ZytoDot
CISH Polymer Detection Kit

For the detection of DIG labeled probes by chromogenic
*in situ* hybridization (CISH)

In vitro diagnostic medical device
according to EU directive 98/79/EC
As of: January 1, 2010 (4.5)

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1. **Scope of Application**

The *ZytoDot* CISH Polymer Detection Kit is designed to be used for the detection of digoxigenin (DIG)-labeled probes in either formalin-fixed, paraffin-embedded tissue or cell samples by chromogenic *in situ* hybridization (CISH).

DIG-labeled probes are detected using primary (unmarked) anti-DIG antibodies, secondary polymerized HRP-conjugated antibodies, and DAB (diaminobenzidine).

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

2. **Safety Precautions and Disposal**

- Read the operating instructions prior to use!
- Do not use the reagents after the expiry date has been reached!
- Avoid any cross-contamination and micro-bacterial contamination of the reagents!
- Some of the system components contain substances (in low concentrations and volumes) that are harmful to health. 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!
- Never pipet solutions with your mouth!
- The disposal of reagents must be carried out in accordance with local regulations!
- A material safety data sheet is available on request for the professional user!
3. **The ZytoDot CISH Polymer Detection Kit**

3.1 **Components**

The kit is made up of the following components:

<table>
<thead>
<tr>
<th>Code</th>
<th>Component</th>
<th>Quantity</th>
<th>Container</th>
</tr>
</thead>
<tbody>
<tr>
<td>BS1</td>
<td>Blocking Solution</td>
<td>4 ml</td>
<td>Dropper bottle, orange cap</td>
</tr>
<tr>
<td>AB1</td>
<td>Mouse-Anti-DIG</td>
<td>4 ml</td>
<td>Dropper bottle, pink cap</td>
</tr>
<tr>
<td>AB2</td>
<td>Anti-Mouse-HRP-Polymer</td>
<td>4 ml</td>
<td>Dropper bottle, violet cap</td>
</tr>
<tr>
<td>SB1a</td>
<td>DAB Solution A</td>
<td>0.3 ml</td>
<td>Dropper bottle, green cap</td>
</tr>
<tr>
<td>SB1b</td>
<td>DAB Solution B</td>
<td>10 ml</td>
<td>Dropper bottle, grey cap</td>
</tr>
<tr>
<td>CS1</td>
<td>Mayer’s Hematoxylin Solution</td>
<td>20 ml</td>
<td>Screw-cap bottle, black</td>
</tr>
<tr>
<td>MT4</td>
<td>Mounting Solution (alcoholic)</td>
<td>4 ml</td>
<td>Glass bottle, brown</td>
</tr>
<tr>
<td></td>
<td>Instruction manual</td>
<td>4 ml</td>
<td>Screw-cap bottle, black</td>
</tr>
</tbody>
</table>

**C-3005-40 (40 tests):** All components are sufficient for 40 reactions.

**C-3005-10 (10 tests):** All components are sufficient for 10 reactions.

3.2 **Storage and Shelf Life**

The components of the kit must be stored at 2…8°C. If these storage conditions are followed, the kit will function, without loss of performance, at least until the expiry date printed on the label.
3.3 Test Material

The *ZytoDot* CISH Polymer Detection Kit has been optimized for the use with formalin-fixed, paraffin-embedded tissue and cell samples. When test material is used that has been fixed or embedded in a different manner (e.g. methanol/glacial-acetic-acid-fixed cells or blood smears) the test protocol may need to be adapted by the user. Our specialists are available to support you whenever adjustments are necessary.

Prior to detection of hybridized digoxigenin-labeled probes we recommend the following procedures:

- **Tissue preparation**: Use 10% neutrally buffered formalin for 24 h at RT. Paraffin embedding should be carried out by standard processing. Prepare 2-5 µm microtome sections.
- **Pretreatment**: The pretreatment (dewax and proteolysis) of the tissue and cell section should be performed using established standard protocols. As a general rule, we recommend that the optimum time for proteolysis will be ascertained in pre-tests.
- **Hybridization**: Hybridization should be carried out in a humidity chamber overnight at 37°C. Wash slides before starting the detection.
- **Quenching**: Incubate slides for 10 min in 3% H₂O₂ in absolute methanol. Quenching can be performed after dewaxing the slides or after hybridization.

3.4 Additional Materials

The following reagents, materials, and equipment are not included in the kit:

Reagents and materials
- Deionized or distilled water
- Ethanol 100%, denatured
- PBS/Tween
- Xylene

Equipment
- Coverslips (22 mm x 22 mm, 24 mm x 32 mm)
- Light microscope
- Staining jars, 50-80 ml
4. The ZytoDot CISH Polymer Detection Kit Protocol

4.1 Preparatory Steps

- **Blocking Solution (BS1)**, **Mouse-Anti-DIG (AB1)**, **Anti-Mouse-HRP-Polymer (AB2)**, **Mayer’s Hematoxylin Solution (CS1)**, **Mounting Solution (alcoholic) (MT4)**: Bring to room temperature before use.

- **Preparation of DAB Solution**: Prior to use, add dropwise **DAB Solution B (SB1b)** in a graduated cup up to 1 ml and add one drop **DAB Solution A (SB1a)**. The solution is stable for 2 h at room temperature (RT).

