# Multicenter Evaluation of a Novel ROS1 Immunohistochemistry Assay (SP384) for Detection of ROS1 Rearrangements in a Large Cohort of Lung Adenocarcinoma Patients

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# Multicenter evaluation of a novel ROS1 immunohistochemistry assay (SP384) for detection of *ROS1* rearrangements in a large cohort of lung adenocarcinoma patients

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**Running title:** ROS1 immunohistochemistry and lung adenocarcinoma

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**Abstract**

**INTRODUCTION:** The detection of a *ROS1* rearrangement in advanced and metastatic lung adenocarcinoma (LUAD) lead to a targeted treatment with tyrosine kinase inhibitors, with favorable progression-free survival and overall survival of the patients. Thus, it is mandatory to screen for the *ROS1* rearrangement in all these patients. *ROS1* rearrangements can be detected using break-apart fluorescence *in situ* hybridization (FISH), which is the "gold standard"; however, ROS1 immunohistochemistry (IHC) can be used as a screening test since it is widely available, easy and rapid to perform, and cost-effective.

**METHODS:** We evaluated the diagnostic accuracy and inter-pathologist agreement of two anti-ROS1 IHC clones, SP384 (Ventana, Tucson, AZ) and D4D6 (Cell Signaling, Danvers, MA), in a training cohort of 51 positive *ROS1* FISH LUAD cases, and then in a large validation cohort of 714 consecutive cases of LUAD from six routine molecular pathology platforms.

**RESULTS:** In the two cohorts, the SP384 and D4D6 clones demonstrate variable sensitivity and specificity rates on the basis of two cutoff points ≥1+ (all % tumor cells and ≥2+ (>30% stained tumor cells). In the validation cohort, the D4D6 yielded the best accuracy for the presence of a *ROS1* rearrangement by FISH. Inter-pathologist agreement was moderate to good (ICC, 0.722–0.874) for the D4D6 clone and good to excellent (ICC, 0.830–0.956) for the SP384 clone.

**CONCLUSIONS:** ROS1 IHC is an effective screening tool for the presence of ROS1 rearrangements. However, users must be acutely aware of the variable diagnostic performance of different anti-ROS1 antibodies prior to implementation into routine clinical practice.

**Keywords:** ROS1; SP384; D4D6; immunohistochemistry; FISH; lung adenocarcinoma.

## INTRODUCTION

Patients with advanced and metastatic lung adenocarcinoma (LUAD) harboring a *ROS1* rearrangement respond effectively to crizotinib, as well as to the other tyrosine kinase inhibitors that are currently used in clinical trials.[1-5] ROS1 screening is thus recommended in most guidelines. *ROS1* rearrangement detection using fluorescence *in situ* hybridization (FISH) is the typical approach that is consistently performed to identify patients with LUAD who would be eligible for these treatments. Based on its high sensitivity and specificity, this method is considered to be the "gold standard" for determining ROS1 positivity.[6, 7] However, given the low incidence of *ROS1* rearrangements in lung adenocarcinoma (1% to 2%),[6] detection of ROS1 protein expression by immunohistochemistry (IHC) can be used as a surrogate, cost-effective screening test that is preferable to FISH analysis for screening the *ROS1* status.[8] Furthermore, the expression of the ROS1 protein, as detected by IHC, may indicate a functional gene product, which is necessary for effective drug targeting.[9] Moreover, FISH testing requires high-level expertise for technical and diagnostic issues, which is not widely available in all laboratories. Finally, the turnaround time for getting the final result can sometimes delay an urgent treatment decision.[10]

Considering these latter constraints, ROS1 IHC was recommended as a frontline screening test prior to confirmatory FISH analysis in *ROS1*-positive cases and to avoid unnecessary FISH analysis in *ROS1*-negative cases.[8] To date, a single commercially available antibody (D4D6 clone, Cell Signaling Technology, Danvers, MA, USA) has been used in several studies and is currently used worldwide in routine clinical practice.[11-13] Given the high sensitivity of IHC relative to FISH or other molecular approaches, such as next generation sequencing (NGS) technologies, 97-98% of tumors that clearly lack ROS1 staining can be safely interpreted as negative for *ROS1* rearrangement.[6, 11-13]

Conversely, the interpretation of a positive ROS1 D4D6 IHC test may be challenging and can lead to false-positive results (related to a patchy pattern and weak intensity of the staining) that do not harbor an underlying *ROS1* rearrangement as detected by FISH.[14-16] Therefore, the variability of specificity reported for the ROS1 D4D6 IHC assay requires caution and subsequent confirmation of the *ROS1* status by a validated method, irrespective of the staining intensity and the percentage

of positive tumor cells.[15, 17] Only the confirmation of a *ROS1* rearrangement by a molecular or cytogenetic method (e.g., FISH, NGS) can be used prior to considering a patient with LUAD for *ROS1*-targeted therapy.[8, 18]

In this context, the development of a new anti-ROS1 antibody (SP384 clone, Ventana, Tucson, AZ, USA) prompted the evaluation of its diagnostic performance to predict the presence of a *ROS1* rearrangement in a routine clinical setting.

