INSTRUCTIONS

ImmunoHisto™ Peroxidase Detection Kit

PIERCE
3747 N. Meridian Road
P.O. Box 117
Rockford, IL 61105

36000

1493.0

Number	Description
36000	**ImmunoHisto™ Peroxidase Detection Kit,** contains sufficient reagents to stain 250-500 tissue sections

Kit Contents:

DAB/Metal Concentrate (10X), 25 ml, store at -20°C (this solution will not freeze at -20°C)

Peroxidase Detection Reagent Pack:

Universal Blocker™ Blocking Buffer in TBS, 250 ml, store at 4°C

ImmunoPure® Peroxidase Suppressor, 2 x 100 ml, store at 4°C

Stable Peroxide Buffer, 250 ml, store at 4°C

BupH™ Tris Buffered Saline, 4 packs (each pack makes 500 ml), store at room temperature

Surfact-Amps® 20 (10% Tween®-20), 10 ml, store at 4°C

Harris Modified Hematoxylin (without acetic acid, mercury-free), 100 ml, store at room temperature

Mounting Medium, 60 ml, store at room temperature

Storage: Upon receipt store individual components as indicated above. The DAB/Metal Concentrate is shipped with an ice pack. The Peroxidase Detection Reagent Pack is shipped at ambient temperature.

Table of Contents

Introduction

The ImmunoHisto™ Peroxidase Detection Kit is a complete system for staining, counterstaining and preserving histological and cytological samples using the Metal Enhanced DAB Substrate for colorimetric detection. Immunohistochemical staining makes it possible to visualize the distribution and localization of specific cellular components within a cell or tissue. Identification of tissue components is achieved using a primary antibody that is specific for the antigen and a horseradish peroxidase-conjugated secondary antibody. Alternatively, a biotinylated secondary antibody and HRP-labeled avidin or streptavidin may be used. Detection is accomplished when the enzyme label is reacted with the Metal Enhanced DAB substrate to yield an intensely colored product that can be analyzed by light microscopy. The Metal Enhanced DAB substrate is a special formulation of cobalt chloride and nickel chloride that produces a dark brown/black precipitate in the presence of HRP. Hematoxylin, a purple nuclear stain, is also included in this kit to counterstain tissue sections, which may improve contrast between background and the colored reaction products.

Important Product Information

- Store the DAB/Metal Concentrate at or below -20°C. This solution is packaged under nitrogen for long-term stability. To use the solution, remove quantity required for use and immediately return bottle to -20°C. Do not allow solution to equilibrate to room temperature. For long-term storage (i.e., >6 months), replace nitrogen by gently bubbling a slow stream of nitrogen into the solution.
- The Stable Peroxide Buffer contains the optimal concentration of hydrogen peroxide. Do not add hydrogen peroxide to the Stable Peroxide Buffer as this will increase background. Storage at -20°C or repeated freeze/thawing will not affect activity of the Stable Peroxide Buffer. For convenience, store the product at 4°C to eliminate the need to thaw.
- To minimize potential microbial contamination, carefully handle the kit reagents and use ultrapure water in all solutions.
- Do not use sodium azide as a preservative for buffers. Sodium azide inhibits HRP activity and could interfere with this system.
- Discard diluted and used solutions along with excess buffer after use.
- Use a humidity chamber set at 20-25°C for all incubations to prevent evaporation. Additionally, completely cover the tissue section with solution during incubations to prevent drying.
- Avoid touching slides and do not allow dust or other debris to contaminate samples, tissues or other material.
- Use acid-cleaned slides. Do not use egg albumin-dipped slides because trace avidin present in the albumin may cause background.
- Adjust the standard protocol when antigen concentrations are too high or low. High antigen concentrations will require less incubation time to obtain optimal staining. To shorten incubation times, increase incubation temperature to 37°C.
- An ABC complex system such as the ImmunoPure® ABC Standard Peroxidase Staining Kit (Product No. 32020) or ImmunoPure® Ultra-Sensitive ABC Standard Peroxidase Staining Kit (Product No. 32050) can increase sensitivity if necessary.

Procedure for Staining Paraffin-Embedded Tissue Section Slides

NOTE: Please read all instructions before beginning the procedure.

A. Materials Required

- Wash Buffer (Tris buffered saline with 0.05% Tween®-20): Dissolve contents of one BupH™ TBS Pack in 500 ml of ultrapure water. Add 2.5 ml of 10% Tween®-20 and mix well. Store reconstituted buffer at 4°C.
- Primary Antibody: Dilute antibody with Universal Blocker™ Blocking Buffer. For best results, empirically determine the optimal dilution for each specific tissue/antigen type being tested. Typical primary antibody dilutions range from 1:5 to 1:100. Amplification methods, such as the ABC Complex, will typically require less primary antibody.
- Biotinylated Secondary Antibody (an HRP-conjugated secondary antibody also may be used): Dilute antibody with Universal Blocker™ Blocking Buffer. For best results, empirically determine the optimal dilution for each specific system being tested. Typical secondary antibody dilutions range from 1:100 to 1:1,000.
- Avidin-HRP or Streptavidin HRP: Dilute conjugate with Universal Blocker™ Blocking Buffer. For best results, empirically determine the optimal dilution for each specific system being tested. Typical conjugate dilutions range from 1:500 to 1:1,000.

