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QuantiGene™ Singleplex Assay

USER GUIDE

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Contents

Product information

How the QuantiGene™ Singleplex assay works

The QuantiGene™ Singleplex Assay is a hybridization-based gene expression assay that utilizes branched DNA for signal amplification. The ability to quantify specific RNA molecules within a sample lies in the design of a QuantiGene™ Probe Set. Each oligonucleotide probe set contains three types of synthetic probes (capture extenders, label extenders, and blocking probes) that hybridize to a contiguous sequence of the target RNA. The capture extenders bind to the capture probes conjugated to the well surface, and via cooperative hybridization, capture the associated target RNA. The label extenders, designed in pairs for improved specificity, have tails that provide the support for the branched DNA (bDNA) signal amplification.

Each bDNA signal amplification unit is constructed through sequential hybridization of 3 oligonucleotides (pre-amplifier, amplifier, and label probe). Each label probe is conjugated with alkaline phosphatase, which mediates the degradation of a chemiluminescent substrate. The resulting luminescence is reported as relative light units (RLU) on a microplate luminometer. The amount of luminescent signal is linearly proportional to the number of RNA molecules present in the sample.

Product description

The QuantiGene™ Singleplex Assay consists of 3 modules, each sold separately:

- **QuantiGene™ Sample Processing Kit**: contains reagents for release and stabilization of sample RNA and DNA from cultured cells, blood (whole blood, PAXgene™ blood, Tempus™ blood, or dried blood spots), or tissues (fresh, frozen or FFPE). This kit is not required if working with purified RNA or DNA samples.
- **QuantiGene™ Singleplex Assay Kit**: contains the generic reagents, plates, and seals required for running the assay.
- **QuantiGene™ Singleplex Probe Set**: contains the custom target-specific pooled probe set to capture user-defined gene of interest.

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

- Cell lysates from cultured cells or 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 incubator must be calibrated for 55°C using the Temperature Validation Kit. See instructions for temperature calibration in the Temperature Validation Kit package insert **Quantigene Incubator Temperature Validation Kit pdf** .
- Capture Probe oligonucleotides are conjugated to the surface of Capture Plate wells. Do not scratch Capture Plate wells with pipette tips.
- When running a new sample type, optimize input by running a dilution series to ensure that 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.
- 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 allow contents from one well to mix to another well.

Required equipment and materials not provided

Unless otherwise indicated, all materials are available through **thermofisher.com**. MLS: Fisher Scientific™ (**fisherscientific.com**) or other major laboratory supplier.

Required Equipment/Material	Source
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)	3054-1002 or equivalent (VistaLab Technologies™) CLS4873 or equivalent (Corning™ Costar™)
Microcentrifuge	MLS
Microplate centrifuge that can achieve 240 x g	Eppendorf™ 5804 and rotor A-2-DWP or equivalent
Nuclease-free water	MLS
Vortex mixer	MLS
QuantiGene™ Incubator Temperature Validation Kit	QS0517
4 inch soft rubber roller or Adhesive Film Applicator	QS0515 or 4333183

(continued)

Required Equipment/Material	Source
Incubator or oven with horizontal air flow, capable of maintaining constant temperatures of 50°C and 55°C, ±1°C	QS0720 or QS0721, or equivalent from 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}	Varioskan™ LUX™ multimode microplate reader (VL0L00D0) or Fluoroskan™ FL Microplate Fluorometer and Luminometer (5200220) or Luminoskan™ Microplate Luminometer (5300330)
Optional Equipment/Material	**Source**
Plate washer that meets or exceeds the following specifications: • 30-200 µL ±5% volume • 96 channels • Angle-dispensing tip • Plate stacker • Automation capable • Minimal dead volume	Wellwash™ Microplate Washer (5165000 or 5165040) or Wellwash™ Versa Microplate Washer (5165010 or 5165050) or BioTek® 405 LS, 405 TS, or ELx405

Contents and storage

QuantiGene™ Singleplex assay kit

The QuantiGene™ Singleplex 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™ Singleplex Assay Kit Package Insert for individual component volumes or quantities.

