RNAscope® Fluorescent Multiplex Kit Quick Guide

For Fresh Frozen Tissues

Introduction

This quick guide is intended for advanced users who are familiar with the procedures in the *RNAscope® Sample Preparation and Pretreatment Guide for Fresh Frozen Tissue, Part 1* (Document No. 320513-USM) and *RNAscope® Fluorescent Multiplex Kit User Manual Part 2* (Document No. 320293-USM). Refer to the user manual for safety guidelines. For every chemical, read the Safety Data Sheet (SDS) and follow handling instructions. Wear appropriate protective eyewear, clothing, and gloves. For the latest services and support information, go to: **https://acdbio.com/technical-support/support-overview**.

Part 1 Prepare and Pretreat Samples

	Workflow Steps	
	PREPARE FRESH FROZEN SECTIONS	1. Remove tissue and cut to fit into cryomolds. 2. Freeze on dry ice or liquid Nitrogen within **5 MIN** of harvest. 3. Embed frozen tissue in cryo-embedding medium and freeze blocks. 4. Store the frozen block in an air-tight container at **–80°C**. **Note:** Embedded tissue may be stored for at least 3 months. 5. Equilibrate block to **–20°C** in a cryostat **~1 HR**. 6. Cut 10–20 µm sections and mount onto SuperFrost® Plus slides. 7. Dry slides at –20°C for **10 MIN**. 8. Store in air-tight slide boxes at **–80°C** until use. OPTIONAL STOPPING POINT (1). Use sectioned tissue within 3 months.
	PREPARE SLIDES ~1 HOUR Fix Sections ↓ Dehydrate Sections ↓ Create Barrier	Fix Sections 1. Chill 200 mL fresh 10% NBF or 4% PFA to **4°C** in a Tissue Tek® Staining Dish. 2. Remove slides from **–80°C**, and place in a Tissue Tek® Slide Rack. 3. *Immediately* immerse slides in the pre-chilled fixative. Fix for **15 MIN** at **4°C**. Dehydrate Sections 1. Have ready 200 mL 50% EtOH, 200 mL 70% EtOH, and 600 mL 100% EtOH in Tissue Tek® Staining Dishes. 2. Remove slides from the fixative and *immediately* place in 50% EtOH for **5 MIN** at **ROOM TEMPERATURE (RT)**. 3. Place the slides in 70% EtOH for 5 MIN at RT. 4. Place the slides in 100% EtOH for 5 MIN at RT. 5. Repeat step 6 with fresh 100% EtOH. OPTIONAL STOPPING POINT (2). Slides may be stored in 100% EtOH at **–20°C** for up to 1 week. Create Barrier 6. Air dry slides for **5 MIN** at **RT** on absorbent paper. Draw 2–4 times around tissue using the Immedge™ hydrophobic barrier pen. Dry completely **~1 MIN**.
	PRETREAT SAMPLES ~30 MIN Prepare Oven and Reagents ↓	Prepare Oven and Reagents (**30 MIN at 40°C**) 1. Set HybEZ™ oven to **40°C** and warm HybEZ™ Humidity Control Tray containing wet Humidifying Paper for **30 MIN** before use. Keep tray warm during assay.

	Apply Protease IV	Apply Protease IV(**30 MIN at RT**) 1. Add ~5 drops of RNAscope® Protease IV to each section for **30 MIN** at **RT**. 2. Place slides into a Tissue-Tek® Slide Rack submerged in 1X PBS. 3. Wash slides in 1X PBS by moving the rack up and down 3–5 times and repeat with fresh 1X PBS. **Note:** If needed, prepare RNAscope® Multiplex assay materials during this step.

