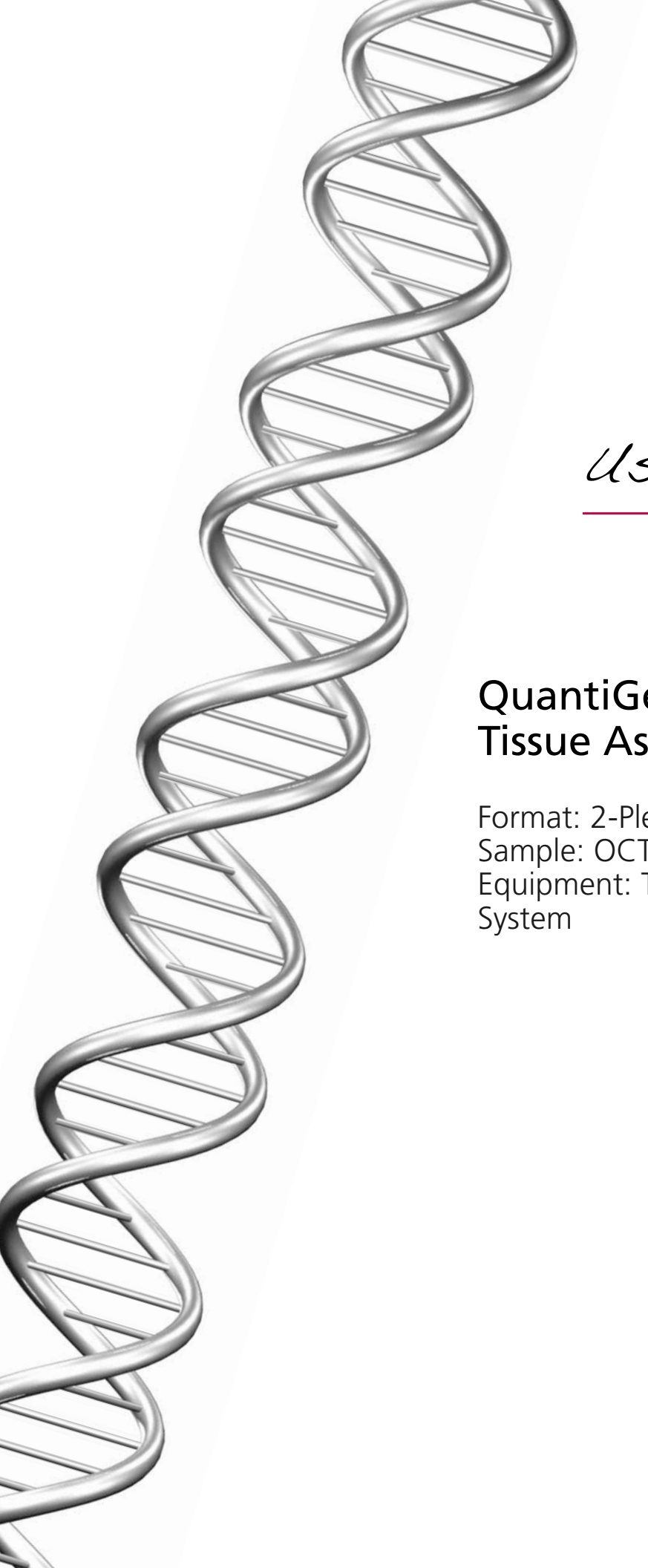

Affymetrix®

User Manual

QuantiGene® ViewRNA ISH Tissue Assay

Format: 2-Plex
Sample: OCT-Embedded Frozen Tissues
Equipment: ThermoBrite Hybridization System

P/N 18960 Rev.A 120414

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Citing QuantiGene ViewRNA in Publications

When describing a procedure for publication using this product, please refer to it as the QuantiGene ViewRNA ISH Tissue Assay.

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Contents

Introduction

About This Manual

This manual provides complete instructions for performing the QuantiGene ViewRNA ISH Tissue Assay for visualization of 2 target RNAs in OCT-embedded frozen tissue samples prepared in accordance with the guidelines provided. This manual provides assay procedures that utilize the ThermoBrite denaturation/hybridization system.

Related User Documents

See the following documents for assay instructions that utilize additional sample types and equipment.

