ZytoDot 2C
SPEC HER2/CEN 17 Probe Kit

For the detection of the human HER2 gene and alpha-satellites of chromosome 17 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 2C SPEC HER2/CEN 17 Probe Kit is designed to be used for the detection of the human HER2 gene as well as chromosome 17 alpha-satellites in either formalin-fixed, paraffin-embedded tissue or cell samples by chromogenic *in situ* hybridization (CISH).

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. **Basic Principles**

The presence of certain nucleic acid sequences in cells or tissue can be detected with *in situ* hybridization using labeled DNA probes. The hybridization results in duplex formation of sequences present in the test object and the specific gene probe.

The ZytoDot 2C SPEC HER2/CEN 17 Probe Kit uses the ZytoDot 2C SPEC HER2/CEN 17 Probe (PD12). The probe contains digoxigenin-labeled polynucleotides, which target sequences of the HER2 gene and DNP-labeled polynucleotides, which target alpha-satellites of the centromere of chromosome 17.

Duplex formation of the labeled probe can be visualized using primary (unmarked) antibodies, which are detected by secondary polymerized enzyme-conjugated antibodies. The enzymatic reactions of the substrates lead to the formation of strong permanent red and green signals that can be visualized by light microscopy at a 40x dry lens.

3. **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!

## 4. The ZytoDot 2C SPEC HER2/CEN 17 Probe Kit

### 4.1 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>PT2</td>
<td>Heat Pretreatment Solution EDTA</td>
<td>500 ml</td>
<td>Screw-cap bottle (large)</td>
</tr>
<tr>
<td>ES1</td>
<td>Pepsin Solution</td>
<td>4 ml</td>
<td>Dropper bottle, white cap</td>
</tr>
<tr>
<td>PD12</td>
<td>ZytoDot 2C SPEC HER2/CEN 17 Probe</td>
<td>0.4 ml</td>
<td>Reaction vessel, brown lid</td>
</tr>
<tr>
<td>WB1</td>
<td>Wash Buffer SSC</td>
<td>500 ml</td>
<td>Screw-cap bottle (large)</td>
</tr>
<tr>
<td>WB5</td>
<td>20x Wash Buffer TBS</td>
<td>2 x 50 ml</td>
<td>Screw-cap bottle</td>
</tr>
<tr>
<td>BS1</td>
<td>Blocking Solution</td>
<td>4 ml</td>
<td>Dropper bottle, orange cap</td>
</tr>
<tr>
<td>AB3</td>
<td>Anti-DIG/Anti-DNP-Mix</td>
<td>4 ml</td>
<td>Dropper bottle, yellow cap</td>
</tr>
<tr>
<td>AB4</td>
<td>Anti-Mouse-HRP-Polymer (plus)</td>
<td>4 ml</td>
<td>Dropper bottle, blue cap</td>
</tr>
<tr>
<td>AB5</td>
<td>Anti-Rabbit-AP-Polymer</td>
<td>4 ml</td>
<td>Dropper bottle, red cap</td>
</tr>
<tr>
<td>SB2a</td>
<td>AP-Red Solution A</td>
<td>0.3 ml</td>
<td>Dropper bottle, red cap (small)</td>
</tr>
<tr>
<td>SB2b</td>
<td>AP-Red Solution B</td>
<td>0.3 ml</td>
<td>Dropper bottle, red cap (small)</td>
</tr>
<tr>
<td>SB2c</td>
<td>AP-Red Solution C</td>
<td>2 ml</td>
<td>Dropper bottle, red cap (medium)</td>
</tr>
<tr>
<td>SB3a</td>
<td>HRP-Green Solution A</td>
<td>0.6 ml</td>
<td>Dropper bottle, green cap (small)</td>
</tr>
<tr>
<td>SB3b</td>
<td>HRP-Green Solution B</td>
<td>10 ml</td>
<td>Dropper bottle, green cap</td>
</tr>
<tr>
<td>SB3c</td>
<td>HRP-Green Solution C</td>
<td>0.6 ml</td>
<td>Dropper bottle, green cap (small)</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>SC2</td>
<td>HER2 Control Slide</td>
<td>2</td>
<td>Plastic pack</td>
</tr>
<tr>
<td></td>
<td>AP-Red reaction vessel</td>
<td>2</td>
<td>Graduated cup, red lid</td>
</tr>
<tr>
<td></td>
<td>HRP-Green reaction vessel</td>
<td>2</td>
<td>Graduated cup, green lid</td>
</tr>
<tr>
<td></td>
<td>Instruction manual</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

**C-3022-40 (40 tests):** Components (PD12), (ES1), (BS1), (AB3), (AB4), (AB5), (SB2a-c), (SB3a-c), (CS1), and (MT4) are sufficient for 40 reactions. Components (PT2) and (WB1) are sufficient for 7 staining jars of 70 ml each. Component (WB5) is sufficient for 27 staining jars of 70 ml each.

