Affymetrix®

User Manual

QuantiGene® 2.0 miRNA Assay

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Contents

Introduction

About This User Manual

Who This Manual is For

This manual is for anyone who has purchased QuantiGene 2.0 Assay Kits to perform the QuantiGene 2.0 miRNA Assay for any of the following sample types:

- Cultured cells
- Whole blood/PAXgene blood
- Fresh, frozen, or formalin-fixed, paraffin-embedded (FFPE) tissues
- Total RNA or purified miRNA

What this Manual Covers

This manual provides:

- Background information about QuantiGene 2.0 miRNA Assay and how it works
- Experimental design and data analysis
- QuantiGene 2.0 miRNA Assay
- Troubleshooting

Contacting Affymetrix

Technical Help

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 1.1 Contacting Affymetrix

Location	Contact Information
North America	1.877.726.6642 option 1, then option 2 pqbhelp@affymetrix.com
Europe	+44 1 628-552550 techsupport_europe@affymetrix.com
Asia	+81 3 6430 430 techsupport_asia@affymetrix.com

QuantiGene 2.0 miRNA Assay Basics

The QuantiGene 2.0 miRNA Assay enables researchers to perform direct detection and quantification of miRNAs at single-base resolution, avoiding biases associated with miRNA or total RNA isolation, cDNA synthesis and PCR amplification. The assay uses validated probe sets that are highly specific for the mature miRNA and do not detect the precursor miRNA. The assay is ideal for target quantification, screening and validation of miRNA array results.

The kits provide the reagents needed to quantify specific miRNA molecules directly from:

- Cultured cell lysates
- Whole blood or PAXgene blood lysates
- Fresh, frozen, or FFPE tissue homogenates
- Purified miRNA or total RNA

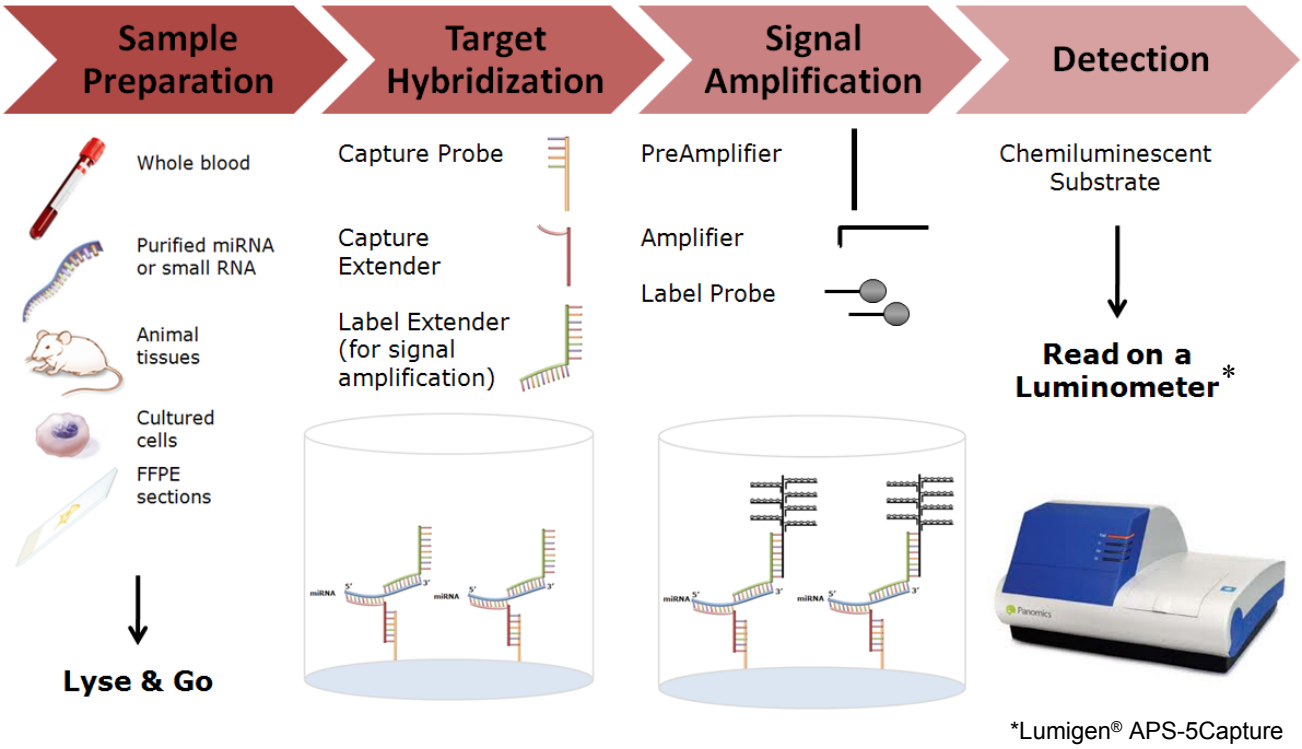
Please refer to the QuantiGene Sample Processing Kit Package Inserts for instructions on preparing cultured cell or blood lysates or tissue homogenates. To prepare miRNA or total RNA, follow standard laboratory methods.

The QuantiGene 2.0 miRNA Assay is a hybridization-based assay performed on 96-well plates. The assay is based on direct from lysate quantification of the miRNA target using novel oligonucleotide chemistry and probe design for specific and efficient capturing of miRNA followed by branched DNA (bDNA) signal amplification.

On the first day, the sample is lysed to release the miRNAs and incubated overnight in the 96-well plates with the target specific probe sets (Capture Extenders – CEs and Label Extenders – LEs).

On the second day the signal amplification tree is built via sequential hybridization of PreAmplifier (PreAmp), Amplifier (Amp) and Alkaline phosphatase label probe (AP-LP). The signal is detected by adding chemiluminescent substrate and using a microplate luminometer for the read out.

How it Works



Assay Specifications

Table 1.2 Assay Specifications

Component	Description
Assay Format	▪ 96 well plate
Sample Types	▪ Purified miRNA ▪ Purified Total RNA ▪ Whole Blood/PAXgene Blood Lysates ▪ Cell Culture Lysates ▪ Plant, Animal and Insect Tissue homogenates
Limit of Detection (Greater than Average Background Signal + 3SD)	▪ 3,000 ~ 60,000 copies/well
Linear range (Entire range must meet accuracy of fold change requirement. Linear regression coefficient {R^2} greater than 0.98.)	▪ 3 Log
% of Spike Recovery	▪ 100 ± 20%, range (80 – 120%)
Accuracy of Fold Change (% of Observed/Expected Fold Change)	▪ 100 ± 20%, range (80 – 120%)
Inter-wells Precision (SD of 3 replicates)	▪ ± 10%
Inter-plates Precision (SD of 3 replicates)	▪ ± 15%

Required Materials

The QuantiGene 2.0 miRNA Assay is a modular kit. Each module below is sold separately in multiple sizes.

- QuantiGene 2.0 Assay Kit
- QuantiGene Sample Processing Kit (not required for purified RNA samples)
- QuantiGene miRNA Probe Set(s)

QuantiGene 2.0 Assay Kit Components

The components of the QuantiGene 2.0 Assay Kit and their recommended storage conditions are listed below. The QuantiGene 2.0 Assay Kit is available in 4 sizes. Refer to the package insert for quantities of individual components supplied. Kits have a shelf life of 6 months from date of receipt (when stored as recommended) and contain the following components:

Table 1.3 QuantiGene 2.0 Assay Kit Components and Their Storage Conditions

Component	Description	Storage
2.0 PreAmplifier (PreAmp 1)	DNA in aqueous buffered solution	–20 °C
2.0 Amplifier (Amp 1)	DNA in aqueous buffered solution	–20 °C
Blocking Reagent	Aqueous buffered solution containing a preservative	–20 °C
Capture Plate	96-well polystyrene plate coated with capture probes	2–8 °C
Label Probe	Oligonucleotide-alkaline phosphatase conjugate in aqueous buffered solution	2–8 °C
2.0 Substrate[a]	Chemiluminescent substrate	2–8 °C
Amplifier/Label Probe Diluent	Aqueous buffered solution with a protein-containing preservative	15–30 °C
Lysis Mixture	Aqueous buffered solution containing a preservative	15–30 °C
Plate Seals	Adhesive-backed foil seal	15–30 °C
Wash Buffer Component 1 (Wash Comp 1)	Aqueous solution	15–30 °C
Wash Buffer Component 2 (Wash Comp 2)	Aqueous buffered solution	15–30 °C

[a] *Lumigen® APS-5*

QuantiGene 2.0 miRNA Assay Accessory Reagents

In addition to QuantiGene 2.0 Assay Kits, two accessory reagents are required to perform QuantiGene 2.0 miRNA assays.

