

ImE ELISA

Learning Objectives

The student will

- Follow oral and written instructions and manage time in the lab efficiently.
- Apply correct terminology regarding microbiological techniques when making observations.
- Use ELISA to detect the presence of specific proteins in a sample.

Background/Theory

ELISA stands for **Enzyme Linked Immunosorbent Assay**. All **Enzyme Immunoassays, EIAs**, involve an antibody molecule whose constant region binds an enzyme, leaving the variable region free to bind its specific antigen, figure 0. The addition of a substrate for the enzyme allows the antigen to be visualized and/or quantified. (OpenStax CNX, 2018) An ELISA can be used to detect a specific antigen or a specific antibody in a sample. This protocol probes for a specific antibody in a sample. This is the same type of test used to determine if a patient has been exposed to a certain disease. If the patient's immune system encounters a pathogen, it will produce antibodies homologous to pathogen antigens. These antigens, present in a viral capsid or a bacterial cell wall, can be purified in the lab and used to probe for the homologous antibody in the patient's blood.

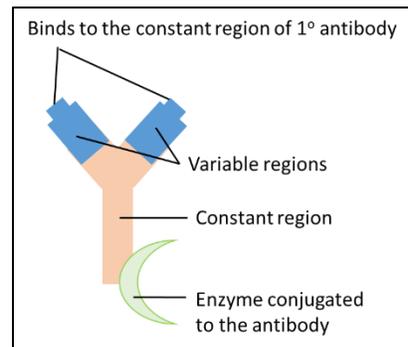


Figure 0. The "enzyme linked" antibody

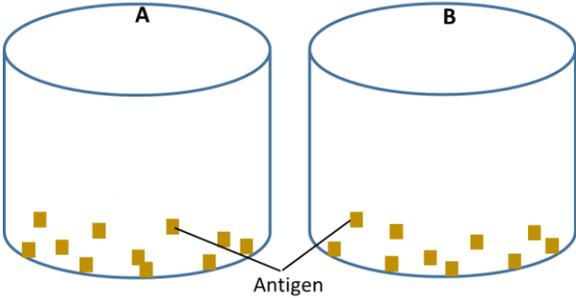
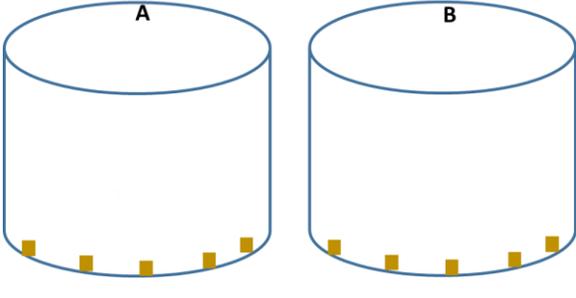
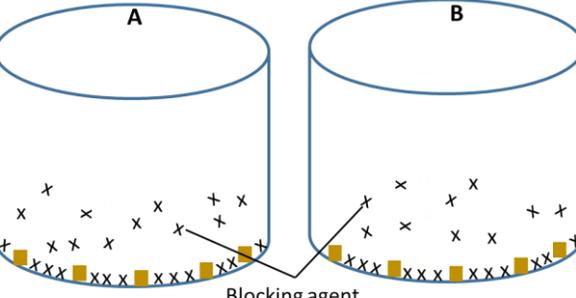
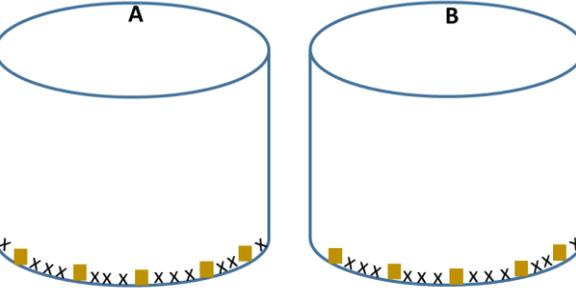
The ELISA takes place in a plastic plate containing numerous small wells. This plate is called a **microtiter plate**. (In this lab you will be performing a simplified test using fewer wells in a microtiter strip.) The first step is to add the antigen prepared from the disease causing organism to the wells, figure 1. The plastic will **adsorb** (bind) the antigen (usually at least part protein) molecules in the solution via hydrophobic interaction. This binding is reflected in the term "Immunosorbent." Because we will be using a purified antigen, this is the only molecule left after the wells are washed, figure 2.

