RNASCOPE® TROUBLESHOOTING TIPS

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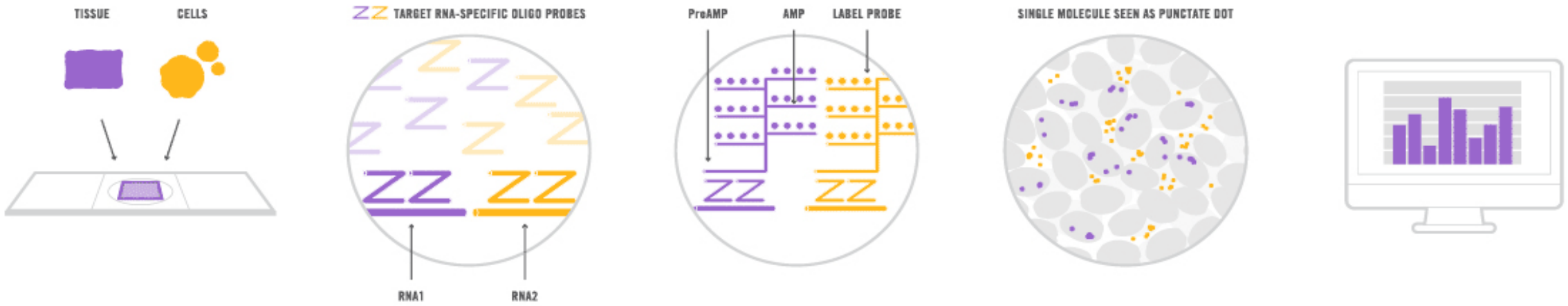
Advanced Cell Diagnostics

TOPICS

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

RNASCOPE® WORKFLOW

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



A BREAKTHROUGH PLATFORM

UNIQUE
Probe Design

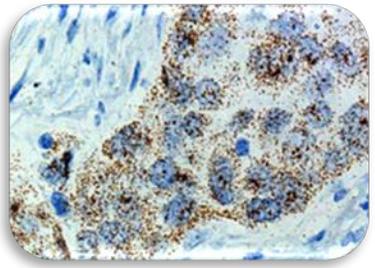
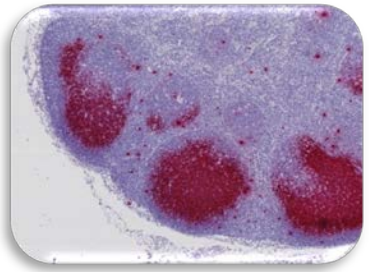
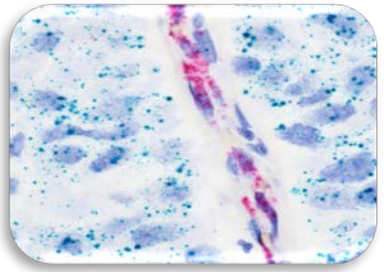
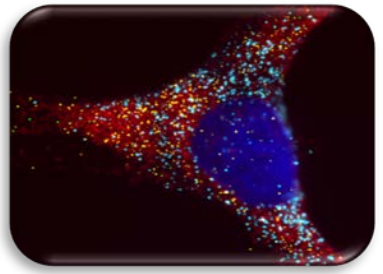
SIGNAL
Amplification + Background Suppression

SINGLE
Molecule Detection in **Single Cells**

ANY
Genome, Gene or Tissue

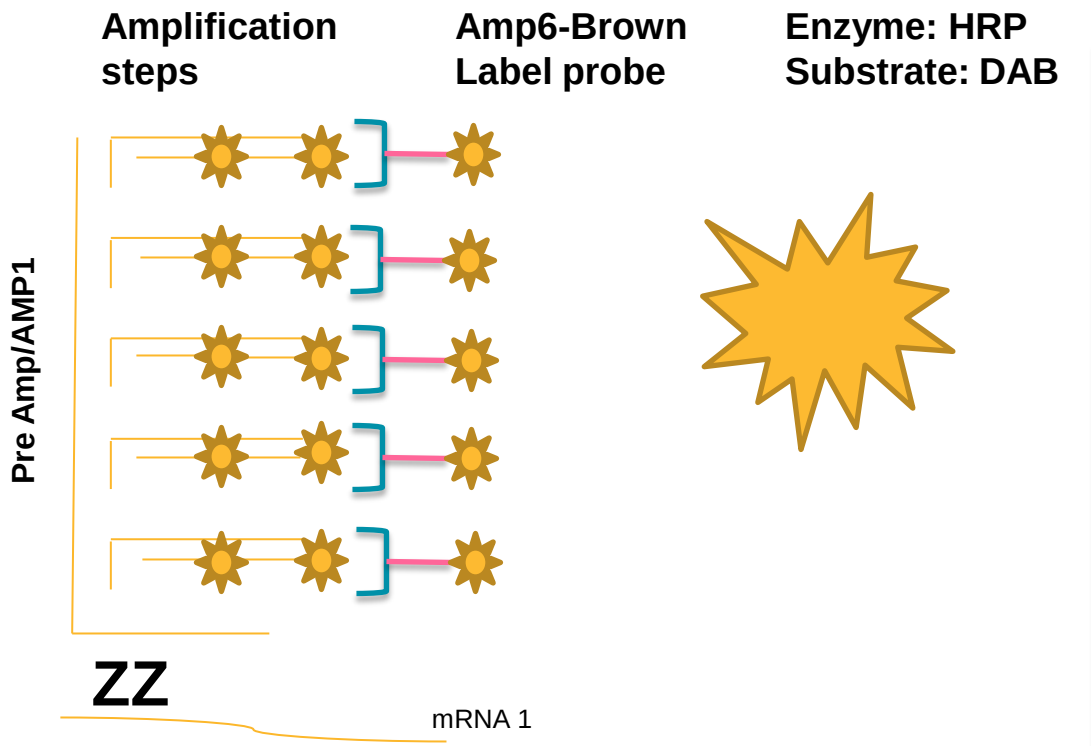
RNASCOPE ® WORKFLOW

RNASCOPE® ASSAY SELECTION

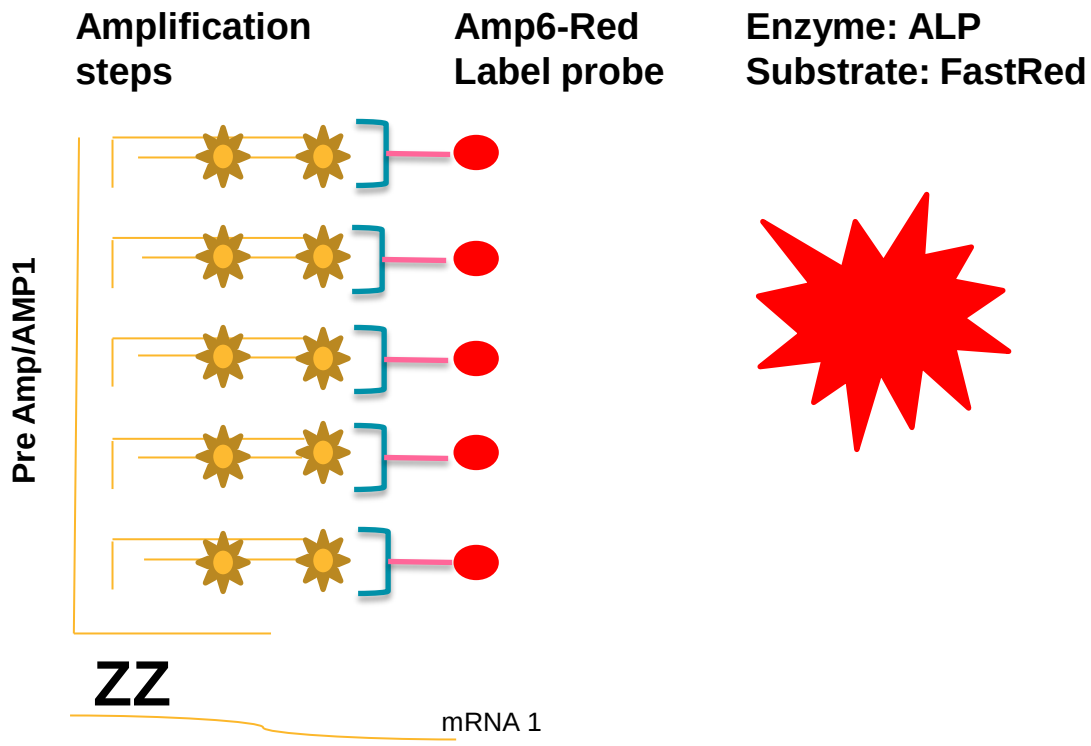
RNAscope Assays	RNAscope 2.0 HD (BROWN) Ventana Systems Leica Bond Rx	RNAscope 2.0 HD (RED) Ventana Systems Leica Bond Rx	RNAscope 2-plex	RNAscope Multiplex – Fluoroscence
Assay type	Chromogenic	Chromogenic	Chromogenic	Fluorescent
Dye used	Diaminobenzene (DAB)-HRP	Fast Red -ALP	HRP-Green, Fast Red -ALP	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

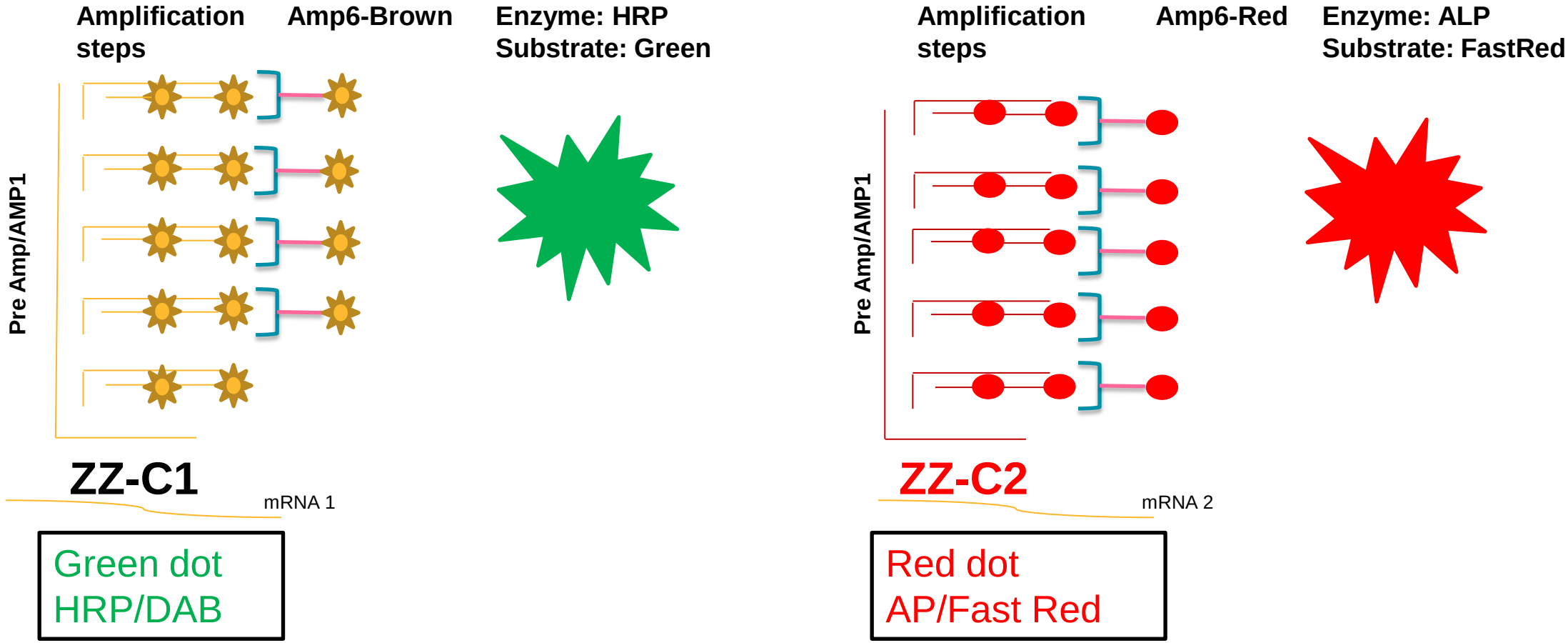


2.0 HD RED

Red dot
AP/Fast Red

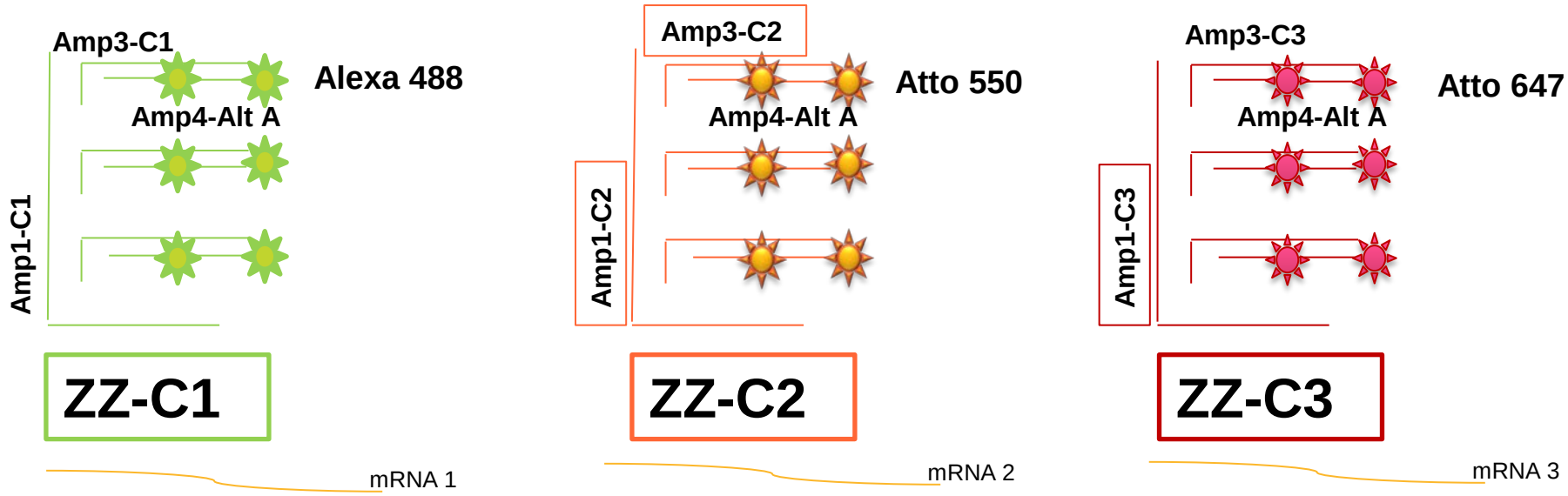
TIP : Do not interchange reagents within Brown/Red assays or across similar 2.0 HD Assays
By default 2.0 HD assays require C1 probes that are ready to use, no further dilution is required

RNASCOPE® 2-PLEX AMPLIFICATION SCHEMATIC



TIP: By default C1 probes are 1X concentration while C2 probes are <u>50X</u>
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

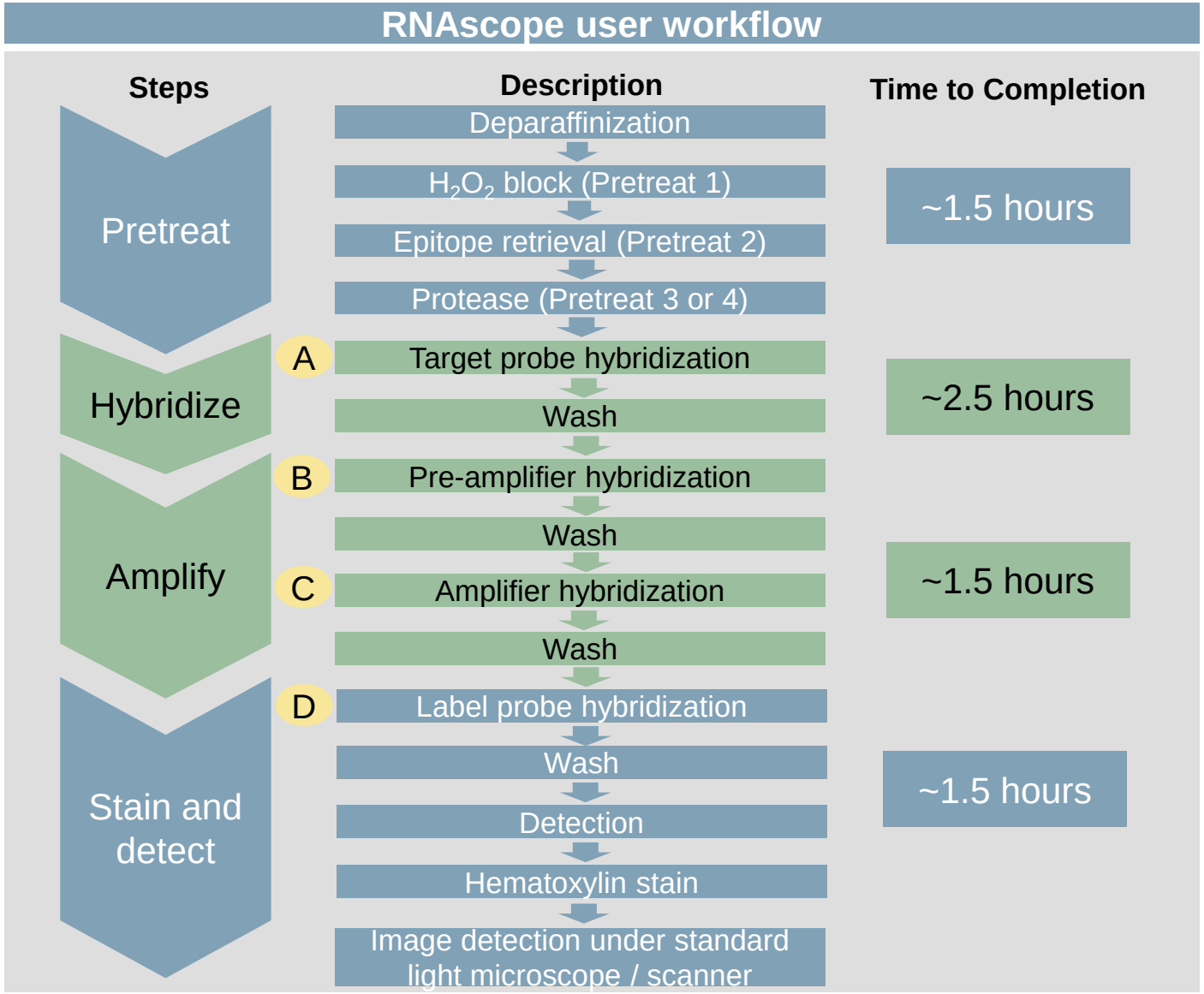
RNASCOPE ® MULTIPLEX FLUORESCENT SCHEMATIC



