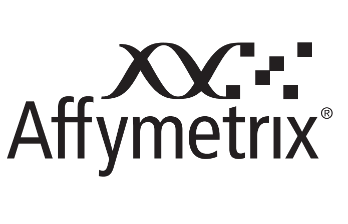

User Manual

QuantiGene® ViewRNA ISH Tissue 2-Plex Assay

Equipment: ThermoBrite Hybridization System

P/N 18849 Rev.B 121014

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Introduction

About This Manual

This manual provides complete instructions for performing the QuantiGene ViewRNA ISH Tissue Assay for visualization of 1 or 2 target RNAs in formalin-fixed paraffin-embedded (FFPE) samples prepared in accordance with the guidelines provided. Protocol modifications, if working with OCT-Embedded Frozen Tissue Sections, are located in the Appendix. This manual provides assay procedures that utilize the ThermoBrite denaturation/hybridization system.

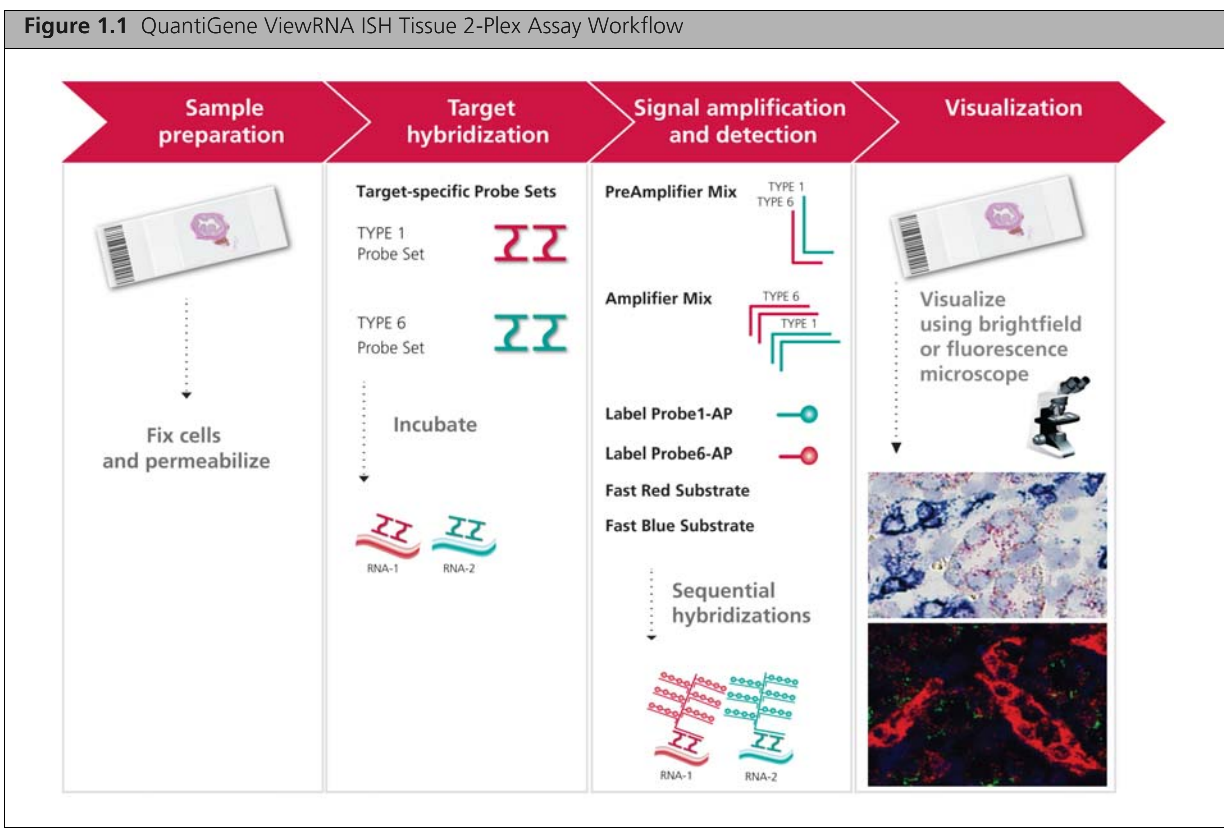
A separate User Manual is available if you are utilizing a tissue culture incubator (w/o CO_2) for hybridization steps.

Assay Overview

In situ hybridization (ISH) techniques are used to visualize DNA or localize RNAs within cells. However, the *in situ* analysis of RNA, in particular, has always been limited by low sensitivity and complicated probe synthesis. The QuantiGene® ViewRNA ISH Tissue 2-Plex Assay, based on highly specific, branched DNA signal amplification technology, has the sensitivity and robustness for simultaneous *in situ* detection of any two target mRNAs within FFPE tissue sections with single-copy sensitivity. The assay design is illustrated and explained in Figure 1.1.

How It Works

Figure 1.1 QuantiGene ViewRNA ISH Tissue 2-Plex Assay Workflow



Sample Preparation. FFPE tissue sections are fixed, then permeabilized to expose mRNA target(s) and allow for accessibility of probe(s).

Target Hybridization. Target-specific Probe Sets hybridize to their respective target mRNAs. Subsequent signal amplification is predicated on specific hybridization of the pair(s) of oligonucleotides (indicated by “II” in the above image) within each probe set to the target sequence. A typical mRNA Probe Set contains 20 oligonucleotide pairs, each with a target-specific region that binds to the target transcript as well as a TYPE-specific sequence upon which subsequent signal amplification is built. For simplicity, only one pair per mRNA target is shown in the figure. TYPE 1 and TYPE 6 Probe Sets are designed to generate red and blue signals, respectively.These separate yet compatible signal amplification systems provide the assay with multiplex capability

Signal Amplification. Signal amplification, using bDNA technology, is achieved via a series of sequential hybridization steps. The PreAmplifiers hybridize to their respective pair of bound Probe Set oligonucleotides then multiple Amplifiers hybridize to their respective PreAmplifier. Next, TYPE-specific Label Probe oligonucleotides, conjugated to alkaline phosphatase, are sequentially hybridized to their corresponding Amplifier molecules. A fully assembled signal amplification “tree” has 400 Label Probe binding sites (a total 8000 for each mRNA molecule)

Detection. The sequential hybridizations of TYPE 6 Label Probe followed by addition of Fast Blue substrate and TYPE 1 Label Probe followed by the addition of Fast Red substrate produce chromogenic precipitates/dots of blue and red color, respectively, wherever the target mRNA molecules are localized. The target mRNAs are visualized using a standard brightfield and/or fluorescent microscope.

Performance Highlights

Table 1.1 Performance Highlights

Specification	Description
Sample types	OCT-embedded frozen tissue or formalin-fixed paraffin-embedded (FFPE) tissue sections ▪ Assay area 20 x 30 mm on standard 25 x 75 mm glass slide ▪ FFPE tissue thickness: 5 ± 1 µm ▪ Fresh frozen thickness: 12 ± 1 µm ▪ FFPE tissue microarray (TMA): Greater than 1 mm diameter and 5 ± 1 µm thickness
Sensitivity	Single RNA molecule per dot
Multiplexing	Simultaneous detection of 2 target RNAs
Detection	Chromogenic and fluorescence
Nuclear stain	Hematoxylin and/or DAPI
Instrumentation	brightfield and/or fluorescence microscope or scanner

Safety Warnings and Precautions

- Formaldehyde is a poison and an irritant. Avoid contact with skin and mucous membranes. Use in a fume hood.
- Ammonium hydroxide is highly volatile. Use in a fume hood.
- Xylene is flammable and an irritant. Avoid inhalation and contact with skin. Use in a fume hood.
- Perform all procedural steps in a well-ventilated area at room temperature unless otherwise noted.
- Discard all reagents in accordance with local, state, and federal laws.

Required Materials

The QuantiGene ViewRNA ISH Tissue 2-Plex Assay is composed of the following 2 modules, each sold separately and available in multiple sizes:

- QuantiGene ViewRNA ISH Tissue 2-Plex Assay Kit
- QuantiGene ViewRNA TYPE 1 and TYPE 6 Probe Sets

QuantiGene ViewRNA ISH Tissue 2-Plex Assay Kit

QuantiGene ViewRNA ISH Tissue 2-Plex Assay Kits are available in two sizes: QVT0012 and QVT0013, sufficient for 24 or 96 assays, respectively. These kits are compatible with Probe Sets designated TYPE 1 and TYPE 6 for mRNAs. Each kit is configured for processing a minimum of 6 or 12 slides, respectively, per experiment

The components of the QuantiGene ViewRNA ISH Tissue 2-Plex Assay Kit and their recommended storage conditions are listed below. Refer to the Package Insert for quantities of individual components supplied. Kits are shipped in 2 parts, based on storage conditions, and have shelf life of 6 months from date of delivery when stored as recommended.

Table 1.2 Assay Kit Components and Their Storage Conditions

Component	Description	Storage
100X Pretreatment Solution	Aqueous buffered solution	2-8 °C
Protease QF[a]	Enzyme in aqueous buffered solution	2-8 °C
Probe Set Diluent QT	Aqueous solution containing formamide, detergent, and blocker	2-8 °C
Label Probe Diluent QF	Aqueous solution containing detergent	2-8 °C
PreAmplifier Mix QT	DNA in aqueous solution containing formamide and detergent	2-8 °C
Amplifier Mix QT	DNA in aqueous solution containing formamide and detergent	2-8 °C
Label Probe 6-AP[a]	Alkaline phosphatase-conjugated oligonucleotide in aqueous buffered solution	2-8 °C
Blue Buffer	Buffer required for preparation for Blue Substrate	2-8 °C
Blue Reagent 1	Blue precipitating substrate component 1 for the detection of alkaline phosphatase activity	2-8 °C
Blue Reagent 2	Blue precipitating substrate component 2 for the detection of alkaline phosphatase activity	2-8 °C
Blue Reagent 3	Blue precipitating substrate component 3 for the detection of alkaline phosphatase activity	2-8 °C
AP Enhancer Solution	Aqueous buffered solution	2-8 °C
Fast Red tablets	Red precipitating substrate for the detection of alkaline phosphatase activity	2-8 °C
Naphthol Buffer	Buffer required for preparation of Red Substrate	2-8 °C
Label Probe 1-AP[a]	Alkaline phosphatase-conjugated oligonucleotide in aqueous buffered saline	2-8 °C
AP Stop QT	Aqueous buffered solution intended for the inactivation of residual LP6-AP activity after the Fast Blue substrate development	15-30 °C
Wash Buffer Component 1 (Wash Comp 1)	Aqueous solution containing detergent	15-30 °C
Wash Buffer Component 2 (Wash Comp 2)	Aqueous buffered solution	15-30 °C

[a] *IMPORTANT! Do not freeze.*

QuantiGene ViewRNA Probe Sets

In addition to the QuantiGene ViewRNA ISH Tissue 2-Plex Assay Kit, QuantiGene ViewRNA TYPE 1 and TYPE 6 Probe Sets, specific to your targets of interest, must be purchased separately. Probe Sets are available in multiple sizes and should be stored at –20 °C. Refer to the Package Insert for quantities provided and design specificities.

