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TECHNICAL NOTE

RNAscope® Multiplex Fluorescent v2 Assay for Cultured Adherent Cells in 96-well Plate Format

Introduction

This Technical Note provides guidelines for sample collection and pretreatment of cultured adherent cells that can be assayed using the RNAscope® Multiplex Fluorescent Kit v2 (Cat. No. 323100). To pretreat the cells, use RNAscope® Protease III (available in the RNAscope® Protease III and Protease IV Reagents, Cat. No. 322340 or RNAscope® Universal Pretreatment Kit Cat No 322380). 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: Cell Preparation

Cell Culture

1. One day before fixation, seed cells in growth medium on a 96-well plate at a density that will allow cells to be 80–90% confluent at the time of fixation.

NOTE: Cells must be firmly adhered to the plate before starting the assay.

Cell Fixation

1. Remove growth medium, and wash with 200 µL/well of 1X PBS. Repeat wash step once.

IMPORTANT! Do not let cells dry out at any time during the assay procedure.

2. Remove 1X PBS, and add 200 µL/well of 10% Neutral Buffered Formalin (NBF). Incubate at **ROOM TEMPERATURE (RT)** for **30 MIN**.
3. Decant NBF into a waste container, and gently rinse plate with 200 µL/well of 1X PBS. Repeat wash step twice.

Dehydrate and Store Cells

1. Remove 1X PBS wash, and add 200 µL/well of 50% EtOH. Incubate at **RT** for **1 MIN**.
2. Remove 50% EtOH, and add 200 uL/well of 70% EtOH. Incubate at **RT** for **1 MIN**.
3. Remove 70% EtOH, and add 200 µL/well of 100% EtOH. Incubate at **RT** for **1 MIN**.
4. Remove 100% EtOH, and replace with 200 µL/well fresh 100% EtOH.

NOTE: Cover 96-well plate with lid and seal with parafilm. Polystyrene bottomed plates can be stored at **–20°C** for up to **3 MONTHS**. Glass bottomed plates can be stored at **4°C** for up to **48 HRS**.

Rehydrate Cells

1. Remove 100% EtOH, and add 200 µL/well of 70% EtOH. Incubate at **RT** for **1 MIN**.
2. Remove 70% EtOH, and add 200 µL/well of 50% EtOH. Incubate at **RT** for **1 MIN**.
3. Remove 50% EtOH, and add 200 µL/well of 1X PBS. Incubate at **RT** for **1 MIN**.
4. Remove 1X PBS, and add 200 µL/well of fresh 1X PBS. Incubate at **RT** for **10 MIN**.

NOTE: During the last incubation, prepare materials for Part 2 and pre-warm **Target Probes** to **40°C**.

Part 2: RNAscope® Pretreatment

Prepare Materials

1. Make 3L 1X Wash Buffer by adding one bottle of 10X Wash Buffer (300 mL) and 2.7 L distilled water into a bottle or beaker.
2. Bring Hybridization Oven to **40°C**.

NOTE: Plate should be inserted inside the HybEZ™ Oven without a Humidity Control Tray.

3. Bring RNAscope® Multiplex Fluorescent Reagent Kit v2 to **RT**.
4. Prepare Mixed Target Probes by adding C1, C2, and C3 Probes in a tube at 50:1:1 volume ratio.

Apply RNAscope® Hydrogen Peroxide

1. Keep the 96-well plate on the bench and add ~3 drops of RNAscope® Hydrogen Peroxide to each well. Make sure to completely cover the cells.
2. Incubate the 96-well plate at **RT** for **10 MIN**.
3. Remove the RNAscope® Hydrogen Peroxide solution from the plate by inverting and gently tapping the plate on absorbent paper.
4. Wash each well with the fresh distilled water. Make sure each well contains liquid.
5. Repeat the wash step with fresh distilled water.

Protease Digestion

1. Remove excess liquid. Place the plate in the HybEZ™ Humidity Control Tray.
2. Add 3 drops of a 1:15 dilution of Protease III to each well. Incubate at **RT** for **10 MIN**.

