This guide is intended to provide healthcare professionals with information on testing for anaplastic lymphoma kinase (ALK) and ROS1 fusions in non-small cell lung cancer (NSCLC).
NSCLC biomarkers

In NSCLC, analysis of epidermal growth factor receptor (EGFR) mutations and ALK and ROS1 gene rearrangements is a prerequisite for determining the appropriate treatment to be used to help improve patient outcomes and survival.1–3

ALK and ROS1 in NSCLC

**ALK rearrangement**

The ALK protein, encoded by the ALK gene, is a transmembrane receptor tyrosine kinase (RTK) which, via ligand-dependent dimerisation, induces signalling linked to cell proliferation and survival.5,6 The natural ligand and consequently the normal function of ALK is not known, but expression patterns across mammalian species suggest a role in the development of the nervous system.5

In 3–5% of patients with NSCLC, ALK gene rearrangement occurs7,8 and provides an oncogenic driver in these tumours.9 The most common ALK rearrangements involve a fusion between the echinoderm microtubule-associated protein-like 4 (EML4) and ALK genes, and were first discovered in NSCLC specimens in 2007.9 The fusion gene is created by an inversion in chromosome 2p, resulting in fusion of the N-terminal portion of the EML4 gene with the sequence coding for the kinase domain of ALK (Figure 1).9 This results in replacement of the extracellular and transmembrane portions of the ALK protein with a coiled-coil portion of the EML4 protein.10


While the physiological ALK protein is located at the plasma membrane, the EML4-ALK fusion protein is localised to the cytoplasm9 and forms constitutive dimers via the coiled-coil portion of EML4.10 This leads to activation and persistent mitogenic signalling (Figure 2).10 This unregulated signalling induces cancer progression through its impact on cell-cycle progression, survival, proliferation and metastasis.6
Several EML4-ALK fusion variants have been identified, all of which contain the sequence coding for the kinase domain of ALK. Additionally, other fusion partners for ALK have been identified, such as TFG and KIF5B.

**ROS1 rearrangement**

ROS1 belongs to the human RTK family and shares a high degree of homology with ALK (Figure 3). The ROS1 gene is located on chromosome 6 and encodes an orphan transmembrane receptor protein with an extracellular N-terminal domain spanning more than 1800 amino acids, making it one of the largest extracellular domains among all human RTKs; a kinase domain and a single transmembrane domain are located within the C-terminal portion. No human ROS1 ligand has been found to date and the physiological function of this orphan receptor is currently unclear.

ROS1 gene rearrangement occurs in 1-2% of patients with NSCLC and is not thought to overlap with mutations in other oncogenic drivers such as ALK or EGFR. In lung cancer, ROS1 fusion partners include FIG, CD74, SLC34A2 and SDC4, with CD74-ROS1 being the most common. In all the known fusion genes, the ROS1 kinase domain is fully retained, with the ROS1 junction point at the mRNA level occurring at the 5’ end of exons 32, 34, 35 or 36 (Figure 4).
The exact mechanism by which ROS1 rearrangements lead to dysregulated kinase activity remains unclear. In contrast to ALK rearrangements, where the fusion partners provide a dimerisation domain that leads to the activation of the kinase, the majority of ROS1 partner proteins lack these domains (Figure 4). It has been suggested ROS1 rearrangements may promote signal transduction programmes which lead to the upregulation of SHP-1 and SHP-2 and activation of pathways commonly involved in promoting cell survival and proliferation.

Patients with tumours expressing ALK or ROS1 fusion proteins comprise distinct molecular subsets of patients with NSCLC for whom personalised treatment with tyrosine kinase inhibitors is indicated. Therefore, tumour specimens from patients with NSCLC should be tested for ALK and ROS1 rearrangements in order to inform treatment decisions.

General testing information

US National Comprehensive Cancer Network (NCCN) clinical guidelines recommend upfront, parallel testing for EGFR, ALK and ROS1 in patients with non-squamous NSCLC at diagnosis of advanced disease. Testing is also recommended for patients with squamous cell carcinoma and a never-/light-smoking history. International guideline recommendations on molecular testing are summarised in Table 1.

### Molecular testing of lung cancers

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Table 1. Current guidelines from the European Society for Medical Oncology and from the College of American Pathologists, the International Association for the Study of Lung Cancer and the Association for Molecular Pathology.
A recommended testing algorithm from an EU expert working group of pathologists is shown below (Figure 5).14

**Testing for ALK and ROS1**

Rapid diagnostic procedures and treatment decisions are essential for patients with advanced NSCLC; therefore, effective collaboration between oncologists and pathologists is key to ensuring all patients are tested at initial diagnosis for all relevant molecular markers.

