ELISA

Protocols

ELISA protocol

An enzyme-linked immunosorbent assay (ELISA) is used to detect the presence of an antigen in a sample. The antigen is immobilized to the well of a plate by adsorption, or captured with a bound, antigen-specific antibody. A detection antibody is then added forming a complex with the antigen, if present. The detection antibody can be covalently linked to an enzyme, or itself be detected by a secondary, enzyme linked antibody. Enzyme substrate is then added to the wells producing a visible signal that is correlated with the amount of antigen and measured by a spectrophotometer.

General Protocol #1

- ***Run time:*** *4 hours – 30 minutes hands-on time* ***Note:*** *A standard curve must be run with each assay for quantitation*
- Allow all reagents to reach room temperature before use. Gently mix all liquid reagents prior to use.
- Add 50-100 µL of prepared standard and sample to wells. Cover plate and incubate at room temperature for 2 hours.
- Thoroughly aspirate or decant solution from wells and discard the liquid.
- Wash wells 4 times using a squirt wash bottle or an automated 96-well plate washer.
- Add 100 µL of diluted detection antibody to wells. Cover plate and incubate at room temperature for 1 hour.
- Thoroughly aspirate or decant solution from wells and discard the liquid.
- Wash wells 4 times.

General Protocol #1

- Add 100 µL of diluted HRP conjugate to each well. Cover plate and incubate at room temperature for 30 minutes.
- Thoroughly aspirate or decant solution from wells and discard the liquid.
- Wash wells 4 times.
- Add 100 µL of chromogenic substrate to each well.
- Develop plate at room temperature in the dark for 30 minutes.
- Add 100 µL of stop solution to each well. The solution in the wells should change from blue to yellow.
- The plate must be evaluated within 30 minutes of stopping the reaction. Read the absorbance of each well at 450 nm and 550 nm. Subtract 550 nm values from 450 nm values to correct for optical imperfections in the microplate.
- Use curve-fitting statistical software to plot a four-parameter logistic curve fit to the standards and then calculate results for the test samples.

General Protocol #2

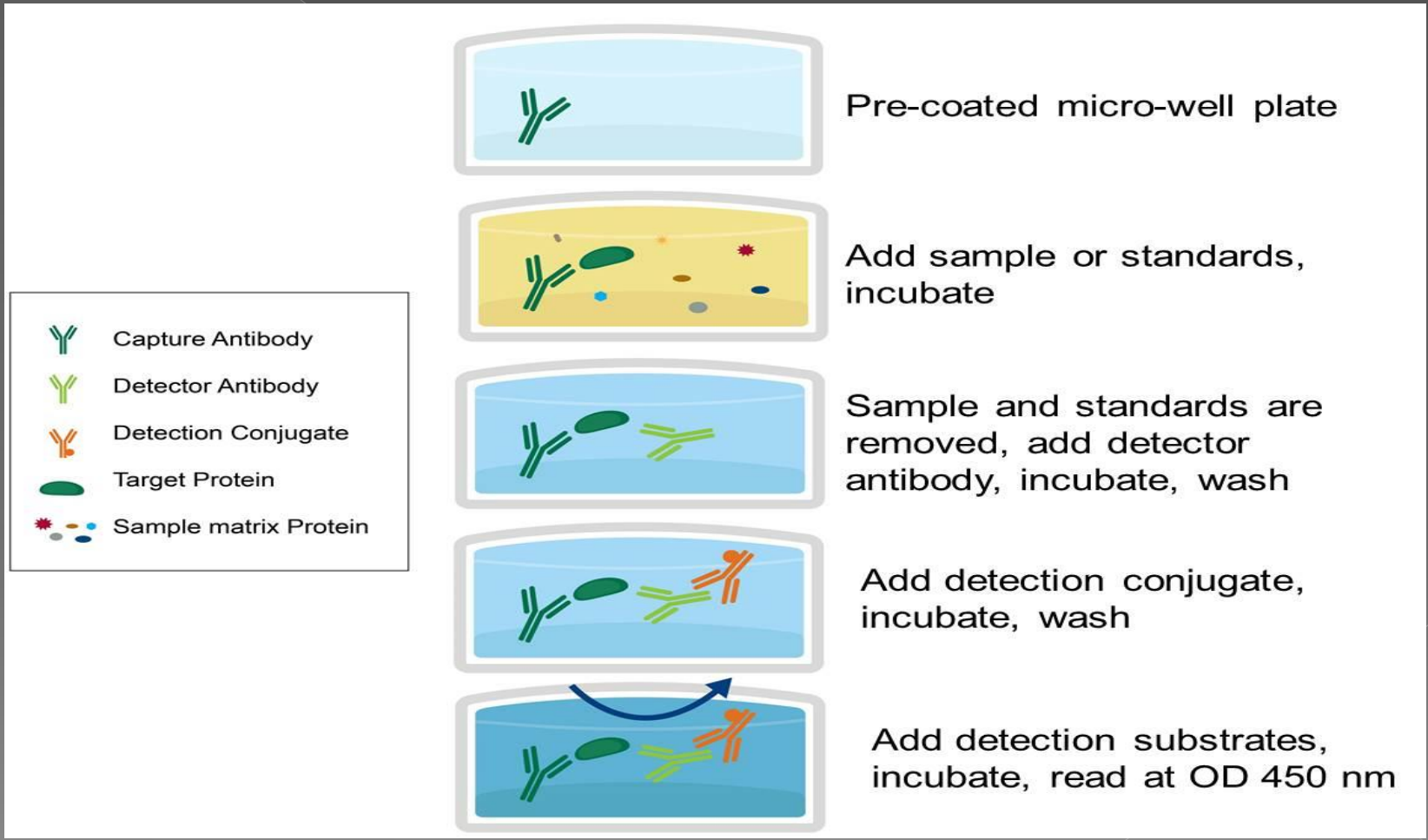
- 100µl peptide (@4µg/ml) in coating buffer is added to individual wells of a microtiter plate. Incubate the plate for 2 hours at 37°C or overnight at 4°C.
- Remove the coating solution and wash the plate three times by filling the wells with 100 µl PBS-0.05%Tween20. The solutions or washes are removed by flicking the plate over a sink. The remaining drops are removed by patting the plate on a paper towel.
- Block the remaining protein-binding sites in the coated wells by adding 100µl blocking buffer, 3% skim milk in PBS per well. Incubate for 1 hour at RT with gentle shaking.
- Wash the plate three times with 100ul PBS-0.05% Tween 20.
- Add 50µl of diluted antibody to each well. Incubate the plate at 37°C for an hour with gentle shaking.

