



## ZytoDot 2C CISH Implementation Kit

**REF** C-3044-10  $\Sigma$  10

**REF** C-3044-40  $\Sigma$  40

For use in chromogenic *in situ* hybridization (CISH)  
procedures

4250380N717V



In vitro diagnostic medical device  
according to IVDR (EU) 2017/746

### 1. Intended use

The ZytoDot 2C CISH Implementation Kit is intended to be used in combination with Digoxigenin/Dinitrophenyl-labeled ZytoDot 2C CISH Probes on formalin-fixed, paraffin-embedded specimens by chromogenic *in situ* hybridization (CISH).

The product is intended for professional use only. All tests using the product should be performed in a certified, licensed anatomic pathology laboratory under the supervision of a pathologist/human geneticist by qualified personnel.

### 2. 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.

### 3. Reagents provided

The ZytoDot 2C CISH Implementation Kit is available in two sizes and is composed of:

Code	Component	Quantity		Container
		40 $\Sigma$	10 $\Sigma$	
PT2	Heat Pretreatment Solution EDTA	500 ml	150 ml	Screw-cap bottle (large)
ES1	Pepsin Solution	4 ml	1 ml	Dropper bottle, white cap
WB1	Wash Buffer SSC	560 ml	210 ml	Screw-cap bottle (large)
WB5	20x Wash Buffer TBS	2x 50 ml	50 ml	Screw-cap bottle
AB14	Anti-DIG/DNP-Mix	4 ml	1 ml	Dropper bottle, yellow cap
AB13	HRP/AP-Polymer-Mix	4 ml	1 ml	Dropper bottle, blue cap
SB6a	AP-Red Solution A	0.4 ml	0.1 ml	Dropper bottle, red cap (small)
SB6b	AP-Red Solution B	15 ml	4 ml	Dropper bottle, red cap
SB7a	HRP-Green Solution A	0.8 ml	0.2 ml	Dropper bottle, green cap (small)
SB7b	HRP-Green Solution B	15 ml	4 ml	Dropper bottle, green cap
CS2	Nuclear Blue Solution	20 ml	4 ml	Screw-cap bottle, black
MT4	Mounting Solution (alcoholic)	4 ml	1 ml	Glass bottle, brown
	AP-Red reaction vessel	2	1	Graduated cup, red lid
	HRP-Green reaction vessel	2	1	Graduated cup, green lid
	Instructions for use	1	1	

**C-3044-10 (10 tests):** Components **ES1**, **AB14**, **AB13**, **SB6a-b**, **SB7a-b**, **CS2**, and **MT4** are sufficient for 10 reactions. Component **PT2** is sufficient for 2 staining jars of 70 ml each. Component **WB1** is sufficient for 3 staining jars of 70 ml each. Component **WB5** is sufficient for 14 staining jars of 70 ml each.

**C-3044-40 (40 tests):** Components **ES1**, **AB14**, **AB13**, **SB6a-b**, **SB7a-b**, **CS2**, and **MT4** are sufficient for 40 reactions. Component **PT2** is sufficient for 7 staining jars of 70 ml each. Component **WB1** is sufficient for 8 staining jars of 70 ml each. Component **WB5** is sufficient for 28 staining jars of 70 ml each.

### 4. Materials required but not provided

- ZytoDot 2C CISH Probe
- 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  $\mu$ l, 1000  $\mu$ l)
- Staining jars or baths
- Timer
- Calibrated thermometer
- Ethanol or reagent alcohol
- Xylene
- Methanol 100%
- Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) 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)

### 5. 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.

## 6. 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. 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 request for the professional user.
- Do not reuse reagents, unless reuse is explicitly permitted!
- Avoid cross-contamination of samples as this may lead to erroneous results.
- The specimens must not be allowed to dry during the hybridization and washing steps.

### Hazard and precautionary statements for AB13, AB14, PT2, SB7b, WB1 and WB5:

The hazard-determining component is a reaction mass of: 5-chloro-2-methyl-4-isothiazolin-3-one [EC no. 247-500-7] and 2-methyl-2H-isothiazol-3-one [EC no. 220-239-6] (3:1).



#### Warning

- |           |  |
|-----------|--|
| H317      | May cause an allergic skin reaction.                                       |
| P261      | Avoid breathing dust/fume/gas/mist/vapours/spray.                          |
| P272      | Contaminated work clothing should not be allowed out of the workplace.     |
| P280      | Wear protective gloves/protective clothing/eye protection/face protection. |
| P302+P352 | IF ON SKIN: Wash with plenty of water.                                     |
| P333+P313 | IF skin irritation or rash occurs: Get medical advice/attention.           |
| P362+P364 | Take off contaminated clothing and wash it before reuse.                   |

### Hazard and precautionary statements for SB7a:

The hazard-determining components are methanol and hydrogen peroxide solution 30 %.



#### Danger

- |                    |  |
|--------------------|--|
| H225               | Highly flammable liquid and vapour.  |
| H301+H311<br>+H331 | Toxic if swallowed, in contact with skin or if inhaled.  |
| H370               | Causes damage to organs.   |
| P210               | Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking. |
| P233               | Keep container tightly closed.   |
| P260               | Do not breathe dust/fume/gas/mist/vapours/spray.   |
| P280               | Wear protective gloves/protective clothing/eye protection/face protection.                     |
| P308+P311          | IF exposed or concerned: Call a POISON CENTER/doctor.  |
| P403+P235          | Store in a well-ventilated place. Keep cool.   |

### Hazard and precautionary statements for CS2:

The hazard-determining component is ethanediol, ethylene glycol.



#### Warning

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|------|--|
| H373 | May cause damage to kidneys through prolonged or repeated exposure if swallowed. |
| P260 | Do not breathe dust/fume/gas/mist/vapours/spray.                                 |
| P314 | Get medical advice/attention if you feel unwell.                                 |

### Hazards and precaution statements for MT4:

The hazard-determining component is xylene.



#### Warning

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|---------------------|--|
| H226                | Flammable liquid and vapour.   |
| H312+H332           | Harmful in contact with skin or if inhaled.  |
| H315                | Causes skin irritation.  |
| H319                | Causes serious eye irritation.   |
| H335                | May cause respiratory irritation.  |
| H373                | May cause damage to organs through prolonged or repeated exposure.   |
| P210                | Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking.                                   |
| P260                | Do not breathe dust/fume/gas/mist/vapours/spray.   |
| P280                | Wear protective gloves/protective clothing/eye protection/face protection.   |
| P305+P351 +<br>P338 | IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. |
| P337+P313           | If eye irritation persists: Get medical advice/attention.  |
| P403+P235           | Store in a well-ventilated place. Keep cool.   |
| EUH208              | Contains methyl 2-methylprop-2-enoate; methyl 2-methylpropenoate; methyl methacrylate. May produce an allergic reaction.         |

### Hazard and precautionary statements of SB6a:

- |      |  |
|------|--|
| H412 | Harmful to aquatic life with long lasting effects. |
| P273 | Avoid release to the environment.                  |

### Special labeling of ES1:

- |        |  |
|--------|--|
| EUH208 | Contains Pepsin A. May produce an allergic reaction. |
| EUH210 | Safety data sheet available on request.              |

## 7. 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/human geneticist to be familiar with the ISH 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/human geneticist 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 performance was validated using the procedures described in the instruction for use of the respective ZytoVision probe and implementation kit. Modifications to these procedures might alter the performance and have to be validated by the user. This IVD is only certified as CE when used as described in this instruction for use within the scope of the intended use.

## 8. 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

## 9. 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  $\leq 0.5 \text{ cm}^3$ .
- Use premium quality paraffin.
- Embedding should be carried out at temperatures lower than 65°C.
- Prepare 3-5  $\mu\text{m}$  microtome sections.
- Use positively charged microscope slides.
- Fix tissue sections for 2-16 h at 50-60°C.

## 10. Preparatory treatment of the device

20x Wash Buffer TBS (WB5) is to be prepared according to the instructions in 11. "Assay procedure". All other kit reagents are ready-to-use. No reconstitution, mixing, or dilution is required.

Bring probe to room temperature (RT) and mix briefly before use.

## 11. Assay procedure

### 11.1 Day 1

#### Preparatory steps

1. Prepare an ethanol series (70%, 90%, and 100% ethanol solutions): Dilute 100% ethanol with deionized or distilled water. These solutions can be stored in suitable containers and can be re-used.
2. Heat Pretreatment Solution EDTA (PT2): Heat to 98°C in a covered staining jar.
3. Preparation of 3% H<sub>2</sub>O<sub>2</sub>: Dilute 1 part 30% H<sub>2</sub>O<sub>2</sub> in 9 parts 100% methanol.
4. ZytoDot 2C CISH Probe: Bring to room temperature before use.

#### Pretreatment (dewax/proteolysis)

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 slides for 3x 3 min in 100% ethanol.
4. Incubate slides for 5 min in 3% H<sub>2</sub>O<sub>2</sub>.
5. Wash slides 2x 1 min in deionized or distilled water.
6. Incubate for 15 min in pre-warmed Heat Pretreatment Solution EDTA (PT2) at 98°C.

*Use eight slides per staining jar (add dummy slides if needed).*

7. Transfer slides immediately to deionized or distilled water and wash for 2x 2 min.
8. Apply (dropwise) Pepsin Solution (ES1) to the specimen and incubate for 5-15 min at 37°C in a humidity chamber.

*ES1 may form precipitates, which do not affect the quality.*

*As a general rule, we recommend to ascertain the optimum time for proteolysis in pre-tests.*

9. Immerse slides in deionized or distilled water.
10. Dehydration in: 70%, 90%, and 100% ethanol, each for 1 min.
11. Air dry sections.

*Note: Make sure to completely dry sections prior to probe application.*

#### Denaturation and hybridization

1. Pipette 10  $\mu\text{l}$  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.*

### 11.2 Day 2

#### Preparatory steps

1. Wash Buffer SSC (WB1): For stringency wash, heat to 80°C in a covered staining jar. **WB1** may form precipitates at 2-8°C, which do not affect the quality and should dissolve when heated.
2. 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 is stable for one week when stored at 2-8°C.*

3. Anti-DIG/DNP-Mix (AB14), HRP/AP-Polymer-Mix (AB13), AP-Red Solution A (SB6a), AP-Red Solution B (SB6b), HRP-Green Solution A (SB7a), HRP-Green Solution B (SB7b), Nuclear Blue Solution (CS2), Mounting Solution (alcoholic) (MT4): Bring to room temperature before use.

*Components **SB7a** and **SB7b** may form precipitates, which do not affect the staining quality.*

#### Post-hybridization and detection

1. Carefully remove the rubber cement or glue.
2. Remove the coverslip by submerging the slides in Wash Buffer SSC (WB1) at room temperature for 5 min.

*WB1 can be reused once. Store at 2-8°C for a maximum of one week.*

3. Wash slides for 5 min in Wash Buffer SSC (WB1) at 80°C.

*Use eight slides per staining jar (add dummy slides if needed).*

4. Wash slides 2x 1 min in deionized or distilled water.
5. Immerse slides in 1x Wash Buffer TBS.
6. Apply Anti-DIG/DNP-Mix (AB14) (1-2 drops per slide) to the slides and incubate for 15 min at 37°C in a humidity chamber.
7. Wash slides 3x 1 min in 1x Wash Buffer TBS.
8. Apply HRP/AP-Polymer-Mix (AB13) (1-2 drops per slide) to the slides and incubate for 15 min at 37°C in a humidity chamber.
9. Wash slides 3x 1 min in 1x Wash Buffer TBS.
10. Prepare AP-Red Solution (working solution): fill 1 ml AP-Red Solution B (**SB6b**) in a graduated cup and add one drop (30  $\mu\text{l}$ ) of AP-Red Solution A (**SB6a**). Mix well.
11. Apply AP-Red Solution (1-2 drops per slide) to the slides and incubate in the dark for 10 min at RT.
12. During the incubation, prepare HRP-Green Solution (working solution): fill 1 ml HRP-Green Solution B (SB7b) in a graduated cup and add two drops (2x 20  $\mu\text{l}$ ) HRP-Green Solution A (SB7a). Mix well.
13. Wash slides for 2 min in deionized or distilled water.
14. Apply HRP-Green Solution dropwise (1-2 drops per slide) to the slides and incubate in the dark for 10 min at room temperature.

15. Wash slides for 2 min in deionized or distilled water.
16. Counterstain specimens for 2 min with **Nuclear Blue Solution (CS2)**.
17. Transfer slides into a staining jar and wash 2 min under cold running tap water.
18. Dehydrate 3x 30 s in 100% ethanol (use very pure ethanol).
19. Incubate slides for 2x 30 s in xylene (use very pure xylene).

*Do not prolong or shorten the incubation time as this might result in loss of signals!*

20. 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)**. Allow 20-30 min for the coverslip to become immobilized.

*Using a pipette tip which has been cut off to increase the size of the opening, can make the pipetting process easier.*

21. Evaluate stained specimens by using light microscopy.

## 12. Interpretation of results

Using the **ZytoDot 2C CISH Implementation Kit**, hybridization signals of Digoxigenin-labeled polynucleotides appear as dark green colored distinct dots, and Dinitrophenyl-labeled polynucleotides appear as bright red colored distinct dots. In interphases or metaphases of normal cells or cells without aberrations of the examined chromosomes, two signals per probe/hapten-label will appear, except for probes targeting X and/or Y chromosomes, resulting in none to two signals per probe/hapten-label, depending on the gender. In cells with chromosomal aberrations, a different signal pattern can be visible in interphases or metaphases. For more details on the interpretation of results, please refer to the instructions for use of the respective **ZytoDot 2C CISH Probe**.

## 13. Recommended quality control procedures

Refer to the instructions for use of the respective **ZytoVision** probe.

## 14. Performance characteristics

Refer to the instructions for use of the respective **ZytoVision** probe.

## 15. Disposal

The disposal of reagents must be carried out in accordance with local regulations.

## 16. Troubleshooting

Any deviation from the operating instructions can lead to inferior staining results or to no staining at all. Please refer to [www.zytovision.com](http://www.zytovision.com) for more information.

### Weak signals or no signals at all

Possible cause	Action
Proteolytic pretreatment not carried out properly	Optimize pepsin incubation time, increase or decrease if necessary
Probe evaporation	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
Counterstaining time too long	Avoid dark counterstaining, because it may obscure positive staining signals
Bluing of counterstain not carried out properly	Use cold running tap water for bluing; do not use warm or hot water, or bluing reagents

### Signals too strong

Possible cause	Action
Proteolytic pretreatment carried out too long	Optimize pepsin incubation time, increase or decrease if necessary

AP-Red Solution incubation time not correct	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
HRP-Green solution incubation time not correct	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

### Red signals too weak

Possible cause	Action
AP-Red Solution was exposed to strong direct light	Prepare and use AP-Red Solution protected from strong direct light
AP-Red Solution was prepared too early	Prepare prior to immediate use
AP-Red Solution incubation time not correct	If required, the incubation time can be extended up to 15 min
Insufficient preparation of chromogenic substrate	Do not increase volume of Solution A

### Green signals too weak

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

### Signals fade or merge

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

### 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

### 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
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**Morphology degraded**

Possible cause	Action
Cell or tissue sample has not been properly fixed	Optimize fixing time and fixative
Proteolytic pretreatment not carried out too long	Decrease pepsin incubation time

**Cross hybridization signals; noisy background**

Possible cause	Action
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 $\mu\text{m}$ microtome sections

**Specimen floats off the slide**

Possible cause	Action
Proteolytic pretreatment too strong	Shorten pepsin incubation time

**17. Literature**

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- Tsukamoto T, et al. (1991) *Int J Dev Biol* 35: 25-32.
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**18. Revision**



[www.zytovision.com](http://www.zytovision.com)

Please refer to [www.zytovision.com](http://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 [helptech@zytovision.com](mailto:helptech@zytovision.com)



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