Component	Description	Storage
Pre-Amplifier Solution	DNA in aqueous buffered solution	2-8 ℃
Amplifier Solution	DNA in aqueous buffered solution	2-8 ℃
Blocking Reagent	Aqueous buffered solution containing a preservative	2-8 ℃
Capture Plate	96-well polystyrene plate coated with capture probes	2-8 ℃
Label Probe	Oligonucleotide-alkaline phosphatase conjugate in aqueous buffered solution	2-8 ℃
Substrate (Lumigen™ APS-5)	Chemiluminescent substrate	2-8 ℃
Label Probe Diluent	Aqueous buffered solution with a protein-containing preservative	15-30 ℃
Lysis Mixture	Aqueous buffered solution containing a preservative	15-30 ℃
Plate Seals (day 1 and day 2)	Adhesive-backed foil plate seal	15-30 ℃
Wash Buffer Component 1	Aqueous solution	15-30 ℃
Wash Buffer Component 2	Aqueous buffered solution	15-30 ℃

QuantiGene™ Singleplex probe set

The QuantiGene™ Singleplex Probe Set is a custom, target-specific component that must be ordered separately. The probe set is available in 200, 1000, and 5000 reaction sizes. Search **the catalog here** or design your own **custom probe set here**.

Component	Description	Storage
QuantiGene™ Singleplex Probe Set (skus QGS-200, QGS-1000, and QGS-5000)	Pre-mixed probe set consisting of target-specific capture extenders, label extenders, and blocking probes	–20 ℃

Before you begin

Before first use

- Calibrate the incubator using the **Temperature Validation Kit** (sku QS0517) to ensure hybridization temperatures are 55±1°C. See instructions in the Temperature Validation Kit package insert. Check temperature routinely or before each run.
- Optimize sample input by running a dilution series to ensure all targets are within the assay's dynamic range. For sample optimization instructions, see Appendix A, "Sample optimization protocol". Follow this procedure when running a particular sample type for the first time, using a luminometer for the first time, or using a new Probe Set for the first time.
- Validate any automated equipment such as plate washers and liquid dispensers.
- Replicates: technical replicates are replicate assays from a single sample and are used to determine assay precision. Biological replicates are replicate assays from biologically-equivalent samples (e.g. cells from the same cell line grown in different wells, subjected to the same treatment, and lysed independently). We recommend running 3 technical replicates of each distinct biological sample.
- Background: assay background is the signal generated by all assay component in the absence of sample input. Include an assay background control in triplicate for each Probe Set used on an individual Capture Plate.
- 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.

Sample preparation

Prior to running the QuantiGene™ Singleplex Assay, ensure you have a lysate or homogenate prepared using one of the following sample processing kits:

SKU	Description	Size
QS0101	Cell Lysate Sample Preparation Kit[1]	2 plates
QS0102	Cell Lysate Sample Preparation Kit	10 plates
QS0103	Cell Lysate Sample Preparation Kit	5 x 10 plates
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
QS0107	FFPE Tissue Sample Processing Kit[3]	10 samples
QS0108	FFPE Tissue Sample Processing Kit	25 samples
QS0109	FFPE Tissue Sample Processing Kit	100 samples
QS0110	Blood Sample Processing Kit	2 plates
QS0111	Blood Sample Processing Kit	10 plates
QS0112	Blood Sample Processing Kit	5 x 10 plates

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

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

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

Assay procedure: day 1

For cell lysate or whole blood lysates

1. Prepare the following reagents:
   a. Lysis mixture: incubate at 37°C for 30 minutes followed by gentle swirling to dissolve any precipitates.

   b. Probe Set(s): thaw, vortex briefly to mix, then briefly centrifuge.

   c. 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, then leave at room temperature until use. If freshly prepared, leave the samples at room temperature and do not put them on ice.

   d. Remove Capture Plate from 4°C and leave at room temperature for 30 minutes. Do not remove from sealed foil pouch until use.

2. Dilute samples: if appropriate, dilute with Diluted Lysis Mixture (1 part Lysis Mixture to 2 parts nuclease-free water, prepared fresh) so that the desired amount of sample is present in a volume of 80 µL/well. See Appendix A, “Sample optimization protocol” for additional support.