Table 1.4 QuantiGene 2.0 miRNA Assay Accessory Components

Accessory Reagent	Description
Required QuantiGene Sample Processing Kit	Contains reagents and instructions for processing different sample types: cultured cells, whole blood/PAXgene blood, fresh or frozen tissues or FFPE samples. Specify sample type when ordering (not required for purified RNA).
Required QuantiGene miRNA target-specific Probe Set(s)	Customer specified miRNA target(s). Each Probe Set contains 1 CE and 1 LE.
Optional QuantiGene miRNA Probe Set(s) for Normalization	Customer specified small RNA(s) or miRNA targets suitable for normalizing. These targets should have characteristics of a traditional housekeeping gene. Target is stably expressed under all experimental conditions evaluated. Each Probe Set contains 1 CE and 1 LE.
Optional miRNA Positive Control (250 pMol, 150 million copies/µl)	Synthetic RNA oligo with exactly the same sequence as the target miRNA can be used as an assay positive control or for accurate determination of miRNA copies in samples.

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

(*Optional*) Upstream Cell Viability Assay

The QuantiGene Cell Viability Reagent provides a simple, reliable and sensitive means for quantifying cell proliferation and viability upstream of QuantiGene, QuantiGene Plex and QuantiGene ViewRNA Plate-Based assays. This homogeneous assay utilizes the non-fluorescent redox dye resazurin, which is converted by metabolically active cells, to resorufin, a highly fluorescent product (Ex 530 – 570 nm; Em 590 – 620 nm).

Table 1.5 QuantiGene Cell Viability Reagent

Component	Description	Storage
QuantiGene Cell Viability Reagent	Non-fluorescent redox dye in aqueous solution	–20 °C

Required Materials Not Provided

Other materials required to perform the QuantiGene 2.0 miRNA Assay that are not included in the assay kit are listed here.

Table 1.6 Required Materials Not Provided

Required Material	Source	Part Number or Model
Adjustable single- and multi-channel precision pipettes for dispensing 1 – 20 µL, 20 – 200 µL and 200 – 1000 µL	Major laboratory supplier (MLS)	
Reagent reservoirs: 25-mL capacity 100-mL capacity	VistaLab Technologies	▪ (P/N 3054-1002) or equivalent ▪ Corning Costar (P/N CLS 4873) or equivalent
Microcentrifuge	Eppendorf	541D or equivalent
Microplate centrifuge that can achieve 240 x g	Eppendorf	5804R and rotor A-2 DWP or equivalent
Vortex mixer	MLS	

Table 1.6 Required Materials Not Provided

Required Material	Source	Part Number or Model
Nuclease-free water	MLS	
Yeast tRNA	Invitrogen	P/N 15401-011
Luminescence detector with the following features: ▪ Sensitivity > 3 x 10^{-21} moles of luciferase ▪ Dynamic range > 8 logs ▪ Well-to-well uniformity ± 5% ▪ Cross-talk < 5 x 10^{-5} ▪ Fluorescent detection module (optional for DNA stain); Ex 480 nm/Em 520 nm **NOTE:** Make sure your luminometer meets or exceeds minimum performance specifications.	Turner BioSystems Molecular Devices	▪ Modulus Microplate Luminometer P/N 9300-001 ▪ LMAX or equivalent
Incubator or oven with the following specifications: ▪ Maintain a constant temperatures of 46 ± 1 °C ▪ Temperature does not vary more than ± 1 °C ▪ Uniform temperature throughout entire incubator	Affymetrix	QS0700, QS0701 (120V) QS0710, QS0711 (220V)
4 inch soft rubber roller or QuantiGene CTC Plate Sealer	Affymetrix Affymetrix	QS0515 QG0400
QuantiGene Incubator Temperature Validation Kit **NOTE:** Highly recommended for temperature validation of incubator every 6 months.	Affymetrix	QS0517
Optional. Plate washer that meets or exceeds the following specifications: ▪ 30 – 200 µL ± 5% volume ▪ 96 or 384 channels ▪ Angle-dispensing tip ▪ Plate stacker ▪ Automation capable ▪ Minimal dead volume	BioTek	ELx 405 model with high throughput pump option

Assay Terminology and Guidelines for Data Analysis

Assay Terminology

Assay Background

A sample well contains all assay components except for target miRNA or sample. The background control is used to determine Limit of Detection (LOD).

Replicates

Technical replicates are replicate assays from a single sample. For example, a cell lysate that is divided into several portions and each portion run in the same assay.

Biological replicates are replicate assays from biologically-equivalent samples. For example, cells grown in different wells that are subjected to the same treatment, lysed independently, then run as distinct samples in the assay.

NOTE: We recommend running 3 technical replicates of each distinct biological sample.

Assay Precision

The Coefficient of Variation (CV) is a measure of assay precision. QuantiGene 2.0 miRNA Assay CVs are typically less than 10% for technical replicates.

To determine the assay CV:

Step	Action
1	Run technical replicates (n=3) of each sample.
2	Calculate the average signal (AVG) of technical replicates from each target miRNA.
3	Calculate the standard deviation (SD) of signals from technical replicates for each target miRNA.
4	Calculate the %CV. %CV = (SD/AVG)*100.

Relative Luminescent Unit (RLU)

The output signal on the luminometer.

Assay Limit of Detection (LOD)

The LOD is the signal above the background plus 3 standard deviations of the background:

To calculate assay limit of detection for each target miRNA:

LOD = AVG RLU of assay background control wells + 3X SD of assay background signals.

Assay signals below LOD should not be used for quantifications.

Limit of Quantification (LOQ)

LOQ is the lowest RLU that exhibits acceptable accuracy of fold change (see *Assay Linearity/Accuracy of Fold Change* below).

Assay Linearity/ Accuracy of Fold Change

Assay linearity is defined as all dilutions that exhibit an accuracy of fold change between 80 and 120%. Assay must exhibit 3 log linearity based on 80 – 120% accuracy of fold change. Typically, the R^2 (linear regression coefficient) value is greater than 0.98.

To determine assay linearity:

Step	Action
1	Run a dilution series of your sample.
2	Subtract the AVG assay background signal from the AVG signal of technical replicates for each target miRNA.
3	Calculate the ratio of background-subtracted AVG RLU from sequential sample dilutions for each target miRNA (80 – 120%). **NOTE:** Quantifiable signals are those signals within the assay's linear range.

Ratio of Background-Subtracted AVG RLU for Each Target miRNA

3-fold serial dilution of the cell lysate (µL)	Signal (background subtracted) (RLU)	Observed fold change	Expected fold change	% Obs/ Exp
60	3,100	3.1	3	103
20	1,000	2.70	3	90
6.6	370			

Guidelines for Assay Optimization and Assay Design

Overview

Here we provide information and guidelines for:

- Optimizing sample lysis
- Optimizing sample input
- Assay controls
- Assay replicates
- Calculations of miRNA copy number (relative and absolute)

Optimizing Lysis Conditions

To determine optimal sample amount for lysis or homogenization:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Follow the recommended amount of cell number or tissue amount per volume of lysis mixture solution or homogenization solution listed in the Sample Processing Kit package insert for the specific sample types. Recommendations are summarized below. To ensure optimal lysis, in the initial experiment, run a test range as indicated in the table.
<table>
<tr><th colspan="3">Recommended Sample Preparation Amount</th></tr>
<tr><th></th><th>Cultured Cells</th><th>Tissue</th></tr>
<tr><td>Recommended</td><td>400 cells/µL of Working Lysis Mixture</td><td>5 mg/300 µL of Working Tissue Homogenization Solution</td></tr>
<tr><td>Test Range</td><td>200, 400, 800 cells/µL of Working Lysis Mixture</td><td>2.5, 5.0, 10 mg/300 µL of Working Tissue Homogenization Solution</td></tr>
</table>
</td></tr>
<tr><td>2</td><td>For each lysate, prepare a 3-fold serial dilution to determine the assay performance. Assay performance is determined by calculating the following.
▪ LOD
▪ LOQ
▪ Assay linearity
▪ % assay CV</td></tr>
<tr><td>3</td><td>Calculate the assay performance for each sample to determine which one had the best performance and use that amount of cells or tissue for future experiments.

