INSTRUCTIONS

April, 2005

-Histofine® Simple Stain MAX PO (R)

Universal Immuno-peroxidase Polymer, Anti-Rabbit
-Histofine® Immunohistochemical staining reagent Store at 2-8°C

Reagents supplied:	17mL x 1 bottle	(170 tests)	Code : 414141F
	17mL x 3 bottles	(500 tests)	Code : 414142F
	17mL x 9 bottles	(1500 tests)	Code : 414144F

Made in Japan

NICHIREI BIOSCIENCES INC.
6-19-20, Tsukiji, Chuo-ku, Tokyo, 104-8402, JAPAN
Phone:81-3-3248-2207 Facsimile:81-3-3248-2243

1. INTRODUCTION

NICHIREI BIOSCIENCES developed a unique immunohistochemical staining system called **Universal Immuno-enzyme Polymer (UIP)** method (US Patent No.6,252,053). This is NICHIREI BIOSCIENCES's original technique. **-Histofine®** Simple Stain MAX PO (R), this provides both high sensitivity and time saving in immunohistochemical applications.

2. PRESENTATION

Liquid. Ready to use.
-Histofine® Simple Stain MAX PO (R) (Universal Immuno-peroxidase Polymer, Anti-Rabbit) is the labeled polymer prepared by combining amino acid polymers with peroxidase and goat anti-rabbit Ig which are reduced to Fab'. It is stored in MOPS (3-Morpholinopropanesulfonic acid) buffer (pH 6.5) containing stabilizer and antibacterial agent.

Description of **-Histofine®** Simple Stain MAX PO (R) (Universal Immuno-peroxidase Polymer, Anti-Rabbit)
IgG fraction purified from immunized goat serum is digested to prepare $F(ab')_2$. Antigen-specific $F(ab')_2$ is affinity-purified with the antigen. Solid-phase absorption is carried out with human serum protein. Peroxidase-labeled amino acid polymer is conjugated to Fab' obtained by reducing $F(ab')_2$.

3. INTENDED USE

FOR RESEARCH USE ONLY.
-Histofine® Simple Stain MAX PO (R) is designed to allow immunohistochemical studies using a user-supplied rabbit primary antibody. This reagent is basically available for formalin fixed paraffin-embedded human tissue sections. Regarding to the application for staining of frozen tissue sections, please read **6. STAINING PROCEDURES.** Also please contact NICHIREI BIOSCIENCES technical service department concerning this reagent for other specimens.

4. PRINCIPLE

The antigen / antibody / Universal Immuno-peroxidase Polymer complex can be prepared by allowing the reagent to react with a rabbit primary antibody bound to the antigen on tissue section. The enzymatic activity of this complex results in a colored deposit, thus staining the antigen site.

5. PRECAUTION WHILE USING OR HANDLING.

1. Before using this reagent, please read these instructions.
2. Do not use reagents after the expiration date.
3. Specimens, before and after fixation, and all other materials exposed to them, should be handled like biohazardous materials with proper precautions.
4. Inhalation or ingestion of the highly allergic fixative formaldehyde is harmful. Wear protective mask. If swallowed, induce vomiting. If skin or eye contact occurs, wash thoroughly with water.
5. Organic reagents are flammable. Do not use near open flame.
6. Never pipette reagents by mouth and avoid their contact with skin, mucous membranes and clothes.
7. Avoid microbial contamination of reagents as incorrect result may occur.
8. Avoid splashing of reagents or generation of aerosols.
9. For research use only. Not for diagnostic use.

6. STAINING PROCEDURES

■ Reagents and Materials required but not provided

- Rabbit primary antibody
- Xylene
- 100% ethanol
- 95% ethanol
- Counter staining solution
- 3% solution of hydrogen peroxide in absolute methanol (Add 1 part of 30% hydrogen peroxide to 9 parts of absolute methanol)
- Phosphate buffered saline (PBS) (pH 7.6±0.2)
  - NaCl 7.75 g
  - K_2HPO_4 1.50 g
  - KH_2PO_4 0.20 g
  - distilled water 1L
- Adhesive for tissue section (0.02% poly-L-lysine, silane or the like)
- Chromogen/substrate reagent
  - Histofine® Simple Stain AEC solution (No preparation, One-bottle)
  500 tests Code: 415182F
  1500 tests Code: 415184F

NICHIREI BIOSCIENCES developed One-bottle, ready to use and no preparation chromogen/substrate called -Histofine® Simple Stain Substrate Solution.

