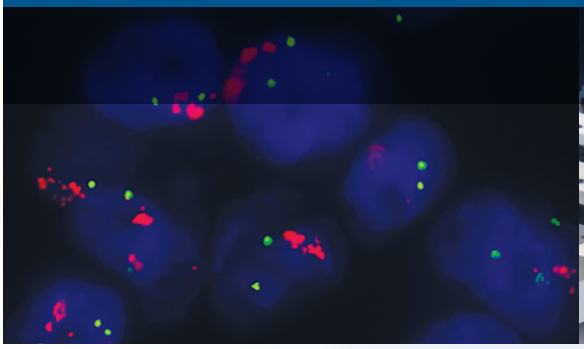
Agilent
Dako

IQFISH Interpretation Guide

ALK, ROS1 and RET IQFISH probes (Dako Omnis)
MET IQFISH probe (Dako Omnis)



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ALK, ROS1, RET and MET IQFISH probes (Dako Omnis)

The purpose of this interpretation guide is to assist in visualizing and analyzing lung tissue FFPE samples that have been hybridized using Agilent's ALK, ROS1, RET, or MET IQFISH probes on the Dako Omnis. This guide covers microscope setup, tissue selection and qualification, signal pattern evaluation, and troubleshooting.

This guide is not intended to aid in the clinical interpretation of any test results. The clinical interpretation should be evaluated within the context of the patient's medical history and other diagnostic laboratory test results by qualified personnel. It is recommended to run control slides concurrently with patient slides, using the same protocol, to monitor assay performance and to assess the accuracy of signal enumeration.

Intended Use

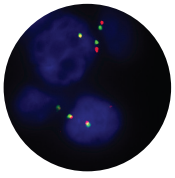
ALK IQFISH Break-Apart Probe (Dako Omnis) is intended for the detection of rearrangements involving the ALK gene by fluorescence in situ hybridization (FISH). The dual-color probe for Dako Omnis is recommended to be used on formalin-fixed, paraffin-embedded (FFPE) lung tissue specimens. (PN: G111800-2)

ROS1 IQFISH Break-Apart Probe (Dako Omnis) is intended for the detection of rearrangements involving the ROS1 gene by fluorescence in situ hybridization (FISH). The dual-color probe for Dako Omnis is recommended to be used on formalin-fixed, paraffin-embedded (FFPE) lung tissue specimens. (PN: G111801-2)

RET IQFISH Break-Apart Probe (Dako Omnis) is intended for the detection of rearrangements involving the RET gene by fluorescence in situ hybridization (FISH). The dual-color probe for Dako Omnis is recommended to be used on formalin-fixed, paraffin-embedded (FFPE) lung tissue specimens. (PN: G111802-2)

MET IQFISH Probe with CEP7 (Dako Omnis) is intended for the detection of the MET gene amplification by fluorescence in situ hybridization (FISH). The dual-color probe for Dako Omnis is recommended to be used on formalin-fixed, paraffin-embedded (FFPE) lung tissue specimens. (PN: G111803-2)

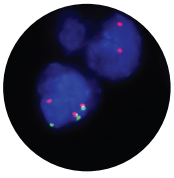
ALK IQFISH Break-Apart Probe (Dako Omnis)



FFPE sample stained with ALK IQFISH dual color, break-apart probe.

Cells show ALK gene rearrangement.

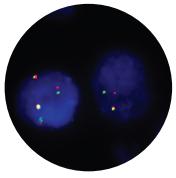
ROS1 IQFISH Break-Apart Probe (Dako Omnis)



FFPE sample stained with ROS1 IQFISH dual color, break-apart probe

Cells show ROS1 gene rearrangement.

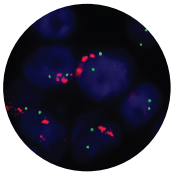
RET IQFISH Break-Apart Probe (Dako Omnis)



FFPE sample stained with RET IQFISH dual color, break-apart probe

Cells show RET gene rearrangement.

MET IQFISH Probe with CEP7 (Dako Omnis)



FFPE sample stained with MET/ CEP7 IQFISH amplification probe

Cells show MET gene amplification.

Filter Recommendation

Filters are individually designed for specific fluorochromes and for each microscope.

