VENTANA ALK (D5F3) CDx Assay

REF 790-4796

06687199001

IVD Σ 50

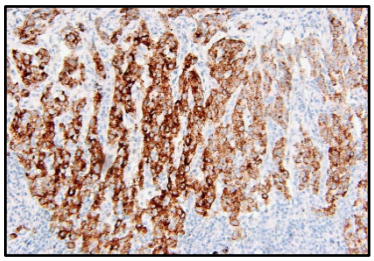

Figure 1. VENTANA ALK (D5F3) CDx Assay staining in non-small cell lung carcinoma.

INTENDED USE

VENTANA ALK (D5F3) CDx Assay is intended for the qualitative detection of the anaplastic lymphoma kinase (ALK) protein in formalin-fixed, paraffin-embedded (FFPE) non-small cell lung carcinoma (NSCLC) tissue stained with a BenchMark XT or BenchMark ULTRA automated staining instrument. It is indicated as an aid in identifying patients eligible for treatment with XALKORI® (crizotinib), ZYKADIA® (ceritinib), or ALECENSA® (alectinib).

This product should be interpreted by a qualified pathologist in conjunction with histological examination, relevant clinical information, and proper controls.

This product is intended for *in vitro* diagnostic (IVD) use.

SUMMARY AND EXPLANATION

The anaplastic lymphoma kinase (ALK) protein is a member of the insulin receptor superfamily of receptor tyrosine kinases.[1] ALK is a type I membrane glycoprotein that is normally expressed in the nervous system.[2] The ALK gene resides at chromosome 2p23 and is constructed of 2 large introns and 26 exons.[1] The molecular pathogenesis of ALK begins with chromosomal rearrangements that partner the 3' coding sequences for the ALK intracellular signaling domain with the 5' promoter elements and coding sequences of other genes. The 5' promoter elements and coding sequences drive overexpression and ligand-independent oligomerization of the chimeric proteins, features common in fusion-type protein tyrosine kinase human neoplasms.

An inversion within chromosome 2p resulting in the formation of a fusion gene product comprising portions of the echinoderm microtubule associated protein-like 4 (EML4) gene and the ALK gene was discovered in 2007 in NSCLC cell lines and archived clinical specimens.[3] A subsequent series of published studies indicated that EML4-ALK inversion events produced at least 9 catalytically active kinase fusion protein variants, each containing the same portion of the ALK C-terminal kinase domain.[4-8] As with ALK gene fusions first identified in anaplastic large-cell lymphoma (ALCL), the EML4-ALK fusion protein was shown to have transforming activity. Consistent with this, EML4-ALK expression in lung alveolar epithelial cells in transgenic mice has been reported to be a potent oncogenic factor.[9]

CLINICAL SIGNIFICANCE

NSCLC is the most common type of lung cancer. There are three common types of NSCLC: adenocarcinoma, squamous cell carcinoma, and large cell carcinoma.

ALK is now recognized as a key oncogenic driver in NSCLC, and although EML4 is the predominant fusion partner, other fusion partner genes have been identified.[10,11] The incidence of ALK gene rearrangements appears to range from 2-7%, translating to approximately 6,000 ALK-positive patients/year in the United States (US) and 40,000 patients/year worldwide.[3,4,7]

Small tissue samples may be easily used in routine immunohistochemistry (IHC), making this technique, in combination with antibodies that detect antigens important for carcinoma interpretation, an effective tool for the pathologist in their diagnosis and prognosis of disease. One important marker in NSCLC is ALK.

The vast majority of ALK gene rearrangements were observed in lung adenocarcinoma specimens compared with squamous or small cell histologies.[3-8] Some evidence suggests a correlation between ALK gene rearrangements and NSCLC in patients of "never or light" smoking status, although this may not be a statistically significant cofactor.[3,4,7,9]

Importantly, ALK gene rearrangements are rarely coincident with EGFR, HER2, or KRAS mutations, demonstrating that ALK positivity is a distinct disease subtype.[9]

XALKORI® is a selective ATP-competitive small-molecule inhibitor of the ALK, ROS1 and c-Met/Hepatocyte Growth Factor Receptor (HGFR) tyrosine kinases and their oncogenic variants (e.g., ALK or ROS1 fusion proteins or c-Met/HGFR mutant variants). XALKORI® has demonstrated concentration-dependent inhibition of ALK and c-Met phosphorylation in cell-based assays using tumor cell lines. It has also demonstrated antitumor activity in mice bearing tumor xenografts expressing EML4- or NPM-ALK fusion proteins or Met.[12,13,14]

ZYKADIA® is a kinase inhibitor. Targets of ZYKADIA® inhibition identified in either biochemical or cellular assays at clinically relevant concentrations include ALK, insulin-like growth factor 1 receptor (IGF-1R), insulin receptor (InsR), and ROS1. Among these, ZYKADIA® is most active against ALK. ZYKADIA®-inhibited autophosphorylation of ALK, ALK-mediated phosphorylation of the downstream signaling protein STAT3, and proliferation of ALK-dependent cancer cells in *in vitro* and *in vivo* assays.

ZYKADIA® inhibited the *in vitro* proliferation of cell lines expressing EML4-ALK and NPM-ALK fusion proteins and demonstrated dose-dependent inhibition of EML4-ALK-positive NSCLC xenograft growth in mice and rats.[15]

The clinical significance of ALK gene rearrangements has been demonstrated in randomized, active controlled, clinical trials of XALKORI®, conducted by Pfizer, and of ZYKADIA®, conducted by Novartis.[14,15]

ALECENSA® is a highly selective and potent ALK and RET tyrosine kinase inhibitor, which inhibits intracellular signaling pathways involved in tumor cell proliferation and survival and therefore, promotes cancer cell death and inhibits tumor cell growth and proliferation.[16] Based on preclinical data, ALECENSA® is not a substrate of the efflux transporters (PGP or BCRP) in the blood brain barrier and can therefore distribute into and be retained within the central nervous system. ALECENSA® induced tumor regression in preclinical mouse xenograft models, including antitumor activity in the brain, and prolonged survival in intracranial tumor animal models.[17] ALECENSA® is well-tolerated and provides a manageable safety profile.[18-20]

XALKORI® is indicated in the United States (US) for the treatment of patients with metastatic NSCLC whose tumors are ALK-positive as detected by an FDA-approved test.

ZYKADIA® is indicated in the US for the treatment of patients with metastatic non-small cell lung cancer (NSCLC), whose tumors are anaplastic lymphoma kinase (ALK)-positive as detected by an FDA-approved test.

ALECENSA® a kinase inhibitor indicated for the treatment of patients with anaplastic lymphoma kinase (ALK)-positive, locally advanced or metastatic non-small cell lung cancer (NSCLC) as detected by an FDA-approved test.

Ventana has demonstrated concordance of VENTANA ALK (D5F3) CDx Assay with the Abbott Vysis ALK Break Apart FISH Probe Kit (ALK FISH) in determining the ALK status of a patient's NSCLC. ALK FISH can present technical challenges in evaluating the staining results. Intrachromosomal re-arrangements can yield subtle signal-splitting, leading to potential false negatives.[21] Recent studies indicate that IHC is sensitive and specific for determining ALK status, and is a viable alternative to ALK FISH.[10,11,21-23] Ventana has developed VENTANA ALK (D5F3) CDx Assay and its associated scoring algorithm to determine ALK status in NSCLC specimens.

Interpretation of VENTANA ALK (D5F3) CDx Assay staining of tissue samples should be made using the recommended scoring algorithm. Histological tissue preparations have the advantage of intact tissue morphology to aid in the interpretation of the ALK positivity of the sample. All histological tests should be interpreted by a pathologist, and the results should be complemented by morphological studies and proper controls and used in conjunction with other clinical and laboratory data. Target antigens of IHC assays are impacted by fixation time, type of fixative, and age of cut slides, so care must be taken to ensure compatibility of specimen preparation prior to staining (refer to the VENTANA ALK (D5F3) CDx Assay Interpretation Guide for Non-Small Cell Lung Carcinoma (NSCLC) (P/N 1012345) and the Specific Limitations section below).

PRINCIPLE OF THE PROCEDURE

VENTANA ALK (D5F3) CDx Assay is a rabbit monoclonal primary antibody that binds to ALK in paraffin-embedded tissue sections. The specific antibody can be visualized using OptiView DAB IHC Detection Kit (Cat. No. 760-700 / 06396500001) followed by the OptiView Amplification Kit (Cat. No. 760-099 / 06396518001 (50 test) or 860-099 / 06718663001 (250 test)). Refer to the appropriate OptiView DAB IHC Detection Kit and OptiView Amplification Kit package inserts for further information.

REAGENT PROVIDED

VENTANA ALK (D5F3) CDx Assay includes a recombinant rabbit monoclonal antibody and contains sufficient reagent for staining 50 slides.

