HiPer® Sandwich ELISA Teaching Kit

Product Code: HTI014

Number of experiments that can be performed: 4

Duration of Experiment: 2 days

Day1-Coating of wells: 15 minutes

Day2- Protocol, observation and result: 5 hours

Storage Instructions:

- The kit is stable for 12 months from the date of manufacture
- Store all the reagents at 2-8°C
- Other kit content can be stored at room temperature (15-25°C)

ISO 9001-2015 CERTIFIED
WHO GMP CERTIFIED
ISO 13485-2016 CERTIFIED

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Aim:

To determine the antigen concentration by Sandwich 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 properties. 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 common ELISA is Sandwich ELISA which can measure the amount of antigen which is sandwiched between two layers of antibodies i.e. capture and detection antibody.

Principle:

In Sandwich ELISA one antibody (capture antibody) is bound to the wells of a microtitre plate. Then one antigen corresponding to the antibody is added and allowed to complex with the bound antibody. Then wells are washed to remove unbound antibodies and a second antibody (detection antibody) labeled with an enzyme e.g. Horseradish peroxidase (HRP) is allowed to bind to the antigen. As a result the antigen is trapped between two layers of antibodies as shown in figure1. Unreacted labeled antibodies are washed out and the amount of antigen immobilized to secondary 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 antigen present in sample.

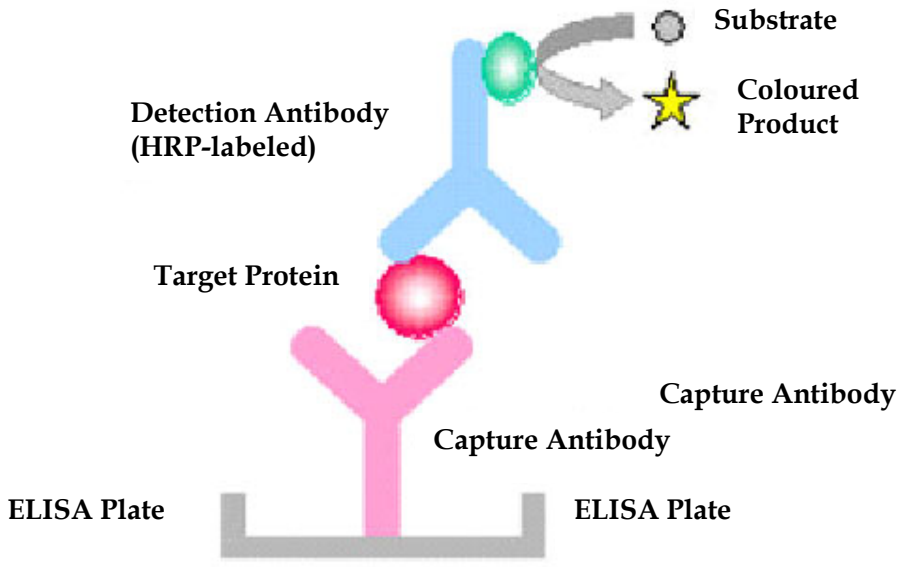


Fig1: In the Sandwich ELISA method an antigen (Target Protein) is sandwiched between two antibodies, Capture and Detection antibody.

Kit Contents:

This kit can be used for the determination of antigen concentration bound to immobilized antibody followed by binding of the antigen to the labeled secondary antibody and its detection by using appropriate substrate.

Table 1:Enlists the materials provided in this kit with their quantity and recommended storage

Sr. No.	Product Code	Materials Provided	Quantity	Storage
			4 expts	
1	TKC130	Standard Antigen (0.4mg/ml)	0.040 ml	2-8 °C
2	TKC132	Capture Antibody	0.3 ml	2-8 °C
3	TKC133	Test Sample 1	2 ml	2-8°C
4	TKC134	Test Sample 2	2 ml	2-8°C
5	TKC135	Test Sample 3	2 ml	2-8°C
6	TKC136	Secondary Antibody – HRP conjugate	0.025 ml	2-8°C
7	TKC137	Blocking Buffer	25 ml	2-8°C
8	TKC138	10X TMB Substrate	5 ml	2-8°C
9	TKC139	Coating Buffer	30 ml	2-8°C
10	TKC140	10X Wash Buffer	40 ml	2-8°C
11	TKC141	Stop Solution	240 ml	2-8°C
12	TKC131	Microtitre plate (Detachable)	1 No.	RT

Materials required but not provided:

Glasswares: Measuring cylinder, Test tubes
Reagents: Distilled water (Recommended Product Code: ML064 –Molecular Biology Grade water)
Other requirements: Blotting paper, Micropipette, Tips, Spectrophotometer, Cuvettes.

