ZYTOVISION

FlexISH

NTRK1/NTRK3 DistinguISH Probe

REF	Z-2314-50	Σ	5 (0.05 ml)
REF	Z-2314-200	Σ	20 (0.2 ml)

For the detection of translocations involving the human NTRK1 gene at 1q23.1 and the human NTRK3 gene at 15q25.3 by fluorescence *in situ* hybridization (FISH)

CE IVD

In vitro diagnostic medical device
according to EU directive 98/79/EC

1. Intended use

The FlexISH NTRK1/NTRK3 DistinguISH Probe (**PL268**) is intended to be used for the detection of translocations involving the human NTRK1 gene at 1q23.1 and the human NTRK3 gene at 15q25.3 in formalin-fixed, paraffin-embedded specimens by fluorescence *in situ* hybridization (FISH). The probe is intended to be used in combination with the FlexISH-Tissue Implementation Kit (Prod. No. Z-2182-5/-20).

Interpretation of the results must be made within the context of the patient´s clinical history with respect to further clinical and pathologic data of the patient by a qualified pathologist.

2. Clinical relevance

The neurotrophic tyrosine receptor kinase genes (NTRK1, NTRK2, and NTRK3) encode a family of receptor tyrosine kinases that serve important roles in cell survival, proliferation, and cellular differentiation in healthy human cells. The tumor types in which NTRK gene fusions have been detected are diverse, and include, e.g., breast cancer, non-small cell lung cancer, sarcoma, melanoma, and thyroid carcinoma. NTRK gene rearrangements result in the fusion of the 3' end of the NTRK gene, encoding the NTRK kinase domain, with the 5' end of various activating genes. The product of the fusion is a chimeric oncoprotein characterized by ligand-independent constitutive activation of the NTRK kinase. The treatment of patients with NTRK fusion-positive cancers with a NTRK inhibitor, such as the FDA-approved drugs larotrectinib or entrectinib, is associated with high response rates, regardless of NTRK gene, fusion partner, and tumor type. Hence, detection of NTRK1 and NTRK3 rearrangements by FISH may be of therapeutic significance.

3. Test principle

The fluorescence *in situ* hybridization (FISH) technique allows for the detection and visualization of specific nucleic acid sequences in cell preparations. Fluorescently-labeled DNA fragments, so called FISH probes, and their complementary target DNA strands in the preparations are co-denatured and subsequently allowed to anneal during hybridization. Afterwards, unspecific and unbound probe fragments are removed by stringency washing steps. After counterstaining the DNA with DAPI, hybridized probe fragments are visualized using a fluorescence microscope equipped with excitation and emission filters specific for the fluorochromes with which the FISH probe fragments have been directly labeled.

4. Reagents provided

The FlexISH NTRK1/NTRK3 DistinguISH Probe is composed of:

- ZyGreen (excitation 503 nm/emission 528 nm) labeled polynucleotides (~10.0 ng/µl), which target sequences mapping in 1q22-q23.1* (chr1:156,245,849-156,781,745) proximal to the NTRK1 breakpoint region and in 15q25.3-q26.1* (chr15:88,825,346-89,475,889) distal to the NTRK3 breakpoint region (see Fig. 1 & Fig. 2).
- ZyOrange (excitation 547 nm/emission 572 nm) labeled polynucleotides (~2.5 ng/µl), which target sequences mapping in 1q23.1* (chr1:156,854,527-157,296,918) distal to the NTRK1 breakpoint region and in 15q25.3* (chr15:87,976,717-88,471,002) proximal to the NTRK3 breakpoint region (see Fig. 1 & Fig. 2).
- ZyBlue (excitation 418 nm/emission 467 nm) labeled polynucleotides, (~70.0 ng/µl), which target sequences mapping in 15q25.3-q26.1* (chr15:87,845,459-89,475,889) harboring the NTRK3 gene region (see Fig. 2.)
- Formamide based hybridization buffer

*according to Human Genome Assembly GRCh37/hg19

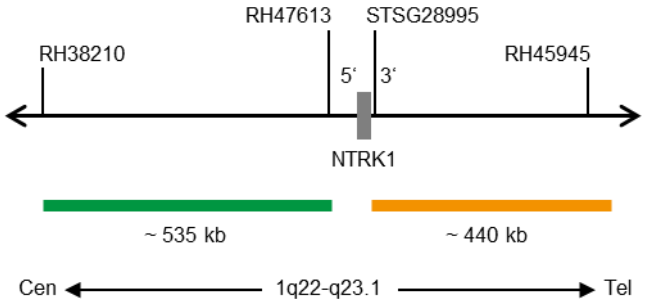

Fig. 1: NTRK1 Probe map (not to scale)

