Affymetrix®

User Manual

QuantiGene® ViewRNA ISH Cell Assay

P/N 18801 Rev.A 110525

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

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Introduction

About This Manual

This manual provides complete instructions for performing the QuantiGene ViewRNA ISH Cell Assay for visualization of up to four target mRNAs in adherent or suspension cultured cells.

This manual provides the following:

- *Required Materials* on page 4
- *Experimental Design and Assay Optimization* on page 9
- *Assay Workflow* on page 15
- *Cover slips in 24-Well Plate Format* on page 15
- *Sample Preparation for Adherent Cells* on page 15
- *QuantiGene ViewRNA ISH Cell Assay Procedure* on page 19
- *Troubleshooting* on page 27
- *Appendix A: Sample Preparation Procedure for Suspension Cells* on page 31
- *Appendix B: Alternate Protocol for Glass Slide Format* on page 33
- *Appendix C: Alternate Protocol for Four-Chambered Dish Format* on page 45
- *Appendix D: Cell Types Validated in QuantiGene ViewRNA ISH Cell Assay with Recommended Pretreatment Conditions* on page 55
- *Appendix E: Templates* on page 57
- *Appendix F: Procedures for Validating the Temperature of a Dry Incubator* on page 59
- *Appendix G:How To Adjust Images For Result Interpretation* on page 63.

Who Should Read This Manual

This manual is for anyone who has purchased the QuantiGene® ViewRNA ISH Cell Assay Kit to perform the QuantiGene ViewRNA fluorescent *in situ* hybridization assay for any of the following sample types:

- Adherent Cultured Cells
- Suspension Cultured Cells

What this Manual Covers

This manual provides recommendations and step-by-step procedures for the following:

- Experimental Design and Assay Optimization
- QuantiGene ViewRNA ISH Cell Assay Procedure
- Alternative protocols for glass slide format (96 assays)
- Alternative protocols for four-chambered dish format (16 assays)
- Troubleshooting

Assay Overview

In situ hybridization techniques are used to visualize DNA or localized 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 Cell Assay incorporates an i*n situ* hybridization technology that has the sensitivity and robustness to visualize up to 4 target mRNAs simultaneously, in single cells, at a single transcript detection level. The QuantiGene ViewRNA ISH Cell Assay, allows both sensitive detection of mRNAs at the individual cell level and the flexibility to quantify gene expression in cultured cells in a variety of formats.

The assay described in this manual is designed for cells grown on glass cover slips in 24-well plate(s) following the assay specifications provided in Table 1.1 on page 2. Protocols for the following formats are listed in the appendices:

- Appendix A: A sample preparation procedure for suspension cells compatible with 24-well plate and glass slide formats; refer to page 31
- Appendix B: Glass Slide Format; refer to page 33
- Appendix C: Four-Chambered Dish Format; refer to page 45

Table 1.1 General Assay Specifications

General Assay Specifications	
Sample Types	Adherent or suspension cells
Format	Cells cultured or adhered to glass cover-slips and then processed using: ▪ 24-Well Plate ▪ Glass slide Cells cultured or adhered in four-chambered dish designed for epifluorescent imaging
Probe	Designed to any targeted mRNA region with average length of 1000 bases
Limit of Detection	Single molecule – appears as single spots/dots in cells
Detection Mode	Fluorescent – Emission at 488, 550, 650 and 740 nm
Multiplexing	Up to 4-plex
Major Instruments Required	
Epifluorescent Microscope System for Image Acquisition	
Light Source	100 Watt mercury light source
Objectives Lens	40X oil immersion (N.A. 1.3)
Filter Sets	▪ Ex 358 nm/ Em 461 nm (DAPI) ▪ Ex 501 nm/ Em 523 nm (FITC) ▪ Ex 554 nm/ Em 576 nm (Cy3) ▪ Ex 644 nm/ Em 669 nm (Cy5) ▪ Ex 740 nm/ Em 764 nm (Cy7)
Cooled-CCD Camera with Computer and Capture Software	▪ 1.3 MP ▪ 6.45 µm pixel size ▪ >65% peak Quantum Efficiency (QE) ▪ 6–10 e- read noise Common models include Exi Aqua (QImaging) and CoolSnap HQ2 (Photometrics)
Incubator	
Incubator must be validated to have 40 ±1 °C. We highly recommend validating the temperature of the incubator every 3 months using QGView Temperature Validation Kit (Affymetrix P/N QV0523).	

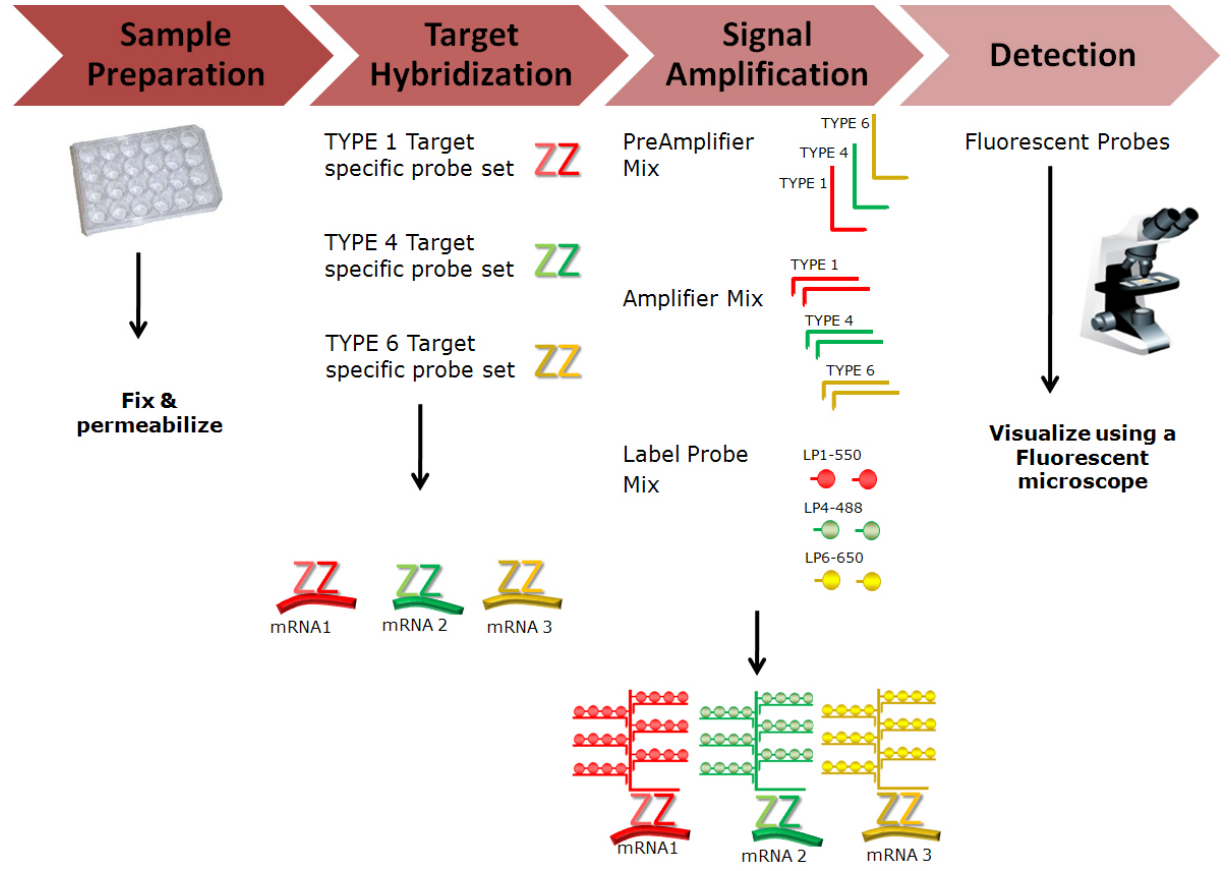
QuantiGene ViewRNA ISH Cell Assay Basics

The QuantiGene ViewRNA ISH Cell Assay is a novel RNA *in situ* hybridization method, based on patent-pending probe design and proprietary branched DNA (bDNA) signal amplification technology, that offers single copy mRNA sensitivity in individual cells in a multiplex assay format. Signal amplification is predicated on specific hybridization of adjacent pairs of oligonucleotides to a target mRNA (see Figure 1.1 below), resulting in excellent signal-to-background ratio.

There are four types of Probe Sets and their corresponding signal amplification systems, designated TYPE 1, 4, 6 and 10. Signal amplification is achieved through sequential hybridization of Pre-Amplifier, Amplifier and Label Probe. Only matched Probe Sets and signal amplification systems of the same TYPE (e.g., TYPE 4 Probe Set, PreAmp 4, Amp 4 and Label Probe 4) can interact and hybridize with each other. Additionally, each Label Probe is conjugated to a specific fluorescent dye, which effectively results in distinct fluorescent detection of Label Probes 1, 4, 6 and 10 at excitation wavelengths of 550, 488, 650 and 740 nm, respectively. Consequently, there are four independent but compatible signal amplification systems that enable fluorescent detection of up to four different target mRNAs in a single assay.

How it Works

Figure 1.1 Schematic of QuantiGene ViewRNA ISH Cell Assay



Sample Preparation. Cells are fixed, permeabilized and digested by protease to allow target accessibility.

Target Hybridization. A target-specific Probe Set hybridizes to each target mRNA. Subsequent signal amplification is predicated on specific hybridization of adjacent pairs of oligonucleotides. A typical Probe Set will contain 20 oligonucleotides pairs. For simplicity, only one pair per target is shown in the figure.

Signal Amplification. Signal amplification, using bDNA technology, is achieved via a series of sequential hybridization steps. The PreAmplifier molecules hybridize to their respective pair of bound Probe Set oligonucleotides, then multiple Amplifier molecules hybridize to their respective PreAmplifier. Next, multiple Label Probe oligonucleotides conjugated to the fluorescent dye hybridize to the corresponding Amplifier molecule. A fully assembled signal amplification "tree" has 400 binding sites for each Label Probe. When all target-specific oligos in the Probe Set bind to the target mRNA transcript, an 8,000 fold amplification occurs for that one transcript.

Detection. Target mRNAs are visualized using a standard fluorescent microscope with the corresponding filter sets (EX: 488, 550 and 650 nm). If using the QuantiGene ViewRNA ISH Cell 740 Module, a filter set is required (EX: 740 nm).

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 http://www.affymetrix.com/panomics.

Table 1.2 Technical Support Contacts

Location	Contact Information
North America	1.877.726.6642 option 1, then option 3; pqbhelp@affymetrix.com
Europe	+44 1 628-552550; techsupport_europe@affymetrix.com
Asia	+81 3 6430 430; techsupport_asia@affymetrix.com

Required Materials

The QuantiGene ViewRNA ISH Cell Assay is composed of the following 4 modules, each sold separately:

- QuantiGene ViewRNA ISH Cell Assay Kit (*for detection of 1–3 mRNAs*)
- QuantiGene ViewRNA Probe Set(s)
- QuantiGene ViewRNA ISH Cell 740 Module (*Optional for 4-plex assay*)
- QuantiGene ViewRNA ISH Cell Accessory Kit (*Optional*)

To perform a basic 1 to 3-plex ViewRNA ISH Assay, the QuantiGene ViewRNA ISH Cell Assay Kit and QuantiGene ViewRNA Probe Set(s) are required. The QuantiGene ViewRNA ISH Cell 740 Module is required to run a 4-plex assay.

QuantiGene ViewRNA ISH Cell Assay Kit

The QuantiGene ViewRNA ISH Cell Assay Kit contains sufficient materials to perform 24 1-plex to 3-plex assays in a 24-well plate format.

IMPORTANT: This kit is compatible with Probe Sets of TYPE 1, 4 and 6. This assay should be run in increments of 6 wells at a time to avoid reagent shortages.

The components of the QuantiGene ViewRNA ISH Cell Assay Kit and their recommended storage conditions are listed below. The QuantiGene ViewRNA ISH Cell Assay Kit is available in one size. Refer to the product insert for quantities of individual components supplied.

Kits have a shelf life of 6 months from date of delivery when stored as recommended.

Table 1.3 QuantiGene ViewRNA ISH Cell Assay Kit Components and Their Storage Conditions

Component	Description	Storage
10X PBS	Aqueous buffered solution	15–30 °C
Detergent Solution QC	Aqueous buffered solution	15–30 °C
Protease QS[a]	Aqueous buffered solution	2–8 °C
Probe Set Diluent[b] QF	Aqueous solution containing formamide and detergent	2–8 °C
Amplifier Diluent[b] QF	Aqueous solution containing formamide and detergent	2–8 °C
Label Probe Diluent[b] QF	Aqueous solution containing detergent	2–8 °C
PreAmplifier Mix	DNA in aqueous buffered solution containing PreAmp1, PreAmp4 and PreAmp6	–20 °C
Amplifier Mix	DNA in aqueous buffered solution containing Amp1, Amp4 and Amp6	–20 °C
Label Probe Mix	Fluorescent dye-labeled oligonucleotides in aqueous buffered solution containing LP1-550, LP4-488 and LP6-650	–20 °C
Wash Buffer Component 1 (Wash Comp 1)	Aqueous solution containing detergent	15–30 °C
Wash Buffer Component 2 (Wash Comp 2)	Aqueous solution	15–30 °C
100X DAPI[c]	Aqueous solution containing DAPI stain	–20 °C

[a] *Do not freeze.*

[b] *WARNING! Probe Set Diluent QF and Amplifier Diluent QF contain formamide, a teratogen, irritant and possible carcinogen. Avoid contact with skin and mucous membranes.*

[c] *WARNING! DAPI is a possible mutagen. Avoid contact with skin and mucous membranes.*

QuantiGene ViewRNA ISH Cell 740 Module

The QuantiGene ViewRNA ISH Cell Assay Kit allows you to visualize up to 3 targets simultaneously. For additional target detection, the QuantiGene ViewRNA ISH Cell 740 Module is available separately and offers the flexibility of expanding the assay to a 4-plex assay.

Table 1.4 QuantiGene ViewRNA ISH Cell 740 Module

Component	Description	Storage
QuantiGene ViewRNA ISH Cell 740 Module	Set of 3 reagents necessary for signal amplification and visualization of target mRNA with a 740 nm filter. Each set is composed of PreAmp10-740, Amp10-740 and LP10-740. Compatible with TYPE 10 Probe Sets.	–20 °C

QuantiGene ViewRNA Probe Sets

In addition to the QuantiGene ViewRNA ISH Cell Assay Kit, QuantiGene ViewRNA Probe Set(s), specific to your mRNA target(s) of interest and necessary for amplification and visualization of your target mRNA(s), must be purchased separately. Probe Sets are available in multiple sizes (30, 110, 360, 1,800 and 3,600 assays). Refer to the Product Insert for more information. Our current probe set catalog can be found at http://www.panomics.com/vrnapsc/.

Table 1.5 QuantiGene ViewRNA Probe Set

Component	Description	Storage
QuantiGene ViewRNA Probe Set	RNA-specific oligonucleotides designed against a target of interest and are compatible with TYPE 1, 4, 6 or 10 Signal Amplifiers.	–20 °C

QuantiGene ViewRNA ISH Cell Assay Accessory Kit (Optional)

The QuantiGene ViewRNA ISH Cell Assay Accessory Kit conveniently provides components and tools that allow you to quickly get started on your QuantiGene ViewRNA fluorescent *in situ* hybridization assay. Components are also available for individual purchase.

Table 1.6 QuantiGene ViewRNA ISH Cell Assay Accessory Components and Storage Conditions

Part Number (P/N)	Accessory Component	Description	Sizes	Storage
QVC0505	Microscope Slides	Non-treated 75 mm x 25 mm glass microscope slides	12 each	15–30 °C
QVC0506	Rectangular Cover Glass	24 mm x 55 mm rectangular, thin pieces of glass for covering and protecting specimens	15 each	15–30 °C
QVC0507	Cover Slips	Non-treated 12 mm, round, thin pieces of glass for direct cell culture	50 each	15–30 °C
QVC0500	Hydrophobic Pen	Wax pen for creating a heat-stable water repellent barrier that keeps reagents localized on samples	each	15–30 °C
QVC0501	Forceps	Straight, fine-tipped forceps for manipulating cover slips	each	15–30 °C
QVC0502	Tissue-Tek Staining Dish (clear)	Plastic buffer reservoir for washing slides	each	15–30 °C
QVC0503	Tissue-Tek Slide Rack	Plastic rack with 24-slide capacity	each	15–30 °C
QVC0504	Tissue-Culture 24-Round Well Plate	Sterile tissue-culture 24-well plate	each	15–30 °C

For ordering information, please visit our website at http://www.panomics.com

Additional Required Materials Not Provided

Other materials required to perform the QuantiGene ViewRNA ISH Cell Assay are listed in the table below.

