

Vysis ALK Break Apart FISH Probe Kit

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REF 06N38-020











30-608495/R2

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Key to Symbols Used

	Manufacturer
	List Number
	Lot Number
	In Vitro Diagnostic Medical Device
	Store at -30°C to -10°C.
	Caution, consult accompanying documents
	Use By
	Consult instructions for use
	Biological Risks
	Authorized Representative

INTENDED USE

The Vysis ALK Break Apart FISH Probe Kit is a qualitative test to detect rearrangements involving the ALK gene via fluorescence in situ hybridization (FISH) in formalin-fixed paraffin-embedded (FFPE) non-small cell lung cancer (NSCLC) tissue specimens to aid in identifying those patients eligible for treatment with XALKORI® (crizotinib). The test is for prescription use only.

SUMMARY AND EXPLANATION OF THE TEST

The Vysis ALK Break Apart FISH Probe Kit uses fluorescence in situ hybridization technology to detect chromosome 2p23 rearrangements. Rearrangement of the ALK locus on 2p23 has been implicated in the development of NSCLC.¹⁻³ The ALK gene codes for a transmembrane glycoprotein with tyrosine kinase activity. In-frame rearrangements with the known fusion partners place the ALK kinase domain under the control of a different gene promoter. This fusion results in a chimeric protein with constitutive tyrosine kinase activity that has been demonstrated to play a key role in controlling cell proliferation.⁴⁻⁶

In NSCLC, the rearrangement of the ALK gene was first identified with the echinoderm microtubule-associated protein-like 4 gene (EML4).¹ In-frame fusions of EML4-ALK genes identified to date include variants containing multiple breakpoints of the EML4 gene occurring at exons 2, 6, 13, 14, 15, 18, and 20 and all variants starting at a portion of the ALK gene encoded by exon 20.^{1-2,5,7-9} Besides the EML4 gene, the ALK gene has also been shown to form fusion partners in NSCLC tumors with TFG and KIF5B.^{4,7}

Several publications using the Vysis ALK Break Apart FISH Probe reported that multiple types of rearrangements were detected involving the ALK gene locus. In NSCLC, the predominant ALK-positive FISH pattern as detected using single interference filter sets [green (FITC), red (Texas red), and blue (4',6-diamidino-2-phenylindole) as well as dual (red/green) and triple (blue, red, green) band-pass filters] was the fusion and split orange and green signals (62%), the second most common pattern was the fusion and single orange (31%), and the final pattern

had single orange and single green signals (7%).¹⁰ The cytogenetic rearrangement patterns seen in ALK-positive tumors reveal the potential for activating chromosomal deletions (single orange), and fusion/truncation, or gene copy number increases in addition to the classic split signal occurring with the rearrangement of ALK with another partner.¹⁰ In another study, a subset of thirty-one patients with FISH positive ALK rearrangements were also tested by PCR and RT-PCR assays that were unable to detect all known ALK fusion partners.¹¹

There are currently no alternative standard methods to the Vysis ALK Break Apart FISH Probe Kit assay for detecting ALK NSCLC. Per the NCCN Guidelines (Version 3.2011) Non-Small Cell Lung Cancer, a big advantage of FISH is that a commercially available probe set is applicable for the detection of ALK-rearrangement in lung adenocarcinomas. The IHC tests used to detect ALK-rearrangement in clinical laboratories worldwide is inadequate for the detection of the majority of ALK-rearranged lung adenocarcinomas.²⁵

Non-small cell lung cancer is the leading cause of cancer death worldwide.^{12,13} With a 5-year morbidity rate of 85–95%, there is a pressing need for improvement in identifying patients most likely to respond to specific treatments.¹³ Tyrosine kinase inhibitors have been demonstrated to reduce lung cancer cell proliferation, resulting in suppression of tumor growth.^{9,14-16}

The therapeutic efficacy of inhibiting ALK in tumors that were selected by ALK positivity using FISH has been demonstrated in an early-phase clinical trial of a small molecule inhibitor of the ALK tyrosine kinase. Additionally, the study reported that sixty-three of eighty-two patients were still receiving therapy at the time of the data cutoff with an estimated probability of progression free survival of 72%.¹¹

BIOLOGICAL PRINCIPLES OF THE PROCEDURE

Fluorescence in situ hybridization (FISH) is a technique that allows the visualization of specific chromosome nucleic acid sequences within a cellular preparation. Specifically, FISH involves the precise annealing of a single-stranded, fluorophore-labeled DNA probe to complementary target sequences. The hybridization of the probe with the cellular DNA region is visible by direct detection using fluorescence microscopy. Formalin-fixed, paraffin-embedded tissue sections are placed on slides. The DNA is denatured to single-stranded form and subsequently allowed to hybridize with the DNA probes. Following hybridization, the unbound probe is removed by a series of washes and the nuclei are counter-stained with DAPI (4,6 diamidino-2-phenylindole), a DNA-specific stain that fluoresces blue. Hybridization of the ALK probe is viewed using a fluorescence microscope equipped with appropriate excitation and emission filters, allowing visualization of the orange and green fluorescent signals.

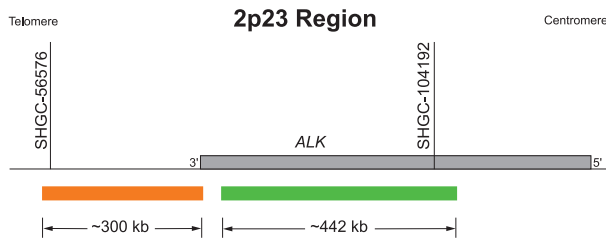
When hybridized with the Vysis ALK Break Apart FISH Probes, the 2p23 ALK region in its native state will be seen as two immediately adjacent or fused (overlapping) orange/green (yellow) signals. However, if a chromosome rearrangement at the 2p23 ALK breakpoint region has occurred, one orange and one green signal separated by at least two signal diameters will be seen. Alternatively, a single orange signal (deletion of green signal) in addition to a fused or broken apart signal may be seen.

