Vysis ALK Break Apart FISH Probe Kit

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REF 06N38

30-608521/R1

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NOTE: Specific instructions for the automated (VP 2000) assay protocol and expanded assay options for specimen handling and processing are highlighted.

Key to Symbols Used

GTIN	Global Trade Item Number
	Manufacturer
REF	Reference Number
LOT	Lot Number
IVD	In Vitro Diagnostic Medical Device
Σ	Contains sufficient for <n> tests
	Temperature Limit
	Caution, consult accompanying documents
	Use By
	Consult instructions for use
	Biological Risks
	Refer to **WARNINGS AND PRECAUTIONS**.
EC REP	Authorized Representative in the European Community

VYSIS ALK BREAK APART FISH PROBE KIT

List No. 06N38-033 (20 assays) / List No. 06N38-053 (50 assays)

CUSTOMER SERVICE: 1-800-553-7042

CUSTOMER SERVICE INTERNATIONAL: CALL YOUR ABBOTT REPRESENTATIVE

This package insert must be read carefully prior to use. Package insert instructions must be followed accordingly. Reliability of assay results cannot be guaranteed if there are any deviations from the instructions in this package insert.

NAME

Vysis ALK Break Apart FISH Probe Kit

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 31 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 2.2013) 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 immunohistochemistry (IHC) tests used to detect *ALK*-rearrangement in clinical laboratories worldwide are inadequate for the detection of most *ALK*-rearranged lung adenocarcinomas.[12]

Non-small cell lung cancer is the leading cause of cancer death worldwide.[13,14] With a 5-year morbidity rate of 85 to 95%, there is a pressing need for improvement in identifying patients most likely to respond to specific treatments.[14] Tyrosine kinase inhibitors have been demonstrated to reduce lung cancer cell proliferation, resulting in suppression of tumor growth.[9,15-17]

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 63 of 82 patients were still receiving therapy at the time of the data cutoff with an estimated probability of progression-free survival of 72%.[11]

In a Phase 3, open-label trial comparing crizotinib with chemotherapy, chromosomal rearrangements of ALK have been associated with marked clinical response to crizotinib, an oral tyrosine kinase inhibitor targeting ALK. Patients were eligible for inclusion in this study if they had locally advanced or metastatic NSCLC that was determined to be ALK positive with the Vysis ALK Break Apart FISH Probe Kit.[18]

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.

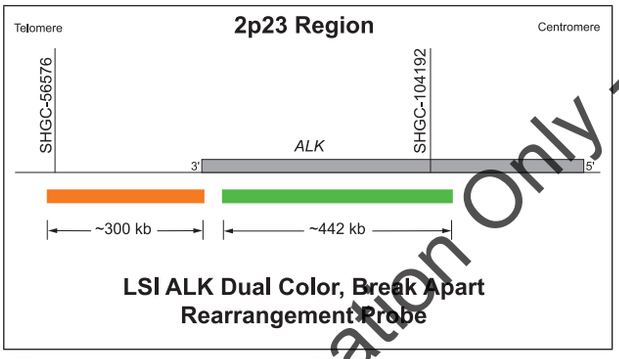
When hybridized with the Vysis ALK Break Apart FISH Probes, the 2p23 *ALK* region in its native state will be seen as 2 immediately adjacent or fused (overlapping) orange/green (yellow) signals. However, if a chromosome rearrangement at the 2p23 *ALK* breakpoint region has occurred, 1 orange and 1 green signal separated by at least 2 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 2 fluorophore-labeled DNA probes in hybridization buffer containing dextran sulfate, formamide, and SSC with blocking DNA:

- Vysis LSI 3′-ALK SpectrumOrange (SO)
- Vysis LSI 5′-ALK SpectrumGreen (SGn)

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.



REAGENTS

Vysis ALK Break Apart FISH Probe Kit

This kit contains sufficient reagents to process 20 or 50 assays dependent on the product ordered. An assay is defined as one 22 mm × 22 mm target area.

1. **Vysis LSI ALK Dual Color Break Apart FISH Probe**
   (20 test kit, 1 vial 200 µL per vial)
   (50 test kit, 1 vial 500 µL per vial)
   SpectrumOrange (50 ng/10 µL) and SpectrumGreen (200 ng/10 µL) fluorophore-labeled DNA probes in hybridization buffer containing dextran sulfate, formamide, and SSC with blocking DNA.
2. **DAPI I Counterstain**
   (20 test kit, 1 vial 300 µL per vial)
   (50 test kit, 1 vial 1000 µL per vial)
   1 µg/mL, DAPI (4′,6-diamidino-2-phenylindole • 2HCl) in phenylenediamine dihydrochloride, glycerol, and phosphate buffered saline mixture.

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

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

Biosafety Statement for Kit Component

- Vysis LSI ALK Dual Color Break Apart FISH Probe

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. These reagents and human specimens should be handled as if infectious, using safe laboratory procedures, such as those outlined in Biosafety in Microbiological and Biomedical Laboratories,19 OSHA Standards on Bloodborne Pathogens,[20] CLSI Document M29-A3,21 and other appropriate biosafety practices.[22] Therefore, all human-sourced materials should be considered 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.[19]
- Decontaminate and dispose of all potentially infectious materials in accordance with local, state, and federal regulations.[22]

- Refer to instrument-specific safety information for the VP 2000 Processor and ThermoBrite instrument.
- Use Vysis Paraffin Pretreatment IV & Post-Hybridization Wash Buffer Kit (List No. 01N31-005) for the Manual Assay. Use VP 2000 Pretreatment Kit (List No. 08N16-001) for the Automated (VP 2000) Assay. Do not interchange between the Manual and Automated Assay Protocols.
- 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 solutions 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 after the dates shown on kit or reagent labels.

