For research use only

September 2011

N-Histofine®
ALK Detection Kit

Package: 20 tests
Storage: Store at 2-8°C

Code: 417071F

PRECAUTIONS

1. Read these instructions carefully before use.
2. Handle specimens with care in view of risks of infection.

KIT COMPONENTS

Vial No.	Reagents	Volume
1	**Blocking Reagent** 3 V/V% Hydrogen peroxide	4 ml ×1
2	**Primary Antibody** anti-ALK mouse monoclonal antibody clone: 5A4	2 ml ×1
3	**Negative Control** Mouse IgG	2 ml ×1
4	**Bridge Reagent**	4 ml ×1
5	**Peroxidase Labeled Empower Reagent**	4 ml ×1
6	**Chromogen Substrate** 3,3'-Diaminobenzidine tetrahydrochloride	0.5 ml ×1
7	**Substrate Buffer Solution**	0.5 ml ×1
8	**Chromogen Reagent** 0.6 V/V% Hydrogen peroxide solution	0.5 ml ×1
9	**ALK Antigen Retrieval Solution A**	150 ml ×1
10	**ALK Antigen Retrieval Solution B**	150 ml ×1

INTENDED USE

This kit uses immunohistochemical (IHC) staining to detect anaplastic lymphoma kinase (ALK) proteins in tumor cells, and to determine the presence or absence of expression of these proteins.

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.[1][2] 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.
In ALCL and inflammatory myofibroblastic tumor, the *ALK* gene has been reported to fuse with genes such as *TFG*, *ATIC, CARS, CLTC, SEC31L1, RANBP2*, and *TPM3*, to form *ALK* fusion genes. The proteins that are produced from these *ALK* fusion genes form dimers, and as a result, the kinase domain of the *ALK* gene was constitutively activated, leading to carcinogenesis.

Recently, fusion of the following genes with the *ALK* gene has been reported: the *EML4* and *KIF5B* genes in non-small cell lung carcinoma, the *SEC31A* and *SQSTM1* genes in ALK-positive large B-cell lymphoma, and the *VCL* gene in renal cell carcinoma.

PRINCIPLE

ALK Detection Kit is composed of reagents for the detection of ALK proteins. The antigen on formalin-fixed paraffin-embedded tissue sections is reacted with Primary Antibody, then reacted with Bridge Reagent. In addition, it is reacted with Peroxidase-Labeled Empowered Reagent. This results in the formation of an antigen・antibody・enzyme complex. Color development is observed when the enzyme in the complex reacts with the substrate. This allows highly sensitive visualization of antigen sites of and reliable confirmation of the presence of antigens by using a light microscope.

Subclass of Primary Antibody: IgG1
Immunogen of Primary Antibody: Recombinant protein consisting of part of the intracellular amino acid sequence (419−520) of the NPM-ALK transcript exhibiting tyrosine kinase activity
Source of Primary Antibody: Culture supernatant of hybridoma

STAINING PROCEDURE

1. Staining Flowchart

Deparaffinization and rehydration
↓
Antigen unmasking treatment
↓
Quenching of endogenous peroxidase
↓
Reaction with Primary Antibody or Negative Control
↓
Reaction with Bridge Reagent
↓
Reaction with Peroxidase Labeled Empower Reagent
↓
Reaction with Chromogen/Substrate Reagent
↓
Counter staining
↓
Mounting

2. Specimen Preparation

For optimal fixation to maintain tissue morphology and antigen activity, it is recommended to use tissue sections as fresh and as small (approx. 1 cm × 1 cm × 0.5 cm) as possible, and the fixatives shown below:

Fixative	Fixing duration
10% formalin or buffered formalin	24–48 hours
20% formalin	12–24 hours

3. Preparation of Sections and Slides

(1) Paraffin-Embedded Tissue Sections

Slice sections into 4-µm thickness and place on poly-L-lysine or silane-coated slides. Place in incubator at 37°C and dry for 24 hours.

(2) Specimen Sample Slides

Prepare 2 slides per specimen as specimen sample slides.
Use one as the Reagent Control slide in the staining process with the Negative Control, Mouse IgG, instead of the Primary Antibody.

