About us

Nichirei Corporation had started bioscience business as a manufacture of Anti-Leukocytes Monoclonal antibodies (CD Antibodies) in 1983. Nichirei Biosciences Inc. (hereinafter called “Nichirei Bio”) was reorganized on April 1, 2005 succeeding Nichirei’s bioscience business adopting the holding company system by its parent company, Nichirei Corporation. Nichirei Bio’s mission is to contribute to the development of the global Bio-industries including medical, cosmetic and healthcare business as a specialized company by offering high-quality products and services based on its advanced technology.

Nichirei Bio’s business field

1. IHC (Immunohistochemistry) products (Histofine®)
   Nichirei Bio has developed and improved patented state-of-the-art technology, UIP (Universal Immuno-enzyme Polymer) method. Nichirei Bio manufactures and supplies Histofine® products including CE marked products adopted the UIP technology. Also for domestic use, Nichirei Bio manufactures and supplies several IVD products for IHC.

2. Diagnostic products (EIA and Lateral Flow Assay kits)
   Nichirei Bio develops, manufactures and distributes several IVD products immuno-reaction applied, especially POC (Point of Care) for rapid diagnostics.

3. Cell-biology products
   Nichirei Bio provides animal sera and media related to cell culture to the customers both in the academic and industrial field.

4. Functional Materials
   Nichirei Bio develops functional materials (powder and extract) from natural raw materials such as Acerola and Camu Camu fruits procured by consolidated subsidiaries and affiliates of the Nichirei group. Nichirei Bio supplies such materials to cosmetics and health food manufacturers.
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**Highlights**

**Two-Step Polymer Detection System**

**N-Histofine® High Stain™ HRP (MULTI)**

*N-Histofine® High Stain™ HRP (MULTI)* is the Two-Step Polymer Detection System for IHC staining providing more amplified staining intensity compared with conventional One-step polymer detection system.

This system is applicable to both of Mouse and Rabbit primary antibodies and is for formalin-fixed, paraffin-embedded tissue sections.

For details, see Page 6.

**DAB Substrate Kit**

**N-Histofine® DAB-2V**

*N-Histofine® DAB-2V* is used as chromogen-substrate reagents for peroxidase-based immunohistochemical staining as well. DAB produces brown precipitates at the site of the target antigen or nucleic acid reacting with peroxidase.

*Higher sensitivity and simplified preparation* are available in comparison with prior *N-Histofine® DAB-3S* kit.

For details, see Page 15.

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### Staining results with *N-Histofine® DAB-2V*

- CD3 Rabbit-Monoclonal antibody (Code: 413591F)
- Actin, Smooth Muscle Mouse-Monoclonal antibody (Code: 412021F)

---

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-Histofine® series application on IHC staining

### Tissue Enzyme Primary antibody Detection systems

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<tr>
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<th>Enzyme</th>
<th>Primary antibody</th>
<th>Detection systems</th>
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<tr>
<td>Human Tissue</td>
<td>HRP</td>
<td>Mouse or Rabbit ab</td>
<td>High Stain™ HRP (MULTI)</td>
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<td>Mouse or Rabbit ab</td>
<td>Simple Stain™ MAX PO (MULTI)</td>
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<td></td>
<td>Mouse ab</td>
<td>Simple Stain™ MAX PO (M)</td>
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<td></td>
<td>Rabbit ab</td>
<td>Simple Stain™ MAX PO (R)</td>
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<td>Goat ab</td>
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<td>HRP</td>
<td>Mouse or Rabbit ab</td>
<td>Simple Stain™ AP (MULTI)</td>
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<td>Simple Stain™ AP (M)</td>
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<td>Simple Stain™ Mouse MAX PO (R)</td>
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<td>Goat ab</td>
<td>Simple Stain™ Mouse MAX PO (G)</td>
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<td>Goat ab</td>
<td>Simple Stain™ Rat MAX PO (G)</td>
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Products
**Detection system for Human tissue sections**

N-Histofine® High Stain™ HRP (MULTI)

This product adopted the UIP (Universal Immuno-enzyme Polymer) method, Nichirei Bio’s patented technology.

### Feature

N-Histofine® High Stain™ HRP (MULTI) is the **Two-Step Polymer Detection System** for IHC staining providing more amplified staining intensity compared with conventional One-step polymer detection system. This system is applicable to both of Mouse and Rabbit primary antibodies and is for formalin-fixed, paraffin-embedded tissue sections. It is the labeled polymer prepared by combining amino acid polymers with multiple molecules of peroxidase (PO) and secondary antibody which is reduced to Fab' fragment. To eliminate background staining, solid-phase adsorption of secondary antibody is conducted with human serum.

### Principle & Procedure

#### Kit components

- High Stain™ Solution A
- High Stain™ Solution B
- Fab' fragment of secondary antibody
- Peroxidase
- Amino-acid polymer

#### Protocol

1. **Add mouse or rabbit primary antibody***.

2. **Add High Stain™ Solution A**.

3. **Add High Stain™ Solution B**.

4. **Add substrate solution***.

**Counterstaining**

**Mounting**

*Primary antibody and substrate solution are not provided in this kit.

### Advantages

1. High intensity of staining.
2. Applicable to both of Mouse and Rabbit primary antibodies.
3. Low expression of antigen is detectable.
4. No background due to unaffected by endogenous biotin (Page 18).
5. Shortened reaction time.

### Contents

- Antibody
- High Stain™ Solution A 10 min
- High Stain™ Solution B 10 min
- DAB chromogen
- Counterstaining
Comparison of Staining Results

**α-Histofine® High Stain™ HRP (MULTI)**

- **CDX-2 Rabbit-Monoclonal antibody** (Code: 418011F)
  - Human colon cancer stained with α-Histofine® High Stain™ HRP (MULTI) and DAB chromogen.
  - Positive reaction is observed in nuclei of sporadic tumor cells.

