Immunohistochemistry PROTOCOLS

Note: The researcher should optimize the precise conditions for a particular assay.

Preparation Of Paraffin-Embedded Tissue Sections

1. Sectioning

Tissue sections (5-7 µm thick) are cut from paraffin-embedded blocks on a microtome and mounted from warm water (40°C) onto adhesive microscope slides. Sections are allowed to dry overnight at room temperature or 40°C. **CAUTION: ANTIGENS MAY BE DESTROYED BY EXPOSURE TO HIGH TEMPERATURES**. (Adhesion of the section to the slide is essential to prevent tissue loss during subsequent incubations and washes).

2. Deparaffinization

Deparaffinize and rehydrate tissue sections using standard methods. It is necessary to completely remove the embedding material before immunostaining. The xylene and alcohol bath solutions should be replaced frequently.

Suggested Reagents and Times for Deparaffinization

• Xylene	➢ 2 to 3 changes, 5 minutes each
• 100% alcohol	➢ 2 changes, 3 minutes each
• 95% alcohol	➢ 2 changes, 3 minutes each
• 80% alcohol	➢ 3 minutes
• 70% alcohol	➢ 3 minutes
• Phosphate buffered saline or Tris buffer	➢ 2 changes, 3 minutes each

PRECAUTION: Once the tissue sections have been rehydrated, do not allow them to dry. Dry the slide around the tissue section with an absorbent wipe. Using a diamond pencil, china marking pencil or nail polish, draw a circle on the microscope slide around the section. This circle will help retain solution on the section during subsequent incubations with reagents.

3. Immunostaining Protocol

1. Tap off excess buffer and wipe around the tissue section with an absorbent wipe. **CAUTION: DO NOT TOUCH THE TISSUE OR ALLOW IT TO DRY.**
2. Antigen retrieval. The fixation and processing steps involved in the preparation of tissues often results in loss of immunoreactivity of antigens. This may be reversed by the use of appropriate antigen retrieval techniques. This is an optional step, see Antigen retrieval in Appendix.
3. Immerse slides into 0.3-3% H_2O_2 and 100% methanol for 10 mins at RT to quench endogenous peroxidase.
4. Wash slides in cold buffer for 5 to 10 minutes. Repeat wash.
5. Apply 1 - 2 drops of pre-blocking agent to the tissue section and allow it to remain in place for 10 to 20 minutes or according to the manufacturer's instructions (see ***Pre-blocking Agents in Appendix***).
6. Tap off the pre-blocking agent and wipe away any excess around the tissue. **CAUTION: DO NOT TOUCH THE TISSUE OR ALLOW IT TO DRY.**
7. Block biotin/avidin (optional, ***see Pre-blocking Agents in Appendix***).
8. Tap off the blocking agents and wipe away any excess around the tissue.
9. Cover each section with diluted primary antibody or negative control. Incubate the slides at room temperature for 1 hour or 4°C for 12 to 24 hours in a closed incubation chamber. *OPTIMAL conditions must be determined by the researcher*.
10. Remove the slides from the incubation chamber and rinse the excess antibody from the slide with a gentle stream of cold PBS or Tris buffer from a wash bottle. Immerse sections in cold buffer for 5 to 10 minutes. Repeat wash.
11. Blot the excess buffer from the slide with an absorbent wipe. **CAUTION: DO NOT TOUCH THE TISSUE OR ALLOW IT TO DRY.** Cover each section with diluted secondary antibody. Incubate the slides in an incubation chamber at room temperature for 1 hour. *OPTIMAL conditions must be determined by the researcher*.
12. Remove the slides from the incubation chamber and rinse the excess antibody from the tissue with a gentle stream of room temperature PBS or Tris buffer. Immerse sections in cold buffer for 5 to 10 minutes. Repeat wash.
13. If using biotinylated secondary antibodies, repeat step 12 using SA-HRP reagent diluted PBS. Incubate 30 minutes at room temperature.
14. Immerse the tissue sections in a freshly prepared chromogen/substrate reagent solution such as DAB/H_2O_2 or AEC/H_2O_2 for 2 to 7 minutes. Remove slides periodically to check for staining development with a low power microscope. Stop stain by rinsing with PBS or TBS.
15. If organic-based mounting media is to be used, dehydrate sections with increasing concentrations of ethanol followed by xylene as in conventional histology. **CAUTION: AEC IS ORGANICALLY SOLUBLE, DO NOT DEHYDRATE SECTIONS.**

