IMMUNOHISTOCHEMISTRY (IHC) - PROTOCOL

Principle

Immunohistochemistry staining is used to identify specific constituents in tissue sections or immobilized cells. To detect the reaction site, the antibody/antigen complex is labeled with an enzyme that can be reacted with a suitable substrate to give a colored product. Proper fixation is crucial to successful staining. Formaldehyde fixation is often used as the routine initial method of choice for tissue and with the immobilized antigen to form an antigen-antibody complex. A second, biotinylated antibody specific for primary antibody reacts with the complex. Streptavidin conjugated to Peroxidase reacts with the ab-ab-ag complex immobilizing the peroxidase at the site of the antigen. Finally, the substrate is added causing a colored precipitate to form on the slide at the location of the antigen. This slide is analyzed using a light microscope or embedded and prepared for electron microscopy.

Reagents Required

- PBS Wash Buffer. Phosphate Buffered Saline (PBS). Use 10x PBS, pH 7.2 (0.2M Potassium Phosphate, 1.5M NaCl). Dilute appropriate volume to 1x with de-ionized water.
- Formaldehyde Fixative. Dilute to 4% in PBS buffer.
- Antibody Dilution Buffer. Prepare 100ml of PBS wash buffer supplemented with 1ml of normal serum of same species as the host for the secondary antibody.
- Biotinylated Secondary Antibody. Prepare dilution of biotinylated secondary antibody in Antibody Dilution Buffer. Use biotinylated secondary antibody conjugate against the same species as the primary antibody.
- Streptavidin Peroxidase. Prepare dilution of Streptavidin Peroxidase in PBS buffer.
- DAB Substrate.
- Hematoxylin Counterstain and Mounting Media

Procedure

1. Grow cells on glass microscope slides, glass cover slips or slide culture chambers. Remove culture medium and gently wash cells 3 times with ice cold PBS. Fix cells by adding a volume of 4% formaldehyde in PBS equal to the original volume of culture medium for 30 minutes on ice. Remove the fixative and wash 3 times for 5 minutes each with PBS. If desired, incubate 5 minutes in 1% H2)2 in PBS to remove endogenous peroxidase activity. Wash the fixed cells 3 times for 5 minutes each with PBS.

2. Prepare appropriate dilution of primary antibody by diluting in Antibody Dilution Buffer. Remove the buffer from the cells. Add a sufficient volume of diluted primary antibody to cover the cells. Incubate with primary antibody for 60 minutes at room temperature. If the primary antibody has a low-affinity for the antigen, incubate at 4ºC overnight. Remove primary antibody solution. Wash 3 times for 5 minutes each with PBS.
3. Remove the buffer from the cells. Add diluted Biotinylated Secondary Antibody and incubate for 30 minutes at room temperature. The optimum dilution may be lot specific. Remove solution. Wash 3 times for 5 minutes each with PBS.

4. Remove the buffer from the cells. Add Diluted Streptavidin Peroxidase and incubate for 30 minutes at room temperature. Remove solution. Wash 3 times for 5 minutes each with PBS.
5. Remove buffer. Add DAB substrate and incubate approximately 10 minutes or until sufficient color develops.
6. Remove solution. Wash 3 times for 2 minutes each with distilled water. Counter stain with hematoxylin for 1 to 5 minutes depending on the concentration and color intensity desired. Wash 3 times for 2 minutes each with distilled water. Dehydrate the cells with 100% ethanol 4 times for 2 minutes each. Clear the cells with xylene 4 times for 2 minutes each. Add 2-3 drops of mounting media add a cover slip and allow to air dry.
7. Observe cells under the microscope. A positive reaction should be visible as a brown precipitate. The nuclei should appear light blue.