The study of immunology is a complex and often abstract endeavor. The key aspects are chemical in nature and therefore difficult to visualize. A number of chemical techniques have been developed to visually represent antibodies and antigens present in the serum, the fluid portion of the blood with the clotting factors removed. The study and use of these techniques is referred to as serology. These tests are performed in vitro, outside of the body, and are primarily used as diagnostic tools to detect disease. Other applications include pregnancy testing and drug testing. These immunological techniques depend upon the principle that an antibody binds only to specific corresponding antigens. The tests are relatively expensive to perform, so this computer simulation will allow you to perform them without the sometimes cost-prohibitive supplies.

Antigens and Antibodies

Antigens are by definition antibody generators. They include proteins, polysaccharides, and various small molecules that stimulate antibody production. Antigens are often molecules that are described as “non-self,” or foreign, to the body. However, there are also “self-antigens” that act as identifier tags, such as the proteins found on the surface of red blood cells. Most often, antigens are a portion of an infectious agent such as a bacterium or a virus, and the antibodies that are produced are the body’s response to the presence of the infectious agent. Antigens are often large, with multiple antigenic sites. We refer to these sites as antigenic determinants, or epitopes. The antibody has a corresponding antigen binding site that has a “lock and key” recognition for the epitope on the antigen. All of the activities presented in this module take advantage of antigen-antibody specificity. They include direct fluorescent antibody technique, Ouchterlony technique (double diffusion), enzyme-linked immunosorbent assay and Western blotting technique.

Positive and Negative Controls

The “lock and key” recognition that antigen and antibody have for each other is much like the specificity that an enzyme and its substrate have for one another. However, with antigen and antibody non-specific binding is sometimes
possible. For this reason, a number of washing steps to remove any non-specific binding that may have occurred are a common theme in the following experiments. In addition, you will also use positive and negative controls to ensure that the test is working accurately. Positive controls include a substance that is known to react positively, thus giving you a standard on which to base your results. Negative controls include substances that should not react. A positive result with a negative control is a “false positive,” which would invalidate all other results. Likewise a negative result with a positive control is a “false negative,” which would also invalidate your results.

Direct Fluorescent Antibody Technique

The direct fluorescent antibody technique uses antibodies to directly detect the presence of antigen. These antibodies have a fluorescent dye molecule attached to them that acts as a visual signal for a positive result. The antigens that are typically tested for are from infectious agents such as bacteria and viruses. In this experiment you will test for the presence of Chlamydia trachomatis, a bacterium that invades the cells of its host, by using fluorescently labeled antibodies to detect the presence of the bacterium. Chlamydia trachomatis is an important infectious agent because it causes a sexually transmitted disease known as Chlamydia. If this disease is left untreated it can lead to sterility in men and women.

In this experiment you will test three patients for the Chlamydia infection. The specimen collected from each patient is an epithelial scraping from the orage of the cervix. Chlamydia trachomatis is an obligate intracellular bacterium, which means that it can only survive inside a host cell. The life cycle of the bacterium has two cellular types. The infectious cell type is the small, dense elementary body that is capable of attaching to the host cell. The reticulate body is a larger, less dense cell that divides actively, also referred to as the vegetative form. The life cycle of Chlamydia begins when the elementary body enters the host cell and continues as the reticulate body divides and the reticulate body releases to infect other cells. The elementary bodies are measured by reacting antigen-specific antibodies to infected cells. The antigen-specific antibodies’ fluorescent dye makes the complex detectable. The sample is viewed with a fluorescent microscope. The presence of ten or more elementary bodies in a field of view with a diameter of 5 millimeters is considered a positive result. The elementary bodies will be stained green inside red host cells.

Choose Exercise 12: Serological Testing from the dropdown menu and click GO. Then click Direct Fluorescent Antibody Test. The opening screen of the Direct Fluorescent Antibody lab will appear in a few seconds (Figure 12.1). Use the Balloons On/Off feature from the Help menu to familiarize yourself with the lab equipment.

