User Manual

QuantiGene® Plex DNA Assay

P/N 17353 Rev.A 0911015

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Introduction

About This User Manual

Who This Manual is For

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

- Cultured cells
- Fresh, frozen, or formalin-fixed, paraffin-embedded (FFPE) tissues
- Genomic DNA

What This Manual Covers

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

- Assay terminology and data analysis guidelines
- QuantiGene Plex DNA assay
- Troubleshooting

Safety Warnings and Precautions

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

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

Contacting Affymetrix

Technical Help

For technical 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.panomics.com.

Table 1.1 Contacting Affymetrix

Location	Contact Information
North America	1.877.726.6642 option 3 pqbhelp@affymetrix.com
Europe	+39.02.95.360.250 techsupport_europe@affymetrix.com
Asia	+86.10.59208157 techsupport_asia@affymetrix.com

About the QuantiGene Plex DNA Reagent System

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

- *QuantiGene Plex DNA Assay Kit.* Contains the generic reagents, plates and seals required for running the assay.
- *QuantiGene DNA 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 genomic DNA samples.

Introduction

The QuantiGene Plex DNA Reagent System is designed to quantitate, in a single well, multiple target-specific DNA molecules (copy number variation) present in:

- Cultured cell lysates
- Fresh or frozen tissue homogenates
- FFPE animal tissue homogenates
- Genomic DNA

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

QuantiGene Plex DNA Assay Specifications

Table 1.2 QuantiGene Plex DNA Assay Specifications

Item	Value
Limit of Detection (LOD)	10,000 copies/well[a]
Limit of Quantitation (LOQ)	20,000 copies/well[b]
Linearity	2.5 logs[c]
Plex Level	3-34 plex
Sample Types	Cultured cells, fresh/frozen tissue (plant or animal), FFPE Tissues
Plate Format	96 well[d]
Automation Compatibility	Yes[e]

[a] *Defined as signal greater than background plus three standard deviations of the background.*

[b] *Defined as the signal just above the lowest signal that obtains an 80-120% spike recovery.*

[c] *Defined as the assay window that consistently achieves a 80-120% accuracy of fold change.*

[d] *Contact Affymetrix for information on a 384-well version.*

[e] *Batch or full automation using standard automation equipment. Contact Technical Support for protocol details and assistance in setting up your automation system.*

QuantiGene Plex DNA Assay Basics

Assay Technology

The QuantiGene Plex DNA assay combines branched DNA (bDNA) signal amplification and multi-analyte profiling beads (xMAP®) technologies to enable the detection and quantitation of multiple DNA targets simultaneously.

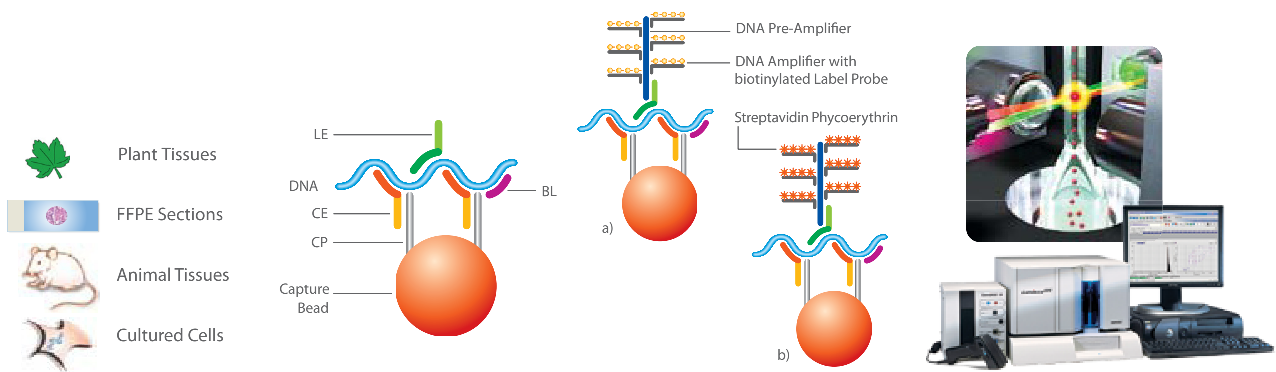
The bDNA assay is a hybridization-based method of target-specific DNA quantitation that amplifies signal rather than target DNA.

The xMAP system combines a flow cytometer, fluorescent-dyed microspheres (beads), lasers and digital signal processing to allow multiplexing of up to 100 unique assays within a single sample. The QuantiGene Plex DNA assay is compatible with all Luminex-based instruments currently available.

Assay Principal

The QuantiGene Plex DNA assay utilizes fluorescent microspheres (Capture Beads) as a support to capture specific DNA molecules. The ability to quantify multiple target-specific DNA molecules in a single sample lies in the design of the Probe Sets. For each DNA 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 DNA, is provided. The CEs discriminate among the different Capture Beads within the bead array while capturing, via cooperative hybridization, the target DNA.

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. Signal is reported as median fluorescence intensity (MFI) and is proportional to the number of target DNA molecules present in the sample.



Step 1: Release Target DNA

Cells are lysed to release DNA. DNA is sheared, (if necessary) and denatured.

Step 2: Target DNA Capture

Specific DNA fragments are captured to their respective beads through a Capture Extender (CE) Capture Probe (CP) interaction during an overnight hybridization at 54°C.

Step 3: Signal Amplification

a) Sequential hybridization of the DNA Pre-Amplifier and DNA Amplifier and biotinylated Label Probe, respectively, for an hour at 50°C.

b) Binding with Streptavidin-conjugated Phycoerythrin (SAPE) at room temperature for 30 minutes.

Step 4: Detection

The sample is analyzed on a Luminex* instrument. The level of SAPE fluorescence is proportional to the amount of DNA targets captured by the bead.

* Bio-Plex suspension array system or other Luminex-based array systems.

QuantiGene Plex DNA Reagent System Contents and Storage Conditions

QuantiGene Plex DNA Assay Kit

The components of the QuantiGene Plex DNA Assay Kit and their recommended storage conditions are listed below. The QuantiGene Plex DNA 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 DNA Assay Kit is supplied in 3 separate parts, based on storage temperature.

Table 1.3 QuantiGene Plex DNA Assay Kit Components and Storage Conditions

Component	Description	Storage
Proteinase K[a]	Proteinase K in aqueous buffered solution	–20 °C
Blocking Reagent	Aqueous buffered solution containing a preservative	–20 °C
Label Probe	Biotinylated oligonucleotide in aqueous buffered solution	–20 °C
DNA Pre-Amplifier	DNA in aqueous buffered solution	–20 °C
DNA Amplifier	DNA in aqueous buffered solution	–20 °C
Amplifier Diluent	Aqueous buffered solution containing protein and a preservative	2-8 °C
Label Probe Diluent	Aqueous buffered solution containing protein and a preservative	2-8 °C
SAPE	Streptavidin-conjugated R-Phycoerythrin	2-8 °C
SAPE Diluent	Aqueous buffered solution containing protein and a preservative	2-8 °C
Lysis Mixture	Aqueous buffered solution containing a preservative	15-30 °C
Neutralization Buffer	Aqueous buffered solution	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 Plates	96-well microplate	15-30 °C
Plate Seals	Adhesive backed foil plate sealer	15-30 °C
Hybridization Plate	96-well, clear polypropylene plate	15-30 °C
Pressure Seals	Clear, pressure-activated seal for use with the overnight hybridization plate	15-30 °C

