



## ZytoDot 2C

### SPEC ROS1 Break Apart Probe

|                       |          |             |
|-----------------------|----------|-------------|
| <b>REF</b> C-3063-100 | $\nabla$ | 10 (0.1 ml) |
| <b>REF</b> C-3063-400 | $\nabla$ | 40 (0.4 ml) |

For the qualitative detection of translocations involving the human ROS1 gene at 6q22.1 by chromogenic *in situ* hybridization (CISH)



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

#### 1. Intended use

The ZytoDot 2C SPEC ROS1 Break Apart Probe (PD43) is intended to be used for the qualitative detection of translocations involving the human ROS1 gene at 6q22.1 in formalin-fixed, paraffin-embedded specimens by chromogenic *in situ* hybridization (CISH). The probe is intended to be used in combination with the ZytoDot 2C CISH Implementation Kit (Prod. No. C-3044-10/-40).

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 ROS1 (Ros proto-oncogene 1, receptor tyrosine kinase) gene is located on 6q22.1 and encodes a receptor tyrosine kinase. Translocations affecting ROS1 have been detected in glioblastoma, cholangiocarcinoma, and non-small cell lung cancer (NSCLC). In NSCLC several ROS1 translocation partners have been detected all of which result in the fusion of variably truncated forms of e.g. TPM3, SDC4, SLC34A2, CD74, EZR, or LRIG3 to the kinase domain of ROS1. GOPC has also been found to be fused to ROS1 in NSCLC. GOPC-ROS1 fusions result from interstitial deletion of approx. 240 kb on 6q22.1. ROS1 rearrangements have been exclusively detected in adenocarcinoma of the lung and are thought to define a molecular subset of NSCLC with distinct clinical characteristics that are similar to those observed in patients with ALK rearranged NSCLC. First evidence suggests that administration of ROS1 kinase inhibitors may represent a very effective therapeutic strategy in NSCLC patients harboring activating ROS1 rearrangements. Accordingly, detection of ROS1 rearrangements using chromogenic *in situ* hybridization might be a helpful tool for the identification of patients likely to respond to ROS1 kinase targeting therapies.

#### 3. Test principle

The chromogenic *in situ* hybridization (CISH) technique allows the detection and visualization of specific nucleic acid sequences in cell preparations. Hapten-labeled nucleotide fragments, so called CISH probes, and their complementary target sequences 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. Duplex formation of the labeled probe can be visualized using primary (unmarked) antibodies, which are detected by secondary polymerized enzyme-conjugated antibodies. The enzymatic reaction with chromogenic substrates leads to the formation of colored precipitates. After counterstaining the nucleus with a nuclear dye, hybridized probe fragments are visualized by light microscopy.

#### 4. Reagents provided

The ZytoDot 2C SPEC ROS1 Break Apart Probe is composed of:

- Digoxigenin-labeled polynucleotides (~0.50 ng/ $\mu$ l), which target sequences mapping in 6q22.1\* (chr6:117,448,964-117,627,255) proximal to the ROS1 breakpoint region (see Fig. 1).
- Dinitrophenyl-labeled polynucleotides (~0.75 ng/ $\mu$ l), which target sequences mapping in 6q22.1\* (chr6:117,659,135-117,871,701) distal to the ROS1 breakpoint region (see Fig. 1).
- Formamide based hybridization buffer

\*according to Human Genome Assembly GRCh37/hg19

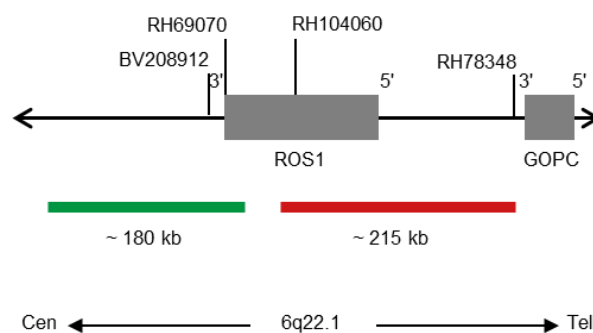


Fig. 1: SPEC ROS1 Probe map (not to scale)

The ZytoDot 2C SPEC ROS1 Break Apart Probe is available in two sizes:

