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 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 SPEC MDM2 Probe is composed of:

- Digoxigenin-labeled polynucleotides (–1.8 ng/µl), which target sequences mapping in 12q15* (chr12:69,190,708-69,430,185) harboring the MDM2 gene region (see Fig. 1).
- Formamide based hybridization buffer
  *according to Human Genome Assembly GRCh37/hg19

5. Materials required but not provided

- ZyroDot CISH Implementation Kit (Prod. No.- C-3018-40)
- 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)!
- 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.

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.
- 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 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
- BS 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 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 94-95°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 CISH Implementation Kit.

13. Interpretation of results

Using the ZytoDot CISH Implementation Kit, the hybridization signals of digoxigenin-labeled polynucleotides appear brown- to dark brown (MDM2 gene region).

Normal situation: In interphases of normal cells or cells without an amplification involving the MDM2 gene region, two distinct dot-shaped brown signals appear (see Fig. 2).

Aberrant situation: In cells with an amplification of the MDM2 gene region or polysomy of chromosome 12, an increased number of brown signals or brown signal clusters will be observed (see Fig. 2).
Analytical specificity:
Analytical sensitivity:
Accuracy:

The performance characteristics of the probe were 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%.

Other signal distribution may be observed in some abnormal samples which might result in a different signal pattern than described above, indicating variant rearrangements. 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- to 630-fold magnification resulting in easily visible signals.
- 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 artifacts.
- 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

<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 fixation 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>Proteolytic pretreatment not carried out properly</td>
<td>Depending on multiple factors, e.g., nature and duration of fixation, 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>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>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 Fixagum, to prevent drying-out of the sample during hybridization</td>
</tr>
<tr>
<td>Insufficient preparation of chromogenic substrate</td>
<td>Instead of using one drop of DAB Solution A use 30 µl</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 fixation, 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>Substrate reaction is too intense</td>
<td>Shorten substrate incubation time; do not heat substrate solution over 25°C; incubate at room temperature only</td>
</tr>
</tbody>
</table>

Signals fade or merge

<table>
<thead>
<tr>
<th>Possible cause</th>
<th>Action</th>
</tr>
</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>
</tbody>
</table>

Fig. 2: Expected results in normal and aberrant nuclei

Normal situation | Low level MDM2 amplification | High level MDM2 amplification

Please note:
- Other signal distribution may be observed in some abnormal samples which might result in a different signal pattern than described above, indicating variant rearrangements. 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.
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- Visualization of signals should be performed at least at 400- to 630-fold magnification resulting in easily visible signals.
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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%.
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

Tissue 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; increase or 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 µm 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

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

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