VENTANA ALK Scoring Interpretation Guide for non-small cell lung carcinoma (NSCLC)

ALK Scoring Interpretation Guide for VENTANA anti-ALK (D5F3) Rabbit Monoclonal Primary Antibody
VENTANA ALK Scoring Interpretation Guide
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1 Introduction to ALK Testing

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 only 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.

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 non-small cell lung carcinoma (NSCLC) cell lines and archived clinical specimens.\(^3\) Studies indicated that EML4-ALK inversion events included at least 9 fusion variants, all containing the same portion of the ALK C-terminal kinase domain, rendering them catalytically active.\(^4\) Consistent with this, EML4-ALK expression in lung alveolar epithelial cells in transgenic mice was a potent oncogenic factor.\(^9\)

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.\(^10,11,12\) The incidence of ALK gene rearrangements appears to range from 2-7%, translating to ~6,000 ALK positive patients/year in the United States and 40,000 patients/year worldwide.\(^3\)\(^-\)\(^8\) However, there are limitations to this estimation, including a small dataset (1500 tumor samples) and the different ALK methodologies used across studies. Notably, 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.\(^3\)\(^-\)\(^7\)\(^,\)\(^9\) Importantly, ALK gene rearrangements are rarely coincident with EGFR, HER2, or KRAS mutations, demonstrating that ALK positivity is a distinct disease subtype.\(^9\)

Pfizer has developed a small molecular kinase inhibitor, XALKORI\(^®\), which inhibits ALK and other kinases. In two clinical trials, ALK-positive locally advanced or metastatic NSCLC patients who were treated with XALKORI\(^®\) exhibited overall response rates of 50% (N=136; 95% CI: 42%, 59%) and 61% (N=119; 95% CI: 52%, 70%), respectively. Thus, determination of ALK status in NSCLC patients is critical for directing patient care. The above-mentioned clinical trials used the Abbott/Vysis FISH (fluorescent in situ hybridization) ALK Break-Apart assay to determine ALK status. However, 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.\(^10\) Recent studies indicate that immunohistochemistry is sensitive and specific for determination of ALK status, and is a viable alternative to ALK FISH.\(^10\)\(^-\)\(^14\) In fact, an ALK IHC positive, FISH negative patient benefitted from treatment with XALKORI\(^®\).\(^15\) However, studies comparing IHC and with FISH have used different clones (D5F3 and 5A4) with different detection systems and scoring methods; thus, a standardized IHC assay is needed.

Ventana has developed the VENTANA anti-ALK (D5F3) Rabbit Monoclonal Primary Antibody, used with the OptiView DAB IHC Detection Kit and OptiView Amplification Kit, as a fully automated immunohistochemistry (IHC) assay on the BenchMark XT or BenchMark GX automated slide stainers. The assay and associated scoring algorithm described here were developed to maximize concordance with FISH in determination of
ALK status, and the sensitivity of the IHC assay enables a reproducible, binary scoring system (Positive or Negative for ALK status) for evaluating the staining results (refer to the package insert for VENTANA anti-ALK (D5F3) Rabbit Monoclonal Primary Antibody, Cat. No. 790-4794 / 06679072001). Ventana used a range of human NSCLC tissue specimens in developing the ALK IHC assay from primary and metastatic tumors, including resections, needle biopsies, bronchial biopsies, and formalin-fixed, paraffin-embedded (FFPE) cell blocks from FNAs.
2 Intended Use Statements for the VENTANA anti-ALK (D5F3) IHC Assay

Intended Use of VENTANA anti-ALK (D5F3)

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 on a VENTANA BenchMark XT or BenchMark GX immunohistochemical automated slide stainer. It is indicated as an aid in identifying patients eligible for treatment with XALKORI® (crizotinib).

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

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

Intended Use of VENTANA ALK 2 in 1 Control Slides

VENTANA ALK 2 in 1 Control Slides consist of formalin-fixed, paraffin-embedded cultured human lung carcinoma cell lines. The slides are intended to be used as assayed, qualitative control material in conjunction with the VENTANA anti-ALK (D5F3) Rabbit Monoclonal Primary Antibody (Cat. No. 790-4794 / 06679072001) for use in monitoring the performance of the immunohistochemical anti-ALK staining process on VENTANA BenchMark XT or BenchMark GX automated slide stainers. This product should be interpreted by a qualified pathologist in conjunction with histological examination and relevant clinical information.