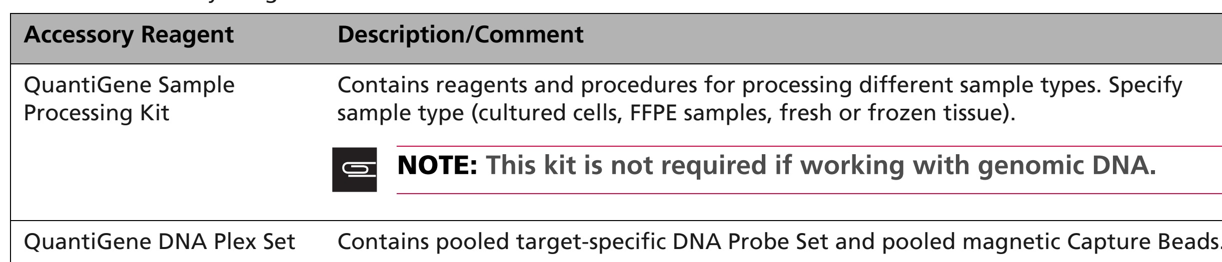
[a] *We recommend storing in an enzyme storage box, such as the NEB Cool Box (New England Biolabs P/N T0400S). NEVER store at –80 °C.*

Accessory Reagents

In addition to QuantiGene Plex DNA Assay Kits, 1-2 additional assay modules are required to perform QuantiGene Plex DNA assays.

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

Table 1.4 Accessory Reagents



Accessory Reagent	Description/Comment
QuantiGene Sample Processing Kit	Contains reagents and procedures for processing different sample types. Specify sample type (cultured cells, FFPE samples, fresh or frozen tissue). **NOTE:** This kit is not required if working with genomic DNA.
QuantiGene DNA Plex Set	Contains pooled target-specific DNA Probe Set and pooled magnetic Capture Beads.

Required Reagents and Equipment Not Provided

Reagents and Equipment

Table 1.5 Reagents and Equipment Not Provided

Item	Source
Adjustable single- and multi-channel precision pipettes for dispensing 1–20 µL, 20–200 µL and 200–1,000 µL	Major Laboratory Supplier (MLS)
Reagent reservoirs: 25 mL capacity 100 mL capacity	VWR (P/N 89094-662 or equivalent) Corning Costar (P/N CLS 4873 or equivalent)
Sonicator 4000 (for processing microplates), or Sonicator XL-2000 1/8'' single probe (for microcentrifuge tube)	Misonix (P/N 431MPX) Misonix (P/N XL-2000)
96-tube rack (if processing microplates)	Qiagen (P/N 19560 and 19566)
Microcentrifuge	Eppendorf 5415D or equivalent
Microplate centrifuge that can achieve 240 x g	Eppendorf 5804R and rotor A-2-DWP or equivalent
Vortex mixer	MLS
Nuclease-free water	MLS
Sodium hydroxide pellets	Sigma (P/N 480848) or equivalent
Microcentrifuge tubes	MLS
ELx405 Select Magnetic Plate Washer (recommended for higher throughput applications) or, Hand-held Magnetic Plate Washer (recommended for lower throughput applications)	BioTek (P/N ELx405UCWVSM) Affymetrix (P/N QP0702)
Shaking incubator with microplate adaptor, capable of maintaining constant temperatures of 50 °C ± 1 °C and 54 °C ± 1 °C	Panomics P/N QP0700 (120V), (holds 6 plates) or Thermo Scientific MaxQ 4450 Digital (holds 6 plates) P/N SHKE4450, 30100 and 30175 (6 required) or LabNet VorTemp 56 (holds 2 plates) P/N S-0256-Q

Table 1.5 Reagents and Equipment Not Provided

Item	Source
Microplate shaker, capable of maintaining a speed of 800 rpm, 3 mm orbit	Labline model 4625 or equivalent
Luminex or Luminex-based instrument	MiraiBio, Bio-Rad or other Luminex instrument provider
Sample Needle Height Align Kit	Luminex P/N CN-0015-01
QuantiGene Incubator Temperature Validation Kit	Panomics (P/N QS0517)
4'' soft, rubber roller	Panomics (P/N QS0515)

Assay Terminology and Data Analysis Guidelines

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 DNA 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 DNA assay.

Assay Precision

The Coefficient of Variation (CV) is a measure of assay precision. QuantiGene Plex DNA 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 signal (AVG) of technical replicates for each target DNA.
3. Calculate the standard deviation (SD) of signals from technical replicates for each target DNA.
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 DNA:

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

Assay signals below LOD should not be used to draw quantitative conclusions about DNA copy number.

Limit of Quantification (LOQ)

LOQ is the lowest MFI that exhibits acceptable accuracy of fold change (see *Assay Linearity/Accuracy of Fold Change* on page 7).

Assay Linearity/Accuracy of Fold Change

Assay linearity is defined as all dilutions that exhibit an accuracy of fold change between 80 and 100%.

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

NOTE: Quantifiable signals are those signals within the assay's linear range.

Table 2.6 Ratio of Background-Subtracted AVG MFI fir Each Target DNA

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			

Guidelines for Assay Optimization, Assay Design, and Data Analysis

Overview

This section provides guidelines for the following:

- Optimizing Sample lysis
- Optimizing Sample input
- Assay controls
- Assay replicates
- Calculation of DNA copy number (relative and absolute)

Optimizing Lysis Conditions

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. Recommendations are summarized below. To ensure optimal lysis, in the initial experiment, run a test range as indicated in the table.

Table 2.7 Recommended sample amount for preparation

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

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.

   Incomplete or poor lysis will produce high assay CV, poor linearity, and poor LOD for all analytes in the plex panel.

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.

To determine the optimal sonication conditions for shearing DNA:

Following the procedure for determining optimal lysis, test different sonication settings for shearing DNA in cell, tissue, or genomic DNA samples. For genomic DNA, the optimal DNA fragment size is 500 base pairs as verified by gel electrophoresis.

Optimizing Sample Input for QuantiGene Plex DNA Assay

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 DNA assay.

- Resulting signal from the sample is above the LOQ. The LOQ is 20,000 DNA copies/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 has contains no sample.
- Resulting signal from the sample is around 100 mean fluorescent intensity (MFI). The Luminex 200 exhibits a range of 2-20,000 MFI at low PMT settings. Hence, 100 MFI should be sufficient to determine DNA copy number variation from 1-100 copies/cell.

Assay Controls

All experiments must have the following controls for determining DNA copy number:

- *Assay background*. A sample well that contains all the assay components except for the sample. The background control is also used to determine data that is below the LOD. Data below the LOD should not be used in downstream calculations for drawing quantitative conclusions.
- *Control or reference sample*. A sample whose DNA copy number has been verified for all targeted genes.
- *Normalization gene.* A target gene with a constant DNA copy number for all assay conditions including the control or reference sample.

Assay Replicates

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

Calculating DNA Copy Number

- Run the reference sample and test samples on the same plate.
- Include the normalization gene as a target in the multiplex panel.

NOTE: The QuantiGene Plex assay can resolve 1 copy/cell differences ranging from 1-4 copies/cell. Since the difference of 3-4 copies is 1/4 copy or 25%, the average assay CV of 15% can resolve a 25% difference. For 7-8 copies, the difference is 1/8 copy or 12% and thus the assay CV must be at least 5% to distinguish 7 copies from 8 copies.