	Alexa-488	**Atto-550**	**Atto-647**
Ex/Em	**495nm/520nm**	**555nm/575nm**	**645nm/670nm**

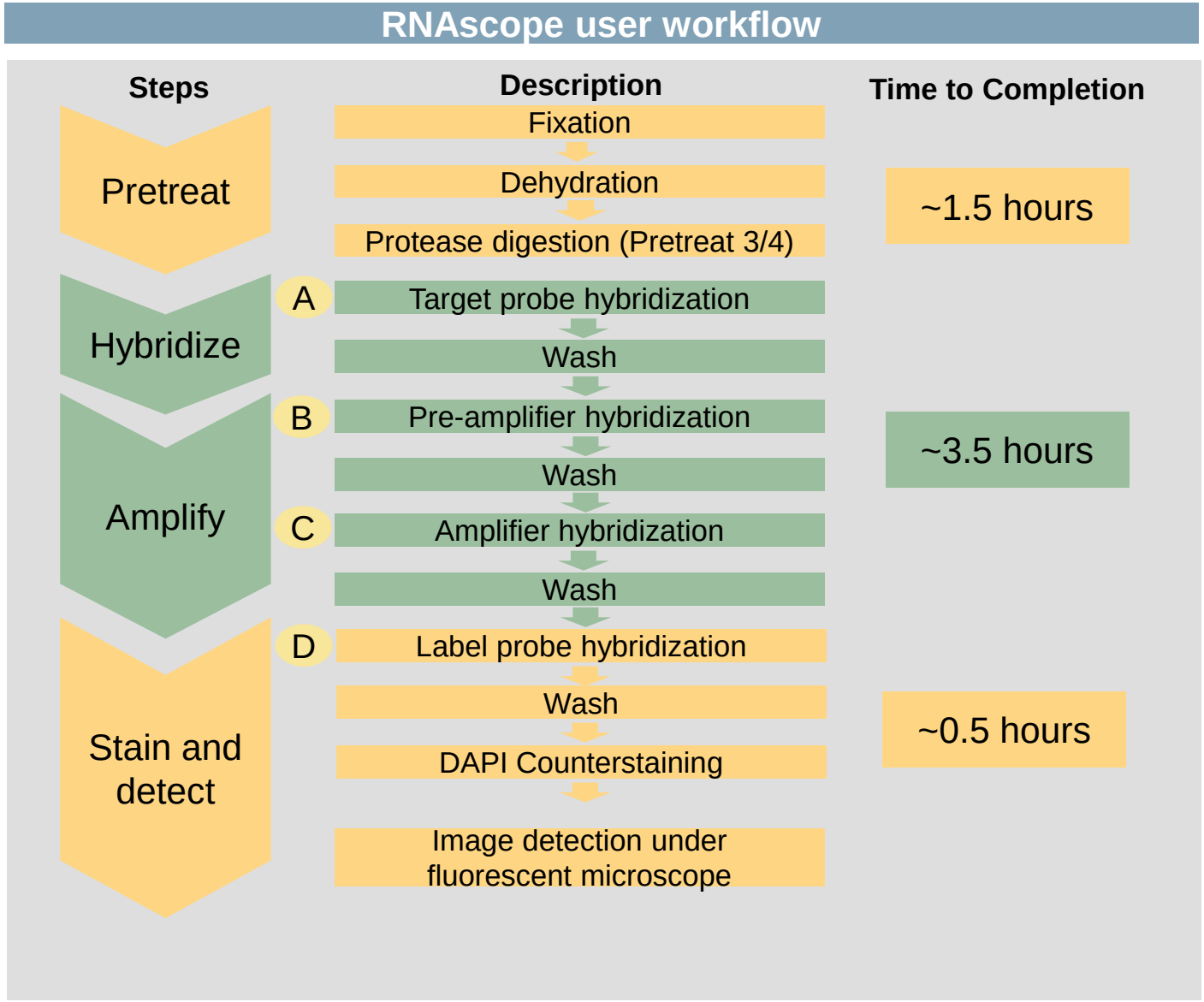
TIP: By default C1 probes are 1X concentration while C2 and C3 probes are <u>50X</u>
To make 3-plex probe mixture at 1X concentration, mix C2 and C3 probes 1:50 with C1 probe
If C2 and C3 are all at 50X concentration, use the "blank-probe-C1" as a diluent and mix at a 1:50 dilution

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

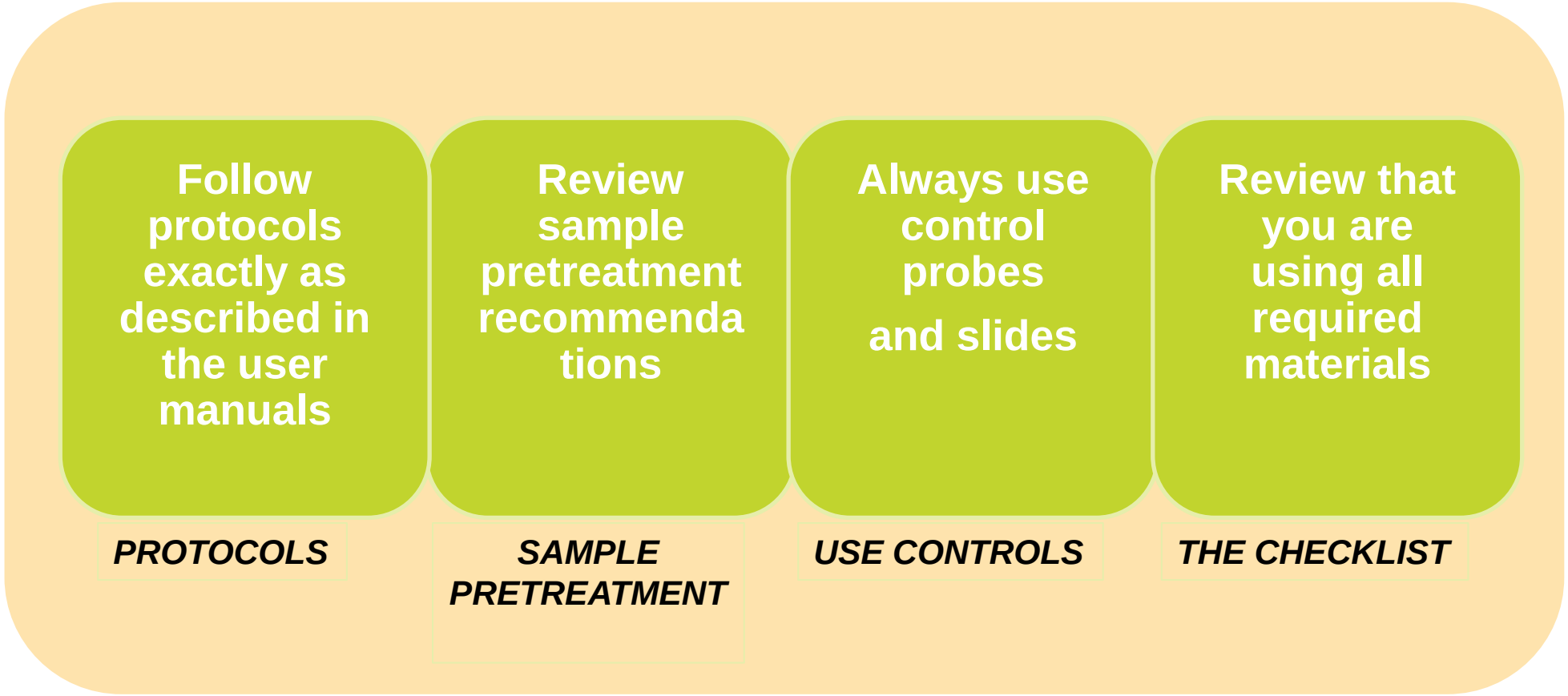
DAY 2

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



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

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

RNASCOPE® REAGENT KIT CONTENTS

OLD

NEW

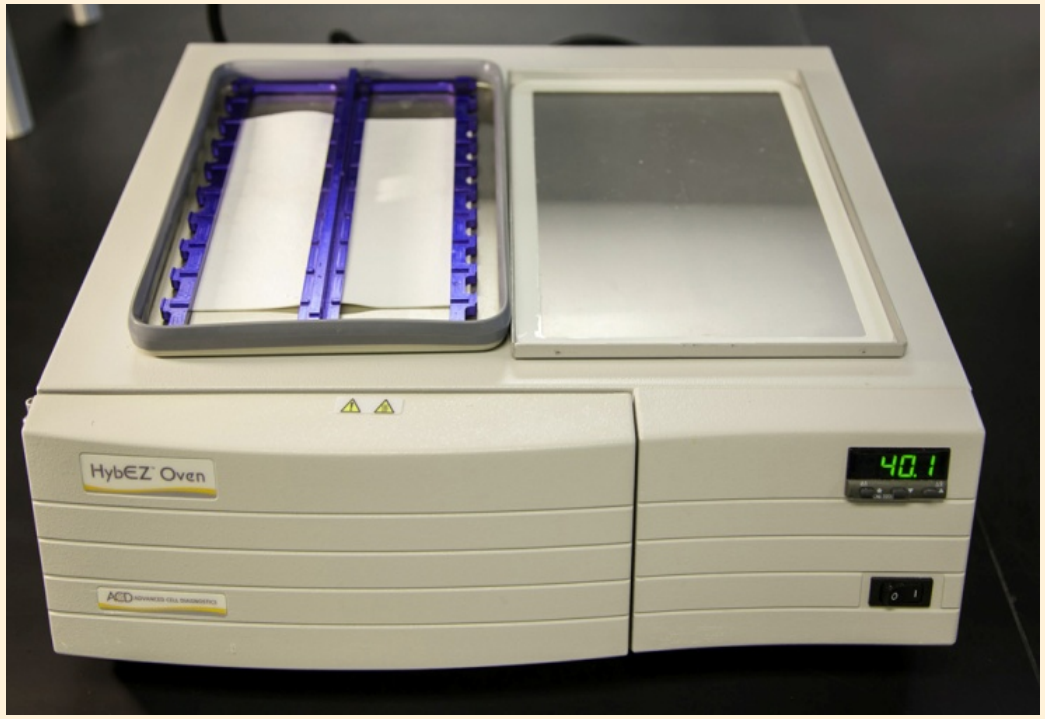
Contents of the reagent kit

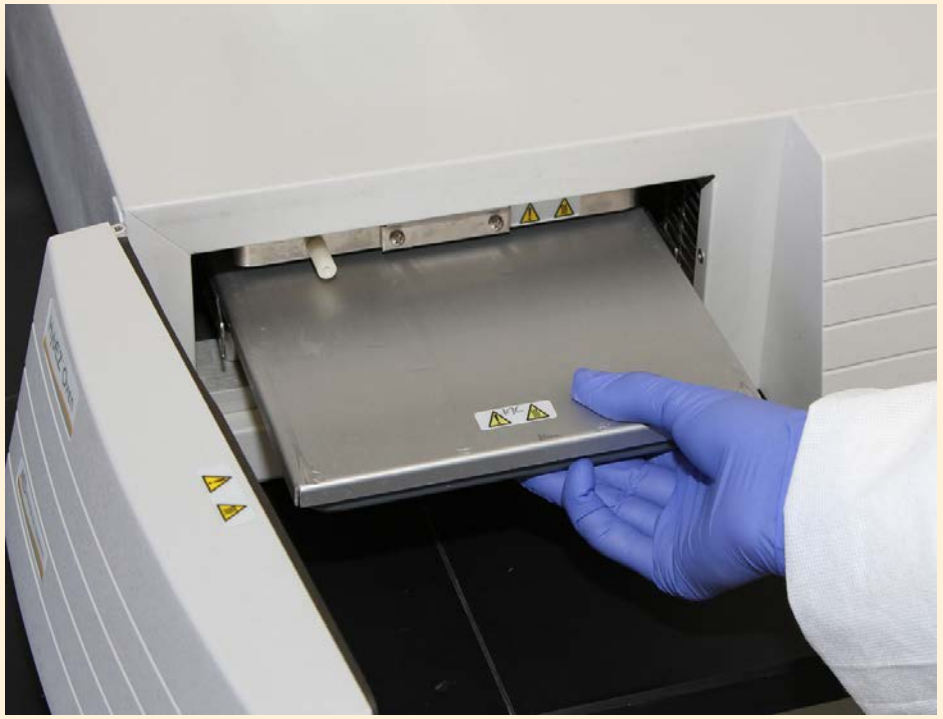
1. Pretreatment reagents
2. RNAscope detection kit
3. Wash buffer

TIP : Warm probes at 40 ºC for 10 minutes before use
TIP :Warm 50x wash buffer at 40 ºC for 20 minutes if you notice a precipitation

HYBEZ HYBRIDIZATION OVEN

☑ ***HyBEZ hybdrization system***



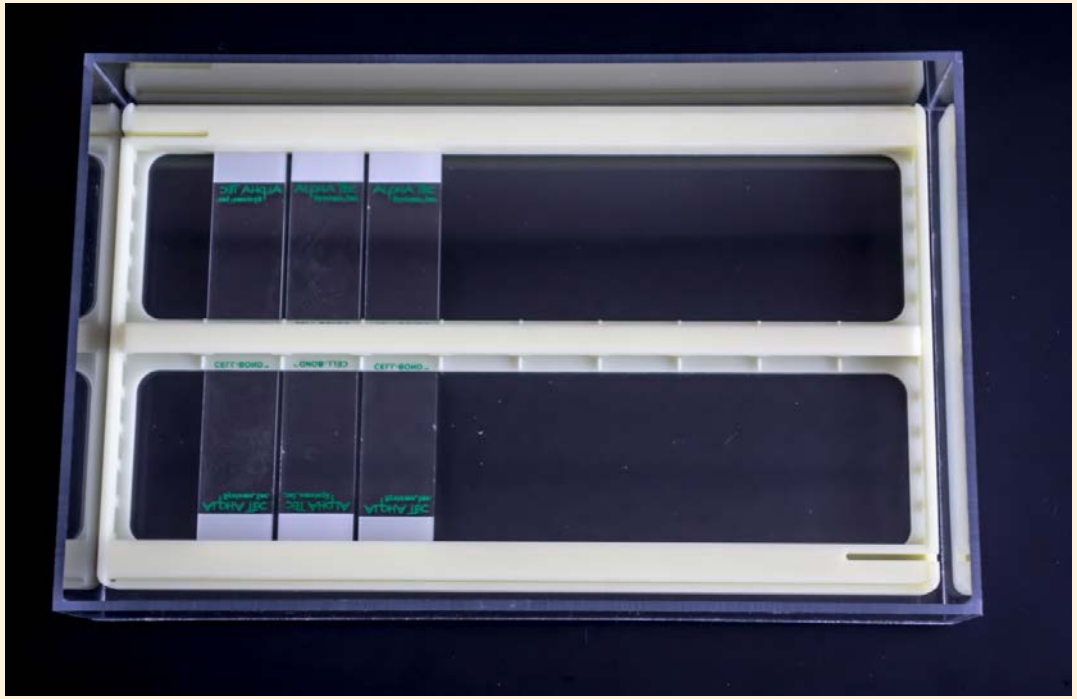


TIP: HybEZ oven is required as it provides both temperature and humidity control, necessary to obtain optimal RNAscope results

