invitrogen

QuantiGene™ Plex 384-well Gene Expression Assay

USER GUIDE

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Product information

IMPORTANT! Before using this product, read and understand the information in the "Safety" appendix in this document.

Product description

How the QuantiGene™ Plex Assay works

The Invitrogen™ QuantiGene™ Plex 384-well Gene Expression Assay enables the multiplexed measurement of gene expression by combining branched DNA (bDNA) signal amplification with Luminex™xMAP™ multi-analyte profiling technology. The bDNA assay is a probe hybridization-based method of target-specific RNA capture and quantitation, amplifying signal rather than the target.

Color-coded fluorescent magnetic microspheres (capture beads) capture specific target RNA molecules through hybridization of a custom oligonucleotide probe set, which consists of 3 types of probes: capture extenders, label extenders, and blocking probes. The probe set hybridizes a contiguous sequence of each target RNA. The capture extenders discriminate between different capture beads based on the sequence of a capture probe conjugated to each bead, and the label extenders have tails that provide the support for the branched DNA signal amplification.

Each amplification unit is constructed through sequential hybridization of bDNA oligonucleotides (pre-amplifier, amplifier, and label probe). The label probe is biotinylated to bind Streptavidin-conjugated R-Phycoerythrin (SAPE). The resulting fluorescence signal is associated with individual capture beads by the Luminex™ instrument, which combines advanced fluidics, optics, and digital signal processing. Signal is reported as median fluorescence intensity (MFI) and is proportional to the number of target RNA molecules present in the sample.

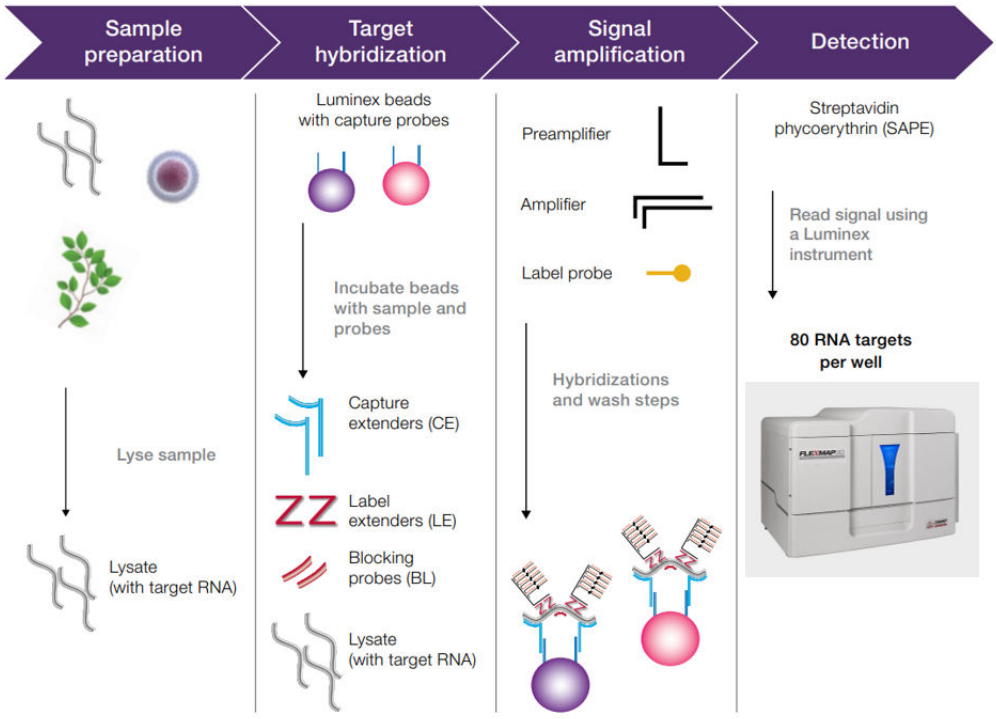

Figure 1 QuantiGene™ Plex 384-well Gene Expression Assay Workflow

Product Description

The QuantiGene™ Plex 384-well Gene Expression Assay consists of 3 modules, each sold separately:

