



ZytoFast PLUS CISH Implementation Kit AP-NBT/BCIP

REF T-1061-40

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For the qualitative detection of digoxigenin-labeled
ZytoFast Probes by chromogenic *in situ* hybridization
(CISH)



In vitro diagnostic medical device
according to EU directive 98/79/EC

1. Intended use

The ZytoFast PLUS CISH Implementation Kit AP-NBT/BCIP is intended to be used in combination with digoxigenin-labeled ZytoFast Probes for the qualitative detection and discrimination of human pathogen viruses, e.g., HPV, EBV, CMV, and the determination of lymphocyte clonality by detecting human Ig-kappa (κ) and Ig-lambda (λ) light chain mRNA in formalin-fixed, paraffin-embedded specimens by chromogenic *in situ* hybridization (CISH). Interpretation of the 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. Clinical relevance

The chromogenic *in situ* hybridization (CISH) is a sensitive and reliable technique to detect specific sequences, e.g., of human pathogens, in formalin-fixed, paraffin-embedded specimens. It offers the advantage of a permanent staining visualized by light microscopy, allowing the additional histopathological evaluation of the specimen.

3. 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 leads to the formation of colored precipitates. After counterstaining the nucleus with a nuclear dye, hybridized probe fragments are visualized by light microscopy.

4. Reagents provided

The ZytoFast PLUS CISH Implementation Kit AP-NBT/BCIP is composed of:

Code	Component	Quantity Σ / 40	Container
PF23	<u>ZytoFast DNA (+) Control Probe</u>	0.1 ml	Reaction vessel, blue lid
PF24	<u>ZytoFast DNA (-) Control Probe</u>	0.1 ml	Reaction vessel, white lid
PF50	<u>ZytoFast 28S rRNA (+) Control Probe</u>	0.1 ml	Reaction vessel, blue lid
PF33	<u>ZytoFast RNA (-) Control Probe</u>	0.1 ml	Reaction vessel, white lid
PT2	<u>Heat Pretreatment Solution EDTA</u>	500 ml	Screw-cap bottle (large)
ES1	<u>Pepsin Solution</u>	4 ml	Dropper bottle, white cap
WB5	<u>20x Wash Buffer TBS</u>	4x 50 ml	Screw-cap bottle
AB11	<u>Rabbit-Anti-Dig</u>	4 ml	Dropper bottle, grey cap
AB12	<u>Anti-Rabbit-AP-Polymer</u>	4 ml	Dropper bottle, green cap
SB4	<u>NBT/BCIP Solution</u>	4 ml	Dropper bottle, blue cap
CS3	<u>Nuclear Red Solution</u>	20 ml	Screw-cap bottle, black
MT4	<u>Mounting Solution (alcoholic)</u>	4 ml	Glass bottle, brown
	Instructions for use	1	

T-1061-40 (40 tests): Components **ES1**, **AB11**, **AB12**, **SB4**, **CS3**, and **MT4** are sufficient for 40 reactions. Components **PF23**, **PF24**, **PF50**, and **PF33** are sufficient for 10 reactions each. Component **PT2** is sufficient for 7 staining jars of 70 ml each. Component **WB5** is sufficient for 57 staining jars of 70 ml each.

5. Materials required but not provided

- Digoxigenin-labeled ZytoFast CISH Probe
- Positive and negative control specimens
- 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 μ l, 1000 μ l)
- Staining jars or baths
- Timer
- Calibrated thermometer
- Ethanol or reagent alcohol
- Xylene
- 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)

6. 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.

7. 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 and potentially infectious. 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 amounts of water!
- A material safety data sheet is available on our homepage (www.zytovision.com).
- Do not reuse reagents.
- Avoid any cross-contamination and micro-bacterial contamination of the reagents!
- The specimens must not be allowed to dry during the hybridization and washing steps!

Special labelling of ES1:

EUH208	Contains Pepsin A. May produce an allergic reaction.
EUH210	Safety data sheet available on request. < 20 % of the mixture consists of ingredient(s) of unknown acute toxicity (inhalation).

Special labelling of SB4:

EUH210	Safety data sheet available on request.
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Hazards and precautionary statements for MT4:

The hazard-determining component is Xylene.



Danger

H226	Flammable liquid and vapour.
H304	May be fatal if swallowed and enters airways.
H302	Harmful if swallowed.
H332	Harmful if inhaled.
H335	May cause respiratory irritation.
H319	Causes serious eye irritation.
H315	Causes skin 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.
P280	Do not breathe dust/fume/gas/mist/vapours/spray.
P303+P361+P353	IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water/shower.
P301+P310	IF SWALLOWED: Immediately call a POISON CENTER/doctor.
EUH208	Contains methyl 2-methylprop-2-enoate; methyl 2-methylpropenoate; methyl methacrylate. May produce an allergic reaction.