Storage:

HiPer® Sandwich ELISA Teaching Kit is stable for 12 months from the date of manufacture without showing any reduction in performance. Store all the reagents at 2-8°C. Other kit content can be stored at room temperature.

Important Instructions:

1. Before starting the experiment the entire procedure has to be read carefully.
2. Always wear gloves while performing the experiment.
3. Bring all the buffers to room temperature before the assay.
4. Use 24 wells per experiment. One plate can be used for 4 experiments. Don't reuse the same wells.
5. Dilute only required amount of buffers to 1X with distilled water before use.
6. Blocking buffer: BSA in PBS.
7. **Coating buffer:** Carbonate bicarbonate buffer.
8. **Stop solution:** Sulphuric acid.
9. **Preparation of 1X TMB substrate:** Take 0.5 ml of 10X TMB substrate and add 4.5 ml of distilled water to it.
10. **Preparation of 1X Wash Buffer:** Take 5 ml of 10X Wash Buffer and add 45 ml of distilled water to it.

Prepare the reagents as indicated below before starting of each experiment:

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

Preparation of dilutions of standard antigen: Concentration of reconstituted antigen is 0.4 mg/ml; dilute this to get a range of standard concentrations using sample diluent, as follows:

No.	Dilutions of Standard Antigen	Concentration of Antigen
1	8 µl of 0.4mg/ml + 4000 µl of sample diluent	800 ng/ml (a)
2	1000 µl of (a) + 1000 µl of sample diluent	400 ng/ml (b)
3	1000 µl of (b)+ 1000 µl of sample diluent	200 ng/ml (c)
4	1000 µl of (c)+ 1000 µl of sample diluent	100 ng/ml (d)
5	1000 µl of (d)+ 1000 µl of sample diluent	50 ng/ml (e)
6	1000 µl of (e)+ 1000 µl of sample diluent	25 ng/ml (f)
7	1000 µl of (f)+ 1000 µl of sample diluent	12.5 ng/ml (g)
8	1000 µl of (g)+ 1000 µl of sample diluent	6.25 ng/ml (h)

Procedure:

Day 1: Coating of wells with Capture antibody

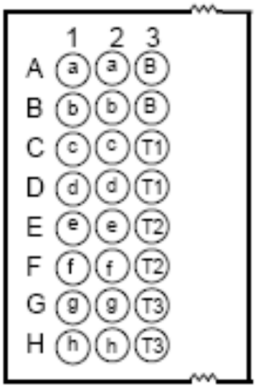
1. Dilute 60µl of capture antibody with 5.94 ml of ready to use coating buffer. Concentration of the capture
   antibody is 10µg/ml.
2. Pipette 200 µl of diluted (1X) capture antibody into each of the 24 wells of the microtitre plate. Gently shake the plate for an even distribution of the capture antibody over the bottom of each well.
3. Incubate the microtitre plate overnight at 4°C.

Day 2: Blocking of the residual binding sites

4. After discarding the well contents rinse the wells with distilled water for three times by draining out the water after each rinse.
5. Add 200 µl of blocking buffer to each well and incubate at room temperature for 1 hour.
6. Rinse the wells three times with distilled water. Each time discard out the water completely by tapping the plate on a blotting paper.

Addition of antigen to wells

7. Prepare standard antigen dilutions as given above.
8. Add 200 µl of standard antigen, test samples and 1X wash buffer to the coated wells (in duplicates).
9. Incubate at room temperature for 30 minutes.
10. Discard the well contents; fill the wells with 1X Wash buffer, allow it to stand for 3 minutes, discard the contents. Repeat this step twice.



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

Addition of HRP labeled antibody

11. Dilute 5 µl of Antibody-HRP conjugate with 5 ml of sample diluent.
12. Add 200 µl of HRP labeled antibody to all the wells.
13. Incubate at room temperature for 30 minutes.
14. Discard the well contents and rinse the wells 3 times with Wash buffer.