- **Cyclin D1 Rabbit-Monoclonal antibody (Clone: SP4)** (Code: 413521F)
  - Human breast cancer (metastasis to liver) stained with α-Histofine® High Stain™ HRP (MULTI) and DAB chromogen.
  - Positive reaction is observed in nuclei of sporadic tumor cells.

- **p40 Rabbit-Polyclonal antibody** (Code: 418101F)
  - Human squamous cell cancer (lung) stained with α-Histofine® High Stain™ HRP (MULTI) and DAB chromogen.
  - Positive reaction is observed in nuclei of tumor cells.

**Product**

Detection system.

<table>
<thead>
<tr>
<th>Product Name</th>
<th>Tests</th>
<th>Volume</th>
<th>Code</th>
<th>For use with</th>
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<tbody>
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Detection system for Human tissue sections

N-Histofine® Simple Stain™ MAX PO

This product adopted the UIP (Universal Immuno-enzyme Polymer) method, Nichirei Bio’s patented technology.

Feature

N-Histofine® Simple Stain™ MAX PO is a detection reagent designed specifically to allow immunohistochemical staining on formalin-fixed paraffin-embedded human tissue sections. It is the labeled polymer prepared by combining amino acid polymers with multiple molecules of peroxidase (PO) and secondary antibody which is reduced to Fab’ fragment. To eliminate background staining, solid-phase adsorption of secondary antibody is conducted with human serum.

Principle & Procedure

Advantages

1. Simplified staining steps*.
2. High sensitivity*.
3. No background due to unaffected by endogenous biotin*.
4. Ready to use.

**Page 18

Staining Results

- Mouse anti-Progesteron Receptor antibody (clone:1A6)
- Rabbit anti-S-100 protein antibody
- Mouse anti-Estrogen Receptor antibody (clone: 1D5)

Product

Detection systems.

<table>
<thead>
<tr>
<th>Product Name</th>
<th>For (slides)</th>
<th>Volume</th>
<th>Code</th>
<th>For use with</th>
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</table>
**Detection system for Human tissue sections**

**N-Histofine® Simple Stain™ AP**

This product adopted the UIP (Universal Immuno-enzyme Polymer) method, Nichirei Bio's patented technology.

### Feature

N-Histofine® Simple Stain™ AP is a detection reagent designed specifically to allow immunohistochemical staining on formalin-fixed paraffin-embedded human tissue sections. It is the labeled polymer prepared by combining amino acid polymer with alkaline phosphatase (AP) and secondary antibody which is reduced to Fab' fragment. To eliminate background staining, solid-phase adsorption of secondary antibody is conducted with human serum.

### Principle & Procedure

1. **Add primary antibody.**
2. **Add polymer.**
3. **Add substrate solution.**
   - 30 min.
4. **Counterstaining**
5. **Mounting**

### Advantages

1. Simplified staining steps*.
2. High sensitivity*.
3. No background due to unaffected by endogenous biotin*.
4. Ready to use.

### Staining Results

**Mouse anti-Muscle Actin antibody**

Human stomach stained with N-Histofine® Simple Stain™ AP (M) and New fuchsin chromogen. Intense staining of smooth muscle in the walls of blood vessel and muscularis mucosae is observed.

**Rabbit anti-S-100 protein antibody**

Human colon stained with N-Histofine® Simple Stain™ AP (R) and New fuchsin chromogen. Cytoplasmic staining of nerve cells scattered in smooth muscle and Auerbach’s plexus is observed.

### Product

Detection systems.

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<th>Code</th>
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<td>Rabbit primary antibody</td>
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</tbody>
</table>

*Page 18
ALK Detection Kit

**Feature**

N-Histofine® ALK Detection Kit detects anaplastic lymphoma kinase (ALK) proteins in tumor cells in paraffin-embedded tissue specimens by IHC staining and determines presence of such protein expression.

**Advantage**

This kit is applicable to low expression of ALK fusion proteins as well.

**Background**

The ALK gene was identified in 1994 as a gene fused to the nucleophosmin (NPM) gene in anaplastic large-cell lymphoma (ALCL) with t(2;5)(p23;q35) translocation. This gene is located at 2p23 and encodes for a receptor-type tyrosine kinase, which belongs to the insulin receptor family. The ALK protein have a kinase domain in its intracellular domain, and its function is associated with the promotion of cell growth and inhibition of apoptosis.

Subsequently, the ALK gene has been reported to form ALK fusion genes fused with ATIC, CLTC, MSN, TPM3, TPM4, TFG, MYH9 and ALO17 genes in ALCL and also ATIC, CAR5, CLTC, DCTN1, TPM3, TPM4, PPFIBP1, RANBP2 and SEC31L1 genes in inflammatory myofibroblastic tumor (IMT).

The proteins produced from these fusion genes are constantly activated by forming dimers and led to cancerous change.

Recently, other ALK fusion genes with EML4 gene, KIF5B gene or KCL1 gene in non-small cell carcinoma of lung, SEC31A gene or SQSTM1 gene in ALK-positive large B-cell lymphoma and VCL gene in renal cell cancer have been also reported.

**Notes for Determination**

Due to this kit detects ALK proteins, ALK fusion proteins as well as full-length ALK protein are reacted. Consequently, results of slight positive to positive for tumors* that infrequently express full-length ALK protein are observed. However, discrimination between ALK fusion proteins and full-length ALK protein is not available. Therefore, considering expression possibility of ALK fusion proteins, confirmation of the presence or absence of ALK fusion genes by using FISH method is preferable in this regard.

