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IMMUNOHISTOCHEMISTRY STAINING HANDBOOK

CAUTION: INVESTIGATIONAL DEVICE
LIMITED BY FEDERAL LAW TO INVESTIGATIONAL USE

FOR RESEARCH ONLY
NOT FOR USE IN DIAGNOSTIC PROCEDURES

TECHNICAL SUPPORT AND ORDERING INFORMATION

Please e-mail technical questions to techsupport@penlabs.com

To purchase additional kits or other products or to obtain a quote, please email sales@penlabs.com

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INTRODUCTION

This immunohistochemistry staining (IHS) kit is designed to determine the localization of target antigens present within paraffin-embedded or frozen tissue preparations for approximately 100 to 150 slides. Briefly, an antigen specific primary antibody is applied to the tissue section slide whereby the primary antibody binds to the target antigen. Once bound, the resulting antigen-antibody complex is detected by means of a biotinylated secondary antibody which is specific for the primary antibody. Enzyme horseradish peroxidase (HRP)-labeled-streptavidin (LAB-SA system) is then added to bind to the biotin residues on the secondary antibody. Localization of the antigen/antibody/enzyme complex is determined by addition of a chromogenic substrate (3,3'- diaminobenzidine techtrachloride (DAB) or aminoethyl carbazole (AEC)). HRP catalyzes the conversion of the soluble substrate into an insoluble chromogen resulting in chromogen deposits (brown for DAB, or red for AEC) within tissue. Hence, the location of the target antigen is determined.

Customers that have used previous lots, please note:

In an attempt to raise the overall quality of our kits, we have implemented a few changes to this product. **Firstly, the procedure for staining with the DAB chromogen has been revised and now uses a two component working solution** (see page 5 for details)**.** Secondly, we have included a heat-induced antigen retrieval step to be used for paraffin-embedded tissues. Thirdly, we have changed the protocol for the dilution of the primary antibody. Finally, we have reformulated the biotinylated secondary antibody and HRP-conjugated streptavidin in order to reduce background.

KIT COMPONENTS

1. 30% hydrogen peroxide solution (2 ml)
2. Normal goat serum blocking solution (15 ml) (thimerosal: 0.2%)
3. Primary antibody (lyophilized powder)
4. Biotinylated secondary antibody solution (15 ml) (thimerosal: 0.2%)
5. Streptavidin-HRP conjugate solution (15 ml) (thimerosal: 0.2%)
6. 40X DAB solution (0.5 ml)
7. 1X DAB substrate buffer for DAB (16.5 ml)
8. 20X AEC solution (1 ml)
9. 20X substrate buffer for AEC (1 ml)
10. 20X hydrogen peroxide (1.5 ml)
11. Mayer's hematoxylin (15 ml)
12. Mounting solution (15 ml)

MATERIALS NOT PROVIDED

1. Xylene (for deparaffinization)
2. Ethanol (for sample rehydration, etc.)
3. Distilled water
4. PBS buffer (0.01 M phosphate buffered saline), pH 7.4
5. TBST (washing steps)
6. Antigen retrieval solution: citrate buffer (0.01M), pH 6.0
   (2.1 gm citric acid monohydrate into 1 L distilled water. Adjust pH to 6.0 with 2N NaOH).
7. Sample tissue slides

IMPORTANT NOTES

1. If unopened and stored at 4^{o}C, the product is stable for one year from production date. Once package has been opened, the expected shelf-life is 3 months.

2. Solutions containing sodium azide or other inhibitors of peroxidase activity should not be used in the staining steps.

3. Do all steps of staining procedure at room temperature except for the primary antibody incubation which we suggest performing overnight at 4°C.

4. Never allow tissue slides to dry. Incubation of slides should be in a moist chamber.

5. Optimal dilution and incubation time of primary antibody should be adjusted depending on the sample preparation for study.

6. The reconstituted primary antibody serum should be stored at 4°C for future use (within two months). For longer term storage, store at -20°C and avoid frequent freeze-thaws.

7. The pH values of DAB and AEC (working conditions) are different (the former is in neutral, and the latter in weak acid). Make sure to add the correct substrate buffer when making the two different substrate-chromogen working solutions.

8. DAB is a suspected carcinogen. Appropriate care should be exercised when using DAB containing solutions including: gloves, eye protection, lab coat, and good laboratory procedures. After completion of the staining procedure, the diluted working solution should be discarded into an equal volume of 3% potassium permanganate ($KMnO_4$), and 2% sodium carbonate in deionized or distilled water. Any containers used in the staining procedure should be washed in the same solution to neutralize the DAB. Dispose in accordance with local and federal regulations.

PREPARATION OF REAGENTS

1. **3% H_2O_2 solution.** (for quenching endogenous peroxidase activity):
   a) For **paraffin embedded tissue** slides: add 1 part of 30% H_2O_2 to 9 parts of ethanol or water.

   b) For **frozen tissue** slides: add 1 part of 30% H_2O_2 to 9 parts of PBS pH 7.4 buffer.