3. Prepare Working Probe Set: combine the following reagents in the order listed. Scale according to the number of assays to be run with required overage. Assay kits include 40% overage.

Reagent	1 well (µL)	48 wells[1] (µL)	96 wells[1] (µL)
Nuclease-free water	12.1	813	1,626
Lysis Mixture	6.6	444	887
Blocking Reagent[2]	1	67	134
QuantiGene™ Singleplex Probe Set	0.3	20.1	40.2
Total	**20**	**1,344**	**2,688**

[1] includes 40% overage

[2] omit for 18S or 28S RNA probe sets. Substitute with nuclease-free water.

4. Prepare the Capture Plate:

   Open the sealed foil pouch and remove the Capture Plate. Vortex Working Probe Set briefly, then dispense 20 µL into each well of the Capture Plate. For fewer than 48 wells, use a single channel pipet. For 48 wells or more, use a multi-channel pipette. Use new tips for each transfer and avoid introducing bubbles.

5. Add sample:
   a. Add 80 µL of sample to each well of the Capture Plate containing Working Probe Set, using a new pipette tip for each transfer. Avoid introducing bubbles and do not mix.

   b. Add 80 µL of Diluted Lysis Mixture to at least 3 wells for background controls.

6. Hybridize target RNA:
   a. Place an adhesive Plate Seal (day 1) on the plate and use the soft rubber roller or an adhesive film applicator to seal.

      Seal very tightly such that the numbers and letters on the edge of the plate are very well defined.

   b. *(Optional)* Centrifuge the Capture Plate at 240 x g for 20 seconds at room temperature to ensure the contents are fully collected at the bottom of each well.

   c. Incubate the Capture Plate at 55±1°C for 16 to 20 hours.

For fresh, frozen, or FFPE tissue homogenates

1. Prepare the following reagents:
   a. Lysis mixture: incubate at 37℃ for 30 minutes followed by gentle swirling to dissolve any precipitates.

   b. Probe Set(s): thaw, vortex briefly to mix, then briefly centrifuge.

   c. Tissue homogenates: if previously frozen, thaw at room temperature followed by incubation at 37 ℃ for 15-30 minutes. Vortex briefly, then leave at room temperature until use.

   d. Remove Capture Plate from 4℃ and leave at room temperature for 30 minutes. Do not remove from sealed foil pouch until use.

2. Dilute samples: if appropriate, dilute with Homogenizing Solutions so that the desired amount of sample is present in a volume of 40 µL/well. See Appendix A, “Sample optimization protocol” for additional support.

3. Prepare Working Probe Set: combine the following reagents in the order listed. Scale according to the number of assays to be run with required overage. Assay kits include 40% overage.

Reagent	1 well (µL)	48 wells[1] (µL)	96 wells[1] (µL)
Nuclease-free water	25.4	1,707	3,414
Lysis Mixture	33.3	2,238	4,476
Blocking Reagent[2]	1	67	134
QuantiGene™ Singleplex Probe Set	0.3	20.1	40.3
Total	**60**	**4,032**	**8,064**

[1] includes 40% overage

[2] omit for 18S or 28S RNA probe sets. Substitute with nuclease-free water.

4. Prepare the Capture Plate:

   Open the sealed foil pouch and remove the Capture Plate. Vortex Working Probe Set briefly, then dispense 60 µL into each well of the Capture Plate. For fewer than 48 wells, use a single channel pipet. For 48 wells or more, use a multi-channel pipette. Use new tips for each transfer and avoid introducing bubbles.

5. Add sample:
   a. Add 40 µL of sample to each well of the Capture Plate containing Working Probe Set, using a new pipette tip for each transfer. Avoid introducing bubbles and do not mix.

b. Add 40 µL of Homogenizing Solution to at least 3 wells for background controls.

6. Hybridize target RNA:

   a. Place an adhesive Plate Seal (day 1) on the plate and use the soft rubber roller or an adhesive film applicator to seal.

