

Buffers and Reagents: (See Direct Elisa protocol buffers and reagents)

For accurate quantitative results, always compare signal of unknown samples against those of a standard curve. Standards (duplicates or triplicates) and blank must be run with each plate to ensure accuracy.

General Procedure:

Coating antigen to microplate

1. Dilute the antigen to a final concentration of 20 µg/ml in PBS or other carbonate buffer. Coat the wells of a PVC microtiter plate with the antigen by pipeting 50 µl of the antigen dilution in the top wells of the plate. Dilute down the plate as required.

Test samples containing pure antigen are usually pipeted onto the plate at less than 2ug/ml. Pure solutions are not essential, but as a guideline, over 3% of the protein in the test sample should be the target protein. (antigen). Antigen protein concentration should not be over 20ug/ml as this will saturate most of the available sites on the microtitre plate.



Ensure the samples contain the antigen at a concentration that is within the detection range of the antibody.



2. Cover the plate with an adhesive plastic and incubate for 2 h at room temperature, or 4°C overnight. The coating incubation time may require some optimization.
3. Remove the coating solution and wash the plate three times 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

4. Block the remaining protein-binding sites in the coated wells by adding 200 µl blocking buffer, 5% non fat dry milk or 5% serum in /PBS, per well. Alternative blocking reagents include BlockACE or BSA.
5. Cover the plate with an adhesive plastic and incubate for at least 2 hrs at room temperature or, if more convenient, overnight at 4°C.
6. Wash the plate twice with PBS.

Incubation with primary and secondary antibody

1. Add 100 µl of diluted primary antibody to each well.
2. Cover the plate with an adhesive plastic and incubate for 2 h at room temperature. This incubation time may require optimization. Although 2 hours is usually enough to obtain a strong signal, if a weak signal is obtained, stronger staining will often observed when incubated overnight at 4°C.
3. Wash the plate four times with PBS.
4. Add 100 µl of conjugated secondary antibody, diluted at the optimal concentration (according to the manufacturer) in blocking buffer immediately before use.
5. Cover the plate with an adhesive plastic and incubate for 1-2 hrs at room temperature.
6. Wash the plate four times with PBS.

Detection

Although many different types of enzymes have been used for detection, horse radish peroxidase (HRP) and alkaline phosphatase (ALP) are the two widely used enzymes employed in ELISA assay. It is important to consider the fact that some biological materials have high levels of endogenous enzyme activity (such as high ALP in alveolar cells, high peroxidase in red blood cells) and this may result in non-specific signal. If necessary, perform an additional blocking treatment with Levamisol (for ALP) or with 0.3% solution of H₂O₂ in methanol (for peroxidase).

ALP substrate

For most applications **pNPP** (p-Nitrophenyl-phosphate) is the most widely used substrate. The yellow color of nitrophenol can be measured at 405 nm after 15-30 min incubation at room temperature. (This reaction can be stopped by adding equal volume of 0.75 M NaOH).

HRP chromogens

The substrate for HRP is hydrogen peroxide. Cleavage of hydrogen peroxide is coupled to oxidation of a hydrogen donor which changes color during reaction.

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 H₂SO₄) and read the optical density at 450 nm.

OPD

(o-phenylenediamine dihydrochloride). The end product is measured at 492 nm. Be aware that the substrate is light sensitive so keep and store it in the dark.

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.

Note: some enzyme substrates are considered hazardous (potential carcinogens), therefore always handle with care and wear gloves.

7. Dispense 100 µl (or 50 µl) of the substrate solution per well with a multichannel pipet or a multipipet.
8. After sufficient color development (if it is necessary) add 100 µl of stop solution to the wells.
9. Read the absorbance (optical density) of each well with a plate reader.

Analysis of data:

Prepare a standard curve from the data produced from the serial dilutions 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.