This study aimed to investigate the correlation between two ROS1 IHC assays and paired *ROS1* FISH analysis as well as inter-pathologist agreement for IHC interpretation in two large-scale multicenter cohorts of LUAD cases.

## PATIENTS AND METHODS

### Case selection

The training cohort was comprised of retrospective archival formalin-fixed paraffin-embedded (FFPE) tumor samples from 51 patients with stage IIIB/IV LUAD and *ROS1* FISH positive. None of the *ROS1* positive cases harbored concurrent *ALK* rearrangements nor *EGFR* or *BRAF* activating mutations. In *ROS1* positive patients, tumor response was evaluated per RECIST 1.1 on target lesions, 3 months after the initiation of crizotinib and was compared with baseline results.

In the validation cohort, 714 cases of consecutive LUAD diagnosed in the Laboratory of Clinical and Experimental Pathology (Pasteur Hospital, Université Côte d'Azur, Nice, France) between 2006 and 2017 were integrated in tissue microarray (TMA) FFPE blocks. Three tissue cores of 600 µm in diameter were taken from different tumor areas within the same FFPE block for each case.

A signed informed consent was obtained from all patients. The study was approved by the local ethics committee (Human Research Ethics Committee, Nice University Hospital Center/hospital-related Biobank BB-0033-00025) and was performed in accordance with the guidelines of the Declaration of Helsinki. *ROS1* FISH positive tissue samples were provided by 6 pathology laboratory centers in France (University Hospitals of Bordeaux, Caen, Nancy, Nice, Rouen and Toulouse) and centralized for IHC and FISH analysis in the Laboratory of Clinical and Experimental Pathology (Nice, France), which is accredited by COFRAC (French National Authority for Accreditation delivery, n°8-3034; www.cofrac.fr) for FISH and IHC testing according to the ISO 15189 norm.

### *ROS1* FISH analysis

Tumor samples were fixed in formaldehyde 10% and paraffin, embedded at each participating center before centralization. Tissue sections (4 µm thick) were stained with hematoxylin eosin and the percentage of tumor cells was assessed independently by three senior thoracic pathologists (VH, MI, PH). Briefly, FISH analysis was conducted using the Abbott Molecular FISH probe (Abbott Molecular Inc., Des Plaines, IL, USA), LSI ROS1 (Tel) SpectrumOrange Probe and LS1 ROS1 (Cen) SpectrumGreen Probe. Additionally, some tumors were assessed for *ROS1* rearrangement using the ZytoLight SPEC ROS1 Dual Colour Break Apart Probe (ZytoVision, Bremerhaven, Germany). FISH was performed according to the manufacturer's recommendations. Interphase signals were manually counted in at least 100 tumor nuclei per case using an epi-fluorescence microscope (Eclipse 80i, Nikon, Champigny-sur-Marne, France) supported in parallel by the PathScan® Viewer software (Excilone, Elancourt, France).

A positive case was defined if more than 15% of tumor cells in more than 100 tumor cells demonstrated a split signal at least 2 signal distances apart or an isolated centromeric 3' (green signal) pattern.

### ROS1 IHC analysis

ROS1 IHC analysis was performed using the D4D6 clone (Cell Signaling Technology, Danvers, MA, USA; CC1 buffer for 64 min, 1:50 dilution for 32 min, ultraView Universal DAB Detection Kit, Ventana, Tucson, AZ, USA) and the SP384 clone (Ventana, Tucson, AZ, USA; CC1 buffer for 64 min, 1:50 dilution for 16 min, OptiView DAB IHC Detection Kit, Ventana) and processed on the Ultra Benchmark autostainer (Ventana) according to the manufacturer's recommendations. Immunostainings were interpreted independently by three senior lung pathologists (VH, MI, PH), blinded to FISH results, for the following criteria: signal intensity (0; no staining, 1+; weak, 2+; moderate; and 3+; strong) and percentage of stained cells, topography and characteristics of the signal (cytoplasmic – diffuse or focal and/or granular; cytoplasmic and membranous; membranous). Results were quantified by multiplying the percentage of ROS1 positive tumor cells by the intensity (H-score, range, 0–300). For each case included on the TMA FFPE blocks, the mean H-score of the three cores was recorded. Non-specific staining of macrophages and type II

pneumocytes was disregarded. ROS1 negative and positive controls were put on each IHC slide (HCL0024, HistoCyte Laboratories Ltd, Newcastle University, UK).

