RNAscope® Assay Protocol for Adherent Cells
Applicable to Coverslips, 96-Well Plates, and Chamber Slides

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1. Cell Culture and Seeding

Format-Specific Instructions

- **Coverslips**:
  - Seed cells at **1×10^5 cells/mL** (50–75% confluency next day) onto **poly-L-lysine-coated coverslips**.
- **96-Well Plates**:
  - Seed cells to achieve **80–90% confluency** at fixation.
- **Chamber Slides**:
  - Seed cells in **4-well chamber slides** (Fisher #08-774-25) for **80–90% confluency**.

General Notes

- Use sterile techniques.
- Allow cells to adhere:
  - **Coverslips**: ≥10 minutes.
  - **All formats**: Incubate overnight.

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2. Fixation

1. **Remove growth medium** and rinse with **1X PBS**:
   - **Coverslips**: Submerge in PBS.
   - **96-Well Plates/Chamber Slides**: Use **100–200 µL/well**.
2. **Fix** with **10% NBF** or **4% PFA** for **30 (if protease) -60 min (if PretreatPro) at RT**.
3. Rinse **3× with 1X PBS**.

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3. Dehydration and Storage

1. **Dehydrate** in ethanol series (1–5 min per step):
    - **50% EtOH → 70% EtOH → 100% EtOH**.
2. **Storage Conditions**:

Format	Storage Solution	Temperature	Duration
Coverslips/Chamber Slides	100% EtOH	-20°C	≤6 months
96-Well (Polystyrene)	100% EtOH	-20°C	≤3 months
96-Well (Glass)	100% EtOH	4°C	≤48 hours

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4. Rehydration

1. Reverse ethanol series:
    - **100% EtOH → 70% EtOH → 50% EtOH → 1X PBS** (1–10 min per step).
2. Rinse **2× with 1X PBS**.

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5. Pretreatment

Hydrogen Peroxide Treatment

1. Apply **RNAscope® Hydrogen Peroxide** (3–8 drops/slide or well) for **10 min at RT**.
2. Wash **2× with PBS**.

Hydrophobic Barrier (Coverslips/Chamber Slides Only)

1. **Coverslips**: Mount on slides using nail polish. Draw barriers around the coverslips with **ImmEdge™ pen**.
2. **Chamber Slides**: Draw barriers along chamber divide lines with **ImmEdge™ pen**.

Protease or PretreatPro Digestion

- **Option 1: Protease III**

1. Dilute **RNAscope Protease III 1:15 in 1X PBS** (*optimize empirically for cell type*).
2. Apply **2–4 drops** to samples; incubate **10 min at RT**.
3. Wash **3× with 1X PBS**.

- **Option 2: PretreatPro**
  1. Heat target retrieval buffer to 100^0C, and incubate the samples for **5 min at 100^0C**
     - **For coverslips, transfer them to a glass staining jar and place a in steamer. After Target Retrieval step, they can be attached to glass slide (using glue or nail polish) for remainder of assay**
  2. Apply **2–4 drops of RTU RNAscope Manual PretreatPro** to samples; incubate **30 min at 40°C**.
  3. Wash **3× with 1X PBS**.

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6. Probe Hybridization and Signal Amplification

Proceed to **Chapter 6 of Multiplex Fluorescent Kit v2 User Manual**.

Critical Notes

- **Do not let cells dry out** during the procedure.
- **Protease/PretreatPro**: Optimize conditions for new cell types.
- **Safety**: Wear PPE; refer to SDS for hazardous reagents.

Support Resources

- **User manual**: RNAscope® Multiplex Fluorescent Kit v2, UM 323100
- **Contact Technical Support**: https://www.bio-techne.com/support/technical-support.