- **QuantiGene™ Sample Processing Kit:** contains reagents for release and stabilization of sample RNA from cultured cells, blood (whole blood, PAXgene™ blood, Tempus™ blood, or dried blood spots), or tissues (fresh, frozen or FFPE). These kits are not required if working with purified RNA samples
- **QuantiGene™ Plex 384-well Assay Kit:** contains the generic reagents, plates, and seals required for running the assay
- **QuantiGene™ Plex Panel:** contains the custom target-specific pooled probe set and associated magnetic capture beads to capture user-defined genes of interest

This user guide contains instructions for using the QuantiGene™ Plex Assay with the following sample types:

- Cell lysates from cultured and primary cells and whole blood
- Tissue homogenates from fresh, frozen, or formalin-fixed, paraffin-embedded (FFPE) tissues
- Purified or *in vitro* transcribed (IVT) RNA

For instructions on preparing cell lysates or tissue homogenates, please refer to the appropriate QuantiGene™ Sample Processing Kit package insert.

Precautions and technical hints

- The shaking incubator must be calibrated for both 54°C & 50°C using the Temperature Validation Kit. The Vortemp 56 requires an inverted plate lid to be placed below the assay plates. See instructions for temperature calibration in the Temperature Validation Kit package insert.
- When running a new sample type, optimize input by running a dilution series to ensure that all target signals are within the dynamic range of the assay.
- Run samples in technical replicates. We recommend a minimum of duplicates, but ideally more in order to calculate intra-assay precision (depending on sample volume availability).
- Use fresh pipette tips when loading samples into each well. Avoid creating bubbles when pipetting. Use a multi-channel pipette whenever possible to achieve optimal assay precision.
- Be careful not to invert the plate or allow contents from one well to mix to another well.
- It is crucial to properly 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. Alternatively, use the ALPS 50 V Heat Sealer with included heat seal. Optimal sealing condition: 170 °C for 3 seconds.

  Note: DO NOT use the Day 2 Plate Seal, otherwise evaporation may occur.
- On day 2 of the assay, turn on and initiate startup protocol of the Luminex™ instrument according to the manufacturer's instructions. Lasers require 30 minutes to warm-up.
- Validate the magnetic plate washer with the QuantiGene™ magnetic separation plate for minimal residual volume and to avoid bead loss.

  Adjust the height of the aspiration needle for an average residual volume of 6 µL or less. The residual volume should not exceed 10 µL. Optimize the settings on the washer to avoid bead loss.

  See Appendix A, “Magnetic plate washer setup” for reference settings on a BioTek ELx405TS with flat magnet (BioTek 7103017).

Required equipment and materials not provided

Unless otherwise indicated, all materials are available through **thermofisher.com**. "MLS" indicates that the material is available from **fisherscientific.com** or another major laboratory supplier.

Required Equipment/Material	Source
Microtiter plate shaker (must have 3 mm orbit at 600-800 rpm)	88880023 or 88880024, QP0706 (IKA™MS3 Digital)
Vortex mixer	MLS
Adjustable single and multi-channel precision pipettes for dispensing 1-20 uL, 20-200 uL, and 200-1000uL	MLS
Reagent reservoirs (25 mL and 100 mL capacity)	3054-1002 or equivalent (VistaLab Technologies™) CLS4873 or equivalent (Corning™Costar™)
Nuclease Free Water (H_2O)	MLS
Plate centrifuge capable of 240 × *g* speeds	MLS
Microcentrifuge	MLS
QuantiGene™ Incubator Temperature Validation Kit	QS0517
4" Soft Rubber Roller	QS0515
FLEXMAP 3D™instrument	APX1342 Luminex Corporation (sold through Thermo Fisher Scientific)
BioTek magnetic Plate Washer, for example BioTek ELx405TS with flat magnet (BioTek 7103017)	BioTek
Use one of the following:	
Labnet VorTemp™ 56 Shaking Incubator	QP0703 or QP0704 (include Temperature Validation Kit)
MaxQ™ 4450 Benchtop Orbital Shaker	SHKE4450 or SHKE4450-1CE
Optional	
Heat sealer	ALPS 50-V Manual Heat Sealer (Cat. No. AB-1443A), use with heat seal AB-0757 (included)

Contents and storage

QuantiGene™ Plex Assay kit (384-well plate)

The QuantiGene™ Plex Assay Kit is supplied in 3 separate boxes based on storage temperature. Storage conditions are listed below. Refer to the product labels for expiration dating, and refer to the QuantiGene™ Plex Assay Kit Package Insert for individual component volumes or quantities.