For the interpretation of IQFISH staining assays, the following combination of filters should be used:

- Specific DAPI filter
- High-quality Cy3/FITC double filter (alternatively specific Cy3 and FITC single filters)

Fluorochrome	Excitation Wavelength	Emission Wavelength
FITC	495 nm	517 nm
Cy3	547 nm	565 nm

Microscope Objective Recommendation

For identifying the relevant tissue/regions for scoring, an oil-immersion 20x objective with a minimum numerical aperture (N.A.) of 0.75 is recommended.

For scoring signal patterns, an oil-immersion 60x or 100x objective with a numerical aperture (N.A.) of 1.4 is recommended.

Scoring Guide

Assessable tissue

- Tissue specimen should be evaluated by a pathologist to identify relevant tumor areas to be scored.
- Avoid areas of heavy inflammation, necrosis and areas where the nuclear boundaries are ambiguous.
- Disregard nuclei with weak signal intensity and non-specific or high background staining.

Quality Control

- Signals must be bright, distinct and easy to evaluate.
- Normal cells allow for an internal control of the staining run.
  - Normal cells should have 1-2 clearly visible green signals and 1-2 clearly visible orange-red signals, indicating that the DNA probes have successfully hybridized to the target region.
  - Due to tissue sectioning, some normal cells will have less than the expected 2 signals of each color.
  - Normal cells undergoing cell division may have more than the normal 1-2 signals of each color.
  - Failure to detect signals in normal cells indicates assay failure, and results should be considered invalid.
- Nuclear morphology must be intact when evaluated using a DAPI filter. Numerous ghost-like cells and a general poor nuclear morphology indicate overdigestion of the specimen, resulting in loss or fragmentation of signals.
  Such specimens should be considered invalid.
- Differences in tissue fixation, processing, and embedding in the user's laboratory may produce variability in results, necessitating regular evaluation of in-house controls.

ALK, ROS1 and RET IQFISH Evaluation

Signal location

- Locate tumor cells on H&E-stained slide. Evaluate the same area on the IQFISH-stained slide.
- Scan several areas of tumor cells to account for possible heterogeneity.
- Select distinct tumor areas for assessment.
- Begin analysis in upper left quadrant of selected area. Scan from left to right, assessing signal pattern in each tumor nucleus.

Signal enumeration

- A minimum of 100 nuclei should be scored per sample.
- The ALK, ROS1, and RET break-apart FISH probes enable detection of gene rearrangements through visible separation of orange-red and green fluorescent signals. (See counting guide.)
  - In nuclei with no rearrangement, orange-red and green signals will overlap or be separated by a distance equal to or less than the diameter of one focus. Typically, a yellow signal is observed in these cases.
  - In nuclei harboring a rearrangement, orange-red and green signals will be separate or one of the signals will be absent.
- Two signals of the same size and color, separated by a distance equal to or less than the diameter of one signal, are counted as one signal.

  Note: For ALK and RET, rearrangement by chromosome inversion is common and can result in a relatively small signal separation distance, so careful attention should be paid when evaluating signal distances.
- Nuclei exhibiting signals of only one color should not be scored.
- Do not score nuclei demonstrating overdigestion.
- Adjust microscope focus to locate all signals in the individual nuclei.

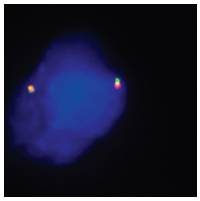
Probe sensitivity and specificity

Sensitivity and specificity were measured for the probes by evaluating the signal pattern of 100 nuclei in nine non-rearranged NSCLC FFPE samples. Because the samples are non-rearranged, only the fusion signal pattern is expected. The fraction of nuclei having the expected signal pattern ranged between 97-100% for the ALK, ROS1, and RET probes.

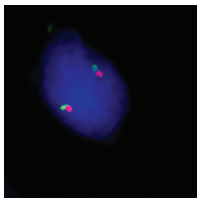
This sensitivity and specificity results in a calculated normal cutoff value of <15%. The normal cutoff value is the maximum number of rearranged signal patterns expected in a sample that does not harbor a rearrangement. Therefore, if a sample scoring gives a fraction of rearranged nuclei higher than 15%, follow up testing to confirm the rearrangement should be performed.

Note: Having a fraction of rearranged nuclei of less than 15% does not mean the sample does not contain a rearrangement, but these tests are not sensitive enough to detect it.

