

ALK Gene Fusion Probe Detection Kit

(CW-002)

Intended use

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

Product content

The kit is based on fluorescence in situ hybridization technology. A nucleic acid probe is labeled with fluorescein. The target gene is detected with homologous complementary to the nucleic acid probe used. Both after denaturation, annealing and renaturation, the hybrid of the target gene and the nucleic acid probe can be formed, and the qualitative, quantitative or relative positioning analysis of the gene to be measured under the microscope can be performed by the fluorescence detection system.

This kit uses the rhodamine fluorescein (RHO)-labeled orange probe (HER2 probe) to detect the HER2 gene, and the fluorescein isothiocyanate (FITC)-labeled green probe (CEP17 probe) to detect chromosome 17 centromere sequence can be used to bind two probes to the target detection area by in situ hybridization. The number of signals corresponding to the CEP17 probe reflects the number of chromosomes at the target area, and the number of signals from the HER2 probe reflects the copy number of the HER2 gene at the target site. By the ratio of the number of HER2 probes and the number of CEP17 probe signals, the amplified state of the HER2 gene in the tissue to be detected can be determined.

Product composition

This kit consists of ALK dual color probe (100 µl/Tube).

Storage condition

Keep sealed away from light at $-20^{\circ}\text{C}\pm 5^{\circ}\text{C}$. This product is valid until the date indicated on the label. Avoid unnecessary repeated freezing and thawing that should not exceed 10 times. After opening, within 24 hours for short-term preservation, keep sealed at $2/8^{\circ}\text{C}$ in dark. For long-term preservation after opening, keep the lid sealed at $-20^{\circ}\text{C} \pm 5^{\circ}\text{C}$ 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 $\leq 1^{\circ}\text{C}$.

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

Testing method

The following reagents are required for the experiment but not provided in this kit

1. 20×SSC (sodium citrate buffer), pH 5.3±0.2

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

2. 20×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

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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 \approx 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-40/0.4xSSC solution, pH 7.0/7.5

Take 0.6mL NP-40 and 4mL 20xSSC, 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 2/8°C, 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

Pre-treatment

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:** Immerse the tissue sections in xylene for 10 minutes, dewax, repeat once, and then immediately immerse 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. **Proteinase treatment:** The tissue slices are immersed in the protease working solution, at 37°C for 5 to 30 minutes.

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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.

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.

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 10 μ l 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.

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.
2. The 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.

Complex dyeing

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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.

FISH results observation

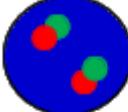
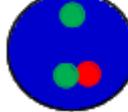
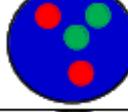
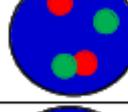
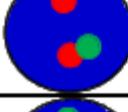
Place the slides under the fluorescence microscope after counterstaining, then under the natural light, use a low -power objective (10×) to locate the NSCLC cell area, and then switch to 40× objective to locate an area where cells are well-distributed. Use high-power objective (60×, 100×) to select the 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

Dual Color Signal Counting Guide

| <ul style="list-style-type: none"> ● 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). |

Common Signal Type Interpretation

| |  ALK single Orange signal  ALK single Green signal  ALK adjacent or fused Orange/Green signal | |
|----|--|---|
| A1 |  | <p>Negative (No gene fusion):</p> <p>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.</p> <p>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.</p> |
| B1 |  | |
| C1 |  | |
| A2 |  | <p>Positive (Gene fusion):</p> <p>Positive signal: These nuclei contain rearranged or "broken apart" signals, 2 or more signal diameters apart.</p> <p>A2: A nucleus can have more than one set of broken apart signals.</p> <p>B2: A nucleus can have fused signal (s) and broken apart signal (s).</p> <p>C2: A nucleus can have a single orange signal (deleted green signal) in addition to fused and/or broken apart signals. <u>Note:</u> A nucleus with signals of only one color should not be enumerated.</p> <p>D2: The same nucleus has fused signals, broken apart signals and deletions.</p> |
| B2 |  | |
| C2 |  | |
| D2 |  | |

| 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 \geq 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.

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- **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 value 1.000 ($p=0.00$) 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 $-20^{\circ}\text{C}\pm 5^{\circ}\text{C}$, 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 Instructions 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.

FAQ and solutions

| Question | Possible 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 conditions | Ensure that the hybridization instrument temperature is 42°C |
| | Low washing temperature | Ensure that the solution temperature of the washing glass slides is up to the required temperature |
| | Washing solution strength is too low | Ensure that the washing solution is prepared according to instructions. (Low SSC concentration and high NP-40 concentration are beneficial to increase the washing solution strength) |
| 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°C and protected away from light, assure its effect |
| No signal or weak signal | Inadequate denaturation of specimens | Assure the temperature of hybridization instrument is set as 83°C, 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°C and protected from light. Place the slides without hybridization at -20°C for long-term storage or at room temperature for short-term storage. Place the hybridized slides at -20°C, 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 |

Precautions

1. This product can be used for both clinical diagnosis and scientific research.
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. Used kits are clinical waste and should be properly disposed of.

References

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