QUICK GUIDE: RNAscope® 2.5 BROWN – **PART 2**

Tissue Pretreatment (Part 2)

Prepare Sections

1. **Turn on HybEZ™ oven** and switch to or ensure **oven** is in **"Hold T" mode** and set at **60°C**.
2. Bake slides for **60 MIN** at **60°C** in oven tray without humidifying paper.

Prepare Materials

1. Reduce HybEZ™ Oven temperature to **40°C** by switching to **"RNAscope" mode**. Place a wet humidifying paper in the Humidity Control Tray and re-insert covered tray into oven.

Apply RNAscope® Protease III

1. Once oven reaches 40°C, place slides in HybEZ™ Slide Rack, and **add 2-4 drops** of **Protease III** to each section. Use enough solution to completely cover the sections.
2. Place the HybEZ™ Slide Rack in the prewarmed oven. Incubate at **40°C** for **30 MIN**.
   *** Prepare RNAscope® 2.5 assay materials during this step.*
3. Wash slides in clear EZ-Batch Wash Tray by **submerging** them in **dH_2O** and tilting tray back and forth 3-5 times. **Repeat** with **fresh dH_2O**.

RNAscope® 2.5 Assay

Workflow Steps	
PREPARE THE MATERIALS ~10-30 MIN	1. Prepare 1.1L of 1X Wash Buffer by adding 1078 mL dH_2O and 22 mL of 50X Wash Buffer to a 1L glass bottle. Mix well. 2. Equilibrate reagents and equipment: • Remove Amp 1-6 from refrigerator. • Warm probes by placing bottles atop the preheated HybEZ™ Oven, then cool to **RT** prior to use.
RUN THE ASSAY ~5 HOURS Hybridize Probe ↓ Hybridize Amp 1 ↓ Hybridize Amp 2 ↓ Hybridize Amp 3 ↓ Hybridize Amp 4 ↓ Hybridize Amp 5 ↓ Hybridize Amp 6 ↓ Detect the Signal ↓ Counterstain the Slides ↓	Hybridize Probe (**2 HRS** at **40°C**) 1. Remove excess liquid from slides and **add ~40µL** of **probe** to each section. 2. Insert sealed tray containing HybEZ™ Slide Rack back into HybEZ™ Oven for **2 HRS** at **40°C**. Remove slide rack. 3. Wash slides in 1X Wash Buffer by tilting the tray for **2 MIN** at **RT**. Repeat with fresh 1X Wash Buffer. Hybridize Amp 1 (**30 MIN** at **40°C**) 1. Remove excess liquid from slides and **add 2-4 drops** of **Amp 1** to each section. 2. Insert sealed tray containing HybEZ™ Slide Rack back into HybEZ™ Oven for **30 MIN** at **40°C**. Remove slide rack. 3. Wash slides in 1X Wash Buffer by tilting the tray for **2 MIN** at **RT**. Repeat with fresh 1X Wash Buffer. Hybridize Amp 2 (**15 MIN** at **40°C**) 1. Remove excess liquid from slides and **add 2-4 drops** of **Amp 2** to each section. 2. Insert sealed tray containing HybEZ™ Slide Rack back into HybEZ™ Oven for **15 MIN** at **40°C**. Remove slide rack. 3. Wash slides in 1X Wash Buffer by tilting the tray for **2 MIN** at **RT**. Repeat with fresh 1X Wash Buffer.

Mount the Slides	Hybridize Amp 3 (**30 MIN** at **40°C**) 1. Remove excess liquid from slides and **add 2-4 drops** of **Amp 3** to each section. 2. Insert sealed tray containing HybEZ™ Slide Rack back into HybEZ™ Oven for **30 MIN** at **40°C**. Remove slide rack. 3. Wash slides in 1X Wash Buffer by tilting the tray for **2 MIN** at **RT**. Repeat with fresh 1X Wash Buffer.
	Hybridize Amp 4 (**15 MIN** at **40°C**) 1. Remove excess liquid from slides and **add 2-4 drops** of **Amp 4** to each section. 2. Insert sealed tray containing HybEZ™ Slide Rack back into HybEZ™ Oven for **15 MIN** at **40°C**. Remove slide rack but do ***not*** place tray back into oven. 3. Wash slides in 1X Wash Buffer by tilting the tray 3-5 times every 30 seconds for **2 MIN** at **RT**. Repeat with fresh 1X Wash Buffer.
	Hybridize Amp 5 (**30 MIN** at **RT**) 1. Remove excess liquid from slides and **add 2-4 drops** of **Amp 5** to each section. 2. Incubate sealed tray containing HybEZ™ Slide Rack for **30 MIN** at **RT**. 3. Wash slides in 1X Wash Buffer by tilting the tray 3-5 times every 30 seconds for **2 MIN** at **RT**. Repeat with fresh 1X Wash Buffer.
	Hybridize Amp 6 (**15 MIN** at **RT**) 1. Remove excess liquid from slides and **add 2-4 drops** of **Amp 6** to each section. 2. Incubate sealed tray containing HybEZ™ Slide Rack for **15 MIN** at **RT**. 3. Wash slides in 1X Wash Buffer by tilting the tray 3-5 times every 30 seconds for **2 MIN** at **RT**. Repeat with fresh 1X Wash Buffer.
	Detect the Signal (**10 MIN** at **RT**) 1. Mix equal volumes of BROWN-A and BROWN-B (pipette 20 µL/section for each one). 2. Remove excess liquid from slides and **pipette 40 µL** of **DAB** onto each tissue section. 3. Incubate sealed tray containing HybEZ™ Slide Rack for **10 MIN** at **RT**. 4. Remove DAB from slides and wash 3-5 times in **dH_2O**.
	Counterstain the Slides 1. Follow Nissl protocol for RNAscope outlined in wet lab recipe/protocol binder (also on lab webpage). Mount the Slides 1. Use 3-4 drops of Permount and coverslip, let air dry overnight at RT.