VENTANA anti-ALK (D5F3) Rabbit Monoclonal Primary Antibody

REF 790-4794

06679072001

IVD Σ 50

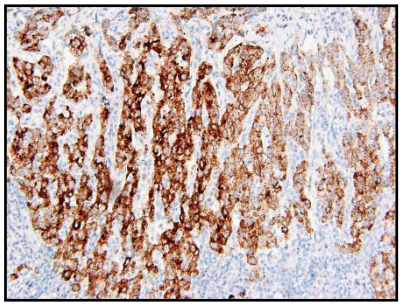

Figure 1. VENTANA anti-ALK (D5F3) antibody expression on non-small cell lung carcinoma.

INTENDED USE

VENTANA anti-ALK (D5F3) Rabbit Monoclonal Primary Antibody (VENTANA anti-ALK (D5F3)) is intended for laboratory use in the detection of the anaplastic lymphoma kinase (ALK) protein in formalin-fixed, paraffin-embedded non-small cell lung carcinoma (NSCLC) tissue stained with the BenchMark series immunohistochemical automated slide stainers. It is indicated as an aid in the assessment of NSCLC patients who might benefit from treatment with XALKORI® (crizotinib).

The clinical interpretation of any staining, or the absence of staining, must be complemented by histological studies and evaluation of proper controls. Evaluation must be made by a qualified pathologist within the context of the patient's clinical history and other diagnostic tests.

This antibody 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] ALK 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 intracellular signaling domain with 5' promoter elements and coding sequences of other genes. The 5' promoter elements and coding sequences drive the overexpression of the chimera and ligand-independent oligomerization, a common feature of 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 included >9 fusion variants, each comprised of the same portion of the ALK C-terminal kinase domain and each resulting in expression of catalytically active kinase fusion protein variants.[4-8] As with fusions involving the ALK gene that had first been 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 was a potent oncogenic factor.[9]

CLINICAL SIGNIFICANCE

Non-small cell lung carcinoma (NSCLC) is the most common type of lung cancer. There are three common types of NSCLC, which include adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. 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.

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]

The vast majority of ALK gene rearrangements were observed in lung adenocarcinoma specimens compared with squamous or small cell histologies.[3-8] There is also evidence that ALK gene rearrangements tend to correlate with patients who are 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 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 and demonstrated antitumor activity in mice bearing tumor xenografts that expressed EML4- or NPM-ALK fusion proteins or Met.

The clinical significance of ALK gene rearrangements was demonstrated in randomized, active controlled, clinical trials of XALKORI conducted by Pfizer.[12] In the United States, XALKORI is indicated 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. Ventana has demonstrated concordance of the VENTANA anti-ALK (D5F3) 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. As stated by Galetta et al., intrachromosomal re-arrangements can yield subtle signal-splitting, leading to potential false negatives.[13] Recent studies indicate that IHC is sensitive and specific for determining ALK status, and is a viable alternative to ALK FISH.[10,11,13-15] In fact, an ALK IHC positive, ALK FISH negative patient benefitted from treatment with XALKORI.[16] Studies comparing IHC with FISH have used different clones with different detection systems and scoring methods. Ventana has developed the VENTANA anti-ALK (D5F3) and scoring algorithm to determine ALK status in NSCLC specimens.

Interpretation of the results of ALK expression on 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 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 Interpretation Guide for VENTANA anti-ALK (D5F3) for Non-Small Cell Lung Carcinoma (NSCLC) P/N 1011879 and the Specific Limitations section below).

PRINCIPLE OF THE PROCEDURE

VENTANA anti-ALK (D5F3) Rabbit Monoclonal Primary Antibody is a rabbit monoclonal primary antibody which 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 anti-ALK (D5F3) includes a recombinant rabbit monoclonal antibody and contains sufficient reagent for staining 50 slides.

One 5 mL dispenser of VENTANA anti-ALK (D5F3) 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.

Refer to the appropriate VENTANA detection kit package insert for detailed descriptions of: (1) Principles of the Procedure, (2) Materials and Reagents Needed but Not Provided, (3) Specimen Collection and Preparation for Analysis, (4) Quality Control Procedures, (5) Troubleshooting, (6) Interpretation of Results, and (7) General Limitations.

MATERIALS REQUIRED BUT NOT PROVIDED

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

Not all products listed in the package insert may be available in all geographies. Consult your local support representative.