Incomplete or poor lysis will produce high assay CV, poor linearity, and poor LOD.

To determine the optimal lysis method for a sample type:

Following the procedure for determining optimal lysis, test different lysis methods, for example, Tissue lyser or liquid nitrogen for plant tissues</td></tr>
</table>

Optimizing Sample Input for QuantiGene miRNA Assay

After you have determined the optimal lysis conditions for sample preparation, use the following guidelines to determine the optimal sample amount/well to use for the QuantiGene 2.0 miRNA assay:

- Resulting signal from the sample is above the LOQ
- Amount of sample is high enough to compensate for sample loading error. For example, if the amount of loaded sample can deviate more than 4 times, then increase the sample input by 4 to ensure detection.

- If the amount of sample is not limiting, use an input that has a signal/background ratio of at least 3-fold. Background is defined as signal from a sample well without sample input.

Assay Replicates

Run all assay samples with a minimum of duplicates and ideally triplicates. Technical replicates are used to calculate assay precision or %CV.

Recommended Assay Controls

All experiments should have the following controls:

- *Assay Background Control*. A sample well that contains all assay components except for the target miRNA. The background control is also used to determine the limit of detection (LOD). Data below the LOD should not be used for quantification.
- *miRNA Positive Control*. miRNA positive controls are provided by Affymetrix to be used as the assay positive control. It is also provided to determine the absolute number of miRNA copies/sample/well. The miRNA copy number can be determined by running an 8-point standard curve and linear curve fitting (see data analysis guideline below). Using the absolute copy number improves data accuracy and precision for inter-day and inter-site experiments and is critical for biomarker discovery and clinical research.
- *Normalization small RNA(s)*. Considerations for the selection of the normalization gene to determine relative fold changes or normalize gene expression data across samples or experiments are listed below:
  - Genes are stably expressed under all experimental conditions evaluated.
  - Relative luminescent signal should be similar to test samples so no dilution factor is required. In situations where samples are limited, select the normalization gene that is highly expressed and which would require only small amount of sample for detection.
  - Examples of recommended small RNAs include:

Human	SNORD43, SNORD44, SNORD48
Mouse	Snord68, Z38 and Z39
Rat	Z38, Z39 and U6

For a list of available Probe Sets for normalization small RNAs, please go to www.affymetrix.com/panomics

Guidelines for Data Analysis

Normalizing Gene Expression Data

To normalize gene expression data:

Step	Action
1	Calculate Average Signal - Background (S-B) for samples and normalization RNAs. Note that background is defined as well containing all assay components except for target miRNA or samples.
2	Calculate Normalized Signal = S-B of sample/S-B of normalization RNAs. **NOTE:** If multiple normalization RNAs are measured, the geometric mean of background-subtracted AVG housekeeping RNA signals may be used for data normalization.

Calculating Relative Fold Change of miRNA Expression

To calculate the relative fold change of target miRNA expression in treated versus untreated samples:

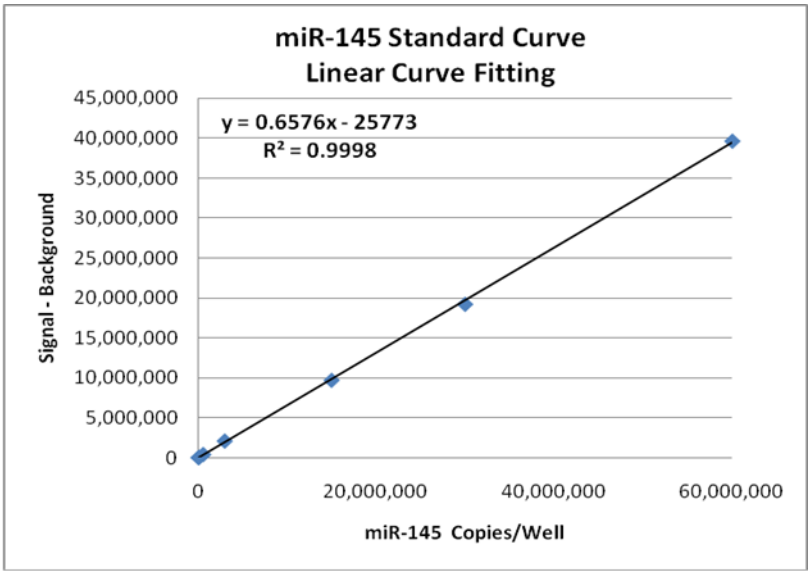
Step	Action
1	Run sample of interest using probe sets of normalization small RNA and target miRNA.
2	Normalize miRNA expression data as described in *Normalizing Gene Expression Data* on page 10.
3	Divide the normalized value for the treated sample by the normalized value for the untreated sample.
4	*Optional*. Several normalization small RNA can be run from the same sample and their Geometric Mean used for normalization purposes.
5	*Optional*. Normalization can also be performed for mRNA using the QuantiGene Assay with the same sample prepared for miRNA. **NOTE:** Given that the temperature of the two assays (mRNA and miRNA) will be different, detection of mRNA and miRNA cannot be performed on the same plate, therefore plan on using separate plates for each.

Calculating miRNA Copy Number/Well

Determination of miRNA copy number can be achieved accurately and easily using the QuantiGene 2.0 miRNA assay. In practice, spike recovery experiments are used to assure specific detection of miRNA copy number in complex sample matrices and cell lysates. The QuantiGene 2.0 miRNA assay has an excellent spike recovery of 100 ± 20%. Determining absolute copy number compared to relative fold changes provides the ability to compare inter-day, inter-lab and inter-group experiments. This makes the QuantiGene 2.0 miRNA assay practical for both clinical and biomarker translational research.

An example is provided to demonstrate how to determine copy number of miR-145 in 8,000 HeLa cells:

Step	Action
1	Use Affymetrix miR-145 Positive Control at 250 pMol (150 million copies/µl) to create an 8-point standard curve in triplicate. ▪ Dilute miR-145 Positive Control in nuclease-free water plus 10 ng/µl Yeast tRNA (Invitrogen # 15401-011) with a final volume of 20 µl/assay well ▪ Include one background point of 0 copies miR-145 Positive Control ▪ Prepare the lowest dilution point at 2 times above the LOD of miRNA probe set (refer to product insert). For miR-145, the probe set LOD is 30,000 copies, so that the lowest dilution point would be 60,000 copies.
2	Run standard curve dilutions and sample lysates.