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

Table 1.7 Required Materials Not Provided

Required Material	Source	Part Number (P/N) or Model
Deionized Water (ddH_2O)	MLS[a]	
100% Ethanol	VWR	89125-188
37% Formaldehyde[b]	Fisher Scientific	F79-1
Prolong® Gold Antifade Reagent	Invitrogen	P36930
Poly-L-Lysine	Sigma	P8920
QG ViewRNA Temperature Validation Kit	Affymetrix	QV0523
Water Bath (capable of maintaining 40 ±1 °C)	MLS	
Pipets (P20, P200 and P1000)	MLS	
Table-top Microtube centrifuge	MLS	
Dry Incubator (capable of maintaining 40 ±1 °C)	▪ VWR ▪ Affymetrix ▪ Affymetrix	▪ 52201-069 ▪ QS0700 (110V) ▪ QS0710 (220V)
Epi-Fluorescent Microscope (upright/inverted; see page 2 for specifications)	MLS	
Fume Hood	MLS	
Optional. Vacuum System (set to aspirate at a rate of ~2 mL/5 sec)	MLS	
The following items are included in the QuantiGene ViewRNA ISH Cell Assay Accessory Kit (Affymetrix P/N QVC0700)		
Microscope Slides	Affymetrix	QVC0505
Cover Slips	Affymetrix	QVC0507
Forceps	Affymetrix	QVC0501
Tissue-Culture 24-Round Well Plate	Affymetrix	QVC0504

[a] MLS = Major Laboratory Supplier

[b] WARNING! Formaldehyde is a poison and irritant. Avoid contact with skin and mucous membranes.

Experimental Design and Assay Optimization

The success of any *in situ* hybridization assay is measured by the maximal specific signal-to-background ratio. In this section, we provide recommendations on experimental design and assay optimization that will ensure your chance of success with the QuantiGene ViewRNA ISH Cell Assay, regardless of whether you are an experienced or first-time user. This manual will guide you through the process of minimizing background signal, while identifying optimal pretreatment conditions, such as fixation time and protease digestion, that will increase specific signals.

Step 1: Assessing Non-Assay Related Background

Background signal can come from a variety of sources, including: (1) auto-fluorescence from the cell type being tested, (2) the materials used to coat the cover slips, and (3) the glass slides. As such, it is important to assess and identify whether any of these reagents or materials are contributing to your background signal. We recommend performing the following experiment to assess the sample background prior to performing any assays. You can avoid doing this step if you use recommended materials listed in the tables on pages 6-7.

Experimental Design

To test for background:

1. Grow cells overnight on a cover slip coated with appropriate matrix protein.
2. After overnight recovery, wash cells once with 1X PBS.
3. Fix with 4% formaldehyde (diluted in 1X PBS) for 10 minutes at room temperature.
4. Stain with 1X DAPI solution (diluted from the stock solution supplied).
5. Mount the cover slip onto a glass microscope slide using Prolong® Gold Antifade Reagent.
6. Observe the samples using an epi-fluorescent microscope with the filter sets intended for future experiment.

Expected Results

The background fluorescence on the glass surface should be minimal with strong, nuclear DAPI staining. No smear, bright spots or debris should be observed in 488, 550, 650 or 740 nm channel. If any of the materials used results in a high level of auto-fluorescence such that it would hinder the detection of your specific signals, we recommend checking the reagents used and verifying that they have been validated for use with the QuantiGene ViewRNA ISH Cell Assay (refer to Table 1.7 on page 7 for recommended suppliers).

Some cell lines will exhibit high auto-fluorescence, particularly in the 488 nm channel. This is a normal biological property of the cells. Should this occur, you can use the 488 nm dye for detecting a highly expressed gene (e.g. housekeeping gene) whereas the gene with lower expression can be detected using the 550 nm or 650 nm dye. If the type of cell line being used is not critical, perform the assay using an alternate cell line.

Step 2: Optimizing Protease Digestion and Fixation Time

The purpose of formaldehyde fixation is to cross-link cells to the glass surface and mRNAs to the cellular structures. The protease digestion unmasks the mRNA in order for the probes to hybridize. The optimal formaldehyde and protease pretreatment conditions can be obtained by titrating the fixation time and protease concentration. In order to detect the target mRNA successfully, it is crucial to identify the optimal condition for fixing and unmasking the target mRNA. Otherwise, the number of target mRNA molecules will be underestimated.

Experimental Design

To identify the optimal pretreatment conditions for the *in situ* hybridization for your cell type, you will initially need to set up a total of 10 samples to run through in a full QuantiGene ViewRNA ISH Cell Assay. Start with a formaldehyde fixation time of 30 minutes and test the range of protease dilutions suggested in Table 2.8 on page 10. For each of the five experimental protease treatments being tested, be sure to include a "**no probe**" negative control. If you do not obtain the desired results with your initial optimization study, we recommend that you repeat the optimization experiment, keeping the same dilution range, but increasing the formaldehyde fixation time to 60 minutes. Refer to Appendix D, on page 55, for cell types validated in QG ViewRNA ISH Cell Assay and their recommended pretreatment conditions.

Using the table below as a guide, prepare the various Working Protease Solutions by diluting Protease in room temperature 1X PBS.

Table 2.8 Suggested Protease Dilutions

Protease Dilution	Formaldehyde Fixation Time (30 minutes)	Formaldehyde Fixation Time (60 minutes)
1:500	(–) Probe and (+) Probe	(–) Probe and (+) Probe
1:1000	(–) Probe and (+) Probe	(–) Probe and (+) Probe
1:2000	(–) Probe and (+) Probe	(–) Probe and (+) Probe
1:4000	(–) Probe and (+) Probe	(–) Probe and (+) Probe
1:8000	(–) Probe and (+) Probe	(–) Probe and (+) Probe

Expected Results

You can expect more than one condition to work and yield signal. The negative controls indicate the background and should have low or no signal. While these initial conditions should work for most samples, keep in mind that some cell types may require pretreatment conditions that fall outside this initial range. When visualizing the *in situ* hybridized samples under the microscope, use the following criteria to select the optimal assay condition:

- **Least Cell Loss**: The majority of cell loss typically occurs after the protease digestion. If the cell loss is less than 50%, proceed with the assay.
- **Lowest Background**: Lowest background inside and outside cells in the "no probe" negative control samples.
- **Highest Signal-to-Background Ratio**: Highest signal-to-background ratio of specific fluorescent dots inside the cell.

The optimal conditions are a balance between (1) minimizing cell loss, (2) minimal impact on cell morphology, and (3) maximum signal-to-background ratio of the detected dots. The maximum signal-to-background ratio should generally be greater than 3. When working with a new cell type, it is important to optimize the formaldehyde fixation time and protease concentration, as each cell type may require different optimal conditions. Once the optimal pretreatment conditions have been identified, the same conditions can be used with different probes with no impact on assay performance.

Examples of Different Optimization Conditions

NOTE: **Refer to Appendix G for information on how to adjust image for interpretation.**

Figure 2.1 Over Digestion by Protease or Under Fixation

- Significant cell loss

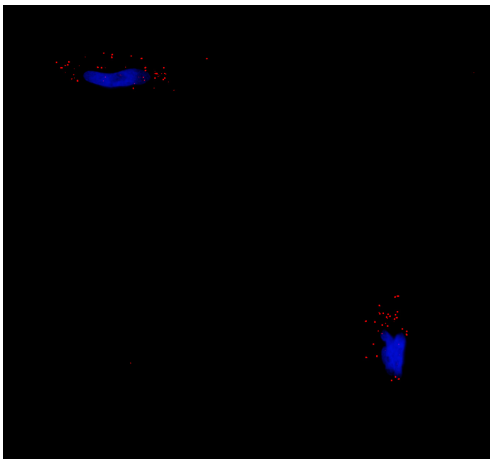


Figure 2.2 Optimum Digestion and Fixation

- Good cell retention
- Bright dots inside the cell

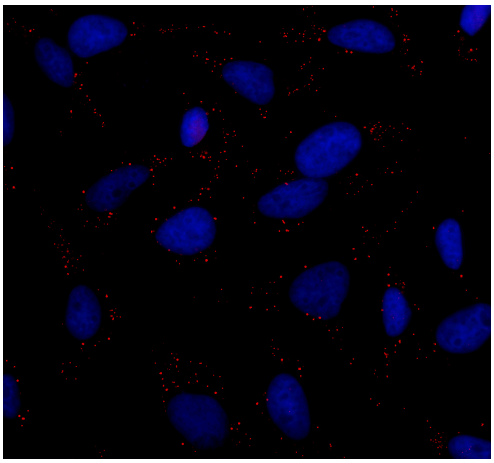
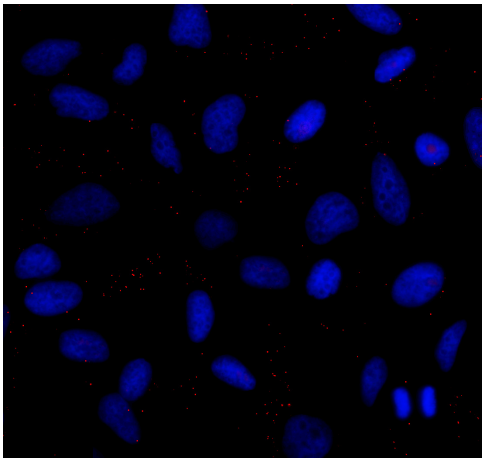


Figure 2.3 Under Digestion by Protease or Over Fixation

- Fewer number of dots and with weak signal inside the cell



Examples of Optimization Conditions

NOTE: Refer to Appendix G for information on how to adjust image for interpretation.

Figure 2.4 ERBB2 mRNAs (TYPE 4)

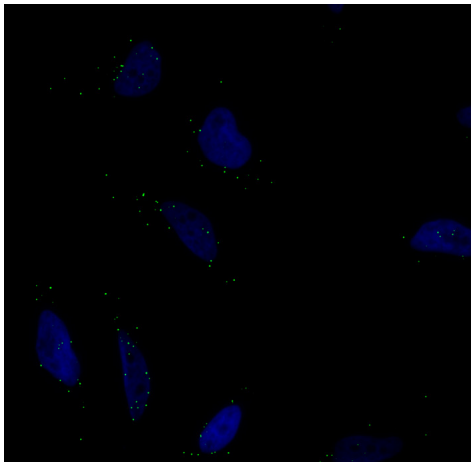


Figure 2.5 HPRT1 mRNAs (TYPE 1)

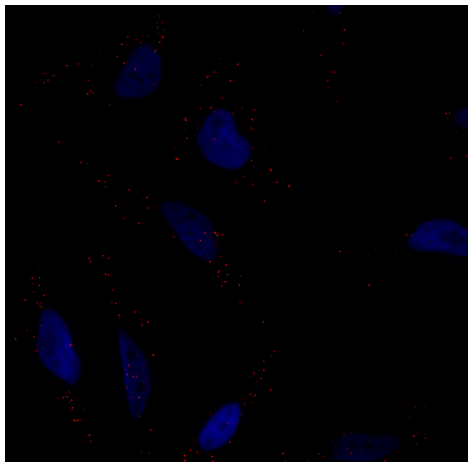
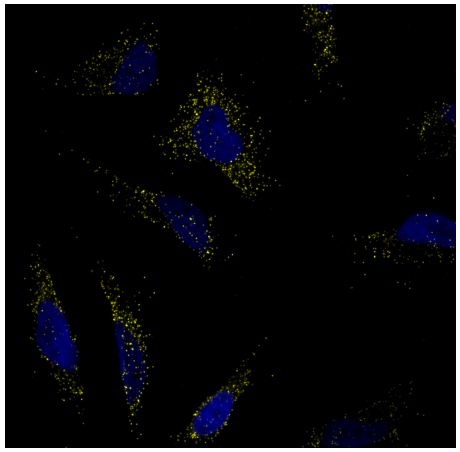


Figure 2.6 PPIB mRNAs (TYPE 6)



Examples of Optimization Conditions

NOTE: **Refer to Appendix G for information on how to adjust image for interpretation.**

Figure 2.7 ACTB mRNAs (TYPE 10)

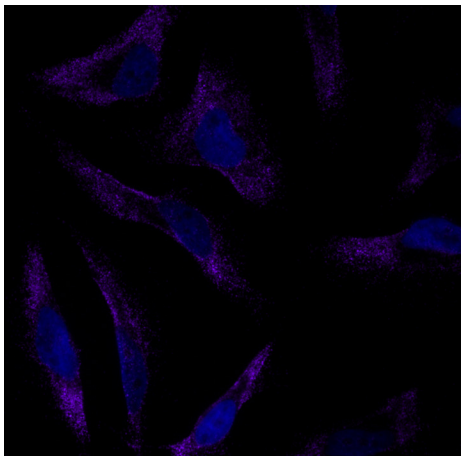


Figure 2.8 4-Plex Merged Image of 4 Different target mRNAs

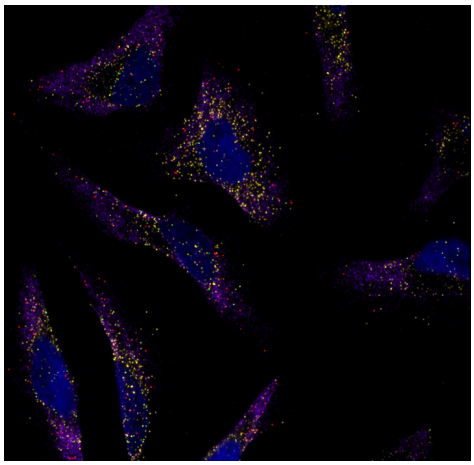
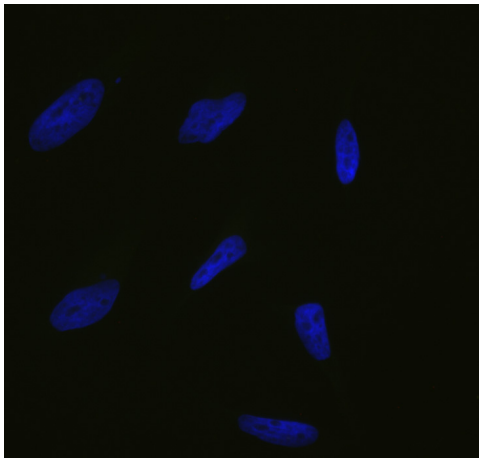


Figure 2.9 No Probe Control (merged image)



NOTE: Images acquired with Olympus IX71 inverted microscope, 40X oil immersion lens, appropriate filter sets and a CCD-cooled camera, Aqua Exi (QImaging). Exposure times: 10 msec for DAPI, 500 msec for 488, 550 and 650 nm and 3000 msec for 740 nm. The exposure time may vary depending on specific imaging system setup.

Interpretation of Results

The QuantiGene ViewRNA ISH Cell Assay offers single-copy mRNA sensitivity in individual cells in a multiplex assay format. The four independent but compatible signal amplification systems enable fluorescent detection of up to four different target mRNAs in a single assay. In a successful QuantiGene ViewRNA ISH Cell Assay, the expression of any given target can be observed as fluorescent signal using an epi-fluorescent microscope equipped with proper filter sets for detecting the specific signal amplification system employed. When mRNA is expressed at low levels, distinct dots dispersed throughout the cytoplasm can be observed. By contrast, when a target mRNA is highly expressed, strong and uniform fluorescent signal is present throughout the cytoplasm. Since the assay offers single-copy sensitivity at single cell level, and each dot represents one target transcript, the level of target gene expression can be quantified by measuring the number of dots per cell.

In the sample images shown above (Figure 2.1 to 2.9), target transcripts with different expression levels in untreated HeLa were detected using the QuantiGene ViewRNA ISH Cell Assay. ERBB2, represented by the green signal, has a relatively low expression level (less than 10 dots per cell) in HeLa cells. As represented by the red fluorescent signal, HPRT1 has a slightly higher expression level (~30 dots per cell) than ERBB2. PPIB, which has an even higher expression level than the previous two transcripts, displays more than 100 yellow dots per cell. In the case of highly expressed genes, such as ACTB, the immense number of transcripts results in homogeneous fluorescent signal throughout the cytoplasm (represented by the purple signal).

For targets such as ERBB2, HPRT1 and even PPIB, quantification of gene expression levels can be performed manually by counting the average number of dots per cell. However, for very high-expressing targets, such as ACTB, quantification should be performed by measuring the total signal intensity level within the whole cell. Highly expressed housekeeping genes, like ACTB or 18S RNA, are useful in defining cell boundaries for software analysis. It is important to note that even in an established cell line, the cell population is never homogeneous due to clonal variations. Hence, the expression levels of a given target within a cell population are expected to be heterogeneous. As such, it is necessary to measure a representative number of cells in the population, and calculate the average. Avoid cells with abnormal (fractured, pinched or split) nuclei appearance and include in your analysis only those cells with normal nuclei having round or oval DAPI counterstaining (blue). Image analysis software should facilitate the quantitation when a large number of samples are handled. The options include CyteSeer (Vala Sciences), MetaMorph (Molecular Devices) and Image J (Freeware from NIH). Contact vendors for specific details on how to quantify the number of dots and the signal intensity at single cell level.

Assay Workflow

About the assay

The complete protocol is sectioned into two parts:.

- Sample Preparation performed at least 2 days prior to the QuantiGene ViewRNA ISH Cell Assay (starting from page 15).
- The QuantiGene ViewRNA ISH Cell Assay, an 8-hour assay that should be run in one day (starting from page 21).