This product is intended for in vitro diagnostic use (IVD).
3 Purpose of Interpretation Training Guide

This guide is intended to provide pathologists with a tool to facilitate the clinical evaluation of formalin-fixed, paraffin-embedded (FFPE) NSCLC tissue sections stained with VENTANA anti-ALK (D5F3) Rabbit Monoclonal Primary Antibody in accordance with the proposed product labeling. The photo images are provided to illustrate the staining patterns and expression levels associated with the VENTANA anti-ALK (D5F3) IHC assay. Images for ALK IHC staining artifacts are also provided. System-level controls, including positive and negative NSCLC cases, and the VENTANA ALK 2 in 1 Control Slides, are also described. Finally, the impact of pre-analytical factors (fixation time and type and delay to fixation) on the ALK IHC assay are discussed and compared with other IHC and ISH assays.

Description of the ALK IHC Assay

Staining will require one serial tissue section for H&E, a second serial tissue section for VENTANA anti-ALK (D5F3), and a third serial tissue section for the Rabbit Monoclonal Negative Control Ig antibody. The assay uses the OptiView DAB IHC Detection Kit and OptiView Amplification Kit, and is run on the BenchMark XT or BenchMark GX platforms. A known ALK positive NSCLC case and ALK negative NSCLC case can serve as system-level controls to ensure acceptable performance of the instrument system and associated reagents for the assay. The VENTANA ALK 2 in 1 Control Slides can also be used as a system-level control (refer to System-Level Control Slides on page 20).

If the sample is inadequate based on the H&E evaluation (e.g., no tumor present), a new sample should be requested. If the system-level controls (positive and negative NSCLC or VENTANA ALK 2 in1 Control Slides) are not acceptable, staining should be repeated. If the slide stained with the Rabbit Monoclonal Negative Control Ig is not acceptable or the VENTANA anti-ALK (D5F3) is not evaluable, then staining also should be repeated. Non-evaluable VENTANA anti-ALK (D5F3) would mean that determination of reactivity was not possible due to staining artifacts present that interfere with clinical interpretation. Samples must be assessed for appropriate tumor morphology and the presence of viable tumor versus necrosis.
4 Clinical Evaluation

Evaluating the VENTANA anti-ALK (D5F3) Rabbit Monoclonal Primary Antibody in NSCLC

For the ALK IHC assay, each case is stained with the VENTANA anti-ALK (D5F3) antibody and a matched Rabbit Monoclonal Negative Control Ig antibody. Neoplastic cells labeled with the ALK IHC assay are evaluated for presence or absence of the DAB signal. The matched negative control slide is used to assess non-specific background staining and degree of background staining known to occur due to specific tissue elements (refer to positive and negative case images below). Please note: All cases must be stained with the OptiView DAB IHC Detection Kit and the OptiView Amplification Kit, because some cases are weakly positive for ALK by DAB detection only.

The scoring algorithm for ALK IHC is provided below in Table I. Representative cases are discussed in the images.

Table 1. Scoring Criteria for Determination of ALK status in NSCLC.

<table>
<thead>
<tr>
<th>Clinical Interpretation</th>
<th>Staining Description</th>
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<td>Positive for ALK</td>
<td>Presence of strong granular cytoplasmic staining in tumor cells (any percentage of positive tumor cells). Known staining elements should be excluded, including: • light cytoplasmic stippling in alveolar macrophages, • cells of neural origin (nerve and ganglion cells), • glandular epithelial staining, and • 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.</td>
</tr>
<tr>
<td>Negative for ALK</td>
<td>Absence of strong granular cytoplasmic staining in tumor cells.</td>
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Positive for ALK

Positive cases stained with the VENTANA anti-ALK (D5F3) IHC assay typically display a strong, granular cytoplasmic signal. In the majority of positive cases, the signal is distributed homogeneously, having a uniform level of intensity throughout the neoplastic portions of the tumor. In some positive cases, however, the signal can be more heterogeneous in staining intensity. Examples of these obviously positive ALK cases, which comprise the majority of positive cases, are shown below.
Examples of Positive Cases with VENTANA anti-ALK (D5F3)
The anti-ALK (D5F3) staining is shown on the left panels, with the matched Rabbit Monoclonal Negative Control Ig staining on the right.

Case 1: Note the homogeneous ALK IHC expression throughout the tumor.

Case 2: Note the homogeneous ALK IHC expression on this case.

Case 3: Note the heterogeneous ALK IHC expression on this case. Strong cytoplasmic staining is still present, but intensity varies.
Some background staining also has been observed within normal mucosa in NSCLC, as well as in necrotic tumor areas; this staining also should not be evaluated as ALK-positive staining. Additionally, staining has been noted in neural cells (including nerve or ganglion cells), and should also be disregarded in slide interpretation. Some staining has also been observed in glandular epithelial cells and rarely in lymphocytic cells.