When you begin the simulation, there will be a shelf at the top of the screen that stores the reagents that you will use for this activity. At the far left is 95% ethyl alcohol that is used as a fixing agent when preparing specimens for microscopy. The bottle labeled Chlamydia F.A. contains the fluorescently-labeled antibodies that are specific for the Chlamydia antigen. The F.A. Mounting bottle contains a special fixative that you will use to preserve the final specimen. The PBS squirt bottle is filled with phosphate-buffered saline to remove any excess antibodies that could bind nonspecifically and lead to erroneous results. The F.A. Buffer is an additional buffer that is designed to remove excess ethyl alcohol after the fixing step. Near the middle of the shelf is a stack of Petri dishes that you will use to house the slides and keep them moist during the incubation step. Moving to the right, there is a stack of glass microscope slides to prepare patient samples and controls. In the next container are cotton-tipped applicators to apply the Chlamydia-fluorescent antibodies to the slide. Next, there’s a stack of filter paper that has been moistened with F.A. buffer. The filter paper will keep the slides moist during the incubation step. Finally, on the far right are bottles containing the patient samples, labeled A, B, and C, and the positive and negative controls, labeled + and –.

Below on the left is the fluorescent microscope to magnify and observe the final preparation. The incubator is on the far right with a timer just to the left. The incubator will automatically open when it is needed. Lower on the screen is the workbench where the microscope slides will be placed along with the Petri dishes. On the bottom is a red biohazard bag for contaminated materials.

Once the samples have been prepared and viewed in the microscope, you will count the number of elementary bodies and record the results in Chart 1 on the next page.

**Activity 1**

Using Direct Fluorescent Antibody Technique to Test for Chlamydia

1. Individually click and drag five slides to the table.
2. The patient samples have been suspended in a small amount of buffer and placed in dropper bottles for ease of dispensing in this simulation. Click on the cap of patient sample A to grab the dropper. Drag the dropper over to the first slide. Release the mouse and the sample will dispense. The dropper will return to the bottle.
3. Repeat this process for the remaining patient samples and the positive and negative controls.
4. Click on the dropper cap of the 95% ethyl alcohol and drag the dropper over to the first slide. Release the mouse and three drops of ethyl alcohol will dispense onto each slide.
5. Set the timer for 5 minutes (compressed time) by pressing the (+) button and selecting Start. The ethyl alcohol will fix the sample to the slide and prevent the sample from being washed off in the subsequent washing steps.
6. Click on the squirt bottle labeled F.A. (Fluorescent Antibody) Buffer and drag it over to the first slide. The squirt bottle will rinse all five slides and remove excess ethyl alcohol.
7. Click on the wooden handle of an applicator stick. Move it over to the bottle labeled Chlamydia F.A., and release the mouse to soak its cotton tip with antibodies that are specific for Chlamydia and labeled with a fluorescent tag.
8. Click and drag the applicator over to the first slide. Release the mouse to apply the Chlamydia fluorescent antibody
to the slide. The applicator will automatically be disposed of in the biohazard bag.

9. Repeat steps 7 and 8 for the remaining slides.

10. Click and drag all five Petri dishes over to the table.

11. A piece of filter paper will be placed in each Petri dish. The filter paper has been moistened with F.A. buffer and will keep the samples from drying out during the incubation step that follows.

12. Click on each slide and drag it over to its Petri dish. Release and the slide will enter the dish and the lid will close.

13. After the last slide is added to the Petri dish, the Petri dishes will be automatically loaded into the incubator.

14. Use the plus (+) button to set the timer to 20 minutes, then select start. During incubation, the antibodies will react with the corresponding antigens that may be present in the sample.

15. The Petri dishes will automatically be removed from the incubator when the time is complete. Click on the squirt bottle of PBS (Phosphate Buffered Saline) and drag it to the first Petri dish. It will automatically squirt and rinse each sample to wash off excess antibodies and prevent non-specific binding of the antigen and antibody. The timer will count down 10 minutes for a thorough washing.

16. Click on the first Petri dish to open the dish and remove the slide. Repeat the process for each dish.

17. Click on each Petri dish and drag it to the biohazardous waste receptacle.

18. Click on the dropper for the F.A. Mounting media and drag it to the first slide. Release the mouse and the dropper will dispense. Click on the dropper to move it to each slide to mount the sample to the slide.

19. Click on a slide and drag it to the fluorescent microscope. Count the number of elementary bodies and record it in Chart 1 below. Recall that the elementary bodies stain green.

20. After recording the results for a slide, drop it into the biohazard bag for disposal. Complete the chart by repeating
these steps for each slide. Then use your results to answer the questions below.

Which patient(s) would be considered positive for Chlamydia?

Are we testing for the presence of antigen or antibody in the patient samples?

Did the controls give the expected results? Explain your answer.