* Large-cell neuroendocrine carcinomas of the lung, small-cell lung carcinomas and rhabdomyosarcomas (particularly alveolar rhabdomyosarcomas).
**Staining Flowchart**

1. Deparaffinization and Rehydration
2. Antigen Unmasking Treatment
3. Quenching of endogenous peroxidase
4. Reaction with Primary Antibody or Negative Control
5. Reaction with Bridge Reagent
6. Reaction with Peroxidase labeled Empower Reagent
7. Reaction with chromogen-substrate reagent
8. Counterstaining
9. Mounting

**Note**
High temperature epitope unmasking is required for formalin fixed paraffin-embedded tissue sections prior to IHC staining.

**References**


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**$\mathcal{N}$-Histofine® ALK Control Slides**

Both formalin fixed paraffin embedded cell lines of NCI-H2228 (Positive) and SK-BR-3 (Negative) are mounted on each slide.

$\mathcal{N}$-Histofine® ALK Control Slides are used as a standard for discrimination of positive tissue blocks possess expressed ALK protein.

This product is used for validation of reagent performance and staining technique in the IHC staining (including cytology staining) with $\mathcal{N}$-Histofine® ALK Detection Kit.

**Product Detection system. Liquid. Ready to use.**

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<td>3</td>
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<td>10</td>
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This product adopted the UIP (Universal Immuno-enzyme Polymer) method, Nichirei Bio’s patented technology.

**Feature**

\(\text{\textit{H}}\)-Histofine® Simple Stain™ Rat MAX PO is a detection reagent designed specifically to allow immunohistochemical staining on formalin-fixed paraffin-embedded rat tissue sections. It is the labeled polymer prepared by combining amino acid polymers with multiple molecules of peroxidase (PO) and secondary antibody which is reduced to Fab’ fragment. To eliminate background staining, solid-phase adsorption of secondary antibody is conducted with rat, human, dog, pig and bovine sera.

**Principle & Procedure**


**Advantages**

1. No reaction to endogenous rat immunoglobulin (Page 25).
2. Simplified staining steps*.
3. High sensitivity*.
4. No background due to unaffected by endogenous biotin*.
5. Ready to use.

**Staining Results**

- **Mouse anti-Vimentin antibody (clone:V9)**
  - Rat colon (treated with high temperature epitope unmasking method) stained with \(\text{\textit{H}}\)-Histofine® Simple Stain™ Rat MAX PO (M) and DAB chromogen. Cytoplasmic staining of fibroblasts and endothelial cells is observed.

- **Mouse anti-PCNA antibody (clone:PC10)**
  - Rat liver stained with \(\text{\textit{H}}\)-Histofine® Simple Stain™ Rat MAX PO (MULTI) and DAB chromogen. Nuclear staining of liver cells is observed.

- **Goat anti-CD3-ε(M-20) antibody**
  - Rat lymph node (treated with high temperature epitope unmasking method) stained with \(\text{\textit{H}}\)-Histofine® Simple Stain™ Rat MAX PO (G) and DAB chromogen. Membrane staining of almost all lymphocytes is observed.

**Product**

Detection systems. Liquid. Ready to use.

<table>
<thead>
<tr>
<th>Product Name</th>
<th>For (slides)</th>
<th>Volume</th>
<th>Code</th>
<th>For use with</th>
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<td>17ml</td>
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<td>170</td>
<td>17ml</td>
<td>414331F</td>
<td>Goat primary antibody</td>
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</table>
Detection system for Mouse tissue sections

N-Histofine® Simple Stain™ Mouse MAX PO

This product adopted the UIP (Universal Immuno-enzyme Polymer) method, Nichirei Bio's patented technology.

Feature

N-Histofine® Simple Stain™ Mouse MAX PO is a detection reagent designed specifically to allow immunohistochemical staining on formalin-fixed paraffin-embedded mouse tissue sections. It is the labeled polymer prepared by combining amino acid polymers with multiple molecules of peroxidase (PO) and secondary antibody which is reduced to Fab' fragment. To eliminate background staining, solid-phase adsorption of secondary antibody is conducted with mouse serum.

Principle & Procedure

[Diagram showing the staining process]

Advantages

1. No reaction to endogenous mouse immunoglobulin (Page 25).
2. Simplified staining steps*.
3. High sensitivity*.
4. No background due to unaffected by endogenous biotin*.
5. Ready to use.

Staining Results

- Rabbit anti-Keratin/Cytokeratin antibody
- Goat anti-CD3-ε(M-20) antibody
- Rat anti-Mouse CD45R/B220

Product

<table>
<thead>
<tr>
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<th>Code</th>
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<td>N-Histofine® Simple Stain™ Mouse MAX PO (G)</td>
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<td>17ml</td>
<td>414311F</td>
<td>Rat primary antibody</td>
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**Staining Results**

Mouse colon stained with **Histofine® MOUSESTAIN KIT** and DAB chromogen.
- Nuclear staining of Epithelium cells is observed.
- Intense staining of smooth muscle and muscularis propria in lamina propria mucosae is observed.

**Product**

Detection system. Liquid. Ready to use.

<table>
<thead>
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<th>Product Name</th>
<th>For (slides)</th>
<th>Volume Code</th>
<th>Code</th>
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<tbody>
<tr>
<td><strong>Histofine® MOUSESTAIN KIT</strong></td>
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Enzyme substrate for peroxidase-based IHC staining

**N-Histofine® DAB-2V**

### Feature

*N-Histofine® DAB-2V* is used as chromogen-substrate reagents for peroxidase-based immunohistochemical staining as well. DAB produces brown precipitates at the site of the target antigen or nucleic acid reacting with peroxidase. Higher sensitivity and simplified preparation are available in comparison with prior *N-Histofine® DAB-3S* kit.

