 3. Dilute 50x stock solutions
Step 4. Dispense reagents for student workstations
Step 5. Set out student workstations

Time Required 1–3 hours

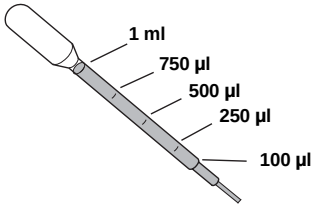
Preparation Timeframe

We recommend rehydrating and diluting the antigen and primary antibody no more than 3 days before the lesson, and the secondary antibody less than 24 hours before the lesson. We also suggest using sterile distilled water to prepare the 1x PBS to avoid contaminating rehydrated reagents. These reagents must be kept on ice or in the refrigerator if prepared more than 4 hours before the lesson.

Note: If you are planning to use this kit for multiple lab sessions over a 1- or 2-week period, we strongly suggest using sterile water to prepare the PBS buffer in order to avoid contaminating reagents. (Water can be sterilized by boiling it in a microwave oven for 5 minutes in a loosely capped bottle; after you remove the bottle from the microwave oven, let it cool, then secure the cap.) Dilute only as much concentrated antibody and antigen as required for each lab session. The rehydrated antibodies are 50x concentrates. Store the remaining concentrated antigen and antibodies in the refrigerator at 4°C. We do not recommend storing the concentrated antibody and antigen for more than 2 weeks, even at 4°C. Do not freeze the solutions.

Volume Measurements

This kit contains graduated disposable plastic transfer pipets (DPTPs) to use for preparing some of the reagents where volumes between 250 microliters (µl) and 5 milliliters (ml) are required. In addition, adjustable- or fixed-volume micropipets are needed to measure 50 µl volumes. The illustration shows the marks on the DPTP corresponding to the volumes to be measured. Volumes over 1 ml will require multiple additions. For each step of the laboratory preparation, use a fresh DPTP or a fresh pipet tip.



Measuring liquids that contain detergents that foam (e.g., the wash buffer) requires that you read the volume at the interface of the liquid and the bubbles.

PROTOCOL I: Step-by-Step Instructor's Advance Preparation Guide

These instructions are for the setup of 12 student workstations of 4 students each.

Supplied Reagents	Quantity
Antigen, chicken gamma globulin, freeze-dried	1 vial
Primary antibody, rabbit anti-chicken polyclonal antibody, freeze-dried	1 vial
Secondary antibody, goat anti-rabbit antibody conjugated to (HRP), freeze-dried	1 vial
HRP enzyme substrate (TMB)	1 bottle
10x phosphate buffered saline (PBS)	1 bottle
10% Tween 20	1 bottle
Required Reagent	
Distilled water, sterile is recommended, see note on page 15	1 L

Step 1. Prepare buffers.

We recommend you use a 100 ml and a 1 liter (L) graduated cylinder for preparing the buffer solutions. You will also need 1 L of distilled water.

Buffer	Volume	Reagent	Used for
1x PBS, 100 ml	90 ml	Distilled water	• Rehydrating antigen, Primary and secondary antibodies to make 50x reagent stock solutions • Diluting 50x antigen to make positive control and "infected" student samples • Negative control • Negative student samples
	10 ml	10x PBS	
Wash Buffer, 900 ml	805 ml	Distilled water	• Dilution of 50x antibody stocks • Plate washing
	90 ml	10x PBS	
	4.5 ml	10% Tween 20	

Step 2. Rehydrate the freeze-dried antigen, primary antibody, and secondary antibody.

Carefully remove the stoppers from the three freeze-dried reagents and use a fresh DPTP to add 0.5 ml 1x PBS to each. Close the stoppers and shake to mix. These solutions are 50x concentrates, or stock solutions. **NOTE: You must <u>not</u> use wash buffer in this step**.

Freeze-Dried Reagent	Protocol for 50x Stock Solution	Used for
Antigen	Add 0.5 ml of 1x PBS to vial	• Positive control • "Infected" student samples
Primary antibody	Add 0.5 ml of 1x PBS to vial	• Primary antibody
Secondary antibody	Add 0.5 ml of 1x PBS to vial	• Secondary antibody

Step 3. Dilute 50x stock reagents.