Table 1.3 ViewRNA Probe Set and Storage Conditions

Component	Description	Storage
QuantiGene ViewRNA TYPE 1 Probe Set	RNA-specific oligonucleotides to your target of interest and compatible with the TYPE 1 Signal Amplification system comprised of: PreAmp Mix QT, Amp Mix QT, Label Probe 1-AP, and Fast Red substrate	–20 °C
QuantiGene ViewRNA TYPE 6 Probe Set	RNA-specific oligonucleotides to your target of interest and compatible with the TYPE 6 Signal Amplification system comprised of: PreAmp Mix QT, Amp Mix QT, Label Probe 6-AP, and Fast Blue Substrate	–20 °C

Required Materials and Equipment Not Provided

Other materials required to perform the QuantiGene ViewRNA ISH Tissue 2-Plex Assay that are not included in the QuantiGene ViewRNA ISH Tissue 2-Plex Assay Kit are listed here.

IMPORTANT: When specified, do not use alternate materials or suppliers.

Table 1.4 QuantiGene ViewRNA Tissue Assay Materials and Equipment Not Provided

Material	Source	Part Number
Tissue Tek Staining Dish (clear color, 3 required) (clear staining dish)	Affymetrix or American Master Tech Scientific	QVC0502 LWT4457EA
Tissue Tek Clearing Agent Dish (green color) (green clearing agent dish)	Affymetrix or American Master Tech Scientific	QVC0503 LWT4456EA
Tissue Tek Vertical 24 Slide Rack	American Master Tech Scientific	LWSRA24
Aluminum slide rack	VWR	100493380
Double-distilled water (ddH_20)	MLS (major laboratory supplier)	
95% Ethanol	VWR	89015-512
10X PBS, pH 7.2-7.4	Bio-Rad Laboratories or Invitrogen	161-0780 700134-032
Gill's Hematoxylin I	American Master Tech Scientific	HXGHE1LT
Histo-Clear or xylene	National Diagnostics Sigma	HS-200 247642
37% Formaldehyde	Fisher Scientific	F79-1
27-30% Ammonium Hydroxide	VWR	JT9726-5
Hydrophobic Barrier Pen	Affymetrix or Vector Laboratories	QVC0500 H4000

Table 1.4 QuantiGene ViewRNA Tissue Assay Materials and Equipment Not Provided (Continued)

Material	Source	Part Number
Ultramount or Advantage Mounting Media	DAKO Innovex	S1964 NB300
Cover Glass, 24 mm x 55 mm	VWR or Affymetrix	48382-138 QVC0501
Optional. DAPI[a]	Invitrogen	D3571
ThermoBrite Humidity Strips	Abbott Molecular	07J68-001
Equipment		
ThermoBrite Denaturation/Hybridization System 110/120 VAC	Abbott Molecular	07J91-010
QuantiGene View Temperature Validation Kit	Affymetrix	QV0523
Water-proof remote probe thermometers, validated for 90-100 °C	VWR	46610-024
1000 mL Glass Beaker	MLS	
Pipettes, P20, P200, P1000	MLS	
Fume hood (for dispensing formaldehyde and ammonium hydroxide)	MLS	
Isotemp Hot Plates	Fisher Scientific	11-300-49SHP (120V) 11-302-49SHP (230V)
Table-top microtube centrifuge	MLS	
Water Bath capable of maintaining 40 ± 1 °C	MLS	
Optional. Microplate Shaker (for washing steps)	VWR	12620-926
Microscope and imaging equipment	See *QuantiGene ViewRNA ISH Tissue 2-Plex Assay Imaging Options on page 6*	

[a] *Required for fluorescence detection*

Microscopy and Imaging Equipment Guidelines for QuantiGene ViewRNA ISH Tissue 2-Plex Assay

A unique benefit of the Affymetrix QuantiGene ViewRNA ISH Tissue 2-Plex Assay is that the stains used to label RNA can be visualized using both brightfield and fluorescence microscopy. The stain colors are described in Table 1.5.

Table 1.5 QuantiGene ViewRNA ISH Tissue 2-Plex Assay Stains

Stain/Detection Molecule	Staining Reagent	Stain Color/Fluorescence
RNA 1 (using TYPE 1 probe)	Fast Red	Red dot/Red dot
RNA 2 (using TYPE 6 probe)	Fast Blue	Aqua blue dot/Far red dot
Nuclear stain	Hemotoxylin/DAPI	Light purplish-blue/Blue

Table 1.6 QuantiGene ViewRNA ISH Tissue 2-Plex Assay Imaging Options

Viewing and Digital Capturing Options	Microscope Type	Recommended Microscope/ System	Required Optics	Recommended Filter
Brightfield viewing	Standard brightfield microscope	▪ Leica DM series ▪ Nikon E series ▪ Olympus BX series ▪ Zeiss Axio Lab/Scope /Imager ▪ Or equivalent	Requires 20 and 40x objectives	Requires neutral density filters and/or color filters for white balancing
Fluorescence viewing and image capture	Microscope with camera and fluorescence options Verify camera does not have infrared blocking filter	▪ Leica DM series ▪ Nikon E series ▪ Olympus BX series ▪ Zeiss Axio Lab/Scope/Imager ▪ Or equivalent	▪ Requires 20 and 40x objectives ▪ Numerical Aperture (NA) ≥0.5	For Fast Red Substrate, use Cy3/TRITC filter set: Excitation 530 ± 20 nm Emission: 590 ± 20 nm Dichroic: 562 nm For Fast Blue Substrate, use custom filter set:[a] Excitation: 630 ± 20 nm Emission: 775 ± 25 nm Dichroic: 750 nm For DAPI filter set Excitation: 387/11 nm Emission: 447/60 nm
Automated image capture in brightfield and/or fluorescence modes	Digital pathology scanner system	▪ Aperio ScanScope AT/XT/CS, use FL version for fluorescence ▪ Leica SCN400-F ▪ Olympus Nanozoomer RS ▪ Or similar	Recommend scanning at 40x when expression is low	Compatible to above

[a] *Recommended vendor: Semrock Cy7-B/Alexa 750 filter modified with excitation filter FF02-628/40-25.*

Assay Guidelines

Tissue Preparation Guidelines

The following are critical guidelines for preparation of FFPE tissue blocks, FFPE tissue slides and TMA slides for use with the QuantiGene ViewRNA ISH Tissue 2-Plex Assay. Samples prepared outside of these guidelines may not produce optimal results.

FFPE Tissue Block Preparation

- Upon removal of tissue, drop 100 mg of tissue into a minimum of 2 mL of fresh 10% Neutral Buffered Formalin (NBF) for 16-24 hr at room temperature. Cut tissue to a maximum of 3 mm thick section to ensure faster diffusion of NBF into tissue. To prevent RNA degradation, place tissues on dry ice or liquid nitrogen if it is not possible to fix the tissue immediately.
- Alternatively, tissues can be fixed in the same way with 4% paraformaldehyde (PFA) for 16-24 hr at room temperature.
- Rinse, dehydrate, and embed in paraffin block.
- Store FFPE tissue blocks at room temperature.

FFPE Tissue Slide Preparation

- Section FFPE tissue to the thickness of 5 ± 1 µm.
- Mount a single section onto one of the following positively-charged glass slides:
  - Leica Non Clipped X-tra® Slides, 1mm White P/N 3800200 or 3800210 (in U.S., Canada, and Asia Pacific region)
  - Tru Scientific TruBond360 P/N 0360W
  - Mercedes StarFrost Platinum P/N MER 7255
- Bake the sections at 60 °C for 16-24 hr.
- Store sections in a slide box at -20°C until use for up to 1 year (avoid freeze/thaw).
- Short term storage or shipping conditions at 4 °C is only recommended up to 2 weeks.

TMA Slide Preparation

- Construct TMA block with the following specifications:
  - Core size: 1.0 mm diameter or greater
  - Maximum TMA area: 20 mm x 30 mm
- Cut TMA sections to a thickness of 5 ± 1 µm.
- Mount a single section onto one of the following positively-charged glass slides:
  - Leica Non Clipped X-tra® Slides, 1mm White P/N 3800200 or 3800210 (in U.S., Canada, and Asia Pacific region)
  - Tru Scientific TruBond360 P/N 0360W
  - Mercedes StarFrost Platinum P/N MER 7255
- Bake the sections at 60 °C for 16-24 hr.
- Store TMAs in a slide box at -20°C until use for up to 1 year (avoid freeze/thaw).
- Short term storage or shipping conditions at 4 °C is only recommended up to 2 weeks.

Experimental Design Guidelines

Assay Controls

We recommend running positive and negative control slides, based on your sample type, in every QuantiGene ViewRNA ISH Tissue 2-Plex Assay. This will allow you to qualify/interpret your results.

Negative Control

This slide undergoes the entire assay procedure and assesses the assay background. The negative control can be one of the following:

- Omitting target Probe Set(s)
- Using a Probe Set designed to the sense strand of target
- Using a target not present in your sample, for example the bacterial gene DapB

Positive Control

This slide undergoes the entire assay procedure using Probe Set(s) for targets that have a consistently high to medium-high homogenous or cell-type specific expression in your sample type. This control ensures the assay procedure has been run successfully.

The following are examples of genes to use:

- Housekeeping genes: ACTB, GAPD, or UBC.
- Housekeeping Pan Panel (pool the individual housekeeping genes together)

Replicates

We recommend running all assays in duplicate.

Recommended Assay Optimization

When working with a new tissue type, we recommend performing the assay optimization procedure as described in *Assay Optimization Procedures on page 27* to identify the optimal pretreatment boiling time and protease digestion time to un-mask the mRNA. Applying the optimal condition will not only provide a favorable environment for the QuantiGene ViewRNA Probe Set to bind to the target mRNA but will also have an impact on the final chromogenic staining quality and tissue morphology. Once identified, the same optimal condition can be used for different Probe Sets. Tissues fixed in 4% PFA may not have the same optimization conditions as tissues fixed in 10% NBF. Therefore, if working with mixed set of samples, some prepared using 4% PFA and other prepared using 10% NBF, separate optimization assays should be run based on the fixative used.

If you are limited on samples for optimization, use the *Assay Optimization Lookup Table on page 33* for a general guideline. If you do not obtain the desired results, we recommend performing the assay optimization procedure.

Probe Set Considerations

Probe Sets of the same TYPE can be combined to create target panel ("pan") or cocktails. For instance, if one wanted to identify epithelial cells, this could easily be accomplished by pooling a panel of cytokeratin probe sets of the same type, such as TYPE 6, KRT5, KRT7, KRT8, KRT10, KRT18, KRT19 and KRT20 into a single assay. We do not recommend combining more than 10 targets for any one signal amplification system, be it TYPE 1 or TYPE 6. Another example might be to create a panel for housekeeping gene that can be used as a positive internal assay control to assess RNA integrity. In this case, you would combine TYPE 1 Probe Sets for UBC, ACTB, PPIB and GAPD into a single assay.