### Reagent preparation

2. Higher sensitivity than that of other DAB substrate kits.
3. Prepared substrate solution is available for two weeks.

### Procedure

1. Absorption and wipe
2. Remove PBS or TBS around tissue section.
3. Apply prepared substrate solution using dropping bottle covering tissue section completely.
4. Wash the slides with tap water, counterstain and mount.

### Advantages

2. Higher sensitivity than that of other DAB substrate kits.
3. Prepared substrate solution is available for two weeks.

### Staining Results

- **Rabbit anti-CD3 antibody** (clone:SP7)
  - Human tonsil cancer
  - Positive reaction is observed in cytomembrane of sporadic tumor cells.

- **Mouse anti-Smooth Muscle Actin** (clone:1A4)
  - Human small intestine
  - Positive reaction is observed in cytoplasm of sporadic smooth muscle cells.

### Product

**Enzyme Substrate system**

<table>
<thead>
<tr>
<th>Product Name</th>
<th>425312F</th>
<th>425314F</th>
<th>Mark in EU</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>N-Histofine® DAB-2V</em></td>
<td>500 tests</td>
<td>1,500 tests</td>
<td></td>
</tr>
<tr>
<td>Reagent A: DAB solution concentrate</td>
<td>1.2 ml x2</td>
<td>30 ml x2</td>
<td></td>
</tr>
<tr>
<td>Reagent B: DAB buffer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tube</td>
<td>x1</td>
<td>x1</td>
<td></td>
</tr>
<tr>
<td>Chip (For Reagent B)</td>
<td>x1</td>
<td>x1</td>
<td></td>
</tr>
<tr>
<td>Dropping bottle</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Addition and reaction of primary antibody**

Remove PBS around tissue sections. Apply primary antibody or negative control (normal serum) to each slide covering sections completely. (at RT or 4°C)

Rinse the slides in PBS. (at RT for 5 min. x 3)

**Addition and reaction of \(\mathbf{N}\)-Histofine® Substrate Kit of Enzyme Substrate Systems**

Remove PBS around tissue sections. Apply \(\mathbf{N}\)-Histofine® Substrate Kit to each slide covering sections completely. (at RT for 5-20 min.)

*User’s substrate solution is available as well.

Wash the slides with distilled water.

**Counter staining**

**Mounting**

As for alcohol soluble chromogens such as AEC, tissue sections are mounted with permanent mounting media, aqueous, without further treatment. As for chromogens insoluble in organic solvent such as DAB, tissue sections are mounted with permanent mounting media, non-aqueous, after washing with distilled water, dehydration in graded series of alcohol and clearing in xylene.

- See instruction for use of primary antibody for further details such as quenching with endogenous peroxidase and high temperature epitope unmasking methods.
- Use moist chamber for slide incubation.
**Histofine® Simple Stain™ AP**

- **Deparaffinization and Rehydration (for paraffin-embedded tissue sections)**
  
  - xylene 3 min.
  - xylene 3 min.
  - 100% ethanol 3 min.
  - 95% ethanol 3 min.
  - PBS 5 min.

  - Remove excess xylene and ethanol at each step.
  - Change xylene and ethanol as appropriate to complete deparaffinization and rehydration.

- **Addition and reaction of primary antibody**

  - Primary antibody or negative control

  - Remove PBS around tissue sections.

  - Apply primary antibody or negative control (normal serum) to each slide covering sections completely.

  - Rinse the slides in PBS. (at RT for 5 min. x 3)

- **Addition and reaction of Histofine® Simple Stain™ AP**

  - Histofine® Simple Stain™ AP

  - Remove PBS around tissue sections.

  - Apply Histofine® Simple Stain™ AP to each slide covering sections completely. (at RT for 30 min.)

  - Rinse the slide in PBS. (at RT for 5 min. x 3)

  - Rinse the slides in TBS. (at RT for 5 min.)

- **Addition and reaction of Histofine® Substrate Kit of Enzyme Substrate Systems**

  - Histofine® New Fuchsin Substrate Kit

  - Remove PBS around tissue sections.

  - Apply Histofine® Substrate Kit to each slide covering sections completely. (at RT for 5-20 min.)

  - Wash the slides with distilled water.

  - User’s substrate solution is available as well.

- **Counter staining**

- **Mounting**

  - As for New Fuchsin substrate kit, tissue sections are mounted with permanent mounting media, aqueous, or air-dried, cleared in xylene for a few seconds and mounted with permanent mounting media, non-aqueous.

  - See instruction for use of primary antibody for further details such as quenching with endogenous peroxidase and high temperature epitope unmasking methods.

  - Use moist chamber for slide incubation.

**Histofine® New Fuchsin Substrate Kit**

- Add 1 drop of Reagent A and 1 drop of Reagent B

- Mix

- Add 2 mL of distilled water

- Mix

- Add 2 drops of Reagent C

- Mix

- Add 1 drop of Reagent D

- Mix

- Use within 30 min.
Advantages of N-Histofine® Simple Stain™ Series

Simplified staining steps

N-Histofine® Simple Stain™

Primary antibody (RT or 4°C)

N-Histofine® Simple Stain™
(RT 30min.)

Protein Blocking (RT 10min.)

Primary antibody (RT or 4°C)

Biotinylated Secondary antibody (RT 10min.)

Streptavidin-enzyme conjugate (RT 5-10 min.)

