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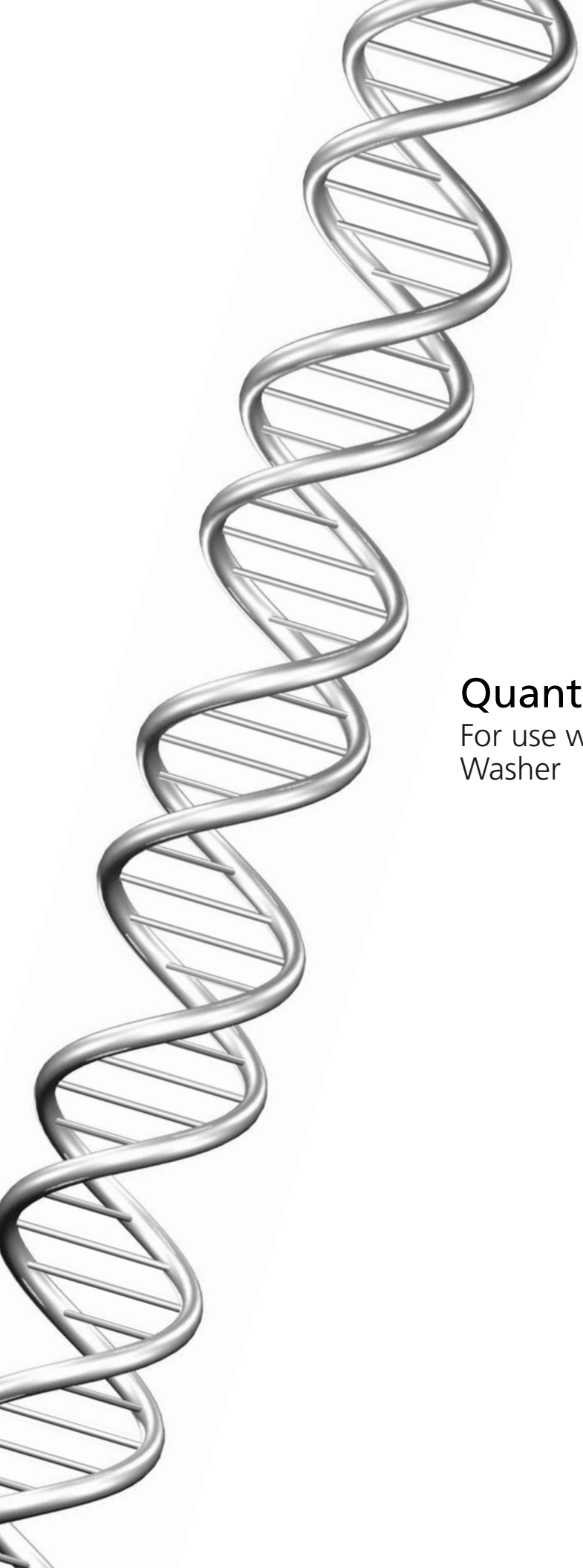

User Manual

QuantiGene Plex Assay

For use with the Hand-Held Magnetic Plate Washer

P/N 16659 Rev.D 061014

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Contents

About This User Manual

This manual is for anyone who has purchased a QuantiGene Plex Assay Kit and QuantiGene Plex Set and intends to perform the QuantiGene Plex assay for any of the following sample types:

- Cultured cells
- Blood samples (whole blood, PAXgene blood, or dried blood spots)
- Fresh, frozen, or formalin-fixed, paraffin-embedded (FFPE) tissues
- Purified RNA, *in vitro* transcripts (IVTs)

Contacting Technical Support

NOTE: For the most current version of user documentation, go to our website at **www.ebioscience.com**

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.ebioscience.com

Location	Contact Information
North America	1-877-726-6642 option 1, then option 3; pqbhelp@affymetrix.com
Europe	+43 1 796 40 40-120 tech@eBioscience.com
Asia	+81 3 6430 430 techsupport_asia@affymetrix.com

About the QuantiGene Plex Assay

The QuantiGene Plex Reagent System consists of 2-3 modules (each sold separately).