Hazards and precautionary statements for WB5:

The hazard-determining components are

- 2-amino-2-(hydroxymethyl)propane-1,3-diolhydrochloride
- 2-amino-2-(hydroxymethyl)-1,3-propanediol
- a mixture 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

H315	Causes skin irritation.
H317	May cause an allergic skin reaction.
H319	Causes serious eye irritation.
H335	May cause respiratory irritation.
P261	Avoid breathing dust/fume/gas/mist/vapours/spray.
P280	Wear protective gloves/ protective clothing/ eye protection/ face protection.
P321	Contaminated work clothing should not be allowed out of the workplace.
P302+P352	IF ON SKIN: Wash with plenty of soap and water.
P333+P313	IF skin irritation or rash occurs: Get medical advice/attention.
P362+P364	Take off contaminated clothing and wash it before reuse.

Hazards and precautionary statements for PF23 and PF24:

The hazard determining component is Formamide.



Danger

H351	Suspected of causing cancer.
H360FD	May damage fertility. May damage the unborn child.
H373	May cause damage to organs through prolonged or repeated exposure.
P201	Obtain special instructions before use.
P202	Do not handle until all safety precautions have been read and understood.
P260	Do not breathe dust/fume/gas/mist/vapours/spray.
P280	Wear protective gloves/protective clothing/eye protection/face protection.
P308+P313	IF exposed or concerned: Get medical advice/attention.
P405	Store locked up.

Hazards and precautionary statements for AB11, AB12, and PT2:

The hazard-determining component is a mixture 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 soap and water.
P333+P313	IF skin irritation or rash occurs: Get medical advice/attention.
P362+P364	Take off contaminated clothing and wash it before reuse.

8. Limitations

- For *in vitro* diagnostic use.
- For professional 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 to be familiar with the CISH 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 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.
- Probes should be used only for detecting target sequences described in the instructions for use of the respective probe.
- The performance was validated using the procedures described in these instructions for use. Modifications to these procedures might alter the performance and have to be validated by the user.

9. 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

10. Preparation of specimens

Recommendations:

- Avoid cross-contamination of samples in any step of preparation 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 $\leq 0.5 \text{ cm}^3$.
- 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.

11. Preparatory treatment of the device

20x Wash Buffer TBS (WB5) is to be prepared according to the instructions in 12. "Assay procedure". All other kit reagents are ready-to-use. No reconstitution, mixing, or dilution is required.

12. Assay procedure

Preparatory steps

- (1) *(Optional) 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) Rabbit-Anti-DIG (AB11), Anti-Rabbit-AP-Polymer (AB12), NBT/BCIP Solution (SB4), Nuclear Red Solution (CS3), 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) Wash slides 2x 1 min in deionized or distilled water at RT.
- (5) Apply (dropwise) Pepsin Solution (ES1) to the specimen and incubate for 10-30 min at 37°C in a humidity chamber.

As a general rule, we recommend to ascertain the optimum time for proteolysis in pre-tests.

- (6) Immerse slides in deionized or distilled water at RT.
- (7) 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).

- (8) Immerse slides in deionized or distilled water at RT.

- (9) *(Optional) Dehydration* in: 70%, 90%, and 100% ethanol, each for 1 min.

- (10) Air dry sections.

Note: Make sure to completely dry sections prior to probe application.

Denaturation and hybridization

- (1) Pipette 10 μl of the ZytoFast 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 package insert 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 Rabbit-Anti-DIG (AB11) (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-Rabbit-AP-Polymer (AB12) (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) Apply NBT/BCIP Solution (SB4) (1-2 drops per slide) to the slides and incubate for 20 min at 37°C in a humidity chamber.
 - (10) Wash slides 3x 1 min in deionized or distilled water at RT.
 - (11) Counterstain specimens for 2-5 min with Nuclear Red Solution (CS3).
 - (12) Transfer slides into a staining jar and wash 2 min under cold running tap water.
 - (13) Dehydrate 3x 30 s in 100% ethanol (use very pure ethanol).
 - (14) Incubate slides for 2x 30 s in xylene (use very pure xylene).
 - (15) Air dry for approximately 2 min.
 - (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). Allow 20-30 min for the coverslip to become immobilized.
 - (17) Evaluate stained specimens by light microscopy.