Probe Description

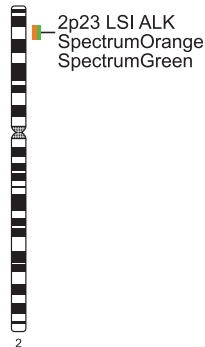
The Vysis LSI ALK Dual Color Break Apart FISH Probe is a mixture that consists of two fluorophore-labeled DNA probes in hybridization buffer containing dextran sulfate, formamide, and SSC with blocking DNA:

- Vysis LSI 3'-ALK SpectrumOrange
- Vysis LSI 5'-ALK SpectrumGreen

The hybridization targets of these probes are on opposite sides flanking the breakpoint of the ALK gene. The 3'-ALK probe that hybridizes telomerically of the breakpoint is approximately 300 kb and is labeled with the SpectrumOrange fluorophore. The 5'-ALK probe that hybridizes centromerically of the breakpoint is approximately 442 kb and is labeled with the SpectrumGreen fluorophore.



LSI ALK Dual Color, Break Apart Rearrangement Probe



REAGENTS

Vysis ALK Break Apart FISH Probe Kit

1. Vysis LSI ALK Dual Color Break Apart FISH Probe

(1 vial, 200 µL per vial) 50 ng/10 µL and 200 ng/10 µL, SpectrumOrange and SpectrumGreen fluorophore-labeled DNA probes in hybridization buffer containing dextran sulfate, formamide, and SSC with blocking DNA.

2. DAPI I Counterstain

(1 vial, 300 µL per vial) 1 µg/mL, DAPI (4',6-diamidino-2-phenylindole · 2HCl) in phenylenediamine dihydrochloride, glycerol, and phosphate buffered saline mixture.

Material Safety Data Sheets (MSDS) on all reagents provided are available from Abbott Molecular Technical Services.

STORAGE INSTRUCTIONS

The Vysis ALK Break Apart FISH Probe Kit must be stored at -30°C to -10°C and protected from light.

Shipping Conditions

The Vysis ALK Break Apart FISH Probe Kit is shipped on dry ice. If you receive reagents that are in a condition contrary to label recommendation, or that are damaged, contact Abbott Molecular Technical Services.

WARNINGS AND PRECAUTIONS

IVD In Vitro Diagnostic Medical Device

For In Vitro Diagnostic Use Only.

- The Vysis ALK Break Apart FISH Probe Kit is intended for use only on 10% neutral buffered formalin-fixed, paraffin-embedded NSCLC tissue.



CAUTION: This preparation contains human sourced and/or potentially infectious components. No known test method can offer complete assurance that products derived from human sources or inactivated microorganisms will not transmit infection. Therefore, all human sourced materials should be considered potentially infectious. It is recommended that these reagents and human sourced specimens should be handled in accordance such as those outlined in Biosafety in Microbiological and Biomedical Laboratories,¹⁷ OSHA Standard

on Bloodborne Pathogens,¹⁸ CLSI Document M29-A3,¹⁹ and other appropriate biosafety practices.²⁰ Therefore, all human sourced materials should be considered potentially infectious.

These precautions include, but are not limited to, the following:

- Wear gloves when handling specimens or reagents.
- Do not pipette by mouth.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in areas where these materials are handled.
- Clean and disinfect spills of specimens by including the use of a tuberculocidal disinfectant such as 1.0% sodium hypochlorite or other suitable disinfectant.^{21,22}
- Decontaminate and dispose of all potentially infectious materials in accordance with local, state, and federal regulations.^{23,24}
- Exposures of the specimens to acids, strong bases or extreme heat, should be avoided. Such conditions are known to damage DNA and may result in FISH assay failure.
- To identify target areas, H & E staining should be conducted on every 10th slide of the same tissue block.
- Proper storage of kit components is essential to ensure the labeled shelf life.
- If any working reagents precipitate or become cloudy, they should be discarded and fresh solutions prepared.
- Fluorophores are readily photobleached by exposure to light. To limit this degradation, handle all solutions and slides containing fluorophores in reduced light.
- Calibrated thermometers are required for measuring temperatures of solutions, water baths, and incubators.
- Always verify the temperature of the pretreatment solution, denaturation solution, and wash buffers prior to each use by measuring the temperature of the solution in the Coplin jar with a calibrated thermometer.
- All hazardous materials should be disposed of according to your institution's guidelines for hazardous disposal.
- Do not use kits or reagents beyond expiration date.
- Failure to follow all procedures for slide denaturation, hybridization, and detection may cause unacceptable or erroneous results.
- Hybridization conditions may be adversely affected by the use of reagents other than those provided by Abbott Molecular.

The Vysis LSI ALK Dual Color Break Apart FISH Probe is classified per applicable 29 CFR 1910.1200 and European Community (EC) Directives as: Toxic (T). The following are the appropriate Risk (R) and Safety (S) phrases:



R41	Risk of serious damage to eyes.
R61	May cause harm to the unborn child.
S45	In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).
S53	Avoid exposure—obtain special instructions before use.

Procedural Notes: Prior to use, thaw reagents at ambient temperature, vortex, and then centrifuge each tube 2 to 3 seconds using a standard bench-top microcentrifuge.

ASSAY PROCEDURE

Materials Provided

- Vysis ALK Break Apart FISH Probe Kit (List No. 06N38-020)

Materials Required But Not Provided

- Vysis Paraffin Pretreatment IV & Post-Hybridization Wash Buffer Kit (List No. 01N31-005)
- ProbeChek ALK Negative Control Slides (List No. 06N38-005)
- ProbeChek ALK Positive Control Slides (List No. 06N38-010)

Laboratory Reagents

- Hemo-De (or equivalent, e.g. d-limonene)
- Hematoxylin and Eosin (H&E) stains
- Immersion oil appropriate for fluorescence microscopy
- Ethanol (100%). Store at room temperature.
- Purified water
- Rubber Cement

Laboratory Materials

- Positively-charged glass microscope slides
- 22 mm x 22 mm glass coverslips
- Microliter pipette tips for 1 to 10 µL volumes (sterile)
- Microliter pipettor for 1 to 10 µL volumes