NOTE: For most cells lines, dilute Protease III to a 1:15 ratio with 1X PBS (for example, 20 µL Protease III with 280 µL 1X PBS). The exact protease dilution factor must be empirically determined for each new cell type.

3. Remove diluted Protease III solution, and add approximately 200 µL/well of 1X PBS. Incubate at **RT** for **2 MIN**.
4. Repeat the wash step twice.

Hybridize the Target Probe

1. Remove 1X PBS, and add 3 drops of pre-mixed Target Probe or Control Probe to each well.
5. Cover the 96-well plate with lid and incubate plate in the HybEZ™ Oven at **40°C** for **2 HRS**.
6. Remove 96-well plate from the HybEZ™ Oven. Decant liquid.
7. Wash cells with 200 µL of 1X Wash Buffer 200 µL per well. Incubate at **RT** for **2 MIN**.
8. Repeat step 5 twice using fresh 1X Wash Buffer each time.

Hybridize Amp 1

1. Remove excess liquid from the plate, and add ~3 drops of RNAscope® Multiplex FL v2 Amp 1 to each well. Make sure to completely cover the cells.
2. Insert the plate into the HybEZ™ Oven for **30 MIN** at **40°C**.
3. Wash the plate with 1X Wash Buffer for **2 MIN** at **RT**. Make sure each well contains liquid.
4. Repeat with fresh 1X Wash Buffer.

Hybridize Amp 2

1. Remove excess liquid from the plate, and add ~3 drops of RNAscope® Multiplex FL v2 Amp 2 to each well. Make sure to completely cover the cells.
2. Insert the plate into the HybEZ™ Oven for **30 MIN** at **40°C**.
3. Wash the plate with 1X Wash Buffer for **2 MIN** at **RT**. Make sure each well contains liquid.
4. Repeat with fresh buffer.

Hybridize Amp 3

1. Remove excess liquid from the plate, and add ~3 drops of RNAscope® Multiplex FL v2 Amp 3 to each well. Make sure to completely cover the cells.
2. Insert the plate into the HybEZ™ Oven for **15 MIN** at **40°C**.

NOTE: Prepare TSA Plus Fluorophores during this step. See the following section.

5. Wash the plate with 1X Wash Buffer for **2 MIN** at **RT**. Make sure each well contains liquid.
6. Repeat with fresh buffer.

Prepare TSA™ Plus Fluorophores

1. Determine the volume of TSA® Plus fluorophore needed (100-120uL uL for each well).
2. Dilute the TSA® Plus fluorophore stocks using the RNAscope® Multiplex TSA buffer provided in the RNAscope® Multiplex Fluorescent Kit v2. Follow these recommendations:

TSA® Plus fluorophore	PerkinElmer Part No.	Recommended dilution range*
TSA® Plus fluorescein	NEL741E001KT or NEL741001KT	1:750–1:3000

TSA® Plus fluorophore	PerkinElmer Part No.	Recommended dilution range*
TSA® Plus Cyanine 3	NEL744E001KT or NEL744001KT	1:750–1:3000
TSA® Plus Cyanine 5	NEL745E001KT or NEL745001KT	1:750–1:3000

*Start with a dilution of 1:1500 and adjust based on signal intensity.

NOTE: Store diluted TSA® Plus fluorophores up to one month at **2–8°C** in the dark.

NOTE: You may replace TSA® Plus fluorophores with certain Opal™ dyes from Perkin Elmer.

TSA® Plus fluorophore	Opal™ Dye	PerkinElmer Reagent Kit
TSA® Plus fluorescein	Opal 520	FP1487001KT: Opal 520 Reagent Pack
TSA® Plus Cyanine 3	Opal 570	FP1488001KT: Opal 570 Reagent Pack
TSA® Plus Cyanine 5	Opal 650 or Opal 690	• FP1496001KT: Opal 650 Reagent Pack • FP1497001KT: Opal 690 Reagent Pack

Develop HRP-C1 signal

1. Remove excess liquid from the plate, and add ~3 drops RNAscope® Multiplex FL v2 HRP-C1 to each well. Make sure to completely cover the cells.
2. Insert the plate into the HybEZ™ Oven for **15 MIN** at **40°C**.
3. Wash the plate with 1X Wash Buffer for **2 MIN** at **RT**. Make sure each well contains liquid.
4. Repeat with fresh 1X Wash Buffer.
5. Remove excess liquid from the plate, and add 100–120 µL diluted TSA® Plus fluorescein to each well.
6. Incubate for **30 MIN** at **40°C**.

