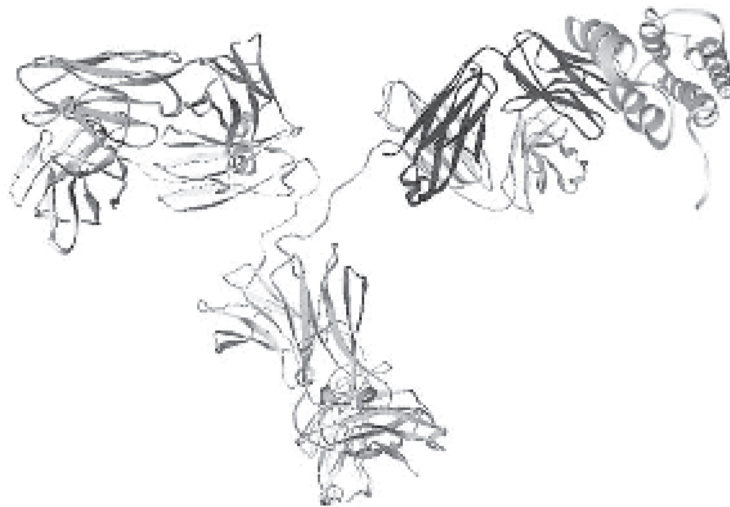

Biotechnology Explorer™

ELISA Immuno Explorer™ Kit

Instruction Manual

Catalog #166-2400EDU

explorer.bio-rad.com

Components of this kit ship in separate containers. Store the bag of reagents in the refrigerator within 1 week of receipt. Note: This kit does not contain any substances of human or disease origin.

Duplication of any part of this document is permitted for classroom use only.

Protocol II: Antigen Detection ELISA

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Student Manual

Introduction

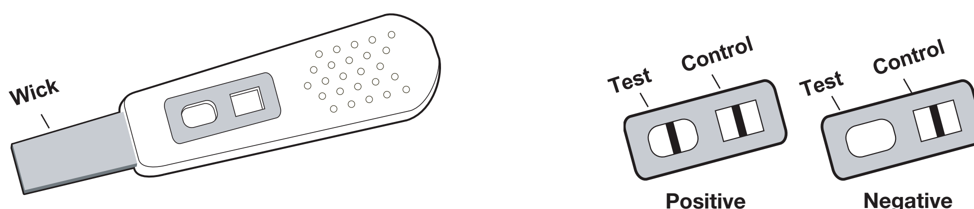
Immunology is the study of the immune system and how the body protects itself against disease. Over 100 years ago, biologists found that animals' internal immune systems respond to invasion by "foreign entities" or antigens. When an invader enters the body, it provokes an immune response that begins with the production of proteins called antibodies. Like magic bullets, antibodies seek out and attach themselves to invading entities (antigens), flagging the invaders for destruction by other cells of the immune system. The antigenic invaders may be any molecules foreign to the body, including components of infectious agents like bacteria, viruses, and fungi. Today, antibodies have become vital scientific tools, used in biotechnology research and to diagnose and treat disease. The number of different antibodies circulating in the blood has been estimated to be between 10^6 and 10^{11}, so there is usually an antibody ready to deal with any antigen. In fact, antibodies make up to 15% of your total blood serum protein. Antibodies are very specific; each antibody recognizes only a single antigen.

You are about to perform an ELISA (enzyme-linked immunosorbent assay). The ELISA relies on antibodies to detect the presence of antigens in liquid samples. Because they are antibody-based, ELISAs are called immunoassays. ELISAs can detect minute amounts of disease agents in samples such as body fluids (before the body has had a chance to mount an immune response). Smallpox virus is an example of a disease agent that can now be detected using an ELISA. If exposure is detected and treated with vaccine within 2–3 days, patients do not develop smallpox. Other applications for ELISA include testing for West Nile virus, HIV coat protein p24, SARS virus, anthrax spores, hormones such as hCG in pregnancy tests, illegal steroids in drug tests, bacteria in food safety tests, and the presence of genetically modified organisms contaminating non-GMO food.

