Fluorescence in situ Hybridization (FISH) protocol including a post-fixation step to be used on formalin-fixed, paraffin-embedded tissue and cell samples with any Zyto*Light* FISH probe:

Test Material

We recommend the following tissue preparation:

- Fixation in 10% neutrally buffered formalin for 24 h at RT. In order to achieve optimum and uniform fixation and paraffin embedding, the sample size should not exceed 0.5 cm^3.
- Standard processing and paraffin embedding: Use premium quality paraffin. Infiltration and embedding should be carried out at temperatures lower than 65 °C.
- Prepare 3-5 µm microtome sections Draw up the sections onto silane-coated or adhesion slides (e.g. HistoBond ®) and fix at 58°C overnight.

Materials and Reagents needed from ZytoVision:

- Zyto*Light* FISH-Tissue Implementation Kit **[Z-2028-5/-20]**
- Zyto*Light* FISH probe
- Formaldehyde Dilution Buffer Set (including 10x $MgCl_2$, 50 ml; 10x PBS, 50 ml) **[PT-0006-100]**

Additional Materials:

- Water bath (37°C, 98°C)
- Xylene
- Neutrally buffered formaldehyde (10% or 37%)
- Hot plate (70°C, 75°C)
- Hybridization oven (heating oven; 37°C)
- Staining jars, 50-80ml
- Humidity chamber
- Pipette (10µl, 30µl)
- Adhesive pistol, including hot adhesive or rubber cement (Fixogum)
- Ethanol 100%, denatured
- Deionized or distilled water
- Drying block
- Coverslip (22mm x 22mm, 24mm x 60mm)
- Fluorescence microscope

1.1 Preparatory Steps:

Day 1:

- *Preparation of two ethanol series (70%, 90%, and 100% ethanol solutions):* Dilute 7, 9, and 10 parts of 100% ethanol with 3, 1, and 0 parts of deionized or distilled water, respectively.
  These solutions can be stored in suitable containers and can be re-used (day 2).

- *Heat Pretreatment Solution Citric* **[PT1]**: Warm to 98°C.

- *Wash Buffer SSC* **[WB1]**: Bring to room temperature.

- *Preparation of 1% Formaldehyde solution:* For 100 ml 1% Formaldehyde solution mix either 2.7 ml of 37% neutrally buffered formaldehyde or 10 ml of 10% neutrally buffered formaldehyde with 10 ml of 10x $MgCl_2$ **[PT4]** and 10 ml of 10x PBS **[PT5]**and adjust volume to 100 ml with deionized or distilled water. Mix thoroughly.

Day 2:

- *Preparation of 1x Wash Buffer A:* Dilute 1 part 25x Wash Buffer A **[WB2]** with 24 parts deionized or distilled water. Fill three staining jars with the 1x Wash Buffer A and pre-warm it to 37°C.

- *DAPI/DuraTect™-Solution* **[MT7]**: Bring to room temperature before use, protect from light.

1.2 Pretreatment (Dewax/Proteolysis/Post-Fixation) [Day 1]:

- Incubate slides for 10 min at 70°C (e.g. on a hot plate).

- Incubate slides for 2x 10 min in xylene.

- Incubate in 100%, 100%, 90%, and 70% ethanol, each for 5 min.

- Wash 2x 2 min in deionized or distilled water.

- Incubate for 15 min in pre-warmed Heat Pretreatment Solution Citric at 98°C.
  We recommend not to use more than six slides per staining jar.

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

- Apply (dropwise) Pepsin Solution **(ES1)** to the tissue/cell section and incubate for approx. 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 tissue/cells, different incubation times may be required. As a general rule, we recommend to ascertain the optimum time for proteolysis in pre-tests.

- Wash for 5 min in Wash Buffer SSC.
- Incubate for 15 min in 1% Formaldehyde solution.
- Wash for 5 min in Wash Buffer SSC.
- Wash for 1 min in deionized or distilled water.
- Dehydration: in 70%, 90%, and 100% ethanol, each for 1 min.
- Air dry sections.

1.3 Denaturation and Hybridization [Day 1]:

- Pipette 10 µl Zyto*Light* FISH probe each onto individual samples.
  A gentle warming of the probe, as well as 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 of the probe to light.
- Avoiding trapped bubbles, cover the samples with a coverslip (22 mm x 22 mm).
  Seal the coverslip, e.g. with a layer of hot glue from an adhesive pistol or with rubber cement.
- Denature the slides at 75°C (±2°C) for 10 min, e.g. on a hot plate.
- Transfer the slide to a humidity chamber and hybridize overnight at 37°C (e.g. in a hybridization oven).
  It is essential that the tissue/cell samples do not dry out during the hybridization step.

1.4 Post-Hybridization and Detection [Day 2]:

- Carefully remove the rubber cement or glue.
- Remove the coverslip by submerging in 1x Wash Buffer A at 37°C for 1-3 min.
- Wash, using 1x Wash Buffer A for 2x 5 min at 37°C .
  The 1x Wash Buffer A should be pre-warmed. Check with a thermometer if necessary.
- Incubate the slides in 70%, 90%, and 100% ethanol, each for 1 min.
- Air dry the samples protected from light.

- Pipette 30 µl DAPI/DuraTect™-Solution 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.

- Carefully remove excess DAPI/DuraTect™-Solution by gently pressing the slide between filter papers.

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