Immunocytochemistry Protocol for Cells on Coverslip –Avidin-Biotin Complex Detection-

This procedure works well with 50 % confluent cells in a 6-well plate and can be performed without coverslips.

Solutions and Reagents

Note: Prepare solutions with Milli-Q or equivalently purified water. Be aware that azide will interfere with the HRP enzyme reaction and should not be added to solutions.

•10X Phosphate Buffered Saline (PBS): 0.58 M sodium phosphate dibasic (Na_2HPO_4), 0.17 M sodium phosphate monobasic (NaH_2PO_4), 0.68 M NaCl. To prepare 1 liter of 10XPBS, use 82.33 g Na_2HPO_4, 23.45 g $NaH_2PO_4H_2O$ and 40 g NaCl. Adjust pH to 7.4.

•4 % Paraformaldehyde: Prepare day of use either from commercial 16 % stock solution or by dissolving paraformaldehyde in 1X PBS by heating at 60°C and stirring until it dissolves. Cool before use.

•100 % Methanol

•Tris Buffered Saline (TBS): 50 mM Tris-HCl (pH 7.4), 150 mM NaCl

•0.2 % Triton X-100: Prepare stock of 20 % Triton in PBS. Rotate tube overnight to dissolve. Dilute to 0.2 %.

•TBS/Triton: 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1 % Triton X-100

•Blocking Buffer: 5.5 % normal goat serum in TBS/Triton

•3 % Bovine Serum Albumin (BSA)

•0.6 % Hydrogen Peroxide: 200 µl 30 % H_2O• in 10 ml TBS

•ABC Reagent: (Vectastain ABC Kit, Vector Laboratories, Inc., Burlingame, CA) Prepare 30 minutes before using.

•DAB Reagent: Add 7 µl of 30 % hydrogen peroxide to 10 ml dH_2O; add this mixture to 10 ml of 1 mg/ml DAB (diaminobenzidine tetrahydrochloride) in PBS, filtrate.

Fixation

1. Prepare 4 % paraformaldehyde and/or cool 100 % methanol to –20°C in a tightly sealed container.

2. Treat cells as desired.

3. Quickly wash cells once with cool or room temperature 1X PBS.

4. Aspirate off PBS completely and immediately fix as appropriate.

5. Aspirate fixative/permeabilizer and wash three times for 5 minutes each with 1 ml TBS/Triton. Rinse with TBS.

Methanol (protein precipitation) Fixation

a. Immerse cells in –20°C 100 % methanol for 10 minutes.

b. Wash slips three times for 2 minutes each with room-temperature TBS. Aspirate off completely and return slips to TBS only. Continue protocol at blocking step.

Paraformaldehyde (cross-linking) Fixation

a. Immerse cells in 4 % paraformaldehyde for 10 minutes at 4°C.

b. Wash coverslips once with TBS. Aspirate completely and then permeabilize cells on coverslips with 0.2 % Triton X-100 for 5 minutes at room temperature or alternatively with –20°C methanol for 5 minutes.

c. Quench cells in fresh 0.1 % sodium borohydride in TBS for 5 minutes. Aspirate off completely and return slips to TBS only. Continue protocol at blocking step.

Blocking

1. Aspirate, then incubate with 1 ml blocking buffer for 45–60 minutes at room temperature.

2. Wash once for 5 minutes with 1 ml TBS.

Staining

1. Aspirate, then incubate with primary antibody at suggested dilution in 3 % BSA in TBS overnight at 4°C.

   Note: When using any primary antibody for the first time, titrate out the primary to determine which dilution allows the strongest specific signal with the least background.

   Note: You may wish to leave one slip for a secondary antibody-only control, and to provide another slip incubated in normal serum from the host animal (rabbit for polyclonals and mouse for monoclonals) instead of the primary antibody. Dilute normal serum to the lowest dilution shown to have no staining by your chosen fixation/permeabilization method.

2. Wash two times for 5 minutes each with 1 ml TBS/Triton. Wash once with TBS.

3. Incubate with biotinylated secondary antibody (diluted appropriately in TBS/3 % BSA; 1:500 for secondary antibody from Vectastain ABC Kit) for 1 hour at room temperature.

4. Wash three times for 5 minutes each with 1 ml of TBS/Triton.

5. Wash once for 5 minutes with 1 ml TBS.

6. Incubate for exactly 30 minutes in 0.6 % hydrogen peroxide at room temperature.

7. Wash three times for 5 minutes each with 1 ml of TBS/Triton. Wash once with TBS.

8. Incubate for 1 hour with 0.5–1.0 ml ABC reagent at room temperature. (Add 2 drops solution A into 5 ml PBS, mix, then add 2 drops solution B, mix.)

9. Wash two times for 5 minutes each with 1 ml TBS.

10. Add 1 ml DAB reagent. Monitor reaction progress under the microscope. Reaction may proceed for 10 minutes.

11. Terminate reaction by adding an equal volume of water.

12. Aspirate and wash once with 1 ml of water.

13. View cells in 6-well plate or mount coverslips with VectaMount (Vector Laboratories, Burlingame, CA) if necessary.