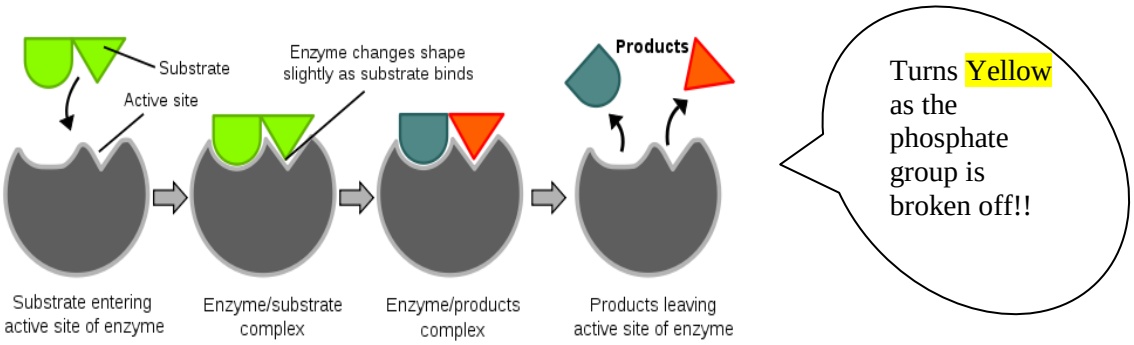
Enzyme Lab - ELISA

Have you ever wondered how scientists know what type of substances you have in your blood? How do they test your blood for certain diseases or molecules that indicate your health status? Enzyme-substrate reactions are one way to determine the amount of substances in blood serum. Remember, an important fact about enzymes is that **they are very specific** about the particular reaction they can catalyze. The reason they are so specific is that they all have a particular shape and will only fit with certain substrate molecules.



There are specific things you should look for when determining if a chemical change is taking place. If any of them are present, it is **evidence of a chemical change**. Check for a **change in temperature**, a **change in color**, **fizzing** (which indicates that a gas is being given off), **glowing, formation of a precipitate**, **smoke** given off, or **fire**.

In this lab, you'll be performing an analysis that is commonly used in science to detect the presence of a molecule. You will be working in pairs or groups to analyze a substance for the presence of a particular protein. We will be using a common scientific set of instructions called a **protocol** to perform an experiment known as an **ELISA** (Enzyme linked immunosorbent assay).

The following information may seem overwhelming but as we work through each step…it will all become very clear!!

Protocol for Ovalbumin (chicken egg) specific antibody ELISA for testing serum samples
Solutions will be prepared in advance but it is important that you understand what the goal is at each step of the process.

Day 1 – Coat the plate with the antigen/protein that we know will react with a specific antibody that we are testing for in the serum. If our bodies have the antibody present, which will be in the serum, then our immune system must have been activated to make that particular antibody by being exposed to the antigen.

Capture: Coat plate with 100 µg/ml ovalbumin protein in 1X phosphate buffered saline (PBS) by adding 25 µl per well of a 48 well ELISA plate. Place the plates in a plastic sandwich bag with a damp paper towel off to the side to prevent evaporation and place the plate in the refrigerator overnight.

Multichannel pipette
Tips
Protein in PBS
Tray for liquid
Plates (96 well plate cut in half to make 2 48 well plates
Sandwich bag
Paper towel

Students: Re-label the plate using a Sharpie as follows:

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
A								
B								
C								
D								
E								
F								

Using the multichannel pipette, set the volume to 25 µl; using the liquid in the tray, be sure each well has the appropriate volume of liquid. You may notice that the liquid doesn't quite cover the bottom of the well. If that occurs, gently tap the sides of the tray to make sure the liquid adheres and covers the bottom of each well. Place plates in a sandwich bag with a damp paper towel and refrigerate overnight.

Day 2

The objective in this step is to fill any spaces on the bottom of the well where our antigen did not bind. The neutral protein in the FBS will bind any empty spaces, creating an even layer across the bottom of the well. This will stop the antibodies in the sera and the secondary antibodies, from binding to anything besides the OVA protein (the targeted antigen).

Students:
Block: Shake off (flip) remaining ova solution into the sink and pat the plate on a paper towel (upside down) to remove any remaining liquid in the wells. Add 100 µl per well of the blocking buffer.
*Note: recipes for buffers may vary; we used 10% FBS in 1XPBS. Place the plate back into the plastic bag with a damp paper towel and place in the refrigerator overnight.

Multichannel pipette
Tips
Tray for liquid
FBS in PBS

*Read plan for Day 3 and set up plate schematic on worksheet for tomorrow! * See attached.

