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qBiomarker Copy Number PCR Handbook

qBiomarker Copy Number PCR Array
qBiomarker Copy Number PCR Assay
For real-time PCR-based, copy number
alteration and variation analysis

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Array Contents

qBiomarker Copy Number PCR Arrays						
Catalog no.	337802					
Number of samples	2	12	24	4	16	64
96-well plates (A, C, D, F formats)	2	12	24	–	–	–
384-well plates (E, G formats)	–	–	–	1	4	16
Rotor-Discs (R format)	2	12	24	–	–	–
Optical Thin-Wall 8-Cap Strips (12 per plate; A, D formats only)	24	144	288	–	–	–
Optical Adhesive Film (1 per plate; C, E, F, G formats only)	2	12	24	1	4	16
Rotor-Disc® Heat Sealing Film (1 per Rotor-Disc; R format only)	2	12	24	–	–	–
384EZLoad Covers (1 set of 4 per plate; E, G formats only)	–	–	–	1 set	4 sets	16 sets
Handbook	1	1	1	1	1	1

Custom qBiomarker Copy Number PCR Arrays				
Catalog no.	337801			
Number of samples	Varies	Varies	Varies	Varies
96-well plates (A, C, D, F formats)	12	24	–	–
384-well plates (E, G formats)	–	–	6	24
Rotor-Discs (R format)	12	24	–	–
Optical Thin-Wall 8-Cap Strips (12 per plate; A, D formats only)	144	188	–	–
Optical Adhesive Film (1 per plate; C, E, F, G formats only)	12	24	6	24
Rotor-Disc Heat Sealing Film (1 per Rotor-Disc; R format only)	12	24	–	–
Handbook	1	1	1	1

Assay Contents

qBiomarker Copy Number PCR Assays	
Catalog no.	337812
Number of samples	200
qBiomarker Copy Number PCR Assay; provided in solution	200 µl
Handbook	1

Mastermix Contents

qBiomarker SYBR® Fluor Mastermix*	(2)	(12)	(24)	(8)	(25 ml)
Catalog no.	337830	337832	337833	337831	337839
Number of array reactions	2 x 96-well	12 x 96-well	24 x 96-well	4 x 384-well	2000 x 25 µl reactions
2x Mastermix, containing HotStart DNA Taq Polymerase, PCR Buffer, dNTP mix (dATP, dCTP, dGTP, dTTP), SYBR Green dye, Fluorescein passive reference dye	2 x 1.35 ml	12 x 1.35 ml	24 x 1.35 ml	8 x 1.35 ml	25 ml

* Suitable for use with the following real-time cyclers: Bio-Rad® models iCycler®, iQ™5, MyiQ™, MyiQ2.

qBiomarker SYBR ROX™ Mastermix*	(2)	(12)	(24)	(8)	(25 ml)
Catalog no.	337820	337822	337823	337821	337829
Number of array reactions	2 x 96-well	12 x 1.35 ml	24 x 96-well	4 x 384-well	200 x 25 µl reactions
2x Mastermix, containing HotStart DNA Taq Polymerase, PCR Buffer, dNTP mix (dATP, dCTP, dGTP, dTTP), SYBR Green dye, ROX passive reference dye	2 x 1.35ml	12 x 1.35 ml	24 x 1.35 ml	8 x 1.35 ml	25 ml

* Suitable for use with the following real-time cyclers: Applied Biosystems® models 5700, 7000, 7300, 7500 (Standard and Fast), 7700, 7900HT (Standard and Fast 96-well block, 384-well block), StepOnePlus™, ViiA 7 (Standard and Fast 96-well block, 384-well block); Eppendorf® Mastercycler® ep *realplex* models 2, 2S, 4, 4S; Stratagene® models Mx3000P®, Mx3005P®, Mx4000®; Takara TP-800.

qBiomarker SYBR ROX FAST Mastermix*	(2)	(12)	(24)	(8)	(25 ml)
Catalog no.	337840	337842	337843	337841	337849
Number of array reactions	2 x 96-well	12 x 1.35 ml	24 x 96-well	4 x 384-well	200 x 25 µl reactions
2x Mastermix, containing HotStart DNA Taq Polymerase, PCR Buffer, dNTP mix (dATP, dCTP, dGTP, dTTP), SYBR Green dye, ROX passive reference dye	2 x 1.35ml	12 x 1.35 ml	24 x 1.35 ml	8 x 1.35 ml	25 ml

* Suitable for use with the Applied Biosystems models 7000, 7300, 7500 (Standard and Fast), 7700, 7900HT (Standard and Fast 96-well block, 384-well block), StepOnePlus; ViiA 7 (Standard and Fast 96-well block, 384-well block); Eppendorf Mastercycler ep realplex with or without ROX filter set; Stratagene models Mx3000, Mx3500, Mx4000; Takara TP-800; Rotor-Gene® Q (QIAGEN), Rotor-Gene 6000.

Storage

qBiomarker Copy Number PCR Arrays are shipped at ambient temperature on blue ice/cold packs or on dry ice depending on the destination and accompanying products. For long-term storage, store qBiomarker Copy Number PCR Arrays at -15°C to -30°C .

qBiomarker Copy Number PCR Assays are shipped at ambient temperature but must be stored at -20°C on arrival and at -15°C to -30°C for long-term storage.

qBiomarker SYBR Mastermixes are shipped on blue ice/cold packs. For long-term storage, keep tubes at -20°C . If the entire volume will not be used at once, we recommend dividing into aliquots and storing at -20°C . Avoid repeated freezing and thawing. If stored under these conditions, qBiomarker SYBR Mastermixes are stable for 6 months after receipt.

Intended Use

qBiomarker Copy Number PCR Arrays, qBiomarker Copy Number PCR Assays, and qBiomarker SYBR Mastermixes are intended for molecular biology applications. These products are not intended for the diagnosis, prevention or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN® products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Technical Assistance

At QIAGEN, we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in sample and assay technologies and the use of QIAGEN products. If you have any questions or experience any difficulties regarding qBiomarker Copy Number PCR Arrays, qBiomarker Copy Number PCR Assays, qBiomarker SYBR Mastermixes, or QIAGEN products in general, please do not hesitate to contact us.

QIAGEN customers are a major source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at QIAGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

For technical assistance and more information, please see our Technical Support Center at www.qiagen.com/support or call one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit www.qiagen.com).

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at www.qiagen.com/us/support/qa-qc-safety-data/safety-data-sheets/ where you can find, view and print the SDS for each QIAGEN kit and kit component.

Quality Control

In accordance with QIAGEN's Quality Management System, each lot of qBiomarker Copy Number PCR Arrays, qBiomarker Copy Number PCR Assays, and qBiomarker SYBR Mastermixes is tested against predetermined specifications to ensure consistent product quality.

Introduction

qBiomarker Copy Number PCR Arrays and Assays are designed for highly sensitive detection and profiling of copy number alterations (CNAs) and copy number variations (CNVs).

CNAs are an important type of genomic aberration in cancer cells. Gene amplifications (which activate oncogenes) and deletions (which inactivate tumor suppressor genes) are important CNAs that affect cancer-related genes, in addition to other genomic changes (such as point mutations, translocations, and inversions). Most of the cancer-related genes affected by CNAs have been defined as key genes in cancer-signaling pathways involved in carcinogenesis and cancer progression.

CNVs are an important source of genetic diversity. De novo, locus-specific mutation rates appear to be much higher for CNVs than for other forms of genetic variation, such as SNPs. CNVs can cause Mendelian diseases through various molecular mechanisms such as gene dosage, gene disruption, gene fusion, and positional effect, and they can also be associated with complex diseases and traits.

CNA and CNV profiling

Quantitative analysis of CNAs or CNVs using qBiomarker Copy Number PCR Arrays is a useful tool for study of the molecular mechanisms of disease and genetic traits and provides the potential for discovery of novel biomarkers.

qBiomarker Copy Number PCR Arrays are available to target key CNAs of cancer-related genes that are known to occur in specific tissue types, gene pathways, and functional groups. The genes are selected from peer-reviewed publications based on their frequency, their function in cancer-signaling pathways, and their association with a cancer phenotype or progression. In addition, qBiomarker Copy Number PCR Arrays are available for CNV profiling that focus on key CNVs known to contribute or correlate with selected complex human diseases, genetic diseases, or traits. Each qBiomarker Copy Number PCR Array enables analysis of up to 95 genes or regions of interest (GOIs or ROIs). The arrays are compatible with most real-time PCR platforms.

Copy number assays

qBiomarker Copy Number PCR Assays provide a method for obtaining specific, accurate, reproducible, and easy-to-interpret copy number change results for an individual gene or region of interest (GOI or ROI). Every assay has been bench-verified and is ready-to-use in microarray follow-up studies, specific target screening, and related studies.