Component	Description	Storage
Proteinase K[1]	Proteinase K in aqueous buffered solution	-20°C
Blocking Reagent	Aqueous buffered solution containing a preservative	-20°C
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
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
Hybridization Plates	384-well round bottom, clear polypropylene plates	15-30°C
Pressure Seals (Day 1)	Clear, pressure-activated seals for use with the Hybridization Plate during the Day 1/overnight hybridization.	15-30°C
Magnetic Separation Plates	384-well flat bottom microplates	15-30°C

(continued)

Component	Description	Storage
Plate Seals (Day 2)	Clear, adhesive plate seals for use with the Magnetic Separation Plate during the Day 2 hybridizations	15-30°C
Heat Seals	Heat seal for use with ALPS 50-V Manual Heat Sealer ALPS 50 V (Cat. No. AB-1443A)	15-30°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™ Plex Panel

The QuantiGene™ Plex Panel includes the target-specific probe set and associated magnetic capture beads. Each panel is supplied in 2 separate boxes based on storage temperature. Refer to the package insert provided with the panel for the gene list and bead identifiers. Do not freeze the capture beads, as they can be damaged if frozen.

Component	Description	Storage
Probe set	Pre-mixed probe set consisting of target-specific capture extenders, label extenders, and blocking probes	-20°C
Capture beads	Pre-mixed set of magnetic Luminex™ xMAP™ capture beads conjugated with capture probes	2-8°C

Before you begin

Before first use

- Validate the magnetic plate washer to ensure proper bead retention. For instructions see Appendix A, “Magnetic plate washer setup”.
- Calibrate the shaking incubator using the Temperature Validation Kit to ensure hybridization temperatures are 54°C and 50°C. See instructions in the Temperature Validation Kit package insert.
- Optimize sample preparation and input by running a dilution series to ensure all targets are within the assay's dynamic range. For sample optimization instructions see Appendix B, “Sample optimization protocol”.

Sample preparation

Prior to running the QuantiGene™ Plex Assay, ensure you have a lysate or homogenate prepared using one of the following sample processing kits (of note, size/plate refers to 96-well plate format):

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 × 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 × 10 Plate
QS0107	FFPE Sample Processing Kit[4]	10 samples

(continued)

Catalog No.	Assay specific reagents	Size
QS0108	FFPE Sample Processing Kit	25 samples
QS0109	FFPE Sample Processing Kit	100 samples

[1] Sufficient for preparing bulk lysates from 1.8 x 10^7 cells.

[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 1.8 × 10^7cells.

[4] A sample is defined as 25-100 mm^2 × 50-60 microns (area × total thickness of FFPE tissue sections)

Assay procedure: day 1

IMPORTANT!

- Tissue homogenates, cell lysates and whole blood lysates must be prepared using the applicable QuantiGene™ Sample Processing Kit.
- Purified RNA samples do not require a QuantiGene™ Sample Processing Kit.
- The day 2 procedure is the same for all sample types.

For cell lysates or whole blood lysates

1. Pre-warm Lysis Mixture at 37°C for 30 minutes followed by gentle swirling.
2. If lysates have been frozen, remove from the freezer and thaw at room temperature followed by incubation at 37°C for 30 min. Following incubation, vortex briefly if samples are in tubes or pipette up and down 5 times if samples are in plates. Leave at room temperature until use.

