# ZytoFast PLUS
# CISH Implementation Kit HRP-DAB

REF T-1063-40 Σ 40

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

4250380N567Z

CE IVD

In vitro diagnostic medical device

according to IVDR (EU) 2017/746

## 1. Intended use

The ZytoFast PLUS CISH Implementation Kit HRP-DAB is intended to be used in combination with Digoxigenin-labeled ZytoFast 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 subsequently 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 ZytoFast PLUS CISH Implementation Kit HRP-DAB is available in one size and is composed of:

| Code | Component | Quantity Σ 40 | Container |
|---|---|---|---|
| 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 |
| AB1 | Mouse-Anti-Dig | 4 ml | Dropper bottle, pink cap |
| AB2 | Anti-Mouse-HRP-Polymer | 4 ml | Dropper bottle, violet cap |
| SB1a | DAB Solution A | 0.3 ml | Dropper bottle, green cap (small) |
| SB1b | DAB Solution B | 10 ml | Dropper bottle, grey cap |
| CS2 | Nuclear Blue Solution | 20 ml | Screw-cap bottle, black |
| MT4 | Mounting Solution (alcoholic) | 4 ml | Glass bottle, brown |
| | Instructions for use | 1 | |

T-1063-40 (40 tests): Components **ES1**, **AB1**, **AB2**, **SB1a-b**, **CS2**, and **MT4** are sufficient for 40 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. Materials required but not provided

- Digoxigenin-labeled ZytoFast CISH Probe
- Positive and negative control tissue
- Microscope slides, positively charged
- Water bath (55°C, 98°C)
- Hybridizer or hot plate
- Hybridizer or humidity chamber in hybridization oven
- Adjustable calibrated pipettes (10 µl, 1000 µl)
- Staining jars or baths
- Timer
- Calibrated thermometer
- Ethanol or reagent alcohol
- Xylene
- Methanol 100%
- Hydrogen peroxide ($H_2O_2$) 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 (100-200x)

## 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 AB1, AB2, PT2, 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 CS2:**

The hazard-determining component is ethanediol, ethylene glycol.

**Warning**

| | |
|---|---|
| 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**

| | |
|---|---|
| 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+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 for SB1a:**

The hazard-determining component is biphenyl-3,3′,4,4′tetrayltetraamine; diaminobenzidine.

**Danger**

| | |
|---|---|
| H350 | May cause cancer. |
| P201 | Obtain special instructions before use. |
| P202 | Do not handle until all safety precautions have been read and understood. |
| P280 | Wear protective gloves/protective clothing/eye protection/face protection. |
| P308+P313 | IF exposed or concerned: Get medical advice/attention. |
| P405 | Store locked up. |

**Hazard and precautionary statements for SB1b:**

The hazard-determining component is imidazole;
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).

**Danger**

| | |
|---|---|
| H317 | May cause an allergic skin reaction. |
| H360D | May damage the unborn child. |
| P201 | Obtain special instructions before use. |
| P261 | Avoid breathing dust/fume/gas/mist/vapours/spray. |
| P280 | Wear protective gloves/protective clothing/eye protection/face protection. |
| P302+P352 | IF ON SKIN: Wash with plenty of water. |
| P308+P313 | IF exposed or concerned: Get medical advice/attention. |
| P362+P364 | Take off contaminated clothing and wash it before reuse. |

**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 as this may lead to erroneous results.
- Fixation in 10% neutrally buffered formalin for 24 h at room temperature (RT, 18°C-25°C).
- Sample size ≤ 0.5 $cm^3$.
- Use premium quality paraffin.
- Embedding should be carried out at temperatures lower than 65°C.
- Prepare 3-5 µm 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.