Principle and Procedure

All qBiomarker Copy Number PCR Assays are designed in unique regions of the genome. A multi-copy reference assay, the qBiomarker Multicopy Reference Copy Number PCR Assay (MRef), is included on each array. The qBiomarker Multicopy Reference Copy Number PCR Assay is also recommended for use with each qBiomarker Copy Number PCR Assay (must be ordered separately, cat. no. VPH000-0000000A). The reference assay recognizes a stable sequence that appears in the human genome over 40 times, and whose copy number is not affected or minimally affected by local genomic changes. Inclusion of this reference assay during testing allows use of the $\Delta\Delta C_T$ method to accurately make copy number calls or relative copy number change calls for specific targets (see *Appendix B: Data Analysis Using the $\Delta\Delta C_T$ Method*).

Each qBiomarker Copy Number PCR Array contains a panel of qBiomarker Copy Number PCR Assays for a set of selected GOIs or ROIs. Four replicates of each assay are included to increase the accuracy of copy number calls using statistical analysis. The arrays are available in 96-well plate, 384-well plate, and Rotor-Disc formats. A 96-well plate array and a Rotor-Disc array each contain 4 replicates of 24 genes (23 target genes plus MRef) for one sample, and a 384-well plate array can either contain 16 replicates of 24 genes (23 target genes plus MRef) for 4 samples, or 4 replicates of 96 genes (95 target genes plus MRef) for one sample. For more details, see page 19.

Description of protocols

The qBiomarker Copy Number PCR procedure (see workflow, page 13) starts with genomic DNA purification. After quality control, the DNA is mixed with the ready-to-use qBiomarker SYBR Mastermix. The mixture is aliquoted into each well of the array plate containing preloaded gene-specific primers. After real-time PCR, the copy-number profile of a particular sample is determined using the $\Delta\Delta C_T$ method, measuring the difference in ΔC_T values (target gene C_T – reference assay C_T) between a test sample genome and a reference genome (see *Appendix B: Data Analysis Using the $\Delta\Delta C_T$ Method*).

The simplicity of the qBiomarker Copy Number PCR Array format and protocol allows routine CNA and CNV profiling in any research laboratory with access to a real-time PCR cyclers.

qBiomarker Copy Number PCR workflow overview

Purify DNA using the QIAamp® DNA Mini Kit, DNeasy® Blood & Tissue Kit, or QIAamp DNA FFPE Tissue Kit (approximately 30 minutes).



Measure DNA concentration and purity by UV spectrophotometry.



Optional: Run quality-control check using DNA QC Plate.



Set up and run real-time PCR using qBiomarker Copy Number PCR Arrays or Assays (2 hours).



Analyze data using the online analysis tools (10–20 minutes).

Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

In addition to the qBiomarker Copy Number PCR Array or Assay and qBiomarker SYBR Mastermix, the following are required:

- For qBiomarker Copy Number PCR Assays: 96-well or 384-well PCR plate with Optical Thin-Wall 8-Cap Strips or Optical Adhesive Film or Rotor-Disc with Rotor-Disc Heat-Sealing Film or PCR tubes
- Purified DNA samples (see *DNA purification*, page 16)
- Spectrophotometer*, cuvettes, and nuclease-free Tris-Cl, pH 8.0, for measuring DNA quality and quantity
- Nuclease-free water (e.g., QIAGEN, cat. nos. 129114, 129115, or 129117); do not use water treated with diethylpyrocarbonate (DEPC)
- Real-time PCR cycler* (see Tables 1 and 2, page 15)
- Single- and multichannel pipets with nuclease-free tips*
- Nuclease-free microcentrifuge tubes
- Centrifuge* and rotor for PCR array plates
- Crushed ice
- **Optional:** DNA QC Plate (QIAGEN, please inquire for cat. no.) for assessing DNA quality
- **Optional:** RT² PCR Array Loading Reservoir (cat. no. 338162) to assist in loading PCR plates

* Ensure that instruments have been checked and calibrated according to the manufacturer's recommendations.

Table 1. Real-time PCR cyclers suitable for use with qBiomarker Copy Number PCR Arrays

Plate format	Plate type	Real-time PCR cyclers
A	96-well	Applied Biosystems models 5700, 7000, 7300, 7500, 7900HT (96-well block), ViiA 7 (96-well block); Bio Rad/MJ Research Chromo4™, iCycler, iQ5, MyiQ, MyiQ2; Stratagene models Mx3000P, Mx3005P; Eppendorf Mastercycler ep realplex models 2, 2S, 4, 4S; TaKaRa TP-800
C	96-well	Applied Biosystems models 7500 Fast (96-well), 7900HT Fast (96-well block), StepOnePlus, ViiA 7 Fast (96-well block)
D	96-well	Bio-Rad CFX96™; Bio-Rad/MJ Research models DNA Engine Opticon®, DNA Engine Opticon 2; Stratagene Mx4000
E	384-well	Applied Biosystems models 7900HT (384-well block), ViiA 7 (384-well block); Bio-Rad CFX384™
F	96-well	Roche® LightCycler® 480 (96-well block)
G	384-well	Roche LightCycler 480 (384-well block)
R	100-well Rotor-Disc	QIAGEN Rotor-Gene Q

Table 2. Real-time PCR cyclers suitable for use with qBiomarker Copy Number PCR Assays

Type of instrument	Real-time PCR cyclers
Instruments that use ROX as a passive reference dye	All Applied Biosystems, TaKaRa, and Stratagene real-time PCR cyclers
Instruments that use fluorescein as a passive reference dye	Bio-Rad iCycler, iQ5, MyiQ, MyiQ2
Instruments that do not require a reference dye	Bio-Rad/MJ Research DNA Engine Opticon, DNA Engine Opticon 2, and Chromo4; Roche LightCycler 480; Eppendorf Mastercycler ep <i>realplex</i> 2, 2S, 4, 4S; QIAGEN Rotor-Gene Q; other instruments that do not require a reference dye

Important Notes

General precautions

We recommend the following guidelines:

- Use sterile pipet tips with filters.
- Store and extract positive materials (specimens, positive controls, and amplicons) separately from all other reagents, and add them to the reaction mix in a spatially separated facility.
- Physically separate the workspaces used for PCR setup and post-PCR processing operations. Decontaminate your PCR workspace and labware (pipets, tube racks, etc.) with UV light before each new use to render any contaminated DNA ineffective in PCR through the formation of thymidine dimers or with 10% bleach to chemically inactivate and degrade any DNA.
- Do not remove the PCR array plate from its protective sealed bag until immediately before use.
- Thaw all components thoroughly at room temperature (15–25°C) before starting an assay.
- When thawed, mix the components (by pipetting repeatedly up and down or by pulse vortexing) and centrifuge briefly.

DNA purification

The kits from QIAGEN shown in Table 3 are recommended for genomic DNA purification from the indicated sample types for use with qBiomarker Copy Number PCR Arrays or Assays.

Table 3. DNA purification kits recommended for use with qBiomarker Copy Number PCR Assays and Arrays

Sample material	Nucleic acid isolation kit	Catalog number (QIAGEN)
Fresh or frozen tissues or cultured cells	QIAamp DNA Mini Kit	51304 or 51306
	DNeasy Blood & Tissue Kit	69504 or 69506
Formalin-fixed, paraffin-embedded (FFPE) tissues	QIAamp DNA FFPE Tissue Kit	56404

Carry out the DNA purification according to the instructions in the kit handbooks, but with the following changes to the kit protocols.

- Always perform the optional RNase A digest to remove RNA. RNA contamination will cause inaccuracies in DNA concentration measurements.
- Elute with nuclease-free water or the elution buffer provided in the DNA purification kit. Do not use water or buffers treated with diethylpyrocarbonate (DEPC).

DNA quality control

All DNA samples should show consistent quality either by UV spectrophotometric measurements or by performance in real-time PCR using the DNA QC Plate.

Measuring DNA concentration and purity by UV spectrophotometry

Dilute samples and measure absorbance in 10 mM Tris-Cl, pH 8.0. An absorbance reading of 1.0 at 260 nm in a 1 cm detection path corresponds to a DNA concentration of 50 µg/ml. All DNA samples should meet the following criteria.

- Concentration, as measured by A_{260} , should be greater than 10 µg/ml.
- A_{260}/A_{280} ratio should be greater than 1.8.
- A_{260}/A_{230} ratio should be greater than 1.7.

Measuring DNA quality with the DNA QC Plate

DNA quality and consistency can be checked more reliably with the DNA QC Plate by real-time PCR measuring 7 reference genes. For a detailed procedure, see *Appendix A: Quality Control of Genomic DNA Using the DNA QC Plate.*"

qBiomarker Copy Number PCR Array plate layouts

Each qBiomarker Copy Number PCR Array shipment includes the array plates/discs and either 12 Optical Thin-Wall 8-Cap Strips (plate formats A and D), one Optical Adhesive Film (plate formats C, E, F, and G), or one Rotor-Disc Heat-Sealing Film (disc format R) per plate/disc.