Table 1.1 Related Manuals for Other Sample Types and Equipment

Document	Format	Sample Type	Equipment
QuantiGene ViewRNA ISH Tissue Assay User Manual	2-Plex	OCT-Embedded Frozen Tissue Sections	Dry Oven and Humidified Incubator
QuantiGene ViewRNA ISH Tissue Assay User Manual	2-Plex	FFPE Tissue Sections	ThermoBrite denaturation/ hybridization system
QuantiGene ViewRNA ISH Tissue Assay User Manual	2-Plex	FFPE Tissue Sections	Dry Oven and Humidified Incubator

See the *QuantiGene ViewRNA ISH Tissue Assay Supplemental Reference Guide* for the following information:

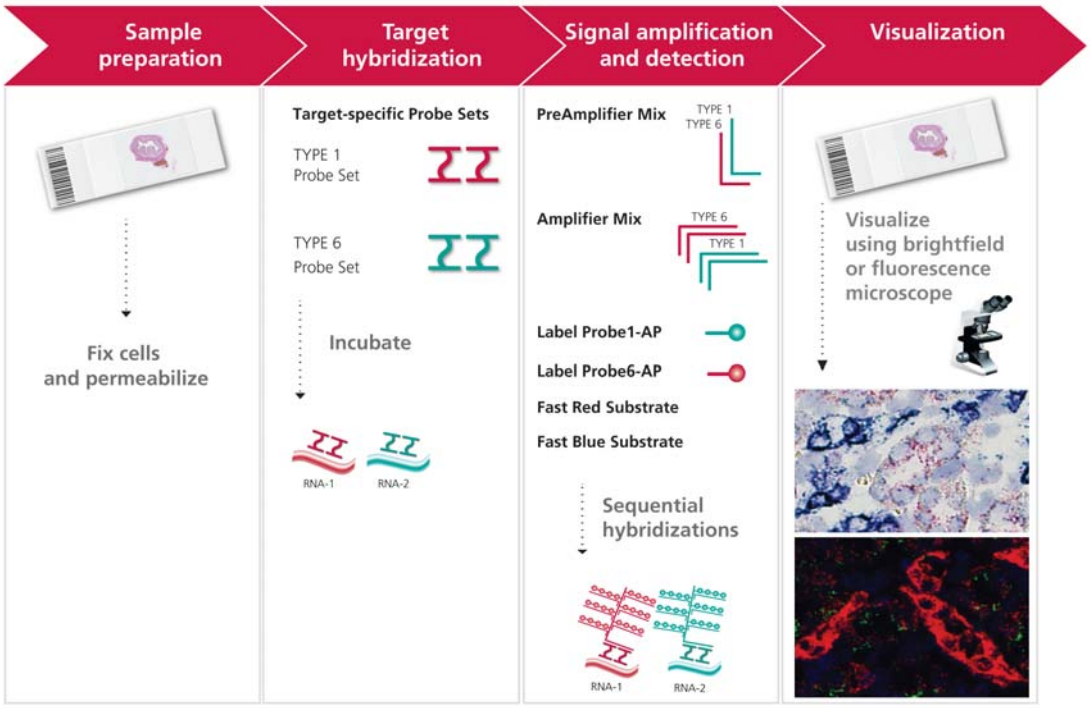
- Guidelines for data interpretation
- Typical results for assay optimization
- Troubleshooting images, examples of backgrounds
- Assay specificity
- Guidance for fluorescence and brightfield imaging
- Procedure for remounting slides
- Procedure for RNase treatment of samples
- Procedure for DNase treatment of samples

Assay Overview

In situ hybridization (ISH) techniques are used to visualize DNA or RNAs within cells in tissue. However, the *in situ* analysis of RNA has always been limited by low sensitivity and difficult probe synthesis. The QuantiGene® ViewRNA ISH Tissue Assay, based on branched DNA signal amplification technology, has the sensitivity and robustness to measure two different genes at single copy sensitivity. The assay design is illustrated and explained in Figure 1.1.

How it Works

Figure 1.1 QuantiGene ViewRNA ISH Tissue Assay Workflow



Sample Preparation. FFPE or frozen tissue sections are fixed, then permeabilized to allow target accessibility.

Target Hybridization. A target-specific Probe Set(s) hybridizes to the target RNA(s). Subsequent signal amplification is predicated on specific hybridization of adjacent Probe Set oligonucleotides indicated by *"II"* in the image above. A typical Probe Set will contain 20 oligonucleotides pairs ("*II*"), for simplicity only two are shown. TYPE 1 Probe Sets will generate red dot patterns while TYPE 6 Probe Sets will generate blue dot patterns. Separate but compatible signal amplification systems enable the multiplex assay.

