Wuhan HealthCare Biotechnology Co., Ltd.
Building #8, Optics Valley Precision Medicine Industry Base,
#9 Gaokeyuan 3rd Road, East Lake High-Tech Zone. 430206, Wuhan, Hubei, China.
Tel#: +86(027)8757-0662 Ext. #812 E-mail: cs@healthcare-bio.com | Website: www.healthcare-biotech.com

**Product Catalogue Number FP002** **For Research Use Only – RUO**

# ALK Gene Amplification Probe Detection Kit

**[Product Name]** ALK Gene Amplification Probe Detection Kit

**[Product Introduction]**

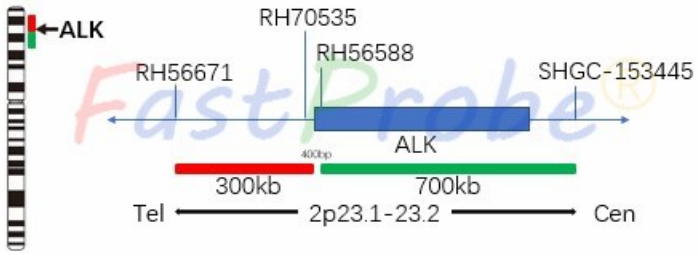
This kit uses Orange Fluorescein and Green Fluorescein labeled ALK probes to detect ALK rearrangements by in situ hybridization.

**[Product Content]**

This kit consists of ALK dual color probe, as shown in Table 1.

**Table 1 Kit composition**

| Component name | Specifications | Quantity | Main components |
|---|---|---|---|
| ALK dual color probe | 100μL/Tube | 1 | ALK Orange probe<br>ALK Green probe |




**[Storage conditions]**

Keep sealed away from light at -20°C±5°C. The product is valid for 12 months. Avoid unnecessary repeated freezing and thawing that should not exceed 10 times. After opening, within 24 hours for short-term preservation, keep sealed at 2-8°C in dark. For long-term preservation after opening, keep the lid sealed at -20°C±5°C away from light.

**[Applicable Instruments]**

1. Fluorescence microscopy imaging systems, including fluorescence microscopy and filter sets suitable for DAPI (367/452), Green (495/517), and Orange (547/565).

2. Automatic hybridization instrument: Strict temperature uniformity is required, and the temperature difference should be ≤1°C.

**[Sample Requirements]**

1. Applicable specimen types: Surgical resection or biopsy tissue paraffin-embedded specimens.

2. Tissue should be fixed in 4% neutral formaldehyde solution within 1 hour after ex vivo. After the tissue is fixed, it is routinely dehydrated and embedded in paraffin.

3. Paraffin section thickness affects the experimental results and slice thickness of 4~5μm is appropriate.

4. It is recommended to choose paraffin-embedded tissue specimens for 5 years preservation time.

**[Related Reagents Preparation]**

①20×SSC, pH 5.3±0.2

Weigh 176g of sodium chloride and 88g of sodium citrate, with 800mL of deionized water to dissolve the aforementioned reagents, at room temperature adjust the pH to 5.3±0.2, with deionized water complete to 1L.
High-pressure steam sterilization, stored at 2~8°C, the shelf life is of 6 months. Discard if the reagent appears cloudy (turbid) or contaminated.

②2×SSC, pH 7.0±0.2

Take 100mL of the above 20xSSC, dilute with 800mL deionized water, mix, adjust the pH to 7.0±0.2 at room temperature, complete to 1L with deionized water, stored at 2~8°C, the shelf life is of 6 months. Discard if the reagent appears cloudy (turbid) or contaminated.

③ Ethanol Solution: 70% ethanol, 85% ethanol
Dilute 700ml, 850ml of ethanol with deionized water to 1L. The shelf life is of 6 months. Discard if the reagent appears cloudy (turbid) or contaminated.

④ Pepsin
Protease Diluent: Take 1000mL distilled water and pour into 1000mL bottle with lid, then add 1.9mL hydrochloric acid (36-38%), mix up and down, adjust to pH ≈2.0, store at 2~8°C for 6 months shelf life.
Protease working solution (0.5%): Take 0.5g pepsin and dissolve in 100mL protease diluent, mix for the current use.

⑤ 0.3% NP-40/0.4 × SSC solution, pH 7.0 ~ 7.5
Take 0.6mL NP-40 and 4mL 20×SSC, add 150mL deionized water, mix, adjust the pH to 7.0 ~ 7.5 at room temperature, with deionized water complete to a volume of 200mL. Stored at 2~8oC, the shelf life is of 6 months. Discard if the reagent appears cloudy (turbid) or contaminated.

