



ZytoFast

human Ig-kappa/Ig-lambda Permanent CISH Kit

REF T-1105-40

Σ 40

For the simultaneous qualitative detection of human Ig-kappa (κ) and Ig-lambda (λ) mRNA by chromogenic *in situ* hybridization (CISH)



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

1. Intended use

The ZytoFast human Ig-kappa/Ig-lambda Permanent CISH Kit is intended to be used for the simultaneous qualitative detection of human Ig-kappa (κ) and Ig-lambda (λ) light chain mRNA in formalin-fixed, paraffin-embedded specimens, such as lymphomas, 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

B cells (a.k.a. B lymphocytes) develop from lymphoid stem cells in the bone marrow. Each clone of B cells expresses a unique antibody molecule, composed of 2 identical heavy and 2 identical light chains, the latter either of κ - or λ -type. Determination of kappa-to-lambda ratio is useful to distinguish between neoplastic and reactive lymphoid proliferations. Polyclonal expression of κ or λ light chains is considered to reflect a reactive hyperplasia in contrast to the monoclonal expression in malignant lymphoma, the most common hematologic malignancy encountered in the Western world. Whereas detection of Ig- κ and Ig- λ by immunohistochemistry often results in excessive background staining, *in situ* hybridization has the advantage of a virtually background-free signal, allowing a safe and simple analysis of the clonality of a given lymphocyte population.

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, unbound and unbound probe fragments are removed by stringency washing steps. Duplex formation of the labeled probe can be visualized using enzyme-conjugated antibodies directed against the probe. 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.

4. Reagents provided

The ZytoFast human Ig-kappa/Ig-lambda Permanent CISH Kit is composed of:

Code	Component	Quantity Σ 40	Container
PF22	<u>ZytoFast human Ig-kappa/Ig-lambda Probe</u>	0.4 ml	Reaction vessel, red lid
PF51	<u>ZytoFast 28S rRNA (+) Control Probe</u>	0.1 ml	Reaction vessel, blue lid
PF7	<u>ZytoFast RNA (-) Control Probe</u>	0.1 ml	Reaction vessel, white lid
ES1	<u>Pepsin Solution</u>	4 ml	Dropper bottle, white cap
WB5	<u>20x Wash Buffer TBS</u>	2x 50 ml	Screw-cap bottle
AB15	<u>Anti-Biotin/DIG-Mix</u>	4 ml	Dropper bottle, yellow cap
SB7a	<u>HRP-Green Solution A</u>	0.8 ml	Dropper bottle, green cap (small)
SB7b	<u>HRP-Green Solution B</u>	15 ml	Dropper bottle, green cap
SB8a	<u>Permanent Red Solution A</u>	0.25 ml	Reaction vessel, brown lid
SB8b	<u>Permanent Red Solution B</u>	15 ml	Dropper bottle, red cap
CS2	<u>Nuclear Blue Solution</u>	20 ml	Screw-cap bottle, black
MT4	<u>Mounting Solution (alcoholic)</u>	4 ml	Glass bottle, brown
	Instructions for use	1	

T-1105-40 (40 tests): Components **ES1**, **PF22**, **AB15**, **SB7a-b**, **SB8a-b**, **CS2** and **MT4** are sufficient for 40 reactions. Components **PF51** and **PF7** are sufficient for 10 reactions each. Component **WB5** is sufficient for 28 staining jars of 70 ml each.

The ZytoFast human Ig-kappa/Ig-lambda Probe is composed of:

- Digoxigenin-labeled oligonucleotides ($\sim 1 \text{ ng}/\mu\text{l}$), which target mRNA sequences encoding Ig-kappa light chain constant regions.
- Biotin-labeled oligonucleotides ($\sim 1 \text{ ng}/\mu\text{l}$), which target mRNA sequences encoding Ig-lambda light chain constant regions.

5. Materials required but not provided

- Positive and negative control specimens
- Microscope slides, positively charged
- Water bath (55°C)
- Hybridizer or hot plate
- Hybridizer or humidity chamber in hybridization oven
- Adjustable calibrated pipettes (10 μl , 100 μl , 1000 μl)
- Staining jars or baths
- Timer
- Calibrated thermometer
- Ethanol or reagent alcohol
- Xylene
- Methanol 100%
- Hydrogen peroxide (H₂O₂) 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)

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).

Hazards and precautionary statements for AB15 and SB7b:

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.

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 SB7a:

The hazard-determining components are Methanol and Hydrogen peroxide solution 30 %.



Danger

H225	Highly flammable liquid and vapour.
H301+H311 +H331	Toxic if swallowed, in contact with skin or if inhaled.
P210	Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking.
P235	Keep cool.
P361+P364	Take off immediately all contaminated clothing and wash it before reuse .
P403+P233	Store in a well-ventilated place. Keep container tightly closed.
P304+P340	IF INHALED: Remove victim to fresh air and keep at rest in a position comfortable for breathing
P270	Do not eat, drink or smoke when using this product.

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 SB8a:

The hazard-determining component is Hydrogen chloride.



