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CE IVD

Human ALK gene fusion detection probe Instructions Manual

[Product Name] Human ALK gene fusion detection probe

[Intended use]

This kit uses fluorescence in situ hybridization to detect the fusion status of ALK gene in non-small cell lung cancer in vitro. The detection samples are surgical resection or biopsy samples of non-small cell lung cancer embedded in 4% neutral formaldehyde fixed paraffin. The product has not been clinically verified in combination with ALK targeted therapeutic drugs, and its clinical detection performance has been confirmed by comparative test with accompanying diagnostic reagents that have been verified by targeted drugs. The test results of the product should not be used as the only basis for the individualized treatment of patients. Clinicians should comprehensively judge the test results in combination with the patient's condition, drug indications, treatment response and other laboratory test indicators.

Anaplastic lymphoma kinase (ALK) was first found as a subtype of anaplastic large cell lymphoma (ALCL). ALK gene is located at chromosome 2p23. Normally, human ALK can be transcribed to produce 6222 BP mRNA, which is composed of 29 exons, encoding 1620 amino acid sequences and 200kda type I transmembrane protein. This protein is a receptor tyrosine kinase (RTK) and a member of RTK insulin superfamily. Subsequently, it was found that many types of ALK gene fusion were found in non-small cell lung cancer, diffuse large B-cell lymphoma and inflammatory myofibroblast tumor, which proved that ALK gene fusion was a strong carcinogenic driver gene.

At present, the incidence rate and mortality rate of lung cancer are first of the malignant tumors in China, of which 80%~85% is non-small-cell lung cancer (NSCLC). Individualized molecular targeted therapy based on epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK) has become a research hotspot of NSCLC. Many clinical studies have proved that ALK inhibitors can benefit NSCLC patients with ALK fusion gene positive. Therefore, it is of great clinical significance to detect ALK gene status in patients with NSCLC.

[Detection principle]

Based on the fluorescence in situ hybridization technology, a nucleotide of the nucleic acid probe is labeled with fluorescein. The detected target gene is homologous and complementary with the used nucleic acid probe. After denaturation, annealing and renaturation, they can form a hybrid between the target gene and the nucleic acid probe. The detected gene is analyzed qualitatively, quantitatively or relatively under the microscope by the fluorescence detection system. The kit adopts rhodamine fluorescein (rho) labeled orange probe and fluorescein isothiocyanate (FITC) labeled green probe. The two probes can be combined with the target detection site by in situ hybridization. Under normal conditions (ALK gene does not fuse), it is displayed as green and orange signals close to each other or yellow signals overlapping each other under fluorescence microscope. When there is gene fusion, the green and red signals are "broken" due to the replacement of recombinant fusion partner genes, and appear as signals far apart. The fusion of ALK gene in the tissues of patients with non-small cell lung cancer was detected in vitro by this broken fluorescence in situ hybridization method, so as to provide a reference basis for the treatment prognosis and medication of patients with non-small cell lung cancer.

[Product Content]

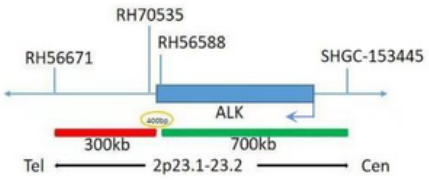
This kit consists of ALK dual color probe, as shown in Table 1.

Table 1 Kit composition

Componentname	Specifications	Quantity	Main components
ALKdualcolorprobe	100μl/Tube	1	ALK Orange probe, ALK Green probe

Table 2 List of reagents not provided

Reagent name	Purity	Reagent name	Purity
Sodium chloride	Analytical purity AR	NP-40	Analytical purity AR
Sodium citrate	Analytical purity AR	Xylene	Analytical purity AR
Anhydrous ethanol	Analytical purity AR	Protease K	≥40 units/g



[Storage conditions]

Keep sealed away from light at -20℃±5℃. 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℃ in dark. For long-term preservation after opening, keep the lid sealed at -20℃±5℃ away from light. See the label of the kit for the production date and expiration date.