ACCESSORIES FOR WASHING STEPS

Tissue Tek washing tray

EZ Batch for slide processing



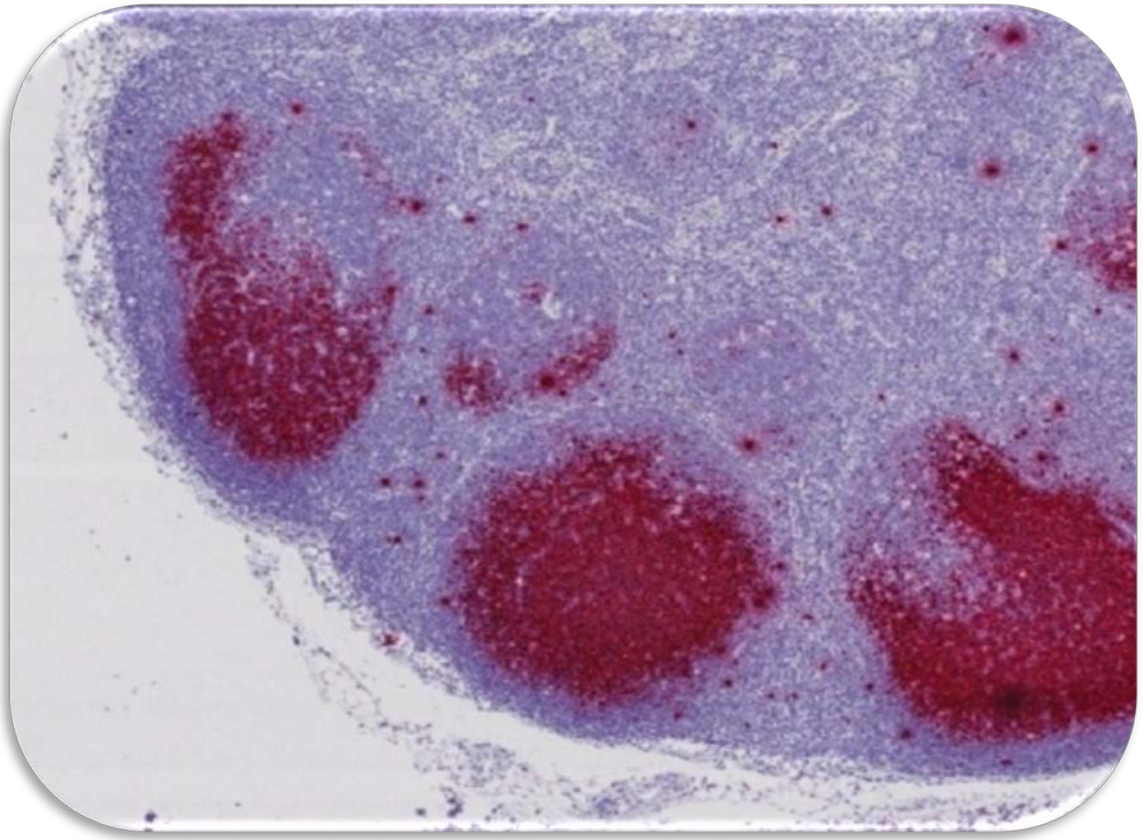
TIP : ACD EZ Batch slide processing tray is easy and convenient for loading multiple slides for hybridization and washing steps.

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

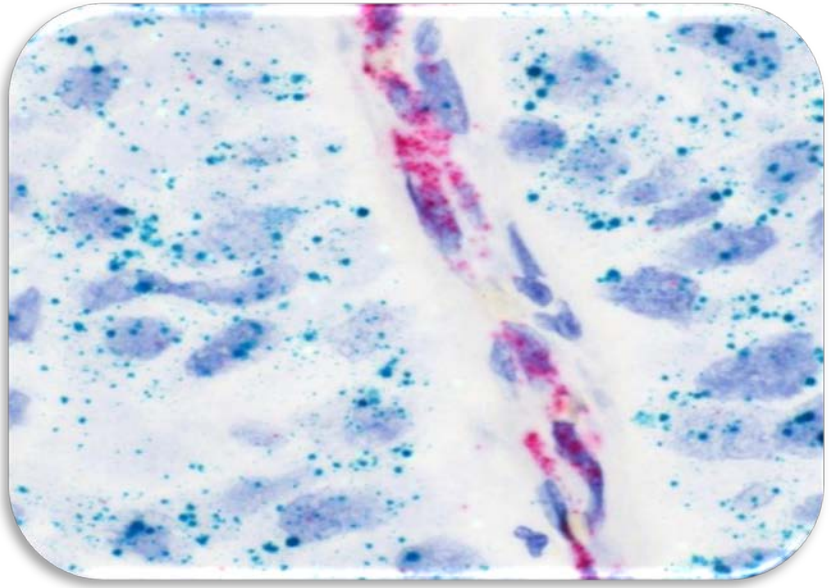
POINTERS FOR RNASCOPE 2.0 RED ASSAY



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

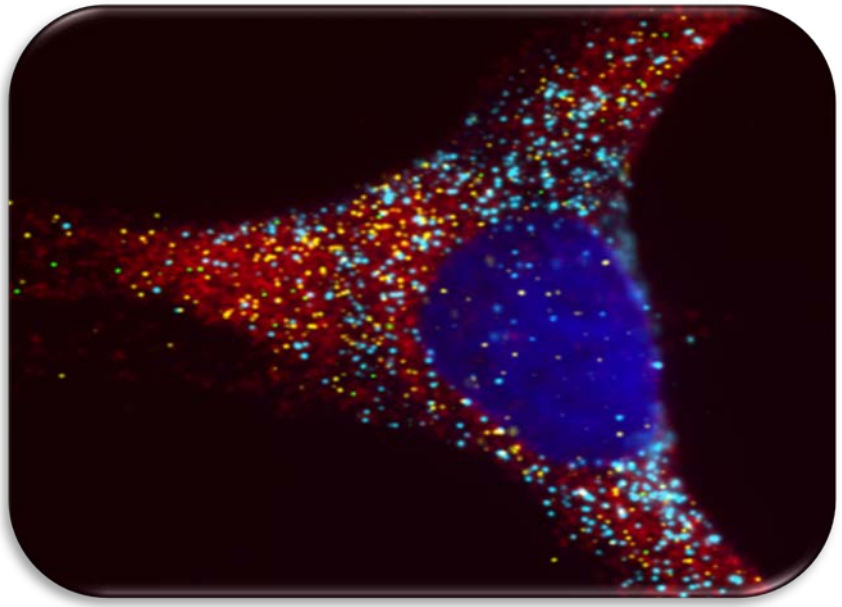


COMPONENTS	MIXING RATIO
Probes C2:C1	1:50
Amp 4B: Amp 4A	1:50
Red-B:Red- A	1:60
Green-B: Green-A	1:50

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



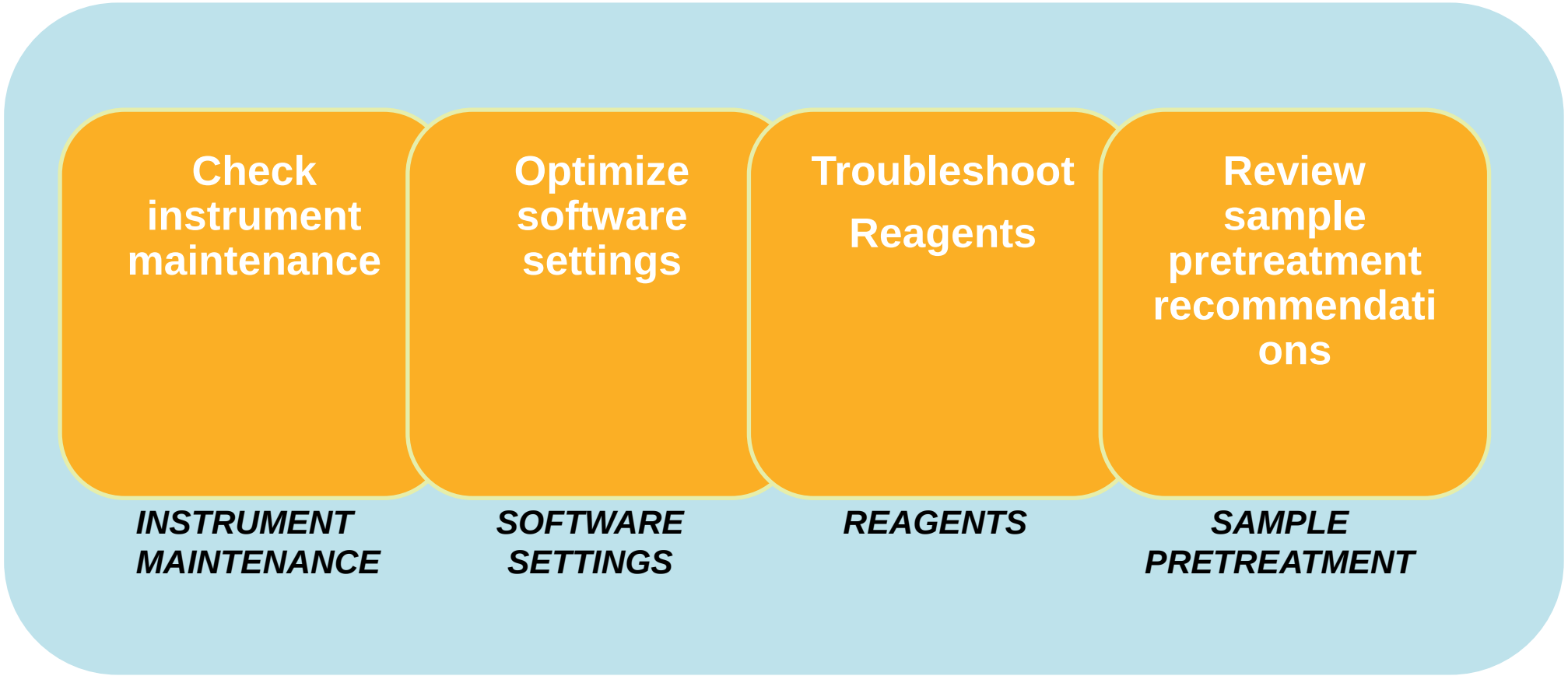
COLOR MODULE OPTIONS

	Channel 1 (C1)	Channel 1 (C2)	Channel 1 (C3)
AMP 4 Alt A	GREEN-Alexa 488	ORANGE-Atto 550	FAR RED-Atto 647
Amp 4Alt 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 <u>50X</u>
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)



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*

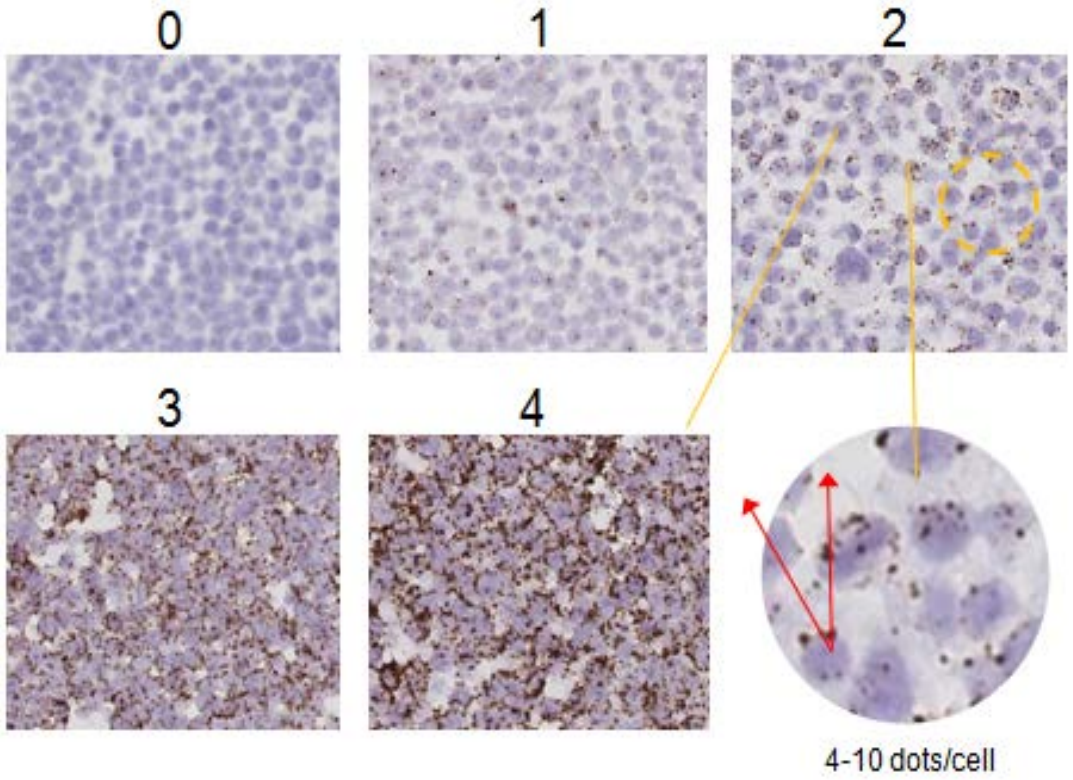
3. *LS Brown and LS Red assays utilize Leica Biosystems' Bond Polymer Refine Detection and Bond Polymer Refine Red Detection kits, respectively*

4. *Do not alter the staining protocol in any way*

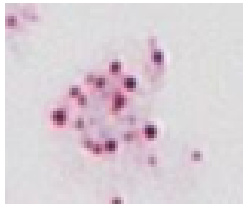
TIP : Visit http://www.acdbio.com/technical-support/downloads/rnascope-ish-guide-troubleshooting/ for more information on tips for LEICA BOND RX® automation assays

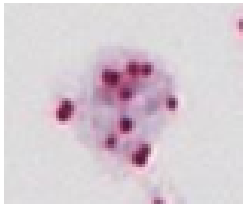
QUALIFY YOUR SAMPLES USING CONTROLS

IMAGE ANALYSIS
RNASCOPE® SCORING GUIDELINE

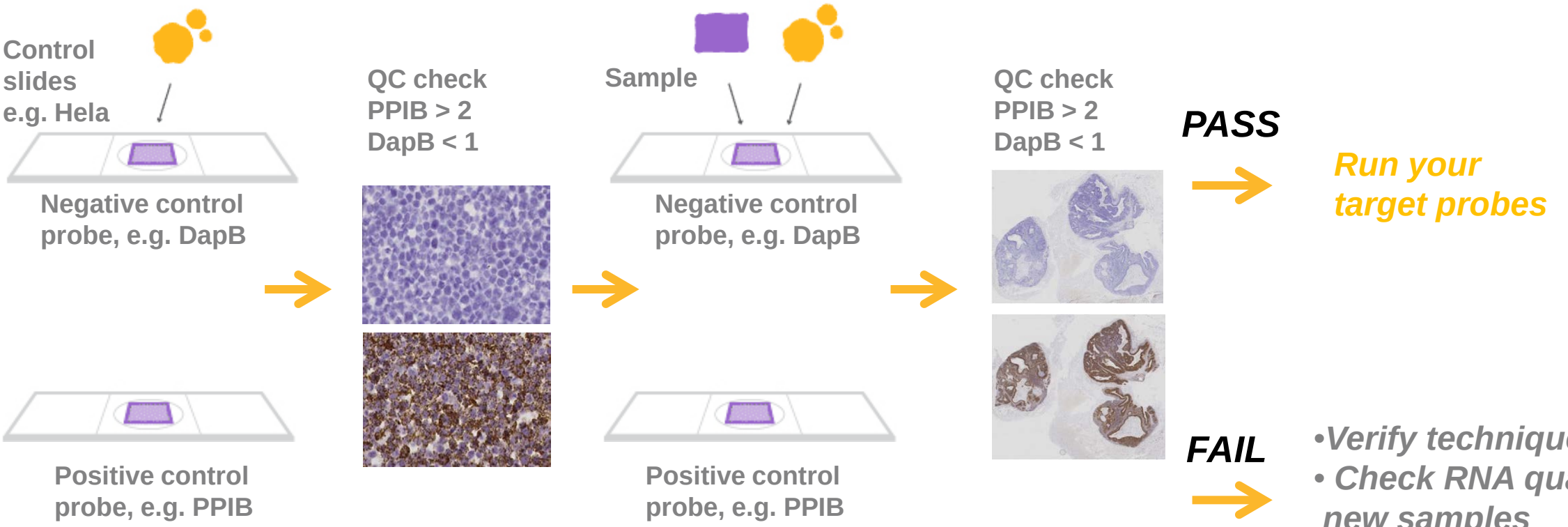


Score	Criteria
0	No staining or <1 dot/ 10 cells*
1	1-3 dots/cell
2	4-9 dots/cell. None or very few dot clusters
3	10-15 dots/cell and <10% dots are in clusters
4	>15 dots/cell and >10% dots are in clusters


