コスモ・バイオ株式会社

ZYTOVISION

ZytoFast PLUS
CISH Implementation Kit
AP-Permanent Red

REF T-1151-40 Σ 40

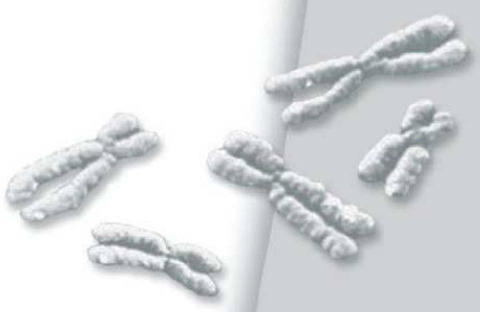
For chromogenic *in situ* hybridization (CISH) using any digoxigenin-labeled ZytoFast CISH Probe

CE

IVD

In vitro diagnostic medical device

according to EU directive 98/79/EC



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As of: April 1, 2012 (5.2)

Contents

1. Scope of Application

The ZytoFast PLUS CISH Implementation Kit AP-Permanent Red is designed to be used for the detection of digoxigenin (DIG)-labeled ZytoFast CISH probes 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 probe.

The ZytoFast PLUS CISH Implementation Kit AP-Permanent Red is to be used with any separately available digoxigenin-labeled ZytoFast 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 the Permanent Red Solution leads to the formation of strong red signals that can be visualized by light microscopy at a 10-20x 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 ZytoFast PLUS CISH Implementation Kit AP-Permanent Red

4.1 Components

The kit is made up of the following components:

Code	Component	Quantity	Container
		Σ 40	
PF23	ZytoFast DNA (+) Control Probe	0.1 ml	Reaction vessel, blue lid
PF24	ZytoFast DNA (-) Control Probe	0.1 ml	Reaction vessel, white lid
PF32	ZytoFast RNA (+) Control Probe	0.1 ml	Reaction vessel, blue lid
PF33	ZytoFast RNA (-) Control Probe	0.1 ml	Reaction vessel, white lid
PT2	Heat Pretreatment Solution EDTA	500 ml	Screw-cap bottle (large)
ES1	Pepsin Solution	4 ml	Dropper bottle, white cap
WB5	20x Wash Buffer TBS	4x 50 ml	Screw-cap bottle
AB11	Rabbit-anti-DIG	4 ml	Dropper bottle, grey cap
AB12	Anti-Rabbit-AP-Polymer	4 ml	Dropper bottle, green cap
SB8a	Permanent Red Solution A	0.1 ml	Reaction vessel, brown lid
SB8b	Permanent Red Solution B	7 ml	Dropper bottle, red cap
CS1	Mayer's Hematoxylin Solution	20 ml	Screw-cap bottle, black
MT4	Mounting Solution (alcoholic)	4 ml	Glass bottle, brown
	Instruction manual	1	

T-1151-40 (40 tests): Components **(ES1)**, **(AB11)**, **(AB12)**, **(SB8a)**, **(SB8b)**, **(CS1)**, and **(MT4)** are sufficient for 40 reactions. Components **(PF23)**, **(PF24)**, **(PF32)**, and **(PF33)** are sufficient for 10 reactions. Component **(PT2)** is sufficient for 7 staining jars of 70 ml each. Component **(WB5)** is sufficient for 57 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 ZytoFast PLUS CISH Implementation Kit AP-Permanent Red 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

- *Digoxigenin-labeled ZytoFast CISH probe*
- *Adhesive pistol, including hot adhesive, or rubber cement (Fixogum)*
- *Ethanol 100%, denatured*
- *Deionized or distilled water*
- *Xylene*

Equipment

- *Water bath (boiling, 55°C)*
- *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!
- ✓ Do not wash slides hybridized with Zyto*Fast* RNA (+) Control Probe **(PF32)** at 55°C as this will reduce signal intensity!

5. The ZytoFast PLUS CISH Implementation Kit AP-Permanent Red Protocol

5.1 Preparatory Steps

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

1x Wash Buffer TBS: Prepare one staining jar with 1x Wash Buffer TBS (prepared using **WB5**) and heat in a water bath to 55°C.

ZytoFast DNA (+) Control Probe **(PF23)**, *ZytoFast DNA (-) Control Probe* **(PF24)**, *ZytoFast RNA (+) Control Probe* **(PF32)**, *ZytoFast RNA (-) Control Probe* **(PF33)**: Bring to hybridization temperature before use.

Pepsin Solution **(ES1)**, *Rabbit-Anti-DIG* **(AB11)**, *Anti-Rabbit-AP-Polymer* **(AB12)**, *Permanent Red Solution A* **(SB8a)**, *Permanent Red Solution B* **(SB8b)**, *Mayer's Hematoxylin Solution* **(CS1)**, *Mounting Solution (alcoholic)* **(MT4)**: Bring to room temperature before use.