Counterstaining

Mounting

Conventional Streptavidin-biotin System

Background staining

Biotin: Streptavidin-enzyme conjugate

Biotinylated secondary antibody

Biotin

High sensitivity

N-Histofine® Simple Stain™

Primary antibody

Tissue section

Antigen

N-Histofine® Simple Stain™

Primary antibody

Tissue section

Antigen

Conventional Streptavidin-biotin System

No background due to unaffected by endogenous biotin

N-Histofine® Simple Stain™

Primary antibody

Tissue section

Antigen

N-Histofine® Simple Stain™

Primary antibody

Tissue section

Antigen

Conventional Streptavidin-biotin System

Background staining

Biotin

Biotinylated secondary antibody

MAX PO, Mouse MAX PO, Rat MAX PO and AP

Contents
Technical Report
**Technical Report 1**

Application of *Histofine® MOUSESTAIN, Mouse MAX and Rat MAX* for *mouse and rat frozen tissue sections*

*Histofine® MOUSESTAIN, Mouse MAX and Rat MAX* for mouse and rat, paraffin embedded tissue sections, are applicable for IHC staining with frozen tissue sections as well by following procedures.

**Staining of frozen tissue sections with MOUSESTAIN KIT**

**MOUSESTAIN KIT** is available for fixed frozen tissues without any change or addition on its procedure. (Principle & Procedure on page 14.)

1. **Frozen tissue sections**
   - Fixed frozen tissues* are only applicable.
   
   *There are two different types of frozen tissues, Fresh frozen tissue and Fixed frozen tissue, used for IHC staining.
   - The fresh frozen tissue should be frozen immediately after the tissue obtained.
   - The fixed frozen tissue should firstly be fixed and then frozen.

2. **Fixative solution**
   - Apply fixative solution appropriate for primary antibody.

3. **Concentration and reaction time of reagents**
   - Apply equivalent concentration and reaction time of the respective reagents to those for paraffin embedded tissue sections.
   - In some preparation of frozen tissue sections, or regarding mouse lineages, tissues or fixing method, background staining may be observed in this regard.

**Staining procedure**

Add primary antibody.  
30 min.  
Add polymer.  
Add substrate solution.  
Counterstaining  
Mounting

**Firstly**  
**Step A** for background staining observed by this staining procedure

**Secondly**  
**Step B**  
**Step A** for background staining still observed by Step A

---

**Staining of frozen tissue sections with *Histofine®* MOUSESTAIN, Mouse MAX and Rat MAX for mouse and rat frozen tissue sections**

Following Step A firstly and Step B-A secondly are recommended before the reaction with substrate solution to eliminate background staining observed by the staining procedure. (Principle & Procedure on pages 12 and 13.)

1. **Frozen tissue sections**
   - Both fixed frozen tissues and fresh frozen tissues are applicable.

2. **Fixative solution**
   - Apply fixative solution appropriate for primary antibody.
1. **Step A**  Adjust reaction time of polymer.

30 min. of reaction time of polymer is designed for paraffin embedded sections.
For frozen tissue sections, apply adequate reaction time* reducing the duration between 10 to 30 min. when some background staining is observed by 30 min. reaction.

*The reaction time depends on mouse or rat lineages, tissues or fixing methods.

---

2. **Step B**  **Step A**  Add 0.2% of glutaraldehyde (GA) solution before the Step A.

Blocking with 0.2% of GA solution for 10 min.* may reduce background staining which is still observed by Step A.
Identify the absence of inhibition on the reaction of applied primary antibody prior to use of the GA solution.

*The effect of the blocking depends on mouse or rat lineages, tissues or fixing methods.

**Dilution with SIGMA G7651**
Dilute 250 times of SIGMA G7651, 50% concentration of GA, with PBS by 0.2% solution.
Others should be equivalent to above dilution.

---

### Background eliminated case by **Step A**

**Detection system** : Simple Stain™ Rat MAX PO (MULTI)
**Tissue sections** : rat normal colon
**Fixative solution** : 4% of PFA (at 4 °C for overnight)
**Primary antibody** : PBS (in place of primary antibody)
**Chromogen** : DAB

---

### Background eliminated case by **Step B**  **Step A**

**Detection system** : Simple Stain™ Mouse MAX PO (R)
**Tissue sections** : mouse normal colon
**Fixative solution** : 4% of PFA (at 4 °C for 10 min.)
**Primary antibody** : PBS (in place of primary antibody)
**Chromogen** : DAB

---

30 min. of reaction time of polymer is designed for paraffin embedded sections.
For frozen tissue sections, apply adequate reaction time* reducing the duration between 10 to 30 min. when some background staining is observed by 30 min. reaction.

*The reaction time depends on mouse or rat lineages, tissues or fixing methods.
IHC Triple-staining Method
The IHC triple-staining method with **Histofine® Simple Stain™ MAX PO (M), Histofine® Simple Stain™ AP (M) and three different murine primary antibodies

I. OBJECTIVE
Detection of three different antigens at different locations within the same tissue section

II. SPECIMENS
20% buffered formalin-fixed and paraffin-embedded tissue section

III. TECHNICAL ADVICE (Staining orders)

1st Detection of Antigen:
Detection of small amount of antigen with BCIP/NBT (Blue)

2nd Detection of Antigen:
Detection of cytoplasmic antigen or large amount of antigen with New Fuchsin (Red)

3rd Detection of Antigen:
Detection of nuclear antigen or moderate to large amount of antigen with DAB (Brown)

IV. STAINING PROCEDURES
1. Deparaffinization and Rehydration
   1-1. Immerse the slide in xylene at RT 3 times for 3 min each.
   1-2. Immerse the slide in 100% ethanol at RT 2 times for 3 min each.
   1-3. Immerse the slide in 95% ethanol at RT 2 times for 3 min each.
   1-4. Rinse the slide in PBS at RT for 5 min.

2. Antigen Retrieval of the 1st primary antibody
   2-1. Refer to the instruction for use of the 1st primary antibodies and conduct proper antigen retrieval depend on the 1st primary antibody with specific buffer, specified temperature and incubation time, if necessary.
   2-2. Allow the slide to cool down at RT for 20 - 60 min.
   The slide should be cooled down slowly.
   Rinse the slide in PBS at RT 3 times for 5 min each.