In evaluating ALK IHC, specific VENTANA anti-ALK staining in NSCLC tumor cells is noted by the presence of a granular cytoplasmic staining pattern. Light granular cytoplasmic stippling in alveolar macrophages may also be present on both the VENTANA anti-ALK (D5F3) stained slides and negative reagent control slides. This staining artifact should NOT be evaluated as ALK-positive staining. Images of these events are shown below.
Cases demonstrating tissue elements and staining artifacts observed with VENTANA anti-ALK (D5F3) IHC assay

The VENTANA anti-ALK (D5F3) staining is shown in the left panel, the Rabbit Monoclonal Negative Control Ig in the right panel.

Case 6: Note punctate granular staining present in glandular epithelial cells when stained with the VENTANA anti-ALK (D5F3) antibody and the less evident staining of glandular epithelial cells noted on the negative control slide. This staining artifact should be excluded in slide interpretation as it is present in normal tissue elements but not in the tumor cells. This case is negative for ALK.

Case 7: Note staining present in apparent neuronal tissue elements when stained with the VENTANA anti-ALK (D5F3) antibody and the lack of staining noted on the negative control slide. This staining should be excluded in slide interpretation as it is present in normal tissue elements, not in the tumor cells. This case is negative for ALK.

Case 8: Note granular stippling present in alveolar macrophages when stained with the VENTANA anti-ALK (D5F3) antibody and the lack of granular staining noted on the negative control slide. This staining should be excluded in slide interpretation as it is present in normal cells, not in the tumor cells. This case is negative for ALK.
Case 9: Note staining of mucin when stained with the VENTANA anti-ALK (D5F3) antibody and the negative control slide. Note the absence of staining in the normal tissue and tumor cells. This case is negative for ALK.

Case 10: Note staining of mucin on both slides. Note that the normal tissue is negative but the tumor cells are positive. This is positive for ALK.

Case 11: Note staining of some lymphocytes when stained with the VENTANA anti-ALK (D5F3) antibody and the less evident staining noted on the negative control slide. This staining should be excluded in slide interpretation as it is present in normal cells. This case is negative for ALK.
Case 12: Note staining of some lymphocytes when stained with the VENTANA anti-ALK (D5F3) antibody and the negative staining results on the negative control slide. This staining should be excluded from slide interpretation as it is present in normal cells. This case is negative for ALK.

Case 13: Note staining of neural tissue when stained with the VENTANA anti-ALK (D5F3) antibody and Rabbit Monoclonal Negative Control Ig. This staining should be excluded in slide interpretation as it is present in normal cells. This case is negative for ALK.
Example of Cases that are Negative for ALK

The vast majority of ALK Negative cases exhibit an absence of DAB signal above background staining from the matched negative control slide.

However, a small percentage of negative cases display a weak, diffuse granular cytoplasmic pattern that is detected above background staining observed on the associated Rabbit Monoclonal Negative Control Ig stained slide. Ventana has estimated these cases represent ~1-2% of all cases stained to date with the ALK IHC assay and are negative by FISH (refer to Challenging Case Examples on page 15).

Examples of Negative Cases with anti-ALK (D5F3)

The VENTANA anti-ALK (D5F3) staining is shown in the panels on the left, the Rabbit Monoclonal Negative Control Ig staining is on the right.

Case 14: Note absence of ALK IHC expression in tumor cells. This case is negative for ALK.

Case 15: Note absence of ALK IHC expression in tumor cells. This case is negative for ALK.
While the vast majority of cases stained with VENTANA anti-ALK (D5F3) IHC assay are clearly positive or negative in their staining results, a few cases have been observed that present a challenge in interpretation.

Case 16: Note presence of weak, diffuse granular cytoplasmic expression on both the slide stained with the VENTANA anti-ALK (D5F3) and the Rabbit Monoclonal Negative Control Ig. Cytoplasmic staining may be more notable on the slide stained with the VENTANA anti-ALK (D5F3) antibody but the staining is not strong in intensity and the granular pattern is not present. The IHC result should be interpreted as negative for ALK.

Case 17: Note presence of weak, diffuse cytoplasmic expression on the slide stained with the VENTANA anti-ALK (D5F3) antibody. Cytoplasmic staining is more notable on the slide stained with the VENTANA anti-ALK (D5F3) antibody compared to the negative control reagent, but is not strong intensity. The IHC result should be interpreted as negative for ALK.
Challenging Case Examples

Staining of VENTANA anti-ALK (D5F3) is on the left, while staining with the Rabbit Monoclonal Negative Control Ig is on the right.