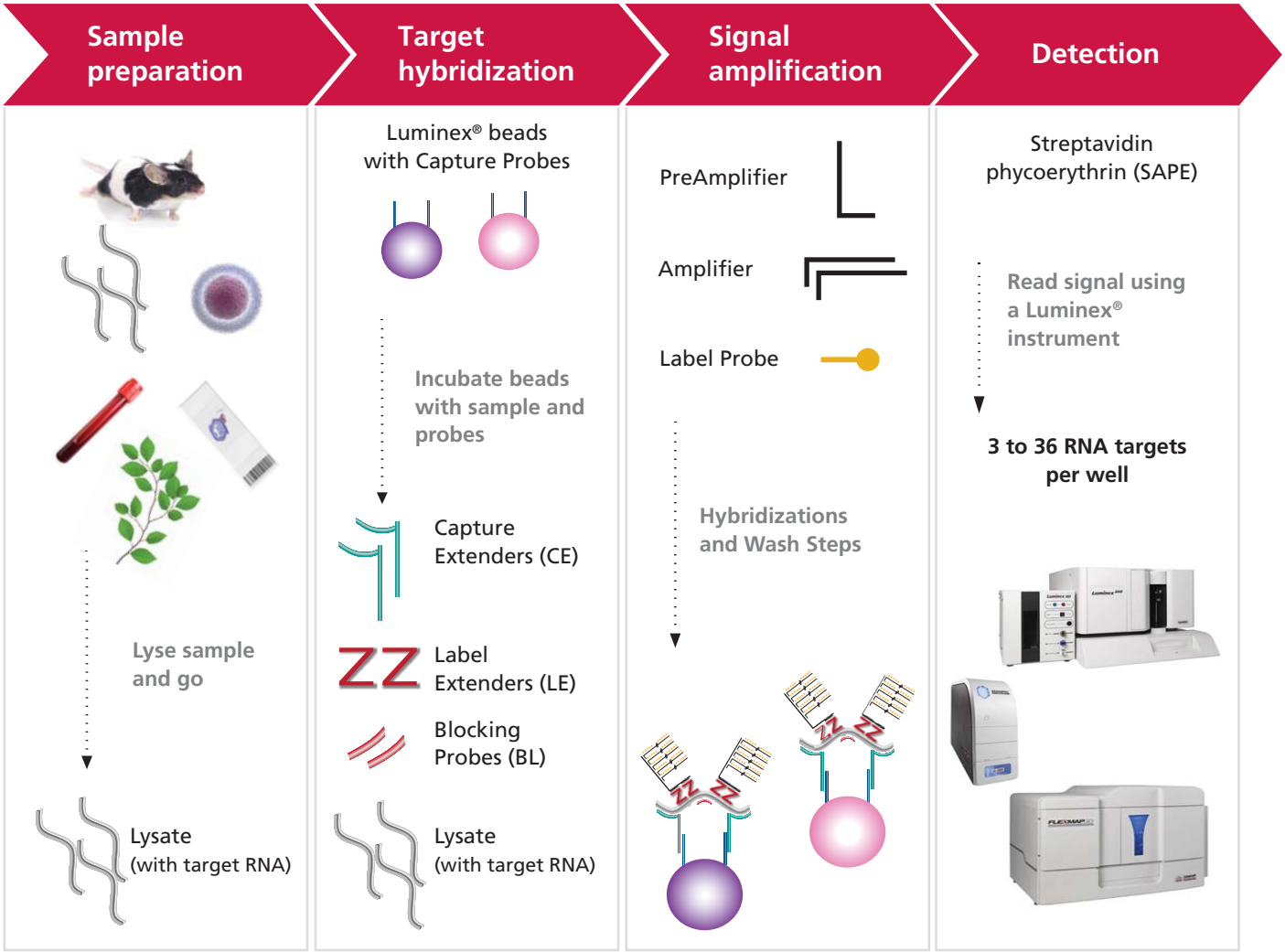
- *QuantiGene Plex Assay Kit.* Contains the generic reagents, plates and seals required for running the assay.
- *QuantiGene Plex Set*. Contains pooled Probe Set for specified targets and associated magnetic Capture Beads for running the assay.
- *QuantiGene Sample Processing Kit.* Contains two reagents and a procedure for release and stabilization of sample DNA and RNA for use in QuantiGene assays. This kit is not required if working with purified RNA samples.

How it Works

The QuantiGene Plex assay combines branched DNA (bDNA) signal amplification and multi-analyte profiling beads (xMAP®) technologies to enable the detection and quantitation of multiple RNA targets simultaneously. The bDNA assay is a hybridization-based method of target-specific RNA quantitation that amplifies signal rather than target RNA, using labeled DNA probes.

The QuantiGene Plex assay utilizes fluorescent microspheres (Capture Beads) as a support to capture specific RNA molecules. The ability to quantify multiple target-specific RNA molecules in a single sample lies in the design of the Probe Sets. For each RNA molecule of interest, an oligonucleotide Probe Set containing three types of synthetic probes, Capture Extenders (CEs), Label Extenders (LEs), and Blockers (BLs) that hybridize and span contiguous sequences of the target RNA, is provided. The CEs discriminate among the different Capture Beads within the bead array while capturing, via hybridization, the target RNA.

Signal amplification is mediated by DNA amplification molecules that hybridize to the tails of the LEs. Each amplification unit contains multiple hybridization sites for biotinylated Label Probes that bind Streptavidin-conjugated R-Phycoerythrin (SAPE). The resulting fluorescence signal associated with individual Capture Beads is read on a Luminex® flow cytometer. The system combines a flow cytometer, fluorescent-dyed microspheres (beads), lasers and digital signal processing to allow multiplexing of up to 36 unique assays within a single sample. Signal is reported as median fluorescence intensity (MFI) and is proportional to the number of target RNA molecules present in the sample.



QuantiGene Plex Assay Kit and Storage Conditions

The components of the QuantiGene Plex Assay Kit and their recommended storage conditions are listed below. The QuantiGene Plex Assay Kit is designed for use with magnetic plate washers and is available in 3 sizes. Refer to the package insert for quantities of individual components supplied. Kit components have a shelf life of 6 months from the date of receipt. Each QuantiGene Plex Assay Kit is supplied in 3 separate boxes, based on storage temperature.

QuantiGene Assay Kit: Box 1- Store at -20 °C

Component	Description	Storage
Proteinase K[1]	Proteinase K in aqueous buffered solution	–20 °C
Blocking Reagent	Aqueous buffered solution containing a preservative	–20 °C

[1] We recommend storing in an enzyme storage box, such as the NEB Cool Box (New England Biolabs P/N T0400S). NEVER store at –80 °C.

QuantiGene Assay Kit: Box 2- Store at 2-8 °C

Component	Description	Storage
Label Probe Solution	Biotinylated oligonucleotide in aqueous buffered solution	2-8 °C
Pre-Amplifier Solution	DNA in aqueous buffered solution	2-8 °C
Amplifier Solution	DNA in aqueous buffered solution	2-8 °C
SAPE	Streptavidin-conjugated R-Phycoerythrin	2-8 °C
SAPE Diluent	Dilution Buffer for SAPE reagent	2-8 °C

QuantiGene Assay Kit: Box 3- Store at Room Temperature

Component	Description	Storage
Lysis Mixture	Aqueous buffered solution containing a preservative	15-30 °C
Wash Buffer Component 1	Aqueous solution	15-30 °C
Wash Buffer Component 2	Aqueous buffered solution	15-30 °C
SAPE Wash Buffer	Aqueous buffered solution	15-30 °C
Magnetic Separation Plate(s)	96-well flat bottom microplate	15-30 °C
Plate Seals	Adhesive backed foil plate sealer	15-30 °C
Hybridization Plate(s)	96-well round bottom, clear polypropylene plate	15-30 °C
Pressure Seals	Clear, pressure-activated seal for use with the overnight Hybridization Plate	15-30 °C

QuantiGene Plex Set and Storage Conditions

The QuantiGene Plex Set includes the Probe Set and Capture Beads. Refer to the package insert for volume of individual components supplied. Each QuantiGene Plex Set is supplied in 2 separate boxes, based on storage temperature. Please note that the Capture Beads can be damaged if frozen.

