ZytoDot 2C
SPEC RET Break Apart Probe

For the qualitative detection of translocations involving the human RET gene at 10q11.21 by chromogenic *in situ* hybridization (CISH)

![CISH](image)

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

1. **Intended use**

   The **ZytoDot 2C SPEC RET Break Apart Probe** (PD44) is intended to be used for the qualitative detection of translocations involving the human RET gene at 10q11.21 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**

   RET (ret proto-oncogene) encodes a tyrosine kinase (TK) receptor. Translocations involving RET were first described in papillary thyroid carcinoma (PTC) where somatic rearrangements result in the fusion of its TK catalytic domain with an N-terminal dimerization domain encoded by various fusion partner genes. More recently, recurrent inversions [inv(10)(p11.2q11.2)] fusing the coiled-coil domains of the kinesin family member 5B (KIF5B) gene to the RET kinase domain have been detected in lung adenocarcinoma. The resulting KIF5B-RET fusion protein can form homodimers through the coiled domains of KIF5B, causing an aberrant activation of the TK of RET, a mechanism known from KIF5B-ALK fusions which is also found in lung adenocarcinoma. Since *in vitro* studies showed transforming activity of KIF5B-RET which could be suppressed by a TK inhibitor, it was assumed that the chimeric oncogene might be a promising molecular target for the treatment of lung cancer. The same holds true for the BCR-RET and FGFR1-OP-RET fusion genes in chronic myelomonocytic leukemia (CMML) generated by two balanced translocations t(10;22)(q11.2;q11.2) and t(6;10)(q27;q11.2), respectively.

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 bystringency 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 RET Break Apart Probe** is composed of:
   - Digoxigenin-labeled polynucleotides (~0.50 ng/µl), which target sequences mapping in 10q11.21* (chr10:43,687,278-43,856,587) distal to the RET breakpoint region (see Fig. 1).
   - Dinitrophenyl-labeled polynucleotides (~0.75 ng/µl), which target sequences mapping in 10q11.21* (chr10:43,340,888-43,510,171) proximal to the RET breakpoint region (see Fig. 1).
   - Formamidase based hybridization buffer

*according to Human Genome Assembly GRCh37/hg19

![ZytoDot 2C SPEC RET Break Apart Probe](image)

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

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

- C-3064-100: 0.1 ml (10 reactions of 10 µl each)
- C-3064-400: 0.4 ml (40 reactions of 10 µl 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 µl, 1000 µl)
   - Staining jars or baths
   - Timer
   - Calibrated thermometer
   - Ethanol or reagent alcohol
   - Xylene
   - Methanol 100%
   - Hydrogen peroxide (H2O2) 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.

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**ZytoDot 2C SPEC RET Break Apart Probe**

2020-07-27
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).
- 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.

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**Danger**

H351 Suspected of causing cancer.

H360DFD 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.

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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
- Alcohol fixatives (when used alone)
- Mercuric chloride
- Formaldehyde/zinc fixative
- Holland’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 ≤ 0.5 cm².
- Use premium quality paraffin.
- Embedding should be carried out at temperatures lower than 65°C.
- Prepare 3-5 μm 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 ZyroDot 2C CISH 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 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, counterstaining, mounting, microscopy) according to the instructions for use of the ZyroDot 2C CISH Implementation Kit.

13. Interpretation of results

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

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

**Aberrant situation:** One RET 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 proximal to the RET breakpoint region (see Fig. 2).

Overlap signals may appear as brown signals.
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

<table>
<thead>
<tr>
<th>Possible cause</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell or tissue sample has not been properly fixed</td>
<td>Optimize fixing time and fixative</td>
</tr>
<tr>
<td>Heat pretreatment, proteolysis, hybridization, denaturation, stringency, wash or antibody-incubation temperature not correct</td>
<td>Check temperature of all technical devices used, using a calibrated thermometer. Use always the same number of slides in solutions with critical temperature</td>
</tr>
<tr>
<td>Probe evaporation</td>
<td>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</td>
</tr>
<tr>
<td>Hybridization time too short</td>
<td>Hybridize for at least 12 h; extend hybridization time if necessary</td>
</tr>
<tr>
<td>Old dehydration solutions</td>
<td>Prepare fresh dehydration solutions</td>
</tr>
<tr>
<td>Incubation with chromogenic substrate too short</td>
<td>Extend incubation time</td>
</tr>
<tr>
<td>Counterstaining time too long</td>
<td>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</td>
</tr>
<tr>
<td>Bluing of counterstain not carried out properly</td>
<td>Use cold running tap water for bluing; do not use warm or hot water, or bluing reagents</td>
</tr>
</tbody>
</table>