Cover slips in 24-Well Plate Format

The format described below allows the *in situ* hybridization assay to be performed on 12 mm cover slips placed in a standard tissue-culture 24-well plate using a temperature validated incubator (refer to *Additional Required Materials Not Provided on page 6*).

Sample Preparation for Adherent Cells

NOTE: If working with suspension cells, refer to Appendix A, *Sample Preparation Procedure for Suspension Cells on page 31*.

Overview

Prior to performing the QuantiGene ViewRNA ISH Cell Assay procedure, adherent cells must be grown and fixed on poly-L-lysine coated 12 mm circular cover slips. Begin the coating procedure the day prior to seeding the cells. The optimal cell density for this assay is 70–90% confluent at the start of the assay.

Important Procedural Notes and Guidelines

- Use sterile cell culture reagents and techniques when preparing poly-L-lysine coated cover slips and culturing cells.
- We recommend the use of a vacuum system for all steps that require aspiration. Alternatively, aspiration can be performed by manually pipetting the solution from the wells.
- Aspirate from the bottom edge of the wells and dispense against the upper edge of the well.

Assay Setup: Sample Preparation

NOTE: The 1X PBS mentioned for the sample preparation for adherent cells is not supplied in the assay kit. Please use sterile cell culture 1X PBS.

I. Prepare poly-L-lysine coated 12 mm circular cover slips in a tissue-culture 24-round well plate.

II. Culture fresh adherent cells on poly-L-lysine coated cover slips in a tissue-culture 24-round well plate.

III. Fix the cells in 4% formaldehyde solution in the tissue-culture 24-round well plate.

I. Prepare poly-L-lysine coated 12 mm circular cover slips in a tissue-culture 24-round well plate:

Step	Task
Step 1. **5 min**	Dilute poly-L-lysine 1:10 (final concentration of 0.01%) by adding 2.5 mL of poly-L-lysine stock solution to 22.5 mL of room temperature (RT) nuclease-free water. Mix well and set aside until use.
Step 2. **20 min**	Place one cover slip into each well of a sterile tissue-culture 24-round well plate. To sterilize the cover slips, dispense 1 mL/well of 70% ethanol and soak at RT for 15 minutes. **NOTE: The glass cover slips tend to float once the solution is added. Should this occur, use a sterile pipette tip to press the floating cover slips down into the solution.**
Step 3. **20 min**	Aspirate off the 70% ethanol and rinse cover slips with 1 mL/well of 100% ethanol. Aspirate off the 100% ethanol and allow the cover slips to air dry completely for 15 minutes.
Step 4. **20 min**	Dispense 1 mL/well of diluted poly-L-lysine, ensuring that the cover slips are completely submerged in the solution. Incubate for 15 minutes at RT.
Step 5. **10 min**	Aspirate off the poly-L-lysine and rinse the cover slips three times, each with 2 mL/well of 1X PBS.
Step 6. **Overnight Incubation**	Aspirate off the final 1X PBS. Allow to air dry, with lid off, in a laminar flow tissue culture hood overnight. Keep air flow on and turn UV light off.
Optional **Storage**	Cover slips may be used directly for cell seeding or stored at 4 °C in the tissue-culture 24-round well plate with lid on and sealed with parafilm for up to 1 month.
Step 7.	Proceed to culturing cells on poly-L-lysine coated cover slips.

II. Culture fresh adherent cells on poly-L-lysine coated cover slips in a tissue-culture 24-round well plate:

Step	Task
Step 1. **5 min**	Wash the adherent cells with 1X PBS and trypsinize. Resuspend the cells in complete cell culture medium.
Step 2. **10 min**	Pellet the cells (200 x g, 5 minutes) at RT, remove the supernatant, and resuspend the cell pellet in fresh complete culture medium.
Step 3. **5 min**	Count and adjust the cell density such that when seeded, 1 mL/well, the culture will reach 70–90% confluence at the start of the assay.
Step 4. **5 min**	Dispense 1 mL/well of diluted cell suspension gently over the poly-L-lysine coated cover slips. Make sure all cover slips are submerged in the cell culture medium.
Step 5. **Overnight Incubation**	Incubate the plate overnight under the recommended growth conditions for the cells.
Step 6.	Proceed to fixation of cells.

III. Fix the cells in 4% formaldehyde solution in the tissue-culture 24-round well plate:

Step	Task
Step 1. **5 min**	In a fume hood, prepare 10 mL of fresh 4% formaldehyde solution by diluting 1.08 mL of a 37% stock formaldehyde with 8.92 mL of 1X PBS. Vortex briefly to mix. **WARNING: Formaldehyde is a poison and irritant. Avoid contact with skin and mucous membranes.**
Step 2. **5 min**	Carefully aspirate off culture medium, avoiding contact with cover slips and cells. Gently rinse cover slips twice, each time with 2 mL/well of 1X PBS.
Step 3. **35 min**	Aspirate off the final 1X PBS wash and add 400 µL/well of freshly prepared 4% formaldehyde, making sure that the cover slips are submerged. Incubate at RT for 30 minutes. **NOTE: If cover slips are not fully submerged, use the forceps to gently press down on the sides of the cover slip. Do not press on the center of the cover slip.** **IMPORTANT: A fixation time of 30 minutes works for most cell lines. However, when using a cell type for the first time, a titration of the fixation time is recommended as the optimal time can vary between cell types. Refer to Experimental Design and Assay Optimization** on page 9 **for detailed instructions.**
Step 4. **10 min**	Aspirate off the formaldehyde solution and gently rinse the cover slips three times, each with 2 mL/well of 1X PBS.
Step 5.	Fixed cells may be used immediately in the *in situ* assay, in which case, proceed to *QuantiGene ViewRNA ISH Cell Assay Procedure* *on page 19*.

III. Fix the cells in 4% formaldehyde solution in the tissue-culture 24-round well plate: (Continued)

Step	Task
Optional **15 min**	Alternatively, samples can be dehydrated for long term storage: **A.** Aspirate off the 1X PBS and replace with 400 µL/well of 50% ethanol. Incubate for 2 minutes at RT. **B.** Aspirate off the 50% ethanol and replace with 400 µL/well of 70% ethanol. Incubate for 2 minutes at RT. **C.** Aspirate off the 70% ethanol and replace with 400 µL/well of 100% ethanol. Incubate for 2 minutes at RT. **D.** Aspirate off the 100% ethanol and replace with 800 µL/well of fresh 100% ethanol. **E.** Seal the plate with parafilm and store the dehydrated cells in 100% ethanol at –20 °C until needed. Dehydrated cells can be stored under these conditions for several weeks. **NOTE:** **Dehydrated cells must be rehydrated prior to being used in the *in situ* assay.**

QuantiGene ViewRNA ISH Cell Assay Procedure

This procedure is designed for adherent cells cultured on cover slips and processed in a 24-round well plate. If an alternative assay format is needed, refer to the following formats listed in the appendices:

- Appendix B: Glass Slide Format; refer to page 33
- Appendix C: Four-Chambered Dish Format; refer to page 45

Important Procedural Notes and Guidelines

- Validate the temperature of the dry incubator using the QG ViewRNA Temperature Validation Kit (Affymetrix P/N QV0523). Refer to Appendix F, on page 59 for procedures for validating the manufacture of a dry oven.
- Before beginning the assay procedure, identify the optimum formaldehyde fixation time and protease concentration for your sample type. If you do not know the optimal conditions, refer to Appendix D, on page 55.
- This assay should be run in increments of 6 wells at a time to avoid reagent shortages.
- Please allocate a 10% overage when using calculations in this manual to account for dead volume.
- Before working with actual samples, practice removing cover slips from tissue-culture 24-well plate with fine tipped forceps and mounting them onto microscope slides as described in *Step 13. on page 25*.
- Before opening reagents supplied in microfuge tubes, briefly centrifuge to collect contents at the bottom of the tube.
- Unless otherwise stated, all reagents prepared should be stored at room temperature until use.
- Discard all reagents in accordance with local, state and federal laws.

Assay Setup

Step	Task
Step 1. **Prepare Incubator** **30 min**	Set a dry incubator to 40 °C. Calibrate and monitor temperature using the QG ViewRNA Temperature Validation Kit (Affymetrix P/N QV0523) according to the manufacturer's instructions.
Step 2. **Prepare Buffers & Other Reagents (for running 24 samples)** **2 min**	Prepare 420 mL of 1X PBS by adding 42 mL of 10X PBS to 378 mL of ddH_20 and mix. **NOTE:** **Scale accordingly if running less than 24 samples.**
Step 3. **1 min**	Place Protease QS on ice.
Step 4. **5 min**	Prepare 630 mL of Wash Buffer by adding components to a 1 L capacity container in the following order and then mixing well: ▪ 624.96 mL ddH_20 ▪ 1.89 mL Wash Comp 1 ▪ 3.15 mL Wash Comp 2 **NOTE:** **Adding the components in the order listed above will ensure against the formation of precipitates.**
Step 5. **1 min**	Pre-warm Probe Set Diluent QF, Amplifier Diluent QF and Label Probe Diluent QF to 40 °C in a water bath for 30 min. **IMPORTANT:** **Probe Set Diluent QF and Amplifier Diluent QF contain formamide, a teratogen, irritant and possible carcinogen. Avoid contact with skin and mucous membranes.**
Step 6. **5 min**	Thaw Probe Set(s), PreAmplifier Mix, Amplifier Mix and Label Probe Mix at RT. Vortex briefly to mix and place tubes on ice until use. Protect Label Probe Mix from light.
Step 7. **5 min**	Thaw the 100X DAPI at RT. Vortex briefly to mix and place tube on ice until use. Protect from light. **IMPORTANT:** **DAPI is a possible mutagen. Avoid contact with skin and mucous membranes.**

Assay Procedure

NOTE: All calculations in this manual are for 1 well. Please scale accordingly.

Step	Task
Step 1. **Rehydration** **25 min**	**NOTE: Rehydration is ONLY necessary when using dehydrated cell samples. Skip this step and proceed directly to Step 2 when using freshly fixed samples.** **A.** Aspirate off the 100% ethanol and replace with 400 µL/well of 70% ethanol. Incubate for 2 minutes at RT. **B.** Aspirate off the 70% ethanol and replace with 400 µL/well of 50% ethanol. Incubate for 2 minutes at RT. **C.** Aspirate off the 50% ethanol and replace with 400 µL/well of 1X PBS. Incubate for 10 minutes at RT.
Step 2. **Permeabilize Cells with Detergent Solution** **15 min**	**A.** Aspirate off the 1X PBS and replace with 400 µL/well of Detergent Solution QC. Cover plate with lid and incubate for 5 minutes at RT. **B.** Aspirate off the Detergent Solution QC and rinse cells twice, each with 2 mL/well of 1X PBS. Allow samples to sit in the final 1X PBS wash while preparing the Working Protease Solution for the next step.
Step 3. **Digest with Working Protease Solution** **25 min**	**IMPORTANT: The optimal protease concentration can vary with cell type. If you do not wish to perform the optimization in Experimental Design and Assay Optimization** **on page 9****, we suggest starting with a 1:4,000 protease dilution in 1X PBS and optimize as needed.** **A.** Prepare the Working Protease Solution by diluting the Protease QS 1:4,000 in 1X PBS.

Working Protease Solution for 1 Well[a]	
Protease QS	0.1 µL
1X PBS	399.9 µL
Total Volume	**400.0 µL**

[a] scale accordingly for other dilution ratios

B. Vortex briefly to mix.
C. Aspirate off the 1X PBS and replace with 400 µL/well of Working Protease Solution. Cover plate with lid and incubate for 10 minutes at RT.
D. Aspirate off the Working Protease Solution and rinse cells three times, each with 2 mL/well of 1X PBS. Allow samples to sit in the final 1X PBS wash while preparing the Working Probe Set Solution for the next step.

<table>
<tr><th>Step</th><th>Task</th></tr>
<tr><td>Step 4.
Hybridization with Probe Set(s)
3 hr 10 min</td><td>
A. Prepare the Working Probe Set Solution by diluting each Probe Set 1:100 in pre-warmed Probe Set Diluent QF.
<table>
<tr><th colspan="2">Working Probe Set Solution for 1 Well</th></tr>
<tr><td>Probe Set (each)</td><td>4 µL</td></tr>
<tr><td>Probe Set Diluent QF (pre-warmed at 40 °C)</td><td>396 µL</td></tr>
<tr><td>Total Volume</td><td>400 µL</td></tr>
</table>
B. Vortex briefly to mix.
NOTE: If using multiple Probe Sets, use the equation below to calculate the Probe Set Diluent QF required per well:
400 µL – (number of Probe Sets x 4 µL) = Total volume of Probe Set Diluent QF per well
C. Aspirate off the 1X PBS and replace with 400 µL/well of the appropriate Working Probe Set Solution. For the “no probe” negative control, use 400 µL/well of pre-warmed Probe Set Diluent QF.
D. Cover plate with lid and incubate at 40 ± 1 °C for 3 hours.
</td></tr>
<tr><td>Step 5.
Wash Cells 3 Times with Wash Buffer
15 min</td><td>
A. Remove plate from the incubator.
B. Aspirate off the Working Probe Set Solution and wash cells three times, each with 2 mL/well of Wash Buffer. Soak cells for 2 minutes between aspirations. Allow samples to sit in the final wash while preparing the Working PreAmplifier Mix Solution.
IMPORTANT: Do not soak samples in Wash Buffer longer than 30 minutes.
</td></tr>
<tr><td>Step 6.
Hybridize with Pre-Amplifier(s)
40 min</td><td>
A. Prepare the Working PreAmplifier Mix Solution by diluting PreAmplifier Mix 1:25 in pre-warmed Amplifier Diluent QF.
<table>
<tr><th colspan="2">3-plex Working PreAmplifier Mix Solution for 1 Well</th></tr>
<tr><td>Amplifier Diluent QF (pre-warmed at 40 °C)</td><td>384 µL</td></tr>
<tr><td>PreAmplifier Mix</td><td>16 µL</td></tr>
<tr><td>Total Volume</td><td>400 µL</td></tr>
</table>
NOTE: If using the QuantiGene ViewRNA ISH Cell 740 Module, prepare the Working PreAmplifier Mix Solution according to the table below.
<table>
<tr><th colspan="2">4-plex Working PreAmplifier Mix Solution for 1 Well</th></tr>
<tr><td>Amplifier Diluent QF (pre-warmed at 40 °C)</td><td>368 µL</td></tr>
<tr><td>PreAmplifier Mix</td><td>16 µL</td></tr>
<tr><td>PreAmp10-740</td><td>16 µL</td></tr>
<tr><td>Total Volume</td><td>400 µL</td></tr>
</table>
B. Vortex briefly to mix.
C. Aspirate off the Wash Buffer and replace with 400 µL/well of Working PreAmplifier Mix Solution.
D. Cover plate with lid and incubate at 40 ± 1 °C for 30 minutes.
</td></tr>
</table>

<table>
<tr><th>Step</th><th>Task</th></tr>
<tr><td>Step 7.
Wash Cells 3 Times with Wash Buffer
15 min</td><td>
A. Remove plate from the incubator.
B. Aspirate off the Working PreAmplifier Mix Solution and wash cells three times, each with 2 mL/well of Wash Buffer. Soak cells for 2 minutes between aspirations. Allow samples to sit in the final wash while preparing the Working Amplifier Mix Solution.

IMPORTANT: Do not soak samples in Wash Buffer longer than 30 minutes.
</td></tr>
<tr><td>Step 8.
Hybridize with Amplifier(s)
40 min</td><td>
A. Prepare the Working Amplifier Mix Solution by diluting Amplifier Mix 1:25 in pre-warmed Amplifier Diluent QF.

<table>
<tr><th colspan="2">3-plex Working Amplifier Mix Solution for 1 Well</th></tr>
<tr><td>Amplifier Diluent QF (pre-warmed at 40 °C)</td><td>384 µL</td></tr>
<tr><td>Amplifier Mix</td><td>16 µL</td></tr>
<tr><td>Total Volume</td><td>400 µL</td></tr>
</table>

NOTE: If using the QuantiGene ViewRNA ISH Cell 740 Module, prepare the Working Amplifier Mix Solution according to the table below.

<table>
<tr><th colspan="2">4-plex Working Amplifier Mix Solution for 1 Well</th></tr>
<tr><td>Amplifier Diluent QF (pre-warmed at 40 °C)</td><td>368 µL</td></tr>
<tr><td>Amplifier Mix</td><td>16 µL</td></tr>
<tr><td>Amp10-740</td><td>16 µL</td></tr>
<tr><td>Total Volume</td><td>400 µL</td></tr>
</table>

B. Vortex briefly to mix.
C. Aspirate off the Wash Buffer and replace with 400 µL/well of Working Amplifier Mix Solution.
D. Cover plate with lid and incubate at 40 ± 1 °C for 30 minutes.
</td></tr>
<tr><td>Step 9.
Wash Cells 3 Times with Wash Buffer
15 min</td><td>
A. Remove plate from the incubator.
B. Aspirate off the Working Amplifier Mix Solution and wash cells three times, each with 2 mL/well of Wash Buffer. Soak cells for 2 minutes between aspirations. Allow samples to sit in the final wash while preparing the Working Label Probe Mix Solution.

