

LAB 14

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

STUDENT GUIDE

GOAL

The goal of this laboratory lesson is to explain the concepts and technique of enzyme linked immunosorbent assay (ELISA).

OBJECTIVES

After completing this lab the student should be able to:

1. Define epitope.
2. Describe how an ELISA works, including an explanation of its sensitivity.
3. Explain the importance of a positive control for ELISA.
4. Explain why the onset of AIDs takes several years for those infected with HIV.
5. Explain why anti-HIV-1 IgG is screened instead of HIV.
6. Explain how HIV targets T_H cells.
7. Describe common causes of false positive and false negative results in ELISA.
8. Compare and contrast a Western immunoblot and an ELISA.
9. Explain why a Western immunoblot is performed to confirm a positive ELISA test for HIV

TIMELINE

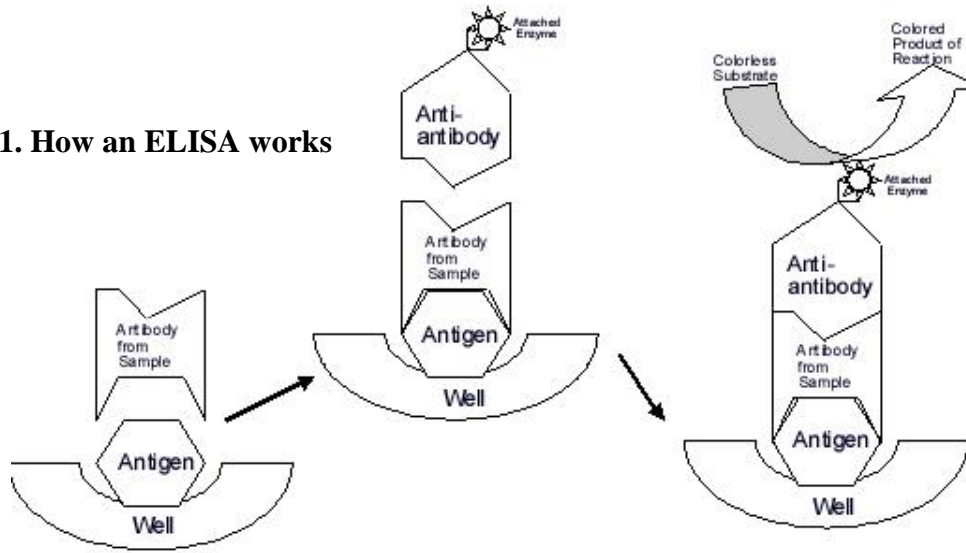
Prep will take 1.5 hours; the lab 1 hour.

BACKGROUND

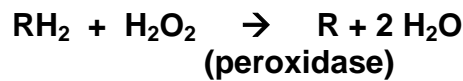
An **epitope** is the area of the antigen that stimulates a specific immune response. There is approximately one epitope, also called an **antigenic determinant**, for each 5 kDa of a protein, but glycoproteins, polysaccharides, lipopolysaccharides, lipids and nucleoproteins all contain epitopes that can elicit immune responses. Epitopes are usually on the surface of antigens and are the site of antibody attachment.

The Enzyme Linked Immunosorbent Assay (ELISA) test is performed by first incubating a specific antigen in the wells of a plastic microtiter plate. The antigen binds to the wells, excess antigen is removed, and the sample suspected to contain antibodies against this antigen is applied to the antigen in the wells. After a wash to remove any unbound sample antibodies, a secondary antibody (an anti-antibody) specific to the antibody being investigated, is applied. This secondary antibody has been conjugated with an enzyme, such as horseradish peroxidase, and after incubation and washing to remove any that is unbound, the enzyme's substrate is added. The slightest amount of linked or sandwiched antibodies in the well will result in detectable amounts of color when the colorless substrate is cleaved to a colored product by the conjugated enzyme. See **Figure 1**, below.

Figure 1. How an ELISA works



The key to this chromogenic reaction (produces a colored product) is the addition of hydrogen peroxide, the substrate for the conjugated enzyme, peroxidase. Hydrogen peroxide is a very reactive and toxic compound that would kill a cell if not for peroxidase. The conjugated enzyme in this experiment is horseradish peroxidase and aminosalicylate (**RH₂** in the equation, below) is the co-substrate, a compound that donates hydrogen atoms in the reaction that converts the hydrogen peroxide to water. The enzyme and co-substrate used in colorimetric assays like ELISA can vary, but all work to form a colored product (**R** in the equation) that can be observed and measured.