- 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.

Vysis LSI ALK Dual Color Break Apart FISH Probe

Danger
Hazard-determining components of labeling: formamide

H360	May damage fertility or the unborn child.
P201	Obtain special instructions before use.
P202	Do not handle until all safety precautions have been read and understood.
P281	Use personal protective equipment as required.
P308+P313	If exposed or concerned: Get medical advice/ attention
P405	Store locked up.
P501	This material and its container must be disposed of in a safe way.

Safety Data Sheet Statement: Important information regarding the safe handling, transport, and disposal of this product is contained in the Safety Data Sheet.

NOTE: Safety Data Sheets (SDS) for all reagents provided in the kit are available upon request from the Abbott Molecular Technical Service Department.

MATERIALS

Materials Provided

- Vysis ALK Break Apart FISH Probe Kit (List No. 06N38-033 [20 Test Kit] or List No. 06N38-053 [50 Test Kit])

Materials Required But Not Provided

- ProbeChek ALK Negative Control Slides (List No. 06N38-005)
- ProbeChek ALK Positive Control Slides (List No. 06N38-010)

Laboratory Reagents

- Hemo-De (or equivalent, eg, 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 × 22 mm glass coverslips
- Microliter pipette tips for 1 to 10 µL volumes (sterile)
- Microliter pipettor calibrated for 1 to 10 µL volumes
- Timer
- Microtome
- Microcentrifuge
- Graduated cylinders
- Purified water bath (37°C to 42°C)
- Diamond-tipped scribe
- Solvent-resistant marker (optional)
- Forceps
- Coplin jars (12 × 50 mL) Suggested type: vertical staining jar
- Calibrated thermometer
- Vortex mixer
- Air incubator/oven (optional)
- Microscope slide box with lid and/or carton slide folders
- ThermoBrite
- ThermoBrite humidity cards

Additional Reagents/Laboratory Materials for Manual Assay Protocol

- Vysis Paraffin Pretreatment IV & Post-Hybridization Wash Buffer Kit (List No. 01N31-005)
- 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

Additional Reagents/Laboratory Materials for Automated (VP 2000) Assay Protocol

- VP 2000 Pretreatment Kit (List No. 08N16-001)
- VP 2000 Processor

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 PROCEDURE

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 (primary and metastatic tumors), such as surgical resections, core needle biopsies, and FFPE cell pellets (eg, fine needle aspirates). 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 2 or more serial paraffin sections, 5 ± 1 µm thick, using a microtome.
2. Float the sections on the surface of a purified water bath set at 37 to 50°C for up to 30 minutes.
3. Mount the sections on positively-charged glass slides.
4. Allow the slide to air-dry.
5. Perform conventional H&E staining for 1 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**.

   NOTE: Include 1 ProbeChek ALK Negative Control slide and 1 ProbeChek ALK Positive Control slide starting with Step 9.
9. Prior to initiating Manual Assay Protocol or Automated (VP 2000) Assay Protocol, bake the unstained specimen and control slides for 2 to 24 hours at 60°C on a ThermoBrite instrument or air incubator/oven.

The Vysis ALK Break Apart FISH Assay may be performed as manual assay or as automated assay. Refer to instructions below.

MANUAL ASSAY PROTOCOL

Working Reagent Preparation for Manual Assay

NOTE: Use Vysis Paraffin Pretreatment IV & Post-Hybridization Wash Buffer Kit (List No. 01N31-005) for the Manual Assay. Use VP 2000 Pretreatment Kit (List No. 08N16-001) for the Automated (VP 2000) Assay. Do not interchange between the Manual and Automated Assay Protocols.

10. **Preparation of Hemo-De** – Fill 3 Coplin jars with 50 mL of Hemo-De. Keep covered when not in use. Store under vented conditions at ambient temperature, and discard after 7 days.
11. **Preparation of Pretreatment Solution** – Fill 1 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 ± 2°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 ± 2°C prior to use. Discard the solution after using 1 day.
12. **Preparation of Protease Solution** – Add 1 vial of Vysis Protease IV (75 mg) to 1 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 1 hour after mixing to ensure that the protease is in solution and confirm that the temperature of the buffer is 37 ± 1°C before use. Discard solution after 1 day.
13. **Preparation of Purified Water** – Fill 1 Coplin jar with 50 mL of purified water. Use at ambient temperature. Replace after each use.
14. **Preparation of Ethanol Solutions (70%, 85%, and 100%)** – Prepare v/v (volume/volume) 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 1 week unless evaporation occurs or the solution becomes diluted or cloudy due to excessive use.

Slide Deparaffinization Procedure

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 8 slides for 12 ± 3 minutes in Vysis Pretreatment Solution that has been previously warmed to 80 ± 2°C.

NOTE: If necessary, 2 slides may be placed back-to-back in each slot of the Coplin jar, with 1 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 8 slides per Coplin jar at 1 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 for 20 ± 4 minutes in Protease Solution that has been previously warmed to 37 ± 1°C.
24. Immerse slides in purified water for 3 minutes.

