General Protocol

Immunohistochemistry Staining Kit

For Research Use Only

Peninsula Laboratories, Inc.
A Member of the Bachem Group
305 Old County Rd., San Carlos, CA 94070
Tel +1 800 922 1516
+1 650 592 5392
Fax +1 650 595 4071

Table of Contents

Introduction

The immunohistochemistry staining (IHS) kit is designed to reveal tissue or cell peptides that react with the antigen-specific primary rabbit (or guinea pig) antibody and are subsequently detected using the secondary biotinylated antibody (goat anti-rabbit or anti-guinea pig IgG) in conjunction with enzyme horseradish peroxidase (HRP)-labeled streptavidin (LAB-SA system). The presence of antigen/antibody/enzyme complex can be revealed by addition of a substrate-chromogen mixture. HRP will catalyze the substrate and convert the chromogen to the desired color deposits: gray for 3,3-diaminobenzidine tetrahydrochloride (DAB), gray-black for nickel intensified DAB, or red for 3-amino-9-ethylcarbazole (AEC), revealing the location of the antigen. The supplied primary antibody is titrated for optimal use for this kit. The LAB-SA system, which conjugates HRP directly to streptavidin, makes the kit more sensitive than the peroxidase-antiperoxidase (PAP) method and is more convenient than a conventional biotin-avidin complex (ABC) system.

The IHS kit can be applied for a variety of sample preparations including frozen or paraffin-embedded tissue sections and fixed cultured cells. The kit provides sufficient reagents for 150 slides.

Materials Provided

- 30% hydrogen peroxide solution (2 ml).
- Normal goat serum blocking solution (16 ml).
- Primary antibody (lyophilized powder).
- Primary antibody working solution bottle (1 bottle).
- Biotinylated secondary antibody solution (16 ml).
- Streptavidin-HRP conjugated solution (16 ml).
- DAB concentrated solution (1.5 ml).
- Substrate concentrated buffer for DAB (1.5 ml).
- Nickel-cobalt concentrated solution (1.5 ml).
- AEC concentrated solution (optional) (1 ml).
- Substrate concentrated buffer for AEC (optional) (1 ml).
- Hydrogen peroxide concentrated solution (2 ml).
- Mayer’s hematoxylin (16 ml).
- Mounting solution (16ml).

Materials Required but not Provided

- Xylene (for deparaffinization).
- Ethanol (for sample rehydration, etc.).
- Distilled water.
- PBS buffer (50 mM), pH 7.4 (sample washing, etc.).

Preparation of Reagents

1. 3% H_2O_2 solution
   a) 3% H_2O_2 in ethanol or water: (for quenching endogenous peroxidase activity in paraffin tissue slides). Add 1 part of 30% H_2O_2 to 9 parts of ethanol or water.
   b) 3% H_2O_2 in PBS: (for quenching endogenous peroxidase activity in frozen tissue slides). Add 1 part of H_2O_2 to 9 parts of PBS buffer.

2. Primary antibody working solution
   a) Reconstitute the lyophilized primary antibody with 16 µl of PBS buffer. Add 16 µl of reconstituted primary antibody serum to 8 ml of PBS buffer in primary antibody working solution bottle and mix vigorously. (1:2,000 dilution).
   b) Optimum dilution should be determined by the investigator.

3. Substrate-chromogen mixture working solution
   a) DAB working solution: Add 1 drop of concentrated substrate buffer for DAB in 1 ml of distilled water. Mix well, then add 1 drop of concentrated DAB solution. Mix well. Add 1 drop of concentrated hydrogen peroxide solution. Mix again.
   b) Nickel-intensified DAB working solution: Add 1 drop of concentrated substrate buffer for DAB in 1 ml of distilled water. Mix well, then add 1 drop of concentrated DAB solution. Mix well. Add 1 drop of concentrated hydrogen peroxide solution and 1 drop of concentrated nickel-cobalt solution. Mix again.
   c) AEC working solution: Add 1 drop of concentrated substrate buffer for AEC in 1 ml distilled water. Mix well. Add 1 drop of concentrated AEC solution and 1 drop of concentrated hydrogen peroxide solution. Mix again.