(3) Specimen Control Slides

a. Positive Control Slide

Slide is prepared with the same method as the specimen sample slides; presence of ALK protein overexpression is confirmed in advance.

b. Negative Control Slide

Slide is prepared with the same method as the specimen sample slides; absence of ALK protein overexpression is confirmed in advance.

Conduct the entire process, from specimen preparation to staining and microscopic observation, after preparing and reviewing specimen control slides, specimen sample slides, and reagent control slides.

4. Procedure

(1) Reagents and Materials required but not provided

・Slide glass (poly-L-lysine or silane-coated)
・Drying apparatus ・Timer
・Staining racks or Coplin jars
・Heat-resistant plastic staining racks or jars
・Water bath
・Thermometer
・Xylene ・95% Ethanol ・100% Ethanol
・Washing container or bottle
・Washing solution (PBS)

PBS (Phosphate-buffered saline, pH 7.6 ± 0.2)

NaCl	7.75 g
K_2HPO_4	1.50 g
KH_2PO_4	0.20 g
Distilled water	1 L

・Purified water
・Counterstaining solution (Hematoxylin)
・Mounting media (Permanent mounting media)
・Test tubes ・Slide stand
・Humidified chamber for slide incubation (Moist chamber)
・Cover glass
・Tissue paper ・Light microscope

(2) Reagent Preparation

1) Preparation of ALK Antigen Retrieval Solution
Measure 1 part ALK Antigen Retrieval Solution A to 1 part ALK Antigen Retrieval Solution B to 8 parts distilled water and mix these well, and place gently in heat-resistant Coplin jar.
Note: Prepare required quantity accordingly and discard after use. Do not apply used solution repeatedly.

2) Preparation of Substrate Solution
Prepare Substrate Solution just before use.
Add 1 drop of Chromogen Substrate and Substrate Buffer Solution, respectively, to 1 ml purified water and mix well. Then, add 1 drop of Chromogen Reagent and mix again. Usage within 30 minutes of preparation is recommended.

3) Other reagents
Apply as supplied.

(3) Deparaffinization and Rehydration

1) Treatment with xylene
(a) Immerse the slides in xylene for 3 minutes.
(b) Remove excess fluid and immerse in xylene for 3 minutes.
(c) Remove excess fluid and immerse in fresh xylene for 3 minutes.

2) Treatment with ethanol
(a) Immerse in 100% ethanol for 3 minutes.
(b) Remove excess fluid and immerse in fresh 100% ethanol for 3 minutes.
(c) Remove excess fluid and immerse in 95% ethanol for 3 minutes.
(d) Remove excess fluid and immerse in fresh 95% ethanol for 3 minutes.

3) Washing
Remove excess fluid and rinse well with PBS (3 minutes in each washing container twice, or use a washing bottle).

(4) Treatment with high-temperature epitope unmasking

1) Preheat water bath to 95–99°C.
Note: Avoid burn injuries.
2) Prepare ALK Antigen Retrieval Solution in heat-resistant plastic staining jar and heat to 95–99 °C in water bath. Close lid loosely to prevent excess water evaporation (Avoid sealing completely or jar may be damaged).
3) Immerse slides in the preheated solution and close the lid loosely.
4) Incubate slides at 95–99°C for 40 minutes after verifying that temperature of the solution is elevated to 95–99°C by thermometer.
5) Remove jar from water bath and remove the lid. Leave slides in the jar at room temperature, 15–25 °C, for 20 minutes to cool slowly.
6) Remove slides from the solution and rinse them in PBS (3 minutes in each washing container twice, or use a washing bottle).

5. Staining Procedure

(1) Treatment with Blocking Reagent, 3 V/V% Hydrogen peroxide solution (removal of endogenous peroxidase)

1) Remove excess fluid around tissue sections on the slides carefully.
2) Apply 2 drops (100 µl) of Blocking Reagent to each slide, covering tissue sections completely; incubate in moist chamber at room temperature for 5 minutes.
3) Rinse slides in PBS (3 minutes in each washing container twice, or use a washing bottle).