The ELISA is used as an initial clinical test of blood for HIV because only inexpensive generic anti-human antibodies need to be used. Many types of HIV antigens are purified and placed in the test well. Then antibodies in the serum sample of the donor are added. If the donor is newly infected, the HIV may have already elicited an immune response of anti-HIV antibodies, but there may not be many HIV particles in the blood – they will be inside infected cells, slowly producing progeny. The titer of HIV may not be high enough to detect, but the anti-HIV antibody titer may be detectable. The ELISA test is extremely sensitive because it is an enzyme-linked test – it only requires a minute amount of bound human antibody epitopes to bind many secondary generic conjugated antibodies, which in turn catalyze the reaction that hydrolyzes substrate molecules to easily detected colored product.

Careful, meticulous lab technique is absolutely required or cross contamination will give false result. In clinical practice, the ELISA is performed in triplicate. If the results are positive, a more sensitive test is performed to verify the results because false positives occur very frequently. There are several reasons why false positives occur. If the technician has poor technique, cross contamination may occur, giving a false positive. Cross-reactivity can also occur in immuno assays. This means that an individual may possess antibodies directed against Human Leukocyte Antigens (HLA), which are present

in the host cells that were used when HIV was propagated in the laboratory by the ELISA test manufacturer. (See the background information on HIV and AIDS given in the appendix of this manual for a discussion of how HIV propagates and steals some of the lipid bilayer of the infected cell for each new viral particle produced.) Women who have had multiple pregnancies may carry HLA antibodies against the HLA of the children that they carried that match the HLA in the test. People that have had organ transplants, blood transfusions, gamma globulin immunization, anti-carbohydrate antibodies, and anti-collagen antibodies (found in gay men, hemophiliacs, those with leprosy, and Africans of both sexes) may all test positive if the ELISA test kit includes any of these antigens along with the HIV antigens. A recent cold or other viral infection can also produce antibodies that cross react with the HIV antigens in the test.

False negatives can also occur. Poor laboratory technique can result in insufficient incubation of the HIV antigen in the test wells, which fails to allow enough antigen to coat the well. Even if the lab technique is adequate, 1/1000 tests give false negative results. One cause is a lack of sero-conversion, the increase in the titer of anti-HIV antibodies that takes some time to occur after the body is first infected. The ELISA does not always detect such low numbers of anti-HIV antibodies. A confirmatory test, such as the clinical immunoblot or Western blot should be performed after an ELISA gives positive results.

A Western immunoblot, illustrated in **Figure 2**, uses a mixture of monoclonal antibodies, each against an epitope of HIV. The HIV epitopes must be present in the patient serum sample in order to get a positive result. In contrast to the ELISA, it is the HIV antigens themselves and not the patient's HIV antibodies that are being tested for. This makes the immunoblot a more definitive test for the presence of HIV. Since monoclonal antibodies are used, this test is more expensive and is used only if an ELISA, a less expensive test, shows a positive result.

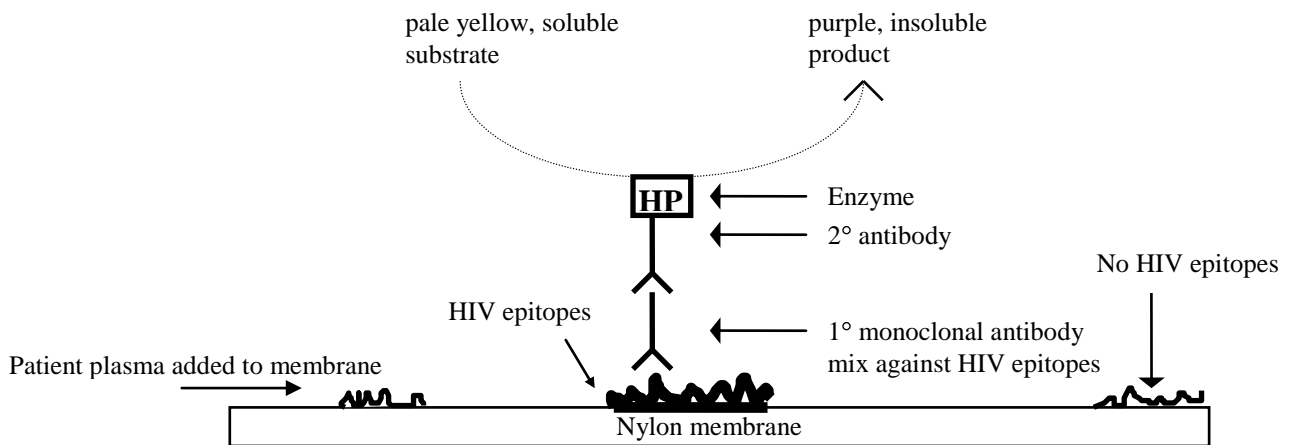


Figure 2. Western (clinical) immunoblot to confirm HIV

LABORATORY OVERVIEW

This lab will be performed in one lab period.

