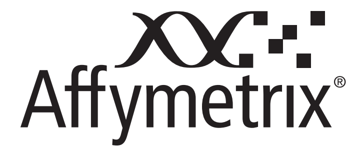


User Manual

QuantiGene® ViewRNA miRNA ISH Cell Assay

Format: 96-well optical-bottom plate

P/N 18842 Rev.A 121010

For research use only.
Not for use in diagnostic procedures.

Trademarks

Affymetrix® , and QuantiGene® are trademarks of Affymetrix, Inc.

All other trademarks are the property of their respective owners.

Limited License

Subject to the Affymetrix terms and conditions that govern your use of Affymetrix products, Affymetrix grants you a non-exclusive, non-transferable, non-sublicensable license to use this Affymetrix product only in accordance with the manual and written instructions provided by Affymetrix. You understand and agree that, except as expressly set forth in the Affymetrix terms and conditions, no right or license to any patent or other intellectual property owned or licensable by Affymetrix is conveyed or implied by this Affymetrix product. In particular, no right or license is conveyed or implied to use this Affymetrix product in combination with a product not provided, licensed, or specifically recommended by Affymetrix for such use.

Citing QuantiGene ViewRNA in Publications

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

Disclaimer

Affymetrix, Inc. reserves the right to change its products and services at any time to incorporate technological developments. This manual is subject to change without notice.

Although this manual has been prepared with every precaution to ensure accuracy, Affymetrix, Inc. assumes no liability for any errors or omissions, nor for any damages resulting from the application or use of this information.

Copyright

© 2012 Affymetrix Inc. All rights reserved.

Contents

Chapter 1 **Introduction** 1

About This Manual 1
Related User Documents 1
Assay Overview 1
How it Works 2
Performance Highlights 3
Safety Warnings and Precautions 3
Required Materials 4
QuantiGene ViewRNA miRNA ISH Cell Assay Kit 4
QuantiGene ViewRNA miRNA Probe Sets 5
QuantiGene ViewRNA TYPE 4 and TYPE 10 Probe Sets 5
QuantiGene ViewRNA ISH Cell 740 Module (Optional) 5
Required Materials and Equipment Not Provided 5
Microscopy and Imaging Equipment Guidelines 7

Chapter 2 **Assay Guidelines** 9

About This Section 9
Assignment of mRNA Targets to Channels When Multiplexing 9
Assessing Non-Assay Related Background 9
Experimental Design Guidelines 10
Recommended Pretreatment Optimization 11

Chapter 3 **QuantiGene ViewRNA miRNA ISH Cell Assay Procedure** 13

About the QuantiGene ViewRNA miRNA ISH Cell Assay Workflow 13
Important Procedural Notes and Guidelines 13
Part 1: Sample Preparation for Adherent Cells 14
A. Preparing of Poly-L-Lysine Coated 96-Well Plate 14
B. Culturing Fresh Adherent Cells on Poly-L-Coated 96-Well Plate. 14
Part 2: Sample Pretreatment and Target Probe(s) Hybridization 16
Part 2 Procedure 16
Part 3: Signal Amplification and Detection 19
Part 3 Procedure 19

Chapter 4 **Troubleshooting** 23

Contacting Technical Support 23
Poor Cell Morphology and Retention 23
High Non-Specific Binding Outside the Cells 23
Weak or No Signal 24
Patchy and Diffused Fast Red Signals 25

High Background Inside Cells 25
Variable Staining Within a Sample 25

Appendix A Sample Preparation Procedure for Suspension Cells 27
About the Preparation for Suspension Cells 27

Appendix B Detecting siRNA with QuantiGene miRNA ISH Cell Assay 29
About Detecting siRNA with QuantiGene miRNA ISH Cell Assay 29

Introduction

About This Manual

This manual provides complete instructions for performing the QuantiGene ViewRNA miRNA ISH Cell Assay for visualization of 1 target miRNA and up to 2 target mRNAs in adherent and suspension cultured cells. The procedures in this manual use samples processed in a 96-well optical-bottom tissue culture plate using a temperature-validated dry incubator.

Related User Documents

Refer to the *QuantiGene ViewRNA miRNA ISH Cell Assay User Manual*, format: Glass Coverslips in 24-Well Plate for assay instructions using cultured cell samples processed on glass coverslips in a 24-well plate.

Refer to the *QuantiGene ViewRNA miRNA ISH Cell Assay Supplemental Reference Guide* for the following information:

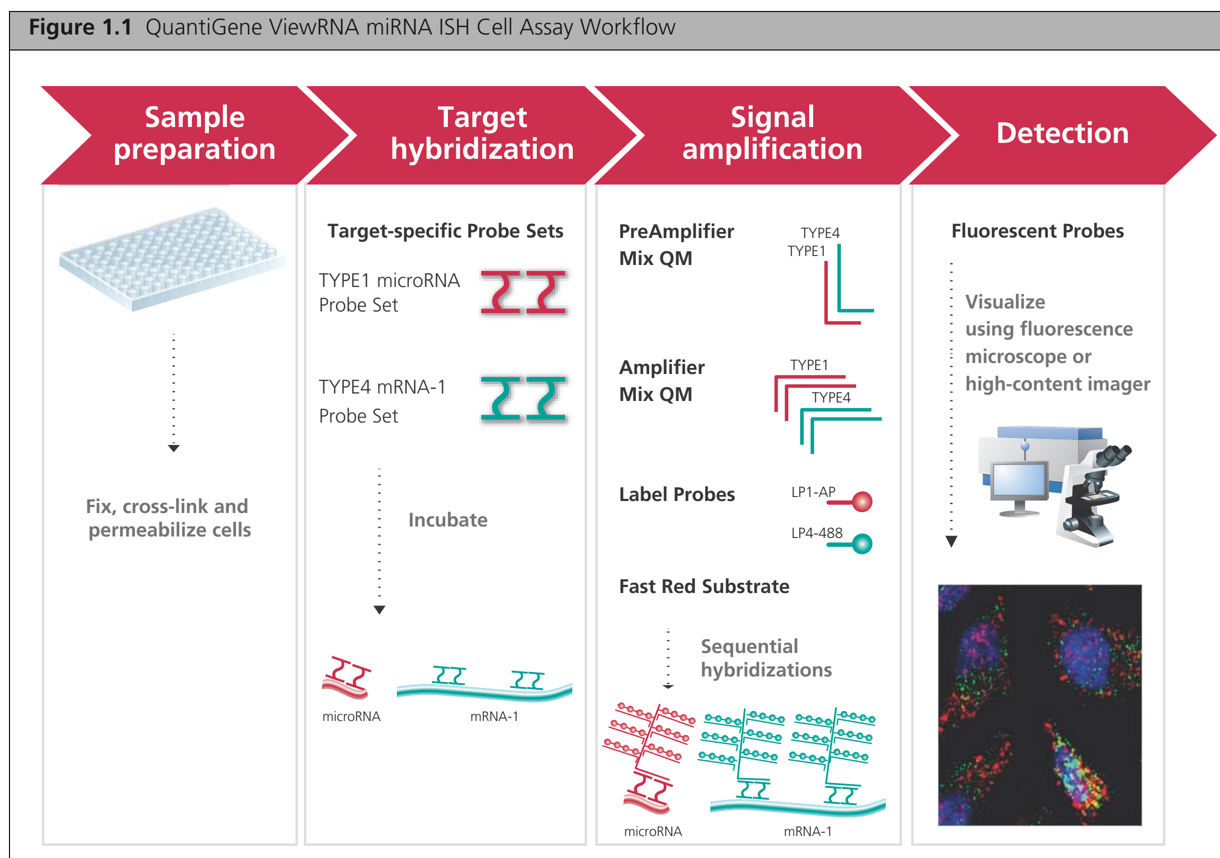
- Guidelines for data interpretation
- Sample images to aid in optimization and troubleshooting

Assay Overview

In situ hybridization (ISH) techniques are used to visualize DNA or localize RNAs within cells. However, the *in situ* analysis of RNA, in particular, has always been limited by low sensitivity and complicated probe synthesis. In the case of miRNAs, these issues are further compounded by the short (~17-27 nt) target sequences, which have a tendency to diffuse out of the cells, limit the affinity and specificity of any probe designs in which standard nucleic acid chemistry is utilized, and in general, negatively affect the overall sensitivity of the assay. The QuantiGene ViewRNA miRNA ISH Cell Assay overcomes these challenges by (1) improving target retention, (2) incorporating a novel probe design with proprietary nucleic acid chemistry, and (3) employing a second generation branched DNA signal amplification system to allow visual detection and validation of miRNA expression down to a 4-nt difference at a single cell resolution. The sensitivity and robustness of the QuantiGene ViewRNA miRNA ISH Cell Assay permit simultaneous *in situ* detection of any miRNA and up to 2 mRNAs within cultured cells manually in an easy-to-handle 96-well plate format. The assay design is illustrated and explained in *Figure 1.1*.