  Note: If using an HRP-conjugated secondary antibody, Avidin-HRP or Streptavidin HRP is not needed.
- Coverslips

B. Method

1. Prepare paraffin-embedded tissue section slides according to standard protocols. (See the Additional Information Section for protocol to de-paraffinize embedded tissues.)
2. Quench endogenous peroxidase activity by incubating tissue for 30 minutes in Peroxidase Suppressor.

   Note: Omit this step if endogenous activity is not a problem or if the antigen will not survive exposure to H_2O_2.
3. Wash slide two times for 3 minutes with Wash Buffer.
4. Add blocking buffer to the slide and incubate for 30 minutes.
5. Blot excess blocker from the tissue sections. Apply the Primary Antibody and incubate tissue for 30 minutes.
6. Wash slide two times for 3 minutes with Wash Buffer.
7. Apply the Biotinylated Secondary Antibody and incubate tissue for 30 minutes. If using an HRP-conjugated secondary antibody, skip to step 10.
8. Wash slides three times for 3 minutes each with Wash Buffer.
9. Incubate the tissue section with the Avidin/Streptavidin-HRP for 30 minutes.
10. Wash slides three times for 3 minutes each with Wash Buffer.
11. Remove the DAB/Metal Concentrate (10X) from -20°C storage and mix well by inverting the bottle. Remove quantity required for use and immediately return bottle to -20°C.
12. Prepare a 1X working solution of the DAB/Metal Concentrate (10X) by adding the Stable Peroxide Buffer and mixing well. For example, to prepare 5 ml of substrate, add 4.5 ml of the Stable Peroxide Buffer to 500 µl of the DAB/Metal Concentrate.

    Note: The 1X substrate solution is stable for several hours at 4°C.
13. Add the Metal Enhanced DAB Substrate Working Solution to the tissue and incubate until the desired staining is achieved. Typical incubations are from 5 to 15 minutes.

    Note: Thick sections may require longer staining times.
14. Wash slide two times for 3 minutes each with Wash Buffer. Counterstain if desired (see Section C).
15. Mount slide with Mounting Media (see Section D).

    Note: The time required to perform this protocol may be reduced with a slight loss in sensitivity by performing the following alterations:

    - Shorten the incubation steps with the primary antibody, the biotinylated secondary antibody and the streptavidin-HRP to 10 minutes each.
    - Instead of incubating washes, rinse tissue with a gentle stream of Wash Buffer several times at each wash step.
    - Because the primary antibody is prepared in Blocking Buffer, combine the incubation steps with the Blocking Buffer and the Primary Antibody.
    - Increase the concentration of the primary antibody and decrease the incubation times to 2 minutes. Optimization of the concentrations may be required.

C. Counterstaining

1. After immunostaining, rinse the slides with distilled water and drain.
2. Add an adequate amount of the hematoxylin stain to the slide to cover the entire tissue surface.
3. Incubate for 1-2 minutes at room temperature.
4. Drain off the hematoxylin and wash slide several times with distilled water.
5. Wash slide in Wash Buffer for 1 minute.
6. Wash slide with distilled water.
7. Mount slide with Mounting Media.

D. Mounting

1. Make sure tissue sections are dry. Place all slides to be mounted on a flat surface and apply 1-3 drops of Mounting Medium to the tissue section.
2. Apply a coverslip on top of the Mounting Medium.
3. Place the slides on a flat surface to set for 1-2 hours at room temperature.

Troubleshooting

Problem	Possible Cause	Solution
Precipitate is brown instead of black/brown	Cobalt and nickel are heavy metals and will separate during storage	Mix by inverting the bottle before use to obtain a homogeneous solution of DAB and metals
Background is dark, reducing the signal-to-noise ratio	DAB/Metal Concentrate was not maintained at -20°C	Store the DAB/Metal Concentrate at or below -20°C to prevent background problems
The DAB/Metal Concentrate contains a precipitate in the bottle	DAB/Metal Concentrate was not maintained at -20°C	If the precipitate does not go into solution upon mixing, do not use the substrate
High background	Too much HRP in the system	Use less antibody – this substrate is 50 times more sensitive than DAB without metals and requires much less antibody for detection

Additional Information

Procedure for De-paraffinizing Embedded Tissues

1. Incubate slides for 5 minutes in clean xylene;* repeat once.
2. Incubate slides for 5 minutes in 100% ethanol; repeat once.
3. Incubate slides for 5 minutes in 95% ethanol: 5% water; repeat once.
4. Incubate slides for 5 minutes in 85% ethanol: 20% water.
5. Incubate slides for 5 minutes in 70% ethanol: 20% water.
6. Incubate slides for 5 minutes in 50% ethanol: 20% water.
7. Incubate slides for 5 minutes in 30% ethanol: 70% water.
8. Incubate slides for 5 minutes in ultrapure water; repeat once.
9. The slides are now ready for immunohistochemistry. Do not allow slides to dry. Store slides in wash buffer until ready to use.

