RNASCOPÉ® TROUBLESHOOTING TIPS

Presented by:
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Senior Scientist

Advanced Cell Diagnostics
TOPICS

• RNAscope® Recommended Workflow
• Tips for RNAscope® Manual and Automation Assays
• Troubleshooting Staining Patterns
• Q&A
RNASCOPE® WORKFLOW

A BREAKTHROUGH PLATFORM

PERMEABILIZE cells or tissue → HYBRIDIZE to target RNA → AMPLIFY signal → VISUALIZE with morphology → QUANTIFY single-cell expression

UNIQUE Probe Design

SIGNAL Amplification + Background Suppression

SINGLE Molecule Detection in Single Cells

ANY Genome, Gene or Tissue
<table>
<thead>
<tr>
<th>Assay Type</th>
<th>Dye Used</th>
<th>Channel (Manual)</th>
<th>Probes Channel (Manual)</th>
<th>Probes Channel (Automation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromogenic</td>
<td>Diaminobenzene (DAB)-HRP</td>
<td>Channel 1</td>
<td>C1 Probes</td>
<td>VS/LS Probes</td>
</tr>
<tr>
<td>Chromogenic</td>
<td>Fast Red -ALP</td>
<td>Channel 1</td>
<td>C1 Probes</td>
<td>VS/LS Probes</td>
</tr>
<tr>
<td>Chromogenic</td>
<td>HRP-Green, Fast Red -ALP</td>
<td>Channel 1, 2</td>
<td>C1, C2 Probes</td>
<td>N/A</td>
</tr>
<tr>
<td>Fluorescent</td>
<td>FITC, Cy3, Cy5,</td>
<td>Channel 1, 2, 3</td>
<td>C1, C2, C3 Probes</td>
<td>N/A</td>
</tr>
</tbody>
</table>
**TIP:** Do not interchange reagents within Brown/Red assays or across similar 2.0 HD Assays

By default 2.0 HD assays require C1 probes that are ready to use, no further dilution is required.
**TIP:** By default C1 probes are 1X concentration while C2 probes are 50X
To make 2-plex probe mixture at 1X concentration, mix C2 probes 1:50 with C1 probes
To view C2 probes only, use the “blank-probe-C1”, as a diluent and mix at a 1:50 dilution
**TIP:** By default C1 probes are 1X concentration while C2 and C3 probes are 50X

To make 3-plex probe mixture at 1X concentration, mix C2 and C3 probes 1:50 with C1 probe

If C2 and C3 are all at 50X concentration, use the “blank-probe-C1” as a diluent and mix at a 1:50 dilution.
**RNAscope® WORKFLOW: CHROMOGENIC ASSAY**

<table>
<thead>
<tr>
<th>Steps</th>
<th>Description</th>
<th>Time to Completion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretreat</td>
<td>Deparaffinization</td>
<td>~1.5 hours</td>
</tr>
<tr>
<td></td>
<td>H₂O₂ block (Pretreat 1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Epitope retrieval (Pretreat 2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Protease (Pretreat 3 or 4)</td>
<td></td>
</tr>
<tr>
<td>Hybridize</td>
<td>Target probe hybridization</td>
<td>~2.5 hours</td>
</tr>
<tr>
<td></td>
<td>Wash</td>
<td></td>
</tr>
<tr>
<td>Amplify</td>
<td>Pre-amplifier hybridization</td>
<td>~1.5 hours</td>
</tr>
<tr>
<td></td>
<td>Wash</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Post-amplifier hybridization</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Wash</td>
<td></td>
</tr>
<tr>
<td>Stain and detect</td>
<td>Amplifier hybridization</td>
<td>~1.5 hours</td>
</tr>
<tr>
<td></td>
<td>Wash</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Label probe hybridization</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Wash</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Detection</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hematoxylin stain</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Image detection under standard light microscope / scanner</td>
<td>~1.5 hours</td>
</tr>
</tbody>
</table>

**TIP:** Detection protocols will vary based on the chromogenic assay used

[Download manuals: http://www.acdbio.com/technical-support/downloads]
**RNAscope® WORKFLOW: FLUORESCENT ASSAY**