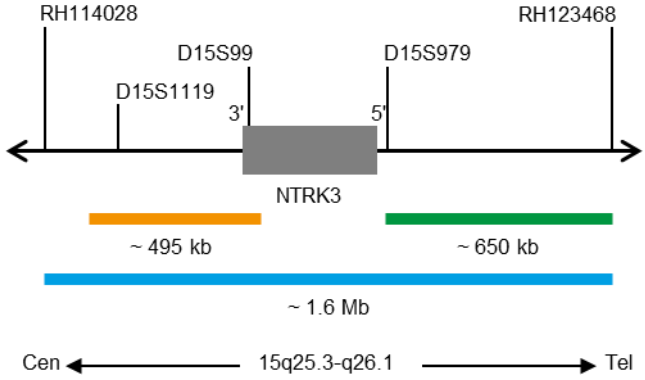

Fig. 2: NTRK3 Probe map (not to scale)

The FlexISH NTRK1/NTRK3 DistinguISH Probe is available in two sizes:

- Z-2314-50: 0.05 ml (5 reactions of 10 µl each)
- Z-2314-200: 0.2 ml (20 reactions of 10 µl each)

5. Materials required but not provided

- FlexISH-Tissue Implementation Kit (Prod. No. Z-2182-5/-20)
- Positive and negative control specimens
- Microscope slides, positively charged
- Water bath (37°C, 98°C)
- Hot plate or hybridizer
- Hybridizer or humidity chamber in hybridization oven
- Adjustable pipettes (10 µl, 25 µl)
- Staining jars or baths
- Timer
- Calibrated thermometer
- Ethanol or reagent alcohol
- Xylene
- Deionized or distilled water
- Coverslips (22 mm x 22 mm, 24 mm x 60 mm)
- Rubber cement, e.g., Fixogum Rubber Cement (Prod. No. E-4005-50/-125) or similar
- Adequately maintained fluorescence microscope (400-1000x)
- Immersion oil approved for fluorescence microscopy
- Appropriate filter sets

6. Storage and handling

Store at 2-8°C in an upright position protected from light.
Use protected from light. Return to storage conditions immediately after use. Do not use reagents beyond expiration date indicated on the label. The device is stable until expiration date indicated on the label when handled accordingly.

7. Warnings and precautions

- The probe should not be exposed to light, especially strong light, for a longer period of time, i.e. all steps should be accomplished, where possible, in the dark and/or using lightproof containers!
- Read the instruction for use prior to use!
- Do not use the reagents after the expiry date has been reached!
- This product contains substances (in low concentrations and volumes) that are harmful to health and potentially infectious. Avoid any direct contact with the reagents. Take appropriate protective measures (use disposable gloves, protective glasses, and lab garments)!
- If reagents come into contact with skin, rinse skin immediately with copious quantities of water!
- A material safety data sheet is available on request for the professional user.
- Do not reuse reagents.
- Avoid cross-contamination of samples as this may lead to erroneous results.

Hazard and precautionary statements:

The hazard determining component is Formamide.

Danger

H319	Causes serious eye irritation.
H351	Suspected of causing cancer.
H360FD	May damage fertility. May damage the unborn child.
H373	May cause damage to organs through prolonged or repeated exposure.
P201	Obtain special instructions before use.
P260	Do not breathe dust/fume/gas/mist/vapours/spray.
P280	Wear protective gloves/protective clothing/eye protection/face protection.
P305+P351+P338	IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P308+P313	IF exposed or concerned: Get medical advice/attention.
P337+P313	If eye irritation persists: Get medical advice/attention.

8. Limitations

- For *in vitro* diagnostic use.
- For professional use only.
- The clinical interpretation of any positive staining, or its absence, must be done within the context of clinical history, morphology, other histopathological criteria as well as other diagnostic tests. It is the responsibility of a qualified pathologist to be familiar with the FISH probes, reagents, diagnostic panels, and methods used to produce the stained preparation. Staining must be performed in a certified, licensed laboratory under the supervision of a pathologist who is responsible for reviewing the stained slides and assuring the adequacy of positive and negative controls.
- Specimen staining, especially signal intensity and background staining, is dependent on the handling and processing of the specimen prior to staining. Improper fixation, freezing, thawing, washing, drying, heating, sectioning, or contamination with other specimens or fluids may produce artefacts or false results. Inconsistent results may result from variations in fixation and embedding methods, as well as from inherent irregularities within the specimen.
- The probe should be used only for detecting loci described in 4. "Reagents provided".
- The performance was validated using the procedures described in this instruction for use. Modifications to these procedures might alter the performance and have to be validated by the user.

9. Interfering substances

Red blood cells present in the specimen might exhibit autofluorescence which hinders signal recognition.