QuantiGene Plex Set- Box 1- Store at - 20 °C

Component	Description	Storage
Probe Set	Premixed probesets- see package insert for gene list	–20 °C

QuantiGene Plex Set: Box 2- Store at 2-8 °C

Component	Description	Storage
Capture Beads	Premixed beads- see identifier for use with gene specific probesets	2-8 °C

Sample Type Specific Reagents

Catalog No.	Assay Specific Reagents	Size
QS0101	Cell Lysate Sample Preparation Kit[1]	2 plate
QS0102	Cell Lysate Sample Preparation Kit	10 plate
QS0103	Cell Lysate Sample Preparation Kit	5 x 10 plate
QS0104	Fresh or Frozen Tissue Sample Processing Kit[2]	10 samples
QS0105	Fresh or Frozen Tissue Sample Processing Kit	25 samples
QS0106	Fresh or Frozen Tissue Sample Processing Kit	100 samples
QS0110	Blood Sample Processing Kit[3]	2 plates
QS0111	Blood Sample Processing Kit	5 plates
QS0112	Blood Sample Processing Kit	5 x 10 Plate
QS0107	FFPE Sample Processing kit[4]	10 samples
QS0108	FFPE Sample Processing kit	25 samples
QS0109	FFPE Sample Processing kit	100 samples

[1] sufficient for preparing bulk lysates from up to approximately 1.8 x 10^7 cells or 2 x 96-well plates containing up to 6 x 10^4 cells/well.

[2] A sample is defined as 5 mg animal tissue or 15 mg plant tissue.

[3] A 2-plate kit is sufficient for preparing bulk lysates from up to approximately 1.8 x 10^7 cells or 2 x 96-well plates containing up to 6 x 10^4 cells/well.

[4] A sample is defined as 25-100 mm^2 x 50-60 micron total thickness of FFPE tissue sections.

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

Required Equipment and Materials Not Supplied

Required Equipment/Material	Source	Part Number
Hand-Held Magnetic Plate Washer	Affymetrix	QP0702
VorTemp™ 56 Shaking Incubator	Labnet International, Inc	
Microtiter Plate shaker	Labline	4625 or equivalent (must have 3 mm orbit with ability to maintain600 rpm)
Luminex 100, Luminex 200, FlexMap 3D, Magpix, Bio-Plex	Luminex Corporation	
Vortex mixer	Major laboratory supplier (MLS)	
Adjustable single and multi channel precision pipettes for dispensing 1-20 µL, 20-200 µL and 200-1000 µL	MLS	
Reagent reservoirs, 25 mL and 100 mL capacity	Vista Labs Corning Costar	3054-1002 or equivalent CLS 4873 or equivalent
Nuclease Free Water (H_2O)	MLS	
Centrifuge	MLS	ability to spin microtiter plate
Microcentrifuge	MLS	
Sample Needle Height Alignment Kit	Luminex	CN-0015-01
QuantiGene Incubator Temperature Validation Kit	Affymetrix	QS0517
4" Soft Rubber Roller	Affymetrix	QS0515

Precautions and Technical Hints

- On Day 2 of the assay, turn on and initiate startup protocol of the Luminex machine according to the manufacturer's instructions. It takes 30 min for the lasers to warm-up.
- If you are running assays on your Luminex instrument that uses both Magnetic Separation Plates (Magnetic Beads) and Filter Plates (Polystyrene Beads) verify the probe height for each plate type before reading. Failure to adjust the probe height can cause damage to the instrument.
- Use fresh pipette tips when loading samples into each well.
- Avoid creating bubbles when pipetting.
- Be careful not to invert the plate or allow contents from one well to mix to another well. The Magnetic Separation Plate is to be inverted only when removing reagents and wash buffer in washing steps
- Use a multi-channel pipette whenever possible to achieve optimal assay precision.
- When running a new sample type, optimize input by running a dilution series to ensure that all target signals are in the linear range of the assay.
- Run all assay samples with a minimum of duplicates and ideally triplicates. Technical replicates are used to calculate assay precision or %CV.
- Ensure that the temperature on the VorTemp instrument has been calibrated using the Temperature Validation Kit.
- The Vortemp 56 must be calibrated for both 54 °C & 50 °C, with an inverted 96 well plate lid in place. See instructions for temperature calibration with Temperature Validation kit

Day 1 Assay Procedure

There are three different procedures for the first day of the QuantiGene Plex assay depending upon the sample type that is used.

- Day 1 for Cell Lysates and Whole Blood Lysates
- Day 1 for Fresh, Frozen, FFPE Tissue Homogenates
- Day 1 for Purified RNA or *in vitro* transcripts (IVTs)

Please note that Day 2 of the assay is the same for all sample types.

Day 1- for Cell Lysates and Whole Blood Lysates

IMPORTANT: Cell and whole blood lysates must be prepared using a QuantiGene Sample Processing Kit for Cell Lysates or Blood Samples.

Step	Action
Step 1.	Pre-warm ***Lysis Mixture*** at 37 °C for 30 minutes followed by gentle swirling.
Step 2.	If cultured lysates have been frozen, remove from the -80 °C freezer and thaw at room temperature followed by incubation at 37 °C for 30 min. For tubes, vortex briefly, for plates, pipette up and down 5 times, then leave at room temperature until use.
Step 3.	***Probe Set & Blocking Reagent*:** Thaw and vortex briefly to mix, then centrifuge Probe Set briefly to collect contents at the bottom of the tube and keep on ice. ***Proteinase K*:** Keep on ice. ***Capture Beads:*** Take out of storage right before use and protect from light.
Step 4.	If appropriate, dilute samples with ***Diluted 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 a volume of 80 µL/assay well. See Optimizing Sample Input at the end of this manual for guidelines.
Step 5	Prepare an appropriate volume of ***Working Bead Mix*** by combining the following reagents in the order listed. Scale according to the number of assays to be run, and include sufficient overage. Use the table below as a guide. Keep Working Bead Mix at room temperature and proteced from light (Do not store on ice).