[Applicable Instruments]

1. Fluorescence microscopy imaging system includes fluorescence microscope and filter sets. The kit is labeled with orange fluorescein, and the filter set compatible with the fluorescent-labeled dye should be selected.

Orange fluorescence: The maximum excitation wavelength is 547nm and the maximum emission wavelength is 565nm.

Green fluorescence: The maximum excitation wavelength is 495nm and the maximum emission wavelength is 517nm.

Fluorescence microscopy imaging system should use a microscope with orange and green channels.

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

[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.

[Test method]

1.Related Reagents Preparation

①20×SSC, pH 5.3±0.2

Sodium chloride	176g
Sodium citrate	88g

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℃, 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℃, 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℃ 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

NP-40	0.6mL
20xSSC	4mL

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.

NP-40	0.2mL
20xSSC	20mL

⑦ Xylene

2. 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℃ overnight (30min at 80℃ 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 95ºC 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 37ºC for 5 to 30 minutes.

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.

3. 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 83°C for 5 minutes (the hybridizer should be preheated to 83ºC) and hybridize at 42°C for 2 to 16 hours.

4. 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 67°C. Shake for 1-3 seconds, remove the coverslip and continue to soak the glass slides for 3 minutes.

② At room temperature, the sections were placed in 0.1% NP-40/2×SSC solution, shaken for 1~3 seconds and immersed for 1~2 minutes.

③ At room temperature, the sections were placed in 70% ethanol and immersed for 1~3 minutes; the sections were dried naturally in the dark.

5. Complex dyeing

The following operations should be performed in a darkroom

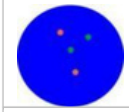
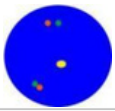
10-15μl DAPI compound dye is dropped in the hybridization area of the glass slide and immediately covered. The suitable filter is selected for glass slide observation under the fluorescence microscope.

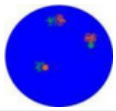
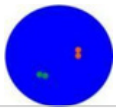
6. FISH results observation

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 3 Dual Color Signal Counting Guide

● Single orange signal ● Single green signal ● 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).

Enumerate 50 tumor cells, record the number of fused or adjacent signals, single orange signal and single green signal in each cell. Enumerate once for each tumor cell, and record the cells with hybridization signal (both orange and green signal), but these cells with no signal, only single color signal, weak signal or extremely diffuse signal should not be counted.

Results determination

Table 4 The Criteria for Judging Positive and Negative ALK Gene Break Apart

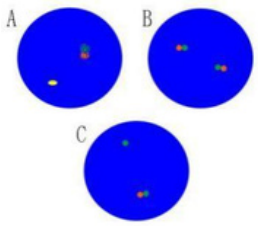
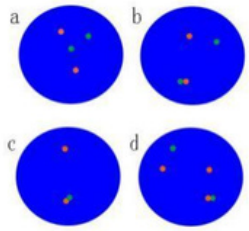
Negative signal（No gene fusion）	
	A. and B. These nuclei contain fused orange and green signals. The signals are either overlapping, adjacent, or are less than two signal diameters apart. C. A single green signal without a corresponding orange signal in addition to fused and/or broken-apart signals indicates 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.
Positive signal（Gene fusion）	
	Positive signal: these nuclei contain rearranged or “broken apart” signals, 2 or more signal diameters apart. Figure a. A nucleus can have more than one set of broken apart signals. Figure b. A nucleus can have fused signal (s) and broken apart signal (s). Figure c. A nucleus can have a single orange signal (deleted green signal) in addition to fused and/or broken apart signals. Note: A nucleus with signals of only one color should not be enumerated. Figure d. The same nucleus has fused signals, broken apart signals and deletions.

