INSTRUCTIONS

April, 2005

N-Histofine® Simple Stain AP (MULTI)

Universal Immuno-Alkaline-Phosphatase Polymer, Anti-Mouse and -Rabbit
N-Histofine® Immunohistochemical staining reagent Store at 2-8°C

Reagents supplied: 17mL x 1 bottle (170 tests) Code : 414261F
17mL x 3 bottles (500 tests) Code : 414262F

Made in Japan

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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. **N-Histofine®** Simple Stain AP (MULTI), this provides both high sensitivity and time saving in immunohistochemical applications.

2. PRESENTATION

Liquid. Ready to use.
N-Histofine® Simple Stain AP (MULTI) (Universal Immuno-Alkaline-phosphatase Polymer, Anti-Mouse and -Rabbit) is the labeled polymer prepared by combining amino acid polymers with Alkaline Phosphatase and goat anti-mouse Ig 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 **N-Histofine®** Simple Stain AP (MULTI) (Universal Immuno-Alkaline-Phosphatase Polymer, Anti-Mouse and -Rabbit) IgG fraction purified from immunized goat serum is digested to prepareF(ab')2.
Antigen-specific $F(ab')_2$ is affinity-purified with the antigen. Solid-phase absorption is carried out with human serum protein. Alkaline phosphatase -labeled amino acid polymer is conjugated to Fab' obtained by reducing $F(ab')_2$.

3. INTENDED USE

FOR RESEARCH USE ONLY.
N-Histofine® Simple Stain AP (MULTI) is designed to allow immunohistochemical studies using a user-supplied mouse primary antibody or rabbit primary antibody. This reagent is basically available for smears and 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-alkaline-phosphatase Polymer complex can be prepared by allowing the reagent to react with a mouse or 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

- Mouse or rabbit primary antibody
- Xylene
- 100% ethanol
- 95% ethanol
- Counterstain solution
- 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
- 0.1M Tris buffered saline (TBS) (pH7.5±0.2)
  solution A 100mL
  solution B 100mL
  distilled water 800mL
  solution A
  $C_4H_{11}NO_3 \cdot CH_3COOH$ 12.11g
  (pH7.5±0.2)
  distilled water 1L
  solution B
  NaCl 87.66 g
  distilled water 1L
- Chromogen/substrate reagent
  N-Histofine® New Fuchsin substrate kit (4-bottles)
  2000 tests Code:415161F
- Blood collection tube containing EDTA
- Negative control reagent
- Cover slips
- Light microscope
- Distilled water
- Humidified chamber for slide incubation
- Mounting media
- Timer
- Staining racks or Coplin jars
- Absorbent wipes
- Drier
- Buffered Formaline Acetone(BFA)
  NaH_2PO_4 15mg
  K_2HPO_4 120mg
  distilled water 30mL
  Acetone 45mL
  Formaline 25mL
- Adhesive for tissue section (0.02% poly-L-lysine, silane or the like)

■ Specimen preparation

[Smears]
(1) Collect blood with a blood collection tube containing EDTA and smear blood on a slide.
(2) Airdry slides well with drier for 20-30minutes.
(3) Fix the cells with BFA at 4°C for 30 seconds.
(4) Rinse slides in tap water for 30-60 seconds.
(5) After excessive water is shaken off, immerse slides in PBS for 5 minutes.

[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

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.

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.

[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.
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.

■ Recommended Staining Procedures

1. 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.

2. Addition and reaction of **N-Histofine®** Simple Stain AP (MULTI) (Universal Immuno-peroxidase Polymer, Anti-Mouse and -Rabbit).
(1) Wipe areas around the sections on the slides carefully.
(2) Apply 2 drops of Simple Stain AP (MULTI) 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.
(4) Rinse them in TBS for 5 minutes.

3. Addition and reaction of chromogen/substrate solution
(1) Wipe areas around the sections on the slides carefully.
(2) Apply 2 drops of the chromogen/substrate solution 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.

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

5. Mounting
In case of New Fuchsin substrate, the tissue sections are mounted with water-soluble mounting media or air-dryed, cleared in xylene for a few seconds and mounted with permanent mounting media.

■ 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.

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. Inadequate incubation of the enzyme and antibody.	3. Change stale chromogen/substrate reagent. 3. Blot off excess solution thoroughly at each stage. 3. 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. Add Levamisole to chromogen/substrate solution. To reduce endogenous enzyme activity chromogen/substrate solution containing 1mM Levamisole is used.
	2. Non -specific binding is 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.

False-positive results may be seen due to nonspecific binding of proteins. Although **N-Histofine**® Simple Stain AP (MULTI) 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 AP (MULTI) 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 AP (MULTI) 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.

11. REFERENCE

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