Hybridization Procedure

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

25. Immerse the slides in 70% ethanol for 1 minute.
26. Immerse the slides in 85% ethanol for 1 minute.
27. Immerse the slides in 100% ethanol for 1 minute.
28. Air-dry the slides for 2 to 5 minutes.
29. Moisten humidity cards with water and place in the card slots of the ThermoBrite instrument. Ensure that the surface of the ThermoBrite instrument is clean and free of debris.
30. Set the denaturation temperature (Melt Temp) to 73°C and the denaturation time (Melt Time) to 3 minutes. Set the hybridization temperature (Hyb Temp) to 37°C and the hybridization time (Hyb Time) from 14 to 24 hours.
31. Thaw probe mixture at ambient temperature, then mix using a vortex mixer, and centrifuge using a microcentrifuge for 2 to 3 seconds. Apply 10 µL of probe mixture to each slide, then immediately apply a coverslip. Ensure no air bubbles are present in the probe mixture prior to applying the coverslip.
32. Seal the coverslip with rubber cement.
33. Place slides on the ThermoBrite instrument and begin the hybridization program. Hybridize the slides 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 instrument 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 1 day, then discard.
35. Pour 50 mL of Wash Buffer II into a Coplin jar. Place the Coplin jar into an ambient temperature water bath prior to heating to prevent breakage of the jar. Allow the jar to warm to 74 ± 1°C for at least 30 minutes prior to use. Use 1 day, then discard.
36. Remove the rubber cement from 1 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.

NOTE: To maintain the proper temperature in Wash Buffer II, wash only 4 slides simultaneously. If there are less than 4 slides, add blank slides to bring the total number to 4. Start timing when the fourth slide is immersed.

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: Ensure the temperature of Wash Buffer II has returned to 74 ± 1°C before washing another 4 slides.

Counterstaining Procedure

39. Air-dry the slide(s) protected from light at ambient temperature.
40. Thaw DAPI I at ambient temperature, then mix using a vortex mixer, and centrifuge using a microcentrifuge for 2 to 3 seconds. Apply 10 µL of DAPI I counterstain to the target area of the slide, apply coverslip, and store protected from light for a minimum of 5 minutes.
41. Perform Slide Evaluation within 24 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 2 weeks 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).

AUTOMATED (VP 2000) ASSAY PROTOCOL

NOTE: Use Vysis Paraffin Pretreatment IV & Post-Hybridization Wash Buffer Kit (List No. 01N31-005) for the Manual Assay. Use VP 2000 Pretreatment Kit (List No. 08N16-001) for the Automated (VP 2000) Assay. Do not interchange between the Manual and Automated Assay Protocols.

NOTE: Minimum of 3 and maximum of 48 specimen or control slides can be processed for each run when Automated (VP 2000) Assay Protocol is used.

43. **VP 2000 Pretreatment Protocol**
Enter and/or confirm the VP 2000 Pretreatment Protocol (**Table 1**) in the Protocol Editor Window. Refer to the Abbott VP 2000 Processor Operations Manual for additional information related to instrument use. Open the VP 2000 Pretreatment Protocol prior to use.

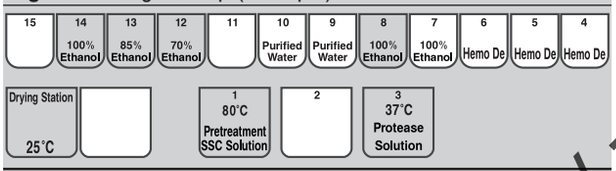
Table 1. VP 2000 Pretreatment Protocol

Step	Description	Basin	Reagent	Time	Temp
1	Hemo-De	4	Hemo-De	5 minutes	Ambient
2	Hemo-De	5	Hemo-De	5 minutes	Ambient
3	Hemo-De	6	Hemo-De	5 minutes	Ambient
4	100% Ethanol	7	100% Ethanol	1 minute	Ambient
5	100% Ethanol	8	100% Ethanol	1 minute	Ambient
6	Pretreatment SSC Solution	1	Pretreatment SSC Solution	25 ± 10 minutes	80°C
7	Purified water	9	Purified water	3 minutes	Ambient
8	Protease Solution	3	Protease Solution	15 ± 5 minutes	37°C
9	Purified water	10	Purified water	3 minutes	Ambient
10	70% Ethanol	12	70% Ethanol	1 minute	Ambient
11	85% Ethanol	13	85% Ethanol	1 minute	Ambient
12	100% Ethanol	14	100% Ethanol	1 minute	Ambient
13	Air dry	Drying Station	-----	-----	Ambient

Working Reagent Preparation for Automated (VP 2000) Assay Protocol

NOTE: Refer to Reagent Map (Figure 1) for the location of reagent basins.