Score = 3


Score = 3

QUALIFY YOUR SAMPLES USING CONTROLS

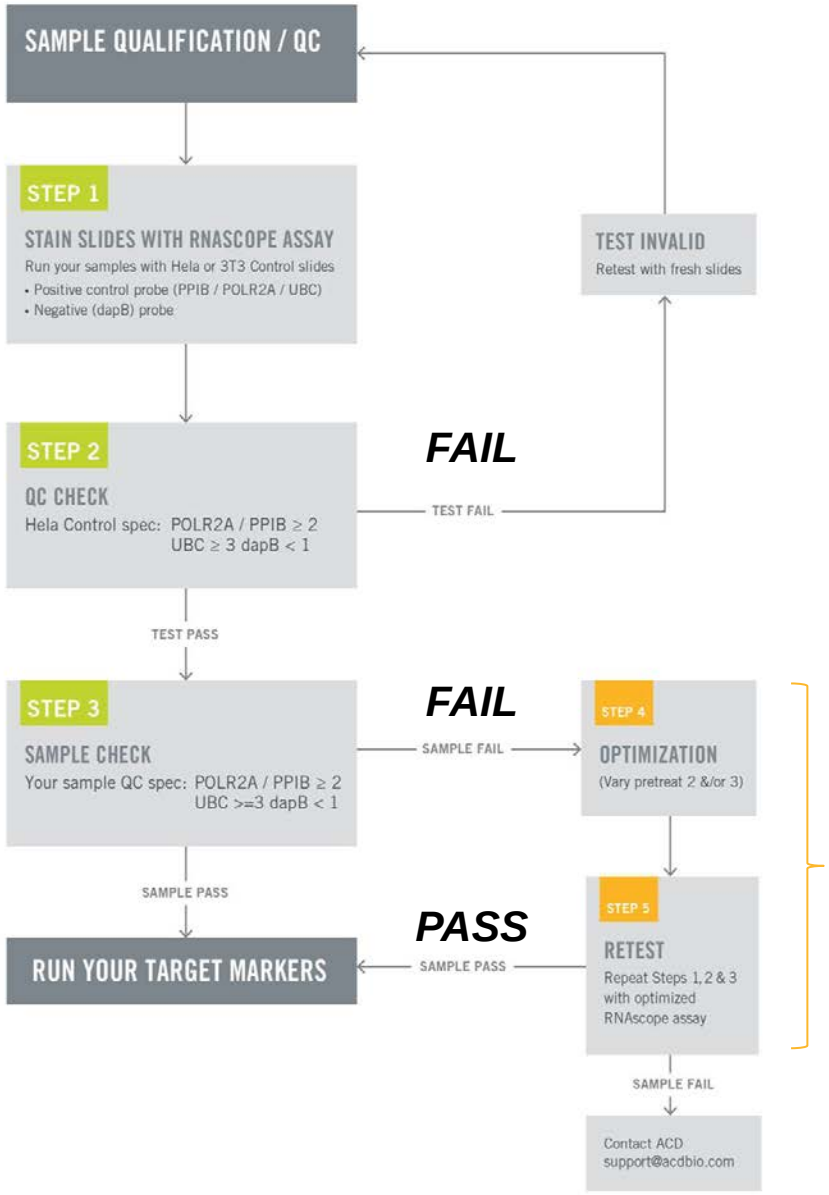


TIP : Always start with standard conditions

OPTIMIZE YOUR ASSAY

Technique check

Sample/ RNA quality check



OPTIMIZE YOUR ASSAY

TIP : Refer to the Troubleshooting Guide

OPTIMIZE YOUR ASSAY

WHY OPTIMIZE YOU 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

☒	Fixation conditions are not optimal
☒	RNA is degraded
☒	Hybridization conditions not optimal
☒	Samples drying during assay
☒	Special tissues sensitive to pretreatment

↓ THE SOLUTIONS

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

NBF: Neutral Buffered Formalin

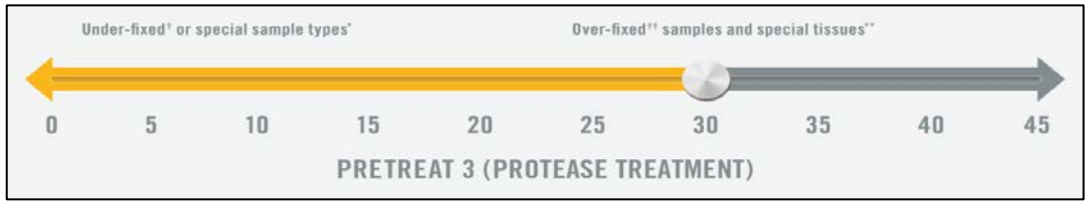
OPTIMIZE YOUR SAMPLE IN 3 EASY STEPS (MANUAL ASSAY)

STEP 1
START WITH STANDARD CONDITIONS

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

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*

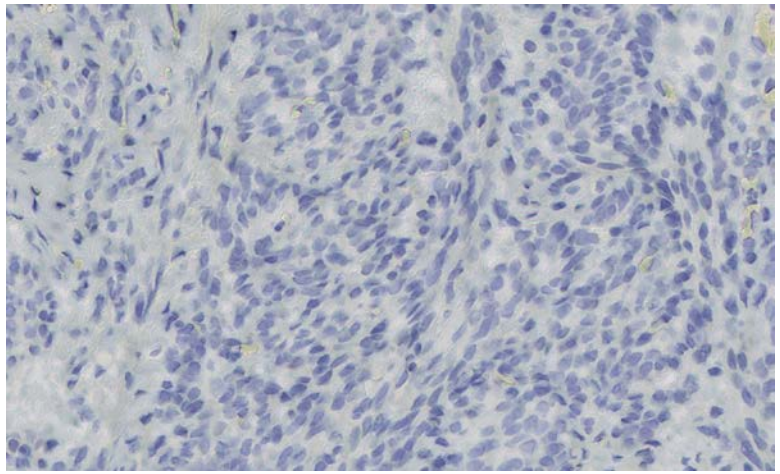
OPTIMIZE YOUR SAMPLE WITH THESE STEPS (AUTOMATED ASSAYS)

	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*

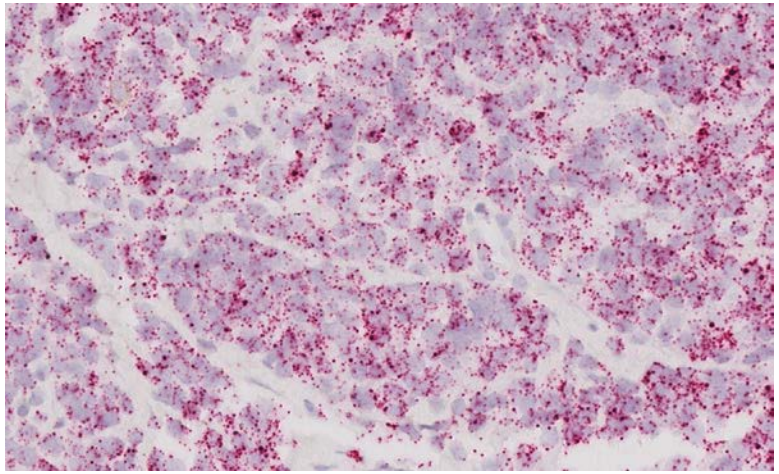
TIP: Refer to the User Manuals for automation assay workflow and pretreatment optimization guideline

EXAMPLE OF SUCCESFUL RNASCOPE® RESULTS

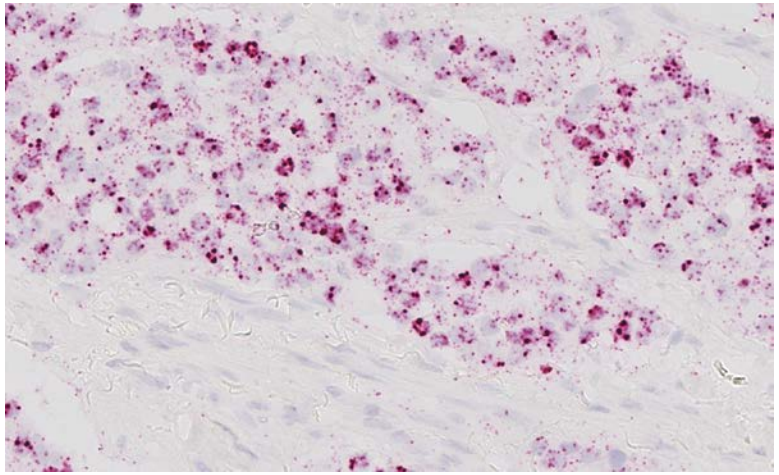
Negative control, DapB



Positive control, PPIB



Target probe



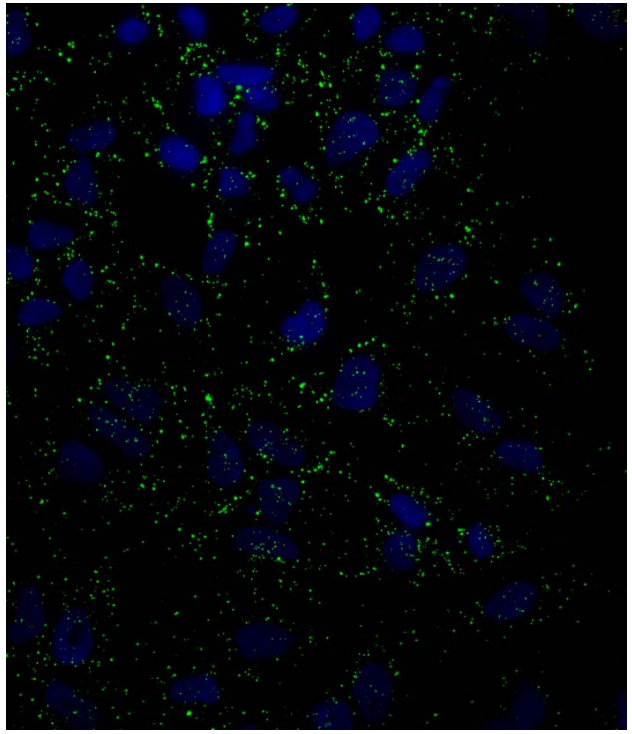
RNAscope 2.0 HD Red Assay

Human breast cancer tissue

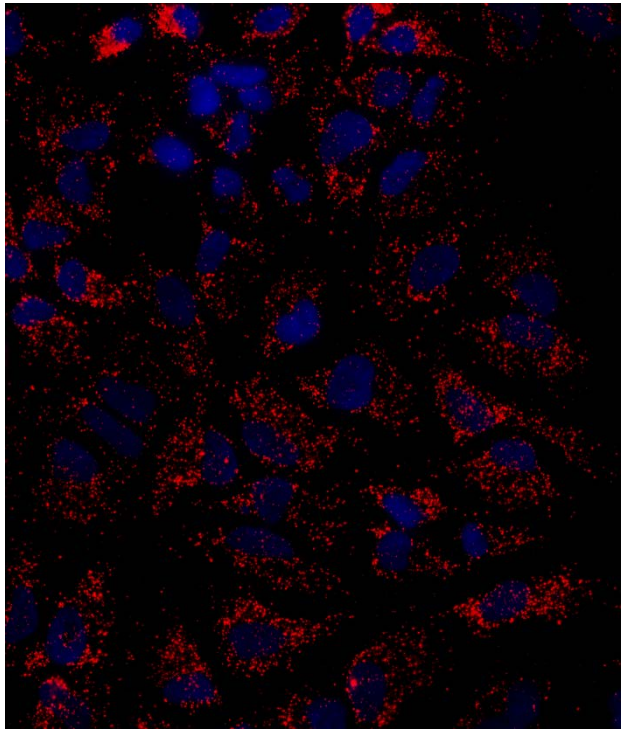
TIP : Visit http://www.acdbio.com/technical-support/downloads/rnascope-ish-guide-troubleshooting/ for more information