Each 384-well array (formats E and G) also includes one set of four 384EZLoad Covers (cat. no. 338125) for each plate. Each 384EZLoad Cover is for single use only.

Plate layouts

The 96-well plate layout for a 23-gene panel (1 plate for 1 sample) has 4 replicates for 23 genes and a multi-copy reference assay (MRef), arranged as shown in Figure 1.



Figure 1. Plate layout for a 23-gene panel on a 96-well plate. The numbers in the cells each represent a single well for gene 1, gene 2, etc.

The 384-well plate layout for a 23-gene panel (1 plate for 4 samples) has 16 replicates for 23 genes and a multi-copy reference assay (MRef), arranged as shown in Figure 2. A set of 4 rows contains 4 replicates for 23 genes.

The 384-well plate layout for a 95-gene panel (1 plate for 1 sample) has 4 replicates of 95 genes and a multi-copy reference assay (MRef), arranged as shown in Figure 3.

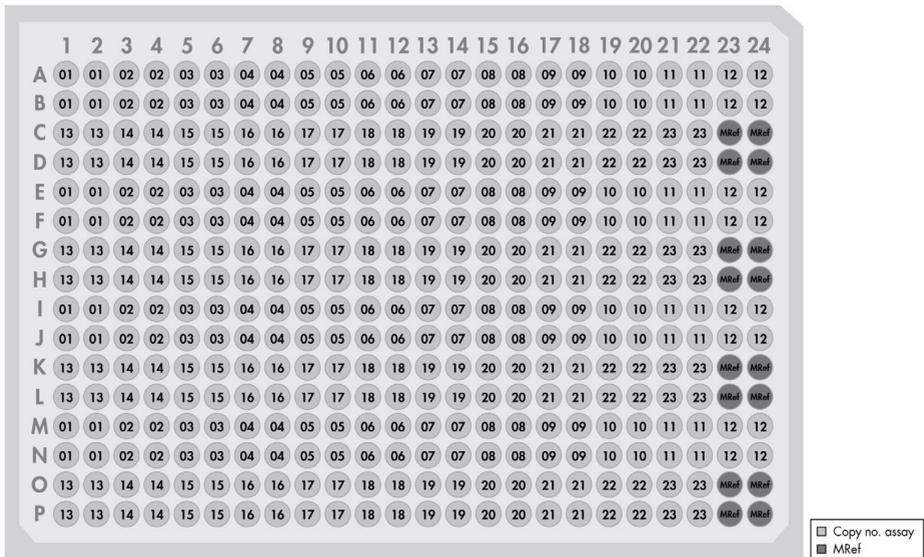


Figure 2. Plate layout for a 23-gene panel on a 384-well plate. The numbers in the cells each represent a single well for gene 1, gene 2, etc. The first set of 4 rows is repeated 3 more times so that one 384-well PCR array plate can be used to assay 4 samples.

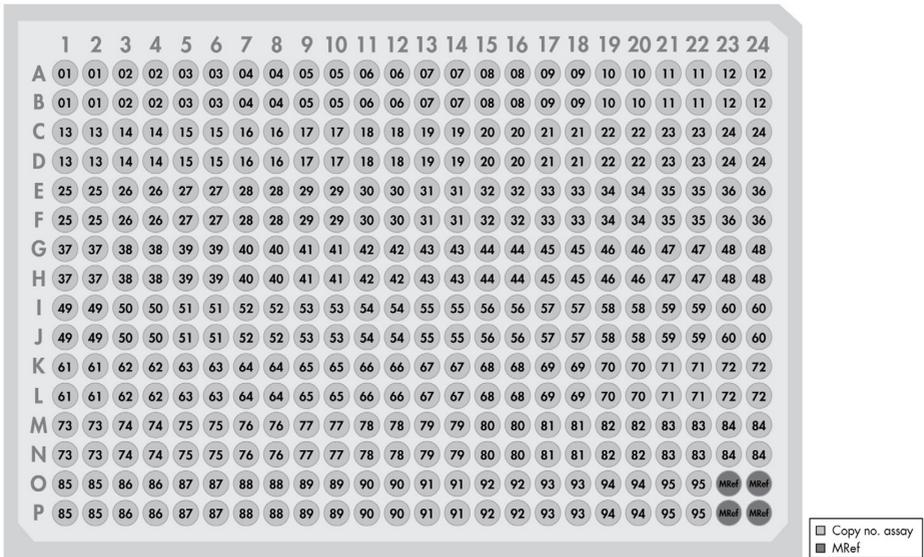


Figure 3. Plate layout for a 95-gene panel on a 384-well plate. The numbers in the cells each represent a single well for gene 1, gene 2, etc. One 384-well PCR array plate can be used to assay one sample.

The 100-well Rotor-Disc layout for a 23-gene panel (1 disc for 1 sample) has 4 replicates of 23 genes and a multi-copy reference assay (MRef), arranged as shown in Figure 4.

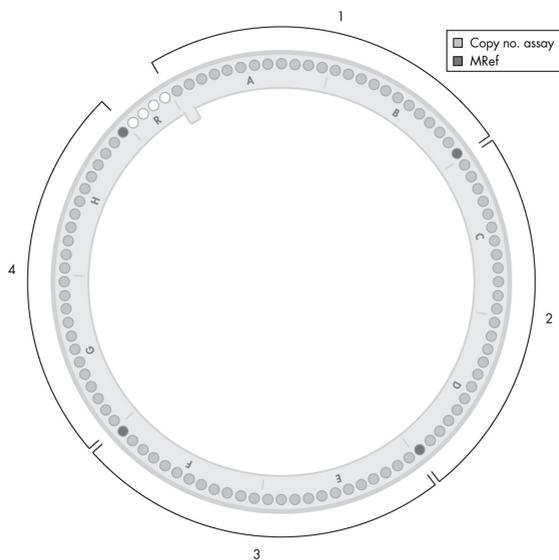


Figure 4. Disc layout for a 23-gene panel on a 100-well Rotor-Disc. One Rotor-Disc can be used to assay one sample.

Protocol: PCR Using qBiomarker Copy Number PCR Array Formats A, C, D, E, F, and G

This protocol is for use with qBiomarker Copy Number PCR Arrays in 96-well and 384-well plates. For use of qBiomarker Copy Number PCR Arrays in Rotor-Discs, see *PCR Using qBiomarker Copy Number PCR Arrays Format R*. For use of individual qBiomarker Copy Number PCR Assays, see *PCR Using qBiomarker Copy Number PCR Assays*.

Important points before starting

- Before beginning the procedure, read *Important Notes*.
- Make sure that the sample cell population contains at least 25% tumor cells for CNA profiling analysis.
- A calibrator sample is a DNA sample with known copy number for the targets of interest. When a calibrator sample or samples are used, we recommend that, if possible, the calibrator sample is from the same tissue type as the test sample. We also recommend that the DNA quality of the calibrator sample should be comparable to that of the test sample (see *Appendix A: Quality Control of Genomic DNA Using the DNA QC Plate*, for details of assessment of sample quality).

For fresh frozen test samples, DNA from a fresh frozen sample should also be used as a calibrator sample. For FFPE test samples, there are 2 options for a calibrator sample. DNA from a fresh frozen sample can be used as a calibrator sample, although the final copy number call may be largely influenced by the quality difference between the test sample and the calibrator sample. Alternatively, DNA from an FFPE sample can be used as a calibrator sample. In this case, it is preferable to include multiple calibrator samples in the study.

- Take time to familiarize yourself with the real-time PCR cycler before starting the protocol. Read the cycler user manual and follow the manufacturer's instructions for proper cycler operation and maintenance.

- The chemically modified hot-start enzyme, along with other proprietary chemical components in the qBiomarker SYBR Mastermix, is essential for successful function of the arrays. Do not replace any of these reagents with other commercial counterparts.
- If a calibrator DNA sample is included, it should be run in the same real-time PCR cycler as the test samples.
- PCR array plates should only be used in the compatible real-time PCR cyclers listed in Table 1. The PCR array plates will not fit properly into incompatible real-time PCR cyclers and may cause damage to the cycler.
- Pipetting accuracy and precision affect the consistency of results. Be sure that all pipets and instruments have been checked and calibrated according to the manufacturer's recommendations.
- Make sure that no bubbles are introduced into the wells of the PCR array during pipetting.
- Do not use DEPC-treated water. Use high-quality, nuclease-free water.
- If precipitates are present in the Mastermix tube, warm the reagents at 42°C for 1 minute and vortex briefly to dissolve. Repeat if necessary.

Procedure

1. Briefly centrifuge the qBiomarker SYBR Mastermix (10–15 seconds) to bring the contents to the bottom of the tube.

Note: As the qBiomarker SYBR Mastermix contains HotStart DNA *Taq* Polymerase that is active only after heat activation, reactions can be prepared at room temperature (15–25°C).

