

Enzyme-Linked Immunosorbent Assay (ELISA)

Which Samples Test Positive for Influenza Antigen?

Materials:

1. **ELISA Plate:** 96-well Maxisorb plate to be coated with influenza proteins.
2. **Influenza Protein Pool (“Labeled FP”):** Contains a mixture of influenza hemagglutinin (HA) and neuraminidase (NA) proteins.
3. **ELISA Coating Buffer (“Coating Buffer”):** This is the buffer that you mix your influenza proteins with to make them stick to – or coat – the ELISA plate.
4. **ELISA Plate Cover:** This is a sticky cover specially made to cover 96-well plates.
5. **ELISA Blocking Buffer (“Block”):** This buffer contains 5% bovine serum albumin (BSA) in 1X PBS, pH 7.4. PBS is “Phosphate Buffered Saline” which is very similar chemically to your blood. It is kept cold until use. ELISA Block is used to block all of the “extra space” in your ELISA wells.
6. **Patient Samples:** Get a fresh aliquot (tube) of all 4 of your patient samples. These have been stored for you in the freezer.
7. **ELISA Positive Control (“E Pos Ctrl”):** This is our positive control serum, and is known to contain *HIGH LEVELS* of anti-influenza antibodies.
8. **ELISA Negative Control (“E Neg Ctrl”):** This is our negative control serum, and is known to contain *NO* anti-influenza antibodies.
9. **Antibody Diluent Buffer (“Ab Diluent”):** This is what you will dilute your patient serum samples with prior to adding them to the ELISA plate.
10. **ELISA Wash Buffer:** 0.05% Tween-20 Detergent in 1X PBS, pH 7.4. This will be located in bottles from which you can take 20 ml in a cup or beaker. This buffer will remove anything that should not be bound to the ELISA wells.
11. **Paper Towels:** Stacks of paper towels will be placed next to the sinks and will be used to pound residual ELISA Wash Buffer liquids from your ELISA plates.
12. **Anti-Human-HRP (“2° Ab”):** This is the secondary antibody that will be used to detect whether or not anti-influenza human antibodies are present. It is conjugated (covalently bound) to horse Radish Peroxidase (HRP), which is the enzyme that we’ll use to “develop” our ELISA plates.
13. **TMB ELISA Substrate (“TMB”):** TMB, or 3,3',5,5'-Tetramethylbenzidine, is the liquid substrate that HRP catalyzes to make a blue color.
14. **ELISA Stop Solution (“Stop”):** This is 1N H₂SO₄. This will change the blue TMB to yellow, and will stop the enzymatic reaction from continuing any further. Otherwise, every well would eventually turn dark blue and you wouldn’t be able to tell the difference among samples.

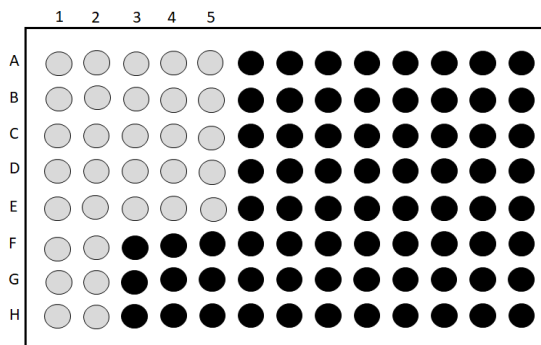
Protocol: Part 1: Coating Your ELISA Plate

✓ You are **STRONGLY ENCOURAGED** to check off each step below as you complete it.

To determine if patients samples have anti-influenza antibodies, we have to coat the wells of our ELISA plates with influenza proteins that can be bound by these antibodies (if present).

1. Obtain the following materials:
 - a. 1 microfuge tube of the **Influenza Protein Pool (labeled “FP”)**
 - b. 1 tube of 5 ml of **ELISA Coating Buffer (labeled “Coating Buffer”)**
 - c. **1 ELISA Plate**
2. Label the **side** of your ELISA plate with your initials, your lab partner’s initials, the date, and your class. There is a frosted area on the side of the plate that is designed for labeling.
3. Prepare 5 ml of Influenza Protein Pool diluted to 1 µg/ml in ELISA Coating Buffer. The stock concentration of the Influenza Protein Pool 150 µg/ml.
 - a. Assume that Concentration is “C” and Volume is “V.” We will use the equation $C_1 * V_1 = C_2 * V_2$.
 - b. C_1 is our final concentration, 1 µg/ml. V_1 is our final volume, 5 ml, which is the same as 5,000 µl. It is best to have the same units on both sides of the equation if possible.
 - c. C_2 is our starting or “stock” concentration, 150 µg/ml. V_2 is the unknown amount of our stock solution that we must add to the ELISA Coating Buffer.
 - d. $1 \mu\text{g/ml} * 5,000 \mu\text{l} = 150 \mu\text{g/ml} * V_2$
 - e. $V_2 = \underline{\hspace{2cm}} \mu\text{l}$ added the 5 ml tube of ELISA Coating Buffer, put the lid on the tube, and mix by inverting 3-5 times.
4. Add 100 µl of Influenza Protein Pool solution to each of the following wells, as shown in the picture:

<input type="radio"/> A1-A5	<input type="radio"/> E1-E5
<input type="radio"/> B1-B5	<input type="radio"/> F1-F2
<input type="radio"/> C1-C5	<input type="radio"/> G1-G2
<input type="radio"/> D1-D5	<input type="radio"/> H1-H2
5. Cover your ELISA Plate with the ELISA Plate Cover, make sure it is well sealed, and check with your teacher to learn if you should:



- a. Store your plate overnight at 4°C (i.e., in the refrigerator) **OR**

- b. Incubate your plate at room temperature for 5 minutes and proceed to Part 2.

Protocol: Part 2: Preparing and Adding Your ELISA Samples to the Plate

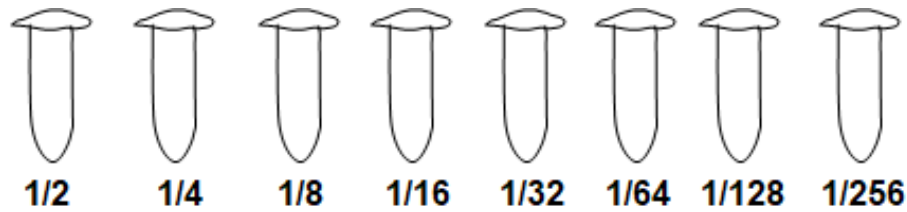
✓ You are **STRONGLY ENCOURAGED** to check off each step below as you complete it.

6. Obtain the following materials:
- 1 tube of 15 ml of **ELISA Blocking Buffer** (labeled “Block”)
 - 1 tube of 5 ml of **Antibody Diluent** (labeled “Ab Diluent”)
 - 1 microfuge tube of the **ELISA Negative Control** (labeled “E Neg Ctrl”)
 - 1 microfuge tube of the **ELISA Positive Control** (labeled “E Pos Ctrl”) at **500ug/mL**.
7. Take the plate cover off of your ELISA plate and save it at your bench.
8. Take your ELISA plate to a sink and fling the contents of the plate into the sink.
9. Return to your bench and add 300 µl of **ELISA Blocking Buffer** to each of your sample wells. These are the same wells that you coated with influenza proteins, so you may want to refer to the map of the plate on the previous page to confirm which wells to block.
- | | |
|----------|----------|
| a. A1-A5 | e. E1-E5 |
| b. B1-B5 | f. F1-F2 |
| c. C1-C5 | g. G1-G2 |
| d. D1-D5 | h. H1-H2 |
10. Set a timer or note the time. Incubate your ELISA in Blocking Buffer for at least 5 minutes at room temperature (though up to 2 hours is acceptable at room temperature) or overnight at 4 °C. Check with your teacher to learn which time and temperature.

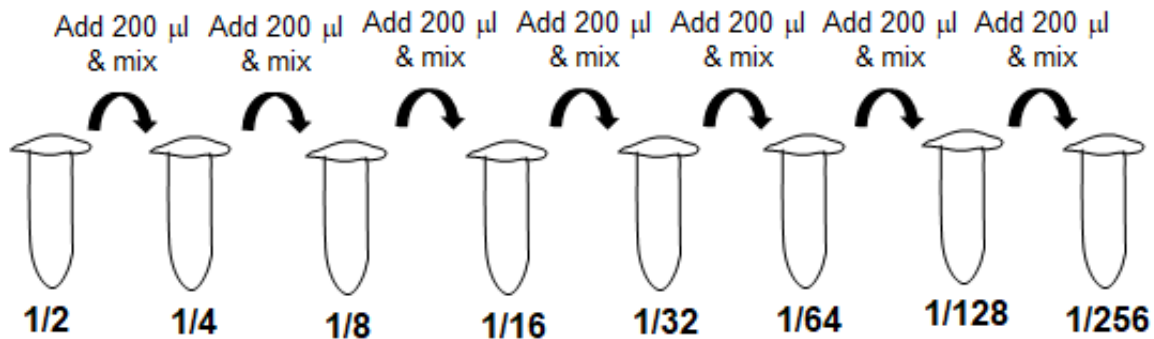
While your plate is blocking, prepare your **ELISA Positive Control**, a patient sample that is known to contain a high level of anti-influenza antibodies (500ug/mL). You will be making **serial dilutions** of your positive control. You will be making 1/2 (pronounced “one to two”) serial dilutions. 1/2 is “1 part in a total of 2 parts,” while 1/4 is “1 part in a total of 4 parts.” Note: Each “step” in the dilution series above is half as concentrated as the previous step.