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>3</td><td>Determine (signal – background) for standard curve dilutions and samples. Background is assay wells of 0 copies miR-145.
<table>
<tr><th colspan="3">Standard Curve</th><th>HeLa Lysate (8,000 cells)</th></tr>
<tr><th>miR-145 (LOD = 30,000 copies)</th><th>AVG Signal</th><th>AVG (Signal – Background)</th><th>AVG (Signal – Background)</th></tr>
<tr><td>0</td><td>38,227</td><td></td><td>4,819,496</td></tr>
<tr><td>60,000</td><td>83,453</td><td>45,226</td><td></td></tr>
<tr><td>120,000</td><td>127,236</td><td>89,009</td><td></td></tr>
<tr><td>600,000</td><td>478,620</td><td>440,393</td><td></td></tr>
<tr><td>3,000,000</td><td>2,164,208</td><td>2,125,981</td><td></td></tr>
<tr><td>15,000,000</td><td>9,767,725</td><td>9,729,498</td><td></td></tr>
<tr><td>30,000,000</td><td>19,289,475</td><td>19,251,248</td><td></td></tr>
<tr><td>60,000,000</td><td>39,714,450</td><td>39,676,223</td><td></td></tr>
</table></td></tr>
<tr><td>4</td><td>Plot a graph of the signal-background (y-axis) and miRNA copy number (x-axis).
Standard curve for miR-145 Positive Control:
 
</td></tr>
</table>

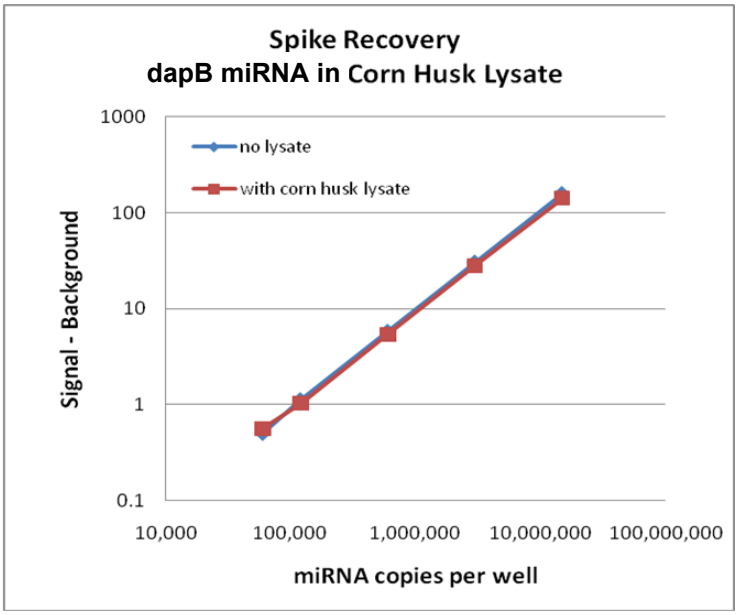
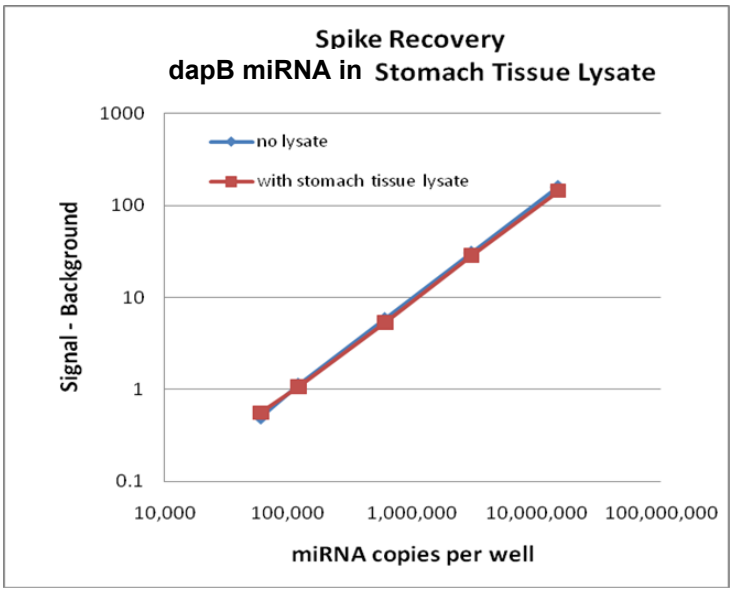
Step	Action
5	Using linear regression curve fitting (Microsoft Excel or other program) to determine the linear equation and regression coefficient (R^2). Note that the R^2 must be equal or greater than 0.96. **To calculate miR-145 copy number /cell using linear curve fitting equation:** **Y** = 0.6576X – 25773 **X** = (Y+25773)/0.6576 If Y value: 4,819,496 (signal – background) Then X value: 4,819,496 + 25773/0.6576 = 7,720,907 copies/ 8,000 cells Therefore, copy number/cell = 7,720,907 copies/8,000 cells = 965 copies/cell

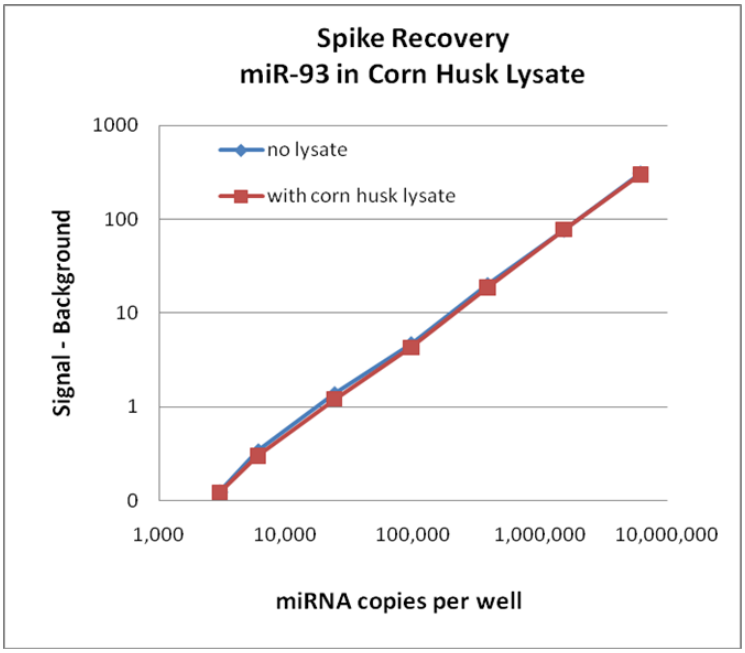
% of Spike Recovery

Spike recovery experiments determine the assay's ability to capture and detect a specific miRNA target in a complex matrices of lysates consisting of proteins, lipids, carbohydrates, and nucleic acids. The QuantiGene 2.0 miRNA Assay must meet the spike recovery of 100 ±20% or a range of 80 – 120%.

To calculate spike recovery:

Step	Action
1	Prepare serial dilutions of miRNA Positive Control (from Affymetrix) in lysates or homogenates for spiked samples. Include 0 miRNA as background. ▪ If preparing spiked samples in lysates, dilute Affymetrix miRNA Positive Control in the lysate of interest with a final volume of 80 µl/assay well. ▪ If preparing spiked samples in homogenates, dilute Affymetrix miRNA Positive Control in the homogenate of interest with a final volume of 40 µl/assay well.
2	Prepare serial dilutions of miRNA Positive Control (from Affymetrix) in assay buffer for unspiked samples. Include 0 miRNA as background. ▪ If preparing unspiked samples for lysates, dilute Affymetrix miRNA Positive Control in Diluted Lysis Mixture plus 2.5 ng/µl of Yeast tRNA (Invitrogen # 15401-011) with a final volume of 80 µl/assay well. ▪ If preparing unspiked samples for homogenates, dilute Affymetrix miRNA Positive Control in Homogenizing Solution plus 5 ng/µl of Yeast tRNA (Invitrogen # 15401-011) with a final volume of 40 µl/assay well.
3	Run assay, acquire signal and determine background-subtracted signal for spiked and unspiked miRNA

Step	Action
4	**To determine % spike recovery:** (spiked S-B/unspiked S-B) x 100 = Must be within 80 – 120% S-B = signal – background (0 miRNA) **NOTE:** Any endogenous miRNA may interfere with the spike recovery results. We have designed a negative miRNA sequence derived from bacterial gene dapB (Affymetrix P/N SMC-10180) to perform spike recovery in various animal and plant lysates. We have also demonstrated the spike recovery of human miR-93 in Corn Husk lysate. See data below. Spike recovery for dapB miRNA from Corn Husk lysate:  Spike recovery for dapB miRNA from Stomach Tissue lysate: 

Step	Action
	Spike recovery for miR-93 miRNA from Corn Husk lysate: 