How it Works

Figure 1.1 QuantiGene ViewRNA miRNA ISH Cell Assay Workflow



Sample Preparation. Cells are fixed and cross-linked to ensure retention of sample and targets. Cells are then permeabilized with detergent and digested with protease to allow target accessibility.

Target Hybridization. Target-specific Probe Sets hybridize to each target miRNA and mRNA. Subsequent signal amplification is predicated on specific hybridization of the pair(s) of oligonucleotides (indicated by "II" in the above image) within each probe set to the target sequence. A typical mRNA Probe Set contains 20 oligonucleotide pairs. By contrast, each miRNA Probe Set consists of only a single oligonucleotide pair synthesized using proprietary nucleic acid chemistry to increase the T_m and enhance binding specificity to miRNAs. For simplicity, only 2 pairs per mRNA target are shown in the figure.

Signal Amplification. Signal amplification, using bDNA technology, is achieved via a series of sequential hybridization steps. The PreAmplifiers hybridize to their respective pair of bound Probe Set oligonucleotides then multiple Amplifiers hybridize to their respective PreAmplifier. Next, multiple Label Probe oligonucleotides, either conjugated to alkaline phosphatase (for miRNA) or to fluorescent ALEXA fluor (for mRNA), hybridize to the corresponding Amplifier molecules. A fully assembled signal amplification "tree" has 400 binding sites for the Label Probe (a total of 400 for each miRNA molecule and 8,000 for each mRNA molecule). These separate, yet compatible, signal amplification systems (TYPE 1, TYPE 4, and TYPE 10) provide the assay with multiplexing capability.

Detection. Following the addition of the Fast Red substrate, alkaline phosphatase breaks down the substrate to form a precipitate wherever the target miRNA molecule is localized. The target miRNA and mRNA are visualized with a standard fluorescent microscope.

Performance Highlights

Table 1.1 Performance Highlights

Specification	Description
Sample types	Adherent or suspension cultured cells
Format	Cultured cells on or adhered to glass cover-slips in a 96-well optical-bottom plate
Sensitivity	miRNAs/mRNA Single molecule that appears as a single spot/dot in cells
Multiplexing	1 miRNA + up to 2 mRNAs (simultaneous detection)
Detection	miRNA: fluorescence or chromogenic mRNAs: fluorescence
Nuclear stain	DAPI
Instrumentation	Fluorescence and brightfield microscope or fluorescence scanner

Safety Warnings and Precautions

- Formaldehyde is a poison and an irritant. Avoid contact with skin and mucous membranes. Use in a fume hood.
- EDC is a cross-linker and can cause respiratory tract, eye and skin irritation as well as target organ damage. Do not breathe dust and use with adequate ventilation. Avoid contact with eyes, skin and clothing.
- Cross-Linking Buffer QM contains 1-methylimidazole, which is corrosive and harmful by ingestion and inhalation. Avoid contact with eyes, skin and mucous membrane.
- Probe Set Diluent QF, PreAmplifier Mix QM, and Amplifier Mix QM contain formamide, a teratogen, irritant and possible carcinogen. Avoid contact with mucous membranes.
- DAPI is a possible mutagen. Avoid contact with skin and mucous membranes.
- Perform all procedural steps in a well-ventilated area at room temperature unless otherwise noted.
- Discard all reagents in accordance with local, state, and federal laws.

Required Materials

The QuantiGene ViewRNA miRNA ISH Cell Assay, for detection of 1 miRNA and 1 mRNA, is composed of 3 modules each sold separately:

- QuantiGene ViewRNA miRNA ISH Cell Assay Kit (for detection of 1 miRNA + 1 mRNA)
- QuantiGene ViewRNA miRNA TYPE 1 Probe Set
- QuantiGene ViewRNA TYPE 4 Probe Set(s)

For detection of 1 miRNA and 2 mRNAs the following additional components are required:

- QuantiGene ViewRNA 740 Module
- QuantiGene ViewRNA TYPE 10 Probe Set

QuantiGene ViewRNA miRNA ISH Cell Assay Kit

QuantiGene ViewRNA miRNA ISH Cell Assay Kit contains sufficient materials to perform 144 (1.5 96-well plates) assays in the 96-well plate format. This kit is compatible with TYPE 1 Probe Sets designated for miRNA, TYPE 4 and TYPE 10 for mRNAs. Each kit is configured for processing in increments of 36 wells per experiment.

The components of the QuantiGene ViewRNA miRNA ISH Cell Assay Kit and their recommended storage conditions are listed below. Refer to the product insert for quantities of individual components supplied. Kits are shipped in 3 parts, based on storage conditions and have a shelf life of 6 months from date of delivery when stored as recommended.

Table 1.2 Assay Kit Components and Their Storage Conditions:

Component	Description	Storage
Label Probe 4-488	ALEXA 488-labeled oligonucleotides in aqueous buffered solution	–20 °C
100X DAPI	Aqueous solution containing DAPI stain	–20 °C
Protease QS[a]	Enzyme in aqueous buffered solution	2-8 °C
Probe Set Diluent QF	Aqueous solution containing formamide and detergent	2-8 °C
PreAmplifier Mix QM	PreAmp 1 and PreAmp 4 in aqueous solution containing formamide and detergent	2-8 °C
Amplifier Mix QM	Amp 1 and Amp 4 in aqueous solution containing formamide and detergent	2-8 °C
Label Probe Diluent QF	Aqueous solution containing detergent	2-8 °C
AP Enhancer Solution	Aqueous buffered solution	2-8 °C
Fast Red Tablets	Red precipitating substrate for the detection of alkaline phosphatase activity	2-8 °C
Naphthol Buffer	Buffer required for preparation of Fast Red Substrate	2-8 °C
Label Probe 1-AP[a]	Alkaline phosphatase-conjugated oligonucleotide in aqueous buffered saline	2-8 °C
10X PBS	Aqueous buffered solution	15-30 °C
Detergent Solution QC	Aqueous solution containing detergent	15-30 °C
Cross-Linking Buffer QM	Aqueous buffered solution containing 1-methylimidazole	15-30 °C
Wash Buffer Component 1 (Wash Comp 1)	Aqueous solution containing detergent	15-30 °C
Wash Buffer Component 2 (Wash Comp 2)	Aqueous buffered solution	15-30 °C

[a] *IMPORTANT! Do not freeze.*

QuantiGene ViewRNA miRNA Probe Sets

QuantiGene ViewRNA miRNA TYPE 1 Probe Sets are available in multiple sizes and should be stored at –20 °C. Refer to the product insert for quantities provided and design specificities. Our current probe set catalog can be found at website to be updated.

Table 1.3 ViewRNA miRNA TYPE 1 Probe Set and Storage Conditions:

Component	Description	Storage
QuantiGene ViewRNA miRNA TYPE 1 Probe Set	RNA-specific oligonucleotides designed against an miRNA target of interest and are compatible with TYPE 1 Signal Amplification System.	–20 °C

QuantiGene ViewRNA TYPE 4 and TYPE 10 Probe Sets

The QuantiGene ViewRNA Probe Sets are available in multiple sizes. Only those designated as TYPE 4 and TYPE 10 are compatible with the QuantiGene ViewRNA miRNA Cell Assay Kit. Refer to the product insert for quantities provided and design specificities. Our current probe set catalog can be found at Panomics.com.

Table 1.4 ViewRNA TYPE 4 and TYPE 10 Probe Set and Storage Conditions:

Component	Description	Storage
QuantiGene ViewRNA TYPE 4 Probe Set	RNA-specific oligonucleotides designed against a transcript target of interest and are compatible with the TYPE 4 Signal Amplification System with detection using ALEXA 488.	–20 °C
QuantiGene ViewRNA TYPE 10 Probe Set (Optional)	RNA-specific oligonucleotides designed against a transcript target of interest and are compatible with the TYPE 10 Signal Amplification System with detection using ALEXA 750.	–20 °C

QuantiGene ViewRNA ISH Cell 740 Module (Optional)

The QuantiGene ViewRNA ISH Cell 740 Module is available separately and offers the flexibility of expanding the assay to a 3-plex assay (1 miRNA + 2 mRNAs).

Table 1.5 ViewRNA ISH Cell 740 Module Contents and Storage Conditions:

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

Required Materials and Equipment Not Provided

Other materials required to perform the QuantiGene ViewRNA miRNA ISH Cell Assay that are not included in the QuantiGene ViewRNA miRNA ISH Cell Assay Kit are listed here.