- Negative control reagent
- Cover slips
- Light microscope
- Distilled water
- Humidified chamber for slide incubation
- Mounting media
- Timer
- Staining racks or Coplin jars
- Absorbent wipes

■ Specimen preparation

[Paraffin-embedded tissue sections]
Specimens may undergo histological disintegration or antigenic denaturation when subjected to highly concentrated fixative or prolonged fixing. Thus, in order to obtain an optimal fixing, maintaining tissue morphology and antigen activity, tissues which are as fresh as possible and small in size (about 1cm x 1cm x 0.5cm) should be used. The fixatives as shown below are recommended.

Fixing reagent	Fixing time
10% formalin or buffered formalin	24-48 hours
20% formalin	12-24 hours

[Frozen tissue sections]
Specimens are embedded in compounds (like OTC compound) and snap-frozen in n-hexsan cooled in dry ice-acetone or liquid nitrogen.

■ Section preparation

[Paraffin embedded tissue sections]
The cut sections should be 3-6 µm and placed on slides. When further treatments are to be done such as Antigen Recovery, Heat-Induced Epitope Retrieval (HIER) or trypsin treatment, the glass slides should be coated with an adhesive like 0.02% poly-L-lysine or silane for tissue sections.

[Frozen tissue sections]
The cryostat sections are mounted on an adhesive (like 0.02% poly-L-lysine) - coated slides and air-dried well. The sections are fixed in 100% acetone for 10 minutes at room temperature or 4% paraformaldehyde-PBS solution for 10 minutes at 4°C and then stained.
The optimal concentration and incubation time of primary antibodies should be determined by the investigation. In some cases, further dilution of primary antibodies may be required to prevent overstaining.
If the sections contain few endogenous peroxidase, few erythrocytes and few granulocytes, quenching of endogenous peroxidase may be omitted.

[Control slides]
A positive control slide, negative control slide and reagent control slide are needed and processed in the same way as the unknown specimen slide to interpret staining results.

■ Deparaffinization and Rehydration

1. Treatment with xylene
(1) Immerse the slides in xylene. After 3 minutes, take out and shake off the excessive xylene in the slides.
(2) Repeat 1.(1) twice using fresh xylene.
2. Treatment with ethanol
(1) Immerse slides in 100% ethanol. After 3 minutes, take out and shake off the excessive 100% ethanol in the slides.
(2) Repeat 2.(1) once with fresh 100% ethanol.
(3) Then, treat them twice with 95% ethanol in the same way as described above.
3. Washing
After excessive ethanol is shaken off, immerse slides in PBS for 5 minutes.

■ Recommended Staining Procedures

1. Quenching of endogenous peroxidase
(1) Wipe areas around the sections on the slides carefully to remove excess solution.
(2) Immerse them in 3% solution of hydrogen peroxide in absolute methanol for 10-15 minutes at room temperature.
(3) Rinse them in fresh PBS for 3 times, each of 5 minutes duration.

2. Addition and reaction of the primary antibody
(1) Wipe areas around the sections on the slides carefully.
(2) Apply 2 drops of primary antibody to specimen slide, positive control slide and negative control slide respectively so as to provide a complete cover of the sections.
(3) To the reagent control slide, apply two drops of negative control reagent (normal serum) in place of primary antibody.
(4) Incubate them at room temperature or 4°C. (Follow the instructions for incubation time data designated in the package insert of primary antibody)
(5) Rinse them in fresh PBS for 3 times, each of 5 minutes duration.

3. Addition and reaction of **-Histofine®** Simple Stain MAX PO (R) (Universal Immuno-peroxidase Polymer, Anti-Rabbit).
(1) Wipe areas around the sections on the slides carefully.
(2) Apply 2 drops of Simple Stain MAX PO (R) to each slide so as to provide a complete cover of the sections. Incubate at room temperature for 30 minutes.
(3) Rinse them in fresh PBS for 3 times, each of 5 minutes duration.

