

RNASCOPE[®] TROUBLESHOOTING TIPS

Presented by:
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Senior Scientist

Advanced Cell Diagnostics

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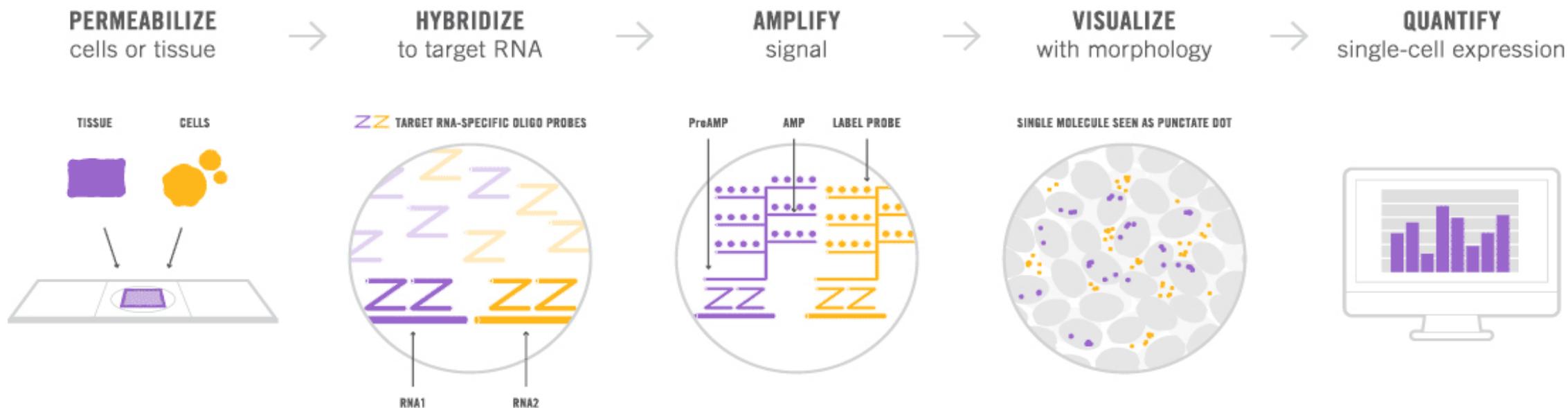




TOPICS

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

RNASCOPE[®] WORKFLOW



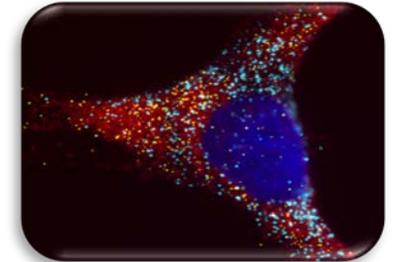
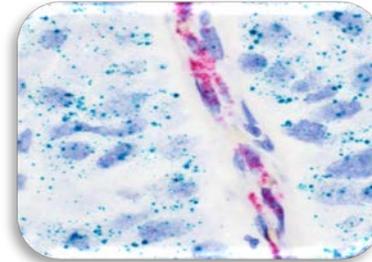
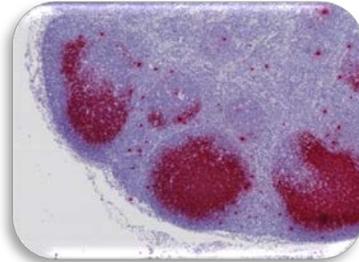
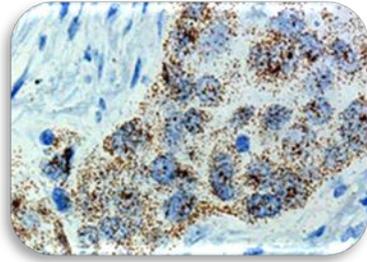
A BREAKTHROUGH PLATFORM





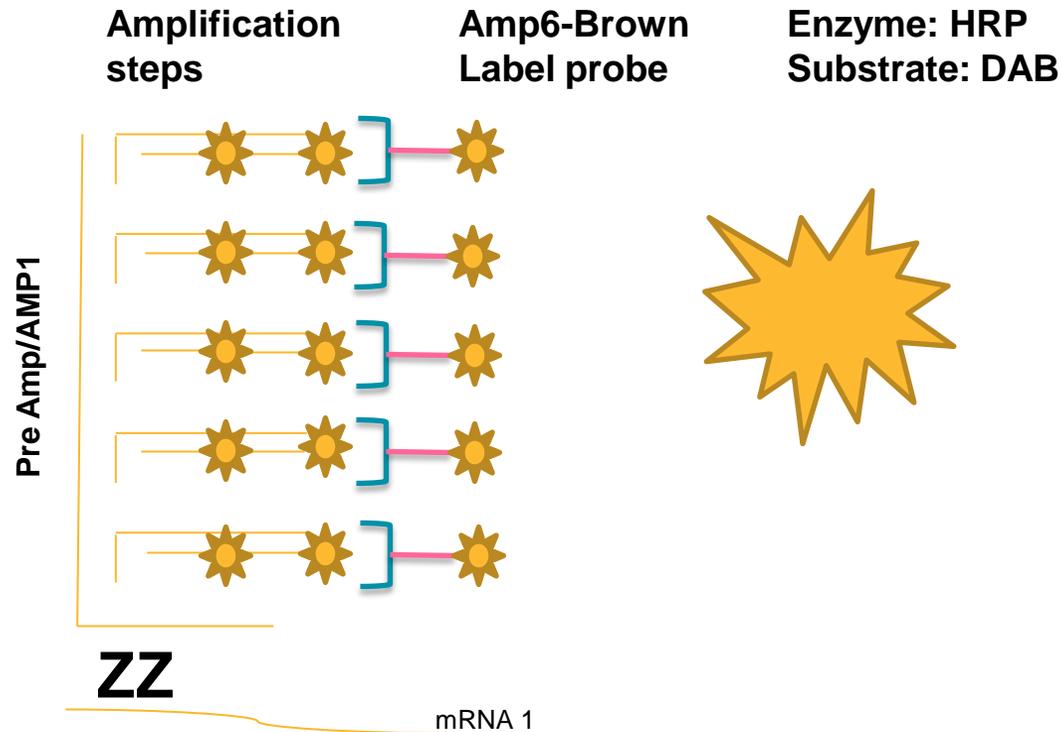
RNASCOPE[®] WORKFLOW

RNASCOPE[®] ASSAY SELECTION



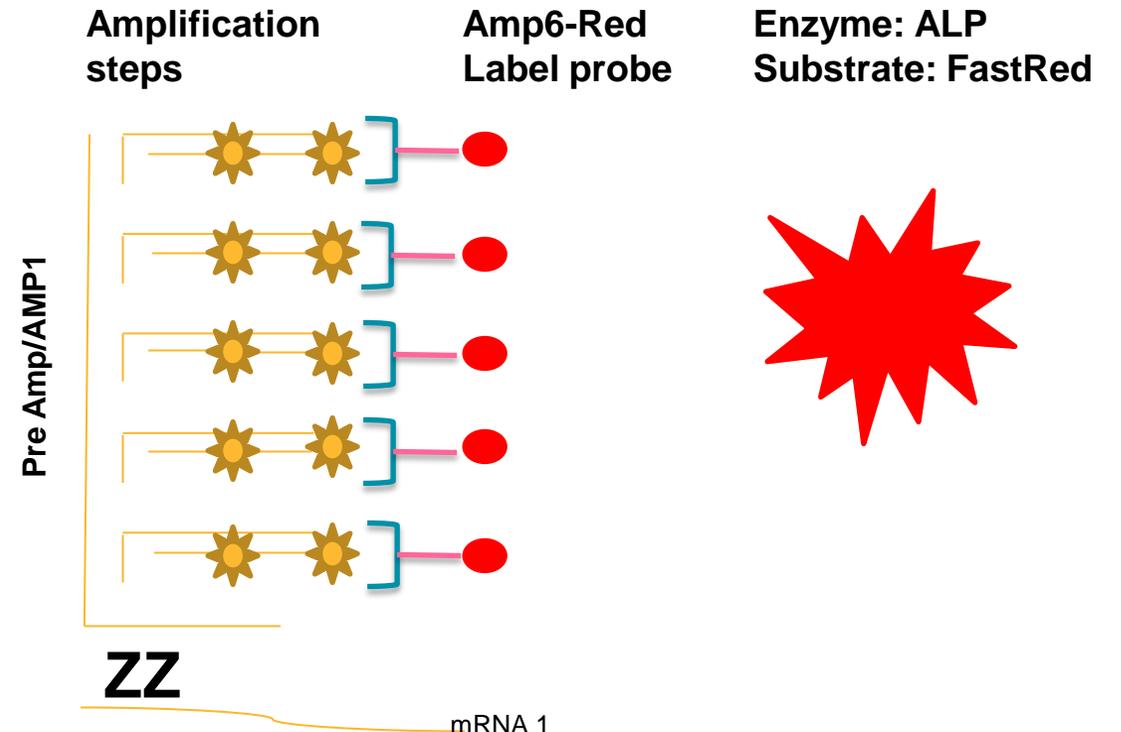
RNAScope Assays	RNAScope 2.0 HD (BROWN) Ventana Systems Leica Bond Rx	RNAScope 2.0 HD (RED) Ventana Systems Leica Bond Rx	RNAScope 2-plex	RNAScope Multiplex – Fluorescence
Assay type	Chromogenic	Chromogenic	Chromogenic	Fluorescent
Dye used	Diaminobenzene (DAB)-HRP	Fast Red -ALP	HRP-Green, Fast Red -ALP	FITC, Cy3, Cy5,
Channel (Manual)	Channel 1	Channel 1	Channel 1, 2	Channel 1, 2, 3
Probes channel (Manual)	C1 Probes	C1 Probes	C1, C2 Probes	C1, C2, C3 Probes
Probes Channel (Automation)	VS/LS Probes	VS/LS Probes	N/A	N/A

RNASCOPE[®] 2.0 HD AMPLIFICATION SCHEMATIC



2.0 HD BROWN

Brown dot
HRP/DAB

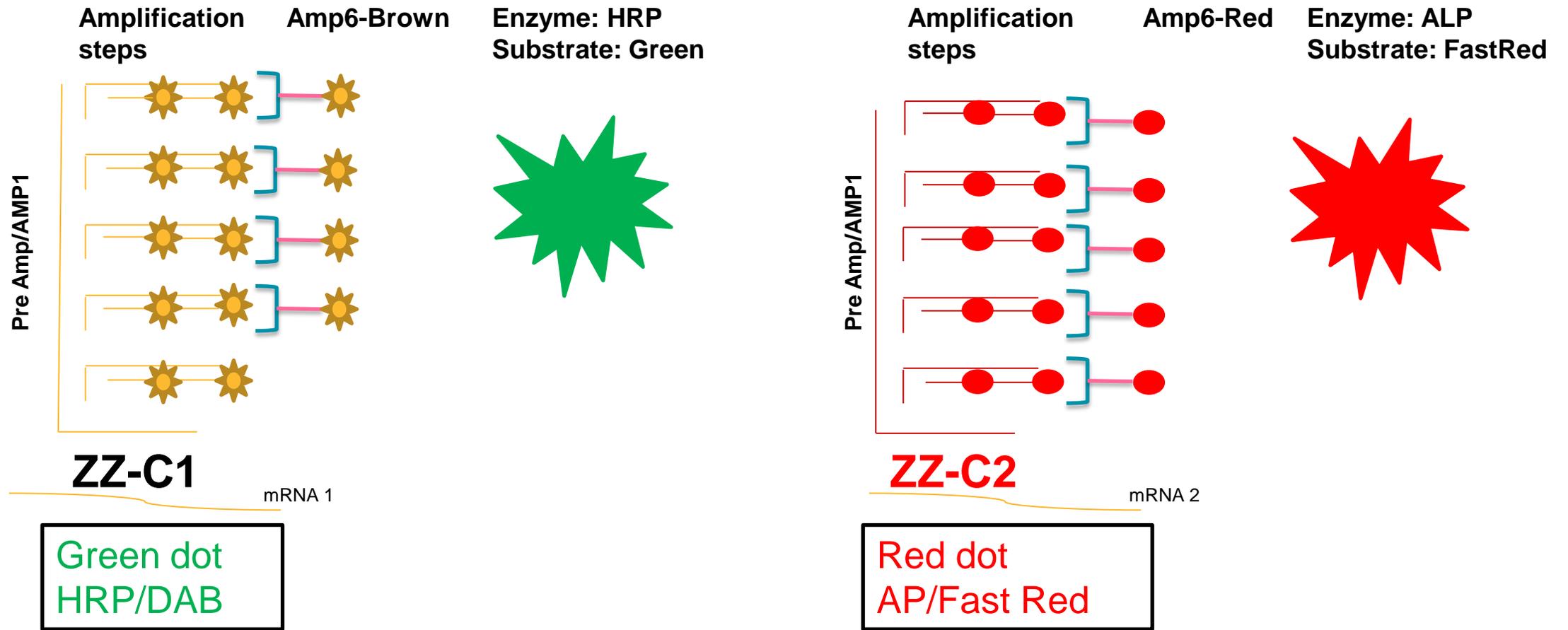


2.0 HD RED

Red dot
AP/Fast Red

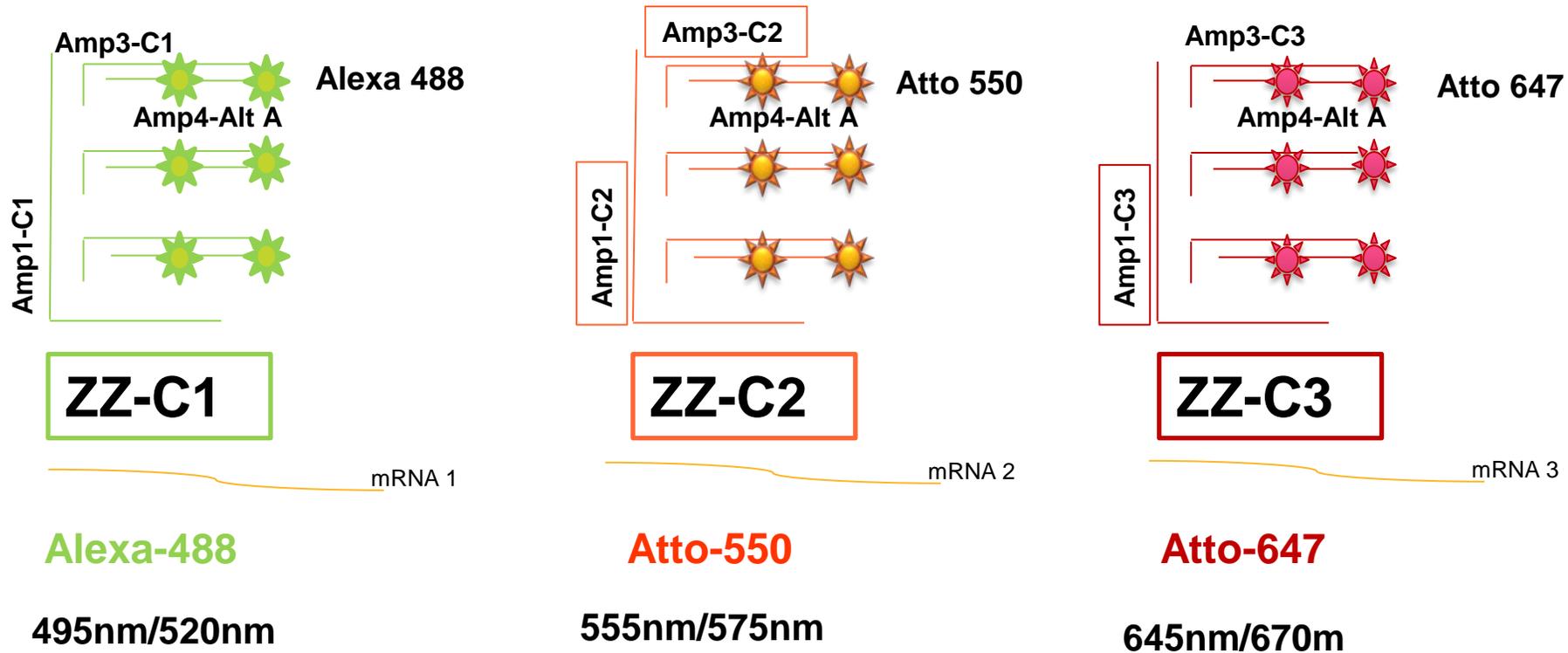
TIP : Do not interchange reagents within Brown/Red assays or across similar 2.0 HD Assays
By default 2.0 HD assays require C1 probes that are ready to use, no further dilution is required