Note: *All of the working solutions should be kept away from light and used within 30 minutes.*

Staining Procedure

1) Sample preparation:
   - Paraffin tissue slides are deparaffinized with xylene and rehydrated in a graded ethanol series. Rinse for 5 minutes in tap water.

   Note: *If quenching of endogenous peroxidase activity is required, incubate the paraffin-embedded tissue sections for 30 minutes in 3% H_2O_2 in either ethanol or water.

   - Frozen tissue slides are air dried and treated with 50% ethanol for 20 minutes. Wash with PBS (two minutes, three times).

   Note: *If quenching of endogenous peroxidase activity is required, incubate the frozen slides for 30 minutes in 3% H_2O_2 in PBS. (3% H_2O_2 in ethanol is not recommended).

2) Rinse well with PBS after quenching step (two minutes, three times).
3) Add 2 drops or 100 µl of normal goat serum blocking solution to each slide. Incubate for 10-20 minutes. Drain or blot excess solution from the slides. Do not rinse.
4) Add 2 drops or 100 µl of the primary antibody working solution to each slide. Incubate for 60 to 120 minutes at room temperature or overnight at 4 °C. Rinse well with PBS (two minutes, three times).
5) Add 2 drops or 100 µl of biotinylated secondary antibody to each slide. Incubate for 30-60 minutes. Rinse well with PBS (two minutes, three times or more).
6) Add 2 drops or 100 µl of streptavidin-HRP conjugate to each slide. Incubate for 30 minutes. Rinse well with PBS (two minutes, three times or more)
7) Add 100 µl of substrate-chromogen mixture working solution to each slide. Development times should be monitored under a microscope. Generally, the developing times of DAB and nickel intensified DAB will be 1-3 minutes and AEC, 3 to 5 minutes. Rinse well with distilled water.
8) Counterstain the slides with 2 drops or 100 µl of Mayer's hematoxylin. Incubate for 1 to 3 minutes. Wash slides in tap water. Immerse and hold slides in PBS until blue (about 30 seconds). Rinse well with distilled water.

---

* Since the antigen may in some cases be destroyed by H_2O_2, treatment should be used only after the incubation with biotinylated secondary antibody. If endogenous peroxidase activity does not present a problem, the quenching step may be omitted.

9) Add 2 drops or 100 µl of mounting solution to the slide and mount cover slip.

Summary of Staining Procedure

Deparaffinized and rehydrated paraffin slides (10-20 minutes)

Rinse with tap water (5 minutes) ↓

Frozen tissue slides are air dried and treated with 50% ethanol (20 minutes)

↓ Wash well with PBS (3x2 minutes)

Quenching of endogenous peroxidase activity (30 minutes)

↓ Rinse well with PBS (3x2 minutes)

Normal goat serum blocking 10-20 minutes

↓ Drain or blot excess solution

Incubation with primary antibody 60-120 minutes at room temperature or overnight at 4 °C

↓ Rinse well with PBS (3x2 minutes)

Incubation with biotinylated second antibody (30-60 minutes)

↓ Rinse well with PBS (3x2 minutes)

Incubation with streptavidin-HRP conjugate (30 minutes)

↓ Rinse well with PBS (3 (or more) x 2 minutes)

Development of substrate-chromogen mixture (monitored under a microscope)

↓ Rinse well with distilled water

Counterstain (1-3 minutes)

↓ Rinse with tap water, PBS and distilled water

Mount with cover slip

Total assay time range is 3.5 hours to 2 days.

This procedure is not intended to replace the preparation steps, precautions and procedures shown in this booklet. Please consult those sections for complete directions.

Notes and Precautions

A) Store the IHS kit at 4 °C, with the exception of the mounting solution. When stored properly, expected shelf life is 2 months.

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

C) Perform all steps of the staining procedure at room temperature except the overnight incubation with the primary antibody, which is to be done at 4 °C.

D) Do not allow tissue slides to dry after deparaffinization or during the peroxidase quenching step. Incubation of slides should be in moist chamber.

E) Optimal dilution and incubation time of primary antibody may be adjusted depending on the sample preparation for study. The remaining reconstituted primary antibody serum can be stored at 4 °C for future use (within one month).