**Statistical analysis**

Statistical analyses assessing agreement between the D4D6 and SP384 antibodies with the ROS1 FISH analysis (gold standard) were performed on dichotomized variables using two positivity cutoff points: IHC ROS1 positive (intensity ≥1+, any percentage of tumor cells) and IHC ROS1 strongly positive (intensity ≥2+, >30% tumor cells), as recently described.[17, 19] Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy were calculated for the comparison between the dichotomized results using each of the two antibodies relative to the FISH results. The Spearman's rank correlation test (*rho*) was used for a correlation analysis between the H-scores as continuous variables of each clone. Intra-class correlation coefficients were calculated for the comparison of H-scores as continuous variables from each of the ROS1 clones, and κ scores were calculated for dichotomized results based on two positivity cutoff points, as described above, to assess the inter-pathologist agreement for each of the clone. The paired Student's t-test was used to assess the correlation between the ROS1 IHC status and RECIST 1.1. All statistical analyses were carried out using R software (version 3.2.2, R Core Team, Vienna, Austria). Any *P*-value <0.05 was assumed to indicate a statistically significant difference.

## RESULTS

### Training cohort

In a clinically selected cohort enriched for tumors with FISH-identified ROS1 rearrangements, the frequency of split or isolated centromeric signals averaged 64% and ranged from 15% to 100%. IHC SP384 showed strong 3+ expression in 40/51 (78%), 2+ moderate in 10/51 (20%) and 1+ weak in 1/51 (2%) of cases (**Table 1, Figure 1**). IHC D4D6 showed a wider range of intensity in 15/51 (30%) with 3+ staining, 16/51 (31%) with 2+ staining and 20/51 (39%) with 1+ staining (**Table 1, Figure 1**). Inter-pathologist agreement was high in interpreting both ROS1 IHC clones (**Supplementary Table S1**).

On the basis of the ≥1+ expression cutoff, both ROS1 IHC clones are 100% sensitive, whereas if ≥2+ tumors are considered positive, the SP384 clone is 98%

sensitive and D4D6 is only 61% sensitive for the detection of a ROS1 rearrangement by FISH (**Table 1**).

H-scores ranged from 5 to 300 and from 50 to 300 when using the D4D6 and SP384 clones, respectively. However, the Spearman's rank correlation was not linear (*rho*=0.508; 95% CI, 0.19–0.72) between the two clones (**Figure 1**, **Supplementary Figure S1**).

Neither the intensity nor the percentage of ROS1 protein expression detected with both SP384 and D4D6 antibodies correlated with the percentage of ROS1 split signals by FISH (**Supplementary Figure S2**).

In most of the cases analyzed with the SP384 clone, a diffuse cytoplasmic staining was observed with or without membranous staining (**Figure 2**). Less frequently, a granular cytoplasmic or a membranous staining alone was noted (**Figure 2**). Corresponding D4D6 IHC staining was weaker and in some cases the percentage of positive tumor cells was lower than those noted in the corresponding tumors stained with the SP384 clone (**Figure 1, Supplementary Figure S1**). ROS1 protein expression was also seen with both D4D6 and SP384 antibodies in non-neoplastic alveolar epithelium at the tumor periphery, in sub-pleural pneumocytes or in areas of type II pneumocyte hyperplasia and bronchiolar metaplasia. However, the expression in this epithelium was weak in intensity in most cases.

In this retrospective study, at the time of clinical care, the vast majority of ROS1 positive patients, as confirmed by FISH, were treated with crizotinib in the second and third line settings. After 3 months, 18% of patients had a complete response, 19% demonstrated a partial response, 19% had stable disease, and 44% had progressive disease (**Supplementary Figure S3**). In total, 56% of patients had disease control after 3 months on crizotinib. However, no significant difference existed between the two IHC clones (**Supplementary Figure S3**) nor between the % of rearranged tumor cells by FISH and the response by RECIST (t-test, *P*-value=0.306). In order to identify putative resistance mechanisms[20], an NGS analysis (Ion AmpliSeq Cancer Hotspot Panel, ThermoFisher Scientific, San Francisco, CA, USA) was performed in two patients with progressive disease and available tumor material. In one patient, we found a mutation in the gene encoding for β-catenin (CTNNB1 p.S45F, VAF 43%). The NGS analysis detected no copy-number

variations. Moreover, these cases had no *ROS1* copy-number gain, as determined by FISH.[20]

**Validation cohort**

A total of 714 cases of LUAD with an unknown *ROS1* rearrangement status were screened on TMA blocks using paired a ROS1 IHC and FISH assay. *ROS1* rearrangements were detected in this screening cohort by FISH at a rate of 9 of 714 cases (1.3%).

On the basis of the ≥1+ expression cutoff, both ROS1 IHC clones are 100% sensitive for the presence of a ROS1 translocation detected by FISH, while the D4D6 clone was deemed more specific and accurate (**Table 1, Figure 3**). Conversely, on the basis of the ≥2+ expression cutoff, the SP384 clone was more sensitive than the D4D6 clone, while the D4D6 was more specific and accurate than SP384 (**Table 1, Figure 3**).

Inter-pathologist agreement ranged from moderate to good (ICC, 0.722–0.874) in interpreting the D4D6 IHC assay and from good to excellent (ICC, 0.830–0.956) for the SP384 IHC assay (**Supplementary Table S1**).