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 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 Solution1 (CC1) (Cat. No. 950-124 / 05279801001)
16. ULTRA Cell Conditioning Solution (ULTRA CC1) (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.

STORAGE

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

To ensure proper reagent delivery and the 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 PREPARATION

Routinely processed, formalin-fixed, paraffin-embedded tissues are suitable for use with this primary antibody when used with VENTANA detection kits and a VENTANA BenchMark series of immunohistochemical automated slide stainers.

Using xenograft models generated from the NCI-H2228 human NSCLC cell-line that is positive for ALK, Ventana has determined that the recommended tissue fixative is 10% neutral buffered formalin (NBF) for a fixation time of at least 6 hours.[17] Fixation times less than 6 hours results in a significant loss of staining intensity for ALK. Zinc formalin fixative also is acceptable for 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).[18]

Fixatives such as 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 to 72 hours), and 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 Interpretation Guide for VENTANA anti-ALK (D5F3) for Non-Small Cell Lung Carcinoma (NSCLC) P/N 1011879 for further discussion on the impact of specimen preparation on the ALK antigen.

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 Interpretation Guide for VENTANA anti-ALK (D5F3) for Non-Small Cell Lung Carcinoma (NSCLC) P/N 1011879 and the Performance Characteristics section below).

WARNINGS AND PRECAUTIONS

1. For *in vitro* diagnostic (IVD) use.
2. For professional use only.
3. 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.
4. Materials of human or animal origin should be handled as biohazardous materials and disposed of with proper precautions.
5. Avoid contact of reagents with eyes and mucous membranes. If reagents come in contact with sensitive areas, wash with copious amounts of water.
6. Avoid microbial contamination of reagents as it may cause incorrect results.
7. Consult local and/or state authorities with regard to recommended method of disposal.
8. For supplementary safety information, refer to the product Safety Data Sheet and the Symbol and Risk Phrase Guide located at www.ventana.com.

STAINING PROCEDURE

VENTANA anti-ALK (D5F3) primary antibody has been developed for use with the BenchMark series immunohistochemical automated slide stainers in combination with Rabbit Monoclonal Negative Control Ig, OptiView DAB IHC Detection Kit, OptiView Amplification Kit and accessory reagents. Refer to Table 1, Table 2, and Table 3 for recommended staining protocols for each stainer.

This antibody has been optimized for specific incubation times but the user must validate results obtained with this reagent. Deviating from the staining protocol in Table 2, Table 3, or Table 3 can result in false positive or false negative results. For the BenchMark ULTRA, an ALK-specific staining procedure has been developed, **U VENTANA ALK (D5F3)**. Prior to selecting protocol conditions from the ALK-specific staining procedure the BenchMark ULTRA must have VSS 12.3 software with the OptiView v5 staining procedure or higher.

The parameters for the automated procedures can be displayed, printed and edited according to the procedure in the instrument's Operator's Manual. Refer to the appropriate VENTANA detection kit package insert for more details regarding immunohistochemistry staining procedures.

Table 1. Recommended Staining Protocol for VENTANA anti-ALK (D5F3) 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 Min (Then select US or EU/Other) Or Negative Control
Counterstain	Hematoxylin II, 4 Minutes
Post Counterstain	Bluing, 4 Minutes

Table 2. Recommended Staining Protocol for VENTANA anti-ALK (D5F3) and Rabbit Monoclonal Negative Control Ig with OptiView DAB IHC Detection Kit and OptiView Amplification Kit on a BenchMark XT instrument.

Procedure Type	Method
IHC Synchronization Option	Selected*
Deparaffinization	Selected
Cell Conditioning (Antigen Unmasking)	Cell Conditioning 1, 92 Minutes, 100°C
Pre-Primary Peroxidase Inhibitor	Selected
Antibody (Primary)	Ventana anti-ALK (D5F3) or Rabbit Mono Neg 16 Minutes, 37°C
OptiView HQ Univ Linker	12 Minutes
OptiView HRP Multimer	12 Minutes
OptiView Amplification	Selected
OV AMP H2O2, OV Amplifier	8 Minutes
OV AMP Multimer	8 Minutes
Counterstain	Hematoxylin II, 4 Minutes
Post Counterstain	Bluing, 4 Minutes

* This selectable step is only applicable when running XT Optiview DAB v4 and is not available with previous software versions.