Figure 1. Reagent Map (Example)



44. **Preparation of Hemo-De** – Fill Basins 4, 5, and 6, each with 500 mL of Hemo-De. Keep covered when not in use. Discard after 7 days.
45. **Preparation of Pretreatment SSC Solution** – Add 500 mL (two 250 mL bottles) Vysis Pretreatment SSC Solution (the bottle with a blue color bar) to Basin 1. Confirm that the set temperature of 80°C has been reached on the Run Screen prior to pretreating the slides. Discard after 1 day of use (maximum of 2 runs).
46. **Preparation of Protease Solution** – Add 1 bottle of Vysis Protease IV Buffer (250 mL) and 1 vial of Vysis Protease IV (750 mg) to Basin 3. Rinse the Vysis Protease IV vial with a small volume of Vysis Protease IV Buffer (250 mL) from the second bottle and add the contents to Basin 3. Add the remaining volume of the second bottle of Vysis Protease IV Buffer to Basin 3. Return the basin to the designated location on the VP 2000. Wait a minimum of 1 hour, stirring 2 to 3 times before starting the assay. Visually ensure that the protease is in solution and confirm that the set temperature of 37°C has been reached on the Run Screen prior to use. Discard solution after 1 day of use (maximum of 2 runs).
47. **Preparation of Purified Water** – Fill Basins 9 and 10, each with 500 mL of purified water. Replace after each run.
48. **Preparation of Ethanol Solutions (70%, 85%, and 100%)** – Prepare v/v (volume/volume) dilutions of 70% and 85% using 100% ethanol and purified water. Store at room temperature in tightly capped containers when not in use.
    - Fill Basins 7, 8, and 14 with 500 mL 100% ethanol
    - Fill Basin 12 with 500 mL 70% ethanol
    - Fill Basin 13 with 500 mL 85% ethanol

Ethanol solutions may be used for 1 week unless evaporation occurs or the solution becomes diluted or cloudy due to excessive use.

Slide Pretreatment

49. Load specimen and/or control slides to the VP 2000 slide basket and attach to the Robotic Arm of the VP 2000. When Pretreatment SSC Solution has reached 80°C, start the VP 2000 Pretreatment Protocol (Step 43, **Table 1**). When VP 2000 Pretreatment Protocol is complete, remove the slide basket from the VP 2000 and remove slides from the slide basket.

Hybridization Procedure

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

50. Moisten humidity cards with water and place in the card slots of the ThermoBrite instrument. Ensure that the surface of the ThermoBrite instrument is clean and free of debris.
51. Set the denaturation temperature (Melt Temp) to 73°C and the denaturation time (Melt Time) to 3 minutes. Set the hybridization temperature (Hyb Temp) to 37°C and the hybridization time (Hyb Time) from 14 to 24 hours.
52. Thaw probe mixture at ambient temperature, then mix using a vortex mixer, and centrifuge using a microcentrifuge for 2 to 3 seconds. Apply 10 µL of probe mixture to each slide, then immediately apply a coverslip. Ensure no air bubbles are present in the probe mixture prior to applying the coverslip.
53. Seal the coverslip with rubber cement.
54. Place slides on the ThermoBrite instrument and begin the hybridization program. Hybridize the slides for 14 to 24 hours.

At the end of the hybridization period, proceed to the **VP 2000 Slide Washing Procedure.**

NOTE: Leave the slides on the ThermoBrite instrument until ready to begin.

VP 2000 Slide Washing Procedure

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

55. VP 2000 Slide Washing Protocol
    Enter the VP 2000 Slide Washing Protocol (**Table 2)** in the Protocol Editor Window. Refer to the Abbott VP 2000 Processor Operations Manual for additional information related to instrument use. Open the VP 2000 Slide Washing Protocol prior to use.

Table 2. VP 2000 Slide Washing Protocol

Step	Description	Basin	Reagent	Time	Temp
1	Wash Buffer II	2	Wash Buffer II	2 minutes	74°C
2	Air Dry	Drying station	-----	30 seconds	Ambient

56. Add 500 mL (two 250 mL bottles) of Wash Buffer II (the bottle with a green color bar) to Basin 2 (heated). Confirm that the set temperature of 74°C has been reached on the Run Screen prior to washing the slides. Discard solution after 1 day of use (maximum of 2 runs).
57. Based on the number of slides hybridized, pour 50 mL of Wash Buffer I (the bottle with an orange color bar) into each of a series of Coplin jars allowing for a maximum of 8 slides per Coplin jar. Use at ambient temperature. Discard solution after 1 day of use.
58. When Wash Buffer II has reached 74°C, remove the rubber cement from slide(s) while minimally disturbing the coverslip, and immerse the slide in ambient temperature Wash Buffer I. Let stand at least 2 minutes to allow the coverslips to float off the slides. Check to ensure all coverslips are floated off or remove coverslips carefully if needed. Once all coverslips are floated off or removed, immediately proceed to the next step.
59. Load specimen and/or control slides to the VP 2000 slide basket. Immediately attach to the Robotic Arm of the VP 2000 and start the VP 2000 Slide Washing Protocol (Step 55, **Table 2**) to move and immerse slide basket into Wash Buffer II at 74°C for 2 minutes, followed by a 30 second drying step at ambient temperature.
60. When VP 2000 Slide Washing Protocol is complete, remove the slide basket from the VP 2000 and remove slides from the slide basket.

Counterstaining Procedure

61. Air-dry the slide(s) protected from light at ambient temperature.
62. Thaw DAPI I at ambient temperature, then mix using a vortex mixer, and centrifuge using a microcentrifuge for 2 to 3 seconds. Apply 10 µL of DAPI I counterstain to the target area of the slide, apply coverslip, and store protected from light for a minimum of 5 minutes.
63. Perform Slide Evaluation within 24 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 2 weeks 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

64. 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

65. Evaluate control slide hybridization adequacy using the following criteria:

Assessing Slide Hybridization Adequacy

- **Nuclear morphology:** Borders of tumor nuclei observed by DAPI should be generally 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 tumor cells.

66. If control slide hybridization adequacy met the hybridization criteria, then repeat slide hybridization adequacy evaluation (Step 65) for all specimen slides. If control slide hybridization adequacy did not meet criteria refer to **Use of Control Slides** section for additional information regarding the use of control slides.

