ZYTOVISION

Zyto*Dot*
CISH Implementation Kit

REF C-3018-40 Σ 40

REF C-3018-10 Σ 10

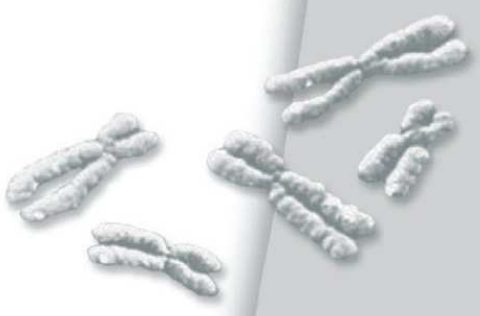
For chromogenic *in situ* hybridization (CISH) using any Zyto*Dot* CISH Probe

CE

IVD

In vitro diagnostic medical device

according to EU directive 98/79/EC



As of: March 1, 2011 (5.0)

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1. Scope of Application

The ZytoDot 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 the 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 CISH Implementation Kit is to be used with any separately available digoxigenin-labeled ZytoDot CISH probe.

Duplex formation of the digoxigenin-labeled probe can be visualized using a primary (unmarked) anti-digoxigenin antibody, which is detected by a secondary polymerized enzyme-conjugated antibody. The enzymatic reaction of DAB (diaminobenzidine) leads to the formation of strong permanent brown 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 CISH Implementation Kit

4.1 Components

The kit is made up of the following components:

Code	Component	Quantity		Container
		40 (Σ)	10	
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	500 ml	150 ml	Screw-cap bottle (large)
WB4	PBS/Tween	2	1	Aluminum pack
BS1	Blocking Solution	4 ml	1 ml	Dropper bottle, orange cap
AB1	Mouse-Anti-DIG	4 ml	1 ml	Dropper bottle, pink cap
AB2	Anti-Mouse-HRP-Polymer	4 ml	1 ml	Dropper bottle, violet cap
SB1a	DAB Solution A	0.3 ml	0.1 ml	Dropper bottle, green cap
SB1b	DAB Solution B	10 ml	2 ml	Dropper bottle, grey cap
CS1	Mayer's Hematoxylin Solution	20 ml	4 ml	Screw-cap bottle, black
MT4	Mounting Solution (alcoholic)	4 ml	1 ml	Glass bottle, brown
	Instruction manual	1	1	

C-3018-40 (40 tests): Components **(ES1)**, **(BS1)**, **(AB1)**, **(AB2)**, **(SB1a-b)**, **(CS1)**, and **(MT4)** are sufficient for 40 reactions. Components **(PT2)** and **(WB1)** are sufficient for 7 staining jars of 70 ml each. Component **(WB4)** is sufficient for 27 staining jars of 70 ml each.

C-3018-10 (10 tests): Components **(ES1)**, **(BS1)**, **(AB1)**, **(AB2)**, **(SB1a-b)**, **(CS1)**, and **(MT4)** are sufficient for 10 reactions. Components **(PT2)** and **(WB1)** are sufficient for 2 staining jars of 70 ml each. Component **(WB4)** 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 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 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 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 CISH probe*
- *Adhesive pistol, including hot adhesive, or rubber cement (Fixogum)*
- *Ethanol 100%, denatured*
- *Deionized or distilled water*
- *Xylene*
- *Hydrogen peroxide (H_2O_2) 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 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 CISH Implementation 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.

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 PBS/Tween*: Add 1 tablet of PBS/Tween **(WB4)** to 1000 ml deionized or distilled water and dissolve. Fill nine staining jars with PBS/Tween.
- *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 3% H_2O_2:* Dilute 1 part of 30% H_2O_2 with 9 parts of 100% methanol.

- *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).

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 in 3x 3 min in 100% ethanol

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

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

6. Place slides in the Heat Pretreatment Solution EDTA **(PT2)** and incubate for 15 min

7. Transfer slides immediately to deionized or distilled water, wash 3x 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. Wash 3x 2 min in deionized or distilled water

10. 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 Zyto*Dot* CISH probe 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). Seal the coverslip, e.g. with a layer of hot glue from an adhesive pistol or with rubber cement

3. Denature the slides at 94-95°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. Incubate slides for 10 min in 3% H_2O_2

6. Wash 3x 2 min in PBS/Tween (prepared using **WB4**)

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

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

9. Apply Mouse-Anti-DIG **(AB1)** dropwise (3-4 drops per slide) to the slides and incubate for 30 min at RT

10. Wash 3x 2 min in PBS/Tween (prepared using **WB4**)

11. Apply Anti-Mouse-HRP-Polymer **(AB2)** dropwise (3-4 drops per slide) to the slides and incubate for 30 min at RT

12. Wash 3x 2 min in PBS/Tween (prepared using **WB4**)

13. 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)**

14. Apply DAB Solution dropwise (3-4 drops per slide) to the slides and incubate for 30 min at RT

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

16. 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.