3. Protein Blocking
   Apply 10% Goat normal serum at RT for 10 min.

4. Add 1st Primary Antibody
   4-1. Apply 1st primary antibody at 37 °C for 1 hour.
   4-2. Rinse the slide in PBS at RT 3 times for 5 min each.

5. Add **Histofine® Simple Stain™ AP (M)
   5-1. Apply **Histofine® Simple Stain™ AP (M) at RT for 30 min.
   5-2. Rinse the slide in PBS at RT 3 times for 5 min each.
   5-3. Rinse the slide in TBS at RT for 5 min.

6. Add BCIP/NBT substrate
   6-1. Apply BCIP/NBT substrate solution.
   Adjust the incubation time by microscopic observation.
   6-2. Wash the slide with distilled water at RT for 5 min.

7. Antigen Retrieval of the 2nd primary antibody
   7-1. Conduct Method-A or Method-B depend on the 2nd primary antibody

Method-A for the 2nd primary antibody NO Antigen Retrieval required
   1) Fill the heat-resistant plastic staining jar with 10 mM Sodium citrate buffer at pH 6.0 and heat to 95 °C.
   2) Immerse the slide in the jar at 95 °C for 10 min*.
   3) Allow the slide to cool down at RT for 20-60 min.
   *Activation treatment for the 2nd primary antibody and the enzyme conjugated polymer of the 1st Detection of Antigen

Method-B for the 2nd primary antibody Antigen Retrieval required
   1) Fill the heat-resistant plastic staining jar with the specific buffer referring the instruction for use of the 2nd primary antibody and heat to 95 °C.
   2) Immerse the slide in the jar at 95 °C for 40 min**.
   3) Allow the slide to cool down at RT for 20-60 min.
   **Activation treatment for the 2nd primary antibody of the 2nd Detection of Antigen, which is able to be combined with the treatment of above Method A

7-2. Rinse the slide in PBS at RT 3 times for 5 min each.

8. Protein Blocking
   Apply 10% Goat normal serum at RT for 10 min.

9. Add 2nd Primary Antibody
   9-1. Apply 2nd primary antibody at 37 °C for 1 hour.
   9-2. Rinse the slide in PBS at RT 3 times for 5 min each.

10. Add **Histofine® Simple Stain™ AP (M)
   10-1. Apply **Histofine® Simple Stain™ AP (M) at RT for 30 min.
   10-2. Rinse the slide in PBS at RT 3 times for 5 min each.
   10-3. Rinse the slide in TBS at RT for 5 min.

11. Add New Fuchsin substrate
   11-1. Apply New Fuchsin substrate solution.
   Adjust the reaction time by microscopic observation.
   11-2. Wash the slide with distilled water at RT for 5 min.

12. Antigen Retrieval of the 3rd primary antibody
   12-1. Conduct method-A or method-B depend on the 3rd primary antibody

Method-A for the 3rd primary antibody NO Antigen Retrieval required
   1) Fill the heat-resistant plastic staining jar with 10 mM Sodium citrate buffer at pH 6.0 and heat to 95 °C.
   2) Immerse the slide in the jar at 95 °C for 10 min*.
   3) Allow the slide to cool down at RT for 20-60 min.
   *Activation treatment for the 2nd primary antibody and the enzyme conjugated polymer of the 2nd Detection of Antigen

Method-B for the 3rd primary antibody Antigen Retrieval required
   1) Fill the heat-resistant plastic staining jar with the specific buffer referring the instruction for use of the 3rd primary antibody and heat to 95 °C.
   2) Immerse the slide in the jar at 95 °C for 40 min**.
   3) Allow the slide to cool down at RT for 20-60 min.
   **Activation treatment for the 3rd primary antibody of the 3rd Detection of Antigen, which is able to be combined with the treatment of above Method A

12-2. Rinse the slide in PBS at RT 3 times for 5 min each.
13. Quenching of Endogenous Peroxidase
13-1. Immerse the slide in 3% H₂O₂ solution in absolute methanol at RT for 10 min.
13-2. Rinse the slide in PBS at RT 3 times for 5 min each.

14. Protein Blocking
   Apply 10% Goat normal serum at RT for 10 min.

15. Add 3rd Primary Antibody
15-1. Apply 3rd primary antibody at 37 °C for 1 hour.
15-2. Rinse the slide in PBS at RT 3 times for 5 min each.

16. Add -Histofine® Simple Stain™ MAX PO (M)
16-1. Apply -Histofine® Simple Stain™ MAX PO (M) at RT for 30 min.
16-2. Rinse the slide in PBS at RT 3 times for 5 min each.

17. Add DAB substrate
17-1. Apply DAB substrate solution.
17-2. Wash the slide with distilled water at RT for 5 min.

18. Mounting
   While the slide is wet by water, apply one drop of permanent mounting media, aqueous, and fix with cover slip.

V. STAINING RESULTS

Case 1: Human Reactive Lymph Node
1. OBJECTIVE
   Observe three types of stained cells in a tissue section.

