F*lex*ISH-
Tissue Implementation Kit

REF Z-2182-20 Σ 20

REF Z-2182-5 Σ 5

For fluorescence *in situ* hybridization (FISH) using any F*lex*ISH probe

CE IVD

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

1. Intended use

The F*lex*ISH-Tissue Implementation Kit is intended to be used in combination with F*lex*ISH probes for the detection of genetic aberrations, e.g., translocations, deletions, amplifications, and chromosomal aneuploidies, in formalin-fixed, paraffin-embedded specimens by fluorescence *in situ* hybridization (FISH).
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

Genetic aberrations, e.g., translocations, deletions and/or amplifications, are associated with various human neoplasms.

3. Test principal

The fluorescence *in situ* hybridization (FISH) technique allows for the detection and visualization of specific nucleic acid sequences in cell preparations. Fluorescently-labeled DNA fragments, so called FISH probes, and their complementary target DNA strands 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. After counterstaining the DNA with DAPI, hybridized probe fragments are visualized using a fluorescence microscope equipped with excitation and emission filters specific for the fluorochromes with which the FISH probe fragments have been directly labeled.

4. Reagents provided

The F*lex*ISH-Tissue Implementation Kit is composed of:

Code	Component	Quantity Σ 20	Quantity Σ 5	Container
PT1	Heat Pretreatment Solution Citric	500 ml	150 ml	Screw-cap bottle (large)
ES1	Pepsin Solution	4 ml	1 ml	Dropper bottle, white cap
WB10	5x F*lex*ISH Wash Buffer	500 ml	150 ml	Screw-cap bottle (large)
MT7	DAPI/DuraTect-Solution	0.8	0.2	Reaction vessel, blue lid
	Instruction manual	1	1	

Z-2182-20 (20 tests): Components **ES1** and **MT7** are sufficient for 20 reactions. Component **WB10** is sufficient for 11x 3 staining jars of 70 ml each. Component **PT1** is sufficient for 7 staining jars of 70 ml each.

Z-2182-5 (5 tests): Components **ES1** and **MT7** are sufficient for 5 reactions. Component **WB10** is sufficient for 3x 3 staining jars of 70 ml each. Component **PT1** is sufficient for 2 staining jars of 70 ml each.

5. Materials required but not provided

- F*lex*ISH probe
- Positive and negative control specimens
- Microscope slides, positively charged
- Water bath (37°C, 98°C)
- Hot plate or hybridizer
- Humidity chamber + hybridization oven or hybridizer
- Adjustable pipettes (10 µl, 30 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 60 mm)
- Rubber cement, e.g., Fixogum Rubber Cement (Prod. No. E-4005-50/-125) or similar
- Adequately maintained fluorescence microscope (400-1000x)
- Immersion oil approved for fluorescence microscopy
- Appropriate filter sets

6. Storage and handling

The components of the kit must be stored at 2-8°C. Additionally, the DAPI/DuraTect-Solution (**MT7**) must be stored protected from light. Return components to storage conditions immediately after use. If these storage conditions are followed, the kit will function, without loss of performance, at least until the expiry date printed on the label. Do not use reagents beyond expiration date indicated on the label.

7. Warnings and precautions

- Read the instruction 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 quantities of water!
- A material safety data sheet is available on request for the professional user.
- Do not reuse reagents.
- The specimens must not be allowed to dry during the hybridization and washing steps!

- DAPI/DuraTect-Solution (**MT7**) should not be exposed to light, especially strong light, for a longer period of time, i.e., all steps should be accomplished, where possible, in the dark and/or using lightproof containers!

The following hazard and precautionary statements are associated with the kit components Heat Pretreatment Solution Citric **(PT1)** and 5x F*lex*ISH Wash Buffer **(WB10)**.

Hazards and precaution statements:

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.
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.

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 FISH 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.
- The probe should be used only for detecting loci described in 4. "Reagents provided".
- The performance was validated using the procedures described in this instruction for use. Modifications to these procedures might alter the performance and have to be validated by the user.

9. Interfering substances

Blood cells present in the specimen might exhibit autofluorescence which hinders signal recognition.

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:

- Fixation in 10% neutrally buffered formalin for 24 h at room temperature (15°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 2-4 µm microtome sections.
- Use positively charged microscope slides.
- Fix for 2-16 h at 50-60°C.

11. Preparatory treatment of the device

5x F*lex*ISH Wash Buffer **(WB10)** is to be pretreated 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

Day 1

Preparatory steps

(1) Preparation of two 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 for up to 160 slides.

(2) Heat Pretreatment Solution Citric **(PT1)**: Fill a staining jar and warm to 98°C.

(3) F*lex*ISH Probe: Bring to room temperature before use, protect from light. Prior to opening the vial, mix by vortexing and spin down briefly.

Pretreatment (dewax/proteolysis)

(1) Incubate slides for 2x 5 min in xylene.

(2) Incubate in 100%, 100%, 90%, and 70% ethanol, each for 2 min.

(3) Wash 2x 2 min in deionized or distilled water.

(4) Incubate for 20 min in pre-warmed Heat Pretreatment Solution Citric (**PT1**) at 98°C.

We recommend not to use more than eight slides per staining jar. After immersing the slides, check the temperature of the Heat Pretreatment Solution Citric inside the jar and start time as soon as the temperature of the solution has reached at least 95°C.

(5) Transfer slides immediately to deionized or distilled water, wash for 2x 2 min and drain off or blot off the water.

(6) Apply (dropwise) Pepsin Solution (**ES1**) to the specimens and incubate for 15 min at 37°C in a humidity chamber.