One 5 mL dispenser of VENTANA ALK (D5F3) CDx Assay contains approximately 70 µg of the rabbit monoclonal (D5F3) antibody.

The antibody is diluted in 0.08 M PBS with 3% carrier protein and 0.05% ProClin 300, a preservative.

Total protein concentration of the reagent is approximately 10 mg/mL. Specific antibody concentration is approximately 14 µg/mL.

MATERIALS AND REAGENTS NEEDED BUT NOT PROVIDED

The following reagents and materials may be required for staining but are not provided:

1. Human appendix or ALK-positive and ALK-negative non-small cell lung carcinoma specimens for use as control tissue
2. Rabbit Monoclonal Negative Control Ig (Cat. No. 790-4795 / 06683380001)
3. Microscope slides, positively charged
4. Drying oven capable of maintaining a temperature of 60°C ± 5°C
5. Bar code labels
6. Xylene (Histological grade)
7. Ethanol or reagent alcohol (Histological grade)
   - 100% solution: Undiluted ethanol or reagent alcohol
   - 95% solution: Mix 95 parts of ethanol or reagent alcohol with 5 parts of deionized water
   - 80% solution: Mix 80 parts of ethanol or reagent alcohol with 20 parts of deionized water
8. Deionized or distilled water
9. OptiView DAB IHC Detection Kit (Cat. No. 760-700 / 06396500001)
10. OptiView Amplification Kit (Cat. No. 760-099 / 06396518001 (50 test) or 860-099 / 06718663001 (250 test))
11. EZ Prep Concentrate (10X) (Cat. No. 950-102 / 05279771001)
12. Reaction Buffer Concentrate (10X) (Cat. No. 950-300 / 05353955001)
13. LCS (Predilute) (Cat. No. 650-010 / 05264839001)
14. ULTRA LCS (Predilute) (Cat. No. 650-210 / 05424534001)
15. Cell Conditioning 1 (CC1) (Cat. No. 950-124 / 05279801001)
16. ULTRA Cell Conditioning Solution (ULTRA CC1) (Cat. No. 950-224 / 05424569001)
17. Hematoxylin II (Cat. No. 790-2208 / 05277965001)
18. Bluing Reagent (Cat. No. 760-2037 / 05266769001)
19. Permanent mounting medium (Permount Fisher Cat. No. SP15-500 or equivalent)
20. Cover glass (sufficient to cover tissue, such as VWR Cat. No. 48393-060)
21. Automated coverslipper (such as the Tissue-Tek SCA Automated Coverslipper)
22. Light microscope
23. Absorbent wipes

Staining reagents, such as VENTANA detection kits and ancillary components, including negative and positive tissue control slides, are not provided.

Reconstitution, Mixing, Dilution, Titration

This antibody is optimized for use on VENTANA BenchMark XT and BenchMark ULTRA instruments in combination with OptiView DAB IHC Detection Kit and OptiView Amplification Kit. No reconstitution, mixing, dilution, or titration is required.

STORAGE

Upon receipt and when not in use, store at 2-8°C. Do not freeze.

To ensure proper reagent delivery and stability of the antibody, replace the dispenser cap after every use and immediately place the dispenser in the refrigerator in an upright position.

Every antibody dispenser is expiration dated. When properly stored, the reagent is stable to the date indicated on the label. Do not use reagent beyond the expiration date.

SPECIMEN COLLECTION AND PREPARATION FOR ANALYSIS

Routinely processed, FFPE tissues are suitable for use with this primary antibody when used with VENTANA detection kits and a VENTANA BenchMark XT or BenchMark ULTRA instrument.

On the basis of xenograft models generated from the NCI-H2228 human NSCLC cell-line, which is positive for ALK, Ventana recommends tissue fixation in 10% neutral buffered formalin (NBF) for at least 6 hours.[24] Fixation times of less than 6 hours result in a significant loss of staining intensity for ALK. Zinc formalin fixative also is acceptable at a fixation time of at least 6 hours. The amount used should be 15 to 20 times the volume of tissue. No fixative will penetrate more than 2 to 3 mm of solid tissue or 5 mm of porous tissue in a 24-hour period. Fixation can be performed at room temperature (15-25°C).[25]

Fixatives such as alcohol-formalin-acetic acid (AFA), PREFER fixative, B5, and other acid and/or alcohol-containing fixatives have demonstrated a loss of staining intensity for ALK at all fixation times tested (1-72 hours). They are not recommended for use with this assay. Delay-to-fixation studies also revealed a loss of staining intensity for ALK when xenograft specimens were not fixed within 6 hours of excision. See the VENTANA ALK (D5F3) CDx Assay Interpretation Guide for Non-Small Cell Lung Carcinoma (NSCLC) (P/N 1012345) for further discussion of the impact of specimen preparation on ALK staining intensity.

Sections should be cut approximately 4 µm thick and mounted on positively-charged glass slides. Slides should be stained promptly, as antigenicity of cut tissue sections may diminish over time and is compromised within 3 months after cutting from the paraffin block (see the VENTANA ALK (D5F3) CDx Assay Interpretation Guide for Non-Small Cell Lung Carcinoma (NSCLC) (P/N 1012345) and the Performance Characteristics section below).

WARNINGS AND PRECAUTIONS

1. For *in vitro* diagnostic (IVD) use.
2. For professional use only.
3. Please note that positively charged slides may be susceptible to environmental stresses resulting in inappropriate staining of any IHC assay (for example, lack of primary antibody or counterstain on the tissue). To better understand the impacts of environmental stresses on IHC positively charged slides, please reference N4629_0313B-Impact of environmental stress on various histology slide types.
4. ProClin 300 solution is used as a preservative in this reagent. It is classified as an irritant and may cause sensitization through skin contact. Take reasonable precautions when handling. Avoid contact of reagents with eyes, skin, and mucous membranes. Use protective clothing and gloves.
5. Materials of human or animal origin should be handled as biohazardous materials and disposed of with proper precautions.
6. Avoid contact of reagents with eyes and mucous membranes. If reagents come in contact with sensitive areas, wash with copious amounts of water.
7. Avoid microbial contamination of reagents as it may cause incorrect results.
8. Consult local and/or state authorities with regard to recommended method of disposal.
9. The impact of prior ALK testing on clinical outcome is unknown.
10. For supplementary safety information, refer to the product Safety Data Sheet and the Symbol and Hazard Guide located at www.ventana.com.

VENTANA ALK (D5F3) CDX ASSAY-SPECIFIC STAINING PROCEDURE

VENTANA ALK (D5F3) CDx Assay has been developed for use on a VENTANA BenchMark XT or BenchMark ULTRA instrument in combination with Rabbit Monoclonal Negative Control Ig, OptiView DAB IHC Detection Kit, OptiView Amplification Kit, and ancillary reagents. An assay-specific staining procedure must be used with VENTANA ALK (D5F3) CDx Assay. Refer to Table 1 or Table 2 for the recommended staining protocols and required staining procedures. Any deviation from recommended test procedures may invalidate expected results. Appropriate controls must be employed and documented. Users who deviate from recommended test procedures must accept responsibility for interpretation of patient results.

Table 1. Recommended staining protocol for VENTANA ALK (D5F3) CDx Assay and Rabbit Monoclonal Negative Control Ig with OptiView DAB IHC Detection Kit and OptiView Amplification Kit on a BenchMark XT instrument.

Staining Procedure: XT VENTANA ALK(D5F3) CDx Assay	
Protocol Step	Parameter Input
Antibody (Primary)	V-ALK (D5F3) Or Rabbit Monoclonal Negative Control Ig
OptiView HQ Univ Linker	12 minutes
Counterstain	Hematoxylin II, 4 minutes
Post Counterstain	Bluing, 4 minutes

Table 2. Recommended staining protocol for VENTANA ALK (D5F3) CDx Assay and Rabbit Monoclonal Negative Control Ig with OptiView DAB IHC Detection Kit and OptiView Amplification Kit on a BenchMark ULTRA Instrument.

Staining Procedure: U VENTANA ALK (D5F3)	
Protocol Step	Parameter Input
Antibody (Primary)	VENTANA ALK Ab-16 minutes (US) Or Negative Control-16 minutes (Rabbit Monoclonal Negative Control Ig)
Counterstain	Hematoxylin II, 4 minutes
Post Counterstain	Bluing, 4 minutes

QUALITY CONTROL PROCEDURES

Rabbit Monoclonal Negative Control Ig

A matched negative reagent control slide must be run for every specimen to aid in the interpretation of results. Rabbit Monoclonal Negative Control Ig (Cat. No. 790-4795 / 06683380001), a negative reagent control antibody, is specifically matched for this assay and is used in place of the primary antibody to evaluate nonspecific staining. The staining procedure for the negative reagent control should equal the primary antibody incubation period. Use of a different negative control reagent, or failure to use the recommended negative control reagent, may cause false results.