<table>
<thead>
<tr>
<th>Steps</th>
<th>Description</th>
<th>Time to Completion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretreat</td>
<td>Fixation</td>
<td>~1.5 hours</td>
</tr>
<tr>
<td></td>
<td>Dehydration</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Protease digestion (Pretreat 3/4)</td>
<td></td>
</tr>
<tr>
<td>Hybridize</td>
<td>A. Target probe hybridization</td>
<td>~3.5 hours</td>
</tr>
<tr>
<td></td>
<td>Wash</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B. Pre-amplifier hybridization</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Wash</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C. Amplifier hybridization</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Wash</td>
<td></td>
</tr>
<tr>
<td>Amplify</td>
<td>D. Label probe hybridization</td>
<td>~0.5 hours</td>
</tr>
<tr>
<td></td>
<td>Wash</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DAPI Counterstaining</td>
<td></td>
</tr>
<tr>
<td>Stain and</td>
<td>Image detection under fluorescent microscope</td>
<td></td>
</tr>
<tr>
<td>detect</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

_TIP: Pretreatment conditions will vary based on sample type_

Download manuals: http://www.acdbio.com/technical-support/downloads
ONE DAY OR TWO DAY ASSAY?

ONE DAY ASSAY

Sample preparation

Sample pretreatment

RNAscope assay

TWO DAY ASSAY

Sample preparation

Sample pretreatment

DAY 1

DAY 2

RNAscope assay

TIP: Review the User Manuals PART 1 and PART 2 for optional stopping points. Refer to the User Manuals for Automation assay workflow.
TIPS FOR RNASCOPE® MANUAL ASSAYS
TIPS FOR MANUAL ASSAYS

Follow protocols exactly as described in the user manuals

Review sample pretreatment recommendations

Always use control probes and slides

Review that you are using all required materials

PROTOCOLS  SAMPLE PRETREATMENT  USE CONTROLS  THE CHECKLIST

**REVIEW THE CHECKLIST:**

| ✔️ | Immedge hydrophobic barrier pen |
| ✔️ | Positive and Negative control probes |
| ✔️ | Hot–Plate for pretreatment/ target retrieval step |
| ✔️ | Superfrost plus slides |
| ✔️ | HybEZ Hybridization system |
| ✔️ | Run RNAscope® control slides |
| ✔️ | Ecomount for 2.0 HD Red & 2-plex chromogenic assay |
| ✔️ | Fresh reagents (ethanol, xylene, 10% NBF) |

**TIP**: Visit www.acdbio.com/go for more information on getting started. Checklist is available on the website and in the manual.
HOT PLATE

Hotplate for retrieval/boiling

TIP: When using a hot plate for pre-treatment step – pay close attention to the TIME and boiling TEMPERATURE.
Contents of the reagent kit

1. Pretreatment reagents
2. RNAscope detection kit
3. Wash buffer

TIP: Warm probes at 40 °C for 10 minutes before use
TIP: Warm 50x wash buffer at 40 °C for 20 minutes if you notice a precipitation
HYBEZ HYBRIDIZATION OVEN

TIP: HybEZ oven is required as it provides both temperature and humidity control, necessary to obtain optimal RNAscope results.
ACCESSORIES FOR WASHING STEPS

- Tissue Tek washing tray
- EZ Batch for slide processing

TIP: ACD EZ Batch slide processing tray is easy and convenient for loading multiple slides for hybridization and washing steps.
### FOLLOW WORKFLOW GUIDELINES (MANUAL)

<table>
<thead>
<tr>
<th>Task</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apply all amplification steps in the right order</td>
<td></td>
</tr>
<tr>
<td>Use “flicking or tapping” technique to remove residual reagent</td>
<td></td>
</tr>
<tr>
<td>Do not let slides dry out</td>
<td></td>
</tr>
<tr>
<td>Make sure the hydrophobic barrier remains intact</td>
<td></td>
</tr>
<tr>
<td>Do not alter the protocol in any way</td>
<td></td>
</tr>
<tr>
<td>Warm probes and wash buffer at 40°C due to precipitation</td>
<td></td>
</tr>
<tr>
<td>Maintain adequate humidity in the Humidity Control Chamber</td>
<td></td>
</tr>
<tr>
<td>Fresh reagents (ethanol, xylene, 10% NBF)</td>
<td></td>
</tr>
</tbody>
</table>