2. Prepare a reaction mix according to Table 4 for each sample.

Recommended amounts of genomic DNA per sample are shown in Table 5.

Table 4. Preparation of reaction mix

Array format	96-well, 23-gene panel (Formats A, C, D, F)	384-well, 23-gene panel (Formats E, G)	384-well, 95-gene panel (Formats E, G)
qBiomarker SYBR Mastermix, 2x	1350 µl	550 µl	2100 µl
Genomic DNA	0.4–1.0 µg (fresh); 0.8–1.2 µg (FFPE)	0.2–0.5 µg (fresh); 0.4–1.0 µg (FFPE)	0.8–2.0 µg (fresh); 1.6–4.0 µg (FFPE)
Nuclease-free water	Variable	Variable	Variable
Total volume	2700 µl*	1100 µl†	4200 µl‡

* This volume provides an excess of 300 µl per sample to allow for some imprecision in pipetting.

† This volume provides an excess of 140 µl per sample to allow for some imprecision in pipetting.

‡ This volume provides an excess of 360 µl per sample to allow for some imprecision in pipetting.

Table 5. Recommended amounts of genomic DNA per sample

Sample type	96-well, 23-gene panel (Figure 1)	384-well, 23-gene panel (Figure 2)	384-well, 95-gene panel (Figure 3)
Fresh tissue	0.4–1.0 µg	0.2–0.5 µg	0.8–2.0 µg
FFPE	0.8–1.2 µg	0.4–1.0 µg	1.6–4.0 µg

- Carefully remove the PCR array plate from its sealed bag.
- Recommended:** Dispense the reaction mix into a RT² PCR Array Loading Reservoir to assist in loading.
- Add reaction mix to each well of the PCR array plate as follows:
 - Note:** Perform pipetting steps as precisely as possible to ensure that each well receives the required volume.
 - Note:** Change pipet tips following each addition to avoid any cross-contamination between the wells.
 - **96-well, 23-gene panel (Formats A, C, D, F):**
Add 25 µl reaction mix to each well of the PCR array plate using a multichannel pipet with 8 tips.

- **384-well, 23-gene panel (Formats E, G):**

Add 10 µl reaction mix to each well of the PCR array plate using a multichannel pipet with 8 tips.

Each 384-well plate (23-gene panel) characterizes 4 samples in separate sets of 96-wells staggered from one another by only 1 well. The spacing between the tips of standard multichannel pipets should allow correct loading of samples into every other row and column. Be sure to load each sample into the correct set of wells using the 384EZLoad Covers provided and see Figure 5 as a guide.

Cover 1 (white) for sample 1

Cover 2 (yellow) for sample 2

A	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
B	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
C	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
D	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
E	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
F	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
G	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
H	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
I	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
J	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
K	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
L	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
M	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
N	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
O	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
P	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4

A	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
B	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
C	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
D	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
E	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
F	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
G	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
H	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
I	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
J	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
K	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
L	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
M	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
N	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
O	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
P	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4

Cover 3 (black) for sample 3

Cover 4 (red) for sample 4

A	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
B	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
C	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
D	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
E	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
F	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
G	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
H	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
I	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
J	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
K	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
L	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
M	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
N	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
O	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
P	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4

A	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
B	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
C	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
D	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
E	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
F	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
G	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
H	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
I	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
J	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
K	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
L	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
M	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
N	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
O	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
P	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4

Figure 5. Loading 384-well, 23-gene panel, formats E, G. 384EZLoad Cover 1 (white): Place 384EZload Cover 1 (white) on the plate. Add 10 µl sample 1 reaction mix to the open wells (odd-numbered wells of rows A, C, E, G, I, K, M, and O). Remove and discard the cover. 384EZLoad Cover 2 (yellow): Place 384EZload Cover 2 (yellow) on the plate. Add 10 µl sample 2 reaction mix to the open wells (even-numbered wells of rows A, C, E, G, I, K, M, and O). Remove and discard the cover. 384EZLoad Cover 3 (black): Place 384EZload Cover 3 (black) on the plate. Add 10 µl sample 3 reaction mix to the open wells (odd-numbered wells of rows B, D, F, H, J, L, N, and P). Remove and discard the cover. 384EZLoad Cover 4 (red): Place 384EZload Cover 4 (red) on the plate. Add 10 µl sample 4 reaction mix to the open wells (even-numbered wells of rows B, D, F, H, J, L, N, and P). Remove and discard the cover.

- **384-well, 95-gene panel (Formats E, G):**

Add 10 μ l reaction mix to each well of the PCR array plate using a multichannel pipet with 8 tips.

Each 384-well plate (95-gene panel) characterizes 1 sample in 4 sets of 96-wells staggered from one another by only 1 well. The spacing between the tips of standard multichannel pipets should allow you to properly load the sample into every other row and column.

6. Tightly seal the PCR array plate with the Optical Thin-Wall 8-Cap Strips (plate formats A and D) or with the Optical Adhesive Film (plate formats C, E, F, and G).
7. Remove any air bubbles in the wells of the PCR array plate by centrifuging the plate at 1000 rpm for 1 minute (96-well plate, formats A, C, D, F) or 2000 rpm for 2 minutes (384-well plate, formats E, G).
8. Place the PCR array plate on ice while setting up the PCR cycling program in step 9.
9. Program the real-time cyler according to Table 6, 7, or 8, depending on the real-time cyler used. If prompted by your cyler software, select "Absolute Quantitation" to begin.

Note: PCR array plates containing reaction mix that will not be processed immediately may be stored wrapped in aluminum foil at -20°C for up to 1 week until ready to run.

Note: Additional instrument specific setup instructions can be found under "Resources/Supplemental Protocols" at www.qiagen.com/us/shop/pcr/primer-sets/qbiomarker-copy-number-pcr-arrays/#resources.

Table 6. Cycling conditions* for Applied Biosystems, Bio-Rad,[†] Stratagene, and Eppendorf[‡] cyclers

Cycles	Duration	Temperature	Comments
1	10 min	95°C	HotStart DNA <i>Taq</i> Polymerase is activated by this heating step.
40	15 s	95°C	Perform fluorescence data collection.
	1 min	60°C	

* Recommended for the following cyclers: Applied Biosystems models 5700, 7000, 7300, 7500, 7700, 7900HT, StepOnePlus, ViiA 7; Bio-Rad models iCycler, iQ5, MyiQ, MyiQ2, CFX96, CFX384; Stratagene models Mx3000P, Mx3005P, Mx4000P; Eppendorf Mastercycler ep realplex models 2, 2S, 4, 4S.

[†] For Bio-Rad models CFX96 and CFX384: adjust the ramp rate to 1°C/s.

[‡] For Eppendorf Mastercycler ep realplex models 2, 2S, 4, and 4S: for the Silver Thermoblock, adjust the ramp rate to 26%; for the Aluminum Thermoblock, adjust the ramp rate to 35%. Refer to instrument specific setup instructions that can be found under "Resources/Supplemental Protocols" at www.qiagen.com/us/shop/pcr/primer-sets/qbiomarker-copy-number-pcr-arrays/#resources.

Table 7. Cycling conditions for Roche LightCycler 480*

Cycles	Duration	Temperature	Comments
1	10 min	95°C	HotStart DNA <i>Taq</i> Polymerase is activated by this heating step.
45	15 s	95°C	Perform fluorescence data collection.
	1 min	60°C	

* Recommended for the Roche LightCycler 480. If using a Roche LightCycler 480, adjust the ramp rate to 1°C/s. Refer to instrument specific setup instructions that can be found under "Resources/Supplemental Protocols" at www.qiagen.com/us/shop/pcr/primer-sets/qbiomarker-copy-number-pcr-arrays/#resources for more information on other required changes to settings for Melt Curve Acquisition.

Table 8. Cycling conditions for Bio-Rad and Takara cyclers and other cyclers†

Cycles	Duration	Temperature	Comments
1	10 min	95°C	HotStart DNA <i>Taq</i> Polymerase is activated by this heating step.
40	15 s	95°C	
	30-40 s	55°C	Perform fluorescence data collection. Different cyclers need different lengths of time to detect the fluorescent signal. Choose the appropriate time for the annealing step (55°C) for your cycler.
	30 s	72°C	

† Recommended for the following cyclers: Bio-Rad/MJ Research models Chromo4, DNA Engine Opticon, DNA Engine Opticon 2; Takara TP-800; all other cyclers excluding Rotor-Gene cyclers.

10. Place 1 PCR array plate in the real-time PCR cycler. Use a compression pad with the optical film-sealed plate formats (plate formats C, E, F, and G) if recommended by the manufacturer. Start the run.
11. After the run is finished, calculate the threshold cycle (C_T) for each well using the real-time cycler software, as described in the following steps.