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4. Addition and reaction of chromogen/substrate reagent
(1) Wipe areas around the sections on the slides carefully.
(2) Apply 2 drops of the chromogen/substrate reagent to each slide so as to provide a complete cover of the sections. Incubate at room temperature for 5-20 minutes.
(3) Rinse them in distilled water for 3 times, each of 5 minutes duration.

5. Counter-staining
(1) Immerse them in the counterstain solution.
(2) Wash them well with tap water.

6. Mounting
In case of alcohol soluble substrates like AEC, the tissue sections are mounted with water-based mounting media without further treatment. In case of alcohol insoluble substrates like DAB, they are mounted with permanent mounting media after washing with water, dehydrated in graded series of alcohol and cleared in xylene.

■ Interpretation of results
1. Microscopic observation
The slides are examined under a light microscope for a positive reaction. It is necessary to make comparison with three types of the control slides for interpreting staining results.

- Positive control slide
A specimen containing the target antigen which is processed in the same way as the unknown specimen.
- Negative control slide
A specimen not containing the target antigen which is processed in the same way as the unknown specimen.
- Reagent control slide
The control specimen is used and processed in the same way as the test specimen except that negative control reagent is used instead of primary antibody. If the slide is stained, it is probably due to non-specific reaction by non-specific protein binding.

7. STORAGE
Store in a dark place at 2-8°C.

8. LIMITATION
Tissue staining is dependent on the handling and processing of the tissue prior to staining. Improper fixation, freezing, thawing, washing, drying, heating, or sectioning may produce artifacts or false-negative results.

Results will not be optimal if old or unbuffered fixatives are used, or excessively heated during embedding or during attachment of sections to slides.

False-positive results may be seen due to nonspecific binding of proteins. Although **N-Histofine®** Simple Stain MAX PO (R) does not require the use of blocking reagent separately, in some cases the application of blocking reagent containing an irrelevant protein, prior to incubation with the primary antibody, may be useful for reducing the background.

9. CONDITION FOR USE
N-Histofine® Simple Stain MAX PO (R) is designed for research use only and is not intended for therapeutic or diagnostic purposes. NICHIREI BIOSCIENCES INC., NICHIREI BIOSCIENCES sales agents and distributors will take no responsibility for **N-Histofine®** Simple Stain MAX PO (R) when used in a way which directly or indirectly violates local regulations or patents. Neither NICHIREI BIOSCIENCES nor its sales agents can be held responsible for any patent infringement which may occur as a result of improper use of the product.

10. TROUBLE SHOOTING

Problem	Possible cause	Solution
○ No staining or only weak staining results on the positive control slide and the unknown specimen slide	1. Drying-out of specimens during staining prior to addition of the reagents.	1. Never allow the tissue to dry out.
	2. The embedding agent is not suitable, or paraffin is not thoroughly removed from paraffin-embedded sections.	2. Select a suitable embedding agent or remove paraffin thoroughly from sections embedded. 2. Change xylene or ethanol as the case may be.
	3. Any trace amount of sodium azide present in the buffer inactivates the peroxidase, making the staining impossible.	3. Use sodium azide free buffer solution. 3. Change buffer solution.
	4. Inadequate incubation of the enzyme and antibody.	4. Change stale chromogen/substrate reagent. 4. Blot off excess solution thoroughly at each stage. 4. Provide sufficient time for reaction with antibody. In particular, primary antibody should be incubated for the time period specified in the insert.
○ The unknown specimen slide is not stained while the positive control slide is stained.	1. Antigen is denatured or masked during fixing or embedding process.	1. Some antigens are sensitive to fixation or embedding. So use less potent fixative and decrease the fixing time. 1. The pretreatment is required for some tissues, in order to reveal the antigen, such as Antigen Recovery, Heat-Induced Epitope Retrieval (HIER) or trypsin treatment.
	2. Antigen is decomposed by autolysis.	2. Use tissues obtained by biopsy or surgery, whenever possible.
	3. Less antigen is present in the sections.	3. Prolong the incubation time.
○ The backgrounds are intensively stained in all the slides.	1. Endogenous enzyme activity was not completely blocked.	1. Ensure that the procedure for quenching of endogenous peroxidase is right.
	2. Non -specific binding compositions are found.	2. Before adding primary antibody, treat with 10% normal goat serum.
	3. Autolysis results in excessive antigens isolated in histological solutions.	3. Obtain fresh tissues whenever available.
	4. Insufficient removal of paraffin.	4. Change xylene or ethanol as the case may be.
	5. Insufficient washing of antibody.	5. Ensure thorough washing of antibody.
	6. A high room temperature accelerates enzyme reactions.	6. Keep room temperature at 15 to 25°C 6. Shorten reaction time.
	7. Drying-out of specimens during staining after of the reagents.	7. Never allow the tissue to dry out.
○ During the reaction, tissue sections come off from the slides.	1. Some antigens require heat induced antigen retrieval procedure or prolonged reaction time with primary antibody, which may render the sections easily come off.	1. Mount tissue sections on slides coated with an adhesive such as 0.02% poly-L-lysine or silane.

