



RNAscope® 2-Plex Detection Reagents Quick Guide

For FFPE Tissues

Introduction

This quick guide is intended for advanced users who are familiar with the procedures in the *Part 1 Sample Preparation Pretreatment Guide for Formalin-Fixed Paraffin-Embedded (FFPE) For RNAscope® 2.5*; see Catalog No. UM 322452 and *RNAscope® 2-Plex Detection Reagents User Manual (Detection only)* (Catalog No. 320494). 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	
<p>PREPARE FFPE SECTIONS</p>	<ol style="list-style-type: none"> Immediately place dissected tissue sample in fresh 10% NBF for 16–32 HRS at ROOM TEMPERATURE (RT). Dehydrate, embed in paraffin, and cut sample into 5 +/- 1 µm sections. Mount sections on Superfrost® Plus slides. <hr/> <p>OPTIONAL STOPPING POINT (1). Use sectioned tissue within 3 months. Store sections with dessicants at RT.</p>
<p>PREPARE SLIDES ~1.5 HOURS</p> <p style="text-align: center;">Bake Slides ↓ Deparaffinize FFPE Sections</p>	<p>Bake Slides</p> <ol style="list-style-type: none"> Bake slides in a dry oven for 1 HR at 60°C. <hr/> <p>OPTIONAL STOPPING POINT (2). Use sectioned tissue within 1 week. Store sections with dessicants at RT.</p> <p>Deparaffinize FFPE Sections</p> <ol style="list-style-type: none"> In a fume hood: <ul style="list-style-type: none"> Fill two Tissue-Tek® Clearing Agent dishes with ~200 mL fresh xylene. Fill two Tissue-Tek® Staining dishes with ~200 mL fresh 100% EtOH. Place slides in a Tissue-Tek® Slide Rack in xylene 2 x 5 MIN. Incubate slides in 100% EtOH 2 x 1 MIN. Remove slides from rack. Air dry slides for 5 MIN at RT. <hr/> <p>OPTIONAL STOPPING POINT (3). Air dry overnight at RT (must use within 24 hrs) or proceed directly to the next step.</p>
<p>PRETREAT SAMPLES ~1–2 HOURS</p> <p style="text-align: center;">Prepare Oven and Reagents ↓ Apply Hydrogen Peroxide ↓ Apply Target Retrieval ↓ Create Barrier ↓ Apply Protease Plus</p>	<p>Prepare Oven and Reagents (30 MIN at 40°C)</p> <ol style="list-style-type: none"> 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. Prepare 700 mL fresh 1X RNAscope® Target Retrieval in a beaker. Cover with foil, bring to a mild boil, and maintain. Do not boil more than 30 MIN before use. <p>Apply Hydrogen Peroxide (10 MIN at RT)</p> <ol style="list-style-type: none"> Add ~5–8 drops of RNAscope® Hydrogen Peroxide to each section for 10 MIN at RT. Place slides into a Tissue-Tek® Slide Rack submerged in distilled water. Wash slides in the distilled water by moving the rack up and down 3–5 times and repeat with fresh distilled water. <p>Apply Target Retrieval</p> <ol style="list-style-type: none"> With a pair of forceps <i>very slowly</i> submerge the slide rack into boiling 1X RNAscope® Target Retrieval solution. Refer to Appendix A of the <i>Part 1, Sample</i>

	<p><i>Preparation and Pretreatment Guide for FFPE Tissue</i> (Cat. No. 322452) for specific pretreatment time, depending on your tissue type.</p> <ol style="list-style-type: none"> Immediately transfer hot slide rack to a staining dish containing distilled water. Wash slides in the distilled water by moving the rack up and down 3–5 times and repeat with fresh distilled water. Wash slides in fresh 100% EtOH by moving the rack up and down 3–5 times, and air dry. <p>Create Barrier</p> <ol style="list-style-type: none"> Draw 2–4 times around tissue using the Immedge™ hydrophobic barrier pen. Dry completely ~2 MIN or OVERNIGHT at RT. <p>Apply Protease Plus</p> <ol style="list-style-type: none"> Remove excess liquid from slides, place in the HybEZ™ Slide Rack, and add ~5 drops of RNAscope® Protease Plus to each section. 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. <p>Note: If needed, prepare RNAscope® 2-Plex assay materials during this step.</p> <ol style="list-style-type: none"> Wash slides in the distilled water by moving the rack up and down 3–5 times and repeat with fresh distilled water.
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Part 2: RNAscope® 2-Plex Assay