Note: If using the Roche LightCycler 480, there are 2 options for data analysis: using the second derivate max setting (in this case, there is no need to calculate the C_T) or using “Fit Points” (in this case, the C_T should be defined manually as described in step 13).
12. Define the baseline by choosing the automated baseline option if the cycler has the adaptive baseline function. If the cycler does not have the adaptive baseline function, set the baseline manually. To set the baseline manually, use the linear view of the amplification plots to determine the earliest visible amplification. Set the cycler to use the readings from cycle number 2 up to 2 cycles before the earliest visible amplification.
13. Manually define the threshold by using the log view of the amplification plots. Choose a threshold value above the background signal but within the lower one-third to lower half of the linear phase of the amplification plot.

Note: Ensure that the threshold values are the same across all qBiomarker Copy Number PCR Array runs in the same analysis. The absolute position of the threshold is less critical than its consistent position across arrays.

14. Export the C_T values for all wells to a blank Excel® spreadsheet.

Data analysis can be conducted at QIAGEN'S GeneGlobe Data Analysis Center using a software-based tool or with a spreadsheet-based tool that can be downloaded from a QIAGEN website.

15. **Note:** The GeneGlobe Data Analysis Center is a web resource for the analysis of real time PCR or NGS data (www.qiagen.com/shop/genes-and-pathways/data-analysis-center-overview-page). To access the center, new users can register online. Once on the site, the data analysis software will be found under "Analysis." The qBiomarker Copy Number PCR Array Data Analysis spreadsheets can be found under "Product Resources/Performance Data" at www.qiagen.com/us/shop/pcr/primer-sets/qbiomarker-copy-number-pcr-arrays/#resources.

Note: If using a 384-well format, download the PCR Array 4x96 384-Well Conversion spreadsheet to dissect a 384-well dataset into the correct 4 sets of 96 genes for each of the 4 samples. For software-based data analysis, this file can be found at the GeneGlobe Data Analysis Center. For spreadsheet-based data analysis, this file can be found under "Product Resources/Performance Data" at www.qiagen.com/us/shop/pcr/primer-sets/qbiomarker-copy-number-pcr-arrays/#resources.

16. **Recommended:** Perform dissociation (melting) curve analysis to verify PCR specificity. Run a melting curve program and generate a first derivative dissociation curve for each well using the real-time cycler software. A single peak should appear in each reaction.

Note: If your cycler does not have a default melting curve program, run the following program instead: 95°C, 1 min; 65°C, 2 min (optics off); 65°C to 95°C at 2°C/min (optics on).

Note: For cycler-specific melting curve analysis settings, please refer to the Instrument Setup Guide for your cycler at www.qiagen.com/us/shop/pcr/primer-sets/qbiomarker-copy-number-pcr-arrays/#resources.

Note: Plates can be stored at -20°C wrapped in aluminum foil and melting curve analysis performed at a later time. When ready to perform melting curve analysis, warm the plate to room temperature ($15\text{--}25^{\circ}\text{C}$), place it in the real-time cycler, and run the melting curve analysis program.

Note: Visually inspect the plate after the run for any signs of evaporation from any of the wells. If evaporation is observed, note which wells are affected, as this may affect the results of data analysis.

Note: Do not open any previously processed qBiomarker Copy Number PCR Array. Removing the Optical Thin-Wall 8-Cap Strips or the Optical Adhesive Film from a qBiomarker Copy Number PCR Array releases PCR product into the air where it may contaminate and affect the results of future real-time PCR experiments.

Protocol: PCR Using qBiomarker Copy Number PCR Array Format R

This protocol is for use with qBiomarker Copy Number PCR Arrays in 100-well Rotor-Discs. For use of qBiomarker Copy Number PCR Arrays in 96-well and 384-well plates, see *PCR Using qBiomarker Copy Number PCR Arrays Formats A, C, D, E, F, and G*, page 23. For use of individual qBiomarker Copy Number PCR Assays, see *PCR Using qBiomarker Copy Number PCR Assays*, page 38.

Important points before starting

- Before beginning the procedure, read “Important Notes,” pages 16-22.
- Make sure that the sample cell population contains at least 25% tumor cells for CNA profiling analysis.
- A calibrator sample is a DNA sample with known copy number for the targets of interest. When a calibrator sample or samples are used, we recommend that, if possible, the calibrator sample is from the same tissue type as the test sample. We also recommend that the DNA quality of the calibrator sample is comparable to that of the test sample (see *Appendix A: Quality Control of Genomic DNA Using the DNA QC Plate* for details of assessment of sample quality).

For fresh frozen test samples, DNA from a fresh frozen sample should also be used as a calibrator sample. For FFPE test samples, there are 2 options for a calibrator sample. DNA from a fresh frozen sample can be used as a calibrator sample, although the final copy number call may be largely influenced by the quality difference between the test sample and the calibrator sample. Alternatively, DNA from an FFPE sample can be used as a calibrator sample. In this case, it is preferable to include multiple calibrator samples in the study.

- Only qBiomarker Copy Number PCR Array Format R and qBiomarker SYBR ROX FAST Mastermix should be used with Rotor-Gene cyclers.

- Take time to familiarize yourself with the real-time PCR cycler before starting the protocol. Read the cycler user manual and follow the manufacturer's instructions for proper cycler operation and maintenance.
- The chemically modified hot-start enzyme, along with other proprietary chemical components in the qBiomarker SYBR Mastermix, is essential for successful function of the arrays. Do not replace any of these reagents with other commercial counterparts.
- If a calibrator DNA sample is included, it should be run in the same real-time PCR cycler as the test samples.
- Pipetting accuracy and precision affects the consistency of results. Be sure that all pipets and instruments have been checked and calibrated according to the manufacturer's recommendations.
- Make sure that no bubbles are introduced into the wells of the PCR array during pipetting.
- Do not use DEPC-treated water. Use high-quality, nuclease-free water.
- If precipitates are present in the Mastermix tube, warm the reagents at 42°C for 1 minute and vortex briefly to dissolve. Repeat if necessary.

Procedure

1. Briefly centrifuge the qBiomarker SYBR Mastermix (10–15 seconds) to bring the contents to the bottom of the tube.

Note: As the qBiomarker SYBR Mastermix contains HotStart DNA Taq Polymerase that is active only after heat activation, reactions can be prepared at room temperature (15–25°C).

2. Prepare a reaction mix according to Table 9 for each sample.

For genomic DNA from fresh tissue samples, it is recommended to start with 0.4–1.0 µg DNA for each sample. Due to the fragmented nature of DNA extracted from FFPE samples, it is recommended to use 0.8–1.2 µg DNA for each sample.

Table 9. Preparation of reaction mix

Array format	Rotor-Disc 100
qBiomarker SYBR Mastermix, 2x	1 150 µl
Genomic DNA	0.4–1.0 µg (fresh) or 0.8–1.2 µg (FFPE)
Nuclease-free water	Variable
Total volume	2300 µl*

* This volume provides an excess of 300 µl per sample to allow for some imprecision in pipetting.

3. Carefully remove the PCR array Rotor-Disc from its sealed bag. Slide the Rotor-Disc into the Rotor-Disc 100 Loading Block using the tab at position A1 and the tube guide holes.

4. Add 20 µl reaction mix to each well of the Rotor-Disc. Proceed to step 5.

Note: Change pipet tips following each pipetting step to avoid cross-contamination between the wells.

Note: Reaction mix can be dispensed manually or using the QIAgility® (www.qiagen.com/goto/QIAgility).

Note: Although wells 97–100 do not contain assays, it is essential to add reaction mix for optimized balancing of the Rotor-Disc.

5. Carefully seal the Rotor-Disc with Rotor-Disc Heat-Sealing Film using the Rotor-Disc Heat Sealer.

For detailed instructions, see the *Rotor-Gene Q User Manual*.

Note: The Rotor-Disc containing PCR components mix may be stored at –20°C wrapped in aluminum foil for up to 1 week if desired.

6. Program the real-time cycler according to Table 10.

Note: Additional instrument specific setup instructions can be found under “Resources/Supplemental Protocols” at www.qiagen.com/us/shop/pcr/primer-sets/qbiomarker-copy-number-pcr-arrays/#resources.

Table 10. Cycling conditions for Rotor-Gene cyclers

Cycles	Duration	Temperature	Comments
1	10 min	95°C	HotStart DNA <i>Taq</i> Polymerase is activated by this heating step.
40	15 s	95°C	
	30 s	60°C	Perform fluorescence data collection.

7. Insert the Rotor-Disc into the Rotor-Disc 100 Rotor and secure with the Rotor-Disc 100 Locking Ring. Start the run.

For detailed instructions, see the *Rotor-Gene Q User Manual*.

8. After the run is finished, calculate the threshold cycle (C_T) for each well using the real-time cycler software. To define the baseline, select "Dynamic Tube" (default analysis setting) to ensure the average background of each well is determined just before amplification commences.

