Immunohistochemistry Paraffin Protocol

Preparation - Perfusion and Paraffin Embedding

1. Fix tissue by perfusing the animal with freshly prepared 4% paraformaldehyde. If perfusion is not possible, incubate freshly dissected tissue in 10% formalin for 4-24 hours. Incubation time is dependent on tissue type and size.

2. Dehydrate by moving tissue through the following wells twice for 30 minutes each:

    1. 70% EtOH
    2. 95% EtOH
    3. 100% EtOH
    4. Xylene

3. Embed tissue in molten paraffin and store at room temperature until ready for sectioning.

4. When ready for sectioning, use a microtome to cut the embedded tissue into 5-20 µm thick sections and float them in a 50° C water bath containing distilled water.

5. Mount sections onto gelatin coated slides and allow them to dry overnight. Slides can be safely stored at room temperature until ready for staining.

Immunofluorescent staining

1. Deparaffinize and rehydrate by immersing the slides through the following wells:

    1. Xylene: Twice for 5 minutes each
    2. 100% EtOH: Twice for 5 minutes each
    3. 95% EtOH: 5 minutes
    4. 70% EtOH: 5 minutes
    5. 50% EtOH: 5 minutes
    6. dH_2O: Rinse until ready for staining. Do not let the tissue dry from this point on.

2. Optional: Perform antigen retrieval described here.

3. Draw a circle on the slide around the tissue with a hydrophobic barrier pen or use rubber cement.

4. Wash the sections twice for 10 minutes with 1% animal serum in PBS-T (PBS with 0.4% Triton X-100). The species of the animal serum is dependent on the host of your secondary antibody. Ex. If using goat anti-mouse secondary, use goat serum.

5. Block any non-specific binding by incubating the tissue sections with 5% serum in PBS-T for 30 minutes at room temperature.

6. Add primary antibody diluted in 1% serum PBS-T and incubate overnight at 4° C. Use the recommended dilution of the antibody as specified on the datasheet.

7. Wash sections twice with 1% serum PBS-T for 10 minutes each.

8. Dilute secondary antibody in 1% serum PBS-T and incubate with sections at room temperature for 1 hour. Use the recommended dilution of the antibody as specified on the datasheet.

9. Wash sections twice with 1% serum PBS-T for 10 minutes each.

10. Optional: Double/Nuclear labeling

    1. Double labeling: If using a second primary antibody and appropriately matched secondary, repeat steps 6-9.

    2. Nuclear labeling: After application of all primary antibodies, DNA binding dyes such as DAPI can be applied, which are used without the need for secondary antibodies. Use the recommended dilution and incubation time as specified on the datasheet. After incubation, wash once for 5 minutes with PBS.

11. Tap off excess wash and apply one drop of anti-fade mounting medium to the slide. Coverslip the tissue sections. Ring the edges of the coverslip with clear fingernail polish to prevent the cells from drying. Allow nail polish to air dry.

12. Slides may now be examined under a microscope with the appropriate fluorescent filter sets. Limiting the amount of time each slide is exposed to the microscope's light will aid in prolonging the signal and prevent photobleaching.

13. Slides can be stored between -20° C and 4° C in a dark slide box or slide book.

Immunochromogenic staining (ABC method with DAB)

1. Deparaffinize and rehydrate by immersing the slides through the following wells:
    1. Xylene: Twice for 5 minutes each
    2. 100% EtOH: Twice for 5 minutes each
    3. 95% EtOH: 5 minutes
    4. 70% EtOH: 5 minutes
    5. 50% EtOH: 5 minutes
    6. dH_2O: Rinse until ready for staining. Do not let the tissue dry from this point on.
2. Optional: Perform antigen retrieval. Refer to antigen retrieval protocol.
3. Draw a circle on the slide around the tissue with a hydrophobic barrier pen or use rubber cement.
4. Wash the sections twice for 10 minutes with 1% animal serum in PBS-T (PBS with 0.4% Triton X-100). The species of the animal serum is dependent on the host of your secondary antibody. Ex. If using goat anti-mouse secondary, use goat serum.
5. Block any non-specific binding by incubating the tissue sections with 5% serum in PBS-T for 30 minutes at room temperature.
    1. Optional: If endogenous peroxidase activity is suspected, quench the tissue with 0.3-3.0% H_2O_2 in PBS or methanol for 15 minutes.
    2. Add primary antibody diluted in 1% serum PBS-T and incubate overnight at 4° C. Use the recommended dilution of the antibody as specified on the datasheet.
    3. Wash sections twice with 1% serum PBS-T for 10 minutes each.
    4. Add a biotinylated secondary antibody and incubate at room temperature for 1 hour. Use the recommended dilution of the antibody as specified on the datasheet.

5. Wash sections twice with 1% serum PBS-T for 10 minutes each.

6. Add ABC-HRP reagent and incubate at room temperature for 1 hour. Follow manufacturer's guidelines for reagent preparation.

7. Wash sections twice in PBS for 10 minutes each.

8. Prepare a working solution of DAB and apply to tissue sections. Monitor the reaction as the chromogenic reaction turns the epitope sites brown. Proceed to the next step when the intensity of the signal is appropriate for imaging. Important: DAB is a carcinogen! Always wear gloves and work in a fume hood when working with DAB. Deactivate and clean work area after use according to manufacturer's instructions.

9. Wash sections twice in PBS for 5 minutes each.

10. To counterstain nuclei, use Hematoxylin according to the manufacturer's instructions. Note: If you are using an aqueous chromogen instead of DAB (i.e. AEC, Fast Red, etc.), skip the following dehydration step and mount in aqueous media instead of organic mounting media.

11. Dehydrate tissue sections by moving slides through the following wells twice for 2 minutes each:

    1. 95% EtOH
    2. 100% EtOH
    3. Xylene

12. Add mounting media to slides and coverslip. The DAB reaction is permanent and stable. Thus, it can be analyzed under a brightfield microscope at any time.