      Seal very tightly such that the numbers and letters on the edge of the plate are very well defined.

   b. *(Optional)* Centrifuge the Capture Plate at 240 x g for 20 seconds at room temperature to ensure the contents are fully collected at the bottom of each well.

   c. Incubate the Capture Plate at 55±1℃ for 16 to 20 hours.

For purified RNA or in vitro transcribed RNA

1. Prepare the following reagents:
   a. Lysis mixture: incubate at 37°C for 30 minutes followed by gentle swirling to dissolve any precipitates.
   b. Probe Set(s): thaw, vortex briefly to mix, then briefly centrifuge.
   c. RNA sample(s). If previously frozen, thaw on ice.
   d. Remove Capture Plate from 4°C and leave at room temperature for 30 minutes. Do not remove from sealed foil pouch until use.

2. Dilute samples: if appropriate, dilute with nuclease-free water so that the desired amount of sample is present in a volume of 20 µL/well. See Appendix A, "Sample optimization protocol" for additional support.

   Note: 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 Working Probe Set: combine the following reagents in the order listed. Scale according to the number of assays to be run with required overage. Assay kits include 40% overage.

Reagent	1 well (µL)	48 wells[1] (µL)	96 wells[1] (µL)
Nuclease-free water	45.4	3,051	6,102
Lysis Mixture	33.3	2,238	4,476
Blocking Reagent[2]	1	67	134
QuantiGene™ Singleplex Probe Set	0.3	20.1	40.3
Total	**80**	**5,376**	**10,752**

[1] includes 40% overage
[2] omit for 18S or 28S RNA probe sets. Substitute with nuclease-free water.

4. Prepare the Capture Plate:

   Open the sealed foil pouch and remove the Capture Plate. Vortex Working Probe Set briefly, then dispense 80 µL into each well of the Capture Plate. For fewer than 48 wells, use a single channel pipet. For 48 wells or more, use a multi-channel pipette. Use new tips for each transfer and avoid introducing bubbles.

5. Add sample:
   a. Add 20 µL of sample to each well of the Capture Plate containing Working Probe Set, using a new pipette tip for each transfer. Avoid introducing bubbles and do not mix.

b. Add 20 µL of nuclease-free water to at least 3 wells for background controls.

6. Hybridize target RNA:

   a. Place an adhesive Plate Seal (day 1) on the plate and use the soft rubber roller or an adhesive film applicator to seal.

      Seal very tightly such that the numbers and letters on the edge of the plate are very well defined.

   b. *(Optional)* Centrifuge the Capture Plate at 240 x g for 20 seconds at room temperature to ensure the contents are fully collected at the bottom of each well.

   c. Incubate the Capture Plate at 55±1°C for 16 to 20 hours.

Assay procedure: day 2

Process plate (signal amplification and detection)

1. About signal amplification and detection:
   a. 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.

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

   c. Do not disturb the contents of the Capture Plate(s) or open the incubator door during incubation steps.

   d. Incubation temperatures must be 55±1°C (Pre-Amplifier and Amplifier hybridizations) or 50±1°C (Label Probe hybridization). Verify temperatures using a QuantiGene™ Incubator Temperature Validation Kit.

2. Warm Pre-Amplifier Solution and Amplifier 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). Keep Label Probe and Label Probe Diluent at room temperature until use.

3. Prepare 1X Wash Buffer:
   a. Add to a 500 mL graduated cylinder, in the following order:

      1. 496 mL nuclease-free water
      2. 1.5 mL Wash Comp 1
      3. 2.5 mL Wash Comp 2

   b. Transfer to a 500 mL bottle and invert to mix. Make 1X Wash Buffer fresh each day. Scale according to the number of plates to be processed. 500 mL is sufficient for 1 Capture Plate.