Signal Amplification. Signal Amplification is achieved via a series of sequential hybridization steps. The PreAmplifier hybridizes to each pair of bound Probe Set oligonucleotides, then multiple Amplifier hybridize to each PreAmplifier. Next, multiple Label Probe oligonucleotides conjugated to alkaline phosphate hybridize to each Amplifier. Each Amplifier branched structure has about 400 binding sites for the Label Probe and each gene typically contains about 20 Amplifiers or 8,000 binding sites.

Detection. The sequential addition of Fast Blue and Fast Red alkaline phosphatase substrates produce precipitate blue and red dots that indicate the presence of the target RNA molecules. Target mRNA is visualized using a standard brightfield or fluorescence microscope.

Performance Highlights

Table 1.2 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: 11 ± 2 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.
- 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 Assay is composed of the following 2 modules, each sold separately and available in multiple sizes:

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

QuantiGene ViewRNA ISH Tissue Assay Kit

QuantiGene ViewRNA ISH Tissue Assay Kits are available in two sizes: QVT0010 and QVT0011, sufficient for 24 or 96 assays respectively. Each kit is configured for processing a minimum of 6 or 12 slides, respectively, per experiment.

The components of the QuantiGene ViewRNA ISH Tissue 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 a shelf life of 6 months from date of delivery when stored as recommended.

Table 1.3 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	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	Alkaline phosphatase-conjugated oligonucleotide in aqueous buffered saline	2-8 °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 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 1.4 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 Assay that are not included in the QuantiGene ViewRNA ISH Tissue Assay Kit are listed here.

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

Table 1.5 QuantiGene ViewRNA Tissue Assay Materials and Equipment

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 Vertical 24 Slide Rack	American Master Tech Scientific	LWSRA24
Aluminum slide rack	VWR	100493380
Double-distilled water (ddH_20)	MLS (major laboratory supplier)	
100% Ethanol	VWR	89125-172
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
37% Formaldehyde	Fisher Scientific	F79-1
27-30% Ammonium Hydroxide	VWR	JT9726-5
Hydrophobic Barrier Pen	Affymetrix or Vector Laboratories	QVC0500 H4000
Ultramount or Advantage Mounting Media	DAKO Innovex	S1964 NB300
Cover Glass, 24 mm x 55 mm	VWR or Affymetrix	48382-138 QVC0501

Table 1.5 QuantiGene ViewRNA Tissue Assay Materials and Equipment (Continued)

Material	Source	Part Number
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
Pipettes, P20, P200, P1000	MLS	
Fume hood (for dispensing formaldehyde and ammonium hydroxide)	MLS	
Table-top microtube centrifuge	MLS	
Water Bath capable of maintaining 40 ± 1 °C	MLS	
Microscope and imaging equipment	See *QuantiGene ViewRNA ISH Tissue Assay Imaging Options on page 7*	

[a] *Required for fluorescence detection*

Microscopy and Imaging Equipment Guidelines for QuantiGene ViewRNA ISH Tissue Assay

A unique benefit of the Affymetrix QuantiGene ViewRNA ISH Tissue 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 1.6.

Table 1.6 QuantiGene ViewRNA ISH Tissue 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	Dark blue dot/Far red dot
Nuclear stain	Hemotoxylin/DAPI	Light blue/Dark blue

Table 2 QuantiGene ViewRNA ISH Tissue 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 OCT-embedded frozen tissue sections for use with the QuantiGene ViewRNA ISH Tissue Assay. Samples prepared outside of these guidelines may not produce optimal results.

Frozen Tissue Preparation

- Upon removal of tissue, immediately snap freeze the tissue using liquid nitrogen then embed into OCT block. If OCT preparation cannot be performed immediately, keep tissues on dry ice during the processing and transport to minimize RNA degradation.
- Store samples at -80 °C until sectioning.

Frozen Tissue Slide Preparation

- Samples should be sectioned at 12 ± 1 µm
- Mount a single section onto one of the following positively-charged glass slides:
  - Leica SurgiPath X-tra P/N 3800200
  - Tru Scientific TruBond360 P/N 0360W
  - Mercedes StarFrost Platinum P/N MER 7200
- Prepared frozen tissue slides should be used immediately in the assay or can be stored at -80 °C for up to 6 months.
- Ship at -80 °C

Experimental Design Guidelines

Assay Controls

We recommend running positive and negative control slides, based on your sample type, in every QuantiGene ViewRNA ISH Tissue 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 25* to identify the optimal 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.