RNASCOPE[®] 2-PLEX AMPLIFICATION SCHEMATIC



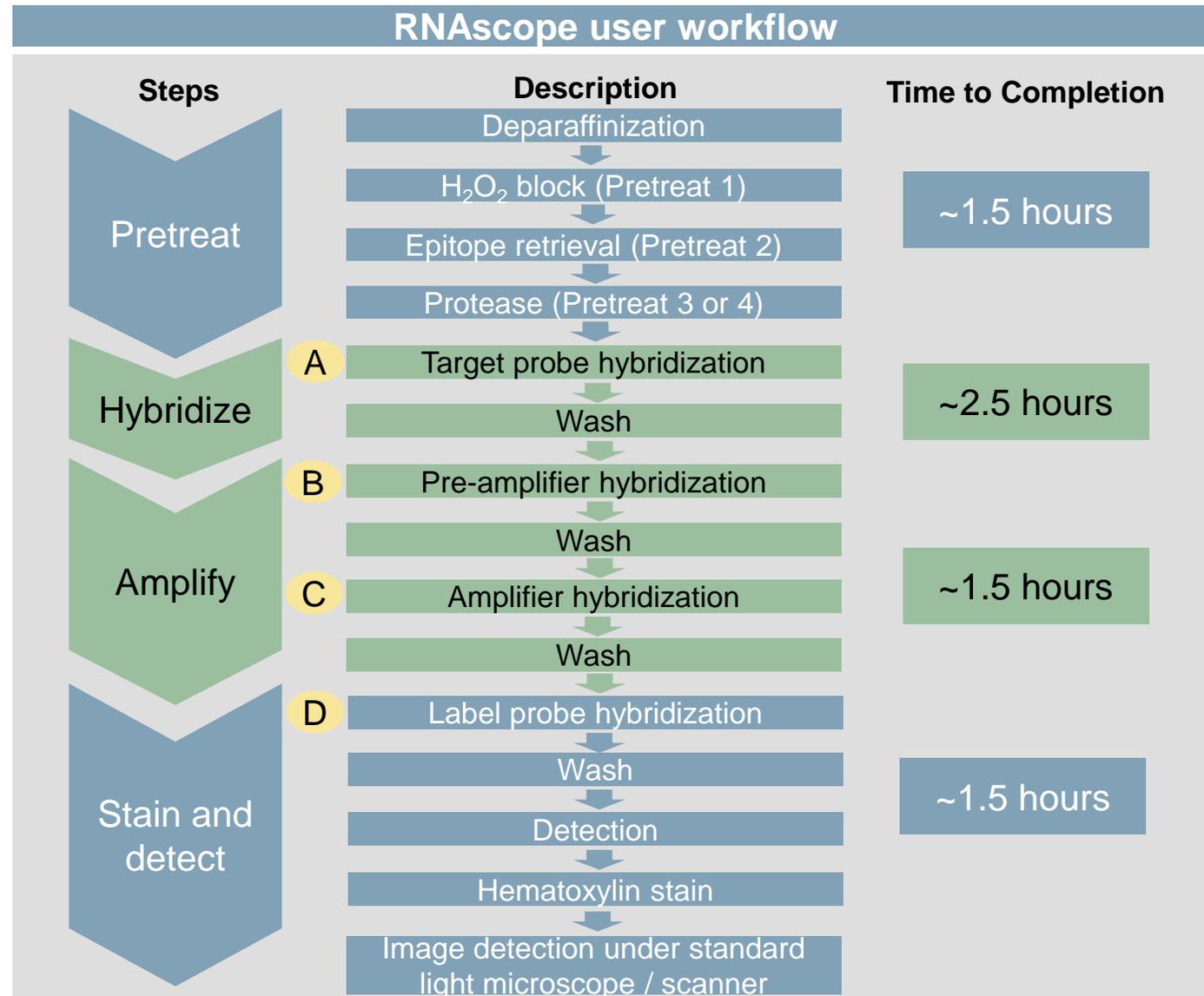
TIP: By default C1 probes are 1X concentration while C2 probes are 50X
To make 2-plex probe mixture at 1X concentration, mix C2 probes 1:50 with C1 probes
To view C2 probes only, use the “blank-probe-C1”, as a diluent and mix at a 1:50 dilution

RNASCOPE[®] MULTIPLEX FLUORESCENT SCHEMATIC



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

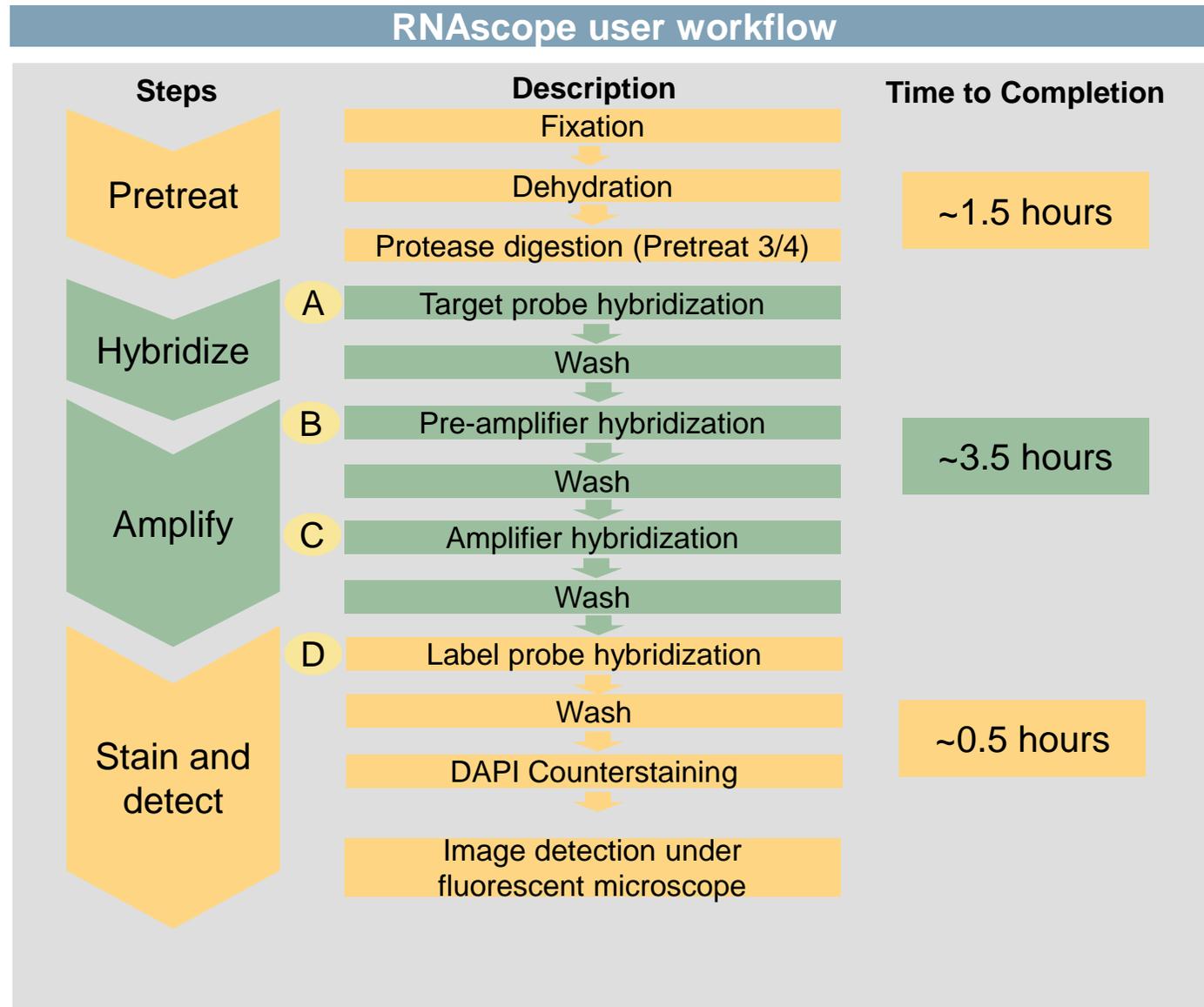
RNASCOPE® WORKFLOW: CHROMOGENIC ASSAY



TIP : Detection protocols will vary based on the chromogenic assay used
 Download manuals: <http://www.acdbio.com/technical-support/downloads>



RNASCOPE® WORKFLOW: FLUORESCENT ASSAY



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



DAY 1

Sample pretreatment



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

PROTOCOLS

Review sample pretreatment recommendations

**SAMPLE
PRETREATMENT**

Always use control probes and slides

USE CONTROLS

Review that you are using all required materials

THE CHECKLIST

TIP : Visit <http://www.acdbio.com/technical-support/downloads/rnascope-ish-guide-troubleshooting/> for more information on tips for manual assays



REVIEW THE CHECKLIST:

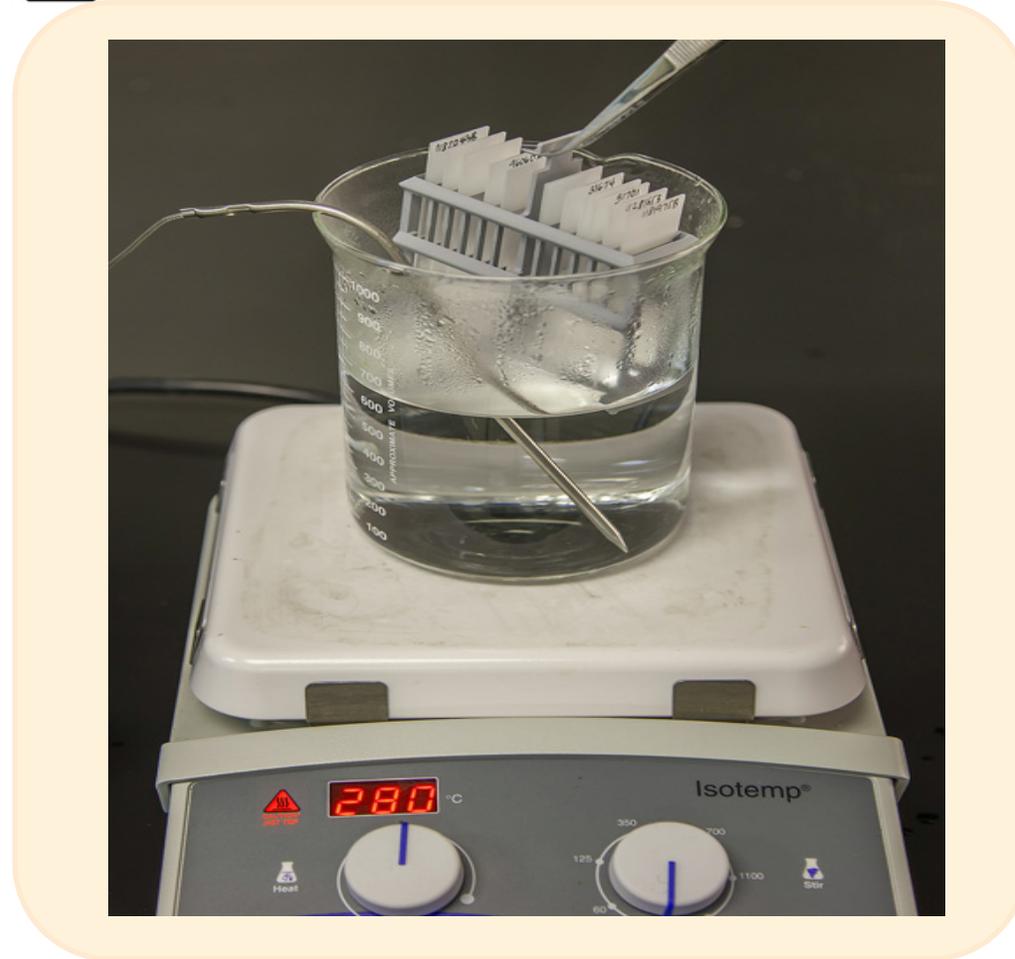
<input checked="" type="checkbox"/>	Immedge hydrophobic barrier pen
<input checked="" type="checkbox"/>	Positive and Negative control probes
<input checked="" type="checkbox"/>	Hot-Plate for pretreatment/ target retrieval step
<input checked="" type="checkbox"/>	Superfrost plus slides
<input checked="" type="checkbox"/>	HybEZ Hybridization system
<input checked="" type="checkbox"/>	Run RNAscope® control slides
<input checked="" type="checkbox"/>	Ecomount for 2.0 HD Red & 2-plex chromogenic assay
<input checked="" type="checkbox"/>	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

RNASCOPE® REAGENT KIT CONTENTS

OLD



NEW



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

☑ **HyBEZ hybridization system**



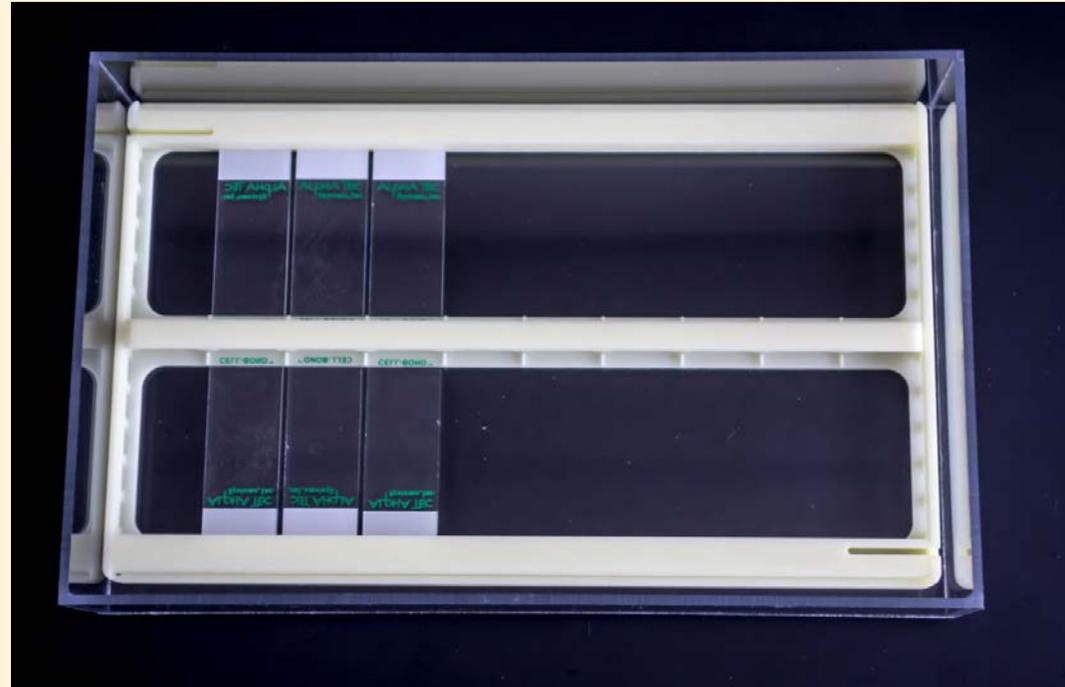
TIP: HybEZ oven is required as it provides both temperature and humidity control, necessary to obtain optimal RNAscope results