*For researchers who prefer to avoid organic solvents, Fisherbrand™ Citrisolv™ Clearing Agent can be used as a substitute.

Related Pierce Products

35000 **ImmunoPure® Peroxidase Suppressor,** 100 ml

28376 **BupH™ Tris Buffered Saline Packs,** 40 packs

28320 **Surfact-Amps®-20 (10% Tween®-20),** 6 x 10 ml

34065 **Metal Enhanced DAB Substrate Kit,** contains 10X Metal Enhanced DAB concentrate and 1X Stable Peroxide Buffer

32020 **ImmunoPure® ABC Standard Peroxidase Staining Kit,** contains Avidin and Biotinylated HRP

32028 **ImmunoPure® ABC Peroxidase Mouse IgG Staining Kit,** contains Avidin**,** Biotinylated Peroxidase, Blocking Buffer, Biotinylated Affinity-purified Horse Anti-Mouse IgG and Mixing bottles with drop dispenser tips

32032	**ImmunoPure® ABC Peroxidase Rabbit IgG Staining Kit,** contains Avidin, Biotinylated Peroxidase, Blocking Buffer, Biotinylated Affinity-purified Goat Anti-Rabbit IgG and mixing bottles with drop dispenser tips
32050	**ImmunoPure® Ultra-Sensitive ABC Standard Peroxidase Staining Kit,** contains Avidin and Biotinylated HRP
32052	**ImmunoPure® Ultra-Sensitive ABC Peroxidase Mouse IgG Staining Kit,** contains Avidin**,** Biotinylated Peroxidase, Blocking Buffer, Biotinylated Affinity-purified Horse Anti-Mouse IgG and Mixing bottles with drop dispenser tips
32054	**ImmunoPure® Ultra-Sensitive ABC Peroxidase Rabbit IgG Staining Kit,** contains Avidin, Biotinylated Peroxidase, Blocking Buffer, Biotinylated Affinity-purified Goat Anti-Rabbit IgG and mixing bottles with drop dispenser tips

Metal Enhanced DAB References

Chan, J.R., *et al.* (2000). Progesterone synthesized by Schwann cells during myelin formation regulates neuronal gene expression. *Mol. Biol. Cell.* **11:**2283-95.

Itoh, H. (2000). Upregulation of HGF activator inhibitor type 1 but not type 2 along with regeneration of intestinal mucosa. *Am. J. Physiol. Gastrointest. Liver Physiol.* **278:**635-43.

Kaushic, C., *et al*. (2000). Effects of estradiol and progesterone on susceptibility and early imune responses to *Chlamydia trachomatis* infection in the female reproductive tract. *Infec. Immunity* **7:**4207-16.

Martin, M.N. and Slovin, J.P. (2000). Purified γ-glutamyl transpeptidases from tomato exhibit high affinity for glutathione and glutathione S-conjugates. *Plant Physiol.* **122:**1417-26.

Scidmore, M.A., *et al.* (2003). Restricted fusion of *Chlamydia trachomatis* vesicles with endocytic compartments during the initial stages of infection. *Infec. Immunity* **2:**973-84.

General References

Adams, J.C. (1977). Technical considerations on the use of horseradish peroxidase as a neuronal marker. *Neuroscience* **2:**141-5.

Adams, J.C. (1981). Heavy metal intensification of DAB-based HRP reaction product. *J. Histochem. Cytochem*. **29:**775.

De Jong, A.S.H., *et al.* (1985). Sensitivity of various visualization methods for peroxidase and alkaline phosphatase activity in immunoenzyme histochemistry. *J. Histochem*. **17:**1119-30.

Graham, R.C. and Karnovsky, M.J. (1966). The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem*. **14:**291-302.

Hsu, S-M., *et al.* (1981). A comparative study of the peroxidase-anti-peroxidase method and avidin-biotin complex method for studying polypeptide hormones with radioimmunoassay antibodies. *Am. J. Clin. Pathol,* **75:**734-8.

Hsu, S-M., *et al.* (1981). Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: A comparison between ABC and unlabelled antibody (PAP) procedures. *J. Histochem. Cytochem.* **29:**577-80.

Hsu, S. and Soban, E. (1982). Color modification of diaminobenzidine (DAB) precipitation by metallic ions and its application for double immunohistochemistry. *J. Histochem. and Cytochem*. **30:**1079-82.

The most current versions of all product instructions are available at *www.piercenet.com*. For a faxed copy, contact customer service (in the USA call 800-874-3723) or your local distributor.