The typical design for a QuantiGene ViewRNA Probe Set consists of 20 pairs of oligos and spans approximately 1,000 bases of the target transcript to achieve maximal sensitivity. The binding of these oligos, side-by-side, to the target sequence serves as base for which the signal amplification is built and is the core of the assay's sensitivity and specificity. By using multiple pairs of oligos in a single Probe set, this ensure that there are many opportunities for the probe to bind to the target's unmasked/accessible

regions so as to achieve the maximal signal amplification possible for that particular RNA target molecule. When working with smaller targets, or applications such as splice variants or RNA fusions, the available number of oligo pairs in the Probe Set is naturally reduced and this will directly impact the sensitivity of the assay. That is, the probes will have fewer opportunities to find the unmasked areas of the target in order to generate signal at that location. In these cases, increasing the Probe Set concentration used in the assay might increase the sensitivity. However, it should be noted that there is always a general trade-off between sensitivity and specificity.

Assignment of Colors to Target mRNAs in 1- vs. 2-Plex Assay

The QuantiGene ViewRNA ISH Tissue Assay has multiplexing capability, allowing *in situ* detection of up to two mRNA targets simultaneously, using the QuantiGene ViewRNA TYPE 1 and/or TYPE 6 Probe Sets. The standard workflow of the assay is designed to automatically assign Fast Red signal to TYPE 1 and Fast Blue signal to TYPE 6 probe sets. While both the Fast Red and Fast Blue signals that form are easily visible under brightfield, the red dots generally have a much higher contrast than the blue dots, especially in the presence of hematoxylin. Thus, when the detection of only 1 target (i.e., 1-plex assay) is desired, we recommend using either TYPE 1 or TYPE 6 Probe Set and developing the signal as Fast Red. See Appendix C, *Modified Protocols for 1-Plex Assay* on page 35 for instructions on how to shorten the length of the assay when developing Fast Red or Fast Blue as a single-plex.

When performing a 2-plex assay, we recommend assigning the TYPE 1 probe set (Fast Red) to the more important target between the two and reserving the TYPE 6 probe set (Fast Blue) for the less critical target, such as a housekeeping gene. Due to the nature of chromogenic assay and the sequential development of Fast Blue then Fast Red signals, large quantities of blue precipitate that are deposited, particularly when a TYPE 6 target is expressed homogenously at high level, have the potential to partially block subsequent hybridization of the TYPE 1 Label Probe and consequently the development of the Fast Red signal. For this reason, the target assigned to Fast Blue should preferably have lower expression than the one assigned to Fast Red to ensure against any potential interference with Fast Red signal development downstream.

Table 2.1 Recommended Assignment of Colors to Target mRNAs in the 2-Plex Assay

TYPE 1 (Fast Red)	TYPE 6 (Fast Blue)
High expression	Low or medium expression
Medium expression	Low expression
Low expression	Low expression

If only medium and high expressing housekeeping targets are available in a particular tissue type and the critical target of interest has low to medium expression, a 2-plex assay can still be performed by assigning Fast Red to the housekeeping target and Fast Blue to the second target. Brightfield detection of the Fast Blue signal for a medium expressing transcript could still be easily done, while fluorescent detection would provide a more sensitive alternative for detecting a low expressing target tagged with Fast Blue.

Fluorescent Mode Guidelines

The advantage of using alkaline phosphatase-conjugated label probe for the enzymatic signal amplification is the availability of substrates with dual property, such as Fast Red and Fast Blue, which allows for both chromogenic and fluorescent detection of the targets. However, for a 2-plex assay in which both Label Probe 1 and Label Probe 6 are conjugated to the same alkaline phosphatase, the enzymes conjugates are unable to differentiate between Fast Red and Fast Blue if both substrates are added simultaneously. As a result, the enzymatic signal amplification has to be performed sequentially in order to direct substrate/color specificity to each target. Additionally, complete inactivation of the first alkaline phosphatase-conjugated label probe (LP6-AP) is necessary, especially when employing fluorescence mode for the detection of the targets. Otherwise, the residual LP6-AP activity can also convert Fast Red substrate in subsequent step into a red signal even at locations where TYPE 1 target is not present, giving a false impression that the Fast Blue and Fast Red signals are colocalized. For this

reason, it is absolutely necessary to quench any residual LP6-AP activity with the QuantiGene ViewRNA AP Stop QT prior to proceeding with the second label probe hybridization and development of the Fast Red color as this will ensure specific signals in fluorescent mode and brighter aqua blue dots in chromogenic mode.

Fast Red has a very broad emission spectrum and its bright signal that can bleed into adjacent Cy5 channel if one uses the standard Cy3/Cy5 filter sets for imaging. For this reason, it is critical that the recommended filter set for Fast Blue detection be used to avoid spectral bleed through of the Fast Red signal into the Fast Blue channel and interfering with Fast Blue detection. Please refer to Table 1.6 on page 6 for exact filter set specifications.

Limitations of Chromogenic *in situ* Assay in Colocalization Studies

When employing the QuantiGene ViewRNA ISH Tissue 2-Plex Assay for colocalization studies, it is crucial to understand the assay's strengths and limitations. By definition, a requisite for *in situ* detection is target accessibility. While the assay, with its branched DNA technology, has the capability to detect RNA molecules down to single-copy sensitivity and the probe sets are designed to maximize the binding opportunities to all accessible regions of the targets, the overall detection for any given target is only as good as the unmasking of the target site is able to provide. This essentially means that *in situ* assays in general are only capable of relative and not absolute detection. That is, not every single molecule of a given target can be detected. So in practice, even if two RNA targets are theoretically expected to be colocalized, only a subset these two transcripts will be detected as being so due to lack of complete target accessibility.

Another factor that can limit the use of this assay for colocalization studies is the nature of chromogenic assay and the sequential development of Fast Blue then Fast Red signals. In chromogenic assay, the enzyme converts the substrate into color precipitates and deposits them at the site where the RNA molecule is localized. Because the Fast Blue and Fast Red substrates are sequentially developed in the QuantiGene ViewRNA ISH Tissue 2-Plex Assay, the Fast Blue precipitates that are formed first and deposited have the potential to partially block subsequent hybridization of the TYPE 1 Label Probe, by masking its binding sites on a nearby/colocalized target and consequently affecting the development of the Fast Red signal. This is yet another form of accessibility issue that needs to be considered when performing colocalization studies and analyzing the data obtained from such studies. Consequently, even when two targets are colocalized, only a subpopulation of the two is actually observed as such because of target accessibility, be it at the probe hybridization step due to incomplete unmasking or at the label probe hybridization step due to masking of the binding site by the deposition of the Fast Blue precipitates.

Guidelines for Working with Tissue Microarrays (TMAs)

Process TMA slides using the same assay procedures but with the following two modifications:

- Increase the initial baking step time from 30 minutes to 60-120 minutes. This additional baking time will increase the tissue attachment to the slide, reducing the risk of the small (>1 mm) core sections falling off during assay procedure.
- Increase the volume/slide of the Protease Working Solution to prevent drying out of tissues at the edges of the TMA.

When designing TMAs to be used in the QuantiGene ViewRNA assay, it is important to understand that only one optimized condition can be used when running the assay. Therefore, if you want multiple tissue types within the same TMA block, we recommend running an optimization procedure on each individual FFPE tissue type to identify the most favorable pretreatment boiling and protease condition. Based on the optimal condition of the tissue morphology, signal strength, and residual cores, you can judge if there is one optimization condition that will be suitable for all the sample types.

QuantiGene ViewRNA ISH Tissue 2-Plex Assay Procedure

About the QuantiGene ViewRNA ISH Tissue 2-Plex Assay Procedure

The QuantiGene ViewRNA ISH Tissue 2-Plex Assay procedure is broken up into 2 parts that are performed over 2 days:

- Part 1: Sample Preparation and Target Probe Set Hybridization (day 1)
- Part 2: Signal Amplification and Detection (day 2)

We do not recommend stopping the procedure at any other point in the assay.

Important Procedural Notes and Guidelines

- Procedure assumes running a maximum of 12 slides at a time.
- Do not mix and match kit components from different kit lots.
- Before beginning procedure, know the pretreatment boiling time and protease digestion time (optimized conditions) for your sample type. If you do not know these optimized conditions, refer to Appendix A, *Assay Optimization Procedures* on page 27.
- Throughout the procedure, dedicate one clear staining dish for fixing in formaldehyde (we recommend labeling this dish). The other two clear staining dishes can be used interchangeably for: 1X PBS, 95% Ethanol, Wash Buffer and Storage Buffer. Rinse staining dishes in between steps with ddH_2O.
- Typical processing times included in the assay procedure assume that preparation for the following step is being done during the incubation periods.

Essential Keys for a Successful Assay

- Prepare samples following *Tissue Preparation Guidelines on page 7*.
- Organize the preparation of the assay before you start:
  - Verify that all materials and equipment are available
  - Be mindful of the incubation times/temperatures, there are small tolerances
  - Double-check all reagent calculations, concentration of reagents is critical
- Employ good washing techniques. Frequently, this washing is performed too gently. Adequate washing is important for consistent low backgrounds.
- Verify and validate temperatures for all equipment using the QuantiGene View Temperature Validation Kit
- DO NOT let tissues dry out where indicated in the procedure
- Incorporate controls, both positive and negative, so that results are unambiguous and can be interpreted. See *Experimental Design Guidelines on page 8*.

Refer to the Quick Reference Guide to quickly get an overview of the assay workflow. Once you become familiar with the procedures, you can rely on this quick guide and a Reagent Preparation Guide for running the assay.

Part 1: Sample Preparation and Target Probe Hybridization

Part 1 Procedure

Step	Action
Step 1. Bake Slides **35 min**	**A.** Use a pencil to label the slides. **B.** Set ThermoBrite at 60 ± 1°C and bake the slides for 30 min with the lid open. **NOTE:** **This increases tissue attachment to the slide.**
Step 2. Prepare Buffers and Reagents While Slides Bake	**A.** Prepare 3 L of 1X PBS: To a 3 L container add 300 mL of 10X PBS and 2.7 L ddH_2O. **B.** Prepare 10% formaldehyde in 1X PBS in a fume hood: To a 200 mL capacity container add 146 ml 1X PBS and 54 mL of 37% formaldehyde and mix well. **C.** Prepare 4% formaldehyde in 1X PBS in a fume hood: To a 200 mL capacity container add 22 mL of 37% formaldehyde to 178 mL 1X PBS and mix well. **D.** Prepare 4 L of Wash Buffer: To a 4 L capacity container add components in the following order and mix well: ▪ 3 L ddH_2O ▪ 36 mL Wash Comp 1 ▪ 10 mL Wash Comp 2 ▪ ddH_2O to 4 L. **E.** Prepare 500 mL of 1X Pretreatment Solution in a 1 L glass beaker: Dilute 5 mL of 100X Pretreatment Solution in 495 mL ddH_2O. **F.** Prepare 200 mL of Storage Buffer: To a 200 mL container add 60 mL of Wash Comp 2 to 140 mL ddH_2O and mix well. **G.** Ensure availability of: ▪ 400 mL Histo-Clear or xylene ▪ 400 mL 95% ethanol (not required if using xylene) ▪ 400 mL ddH_2O **H.** Prewarm 40 mL of 1X PBS and Probe Set Diluent QT to 40 ± 1 °C. **I.** Thaw Probe Set(s). Place on ice until use. **J.** *Optional.* If using a microplate shaker for the washes, set the speed to 550 rpm. Simply place a slide rack in a clear staining dish containing the appropriate reagent, insert the slides into the rack, manually lift the rack up and down 10 times and then place the entire staining dish on the platform of the microplate shaker equipped with a non-skid pad and shake for the recommended amount of time.
Step 3. Fix Slides **1 hr 3 min**	**A.** In a fume hood, pour 200 mL of 10% formaldehyde into clear staining dish. **B.** Insert slides into an empty slide rack. **C.** Submerge the slides into the 10% formaldehyde solution and fix for 1 hour at room temperature (RT) in a fume hood. **D.** Remove the slide rack from the 10% formaldehyde and wash twice with 1X PBS, each time with 200 mL for 1 min with frequent agitation. **E.** Remove each slide and flick it to remove the 1X PBS. Tap the slide on its edge then wipe the backside on a laboratory wipe to eliminate excess 1X PBS. Place the slides face up on a paper towel to air dry. Make sure the slides are completely dry before going to the next step.