Probe Set Considerations

QuantiGene ViewRNA Probe Sets, TYPE specific, are designed to a specified region of a target RNA. TYPE 1 Probe Set(s) will be visualized using a Fast Red substrate, alkaline phosphatase breaks down the substrate to form a dark red precipitate wherever the target RNA molecule is localized. This vivid red color is easily visible to the eyes in brightfield. TYPE 6 Probe Set(s) will be visualized using a Fast Blue substrate, alkaline phosphatase breaks down the substrate to form a dark blue precipitate wherever the target RNA molecule is localized. The dark blue color is visible to the eyes in brightfield; however the contrast, especially when expression is very low and in the presence of hematoxylin nuclear staining, may make it more difficult to visualize under lower magnifications (10X). Our recommendation, when defining which target(s) will be of a particular TYPE, is to assign the lowest expressing target to TYPE 1 and the higher expressing target to TYPE 6. This recommendation is based on the easy of viewing by eye under brightfield using 10X magnification.

Probe Sets, TYPE specific, can be combined to create “pan” panels or target cocktails. For example, if you wanted to identify epithelial cells, you could combining several cytokeretins. To do this, you would add, for example, TYPE 1 Probe Sets for KRT5, KRT7, KRT8, KRT10, KRT18, KRT19 and KRT20 into a single assay. We do not recommend combining more than 10 targets for a specific signal amplification system (for example, TYPE 1 or TYPE 6) in a single assay. Another example might be creating a pan housekeeping panel as a positive internal assay control to assess RNA integrity. In this case, you might add TYPE 6 Probe Sets for UBC, ACTB, PPIB and GAPD into a single assay well.

Typically, QuantiGene ViewRNA Probe Set designs cover approximately 1,000 bases and contain 20 pairs of oligos to achieve maximal sensitivity. The requirement for a pair of oligos to bind, side-by-side, in order to build the signal amplification system, is at the core of the assays sensitivity and specificity. The use of multiple pairs of oligos in a Probe Set ensure that we have many opportunities for binding to the specific targets’ unmasked regions (accessible regions), and generating signal at that location. If you are working with smaller targets, or applications such as splice variants or RNA fusions, there are only a few available pairs of oligos in a Probe Set and this will directly impact assay sensitivity. That is, probes will have fewer opportunities to find unmasked areas of the target and generate signal at that location. In these cases, increasing the Probe Set concentration used in the assay might increase the sensitivity.

QuantiGene ViewRNA ISH Tissue Assay Procedure

About the QuantiGene ViewRNA ISH Tissue Assay Procedure

The QuantiGene ViewRNA ISH Tissue 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 protease digestion time (optimized condition) for your sample type. If you do not know these optimized conditions, refer to Appendix A, *Assay Optimization Procedures* on page 25.
- 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, 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.

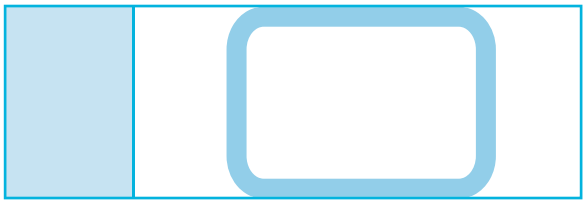
Essential Keys for a Successful Assay

- Prepare samples following *Tissue Preparation Guidelines on page 9*.
- 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 with 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 ambiguous results can be interpreted. See *Experimental Design Guidelines on page 9*.

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. Tissue Pretreatment **16-18 hr**	**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 chilled 4%formaldehyde in 1X PBS in a fume hood: To a 200 mL capacity container add 178 mL 1X PBS and 22 mL of 37% formaldehyde and mix well. Refrigerate at 4 °C for 1 hr. **C.** Insert slides into an empty slide rack and submerge into a clear staining dish containing chilled 4% formaldehyde. Incubate 16-18 hour at 4C. **D.** Remove the slide rack from the chilled 4% formaldehyde and submerge it into a clear staining dish containing 200 mL of 1X PBS. Incubate for 1 min with frequent agitation. **E.** Decant the 1X PBS, refill with 200 mL of fresh 1X PBS and incubate for 1 min with frequent agitation. **F.** Pour 4% formaldehyde back into the 200 mL container and save for later use. Store at RT.
Step 2. Tissue Dehydration **65 min**	**A.** Ensure availability of: ▪ 200 mL 100% ethanol ▪ 200 mL 70% ethanol. To a 200 mL capacity container add 140 mL of 100% ethanol to 60 mL of ddH_2O ▪ 200 mL of 50% ethanol. To a 200 mL capacity container add 100 mL of 100% ethanol to 100 mL of ddH_2O **B.** Pour 200 mL of 50% ethanol into a clean clear staining dish. **C.** Remove the slide rack from the 1X PBS and submerge it into the 50% ethanol. Move slide rack up and down 3 times. Cover with lid and incubate for 10 min at RT without agitation. **D.** Pour 200 mL of 70% ethanol into a clean clear staining dish. **E.** Remove the slide rack from the 50% ethanol and submerge it into the 70% ethanol. Move slide rack up and down 3 times. Cover with lid and incubate for 10 min at RT without agitation. **F.** Pour 200 mL of 100% ethanol into a clean clear staining dish. **G.** Remove the slide rack from the 70% ethanol and submerge it into the 100% ethanol. Move slide rack up and down 3 times. Cover with lid and incubate for 10 min at RT without agitation. **H.** Remove the slides from the slide rack and place them face up on a paper towel to air dry for 5 min at RT. Discard all ethanol solutions. **I.** Use a pencil to label the slides. **J.** Set ThermoBrite at 60 ± 1°C and bake the slides for 30 min with the lid open.
Step 3. 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. 