4. Pre-Amplifier Hybridization:
   a. Wash the Capture Plate (manual washing):
      1. Remove the Capture Plate from the incubator and remove the Plate Seal.
      2. Add 200 µL/well of 1X Wash Buffer.
      3. Invert the Capture Plate over an appropriate receptacle (for example, a biohazard container) and expel the contents forcibly.
      4. Firmly tap the inverted plate on a clean paper towel to dry.
      5. Repeat the wash two more times using 300 µL/well of 1X Wash Buffer.
   b. Remove all 1X Wash Buffer:
      1. Invert the Capture Plate on a clean, dry paper towel.
      2. Centrifuge at 240 x g for 1 minutes at room temperature. Use maximum acceleration and brake settings available.
   c. Transfer Pre-Amplifier Solution to a 25 mL reagent reservoir and pipet 100 µL using a multi-channel pipette into each well.
   d. Seal the Capture Plate with a Plate Seal (day 2) and incubate at 55±1℃ for 60 minutes.
5. Amplifier Hybridization:
   a. Repeat wash procedure from 4a and 4b on page 19.
   b. Transfer Amplifier Solution to a 25 mL reagent reservoir and pipet 100 µL using a multi-channel pipette into each well.
   c. Seal the Capture Plate with a Plate Seal (day 2) and incubate at 55±1℃ for 60 minutes.
6. Label Probe Hybridization:

   Note: if you are using a single incubator, adjust the temperature to 50±1℃. Verify the temperature using a temperature validation kit.

   a. Prepare Label Probe Working Reagent:
      1. Centrifuge Label Probe briefly to collect contents at the bottom of the tube.
      2. Add 11 µL of Label Probe to 11 mL of Label Probe Diluent
      3. Invert to mix and keep at room temperature until use.
   b. Repeat wash procedure from 4a and 4b on page 19.
   c. Transfer Label Probe Working Solution to a 25 mL reagent reservoir and pipet 100 µL using a multi-channel pipette into each well.
   d. Seal the Capture Plate with a Plate Seal (day 2) and incubate at 50±1℃ for 60 minutes.

e. During this incubation remove 2.0 Substrate from 4°C and allow it to warm to room temperature.

7. Add Substrate and Detect signal:
   a. Repeat wash procedure from 4a and 4b on page 19.
   b. Add 100 µL of Substrate to each well of the Capture Plate.
   c. Seal the Capture Plate with a Plate Seal (day 2) and incubate at room temperature for 5 minutes protected from light.
   d. 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 of adding Substrate.

Analyze results

Calculating assay precision

The coefficient of variation (CV) is a measure of assay precision. QuantiGene™ Singleplex 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 mean signal of technical replicates.
3. Calculate the standard deviation among technical replicate signals.
4. Calculate the %CV by dividing the standard deviation by the mean.

Calculating assay limit of detection

Calculate assay limit of detection (LOD) as follows: LOD=AVG + 3*STDEV, where AVG is the average of assay background control signals, and STDEV is the standard deviation 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 average assay background signal from the average signal of technical replicates.
3. Use one of the following methods:
   a. Plot background-subtracted average signal versus the amount of sample used. A straight line (R^2 >0.95) indicates you are operating in the linear range of the assay

b. Calculate the ratio of background-subtracted average 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 average 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 average assay background signal from the average signal of technical replicates.
2. Divide the background-subtracted average signals by the background-subtracted average signal of the housekeeping RNA. Note: if multiple housekeeping RNAs are measured, the geometric mean of background-subtracted average 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 a sample of interest versus a reference sample:

1. Normalize gene expression data as described above.
2. Divide the normalized value for the sample of interest (treated) by the normalized value for the reference sample (untreated).

Troubleshooting

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	Label Probe in Label Probe diluent.
Incorrect incubation temperature	Verify incubation temperatures using a QuantiGene™ Incubator Temperature Validation Kit.
Inappropriate hybridization temperature	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	Check for expiration dates on product labels.
Luminometer does not have the required sensitivity	Only use luminometers that meet or exceed the minimum performance specifications.

Troubleshooting non-uniform signal across the plate

Probable Cause	Recommended Action
Temperature gradients within the incubator	Verify that the incubator maintains a constant, even temperature. 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.

(continued)

Probable Cause	Recommended Action
Incomplete sealing during overnight hybridization	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.

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	Check for expiration dates on product labels.
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.

Troubleshooting well-to-well variation (high intra-plate 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 ℃ 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.

Troubleshooting day-to-day variation (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 Substrate.
Non-constant time between addition of 2.0 Substrate and plate read	Make sure that time between addition of Substrate and plate read is consistent.