Step	Action
Step 4. Deparaffinization **30 min**	If using Histo-Clear: **A.** Pour 200 mL of Histo-Clear into a green clearing dish and insert an empty slide rack. **B.** Set the ThermoBrite to 80 ± 1 °C. **C.** Bake the slides on the ThermoBrite with the lid open at 80 °C for 3 min to melt the paraffin. **D.** Immediately insert the warm slides in the Histo-Clear and agitate frequently by moving the rack up and down for 5 min at RT. **E.** Discard the used Histo-Clear and refill with another 200 mL of fresh Histo-Clear. Agitate frequently by moving the rack up and down for another 5 min at RT. **F.** Remove the slide rack from the Histo-Clear and wash the slides twice, each time with 200 mL of 95% ethanol for 1 min with frequent agitation. **G.** Remove the slides from the rack and place them face up on a paper towel to air dry for 5 min at RT. If using xylene: **A.** In a fume hood, pour 200 mL of xylene into a green clearing agent dish. **B.** Load the slides into a slide rack and transfer the rack to the green clearing dish containing the xylene. **C.** Incubate the slides in a fume hood, at RT in xylene for 5 min with frequent agitation. **D.** Discard the used xylene and refill with another 200 mL of fresh xylene. Agitate frequently by moving the rack up and down for another 5 min at RT. **E.** Remove the slide rack from the xylene, and wash the slides twice, each time with 200 mL of 95% ethanol for 1 min with frequent agitation. **F.** Remove the slides from the rack and place them face up on a paper towel to air dry for 5 min at RT.
Step 5. Draw Hydrophobic Barrier **1 hr**	**A.** Dab the hydrophobic pen on a paper towel several times before use to ensure proper flow of the hydrophobic solution. **B.** To create a hydrophobic barrier, place the slide over the template image below, making sure that the tissue sections fall inside the blue rectangle, and lightly trace the thick blue rectangle 2 to 4 times with the Hydrophobic Barrier Pen to ensure a solid seal. Allow for barrier to dry at RT for 20-30 min.

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>Step 6. Tissue Pretreatment

10-25 min, depending on optimized time</td><td>A. Tightly cover the beaker containing the 500 mL of 1X Pretreatment Solution with aluminum foil, place it on a hot plate and heat the solution to a temperature of 95 °C. Use a water-proof probe thermometer to measure and maintain the temperature of the solution at 90-95 °C during the pretreatment period.

NOTE: The temperature will drop a few degrees as the samples are added, so it is prudent to have the temperature be on the higher end of this range when adding the slides

B. Load the slides into the slide rack.
C. Using a pair of forceps, submerge the slide rack into the heated 1X Pretreatment Solution. Cover the glass beaker with aluminum foil and incubate at 90-95 °C for the optimal time as determined in Assay Optimization Procedures on page 27.
D. After the pretreatment, remove the slide rack with forceps, submerge it into a clearing dish containing 200 mL of ddH$_2$0 and wash for 1 min with frequent agitation.
E. Repeat the wash one more time with another 200 mL of fresh ddH$_2$0.
F. Transfer the slide rack to a clear staining dish containing 1X PBS.

IMPORTANT: From this point forward do not let the tissue sections dry out.</td></tr>
<tr><td>Step 7. Protease Digestion and Fixation

30-50 min, depending on optimized time</td><td>A. Set the ThermoBrite to 40 ± 1 °C and insert two ThermoBrite Humidity strips.
B. Using the table below as a guide, prepare the Working Protease Solution by diluting the Protease QF 1:100 in prewarmed 1X PBS. Scale reagents according to the number of assays to be run. Include one slide volume overage.

<table><tr><th colspan="2">Working Protease Solution per Slide</th></tr><tr><th>Reagent</th><th>Volume</th></tr><tr><td>Protease QF</td><td>4 µL</td></tr><tr><td>1X PBS (prewarmed to 40 °C)</td><td>396 µL</td></tr><tr><td>Total volume</td><td>400 µL</td></tr></table>
C. Remove each slide and flick it to remove excess 1X PBS. Tap the slide on its edge then wipe the backside on a laboratory wipe.
D. Place the slides, face up, flat on a lab bench and immediately add 400 µL of the Working Protease Solution onto the tissue section.
E. Transfer the slides to the ThermoBrite. Close the lid and incubate at 40 °C for the optimal time as determined in the in the Assay Optimization Procedures on page 27.
F. Pour 200mL of 1X PBS into a clear staining dish and insert an empty slide rack into it.
G. After the incubation, decant the Working Protease Solution from the slides, insert the slides into a rack and wash gently by moving the rack up and down for 1 min.
H. Repeat the wash one more time with another 200 mL of fresh 1X PBS.
I. Transfer the slide rack to a clear staining dish containing 200 mL of 4% formaldehyde and fix for 5 min at RT under a fume hood.
J. Wash the slides twice, each time with 200 mL of fresh 1X PBS for 1 min with frequent agitation.
K. Transfer the 4% formaldehyde solution to a 200 mL capacity container and keep for later use in Step 24. Apply Fast Red Substrate.</td></tr>
</table>

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>Step 8. Target Probe Set Hybridization

2 hr 10 min</td><td>
A. Using the table below as a guide, prepare the Working Probe Set Solutions by diluting the QuantiGene ViewRNA Probe Set(s) 1:40 in prewarmed Probe Set Diluent QT and briefly vortex. Scale reagents according to the number of assays to be run. Include one slide volume overage.

<table>
<tr><th colspan="2">Working Probe Set Solution per Slide</th></tr>
<tr><th>Reagent</th><th>Volume</th></tr>
<tr><td>Probe Set Diluent QT (prewarmed to 40 °C)</td><td>380 µL</td></tr>
<tr><td>QuantiGene ViewRNA TYPE 1 Probe Set</td><td>10 µL</td></tr>
<tr><td>QuantiGene ViewRNA TYPE 6 Probe Set</td><td>10 µL</td></tr>
<tr><td>Total volume</td><td>400 µL</td></tr>
</table>

B. Remove each slide and flick it to remove 1X PBS. Tap the slide on its edge then wipe the backside on a laboratory wipe.
C. Place the slides, face up, flat on the lab bench and immediately add 400 µL Working Probe Set Solution to each tissue section.
D. Transfer the slides to the ThermoBrite, close the lid and incubate at 40 °C for 2 hr.
</td></tr>
<tr><td>Step 9. Wash Slides

10 min</td><td>
A. Insert an empty slide rack into a clear staining dish containing 200 mL of Wash Buffer.
B. After incubation, decant the Working Probe Set Solution from the slides and insert them into the slide rack.
C. Wash the slides at RT for 2 min with frequent agitation.
D. Repeat the wash two more times, for a total of 3 washes, each time with 200 mL of fresh Wash Buffer at RT for 2 min with constant and vigorous agitation.
</td></tr>
<tr><td>Step 10. Stop Point

1 min</td><td>
A. Store slides in a clear staining dish containing 200 mL of Storage Buffer for up to 24 hours at RT.
B. The following reagent preparations should be stored at RT for use in Part 2:
▪ 4% formaldehyde
▪ 1X PBS
▪ Wash Buffer
C. All other reagent and solution preparations should be discarded.
D. Rewet the ThermoBrite Humidity Strips in ddH_2O.
E. When you are ready to continue the assay, proceed to Step 11. Prepare Additional Buffers and Reagents on page 16.
</td></tr>
</table>

Part 2: Signal Amplification and Detection

Part 2 Procedure

Step	Action
Step 11. Prepare Additional Buffers and Reagents **10 min**	**A.** Prepare 1 L of 0.01% ammonium hydroxide in ddH_2O: In a fume hood, add 0.33 mL 30% ammonium hydroxide to 999.67 mL ddH_2O and mix well. **B.** Ensure availability of 200 mL Gill's Hematoxylin. Pour into a clear staining dish and store at RT away from light until use. **C.** If you plan on using fluorescence detection, prepare 200 mL DAPI. The final dilution of DAPI should be 3.0 µg/mL in 1X PBS. Store in the dark at 4 °C until use or place on ice. **D.** Prewarm PreAmplifier Mix QT, Amplifier Mix QT, and Label Probe Diluent QF buffers to 40 °C. **E.** Place Label Probe 1-AP, Label Probe 6-AP, and Blue reagents on ice. **F.** Bring Fast Red Tablets, Naphthol Buffer, AP Enhancer Solution, and Blue Buffer to RT.
Step 12. Wash Slides **5 min**	**A.** Remove the slides from Storage Buffer. **B.** Wash slides twice, each time with 200 mL of fresh Wash Buffer for 2 min with constant agitation.
Step 13. PreAmp Hybridization **30 min**	**A.** Set the ThermoBrite to 40 ± 1 °C and insert twoThermoBrite Humidity strips. **B.** Swirl PreAmplifier Mix QT bottle briefly to mix the solution. **C.** Remove each slide and flick it to remove the Wash Buffer. Tap the slide on its edge then wipe the backside on a laboratory wipe. Place slides face up, flat on the lab bench and immediately add 400 µL of PreAmplifier Mix QT directly to each tissue section. **D.** Transfer slides in the ThermoBrite. Close the lid and incubate at 40 °C for 25 min.
Step 14. Wash Slides **10 min**	**A.** Insert an empty slide rack into a clear staining dish containing 200 mL of Wash Buffer. **B.** After incubation, decant the PreAmplifier Mix QT from the slides and insert them into the slide rack. **C.** Wash the slides 3 times, each time with 200 mL of fresh Wash Buffer at RT for 2 min with constant and vigorous agitation.
Step 15. Amp Hybridization **20 min**	**A.** Swirl Amplifier Mix QT bottle briefly to mix the solution. **B.** Remove each slide and flick it to remove the Wash Buffer. Tap the slide on its edge then wipe the backside on a laboratory wipe. Place slides face up, flat on the lab bench and immediately add 400 µL of Amplifier Mix QT directly to each tissue section. **C.** Transfer slides in the ThermoBrite. Close the lid and incubate at 40 °C for 15 min.
Step 16. Wash Slides **10 min**	**A.** Insert an empty slide rack into a clear staining dish containing 200 mL of Wash Buffer. **B.** After incubation, decant the Amplifier Mix QT from the slides and insert them into the slide rack. **C.** Wash the slides 3 times, each time with 200 mL of fresh Wash Buffer at RT for 2 min with constant and vigorous agitation.

