RNAscope® RED Assay and Immunofluorescence

Introduction

This Technical Note provides guidelines for the preparation of fixed frozen tissue that can be assayed using an RNAscope® 2.5 HD Detection Kit – RED (Cat. No. 322360) combined with immunofluorescence. The required RNAscope® Pretreat Reagents are RNAscope® Hydrogen Peroxide and Protease Plus (Cat. No. 322330), and Target Retrieval (Cat. No. 322000). Read the Safety Data Sheet (SDS) available on the website and follow handling instructions. Wear appropriate protective eyewear, clothing, and gloves. For the latest services and support information, go to: www.acdbio.com/support.

Workflow

Part 1: Prepare the Tissue Sections

Fix Sample

1. If needed, perfuse tissue with freshly prepared 4% paraformaldehyde (PFA) in 1X PBS, or go directly to step 2.
2. Dissect tissue and place in freshly prepared 4% PFA for **24 HRS** at **4°C**.

Freeze Tissue

1. Immerse the tissue in 10% sucrose in 1X PBS at **4°C** until the tissue sinks to the bottom of the container (approximately 18 HRS for brain tissue).
2. Repeat this step with 20% sucrose in 1X PBS, followed by 30% sucrose in 1X PBS, each time allowing the tissue to sink to the bottom of the container.
3. Place the tissue in a cryomold filled with Optimal Cutting Temperature (OCT) embedding media.
4. Freeze the tissue by placing the container in dry ice, or immersing it using forceps in liquid nitrogen.
5. Store the frozen tissue in an airtight container at **–80°C**.

Prepare Sections

1. Before tissue sectioning, equilibrate the tissue blocks at **–20°C** for at least **1 HR** in a cryostat.
2. Section the blocks by cutting 7–15 µm sections. Mount the sections on SuperFrost® Plus slides (Fisher Scientific #12-550-15).

IMPORTANT! Use only SuperFrost® Plus slides. Other slide types may result in tissue loss.

3. Air dry the slides for **20 MIN** at **–20°C**.

NOTE: If slides are not used immediately, store the sections at **–80°C** for **< 3 MONTHS**.

Bake Slides

1. Bake slides in a dry air oven for **30 MIN** at **60°C**.
2. Wash the slides with 200 mL 1X PBS in a Tissue-Tek® slide rack for **5 MIN** while moving the rack up and down to remove OCT.
3. Proceed with the RNAscope® Assay protocol.

Part 2: RNAscope® Pretreatment

Prepare Materials

1. Bring HybEZ™ Oven to **40°C**.
2. Place a wet humidifying paper in the Humidity Control Tray, leaving the HybEZ™ Slide Rack on bench. Insert the covered tray into the oven and close the oven door. The tray should be pre-warmed for at least **30 MIN** before use. Keep tray warm during the assay.

3. Prepare 700 mL fresh 1X Target Retrieval in a beaker. Cover with foil, and bring temperature to **98–102°C**. Maintain this temperature.

Apply Hyrdrogen Peroxide

1. Add 2–4 drops of RNAscope® Hydrogen Peroxide to each section for **10 MIN** at **RT**. Use enough solution to completely cover the sections.

Apply Target Retrieval

1. With a pair of forceps *very slowly* submerge the slide rack into the hot 1X RNAscope® Target Retrieval solution for **5 MIN**.

NOTE: Depending on tissue type, boiling time may need to be adjusted.

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. Air dry.

Create Barrier

1. Draw 2–4 times around section using the Immedge™ hydrophobic barrier pen. Let the barrier dry completely **~1 MIN** or **OVERNIGHT** at **RT.**

Apply Protease Plus

1. Place slides in the HybEZ™ Slide Rack, and add 2–4 drops of RNAscope® Protease Plus to each section. Use enough solution to cover each tissue section completely.
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 HD assay materials during this incubation.

3. Place slides in a Tissue-Tek® Slide Rack submerged in distilled water
4. Wash the slides by moving the rack up and down 3–5 times. Repeat with fresh distilled water.