Day 3

Now that we have prepared the plates with the antigen that will bind to the antibody we are testing for, we are ready to test our serum. Be sure to orient your plate so that there are 8 columns across and 6 rows down. See your schematic for help.

Test serum: The diluent (10% FBS in 1XPBS) should be placed in the wells first. Always add the smaller volume of liquid to the larger volume. The diluent is in the white tray.

The serum was collected from mice that were immunized with OVA protein. This means they should have antibodies that are specific for the OVA protein that we put in the wells on the first day. There are four different tubes of serum:

1. **1x** From mice immunized once with OVA protein and blood collected 38 days later.
2. **2x** From mice immunized twice with OVA protein, on days 0 and 30. Blood was collected 8 days after the second immunization (day 38).
3. **N** From mice that were not immunized referred to as *naïve* mice.
4. **C** This will be our positive control

Students:

-Take the plate out of the Ziploc bag. Keep the bag. Flip the plate in the sink to remove the blocking buffer.

-Wash the plate 2 times by pouring the ELISA wash solution onto the plate, completely covering each well. Flip the plate in the sink. Repeat. To wash: use the small cup and fill it ¾ full with the washing solution, pour the solution over the wells and then flip it out into the sink.

(Washing solution- 1x PBS with Tween and water)

-After the second wash, invert the plate and pat it on a paper towel, to remove excess liquid.

-Important do not let the plate dry out completely, the next solution should be added immediately.

- Next add 148.5 µl of the diluent to the top row (**Row A**) of the plate and then add 50ul to every row below the first row (Rows B-F).

This will need to be done in two steps. First add 145 µl with the multi-channel pipette to the first row and then add 50 µl to each row below Row A. Then using the single pipette add 3.5 µl of the 10% FBS to each well in Row A.

-Add serum to the wells in row A as follows: **Be sure to use a new tip for each sample.**

A1 Add 1.5µl of the control serum to this well. (+ control)
A2 leave blank (- control)
A3 and A4 Add 1.5 µl to each well of the N serum
A5 and A6 Add 1.5 µl to each well of the 1x serum
A7 and A8 Add 1.5 µl to each well of the 2x serum

The samples are done in duplicate to confirm positive or negative results.

-Once the serum is in the top wells, Row A should have a total of 150µl of liquid. (Serum +10%FCS in PBS.) All other rows should have a total of 50µl.

-Place new tips on the multi-channel pipette and set it to 100ul. Put the tips into row A and mix by pipetting up and down 5 times. On the fifth time leave the tip full, containing 100ul, and move to the next row just below that row and do exactly the same mix technique using 100µl from the previous well. Pipette up and down 5 times then transfer 100ul to the next well. There will be 50ul remaining in each well. When you get to the last row, throw away the tips including the 100ul of liquid that remains. This is a serial dilution of 1.5 fold.

-Place the plate into the plastic Ziploc and re-wet the paper towel inside, if necessary. Place in the refrigerator overnight.

Day 4
Overnight, any antibodies that were specific for the antigen that we coated our plates with will bind to the antigen. **In this step we will be adding a secondary antibody that has an enzyme attached to it that we know will bind to the antibody that attached to our antigen.**

Detection:
*Teacher to make solution the morning of the experiment and placed in a central location for access. [Dilute antibodies: Use alkaline phosphatase conjugated antibodies for detection –dilute antibodies 1:2000 in diluents (10% FCS in PBS). Make enough to add 50 µl per well = ~5.1 ml per plate total volume.]

Students:
Wash plates 2X using wash solution and add 50 µl detection antibody per well. Place back in refrigerator.

Day 5

*Teacher to make solution just prior to experiment and be sure to store in a dark place.

Substrate: Substrate, 1 aliquot is added to 22.5ml glycine buffer. Make enough to add 100 µl per well ~ 11 ml/plate. Mix and store at RT in the dark.