The following fixatives are incompatible with ISH:

- Bouin's fixative
- B5 fixative
- Acidic fixatives (e.g., picric acid)
- Zenker's fixative
- Alcohols (when used alone)
- Mercuric chloride
- Formaldehyde/zinc fixative
- Hollande's fixative
- Non-buffered formalin

10. Preparation of specimens

Prepare specimens as described in the instructions for use of the FlexISH-Tissue Implementation Kit.

11. Preparatory treatment of the device

The product is ready-to-use. No reconstitution, mixing, or dilution is required. Bring probe to room temperature (18-25°C) before use, protect from light. Prior to opening the vial, mix by vortexing and spin down briefly.

12. Assay procedure

Specimen pretreatment
Perform specimen pretreatment (dewaxing, proteolysis) according to the instructions for use of the FlexISH-Tissue Implementation Kit.

Denaturation and hybridization

1. Pipette 10 µl of the probe onto each pretreated specimen.
2. Cover specimens with a 22 mm x 22 mm coverslip (avoid trapped bubbles) and seal the coverslip.

We recommend using rubber cement (e.g., Fixogum) for sealing.

3. Place slides on a hot plate or hybridizer and denature specimens for 10 min at 75°C.
4. Perform hybridization for 2 h up to 16 h (i.e. overnight) at 37°C by either transferring the slides to a hybridizer or to a humidity chamber and a hybridization oven.

It is essential that specimens do not dry out during the hybridization step.

Post-hybridization
Perform post-hybridization processing (washing, counter-staining, fluorescence microscopy) according to the instructions for use of the FlexISH-Tissue Implementation Kit.

13. Interpretation of results

With the use of appropriate filter sets, the hybridization signals of the probe appear green (proximal to the NTRK1 and distal to the NTRK3 breakpoint region), orange (distal to the NTRK1 and proximal to the NTRK3 breakpoint region), and blue (proximal and distal to the NTRK3 breakpoint region).

Normal situation: In interphases of normal cells or cells without a rearrangement involving the NTRK1 or NTRK3 gene region, four green/orange fusion signals appear when using an appropriate dual bandpass filter set, and two blue signals appear when using an appropriate single bandpass filter set (see Fig. 3).

Aberrant situation: One NTRK1 gene region affected by a translocation is indicated by one separate green signal and one separate orange signal not co-localizing with blue signals. Loss of one green signal resulting in an isolated orange signal is the result of a deletion proximal to the NTRK1 breakpoint region. One NTRK3 gene region affected by a translocation is indicated by one separate green signal and one separate orange signal, each co-localizing with a blue signal. Loss of one green signal resulting in an isolated orange signal co-localizing with a blue signal is the result of a deletion distal to the NTRK3 breakpoint region (see Fig. 3).

Overlapping green and orange signals may appear as yellow signals.

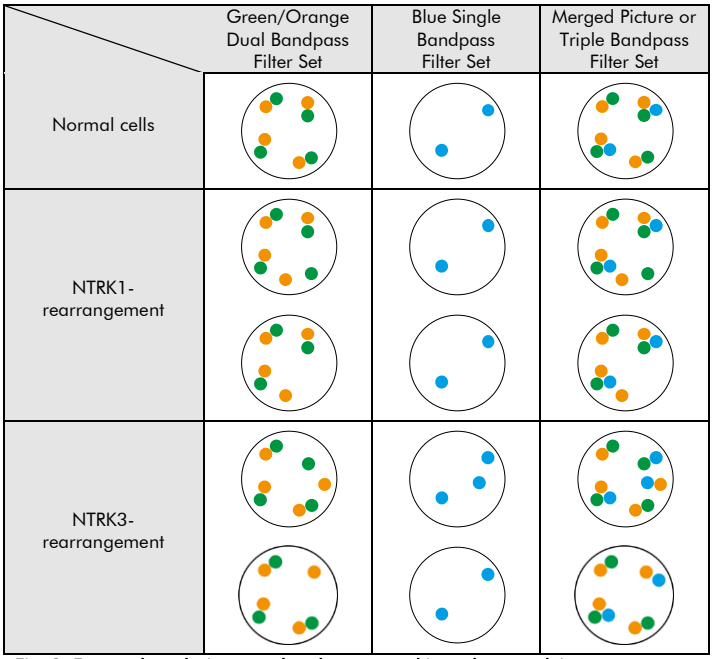

Fig. 3: Expected results in normal and rearranged interphase nuclei

Genomic aberrations due to small deletions, duplications or inversions might result in inconspicuous signal patterns.
Other signal distribution may be observed in some abnormal samples which might result in different signal patterns than described above, indicating variant rearrangements. Unexpected signal patterns should be further investigated.

