ZytoDot® 2C Practical Procedure – Technical Tips and Tricks:

Technical tips & tricks which should be considered before performing the ZytoDot® 2C CISH method:

Preparatory Steps:

- Please, first check all temperatures of the used technical devices with a thermometer, as we know this can widely differ from the temperatures which are seen on the display of the respective devices.
- Please, set in advance the following conditions: In order to standardize, it is recommended to always use 8 slides during the heat pretreatment and stringency washing (per 70 ml staining jar). If processing less slides, place empty slides in the jar as placeholders together with the specimen slides.
- Regularly change the ethanol and xylene solutions for dewaxing. We recommend changing these solutions every 150 to 200 slides. Otherwise, the deparaffinization might be insufficient and could interfere with the CISH results.

Preparation of Specimens:

- Fixation should be carried out in 10% neutrally buffered formalin (37% buffered formaldehyde solution is called formalin 100%, 10% formalin solution corresponds to 4% formaldehyde solution) for 24h at room temperature (18-25°C). Infiltration and embedding should be carried out at temperatures lower than 65°C using premium quality paraffin. The mounted tissue section should be fixed on the slide for 2-16h at 50-60°C.
- For best results, the sample size should not exceed 0.5 cm^2 and should have a thickness of 3-5 μm.
- The samples should be drawn up onto positively charged slides which are compatible with CISH (e.g. Histobond). Using slides that are not positively charged leads to very weak connection between tissue section and slide so that the tissue may float off during pretreatment. As an alternative, glass slides can be APES-treated (silanized) or coated by poly-L-lysine.

Proteolysis:

- The pepsin digestion time is a crucial step and the digestion time needs to be adapted. For every tissue type, the optimal pepsin digestion time differs due to specimen variability (tissue/cell type, nature and duration of fixation, thickness of section) and needs to be determined first. This can be done by performing a pepsin digestion time series: Try e.g. 3 different pepsin digestion times (e.g. 5, 10, and 15 minutes) in order to find the most appropriate one. Additionally, we suggest to apply the pepsin solution directly onto the slides after taking it out of the fridge (this is more convenient instead of bringing the solution to room temperature before use and will prevent pepsin from autodigestion).
- For standardization incubation of pepsin should be carried out at 37°C (enzyme optimum) and not at room temperature.
- Please note that the incubation time and temperature of heat pretreatment, pepsin digestion and denaturation are settings that depend on each other. Altering one of these three steps might requires adaptation of the other two steps as well.

Hybridization and Detection:

- Make sure that the specimens are completely dry before applying the probe. Otherwise, this may cause inconsistent results.
- After hybridization, it is absolutely mandatory to remove the coverslip before performing the stringency washing step! Incubating the slide with coverslip and probe at 80°C will result in denaturation of probe and target hybrids and thus weak signals.
- HRP-Green and AP-Red Solutions should be prepared shortly before use! It is also important to stick to the volumes indicated in the manual when mixing the Solution A with Solution B (always drop Solution A into Solution B). It might be convenient to pipette the volumes instead of dropping them.
- Readily mixed HRP-Green and AP-Red is light sensitive. Therefore, it is necessary to protect it from light. Exposure to light weakens the green and red signals.
- After application of the chromogens it is crucial to stick exactly to the indicated incubation times! Fading of the signals or signal loss is possible.
- Make sure that the tap water you use for washing after counterstaining is cold (max. 25°C). Using warm tap water could weaken the signals.
- Do not wash in any buffer after the incubation with chromogenic substrates because this may lead to missing or weak signals. For this reason always wash in distilled or deionized water.

Stringency Washing:

- It is mandatory to maintain the recommended stringency washing conditions of 80°C for 5 min.

Evaluation:

- In order to optimally balance the contrast and the resolution of the image, it is necessary to setup the Köhler illumination.
- Visualization of signals should be performed using at least 400-fold magnification for evaluating Zyto*Dot* 2C amplification probes or 630-fold magnification for evaluation of Zyto*Dot* 2C Break Apart or deletion probes.
- For further information refer to „Microscopy and Filters – Technical Tips and Tricks“