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>Step 17. Label Probe 6-AP Hybridization

20 min</td><td>
A. Briefly vortex and spin down Label Probe 6-AP before using.
B. Using the table below as a guide, prepare Working Label Probe 6-AP Solution by diluting 1:1000 in prewarmed Label Probe Diluent QF and briefly vortexing to mix. Scale reagents according to the number of assays to be run. Include one slide volume overage.
<table>
<tr><th colspan="2">Working Label Probe 6-AP Solution Per Slide</th></tr>
<tr><th>Reagent</th><th>Volume</th></tr>
<tr><td>Label Probe Diluent QF (prewarmed to 40 °C)</td><td>399.6 µL</td></tr>
<tr><td>Label Probe 6-AP</td><td>0.4 µL</td></tr>
<tr><td>Total volume</td><td>400 µL</td></tr>
</table>
C. Remove each slide and flick it to remove the Wash Buffer. Tap the slide on its edge then wipe the backside on a laboratory wipe. Place slides face up, flat on the lab bench and immediately add 400 µL of Working Label Probe 6-AP solution directly to each tissue section.
D. Transfer the slides in the ThermoBrite. Close the lid and incubate at 40 °C for 15 min.
</td></tr>
<tr><td>Step 18. Wash Slides

15 min</td><td>
A. Insert an empty slide rack into a clear staining dish containing 200 mL of Wash Buffer.
B. After incubation, decant the Working Label Probe 6-AP Solution from the slides and insert them into the slide rack.
C. Wash the slides 3 times, each time with 200 mL of fresh Wash Buffer at RT for 3 min with constant and vigorous agitation.
</td></tr>
<tr><td>Step 19. Apply Fast Blue Substrate

40 min</td><td>
A. Prepare the Fast Blue Substrate: in a 15 mL conical tube, add 5 mL of Blue Buffer. Add 105 µL of Blue Reagent 1, vortex, add 105 µL of Blue Reagent 2, vortex, and add 105 µL Blue Reagent 3, then briefly vortex. Protect from light by wrapping in aluminum foil until use.
B. Remove each slide and flick it to remove the Wash Buffer. Tap the slide on its edge then wipe the backside on a laboratory wipe. Place slides face up, flat on an aluminum slide rack.
C. Immediately add 400 µL Fast Blue Substrate and incubate in the dark at RT for 30 min.
</td></tr>
<tr><td>Step 20. Wash Slides

5 min</td><td>
A. Insert an empty slide rack into a clear staining dish containing 200 mL of Wash Buffer.
B. After incubation, decant the Fast Blue Substrate from the slides and insert them into the slide rack.
C. Wash the slides twice, each time with 200 mL of fresh Wash Buffer at RT for 2 min with frequent agitation.
</td></tr>
<tr><td>Step 21. Quenching of LP6-AP

35 min</td><td>
A. Remove each slide and flick it to remove the Wash Buffer. Tap the slide on its edge then wipe the backside on a laboratory wipe. Place slides flat, face up on an aluminum slide rack.
B. Immediately add 400 µL of the AP Stop QT and incubate in the dark at RT for 30 min.
C. Insert an empty slide rack into a clear staining dish containing 200 mL of 1X PBS.
D. After incubation, decant the AP Stop Buffer from the slides and insert them into the slide rack.
E. Wash the slides twice, each time in 200 mL of fresh 1X PBS at RT for 1 min with frequent agitation.
F. Replace the 1X PBS with 200 mL of fresh Wash Buffer and rinse the slides from any residual PBS by moving the slide rack up and down for 1 min.
</td></tr>
</table>

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>Step 22. Label Probe 1-AP Hybridization

20 min</td><td>
A. Briefly vortex and spin down Label Probe 1-AP before using.
B. Using the table below as a guide, prepare Working Label Probe 1-AP Solution by diluting 1:1000 in prewarmed Label Probe Diluent QF and briefly vortexing to mix. Scale reagents according to the number of assays to be run. Include one slide volume overage.
<table>
<tr><th colspan="2">Working Label Probe 1-AP Solution Per Slide</th></tr>
<tr><th>Reagent</th><th>Volume</th></tr>
<tr><td>Label Probe Diluent QF (prewarmed to 40 °C)</td><td>399.6 µL</td></tr>
<tr><td>Label Probe 1-AP</td><td>0.4 µL</td></tr>
<tr><td>Total volume</td><td>400 µL</td></tr>
</table>
C. Remove each slide and flick it to remove the Wash Buffer. Tap the slide on its edge then wipe the backside on a laboratory wipe. Place slides face up, flat on the lab bench and immediately add 400 µL of Working Label Probe 1-AP solution directly to each tissue section.
D. Transfer the slides to the ThermoBrite. Close the lid and incubate at 40 °C for 15 min.
</td></tr>
<tr><td>Step 23. Wash Slides

15 min</td><td>
A. Insert an empty slide rack into a clear staining dish containing 200 mL of Wash Buffer.
B. After incubation, decant the Working Label Probe 1-AP Solution from the slides and insert them into the slide rack.
C. Wash the slides 3 times, each time with 200 mL of fresh Wash Buffer at RT for 3 min with constant and vigorous agitation.
</td></tr>
<tr><td>Step 24. Apply Fast Red Substrate

1 hr</td><td>
A. Remove each slide and flick it to remove the Wash Buffer. Tap the slide on its edge then wipe the backside on a laboratory wipe. Place slides face up, flat on the lab bench.
B. Immediately add 400 µL of the AP-Enhancer Solution to each tissue section (pipet directly from bottle) and incubate at RT for 5 min while preparing the Fast Red Substrate.
C. Prepare the Fast Red Substrate: in a 15 ml conical tube, add 5 ml of Naphthol Buffer and one Fast Red Tablet. Vortex at high speed to completely dissolve the tablet. Protect from light by wrapping the tube in aluminum foil until use.
D. Decant the AP Enhancer Solution and flick the slide twice to completely remove any excess AP Enhancer Solution. Tap the slide on its edge then wipe the backside on a laboratory wipe. Immediately add 400 µL of Fast Red Substrate onto each tissue section.
E. Transfer the slides to the ThermoBrite. Close the lid and incubate at 40 °C for 30 min.
F. Insert an empty slide rack into a clear staining dish containing 200 mL of 1X PBS.
G. After incubation, decant the Fast Red Substrate from the slides and insert them into the slide rack.
H. Rinse off the excess Fast Red Substrate from the slides by moving the slide rack up and down for 1 min.
I. Fix the slide, under a fume hood, for 5 min in 200 mL of 4% formaldehyde (saved from Step 7. Protease Digestion and Fixation).
J. Rinse off the residual formaldehyde by transferring the slide rack to a clear staining dish containing 200 mL of fresh 1X PBS and washing it for 1 min with frequent agitation.
</td></tr>
</table>

Step	Action
Step 25. Counterstain **50 min**	**A.** Transfer the slide rack to the clear staining dish containing the 200 mL of Gill's Hematoxylin and stain for 5-10 sec at RT. **B.** Wash the slides 3 times, each with 200 mL of fresh ddH_2O for 1 min by moving the rack up and down to remove the excess Gill's Hematoxylin. **C.** Pour off the ddH_2O, refill with 200 mL of 0.01% ammonium hydroxide and incubate the slides for 10 sec. Unused 0.01% ammonium hydroxide can be stored at RT for up to one month. **D.** Wash the slides once in 200 mL of fresh ddH_2O by moving the rack up and down for 1 min. **E.** *Optional*. If you plan to view slides using the fluorescent microscope, then move slide rack into a clear staining dish containing 200 mL DAPI staining solution. Incubate the slides for 1 min. Decant DAPI staining solution, and rinse the slides with 200 mL fresh ddH_2O by moving the slide rack up and down for 1 min. **F.** Remove the slides from the slide rack and flick to remove the excess ddH_2O. Tap the slide on its edge then wipe the backside on a laboratory wipe. Place them face up onto paper towel to air dry in the dark. **G.** Ensure that slide sections are completely dry before mounting (about 20 min).
Step 26. Add Coverslip and Image **20 min**	If using DAKO Ultramount mounting medium: **A.** Dab the first 2-3 drops of mounting medium onto a paper towel to remove bubbles. **B.** Add a minimum of 2 drops of DAKO Ultramount mounting medium to tissue section without making any bubbles. Use a pipette tip to draw out any air bubbles in the droplets. **C.** Slowly place the cover glass onto the specimen slide at an angle. Make sure the cover glass comes into contact with the mounting medium first before completely releasing the cover glass to overlap with the glass slide. **D.** After mounting, place the slide on its edge on a laboratory wipe to remove excess mounting medium. Image the results under a brightfield and/or fluorescence microscope. **E.** Store the mounted slides at 4 °C to avoid bubble formation over time. If using Innovex Advantage mounting medium: **A.** Place a 24 mm x 55 mm cover glass horizontally onto a clean, flat surface. **B.** Dab the first 2-3 drops of mounting media onto a paper towel to remove bubbles. **C.** Add 2 drops of the Innovex Advantage medium directly onto the middle of the cover glass. Use a pipette tip to draw out any air bubbles in the droplets. **D.** Invert the specimen slide and slowly place it onto the mounting medium at an angle. Make sure the tissue comes into contact with the mounting medium first before completely letting go of the glass slide to overlap with the cover glass. **E.** After mounting, flip the slide over and place it on its edge on a laboratory wipe to soak up and remove excess mounting medium. Allow slide to dry at RT, in the dark for 15 min. Do not bake slides to speed up the drying process. **F.** To prevent bubble formation, seal all 4 edges of the cover glass with a flat black-colored nail polish, as iridescent and colored ones tend to give off autofluorescence and interfere with fluorescent imaging. **G.** Image the results under brightfield and/or fluorescence microscope. **H.** Store slides at RT.

Troubleshooting

Contacting Technical Support

For technical support, contact the appropriate resource provided below based on your geographical location. For an updated list of FAQs and product support literature, visit our website at www.affymetrix.com/panomics.