EXAMPLE OF SUCCESFUL RNASCOPE® RESULTS

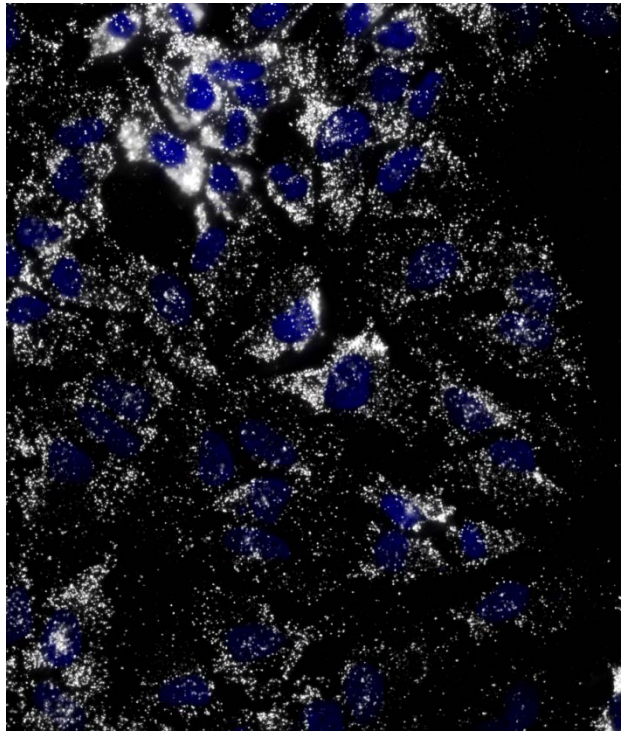
Hs POLR2A/Alexa 488



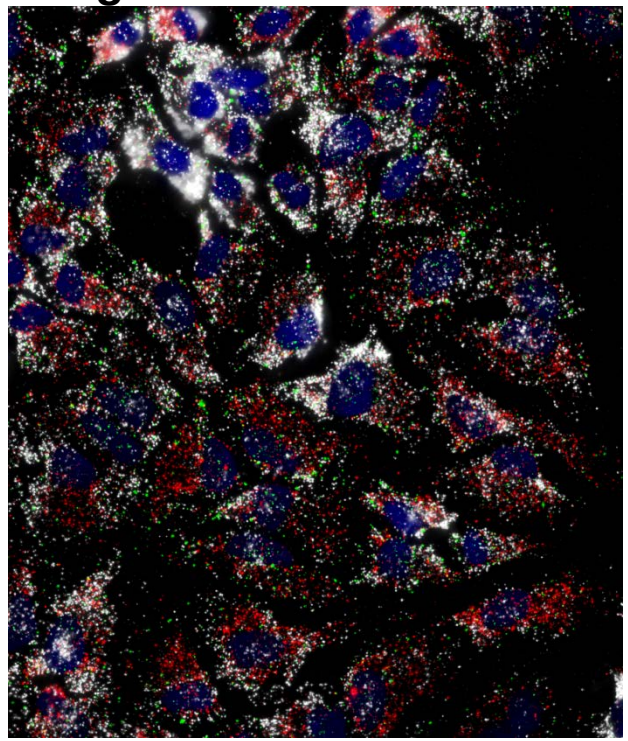
Hs PPIB/Atto 550



Hs UBC/Atto 647



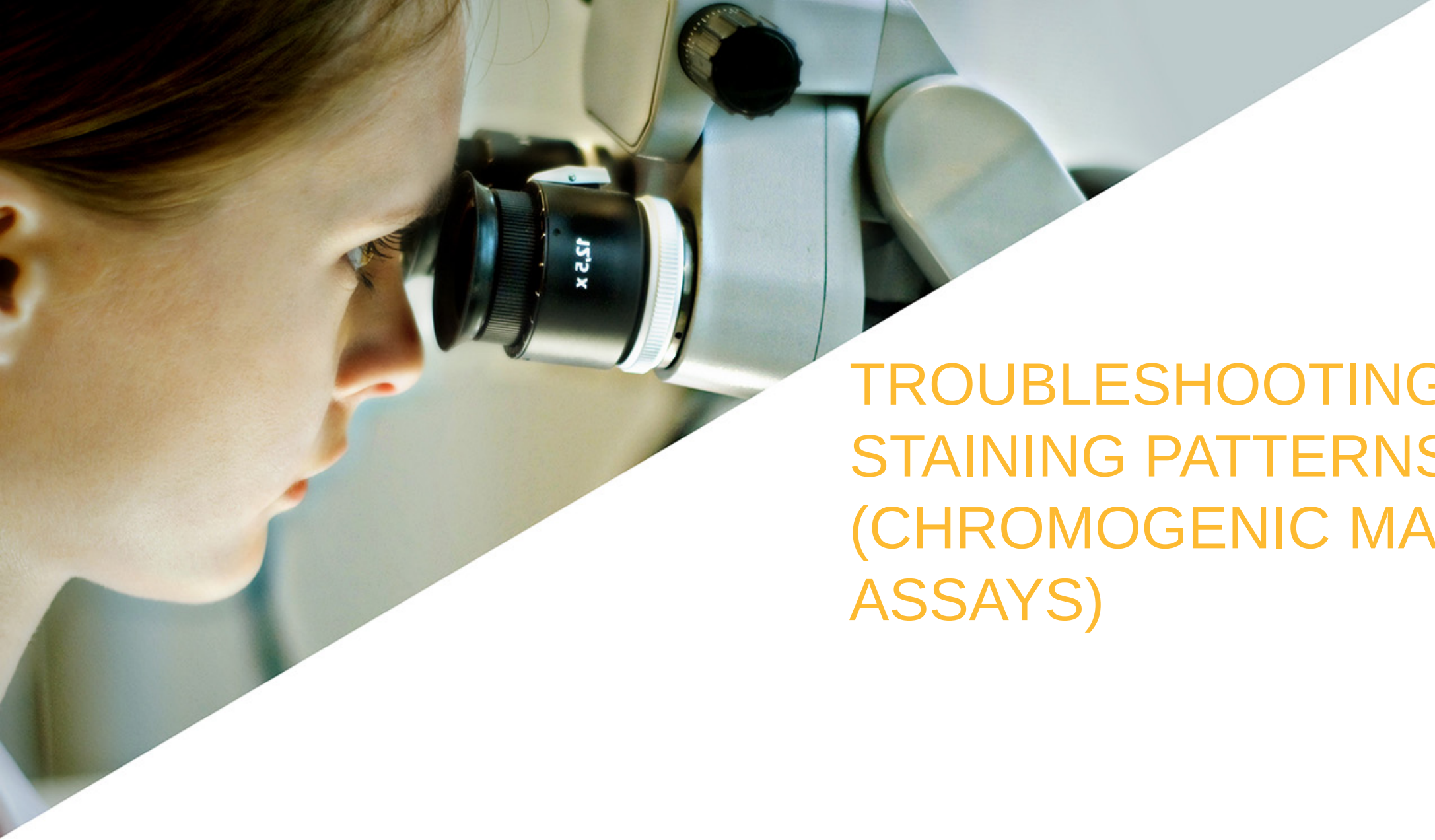
Merged



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**

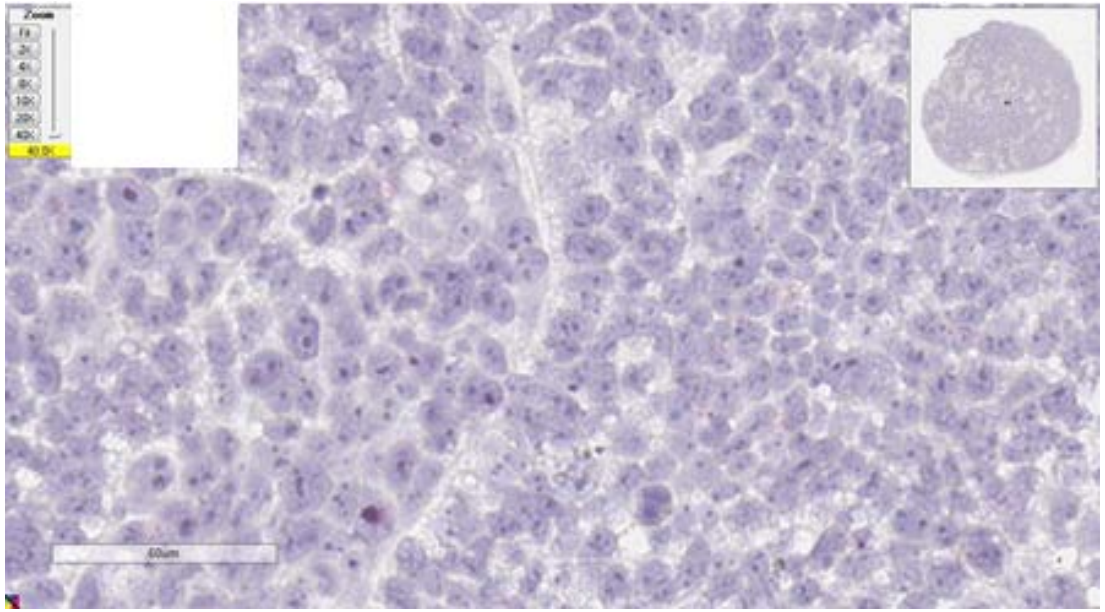
PROBABLE CAUSE	***SUGGESTED ACTION***
Suboptimal fixation •*Over fixation* •*Under fixation*	*Prepare samples according to ACD recommendation* *Optimize pretreatment conditions*
Hybridization temperature not optimal	*Use HybEZ when performing RNAscope* *HybEZ temperature should be at 40°C*
Reagents used in the wrong sequence	*Apply reagents in the correct order*
Gene of interest no expressed	*Check positive control for technical accuracy of the assay*

TROUBLESHOOTING: **SAMPLE DIGESTION**

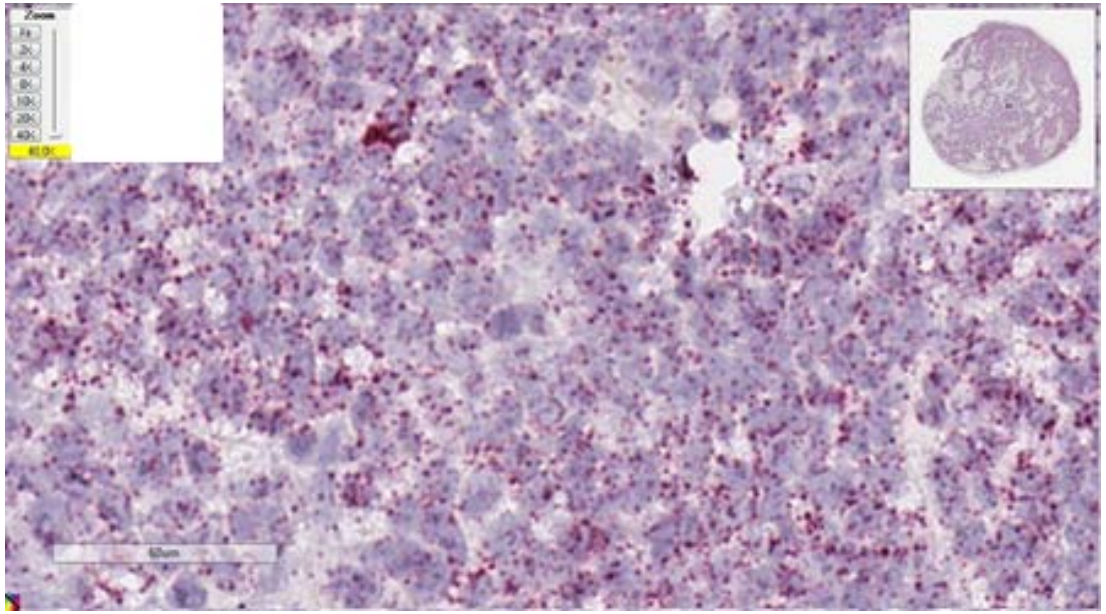
OPTIMAL DIGESTION

XENOGRAFT TISSUE

*8 min Pretreat 2 , 30 min Pretreat 3



DapB



Hs-PPIB

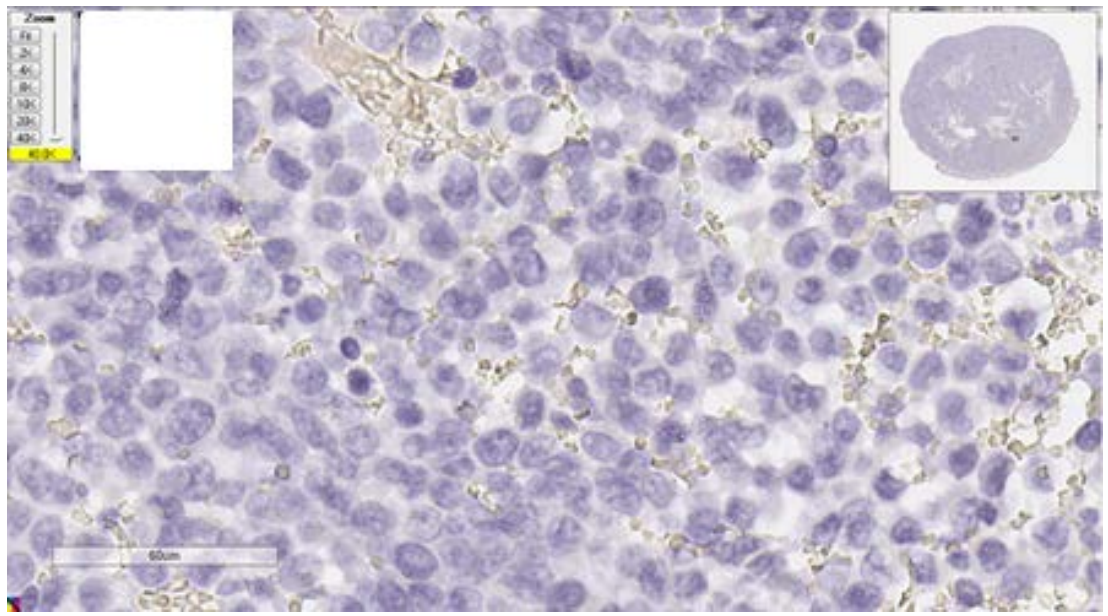
***Conditions used for manual assays**

TROUBLESHOOTING: **SAMPLE DIGESTION**

UNDER DIGESTION

XENOGRAFT TISSUE

*8 min Pretreat 2 , 15 min Pretreat 3



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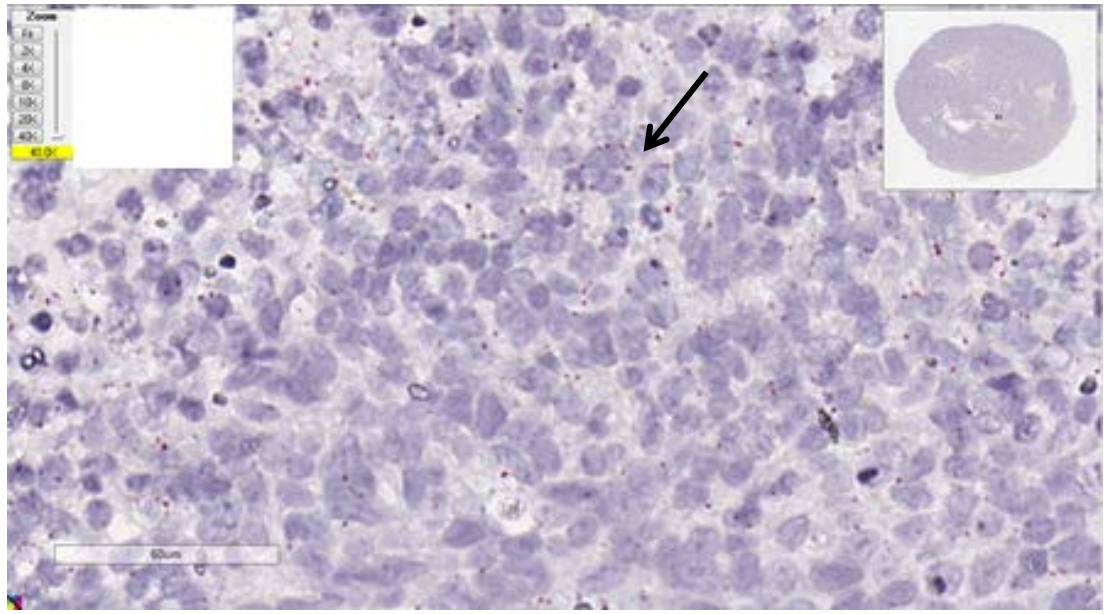


Hs-PPIB

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

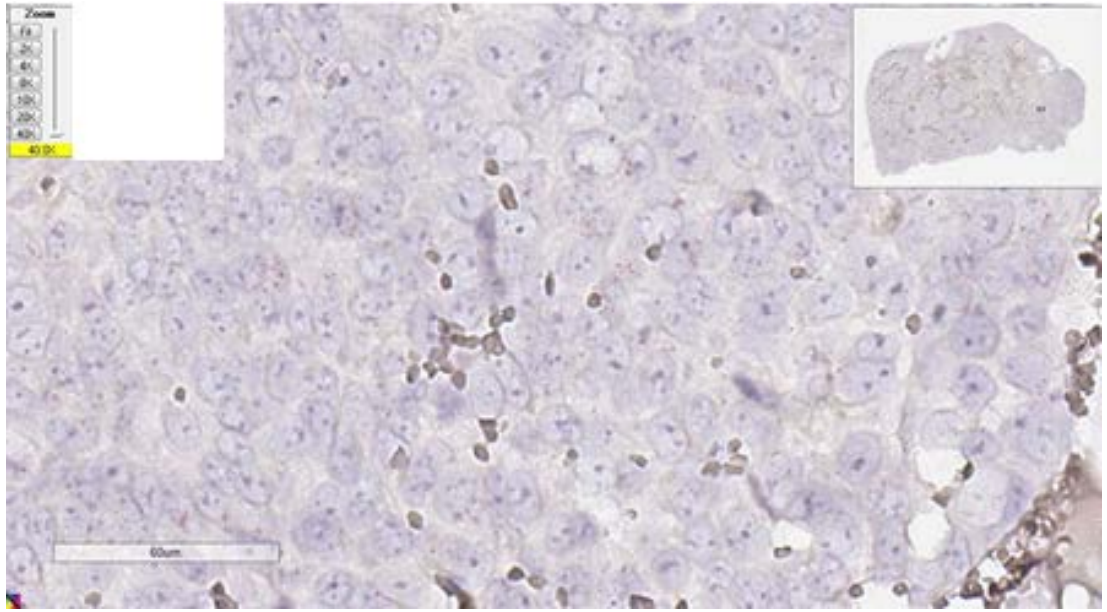
***Conditions used for manual assays**

TROUBLESHOOTING: **SAMPLE DIGESTION**

OVER DIGESTION

XENOGRAFT TISSUE

*15 min Pretreat 2 , 30 min Pretreat 3



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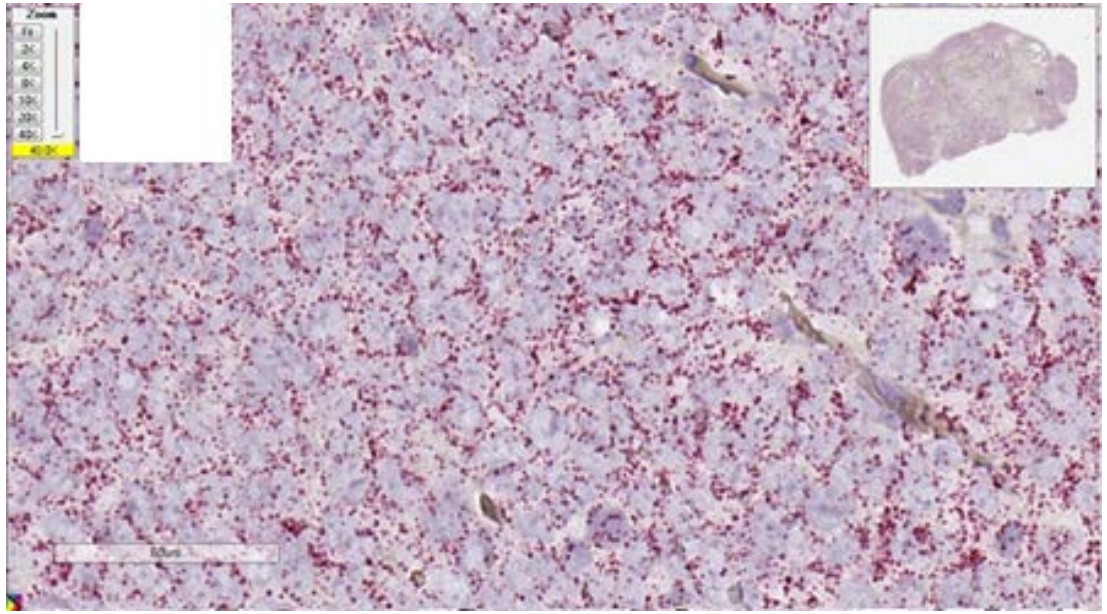