Table 3. Recommended Staining Protocol for VENTANA anti-ALK (D5F3) and Rabbit Monoclonal Negative Control Ig with OptiView DAB IHC Detection Kit and OptiView Amplification Kit on a BenchMark GX instrument.

Procedure Type	Method
Deparaffinization	Selected
Cell Conditioning (Antigen Unmasking)	Cell Conditioning 1, 92 Minutes, 100°C
Pre-Primary Peroxidase Inhibitor	Selected
Antibody (Primary)	Ventana anti-ALK (D5F3) or Rabbit Mono Neg 16 Minutes, 37°C
OptiView HQ Univ Linker	12 Minutes
OptiView HRP Multimer	12 Minutes
OptiView Amplification	Selected
OV AMP H2O2, OV Amplifier	8 Minutes
OV AMP Multimer	8 Minutes
Counterstain	Hematoxylin II, 4 Minutes
Post Counterstain	Bluing, 4 Minutes

Due to variation in tissue fixation and processing, as well as general lab instrument and environmental conditions, it may be necessary to increase or decrease the primary antibody incubation, cell conditioning or protease pretreatment based on individual specimens, detection used, and reader preference when using the BenchMark XT and GX staining procedures.

CONTROLS

Rabbit Monoclonal Negative Control Ig

The matched negative reagent control must be run for every specimen to aid in the interpretation of results. A negative reagent control is used in place of the primary antibody to evaluate nonspecific staining. The slide should be stained with Rabbit Monoclonal Negative Control Ig (Cat. No. 790-4795 / 06683380001). This Rabbit Monoclonal Negative Control Ig is specifically matched for this assay. The staining procedure for the negative reagent control should equal the primary antibody incubation period. Use of no negative control reagent, or other negative control reagents, can result in false results.

Tissue Run Controls

System level controls must be run with patient samples. Examples of positive control tissues for this antibody are ALK positive non-small cell lung carcinomas or benign appendix.[19]

Positive/Negative Tissue Control

A positive and 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 anti-ALK (D5F3) staining procedure performed. 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. NSCLC cases with staining that is representative of a clinically positive as well as a clinically negative result are suitable for optimal quality control and to detect minor levels of reagent degradation or out of specification issues which could be instrument related. The positive staining tissue components (granular cytoplasmic staining of neoplastic cells) are used to confirm that the antibody was applied and the instrument functioned properly.

Appendix may also be used as both an ALK-positive and negative system-level control tissue for the VENTANA anti-ALK (D5F3). The scoring criteria in Table 5 below should be used when assessing ALK staining in appendix tissue.

Table 4. Appendix tissue control evaluation criteria. Representative images are provided in the Interpretation Guide for VENTANA anti-ALK (D5F3) for appendix P/N 1011879.

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 show positive staining.

Appendix and NSCLC Controls

The presence of an appropriately colored reaction product within the cytoplasm of the target cells is indicative of positive reactivity. If the target cells fail to demonstrate appropriate staining or demonstrates a change in clinical diagnostic interpretation, any results with the test specimens should be considered invalid.

In markedly inflamed appendix, an increase in specific staining of neural/neuroendocrine structures and histiocytes in the lymphoid tissue were observed when stained with VENTANA anti-ALK (D5F3). This may be due to reactive hyperplasia of neural structures or drop out of other normal structures due to the inflammation. These structures were confirmed to be neural/neuroendocrine structures and histiocytes by additional antibody stains (S100, Synaptophysin, CD68). See Interpretation Guide (P/N 1011879) for representative images.

Negative Reagent Control

Nonspecific staining, if present, will have a diffuse appearance. Sporadic light staining of connective tissue may also be observed in tissue sections that are excessively formalin fixed. 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. Refer to VENTANA anti-ALK (D5F3) Scoring Interpretation Guide P/N 1011879 for discussion on acceptable levels of background staining for this assay.

STAINING INTERPRETATION / EXPECTED RESULTS

Patient Tissue

Patient tissue will be evaluated according to the ALK scoring algorithm. Refer to the VENTANA anti-ALK (D5F3) Scoring Interpretation Guide P/N 1011879 for specifics and images.

The scoring algorithm for VENTANA anti-ALK (D5F3) is provided in Table 5.

Table 5. Scoring algorithm for VENTANA anti-ALK (D5F3). Representative images are provided in the Interpretation Guide P/N 1011879.