Table 4.1 Technical Support Contacts

Location	Contact Information
North America	1.877.726.6642 option 1, then option 3; pqbhelp@affymetrix.com
Europe	+44 1628-552550; techsupport_europe@affymetrix.com
Asia	+81 3 6430 430; techsupport_asia@affymetrix.com

Weak or No Signals

Table 4.2 Troubleshooting Weak or No Signal

Probable Cause	Recommended Action
Incorrect pretreatment conditions	Repeat pretreatment assay optimization procedure to determine optimal boiling time and protease digestion time.
Sample preparation, over-fixation	Make sure that freshly-dissected tissues are fixed in 10% neutral buffered formalin (NBF) or 4% paraformaldehyde (PFA) for 16-24 hr.
Improper fixation, reagents, or concentrations	Make sure correct concentration of NBF was used to fix the slides in respective steps.
Tissue dries up during hybridization steps	ThermoBrite recommendations: ▪ Prewet the ThermoBrite Humidity strips inside the ThermoBrite before starting hybridization ▪ Make sure the ThermoBrite is placed on a level bench. ▪ Calibrate the ThermoBrite to 40°C using QuantiGene View Temperature Validation Kit (Affymetrix P/N QV0523). ▪ Close the ThermoBrite lid during hybridization steps. Prevent sections from drying out by: ▪ Preparing enough reagents and use the recommended volumes for each step of the assay. ▪ Ensuring that you have a solid seal when drawing your hydrophobic barriers. ▪ Adding all working reagents onto the slides before moving them to the 40°C ThermoBrite.
Tissue dries up during processing	Keep tissue section moist starting from the pretreatment boiling step by: ▪ Adding respective reagents immediately after decanting solution from the slides. ▪ Limiting tissue exposure to air for too long before adding hybridization reagents. ▪ Adding all working reagents onto the slides before moving them to the 40°C ThermoBrite.
Tissue over-fixed after protease digestion	Make sure the tissue sections are not fixed for more than 5 min in 4% formaldehyde after protease digestion.
Reagents applied in wrong sequence	Apply target Probe Set (s), PreAmplifier Mix QT, Amplifier Mix QT, Label Probe-AP, and substrates in the correct order.

Table 4.2 Troubleshooting Weak or No Signal (Continued)

Probable Cause	Recommended Action
Incorrect storage condition	Store the components at the storage condition as written on the component label or kit boxes.
Hybridization temperature not optimal	Calibrate the ThermoBrite at 40°C using a QuantiGene View Temperature Validation Kit (Affymetrix P/N QV0523).
Probe Set hybridization temperature, time, and/or concentration not optimal	Decrease hybridization temperature from 40 to 38 °C and increase Probe Set concentration by diluting target Probe Set 1:30 instead of 1:40 and hybridize for 2 hr.
Label Probe-AP concentration too low	▪ Verify that the correct concentrations were used. ▪ Increase the recommended concentrations for Label Probe-AP. If this is necessary, it may result in higher backgrounds.
Mounting solution contained alcohol	Use DAKO Ultramount or Innovex Advantage mounting media to mount your tissue. Avoid any mounting solution containing alcohol.
Fast Red and Fast Blue Substrate solutions not freshly prepared	Prepare Fast Red and Fast Blue Substrate solutions immediately before use.
Gene of interest is not expressing	Verify expression using other tissue lysate methods such as QuantiGene, QuantiGene Plex assay or Affymetrix array. Run the same Probe Set on known samples that have been validated to express the gene of interest.
RNA in tissue is degraded	Verify tissue fixation: ▪ Ensure tissue was freshly harvested and immediately fixed in 10% NBF or 4% PFA for 16-24 hr. ▪ Ensure FFPE blocks and sections were stored correctly. Use a positive control Probe Set such as one used for a housekeeping gene or a housekeeping gene panel (ACTB, GAPD and UBC) to assess RNA integrity, using a 1-plex assay format.
Dark hematoxylin stain reduces visibility for the Blue dots	▪ Reduce hematoxylin staining time to 5 sec. Tissues with lower cell density require longer hematoxylin incubation than tissues that have higher cell density. It may be helpful to titrate incubation times. ▪ Increase brightness of lamp during viewing. ▪ View under 40X objective. ▪ Image under fluorescent mode.

Diffused Signals

Table 4.3 Troubleshooting diffused signals

Probable Cause	Recommended Action
Tissue dries up during processing	Keep tissue section moist starting from the pretreatment boiling step by: ▪ Adding respective reagents immediately after decanting solution from slides. ▪ Limiting tissue exposure to air for too long before adding hybridization reagents. ▪ Adding all working reagents onto the slides before moving them to the 40 °C ThermoBrite.
Insufficient washing in 1X PBS	Make sure tissues are washed in 1X PBS twice after protease digestion and twice again after subsequent fixing in 4% formaldehyde.
Fast Red substrate not freshly prepared	Prepare Fast Red substrate immediately before use.
Slides are not dried before mounting	Ensure that slide sections are completely dry before mounting (about 20 min).

Table 4.3 Troubleshooting diffused signals

Probable Cause	Recommended Action
Mounting solution contained alcohol	Use DAKO Ultramount or Innovex Advantage mounting media to mount your tissue. Avoid any mounting solution containing alcohol.

Diffused Background Signals in both ± Probe Samples

Table 4.4 Troubleshooting Diffused Background Signal in Both ± Probe Samples

Probable Cause	Recommended Action
Endogenous alkaline phosphatase activity	▪ Verify by incubating protease-treated sample with Fast Blue Substrate. If endogenous AP activity is present, diffused signals (that can be weak or strong) will appear. ▪ Inactivate endogenous AP with 0.2 M HCl for 10 min at RT before the protease step. Wash samples twice with 1X PBS before proceeding to protease digestion.

Poor Cell Morphology

Table 4.5 Troubleshooting poor cell morphology

Probable Cause	Recommended Action
Incorrect pretreatment conditions	See *Optimization Experimental Design Layout on page 27*.
Sample preparation not fixed properly	Make sure that freshly-dissected tissues are fixed in 10% NBF or 4% PFA for 16-24 hours.
Section thickness not optimal	Make sure tissues are sectioned at 5 ± 1 µm thick.

Tissue Detachment from Slide

Table 4.6 Troubleshooting tissue detachment from slides

Probable Cause	Recommended Action
Insufficient baking of slides	▪ Verify that 30 min baking step was done. ▪ It may be necessary to increase baking time to 1 hr.
Incorrect pretreatment conditions	Perform full assay optimization procedure to determine optimal boiling time and protease digestion time.
Improper fixation, reagents, or concentrations	Make sure the correct concentration of NBF was used to fix the slides in the respective steps.
Temperature of pretreatment condition too high	Make sure the temperature is within the tolerance range of 90-95 °C. For fatty, soft tissue such as breast, adjust to 90 °C.
Protease treatment is too long or at too high concentration	Reduce protease concentration and/or incubation time.

High Non-Specific Binding on Glass Slide

Table 4.7 Troubleshooting non-specific binding to slide

Probable Cause	Recommended Action
Incompatible glass slide	Use the glass slides from the following recommended vendors: ■ Leica Non Clipped X-tra® Slides, 1mm White P/N 3800200 or 3800210 ■ Tru Scientific TruBond360 P/N 0360W ■ Mercedes StarFrost Platinum P/N MER 7255 Prevalidate each new batch of slides by running the entire assay, including Probe Set(s), on empty slides (without fixed tissues) to determine if the slides are suitable for the assay. Decrease Probe Set concentration by diluting target Probe Set 1:50 instead of 1:40 and hybridize for 3 hr at 40 °C.
Insufficient washing	■ Move the slide rack up and down with frequent agitation. ■ Increase wash incubation time by 1 min per wash.

Pink Non-Specific Background Where Paraffin Used to Be

Table 4.8 Troubleshooting Pink or Blue Background Where Paraffin was Present

Probable Cause	Recommended Action
Incomplete removal of paraffin	■ Be sure to use fresh Histo-Clear or xylene for the indicated amount of time during the dewaxing step. ■ Use 3 changes of Histo-Clear or xylene instead of 2.
Polymerization of poor quality paraffin	Melt the paraffin at 60 °C instead of 80 °C for 3 min and remove paraffin using 3 changes of fresh Histo-Clear.

Hydrophobic Barrier Falls Off

Table 4.9 Troubleshooting hydrophobic barrier problems

Probable Cause	Recommended Action
Incompatible glass slide	Use the glass slides from the following recommended vendors: ■ Leica Non Clipped X-tra® Slides, 1mm White P/N 3800200 or 3800210 ■ Tru Scientific TruBond360 P/N 0360W ■ Mercedes StartFrost Platinum P/N MER 7255 Prevalidate each new batch of slides by drawing a hydrophobic barrier onto an empty slide (without fixed tissue), allow it to dry for 20-30 min, boil in pretreatment solution for 40 min to determine if the hydrophobic barrier is intact and the slides are suitable for the assay.
Incorrect hydrophobic pen	Use Hydrophobic Barrier Pen (Affymetrix QVC0500 or Vector Laboratories H4000).
Hydrophobic barrier was not dried completely	Allow 20-30 min for hydrophobic barrier to dry completely before proceeding to the next step.

High Background

Table 4.10 Troubleshooting high background

Probable Cause	Recommended Action
Tissue dries up during hybridization steps	Keep tissue section moist starting from the pretreatment boiling step by: ■ Adding respective reagents immediately after decanting solution from the slides. ■ Limiting tissue exposure to air for too long before adding hybridization reagents. ■ Adding all working reagents onto the slides before moving them to the 40°C ThermoBrite.
Incomplete removal of paraffin	Use fresh Histo-Clear solution. Immediately submerge the warm slides into the Histo-Clear solution after 80 °C baking and move the slide rack up and down with frequent agitation.
Incorrect pretreatment conditions	Repeat pretreatment assay optimization procedure to determine optimal boiling time and protease digestion time.
Insufficient washing	■ Move the slide rack up and down with frequent agitation. ■ Increase wash incubation time by 1 min per wash.
Concentration of hybridization reagents was too high	Double check the dilution calculation for all working solutions.
Hybridization temperature not optimal	Calibrate the ThermoBrite at 40 °C using the QuantiGene View Temperature Validation Kit (Affymetrix P/N QV0523).
Label Probe-AP concentration too high	■ Verify that the correct concentrations were used. ■ Decrease the recommended concentration for Label Probe-AP.

Fast Red Signal for TYPE 1 Target is Weak or Different in 2-Plex Versus 1-Plex

Table 4.11 Troubleshooting Weak or Different Fast Red Signal for TYPE 1 Target in 2-Plex Versus 1-Plex

Probable Cause	Recommended Action
Cross-inhibition of LP1-AP by Fast Blue precipitate	Assign lower expressing target to TYPE 6 (Fast Blue) and higher expressing target to TYPE 1 (Fast Red).
Colocalization of TYPE 1 and TYPE 6 targets	Perform assay as a 1-plex for each target.
	Assign lower expressing target to TYPE 6 (Fast Blue) and higher expressing target to TYPE 1 (Fast Red).
	If colocalization study is desired, try reducing development time for Fast Blue from 30 min to 10-15 min.

TYPE 1 Target Signals are also Observed in the Channel for TYPE 6 Target

Table 4.12 Troubleshooting TYPE 1 Target Signals Observed in the Channel for TYPE 6 Target

Probable Cause	Recommended Action
Spectral bleed through of Fast Red signal	Check to make sure that the filter set for Fast Blue is as recommended.
Incorrect filter set for Fast Blue signal	Use the correct filter set. Refer to *QuantiGene ViewRNA ISH Tissue 2-Plex Assay Imaging Options* for the specifications of the filter set recommended for Fast Blue.