Hs-PPIB

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

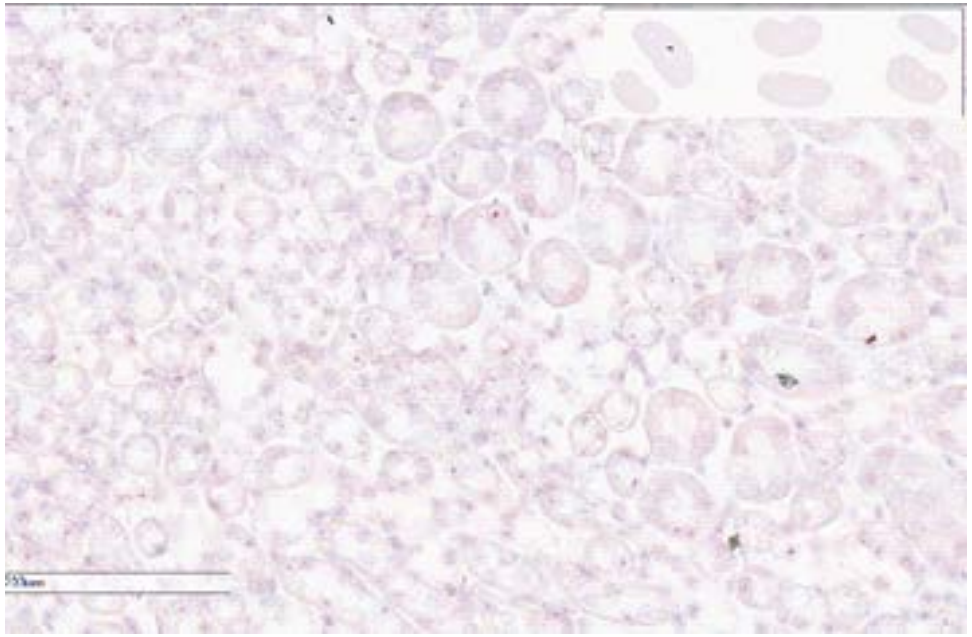
*Conditions used for manual assays

TROUBLESHOOTING: **BACKGROUND STAINING**

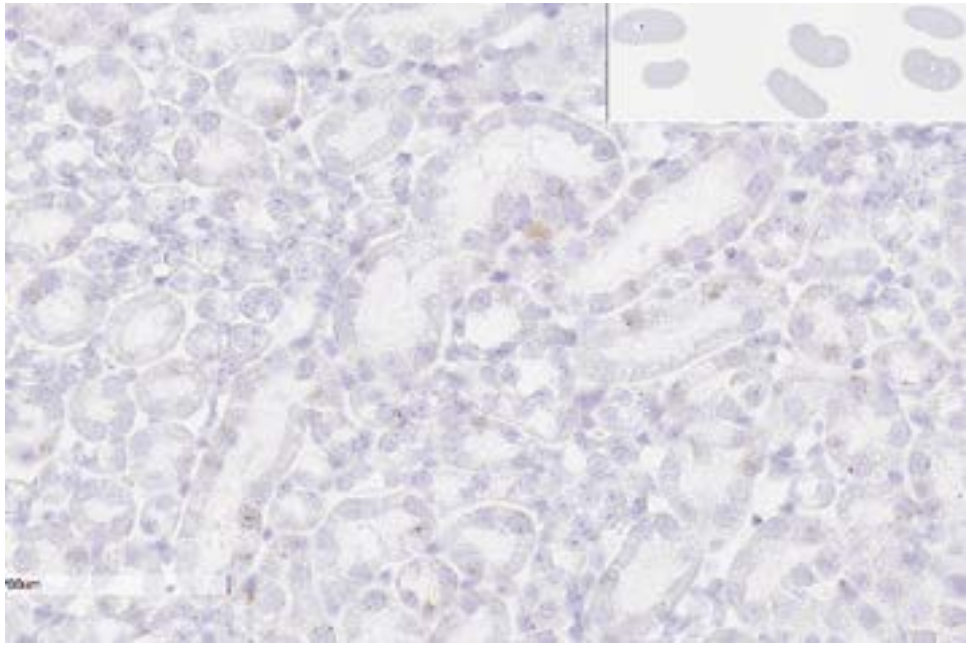
HIGH BACKGROUND

KIDNEY FFPE TISSUE

DapB



*15 min Pretreat 2 , 30 min Pretreat 3



*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*

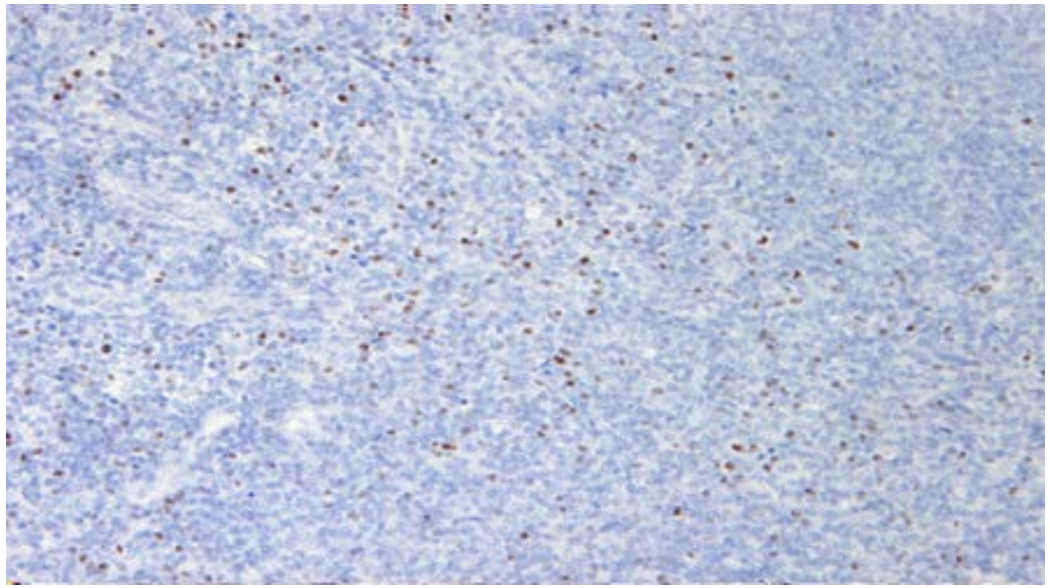
***Conditions used for manual assays**

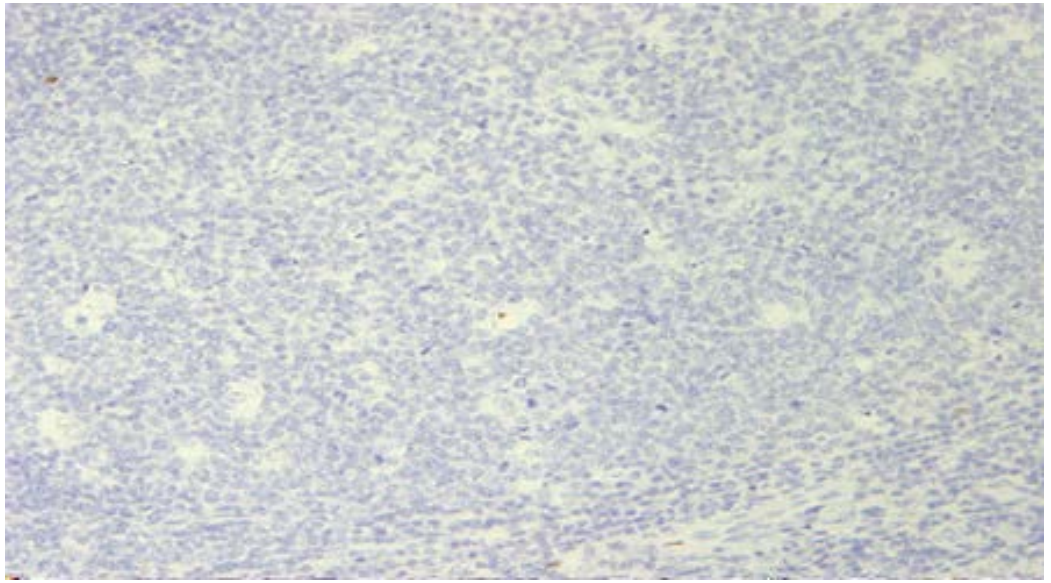
TROUBLESHOOTING: **BACKGROUND STAINING**

NUCLEAR HAZY BACKGROUND

HUMAN TONSIL FFPE TISSUE

DapB





*15 min ER2, 30 min Protease

*20 min ER2, 30 min Protease

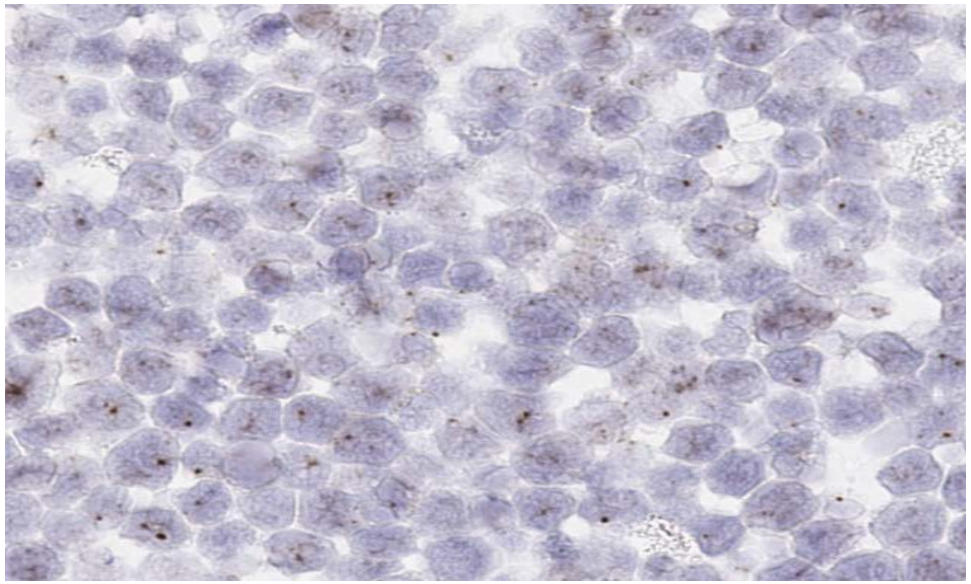
***Assay**: RNAscope LS BROWN (LEICA BOND RX)*
***Issue**: Nuclear hazy background, under pretreatment*
***Optimization**: Increase ER2 time in increments of 5 mins and protease in increments of 10 mins*
***Result**: Clean background*

