

Section B and C

Volume-30

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13. METHODS IN BIOLOGY

B. HISTOCHEMICAL AND IMMUNOTECHNIQUES

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

Materials and equipment:

Antigen, for example, human serum albumin, HAS

15. 05 M carbonate—bicarbonate buffer, pH 9.6

Phosphate-buffered saline, PBS containing 0.05% Tween-20 (PBS-Tween)

Hydrogen peroxide (30%)

0.18 M phosphate-Citrate buffer, pH 4.0

2, 2'-azino-bis (3'-ethylbenzthiazoline sulphonic acid), ABTS

Casein

Bovine serum albumin, BSA

Normal sheep serum

Sodium fluoride (80 mg in 25 ml distilled water)

Horse radish peroxidase—anti-immunoglobulin conjugate; for example,

Sheep anti-mouse Ig conjugate

Test sera; for example,

Sera from mice immunized with HAS Enzyme immunoassay micro-litre plates

ELISA reader

Preparation in advance:

Preparation of enzyme substrate:

Prepare this just before adding to the plates.

1. Add 50 mg ABTS to 100 ml 0.18 M phosphate-citrate buffer, pH 4.0.

2. Add 30 μ l of 30% hydrogen peroxide

Hydrogen peroxide is gradually lost from the stock solution with storage. Therefore it is advisable to calculate the exact amount needed to be added each week.

3. Make a 1:1000 dilution of hydrogen peroxide by adding 50 μ l H₂O₂ to 50 ml distilled water.

4. Determine the absorbance at 240 nm in a 1 cm cell against a distilled water blank.

5. The % concentration of original H₂O₂ = absorbance₂₄₀ x 77.98.

6. Volume of original H₂O₂ solution needed per 100 ml substrate solution.

$$= \frac{1}{\%concentration} ml$$

For example, absorbance of 1:1000 dilution of H₂O₂ = 0.37, therefore % concentration = 0.37 x 77.98 = 28.9%, therefore, volume of H₂O₂ needed per 100 ml = 1/28.9 = 0.035 ml.

Method:

1. Dissolve the antigen in carbonate—bicarbonate buffer. The optimum concentration should be determined for each antigen but a concentration of 5-10 μg ml⁻¹ should give acceptable results for most antigens.

2. Add 200 μl to each well of a micro-ELISA plate and incubate overnight at 4° in a humid chamber.

3. Wash to remove unbound antigen and fill the wells with 250 μl 1% w/v casein to block any remaining protein-binding sites (gelatin, BSA or skimmed milk powder are often used instead of casein).

4. Incubate at room temperature for 1 h.

5. Wash the plates two times with PBS—Tween by filling, then inverting and shaking the plates.

6. Dilute the test sera in PBS-Tween containing 1% BSA. (The optimum dilution must be determined in advance; it will generally be about 1:1000.)

7. Add 200 μl diluted test serum and incubate for 2 h at room temperature in a humid chamber.

8. Wash the plates three times with PBS—Tween.

9. Prepare the peroxidase- antibody conjugate by mixing 100 mg casein, 1 ml sheep serum, 100 μl Tween 20 with 50 μl peroxidase—antibody and adjust to a final volume of 100 ml with PBS. Allow to dissolve with gentle stirring. (The exact dilution of conjugate will vary and must be determined by experiment. As a guide, this will generally be between 1:1000 to 1:10,000 for good antibody preparations.)

10. Add 200 μl diluted conjugate to each well.

11. Incubate at room temperature for 1 h.

12. Wash three times with PBS—Tween.

13. Prepare the substrate solution and add 200 μl substrate to each well. Leave in the dark at room temperature for the color to develop, usually 10-30 min.

14. Stop the reaction by adding 50 μ l sodium fluoride solution to each well.

15. Quantitate the color reaction in an ELISA reader set at 650 nm.

Technical notes:

1 Strictly, each assay should include dilutions of a standard reference serum for the calibration of unknown samples. In practice, however, the test is reasonably reproducible and some workers record their results directly in absorbance units.

2 The same assay could be performed with radiolabelled antibody. In this case flexible polystyrene plates should be used so that each well may be punched out and the bound radioactivity measured by spectrometer after step 12, instead of processing for enzyme activity.

3 An alternative substrate for the peroxidase enzyme is 34 mg 0-phenylene diamine and 50 μ l hydrogen peroxide (20 volumes) to 100 ml 0.1 M citrate—phosphate buffer, pH 5.0. The reaction is stopped by the addition of 50 μ l 12.5% sulphuric acid and the absorbance measured at 492 nm.

4 If an alkaline phosphatase-labelled enzyme is used, the substrate should be made up as follows: 50 mg 4-nitrophenyl phosphate in 50 ml diethanolamine buffer pH 9.8 . The reaction is stopped by the addition of 50 μ l 3 M NaOH and the absorbance is measured at 405 nm.

5 Material from detergent- solubilized cells binds very poorly to ELISA plates because of the surfactant effect; for example, protein dissolved in <0.1% Triton X-100 shows little and variable binding; >0.1% detergent inhibits binding completely. The problem of poor adherence may be overcome (for many antigens) by denaturation with Benin's fixative: add 50 (ill antigen solution to each well (approximately 40 (Lig ml⁻¹ initial protein concentration) and 200 μ l Beuin's fluid. Centrifuge at 500 g for 10 min, remove the fixative, and wash once with 50% w/v ethanol and twice with phosphate-buffered saline (PBS). Block plates with PBS containing 3% w/v BSA and 0.01% w/v thiomersal for 1 h. Such plates can be stored at 4° for 1 week. This does not work for all cell-derived antigens and needs to be determined empirically.

IMMUNO- OR WESTERN BLOTTING

In this technique a sheet of nitrocellulose is placed against the surface of an SDS-PAGE protein fractionation gel and a current applied across the gel (at right angles to its face), thus causing the proteins to move out of the gel and onto the nitrocellulose where they bind firmly by non-covalent forces. Two variants of the basic apparatus are available to accomplish this electro..