



Indirect Standard ELISA Protocol

I. Apply Antigen

1. Prepare an antigen solution in 0.05M Sodium carbonate buffer, pH 9.6* (1-10 μ l /ml depending on antigen nature). Calculate total volume sufficient for coating all assay plates. Coat each well with 100 μ l of antigen solution. Cover the plate with adhesive film and incubate either at +4°C overnight or at RT for 2 hours (alternatively at 37°C for 1 hour) with shaking plates on a rocking platform at least for 30 min at moderate speed.
2. Wash the plates 3 times with PBS-T* using an automatic washer, or empty plates by shaking coating solution out of wells and filling wells with 200 μ l of PBS-T three times. As a final step, tap plates on paper towels to remove excess buffer.

II. Block plate

1. Block wells by adding 200 μ l of blocking solution (0.25% Gelatin in PBS-T), seal and incubate for 1 hour at RT on rocking platform.
2. Wash the plates 3 times with PBS-T, as in Section I, step 2, of this protocol.

III. React Primary Antibody

1. Add 100 μ l diluted with blocking solution primary antibody (hybridoma culture supernatant can be diluted 1:3) to each well, seal plates and incubate for 1 hour at RT at rocking platform.
2. Wash plates 3 times with PBS-T, as in Section I, step 2, of this protocol.

IV. Apply Secondary Antibody

1. Dilute secondary anti-mouse antibody with blocking solution at dilution as recommended by manufacturer. Add 100 μ l per each well, seal and incubate for 1 hour at RT on rocking platform.
2. Wash plates 3 times with PBS-T, as in Section I, step 2, of this protocol.

V. Add chromogenic substrate and develop

1. Prepare TMB* solution in 0.1M Sodium acetate buffer, pH 5.2* and filter through 0.45 μ m filter. Add 30% hydrogen peroxide to final concentration of 0.01% acetate. Immediately add 100 μ l per each well and allow to develop at RT for 5, 15, 30 min.
2. If desired, after color development add 50 μ l of stop solution, 10% (v/v) phosphoric acid.
3. Read plates with a plate reader.

*SOLUTIONS:

1) Coating Buffer, 0.05M Sodium carbonate buffer, pH 9.6
1.59g Na₂CO₃ + 2.93g NaHCO₃ + 0.1g NaN₃
Adjust to 1L by dH₂O. Keep at +4°C

3) 3M Sodium Acetate, pH 5.2
408.3g of Sodium Acetate·3H₂O dissolve in
800ml dH₂O, adjust pH to 5.2 with glacial acetic
acid.
Adjust volume to 1L, autoclave.

2) PBS-T, Phosphate Buffered Saline, pH 7.4,
containing 0.05% Tween-20

4) TMB – 3',3',5,5'-tetramethylbenzidine
Stock solution -100mg TMB dissolve in 10ml of
DMSO. Aliquot and store in dark vials at +4°C
For developing mix *ex tempore* 100 μ l of TMB
and 9.9 ml of 0.1M Sodium acetate