13. Interpretation of results

Using the ZytoFast PLUS CISH Implementation Kit AP-NBT/BCIP, hybridization of digoxigenin-labeled oligonucleotides results in blue-violet colored precipitates. Counterstaining the samples with Nuclear Red Solution (CS3) will result in nuclei stained in light red.

Depending on the ZytoFast CISH 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 package insert accompanying the probe.

Please note:

- Visualization of signals should be performed using at least a 100-fold magnification resulting in easily visible signals.
- Do not evaluate areas of necrosis, overlapping nuclei, over-digested nuclei and nuclei with weak signal intensity.
- A negative or unspecific result can be caused by multiple factors (see chapter 17 "Troubleshooting").

- In order to correctly interpret the results, the user must validate this product prior to use in diagnostic procedures according to national and/or international guidelines.

14. Recommended quality control procedures

In order to monitor correct performance of processed specimens and test reagents, each assay should be accompanied by internal and external controls. If internal and/or external controls fail to demonstrate appropriate staining, results with patient specimens must be considered invalid.

The *ZytoFast* 28S rRNA (+) Control Probe (PF50) consists of digoxigenin-labeled oligonucleotides targeting the sequences of a large subunit of ribosomal RNA (28S rRNA). Strong hybridization signals within the nuclei with additional light cytoplasmic staining verify the integrity of cellular mRNA in specimens.

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

The *ZytoFast* RNA (-) Control Probe (PF33) and *ZytoFast* DNA (-) Control Probe (PF24) consist of a set of digoxigenin-labeled 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 unspecific background staining within specimens.

15. Performance characteristics

Refer to the instructions for use of the respective probe.

16. Disposal

The disposal of reagents must be carried out in accordance with local regulations.

17. Troubleshooting

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

Weak signals or no signals at all

Possible cause	Action
Cell or tissue sample has not been properly fixed	Optimize fixing time and fixative
Heat pretreatment, proteolysis, hybridization, denaturation, stringency wash or antibody-incubation temperature not correct	Check temperature of all technical devices used, using a calibrated thermometer. Use always the same number of slides in solutions with critical temperature
Proteolytic pretreatment not carried out properly	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. Ascertain the optimum time for pepsin incubation in pre-tests
Hybridization time too short	Hybridize for at least 1 h; extend hybridization time if necessary
Too low concentrated Wash Buffer	Check concentration of Wash Buffer
Old dehydration solutions	Prepare fresh dehydration solutions
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
Incubation temperature for color substrates not correct	Check temperature of all technical devices used, using a calibrated thermometer
Counterstaining time too long	The counterstaining time depends on the nature of the specimen and should be optimized accordingly. Avoid dark counterstaining, because it may obscure positive staining signals
No target sequences available	Use <i>ZytoFast</i> 28S rRNA (+) Control Probe (PF50) or <i>ZytoFast</i> DNA (+) Control Probe (PF23) to verify pepsin incubation time. Use verified positive tissue to confirm test performance

Signals too strong

Possible cause	Action
Proteolytic pretreatment carried out too long	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. Ascertain the optimum time for pepsin incubation in pre-tests
Substrate reaction is too intense	Shorten substrate incubation time; do not heat substrate solution above temperatures given in the instructions for use

Signals fade or merge

Possible cause	Action
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

Uneven / in some parts only very light staining

Possible cause	Action
Incomplete dewaxing	Use fresh solutions; check duration of dewaxing
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

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

Tissue 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
Stringency wash temperature not correct	Check temperature of the technical devices used, using a calibrated thermometer. Use always the same number of slides in the jar. We recommend not to use more than eight slides per jar for heat incubation steps
Slides not thoroughly rinsed	Use fresh and sufficient wash buffer 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
Prolonged substrate incubation time	Shorten substrate incubation time
Incomplete dewaxing	Use fresh solutions; check duration of dewaxing
Proteolytic pretreatment too strong	Optimize pepsin incubation time
Slides cooled to room temperature before hybridization	Transfer the slides quickly to hybridization temperature

Tissue-antibody interaction	Use <i>ZytoFast</i> RNA (-) Control Probe (PF33) or <i>ZytoFast</i> DNA (-) Control Probe (PF24) to ascertain tissue-specific background staining
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Overlapping signals

Possible cause	Action
Inappropriate thickness of tissue sections	Prepare 3-5 μm microtome sections

Specimen floats off the slide

Possible cause	Action
Unsuitable slide coating	Use appropriate (positively charged) slides
Proteolytic pretreatment too strong	Shorten pepsin incubation time

18. Literature

- Isola J, Tanner M (2004) *Methods Mol Med* 97: 133-44.
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- Tsukamoto T, et al. (1991) *Int J Dev Biol* 35: 25-32.
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