Chapter 8
Translocation Testing of Lung Cancer Biomarkers

Sanja Dacic

Specimen Requirements

Specimen requirements are common to FISH and other DNA-based molecular assays. Fresh, frozen, formalin-fixed paraffin embedded (FFPE), and alcohol-fixed specimens are suitable for analysis. The most common fixatives used in clinical practice are 10% neutral buffered formalin and 70% ethanol alcohol. Both result in great morphology preservation in addition to good DNA/RNA preservation. Unlike PCR-based assays, alcohol fixatives can be a problem for FISH assays. Most problematic fixatives include acidic solutions (Bouins), heavy metal fixatives (Zenker, B5), and bone decalcifying solutions that result in technically suboptimal assays and therefore should be avoided. Time of fixation may also have impact on the assay quality. Based on the experience with *HER2* testing, formalin fixation of 6–12 h is recommended for small biopsy specimens and 8–18 h for large surgical resection specimens [1]. Many laboratories can perform successful testing on rapidly process specimens with fixation time of only 4 h. Similarly, specimens fixed between 24 and 48 even 72 h can give optimal results. Cytology specimens are suitable for gene rearrangement testing. Most of the laboratories prefer cell blocks. Although smear preparations can be used as well, FISH analysis requires non-overlapping tumor cells which could be an issue with DAPI staining. Specimen adequacy for testing in terms of processing, fixation, and tumor cellularity should be determined by each laboratory based on their internal validation.

S. Dacic, MD, PhD
Department of Pathology, University of Pittsburgh Medical Center, Pittsburgh, PA, USA
e-mail: dacics@upmc.edu

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Other Technical Considerations

FFPE tissue sections used for FISH analysis are usually 4–5 microns thick and should be placed on the charged ("+") slides to prevent tissue detachment during specimen processing. Laboratories should decide what type of glass slides should be used, but several technical details should be taken into consideration. Slides with a heavy coating or slides designed for microdissection should not be used for FISH as they frequently result in a poor hybridization and tissue detachment, respectively.

FISH protocols include several steps including deparaffinization, pretreatment/target retrieval, probe and target DNA denaturation, hybridization, post-hybridization washes, detection, and interpretation. Probe and target DNA denaturation is a critical step that may require modifications depending on the tissue size and preservation, duration, and type of fixation. This is particularly true for small biopsy and cytology specimens. Time of digestions is critical and should be standardized to maintain nuclear morphology. Overdigested tissue samples frequently show chromatin artifactual "split signals" that could be interpreted as false-positive findings. Hybridization and washing steps should be standardized and protocols should be established for every probe and specimen type. Automated tissue processing and standardized commercially available tissue digestion kits can improve consistency of the FISH assay. Typically, FISH assays designed for detection of gene rearrangements take up to 2 days, although depending on the probe robustness same-day assay is possible. A variety of amplification steps are available for enhancing weak signals. Most of the probes today are of excellent quality and such steps are not necessary.

Type of Probes

Two main FISH assay strategies are used for detection of chromosomal translocations: fusion probes and break-apart probes. Fusion strategy uses two probes, one localizing centromeric to one chromosomal break point and the second probe localizing telomeric to the reciprocal break point. Translocation negative cells show separated or "split" signals (e.g., two green and two red signals) (Fig. 8.1). In contrast, translocation positive cells show a "fusion" signal (e.g., one yellow, one green, one red signal). Break-apart assay uses two probes localizing proximal and distal to

only one of the two breakpoints of interest. The two probes are in close proximity to one another, and therefore, normal cells show fusion signals only (Fig. 8.2). Translocation positive cells show at least one pair of split signal (e.g., one fusion, one green, one red). The disadvantage of break-apart probes is that the fusion partner is not identified.