ACCESSORIES FOR WASHING STEPS

Tissue Tek washing tray



EZ Batch for slide processing



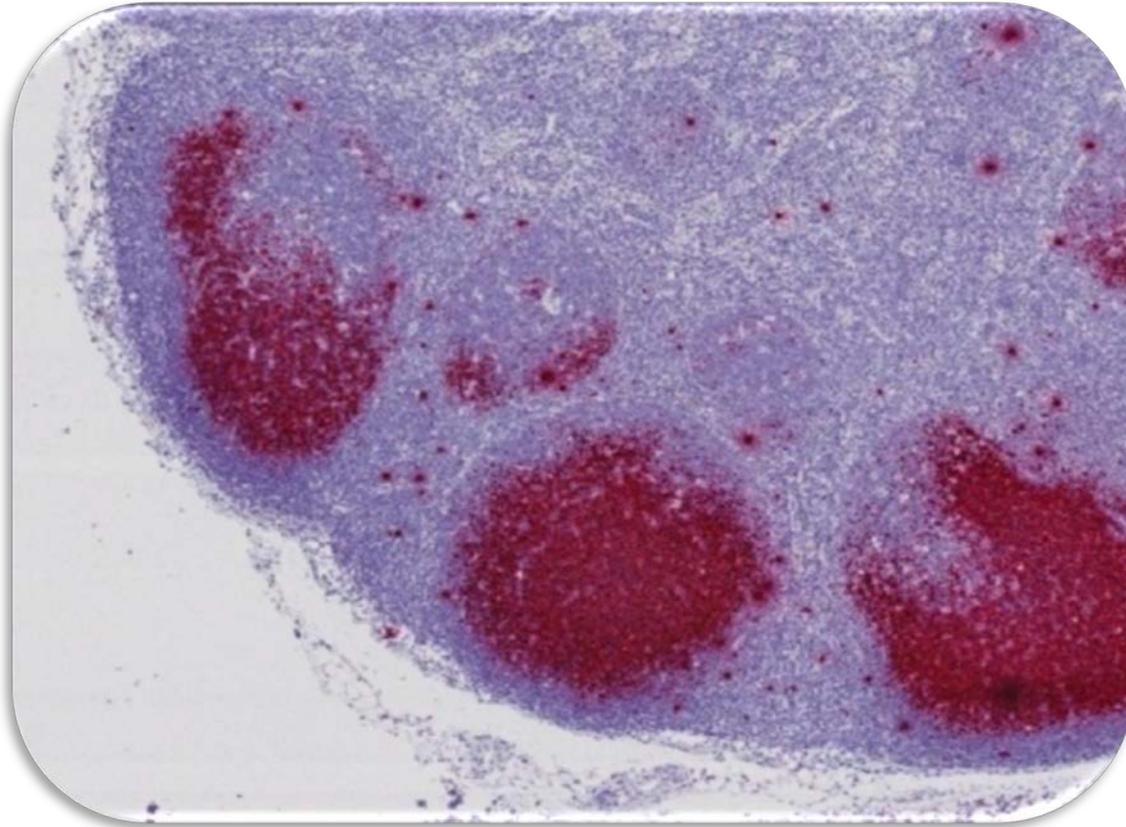
TIP : ACD EZ Batch slide processing tray is easy and convenient for loading multiple slides for hybridization and washing steps.

FOLLOW WORKFLOW GUIDELINES (MANUAL)

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

TIP : Visit <http://www.acdbio.com/technical-support/downloads/rnascope-ish-guide-troubleshooting/> for more information on tips for automation assays

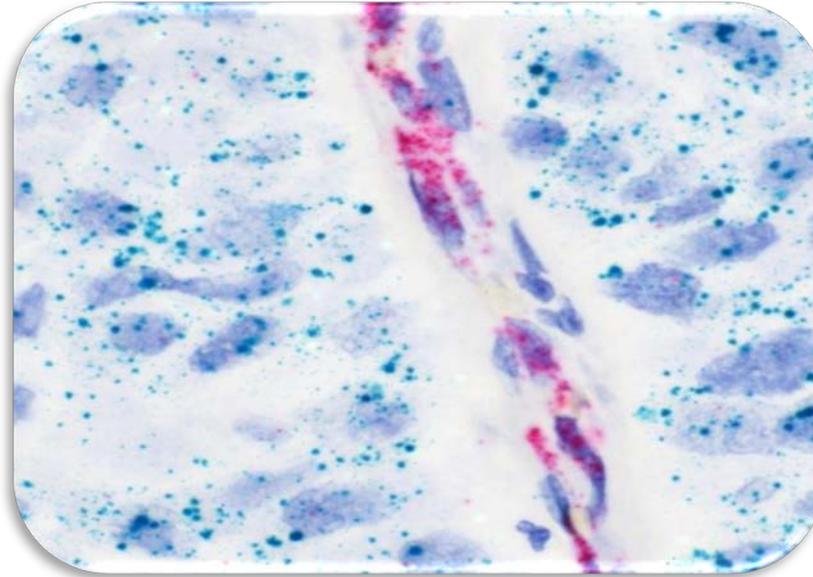
POINTERS FOR RNASCOPE 2.0 RED ASSAY



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**

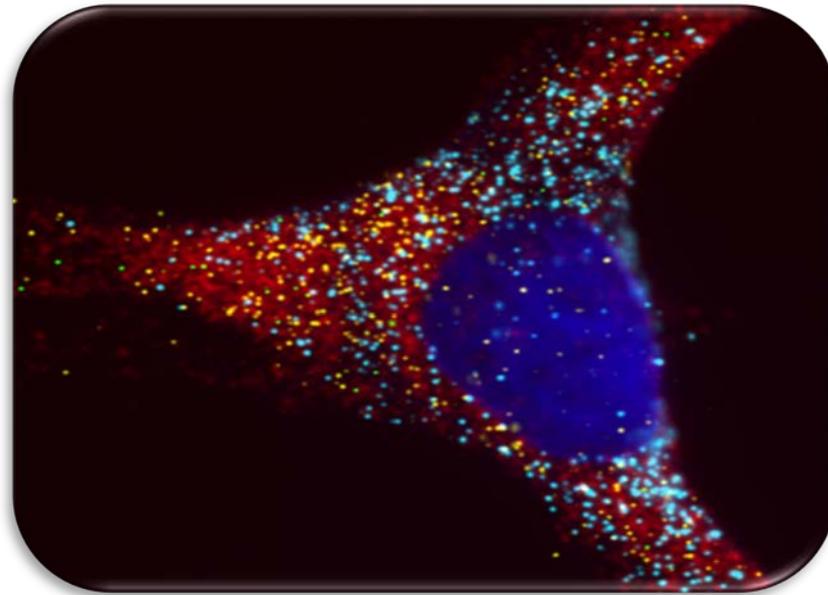
POINTERS FOR RNASCOPE® 2-PLEX CHROMOGENIC ASSAY



COMPONENTS	MIXING RATIO
Probes C2:C1	1:50
Amp 4B: Amp 4A	1:50
Red-B:Red- A	1:60
Green-B: Green-A	1:50

- 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

POINTERS FOR RNASCOPE® MULTIPLEX FLUORESCENT ASSAY



COLOR MODULE OPTIONS

	Channel 1 (C1)	Channel 2 (C2)	Channel 3 (C3)
AMP 4 Alt A	GREEN-Alexa 488	ORANGE-Atto 550	FAR RED-Atto 647
Amp 4 Alt B	ORANGE-Atto 550	GREEN-Alexa 488	FAR RED-Atto 647
Amp 4 Alt C	ORANGE-Atto 550	FAR RED-Atto 647	GREEN-Alexa 488

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

**INSTRUMENT
MAINTENANCE**

Optimize
software
settings

**SOFTWARE
SETTINGS**

Troubleshoot
Reagents

REAGENTS

Review
sample
pretreatment
recommendations

**SAMPLE
PRETREATMENT**

TIP : Visit <http://www.acdbio.com/technical-support/downloads/rnascope-ish-guide-troubleshooting/> for more information on tips for automation assays



POINTERS FOR RNASCOPE® ON THE VENTANA DISCOVERY® XT OR ULTRA ASSAY

1 *Check Instrument Maintenance:*

- Perform instrument maintenance
- Perform decontamination protocol every three months (prevents microbial growth)
- Use appropriate buffers for RNAscope assay, remove or purge before a run

2 *Optimize Software Settings:*

- *Uncheck the **Slide Cleaning** option (ULTRA only)
- Use appropriate hybridization temperature (different for XT versus ULTRA)

***TIP: *This is a cleaning step in Ventana Equipment may cause the slides to dry out
Refer to User Manual for details***



POINTERS FOR RNASCOPE® (LEICA BOND RX®)

1

Do not shake the contents in the containers as this will form bubbles

2

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

3

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

4

Do not alter the staining protocol in any way

TIP : Visit <http://www.acdbio.com/technical-support/downloads/rnascope-ish-guide-troubleshooting/> for more information on tips for LEICA BOND RX® automation assays

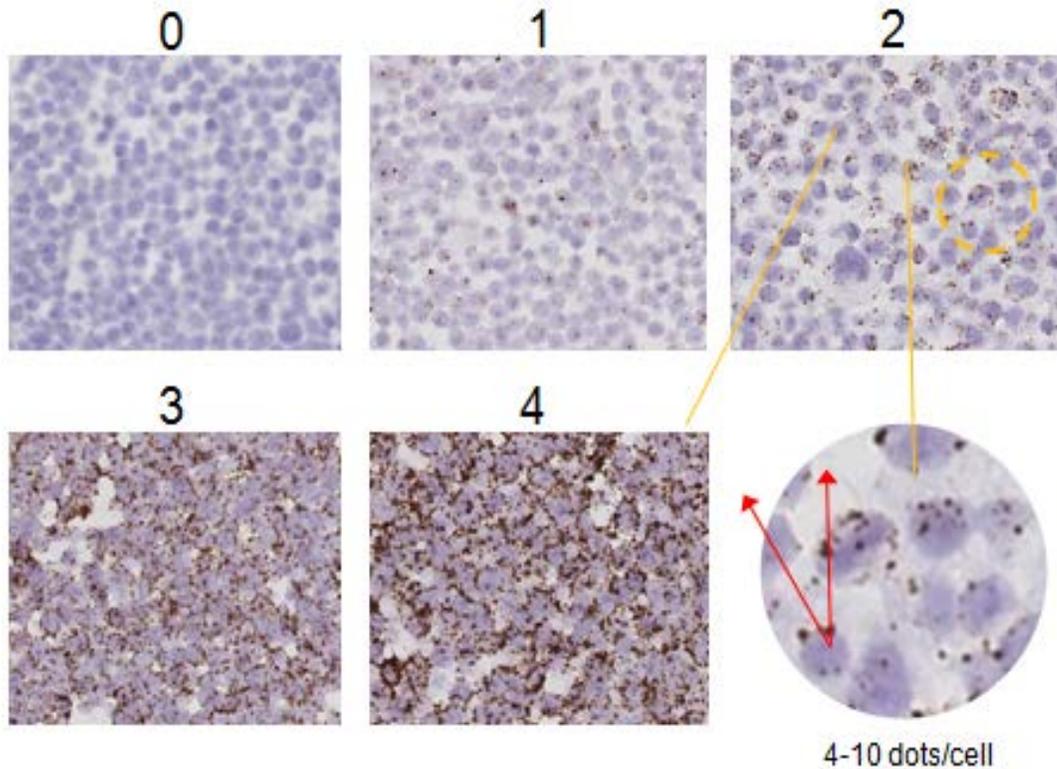




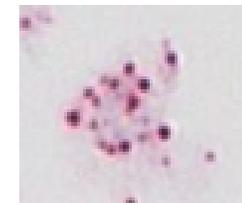
QUALIFY YOUR SAMPLES USING CONTROLS

IMAGE ANALYSIS

RNASCOPE® SCORING GUIDELINE



Score	Criteria
0	No staining or <1 dot/ 10 cells*
1	1-3 dots/cell
2	4-9 dots/cell. None or very few dot clusters
3	10-15 dots/cell and <10% dots are in clusters
4	>15 dots/cell and >10% dots are in clusters

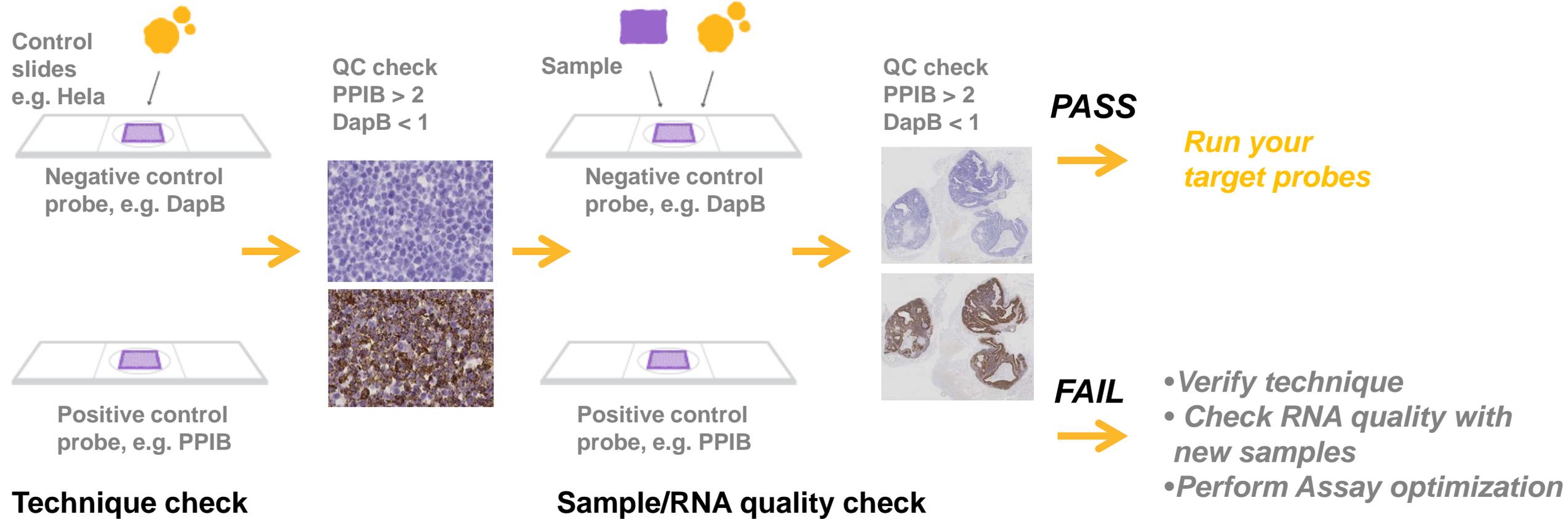


Score = 3



Score = 3

QUALIFY YOUR SAMPLES USING CONTROLS



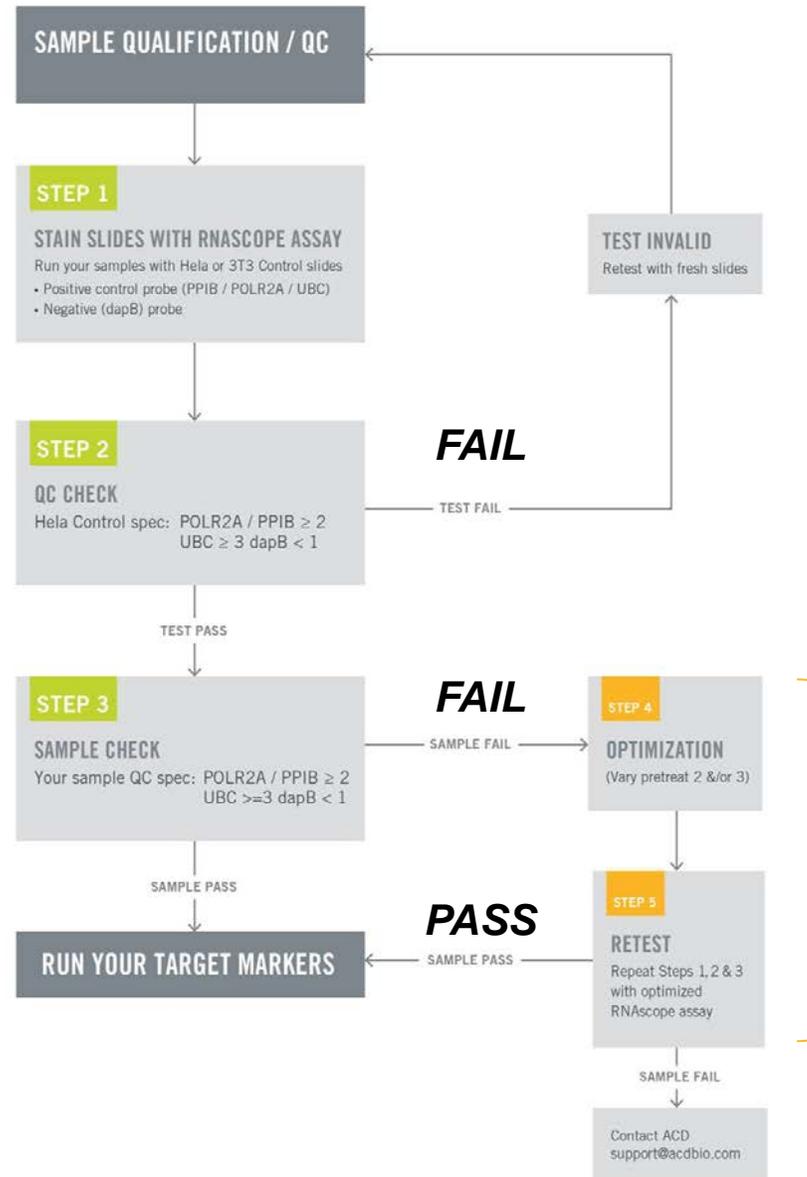
TIP : Always start with standard conditions