F) The pH values of DAB and AEC (working conditions) are different: neutral and weak acid, respectively. Be careful to add the correct substrate buffer concentrate when preparing the corresponding working solutions.

G) DAB and nickel are suspected carcinogens. Appropriate care should be exercised when using these reagents 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.

Troubleshooting

Possible causes of overstaining

1. Concentration of primary antibody was too high.
2. Incubation time is too long.
3. Concentration of the secondary and/or subsequent label reagents was too high.
4. Reaction temperature was too high.
5. Substrate incubation time was too long.

Possible causes of weak staining

1. Too much rinse buffer was left on slides causing excessive dilution of reagents.
2. Concentration of antibody or linker was too low or incubation time is too short.
3. Deparaffinization was not complete (possibly accompanied by high background).
4. Improper substrate preparation (i.e. contaminated by sodium azide or other inhibitor of peroxidase) or substrate was too old.
5. Incompatible counterstain or mounting media that dissolved the reaction product.

Possible causes of zero staining

1. Incorrect procedure.
2. Tissue dried out during staining protocol.
3. No antigen present in tissue.

Possible causes of high background

1. Endogenous peroxidase activity not blocked.
2. Nonspecific protein binding or endogenous biotin in tissue.
3. Incomplete deparaffinization.
4. Inadequate rinsing of slides.
5. Improper reagent dilution (too concentrated).
6. Overdevelopment of substrate.
7. Tissue dried out during staining protocol.

References

1. A.H. Coons
   Fluorescent antibody methods. General cytochemical methods. p. 399-422, ed. J. Danielli. Academic Press, New York (1958)
2. M. Schultzberg et al.
   Distribution of peptide- and catecholamine-containing neurons in the gastro-intestinal tract of rat and guinea-pig: immunohistochemical studies with antisera to substance P, vasoactive intestinal polypeptide, enkephalins, somatostatin, gastrin/cholecystokinin, neurotensin and dopamine beta-hydroxylase.
   Neuroscience **5**, 689-744 (1980)
3. K. Jessen et al.
   Immunohistochemical studies of the enteric nervous system in tissue culture and in situ: localization of vasoactive intestinal polypeptide (VIP), substance-P and enkephalin immunoreactive nerves in the guinea-pig gut.
   Neuroscience **5**, 1717-1735 (1980)
4. J. Haaijman
   Labeling of proteins with fluorescent dyes: quantitative aspects of immunofluorescence microscopy. Immunohistochemistry. p. 47-85, ed. A. Cuello. John Wiley & Sons (1984)
5. Y.N. Wang et al.
   Immunocytochemical localization of neuropeptide Y-like immunoreactivity in adrenergic and non-adrenergic neurons of the rat gastrointestinal tract.
   Peptides **8**, 145-151 (1987)
6. R.M. Kobayashi et al.
   Regional distribution of neurotensin and somatostatin in rat brain.
   Brain Res. **126**, 584-588 (1977)
7. R.N. Burgus et al.
   Primary structure of somatostatin, a hypothalamic peptide that inhibits the secretion of pituitary growth hormone.
   Proc. Natl. Acad. Sci. USA **70**, 684-688 (1973)

Ordering Information

For additional kits or our most current catalog products, please visit our web site at www.bachem.com or call us at:

Tel +1 800 922 1516
+1 650 592 5392
Fax +1 650 595 4071

Technical Support

If you need technical information or assistance with assay procedures, please call our Technical Service Department at (800) 922-1516 or (650) 592-5392. Our staff will be happy to answer your questions about this or any other products.

Guarantee and Limitation of Remedy

The Peninsula Laboratories, Inc. makes no guarantee of any kind, expressed or implied, which extends beyond the description of the material in this kit, except that these materials and this kit will meet our specifications at the time of delivery. Customer's remedy and Peninsula Laboratories, Inc.'s sole liability hereunder are limited at Peninsula Laboratories, Inc.'s option to either refund the purchase price or to replace the material that does not meet our specifications. By the acceptance of our products, the customer indemnifies and holds Peninsula Laboratories, Inc. harmless against, and assumes all liability for, the consequences of its use or misuse by the customer, its employees or others.