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

ELISA IMMUNOASSAY FOR DISEASE DETECTION

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 in such as the "Starlink" corn protein can all be observed at very low levels with an ELISA assay .

In this experiment, you will use the ELISA assay to detect the presence of an antigen, such as a disease-related substance, from a sample of body fluid. Each student will be given a solution that represents body fluid, and the fluid will be exchanged with three other individuals. The ELISA test will used to detect a simulated disease agent in the exchanged fluids. The class set of data will be analyzed to determine the identity of the original carrier(s) of the disease.

This is similar to the type of assay that is used for the AIDS test to detect HIV. Epidemiology studies, such as those carried out by the Center for Disease Control (CDC) in Atlanta, can be carried out to investigate the origin of diseases, and to stop the spread of disease.

THE ELISA IMMUNOASSAY FOR DISEASE DETECTION

OBJECTIVES:

1. To be able to describe an ELISA test and explain the function of each reagent in the ELISA procedure.
2. To perform and interpret an ELISA test.
3. To describe applications using the ELISA test.

I. INTRODUCTION

Assays that 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:

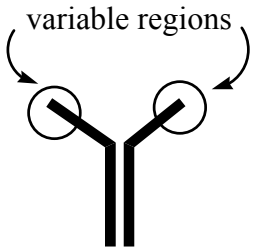

Figure 1. Antibody structure

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. The person will never have the mumps again!

In this laboratory, you will use the ELISA assay to detect the presence of an antigen, such as a disease-related agent, from a sample of "body fluid". You will exchange body fluid with three other individuals, and then the ELISA will be used to detect the simulated disease agent in the body fluids. Finally, you will analyze the class data set to determine the identity of the original carrier(s) of the disease. This assay is similar to the type of assay used for the AIDS test to detect infection with the HIV virus. Epidemiological studies, such as those carried out the Center for Disease Control in Atlanta, are used to investigate the origins of diseases and to help stop the spread of diseases.

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 immunosorbant assay**, or ELISA. The assay scheme is depicted in Figure 2.

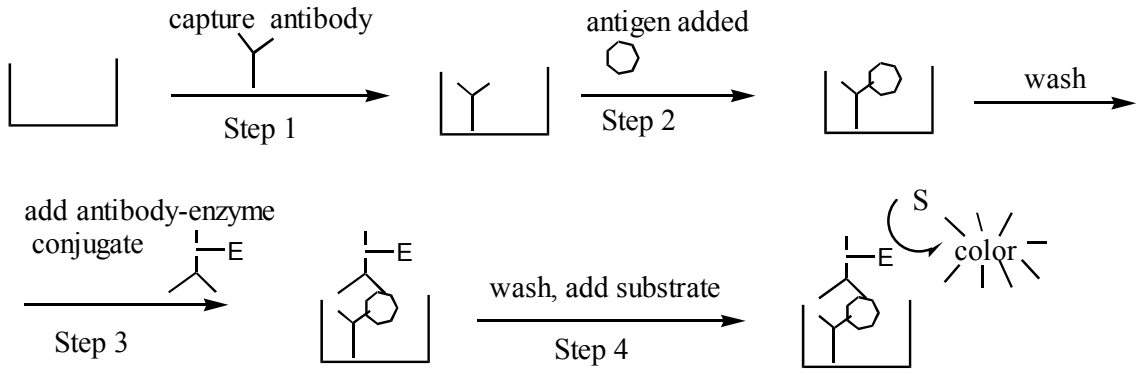

Figure 2. The ELISA assay scheme

In this assay, a plastic well is first coated with a "capture" (or primary) antibody, which is specific for the antigen (the antibody sticks to the plastic well because of hydrophobic patches on the antibody). Second, a sample containing the antigen (substance to be detected) is added to the well. The antigen is 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 gluteraldehyde. 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.

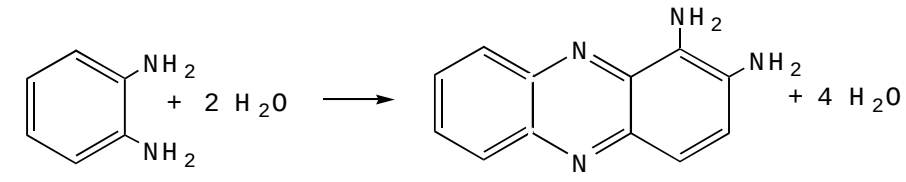

Figure 3. Conversion of OPD to a soluble colored product.

Standards: It is a common practice in scientific investigations to have a positive and a negative control. The positive control is a sample that is known to have the material being tested. The negative control is a sample that you are certain does not have any of the material being tested. You will have both controls, which are a tube that has only buffer in it and a tube that has a high concentration of the simulated disease agent.

II. EXPERIMENTAL PROCEDURE

Each student should have these items before starting the experiment:

Glass tube containing "body solution" (4 mL of solution)
Empty glass tube
Transfer pipet

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

- A microtiter plate that is 4 x 8 rows (each student gets 1 x 8 rows)
- Two squirt bottles with TBT buffer (500 mL each)
- A rack with marker, and positive and negative control solutions in glass tubes (1 mL each).
- Micropipettor, 100 uL fixed volume
- Tips for the micropipettor
- A jar of anti-IgA-HRP antibody (5-10 mL of solution)
- A jar of OPD color reagent (5-10 mL of solution)

Exchanging body solutions:

1. At the start of the laboratory, you will receive a transfer pipet, an empty tube, and a tube containing a solution that represents your "body fluids". Label the two tubes as "sharing" and "non-sharing", and with your initials, using the marker provided.