OPTIMIZE YOUR ASSAY

Technique check

Sample/
RNA quality check



OPTIMIZE YOUR ASSAY →

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
- 4% PFA ≤ 24 hours /RT
- 4% PFA <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

<input checked="" type="checkbox"/>	Fixation conditions are not optimal
<input checked="" type="checkbox"/>	RNA is degraded
<input checked="" type="checkbox"/>	Hybridization conditions not optimal
<input checked="" type="checkbox"/>	Samples drying during assay
<input checked="" type="checkbox"/>	Special tissues sensitive to pretreatment

↓ THE SOLUTIONS

<input checked="" type="checkbox"/>	Fix samples as recommended. E.g., for FFPE use 10% NBF RT, 16-32 hrs
<input checked="" type="checkbox"/>	Acquire new samples and assess RNA quality
<input checked="" type="checkbox"/>	Use the HybEZ hybridization oven only
<input checked="" type="checkbox"/>	Use Immedge pen and add adequate reagents to avoid drying
<input checked="" type="checkbox"/>	Start with standard pretreatment, then optimize conditions accordingly

OPTIMIZE YOUR SAMPLE IN 3 EASY STEPS (MANUAL ASSAY)

STEP 1 START WITH STANDARD CONDITIONS

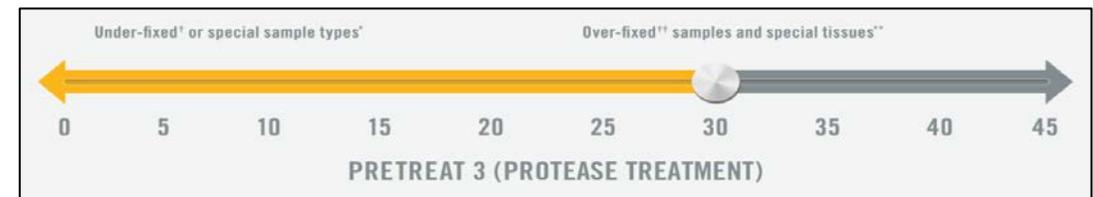


Observe Staining Pattern -
*High background, over-digested? = **under fixed***
*No signal/weak signal, under-digested? = **over fixed***

STEP 2 ADJUST PRETREATMENT 2, BOILING TIME



STEP 3 ADJUST PRETREATMENT 3/4, PROTEASE TIME*



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

VENTANA XT/ULTRA

*OVER FIXED/
UNDER DIGESTED*

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

Increase Pretreat 2/3 and/or CC time

*UNDER FIXED/
OVER DIGESTED*

*Reduce temp to 88°C, this improves
morphology and reduces background*

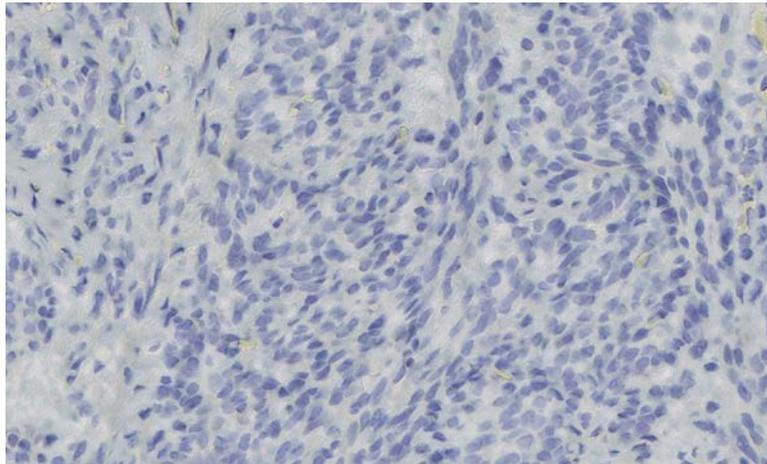
Decrease Pretreat 2/3 and/or CC time

***TIP: Refer to the User Manuals for automation assay workflow
and pretreatment optimization guideline***

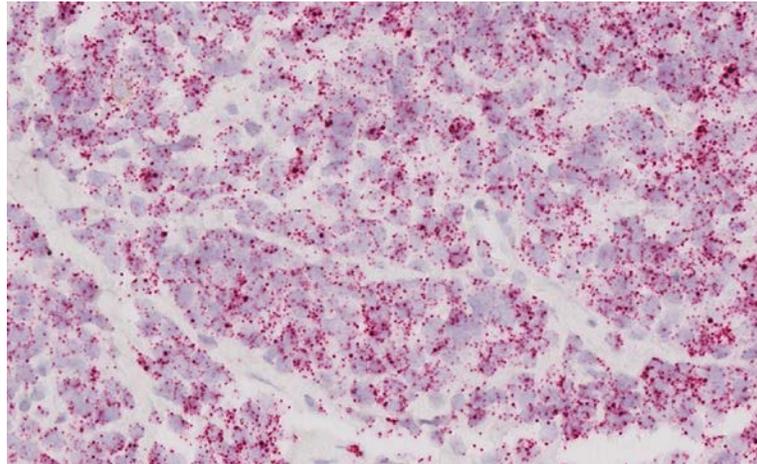


EXAMPLE OF SUCCESSFUL RNASCOPE[®] RESULTS

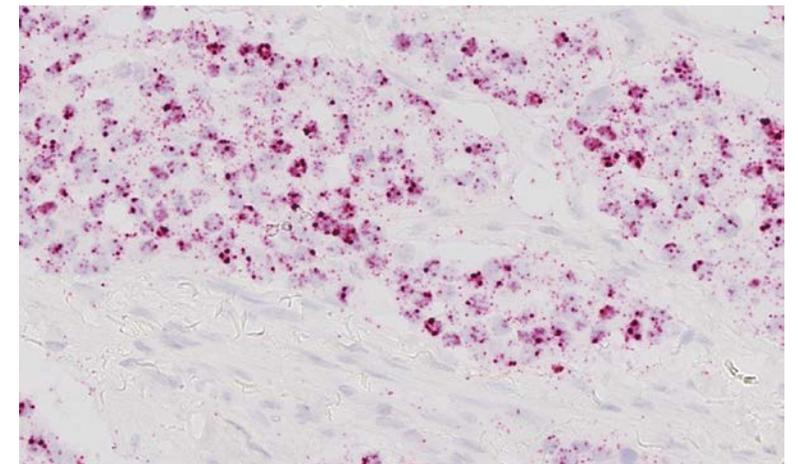
Negative control, DapB



Positive control, HsPPIB



Target probe



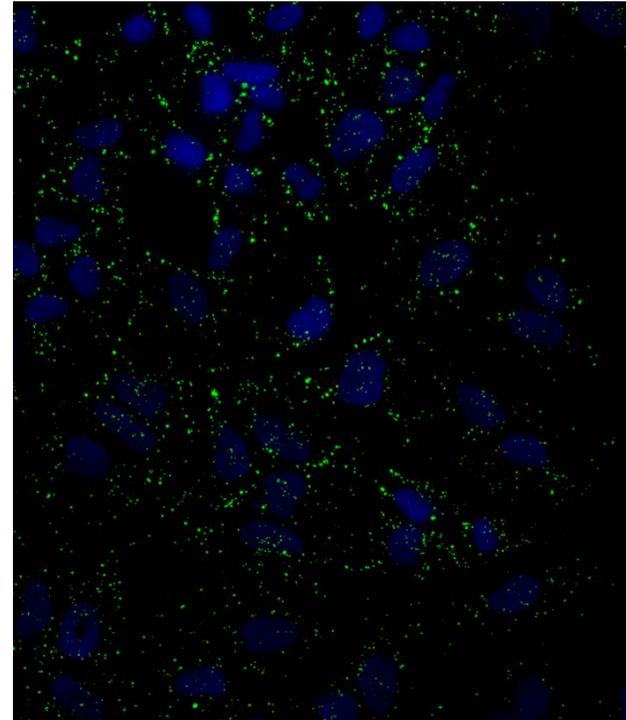
RNAscope 2.0 HD Red Assay

Human breast cancer tissue

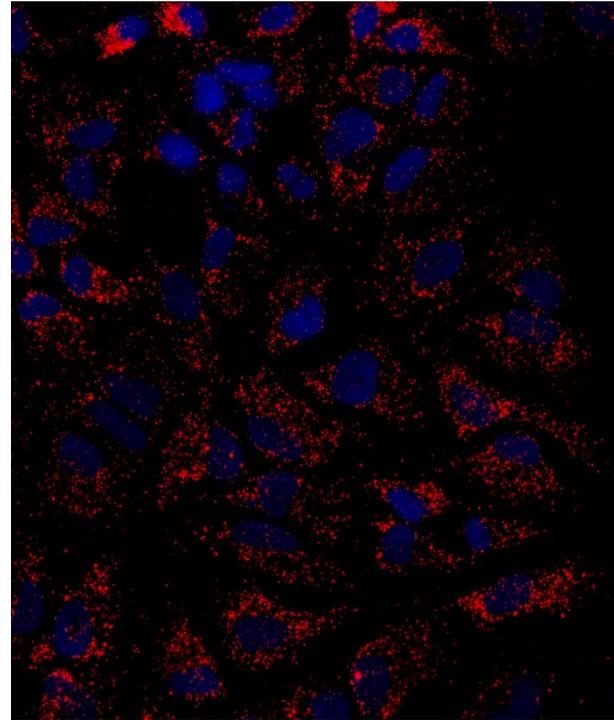
TIP : Visit <http://www.acdbio.com/technical-support/downloads/rnascope-ish-guide-troubleshooting/> for more information

EXAMPLE OF SUCCESSFUL RNASCOPE[®] RESULTS

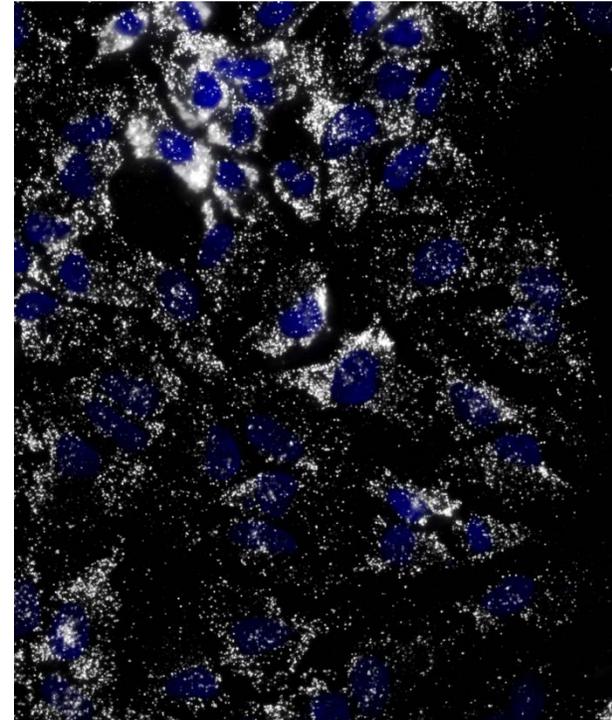
Hs POLR2A/Alexa 488



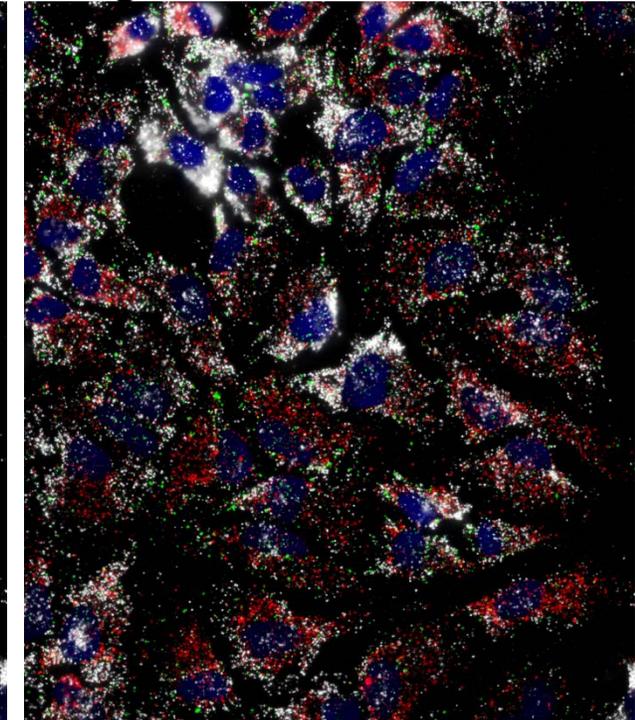
Hs PPIB/Atto 550



Hs UBC/Atto 647



Merged



RNAscope Multiplex Fluorescent Assay
Amp 4 ALT A*

Human HeLa Cell Line

TIP: Use different AMP4 ALT reagents (A, B, C) for alternative color combinations



TROUBLESHOOTING STAINING PATTERNS (CHROMOGENIC MANUAL ASSAYS)

