

Buffers and reagents

Bicarbonate/carbonate coating buffer (100 mM)

Antigen or antibody should be diluted in coating buffer to immobilize them to the wells:

3.03 g Na₂CO₃,
6.0 g NaHCO₃
1000 ml distilled water
pH 9.6,

PBS

1.16 g Na₂HPO₄,
0.1 g KCl,
0.1 g K₃PO₄,
4.0 g NaCl (500 ml distilled water) pH 7.4.

Blocking solution

Commonly used blocking agents are 1% BSA, serum, non-fat dry milk, casein, gelatin in PBS.

Wash solution

Usually PBS or Tris-buffered saline (pH 7.4) with detergent such as 0.05% (v/v) Tween20 (TBST).

Antibody dilution buffer

Primary and secondary antibody should be diluted in 1x blocking solution to reduce non-specific binding.

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 2µg/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 20µg/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 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

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

6. Wash the plate twice with PBS.

Incubation with the antibody

7. Add 100 μ l of the antibody, diluted at the optimal concentration (according to the manufacturer's instructions) in blocking buffer immediately before use.
8. 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 be observed when incubated overnight at 4°C.
9. Wash the plate four times with PBS.

Detection

10. Dispense 100 μ l (or 50 μ l) of the substrate solution per well with a multichannel pipet or a multipipet.
11. After sufficient color development (if it is necessary) add 100 μ l of stop solution to the wells.
12. Read the absorbance (optical density) of each well with a plate reader.
Note: some enzyme substrates are considered hazardous (potential carcinogens), therefore always handle with care and wear gloves.

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.