2. **Primary antibody working solution.**
   a) Reconstitute the lyophilized primary antibody with 50 µl of distilled water.

   b) Due to inherent differences in tissue preparation, a dilution series of 1:250, 1:1000, 1:2000, 1:4000, 1:8000 and 1:16000 in normal goat serum blocking solution is strongly recommended to ascertain the optimum antibody dilution, particularly for the initial experiment.

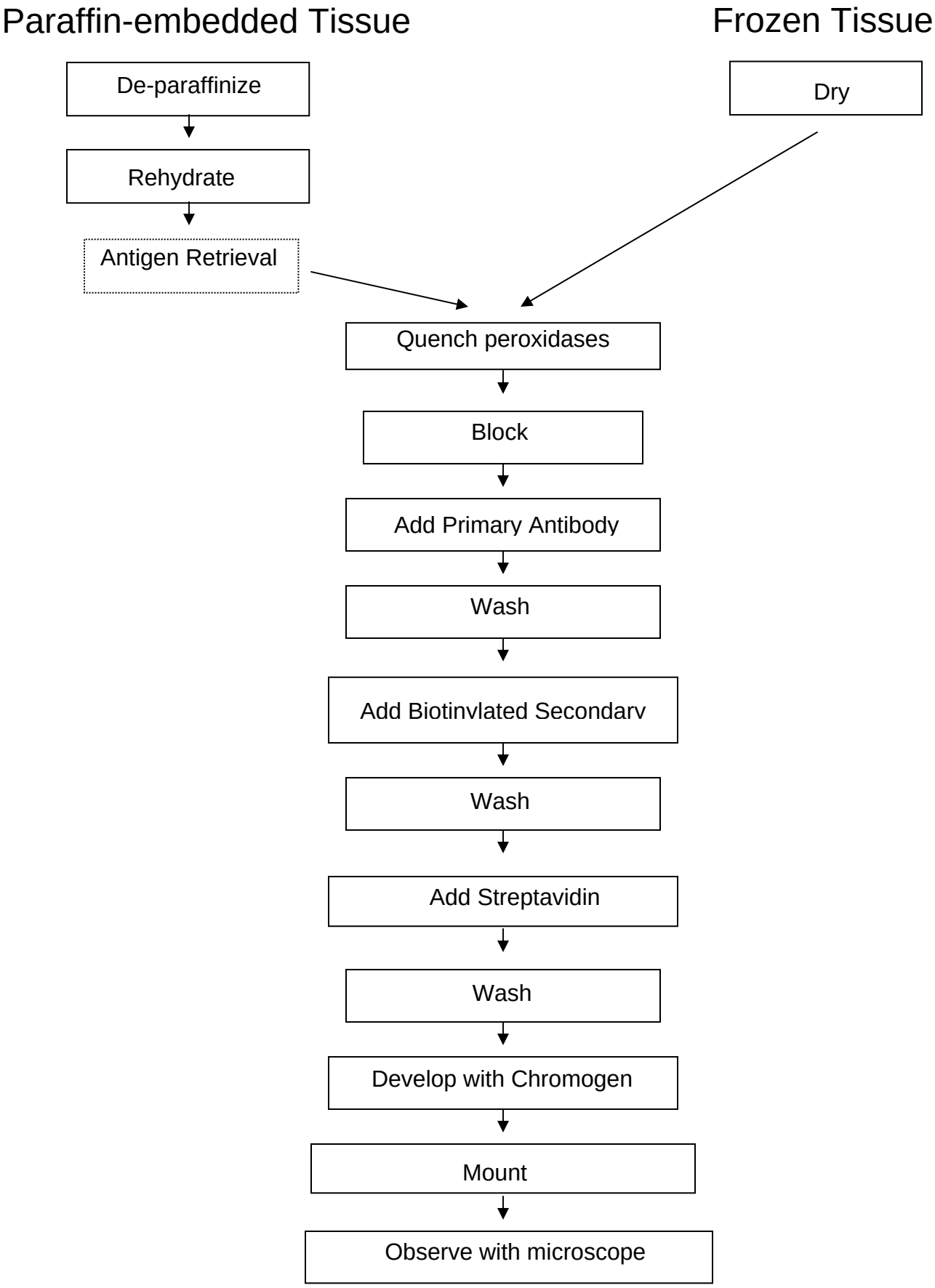
3. **Substrate-chromogen working solution. Note: Our DAB staining procedure has changed. Our kit now uses an improved 2-solution substrate-chromogen working solution.**

   a) To make **DAB** working solution:
      i. Add 25 µl of 40X DAB solution and 1 ml 1X DAB substrate buffer
      ii. Mix well.

   b) To make **AEC** working solution:
      i. Add 1 drop of 20X substrate buffer for AEC to 1 ml distilled water and mix.
      ii. Add 1 drop of 20X AEC solution and 1 drop of 20X hydrogen peroxide.
      iii. Mix well.