Describe the significance of the washing steps.

---

<table>
<thead>
<tr>
<th>CHART 1</th>
<th>Direct Fluorescent Antibody Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>Number of Elementary Bodies</td>
</tr>
<tr>
<td>Patient A</td>
<td></td>
</tr>
<tr>
<td>Patient B</td>
<td></td>
</tr>
<tr>
<td>Patient C</td>
<td></td>
</tr>
<tr>
<td>Positive control</td>
<td></td>
</tr>
<tr>
<td>Negative control</td>
<td></td>
</tr>
</tbody>
</table>

Ouchterlony Technique

Ouchterlony technique is also known as double diffusion. In this technique, antigen and antibody diffuse toward each other in a semi-solid medium made up of clear, clarified agar. When the antigen and antibody are in optimal proportions, cross-linking of the antigen and antibody occurs, forming an insoluble precipitate called a precipitin line. These lines can then be used to visually identify and look for similarities between antigens. If optimum proportions have not been met—for example, if there is excess antigen or excess antibody—then no visible precipitate will form. This technique provides easily visible evidence of the binding between antigen and antibody, and sophisticated equipment is not needed to observe the antigen-antibody reaction.

The Ouchterlony technique is designed to determine if antigens are identical, related, or unrelated. Antigens that are related may have some but not all antigenic determinants in common. Antigens are said to have identity if they are identical. In the case of identity, precipitin lines diffuse into each other to completely fuse and form an arc. Antigens can also have partial identity if they are similar or if they are related. In the case of partial identity, a spur pointing toward the more similar antigen forms in addition to the arc. Antigens that have no similarities or are unrelated have a relationship referred to as non-identity. In this case the lines intersect to form two spurs that resemble an “X.”

In Ouchterlony technique, holes are punched into the agar to form wells. The wells are then loaded with either antigen or antibody, which are allowed to diffuse toward each other. Often the same antigen is placed in adjacent wells to assess the purity of an antigen preparation. In this case a smooth arc with no spurs should be seen, as the antigens are identical. Multiple antibodies can also be placed in a center well. The antibodies will diffuse out in all directions and react with the antigens that are placed in the surrounding wells. In this simulation, you will use human and bovine albumin as the antigens, and the antibodies will be made in goats against albumin from either humans or cows (bovine). The goals are to identify an unknown antigen and to observe the patterns produced by the various relationships: identity, partial identity, and non-identity.

From the Experiment menu, select Ouchterlony Double Diffusion. You will see the opening screen for the Ouchterlony Technique lab (Figure 12.2). Use the Balloons On/Off feature from the Help menu to familiarize yourself with the equipment on the screen.

In the upper left area of the screen there are several drop-Per bottles containing either antibodies or antigen. Starting from the far left, the bottle labeled Goat A-H, for goat anti-human albumin, contains antibodies made in a goat against human albumin. The next bottle is labeled Goat A-B, for goat anti-bovine albumin, and contains antibodies made in a goat against bovine albumin. The next three bottles contain various antigens: BSA (bovine serum albumin), HSA (human serum albumin) and an unknown antigen.

Below the top shelf, there is a stack of Petri dishes that contain clear agar that has been clarified to remove substances that could inhibit the diffusion. Slightly below is a workbench where you will set up the reactions in the Petri dishes. On the far right is a well cutter that will punch holes in the agar.

Just below the well cutter is a timer to time the diffusion process. When the time has elapsed and the diffusion has progressed, you will record your results in Chart 2 below.

ACTIVITY 2

Comparing Samples with Ouchterlony Double Diffusion

1. Click and drag a Petri dish over to the workbench. The lid will open to reveal an enlarged view of the inside of the Petri dish.
2. Click and drag the well cutter to the enlarged view of the Petri dish. Punch a hole in the middle of the Petri dish by releasing the mouse button. Now punch four more holes around the center hole.

3. After the fifth well is punched, the well cutter will return to the shelf and the wells will be labeled 1 through 5.

4. Click on the dropper of the bottle labeled Goat A-H. This bottle contains the goat anti-human albumin, which is an anti-serum containing antibodies produced by goats against human albumin. Drag the dropper to well 1 and fill it with a sample.

5. Repeat this procedure for the bottle labeled Goat A-B, which contains the goat anti-bovine albumin. This is an anti-serum containing antibodies produced by goats against bovine (cow) albumin. Release the mouse to also add this solution to well 1.

