ZYTOVISION

ZytoDot 2C
CISH Polymer Detection Kit

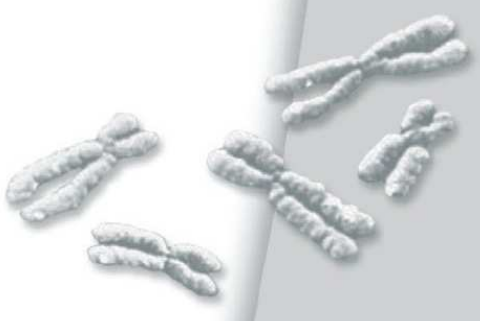
REF C-3028-40 Σ 40

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

研究用です。診断用には使用できません

In vitro diagnostic medical device

according to EU directive 98/79/EC



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Contents

1. Scope of Application

The ZytoDot 2C CISH Polymer Detection Kit is designed to be used for the detection of digoxigenin (DIG)-labeled and dinitrophenyl (DNP)-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 HRP-Green solution. DNP-labeled probes are detected using primary (unmarked) anti-DNP antibodies, secondary polymerized AP-conjugated antibodies, and AP-Red solution.

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 2C CISH Polymer Detection Kit

3.1 Components

The kit is made up of the following components:

Code	Component	Quantity Σ 40	Container
WB5	20x Wash Buffer TBS	2x 50 ml	Screw-cap bottle
BS1	Blocking Solution	4 ml	Dropper bottle, orange cap
AB3	Anti-DIG/Anti-DNP-Mix	4 ml	Dropper bottle, yellow cap
AB4	Anti-Mouse-HRP-Polymer (plus)	4 ml	Dropper bottle, blue cap
AB5	Anti-Rabbit-AP-Polymer	4 ml	Dropper bottle, red cap
SB2a	AP-Red Solution A	0.3 ml	Dropper bottle, red cap (small)
SB2b	AP-Red Solution B	0.3 ml	Dropper bottle, red cap (small)
SB2c	AP-Red Solution C	2 ml	Dropper bottle, red cap (medium)
SB3a	HRP-Green Solution A	0.6 ml	Dropper bottle, green cap (small)
SB3b	HRP-Green Solution B	10 ml	Dropper bottle, green cap
SB3c	HRP-Green Solution C	0.6 ml	Dropper bottle, green cap (small)
CS1	Mayer's Hematoxylin Solution	20 ml	Screw-cap bottle, black
MT4	Mounting Solution (alcoholic)	4 ml	Glass bottle, brown
	AP-Red reaction vessel	2	Graduated cup, red lid
	HRP-Green reaction vessel	2	Graduated cup, green lid
	Instruction manual	1	

Components **(BS1)**, **(AB3)**, **(AB4)**, **(AB5)**, **(SB2a-c)**, **(SB3a-c)**, **(CS1)**, and **(MT4)** are sufficient for 40 reactions. Component **(WB5)** is sufficient for 27 staining jars of 70 ml each.

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 2C 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/dinitrophenyl-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 5 min in 3% H_2O_2 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

- *Ethanol 100%, denatured*
- *Deionized or distilled water*
- *Xylene*

Equipment

- *Hybridization oven (heating oven)*
- *Staining jars, 50-80 ml*
- *Humidity chamber*
- *Coverslips (22 mm x 22 mm, 24 mm x 32 mm)*
- *Light microscope*

4. The ZytoDot 2C CISH Polymer Detection Kit Protocol

4.1 Preparatory Steps

- *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.

4.2 Detection

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

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 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

5. Wash 3x 2 min in 1x Wash Buffer TBS (prepared using**WB5**)

6. 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

7. Wash 3x 2 min in 1x Wash Buffer TBS (prepared using**WB5**)

8. 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

9. Wash 3x 2 min in 1x Wash Buffer TBS (prepared using**WB5**)

10. 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 (i.e. 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

11. 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)

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

13. During the wash step, prepare HRP-Green Solution by adding two drops of HRP-Green Solution A (**SB3a**) in a graduated cup (i.e. 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

14. 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)

15. Wash 2 min in 1x Wash Buffer TBS (prepared using **WB5**)

16. Counterstain the tissue or cell samples for 8-10 s with Mayer's Hematoxylin Solution (**CS1**)

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

5. 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 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 Zyto*Dot* CISH system by ZytoVision.

For troubleshooting, please refer to chapter 7.

6. Literature

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7. 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
Streaks on the slide after stopping the pepsin treatment	Precipitation	Wash section immediately in deionized or distilled water
Weak signal or no signal	No target sequence in test material	Use controls
	Cell or tissue sample has not been fixed properly	Optimization of fixation time
	Over- or underdigested tissue	Optimization of proteolytic pretreatment time
	Denaturation temperature not correct	Check temperature; adjust temperature if necessary
	Hybridization temperature not correct	Check temperature; adjust temperature if necessary
	Hybridization time too short	Hybridize at least 12 h; extend hybridization time if necessary
	Incubation with chromogenic substrate too short	Extend incubation time
	Counterstaining too dark	Reduce counterstaining time
Green signals fades or merges	An aqueous mounting solution has been used	Use only xylene-based mounting solutions
	Incubation time in the single alcohol components of the dehydration series too long	Do not excess incubation times of 30 s per alcohol step (better: rinse carefully)
	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
	Slides were air dried before mounting	Apply mounting solution immediately after dehydration series
	Denaturation too strong	Reduce denaturation temperature to 76°C
Weak red signals	Incubation time in xylene components before mounting too long	Do not excess incubation times of 30 s per xylene step
	Precipitates in AP-Red solution	Warm AP-Red solution for 10-15 min at 37°C and mix thoroughly by inversion
Uneven / in some parts only very light staining	Incomplete dewaxing	Use fresh solutions; check length of dewaxing times

Cross hybridization signals; strong background staining	Probe concentration per area too high	Reduce probe volume per section/area, distribute probe dropwise to avoid local concentration
	Proteolytic pretreatment too strong	Optimization of incubation time
	Dehydration of sections between the individual incubation steps	Prevent dehydration
	Washing temperature following hybridization too low	Check temperature
	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	Proteolytic pretreatment too strong	Shortening of incubation time
	Unsuitable slide coating	Use appropriate slides

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