Label one 30 ml bottle for each of the diluted solutions below. Use a fresh DPTP to add the contents of the appropriate 50x concentrated stock to the corresponding 30 ml bottle.

Diluted solution	Volume	Reagent	Used for
Positive control (1x antigen), label one 30 ml bottle	7.5 ml	1x PBS	Positive control
	150 µl	50x antigen stock	
	NOTE: you must not add any buffer containing Tween 20 to the antigen, or the experiment will not work.		
1x primary antibody, label one 30 ml bottle	24.5 ml	Wash buffer	Primary antibody
	0.5 ml	50x primary antibody stock	
	• Use the DPTP to rinse out the vial with some of the diluted reagent to ensure that all of the stock solution is used. • Close the cap and shake to mix.		
1x secondary antibody, label one 30 ml bottle	24.5 ml	Wash buffer	Secondary antibody
	0.5 ml	50x secondary antibody stock	
	• Dilute the secondary antibody less than 24 hours before the start of the lesson. Use the DPTP to rinse out the vial with some of the diluted reagent to ensure that all of the stock solution is used. • Close the cap and shake to mix.		

Step 4. Dispense reagents for student workstations.

Tubes	Description	Label	Contents (Each Tube)	
Violet tubes, 12	Positive control	"+"	0.5 ml	positive control solution (1x antigen)
Blue tubes, 12	Negative control	"–"	0.5 ml	1x PBS
Green tubes, 12	Primary antibody	"PA"	1.5 ml	1x primary antibody solution
Orange tubes, 12	Secondary antibody	"SA"	1.5 ml	1x secondary antibody solution
Brown tubes, 12	Enzyme substrate	"SUB"	1.5 ml	HRP enzyme substrate (TMB)
	Note: TMB is light sensitive, so it is important to use the dark tubes to store this reagent.			
Yellow tubes, # depends on # of students (1–3 tubes)	"Infected" student sample(s) (6.6x antigen)	Determined by instructor	100 µl	50x antigen stock solution
			650 µl	1x PBS
	The number of students in the class will determine the number of "infected" samples you mix with the blanks. For a result where about half the students become infected, we recommend making one infected sample per 16 students. (Note: if your class is less than 10 students, use a single sample and perform just 2 rounds of sharing.) **Note: You must not add any buffer containing Tween 20 to the antigen, or the experiment will not work**. You may want to keep the infected samples separate from the blank samples until the lesson to keep track of who receives them.			

Tubes	Description	Label	Contents (Each Tube)
Yellow tubes, # depends on # of students	Blank (uninfected) student samples	Determined by instructor*	750 µl 1x PBS
	Make enough blank yellow tubes for your student number minus the "infected" samples. **Note: You must use 1x PBS and not wash buffer for the blank students samples, or the experiment will not work.**		
* (Optional) For your own information, you may wish to number each tube of student sample (infected and blank) from 1 to 48 and record which numbered tubes contain the infected samples.			

Step 5. Set out student workstations.

Student Workstation Checklist

One workstation serves 4 students.

Item (Label)	Contents	Number	(✔)
Yellow tubes	Student test 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	❑

Note on sharing protocol: Make sure that the students share “body fluids” with students from other parts of the room, not just with their near neighbors. The best way to do this is to have orderly sharing: Tell the students to share with one other person, then return to their lab station. After all students are finished with the first sharing and are back in place, then tell them to share with a different person. The degree of sharing will depend on your class size. We recommend one "infected" sample per 16 students. If your class size is less than 10, use a single "infected" sample and perform just two rounds of sharing.

Stopping points: Although this procedure is designed to fit into a single lesson period, you may stop the laboratory activity after sharing the “body fluids” and place all the reagents in the refrigerator at 4°C overnight. Alternatively, if you wish to stop during the ELISA you may add wash buffer to the microplate wells at any stage after the addition of antigen and prior to the addition of enzyme substrate. Place the microplate strips and all the reagents in the refrigerator at 4°C overnight.

Setting Up The Activity to Test for a Specific Disease (e.g., HIV)

Appendix C provides information on a variety of diseases that can be diagnosed using ELISA. In addition, for each disease, we provide a table describing what the reagents for the activity represent in a real-world diagnostic ELISA. Below is an example of a diagnostic test to detect HIV viral proteins in a patient's blood sample.

