Sample Preparation Technical Note for Fixed Frozen Tissue Using RNAscope® 2.5 Chromogenic Assay – **PART 1**

Tissue Section Prep and Pretreatment

Fix Sample

1. Perfuse tissue with freshly prepared **RNAse-free 4%** paraformaldehyde **(PFA)** in 1X PBS.
2. Dissect tissue and place in freshly prepared **RNAse-free 4% PFA** for **24 HRS** at **4°C**.

Freeze Tissue

1. Immerse the tissue in **10% sucrose** in 1X PBS at **4°C until the tissue sinks** to the bottom of the container (~18 HRS for brain tissue).
2. Immerse the tissue in **20% sucrose** in 1X PBS at **4°C until the tissue sinks** to the bottom of the container.
3. Immerse the tissue in **30% sucrose** in 1X PBS at **4°C until the tissue sinks** to the bottom of the container.
4. **Flash freeze** the tissue in **OCT** (Optimal Cutting Temperature) embedding media with crushed **dry ice** (or liquid nitrogen).
5. Store tissue blocks in an airtight container at **-80°C**.

Prepare Sections

1. Before sectioning, **equilibrate** the tissue blocks at **-20°C** for at least **1 HR** in a cryostat.
2. Section the blocks by cutting the sections to a thickness of **20 µm**. Mount the sections on SuperFrost® Plus slides.
3. Air dry the slides at **RT while sectioning** and overnight at **-80°C**. If all the slides are not used immediately, store them at **-80°C** for up to **3 MONTHS**.

Run the Assay

1. Day of RNAscope® assay: **Turn on HybEZ™ oven** and switch to **"Hold T" mode** and set at **60°C**.
2. Once oven reaches 60°C, **wash slides** with 200 mL **1X PBS** in a Tissue-Tek® slide rack for **5 MIN** while **moving the rack GENTLY up and down** to remove OCT.
3. Bake slides for **30 MIN** at **60°C** in oven tray without humidifying paper.
4. Post-fix the slides by immersing them in prechilled 4% PFA in 1X PBS for **15 MIN*** at **RT**.
   ***Post-fix slides for **30 MIN** if underfixed.

Prepare Materials

1. Prepare **250 mL** each of **50% EtOH** and **70% EtOH**, and **2X 250 mL** of **100% EtOH**.
2. Fill food steamer with H_2O and plug it in – DO NOT turn it on yet!
3. Prepare **250 mL** fresh **1X Target Retrieval** (**225 mL dH_2O + 25 mL 10X Target Retrieval**) in a **staining dish**.
4. Prepare **3X staining dish** filled with **dH_2O** and **1X staining dish** filled with **100% EtOH**.
5. Place **one dH_2O** staining dish and **1X Target Retrieval** staining dish **inside food steamer** and replace food steamer lid.
6. Insert thermometer probe into 1X Target Retrieval (through hole in food steamer lid).

Dehydrate the tissue

1. Remove slides from 4% PFA, and immerse them in **50% EtOH** for **5 MIN** at **RT.**
   TURN ON FOOD STEAMER AS SOON AS SLIDES ARE IN 50% EtOH
2. Remove slides from 50% EtOH, and immerse them in **70% EtOH** for **5 MIN** at **RT.**
3. Remove slides from 70% EtOH, and immerse them in **100% EtOH** for **5 MIN** at **RT.**
4. Remove slides from 100% EtOH, and immerse them in **fresh 100% EtOH** for **5 MIN** at **RT.**

Dry the slides

1. Remove slides from 100% EtOH, and let them **air dry** for **5 MIN** at **RT.**

Apply RNAscope® Hydrogen Peroxide

1. Add **2-4 drops H_2O_2 to each section** and let sit for **10 MIN*** at **RT**. Use enough solution to completely cover the sections. Then rinse 1X in 1st staining dish with RT dH_2O.
   ***5 MIN** with **H_2O_2** may be sufficient for *newer tissue* – use *half Target Retrieval time* too.
2. Remove **dH_2O** staining dish from food steamer.

Apply RNAscope® Target Retrieval

1. **Slowly submerge** Tissue-Tek® slide rack containing **slides into boiling 1X Target Retrieval** solution and keep them in solution for **5 MIN only***. Check Target Retrieval temperature – must be between **98-102°C**.
   ***2.5 MIN** in **Target Retrieval** may be sufficient for *newer tissue* – use *half H_2O_2 time* too.
2. *Immediately* **submerge hot slide rack slowly** into 2nd **staining dish with HOT dH_2O**.
3. Wash slides in **dH_2O** by **gently** moving rack **up and down 3-5 times. Repeat** with **fresh dH_2O** (3rd staining dish).
4. Rinse slides in fresh **100% EtOH** by **gently** moving rack **up and down 3-5 times**. Air dry.

Create Barrier

1. **Draw 2-4 times** around desired tissue sections using Immedge™ **hydrophobic barrier** pen. Let barrier **dry** completely ~**1 MIN** or **OVERNIGHT** at **RT**.