TIP:
• Use Ecomount or PERTEX as the mounting medium
• Do not dehydrate sample with alcohol, to avoid a diffused signal
• Samples should be dried in a 60 degree oven for 15 minutes before mounting
TIP: By default C1 probes are 1X concentration while C2 probes are 50X
To make 2-plex probe mixture at 1X concentration, mix C2 probes 1:50 with C1 probes
To view C2 probes only, use the “blank-probe-C1”, as a diluent and mix at a 1:50 dilution
Use Ecomount or PERTEX as the mounting medium
• Do not dehydrate sample with alcohol, to avoid a diffused signal
• Samples should be dried in a 60 degree oven for 15 minutes before mounting
**COLOR MODULE OPTIONS**

<table>
<thead>
<tr>
<th></th>
<th>Channel 1 (C1)</th>
<th>Channel 2 (C2)</th>
<th>Channel 3 (C3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP 4 Alt A</td>
<td><strong>GREEN-Alexa 488</strong></td>
<td><strong>ORANGE-Atto 550</strong></td>
<td><strong>FAR RED-Atto 647</strong></td>
</tr>
<tr>
<td>Amp 4 Alt B</td>
<td><strong>ORANGE-Atto 550</strong></td>
<td><strong>GREEN-Alexa 488</strong></td>
<td><strong>FAR RED-Atto 647</strong></td>
</tr>
<tr>
<td>Amp 4 Alt C</td>
<td><strong>ORANGE-Atto 550</strong></td>
<td><strong>FAR RED-Atto 647</strong></td>
<td><strong>GREEN-Alexa 488</strong></td>
</tr>
</tbody>
</table>

**TIP:** By default C1 probes are 1X concentration while C2 and C3 probes are 50X
To make 3-plex probe mixture at 1X concentration, mix C2 and C3 probes 1:50 with C1 probe
If C2 and C3 are all at 50X concentration, use the “blank-probe-C1” as a diluent and mix at a 1:50 dilution.
TIPS FOR RNASCOPE® AUTOMATED ASSAYS
TIPS FOR AUTOMATION ASSAYS (VENTANA® SYSTEMS)

Check instrument maintenance

Optimize software settings

Troubleshoot Reagents

Review sample pretreatment recommendations

INSTRUMENT MAINTENANCE
SOFTWARE SETTINGS
REAGENTS
SAMPLE PRETREATMENT

### Check Instrument Maintenance:

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>![Check]</td>
<td>Perform instrument maintenance</td>
</tr>
<tr>
<td>![Check]</td>
<td>Perform decontamination protocol every three months (prevents microbial growth)</td>
</tr>
<tr>
<td>![Check]</td>
<td>Use appropriate buffers for RNAscope assay, remove or purge before a run</td>
</tr>
</tbody>
</table>

### Optimize Software Settings:

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>![Check]</td>
<td>*Uncheck the Slide Cleaning option (ULTRA only)</td>
</tr>
<tr>
<td>![Check]</td>
<td>Use appropriate hybridization temperature (different for XT versus ULTRA)</td>
</tr>
</tbody>
</table>

**TIP:** *This is a cleaning step in Ventana Equipment may cause the slides to dry out. Refer to User Manual for details*
Do not shake the contents in the containers as this will form bubbles.

LS Amp 1, LS Amp 3, 10X LS Wash Buffer, and all target probes require warming up at 40°C for 30 mins.

LS Brown and LS Red assays utilize Leica Biosystems’ Bond Polymer Refine Detection and Bond Polymer Refine Red Detection kits, respectively.

Do not alter the staining protocol in any way.

QUALIFY YOUR SAMPLES USING CONTROLS
# IMAGE ANALYSIS

## RNASCOPE® SCORING GUIDELINE

<table>
<thead>
<tr>
<th>Score</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No staining or &lt;1 dot/ 10 cells*</td>
</tr>
<tr>
<td>1</td>
<td>1-3 dots/cell</td>
</tr>
<tr>
<td>2</td>
<td>4-9 dots/cell. None or very few dot clusters</td>
</tr>
<tr>
<td>3</td>
<td>10-15 dots/cell and &lt;10% dots are in clusters</td>
</tr>
<tr>
<td>4</td>
<td>&gt;15 dots/cell and &gt;10% dots are in clusters</td>
</tr>
</tbody>
</table>

* *No staining* refers to the absence of any visible signal in the tissue section.

