Sandwich ELISA assay:

ELISA (enzyme-linked immunosorbent assay) is a plate-based assay technique designed for detecting and quantifying peptides, proteins, antibodies and hormones. In ELISA test, an antigen must be immobilized to a solid surface and then complexed with an antibody that is linked to an enzyme. Detection is accomplished by assessing the conjugated enzyme activity via incubation with a substrate to produce a measurable product. The most crucial element of the detection strategy is a highly specific antibody-antigen interaction .

ELISAs can be performed with a number of modifications to the basic procedure: direct, indirect, sandwich or competitive. The sandwich ELISA is one of the most useful immunoassay formats and it is designed for detection of soluble antigens. This type of assay is useful where a single species antiserum is available and where antigen does not attach well to plates.

Workflow:

Coating → **Blocking** → **Detection** → **Read result**

Materials and reagents:

1. Carbonate Coating Buffer

Anhydrous Na_2CO_3, 1.5 g

Anhydrous $NaHCO_3$, 2.93 g

Distilled water, 1 liter, pH to 9.6

2. Blocking buffer

Phosphate Buffered Saline (PBS) containing 1% w/v BSA or 10 % fetal bovine serum

Filter before use to remove particulates

3. Wash buffer

Phosphate Buffered Saline containing 0.05% v/v Tween®-20

Recommended Substrates and Stop Solutions

for use with HRP-conjugated antibodies. Stop with 0.2M H_2SO_4.

for use with alkaline phosphatase-conjugated antibodies. Stop with 1M NaOH

Reagent Preparation

1. Standard Solutions:

-10,000 pg/mL: Add 1 mL of sample diluent buffer into one tube of standard (10 ng per tube) and mix thoroughly. Note: Store this solution at 4°C for up to 12 hours (or -20°C for 48 hours) and avoid freeze thaw cycles.

-5,000 pg/mL: Mix 0.3 mL of 10,000 pg/mL with 0.3 mL of sample diluent buffer and mix thoroughly.

-2,500 pg/mL: Mix 0.3 mL of 5,000 pg/mL with 0.3 mL of sample diluent buffer and mix thoroughly.

-Perform similar dilutions until the standard solutions with these concentrations (pg/mL) are made: 1,250, 625, 312, 156 and 78.

-Add 100 µL of each of the diluted standard solutions to the appropriate empty wells. Repeat in duplicate or triplicate

for accuracy .

Note: The standard solutions are best used within 2 hours.

2.Avidin-Biotin-Peroxidase (ABC):

-Calculate the total volume needed for the assay by multiplying 0.1 mL/well and the number of wells required. Add 2-3 extra wells to the calculated number of wells to account for possible pipetting errors.

-Generate the required volume of diluted ABC solution by performing a 1:100 dilution (For each 1 µL concentrated ABC solution, add 99 µL ABC dilution buffer) and mixing thoroughly.

Note: The diluted ABC solution should not be prepared more than 1 hour prior to the experiment.

3.Biotinylated Antibody:

-Calculate the total volume needed for the assay by multiplying 0.1 mL/well and the number of wells required. Add 2-3 extra wells to the calculated number of wells to account for possible pipetting errors.

-Generate the required volume of diluted antibody by performing a 1:100 dilution (For each 1 µL concentrated antibody, add 99 µL antibody dilution buffer) and mixing thoroughly.

Equipment and supplies:

1. ELISA plate readers:

2.Pipettes:

-Single-channel, fixed-volume, and adjustable-volume (1–20 µL, 10–100 µL, 20–200 µL, etc.) • Multichannel, 8- and 12-channel.

3. Washer systems:

- Manual systems that wash one row or column at a time • Semiautomated systems that handle one strip or plate at a time • Fully automated systems that can process multiple plates.

4. Incubators (plate shaker incubator)

Safety (Warning and Biohazard consideration):

-Samples of tissue, serum or blood origin should be handled to guidelines for prevention of transmission of blood borne diseases.

-Some enzyme substrates are considered hazardous, due to potential carcinogenicity. Handle with care and refer to Material Safety Data Sheets for proper handling precautions.

-Wear appropriate protective clothing, gloves, and eyewear necessary to avoid any accidental contact with reagents.

-Reagents which contain preservatives may be toxic if ingested, inhaled, or in contact with skin.

Procedure:

1. Capture Antibody Coating:

- Dilute the capture antibody to a final concentration of 1-10 µg/mL in bicarbonate/carbonate antigen coating buffer (100 mM NaHCO3 in deionized water; pH adjusted to 9.6).

- Pipette 100 µL of diluted antibody to each well of a microtiter plate.