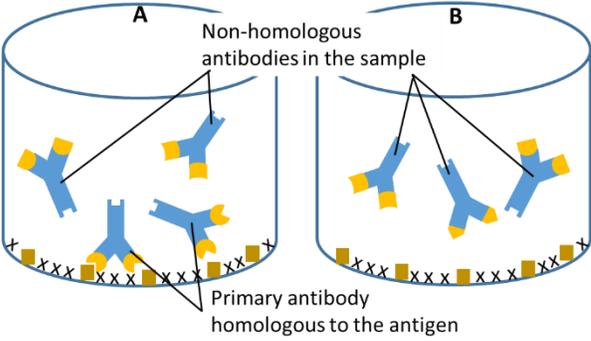
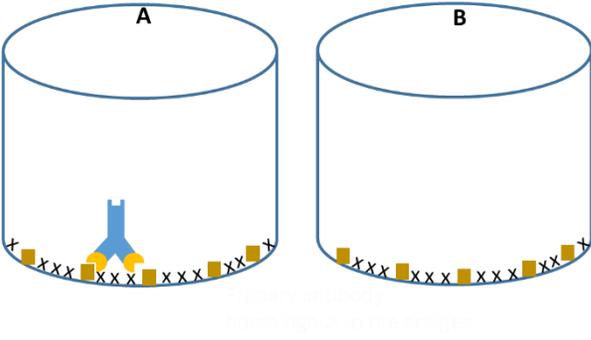
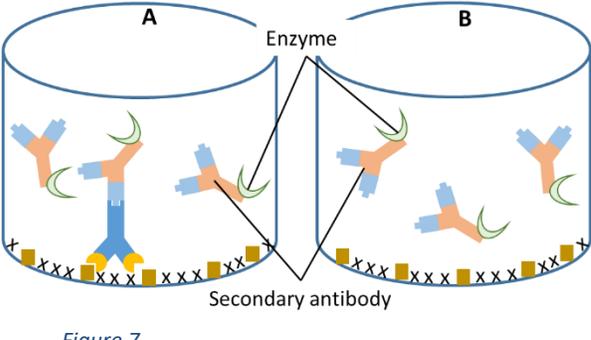
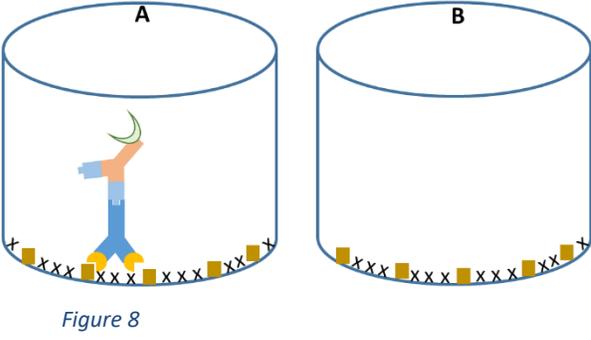
In the next step a blocking agent is added, figure 3. Common blocking agents are gelatin, skim milk or bovine serum albumin. These substances contain a variety of proteins that will fill in the spaces between the antigen molecules in the well preventing any non-specific antibody interaction with the plastic well in later steps. This is followed by another gentle wash, figure 4.

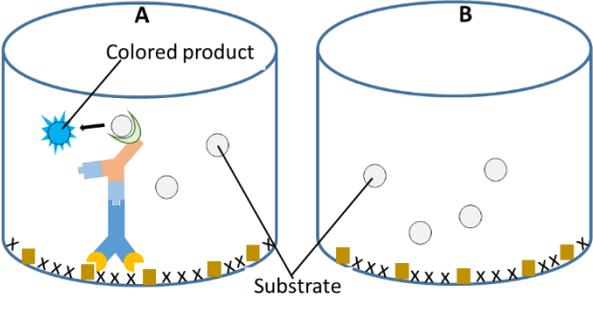
The sample is added in the third step, figure 5. In our scenario the sample would be the patient's blood serum sample. Any serum sample contains a large number of antibodies specific for a variety of antigens. Only the IgG antibody homologous to the antigen, antibodies made in response to the pathogen, will bind. This homologous antibody is called the **primary antibody**. Other non-homologous antibodies will not bind to the antigen and will be washed away. The primary antibody confers **specificity** to the assay. At this point the test is either positive or negative. The remaining steps will make the result visible.