6. Click on the dropper for the bottle labeled BSA, which contains bovine serum albumin. Drag the dropper over to well 2 and release to dispense BSA.

7. Dispense BSA into well 3 using the same procedure as step 6.

8. Click on the dropper of the bottle labeled HSA, which contains human serum albumin. Drag the dropper over to well 4 and release to dispense HSA.

9. Click on the dropper for the bottle labeled Unknown and drag it over to well 5. Release the mouse to dispense the unknown into well 5.

10. Use the plus (+) button to set the timer to 16 hours. Click on the Start button to start the timer. The antigen and antibodies will diffuse toward each other and form a precipitate detected as a precipitin line.

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**FIGURE 12.2 Opening screen of the Ouchterlony Technique lab.**

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<table>
<thead>
<tr>
<th>CHART 2</th>
<th>Ouchterlony Double Diffusion Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wells</td>
<td>Identity</td>
</tr>
<tr>
<td>2 and 5</td>
<td></td>
</tr>
<tr>
<td>2 and 3</td>
<td></td>
</tr>
<tr>
<td>3 and 4</td>
<td></td>
</tr>
<tr>
<td>4 and 5</td>
<td></td>
</tr>
</tbody>
</table>
Examine the precipitin lines that formed. Fill out Chart 2 by determining if each pair has identity, partial identity, or non-identity.

What is the identity of the unknown? How do you know this?

Which components are the antigens in this experiment?

What role does albumin usually play in serum?

Why do you think the agar needs to be clear and clarified?

---

**Enzyme-Linked Immunosorbent Assay (ELISA)**

ELISA is an acronym for enzyme-linked immunosorbent assay. The assay is considered enzyme-linked because an enzyme is chemically linked to an antibody in both the direct and indirect versions of the test. Immunosorbent refers to the fact that either antigens or antibodies are being adsorbed (stuck) to plastic. ELISA can test for the presence of antigen or antibody. If the test is designed to detect an antigen or antigens, it is a direct ELISA because it is directly looking for the foreign substance. An indirect ELISA is designed to detect antibodies that the patient has made against the antigen. Seroconversion occurs when a patient goes from testing negative for a specific antibody to testing positive for the same antibody.

In the direct ELISA, the 96-well-microtiter plate is coated with antigens. The large number of wells makes it easy to test many samples at a time. The patient serum sample is added to test for the presence of antibodies that bind to the antigens on the plate. The secondary antibody that is added has an enzyme linked to it that binds to the constant region of the primary antibody if it is present in the patient sample. The constant region of an antibody has the same sequence of amino acids within a class of antibodies, e.g., IgG would have the same constant region. The variable region of an antibody provides the diversity of antibodies and is the site to which the antigen binds. The configuration that forms in the indirect ELISA is antigen, antibody, and secondary antibody. Just as in the direct ELISA, the addition of substrate is used to determine if the sample is positive for the presence of antibody.

In this simulation, you will use the indirect ELISA to test for the presence of antibodies made against Human Immunodeficiency Virus (HIV). You will use positive and negative controls to verify the results.

From the Experiment menu, select Indirect ELISA. You will see the opening screen for the lab (Figure 12.3). Use the Balloons On/Off feature from the Help menu to familiarize yourself with the equipment on the screen.

At the left hand corner of the screen in an open cabinet are two pipettors that will be used to dispense small volumes. The pipettor on the left is a multi-channel pipettor that can dispense eight samples at once, and the other is a 100µl pipettor. In the same cabinet is a 96-well microtiter plate, a plastic tray that has 12 wells across and 8 wells down. The closed cabinet to the right contains the patient samples and controls that will be used later in the lab. It will automatically open when necessary. On the far right is a microtiter plate reader that will detect the optical density of the samples after the reaction has occurred. Just below the plate reader is a pipette tip dispenser that contains single-use, disposable pipette tips. The Washing Buffer will be used to remove excess reagents and reduce non-specific binding. The Antigens bottle contains the HIV antigens that will be adsorbed (stuck) to the plastic of the microtiter plate. The Developing Buffer bottle contains the secondary antibody to which the enzyme is conjugated. The Substrates bottle contains the substrates that the enzyme converts from a colorless compound to a colored compound.

At the lower left of the screen is a sink that will be used to remove liquids from the microtiter plate. Next to the sink is a blotter that will be used to remove the last amount of liquid from the wells of the microtiter plate. Next to the blotter is the workbench. Below the workbench is the data table for recording the optical density of each sample. In the lower right corner is a biohazard bag for the disposal of biohazardous waste.