Colocalized Fast Blue and Fast Red Signals When Only TYPE 6 Probe Set is Used in the 2-Plex Assay

Table 4.13 Troubleshooting Colocalized Fast Blue and Fast Red Signals When Using Only TYPE 6 in a 2-Plex Assay

Probable Cause	Recommended Action
Residual LP6-AP activity	▪ Do not omit *Step 21. Quenching of LP6-AP*. ▪ Be sure to quench LP6-AP activity with AP Stop QT for the entire 30 min.

Assay Optimization Procedures

About the Optimization and Typical Results

The QuantiGene ViewRNA ISH Tissue 2-Plex Assay procedure is broken up into 2 parts that are performed over 2 days:

- Part 1: Sample Preparation and Target Probe Set Hybridization (day 1)
- Part 2: Signal Amplification and Detection (day 2)

We do not recommend stopping the procedure at any other point in the assay.

The two conditions to be optimized, tissue pretreatment boiling time and protease digestion time are included in Part 1: Sample Preparation.

Optimization Procedure Overview

You will need to prepare ten, 5 ± 1 µm thick FFPE tissue sections from a block, or blocks which were prepared in the same way (fixation time, section thickness, and tissue type) as the FFPE tissue of your interest. Each slide will be treated with a different set of conditions as described in Table A.1. With the exception of Slide 7, hybridize every slide with medium expression housekeeping genes, for example, ACTB and GAPD. These control targets should have consistent homogenous expression in your samples. Once an optimal assay condition is determined for your sample type, apply those conditions to your targets of interest.

Optimization Experimental Design Layout

Table A.1 Optimization Experiment Setup

Protease Incubation Time (min)	Pretreatment Boiling Time (min)			
	0	**5**	**10**	**20**
0	Slide 1 with probe			
10		Slide 2 with Probe	Slide 5 with probe	Slide 9 with probe
20		Slide 3 with probe	Slide 6 with probe Slide 7 with no probe	Slide 10 with probe
40		Slide 4 with probe	Slide 8 with probe	

Important Procedural Notes and Guidelines

- Procedure assumes running a maximum of 12 slides at a time.
- Do not mix and match kit components from different kit lots.
- Throughout the procedure, dedicate one clear staining dish for fixing in formaldehyde (we recommend labeling this dish). The other two clear staining dishes can be used interchangeably for: 1X PBS, 95% Ethanol, Wash Buffer and Storage Buffer. Rinse staining dishes in between steps with ddH_2O.
- Typical processing times included in the assay procedure assume that preparation for the following step is being done during the incubation periods.

Essential Keys for a Successful Assay

- Prepare samples following the *Tissue Preparation Guidelines on page 7*.
- Organize the preparation of the assay before you start:
  - Verify that all materials and equipment are available
  - Be mindful of the incubation times/temperatures, there are small tolerances
  - Double-check all reagent calculations, concentration of reagents is critical
- Employ good washing techniques. Frequently, this washing is performed too gently. Adequate washing is important for consistent low backgrounds.
- Verify and validate temperatures for all equipment
- DO NOT let tissues dry out where indicated in the procedure

- Incorporate controls, both positive and negative, so that results are unambiguous and can be interpreted. See *Experimental Design Guidelines on page 8*.

Sample Preparation and Target Probe Hybridization

Procedure

Step	Action
Step 1. Bake Slides **35 min**	**A.** Use a pencil to label the slides. **B.** Set ThermoBrite to 60 ± 1°C and bake the slides for 30 min with the lid open. **NOTE:** This increases tissue attachment to the slide.

Step	Action
Step 2. Prepare Buffers and Reagents While Slides Bake	**A.** Prepare 3 L of 1X PBS: To a 3 L container add 300 mL of 10X PBS and 2.7 L ddH_2O. **B.** Prepare 10% formaldehyde in 1X PBS in a fume hood: To a 200 mL capacity container add 146 mL 1X PBS and 54 mL of 37% formaldehyde and mix well. **C.** Prepare 4% formaldehyde in 1X PBS in a fume hood: To a 200 mL capacity container add 22 mL of 37% formaldehyde to 178 mL 1X PBS and mix well. **D.** Prepare 4 L of Wash Buffer: To a 4 L capacity container add components in the following order and mix well: - 3 L ddH_2O - 36 mL Wash Comp 1 - 10 mL Wash Comp 2 - ddH_2O to 4 L **E.** Prepare 500 mL of 1X Pretreatment Solution in a 1 L glass beaker: Dilute 5 mL of 100X Pretreatment Solution in 495 mL ddH_2O. **F.** Prepare 200 mL of Storage Buffer: To a 200 mL container add 60 mL of Wash Comp 2 to 140 mL ddH_2O and mix well. **G.** Ensure availability of: - 400 mL Histo-Clear or xylene - 400 mL 95% ethanol (not required if using xylene) - 400 mL ddH_2O **H.** Prewarm 40 mL of 1X PBS and Probe Set Diluent QT to 40 ± 1 °C. **I.** Thaw Probe Set(s). Place on ice until use.
Step 3. Fix Slides **1 hr 5 min**	**A.** In a fume hood, pour 200 mL of 10% formaldehyde into clear staining dish. **B.** Insert the baked slides into an empty slide rack. **C.** Submerge the slides into the 10% formaldehyde solution and fix for 1 hr at room temperature (RT) in a fume hood. **D.** Remove the slide rack from the 10% formaldehyde solution and wash twice with 1X PBS, each time with 200 mL for 1 min with frequent agitation. **E.** Remove each slide from the rack and flick it to remove the 1X PBS. Tap the edge and wipe the backside of the slide against a laboratory wipe to eliminate excess 1X PBS. Place the slides face up on a paper towel to air dry. Make sure the slides are completely dry before going to the next step.

Step	Action
Step 4. Deparaffinization **30 min**	If using Histo-Clear: **A.** Pour 200 mL of Histo-Clear into a green clearing agent dish and insert an empty slide rack. **B.** Set ThermoBrite to 80 ± 1 °C. **C.** Bake the slides on the ThermoBrite with the lid open at 80 °C for 3 min to melt the paraffin. **D.** Immediately insert the warm slides in the Histo-Clear and agitate frequently, by moving the rack up and down for 5 min at RT. **E.** Discard the used Histo-Clear and refill with another 200 mL of fresh Histo-Clear. Agitate frequently by moving the rack up and down for another 5 min at RT. **F.** Remove the slide rack from the Histo-Clear and wash the slides twice, each time with 200 mL of 95% ethanol for 1 min with frequent agitation. **G.** Remove the slides from the rack and place them face up on a paper towel to air dry for 5 min at RT. If using xylene: **A.** Pour 200 mL of xylene (in a fume hood) into green clearing agent dish. **B.** Load the slides into the slide rack and transfer the slide rack to the green clearing dish containing 200 mL xylene. **C.** Incubate in a fume hood at RT for 5 min with frequent agitation. **D.** Discard the used xylene and refill with another 200 mL of fresh xylene. Agitate frequently by moving the rack up and down for another 5 min at RT. **E.** Remove the slide rack from the xylene, and wash the slides twice, each time with 200 mL of 95% ethanol for 1 min with frequent agitation. **F.** Remove the slides from the rack and place them face up on a paper towel to air dry for 5 min at RT.
Step 5. Draw Hydrophobic Barrier **1 hr**	**A.** Dab the hydrophobic pen on a paper towel several times before use to ensure proper flow of the hydrophobic solution. **B.** To create a hydrophobic barrier, place the slide over the template image below, tissue sections should fall inside blue rectangle, and lightly trace the thick blue rectangle 2 to 4 times with the Hydrophobic Barrier Pen to ensure a solid seal. Allow for barrier to dry at RT for 20-30 min.

Step	Action
Step 6. Tissue Pretreatment **25 min**	**A.** Tightly cover the beaker containing the 500 mL of 1X Pretreatment Solution with aluminum foil, place it on a hot plate and heat the solution to a temperature of 95 °C. Use a waterproof probe thermometer to measure and maintain the temperature of the solution at 90-95 °C during the pretreatment period. **NOTE: The temperature will drop a few degrees as the samples are added, so it is prudent to have the temperature be on the higher end of this range when adding the slides.** **B.** Set slide 1 aside on the lab bench. **C.** Load slides 9 and 10 into the slide rack. **D.** Using a pair of forceps, submerge the slide rack into the heated 1X Pretreatment Solution. Cover the glass beaker with aluminum foil and incubate for 10 min at 90-95 °C. **E.** At the end of 10 min, use forceps to add slides 5, 6, 7, and 8 into the 90-95 °C 1X Pretreatment Solution. Re-cover the glass beaker with aluminum foil and incubate for 5 min. **F.** At the end of 5 min, use forceps to add slides 2, 3, and 4 into the 90-95 °C 1X Pretreatment Solution. Re-cover the glass beaker with aluminum foil and incubate for 5 min. **G.** Using a pair of forceps, remove the slide rack loaded with slides and submerge it into a clearing dish containing 200 mL ddH_2O. and wash for 1 min with frequent agitation. **H.** Repeat the wash once more with another 200 mL of fresh ddH_2O. **I.** Transfer the slide rack to a clear staining dish containing 1X PBS. **IMPORTANT: From this point forward do not let the tissue sections dry out.**

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>Step 7. Protease Digestion and Fixation

50 min</td><td>
A. Set the ThermoBrite to 40 ± 1 °C and insert two wet ThermoBrite Humidity strips.
B. Using the table below as a guide, prepare the Working Protease Solution by diluting the Protease QF 1:100 in prewarmed 1X PBS. Scale reagents according to the number of assays to be run. Include one slide volume overage.

