Biol 465
Immunology

Lab #4, Part 1: The ELISA assay

We have had a thorough introduction to antibody structure and formation in developing B-cells. We should now have a better understanding of how a given antibody is formed to have specificity for one or a few related antigens and to have one of 5 different constant regions which will give the antibody different effector functions.

Today's lab will be the third that shows the utility of Mother Nature's design on antibodies. We will perform an assay called an **e**nzyme-**l**inked **i**mmuno**s**orbent **a**ssay (**ELISA**). The assay is used in medical laboratories for diagnosis, in research laboratories, in drug testing and many other applications in which the goal is to test for the presence of a specific substance (referred to as an antigen) in a variety of sample types (e.g. blood, urine, water air). One or more of the pregnancy tests we used, may have used ELISA technology, but we did not spend much time on the details of ELISA last week.

The ELISA procedure relies on specific antigen-antibody interactions followed by an enzymatic detection method. In the one type of ELISA, a specific antibody (called the primary antibody) is added to a special type of tube or plate, which will allow stable binding (adsorption) to the wells of the plate.* Next, a liquid sample (e.g. urine, blood, water to be tested) is introduced to the same well of the plate. If the antigen is present, it will bind to the antigen binding site of the adsorbed antibody. * A second antibody (called the secondary antibody) that is specific for different epitope of the antigen of interest is then added. The secondary antibody is covalently linked to an enzyme. If the antigen of interest is present, it will be immobilized on the primary antibody and retained in the well. The secondary, enzyme-linked antibody will be retained in the well through the second antigen-antibody interaction. The final step in ELISA is addition of a substrate for the enzyme. The enzymatic reaction must alter the substrate to make it detectable in some way (the substrate is often chromogenic or color-generating). At the end of an ELISA one should be able to "see" either by eye or by other detection systems, color development, only in the samples that had the antigen of interest.

* The wells of an ELISA plate are washed with gentle buffers between each of the additions of new reagents. (See step by step description of ELISA, next page.)

There are many, many variations and applications of ELISA (see attached and animations).

http://www.immunospot.com/index.php?id=164

http://www.sumanasinc.com/webcontent/animations/content/ELISA.html

In our lab today we will **simulate** ELISA used to detect an HIV antigen in mock student samples. Early detection of HIV infection is essential for life-prolonging therapies to be effective. Early detection of HIV is also key in preventing the spread of the virus to others. ELISA-based detection methods are easy to perform (given the availability of a primary antibody for the antigen) and rapid, and are common tools in detection of HIV antigens (or HIV antibodies produced as a result of HIV infection).

Today we will simulate a typical ELISA assay to detect the HIV capsid p24 protein in mock serum samples from each student.

Materials

- Student test samples (yellow tubes labeled 1-8) **take 1/student**
- Purified HIV capsid protein (+ control) (violet tubes) **take 1/pair**
- Some other purified protein (- control) (blue tubes) **take 1/pair**
- Primary mouse anti-HIV capsid p24 antibody (green tubes) **take 1/pair**
- Enzyme-linked secondary anti-mouse antibody (orange tubes) **take 1/pair**
- Substrate for enzyme (Horseradish peroxidase) (brown tube) **take 1/pair**
- 12-well ELISA microplate strips **take 1/pair**

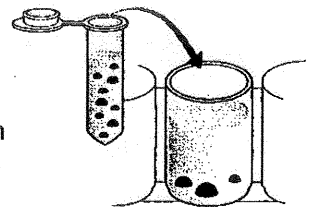
- Racks for reagents
- Wash buffer
- Stack of paper towels
- Micropipettor/tips

See attached protocol. Each student will check their own sample in triplicate using one 12-well microplate strip. Skip a well between your + control, your – control and your sample wells. You should use 11 of the wells.