IMPORTANT! Proceed to the RNAscope® protocol using the *RNAscope® 2.5 HD Detection Kit (Red) User Manual Part 2* (Document No. 322360-USM) available at **http://www.acdbio.com/technical-support/user-manuals.**

Part 3: Immunofluorescence

IMPORTANT! Proceed to immunofluorescence assay after RNAscope® 2.5 HD RED assay.

Prepare Materials

1. Prepare **0.2 M PB**: Dissolve 184 g Na phosphate dibasic and 42 g Na phosphate monobasic in 1 L distilled water. Bring to 8 L with water, and check that pH is 7.2–7.4.
2. Prepare **PBST**: Add 18 g NaCl and 3 mL Triton X-100 to 1 L 0.2 M PB and 1 L distilled water.

Block Tissue

1. After the chromogen development step of the RNAscope® assay, wash the slides by moving the slide rack up and down 3–5 times in distilled water. Repeat with fresh distilled water.
2. Wash slides in PBST for **5 MIN**. Repeat twice with fresh PBST each time.
3. Incubate tissue in 4% serum in PBST for **1 HR** at **RT**.

NOTE: Use serum from the species the secondary antibody was raised in.

Antibody Staining

1. Add the primary antibody diluted in PBST to the sections. Incubate **OVERNIGHT** at **4°C**, or using conditions that have been optimized for antibody incubation.

NOTE: You can mix two different primary antibodies in PBST.

2. Wash slides with PBST for **5 MIN**. Repeat twice with fresh PBST each time.
3. Add secondary antibody diluted in PBST to the sections, and incubate **1 HR** at **RT**.

NOTE: If you used two primary antibodies, incubate sections in the 1st secondary antibody, wash sections three times in PBST, followed by the 2nd secondary antibody for another **HR** at **RT**.

4. Wash slides with PBST for **5 MIN**. Repeat twice with fresh PBST each time.
5. Wash slides with **0.1 M PB** by moving the slide rack up and down 3–5 times.

Mount the Slides

1. Remove excess liquid from the slides, and add ~4 drops of DAPI to each slide. Incubate for **30 SEC** at **RT.**
2. Remove DAPI and *immediately* place 1–2 drops of Prolong Gold antifade mounting medium on the slide (not provided).
3. Carefully place a 24 mm x 50 mm glass coverslip over the tissue section. Avoid trapping air bubbles.
4. Dry slides for at least **30 MIN** in the dark before imaging.
5. Store slides at **2–8°C** in the dark for up to two weeks.

Evaluate the Results

You will need to use a fluorescent or confocal microscope to visualize immunofluorescence. The RNAscope® assay should produce clear, intense, red punctate dots. Puncta can fill a large portion of the cytoplasm when a robust signal is detected.

Obtaining Support

For the latest services and support information, go to: **https://acdbio.com/technical-support/support-overview**.

At the website, you can:

- Access telephone and fax numbers to contact Technical Support and Sales.
- Search through FAQs.
- Submit a question directly to Technical Support.

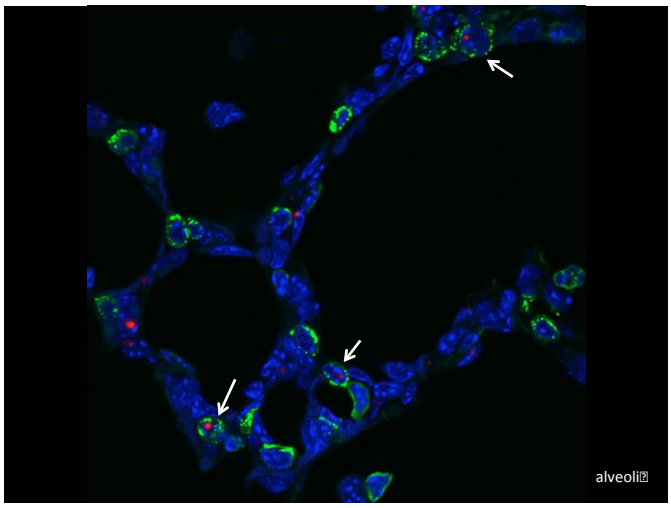

Figure 1. Red RNAscope® signal, green immunohistochemistry signal, and blue DAPI signal on postnatal day 4 wild type mouse lung. White arrows point to cells stained with both red and green.

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