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Colorimetric FFPE-- Trypsin Digestion

Staining using a purified/biotinylated primary antibody

Research Use Only

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Materials

- 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%)
- 2.5% Trypsin (Fisher Scientific)
- Endogenous peroxidase blocking solution: 0.3% H_2O_2 diluted in ddH_2O
- 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 (2-step protocol)
- Secondary antibody: biotinylated (for 3-step protocol)
- Amplification reagent: avidin-horseradish peroxidase (cat. no. 18-4100)
- Visualization reagents: H_2O_2 30% Stock, Diaminobenzidine (DAB) 1% stock (Sigma chemical)
- Nuclear counterstain: Hematoxylin (Fisher Scientific)
- Mounting medium: Permount (Fisher Scientific)

Accessories

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

Methods

1. Cut and mount paraffin-embedded specimens on Superfrost plus slides. Slides are placed in plastic vertical slide holders.
2. Heat slide 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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Be sure to adequately mix solutions when moving the slides through the reagents. Remove bubbles from collecting on the slides by dipping the slide holder, containing 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. Completely 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. Block endogenous peroxidase by submerging slides in 0.3% H_2O_2 for 15-40 min at room temperature.
   Note: Individual tissues will require optimization of incubation times depending on the amount of endogenous peroxidase present in the tissue.
7. Gently wash slides 3 times in PBS (5 min/wash).
8. 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.
9. Using forceps, gently lift and remove the parafilm without disturbing the tissue section. Immerse the slide with the tissue into a coplin jar containing PBS. Gently agitate using an orbital shaker set to low speed, changing the PBS wash solution 2 more times for a total of 3 washes (5 min/wash).
10. Dilute the primary antibody, at manufacturer's recommended dilution, in blocking reagent. Overlay the primary antibody solution on the tissue and cover with parafilm as described in step 8. Incubate in a humidified chamber overnight at 4°C.
11. Gently wash the tissue 3 times in PBS (5 min/wash) as described in step 9.
    If using an unconjugated primary antibody, continue to step 12 (3-step protocol).
    If using a biotinylated primary antibody, continue to step 14 (2-step protocol).
12. Dilute the biotinylated secondary antibody, at manufacturer's recommended dilution, in blocking reagent. Overlay the secondary antibody solution on the tissue and cover with parafilm as described in step 8. Incubate in a humidified chamber for 1 hour at room temperature.
13. Gently wash the tissue 3 times in PBS (5 min/wash) as described in step 9.
14. Dilute the amplification reagent avidin-horseradish peroxidase, at recommended dilution, in blocking reagent and overlay on to the tissue. Cover the tissue with arafilm and incubate for 30 min at room temperature in a humidified chamber.
15. Wash the slides 3 times in PBS.
16. Prepare the visualization/development solution by adding 500 µL of DAB stock and 2.5 µL 30% H_2O_2 to 50 mL 1X PBS pH 7.4. Incubate slides in DAB until brown staining is detected, monitoring carefully using a brightfield microscope.

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NOTE: DAB is a suspected carcinogen, see manufacturer's recommendations for correct handling and disposal.

17. When development is complete, wash the tissue sections by immersing the slides into a coplin jar containing ddH_2O.
18. Optional: Nuclei can be counterstained using hematoxylin, again monitor until desired staining is achieved. Once nuclei are stained, immerse the slides into a coplin jar containing ddH_2O. Hold the coplin jar under a trickle of water to remove excess dye, but taking care not to damage the tissue section.
19. Dehydrate the tissue sections by incubation the slides in reverse order of hydration:
    70% ETOH (5 min)
    90% ETOH (5 min)
    100% ETOH (2x5 min each)
    Histoclear II (3x5 min each)
20. Mount and coverslip using 3 drops of Permount (Fisher Scientific). Allow slides to dry for 1-2 hours before visualizing using a brightfield microscope

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