- Prep
- Addition of simulated HIV antigen to the wells of a micro-titer plate
- Washing of wells and addition of simulated HIV antibodies or negative simulated serum samples
- Washing and addition of secondary antibody conjugated to horseradish peroxidase (HP) enzyme
- Color detection of bound secondary antibodies with HP substrate solution

SAFETY GUIDELINES

Good Laboratory Practice requires wearing safety glasses, lab coat and gloves.

MATERIALS

Per class:

Water bath set at 37°C

EDVOTEK Kit #271

HIV antigens (simulated –
component A)

Positive control (1° antibodies –
component B)

Donor 1 serum (simulated –
component C)

Donor 2 serum (simulated –
component D)

Anti-IgG-peroxidase conjugate
(2° antibody – **component E**)

Hydrogen peroxide (**component F**)

Amiosalicylic acid (peroxide co-
substrate – **component G**)

Phosphate buffered saline
concentrate (**component H**)

Microtiter plates

Microcentrifuge tubes – 1.5 ml

Two 50 ml plastic tubes for
mixing 2° antibody and substrate

Scissors

Sharpie markers

Colored pencils

Tubes or bottles to hold 25 ml – one per student up to 10 per class (for PBS)

Beakers for waste solutions – one per two students

1 Corning bottle to hold 270 ml

Graduated cylinder to measure 270 ml

Electric serological pipette pumps – one per two students

1 ml serological pipettes – one per student

1 25 ml serological pipette

Bottles of dH₂O – one per two students

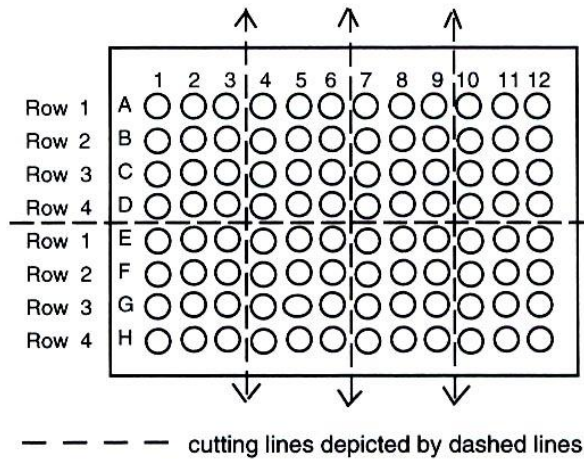
PROCEDURE

Each student can perform his or her own lab. All prep should be done so that everyone receives each of the reagents.

Part I. Prep

Team 1

Make sure the water bath for incubation is turned on and set at 37°C. Cut the micro-titer plates, as shown below, so that each student receives a small micro-titer plate with 12 wells – 4 rows of three wells. Distribute to class members. (If there are only 4 Teams in the class, do the Team 5 prep, also.)



Team 2

Aliquot reagents A-D for each class member into 1.5 ml microcentrifuge tubes as given below and distribute one set to each person.

Label on Tube	Amount (ml)	Component
HIV	1.4	HIV antigens (simulated – component A)
+	0.4	Positive control (1° antibodies – component B)
DS #1	0.4	Donor 1 serum (simulated – component C)
DS #2	0.4	Donor 2 serum (simulated – component D)

Team 3

Prepare the phosphate buffered saline (PBS) by measuring 270 ml of dH₂O and adding to a labeled Corning bottle. Add all of **Component H**, Phosphate Buffered Saline concentrate, to the water. Invert to mix. Label one small bottle for each person as “PBS” and dispense 25 ml into each and distribute one to each class member. Give the bottle of all remaining diluted PBS to Team 4.

Team 4

Prepare the 2°antibody solution.

1. Add 0.3 ml of diluted PBS (from Team 3) to the tube of Anti-IgG-peroxidase conjugate – **component E**. Mix thoroughly by tapping and inverting the tube.

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2. Place 15 ml of diluted PBS (from Team 3) into the 50 ml plastic tube that came in the kit. Label the tube “2° Antibody.”
3. Transfer the entire contents of **component E** to the 15 ml of PBS in the labeled 50 ml tube and invert to mix.
4. Label a 1.5 ml microcentrifuge tube for each student as ‘2° Antibody’ and dispense 1.4 ml to each.
5. Give the remaining PBS (from Team 3) to Team 5 (or Team 1, if there are only 4 Teams).