Workflow Steps	
<p>PREPARE THE MATERIALS ~10–30 MIN</p>	<ol style="list-style-type: none"> Prepare 3 L of 1X Wash Buffer by adding 2.94 L distilled water and 1 bottle (60 mL) of RNAscope® Wash Buffer (50X) to a large carboy. Mix well. Prepare 50% Hematoxylin and 0.02% Ammonia water. Add 200 mL xylene to a Tissue Tek® Clearing Agent Dish. Equilibrate reagents and equipment: <ul style="list-style-type: none"> Place Amp 1–6 at RT. Ensure HybEZ™ Oven and prepared Humidity Control Tray are at 40°C. <p>Prepare RNAscope® Probes</p> <ol style="list-style-type: none"> Warm probes for 10 MIN at 40°C, then cool to RT. Briefly spin the C2 probe. 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. <p>Note: Mixed probes can be stored at 4°C for up to 6 months.</p>
<p>RUN THE ASSAY ~7 HOURS</p> <p>Hybridize Probe ↓ Hybridize Amp 1 ↓ Hybridize Amp 2 ↓ Hybridize Amp 3 ↓ Hybridize Amp 4 ↓</p>	<p>Hybridize Probe (2 HRS at 40°C)</p> <ol style="list-style-type: none"> Remove excess liquid from slides, place in the HybEZ™ Slide Rack, and add ~4 drops probe to each section. Insert sealed tray containing HybEZ™ Slide Rack back into the HybEZ™ Oven for 2 HRS at 40°C. Remove slide rack. Wash slides in 1X Wash Buffer for 2 MIN at RT. Repeat with fresh 1X Wash Buffer. <p>Hybridize Amp 1 (30 MIN at 40°C)</p> <ol style="list-style-type: none"> Remove excess liquid from slides, place in the HybEZ™ Slide Rack, and add ~4 drops Amp 1 to each section. Insert sealed tray containing HybEZ™ Slide Rack into the HybEZ™ Oven for 30 MIN at 40°C. Remove slide rack. Wash slides in 1X Wash Buffer for 2 MIN at RT. Repeat with fresh 1X Wash Buffer.

<p style="text-align: center;">Hybridize Amp 5 ↓ Hybridize Amp 6 ↓ Detect the Red Signal ↓ Detect the Green Signal ↓ Counterstain the Slides ↓ Mount the Slides</p>	<p>Hybridize Amp 2 (15 MIN at 40°C)</p> <ol style="list-style-type: none"> 1. Remove excess liquid from slides, place in the HybEZ™ Slide Rack, and add ~4 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. 3. Wash slides in 1X Wash Buffer for 2 MIN at RT. Repeat with fresh buffer. <p>Hybridize Amp 3 (30 MIN at 40°C)</p> <ol style="list-style-type: none"> 1. Remove excess liquid from slides, place in the HybEZ™ Slide Rack, and add ~4 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. <p>Hybridize Amp 4 (15 MIN at 40°C)</p> <ol style="list-style-type: none"> 1. Remove excess liquid from slides, place in the HybEZ™ Slide Rack, and add ~4 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 <i>not</i> place tray back into the oven. 3. Wash slides in 1X Wash Buffer for 2 MIN at RT. Repeat with fresh buffer. <p>Hybridize Amp 5 (30 MIN at RT)</p> <ol style="list-style-type: none"> 1. Remove excess liquid from slides, place in the HybEZ™ Slide Rack, and add ~4 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. <p>Hybridize Amp 6 (15 MIN at RT)</p> <ol style="list-style-type: none"> 1. Remove excess liquid from slides, place in the HybEZ™ Slide Rack, and add ~4 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. <p>Detect the Red Signal (30 MIN at RT)</p> <ol style="list-style-type: none"> 1. Briefly spin RNAscope® Fast Red B and mix a 1:60 ratio of Fast Red B to RNAscope® Fast Red A (2.0 µL of Fast Red B to 120 µL of Fast Red A per section). 2. Remove excess liquid from slides, place in the HybEZ™ Slide Rack, and pipette ~120 µL of Fast Red solution onto each tissue section. 3. Incubate sealed tray containing HybEZ™ Slide Rack for 30 MIN at RT. 4. Wash slides in 1X Wash Buffer for 2 MIN at RT. Repeat with fresh buffer. <p>Detect the Green Signal (10 MIN at RT)</p> <ol style="list-style-type: none"> 1. Briefly spin RNAscope® Green B and mix a 1:50 ratio of Green B to RNAscope® Green A (2.4 µL of Green B to 120 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 3–5 times in distilled water. <p>Counterstain the Slides (2 MIN at RT)</p> <ol style="list-style-type: none"> 1. Place slides in 50% Hematoxylin 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. <p>Mount the Slides</p>
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	<ol style="list-style-type: none"> 1. Dry slides in a 60°C dry oven for 15 MIN. 2. Cool the slides at RT ~5 MIN. 3. Dip the slides into fresh pure xylene and immediately place 1–2 drops of EcoMount on the slide before the xylene dries. 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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