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>Step 4. Prepare Buffers and Reagents

1 hr</td><td>
A. 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.
B. 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.
C. Prewarm 40 mL of 1X PBS and Probe Set Diluent QT to 40 ± 1 °C.
D. Thaw Probe Set(s). Place on ice until use.
</td></tr>
<tr><td>Step 5. Protease Digestion and Fixation

30-50 min, depending on optimized time</td><td>
IMPORTANT: From this point forward, do not let tissue sections dry out.
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. Place the slides flat face up on the lab bench and immediately add 400 µL of the Working Protease Solution onto the tissue section.
D. Place the slides in the ThermoBrite. Close the lid and incubate at 40 °C for the optimal time as determined in the Assay Optimization Procedures on page 25.
E. Pour 200 mL of 1X PBS into a clear staining dish and insert an empty rack into it.
F. After incubation, decant the Working Protease Solution from the slides, insert them into the slide rack and rinse by moving up and down for 1 min.
G. Decant the 1X PBS, refill with 200 mL of fresh 1X PBS and rinse by moving slide rack up and down for 1 min.
H. Transfer the slide rack into the clear staining dish containing 4% formaldehyde and incubate under a fume hood for 5 min at RT.
I. Decant the clear staining dish containing 1X PBS and refill with 200 mL of fresh 1X PBS.
J. 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.
K. Decant the 1X PBS, refill with 200 mL of fresh 1X PBS and rinse by moving slide rack up and down for 1 min.
L. Transfer the 4% formaldehyde solution to a 200 mL capacity container, keep for later use.
</td></tr>
</table>

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>Step 6. 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 flat face up on the lab bench and immediately add 400 µL Working Probe Set Solution to each tissue section.
D. Place the slides in the ThermoBrite, close the lid and incubate at 40 °C for 2 hr.
</td></tr>
<tr><td>Step 7. 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. Incubate the slides in Wash Buffer at RT for 2 min with frequent agitation. 
D. Decant the Wash Buffer, refill with 200 mL fresh Wash Buffer and incubate the slides at RT for 2 min with frequent agitation. Repeat this step one more time for a total of 3 washes.
</td></tr>
<tr><td>Step 8. 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. When you are ready to continue the assay, proceed to *Step 9. Prepare Additional Buffers and Reagents on page 15*.
</td></tr>
</table>

Part 2: Signal Amplification and Detection

Part 2 Procedure

Step	Action
Step 9. 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 10. Wash Slides **5 min**	**A.** Remove the slides from Storage Buffer, transfer slide rack to clear staining dish containing Wash Buffer, and incubate for 2 min with frequent agitation. **B.** Decant Wash Buffer, refill with 200 mL fresh Wash Buffer, and incubate for 2 min with frequent agitation.
Step 11. PreAmp Hybridization **30 min**	**A.** Set the ThermoBrite to 40 ± 1 °C and rewet the ThermoBrite Humidity strips with ddH_2O. **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 flat face up on the lab bench and immediately add 400 µL of PreAmplifier Mix QT directly to each tissue section. **D.** Place slides in the ThermoBrite. Close the lid and incubate at 40 °C for 25 min.
Step 12. 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.** Incubate the slides in Wash Buffer at RT for 2 min with frequent agitation. **D.** Decant the Wash Buffer, refill with 200 mL fresh Wash Buffer and incubate the slides at RT for 2 min with frequent agitation. Repeat this step one more time for a total of 3 washes.
Step 13. 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 flat face up on the lab bench and immediately add 400 µL of Amplifier Mix QT directly to each tissue section. **C.** Place slides in the ThermoBrite. Close the lid and incubate at 40 °C for 15 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 Amplifier Mix QT from the slides and insert them into the slide rack. **C.** Incubate the slides in Wash Buffer at RT for 2 min with frequent agitation. **D.** Decant the Wash Buffer, refill with 200 mL fresh Wash Buffer and incubate the slides at RT for 2 min with frequent agitation. Repeat this step one more time for a total of 3 washes.