## 11. Assay procedure

### 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 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.*

(4) *1x Wash Buffer TBS*: For stringency wash, heat to 55°C in a covered staining jar.
(5) *ZytoFast CISH Probe*: Before use, bring to hybridization temperature and mix thoroughly.
(6) *Preparation of 3% $H_2O_2$:* Dilute 1 part 30% $H_2O_2$ in 9 parts 100% methanol.
(7) *Mouse-Anti-DIG* (**AB1**), *Anti-Mouse-HRP-Polymer* (**AB2**), DAB Solution A (**SB1a**), DAB Solution B (**SB1b**), *Nuclear Blue Solution* (**CS2**), *Mounting Solution (alcoholic)* (**MT4**): Bring to RT (18°C-25°C) 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% $H_2O_2$.
(5) Wash slides 2x 1 min in deionized or distilled water at RT.
(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 at RT.
(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 µl of the Zyto*Fast* CISH 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 75°C.
(4) Transfer slides to a humidity chamber and hybridize (e.g., in a hybridization oven) for 1 h at
37°C for DNA targeting* probes or at
55°C for RNA targeting* probes.

**Please refer to the instructions of use accompanying the probe. It is essential that specimens do not dry out during the hybridization step.*

### Post-hybridization and detection

(1) Carefully remove the rubber cement or glue.
(2) Remove the coverslip by submerging the slides in 1x Wash Buffer TBS at RT for 5 min.
(3) Wash slides for 5 min in 1x Wash Buffer TBS at 55°C.

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

(4) Wash slides for 5 min in 1x Wash Buffer TBS at RT.
(5) Apply Mouse-Anti-DIG (**AB1**) (1-2 drops per slide) to the slides and incubate for 30 min at 37°C in a humidity chamber.
(6) Wash slides 3x 1 min in 1x Wash Buffer TBS at RT.
(7) Apply Anti-Mouse-HRP-Polymer (**AB2**) (1-2 drops per slide) to the slides and incubate for 30 min at 37°C in a humidity chamber.
(8) Wash slides 3x 1 min in 1x Wash Buffer TBS at RT.
(9) Prepare DAB Solution (working solution): fill 1 ml DAB Solution B (**SB1b**) in a graduated cup and add one drop (30 µl) DAB Solution A (**SB1a**). Mix well.
(10) Apply DAB Solution (1-2 drops per slide) to the slides and incubate for 20 min at 37°C in a humidity chamber.
(11) Wash slides 3x 1 min in deionized or distilled water at RT.
(12) Counterstain specimens for 2-5 min with Nuclear Blue Solution (**CS2**).
(13) Transfer slides into a staining jar and wash 2 min under cold running tap water.
(14) Dehydrate 3x 30 s in 100% ethanol (use very pure ethanol).
(15) Incubate slides for 2x 30 s in xylene (use very pure xylene).
(16) Air dry for approximately 2 min.
(17) 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.*

(18) Evaluate stained specimens by light microscopy.

## 12. Interpretation of results

Using the Zyto*Fast* PLUS CISH Implementation Kit HRP-DAB, hybridization of Digoxigenin-labeled oligonucleotides appear as brown colored precipitates. Counterstaining the samples with Nuclear Blue Solution (**CS2**) will result in nuclei stained in light violet-blue.

Depending on the Zyto*Fast* probe applied, a positive reactivity in the target cells is found either within the cytoplasm or the nucleus, respectively. For a more detailed description of the expected signal pattern, please refer to the instructions of use accompanying the Zyto*Fast* 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 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 |
| Insufficient preparation of chromogenic substrate | Instead of preparing the color substrates by dropping, use a pipette |
| 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 fade or merge**

| Possible cause | Action |
|---|---|
| An unsuitable mounting solution has been used | Use only the mounting solution provided with the kit or recommended by the instructions for use. Use solutions free of any impurities; do not use coverslip tape |

**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 |

**Morphology degraded**

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

**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 not carried out properly | 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 µm microtome sections |

**Specimen floats off the slide**

| Possible cause | Action |
|---|---|
| Proteolytic pretreatment too strong | Shorten pepsin incubation time |

## 17. 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.


## 18. Revision

| Revision | Description of the change |
|---|---|
| 1.2.1 | 11. Assay procedure<br>The pretreatment steps are now to be performed in a reversed order: From now on, incubation in the Heat Pretreatment Solution EDTA has to be performed before application of the Pepsin Solution. In addition, dehydration of the slides in an ethanol series after the pretreatment is now obligatory in order to reduce the drying time. |

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Please refer to 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.
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