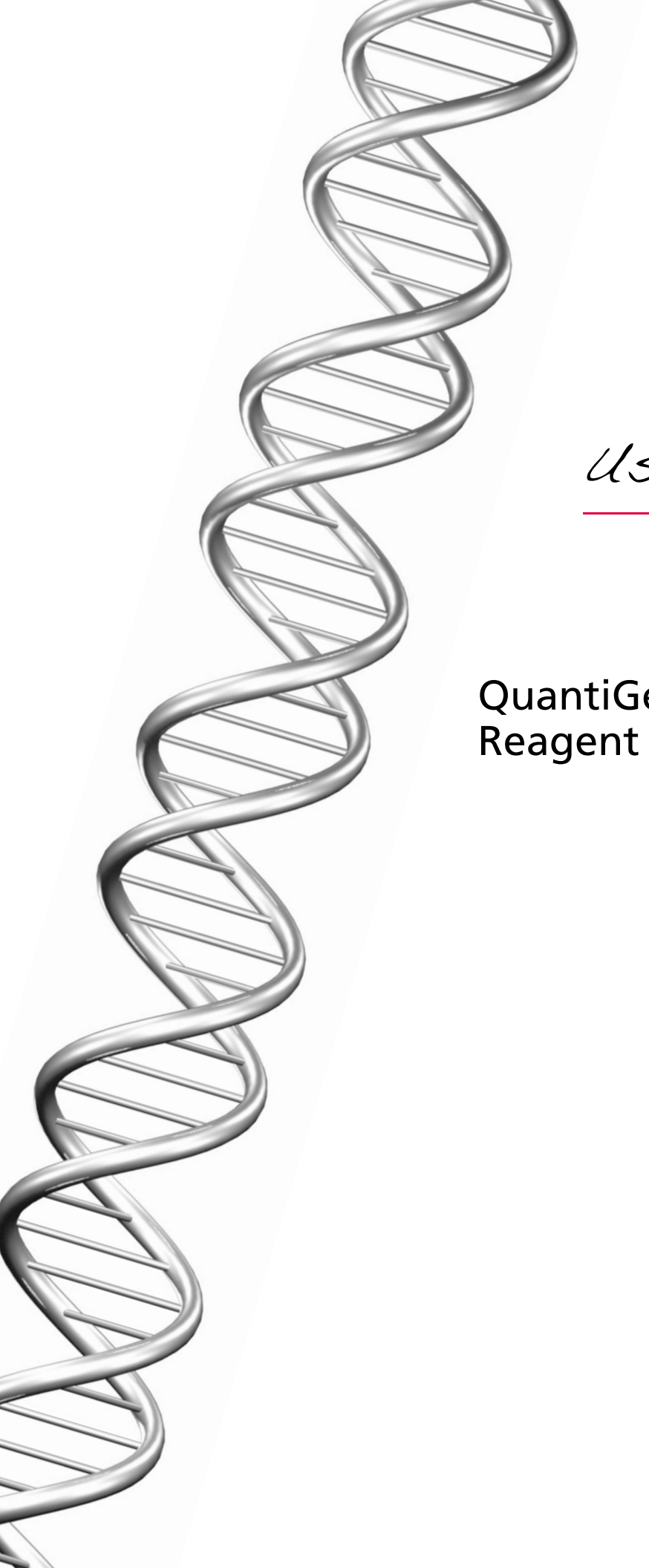

Affymetrix®

User Manual

QuantiGene® 2.0 Reagent System

P/N 13074 Rev.C 100111

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When describing a procedure for publication using this product, please refer to it as the QuantiGene 2.0 Reagent System.

If a paper cites a QuantiGene product and is published in a research journal, the lead author(s) may receive a travel stipend for use at a technology conference or tradeshow by sending a copy of the paper to our technical support group at pqbhelp@affymetrix.com or via fax at (510) 818-2610.