- C-3063-100: 0.1 ml (10 reactions of 10  $\mu$ l each)
- C-3063-400: 0.4 ml (40 reactions of 10  $\mu$ l each)

#### 5. Materials required but not provided

- ZytoDot 2C CISH Implementation Kit (Prod. No. C-3044-10/-40)
- Positive and negative control specimens
- Microscope slides, positively charged
- Water bath (80°C, 98°C)
- Hybridizer or hot plate
- Hybridizer or humidity chamber in hybridization oven
- Adjustable pipettes (10  $\mu$ l, 1000  $\mu$ l)
- Staining jars or baths
- Timer
- Calibrated thermometer
- Ethanol or reagent alcohol
- Xylene
- Methanol 100%
- Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) 30%
- Deionized or distilled water
- Coverslips (22 mm x 22 mm, 24 mm x 32 mm)
- Rubber cement, e.g., Fixogum Rubber Cement (Prod. No. E-4005-50/-125) or similar
- Adequately maintained light microscope (400-630x)

#### 6. Storage and handling

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

## 7. Warnings and precautions

- Read the instructions 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)!
- Report any serious incident that has occurred in relation to the product to the manufacturer and the competent authority according to local regulations!
- If reagents come into contact with skin, rinse skin immediately with copious amounts of water!
- A material safety data sheet is available on our homepage ([www.zytovision.com](http://www.zytovision.com)).
- Do not reuse reagents, unless reuse is explicitly permitted!
- Avoid any cross-contamination and micro-bacterial contamination of the reagents!
- The specimens must not be allowed to dry during the hybridization and washing steps!

### Hazard and precautionary statements:

The hazard-determining component is formamide.



### Danger

|           |  |
|-----------|--|
| 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.                                    |
| P202      | Do not handle until all safety precautions have been read and understood.  |
| P260      | Do not breathe dust/fume/gas/mist/vapours/spray.                           |
| P280      | Wear protective gloves/protective clothing/eye protection/face protection. |
| P308+P313 | IF exposed or concerned: Get medical advice/attention.                     |
| P405      | Store locked up.   |

## 8. Limitations

- For *in vitro* diagnostic use.
- For professional use only.
- For non-automated 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 CISH 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 chapter 4. "Reagents provided".
- The performance was validated using the procedures described in these instructions for use. Modifications to these procedures might alter the performance and have to be validated by the user.

## 9. Interfering substances

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

Recommendations:

- Avoid cross-contamination of samples in any step of preparation as this may lead to erroneous results.
- Fixation in 10% neutrally buffered formalin for 24 h at room temperature (18-25°C).
- Sample size  $\leq 0.5 \text{ cm}^3$ .
- Use premium quality paraffin.
- Embedding should be carried out at temperatures lower than 65°C.
- Prepare 3-5  $\mu\text{m}$  microtome sections.
- Use positively charged microscope slides.
- Fix tissue sections for 2-16 h at 50-60°C.

## 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) and mix briefly before use.

## 12. Assay procedure

### Specimen pretreatment

Perform specimen pretreatment (e.g., dewaxing, proteolysis) according to the instructions for use of the [ZytoDot 2C CISH Implementation Kit](#).

### Denaturation and hybridization

1. Pipette 10  $\mu\text{l}$  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 5 min at 79°C.
4. Transfer slides to a humidity chamber and hybridize overnight at 37°C (e.g., in a hybridization oven).

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

### Post-hybridization

Perform post-hybridization processing (washing, detection, counter-staining, mounting, microscopy) according to the instructions for use of the [ZytoDot 2C CISH Implementation Kit](#).

## 13. Interpretation of results

Using the [ZytoDot 2C CISH Implementation Kit](#), hybridization signals of Digoxigenin-labeled polynucleotides appear as dark green colored distinct dots (proximal to the ROS1 breakpoint region), and Dinitrophenyl-labeled polynucleotides appear as bright red colored distinct dots (distal to the ROS1 breakpoint region).

**Normal situation:** In interphases of normal cells or cells without a translocation involving the ROS1 gene region, two red/green fusion signals appear (see Fig. 2).

**Aberrant situation:** One ROS1 gene region affected by a translocation is indicated by one separate green signal and one separate red signal. Isolated green signals are the result of deletions distal to the ROS1 breakpoint region or are due to unbalanced translocations affecting this chromosomal region (see Fig. 2).