- Timer
- Microtome
- Microcentrifuge
- Graduated cylinders
- Static or circulating water baths (37°C)
- Circulating water baths (74°C and 80°C) Note: Static water baths do not provide adequate temperature control for higher temperature.
- Purified water bath (37°C to 42°C)
- Diamond-tipped scribe
- Solvent Resistant Marker (optional)
- Forceps
- Disposable syringe (5 mL)
- Coplin jars (12 x 50 mL) Suggested type: vertical staining jar
- Fluorescence microscope equipped with recommended filter(s) (Refer to next section)
- Calibrated thermometer
- Vortex mixer
- Microscope slide box with lid and/or carton slide folders
- ThermoBrite® (List No. 7J68-020)
- ThermoBrite humidity cards (List No. 7J68-001)

Microscope Equipment and Accessories

Microscope An epi-illumination fluorescence microscope is required for viewing the hybridization results. The microscope should be checked to confirm it is operating properly to ensure optimum viewing of FISH assay specimens. A microscope used with general DNA stains such as DAPI, propidium iodide, and quinacrine may not function adequately for FISH assays. Routine microscope cleaning and periodic maintenance by the manufacturer's technical representative, especially alignment of the mercury lamp, are advisable.

Excitation Light Source A 100-watt mercury lamp is the recommended excitation source. Record the number of hours that the bulb has been used and replace the bulb before it exceeds the rated time. Ensure that the lamp is properly aligned.

Objectives Use oil immersion fluorescence objectives with numeric apertures ≥ 0.75 when using a microscope with a 100-watt mercury lamp. A 10X to 25X objective, in conjunction with 10X eyepieces, is suitable for scanning the specimen to select regions for enumeration. For enumeration of FISH signals, satisfactory results can be obtained with a 60X to 100X oil immersion achromat type objective.

Immersion Oil The immersion oil used with oil immersion objectives should be one formulated for low auto fluorescence and specifically for use in fluorescence microscopy.

Filters Hybridization of the ALK probes to their target regions of the DNA is marked by orange and green fluorescence. All of the other DNA present will fluoresce blue as a result of the DAPI I Counterstain. Single and dual-bandpass fluorescence microscope filter sets optimized for use with the FISH DNA probe kits are available from Abbott Molecular for most microscope models.

The recommended filters for use with the Vysis ALK Break Apart FISH Probe Kit are the Vysis Dual Band (V2) – Green, Orange Filter, the Vysis Single Band DAPI filter, the Vysis Single Band Orange Filter, and the Vysis Single Band Green Filter.

ASSAY PROTOCOL

Refer to the **Warnings and Precautions** section of this package insert before preparing samples.

Specimen Collection and Processing

The following procedure has been optimized for use on FFPE lung cancer tissue specimens. Exposure of the specimens to acids, such as decalcifying agents, strong bases, and extreme heat should be avoided. Such conditions are known to damage DNA and may result in FISH assay failures.

Use lung cancer tissue specimens that were fixed in formalin (10% neutral buffered formalin) and that are well processed and produce good tissue sections. The preferred fixation duration for tissue samples is 6 to 48 hours.

Slide Preparation of NSCLC FFPE Tissue Specimens

Note: Start processing specimens for which only slides rather than specimen blocks are available at Step 5.

1. Cut two or more serial paraffin sections, $5 \pm 1 \mu\text{m}$ thick, using a microtome.
2. Float the sections on the surface of a purified water bath set at $40 \pm 2^\circ\text{C}$.
3. Mount the sections on positively-charged glass slides.
4. Allow the slide to air-dry.

5. Perform conventional H&E staining for one specimen slide.

Note: The specimen slide used for the assay procedure should be within 10 serial sections of the H&E slide.

Note: Step 6 to be performed by a pathologist.

6. Examine and mark the largest possible area of tumor on the H&E slide, excluding necrotic areas, in situ carcinoma areas, and small cell carcinoma areas using a solvent resistant marker or diamond-tipped glass scribe.
7. Using a glass scribe, transfer the mark from the H&E slide to the corresponding areas of the unstained slide by marking the glass slide opposite the tissue section.
8. Store prepared slides at ambient temperature until ready to bake prior to Slide Deparaffinization Procedure.

Working Reagent Preparation

9. **Preparation of Hemo-De** - Fill three Coplin jars with 50 mL of Hemo-De. Keep covered when not in use. Store under vented conditions at ambient temperature and discard after seven days.
10. **Preparation of Pretreatment Solution** - Fill one Coplin jar with 50 mL of Pretreatment Solution. Transfer the Coplin jar to a circulating water bath at ambient temperature and bring the temperature of the water bath to $81 \pm 2^\circ\text{C}$ (slightly higher than the desired temperature inside of the Coplin jar) prior to deparaffinizing the slides. Ensure the temperature of the solution has reached $80 \pm 2^\circ\text{C}$ prior to use. Discard the solution after using one day.
11. **Preparation of Protease Solution** - Add one vial of Vysis Protease IV to one bottle of Vysis Protease IV Buffer. Rinse the vial with a small volume of Vysis Protease IV Buffer and return to the bottle of Vysis Protease IV Buffer. Replace the cap and gently invert several times to mix. Transfer the prepared solution to Coplin jar, and place the Coplin jar in a 37°C water bath. Wait a minimum of one hour after mixing to ensure that the protease is in solution and confirm that the temperature of the buffer is $37 \pm 1^\circ\text{C}$ before use. Discard solution after one day.
12. **Preparation of Purified Water** - Fill one Coplin jar with 50 mL of purified water. Use at ambient temperature. Replace after each use.
13. **Preparation of Ethanol Solutions (70%, 85%, and 100%)** - Prepare v/v dilutions of 70% and 85% using 100% ethanol and purified water. Store at room temperature in tightly capped containers when not in use. Solutions may be used for one week unless evaporation occurs or the solution becomes diluted or cloudy due to excessive use.

Slide Deparaffinization Procedure

Note: Include one ProbeChek Negative Control slide and one ProbeChek Positive Control slide starting with Step 14.