TROUBLESHOOTING: NO STAINING OBSERVED

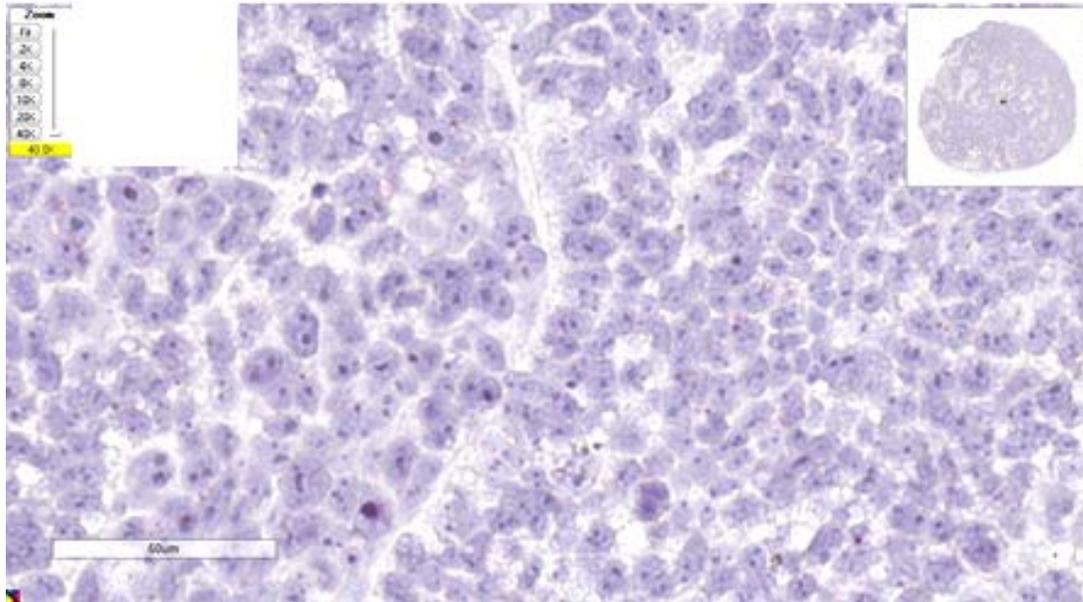
<i>PROBABLE CAUSE</i>	<i>SUGGESTED ACTION</i>
<i>Suboptimal fixation</i> <ul style="list-style-type: none">• <i>Over fixation</i>• <i>Under fixation</i>	<i>Prepare (fix) samples according to ACD recommendation</i> <i>Optimize pretreatment conditions</i>
<i>Hybridization temperature not optimal</i>	<i>Use HybEZ when performing RNAscope</i> <i>HybEZ temperature should be at 40°C</i>
<i>Reagents used in the wrong sequence</i>	<i>Apply reagents in the correct order</i>
<i>Gene of interest not expressed</i>	<i>Check positive control for technical accuracy of the assay</i>