Team 5 (or Team 1, if there are only 4 Teams)

Peroxidase Substrate Solution (Prepare just before the final incubation step in the procedure.)

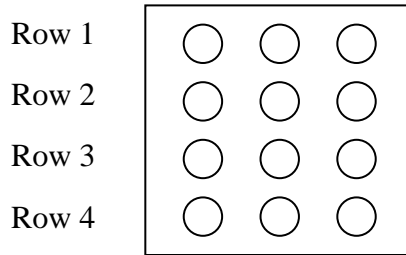
1. Dispense 13.5 ml of the dilute PBS (from Team 3) to a plastic tube provided in the kit.
2. Add all of the Aminosalicylic acid (peroxide co-substrate – **component G**). Cap and mix thoroughly by shaking and vortexing. There may be undissolved material remaining.
3. Add 1.5 ml of the Hydrogen peroxide (**component F**). Cap and mix.
4. Dispense 1.4 ml into 1.5 ml microcentrifuge tubes labeled “Substrate” and dispense one tube to each student during the 15 minute incubation with the 2° antibody.

Each person will need the following:

- One tube of simulated HIV serum (the antigens)
- One tube of positive control serum
- One tube of simulated donor serum #1 (the antibodies)
- One tube of simulated donor serum #2 (the antibodies)
- One tube of 2° antibody solution (the anti-antibodies)
- 25 ml PBS
- Substrate solution (to be mixed immediately prior to use)
- P-1000 automatic micropipetter and sterile tips
- P-100 automatic micropipetter and sterile tips
- Beaker for used tips (share with your partner)
- Beaker for waste solutions (share with your partner)

Part II. Experimental Procedure

1. Label a beaker for waste solutions.
2. Label your micro-titer plate with your team number. Label the wells of your micro-titer plate as shown:

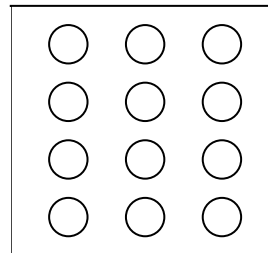


3. Into all 12 wells, add 100 μ l of HIV serum (the antigens).
4. Incubate for 5 minutes at room temperature.
5. Using a micropipetter and sterile tips, remove all the HIV antigens and dispose into the waste beaker.
6. Add 200 μ l PBS buffer to the wells in all rows to wash.
7. Remove all the liquid from each well, changing tips each time. Dispose the liquid into the waste beaker.

(In research labs, the next step would be to add a blocking solution of protein, such as BSA to saturate all sites not occupied in the well with the antigen. The kit manufacturers have designed this experiment so that it works without this important step.)

8. Use an automatic micropipetter to add the following reagents. Be sure you use a new tip for each solution.

- Row 1: 100 μ l PBS (negative control)
- Row 2: 100 μ l “+” (positive control)
- Row 3: 100 μ l DS #1
- Row 4: 100 μ l DS #2



9. Incubate for 15 minutes by floating your micro-titer plate in a 37°C water bath.
(NOTE: be careful that your lab coat sleeve does not tip over another Team’s plate!)

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10. Remove all the solutions from each well. Be sure to change tips when you change rows or you will cross contaminate and get false results.
11. Add 200 μ l PBS to each well without touching the wells with the tip. If you touch the tip to the wells, change tips.
12. Remove the PBS from each well using a different tip for each row.
13. Add 100 μ l of the 2^o antibody solution to each well. If you touch the tip to the wells, change tips.
14. Incubate for 15 minutes by floating your micro-titer plate in a 37°C water bath.
This is the time to mix the substrate solution.
15. Remove all the liquid from each well using a different tip for each row.
16. Add 200 μ l PBS to each well without touching the wells with the tip.
17. Remove the PBS from each well using a different tip for each row.
18. Add 100 μ l substrate solution to each well.
19. Incubate by floating your micro-titer plate in a 37°C water bath for 5 minutes, which should be enough time to see results.
20. Draw your results in your notebook or use the scanner to record all ELISA results from the class.

DATA ANALYSIS

Label your results and describe what you observe in each well.

QUESTIONS

1. Define epitope.
2. Explain how the ELISA test works and why is it so sensitive.
3. Why can the onset of AIDS take several years?
4. Why is anti-HIV-1 IgG screened instead of the virus itself?
5. Why are there so many immunological variants of HIV?
6. What can cause a false positive in an ELISA?
7. What can cause a false negative in an ELISA?
8. Compare and contrast a Western immunoblot and an ELISA.
9. Explain why a Western immunoblot is performed to confirm a positive ELISA test for HIV.