⑥ Diamidinophenylindole (DAPI) complex dye agent. Please use the commercially available DAPI staining complex containing anti-quenching agent.

⑦ Xylene

**[Operation Instructions]**

**1. Hybridization pretreatment**

① Sectioning: Neutral formalin fixed paraffin-embedded tissue sections are placed on clean glass slides.
② Baking: The tissue slices are placed on the baking machine 65°C overnight (30min at 80°C baking for old slices).
③ Dewaxing: Tissue sections are soaked during 10 minutes in xylene dye tank for dewaxing, repeated once, and then immediately immersed in 100% ethanol for 5 minutes.
④ Rehydration: At room temperature, the tissue slices are placed in 100% ethanol, 85% ethanol and 70% ethanol for 2 minutes, and then immersed in deionized water for 3 minutes. After taking out the slices, remove by absorption the excess moisture around the tissue slices with sterile clean tissue paper.
⑤ Water treatment: Under 95°C water bath, the tissue slices are soaked in deionized water for 30 to 40 minutes (deionized water is preheated by water bath).

⑥ Washing: At room temperature, the tissue sections are soaked in 2xSSC solution, rinse twice for 5 minutes each.
⑦ Protease treatment: The tissue slices are immersed in the protease working solution, at 37°C for 5 to 30 minutes.

*Protease action time depends on the slice thickness. In order to achieve proteins full digestion and do not affect the tissue morphology, proteases can also digest proteins surrounding the target DNA, increasing the probe and the target DNA binding opportunities and improving the hybridization rate. If the protease concentration is too high, the digestion time subsequently will be too long; or if the incubation temperature is too high, it will destroy the cell structure, resulting in tissue sections falls off, disappearance of nuclei or unclear nuclei. Insufficient protease digestion will affect tissue permeability, signal intensity and hybridization rate with too strong auto-fluorescence under the microscope.*

⑧ Washing: At room temperature, the tissue sections are soaked in 2xSSC solution, rinse twice for 5 minutes each.
⑨ Dehydration: The tissue slices are placed in order in 70% ethanol, 85% ethanol and 100% ethanol for 2 minutes each, take out and air dry.

**2. Denaturation and Hybridization**

The following operations should be performed in a darkroom.

① Take ALK dual-color probe at static room temperature for 5 minutes. Briefly centrifuge manually (do not use vortex or shaker instrument). Take 10μl droplet in the cell and drop in the hybridization zone, immediately cover 22mmx22mm glass slide area; spread evenly without bubbles the probe under the glass slide covered area and seal edges with rubber (edge sealing must be thorough to prevent dry film from affecting the test results during hybridization).

② Place the glass slide in the hybridization instrument, denature at 85°C for 5 minutes (the hybridizer should be preheated to 85°C) and hybridize at 42°C for 2 to 16 hours.

**3. Washing**

The following operations should be performed in a darkroom.

① Take out the hybridized glass slides, remove the rubber on the coverslip and immediately place the slides in a 0.3% NP-40/0.4x SSC solution at 60°C. Shake for 1-3 seconds, remove the coverslip and continue to soak the glass slides for 3 minutes.

②The glass slides are placed in deionized water at 37°C, shaken for 1-3 seconds, soaked for 3 minutes and air dried in the dark.

**4. Complex dyeing**

The following operations should be performed in a darkroom

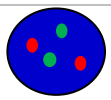
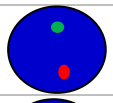
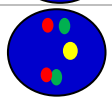
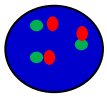
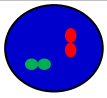
10μl DAPI compound dye is dropped in the hybridization area of the glass slide and immediately covered. The suitable filter is selected for glass slide observation under the fluorescence microscope.

**5. FISH results observation**

Place the slides under the fluorescence microscope after counterstaining, then under the natural light, use a low-power objective (10×) to locate the NSCLC cell area, and then switch to 40× objective to locate an area where cells are well-distributed. Use high-power objective (60×, 100×) to select the cells which have same size of nuclei, complete nuclei boundary, well DAPI staining, no overlapping of nuclei and show clear signal, randomly choose at least 50 tumor cells, enumerate the orange and green signal in these nuclei.