**Activity 3**

**Using Indirect ELISA to Test for HIV**

1. Click on the 96-well-microtiter plate and hold down the mouse button to drag it over to the workbench. Release the button to drop it there.

2. Move the multi-channel pipettor to the pipette tip dispenser. Release the mouse to insert the tips.
3. Bring the multi-channel pipettor to the bottle labeled antigens. Release the mouse to add the antigen solution for dispensing.

4. Position the multi-channel pipettor directly over the 96-well-microtiter plate. Release the mouse to dispense the liquid into the wells. You are filling up one row of the plate.

5. Click and drag the multi-channel pipettor to the biohazard bin for removal and disposal of the tips.

6. Using the plus (+) button, set the timer to 14 hours. Click on the Start button to start the timer.

7. After the simulated 14 hours have passed, click on the bottle of Washing Buffer and drag it over to the 96-well-microtiter plate. Release the mouse and the solution will squirt into the tray. The washing buffer is used to remove excess antigens that are not adsorbed (stuck) to the plate.

8. Click on the 96-well-microtiter plate and drag it to the sink. Release the mouse and the contents of the tray will be dumped into the sink.

9. Repeat the washing steps twice by repeating steps 7 and 8 twice.

10. Click and drag the 96-well-microtiter plate over to the blotter. Release the mouse and the plate will be pressed to the surface of the blotter to remove the last amount of liquid from the wells.

11. Click on the 100μl pipettor and drag it over to the tip dispenser. Release the mouse to place a tip onto the pipettor.

12. Click and drag the 100μl pipettor over to the positive control bottle, labeled +. Release the mouse and the sample will be drawn up into the pipette tip.

13. Click and drag the 100μl pipettor over to the 96-well-microtiter plate. Release the mouse and the sample will be dispensed into the wells of the plate. The tip will automatically be removed and disposed of in the biohazardous waste.

14. Repeat the process for each of the samples.

15. An enlargement of the wells will appear to show you where you placed the samples. Click on the (X) to close the pop-up window.

16. Set the timer for 1 hour using the (+) button. Click on the Start button to start the timer. This process will allow the antigens to react with each of the samples.
17. After the time has elapsed, click on the Washing Buffer and drag it over to the 96-well-microtiter plate. Release the mouse and the solution will squirt into the tray. The washing buffer is used to remove any non-specific binding that may have occurred.

18. Click on the 96-well-microtiter plate and drag it to the sink. Release the mouse and the contents of the tray will be dumped into the sink.

19. Repeat the washing steps twice by repeating steps 17 and 18 twice.

20. Click and drag the 96-well-microtiter plate over to the blotter. Release the mouse and the plate will be pressed to the surface of the blotter to remove the last amount of liquid from the wells.

21. Click on the multi-channel pipettor and drag it to the tip dispenser. Release the mouse to attach the tips to the multi-channel pipettor.

22. Click and drag the multi-channel pipettor to the developing buffer. Release the mouse and the buffer will be drawn up into the tips. The developing buffer contains the conjugated secondary antibody.

23. Click on the multi-channel pipettor and drag it to the 96-well-microtiter plate. Release the mouse and the solution will dispense into the wells. Click on the multi-channel pipettor and drag it to the biohazardous waste.

24. Set the timer for 1 hour, and then press Start. This will allow the conjugated secondary antibody to bind to the primary antibody if it is present in the sample.

25. Click on the multi-channel pipettor and drag it to the tip dispenser. Release the mouse to attach tips to the pipettor.

26. Click and drag the multi-channel pipettor to the Substrate bottle. Release the mouse and the solution will be drawn up into the tips.

27. Click on the multi-channel pipettor and drag it to the 96-well-microtiter plate. The substrate will be added to the wells.

28. Click on the multi-channel pipettor and drag it to the biohazardous waste. Release the mouse to release the tips.

29. An enlargement of the wells will appear. The development will progress over time. Click on a well and the optical density will appear in the window of the microtiter plate reader. Click on the Record button to record the optical density in the Data Table, then record the test results in Chart 3 using the following guidelines:
   • A result of <0.300 is read as negative for HIV-1.
   • A result of 0.300 – 0.499 is read as indeterminate, need to retest.
   • A result of >0.500 is read as positive for HIV-1.