14. Bake the unstained specimen and control slides for 2 to 24 hours at 60°C on a ThermoBrite.
15. Immerse slides in the first Coplin jar containing Hemo-De for 5 minutes at ambient temperature.
16. Repeat Step 15 twice using fresh Hemo-De each time.
17. Dehydrate slides in 100% ethanol for 1 minute at ambient temperature. Repeat in a second Coplin jar of 100% ethanol.
18. Allow slides to air dry for 2 to 5 minutes (optional).

Slide Pretreatment

19. Immerse up to eight slides in Vysis Pretreatment Solution which has been previously warmed to $80 \pm 2^\circ\text{C}$ for 12 ± 3 minutes.

Note: If necessary, two slides may be placed back-to-back in each slot of the Coplin jar, with one slide placed in each end slot. For slides in the end slots, the side of the slide with the tissue section must face the center of the jar, for a maximum of eight slides per Coplin jar at one time.

20. Immerse slides in purified water for 3 minutes.

Protease Pretreatment

21. Remove slides from the purified water.
22. Remove excess water by blotting the edges of the slide on a paper towel.
23. Immerse slides in Protease Solution previously warmed to $37 \pm 1^\circ\text{C}$ for 20 ± 2 minutes.
24. Immerse slides in purified water for 3 minutes.

Hybridization Procedure

A ThermoBrite should be used for the denaturation and hybridization steps. Refer to the ThermoBrite Operators Manual for instructions on instrument use.

25. Immerse the slides in 70% ethanol for one minute.
26. Immerse the slides in 85% ethanol for one minute.
27. Immerse the slides in 100% ethanol for one minute.
28. Air-dry the slides for 2 to 5 minutes.
29. Moisten a humidity card with water and place in the card slots of the ThermoBrite. Ensure that the surface of the ThermoBrite is clean and free of debris.
30. Set the denaturation temperature (Melt Temp) to 73°C and the denaturation time (Melt Time) to three minutes. Set the hybridization temperature (Hyb Temp) to 37°C and the hybridization time (Hyb Time) from 14 to 24 hours.
31. Apply 10 µL of probe mixture to a slide and immediately apply a coverslip. Ensure no air bubbles are in the probe mixture prior to applying the coverslip.
32. Seal the coverslip with rubber cement.
33. Place slides on the ThermoBrite and begin the hybridization program. Hybridize the slides overnight for 14 to 24 hours.

At the end of the hybridization period, proceed to the **Slide Washing Procedure**.

Note: Leave the slides on the ThermoBrite until ready to begin.

Slide Washing Procedure

Note: Hybridized slides must be washed on the day hybridization was completed.

34. Pour 50 mL of Wash Buffer I into a Coplin jar. Use at ambient temperature. Use one day, then discard.
35. Pour 50 mL of Wash Buffer II into a Coplin jar. Place the Coplin jar into a room temperature water bath prior to heating to prevent breakage of the jar. Allow the jar to warm to 74 ± 1°C before using for at least 30 minutes prior to use. Use one day, then discard.
36. Remove the rubber cement from one slide while minimally disturbing the coverslip, and immerse the slide in ambient temperature Wash Buffer I. Repeat with the other slides and let stand 2 to 5 minutes to allow the coverslips to float off the slides.
37. Immediately immerse the slide in Wash Buffer II at 74 ± 1°C. Gently agitate for 1 to 3 seconds. Repeat with the other slides.
38. Remove the slides after 2 minutes.

Note: To maintain the proper temperature in Wash Buffer II, wash only four slides simultaneously. If there are less than four slides, add blank slides to bring the total number to four. Start timing when the fourth slide is immersed.

Note: Ensure the temperature of Wash Buffer II has returned to 74 ± 1°C before washing another four slides.

Counterstaining Procedure

39. Air-dry the slide(s) protected from light at ambient temperature.
40. Apply 10 µL of DAPI counterstain to the target area of the slide, apply coverslip, and store protected from light for a minimum of 5 minutes.
41. Enumerate specimens under a fluorescence microscope within 4 hours or store at -20°C (± 10°C).

Archiving Procedure (optional)

Store the hybridized slides at -20°C (± 10°C) while protecting from light. Under these conditions, the slides can be stored for up to one week after the application of DAPI I Counterstain without significant loss in fluorescence signal intensity.

Note: Allow slides to come to ambient temperature prior to viewing.

Slide Examination

42. View slides using a suitable filter set on an optimally performing fluorescence microscope (Refer to Microscope Equipment and Accessories – Filters section of this package insert).

INTERPRETATION AND RESULT REPORTING

Quality Control

Assessing Slide Hybridization Adequacy

43. Evaluate control slide hybridization adequacy using the following criteria:
 - **Nuclear morphology:** Borders of tumor nuclei observed by DAPI should generally be distinguishable, and nuclei should have good integrity.

- **Background:** The background should not contain particles that interfere with enumeration.

Note: Fluorescent haze or glow may be noticeable outside of the nuclei, but as long as the fluorescent haze/glow does not cover the nuclei and make enumeration difficult, it is acceptable.

- **Probe signal intensity:** The signals should be bright, distinct, and easily evaluable. Signals should be in bright, compact, round or oval shapes. Overly diffuse signals should be avoided.
- The majority of the target viewing area should meet these quality criteria.
- The target viewing area must contain at least 50 evaluable cells.
- If control slide hybridization adequacy met the hybridization criteria, then repeat slide hybridization adequacy evaluation (step 44) for all specimen slides. If control slide hybridization adequacy did not meet criteria, refer to **Quality Control, Use of Control Slides** section for additional information regarding the use of control slides.

Slide Evaluation

44. Locate Target Viewing Area

- Use the H&E stained slide to confirm the target area prior to viewing the FISH slides.
- Use a 10X to 25X objective and the DAPI bandpass filter to locate the hybridized area of interest.
- Avoid areas of necrosis and where the nuclear borders are ambiguous. Skip nuclei with insufficient counterstain to determine the nuclear border.

45. Assess Target Area

- Using a 60X to 100X objective, use the prescribed filters to examine the quality of ALK signals and quality of tissue morphology. Adjust the depth of the focus and become familiar with the size and shape of the target signals and noise (debris). Verify that background appears dark and relatively free of strong fluorescence that can make enumeration difficult.
- Scan the entire scribed area(s). Observe the signal distribution among tumor cells during scanning in order to select a representative area for enumeration.