**[Signal classification and enumeration]**

**Table 2 Dual Color Signal Counting Guide**

| ●Single orange signal<br>●Single green signal<br>●Adjacent or fused orange green signals | |
|---|---|
|  | Individual orange or green signals are considered as single signals. |
|  | Diffuse signals can have a fuzzy or elongated DNA fiber appearance. |
|  | Orange and green signals are adjacent that the distance is less than two signal diameters, or are overlapping, which are considered as one fused signal. Multiple fused and/ or broken apart signals may be observed in a single nucleus. |
|  | If diffuse signal is adjacent or connected with a fiber signal, they should be recorded as one fused signal. Multiple fused and/or broken apart signals may be observed in a single nucleus. |
|  | Two signals of the same color that have the same size are adjacent, separated by a distance less than two signal diameters, which should be recorded as one signal, (this is a split signal). |

[Common Signal Type Interpretation]

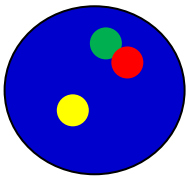
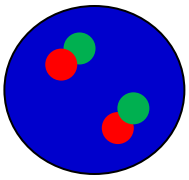
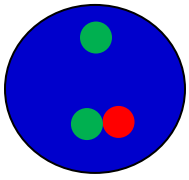
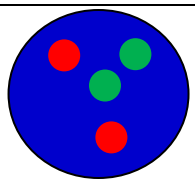
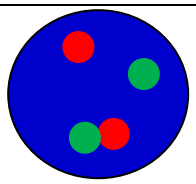
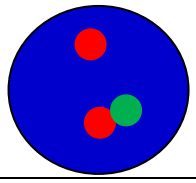
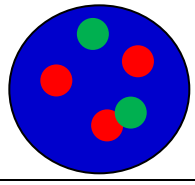
<table>
<tr><td colspan="2">ALK single Orange signal ALK single Green signal ALK adjacent or fused Orange/Green signal</td></tr>
<tr><td>A1<br>  </td><td rowspan="3">Negative (No gene fusion):<br><br>A1. and B1: These nuclei contain fused orange and green signals. The signals are either overlapping, adjacent, or are less than two signal diameters apart.<br><br>C1: A single green signal without a corresponding orange signal in addition to fused and/or broken-apart signals indicates a deletion of the orange portion of the ALK probe and is considered negative. The target area of the drug is located within the area targeted by the orange probe.</td></tr>
<tr><td>B1<br>  </td></tr>
<tr><td>C1<br>  </td></tr>
<tr><td>A2<br>  </td><td rowspan="4">Positive (Gene fusion):<br><br>Positive signal: These nuclei contain rearranged or “broken apart” signals, 2 or more signal diameters apart.<br><br>A2: A nucleus can have more than one set of broken apart signals.<br><br>B2: A nucleus can have fused signal (s) and broken apart signal (s).<br><br>C2: A nucleus can have a single orange signal (deleted green signal) in addition to fused and/or broken apart signals.<br>Note: A nucleus with signals of only one color should not be enumerated.<br><br>D2: The same nucleus has fused signals, broken apart signals and deletions.</td></tr>
<tr><td>B2<br>  </td></tr>
<tr><td>C2<br>  </td></tr>
<tr><td>D2<br>  </td></tr>
</table>

**Table 3 Determination of Cells as Positive or Negative**

| Signal type | Number of adjacent or fused signals | Number of single orange signals | Number of single green signals | Cell determination |
|---|---|---|---|---|
| A1, B1 | ≥1 | 0 | 0 | Negative |
| C1 | ≥1 | 0 | ≥1 | Negative |
| A2, B2, D2 | ≥0 | ≥1 | ≥1 | Positive |
| C2 | ≥1 | ≥1 | 0 | Positive |

Enumerate the positive cells and negative cells based on the determination methods described in Table 2 and 3, calculate Ratio value, Ratio = number of positive cells / total number of enumerated cells × 100%.

**① Positive results**

The tissue specimen is considered positive for ALK Gene Break Apart if Ratio > 50%.

**② Negative results**

The tissue specimen is considered negative for ALK Gene Break Apart if Ratio < 10%.

**③ Indeterminate results**

If Ratio is between 10%-50%, choose another cell area to enumerate 50 cells once more, add the first and second cell count readings together and calculate the final Ratio. If Ratio < 15%, the tissue specimen is considered negative, if Ratio ≥ 15%, the tissue specimen is considered positive.

**Quality control**

It is recommended to simultaneously select the specimens with known positive and negative ALK Gene Break Apart as the external control slides.

This kit uses dual-color probe to detect ALK Gene Break Apart, the hybridization is regarded as successful when 75% cell nuclei in the tissue show the dual color signal, and the dual color signal are taken as control by each other, tumor cell and non-tumor cell are taken as control by each other.