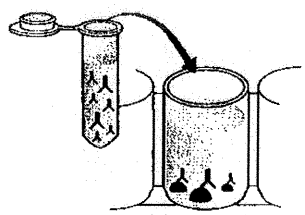
+ + + skip - - -skip sample, sample, sample

The main steps in this antigen detection ELISA are:

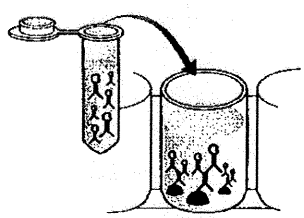
1. Add your sample and control samples to the wells in a microplate strip. Your samples contain many proteins and may or may not contain the antigen. Incubate for 5 minutes to allow all the proteins in the samples to bind to the plastic wells via hydrophobic interaction. This is called an immunosorbent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



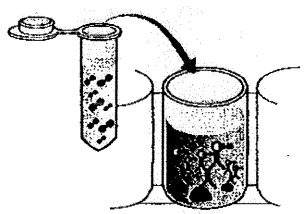
2. Add primary antibody to the wells and incubate. The antibodies will seek out the antigen from the many proteins bound to the well. If your sample contains the antigen, the antibodies will bind it tightly and remain in the well.



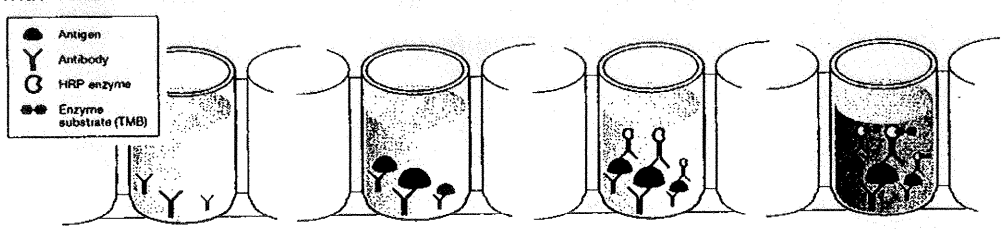
3. Detect the bound antibodies with HRP-labeled secondary antibody. If the primary antibodies have bound to the antigen, the secondary antibodies will bind tightly to the primary antibodies.



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

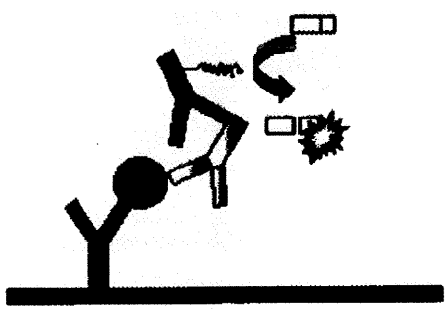


In an **antigen capture** assay, primary antibody is bound in the plastic wells, antigen is captured by the immobilized primary antibody, and the captured antigen is detected by a secondary antibody, also linked to HRP, that turns the assay solution blue upon reaction with TMB.

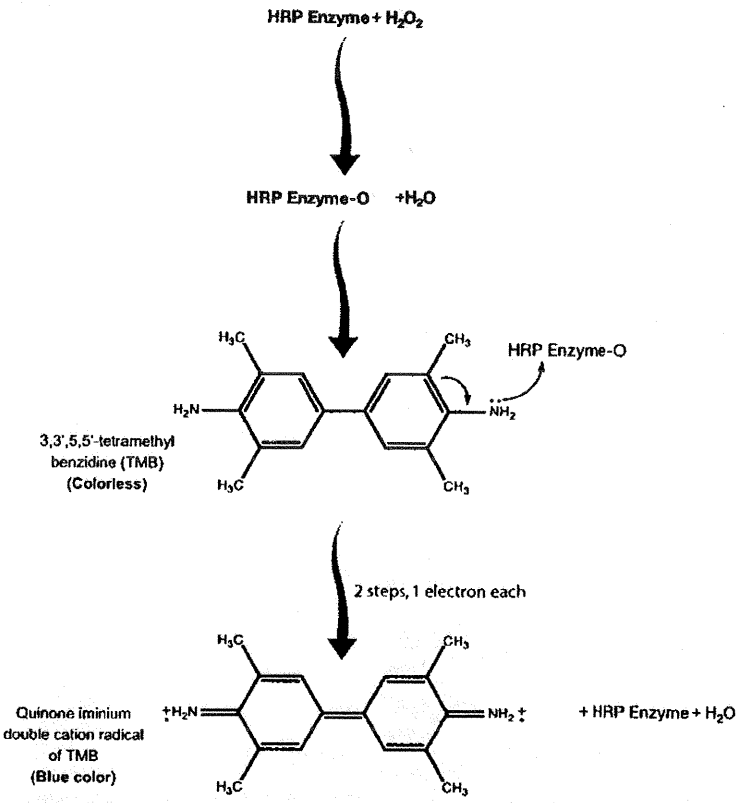


Antigen capture ELISA.