System-Level Controls

System-level controls must be run with patient samples. They can be either human appendix[26] or known ALK-positive/negative NSCLC tissue samples.

Control tissue should be autopsy, biopsy, or surgical specimens prepared and fixed as soon as possible in a manner identical to test sections. Such tissue may monitor all steps of the analysis, from tissue preparation through staining. Use of a tissue section fixed or processed differently from the test specimen provides control for all reagents and method steps except fixation and tissue preparation.

Appendix or ALK-Positive/Negative NSCLC Tissue Controls

An ALK-positive and an ALK-negative control tissue fixed and processed in the same manner as the patient specimens can be run for each set of test conditions and with every VENTANA ALK (D5F3) CDx Assay staining procedure performed.

NSCLC cases with staining representative of clinically ALK-positive and clinically ALK-negative results are suitable for optimal quality control, including detection of minor levels of reagent degradation or instrument out-of-specification issues.

Human appendix tissue contains positive and negative staining elements for the ALK protein and is also suitable for use as a system-level control. The positive staining tissue components are used to confirm that the antibody was applied and the instrument functioned properly; the negative staining elements are used to detect minor levels of reagent degradation or instrument out-of-specification issues

Appropriate staining of ALK-positive and negative NSCLC and appendix tissue components is described in Table 3 and Table 4, and in the VENTANA ALK (D5F3) CDx Assay Interpretation Guide for Non-Small Cell Lung Carcinoma (NSCLC) (P/N 1012345).

Known positive and known negative tissue controls should be utilized only for monitoring the correct performance of processed tissues and test reagents.

Assay Verification

Prior to initial use of an antibody or staining system in a diagnostic procedure, the specificity of the antibody should be verified by testing it on a series of tissues with known IHC performance characteristics representing ALK-positive and -negative tissues (refer to the Quality Control Procedures previously outlined in this section of the product insert and to the Quality Control recommendations of the College of American Pathologists Laboratory Accreditation Program, Anatomic Pathology Checklist[27] or the CLSI Approved Guideline[28]). These quality control procedures should be repeated for each new antibody lot, or whenever there is a change in assay parameters. NSCLC tissues with known ALK status, or human appendix samples, are suitable for assay verification.

INTERPRETATION OF RESULTS

The VENTANA automated immunostaining procedure causes a brown colored DAB reaction product to precipitate at the antigen sites localized by the VENTANA ALK (D5F3) CDx Assay antibody. A qualified pathologist experienced in IHC procedures must evaluate system-level controls and qualify the stained product before interpreting results.

Positive/Negative System-Level Tissue Controls

The stained positive and negative tissue controls should be examined to ascertain that all reagents are functioning properly. The presence of an appropriately colored reaction product on the positive control tissue within the cytoplasm of the target cells is indicative of positive reactivity.

If the positive or negative tissue controls fail to demonstrate appropriate staining or demonstrate a change in clinical diagnostic interpretation, any results with the test specimens should be considered invalid.

Table 3. Appendix tissue control evaluation criteria. Representative images are provided in the VENTANA ALK (D5F3) CDx Assay Interpretation Guide for Non-Small Cell Lung Carcinoma (NSCLC) (P/N 1012345).

Acceptable	Unacceptable
Presence of strong granular cytoplasmic staining in ganglion cells. (See note)	Absence of strong granular cytoplasmic staining in ganglion cells.
Absence of strong granular cytoplasmic staining in glandular epithelial cells, muscle, and lymphoid tissue (scant or rare staining of lymphoreticular cells may be observed in lymphoid aggregates).	Excessive non-specific background staining of glandular epithelial cells, muscle, or lymphoid tissue that interferes with scoring.

Note: The nerve in appendix muscular layers shows positive staining.

Negative Reagent Control

Nonspecific staining, if present, will have a diffuse appearance and can be evaluated using the negative reagent control slide stained with Rabbit Monoclonal Negative Control Ig. Intact cells should be used for interpretation of staining results, as necrotic or degenerated cells often stain nonspecifically. If background staining is excessive, results from the test specimen should be considered invalid. Examples of acceptable levels of background staining for this assay can be found in the VENTANA ALK (D5F3) CDx Assay Interpretation Guide for Non-Small Cell Lung Carcinoma (NSCLC) (P/N 1012345).

Patient Tissue

Patient tissue must be evaluated according to the VENTANA ALK (D5F3) CDx Assay scoring algorithm provided in Table 4. Refer to the VENTANA ALK (D5F3) CDx Assay Interpretation Guide for Non-Small Cell Lung Carcinoma (NSCLC) (P/N 1012345).

Table 4. Scoring algorithm for VENTANA ALK (D5F3) CDx Assay. Representative images are provided in the VENTANA ALK (D5F3) CDx Assay Interpretation Guide for Non-Small Cell Lung Carcinoma (NSCLC) (P/N 1012345).

Clinical Interpretation	Staining Description
Positive for ALK	Presence of strong granular cytoplasmic staining in tumor cells (any percentage of positive tumor cells). Certain staining elements should be excluded, including: · light cytoplasmic stippling in alveolar macrophages, · cells of neural origin (nerve and ganglion cells), · glandular epithelial staining, and · scattered lymphoreticular cells within lymphocytic infiltrates. Some background staining also may be observed within normal mucosa in NSCLC (including mucin) and in necrotic tumor areas, which should be excluded from the clinical evaluation.
Negative for ALK	Absence of strong granular cytoplasmic staining in tumor cells.

GENERAL LIMITATIONS

1. IHC is a multiple step diagnostic process that requires specialized training in the selection of the appropriate reagents, tissue selection, fixation, processing, preparation of the IHC slide, and interpretation of the staining results.
2. Tissue staining is dependent on the handling and processing of the tissue prior to staining. Improper fixation, freezing, thawing, washing, drying, heating, sectioning, or contamination with other tissues or fluids may produce artifacts, antibody trapping, or false negative results. Inconsistent results may result from variations in fixation and embedding methods, or from inherent irregularities within the tissue.
3. Excessive or incomplete counterstaining may compromise proper interpretation of results.
4. The clinical interpretation of any positive staining, or its absence, must be evaluated within the context of clinical history, morphology, and other histopathological criteria. The clinical interpretation of any staining, or its absence, must be complemented by morphological studies and system-level controls as well as other diagnostic tests. It is the responsibility of a qualified pathologist to be familiar with the antibodies, reagents, and methods used to interpret the stained preparation. Staining must be performed in a certified licensed laboratory under the supervision of a pathologist who is responsible for reviewing the stained slides and assuring the adequacy of positive and negative controls.
5. Ventana Medical Systems, Inc. provides antibodies and reagents at optimal dilution for use when the provided instructions are followed. Any deviation from recommended test procedures may invalidate expected results. Appropriate controls must be employed and documented. Users who deviate from recommended test procedures must accept responsibility for interpretation of patient results.
6. This product is not intended for use in flow cytometry, performance characteristics have not been determined.
7. Reagents may demonstrate unexpected reactions in previously untested tissues. The possibility of unexpected reactions even in tested tissue groups cannot be completely eliminated because of biological variability of antigen expression in neoplasms, or other pathological tissues.[29]
8. Tissues from persons infected with hepatitis B virus and containing hepatitis B surface antigen (HBsAg) may exhibit nonspecific staining with horseradish peroxidase.[30]
9. False positive results may be seen because of non-immunological binding of proteins or substrate reaction products. They may also be caused by pseudoperoxidase activity (erythrocytes), endogenous peroxidase activity (cytochrome C), or endogenous biotin (example: liver, brain, breast, kidney) depending on the type of immunostain used.[31]
10. As with any IHC test, a negative result means that the antigen was not detected, not that the antigen was absent in the cells or tissue assayed.