FISH test would be considered as failure in case of the descriptions below and needs to repeat again, including: (1) the positive control or negative control presents no expected results; (2) tumor lesions is too small to observe two tumor region and enumerate; (3) the cells available to enumerate < 75%; (4) more than 10% signal are located outside the cell nuclei; (5) the cell nuclei structure is hard to recognize; (6) strong auto-fluorescence exists.

***Note***: The above requirements should be met in the same experiment, otherwise, the experiment is invalid and it should be re-performed.

***Note***: Perform quality control for each experiment.

**Technological specification**

- **Appearance**: The outer package should be complete without damage, with complete and clear labeling, each tube of liquid reagent should be clearly labeled and no leakage.
- **Fluorescence signal intensity**: After effective hybridization with peripheral blood (or the culture solution) lymphocyte, the probe should present fluorescence signal visible to naked eye under fluorescence microscope.
- **Coincidence rate of negative references**: Perform the detection of formalin-fixed, paraffin-embedded tissue sections from 5 non-small cell lung cancer (NSCLC) patients with negative ALK Gene Break Apart, analyze the fluorescence signal, the test results should be judged as negative.
- **Coincidence rate of positive references**: Perform the detection of formalin-fixed, paraffin-embedded tissue sections from 5 NSCLC patients with positive ALK Gene Break Apart, analyze the fluorescence signal, the test results should be judged as positive.
- **Sensitivity**: Perform the detection of sensitivity references (Karyotype references), analyze 100 chromosomes 2 of 50 cells at metaphase, the test result should show 1 green signal and 1 orange signal in at least 98 chromosomes 2.
- **Specificity**: Perform the detection of specificity references (Karyotype references), analyze 100 chromosomes 2 of 50 cells at metaphase, the test result should show 1 specific green signal and 1 specific orange signal at the target area of at least 98 chromosomes 2.

- **Clinical study**: The clinical study of this kit, based on the synchronous blind trial, takes The Vysis ALK Break Apart FISH Probe Kit produced by Abbott Molecular Inc. as the reference kit, 1189 valid specimens in total are tested, and the test results show that the positive coincidence rate, negative coincidence rate and general coincidence rate all reach up to 100%, Kappa value1.000 (p=0.00) and the excellent consistency between the two kits. The clinical test results show that our kit is well consistent with the similar product on the market, reliable, safe, convenient and stable, which presents high clinical application value.
- **Stability**: The stability test result shows that, ALK Gene Break Apart FISH Probe Kit can be stored for 16 months at -20°C±5°C, and perform acceleration testing at 37°C for 8 days, which does not influence the performance of the kit.

**Product Use Limitations**

- The kit only applies to specified specimen types. This kit is applied only for formalin-fixed, paraffin-embedded human non-small cell lung cancer (NSCLC) tissue specimens collected by surgical resection or biopsy. Other types of tissue specimens should not be used, and for the FFPE tissue specimens with long-term storage, the test performance should not be evaluated according to this Instruction for Use.
- It is necessary to operate according to the procedures provided by Instruction for Use, modifications to these procedures may alter the performance of the assay.
- This kit was not evaluated together with the target drug to ALK gene in clinic, but only the detection performance for ALK gene was evaluated.
- This kit is only for detecting ALK Gene Break Apart, and should not be the sole basis for treatment, prognosis estimation or other clinical management for NSCLC patients, the comprehensive evaluation should be performed in the context of the patient's medical history and other diagnostic laboratory test results.
- The product is to be used by professionally trained personnel only.

**[Troubleshooting]**

The common factors influencing test results and the handling methods are shown in Table 4:

Table 4 Frequent problems and handling methods

| Problem | Probable cause | Recommended solution |
|---|---|---|
| **Strong background of slides** | Inadequate wash of glass slide before preparation of specimens | Wash the glass slide using the absolute ethyl alcohol. |
| | Inadequate wash after hybridization | Assure that the wash buffer is prepared in line with Instruction For Use, assure the correct pH value and temperature of wash buffer, remove the coverslip and repeat the washing steps. |
| | Improper use of filter sets | Replace with suitable filter sets to reduce the background light. |
| | Improper hybridization condition | Assure the temperature of hybridization instrument is set as 42ºC. |
| | The temperature is too low when washing | Assure that the wash buffer reaches to the required temperature when washing the slides. |
| | The washing intensity of wash buffer is too low | Assure the wash buffer is prepared in line with Instruction For Use. (low SSC concentration or high NP-40 concentration would help improving the washing intensity of wash buffer). |
| **Weak counterstaining** | Weak counterstaining | Remove coverslip, at room temperature, immerse the slides in the wash buffer containing 2 × SSC/ 0.1%NP-40 for 5 minutes. And then sequentially immerse the slides in 70%, 85% and 100% ethanol solution for 1 minutes respectively, and then perform the counterstaining. |
| | The counter stain has been kept under long-term storage or excessive light | Assure the counter stain is stored at -20ºC and protected away from light, assure its effect. |