Slide Evaluation

67. **Locate Target Viewing Area**

- If necessary, 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.

68. **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.

69. **Select and Enumerate Cells Within Target Area**

- Select an area of good nuclear distribution (ie, 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 of this step until 50 tumor cells have been enumerated.
- Stop when 50 tumor cells selected from representative areas have been enumerated.

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

70. **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 2**.
- 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 <2 signal diameters apart are considered as a single fused signal (**Figure 3, Panel 1**).
  - There is a single green signal without a corresponding orange signal (**Figure 3, Panel 1**).
- Cells are considered positive (re-arranged) when:
  - At least 1 set of orange and green signals are 2 or more signal diameters apart (**Figure 3, Panel 2**).
  - There is a single orange signal without a corresponding green signal in addition to fused and/or broken apart signals (**Figure 3, Panel 2**).

Figure 2. *ALK* Signal Enumeration Guide

- Single orange signal
- Single green signal
- Adjacent or fused orange green signals

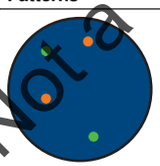
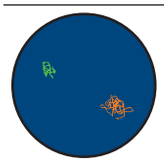
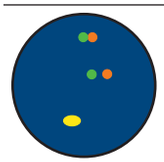
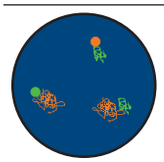
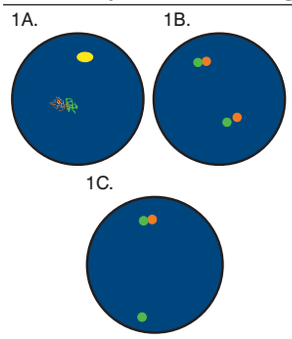
Panel 1: Typical Signal Patterns	**Guidelines**:
	A. Individual orange or green signals are considered as single signals.
	B. Diffuse signals can have a fuzzy or elongated DNA fiber appearance and should be recorded as a single signal.
	C. Adjacent orange and green signals that are <2 signal diameters apart or are overlapping are considered as 1 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 1 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 <2 signal diameters should be recorded as 1 signal (this is a split signal).

Figure 3. *ALK* Signal Enumeration Guide: Signal Profiles

Signal Profile 1: Negative

Panel 1. Adjacent or fused orange and green signals



A. and B. These examples contain fused orange and green signals. The signals are either overlapping, adjacent or are < 2 signal diameters apart.

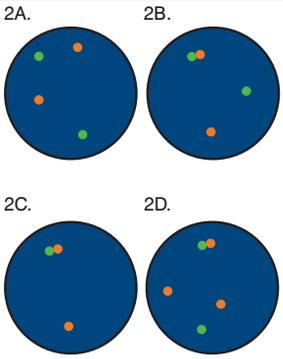
NOTE: Overlapping signals may appear as yellow signals.

C. A single green signal without a corresponding orange signal in addition to a fused signal (overlapping, adjacent, or are < 2 signal diameters apart) 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.

NOTE: Nuclei containing signals of only 1 color should not be enumerated.

Signal Profile 2: Positive

Panel 2: Broken apart or deleted green



These nuclei contain rearranged or "broken apart" signals, 2 or more signal diameters apart.

A. A nucleus can have more than 1 set of broken apart signals.

B. A nucleus can have fused signal(s) and broken apart signal(s).

C. A nucleus can have a single orange signal (deleted green signal) in addition to fused and/or broken apart signals.

NOTE: Nuclei containing signals of only 1 color should not be enumerated.

D. The same nucleus may have fused signals, broken apart signals and deletions.

Recording of Signal Enumeration

71. 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 1 color (without a fused and/or broken apart signal). Score only those nuclei with 1 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

72. Classify each nucleus according to **Table 3**.

Table 3. 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

73. Determine the number of cells classified as negative.

74. Determine the number of cells classified as positive.

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

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

77. 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** under **INTERPRETATION AND RESULT REPORTING – Quality Control**.

- If there are fewer than 50 tumor nuclei within the scribed area that can be enumerated for a specimen slide, the specimen slide is uninformative.
- For uninformative specimen slides, repeat the assay with new slides.

Use of Control Slides

- Control slides must be run concurrently with specimen slides to monitor assay performance and to assess the accuracy of signal enumeration. Control slides should be processed with specimen slides, beginning at slide baking step (step 9).
- 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 in **Table 4.**

Table 4. Established Range for ProbeChek ALK Control Slides

Acceptable specification range established for ProbeChek ALK Negative Control Slides	0 – 8% (0 – 4 positive cells)
Acceptable specification range established for ProbeChek ALK Positive Control Slides	20 – 62% (10 – 31 positive cells)

- 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.

A **Tips and Troubleshooting Guide** is provided in **Appendix A.**

LIMITATIONS

- **FOR IN VITRO DIAGNOSTIC USE ONLY**
- Optimal performance of this test requires appropriate specimen handling, preparation, and storage as described in this package insert.
- 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 tumor 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 for all methods is 15% using NSCLC 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 8) 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 were 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 × 20 metaphase spreads per lot × 6 slide lots). Refer to **Table 5**.

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 × 6 slide lots). Refer to **Table 6**.