Part 2: RNAscope® Fluorescent Multiplex Assay

	Workflow Steps	
	PREPARE THE MATERIALS ~10–30 MIN	1. Prepare 3 L of 1X Wash Buffer by adding 2.94 L distilled water and 1 bottle (60 mL) of 50X Wash Buffer to a large carboy. Mix well. 2. Place Amp 1–4-FL reagents at **RT**. 3. Ensure HybEZ™ Oven and prepared Humidity Control Tray are at **40°C**. Prepare Probes 1. Warm probes for **10 MIN** at **40°C**, then cool to **RT**. 2. Briefly spin the C2 and C3 probes. 3. Pipette 1 volume of C2 and 1 volume of C3 probes to 50 volumes of C1 probe into a tube. Invert the tube several times to mix. **Note:** Mixed probes can be stored at **4°C** for up to 6 months.
	RUN THE ASSAY ~7 HOURS Hybridize Probe ↓ Hybridize Amp 1-FL ↓ Hybridize Amp 2-FL ↓ Hybridize Amp 3-FL ↓ Hybridize Amp 4-FL ↓ Counterstain and Mount the Slides	Hybridize Probe (**2 HRS at 40°C**) 1. Remove excess liquid from slides, place in the HybEZ™ Slide Rack, and add ~4 drops probe to each section. 2. Insert sealed tray containing HybEZ™ Slide Rack back into the HybEZ™ Oven for **2 HRS** at **40°C**. Remove slide rack. 3. Wash slides in 1X Wash Buffer for **2 MIN** at **RT**. Repeat with fresh 1X Wash Buffer. Hybridize Amp 1-FL (**30 MIN at 40°C**) 1. Remove excess liquid from slides, place in the HybEZ™ Slide Rack, and add ~4 drops Amp 1-FL to each section. 2. Insert sealed tray containing HybEZ™ Slide Rack into the HybEZ™ Oven for **30 MIN** at **40°C**. Remove slide rack. 3. Wash slides in 1X Wash Buffer for **2 MIN** at **RT**. Repeat with fresh 1X Wash Buffer. Hybridize Amp 2-FL (**15 MIN at 40°C**) 1. Remove excess liquid from slides, place in the HybEZ™ Slide Rack, and add ~4 drops Amp 2-FL to each section. 2. Insert sealed tray containing HybEZ™ Slide Rack into the HybEZ™ Oven for **15 MIN** at **40°C**. Remove slide rack. 3. Wash slides in 1X Wash Buffer for **2 MIN** at **RT**. Repeat with fresh buffer. Hybridize Amp 3-FL (**30 MIN at 40°C**) 1. Remove excess liquid from slides, place in the HybEZ™ Slide Rack, and add ~4 drops Amp 3-FL to each section. 2. Insert sealed tray containing HybEZ™ Slide Rack into the HybEZ™ Oven for **30 MIN** at **40°C**. Remove slide rack. 3. Wash slides in 1X Wash Buffer for **2 MIN** at **RT**. Repeat with fresh buffer. Hybridize Amp 4-FL (**15 MIN at 40°C**) 1. Remove excess liquid from slides, place in the HybEZ™ Slide Rack, and add ~4 drops Amp 4-FL to each section. **Note:** Use either Amp 4-FL–Alt A, Alt B, or Alt C. 2. Insert sealed tray containing HybEZ™ Slide Rack into the HybEZ™ Oven for **15 MIN** at **40°C**. Remove slide rack, but do *not* place tray back into the oven.

		3. Wash slides in 1X Wash Buffer for **2 MIN** at **RT**. Repeat with fresh buffer. Counterstain and Mount the Slides 1. Remove excess liquid from the slides and add ~4 drops of DAPI to each section. 2. Incubate for **30 SEC** at **RT**. 3. Remove DAPI and *immediately* place 1–2 drops of fluorescent mounting medium on the slide. Place coverslip over section. 4. Store slides in the dark at **4°C**.
	EVALUATE THE RESULTS	Examine tissue sections *immediately or within a few days* under a fluorescent microscope at 20–40X magnification.

Troubleshooting

For troubleshooting information, please contact technical support at **support@acdbio.com**.

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Headquarters
7707 Gateway Blvd Suite 200, Newark, CA 94545 Phone 1-510-576-8800 Toll Free 1-877-576-3636
For support, email **support@acdbio.com**
www.acdbio.com