Detecting p24 HIV-1 Capsid Protein.

Tube Description	Tube Color	Actual Tube Contents	Simulated Tube Contents
Student samples (unknowns)	Yellow	1x antigen or	Sample derived from patient's blood 1x PBS
Primary antibody	Green	1x primary antibody	Anti-p24 capsid protein antibody from mouse
Secondary antibody	Orange	1x secondary antibody	Anti-mouse immunoglobulin antibody conjugated to HRP
Positive control	Violet	1x antigen	Heat-inactivated viral antigen (p24 protein)
Negative control	Blue	1x PBS	HIV negative human serum

Instructor's Answer Key and Discussion Points

Pre-Lab Focus Questions

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

   The immune system includes physical barriers, such as the skin and mucous membranes that prevent pathogens from entering the body, and cellular responses, such as circulating macrophages that respond to foreign invaders. Our acquired immune system mounts a specific antibody response when the body is exposed to a foreign invader, and our immune cells attack the invader.

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

   Doctors use the immune response when we are vaccinated against diseases. Our immune system remembers the pathogens to which we have been exposed, and the next time we are exposed to the pathogens our immune system attacks them more quickly and efficiently. Doctors take advantage of this priming effect by exposing us to inactivated pathogens (killed or weakened organisms that cannot make us sick) so that if we are later exposed to the live pathogen, our body will mount a strong and immediate antibody response, reducing or eliminating the chance that it will make us sick.

3. **What are some ways that diseases spread?**

Diseases Can Spread Through:	Examples:
Exchange of bodily fluids	• HIV, SARS, Epstein-Barr virus (cause of mononucleosis), STDs
Ingestion of contaminated food or water	• *E. coli* O157:H7, prions that cause Creutzfeldt-Jakob and mad cow diseases, protozoa that cause giardiasis, nematodes that cause trichinosis
Inhalation	• Viruses that cause the flu, bacteria that cause tuberculosis
Vector transfer	• Mosquito-borne diseases (malaria, West Nile virus, dengue fever, yellow fever), tick-borne diseases (Lyme disease, Rocky Mountain spotted fever)

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

   Diseases that attack the immune system include autoimmune diseases (e.g., rheumatoid arthritis, lupus, asthma, eczema, SCID) and AIDS. An extensive list can be found in Appendix A.

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

   Problems with the immune system fall into three categories: hypersensitivity, immunodeficiency, and autoimmune diseases. Hypersensitivity occurs when the immune system overreacts to an antigen; hypersensitivity reactions include anaphylactic reactions, allergies, and contact sensitivity (e.g., reaction to poison ivy). Immunodeficiency means that an individual cannot mount an effective immune response. Immunodeficiency may be genetic (e.g., SCID or "bubble boy" disease) or induced by a disease (e.g., immunodeficiency from HIV infection) or by immunosuppressive drugs (e.g., drugs given after organ transplant to prevent rejection). Autoimmune disease results from the immune system inappropriately mounting an immune response to

itself, for example, diseases like lupus, rheumatoid arthritis, multiple sclerosis (MS), insulin-dependent diabetes, and celiac disease.

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

   Immunosuppressive drugs (like prednisolone and cyclosporine) prevent the body from treating the transplanted organ as a foreign invader; availability of immunosuppressive drugs is largely responsible for the success of transplantation as a treatment for organ failure. Organs are rejected when the body mounts a strong immune response to the transplant. On the negative side, the action of immunosuppressive drugs is not specific and they suppress all immunological reactions. As a result, transplant recipients are very vulnerable to infections.

7. **Why is rapid detection of disease exposure important?**

   Rapid detection of disease exposure is important for several reasons. For many diseases, detecting the infection and beginning treatment early may reduce the severity of the symptoms or even prevent the disease completely. Rapid detection of disease exposure is also important to prevent further spread of the disease.

8. **What does ELISA stand for?**

   Enzyme-linked immunosorbent assay.

9. **Why are enzymes used in this immunoassay?**

   Enzymes provide a way to see whether the primary antibody has attached to its target (antigen) in the microplate well. Primary and secondary antibodies are invisible, so a detection method is necessary. The enzyme HRP is linked to the secondary antibody. HRP reacts with a colorless substrate in a chemical reaction that turns blue. If the secondary antibody is present in the well, the color change indicates a positive result.

