ZYTOVISION

Zyto*Fast*
HPV type 31/33 Probe
(Digoxigenin-labeled)

REF T-1057-400 Σ 40 (0.4 ml)

For the qualitative detection of
human papillomavirus (HPV) type 31/33 DNA
by chromogenic *in situ* hybridization (CISH)

CE IVD

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

1. Intended use

The Zyto*Fast* HPV type 31/33 Probe (**PF27**) is intended to be used for the qualitative detection of human papillomavirus (HPV) type 31/33 DNA in formalin-fixed, paraffin-embedded specimens by chromogenic *in situ* hybridization (CISH). The probe is intended to be used in combination with the Zyto*Fast* PLUS CISH Implementation Kit HRP-DAB (Prod. No. T-1063-40).

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

At least 50 percent of sexually active men and women acquire some form of genital HPV infection at some point in their life. A subset of these HPV genotypes causes anogenital warts, which can be either benign or cancerous. Consequently, HPV types are designated "low risk" (LR) or "high risk" (HR), based on whether they are known to cause benign or cancerous lesions.
Most of the approx. 30 identified genital HPV types, predominantly types 6 and 11, are called "low-risk" types, and may cause mild Pap test abnormalities or genital warts. Until now, approximately 10-15 HPV types are associated with lesions that can progress to cancer. Among those are the HPV types 16/18/31/33/35/39/45/51/52/56/58/59/66/68/82. These cancer-associated HPV types are designated as high-risk HPV types. The infection with the HPV HR-types can lead to development of cancer of the oropharynx, cervix, vulva, vagina, anus, or penis. The majority of malignant cervical carcinomas (approx. 70%) occur as a result of infections with HPV types 16 or 18.

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 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 Zyto*Fast* HPV type 31/33 Probe is composed of:

- Digoxigenin-labeled oligonucleotides (~ 0.6 ng/µl) specific for HPV type 31/33, which target DNA sequences encoding for HPV 31/33 proteins E6, E7, and/or L1.
  The probe also targets the respective RNA sequences of E6, E7, and/or L1 proteins, which are expressed during some stages of infection.
- Formamide based hybridization buffer

The Zyto*Fast* HPV type 31/33 Probe is available in one size:

- T-1057-40: 0.4 ml (40 reactions of 10 µl each)

5. Materials required but not provided

- Zyto*Fast* PLUS CISH Implementation Kit HRP-DAB (Prod. No. T-1063-40) or
- 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, 100 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!

Hazard and precautionary statements:

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.
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.

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 4. "Reagents provided".
- 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 as this may lead to erroneous results.
- Fixation in 10% neutrally buffered formalin for 24 h at room temperature (18-25°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

The product is ready-to-use. No reconstitution, mixing, or dilution is required. Bring probe to hybridization temperature (37°C) and mix thoroughly before use.

12. Assay procedure

Specimen pretreatment
Perform specimen pretreatment (e.g., dewaxing, proteolysis) according to the instructions for use of the Zyto*Fast* PLUS CISH Implementation Kit.

Denaturation and hybridization

1. Pipette 10 µl of the 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 1 h at 37°C (e.g., in a hybridization oven).

It is essential that specimens do not dry out during the hybridization step.

Post-hybridization and detection
Perform post-hybridization processing (washing, detection, counter-staining, mounting, microscopy) according to the instructions for use of the respective Zyto*Fast* PLUS CISH Implementation Kit.

13. Interpretation of results

Using the Zyto*Fast* PLUS CISH Implementation Kits, hybridized Digoxigenin-labeled oligonucleotides appear as brown pattern when detected by horseradish peroxidase (HRP) and DAB.

The staining pattern in the nucleus can be observed as distinct dot-shaped signals in case of integrated HPV, or as a strong and homogeneous nuclear staining in case of episomal HPV. A cytoplasmic staining is observed when RNA sequences of HPV are detected.

Please note:

- Visualization of signals should be performed using a set of objectives ranging from an at least 200-fold to 630-fold magnification. The presence of the episomal staining pattern is usually detected clearly by an objective with 200-fold magnification, whereas the detection of the integrated HPV pattern requires a greater magnification, preferably 630-fold.
- 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 controls: In unclear cases, DNA Control Probes should be used for further clarification.
External controls: Validated positive and negative control specimens.

15. Performance characteristics

The performance of the ZytoFast HPV type 31/33 Probe was assessed by performing chromogenic *in situ* hybridizations (CISH) on eight formalin-fixed, paraffin-embedded (FFPE) tissue specimens using each Zyto*Fast* PLUS CISH Implementation Kit and subsequently comparing the results with results obtained by a CE-labeled reference IVD.

Analytical sensitivity: The analytical sensitivity was calculated as 100%.

Analytical specificity: The analytical specificity was calculated as 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
Heat pretreatment, proteolysis, hybridization, denaturation or stringency wash 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
Insufficient preparation of chromogenic substrate	Instead of preparing the color substrates by dropping, use a pipette
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 positive control probes 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 solution above the temperature 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 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
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
Tissue-antibody interaction	Use negative control probes 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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