Adding the **secondary antibody** allows visualization of the result, figure 7. The secondary antibody is commercially prepared and takes advantage of the constant region of the Ig molecule. You will recall that the constant region is so named because it is the same for all Ig molecules of the same class in the same organism. For example, one can inject a goat with human IgG and the goat will make antibodies that will recognize the constant region of the human IgG. The secondary antibody is essentially an antibody to an antibody. An enzyme is then conjugated to the secondary antibody. This is

where the “Enzyme Linked” part of the name comes from. In our case, the enzyme used is horseradish peroxidase (HRP). The following wash step is very important. All unbound secondary antibody must be removed, figure 8.

Molecular Representaiton	Description
 <p>Figure 1</p>	<p><i>Figure 1</i> Purified antigen is added to all the wells.</p>
 <p>Figure 2</p>	<p><i>Figure 2</i> After the wash step, only bound antigen is left.</p>
 <p>Figure 3</p>	<p><i>Figure 3</i> The blocking agent is added. It fills in the spaces between the antigen molecules in the well preventing any non-specific antibody interaction with the plastic well in later steps.</p>
 <p>Figure 4</p>	<p><i>Figure 4</i> The wash step removes excess blocking agent.</p>

Molecular Representaiton	Description
 <p data-bbox="300 598 381 630"><i>Figure 5</i></p>	<p data-bbox="896 247 998 279"><i>Figure 5</i></p> <p data-bbox="896 283 1396 451">The sample is added. Well A represents a sample from a patient who has been exposed to the antigen and well B represents a sample from a patient not exposed.</p>
 <p data-bbox="300 1014 381 1045"><i>Figure 6</i></p>	<p data-bbox="896 663 998 695"><i>Figure 6</i></p> <p data-bbox="896 699 1396 909">After washing, unbound non-homologous antibodies will be washed away. (For simplicity sake, only one bound primary antibody is shown. In reality, many of the antigen molecules will have the primary antibody bound in sample A.)</p>
 <p data-bbox="300 1409 381 1440"><i>Figure 7</i></p>	<p data-bbox="896 1079 998 1110"><i>Figure 7</i></p> <p data-bbox="896 1115 1396 1251">The conjugated secondary antibody is added to the wells. It will bind to any human IgG. This is why the previous wash step is so important.</p>
 <p data-bbox="300 1778 381 1810"><i>Figure 8</i></p>	<p data-bbox="896 1470 998 1501"><i>Figure 8</i></p> <p data-bbox="896 1505 1339 1568">Following the wash step, all unbound secondary antibody is removed.</p>

Molecular Representaiton	Description
 <p data-bbox="300 562 381 590">Figure 9</p>	<p data-bbox="894 247 992 275"><i>Figure 9</i></p> <p data-bbox="894 281 1419 415">The substrate is added. The enzyme in well A will catalyze a color change in the substrate. Well B will not show a color change within the 5 minute time limit.</p>

The final step is the addition of the **substrate**, figure 9. In the wells that contain bound secondary antibody conjugated with HRP, the HRP will catalyze a color change in the substrate. The wells that do not have secondary antibody and consequently no HRP, will show no color change.

The method described above is called the **Antibody Capture Method** because you use the purified antigen to “capture” the corresponding antibody if it is present. Because the antigen is used to detect the antibody, the Antibody Capture Method is an **indirect ELISA**. The antibody capture method is used to screen for HIV exposure. If the patient has been exposed to HIV, antibodies to HIV are present in the patient’s blood. A component of the viral capsid is used as the antigen.

In the sandwich ELISA, an antibody is used to probe for an antigen in the patient sample. This can also be referred to as the Antigen Capture ELISA. In HIV screening, both ELISA versions are often used together.

Application: THE MMR TITER

Excerpt from (OpenStax CNX, 2018)

The MMR vaccine is a combination vaccine that provides protection against measles, mumps, and rubella (German measles). Most people receive the MMR vaccine as children and thus have antibodies against these diseases. However, for various reasons, even vaccinated individuals may become susceptible to these diseases again later in life. For example, some children may receive only one round of the MMR vaccine instead of the recommended two. In addition, the titer (amount of antibody) of protective antibodies in an individual’s body may begin to decline with age or as the result of some medical conditions.

