7. ANTIGEN CAPTURE ELISA TEST

Aim:

To determine the antigen concentration by Antigen Capture ELISA method.

Introduction:

Enzyme linked immunosorbent assay or ELISA is a sensitive immunological technique to detect the presence of a specific antigen (Ag) or antibody (Ab) in a biological sample. It utilizes the dual properties of antibody molecules being specific in reactivity and their ability to be conjugated to active molecules such as enzymes. An enzyme conjugated with an antibody reacts with a chromogenic colourless substrate to generate a coloured reaction product. ELISA is extensively used for diagnostic purpose which utilizes the dual. It requires an immobilized antigen/antibody bound to a solid support (e.g. microtitre plate or membrane). There are different types of ELISAs for the detection of a protein of interest in a given sample. One of the most sensitive ELISA is Antigen Capture ELISA which can measure the concentration of antigen.

Principle:

In Antigen Capture ELISA method, an antibody is bound to the wells of a microtitre plate. Then one unlabeled antigen and one enzyme labeled antigen (Horseradish peroxidase (HRP)) corresponding to the antibody are added and allowed to complex with the bound antibody. As a result, there is a competition between the labeled and unlabeled antigen for the immobilized antibody. Unreacted antigens are washed out and the amount of labeled antigen bound to the immobilized antibody is detected by using H_2O_2 as substrate and Tetramethylbenzidine (TMB) as a chromogen. HRP acts on H_2O_2 to release nascent oxygen, which oxidizes TMB to TMB oxide, which gives, a blue coloured product. The intensity of the colour is measured using a spectrophotometer at 450 nm. The developed colour is directly proportional to the amount of labeled antigen present in the sample and the amount of labeled antigen that binds is inversely proportional to the amount of unlabeled antigen in the reaction mixture. As a result the estimate of label in the microtitre well decreases with increase in the antigen concentration in the standard or test sample.



Figure 1: In Antigen Capture ELISA, an antibody is bound to the well and a labeled and unlabeled antigen are allowed to compete for the binding to the antibody. The concentration of antigen can be determined from the intensity of the colour developed in the product.

Materials required:

Sl No.	Materials	Remarks
1	Unlabeled antigen (1mg/ml)	All the materials were
2	Antibody	provided in HiPer®
3	Test Sample 1	Antigen Capture
4	Test Sample 2	ELISA testing Kit.
5	Test Sample 3	
6	HRP labeled antigen	
7	Blocking Buffer (BSA in PBS)	
8	10X TMB substrate	
9	Coating Buffer (Carbonate bicarbonate	
	buffer)	
10	10X Wash Buffer (PBS with Tween 20)	
11	Stop Solution (Sulphuric acid)	
12	Microtitre plate (Detachable)	
13	Measuring cylinder	
14	Test tubes	
15	Blotting paper	
16	Micropipettes and Tips	
17	Distilled water	

Prepare the reagents as indicated below before starting each experiment:

Preparation of 1XTMB substrate: Take 0.5 ml of 10XTMB substrate and add 4.5 ml of distilled water to it.

Preparation of 1X Wash Buffer: Take 5 ml of 10X Wash Buffer and add 45 ml of distilled water to it.

Preparation of sample diluent: Take 1 ml of blocking buffer and make up the volume to 30 ml with 1X PBST. Use this to dilute standard antigen and HRP labeled antigen.

Preparation of dilutions of standard antigen:

Concentration of standard antigen is 1 mg/ml; dilute this to get a range of concentrations using sample diluent, as follows:

No.	Dilutions of Standard Antigen	Concentration of Antigen
1	40µl of 1mg/ml (stock)+ 960 µl of sample diluent	40µg/ml (a)
2	$400\mu l \text{ of } (a) + 1600 \mu l \text{ of sample diluent}$	8µg/ml (b)
3	1000 μ l of (b)+ 1000 μ l of sample diluent	4 µg/ml (c)
4	1000 μ l of (c)+ 1000 μ l of sample diluent	2 µg/ml (d)
5	1000 μ l of (d)+ 1000 μ l of sample diluent	1 µg/ml (e)
6	1000 μ l of (e)+ 1000 μ l of sample diluent	0.5 µg/ml (f)
7	1000 µl of (f)+ 1000 µl of sample diluent	0.25 µg/ml (g)
8	1000 µl of (g)+ 1000 µl of sample diluents	0.125 µg/ml (h)