NOTE: If the DNA copy number is not known for a reference sample, relative fold changes can be used to estimate the copy number.

An example is provided to demonstrate how to calculate DNA copy number. In this experiment, a background, reference, and 3 samples are tested using a 4-plex panel containing a normalization gene and 3 test genes. The example shows how to calculate the DNA copy number for the three test genes.

To calculate the DNA copy number:

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)	4.3	6.3	3	5
Reference sample	59.8	85.8	51.2	52.3
Test sample 1	71.7	49.5	127.7	39
Test sample 2	62	92.8	107	5.5
Test sample 3	55	20	52	89.5

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
Reference sample	55.5	79.5	48.2	47.3
Test sample 1	67.4	43.2	124.7	34
Test sample 2	57.7	86.5	104	0.5
Test sample 3	50.7	50	49	84.5

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
Reference sample	1	1.43	0.87	0.85
Test sample 1	1	0.64	1.85	0.5
Test sample 2	1	1.5	1.8	0.01
Test sample 3	1	0.99	0.97	1.67

4. For each test sample, divide the normalized signal by the reference sample for each test gene. This corrects for the differences of detection efficiency between the test genes in the multi-gene analysis.

Sample	Normalization Gene	Test Gene 1	Test Gene 2	Test Gene 3
Background (no sample)	na	na	na	na
Reference sample	1	1	1	1
Test sample 1	1	0.45	2.13	0.59
Test sample 2	1	1.05	2.08	0.01
Test sample 3	1	0.69	1.11	1.96

5. Based on the known copy number of each gene in the reference sample, take that number and multiply it by the normalized data in step 4.

Sample	Normalization Gene	Test Gene 1	Test Gene 2	Test Gene 3
Known copy number of reference sample	2	2	2	1
Reference sample	2	2	2	1
Test sample 1	2	0.89	4.26	0.59
Test sample 2	2	2.09	4.15	0.01
Test sample 3	2	1.38	2.23	1.96

6. *Optional.* Round to nearest whole number to obtain copy number/target/cell.

Sample	Normalization Gene	Test Gene 1	Test Gene 2	Test Gene 3
Known DNA copy number/cell of reference sample	2	2	2	1
Reference sample	2	2	2	1
Test sample 1	2	1	4	1
Test sample 2	2	2	4	0
Test sample 3	2	1	2	2

QuantiGene Plex DNA Assay Procedure

About the Assay Procedure

A general outline of the assay procedure is as follows:

Sample Preparation

Refer to the appropriate QuantiGene Sample Processing Kit Package Insert for sample type-specific instructions for preparing cultured cell lysates or tissue homogenates. Follow standard laboratory methods for purification of DNA.

Use samples immediately in QuantiGene or QuantiGene Plex assays, or store at –80 °C until use.

Instrument and Equipment Setup

- Setup Luminex instrument
- Validate Luminex instrument setup and operation
- Validate shaking incubator setup and operation
- Setup and operation of BioTek ELx 405 Magnetic Plate Washer

Target DNA Capture (Day 1)

- Dilute samples, if appropriate
- Sonicate samples to fragment DNA
- Denature samples in the presence of DNA Probe Set
- Prepare Working Bead Mix
- Dispense Working Bead Mix, samples and controls into Hybridization Plate
- Hybridize samples overnight

Signal Amplification and Detection of Target DNA (Day 2)

- Transfer samples to Magnetic Separation Plate, and wash away unbound material
- Sequentially hybridize the DNA Pre-Amplifier, DNA Amplifier, Label Probe and SAPE
- Analyze samples using a Luminex-based instrument

Instrument and Equipment Setup

Luminex Setup and Operation

Follow the manufacturer's recommended protocol for general operation and maintenance for your instrument. See *Alternative Magnetic Plate Washers* on page 33 for information on cleaning maintenance of your instrument.

The QuantiGene Plex DNA assay is optimized for use at the low sensitivity setting. The sensitivity setting is performed during calibration. Refer to the table in *Setting Up Luminex Instrument for QuantiGene Plex DNA Assays* on page 35 for information on setting up your Luminex instrument.

IMPORTANT: Refer to the QuantiGene DNA Plex Set Product Insert for the target—bead region associations for your panel.

Adjusting Luminex Probe Height for Use with Magnetic Separation Plates

If you are running assays on your Luminex instrument that use both Magnetic Separation Plates and Filter Plates, it is critical that you verify/adjust the probe height, for each plate type, before reading.

To adjust the probe height:

1. Place 2 x 5 mm discs (from the sample needle height align kit) into H12 well position of the Affymetrix Magnetic Separation Plate.
2. In the needle adjustment setting, set the well used for needle adjustment to the H12 position. For Bio-Plex software, the needle adjustment setting will move the plate so that the needle hits the H12 position.
3. Adjust the needle as described in the instruction manual provided with your Luminex instrument.

Validating Luminex Instrument Calibration and Setup

! **IMPORTANT: Perform this procedure before you run the assay, to ensure the Luminex instrument is setup correctly for your QuantiGene Plex DNA assay.**

To verify the instrument calibration and setup:

1. Setup the Luminex instrument according to the guidelines in *Setting Up Luminex Instrument for QuantiGene Plex DNA Assays* on page 35. Confirm that the Luminex Probe height is adjusted for use with magnetic separation plates.
2. Define a protocol with the appropriate bead regions and set to read 2 wells.

   ! **IMPORTANT: Refer to the QuantiGene DNA Plex Set Package Insert for the target—bead associations for your panel.**

3. Vortex Capture Beads at maximum speed for 30 seconds.
4. Add 2.5 µL of Capture Beads to 250 µL of SAPE Wash Buffer. Vortex to mix.
5. Add 100 µL of the Capture Bead mixture into each of 2 wells on the Magnetic Separation Plate.
6. Place plate on the magnetic plate washer and perform a total of 15 washes to simulate a real assay.
7. Add 130 µL SAPE Wash Buffer per well. Shake for 2 minutes at 800 rpm.
8. Insert the Magnetic Separation Plate into the instrument and read the 2 wells.
9. View the window with the bead regions and DD gate. The expected results are:
   - Signals for the expected beads show up on the bead map
   - Average bead count is greater than 50/region
   - Single peak in the DD gate window with signals within the set DD gate region.

Setting up and Validating the Recommended Shaking Incubators

This procedure is for the VorTemp 56 incubator (holds up to 2 plates).

To setup the VorTemp 56 incubator and to validate the temperature:

1. Place an inverted plate lid into each of the two plate carriers in the VorTemp Shaking Incubator.

! **IMPORTANT: With the inverted plate lids in place, the Vortemp digital display and the actual temperature measured by the QuantiGene Incubator Validation Kit thermocouple (inserted into the mock hybridization plate) may differ by 4 °C or more.**

2. Set the shaking speed to 600 rpm.

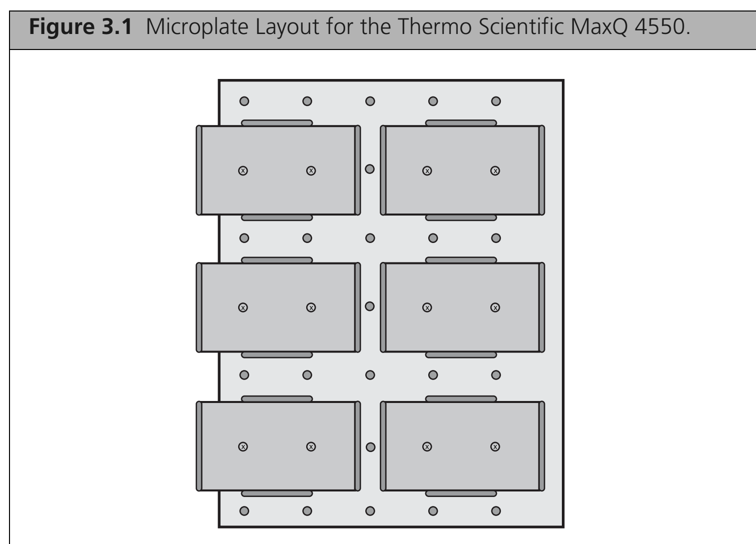
3. Set the temperature for the assay.
4. Validate the temperature with the QuantiGene Incubator Validation Kit
5. If running a single plate, place another sealed plate in the second position for balance.
6. Validate the temperature at least once per month.