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

Table 5. Analytical Sensitivity

	No. of Metaphase Chromosome Signals		Sensitivity	
Probe	Total True Positive	Total Expected	Point Estimate (%)	95% CI
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 6. Analytical Specificity

	No. of Metaphase Chromosome Spreads			Specificity	
Probe	Total False Positive	Total True Positive	Total Expected	Point Estimate (%)	95% CI
Vysis LSI 3′-ALK SO	0	120	120	100.0	(97.0, 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 3 days of incubation with the selected organisms at 35 to 37°C.

Control Slide Reproducibility Using Manual Method

Control slide reproducibility was evaluated using 3 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 3 readers for a total of 90 data points (3 lots × 5 runs × 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 7** and **Table 8**.) Therefore, it was demonstrated that ProbeChek 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 7. Reproducibility of ProbeChek ALK Negative Control Slides

	No. of Observations with the Percent *ALK* Rearrangement		
Readers	Within Specification	Outside Specification	Total
1	15	0	15
2	15	0	15
3	15	0	15

Fisher-Freeman-Halton *P* value = 1.00

Table 8. Reproducibility of ProbeChek ALK Positive Control Slides

	No. of Observations with the Percent *ALK* Rearrangement		
Readers	Within Specification	Outside Specification	Total
1	15	0	15
2	15	0	15
3	15	0	15

Fisher-Freeman-Halton *P* value = 1.00

Control Slide Reproducibility Using Automated Method

Reproducibility of the ProbeChek ALK Negative and Positive Control Slides was evaluated at 3 internal laboratories. A run consisted of 2 negative and 2 positive control slides. Each control slide was processed on a VP 2000 Processor and evaluated at each of the 3 laboratories, for 5 runs on 5 non-consecutive days, over a minimum of 20 days. Each laboratory evaluated 20 control slides, which yielded 20 slide evaluation results. This resulted in a total of 60 slide evaluations.

The percentage of control slides within the established range was calculated to be 100.00% (60/60) (95% CI 93.98 to 100.00). The results are provided in **Table 9.**

Table 9. Percent ProbeChek Control Slides (ALK Positive and Negative Combined) within Established Range Using VP 2000 Slide Processing

No. of Control Slide Results	No. of Control Slide Results Within Established Range	Percent (%) of Control Slide Results Within Established Range	Two-sided 95% CI
60	60	100.00	(93.98, 100.00)

Tissue Reproducibility Using Manual Slide Processing

Tissue reproducibility was evaluated using FFPE lung tumor sections. This study was conducted using 6 serial sections (5 µm) prepared from twenty NSCLC FFPE specimen blocks. The panel included 3 positive specimens with > 50% of the cells with *ALK* rearrangement, 3 specimens falling within the range of 10% to 50% cells with the *ALK* rearrangement and 14 negative specimens with < 10% cells with the *ALK* rearrangement. Two slides were prepared from each specimen and each slide was evaluated by 2 readers. Between-reader (**Table 10**) and between-slide reproducibility (**Table 11**) 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 10. Between-Reader Reproducibility

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

Fisher-Freeman-Halton *P* value: 1.00

Table 11. Between-Slide Reproducibility

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

Fisher-Freeman-Halton *P* value: 1.00

External Reproducibility Using Manual Slide Processing

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

Three lots of the Vysis ALK Break Apart FISH Probe Kit reagents were used in the evaluation. A run consisted of 1 replicate each of a ProbeChek ALK Negative Control slide, a ProbeChek ALK Positive Control slide and each panel member. Each of the 3 clinical sites tested the reproducibility panel using 2 of the 3 clinical lots. Each of the 2 technologists at each of the 3 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 control 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 2 readers.

The overall kappa coefficient was 0.92 (95% CI 0.85 to 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. The results are found in **Table 12**. The overall percent agreement (PA) between all reader results was 97.64% (95% CI 96.25 to 98.52). The positive percent agreement (PPA) was 96.46% (95% CI 94.40 to 97.78) and the negative percent agreement (NPA) was 100.00% (95% CI 98.42 to 100.00). The results are found in **Table 13**. 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 **Table 14** and **Table 15**, respectively.

Table 12. Overall Reproducibility Using Manual Slide Processing

	Number of Slides Across Sites/Lots/Runs/Readers		
Panel Member	Negative	Positive	Total
1	1	59	60
2	0	60	60
3	2	58	60
4	60	0	60
5	60	0	60
6	4	56	60

Kappa Statistic: 0.92 (95% CI 0.85 to 0.98)

Table 13. Percent Agreement Between All Readers with Expected Results Using Manual Slide Processing

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

PA: 97.64 (95% CI 96.25 to 98.52)

PPA: 96.46 (95% CI 94.40 to 97.78)

NPA: 100.00 (95% CI 98.42 to 100.00)

Table 14. Reproducibility by Site Using Manual Slide Processing

		Number of Slides Across Lots/Runs/Readers		Kappa Analysis			
Site	Panel Member	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 15. Reproducibility by Lot Using Manual Slide Processing

		Number of Slides Across Sites/Runs/Readers		Kappa Analysis			
Lot	Panel Member	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				

External Reproducibility Using VP 2000 Slide Processing

Reproducibility of the Vysis ALK Break Apart FISH Probe Kit using VP 2000 slide processing was evaluated at 3 external laboratories by testing a coded, randomized 18-member specimen panel (6 unique specimens, 3 slides each) that consisted of 4 unique ALK-positive NSCLC FFPE tissue specimens with varying levels of positivity (panel members 1, 2, 3, and 6) and 2 unique ALK-negative NSCLC FFPE tissue specimens (panel members 4 and 5).