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

18. Dehydration: in 70%, 85%, 95%, and 2x 100% ethanol, each for 2 min

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

Air dry sections for approximately 15 min

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)** 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

The CISH hybridization signal of one single copy of a gene (or a centromeric region) appears as a DAB brown-colored distinct dot-shaped signal, which can be clearly distinguished from the background counterstained with hematoxylin. Visualization of signals should be performed using a 40x objective resulting in easily visible signals. At 20x, dots are small but still clearly recognizable.

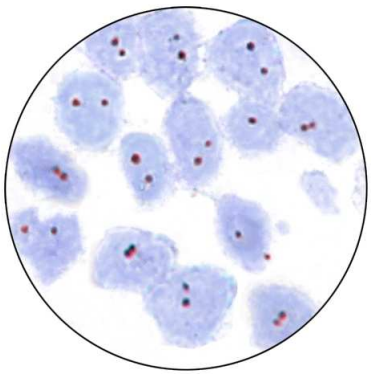
Prior to signal enumeration, the tissue/cell section should be scanned for any possible intratumoral heterogeneity using a 10x or 20x objective. In case of heterogeneity, an area representative for the amplification status has to be chosen. For signal enumeration, areas of necrosis, overlapping nuclei,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

In normal diploid nuclei without chromosome aberrations, 2 dot-shaped signals with smooth, rounded edges will be visible per nucleus (see fig. **1**), 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.

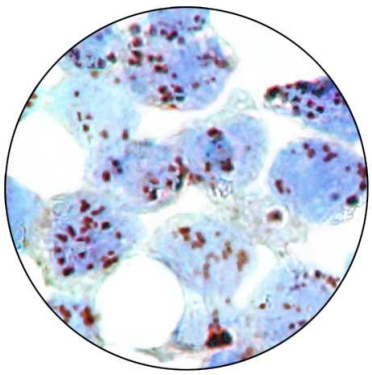
In cells with chromosomal aberrations, a different signal pattern can be visible.

In case of low gene amplifications or gain of chromosomes, multiple dots or small clusters will be visible in the nuclei. Small clusters are irregularly shaped signals comprising an area of up to 5 dots (see fig. **2**). As a reference, a single dot of a normal cell of the same slide must be used.

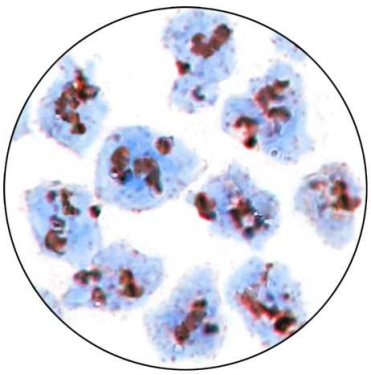
In case of high gene amplifications, a large number of dots or large clusters, comprising an area greater than 5 dots, will be visible in the nuclei (see fig. **3**). As a reference, a single dot of a normal cell of the same slide must be used.



1) 2 Signals/dots



2) Multiple dots and small clusters



3) Large clusters

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 and gene copy number is known. A negative or unspecific result can be caused by multiple factors. For troubleshooting, please refer to chapter 8.

7. Literature

Isola J, Tanner M (2004) *Methods Mol Med* **97**: 133-44.

Speel EJ, et al. (1994) *J Histochem Cytochem* **42**: 1299-307.

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

Wilkinson DG: In Situ Hybridization, A Practical Approach, *Oxford University Press* (1992) ISBN 0 19 963327 4.

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
Streaks on the slide after stopping the pepsin treatment	Precipitation	Wash section immediately in deionized or distilled water
Weak signal or no signal at all	No target sequences available	Use controls
	Cell or tissue sample has not been properly fixed	Optimization of fixing time
	Proteolytic pretreatment not carried out properly	Optimization of incubation time
	Denaturing temperature not correct	Check temperature; increase or decrease if necessary
	Hybridization temperature not correct	Check temperature
	Hybridization time too short	Extension of hybridization time
	Incubation with chromogenic substrate too short	Extension of incubation time
	Too dark counterstaining	Optimize counterstaining time
Uneven and in some parts only very light staining	Incomplete dewaxing	Use fresh solutions; check length of dewaxing times
Cross hybridization signals; strong background staining	Probe volume 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
Section floats off the slide	Proteolytic pretreatment too strong	Shortening of incubation time
	Unsuitable slide coating	Use appropriate slides

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