**C-3022-10 (10 tests):** Components (PD12), (ES1), (BS1), (AB3), (AB4), (AB5), (SB2a-c), (SB3a-c), (CS1), and (MT4) are sufficient for 10 reactions. Components (PT2) and (WB1) are sufficient for 2 staining jars of 70 ml each. Component (WB5) is sufficient for 14 staining jars of 70 ml each.
4.2 Storage and Shelf Life

The components of the kit must be stored at 2...8°C except for the probe solution (PD12), which must be stored at -16...-22°C; a short-time storage at 2...8°C is possible.

If these storage conditions are followed, the kit will function, without loss of performance, at least until the expiry date printed on the label.

4.3 Test Material

The ZytoDot 2C SPEC HER2/CEN 17 Probe 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.

We recommend the following tissue preparation:

✔️ Fixation in 10% neutrally buffered formalin for 24 h at RT

*In order to achieve optimum and uniform fixation and paraffin embedding, the sample size should not exceed 0.5 cm$^3$.*

✔️ Standard processing and paraffin embedding

*Use premium quality paraffin. Infiltration and embedding should be carried out at temperatures lower than 65°C.*

✔️ Prepare 2-5 µm microtome sections

*Draw up the sections onto silane-coated or adhesion slides (e.g. HistoBond®) and fix for 2-16 h at 50-60°C.*

A HER2 Control Slide (SC2) may be used as control:

*Control slides were pre-baked for 15 min at 58°C. If desired, tissue sample can be mounted next to the control cell lines. In either case, control slides have to be baked at 60°C for 2-16 h.*

4.4 Additional Materials

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

Reagents and materials

- Adhesive pistol, including hot adhesive, or rubber cement (Fixogum)
- Ethanol 100%, denatured
- Deionized or distilled water
- Xylene
- Hydrogen peroxide (H$_2$O$_2$) 30%
- Methanol 100%

- 3 -
Equipment

- Water bath (80°C, boiling)
- Hot plate
- Hybridization oven (heating oven)
- Staining jars, 50-80 ml
- Humidity chamber
- Pipet (10 µl, 1000 µl)
- Coverslips (22 mm x 22 mm, 24 mm x 32 mm)
- Light microscope

4.5 Important Information

The following should be kept in mind:

✓ The tissue and cell sections must not be allowed to dry during the hybridization and washing steps!

✓ The temperature for denaturating and washing, described in the protocol, should be used as a guide. Dependent upon the age and the fixation step of the sample material, an increase or decrease in temperature of the denaturing or wash steps can lead to better hybridization results!

5. The ZytoDot 2C SPEC HER2/CEN 17 Probe Kit Protocol

5.1 Preparatory Steps

Day 1:

- Preparation of ethanol series (70%, 85%, 95%, and 100% ethanol solutions): Dilute 7, 8.5, 9.5, and 10 parts of 100% ethanol with 3, 1.5, 0.5, and 0 parts of deionized or distilled water, respectively. These solutions can be stored in suitable containers and re-used.

- Heat Pretreatment Solution EDTA (PT2): Heat in a covered staining jar standing in a boiling water bath to at least 95°C.

- Pepsin Solution (ES1): Bring to room temperature before use.

- Preparation of 3% H₂O₂: Dilute 1 part of 30% H₂O₂ with 9 parts of 100% methanol.

Day 2:

- Wash Buffer SSC (WB1): Prepare two staining jars with Wash Buffer SSC, one at room temperature (RT), the other heated to 75°C (depending on the
number of slides, the temperature should be increased by 1°C per slide for more than two slides, but not exceed 80°C).

- **Preparation of 1x Wash Buffer TBS:** Dilute 1 part of 20x Wash Buffer TBS (WB5) in 19 parts deionized or distilled water. Diluted 1x Wash Buffer TBS lasts for one week when stored at 2-8°C.

- **Blocking Solution (BS1), Anti-DIG/Anti-DNP-Mix (AB3), Anti-Mouse-HRP-Polymer (plus) (AB4), Anti-Rabbit-AP-Polymer (AB5), Mayer’s Hematoxylin Solution (CS1), Mounting Solution (alcoholic) (MT4):** Bring to room temperature before use.