46. Select and Enumerate Cells Within Target Area

- Select an area of good nuclear distribution (i.e., where individual nuclei can be distinguished) and ensure areas chosen for enumeration are representative of the signal distribution observed.
- Using a 60X to 100X objective and prescribed filters, begin analysis of the cells selected for enumeration and record signals in each cell.
- Move to the next representative area for enumeration.
- Repeat bullets 2 and 3 until 50 cells have been enumerated.
- Stop when 50 cells selected from representative areas were enumerated.

Note: The field diaphragm may be narrowed around the cells of interest to aid in enumeration.

47. Signal Enumeration Rules

- Focus up and down to find all of the signals present in the nucleus. Enumerate the signals within the nuclear boundary of each selected interphase tumor cell according to the guidelines provided in Figure 1.
- Cells are considered negative (non-rearranged) when:
 - Orange and green signals are adjacent or fused (appear yellow under the Orange/Green V2 filter). Orange and green signals that are less than two signal diameters apart are considered as a single fused signal (Figure 2, Panel 1).
 - There is a single green signal without a corresponding orange signal (Figure 2, Panel 1).
- Cells are considered positive (re-arranged) when:
 - At least one set of orange and green signals are two or more signal diameters apart (Figure 2, Panel 2).
 - There is a single orange signal without a corresponding green signal in addition to fused and/or broken apart signals (Figure 2, Panel 2).

Figure 1
ALK Signal Enumeration Guide

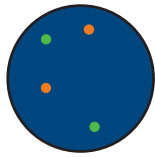
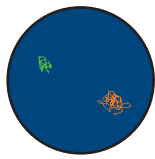
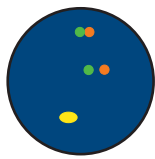
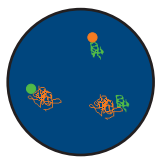
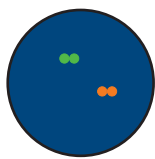
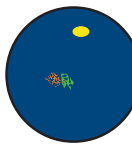

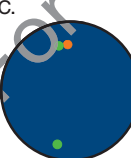
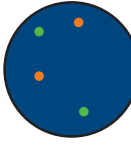
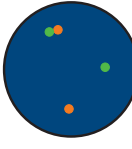
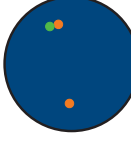
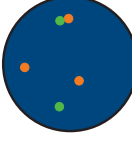
<ul style="list-style-type: none"> ● Single orange signal ● Single green signal ● Adjacent or fused orange green signals 	
Panel 1: Typical Signal Patterns	Guidelines:
	A. Individual orange or green signals are considered as single signals.
	B. Diffuse signals can have a fuzzy or elongated DNA fiber appearance and should be recorded as a single signal.
	C. Adjacent orange and green signals that are less than two signal diameters apart or are overlapping are considered as one whole fused signal. Multiple fused and/or broken apart signals may be observed in a single nucleus.
	D. If diffuse signals are adjacent or connected by a fiber, they should be recorded as one fused signal. Multiple fused and/or broken apart signals may be observed in a single nucleus.
	E. Two signals of the same color that are the same size and separated by a distance less than two signal diameters should be recorded as one signal, (this is a split signal).

Figure 2
ALK Signal Enumeration Guide

Signal Profile 1: Negative	
Panel 1. Adjacent or fused orange and green signals	
1A. 	A. and B. These examples contain fused orange and green signals. The signals are either overlapping, adjacent, or are less than two signal diameters apart.
1B. 	
1C. 	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.
Nuclei containing signals of only one color should not be enumerated.	

Signal Profile 2: Positive	
Panel 2: Broken apart or deleted green	
2A. 	<p>These nuclei contain rearranged or "broken apart" signals, 2 or more signal diameters apart.</p> <p>A. A nucleus can have more than one set of broken apart signals.</p> <p>B. A nucleus can have fused signal(s) and broken apart signal(s).</p> <p>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.</p> <p>D. The same nucleus may have fused signals, broken apart signals and deletions.</p>
2B. 	
2C. 	
2D. 	

Recording of Signal Enumeration

48. Record signal patterns for 50 nuclei.

- For each nucleus, record the number of fused (adjacent) signals.
- For each nucleus, record the number of single orange signals.
- For each nucleus, record the number of single green signals.
- An individual cell is counted only once regardless of the number of rearrangements and/or deletions that it contains.
- Do not score nuclei with no signals or with signals of only one color (without a fused and/or broken apart signal). Score only those nuclei with one or more FISH signals of each color.
- Do not enumerate a nucleus if it contains signals that are weak or overly diffuse.

Results Recording for ALK Status

49. Classify each nucleus according to the Table 1.

Table 1
Classification of Cells as Positive or Negative

Signal Profile	No. of Adjacent or Fused Signals	No. of Single Orange Signals	No. of Single Green Signals	Cell Classification
1A, 1B	≥ 1	0	0	Negative
1C	≥ 1	0	≥ 1	Negative
2A, 2B, 2D	≥ 0	≥ 1	≥ 1	Positive
2C	≥ 1	≥ 1	0	Positive

50. Determine the number of cells classified as negative.

51. Determine the number of cells classified as positive.

52. A sample is considered negative if <5 cells out of 50 (<5/50 or <10%) are positive.

53. A sample is considered positive if >25 cells out of 50 (>25/50 or >50%) are positive.

54. A sample is considered equivocal if 5 to 25 cells (10 to 50%) are positive. If the sample is equivocal, a second reader should evaluate the slide.

- The first and second cell count readings are added together and a percent is calculated out of 100 cells (average percent of positive cells).
- If the average percent positive cells is <15% (<15/100), the sample is considered negative.
- If the average percent positive cells is ≥15% (≥15/100), the sample is considered positive.

Uninformative Result:

Designate a specimen as Uninformative if the specimen failed the quality checks as described in the section **Assessing Slide Hybridization Adequacy**.

- If there are fewer than 50 tumor nuclei within the scribed area that can be enumerated for a specimen, the specimen is uninformative.