Order of addition	Reagent	1 Well (µL)	48 Wells[1] (µL)	96 Wells[1] (µL)
1	***Nuclease-free Water***	5.2	349	697
2	***Lysis Mixture***	6.6	442	884
3	***Blocking Reagent***	2	134	268
4	***Proteinase K***	0.2	13	27
5	***Capture Beads***- vortex 30 seconds prior to adding	1	67	134
6	***Probe Set***	5	335	670
Total		20	1,340	2,680

[1] Includes 40% overage to enable use of reagent reservoir and multichannel pipette.

Step	Action
Step 6.	Vortex ***Working Bead Mix*** for 10 seconds, then pipette 20 µL into each well of the ***Hybridization Plate***. **For fewer than 48 wells:** Dispense 20 µL of Working Bead Mix into each well of the ***Hybridization Plate*** using a single channel pipette. **For 48 or more wells:** Transfer ***Working Bead Mix*** to a 25-mL reagent reservoir using a single channel pipette. Do not pour or reagent shortage will occur. Using a multichannel pipette and new tips for each transfer, dispense 20 µL ***Working Bead Mix*** into each well of the ***Hybridization Plate***.
Step 7.	Add 80 µL lysate or diluted lysate to each well of the ***Hybridization Plate*** containing ***Working Bead Mix***. The total final volume in each well will be 100 µL. Load each sample using a new pipette tip. Background Controls- Add 80 µL of diluted Lysis Mixture (1 volume ***Lysis Mixture*** plus 2 volumes nuclease-free water) to 3 wells.
Step 8.	Seal the ***Hybridization Plate*** using a ***Pressure Seal***: Remove the backing of the Pressure Seal, center and place onto the Hybridization Plate. Using a soft-rubber roller, apply firm even pressure across the seal. Ensure that the plate has been completely sealed to prevent evaporation.
Step 9.	Place an inverted ***Plate Lid*** (not provided) into the VorTemp 56 shaking incubator. Then place the ***Hybridization Plate*** onto the inverted Plate Lid and Incubate for 18-22 hours at 54 °C ± 1 °C at 600 rpm. Ensure the VorTemp has been calibrated using the Temperature Validation Kit.
Step 10.	After incubation, proceed to Day 2 of the assay.

Day 1- for Fresh, Frozen or FFPE Tissue Homogenates

IMPORTANT: Tissue Homogenates must be prepared using a QuantiGene Sample Processing Kit for Fresh, Frozen or FFPE Tissue Homogenates.

Step	Action
Step 1.	Pre-warm ***Lysis Mixture*** at 37 °C for 30 minutes followed by gentle swirling.
Step 2.	If the tissue homogenates have been frozen, remove from freezer and thaw at room temperature followed by incubation at 37 °C for 30 min. Vortex the sample after incubation.
Step 3.	***Probe Set & Blocking Reagent*:** Thaw and vortex briefly to mix, then centrifuge Probe Set briefly to collect contents at the bottom of the tube and keep on ice. ***Proteinase K*:** Keep on ice. ***Capture Beads:*** Take out of storage right before use and protect from light.
Step 4.	If appropriate, dilute tissue homogenates with ***Homogenizing Solution*** so that the desired amount of sample is present in a volume of 40 µL/assay well. See Optimizing Sample Input at the end of this manual for guidelines.

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>Step 5.</td><td>Prepare an appropriate volume of Working Bead Mix by combining the following reagents in the order listed. Scale according to the number of assays to be run, and include sufficient overage. Use the table below as a guide. Keep Working Bead Mix at room temperature and protected from light (Do not store on ice).
<table>
<tr><th>Order of addition</th><th>Reagent</th><th>1 Well (µL)</th><th>48 Wells[1] (µL)</th><th>96 Wells[1] (µL)</th></tr>
<tr><td>1</td><td>Nuclease-free water</td><td>18.5</td><td>1,240</td><td>2,479</td></tr>
<tr><td>2</td><td>Lysis Mixture</td><td>33.3</td><td>2,231</td><td>4,462</td></tr>
<tr><td>3</td><td>Blocking Reagent</td><td>2</td><td>134</td><td>268</td></tr>
<tr><td>4</td><td>Proteinase K</td><td>0.2</td><td>13</td><td>27</td></tr>
<tr><td>5</td><td>Capture Beads- Vortex 30 seconds prior to adding</td><td>1</td><td>67</td><td>134</td></tr>
<tr><td>6</td><td>Probe Set</td><td>5</td><td>335</td><td>670</td></tr>
<tr><td>Total</td><td></td><td>60</td><td>4,020</td><td>8,040</td></tr>
</table>
[1] Includes 40% overage to enable use of reagent reservoir and multichannel pipettor.</td></tr>
<tr><td>Step 6.</td><td>Vortex Working Bead Mix for 10 seconds to mix, then dispense into the Hybridization Plate.

For fewer than 48 wells:
Dispense 60 µL of Working Bead Mix into each well of the Hybridization Plate using a single channel pipette.

For 48 or more wells:
Pour Working Bead Mix into a 25-mL reagent reservoir. Pipette any remaining solution into the reagent reservoir or shortage may occur. Using a multichannel pipette and new tips for each transfer, dispense 60 µL of Working Bead Mix into each well of the Hybridization Plate.</td></tr>
<tr><td>Step 7.</td><td>Add 40 µL tissue homogenate or diluted tissue homogenate to each well of the Hybridization Plate containing Working Bead Mix. Load each sample using a new pipette tip.

Background Controls- Add 40 µL of Homogenizing Solution to 3 wells for assay background.</td></tr>
<tr><td>Step 8.</td><td>Seal the Hybridization Plate using a Pressure Seal:

Remove the backing of the Pressure Seal, center and place onto the Hybridization Plate. Using a soft-rubber roller, apply firm even pressure across the seal. Ensure that the plate has been completely sealed to prevent evaporation.</td></tr>
<tr><td>Step 9.</td><td>Place an inverted Plate Lid (not provided) to the VorTemp 56 shaking incubator. Then place the Hybridization Plate onto the inverted Plate Lid and Incubate for 18-22 hours at 54 °C ± 1 °C at 600 rpm. Ensure the VorTemp has been calibrated using the Temperature Validation Kit.</td></tr>
<tr><td>Step 10.</td><td>After incubation, proceed to Day 2 of the assay.</td></tr>
</table>