General Protocol #2

- Wash the plate six times with 100ul PBS-0.05%Tween 20.
- Add 50µl of conjugated secondary antibody, diluted at the optimal concentration (according to the manufacturer) in blocking buffer immediately before use. Incubate at 37℃ for an hour.
- Wash the plate six times with 100ul PBS-0.05%Tween20.
- Prepare the substrate solution by mixing acetic acid, TMB and 0.03% H2O2 with the volume ratio of 4:1:5.
- Dispense 50µl of the substrate solution per well with a multichannel pipe. Incubate the plate at 37℃ in dark for 15-30mins.
- After sufficient color development, add 100µl of stop solution to the wells (if necessary).
- Read the absorbance (optical density at 450nm) of each well with a plate reader.

General Protocol

Enzyme-Linked ImmunoSorbent Assay (ELISA)



Coating with capture antibody

- Coat the wells of a PVC microtiter plate with the capture antibody at 1–10 µg/mL concentration in carbonate/bicarbonate buffer (pH 9.6).

  Unpurified antibodies (eg ascites fluid or antiserum) may require increased concentration of the sample protein (try 10 µg/mL) to compensate for the lower concentration of specific antibody.
- Cover the plate with adhesive plastic and incubate overnight at 4°C.
- Remove the coating solution and wash the plate twice by filling the wells with 200 µL PBS. The solutions or washes are removed by flicking the plate over a sink. The remaining drops are removed by patting the plate on a paper towel.

Blocking and adding samples

- Block the remaining protein-binding sites in the coated wells by adding 200 µL blocking buffer (5% non-fat dry milk/PBS) per well.
- Cover the plate with adhesive plastic and incubate for at least 1–2 h at room temperature or overnight at 4°C.
- Wash the plate twice with 200 µL PBS.
- Add 100 µL of diluted samples to each well. Always compare signal of unknown samples against those of a standard curve. Run standards (duplicates or triplicates) and blank with each plate. Incubate for 90 min at 37°C.

  Ensure concentration of standards spans the most dynamic detection range of antibody binding. You may need to optimize the concentration range to obtain a suitable standard curve. Always run samples and standards in duplicate or triplicate.
- Remove samples and wash the plate twice with 200 µL PBS.

Incubation with detection and secondary antibody

- Add 100 µL of diluted detection antibody to each well.

  Check that the detection antibody recognizes a different epitope on the target protein to the capture antibody. This prevents interference with antibody binding. Use a tested matched pair whenever possible.
- Cover the plate with adhesive plastic and incubate for 2 h at room temperature.
- Wash the plate four times with PBS.
- Add 100 µL of conjugated secondary antibody, diluted in blocking buffer immediately before use.
- Cover the plate with adhesive plastic and incubate for 1–2 h at room temperature.
- Wash the plate four times with PBS.

Detection

- Horse radish peroxidase (HRP) and alkaline phosphatase (ALP) are the two most widely used enzymes for detection in ELISA assays.
- Consider that some biological materials have high levels of endogenous enzyme activity (such as high ALP in alveolar cells, high peroxidase in red blood cells) that may result in nonspecific signal. If necessary, perform an additional blocking treatment with levamisol (for ALP) or 0.3% H2O2 in methanol (for peroxidase).
- **ALP substrate**
  P-Nitrophenyl-phosphate (pNPP) is the most widely used substrate for most applications. Measure the yellow color of nitrophenol at 405 nm after 15–30 min incubation at room temperature and stop the reaction by adding equal volume of 0.75 M NaOH.
- **HRP chromogenes**
  The substrate for HRP is hydrogen peroxide. Cleavage of hydrogen peroxide is coupled to oxidation of a hydrogen donor which changes color during reaction.

Detection

TMB (3,3',5,5'-tetramethylbenzidine)
Add TMB solution to each well, incubate for 15–30 min, add equal volume of stopping solution (2 M H_2SO_4) and read the optical density at 450 nm.
OPD (o-phenylenediamine dihydrochloride)
The end product is measured at 492 nm. Keep and store the substrate it in the dark as it is light sensitive.
ABTS (2,2'-azino-di-[3-ethyl-benzothiazoline-6 sulfonic acid] diammonium salt)

The end product is green and the optical density can be measured at 416 nm.
Always handle with care and wear gloves as some enzyme substrates are considered hazardous (potential carcinogens).

Data analysis

- Prepare a standard curve from the serial dilutions data with concentration on the x axis (log scale) vs absorbance on the Y axis (linear). Interpolate the concentration of the sample from this standard curve.

References

- https://www.abcam.com
- https://www.sinobiological.com
- https://www.bio-rad
- https://www.thermofisher.com