4-10 dots/cell

Score = 3

Score = 3
QUALIFY YOUR SAMPLES USING CONTROLS

Control slides e.g. Hela

Negative control probe, e.g. DapB

QC check
PPIB > 2
DapB < 1

Sample

Negative control probe, e.g. DapB

QC check
PPIB > 2
DapB < 1

PASS

Run your target probes

FAIL

• Verify technique
• Check RNA quality with new samples
• Perform Assay optimization

Technique check

Sample/RNA quality check

TIP : Always start with standard conditions
OPTIMIZE YOUR ASSAY

Technique check

Sample/RNA quality check

TIP: Refer to the Troubleshooting Guide
OPTIMIZE YOUR ASSAY
WHY OPTIMIZE YOUR RNASCOPE® ASSAYS?

Under fixed when using the following conditions:
- 4% PFA/24 hours/4°C
- 10% NBF/24 hours /4°C

Over fixed when using the following conditions:
- 10% NBF > 48 hours /RT
- 10% NBF > 48 hours /4°C

Special sample types:
- Xenograft
- Cultured cells
- Cell pellet

Special Tissues:
- Liver
- Muscle
- Retina
- Lymphoid tissues (e.g. spleen, tonsil, lymph node)
## FACTORS AFFECTING RNASCOPE® ASSAY PERFORMANCE

<table>
<thead>
<tr>
<th>Issue</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixation conditions are not optimal</td>
<td>Fix samples as recommended. E.g., for FFPE use 10% NBF RT, 16-32 hrs</td>
</tr>
<tr>
<td>RNA is degraded</td>
<td>Acquire new samples and assess RNA quality</td>
</tr>
<tr>
<td>Hybridization conditions not optimal</td>
<td>Use the HybEZ hybridization oven only</td>
</tr>
<tr>
<td>Samples drying during assay</td>
<td>Use Immedge pen and add adequate reagents to avoid drying</td>
</tr>
<tr>
<td>Special tissues sensitive to pretreatment</td>
<td>Start with standard pretreatment, then optimize conditions accordingly</td>
</tr>
</tbody>
</table>

NBF: Neutral Buffered Formalin
**OPTIMIZE YOUR SAMPLE IN 3 EASY STEPS (MANUAL ASSAY)**

**STEP 1**
START WITH STANDARD CONDITIONS

**STEP 2**
ADJUST PRETREATMENT 2, BOILING TIME

**STEP 3**
ADJUST PRETREATMENT 3/4, PROTEASE TIME*

**Observe Staining Pattern** -
High background, over-digested? = **under fixed**
No signal/weak signal, under-digested? = **over fixed**

---

**TIP**: For cultured cells, protease is diluted 1:15 in 1X PBS

* For fresh frozen samples, only protease pretreatment is required and is performed at room temperature and **NOT** at 40°C
OPTIMIZE YOUR SAMPLE WITH THESE STEPS
(AUTOMATED ASSAYS)

LEICA BOND RX

OVER FIXED/ UNDER DIGESTED
Increase ER2 time in increments of 5 mins and protease in increments of 10 mins

UNDER FIXED/ OVER DIGESTED
Reduce temp to 88°C, this improves morphology and reduces background

VENTANA XT/ULTRA

Increase Pretreat 2/3 and/or CC time

Decrease Pretreat 2/3 and/or CC time

TIP: Refer to the User Manuals for automation assay workflow and pretreatment optimization guideline
EXAMPLE OF SUCCESSFUL RNASCOPE® RESULTS

Negative control, DapB
Positive control, HsPPIB
Target probe

RNAzcope 2.0 HD Red Assay
Human breast cancer tissue

EXAMPLE OF SUCCESSFUL RNASCOPE® RESULTS

Hs POLR2A/Alexa 488  Hs PPIB/Atto 550  Hs UBC/Atto 647  Merged

RNAscope Multiplex Fluorescent Assay
Amp 4 ALT A*

TIP: Use different AMP4 ALT reagents (A, B, C) for alternative color combinations
TROUBLESHOOTING STAINING PATTERNS (CHROMOGENIC MANUAL ASSAYS)
# Troubleshooting: No Staining Observed

