

California Lutheran University's Enriched Science (Clues) and California State University Program for Education and Research in Biotechnology (C-SUPERB)

ELISA IMMUNOASSAY FOR HUMAN IgA

The ELISA assay is a method to detect very small amounts of almost any substance. For example, pesticide residues, progesterone in urine, and transgenic proteins such as the "Starlink" corn protein can all be observed at very low levels.

The ELISA test uses antibodies that are specific for the substance to be measured. The antibodies bind only to that substance, so the assay is very specific. The antibodies also have an enzyme (a protein that does a chemical reaction) attached to them. After the antibodies are bound, the substrate (reactant for the enzyme) is added, and the enzyme converts the reactant (colorless) into a colored product. The amount of colored product is therefore directly related to the amount of substance present.

The ELISA assay is very versatile. It is used for over-the-counter pregnancy tests, where it detects progesterone in urine, which increases a lot in the first few weeks of pregnancy. An ELISA test is used by some supermarkets to ensure that produce does not have pesticide residues. The ELISA assay is used in many biochemical research labs, too.

THE ELISA IMMUNOASSAY TO FIND LEVELS OF IgA IN HUMAN SALIVA

OBJECTIVES:

1. To use an ELISA assay to find the concentration of IgA in human saliva.
2. To understand the structure and the binding of antibodies.
3. To understand the function of the linked enzyme in this assay.
4. To understand a "standard curve" - how to find the concentration of an unknown sample by comparison with known standard samples.

I. INTRODUCTION

Assays which utilize **antibodies** to detect specific molecules are called immunoassays, because antibodies are one of the main components of our immune system. Immunoassays can detect small amounts of biomolecules, drugs, and pesticides, and there are many applications for these assays in clinical, industrial and research settings. Many different types of samples may be tested, including proteins, carbohydrates, drugs, nucleic acids, and lipids. Immunoassays are also very sensitive, and they can usually detect substances in the ng/mL range (1 ng of the substance in 1 mL of solution, which is one part per million). Immunoassays are usually very economical, because many samples may be assayed in one 96 well microtiter plate.

All immunoassays use antibodies to bind the test molecule to be detected. The test molecule is called an **antigen**. Antibodies are proteins that bind one particular antigen very specifically. Antibodies are "Y" shaped molecules, with "variable" regions at the two ends of the Y. See figure 1 below.

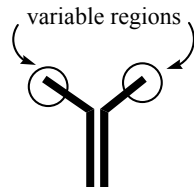


Figure 1. Antibody structure

The variable regions bind only one specific antigen, and there are different antibodies for every antigen. An example of antigen for the immune system would be a protein on the outside of the mumps virus. Once someone has had the mumps, or been vaccinated, they have antibodies that recognize (bind) the mumps virus, and they alert other immune cells to destroy the virus. That way that person will never have the mumps again!

In our ELISA assay we can choose the antibody, and that will determine what the immunoassay detects. In this experiment, we will determine the concentration of human IgA antibody in saliva. Therefore, our antigen is ALSO an antibody, which can be a little confusing. The IgA antibody is a protein produced in response to pathogens and other "foreign" substances coming in contact with the gastro-intestinal tract. The IgA antibody is often one of the antibodies that increases when an individual is responding to a bacterial or viral infection, or when oral vaccines are ingested. Therefore, the level of IgA can be used to help determine if someone is fighting an infection. However, the daily concentration of IgA varies tremendously in any individual, and depends primarily on how much saliva has recently been produced and the hydration state of the individual. Don't be alarmed if your IgA value determined today is low or high; it does not reflect the long-term ability of your immune system.

Theory of the ELISA Reaction:

There are a number of different methods that utilize antibodies in an immunoassay, but we will be using the **enzyme-linked immunosorbent assay**, or ELISA. The assay scheme is depicted in Figure 2.