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Contents

Chapter 1 Introduction 1

About This Manual 1
Who Should Read this Manual 1
What this Manual Covers 1
Safety Warnings and Precautions 1
Contacting Affymetrix 1
Technical Help 1
QuantiGene QuantiGene 2.0 Reagent System Basics 1
How it Works 2
Required Materials 3
QuantiGene QuantiGene 2.0 Reagent System Kit Components 3
QuantiGene 2.0 Reagent System Accessory Reagents 3
Required Materials Not Provided 4

Chapter 2 Experimental Design and Assay Optimization 5

Overview 5
Optimizing Sample Input 5
Replicates 5
Recommended Assay Controls 5
Assay Background Control 5
Use of Housekeeping Genes 5
Data Analysis Guidelines 6
Calculating Assay Precision 6
Calculating Assay Limit of Detection 6
Determining Assay Linearity 6
Normalizing Gene Expression Data 6
Calculating Fold-Change of Gene Expression 6

Chapter 3 QuantiGene 2.0 Assay Procedure 7

Assay Workflow 7
Capturing Target RNA 7
About Capturing Target RNA 7
Capturing Target RNA from Cultured Cell or Blood Lysates 7
Capturing Target RNA from Fresh, Frozen, or FFPE Tissue Homogenates 9
Capturing Target RNA from Total RNA, Purified mRNA, or In Vitro Transcribed RNA 11
Signal Amplification and Detection 13
About Signal Amplification and Detection 13
Preparing 1X Wash Buffer 14
Hybridizing the 2.0 PreAmplifier 14
Hybridizing the 2.0 Amplifier 14

Hybridizing the Label Probe 15
Adding Substrate and Detecting Signal 16

Chapter 4 Troubleshooting 17
Low Assay Signal or Poor Sensitivity 17
Non-Uniform Signal Across the Plate 17
High Background Signal 18
Well-To-Well Variation 18
Day-To-Day Variation 19

Appendix A Alternative Capture Plate Washing Method 21
Automated Washing Procedure 21

Appendix B Capture Plate Dimensions 23
About Capture Plate Dimensions 23

Appendix C Blank Plate Map 25

Introduction

About This Manual

Who Should Read this Manual

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

- Cultured cells
- Whole blood
- Fresh, frozen, or formalin-fixed, paraffin-embedded (FFPE) animal tissues
- Purified RNA

What this Manual Covers

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

- Experimental design and data analysis
- QuantiGene 2.0 assay
- Troubleshooting

Safety Warnings and Precautions

All chemicals should be considered potentially hazardous. We recommend that this product and its components be handled by those trained in laboratory techniques and used according to the principles of good laboratory practice.

For research use only. Not for use in diagnosis of disease in humans or animals.

Contacting Affymetrix

Technical Help

For technical questions, please contact our technical support group by telephone at 1-877-726-6642 option 3 or email at techsupport@panomics.com (US and Canada). In Europe, contact techsupport_europe@panomics.com. For an updated list of FAQs and product support literature, visit our website at www.panomics.com

QuantiGene QuantiGene 2.0 Reagent System Basics

The QuantiGene 2.0 Reagent System is designed to quantitate target-specific RNA molecules directly from:

- Cultured cell lysates
- Whole blood, PAXgene blood RNA, or dried blood spot lysates
- Fresh or frozen animal tissue homogenates
- FFPE animal tissue homogenates
- Total RNA, mRNA, or in vitro transcribed RNA preparations

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

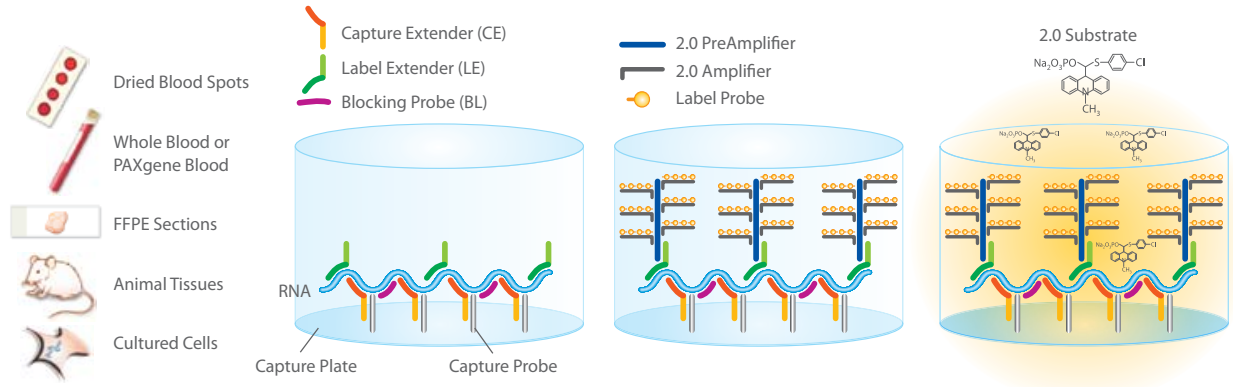
The QuantiGene 2.0 assay is a hybridization-based assay performed on 96-well plates.

