RNAscope® 2.5 HD Duplex Detection Kit (GREEN/RED) Quick Guide

For FFPE Tissues

Introduction

This quick guide is intended for advanced users who are familiar with the procedures in the *Formalin-Fixed Paraffin-Embedded (FFPE) Sample Preparation Pretreatment Guide User Manual, Part 1* (Catalog No. 322452-USM) and *RNAscope® 2.5 HD Duplex Detection Kit User Manual, Part 2* (Catalog No. 322500-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: **www.acdbio.com/support**.

Part 1 Prepare and Pretreat Samples

	Workflow Steps	
	PREPARE FFPE SECTIONS	1. Immediately place dissected tissue sample in fresh 10% NBF for **16–32 HRS** at **ROOM TEMPERATURE (RT)**. 2. Dehydrate, embed in paraffin, and cut the sample into 5 +/− 1 µm sections. Mount sections on Superfrost® Plus slides. OPTIONAL STOPPING POINT (1). Use sectioned tissue within 3 months. Store sections with desiccants at RT.
	PREPARE SLIDES ~1.5 HOURS Bake Slides ↓ Deparaffinize FFPE Sections	Bake Slides 1. Bake slides in a dry oven for **1 HR** at **60°C**. OPTIONAL STOPPING POINT (2). Use sectioned tissue within 1 week. Store sections with dessicants at RT. Deparaffinize FFPE Sections 1. In a fume hood: • Fill two Tissue-Tek® Clearing Agent dishes with ~200 mL fresh xylene. • Fill two Tissue-Tek® Staining dishes with ~200 mL fresh 100% EtOH. 2. Place slides in a Tissue-Tek® Slide Rack in xylene **2 x 5 MIN**. 3. Incubate slides in 100% EtOH **2 x 1 MIN**. 4. Remove slides from the rack. Air dry slides for **5 MIN** at **RT**. OPTIONAL STOPPING POINT (3). Air dry overnight at RT (must use within 24 hrs) or proceed directly to the next step.
	PRETREAT SAMPLES ~1–2 HOURS Prepare Oven and Reagents ↓ Apply RNAscope® Hydrogen Peroxide ↓	**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 the assay. 2. Prepare 700 mL fresh 1X Target Retrieval in a beaker. Cover with foil, bring to a mild boil, and maintain. Do not boil more than **30 MIN** before use. **Apply Hydrogen Peroxide (10 MIN at RT)** 1. Add ~5–8 drops of Hydrogen Peroxide to each section for **10 MIN** at **RT**. 2. Place slides into a Tissue-Tek® Slide Rack submerged in distilled water. 3. Wash slides in the distilled water by moving the rack up and down 3–5 times and repeat with fresh distilled water.

	↓ Perform Target Retrieval ↓ Create Hydrophobic Barrier ↓ Apply Protease Plus	**Perform RNAscope® Target Retrieval** 1. With a pair of forceps *very slowly* submerge the slide rack into the boiling 1X Target Retrieval solution. Refer to Appendix A of the *Formalin-Fixed Paraffin-Embedded (FFPE) Sample Preparation Pretreatment Guide User Manual, Part 1* (Cat. No. 322452) for specific pretreatment time, depending on your tissue type. 2. *Immediately* transfer hot slide rack to a staining dish containing distilled water. 3. Wash slides in the distilled water by moving the rack up and down 3–5 times and repeat with fresh distilled water. 4. Wash slides in fresh 100% EtOH by moving the rack up and down 3–5 times and air dry. **Create Hydrophobic Barrier** 1. Draw 2–4 times around tissue using the Immedge™ hydrophobic barrier pen. Dry completely **~2 MIN** or **OVERNIGHT** at **RT**. **Apply Protease Plus** 1. Place slides in the HybEZ™ Slide Rack, and add ~5 drops of Protease Plus to each section. 2. Place the HybEZ™ Slide Rack in the prewarmed HybEZ™ Humidity Control Tray. Seal tray and insert back into the HybEZ™ Oven. Incubate at **40°C** for **30 MIN**. **Note:** If needed, prepare RNAscope® 2.5 assay materials during this step. 3. Wash slides in the distilled water by moving the rack up and down 3–5 times and repeat with fresh distilled water.

Part 2: RNAscope® 2.5 Duplex 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. Warm 50X Wash Buffer up to **40°C** for **10–20 MIN** before making 1X Wash Buffer. 2. Prepare 50% Hematoxylin and 0.02% Ammonia water. 3. Equilibrate remove Amps 1–10 from the refrigerator and keep at **RT** **Prepare Probes** 4. Warm probes for **10 MIN** at **40°C**, then cool to **RT**. 5. Briefly spin the C2 probe. 6. Mix 1:50 ratio of C2 probe to C1 probe by pipetting 1 volume of C2 probe to 50 volumes of C1 probe into a tube. Invert the tube several times. 7. Mixed probes can be stored at **4°C** for up to 6 months.
	RUN THE ASSAY ~7 Hours Hybridize Probe ↓ Hybridize Amp 1 ↓ Hybridize Amp 2 ↓ Hybridize Amp 3 ↓ Hybridize Amp 4	**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 the 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 (30 MIN at 40°C)** 1. Remove excess liquid from slides, place in the HybEZ™ Slide Rack, and add ~4 drops Amp 1 to each section. 2. Insert the 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 5
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Hybridize Amp 6
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Detect the Red Signal
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Hybridize Amp 7
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Hybridize Amp 8
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Hybridize Amp 9
↓
Hybridize Amp 10
↓
Detect the Green Signal
↓
Counterstain the Slides
↓
Mount the Slides