Antigen capture
(more common to use 1° Antibody; Enz-linked 2° Antibody)



Colorimetric detection: Secondary antibodies for ELISA are linked to enzymes. Detection of secondary antibodies that are bound to primary antibodies occurs by an enzyme-substrate reaction. In this kit, the secondary antibody is linked to horseradish peroxidase (HRP). In the presence of hydrogen peroxide (H_2O_2), HRP catalyzes the oxidation of the chromogenic substrate 3,3',5,5'-tetramethylbenzidene (TMB). This oxidation of TMB by HRP forms a blue product. Note: TMB is light sensitive, and the assay results should be determined 5–10 minutes after the substrate is added to the wells. If the microplate strips sit longer, nonspecific color may develop. Color that develops after the 5-minute incubation should not be considered in the assay results. After 20–30 minutes, the blue color may begin to fade as TMB precipitates out of solution.



Colorimetric Detection: Oxidation of TMB by HRP

Controls: Controls are always run side by side with actual samples to make sure that the procedure is working correctly. Controls can resolve ambiguous results that occur due to human error or contaminated reagents; controls must be included in any valid ELISA. For the negative control, the antigen or primary antibody is either omitted (as in this kit) or the antigen is replaced by a factor that will not bind specifically to the antibody. The positive control always contains the target antigen or antibody. A negative sample that gives a positive assay result is called a **false positive**. A positive sample that gives a negative assay result is called a **false negative**.

Many diagnostic assays give a percentage of false positive or false negative results, so confirmation of diagnosis by a second type of assay is important. For example, immunoassays for antibodies to human immunodeficiency virus (HIV) can give either false positive or false negative results. False positives can result from recent vaccinations, and false negatives can result from immunosuppression (e.g., from drugs given after transplants) or from administering the test too soon after infection with HIV. (Antibodies against HIV do not appear until some weeks after HIV infection; the appearance of specific antibodies is called seroconversion.) Because of this, positive HIV ELISA results are always confirmed by western blot (see page 91).

Laboratory Guide

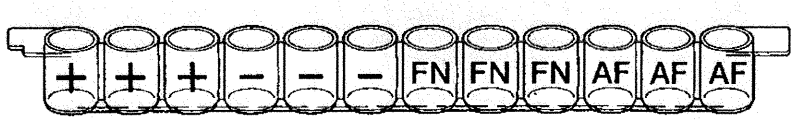
Student Workstation Checklist

One workstation serves 4 students.

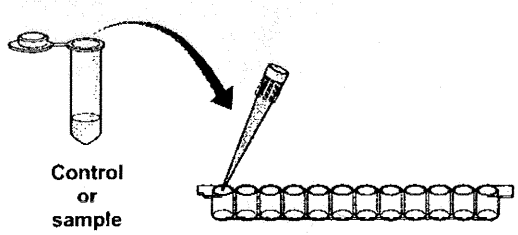
Items	Contents	Number	(✔)
Yellow tubes	Student samples (0.25 ml)	4	❑
Violet tube (+)	Positive control (0.5 ml)	1	❑
Blue tube (--)	Negative control (0.5 ml)	1	❑
Green tube (PA)	Primary antibody (1.5 ml)	1	❑
Orange tube (SA)	Secondary antibody (1.5 ml)	1	❑
Brown tube (SUB)	Enzyme substrate (1.5 ml)	1	❑
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		1	❑
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

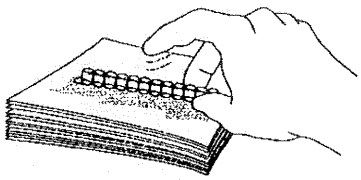
1. Label the yellow tubes with each student's initials.
2. Label the outside wall of each well of your 12-well strip. Two students may share a strip of 12 wells. On each strip label the first three wells with a "+" for the positive controls and the next three wells with a "–" for the negative controls. 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:



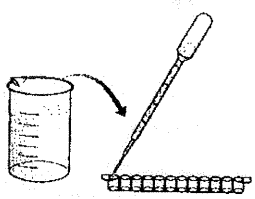
3. Bind the antigen to the wells:
   a. Use a pipet to transfer 50 µl of the positive control (+) from the violet tube into the three "+" wells.
   b. Use a fresh pipet tip to transfer 50 µl of the negative control (–) from the blue tube into the three "–" wells.
   c. Use a fresh pipet tip for each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



