ADVANCED CELL DIAGNOSTICS

RNAscope® 2.5 Duplex Detection Kit (Chromogenic) Quick Guide

For FFPE Tissues

Document Number : 322500- QKG

Introduction

Note: Due to the length of this procedure (~14 hours), we recommend using the stopping point following probe hybridization. Slides should be stored in 5X SSC. Please see the user manual 322500-USM for further information.

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 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 dessicants 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 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 Hydrogen Peroxide ↓ Apply Target Retrieval ↓ Create Barrier ↓ Apply Protease Plus	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. 2. Prepare 700 mL fresh 1X Target retrieval solution 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 Hyrdrogen 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. Apply Target Retrieval (**Pretreat 2**) 1. With a pair of forceps *very slowly* submerge the slide rack into boiling 1X Target Retrieval solution. Refer to Appendix A of the *Part 1, Sample Preparation and Pretreatment Guide for FFPE Tissue* (Doc. No.322452-USM) 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.

		4. Wash slides in fresh 100% EtOH for immediately bymoving the rack up and down 3–5 times and air dry. Create Barrier 1. Draw 2–4 times around tissue using the Immedge™ hydrophobic barrier pen. Dry completely ~**2 MIN** at **RT**. Apply Protease Plus 1. Place in the HybEZ™ Slide Rack, and add 4–8 drops of Protease Plus) to cover each section. 2. Place the HybEZ™ Slide Rack in the pre-warmed 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 Duplex 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. Warm 50X wash buffer for 20 minutes before preparing 1X wash buffer solution 2. 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. 3. Prepare 50% Hematoxylin. 4. Equilibrate reagents and equipment: • Place Amps 1–10 at **RT**. Prepare Probes 1. Warm probes for **10 MIN** at **40°C**, then cool to **RT**. 2. Briefly spin the C2 probe. 3. 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. **Note:** 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 Amp 5 ↓ Hybridize Amp 6 ↓ Detect the Red Signal ↓ Hybridize Amp 7	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. OPTIONAL STOPPING POINT (4). The slides can be stored in 5X SSC overnight at RT. Hybridize Amp 1 (**30 MIN at 40°C**) 1. Remove slides from SSC and wash in 1X Wash Buffer for 1–2 times, remove excess liquid from slides, place in the HybEZ™ Slide Rack, and add ~4–8 drops Amp 1 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 (**15 MIN at 40°C**) 1. Remove excess liquid from slides, place in the HybEZ™ Slide Rack, and add ~4–8 drops Amp 2 to each section. 2. Insert sealed tray containing HybEZ™ Slide Rack into the HybEZ™ Oven for **15 MIN** at **40°C**. Remove slide rack.

↓
Hybridize Amp 8
↓
Hybridize Amp 9
↓
Hybridize Amp 10
↓
Detect the Green Signal
↓
Counterstain the Slides
↓
Mount the Slides

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 ~4–8 drops Amp 3 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 (**15 MIN at 40°C**)

1. Remove excess liquid from slides, place in the HybEZ™ Slide Rack, and add ~4–8 drops Amp 4 to each section.
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.

Hybridize Amp 5 (**30 MIN at RT**)

1. Remove excess liquid from slides, place in the HybEZ™ Slide Rack, and add ~4–8 drops 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 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 ~4–8 drops 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 for **2 MIN** at **RT**. Repeat with fresh buffer.

Detect the Red Signal (**10 MIN at RT**)

1. Briefly spin Red-B and mix a 1:60 ratio of Red-B to Red-A (2.5 µL of Red-B to 150 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**. Keep slide in dark.
4. Wash slides in 1X Wash Buffer for **2 MIN** at **RT**. Repeat with fresh buffer.

Hybridize Amp 7 (**15 MIN at 40°C**)

1. Remove excess liquid from slides, place in the HybEZ™ Slide Rack, and add ~4–8 drops Amp 7 to each section.
2. Incubate 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 ~4–8 drops Amp 8 to each section.
2. Incubate 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 ~4–8 drops Amp 9 to each section.
2. Incubate 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 ~4–8 drops Amp 10 to each section. 2. Incubate 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 Green Signal (**10 MIN at RT**) 1. Briefly spin Green B and mix a 1:50 ratio of Green -B to Green -A (3 µL of Green -B to 150 µL of Green -A per section). 2. Remove excess liquid from slides, place in the HybEZ™ Slide Rack, and pipette ~150 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 briefly in distilled water (<30 seconds). Counterstain the Slides (**30 SEC at RT**) 1. Place slides in 50% Hematoxylin for **30 SEC** at **RT**. 2. Wash quickly in tap water and repeat wash once or twice. Do not let stained section sit in water for more than 30 seconds for each wash. Mount the Slides 1. Dry slides in a **60°C** dry oven for **15–30 MIN**. 2. Cool the slides at **RT ~5 MIN**. 3. Place 1–2 drops of VectaMount on the slide. Place coverslip over section. 4. Air dry for **5 MIN**.
	EVALUATE THE RESULTS	Examine tissue sections under a standard bright field microscope at 20–40X magnification.

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