## DISCUSSION

This is the first study to assess the diagnostic performance of a newly developed anti-ROS1 antibody (SP384 clone) by comparison to two diagnostic ROS1 assays that are validated for a clinical setting (e.g., D4D6 IHC and ROS1 break-apart FISH assays) in two large-scale multicenter cohorts of LUAD cases.

We considered two cutoffs for positivity and showed that none of the ROS1 IHC assays demonstrated 100% accuracy in detection of *ROS1* rearrangements as identified by FISH. In the validation cohort of ROS1-positive LUAD cases, while these two clones demonstrate similar sensitivity around the ≥1+ cutoff, the sensitivity rate dropped at 61% for the D4D6 clone when the ≥2+ cutoff was applied. In addition, the intensity and the percentage of stained tumor cells were higher when using the SP384 clone than with the D4D6 clone. Inter-reader agreement varied from moderate to good when interpreting the D4D6 assay, while it was deemed good to excellent for the SP384 clone, as recently reported.[19] Most of the cases analyzed with the SP384 clone showed diffuse cytoplasmic staining, with or without membranous labeling.

It is noteworthy that the percentage and the intensity of SP384 and D4D6 IHC did not correlate with the percentage of *ROS1* FISH split signals, as had previously been reported.[12] No significant difference was noted between the different ROS1 IHC assays and the RECIST 1.1 subgroups in patients treated with crizotinib.

In our screening cohort, which was drawn from a clinical molecular diagnostics practice, ROS1 rearrangement was detected in 1.3% of tumors, compared to 1–2% of unselected LUAD cases, as previously reported.[6] Around the ≥1+ cutoff, sensitivity was similar for both IHC assays; however, the specificity of the D4D6 clone (97.87%) was higher than that of the SP384 clone (87.38%). We also explored the recently described ≥2+ cutoff in >30% tumor cells.[17, 19] Whereas the sensitivity of the D4D6 clone declined to 66.67% under these criteria, and was 88.89% for the SP384 antibody, the specificity was still higher for the D4D6 assay (99.57%) than the SP384 clone (91.06%).

The average H-score of the SP384 clone was higher that the D4D6 antibody, which led to easier interpretation. However, if an algorithm for *ROS1* testing that is similar to that established for *ALK* status testing were used and if the ≥1+ cutoff were to be used for both IHC clones, this could lead to an increase of nearly 10% in unnecessary FISH testing based on screening with the SP384 clone. Moreover, in

our study, the ≥2+ cutoff in >30% tumor cells did not improve the sensitivity and specificity rates of either IHC assay.

When newly developed diagnostic assays for the detection of rare events such as *ROS1* rearrangements are considered for implementation in a routine clinical setting, our findings highlight the importance of their evaluation in large-scale cohorts, and the necessity of both positive and negative cases. As accurate detection of *ROS1* rearrangements in clinical tumor samples is mandatory for optimal targeted treatment, investigation of new testing modalities should not be limited to positive samples only.[18]

Previous studies reported ROS1 D4D6 IHC as being highly sensitive, but less specific compared with FISH for the detection of *ROS1* rearrangements. ROS1 D4D6-positive tumors, especially when the tumor is stained with moderate to strong intensity or a diffuse pattern, are recommended to undergo FISH to confirm the presence of *ROS1* rearrangement.[12-14] Besides the assessment of the *ROS1* rearrangement status, it is mandatory to systematically detect other genomic alterations in advanced and metastatic lung adenocarcinoma. This can be performed by molecular biology (e.g., *EGFR* mutation) and by molecular and/or IHC approaches (e.g., *BRAFV600* mutation, *ALK* rearrangement). Moreover, PD-L1 IHC is now required in this population. Additionally, the evaluation of other genomic alterations (e.g., *RET* rearrangement, *MET* mutations, *HER2* mutations, *NTRK* rearrangement) can be required if the tumors are *EGFR, ALK, ROS1* and *BRAF* wild-type and with less than 50% of PD-L1 positive tumor cells. Moreover, recent clinical trials for immunotherapy associated with the assessment of the tumor mutation burden (TMB) have indicated some promising results for the systemic use of this predictive biomarker in the near future.[21, 22] In this context, one challenge is to be able to perform these analyses on smaller tissue biopsies and/or samples with low proportions of tumor cells (less than 20%). Considering that a minimum number of tissue sections are required for the diagnosis of NSCLC, it is critical to conserve tissue as much as possible for ancillary analyses (different predictive IHC, targeted sequencing analyses or NGS panels – potentially including TMB assessment). In this context, it is of interest to economize and only use tissue sections for *ROS1* FISH analysis in IHC positive cases. More importantly, the feasibility of using ROS1 IHC for *ROS1* status assessment has many advantages in comparison with the *ROS1* FISH.

IHC is available in all pathology laboratories and can easily be conducted. The turnaround time to get the results is much shorter than the FISH approach. IHC is a cost-effective technology. Finally, shipment and archiving of stained slides can be done at room temperature.