Which patient(s) would be considered positive for HIV?

<table>
<thead>
<tr>
<th>CHART 3</th>
<th>Indirect ELISA Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>Optical Density</td>
</tr>
<tr>
<td>Patient A</td>
<td></td>
</tr>
<tr>
<td>Patient B</td>
<td></td>
</tr>
<tr>
<td>Patient C</td>
<td></td>
</tr>
<tr>
<td>Positive control</td>
<td></td>
</tr>
<tr>
<td>Negative control</td>
<td></td>
</tr>
</tbody>
</table>

Are we testing for the presence of antigen or antibody in the patient samples?

What key substance is present in the “Developing Buffer”?

What does seroconversion mean? Which patient would be positive for seroconversion?

**Western Blotting Technique**

Southern blotting was developed by Ed Southern in 1975 to identify DNA. A variation of this technique, developed to identify RNA, was named Northern blotting, thus continuing the directional theme. Western blotting, another variation that identifies proteins, is named following the same tradition.

The full procedure involves using an electrical current to separate proteins on the basis of their size and charge. This technique uses gel electrophoresis to separate the proteins in a gel matrix. Because the resulting gel is fragile and would be difficult to use in further tests, the proteins are then trans-
ferred to a nitrocellulose membrane. The original Western blot technique used blotting to transfer the proteins, but now electricity is also used for the transfer of the proteins to nitrocellulose strips. These strips are commercially available, which eliminates the need for the electrophoresis and transfer equipment. This simulation will pick up the procedure with the HIV (Human Immunodeficiency Virus) antigens already transferred to nitrocellulose and cut into strips.

Western blotting is also known as immunoblotting. This is because the proteins that are transferred, or blotted, onto the membrane are later treated with antibodies. In a manner similar to the ELISA technique, the secondary antibodies have an enzyme attached to them that allows the use of color to detect a particular protein. In the ELISA technique, the antigens are treated with a mixture of antibodies, whereas the Western blot has a discrete protein band that represents the specific antigen that the antibody is recognizing.

The initial test for HIV is the ELISA, which is less expensive and easier to perform than the Western blot. The Western blot is used as a confirmatory test for a positive ELISA because the ELISA is prone to false positive results. The bands from a positive Western blot are from antibodies binding to specific proteins and glycoproteins from the human immunodeficiency virus. A positive result from the Western blot is determined by the presence of particular protein bands.

From the Experiment menu, select Western Blot. You will see the opening screen for the lab (Figure 12.4). Use the Balloons On/Off feature from the Help menu to familiarize yourself with the equipment on the screen.

At the left there is a shelf that contains Washing Buffer that will be used to wash away excess reagents and reduce non-specific binding. The bottle labeled Developing Buffer contains the secondary antibody to which the enzyme alkaline phosphatase is conjugated. The Substrates in the next bottle are tetramethyl benzidine and hydrogen peroxide, the specific reactants that are used by the alkaline phosphatase enzyme found in the developing buffer. The right hand shelf has the three patient samples (A, B, and C) and the positive and negative controls. Below this shelf is a stack of nitrocellulose strips that have been prepared with the HIV antigens, and a stack of troughs that will be used to house the nitrocellulose strips during the reaction. These strips are commercially available. Usually, they are stored in a plastic bag with a solution to keep them from drying out. However, for the ease of the simulation they will be kept out of the bag.

Moving down the screen, there is a rocking apparatus that will be used to gently mix the samples. The timer for the rocking apparatus is just below the apparatus. Just to the right
is a workbench with a tray that will be used to transfer the troughs to the rocking apparatus. Below the workbench is the data table. The protein bands present will be recorded by clicking Record Data for each sample. In the far right corner is a biohazard bag used to dispose of the contaminated materials.

**ACTIVITY 4**

**Using Western Blot Strips to Detect Anti-HIV Antibodies**

1. Click a trough from the shelf to place it on the tray on the workbench. Repeat this process until there are five troughs on the tray.

2. Click on the stack of nitrocellulose strips, then release the mouse and a strip will drop into the trough. Repeat this process to place a nitrocellulose strip in each trough.

3. Click on the dropper cap of the patient sample labeled A. The dropper will appear. Then drag the dropper over to the first trough. Release the mouse and the sample will dispense. The dropper will return to the bottle. This step adds the antibody from patient A to the nitrocellulose strip.

4. Repeat this step for the remaining anti-sera: patient B sample, patient C sample, and the positive and negative controls.