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

    Controls are needed to make sure that the experiment worked. If there are no positive controls and the sample is negative, we can't know if the sample was truly negative or if the assay didn't work. Conversely, without a negative control, there is no way of knowing if all samples (positive or not) would have given a positive result.

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?**

   In either case, all the proteins present in the sample bind to the plastic wells.

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

   Washing removes any proteins that have not bound to the plastic wells and any antibodies that have not bound to their targets, thus preventing unbound proteins (either antigen or antibodies) from giving false positive results.

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?**

   If the sample contained the antigen, the primary antibody bound the antigen. If it did not contain the antigen, the primary antibody did not bind and was flushed out in the wash step.

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?**

   If the sample contained antigen, the secondary antibody bound to the primary antibodies already bound to antigen in the wells. If the test sample did not contain antigen, primary antibody did not bind in the wells, so the secondary antibody had nothing to bind and was flushed out in the wash step.

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?**

   A negative result does not necessarily mean that you do not have the disease. It could be a false negative. The ELISA may not be sensitive enough to detect very low levels of disease agent, such as the levels that might be present soon after infection. Another cause of false negatives is experimental error, such as putting a negative control into a well where you thought you were putting an experimental sample.

6. **Why did you assay your samples in triplicate?**

   Assaying the samples in triplicate is another control. If you do not get the same result in all triplicate wells, you have a problem with your experimental technique or you have made a pipetting error. In a clinical laboratory, the experiment would have to be repeated. If this error occurs in this activity, take the result of the two matching wells since this is probably correct.

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

   Test kits that are based on the same principles as the ELISA include home pregnancy and ovulation tests, and tests for the presence of illegal drugs such as marijuana and cocaine.

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?**

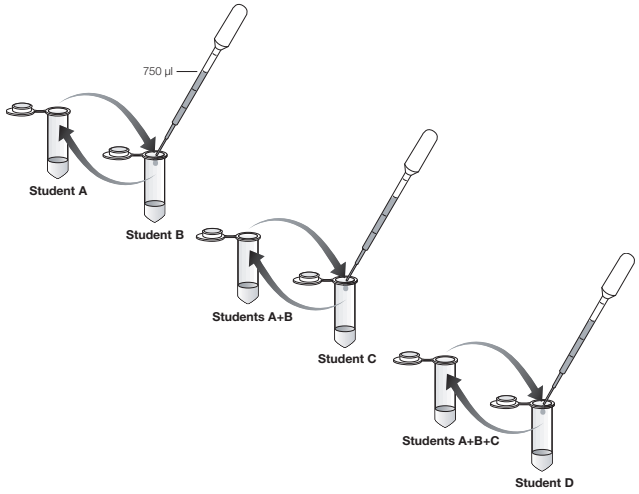
   Having intimate contact with another person means that you are exposed to any germs that a person may have contracted from any previous intimate contacts.

Laboratory Quick Guide

ELISA for Tracking Disease Outbreaks
Student Workstation Checklist

One workstation serves 4 students.

Item (Label)	Contents	Number	(✔)
Yellow tubes	Student test 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	❑



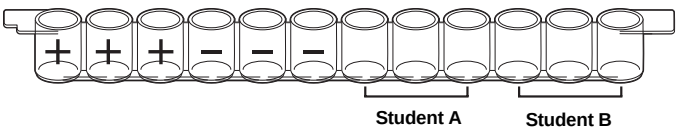
1. Label a yellow tube and a plastic transfer pipet with your initials.
2. Use the pipet to transfer all your "bodily fluid" sample into the tube of another student. Gently mix the samples, then take back half of the shared sample (750 µl) to your own tube. Write down the name of the student next to "Sharing Partner #1".
3. When instructed to do so, repeat the sharing protocol two more times. Discard this transfer pipet after this step.

   Optional stopping point: Samples may be stored at 4°C overnight.
4. Label your 12-well strip. On each strip label the first 3 wells with a "+" for the positive controls and the next 3 wells with a "–" for the negative controls. Label the remaining wells with your and your lab partner's initials (3 wells each).