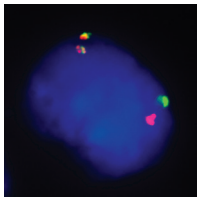
Break-apart Counting Guide



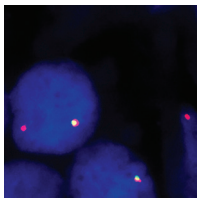
In nuclei with no rearrangement, orange-red and green signals will overlap or be within two foci distance apart. Typically, a yellow signal is observed in these cases.



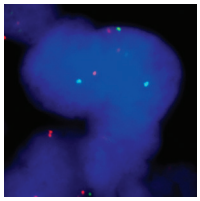
In nuclei with no rearrangement, orange-red and green signals will overlap or be separated by a distance equal to or less than the diameter of one focus.



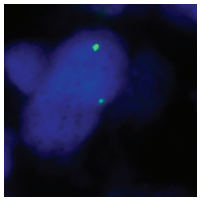
In nuclei harboring a rearrangement, orange-red and green signals will be separate.



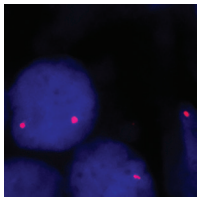
In nuclei harboring a rearrangement, orange-red and green signals will be separate. In this case, the green signal is lost, indicating a rearrangement.



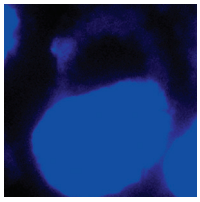
Do not score (nuclei are overlapping, not all areas of nuclei are visible).



Do not score nuclei with only green signals.



Do not score nuclei with only orange-red signals.



Do not score (overdigested nuclei).

MET IQFISH Evaluation

Signal location

- Locate tumor cells on H&E-stained slide. Evaluate the same area on the IQFISH-stained slide.
- Scan several areas of tumor cells to account for possible heterogeneity.
- Select distinct tumor areas for assessment.
- Begin analysis in upper left quadrant of selected area. Scan from left to right, assessing signal pattern in each tumor nucleus.

Signal enumeration

- Count MET (orange-red) and CEP7 (green) signals in representative tumor areas. (See counting guide.)
- Calculate the MET/CEP7 ratio by dividing the total number of MET signals by the total number of CEP7 signals.
- Two signals of the same size, separated by a distance equal to or less than the diameter of one signal, are counted as one signal.
- Nuclei with high levels of MET (orange-red) gene amplification may exhibit formation of signal clusters. Estimate the MET signal number. MET clusters may obscure CEP7 (green) signals. You can check this using a specific FITC filter.
- Nuclei exhibiting signals of only one color should not be scored.
- Do not score nuclei demonstrating overdigestion.
- Adjust microscope focus to locate all signals in the individual nuclei.

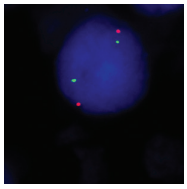
Probe sensitivity and specificity

Sensitivity and specificity were measured for the probe by evaluating the ratio of MET to CEP7 probe signal for 100 nuclei in nine NSCLC FFPE samples that lacked MET amplification. The ratio of MET to CEP7 signals in these samples is expected to be <2. The fraction of nuclei having this expected ratio ranged between 97-100%.

This sensitivity and specificity results in a calculated normal cutoff value of <15%. The normal cutoff value is the maximum number of nuclei expected to have a MET to CEP7 ratio >2 in a sample that does not harbor a MET amplification. Therefore, if a sample scoring gives a fraction nuclei with ratio >2 that is higher than 15% follow up testing to confirm the MET amplification should be performed.

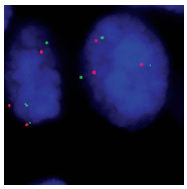
Note: Having a fraction of MET amplified nuclei of less than 15% does not mean the sample does not contain a MET amplification, but this test is not sensitive enough to detect it.

MET Counting Guide



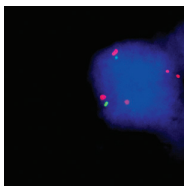
Two green signals indicates the presence of two copies of chromosome 7.
Two orange-red signals indicate the presence of two copies of the MET gene.

The ratio of MET to CEP7 is 2/2 = 1; non-amplified.