TROUBLESHOOTING: SAMPLE DIGESTION

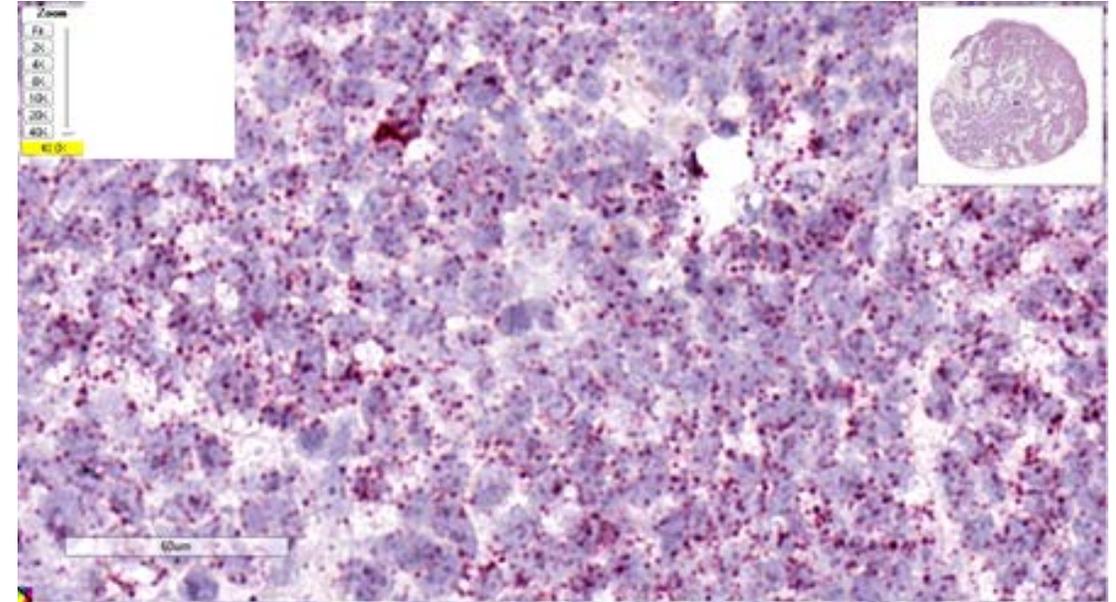
OPTIMAL DIGESTION

XENOGRAFT TISSUE

*8 min Pretreat 2 , 30 min Pretreat 3



DapB



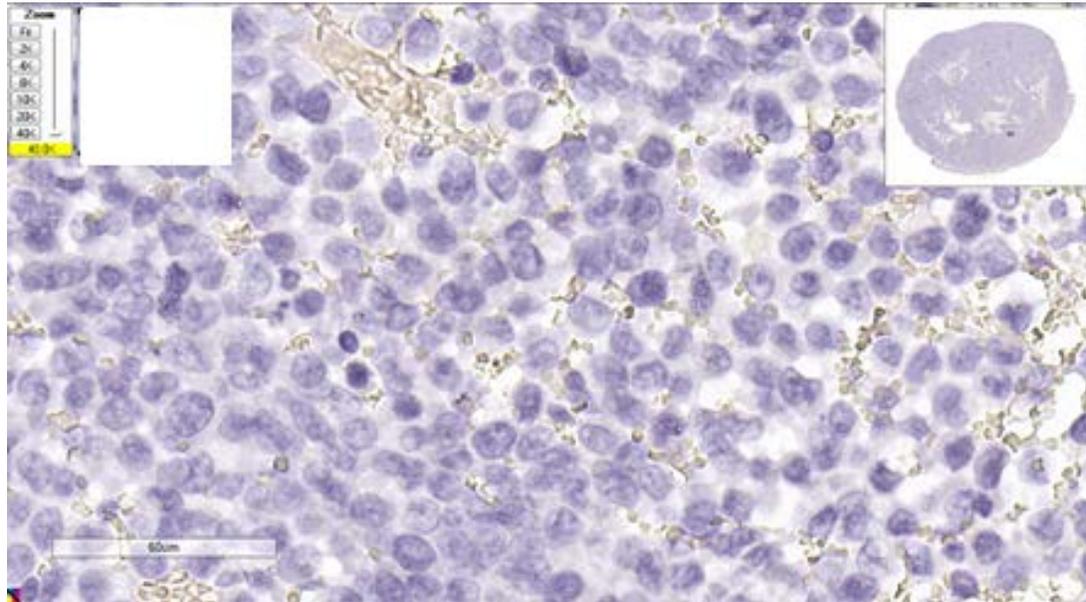
Hs-PPIB

**Conditions used for manual assays*

TROUBLESHOOTING: SAMPLE DIGESTION

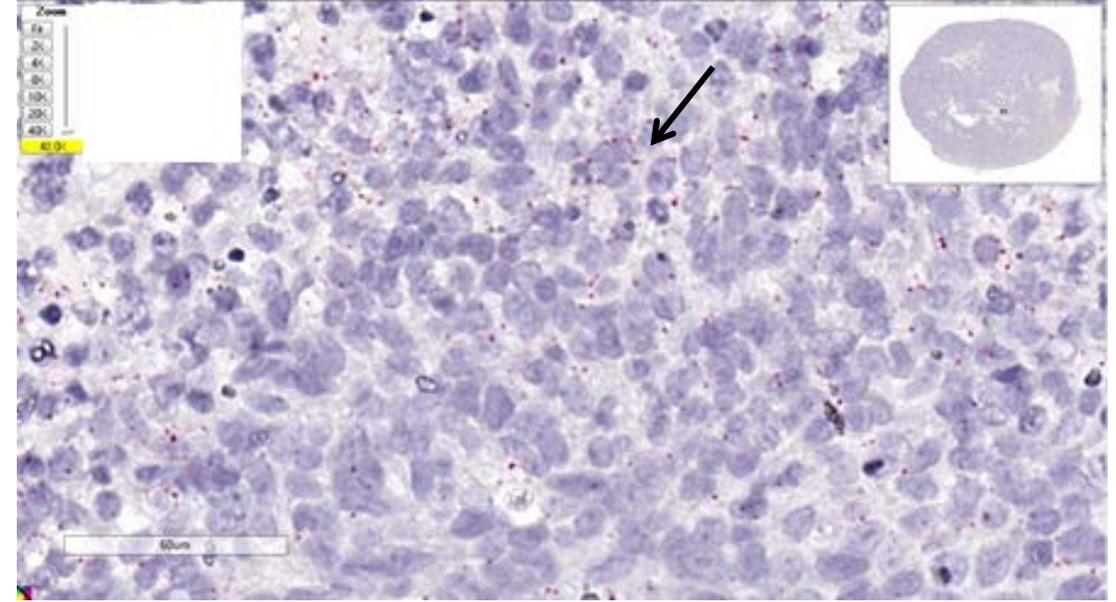
UNDER DIGESTION

*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

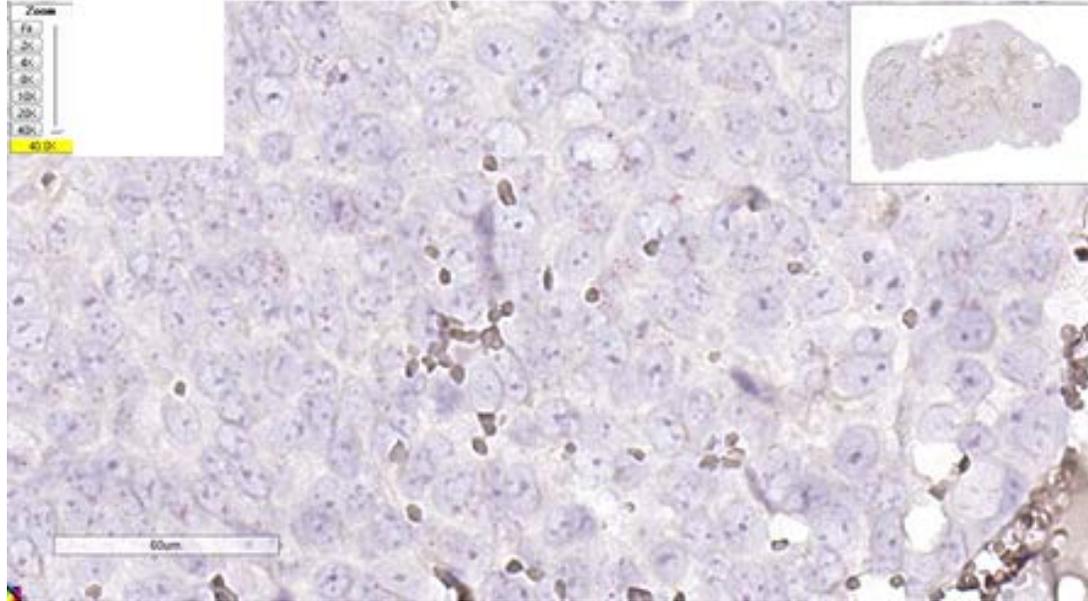
*Conditions used for manual assays

TROUBLESHOOTING: SAMPLE DIGESTION

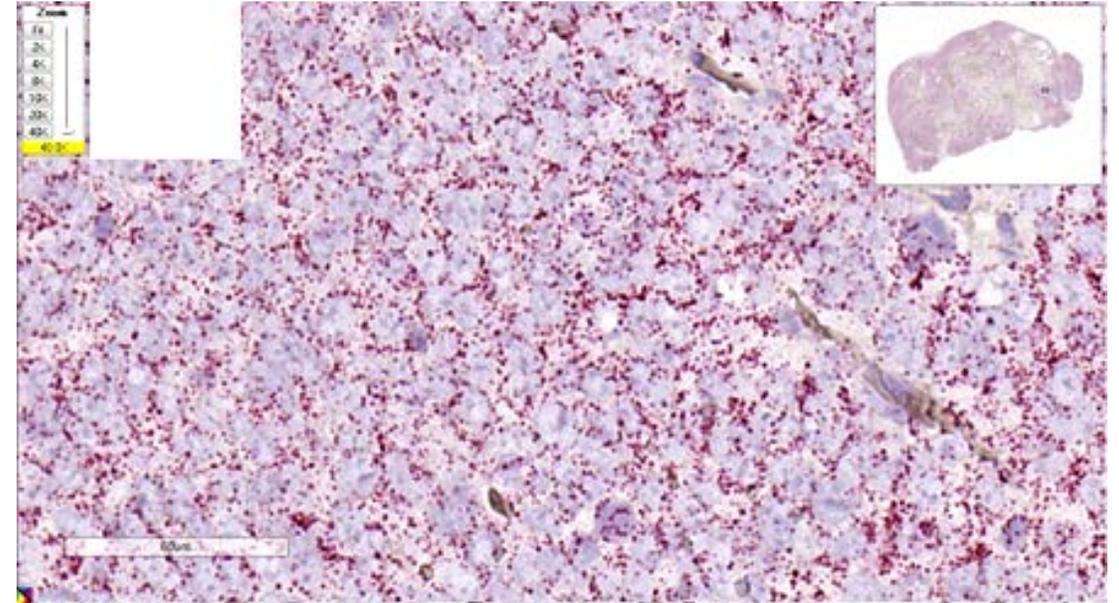
OVER DIGESTION

XENOGRAFT TISSUE

*15 min Pretreat 2 , 30 min Pretreat 3



DapB



Hs-PPIB

Assay: RNAscope 2.0 HD RED

Issue: Nuclear background, over pretreatment

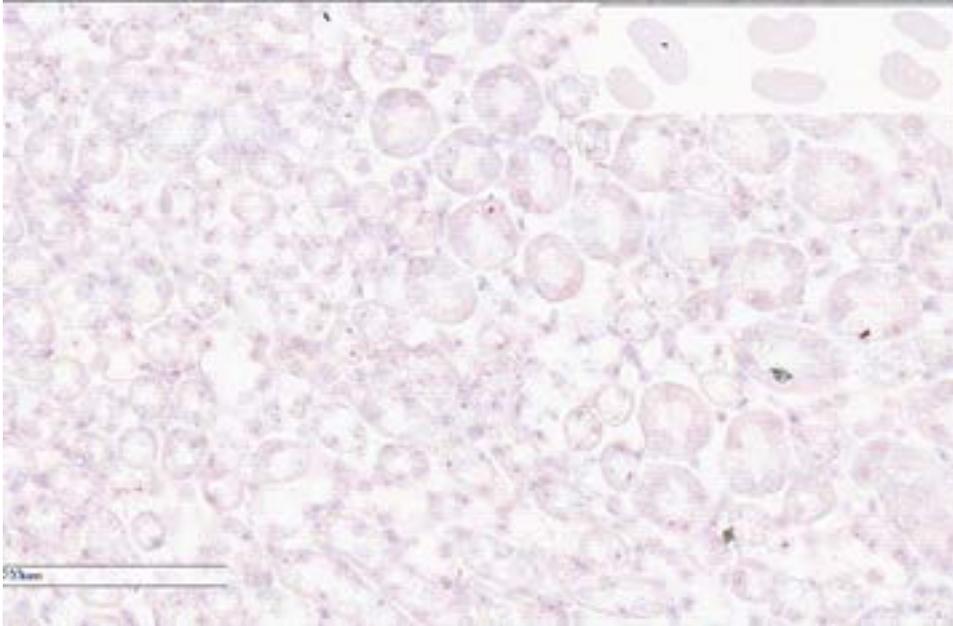
Solution: Decrease pretreatment

*Conditions used for manual assays

TROUBLESHOOTING: BACKGROUND STAINING

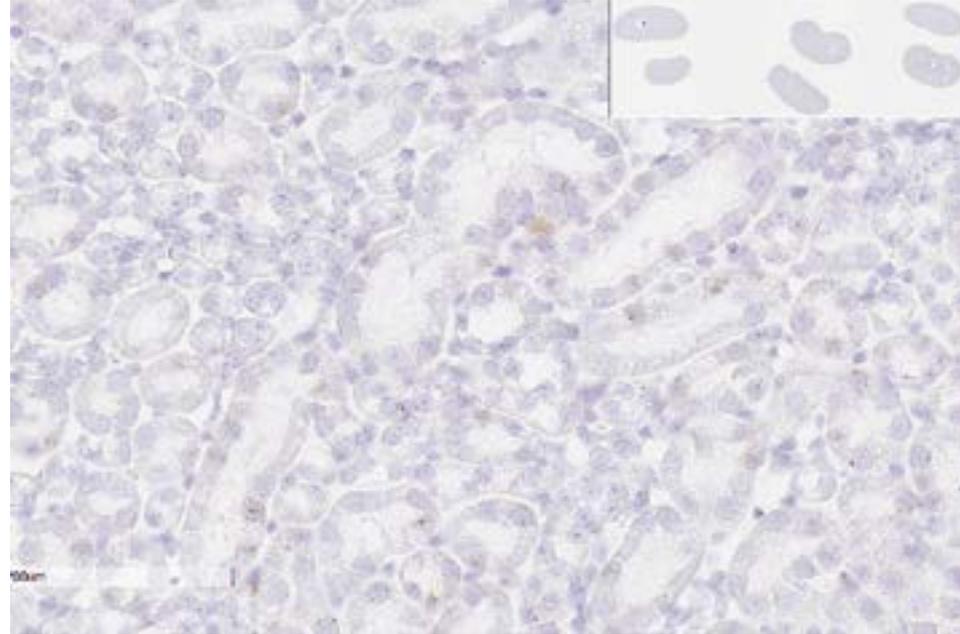
HIGH BACKGROUND

DapB



*15 min Pretreat 2 , 30 min Pretreat 3

KIDNEY FFPE TISSUE



*7 min Pretreat 2 , 30 min Pretreat 3

Assay: RNAscope 2.0 HD BROWN

Issue: High background, over pretreatment

Optimization: Decrease pretreatment 2 (boiling) conditions

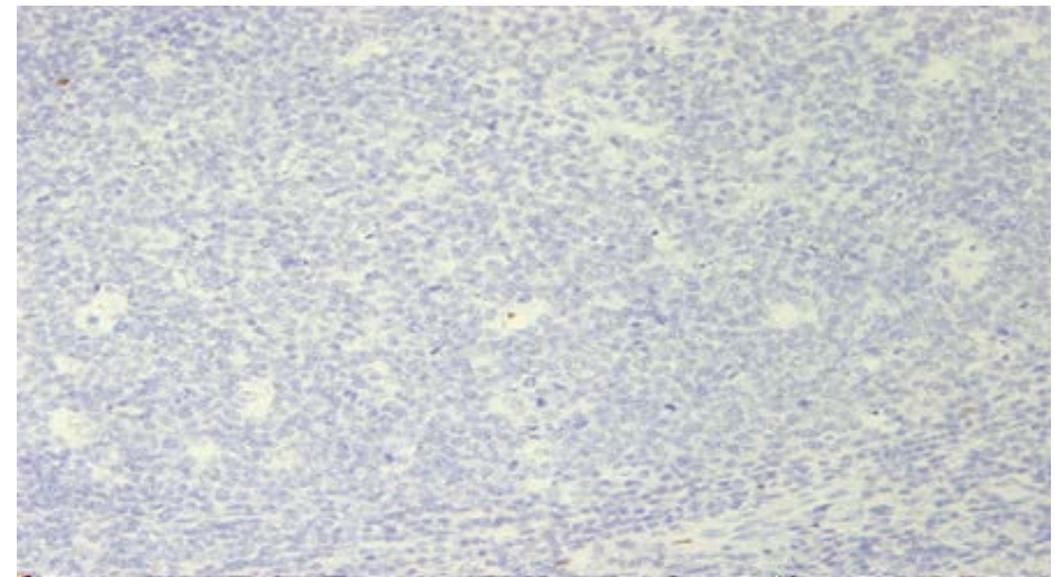
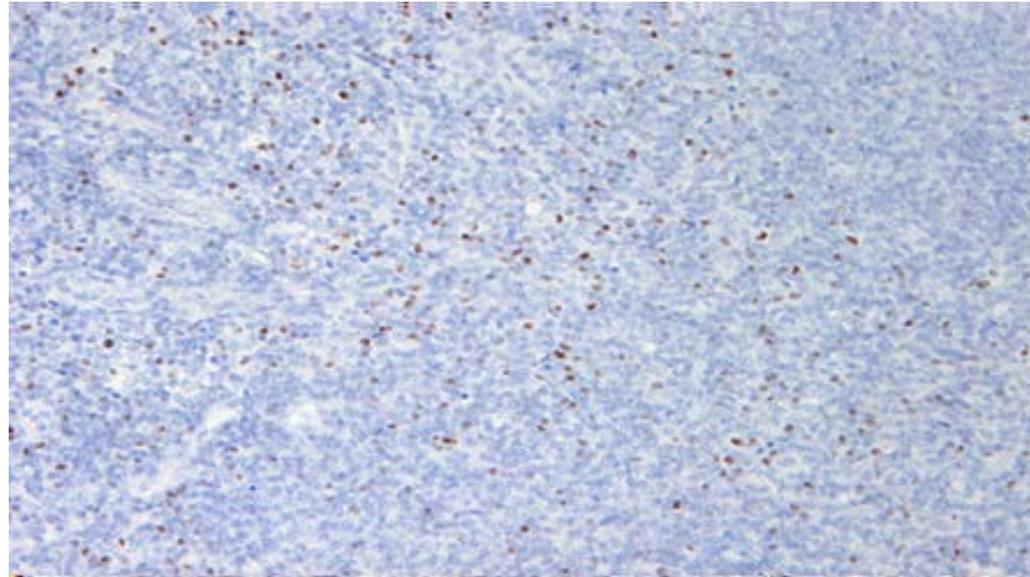
Result: Clean background

TROUBLESHOOTING: BACKGROUND STAINING

NUCLEAR HAZY BACKGROUND

HUMAN TONSIL FFPE TISSUE

DapB



*15 min ER2, 30 min Protease

*20 min ER2, 30 min Protease

Assay: RNAscope 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

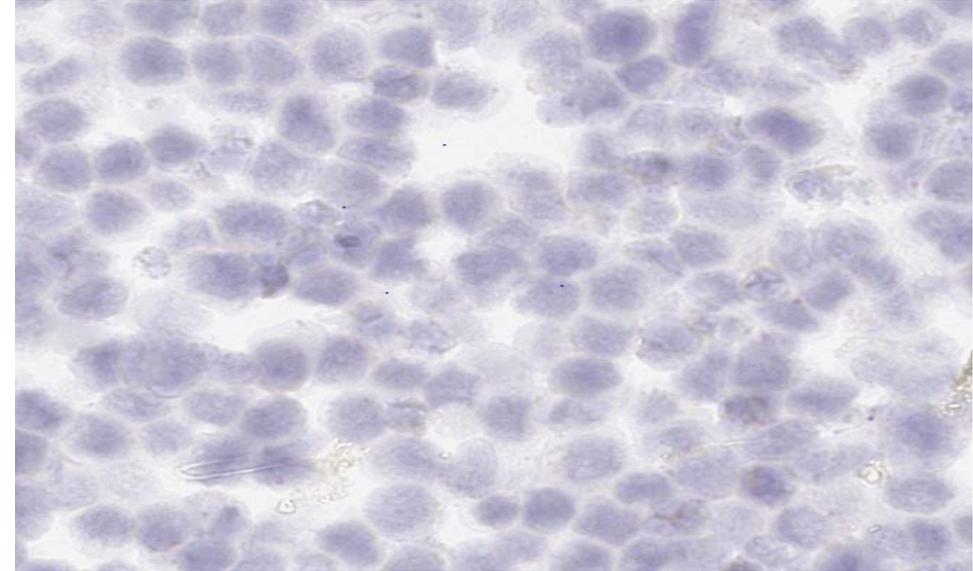
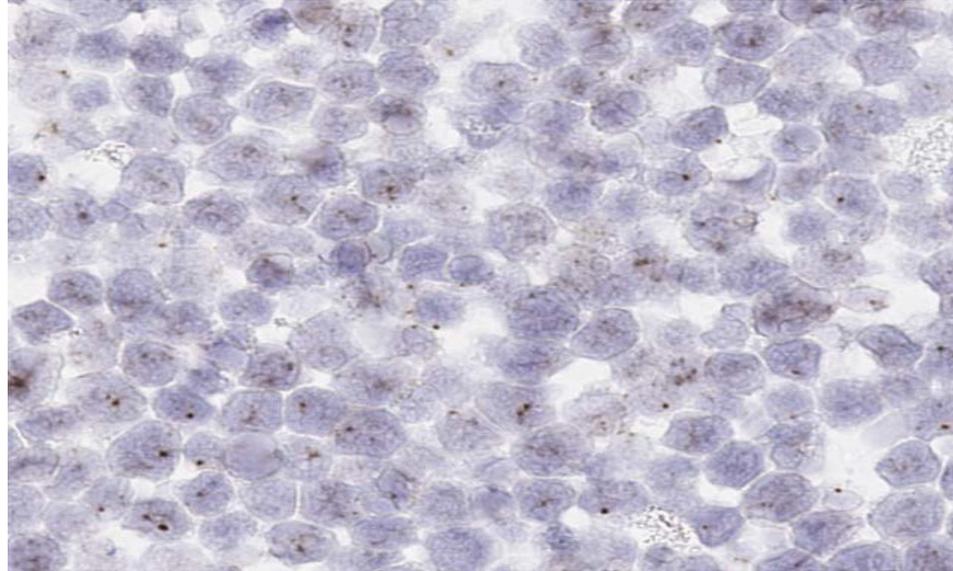
Result: Clean background

TROUBLESHOOTING: ASSAY WORKFLOW

HIGH BACKGROUND/DRYING

FFPE HELA PELLET

DapB



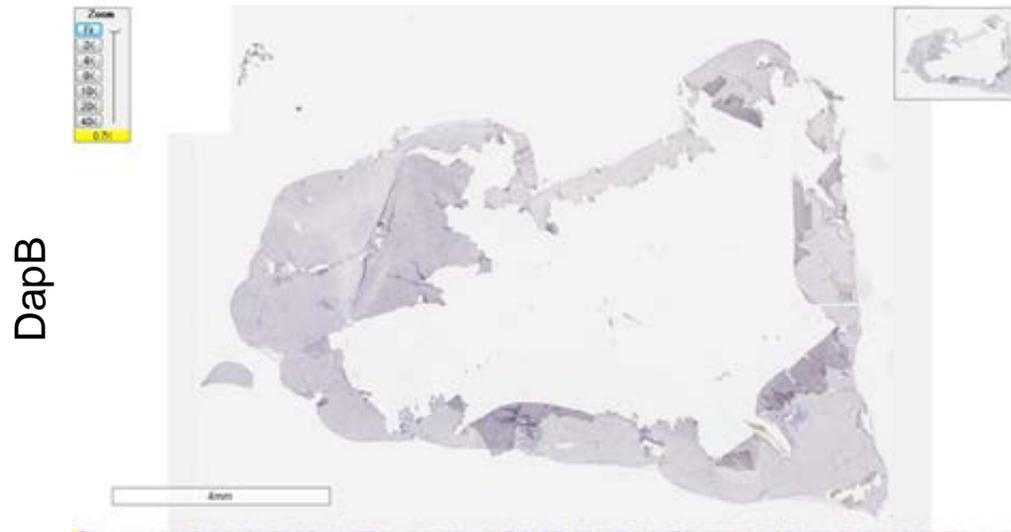
15 min Pretreat 2 , 30 min Pretreat 3

BACKGROUND TYPE	PROBABLE CAUSE	SUGGESTED ACTION
Cytoplasmic and nuclear	<ul style="list-style-type: none"> •Samples drying between amplification steps 	<ul style="list-style-type: none"> •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	<ul style="list-style-type: none"> •Incomplete paraffin removal •Suboptimal tissue preparation 	<ul style="list-style-type: none"> •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**

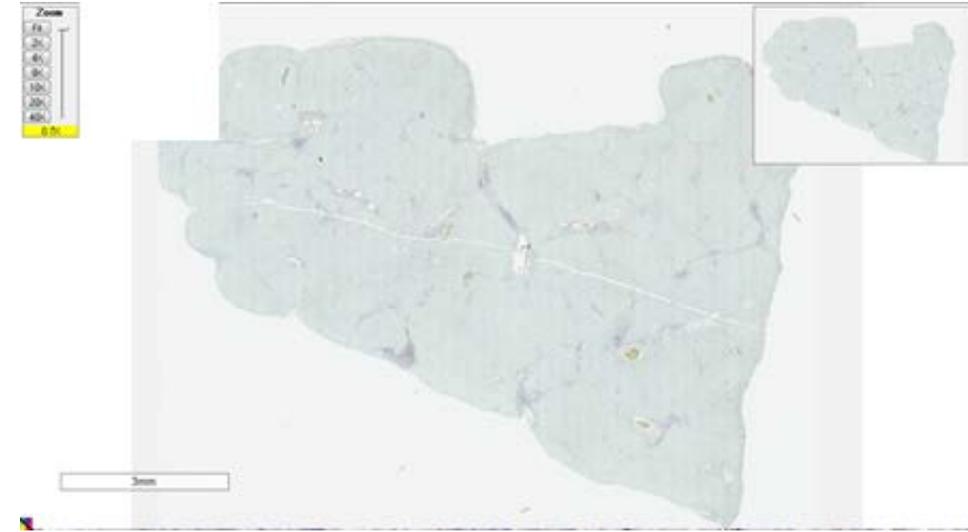
TROUBLESHOOTING: SAMPLE PREPARATION

SAMPLE FALLING OFF



Standard baking protocol

XENOGRAFT FFPE TISSUE



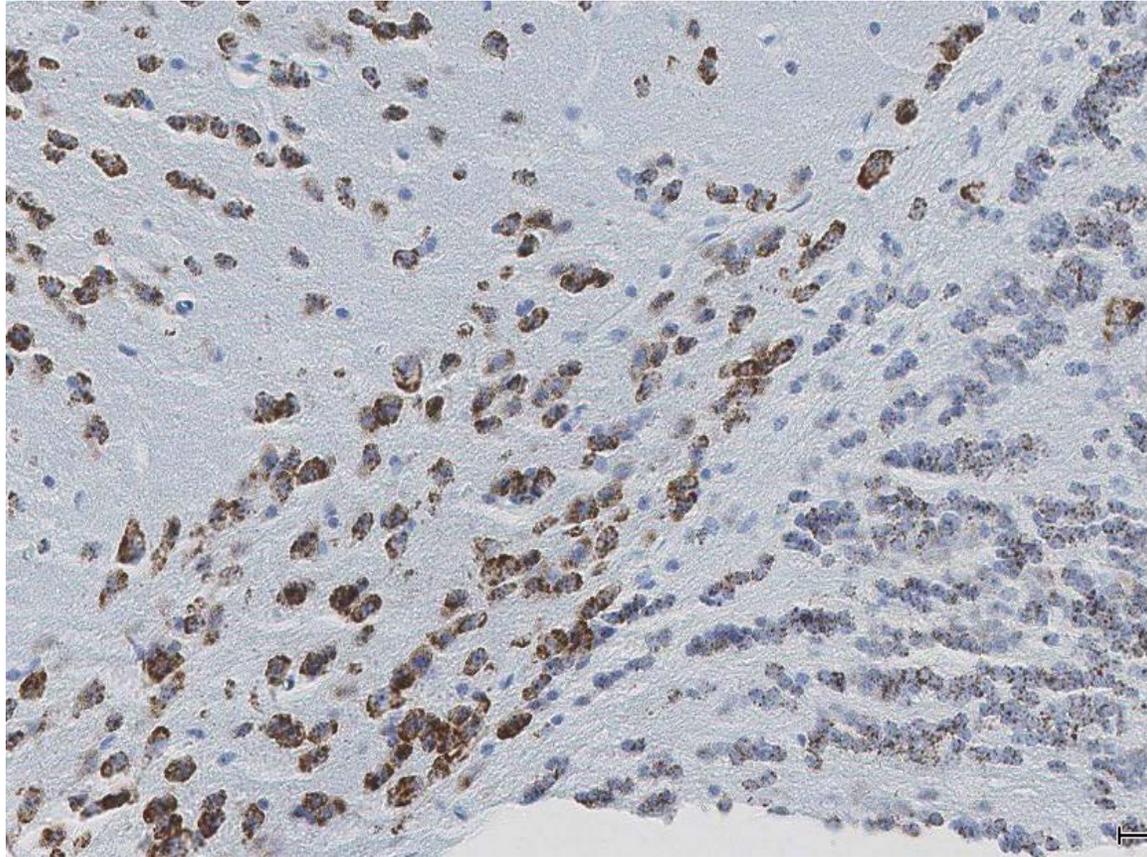
Increased baking by 1 hour

ISSUE	PROBABLE CAUSE	SUGGESTED ACTION
Tissue detaches from slides	•Wrong slides used	•Use only SuperFrost® Plus slides
	•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