Depending on multiple factors, e.g., nature and duration of fixing, thickness of sections, and nature of specimens, different incubation times may be required. As an incubation guideline, we recommend an incubation time of 2-30 min. As a general rule, we recommend to ascertain the optimum time for proteolysis in pre-tests.

(7) Wash for 2x 2 min in deionized or distilled water.

(8) Dehydration: in 70%, 90%, and 100% ethanol, each for 1 min.

(9) Air dry sections.

Make sure to completely dry sections prior to probe application since residual moisture may reduce signal intensity and/or affect specimen morphology.

Denaturation and hybridiziation

(1) Pipette 10 µl of the F*lex*ISH Probe onto each pretreated specimen.

Avoid long exposure of the probe to light.

(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 10 min at 75°C.

(4) Perform hybridization for 2 h up to 16 h (i.e. overnight) at 37°C by either transferring the slides to a hybridizer or to a humidity chamber and a hybridization oven.

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

Day 1 or Day 2

Preparatory steps

(1) Preparation of 1x F*lex*ISH Wash Buffer: Dilute 1 part 5x F*lex*ISH Wash Buffer (**WB10**) with 4 parts deionized or distilled water. Fill three staining jars with the 1x F*lex*ISH Wash Buffer, pre-warm one jar to 72°C and keep two jars at room temperature.

(2) DAPI/DuraTect-Solution (**MT7**): Bring to room temperature before use, protect from light.

Post-hybridziation and detection

(1) Carefully remove the rubber cement or glue.

(2) Remove the coverslips by submerging in 1x F*lex*ISH Wash Buffer at room temperature for 1-2 min.

To facilitate the removal of the coverslip, this step can alternatively be performed for 2 min at 37°C.

(3) Wash using 1x F*lex*ISH Wash Buffer for 10 min at 72°C.

The 1x FlexISH Wash Buffer should be pre-warmed. Check with a thermometer if necessary. Do not use more than eight slides per staining jar.

(4) Wash using 1x F*lex*ISH Wash Buffer for 3 min at room temperature.

(5) Incubate the slides in 70%, 90%, and 100% ethanol, each for 1 min.

(6) Air dry the samples protected from light.

(7) Pipette 25 µl DAPI/DuraTect-Solution **(MT7)** onto the slides. Avoiding trapped bubbles, cover the samples with a coverslip (24 mm x 60 mm). Incubate in the dark for 15 min.

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

(8) Store the slides in the dark. For longer storage periods, this should take place at 2-8°C.

(9) Evaluation of the sample material is carried out by fluorescence microscopy. Filter sets for the following wavelength ranges are required:

Fluorescent dye	Excitation	Emission
ZyBlue	418 nm	467 nm
ZyGreen	503 nm	528 nm
ZyGold	532 nm	553 nm
ZyOrange	547 nm	572 nm
ZyRed	580 nm	599 nm

13. Interpretation of results

With the use of appropriate filter sets in interphases or metaphases of normal cells or cells without aberrations of chromosomes, two signals per probe/fluorescence label appear, except for probes targeting X and/or Y chromosomes, resulting in none to two signals per probe/fluorescence label, depending on the gender. In cells with chromosomal aberrations, a different signal pattern can be visible in interphases or metaphases. For more details on the interpretation of results, please refer to the respective probe manual.

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: Non-neoplastic cells within the specimen that exhibit normal signal pattern, e.g., fibroblasts.

External control: Validated positive and negative control specimens.

15. Performance characteristics

Refer to the instruction 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
No target sequences available	Use appropriate controls
Cell or tissue sample has not been properly fixed	Optimize fixing time and fixative
Heat pretreatment, hybridization, proteolysis, denaturation, or stringency wash temperature not correct	Check temperature of all technical devices used, using a calibrated thermometer
Proteolytic pretreatment not carried out properly	Optimize pepsin incubation time, increase or decrease if necessary
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.
Too low concentrated F*lex*ISH Wash Buffer	Check concentration of F*lex*ISH Wash Buffer
Old dehydration solutions	Prepare fresh dehydration solutions
Fluorescence microscope wrongly adjusted	Adjust correctly
Inappropriate filter sets used	Use filter sets appropriate for the fluochromes of the probe. *Triple-bandpass filter sets provide less light compared to single or dual-bandpass filter sets. Consequently, the signals may appear fainter using these triple-bandpass filter sets.*
Too strong beam of light while handling probes/slides	Accomplish hybridization and washing steps in the dark

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
Insufficient drying before probe application	Extend air-drying

Cross hybridization signals; noisy background

Possible cause	Action
Incomplete dewaxing	Use fresh solutions; check duration of dewaxing
Proteolytic pretreatment too strong	Optimize pepsin incubation time
Probe volume per area too high	Reduce probe volume per section/area, distribute probe dropwise to avoid local concentration
Slides cooled to room temperature before hybridization	Transfer the slides quickly to 37°C
Too high concentrated stringency F*lex*ISH Wash Buffer	Check concentration of F*lex*ISH Wash Buffer
Washing temperature following hybridization too low	Check temperature; increase if necessary
Dehydration of sections between the individual incubation steps	Prevent dehydration by sealing the slides and performing incubation in humid environment

Overlapping signals

Possible cause	Action
Inappropriate thickness of tissue sections	Prepare 2-4 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

Weak counterstain

Possible cause	Action
Low concentrated DAPI	Use DAPI/DuraTect-Solution (ultra) (Prod. No. MT-0008-0.8) instead
DAPI incubation time too short	Adjust DAPI incubation time

18. Literature

- Kievits T, et al. (1990) *Cytogenet Cell Genet* **53**: 134-6.
- Wilkinson DG: In Situ Hybridization, A Practical Approach, Oxford University Press (1992) ISBN 0 19 963327 4.

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