IMPORTANT: Do not soak samples in Wash Buffer longer than 30 minutes.
</td></tr>
</table>

<table>
<tr><th>Step</th><th>Task</th></tr>
<tr><td>Step 10.
Hybridize with Label Probe(s)
40 min</td><td>
IMPORTANT: Protect samples from light during this and all subsequent steps.

A. Prepare the Working Label Probe Mix Solution by diluting Label Probe Mix 1:25 in pre-warmed Label Probe Diluent QF.

<table>
<tr><th colspan="2">3-plex Working Label Probe Mix Solution for 1 Well</th></tr>
<tr><td>Label Probe Diluent QF (pre-warmed at 40 °C)</td><td>384 µL</td></tr>
<tr><td>Label Probe Mix</td><td>16 µL</td></tr>
<tr><td>Total Volume</td><td>400 µL</td></tr>
</table>

NOTE: If using the QuantiGene ViewRNA ISH Cell 740 Module, prepare the Working Label Probe Mix Solution according to the table below.

<table>
<tr><th colspan="2">4-plex Working Label Mix Solution for 1 Well</th></tr>
<tr><td>Label Probe Diluent QF (pre-warmed at 40 °C)</td><td>368 µL</td></tr>
<tr><td>Label Probe Mix</td><td>16 µL</td></tr>
<tr><td>LP10-740</td><td>16 µL</td></tr>
<tr><td>Total Volume</td><td>400 µL</td></tr>
</table>

B. Vortex briefly to mix. Protect from light.
C. Aspirate off the Wash Buffer and replace with 400 µL/well of Working Label Probe Mix Solution.
D. Cover plate with lid and incubate at 40 ± 1 °C for 30 minutes.
</td></tr>
<tr><td>Step 11.
Wash Cells 3 Times with Wash Buffer
25 min</td><td>
A. Remove plate from the incubator.
B. Aspirate off the Working Label Probe Mix Solution and wash cells three times, each with 2 mL/well of Wash Buffer. Soak cells for 2 minutes for the first two washes and 10 minutes for the final wash. Allow samples to sit in the final wash while preparing the Working DAPI Solution.

IMPORTANT: Do not soak samples in Wash Buffer longer than 30 minutes.
</td></tr>
<tr><td>Step 12.
DAPI-Staining
5 min</td><td>
A. Prepare Working DAPI Solution by diluting the 100X DAPI 1:100 in 1X PBS.

<table>
<tr><th colspan="2">Working DAPI Solution for 1 Well</th></tr>
<tr><td>1X PBS</td><td>396 µL</td></tr>
<tr><td>100X DAPI</td><td>4 µL</td></tr>
<tr><td>Total Volume</td><td>400 µL</td></tr>
</table>

B. Vortex briefly to mix. Protect from light.
C. Aspirate off the Wash Buffer and replace with 400 µL/well of Working DAPI Solution. Incubate at RT for 1 minute.
D. Aspirate off DAPI Working Solution and wash cells once with 2 mL/well of 1X PBS.
E. Add 400 µL/well of fresh 1X PBS.
</td></tr>
</table>

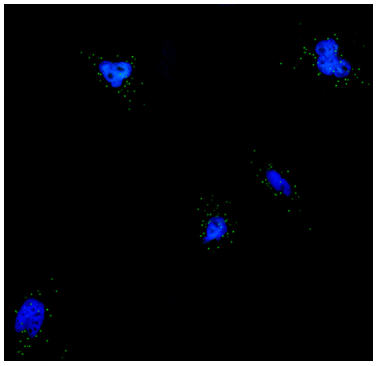
<table>
<tr><th>Step</th><th>Task</th></tr>
<tr><td>Step 13.
Mounting on Glass Slide
20 min</td><td>
A. For easy identification of samples, label the frosted area of the glass slides with a pencil.
B. Place a small drop (10–15 µL/sample) of Prolong® Gold Antifade Reagent onto a microscope slide (one microscope slide can hold 2 samples). Avoid air bubbles.
C. Remove a cover slip from the 24-well plate with fine tipped forceps. Dab the edge of the cover slip gently on a dry laboratory wipe to remove excess 1X PBS. Mount the cover slip, cell side facing DOWN, onto the spot of mounting medium. Avoid air bubbles.
D. Repeat step 13A–C until all cover slips are mounted onto the slides.
</td></tr>
<tr><td>Step 14.
Image Samples</td><td>
A. Samples may be viewed under a microscope immediately after mounting. However, fluorescent signals will be enhanced after the mounting medium is completely cured.
B. Cure the slides at RT, protected from light, for several hours to overnight. Samples can then be stored at 2–8 °C protected from light. Fluorescent signals will be stable for up to one week when properly stored.
C. Prior to viewing the samples under the microscope, gently wipe the cured cover slips with a laboratory wipe soaked with 70% ethanol to remove any crystallized salt that may have formed on the cover slips from the 1X PBS.
D. For viewing samples, use appropriate filter sets.
<table>
<tr><th>Probe Set</th><th>Filter Set</th></tr>
<tr><td>TYPE 4</td><td>FITC (488 nm)</td></tr>
<tr><td>TYPE 1</td><td>Cy3 (550 nm)</td></tr>
<tr><td>TYPE 6</td><td>Cy5 (650 nm)</td></tr>
<tr><td>TYPE 10</td><td>Cy7 (740 nm)</td></tr>
</table>
NOTE: Signals of LP4-488 and LP1-550 are visible to unaided eyes under microscope, and appear as green and red dots, respectively. Signals of LP6-650 and LP-740 are invisible and require CCD-cooled camera to capture the image.
</td></tr>
</table>

NOTE: Remaining reagents sent in the kit can be stored as recommended for up to six months from date of purchase.

Troubleshooting

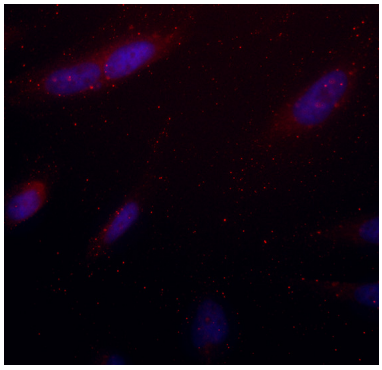
Poor Cell Retention

Table 5.9 Troubleshooting Poor Cell Retention

Figure 5.1	Probable Cause	Recommended Actions
	Excess protease treatment or inadequate fixation	▪ Titrate fixation time and protease concentrations.
	Poor cell adhesion to the glass surface	▪ Try coating the cover slip surfaces with different extracellular matrices such as MatriGel, collagen or poly-D-lysine. ▪ Use protocol for suspension cells if cell adhesion is extremely poor.
	Cell detachment during aspiration and dispensing of the solutions	▪ Avoid aspirating and dispensing solutions directly on top of the cells. Dispense and aspirate reagents at a slower speed.

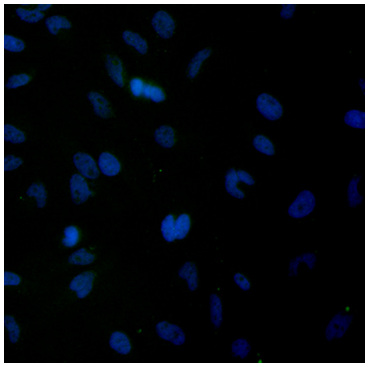
Non-Specific Dots Outside the Cells

Table 5.10 Troubleshooting Non-Specific Dots Outside the Cells

Figure 5.2	Probable Cause	Recommended Actions
	Non-specific binding of Probe Sets, PreAmps, Amps or Label Probes to glass surface	▪ Verify coating materials by running the assay without cells in the presence and absence of coating materials.
	Insufficient washing	▪ Increase soaking time by 5 minutes for each washing step.
	Samples were allowed to dry	▪ Make sure sufficient solution is covering the entire sample at all times during the assay. If using glass slide format: ▪ Make sure to wet the humidifying strips inside the ThermoBrite before starting hybridization. ▪ Make sure the ThermoBrite is placed on a leveled bench and the lid is closed during the assay. ▪ Add all reagents to slides **before** transferring the slides to the ThermoBrite.

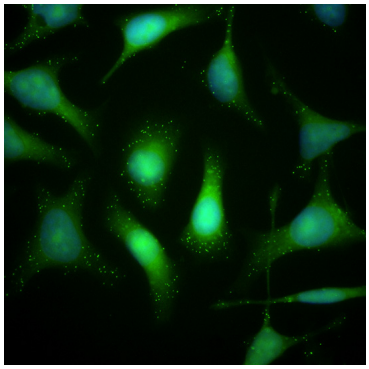
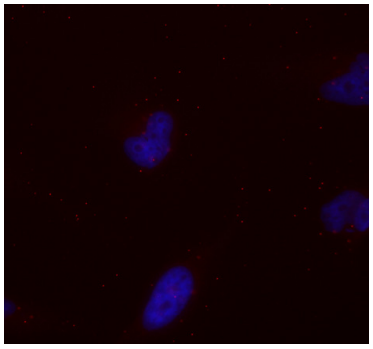
Weak or No Signal

Table 5.11 Troubleshooting Weak or No Signal

Figure 5.3	Probable Cause	Recommended Actions
	Protease digestion not optimal	▪ Titrate protease concentrations.
	Fixation time not optimal	▪ Titrate formaldehyde fixation time.
	Inaccurate hybridization temperature	▪ Hybridization reactions must be carried out at 40 ± 1 °C. ▪ Use a QG ViewRNA Temperature Validation Kit (Affymetrix P/N QV0523) to verify and monitor the temperature.
	Target is not expressed in the cells being assayed	▪ Use positive control probe set such as one for a housekeeping gene to validate the procedure.
	Samples left too long in Wash Buffer	▪ Follow protocol for appropriate washing times.
	Photo-bleaching of fluorescent signals	▪ Keep Label Probe Mix in dark during experiment. ▪ After adding Label Probe Mix Solution, protect samples from light. ▪ Minimize exposure of samples to fluorescent light when viewing under the microscope.
	Incorrect use of Probe Set(s)	▪ Ensure that the Working Solutions are prepared properly.
	Incorrect use of PreAmp, Amp and/or LP	▪ Make sure that the Probe Set, PreAmp-Mix, Amp Mix and Label Probe Mix are added in the correct order and to the appropriate samples.
	Reagents did not reach the cells	▪ Make sure cell seeded cover slips are facing up during the assay. ▪ If you lose track of which side of the cover slip contains the cells, check it under the microscope by scraping a small surface area with fine tipped forceps.
	Inappropriate microscope setup or operation	▪ Ensure that your microscope is in good working order and that your light source, objectives, filters and exposure times for image acquisition are selected properly (see microscope specification on page 2).

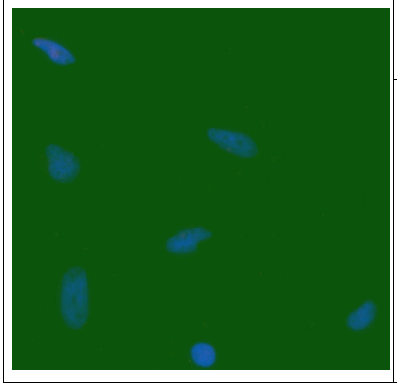
High Background Inside the Cells

Table 5.12 Troubleshooting High Background Inside the Cells

Figure 5.4	Probable Cause	Recommended Actions
	Insufficient washing after formaldehyde fixation	▪ Increase washing volume or soaking time by 5 minutes for each washing step.
	Auto-fluorescence	▪ Some cell lines may exhibit high auto-fluorescence, particularly in the 488 nm wavelength. This is a normal biological property of the cell. Should this occur, use 488 nm channel for detecting a highly expressed gene.
Figure 5.5	**Probable Cause**	**Recommended Actions**
 No probe negative control sample image	Insufficient washing after hybridization	▪ Increase washing volume or soaking time by 5 minutes for each washing step.
	Samples were allowed to dry	▪ Make sure sufficient solution is covering the entire sample at all times during the assay. ▪ Make sure to wet the humidifying strips inside the ThermoBrite before starting hybridization. ▪ Make sure the ThermoBrite is placed on a leveled bench and the lid is closed during the assay. ▪ Add all reagents to slides **before** transferring the slides to the ThermoBrite.

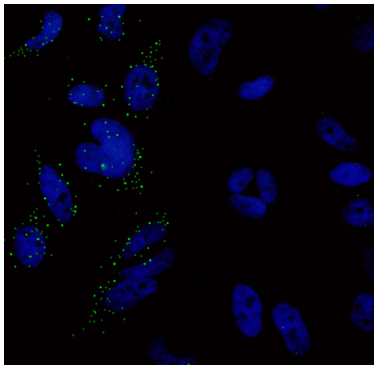
High Homogeneous Background On the Slide

Table 5.13 Troubleshooting High Homogeneous Background On the Slide

Figure 5.6	Probable Cause	Recommended Actions
	Incorrect filter setup	▪ Check filter specification and replace filters used in the imaging system if necessary.
	Auto-fluorescence from the nail polish used in the Glass Slide Format or from coating material	▪ Under a microscope, check nail polish on a glass slide for auto-fluorescent properties. ▪ Make sure the coating material used does not have auto-fluorescent properties by comparing coated and non-coated glass surfaces under a microscope.

Variable Staining Within a Sample

Table 5.13 Troubleshooting Variable Staining Within a Sample

Figure 5.7	Probable Cause	Recommended Actions
	Samples were allowed to dry	▪ Make sure sufficient solution is covering the entire sample at all times during the assay. If using glass slide format: ▪ Make sure to wet the humidifying strips inside the ThermoBrite before starting hybridization. ▪ Make sure the ThermoBrite is placed on a leveled bench and the lid is closed during the assay. ▪ Add all reagents to slides **before** transferring the slides to the ThermoBrite.
	Insufficient mixing of reagents	▪ Pre-warm all hybridization buffers to dissolve any precipitates before use. ▪ Briefly vortex all working hybridization solutions before use.

Cover Slip Falls Off During the Assay (For Cover Sips Mounted on Glass Slide Format only)

Table 5.14 Troubleshooting Cover Slip Falls Off During the Assay

Recommended Actions
▪ Make sure the nail polish between the cover slip and microscope slide is completely dried before starting the assay by extending the nail polish drying time to 20–30 minutes. ▪ Increase the amount of nail polished used on the slide. ▪ Change to a new source.

Appendix A

Sample Preparation Procedure for Suspension Cells

Overview

Suspension cells are fixed in suspension and then spotted directly on the poly-L-lysine coated glass microscope slides or glass cover slips (for instructions on how to prepare poly-L-lysine coated glass cover slips, refer to *Assay Setup: Sample Preparation* *on page 15*). Cells can be prepared on the same day for use in the *in situ* hybridization assay.

This procedure is compatible with 24-well plate and glass slide formats.

Important Procedural Notes and Guidelines

- A vacuum system is recommended for all steps that require aspiration. Alternatively, aspiration can be performed by manually pipetting the solution from the wells.
- Aspirate from the bottom edge of the wells and dispense against the upper edge of the well.

NOTE: The 1X PBS mentioned for the sample preparation for adherent cells is not supplied in the assay kit. Please use sterile cell culture 1X PBS.

Procedure

Step	Action
Step 1. **Set Incubator to 50 °C** **1 min**	**A.** Set incubator to 50 ± 1 °C.
Step 2. **Fix cells in 4% formaldehyde solution** **90 min**	**A.** Under a fume hood, prepare fresh 4% formaldehyde solution by adding 300 µL of 37% formaldehyde to 2475 µL of 1X PBS. Vortex briefly to mix. **B.** Disperse suspension cells to single cells by pipetting up and down with a 10 mL pipette. **C.** Aliquot 2.5-5 million suspended cells into a 15 mL centrifuge tube. Spin down cells (200 x g, 5 minutes), aspirate off culture medium and resuspend cell pellet with 5 mL of 1X PBS. **D.** Spin down cells (200 x g, 5 minutes), aspirate off 1X PBS solution and resuspend cell pellet with 1 mL of 4% formaldehyde. **E.** Incubate for 60 minutes at RT (briefly vortex the tube every 20 minutes during the incubation to resuspend cells). **IMPORTANT:** **For suspension cells, we recommend starting with a fixation time of 60 minutes. However, when using a new cell type for the first time, a titration of the fixation time is recommended as the optimal time can vary between cell types.** **F.** Transfer cell solution to a 1.5 mL microcentrifuge tube. Spin down cells (15,000 x g, 1 minute), gently decant formaldehyde solution and wash cell pellet three times, each with 1 mL of 1X PBS (15,000 x g, 1 minute). **G.** Spin down cells (15,000 x g, 1 minute), gently decant the final 1X PBS wash and resuspend cell pellet thoroughly with 150 µL of 1X PBS using 200 µl pipette to obtain a single-cell suspension. Adjust cell concentration to 1000 cells/µl.

