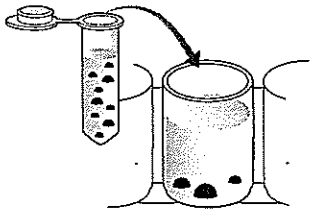
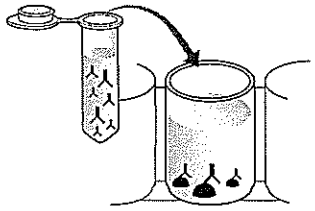
The main steps in this antigen detection ELISA are:

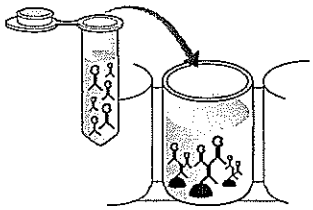
1. Add your sample and control samples to the wells in a microplate strip. Your samples contain many proteins and may or may not contain the antigen. Incubate for 5 minutes to allow all the proteins in the samples to bind to the plastic wells via hydrophobic interaction. This is called an immunosorbent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



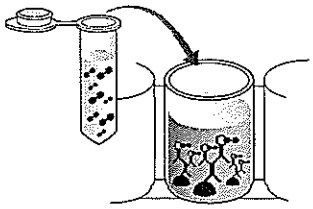
2. Add primary antibody to the wells and incubate. The antibodies will seek out the antigen from the many proteins bound to the well. If your sample contains the antigen, the antibodies will bind it tightly and remain in the well.



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.

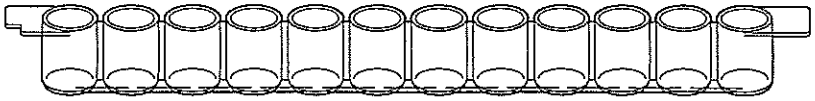


General Introduction to This ELISA Kit

To create a relevant and meaningful classroom context for this activity, the in-depth information in Appendices A and B provides background vocabulary and factual and conceptual lecture points. In addition, useful reading and web sites are included in Appendix E. Of course, there is no substitute for a good textbook and the knowledge and expertise of the instructor.

The following section briefly describes the technical and conceptual points that are directly related to the laboratory activities in this curriculum. Student understanding of these points is extremely important to a successful outcome.

Microplate strips: Microplates are made of polystyrene which adsorbs (binds) proteins by hydrophobic interaction. The plates provided in this kit have 96 wells, arranged in 8 removeable rows of 12-well strips. Two students share one strip. Each well holds approximately 250 microliters (µl).



Antigen: In this kit, the antigen is chicken gamma-globulin (purified from egg yolks) which serves as a generic representative of any hypothetical antigen, protein or otherwise.

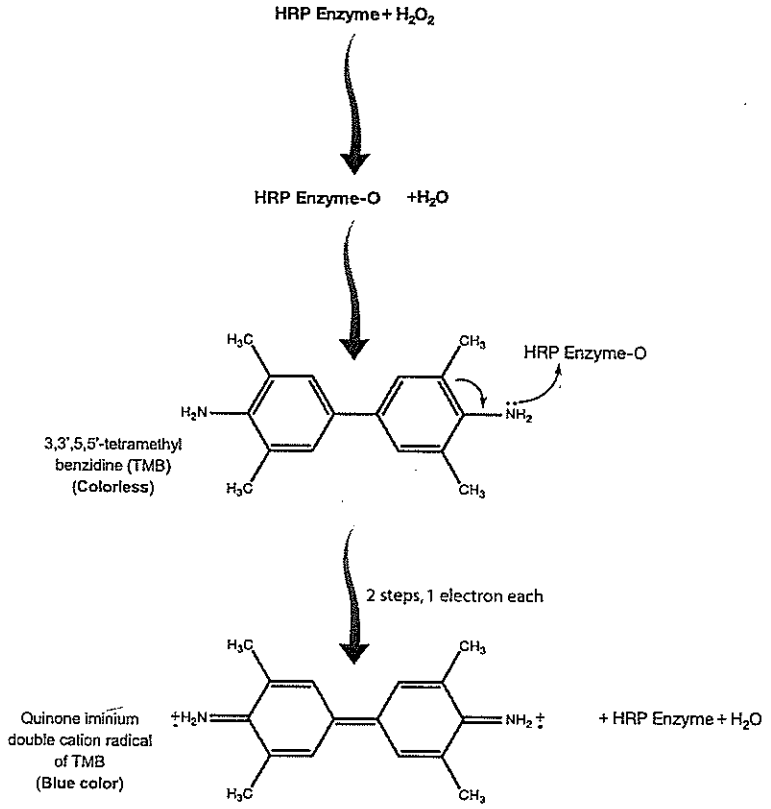
Incubation times: The rate of binding depends on the incubation temperature and the concentrations of the reagents. This kit has been optimized so that each incubation can be performed for 5 minutes at room temperature. Exceeding this time or temperature will cause an increase in color intensity and possibly some background color in the negative controls.