Use of Control Slides

- Control slides must be run concurrently with patient slides to monitor assay performance and to assess the accuracy of signal enumeration. Control slides should be processed with specimen slides, beginning at **Slide Deparaffinization Procedure** step 14 (baking at 60°C).
- Control slides should be run on each day of FISH testing and with each new kit lot.
- The established range for acceptable test performance for ProbeChek ALK Control Slides are specified on each lot-specific Certificate of Analysis included with the control slide kit.
- If a control slide fails to meet any of the acceptance criteria, the assay may not have been performed properly or the ALK Break Apart FISH Probe Kit components may have performed inadequately. In no case should FISH results be reported if either control slide fails. A repeat analysis with fresh control slides and clinical specimen slide(s) will be necessary.

Tips and Troubleshooting

When viewing the results of a FISH assay, ensure that the microscope is properly aligned and functioning optimally.

The following table lists some less than optimal results that may be encountered using the LSI probes. Probable causes and suggestions to improve assay performance are included.

Problem	Probable Cause	Possible Solution
No signal or weak signals	Inappropriate filter set used to view slides	Use recommended filters.
	Microscope not functioning properly	Call microscope manufacturer's technical representative.
	Improper lamps (i.e., Xenon or Tungsten)	Use a mercury lamp (100-watt recommended).
	Mercury lamp too old	Replace with a new lamp.
	Mercury lamp misaligned	Realign lamp.
	Dirty or cracked collector lenses	Clean or replace lens.
	Dirty or broken mirror in lamp house	Clean or replace mirror.
	Inappropriate hybridization time	Verify hybridization time.
	Inappropriate post-hybridization wash temperature	Verify temperature of Wash Buffer II.
	Air bubbles trapped under coverslip prevented probe access	Apply coverslip by first touching the surface of the probe mixture.
	Inadequate protease digestion	Verify temperature of the Protease Solution.
Section over fixed (cell boundaries will be distinct)	Prolonged tissue fixation times may lead to progressive degradation of signal intensity and may require longer digestion times.	
Uninformative Result	Too few nuclei (< 50) available for enumeration	Repeat assay with new slide.
Noisy background	Inadequate wash stringency	Verify temperature of the Wash Buffer II.

Problem	Probable Cause	Possible Solution
Variation of signal intensity across tissue section	Probe unevenly distributed on slide due to air bubbles under coverslip	Repeat assay on next adjacent section of same tissue block and make sure no air bubbles are trapped under coverslip.
		Apply coverslip by first touching the surface of the probe mixture.
Tissue loss or tissue morphology degraded	Tissue section under-fixed (poor DAPI staining)	Verify protease digestion time.
	DNA loss (poor DAPI staining)	Verify fixation conditions.
	Inappropriate slides used	Use positively-charged slides.
	Improper slide baking	Verify temperature of ThermoBrite.
	Over pretreatment	Verify time and temperature Vysis Pretreatment Solution.
	Over denaturation (Melt Time)	Verify Melt time.
	Tissue section was torn when removing coverslip after hybridization	Allow additional time for coverslip to soak off in wash buffer.

LIMITATIONS OF THE PROCEDURE

- **FOR IN VITRO DIAGNOSTIC USE ONLY.**
- Optimal performance of this test requires appropriate specimen handling, preparation, and storage as described in these instructions for use.
- The Vysis ALK Break Apart FISH Probe Kit has been optimized only for identifying and quantifying rearrangements of the ALK gene from formalin-fixed, paraffin-embedded human NSCLC tissue specimens. The assay should be performed only on 10% neutral buffered formalin FFPE human lung cancer tissue. Other types of specimens or fixatives should not be used.
- The performance of the Vysis ALK Break Apart FISH Probe Kit was established using the procedures provided in this package insert only. Modifications to these procedures may alter the performance of the assay.
- The clinical interpretation of any test results should be evaluated within the context of the patient's medical history and other diagnostic laboratory test results.
- FISH assay results may not be informative if the specimen quality and/or specimen slide preparation is inadequate.
- Technologists performing the FISH signal enumeration must be capable of visually distinguishing between the orange, green, and yellow signals.

EXPECTED VALUES

Normal Cutoff

The normal cutoff value is defined as the maximum amount of scoreable interphase nuclei with a specific abnormal signal pattern at which a specimen is considered negative for that signal pattern. The normal cutoff value is expressed in terms of a percentage or the actual number of nuclear FISH patterns positive for rearrangement per the standard number of nuclei tested. The normal cutoff was established as 15% using NSCLC FFPE tissue specimens.

SPECIFIC PERFORMANCE CHARACTERISTICS

Probe Localization on Metaphase Chromosomes

The location of hybridization of the Vysis ALK Break Apart FISH Probe was evaluated on metaphase spreads (a total of eight) from cultured lymphocyte slide preparations in conjunction with the inverted DAPI chromosome banding technique.

The Vysis LSI 3'-ALK SpectrumOrange and Vysis LSI 5'-ALK SpectrumGreen probes, components of the Vysis LSI ALK Dual Color Break Apart FISH Probe were shown to hybridize to the intended locus (2p23) on a total of 8 metaphase spreads and to no other locations.

Analytical Sensitivity and Specificity

Analytical sensitivity is defined as the percentage of chromosome targets with the expected normal signal pattern. Analytical specificity is defined as the percentage of signals that hybridize to the correct locus and no other location.

The analytical sensitivity and analytical specificity of the Vysis LSI 3'-ALK SpectrumOrange and Vysis LSI 5'-ALK SpectrumGreen FISH probes was evaluated using metaphase chromosomes prepared from 6 peripheral blood cultures of karyotypically normal specimens from 5 individual donors (6 slide lots).

For the analytical sensitivity calculation, the signals for Vysis LSI 3'-ALK SO and Vysis LSI 5'-ALK SGN FISH probes were enumerated for each metaphase spread (normal = 2 signals). In total, 240 signals were expected for each probe (2 signals per cell x 20 metaphase spreads per lot x 6 slide lots). Refer to Table 2.

For the analytical specificity calculation, the number of metaphase spreads with the expected signal pattern was enumerated. In total, 120 metaphase spreads were evaluated (20 metaphase spreads x 6 slide lots). Refer to Table 3.