Clinical Interpretation	Staining Description
Positive for ALK	Presence of strong granular cytoplasmic staining in tumor cells (any percentage of positive tumor cells). Certain staining artifacts 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 infiltrate. Some background staining also may be observed within normal mucosa in NSCLC (including mucin) and in necrotic tumor areas, which also should be excluded from the clinical evaluation.
Negative for ALK	Absence of strong granular cytoplasmic staining in tumor cells.

GENERAL LIMITATIONS

1. Immunohistochemistry is a multiple step diagnostic process that requires specialized training in the selection of the appropriate reagents, tissue selections, fixation, processing, preparation of the immunohistochemistry 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.[20] Contact your local support representative with documented unexpected reactions.
8. Tissues from persons infected with hepatitis B virus and containing hepatitis B surface antigen (HBsAg) may exhibit nonspecific staining with horseradish peroxidase.[21]
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.[22]
10. As with any immunohistochemistry 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 anti-ALK (D5F3) has been optimized on VENTANA BenchMark series of immunohistochemical automated slide stainers in combination with the OptiView DAB IHC Detection Kit at a 16 minute primary antibody incubation time with the OptiView Amplification option selected in the protocol.
2. The 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. Patient tissue must be stained within 3 months of sectioning. Loss of staining performance has been observed with the VENTANA anti-ALK (D5F3) on sections that have been stored at room temperature for longer than 3 months.
4. Ventana recommends that samples be fixed at least 6 hours in 10% NBF or zinc formalin. Fixation times and types other than recommended can lead to false negative results. Fixatives such as AFA, Prefer fixative, B5, and other alcohol fixatives have demonstrated a loss of specific ALK protein expression. Refer to the VENTANA anti-ALK (D5F3) Scoring Interpretation Guide P/N 1011879 for further discussion.
5. Some staining artifacts have been noted with the VENTANA anti-ALK (D5F3). Light granular cytoplasmic stippling in alveolar macrophages may be present on both the anti-ALK and negative reagent control stained slides, indicating this is an artifact of the detection system and should not be evaluated as anti-ALK positive staining. In addition, punctate staining has been observed on necrotic areas of tumor which should also be disregarded during patient sample evaluation. Staining of neural tissue including nerve has been observed with the antibody as well as some staining of occasional cells within infiltrating lymphocytes. Refer to the VENTANA anti-ALK (D5F3) Scoring Interpretation Guide P/N 1011879 for further discussion.
6. Slight variability in staining intensity may be observed on the tissue controls due to the OptiView Amplification Kit. Refer to the Interpretation Guide for examples of acceptable staining performance.
7. The correlation of VENTANA anti-ALK (D5F3) Rabbit Monoclonal Primary Antibody to XALKORI clinical outcome has not been established.

TROUBLESHOOTING

If inappropriate staining is observed on either the system-level human NSCLC or appendix tissue controls, or patient specimens, ensure that instrument maintenance procedures have been followed for the BenchMark ULTRA, BenchMark XT or BenchMark GX instrument. If no technical issues or deviations are noted then prior to conducting a repeat run, please contact your local support representative.

PERFORMANCE CHARACTERISTICS

Staining tests for analytical studies were conducted and the results are listed in the following sections.

Specificity

Table 6. Specificity of VENTANA anti-ALK (D5F3) was determined by testing formalin-fixed, paraffin-embedded 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

Tissue	# positive / total cases	Tissue	# positive / total cases
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
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 Few glial cell 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.

Sensitivity

Table 7. Sensitivity of VENTANA anti-ALK (D5F3) was determined by testing a variety of formalin-fixed, paraffin-embedded neoplastic tissues. Results were consistent with reported ALK expression in scientific literature.

Pathology	# 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

Platform Concordance (Bridging Study)

The VENTANA anti-ALK (D5F3) antibody was initially released on the BenchMark XT and GX. To demonstrate equivalent performance of the assay between the BenchMark ULTRA and BenchMark XT, a bridging study was performed. This study evaluated ALK clinical status (based on the ALK scoring algorithm found in Table 5) in 184 unique NSCLC specimens stained with the VENTANA anti-ALK (D5F3) across both platforms. The resulting stained slides were blinded and randomized then evaluated by three pathologists. Results of platform concordance for this study can be found in Table 8 and Table 9.