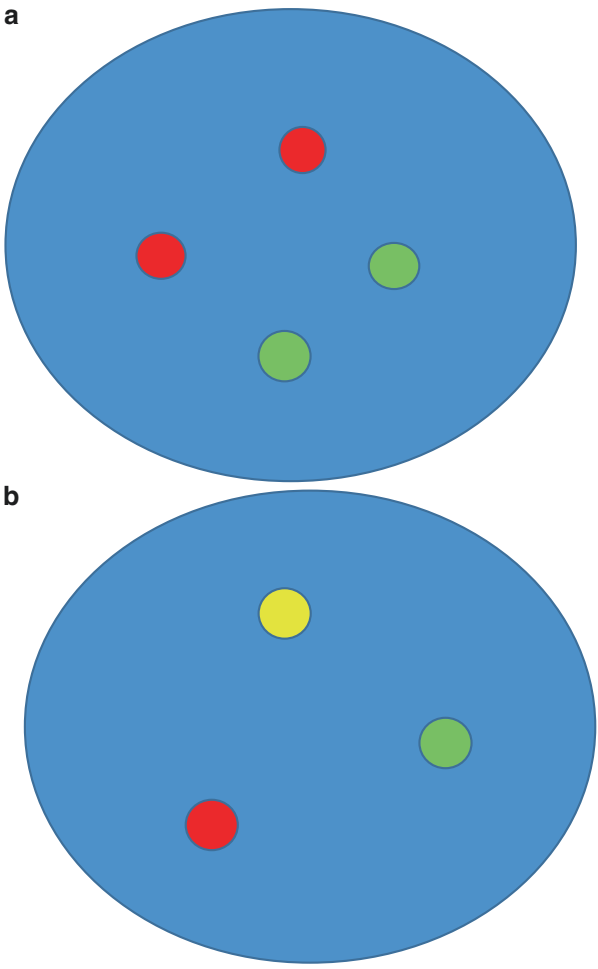

Fig. 8.1 (**a**) Fusion probe FISH assay negative for translocation ("split signal"). (**b**) Fusion probe FISH assay positive for translocation ("fusion signal")

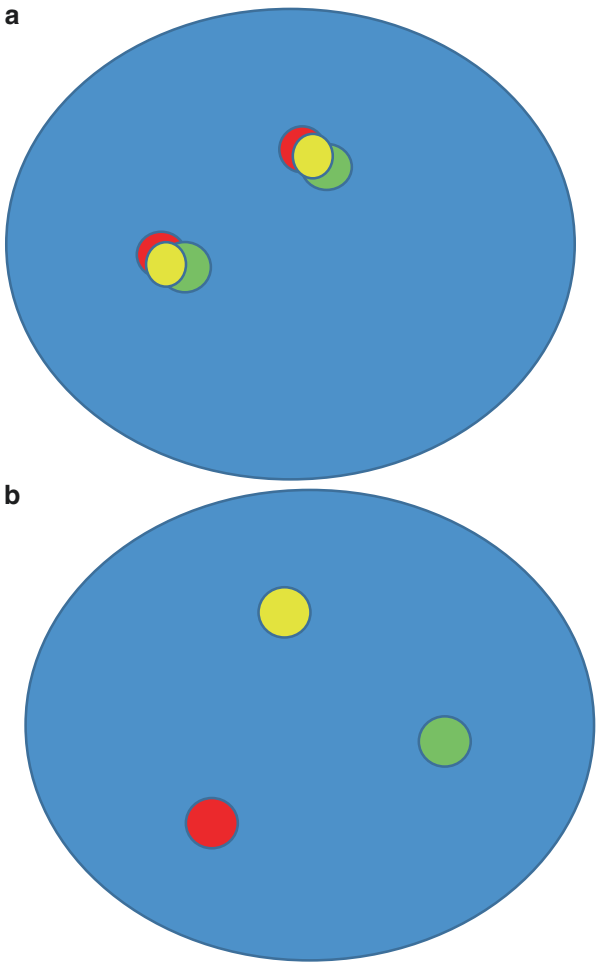

Fig. 8.2 (**a**) Break-apart probe FISH assay negative for translocation ("fusion signal"). (**b**) Break-apart probe FISH assay positive for translocation ("split signal")

Interpretation

The identification of non-overlapping cells/nuclei is the first step in the interpretation. It is essential that a pathologist identify adequate tumor areas on the routine H&E slide that is adjacent to section submitted for FISH analysis. An experienced cytotechnologist who has undergone specific FISH training in solid tumors should analyze the slides. Interpretation should be performed in areas of the slide with good signal, in which at least 50% of all nuclei are easily analyzable, with minimal background and autofluorescence. The FISH signal intensity should be consistently greater than background intensity.

Common Clinical Assays for Gene Rearrangements in Lung Cancer

ALK Gene Rearrangement

FISH Probe Design

The most common of *ALK* rearrangements involve a pericentric inversion on the short arm of chromosome 2, inv. [2] (p21p23), which creates a fusion gene encoding the amino-terminal portion of *EML4* (2p21) and the intracellular region of *ALK* (2p23), genes that are normally approximately 13 Mb apart [2]. Although the *EML4-ALK* fusion is the most common, other less common variant fusions have been reported, including translocations with other chromosomes (*KIF5B-ALK*, *TFG-ALK*) [3, 4]. The Vysis LSI *ALK* break-apart FISH probe kit (Abbott Molecular) was used to identify patients with *ALK* rearrangement positive NSCLC in the first clinical trials with ALK inhibitors, and therefore, the US Food and Drug Administration (FDA) approved this commercially available assay as a companion diagnostics for detection of *ALK* rearrangements in lung cancer [5]. The break-apart probe is designed by labeling the 3′(telomeric) 300-kb part fusion breakpoint with orange fluorochrome (Spectrum Orange, often referred as red) and the 5′ 442′-kb probe by a green signal (SpectrumGreen) (Fig. 8.3).