   Do not store on ice prior to use.
3. Handle the reagents listed below as follows:
   a. Probe Set & Blocking Reagent: Thaw and vortex briefly to mix, then centrifuge Probe Set briefly to collect contents at the bottom of the tube.
   b. Proteinase K: Keep on ice.
   c. Capture Beads: Take out of storage right before use and protect from light.
4. If samples require dilution, dilute with Diluted Lysis Mixture (dilute 1 volume Lysis Mixture plus 2 volumes Nuclease-free Water, prepared fresh) so that the desired amount of sample is present in a volume of 50 µL/assay well. In order to optimize sample input, please see “Optimize sample input” on page 29.

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. Keep Working Bead Mix at room temperature and protected from light. Do not store on ice.

Order	Reagent	2 to 64-plex		65 to 80-plex	
		1 Well (µL)	384 Well (µL)[1]	1 Well (µL)	384 Well (µL)[1]
1	Nuclease-free Water	6.18	3,085	5.38	2,686
2	Lysis Mixture	6.67	3,330	6.67	3,330
3	Blocking Reagent	2.00	998	2.00	998
4	Proteinase K	0.20	100	0.20	100
5	Capture Beads (**vortex 30 seconds before adding**)	0.75	374	0.75	374
6	Probe Set	4.20	2,097	5.00	2,496
	Total	20.00	9,984	20.00	9,984

[1] Includes 30% overage to enable use of reagent reservoir and multichannel pipette.

6. Vortex Working Bead Mix for 10 seconds, then pipette 20 µL into each well of the Hybridization Plate.

7. Add 50 µL of lysate or diluted lysate to each well of the Hybridization Plate containing Working Bead Mix. The total final volume in each well will be 70 µL. Load samples using a multichannel pipette, if possible. There is no need for mixing - try to avoid introducing bubbles.

   Background Controls: Add 50 µL of Diluted Lysis Mixture (1 volume Lysis Mixture plus 2 volumes Nuclease-free Water) to at least 3 wells containing Working Bead Mix.

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.

   Alternatively, use the ALPS 50 V heat sealer with included heat seal. Optimal sealing condition: 170 °C for 3 seconds

   Note: DO NOT use the Day 2 Plate Seal, otherwise evaporation may occur.

9. Place the Hybridization Plate in the MaxQ™ shaking incubator, then incubate for 18-22 hours at 54°C±1°C at 300 rpm. Ensure the incubator has been calibrated using the Temperature Validation Kit. If using a VorTemp™ 56, ensure there is an inverted plate lid in place, as explained by the package insert, then incubate for 18-22 hours at 54°C±1°C at 800 rpm.

10. After incubation, proceed to “Assay procedure: day 2” on page 20.

For fresh, frozen, or FFPE tissue homogenates

1. Pre-warm Lysis Mixture at 37°C for 30 minutes followed by gentle swirling.

2. If tissue homogenates have been frozen, remove from the freezer and thaw at room temperature followed by incubation at 37°C for 30 min. Following incubation, vortex briefly if samples are in tubes or pipette up and down 5 times using a multi-channel pipette if samples are in plates. Leave at room temperature until use.

3. Handle the reagents listed below as follows:
   a. Probe Set & Blocking Reagent: Thaw and vortex briefly to mix, then centrifuge Probe Set briefly to collect contents at the bottom of the tube.

   b. Proteinase K: Keep on ice.

   c. Capture Beads: Take out of storage right before use and protect from light when possible.

4. If samples require dilution, dilute with Homogenization Solution so that the desired amount of sample is present in a volume of 38.75 µL/assay well. In order to optimize sample input, please see “Optimize sample input” on page 29.

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. Keep Working Bead Mix at room temperature and protected from light when possible. Do not store on ice.

Order	Reagent	2 to 64-plex		65 to 80-plex	
		1 Well (µL)	384 Well (µL)[1]	1 Well (µL)	384 Well (µL)[1]
1	Nuclease-free Water	0.80	399	—	—
2	Lysis Mixture	23.30	11,631	23.30	11,631
3	Blocking Reagent	2.00	998	2.00	998
4	Proteinase K	0.20	100	0.20	100
5	Capture Beads (**vortex 30 seconds before adding**)	0.75	374	0.75	374
6	Probe Set	4.20	2,097	5.00	2,496.00
	Total	31.25	15,599	31.25	15,599

[1] Includes 30% overage to enable use of reagent reservoir and multichannel pipette.