The ability to quantify specific RNA molecules within a sample lies in the design of a QuantiGene 2.0 Probe Set. Each oligonucleotide probe set contains three types of synthetic probes, Capture Extenders (CEs), Label Extenders (LEs), and Blockers (BLs) that hybridize to contiguous sequences of the target RNA. The CEs bind to the capture oligonucleotides conjugated to the well surface, and via cooperative hybridization, capture the associated target RNA.

Signal amplification is mediated by DNA amplification molecules that hybridize to the tails of the LEs. Each amplification unit contains hybridization sites for 400 alkaline phosphatase conjugated Label Probes, which can then be detected by the alkaline phosphatase mediated degradation of a chemiluminescent substrate. Luminescence is reported as relative light units (RLUs) on a microplate luminometer. The amount of luminescent signal is linearly proportional to the number of RNA molecules present in the sample.

How it Works

Figure 1.1 QuantiGene QuantiGene 2.0 Reagent System Basics



Step 1: Release Target RNA	Step 2: Target RNA Capture	Step 3: Signal Amplification	Step 4: Detection
Cells are lysed to release RNA.	Probe Set design determines the specificity of the target RNA capture. Probe Set oligonucleotides (CEs, LEs, BLs) bind a contiguous region of the target RNA and the CEs (Capture Extenders), by cooperative hybridization, selectively capture target RNA to the 96-well Capture Plate during an overnight incubation.	Signal amplification is performed via sequential hybridization of 2.0 Pre-Amplifier to Probe Set LEs (Label Extenders), 2.0 Amplifier to the 2.0 Pre-Amplifier and Label Probe to the 2.0 Amplifier. The number of LEs determines assay sensitivity.	Addition of a chemilumigenic 2.0 Substrate* generates a luminescent signal that is proportional to the amount of target mRNA present in the sample. *Lumigen® APS-5

Required Materials

QuantiGene QuantiGene 2.0 Reagent System 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 product insert for quantities of individual components supplies. Kit components have a shelf life of 6 months from date of receipt and contains the following components:

Table 1.1 QuantiGene 2.0 Reagent System 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 Reagent System Accessory Reagents

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

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

Table 1.2 QuantiGene 2.0 Reagent System Accessory Components

Accessory Reagent	Description
QuantiGene Sample Processing Kit	Contains reagents and instructions for processing different sample types. Specify sample type (cultured cells, fresh or frozen animal tissue, FFPE samples or blood samples).
QuantiGene 2.0 target-specific and housekeeping Probe Sets	Each Probe Set contains CEs, LEs, and BLs.

Required Materials Not Provided

Other materials required to perform the QuantiGene QuantiGene 2.0 Reagent System that are not included in the are listed here.

Table 1.3 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, divided 25-mL capacity 100-mL capacity	VistaLab Technologies	 (P/N 3054-1004) or equivalent (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	
Nuclease-free water	MLS	
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 **IMPORTANT: 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, capable of maintaining constant temperatures of 50 and 55 °C ±1 °C	Hybaid VWR	Hybridization oven model 9270 Economy incubator model 1500E, 1500EM or equivalent
4 inch soft rubber roller or QuantiGene CTC Plate Sealer	Affymetrix Affymetrix	QS0515 QG0400
QuantiGene Incubator Temperature Validation Kit	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

Experimental Design and Assay Optimization

Overview

Here we provide information and guidelines for:

- Optimizing sample input
- Replicate recommendations
- Assay background controls
- Housekeeping genes
- Data analysis

Optimizing Sample Input

The QuantiGene 2.0 assay has a linear dynamic range of greater than 3.5 logarithms and can detect 200 copies of target RNA. When running a sample type for the first time, using a luminometer for the first time, and/or using new target-specific Probe Sets, optimize sample input to ensure that assay signals exhibit a linear dose response.