The following procedure is for the Thermo Scientific MaxQ 4450 Digital Shaking Incubator (holds up to 6 plates).

To setup the Thermo Scientific MaxQ 4450 incubator and to validate the temperature:

1. Verify that the plate holders are assembled in the format shown in Figure 3.1.

Figure 3.1 Microplate Layout for the Thermo Scientific MaxQ 4550.



2. Set the shaking speed to 300 rpm.
3. Set the temperature for the assay.
4. Validate the temperature with the QuantiGene Incubator Validation Kit.
5. Validate the temperature at least once per month.

Setting Up and Operating the BioTek ELx 405 Magnetic Plate Washer

Recommendations

- Follow the manufacturer's instructions for detailed operating and maintenance procedures.
- For priming, prepare at least 200 mL each of QuantiGene Plex Wash Buffer and 200 mL of SAPE Wash Buffer.
- Use Affymetrix Magnetic Separation Plates (provided in the QuantiGene Plex DNA Assay Kit and also sold separately).
- Reserve Buffer A container for QuantiGene Plex Wash Buffer and SAPE Wash Buffer.
- Reserve Buffer B container for distilled water (for rinsing).

IMPORTANT: Separate purchase of additional SAPE Wash Buffer and Wash Buffer components will be necessary to accommodate the larger priming volume required for the BioTek Elx 405 magnetic plate washer.

Creating a Wash Program

NOTE: Instructions on how to create wash, soak, and link programs can also be found in the BioTek ELx 405 Magnetic Plate Washer user guide.

IMPORTANT: Always follow the vendors update procedures for creating a magnetic plate wash program.

To create a wash program:

1. Create SOAK 01 program.

   Go to the LED window on the washer and follow the sequence Main Menu >Define >Create > Soak > Select Soak Program >Enter Program Name > 60 Seconds.

2. Create WASH 01 program.

   Go to the LED window on the washer and follow the sequence Main Menu >Define >Create > Wash > Select Wash Program >Enter Program Name > Method, Dispense, Aspirate and then enter then following data:

   - Wash buffer = buffer A
   - Plate type = 96
   - Cycles = 3
   - Soak = 15 sec
   - Dispense volume = 100 µL
   - Dispense flow rate = 1
   - Dispense height = 120
   - Horizontal dispense position = -30
   - Horizontal Y dispense position = 00
   - Aspirate height = 46
   - Horizontal aspirate position = -45
   - Horizontal Y aspirate position = 10
   - Aspirate rate = 01
   - Aspiration delay = 0000 msec
   - Crosswise aspiration = No
   - Final aspiration = Yes
   - Aspiration delay = 0000 msec

3. Create LINK 01 program. This links the SOAK 01 to the WASH 01 program to create the QuantiGene Plex wash program.

   Go to the LED window on the washer and follow the sequence Main Menu >Define >Create > More > Link > Enter SOAK 01 > Enter WASH 01.

   IMPORTANT: The wash program settings provided are a very good starting point, however there can be unit to unit variations that may impact performance. We have found the most critical settings to be the Horizontal Aspiration Position and the Horizontal Y Aspiration Position. If residual volume of buffer is greater than 10 µL per well for a 96-well plate, adjust Horizontal Aspiration Position and the Horizontal Y Aspiration Position.

Washing QuantiGene Plex Assay Plates with BioTek ELx 405 Magnetic Plate Washer

To wash plates:

1. Turn on the plate washer.
2. Run a RINSE followed by a PRIME_200 program with distilled water in Buffer B container.
   A. To RINSE, go to Main Menu >Run > Maint >Day_Rinse >Rinse.
   B. To PRIME, go to Main Menu >Run >Prime >Prime_200 (default set by the manufacturer).
3. Place QuantiGene Wash Buffer in Buffer A container.
4. Run another PRIME_200 program.
5. Select the QuantiGene Plex wash program.
   Go to Main Menu >More >LINK 01 (as defined in *Creating a Wash Program* on page 16)
6. Place Magnetic Separation Plate on top of the plate holder and press Start.
7. When switching to SAPE Wash Buffer, place it in Buffer A container and repeat steps 4-5.
8. When finished washing, place distilled water in Buffer A container and run the PRIME_200 program 3 times.

Capturing Target DNA (Day 1)

About Capturing Target DNA

This section provides two protocols for capturing target DNA based on sample type:

- Cultured cell lysate
- Fresh, frozen, FFPE tissue homogenates, or genomic DNA preparations

Refer to the appropriate protocol for your sample type.

Capturing Target DNA from Cultured Cell Lysate

> **IMPORTANT: Cell lysate must be prepared using the QuantiGene Sample Processing Kit for Cultured Cells.**

To capture target DNA from cultured cell lysates:

1. Prepare the following reagents:
   - NaOH

     > **WARNING: Explosion hazard. Never add water to NaOH pellets. May cause burns. Use caution. Dissolution of NaOH in water is exothermic.**

     A. Prepare 10 M NaOH by slowly dissolving 4 g of NaOH pellets in 6 mL nuclease-free water.

     B. Adjust the volume to 10 mL using nuclease-free water.

     C. Store at room temperature (good for up to 1 year).

     D. Weekly, prepare 2.5 M NaOH solution by adding 1 mL of 10 M NaOH to 3 mL nuclease-free water.
   - DNA Probe Set and Blocking Reagent. Thaw, vortex briefly to mix, then centrifuge briefly to collect contents at the bottom of the tube.
   - Capture Beads. Take out of 4 °C storage right before use and protect from light.
   - Proteinase K. Take out of -20 °C storage right before use and place on ice.
   - Cultured cell lysates. If frozen, thaw at room temperature followed by incubation at 37 °C for 30 minutes. For tubes, vortex briefly, for plates, pipette up and down 5 times, then leave at room temperature until use.

     > **IMPORTANT: Do not put samples back on ice.**

2. Pre-warm Lysis Mixture at 37 °C for 30 minutes followed by gentle swirling.
3. If appropriate, dilute cell lysates with diluted Lysis Mixture (1 volume Lysis Mixture plus 2 volumes nuclease-free water) so that the desired amount of sample is present in 58 µL/well. For triplicates with overage, prepare 260 µL/sample.

> **IMPORTANT: Always verify that assay signals are within both the instrument and assay linear ranges. For more information, see** ***Guidelines for Assay Optimization, Assay Design, and Data Analysis* on page 8**.

4. Shear samples with one of the following methods:

   Method 1:

   A. Transfer 260 µL of sample into 1.5 mL microcentrifuge tube.

B. Using a Misonix XL-2000 sonicator with a 1/8 inch probe, power output = 6, sonicate samples for 10 seconds. The tip of the probe should be approximately 5 mm below the solution level.