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

Table 1.6 QuantiGene ViewRNA miRNA ISH Cell Assay Materials and Equipment Required but not Provided:

Material	Source	Part Number
Double-Distilled Water (ddH_2O)	MLS[a]	
100% Ethanol (200 proof)	VWR	89125-188
37% Formaldehyde	Fisher Scientific	F79-1
Poly L-Lysine	Sigma	P8920
Sterile Nuclease-Free Water	MLS	
Complete Cell Culture Medium	MLS	
Sterile Culture Grade 1X PBS	MLS	
EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride)	Pierce Biotechnology	22980
96-Well Optical-Bottom Plate	Nunc	165305 (polystyrene) 164588 (glass)
Equipment		
QuantiGene View Temperature Validation Kit or QuantiGene Incubator Temperature Validation Kit	Affymetrix	QV0523 QS0517
Water Bath (capable of maintaining 40 ± 1 °C)	MLS	
Pipettes (P20, P200, P1000, multichannel P200)	MLS	
Table-Top Microtube Centrifuge	MLS	
Platform Rocker	MLS	
Dry Incubator (capable of maintaining 40 ± 1 °C)	Affymetrix	QS0704 (1.6 ft^3; 120V) QS0700 (3 ft^3; 120V) QS0701 (5 ft^3; 120V) QS0712 (1.6 ft^3; 220V) QS0710 (3 ft^3; 220V) QS0711 (5 ft^3; 220V)
Microscope and Imaging Equipment	See *QuantiGene ViewRNA miRNA ISH Cell Assay Imaging Options on page 7*	
Fume Hood	MLS	
Laminar Flow Hood	MLS	
Optional Vacuum System (set to aspirate at a rate of ~2 mL/5 sec)	MLS	
Optional Aspirator 8-Channel Aspirator Adapter	 VWR VWR	 8909-050 29443-002

[a] *Major Laboratory Supplier*

Microscopy and Imaging Equipment Guidelines

Table 1.7 QuantiGene ViewRNA miRNA ISH Cell Assay Imaging Options

Viewing and Digital Capturing Options	Microscope Type	Recommended Microscope/ System	Required Optics	Recommended Filter
Brightfield viewing	Standard brightfield microscope	▪ Leica DM series ▪ Nikon TE2000/Ti series ▪ Olympus IX series ▪ Zeiss Axio Vert/Observer ▪ Or equivalent ▪ Most HCS scanners are OK if correct filters are used in conjunction with compatible light source[a]	20X (N.A. 0.5 or 0.75) air objectives	Requires neutral density filters and/or color filters for white balancing
Fluorescence viewing and image capture	Microscope with camera and fluorescence options Fluorescence detection requires high-resolution, high-sensitivity cooled-CCD camera: 6.45 µm pixel size or smaller >65% peak Quantum Efficiency 6-10 electrons read noise Common models include: CoolSnap HQ2 (Photometrics) Orca R2 (Hamamatsu) Retiga Exi (QImaging)		20X (N.A.0.5 or 0.75) air objectives	For Fast Red Substrate: Excitation: 530/40 nm Emission: 590/40 nm (e.g., Semrock Cy3-4040c) For ALEXA 488: Excitation: 485/20 nm Emission: 524/24 nm (e.g., Semrock FITC-2024B) For ALEXA 750: Excitation: 708/75 nm Emission: 809/81 nm (e.g., Semrock Cy7-B) For DAPI filter set Excitation: 387/11 nm Emission: 447/60 nm (e.g., Semrock DAPI-11060B)

[a] Light source must have sufficient output at 700-775 nm with removable IR blocking filter.

Assay Guidelines

About This Section

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 (a) assignment of mRNA targets to the available channels when multiplexing, (b) assessing for non-assay related background (c) experimental design and (d) pretreatment assay optimization that will ensure your chance of success with the QuantiGene ViewRNA miRNA ISH Cell Assay.

Assignment of mRNA Targets to Channels When Multiplexing

The QuantiGene ViewRNA miRNA ISH Cell Assay has multiplexing capability and allows for *in situ* detection of any miRNA and up to 2 mRNAs. However, when multiplexing 2 mRNAs, it is helpful to have some knowledge of the expression level of the two targets, as this will assist in the proper assignment of the mRNA targets to the available channels (FITC or Cy7) in order to achieve the best signal detection. While the FITC channel can be utilized for any mRNA target with low, medium, or high expression, the Cy7 channel should be reserved for mRNA targets with high expression due to its inherent lower sensitivity. It should be noted that the dimmer property of the signals typically associated with Cy7 channel is not assay-related, but rather a product of the innate characteristic of many fluorophores that emit light in the far red region (for example, ALEXA 750) and the limitations of conventional instruments (that is, camera and light source) used for this channel. Nonetheless, the following guidelines will ensure proper signal detection of your targets when performing multiplex assay.

Table 2.1 Guidelines for Proper Signal Detection:

Target	Probe Set	PreAmp	Amp	Label Probe	Substrate	Detection Signal	Recommended Target
miRNA	TYPE 1	PreAmp 1	Amp 1	LP1-alkaline phosphatase	Fast Red	Fast Red	Any miRNA with low, medium or high expression
mRNA #1	TYPE 4	PreAmp 4	Amp 4	LP4-ALEXA 488	None	ALEXA 488	Any mRNA with low, medium or high expression
mRNA #2	TYPE 10	PreAmp 10	Amp 10	LP10-ALEXA 750	None	ALEXA 750	Any high expressing transcript

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 plate, and (3) the 96-well plate. 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 the recommended materials listed in *Table 1.6 on page 6*.

Table 2.2 Procedure for Assessing Non-Assay Related Background:

Step	Action
1	Grow cells overnight on a 96-well optical-bottom plate 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 min at room temperature.
4	Stain with 1X DAPI solution (diluted in 1X PBS from the 100X stock solution supplied in the kit) for 5 min.
5	Observe the samples using a fluorescent microscope or scanner with the filter sets intended for future experiment.

Expected Results

The background fluorescence on the optical-bottom surface of the plate should be minimal with strong, nuclear DAPI staining. Some smear, bright spots or debris should be observed in the 488 nm, Fast Red, or 750 nm detection 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 miRNA ISH Cell Assay (see *Table 1.6 on page 6* 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. If this occurs, the 488 nm dye can still be used for detecting your mRNA of interest since the specific signals are generally much brighter than the autofluorescence. Alternatively, if the type of cell line being used is not critical, perform the assay using a different cell line.

Experimental Design Guidelines

Control Cell Lines

We recommend running positive and negative control cell lines, based on your sample type, in every QuantiGene ViewRNA miRNA ISH Cell Assay. This will allow you to appropriately qualify and interpret your results. Examples include:

- Isogenic cell lines (+/-) miRNA target expression
- Cell line (+/-) known treatment that induces up- or down-regulation of miRNA expression

Negative Assay Control

- This sample undergoes the entire assay procedure with the exception of adding the target Probe Set(s). Alternatively, a Probe Set designed against a target not present in your cell line, for example, bacterial target DapB. This control permits the assessment of assay background.

Positive Assay Control

- This sample undergoes the entire assay procedure using Probe Set(s) against targets with high to medium high expression and are either expressed ubiquitously (for example, Let-7a for miRNA and ActB for mRNA) or specifically in your cell type. This control ensures that the assay procedure has been performed successfully.

Replicates

We recommend running all assays in duplicate.

Recommended Pretreatment Optimization

Critical to any *in situ* assay is the balance between the cross-linking of cells to the glass surface and target molecules to the cellular structures by chemical fixatives and the subsequent unmasking of the miRNA and mRNA targets by protease digestion for the probes to hybridize. Because the optimal pretreatment conditions are largely sample dependent, we recommend running a duplex assay, that is, 1 miRNA + 1 mRNA and titrating the fixation time and protease concentration as described in *Table 2.3* to empirically determine the optimal condition for any new cell type to ensure an optimal balance between cell retention and target accessibility.

When optimizing the pretreatment conditions for your cell type, choose a pair of targets with medium to medium-high levels of expression. This will avoid possible signal saturation that may be associated with extremely high expressing targets and allow for detectable changes in the signals to be assessed as a function of the different pretreatment conditions. In general, a housekeeping gene with medium expression, such as PPIB, can be used as the mRNA target for this purpose and Let-7a can be employed as the miRNA counterpart. Once the optimal pretreatment conditions have been identified for a cell type, the same conditions can be used with different probes with no impact on assay performance.

Table 2.3 Recommended Pretreatment Optimization Conditions:

Protease Dilution	Formaldehyde Fixation Time	
	30 min	60 min
1:1000	+/- probe	+/- probe
1:2000	+/- probe	+/- probe
1:4000	+/- probe	+/- probe
1:5000	+/- probe	+/- probe
1:6000	+/- probe	+/- probe
1:8000	+/- probe	+/- probe

Selecting the Optimal Conditions

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 conditions:

- *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.
- *Least Impact on Cell Morphology and Structure*. Cell morphology and structure is most affected by the protease digestion. A protease condition that sufficiently exposes the targets without loss of nuclei or morphology is desired.
- *Highest Signal-to-Background Ratio*. Highest signal-to-background ratio of specific fluorescent dots inside the cell. The average signal-to-background ratio should generally be greater than 3 for mRNA and 8 for miRNA signals.