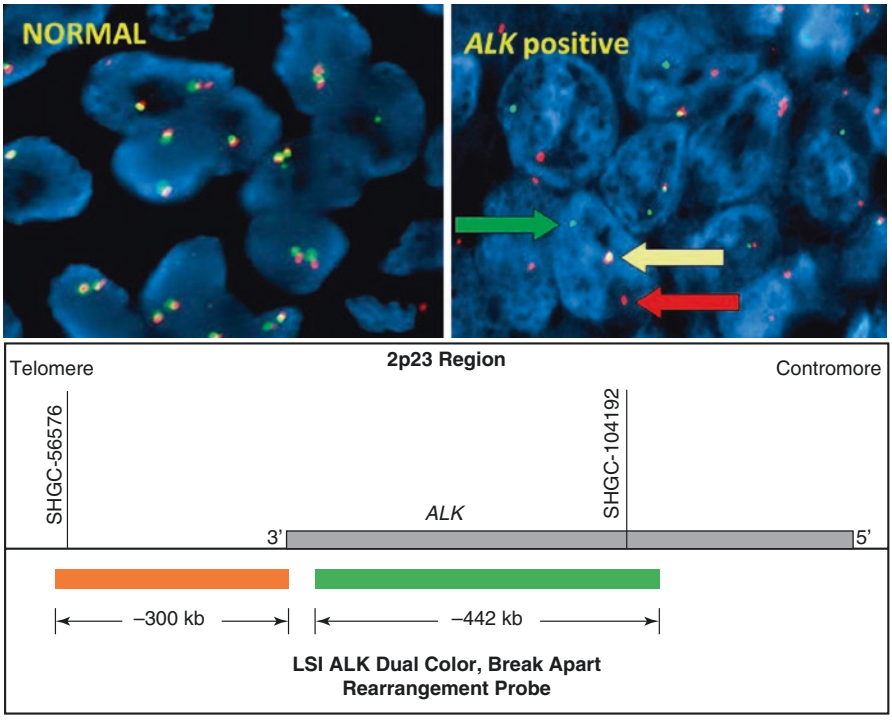

Fig. 8.3 Break-apart *ALK* FISH probe

Interpretation

The interpretation of *ALK* FISH assay may be challenging because the 5′ and 3′ probes are genetically very close [6–9]. Therefore, cells with normal pattern show fused signals. For the same reason in some cases split signal can be very narrow that the signals seems to be very close and fused in otherwise *ALK* rearranged cells. In the interpretation of *ALK* FISH, it is essential to pay attention to distance between the signals which should measure at least two signal diameters in cases positive for translocation. Any distance that is less than two signal diameter is considered to represent lack of gene rearrangement. A minimum of 50 tumor cells should be scored if there is one scorer, and a minimum of 100 tumor cells is needed if there are two scorers.

The assay is considered to be positive for *ALK* rearrangement if at least 15% of tumor cells show rearrangement. FISH patterns that are considered to represent gene rearrangement include split pattern and isolated 3′ pattern (Fig. 8.4). The number of accompanied fused 5′-3′ signals in the cell is not important for pattern classification. Isolated 5′ pattern may also be identified and is considered to represent nonfunctional reciprocal fusion product. Although this pattern has been reported to be associated with a rare *BIRC6-ALK* fusion, it should not be interpreted as rearrangement positive [10, 11]. FISH criteria particularly in respect to isolated 3′