Note: All of the above substrate-chromogen mixture working solutions should be kept away from light and used within 30 minutes.

STAINING PROCEDURE OVERVIEW



STAINING PROCEDURE

Please read "IMPORTANT NOTES" on page 4 and "PREPARATION OF REAGENTS" on page 5 before starting.

1. **Prepare sample tissue slides.**
   a) **For <u>paraffin-embedded</u> tissue slides**:
      - **i. De-wax with xylene.**
      - **ii. Re-hydrate in a graded ethanol series.**
      - **iii. Rinse for 5 minutes in tap water.**
      - **iv. Perform heat-induced antigen retrieval (recommended)**
        a. Place slides in a Coplin jar or plastic slide container.
        b. Fill with antigen retrieval buffer (0.01M Citrate buffer, pH 6.0).
        c. Place container in a steamer (rice cooker) with 150 ml of water.
        d. Press “cook” button.
        e. After it’s done (10 to 20 minutes), remove container from cooker and open lid.
        f. Allow to cool for at least 20 minutes.
        g. Wash once with TBST.
      - (Optional) *For quenching of endogenous peroxidase activity, incubate the paraffin-embedded tissue sections for 10 to 30 minutes in 3% H_2O_2 in either ethanol or water.

*In some cases, the antigen may be destroyed by treatment with H_2O_2. If endogenous peroxidase activity does not present a problem, the quenching step may be omitted.

b) **For <u>frozen</u> tissue slides:**

i. **Air dry and treat with 50% ethanol for 20 minutes.**

ii. **Wash with PBS in 3 changes for 2 minutes each.**

- (Optional) *For quenching of endogenous peroxidase activity, incubate the frozen slides for 30 minutes in 3% H_2O_2 in PBS.

2. **Rinse thoroughly with TBST in 3 changes for 2 minutes each at room temperature on shaker.**

3. **Add 2 drops or 100 µl of normal goat serum blocking solution to each slide and incubate for 10 to 20 minutes.** Drain or blot excess solution from the slides. Do not rinse.

4. **Add 2 drops or 100 µl of the diluted primary antibody solution to each slide (please see Preparation of Reagents, Item 2b) and incubate for 60 to 120 minutes at room temperature or overnight at 4°C (recommended).**

5. **Rinse thoroughly with TBST in 3 changes for 2 minutes each at room temperature on a shaker.**

6. **Add 2 drops or 100 µl of biotinylated secondary antibody to each slide and incubate for 30 to 60 minutes.**

7. **Rinse thoroughly with TBST in 3 changes for 2 minutes each at room temperature on a shaker.**

8. **Add 2 drops or 100 µl of streptavadin-HRP conjugate to each slide and incubate for 30 minutes.**

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* In some cases, the antigen may be destroyed by treatment with H_2O_2. If endogenous peroxidase activity does not present a problem, the quenching step may be omitted.

9. **Rinse thoroughly with TBST in 3 changes for 2 minutes each at room temperature on a shaker.**

10. **Add 100 µl of substrate-chromogen mixture working solution to each slide.** Color development should be monitored under a microscope. Generally, the development times are 1 to 3 minutes for DAB and 3 to 5 minutes for AEC. Rinse well with distilled water.

11. **Counterstain the slides with 2 drops or 100 µl of Mayer's hematoxylin and incubate for 1 to 3 minutes.**

12. **Wash slides in tap water.**

13. **Immerse slides into PBS (pH 7.4) until blue (about 30 seconds).**

14. **Rinse well with distilled water.**

15. **Add 2 drops or 100 µl of mounting solution to the slide and mount with coverslip.**

TROUBLESHOOTING

1. Possible causes of overstaining:

a) Too high of a concentration of primary antibody.
b) Incubation time too long.
c) Concentration of linker or label reagents too high.
d) Reaction temperature too high.
e) Substrate incubation time too long.

2. Possible causes of weak staining:

a) Too much rinse buffer left on slides causing excessive dilution of reagents.
b) Concentration of antibody or linker too low or incubation time too short.
c) Incomplete deparaffinization
d) Improper substrate preparation (i.e. contaminated by sodium azide or other inhibitor of peroxidase) or substrate too old.
e) Incompatible counterstain or mounting media that dissolves reaction product.

3. Possible causes of zero staining:

a) Primary antibody does not recognize antigen.
b) Antigen not present in tissue.
c) Tissue dried out during staining protocol.

4. Possible causes of high background:

a) Endogenous peroxidase activity not blocked.
b) Nonspecific protein binding or endogenous biotin in tissue.
c) Incomplete de-waxing.
d) Inadequate rinsing of slides.
e) Over development of substrate.
f) Tissue dried out during staining protocol.

GUARANTEE AND LIMITATION OF REMEDY

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