Different testing approaches, such as DNA-based hybrid capture library preparation followed by NGS, RNA-based anchored multiplex polymerase chain reaction library preparation followed by NGS or nCounter technologies could allow the simultaneous detection of gene rearrangements such *ALK, ROS1, RET*, or *NTRK*.[18, 23, 24] Thus, it might be questionable whether the development of IHC assays for the detection of these alterations should continue, in particular for *ROS1* rearrangement. However, some difficulties can be encountered for the NGS approach in comparison with the IHC method in a clinical setting.[25] Depending on the quantity and quality of nucleic acids present in the FFPE tissue biopsy, NGS can give false negative results, in particular in small specimens and/or when a low percentage of tumor cells are present. Moreover, the technology is not available in all laboratories and requires specific and high-level expertise. The time lag for results is also much longer than for IHC testing. Finally, the accreditation is certainly easier to obtain for an IHC test than for a test developed on an NGS platform.

Assuming that the implementation of the SP384 IHC assay in the testing algorithm of advanced and metastatic LUAD patients can be considered, following the results showed in the present work and probably other forthcoming studies, independent validation studies need to be performed. In addition, it will be of interest to further assess the performance of the SP384 clone on cytology samples such as cellblocks.[26, 27] Finally, as a perspective, the ROS1 IHC assay could be a good candidate to be associated with other “predictive” antibodies such as the anti-ALK, anti-BRAFV600E and anti-PD-L1 in a chromogenic multiplex IHC assay for small tissue biopsies.[28]

In conclusion, ROS1 IHC testing is an effective screening tool for the presence of *ROS1* rearrangements. However, users must be aware of the variable diagnostic performance of different commercialized anti-ROS1 antibodies before incorporation into the diagnostic molecular pathology workup of LUAD cases.

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**Conflict of interests:**

I.R. has received honoraria from AstraZeneca, MSD, Bristol-Myers Squibb, Boehringer-Ingelheim, Roche and Takeda.

J.M has received research grant and honoraria from Roche, AstraZeneca and Bristol-Myers Squibb, and honoraria from Boehringer-Ingelheim, MSD, PharmaMar, Pfizer, Takeda.

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The remaining authors have declared no conflict of interests.

## Legend to Figures

**Figure 1. Representative images of ROS1 IHC using the D4D6 and the SP384 antibodies in the training cohort. (A)** Strong diffuse staining with the SP384 antibody, and **(B)** weak to moderate diffuse staining with the D4D6 antibody in the same case as in (A). **(C)** Weak to moderate diffuse staining with the SP384 antibody, and **(D)** weak focal staining with the D4D6 antibody in the same case as in (C). A *ROS1* rearrangement was identified in both cases (A-B, and C-D) by FISH. (Scale bars are shown on the figures).

**Figure 2. Representative immunostaining localization in tumor cells using the SP384 antibody. (A)** Diffuse cytoplasmic. **(B)** Membranous and cytoplasmic. **(C)** Membranous. **(D)** Cytoplasmic granular. Scale bars are shown on the figures.

**Figure 3. Representative comparative images between the ROS1 IHC using the D4D6 and the SP384 antibodies and the ROS1 FISH analysis in the validation cohort.**

**(A)** Strong diffuse (3+) staining with the D4D6 and SP384 antibodies in a LUAD case positive by ROS1 FISH.

**(B)** Weak diffuse staining (1+) with the D4D6 antibody but strong staining (3+) with the SP384 clone in a LUAD case positive by ROS1 FISH.

**(C)** Weak diffuse staining (1+) with the D4D6 antibody and moderate staining (2+) with the SP384 clone in a LUAD case positive by ROS1 FISH.

**(D)** Lack of staining with both D4D6 and SP384 clones in a LUAD negative by ROS1 FISH.

**(E)** No staining with the D4D6 clone in a LUAD case moderately stained (2+) with SP384 clone and positive by ROS1 FISH.

**(F)** No staining with the D4D6 clone in a LUAD case strongly stained (3+) with the SP384 clone but revealed negative by ROS1 FISH.

Red circles; positive tumor cells by FISH; green circles, negative tumors cells by ROS1 FISH.

## Legend to Supplementary Figures

**Supplementary Figure S1.** Distribution plot of H-scores for each anti-ROS1 antibody.

**Supplementary Figure S2.** Correlation analysis between the H-scores of **(A)** D4D6 or **(B)** SP384 clones and the percentage of tumor cells harboring *ROS1* rearrangement by FISH. *Note: the X-axis in (A) is manually set to have a maximum of 300 to match with SP384.*

**Supplementary Figure S3.** Correlation analysis between response assessments by RECIST 1.1 after 3 months under crizotinib and the IHC status by D4D6 (paired Student's t-test; P=0.002) and SP384 (paired Student's t-test; P=0.006) clones. Abbreviations: Progressive Disease (PD), Stable Disease (SD), Partial Response (PR), Complete Response (CR).