If you choose to bypass the recommended pretreatment assay optimization, note that the assay protocol provided in this user manual serves only as a starting point. Understanding the following trends will aid in any future pretreatment assay optimization study. Refer to the *QuantiGene ViewRNA miRNA ISH Cell Assay Reference Guide* for more information.

- Under fixation causes a loss of cells and target molecules and results in no or low signals.
- Over fixation prevents proper unmasking of the targets and results in no or low signals.
- For optimal target retention, miRNA targets (shorter length) typically requires longer fixation time than the mRNA targets (longer length).
- Given the same fixation condition, mRNA targets require stronger protease treatment than miRNA targets for optimal signal detection.

- The current assay conditions favor miRNA detection; to improve mRNA detection, a trade-off in miRNA signal may be necessary.

QuantiGene ViewRNA miRNA ISH Cell Assay Procedure

About the QuantiGene ViewRNA miRNA ISH Cell Assay Workflow

The QuantiGene ViewRNA miRNA ISH Cell Assay workflow is sectioned into 3 parts that are performed over the course of 4 days:

- Part 1: Sample Preparation (performed at least 2 days prior to the QuantiGene ViewRNA miRNA ISH Cell Assay)
- Part 2: Sample Pretreatment and Target Probe(s) Hybridization
- Part 3: Signal Amplification and Detection

Important Procedural Notes and Guidelines

- Use sterile cell culture reagents and techniques when preparing poly-L-lysine coated 96-well plate and culturing cells.
- Although the assay is compatible with both glass-bottom and polystyrene plates (that are qualified for imaging studies), the highest signal-to-noise ratios are achieved with glass-bottom plates.
- Sample dehydration and rehydration on glass-bottom plates tend to weaken the adhesive between the glass bottom and the polystyrene plate tending to make the plate leaky.
- Avoid reagent shortage by running the assay in increments of 36 wells per experiment.
- Aspirate and dispense slowly and gently to reduce cell loss. Remove well contents slowly, against the well wall and add contents against the upper well wall. For optimal cell retention, remove well contents using a 200 µL multichannel pipette or an aspirator with an 8-channel adaptor connected to a vacuum.

Before You Start

- Validate the temperature of the dry incubator using either the QuantiGene ViewRNA Temperature Validation Kit (Affymetrix P/N QV0523) or the QuantiGene Incubator Temperature Validation Kit (Affymetrix P/N QS0517).
- Make sure that the EDC is fresh and properly stored in desiccant.
- Identify the optimum formaldehyde fixation time and protease concentration for your sample type.

Part 1: Sample Preparation for Adherent Cells

Adherent cells that are used in the QuantiGene ViewRNA miRNA ISH Cell Assay must be cultured and fixed on poly-L-lysine coated 96-well optical-bottom tissue culture plate. 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.

NOTE: If working with suspension cells, refer to *Sample Preparation Procedure for Suspension Cells on page 27*.

A. Preparing of Poly-L-Lysine Coated 96-Well Plate .

Step	Action
Step 1. Prepare Reagents **5 min**	**A.** Prepare 11 mL of a 0.01% poly-L-lysine solution: Mix 1.1 mL of the poly-L-lysine stock solution + 9.9 mL of nuclease-free water. Set aside until use. **B.** Ensure availability of: ▪ 1X PBS (sterile) ▪ Sterile 96-well optical-bottom tissue culture plate
Step 2. Coat Plate **20 min**	Dispense 100 µL/well of diluted poly-L-lysine and cover plate. Incubate for 15 min at RT.
Step 3. Wash Plate **10 min**	Expel the poly-L-lysine by inverting the plate over an appropriate receptacle and assertively flicking the solution from the wells. Wash 3 times with 150 µL/well 1X PBS for each wash.
Step 4. Incubate Overnight	Expel 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 to prevent the tissue culture plastic from becoming brittle and cracking during the assay. **NOTE:** Coated plates may be used directly for cell seeding or stored at 4 °C with lid on and sealed with parafilm for up to 1 month.
Step 5.	Proceed to culturing cells on poly-L-lysine coated plate.

B. Culturing Fresh Adherent Cells on Poly-L-Coated 96-Well Plate.

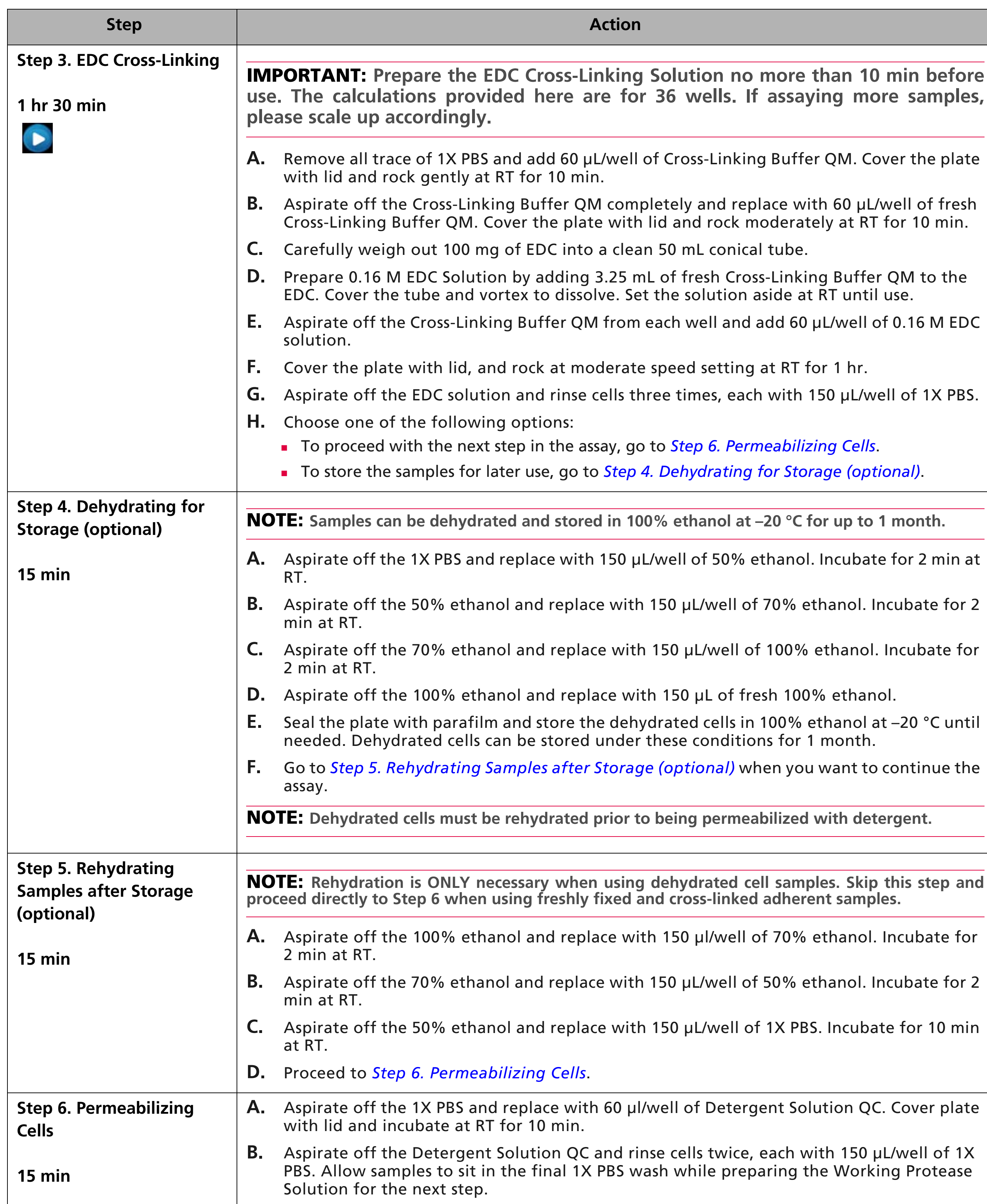
Step	Action
Step 1. Dissociate Cells from Culture Flask with Trypsin **5 min**	Wash the adherent cells with 1X PBS and treat with trypsin. Resuspend the cells in complete culture medium.
Step 2. Remove Trypsin **10 min**	Pellet the cells (200 x g, 5 min) at RT, remove the supernatant, and resuspend the cell pellet in fresh complete culture medium
Step 3. Count and Adjust Cell Density **10 min**	Count and adjust the cell density such that when seeded at 100 µL/well, the culture will reach 70-90% confluence at the start of the assay.

Step	Action
Step 4. Seed Cells on Coated Plate **5 min**	Dispense 100 µL/well of the adjusted cell suspension.
Step 5. Incubate Overnight	Incubate the plate overnight under the recommended growth conditions for the cells.
Step 6.	Proceed to *Part 2: Sample Pretreatment and Target Probe(s) Hybridization on page 16*.