For each probe, the analytical sensitivity was calculated to be 100.0% (240/240)(95% CI 98.5 – 100.0) and the analytical specificity was calculated to be 100% (120/120)(95% CI 97.0 – 100.0).

Table 2
Analytical Sensitivity

Probe	No. of Metaphase Chromosome Signals			Sensitivity	
	Total True Positive	Total Expected	Point Estimate (%)	95% Confidence Interval	
	Vysis LSI 3'-ALK SO	240	240	100.0	(98.5, 100.0)
Vysis LSI 5'-ALK SGN	240	240	100.0	(98.5, 100.0)	

Table 3
Analytical Specificity

Probe	No. of Metaphase Chromosome Spreads			Specificity	
	Total False Positive	Total True Positive	Total Expected	Point Estimate (%)	95% Confidence Interval
	Vysis LSI 3'-ALK SO	0	120	120	100.0
Vysis LSI 5'-ALK SGN	0	120	120	100.0	(97.0, 100.0)

Microbial Contamination

The Vysis ALK Break Apart FISH Probe Kit met the requirements for a microbiologically uncontrolled product per "Guideline for the Manufacture of In Vitro Diagnostic Products," 1/10/1994, as none of the reagents would sustain growth of the selected microorganisms and in fact killed the applied inoculum of microorganisms as referenced by the lack of growth upon subculture. Additionally, upon testing the reagents in the normal QC procedure, all the reagents performed satisfactorily even after three days of incubation with the selected organisms at 35 to 37°C.

Control Slide Reproducibility

Control slide reproducibility was evaluated using three lots of both the ProbeChek ALK Negative Control Slides and ProbeChek ALK Positive Control Slides. Each lot was run on 5 non-consecutive days over a 23-day time period and evaluated by three readers for a total of 90 data points (3 lots x 5 runs x 3 readers = 45 evaluations per control slide type).

For each specimen, the signal patterns of 50 nuclei were evaluated by counting the number of fused signals, single orange signals, and single green signals present for each target by each reader.

There was no statistical difference in FISH classification between 3 readers by the Fisher-Freeman-Halton test at the significance level of 0.05. (Refer to Table 4 and Table 5). Therefore, it was demonstrated that Probe Check ALK Negative Control Slides and ProbeChek ALK Positive Control Slides could be reproducibly classified. All slides in this study were found to be within specifications.

Table 4
Reproducibility of ProbeChek ALK Negative Control Slides

Readers	Number of Observations with the Percent ALK Rearrangement		Total
	Within Specification ($\leq 8\%$)	Outside Specification ($> 8\%$)	
1	15	0	15
2	15	0	15
3	15	0	15

Fisher-Freeman-Halton p -value = 1.00

Table 5
Reproducibility of ProbeChek ALK Positive Control Slides

Readers	Number of Observations with the Percent ALK Rearrangement		Total
	Within Specification ($\geq 20\%$)	Outside Specification ($< 20\%$)	
1	15	0	15
2	15	0	15
3	15	0	15

Fisher-Freeman-Halton p -value = 1.00

Tissue Reproducibility

Tissue reproducibility was evaluated using FFPE lung tumor sections. This study was conducted using six serial sections (5 μ m) prepared from twenty NSCLC FFPE specimen blocks. The panel included three positive specimens with > 50% of the cells with ALK rearrangement, three specimens falling within the range of 10% to 50% cells with the ALK rearrangement and fourteen negative specimens with < 10% cells with the ALK rearrangement. Two slides were prepared from each specimen and each slide was evaluated by two readers. Between-reader (Table 6) and between-slide reproducibility (Table 7) were evaluated.

The Vysis ALK Break Apart FISH Probe Kit was shown to be reproducible based upon the between-reader and between-slide analyses resulting in a Fisher-Freeman-Halton p -value of 1.00.

Table 6
Between-Reader Reproducibility

	Number of Panel Members		Total
	Negative	Positive	
Reader 1	14	6	20
Reader 2	14	6	20
Reader 3	14	6	20

Fisher-Freeman-Halton p -value: 1.00

Table 7
Between-Slide Reproducibility

	Number of Panel Members		Total
	Negative	Positive	
Slide 1	14	6	20
Slide 2	15	5	20
Slide 3	14	6	20

Fisher-Freeman-Halton p -value: 1.00

External Reproducibility

Reproducibility of the Vysis ALK Break Apart FISH Probe Kit was evaluated at three external laboratories by testing a coded, randomized 12-member specimen panel (6 unique specimens, 2 slides each) that consisted of four unique ALK-positives with varying levels of positivity (Panel Member 1, 2, 3, and 6) and two unique ALK negative NSCLC FFPE tissue specimens (Panel Member 4 and 5).

Three lots of the Vysis ALK Break Apart FISH Probe Kit reagents were used in the evaluation. A run consisted of one replicate each of a ProbeChek Negative Control slide, a ProbeChek Positive Control slide and each panel member. Each of the three clinical sites tested the Reproducibility Panel using two of the three clinical lots. Each of the two technologists at each of the three testing sites enumerated 6 study specimens along with control slides once a day, for 5 non-consecutive days, per reagent lot over a period of 20 days. Each site evaluated 120 specimen slides for a total of 360. This resulted in 240 enumerations at each site for a minimum of 720 enumerations. Each site evaluated 40 controls slides (20 positive and 20 negative slides) for a total of 120. This resulted in 80 enumerations at each site for a minimum of 240 enumerations. For each panel member and control slides, the signal patterns of 50 nuclei were enumerated by two readers. The overall kappa coefficient was 0.92 (95% CI 0.85 – 0.98). The Z-Score of 27.08, which is greater than 1.96, showed the kappa coefficient is significantly different from zero at a 0.05 level of significance. These results are found in Table 8. The overall percent agreement (PA) between all reader results was 97.64% (95% CI 96.25 – 98.52). The positive percent agreement (PPA) was 96.46% (95% CI 94.40 – 97.78) and the negative percent agreement (NPA) was 100.00% (95% CI 98.42 – 100.00). The results are found in Table 9. The kappa coefficient demonstrated the reproducibility for each site, ranging from 0.83 to 0.96, and for each lot, ranging from 0.86 to 0.96. The results are found in Tables 10 and 11, respectively.