C. Wash the tip between samples by sonicating in sterile distilled water for 10 seconds.

Method 2 (for high-throughput shearing with microplate homogenization):

A. Transfer samples into a 96-tube rack (Qiagen P/N 19566 and 19560).

B. Using a Misonix 4000 sonicator with microplate horn (Misonix 431 MPX), amplitude = 100%, sonicate for 6 minutes. Follow manufacturer's instructions to avoid overheating.

NOTE: Shearing will not affect relative fold change. A similar result is obtained if you normalize to the normalization gene and reference sample.

Table 3.8 Effect of Shearing on Relative Sensitivity of the Assay

Shearing Method	Relative MFI Signal
Unsheared[a]	10
Sheared with microplate horn	80
Sheared with single probe sonicator	100

[a] Unsheared samples refer to samples prepared using the QuantiGene Sample Processing Kit for Cultured Cells.

5. For each assay well, mix the following reagents:
   - 58 µL cell lysate (at 400 cells/µL)
   - 5 µL DNA Probe Set
   - 5 µL 2.5 M NaOH solution

   For triplicates, prepare enough sample for 4 wells to provide overage.

   Include 3 assay wells for background control by replacing cell lysate with Diluted Lysis Mixture (1 volume Lysis Mixture + 2 volumes nuclease-free water).
6. Incubate samples at room temperature for 30 minutes.
7. Add 12 µL Neutralization Buffer per assay well.
8. 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 appropriate overage. Use the table below as a guide.

IMPORTANT: Vortex (at maximal speed) Capture Beads for 30 seconds before addition.

Table 3.9 Working Bead Mix

Order of addition	Reagent	1 Well (µL)	48 Wells[a] (µL)	96 Wells[a] (µL)
1	Nuclease-free water	3.1	208	417
2	Lysis Mixture	13.7	921	1841
3	Blocking Reagent	2	134	269
4	Proteinase K	0.2	13	27
5	Capture Beads	1	67	134
Total		20	1343	2688

[a] Includes 40% overage to enable use of reagent reservoir and multichannel pipettor.

9. Vortex Working Bead Mix for 30 seconds to mix, then dispense into the Hybridization Plate.

   For fewer than 48 wells:

Using a single channel pipette and a new tip for each transfer, dispense 20 µL Working Bead Mix into each well of the Hybridization Plate.

For 48 wells or more:

A. Using a single channel pipette, transfer Working Bead Mix to a 25-mL reagent reservoir.

NOTE: Do not pour or reagent shortage will occur.

B. Using a multichannel pipette and new tips for each transfer, dispense 20 µL of Working Bead Mix into each well of the Hybridization Plate.

IMPORTANT: Include 3 wells for assay background control.

10. Using a new pipette tip for each transfer, add 80 µL neutralized cell lysate to each well of the Hybridization Plate containing Working Bead Mix.

IMPORTANT: Add 80 µL of Background Control, prepared in step 5, to 3 wells.

11. Seal the Hybridization Plate using a Pressure Seal:

A. Center the Pressure Seal on the Hybridization Plate.

B. Using a soft-rubber roller, apply firm, even pressure across the seal.

Alternatively, if using Heat-Sealing Foils (purchased separately, Affymetrix P/N QP0542) and an ABgene heat sealer (ABGene AB 0384or AB 0563/1000):

A. Center the seal on the Hybridization Plate with white side facing up and silver side contacting the surface of the plate.

B. Place the Hybridization Plate squarely onto the accessory plate carrier.

C. Press down firmly to seal for 5 full seconds.

D. Turn plate 180 degrees and repeat step c.

12. Place the Hybridization Plate into the shaking incubator.

Incubate for 18-22 hours at 54 °C ± 1 °C and appropriate shaking rpm.

IMPORTANT: For correct setup and operation of recommend shaking incubators, refer to *Setting up and Validating the Recommended Shaking Incubators* on page 14.

Capturing Target DNA from Fresh, Frozen, or FFPE Tissue, or Genomic DNA

> **IMPORTANT:** Tissue homogenates must be prepared using the QuantiGene Sample Processing Kit for tissue.

To capture target DNA from tissue homogenates or genomic DNA:

1. Prepare the following reagents:
   - NaOH:

     > **WARNING:** Explosion hazard. Never add water to NaOH pellets. May cause burns. Use caution. Dissolution of NaOH in water is exothermic.

     A. Prepare 10 M NaOH by slowly dissolving 4 g of NaOH pellets in 6 mL nuclease-free water.

     B. Adjust the volume to 10 mL using nuclease-free water.

     C. Store at room temperature (good for up to 1 year).

     D. Weekly, prepare 2.5 M NaOH solution by adding 1 mL of 10 M NaOH to 3 mL nuclease-free water.
   - DNA Probe Set and Blocking Reagent. Thaw, vortex briefly to mix, then centrifuge briefly to collect contents at the bottom of the tube.
   - Capture Beads. Take out of 4 °C storage right before use and protect from light.
   - Proteinase K. Take out of -20 °C storage right before use and place on ice.
   - Tissue homogenates. If frozen, thaw at room temperature followed by incubation at 37 °C for 30 minutes. For tubes, vortex briefly, for plates, pipette up and down 5 times, then keep at room temperature until use.
   - DNA samples. If frozen, thaw on ice. Vortex briefly before use.
2. Pre-warm Lysis Mixture at 37 °C for 30 minutes followed by gentle swirling.
3. 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.

   If appropriate, dilute genomic DNA in nuclease-free water so that the desired amount is present in a volume of 40 µL/well.

   > **IMPORTANT:** Always verify that assay signals are within both the instrument and assay linear ranges. For more information, see *Guidelines for Assay Optimization, Assay Design, and Data Analysis* on page 8.

4. Shear samples to an average size of 500 base pairs. The fragment size of the DNA can be verified by agarose gel electrophoresis.

   > **NOTE:** Shearing is not required for FFPE or plant homogenates prepared with Affymetrix Sample Processing Kit.

   Shearing can be performed with one of the following methods:

   Method 1:

   A. Transfer samples into 1.5 mL microcentrifuge tubes.

   B. Using a Misonix XL-2000 sonicator with a 1/8 inch probe, power output = 6, sonicate samples for 10 seconds. The tip of the probe should be approximately 5 mm below the solution level.

   C. Wash the tip between samples by sonicating in sterile distilled water for 10 seconds.

   Method 2 (for high-throughput shearing with microplate homogenization):

   A. Transfer samples into a 96-tube rack (Qiagen P/N 19566 and 19560).

B. Using a Misonix 4000 sonicator with microplate horn (Misonix 431 MPX), amplitude = 100%, sonicate for 6 minutes. Follow manufacturer's instructions to avoid overheating.

NOTE: Shearing will not affect relative fold change. A similar result is obtained if you normalize to the normalization gene and reference sample.

Table 3.10 Effect of Shearing on Relative Sensitivity of the Assay Using Genomic DNA

Shearing Method	Relative MFI Signal
Unsheared	10
Sheared with microplate horn	80
Sheared with single probe sonicator	100

Table 3.11 Effect of Shearing on Relative Sensitivity of the Assay Using Fresh or Frozen Tissue

Shearing Method	Relative MFI Signal
Unsheared[a]	40
Sheared with microplate horn	100
Sheared with single probe sonicator	100

[a] *Unsheared samples refer to samples prepared using the QuantiGene Sample Processing Kit for Tissues.*

5. For each assay well, mix the following reagents:
   - 40 µL sample
   - 18 µL Lysis Mixture
   - 5 µL Probe Set
   - 5 µL 2.5 M NaOH solution

   For triplicates, prepare enough sample for 4 wells to provide overage.