Optional: Select "Ignore First." Fluorescent signal from the initial cycles may not be representative of the remainder of the run. Thus, better results may be achieved if the initial cycles are ignored. Up to 5 cycles can be ignored.

Optional: Select "Noise Slope Correction." Selection of this option can improve data whose baseline (initial cycles) is noticeably sloped. "Noise Slope Correction" improves the data when raw data backgrounds are observed to slope upward or downward before the takeoff point (C_T).

Note: Ensure that the settings are the same across all qBiomarker Copy Number PCR Array runs in the same analysis.

9. Manually define the threshold by using the log view of the amplification plots. Choose a threshold value above the background signal but within the lower one-third to lower half of the linear phase of the amplification plot.

Note: Ensure that the threshold values are the same across all qBiomarker Copy Number PCR Array runs in the same analysis. The absolute position of the threshold is less critical than its consistent position across arrays.

10. Export the C_T values for all wells to a blank Excel spreadsheet. Data analysis can be conducted at QIAGEN'S GeneGlobe Data Analysis Center using a software-based tool or with a spreadsheet-based tool that can be downloaded from a QIAGEN website.

Note: The GeneGlobe Data Analysis Center is a web resource for the analysis of real time PCR or NGS data (www.qiagen.com/shop/genes-and-pathways/data-analysis-center-overview-page). To access the center, new users can register online. Once on the site, the data analysis software will be found under "Analysis." The qBiomarker Copy Number PCR Array Data Analysis spreadsheets can be found under "Product Resources/Performance Data" at www.qiagen.com/us/shop/pcr/primer-sets/qbiomarker-copy-number-pcr-arrays/#resources.

Note: If using a 384-well format, download the PCR Array 4x96 384-Well Conversion spreadsheet to dissect a 384-well dataset into the correct 4 sets of 96 genes for each of the 4 samples. For software-based data analysis, this file can be found at the GeneGlobe Data Analysis Center. For spreadsheet-based data analysis, this file can be found under "Product Resources/Performance Data" at www.qiagen.com/us/shop/pcr/primer-sets/qbiomarker-copy-number-pcr-arrays/#resources.

11. **Recommended:** Perform dissociation (melting) curve analysis to verify PCR specificity. Run a melting curve program and generate a first derivative dissociation curve for each well using the real-time cycler software. A single peak should appear in each reaction.

Note: Melting curve analysis can be added during creation of the Rotor-Gene Q PCR program.

Note: For Rotor-Gene Q melting curve analysis settings, refer to the setup instructions that can be found under "Resources/Supplemental Protocols" at www.qiagen.com/us/shop/pcr/primer-sets/qbiomarker-copy-number-pcr-arrays/#resources.

Note: Rotor-Discs can be stored at -20°C wrapped in aluminum foil and melting curve analysis performed at a later time. When ready to perform melting curve analysis, warm

the plate to room temperature (15–25°C), place it in the real-time cycler, and run the melting curve analysis program.

Note: Visually inspect the Rotor-Disc after the run for any signs of evaporation from any of the wells. If evaporation is observed, note which wells are affected, as this may affect the results of data analysis.

Note: Do not open any previously processed qBiomarker Copy Number PCR Array. Removing the film from qBiomarker Copy Number PCR Arrays releases PCR product into the air where it may contaminate and affect the results of future real-time PCR experiments.

Protocol: PCR Using qBiomarker Copy Number PCR Assays

This protocol is for use with qBiomarker Copy Number PCR Assays. For use of qBiomarker Copy Number PCR Arrays in 96-well and 384-well plates, see *PCR Using qBiomarker Copy Number PCR Array Formats A, C, D, E, F, G*, page 23. For use of qBiomarker Copy Number PCR Arrays in Rotor-Discs, see *PCR Using qBiomarker Copy Number PCR Arrays Format R*, page 32.

Important points before starting

- Before beginning the procedure, read “Important Notes,” pages 16–22.
- Make sure that the sample cell population contains at least 25% tumor cells for CNA profiling analysis.
- A calibrator sample is a DNA sample with known copy number for the targets of interest. When a calibrator sample or samples are used, we recommend that, if possible, the calibrator sample is from the same tissue type as the test sample. We also recommend that the DNA quality of the calibrator sample is comparable to that of the test sample (see *Appendix A: Quality Control of Genomic DNA Using the DNA QC Plate* for details of assessment of sample quality).

For fresh frozen test samples, DNA from a fresh frozen sample should also be used as a calibrator sample. For FFPE test samples, there are 2 options for a calibrator sample. DNA from a fresh frozen sample can be used as a calibrator sample, although the final copy number call may be largely influenced by the quality difference between the test sample and the calibrator sample. Alternatively, DNA from an FFPE sample can be used as a calibrator sample. In this case, it is preferable to include multiple calibrator samples in the study.

- Take time to familiarize yourself with the real-time PCR cycler before starting the protocol. Read the cycler user manual and follow the manufacturer's instructions for proper cycler operation and maintenance.
- The chemically modified hot-start enzyme, along with other proprietary chemical components in the qBiomarker Copy Number Reference Mix and qBiomarker SYBR Mastermix, is essential for successful function of the assays. Do not replace any of these reagents with other commercial counterparts.
- If a calibrator DNA sample is included, it should be run in the same real-time PCR cycler as the test samples.
- Pipetting accuracy and precision affect the consistency of results. Be sure that all pipets and instruments have been checked and calibrated according to the manufacturer's recommendations.
- Make sure that no bubbles are introduced into the wells of the PCR plate during pipetting.
- Do not use DEPC-treated water. Use high-quality, nuclease-free water.
- If precipitates are present in the Mastermix tube, warm the reagents at 42°C for 1 minute and vortex briefly to dissolve. Repeat if necessary.

Procedure

1. Briefly centrifuge the qBiomarker SYBR Mastermix (10–15 seconds) to bring the contents to the bottom of the tube.

Note: As the qBiomarker SYBR Mastermix contains HotStart DNA *Taq* Polymerase that is active only after heat activation, reactions can be prepared at room temperature (15–25°C).

2. Prepare a reaction mix according to Table 11.

For genomic DNA from fresh or frozen tissue samples, it is recommended to start with 4 ng DNA per well if setting up reactions in 96-well plates or 100-well Rotor-Discs or 2 ng DNA per well if setting up reactions in 384-well plates. Due to the fragmented nature of DNA extracted from FFPE samples, it is recommended to use 8–20 ng DNA per

well if setting up reactions in 96-well plates or 100-well Rotor-Discs or 4–10 ng DNA per well if setting up reactions in 384-well plates.

Note: It is recommended to prepare a reaction mix for a minimum of 4 replicate reactions for each sample. Prepare 5% more mix than is required to allow for imprecision in pipetting.

Table 11. Preparation of reaction mix

Format	96-well plate	Rotor-Disc	384-well plate
Number of reactions	4	4	4
qBiomarker SYBR Mastermix, 2x	50 µl	40 µl	20 µl
Genomic DNA	16 ng (fresh); 32–80 ng (FFPE)	16 ng (fresh); 32–80 ng (FFPE)	8 ng (fresh); 16–40 ng (FFPE)
qBiomarker Copy Number PCR Assay	5 µl	4 µl	2 µl
Nuclease-free water	Variable	Variable	Variable
Total volume	100 µl	80 µl	40 µl

3. Add 25 µl reaction mix to each well of a 96-well plate or 20 µl reaction mix to each well of a Rotor-Disc 100 or 10 µl reaction mix to each well of a 384-well plate using a single-channel pipet.

Note: Change pipet tips following each addition to avoid any cross-contamination between the wells.

4. Tightly seal the PCR plate with Optical Thin-Wall 8-Cap Strips or Optical Adhesive Film. If using a Rotor-Disc or Strip Tubes, tightly seal using the Rotor-Disc Heat Sealing Film or caps.
5. Remove any air bubbles in any of the plate wells by centrifuging the plate at 1000 rpm for 1 minute (96-well plate, formats A, C, D, F), 2000 rpm for 2 minutes (384-well plate, formats E, G).

This step is not necessary for Rotor-Disc, format R.

6. Place the PCR plate on ice while setting up the PCR cycling program in step 7.

7. Program the real-time cycler according to Table 12, 13, 14, or 15, depending on the real-time cycler used. If prompted by your cycler software, select “Absolute Quantitation” to begin.

Note: Additional instrument specific setup instructions can be found under “Resources/Supplemental Protocols” at www.qiagen.com/us/shop/pcr/primer-sets/qbiomarker-copy-number-pcr-arrays/#resources.

Table 12. Cycling conditions* for Applied Biosystems, Bio-Rad,† Stratagene, and Eppendorf‡ cyclers

Cycles	Duration	Temperature	Comments
1	10 min	95°C	HotStart DNA <i>Taq</i> Polymerase is activated by this heating step.
40	15 s 1 min	95°C 60°C	Perform fluorescence data collection.