- Cover the plate with adhesive plastic and incubate at 4°C overnight (or 37°C for 30 min)

- Remove the coating solution and wash the plate 3X with 200 µL PBS (Phosphate Buffered Saline) buffer (10 mM Na2HPO4 and 1.8 mM NaH2PO4 in deionized water with 0.2% Tween 20; pH Adjusted to 7.4) with for 5 minutes each time. The coating/washing solutions can be removed by flicking the plate over a sink. The remaining drops can be removed by patting the plate on a paper towel or by aspiration. Do not allow the wells to dry out at any time.

2. Blocking:

-Pipette 200 µL blocking buffer (5% w/v non-fat dry milk in PBS buffer) per well to block residual protein-binding sites. Alternatively, BSA or Block ACE can be used to replace non-fat dry milk.

-Cover the plate with adhesive plastic and incubate for 1-2 hour(s) at 37°C (or at 4°C overnight).

-Remove the blocking solution and wash the plate 2X with 200 µL PBS for 5 minutes each time. Flick the plate and pat the plate as described in the coating step.

3. Sample (Antigen) Incubation:

-Serially dilute the sample with blocking buffer immediately before use. The optimal dilution should be determined by a titration assay according to the antibody manufacturer.

-Pipette 100 µL of each of the diluted sample solutions and control to each empty well. Repeat in duplicate or triplicate for accuracy. The negative control should be species- and isotype-matched as well as nonspecific immunoglobulin diluted in PBS buffer.

-Cover the plate with adhesive plastic and incubate for 2 hours at room temperature.

-Remove the content in the wells and wash them 3X with 200 µL PBS buffer for 5 minutes each time. Flick the plate and pat the plate as described in the coating step.

5. Biotinylated Antibody Incubation:

-Pipette 100 µL of diluted antibody to the wells with control, standard solutions and diluted samples.

-Cover the plate with adhesive plastic and incubate for 1 hour at 37°C (or 2 hours at room temperature).
These incubation times should be sufficient to receive a strong signal. However, if a weak signal is observed,

perform incubation overnight at 4°C for a stronger signal.

-Remove the content in the wells and wash them 3X with 200 µL PBS for 5 min each time. Flick the plate and pat the plate as described in the coating step.

6. ABC Incubation (enzyme conjugate|):

-Pipette 100 µL of diluted ABC solution to the wells with control, standard solutions and diluted samples.

-Cover the plate with adhesive plastic and incubate for 0.5 hour at 37°C.

-Remove the content in the wells and wash them 3X with 200 µL PBS buff er for 5 min each time. Flick the plate and pat the plate as described in the coating step.

7. Substrate Preparation:

Prepare the substrate solution immediately before use or bring the pre-made substrate to room temperature. The two widely used enzymes for signal detection are horse radish peroxidase (HRP) and alkaline phosphatase (AP), and their corresponding substrates, stopping solutions, detection absorbance wavelengths and color developed are as follows :

Enzyme	Substrate	Stop Solution	Absorbance (nm)	Color Developed
HRP	TMB	2M H2SO4	450	Yellow
AP	pNPP	0.75M NaOH	٤٠٥	Yellow

* TMB: 3,3’,5,5’-tetramethylbenzidine; pNPP: p-nitrophenyl-phosphate

Note:

- The TMB substrate must be kept at 37°C for 30 min before use.

- Hydrogen peroxide can also act as a substrate for HRP.

- Sodium azide is an inhibitor of HRP. Do not include the azide in buffers or wash solutions if HRP-labeled conjugate is used for detection .

8. Signal Detection:

-Pipette 90 µL of substrate solution to the wells with the control, standard solutions and diluted samples.

-Incubate the plate at 37°C in the dark. If TMB is used, shades of blue will be observed in the wells with the most concentrated solutions. Other wells may show no obvious color.

-Color should be developed in positive wells after 15 min. After sufficient color development, pipette 100 µL of stop solution to the appropriate wells (if necessary).

-Read the absorbance (OD: Optical Density) of each well with a plate reader.

9. Data Analysis:

-Prepare a standard curve using the data produced from the diluted standard solutions.

Use absorbance on the Y-axis (linear) and concentration on the X-axis (log scale).

-Interpret the sample concentration from the standard curve.

References:

1.https://www.bosterbio.com/Antibody and ELISA experts

2.https:// www.hycultbiotech.com/SANDWICH ELISA

3. Engvall E and Perlmann P (1971). Enzyme linked immunosorbent assay (ELISA) quantitative assay of immunoglobulin G. Immunochemistry, v8 p871-875.

4.https:// www.thermo.com/ ELISA technical guide and protocols

شناسنامه سند: **CoreLab.112**	
نام سند	پروتکل استاندارد Sandwich Elisa
تاریخ صدور	۱۳۹۹/۸/۱۰
نام کامل فایل	Sandwich Elisa (Sandwich Enzyme-Linked Immunosorbent Assay)
شرح سند	روش Sandwich Elisa (Sandwich Enzyme-Linked Immunosorbent Assay) را شرح می‌دهد.
تهیه کننده	شهربانو نادری