   Include 3 assay wells for background control by replacing tissue homogenate with homogenizing solution or water if analyzing purified genomic DNA.
6. Incubate at room temperature for 30 minutes.
7. Add 12 µL Neutralization Buffer per assay well.
8. 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 appropriate overage. Use the table below as a guide.

IMPORTANT: Vortex (at maximal speed) Capture Beads for 30 seconds before addition.

Table 3.12 Working Bead Mix

Order of addition	Reagent	1 Well (µL)	48 Wells[a] (µL)	96 Wells (µL)
1	Nuclease-free water	1.8	121	242
2	Lysis Mixture	15	1008	2016
3	Blocking Reagent	2	134	268
4	Proteinase K	0.2	13	27
5	Capture Beads	1	67	134
Total		20	1343	2687

[a] *Includes 40% overage to enable use of reagent reservoir and multichannel pipettor.*

9. Vortex Working Bead Mix for 30 seconds to mix, then dispense into the Hybridization Plate.

For fewer than 48 wells:

Using a single channel pipette and a new tip for each transfer, dispense 20 µL Working Bead Mix into each well of the Hybridization Plate.

For 48 wells or more:

A. Using a single channel pipette, transfer Working Bead Mix to a 25-mL reagent reservoir.

NOTE: Do not pour or reagent shortage will occur.

B. Using a multichannel pipette and new tips for each transfer, dispense 20 µL of Working Bead Mix into each well of the Hybridization Plate.

IMPORTANT: Include 3 wells for assay background control.

10. Using a new pipette tip for each transfer, add 80 µL neutralized tissue homogenate or genomic DNA to each well of the Hybridization Plate containing Working Bead Mix.

IMPORTANT: Add 80 µL of Background Control, prepared in step 5, to 3 wells.

11. Seal the Hybridization Plate using a Pressure Seal:

A. Center the Pressure Seal on the Hybridization Plate.

B. Using a soft-rubber roller, apply firm, even pressure across the seal.

Alternatively, if using Heat-Sealing Foils (purchased separately, Affymetrix P/N QP0542) and an ABgene heat sealer (ABGene AB 0384or AB 0563/1000):

A. Center the seal on the Hybridization Plate with white side facing up and silver side contacting the surface of the plate.

B. Place the Hybridization Plate squarely onto the accessory plate carrier.

C. Press down firmly to seal for 5 full seconds.

D. Turn plate 180 degrees and repeat step c.

12. Place the Hybridization Plate into the shaking incubator.

Incubate for 18-22 hours at 54 °C ± 1 °C and appropriate shaking rpm.

IMPORTANT: For correct setup and operation of recommend shaking incubators, refer to *Setting up and Validating the Recommended Shaking Incubators* on page 14.

Signal Amplification and Detection of DNA Targets (Day 2)

About this Procedure

These instructions are for processing one 96-well plate using multichannel pipettes, reagent reservoirs, and a magnetic plate washer. To process fewer or more wells, scale reagents accordingly.

Before You Start

Bring Amplifier Diluent, Label Probe Diluent, and SAPE Diluent to room temperature. **Warm Amplifier Diluent to 37 °C for 20 minutes** to dissolve any precipitates, and mix well by inversion before use.

Prepare Wash Buffer

To prepare Wash Buffer:

1. Add to a 250-mL graduated cylinder, in this order:
   - 150 mL nuclease-free water
   - 0.6 mL Wash Buffer Component 1
   - 10 mL Wash Buffer Component 2

   Bring the volume to 200 mL with nuclease-free water.

   NOTE: Scale preparation according to the number of plates to be processed and the prime volume required for setup of the operation of the magnetic plate washer. For each plate, plan for 160 mL Wash Buffer (this does not include priming volume).

2. Transfer to a 250-mL bottle and invert to mix.

   Do not store unused Wash Buffer. Make Wash Buffer fresh daily.

Prepare Magnetic Plate Washer

Prepare the BioTek Magnetic Plate washer as described in *Setting Up and Operating the BioTek ELx 405 Magnetic Plate Washer* on page 15.

For other magnetic plate washers, see *Alternative Magnetic Plate Washers* on page 33.

If you are using the hand-held magnetic washer (for low throughput use), refer to the package insert for operating details.

IMPORTANT Precautions

IMPORTANT: Avoid splashing and cross-contamination during all wash steps.

IMPORTANT: Minimize the exposure of Capture Beads to room light.

Hybridizing the DNA Pre-Amplifier

To hybridize the DNA Pre-Amplifier:

1. Prepare DNA Pre-Amplifier Working Reagent:
   - A. Centrifuge the DNA Pre-Amplifier briefly to collect the contents at the bottom of the tube.
   - B. Add 36 µL of DNA Pre-Amplifier to 12 mL of Amplifier Diluent.

C. Invert several times to mix.

> **IMPORTANT: The Amplifier Diluent is a viscous solution. Pipette carefully to ensure that the entire contents are expelled from the pipette and that the working reagent is completely transferred to the reagent reservoir.**

2. Transfer the overnight hybridization mixture to the Magnetic Separation Plate:

A. Remove the Hybridization Plate from the shaking incubator, and centrifuge at 240 x g for one minute.

> **IMPORTANT: Adjust temperature of shaking incubator to 50 °C ± 1 °C. Verify temperature using a QuantiGene Incubator Temperature Validation Kit.**

B. Pipette up and down 5 times, then completely transfer the hybridization mixture to the Magnetic Separation Plate.

C. Immediately proceed to the next step.

3. Wash away the unbound sample using the magnetic plate washer:

A. Place the Magnetic Separation Plate on the plate holder of the plate washer.

B. Select the appropriate program.

C. Click **Start**.

D. When the wash is complete, immediately proceed to the next step.

> **IMPORTANT: Wash program takes approximately 5 minutes for an entire plate. Do not allow plate to dry as this will result in high background.**

4. Start the DNA Pre-Amplifier hybridization:

A. Transfer DNA Pre-Amplifier Working Reagent to a 25 mL-capacity reagent reservoir.

B. Add 100 µL of DNA Pre-Amplifier Working Reagent to each assay well.

C. Seal the Magnetic Separation Plate with a foil Plate Seal.

D. Shake at 800 rpm for 1 minute at room temperature to ensure beads are resuspended.

E. Place the Magnetic Separation Plate into a recommended shaking incubator, and incubate for 1 hour at 50 °C ± 1 °C and appropriate shaking rpm.

> **IMPORTANT: For correct setup and operation of recommend shaking incubators, refer to** ***Setting up and Validating the Recommended Shaking Incubators* on page 14**.

Hybridizing the DNA Amplifier

To hybridize the DNA Amplifier:

1. Prepare DNA Amplifier Working Reagent:

A. Centrifuge the DNA Amplifier briefly to collect the contents at the bottom of the tube.

B. Add 36 µL of DNA Amplifier to 12 mL of Amplifier Diluent.

C. Invert several times to mix.

2. Wash away the unbound DNA Pre-Amplifier using the magnetic plate washer:

A. Remove the Magnetic Separation Plate from the shaking incubator.

B. Remove the foil Plate Seal.

C. Place the Magnetic Separation Plate on the plate tray of the plate washer.

D. Select the appropriate program.

- **E.** Click **Start**.
- **F.** When the wash is complete, immediately proceed to the next step.