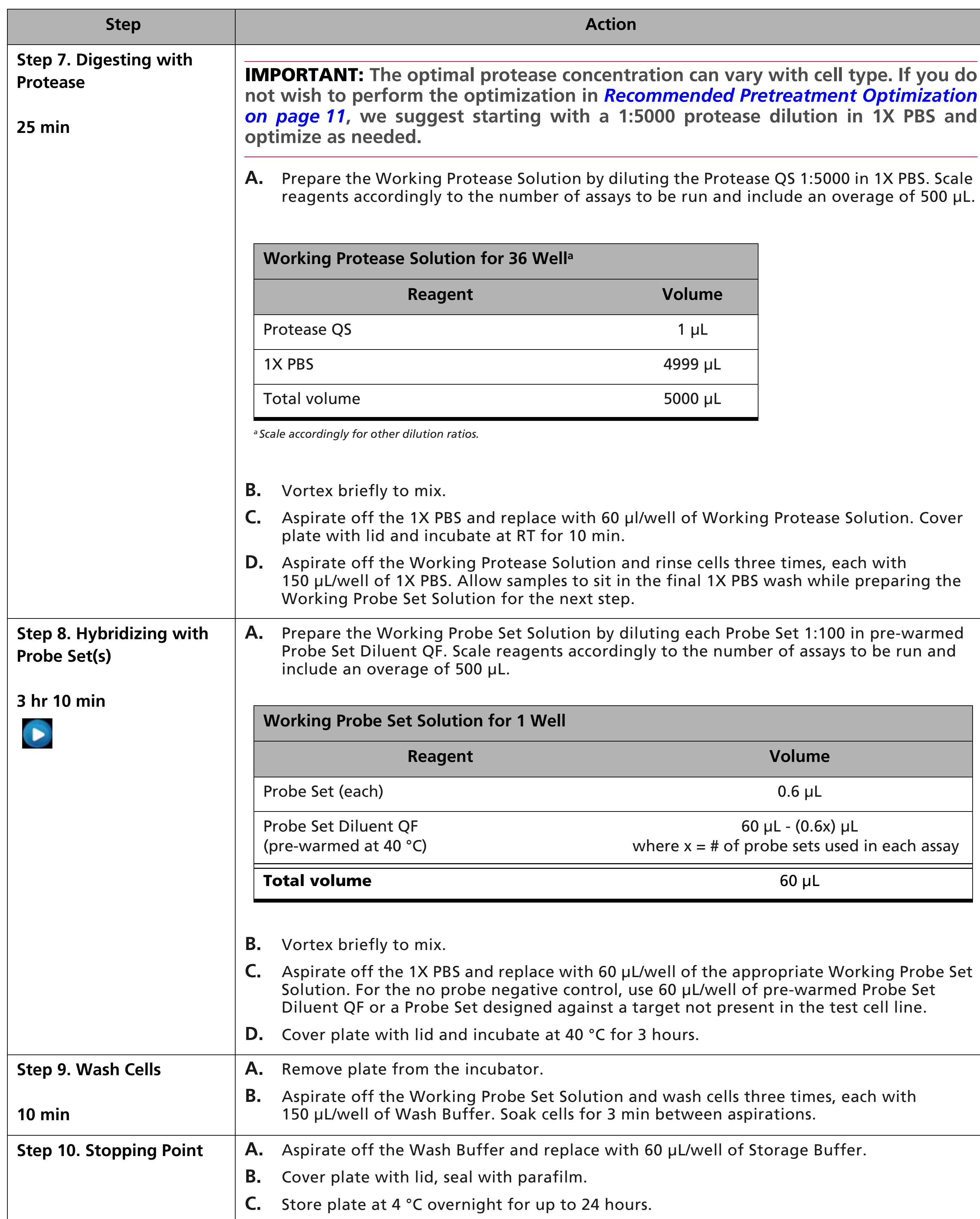
Part 2: Sample Pretreatment and Target Probe(s) Hybridization

Part 2 Procedure

Step	Action
Step 1. Sample Fixation **1 hr 15 min**	**A.** Prepare 1L of 1X PBS: Mix 100 mL of 10X PBS + 900 mL of ddH_20 water. This will be used throughout the assay. **B.** In a fume hood, prepare 6.5 mL of fresh 4% formaldehyde solution: Dilute 700 µL of a 37% formaldehyde stock + 5.8 mL of 1X PBS. Vortex briefly to mix. **C.** Carefully aspirate off culture medium, avoiding contact with the cells. Gently wash cells twice, each time with 150 µL/well of 1X PBS. **D.** Aspirate off the final 1X PBS wash and add 60 µL/well of freshly prepared 4% formaldehyde. Cover plate with lid and incubate at RT for 50 min under a fume hood. **E.** Aspirate off the formaldehyde solution and gently rinse the cells three times, each with 150 µL/well of 1X PBS.
Step 2. Preparing Buffers and Reagents during Sample Fixation **0 min**	**A.** Set a dry incubator to 40 ± 1 °C. Calibrate and monitor temperature using the QG ViewRNA Temperature Validation Kit (Affymetrix P/N QV0523) or the QuantiGene Incubator Temperature Validation Kit (Affymetrix P/N QS0517) according to the manufacturer's instructions. **B.** Equilibrate EDC to RT **C.** Pre-warm Probe Set Diluent QF to 40 °C. **D.** Thaw Probe Set(s). Vortex briefly to mix, centrifuge to collect contents to the bottom of the tubes, and place tubes on ice until use. **E.** Place Protease QS on ice. **F.** Prepare 800 mL of Wash Buffer by adding components to a 1 L capacity container in the following order, to prevent formation of precipitates, and then mixing well: **1)** 793.6 mL ddH_20 **2)** 2.4 mL Wash Comp 1 **3)** 4 mL Wash Comp 2 **G.** Prepare 6.5 mL of Storage Buffer: Mix 1.95 mL of Wash Comp 2 + 4.55 mL of nuclease-free water. **H.** Ensure availability of the following: ▪ 1X PBS ▪ Cross-Linking Buffer QM ▪ Detergent Solution QC ▪ 50%, 70% and 100% ethanol ▪ Platform rocker (moderate speed setting)



Step	Action
Step 3. EDC Cross-Linking **1 hr 30 min**	**IMPORTANT: Prepare the EDC Cross-Linking Solution no more than 10 min before use. The calculations provided here are for 36 wells. If assaying more samples, please scale up accordingly.** **A.** Remove all trace of 1X PBS and add 60 µL/well of Cross-Linking Buffer QM. Cover the plate with lid and rock gently at RT for 10 min. **B.** Aspirate off the Cross-Linking Buffer QM completely and replace with 60 µL/well of fresh Cross-Linking Buffer QM. Cover the plate with lid and rock moderately at RT for 10 min. **C.** Carefully weigh out 100 mg of EDC into a clean 50 mL conical tube. **D.** Prepare 0.16 M EDC Solution by adding 3.25 mL of fresh Cross-Linking Buffer QM to the EDC. Cover the tube and vortex to dissolve. Set the solution aside at RT until use. **E.** Aspirate off the Cross-Linking Buffer QM from each well and add 60 µL/well of 0.16 M EDC solution. **F.** Cover the plate with lid, and rock at moderate speed setting at RT for 1 hr. **G.** Aspirate off the EDC solution and rinse cells three times, each with 150 µL/well of 1X PBS. **H.** Choose one of the following options: - To proceed with the next step in the assay, go to *Step 6. Permeabilizing Cells*. - To store the samples for later use, go to *Step 4. Dehydrating for Storage (optional)*.
Step 4. Dehydrating for Storage (optional) **15 min**	**NOTE: Samples can be dehydrated and stored in 100% ethanol at –20 °C for up to 1 month.** **A.** Aspirate off the 1X PBS and replace with 150 µL/well of 50% ethanol. Incubate for 2 min at RT. **B.** Aspirate off the 50% ethanol and replace with 150 µL/well of 70% ethanol. Incubate for 2 min at RT. **C.** Aspirate off the 70% ethanol and replace with 150 µL/well of 100% ethanol. Incubate for 2 min at RT. **D.** Aspirate off the 100% ethanol and replace with 150 µL 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 1 month. **F.** Go to *Step 5. Rehydrating Samples after Storage (optional)* when you want to continue the assay. **NOTE: Dehydrated cells must be rehydrated prior to being permeabilized with detergent.**
Step 5. Rehydrating Samples after Storage (optional) **15 min**	**NOTE: Rehydration is ONLY necessary when using dehydrated cell samples. Skip this step and proceed directly to Step 6 when using freshly fixed and cross-linked adherent samples.** **A.** Aspirate off the 100% ethanol and replace with 150 µl/well of 70% ethanol. Incubate for 2 min at RT. **B.** Aspirate off the 70% ethanol and replace with 150 µL/well of 50% ethanol. Incubate for 2 min at RT. **C.** Aspirate off the 50% ethanol and replace with 150 µL/well of 1X PBS. Incubate for 10 min at RT. **D.** Proceed to *Step 6. Permeabilizing Cells*.
Step 6. Permeabilizing Cells **15 min**	**A.** Aspirate off the 1X PBS and replace with 60 µl/well of Detergent Solution QC. Cover plate with lid and incubate at RT for 10 min. **B.** Aspirate off the Detergent Solution QC and rinse cells twice, each with 150 µL/well of 1X PBS. Allow samples to sit in the final 1X PBS wash while preparing the Working Protease Solution for the next step.