Table4 DeterminationofCellsasPositiveorNegative

Signaltype	Numberofadjacent orfusedsignals ≥1	≥1	Numberofsingleorange signals	Number of greensignals	single	Celldetermination
A、B	≥0	≥1	0	0 ≥1	≥1 0	Negative
C			≥1			Negative
a、b、d						Positive
c						Positive

Enumerate the positive cells and negative cells based on the determination methods described in Table 4 and 5, 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.

[Interpretation of test results]

1. In this kit, a two-color probe is used to detect the fusion status of ALK gene. When ≥ 75% of the nuclei in the tissue show two-color signals, it is regarded as successful hybridization, and the two-color signals are compared with each other, and cancer cells and non cancer cells are compared with each other.

2. Fish test shall be deemed to have failed in the following cases and shall be retested, including: ① the positive or negative control sample does

not show the expected results; ② The tumor focus is too small to observe and count two tumor areas; ③ Cells with countable signals < 75%; ④ More than 10% of the fluorescence signals were located outside the nucleus; ⑤ The nuclear structure is difficult to distinguish; ⑥ It has strong spontaneous fluorescence.

3. The common factors affecting the test results and treatment methods in the experiment are shown in Table 6:

Table 6 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 42℃.
	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 -20℃ 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 83℃, 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 -20℃ and protected from light. Place the slides without hybridization at -20℃ for long-term storage or at room temperature for short-term storage. Place the hybridized slides at -20℃, 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.

[Product performance index]

1. The outer package of the kit shall be complete without damage, and the marks shall be complete and clear; Each liquid reagent shall be clearly marked without leakage.

2. After the probe effectively hybridizes with peripheral blood (or its culture medium) lymphocytes, it should send out fluorescence signals that can be recognized by the naked eye under the fluorescence microscope.

3. The paraffin sections (negative reference) of 5 patients with non-small cell lung cancer with negative ALK gene fusion were detected and the fluorescence signals were analyzed. The results were negative.

4. Paraffin sections (positive reference) of 5 patients with non-small cell lung cancer with ALK gene fusion positive were detected, and the fluorescence signals were analyzed. The results were all positive.

5. Detect the sensitivity reference (karyotype reference), analyze 100 chromosomes 2 of 50 cells in metaphase, and at least 98 chromosomes 2 show a green fluorescence signal and a red fluorescence signal.

6. Detect the specific reference (karyotype reference), analyze 100 chromosomes 2 of 50 cells in metaphase division phase, and at least 98 chromosomes 2 show a specific green fluorescence signal and a specific red fluorescence signal in the target area.

The clinical study of this kit adopts the experimental design of synchronous blind method, and uses the concomitant diagnostic reagent (Abbott molecular's "ALK gene recombination detection kit (fluorescence in situ hybridization)" (Registration Certificate No.: gxzz 20143405183) that has been verified by targeted drugs as the comparison reagent. A total of 1189 effective samples have been tested, and the positive coincidence

rate of this kit The negative coincidence rate and overall coincidence rate were 100%, and the kappa value was 1.000 (P = 0.00).

[Precautions]

1. Please read this manual carefully before testing. The testing personnel shall receive professional technical training, and the signal counting personnel must be able to observe and distinguish orange and green signals.
2. When testing clinical samples, when it is difficult to count the hybridization signal and the samples are not enough to repeat the retest, the test will not provide any test results. If the amount of cells is insufficient for analysis, the test will not provide test results.
3. Xylene, formamide and DAPI re dye used in this experiment have potential toxicity or carcinogenicity. It is necessary to operate in the fume hood and wear masks and gloves to avoid direct contact.

The results of this kit will be affected by various factors of the sample itself, but also limited by enzyme digestion time, hybridization temperature and time, operating environment and the limitations of current molecular biology technology, which may lead to wrong ALK gene fusion results. Users must understand the potential errors and accuracy limitations that may exist in the detection process.

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[Basic information]

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