<table>
<thead>
<tr>
<th>Probable Cause</th>
<th>Suggested Action</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Suboptimal fixation</strong></td>
<td>Prepare (fix) samples according to ACD recommendation</td>
</tr>
<tr>
<td>• Over fixation</td>
<td>Optimize pretreatment conditions</td>
</tr>
<tr>
<td>• Under fixation</td>
<td></td>
</tr>
<tr>
<td><strong>Hybridization temperature not optimal</strong></td>
<td>Use HybEZ when performing RNAscope</td>
</tr>
<tr>
<td></td>
<td>HybEZ temperature should be at 40°C</td>
</tr>
<tr>
<td><strong>Reagents used in the wrong sequence</strong></td>
<td>Apply reagents in the correct order</td>
</tr>
<tr>
<td><strong>Gene of interest not expressed</strong></td>
<td>Check positive control for technical accuracy of the assay</td>
</tr>
</tbody>
</table>
TROUBLESHOOTING: SAMPLE DIGESTION

OPTIMAL DIGESTION

*8 min Pretreat 2, 30 min Pretreat 3

DapB

Hs-PPIB

XENOGRAFT TISSUE

*Conditions used for manual assays
TROUBLESHOOTING: SAMPLE DIGESTION

UNDER DIGESTION

*8 min Pretreat 2, 15 min Pretreat 3

DapB

XENOGRAFT TISSUE

Hs-PPIB

Assay: RNAscope 2.0 HD RED
Issue: Strong hematoxylin, under pretreatment, weak PPIB
Solution: Increase pretreatment

*Conditions used for manual assays
TROUBLESHOOTING: SAMPLE DIGESTION

OVER DIGESTION

Assay: RNAscope 2.0 HD RED
Issue: Nuclear background, over pretreatment
Solution: Decrease pretreatment

*Conditions used for manual assays
TROUBLESHOOTING: BACKGROUND STAINING

HIGH BACKGROUND

Assay: RNAscope 2.0 HD BROWN
Issue: High background, over pretreatment
Optimization: Decrease pretreatment 2 (boiling) conditions
Result: Clean background

*Conditions used for manual assays
TROUBLESHOOTING: BACKGROUND STAINING

**NUCLEAR HAZY BACKGROUND**

**HUMAN TONSIL FFPE TISSUE**

*15 min ER2, 30 min Protease

*20 min ER2, 30 min Protease

---

**Assay:** RNAsecope LS BROWN (LEICA BOND RX)

**Issue:** Nuclear hazy background, under pretreatment

**Optimization:** Increase ER2 time in increments of 5 mins and protease in increments of 10 mins

**Result:** Clean background

---

*Conditions LEICA BOND RX automated assays*
## TROUBLESHOOTING: ASSAY WORKFLOW

### HIGH BACKGROUND/DRYING

<table>
<thead>
<tr>
<th>DapB</th>
<th>15 min Pretreat 2, 30 min Pretreat 3</th>
</tr>
</thead>
</table>

### FFPE HEla PELLET

### BACKGROUND TYPE | PROBABLE CAUSE | SUGGESTED ACTION |
|--------------------|---------------|------------------|
| Cytoplasmic and nuclear | • Samples drying between amplification steps | • Completely cover tissue when applying reagents  
• Process slides one at a time to prevent drying  
• Ensure HybEZ Oven is at the appropriate temperature  
• Use the Immedge® hydrophobic barrier pen |
| Extracellular | • Incomplete paraffin removal  
• Suboptimal tissue preparation | • Use fresh/unused EtOH and Xylene and agitate slides during incubation steps  
• Prepare tissue samples according to ACD recommended procedures |

*Conditions used for manual assays*
# Troubleshooting: Sample Preparation

## Sample Falling Off

<table>
<thead>
<tr>
<th>ISSUE</th>
<th>PROBABLE CAUSE</th>
<th>SUGGESTED ACTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue detaches from slides</td>
<td>• Wrong slides used</td>
<td>• Use only <a href="#">SuperFrost® Plus</a> slides</td>
</tr>
</tbody>
</table>
|                              | • Suboptimal tissue preparation               | • Prepare tissue samples according to ACD recommended procedures  
|                              |                                                | • Bake slides for a longer time (up to overnight)   
|                              |                                                | • Reduce boiling time                                |