To determine whether the titer of antibody in an individual’s bloodstream is sufficient to provide protection, an MMR titer test can be performed. The test is a simple immunoassay that can be done quickly with a blood sample. The results of the test will indicate whether the individual still has immunity or needs another dose of the MMR vaccine.

Submitting to an MMR titer is often a pre-employment requirement for healthcare workers, especially those who will frequently be in contact with young children or immunocompromised patients. Were a healthcare worker to become infected with measles, mumps, or rubella, the individual could easily pass these diseases on to susceptible patients, leading to an outbreak. Depending on the results of the MMR titer, healthcare workers might need to be revaccinated prior to beginning work.

To determine the MMR titer, a quantitative Antibody Capture ELISA is performed. A serial dilution is performed on the patient’s serum sample. Each 2-fold dilution is tested and the most dilute sample still showing a positive result is identified. From that information, the antibody titer is determined. Because the health risk to a fetus is great, this test is routinely run on pregnant women to ensure they remain immune to these three diseases.

Application: HIV screening test

Excerpt from (Centers for Disease Control and Prevention, Division of HIV/AIDS Prevention, 2018)

An estimated 1.1 million people in the United States are living with HIV, including about 162,500 people who are unaware of their status. Approximately 40% of new HIV infections are transmitted by people who are living with undiagnosed HIV. For those who are living with undiagnosed HIV, testing is the first step in maintaining a healthy life and reducing the spread of HIV.

Clinical trials sponsored by the National Institutes of Health show a clear personal health advantage to being diagnosed with HIV early and starting therapy right away. This information further highlights the importance of routine HIV testing and its potential impact on better health outcomes.

HIV tests are very accurate, but no test can detect the virus immediately after infection. How soon a test can detect infection depends upon different factors, including the type of test being used. There are three types of HIV diagnostic tests: nucleic acid tests (NAT), antigen/antibody tests, and antibody tests.

- **NATs** look for the actual virus in the blood. This test is very expensive and is not routinely used for HIV screening unless the person recently had a high-risk exposure or a possible exposure with early symptoms of HIV infection.
- **Antigen/antibody tests** look for both HIV antibodies and antigens. Antigens are foreign substances that cause your immune system to activate. If you're infected with HIV, an antigen called p24 is produced even before antibodies develop. Tests that detect both antigen and antibodies are recommended for testing done in labs and are now common in the United States. There is also a rapid antigen/antibody test available.
- **Antibody tests** detect the presence of antibodies, proteins that a person's body makes against HIV, not HIV itself. Most rapid tests and home tests are antibody tests.

An initial HIV test usually will either be an antigen/antibody test or an antibody test. If the initial HIV test is a rapid test and it is positive, the individual will be sent to a health care provider to get follow-up testing. If the initial HIV test is a laboratory test and it is positive, the laboratory will usually conduct follow-up testing on the same blood sample as the initial test. Although HIV tests are generally very accurate, follow-up testing allows the health care provider to be sure the diagnosis is right.

Khan Academy has a good video summarizing HIV testing called *Diagnosing HIV - Concepts and tests*. It can be found at the QR to the right or <https://youtu.be/3CjCAeGhtHA> (Punwani, 2015). This video explains why the combination antigen/antibody testing is preferred. Testing for both decreases the window of time after the initial infection when an infected person falsely tests negative.



Experiment/Exercise

Materials per student pair

Microtiter strip

1 flask of wash buffer (share with another pair) with one pipette

9 pasture pipettes with one reusable bulb

Stack of paper towels 1 inch high

One set of reagents (share with another pair)

Microtube with purified antigen (AG)

Microtube with blocking agent, skim milk (BA)

Microtube with secondary antibody (SA)

Microtube with substrate (SUB)

Microtube positive control (+)

Microtube negative control (-)

2 microtubes with patient samples (numbered)