- **Preparation of AP-Red Solution:** Prior to immediate use, mix one drop of AP-Red Solution A (SB2a) and one drop of AP-Red Solution B (SB2b) in a graduated cup (e.g. AP-Red reaction vessel) and incubate up to 3 min in the dark. Add six drops of AP-Red Solution C (SB2c), fill up to 1 ml with deionized or distilled water and mix well.

- **Preparation of HRP-Green Solution:** Prior to immediate use, add two drops HRP-Green Solution A (SB3a) in a graduated cup (e.g. HRP-Green reaction vessel) and fill up to 1 ml with HRP-Green Solution B (SB3b). Add two drops of HRP-Green Solution C (SB3c) and mix well.

### 5.2 Pretreatment (Dewax/Proteolysis) [day 1]

1. Incubate slides for 10 min at 70°C (e.g. on a hot plate)
2. Incubate slides for 2x 5 min in xylene
3. Incubate for 3x 3 min in 100% ethanol
   - Alternatively, dewaxing protocols routinely used in immunohistochemistry procedures, e.g. 2x 15 min xylene, 2x 5 min 100% ethanol, 2x 5 min 96% ethanol, 1x 5 min 70% ethanol, can be used.
4. Wash 3x 2 min in deionized or distilled water
5. Incubate slides for 5 min in 3% H₂O₂
6. Wash 3x 2 min in deionized or distilled water
7. Heat Heat Pretreatment Solution EDTA (PT2) in a covered staining jar standing in a boiling water bath to at least 95°C
8. Place slides in the Heat Pretreatment Solution EDTA (PT2) and incubate for 15 min
9. Transfer slides immediately to deionized or distilled water, wash 3x 2 min and drain off or blot off the water
10. Apply (dropwise) Pepsin Solution (ES1) to the tissue/cell section and incubate for 5 min at RT in a humidity chamber

* 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. 
As an incubation guideline, we recommend an incubation time of 3-10 min for tissue and cell samples. As a general rule, we recommend to ascertain the optimum time for proteolysis in pre-tests.

11. Wash 3x 2 min in deionized or distilled water
12. Dehydration: in 70%, 85%, 95%, and 2x 100% ethanol, each for 2 min
Air dry sections.

5.3 Denaturation and Hybridization [day 1]

1. Vortex the ZytoDot 2C SPEC HER2/CEN 17 Probe (PD12) and pipette 10 µl each onto individual samples
   Distribute dropwise on the whole target area to avoid local concentration of probe. Alternatively, add probe to the center of a coverslip and place coverslip upside down on target area. A gentle warming of the probe, as well as using a pipette tip, which has been cut off to increase the size of the opening, can make the pipetting process easier.

2. Avoiding trapped bubbles, cover the samples with a coverslip (22 mm x 22 mm; 24 mm x 32 mm). Seal the coverslip, e.g. with a layer of hot glue from an adhesive pistol or with rubber cement

3. Denature the slides at 78-80°C for 5 min, e.g. on a hot plate

4. Transfer the slides to a humidity chamber and hybridize overnight at 37°C (e.g. in a hybridization oven)
   It is essential that the tissue/cell samples do not dry out during the hybridization step.

5.4 Post-Hybridization and Detection [day 2]

1. Carefully remove the rubber cement or glue

2. Remove the coverslip by submerging in Wash Buffer SSC (WB1) at RT for 5 min

3. Wash 5 min in Wash Buffer SSC (WB1) at 75-80°C
   The Wash Buffer SSC should be pre-heated. Increase temperature by 1°C per slide for more than 2 slides, but do not exceed 80°C. Check with a thermometer if necessary.

