Influenza Outbreak Investigation
ELISA Teacher Preparation

Note: It is extremely helpful if students have had experience with micropipetting and serial dilutions prior to performing ELISAs. Practice activities are found under "Micropipetting & Serial Dilution Practice."

Reagent Preparation for Influenza Outbreak Investigation Kit ELISA

- **Assumes 32 patient samples**
- **Assumes students work in 8 groups of 4, with each group testing 4 patient samples**

This ELISA Can be Performed One of Two Ways:

- Patient samples are binary, either antibody-positive or antibody negative, or
- Patient samples are trinary:
    - The immune response of individuals vaccinated recently with the current vaccine are 'boosted' when exposed to virus in the scenario, resulting in high levels of anti-influenza antibodies. These individuals have mild influenza symptoms and test influenza-positive by PCR.
      NOTE: This curriculum was created in 2018, so 'recent vaccination' corresponds to the 2017 vaccine formulation. Vaccination '4 years ago' corresponds to the California strain vaccine.
    - The immune response of individuals vaccinated with an older influenza vaccine do not have a strong memory response, resulting in low levels of anti-influenza antibodies. These individuals have no influenza symptoms and test influenza-negative by PCR.
    - Those individuals who have never been vaccinated against influenza and were recently infected during the current outbreak have not had sufficient time to generate an anti-influenza antibody response and as a result test negative for anti-influenza antibodies by ELISA. These individuals have severe influenza symptoms and test influenza-positive by PCR.
    - Individuals who are not infected by influenza, regardless of symptoms, test negative by ELISA and test negative by PCR.

Rationale:

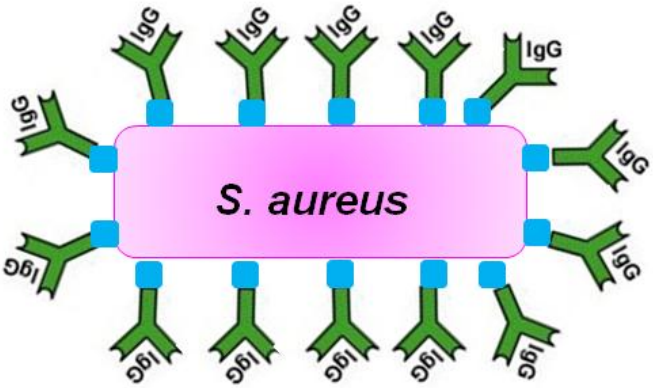
When performing an indirect ELISA to measure the amount of antibody present in a sample (such as anti-influenza antibodies), one typically performs the following steps:

1. Coat the plate with the antigen of interest
2. Block the 'extra space' on the plate with an irrelevant protein, such as casein (in dry milk) or bovine serum albumin
3. Add your sample(s) that you are testing for the presence of antibodies, such as a human serum sample being tested for anti-influenza antibodies, or add your positive or negative controls
4. Wash away any unbound sample
5. Add a secondary antibody conjugated to an enzyme like horse radish peroxidase (HRP)
6. Wash away any unbound secondary antibody
7. Add the substrate for the enzyme (typically TMB)

8. Add stop solution to stop the enzymatic reaction (such as with the addition of 1 M H_2SO_4 when using TMB)
9. Read then optical density on a spectrophotometer
10. Analyze your data

In the *Influenza Outbreak Investigation* ELISA, students use mock solutions (either 1X Phosphate Buffered Saline (PBS) or dH_2O) for the coating (step 1), blocking (step 2) and secondary antibody (step 5) steps in the procedure. The "patient samples" and the "positive control" are dilutions of Protein A-HRP in 0.2M carbonate-bicarbonate, pH 9.4, often referred to as "1X Carb/Bicarb buffer." This reduces the cost of the ELISA (compared to traditional ELISAs performed with antibodies), reduces the number of different solutions that teachers must prepare, and is fairly resistant to student errors.

Protein A is produced by *Staphylococcus aureus,* a Gram-positive, round-shaped bacterium. The protein serves as a defense mechanism against antibodies by binding to the constant region of the antibody, preventing the variable (antigen-binding) regions of the antibody from binding to and neutralizing the bacterium during infection. This is illustrated in the image below and by viewing the protein structure of an antibody bound to Protein A, PDB ID 1L6X.



[https://www.ncbi.nlm.nih.gov/Structure/pdb/1L6X]. Conjugation of Protein A to horse radish peroxidase (HRP) permits the use of Protein A-HRP for applications like ELISA without the need for a species-specific secondary antibody. In fact, Protein A also binds to immunoglobulins from many different species, including mice, rabbit, cat, dog and guinea pig [http://sevierlab.vet.cornell.edu/resources/TR0034-Ab-binding-proteins.pdf].