QuantiGene 2.0 miRNA Assay Procedure

Assay Workflow

Table 3.1

Step	Tasks
1	Prepare samples[a]
2	Capture miRNA ▪ Dilute samples ▪ Prepare Working Probes Sets ▪ Dispense Working Probe Sets, samples, and controls into Capture Plate ▪ Hybridize overnight
3	Amplify and detect signal ▪ Wash away unbound material ▪ Sequentially hybridize 2.0 PreAmp, Amp, and Label Probe ▪ Add 2.0 Substrate, incubate, and read signal

[a] *For a procedure refer to Appropriate QuantiGene Sample Processing Kit for preparing cultured cell lysate, blood lysates and tissue homogenates. Follow standard laboratory methods for purification of RNA. Use samples immediately, or store at –80 °C until ready to use.*

Capturing Target miRNA

Refer to the appropriate procedure for your sample type:

- Cultured cell or blood lysates
- Fresh, frozen or FFPE tissue homogenates
- Total RNA or purified miRNA

Capturing Target miRNA from Cultured Cell or Whole Blood/ PAXgene Blood

To capture target miRNA from cultured cell or blood lysates:

Step	Action
1	Prepare reagents: ▪ *Probe Set(s) and Blocking Reagent*. Thaw, vortex briefly to mix, then briefly centrifuge to collect contents at the bottom of the tubes. Keep on ice until use. ▪ *Cultured cell or whole blood/PAXgene blood lysate(s)*. If previously frozen, thaw at room temperature followed by incubation at 37 °C for 15 – 30 minutes. Vortex briefly, then leave at room temperature until use. ▪ *Lysis Mixture*. Re-dissolve any precipitates by incubating at 37 °C for 30 minutes followed by gentle swirling. ▪ Remove Capture Plate from 4 °C and place on the benchtop to warm completely to room temperature (approximately 30 minutes). Do not remove the plate from the sealed foil pouch.

To capture target miRNA from cultured cell or blood lysates:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>2</td><td>Determine sample input.

Estimate sample input based on this chart and the LOD of miRNA target-specific probe set from the package insert.

<table>
<tr><th colspan="3">Recommended Sample Input
(based on LOD = 3,000 Copies of miRNA Probe Set)</th></tr>
<tr><th>miRNA (copies per cell)</th><th>Cultured Cells
(number of cells)</th><th>Whole Blood/PAXgene
Blood Lysate (µL)</th></tr>
<tr><td>< 10</td><td>6,000</td><td>80[a]</td></tr>
<tr><td>100</td><td>600</td><td>80</td></tr>
<tr><td>> 1,000</td><td>60</td><td>8</td></tr>
</table>

[a] May not have sensitivity required.

If appropriate, based on the expression level of target miRNA of interest, dilute sample with Diluted Lysis Mixture (1 volume of Lysis Mixture plus 2 volumes of nuclease-free water, prepared fresh) so that the final desired amount of sample present in a volume of 80 µl/assay well.</td></tr>
<tr><td>3</td><td>Prepare Working Probe Set:

Prepare an appropriate volume of Working Probe Set by combining the following reagents in the order listed. Scale according to the number of assays to be run.

NOTE: Include 3 wells for assay background controls.

<table>
<tr><th colspan="4">Preparation of Working Probe Sets</th></tr>
<tr><th>Reagent</th><th>1 Well (µL)</th><th>48 Wells[a] (µL)</th><th>96 Wells[a] (µL)</th></tr>
<tr><td>Nuclease-free Water</td><td>11.7</td><td>786.0</td><td>1,573.0</td></tr>
<tr><td>Lysis Mixture</td><td>6.7</td><td>447</td><td>893.0</td></tr>
<tr><td>Blocking Reagent</td><td>1.0</td><td>67.0</td><td>134.0</td></tr>
<tr><td>CE</td><td>0.3</td><td>20.1</td><td>40.2</td></tr>
<tr><td>LE</td><td>0.3</td><td>20.1</td><td>40.2</td></tr>
<tr><td>Total</td><td>20.0</td><td>1,340.2</td><td>2,680.4</td></tr>
</table>

[a] Includes 40% overage</td></tr>
</table>

To capture target miRNA from cultured cell or blood lysates:

Step	Action
4	Prepare the Capture Plate: **A.** After the Capture Plate reaches room temperature (minimum of 30 minutes at room temperature), open the sealed foil pouch and remove the Capture Plate. **B.** Vortex Working Probe Set briefly to mix, then dispense into the Capture Plate. **C.** For fewer than 48 wells: Using a single channel pipette and a new tip for each transfer, dispense 20 µL of Working Probe Set into each assay well. Avoid introducing bubbles. **D.** For 48 wells or more: ▪ Using a single channel pipette, transfer Working Probe Set to a 25-mL divided reagent reservoir. **NOTE:** Do not pour or reagent shortage will occur. ▪ Using a multichannel pipette and new tips for each transfer, dispense 20 µL of Working Probe Set into each assay well. Avoid introducing bubbles. **IMPORTANT:** Capture Probe oligonucleotides are conjugated to the surface of Capture Plate wells. Do not scratch Capture Plate wells with pipette tips.
5	Add sample to the Capture plate: Using a new pipette tip for each transfer, add 80 µL of sample to each well of the Capture Plate containing Working Probe Set. Avoid introducing bubbles. Do not mix. **IMPORTANT:** Add 80 µL of Diluted Lysis Mixture (1 volume Lysis Mixture plus 2 volumes nuclease-free water) to 3 wells for the assay background controls. Run background wells for each Probe Set used.
6	Bind target miRNA: **A.** Place an adhesive Plate Seal squarely on the plate and seal tightly. **IMPORTANT:** Complete and uniform sealing of the overnight hybridization plate is essential. Use a soft rubber roller or the QuantiGene CTC Plate Sealer. Letters and numbers on the Capture Plate should be clearly defined beneath the Plate Seal. **B.** Centrifuge the Capture Plate at 240 x g for 20 seconds at room temperature to ensure the contents contact the bottom of the well. **C.** Immediately place the Capture Plate in a 46 ± 1 °C incubator to begin the overnight (16–20 hour) hybridization. **IMPORTANT:** Temperature must be 46 ± 1 °C for miRNA assay. Verify temperature using a QuantiGene Incubator Temperature Validation Kit.

Capturing Target miRNA from Fresh, Frozen or FFPE Tissue Homogenates

To capture target miRNA from fresh, frozen or FFPE tissue homogenates:

Step	Action
1	Prepare reagents: ▪ *Probe Set(s) and Blocking Reagent*. Thaw, vortex briefly to mix, then briefly centrifuge to collect contents at the bottom of the tubes. Keep on ice until use. ▪ *Tissue homogenates*. If previously frozen, thaw at room temperature followed by incubation at 37 °C for 15 – 30 minutes. Vortex briefly, then leave at room temperature until use. ▪ *Lysis Mixture*. Re-dissolve any precipitates by incubating at 37 °C for 30 minutes followed by gentle swirling. ▪ Remove Capture Plate from 4 °C and place on the benchtop to warm completely to room temperature (approximately 30 minutes). Do not remove the plate from the sealed foil pouch.

To capture target miRNA from fresh, frozen or FFPE tissue homogenates:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>2</td><td>Determine sample input.

Estimate sample input based on this chart and the LOD of miRNA target-specific probe set from the package insert.

<table>
<tr><th colspan="2">Recommended Sample Input
(based on LOD = 3,000 Copies of miRNA Probe Set)</th></tr>
<tr><th>miRNA (copies per cell)</th><th>Tissue Homogenate (µL)</th></tr>
<tr><td>< 10</td><td>40[a]</td></tr>
<tr><td>100</td><td>40</td></tr>
<tr><td>> 1,000</td><td>4</td></tr>
</table>
[a] May not have sensitivity required.