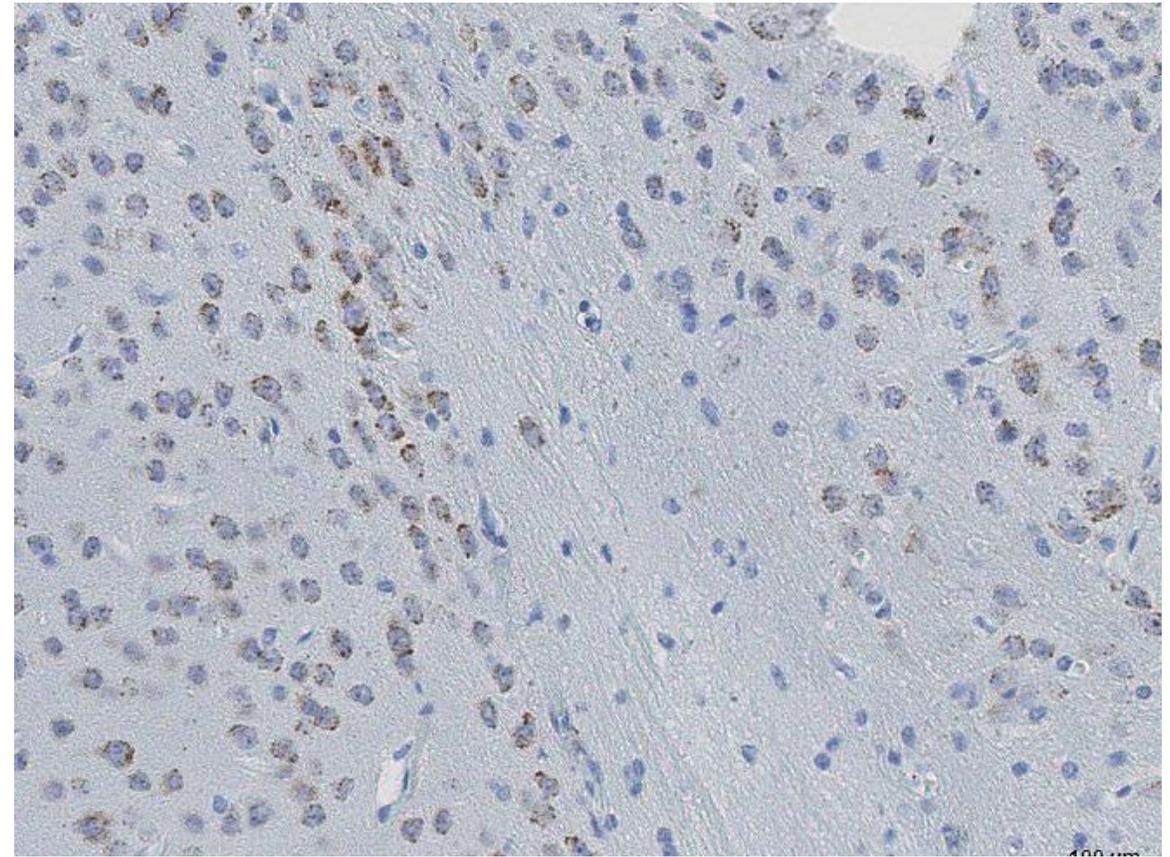
24 hours fixation/**Optimal**



Sample: FFPE brain sample

3 weeks fixation/**Over fixed**

Synaptophysin



Assay: RNAscope 2.0 HD Brown

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

TROUBLESHOOTING: OTHER ISSUES

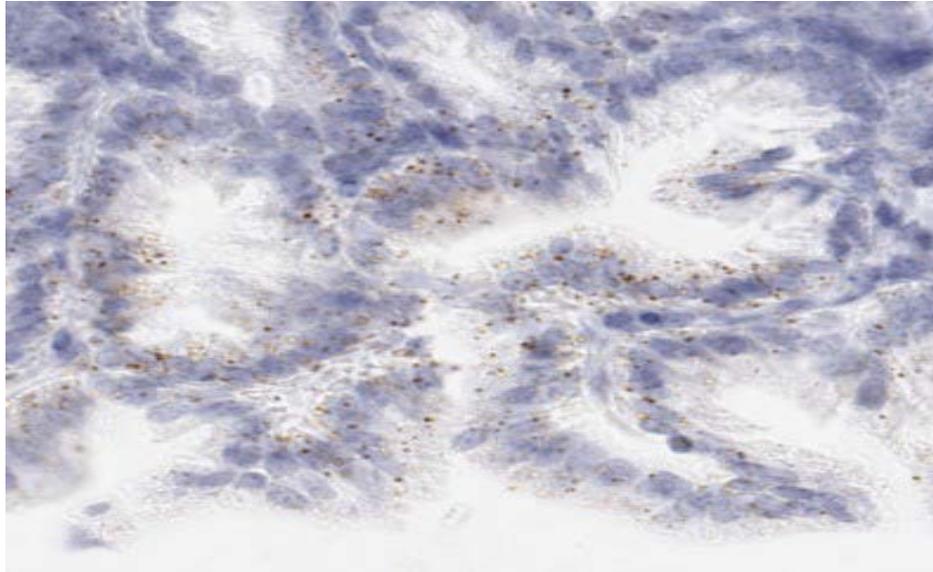
ISSUE	PROBABLE CAUSE	SUGGESTED ACTION
Unknown tissue preparation method	<ul style="list-style-type: none"> •Sample provider/clinical site/vendor did not provide detailed instructions 	<ul style="list-style-type: none"> •Follow the appropriate Tissue Specimen Preparation and Assay Optimization Guidelines/Technotes*
		<ul style="list-style-type: none"> •Start with standard conditions •Optimize your assay
Diffused Signal (RED)	<ul style="list-style-type: none"> •Sample not completely dried •Alcohol used to dehydrate sample •Too much Ecomount mounting medium used 	<ul style="list-style-type: none"> •Dry sample as recommended (prolonged drying i.e. overnight, may be required) •Do not dehydrate samples, dry at 60°C, 15 min •Use Ecomount sparingly and as recommended

TIP: Applies to all samples used with RNAscope®



TROUBLESHOOTING: UNDER FIXATION

Positive control, Rn PPIB



Sample: Flash Frozen followed by FFPE sample preparation (fixation), Rat intestines

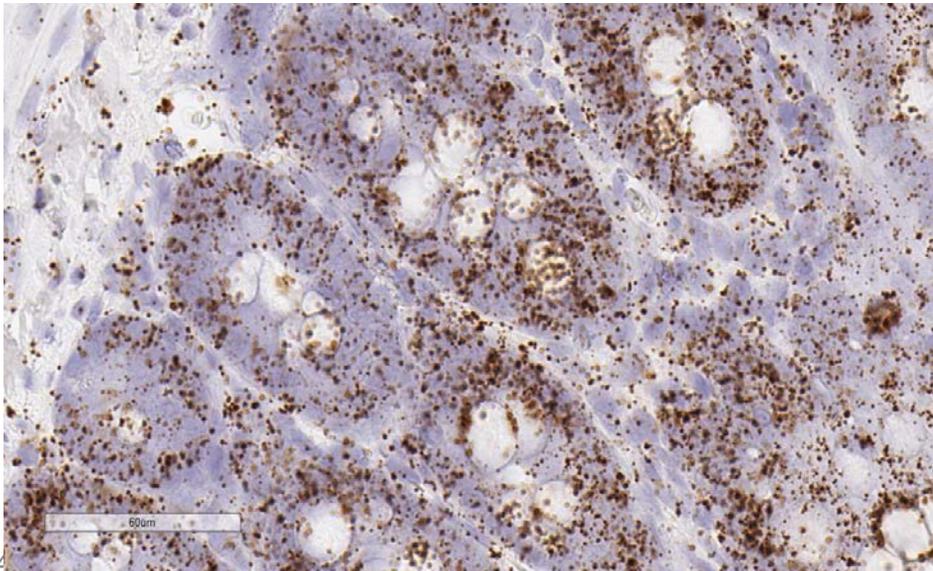
Assay: RNAscope 2.0 HD Brown

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

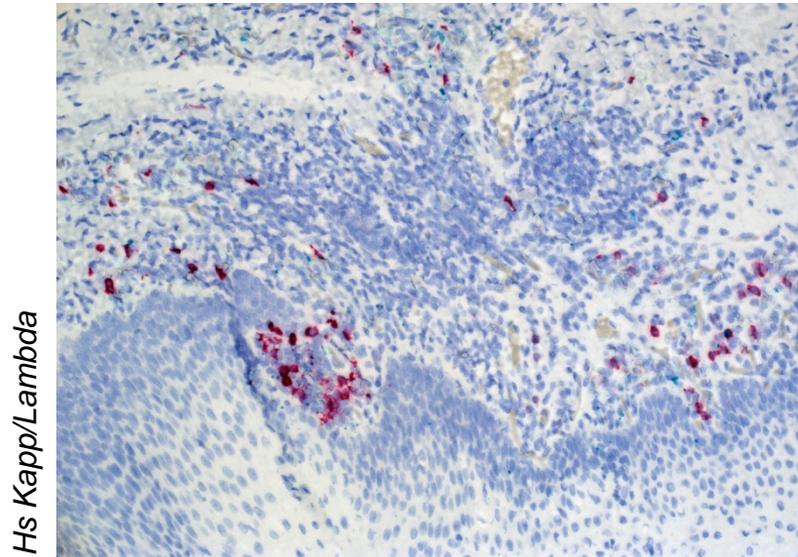
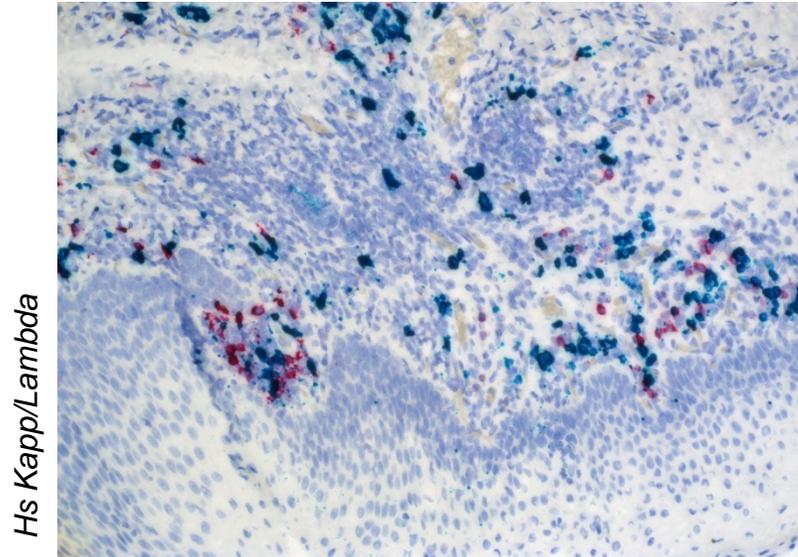
Positive control, Rn PPIB



TIP : Refer to the Troubleshooting Guide

<http://www.acdbio.com/technical-support/downloads/rnascope-ish-guide-troubleshooting/>

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

RNASCOPE® PRETREATMENT GUIDE: MANUAL ASSAYS

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 **PRETREAT 2** and/or **PRETREAT 3 TIME** based on your tissue type (see Table 2).
NOTE: Certain Xenografts and Cell Pellets, require very mild pretreatment (**PRETREAT 2** for 8 min, **PRETREAT 3** for 15 min).

Table 1. Tissue Pretreatment Guidelines

Reagent	Mild	Standard	Extended
Pretreat 2	15 min	15 min	30 min
Pretreat 3	15 min	30 min	30 min

Table 2. Tissue Pretreatment Table

Species	Tissue type	Pathology	Pretreat Condition	Species	Tissue type	Pathology	Pretreat Condition
Mouse / Rat	Intestine	Normal	Standard	Human	Cervical	Normal	Standard
	Intestine	Tumor	Standard		Cervical dysplasia	Abnormal	Standard
	Embryo	Normal	Standard		Brain	Tumor	Standard
	Brain	Normal	Standard		Brain	Normal	Standard
	Spleen	Normal	Mild		Head	Cancer	Standard
	Eye/Retina	Normal	Standard		Neck	Cancer	Standard
	Liver	Normal	Extended		Liver	Cancer	Standard
	Kidney	Normal	Standard		Kidney	Normal	Standard
Human	Breast	Tumor	Standard		Skin	Normal	Standard
	Colon	Tumor	Standard		Melanoma	Tumor	Standard
	Colon	Normal	Standard		Nevus	Benign	Standard
	Lung	Tumor	Standard		Placenta	Normal	Standard
	Lung	Normal	Standard		Skin (TMA*)	Normal	Standard
	Prostate	Tumor	Standard		Breast (TMA)	Normal	Standard
	Prostate	Normal	Standard		Melanoma (TMA)	Normal	Standard
	Lymph node	Tumor	Mild		Nevus (TMA)	Benign	Standard
	Lymph node	Normal	Mild	Stomach (TMA)	Normal	Standard	
	Tonsil	Normal	Mild	Stomach (TMA)	Tumor	Standard	
	Pancreas	Normal	Standard	Cell pellets**	—	Mild	
	Cervical	Cancer	Standard	HeLa cells† (ACD control)	—	Standard	

* Tissue Microarray
 ** Fixed with 10% NBF
 † Fixed with 10% Formaldehyde/PBS

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



RNASCOPE® PRETREATMENT GUIDE: VENTANA DISCOVERY® ULTRA SYSTEMS



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 2&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

Reagent	Mild	Standard	Extended
Pretreatment 2/3	8/8 min	12/12 min	12/12 min
Boiling time (CC)	7 min	15 min	30 min