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>Step 15. 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 flat face up on the lab bench and immediately add 400 µL of Working Label Probe 6-AP solution directly to each tissue section.
D. Place the slides in the ThermoBrite. Close the lid and incubate at 40 °C for 15 min.
</td></tr>
<tr><td>Step 16. 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. Incubate the slides in Wash Buffer at RT for 3 min with frequent agitation.
D. Decant the Wash Buffer, refill with 200 mL fresh Wash Buffer and incubate the slides at RT for 3 min with frequent agitation. Repeat this step one more time for a total of 3 washes.
</td></tr>
<tr><td>Step 17. 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. Store solution away from direct light 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 flat face up on an aluminum slide rack.
C. Immediately add 400 µL Fast Blue Substrate and incubate in the dark at RT for 30 min.
NOTE: Fast Blue Substrate precipitate inactivates Label Probe 6-AP.
</td></tr>
<tr><td>Step 18. 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. Incubate the slides in Wash Buffer at RT for 2 min with frequent agitation.
D. Decant the Wash Buffer, refill with 200 mL fresh Wash Buffer and incubate the slides at RT for 2 min with frequent agitation.
</td></tr>
</table>

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>Step 19. 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 flat face up on the lab bench and immediately add 400 µL of Working Label Probe 1-AP solution directly to each tissue section.
D. Place the slides in the ThermoBrite. Close the lid and incubate at 40 °C for 15 min.
</td></tr>
<tr><td>Step 20. 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. Incubate the slides in Wash Buffer at RT for 3 min with frequent agitation.
D. Decant the Wash Buffer, refill with 200 mL fresh Wash Buffer and incubate the slides at RT for 3 min with frequent agitation. Repeat this step one more time for a total of 3 washes.
</td></tr>
<tr><td>Step 21. 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 flat face up 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.
D. Decant the AP Enhancer Solution by flicking. 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. Place the slides in 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. Move the slide rack up and down several times for 1 min to rinse off the Fast Red Substrate.
I. Retrieve 200 mL of 4% formaldehyde (used previously) and pour in the clear staining dish labeled for formaldehyde.
J. Move the slide rack to the clear staining dish containing 200 mL of 4% formaldehyde and incubate for 5 min under a fume hood.
K. Rinse off the residual formaldehyde by transferring the slide rack to a clear staining dish containing fresh 1X PBS. Move the slide rack up and down several times for 1 min.
</td></tr>
</table>

Step	Action
Step 22. Counterstain **50 min**	**A.** Transfer the slide rack to the clear staining dish containing the 200 mL of Gill's Hematoxylin. Incubate slides for 1 min at RT. **B.** After incubation, transfer the slide rack to a clear staining dish containing ddH_2O. Move the slide rack up and down several times to rinse off the Gill's Hematoxylin. **C.** Decant the ddH_2O, refill with 200 mL fresh ddH_2O and move slide rack up and down several times. Repeat this step one more time. **D.** Decant the ddH_2O, refill with 200 mL 0.01% ammonium hydroxide and incubate for 10 sec. **E.** Decant 0.01% ammonium hydroxide, refill with fresh ddH_2O and move slide rack up and down several times. Unused 0.01% ammonium hydroxide solution can be stored at RT for up to one month. **F.** *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, refill with 200 mL fresh ddH_2O and move the slide rack up and down several times to rinse off DAPI solution. **G.** Remove the slides from the slide rack and decant the ddH_2O by flicking. Tap the slide on its edge then wipe the backside on a laboratory wipe. Place them face up onto a paper towel to air dry. **H.** Ensure that slide sections are completely dry before mounting (about 20 min).
Step 23. 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 remove excess mounting medium. Allow slides to dry at RT 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 nail polish. **G.** Image the results under a 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 4.7 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 4.1 Troubleshooting Weak or No Signal

Probable Cause	Recommended Action
Incorrect protease digestion conditions	Repeat assay optimization procedure to determine optimal protease digestion time.
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 protease digestion 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.
Incorrect storage condition	Store the components at the storage condition as written on the component label or kit boxes.