Table 8

**Overall Reproducibility
Number of Slides Across Sites/Lots/Runs/Readers**

	Negative	Positive	Total
Panel Member 1	1	59	60
Panel Member 2	0	60	60
Panel Member 3	2	58	60
Panel Member 4	60	0	60
Panel Member 5	60	0	60
Panel Member 6	4	56	60

Kappa Statistic: 0.92 (0.85, 0.98)

Table 9

Percent Agreement Between All Readers with Expected Results

Reader Results	Expected Results		Total
	Positive	Negative	
Positive	463	0	463
Negative	17	240	257
Total	480	240	720

PA: 97.64 (95%CI: 96.25, 98.52)

PPA: 96.46 (95%CI: 94.40, 97.78)

NPA: 100.00 (95%CI: 98.42, 100.00)

**Table 10
Reproducibility by Site**

Site	Panel Member	Number of Slides Across Lots/Runs/Readers		Kappa Analysis			
		Negative	Positive	Kappa	95% CI	Standard Error	Z-Score
1	1	0	20	0.96	(0.83, 1.00)	0.068	14.21
	2	0	20				
	3	0	20				
	4	20	0				
	5	20	0				
	6	1	19				
2	1	0	20	0.96	(0.83, 1.00)	0.068	14.21
	2	0	20				
	3	0	20				
	4	20	0				
	5	20	0				
	6	1	19				
3	1	1	19	0.83	(0.72, 0.94)	0.056	14.90
	2	0	20				
	3	2	18				
	4	20	0				
	5	20	0				
	6	2	18				

Table 11

Reproducibility by Lot

Lot	Panel Member	Number of Slides Across Sites/Runs/Readers		Kappa Analysis			
		Negative	Positive	Kappa	95% CI	Standard Error	Z-Score
1	1	0	20	0.86	(0.75, 0.98)	0.059	14.75
	2	0	20				
	3	2	18				
	4	20	0				
	5	20	0				
	6	2	18				
2	1	0	20	0.96	(0.83, 1.00)	0.068	14.21
	2	0	20				
	3	0	20				
	4	20	0				
	5	20	0				
	6	1	19				
3	1	1	19	0.93	(0.80, 1.00)	0.065	14.34
	2	0	20				
	3	0	20				
	4	20	0				
	5	20	0				
	6	1	19				

Clinical Trial Information

The use of single-agent XALKORI in the treatment of locally advanced or metastatic ALK-positive NSCLC was investigated in 2 multi-center, single-arm studies (Studies A and B). Patients enrolled into these studies had received prior systemic therapy, with the exception of 15 patients in Study B who had no prior systemic treatment for locally advanced or metastatic disease. Data for Study B is not shown as ALK-positivity was identified using a number of local assays.

In Study A, ALK-positive NSCLC was identified using the Vysis ALK Break Apart FISH Probe Kit. The primary efficacy endpoint in both studies was Objective Response Rate (ORR) according to Response Evaluation Criteria in Solid Tumors (RECIST). Response was evaluated by the investigator and by an independent radiology review panel. Duration of Response (DR) was also evaluated. Patients received 250 mg of XALKORI orally twice daily.

Demographic and disease characteristics for Study A is provided in Table 12.

Table 12
Demographic and Disease Characteristics in Study A

Characteristics	N= 136
Sex, n (%)	
Male	64 (47)
Female	72 (53)
Age (years)	
Median (range)	52 (29 - 82)
Race, n (%)	
White	87 (64)
Black	5 (4)
Asian	43 (32)
Other	1 (1)
ECOG Performance Status (PS) at baseline, n (%)	
0	37 (27)
1	74 (54)
2 - 3 ^a	25 (18)
Smoking status, n (%)	
Never smoked	92 (68)
Former smoker	39 (29)
Current smoker	5 (4)
Disease stage, n (%)	
Locally advanced	9 (7)
Metastatic	127 (93)
Histological classification, n (%)	
Adenocarcinoma	130 (96)
Large cell carcinoma	1 (1)
Squamous cell carcinoma	0
Adenosquamous carcinoma	3 (2)
Other	2 (2)
Prior systemic therapy for locally advanced or metastatic disease — number of regimens, n (%)	
1	13 (10)
2	37 (27)
3	37 (27)
≥4	49 (36)

^a Includes 1 patient with an ECOG PS of 1 at screening but was 3 at baseline

One hundred thirty-six patients with locally advanced or metastatic ALK-positive NSCLC from Study A were analyzed at the time of data cutoff. The median duration of treatment was 22 weeks. Based on investigator assessments, there was 1 complete and 67 partial responses for an ORR of 50% (95% CI: 42%, 59%). Seventy-nine percent of objective tumor responses were achieved during the first 8 weeks of treatment. The median response duration was 41.9 weeks. Efficacy data from Study A are provided in Table 13.

Table 13
Locally Advanced or Metastatic ALK-Positive NSCLC Efficacy Results from Study A^a using the Vysis ALK Break Apart FISH Probe Kit

Efficacy Parameter	N=136
ORR (CR + PR) ^b [% (95% CI)]	50% (42%, 59%)
Number of Responders	68
Duration of Response ^c [Median (range) weeks]	41.9 (6.1+, 42.1+)

^a Response was assessed by the Investigator.

^b One patient was not evaluable for response.

^c Preliminary estimate using Kaplan-Meier method.

+ Censored values

CR = Complete Response

PR = Partial Response

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Technical Assistance:


For technical assistance, call Abbott Molecular Technical Services +1-800-553-7042 in the US and from outside the US +49-6122-580 or visit the Abbott Molecular website at <http://www.abbottmolecular.com>.

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Manufacturer's Address

 Abbott Molecular Inc.
1300 East Touhy Avenue
Des Plaines, IL 60018 USA

Authorized Representative's
Address (AR)

 ABBOTT GmbH &
Co. KG
Max-Planck-Ring 2
65205 Wiesbaden
Germany



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