11. Obtain 8 microfuge tubes and label them with your initials and as follows:
- | | |
|--------------------|----------|
| a. 1/2 (250 ug/mL) | e. 1/32 |
| b. 1/4 (125 ug/mL) | f. 1/64 |
| c. 1/8 (etc) | g. 1/128 |
| d. 1/16 | h. 1/256 |

12. Arrange the tubes in your microfuge tube rack in the order they are listed above so that they are all in a row with the 1/2 tube on the left and the 1/256 tube on the right.



13. Add 200 μ l of **Antibody Diluent** to each of the labeled microfuge tubes.
14. Add 200 μ l of your **ELISA Positive Control** to the 1/2 tube, mix by pipetting up and down three times, and then discard your pipette tip. This is your first 1:2 dilution – 1 part (200 μ l) of positive control into a total of 2 parts (200 μ l Positive Control + 200 μ l Diluent = 400 μ l total).
15. For each of the following serial dilutions, you may use the same pipette tip.
- Take 200 μ l from the 1/2 tube, add it to the 1/4 tube, and mix by pipetting up and down three times.
 - Take 200 μ l from the 1/4 tube, add it to the 1/8 tube, and mix by pipetting up and down three times.
 - Continue to take 200 μ l from one tube in the serial dilution series, add it to the next one, mix, and then move on to the next dilution until you reach the last tube, the 1/256 tube.



16. Each group of students will be testing samples from four patients. **Write your Sample IDs below.**
- Sample ID: _____
 - Sample ID: _____
 - Sample ID: _____
 - Sample ID: _____
17. Think ahead about how you will add samples to your plate by looking at the plate plan in Table 1. **DO NOT add samples yet!!!** Notice that you will add your positive control samples to the plate in **duplicate** (i.e., two wells of the same sample) and you will load your patient samples and negative controls in **triplicate** (i.e., three wells of the same sample).

18. Fill in your sample IDs in **Table 1: ELISA Plate Plan** below. For example, if your first patient sample identification (ID) is “A444,” write “A444” on the line “ID: _____” for the cells labeled “Sample #1, Replicate #1;” “Sample #1, Replicate #2;” & “Sample #1, Replicate #3.”

19. Take your ELISA plate to the sink and fling the contents into the sink. Pound the plate on a stack of paper towels to help remove the last of the Blocking Buffer.

Table 1: ELISA Plate Plan

	1	2	3	4	5
A	Positive Control 1/2 Replicate #1	Positive Control 1/2 Replicate #2	Negative Control Replicate #1	Negative Control Replicate #2	Negative Control Replicate #3
B	Positive Control 1/4 Replicate #1	Positive Control 1/4 Replicate #2	Sample #1: ID: _____ Replicate #1	Sample #1: ID: _____ Replicate #2	Sample #1: ID: _____ Replicate #3
C	Positive Control 1/8 Replicate #1	Positive Control 1/8 Replicate #2	Sample #2: ID: _____ Replicate #1	Sample #2: ID: _____ Replicate #2	Sample #2: ID: _____ Replicate #3
D	Positive Control 1/16 Replicate #1	Positive Control 1/16 Replicate #2	Sample #3: ID: _____ Replicate #1	Sample #3: ID: _____ Replicate #2	Sample #3: ID: _____ Replicate #3
E	Positive Control 1/32 Replicate #1	Positive Control 1/32 Replicate #2	Sample #4: ID: _____ Replicate #1	Sample #4: ID: _____ Replicate #2	Sample #4: ID: _____ Replicate #3
F	Positive Control 1/64 Replicate #1	Positive Control 1/64 Replicate #2	Empty	Empty	Empty
G	Positive Control 1/128 Replicate #1	Positive Control 1/128 Replicate #2	Empty	Empty	Empty
H	Positive Control 1/256 Replicate #1	Positive Control 1/256 Replicate #2	Empty	Empty	Empty

20. Use **Table 1: ELISA Plate Plan** above to add 100 µl of the correct sample to each well.

- For example, add 100 µl of the **1/2 ELISA Positive Control** to well A1 (labeled “Positive Control #1: 1/2” in Table 1) and 100 µl of the 1/2 ELISA Positive Control to well A2 (labeled “Positive Control #1: 1/2” in Table 1).
- Add 100 µl per well for each of your **samples** in the proper locations in **triplicate**. For example, add 100 µl of your first patient sample to well B3 (labeled “Sample #1, Replicate #1” in Table 1), 100 µl of first patient sample to well B4 (labeled

“Sample #1, Replicate #2” in Table 1) and 100 µl of first patient sample to well B5 (labeled “Sample #1, Replicate #3” in Table 1).