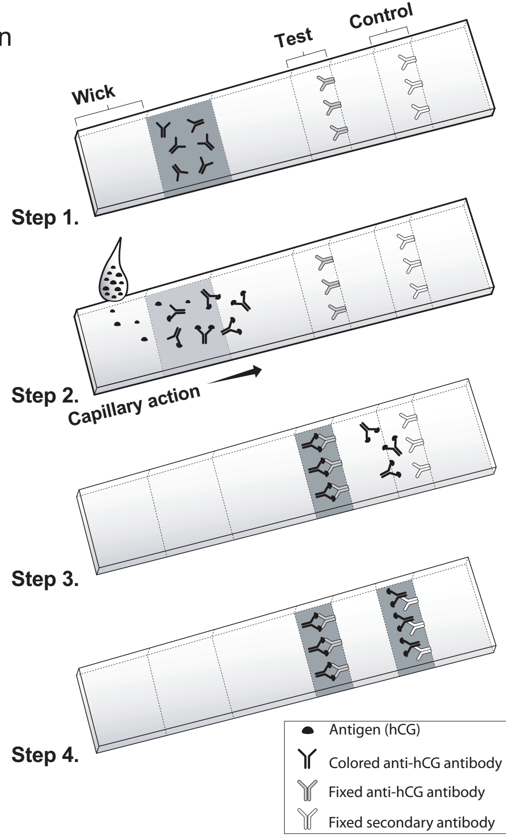
Where Is ELISA Used in the Real World?

With its rapid test results, the ELISA has had a major impact on many aspects of medicine and agriculture. ELISA is used for such diverse purposes as home pregnancy tests, disease detection in people, animals, and plants, detecting illegal drug use, testing indoor air quality, and determining if food is labeled accurately. For new and emerging diseases like severe acute respiratory syndrome (SARS), one of the highest priorities of the US Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO) has been to develop an ELISA that can quickly and easily verify whether patients have been exposed to the virus.

Over-the-counter kits that are based on the same principles as this ELISA activity include home pregnancy and ovulation tests, and tests for the presence of illegal drugs like marijuana and cocaine.

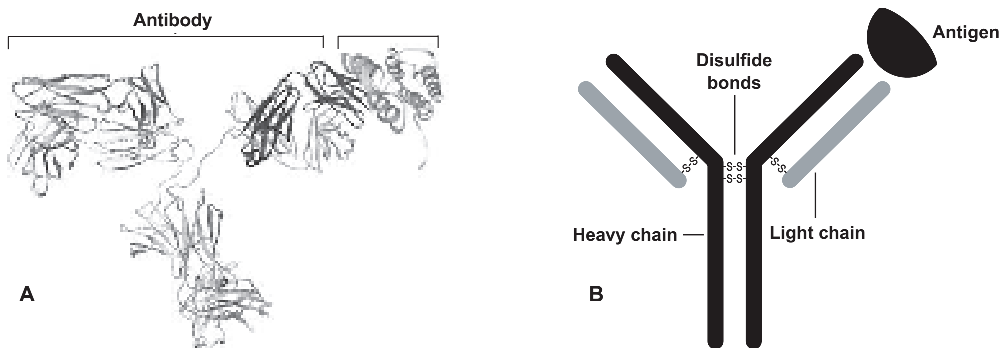


Some tests give positive or negative results in a matter of minutes. For example, home pregnancy dipstick tests detect levels of human chorionic gonadotropin (hCG), a hormone that appears in the blood and urine of pregnant women within days of fertilization. The wick area of the dipstick is coated with anti-hCG antibody labeled with a pink compound (step 1). When the strip is dipped in urine, if hCG is present it will bind to the pink antibody, and the pink hCG-antibody complex will migrate up the strip via capillary action (step 2). When the pink complex reaches the first test zone, a narrow strip containing an unlabeled fixed anti-hCG antibody, the complex will bind and concentrate there, making a pink stripe (step 3). The dipsticks have a built-in control zone containing an unlabeled secondary antibody that binds unbound pink complex (present in both positive and negative results) in the second stripe (step 4). Thus, every valid test will give a second pink stripe, but only a positive pregnancy test will give two pink stripes.



How Are Antibodies Made?

When exposed to antigens, all mammals generate an immune response and produce antibodies, proteins that recognize and bind tightly to the specific antigens. Each antibody recognizes only a single antigen. Animals such as goats, rabbits, and mice can be injected with an antigen and, after a period of time, their serum will contain antibodies that specifically recognize that antigen. If the antigen was a disease-causing agent, the antibodies can be used to develop diagnostic tests for the disease. In an immunoassay, the antibodies used to recognize antigens like disease agents are called primary antibodies.