<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>Step 7. Digesting with Protease

25 min</td><td>

IMPORTANT: **The optimal protease concentration can vary with cell type. If you do not wish to perform the optimization in *Recommended Pretreatment Optimization on page 11*, we suggest starting with a 1:5000 protease dilution in 1X PBS and optimize as needed.**

A. Prepare the Working Protease Solution by diluting the Protease QS 1:5000 in 1X PBS. Scale reagents accordingly to the number of assays to be run and include an overage of 500 µL.

<table>
<tr><th colspan="2">Working Protease Solution for 36 Well[a]</th></tr>
<tr><th>Reagent</th><th>Volume</th></tr>
<tr><td>Protease QS</td><td>1 µL</td></tr>
<tr><td>1X PBS</td><td>4999 µL</td></tr>
<tr><td>Total volume</td><td>5000 µL</td></tr>
</table>

[a] *Scale accordingly for other dilution ratios.*

B. Vortex briefly to mix.

C. Aspirate off the 1X PBS and replace with 60 µl/well of Working Protease Solution. Cover plate with lid and incubate at RT for 10 min.

D. Aspirate off the Working Protease Solution and rinse cells three times, each with 150 µL/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.

</td></tr>
<tr><td>Step 8. Hybridizing 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. Scale reagents accordingly to the number of assays to be run and include an overage of 500 µL.

<table>
<tr><th colspan="2">Working Probe Set Solution for 1 Well</th></tr>
<tr><th>Reagent</th><th>Volume</th></tr>
<tr><td>Probe Set (each)</td><td>0.6 µL</td></tr>
<tr><td>Probe Set Diluent QF
(pre-warmed at 40 °C)</td><td>60 µL - (0.6x) µL
where x = # of probe sets used in each assay</td></tr>
<tr><td>Total volume</td><td>60 µL</td></tr>
</table>

B. Vortex briefly to mix.

C. Aspirate off the 1X PBS and replace with 60 µL/well of the appropriate Working Probe Set Solution. For the no probe negative control, use 60 µL/well of pre-warmed Probe Set Diluent QF or a Probe Set designed against a target not present in the test cell line.

D. Cover plate with lid and incubate at 40 °C for 3 hours.

</td></tr>
<tr><td>Step 9. Wash Cells

10 min</td><td>

A. Remove plate from the incubator.

B. Aspirate off the Working Probe Set Solution and wash cells three times, each with 150 µL/well of Wash Buffer. Soak cells for 3 min between aspirations.

</td></tr>
<tr><td>Step 10. Stopping Point</td><td>

A. Aspirate off the Wash Buffer and replace with 60 µL/well of Storage Buffer.

B. Cover plate with lid, seal with parafilm.

C. Store plate at 4 °C overnight for up to 24 hours.

</td></tr>
</table>

Part 3: Signal Amplification and Detection

Part 3 Procedure

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>Step 11. Prepare Additional Buffers and Reagents

10 min</td><td>
A. Prewarm PreAmplifier Mix QM, Amplifier Mix QM, and Label Probe Diluent QF to 40 °C.
B. Place Label Probe 1-AP on ice.
C. Thaw Label Probe 4-488 at RT. Vortex briefly to mix and place tubes on ice until use. Protect Label Probe from light.
D. Thaw 100X DAPI briefly at RT. Vortex to mix. Prepare 6.5 mL of 1X DAPI: Mix 65 µL of DAPI + 6435 µL of 1X PBS and place on ice until use. Protect from light.
E. Equilibrate the following reagents to RT:
AP Enhancer (3 mL/36 wells)
Fast Red Tablets (protect from light) (1 tablet/36 wells)
Naphthol Buffer (5 mL/36 wells)
F. Ensure availability of the following reagents:
Wash Buffer
1X PBS
</td></tr>
<tr><td>Step 12. Hybridizing with PreAmplifier(s)

60 min</td><td>
A. Swirl the prewarmed PreAmplifier Mix QM briefly to mix the content. Prepare the Working PreAmplifier Mix Solution as instructed in the table below. Scale reagents according to the number of assays to be run and include overage of 500 µL.
<table>
<tr><th rowspan="2">Reagent</th><th colspan="2">Working PreAmplifier Mix Solution for 1 Well</th></tr>
<tr><th>2-Plex
(1 miRNA + 1 mRNA)</th><th>3-Plex
(1 miRNA + 2 mRNAs)</th></tr>
<tr><td>PreAmplifier Mix QM
(pre-warmed at 40 °C)</td><td>60 µL</td><td>57.6 µL</td></tr>
<tr><td>PreAmplifier 10-740</td><td>—</td><td>2.4 µL</td></tr>
<tr><td>Total volume</td><td>60 µL</td><td>60 µL</td></tr>
</table>
B. Aspirate off the Storage Buffer and wash cells two times, each with 150 µL/well of Wash Buffer.
C. Remove the Wash Buffer and replace with 60 µL/well of Working PreAmplifier Mix Solution.
D. Cover plate with lid and incubate at 40 °C for 60 min.
</td></tr>
<tr><td>Step 13. Wash Cells

10 min</td><td>
A. Remove plate from the incubator.
B. Aspirate off the Working PreAmplifier Mix Solution and wash cells three times, each with 150 µL/well of Wash Buffer. Soak cells for 3 min between aspirations. Allow samples to sit in the final wash while preparing the Working Amplifier Mix Solution.
</td></tr>
</table>

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>Step 14. Hybridizing with Amplifier(s)

60 min</td><td>A. Swirl the prewarmed Amplifier Mix QM briefly to mix the content. Prepare the Working Amplifier Mix Solution as instructed in the table below. Scale reagents according to the number of assay to be run and include overage of 500 µL.

<table>
<tr><th rowspan="2">Reagent</th><th colspan="2">Working Amplifier Mix Solution for 1 Well</th></tr>
<tr><th>2-Plex
(1 miRNA + 1 mRNA)</th><th>3-Plex
(1 miRNA + 2 mRNAs)</th></tr>
<tr><td>Amplifier Mix QM
(pre-warmed at 40 °C)</td><td>60 µL</td><td>57.6 µL</td></tr>
<tr><td>Amplifier 10-740</td><td>—</td><td>2.4 µL</td></tr>
<tr><td>Total volume</td><td>60 µL</td><td>60 µL</td></tr>
</table>

B. Aspirate off the Wash Buffer and replace with 60 µL/well of Working Amplifier Mix Solution.
C. Cover plate with lid and incubate at 40 °C for 60 min.</td></tr>
<tr><td>Step 15. Wash Cells

10 min</td><td>A. Remove plate from the incubator.
B. Aspirate off the Working Amplifier Mix Solution and wash cells three times, each with 150 µL/well of Wash Buffer. Soak cells for 3 min between aspirations. Allow samples to sit in the final wash while preparing the Working Label Probe Mix Solution.</td></tr>
<tr><td>Step 16. Hybridizing with Label Probe(s)

60 min</td><td>IMPORTANT: Protect samples from light, by covering reagents and plate with aluminum foil, during this and all subsequent steps.

A. Prepare the Working Label Probe Mix Solution by diluting the LP4-488 1:100 and the LP1-AP 1:1500 (and LP10-740 1:25) in pre-warmed Label Probe Diluent QF. Scale reagents according to the number of assay to be run and include overage of 500 µL.