Day 1- for Purified RNA or *in vitro* Transcribed RNA

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>Step 1.</td><td>Pre-warm Lysis Mixture at 37 °C for 30 minutes followed by gentle swirling.</td></tr>
<tr><td>Step 2.</td><td>Probe Set & Blocking Reagent: Thaw and vortex briefly to mix, then centrifuge Probe Set briefly to collect contents at the bottom of the tube. Keep on ice.
Capture Beads: Take out of storage right before use and protect from light.</td></tr>
<tr><td>Step 3.</td><td>Remove RNA from freezer and thaw on ice, Vortex briefly before use. If appropriate, dilute RNA using nuclease-free water so that the desired amount of RNA present is 20 µL. See Optimizing Sample Input at the end of this manual for guidelines. The typical sample input range from 50-500 ng/well.</td></tr>
<tr><td>Step 4.</td><td>Prepare an appropriate volume of Working Bead Mix by combining the following reagents in the order listed into a an appropriate sized tube. Scale according to the number of assays to be run, and include appropriate overage. Use the table below as a guide. Keep Working Bead Mix at room temperature and protected from light (Do not store on ice).
<table>
<tr><th>Order of addition</th><th>Reagent</th><th>1 Well (µL)</th><th>48 Wells[1] (µL)</th><th>96 Wells[1] (µL)</th></tr>
<tr><td>1</td><td>Nuclease-free water</td><td>38.7</td><td>2593</td><td>5186</td></tr>
<tr><td>2</td><td>Lysis Mixture</td><td>33.3</td><td>2231</td><td>4,462</td></tr>
<tr><td>3</td><td>Blocking Reagent</td><td>2</td><td>134</td><td>268</td></tr>
<tr><td>5</td><td>Capture Beads- Vortex 30 seconds prior to adding</td><td>1</td><td>67</td><td>134</td></tr>
<tr><td>6</td><td>Probe Set</td><td>5</td><td>335</td><td>670</td></tr>
<tr><td>Total</td><td></td><td>80</td><td>5,360</td><td>10,720</td></tr>
</table>
[1] Includes 40% overage to enable use of reagent reservoir and multichannel pipettor.</td></tr>
<tr><td>Step 5.</td><td>Vortex Working Bead Mix for 10 seconds to mix, then dispense into the Hybridization Plate.

For fewer than 48 wells:
Dispense 80 µL of Working Bead Mix into each well of the Hybridization Plate using a single channel pipette.

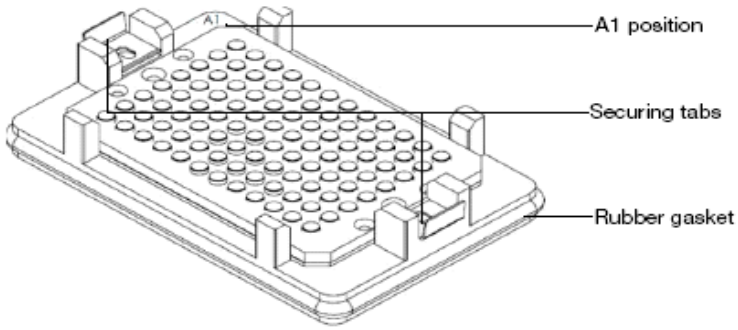
For 48 or more wells:
Pour Working Bead Mix to a 25 mL reagent reservoir. Pipette any remaining solution into the reagent reservoir or shortage may occur. Using a multichannel pipette and new tips for each transfer, dispense 80 µL of Working Bead Mix into each well of the Hybridization Plate.</td></tr>
<tr><td>Step 6.</td><td>Add 20 µL of RNA sample to each well of the Hybridization Plate containing Working Bead Mix. Load each sample using a new pipette tip.

Background Controls- Add 20 µL of Nuclease Free Water to 3 wells. For IVT RNA background controls, add 20µL of nuclease free water containing 200 ng/µL yeast tRNA.</td></tr>
<tr><td>Step 7.</td><td>Seal the Hybridization Plate using a Pressure Seal:

Remove the backing of the Pressure Seal, center and place onto the Hybridization Plate. Using a soft-rubber roller, apply firm even pressure across the seal. Ensure that the plate has been completely sealed to prevent evaporation.</td></tr>
<tr><td>Step 8.</td><td>Place an inverted Plate Lid (not provided) into the VorTemp 56 shaking incubator. Then place the Hybridization Plate onto the inverted Plate Lid and Incubate for 18-22 hours at 54 °C ± 1 °C at 600 rpm. Ensure the VorTemp has been calibrated using the Temperature Validation Kit.</td></tr>
<tr><td>Step 9.</td><td>After incubation, proceed to Day 2 of the assay.</td></tr>
</table>

Day 2 Assay Procedure

These instructions are for processing one 96 well plate using a multi-channel pipettes and reagent reservoirs. To process fewer or more wells, scale reagents accordingly. During Day 2 of the assay, turn on and calibrate the Luminex machine according to the manufacturer's instructions. It takes 30 min for the lasers to warm-up. Create a protocol prior to assay completion. See Setup of Luminex Protocol.