Table 8. Concordance results between BenchMark XT and BenchMark ULTRA

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

Table 9. Concordance rate between BenchMark XT and BenchMark ULTRA

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

Repeatability and Intermediate Precision Studies

Repeatability and intermediate precision of the VENTANA anti-ALK (D5F3) were evaluated on the BenchMark ULTRA, XT, and GX automated slide stainers 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 both the BenchMark XT and BenchMark ULTRA platforms. 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-platform precision testing, 2 replicate slides from each of the NSCLC specimens were stained with the VENTANA anti-ALK (D5F3) across three BenchMark XT or three BenchMark ULTRA automated slide stainers. For Inter-Day precision, 2 replicate slides from each of the NSCLC specimens were stained with the VENTANA anti-ALK (D5F3) on a single BenchMark XT or BenchMark ULTRA slide stainer across 5 non-consecutive days. All slides were blinded, randomized within each instrument cohort. Each cohort was evaluated individually by a pathologist using the VENTANA anti-ALK (D5F3) scoring algorithm (provided in Table 5). Each replicate NSCLC specimen produced equivalent ALK IHC staining results. A summary of the results can be found in Table 10 and Table 11.

An inter-platform study was performed comparing the performance of the VENTANA anti-ALK (D5F3) on the BenchMark XT and BenchMark GX automated slides stainers. In this study two multi-tissue blocks each containing 8 NSCLC specimens (3 ALK-positive, 1 ALK-negative per block) were evaluated. For this comparison 5 replicate slides were stained on three BenchMark XT and three BenchMark GX automated slide stainers. These slides were evaluated for appropriate staining based on the VENTANA anti-ALK (D5F3) IHC Scoring Algorithm found in Table 5. Each replicate NSCLC specimen produced equivalent ALK IHC staining results between the two platforms. A summary of the results can be found in Table 11.

Table 10. Repeatability and Intermediate Precision of VENTANA anti-ALK (D5F3) on individual NSCLC specimens on the BenchMark XT.

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 instruments)	60	100% (97.9-100%)
Inter-Day Precision (5 non-consecutive days)	100	100% (98.7-100%)

Table 11. Repeatability and Intermediate Precision of VENTANA anti-ALK (D5F3) on individual NSCLC specimens on the BenchMark ULTRA.

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 instruments)	60	100% (94.0-100.0%)
Inter-Day Precision (5 non-consecutive days)	100	100% (96.3-100.0%)

Table 12. Inter-Platform Precision of VENTANA anti-ALK (D5F3) on multi-tissue block NSCLC specimens using the BenchMark XT and BenchMark GX

NSCLC Tissue Precision	N= Total Slides Evaluated in the Cohort	Overall Percent Agreement for ALK Status
Inter-Platform Precision BenchMark XT to BenchMark GX (across 3 instruments)	30	100%

Inter-Reader Precision Studies

An Inter-Reader Precision Study was performed on a cohort of cases from an on-going, 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 the VENTANA anti-ALK (D5F3) on the BenchMark XT. The cases were blinded for ALK FISH status, randomized, and provided to three readers, who evaluated the ALK IHC staining results per the VENTANA anti-ALK (D5F3) Scoring algorithm provided in Table 5. Inter-reader precision rates from the study demonstrated excellent agreement rates between readers. The results provided in Table 13 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 which were stained with VENTANA anti-ALK (D5F3) on the BenchMark ULTRA. The cases were blinded, randomized and provided to three readers, who evaluated the ALK IHC staining results per the VENTANA anti-ALK (D5F3) Scoring Algorithm provided in Table 5. An excellent Inter-Reader precision agreement rate between readers was demonstrated. Table 13 reflects the inter-reader precision rates from this study.

Table 13. Inter-Reader Precision for ALK status in NSCLC specimens obtained from clinical method comparison Cohort # 1 stained with the VENTANA anti-ALK (D5F3) on the BenchMark XT.

Inter-Reader Precision	Average Positive Agreement (95% CI)	Average Negative Agreement (95% CI)	Overall Percent Agreement (95% CI)
Average of all three reader comparisons	97.6% (95.0-99.5%)	99.5% (98.9-99.9%)	99.1% (98.2-99.8%)
Reader 1 vs Reader 2	99.1% (97.1-100%)	99.8% (99.4-100%)	99.7% (98.2-99.9%)
Reader 1 vs Reader 3	96.3% (92.3-99.2%)	99.2% (98.3-99.8%)	98.6% (96.6-99.5%)
Reader 2 vs Reader 3	97.2% (93.5-100%)	99.4% (98.6-100%)	99.0% (97.1-99.7)

Table 14. Inter-Reader Precision for ALK status in NSCLC specimens stained with the VENTANA anti-ALK (D5F3) on the BenchMark ULTRA.