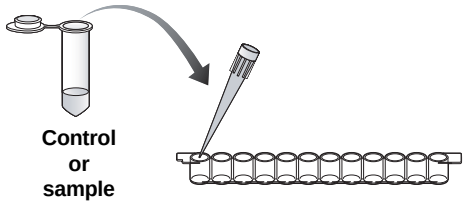
Sharing Partner #1 _____

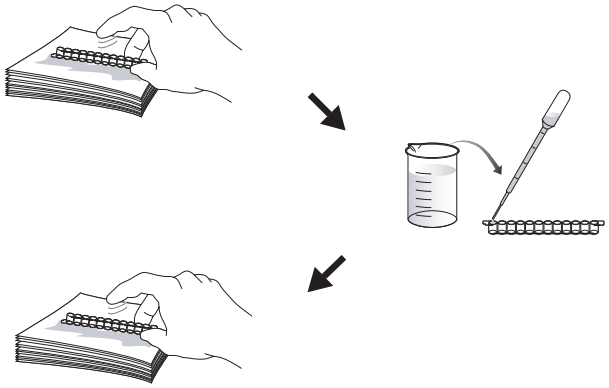
Sharing Partner #2 _____

Sharing Partner #3 _____



5. Use a fresh pipet tip to transfer 50 µl of the positive control (+) into the three "+" wells.
6. Use a fresh pipet tip to transfer 50 µl of the negative control (–) into the three "–" wells.
7. Transfer 50 µl of each of your team's samples from step 3 into the appropriately initialed three wells, using a fresh pipet tip for each sample.
8. Wait 5 minutes while all the proteins in the samples bind to the plastic wells.
9. WASH:
   a. Tip the microplate strip upside down onto the paper towels, and gently tap the strip a few times upside down. Make sure to avoid samples splashing back into wells.
   b. Discard the top paper towel.
   c. Use a fresh transfer pipet to fill each well with wash buffer, taking care not to spill over into wells. Note: the same transfer pipet is used for all washing steps.
   d. Tip the microplate strip upside down onto the paper towels and tap.
   e. Discard the top 2–3 paper towels.

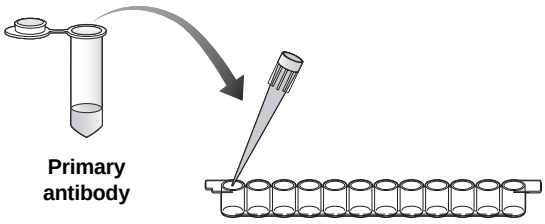




WASH

10. Repeat wash step 9.

11. Use a fresh pipet tip to transfer 50 µl of primary antibody (PA) into all 12 wells of the microplate strip.

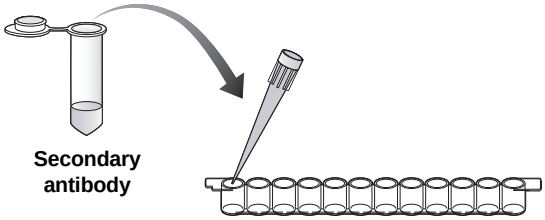


12. Wait 5 minutes for the antibodies to bind to their targets.

13. Wash the unbound primary antibody out of the wells by repeating all of wash step 9 **two** times.

WASH 2x

14. Use a <u>fresh</u> pipet tip to transfer 50 µl of secondary antibody (SA) into all 12 wells of the microplate strip.

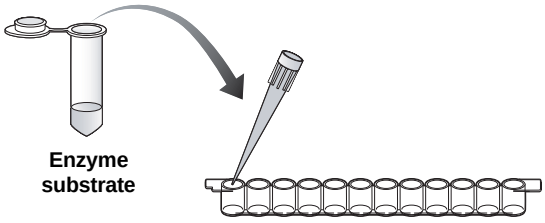


15. Wait 5 minutes for the antibodies to bind to their targets.

16. Wash the unbound secondary antibody out of the wells by repeating wash step 9 **three** times.

WASH 3x

17. Use a <u>fresh</u> pipet tip to transfer 50 µl of enzyme substrate (SUB) into all 12 wells of the microplate strip.



18. Wait 5 minutes. Observe and record the results.