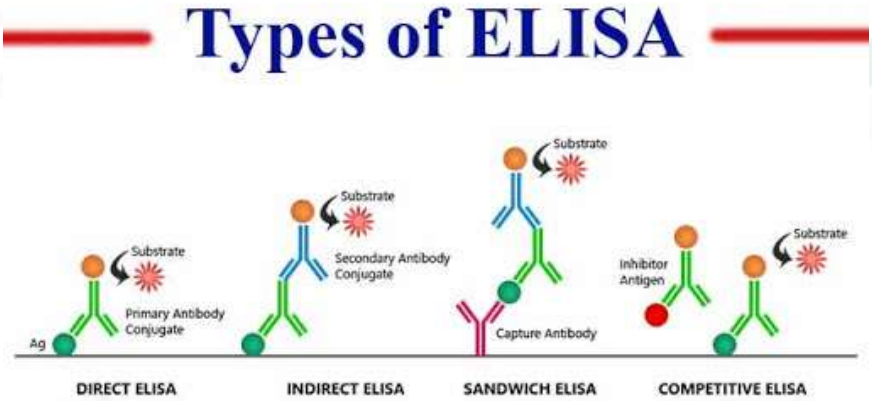
ELISA



Indirect ELISA assay:

ELISA is a highly sensitive test technique based on immunological reactions that combine the specific reaction of antigens and antibodies with the efficient catalytic action of enzymes on substrates. Since the reaction of the antigen and the antibody is carried out in the well of a solid phase carrier-polystyrene microliter plate, after each reagent is added, the excess free reactant can be removed by washing to ensure the specificity and stability of the test result. In ELISA, indirect ELISA is useful for antibody screening, epitope mapping, and protein quantification. The secondary antibody serves to enhance the signal of the primary antibody, which makes it more sensitive than direct ELISA. However, it also produces a higher background signal and potentially decreases the overall signal.

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.

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. Antigen Coating:

- Dilute purified antigens to a final concentration of 1-10 µg/mL in bicarbonate/carbonate antigen-coating buffer (100 mM $NaHCO_3$ in deionized water; pH adjusted to 9.6).

-Pipette 100 µL of diluted antigen 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 Na_2HPO_4 and 1.8 mM NaH_2PO_4 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. Primary Antibody Incubation:

Serially dilute the primary antibody of choice with blocking buffer. The optimal dilution should be determined by a titration assay according to the antibody manufacturer.

-Pipette 100 µL of each diluted antibody per well. Repeat in duplicate or triplicate for accuracy. The negative control should be species- and isotype-matched as well as non-specific immunoglobulin diluted in PBS buffer.

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

4. Secondary Antibody Incubation:

Serially dilute the conjugated secondary antibody 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 diluted secondary antibody solution to each well.

- 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 min each time. Flick the plate and pat the plate as described in the coating step.

5. 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 .

6. Signal Detection:

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

-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 wells (if necessary).

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

7. 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.110

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تاریخ صدور	۱۳۹۹/۸/۱۰
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	روش استاندارد تست Indirect ELISA (Indirect Enzyme-Linked Immunosorbent)را شرح می‌دهد.
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