3. Start the DNA Amplifier hybridization:

- **A.** Transfer DNA Amplifier Working Reagent to a 25 mL-capacity reagent reservoir.
- **B.** Add 100 µL of DNA Amplifier Working Reagent to each assay well.
- **C.** Seal the Magnetic Separation Plate with a foil Plate Seal.
- **D.** Shake at 800 rpm for 1 minute at room temperature to ensure beads are resuspended.
- **E.** Place the Magnetic Separation Plate into a recommended shaking incubator, and incubate for 1 hour at **50 °C ± 1 °C** and 600 rpm.

Hybridizing the Label Probe

To hybridize the Label Probe:

1. Prepare the Label Probe Working Reagent:

- **A.** Centrifuge Label Probe briefly to collect the contents at the bottom of the tube.
- **B.** Add 36 µL of Label Probe to 12 mL of Label Probe Diluent.
- **C.** Vortex for 15 seconds to mix.

2. Wash away the unbound DNA Amplifier using the magnetic plate washer:

- **A.** Remove the Magnetic Separation Plate from the shaking incubator.
- **B.** Remove the foil Plate Seal.
- **C.** Place the Magnetic Separation Plate on the plate tray of the plate washer.
- **D.** Select the appropriate program.
- **E.** Click **Start**.
- **F.** When the wash is complete, immediately proceed to the next step.

3. Hybridize the Label Probe:

- **A.** Transfer the Label Probe Working Reagent to a 25 mL-capacity reagent reservoir.
- **B.** Add 100 µL of the Label Probe Working Reagent to each assay well.
- **C.** Seal the Magnetic Separation Plate with a foil Plate Seal.
- **D.** Shake at 800 rpm for 1 minute at room temperature to ensure beads are resuspended.
- **E.** Place the Magnetic Separation Plate into a recommended shaking incubator, and incubate for 1 hour at **50 °C ± 1 °C** and 600 rpm.

Binding the SAPE

To bind SAPE:

1. Prepare the SAPE Working Reagent:

- **A.** Briefly vortex SAPE to mix, then briefly centrifuge to collect the contents at the bottom of the tube.
- **B.** Add 36 µL of SAPE to 12 mL of SAPE Diluent.
- **C.** Vortex for 15 seconds to mix, and protect from light.

2. Wash away the unbound Label Probe using the magnetic plate washer:

- **A.** Remove the Magnetic Separation Plate from the shaking incubator.
- **B.** Remove the foil Plate Seal.
- **C.** Place the Magnetic Separation Plate on the plate tray of the plate washer.

D. Select the appropriate program.
E. Click **Start**.
F. When the wash is complete, immediately proceed to the next step.

3. Bind the SAPE:
   A. Transfer the SAPE Working Reagent to a 25 mL-capacity reagent reservoir.
   B. Add 100 µL of the SAPE Working Reagent to each assay well.
   C. Seal the Magnetic Separation Plate with a foil Plate Seal.
   D. Completely wrap the Magnetic Separation Plate with aluminum foil.
   E. Place on a shaking platform at **room temperature** and shake at 800 rpm for 1 minute followed by 600 rpm for **30 minutes**.

Detecting the Signal

To detect the signal:

1. Wash away the unbound SAPE using the magnetic plate washer:
   A. Replace QuantiGene Plex Wash Buffer with SAPE Wash Buffer in Buffer A container.
   B. Prime with SAPE Wash Buffer.
   C. Remove the Magnetic Separation Plate from the shaking platform.
   D. Remove the foil Plate Seal.
   E. Place the Magnetic Separation Plate on the plate tray of the plate washer.
   F. Select the appropriate program.
   G. Click **Start**.
   H. When the wash is complete, immediately proceed to the next step.
2. Prepare the plate for analysis on a Luminex instrument.

   IMPORTANT: Verify the probe height in the Luminex instrument is set appropriately for use with Magnetic Separation Plates. See *Adjusting Luminex Probe Height for Use with Magnetic Separation Plates* on page 14.

   A. Add 130 µL of SAPE Wash Buffer to each assay well.
   B. Seal the Magnetic Separation Plate with a foil Plate Seal.
   C. Wrap the Magnetic Separation Plate in aluminum foil.

   NOTE: At this point, the plate can be stored at room temperature in the dark for up to 2 hours or at 4 °C for 24 hours (without shaking). Proceed to the next step when you are ready to read the plate.

   D. Place on a shaking platform at **room temperature** and 800 rpm for 2-3 minutes, then read immediately.

   IMPORTANT: If running more than 1 plate at a time, leave the 2nd plate at room temperature in the dark (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 2-3 minutes, then read immediately.

   NOTE: See *Setting Up Luminex Instrument for QuantiGene Plex DNA Assays* on page 35 for setting up the Luminex reader.

Troubleshooting

Low Assay Signal or Poor Sensitivity:

Table 4.13 Troubleshooting Low Assay Signal or Poor Performance

Probable Cause	Recommended Actions
Number of DNA target copies below limit of detection	Increase the sample input and/or perform a recommended method for DNA shearing.
Incomplete cell lysis and/or DNA shearing	See *Guidelines for Assay Optimization, Assay Design, and Data Analysis* on page 8.
Signal amplification reagent is incorrectly prepared	Carefully add the correct amounts of DNA Pre-Amplifier, DNA Amplifier, Label Probe and SAPE to the appropriate Diluent and mix thoroughly
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.
Instrument needle is partially clogged	Replace or clean needle according to the manufacturer's recommendations.
Significant DNA degradation	Run a 1% agarose gel to verify DNA integrity.
Incomplete DNA denaturation	Use freshly prepared NaOH.

High Background Signal

Table 4.14 Troubleshooting High Background Signal

Probable Cause	Recommended Actions
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)

Table 4.15 Troubleshooting Low Assay Precision (High CV)

Probable Cause	Recommended Actions
Inaccurate pipetting	▪ Use only calibrated, precision pipettes ▪ Affix tips securely ▪ Use a new tip for each transfer ▪ Pipette slowly and carefully, avoiding bubbles
Residual Wash Buffer	Set the magnetic washer so that there is only 5–10 µL of residual volume for each wash step.
Non-homogenous 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	See *Guidelines for Assay Optimization, Assay Design, and Data Analysis* on page 8
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

Table 4.16 Troubleshooting Low Bead Count

Probable Cause	Recommended Actions
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.
Instrument needle is partially clogged	Replace or clean needle according to the manufacturer's recommendations.
Luminex or BioPlex system is clogged	Refer to the troubleshooting guide provided with the system.
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.

Poor Assay Linearity

Table 4.17 Troubleshooting Poor Assay Linearity

Probable Cause	Recommended Actions
Incomplete cell lysis	See *Guidelines for Assay Optimization, Assay Design, and Data Analysis* on page 8
Inadequate sample preparation	Refer to the appropriate sample processing kit product inserts for detailed procedures and troubleshooting.
Instrument saturation	Signals >20,000 MFI on Luminex instruments are saturated.
Assay saturation	Perform serial dilution of sample to ensure appropriate fold change is observed. See *Guidelines for Assay Optimization, Assay Design, and Data Analysis* on page 8 for more information.