Preparation of Permanent Red Solution: Prior to immediate use, add 16 µl of Permanent Red Solution A **(SB8a)** in a graduated cup, fill up to 1 ml with Permanent Red Solution B **(SB8b)** and mix well.

5.2 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 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. Air dry slides

5. Apply (dropwise) Pepsin Solution **(ES1)** to the tissue/cell section and incubate for 10-20 min at 37°C 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 a general rule, we recommend to ascertain the optimum time for proteolysis in pre-tests.

6. Immerse slides 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. Immerse slides in deionized or distilled water and drain off or blot off the water

5.3 Denaturation and Hybridization

1. Vortex the Zyto*Fast* 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.

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 75°C for 5 min, e.g. on a hot plate

4. Transfer the slides to a humidity chamber and hybridize (e.g. in a hybridization oven) for 60 min at:

37°C for DNA-targeting probes or at
55°C for RNA-targeting probes

It is essential that the tissue/cell samples do not dry out during the hybridization step.

5.4 Post-Hybridization and Detection

1. Carefully remove the rubber cement or glue

2. Remove the coverslip by submerging in 1x Wash Buffer TBS (prepared using **WB5**)

3. Wash 5 min in 1x Wash Buffer TBS (prepared using **WB5**) at 55°C

*Do **NOT** perform this step on slides hybridized with ZytoFast RNA (+) Control Probe* **(PF32)** *as this will reduce signal intensity!*

1x Wash Buffer TBS must be sufficiently prewarmed. Check with a thermometer if necessary.

4. Wash 5 min in 1x Wash Buffer TBS (prepared using **WB5**) at RT

5. Apply Rabbit-Anti-DIG **(AB11)** dropwise (3-4 drops per slide) to the slides and incubate for 30 min at 37°C in a humidity chamber

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

7. Apply Anti-Rabbit-AP-Polymer **(AB12)** dropwise (3-4 drops per slide) to the slides and incubate for 30 min at 37°C in a humidity chamber

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

9. During the incubation, prepare Permanent Red Solution by adding 16 µl of Permanent Red Solution A **(SB8a)** in a graduated cup, fill up to 1 ml with Permanent Red Solution B **(SB8b)** and mix well

10. Apply Permanent Red Solution dropwise (3-4 drops per slide) to the slides and incubate for 15-30 min at 37°C

It is recommended that you check the color development in intervals of approx. 5-10 min using a microscope.

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

12. Counterstain the tissue or cell samples for 10-30 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.

13. Wash 2 min in running tap water

14. Dehydration: 3x 100% ethanol, each for 30 s

15. Incubate 2x 30 s in xylene

16. 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

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

6. Interpretation of Results

The Zyto*Fast* PLUS CISH Implementation Kit AP-Permanent Red procedure causes red colored precipitates within the cells targeted by the Zyto*Fast* CISH probe, which can be clearly distinguished from the background. Counterstaining the samples using Mayer's Hematoxylin Solution **(CS1)** will result in nuclei stained blue.

Depending on the target being RNA or DNA, a positive reactivity in the target cells is indicated by a red colored reaction product either within the cytoplasm or the nucleus, respectively. For a more detailed description of the expected signal pattern, please refer to the package insert accompanying the probe.

Visualization of signals should be performed by light microscopy using a 10x or 20x objective. For signal evaluation, necrotic, degenerated or over-digested cells should be avoided as these cells often stain nonspecifically.

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 containing both true positive and negative staining cells.

A negative or unspecific result can be caused by multiple factors. For trouble-shooting, please refer to chapter 8.

The Zyto*Fast* RNA (+) Control Probe **(PF32)** consists of poly-dT oligonucleotides targeting the poly(A) tails of mRNAs. Strong red hybridization signals within the cytoplasm of cells verify the integrity of cellular mRNA in specimens.

The Zyto*Fast* DNA (+) Control Probe **(PF23)** consists of oligonucleotides targeting human Alu repetitive sequences. Strong hybridization signals within the nuclei of cells verify the integrity of cellular DNA in specimens.

The Zyto*Fast* RNA (-) Control Probe **(PF33)** and Zyto*Fast* DNA (-) Control Probe **(PF24)** consist of a set of random sequence oligonucleotides with GC contents of 40-70% without known consensus to any naturally occurring sequences. These probes should not result in positive staining signals and are to be used to assess the unspecific background staining within specimens.

7. Literature

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 present or target sequences below detection limit	Use controls
	Low amount of target sequences	Extension of hybridization and antibody incubation times
	Cell or tissue sample has not been properly fixed	Optimization of fixing time
	Proteolytic pretreatment not carried out properly	Optimization of incubation time
	Denaturation 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
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	Incomplete dewaxing	Use fresh solutions; check length of dewaxing times
	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

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