Cultures

None

Procedure

1. Each and every step in this protocol is important. If you skimp on the wash steps or the incubation times, your assay will lack either specificity or sensitivity yielding erroneous results.
2. Label the first three wells of the microtiter strip "+" for the positive control, the next three "-" for the negative control, wells 7, 8 and 9 with one sample number and the final 3 wells with the second sample number.
3. Add antigen (AG) to the wells.
 - a. With a clean Pasteur pipette, add one drop (approximately 50 μ L) purified homologous antigen to each well. Discard the pipette into the benchtop disposal container.
 - b. Allow the strip to incubate at room temperature for 5 minutes.
4. Wash 2x.
 - a. Turn the microtiter strip over on the paper towels to drain out the antigen solution.
 - b. With a clean pipette fill each well with wash buffer. Turn the strip over onto a fresh paper towel to drain it off. Tap the strip on the paper towel a couple of times.
 - c. Discard the top 2-3 paper towel layers and repeat this wash a second time with fresh buffer. In each wash step, there will be bubbles left in the wells. This will not affect your results. Return the pipette to the wash buffer flask. It can be used for wash buffer throughout the assay.
5. Add the blocking agent (BA).
 - a. With a clean pipette, add one drop, approximately 50 μ L, blocking agent (BA) to each well. Discard the pipette into the bench disposal container.
 - b. Allow the strip to incubate at room temperature for 5 minutes.
6. Wash 2x
 - a. Turn the microtiter strip over on the paper towels to drain out the blocking agent.
 - b. Using the wash buffer pipette, fill each well with wash buffer. Turn the strip over onto a fresh paper towel to drain it off. Tap the strip on the paper towel a couple of times.
 - c. Discard the top 2-3 paper towel layers and repeat this wash a second time with fresh buffer. Return the pipette to the wash buffer flask.
7. Add the controls and the samples. In this step you will treat the wells differently.
 - a. With a clean pipette, add one drop, approximately 50 μ L, of the positive control solution to the corresponding wells. Discard the pipette into the benchtop disposal container.
 - b. With a clean pipette, add one drop of the negative control solution to the corresponding wells. Discard the pipette into the benchtop disposal container.
 - c. Add the samples to the corresponding wells using a fresh pipette for each sample.
 - d. Allow the strip to incubate at room temperature for 5 minutes.
8. After 5 minutes, gently wash the wells twice as in steps 4 and 6.
9. Add the secondary antibody (SA).
 - a. With a clean pipette, add one drop, approximately 50 μ L, of the secondary antibody (SA) solution to each well. Discard the pipette into the benchtop disposal container.
 - b. Allow the strip to incubate at room temperature for 5 minutes.
10. Wash the wells as in step 4 and 6 EXCEPT this time you will gently **wash THREE times**. It is very important that every bit of unbound secondary antibody is removed.

11. Add one drop of the substrate (SUB) to each of the wells with a fresh pipette. Watch for a color change to blue within 5 minutes. You may even see a color change immediately. After 5 minutes exposure to light will cause the substrate to turn blue spontaneously regardless of the presence of the enzyme. Any color change after this point, is not a valid positive.
12. Check your result with the instructor. They will initial your data sheet.
13. All microtubes and microtiter strips can be placed in the benchtop containers after use. Please do not dispose of the rubber pipette bulbs. They can be cleaned and reused. Place them in the beaker in the tube disposal tub.

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Lab Report: ELISA Test

Name _____

Lab Section _____

Data and Observations

	Well 1	Well 2	Well 3	Well 4	Well 5	Well 6	Well 7	Well 8	Well 9	Well 10	Well 11	Well 12
Sample +/- or patient #												
Color formed												
Result +/-												

Post Lab Questions

1. What is the purpose of the + control? What specific molecule, suspended in PBS (phosphate buffered saline) is present in the positive control?
2. What is the purpose of the negative control? Considering the components of the positive control, what should the negative control be?
3. This test has a specificity of over 99%. It is so specific that we only need to use clean pipettes and microtiter strips. They do not need to be sterile. What accounts for this very high specificity?
4. What would be the error resulting from the following scenarios? Potential false positives or potential false negatives? Loss of specificity or loss of sensitivity?
 - a. Only washing the wells twice after the secondary antibody incubation.
 - b. Using the pipette from the + control to add the negative control solution and both the patient samples to the wells.

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- c. Using the pipette form the – control to add both patient samples to the wells.
 - d. Washing the wells too vigorously.
 - e. The blocking agent is left out.
5. In the combination antibody/antigen test commonly used for HIV screening, the antibody is detected by a _____ ELISA and the p24 antigen is detected by a _____ ELISA. Using the combination of these two methods results in greater **specificity/sensitivity** (circle one).
6. What does MMR stand for? What is an MMR antibody titer testing for? Why is this test necessary for some pregnant women?

References

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