Guidelines for *in situ* hybridization on fresh frozen material using the miRCURY LNA™ ISH Optimization kits

These guidelines are a supplement to the miRCURY LNA™ microRNA ISH Optimization Kit (FFPE) manual V2.0. The manual can be downloaded from http://www.exiqon.com/mirna-ish-kit.

ISH protocols vary extensively due to different equipment set-up and Lab routines. This standard procedure guides the process of a manual *In Situ* Hybridization on human tissue using a DAKO Hybridizer (S2450).

Note that this protocol and the Exiqon miRCURY LNA™ microRNA ISH Optimization kits are not validated for use on Fresh Frozen material, and will likely require additional optimization. Please see page 23 -28 in the miRCURY LNA™ microRNA ISH Optimization Kit (FFPE) manual V2.0 for tips and troubleshooting.

General recommendations

Every step in the following procedure must take place in a clean and nuclease free environment. The operator must wear gloves during the entire process, and only use dry-sterilized glassware etc. (see below). in order to maintain RNase-free conditions. Use only ultrapure water or RNase free milli-Q H_2O in all reagents. All buffers/reagents applied in the assay must be RNase negative.

All glassware, including slides and coverslips, should be washed in a dishwasher. Wrap the items in aluminum foil so the surface or openings are covered and place a piece of indicator tape on the surface of the foil. Place items in an appropriate oven to be dry-sterilized for 8 hours at 180°C.

Note: for optimal *In situ* hybridization results on mouse tissue, it is recommended that the tissue specimens are obtained from perfusion fixed mice (see separate Guidelines for perfusion fixation). Before OCT-embedding, the fixed tissue samples must be cryo-protected with 20-30% sucrose in PBS overnight at 4°C. OCT-embedding/snap-freezing can be performed in isopentane at -80°C or liquid nitrogen. Store OCT-embedded samples at -80°C.)

microRNA ISH protocol

Before starting the experiment, please refer to the miRCURY LNA™ microRNA ISH Optimization Kit (FFPE) manual pages12-18. Note the recommendations regarding optimization of the protocol using control probes. When using Fresh frozen samples, Step 1 (deparaffinization of FFPE slides) in the protocol is replaced by the following Steps:

Step 1a Cryo sectioning and fixation	Cut 10µm thick sections from OCT embedded FrFr blocks, mount them on a clean, non-contaminated, SuperFrost®Plus slides and let them air-dry for app. 15min (depending on the size of the tissue).Submerge the slides in 10% Neutral buffered Formalin (NBF) and leave it over night (o/n) at room temperature (RT).
Step 1b Wash	Transfer slides into container(s) with PBS and rinse for 3 x 3min (using fresh PBS for each wash).

Proceed to Step 2, page 19 of the miRCURY LNA™ microRNA ISH Optimization Kit (FFPE) manual V2.0.

Follow the rest of the protocol as described.

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