<table>
<tr><th rowspan="2">Reagent</th><th colspan="2">Working Label Probe Mix Solution for 1 Well</th></tr>
<tr><th>2-Plex
(1 miRNA + 1 mRNA)</th><th>3-Plex
(1 miRNA + 2 mRNAs)</th></tr>
<tr><td>Label Probe Diluent QF
(pre-warmed at 40 °C)</td><td>59.36 µL</td><td>56.96 µL</td></tr>
<tr><td>LP1-AP</td><td>0.04 µL</td><td>0.04 µL</td></tr>
<tr><td>LP4-488</td><td>0.6 µL</td><td>0.6 µL</td></tr>
<tr><td>LP10-740</td><td>—</td><td>2.4 µL</td></tr>
<tr><td>Total volume</td><td>60 µL</td><td>60 µL</td></tr>
</table>

B. Vortex briefly to mix.
C. Aspirate off the Wash Buffer and replace with 60 µL/well of Working Label Probe Mix Solution.
D. Cover plate with lid and incubate at 40 °C for 60 min.</td></tr>
<tr><td>Step 17. Wash Cells

20 min</td><td>A. Remove plate from incubator.
B. Aspirate off the Working Label Probe Mix Solution and wash cells three times, each with 150 µL/well of Wash Buffer. Soak cells for 3 min for the first two washes and 10 min for the final wash. Protect from light.</td></tr>
</table>

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>Step 18. Developing with Fast Red Substrate

1 hr 20 min</td><td>NOTE: Volumes indicated for this step are for 36 wells. Please scale up accordingly.
A. Aspirate off the final Wash Buffer.
B. Immediately add 60 µL/well of AP Enhancer Solution and incubate at RT for 5 min while preparing the Fast Red Substrate.
C. Prepare the Fast Red Substrate: Add 5 mL of Naphthol Buffer + 1 Fast Red tablets into a 15 mL conical tube. Vortex on high speed to completely dissolve the tablets. Protect from light.
D. Aspirate off the AP Enhancer completely and add 60 µL/well of the Fast Red Substrate. Cover plate with lid and incubate at 40 °C for 45 min.
E. Meanwhile, in a hood, prepare 6.5 mL of 4% formaldehyde solution: Mix 700 µL of 37% formaldehyde stock + 5.8 mL of 1X PBS. Set aside until use.
F. Remove plate from incubator.
G. Aspirate off the Fast Red Substrate and wash cells twice, each with 150 µL/well 1X PBS.
H. Aspirate off the final 1X PBS wash and fix cells in 60 µL/well of 4% formaldehyde at RT, in the hood for 10 min. Protect from light.
I. Aspirate off the 4% formaldehyde and wash cells three times, each with 150 µL/well of 1X PBS.</td></tr>
<tr><td>Step 19. DAPI Staining

5 min</td><td>A. Aspirate off the 1X PBS and replace with 60 µL/well of Working DAPI Solution. Incubate at RT for 5 min. Protect from light.
B. Aspirate off Working DAPI Solution and wash cells once with 150 µL/well of 1X PBS.
C. Add 150 µL/well of fresh 1X PBS.
D. Cover plate with aluminum foil to protect from light.</td></tr>
<tr><td>Step 20. Image Samples</td><td>A. Samples may be viewed under a microscope or imaged immediately.
B. Prior to viewing or imaging the samples, gently wipe the bottom of the plate with a microfiber cloth to remove any grease or film.
C. For viewing samples, use appropriate filter sets.

<table><tr><th>Probe Set</th><th>Filter Set</th></tr><tr><td>TYPE 1</td><td>Fast Red: EX 530/40 nm; EM 590/40 nm</td></tr><tr><td>TYPE 4</td><td>ALEXA 488: EX 485/20 nm; EM 524/24 nm</td></tr><tr><td>TYPE 10</td><td>ALEXA 750: EX 708/75 nm; EM 809/81 nm</td></tr></table>
NOTE: Signals of LP1-Fast Red and LP4-488 are visible to unaided eyes under the microscope and appear as red and green dots, respectively. Signal of LP10-740 is invisible and require CCD-cooled camera to capture the image.</td></tr>
</table>

Troubleshooting

Contacting Technical Support

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

Table 4.1 Technical Support Contacts

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

Poor Cell Morphology and Retention

Table 4.2 Troubleshooting Poor Cell Morphology and Retention

Probable Cause	Recommended Action
Excess protease treatment or inadequate fixation	Titrate fixation time and protease concentration.
Poor cell adhesion to the glass/ polystyrene surface of the plate	Check to make sure that the poly-L-lysine is fresh and has not expired.
	Try coating the coverslips with different extracellular matrices such as MatriGel, collagen or poly-D-lysine.
	Use protocol for suspension cells if cell adhesion is extremely poor.
Cells were not seeded/spotted at optimal density	Increase the number of cells seeded/spotted so that the density is between 70-90% at the beginning of the assay.
Cells were dislodged by vigorous dispensing or aspiration during washes	Slowly aspirate from the bottom edge of the wells and gently dispense against the upper edge of the well to minimize cells loss or displacement.

High Non-Specific Binding Outside the Cells

Table 4.3 Troubleshooting Non-Specific Binding Outside Cells

Probable Cause	Recommended Action
Non-specific binding of Probe Set(s), PreAmps, Amps or Label Probes to glass/polystyrene 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 min for each washing step.
Samples were allowed to dry	Make sure sufficient solution is covering the entire sample at all times during the assay.

Weak or No Signal

Table 4.4 Troubleshooting Weak Signal:

Probable Cause	Recommended Action
Protease digestion not optimal	Titrate protease concentrations; generally higher protease concentration is required for mRNA target and lower protease concentration for miRNA target.
Fixation time not optimal, over- or under-fixation	Titrate formaldehyde fixation time; generally longer fixation time is required for miRNA target and shorter fixation time for mRNA target.
Cross-linking not optimal	Check to make sure that EDC is fresh and properly stored in desiccant at -20 °C.
	Equilibrate EDC to RT before opening the bottle to avoid moisture absorption.
	Prepare EDC cross-linking solution just before use.
	Ensure that EDC is prepared at the proper concentration.
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 either expressed at a very low level or not expressed at all in the cells being assayed	Use positive control cell line known to express your miRNA target to validate the procedure.
	Use positive control probe set against a target with medium to high ubiquitous expression (housekeeping gene) or against a target that is your cell-type specific with medium to high expression.
Probe Set concentration too low (miRNA target only)	Increase the recommended concentration by diluting 1:75 instead of 1:100.
Samples left too long in Wash Buffer	Follow protocol for appropriate washing times.
	Do not leave samples in Wash Buffer longer than 30 min.
Samples left too long in Storage Buffer	Follow protocol for appropriate storage time and temperature.
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 Probes are added in the correct order and to the appropriate samples.
Accidental Omission of AP-Enhancer (miRNA target only)	Check to make sure AP-Enhancer is warmed up to RT and added to samples.
Insufficient Fast Red Incubation Time (miRNA target only)	Make sure that Naphthol Buffer and Fast Red Tablets are at RT and not cold when substrate solution is being prepared.
	Increase Fast Red Substrate incubation time to 60 min for low expressing miRNA targets.
Reagents did not reach the cells	Make sure cell seeded coverslips are faced up during the assay.
	If you lose track of which side of the coverslip contains the cells, check it under the microscope by scraping a small surface area with a 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 (refer to *Microscopy and Imaging Equipment Guidelines on page 7*.)

Patchy and Diffused Fast Red Signals

Table 4.5 Troubleshooting Patchy and Diffused Fast Red Signals

Probable Cause	Recommended Action
Accidental Omission of AP-Enhancer	Check to make sure that the AP-Enhancer step has not been left out.
Incomplete Removal of AP-Enhancer	Ensure that the AP-Enhancer is completely aspirated off before adding the Fast Red Substrate
Fast Red Substrate not freshly prepared	Prepare Fast Red substrate immediately before use.
Insufficient Fast Red incubation time	Increase the incubation time by 15 min.
Insufficient post-label fixation	Ensure that the formaldehyde is fresh and post-label fixation is for the entire 10 min.
Endogenous alkaline phosphatase activity in cell line	Treat cell line with a solution of 0.2 M HCl and 3 M NaCl for 10 min at RT and then wash twice each time with 2 mL of 1X PBS for 1 min before proceeding to the detergent treatment step.

High Background Inside Cells

Table 4.6 Troubleshooting High Background Inside Cells

Probable Cause	Recommended Action
Insufficient washing after formaldehyde fixation	Increase washing volume or soaking time by 5 min for each washing step.
Insufficient washing after hybridization	Increase washing volume or soaking time by 5 min 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. If this occurs, the 488 nm channel can still be used especially for detecting a highly expressed transcript.
	If the cell type is not critical, switch to another cell line with lower autofluorescence.
	Switch to using 740 module for detection of the mRNA target.
	Check to make sure that the plate itself is not emiting high level of autofluorescence. Use plates recommended in *Table 1.6 on page 6*.
Samples were allowed to dry	Make sure sufficient solution is covering the entire sample at all times during the assay.

