Preparing PBMC and Non-Adherent Cells for the RNAscope® Fluorescent Multiplex Assay

Introduction

This Technical Note provides guidelines to prepare Peripheral Blood Mononuclear Cells (PBMC) and non-adherent cells that can be assayed using an RNAscope® Fluorescent Multiplex Detection Kit. The required RNAscope® Pretreat Reagent is Protease III (available in RNAscope® Protease III and Protease IV Reagents, Cat. No. 322340 or RNAscope® Universal Pretreatment Kit Cat. No. 322380). RNAscope® PBMC Preparation Reagents are also required (Cat. No. 320970; includes Cell Prep and PBMC Wash reagents). Material required but not provided by ACD includes 100% EtOH, Histopaque® 1077 (Sigma-Aldrich), Superfrost® Plus slides (Fisher), and 10% NBF. Refer to the user Safety Data Sheet (SDS) available on the ACD website.

Part 1: Prepare Samples

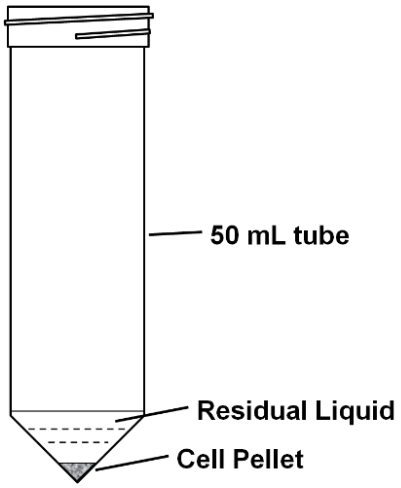
PBMC Collection

Reagent Preparation

1. Add 3 mL of Histopaque® 1077 (or other Ficoll solution) to a 15 mL conical centrifuge tube and bring the solution to **ROOM TEMPERATURE (RT)**.
2. Prepare PBMC Prep/PBS solution by dissolving 50X CellPrep (stock should be frozen at **-20°C**) in 1X PBS. Prepare 50 mL for each 5 mL blood sample.

PBMC Purification

1. Transfer 5 mL of blood to a 15 mL conical tube.
2. Carefully layer the blood sample onto the Histopaque® 1077 solution.
3. Centrifuge at **RT** in a horizontal rotor (swing-out head) for **20 MIN** at 800 RCF (with minimal acceleration/break).
4. Carefully remove the upper phase (plasma phase) with a pipette or aspiration device, leaving ~0.5 cm above the PBMC layer.
5. Use a 1 mL pipette to transfer the PBMC layer to the 50 mL polypropylene tube containing 40 mL PBMC Prep/PBS. Pipette up and down several times to minimize cell loss in the pipette tip.
6. Centrifuge at **RT** for **10 MIN** at 250 RCF (with maximum acceleration/break).
7. Aspirate supernatant without disturbing the cell pellet, leaving ~ 5 mL liquid.
8. Resuspend cell pellet with remaining liquid by pipetting up and down 10 times then transfer to a new 15 mL tube.
9. Wash the 50 mL tube with 5 mL PBMC Prep/PBS solution and transfer the solution to the 15 mL tube



containing resuspended cells to minimize cell loss.

10. Centrifuge at **RT** at 250 RCF for **10 MIN** (with maximum acceleration/break).
11. Aspirate supernatant leaving as little liquid as possible without touching the cell pellet.

Non-adherent Cell Collection

1. Harvest cells by centrifuging at **RT** at 250 RCF for **10 MIN** in a 50 mL polypropylene tube.
2. Aspirate supernatant without disturbing the cell pellet.
3. Wash with 40 mL 1X PBS by resuspending cells and centrifuging at RT at 250 RCF.
4. Aspirate supernatant, leaving as little liquid as possible without touching the cell pellet

Cell Fixation

1. Resuspend cells in 5 mL of 10% NBF. Gently pipette up and down 10 times to completely break apart the cell pellet.
2. Incubate the tube in a **37°C** water bath for **1 HR**.

Post-Fixation Wash and Storage

1. Centrifuge at 250 RCF for **10 MIN** to pellet the cells.
2. Remove supernatant without disturbing the pellet.
3. Resuspend cells in 10 mL PBMC-Wash, and centrifuge at 250 RCF for **10 MIN**.
4. Resuspend cells in 10 mL 70% EtOH. Pipette up and down 10 times to completely break apart the cell pellet.
5. Incubate at **RT** for **10 MIN** and transfer to **4°C**.

NOTE: The cells can be stored in 70% EtOH at **4°C** for up to **7 days**.

Slide Preparation

1. Adjust the cell density with 70% EtOH to1X10^6 cells/mL.
2. Mix well by pipetting. Transfer 1 mL cell suspension to each pre-assembled cyto-centrifuge cartridge.

NOTE: Cell density and volume described here is based on the Hettich cyto-centrifuge with an 8 mL funnel chamber. If other cyto-centrifuge systems are used, adjust the cell density and volume to achieve a single cell layer after cyto-centrifuge.

3. Cyto-centrifuge at 800 RCF for **20 MIN**.
4. Carefully remove supernatant completely with pipette, disassemble cyto-centrifuge cartridge.
5. Air dry slides for **20 MIN**.
6. Immerse slides in 50% EtOH. Incubate at **RT** for **5 MIN**.
7. Remove 50% EtOH and replace with 70% EtOH. Incubate at **RT** for **5 MIN**.
8. Remove 70% EtOH and replace with 100% EtOH. Incubate at **RT** for **5 MIN**.
9. Remove 100% EtOH and replace with fresh 100% EtOH. Incubate at **RT** for **5 MIN**.

NOTE: The slides can be stored in 100% EtOH at **-20°C** for up to **1 MONTH**.

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. Re-insert the covered tray into the oven and close the oven door. The tray should be pre-warmed for at least **20 MIN** before use.

Create a Hydrophobic Barrier

1. Remove slides from 100% EtOH and dry at **37°C** for **30 MIN** on a slide warmer.
2. Draw 2–4 times around the cell spot using the Immedge™ hydrophobic barrier pen. Let the barrier dry completely **~1 MIN**.

Add Protease III

1. Place the slides on the HybEZ™ Slide Rack.
2. Add 2–4 drops Protease III. Use enough solution to completely cover the cell spot.
3. Place the slide rack in the pre-warmed Humidity Control Tray, close lid, and incubate the tray in the HybEZ™ Oven for **30 MIN** at **40°C**

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4. Take slides out of the oven and one at a time tap/flick to remove excess Protease III. Do not let sample dry out.
5. Submerge the slides in a Coplin jar containing 1X PBS.
6. Remove 1X PBS, replace with fresh 1X PBS, and incubate at **RT** for **1 MIN**.

IMPORTANT! Proceed to the RNAscope® protocol using the *RNAscope® Fluorescent Multiplex Kit User Manual Part 2* (Cat. No. 320293) available at **http://www.acdbio.com/technical-support/user-manuals.**

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.

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