ELISA Protocol

Materials:

• 96-Well Microtiter Plates, Eppendorf Tubes
• Twelve-Channel Pipettor
• 1mL Adjustable Pipettor
• Humid Chamber
• Wash Bottle or ELISA Plate Washer
• ELISA Plate Reader, TMB

Buffer Formulations:

Carbonate Buffer

1 Liter – pH 9.5 – Store at 4°C

• 1.59g Na_2CO_3 , 2.93g $NaHCO_3$, 2mL 10% NaN_3, DI H_2O to 1L

Wash Buffer

• PBS, 0.02% Thimerosal, 0.05% Tween-20

Blocking Buffer

• PBS, 0.1% BSA, 0.02% Thimerosal, Store in a dark bottle

Plate Design

Mark each column with the project number. The first three rows are reserved for 3 dilutions of the pre-bleed and the following 5 rows are for dilutions of the bleed. See chart below:

1	2	3	4	5	6	7	8	9	10	11	12
A			1:**1**,000			**Pre-Bleed**					
B			1:**5**,000								
C			1:**25**,000								
D			1:**1**,000			**Bleed**					
E			1:**5**,000								
F			1:**25**,000								
G			1:**125**,000								
H			1:**625**,000								

DAY ONE

Antigen Preparation

1. The working peptide antigen concentration is 10µg/mL: Dissolve 1mg peptide in 200µL DI H_2O (5mg/mL). Add 2µL peptide to 1mL of 50mM carbonate buffer.
2. The working protein antigen concentration is 40µg/mL: Concentrate protein to 4mg/mL and dialyze against 50mM carbonate buffer overnight. Then dilute the protein to 40ug/mL with carbonate buffer.

Coating

3. Add 100µL of antigen at the recommended dilutions above to each well on the plate. Incubate at room temperature for 24 hours in a humid chamber.

a. Humid chamber: Place wet paper towels in the bottom of a bin and stack the plates on top of the towels. Cover with a lid.

DAY TWO

Blocking

4. Remove coating solution and rinse twice (2) with DI H_2O

5. Flip dry on a stack of paper towels – slap to remove all traces of liquid from the wells.

6. Add 280µL blocking buffer to each well. Incubate in a humid chamber at room temperature for two (2) hours.

a. More blocking buffer is better, about 200-300µL per well, but make sure not to overfill each well.

Serum Sample

7. Dilute serum (pre-bleed and bleed in separate Eppendorf tubes) with blocking buffer to 1:1000 (1µL serum to 1mL blocking buffer).

8. Add 125µL of 1:1000 diluted pre-immune serum to row A of the plate.

9. Using a twelve-channel pipettor, take 25µL from row A and add to row B, mixing with the pipettor. Repeat with rows B and C, removing 25µL from row B and mixing it with row C. Remove 25µL from row C and discard.

10. Add 125µL of 1:1000 diluted serum to row D of the plate.

11. Using a twelve-channel pipettor make a series of 5 dilutions for rows E through H as described above for the pre-bleed, discarding 25µL after row H.

12. Incubate for one hour at room temperature.

Secondary Antibody

13. Dispose of the serum samples and wash the wells with the wash buffer using a wash bottle or an ELISA plate washer one (1) time. Follow with one wash using DI H_2O. Drain the remaining liquid on a stack of paper towels.

14. Dilute the HRP conjugated secondary to the dilution recommended below with the blocking buffer.

a. Goat anti-rabbit IgG: 1:20000

b. anti-goat IgG: 1:10000

c. anti-mouse IgG: 1:5000

d. *Note*: Dilutions are only recommendations. Optimal dilutions will vary for each secondary and should be determined for each new lot.

15. Incubate at room temperature for one (1) hour.

Development

16. Dispose of the secondary and wash the wells with the wash buffer one (1) time. Follow with one wash using DI H_2O. Drain the remaining liquid on a stack of paper towels.

17. Add TMB per manufacturer instructions.

a. Positive wells will change to a blue color depending on signal intensity.

18. Add 90µL of 1M HCl to each well (or recommended media) to stop the color development and read immediately on a microtiter plate reader at A_{450}.

a. The blue will change to yellow when the reaction is stopped.

19. Read multiple plates in the order the color development was stopped.

a. It is not recommended to have more than two plates developing at the same time. Have two timers so that the reaction is stopped after 5 minutes.