SPECIFIC LIMITATIONS

1. VENTANA ALK (D5F3) CDx Assay has been approved on the VENTANA BenchMark XT and BenchMark ULTRA instruments with the OptiView DAB IHC Detection Kit and the OptiView Amplification Kit and is not approved with any other detection or automated staining instruments.
2. A patient specimen slide should be stained with Rabbit Monoclonal Negative Control Ig (Cat. No. 790-4795 / 06683380001). Other negative control reagents are not suitable for this assay.
3. This assay has not been validated for use with cytology smears or decalcified specimens.
4. Patient tissue should be stained within 3 months of sectioning from the tissue block. Loss of staining performance has been observed with VENTANA ALK (D5F3) CDx Assay on sections that have been stored at room temperature for longer than 3 months.
5. Ventana recommends that samples be fixed at least 6 hours in 10% NBF or zinc formalin. Use of fixation times or fixative types other than those recommended can lead to false negative results. Fixatives such as AFA, PREFER fixative, B5, and other acid and/or alcohol-containing fixatives have demonstrated a loss of specific ALK protein staining. Refer to the VENTANA ALK (D5F3) CDx Assay Interpretation Guide for Non-Small Cell Lung Carcinoma (NSCLC) (P/N 1012345) for further discussion.
6. Some staining artifacts have been noted with VENTANA ALK (D5F3) CDx Assay. Light granular cytoplasmic stippling in alveolar macrophages may be present on both the VENTANA ALK (D5F3) CDx Assay- and negative reagent control-stained slides, indicating that it is an artifact of the detection system and should not be evaluated as ALK-positive staining. In addition, punctate staining has been observed on necrotic tumor areas; such staining should also be disregarded during patient sample evaluation. Staining of neural tissue, including nerve, and of occasional cells within infiltrating lymphocytes has been observed with VENTANA ALK (D5F3) CDx Assay antibody. Refer to the VENTANA ALK (D5F3) CDx Assay Interpretation Guide for Non-Small Cell Lung Carcinoma (NSCLC) (P/N 1012345) for further discussion.
7. Slight variability in overall staining intensity may be observed on system-level (tissue) controls due to the OptiView Amplification Kit. Refer to the VENTANA ALK (D5F3) CDx Assay Interpretation Guide for Non-Small Cell Lung Carcinoma (NSCLC) (P/N 1012345) for examples of acceptable staining performance.

PERFORMANCE CHARACTERISTICS

Specificity/Sensitivity

Analytical specificity and sensitivity were determined by staining multiple cases of normal and neoplastic human tissue with VENTANA ALK (D5F3) CDx Assay. The results are listed in Table 5 and Table 6.

Table 5. Specificity/sensitivity of VENTANA ALK (D5F3) CDx Assay in normal tissue. Testing used FFPE normal tissues.

Tissue	# Positive / Total Cases	Tissue	# Positive / Total Cases
Cerebrum	0/3*	Thymus	0/3
Cerebellum	0/3	Myeloid (bone marrow)	0/3
Adrenal gland	0/3	Lung	0/3
Ovary	0/3	Heart	0/3
Pancreas	0/3	Esophagus	0/3
Parathyroid gland	0/3	Stomach	0/3
Hypophysis	0/3**	Small intestine	0/3***
Testis	0/3	Colon	0/3***
Thyroid	0/3	Liver	0/3
Breast	0/3	Salivary gland	0/3
Spleen	0/3	Kidney	0/3

Tissue	# Positive / Total Cases	Tissue	# Positive / Total Cases
Tonsil	0/3	Prostate	0/3
Endometrium	0/3	Cervix	0/3
Skeletal muscle	0/4	Skin	0/3
Nerve (sparse)	0/3	Mesothelium and lung	0/3

* 2/3 A few glial cells in the cerebrum showed weak-to-moderate positivity.

** 3/3 Hypophysis stained weakly.

*** Ganglion cells within 4/6 intestinal tissues stained positive for ALK at varying intensities.

Table 6. Specificity/sensitivity of VENTANA ALK (D5F3) CDx Assay in neoplastic tissue. Testing used a variety of formalin-fixed, paraffin-embedded neoplastic tissues.

Tissue	# Positive / Total Cases
Glioblastoma	0/1
Atypical meningioma	0/1
Malignant ependymoma	0/1
Malignant oligodendroglioma	0/1
Serous ovarian adenocarcinoma	1/1
Ovarian adenocarcinoma	0/1
Islet cell carcinoma	0/1
Pancreatic adenocarcinoma	0/1
Seminoma	0/1
Embryonal carcinoma	0/1
Medullary carcinoma	0/1
Papillary carcinoma	0/1
Breast intraductal carcinoma	0/1
Breast invasive ductal carcinoma	0/2
Diffuse B-cell lymphoma	0/3
Lung small cell undifferentiated carcinoma	0/1
Lung squamous cell carcinoma	0/1
Lung adenocarcinoma	0/1
Esophageal squamous cell carcinoma	0/1
Esophageal adenocarcinoma	0/1
Gastric mucinous adenocarcinoma	0/1
Gastrointestinal adenocarcinoma	0/1
Malignant interstitialoma	0/1
Rectal adenocarcinoma	0/1
Rectal malignant interstitialoma	0/1
Hepatocellular carcinoma	0/1
Hepatoblastoma	1/1
Renal clear cell carcinoma	0/1
Prostatic adenocarcinoma	0/2
Leiomyoma	0/1
Endometrial adenocarcinoma	0/1
Endometrial clear cell carcinoma	0/1
Uterine squamous cell carcinoma	0/2
Embryonal rhabdomyosarcoma	0/1
Anal malignant melanoma	0/1
Basal cell carcinoma	0/1
Squamous cell carcinoma	0/1
Neurofibroma	0/1
Retroperitoneal neuroblastoma	1/1
Malignant mesothelioma	0/1
Hodgkin lymphoma	0/1
Anaplastic large cell lymphoma	0/1
Bladder transitional cell carcinoma	0/1
Low grade leiomyosarcoma	0/1
Osteosarcoma	0/1
Spindle cell rhabdomyosarcoma	0/1
Intermediate grade leiomyosarcoma	0/1

BenchMark XT to BenchMark ULTRA Concordance

To demonstrate equivalent performance of the assay between the BenchMark XT and BenchMark ULTRA, a concordance study was performed. This study evaluated ALK clinical status (based on the ALK scoring algorithm found in Table 4) in 184 unique NSCLC specimens stained with VENTANA ALK (D5F3) CDx Assay across both BenchMark instruments. The resulting stained slides were blinded and randomized then evaluated by three pathologists. Results of concordance for this study can be found in Table 7 and Table 8.

Table 7. ALK status comparison in NSCLC specimens determined using VENTANA ALK (D5F3) CDx Assay stained on the BenchMark XT instrument vs VENTANA ALK (D5F3) CDx Assay stained using the BenchMark ULTRA instrument.

VENTANA ALK (D5F3) CDx Assay concordance between the BenchMark XT instrument and BenchMark ULTRA instrument			
BenchMark ULTRA instrument	BenchMark XT instrument		
	Positive	Negative	Total
Positive	85	1	86
Negative	1	97	98
Total	86	98	184

Table 8. Concordance of ALK status between BenchMark XT and BenchMark ULTRA instruments.

Concordance Agreement Rates	Positive Percent Agreement (95% CI)	Negative Percent Agreement (95% CI)	Overall Percent Agreement (95% CI)
Concordance between BenchMark XT instrument and BenchMark ULTRA instrument	98.8% (93.7-99.8%)	99.0% (94.4-99.8%)	98.9% (96.1-99.7%)

Tissue Thickness

Tissue thickness was evaluated using 4 unique cases of human NSCLC (3 ALK-positive and 1 ALK-negative) and 4 unique cases of human appendix. Tissues were sectioned and tested in duplicate at 3, 4, 5, 6, and 7 microns. All tissue thicknesses demonstrated appropriate specific staining for ALK and appropriate background levels with VENTANA ALK (D5F3) CDx Assay. No specimens exhibited a change in clinical ALK status within this range of thickness. Ventana recommends that specimens be cut at 4-6 microns for the assay.

Repeatability and Intermediate Precision Studies

Repeatability and intermediate precision of VENTANA ALK (D5F3) CDx Assay were evaluated on the BenchMark XT and BenchMark ULTRA instruments in combination with the OptiView DAB IHC Detection and OptiView Amplification kits. Ten unique NSCLC tissue specimens (5 ALK-positive and 5 ALK-negative) were evaluated on the BenchMark XT and BenchMark ULTRA instruments. For Intra-Day precision, 5 replicate slides from each of the NSCLC specimens were stained across a single BenchMark XT or a single BenchMark ULTRA instrument. For intra-instrument precision testing, 3 replicate slides from each of the NSCLC specimens were stained with VENTANA ALK (D5F3) CDx Assay across three BenchMark XT instruments while 2 replicate slides from each of the NSCLC specimens were stained across three BenchMark ULTRA instruments. For Inter-Day precision, 2 replicate slides from each of the NSCLC specimens were stained with VENTANA ALK (D5F3) CDx Assay on a single BenchMark XT or BenchMark ULTRA instrument across 5 non-consecutive days. All slides were blinded, randomized within each instrument tissue cohort. Each cohort was evaluated individually by a pathologist using the VENTANA ALK (D5F3) CDx Assay scoring algorithm (provided in Table 4). Each replicate NSCLC specimen produced equivalent ALK IHC staining results. A summary of the results for repeatability and intermediate precision for the BenchMark XT and BenchMark ULTRA instruments can be found in Table 9 and Table 10, respectively.