**Table 1.** Comparative performance analyses between *ROS1* FISH and SP384 and D4D6 IHC clones.

| **Training cohort** | **SP384 IHC** | | | | **D4D6 IHC** | | | |
|---|---|---|---|---|---|---|---|---|
| **FISH ROS1** | **Strong** 3+ | **Moderate** 2+ | **Weak** 1+ | **Negative** 0 | **Strong** 3+ | **Moderate** 2+ | **Weak** 1+ | **Negative** 0 |
| Positive n = 51 | 40 (78%) | 10 (20%) | 1 (2%) | 0 (0%) | 15 (30%) | 16 (31%) | 20 (39%) | 0 (0%) |
| **Sensitivity** Cutoff ≥1+ Cutoff ≥2+ | 100% (95% CI, 93.02%–100%) 98.04% (95% CI, 89.55%–99.95% | | | | 100% (95% CI, 93.02%–100%) 60.78% (95% CI, 46.11%–74.16%) | | | |
| **Validation cohort** | **SP384 IHC** | | | | **D4D6 IHC** | | | |
| **FISH ROS1** | | | | | | | | |
| Positive n = 9 (1.3%) | 6 (67%) | 2 (22%) | 1 (11%) | 0 (0%) | 3 (33%) | 3 (33%) | 3 (33%) | 0 (0%) |
| Negative n = 705 (98.7%) | 0 (0%) | 63 (9%) | 26 (4%) | 616 (87%) | 0 (0%) | 3 (1%) | 12 (2%) | 690 (97%) |
| **Cutoff ≥1+** | | | | | | | | |
| Sensitivity | 100% (95% CI, 66.37%–100%) | | | | 100% (95% CI, 66.37%–100.00%) | | | |
| Specificity | 87.38% (95% CI, 84.70%–89.74% | | | | 97.87% (95% CI, 96.51–98.80%) | | | |
| PPV | 9.18% (95% CI, 7.69%–10.94%) | | | | 37.50% (95% CI, 26.67%–49.75%) | | | |
| NPV | 100% | | | | 100% | | | |
| Accuracy | 87.54% (95% CI, 84.89%–89.87%) | | | | 97.90% (95% CI, 96.56%–98.82%) | | | |
| **Cutoff ≥2+** | | | | | | | | |
| Sensitivity | 88.89% (95% CI, 51.75%–99.72%) | | | | 66.67% (95% CI, 29.93%–92.51%) | | | |
| Specificity | 91.06% (95% CI, 88.71%–93.06%) | | | | 99.57% (95% CI, 98.76%–99.91%) | | | |
| PPV | 11.27% (95% CI, 8.37%–15.01%) | | | | 66.67% (95% CI, 37.12%–87.14%) | | | |
| NPV | 99.84% (95% CI, 99.02%–99.98%) | | | | 99.57% (95% CI, 98.93%–99.83%) | | | |
| Accuracy | 91.04% (95% CI, 88.70%–93.03%) | | | | 99.16% (95% CI, 98.18%–99.69%) | | | |

Abbreviations: FISH, Fluorescence in situ hybridization; NPV, Negative Predictive Value; PPV, Positive Predictive Value.

**References**


1. Shaw AT, Felip E, Bauer TM, et al. Lorlatinib in non-small-cell lung cancer with ALK or ROS1 rearrangement: an international, multicentre, open-label, single-arm first-in-man phase 1 trial. *Lancet Oncol* 2017;18:1590-1599.
2. Li Z, Shen L, Ding D, et al. Efficacy of Crizotinib among Different Types of ROS1 Fusion Partners in Patients with ROS1-Rearranged Non-Small Cell Lung Cancer. *J Thorac Oncol* 2018;13:987-995.
3. Sgambato A, Casaluce F, Maione P, et al. Targeted therapies in non-small cell lung cancer: a focus on ALK/ROS1 tyrosine kinase inhibitors. *Expert Rev Anticancer Ther* 2018;18:71-80.
4. Shaw AT, Ou SH, Bang YJ, et al. Crizotinib in ROS1-rearranged non-small-cell lung cancer. *N Engl J Med* 2014;371:1963-1971.
5. Mazieres J, Zalcman G, Crino L, et al. Crizotinib therapy for advanced lung adenocarcinoma and a ROS1 rearrangement: results from the EUROS1 cohort. *J Clin Oncol* 2015;33:992-999.
6. Bubendorf L, Buttner R, Al-Dayel F, et al. Testing for ROS1 in non-small cell lung cancer: a review with recommendations. *Virchows Arch* 2016;469:489-503.
7. Jurmeister P, Lenze D, Berg E, et al. Parallel screening for ALK, MET and ROS1 alterations in non-small cell lung cancer with implications for daily routine testing. *Lung Cancer* 2015;87:122-129.
8. Lindeman NI, Cagle PT, Aisner DL, et al. Updated Molecular Testing Guideline for the Selection of Lung Cancer Patients for Treatment With Targeted Tyrosine Kinase Inhibitors: Guideline From the College of American Pathologists, the International Association for the Study of Lung Cancer, and the Association for Molecular Pathology. *J Thorac Oncol* 2018;13:323-358.
9. Davies KD, Doebele RC. Molecular pathways: ROS1 fusion proteins in cancer. *Clin Cancer Res* 2013;19:4040-4045.
10. Ilie M, Hofman P. The potential value of immunohistochemistry as a screening tool for oncogenic targets of personalized lung cancer therapy. *J Oncopathol* 2013;1:1.
11. Yoshida A, Tsuta K, Wakai S, et al. Immunohistochemical detection of ROS1 is useful for identifying ROS1 rearrangements in lung cancers. *Mod Pathol* 2014;27:711-720.