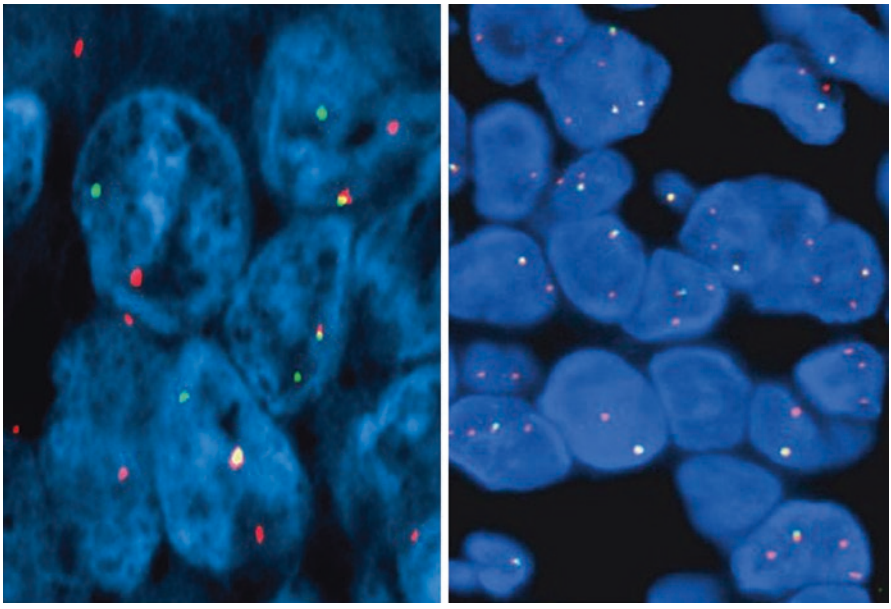

Fig. 8.4 Break-apart *ALK* FISH translocation patterns

("single orange") have been recently challenged [12, 13]. Cases classified as rearrangement positive based on isolated 3′ showed a higher rate of fusion negative cases by NGS and IHC than the group with a FISH split signal indicating that these cases may be FISH false positive [13, 14]. Isolated 3′ may be a result of technical factors such as nuclear sectioning causing loss of the 5′ (green) probe binding site, or simply observer error. Technical errors can't be reliably excluded in a case with a lower percentage of nuclei positive for rearrangement. Overall, cases with atypical signal patterns should be tested by another method such as IHC or NGS. RT-PCR may also be considered if the assay is designed to cover a large number of known fusions [15–20].

Another major source for false interpretations is the cases in which the rate of rearrangement positive cells falls within the range of 10–20%. In those cases, it is essential to recount the tumor cells and to perform a different assay as indicated above.

ALK false-negative results may also occur and are most likely caused by the complex gene rearrangements and cryptic insertions [14, 21, 22]. Recently, Wiesner et al. identified a novel *ALK* transcript, ALK^{ATI}, which arises independently of genomic aberrations at the *ALK* locus through alternative transcription initiation and which can be detected by ALK IHC, but not FISH [23]. Preliminary data showed that the patients with ALK^{ATI} may benefit from ALK inhibitors.

ALK RT-PCR

RT-PCR for detection of *ALK* rearrangements provides detailed information about *ALK* fusion partners. The risk of false-negative results and high failure rate for RNA-based assays on FFPE tissue samples make implementation of this assay in clinical practice difficult. In addition, this assay is designed to detect only known fusion partners and fusions with unknown partners would remain undetectable [1, 3, 4, 18, 24, 25].

Other Gene Rearrangements

The other most commonly identified gene fusions identified in lung carcinoma include genes *ROS1*, *RET*, *NTRK1*, and *NRG1*. Similar to *ALK*, break-apart FISH assay is currently the most commonly used method for detection of *ROS1* fusions (Fig. 8.5) [26–28]. *RET* rearrangement is also detectable by FISH or RT-PCR [26, 29–34]. Targeted NGS identified *NTRK1* rearrangements resulting in oncogenic fusion products *MPRIP-NTRK1* and *CD74-NTRK1* [35]. Whole transcriptome sequencing identified *CD74-NRG1* fusion that was also identified by RT-PCR [36].

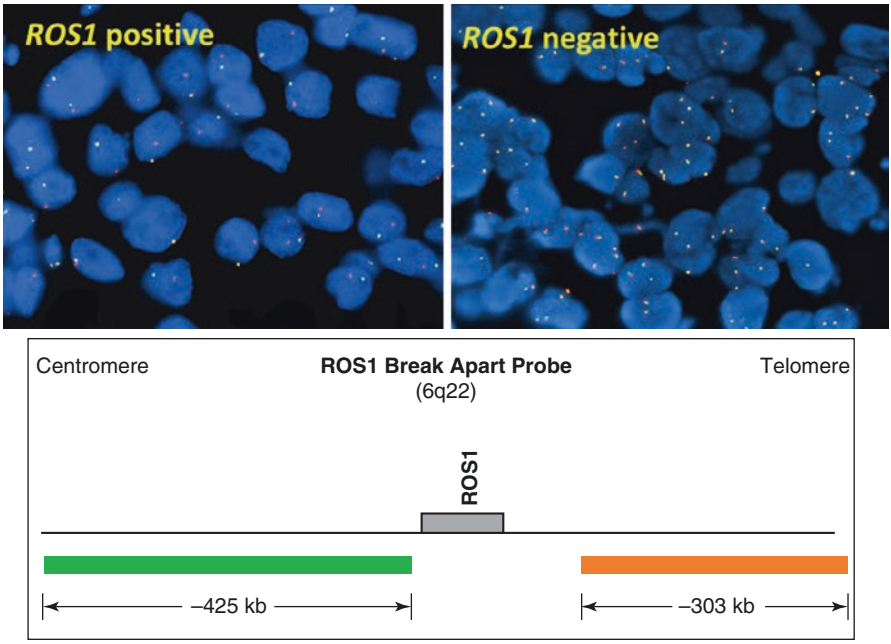

Fig. 8.5 Break-apart *ROS1* FISH probe

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