4. Appendix

Reagent Materials Required

- Primary antibody for immunohistochemistry
- Secondary antibody: an antibody, conjugated with biotin or HRP, against a species immunoglobulin that matches the host species of the primary antibody.
- Streptavidin-HRP: This enzyme conjugate is needed if a biotinylated secondary antibody is used.
- Negative or isotype Control: Normal serum from the same animal or species providing the primary antiserum or the same immunoglobulin isotype. Prepare same as primary antiserum; use same diluent and working dilution.
- Antisera Diluent: Use to dilute primary and secondary antisera; Phosphate buffered saline (PBS), pH 7.4 or Tris/Saline buffer (TBS), pH 7.6. In some cases, 0.25% bovine serum albumin may be added to reduce background.
- Antigen retrieval Methods: Certain paraffin-embedded sections need to have an antigen retrieval procedure to uncover antigenic sites from tissue sections. In many cases incubation times will vary depending upon the particular processing method that has been used, or for different antigens.
- These methods provide a general procedure for use with the majority of ***ImmunoKontact*** reagents. In some cases specific recommendations are provided on product datasheets, and these methods should always be used in conjunction with product and batch specific information provided with each vial. The researcher must optimise for the conditions of concentration, time and temperature. Commonly antigen retrieval methods are indicated below.

Proteolytic antigen retrieval using Trypsin

Reagents:

Calcium Chloride 0.1g
Trypsin (Sigma Type II) 0.1g
Distilled Water 100ml
Sodium Hydroxide (0.1M)

Method:

1. Dissolve calcium chloride in distilled water and pH to 7.8 with sodium hydroxide. Store at 37°C.
2. Dissolve trypsin in calcium chloride solution.
3. Place sections in Trypsin solution at 37°C and incubate for pre-determined optimum time (approx. 20-30 mins).
4. Wash in TBS and proceed with staining.

Proteolytic antigen retrieval using pronase

Reagents:

Calcium Chloride 0.1g
Pronase 0.1g
Distilled Water 100ml
Sodium Hydroxide (0.1M)

Method:

5. Dissolve calcium chloride in distilled water.
6. Dissolve pronase in calcium chloride solution and pH to 7.8 with sodium hydroxide.
7. Place sections in pronase solution at room temperature and incubate for pre-determined optimum time (approx. 10 mins).
8. Wash in TBS and proceed with staining.

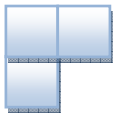
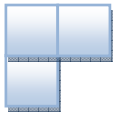
Heat Treatment using citrate buffer

Reagents

0.01M citrate buffer pH 6.0

Method:

9. Place slides in suitable container of prepared citrate buffer, cover with clingfilm (vented) and heat to 90oC (Please note citrate buffer must boil). If the STUF/buffer evaporates after 5 minutes, top up with hot distilled water.
10. Let sections stand for 15 minutes to cool.
11. Wash in TBS and proceed with staining.



- Pre-blocking Agents: A reagent solution that serves to block nonspecific antigenic sites found on tissue.
  - H_2O_2 methanol blocking solution: Contains 0.3-3% H_2O_2 and 100% methanol to block endogenous peroxidase.
  - Normal serum blocking solution: Contains normal serum from host species providing second antibody. 1-10% normal serum in combination with 0.25% bovine serum albumin in PBS pH 7.4 or Tris buffer pH 7.6. Block may contain detergent if antigens are internal (0.1-0.3% triton X-100 are typical).
  - Biotin/Avidin blocking solution: It is necessary to use a commercially available kit to block endogenous biotin and avidin when using a biotinylated secondary antibody on certain tissues that are rich in biotin and avidin such as liver, kidney and small intestine.
- Rinsing/Washing Buffer
  - PBS, pH 7.4
  - Tris buffer pH 7.6
- Chromogen
  - DAB dissolved in Tris buffer pH 7.6
  - AEC dissolved in acetate buffer. CAUTION: the chromogens are carcinogenic; please handle and dispose of in an appropriate manner in compliance with local and federal regulations
- Substrate reagent
  - Hydrogen peroxide
- Dehydration reagents (for DAB). Ethanol baths of increasing concentration: 70, 80, 95, and 100% ethanol and xylene baths
- Mounting Media
  Water-based mounting media (can be used with both DAB and AEC).
  Permanent organic-based mounting media (for DAB only). Tissue must be dehydrated prior to use.