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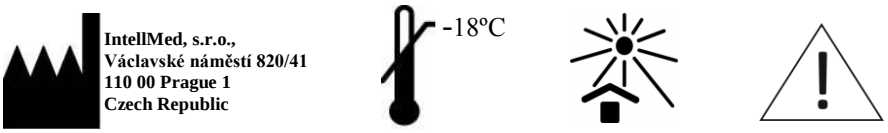
FLUORESCENCE IN SITU HYBRIDIZATION (FISH)

Instructions for use



Kit consists of:
Directly marked probe in hybridization buffer
(Green, Orange, etc. depending on the kit type)

Further necessary chemicals and equipment:

- **Ethanol**
- **Purified water (deionized or distilled)**
- **Acetic acid and methanol**
- **Rubber cement**
- **Moist chamber**
- **Water bath**
- **Hotplate (thermoblock)**
- **Incubator (37 °C)**
- **Mounting medium with DAPI**
- **Fluorescence microscope with corresponding filter** (emission/excitation maximum for orange probes is 588nm/559nm, emission/excitation maximum for green probes is 524nm/497nm)
- **70%, 85%, and 96% ethanol -** In case classical colouring vessels are used (Copplin jar), 70 ml of each ethanol solution is sufficient
- **0.2M HCl**
- **20xSSC, pH 5.3**
- **2xSSC, pH 7.0**
- **1M NaSCN**
- **0,9% NaCl, pH 2.0**
- **Pepsine**
- **10% formaldehyde**
- **Denaturant solution:** 49 ml formamide, 7 ml 20xSSC, 14 ml purified water to be modified pH 7-8, store at temperature of 2-8°C (max. 7 days); heat up to 73±1 °C before use.
- **Washing solution I (0.4x SSC / 0.3% NP-40):** 20 ml 20xSSC, 3 ml NP-40, add purified water up to 1 litre total volume**,** modify pH to 7.0, store at laboratory temperature (max. 6 months); heat up to 73±1°C before use.
- **Washing solution II (2x SSC / 0.1% NP-40):** 100 ml 20xSSC, 1 ml NP-40, add purified water up to 1 litre total volume, modify pH to 7.0, store at laboratory temperature (max. 6 months).

Procedure:

- If we use raw material (tissue, cell line, etc.), it is necessary to fix the preparation for 10 minutes in a mixture of methanol and acetic acid in a ratio of 3:1 after its application onto the microscopic slide (coating, imprint, cytospin). The fixation mixture is always to be prepared just before use. Let the preparation dry naturally after fixation. For next steps see Procedure for raw material.
- If we use paraffin slices, it is first necessary to deparaffinize and pretreat material (see Procedure for FFPE)

Pre-treatment procedure for raw material

1. Incubate the preparations in denaturation buffer for 5 minutes at a temperature of 73 ± 1°C.
2. Immediately after removing, immerse the preparation into 70% ethanol for 1 minute, then into 85% ethanol for one minute, and lastly into 96% ethanol where the preparation can stay until we are ready to apply the probe (however wait, at least one minute).
3. Remove the slides from ethanol, dry slightly by attaching the edge of each slide to an absorbent pad, set them onto the thermoblock with a temperature of 45-50 °C, and let the remaining ethanol vaporize (3-5 minutes).

Pre-treatment procedure for FFPE

1. De-paraffinize slides in xylene for 7 minutes 3x.
2. Re-hydrate in 96% ethanol for 5 minutes 2x.
3. Dry slides by attaching the edge of each slide to an absorbent pad, set them onto the thermoblock with a temperature of 45-50 °C, and let the remaining ethanol vaporize (3-5 minutes).
4. Pre-treate with 0,2M HCl for 20 min, then wash in deionized water for 3 min and 2xSSC for 3 min.
5. Place slides in 1M sodium thiocyanate at 80°C for 20 min, then wash in deionized water for 1 min and 2xSSC for 5 min 2x.
6. Digest in 0,05% pepsine in 0,9% NaCl, pH 2.0 at 37°C for 40 min, then wash in 2xSSC for 5 min 2x.
7. Pre-treate in 10% formaldehyde for 10 min, wash in 2xSSC for 5 min 2x and in deionized water for few seconds and then dry slides onto the termoblock at 45-50 °C for 10 min.