Danger

H314	Causes severe skin burns and eye damage.
P260	Do not breathe dust/fume/gas/mist/vapours/spray.
P280	Wear protective gloves/ protective clothing/ eye protection/ face protection.
P301+P330+ P331	IF SWALLOWED: rinse mouth. Do NOT induce vomiting.
P303+P361+ P353	IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water/shower.
P305+P351+ P338	IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P310	Immediately call a POISON CENTER/doctor

Hazards and precautionary statements for CS2:

The hazard-determining component is Ethylene glycol.

**Warning**

H373	May cause damage to organs through prolonged or repeated exposure.
P260	Do not breathe dust/fume/gas/mist/vapours/spray.
P314	Get medical advice/attention if you feel unwell.

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) 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.
- (3) 1x Wash Buffer TBS: For stringency wash, heat to 55°C in a covered staining jar.
- (4) Preparation of 3% H₂O₂: Dilute 1 part 30% H₂O₂ with 9 parts 100% methanol.
- (5) ZytoFast CISH Probe: Before use, bring to hybridization temperature (55°C) and mix thoroughly.
- (6) Anti-Biotin/DIG-Mix (AB15), HRP-Green Solution A (SB7a), HRP-Green Solution B (SB7b), Permanent Red Solution A (SB8a), Permanent Red Solution B (SB8b), 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% H₂O₂.
- (5) Wash slides 2x 1 min in deionized or distilled water at RT.
- (6) Apply (dropwise) Pepsin Solution (ES1) to the specimen and incubate for 20-50 min at 37°C in a humidity chamber.

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

- (7) Immerse slides in deionized or distilled water at RT.
- (8) (Optional) Dehydration in: 70%, 90%, and 100% ethanol, each for 1 min.
- (9) 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 for 2 h at 55°C (e.g., in a hybridization oven).

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 Anti-Biotin/DIG-Mix (AB15) (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) During the washing steps, prepare HRP-Green Solution (working solution): fill 1 ml HRP-Green Solution B (SB7b) in a graduated cup and add two drops (2x 20 μl) HRP-Green Solution A (SB7a). Mix well.
- (8) Apply HRP-Green Solution (1-2 drops per slide) to the slides and incubate for 15 min at RT.
- (9) Wash slides 3x 1 min in deionized or distilled water.

- (10) During the washing steps, prepare Permanent Red Solution (working solution): fill 1 ml Permanent Red Solution B (SB8b) in a graduated cup and add 16 µl Permanent Red Solution A (SB8a). Mix well.
- (11) Apply Permanent Red Solution (1-2 drops per slide) to the slides and incubate for 15 min at 37°C in a humidity chamber.
- (12) Blot off Permanent Red Solution, **but do not rinse!**
- (13) Apply Permanent Red Solution (1-2 drops per slide) to the slides and incubate again for 15 min at 37°C in a humidity chamber.
- (14) Wash slides 3x 1 min in deionized or distilled water at RT.
- (15) Counterstain specimens for 2-5 min with Nuclear Blue Solution (CS2).
- (16) Transfer slides into a staining jar and wash 2 min under cold running tap water.
- (17) Dehydrate 3x 30 s in 100% ethanol (use very pure ethanol).
- (18) Incubate slides for 2x 30 s in xylene (use very pure xylene).
- (19) 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.
- (20) Evaluate stained specimens by light microscopy.

13. Interpretation of results

The ZytoFast human Ig-kappa/Ig-lambda Permanent CISH Kit procedure causes green and/or red colored precipitates within the cells targeted by the ZytoFast human Ig-kappa/Ig-lambda Probe (PF22), which can be clearly distinguished from the background.

A positive reactivity for Ig-kappa (κ) mRNA in plasma B-cells is indicated by green cytoplasmic staining.

A positive reactivity for Ig-lambda (λ) mRNA in plasma B-cells is indicated by red cytoplasmic staining.

Counterstaining the samples with Nuclear Blue Solution (CS2) will result in nuclei stained in light violet-blue.

In lymphoid tissue, the normal kappa-to-lambda ratio is roughly 2:1, an indication for monoclonality is given if the kappa-to-lambda ratio is >3:1 or <0.3:1.

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 (PF51) consists of biotin-labeled oligonucleotides targeting the sequences of a large subunit of ribosomal RNA (28S rRNA). Strong red hybridization signals within the nuclei with additional light cytoplasmic staining verify the integrity of cellular mRNA in specimens.

The ZytoFast RNA (-) Control Probe (PF7) consists of a set of biotin-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

The performance of the ZytoFast human Ig-kappa/Ig-lambda Permanent CISH Kit was assessed by performing chromogenic *in situ* hybridizations (CISH) on light-chain restriction positive and negative tissue specimens. Light-chain restriction status of included tissue specimens was assessed by reference methods prior to this study.

Analytical sensitivity: The analytical sensitivity was calculated to be 100%.

Analytical specificity: The analytical specificity was calculated to be 100%.

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
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 2 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
Insufficient preparation of chromogenic substrate	Instead of using two drops of HRP Green Solution A use 40 µl; use calibrated pipettes
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 <u>ZytoFast 28S rRNA (+) Control Probe (PF51)</u> to verify pepsin incubation time. Use verified positive tissue to confirm test performance
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 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 solutions 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
Tissue-antibody interaction	Use <i>ZytoFast</i> RNA (-) Control Probe (PF7) to ascertain tissue-specific background staining

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

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