6. Vortex Working Bead Mix for 10 seconds, then pipette 31.25 µL into each well of the Hybridization Plate.

7. Add 38.75 µL of tissue homogenate or diluted tissue homogenate to each well of the Hybridization Plate containing Working Bead Mix . The total final volume in each well will be 70 µL. Load samples using a multichannel pipette, if possible. There is no need for mixing - try to avoid introducing bubbles. . Background Controls: add 38.75 µL of Homogenizing Solution to at least 3 wells containing Working Bead Mix.

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.

   Alternatively, use the ALPS 50 V heat sealer with included heat seal. Optimal sealing condition: 170 °C for 3 seconds

   Note: DO NOT use the Day 2 Plate Seal, otherwise evaporation may occur.

9. Place the Hybridization Plate in the MaxQ™ shaking incubator, then incubate for 18-22 hours at 54°C±1°C at 300 rpm. Ensure the incubator has been calibrated using the Temperature Validation Kit. If using a VorTemp™ 56, ensure there is an inverted plate lid in place, as explained by the package insert, then incubate for 18-22 hours at 54°C±1°C at 800 rpm.

10. After incubation, proceed to “Assay procedure: day 2” on page 20.

For purified RNA or in vitro transcribed RNA

1. Pre-warm Lysis Mixture at 37°C for 30 minutes followed by gentle swirling.

2. 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 is present in 20 µL. See “Optimize sample input” on page 29 for guidelines. The typical sample input range is 50-500 ng/well.

3. Handle the reagents listed below as follows:
   a. Probe Set & Blocking Reagent: Thaw and vortex briefly to mix, then centrifuge Probe Set briefly to collect contents at the bottom of the tube.
   b. Capture Beads: Take out of storage right before use and protect from light when possible.

4. 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. Keep Working Bead Mix at room temperature and protected from light when possible. Do not store on ice.

Order	Reagent	2 to 64-plex		65 to 80-plex	
		1 Well (µL)	384 Well (µL)[1]	1 Well (µL)	384 Well (µL)[1]
1	Nuclease-free Water	19.75	9,859	18.95	9,460
2	Lysis Mixture	23.30	11,631	23.30	11,631
3	Blocking Reagent	2.00	998	2.00	998
4	Capture Beads (**vortex 30 seconds before adding**)	0.75	374	0.75	374
5	Probe Set	4.20	2,097	5.00	2,496
	Total	50.00	24,959	50.00	24,959

[1] Includes 30% overage to enable use of reagent reservoir and multichannel pipette.

5. Vortex Working Bead Mix for 10 seconds, then pipette 50 µL into each well of the Hybridization Plate.

6. Add 20 µL of RNA sample to each well of the Hybridization Plate containing Working Bead Mix . The total final volume in each well will be 70 µL. Load samples using a multichannel pipette, if possible. There is no need for mixing - try to avoid introducing bubbles.

   Background Controls: Add 20 µL of Nuclease Free Water to at least 3 wells containing Working Bead Mix.

7. 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.

   Alternatively, use the ALPS 50 V heat sealer with included heat seal. Optimal sealing condition: 170 °C for 3 seconds

   Note: DO NOT use the Day 2 Plate Seal, otherwise evaporation may occur.

8. Place the Hybridization Plate in the MaxQ™ shaking incubator, then incubate for 18-22 hours at 54°C±1°C at 300 rpm. Ensure the incubator has been calibrated using the Temperature Validation Kit. If using a VorTemp™ 56, ensure there is an inverted plate lid in place, as explained by the package insert, then incubate for 18-22 hours at 54°C±1°C at 800 rpm.