- c. Add 100 µl per well for your **ELISA Negative Control** in Wells A3, A4 and A5.
21. Cover your plate with your plate cover and ask your teacher how long to let your samples incubate:
- a. Overnight at 4°C (i.e., in the refrigerator) **OR**
 - b. At 37°C for 15 minutes.

Protocol: Part 3: Completing Your ELISA & Developing Your Plate

✓ You are **STRONGLY ENCOURAGED** to check off each step below as you complete it.

22. After the incubation of your patient and control samples is complete, take your ELISA plate to the sink and fling the contents into the sink. Pound the plate on a stack of paper towels to help remove the last of the samples.
23. Wash your ELISA plate by adding 200 µl of **ELISA Wash Buffer** to all wells that have control and patient samples. These are the same wells noted in Table 1: ELISA Plate Plan and in the image on the second page of this protocol.
24. Remove the Wash Buffer by flinging the contents of the plate in a sink and then pound out the excess on a stack of paper towels.
25. Repeat this process two more times (for a total of three washes) and let the third wash sit while you complete the next step.
26. Obtain the following reagents:
- a. 1 tube of 5 ml of **Secondary Antibody, Anti-Human-HRP**, labeled “**2° Ab.**”
 - b. 1 tube of 5 ml of **TMB ELISA Substrate (labeled “TMB”)**
 - c. 1 tube of 5 ml of **ELISA Stop Solution (labeled “Stop”)** (**NOTE:** This Step is Optional – Check with your teacher to learn if you need this tube).
27. Discard the ELISA Wash Buffer into the sink, and pound the plate on paper towels to remove any residual Wash Buffer.
28. Add 100 µl of **Secondary Antibody, “2° Ab,”** to each of your sample wells. These are the same wells that you added Wash Buffer to. Refer to Table 1: ELISA Plate Plan or the map on the second page of this protocol if needed. This secondary antibody will bind to any anti-influenza human antibodies if they are present.

29. Let the Anti-Human-HRP incubate in your ELISA plate for at least 5 minutes at room temperature.
30. After the incubation is complete, take your ELISA plate to the sink and fling the contents into the sink. Pound the plate on a stack of paper towels to help remove the last of the Protein A-HRP.
31. 200 μ l of **ELISA Wash Buffer** to all wells that have control samples and patient samples. These are the same wells noted in Table 1: ELISA Plate Plan and in the image on the second page of this protocol.
32. Remove the Wash Buffer by flinging the contents of the plate in a sink and then pound out the excess on a stack of paper towels.
33. Repeat for a total of 3 washes.
34. After your last wash, add 100 μ l of **ELISA TMB Substrate** to all wells that have control samples and patient samples. These are the same wells noted in Table 1: ELISA Plate Plan and in the image on the second page of this protocol.
35. Let the ELISA TMB Substrate incubate in your ELISA plate ***until your positive control samples are blue***. The most concentrated positive controls, the 1/2 dilutions in wells A1 and A2, should be very dark blue. The other positive control samples will get progressively lighter blue as the positive control becomes more dilute. The longer you allow the plate to develop, the darker the blue colors will become.
36. If your classroom has a plate reader, read the blue color at 605nm or 650nm. Different TMB providers give different instructions. Either wavelength will work.
37. If you are using the **ELISA Stop Solution ("Stop")**, stop the reaction by adding 100 μ l of the ELISA Stop Solution to each well that contains a sample.
38. If your classroom has a plate reader, read the yellow color at 450nm.
39. Record your sample IDs in **Table 2: Antibody Responses to Influenza** and note which samples were positive and which samples were negative for influenza antibodies. Remember that the Negative Control has no anti-influenza antibodies and the 1/2 dilution of the Positive Control has a high level of anti-influenza antibodies. Each patient might have different levels of antibodies (i.e., different colors of blue). You can estimate the amount of antibodies present by comparing the patient samples to the dilutions of the positive control samples.
40. To calculate the concentration of protein in the patient samples, you must first construct a standard curve by graphing the OD (optical density) readings of your standard curve samples (y axis) vs the known protein concentration in each sample (x-axis). Then generate the equation of the line that best fits your graph. You can use the equation of the line to calculate your unknown protein concentrations.

Table 2: Antibody Responses to Influenza

Sample Name	Positive or Negative for Influenza?
Sample ID:	
Sample ID:	
Sample ID:	
Sample ID:	