Variable Staining Within a Sample

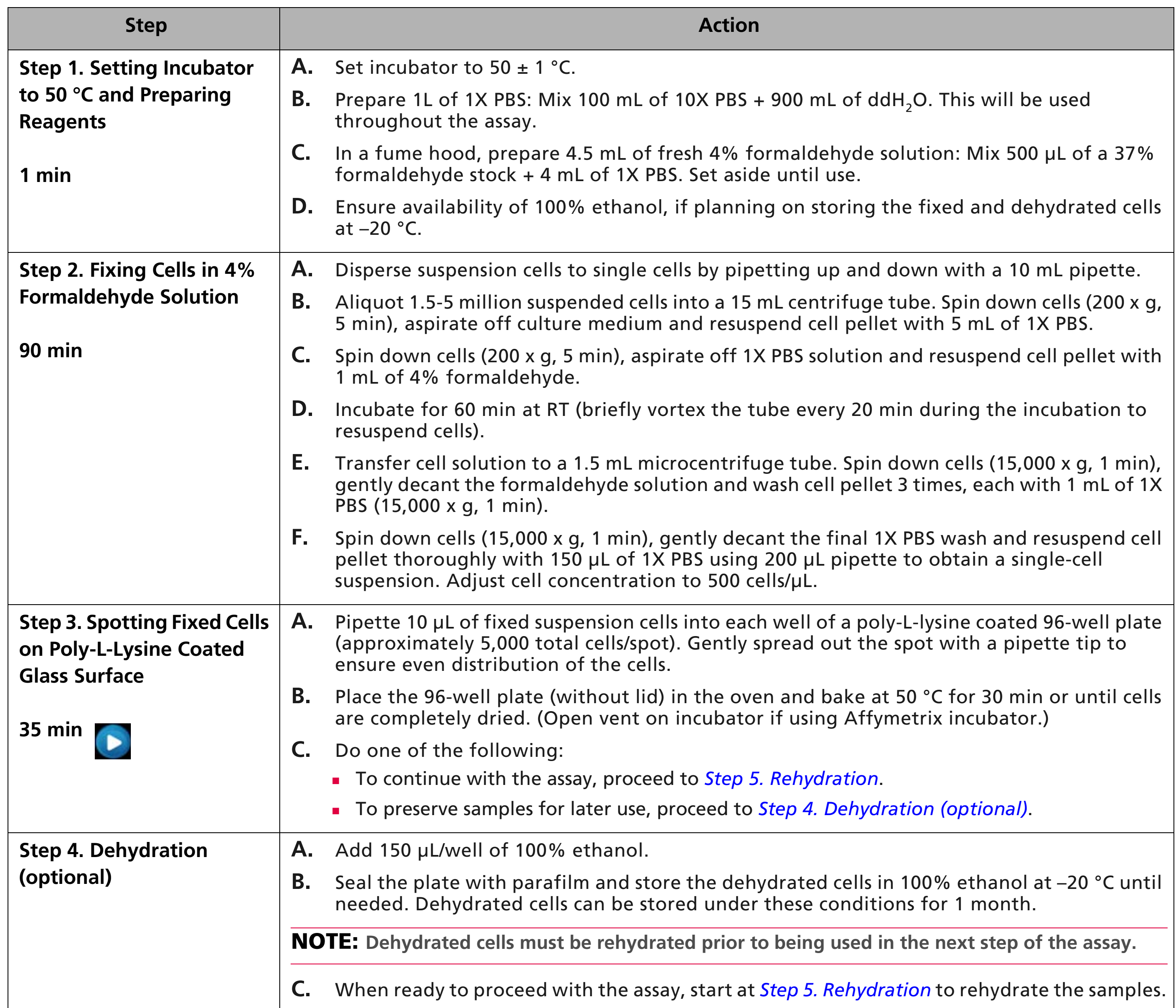
Table 4.7 Troubleshooting Variable Staining Within a Sample

Probable Cause	Recommended Action
Sample were allowed to dry	Make sure sufficient solution is covering the entire sample at all times during the assay.
Insufficient mixing of reagents	Prewarm all hybridization buffers to dissolve any precipitates before use.
	Briefly vortex all working hybridization solutions before use.

Sample Preparation Procedure for Suspension Cells

About the Preparation for Suspension Cells

Suspension cells that are used in the QG ViewRNA miRNA ISH Cell Assay must be fixed in suspension and then spotted directly on poly-L-line coated 96-well optical-bottom plate (for instructions on how to prepare poly-L-lysine coated 96-well optical bottom plate, refer to *Part 1: Sample Preparation for Adherent Cells on page 14*.) Cells can be prepared on the same day for use for the *in situ* hybridization assay in 96-well optical-bottom plate format.



Step	Action
Step 1. Setting Incubator to 50 °C and Preparing Reagents **1 min**	**A.** Set incubator to 50 ± 1 °C. **B.** Prepare 1L of 1X PBS: Mix 100 mL of 10X PBS + 900 mL of ddH_2O. This will be used throughout the assay. **C.** In a fume hood, prepare 4.5 mL of fresh 4% formaldehyde solution: Mix 500 µL of a 37% formaldehyde stock + 4 mL of 1X PBS. Set aside until use. **D.** Ensure availability of 100% ethanol, if planning on storing the fixed and dehydrated cells at –20 °C.
Step 2. Fixing Cells in 4% Formaldehyde Solution **90 min**	**A.** Disperse suspension cells to single cells by pipetting up and down with a 10 mL pipette. **B.** Aliquot 1.5-5 million suspended cells into a 15 mL centrifuge tube. Spin down cells (200 x g, 5 min), aspirate off culture medium and resuspend cell pellet with 5 mL of 1X PBS. **C.** Spin down cells (200 x g, 5 min), aspirate off 1X PBS solution and resuspend cell pellet with 1 mL of 4% formaldehyde. **D.** Incubate for 60 min at RT (briefly vortex the tube every 20 min during the incubation to resuspend cells). **E.** Transfer cell solution to a 1.5 mL microcentrifuge tube. Spin down cells (15,000 x g, 1 min), gently decant the formaldehyde solution and wash cell pellet 3 times, each with 1 mL of 1X PBS (15,000 x g, 1 min). **F.** Spin down cells (15,000 x g, 1 min), 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 500 cells/µL.
Step 3. Spotting Fixed Cells on Poly-L-Lysine Coated Glass Surface **35 min**	**A.** Pipette 10 µL of fixed suspension cells into each well of a poly-L-lysine coated 96-well plate (approximately 5,000 total cells/spot). Gently spread out the spot with a pipette tip to ensure even distribution of the cells. **B.** Place the 96-well plate (without lid) in the oven and bake at 50 °C for 30 min or until cells are completely dried. (Open vent on incubator if using Affymetrix incubator.) **C.** Do one of the following: ▪ To continue with the assay, proceed to *Step 5. Rehydration*. ▪ To preserve samples for later use, proceed to *Step 4. Dehydration (optional)*.
Step 4. Dehydration (optional)	**A.** Add 150 µL/well of 100% ethanol. **B.** 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 1 month. **NOTE: Dehydrated cells must be rehydrated prior to being used in the next step of the assay.** **C.** When ready to proceed with the assay, start at *Step 5. Rehydration* to rehydrate the samples.

Step	Action
Step 5. Rehydration	**A.** If the samples were from *Step 4. Dehydration (optional)*, aspirate off the 100% ethanol and replace with 150 µl/well of 70% ethanol. Incubate for 2 min at RT. **B.** If the samples were baked but not stored in 100% ethanol, add 150 µl/well of 70% ethanol. Incubate for 2 min at RT. **C.** Aspirate off the 70% ethanol and replace with 150 µL/well of 50% ethanol. Incubate for 2 min at RT. **D.** Aspirate off the 50% ethanol and replace with 150 µL/well of 1X PBS. Incubate for 10 min at RT. **E.** To continue with the assay, go to *Step 3. EDC Cross-Linking on page 17* to proceed with cross-linking and permeabilization.

Detecting siRNA with QuantiGene miRNA ISH Cell Assay

About Detecting siRNA with QuantiGene miRNA ISH Cell Assay

The QuantiGene ViewRNA miRNA ISH Cell Assay kit can also be used to detect siRNA in cultured cells providing researchers in the field of RNAi with a useful and sensitive tool for simultaneous *in situ* analysis of siRNA and its mRNA target sequences. Because siRNA sequences used to transfect cells for knockdown study are mostly double-stranded, a slight modification to the protocol is necessary to denature the double-stranded siRNA molecule to allow accessibility between the probe and siRNA target sequence.

We recommend using DeliverX and DeliverX Plus siRNA transfection reagents (Affymetrix) for high transfection efficiency and low cellular toxicity. Please apply the following modifications when using the QuantiGene ViewRNA miRNA ISH Cell Assay for detecting siRNA.

Step	Action
Step 1. Prepare Buffer and Reagents	**A.** Set a dry incubator to 75 °C. **B.** Prepare 6.5 mL of Denaturing Solution (2X SSC/50% Formamide). **C.** Place the Denaturing Solution in a capped bottle. **D.** Place 50 mL of 1X PBS in a capped bottle. **E.** Preheat the 1X PBS and Denaturing Solution to 75 °C.
Step 2. Pretreatment of Cells	24-hr post-transfection, fix, permeabilize and digest cells with protease according to *Part 2: Sample Pretreatment and Target Probe(s) Hybridization on page 16*. Follow the protocol exactly up to *Step D* in *Step 7. Digesting with Protease on page 18* (that is, protease treatment followed by 3 washes in 1X PBS).
Step 3. Denaturation	**A.** Remove the 1X PBS from cells and add 60 µL/well of preheated Denaturing Solution. **B.** Cover plate with lid and incubate at 75 °C for 15 min.
Step 4. Wash Cells	**A.** Aspirate off the Denaturing Solution. **B.** Wash cells 3 times, each with 150 µL/well of preheated 1X PBS.
Step 5. Target Probe(s) Hybridization	Proceed with the assay starting from *Step 8. Hybridizing with Probe Set(s) on page 18*.