Step	Action
Step 3. **Spot fixed cells on poly-L-lysine coated glass surface** **35 min**	**A.** (*For glass slide only*) Draw two hydrophobic barriers on poly-L-lysine coated microscope slides (see figure immediately below) using the templates as a guide (see Appendix E, *Templates on page 57*) and a hydrophobic pen (Affymetrix P/N QVC0500; included in the QuantiGene ViewRNA ISH Cell Assay Accessory Kit, Affymetrix P/N QVC0700). Allow the hydrophobic barrier to dry for 1 minute. **NOTE: Ensure a strong hydrophobic barrier is achieved. This may require the barrier to be drawn multiple times.**  **B.** Pipette 20 µl of fixed suspension cells on poly-L-lysine coated microscope glass slide (Thermo Scientific P/N P4981-001) or glass cover slip (~20,000 total cells/ spot). Gently spread out the spot with a pipette tip to ensure even distribution of the cells. **C.** Bake cells in dry oven at 50 ±1 °C for 30 minutes or until cells are completely dried (Open vent on incubator if using Affymetrix incubator). **D.** Proceed to the *in situ* hybridization Assay Procedure starting with the Rehydration step. Alternatively, sample slides can be stored in 100% ethanol at –20°C for up to 1 month.

Appendix B

Alternate Protocol for Glass Slide Format

Overview

This alternate QuantiGene ViewRNA ISH Cell Assay protocol allows the *in situ* hybridization assay to be performed on 12 mm cover slips mounted on standard microscope slides (64 mm x 25 mm) using a ThermoBrite System (Abbott Molecular P/N 07J91-010, 110V). Signals can be visualized using an upright/inverted fluorescent microscope; refer to page 2 for specifications.

If you choose to use this alternate protocol, additional volumes of the following reagents are required and will need to be purchased separately from the QuantiGene ViewRNA ISH Cell Assay Kit:

- 10X PBS
- Detergent Solution QC
- Wash Buffer Component 1
- Wash Buffer Component 2

Additional Required Materials Not Provided

Other materials that may also be required to perform the QuantiGene ViewRNA ISH Cell Assay are listed in the table below. When specified, **do not use** alternate materials or suppliers.

Table B.1 Required Materials Not Provided

Required Material	Supplier	Part Number or Model
Deionized Water (ddH_2O)	MLS[a]	
100% Ethanol	VWR	89125-188
37% Formaldehyde[b]	Fisher Scientific	F79-1
Prolong® Gold Antifade Reagent	Invitrogen	P36930
Nail Polish—viscous, flat black color	various	
Poly-L-Lysine	Sigma	P8920
10X PBS	Affymetrix	QVC0508
Detergent Solution QC	Affymetrix	QVC0509
Wash Buffer Components (Includes Wash Buffer Component 1 and Wash Buffer Component 2)	Affymetrix	QVC0507
ThermoBrite System (capable of maintaining 40 ± 1 °C)	Abbot Molecular	0731-010 (110V)
Humidifying Strips	Abbot Molecular	30-144115
QG ViewRNA Temperature Validation Kit	Affymetrix	QV0523
Pipets (P20, P200 and P1000)	MLS	
Water Bath (capable of maintaining 40 ± 1 °C)	MLS	
Table-top MicroTube Centrifuge	MLS	
Epi-Fluorescence Microscope (see page 2 for specifications)	MLS	
Optional Vacuum System (set to operate at a rate of ~2 mL/5 sec)	MLS	

[a] MLS = Major Laboratory Supplier
[b] WARNING! Formaldehyde is a poison and irritant. Avoid contact with skin and mucous membranes.

Table B.2 Required Materials Not Provided (Included in the QuantiGene ViewRNA Cell Assay Accessory Kit)

Required Material	Sizes	Supplier	Part Number or Model
The following items are included in the QuantiGene ViewRNA ISH Cell Assay Accessory Kit (Affymetrix, P/N QVC0700). Components are also available for individual purchase.			
Microscope Slides	12 each	Affymetrix	QVC0505
Rectangular Cover Glass	15 each	Affymetrix	QVC0506
Cover Slips	50 each	Affymetrix	QVC0507
Hydrophobic Pen	each	Affymetrix	QVC0500
Forceps	each	Affymetrix	QVC0501
Tissue-Tek Staining Dish (clear)	each	Affymetrix	QVC0502
Tissue-Tek Slide Rack	each	Affymetrix	QVC0503
Tissue-Culture 24-Round Well Plate	each	Affymetrix	QVC0504

Sample Preparation for Adherent Cells

NOTE: See Appendix A for Suspension Cells Protocol on on page 31.

Overview

Prior to performing the QuantiGene ViewRNA ISH Cell Assay procedure, adherent cells must be grown and fixed on poly-L-lysine coated 12 mm circular cover slips. Begin the coating procedure the day prior to seeding the cells. The optimal cell density for this assay is 70–90% confluent at the start of the assay.

Important Procedural Notes and Guidelines

- Before working with actual samples, practice removing cover slips from tissue-culture 24-round well plate with fine tipped forceps and mounting them onto microscope slides as described in *Step 1. on page 38*.
- Use sterile cell culture reagents and techniques when preparing poly-L-lysine coated cover slips and culturing cells.
- A vacuum system is recommended for all steps that require aspiration. Alternatively, aspiration can be performed by manually pipetting the solution from the wells.
- Please allocate a 10% overage when using calculations in this manual to account for dead volume.
- Aspirate from the bottom edge of the wells and dispense against the upper edge of the well.

NOTE: The 1X PBS mentioned for the sample preparation for adherent cells is not supplied in the assay kit. Please use sterile cell culture 1X PBS.

Assay Setup: Sample Preparation

I. Prepare poly-L-lysine coated 12 mm circular cover slips in a tissue-culture 24-round well plate.

II. Culture fresh adherent cells on poly-L-lysine coated cover slips in a tissue-culture 24-round well plate.

III. Fix cells in 4% formaldehyde solution in the tissue-culture 24-round well plate.

IV. Dehydrate cells in ethanol in the tissue-culture 24-round well plate.

I. Prepare poly-L-lysine coated 12 mm circular cover slips in a tissue-culture 24-round well plate:

Step	Task
Step 1. **5 min**	Dilute poly-L-lysine 1:10 (final concentration of 0.01%) by adding 2.5 mL of poly-L-lysine stock solution to 22.5 mL of room temperature (RT) nuclease-free water. Mix well and set aside until use.
Step 2. **20 min**	Place one cover slip into each well of a sterile tissue-culture 24-round well plate. To sterilize the cover slips, dispense 1 mL/well of 70% ethanol and soak at RT for 15 minutes. **NOTE: The glass cover slips tend to float once the solution is added. Should this occur, use a sterile pipette tip to press the floating cover slips down into the solution.**
Step 3. **20 min**	Aspirate off the 70% ethanol and rinse cover slips with 1 mL/well of 100% ethanol. Aspirate off the 100% ethanol and allow the cover slips to air dry completely for 15 minutes.
Step 4. **20 min**	Dispense 1 mL/well of diluted poly-L-lysine, ensuring that the cover slips are completely submerged in the solution. Incubate for 15 minutes at RT.
Step 5. **10 min**	Aspirate off the poly-L-lysine and rinse the cover slips three times, each with 2 mL/well of 1X PBS.
Step 6. **Overnight Incubation**	Aspirate off the final 1X PBS. Allow to air dry, with lid off, in a laminar flow tissue culture hood overnight. Keep air flow on and turn UV light off.
Optional **Storage**	Cover slips may be used directly for cell seeding or stored at 4 °C in the tissue-culture 24-round well plate with lid on and sealed with parafilm for up to 1 month.
Step 7.	Proceed to culturing cells on poly-L-lysine coated cover slips.

II. Culture fresh adherent cells on poly-L-lysine coated cover slips in a tissue-culture 24-round well tissue plate:

Step	Task
Step 1. **5 min**	Wash the adherent cells with 1X PBS and trypsinize. Resuspend the cells in complete cell culture medium.
Step 2. **10 min**	Pellet the cells (200 x g, 5 minutes) at RT, remove the supernatant, and resuspend the cell pellet in fresh complete culture medium.
Step 3. **5 min**	Count and adjust the cell density such that when seeded, 1 mL/well the culture will reach 70–90% confluence at the start of the assay.
Step 4. **5 min**	Dispense 1 mL/well of diluted cell suspension gently over the poly-L-lysine coated cover slips. Make sure all cover slips are submerged in the cell culture medium.
Step 5. **Overnight Incubation**	Incubate the plate overnight under the recommended growth conditions for the cells.
Step 6.	Proceed to fixation of cells.

III. Fix the cells in 4% formaldehyde solution in the tissue-culture 24-round well plate:

Step	Task
Step 1. **5 min**	In a fume hood, prepare 10 mL of fresh 4% formaldehyde solution by diluting 1.08 mL of a 37% stock formaldehyde with 8.92 mL of 1X PBS. Vortex briefly to mix. **WARNING: Formaldehyde is a poison and irritant. Avoid contact with skin and mucous membranes.**
Step 2. **5 min**	Carefully aspirate off culture medium, avoiding contact with cover slips and cells. Gently wash cover slips twice, each time with 2 mL/well of 1X PBS.
Step 3. **35 min**	Aspirate off the final 1X PBS wash and add 400 µL/well of freshly prepared 4% formaldehyde, making sure that the cover slips are submerged. Incubate at RT for 30 minutes. **NOTE: If cover slips are not fully submerged, use the forceps to gently press down on the sides of the cover slip. Do not press on the center of the cover slip.** **IMPORTANT: A fixation time of 30 minutes works for most cell lines. However, when using a new cell type for the first time, a titration of the fixation time is recommended as the optimal time can vary between cell types. Refer to Experimental Design and Assay Optimization** **on page 9** **for detailed instructions.**
Step 4. **10 min**	Aspirate off the formaldehyde solution and gently rinse the cover slips three times, each with 2 mL/well of 1X PBS.
Step 5.	Proceed to dehydration of cells.

IV. Dehydrate cells in ethanol in a tissue-culture 24-round well plate:

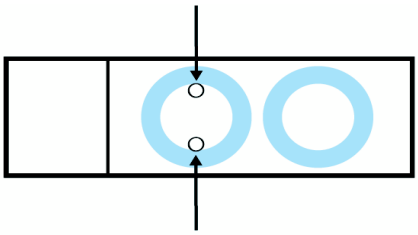
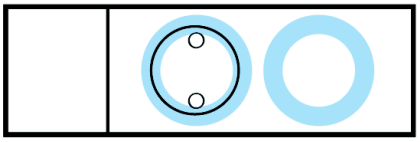
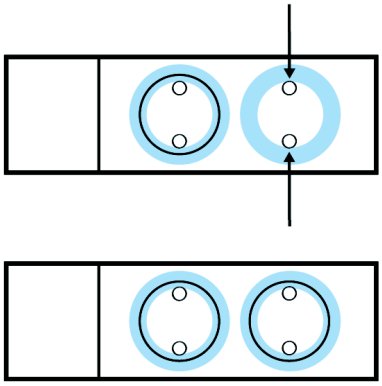
Step	Task
Step 1. **Dehydrate cells in tissue-culture 24-round well plate** **15 min**	**A.** Aspirate off the 1X PBS and replace with 400 µL/well of 50% ethanol. Incubate for 2 minutes at RT. **B.** Aspirate off the 50% ethanol and replace with 400 µL/well of 70% ethanol. Incubate for 2 minutes at RT. **C.** Aspirate off the 70% ethanol and replace with 400 µL/well of 100% ethanol. Incubate for 2 minutes at RT. **D.** Aspirate off the 100% ethanol and replace with 800 µL/well of fresh 100% ethanol. **E.** Cell samples may be used directly in the *in situ* hybridization assay. Alternatively, seal the plate with parafilm and store the dehydrated cells in 100% ethanol at –20 °C until needed. Dehydrated cells can be stored under these conditions for several weeks. **NOTE: Dehydrated cells must be rehydrated prior to being used in the *in situ* assay.**
Step 2.	Proceed to Assay Setup.

Assay Setup

Step	Task
Step 1. **Prepare ThermoBrite** **30 min**	Wet the humidifying strips with ddH_20 thoroughly and set ThermoBrite to 40 °C. Calibrate and monitor ThermoBrite temperature using the QG ViewRNA Temperature Validation Kit (Affymetrix P/N QV0523) according to the manufacturer's instruction.
Step 2. **Prepare Buffers & Other Reagents (for running 12 slides)** **2 min**	Prepare 1,900 mL of 1X PBS by adding 190 mL of 10X PBS to 1,710 mL of ddH_20 and then mixing well.
Step 3. **5 min**	Prepare 250 mL of 100%, 70% and 50% ethanol solutions.
Step 4. **1 min**	Place Protease QS on ice.
Step 5. **5 min**	Prepare 3.2 L of Wash Buffer by adding components to a 4 L capacity container in the following order and then mixing well: ▪ 3,174.4 mL ddH_20 ▪ 9.6 mL Wash Comp 1 ▪ 16 mL Wash Comp 2 **NOTE:** **Adding the components in the order listed above will ensure against the formation of precipitates.**
Step 6. **1 min**	Pre-warm Probe Set Diluent QF, Amplifier Diluent QF and Label Probe Diluent QF to 40 °C in a water bath for 30 min. **WARNING:** **Probe Set Diluent QF and Amplifier Diluent QF contain formamide, a teratogen, irritant and possible carcinogen. Avoid contact with skin and mucous membranes.**
Step 7. **5 min**	Thaw Probe Set(s), PreAmplifier Mix, Amplifier Mix and Label Probe Mix at RT. Vortex briefly to mix and place tubes on ice until use. Protect Label Probe Mix from light.
Step 8. **5 min**	Thaw the 100X DAPI at RT. Vortex briefly to mix and place tube on ice until use. Protect from light. **WARNING:** **DAPI is a possible mutagen. Avoid contact with skin and mucous membranes.**

Assay Procedure

NOTE: All calculations in this section are for 1 cover slip. Please scale accordingly.

Step	Action
Step 1. Glue Cover Slips onto Microscope Slides (for adherent cell samples only. For suspension cells go directly to Step 2) **40 min**	**A.** Using the real-size templates provided on Appendix E, on page 57 as a guide, draw two hydrophobic barriers (see figure immediately below) on each side. Allow the hydrophobic barrier to dry for 1 minute. **NOTE:** Ensure a strong hydrophobic barrier is achieved. This may require the barrier to be drawn multiple times.  **B.** Using fine tipped forceps, transfer a cover slip from the 24-well plate, cell side facing up, to a dry laboratory wipe. Allow the ethanol to completely evaporate off from the cover slip. **C.** Place two small droplets of nail polish onto a glass microscope slide approximately 12 mm apart (see arrows in figure immediately below). **NOTE:** Select a nail polish that is viscous in order to prevent spreading and black in color with a flat finish to minimize auto-fluorescence.  **D.** Transfer the dried cover slip, with cell side up onto the glass slide (see figure immediately below). Gently press on the top of the cover slip with forceps to create a permanent seal. Do not press in the center of the cover slip.  **E.** Repeat Step 1B–D in order to glue a second cover slip onto the microscope slide (see figure immediately below).  **F.** Allow the nail polish to dry at RT for at least 15 minutes. **G.** For easy identification of samples, label the frosted area of the glass slides with a pencil only. If a permanent marker is used, the writing will be dissolved by the reagents. **NOTE:** See Appendix E, on page 57 for templates to aid in gluing cover slip onto microscope slides.

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>Step 2.
Rehydration
20 min</td><td>
A. Insert slides, evenly spaced, into the Tissue-Tek Vertical 24-Slide Rack (labeled ends up).
B. Rehydrate cells by submerging slides into a Tissue-Tek Staining Dish containing 250 mL of 100% ethanol. Incubate for 2 minutes at RT.
C. Decant 100% ethanol and replace with 250 mL of 70% ethanol. Incubate for 2 minutes at RT.
D. Decant 70% ethanol and replace with 250 mL of 50% ethanol. Incubate for 2 minutes at RT.
E. Decant 50% ethanol and replace with 250 mL of 1X PBS. Incubate for 10 minutes at RT.
</td></tr>
<tr><td>Step 3.
Permeabilize cells with Detergent Solution
8 min</td><td>
A. Decant 1X PBS and replace with 250 mL of Detergent Solution QC. Incubate for 5 minutes at RT.
B. Decant Detergent Solution QC and replace with 250 mL of 1X PBS. Gently agitate slides up and down 5 times.
C. Decant 1X PBS and replace with fresh 250 mL of 1X PBS. Gently agitate slides up and down 5 times.
</td></tr>
<tr><td>Step 4.
Digest with Working Protease Solution
20 min</td><td>
IMPORTANT: The optimal protease concentration can vary with cell type. If you do not wish to perform the optimization in Experimental Design and Assay Optimization on page 9, we suggest starting with 1:1,000 protease dilution in 1X PBS and optimize as needed.