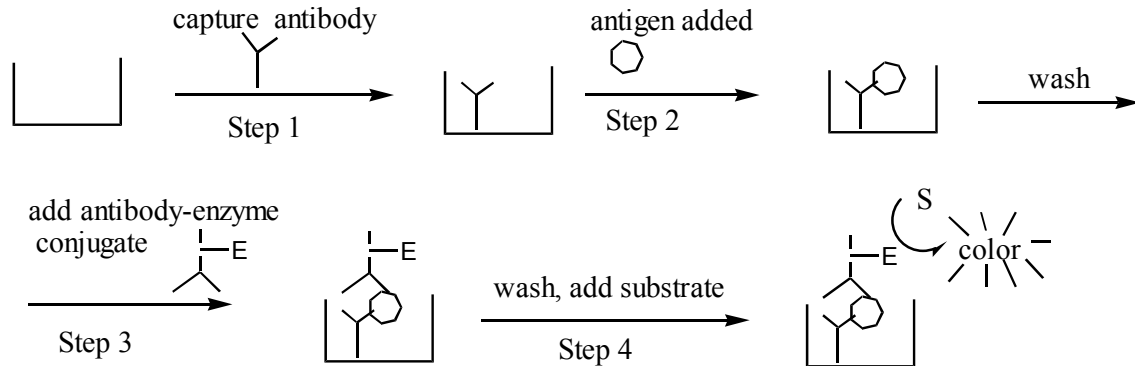


Figure 2. The ELISA assay scheme

In this assay, a plastic well is first coated with a "capture" (or primary) antibody, which is specific for the antigen. Second, a sample containing the antigen (substance to be detected) is added to the well. The antigen will be bound by the capture antibody, while other molecules remain in solution. Third, the well is washed free of unbound molecules, and a second, enzyme-linked antibody (secondary antibody) is introduced. This enzyme-antibody is also specific for the antigen and binds to the "captured" antigen to form a "sandwich". Fourth, the excess antibody-enzyme conjugate is washed away. Then a colorless substrate is added, which is converted by the attached enzyme to a colored product that may be measured by a spectrophotometer.

Antibody-Enzyme conjugate. The enzyme-labeled antibody is formed by covalently linking an enzyme called horseradish peroxidase (HRP) to the antibody with glutaraldehyde. This conjugate retains both enzyme activity and antibody binding ability. The enzyme portion converts the colorless substrate o-phenylenediamine (OPD) to a colored product, which is measured at a wavelength of 405 nm. The reaction is shown in figure 3.

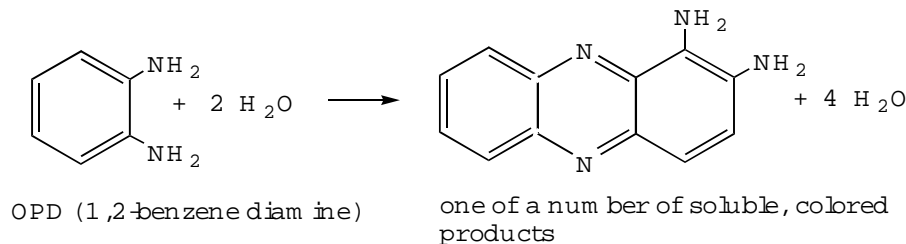


Figure 3. Conversion of OPD to a soluble colored product.

Standard curve. Because of the large number of steps in the assay and the variability in reagents, a standard curve must be constructed with every batch of samples. Furthermore, the samples must be diluted to fall within the accuracy limits of the standard curve (the area where the "curve" is linear). Thus, an IgA standard curve will be produced, along with two different dilutions of your saliva. Finally, to ensure accurate values for your saliva dilutions, each dilution will be done in triplicate.

II. EXPERIMENTAL PROCEDURE

A. Check to see that each station (for four students) has the following:

- A microtiter plate that is 8 x 8 rows (each student uses 2 x 8 rows)
- Two squirt bottles with TBT buffer (500 mL each)
- A set of IgA standards, at 10,000, 2700, 900, 300, 90, 30, 10 and 5 ng/mL (1 mL each)
- A test tube rack with an attached Sharpie marker and 8 glass test tubes
- 8 squares of parafilm
- A microfuge tube rack and 4 microfuge tubes
- 8 large pipets for making dilutions
- Micropipettor, 100 uL fixed volume
- Tips for the micropipettor
- A jar of anti-IgA-HRP antibody (10 mL)
- A jar of OPD color reagent (10 mL)

There should also be one microcentrifuge and one plate reader for the class.