(2) Addition and Reaction of Primary Antibody, Anti-human ALK Mouse Monoclonal Antibody (5A4), or Negative Control, Mouse IgG

1) Remove excess fluid around tissue sections on the slides carefully.
2) Apply 2 drops (100 µl) of Primary Antibody on specimen, Positive Control, and Negative Control Slides, covering tissue sections completely. For the Reagent Control Slide, apply 2 drops (100 µl) of Negative Control instead of Primary Antibody.
3) Incubate slides in moist chamber at room temperature for 30 minutes.
4) Rinse slides in PBS (3 minutes in each washing container twice, or use a washing bottle).

(3) Addition and Reaction of Bridge Reagent

1) Remove excess fluid around tissue sections on the slides carefully.
2) Apply 2 drops (100 µl) of Bridge Reagent, covering tissue sections completely.
3) Incubate slides in moist chamber at room temperature for 15 minutes.
4) Rinse slides in PBS (3 minutes in each washing container twice, or use a washing bottle).

(4) Addition and Reaction of Peroxidase-Labeled Empower Reagent

1) Remove excess fluid around tissue sections on the slides carefully.
2) Apply 2 drops (100 µl) of Peroxidase-Labeled Empower Reagent, covering tissue sections completely.
3) Incubate slides in moist chamber at room temperature for 30 minutes.
4) Rinse slides in PBS (3 minutes in each washing container twice, or use a washing bottle).

(5) Addition and Reaction of Substrate Solution

1) Remove excess fluid around tissue sections on the slides carefully.
2) Apply 2 drops (100 µl) of Substrate Solution prepared in advance, covering tissue sections completely.
3) Incubate slides in moist chamber at room temperature for 10 minutes.
4) Rinse slides with purified water.

(6) Counterstaining

1) Immerse slides in counterstaining solution.
2) Wash slides under running water.

(7) Mounting

Mount tissue sections with permanent mounting media after dehydration and clearing in xylene.

INTERPRETATION OF STAINING RESULTS

1. Procedure

Examine slides under light microscope for positive staining results. Interpret staining results by comparing with the following 3 control slides:

a. Positive Control Slide
ALK protein overexpression in tumor cells as positive result is observed.

b. Negative Control Slide
ALK protein overexpression in tumor cells as positive result is not observed.

c. Reagent Control Slide
ALK protein overexpression in tumor cells as positive result is not observed. There is also considerable non-specific reaction by non-specific protein binding when this slide is stained.

Caution

This kit reacts not only with ALK fusion proteins but also with the full-length ALK protein. That is why this kit shows slight positivity or positivity for tumors*1 that rarely express the full-length ALK protein. This kit cannot distinguish between ALK fusion proteins and the full-length ALK protein. Therefore, in samples, which show a positivity with this kit, a confirmation of the presence or absence of *ALK* fusion genes by using the fluorescence in situ hybridization (FISH) or RT-PCR method is preferable.

*1: Large-cell neuroendocrine carcinomas of the lung, small-cell lung carcinomas, and also rhabdomyosarcomas (particularly alveolar rhabdomyosarcomas).

2. Notes for Interpretation

a. Interpret staining results consistently by comparing the staining results of each specimen control slide.
b. Complete removal of embedding agent is important to achieve clear staining. Residual paraffin causes strengthening of background staining.
c. False positive results may be observed in general due to non-immunological binding of proteins and substrate reaction products. False positive results are caused by red blood cells and may be caused by reaction of peroxidase or reaction of endogenous peroxidase by cytochrome C as well.
d. Interpret staining results of necrotized areas of specimen tissue carefully by comparing with negative control slides, as it is easy for the antigen to bind non-specifically, which is liable to be a cause of non-specific staining.
e. Interpret staining results carefully by comparing with negative control slides, as the interstitial line of collagen possesses hydrophobic properties after fixing and is easily bound with antibody, and positively charged antibody, negatively charged for its own, which is liable to be a cause of non-specific staining.
f. Some granulocytes and macrophages possess Fc receptors on their cytomembrane surfaces; these are likely to bind with the Fc domain of the antibody. Interpret staining results carefully by consistently comparing with negative control slides, as some staining may be found away from the original, specific reaction site of the antibody.