Co-denaturation and hybridization

1. Apply the probe in a quantity as to overlay the test sample, and cover with cleaned cover glass (for a cover glass with dimensions of max. 22×22 mm use 10 µl of probe). It is necessary to coat the cover glass with suitable rubber cement after gluing.
2. Denature prepared slides at 85°C for 1 minute for FFPE or at 75°C for 1 minute for raw material.
3. Incubate over night at 37 °C in moist chamber.

Washing off of unbound probe

4. Unglue the cover glass and immerse the preparation in washing solution I (0.4x SSC/ 0,3% NP-40) heated up to 73± 1 °C. Slightly shake the glass with the preparation in the solution for about 3-5 secs. Incubate for 2 minutes.
5. Transfer to washing solution II (2x SSC/0,1% NP-40), shake again for about 3-5 secs, and incubate for 30 seconds.
6. Dry slightly by attaching the edge of the glass to an absorbent pad, and let dry naturally out of the light.
7. Apply mounting medium/DAPI (for a cover glass with dimensions of max. 22×22 mm use 10 µl of DAPI). The goal is to colour the nuclei in such a way as to be able to observe them using a fluorescence microscope. If you do not use the commercially available DAPI dye-stuff/mounting medium, it is possible to colour the preparations using the DAPI dye-stuff only, or using Hoechst dye-stuff, respectively. Any superfluous dye-stuff must be washed off, and a mounting medium e.g. buffered glycerol must be used.
8. Cover using a cover glass and examine using a fluorescence microscope.

Possible problems and their solutions:

Problem	Possible solution
Cross hybridization	Increase the temperature of washing solution I by 2 °C. Decrease the denaturation temperature by 2 °C.
Weak or no signals	Increase the denaturation temperature by 2 °C. Prolong the hybridization time. Shorten the washing time. Verify the pH of the solutions. Verify that the preparation with the probe or the probe itself was not subject to direct illumination. Verify that the probe was stored at –20°C. Immerse the preparation after fixation into 70% acetic acid for a maximum of 1 minute, and let dry naturally thereafter. This step is recommended especially for hybridization for mitotic preparations where complete elimination of cytoplasmic membranes was not achieved. The preparation is of an earlier date, and was not stored at –80°C (does not apply to paraffin slices).
Diffuse signals	Decrease the denaturation temperature by 2 °C. Shorten the denaturation time by a few seconds. Verify the pH of the solutions.
Bad sample morphology	Decrease the denaturation temperature by 2 °C. Shorten the denaturation time by a few seconds. Prolong the preparation fixation time.
Impurities on the background or "fogging" of the sample after hybridization and rinsing	Mixing of water solution with mounting medium took place. After hybridization and washing of the sample, let the preparation dry thoroughly. Verify the pH of the washing solutions and the denaturation buffer. Prolong the washing time after hybridization.

Safety information:

DNA probes contain:

Formamid - teratogenic. Do not contact skin with probes. Wear protective clothing and gloves when manipulate with probe.

R61
S24, S 25, S35, S36, S 37, S 39, S 45, S 53

Following dangerous substances are needed for using of DNA probes. These sustances are not included or distributed in kit:

NP-40, contained in washing solutions, is irritant as well as **DAPI** counterstain. Do not contact these solutions with skin or do not inhale. Wear protective clothing and gloves.

R 36, R 38, R 37 S 26, S 27, S 28, S 29, S 30, S 33, S 46,

Disposal of unused product:

Unused reagents and waste is special waste which is disposed according with national and local regulations.