For the qualitative detection of translocations involving the human MALT1 gene at 18q21.32 by chromogenic in situ hybridization (CISH)

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

1. Intended use

The ZyroDot 2C SPEC MALT1 Break Apart Probe (PD52) is intended to be used for the qualitative detection of translocations involving the human MALT1 gene at 18q21.32 in formalin-fixed, paraffin-embedded specimens by chromogenic in situ hybridization (CISH). The probe is intended to be used in combination with the ZyroDot 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 MALT1 (MALT1 paracaspase, a.k.a. MLT) gene encodes a human paracaspase and is often rearranged in MALT lymphomas accounting for 5-10% of all B-cell non Hodgkin lymphomas (NHL). The most common translocations affecting the MALT1 gene are t(11;18)(q22.2;q21.3) and t(14;18)(q32.3;q21.3) occurring in 50% and 15-20% of MALT lymphomas, respectively. These translocations lead to the expression of BIRC3-MALT1 (a.k.a. API2-MALT1) and IGH-MALT1 fusion proteins, resulting in constitutive activation of the NF-kB signaling pathway which controls the expression of numerous anti-apoptotic and proliferation-promoting genes. The translocation t(11;18)(q22.2;q21.3) is mainly found in pulmonary and gastric lymphomas, whereas t(14;18) (q32.3;q21.3) occurs more frequently in non-gastrointestinal MALT lymphomas, e.g., of the skin and salivary glands. The presence of a t(11;18)(q22.2;q21.3) correlates with unresponsiveness to eradication of Helicobacter pylori in gastric MALT lymphomas. Hence, detection of MALT1 translocations by CISH may be a supportive tool to identify patients eligible for an anti-H. pylori therapy.

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 ZyroDot 2C SPEC MALT1 Break Apart Probe is composed of:

- Digoxigenin-labeled polynucleotides (~0.50 ng/µl), which target sequences mapping in 18q21.31-q21.32* (chr18:56,021,766-56,620,885) proximal to the MALT1 breakpoint region (see Fig. 1).
- Dinitrophenyl-labeled polynucleotides (~0.75 ng/µl), which target sequences mapping in 18q21.32* (chr18:55,578,145-56,724,408) distal to the MALT1 breakpoint region (see Fig. 1).
- Formamide based hybridization buffer

*aaccording to Human Genome Assembly GRCh37/hg19

5. Materials required but not provided

- ZyroDot 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.

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!)

ZyroDot 2C SPEC MALT1 Break Apart Probe

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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 ≤ 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 ZytoDot 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, 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 MALT1 breakpoint region), and Dinitrophenyl-labeled polynucleotides appear as bright red colored distinct dots (distal to the MALT1 breakpoint region).

Normal situation: In interphases of normal cells or cells without a translocation involving the MALT1 gene region, two red/green fusion signals appear (see Fig. 2).

Aberrant situation: One MALT1 gene region affected by a translocation is indicated by one separate green signal and one separate red signal (see Fig. 2).

Overlapping signals may appear as brown signals.
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 ≤ 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.
### Green signals too weak

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

### Signals fade or merge

<table>
<thead>
<tr>
<th>Possible cause</th>
<th>Action</th>
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</thead>
<tbody>
<tr>
<td>An unsuitable mounting solution has been used</td>
<td>Use only the mounting solution provided with the kit or xylene-based mounting solutions free of any impurities; do not use coverslip tape</td>
</tr>
<tr>
<td>Sections were not dehydrated properly</td>
<td>Use fresh ethanol and xylene solutions; use only xylene of &quot;pure&quot; quality</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Possible cause</th>
<th>Action</th>
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</thead>
<tbody>
<tr>
<td>Incomplete dewaxing</td>
<td>Use fresh solutions; check duration of dewaxing times</td>
</tr>
<tr>
<td>Reagent volume too small</td>
<td>Ensure that the reagent volume is large enough to cover the tissue area</td>
</tr>
<tr>
<td>Air bubbles caught before hybridization or during mounting</td>
<td>Avoid air bubbles</td>
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</tbody>
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### Inconsistent results

<table>
<thead>
<tr>
<th>Possible cause</th>
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<tbody>
<tr>
<td>Insufficient drying before probe application</td>
<td>Extend air-drying</td>
</tr>
<tr>
<td>Too much water/wash buffer on tissue prior to application of pepsin, antibodies and/or color substrates</td>
<td>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</td>
</tr>
<tr>
<td>Variations in tissue fixation and embedding methods</td>
<td>Optimize fixation and embedding methods</td>
</tr>
<tr>
<td>Variations in tissue section thickness</td>
<td>Optimize sectioning</td>
</tr>
</tbody>
</table>

### Morphology degraded

<table>
<thead>
<tr>
<th>Possible cause</th>
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</thead>
<tbody>
<tr>
<td>Cell or tissue sample has not been properly fixed</td>
<td>Optimize fixing time and fixative</td>
</tr>
<tr>
<td>Proteolytic pretreatment not carried out properly</td>
<td>Optimize pepsin incubation time; decrease if necessary</td>
</tr>
</tbody>
</table>

### Cross hybridization signals; noisy background

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Stringency wash temperature not correct</td>
<td>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</td>
</tr>
<tr>
<td>Slides not thoroughly rinsed</td>
<td>Use fresh and sufficient wash buffer and deionized or distilled water where indicated</td>
</tr>
<tr>
<td>Sections dried out any time during or after hybridization</td>
<td>Avoid sections being dried out; use humidity chamber; seal coverslip properly</td>
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### Prolonged substrate incubation time

<table>
<thead>
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<tbody>
<tr>
<td>Incomplete dewaxing</td>
<td>Use fresh solutions; check duration of dewaxing</td>
</tr>
<tr>
<td>Proteolytic pretreatment too strong</td>
<td>Optimize pepsin incubation time</td>
</tr>
<tr>
<td>Slides cooled to room temperature before hybridization</td>
<td>Transfer the slides quickly to hybridization temperature</td>
</tr>
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</table>

### Overlapping signals

<table>
<thead>
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<tr>
<td>Inappropriate thickness of tissue sections</td>
<td>Prepare 3-5 µm microtome sections</td>
</tr>
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</table>

### Specimen floats off the slide

<table>
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</thead>
<tbody>
<tr>
<td>Unsuitable slide coating</td>
<td>Use appropriate (positively charged) slides</td>
</tr>
<tr>
<td>Proteolytic pretreatment too strong</td>
<td>Shorten pepsin incubation time</td>
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</tbody>
</table>

### 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.

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

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