2. SPECIMENS
   Human Reactive Lymph Node

3. Used primary antibodies, antigen retrievals, detections and chromogens
   1st Detection of Antigen:
   Primary Antibody: CD8
   Antigen Retrieval: 1mM buffered EDTA at pH 8.0, 95 °C for 40 min.
   Detection system: -Histofine® Simple Stain™ AP (M)
   Chromogen: BCIP/NBT (Blue)

   2nd Detection of Antigen:
   Primary Antibody: CD4
   Antigen Retrieval: 1mM buffered EDTA at pH 8.0, 95 °C for 40 min.
   Detection system: -Histofine® Simple Stain™ AP (M)
   Chromogen: New Fuchsin (Red)

   3rd Detection of Antigen:
   Primary Antibody: CD20cy
   Antigen Retrieval: 10mM Sodium citrate buffer at pH 6.0, 95 °C for 40 min.
   Detection system: -Histofine® Simple Stain™ MAX PO (M)
   Chromogen: DAB (Brown)

4. Staining Images

Case 2: Cervical Squamous Cell Carcinoma
1. OBJECTIVE
   Observe three types of stained cells in a tissue section.

2. SPECIMENS
   Cervical Squamous Cell Carcinoma

3. Used primary antibodies, antigen retrievals, detections and chromogens
   1st Detection of Antigen:
   Primary Antibody: Beta-catenin
   Antigen Retrieval: 1mM buffered EDTA at pH 8.0, 95 °C for 40 min.
   Detection system: -Histofine® Simple Stain™ AP (M)
   Chromogen: BCIP/NBT (Blue)

   2nd Detection of Antigen:
   Primary Antibody: Cytokeratin (AE1/AE3)
   Antigen Retrieval: 10mM Sodium citrate buffer at pH 6.0, 95 °C for 40 min.
   Detection system: -Histofine® Simple Stain™ AP (M)
   Chromogen: New Fuchsin (Red)

   3rd Detection of Antigen:
   Primary Antibody: Ki-67 antigen
   Antigen Retrieval: 10mM Sodium citrate buffer at pH 6.0, 95 °C for 40 min.
   Detection system: -Histofine® Simple Stain™ MAX PO (M)
   Chromogen: DAB (Brown)

4. Staining Images

VI. ADVICE FOR STAINING

VI-1. Staining Schedule
   Two-day separated completion of all the steps of the IHC triple-staining is available under the following conditions.
   1st day
   The reaction condition of 1st primary antibody should be at 4 °C for overnight.
   2nd day
   The reaction condition of both 2nd and 3rd primary antibodies should be at 37 °C for 1 hour.
### VI-2. Chromogens Preparations

#### 1. BCIP/NBT substrate solution

1-1. Reagents preparation

<table>
<thead>
<tr>
<th>Substrate buffer</th>
<th>(store at 2-8 °C):</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mM Tris-HCl Buffer (100 mM sodium chloride, 50mM MgCl₂), pH 9.5</td>
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<tr>
<td>Adjust pH with HCl.</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>NBT stock solution</th>
<th>(store at −20 °C):</th>
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</thead>
<tbody>
<tr>
<td>Dissolve 75 mg of NBT (Nitro Blue Tetrazolium, SIGMA) in 1 ml of 70% N,N-dimethylformamid.</td>
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</table>

<table>
<thead>
<tr>
<th>BCIP stock solution</th>
<th>(store at −20 °C):</th>
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</thead>
<tbody>
<tr>
<td>Dissolve 50 mg of BCIP (5-Bromo-4-Chloro-3-Indolyl Phosphate-p-Toluidine salt, SIGMA) in 1 ml of N,N-dimethylformamid.</td>
<td></td>
</tr>
</tbody>
</table>

1-2. Substrate solution preparation

Add 5 μl of BCIP stock solution and 6.5 μl of NBT stock solution to 1.5 ml of Substrate buffer and mix well.

Use the solution within 30 min after preparation.

#### 2. New Fuchsin substrate solution

2-1. Reagents preparation

<table>
<thead>
<tr>
<th>Naphthol AS-BI phosphate solution</th>
<th>(Use within 30 min after preparation):</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dissolve 10 mg of naphthol AS-BI phosphoric acid (SIGMA) in 100 μl of N,N-dimethylformamid.</td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>New Fuchsin solution</th>
<th>(store at 2-8 °C):</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dissolve 4.0 g of New Fuchsin powder (MERCK) in 100 ml of 2N HCl and filter the solution.</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>4 % Sodium Nitrite solution</th>
<th>(Use within 30 min after preparation):</th>
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</thead>
<tbody>
<tr>
<td>Dissolve 40 mg of Sodium Nitrite in 1ml of distilled water</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>0.2 M Tris-HCl buffer</th>
<th>(store at room temperature):</th>
</tr>
</thead>
<tbody>
<tr>
<td>200mM Tris-HCl buffer, pH 8.2-8.3</td>
<td></td>
</tr>
<tr>
<td>Adjust pH with HCl.</td>
<td></td>
</tr>
</tbody>
</table>

2-2. Substrate solution preparation

Mix 100 μl of New Fuchsin solution and 100 μl of 4 % Sodium Nitrite solution and incubate for 1 min.

Add 40ml of 0.2N Tris-HCl buffer to the mixture.

Add 100 μl of Naphthol AS-BI phosphate solution to the mixture while stirring constantly.

Use the solution immediately after Filtration.

#### 3. DAB substrate solution

Dissolve and mix following reagents and stir the solution.
Use the solution within 30 min after preparation.

- 10 mg of 3,3’-Diaminobenzidine, tetrahydrochloride
- 50 ml of 0.05 M Tris-HCl buffer, 15mM NaN₃ pH 7.6
- 50 μl of 5 % H₂O₂ in distilled water
- 34 mg of Imidazole
Advantages of Immunohistochemical polymer detection systems designed for mouse & rat tissue sections

Introduction

When the immunohistochemical detection systems for human tissue sections are used for staining on mouse and rat tissue sections, background staining may be caused due to such reactivity with endogenous immunoglobulins of mouse and rat in the tissue sections. Therefore, the immunohistochemical detection systems designed for staining on mouse and rat tissue sections were developed. Background staining is compared among those detection systems for mouse, rat, and human tissue sections on mouse/rat tissue sections.

