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IHC FFPE Tissue Trypsin Digestion Antigen Retrieval—Indirect Method

Staining using a purified/biotinylated antibody

Research Use Only

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Materials

- Tris buffered saline (TBS) (50 mM Tris, 150 mM NaCl, pH 7.4) or phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4)
- Clearing and rehydration/dehydration reagents: Histoclear II or Xylene, 100% Ethanol (Histology grade: 100%, 90%, and 70%)
- Antigen retrieval reagent: 2.5% Trypsin (Fisher Scientific)
- Blocking reagent: IHC/ICC Blocking Buffer-Low Protein (cat. no. 00-4953) or High Protein (cat. no. 00-4952) or 10% Normal Serum (from the species from which the secondary antibody was made) in PBS
- Primary antibody: purified or biotinylated format
- Secondary antibody/Visualization reagent: biotinylated and streptavidin-conjugated fluorophore (for 3-step protocol) or fluorophore-conjugated antibody (for 2-step protocol)
- Nuclear counterstain: DAPI or DRAQ5 (cat. no. 65-0880)
- Mounting medium: Fluoromount-G (cat. no. 00-4958) or Fluoromount-G with DAPI (cat. no. 00-4959)

Accessories

- Humidified container in which to place the samples during incubations
- Coplin jars
- Parafilm
- Glass coverslips (size appropriate to tissue section size)
- Clear nail polish

Methods

1. Paraffin-embedded sections are cut and mounted on Superfrost plus slides. Slides are placed in plastic vertical slide holders.
2. Slides are heated for 20 min for 50-60°C in a dry oven to facilitate attachment of tissue and soften the paraffin.
   NOTE: To prevent damage to target antigens temperature should not exceed 60°C.
3. Remove paraffin and rehydrate tissue using the following slide wash/incubation sequence:
   Histoclear II (3 x 5 min each)
   100% Ethanol (2 x 5 min each)
   90% Ethanol (5 min)
   70% Ethanol (5 min)
   ddH2O (5 min)

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When moving the slides through the solutions, be sure to adequately mix the reagents and remove bubbles collecting on the slides by dipping the slide holder, with slides, up and down in the solution several times. From this point on, it is critical that the tissue does not dry out as this will result in difficulty interpreting staining results.

4. Submerge slides in 0.1% Trypsin diluted in 1X PBS for 10-20 min at 37°C. Incubation time must be optimized and is antigen and tissue dependent.
5. Wash the slides in a coplin jar with 1X PBS for 5 min using gentle agitation in an orbital shaker set to low speed.
6. Cover the tissue with blocking reagent for 1 hour at room temperature (100 µL/ tissue section). To limit evaporation of blocking reagent and help evenly spread the blocking solution over the tissue, use forceps to gently overlay the tissue section with a piece of parafilm cut to the dimension of the tissue. It is not necessary to stretch the parafilm or cover the edges of the slide.
   NOTE: PAP Pen ink can interfere with eFluor® Nanocrystal signal and is not recommended.
7. Using forceps, gently lift and remove the Parafilm without disturbing the tissue section and immerse the slide, with the tissue, into a coplin jar containing PBS. Using an orbital shaker set to low speed, gently agitate, changing the PBS wash solution 2 more times for a total of 3 washes (5 min/wash).
8. Dilute the primary, at manufacturer's recommended dilution, in blocking reagent. Overlay the primary antibody solution on the tissue and cover with parafilm as described in step 6. Incubate in a humidified chamber overnight at 4°C.
9. Gently wash the tissue 3 times in PBS (5 min/wash) as described in step 7.
   If using an unconjugated primary antibody, continue to step 10 (3-step protocol).
   If using a biotinylated primary antibody, continue to step 12 (2-step protocol).
10. Dilute the secondary antibody, at manufacturer's recommended dilution, in blocking reagent. If using a fluorophore-conjugated antibody, protect from light. Overlay the secondary antibody solution on the tissue and cover with parafilm as described in step 6. Incubate in a humidified chamber for 1 hour at room temperature.
11. Gently wash the tissue 3 times in PBS or TBS (5 min/wash) as described in step 7.
12. Dilute Streptavidin conjugate, at manufacturer's recommended dilution, in blocking reagent, protecting from light. Overlay the visualization reagent on the tissue and cover with parafilm as described in step 6. Incubate in a humidified chamber for 30 min at room temperature.
13. Gently wash the tissue 3 times in PBS or TBS (5 min/wash) as described in step 7.
14. Optional: Nuclei can be counterstained using DAPI (see step 15) or DRAQ5. It is necessary to select a counterstaining agent with a fluorescent emission spectra that does not overlap with the other fluorophores used in the experiment.

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15. Mount and coverslip using Fluoromount-G or Fluoromount-G with DAPI. Seal the edge of the coverglass with clear nail polish.
16. Allow slides to dry for 1-2 hours before visualizing.
17. Slides can be stored at 4°C protected from light if needed.

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