In addition, repeatability of VENTANA ALK (D5F3) CDx Assay staining on human appendix (system-level control) was also evaluated. Eight unique human appendix tissues were used for this study. For Intra-Day precision, 13 replicate slides from two multi-tissue blocks containing 4 appendix specimens were stained on a single BenchMark XT instrument. For Inter-Instrument precision, 5 replicate slides from two multi-tissue blocks containing 4 appendix specimens each were stained with VENTANA ALK (D5F3) CDx Assay across three BenchMark XT instruments. For Inter-Day precision, 5 replicate slides from each of two multi-tissue blocks containing 4 appendix specimens were stained with VENTANA ALK (D5F3) CDx Assay on a single BenchMark XT instrument across 5 non-consecutive days. All slides were evaluated by a pathologist using the VENTANA ALK (D5F3) CDx Assay scoring guide for appendix control tissue (provided in Table 3). Each replicate appendix specimen produced equivalent ALK IHC staining results. The overall percent agreement for intra-day and inter-instrument (across 3 instruments) repeatability was 100%, while the inter-day repeatability (across 5 non-consecutive days) was 98%.

Table 9. Repeatability and intermediate precision of VENTANA ALK (D5F3) CDx Assay on individual NSCLC specimens stained on the BenchMark XT instrument.

NSCLC Tissue Repeatability/Precision	N= Total Slides Evaluated in the Cohort	Overall Percent Agreement for ALK Status (95% CI)
Intra-Day Repeatability	50	100% (97.5-100%)
Intra-Platform Precision (across 3 BenchMark XT instruments)	90	100% (97.9-100%)
Inter-Day Precision (5 non-consecutive days)	100	100% (98.7-100%)

Table 10. Repeatability and intermediate precision of VENTANA ALK (D5F3) CDx Assay on individual NSCLC specimens stained on the BenchMark ULTRA instrument.

NSCLC Tissue Repeatability/Precision	N= Total Slides Evaluated in the Cohort	Overall Percent Agreement for ALK Status (95% CI)
Intra-Day Repeatability	50	100% (92.9-100.0%)
Intra-Platform Precision (across 3 BenchMark ULTRA instruments)	60	100% (94.0-100.0%)
Inter-Day Precision (5 non-consecutive days)	100	100% (96.3-100.0%)

Lot-to-Lot Reproducibility

Lot-to-lot reproducibility of VENTANA ALK (D5F3) CDx Assay was determined by testing three lots of VENTANA ALK (D5F3) CDx Assay across 38 unique NSCLC cases (21 ALK-positive specimens (from 18 unique cases) and 20 ALK-negative NSCLC tissue specimens) on the BenchMark XT instrument using the OptiView DAB IHC Detection and OptiView Amplification kits. All cases were stained in duplicate with each of the three lots of primary antibody. Slides were blinded and randomized prior to evaluation for clinical status as determined by the VENTANA ALK (D5F3) CDx Assay scoring algorithm (provided in Table 4) by three pathologists. All three lots of antibody exhibited greater than 90% concordant staining results for ALK status across the 41 NSCLC tissue specimens evaluated. Results are reported as overall percent agreement, average positive agreement, and average negative agreement rates. The overall percent agreement rate between lots was 99.2%; therefore, VENTANA ALK (D5F3) CDx Assay is reproducible in its staining results across antibody lots. Results can be found in Table 11.

Lot-to-lot reproducibility of VENTANA ALK (D5F3) CDx Assay using the BenchMark ULTRA instrument was determined by testing three lots of VENTANA ALK (D5F3) CDx Assay across 30 unique NSCLC cases (15 ALK-positive specimens and 15 ALK-negative NSCLC tissue specimens) using the OptiView DAB IHC Detection and OptiView Amplification kits. All cases were stained in duplicate with each of the three lots of primary antibody. Slides were blinded and randomized prior to evaluation for clinical status as determined by the VENTANA ALK (D5F3) CDx Assay scoring algorithm (provided in Table 4) by a pathologist. All three lots of antibody exhibited greater than 90% concordant staining results for ALK status across the 30 NSCLC tissue specimens evaluated. Results are reported as overall percent agreement, average positive agreement, and average negative agreement rates. The overall percent agreement rate between lots was 99.1%; therefore, VENTANA ALK (D5F3) CDx Assay is reproducible in its staining results across antibody lots. Results can be found in Table 12.

Lot-to-lot reproducibility of VENTANA ALK (D5F3) CDx Assay was also evaluated using 12 unique human appendix tissue specimens. Reproducibility was determined by testing three lots of antibody in combination with three lots of OptiView DAB IHC Detection and OptiView Amplification Kits across three BenchMark XT instruments. The overall agreement rate for appropriate positive and negative staining elements of the appendix using VENTANA ALK (D5F3) CDx Assay was 100%.

Table 11. Lot-to-lot reproducibility agreement rates across 41 NSCLC tissue specimens. Twenty-one ALK-positive specimens (from 18 unique cases) and 20 ALK-negative specimens were tested.

Lot –to-Lot Reproducibility Agreement Rates	Average Positive Agreement (95% CI)	Average Negative Agreement (95% CI)	Overall Percent Agreement (95% CI)
Average of all three lot to lot comparisons	99.2% (97.4-100%)	99.1% (96.8-100.0)	99.2% (97.5-100%)

Table 12. Lot-to-lot reproducibility agreement rates across 30 NSCLC tissue specimens. Fifteen ALK-positive specimens and 15 ALK-negative specimens were tested.

Lot –to-Lot Reproducibility Agreement Rates	Positive Percent Agreement (95% CI)	Negative Percent Agreement (95% CI)	Overall Percent Agreement (95% CI)
Average of all three lot to lot comparisons	98.9% (96.8-99.6%)	99.3% (97.3-99.8%)	99.1% (97.9-99.6%)

Inter-Reader Precision Studies

Several inter-reader precision studies were performed: two studies on the BenchMark XT instrument, and one on the BenchMark ULTRA instrument.

In BenchMark XT Inter-Reader Precision Study 1, three pathologists evaluated a total of 185 unique cases. The 185 cases correlated with 100 ALK-positive and 100 ALK-negative blocks that were stained with VENTANA ALK (D5F3) CDx Assay. The cases were blinded and randomized prior to evaluation for ALK IHC staining results per the VENTANA ALK (D5F3) CDx Assay scoring algorithm provided in Table 4. The results provided in Table 13 below reflect the inter-reader precision rates for unique cases from the study cohort.

Table 13. Inter-Reader Precision Study 1 on the BenchMark XT instrument.

Inter-Reader Precision	Average Positive Agreement (95% CI)	Average Negative Agreement (95% CI)	Overall Percent Agreement (95% CI)
Average of all three readers comparisons	98.8% (97.3-100%)	99.0% (97.7-100%)	98.9% (97.4-100%)

BenchMark XT Inter-Reader Precision Study 2 was performed on a cohort of cases from a randomized clinical study of ALK-positive NSCLC patient specimens enrolled with the Abbott Vysis ALK Break Apart FISH Probe Kit. Approximately 300 cases were stained with VENTANA ALK (D5F3) CDx Assay on the BenchMark XT instrument. The cases were blinded for ALK FISH status, randomized, and provided to three readers, who evaluated the ALK IHC staining results per the VENTANA ALK (D5F3) CDx Assay scoring algorithm provided in Table 4. The results provided in Table 14 reflect the inter-reader precision rates from this clinical trial cohort.

For the BenchMark ULTRA inter-reader precision study, a cohort of 184 unique NSCLC cases was evaluated. The cohort consisted of 90 ALK-positive and 94 ALK-negative cases that were stained with VENTANA ALK (D5F3) CDx Assay on the BenchMark ULTRA instrument. The cases were blinded, randomized, and provided to three readers, who evaluated the ALK IHC staining results per the VENTANA ALK (D5F3) CDx Assay scoring algorithm provided in Table 4. An excellent inter-reader precision agreement rate between readers was demonstrated. Table 15 reflects the inter-reader precision rates from this study.

Table 14. BenchMark XT Inter-Reader Precision Study 2 for ALK status in NSCLC specimens obtained from clinical method comparison Cohort # 1.

Inter-Reader Precision	Average Positive Agreement (95% CI)	Average Negative Agreement (95% CI)	Overall Percent Agreement (95% CI)
Average of all three readers comparisons	97.6% (95.0-99.5%)	99.5% (98.9-99.9%)	99.1% (98.2-99.8%)

Table 15. BenchMark ULTRA Inter-Reader Precision Study for ALK status in NSCLC specimens stained with VENTANA ALK (D5F3) CDx Assay.

