ZYTOVISION

ZytoFast PLUS
CISH Implementation Kit AP-NBT/BCIP

REF T-1061-40 Σ 40

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

For research use only.
Not for use in diagnostic procedures.

1. Intended use

The ZytoFast PLUS CISH Implementation Kit AP-NBT/BCIP is intended to be used in combination with Digoxigenin-labeled ZytoFast Probes on formalin-fixed, paraffin-embedded specimens by chromogenic *in situ* hybridization (CISH).

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 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 AP-NBT/BCIP 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
AB11	Rabbit-Anti-Dig	4 ml	Dropper bottle, grey cap
AB12	Anti-Rabbit-AP-Polymer	4 ml	Dropper bottle, green cap
SB4	NBT/BCIP Solution	4 ml	Dropper bottle, blue cap
CS3	Nuclear Red Solution	20 ml	Screw-cap bottle, black
MT4	Mounting Solution (alcoholic)	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. 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 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 µl, 1000 µl)
- 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)

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 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 labeling of ES1:

EUH208	Contains Pepsin A. May produce an allergic reaction.
EUH210	Safety data sheet available on request.

Special labeling of SB4:

EUH210	Safety data sheet available on request.

Hazard and precautionary 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 AB11, AB12, PT2, and WB5

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 water.
P333+P313	IF skin irritation or rash occurs: Get medical advice/attention.
P362+P364	Take off contaminated clothing and wash it before reuse.

7. Limitations

- For research use only.
- For professional use only.
- 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.

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 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 ≤ 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 12. "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) *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) 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).

(6) Transfer slides immediately to deionized or distilled water and wash for 2x 2 min.
(7) 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.

(8) Immerse slides in deionized or distilled water at RT.
(9) 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.

Using a pipette tip which has been cut off to increase the size of the opening, can make the pipetting process easier.

(17) Evaluate stained specimens by light microscopy.

12. Interpretation of results

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

Depending on the ZytoFast 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 of the respective ZytoFast 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.

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 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 or 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

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

17. 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.
- Wilkinson DG: In Situ Hybridization, A Practical Approach, *Oxford University Press* (1992), ISBN 0 19 963327 4.

Our experts are available to answer your questions.
Please contact helptech@zytovision.com

ZytoVision GmbH
Fischkai 1
27572 Bremerhaven/ Germany
Phone: +49 471 4832-300
Fax: +49 471 4832-509
www.zytovision.com
Email: info@zytovision.com