| | | |
|---|---|---|
| **No signal or weak signals** | Inadequate denaturation of specimens | Assure the temperature of hybridization instrument is set as 83ºC, at least 10 minutes in advance is needed to preheat hybridization instrument. |
| | The probe mixture and hybridization buffer were not mixed sufficiently before use | Blow the probe mixture and mix the probe sufficiently, centrifuge for a short time. |
| | The probe mixture on tissue slides dries too fast | After dropping probe mixture the target area should be covered by coverslip immediately, when washing the slides you can only remove one coverslip at a time, and dip it into wash buffer immediately before removing next coverslip. |
| | Air bubbles formed under coverslip during hybridization | The coverslip should cover the probe mixture in order to gently squeeze out air bubbles. |
| | Inappropriate hybridization condition | Ensure to comply with the time and temperature required by hybridization and do not leave gaps when sealing the slides with rubber cement. The hybridization time should be adjusted according to the situation. |
| | Improper wash buffer or incorrect washing conditions | Be sure to follow the requirements of Instruction for Use to formulate the wash buffer. Ensure that the temperature of wash buffer reaches to the temperature predetermined in washing step. The thermometer and pH meter should be accurately calibrated. Remove coverslip before immersing the slide into wash buffer. |
| | Inappropriate storage of probe or specimens slides | Make sure that the probe mixture is stored at -20ºC and protected from light. Place the slides without hybridization at -20ºC for long-term storage or at room temperature for short-term storage. Place the hybridized slides at -20ºC, away from light, and store for less than 6 months. |
| | Incorrect use of DAPI counter stain, excessively high brightness of counter stain | Remove the coverslip, immerse the slides in 2 × SSC/ 0.1%NP-40 for 5 minutes at room temperature. Sequentially immerse the slides in 70%, 85% and 100% ethanol solution for 1 minutes respectively, and then perform the counterstaining after air drying the slides. |
| | Inappropriate filter sets were selected for observation | Use correct filter sets to observe the probe fluorescence. For the detailed information, please consult the technical service department of Wuhan HealthCare Biotechnology Co., Ltd. |

If there are other problems, please contact our technical support at: cs@healthcare-bio.com

**[Precautions]**

① This product is only used for research use only.

② The results of this kit will be affected by various factors of the sample itself, but also limited by hybridization temperature and time, operating environment and the limitations of current molecular biology technology, which may lead to wrong results.

③ Users must understand the potential errors and accuracy limitations that may exist in the detection process.

④ All chemicals are potentially dangerous. Avoid direct contact and wastes should be properly disposed off.

**Reference**


- Leighl NB., Rekhtman N, Biermann WA., and Huang J, *et al*. Molecular Testing for Selection of Patients With Lung Cancer for Epidermal Growth Factor Receptor and Anaplastic Lymphoma Kinase Tyrosine Kinase Inhibitors: American Society of Clinical Oncology Endorsement of the College of American Pathologists/ International Society for the Study of Lung Cancer/Association of Molecular Pathologists Guideline. Journal of Clinical Oncology, 2014,32: 3673-3679.
- Hatanaka H. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. Nature, 2007, 448 (7153): 561-566.

- Choi Y L, Soda M, and Yamashita Y, *et al*. EML4-ALK mutations in lung cancer that confer resistance to ALK inhibitors. New England Journal of Medicine, 2010, 363 (18): 1734-1739.
- Wong D, Leung E K, and Tam I, *et al*. The EML4-ALK fusion gene is involved in various histologic types of lung cancers from nonsmokers with wild-type EGFR and KRAS. Cancer, 2009, 115 (8): 1723-1733.
- Zhang X, Zhang S, and Yang X, *et al*. Fusion of EML4 and ALK is associated with development of lung adenocarcinomas lacking EGFR and KRAS mutations and is correlated with ALK expression. Molecular Cancer, 2010, 9: 188.
- Shaw A T, Yeap B Y, and Mino-Kenudson M, *et al*. Clinical features and outcome of patients with non-small-cell lung cancer who harbor EML4-ALK. Journal of Clinical Oncology, 2009, 27 (26): 4247-4253.


**[Manuscript version and approval date]**
Manual version: V1.1 reviewed on 24 December 2021
Approval date: 03 January 2018