If appropriate, based on the expression level of target miRNA of interest, dilute tissue homogenates with Homogenizing Solution so that the desired amount of sample present in a volume of 40 µl/assay well.</td></tr>
<tr><td>3</td><td>Prepare Working Probe Set:

Prepare an appropriate volume of Working Probe Set by combining the following reagents in the order listed. Scale according to the number of assays to be run, and include 40% overage.

NOTE: Include 3 wells for assay background controls.

<table>
<tr><th colspan="4">Preparation of Working Probe Sets</th></tr>
<tr><th>Reagent</th><th>1 Well (µL)</th><th>48 Wells[a] (µL)</th><th>96 Wells[a] (µL)</th></tr>
<tr><td>Nuclease-free Water</td><td>25.1</td><td>1,682.0</td><td>3,363.0</td></tr>
<tr><td>Lysis Mixture</td><td>33.3</td><td>2,233.0</td><td>4,467.0</td></tr>
<tr><td>Blocking Reagent</td><td>1.0</td><td>67.0</td><td>134.0</td></tr>
<tr><td>CE</td><td>0.3</td><td>20.1</td><td>40.2</td></tr>
<tr><td>LE</td><td>0.3</td><td>20.1</td><td>40.2</td></tr>
<tr><td>Total</td><td>60.0</td><td>4,022.2</td><td>8,044.4</td></tr>
</table>
[a] Includes 40% overage</td></tr>
</table>

To capture target miRNA from fresh, frozen or FFPE tissue homogenates:

Step	Action
4	Prepare the Capture Plate: **A.** After the Capture Plate reaches room temperature (minimum of 30 minutes at room temperature), open the sealed foil pouch and remove the Capture Plate. **B.** Vortex Working Probe Set briefly to mix, then dispense into the Capture Plate. **C.** For fewer than 48 wells: Using a single channel pipette and a new tip for each transfer, dispense 60 µL of Working Probe Set into each assay well. Avoid introducing bubbles. **D.** For 48 wells or more: ▪ Using a single channel pipette, transfer Working Probe Set to a 25-mL divided reagent reservoir. **IMPORTANT:** Do not pour or reagent shortage will occur. ▪ Using a multichannel pipette and new tips for each transfer, dispense 60 µL of Working Probe Set into each assay well. Avoid introducing bubbles. **IMPORTANT:** Capture Probe oligonucleotides are conjugated to the surface of Capture Plate wells. Do not scratch Capture Plate wells with pipette tips.
5	Add sample to the Capture plate: Using a new pipette tip for each transfer, add 40 µL of sample to each well of the Capture Plate containing Working Probe Set. Avoid introducing bubbles. Do not mix. **IMPORTANT:** Add 40 µL of Homogenizing Solution to 3 wells for the assay background controls. Run background wells for each Probe Set used.
6	Bind target miRNA: **A.** Place an adhesive Plate Seal squarely on the plate and seal tightly. **IMPORTANT:** Complete and uniform sealing of the overnight hybridization plate is essential. Use a soft rubber roller or the QuantiGene CTC Plate Sealer. Letters and numbers on the Capture Plate should be clearly defined beneath the Plate Seal. **B.** Centrifuge the Capture Plate at 240 x g for 20 seconds at room temperature to ensure the contents contact the bottom of the well. **C.** Immediately place the Capture Plate in a 46 ± 1 °C incubator to begin the overnight (16–20 hour) hybridization. **IMPORTANT:** Temperature must be 46 ± 1 °C for miRNA assay. Verify temperature using a QuantiGene Incubator Temperature Validation Kit.

Capturing Target miRNA from Total RNA or Purified miRNA

To capture target miRNA from purified RNA preparations:

Step	Action
1	Prepare reagents: ▪ *Probe Set(s) and Blocking Reagent*. Thaw, vortex briefly to mix, then briefly centrifuge to collect contents at the bottom of the tubes. Keep on ice until use. ▪ *RNA sample(s)*. If previously frozen, thaw on ice. ▪ *Lysis Mixture*. Re-dissolve any precipitates by incubating at 37 °C for 30 minutes followed by gentle swirling. ▪ Remove Capture Plate from 4 °C and place on the benchtop to warm completely to room temperature (approximately 30 minutes). Do not remove the plate from the sealed foil pouch.

To capture target miRNA from purified RNA preparations:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>2</td><td>Determine sample input.

Estimate sample input based on this chart and the LOD of miRNA target-specific probe set from the package insert.

<table>
<tr><th colspan="2">Recommended Sample Input
(based on LOD = 3,000 Copies of miRNA Probe Set)</th></tr>
<tr><th>miRNA (copies per cell)</th><th>Total RNA (ng)</th></tr>
<tr><td>< 10</td><td>100[a]</td></tr>
<tr><td>100</td><td>10</td></tr>
<tr><td>> 1,000</td><td>1</td></tr>
</table>
[a] 100 ng of RNA is approximately equivalent to 5,000 cells.

Dilute RNA in nuclease-free water so that the desired amount of RNA is present in a volume of 20 µL/assay well based on expression level of target miRNA of interest.</td></tr>
<tr><td>3</td><td>Prepare Working Probe Set:

Prepare an appropriate volume of Working Probe Set by combining the following reagents in the order listed. Scale according to the number of assays to be run, and include 40% overage.

IMPORTANT: Include 3 wells for assay background controls.

<table>
<tr><th colspan="4">Preparation of Working Probe Sets</th></tr>
<tr><th>Reagent</th><th>1 Well (µL)</th><th>48 Wells[a] (µL)</th><th>96 Wells[a] (µL)</th></tr>
<tr><td>Nuclease-free Water</td><td>45.1</td><td>3,022.0</td><td>6,043.0</td></tr>
<tr><td>Lysis Mixture</td><td>33.3</td><td>2,233.0</td><td>4,467.0</td></tr>
<tr><td>Blocking Reagent</td><td>1.0</td><td>67.0</td><td>134.0</td></tr>
<tr><td>CE</td><td>0.3</td><td>20.1</td><td>40.2</td></tr>
<tr><td>LE</td><td>0.3</td><td>20.1</td><td>40.2</td></tr>
<tr><td>Total</td><td>80.0</td><td>5362.2</td><td>10,724.4</td></tr>
</table>
[a] Includes 40% overage</td></tr>
</table>

To capture target miRNA from purified RNA preparations:

Step	Action
4	Prepare the Capture Plate: **A.** After the Capture Plate reaches room temperature (minimum of 30 minutes at room temperature), open the sealed foil pouch and remove the Capture Plate. **B.** Vortex Working Probe Set briefly to mix, then dispense into the Capture Plate. **C.** For fewer than 48 wells: Using a single channel pipette and a new tip for each transfer, dispense 80 µL of Working Probe Set into each assay well. Avoid introducing bubbles. **D.** For 48 wells or more: ▪ Using a single channel pipette, transfer Working Probe Set to a reagent reservoir. **IMPORTANT:** Do not pour or reagent shortage will occur. ▪ Using a multichannel pipette and new tips for each transfer, dispense 80 µL of Working Probe Set into each assay well. Avoid introducing bubbles. **IMPORTANT:** Capture Probe oligonucleotides are conjugated to the surface of Capture Plate wells. Do not scratch Capture Plate wells with pipette tips.
5	Add sample to the Capture plate: Using a new pipette tip for each transfer, add 20 µL of sample to each well of the Capture Plate containing Working Probe Set. Avoid introducing bubbles. Do not mix. **IMPORTANT:** Add 20 µL of nuclease-free water to 3 wells for the assay background controls. Run background wells for each Probe Set used.
6	Bind target miRNA: **A.** Place an adhesive Plate Seal squarely on the plate and seal tightly. **IMPORTANT:** Complete and uniform sealing of the overnight hybridization plate is essential. Use a soft rubber roller or the QuantiGene CTC Plate Sealer. Letters and numbers on the Capture Plate should be clearly defined beneath the Plate Seal. **B.** Centrifuge the Capture Plate at 240 x g for 20 seconds at room temperature to ensure the contents contact the bottom of the well. **C.** Immediately place the Capture Plate in a 46 ± 1 °C incubator to begin the overnight (16–20 hour) hybridization. **IMPORTANT:** Temperature must be 46 ± 1 °C for miRNA assay. Verify temperature using a QuantiGene Incubator Temperature Validation Kit.