Case 18: Cytoplasmic staining is more notable on the slide stained with the VENTANA anti-ALK (D5F3) antibody compared to the negative control reagent but the IHC result should be interpreted as negative. This case should be scored as negative for ALK IHC due to lack of strong, granular cytoplasmic staining.

Case 19: Note presence of diffuse cytoplasmic expression on the slide stained with the VENTANA anti-ALK (D5F3) antibody. Cytoplasmic staining is more prevalent on the slide stained with the VENTANA anti-ALK (D5F3) antibody compared to the negative control reagent but the IHC result should be interpreted as negative. This case should be scored as negative for ALK IHC due to lack of strong granular cytoplasmic staining.

Case 20: Note presence of diffuse cytoplasmic expression on the slide stained with the VENTANA anti-ALK (D5F3) antibody. Cytoplasmic staining is more prevalent on the slide stained with the VENTANA anti-ALK (D5F3) antibody compared to the negative control reagent but the IHC result should be interpreted as negative. This case should be scored as negative for ALK IHC due to lack of strong granular cytoplasmic staining.
Case 21: Note presence of diffuse cytoplasmic expression on the slide stained with the VENTANA anti-ALK (D5F3) antibody. Cytoplasmic staining is more prevalent on the slide stained with the VENTANA anti-ALK (D5F3) antibody compared to the negative control reagent but the IHC result should be interpreted as negative. This case should be scored as negative for ALK IHC due to lack of strong granular cytoplasmic staining.

Case 22: Note presence of membrane cytoplasmic expression on the slide stained with the VENTANA anti-ALK (D5F3) antibody. Although staining is present, it is not the typical strong granular cytoplasmic staining pattern but rather membrane/cytoplasmic staining. Staining is more prevalent on the slide stained with the VENTANA anti-ALK (D5F3) antibody compared to the negative control reagent but the IHC result should be interpreted as negative. This case should be scored as negative for ALK IHC.
Pre-Analytical Conditions and Impact on the VENTANA anti-ALK (D5F3) Assay

Ventana has conducted studies using the H2228 cell line (positive for ALK) generated as xenograft tumors in SCID mice as a model system for determining the impact of pre-analytical factors on the assay. The tumors were harvested and fixed with different fixatives across a range of times and stained with the VENTANA anti-ALK (D5F3) assay. Consistent with ASCO/CAP guidelines for HER2 testing, tissues must be fixed using 10% neutral buffered formalin (NBF) for a period of at least 6 hours, for optimal ALK IHC staining results. Zinc formalin also yielded acceptable staining results at >6 hour timepoints, and can be used with the ALK assay. However, fixation times below 6 hours (under-fixation) in NBF and in Zinc formalin resulted in a significant decrease in staining intensity for ALK.

Fixatives other than NBF and Zinc formalin, including AFA, B5, and Prefer, also were tested and should not be used with the VENTANA anti-ALK (D5F3) IHC assay as the staining results were severely compromised. Intensity of the ALK IHC assay was dramatically decreased under all timepoints tested with AFA, B5, and Prefer fixatives. In addition, fixing in 95% alcohol for as little as one hour resulted in a significant negative impact to ALK staining intensity and should not be performed with this assay.

Ventana also investigated the impact that delay to fixation has on the VENTANA anti-ALK (D5F3) staining results. Xenograft samples were excised and left un-fixed for times ranging from 30 minutes to 24 hours, then fixed for 12 hours in 10% NBF. The staining results indicated that ALK intensity was compromised if the time to fixation in NBF was delayed >6 hours.

Finally, it is important to emphasize that the ALK protein (as detected with D5F3 but also 5A4) appears to be more sensitive to pre-analytical factors when compared with other lung markers detected by IHC (TTF1 and EGFR) using the xenograft models. Representative data are shown below. Ventana emphasizes that fixation conditions for human lung specimens be carefully monitored and controlled to ensure optimal staining results with the ALK IHC assay.

Fixative Examples

The images below demonstrate the impact of fixation conditions on the ALK IHC assay compared with other markers. VENTANA anti-ALK (D5F3) Rabbit Monoclonal Primary Antibody staining is shown on the images below with various fixation types and times. Staining with VENTANA anti-TTF-1 (SP141) and VENTANA anti-EGFR (5B7) antibodies are also shown at those same conditions.
H2228 Xenograft tumor fixed in 10% NBF for 1 hour (left) and 12 hours (right) was stained with the VENTANA anti-ALK (D5F3) IHC assay. >6 hour fixation is optimal for ALK IHC.