9. After incubation, proceed to“Assay procedure: day 2” on page 20.

Assay procedure: day 2

Setup of the Luminex™ protocol

Please refer to the QuantiGene™ Plex panel package insert for specific bead regions when setting up your protocol in the Luminex™ xPONENT™ software. If given the option between calibrating with Low or 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
50 µL	5 ,000 - 25,000	45 sec	50

If there is a malfunction of the instrument or software during the run, the plate can be reprocessed on the Luminex™ instrument. Remove the plate from the instrument, insert on the Magnetic Plate Washer, wait 2 min, then aspirate the solution. Resuspend the beads in 50 µL of SAPE Wash Buffer, seal the plate, wrap in foil, and shake at 600 rpm for 3 min at room temperature. The assayed samples may take longer to read since there will be fewer beads in each previously read well due to consumption from the initial run.

Process Plate

These instructions are for processing one 384-well plate using a multi-channel pipettes and reagent reservoirs. A liquid-handler with 384-well pipetting head is highly recommended for processing multiple plates.

Scale reagents accordingly to process a different number of wells. Prior to completing the QuantiGene™ Plex Assay on Day 2, allow plenty of time to warm up and calibrate the Luminex™ instrument. The lasers require 30 minutes to warm

up. Additionally, ensure your protocol is set up correctly in xPONENT™. See Setup of Luminex™ Protocol for more details and settings.

1. 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.

2. Prepare 1X Wash Buffer: add 3 mL Wash Buffer Component 1 and 50 mL Wash Buffer Component 2 to 950 mL Nuclease Free Water. This volume is sufficient for 1 plate. Scale wash buffer volumes according to the number of wells or plates to be processed. Prime the plate washer twice with Nuclease Free Water, and then once with Wash Buffer.

3. Remove the Hybridization Plate from the shaking incubator and adjust temperature to 50°C ± 1°C.

4. Centrifuge Hybridization Plate at 240 × *g* for one minute at room temperature. Remove the pressure seal and, using a multichannel pipette, pipette up and down 5 times, then completely transfer from the Hybridization Plate to the Magnetic Separation Plate.

5. Wash the plate:
   a. Place the Hybridization Plate on a plate washer with the appropriate magnet.

   b. Run a preset washing program with the following parameters (see Appendix A, “Magnetic plate washer setup”):
      - Soak 2 minutes to allow the magnetic beads to accumulate on the bottom of each well.
      - 3 washes with dispensing volume of 80 µL for each wash. Soak 15 seconds between washes.

6. Pre-Amplifier Hybridization:
   a. Transfer Pre-Amplifier Solution to a 25-mL reagent reservoir and pipette 30µL using a multichannel pipette into each well.

   b. Seal the Magnetic Separation Plate with a Day 2 Plate Seal.

   c. Place the Magnetic Separation Plate into the MaxQ™ shaking incubator, then incubate for 1 hour at 50°C±1°C with shaking at 300 rpm. Alternatively, place the Magnetic Separation Plate into the VorTemp™ 56 shaking incubator, then incubate for 1 hour at 50°C±1°C with shaking at 800 rpm. Ensure the incubator has been calibrated using the Temperature Validation Kit.

7. After the 1 hour Pre-Amplifier incubation, remove the Magnetic Separation Plate from the shaking incubator, remove the seal, insert the plate into the Magnetic Plate Washer and repeat the washing procedure from Step 5.

8. Amplifier Hybridization:
   a. Transfer Amplifier Solution to a 25-mL reagent reservoir and pipette 30 µL using a multichannel pipette into each well.

   b. Seal the Magnetic Separation Plate with a Day 2 Plate Seal.

   c. Place the Magnetic Separation Plate into the MaxQ™ shaking incubator, then incubate for 1 hour at 50°C±1°C with shaking at 300 rpm. Alternatively, place the Magnetic Separation Plate into the VorTemp™ 56 shaking incubator, then incubate for 1 hour at 50°C±1°C with shaking at 800 rpm. Ensure the incubator has been calibrated using the Temperature Validation Kit.

9. After the 1 hour Amplifier incubation, remove the Magnetic Separation Plate from the shaking incubator, remove the seal, insert the plate into the Magnetic Plate Washer and repeat the washing procedure from Step 5.