Procedure:

Day 1: Coating of wells with antibody

- 1. Dilute 50 μ l of antibody with 4.95 ml of coating buffer. Concentration of the diluted antibody is 1 μ g/ml.
- 2. Pipette 200 µl of diluted antibody into each wells of the microtitre plate (24 wells). Tap or shake the plate so that the antibody solution is evenly distributed over the bottom of each well.
- 3. Incubate the wells overnight at 4° C.

Day 2: Blocking the residual binding sites on the wells

- 4. Discard the well contents. Rinse the wells with distilled water for three times by draining out the water after each rinse.
- 5. Fill each well with 200 μ l of blocking buffer and incubate at room temperature for 1 hour.
- 6. Rinse the plate three times (as given above) with distilled water. Drain out the water completely by tapping the plate on a blotting paper.

Addition of antigen to the wells

- 7. Prepare standard and test antigen dilutions as given above.
- 8. Add 100 μ l of standard antigen, test samples and 1X PBST to the coated wells (in duplicates) as shown in the following picture.



a to h – various dilutions of standard antigen, T1, T2 and T3 – Three test samples, B – 1X PBST (Blank)

Addition of HRP labeled antigen

- 9. Dilute 3 µl of HRP labeled antigen with 3 ml of sample diluent
- 10. Add 100 μ l of HRP labeled antigen to all the wells.
- 11. Incubate at room temperature for 30 minutes.
- 12. Discard the well contents; fill the wells with 1X PBST, allow it to stand for 3 minutes, discard the contents. Repeat this step twice.

Addition of substrate and measurement of absorbance

- 13. Dilute required amount of 10 X TMB/H₂O₂ (substrate) solutions to 1X using distilled water.
- 14. Add 200 µl of 1X substrate to each well.
- 15. Incubate at room temperature for 10 minutes.
- 16. Add 100 μ l of 1X stop solution to each well.
- 17. Read the absorbance at 450 nm by ELISA plate reader.



Observation and Result:

Look for the development of blue colour in the wells at the end of the experiment. Read the absorbance at 450 nm by ELISA plate reader and record the readings as follows:

Sample	Concentration (µg /ml)	A	450	Average A ₄₅₀
a	40 µg /ml	0.059	0.066	0.062
b	8 μg /ml	0.108	0.112	0.110
с	4 µg /ml	0.129	0.172	0.150
d	2 µg /ml	0.183	0.214	0.198
e	1 μg /ml	0.256	0.239	0.247
f	0.5 µg /ml	0.273	0.576	0.424
g	0.25 µg /ml	0.586	0.501	0.543
h	0.125 µg /ml	0.477	0.655	0.566
T1		0.284	0.191	0.237
T2		0.279	0.387	0.333
T3		0.594	0.602	0.598
Blank (1XPBST)		0.698	0.685	0.691

Calculation of antigen concentration in test sample:

Calculate the average A450 for each of the samples (standard and test) and plot A450 of standards on Y axis (linear scale) versus the concentration of antigen in μ g/ml on X axis (log scale) on a semilog graph sheet as shown below:



Calculation of antigen concentration:

From the standard curve, determine the concentration of antigen in the test samples and record the readings as below:

Test Sample	Concentration (mg/ml)
T1	0.0049
T2	0.0038
T3	0.0007

Interpretation:

By performing Antigen Capture ELISA, concentrations of the test antigens can be detected from the standard curve. When unlabeled antigen (test antigen or varying amounts of standard antigen) and fixed amount of labeled antigen are added to the antibody immobilized on the wells, the two antigens compete to bind to the immobilized antibody. The amount of labeled antigen bound is then estimated by a chromogenic assay. The amount of labeled antigen that binds to the immobilized antibody is inversely proportional to the amount of unlabeled antigen in the reaction mixture.

Flowchart of antigen captures ELISA:

Observation and Result: