

ZytoDot 2C CISH Implementation Kit

REF C-3044-40 ∇_{Σ} 40

REF C-3044-10 ∇_{Σ} 10

For chromogenic *in situ* hybridization (CISH) using any
ZytoDot 2C CISH Probe



IVD

In vitro diagnostic medical device

according to EU directive 98/79/EC



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1. Scope of Application

The ZytoDot 2C CISH Implementation Kit is designed to be used for the detection of human DNA sequences 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 CISH Implementation Kit is to be used with any separately available digoxigenin (DIG) and dinitrophenyl (DNP) labeled ZytoDot 2C CISH probe.

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 CISH Implementation Kit

4.1 Components

The kit is made up of the following components:

Code	Component	Quantity		Container
		40	10	
PT2	<u>Heat Pretreatment Solution EDTA</u>	500 ml	150 ml	Screw-cap bottle (large)
ES1	<u>Pepsin Solution</u>	4 ml	1 ml	Dropper bottle, white cap
WB1	<u>Wash Buffer SSC</u>	500 ml	150 ml	Screw-cap bottle (large)
WB5	<u>20x Wash Buffer TBS</u>	2x 50 ml	50 ml	Screw-cap bottle
AB14	<u>Anti-DIG/DNP-Mix</u>	4 ml	1 ml	Dropper bottle, yellow cap
AB13	<u>HRP/AP-Polymer-Mix</u>	4 ml	1 ml	Dropper bottle, blue cap
SB6a	<u>AP-Red Solution A</u>	0.4 ml	0.1 ml	Dropper bottle, red cap (small)
SB6b	<u>AP-Red Solution B</u>	15 ml	4 ml	Dropper bottle, red cap
SB7a	<u>HRP-Green Solution A</u>	0.8 ml	0.2 ml	Dropper bottle, green cap (small)
SB7b	<u>HRP-Green Solution B</u>	15 ml	4 ml	Dropper bottle, green cap
CS2	<u>Nuclear Blue Solution</u>	20 ml	4 ml	Screw-cap bottle, black
MT4	<u>Mounting Solution (alcoholic)</u>	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
	Instruction manual	1	1	

C-3044-40 (40 tests): Components **(ES1)**, **(AB14)**, **(AB13)**, **(SB6a-b)**, **(SB7a-b)**, **(CS2)**, 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-3044-10 (10 tests): Components, **(ES1)**, **(AB14)**, **(AB13)**, **(SB6a-b)**, **(SB7a-b)**, **(CS2)**, 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. 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 CISH Implementation 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 cm³.
- ✓ Standard processing and paraffin embedding
Use premium quality paraffin. Infiltration and embedding should be carried out at temperatures lower than 65°C.
- ✓ Prepare 3-5 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.

4.4 Additional Materials

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

Reagents and materials

- ZytoDot 2C CISH probe
- Adhesive pistol, including hot adhesive, or rubber cement (Fixogum)
- Ethanol 100%, denatured
- Deionized or distilled water
- Xylene
- Hydrogen peroxide (H₂O₂) 30%
- Methanol 100%

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 denaturing 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 CISH Implementation Kit Protocol

5.1 Preparatory Steps

Day 1:

- *Preparation of ethanol series (70%, 90%, and 100% ethanol solutions):* Dilute 7, 9, and 10 parts of 100% ethanol with 3, 1, 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.
- *Anti-DIG/DNP-Mix (AB14), HRP/AP-Polymer-Mix (AB13), Nuclear Blue Solution (CS2), Mounting Solution (alcoholic) (MT4):* Bring to room temperature before use.
- *Preparation of AP-Red Solution:* Prior to immediate use, add one drop (30 µl) of AP-Red Solution A (SB6a) in a graduated cup (e.g. AP-Red reaction vessel), fill up to 1 ml with AP-Red Solution B (SB6b) and mix well.

*Do not expose to **strong** direct light.*

- *Preparation of HRP-Green Solution:* Prior to immediate use, add two drops (2x 20 μ l) HRP-Green Solution A (SB7a) in a graduated cup (e.g. HRP-Green reaction vessel), fill up to 1 ml with HRP-Green Solution B (SB7b) 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. Incubate slides for 5 min in 3% H₂O₂
 5. Wash 2x 1 min in deionized or distilled water
 6. Place slides in the pre-heated Heat Pretreatment Solution EDTA (PT2) and incubate for 15 min
 7. Transfer slides immediately to deionized or distilled water, wash 2x 2 min and drain off or blot off the water
 8. 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.
 9. Immerse slides in deionized or distilled water and drain off or blot off the water
 10. Dehydration: in 70%, 90%, and 100% ethanol, each for 1 min
- Air dry sections.