A) Structure of IgG bound to the HIV capsid protein p24 as determined by X-ray crystallography (Harris et al. 1998, Momany et al. 1996). These structures can be downloaded from the Protein Data Bank (www.pdb.ufmg.br, (Berman et al. 2000) using the PDB identification codes 1IGY and 1AFV and manipulated using free online software such as Rasmol and Protein Explorer. **B)** A commonly used representation of an antibody bound to an antigen.

Secondary antibodies recognize and bind to primary antibodies in an immunoassay. They are prepared by injecting antibodies produced by one species of animal into another species. This works because the antibodies produced by different species are different enough from each other that they will provoke an immune response. For example, if you want a secondary antibody that will recognize a human primary antibody, inject human antibodies into an animal like a rabbit. After the rabbit immune response, the rabbit serum will contain antibodies that recognize and bind to human antibodies. Secondary antibodies are frequently labeled to make them visible.

In this experiment, the secondary antibodies you will be working with are conjugated to an enzyme named horseradish peroxidase (HRP); HRP in the presence of its substrate, TMB, produces a blue color.

Controls in Immunoassays

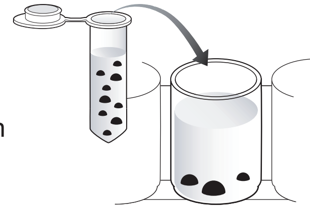
For any immunoassay to be valid, it must include both positive and negative controls, i.e., samples that will give known results. Controls are always run side by side with experimental samples. If you do not run a positive control and the experiment gives negative results, how can you be sure the results are truly negative? What if the assay simply did not work? If a positive sample gives a negative assay result, it is called a **false negative**. Conversely, if you do not run a negative control and the experiment gives all positive results, how can you be sure the results are truly positive? What if the assay was contaminated with antigen? If a negative sample gives a positive assay result, it is called a **false positive**.

Controls are also needed to guard against experimental error and to ensure that the assay is working correctly. There can be problems with reagents, which can degrade due to age or poor storage conditions. Operators can make mistakes by choosing the wrong reagents, making errors in dilutions or in pipetting, or failing to remove unbound reagents. Poor record keeping is another source of false assay results. Most of these possibilities can be checked for within the assay with the appropriate controls.

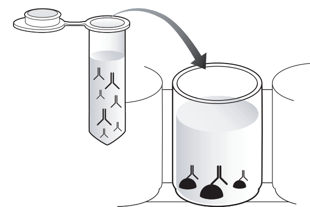
Now let's put this all together.

The main steps in this antigen detection ELISA are:

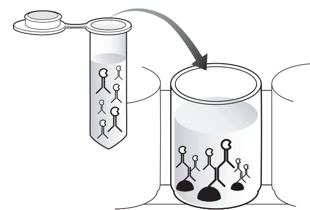
1. Add your sample and control samples to the wells in a microplate strip. Your samples contain many proteins and may or may not contain the antigen. Incubate for 5 minutes to allow all the proteins in the samples to bind to the plastic wells via hydrophobic interaction. This is called an immunosorbent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



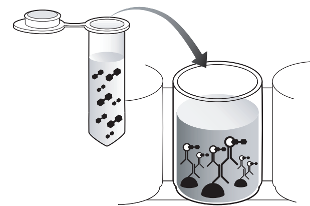
2. Add primary antibody to the wells and incubate. The antibodies will seek out the antigen from the many proteins bound to the well. If your sample contains the antigen, the antibodies will bind it tightly and remain in the well.



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.



Pre-Lab Focus Questions

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

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

3. How are the antibodies in your body made?

4. How are antibodies that are used in ELISA made?

5. Why is a rapid antigen detection test necessary?

6. What does ELISA stand for?

7. Why are enzymes used in this immunoassay?

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

Laboratory Guide

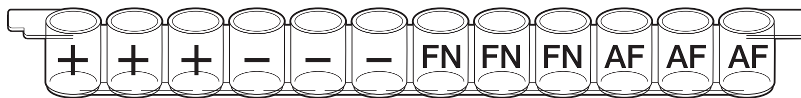
Student Workstation Checklist

One workstation serves 4 students.