Table 4.1 Troubleshooting Weak or No Signal

Probable Cause	Recommended Action
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 hrs. ▪ Ensure FFPE blocks and sections were stored correctly. In the Fast Blue detection channel, use a positive control Probe Set such as a pan housekeeping panel, which might include TYPE 6 Probe Sets for ACTB, GAPD and UBC, to assess RNA integrity. In that same assay, the Fast Red channel would be used to detect target of interest using a TYPE 1 Probe Set.
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 4.2 Troubleshooting diffused signals

Probable Cause	Recommended Action
Tissue dries up during processing	Keep tissue section moist starting from the protease digestion 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).
Mounting solution contained alcohol	Use DAKO Ultramount or Innovex Advantage mounting media to mount your tissue. Avoid any mounting solution containing alcohol.

Poor Cell Morphology

Table 4.3 Troubleshooting poor cell morphology

Probable Cause	Recommended Action
Incorrect protease digestion conditions	See *Experimental Design Layout for Optimization on page 25*.
Section thickness not optimal	Make sure tissues are sectioned at 12 ± 1 µm thick.

Tissue Detachment from Slide

Table 4.4 Troubleshooting tissue detachment from slides

Probable Cause	Recommended Action
Insufficient baking and fixing of slides	▪ Verify that 16-18 hours of chilled 4% formaldehyde fixing was done. ▪ Verify that 30 min baking step was done. ▪ It may be necessary to increase baking time to 1 hr.
Incorrect protease digestion conditions	See *Experimental Design Layout for Optimization on page 25*.
Improper fixation, reagents, or concentrations	Make sure the correct concentration of NBF was used to fix the slides in the respective steps.
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 4.5 Troubleshooting non-specific binding to slide

Probable Cause	Recommended Action
Incompatible glass slide	Use the glass slides from the following recommended vendors: ▪ Leica Surgipath X-tra P/N 3800200 ▪ Tru Scientific TruBond360 P/N 0360W ▪ Mercedes StarFrost Platinum P/N MER 7200 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. 

Hydrophobic Barrier Falls Off

Table 4.6 Troubleshooting hydrophobic barrier problems

Probable Cause	Recommended Action
Incompatible glass slide	Use the glass slides from the following recommended vendors: ▪ Leica Surgipath X-tra P/N 3800200 ▪ Tru Scientific TruBond360 P/N 0360W ▪ Mercedes StartFrost Platinum P/N MER 7200 Prevalidate each new batch of slides by running the entire assay, including Probe Set, on empty slides (without fixed tissues) to determine if 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 4.7 Troubleshooting high background

Probable Cause	Recommended Action
Tissue dries up during hybridization steps	Keep tissue section moist starting from the protease treatment 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.
Incorrect protease digestion conditions	See *Experimental Design Layout for Optimization on page 25*.
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.

Assay Optimization Procedures

About the Optimization and Typical Results

The QuantiGene ViewRNA ISH Tissue 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 condition to be optimized, protease digestion time, is included in Part 1: Sample Preparation.

An example of typical results for both brightfield and fluorescence detection, can be found in the *QuantiGene ViewRNA ISH Tissue Assay Supplemental Reference Guide*.

Optimization Procedure Overview

You will need to prepare five, 12 ± 1 µm thick OCT-embedded frozen tissue sections from a block, or blocks which were prepared in the same way (section thickness and tissue type) as the OCT-embedded frozen tissue of your interest. Each slide will be treated with a different protease incubation time as described in Table A.1. With the exception of Slide 4, 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.

Experimental Design Layout for Optimization

Table A.1 Optimization Experiment Setup

Protease Incubation Time (min)	Sample
0	Slide 1 with probe
10	Slide 2 with Probe
20	Slide 3 with probe, Slide 4 without probe
40	Slide 5 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, 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.

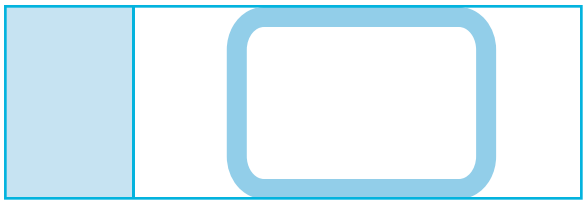
Essential Keys for a Successful Assay

- Prepare samples following the *Tissue Preparation Guidelines on page 9*.
- 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 ambiguous results can be interpreted. See *Experimental Design Guidelines on page 9*.