Overlapping signals may appear as brown signals.

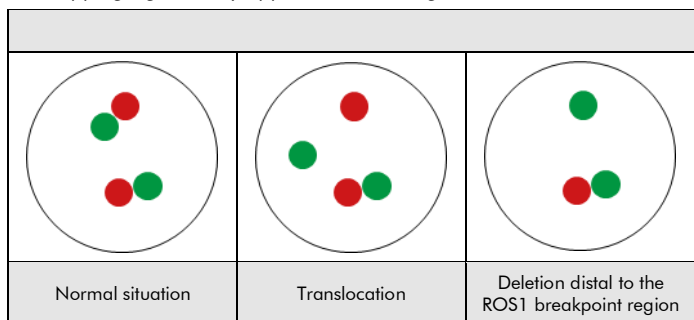


Fig. 2: Expected results in normal and aberrant nuclei

Genomic aberrations due to small deletions, duplications or inversions might result in inconspicuous signal patterns.

Other signal patterns than those described above may be observed in some abnormal samples. These unexpected signal patterns should be further investigated.

**Please note:**

- Due to decondensed chromatin, single CISH signals can appear as small signal clusters. Thus, two or three signals of the same size, separated by a distance  $\leq 1$  signal diameter, should be counted as one signal.
- Prior to signal enumeration, the specimen should be scanned for any possible intratumoral heterogeneity at 100- to 200-fold magnification.
- Visualization of signals should be performed at least at 400-fold magnification resulting in easily visible signals. A 630-fold magnification is recommended for probes detecting chromosomal breaks. Do not use contrast enhancing filter lenses as this might distort the signal color. To obtain signals in bright colors, open the aperture diaphragm. Be sure to focus up and down when evaluating a nucleus, as red and green signals might be located on top of each other.
- Do not evaluate areas of necrosis, overlapping nuclei, over-digested nuclei and nuclei with weak signal intensity.
- Due to mitosis, additional signals may be visible even in a small percentage of non-neoplastic cells. Occasionally, nuclei with missing signals may be observed in paraffin-embedded specimens due to cutting artefacts.
- A negative or unspecific result can be caused by multiple factors (see chapter 17. "Troubleshooting").
- 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**

The performance of the probe was determined by comparison against the corresponding IVD approved FISH probe. The concordance was 100%.

**Accuracy:** The accuracy was calculated as 100%.

**Analytical sensitivity:** The analytical sensitivity was calculated as 100%.

**Analytical specificity:** The analytical specificity was calculated as 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  |
|---|---|
| Cell or tissue sample has not been properly fixed   | Optimize fixing time and fixative   |
| Heat pretreatment, proteolysis, hybridization, denaturation, stringency wash or antibody-incubation temperature not correct | Check temperature of all technical devices used, using a calibrated thermometer. Use always the same number of slides in solutions with critical temperature  |
| Proteolytic pretreatment not carried out properly   | Depending on multiple factors, e.g., nature and duration of fixing, thickness of sections, and nature of tissue/cells, different incubation times may be required. Ascertain the optimum time for pepsin incubation in pre-tests  |
| Hybridization time too short  | Hybridize for at least 12 h; extend hybridization time if necessary   |
| Old dehydration solutions   | Prepare fresh dehydration solutions   |
| 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 |
| Incubation with chromogenic substrate too short   | Extend incubation time  |
| Counterstaining time too long   | The counterstaining time depends on the nature of the specimen and should be optimized accordingly. Avoid dark counterstaining, because it may obscure positive staining signals  |
| Bleuing of counterstain not carried out properly  | Use cold running tap water for bleuing; do not use warm or hot water, or bleuing reagents   |

**Signals too strong**

| Possible cause                                 | Action   |
|--|--|
| Proteolytic pretreatment carried out too long  | Depending on multiple factors, e.g., nature and duration of fixing, thickness of sections, and nature of tissue/cells, different incubation times may be required. Ascertain the optimum time for pepsin incubation in pre-tests |
| AP-Red Solution incubation time not correct    | If required, the incubation time can be shortened down to 5 min. Do not heat substrate solution over 25°C; incubate at room temperature only   |
| HRP-Green solution incubation time not correct | If required, the incubation time can be shortened down to 7 min. Do not heat substrate solution over 25°C; incubate at room temperature only   |

