Sandwich ELISA Protocol

Statement: This experimental protocol serves as a general guidance document, and the reagents mentioned herein may be substituted according to specific circumstances. Regardless of the situation, we strongly recommend conducting preliminary experiments prior to formal implementation to verify the applicability and feasibility of the protocol within your laboratory environment.

Materials Required

- High-binding 96-well ELISA microplate
- Capture antibody (Specific antibody against the target antigen)
- Standard antigen (purified protein for standard curve)
- Detection antibody (different epitope, preferably polyclonal or monoclonal from another species)
- Enzyme-conjugated secondary antibody (e.g., HRP-conjugated anti-species antibody)
- Coating buffer (e.g., PBS or carbonate-bicarbonate buffer, pH 9.6)
- Blocking buffer (e.g., 1-5% BSA or non-fat dry milk in PBS)
- Wash buffer (PBS + 0.05-0.1% Tween-20, pH 7.4)
- Substrate solution (e.g., TMB for HRP)
- Stop solution (e.g., 1M H_2SO_4 or 1N HCl for TMB)
- Microplate reader (capable of measuring absorbance at appropriate wavelength)

1. Coating

a. Dilute the capture antibody in coating buffer according to manufacturer recommendations (usually 1-10 µg/mL).

b. Add 100 µL per well to the ELISA plate.

c. Cover the plate and incubate overnight at 4°C or 2 hours at 37°C or room temperature (RT)

d. Wash 3-5 times with wash buffer (300 µL/well) and tap the plate gently on absorbent paper after each wash.

Note: The choice of coating buffer and incubation time significantly affects antibody binding efficiency. Ensure the solution is well-mixed before adding to the wells

2. Blocking

a. Add 200-300 µL of blocking buffer per well.

b. Incubate for 1-2 hours at RT or overnight at 4°C.

c. Wash 3-5 times with wash buffer (300 µL/well) and tap the plate gently on absorbent paper after each wash.

Note: Blocking prevents nonspecific binding and must be compatible with both capture and detection antibodies. Avoid using BSA if biotin-streptavidin systems are used.

3. Samples and Standards Incubation

a. Prepare serial dilutions of the purified standard antigen with known concentrations in blocking buffer.

b. Dilute samples appropriately in blocking buffer.

c. Add 100 µL per well (standards and samples in duplicate or triplicate). Include a blank (blocking buffer only).

d. Incubate for 1-2 hours at RT or 37°C (or overnight at 4°C for low-abundance antigens).

e. Wash 3-5 times with wash buffer (300 µL/well) and tap the plate gently on absorbent paper after each wash.

Note: Vortex samples before use to ensure homogeneity and optimize sample dilution to fall within the linear range of the standard curve.

4. Detection Antibody Incubation

a. Dilute the detection antibody in blocking buffer (follow manufacturer's recommended concentration).

b. Add 100 µL per well.

c. Incubate for 1-2 hours at RT or 37°C .

d. Wash 3–5 times with wash buffer (300 µL/well) and tap the plate gently on absorbent paper after each wash.

Note: Optimize antibody concentration, too high a concentration may lead to non-specific binding, while too low may reduce sensitivity. Ensure the antibody solution is well-mixed before adding to the wells. Ensure that the detection antibody recognizes a different epitope than the capture antibody and doesn't cross-react with the plate matrix.

5. Secondary Antibody Incubation (if needed)

If using an unlabeled detection antibody:

a. Use an enzyme-conjugated secondary antibody raised against the species of the detection antibody.

b. Dilute in blocking buffer.

c. Add 100 µL per well.

d. Incubate for 30-60 minutes at RT.

e. Wash 3–5 times with wash buffer (300 µL/well) and tap the plate gently on absorbent paper after each wash.

Note: If using a directly labeled detection antibody, skip this step.

6. Color development and Plate Reading

a. Prepare fresh substrate solution (e.g., TMB for HRP).

b. Add 100 µL per well.

c. Incubate 5-30 minutes at RT in the dark (monitor color development)

Note: The time required depends on the sensitivity of the system and concentration of the target antigen.

d. Add 50-100 µL stop solution (e.g., 1M H_2SO_4) per well.

e. Read absorbance immediately using a microplate reader (e.g., 450 nm for TMB substrate) .

f. Optionally measure at 650 nm as a reference wavelength to correct for optical interference.

7. Data Analysis

a. Plot the absorbance values of the standards against their known concentrations to generate a standard curve.

b. Use the standard curve to calculate the concentration of the target antigen in the samples.