NOTE: You can mix and match channels and fluorophores. Do not assign the same fluorophore to more than one channel.

7. Wash the plate in 1X Wash Buffer for **2 MIN** at **RT**. Repeat with fresh buffer.
8. Remove excess liquid from the plate, and add ~3 drops RNAscope® Multiplex FL v2 HRP blocker to each well.
9. Insert the plate into the HybEZ™ Oven for **15 MIN** at **40°C**.
10. Wash the plate with 1X Wash Buffer for **2 MIN** at **RT**. Repeat with fresh buffer.

Develop HRP- C2 signal

1. Remove excess liquid from the plate, and add ~3 drops RNAscope® Multiplex FL v2 HRP-C2 to each well. Make sure to completely cover the cells.
2. Insert the plate into the HybEZ™ Oven for **15 MIN** at **40°C**.
3. Wash the plate with 1X Wash Buffer for **2 MIN** at **RT**. Make sure each well contains liquid.
4. Repeat with fresh 1X Wash Buffer.
5. Remove excess liquid from the plate, and add 100-120µL diluted TSA® Plus Cyanine 3 to each well,
6. Incubate for **30 MIN** at **40°C**.

NOTE: You can mix and match channels and fluorophores. Do not assign the same fluorophore to more than one channel.

7. Wash the plate in 1X Wash Buffer for **2 MIN** at **RT**. Repeat with fresh buffer.
8. Remove excess liquid from the plate, and add ~3 drops RNAscope® Multiplex FL v2 HRP blocker to each well.
9. Insert the plate into the HybEZ™ Oven for **15 MIN** at **40°C**.
10. Wash the plate with 1X Wash Buffer for **2 MIN** at **RT**. Repeat with fresh buffer.

Develop HRP-C3 signal

1. Remove excess liquid from the plate, and add ~3 drops RNAscope® Multiplex FL v2 HRP-C3 to each well. Make sure to completely cover the cells.
2. Insert the plate into the HybEZ™ Oven for **15 MIN** at **40°C**.
3. Wash the plate with 1X Wash Buffer for **2 MIN** at **RT**. Make sure each well contains liquid.
4. Repeat with fresh 1X Wash Buffer.
5. Remove excess liquid from the plate, and add 100–120 µL diluted TSA® Plus Cyanine 5 to each well.
6. Incubate for **30 MIN** at **40°C**

NOTE: You can mix and match channels and fluorophores. Do not assign the same fluorophore to more than one channel.

7. Wash the plate in 1X Wash Buffer for **2 MIN** at **RT**. Repeat with fresh buffer.

8. Remove excess liquid from the plate, and add ~3 drops RNAscope® Multiplex FL v2 HRP blocker to each well.
9. Insert the plate into the HybEZ™ Oven for **15 MIN** at **40°C**.
10. Wash the plate with 1X Wash Buffer for **2 MIN** at **RT**. Repeat with fresh buffer.

Counterstain with DAPI

1. Remove excess liquid from the plate, and add ~2 drops of DAPI to each well.
2. Incubate for **30 SEC** at **RT**.
3. Remove DAPI, and wash with 200 µl/well of 1X PBS.
4. Remove 1X PBS, and replace with fresh 1X PBS.

NOTE: You may store the plate at **4°C** for up to **48 HRS** with an adhesive seal before imaging.

Evaluate the Results

Examine the plate under a fluorescent microscope at 20–40X magnification using appropriate filter sets.

TSA® Plus fluorophore	Filter setting
TSA® Plus fluorescein	FITC
TSA® Plus Cyanine 3	Cy3
TSA® Plus Cyanine 5	Cy5

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.

IMPORTANT! This protocol has not been through ACD's complete development and verification process, and is for limited distribution only.

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