11. REFERENCE
(1) Kimura, N., *et al*: Synaptotagmin I Expression in Mast Cells of Normal Human Tissues, Systemic Mast Cell Disease, and a Human Mast Cell Leukemia Cell Line. J. Histochem. Cytochem. 49: 341-346, 2001.

(2) Naito, Z., *et al*: Expression and accumulation of lumican protein in uterine cervical cancer cells at the periphery of cancer nests. Int. J. Oncol. 20: 943-948, 2002.

(3) Hoshino, Y., *et al*: Maximal HIV-1 Replication in Alveolar Macrophages during Tuberculosis Requires both Lymphocyte Contact and Cytokines. J. Exp. Med. 195: 495–505, 2002.

(4) Sawada, H., *et al*: Characterization of an Anti-Decorin Monoclonal Antibody, and Its Utility. J. Biochem. 132: 997–1002, 2002.

(5) Ozaki, K., *et al*: Mast Cell Tumors of the Gastrointestinal Tract in 39 Dogs. Vet. Pathol. 39:557-564, 2002.

(6) Zen, Y., *et al*: Lipopolysaccharide Induces Overexpression of MUC2 and MUC5AC in Cultured Biliary Epithelial Cells. Possible Key Phenomenon of Hepatolithiasis. Am. J. Pthol. 161: 1475-1484, 2002.

(7) Sawada, H., *et al*: Case report: Altered decorin expression of systemic sclerosis by UVA1 (340–400 nm) phototherapy: Immunohistochemical analysis of 3 cases. BMC Dermatol. 3:2, 2003.

(8) Takagi-Morishita, Y., *et al*: Mouse Uterine Epithelial Apoptosis is Associated with Expression of Mitochondrial Voltage-Dependent Anion Channels, Release of Cytochrome c from Mitochondria, and the Ratio of Bax to Bcl-2 or Bcl-X^1. Biol. Reprod. **68**: 1178–1184, 2003.

(9) Morimoto, R., *et al*: Co-expression of vesicular glutamate transporters (VGLUT1 and VGLUT2) and their association with synaptic-like microvesicles in rat pinealocytes. J. Neurochem. 84: 382-391, 2003.

(10) Nakatani, K., *et al*: Cytoglobin/STAP, its unique localization in splanchnic fibroblast-like cells and function in organ fibrogenesis. Lab. Invest. 84: 91-101, 2003.

(11) Kitada, M., *et al*: Translocation of Glomerular p47phox and p67phox by Protein Kinase C-beta Activation is Required for Oxidative Stress in Diabetic Nephropathy. Diabetes. 52: 2603-2614, 2003.

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Distributor
COSMO BIO CO., LTD.
Inspiration for Life Science
TOYO 2CHOME, KOTO-KU, TOKYO, 135-0016, JAPAN
http://www.cosmobio.co.jp e-mail : export@cosmobio.co.jp
Phone : +81-3-5632-9617 FAX : +81-3-5632-9618