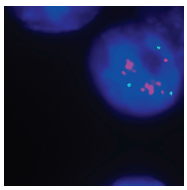
Three green signals indicate the presence of three copies of chromosome 7.
Three orange-red signals indicate the presence of three copies of the MET gene.

The ratio of MET to CEP7 is 3/3 = 1; non-amplified.



Two green signals indicate the presence of two copies of chromosome 7.
Five orange-red signals indicate the presence of five copies of the MET gene.

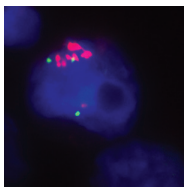
The ratio of MET to CEP7 is 5/2 = 2.5; amplified.



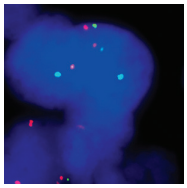
Three green signals indicate the presence of three copies of chromosome 7.

Approximately 12 orange-red signals indicate the presence of 12 copies of the MET gene (cluster estimation).

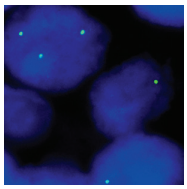
The ratio of MET to CEP7 is 12/3 = 4; amplified.



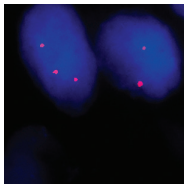
Cluster of orange-red signals hiding green signals. Check the green signals with a specific FITC filter, or do not score.



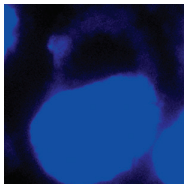
Do not score (nuclei are overlapping, not all areas of nuclei are visible).



Do not score nuclei with only green signals.



Do not score nuclei with only orange-red signals.



Do not score (overdigested nuclei).

Troubleshooting

Agilent Dako

Problem	Probable Cause	Suggested Action
1. No signals or weak signals	1a. Reagents have been exposed to high temperatures during transport or storage	1a. Check storage conditions. Ensure that dry ice was present when the IQFISH probe mix and pepsin shipments were received. Ensure that the reagents have been stored as specified.
	1b. Microscope not functioning properly - Inappropriate filter set - Improper lamp - Mercury lamp too old - Dirty and/or cracked collector lenses - Unsuitable immersion oil	1b. Check the microscope and ensure that the used filters are suitable for use with the probe mix fluorochromes, and that the mercury lamp is correct and has not been used beyond expected lifetime. In case of doubt, please contact your local microscope vendor.
	1c. Faded signals	1c. Avoid long microscopic examination and minimize exposure to strong light sources.
	1d. Buffers identified incorrectly	1d. Replace bulk buffers and ensure correct registration (at Workstation) and identification (at Touch Screen) of bulk buffers. Consult Dako Omnis Basic User Guide for further details. Please note: ISH Pre-Treatment Solution is green and ISH Stringent Wash Buffer is yellow.
	1e. Incomplete mixing of probe prior to loading onto the Omnis	1e. Inspect probe prior to loading onto the Omnis to ensure homogeneity. A gradient of pink color in the vial indicates incomplete mixing. Ensure mixing device is working properly.
2. Areas without signal	2. Air bubbles caught during mounting	2. Avoid air bubbles. If observed, gently tap them away using forceps
3. Excessive background staining	3a. Inappropriate tissue fixation	3a. Ensure that only formalin-fixed, paraffin-embedded tissue sections are used.
	3b. Prolonged exposure of hybridized section to strong light	3b. Avoid long microscopic examination and minimize exposure to strong light.
4. Poor tissue morphology	4a. Incorrect pepsin treatment	4a. Adjust pepsin incubation time. Please note: Users that deviate from the recommended incubation time (30 minutes) must validate the appropriateness of the resulting stain. It is the responsibility of a qualified pathologist, familiar with the ISH probes, and methods used, to interpret the stain.
	4b. Too long pepsin treatment or very thin section thickness may cause ghost cells or donut cells to appear	4b. Attempt a staining protocol with shorter pepsin incubation time. The recommended section thickness is 4-6 µm.
5. High level of green auto fluorescence on slide including areas without FFPE tissue	5. Use of expired or non-recommended glass slides	5. Use Dako Flex or Superfrost Plus slides. Ensure that the glass slides have not passed the expiration date.

IQFISH Probes are CE marked under the European In Vitro Diagnostic Directive (99/79/EC). These products are not approved for sale in the U.S.

This information is subject to change without notice.

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