Blocking: Blocking agents are added after antigen adsorption to prevent nonspecific binding of antibodies to the plastic, which would produce false positive results. The blocking agent may be a protein or a detergent (or both). Common blocking agents include Tween 20 (a nonionic detergent that is used in this kit), nonfat dry milk, gelatin, and bovine serum albumin (BSA). Although Tween 20 is a sufficient block for this protocol, you may wish to add the following blocking step for teaching purposes: have the students add 50 µl of 1% gelatin in wash buffer to their wells for 15 min after the addition of the antigen and then perform a wash step.

Primary (1°) antibodies: The antibodies that recognize and bind to the antigen in an immunoassay are primary antibodies. In this kit, the primary antibody is a polyclonal rabbit antibody raised against chicken gamma-globulin. In the ELISA antibody test starting on page 55, this primary antibody simulates human antibodies in a sample of human serum.

Secondary (2°) antibodies: Secondary antibodies recognize and bind to primary antibodies. They are made in animals of a different species than that used to make the primary antibody. For this kit, goats were immunized with rabbit IgG to make the secondary antibodies.

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

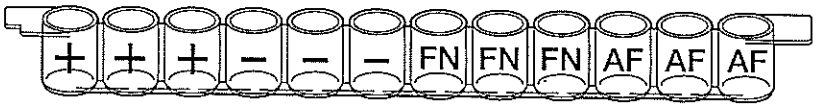
Student Workstation Checklist

One workstation serves 4 students.

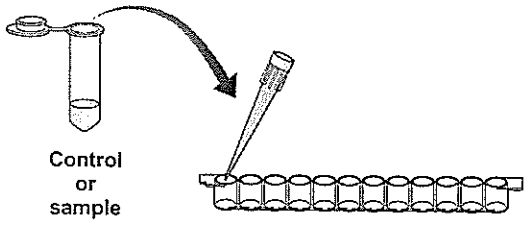
Items	Contents	Number	(✔)
Yellow tubes	Student samples (0.25 ml)	4	❑
Violet tube (+)	Positive control (0.5 ml)	1	❑
Blue tube (–)	Negative control (0.5 ml)	1	❑
Green tube (PA)	Primary antibody (1.5 ml)	1	❑
Orange tube (SA)	Secondary antibody (1.5 ml)	1	❑
Brown tube (SUB)	Enzyme substrate (1.5 ml)	1	❑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

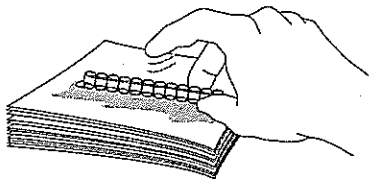
1. Label the yellow tubes with each student's initials.
2. Label the outside wall of each well of your 12-well strip. Two students may share a strip of 12 wells. On each strip label the first three wells with a "+" for the positive controls and the next three wells with a "–" for the negative controls.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