Due to an increasing number of molecular tests that should be performed on NSCLC specimens, tissue sample size should be maximised whenever possible. Tissue handling, processing and sectioning should be standardised to minimise wastage and optimised for the staining procedures and molecular tests required for NSCLC. Histological and cytological specimens are both potentially suitable for ALK and ROS1 testing. If the initial tissue sample is small, three to four spare sections should be cut upfront to avoid tissue loss from recutting.25

ALK+ and ROS1+ NSCLC can be identified using fluorescence in-situ hybridisation (FISH), immunohistochemistry (IHC), reverse transcription-polymerase chain reaction (RT-PCR) and next-generation sequencing (NGS). Participating in external quality assessment programmes is recommended to verify that the selected test is performing as expected.26
Fluorescence *in-situ* hybridisation

**ALK**

FISH involves the hybridisation of a fluorophore-labelled single-stranded DNA probe which is complementary in sequence to the genetic region of interest. The fluorophore signal can be visualised using a fluorescence microscope.

In NSCLC specimens, ALK gene rearrangements can be detected using a break-apart FISH probe assay. The assay uses two fluorophore-labelled probes that flank the break point of the ALK gene, one on the 3’ segment (orange) and one on the 5’ segment (green).

**Positive for ALK**
- If rearrangement has occurred, nuclei will contain ‘broken apart’ orange and green signals, which appear separated by at least two signal diameters.
- If deletion has occurred, nuclei will contain single orange signals.

**Negative for ALK**
- If no activating rearrangement or deletion in the ALK gene locus has occurred, nuclei will contain fused orange and green signals (either overlapping, adjacent, or less than two signal diameters apart) or nuclei will contain single green signals, in addition to fused signals.

![Figure 6. FISH for the detection of ALK rearrangement](image)

**ROS1**

Similar to the principles used for ALK gene rearrangement testing, most FISH assays for ROS1 use a dual colour break-apart probe design which labels the 3’ (green) and 5’ (orange) segments of the fusion breakpoint.

**Negative for ROS1**
- If no rearrangement in the ROS1 gene locus has occurred, nuclei will contain fused orange and green signals.

**Positive for ROS1**
Two positive rearrangement patterns can occur:
- Typical: one fusion signal (native ROS1) and ‘broken apart’ green and orange signals are seen.
- Atypical: one fusion signal (native ROS1) and one green signal without the corresponding orange signal are seen.
A specimen is positive for ALK or ROS1 rearrangement if >25/50 tumour cells are judged positive. A specimen is negative for ALK or ROS1 rearrangement if <5/50 are judged positive. If there is uncertainty, a second count should be conducted and an average calculated. If the average proportion of positive cells is ≥15% (of 100 cells) the sample is considered positive.25

During analysis, only intact cells with non-overlapping nuclei should be scored. To optimise FISH results, use of sections older than 6 months is not advised as this may result in poor hybridisation.14

Immunohistochemistry

IHC detects the expression of ALK and ROS1 protein and is a valuable screening tool for testing NSCLC samples for ALK and ROS1 rearrangement.

ALK

One of the challenges with IHC is that even in ALK-rearranged NSCLC, ALK protein expression is relatively low. Standard detection methodology, as used in the identification of ALK-rearranged anaplastic lymphomas, is inadequate in NSCLC. Thus in NSCLC, primary anti-ALK antibodies are often used in conjunction with enhanced detection systems for signal amplification.25

The VENTANA ALK (D5F3) CDx assay is an FDA-approved companion diagnostic and CE-IVD-labelled ALK IHC assay.

According to the package insert:

- A specimen is **positive** for ALK if there is strong, granular, cytoplasmic, brown staining in the tumour cells (any percentage of positive tumour cells); staining is usually homogenous, with a uniform level of intensity throughout the neoplastic portions of the tumour.

- A specimen is **negative** for ALK in the absence of strong, granular, cytoplasmic staining in the tumour cells.

Figure 8. IHC for the detection of ALK rearrangement
Pathologists should be aware of various artefacts that may lead to false-positive ALK staining, including:

- light cytoplasmic stippling in alveolar macrophages
- cells of neuronal origin
- glandular epithelial staining
- cells with lymphocytic infiltrate
- normal mucosa in NSCLC (including mucin)
- necrotic tumour areas.