Step	Action
Step 1. Setup	Warm ***Pre-Amplifier Solution, Amplifier Solution, and Label Probe Solution*** at 37 °C for 30 minutes to dissolve any precipitates, and mix well by inversion before use. Leave at room temperature until ready to use (solutions are viscous). Bring ***SAPE Diluent*** to room temperature.
Step 2. Prepare Wash Buffer	Add 0.6 mL ***Wash Buffer Component 1*** and 10 mL ***Wash Buffer Component 2*** to 189 mL Nuclease Free Water. This volume is sufficient for 1 plate. Scale preparation according to the number of wells or plates to be processed.
Step 3. Adjust VorTemp temperature	Remove the ***Hybridization Plate*** from the VorTemp incubator and adjust temperature to 50 °C ± 1 °C using previously calibrated temperature setting. Restart VorTemp to ensure temperature is at 50 °C ± 1 °C.
Step 4. Transfer to Magnetic Separation Plate	Centrifuge ***Hybridization Plate*** at 240 x *g* for one minute at room temperature. Remove the plate seal and pipette up and down 5 times, then completely transfer from the ***Hybridization Plate*** to the ***Magnetic Separation Plate.*** Use a multichannel pipette, one column at a time and change tips after each transfer.
Step 5. Wash Plate	**A.** Insert the ***Magnetic Separation Plate*** into the Hand-Held Magnetic Plate Washer so that the A1 location of the 96-Well Plate matches up with the A1 Position noted on the washer. **B.** Lock the 96-Well Plate in place by pushing the 2 securing tabs, located on each end of the washer, towards the Magnetic Separation Plate until they overlap the skirt of the plate. Verify that the Magnetic Separation Plate is securely locked by holding the assembly in the palm of your hand and gently pulling up on the 96-Well Plate. **C.** Wait 1 min to allow the Magnetic Beads to accumulate on the bottom of each well. **D.** Remove the solution in the wells by quickly inverting the assembly over a sink or waste container and gently blot onto several layers of paper towels to remove any residual solution. Do not remove the Magnetic Separation Plate from the Hand-Held Magnetic Plate Washer.  **E.** Add 100 µL of 1X ***Wash Buffer*** into each well. **F.** Wait 15 sec to allow the Magnetic Beads to accumulate on the bottom of each well. **G.** Remove the Wash Buffer in the wells by quickly inverting the assembly over a sink or waste container. Repeat Actions E-G two more times for a total of three washes. After the last wash, blot the 96-Magnetic Separation Plate onto several layers of paper towels to remove any residual solution.
Step 6. Pre-Amplifier Hybridization	Transfer ***Pre-Amplifier Solution*** to a 25 mL-capacity reagent reservoir and pipette 100 µL using a multi-channel pipette into each well. Seal the ***Magnetic Separation Plate*** with a foil Plate Seal. Remove the Magnetic Separation Plate from the Hand Held Magnetic Plate Washer. Shake at 800 rpm for 1 minute at room temperature to ensure beads are resuspended. Place the ***Magnetic Separation Plate*** into the VorTemp shaking incubator, and incubate for 1 hour at 50 °C ± 1 °C with shaking at 600 rpm.

Step	Action
Step 7. Wash Plate	After 1 hour incubation, remove plate from VorTemp, remove seal, insert plate into Hand-Held Magnetic Plate Washer, repeat washing procedure from Step 6.
Step 8. Amplifier Hybridization	Transfer ***Amplifier Solution*** to a 25 mL-capacity reagent reservoir and pipette 100 µL using a multi-channel pipette into each well. Seal the ***Magnetic Separation Plate*** with a foil Plate Seal. Remove the Magnetic Separation Plate from the Hand Held Magnetic Plate Washer. Shake at 800 rpm for 1 minute at room temperature to ensure beads are resuspended. Place the ***Magnetic Separation Plate*** into the VorTemp shaking incubator, and incubate for 1 hour at 50 °C ± 1 °C with shaking at 600 rpm.
Step 9. Wash Plate	After the 1 hour incubation, remove plate from VorTemp, remove seal, insert plate into Hand-Held Magnetic Plate Washer, repeat washing procedure from Step 6.
Step 10. Hybridize Label Probe	Transfer ***Label Probe Solution*** to a 25 mL-capacity reagent reservoir and pipette 100 µL of to each assay well using a multi-channel pipette. Seal the ***Magnetic Separation Plate*** with a foil Plate Seal. Remove the Magnetic Separation Plate from the Hand Held Magnetic Plate Washer. Shake at 800 rpm for 1 minute at room temperature to ensure beads are resuspended. Place the Magnetic Separation Plate into the VorTemp and incubate for 1 hour at 50 °C ± 1 °C at 600 shaking rpm.
Step 11. Prepare SAPE Working Reagent	Briefly vortex ***SAPE*** to mix, then briefly centrifuge to collect the contents at the bottom of the tube. In a 15 mL tube, add 36 µL of ***SAPE*** to 12 mL of ***SAPE Diluent.*** Vortex for 15 seconds to mix, and protect from light.
Step 12. Wash Plate	After the 1 hour incubation, repeat the wash procedure from Step 6.
Step 13. Bind SAPE	Transfer the ***SAPE Working Reagent*** to a 25 mL-capacity reagent reservoir and pipette 100 µL into each assay well using a multi-channel pipette. Seal the ***Magnetic Separation Plate*** with a foil Plate Seal. Remove the Magnetic Separation Plate from the Hand Held Magnetic Plate Washer. Place on a shaking platform at room temperature and shake at 800 rpm for 1 minute followed by 600 rpm for 30 minutes.
Step 14. Wash Plate with SAPE Wash Buffer	Repeat the wash procedure from Step 6 but use ***SAPE Wash Buffer*** during this step.
Step 15. Prepare Plate for Analysis	Add 130 µL of SAPE Wash Buffer to each assay well. Seal the Magnetic Separation Plate with a foil Plate Seal. Remove the Magnetic Separation Plate from the Hand Held Magnetic Plate Washer and wrap the plate in aluminum foil. Place the plate on the Microtiter Plate Shaker and shaker at 800 rpm for 3 minutes at room temperature. Read plate immediately on Luminex instrument. If running more than 1 plate at a time, leave the 2nd plate at room temperature (without shaking). Once the 1st plate has been read and the instrument wash protocol has been completed, place the 2nd plate on a shaker platform at room temperature shaking at 800 rpm for 3 minutes, then read immediately. The plate can be stored at room temperature for up to 2 hours or at 4 °C for 24 hours (without shaking).

Setup of the Luminex Protocol

Please refer to the Product Insert for specific bead regions when entering the information into the Luminex acquisition software (xPonent®, Bio-Plex®, MasterPlex®, StarStation®).

The Luminex system allows for calibration of Low and High RP1 target values. We recommend RP1 Low target value settings for the QuantiGene Plex Assay.

Use the following parameters to complete protocol definition.