*Conditions used for manual assays*
TROUBLESHOOTING SUB-OPTIMAL FIXATION CONDITIONS

24 hours fixation/Optimal

Sample: FFPE brain sample

Assay: RNAscope 2.0 HD Brown

3 weeks fixation/Over fixed

Synaptophysin

TIP: Sample fixation has a great effect on the success of your assay
Solution: Increase pretreatment for better target accessibility
## TROUBLESHOOTING: OTHER ISSUES

<table>
<thead>
<tr>
<th>ISSUE</th>
<th>PROBABLE CAUSE</th>
<th>SUGGESTED ACTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unknown tissue preparation method</td>
<td>• Sample provider/clinical site/vendor did not provide detailed instructions</td>
<td>• Follow the appropriate Tissue Specimen Preparation and Assay Optimization Guidelines/Technotes*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Start with standard conditions</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Optimize your assay</td>
</tr>
<tr>
<td>Diffused Signal (RED)</td>
<td>• Sample not completely dried</td>
<td>• Dry sample as recommended (prolonged drying i.e. overnight, may be required)</td>
</tr>
<tr>
<td></td>
<td>• Alcohol used to dehydrate sample</td>
<td>• Do not dehydrate samples, dry at 60°C, 15 min</td>
</tr>
<tr>
<td></td>
<td>• Too much Ecomount mounting medium used</td>
<td>• Use Ecomount sparingly and as recommended</td>
</tr>
</tbody>
</table>

*TIP: Applies to all samples used with RNAscope®*
Sample: Flash Frozen followed by FFPE sample preparation (fixation), Rat intestines

Assay: RNAscope 2.0 HD Brown

Issue: Weak staining, destroyed morphology, FFPE sample is under fixed

Optimization: Fixation according to recommended guidelines for FFPE samples

Result: Strong staining for positive control, PPIB, intact morphology

**TROUBLESHOOTING: GREEN SIGNAL FADING**

**Sample:** FFPE human tonsil sample

**Assay:** RNAscope 2-plex assay

**Issue:** Green signal faded

**Probable cause:**
- Hematoxylin or associated low pH
- Bluing with ammonia water

**Solution:**
- Use hematoxylin briefly as recommended (30 secs)
- Use tap water instead of ammonia water
TIP: Refer to the user manual for tissue specific pretreatment guidelines.
Tissue Pretreatment Guidelines

Follow the recommended pretreatment conditions based on your tissue type for:

- Any new or previously untested FFPE tissues
- Samples prepared suboptimally

Guidelines for Optimal Tissue Pretreatment

- Test representative samples with positive and negative control probes. [Controls should be: Positive = uniform signal; negative = blank].
- Fix sample in FRESH 10% NBF for 16-32 hours at ROOM TEMPERATURE. **NOTE:** Do not fix at 4°C. DO NOT fix for <16 hrs or >32 hrs. Refer to Table 1 for under/over-fixed tissue pretreatment guidelines.
- Vary PRETREATMENT 2A.3 and/or CELL CONDITION (Boiling time) TIME based on your tissue type (see Table 2).
- This tissue optimization guide is recommended for Ventana Discovery platform only.

Table 1. Tissue Pretreatment Guidelines

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Mild</th>
<th>Standard</th>
<th>Extended</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretreatment 2A.3</td>
<td>8-10 min</td>
<td>12/12 min</td>
<td>12/12 min</td>
</tr>
<tr>
<td>Boiling time (CC)</td>
<td>7 min</td>
<td>15 min</td>
<td>30 min</td>
</tr>
</tbody>
</table>

Table 2. Tissue Pretreatment Table

For information about species or tissue type not listed here, contact support at support@acdbio.com.

