

# Peroxidase-Anti-Peroxidase

## *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 Technical 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. 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 H<sub>2</sub>O<sub>2</sub>, fix tissue in 4% OsO<sub>4</sub> for thirty minutes and embed in epoxy (*example: Poly/Bed, Araldite*).

## Post-embedding 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 filter paper.

1. 3% of H<sub>2</sub>O<sub>2</sub> 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 OsO<sub>4</sub> - 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 H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub> 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 see Technical 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% H<sub>2</sub>O<sub>2</sub>. 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 H<sub>2</sub>O<sub>2</sub>. The result will be a blue end product which should be photographed immediately because it diffuses rapidly.

**Hanker-Yates Reagent** Please see Technical 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, 1982.

Autoradiography and Immunocytochemistry, M.A. Williams, North-Holland Publishing Company, New York City, NY, USA, 1977.

Immunohistochemistry, A.C. Cuellar, 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.

## Reference Bibliography

*References selected primarily for their method aspects.*

1. Baker, B.L., Dermody, W.C. and Reel, J.R., 1975, Distribution of gonadotropin-releasing hormone in the rat brain as observed with immunocytochemistry. *Endocrinology*, 97, 125-135.
2. Bocker, W., 1974, Use of a triple layer enzyme method as an alternative to immunofluorescence for detection of tissue antigens. *Beitr. Path.*, 153, 410-414.
3. Brooks, J.J., 1982, Immunohistochemistry of soft tissue tumors. *Hum. Path.*, 13 (11), 969-974.
4. Burns, A., 1975, Background staining and sensitivity of the unlabeled antibody-enzyme (PAP) method. Comparison with peroxidase-labeled sandwich methods using formalin-fixed paraffin embedded material. *Histochemistry*, 43, 291-294.
5. Dell'Orto, P., et al., 1982, Immunohistochemical localization of immunoglobulins and lysozyme in epoxy-embedded lymph nodes. *J. Histochem. and Cytochem.*, 30 (7), 630-636.
6. Erlandsen, S.L., Parsons, J.A., Burke, J.P., Redlick, J.A., Van Orden, D.E., and Van Orden, L.S., 1975, A modification of the unlabeled antibody enzyme method using heterologous antisera for the light microscope and ultrastructural localization of insulin, glucagon, and growth hormone. *J. Histochem. and Cytochem.*, 23, 666-677.
7. Halmi, N.S. and Duello, T., 1976, "Acidophilic" pituitary tumors. *Arch. Path. Lab. Med.*, 100, 346-351.
8. Marucci, A.A. and Dougherty, R.M., 1975, Use of the unlabeled antibody immunohistochemical technique for the detection of human antibody. *J. Histochem. Cytochem.* 23, 618-623.
9. Mason, D.Y., Farrel, C. and Taylor, C.R. 1975, The detection of intracellular antigens in human leucocytes by immunoperoxidase staining. *Brit. J. Haematol.*, 31, 361-369.
10. Maynard, J.H. 1984, Rapid immunoperoxidase staining. *J. Histotech.*, 7 (4), 187-189.
11. Moriarty, G.C., Moriarty, C.M. and Sternberger, L.A., 1973, Ultrastructural immunocytochemistry with unlabeled antibodies and the peroxidase-anti-peroxidase complex. A technique more sensitive than radioimmunoassay. *J. Histochem. Cytochem.*, 21, 825-833.
12. Palmer, P.E., Delellis, R.E. and Wolfe, H.J., 1974, Immunohistochemistry of liver in  $\alpha_1$ -antitrypsin deficiency. A comparative study. *Am. J. Clin. Path.*, 62, 350-354.
13. Pedraza, M.A., et al., 1984, Immunoperoxidase methods with plastic embedded materials. *Lab. Med.*, 15 (2), 113-115.
14. Pelliniem, L.J. and Van Noorden, S., 1977, A streamlined technique for on-grid electron immunocytochemistry. *Histochem.*, 53, 271-272.
15. Petrali, J.P., Hinton, D.M., Moriarty, G.C. and Sternberger, L.A., 1974, The unlabeled antibody enzyme method of immunocytochemistry. Quantitative comparison of sensitivities with and without peroxidase-anti-peroxidase complex. *J. Histochem. Cytochem.*, 22, 782-801.
16. Pickel, V.M., Joh, T.H. and Reis, D.J., 1975, Ultrastructural localization of tyrosine hydroxylase in noradrenergic neurons of brain. *Proc. Nat. Acad. Sci. USA*, 72, 659-663.
17. Said, J.W., 1983, Immunohistochemical localization of keratin proteins in tumor diagnosis. *Hum. Path.*, 14 (12), 1017-1019.
18. Silverman, A.J. and Desnoyers, P., 1976, Ultrastructural immunocytochemical localization of luteinizing hormone-releasing hormone (LH-RH) in the median eminence of the guinea pig. *Cell. Tiss. Res.*, 169, 157-166.
19. Sternberger, L.A., 1974, "Immunocytochemistry", Prentice-Hall, Englewood Cliffs, NJ, USA.
20. Sternberger, L.A., Hardy, P.H., Jr., Cuculis, J.J. and Meyer, H.G., 1970, The unlabeled antibody enzyme method of immunohistochemistry. Preparation and properties of soluble antigen-antibody complex (horseradish peroxidase-anti-horseradish peroxidase) and its use in identification of spirochetes. *J. Histochem. Cytochem.*, 18, 315-353.
21. Aternberger, L.A. and Petrali, J.P., 1975, Quantitative immunocytochemistry of pituitary receptors for luteinizing hormone-releasing hormone. *Cell Tiss. Res.*, 162, 141-176.
22. Vandesande, F. and Dierickx, K., 1975, Identification of the vasopressin producing and of the oxytocin producing neurons in the hypothalamic magnocellular neurosecretory system of rat. *Cell Tiss. Res.*, 164, 153-162.

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