4.2 Detection

1. Wash 3x 2 min in a PBS/Tween buffer (not provided)
2. Apply **Blocking Solution (BS1)** dropwise (3-4 drops per slide) to the slides and incubate for 10 min at RT
3. Blot off **Blocking Solution (BS1)**, but do not rinse!
4. Apply **Mouse-Anti-DIG (AB1)** dropwise (3-4 drops per slide) to the slides and incubate for 30 min at RT
5. Wash 3x 2 min in a PBS/Tween buffer (not provided)
6. Apply **Anti-Mouse-HRP-Polymer (AB2)** dropwise (3-4 drops per slide) to the slides and incubate for 30 min at RT
7. Wash 3x 2 min in a PBS/Tween buffer (not provided)
8. During the wash steps, prepare DAB Solution by adding **DAB Solution B (SB1b)** dropwise in a graduated cup up to 1 ml and add one drop **DAB Solution A (SB1a)**
9. Apply DAB Solution dropwise (3-4 drops per slide) to the slides and incubate for 30 min at RT
10. Transfer slides into a staining jar and wash 2 min in running tap water
11. Counterstain the tissue or cell samples for 8-10 s with **Mayer’s Hematoxylin Solution (CS1)**
12. Transfer slides into a staining jar and wash 2 min in running tap water
13. Dehydration: in 70%, 85%, 95%, and 2x 100% ethanol, each for 2 min
14. Incubate 2x 2 min in xylene (use very pure xylene)

Air dry sections for approximately 15 min

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15. Avoiding trapped bubbles, cover the samples with a coverslip (22 mm x 22 mm; 24 mm x 32 mm) by using Mounting Solution (alcoholic) (MT4) and air dry the slides for approx. 30 min

16. Evaluation of the sample material is carried out by light microscopy
5. Interpretation of Results

Probes labeled with digoxigenin (DIG) will result in permanent DAB brown-colored distinct dot-shaped signals, which can be clearly distinguished from the background counterstained with hematoxylin.

In normal diploid nuclei without chromosome aberrations, 2 dot-shaped signals with smooth, rounded edges will be visible per nucleus, except for probes targeting sex chromosomes resulting in 0 to 2 dot-shaped signals per probe, depending on the gender. 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 tissue sections.

Visualization of signals should be performed using at least a 40x objective resulting in easily visible signals.

The counterstaining time depends on the nature of the tissue or cells used and should therefore be optimized. Avoid dark counterstaining, as this may obscure positive staining signals. Absent or weak signals may be the result of a too short incubation with Mayer’s Hematoxylin Solution.

The final experimental results are also strongly influenced by the preceding experimental steps, i.e., tissue fixation, pretreatment, denaturation of DNA probe, hybridization, and washing. For a particularly user-friendly performance we recommend the use of a ZytoDot CISH system by ZytoVision.

For troubleshooting, please refer to chapter 7.
6. Literature


7. Problems and Possible Causes

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

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible cause</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streaks on the slide after stopping the pepsin treatment</td>
<td>Precipitation</td>
<td>Wash section immediately in deionized or distilled water</td>
</tr>
<tr>
<td>Weak signal or no signal at all</td>
<td>No target sequences available</td>
<td>Use controls</td>
</tr>
<tr>
<td></td>
<td>Cell or tissue sample has not been properly fixed</td>
<td>Optimization of fixing time</td>
</tr>
<tr>
<td></td>
<td>Proteolytic pretreatment not carried out properly</td>
<td>Optimization of incubation time</td>
</tr>
<tr>
<td></td>
<td>Denaturing temperature not correct</td>
<td>Check temperature; increase or decrease if necessary</td>
</tr>
<tr>
<td></td>
<td>Hybridization temperature not correct</td>
<td>Check temperature</td>
</tr>
<tr>
<td></td>
<td>Hybridization time too short</td>
<td>Extension of hybridization time</td>
</tr>
<tr>
<td></td>
<td>Incubation with chromogenic substrate too short</td>
<td>Extension of incubation time</td>
</tr>
<tr>
<td></td>
<td>Too dark counterstaining</td>
<td>Optimize counterstaining time</td>
</tr>
<tr>
<td>Uneven and in some parts only very light staining</td>
<td>Incomplete dewaxing</td>
<td>Use fresh solutions; check length of dewaxing times</td>
</tr>
<tr>
<td>Cross hybridization signals; strong background staining</td>
<td>Probe volume per area too high</td>
<td>Reduce probe volume per section/area, distribute probe dropwise to avoid local concentration</td>
</tr>
<tr>
<td></td>
<td>Proteolytic pretreatment too strong</td>
<td>Optimization of incubation time</td>
</tr>
<tr>
<td></td>
<td>Dehydration of sections between the individual incubation steps</td>
<td>Prevent dehydration</td>
</tr>
<tr>
<td></td>
<td>Washing temperature following hybridization too low</td>
<td>Check temperature</td>
</tr>
<tr>
<td>Section floats off the slide</td>
<td>Proteolytic pretreatment too strong</td>
<td>Shortening of incubation time</td>
</tr>
<tr>
<td></td>
<td>Unsuitable slide coating</td>
<td>Use appropriate slides</td>
</tr>
</tbody>
</table>