***Conditions LEICA BOND RX automated assays**

TROUBLESHOOTING: **ASSAY WORKFLOW**

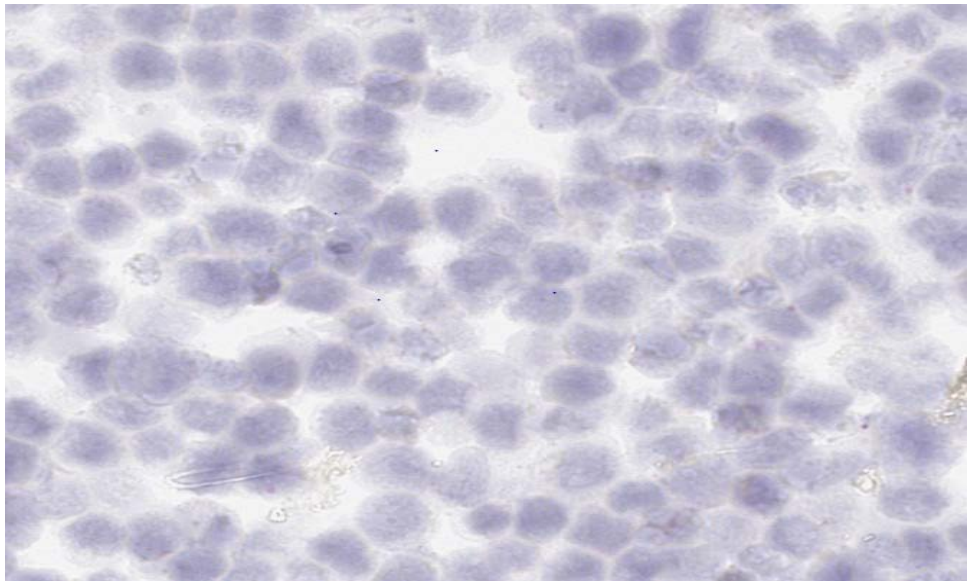
HIGH BACKGROUND/DRYING

DapB



15 min Pretreat 2 , 30 min Pretreat 3

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

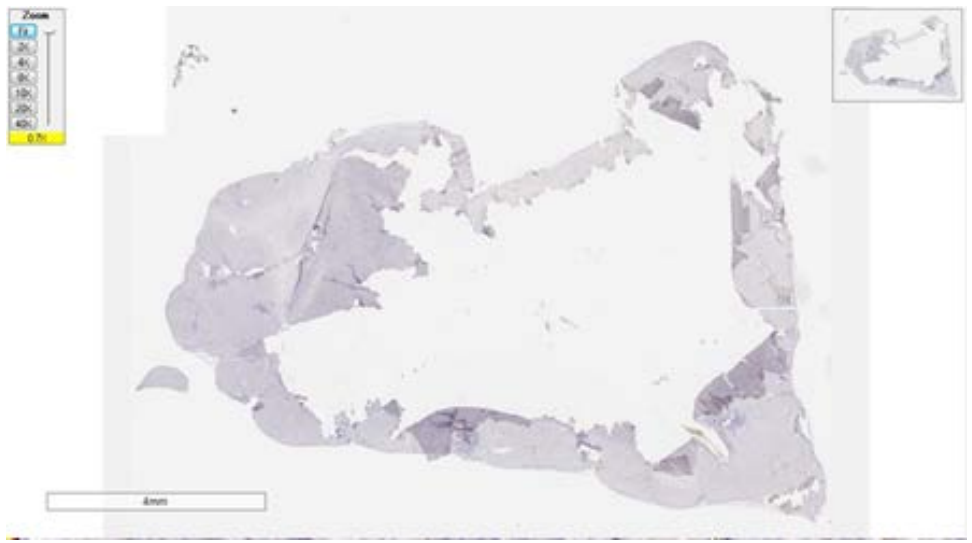
***Conditions used for manual assays**

TROUBLESHOOTING: **SAMPLE PREPARATION**

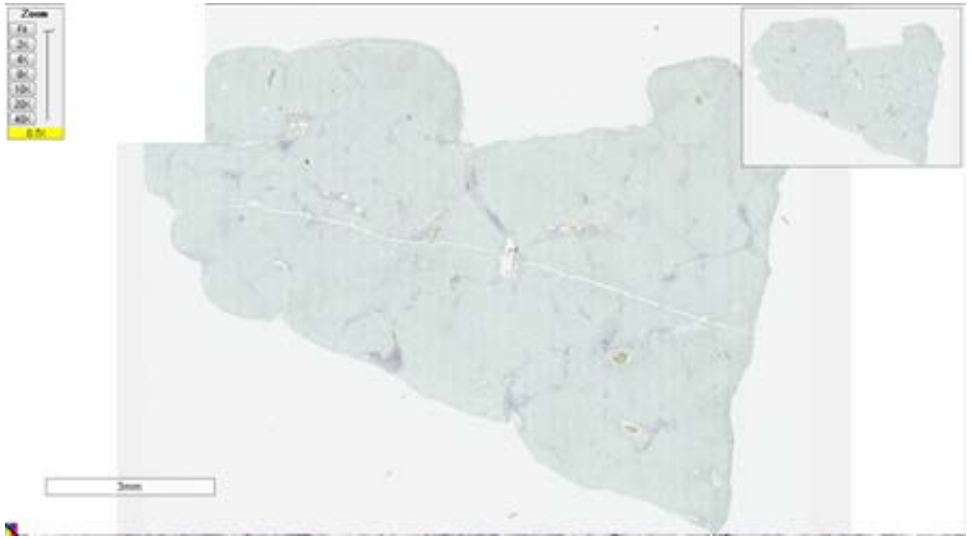
SAMPLE FALLING OFF

XENOGRAFT FFPE TISSUE

DapB



Standard baking protocol



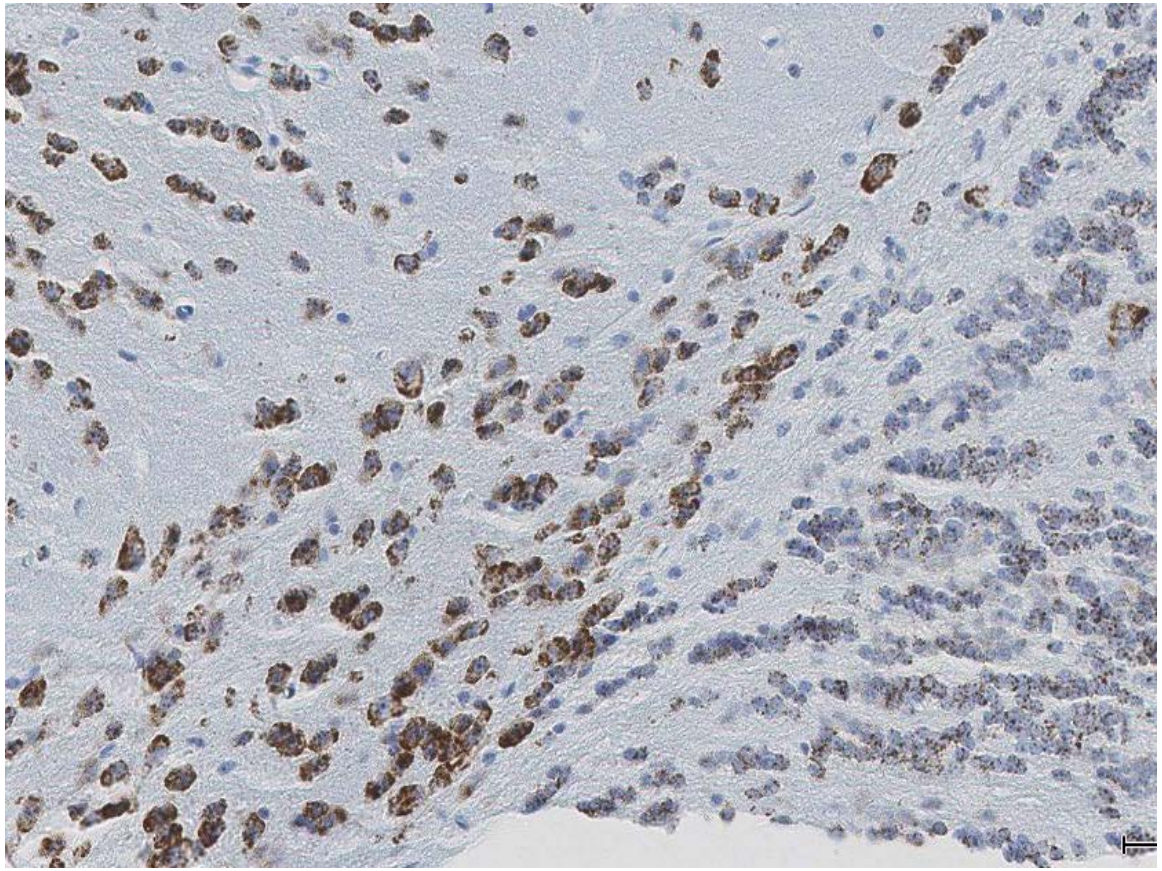
Increased baking by 1 hour

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**

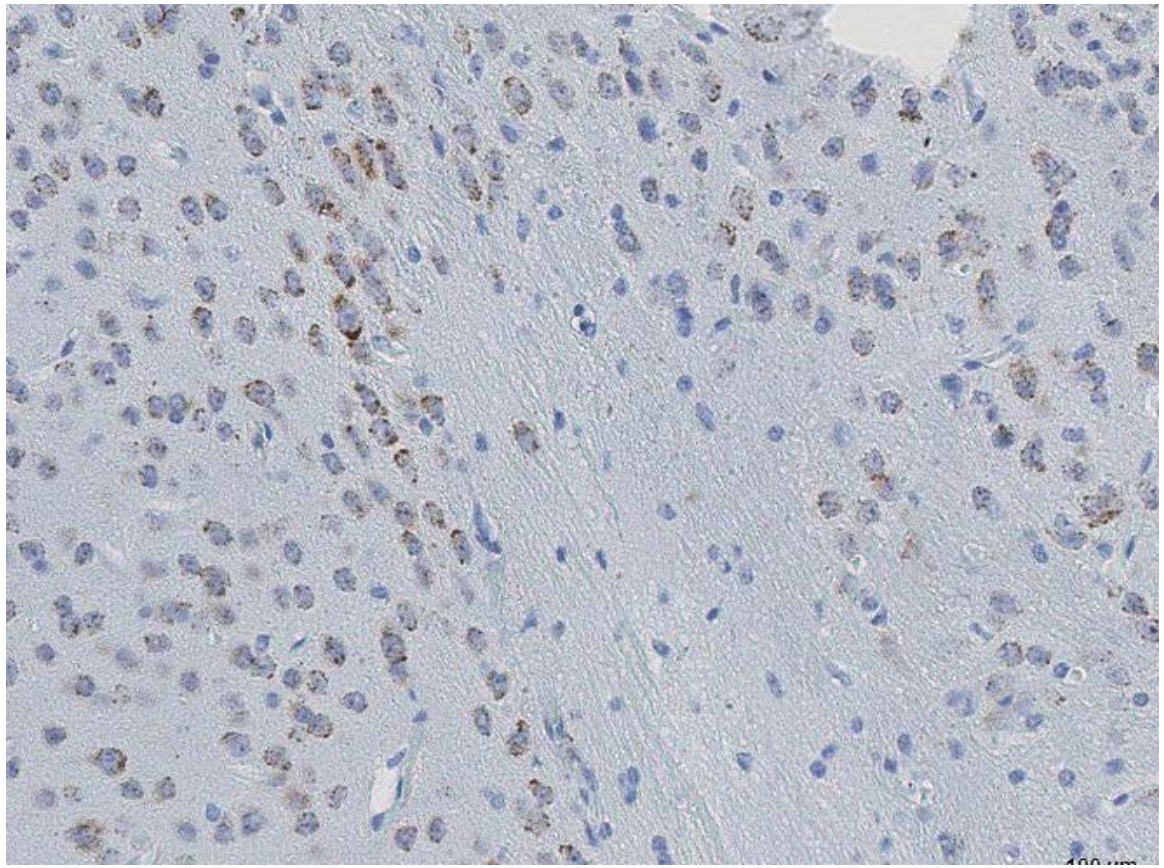
TROUBLESHOOTING **SUB-OPTIMAL FIXATION CONDITIONS**

24 hours fixation/**Optimal**



Sample: FFPE brain sample

3 weeks fixation/**Over fixed**



Assay: RNAscope 2.0 HD Brown

Synaptophysin

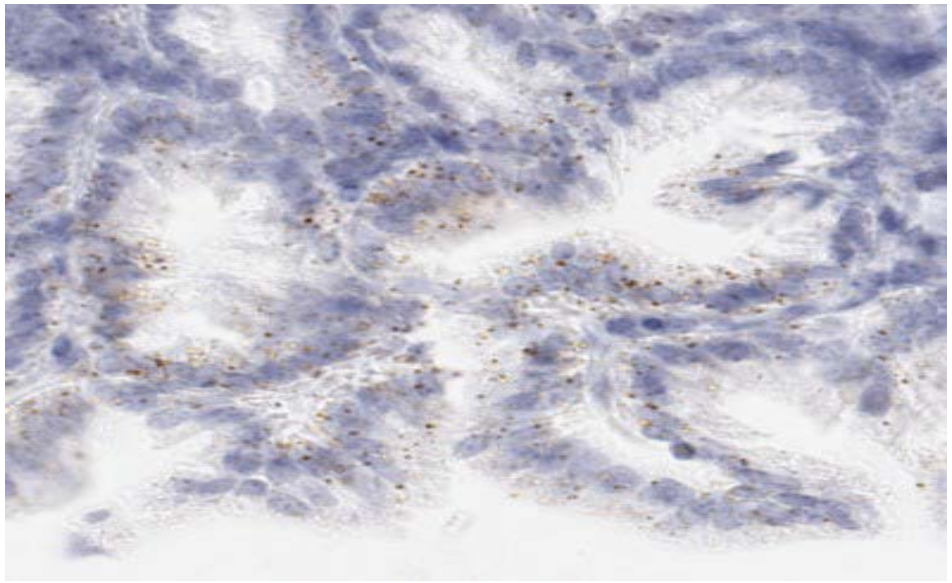
TIP: Sample fixation has a great effect on the success of your assay
Solution: Increase pretreatment for better target accessibility

TROUBLESHOOTING: **OTHER ISSUES**

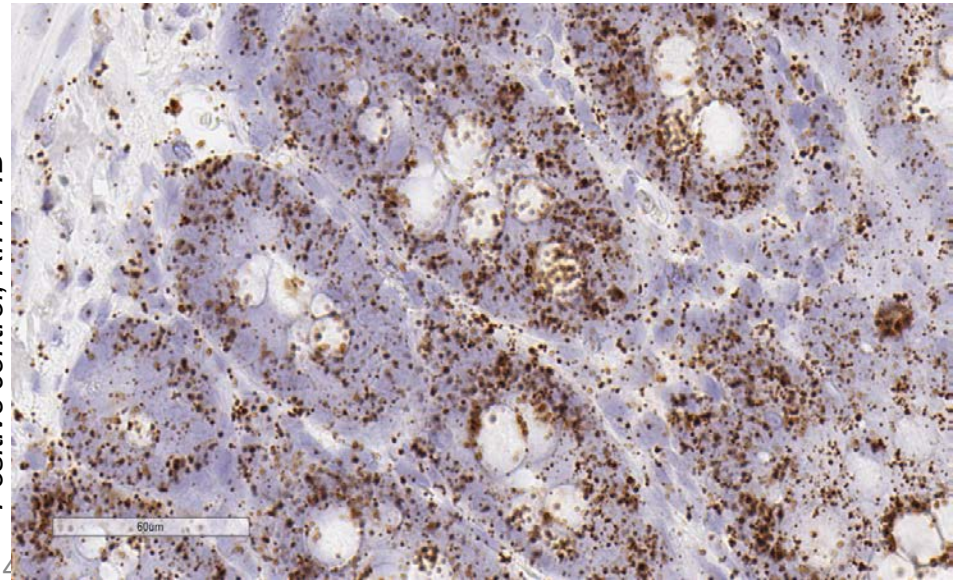
ISSUE	PROBABLE CAUSE	SUGGESTED ACTION
Unknown tissue preparation method	•Sample provider/clinical site/vendor did not provide detailed instructions	•Follow the appropriate Tissue Specimen Preparation and Assay Optimization Guidelines/Technotes*
		•Start with standard conditions •Optimize your assay
Diffused Signal (RED)	•Sample not completely dried •Alcohol used to dehydrate sample •Too much Ecomount mounting medium used	•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



Positive control, Rn PPIB

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

Assay: RNAscope 2.0 HD Brown

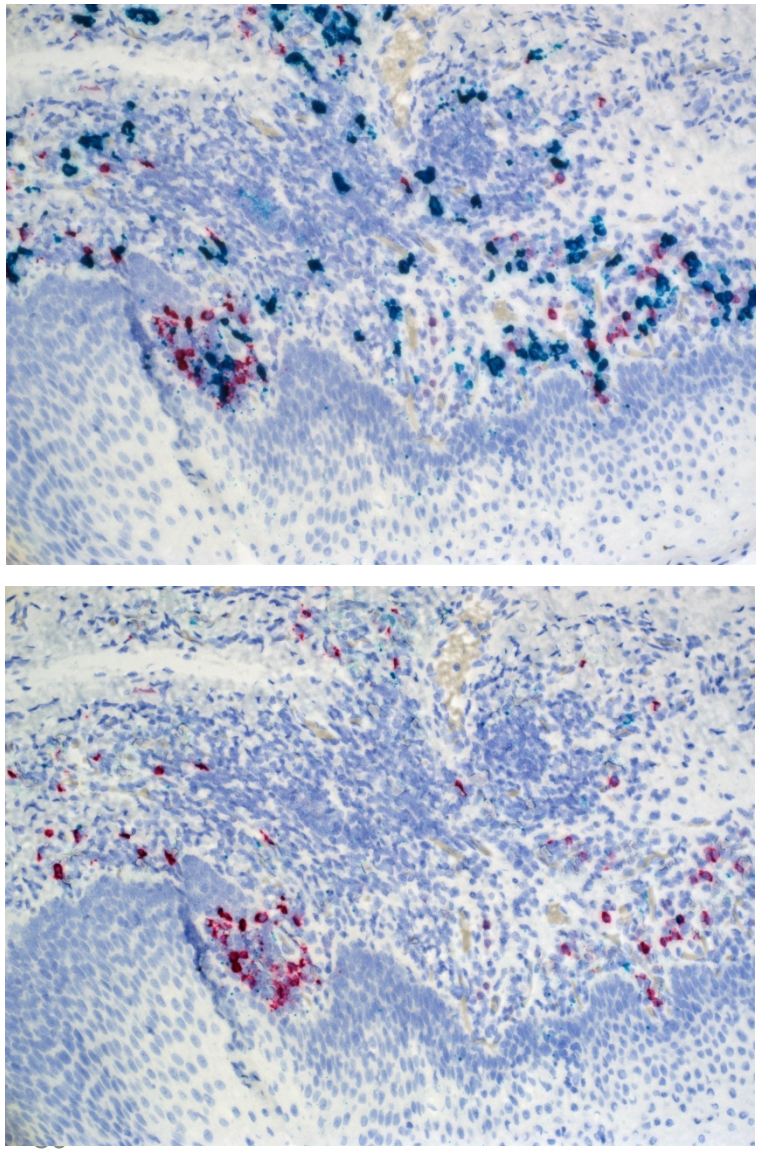
Issue: Weak staining, destroyed morphology, FFPE sample is under fixed

Optimization: Fixation according to recommended guidelines for FFPE samples

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

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 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
Mouse / Rat	Intestine	Normal	Standard
	Intestine	Tumor	Standard
	Embryo	Normal	Standard
	Brain	Normal	Standard
	Spleen	Normal	Mild
	Eye/Retina	Normal	Standard
	Liver	Normal	Extended
	Kidney	Normal	Standard
Human	Breast	Tumor	Standard
	Colon	Tumor	Standard
	Colon	Normal	Standard
	Lung	Tumor	Standard
	Lung	Normal	Standard
	Prostate	Tumor	Standard
	Prostate	Normal	Standard
	Lymph node	Tumor	Mild
	Lymph node	Normal	Mild
	Tonsil	Normal	Mild
	Pancreas	Normal	Standard
	Cervical	Cancer	Standard
Human	Cervical	Normal	Standard
	Cervical dysplasia	Abnormal	Standard
	Brain	Tumor	Standard
	Brain	Normal	Standard
	Head	Cancer	Standard
	Neck	Cancer	Standard
	Liver	Cancer	Standard
	Kidney	Normal	Standard
	Skin	Normal	Standard
	Melanoma	Tumor	Standard
	Nevus	Benign	Standard
	Placenta	Normal	Standard
	Skin (TMA*)	Normal	Standard
	Breast (TMA)	Normal	Standard
	Melanoma (TMA)	Normal	Standard
	Nevus (TMA)	Benign	Standard
	Stomach (TMA)	Normal	Standard
	Stomach (TMA)	Tumor	Standard
	Cell pellets**	—	Mild
	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
Mouse / Rat	Intestine	Normal	Standard
	Intestine	Tumor	Standard
	Embryo	Normal	Standard
	Brain	Normal	Standard
	Spleen	Normal	Mild
	Testis	Normal	Mild
	Liver	Normal	Extended
	Kidney	Normal	Extended
Human	Breast	Tumor	Standard
	Colon	Tumor	Standard
	GI tract	Normal	Standard
	Lung	Tumor	Standard
	Lung	Normal	Standard
	Prostate	Tumor	Standard
	Prostate	Normal	Standard
	Lymph node	Tumor	Mild
	Lymph node	Normal	Mild
	Tonsil	Normal	Mild
	Pancreas	Normal	Standard
	Cervical	Cancer	Standard

Species	Tissue type	Pathology	Pretreat Condition
Human	Head and Neck	Normal	Standard
	Cervical dysplasia	Abnormal	Standard
	Brain and spinal cord	Tumor	Standard
	Muscle	Normal	Extended
	Liver	Cancer	Extended
	Thymus	Cancer	Standard
	Heart	Cancer	Standard
	Kidney	Normal	Standard
	Skin	Normal	Standard
	Melanoma	Tumor	Standard
	Nevus	Benign	Standard
	Placenta	Normal	Standard
	Skin (TMA*)	Normal	Standard
	Breast (TMA)	Normal	Standard
	Melanoma (TMA)	Normal	Standard
	Nevus (TMA)	Benign	Standard
	Stomach (TMA)	Normal	Standard
	Stomach (TMA)	Tumor	Standard
	Cell pellets**	—	Mild
	HeLa cells† (ACD control)	—	Standard

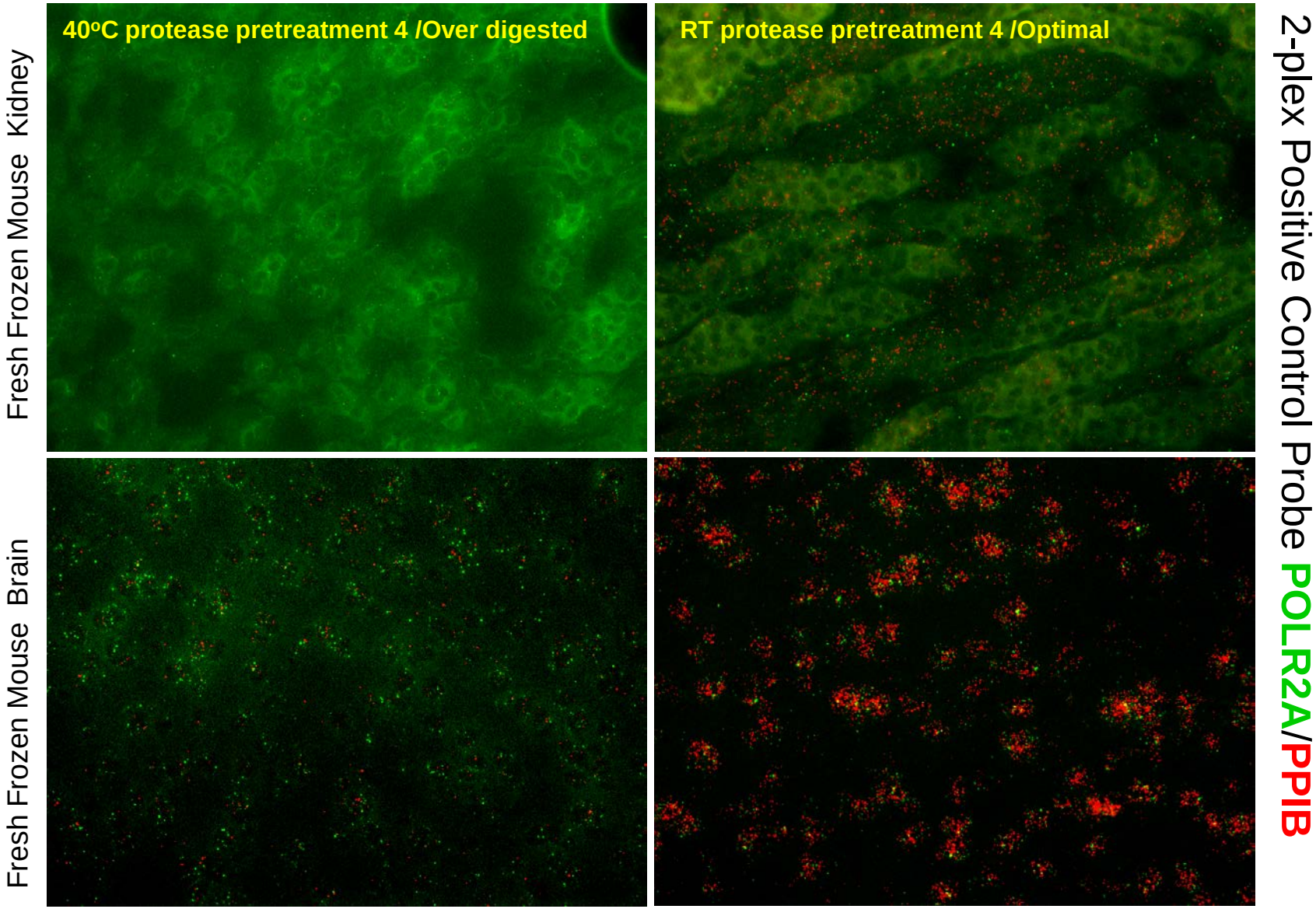
* Tissue Microarray
** Fixed with 10% NBF
† Fixed with 10% Formaldehyde/PBS