A run consisted of 1 replicate each of a ProbeChek ALK Negative Control slide and a ProbeChek ALK Positive Control slide, and 3 replicates of each panel member. The technicians at each of the 3 sites enumerated 3 replicates each of the 6 panel specimens along with control slides once a day, for 5 nonconsecutive days over a period of at least 20 days. On each testing day, 1 technician at each of the sites processed the specimen and control slides using the VP 2000 Processor. A second technician at each of the sites enumerated each of the specimen slides and controls. Each site evaluated 90 specimen slides for a total of 270 results.

The overall kappa coefficient was 0.94 (95% CI 0.85 to 1.00). The z score of 21.86, which is greater than 1.96, showed the kappa coefficient is significantly different from zero at a 0.05 level of significance. The results are found in **Table 16.**

The overall percent agreement (PA) between all reader results was 99.25% (95% CI 97.32 to 99.80). The positive percent agreement (PPA) was 98.88% (95% CI 96.00 to 99.69) and the negative percent agreement (NPA) was 100.00% (95% CI 95.91 to 100.00). The results are found in **Table 17.**

Table 16. Overall Reproducibility Using VP 2000 Slide Processing

	Number of Slides Across Sites/Days		
Panel Member	**Negative**	**Positive**	**Total**
1	0	45	45
2	0	45	45
3	0	44	44[a]
4	45	0	45
5	45	0	45
6	2	42	44[a]
Kappa Statistic: 0.94 (95% CI 0.85 to 1.00)			

[a] One specimen was uninformative.

Table 17. Percent Agreement Across Sites/Days/Panels with Expected Results Using VP 2000 Slide Processing

	Expected Results		
Results	**Positive**	**Negative**	**Total**
Positive	176	0	176
Negative	2	90	92
Total	178	90	268[a]
PA: 99.25 (95% CI 97.32 to 99.80)			
PPA: 98.88 (95% CI 96.00 to 99.69)			
NPA: 100.00 (95% CI 95.91 to 100.00)			

[a] Two specimens were uninformative.

VP 2000 Pretreatment Kit Reproducibility

Lot-to-lot reproducibility of the VP 2000 Pretreatment Kit was evaluated internally by testing FFPE lung tumor sections, consisting of 2 unique ALK-positive NSCLC FFPE tissue specimens with varying levels of positivity and 1 unique ALK-negative NSCLC FFPE tissue specimen.

Reproducibility was evaluated using 3 unique lots of VP 2000 Pretreatment Kit reagents, tested over 3 days (1 lot per day) using a VP 2000 Processor. For each lot, 6 blinded specimen slides (3 panel members, run in duplicate) were each evaluated twice by independent readers for a total of 36 results.

The overall percent agreement (PA) with the expected result across lots was 100.00% (95% CI 90.26% to 100.00%). The positive percent agreement (PPA) was 100.00% (95% CI 85.75% to 100.00%). The negative percent agreement (NPA) was 100.00% (95% CI 73.54% to 100.00%).

The results are provided in **Table 18.**

Table 18. Overall Percent Agreement (Across Lots/Panel Members) with Expected Results for the VP 2000 Pretreatment Kit.

	Expected Results		
Results	**Positive**	**Negative**	**Total**
Positive	24	0	24
Negative	0	12	12
Total	24	12	36

Agreements	Pt. Est.	Ratio	Exact 95.00% CI
PA	100.00%	36/36	(90.26%, 100.00%)
PPA	100.00%	24/24	(85.75%, 100.00%)
NPA	100.00%	12/12	(73.54%, 100.00%)

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 are 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 are provided in **Table 19**.

Table 19. 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 regimes, 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% to 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 20**.

Table 20. 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

Concordance Between Manual and Automated Methods

The concordance between the manual and automated slide processing methods was evaluated by testing FFPE NSCLC specimens with both manual and automated VP 2000 slide processing at 3 external laboratories. The comparator test method used in the analyses was the VP 2000 slide processing method, and the reference method was manual slide processing.

A total of 235 specimens were used, including 125 ALK-positive and 110 ALK-negative specimens. Four slides of each specimen were tested; 1 slide was processed using the manual slide processing method, and the other 3 slides were processed (1 slide for each site) using the VP 2000 slide processing method.

The agreement analysis showed an average PA of 95.66% (95% CI 93.92 to 97.26), an average PPA of 98.47% (95% CI 96.88 to 99.69) and an average NPA of 92.96% (95% CI 89.81 to 95.79). Results are presented in **Table 21.**

Table 21. Agreement Analysis for VP 2000 Slide Processing vs. Manual Slide Processing

Site	PPA (%) (n/N)	NPA (%) (n/N)	PA (%) (n/N)[a]
1	98.04 (100/102)	100.00 (113/113)	99.07 (213/215)
2	99.13 (114/115)	93.86 (107/114)	96.51 (221/229)
3	98.18 (108/110)	85.09 (97/114)	91.52 (205/224)
	PPA (%) (95% CI)[b]	**NPA (%) (95% CI)[b]**	**PA (%) (95% CI)[b]**
Average Across All Sites	98.47 (96.88, 99.69)	92.96 (89.81, 95.79)	95.66 (93.92, 97.26)

[a] The reduced number of specimen slides results (N) was due to uninformative results and limited slide availability.
[b] 95% CI was derived using the bootstrap method.