For each sample type, we provide recommendations for typical starting sample inputs depending on expression level of target RNA (see *Capturing Target RNA* on page 7). Using these recommendations as a guide, perform a 2- to 4-fold dilution series of your sample and verify that the resulting assay signals are linearly proportional to sample input. For more information, see *Determining Assay Linearity* on page 6.

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 QuantiGene 2.0 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 QuantiGene 2.0 assay.

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

Recommended Assay Controls

Assay Background Control

Assay background is the signal generated by all assay components in the absence of sample input. Include an assay background control, in triplicate, for each Probe Set used on an individual Capture Plate.

Use of Housekeeping Genes

A housekeeping gene is a target gene that is stably expressed under all experimental conditions evaluated. Signals from housekeeping genes can be used to normalize gene expression data across samples. Measure one or more housekeeping genes in triplicate for each sample.

For a list of available 2.0 Housekeeping Gene Probe Sets, please go to www.panomics.com.

Data Analysis Guidelines

Calculating Assay Precision

The Coefficient of Variation (CV) is a measure of assay precision. QuantiGene 2.0 Assay CVs are typically less than 15% for technical replicates.

To determine the assay CV:

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

Calculating Assay Limit of Detection

Calculate assay limit of detection (LOD) as follows:

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

Assay signals below LOD should not be used to draw quantitative conclusions about gene expression.

Determining Assay Linearity

To determine assay linearity:

1. Run a dilution series of your sample.
2. Subtract the AVG assay background signal from the AVG signal of technical replicates.
3. Use one of the following methods:
   - Plot background-subtracted AVG signal versus the amount of sample used. A straight line (R^2 ³ 0.95) indicates you are operating in the linear range of the assay.
   - Calculate the ratio of background-subtracted AVG RLU from sequential sample dilutions. Observed values should be within 20% of the expected ratio.

     For example, for a 2-fold sample dilution, the expected ratio of background-subtracted AVG RLU is 2 ± 20%, so the observed ratio should be between 1.6 and 2.4.

Normalizing Gene Expression Data

To normalize gene expression data:

1. For the gene of interest, subtract the AVG assay background signal from the AVG signal of technical replicates.
2. Divide the background-subtracted, AVG signals by the background-subtracted, AVG signal of the housekeeping RNA.

NOTE: If multiple housekeeping RNAs are measured, the geometric mean of background-subtracted AVG housekeeping RNA signals may be used for data normalization.

Calculating Fold-Change of Gene Expression

To calculate fold-change of gene expression of target RNA in treated versus untreated samples:

1. Normalize gene expression data as described *Normalizing Gene Expression Data* on page 6.
2. Divide the normalized value for the treated sample by the normalized value for the untreated sample.

QuantiGene 2.0 Assay Procedure

Assay Workflow

Table 3.4

Step	Tasks	For a procedure refer to...
1	Prepare samples	Appropriate QuantiGene Sample Processing Kit package insert for preparing cultured cell, whole 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.
2	Capture RNA ▪ Dilute samples ▪ Prepare Working Probes Sets ▪ Dispense Working Probe Sets, samples, and controls into Capture Plate ▪ Hybridize overnight	▪ *Capturing Target RNA from Cultured Cell or Blood Lysates* on page 7 ▪ *Capturing Target RNA from Fresh, Frozen, or FFPE Tissue Homogenates* on page 9 ▪ *Capturing Target RNA from Total RNA, Purified mRNA, or In Vitro Transcribed RNA* on page 11
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	*Signal Amplification and Detection* on page 13

Capturing Target RNA

About Capturing Target RNA

This following provides procedures for capturing target RNA, based on the following sample type:

- Cultured cell and blood lysates
- Fresh, frozen, or FFPE tissue homogenates
- Total RNA, mRNA, or in vitro transcribed RNA preparations

Refer to the appropriate procedure for your sample type.