Materials & Methods

Materials

- Formalin-fixed paraffin-embedded mouse and rat tissue sections
- 
  - Histofine® Simple Stain™ Mouse MAX PO (Rat): rat primary antibody for mouse tissue sections
- 
  - Histofine® Simple Stain™ Rat MAX PO (M): mouse primary antibody for rat tissue sections
- 
  - Histofine® Simple Stain™ MAX PO (M): mouse primary antibody for human tissue sections

Methods

To compare background staining between polymers, immunohistochemical staining on mouse tissue sections was conducted with 
  - Histofine® Simple Stain™ Mouse MAX PO (Rat) and 
  - Histofine® Simple Stain™ MAX PO (M). (Fig.1)

To compare background staining between polymers, immunohistochemical staining on rat tissue sections was conducted with 
  - Histofine® Simple Stain™ Rat MAX PO (M) and 
  - Histofine® Simple Stain™ MAX PO (M). (Fig.2)

PBS was used in place of primary antibody to identify the background staining caused by polymers. DAB solution was used for brown color development.

Steps of immunohistochemical staining

1. Quenching of endogenous peroxidase
2. Incubation with PBS for 30 min.
3. Incubation with polymer for 30 min.
4. Incubation with DAB solution for 10 min.
5. Counterstaining
6. Mounting

Results

It was found that the background staining by endogenous immunoglobulins was not observed when the immunohistochemical staining was conducted with those polymer detection systems designed for mouse and rat.

Fig.1 Staining comparison of Mouse tissue sections

Fig.2 Staining comparison of Rat tissue sections
1. Staining is not observed or weak staining is observed on positive control slide and specimen slide.

- 1) Drying-out of tissue sections during staining prior to addition of reagents.
- 2) Embedding agent used is not suitable, or paraffin is not thoroughly removed from paraffin-embedded tissue sections.
- 3) Any trace amount of sodium azide present in buffer inactivates peroxidase, such staining may not be available.
- 4) Inadequate incubation of enzyme and antibody.

2. Specimen slide is not stained while positive control slide is stained.

- 1) Antigen is denatured or masked during fixing or embedding process.
- 2) Antigen is decomposed by autolysis.
- 3) Amount of antigen is few in tissue sections.

3. Backgrounds are intensively stained on all the slides.

   - Peroxidase staining
     - 1) Endogenous enzyme activity was not completely blocked.
     - 2) Non-specific staining is found.
   - Alkaline phosphatase staining
     - 1) Endogenous enzyme activity was not completely blocked.
     - 2) Non-specific staining is found.

4. During reaction, tissue sections come off from slides.

   - 1) Heat induced antigen retrieval procedure some antigens required or prolonged reaction time with primary antibody may promote tissue sections come off easier.

---

### Problem Possible cause Solution

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Staining is not observed or weak staining is observed on positive control slide and specimen slide.</td>
<td>1) Drying-out of tissue sections during staining prior to addition of reagents. 2) Embedding agent used is not suitable, or paraffin is not thoroughly removed from paraffin-embedded tissue sections. 3) Any trace amount of sodium azide present in buffer inactivates peroxidase, such staining may not be available. 4) Inadequate incubation of enzyme and antibody.</td>
<td>• Prevent tissue sections from drying out. • Use suitable embedding agent or remove paraffin thoroughly from tissue sections embedded. • Change xylene or ethanol in some cases. • Use sodium azide free buffer solution. • Change buffer solution.</td>
</tr>
<tr>
<td>2. Specimen slide is not stained while positive control slide is stained.</td>
<td>1) Antigen is denatured or masked during fixing or embedding process. 2) Antigen is decomposed by autolysis. 3) Amount of antigen is few in tissue sections.</td>
<td>• Change stale chromogen-substrate reagent. • Remove excess solution thoroughly at each stage. • Allow antibody sufficient time to react. In particular, primary antibody should be incubated for the specified time in its instruction for use. • Some antigens are sensitive to fixation or embedding. Therefore use less potent fixative and shorten the fixing time. • Heat-Induced Epitope Retrieval or treatment with proteolytic enzyme protease may be required for some tissues to reveal antigens before staining. • Use tissues obtained by biopsy or surgery. • Prolong reaction time of reagent at each step.</td>
</tr>
<tr>
<td>3. Backgrounds are intensively stained on all the slides.</td>
<td>1) Endogenous enzyme activity was not completely blocked. 2) Non-specific staining is found.</td>
<td>• Ensure treatment with 3% of hydrogen peroxide added methanol to inactivate endogenous peroxidase activity. • Before adding primary antibody, treat with 10% normal goat or rabbit serum as follows.</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Product name</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Simple Stain™ MAX PO (M)</td>
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<td>Add Levamisole to chromogen-substrate solution. To reduce endogenous enzyme activity, chromogen-substrate solution containing 1mM Levamisole should be used.</td>
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<tr>
<td>4. During reaction, tissue sections come off from slides.</td>
<td>1) Heat induced antigen retrieval procedure some antigens required or prolonged reaction time with primary antibody may promote tissue sections come off easier.</td>
<td>• Use fresh tissues whenever available. • Change xylene or ethanol in some cases. • Ensure thorough washing of antibody. • Keep room temperature at 15 to 25°C. • Shorten reaction time of enzyme. • Prevent tissue sections from drying out. • Mount tissue sections on slides coated with an adhesive such as 0.02% poly-L-lysine or silane.</td>
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References

Human Tissue Sections


Mouse • Rat Tissue Sections


ALK Detection KIT

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<th>Code</th>
<th>For use with</th>
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For Human Tissue Sections

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Histofine® ALK Control Slides

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Storage

Store in a dark place at 2 to 8 °C.