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%)

Concordance with ALK FISH

Three cohorts were used to compare the staining results from the VENTANA anti-ALK (D5F3) Rabbit Monoclonal Primary Antibody with ALK FISH in terms of ALK clinical status. The cohorts included a range of human NSCLC tissue samples from primary and metastatic tumors, including resections, needle biopsies, bronchial biopsies, and formalin-fixed, paraffin-embedded (FFPE) cell blocks from FNAs. All studies were scored using the scoring algorithm (described in Table 5).

Concordance Study 1

A study was conducted in an external laboratory comparing the VENTANA anti-ALK (D5F3) Rabbit Monoclonal Primary Antibody with retrospective Abbott Vysis ALK Break Apart FISH Probe Kit data. The external site stained approximately 100 NSCLC cases using the VENTANA anti-ALK (D5F3) Rabbit Monoclonal Primary Antibody on a BenchMark XT instrument. The VENTANA anti-ALK (D5F3) demonstrated >98% overall percent agreement with the retrospective Abbott Vysis ALK Break Apart FISH Probe Kit data on this NSCLC sample cohort. The results are detailed in Table 14 and Table 15. Note that 86 of the 100 cases had available FISH data and sufficient tumor present for comparison with the ALK IHC result.

Table 15. VENTANA anti-ALK (D5F3) Compared to Abbott Vysis ALK Break Apart FISH Probe Kit.

VENTANA anti-ALK (D5F3) Comparison to Abbott Vysis ALK Break Apart FISH Probe Kit			
VENTANA anti-ALK (D5F3)	Abbott Vysis ALK Break Apart FISH Probe Kit		
	Positive	Negative	Total
Positive	10	0	10
Negative	1	75	76
Total	11	75	86

Table 16. Percent Overall, Positive, and Negative Agreement Rates for VENTANA anti-ALK (D5F3) Compared to Abbott Vysis Break Apart FISH Probe Kit.

Percent Overall, Positive, and Negative Agreement Rates			
Rate	n/N	%	95% CI [a]
Overall Percent Agreement	85/86	98.8	93.7, 99.8
Positive Percent Agreement	10/11	90.9	62.3, 98.4
Negative Percent Agreement	75/75	100.0	95.1, 100.0
[a] Two-sided 95% confidence interval calculated using the score method.			

Note that preparation of tissue specimen from this study was not verified as having followed the specimen preparation procedures recommended for this assay.

Concordance Study 2

A study was conducted in a second external laboratory comparing the VENTANA anti-ALK (D5F3) Rabbit Monoclonal Primary Antibody with Abbott Vysis ALK Break Apart FISH Probe Kit data on 73 NSCLC cases (cut within one week of staining). The external site stained the cases using the VENTANA anti-ALK (D5F3) Rabbit Monoclonal Primary Antibody on a BenchMark XT instrument. The VENTANA anti-ALK (D5F3) demonstrated >93% overall percent agreement with the retrospective Abbott Vysis ALK Break Apart FISH Probe Kit data on this NSCLC sample cohort. The results are detailed in Table 17 and Table 18.

Table 17. VENTANA anti-ALK (D5F3) Compared to Abbott Vysis ALK Break Apart FISH Probe Kit.

VENTANA anti-ALK (D5F3) Comparison to Abbott Vysis ALK Break Apart FISH Probe Kit			
VENTANA anti-ALK (D5F3)	Abbott Vysis ALK Break Apart FISH Probe Kit		
	Positive	Negative	Total
Positive	2	4	6
Negative	0	56	56
Total	2	60	62

Table 18. Percent Overall, Positive, and Negative Agreement Rates for VENTANA anti-ALK (D5F3) Compared to Abbott Vysis Break Apart FISH Probe Kit.

Percent Overall, Positive, and Negative Agreement Rates			
Rate	n/N	%	95% CI [a]
Overall Percent Agreement	58/62	93.5%	84.6-97.5
Positive Percent Agreement	2/2	100%	34.2-100.0
Negative Percent Agreement	56/60	93%	84.1-97.4
[a] Two-sided 95% confidence interval calculated using the score method.			

Of the four discordant (FISH negative, ALK IHC positive) cases, additional unstained slides were tested with another ALK (different clone and detection system). Three of the four cases agreed with the VENTANA anti-ALK (D5F3) in terms of ALK IHC staining detected.