Hybridize Amp 2 (15 MIN at 40°C)

1. Remove excess liquid from slides, place in the HybEZ™ Slide Rack, and add ~4 drops Amp 2 to each section.
2. Insert the 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 (30 MIN at 40°C)

1. Remove excess liquid from slides, place in the HybEZ™ Slide Rack, and add ~4 drops Amp 3 to each section.
2. Insert the 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 (15 MIN at 40°C)

1. Remove excess liquid from slides, place in the HybEZ™ Slide Rack, and add ~4 drops Amp 4 to each section.
2. Insert the sealed tray containing HybEZ™ Slide Rack into the HybEZ™ Oven for **15 MIN** at **40°C**. Remove slide rack, but do *not* place the tray back into the oven.
3. Wash slides in 1X Wash Buffer for **2 MIN** at **RT**. Repeat with fresh buffer.

Hybridize Amp 5 (30 MIN at RT)

1. Remove excess liquid from slides, place in the HybEZ™ Slide Rack, and add ~4 drops Amp 5 to each section.
2. Incubate the sealed tray containing HybEZ™ Slide Rack for **30 MIN** at **RT**.
3. Wash slides in 1X Wash Buffer for **2 MIN** at **RT**. Repeat with fresh buffer.

Hybridize Amp 6 (15 MIN at RT)

1. Remove excess liquid from slides, place in the HybEZ™ Slide Rack, and add ~4 drops Amp 6 to each section.
2. Incubate the sealed tray containing HybEZ™ Slide Rack for **15 MIN** at **RT**.
3. Wash slides in 1X Wash Buffer for **2 MIN** at **RT**. Repeat with fresh buffer.

Detect the Signal (10 MIN at RT)

1. Briefly spin RED-B and mix 1 volume of RED-B to 60 volumes of RED-A (must use within **3–5 MIN**). For examples add 2.5 µL of Red-B to150 µL of Red-A per section.
2. Remove excess liquid from slides, place in the HybEZ™ Slide Rack, and pipette ~120 µL of RED solution onto each tissue section.
3. Incubate sealed tray containing HybEZ™ Slide Rack for **10 MIN** at **RT**.
4. Remove solution from slides and wash 3–5 times in distilled water.

Hybridize Amp 7 (15 MIN at 40°C)

1. Remove excess liquid from slides, place in the HybEZ™ Slide Rack, and add ~4 drops Amp 7 to each section.
2. Incubate the sealed tray containing HybEZ™ Slide Rack for **15 MIN** at **40°C**.
3. Wash slides in 1X Wash Buffer for **2 MIN** at **RT**. Repeat with fresh buffer.

Hybridize Amp 8 (30 MIN at 40°C)

1. Remove excess liquid from slides, place in the HybEZ™ Slide Rack, and add ~4 drops Amp 8 to each section.
2. Incubate the sealed tray containing HybEZ™ Slide Rack for **30 MIN** at **40°C**.
3. Wash slides in 1X Wash Buffer for **2 MIN** at **RT**. Repeat with fresh buffer.

Hybridize Amp 9 (30 MIN at RT)

1. Remove excess liquid from slides, place in the HybEZ™ Slide Rack, and add ~4 drops Amp 9 to each section.
2. Incubate the sealed tray containing HybEZ™ Slide Rack for **30 MIN** at **RT**.

		3. Wash slides in 1X Wash Buffer for **2 MIN** at **RT**. Repeat with fresh buffer. **Hybridize Amp 10 (15 MIN at RT)** 1. Remove excess liquid from slides, place in the HybEZ™ Slide Rack, and add ~4 drops Amp 10 to each section. 2. Incubate the sealed tray containing HybEZ™ Slide Rack for **15 MIN** at **RT**. 3. Wash slides in 1X Wash Buffer for **2 MIN** at **RT**. Repeat with fresh buffer. **Detect the Signal (10 MIN at RT)** 1. Briefly spin GREEN-B and mix 1 volume of Green-B to 50 volumes of GREEN-A (must use within **3–5 MIN**). For examples add 3 µL of GREEN-B to150 µL of GREEN-A per section. 2. Remove excess liquid from slides, place in the HybEZ™ Slide Rack, and pipette ~120 µL of GREEN solution onto each tissue section. 3. Incubate sealed tray containing HybEZ™ Slide Rack for **10 MIN** at **RT**. 4. Remove solution from slides and wash **5 MIN** with 1X wash buffer 5. Rinse quickly with distilled water. **Counterstain the Slides (30 Sec at RT)** 1. Place slides in 50% Hematoxylin I for **30 Sec** at **RT**. Wash 3–5 times in distilled water and repeat with fresh distilled water. 2. Wash slides **10 SEC** in 0.02% Ammonia water, and then wash 3–5 times in distilled water. **Mount the Slides** 1. Dry slides in a **60°C** dry oven for **15 MIN**. 2. Dip the slides into fresh pure xylene and immediately place 1–2 drops of EcoMount on the slide before the xylene dries. Place a coverslip over the section. 3. Air dry for **30 MIN**.
	EVALUATE THE RESULTS	Examine tissue sections under a standard bright field microscope at 20–40X magnification

Troubleshooting

For troubleshooting information, please contact technical support at support.acd@bio-techne.com.

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