Table 2. Tissue Pretreatment Table

Species	Tissue type	Pathology	Pretreat Condition	Species	Tissue type	Pathology	Pretreat Condition
Mouse / Rat	Intestine	Normal	Standard	Human	Head and Neck	Normal	Standard
	Intestine	Tumor	Standard		Cervical dysplasia	Abnormal	Standard
	Embryo	Normal	Standard		Brain and spinal cord	Tumor	Standard
	Brain	Normal	Standard		Muscle	Normal	Extended
	Spleen	Normal	Mild		Liver	Cancer	Extended
	Testis	Normal	Mild		Thymus	Cancer	Standard
	Liver	Normal	Extended		Heart	Cancer	Standard
	Kidney	Normal	Extended		Kidney	Normal	Standard
Human	Breast	Tumor	Standard		Skin	Normal	Standard
	Colon	Tumor	Standard		Melanoma	Tumor	Standard
	GI tract	Normal	Standard		Nevus	Benign	Standard
	Lung	Tumor	Standard		Placenta	Normal	Standard
	Lung	Normal	Standard		Skin (TMA*)	Normal	Standard
	Prostate	Tumor	Standard		Breast (TMA)	Normal	Standard
	Prostate	Normal	Standard		Melanoma (TMA)	Normal	Standard
	Lymph node	Tumor	Mild		Nevus (TMA)	Benign	Standard
	Lymph node	Normal	Mild	Stomach (TMA)	Normal	Standard	
	Tonsil	Normal	Mild	Stomach (TMA)	Tumor	Standard	
Pancreas	Normal	Standard	Cell pellets**	—	Mild		
Cervical	Cancer	Standard	HeLa cells† (ACD control)	—	Standard		

* Tissue Microarray
 ** Fixed with 10% NBF
 † Fixed with 10% Formaldehyde/PBS

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

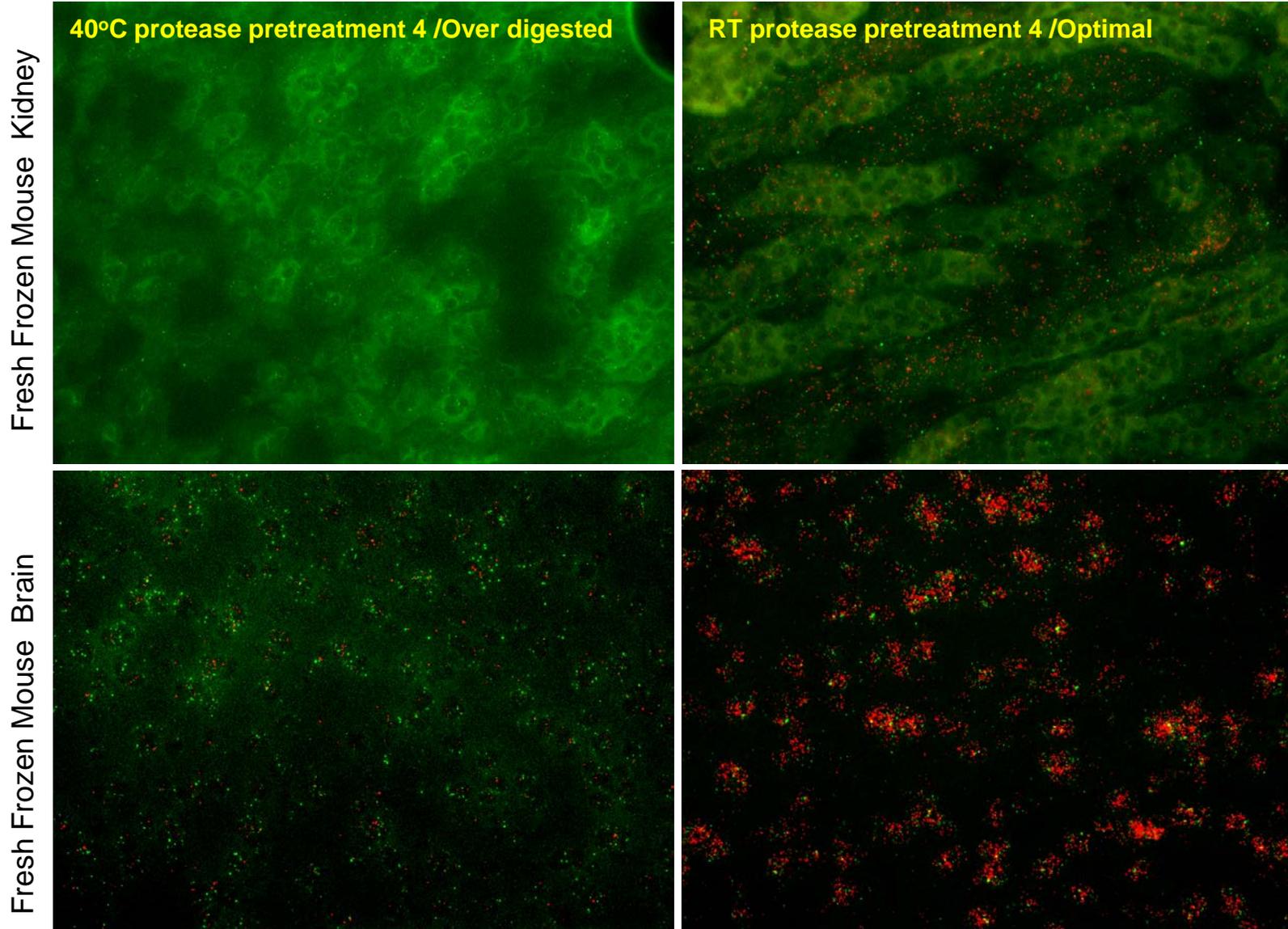


TIP : Refer to the user manual for tissue specific pretreatment guidelines



TROUBLESHOOTING TIPS MULTIPLEX FLUORESCENT ASSAY

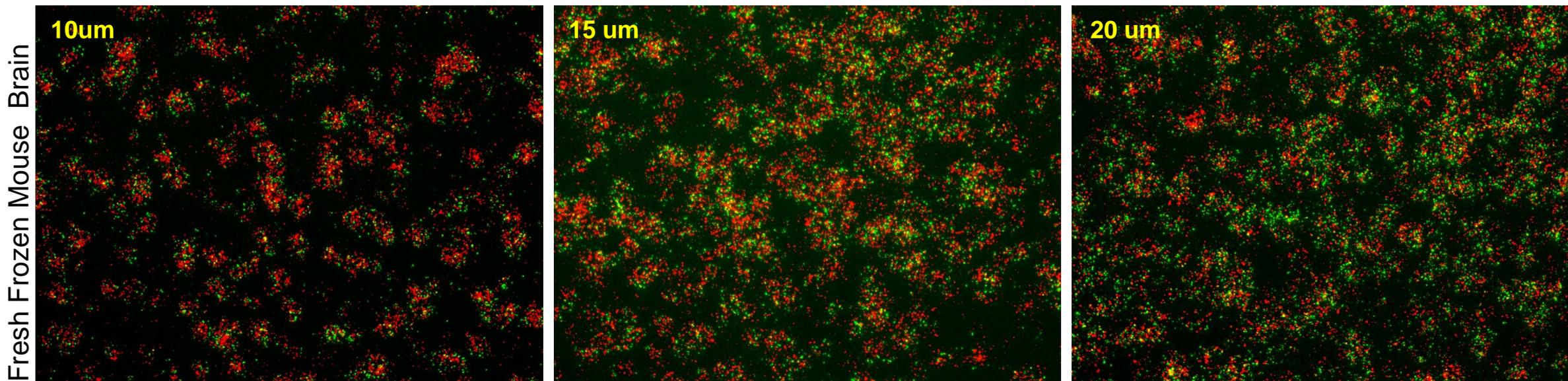
TROUBLESHOOTING: SAMPLE DIGESTION



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

TROUBLESHOOTING: SAMPLE DIGESTION

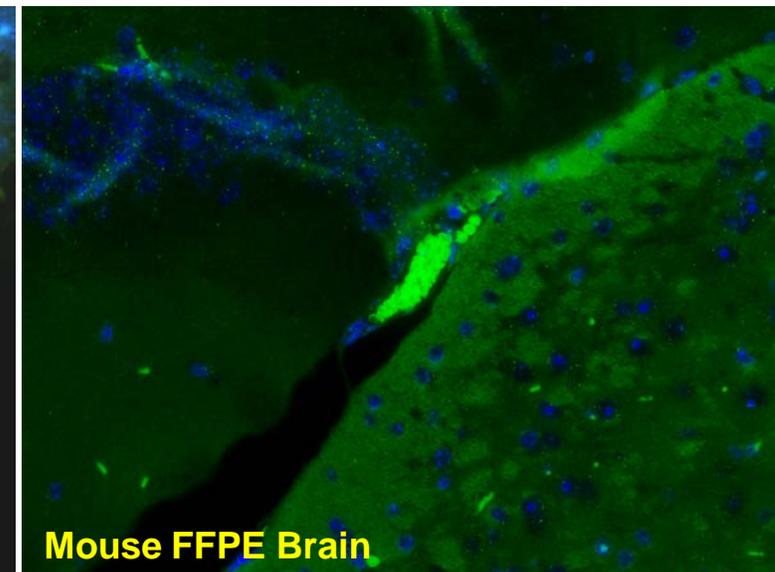
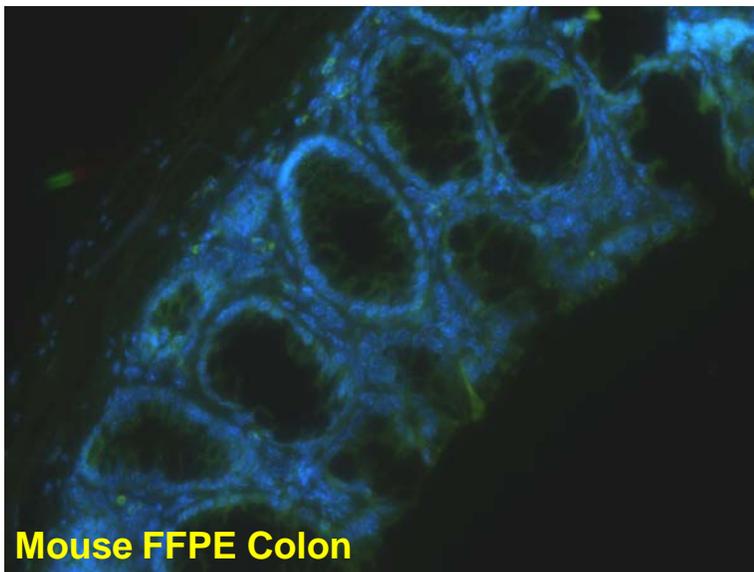
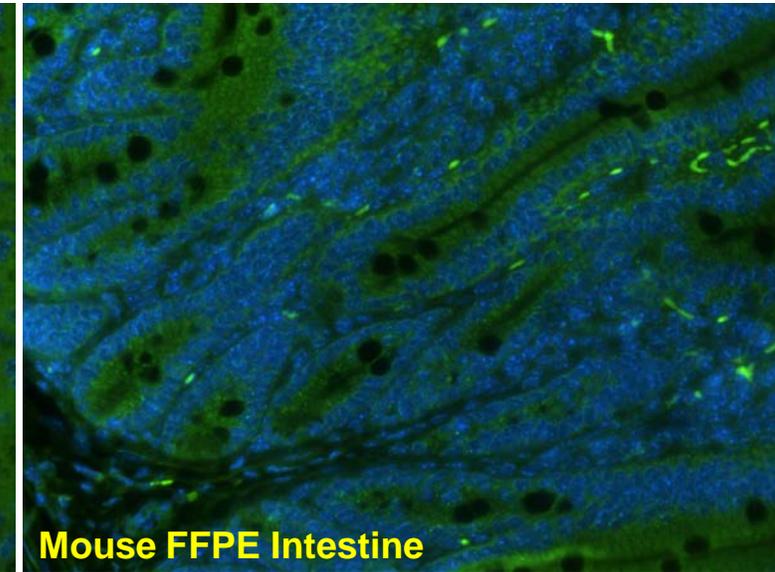
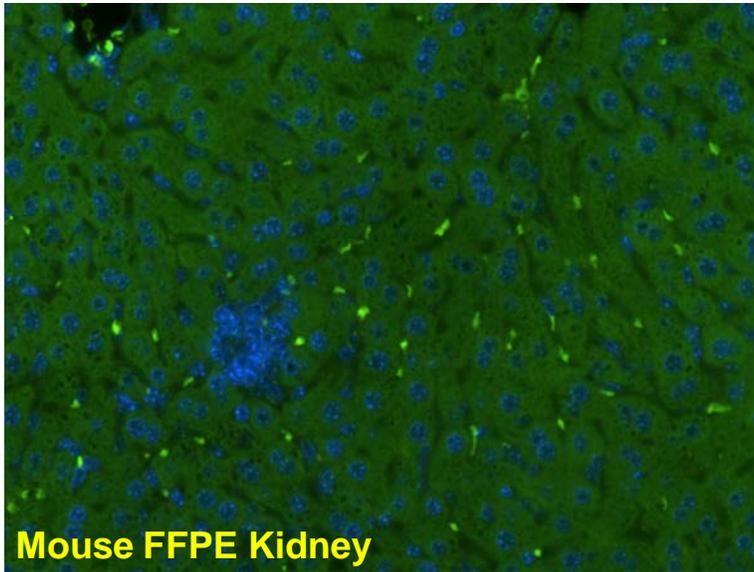
2-plex Mouse Positive Control Probe Mm **POLR2A**/**PPIB**



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

TIP: Sample thickness can affect signal in your samples
Solution: Use recommended sample thickness, 10-20um

TROUBLE SHOOTING AUTOFLUORESCENCE



TIP: FFPE sample have inherent autofluorescence

Solution: Use appropriate background correction software to reduce autofluorescence

MULTIPLEX FLUORESCENT ASSAY 101—PROBLEMS AND SOLUTIONS

SOURCE	ISSUE	PROBLEM	SOLUTION
Microscopy	No/weak signal Nonspecific signal	<ol style="list-style-type: none">1. Wrong filter setting/longer emission cut off2. Wrong exposure3. Inappropriate imaging enhancing with software	<ol style="list-style-type: none">1. Use correct filter settings2. Do not use using autoexposure at first, verify signal with naked eye3. Use known image enhancing software e.g. Nuance
Sample	No/weak signal	<ol style="list-style-type: none">1. Compromised RNA quality2. Sample preparation (high autofluorescence background on the sample)	<ol style="list-style-type: none">1. Use new sample with good RNA quality2. Follow the pretreatment guideline recommended3. Always perform assay with 3-plex positive control and 3plex negative probes to assess RNA quality4. Always check signal with naked eye under objective lens first

MULTIPLEX FLUORESCENT ASSAY 101—TIPS AND TRICKS

- 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

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

SUMMARY

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

The screenshot shows the top navigation bar of the ACD website. The ACD logo is on the left, followed by the tagline "Provider of the most sensitive and specific RNA in situ hybridization technology". To the right are buttons for "Contact Us" and "Quote Builder", and a search bar labeled "Search entire website". Below this is a horizontal menu with tabs for "Science", "Applications", "Products", "Biopharma", "Support", and "Company". A red star and arrow point to the "Support" tab. The "Support" dropdown menu is open, listing various resources: "Technical Support Overview", "Training Webinars", "Download Manuals" (with sub-items: "User Manual Selection Guide", "Manuals & MSDS", "Positive & Negative Control Images", "Tissue Requirements & Troubleshooting Tips"), "Getting Started", "Troubleshooting Guide", "Frequently Asked Questions (FAQs)", "Online Training Videos", and "Product Literature" (with sub-items: "Brochures", "Probe Lists", "Publication List", "Application Reviews", "Researcher in the Spotlight", "Scientific Posters"). Below the navigation bar are two large banners: "ACD CANCER APPLICATIONS VISUALIZING THE... ISCRIPTOME" and "HOW IT WORKS" / "WHY RNA?". At the bottom of the page are buttons for "CHOOSE ASSAY" and "DOWNLOAD MANUALS".

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

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QUESTIONS?



PLEASE COMPLETE THE WEBINAR SURVEY, WE VALUE YOUR FEEDBACK



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