A. Prepare diluted Working Protease Solution in 1X PBS.

<table>
<tr><th>Working Protease Solution for 1 Cover Slip[a]</th><th></th></tr>
<tr><td>Protease QS</td><td>0.1 µL</td></tr>
<tr><td>1X PBS</td><td>99.9 µL</td></tr>
<tr><td>Total Volume</td><td>100.0 µL</td></tr>
</table>
[a] scale accordingly for other dilution ratios

B. Vortex briefly to mix.
C. Remove slides from 1X PBS. Drain solution from the surface of each slide by dabbing the edge on a laboratory wipe until most of the solution is removed from the hydrophobic barrier. Place slides on a flat surface and add 100 µL/sample of the Working Protease Solution. Incubate for 10 minutes at RT.
D. Quickly decant Working Protease Solution and submerge slides into a Tissue-Tek Staining Dish containing 250 mL of 1X PBS. Gently agitate up and down 5 times.
E. Decant 1X PBS and replace with fresh 250 mL of 1X PBS. Gently agitate slides up and down 5 times.
F. Repeat Step 4E once.
</td></tr>
</table>

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>Step 5.
Hybridization with Probe Set(s)
3 hr 10 min</td><td>
A. Set ThermoBrite to 40°C and insert the pre-soaked humidifying strips.
B. Prepare Working Probe Set Solution by diluting Probe Set(s) 1:100 in pre-warmed Probe Set Diluent QF.
<table>
<tr><th colspan="2">Working Probe Set Solution for 1 Cover Slip</th></tr>
<tr><td>Probe Set (each)</td><td>0.8 µL</td></tr>
<tr><td>Probe Set Diluent QF (pre-warmed at 40 °C)</td><td>79.2 µL</td></tr>
<tr><td>Total Volume</td><td>80.0 µL</td></tr>
</table>
C. Vortex briefly to mix.
NOTE: If using multiple Probe Sets, use the equation below to calculate the Probe Set Diluent QF required per cover slip:
80 µL – (number of Probe Sets x 0.8 µL) = Total volume of Probe Set Diluent QF per cover slip
D. Remove slides from 1X PBS. Drain solution from the surface of each slide by dabbing the edge on a laboratory wipe until most of the solution is removed from the hydrophobic barrier. Immediately add 80 µL/sample of the appropriate Working Probe Set Solution. For the “no probe” negative control, use 80 µL/sample of pre-warmed Probe Set Diluent QF.
E. Carefully transfer slides to the ThermoBrite System. Close the lid and incubate at 40 ± 1 °C for 3 hours.
</td></tr>
<tr><td>Step 6.
Wash Slides 3 Times with Wash Buffer
8 min</td><td>
A. Decant off the Working Probe Set Solution and submerge slides into a Tissue-Tek Staining Dish containing 250 mL of Wash Buffer. Incubate slides for 2 minutes at RT with occasional agitation.
B. Replace with fresh Wash Buffer and incubate the slides at RT for 2 minutes with occasional agitation.
C. Repeat Step 6B once.
IMPORTANT: Do not soak samples in Wash Buffer longer than 30 minutes.
</td></tr>
</table>

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>Step 7.
Hybridize with Pre-Amplifier(s)
40 min</td><td>
A. Prepare the Working PreAmplifier Mix Solution by diluting PreAmplifier Mix 1:25 in pre-warmed Amplifier Diluent QF.
<table>
<tr><th colspan="2">3-plex Working PreAmplifier Mix Solution for 1 Cover Slip</th></tr>
<tr><td>Amplifier Diluent QF (pre-warmed at 40 °C)</td><td>76.8 µL</td></tr>
<tr><td>PreAmplifier Mix</td><td>3.2 µL</td></tr>
<tr><td>Total Volume</td><td>80 µL</td></tr>
</table>
NOTE: If using the QuantiGene ViewRNA ISH Cell 740 Module, prepare the Working PreAmplifier Mix Solution according to the table below.
<table>
<tr><th colspan="2">4-plex Working PreAmplifier Mix Solution for 1 Cover Slip</th></tr>
<tr><td>Amplifier Diluent QF (pre-warmed at 40 °C)</td><td>73.6 µL</td></tr>
<tr><td>PreAmplifier Mix</td><td>3.2 µL</td></tr>
<tr><td>PreAmp10-740</td><td>3.2 µL</td></tr>
<tr><td>Total Volume</td><td>80 µL</td></tr>
</table>
B. Vortex briefly to mix.
C. Remove slides from Wash Buffer. Drain solution from the surface of each slide by dabbing the edge on a laboratory wipe until most of the solution is removed from the hydrophobic barrier. Immediately add 80 µL/sample of Working PreAmplifier Mix Solution.
D. Carefully transfer slides to the ThermoBrite System. Close the lid and incubate slides at 40 ± 1 °C for 30 minutes.
</td></tr>
<tr><td>Step 8.
Wash Slides
3 Times with Wash Buffer
8 min</td><td>
A. Decant off the Working PreAmplifier Mix Solution and submerge slides into a Tissue-Tek Staining Dish containing 250 mL of Wash Buffer. Incubate for 2 minutes at RT with occasional agitation.
B. Replace with fresh Wash Buffer and incubate the slides for another 2 minutes at RT with occasional agitation.
C. Repeat Step 8B once.
IMPORTANT: Do not soak samples in Wash Buffer longer than 30 minutes.
</td></tr>
</table>

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>Step 9.
Hybridize with Amplifier(s)
40 min</td><td>
A. Prepare the Working Amplifier Mix Solution by diluting Amplifier Mix 1:25 in pre-warmed Amplifier Diluent QF.
<table>
<tr><th colspan="2">3-plex Working Amplifier Mix Solution for 1 Cover Slip</th></tr>
<tr><td>Amplifier Diluent QF (pre-warmed at 40 °C)</td><td>76.8 µL</td></tr>
<tr><td>Amplifier Mix</td><td>3.2 µL</td></tr>
<tr><td>Total Volume</td><td>80 µL</td></tr>
</table>
IMPORTANT: If using the QuantiGene ViewRNA ISH Cell 740 Module, prepare the Working Amplifier Mix Solution according to the table below.
<table>
<tr><th colspan="2">4-plex Working Amplifier Mix Solution for 1 Cover Slip</th></tr>
<tr><td>Amplifier Diluent QF (pre-warmed at 40 °C)</td><td>73.6 µL</td></tr>
<tr><td>Amplifier Mix</td><td>3.2 µL</td></tr>
<tr><td>Amp10-740</td><td>3.2 µL</td></tr>
<tr><td>Total Volume</td><td>80 µL</td></tr>
</table>
B. Vortex briefly to mix.
C. Remove slides from Wash Buffer. Drain solution from the surface of each slide by dabbing the edge on a wipe until most of the solution is removed from the hydrophobic barrier. Immediately add 80 µL/sample of Working Amplifier Mix Solution.
D. Carefully transfer slides to the ThermoBrite System. Close the lid and incubate slides at 40 ± 1 °C for 30 minutes.
</td></tr>
<tr><td>Step 10.
Wash Cells
3 Times with Wash Buffer
8 min</td><td>
A. Decant off the Working Amplifier Mix Solution and submerge slides into a Tissue-Tek Staining Dish containing 250 mL of Wash Buffer. Incubate for 2 minutes at RT with occasional agitation.
B. Replace with fresh Wash Buffer and incubate the slides for another 2 minutes at RT with occasional agitation.
C. Repeat Step 10B once.
IMPORTANT: Do not soak samples in Wash Buffer longer than 30 minutes.
</td></tr>
</table>

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>Step 11.
Hybridize with Label Probe(s)
40 min</td><td>
IMPORTANT: Protect samples from light during this and all subsequent steps.

A. Prepare the Working Label Probe Mix Solution by diluting Label Probe Mix 1:25 in pre-warmed Label Probe Diluent QF.

<table>
<tr><th colspan="2">3-plex Working Label Probe Mix Solution for 1 Cover Slip</th></tr>
<tr><td>Label Probe Diluent QF (pre-warmed at 40 °C)</td><td>76.8 µL</td></tr>
<tr><td>Label Probe Mix</td><td>3.2 µL</td></tr>
<tr><td>Total Volume</td><td>80 µL</td></tr>
</table>

NOTE: If using the QuantiGene ViewRNA ISH Cell 740 Module, prepare the Working Label Probe Mix Solution according to the table below.

<table>
<tr><th colspan="2">4-plex Working Label Mix Solution for 1 Cover Slip</th></tr>
<tr><td>Label Probe Diluent QF (pre-warmed at 40 °C)</td><td>73.6 µL</td></tr>
<tr><td>Label Probe Mix</td><td>3.2 µL</td></tr>
<tr><td>LP10-740</td><td>3.2 µL</td></tr>
<tr><td>Total Volume</td><td>80 µL</td></tr>
</table>

B. Vortex briefly to mix. Protect from light.
C. Remove slides from Wash Buffer. Drain solution from the surface of each slide by dabbing the edge on a laboratory wipe until most of the solution is removed from the hydrophobic barrier. Immediately add 80 µL/sample of Working Label Probe Mix Solution.
D. Carefully transfer slides to the ThermoBrite System. Close the lid and incubate slides at 40 ± 1 °C for 30 minutes.
</td></tr>
<tr><td>Step 12.
Wash Cells 3 Times with Wash Buffer
16 min</td><td>
A. Decant off the Working Label Probe Mix Solution and submerge slides into a Tissue-Tek Staining Dish containing 250 mL of Wash Buffer. Incubate for 2 minutes at RT with occasional agitation.
B. Replace with fresh Wash Buffer and incubate the slides for another 2 minutes at RT with occasional agitation.
C. Replace with fresh Wash Buffer and incubate the slides for 10 minutes at RT with occasional agitation.

IMPORTANT: Do not soak samples in Wash Buffer longer than 30 minutes.
</td></tr>
<tr><td>Step 13.
DAPI-Staining
5 min</td><td>
A. Prepare Working DAPI Solution by diluting the 100X DAPI 1:100 in 1X PBS.

<table>
<tr><th colspan="2">Working DAPI Solution for 1 Cover Slip</th></tr>
<tr><td>1X PBS</td><td>99 µL</td></tr>
<tr><td>100X DPAPI</td><td>1 µL</td></tr>
<tr><td>Total Volume</td><td>100 µL</td></tr>
</table>

B. Vortex briefly to mix. Protect from light.
C. Remove slides from Wash Buffer. Drain solution from the surface of each slide by dabbing the edge on a laboratory wipe until most of the solution is removed from the hydrophobic barrier. Add 100 µL/sample of Working DAPI Solution and incubate at RT for 1 minute.
D. Decant off Working DAPI Solution and submerge slides in 250 mL of 1X PBS. Gently agitate slides up and down 5 times.
</td></tr>
</table>

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>Step 14.
Mounting on Glass Slide
10 min</td><td>A. Remove slides from the slide rack and place them on a dry paper towel.
B. Add one drop (10–15 µL/sample) of Prolong® Gold Antifade Reagent on each sample and cover both of the circular cover slips with a rectangular 24 mm x 55 mm cover glass. Avoid air bubbles.</td></tr>
<tr><td>Step 15.
Image Samples</td><td>A. Samples may be viewed under microscope immediately after mounting. However, fluorescent signals will be enhanced after the mounting medium is completely cured.
B. Cure the slides at RT protected from light for several hours to overnight. Samples can then be stored at 2–8 °C protected from light. Fluorescent signals will be stable for up to one week when properly stored.
C. For viewing samples, use appropriate filter sets.

<table>
<tr><th>Probe Set</th><th>Filter Set</th></tr>
<tr><td>TYPE 4</td><td>FITC (488 nm)</td></tr>
<tr><td>TYPE 1</td><td>Cy3 (550 nm)</td></tr>
<tr><td>TYPE 6</td><td>Cy5 (650 nm)</td></tr>
<tr><td>TYPE 10</td><td>Cy7 (740 nm)</td></tr>
</table>

NOTE: Signals of LP4-488 and LP1-550 are visible to unaided eyes under microscope, and appear as green and red dots, respectively. Signals of LP6-650 and LP-740 are invisible and require CCD-cooled camera to capture the image.</td></tr>
</table>

Appendix C

Alternate Protocol for Four-Chambered Dish Format

This alternate QuantiGene ViewRNA ISH Cell Assay protocol allows the *in situ* hybridization assay to be performed on a glass bottom, 35 mm, four-chambered tissue culture dish (Affymetrix P/N QVC0510)using a temperature-validated incubator. Signals can be visualized using an inverted fluorescent microscope; refer to page 2 for specifications.

NOTE: One QuantiGene ViewRNA ISH Cell Assay Kit contains sufficient reagents to process 16 samples in this format.

Additional Required Materials Not Provided

Other materials that may also be required to perform the QuantiGene ViewRNA ISH Cell Assay are listed in the table below. When specified, **do not use** alternate materials or suppliers.

Table C.3 Required Materials Not Provided

Required Material	Supplier	Part Number or Model
Deionized Water (ddH_2O)	MLS[a]	
100% Ethanol	VWR	89125-188
37% Formaldehyde[b]	Fisher Scientific	F79-1
Prolong® Gold Antifade Reagent	Invitrogen	P36930
Poly-L-Lysine	Sigma	P8920
QG ViewRNA Temperature Validation Kit	Affymetrix	QV0523
Four-Chambered Dish	Affymetrix	QVC0510
Pipets (P20, P200 and P1000)	MLS	
Water Bath (capable of maintaining 40 ±1 °C)	MLS	
Table-top MicroTube Centrifuge	MLS	
Dry Incubator (capable of maintaining 40 ±1 °C)	▪ VWR ▪ Affymetrix ▪ Affymetrix	▪ 52201-069 ▪ QS0700 (110V) ▪ QS0710 (220V)
Fume Hood	MLS	
Inverted Epi-Fluorescence Microscope (see page 2 for specifications)	MLS	

[a] MLS = Major Laboratory Supplier

[b] WARNING! Formaldehyde is a poison and irritant. Avoid contact with skin and mucous membranes.

Sample Preparation for Adherent Cells

Overview

Prior to performing the QuantiGene ViewRNA ISH Cell Assay, adherent cells must be grown and fixed on poly-L-lysine coated glass bottom chambered dishes. Begin the coating procedure the day prior to seeding the cells. The optimal cell density for this assay is 70–90% confluent at the start of the assay.

Important Procedural Notes and Guidelines

- Use sterile cell culture reagents and techniques when preparing poly-L-lysine coated chambered dishes and culturing cells.
- The bottom of the dish is glass. Handle with care.
- Please allocate a 10% overage when using calculations in this manual to account for dead volume.

NOTE: Before fixation, cell attachment may be weak. Aspiration and dispensing of contents from and into the chambers should be performed slowly and gently with a 1 mL pipette. Aspirate from the corners and dispense against the outer chamber wall. After fixation, contents of the well can be expelled by inverting the dish over an appropriate receptacle and then inverting the dish on a clean dry paper towel for 1–2 seconds. To save time, wash step dispenses can be performed using a pipet-aid equipped with a 5 mL disposable pipette and slowly dispensing 1 mL into each chamber against the outer chamber wall. Alternatively, use a repeater pipettor

NOTE: The 1X PBS mentioned for the sample preparation for adherent cells is not supplied in the assay kit. Please use sterile cell culture 1X PBS.

Assay Setup: Sample Preparation

I. Prepare poly-L-lysine coated chambered dish.

II. Culture fresh adherent cells on poly-L-lysine coated chambered dish.

III. Fix cells with 4% formaldehyde solution in the chambered dish.

I. Prepare poly-L-lysine coated chambered dish:

Step	Task
Step 1. **5 min**	Dilute poly-L-lysine 1:10 (final concentration of 0.01%) by adding 0.25 mL of poly-L-lysine stock solution to 2.25 mL of room temperature (RT) nuclease-free water. Mix well and set aside until use.
Step 2. **15 min**	Dispense 600 µL/chamber of diluted poly-L-lysine and incubate for 15 minutes at RT.
Step 3. **5 min**	Aspirate off the poly-L-lysine and rinse three times, each with 1 mL/chamber of 1X PBS.
Step 4. **Overnight Incubation**	Aspirate off the final 1X PBS. Allow to air dry, with lid off, in a laminar flow tissue culture hood overnight. Keep air flow on and turn UV light off.
Optional **Storage**	Coated dishes may be used immediately for cell seeding or stored at 4 °C under sterile conditions. Coated dishes can be stored up to 1 month.
Step 5.	Proceed to culturing cells on poly-L-lysine coated chambered dishes.

II. Culture fresh adherent cells on poly-L-lysine coated chambered dish:

Step	Task
Step 1. **5 min**	Wash the adherent cells with 1X PBS and trypsinize. Resuspend the cells in complete cell culture medium.
Step 2. **10 min**	Pellet the cells (200 x g, 5 minutes) at RT, remove the supernatant, and resuspend the cell pellet in fresh complete culture medium.

II. Culture fresh adherent cells on poly-L-lysine coated chambered dish: (Continued)

Step	Task
Step 3. **5 min**	Count and adjust the cell density such that when seeded, 600 µl/chamber, the culture will reach 70–90% confluence at the start of the assay.
Step 4. **2 min**	Dispense 600 µL/chamber of diluted cell suspension.
Step 5. **Overnight Incubation**	Incubate the plate overnight under the recommended growth conditions for the cells.
Step 6.	Proceed to fixation of cells.