Sample Preparation and Target Probe Hybridization

Procedure

Step	Action
Step 1. Tissue Pretreatment **16-18 hr**	**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 chilled 4%formaldehyde in 1X PBS in a fume hood: To a 200 mL capacity container add 178 mL 1X PBS and 22 mL of 37% formaldehyde and mix well. Refrigerate at 4 °C for 1 hr. **C.** Insert slides into an empty slide rack and submerge into a clear staining dish containing chilled 4% formaldehyde. Incubate 16-18 hour at 4C. **D.** Remove the slide rack from the chilled 4% formaldehyde and submerge it into a clear staining dish containing 200 mL of 1X PBS. Incubate for 1 min with frequent agitation. **E.** Decant the 1X PBS, refill with 200 mL of fresh 1X PBS and incubate for 1 min with frequent agitation. **F.** Pour 4% formaldehyde back into the 200 ml container and save for later use.

Step	Action
Step 2. Tissue Dehydration **65 min**	**A.** Ensure availability of: ▪ 200 mL 100% ethanol ▪ 200 mL 70% ethanol. To a 200 mL capacity container add 140 mL of 100% ethanol to 60 mL of ddH_2O ▪ 200 mL of 50% ethanol. To a 200 mL capacity container add 100 mL of 100% ethanol to 100 mL of ddH_2O **B.** Pour 200 mL of 50% ethanol into a clean clear staining dish. **C.** Remove the slide rack from the 1X PBS and submerge it into the 50% ethanol. Move slide rack up and down 3 times. Cover with lid and incubate for 10 min at RT without agitation. **D.** Pour 200 mL of 70% ethanol into a clean clear staining dish. **E.** Remove the slide rack from the 50% ethanol and submerge it into the 70% ethanol. Move slide rack up and down 3 times. Cover with lid and incubate for 10 min at RT without agitation. **F.** Pour 200 mL of 100% ethanol into a clean clear staining dish. **G.** Remove the slide rack from the 70% ethanol and submerge it into the 100% ethanol. Move slide rack up and down 3 times. Cover with lid and incubate for 10 min at RT without agitation. **H.** Remove the slides from the slide rack and place them face up on a paper towel to air dry for 5 min at RT. Discard all ethanol solutions. **I.** Use a pencil to label the slides. **J.** Set ThermoBrite to 60 ± 1°C and bake the slides for 30 min with the lid open.
Step 3. 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 4. Prepare Buffers and Reagents **1 hr**	**A.** 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. **B.** 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. **C.** Prewarm 40 mL of 1X PBS and Probe Set Diluent QT to 40 ± 1 °C. **D.** Thaw Probe Set(s). Place on ice until use.

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>Step 5. Protease Digestion and Fixation

50 min</td><td>
IMPORTANT: From this point forward, do not let tissue sections dry out.

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. Place slide 5 face up on a clean, flat surface and add 400 µL of the Working Protease Solution onto the tissue section.
E. Carefully move the slide onto the ThermoBrite, close the lid and incubate for 20 min at 40 °C.
F. After 19 min, place slides 3 and 4 face up on a clean, flat surface and add 400 µL of the Working Protease Solution onto the tissue section.
G. Carefully move the slides onto the ThermoBrite, close the lid and incubate for 10 min.
H. Wait 9 min, then place slide 2 face up on a clean, flat surface and add 400 µL of Working Protease Solution onto the tissue section.
I. Carefully move the slide onto the ThermoBrite, close the lid and incubate for 10 min.
J. Pour 200 mL of 1X PBS into a clear staining dish and insert an empty rack into it.
K. 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.
L. Retrieve slide 1 and add to slide rack in PBS. There should be 5 slides in the slide rack.
M. Decant the 1X PBS, refill with 200 mL of fresh 1X PBS and rinse by moving slide rack up and down for 1 min.
N. Transfer the slide rack into the clear staining dish containing 4% formaldehyde and incubate under a fume hood for 5 min at RT.
O. Decant the clear staining dish containing 1X PBS and refill with 200 mL of fresh 1X PBS.
P. 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.
Q. Decant the 1X PBS, refill with 200 mL of fresh 1X PBS and rinse by moving slide rack up and down for 1 min.
R. Transfer the 4% formaldehyde solution to a 200 mL capacity container, keep for later use.
S. Proceed to *Step 6. Target Probe Set Hybridization on page 14* to continue the assay procedure.
</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 B.1 Tissue Slide Templates