### Signals too strong

<table>
<thead>
<tr>
<th>Possible cause</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteolytic pretreatment carried out too long</td>
<td>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</td>
</tr>
<tr>
<td>AP-Red Solution incubation time not correct</td>
<td>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</td>
</tr>
<tr>
<td>HRP-Green solution incubation time not correct</td>
<td>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</td>
</tr>
</tbody>
</table>

### Red signals too weak

<table>
<thead>
<tr>
<th>Possible cause</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP-Red Solution was exposed to strong direct light</td>
<td>Prepare and use AP-Red Solution protected from strong direct light</td>
</tr>
<tr>
<td>AP-Red Solution was prepared too early</td>
<td>Prepare prior to immediate use</td>
</tr>
<tr>
<td>AP-Red Solution incubation time not correct</td>
<td>If required, the incubation time can be extended up to 15 min</td>
</tr>
</tbody>
</table>

### 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.
**Insufficient preparation of chromogenic substrate**
- Do not increase volume of Solution A

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

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

**Uneven or in some parts only very light staining**
- **Possible cause**
  - Incomplete dewaxing
  - Reagent volume too small
  - Air bubbles caught before hybridization or during mounting
- **Action**
  - Use fresh solutions; check duration of dewaxing times
  - Ensure that the reagent volume is large enough to cover the tissue area
  - Avoid air bubbles

**Inconsistent results**
- **Possible cause**
  - Insufficient drying before probe application
- **Action**
  - Extend air-drying

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

**Cross hybridization signals, noisy background**
- **Possible cause**
  - Stringency wash temperature not correct
  - Slides not thoroughly rinsed
- **Action**
  - 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
  - Use fresh and sufficient wash buffer and deionized or distilled water where indicated

**Sections dried out any time during or after hybridization**
- **Possible cause**
  - Prolonged substrate incubation time
  - Incomplete dewaxing
  - Proteolytic pretreatment too strong
- **Action**
  - Shorten substrate incubation time
  - Use fresh solutions; check duration of dewaxing
  - Optimize pepsin incubation time

**Specimen floats off the slide**
- **Possible cause**
  - Unsuitable slide coating
- **Action**
  - Use appropriate (positively charged) slides

**Overlapping signals**
- **Possible cause**
  - Inappropriate thickness of tissue sections
- **Action**
  - Prepare 3-5 µm microtome sections

**Specimen too strongly stained**
- **Possible cause**
  - Prolonged washing steps after staining with HRP-Green
- **Action**
  - Shorten washing steps after staining with HRP-Green

**Specimen too weakly stained**
- **Possible cause**
  - too much water/wash buffer on tissue prior to application of pepsin, antibodies and/or color substrates
- **Action**
  - 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

**Specimen takes on a brownish color**
- **Possible cause**
  - Use fresh solutions; check duration of dewaxing times
  - Ensure that the reagent volume is large enough to cover the tissue area
- **Action**
  - Avoid air bubbles

**Specimen takes on an orange color**
- **Possible cause**
  - Overlapping signals
- **Action**
  - Do not increase volume of Solution A

**Specimen shows too many signals**
- **Possible cause**
  - Reagent volume too small
  - Use fresh solutions; check duration of dewaxing times
- **Action**
  - Ensure that the reagent volume is large enough to cover the tissue area

**Specimen shows no signals**
- **Possible cause**
  - Specimen floats off the slide
  - Specimen too weakly stained
- **Action**
  - Use appropriate (positively charged) slides
  - Shorten washing steps after staining with HRP-Green

**Specimen shows very weak signals**
- **Possible cause**
  - Stringency wash temperature not correct
  - Slides not thoroughly rinsed
- **Action**
  - 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
  - Use fresh and sufficient wash buffer and deionized or distilled water where indicated

18. **Literature**

19. **Revision**

Please refer to 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 help@zytovision.com.

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