4. Wash 3x 2 min in deionized or distilled water

5. Apply Blocking Solution (BS1) dropwise (3-4 drops per slide) to the slides and incubate for 10 min at RT

6. Blot off Blocking Solution (BS1), but do not rinse!

7. Apply Anti-DIG/Anti-DNP-Mix (AB3) dropwise (3-4 drops per slide) to the slides and incubate for 30 min at 37°C in a humidity chamber
8. Wash 3x 2 min in 1x Wash Buffer TBS (prepared using WB5)
9. Apply Anti-Mouse-HRP-Polymer (plus) (AB4) dropwise (3-4 drops per slide) to the slides and incubate for 30 min at 37°C in a humidity chamber
10. Wash 3x 2 min in 1x Wash Buffer TBS (prepared using WB5)
11. Apply Anti-Rabbit-AP-Polymer (AB5) dropwise (3-4 drops per slide) to the slides and incubate for 30 min at 37°C in a humidity chamber
12. Wash 3x 2 min in 1x Wash Buffer TBS (prepared using WB5)
13. During the wash steps, prepare AP-Red Solution by mixing one drop of AP-Red Solution A (SB2a) and one drop of AP-Red Solution B (SB2b) in a graduated cup (e.g. AP-Red reaction vessel) and incubate up to 3 min in the dark. Add six drops of AP-Red Solution C (SB2c), fill up to 1 ml with deionized or distilled water and mix well
14. Apply AP-Red Solution dropwise (3-4 drops per slide) to the slides and incubate for 10 min at RT in the dark. If required, the incubation time can be shortened or extended (7-15 min)
15. Transfer slides into a staining jar and wash 2 min in running tap water
16. During the wash step, prepare HRP-Green Solution by adding two drops of HRP-Green Solution A (SB3a) in a graduated cup (e.g. HRP-Green reaction vessel) and fill up to 1 ml with HRP-Green Solution B (SB3b). Add two drops of HRP-Green Solution C (SB3c) and mix well
17. Apply HRP-Green Solution dropwise (3-4 drops per slide) to the slides and incubate for 3 min at RT in the dark. If required, the incubation time can be shortened or extended (2-5 min)
18. Wash 2 min in 1x Wash Buffer TBS (prepared using WB5)
19. Counterstain the tissue or cell samples for 8-10 s with Mayer’s Hematoxylin Solution (CS1)

   The counterstaining time depends on the nature of tissue/cell used and should therefore be optimized. Avoid dark counterstaining, because it may obscure positive staining signals.

20. Transfer slides into a staining jar and wash 2 min in running tap water
21. Dehydration: 3x 30 s in 100% ethanol (use very pure ethanol)
22. Incubate 2x 30 s in xylene (use very pure xylene)

   Do not prolong the incubation time as this might result in loss of signals!
23. Avoiding trapped bubbles, cover the samples immediately 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
24. Evaluation of the sample material is carried out by light microscopy
6. Interpretation of Results

6.1 CISH Results

The CISH hybridization signal of one single copy of a HER2 gene appears as a dark green-colored distinct dot-shaped signal, the signal of one single copy of a chromosome 17 centromeric region appears as bright red-colored distinct dot-shaped signal, which can be clearly distinguished from the background counterstained with hematoxylin. Visualization of signals should be performed using at least a 40x objective resulting in easily visible signals.

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.

Prior to signal enumeration, the tissue/cell section should be scanned for any possible intratumoral heterogeneity using a 10x or 20x objective. In case of heterogeneity, an area representative for the amplification status has to be chosen. For signal enumeration, areas of necrosis, overlapping nuclei, and nuclei with weak signal intensity should be avoided.

In normal diploid nuclei without gene amplification, 2 green and 2 red dot-shaped signals with smooth, rounded edges will be visible per nucleus (see fig. 1). 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.

In case of low gene amplifications (see fig. 3) or chromosome 17 aneusomy (see fig. 2), green HER2 gene specific signals will be visible as multiple dots or small clusters. Small clusters are irregularly shaped signals comprising an area of up to 5 dots. As a reference, a single green dot of a normal cell of the same slide must be used. Additionally, red signals of the centromere 17 control will be visible.

In case of high gene amplifications, a large number of green dots or large clusters, comprising an area greater than 5 dots, will be visible in the nuclei (see fig. 4). As a reference, a single green dot of a normal cell of the same slide must be used. Red signals might be overlaid and might not be visible any more.

In order to judge the specificity of the hybridization signals and to confirm the correct performance of the method, any hybridization should be accompanied by controls. We recommend using at least one control sample, in which the chromosome 17 and HER2 gene copy number is known. The polynucleotides contained in the ZytoDot 2C SPEC HER2/CEN 17 Probe (PD12) which recognize the alpha-satellite-sequences of the centromere of chromosome 17 function in themselves as an internal control that a successful hybridization has occurred, as well as proving the integrity of the cellular DNA. A negative or unspecific result can be caused by multiple factors. For troubleshooting, please refer to chapter 8.
6.2 Positive Control

The included HER2 Control Slides (SC2) should be used for monitoring the correct performance of the CISH experiment. For use as an on-slide-control simply mount tissue sample of interest next to the cell lines of the control slide before baking the slide.

The HER2 Control Slide (SC2) contains four different cell lines (see below) affected by different levels of HER2 amplification and one tissue (canine myocardial muscle). Cells have been embedded in red-colored paraffin, fixed in 10% neutral buffered formalin (24 h, pH 7.0), and mounted on positively charged slides in a thickness of 4 μm. Slides were pre-baked for 15 min at 58°C.

