ZYTOVISION

ZytoFast
human Ig-kappa/Ig-lambda CISH Kit

REF T-1005-40 Σ 40

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

CE IVD

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

1. Intended use

The ZytoFast human Ig-kappa/Ig-lambda 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, unspecific 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 CISH Kit is available in one size and is composed of:

Code	Component	Quantity Σ 40	Container
PF22	ZytoFast human Ig-kappa/Ig-lambda Probe	0.4 ml	Reaction vessel, red lid
ES1	Pepsin Solution	4 ml	Dropper bottle, white cap
WB5	20x Wash Buffer TBS	2x 50 ml	Screw-cap bottle
AB15	Anti-Biotin/DIG-Mix	4 ml	Dropper bottle, yellow cap
SB4	NBT/BCIP Solution	4 ml	Dropper bottle, blue cap
SB5	AEC Solution	4 ml	Dropper bottle, red cap
CS4	Nuclear Green Solution	20 ml	Screw-cap bottle, black
MT5	Mounting Solution (aqueous)	4 ml	Dropper bottle, transparent cap
	Instructions for use	1	

T-1005-40 (40 tests): Components **ES1**, **PF22**, **AB15**, **SB4**, **SB5**, **CS4**, and **MT5** are sufficient for 40 reactions. 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 (~ 1 ng/µl), which target mRNA sequences encoding Ig-kappa light chain constant regions.
- Biotin-labeled oligonucleotides (~ 1 ng/µl), 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, 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)

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 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 AB15, CS4, 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.

Hazard and precautionary statements for SB5:

The hazard-determining component is: N,N-dimethylformamide, dimethyl formamide.

Danger

H226	Flammable liquid and vapour.
H319	Causes serious eye irritation.
H332	Harmful if inhaled.
H360D	May damage the unborn child.
P201	Obtain special instructions before use.
P210	Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking.
P308+P313	IF exposed or concerned: Get medical advice/attention.
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.

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 ≤ 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.

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% H_2O_2:* Dilute 1 part 30% H_2O_2 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**), *NBT/BCIP Solution* (**SB4**), *AEC Solution* (**SB5**), *Nuclear Green Solution* (**CS4**), *Mounting Solution (aqueous)* (**MT5**): 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) Apply (dropwise) Pepsin Solution (**ES1**) to the specimen and incubate for 20-50 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.

(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 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 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) Apply AEC Solution (**SB5**) (1-2 drops per slide) to the slides and incubate for 15 min at RT.

*Do not expose to **strong** direct light.*

(8) Wash slides 3x 1 min in deionized or distilled water at RT.

(9) Apply NBT/BCIP Solution (**SB4**) (1-2 drops per slide) to the slides and incubate for 30 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 Green Solution (**CS4**).

(12) Wash slides 3x 1 min in deionized or distilled water at RT, and drain off or blot off the water.

(13) Avoiding trapped bubbles, cover the samples with a coverslip (22 mm x 22 mm; 24 mm x 32 mm) by using Mounting Solution (aqueous) (**MT5**).

(14) Evaluate stained specimens by light microscopy.

13. Interpretation of results

Using the Zyto*Fast* human Ig-kappa/Ig-lambda CISH Kit, hybridized Digoxigenin-labeled κ-oligonucleotides appear as a bright red pattern (AEC), Biotin-labeled λ-oligonucleotides appear as a strong blue-violet pattern (NBT/BCIP).

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

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

Counterstaining the samples with Nuclear Green Solution (**CS4**) will result in nuclei stained in light green.

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.

Internal control: A plasma B-cell within the specimen should show either Ig-kappa or Ig-lambda staining pattern. Cells within the specimen not belonging to the B-cell type, e.g. fibroblasts, should show no staining pattern.
External control: Validated positive and negative control specimens.

15. Performance characteristics

The performance of the ZytoFast human Ig-kappa/Ig-lambda 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
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 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 aqueous 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 sitting 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

18. Literature

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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