CAUTIONS

1. Cautions for Handling

a. Handle specimens with adequate care due to the possibility of infection risk.
b. Handle specimen tissues with adequate care due to the possibility of infection risks such as HIV and HBV.
c. Avoid contact of reagents with skin.
d. Due to its mutagenicity, handle the Chromogen Substrate, 3,3′-Diaminobenzidine·4HCl, with adequate care .
e. Due to the hydrogen peroxide solution contained, handle the Blocking and Chromogen Reagents with adequate care.

2. Notes for Use

a. Bring all reagents to room temperature (15–25°C) before use.
b. Do not use reagents after their expiry dates.
c. Do not allow tissue sections to dry out at any point during staining. Placing tissue sections in a moist chamber during incubation with reagents is sufficient to prevent drying.
d. Replace xylene and ethanol used for deparaffinization every 40 slides.
e. Do not allow paraffin temperatures to exceed 58°C for embedding tissues due to antigens being sensitive to heat.
f. Do not use reagents mixed with different lots, different lots, manufacturers, or as combinations.

3. Waste Management

a. Primary Antibody and Negative Control should be disposed of by flushing down the drain with copious amounts of water, as there is a risk of explosion by reaction of their sodium azide content with the copper/lead in drainpipes.
b. Prepared Substrate Solution containing DAB, as well as unused solutions and components should be disposed of as hazardous chemicals in accordance with all applicable rules and regulations.
c. Apparatus, reagents, and reagent containers that come in contact with specimen tissues should be sterilized in an autoclave at 120°C for 20 minutes or immersed in disinfectant such as 1 V/V% hypochlorous acid overnight due to infection risks.

TROUBLE SHOOTING

Problem	Possible cause	Solution
No staining or only weak staining on the Positive Control slide and the unknown specimen slide.	1. Tissue section is dry.	1) Place tissue section in moist chamber after moisturizing to prevent drying.
	2. The embedding agent is not suitable, or paraffin has not been thoroughly removed from paraffin-embedded sections.	1) Select a suitable embedding agent or remove paraffin thoroughly from embedded tissue. 2) Change xylene or ethanol solution as the case may be.
	3. Insufficient antigen unmasking treatment (high-temperature epitope unmasking) or antibody reaction.	1) Adjust temperature of Antigen Unmasking Solution to 95－99℃. 2) Incubate for 40 minutes for accurate reaction.
	4. Inadequate incubation of the enzyme and antibody.	1) Change stale Chromogen/Substrate Reagent. 2) Wipe off excess fluid thoroughly at each stage. 3) Provide sufficient time for reaction with antibody. In particular, Primary Antibody should be incubated for the period specified in the package 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. Use less potent fixative and decrease fixing time.
	2. Antigen is decomposed by autolysis.	1) Use tissues obtained by biopsy or surgery whenever possible.
	3. Less antigen is present in the sections.	1) 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 correct.
	2. Autolysis results in excessive antigens isolated in histological solutions.	1) Obtain fresh tissues whenever available.
	3. Insufficient removal of paraffin.	1) Change xylene or ethanol as the case may be.
	4. Insufficient washing of antibody.	1) Ensure thorough washing of antibody.
	5. A high room temperature accelerates enzyme reactions.	1) Keep room temperature at 15–25℃. 2) Shorten reaction time.
	6. Drying-out of specimens during staining after addition of the reagents.	1) Never allow the tissue to dry out.
During the reaction, tissue sections detach from the slides.	1. Some antigens require heat-induced antigen retrieval procedures or prolonged reaction time with the Primary Antibody, which may cause the sections to detach easily.	1) Mount tissue sections on slides coated with an adhesive such as 0.02% poly-L-lysine or silane.

STORAGE AND EXPIRATION

・Storage: Store at 2–8°C
・Expiration: Indicated on the labels of each vial

REFERENCES

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3. Takeuchi K., et al: KIF5B-ALK, a novel fusion oncokinase identified by an immunohistochemistry-based diagnostic system for ALK-positive lung cancer. Clin Cancer Res. 15(9): 3143-3149, 2009
4. Takeuchi K., et al: Pulmonary inflammatory myofibroblastic tumor expressing a novel fusion, PPFIBP1-ALK: reappraisal of anti-ALK immunohistochemistry as a tool for novel ALK-fusion identification. Clin Cancer Res. 17(10): 3341-3348, 2011

INTELLECTUAL PROPERTY

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