Inter-Reader Precision	Average Positive Agreement (95% CI)	Average Negative Agreement (95% CI)	Overall Percent Agreement (95% CI)
Average of all three Readers comparisons	98.4% (96.5-99.6%)	98.6% (96.9-99.7%)	98.5% (96.7-99.6%
Reader 1 vs Reader 2	98.9% (96.8-100%)	98.9% (97.0-100%)	98.9% (96.0-99.7%)
Reader 1 vs Reader 3	98.8% (96.7-100%)	99.0% (97.2-100%	98.9% (96.0-99.7%)
Reader 2 vs Reader 3	97.6% (94.7-99.4%)	97.9% (95.4-99.5%)	97.8% (94.4-99.1%)

Inter-Laboratory Reproducibility Study on BenchMark XT

The BenchMark XT Inter-Laboratory Reproducibility Study for VENTANA ALK (D5F3) CDx Assay was completed to demonstrate reproducibility of the assay in determining ALK clinical status on the BenchMark XT instrument, using NSCLC (6 ALK-positive and 6 ALK-negative) tissue specimens run across 3 reagent lots, 3 instruments and 5 non-consecutive days at three external laboratories. The specimens were randomized and evaluated by a total of 6 readers (2 readers/site) who were blinded to the ALK clinical status of the cohort. This cohort contained 180 slides generated from 12 NSCLC cases positive and negative for ALK expression by IHC and FISH. These cases were stained in replicate over 21 days at the 3 laboratories. See Table 16 for results. The acceptability rate for morphology and background in these studies was 100%. The data indicate excellent agreement in assay reproducibility across 3 sites and 6 readers.

Table 16. The ALK BenchMark XT ILR analysis provided the average positive agreement (APA) and average negative agreement (ANA) for between-site, between-reader, and between-run (day) comparisons performed pairwise utilizing evaluable observations. Inter-Laboratory Reproducibility: Agreement rates for VENTANA ALK (D5F3) CDx Assay on BenchMark XT instrument (n = 180 slides evaluated).

Agreement Rates for Inter-Laboratory Reproducibility (ALK Clinical Status)	Average Positive Agreement (95% CI)	Average Negative Agreement (95% CI)	Overall Percent Agreement (95% CI)
Between-site (3 sites)	93.8% (76.2-100%)	94.9% (79.2-100%)	94.4% (83.3-100%)
Between-day (5 non-consecutive days)	99.1% (96.4-100%)	99.2% (96.9-100%)	99.2% (97.5-100%)
Between-reader (2 readers/site)	98.8% (95.2-100%)	99.0% (95.8-100%)	98.9% (96.7-100%)

Inter-Laboratory Reproducibility Study on BenchMark ULTRA

The BenchMark ULTRA Inter-Laboratory Reproducibility Study for VENTANA ALK (D5F3) CDx Assay was completed to demonstrate reproducibility of the assay in determining ALK

clinical status on the BenchMark ULTRA instrument, using NSCLC (7 ALK-positive and 7 ALK-negative) tissue specimens run across 3 reagent lots, 3 instruments and 5 non-consecutive days at three external laboratories on the BenchMark ULTRA. The specimens were randomized and evaluated by a total of 6 readers (2 readers/site) who were blinded to the ALK clinical status of the cohort. This cohort contained 210 slides generated from 14 NSCLC cases positive and negative for ALK expression by IHC and FISH. These cases were stained in replicate over 21 days at the 3 laboratories. See Table 17 for results. The overall final staining acceptability rate for all data pooled was 99%. The acceptability rate for morphology and background in these studies was 100%. The data indicate excellent agreement in assay reproducibility across 3 sites and 6 readers. See Table 18 for results.

The ALK ULTRA ILR analysis provided the positive percent agreement (PPA) and negative percent agreement (NPA) across all evaluable observations obtained from the study by pooling all sites, readers, and days, when using the consensus score as a reference standard.

Table 17. Inter-laboratory reproducibility: Agreement rates for VENTANA ALK (D5F3) CDx Assay on the BenchMark ULTRA instrument (n = 210 slides evaluated).

Agreement Rates for Inter-Laboratory Reproducibility (ALK Clinical Status)	Positive Percent Agreement (95% CI)	Negative Percent Agreement (95% CI)	Overall Percent Agreement (95% CI)
Across all evaluable observations	92.8% (88.4-95.6%)	100.0% (98.2-100.0%)	96.4% (94.1-97.8%)

Table 18. Inter-laboratory reproducibility: Inter-reader agreement rates for VENTANA ALK (D5F3) CDx Assay on the BenchMark ULTRA instrument (n = 210 slides evaluated).

Agreement Rates for Inter-Laboratory Reproducibility (ALK Clinical Status) between reader agreement rates	Average Positive Agreement (95% CI)	Average Negative Agreement (95% CI)	Overall Percent Agreement (95% CI)
Readers A1 vs A2	97.0% (87.5-100%)	97.3% (89.5-100%)	97.1% (90.2-99.2%)
Readers B1 vs B2	93.5% (71.4-100%)	94.7% (81.0-100%)	94.2% (86.0-97.7%)
Readers C1 vs C2	92.3% (66.7-100%)	93.2% (74.7-100%)	92.8% (84.1-96.9%)
Overall	94.3% (75.6-100%)	95.1% (81.6-100%)	94.7% (84.1-100%)

Method Comparison Study on BenchMark XT Instrument

The Method Comparison Study cohorts were generated from two independent, randomized clinical trials of crizotinib (designated Trial #1 and Trial #2) that enrolled patients with ALK-positive NSCLC. ALK status for these patients was determined using the Vysis ALK Break Apart FISH Probe Kit clinical trial assay at multiple central laboratories. Valid Vysis ALK FISH results were obtained for a total of 1644 NSCLC tissue specimens (1018 and 626 specimens for Trials #1 and #2, respectively).

In the VENTANA ALK (D5F3) CDx Assay Method Comparison Study, specimens from patients screened for Trials #1 and #2 were sent to a central laboratory for staining with VENTANA ALK (D5F3) CDx Assay and evaluation for ALK IHC status based on the VENTANA ALK (D5F3) CDx Assay scoring algorithm criteria (Table 4). Of the specimens yielding valid Vysis ALK FISH results in clinical trial screening, 933 specimens from Trial #1 (Table 19) and 598 specimens from Trial #2 (Table 21) also yielded valid results for VENTANA ALK (D5F3) CDx Assay.

The numbers of specimens yielding ALK-positive and ALK-negative results for each assay are shown in Table 19 and Table 21 for the Trial #1 and #2 cohorts, respectively. The agreement rates between the two assays are shown in Table 20 and Table 22 for the Trial #1 and #2 cohorts, respectively. The reported positive and negative percent agreement rates were 86.0% and 96.3%, respectively, for Trial #1 (Table 20) and 92.7% and 94.8%, respectively, for Trial #2 (Table 22).

Table 19. ALK status comparison in NSCLC specimens (cohort from Trial #1) determined using VENTANA ALK (D5F3) CDx Assay vs. Vysis ALK Break Apart FISH Probe Kit.

ALK Status		Vysis ALK Break Apart FISH Probe Kit		
		Positive	Negative	Total
VENTANA ALK (D5F3) CDx Assay	Positive	154	28	182
	Negative	25	726	751
	Total	179	754	933

Table 20. Agreement rates between VENTANA ALK (D5F3) CDx Assay and Vysis ALK Break Apart FISH Probe Kit in Trial #1.

Agreement Rates between ALK Assays	Positive Percent Agreement (95% CI)	Negative Percent Agreement (95% CI)	Overall Percent Agreement (95% CI)
VENTANA ALK (D5F3) CDx Assay and Vysis ALK Break-Apart FISH Probe Kit	86.0% (80.2-90.4%)	96.3% (94.7-97.4%)	94.3% (92.6-95.6%)

Table 21. ALK status comparison cohort from Trial #2 in NSCLC specimens determined using VENTANA ALK (D5F3) CDx Assay vs. Vysis ALK Break Apart FISH Probe Kit.

ALK Status		Vysis ALK Break Apart FISH Probe Kit		
		Positive	Negative	Total
VENTANA ALK (D5F3) CDx Assay	Positive	179	21	200
	Negative	14	384	398
	Total	193	405	598

Table 22. Agreement rates between VENTANA ALK (D5F3) CDx Assay and Vysis ALK Break Apart FISH Probe Kit in Trial #2.

Agreement Rates between ALK Assays	Positive Percent Agreement (95% CI)	Negative Percent Agreement (95% CI)	Overall Percent Agreement (95% CI)
VENTANA ALK (D5F3) CDx Assay and Vysis ALK Break Apart FISH Probe Kit	92.7% (88.2-95.6%)	94.8% (92.2-96.6%)	94.1% (92.0-95.8%)

Note that tissue specimens used in Trial #1 and Trial #2 were not verified as having been prepared according to the specimen preparation procedures recommended for VENTANA ALK (D5F3) CDx Assay.

