Chromogenic in situ Hybridization (CISH) protocol including a post-fixation step to be used on formalin-fixed, paraffin-embedded tissue and cell samples with any ZytoDot 2C CISH 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 sections onto silane-coated or adhesion slides and fix at 58°C overnight.

Materials and Reagents needed from ZytoVision:

- ZytoDot 2C CISH Implementation Kit [C-3044-40]
- ZytoDot 2C probe
- Formaldehyde Dilution Buffer Set (including 10x $MgCl_2$, 50 ml; 10x PBS, 50 ml) [PT-0006-100]

Additional Materials:

- Water bath (80°C, 98°C)
- Xylene
- Neutrally buffered formaldehyde (10% or 37%)
- Hydrogen peroxide (H_2O_2), 30%
- Ethanol, 100%, denatured
- Hot plate (70°C, 75°C)
- Hybridization oven (heating oven; 37°C)
- Staining jars, 50-80 ml
- Humidity chamber
- Pipette (10 µl, 30 µl)
- Adhesive pistol, including hot adhesive, or rubber cement (Fixogum)
- Deionized or distilled water
- Coverslips (22 mm x 22 mm , 24 mm x 60 mm)
- Light microscope

Preparatory Steps:

Day 1:

- Preparation of an 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 re-used.

- Preparation of 1x Wash Buffer TBS: Dilute 1 part 20x Wash Buffer TBS [WB5] with 19 parts deionized or distilled water.

- Preparation of 3% H_2O_2: Dilute 1 part of 30% H_2O_2 with 9 parts of 100% methanol.

- Heat Pretreatment Solution **EDTA [PT2]:** Heat in a covered staining jar standing in a boiling water bath to at least 98°C.

- 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.

- Pepsin Solution **[ES1]:** Bring to room temperature before use.

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 5 min in xylene.

- Incubate for 3x 3 min in 100% ethanol
  Alternatively, dewaxing protocols routinely used in immunohistochemistry procedures, e.g. 2x 15 min xylene, 2x 5 min 100% ethanol, 2x 5 min 96% ethanol, 1x 5 min 70% ethanol, can be used.

- Incubate slides for 5 min in 3% H_2O_2.

- Wash 2x 1 min in deionized or distilled water.

- Place slides in the pre-heated Heat Pretreatment Solution EDTA and incubate for 15 min.
  Use eight slides per staining jar (add dummy slides if needed)

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

- Apply (dropwise) Pepsin Solution to the tissue/cell section and incubate for 5-15 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.

- Incubate slides for 5 min in 1x Wash Buffer TBS.
- Incubate slides for 5 min in 1% Formaldehyde solution.
- Incubate slides for 5 min in 1x Wash Buffer TBS.
- Wash 2x 1 min in deionized or distilled water.
- Dehydration: in 70%, 90%, and 100% ethanol, each for 1 min.
- Air dry specimens.

Denaturation and Hybridization [Day 1]:

- Pipette 10 µl Zyto*Dot* 2C 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.
- 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 79 °C for 5 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).

Detection [Day 2]:

From here on the instructions of the second day protocol of the Zyto*Dot* 2C CISH Implementation Kit [Product No.: C-3044-40] can be followed.