**TIP:** Refer to the user manual for tissue specific pretreatment guidelines.
TROUBLESHOOTING TIPS
MULTIPLEX FLUORESCENT ASSAY
TIP: Pretreatment temperature has a great effect on the success of your assay
Solution: Perform pretreatment at RT to avoid over digestion of your sample
TIP: Sample thickness can affect signal in your samples
Solution: Use recommended sample thickness, 10-20um

TROUBLESHOOTING: SAMPLE DIGESTION

Experiment condition: 10% NBF, 15 min Fixation, Pretreatment 4, RT

2-plex Mouse Positive Control Probe Mm POLR2A/PPIB
TROUBLE SHOOTING AUTOFLUORESCENCE

**TIP**: FFPE samples have inherent autofluorescence.

**Solution**: Use appropriate background correction software to reduce autofluorescence.

Mouse FFPE Kidney

Mouse FFPE Intestine

Mouse FFPE Colon

Mouse FFPE Brain
## MULTIPLEX FLUORESCENT ASSAY 101—PROBLEMS AND SOLUTIONS

<table>
<thead>
<tr>
<th>SOURCE</th>
<th>ISSUE</th>
<th>PROBLEM</th>
<th>SOLUTION</th>
</tr>
</thead>
</table>
| Microscopy     | No/weak signal Nonspecific signal | 1. Wrong filter setting/longer emission cut off  
2. Wrong exposure  
3. Inappropriate imaging enhancing with software | 1. Use correct filter settings  
2. Do not use using autoexposure at first, verify signal with naked eye  
3. Use known image enhancing software e.g. Nuance |
| Sample         | No/weak signal              | 1. Compromised RNA quality  
2. Sample preparation (high autofluorescence background on the sample) | 1. Use new sample with good RNA quality  
2. Follow the pretreatment guideline recommended  
3. Always perform assay with 3-plex positive control and 3plex negative probes to assess RNA quality  
4. Always check signal with naked eye under objective lens first |
• Be aware of the suggested filter settings for your microscope
• Use the suggested pretreatment condition
• Use the sample preparation protocol (PART 1) for your samples for optimal results
• Always run a 3-plex positive control and negative control to assess RNA quality and to verify microscope setting are appropriate
• Always evaluate the results by eye first before capturing images
FREQUENTLY ASKED QUESTIONS
RNAscope® assay compatibility with different tissues
RNAscope manual assay can be used with FFPE, fresh-frozen, fixed-frozen and cultured cells. RNAscope automated assays are primarily supported with the FFPE tissue. Please refer to the User Manual Selection Guide: http://www.acdbio.com/technical-support/downloads

Key differences between RNAscope® ISH assay and IHC for FFPE samples
No cooling is required during Epitope retrieval, users should directly put the slides in water at room temperature, dehydrate and proceed to Pretreatment 3 step as per the manual Part 1

TIP: Visit www.acdbio.com/support for additional FAQs
1. **RNAscope® recommended workflow for**
   - Manual assays
   - Automated assays

2. **Tips for RNAscope® manual and automation assays**
   - Check instrument maintenance
   - Optimize software settings
   - Optimize your assay

3. **Troubleshooting staining patterns**
   - High background, no signal, sample detachment
   - Optimizing with Pretreatment 2 and 3 optimization (MANUAL)
   - Adjusting ER2, protease time and hyb temperature changes (LEICA)
   - Offline/online pretreatment optimization (CC and pretreat 2/3) (VENTANA)
VISIT THE SUPPORT PAGE TO LEARN MORE

Support tab

TIP: Visit www.acdbio.com/technical-support/support-overview
CONTACT ACD SUPPORT

- Support via email –support@acdbio.com
- Support via phone-1-877-376-3636, option 3
  - Time 8:00am-6:00pm PST
- Support Resources available on website www.acdbio.com

<table>
<thead>
<tr>
<th>Manuals</th>
<th>Getting Started</th>
<th>FAQs</th>
<th>Videos</th>
<th>Product Literature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Download manuals, technical notes and MSDS.</td>
<td>Simple tips &amp; tricks for you to get the best RNAScope result from day 1.</td>
<td>Browse through our product frequently asked questions or add one of your own.</td>
<td>View our product and workflow videos on our Video page.</td>
<td>Find RNAScope publication lists, gene lists and download product brochures.</td>
</tr>
</tbody>
</table>
QUESTIONS?

PLEASE COMPLETE THE WEBINAR SURVEY, WE VALUE YOUR FEEDBACK