

Protocol for Immunohistochemistry

Sacrifice and tissue processing

Rats are deeply anesthetized with pentobarbital sodium (Pental). Brain is fixed by transcardial perfusion, first with 50 ml of phosphate buffered saline (0.02M PBS, pH 7.4) containing heparin (5U/ml), then with 220 ml of ice-cold 4% paraformaldehyde in 0.1M PBS, pH 7.5 containing sucrose 4%. Brain is cut in coronal blocks and further fixed by immersion in the same fixative, refrigerated, for 1-2 hours. Brain blocks are transferred to 12% sucrose in 0.1M PBS and sectioned in a cryostat within 10 days. Brain sections, 30 5m thick, are floated in 0.1M PBS and then preserved in a cryopreservation buffer at -20°C. The cryopreservation buffer contains 40% ethylene glycol and 1% polyvinylpyrrolidone in 0.1M potassium acetate buffer, pH 6.5.

Staining procedure

1. Floating sections are rinsed in 0.02M PBS, 2x 5 minutes.
2. Endogenous peroxidase activity is quenched by incubation with 0.2 % hydrogen peroxide in 0.1M phosphate buffer pH 7.3 containing 0.2% Triton X-100 for 25 minutes at room temperature.
3. Sections are rinsed in 0.02M PBS, 2x 5 minutes.
4. Sections are incubated with the primary antiserum in a medium containing 0.3% Triton X-100, 0.05% Tween 20, 4% normal donkey serum (NDS), for 1 hour at room temperature and then overnight refrigerated.
5. Sections are rinsed in 0.02M PBS, containing 4% NDS, 2x 5 minutes.
6. At this point, staining may proceed with various types of secondary antibodies. Two alternative procedures are described here:

Protocol A Sections are incubated with biotinylated donkey anti-rabbit (from Chemicon USA, catalog number AP182B) diluted 1:400 in 0.02M PBS, containing 0.3% Triton X-100, 0.05% Tween 20, and 4% NDS, for 1 hour at room temperature and then overnight refrigerated.

7. Sections are rinsed in 0.02M PBS containing 4% NDS, 2x 5 min.
8. Sections are incubated with extravidin-peroxidase (Sigma Catalog number E2886) diluted 1:100 in 0.02M PBS, for 45 minutes at room temperature.
9. Sections are rinsed in 0.02M PBS, 3x 5 minutes.

Protocol B Sections are incubated with horseradish peroxidase labeled donkey anti-rabbit (from Chemicon USA, catalog number AP182P), 1:400 in 0.02M PBS, containing 0.3% Triton X-100, 0.05% Tween 20, and 4% NDS for 1 h at room temperature and then refrigerated overnight.

7. Sections are rinsed in 0.02M PBS containing 4% NDS, 2x 5 min.
8. Sections are rinsed in 0.02M PBS, 2x 5 minutes.

Color development

1. Sections are incubated with a solution of diaminobenzidine (Sigma catalog number D5637) at the concentration of 0.0125% and containing 0.05% nickel ammonium sulfate for 10 minutes at room temperature.
2. Sections are transferred to the same DAB solution but with added hydrogen peroxide at a final concentration of 0.0015%. Duration of incubation should be adjusted by the end user.
3. Sections are rinsed in 0.02M PBS, 4x 10 minutes.
4. Sections are mounted on glass slides (gelatinized or coated by any other type of adhesive material) and allowed to dry.
5. Sections are dehydrated in ascending series of ethanol concentrations (70%, 90%, 100%, 5 minutes in each), delipidated in xylene (10 minutes) and coverslipped in Permount (or any other xylene diluted adhesive).

Antigen retrieval

If the final result is negative, e.g. no staining, then antigen retrieval is recommended. Keep a stock of 0.1% trypsin (Sigma type II, catalog no. T-8128) dissolved in 0.02M PBS in frozen aliquots. For antigen retrieval, dilute the stock 1:100 to obtain a final trypsin concentration of 0.001%. Also add CaCl_2 to a final concentration of 0.001%. Incubate the sections in this solution for 1-2 minutes at room temperature and then rinse twice in 0.02M PBS. Add trypsin-inhibitor (Sigma type II-S, catalog no. T-9128) to the primary antibody solution. Keep a stock of 0.1% trypsin inhibitor diluted in 0.02M PBS in frozen aliquots. Dilute the stock 1:100 into the primary antibody solution.