Signal Amplification and Detection

These instructions are for processing a single Capture Plate using multichannel pipettes and reagent reservoirs. To process more than one Capture Plate, scale reagents accordingly. If using a 50-plate kit, scale reagent preparations for a minimum of 10 plates per run, or reagent shortages will occur.

IMPORTANT: Do not let the Capture Plate(s) stand dry for more than 5 minutes at any point in this procedure.

IMPORTANT: Incubation temperatures must be 46 ± 1 °C. Verify temperatures using a QuantiGene Incubator Temperature Validation Kit.

IMPORTANT: If using a recommended plate washer, centrifugation of the Capture Plate after washing steps is not necessary.

Preparing Wash Buffer

To prepare the wash buffer:

Step	Action
1	Add to a 500-mL graduated cylinder, in this order: **A.** 400 mL double-distilled water (ddH_2O) **B.** 1.5 mL Wash Comp 1 **C.** 2.5 mL Wash Comp 2 **D.** Bring volume to 500 mL with ddH_2O. **IMPORTANT:** Scale preparation according to the number of plates to be processed. 500 mL is sufficient for processing one Capture Plate.
2	Transfer to a 500-mL bottle and invert to mix. Do not store unused Wash Buffer. Make Wash Buffer fresh daily

Hybridizing the 2.0 PreAmplifier

To hybridize the 2.0 PreAmplifier:

Step	Action
1	Prepare PreAmp Working Reagent: **A.** Thaw 2.0 PreAmp, then centrifuge briefly to collect the contents at the bottom of the tube. **B.** Add 11 µL of 2.0 PreAmp to 11 mL of Amplifier/Label Probe Diluent. **C.** Invert to mix. **D.** Keep at room temperature until use.
2	Wash the Capture Plate: **A.** Remove the Capture Plate from the incubator and remove the Plate Seal. **B.** Add 200 µL/well of Wash Buffer. **C.** Invert the Capture Plate over an appropriate receptacle (for example, a BioHazard container) and expel the contents forcibly. **D.** Firmly tap the inverted plate on a clean paper towel to dry. **E.** Repeat steps 2B – 2D two more times using 300 µL/well of Wash Buffer. **IMPORTANT:** For recommendations on automated plate washing, see *Alternative Plate Washing Method* on page 31.
3	Remove all traces of Wash Buffer: **A.** Place inverted plate with a dry paper towel into the centrifuge and centrifuge at 240 x g for 1 minute at room temperature. Use maximum acceleration and brake settings available. **IMPORTANT:** Do not exceed 240 x g for 1 minute. **B.** Proceed to the next step immediately.
4	Add 100 µL of 2.0 PreAmp Working Reagent to each well of the Capture Plate.
5	Seal the Capture Plate with a Plate Seal and incubate at 46 ± 1°C for 60 minutes

Hybridizing the 2.0 Amplifier

To hybridize the 2.0 Amplifier:

Step	Action
1	Prepare 2.0 Amp Working Reagent: **A.** Thaw 2.0 Amp, then centrifuge briefly to collect the contents at the bottom of the tube. **B.** Add 11 µL of 2.0 Amp to 11 mL of Amplifier/Label Probe Diluent. **C.** Invert to mix. **D.** Keep at room temperature until use.
2	Wash the Capture Plate: **A.** Remove the Capture Plate from the incubator and remove the Plate Seal. **B.** Add 200 µL/well of Wash Buffer. **C.** Invert the Capture Plate over an appropriate receptacle (for example, a BioHazard container) and expel the contents forcibly. **D.** Firmly tap the inverted plate on a clean paper towel to dry. **E.** Repeat steps 2B – 2D two more times using 300 µL/well of Wash Buffer.
3	Remove all traces of Wash Buffer: **A.** Place inverted plate with a dry paper towel into the centrifuge and centrifuge at 240 x g for 1 minute at room temperature. Use maximum acceleration and brake settings available. **IMPORTANT:** Do not exceed 240 x g for 1 minute. **B.** Proceed to the next step immediately.
4	Add 100 µL of 2.0 Amp Working Reagent to each well of the Capture Plate.
5	Seal the Capture Plate with a Plate Seal and incubate at 46 ± 1 °C for 60 minutes.

Hybridizing the Label Probe

To hybridize the Label Probe:

Step	Action
1	Prepare Label Probe Working Reagent: **A.** Centrifuge Label Probe briefly to collect the contents to the bottom of the tube. **B.** Add 11 µL of Label Probe to 11 mL of Amplifier/Label Probe Diluent. **C.** Invert to mix. **D.** Keep at room temperature until use.
2	Wash the Capture Plate: **A.** Remove the Capture Plate from the incubator and remove the Plate Seal. **B.** Add 200 µL/well of Wash Buffer. **C.** Invert the Capture Plate over an appropriate receptacle (for example, a BioHazard container) and expel the contents forcibly. **D.** Firmly tap the inverted plate on a clean paper towel to dry. **E.** Repeat steps 2B – 2D two more times using 300 µL/well of Wash Buffer.
3	Remove all traces of Wash Buffer: **A.** Place inverted plate with a dry paper towel into the centrifuge and centrifuge at 240 x g for 1 minute at room temperature. Use maximum acceleration and brake settings available. **IMPORTANT:** Do not exceed 240 x g for 1 minute. **B.** Proceed to the next step immediately.
4	Add 100 µL of Label Probe Working Reagent to each well of the Capture Plate.

To hybridize the Label Probe:

Step	Action
5	Seal the Capture Plate with a Plate Seal and incubate at 46 ± 1 °C for 60 minutes. **IMPORTANT:** During this incubation, remove 2.0 Substrate from 4 °C and allow it to warm to room temperature.

Adding the 2.0 Substrate and Detecting Signal

To add the 2.0 substrate and detecting signal:

Step	Action
1	Wash the Capture Plate: **A.** Remove the Capture Plate from the incubator and remove the Plate Seal. **B.** Add 200 µL/well of Wash Buffer. **C.** Invert the Capture Plate over an appropriate receptacle (for example, a BioHazard container) and expel the contents forcibly. **D.** Firmly tap the inverted plate on a clean paper towel to dry. **E.** Repeat steps 2B – 2D two more times using 300 µL/well of Wash Buffer.
2	Remove all traces of Wash Buffer: **A.** Place inverted plate with a dry paper towel into the centrifuge and centrifuge at 240 x g for 1 minute at room temperature. Use maximum acceleration and brake settings available. **IMPORTANT:** Do not exceed 240 x g for 1 minute. **IMPORTANT:** Ensure that 2.0 Substrate is at room temperature before use and that the luminometer is ready for use. **B.** Proceed to the next step immediately.
3	Add 100 µL of 2.0 Substrate to each well of the Capture Plate.
4	Seal the Capture Plate with a Plate Seal and incubate at room temperature for 5 minutes.
5	Remove the Plate Seal, place the Capture Plate in the luminometer, and read. Set integration (read) time to 0.2 seconds. For best results, read plate within 15 minutes. **NOTE:** Depending upon luminometer used, some adjustments in integration time may be required to obtain better signal to background ratio and linearity.

Troubleshooting

Low Assay Signal or Poor Sensitivity

Table 4.1 Troubleshooting Low Assay Signal or Poor Sensitivity

Probable Cause	Recommended Action
Number of target miRNA molecules below limit of detection	Increase the sample input. Verify complete cell lysis (see *Optimizing Sample Input for QuantiGene miRNA Assay* on page 9).
Signal amplification reagent incorrectly prepared	Dilute 2.0 PreAmp, 2.0 Amp, and Label Probe in Amplifier/Label Probe diluent.
Inappropriate hybridization temperature	Hybridization reactions must be carried out at 46 ± 1 °C as directed in protocol. Use a QuantiGene Incubator Temperature Validation Kit to verify and monitor the temperature.
Inactivation of alkaline phosphatase	Do not exceed 50 °C after the addition of Label Probe. Do not allow the Capture Plate to stand dry for more than 5 minutes once the signal amplification and detection procedure has started.
Expired reagents were used	Reagents are good for up to 6 months from date of receipt.
Luminometer does not have the required sensitivity	Only use luminometers that meet or exceed the minimum performance specifications (see *Optimizing Sample Input for QuantiGene miRNA Assay* on page 9).