A Sample optimization protocol

Optimizing sample input

Optimal QuantiGene™ Singleplex 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. 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:

Sample Type	Recommendation	Test Range
Cultured cells	400 cells/µL Working Lysis Mixture	200, 400, and 800 cells/µL Working Lysis Mixture
Tissue	5 mg tissue/300 µL Working Homogenization Solution	2.5, 5.0, and 10mg tissue/300 µL Working Homogenization Solution

2. For each lysate or tissue homogenate, prepare a 2 to 4-fold serial dilution to determine the assay performance, as determined by calculating the LOD (limit of detection, LOQ (limit of quantification), Assay linearity, and %CV (coefficient of variation). Please refer to the Glossary for detailed calculation instructions.

3. Calculate the assay performance for each sample/dilution to determine which one had the best performance and use that amount of cells or tissue for future experiments. The QuantiGene™ Singleplex Assay has a linear dynamic range of >3.5 logs and can detect 200 copies of target RNA.

B

Automated capture plate washing

Bio-Tek ELx405R washer settings

Program 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 and after the Pre-Amplifier and Amplifier hybridizations. Use Link 2 to wash the Capture Plates after the Label Probe hybridization. Please note automated washing of plates will require additional wash buffer than what is provided in the assay kit.

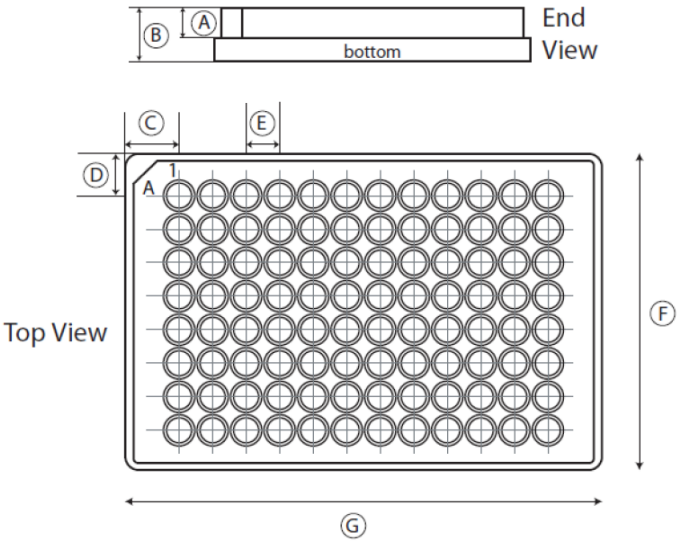
ELx405R Washer Settings:

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

(continued)

Parameter	Program		
	D3	44	45
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
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



Ⓐ 10.8 mm, Well depth
Ⓑ 14.3 mm, Plate height
Ⓒ 14.0 mm, Well A1 x-offset
Ⓓ 11.2 mm, Well A1 y-offset
Ⓔ 9.0 mm, Well-to-well offset
Ⓕ 85.5 mm, Plate width
Ⓖ 127.8 mm, Plate length

Plate map

QuantiGene Singleplex 96-well plate map

This plate map is for samples (cell lysates, tissue homogenates, purified RNA, etc.) in triplicate with 15 samples in total.

	1	2	3	4	5	6	7	8	9	10	11	12
A	BKD	BKD	BKD	S4	S4	S4	S8	S8	S8	S12	S12	S12
B	S1	S1	S1	S5	S5	S5	S9	S9	S9	S13	S13	S13
C	S2	S2	S2	S6	S6	S6	S10	S10	S10	S14	S14	S14
D	S3	S3	S3	S7	S7	S7	S11	S11	S11	S15	S15	S15
E	BKD	BKD	BKD	S4	S4	S4	S8	S8	S8	S12	S12	S12
F	S1	S1	S1	S5	S5	S5	S9	S9	S9	S13	S13	S13
G	S2	S2	S2	S6	S6	S6	S10	S10	S10	S14	S14	S14
H	S3	S3	S3	S7	S7	S7	S11	S11	S11	S15	S15	S15

target probe
hkg probe

D

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.

Biological hazard safety

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

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  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

    Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

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