There were also 10 cases where FISH results were undetermined or was not performed. Four of these cases were positive by the VENTANA anti-ALK (D5F3) and the other ALK

clone, and six were negative by ALK IHC. There was one case that was positive by FISH but not enough sample was available to stain with IHC.

Concordance Study 3

In this study, approximately 300 cases from an on-going, global clinical study of ALK positive NSCLC patients enrolled with the Abbott Vysis ALK Break Apart FISH Probe Kit were stained with the VENTANA anti-ALK (D5F3) Rabbit Monoclonal Antibody assay. This is the same cohort that was previously discussed for the Inter-Reader Precision Study found on page 6 of this package insert. Of the approximately 300 cases, some were categorized as "uninformative" by FISH or "FISH assay not performed" and were stained and evaluated for informational purposes only.

The cases were blinded for FISH status, randomized, and provided to two readers, who evaluated the staining results. Results were compared with the FISH status obtained from the global clinical study.

The results of the comparison of ALK IHC with ALK FISH are shown in Table 19 below.

Table 19. Agreement of VENTANA anti-ALK (D5F3) with Abbott Vysis ALK Break Apart FISH Probe Kit as evaluated by 2 pathologists.

VENTANA anti-ALK (D5F3) Comparison to Abbott Vysis ALK Break Apart FISH Probe Kit				
VENTANA anti-ALK (D5F3)		Abbott Vysis ALK Break Apart FISH Probe Kit		
Reader		Positive	Negative	Total
Reader 1	Positive	37	13	50
	Negative	11	223	234
	Total	48	236	284
Reader 2	Positive	37	12	49
	Negative	11	225	236
	Total	48	237	285

Note that preparation of tissue specimens from this study was not verified as having followed the specimen preparation procedures recommended for this assay.

Discrepant cases that were VENTANA anti-ALK (D5F3) anti-ALK positive, ALK FISH negative:

- There were 4 cases evaluated by at least one reader as ALK IHC positive, FISH negative. Upon consensus review, it was determined that they should be evaluated as IHC negative. These cases had focal cytoplasmic/membrane staining and are explained in the Interpretation Guide.
- There were 9 ALK IHC positive, ALK FISH negative cases that were considered true discrepant cases.

Of the 9 discordant cases, 7 had unstained slides that were available for additional ALK diagnostic testing (molecular testing and IHC testing using a different clone and detection system). These additional testing results indicated that the majority of discrepant cases favored the positive IHC evaluation for ALK status when ALK FISH was negative. (Note that cut slides from these cases exceeded the recommended 3 months).

Discrepant cases that were VENTANA anti-ALK (D5F3) negative, ALK FISH positive:

- There were 11 cases that were positive by FISH but negative by the VENTANA anti-ALK (D5F3). 10 cases had unstained slides that were available for the additional ALK diagnostic testing with molecular techniques and IHC. These additional testing results indicated that the majority of cases that were negative by the VENTANA anti-ALK (D5F3) were also negative by another ALK IHC system, but were positive by one or more molecular assays. Note that cut slides from these cases exceeded the recommended 3 months for ALK IHC.
- Finally, there were 14 cases in the cohort that were uninformative by FISH (no result was obtained). Of these, 3 were evaluated as positive by both readers by the VENTANA anti-ALK (D5F3). In addition, there were 19 cases where the FISH assay could not be performed, based on the H&E slide (usually due to the tumor content being insufficient). Of these, both readers evaluated the ALK IHC staining results as positive in 4 cases. Therefore, on average, 21% of the cases where FISH results were not obtained had a positive ALK status by the VENTANA anti-ALK (D5F3).

Conclusion

The VENTANA anti-ALK (D5F3) Rabbit Monoclonal Primary Antibody is reproducible in its staining results for clinical ALK status on the BenchMark ULTRA, BenchMark XT and Benchmark GX platforms. The binary scoring algorithm is highly reproducible across readers. The assay is concordant with the Abbott Vysis ALK Break Apart FISH Probe Kit for ALK status. The VENTANA anti-ALK (D5F3) Rabbit Monoclonal Primary Antibody may be used in identifying patients eligible for treatment with XALKORI (crizotinib). Specimen preparation and cut slide stability recommendations from Ventana must be followed to obtain appropriate staining results.

All studies met their acceptance criteria.

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