10. Label Probe Hybridization:
    a. Transfer Label Probe Solution to a 25-mL reagent reservoir and pipette 30 µL using a multichannel pipette into each well.

    b. Seal the Magnetic Separation Plate with a Day 2 Plate Seal.

    c. Place the Magnetic Separation Plate into the MaxQ™ shaking incubator, then incubate for 1 hour at 50°C±1°C with shaking at 300 rpm. Alternatively, place the Magnetic Separation Plate into the VorTemp™ 56 shaking incubator, then incubate for 1 hour at 50°C±1°C with shaking at 800 rpm. Ensure the incubator has been calibrated using the Temperature Validation Kit.

11. During the Label Probe Incubation, 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 135 µL of SAPE to 15 mL of SAPE Diluent to make the SAPE Working Reagent. Vortex for 15 seconds to mix, and protect from light.

12. After the 1 hour Label Probe incubation, remove the Magnetic Separation Plate from the shaking incubator, remove the seal, insert the plate into the Magnetic Plate Washer and repeat the washing procedure from Step 5.

13. Bind SAPE:
    a. Transfer the SAPE Working Reagent to a 25-mL reagent reservoir and pipette 30 µL into each assay well using a multichannel pipette.

    b. Seal the Magnetic Separation Plate with a Day 2 Plate Seal. Cover or wrap in foil to protect from light. Place on a shaking platform at room temperature and shake at 800 rpm for 1 minute followed by 600 rpm for 30 minutes.

c. Prime the plate washer twice with Nuclease Free Water, and then once with SAPE Wash Buffer. Make sure that there is 500 mL of SAPE Wash Buffer for the washer. This volume is sufficient for 1 plate.

14. After the 30 minute SAPE incubation, remove the Magnetic Separation Plate from the plate shaker, remove the seal, insert the plate into the Magnetic Plate Washer and repeat the washing procedure from Step 5 using SAPE Wash Buffer instead of the QuantiGene™ Plex Wash Buffer.

15. Prepare the plate for analysis

    a. Add 50 µL of SAPE Wash Buffer to each assay well using a multichannel pipette.

    b. Seal the Magnetic Separation Plate with a Day 2 Plate Seal. Wrap or cover the plate with aluminum foil to protect from light.

    c. Place the Magnetic Separation Plate on the Microtiter Plate Shaker and shake at 600 rpm for 3 minutes at room temperature. Read plate immediately on Luminex™ instrument.

Note: If running more than 1 plate at a time, leave the 2nd plate at room temperature (without shaking and protected from light). 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 600 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).

Analyze results

An example is provided for calculating gene expression fold changes. Target signals must be in the linear range of the assay. Signals over 45,000 MFI on the Luminex™ FLEXMAP 3D™ may be saturating.

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 4	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 type	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 4	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 reference gene signal (background subtracted). This will correct for sample preparation, sample input and deviations between wells, plates, and experiments.

Sample type	Normalization gene	Test gene 1	Test gene 2	Test gene 3
Background (no sample	—	—	—	—
Untreated sample	1	7.83	0.04	7.61
Treated sample 1	1	1.75	0.06	3.64
Treated sample 4	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 type	Normalization gene	Test gene 1	Test gene 2	Test gene 3
Background (no sample	—	—	—	—
Untreated sample	1	1	1	1
Treated sample 1	1	0.22	1.57	0.48
Treated sample 4	1	0.56	1.14	0.27
Treated sample 3	1	0.43	0.89	0.91

Note: A cloud-based software tool is available at **apps.thermofisher.com/apps/quantigene**. In addition, the data can be exported from the software tool to the Applied Biosystems™ Transcriptome Analysis Console (TAC) software for advanced analysis and visualization. When combined with TAC, the analysis allows for better visualization and interpretation using tools like scatter and volcano plots, hierarchical clustering, and link outs to publicly available annotations. Download a free copy of TAC at **www.thermofisher.com/us/en/home/life-science/microarray-analysis/microarray-analysis-instruments-software-services/microarray-analysis-software/affymetrix-transcriptome-analysis-console-software.html** .