B. Procedure:

1. Prepare dilutions of your saliva as follows: Dribble about 0.5 mL of saliva into a sterilized microfuge tube.
2. Snap the lid on, and spin the samples in the microcentrifuge for two minutes to pellet any crud. Do not disturb the "food" pellet that is formed at the bottom of the microfuge tube.
3. Use the micropipets to pipet 100 uL (0.100 mL) of the clear saliva into 9.9 mL of TBT buffer in a glass test tube. Mix thoroughly by capping the tube with Parafilm and inverting several times. This is a 1:100 dilution of saliva.
4. Make a 1:500 dilution by adding 1.0 mL of the 1:100 dilution into 4.0 mL of TBT buffer. Save these two dilutions.
5. Take a two row microtiter plate and empty out the TBT buffer by snapping it into the sink and then slapping it upside down onto a pile of paper towels (this is called "whapping"). This dislodges all of the buffer.
6. Now load the plate with your saliva dilutions and the IgA standards. First, use the micropipets to pipet 0.10 mL (100 uL) of the 1:100 saliva dilution in wells 1 through 3. Next, pipet 0.10 mL of the 1:500 dilution into wells 4 through 6. Then pipet TBT buffer only into wells 7 and 8. See figure 4 on the next page.

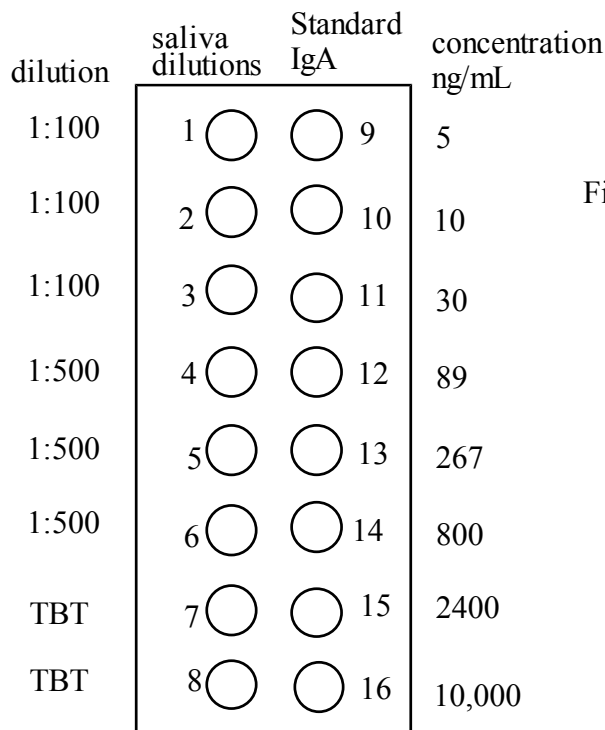
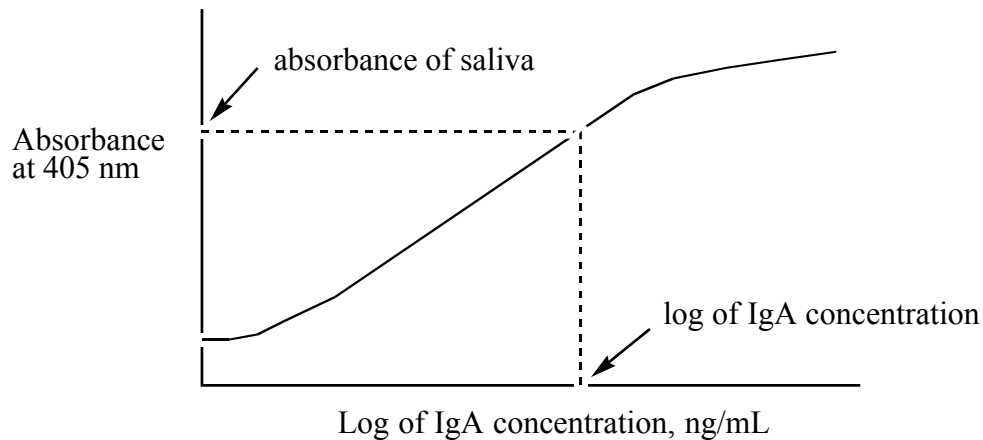


Figure 4. Diagram of samples in microtiter wells.