Capturing Target RNA from Cultured Cell or Blood Lysates

To capture target RNA from cultured cell or blood lysates:

1. Prepare the following reagents:
   - Probe Set(s) and Blocking Reagent. Thaw, vortex briefly to mix, then briefly centrifuge to collect contents at the bottom of the tubes.
   - Cultured cell or whole 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 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.

2. If appropriate, based on the expression level of target or housekeeping RNA of interest, dilute samples with Dilute Lysis Mixture (1 volume of Lysis Mixture plus 2 volumes nuclease-free water, prepared fresh) so that the desired amount of sample is present in volume of 80 µL/assay well. Use the table below as a guide and scale dilutions according to the number of assays to be run.

Table 3.1 Recommended input for different preparations

Recommended Sample Input		
RNA (copies per cell)	**Cultured Cells (number of cells)**	**Whole Blood Lysate (µL)**
1	6,000	80[a]
10	600	80
100	60	8
≥ 1,000	≤ 6	≤ 0.8

[a] *May not have sensitivity required.*

3. 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. For a guide, use the table below that corresponds to the date of your Probe Set.

IMPORTANT: **Include 3 wells for assay background controls.**

Table 3.2 For QuantiGene 2.0 Probe Sets Received Before December 31, 2007

Reagent	1 Well (µL)	48 Wells[a] (µL)	96 Wells[a] (µL)
Nuclease-free water	12.1	813	1,626
Lysis Mixture	6.6	444	887
Blocking Reagent[b]	1	67	134
CEs	0.1	6.7	13.4
LEs	0.1	6.7	13.4
BLs	0.1	6.7	13.4
Total	20	1,344	2,688

[a] *Includes 40% overage.*

[b] *Omit for 18S or 28S RNA robe Sets. Substitute volume with nuclease-free water.*

Table 3.3 For QuantiGene 2.0 Probe Sets Received After January 1, 2008

Reagent	1 Well (µL)	48 Wells[a] (µL)	96 Wells[a] (µL)
Nuclease-free water	12.1	813	1,626
Lysis Mixture	6.6	444	887
Blocking Reagent[b]	1	67	134
2.0 Probe Set	0.3	20.1	40.2
Total	20	1,344	2,688

[a] *Includes 40% overage.*

[b] *Omit for 18S or 28S RNA robe Sets. Substitute volume with nuclease-free water.*

4. Prepare the Capture Plate:

   A. Open the sealed foil pouch and remove the Capture Plate.

B. Vortex Working Probe Set briefly to mix, then dispense into the Capture Plate.

For fewer than 48 wells:

Using a single channel pipette and a new tip for each transfer, dispense 20 µL Working Probe Set into each assay well. Avoid introducing bubbles.

For 48 wells or more:

A. 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.

B. 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. Using a new pipette tip for each transfer, add 80 µL sample to each well of the Capture Plate containing Working Probe Set. Avoid introducing bubbles. Do not mix.

IMPORTANT: Add 80 µL of Dilute Lysis Mixture (1 volume Lysis Mixture plus 2 volumes nuclease-free water) to 3 wells for assay background controls.

6. Bind target RNAs:

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 55 ±1 °C incubator to begin the overnight (16–20 hour) hybridization.

IMPORTANT: Temperature must be 55 ± 1 °C. Verify temperature using a QuantiGene Incubator Temperature Validation Kit.

Capturing Target RNA from Fresh, Frozen, or FFPE Tissue Homogenates

To capture target RNA from tissue homogenates

1. Prepare the following reagents:

- Probe Set(s) and Blocking Reagent. Thaw, vortex briefly to mix, then centrifuge briefly to collect contents at the bottom of the tubes.
- Tissue homogenates. If previously frozen, thaw at room temperature followed by incubation at 37 °Cfor 15–30 minutes. Vortex briefly, then leave at room temperature until use.
- Lysis Mixture. Re-dissolve any precipitates by incubating at 37 °C 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.

2. If appropriate, based on the expression level of the target or housekeeping RNA of interest, dilute tissue homogenates with Homogenizing Solution so that the desired amount of sample is present in a volume of 40 µL/assay well. Use the table below as a guide and scale dilutions according to the number of assays to be run.