Sample Size	DD Gate	Timeout	Bead Event/Bead Region
100 µL	5,000 - 25,000	45 sec	100

NOTE: **If there is a malfunction of the instrument or software during the run, the 96-well plate can be reprocessed on the Luminex instrument. Remove the 96-well plate from the instrument, insert the 96-well plate back into the Hand-Held Magnetic Plate Washer, wait 1 min, then remove the solution in the wells by quickly inverting the assembly over a sink. Tap the assembly onto several layers of paper towels to remove any residual solution. Resuspend the beads in 130 µL of SAPE Wash Buffer, remove from the Hand-Held Magnetic Plate Washer, seal the plate, wrap in foil and shake at 800 rpm for 3 min at room temperature. The assayed samples may take longer to read since there will be less beads in the 96-well plate.**

Analyzing Results

An example is provided for calculating gene expression fold changes. Target signals must be in the linear range of the assay. Signals over 20,000 MFI may not be linear on a Luminex 100/200 system.

1. For each sample, determine the average signal (MFI) for all genes.

Sample Type	Normalization Gene	Test Gene 1	Test Gene 2	Test Gene 3
Background (no sample)	6.3	8	6.8	6
Untreated sample	2727	21315	117.5	20710.5
Treated sample 1	2551.5	4449.5	169.3	9260.5
Treated sample 2	2741.5	11986	133.3	5547
Treated sample 3	3020.5	10141.3	115.5	20959.8

2. For each sample, subtract the average background signal for each gene.

Sample	Normalization Gene	Test Gene 1	Test Gene 2	Test Gene 3
Background (no sample)	0	0	0	0
Untreated sample	2720.7	21307	110.7	20704.5
Treated sample 1	2545.2	4441.5	162.5	9254.5
Treated sample 2	2735.2	11978	126.5	5541
Treated sample 3	3014.2	10133.3	108.7	20953.8

3. For each sample, divide each test gene signal (background subtracted) by the normalization gene signal (background subtracted). This will correct for sample preparation, sample input and deviations between wells, plates, and experiments.

Sample	Normalization Gene	Test Gene 1	Test Gene 2	Test Gene 3
Background (no sample)	na	na	na	na
Untreated sample	1	7.83	0.04	7.61
Treated sample 1	1	1.75	0.06	3.64
Treated sample 2	1	4.37	0.05	2.03
Treated sample 3	1	3.36	0.04	6.95

4. For each test gene, calculate **Fold Change** by dividing the normalized value for the treated samples by the normalized value for the untreated sample.

Sample	Normalization Gene	Test Gene 1	Test Gene 2	Test Gene 3
Background (no sample)	na	na	na	na
Untreated sample	1	1	1	1
Treated sample 1	1	0.22	1.57	0.48
Treated sample 2	1	0.56	1.14	0.27
Treated sample 3	1	0.43	0.89	0.91

Assay Terminology

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 Plex 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 Plex assay.

Assay Precision

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

To determine the assay CV:

1. Run technical replicates of each sample.
2. Calculate the average background-subtracted signal (AVG) of technical replicates for each target RNA.
3. Calculate the standard deviation (SD) of signals from technical replicates for each target RNA.
4. Calculate the %CV.

   %CV = (SD/AVG)*100.

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 RNA:

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

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

Limit of Quantification (LOQ)

LOQ is the lowest MFI that exhibits acceptable accuracy of fold change. Quantifiable signals are those signals within the assay's linear range.

Assay Linearity/Accuracy of Fold Change

Assay linearity is defined as all dilutions that exhibit an accuracy of fold change between 80 and 120%.

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 for each target RNA.
3. Calculate the ratio of background-subtracted AVG MFI from sequential sample dilutions for each target RNA. Observed values should be within 20% of the expected ratio of 100% (80%-120%).

3-fold serial dilution of the cell lysate (µL)	Signal (background subtracted) (MFI)	Observed fold change	Expected fold change	% Obs/Exp
60	3100	3.10	3	103
20	1000	2.70	3	90
6.6	370			

Troubleshooting

Observation	Probable Cause	Recommend Solution
Low Assay Signal or Poor Sensitivity	Number of RNA transcripts below limit of detection	Increase the sample input.
	Incomplete cell lysis	Refer to the appropriate sample processing kit product inserts for detailed procedures.
	Expired reagents were used	Reagents are good for 6 months from date of receipt.
	Sub-optimal assay conditions	Follow the recommended incubation times and temperature. Shake the Magnetic Separation Plate during all incubations.
	Photobleaching of SAPE	Protect SAPE from light throughout the procedure.
	Incorrect wash buffer was used	Use SAPE Wash Buffer to wash away unbound SAPE.
	Significant RNA degradation	Refer to the appropriate sample processing kit package inserts for detailed procedures and troubleshooting.
High Background Signal	Sub-optimal assay conditions	Follow the recommended incubation times and temperature. Shake the Magnetic Separation Plate during all incubations.
	Poor washing	Set up the magnetic washer with 5-10 µL of residual volume for each wash step. Verify washing program on the magnetic washer.
Low Assay Precision (High CV)	Inaccurate pipetting	▪ Use only calibrated, precision pipettes ▪ Affix tips securely ▪ Use a new tip for each transfer ▪ Pipette slowly and carefully, avoiding bubbles
	Non-homogeneous samples	Warm samples to 37 °C to dissolve any precipitate, and vortex briefly before use. If samples contain particulates, centrifuge at high speed for 15 minutes, then transfer supernatants to a new tube and repeat centrifugation and transfer step before use.
	Incomplete cell lysis	Refer to the appropriate sample processing kit product inserts for detailed procedures.

Observation	Probable Cause	Recommend Solution
	Instrument needle is partially clogged	Replace or clean the needle according to the manufacturer's recommendations.
	Bubble introduction into Luminex fluidics	Check Luminex probe for proper height, then run instrument debubbling protocol. Make sure every well contains 130 µL of SAPE Wash Buffer and verify the Luminex sample size is set to 100 µL.
	Using buffers containing precipitates	Eliminate precipitates by warming to 37 °C for 30 minutes followed by gentle swirling. If precipitate remains, continue with the incubation.
Low Bead Count	Capture Beads settled or clumped in stock tube	Vortex Capture Beads for 30 seconds immediately prior to adding to Working Bead Mix.
	Capture Beads were not resuspended prior to transfer to the Magnetic Separation Plate	Pipette up and down to resuspend the Capture Beads in the Hybridization Plate prior to transfer of the hybridization mixture to the Magnetic Separation Plate.
	Magnetic Separation Plate not shaken enough prior to reading	Shake the Magnetic Separation Plate at 800 rpm for at least two minutes to resuspend the beads before reading the plate.
	Incorrect Luminex probe height	Adjust the height of the probe following the procedures supplied with your Luminex system.
Poor Assay Linearity	Incomplete cell lysis	Refer to the appropriate sample processing kit product inserts for detailed procedures.
	Inadequate sample preparation	Refer to the appropriate sample processing kit product inserts for detailed procedures.
	Instrument saturation	Signals >20,000 MFI on a Luminex 100/200 instrument may be saturated. The FlexMap and MagPix have a higher range prior to saturation.
	Assay saturation	Perform serial dilution of sample to ensure appropriate fold change is observed.