<u>Preparation of Solutions:</u>

1. **1X PBS**: PBS is provided as a 10X stock solution (i.e., 10-times more concentrated than needed for the experiment). To dilute, mix 1 part 10X PBS with 9 parts dH_2O. Assuming that 1X PBS is used for all steps in the protocol listed below (instead of dH_2O), in a class of 8 groups of 4 students each prepare 500 ml of 1X PBS by mixing 50 ml of 10X PBS with 450 ml of dH_2O. Store in a bottle with a lid until use. Shelf life: 1-2+ years at room temperature.
2. **Wash Buffer**: The Wash Buffer (1X PBS / 0.05% Tween-20) is provided in the kit. Store in a bottle with a lid until use. Shelf life: 6 months at room temperature.
3. **Positive Samples and Positive Control**:

a. Assuming Binary Results: Antibody Positive / Antibody Negative: Prepare 15 ml of a 1:5,000 dilution of Protein A-HRP by adding 3.0 μl of Protein A-HRP to 15 ml of 1X Carb/Bicarb Buffer.
b. Assuming Trinary Results: High Antibody Positive / Low Antibody Positive / Antibody Negative:
    i. Prepare 10 ml of a 1:5,000 dilution of Protein A-HRP by adding 2.0 μl of Protein A-HRP to 10 ml of 1X Carb/Bicarb Buffer.
    ii. Prepare 10 ml of a 1:10,000 dilution of Protein A-HRP by adding 1.0 μl of Protein A-HRP to 10 ml of 1X Carb/Bicarb Buffer.

Solutions Provided in the Kit (Per Class):

1. 50 ml 10X PBS (store at room temperature up to 2+ years)
2. 400mL PBS-Tween = 1X PBS + 0.05% Tween 20 (ELISA Wash Buffer) (store at room temperature up to 6+ months)
3. 5 μl Protein A-HRP (can be stored at 4°C or -20°C, but do not repeatedly freeze/thaw, light protected)
4. 30 ml 1X Carb/Bicarb Buffer (store at 4°C up to 2+ years)
5. 50 ml TMB (store at 4°C up to 2 years, light protected)
6. 50 ml 1 M H_2SO_4 **(Optional – not provided in the kit).** ***Additional safety precautions should be taken when handling sulfuric acid.*** (store at room temperature up to 2+ years)

Assuming Binary Results: Antibody Positive / Antibody Negative:

	What tube SAYS	**What tube IS (Volume/Group)**
Coating Buffer	"Coating Buffer"	1X PBS or dH_2O **(5 ml)**
Flu Proteins	"FP"	1X PBS or dH_2O **(50 ul)**
Block	"Block"	1X PBS or dH_2O **(15 ml)**
Patient Samples	Varies, Patient ID, See Key	Positive Sample: 1:5,000 Protein A-HRP in Carb/Bicarb **(350 ul/each)** Negative Sample : 1X PBS or dH_2O **(350 ul/each)**
Positive Control	"Pos Ctrl"	1:5,000 Protein A-HRP in Carb/Bicarb **(250 ul/each)**
Negative Control	"Neg Ctrl"	1X PBS or dH_2O **(250 ul)**
Antibody Diluent	"Ab Diluent"	Carb/Bicarb Buffer **(2 ml)**
Wash Buffer	"Wash Buffer"	PBS-Tween **(50 ml)** This can be distributed in beakers or bottles instead of tubes.

Anti-Human HRP (2^o Ab)	"2^o Ab"	1X PBS **(5 ml)**
TMB	"TMB"	TMB **(5 ml)**

Assuming Trinary Results: High Antibody Positive / Low Antibody Positive / Antibody Negative:

	What tube SAYS	**What tube IS (Volume/Group)**
Coating Buffer	"Coating Buffer"	1X PBS or dH_2O **(5 ml)**
Flu Proteins	"FP"	1X PBS or dH_2O **(50 ul)**
Block	"Block"	1X PBS or dH_2O **(15 ml)**
Patient Samples	Varies, Patient ID, See Key	High-Positive Sample: 1:5,000 Protein A-HRP in Carb/Bicarb **(350 ul/each)** Low-Positive Sample: 1:10,000 Protein A-HRP in Carb/Bicarb **(350 ul/each)** Negative Sample : 1X PBS or dH_2O **(350 ul/each)**
Positive Control	"Pos Ctrl"	1:10,000 Protein A-HRP in Carb/Bicarb **(250 ul/each)**
Negative Control	"Neg Ctrl"	1X PBS or dH_2O **(250 ul)**
Antibody Diluent	"Ab Diluent"	Carb/Bicarb Buffer **(2 ml)**
Wash Buffer	"Wash Buffer"	PBS-Tween **(50 ml)**
Anti-Human HRP (2^o Ab)	"2^o Ab"	1X PBS **(5 ml)**
TMB	"TMB"	TMB **(5 ml)**
Stop Solution (Optional)	"Stop"	1 M H_2SO_4 **(5 ml)** **NOT PROVIDED IN KIT**

Teacher Tips:

- Protein A-HRP dilutions can be made up to 3 days in advance, though the signal will be stronger if the reagent is diluted the day that it is used. It is best to keep the solution light protected until use, such as by wrapping the tube in aluminum foil, and stored at 4C (i.e., in the refrigerator).
- To save pipette tips, consider having students use a transfer pipette to add Wash Buffer and/or Block to their plates. It is not critical that the volumes used for these steps be accurate.
- ELISA plates are typically coated overnight at 4^oC. When students add their patient and control samples (which is when this ELISA plate is actually being coated), the plates can be incubated at 37^oC for 15-60 minutes or up to 3 days at 4^oC with only minimal changes in signal intensity.