Table 3.1 Recommended input for different preparations

Recommended Sample Input	
RNA (copies per cell)	**Tissue Homogenate (µL)**
1	40[a]
10	40
100	4
≥ 1,000	≤ 0.4

[a] *May not have sensitivity required.*

3. 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. For a guide, use the table below that corresponds to the date of your Probe Set.

IMPORTANT: **Include 3 wells for assay background controls.**

Table 3.2 For QuantiGene 2.0 Probe Sets Received Before December 31, 2007

Reagent	1 Well (µL)	48 Wells[a] (µL)	96 Wells[a] (µL)
Nuclease-free water	25.4	1,646	3,293
Lysis Mixture	33.3	2,238	4,476
Blocking Reagent[b]	1	67	134
CEs	0.1	6.7	13.4
LEs	0.1	6.7	13.4
BLs	0.1	6.7	13.4
Total	60	4,032	8,064

[a] *Includes 40% overage.*

[b] *Omit for 18S or 28S RNA robe Sets. Substitute volume with nuclease-free water.*

Table 3.3 For QuantiGene 2.0 Probe Sets Received After January 1, 2008

Reagent	1 Well (µL)	48 Wells[a] (µL)	96 Wells[a] (µL)
Nuclease-free water	25.4	1,646	3,293
Lysis Mixture	33.3	2,238	4,476
Blocking Reagent[b]	1	67	134
2.0 Probe Set	0.3	20.1	40.2
Total	60	4,032	8,064

[a] *Includes 40% overage.*

[b] *Omit for 18S or 28S RNA robe Sets. Substitute volume with nuclease-free water.*

4. Prepare the Capture Plate:

   A. Open the sealed foil pouch and remove the Capture Plate.

B. Vortex Working Probe Set briefly to mix, then dispense into the Capture Plate.

For fewer than 48 wells:

Using a single channel pipette and a new tip for each transfer, dispense 60 µL Working Probe Set into each assay well. Avoid introducing bubbles.

For 48 wells or more:

A. 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.

B. 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. Using a new pipette tip for each replicate, transfer 40 µL 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 assay background controls.

6. Bind target RNAs:

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 55 ±1 °C incubator to begin the overnight (16–20 hour) hybridization.

IMPORTANT: Temperature must be 55 ± 1 °C. Verify temperature using a QuantiGene Incubator Temperature Validation Kit.

Capturing Target RNA from Total RNA, Purified mRNA, or In Vitro Transcribed RNA

To capture target RNA from purified RNA preparations:

1. Prepare the following reagents:
   - Probe Set(s) and Blocking Reagent. Thaw, vortex briefly to mix, then centrifuge briefly to collect contents at the bottom of the tubes.
   - RNA sample(s). If previously frozen, thaw on ice.
   - Lysis Mixture. Re-dissolve any precipitates by incubating at 37 °C 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.

2. 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 or housekeeping RNA of interest (for total RNA or mRNA). Use the table below as a guide and scale dilutions according to the number of assays to be run.

Table 3.1 Recommended input for different preparations

Recommended Sample Input		
Target RNA (copy number/cell)	**Total RNA (ng)**	**PolyA+ RNA (pg)**
1	100	2,000
10	10	200
100	1	20
≥ 1,000	≤ 0.1	≤ 2

For in vitro transcribed (IVT) RNA, the recommended sample input is > 1000 RNA copies per well. We recommend including 200 ng/µL yeast tRNA in IVT dilutions to minimize RNA loss.

3. 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. For a guide, use the table below that corresponds to the date of your Probe Set.

! IMPORTANT: Include 3 wells for assay background controls.