* Recommended for the following cyclers: Applied Biosystems models 5700, 7000, 7300, 7500, 7700, 7900HT, StepOnePlus, ViiA 7; Bio-Rad models iCycler, iQ5, MyiQ, MyiQ2, CFX96, CFX384; Stratagene models Mx3000P, Mx3005P, Mx4000P; Eppendorf Mastercycler ep *realplex* models 2, 2S, 4, 4S.

† For Bio-Rad models CFX96 and CFX384: adjust the ramp rate to 1°C/s.

‡ For Eppendorf Mastercycler ep *realplex* models 2, 2S, 4, and 4S: for the Silver Thermoblock, adjust the ramp rate to 26%; for the Aluminum Thermoblock, adjust the ramp rate to 35%. Refer to instrument specific setup instructions that can be found under “Resources/Supplemental Protocols” at www.qiagen.com/us/shop/pcr/primer-sets/qbiomarker-copy-number-pcr-arrays/#resources.

Table 13. Cycling conditions for Roche LightCycler 480

Cycles	Duration	Temperature	Comments
1	10 min	95°C	HotStart DNA <i>Taq</i> Polymerase is activated by this heating step.
45	15 s 1 min	95°C 60°C	Perform fluorescence data collection.

Recommended for the Roche LightCycler 480. If using a Roche LightCycler 480, adjust the ramp rate to 1°C/s. Refer to instrument specific setup instructions that can be found under “Resources/Supplemental Protocols” at www.qiagen.com/us/shop/pcr/primer-sets/qbiomarker-copy-number-pcr-arrays/#resources for more information on other required changes to settings for Melt Curve Acquisition.

Table 14. Cycling conditions for Rotor-Gene cyclers

Cycles	Duration	Temperature	Comments
1	10 min	95°C	HotStart DNA <i>Taq</i> Polymerase is activated by this heating step.
40	15 s	95°C	Perform fluorescence data collection.
	30 s	60°C	

Table 15. Cycling conditions for Bio-Rad and Takara cyclers and other cyclers*

Cycles	Duration	Temperature	Comments
1	10 min	95°C	HotStart DNA <i>Taq</i> Polymerase is activated by this heating step.
40	15 s	95°C	Perform fluorescence data collection. Different cyclers need different lengths of time to detect the fluorescent signal. Choose the appropriate time for the annealing step (55°C) for your cycler.
	30-40 s	55°C	
	30 s	72°C	

* Recommended for the following cyclers: Bio-Rad/MJ Research models Chromo4, DNA Engine Opticon, DNA Engine Opticon 2; Takara TP-800; all other cyclers.

- Place the PCR plate/disc in the real-time PCR cycler. Use a compression pad with the optical film-sealed plate formats (plate formats C, E, F, and G) if recommended by the manufacturer. Start the run.
- After the run is finished, calculate the threshold cycle (C_T) for each well using the real-time cycler software.

For assays performed using a Rotor-Gene cycler, follow steps 8 and 9. For assays performed using all other cyclers, follow steps 11–13.

- Export the C_T values for all wells to a blank Excel spreadsheet. Data analysis can be conducted at QIAGEN'S GeneGlobe Data Analysis Center using a software-based tool or with a spreadsheet-based tool that can be downloaded from a QIAGEN website.

Note: The GeneGlobe Data Analysis Center is a web resource for the analysis of real time PCR or NGS data (www.qiagen.com/shop/genes-and-pathways/data-analysis-

center-overview-page). To access the center, new users can register online. Once on the site, the data analysis software will be found under “Analysis.” The qBiomarker Copy Number PCR Array Data Analysis spreadsheets can be found under “Product Resources/Performance Data” at www.qiagen.com/us/shop/pcr/primer-sets/qbiomarker-copy-number-pcr-arrays/#resources.

Note: If using a 384-well format, download the PCR Array 4x96 384-Well Conversion spreadsheet to dissect a 384-well dataset into the correct 4 sets of 96 genes for each of the 4 samples. For software-based data analysis, this file can be found at the GeneGlobe Data Analysis Center. For spreadsheet-based data analysis, this file can be found under “Product Resources/Performance Data” at www.qiagen.com/us/shop/pcr/primer-sets/qbiomarker-copy-number-pcr-arrays/#resources.

11. **Recommended:** Perform dissociation (melting) curve analysis to verify PCR specificity. Run a melting curve program and generate a first derivative dissociation curve for each well using the real-time cycler software. A single peak should appear in each reaction.

Note: If your cycler does not have a default melting curve program, run the following program instead: 95°C, 1 min; 65°C, 2 min (optics off); 65°C to 95°C at 2°C/min (optics on).

Note: For cycler-specific melting curve analysis settings, please refer to instrument specific setup instructions that can be found under “Resources/Supplemental Protocols” at www.qiagen.com/us/shop/pcr/primer-sets/qbiomarker-copy-number-pcr-arrays/#resources.

Note: Plates/discs can be stored at –20°C wrapped in aluminum foil and melting curve analysis performed at a later time. When ready to perform melting curve analysis, warm the plate/disc to room temperature (15–25°C), place it in the real-time cycler, and run the melting curve analysis program.

Note: Visually inspect the plate/disc after the run for any signs of evaporation from any of the wells. If evaporation is observed, note which wells are affected, as this may affect the results of data analysis.

Note: Do not open any previously processed qBiomarker Copy Number PCR Assay plates/discs. Removing the strips or film releases PCR product into the air where it may contaminate and affect the results of future real-time PCR experiments.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: www.qiagen.com/FAQ/FAQlist.aspx. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit www.qiagen.com).

Comments and suggestions

C_t values very high across all wells for a sample

- | | | |
|----|---------------------------|---|
| a) | Cycling program incorrect | Make sure to program the real-time PCR cycler with the temperature profile shown in the protocols. Repeat the PCR with corrected settings if necessary. |
| b) | Poor DNA quality | Check the DNA quality using the DNA QC Plate (see "Appendix A: Quality Control of Genomic DNA Using the DNA QC Plate," page 47) or on an agarose gel* to see if the DNA is degraded. If the DNA is not degraded, it could be of insufficient purity. We recommend using one of the kits indicated in Table 3, page 17, for isolation of high-quality DNA. |
| c) | Too little DNA used | Make sure that the DNA has been properly quantified. During the DNA purification process, it is essential to perform an RNase digestion. RNA contamination in the DNA sample will lead to overestimation of DNA quantity.

Note that larger amounts of DNA are recommended when working with FFPE samples. |

Potentially amplified genes have lower copy number and potentially deleted genes have higher copy number

- | | |
|----------------------|--|
| Non-recurrent events | Copy number loss of large chromosomal segments containing the potentially amplified genes can occur when the genes are unrelated to the tumor growth. Similarly, copy number gain can occur for tumor suppressor genes. Chromosomes or chromosome segments containing tumor suppressor genes that are inactivated by point mutations can also show copy number gain. |
|----------------------|--|

Comments and suggestions

Copy number is lower than expected in heterogeneous samples

Non-tumor cells in the sample In the presence of non-tumor cells that have normal diploid genomes, the copy number call is dependent on the copy number of the target gene in the cancer cells and the amount of non-tumor cells in the heterogeneous sample. If the percentage of non-tumor cells in the sample can be estimated, the copy number for a gene can be estimated based on Table 16.

Table 16. Evaluating gene copy number for cancer cells in the presence of non-tumor cells

Gene copy number of cancer cells	Expected copy number in sample with indicated percentage of normal cells				
	12.5%	25%	50%	75%	87.5%
0	0.25	0.5	1	1.5	1.75
3	2.9	2.8	2.5	2.3	2.1
4	3.8	3.5	3.0	2.5	2.3
6	5.5	5.0	4.0	3.0	2.5
8	7.3	6.5	5.0	3.5	2.8
10	9.0	8.0	6.0	4.0	3.0
12	10.8	9.5	7.0	4.5	3.3
14	12.5	11.0	8.0	5.0	3.5
16	14.3	12.5	9.0	5.5	3.8
18	16.0	14.0	10.0	6.0	4.0
20	17.8	15.5	11.0	6.5	4.3
22	19.5	17.0	12.0	7.0	4.5
24	21.3	18.5	13.0	7.5	4.8
26	23.0	20.0	14.0	8.0	5.0
28	24.8	21.5	15.0	8.5	5.3
30	26.5	23.0	16.0	9.0	5.5
32	28.3	24.5	17.0	9.5	5.8

Appendix A: Quality Control of Genomic DNA Using the DNA QC Plate

Sample DNA quality can affect the performance of qBiomarker Copy Number PCR Arrays and Assays. For DNA extracted from FFPE samples, different degrees of cross-linking and fragmentation may result in suboptimal PCR results. When uncertain of the sample quality, it is recommended to check the DNA quality first using the DNA QC Plate.