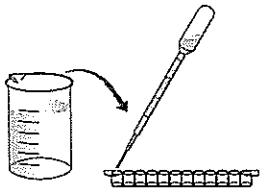
3. Bind the antigen to the wells:
   a. Use a pipet to transfer 50 µl of the positive control (+) from the violet tube into the three "+" wells.
   b. Use a fresh pipet tip to transfer 50 µl of the negative control (–) from the blue tube into the three "–" wells.
   c. Use a fresh pipet tip for each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



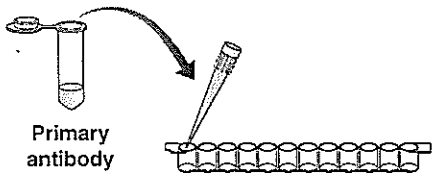
4. Wait 5 minutes while all the proteins in the samples bind to the plastic wells.
5. Wash the unbound sample out of the wells:
    a. Tip the microplate strip upside down onto the paper towels so that the samples drain out, then gently tap the strip a few times upside down on the paper towels. Make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



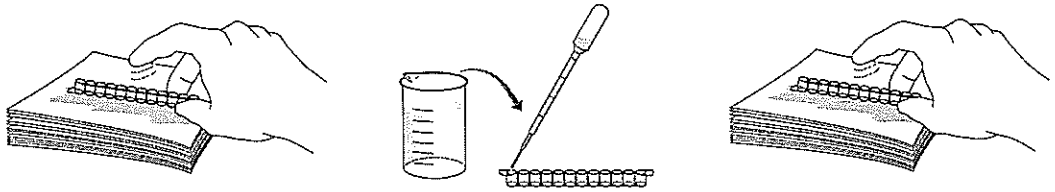
    b. Discard the top paper towel.
    c. Use a transfer pipet filled with wash buffer from the beaker to fill each well with wash buffer taking care not to spill over into neighboring wells. The same transfer pipet will be used for all washing steps..



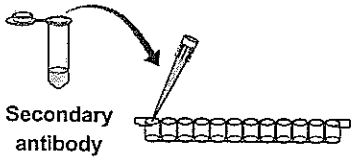
    d. Tip the microplate strip upside down onto the paper towels so that the wash buffer drains out, then gently tap the strip a few times upside down on the paper towels.
    e. Discard the top 2–3 paper towels.
6. Repeat wash step 5.
7. Use a <u>fresh</u> pipet tip to transfer 50 µl of primary antibody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



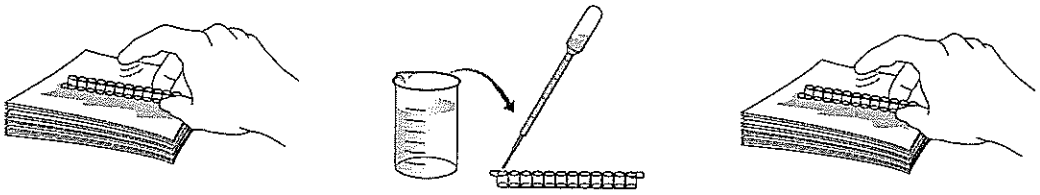
8. Wait 5 minutes for the primary antibody to bind.
9. Wash the unbound primary antibody out of the wells by repeating wash step 5 **two** times.



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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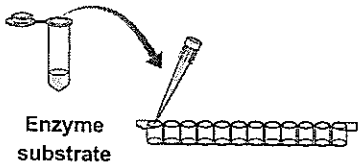
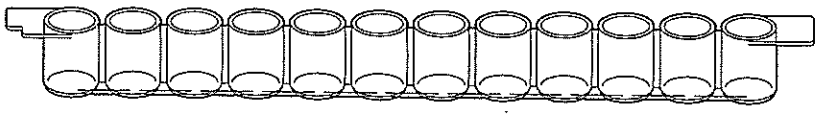
13. Use a fresh pipet tip to transfer 50 µl of enzyme substrate (SUB) from the brown tube into all 12 wells of the microplate strip.



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?