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**

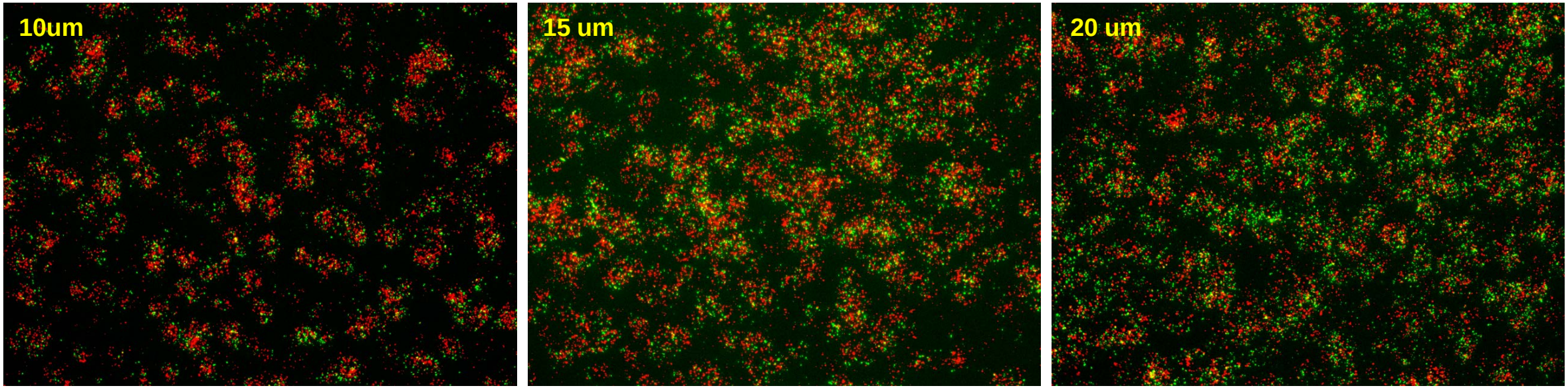


TIP: Pretreatment temperature has a great effect on the success of your assay
Solution: Perform pretreatment at RT to avoid over digestion of your sample

TROUBLESHOOTING: **SAMPLE DIGESTION**

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

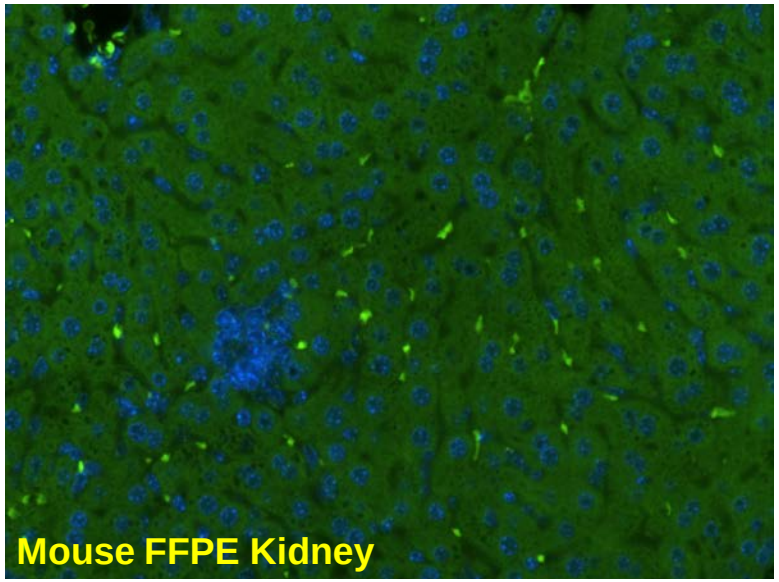
Fresh Frozen Mouse Brain

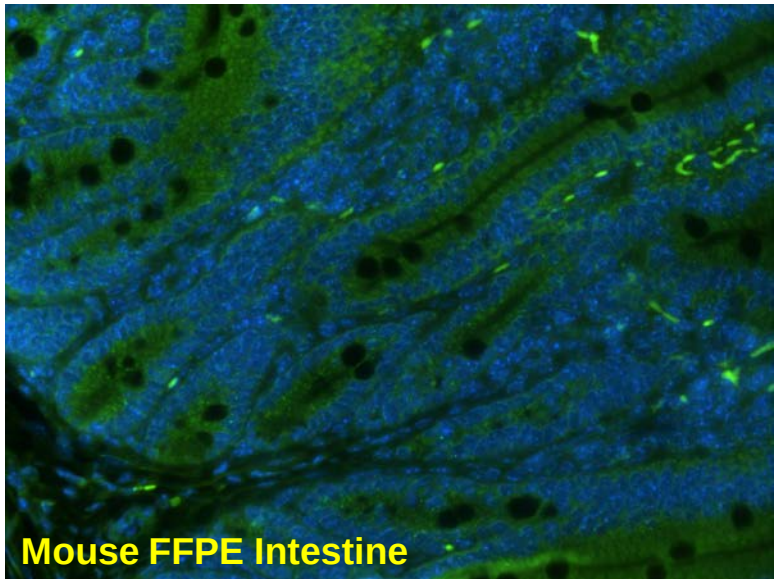


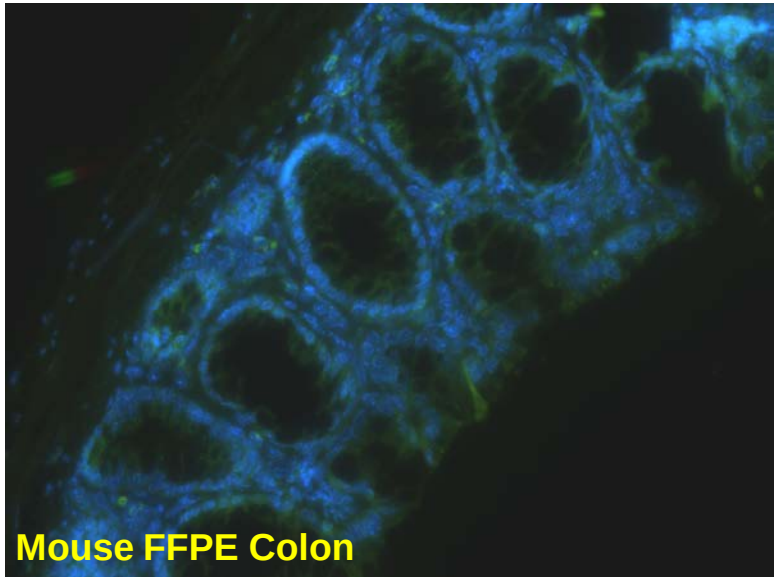
Experiment condition: 10% NBF, 15 min Fixation, Pretreatment 4, RT

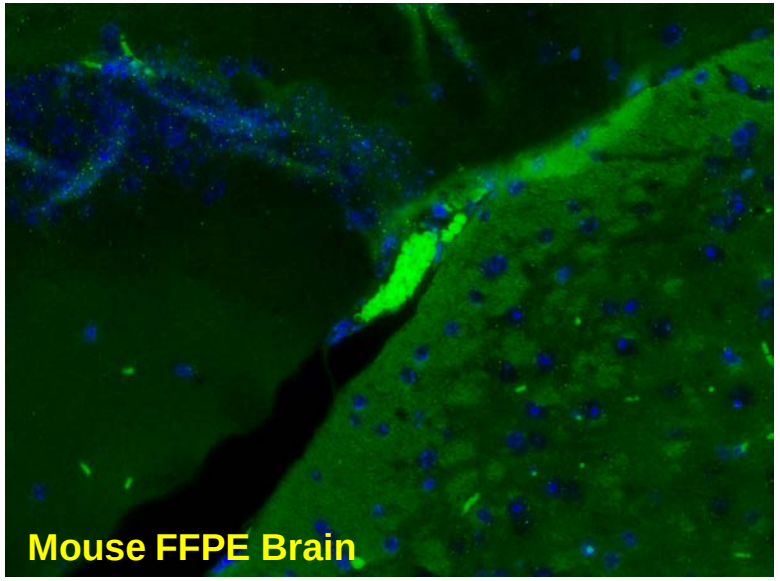
TIP: Sample thickness can signal in your samples
Solution: Use recommended sample thickness, 10-20um

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	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

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

  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

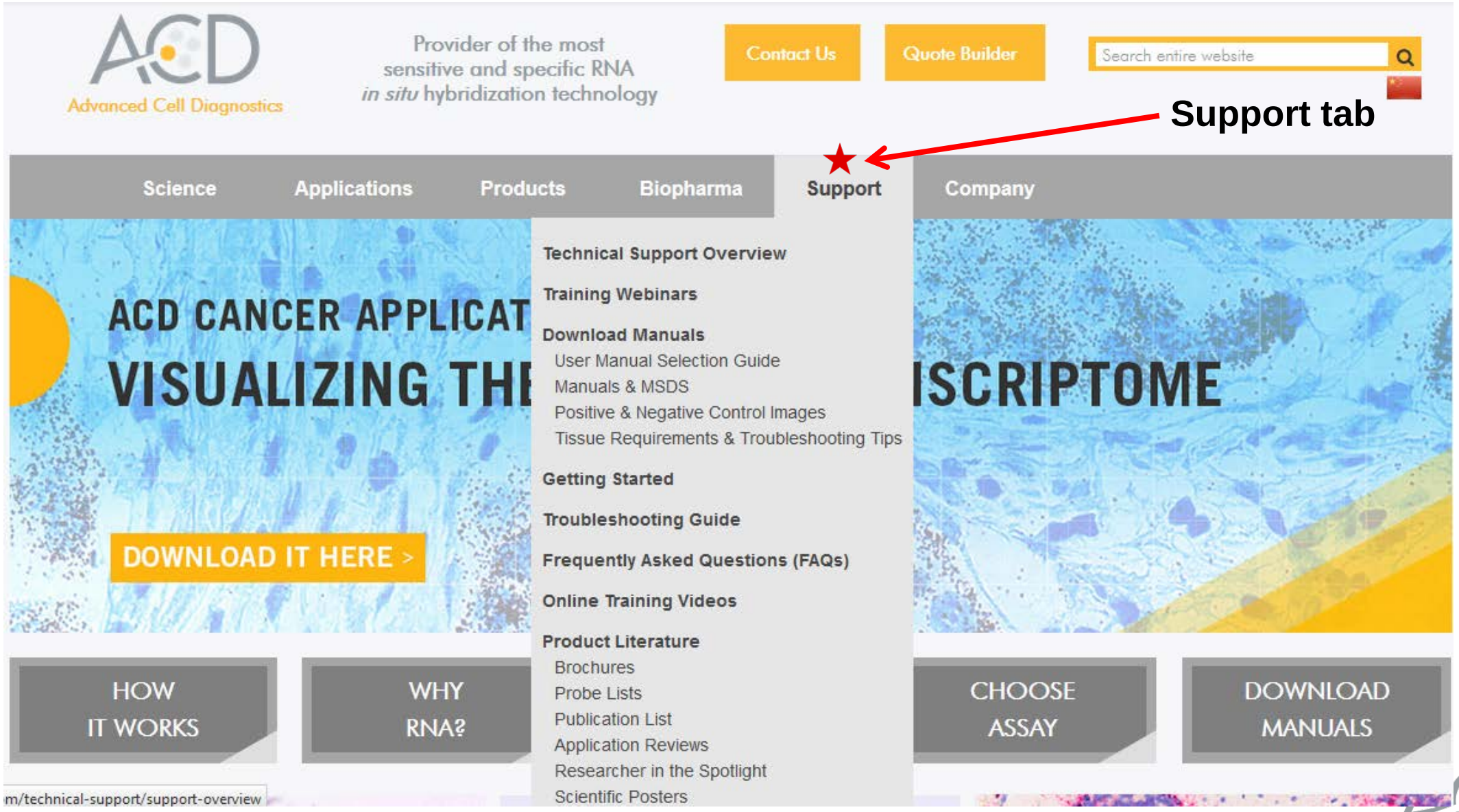
SUMMARY

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 hybridization temperature changes (LEICA)*
   - *Offline/online pretreatment optimization (CC and pretreat 2/3) (VENTANA)*

VISIT THE SUPPORT PAGE TO LEARN MORE



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

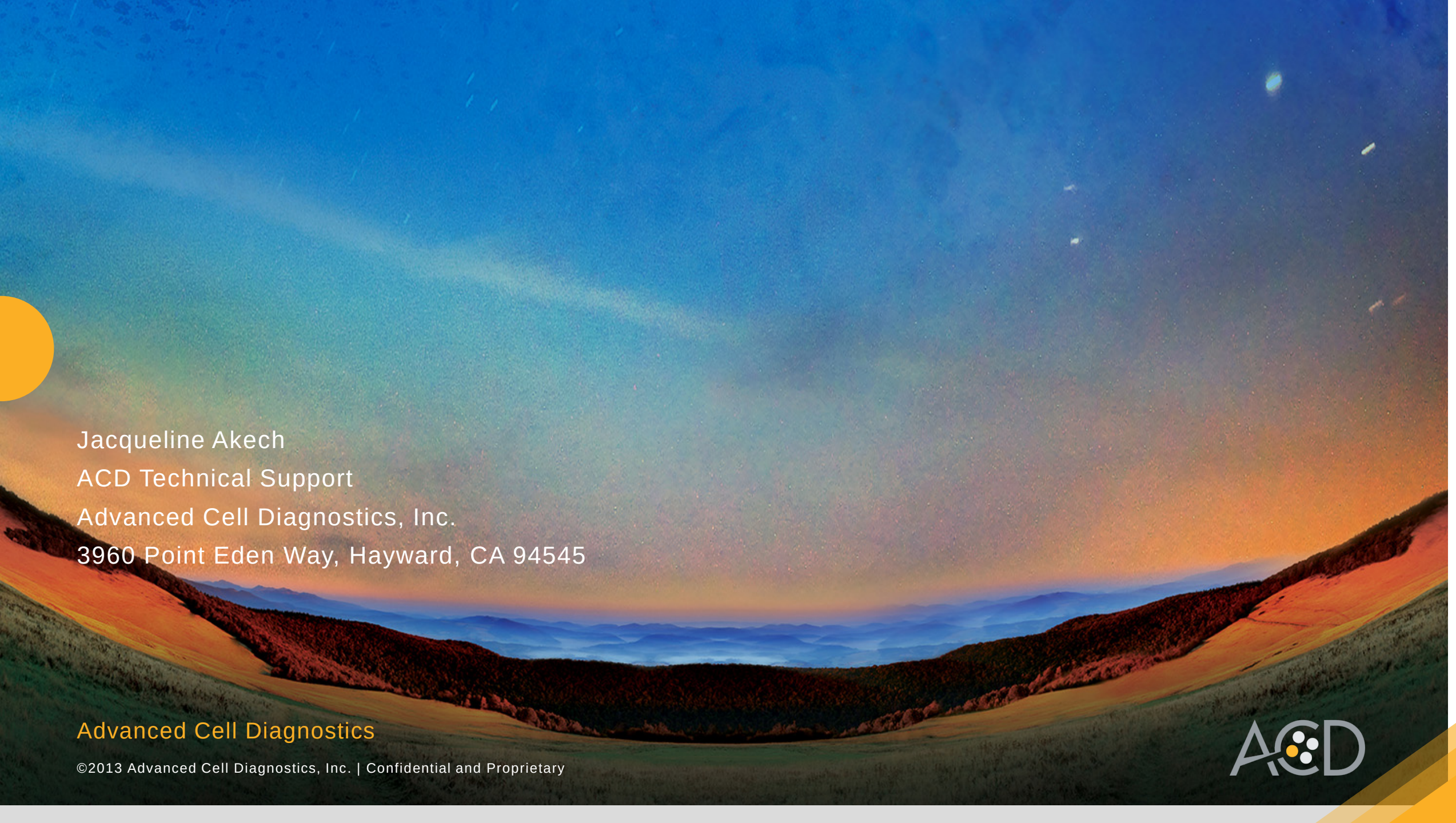
CONTACT ACD SUPPORT

- 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

Manuals	Getting Started	FAQs	Videos	Product Literature
Download manuals, technical notes and MSDS.	Simple tips & tricks for you to get the best RNAscope result from day1.	Browse through our product frequently asked questions or add one of your own.	View our product and workflow videos on our Video page.	Find RNAscope publication lists, gene lists and download product brochures.
Go →	Go →	Go →	Go →	Go →

QUESTIONS?

PLEASE COMPLETE THE WEBINAR SURVEY, WE VALUE YOUR FEEDBACK



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Advanced Cell Diagnostics