Table 3.2 For QuantiGene 2.0 Probe Sets Received Before December 31, 2007

Reagent	1 Well (µL)	48 Wells[a] (µL)	96 Wells[a] (µL)
Nuclease-free water	45.4	3,051	6,102
Lysis Mixture	33.3	2,238	4,476
Blocking Reagent[b]	1	67	134
CEs	0.1	6.7	13.4
LEs	0.1	6.7	13.4
BLs	0.1	6.7	13.4
Total	80	5,376	10,752

[a] *Includes 40% overage.*
[b] *Omit for 18S or 28S RNA robe Sets. Substitute volume with nuclease-free water.*

Table 3.3 For QuantiGene 2.0 Probe Sets Received After January 1, 2008

Reagent	1 Well (µL)	48 Wells[a] (µL)	96 Wells[a] (µL)
Nuclease-free water	45.4	3,051	6,102
Lysis Mixture	33.3	2,238	4,476
Blocking Reagent[b]	1	67	134
2.0 Probe Set	0.3	20.1	40.2
Total	80	5,376	10,752

[a] *Includes 40% overage.*
[b] *Omit for 18S or 28S RNA robe Sets. Substitute volume with nuclease-free water.*

4. Prepare the Capture Plate:

A. Open the sealed foil pouch and remove the Capture Plate.

B. Vortex Working Probe Set briefly to mix, then dispense into the Capture Plate.

For fewer than 48 wells:

Using a single channel pipette and a new tip for each transfer, dispense 80 µL Working Probe Set into each assay well. Avoid introducing bubbles.

For 48 wells or more:

A. 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.

B. 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. Using a new pipette tip for each replicate, transfer 20 µL 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 assay background controls.

6. Bind target RNAs:

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 55 ±1 °C incubator to begin the overnight (16–20 hour) hybridization.

IMPORTANT: Temperature must be 55 ± 1 °C. Verify temperature using a QuantiGene Incubator Temperature Validation Kit.

Signal Amplification and Detection

About 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. If you are processing fewer than 96 wells, use the protocol for processing a partial plate, located on the Panomics website at www.panomics.com.

- Do not let the Capture Plate(s) stand dry for more than 5 minutes at any point in this procedure.
- Do not disturb the contents of the Capture Plate(s) or open the incubator door during incubation steps.
- Incubation temperatures must be 55 ± 1 °C (2.0 PreAmp and 2.0 Amp hybridization) or 50 ± 1 °C (Label Probe hybridization). Verify temperatures using a QuantiGene Incubator Temperature Validation Kit.

Preparing 1X Wash Buffer

To prepare 1X Wash Buffer:

1. Add to a 500-mL graduated cylinder, in this order:
   - 496 mL nuclease-free water
   - 1.5 mL Wash Comp 1
   - 2.5 mL Wash Com 2

   NOTE: Scale preparation according to the number of plates to be processed. One-half liter is sufficient for processing 1 Capture Plate.

2. Transfer to a 500-mL bottle and invert to mix. Do not store unused 1X Wash Buffer. Make 1X Wash Buffer fresh daily.

Hybridizing the 2.0 PreAmplifier

To hybridize the 2.0 PreAmp:

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 1X 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 1X Wash Buffer.

   NOTE: For recommendations for automated plate washing see, *Alternative Capture Plate Washing Method* on page 21.

3. Remove all traces of 1X Wash Buffer:
   A. Invert the Capture Plate on a clean, dry paper towel.
   B. 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.

   C. 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 55 ± 1 °C for 60 minutes.

Hybridizing the 2.0 Amplifier

To hybridize the 2.0 Amp:

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 1X 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 1X Wash Buffer.
3. Remove all traces of 1X Wash Buffer:
   A. Invert the Capture Plate on a clean, dry paper towel.
   B. 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.**

   C. 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 55C ± 1 °C for 60 minutes.

Hybridizing the Label Probe

To hybridize the Label Probe:

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 2.0 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.

   IMPORTANT: **If you are using a single incubator, adjust the temperature to 50 ± 1 °C. Verify the temperature using a QuantiGene Incubator Temperature Validation Kit.**

   B. Add 200 µL/well of 1X 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 1X Wash Buffer.
3. Remove all traces of 1X Wash Buffer:
   A. Invert the Capture Plate on a clean, dry paper towel.
   B. 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.**

C. Proceed to the next step immediately.

4. Add 100 µL of Label Probe Working Reagent to each well of the Capture Plate.
5. Seal the Capture Plate with a Plate Seal and incubate at 50 ± 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 Substrate and Detecting Signal

To detect signal:

1. Wash the Capture Plate:
   A. Remove the Capture Plate from the incubator and remove the Plate Seal.
   B. Add 200 µL/well of 1X 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 1b–1d two more times using 300 µL/well of 1X Wash Buffer.
2. Remove all traces of 1X Wash Buffer:
   A. Invert the Capture Plate on a clean, dry paper towel.
   B. 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.

