

Cancer Biology 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 Cancer Biology Kit ELISA

- Assumes 12 patient samples (tested in duplicate)
- Assumes students work in 8 groups of 4, with each group testing 3 patient samples

This ELISA Can be Performed One of Two Ways:

- Patient samples are binary, either biomarker-positive or biomarker-negative, or
- Patient samples are trinary:
 - Breast cancer patients in whom the process of metastasis is well underway will have high levels of the metastasis biomarker CA 27.29 in their serum.
 - Breast cancer patients in whom the process of metastasis is only recently underway will have lower levels of the metastasis biomarker CA 27.29 in their serum.
 - Breast cancer patients whose cancer has not undergone the process of metastasis will have no or very low levels of CA 27.29 in their serum.

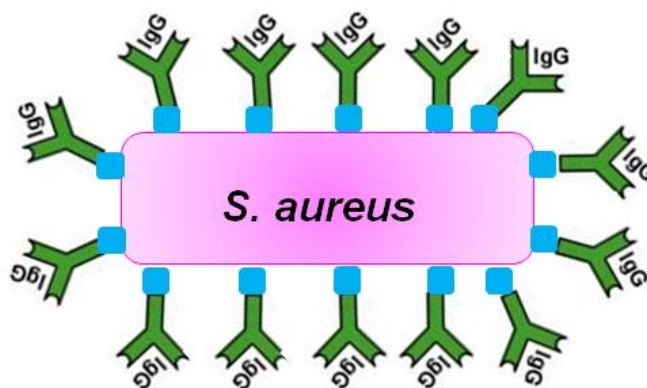
Rationale:

When performing a ‘sandwich’ ELISA to measure the amount of biomarker present in a sample, one typically performs the following steps:

1. **Coat** the plate with the primary antibody that binds to the biomarker (i.e., anti-biomarker antibody; sometimes called the coating antibody)
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 the biomarker, such as a human serum sample being tested for CA 27.29, and add your positive and 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** the optical density in each well on a spectrophotometer or estimate by eye
10. **Analyze** your data

In the *Cancer Biology* ELISA, students use mock solutions (either 1X Phosphate Buffered Saline (PBS) or dH₂O) 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>].

Preparation of Solutions:

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 dH₂O. Assuming that 1X PBS is used for all steps in the protocol listed below (instead of dH₂O), 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 dH₂O. 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: Biomarker Positive / Biomarker Negative: Prepare 12 ml of a 1:10,000 dilution of Protein A-HRP by adding 1.2 µl of Protein A-HRP to 12 ml of 1X Carb/Bicarb Buffer.
 - b. Assuming Trinary Results: High Biomarker Positive / Low Biomarker Positive / Biomarker Negative:
 - i. Prepare 7 ml of a 1:5,000 dilution of Protein A-HRP by adding 1.4 µl of Protein A-HRP to 7 ml of 1X Carb/Bicarb Buffer.
 - ii. Prepare 5 ml of a 1:10,000 dilution of Protein A-HRP by adding 0.5 µl of Protein A-HRP to 5 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. 400 ml of ELISA wash buffer (1X PBS + 0.05% Tween-20 - store at room temperature 6+ months unless it gets contaminated)
3. 5 µl Protein A-HRP (can be stored at 4°C or -20°C, but do not repeatedly freeze/thaw, light protected)
4. 40 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 H₂SO₄ **(Optional)**. ***Additional safety precautions should be taken when handling sulfuric acid.*** (store at room temperature up to 2+ years)

Labeling of Solutions and Samples:

Assuming Binary Results: Biomarker Positive / Biomarker Negative:

	What tube SAYS	What tube IS (Volume/Group)
Coating Buffer	"Coating Buffer"	1X PBS or dH ₂ O (5 ml)
Anti-Biomarker Ab (Primary Ab)	"1° Ab"	1X PBS or dH ₂ O (50 ul)
Block	"Block"	1X PBS or dH ₂ O (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 ₂ O (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 ₂ O (250 ul)
Diluent Buffer	"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-Biomarker HRP (Secondary Ab)	"2° Ab"	1X PBS (5 ml)
TMB	"TMB"	TMB (3 ml)

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

	What tube SAYS	What tube IS (Volume/Group)
Coating Buffer	"Coating Buffer"	1X PBS or dH ₂ O (5 ml)
Anti-Biomarker Ab (Primary Ab)	"1° Ab"	1X PBS or dH ₂ O (50 ul)
Block	"Block"	1X PBS or dH ₂ O (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 ₂ O (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 ₂ O (250 ul)
Diluent Buffer	"Diluent"	Carb/Bicarb Buffer (2 ml)
Wash Buffer	"Wash Buffer"	PBS-Tween (50 ml)
Anti-Biomarker HRP (Secondary Ab)	"2° Ab"	1X PBS (5 ml)
TMB	"TMB"	TMB (3 ml)
Stop Solution (Optional)	"Stop"	1 M H ₂ SO 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 4°C (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°C. 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°C for 15-60 minutes (it is crucial to do the shorter plate coating at 37°C) or up to 3 days at 4°C with only minimal changes in signal intensity.