The HER2 Control Slide (SC2) is designed as follows:

<p>| | | | | |</p>
<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

1: no HER2 amplification, 1-2 HER2 and 1-2 CEN 17 signals per nucleus
2: no HER2 signals, no CEN 17 signals
3: no HER2 amplification, 1-2 HER2 and 1-2 CEN 17 signals per nucleus
4: chromosome 17 aneusomy, 3-6 HER2 and 3-6 CEN 17 signals per nucleus
5: high level HER2 amplification, large HER2 cluster per nucleus
Prior to use of HER2 Control Slide (SC2), remove the label, then name the slide with a pencil, and, if desired, mount tissue sample of interest for on-slide-control, bake slide at 60°C for a minimum of 2 h up to 16 h, and proceed with chapter 5.2 Pretreatment (Dewax/Proteolysis) using a pepsin incubation time of 10 min.

7. Literature

8. 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</td>
<td>No target sequence in test material</td>
<td>Use controls</td>
</tr>
<tr>
<td></td>
<td>Cell or tissue sample has not been fixed properly</td>
<td>Optimization of fixation time</td>
</tr>
<tr>
<td></td>
<td>Over- or underdigested tissue</td>
<td>Optimization of proteolytic pretreatment time</td>
</tr>
<tr>
<td></td>
<td>Denaturation temperature not correct</td>
<td>Check temperature; adjust temperature if necessary</td>
</tr>
<tr>
<td></td>
<td>Hybridization temperature not correct</td>
<td>Check temperature; adjust temperature if necessary</td>
</tr>
<tr>
<td></td>
<td>Hybridization time too short</td>
<td>Hybridize at least 12 h; extend hybridization time if necessary</td>
</tr>
<tr>
<td></td>
<td>Incubation with chromogenic substrate too short</td>
<td>Extend incubation time</td>
</tr>
<tr>
<td></td>
<td>Counterstaining too dark</td>
<td>Reduce counterstaining time</td>
</tr>
<tr>
<td>Green signals fades or merges</td>
<td>An aqueous mounting solution has been used</td>
<td>Use only xylene-based mounting solutions</td>
</tr>
<tr>
<td></td>
<td>Incubation time in the single alcohol components of the dehydration series too long</td>
<td>Do not excess incubation times of 30 s per alcohol step (better: rinse carefully)</td>
</tr>
<tr>
<td></td>
<td>Sections were not dehydrated properly</td>
<td>Use fresh ethanol and xylene solutions; use only xylene of “pure” quality</td>
</tr>
<tr>
<td></td>
<td>Substrate reaction is too intensive</td>
<td>Shorten substrate incubation time; do not heat substrate solution over 25°C; incubate at room temperature only</td>
</tr>
<tr>
<td></td>
<td>Slides were air dried before mounting</td>
<td>Apply mounting solution immediately after dehydration series</td>
</tr>
<tr>
<td></td>
<td>Denaturation too strong</td>
<td>Reduce denaturation temperature to 76°C</td>
</tr>
<tr>
<td>Weak red signals</td>
<td>Incubation time in xylene components before mounting too long</td>
<td>Do not excess incubation times of 30 s per xylene step</td>
</tr>
<tr>
<td></td>
<td>Precipitates in AP-Red solution</td>
<td>Warm AP-Red solution for 10-15 min at 37°C and mix thoroughly by inversion</td>
</tr>
<tr>
<td>Uneven / 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 concentration 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>--------------------------------------</td>
<td>----------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Proteolytic pretreatment too strong</td>
<td>Optimization of incubation time</td>
<td></td>
</tr>
<tr>
<td>Dehydration of sections between the individual incubation steps</td>
<td>Prevent dehydration</td>
<td></td>
</tr>
<tr>
<td>Washing temperature following hybridization too low</td>
<td>Check temperature</td>
<td></td>
</tr>
<tr>
<td>Prolonged substrate incubation time</td>
<td>Shorten substrate incubation time</td>
<td></td>
</tr>
<tr>
<td>Endogenous levamisole-resistant alkaline phosphatase</td>
<td>Additional blocking with Bouin’s Solution or 1M citric acid free acid for 1-10 min</td>
<td></td>
</tr>
<tr>
<td>Section floats off the slide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated slide coating</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unsuitable slide coating</td>
<td>Use appropriate slides</td>
<td></td>
</tr>
<tr>
<td>Proteolytic pretreatment too strong</td>
<td>Shortening of incubation time</td>
<td></td>
</tr>
<tr>
<td>Untreated slide coating</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>