Alternative Magnetic Plate Washers

Magnetic Plate Washer

This appendix provides information for the Bio-Rad, Bio-Plex Pro, Pro II and Tecan HydroFlex magnetic plate washers.

Recommendations

- Follow the manufacturer's instructions for detailed operating and maintenance procedures.
- For priming, prepare at least 40 mL of QuantiGene Plex Wash Buffer and 80 mL of SAPE Wash Buffer for priming. Two primes of 40 mL each are required for the SAPE Wash Buffer.
- Use Affymetrix Magnetic Separation Plates. Use of alternative plates may negatively impact assay performance.
- Reserve Liquid 1 container (connected to channel 1) for QuantiGene Plex Wash Buffer and SAPE Wash Buffer.
- Reserve Liquid 2 container (connected to channel 2) for distilled water for rinsing.

Setup and Operation

Setting Up and Operating the Plate Washer

1. Add QuantiGene Plex Wash Buffer to Liquid 1 container and distilled water to Liquid 2 container.
2. Turn on the magnetic plate washer.
3. Run a **Rinse** followed by **Prime** step.
4. Select the **QGPlex** program.

   If the magnetic plate washer does not have the QGPlex program, see *Creating a Program* on page 33.
5. Click **Start**.
6. Select the column of sample wells, or entire plate, to be washed.
7. When switching from QuantiGene Plex Wash Buffer to SAPE Wash Buffer, put SAPE Wash Buffer in Liquid 1 container and run the **Prime** step twice.
8. After the completion of each wash, or at the end of the day, run a **Rinse** step to prevent clogging of the aspirate/dispense needles.
9. Before shutting down, replace Liquid 1 container with distilled water and run the **Prime** step 3 times.

Creating a Program

If the Bio-Rad Bio-Plex Pro, Bio-Plex Pro II, or Tecan HydroFlex wash station does not have a QGPlex program, use the Bio-Plex Pro II Hydro Control software to create one.

To create a program:

1. Select **New program** from the **File** menu and name the file **QGPlex**.
2. Select **Plate mode**.

3. Set/verify plate parameters. Go to Plate > managing plate > define plate >select default plate or create new plate >edit >plate definition >plate parameter.

Table A.1 Plate Parameters

Plate Definition (Plate Parameter)	Bio-Rad, Bio-Plex Pro Wash Station* (μM)	Bio-Rad, Bio-Plex Pro Wash Station† (μM)	Tecan HydroFlex (μM)
Aspiration Y offset 1	-2200	-1700	-1700
Aspiration Y offset 2	2200	2200	2200
Dispense Y offset	2400	2400	2400
Z position bottom	4000	4000	4000
Z position overflow	15000	15000	15000

** For units that have the 96 individual magnet configuration.*
† For units that have the 9 row magnet configuration.

! **IMPORTANT: The plate parameter settings provided are a very good starting point, however there may be unit to unit variations that may impact performance. We have found the most critical settings to be the Aspirate Y offset 1. If residual volume of buffer is greater than 10 µL per well adjust the Aspirate Y offset 1 up or down.**

4. Add a **Cycle** step and set to 1.
5. Add a **Soak** step and set to **1 minute**, 1**0 sec**.
6. Add an **Aspirate** step and set:
   - Mode = Normal
   - Z-Position = custom, 4.2 mm[1]
   - Time = 2s
   - Head speed = 10 mm/s
   - Aspirate rate = 1
7. Add a **Cycle** step and set to 3.
8. Add a **Dispense** step and set:
   - Z-Position = Overflow
   - Volume = 100 µL
   - Channel = 1
   - Dispension rate = 300 µl/s
9. Add a **Soak** step and set to 20 sec.
10. Add an **Aspirate** step and set:
    - Mode = Normal
    - Z-Position = custom, 4.2 mm[1]
    - Time = 1s
    - Head speed = 10 mm/s
    - Aspirate rate = 1

[1] For a small number of units sold before November 2008, designated Series A, the 4.2 mm setting should be changed to 5.0 mm. If you are unsure, please contact your Bio-Rad representative

Setting Up Luminex Instrument for QuantiGene Plex DNA Assays

Instrument Settings

Calibrate the instrument using the standard PMT setting (may be referred to as low by some suppliers). The key information in this table is related to the bead calibration required and setting of the DD gate. Because of continual software updates, we recommend that you contact your Luminex instrument supplier and verify the latest recommendations for setup to magnetic xMAP/Luminex beads.

When beads are injected into the flow cell, a small percentage can clump and go through the flow cell as doublets. The DD gate of Doublet Discriminator gate allows for discrimination of doublet formation. When initially setting the DD gate, follow the recommendations in the table below. In some cases, you might need to adjust the DD gate around the largest peak which represents the singlet beads. Adjustments can be made during the processing of the first sample.

IMPORTANT: Use SAPE Wash Buffer. The bead position on the DD gate might change if the incorrect buffer is used to suspend the Capture Beads during reading.

Table B.1 Luminex Instrument Settings

Software / Instrument Platform and Supplier	Type of Bead Calibration	DD gate	Sample Size (µL)	Timeout (sec)	Bead Events/ Bead region
xPonent/Luminex v3.0	**Magnetic**[*] PROTOCOL> BEAD TYPE, select magnetic	Automatically set	100	40	100
IS100/Luminex v2.3	**Standard** NEW BATCH> CREATE ASSAY TEMPLATE>Acq. Detail, enter DD gate value	5,000–25,000	100	40	100
MasterPlex CT/MiraiBio v1.0	**Standard** ACQUISITION SET-UP>SET-UP, enter DD gate value	5,000–25,000	100	40	100
MasterPlex CT/MiraiBio v1.2	**Magnetic**[a] ACQUISITION TEMPLATE SET-UP>BEAD TYPE,select Magnetic	Automatically set	100	40	100
Bio-Plex 4.0/Bio-Rad	**Standard** RUN PROTOCOL> ADVANCE WINDOW SETTING, enter DD gate values	5,000–25,000	100	45	100
Bio-Plex 5.0/Bio-Rad	**Standard** SELECT ANALYTE> EDIT PANEL, select MagFlex	Automatically set	100	45	100
Applied Station/Applied Cytometry	**Standard** TEMPLATE SET-UP> enter DD gate value	5,000–25,000	100	40	100

[*] *You must use MagPex calibrator beads for calibration (MagPlex Calibration Beads, Luminex P/N MCAL1-05 or Affymetrix P/N PC0622 and MagPlex Control Beads, Luminex P/N MCON1-05 or Affymetrix P/N PC0623).*

Recommended Maintenance for Luminex Instruments

About Luminex Maintenance

For optimal results, we strongly recommend that you clean the sample probe/needle on a regular basis according to the manufacturer's recommendations. Probe/needles that have rusty or salt deposits should be replaced (Luminex P/N CN-0007-01).

After Each Run

Clean the probe/needle after each run as indicated in the following table.

Table C.1 Steps for Cleaning the Probe After Each Run

Step	Number of Times	Solution
Sanitize	2x	20% bleach
Backflush	3x	none
Alcohol wash	3x	70% ethanol
Wash	4x	Distilled water

Before Shut Down

Clean the probe/needle before shutting down the instrument as indicated in the following table.

Table C.2 Steps for Cleaning the Probe/Needle

Step	Number of Times	Solution
Sanitize	2x	20% bleach
Backflush	3x	none
Alcohol wash	3x	70% ethanol
Wash	4x	Distilled water
Soak	1x	Water

Blank Plate Maps

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

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
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