Crizotinib Clinical Outcome Study

The clinical efficacy analysis for VENTANA ALK (D5F3) CDx Assay as a diagnostic device for selection of patients who might benefit from treatment with crizotinib, an ALK-targeted agent, was based on Trial #1. These patients were tested with VENTANA ALK (D5F3) CDx Assay under the Method Comparison Study as well as an additional study. Trial #1 was a multicenter, multinational, randomized, open-label, Phase 3 efficacy and safety study of crizotinib vs. first-line chemotherapy (pemetrexed/cisplatin or pemetrexed/carboplatin) in previously untreated patients with ALK-positive advanced non-squamous NSCLC. The Vysis ALK Break Apart FISH Probe Kit (ALK FISH) was used to

determine ALK positivity and trial eligibility for Trial #1. Based on the Vysis ALK FISH assay results, 343 patients were in the randomized set (172 in the crizotinib arm and 171 in the chemotherapy arm). In the VENTANA ALK (D5F3) CDx Assay Clinical Outcome Study, tissue specimens from Trial #1 were retrospectively tested with VENTANA ALK (D5F3) CDx Assay. Of the 343 patients enrolled in Trial #1, 133 had been tested with VENTANA ALK (D5F3) CDx Assay under the Method Comparison Study protocol, and an additional 39 patients had been tested under a separate study protocol, for a total of 172 patients tested with VENTANA ALK (D5F3) CDx Assay. Of these patients, 166 were diagnosed as ALK-positive or ALK-negative by ALK (D5F3) IHC. The overall efficacy results for these patients are summarized according to the VENTANA ALK (D5F3) CDx Assay results in Table 23.

Table 23. Clinical benefit of crizotinib (progression-free survival) for patients enrolled in Trial #1.

ALK Status		HR [a]	SE [a]	95% CI [a]	Sample Size	
					Chemotherapy Arm	Crizotinib Arm
Total Enrolled	FISH+	0.454	0.139	(0.346, 0.596%)	171	172
ALK IHC Tested	FISH+ [b]	0.407	0.214	(0.267, 0.618%)	82	90
	FISH+/IHC+	0.401	0.237	(0.252, 0.639%)	63	78
	FISH+/IHC-	1.711	0.703	(0.431, 6.789%)	17	8

[a] Observed hazard ratio (HR) for progression-free survival (PFS) of crizotinib versus chemotherapy, standard error (SE), and 2-sided 95% confidence interval (CI). Results were estimated using a stratified Cox model with the following strata: race, brain metastasis, and ECOG score.

[b] For two ALK FISH+ patients in the chemotherapy arm and 4 patients in the crizotinib arm, no positive or negative ALK IHC result was obtained.

Additional imputation analyses were performed to include patients with missing or invalid VENTANA ALK (D5F3) CDx Assay test results and to evaluate the robustness of study conclusions. Statistical analysis of discordant patients not enrolled in Trial #1 involved simulation of a range of possible outcomes for these patients. Results from all of the hypothetical analyses were generally similar to those from the primary efficacy analysis.

FISH+/IHC- Discordant Cases from Trial #1:

Method Comparison Study

In the Method Comparison Study (Table 19 and Table 20), 25 patients from Trial #1 were evaluated as FISH+/IHC-. The median Vysis ALK FISH score (% tumor cells positive for ALK gene rearrangement) for these cases was 20% (mean 31.6%, SD 21.58%), and for 14 of these cases, the FISH score was 25% or less. While all of these cases had Vysis ALK FISH scores above the 15% cut-off for ALK positivity, their scores were in the FISH equivocal zone (10%–50%). In contrast, the median FISH score observed for all enrolled patients tested with IHC was 58% (mean 56.9%, SD 21.97%).

Clinical Outcome Study

In the Clinical Outcome Study, 25 patients enrolled into Trial #1 were evaluated as IHC- (see last row of Table 23). Eight of these cases were randomized to the crizotinib arm of the clinical study. Of these patients, five had FISH scores very close to the FISH cut-off (15%–18% of tumor cells positive for ALK gene rearrangement) and also exhibited objective progression or stable disease/no response. Two of the 8 patients had FISH scores outside the FISH equivocal zone (66% and 72% of tumor cells positive for ALK gene rearrangement) and exhibited a partial objective tumor response. The eighth IHC- patient was FISH- and was enrolled erroneously; this patient exhibited an indeterminate response.

FISH-/IHC+ Discordant Cases from Trial #1

In the VENTANA ALK (D5F3) CDx Assay Method Comparison Study, 28 cases screened for Trial #1 were evaluated as FISH-/IHC+. Since FISH was the clinical trial assay, and only FISH+ cases were enrolled into Trial #1, no outcome data are available on the FISH-/IHC+ discordant cases.

Ceritinib Clinical Outcome Study

The clinical efficacy analysis of VENTANA ALK (D5F3) CDx Assay as a diagnostic device for selection of patients who might benefit from treatment with ceritinib was based on an open-label, randomized active-control multi-center Phase 3 study (Trial A2301) of oral ceritinib. This study compared the clinical efficacy and safety of ceritinib treatment to that of chemotherapy [(platinum-based doublet with pemetrexed followed by pemetrexed maintenance in patients without progressive disease after 4 cycles] in previously untreated adult patients with ALK-positive, locally advanced or metastatic, non-squamous NSCLC. VENTANA ALK (D5F3) CDx Assay was used on the BenchMark XT instrument to test a total of 1778 patients for Trial A2301 eligibility, which required a positive ALK status. The study A2301 enrolled patients based on the VENTANA ALK (D5F3) CDx Assay irrespective of prior ALK status. A total of 376 patients whose tumors yielded ALK-positive results from the assay were in the randomized set (189 in the ceritinib arm and 187 in the chemotherapy arm). The overall efficacy results for the ceritinib-treated patients are summarized in Table 24. Ceritinib demonstrated a statistically significant and clinically meaningful benefit over chemotherapy, with a 45% risk reduction in PFS per BIRC (HR=0.55; 95% CI: 0.42, 0.73; p <.001), for patients selected using the VENTANA ALK (D5F3) CDx assay. The median PFS per BIRC assessment was 16.6 months (95% CI: 12.6, 27.2) and 8.1 months (95% CI: 5.8, 11.1) for the ceritinib and chemotherapy arms, respectively.

Table 24. Clinical benefit of ceritinib (progression-free survival) for patients randomized in Trial A2301.

Progression-Free Survival	ZYKADIA (N=189)	Chemotherapy (N=187)
Median, months (95% CI)	16.6 (12.6, 27.2)	8.1 (5.8, 11.1)
HR (95% CI) [a]	0.55 (0.42, 0.73)	
p-valued [b]	<0.0001	

HR=hazard ratio; CI=confidence interval; BIRC=Blinded Independent Review Committee; NR=not reached; NE=not estimable

[a] Cox proportional hazards model stratified by brain metastases (absence or presence), WHO performance status (0 vs. ≥ 1), and prior adjuvant chemotherapy (absence vs. presence). [b] Log-rank test stratified by brain metastases (absence vs. presence), WHO performance status (0 vs. ≥ 1), and prior adjuvant chemotherapy (absence vs. presence).

Staining acceptability rates for VENTANA ALK (D5F3) CDx Assay in the intent-to-diagnose population (the 1778 patients tested with the assay) are reported in Table 25. The rates of acceptable morphology and acceptable background for VENTANA ALK (D5F3) CDx Assay-stained slides are also reported. For 122 specimens, the initial VENTANA ALK (D5F3) CDx Assay staining attempt failed, and another staining attempt was performed. On the final staining attempt, 48 of the 122 specimens remained unacceptable (1 due to invalid run control, 30 due to unacceptable H&E, 12 due to unacceptable negative reagent control, 1 due to unacceptable background, 2 due to unacceptable background and morphology, and 2 due to unevaluable IHC slide). VENTANA ALK (D5F3) CDx Assay demonstrated high initial and final overall staining acceptability rates; 93.1% and 97.3% respectively. Final morphology and background acceptability rates were 99% or greater.

Table 25. Initial and final VENTANA ALK (D5F3) CDx Assay staining performance characteristics for NSCLC study specimens screened for enrollment into Trial A2301.

Evaluated Staining Attributes	Acceptability rate % (n/N) (95% CI)	
	Initial*	Final**
Overall ALK IHC staining acceptability rate	93.1% (1656/1778) (91.9-94.2%)	97.3% (1730/1778) (96.4-98.0%)
Background staining	99.0% (1655/1672) (98.4-99.4%)	99.8% (1727/1730) (99.5-99.9%)
Morphology	99.0% (1657/1674) (98.4-99.4%)	99.9% (1728/1730) (99.6-100%)

* Initial staining attempt ** Final staining attempt

Alectinib Clinical Outcome Study

The clinical efficacy analysis of VENTANA ALK (D5F3) CDx Assay as a diagnostic device for selection of patients who might benefit from treatment with alectinib was based on an open-label, randomized active-control multi-center Phase 3 study (Trial #BO28984) of oral alectinib. This study compared the clinical efficacy and safety of alectinib treatment to that of crizotinib in previously untreated adult patients with ALK-positive, locally advanced or metastatic, NSCLC. VENTANA ALK (D5F3) CDx Assay was used on the BenchMark XT instrument to test a total of 1239 patients for Trial #B028984 eligibility, which required a positive ALK status by central testing. A total of 303 patients whose tumors yielded ALK-positive results from the assay were randomized and analyzed for efficacy (152 in the alectinib arm and 151 in the crizotinib arm). The overall efficacy results are summarized in Table 26. Alectinib demonstrated a statistically significant and clinically meaningful benefit over crizotinib with a 47% risk reduction in PFS per IRC (HR= 0.53, 95% CI: 0.38,0.73; p<0.0001) for patients selected using the VENTANA ALK (D5F3) CDx assay. The median PFS per IRC assessment was 25.7 months (95% CI: 19.9, NE) and 10.4 months (95% CI: 7.7, 14.6) for the alectinib and crizotinib arms, respectively.