III. Fix the cells in 4% formaldehyde solution in the chambered dish:

Step	Task
Step 1. **5 min**	(For each four-chambered dish) In a fume hood, prepare 3 mL of fresh 4 % formaldehyde solution by diluting 324 µL of a 37% stock formaldehyde with 2.676 mL of 1X PBS and then mixing well. **WARNING: Formaldehyde is a poison and irritant. Avoid contact with skin and mucous membranes.**
Step 2. **5 min**	Gently aspirate off culture medium and rinse cells twice, each time with 1 mL/chamber of 1X PBS.
Step 3. **35 min**	Aspirate off the final 1X PBS wash and add 600 µL/chamber of freshly prepared 4% formaldehyde solution. Cover dish with lid and incubate at RT for 30 minutes. **IMPORTANT: A fixation time of 30 minutes works for most cell lines. However, when using a new cell type for the first time, a titration of the fixation time is recommended as the optimal time can vary between cell types. Refer to Experimental Design and Assay Optimization** on page 9 **for detailed instructions.**
Step 4. **5 min**	Aspirate off the formaldehyde solution and gently rinse the cells three times, each with 1 mL/chamber of 1X PBS.
Step 5.	Fixed cells may be used immediately for the *in situ* assay, in which case, proceed to *Assay Setup* *on page 48*.
Optional **15 min**	Alternatively, samples can be dehydrated for long term storage. **IMPORTANT: Sample dehydration and rehydration on the glass-bottom chambered dish tends to weaken the adhesive between the glass bottom and the plastic dish. Hence, the chambered dish may become leaky.** **A.** Aspirate off the 1X PBS and replace with 600 µL/chamber of 50% ethanol. Incubate for 2 minutes at RT. **B.** Aspirate off the 50% ethanol and replace with 600 µL/chamber of 70% ethanol. Incubate for 2 minutes at RT. **C.** Aspirate off the 70% ethanol and replace with 600 µL/chamber of 100% ethanol. Incubate for 2 minutes at RT. **D.** Aspirate off the 100% ethanol and replace with 600 µL/chamber of fresh 100% ethanol. **E.** Seal the chambered dish with parafilm and store the dehydrated cells in 100% ethanol at –20 °C until needed. Dehydrated cells can be stored under these conditions for several weeks. **NOTE: Dehydrated cells must be rehydrated prior to being used in the *in situ* assay.**
Step 6.	Proceed to Assay Setup.

Assay Setup

Step	Task
Step 1. **Prepare Incubator** **30 min**	Set a dry incubator to 40 °C. Calibrate and monitor incubator temperature using the QG ViewRNA Temperature Validation Kit (Affymetrix P/N QV0523) according to the manufacturer's instructions.
Step 2. **Prepare Buffers & Other Reagents (for one Four-chambered dish)** **2 min**	Prepare 40 mL of 1X PBS by adding 4 mL of 10X PBS to 36 mL of ddH_20 and then mixing well. **NOTE:** **Scale accordingly if running less than 4 chambers in the dish.**
Step 3. **1 min**	Place Protease QS on ice.
Step 4. **5 min**	Prepare 53 mL of Wash Buffer by adding components to a 100 mL capacity container in the following order and then mixing well: ▪ 52.6 mL ddH_20 ▪ 159 µL Wash Comp 1 ▪ 265 µL Wash Comp 2 **NOTE:** **Adding the components in the order listed above will ensure against the formation of precipitates.**
Step 5. **1 min**	Pre-warm Probe Set Diluent QF, Amplifier Diluent QF and Label Probe Diluent QF to 40 °C in a water bath for 30 min. **IMPORTANT:** **Probe Set Diluent QF and Amplifier Diluent QF contain formamide, a teratogen, irritant and possible carcinogen. Avoid contact with skin and mucous membranes.**
Step 6. **5 min**	Thaw Probe Set(s), PreAmplifier Mix, Amplifier Mix and Label Probe Mix at RT. Vortex briefly to mix and place tubes on ice until use. Protect Label Probe Mix from light.
Step 7. **5 min**	Thaw the 100X DAPI at RT. Vortex briefly to mix and place tube on ice until use. Protect from light. **IMPORTANT:** **DAPI is a possible mutagen. Avoid contact with skin and mucous membranes.**

Assay Procedure

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>Step 1.
Rehydration
17 min</td><td>NOTE: Rehydration is ONLY necessary when using dehydrated cell samples. Skip this step and proceed directly to Step 2 when using freshly fixed samples.

A. Aspirate off the 100% ethanol and replace with 600 µL/chamber of 70% ethanol. Incubate for 2 minutes at RT.
B. Aspirate off the 70% ethanol and replace with 600 µL/chamber of 50% ethanol. Incubate for 2 minutes at RT.
C. Aspirate off the 50% ethanol and replace with 600 µL/chamber of 1X PBS. Incubate for 10 minutes at RT.</td></tr>
<tr><td>Step 2.
Permeabilize cells with Detergent Solution
7 min</td><td>A. Invert dish over appropriate receptacle to gently expel the 1X PBS, then invert dish on a clean, dry paper towel for 1–2 seconds.
B. Immediately add 600 µL/chamber of Detergent Solution QC. Cover dish with lid and incubate for 5 minutes at RT.
C. Expel the Detergent Solution QC from the dish as described in Step 2A and rinse dish twice, each with 1 mL/chamber of 1X PBS.</td></tr>
<tr><td>Step 3.
Digest with Working Protease Solution
15 min</td><td>IMPORTANT: The optimal protease concentration can vary with cell type. If you do not wish to perform the optimization in Experimental Design and Assay Optimization on page 9, we suggest starting with 1:4,000 protease dilution in 1X PBS and optimize as needed.

A. Prepare diluted Working Protease Solution in 1X PBS.

<table>
<tr><th colspan="2">Working Protease Solution for 1 Chamber[a]</th></tr>
<tr><td>Protease QS</td><td>0.15 µL</td></tr>
<tr><td>1X PBS</td><td>599.85 µL</td></tr>
<tr><td>Total Volume</td><td>600.0 µL</td></tr>
</table>
[a] scale accordingly for other dilution rations

B. Vortex briefly to mix.
C. Invert dish over appropriate receptacle to gently expel the 1X PBS, then invert dish on a clean, dry paper towel for 1-2 seconds. Immediately add 600 µL/chamber of Working Protease Solution. Cover dish with lid and incubate for 10 minutes at RT.
D. Expel the Working Protease Solution from the dish and rinse dish three times, each with 1 mL/chamber of 1X PBS. Allow samples to sit in the final 1X PBS wash while preparing the Working Probe Set Solution.</td></tr>
</table>

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>Step 4.
Hybridization with Probe Set(s)
3 hr 5 min</td><td>
A. Prepare Working Probe Set Solution by diluting Probe Set(s) 1:100 in pre-warmed Probe Set Diluent QF.
<table>
<tr><th colspan="2">Working Probe Set Solution for 1 Chamber</th></tr>
<tr><td>Probe Set (each)</td><td>6 µL</td></tr>
<tr><td>Probe Set Diluent QF (pre-warmed at 40 °C)</td><td>594 µL</td></tr>
<tr><td>Total Volume</td><td>600 µL</td></tr>
</table>
B. Vortex briefly to mix.
NOTE: If using multiple Probe Sets, use the equation below to calculate the Probe Set Diluent QF required per chamber:
600 µL – (number of Probe Sets x 6 µL) = Total volume of Probe Set Diluent QF per chamber
C. Invert dish over appropriate receptacle to gently expel the 1X PBS, then invert dish on a clean, dry paper towel for 1–2 seconds. Immediately add 600 µL/chamber of the appropriate Working Probe Set Solution. For the “no probe” negative control, use 600 µL/chamber of pre-warmed Probe Set Diluent QF.
D. Cover the dish and incubate at 40 ± 1 °C for 3 hours.
</td></tr>
<tr><td>Step 5.
Wash Cells 3 Times with Wash Buffer
7 min</td><td>
A. Remove dish from the incubator.
B. Invert dish over appropriate receptacle to gently expel the Working Probe Set Solution, then invert dish on a clean, dry paper towel for 1–2 seconds. Wash dish three times, each with 1 mL/chamber of Wash Buffer. Soak sample for 2 minutes during each wash. Allow samples to sit in the final wash while preparing the Working PreAmplifier Mix Solution.
IMPORTANT: Do not soak samples in Wash Buffer longer than 30 minutes.
</td></tr>
<tr><td>Step 6.
Hybridize with Pre-Amplifier(s)
35 min</td><td>
A. Prepare the Working PreAmplifier Mix Solution by diluting PreAmplifier Mix 1:25 in pre-warmed Amplifier Diluent QF.
<table>
<tr><th colspan="2">3-plex Working PreAmplifier Mix Solution for 1 Chamber</th></tr>
<tr><td>Amplifier Diluent QF (pre-warmed at 40 °C)</td><td>576 µL</td></tr>
<tr><td>PreAmplifier Mix</td><td>24µL</td></tr>
<tr><td>Total Volume</td><td>600 µL</td></tr>
</table>
IMPORTANT: If using the QuantiGene ViewRNA ISH Cell 740 Module, prepare the Working PreAmplifier Mix Solution according to the table below.
<table>
<tr><th colspan="2">4-plex Working PreAmplifier Mix Solution for 1 Chamber</th></tr>
<tr><td>Amplifier Diluent QF (pre-warmed at 40 °C)</td><td>552 µL</td></tr>
<tr><td>PreAmplifier Mix</td><td>24 µL</td></tr>
<tr><td>PreAmp10-740</td><td>24 µL</td></tr>
<tr><td>Total Volume</td><td>600 µL</td></tr>
</table>
B. Vortex briefly to mix.
C. Invert dish over appropriate receptacle to gently expel the Wash Buffer, then invert dish on a clean, dry paper towel for 1–2 seconds. Immediately add 600 µL/chamber of Working PreAmplifier Mix Solution.
D. Cover the dish and incubate slides at 40 ± 1 °C for 30 minutes.
</td></tr>
</table>

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>Step 7.
Wash Cells 3 Times with Wash Buffer
7 min</td><td>
A. Remove dish from the incubator.
B. Invert dish over appropriate receptacle to gently expel the Working PreAmplifier Mix Solution, then invert dish on a clean, dry paper towel for 1–2 seconds. Wash dish three times, each with 1 mL/chamber of Wash Buffer. Soak sample for 2 minutes during each wash. Allow samples to sit in the final wash while preparing the Working Amplifier Mix Solution.

IMPORTANT: Do not soak samples in Wash Buffer longer than 30 minutes.
</td></tr>
<tr><td>Step 8.
Hybridize with Amplifier(s)
35 min</td><td>
A. Prepare the Working Amplifier Mix Solution by diluting Amplifier Mix 1:25 in pre-warmed Amplifier Diluent QF.

<table>
<tr><th colspan="2">3-plex Working Amplifier Mix Solution for 1 Chamber</th></tr>
<tr><td>Amplifier Diluent QF (pre-warmed at 40 °C)</td><td>576 µL</td></tr>
<tr><td>Amplifier Mix</td><td>24 µL</td></tr>
<tr><td>Total Volume</td><td>600 µL</td></tr>
</table>

IMPORTANT: If using the QuantiGene ViewRNA ISH Cell 740 Module, prepare the Working Amplifier Mix Solution according to the table below.

<table>
<tr><th colspan="2">4-plex Working Amplifier Mix Solution for 1 Chamber</th></tr>
<tr><td>Amplifier Diluent QF (pre-warmed at 40 °C)</td><td>552 µL</td></tr>
<tr><td>Amplifier Mix</td><td>24 µL</td></tr>
<tr><td>Amp10-740</td><td>24 µL</td></tr>
<tr><td>Total Volume</td><td>600 µL</td></tr>
</table>

B. Vortex briefly to mix.
C. Invert dish over appropriate receptacle to gently expel the Wash Buffer, then invert dish on a clean, dry paper towel for 1–2 seconds.
D. Cover dish with lid and incubate slides at 40 ± 1 °C for 30 minutes.
</td></tr>
<tr><td>Step 9.
Wash Cells 3 Times with Wash Buffer
7 min</td><td>
A. Remove dish from the incubator.
B. Invert dish over appropriate receptacle to gently expel the Working Amplifier Mix Solution, then invert dish on a clean, dry paper towel for 1–2 seconds. Wash dish three times, each with 1 mL/chamber of Wash Buffer. Soak sample for 2 minutes during each wash. Allow samples to sit in the final wash while preparing the Working Amplifier Mix Solution. Immediately add 600 µL/chamber of Working Label Probe Mix Solution.

IMPORTANT: Do not soak samples in Wash Buffer longer than 30 minutes.
</td></tr>
</table>

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>Step 10.
Hybridize with Label Probe(s)
35 min</td><td>
IMPORTANT: Protect samples from light during this and all subsequent steps.

A. Prepare the Working Label Probe Mix Solution by diluting Label Probe Mix 1:25 in pre-warmed Label Probe Diluent QF.

<table>
<tr><th colspan="2">3-plex Working Label Probe Mix Solution for 1 Chamber</th></tr>
<tr><td>Label Probe Diluent QF (pre-warmed at 40 °C)</td><td>576 µL</td></tr>
<tr><td>Label Probe Mix</td><td>24 µL</td></tr>
<tr><td>Total Volume</td><td>600 µL</td></tr>
</table>

IMPORTANT: If using the QuantiGene ViewRNA ISH Cell 740 Module, prepare the Working Label Probe Mix Solution according to the table below.

<table>
<tr><th colspan="2">4-plex Working Label Mix Solution for 1 Chamber</th></tr>
<tr><td>Label Probe Diluent QF (pre-warmed at 40 °C)</td><td>552 µL</td></tr>
<tr><td>Label Probe Mix</td><td>24 µL</td></tr>
<tr><td>LP10-740</td><td>24 µL</td></tr>
<tr><td>Total Volume</td><td>600 µL</td></tr>
</table>

B. Vortex briefly to mix. Protect from light.
C. Invert dish over appropriate receptacle to gently expel the Wash Buffer, then invert dish on a clean, dry paper towel for 1–2 seconds. Immediately add 600 µL/chamber of Working Label Probe Mix Solution.
D. Cover dish with lid and incubate slides at 40 ± 1 °C for 30 minutes.
</td></tr>
<tr><td>Step 11.
Wash Cells 3 Times with Wash Buffer
15 min</td><td>
A. Remove dish from the incubator.
B. Invert dish over appropriate receptacle to gently expel the Working Label Probe Mix Solution, then invert dish on a clean, dry paper towel for 1–2 seconds. Wash dish three times, each with 1 mL/chamber of Wash Buffer. Soak samples for 2 minutes for the first two washes and 10 minutes for the final wash. Allow samples to sit in the final wash while preparing the Working DAPI Solution.

IMPORTANT: Do not soak samples in Wash Buffer longer than 30 minutes.
</td></tr>
<tr><td>Step 12.
DAPI-Staining
5 min</td><td>
A. Prepare Working DAPI Solution by diluting the 100X DAPI 1:100 in 1X PBS.

<table>
<tr><th colspan="2">Working DAPI Solution for 1 Chamber</th></tr>
<tr><td>1X PBS</td><td>6 µL</td></tr>
<tr><td>100X DAPI</td><td>594 µL</td></tr>
<tr><td>Total Volume</td><td>600 µL</td></tr>
</table>

B. Vortex briefly to mix. Protect from light.
C. Invert dish over appropriate receptacle to gently expel the Wash Buffer, then invert dish on a clean, dry paper towel for 1–2 seconds. Immediately add 600 µL/chamber of Working DAPI Solution and incubate at RT for 1 minute.
D. Expel Working DAPI Solution from the dish. Wash dish once with 1 mL/chamber of 1X PBS.
E. Add 600 µL/chamber of 1X PBS.
F. (Optional) Replace 1X PBS with a thin layer of Prolong Gold Antifade Reagent to preserve a better fluorescent signal.
</td></tr>
</table>

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>Step 13.
Image
Samples</td><td>
A. Samples may be viewed under microscope immediately.
B. Dishes may be sealed with parafilm and stored, protected from light, at 2–8°C. Fluorescent signals will be stable for up to one week when stored properly.
<table>
<tr><th>Probe Set</th><th>Filter Set</th></tr>
<tr><td>TYPE 4</td><td>FITC (488 nm)</td></tr>
<tr><td>TYPE 1</td><td>Cy3 (550 nm)</td></tr>
<tr><td>TYPE 6</td><td>Cy5 (650 nm)</td></tr>
<tr><td>TYPE 10</td><td>Cy7 (740 nm)</td></tr>
</table>
NOTE: Signals of LP4-488 and LP1-550 are visible to unaided eyes under microscope, and appear as green and red dots, respectively. Signals of LP6-650 and LP-740 are invisible and require CCD-cooled camera to capture the image.
</td></tr>
</table>

Appendix D

Cell Types Validated in QuantiGene ViewRNA ISH Cell Assay with Recommended Pretreatment Conditions

Table D.1 Validated Cell Types

Cell Type	Adherent/Suspension	4% Formaldehyde Fixation (in minutes)	Protease Treatment (Dilution Factor)
HeLa	Adherent	30	1:4000–1:8000
293		30	1:4000–1:8000
A549		30	1:4000–1:8000
HuH7		30	1:4000–1:8000
C2C12		30	1:4000–1:8000
HepG2		30	1:4000–1:8000
SK-N-SH		30	1:4000–1:8000
SK-MEL-24		30	1:4000–1:8000
WiDr		30	1:4000–1:8000
Primary Macrophage		30	1:4000–1:8000
Primary Islet Cells		30	1:4000–1:8000
Jurkat	Suspension	60	1:4000–1:8000
K562		60	1:4000–1:8000
THP-1		60	1:4000–1:8000

Important Procedural Notes and Guidelines

- The suggested range of protease dilution provided in this appendix for each cell type serves only as an initial reference. The optimal protease dilution will still need to be determined empirically since the specific protease activity can vary from the length of storage even under the recommended condition and there is acceptable range of specific protease activity between lots.
- The recommended protease dilution is for the 24-well plate format. Be sure to increase the protease dilution to 1:1000–1:2000 when using the slide format.

