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CHROMOGENIC IMMUNOHISTOCHEMISTRY STAINING OF FROZEN TISSUE (IHC-F)

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TISSUE PREPARATION - PERFUSION AND FIXATION

Note: **This portion of the protocol can be skipped if you are working with pre-mounted slides. The technique described below utilizes formaldehyde-based fixation before the tissue is frozen and sectioned. Tissues can also be fixed following snap freezing and sectioning. See the next section of this protocol for more information on cryopreservation.**

1. Fix tissue by perfusing the animal with freshly prepared 4% paraformaldehyde or by immersing it in 4% paraformaldehyde for 4-24 hours at room temperature. Fixation temperature and time may require optimization depending on the tissue type and size.

    Optional: Alternate fixation methods may be used and result in better performance depending on the protein target. Please see Application Notes section on the datasheet of the product you are using for further information, if available.

2. Cryoprotect the tissue by directly perfusing a sucrose solution or by first dissecting the tissue and allowing it to sink overnight in a 30% sucrose/ 70% fixative solution.

3. Embed tissue in OCT cryostat sectioning medium and store at -80° C until ready for sectioning. Tissue can be safely stored for 6-12 months.

4. When ready for sectioning, move the embedded tissue directly into the cryostat and use OCT medium to mount it to the chuck. Allow the temperature of the tissue to equilibrate with the cryostat.

5. Cut the tissue in 5-20 µm thick sections. Mount tissue sections onto gelatin or poly-L-lysine coated slides by placing the cold sections onto warm slides. Slides can be safely stored for 6-12 months at -80° C until ready for staining.

TISSUE PREPARATION – CYROPRESERVATION

Note: **This portion of the protocol can be skipped if you are working with pre-mounted tissue slides. The technique described below utilizes frozen tissues that are fixed after snap freezing and sectioning with a cryostat.**

1. After dissection, immediately snap freeze tissue with isopentane cooled by liquid nitrogen. To do this, prepare a small dewar of liquid nitrogen. Take an aluminum can cut in half and fill with isopentane. Float the can in the liquid nitrogen until the isopentane is cooled. Quickly dissect the tissue, wrap in aluminum foil, and place in the cooled isopentane. After the tissue is frozen, place it in dry ice and move to -80° C until ready for cutting.

2. Embed tissue in OCT compound by slowly layering the compound so that the tissue does not thaw. Move the embedded tissue directly into the cryostat and use OCT medium to mount it to the chuck. Allow the temperature of the tissue to equilibrate with the cryostat.

3. Cut the tissue in 5-20 µm thick sections. Mount tissue sections onto gelatin or poly-L-lysine coated slides by placing the cold sections onto warm slides. Slides can be safely stored for 6-12 months at -80° C until ready for fixing. Uncut tissue can be restored at -80°C.

4. Remove slides from freezer and fix with cold fixative (acetone or methanol) for 10 minutes. Proceed to staining

BLOCKING NON-SPECIFIC BINDING

1. Warm slides to room temperature and wash slides twice with PBS.

   Note: Fixation may result in epitope masking and non-specific background that can impact specific labeling. If necessary, a protocol for antigen retrieval can be performed at this time. However, many antigen retrieval methods are too harsh on cryostat cut tissue sections.

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

3. 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.

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

   Optional: If endogenous peroxidase activity is suspected, quench the tissue with 0.3-3.0% H2O2 in PBS or methanol for 15 minutes.

CHROMOGENIC IMMUNOHISTOCHEMISTRY STAINING OF FROZEN TISSUE (IHC-F)

ANTIBODY STAINING

1. Add the primary antibody diluted in 1% animal serum in PBS-T and incubate at room temperature for 1-2 hours. Continue the incubation overnight at 4°C. in a humidified chamber. Note: Use the recommended dilution of the antibody specified on the datasheet. If not specified, the recommended starting dilution is 2-5 µg/ml. For more information on primary antibody selection, please read our IHC Primary Antibody Selection Guide.

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

3. Add a biotinylated secondary antibody and incubate at room temperature for 1 hour. Use the recommended dilution of the antibody specified on the datasheet.

   Note: For help selecting the optimal secondary antibody, please read our Secondary Antibody Handbook

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

DETECTION

1. If using the ABC method, then add Add ABC-HRP reagent and incubate at room temperature for 1 hour. Follow manufacturer's guidelines for reagent preparation.

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

3. Prepare a working solution of DAB and apply to tissue sections. Monitor the reaction as the chromogenic reaction turns the epitope sites brown (time of color development may vary from few seconds to 10 minutes). 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.

4. As soon as the section develop, immerse them in deionized water for 2 minutes each.

5. If nuclear counterstaining is desired, 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.

DEHYDRATION AND MOUNTING

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

   a. 95% Ethanol, two times for 10 seconds each
   b. 100% Ethanol, two times for 10 seconds each
   c. Xylene, two times for 10 second each

2. Add mounting media to slides and top with coverslips. The DAB reaction is permanent and stable and can be analyzed under a brightfield microscope at any time.