IMMUNOHISTOCHEMISTRY, FROZEN

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Introduction

Immunohistochemistry is used to identify the location and distribution of target antigens in cells or tissues by staining with a specific antibody. The antibody is conjugated to either a fluorescent or enzymatic label, and the location of the label seen through a microscope approximates the position of the target antigen.
Most tissues cannot be viewed under a microscope because they are too thick allow light to be transmitted. The tissue can be sliced into very thin sections provided they are first processed to prevent cell damage. Valuable time can be saved skipping the fixation and dehydration steps required for paraffin embedding, by freezing the tissue in a modified microtome, the cryostat. In addition, frozen sections will more often retain their enzyme and antigen functions.
Frozen tissue slides are ideal for rapidly identifying cellular localization of RNA or protein. First, tissue is excised then sectioned and immediately frozen by liquid nitrogen and then stored in -80ºC. After staining with the appropriate antibodies localization of the target antigens can be identified.

Materials / reagents

- **Cold fixative:**
  acetone (-20℃) or alcohol or formal alcohol, formalin, etc.
- **Blocking buffer :**
  10% serum from host species of secondary antibody diluted in PBS
  or 2% Bovine Serum Albumine (BSA) diluted in PBS
- **Antibody buffer:**
  PBS with 2% BSA
- **Diaminobenzidine (DAB) solution:**
  0.5 mg/ml diaminobenzidine (DAB) in PBS

Procedure

Preparation of frozen tissues

1. Label base mold and partially fill the mold with frozen tissue matrix.
2. Remove desired tissues, trim and cut tissue no more than 5 mm thick.
3. Place in pre-labeled base molds filled with frozen tissue matrix. Arrange tissue in the matrix near the bottom so tissue is easily exposed when sections are cut.
4. Place a stainless steel beaker containing 2-methylbutane in liquid nitrogen and allow to cool adequately.
5. Place base mold with tissue into the beaker with cold 2-methylbutane and quickly immerse the block. Allow the tissue matrix to solidify completely and remove block from 2-methylbutane and place on dry ice or in the -20℃ cryostat.
6. Store blocks in the -80℃ freezer until ready for sectioning.

Sectioning of frozen tissues

1. Before cutting sections allow the temperature of the block to equilibrate to the temperature of the cryostat (typically -20℃).
2. Place the tissue block on the cryostat specimen disk. Adjust the positioning of the block to align the block with the knife blade. Cut tissue block until the desired tissue is exposed.
3. Cut sections of the desired thickness (usually 5 µm), place the sections on a superfrost slide and dry overnight at room temperature.
4. Fix slides by immersion in cold suitable fixative, air dry at room temperature and proceed to staining.
5. Alternatively, the frozen section slides can be stored for a short period of time at -70℃ in a sealed slide box. When ready to stain, remove slides from freezer and warm to -20℃ in the cryostat or -20℃ freezer, fix for 2 minutes in cold fixative and allow to come to room temperature to continue with the staining.

Standard immunohistochemical staining procedure for frozen sections

Please read entire procedure before staining sections. Perform all incubations in a humidified chamber and do not allow sections to dry out. Isotype and system controls should also be run and must be matched to the isotype of each primary antibody to be tested..

1. Label slides with a solvent resistant pen and demarcate the tissue if required.
2. Rinse slides 3x in PBS, to remove the tissue-freezing matrix.
3. Block endogenous peroxidase activity by incubating the slides in 0.3% H_2O_2 solution in PBS for 10 minutes.
4. Rinse slides 3x in PBS, 2 minutes each time.
5. Block non-specific binding by incubating with blocking buffer for 30-60 min at room temperature in a humidified chamber.
6. Remove blocking buffer.
7. Dilute the primary antibody in the antibody buffer. Apply the diluted antibody to the tissue sections on the slide. Incubate for 1 hour at room temperature in a humidified chamber.
8. Rinse slides 3x in PBS, 2 minutes each time.
9. Dilute the secondary antibody in the antibody buffer. Apply to the tissue sections on the slide and

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incubate for 30 minutes at room temperature.

10. Rinse slides 3x in PBS, 2 minutes each time.
11. According to the second antibody an appropriate conjugate should be used.
12. Rinse slides 3x in PBS, 2 minutes each time.
13. Prepare DAB solution. For blue stain, mix two parts DAB solution + one part 3% $NiCl_2$. For orange-brown stain, use the DAB solution without $NiCl_2$.
14. Apply the appropriate DAB solution and allow slides to incubate for 5 minutes or until the desired color intensity is reached.
15. Transfer slide to a glass dish. Add ~5 µl 0.3% H_2O_2. It may take seconds to several minutes for staining to occur, depending on the abundance of the antigen and the quality of the antibody. Observe the extent of staining under a stereomicroscope and, when the color is appropriately developed, stop the reaction by washing with several changes of PBS.

Safety

- Samples of tissue, serum or blood origin should be handled to guidelines for prevention of transmission of blood borne diseases.
- Some enzyme substrates are considered hazardous, due to potential carcinogenicity. Handle with care and refer to Material Safety Data Sheets for proper handling precautions.
- Wear appropriate protective clothing, gloves, and eyewear to avoid any accidental contact with reagents.
- Reagents which contain preservatives may be toxic if ingested, inhaled, or in contact with skin.
- Use extreme caution, DAB is a suspect carcinogen. Handle with care. Wear gloves, lab coat and eye protection.
- Formalin has been implicated as a causative agent for strong allergy reactions (contact dermatitis with prolonged exposure) and may be a carcinogen. It should be used with care and always in a well ventilated environment.

Notes

- This protocol is to be used for research purposes only.
- When making buffer(s) fresh every time, there is no need to add sodium azide to the buffer.
- Please note that this is a general protocol. Optimal dilutions for the primary and secondary antibodies, cells preparation, controls, as well as incubation times will need to be determined empirically and may require extensive titration. Ideally, one would use the primary antibody as recommended in the product data sheet.
- The appropriate negative and positive controls should always be included.
- Pre-incubation of the sample with 5% BSA for 10 min. prior to the primary antibody reaction may decrease background staining. For best results with animal tissues, use 5 to 10% normal serum from the same species as the host of the secondary antibody.
- Allow the slides to drain, shake off excess fluid with a brisk motion and carefully wipe each slide around the sections.
- Dilute the primary antibody or negative control reagent to its optimal dilution. The antibody buffer alone may be used as a negative control. A positive control slide (a tissue known to contain the antigen under study) should also be run.
- If at the preparation of the frozen tissues the block is left in 2-methylbutane too long, the block may crack.

Technical assistance

For technical assistance and more information please contact Hbt directly at support@hbt.nl.

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