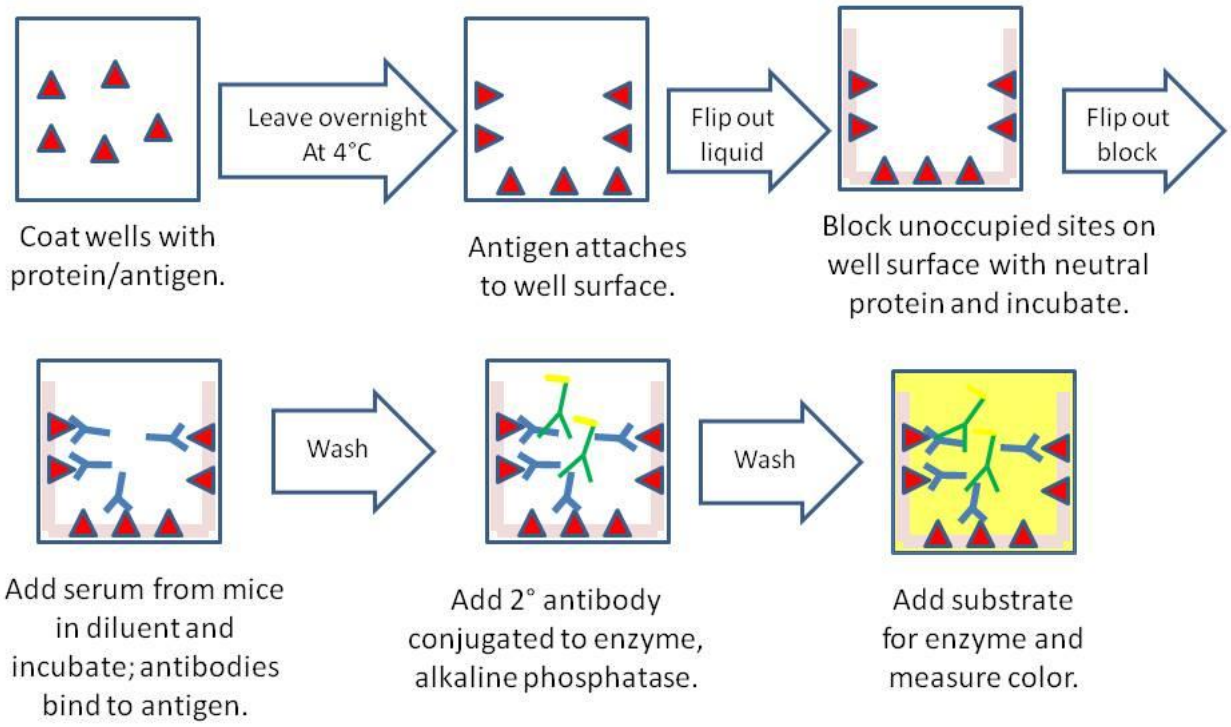
Students:
Wash plates 2X using wash solution.

Development:
Add 100 µl substrate per well. Wait about 10 to 15 minutes and watch the plate to see how the enzyme and substrate react to turn the liquid in the wells yellow. Record data on your worksheet and complete the analysis questions and conclusion.

Notes for Teachers:

- ✓ Make a class set of the lab for students to read before starting the lab.
- ✓ Practice with the multichannel pipettes using water with some food coloring before starting the lab so students have an idea of how to check the volumes, etc.
- ✓ Daily, place the instructions on the board and review the process.
- ✓ With the exception of Day 3 when the serum is tested, one tray of solution works best and is most efficient.
- ✓ As the students become proficient with the pipettes, this process moves fairly quickly so have other work or instruction planned for days 1, 2, and 4.
- ✓ Use multichannel pipettes to add reagent and do serial dilutions
- ✓ Do not allow wells on the ELISA plate to dry out – prep reagent for the next step before you begin washing and setup to add the reagent close to the ELISA washer.
- ✓ To prevent evaporation ELISA plates should be in sealed plastic bags with a damp paper towel.
- ✓ All antibodies must be immediately returned to 4°C – especially enzyme conjugated antibodies.
- ✓ Consider taking pictures of the plates for the students with a camera.

Indirect ELISA



ELISA
Enzyme Linked Immunosorbent Assay

Name: _____
Hour: _____

Indicate on the table, how you set up your experiment based on Day 3 of the Protocol:

	1	2	3	4	5	6	7	8
A								
B								
C								
D								
E								
F								

Document the results of your experiment by coloring in the wells to match your plate:

	1	2	3	4	5	6	7	8
A								
B								
C								
D								
E								
F								

Analysis

1. When the substrate, the final step in the experiment, was added to the plate, did a chemical reaction take place? How did you know?

2. How do you know which wells had more of the target protein than the others?

3. What is the control in an experiment?

4. What is the independent variable in an experiment?

5. What is the dependent variable in an experiment?

6. Define substrate.

7. Define enzyme.

8. Explain, in your own words, why we did this experiment.

Conclusion

This particular assay is used to test human blood for HIV. What would you want your wells to look like if the test was on your blood? Why? Explain!