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TECHNICAL ASSISTANCE

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APPENDIX A. TIPS AND TROUBLESHOOTING GUIDE

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	Method	Probable Cause	Possible Solution
• No signal or weak signals	• Slide Evaluation	• Incorrect immersion oil used	• Confirm that immersion oil is appropriate for fluorescence microscopy.
		• Improper lamps (ie, Xenon or Tungsten)	• Use a mercury lamp (100-watt recommended).
		• Mercury lamp misaligned	• Realign lamp.
		• Mercury lamp has too many hours	• Replace with a new lamp.
		• Inappropriate filter set used to view slides	• Use recommended filters.
		• Fluorescent filter degraded	• Replace fluorescent filter.
		• Dirty or cracked collector lenses	• Clean or replace lens.
		• Dirty or broken mirror in lamp house	• Clean or replace mirror.
		• Degraded component(s) in the light path	• Call microscope manufacturer's technical representative and replace component(s) as needed.
		• Microscope not functioning properly	• Call manufacturer's technical representative.
	• Manual Slide Processing or VP 2000 Slide Processing	• Section overfixed (cell boundaries will be distinct)	• Prolonged tissue fixation times may lead to progressive degradation of signal intensity and may require longer digestion times.
		• Inadequate slide pretreatment	• Verify time and temperature of the Pretreatment Solution. • Adjust time for the Pretreatment Solution within the allowed range.
		• Inadequate protease digestion	• Verify time and temperature of the Protease Solution. • Adjust time for the Protease Solution within the allowed range.
		• Air bubbles trapped under coverslip prevented probe access	• Apply coverslip by first touching the surface of the probe mixture.
		• Inappropriate hybridization time	• Verify hybridization time.
		• Inappropriate post-hybridization wash temperature	• Verify temperature of Wash Buffer II.
	• VP 2000 Slide Processing	• Incorrect VP 2000 protocol used	• Verify VP 2000 protocol.
		• VP 2000 reagent basin(s) filled incorrectly	• Refer to **Working Reagent Preparation** section for filling instructions.
		• VP 2000 reagent(s) placed in incorrect locations	• Verify VP 2000 reagent locations match Reagent Map.
• Uninformative Result	• Slide Evaluation	• Too few tumor nuclei available for enumeration	• Repeat assay with new slide.
	• VP 2000 Slide Processing	• Incorrect VP 2000 protocol used	• Verify VP 2000 protocol.
		• VP 2000 reagent basin(s) filled incorrectly	• Refer to **Working Reagent Preparation** section for filling instructions.
		• VP 2000 reagent(s) placed in incorrect locations	• Verify VP 2000 reagent locations match Reagent Map.

APPENDIX A. TIPS AND TROUBLESHOOTING GUIDE (CONTINUED)

Problem	Method	Probable Cause	Possible Solution
• Noisy (high) background	• Manual Slide Processing or VP 2000 Slide Processing	• Inadequate wash stringency	• Verify time and temperature of Wash Buffer II.
	• Slide Evaluation	• Incorrect immersion oil used	• Confirm that immersion oil is appropriate for fluorescence microscopy.
		• Fluorescent filter degraded	• Replace fluorescent filter.
• Variation of signal intensity across tissue section	• Manual Slide Processing or VP 2000 Slide Processing	• 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.
		• Air bubbles or immersion oil trapped under coverslip after application of DAPI	Reapply the coverslip. If the coverslip is to be removed and reapplied, then follow this protocol: 1. Immerse the slides in 70% ethanol for up to 5 minutes or until the coverslip is released. 2. Immerse the slides in 85% ethanol for 3 minutes. 3. Immerse the slides in 100% ethanol for 3 minutes. 4. Air-dry the slides protected from light at ambient temperature for 2 to 5 minutes. 5. Apply 10 µL of DAPI to each slide, re-apply coverslip, and store protected from light for a minimum of 5 minutes. 6. Enumerate specimens under a fluorescence microscope within 24 hours or store at −20±10°C.
• Tissue loss, low cellularity, or tissue morphology degraded	• Manual Slide Processing or VP 2000 Slide Processing	• Inappropriate slides used	• Use positively-charged slides.
		• Tissue section under-fixed (poor DAPI staining)	• Verify protease digestion time. • Decrease protease digestion time within allowed range.
		• Improper slide baking	• Verify temperature of ThermoBrite instrument. • Increase baking time within the allowed range.
		• DNA loss (poor DAPI staining)	• Verify fixation conditions.
		• Overpretreatment	• Verify time and temperature Pretreatment Solution. • Decrease time of the Pretreatment Solution within the allowed range.
		• Overdigestion (protease solution)	• Verify temperature of the Protease Solution. • Decrease digestion time for the Protease Solution within the allowed range.
		• Overdenaturation	• Verify denaturation time.
		• Tissue section was torn when removing coverslip after hybridization	• Allow additional time for coverslip to soak off in wash buffer.
• 22 mm × 22 mm coverslip is unable to cover the entire tissue sections	• Manual Slide Processing or VP 2000 Slide Processing	• Tissue sections are too large.	• Use larger coverslips with adjusted probe or DAPI volumes: Use 1 coverslip of 22 mm × 30 mm with 14 µL probe or DAPI; or use 2 coverslips each of 22 mm × 22 mm with 10 µL probe or DAPI under each coverslip; or use 1 coverslip of 24 mm × 50 mm with 20 µL probe or DAPI.