7. Pipet 0.100 mL (100 uL) of the standard IgA solutions into wells 9 through 16, starting with the lowest concentration (well 9) and moving to the highest concentration (well 16). **You do not have to change pipet tips for each new concentration as long as you move from low concentration to high concentration.**
8. Allow the samples to sit in the plate for 20 minutes. Then invert the plate and snap the samples into the sink. Immediately squirt TBT buffer into all the wells. **Do this gently; don't get lots of foam in the wells.** Allow the extra buffer to drip into the sink. Don't worry about being sloppy and dripping a lot, but do make sure that the wells are filled to the top with buffer, not bubbles!
9. Allow the plate to sit for 2 minutes, and then whap the buffer out of the wells. Wash the wells three more times with TBT buffer, letting the plate sit for two minutes each time and then whapping the buffer. After the last wash, leave the plate empty.
10. Add 100 uL (0.100 mL) of the anti-IgA-HRP conjugate to each well. Incubate for 20 minutes at room temperature.
11. Empty the plate by whapping, and then wash the plate four times with TBT buffer as in step 6. After the last wash, leave the plate empty.
12. Add 100 uL (0.100 mL) of the OPD substrate to each well, and allow it to incubate until the color is formed. **Ask your teacher when to measure the absorbance of your plate.** The reaction may take as little as 2 minutes or as long as 15 minutes. (Teacher note: monitor the absorbance, and when it reaches about 0.800 for the 10,000 ng/mL standard, read all the wells).
13. Average the absorbance of the two wells with buffer (wells 7 and 8). Subtract this absorbance from all your values, both your saliva and the standards. Then plot the adjusted standard IgA

absorbances versus the log of the concentration of the IgA in ng/mL. Note that the IgA concentrations below 1 ng/mL will have negative log values. The data points will probably lie on a slight sigmoid curve, so do not try to fit a straight line through them. See Figure 5 on the next page for a sample graph.



14. Use the standard curve to determine the concentration of IgA in your saliva. Note that the value from the graph is the log of the IgA concentration, and be sure to convert back. Then, multiply by your dilution factor (100 or 500) to obtain the concentration of IgA in your saliva.

Sample calculation: If your 1:100 saliva dilution had a absorbance of 0.545, you could find that on the y axis, and then draw a line over to the curve. Then draw a line down to the X axis, and this is the log of the IgA concentration. If you do an inverse log (10^x), you have the concentration of IgA in the diluted saliva. Multiply that concentration by 100 to find the IgA concentration in your undiluted saliva.

IV. EXERCISES:

1. Why was it important to wash the microtiter plate after the addition of each reagent?
2. Did your triplicate values agree well? In other words, did you measure the same concentration for each of your 1:100 dilutions, and the same for each of your 1:500 dilutions?
3. What about the averages for the 1:100 and 1:500 dilutions? If these didn't match, which dilution do you think was more accurate, and why? Can you suggest a reason why the dilutions might not agree?
4. How could you improve the accuracy of this assay?
5. Do you think this particular assay is quantitative enough for a hospital laboratory?

How to prepare the ELISA assay materials (optional!)

1. The antibody-coated microtiter plate was prepared by adding 100 μ L of affinity-purified specific anti-IgA human antibody at a concentration of 2 μ g/mL in 0.1 M carbonate buffer, pH 9, to each well and incubating 1-2 hours at room temperature, or overnight at 4 $^{\circ}$ C. The antibody was removed and the wells were coated with 2% BSA for 1-2 hours at room temperature, or overnight at 4 $^{\circ}$ C. Finally, the BSA was recovered and the wells flooded with TBT. Store the plate at 4 $^{\circ}$ C. The plates are still good after 3 weeks of storage at 4 $^{\circ}$ C.
2. TBT buffer: 0.15 M NaCl, 0.010 M Tris, pH 7.4, 0.01 % Triton X100.
3. Human IgA standards: prepared at 10,000, 2400, 800, 267, 89, 30, 10, and 5 ng/mL. Note that the standard concentrations may be changed.
4. Anti-human IgA-HRP antibody conjugate (available commercially) diluted 1:2500 in TBT.
5. OPD substrate:
 - 200 mL of 0.10 M NaCitrate, pH 4.7
 - 200 mg of o-phenylenediamine (keep desiccated)
 - 0.85 mL 3% H₂O₂

Store at 4 $^{\circ}$ C for up to 5 days. Discard if the solution turns pale yellow.