<table>
<tr><th colspan="2">Working Protease Solution per Slide</th></tr>
<tr><th>Reagent</th><th>Volume</th></tr>
<tr><td>Protease QF</td><td>4 µL</td></tr>
<tr><td>1X PBS (prewarmed to 40 °C)</td><td>396 µL</td></tr>
<tr><td>Total volume</td><td>400 µL</td></tr>
</table>
C. Leave slide 1 on the lab bench as it is excluded from this step.
D. Remove slides 4 and 8 and flick off excess 1X PBS. Tap the slides on their edges then wipe the backside on a laboratory wipe. Leave remaining slides in 1X PBS until appropriate incubation time.
E. Place the slides face up on a clean, flat surface and add 400 µL of the Working Protease Solution onto the tissue section.
F. Carefully move the slides onto the ThermoBrite, close the lid and incubate for 20 min at 40 °C.
G. After 19 min, remove slides 3, 6, 7, and 10 from the clear staining dish and flick off excess 1X PBS. Tap the slides on their edges, then wipe the backside on a laboratory wipe.
H. Place the slides face up on a clean, flat surface and add 400 µL of the Working Protease Solution onto the tissue section.
I. Carefully move the slides onto the ThermoBrite, close the lid and incubate for 10 min.
J. Wait 9 min, then remove slides 2, 5, and 9 from the clear staining dish and flick off excess 1X PBS. Tap the slides on their edges, then wipe the backside on a laboratory wipe.
K. Place the slides face up on a clean, flat surface and add 400 µL of Working Protease Solution onto the tissue sections.
L. Carefully move the slides onto the ThermoBrite, close the lid and incubate for 10 min.
M. Pour 200 mL of 1X PBS into a clear staining dish and insert an empty rack into it.
N. At the end of 10 min (40 min total incubation time), decant the Working Protease Solution from the slides, insert them into the slide rack and rinse by moving up and down for 1 min.
O. Retrieve slide 1 and add to slide rack in PBS. There should be 10 slides in the slide rack.
P. Decant the 1X PBS, refill with 200 mL of fresh 1X PBS and rinse by moving slide rack up and down for 1 min.
Q. Transfer the slide rack into the clear staining dish containing 4% formaldehyde and incubate under a fume hood for 5 min at RT.
R. Decant the clear staining dish containing 1X PBS and refill with 200 mL of fresh 1X PBS.
S. Transfer the slide rack from the 4% formaldehyde solution to the clear staining dish containing 1X PBS, and incubate for 1 min with frequent agitation.
T. Decant the 1X PBS, refill with 200 mL of fresh 1X PBS and rinse by moving slide rack up and down for 1 min.
U. Transfer the 4% formaldehyde solution to a 200 mL capacity container, keep for later use.
V. Proceed to Step 8. Target Probe Set Hybridization on page 15 to continue the assay procedure.
</td></tr>
</table>

Assay Optimization Lookup Table

The table below contains a list of the tissues that we prepared according to the guidelines outlined in this manual using 10% NBF (*Tissue Preparation Guidelines on page 7*) and optimized using the recommended pretreatment assay optimization procedure. You can use this table as a guideline to minimize the number of conditions if you do not have sufficient slides to perform the recommended pretreatment optimization procedure. These guidelines are specific to tissues prepared using 10% NBF and may not be applicable to tissues prepared using 4% PFA. You must also include a negative control slide (without Probe Set) to ensure no background is visible and well-defined cell morphology is achieved.

If your tissue type is not listed in the Table B.1, and you have only a few slides available for optimization, Table B.2 lists recommended boiling and protease incubation times.

Table B.1 Assay Optimization Lookup Table

Tissue Information		Optimal Conditions		Range for Tolerance Conditions
Species	**Type**	**Boiling at 90-95 °C (min)**	**Protease at 40 ± 1 °C (min)**	**(Boiling, Protease) min**
Human	Brain	20	10	(10,10)(10,20)
	Breast	20	15	(25,15)(30,20)(25,20)
	Colon	5	20	(5,10)
	Kidney	20	10	
	Liver	20	20	(10,20)
	Lung	10	20	
	Osteoarthritic tissue	20	20	
	Pancreas	10	10	(10,20)(5,10)
	Prostate	10	20	(5,10)(20,10)(10,10)
	Salivary gland	10	10	(5,10)
	Skin	5	10	
	Tonsil	10	20	
	Thyroid	10	20	
Rat	Kidney	10	20	(10,10)(20,20)
	Liver	10	20	
	Thyroid	10	20	

Table B.1 Assay Optimization Lookup Table (Continued)

Tissue Information		Optimal Conditions		Range for Tolerance Conditions
Species	**Type**	**Boiling at 90-95 °C (min)**	**Protease at 40 ± 1 °C (min)**	**(Boiling, Protease) min**
Mouse	Bone	20	20	
	Brain	10	10	
	Heart	10	40	
	Kidney	20	20	(10,20)
	Liver	20	20	(10,20)
	Lung	10	20	
	Retina	10	10	
Salmon	Heart	10	10	
	Muscle	10	20	

Table B.2 Recommended Boiling and Protease Incubation Times for Limited Optimization

Number of Available Slides	Boiling Times (min)	Protease Incubation Times (min)
3	5	10
	10	10
	10	20
5	5	10
	5	20
	10	10
	10	20
	20	10
7	5	10
	5	20
	10	10
	10	20
	20	10
	20	20
	0	0

Modified Protocols for 1-Plex Assay

About this Appendix

This appendix provides modified and shortened assay procedures for performing 1-plex assay using the QuantiGene ViewRNA ISH Tissue 2-Plex Kit. Whether preference for target detection is Fast Red or Fast Blue, both TYPE 1 and TYPE 6 probe sets can be used.

Table C.1 Modified 1-Plex Protocol for Fast Red Detection Using TYPE 1 or TYPE 6 Probe Set

Probe Set Designation	Modified Protocol
TYPE 1	a. Perform the assay as directed up through *Step 16. Wash Slides*. b. Omit *Step 17. Label Probe 6-AP Hybridization* - *Step 21. Quenching of LP6-AP*. c. Continue with *Step 22. Label Probe 1-AP Hybridization* - *Step 26. Add Coverslip and Image* of the assay.
TYPE 6	a. Perform the assay as directed up through *Step 18. Wash Slides*. b. Omit *Step 19. Apply Fast Blue Substrate* - *Step 23. Wash Slides*. c. Continue with *Step 24. Apply Fast Red Substrate* - *Step 26. Add Coverslip and Image* of the assay.

Table C.2 Modified 1-Plex Protocol for Fast Blue Detection Using TYPE 1 or TYPE 6 Probe Set

Probe Set Designation	Modified Protocol
TYPE 1	a. Perform the assay as directed up through *Step 16. Wash Slides*. b. For *Step 17. Label Probe 6-AP Hybridization*, replace Label Probe 6-AP with Label Probe 1-AP. c. Continue with *Step 18. Wash Slides* - *Step 20. Wash Slides* of the assay. d. Omit *Step 21. Quenching of LP6-AP* - *Step 24. Apply Fast Red Substrate*. e. Continue with *Step 25. Counterstain* - *Step 26. Add Coverslip and Image* of the assay.
TYPE 6	a. Perform the assay as directed up through *Step 20. Wash Slides* of the assay. b. Omit *Step 21. Quenching of LP6-AP* - *Step 24. Apply Fast Red Substrate*. c. Continue with *Step 25. Counterstain* - *Step 26. Add Coverslip and Image* of the assay.

Using Frozen Tissues with the QG ViewRNA ISH Tissue 2-Plex Assay

About This Appendix

This appendix provides procedural modifications for running the QuantiGene ViewRNA ISH Tissue 2-Plex Assay on fresh frozen or OCT-embedded frozen tissue sections and should be used in conjunction with the QuantiGene ViewRNA ISH Tissue 2-Plex User Manual. This modified assay protocol has been validated on only a limited number of OCT-embedded frozen tissue samples and may or may not apply to those not included in list below:

- Mouse and rat spinal chord
- Mouse brain
- Mouse eye balls
- Mouse lung
- Mouse liver
- Human skin
- Mouse skin
- Mouse duodenum

Important Procedural Notes

- Samples should be freshly sectioned at 12 ± 1 µm and mounted onto Fisherbrand Superfrost Plus (white label) microscope slides (Fisher Scientific, Cat # 12-550-15); avoid other colored labels as they tend to give high background.
- Prepared frozen tissue slides should be used immediately in the assay or can be stored at –80 °C for up to 6 months.
- Optimization should be done for Protease only. There is no pretreatment boiling step required.

Modifications to Part 1: Sample Preparation and Target Probe Hybridization

The following procedural steps would replace steps 1-6 in the *QuantiGene ViewRNA ISH Tissue 2-Plex User Manual*.

Step	Action
Step 1. Prepare 4% formaldehyde in 1X PBS	To a 200 mL capacity container add 178 mL of 1X PBS and 22 mL of 37% formaldehyde. Mix well and refrigerate at 4 °C for 1 hour.
Step 2. Tissue Pretreatment	Pour chilled 4% formaldehyde into a clear staining dish and insert an empty slide rack into the solution. Insert frozen tissue slides into the slide rack and incubate for 16-18 hours at 4 °C

Step	Action
Step 3. Prepare reagents	**A.** 2L of 1X PBS: To a 2 L container add 200 mL of 10X PBS and 1.8 L ddH_20. **B.** 50% ethanol: To a 200 mL capacity container add 100 mL of ddH_2O and 100 mL 100% ethanol and mix well. **C.** 70% ethanol: To a 200 mL capacity container add 60 mL of ddH_2O and 140 mL 100% ethanol and mix well. **D.** Wash Buffer: To a 3 L capacity container add components in the following order to prevent precipitation from forming and then mix well: 2.5 L ddH_2O, 27 mL Wash Comp 1, 7.5 mL Wash Comp 2 and ddH_2O to 3 L. **E.** Storage Buffer (for optional stop point): To a 200 mL container add 60 mL of Wash Comp 2 to 140 mL ddH_2O and mix well. **F.** Ensure the availability of 200 mL of 100% ethanol. **G.** Prewarm 40 mL of 1X PBS and Probe Set Diluent QF to 40 °C. **H.** Thaw Probe Set(s). Place on ice until use
Step 4. Wash with 1X PBS	**A.** Remove slide rack from the 4% formaldehyde and submerge it into a clear staining dish containing 200 mL of 1X PBS. Incubate for 1 minute with constant agitation. **B.** Decant the 1X PBS, refill with 200 mL of fresh 1X PBS and incubate for 1 minute with constant agitation.
Step 5.Tissue dehydration	**A.** Pour 200 mL of 50% ethanol into a clean clear staining dish. **B.** Remove the slide rack from the 1X PBS and submerge it into the 50% ethanol. Incubate for 10 minutes at room temperature without agitation. **C.** Pour 200 mL of 70% ethanol into a clean clear staining dish. **D.** Remove the slide rack from the 50% ethanol and submerge it into the 70% ethanol. Incubate for 10 minutes at room temperature without agitation. **E.** Pour 200 mL of 100% ethanol into a clean clear staining dish. **F.** Remove the slide rack from the 70% ethanol and submerge it into the 100% ethanol. Incubate for 10 minutes at room temperature without agitation. **G.** Remove the slides from the slide rack and bake them at 60 ± 1 °C for 30 minutes. This can be done using a dry incubator or using the Thermobrite instrument with the lid open. **NOTE: Following the baking step, we recommend you begin the protease digestion and fixation step within 1 hr.**

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>Step 6. Draw a hydrophobic barrier</td><td>A. Dab the hydrophobic pen on a paper towel several times before use to ensure proper flow of the hydrophobic solution.
B. To create a hydrophobic barrier, place the slide over the template image below, making sure that the tissue sections fall inside the blue rectangle, and lightly trace the thick blue rectangle 2 to 4 times with the Hydrophobic Barrier Pen to ensure a solid seal. Allow for barrier to dry at RT for 20-30 min.

IMPORTANT: Consistently draw hydrophobic barrier size as indicated in template, even if using smaller tissue sections.

IMPORTANT: Draw the barrier 2-4 times to ensure a solid seal.</td></tr>
<tr><td>Step 7. Protease Digestion and Fixation</td><td>Continue the remainder of the assay procedure, unchanged from the QuantiGene ViewRNA ISH Tissue 2-Plex User Manual, from step 7: Protease Digestion and Fixation.</td></tr>
</table>

Templates for Drawing the Hydrophobic Barrier

NOTE: To ensure templates print to the correct size, make sure that you select none under the page scaling option in the print dialog box.

Figure E.1 Tissue Slide Templates