5. Click on the tray that is holding the five troughs and drag it to the rocking apparatus.

6. Set the timer for 60 minutes using the plus (+) button, and then click Start. The gentle rocking action will allow the antibodies to react with the antigens bound to the nitrocellulose.

7. When the time has elapsed, click on the tray and drag it back to the workbench.

8. Click on the first trough and drag it to the biohazardous waste container. The trough will tilt and drain off the liquid. Repeat this process for each trough.

9. Click on the squirt bottle of Washing Buffer and drag it over to the first trough. The bottle will add washing buffer to each trough. Then each trough will be automatically drained into the biohazardous waste container.

10. Repeat the washing steps two more times by adding washing buffer to the first trough. The washing and rinsing steps will automatically repeat each time. These washing steps are performed to remove any non-specific binding of secondary conjugated antibodies. Excess secondary conjugated antibody could react erroneously with substrate and give a false positive result.

11. Click on the dropper bottle of Developing Buffer and add this solution to the first trough by dragging the dropper to the trough and releasing the mouse. Move the dropper over to the next trough to add developing buffer to each trough.

12. Click on the tray and drag it to the rocking apparatus. Set the timer for 60 minutes using the (+) button, and then press Start. This will allow the conjugated secondary antibody to bind to the primary antibody if it is present in the sample.

13. When the time has elapsed, click on the tray and drag it back to the workbench.

14. Click on the first trough and drag it to the biohazardous waste container. The trough will tilt and drain off the liquid. Repeat this process for each trough.

15. Click on the squirt bottle of Washing Buffer and drag it over to the first trough. The bottle will add washing buffer to each trough as it passes over the trough. Each trough will be automatically drained into the biohazardous waste container.

16. Repeat the washing steps two more times by adding washing buffer to the first trough. The washing and rinsing steps will automatically repeat each time. These washing steps are performed to remove any non-specific binding of secondary conjugated antibodies. Excess secondary conjugated antibody could react erroneously with substrate and give a false positive result.

17. Click on the dropper for the bottle labeled Substrates. Then drag the dropper over to the first trough and release the mouse button to dispense the substrates (tetramethyl benzidine and hydrogen peroxide) into the trough. Move the dropper over to the next trough and repeat until the substrates have been added to each trough. The substrates are the chemicals that are being changed by the enzyme that is linked to the antibody.

18. Click on the tray and drag it to the rocking apparatus.

19. Set the timer for 10 minutes using the (1) button. Click the Start button to start the timer.

20. Click on the tray and drag it to the workbench. Click on the nitrocellulose strip inside the trough to visualize each result. Then click Record Data to enter the results in the data table. The bands are a result of the enzyme that is linked to the secondary antibody reacting with the substrates and creating the color.

21. Use the criteria below to report an overall result for the patient or control samples. The antigens that are present on the nitrocellulose strips are the following proteins from HIV:

<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>gp 160</td>
<td>Glycoprotein 160, a viral envelope precursor</td>
</tr>
<tr>
<td>gp 120</td>
<td>Glycoprotein 120, a viral envelope protein that binds to CD4</td>
</tr>
<tr>
<td>p55</td>
<td>A precursor to the viral core protein p24</td>
</tr>
<tr>
<td>gp41</td>
<td>A final envelope protein</td>
</tr>
<tr>
<td>p31</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>p24</td>
<td>Viral core protein</td>
</tr>
</tbody>
</table>
The criteria for reporting a positive result varies slightly from agency to agency. The Centers for Disease Control and Prevention recommend the following criteria that we will use for this simulation.

<table>
<thead>
<tr>
<th>Observation</th>
<th>Reported Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>No bands are present.</td>
<td>Negative</td>
</tr>
<tr>
<td>Either p31 or p24 are present and gp 160 or gp 120 are present.</td>
<td>Positive</td>
</tr>
<tr>
<td>Bands are present, but they do not match the criteria for a positive result</td>
<td>Indeterminate</td>
</tr>
</tbody>
</table>

Patients that are deemed indeterminate after multiple testing should be monitored and tested again at a later date.

Fill in Chart 4 using the information in the data table to determine the reported results for each patient and control sample.

What are bound to the nitrocellulose initially, antigens or antibodies?

Compare and contrast the indirect ELISA and the Western blot technique.

Which test do you think would be more specific, the indirect ELISA or the Western blot technique? Why?