Appendix E

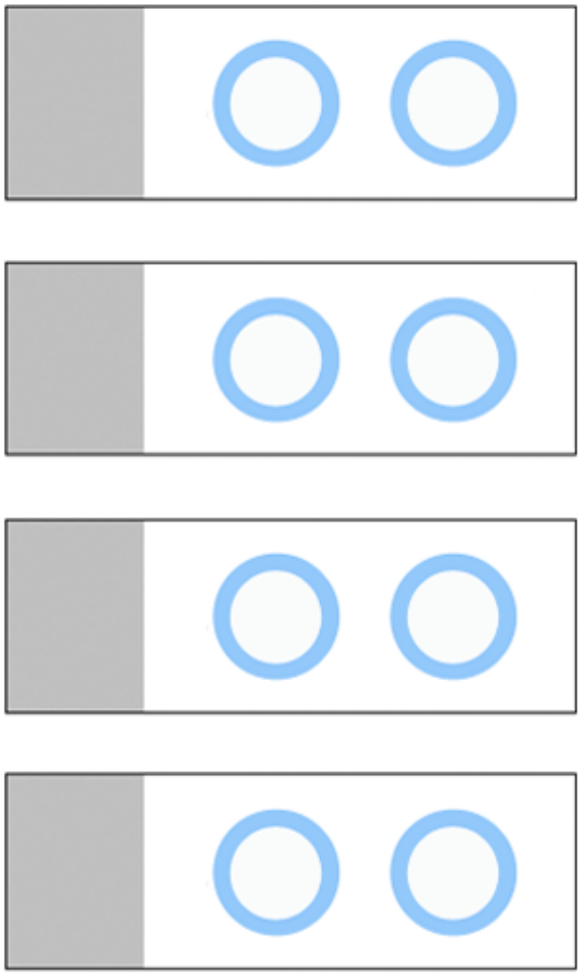
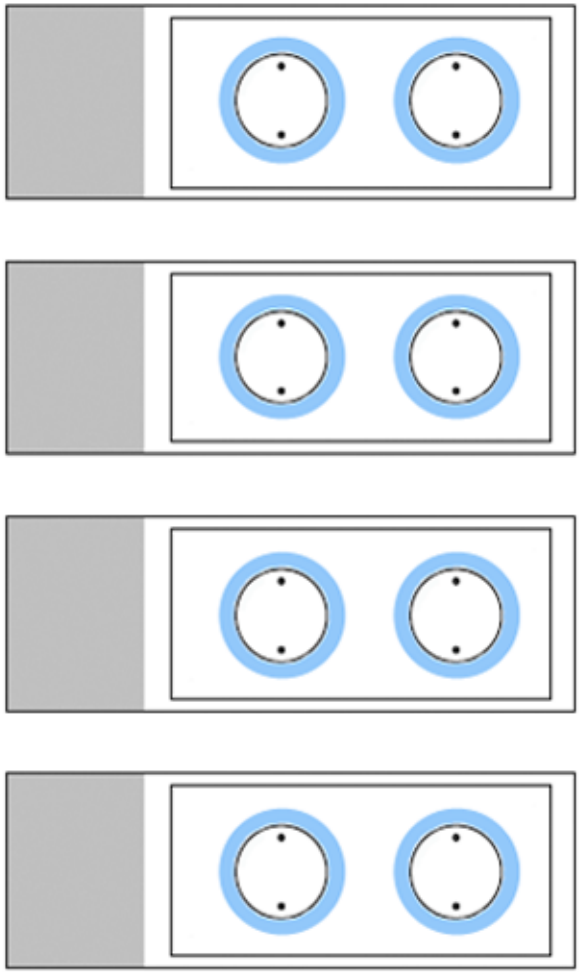
Templates

Important Procedural Notes and Guidelines

Use the templates provided below to:

- Draw hydrophobic rings
- Glue 12 mm cover slips and mount 24 x 55 mm cover glass

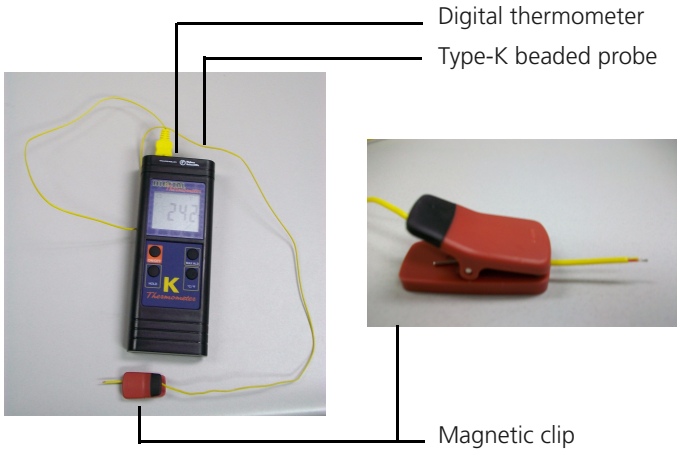
Table E.2 Slide templates

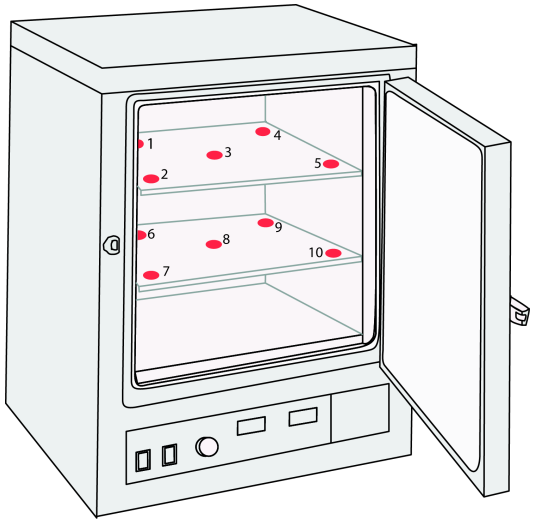
Templates for Drawing Hydrophobic Rings	Templates for Gluing 12 mm Cover Slips and Mounting 24 x 55 mm Cover Glass
	

Appendix F

Procedures for Validating the Temperature of a Dry Incubator

Procedure (for using QC ViewRNA Temperature Validation Kit, Affymetrix QV0523)

Step	Action
Step 1. **Assemble Kit**	**A.** Insert the battery to activate the digital thermometer. **B.** Slide the Type-K beaded probe through the magnetic clip as shown in figure below. Make sure the Type-K beaded probe is pointing upward so that it is not in contact with any metal surface when put inside the incubator. **C.** Insert the Type-K beaded probe into the digital thermometer. Please refer to the manufacturer's instruction manual for thermometer specifications. **D.** Turn on the digital thermometer device. **Figure F.1** Assembled Kit 
Step 2. **Prepare Dry Incubator**	**A.** Turn on the dry incubator. **B.** Set the temperature to 40 °C. **C.** Allow the dry incubator to equilibrate.

Step	Action
Step 3. Measure and Adjust Dry Incubator Temperature	**A.** Place the assembled Type-K beaded probe inside the incubator (see figure below). **Figure F.2** Complete Setup for Dry Incubator  **B.** Close the door making sure there is sufficient slack in the wiring. **C.** Wait for temperature to equilibrate. **D.** Record the temperature. **E.** If necessary, adjust the dry incubator temperature so that the digital thermometer reads 40 °C. **NOTE:** Incubator specification is 40 ±1 °C.
Step 4. Assess Dry Incubator Temperature Uniformity	**A.** Repeat Steps 2–3 to measure the temperature at multiple regions in the incubator to determine temperature uniformity (see figure F.3 below). Remember to wait for the temperature to equilibrate before recording the measurements. **Figure F.3** Dry Incubator Uniformity Test Positions  **IMPORTANT:** The temperature for all positions should be 40 ±1 °C.
Step 5. Assess Ramp-Up Time	**A.** Open the incubator door for 1 min then close the door and measure the time needed for temperature to return to 40 °C. **B.** Repeat the previous step two more times. **IMPORTANT:** Do not use the incubator for the assay if it takes more than 5 minutes to return to 40 °C **or if it overshoots by more than 2 °C during recovery.**

Step	Action
Step 6. ***Optional*** **Perform Additional Temperature Equilibrium Test**	If no alternative assay format, not covered in the QuantiGene ViewRNA ISH Cell Assay User Manual, must be used, we recommend additional testing to evaluate the temperature equilibrium profile of the alternative assay format to ensure optimal conditions during hybridization **A.** Prewarm Amplifier Diluent to 40 °C. Dispense the exact volume of Amplifier Diluent that will be used in the assay to the new assay format. **B.** Secure the Type-K beaded probe so that the probe is fully submerged in the Amplifier Diluent solution. Cover with lid if applicable. **C.** Record the ambient temperature of the solution then place the device into the 40 °C incubator. Measure the temperature at 10, 15, 20, 25 and 30 min. **IMPORTANT: Amplifier Diluent temperature should reach at least 38 °C by 15 minutes and stabilize at 40 ±1 °C at subsequent time points. Do not use the new assay format if the temperature profile does not meet these criteria.**
Step 7. **Pass/Fail Criteria & Regular Check-Up**	The incubator must meet ALL validation criteria in Steps 3–6. Perform tests once every three months.

Appendix G

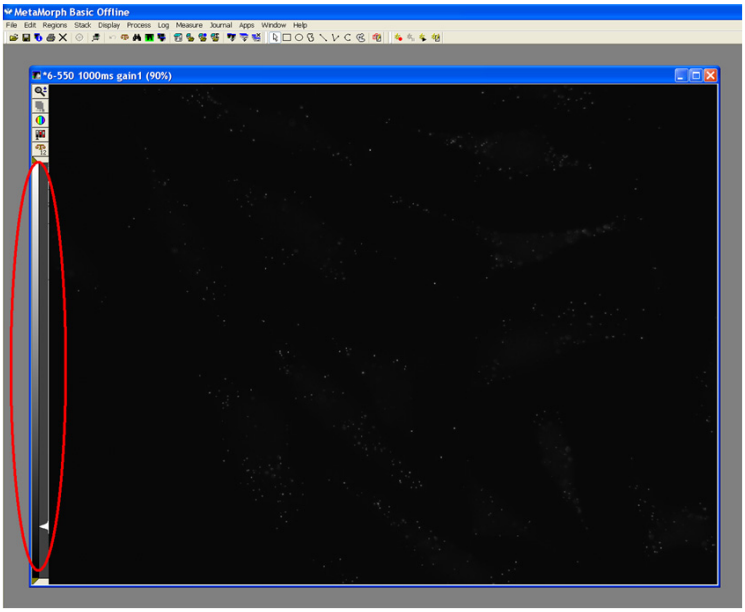
How To Adjust Images For Result Interpretation

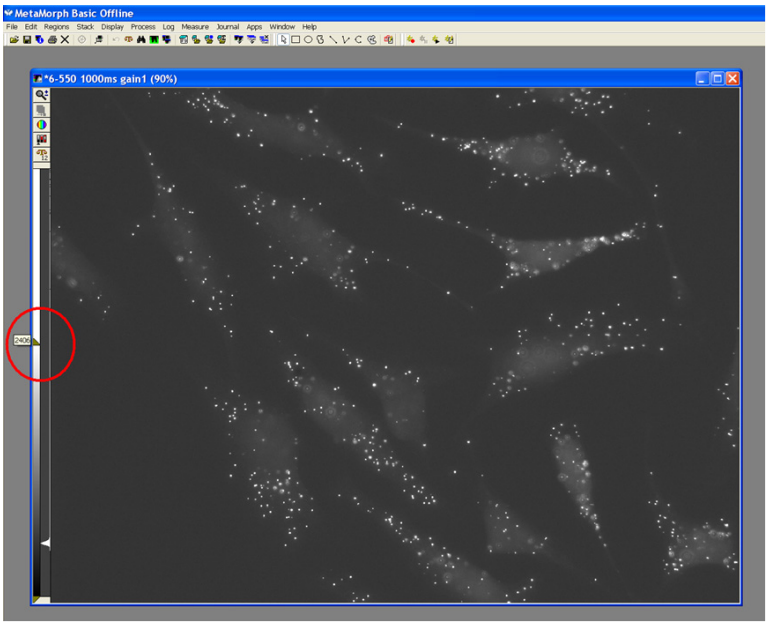
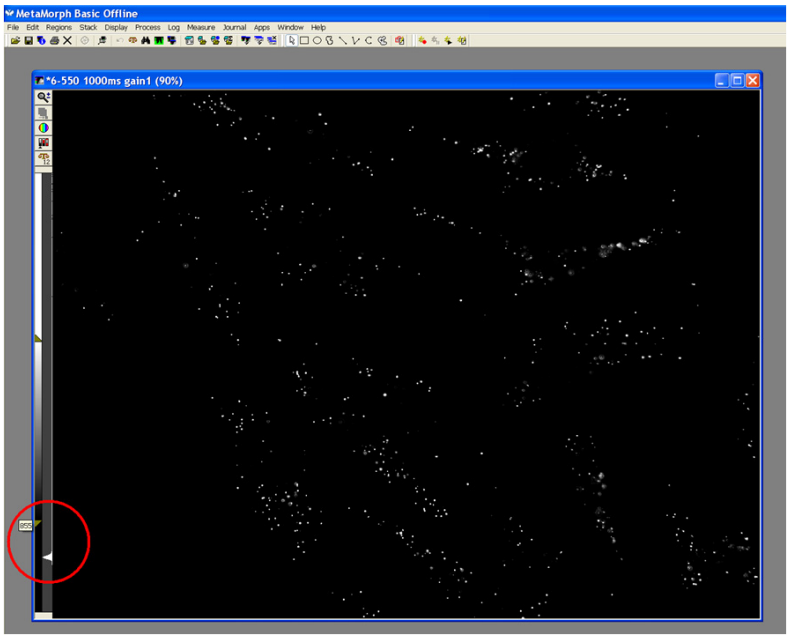
Overview

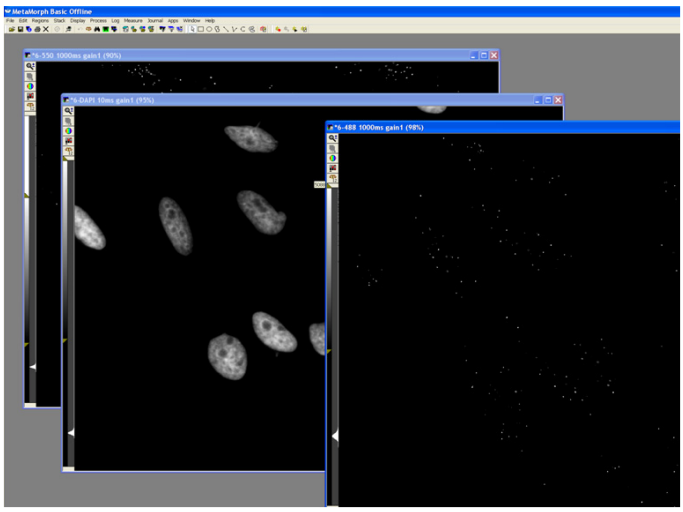
Captured raw images may not always displayed optimally for visualization. To display the images for optimal visualization, the image intensity thresholds may have to be adjusted.

Please note that for image interpretation and comparison within experiments, it is necessary to apply the same threshold settings for images of the same channel and sample type.

The following protocol provides a step-by-step guideline on how to adjust the image intensity threshold for viewing using MetaMorph imaging software (other imaging software have similar features). Note that this does not alter the absolute signal intensity value of the raw image, and hence does not impact the quantification of signal intensity.

Step	Task
Step 1.	Obtain the image and its corresponding image histogram (see red circle below) using any compatible image viewing software. 

Step	Task
Step 2.	For each individual channel, adjust the **upper threshold** (see red circle below) until visible spots are observed (e.g. lowering the upper threshold will enhance the signal, whereas increasing the upper threshold will diminish the signal). 
Step 3.	Adjust the **lower threshold** (see red circle below) to eliminate background. Repeat Step 2 for finer adjustments of the upper threshold if necessary. 
Step 4.	Note the adjustments made to the image and apply the same threshold settings to other images of the same channel and experiment for comparison.

Step	Task
Step 5.	Repeat Steps 2–4 to adjust the threshold for other channels. 
Step 6.	Generate overlaid images of all channels for each sample if desired.