Worksheet for the ELISA to measure IgA in human saliva

1. Record your absorbance values for each of your wells. Label the wells with the saliva dilution, or the concentration of IgA in the well.

- | | |
|----------|-----------|
| 1. _____ | 9. _____ |
| 2. _____ | 10. _____ |
| 3. _____ | 11. _____ |
| 4. _____ | 12. _____ |
| 5. _____ | 13. _____ |
| 6. _____ | 14. _____ |
| 7. _____ | 15. _____ |
| 8. _____ | 16. _____ |

2. Average the absorbance values for the wells with TBT buffer: _____ + _____ = _____

3. Subtract the TBT buffer absorbance from all your other values. Record the values in the new table below. Explain here why you are doing this step:

Adjusted values for the ELISA assay:

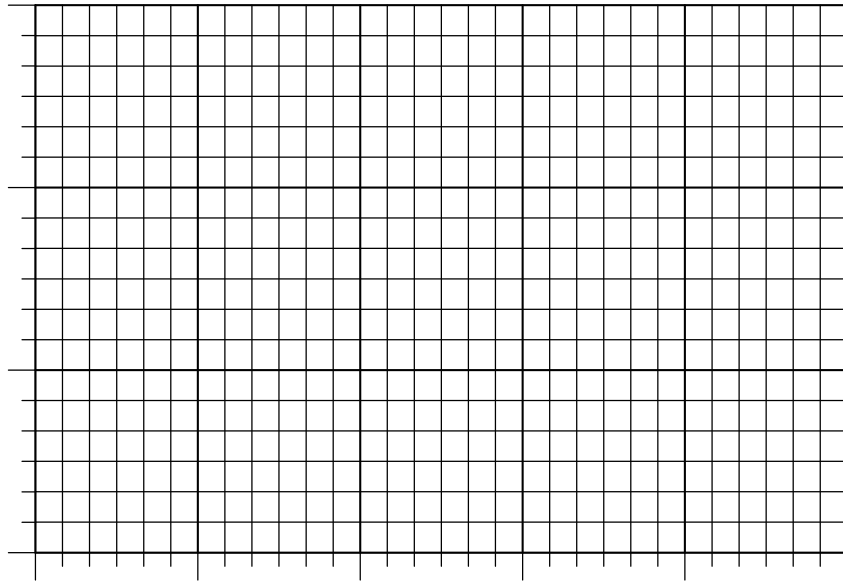
- | | |
|----------|-----------|
| 1. _____ | 9. _____ |
| 2. _____ | 10. _____ |
| 3. _____ | 11. _____ |
| 4. _____ | 12. _____ |
| 5. _____ | 13. _____ |
| 6. _____ | 14. _____ |
| 7. _____ | 15. _____ |
| 8. _____ | 16. _____ |

4. Find the log of the concentration of the IgA standards:

IgA concentration Log of the IgA concentration:

- | | |
|----------|----------|
| 9 _____ | 9 _____ |
| 10 _____ | 10 _____ |
| 11 _____ | 11 _____ |
| 12 _____ | 12 _____ |
| 13 _____ | 13 _____ |
| 14 _____ | 14 _____ |
| 15 _____ | 15 _____ |
| 16 _____ | 16 _____ |

5. Plot the log of the IgA concentration on the x axis versus the absorbance at that concentration on the y axis. This is your IgA standard curve. Label the graph completely, with a title, and the value and units on each axis.



6. Determine the concentration of IgA in your saliva from the graph.
- a. Find the average absorbance of your three 1:100 dilutions.
 - b. Find the corresponding log of IgA concentration
 - c. Convert the log of the IgA concentration into concentration by finding the inverse log (10^x) value of the concentration.
 - d. Repeat steps a through c for the 1:500 dilutions.

7. Answer the questions below:

- a. Why was it important to wash the microtiter plate after the addition of each reagent?
- b. Did your triplicate values agree well? In other words, did you measure the same concentration for each of your 1:100 dilutions, and the same for each of your 1:500 dilutions?
- c. What about the averages for the 1:100 and 1:500 dilutions? If these didn't match, which dilution do you think was more accurate, and why? Can you suggest a reason why the dilutions might not agree?
- d. How could you improve the accuracy of this assay?
- e. Do you think this particular assay is quantitative enough for a hospital laboratory?