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TECHNICAL DATA SHEET 194A

Peroxidase-Anti-Peroxidase

Page 1 of 3

Procedures and References

There are many variations for the PAP method. We are offering these protocols as a guide and as a convenience. Specific situations may require alterations to one or more of the given procedures. Please see Data Sheet #194 for additional information.

PAP staining for Light Microscopy (LM) is best performed on freshly deparaffinized sections. JB-4™*, Immuno-Bed*, and other acrylic embedding materials can be used after etching or treating with Poly/Solv*. Staining for Electron Microscopy (EM) can be performed before embedding or directly on ultra-thin epoxy sections.

All undiluted antisera and wash solutions should be sterile filtered whenever they are used for EM. Sera, frozen after filtration, do not have to be refiltered upon thawing. Filtration is not necessary for LM PAP procedures. The PAP reagent should *never* be filtered even when it is used for EM.

All antisera, including PAP, are diluted in 0.05 M Tris Buffer* at pH = 7.6 and should contain 1% of normal serum from the species that donates the secondary antiserum.

Phosphate buffered saline (PBS), Tris-saline 1:10 buffer and 0.85% buffered saline have also been used as buffers for PAP procedures. Undiluted PAP is best stored, quick-frozen, in small aliquots. Once diluted, PAP should not be reused.

LM Staining:

3-5 drops of reagents are placed directly on the sections in each step:

1. Deparaffinize fixed section(s) and bring through graded alcohol to water. Carefully blot off excess water with absorbent paper without touching sections.
2. Block endogenous peroxidase activity with 0.3% Hydrogen Peroxide (diluted with buffer).
3. Rinse with buffer and place in buffer bath for 5 minutes. Blot dry as described in step 1.
4. Block background staining by adding normal serum from animal donating the secondary antibody. Incubate for 20 minutes.
5. Decant off sera and add primary antisera diluted as described above. Incubate overnight at 4°C or for 20-60 minutes at room temperature using a lower dilution.
6. Rinse three times with buffer - five minutes for each rinse. Blot as described above.
7. Add secondary antiserum. Incubate for 20 minutes.
8. Rinse three times with buffer. Blot as described above.
9. Add PAP. Incubate for 20 minutes.
10. Rinse with buffer and place in buffer bath for 10 minutes. Blot dry as described above.
11. Apply substrate mixture (see below) to get colored end product.
12. Counterstain with hematoxylin* or other nuclear stain and coverslip.

Pre-embedment Staining for EM:

Place staining and wash solutions in small tubes. Vibratome®* sections are wrapped around the broken end of a wooden applicator for the transfer from tube to tube. Use tweezers* for transferring normal blocks. Procedure is identical to that used for LM staining.

After staining with substrate and H₂O₂, fix tissue in 4% OsO₄* for thirty minutes and embed in epoxy (example: Poly/Bed*, Araldite*).

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Post-embedment Staining for EM:

Place sections on nickel grids*. Except for steps 1 and 8-10 below, stain by floating grids on two drops of solution held in shallow depressions in parafilm* in covered petri dishes. Wash using a stream from a plastic wash bottle. Blot grids dry by edgewise contact with Whatman No. 4 filter paper.

1. 3% of H₂O₂ on glass slides, covered for three minutes. Wash with buffer.
2. 3% of normal goat serum - 5 minutes. Blot, but don't wash.
3. Primary antiserum - 48 hours at 2-5°C. Return to room temperature for last 1 or 2 hours. Seal grids* into petri dishes with parafilm* or teflon tape for this incubation to prevent drying out. Wash with buffer. Blot.
4. 3% of normal serum - 5 minutes. Blot, but don't wash.
5. Secondary antisera - 5 minutes. Wash with buffer, but don't blot.
6. 3% of normal serum - 5 minutes. Blot, but don't wash.
7. PAP - 5 minutes. Wash with buffer, but don't blot.
8. Substrate mixture (see below) to get colored end product.
9. Fix in 4% of OsO₄* - 30 to 60 minutes.

Substrate Solutions - Electron Donors:

Diaminobenzidine Tetrahydrochloride (DAB•4HCl)*: Prepare immediately before use - for LM - 0.05% of DAB•4HCl, 0.01% of H₂O₂ in buffer (0.05 M Tris Buffer*, pH = 7.6) for 8 minutes or until specific staining is visible and the background is barely visible. Never stain for longer than 8 minutes. Wash three times in water for two minutes each time. For EM, suspend grids* with forceps for three minutes in beaker* containing 0.0125% of DAB•4HCl and 0.0025% of H₂O₂ in buffer (we suggest 0.05M Tris Buffer* at pH = 7.6). Stir solution using a magnetic stirrer. Suspend grids* in water for 30 minutes under agitation. Wash each grid with water from a spray bottle. Please write for Data Sheet #164 for more information.

3-Amino-9-ethylcarbazole (AEC)*: Dissolve 20mg of AEC into 0.5ml of N,N-dimethylformamide*. Add 9.5ml of 0.05 M Acetate Buffer (pH = 5.0). Add three drops of 3% H₂O₂. Acetate Buffer - 21ml of 0.05 N acetic acid* and 79ml of 0.05 M sodium acetate*. Produces a red, water soluble reaction product.

4-Chloro-1-naphthol (CN)*: Dissolve 3mg of CN in 0.5ml of 100% Ethanol. Add 9.5ml of Tris-HCl Buffer. Add three drops of H₂O₂. The result will be a blue end product which should be photographed immediately because it diffuses rapidly.

Hanker-Yates Reagent*: Please write for Data Sheet #204.

Other Electron Donors*: 2,2'-Azino-di-(3-ethylbenzylthiazoline sulfonic acid)-6-diammonium salt*, Guaiacol (syn: o-Methoxyphenol), 4-Hydroxyphenol acetic acid, 3-methyl-2-benzothiazoline hydrazone HCl (MBTH), and 3-(p-Hydroxyphenyl) propionic acid have been demonstrated as useful substitutes for DAB•4HCl. Please see the references given below and current journals for additional information.

We highly recommend the following resources be studied before attempting these procedures:

- "Manual of Immunoperoxidase Techniques"* , R.J. Wordinger, et al., American Society of Clinical Pathologists Press, Chicago, IL, USA, 9182.
- "Autoradiography and Immunocytochemistry"* , M.A. Williams, North-Holland Publishing Company, New York City, NY, USA, 1977.
- "Immunohistochemistry", A.C. Cuello, ed., Wiley and Sons, New York City, NY, USA, 1983.
- "Immunocytochemistry: Practical Applications in Pathology and Biology", J.M. Polak and S. Van Noorden, eds., John Wright and Sons, Bristol, UK, 1983.
- "Immunochemistry in Practice", A. Johnstone and R. Thorpe, Blackwell Scientific Publications, Oxford, UK, 1982.

*Available from Polysciences, Inc.

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