12. Sholl LM, Sun H, Butaney M, et al. ROS1 immunohistochemistry for detection of ROS1-rearranged lung adenocarcinomas. *Am J Surg Pathol* 2013;37:1441-1449.
13. Mescam-Mancini L, Lantuejoul S, Moro-Sibilot D, et al. On the relevance of a testing algorithm for the detection of ROS1-rearranged lung adenocarcinomas. *Lung Cancer* 2014;83:168-173.
14. Cha YJ, Lee JS, Kim HR, et al. Screening of ROS1 rearrangements in lung adenocarcinoma by immunohistochemistry and comparison with ALK rearrangements. *PLoS One* 2014;9:e103333.
15. Luk PP, Selinger CI, Mahar A, et al. Biomarkers for ALK and ROS1 in Lung Cancer: Immunohistochemistry and Fluorescent In Situ Hybridization. *Arch Pathol Lab Med* 2018.
16. Rogers TM, Russell PA, Wright G, et al. Comparison of methods in the detection of ALK and ROS1 rearrangements in lung cancer. *J Thorac Oncol* 2015;10:611-618.
17. Selinger CI, Li BT, Pavlakis N, et al. Screening for ROS1 gene rearrangements in non-small-cell lung cancers using immunohistochemistry with FISH confirmation is an effective method to identify this rare target. *Histopathology* 2017;70:402-411.
18. Davies KD, Le AT, Sheren J, et al. Comparison of Molecular Testing Modalities for Detection of ROS1 Rearrangements in a Cohort of Positive Patient Samples. *J Thorac Oncol* 2018;13:1474-1482.
19. Hanlon Newell A, Liu W, Bubendorf L, et al. MA26.07 ROS1 (SP384) Immunohistochemistry Inter-Reader Precision Between 12 Pathologists. *J Thorac Oncol* 2018;13:S452-S453.
20. McCoach CE, Le AT, Gowan K, et al. Resistance Mechanisms to Targeted Therapies in ROS1(+) and ALK(+) Non-small Cell Lung Cancer. *Clin Cancer Res* 2018;24:3334-3347.
21. Heeke S, Hofman P. Tumor mutational burden assessment as a predictive biomarker for immunotherapy in lung cancer patients: getting ready for prime-time or not? *Transl Lung Cancer Res* 2018;7:631-638.
22. Hellmann MD, Ciuleanu TE, Pluzanski A, et al. Nivolumab plus Ipilimumab in Lung Cancer with a High Tumor Mutational Burden. *N Engl J Med* 2018;378:2093-2104.

23. Rossi G, Jocolle G, Conti A, et al. Detection of ROS1 rearrangement in non-small cell lung cancer: current and future perspectives. *Lung Cancer (Auckl)* 2017;8:45-55.
24. Reguart N, Teixido C, Gimenez-Capitan A, et al. Identification of ALK, ROS1, and RET Fusions by a Multiplexed mRNA-Based Assay in Formalin-Fixed, Paraffin-Embedded Samples from Advanced Non-Small-Cell Lung Cancer Patients. *Clin Chem* 2017;63:751-760.
25. Hofman V, Lassalle S, Bence C, et al. Any Place for Immunohistochemistry within the Predictive Biomarkers of Treatment in Lung Cancer Patients? *Cancers (Basel)* 2018;10.
26. Lozano MD, Echeveste JI, Abengozar M, et al. Cytology Smears in the Era of Molecular Biomarkers in Non-Small Cell Lung Cancer: Doing More With Less. *Arch Pathol Lab Med* 2018;142:291-298.
27. Vlajnic T, Savic S, Barascud A, et al. Detection of ROS1-positive non-small cell lung cancer on cytological specimens using immunocytochemistry. *Cancer Cytopathol* 2018;126:421-429.
28. Ilie M, Beaulande M, Hamila M, et al. Automated chromogenic multiplexed immunohistochemistry assay for diagnosis and predictive biomarker testing in non-small cell lung cancer. *Lung Cancer* 2018;124:90-94.