   C. Proceed to the next step immediately.

> **NOTE:** Ensure that 2.0 Substrate is at room temperature before use.

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.

Troubleshooting

Low Assay Signal or Poor Sensitivity

Table 4.1 Troubleshooting Low Assay Signal or Poor Sensitivity

Probable Cause	Recommended Action
Number of target RNA molecules below limit of detection	Increase the sample input.
Signal amplification reagent incorrectly prepared	Dilute 2.0 PreAmp, 2.0 Amp, and Label Probe in Amplifier/Label Probe diluent.
Incorrect incubation temperature	Verify incubation temperatures using a QuantiGene Incubator Temperature Validation Kit.
Inappropriate hybridization temperature	Hybridization reactions must be carried out at 40 ±1 °C. 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 page 9).

Non-Uniform Signal Across the Plate

Table 4.2 Troubleshooting Non-Uniform Signal

Probable Cause	Recommended Action
Temperature gradients within the incubator	Verify that the incubator maintains a constant, even temperature. 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.
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 20 minutes after the addition of sample	Do not let the Capture Plate sit at room temperature for longer than 20 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.

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 Capture 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			
Aspiration			
Aspirate height		32	32

Table A.1 ELx405R Washer Settings

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

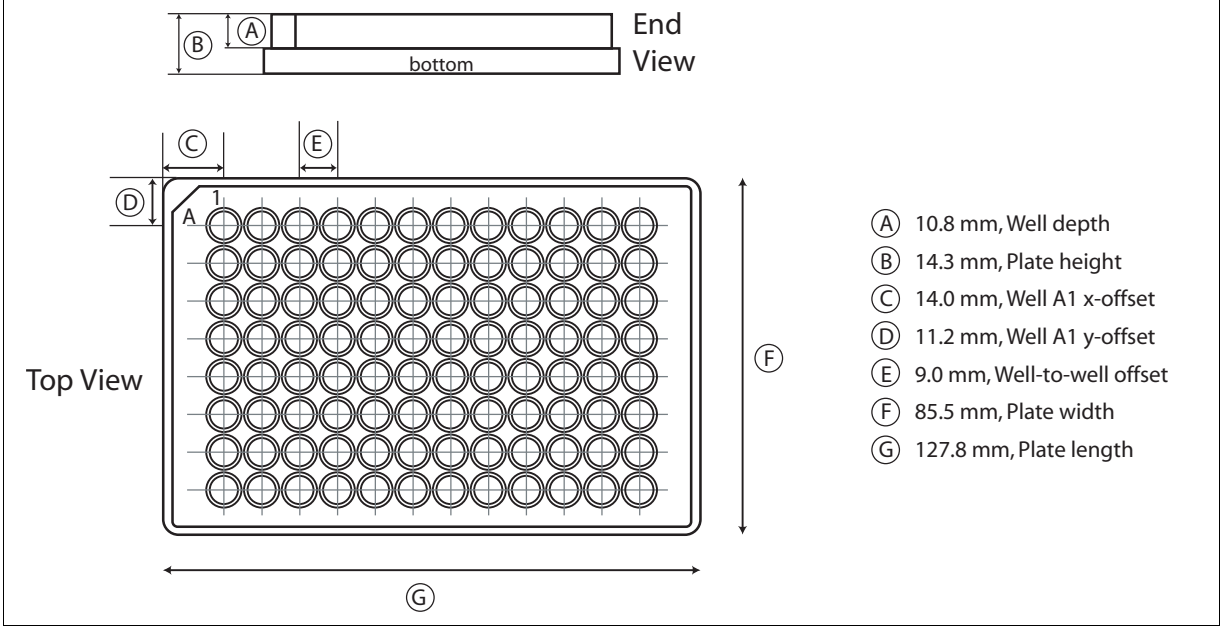
Capture Plate Dimensions

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