Validating the Hand-Held Magnetic Plate Washer

The Hand-Held Magnetic Plate Washer is designed for use with the QuantiGene Plex Assay configured for the Magnetic Separation Plate. This device uses magnetic separation to enable quick and easy processing of wash steps after each incubation. This section describes how to validate the hand held washer prior to running an experiment and how to operate the device when performing washes.

IMPORTANT: **Failure to completely read and follow the instructions for validating and using the Hand-Held Magnetic Plate Washer could result in assay failure.**

IMPORTANT: **Do not substitute another plate type for the Magnetic Separation Plate (P/N 10056) included in the QuantiGene Plex Kit. This plate is specifically for use with the Hand-Held Magnetic Plate Washer (P/N QP0702). Other plate types can result in assay failure or poor assay performance. After reading all instructions in this document, we recommend that you perform a series of practice washes using the Hand-Held Magnetic Plate Washer**

Step	Action
Step 1. Set Up Luminex Instrument	Set up the Luminex instrument according to the guidelines provided. Define a protocol with the appropriate bead regions and set to read 2 wells. Refer to the Product Insert for the target-bead population of the panel.
Step 2. Prepare Magnetic Separation Plate	Vortex Capture Beads at maximum speed for 30 seconds. Add 2.5 µL of Capture Beads to 250 µL of SAPE Wash Buffer. Vortex to mix. Add 100 µL of the Capture Bead mixture into each of 2 wells on the Magnetic Separation Plate.
Step 3. Perform Wash Steps	**A.** Perform a series of wash steps using the Wash Buffer to simulate the multiple wash steps in the assay. No incubation needed. **B.** After the final wash step, add 130 µL SAPE Wash Buffer to each well. Cover the Magnetic Separation Plate with a plate seal, place on a shaking platform at room temperature and shake for 2 to 5 min to completely resuspend the Magnetic Beads.
Step 4. Read Magnetic Separation Plate	**A.** Insert the Magnetic Separation Plate into the instrument and read the 2 wells. Make sure the probe height is set properly for the Magnetic Separation Plate. **B.** View the window with the bead regions and Doublet Discriminator (DD) gate. The expected results are: ▪ Signals for the expected beads should show up on the bead map. ▪ Average bead count should be greater than 50/region. ▪ The main single peak in the DD gate window should be within the set DD gates. **NOTE:** **If you are running assays on your Luminex instrument that uses both Assay Plates for Magnetic Beads and Filter Plates for Polystyrene Beads, verify the probe height for each plate type before reading.**

Optimizing Sample Input

Optimal QuantiGene Plex assay performance depends on the complete release and stabilization of the RNA from the cells and protein complexes. Incomplete cell lysis may result in poor assay precision, high CV value or non-linear assay. If any of these conditions occur, your samples may not be completely lysed. Complete cell lysis depends on the correct ratio of cells to lysis solution (Working Lysis Mixture or Working Homogenization Solution) and the method used to lyse the cells or homogenize tissue.

To determine optimal sample amount for lysis or homogenization:

1. 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. Example recommendations are summarized below for cultured cells and animal tissues. To ensure optimal lysis, in the initial experiment, run a test range of sample preparations as indicated in the table.

	Cultured Cells	Tissue
Recommended	400 cells/µL Working Lysis Mixture	5 mg[1]/300 µL Working Tissue Homogenization Solution
Test Range	200, 400, 800 cells/µL Working Lysis Mixture	2.5[1], 5.0[1], 10 mg[1]/300 µL Working Tissue Homogenization Solution

[1] *Wet tissue*

2. 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
3. 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.

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. Procedures for these lysis methods can be found in the Sample Processing Kit Package Insert.

After you have determined the optimal lysis conditions for sample preparation, use the following guidelines to determine the optimal sample amount to use for the QuantiGene Plex assay.

- Resulting signal from the sample is above the LOQ. The LOQ is ≤ 2,000-4,000 transcripts/well.
- 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 10-fold. Background is defined as signal from a sample well that contains no sample.
- Ensure signal from samples are within the assay and instrument linear range. Luminex instruments exhibit saturation at approximately 20,000 MFI. In our experience, assay linear working ranges are approximately 1.5 k-1,500 k RNA transcripts.

Example Plate Layout for Used for Training

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1 Undiluted	Sample 2 Undiluted	Sample 3 Undiluted	Sample 4 Undiluted	RNA 1000 ng	Blank water						
B	Sample 1 Undiluted	Sample 2 Undiluted	Sample 3 Undiluted	Sample 4 Undiluted	RNA 1000 ng	Blank water						
C	Sample 1 1:4 dil	Sample 2 1:4 dil	Sample 3 1:4 dil	Sample 4 1:4 dil	RNA 250 ng	Blank lysis mix						
D	Sample 1 1:4 dil	Sample 2 1:4 dil	Sample 3 1:4 dil	Sample 4 1:4 dil	RNA 250 ng	Blank lysis mix						
E	Sample 1 1:16 dil	Sample 2 1:16 dil	Sample 3 1:16 dil	Sample 4 1:16 dil	RNA 62.5 ng							
F	Sample 1 1:16 dil	Sample 2 1:16 dil	Sample 3 1:16 dil	Sample 4 1:16 dil	RNA 62.5 ng							
G	Sample 1 1:64 dil	Sample 2 1:64 dil	Sample 3 1:64 dil	Sample 4 1:64 dil	RNA 15.63 ng							
H	Sample 1 1:64dil	Sample 2 1:64dil	Sample 3 1:64dil	Sample 4 1:64dil	RNA 15.63 ng							

Blank Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												