**ROS1**

IHC is an effective screening tool for ROS1+ NSCLC, with a sensitivity of 100% in most studies and a specificity of 92–100% using the ROS1 (D4D6) rabbit monoclonal antibody (Cell Signaling Technology, Danvers, MA, USA).14

- A specimen is **positive** for ROS1 when fine granular cytoplasmic staining is seen (Figure 9). However, the staining pattern may vary depending on the function and subcellular location of the gene fusion partner; for example, the CD74–ROS1 fusion is associated with globular staining, whereas the EZR–ROS1 fusion is associated with membranous staining.27, 28

Of note, false-positive staining may occur in:

- non-neoplastic hyperplastic type II pneumocytes where weak ROS1 expression can occasionally occur
- alveolar macrophages
- osteoclast-type giant cells in bone metastases where strong, granular cytoplasmic staining has been detected.

A positive control slide should be included with every ALK or ROS1 test staining run to confirm reagents are functioning properly and guard against false-negative results. A negative control slide should also be included, to check for background staining and confirm the absence of target antigen labelling.
Reverse transcription-polymerase chain reaction

ALK

A number of different RT-PCR-based approaches can be used to detect the presence of aberrant ALK mRNA transcripts. These methods are highly sensitive and specific, rapid and require relatively little sample material.

RT-PCR-based approaches for identifying ALK+ tumours include:

- assays to detect unbalanced expression of the 5’ and 3’ ALK mRNA regions\textsuperscript{29,30}
- multiplex PCR assays using primer pairs specific to known ALK fusion variants.\textsuperscript{31}

There are many EML4-ALK fusion variants and while the breakpoint of ALK is constantly located before the 5’ end of exon 20 where the coding sequence of the kinase domain starts, the breakpoint of EML4 has been observed in various exons. Therefore, multiplex primer sets should be designed to detect all the possible EML4-ALK variants. The AmoyDx EML4-ALK Fusion Gene Detection Kit is a CE-IVD-labelled RT-PCR assay to detect EML4-ALK fusion.

ROS1

RT-PCR has been successfully utilised to identify aberrant ROS1 mRNA transcripts with a sensitivity of 100% and a specificity of 85-100%, using FISH as the reference standard method.\textsuperscript{14,32,33}

RT-PCR-based approaches for identifying ROS1+ tumours include:

- multiplex PCR assays using primer sets specific to known ROS1 fusion variants\textsuperscript{34,35}
- assays which utilise a dual capture and reporter probe system to detect known ROS1 fusion gene transcripts.\textsuperscript{34,36}

The AmoyDx ROS1 Gene Fusions Detection Kit is a CE-IVD-labelled RT-PCR assay to detect 14 ROS1 gene fusions.

Next-generation sequencing

The rapid development of technologies for large-scale sequencing has facilitated high-throughput molecular analysis that offers various advantages over traditional sequencing, including the ability to fully sequence large numbers of genes in a single test and simultaneously detect a wide array of different genomic alterations, including gene fusions.

Several NGS strategies for detecting gene fusions have been developed, including hybrid-capture-based target enrichment, multiplex amplicon RNA massive parallel sequencing, personalised analysis of rearranged ends (PARE) and anchored multiplex PCR (AMP).\textsuperscript{37-40} A number of commercial NGS panels covering ALK and ROS1 gene fusions are available, including the Thermo Fisher Oncomine Fusion panel and the ArcherDx FusionPlex™ v2 panel.
External quality assessment

Laboratories conducting molecular testing of NSCLC specimens should consider participating in external quality assessment (EQA) programmes, which can help to ensure and enhance proficiency in molecular testing. Below is a list of websites of medical and pathology societies which conduct EQA programmes.

**ESP EQA**
http://lung.eqascheme.org

**Italian AIOM SIAPEC**
https://testbiomolecolari.it/tags/nsclc?destination=taxonomy/term/36

**German DGP/QUIP**
http://www.pathologie-dgp.de/pathologie/quip-qualitaetssicherung/alk-ish/

**UK NEQAS**
https://www.ukneqas-molgen.org.uk/molecular-pathology

**NordiQC**

**French AFAQAP**
https://www.afaqap.fr/search/node/ALK

**Spanish SEAP**
https://www.seap.es/modulo-de-calidad-alkanza

Useful resources and publications

[www.ALKTesting.org](http://www.ALKTesting.org)


Vysis ALK Break Apart FISH assay

ALK Break Apart FISH protocol for cytological specimens (courtesy of Professor Lukas Bubendorf, Institute of Pathology, University Hospital Basel, Switzerland)

VENTANA ALK (DSF3) CDx assay
http://www.ventana.com/product/1816?type=2326


AmoyDx ROS1 Gene Fusions Detection Kit

AmoyDx EML4-ALK Fusion Gene Detection Kit
References