# Figure 1

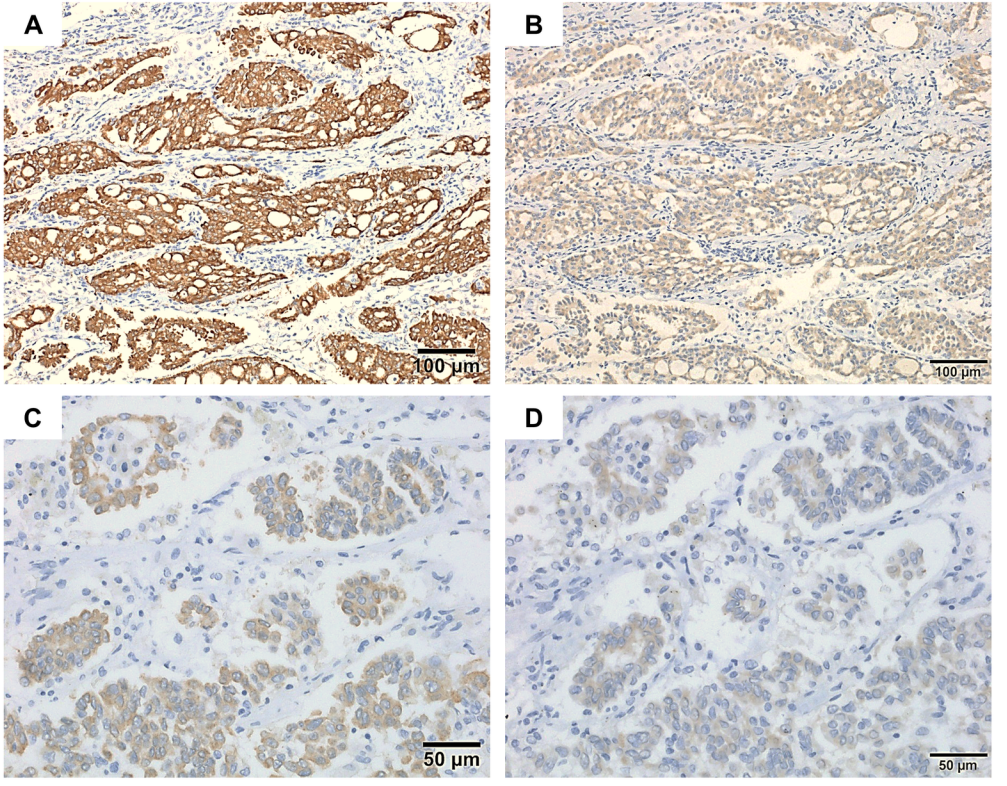

Figure 2

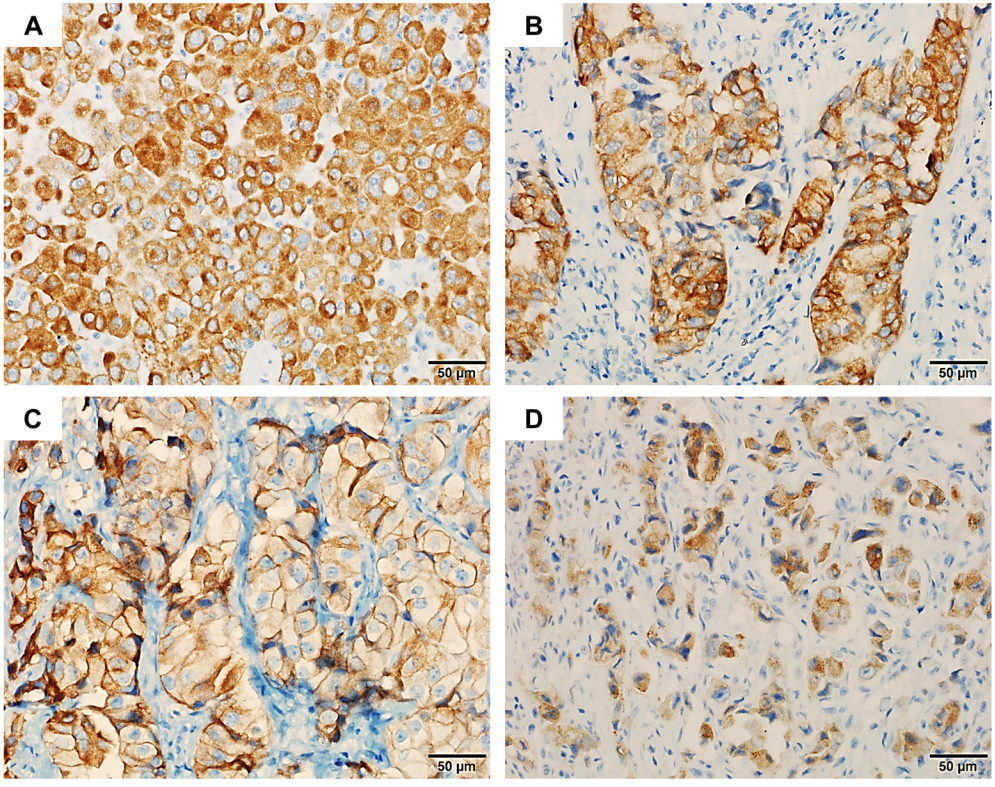

Figure 3