Table 26. Clinical benefit of alectinib or crizotinib (progression-free survival) for patients enrolled in Trial #BO28984.

Progression-Free Survival IRC-assessed	Alectinib (N=152)	Crizotinib (N=151)
Median, months (95% CI) [a]	25.7 (19.9,NE)	10.4 (7.7, 14.6)
HR (95% CI) [b]	0.53 (0.38, 0.73)	
p-valued [c]	< 0.0001	

HR=hazard ratio; CI=confidence interval; IRC=Independent Review Committee; NE=not estimable

[a] Estimated using the Kaplan-Meier method.

[b] Hazard ratio was estimated by Cox regression, stratified for covariates Race (Asian vs Non-Asian) and CNS metastases at baseline (presence/absence) by IRC

[c] Based on the stratified log-rank test (same stratification as [b]).

Results for PFS as determined by investigator assessment were consistent with those observed by IRC (HR=0.48 [95% CI: 0.35-0.66], stratified log-rank p<0.0001). Staining acceptability rates for VENTANA ALK (D5F3) CDx Assay in the intent-to-diagnose population (the 1239 patients tested with the assay) were comparable to results in trial A2301.

Refer to Drugs@FDA for the most recent therapeutic product labeling.

Conclusion

VENTANA ALK (D5F3) CDx Assay is reproducible in its staining results for clinical ALK status on the BenchMark XT and BenchMark ULTRA instruments. The binary scoring algorithm is highly reproducible across readers. The assay is concordant with Vysis ALK Break Apart FISH Probe Kit for ALK status. VENTANA ALK (D5F3) CDx Assay may be used in identifying patients eligible for treatment with XALKORI® (crizotinib), ZYKADIA® (ceritinib), or ALECENSA® (alectinib).

REFERENCES

1. Kutok JL, Aster JC, et al. Molecular biology of anaplastic lymphoma kinase-positive anaplastic large-cell lymphoma. J Clin. Onc. 2002;20(17):3691-3702.
2. Iwahara T, et al. Molecular characterization of ALK, a receptor tyrosine kinase expressed specifically in the nervous system. Oncogene. 1997;14:439-49.
3. Soda M, et al. Identification of the transforming EML4−ALK fusion gene in non-small-cell lung cancer. Nature 2007;448(7153)561-66.
4. Inamura K, et al. EML4–ALK fusion is linked to histological characteristics in a subset of lung cancers. J Thorac Oncol. 2008;3(1):13-17.
5. Choi YL, et al. Identification of novel isoforms of the EML4-ALK transforming gene in non-small cell lung cancer. Cancer Res. 2008;68(13):4971-76.
6. Koivunen JP, et al. EML4–ALK fusion gene and efficacy of an ALK kinase inhibitor in lung cancer. Clin Cancer Res. 2008;14(13):4275-83.
7. Shinmura K, et al. EML4−ML4−ALK fusion transcripts, but no NPM-, TPM3−, CLTC−, ATIC−, or TFG-ALK fusion transcripts, in non-small cell lung carcinomas. Lung Cancer. 2008;61:163-9.
8. Takeuchi K, et al. Multiplex reverse transcription−PCR screening for EML4−ALK fusion transcripts. Clin Cancer Res. 2008;14(20):6618−24.
9. Shaw AT, et al. Clinical features and outcome of patients with non-small-cell lung cancer who harbor EML4-ALK. J Clin Oncol. 2009;27(26):4247-53.
10. Yi ES, et al. Correlation of IHC and FISH for ALK gene rearrangement in non-small cell lung carcinoma: IHC score algorithm for FISH. J Thorac Oncol. 2011;6(3):459-65.
11. McLeer-Florin A, et al. Dual IHC and FISH testing for ALK gene rearrangement in lung adenocarcinomas in a routine practice: a French study. J of Thorac Oncol. 2012;7(2):348-54.
12. Christensen JG, et al. Cytoreductive antitumor activity of PF-2341066, a novel inhibitor of anaplastic lymphoma kinase and c-Met, in experimental models of anaplastic large-cell lymphoma. Mol Cancer Ther. 2007;6(12 Pt 1):3314-22.
13. Yamazaki S, et al. Pharmacokinetic/pharmacodynamic modeling of crizotinib for anaplastic lymphoma kinase inhibition and antitumor efficacy in human tumor xenograft mouse models. J Pharmacol Exp Ther. 2012;340(3):549-57.
14. XALKORI package insert. Pfizer. Document ID:6427427a-821b-48b4-8f06-0477f0ae4e36. LAB-0441-6.0.June 2014.
15. ZYKADIA package insert Novartis. Document ID:T2015-114/T2015-115. July 2015.
16. H Sakamoto, et al. CH5424802, a selective ALK inhibitor capable of blocking the resistant gatekeeper mutant. Cancer Cell. 2011;19(5):679-90.
17. Kodama T, et al. Antitumor activity of the selective ALK inhibitor alectinib in models of intracranial metastasis. Cancer Chemother Pharmacol. 2014;75(5):1023-28.
18. Gadgeel S, et al. Safety and activity of alectinib against systemic disease and brain metastases in ptients with crizotinib-resistant ALK-rearranged non-small-cell lung cancer (AF-002JG): results from the dose fining prtion of a phase ½ study. Lancet Oncol.2014;15(10):1119-28.
19. Shaw A, et al. Alectinib in ALK-positive, crizotinib-resistant, non-small-cell lung cancer: a single-group, multicenter, phase 2 trial. Lancet Oncol.2016;17(2):234-42.
20. ALECENSA package insert
21. Galetta D, et al. The emerging role of ALK inhibitors in the treatment of advanced non-small cell lung cancer. Expert Opin Ther Targets. 2012 Suppl 2: SS45-54.
22. Jokoji R, et al. Combination of morphological feature analysis and immunohistochemistry is useful for screening of EML4-ALK-positive lung adenocarcinoma. J Clin Pathol. 2010;63(12):1066-70.
23. Mino-Kenudson M, et al. A novel, highly sensitive antibody allows for the routine detection of ALK-rearranged lung adenocarcinomas by standard immunohistochemistry. Clin Cancer Res. 2010;16(5):1561-71.
24. Sheehan DC, Hrapchak BB. Theory and Practice of Histotechnology, 2nd edition. St. Louis: C.V. Mosby Company; 1980.
25. Carson F, Hladik C. Histotechnology: A Self Instructional Text, 3rd edition. Hong Kong: American Society for Clinical Pathology Press; 2009.
26. NordiQC: Nordic Immunohistochemical Quality Control Anaplastic Lymphoma Kinase (ALK) (lung protocol). Assessment Run 39 2013. http://www.nordiqc.org/Run-39-B16-H4/Assessment/Run39_ALK.pdf. 07-12-2013. Accessed February 26, 2014.
27. College of American Pathologists Laboratory Accreditation Program, Anatomic Pathology Checklist, 2011.
28. CLSI. Quality Assurance for Design Control and Implementation of Immunohistochemistry Assays: Approved Guideline-Second Edition. CLSI document I/LA28-A2 (ISBN 1-56238-745-6). CLSI, 940 West Valley Road, Suite 1400, Wayne, PA 19087-1898 USA, 2011.
29. Herman GE, Elfont EA. The taming of immunohistochemistry: the new era of quality control. Biotech Histochem. 1991;66(4):194-99.
30. Omata M, et al. Nonimmunologic binding of horseradish peroxidase to hepatitis B surface antigen. A possible source of error in immunohistochemistry. Am J Clin Pathol. 1980;73(5):626-32.
31. Nadji M, Morales AR. Immunoperoxidase: part 1. The technique and its pitfalls. Lab Med. 1983;14:767.

INTELLECTUAL PROPERTY

Sold under license from
Cell Signaling TECHNOLOGY®

CONTACT INFORMATION

Ventana Medical Systems, Inc.
1910 E. Innovation Park Drive
Tucson, Arizona 85755
USA
+1 520 887 2155
+1 800 227 2155 (USA)
www.ventana.com