Troubleshooting

Observation	Possible cause	Recommended action
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	Refer to the appropriate sample processing kit product inserts for detailed procedures. 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 voiding 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	Possible cause	Recommended action
Low assay precision (high CV) *(continued)*	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 50 µL of SAPE Wash Buffer and verify the Luminex™ sample size is set to 50 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 >45,000 MFI on a Luminex™FLEXMAP 3D™ instrument may be saturated.
	Assay saturation	Perform serial dilution of sample to ensure appropriate fold change is observed.
Malfunction of Luminex™ FLEXMAP 3D™ during acquisition	—	Remove the plate from the instrument, place it on the Magnetic Plate Washer, wait 2 minutes, then aspirate the solution. Resuspend the beads in 50 µL of SAPE Wash Buffer, seal the plate, wrap in foil and shake at 600 rpm for 3 minutes at room temperature. The assayed samples may take longer to read since there will be fewer beads in each previously read well due to consumption from the initial run.

A Magnetic plate washer setup

Magnetic plate washer settings

The following settings are for reference only as a starting point. For plate washer from other manufacturers, adjust accordingly. Always validate your own unit before experiment.

Example: Biotek ELx405TS with flat magnet 7103017

- Settings:
  - 1. "Move" carrier home - soak 2 minutes
  - 2. "Wash" 80 µL of buffer for 1 cycle
  - Travel rate = 1 CW
  - Volume = 80 µL/well
  - Buffer A
- Dispense settings:
  - Z = 105 (13.34 mm above carrier)
  - X = -15 (0.69 mm left of carrier)
  - Y = 0 (Center of the well)
  - Delay start of vaccum until 80 µL/wells is dispersed.
- Aspiration settings:
  - Travel rate = 1 CW
  - Z= 30 (3.81 mm above carrier)
  - X= -9 (0.41 mm left of center)
  - Y= 4 (0.30 mm front of center)
  - 3. “Move” carrier home – soak 15 seconds
  - 4. “Wash” (same as above)
  - 5. “Move” carrier home – soak 15 seconds
  - 6. “Wash” (Same as above)
  - 7. “Move” carrier home -soak 15 seconds
  - 8. “Aspirate” (same aspiration conditions as in the wash part)
  - Travel Rate : 1 CW
  - Delay 0 msec

To test the setup, run the program with Wash Buffer. Then using pipette, manually check the residual volume of 10 wells in random positions. The average residual volume should be about 6 µL or less, and none of the wells should exceed 10 µL.

B Sample optimization protocol

Optimize 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 values, or non-linear results. 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 the tissue.

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. Make sure that your sample is completely lysed or homogenized.

2. For each lysate or homogenate, prepare a 4-fold, 4-point serial dilution in Diluted Lysis Mixture or Tissue Homogenization Solution, respectively, to determine the assay performance. Make sure to account for sufficient sample input volume (technical replicates). Assay performance is determined by calculating the following:
   - LOD (Assay limit of detection)
   - LOQ (Limit of Quantification)
   - Assay linearity
   - %CV (Coefficient of Variation)

   Please refer to the Glossary on page 34 for detailed calculation instructions.

3. Calculate the assay performance for each sample (input) to determine which one had the best performance and use that amount of cells or tissue for future experiments.

Determine the optimal lysis method for a sample type

Following the procedure for determining optimal lysis, test different lysis methods. For example, tissue lyser vs. 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 between 4,000 and 8,000 RNA transcripts per 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 5-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™ FLEXMAP 3D™ instruments exhibit saturation starting around 45,000 MFI.

C

Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, and so on). To obtain SDSs, see the "Documentation and Support" section in this document.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with sufficient ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if needed) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

WARNING! HAZARDOUS WASTE (from instruments). Waste produced by the instrument is potentially hazardous. Follow the guidelines noted in the preceding General Chemical Handling warning.

WARNING! 4L Reagent and Waste Bottle Safety. Four-liter reagent and waste bottles can crack and leak. Each 4-liter bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position.

Biological hazard safety

WARNING! Potential Biohazard. Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at:
  https://www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2009-P.pdf
- World Health Organization, *Laboratory Biosafety Manual*, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at:
  www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf

Glossary

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 avereage 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 = (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/accu- -racy 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	—	—	—

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