Addition of substrate and measurement of absorbance

15. Add 200 µl of 1X TMB substrate to each well.
16. Incubate at room temperature for 10 minutes.
17. Add 100 µl of Stop Solution to each well.
18. Transfer the contents of each well to individual tubes containing 2 ml of Stop Solution.
19. Prepare substrate blank by adding 200 µl of 1X substrate solution to 2.1 ml of Stop Solution.
20. Read the absorbance at 450 nm after blanking the spectrophotometer with substrate blank.

Flowchart:

Coating of wells with capture antibody

Addition of antigen

Addition of HRP-labeled antibody

Appearance of coloured product upon reaction with the substrate

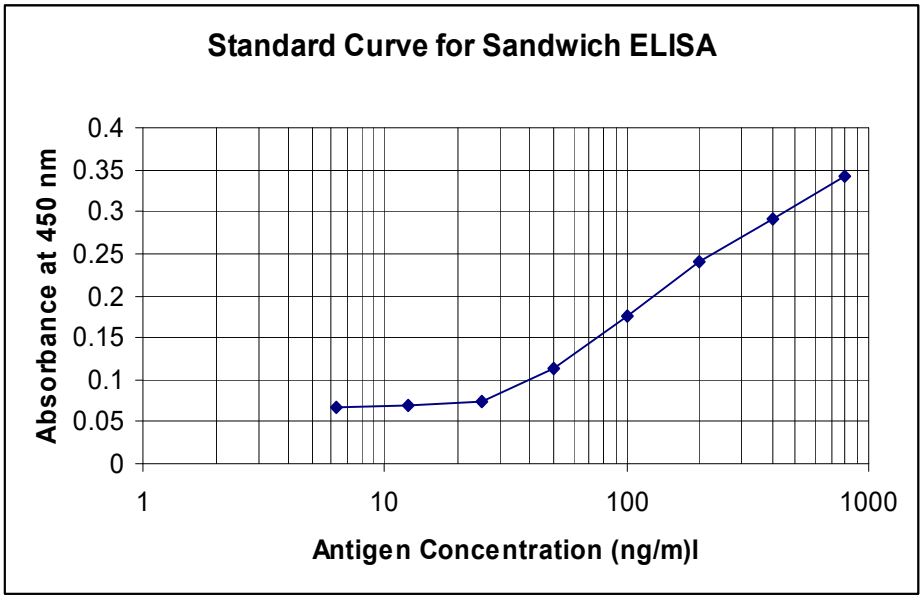
Observation and Result:

Look for the development of blue colour in the wells at the end of the experiment. Read the absorbance at 450 nm after blanking the spectrophotometer with substrate blank and record the readings as follows:

Sample	Concentration (ng/ml)	A_{450}	Average A_{450}
a			
b			
c			
d			
e			
f			
g			
h			
T1			
T2			
T3			
Blank (1X wash buffer)			

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 ng/ml on X axis (log scale) on a semi-log graph sheet as shown below:



Calculation of antigen concentration:

Calculate the concentration of antigen in mg/ml, in each of the test samples as follows:
Concentration of antigen in the sample:

$$\frac{\text{Concentration in ng/200 µl (from the graph) X Dilution factor}}{10^6} = \_\_\_\_\_ \text{ mg/ml}$$

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

Test Sample	Concentration (mg/ml)
1	
2	
3	

Interpretation:

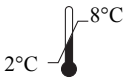
By performing Sandwich ELISA, concentrations of the test antigens (which are sandwiched between two antibodies- capture and detection) can be detected from the standard curve.

Troubleshooting Guide:

Sr.No	Problem	Probable Cause	Solution
1	No signal	Omission of any step	Prepare a check-list for the steps followed
2	High background	Insufficient washing or Secondary antibody concentration is high or Contamination in buffer	Wash plates thoroughly after incubation with Secondary antibody. Decrease the antibody concentration. Use freshly prepared buffer

Technical Assistance:

At HiMedia we pride ourselves on the quality and availability of our technical support. For any kind of technical assistance mail at mb@himedialabs.com



Storage temperature

Do not use if package is damaged

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