The DNA QC Plate is designed to measure the C_T values of 7 reference genes. When the DNA is highly cross-linked or fragmented, the C_T values from these 7 genes will be much higher than those from the same amount of high-quality DNA. DNA QC Plates are available in formats A, C, D, and F (96-well plates; Figure 6); formats E, G (384-well plates; see Product Sheet or Web site for plate layout); or format R (Rotor-Disc 100; see Product Sheet or Web site for plate layout). Each 96-well format DNA QC Plate can be used for quality control of 12 DNA samples. Each 384-well format DNA QC Plate can be used for quality control of 48 DNA samples. Each Rotor-Disc 100 format DNA QC Plate can be used for quality control of 12 DNA samples.

	1	2	3	4	5	6	7	8	9	10	11	12
A	BRAF											
B	KRAS											
C	HRAS											
D	NRAS											
E	MEK1											
F	PIK3CA											
G	PTEN											
H	SMPC											

Figure 6. Plate layout for DNA QC Plate, 96-well plate. The reference genes are indicated for each well.

Procedure

1. Prepare a reaction mix according to Table 17.

Note: Use the qBiomarker Probe Mastermix included with the DNA QC Plate.

Note: It is recommended to prepare a reaction mix for 8.4 replicate PCRs for each sample. Prepare more mix than is required so as to allow for imprecision in pipetting.

Table 17. Preparation of reaction mix for 8.4 replicate PCRs

Number of reactions	96-well plate	384-well plate	Rotor-Disc 100
qBiomarker Probe Mastermix, 2x (included with DNA QC Plate)	105 μ l	42 μ l	84 μ l
Genomic DNA	40 ng	16 ng	32 ng
Nuclease-free water	Variable	Variable	Variable
Total volume	210 μl	84 μl	168 μl

2. Carefully remove the DNA QC Plate from its sealed bag.
3. **Recommended:** Dispense the reaction mix into a RT² PCR Array Loading Reservoir to assist in loading.

4. Add reaction mix to each well of the DNA QC Plate as follows.
 - For 96-well plate:** add 25 μ l reaction mix.
 - For 384-well plate:** add 10 μ l reaction mix.
 - For Rotor-Disc 100:** add 20 μ l reaction mix.

Note: Change pipet tips following each addition to avoid any cross-contamination between the wells.
5. Carefully but tightly seal the DNA QC Plate with Optical Thin-Wall 8-Cap Strips (formats A and D), Optical Adhesive Film (formats C, E, F, and G), or Rotor-Disc Heat-Sealing Film (format R).
6. Remove any air bubbles in any of the plate wells by centrifuging the plate at 1000 rpm for 1 minute (96-well plate; formats A, C, D, F) and 2000 rpm for 2 min (384-well plate; formats E, G).

This step is not necessary for the Rotor-Disc, format R.
7. Place the DNA QC Plate on ice while setting up the PCR cycling program in step 8.
8. Program the thermal cycler according to the manufacturer's instructions, using the conditions outlined in Table 18.

Table 18. Cycling conditions

Cycles	Duration	Temperature	Comments
1	10 min	95°C	Initial PCR activation step
40	15 s	95°C	Denaturation step
	60 s	60°C	Annealing and extension. Detect and record FAM™ fluorescence from every well during the annealing/extension step of each cycle.

-
9. Place the DNA QC Plate in the real-time PCR cycler. Use a compression pad with the optical film-sealed plate formats (plate formats C, E, F, and G) if recommended by the manufacturer. For format R, insert the Rotor-Disc 100 into the Rotor-Disc 100 Rotor and secure with the Rotor-Disc 100 Locking Ring. Start the run.
 10. After the run is finished, calculate the threshold cycle (C_T) for each well using the real-time cycler software, as described in the following steps.

Note: Ensure the settings are the same across all PCR assay runs in the same analysis.

11. **For formats A, C, D, E, F, G:** We highly recommend manually setting the baseline and threshold values (see Table 19 for examples). To define the baseline value, use the linear view of the amplification plots and set the instrument to use the readings from cycle 5 up to 2 cycles before the earliest visible amplification, usually around cycle 15, but not greater than cycle 20.

To define the threshold value, use the log view of the amplification plots and place the threshold value above the background signal but within the lower half to one-third of the linear phase of the amplification plot.

12. **For format R:** To define the baseline value, select "Ignore First." Manually define the threshold value by using the log view of the amplification plots. Select a threshold value above the background signal. The threshold value should be in the lower half to one-third of the linear phase of the amplification plot. A threshold setting of 0.03 is recommended as a reference.

Fluorescent signal from the initial cycles may not be representative of the remainder of the run. Therefore, better results may be achieved if the initial cycles are ignored. Up to 5 cycles can be ignored.

Table 19 shows typical settings for selected real-time PCR cyclers. Actual settings may differ.

Table 19. Typical baseline and threshold settings

Instruments	Baseline	Threshold
Applied Biosystems 7900HT	5–18 cycles	0.1
Applied Biosystems 7500	5–18 cycles	0.1
Stratagene Mx3000P, Mx3005P	Varies	0.1

13. Export the C_T values for all wells to a blank Excel spreadsheet.

Data analysis

Data analysis can be conducted at QIAGEN'S GeneGlobe Data Analysis Center using a software-based tool or with a spreadsheet-based tool that can be downloaded from a QIAGEN website.

Note: The GeneGlobe Data Analysis Center is a web resource for the analysis of real-time PCR or NGS data (www.qiagen.com/shop/genes-and-pathways/data-analysis-center-overview-page). To access the center, new users can register online. Once on the site, the data analysis software can be found under "Analysis." The qBiomarker Human DNA QC PCR Array data analysis spreadsheets can be found under "Product Resources/Performance Data" at www.qiagen.com/us/shop/pcr/primer-sets/qbiomarker-copy-number-pcr-arrays/#resources.

Ordering Information

Product	Contents	Cat. no.
qBiomarker Copy Number PCR Array*	Arrays of assays for real-time PCR-based copy number alteration and variation profiling; available in 96-well, 384-well, and Rotor-Disc formats	Varies
qBiomarker Copy Number PCR Assay (200)*	For 200 reactions; Mix of 2 gene-specific primers provided in solution	337812
qBiomarker SYBR Fluor Mastermix (2)†	For 2 x 96 assays in 96-well plates	337830
qBiomarker SYBR ROX Mastermix (2)†	For 2 x 96 assays in 96-well plates	337820
qBiomarker SYBR ROX FAST Mastermix (2)†	For 2 x 96 assays in 96-well plates	337840
Related products		
DNA QC Plate	Array of assays for evaluating DNA quality for real-time PCR-based arrays; available in 96-well, 384-well, and Rotor-Disc formats	Inquire

* QIAGEN reserves the right to occasionally redesign individual qBiomarker Copy Number PCR Assays for improved performance. This revision history can be accessed by contacting Technical Services and supplying the batch numbers from your assays and arrays.

† Larger sizes available; please inquire.

Nuclease-Free Water (10 x 50 ml)	10 x 50 ml nuclease-free water prepared without the use of DEPC (diethylpyrocarbonate)	129114
Nuclease-Free Water (1000 ml)	1000 ml nuclease-free water prepared without the use of DEPC (diethylpyrocarbonate)	129115
Nuclease-Free Water (5 liters)	5 liters nuclease-free water prepared without the use of DEPC (diethylpyrocarbonate)	129117
RT ² PCR Array Loading Reservoir	12 x 5 ml capacity, irradiation-sterilized loading reservoirs	338162
384EZLoad Covers (4)	Pack of 4 color-coded covers for loading 384-well plates	338125

DNA purification kits

QIAamp DNA Mini Kit (50)	For 50 DNA preps: 50 QIAamp Mini Spin Columns, QIAGEN Proteinase K, Reagents, Buffers, Collection Tubes (2 ml)	51304
QIAamp DNA Mini Kit (250)	For 250 DNA preps: 250 QIAamp Mini Spin Columns, QIAGEN Proteinase K, Reagents, Buffers, Collection Tubes (2 ml)	51306
DNeasy Blood & Tissue Kit (50)	For 50 DNA preps: 50 DNeasy Mini Spin Columns, Proteinase K, Buffers, Collection Tubes (2 ml)	69504
DNeasy Blood & Tissue Kit (250)	For 250 DNA preps: 250 DNeasy Mini Spin Columns, Proteinase K, Buffers, Collection Tubes (2 ml)	69506
QIAamp DNA FFPE Tissue Kit (50)	For 50 DNA preps: 50 QIAamp MinElute® Columns, Proteinase K, Buffers, Collection Tubes (2 ml)	56404
RNase A (17,500 U)	2.5 ml (100 mg/ml; 7000 units/ml, solution)	19101

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Document Revision History

R6	Updated template.
11/2018	Updated data analysis procedure.

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