Please note:

- Due to decondensed chromatin, single FISH signals can appear as small signal clusters. Thus, two or three signals of the same size, separated by a distance ≤ 1 signal diameter, should be counted as one signal.
- Do not evaluate overlapping nuclei.
- Do not count over-digested nuclei (recognized by dark areas visible inside of the nuclei).
- Do not count nuclei with strong auto-fluorescence, which hinders signal recognition.
- A negative or unspecific result can be caused by multiple factors (see chapter 17).
- In order to correctly interpret the results, the user must validate this product prior to use in diagnostic procedures according to national and/or international guidelines.

14. Recommended quality control procedures

In order to monitor correct performance of processed specimens and test reagents, each assay should be accompanied by internal and external controls. If internal and/or external controls fail to demonstrate appropriate staining, results with patient specimens must be considered invalid.

Internal control: Non-neoplastic cells within the specimen that exhibit normal signal pattern, e.g., fibroblasts.

External control: Validated positive and negative control specimens.

15. Performance characteristics

Accuracy: The location of hybridization of the probe was evaluated on metaphase spreads of a karyotypically normal male. In all tested specimens the probe hybridized solely to the expected loci. No additional signals or cross-hybridizations were observed. Therefore, the accuracy was calculated to be 100%.

Analytical sensitivity: For the analytical sensitivity assessment, the probe was evaluated on metaphase spreads of karyotypically normal males. All nuclei showed the expected normal signal pattern in all tested specimens. Therefore, the analytical sensitivity was calculated to be 100%.

Analytical specificity: For the analytical specificity assessment, the probe was evaluated on metaphase spreads of karyotypically normal males. In all tested specimens, all signals hybridized solely to the expected target loci and no other loci. Therefore, the analytical specificity was calculated to be 100%.

16. Disposal

The disposal of reagents must be carried out in accordance with local regulations.

17. Troubleshooting

Any deviation from the operating instructions can lead to inferior staining results or to no staining at all.

Weak signals or no signals at all

Possible cause	Action
No target sequences available	Use appropriate controls
Specimen has not been properly fixed	Optimize fixing time and fixative
Heat pretreatment, proteolysis, denaturation, hybridization, or stringency wash temperature not correct	Check temperature of all technical devices used, using a calibrated thermometer
Proteolytic pretreatment not carried out properly	Optimize pepsin incubation time, increase or decrease if necessary
Probe evaporation	When using a hybridizer, the use of the wet stripes/water filled tanks is mandatory. When using a hybridization oven, the use of a humidity chamber is required. In addition, the coverslip should be sealed completely, e.g., with Fixogum, to prevent drying-out of the sample during hybridization.
Too low concentrated stringency wash buffer	Check concentration of stringency wash buffer
Old dehydration solutions	Prepare fresh dehydration solutions
Fluorescence microscope wrongly adjusted	Adjust correctly
Inappropriate filter sets used	Use filter sets appropriate for the fluochromes of the probe. *Triple-bandpass filter sets provide less light compared to single or dual-bandpass filter sets. Consequently, the signals may appear fainter using these triple-bandpass filter sets.*
Photo-damage of the probes/fluorophores	Accomplish hybridization and washing steps in the dark

Cross hybridization signals; noisy background

Possible cause	Action
Incomplete dewaxing	Use fresh solutions; check duration of dewaxing
Proteolytic pretreatment too strong	Optimize pepsin incubation time
Probe volume per area too high	Reduce probe volume per section/area, distribute probe dropwise to avoid local concentration
Slides cooled to room temperature before hybridization	Transfer the slides quickly to 37°C
Too high concentrated stringency wash buffer	Check concentration of stringency wash buffer
Washing temperature following hybridization too low	Check temperature; increase if necessary
Dehydration of sections between the individual incubation steps	Prevent dehydration by sealing the slides and performing incubation in humid environment

Overlapping nuclei

Possible cause	Action
Inappropriate thickness of specimen sections	Prepare 2-4 µm microtome sections

Tissue morphology degraded

Possible cause	Action
Specimen has not been properly fixed	Optimize fixing time and fixative
Proteolytic pretreatment not carried out properly	Optimize pepsin incubation time
Insufficient drying before probe application	Extend air-drying

Specimen floats off the slide

Possible cause	Action
Unsuitable slide coating	Use appropriate (positively charged) slides
Proteolytic pretreatment too strong	Shorten pepsin incubation time

Weak counterstain

Possible cause	Action
Low concentrated DAPI solution	Use DAPI/DuraTect-Solution (ultra) (Prod. No. MT-0008-0.8) instead
DAPI incubation time too short	Adjust DAPI incubation time

18. Literature

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Our experts are available to answer your questions.
Please contact helptech@zytovision.com

ZytoVision GmbH
Fischkai 1
27572 Bremerhaven/ Germany
Phone: +49 471 4832-300
Fax: +49 471 4832-509
www.zytovision.com
Email: info@zytovision.com