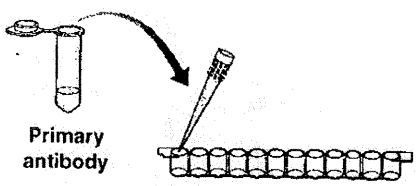
4. Wait 5 minutes while all the proteins in the samples bind to the plastic wells.
5. Wash the unbound sample out of the wells:
    a. Tip the microplate strip upside down onto the paper towels so that the samples drain out, then gently tap the strip a few times upside down on the paper towels. Make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



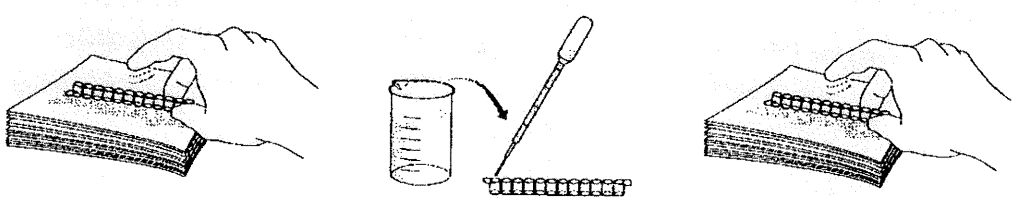
    b. Discard the top paper towel.
    c. Use a transfer pipet filled with wash buffer from the beaker to fill each well with wash buffer taking care not to spill over into neighboring wells. The same transfer pipet will be used for all washing steps..



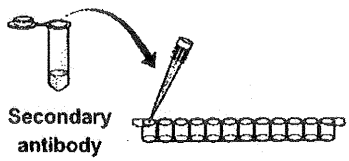
    d. Tip the microplate strip upside down onto the paper towels so that the wash buffer drains out, then gently tap the strip a few times upside down on the paper towels.
    e. Discard the top 2–3 paper towels.
6. Repeat wash step 5.
7. Use a <u>fresh</u> pipet tip to transfer 50 µl of primary antibody (PA) from the green tube into all 12 wells of the microplate strip.



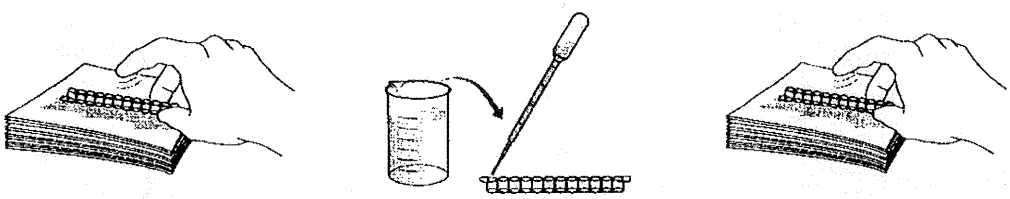
8. Wait 5 minutes for the primary antibody to bind.
9. Wash the unbound primary antibody out of the wells by repeating wash step 5 **two** times.



10. 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.



11. Wait 5 minutes for the secondary antibody to bind.
12. Wash the unbound secondary antibody out of the wells by repeating wash step 4 **three** times.



The secondary antibody is attached to an enzyme (HRP) that chemically changes TMB (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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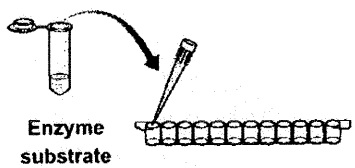
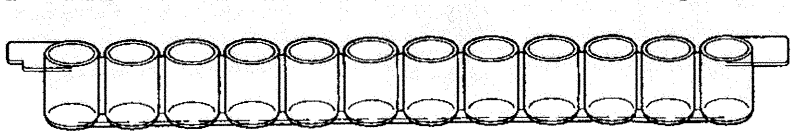
13. Use a fresh pipet tip to transfer 50 µl of enzyme substrate (SUB) from the brown tube into all 12 wells of the microplate strip.



14. 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 1. In each of the wells, put a "+" if the well turned blue and a "–" if there is no color change.



Is your sample positive? Explain your answer.

Post-Lab Focus Questions

1. Did your sample contain the antigen?

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

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

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

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

6. If the sample gave a negative result for the antigen, does this mean that the antigen is not present? What reasons could there be for a negative result when the antigen is actually present?

7. Why did you assay your samples in triplicate?

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