5.3 Denaturation and Hybridization [day 1]

1. Vortex the ZytoDot 2C CISH Probe and pipette 10 μ l 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). 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 2x 1 min in deionized or distilled water
5. Immerse slides in 1x Wash Buffer TBS (prepared using **WB5**) and drain off or blot off the 1x Wash Buffer TBS
6. Apply Anti-DIG/DNP-Mix (AB14) dropwise (3-4 drops per slide) to the slides and incubate for 15 min at 37°C in a humidity chamber
7. Wash 3x 1 min in 1x Wash Buffer TBS (prepared using **WB5**) and drain off or blot off the 1x Wash Buffer TBS
8. Apply HRP/AP-Polymer-Mix (AB13) dropwise (3-4 drops per slide) to the slides and incubate for 15 min at 37°C in a humidity chamber
9. During the incubation, prepare AP-Red Solution by adding one drop (30 µl) of AP-Red Solution A (SB6a) in a graduated cup (e.g. AP-Red reaction vessel), fill up to 1 ml with AP-Red Solution B (SB6b) and mix well
Do not expose to strong direct light.
10. Wash 3x 1 min in 1x Wash Buffer TBS (prepared using **WB5**)
11. Apply AP-Red Solution dropwise (3-4 drops per slide) to the slides and incubate for 10 min at RT (protect from strong direct light). If required, the incubation time can be shortened or extended (7-15 min)
12. During the incubation, prepare HRP-Green Solution by adding two drops (2x 20 µl) of HRP-Green Solution A (SB7a) in a graduated cup (e.g. HRP-Green reaction vessel), fill up to 1 ml with HRP-Green Solution B (SB7b) and mix well
13. Wash 2 min in deionized or distilled water and drain off or blot off the water

14. Apply HRP-Green Solution dropwise (3-4 drops per slide) to the slides and incubate for 10 min at RT (protect from strong direct light). If required, the incubation time can be shortened or extended (5-15 min)

15. Wash 2 min in deionized or distilled water

16. Counterstain the tissue or cell samples for 2 min with Nuclear Blue Solution (CS2)

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.

17. Transfer slides into a staining jar and wash 2 min in running tap water

18. Dehydration: 3x 30 s in 100% ethanol (use very pure ethanol)

19. Incubate 2x 30 s in xylene (use very pure xylene)

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

20. 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

21. Evaluation of the sample material is carried out by light microscopy

6. Interpretation of Results

Probes labeled with digoxigenin (DIG) will result in permanent dark-green signals when using HRP-Green solution, probes labeled with dinitrophenyl (DNP) will result in permanent bright-red signals when using AP-Red solution.

By using appropriate probes, 2 green and 2 red distinct dot-shaped signals with smooth, rounded edges will be visible per nucleus in normal diploid cells. 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.

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.

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 Nuclear Blue 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 8.

7. Literature

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8. Problems and Possible Causes

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

Problem	Possible cause	Action
Poor tissue morphology	Overdigested tissue	Shorten the pepsin incubation time
Weak signals or no signals	No target sequence in test material	Use control slides
	Cell or tissue sample has not been fixed properly	Optimization of fixation time; check quality of fixative and its compatibility with <i>in situ</i> -hybridization systems
	Over- or underdigested tissue	Optimization of proteolytic pretreatment time
	Denaturation temperature not correct	Check temperature; adjust temperature if necessary
	Hybridization temperature not correct	
	Stringency wash temperature not correct	
	Antibody incubation temperature not correct	Hybridize for at least 12 h; extend hybridization time if necessary
	Hybridization time too short	
	Incubation with chromogenic substrate too short	Extend incubation time
	Chromogenic substrates were prepared too early	Prepare chromogenic substrates prior to immediate use
	Counterstaining too dark	Reduce counterstaining time
Insufficient preparation of chromogenic substrates	Instead of using one drop of AP-Red Solution A use 30 μ l, and instead of using two drops of HRP-Green Solution A use 40 μ l, and fill up to 1 ml with the respective Solution B	
Weak red signals	AP-Red Solution was exposed to strong direct light	Do not prepare AP-Red Solution or perform staining in direct strong light
Weak green signals	Incubation times of any washing steps after staining with HRP-Green too long	Do not exceed given incubation times
	Counterstaining time too long	Reduce counterstaining time
Green signals fades or merges	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
	Substrate reaction is too intensive	Shorten substrate incubation time; do not heat substrate solution over 25°C; incubate at room temperature only

Uneven / in some parts only very light staining	Incomplete dewaxing	Use fresh solutions; check length 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
Strong background staining	Slides not thoroughly rinsed	Use fresh wash buffers 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
	Washing temperature following hybridization too low	Check temperature; adjust temperature if necessary
	Prolonged substrate incubation time	Shorten substrate incubation time
	Endogenous levamisole-resistant alkaline phosphatase	Additional blocking with Bouin's Solution or 1M citric acid free acid for 1-10 min
Section floats off the slide	Unsuitable slide coating	Use silane-coated or adhesion slides



ZytoVision GmbH · Fischkai 1
D - 27572 Bremerhaven · Germany
Phone: +49 (0)471/4832 - 300
Fax: +49 (0)471/4832 - 509
www.zytovision.com
info@zytovision.com



Your local distributor