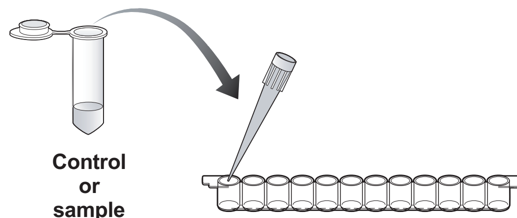
Items	Contents	Number	(✓)
Yellow tubes	Student samples (0.25 ml)	4	❑
Violet tube (+)	Positive control (0.5 ml)	1	❑
Blue tube (–)	Negative control (0.5 ml)	1	❑
Green tube (PA)	Primary antibody (1.5 ml)	1	❑
Orange tube (SA)	Secondary antibody (1.5 ml)	1	❑
Brown tube (SUB)	Enzyme substrate (1.5 ml)	1	❑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

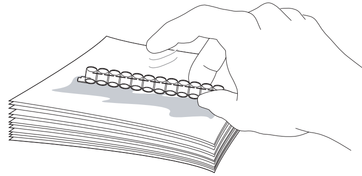


3. Bind the antigen to the wells:
   a. Use a pipet to transfer 50 µl of the positive control (+) from the violet tube into the three "+" wells.
   b. Use a fresh pipet tip to transfer 50 µl of the negative control (–) from the blue tube into the three "–" wells.
   c. Use a fresh pipet tip for each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

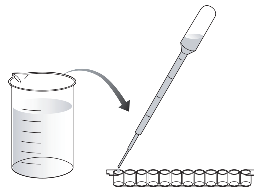


PROTOCOL II
Antigen Detection ELISA

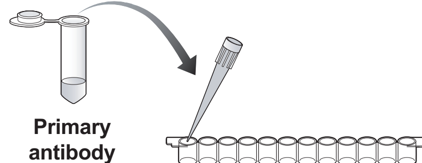
4. Wait 5 minutes while all the proteins in the samples bind to the plastic wells.
5. Wash the unbound sample out of the wells:
    a. Tip the microplate strip upside down onto the paper towels so that the samples drain out, then gently tap the strip a few times upside down on the paper towels. Make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



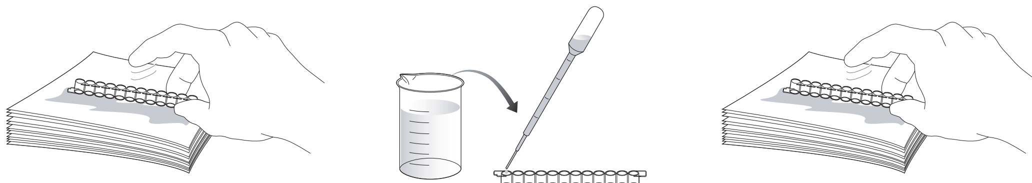
    b. Discard the top paper towel.
    c. Use a transfer pipet filled with wash buffer from the beaker to fill each well with wash buffer taking care not to spill over into neighboring wells. The same transfer pipet will be used for all washing steps..



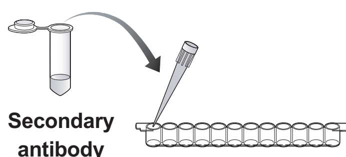
    d. Tip the microplate strip upside down onto the paper towels so that the wash buffer drains out, then gently tap the strip a few times upside down on the paper towels.
    e. Discard the top 2–3 paper towels.
6. Repeat wash step 5.
7. Use a fresh pipet tip to transfer 50 µl of primary antibody (PA)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



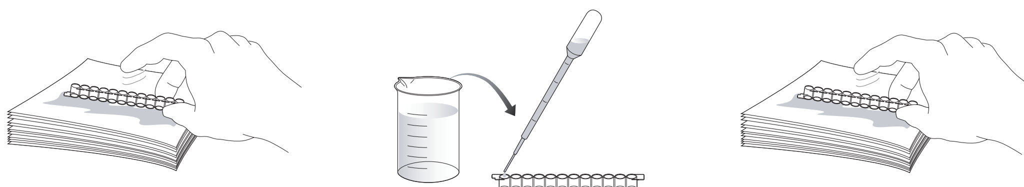
8. Wait 5 minutes for the primary antibody to bind.
9. Wash the unbound primary antibody out of the wells by repeating wash step 5 **two** times.



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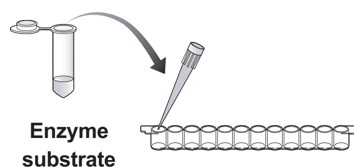
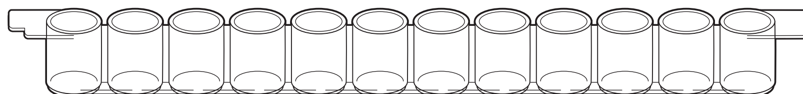
13. Use a fresh pipet tip to transfer 50 µl of enzyme substrate (SUB) from the brown tube into all 12 wells of the microplate strip.



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?