Non-Uniform Signal Across the Plate

Table 4.2 Troubleshooting Non-Uniform Signal Across the Plate

Probable Cause	Recommended Action
Temperature gradients within the incubator	Verify that the incubator maintains a constant, even temperature throughout the incubator. Avoid opening and closing the incubator door during hybridization steps.
Temperature gradients on Capture Plate at time of reading	Read plate at room temperature. If luminometer has heating capability, ensure that this function is turned off and indicates room temperature.
Incomplete sealing during overnight hybridization	Use the CTC Plate Sealer for robust plate sealing (Affymetrix P/N QG0400). Ensure numbers and letters are clearly visible from under the foil seal. Verify that the supplied plate seal was used.
Capture Plates exposed to moisture prior to the assay	Allow the Capture Plate to come to room temperature for 30 minutes before opening the sealed foil pouch to avoid condensation.
Variable salt concentrations	Hybridization is affected by salt. When diluting samples, always use the appropriate diluent.

High Background Signal

Table 4.3 Troubleshooting High Background Signal

Probable Cause	Recommended Action
Residual Wash Buffer	Ensure that the plate wash method completely removes all residual Wash Buffer prior to moving to the next step in the procedure.
Incorrect temperature in the incubator	Verify incubation temperatures using a QuantiGene Incubator Temperature Validation Kit.
Expired reagents were used	Reagents are good for 6 months from the date of receipt.
Capture Plate sat at room temperature longer than 10 minutes after the addition of sample	Do not let the Capture Plate sit at room temperature for longer than 10 minutes after the addition of the overnight hybridization mixture.
Capture Plate sat at room temperature for longer than 10 minutes before washing (2nd day)	Wash the Capture Plate within 10 minutes after removal from the incubator.
Cross-talk between wells	Only use Luminometers with cross-talk < 0.001%. Reduce integration time on Luminometer to minimize cross-talk. Move high-expressing samples away from background wells.

Well-To-Well Variation

Table 4.4 Troubleshooting Assay CVs

Probable Cause	Recommended Action
Residual Wash Buffer	Ensure that the plate wash method completely removes all residual Wash Buffer prior to moving to the next step in the procedure.
Scratching of the capture well surface	Minimize contact with the Capture Plate well surfaces during all addition and washing steps.
Cross-talk among neighboring wells during reading	Only use luminometers with cross-talk < 0.001%.
Variable salt concentrations	Hybridization is affected by salt. When diluting samples, always use the appropriate diluent.
Inaccurate pipetting	▪ Only use calibrated, precision pipettes ▪ Affix tips securely ▪ Use a new tip for each transfer ▪ Pipet slowly and carefully, avoiding bubbles
Non-homogenous samples	Warm samples to 37 °C to dissolve any precipitates and vortex briefly before use.
Samples too viscous to pipet accurately	Dilute samples 1 – 2 in the appropriate diluent before use.

Day-To-Day Variation

Table 4.5 Troubleshooting High Inter-Plate CVs

Probable Cause	Recommended Action
Variable incubation temperatures	Keep incubation temperatures consistent.
Variable incubation times	Keep incubation times consistent, especially for incubation with 2.0 Substrate.
Non-constant time between addition of 2.0 Substrate and plate read	Make sure that time between addition of 2.0 Substrate and plate read is consistent.

Alternative Plate Washing Method

Automated Washing Procedure

NOTE: Automated washing of plates might require the purchase of additional Wash Buffer.

Program the BIO-TEK ELx405R washer with settings for the dispense program D3 and the wash programs 44 and 45. Link the dispense program D3 to the wash programs 44 and 45 to yield Link 1 and 2, respectively. Use Link 1 to wash the Capture Plates after the overnight hybridization of the sample with the target-specific Probe Set, after the 2.0 Pre-Amplifier hybridization and the 2.0 Amplifier hybridization. Use Link 2 to wash the Capture Plates after the Label Probe hybridization.

Table A.1 ELx405R Washer Settings

Parameter	Program		
	D3	44	45
Method			
Number of cycles		3	5
Soak/Shake		Yes	Yes
Soak duration		10 seconds	10 seconds
Shake before soak		No	No
Prime after soak		No	No
Prime volume			
Prime flow rate			
Dispense			
Dispense volume	290	395	395
Dispense flow rate	5	5	5
Dispense height	115	115	115
Horizontal dispense position	10	10	10
Horizontal Y dispense position	0	0	0
Bottom wash first	No	No	No
Bottom dispense volume			
Bottom flow rate			
Bottom dispense height			
Bottom dispense position			
Prime	No	No	No
Prime volume			
Prime flow rate			

Table A.1 ELx405R Washer Settings

Parameter	Program		
	D3	44	45
Aspiration			
Aspirate height		32	32
Horizontal aspirate position		-45	-45
Horizontal Y aspirate position		0	0
Aspirate rate		5	5
Aspirate delay		0	0
Crosswise aspirate		No	No
Crosswise aspirate on			
Crosswise height			
Crosswise horizontal position			
Final aspirate		Yes	Yes
Final aspirate delay		2 seconds	2 seconds

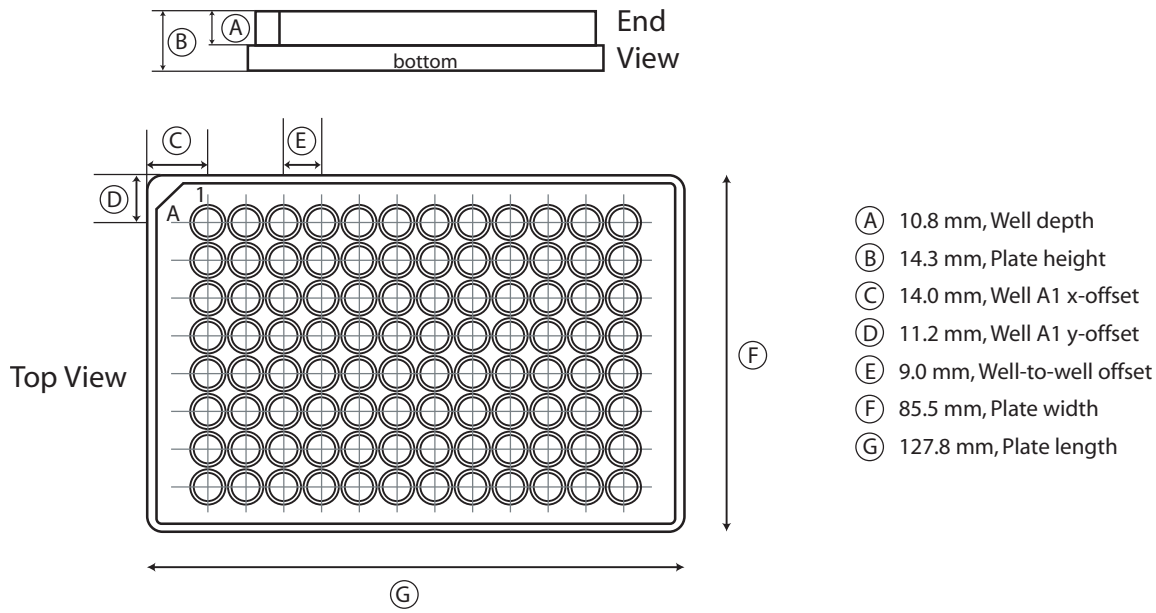
Capture Plate Dimensions

About Capture Plate Dimensions

We provide the Capture Plate dimensions to enable you to setup and validate alternative automated plate washers.

NOTE: The Capture Plate construction adheres to the Society for Biomolecular screening standards.

Figure B.1



Blank Plate Map