H2228 Xenograft tumor fixed in Zinc Formalin for 1 hour (left) and 12 hours (right) was stained with VENTANA anti-ALK (D5F3) IHC assay. >6 hour fixation is optimal for ALK IHC.

H2228 Xenograft tumor fixed in 10% NBF for 1 hour (left) and 12 hours (right) was stained with the anti-TTF-1 (SP141) from Venta na. Note there is less of a difference in staining intensity between the times for this marker compared with the ALK images above.
Fixation with Prefer, Bouin’s, AFA, and 95% ethanol also are not recommended for the VENTANA anti-ALK (D5F3) assay. VENTANA anti-ALK (D5F3) staining is shown at 12 hours in AFA and 95% ethanol, compared with TTF1 and EGFR (see above).
Cut Slide Stability

Ventana has determined that the ALK IHC assay should not be performed on cut slides that have been stored longer than 3 months. The staining intensity of the assay diminishes, first observed at 3 months after cutting, when stored at room temperature (although none of the ALK positive cases tested at that time point changed its status from ALK positive to ALK negative). Examples are shown below. Ventana has not tested the impact of cut slide stability combined with different fixatives, and 3 months may not be the optimal stability for fixatives other than NBF.

Although both slides are positive for ALK, note diminished staining intensity on the slide stored ambient (room temperature) for 3 months (right panel) compared to the freshly sectioned stained slide (left panel).

Other Information Regarding the VENTANA anti-ALK (D5F3) IHC Assay

Reproducibility of the VENTANA anti-ALK (D5F3) IHC Assay

The advantage of the VENTANA anti-ALK (D5F3) IHC Assay is that the use of the OptiView DAB IHC Detection Kit and OptiView Amplification Kit enables the vast majority of NSCLC cases to easily be interpreted as a “Positive” or “Negative” Result. The enhanced sensitivity of the assay means that the reader does not need to provide a semi-quantitative assessment of percent tumor cell staining or staining intensities, as is the case for other biomarkers assessed by IHC assays. However, Ventana recognizes that the OptiView Amplification Kit is new technology for many pathologists. One factor that is apparent in more sensitive detection systems is that there can be more slide-to-slide variability in total staining intensity, compared with DAB only.

System-Level Control Slides

System-level control slides are available from Ventana to be used with the VENTANA anti-ALK (D5F3) IHC assay. Control slides for the ALK IHC test are derived from cultured human NSCLC cell lines that are ALK-positive or ALK-negative. The cells are fixed in NBF and suspended in an agarose matrix. The agarose pellets are then fixed in NBF and embedded in a paraffin block where ~4 µm sections are cut and mounted on a positively charged glass slide. The control slides are packaged as a 10 slide kit configuration and sold separately.
The control slides for the Ventana ALK IHC test consist of a positive (H2228) and a negative (CALU-3) cell line and are intended to be used as system-level controls, to ensure proper function of the reagents and instrument. Note that the CC1 (cell conditioning) conditions for the cell line staining procedure are different from those needed for human lung tissue to prevent background staining and impact to morphology. The images below demonstrate the expected staining outcome for the control slides.

**VENTANA ALK 2 in 1 Control Slide**

Positive cell line (left) and negative cell line (right) staining result.

Positive staining on the H2228 cell line is defined as the presence of strong cytoplasmic staining in $\geq 90\%$ of the cells. Negative staining on the CALU-3 cell line is defined as the absence of strong cytoplasmic staining in $\geq 90\%$ of the cells. Cells may have light granular cytoplasmic staining and this should be considered as acceptable background. Staining of remnant agarose is a known artifact and should be disregarded in slide evaluation. The images below show the staining observed on the CALU-3 cell line and the staining of remnant agarose artifact.

Both images above depict the CALU-3 cell line stained with anti-ALK (D5F3). Note the presence of light granular cytoplasmic staining. Both are acceptable staining results with the anti-ALK (D5F3) antibody.

The image below depicts the remnant agarose that may be present on the control cell lines and should be disregarded when interpreting these slides.
If the recommended protocol for the VENTANA ALK 2 in 1 Control Slides is not followed then staining on the control slides may not be acceptable. The images below show unacceptable staining results for the control slides if the cell conditioning time is longer than the recommended 16 minutes.

Example of overstaining for VENTANA ALK 2 in 1 Control Slides:

The above images depict the presence of cytoplasmic staining on the CALU-3 negative cell line for 92 minute cell conditioning time selected for CC1 in the protocol.
5 References