**Red signals too weak**

| Possible cause                                     | Action   |
|--|--|
| AP-Red Solution was exposed to strong direct light | Prepare and use AP-Red Solution protected from strong direct light |
| AP-Red Solution was prepared too early             | Prepare prior to immediate use                                     |
| AP-Red Solution incubation time not correct        | If required, the incubation time can be extended up to 15 min      |

|   |                                      |
|---|--------------------------------------|
| Insufficient preparation of chromogenic substrate | Do not increase volume of Solution A |
|---|--------------------------------------|

**Green signals too weak**

| Possible cause  | Action  |
|---|---|
| Incubation time of any washing steps after staining with HRP-Green too long | Do not exceed given incubation times                          |
| HRP-Green solution incubation time not correct                              | If required, the incubation time can be extended up to 15 min |
| Insufficient preparation of chromogenic substrate                           | Do not increase volume of Solution A                          |

**Signals fade or merge**

| Possible cause                                | Action  |
|---|---|
| An unsuitable mounting solution has been used | Use only the mounting solution provided with the kit or xylene-based mounting solutions free of any impurities; do not use coverslip tape |
| Sections were not dehydrated properly         | Use fresh ethanol and xylene solutions; use only xylene of "pure" quality   |

**Uneven or in some parts only very light staining**

| Possible cause   | Action  |
|--|---|
| Incomplete dewaxing  | Use fresh solutions; check duration of dewaxing times                   |
| Reagent volume too small                                   | Ensure that the reagent volume is large enough to cover the tissue area |
| Air bubbles caught before hybridization or during mounting | Avoid air bubbles   |

**Inconsistent results**

| Possible cause  | Action   |
|---|--|
| Insufficient drying before probe application  | Extend air-drying  |
| Too much water/wash buffer on tissue prior to application of pepsin, antibodies and/or color substrates | Ensure that excess liquid is removed from tissue section by blotting or shaking it off the slide. Small amounts of residual water/wash buffer do not interfere with the test |
| Variations in tissue fixation and embedding methods   | Optimize fixation and embedding methods  |
| Variations in tissue section thickness  | Optimize sectioning  |

**Morphology degraded**

| Possible cause                                    | Action   |
|---|--|
| Cell or tissue sample has not been properly fixed | Optimize fixing time and fixative                      |
| Proteolytic pretreatment not carried out properly | Optimize pepsin incubation time; decrease if necessary |

**Cross hybridization signals; noisy background**

| Possible cause                          | Action   |
|---|--|
| Stringency wash temperature not correct | Check temperature of the technical devices used, using a calibrated thermometer. Use always the same number of slides in the jar. We recommend not to use more than eight slides per jar for heat incubation steps |
| Slides not thoroughly rinsed            | Use fresh and sufficient wash buffer and deionized or distilled water where indicated  |

|   |   |
|---|---|
| Sections dried out any time during or after hybridization | Avoid sections being dried out; use humidity chamber; seal coverslip properly |
| Prolonged substrate incubation time                       | Shorten substrate incubation time   |
| Incomplete dewaxing                                       | Use fresh solutions; check duration of dewaxing                               |
| Proteolytic pretreatment too strong                       | Optimize pepsin incubation time   |
| Slides cooled to room temperature before hybridization    | Transfer the slides quickly to hybridization temperature                      |

**Overlapping signals**

| Possible cause                             | Action                                       |
|--|--|
| Inappropriate thickness of tissue sections | Prepare 3-5 $\mu\text{m}$ microtome sections |

**Specimen floats off the slide**

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

**18. Literature**

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- Takeuchi K, et al. (2012) *Nat Med* 18: 378-81.
- Wilkinson DG: *In Situ Hybridization, A Practical Approach*, Oxford University Press (1992) ISBN 0 19 963327 4.

**19. Revision**

Please refer to [www.zytovision.com](http://www.zytovision.com) for the most recent instructions for use as well as for instructions for use in different languages.

Our experts are available to answer your questions.  
Please contact [helptech@zytovision.com](mailto:helptech@zytovision.com)



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

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