2. Divide the body fluid solution equally into the sharing tube and the second, non-sharing tube. Set the non-sharing tube in the rack. Use the sharing tube for the rest of the procedure below. The non-sharing tube will be used for later analysis.

3. Find one other student in your class and exchange your solution from your sharing tube with that student. Use the transfer pipet to draw up half of your solution, while your partner draws up half of their solution. Then place your solution in your partner's tube. Your partner will place their solution in your tube. You and your partner need to exchange fluids at the same time. Then mix the "shared" solution briefly by swirling the tube. Record the name of the person with whom you exchanged fluids.

4. After the instructor tells you to go ahead with round 2, find a different student to exchange your sharing solution with, and record the name of our second contact. NOTE: Be sure to chose students from all over the class, not just ones in your immediate area.

5. Optional: for classes of larger than 16 students. At the instructor's signal to begin round 3, exchange your sharing solution with a third student. Record the name of your third contact.

ELISA assay:

6. After all three contacts have been completed, you will do an ELISA test on your sharing tube and non-sharing tube fluids.

7. Take one row of the 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.

8. Now load the plate with your body fluid and the control. Load your solutions as shown in Figure 4 on the next page. Test each of the solutions twice. To load the wells, pipet 100 uL of each sample into each well, using the micropipets and tips provided. Use a new tip for each sample.

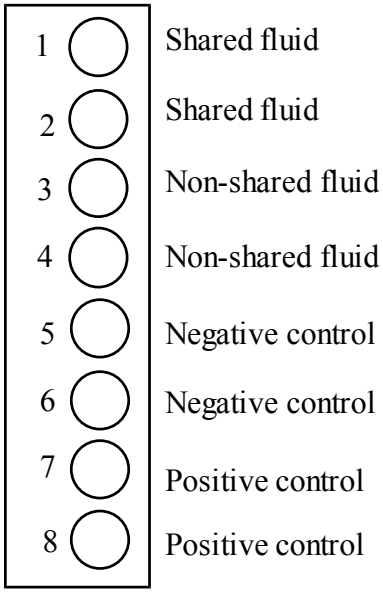

Figure 4. Diagram of samples in microtiter wells.

9. 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!

10. 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.

11. Add 100 uL (0.100 mL) of the anti-IgA-HRP conjugate to each well. Incubate for 20 minutes at room temperature.

12. 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.

13. Add 100 uL (0.100 mL) of the OPD substrate to each well, and allow it to incubate until the color is formed Be patient; the color may take as long as fifteen minutes to develop.

14. Make a chart of who is positive and negative in the class, and use it to find the original person who was infected.

EXERCISES:

1 Which person had the "infected" tube? How did you figure it out?

2. Why were positive and negative controls needed?

3. What was the purpose of washing the plates between the addition of each reagent?

4. Explain what is meant by a false positive test. Describe one error that would result in a false positive test.

5. Explain what is meant by a false negative test. Name one error that would result in a false negative test.

6. After doing this lab, would you agree or disagree with the following statement: "When you have sex with someone, you are also having sex with everyone that they have previously had sex with". Explain your answer.

7. How can you protect yourself from sexually transmitted diseases?

How to prepare the ELISA assay materials (optional!)

1. The antibody-coated microtiter plate was prepared by adding 100 uL of affinity-purified specific anti-IgA human antibody at a concentration of 2 ug/mL in 0.1 M carbonate buffer, pH 9, to each well and incubating 1-2 hours at room temperature, or overnight at 4 °C. The antibody was removed and the wells were coated with 2% BSA for 1-2 hours at room temperature, or overnight at 4 °C. Finally, the BSA was recovered and the wells flooded with TBT. Store the plate at 4 °C. The plates are still good after 3 weeks storage at 4 °C.

2. TBT buffer: 0.15 M NaCl, 0.010 M Tris, pH 7.4, 0.01 % Triton X100.

3. The negative control tubes are just TBT buffer. The positive control and the infected tube are a solution of 5 ug/mL IgA in TBT.

4. Anti-human IgA-HRP antibody conjugate (available commercially) diluted 1:2500 in TBT.

5. OPD substrate: 200 mL of 0.10 M sodium citrate, pH 4.7
200 mg of o-phenylenediamine (keep desiccated)
0.85 mL 3% H_2O_2

Store at 4 °C for up to 5 days. Discard if the solution turns pale yellow.

Worksheet for the ELISA used in an epidemiological study

1. Record the names of the partners with whom you exchanged body fluid:

Exchange #1 _____

Exchange #2 _____

Exchange #3 _____

2. Record your ELISA results for your shared tube and your non-shared tube.

Shared tube: Non-shared tube:

3. Enter your results for your shared tube on the board, and copy them here:
Classmates who were positive: Classmates who were negative:

4. Determine who was the person with the infected tube. Describe how you figured this out.

5. Answer the questions below:
   a. Why were positive and negative controls needed?
   b. What was the purpose of washing the plates between the addition of each reagent?
   c. Explain what is meant by a false positive test. Describe one error that would result in a false positive test.
   d. Explain what is meant by a false negative test. Name one error that would result in a false negative test.
   e. After doing this lab, would you agree or disagree with the following statement: "When you have sex with someone, you are also having sex with everyone that they have previously had sex with". Explain your answer.
   f. How can you protect yourself from sexually transmitted diseases?