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January 2020

# Microbial DNA qPCR Handbook

Microbial DNA qPCR Array

Microbial DNA qPCR Assay/Multi-Assay Kits

For real-time PCR-based profiling/detection of microbial species, antibiotic resistance genes or virulence factor genes

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# Kit Contents

<b>Microbial DNA qPCR Array Kit</b> <b>Catalog no. 330261: BAID-####X(R/F)X-##</b> <b>Format</b>	<b>A</b>	<b>C</b>	<b>D</b>	<b>E</b>	<b>F</b>	<b>G</b>	<b>R</b>
96-well plate containing dried assays	1	1	1	–	1	–	–
384-well plate containing dried assays	–	–	–	1	–	1	–
Optical Thin-Wall 8-Cap Strips (12 per plate)	12	–	12	–	–	–	–
Rotor-Disc® 100 containing dried assays	–	–	–	–	–	–	1
Optical Adhesive Film (1 per plate)	–	1	–	1	1	1	–
384EZLoad Covers* (1 set of 4 per plate)	–	–	–	1	–	1	–
Rotor-Disc Heat Sealing Film (1 per Rotor-Disc)	–	–	–	–	–	–	1
Microbial qPCR Mastermix (tube)†	1	1	1	2	1	2	1

\* Must be purchased separately for custom arrays; for single use only.

† Microbial qPCR Mastermix is available with ROX™ or fluorescein passive reference dye.

<b>Microbial DNA qPCR Assay</b> <b>Catalog no. 330025: BPXX####X(R/F)</b> <b>Number of samples</b>	<b>(100)</b> <b>100</b>
Microbial DNA qPCR Assay	1
Microbial qPCR Mastermix (tube)	1

<b>Microbial DNA qPCR Assay/Multi-Assay Kits</b> <b>Catalog no. 330033: BBXX-####X(R/F)</b> <b>330043: BBID-####Z(R/F)-3/4/5/6/7</b> <b>Number of samples</b>	<b>(20)</b> <b>20</b>
Microbial DNA qPCR Assay	Varies
Positive PCR Control Assay	1
Microbial DNA Positive Control (tubes)	1
Microbial qPCR Mastermix (tubes)	1 or 2
Microbial DNA-Free Water (tubes)	1 or 2

## Cyclers for use with array format

Format	Suitable real-time cyclers	Plate
A	Applied Biosystems® (Standard 96-well block) 5700, 7000, 7300, 7500, 7900HT, QuantStudio, ViiA™ 7; Bio-Rad® Chromo4™, iCycler®, iQ™5, MyiQ™, MyiQ2; Eppendorf® Mastercycler® ep <i>realplex</i> 2, 2S, 4, 4S; Agilent® Mx3005P®, Mx3000P®	96-well
C	Applied Biosystems (Fast 96-well block) 7500 Fast, 7900HT Fast, StepOnePlus™, QuantStudio Fast, ViiA 7 Fast	96-well
D	Bio-Rad CFX96™, Opticon®, Opticon 2; Agilent Mx4000®	96-well
E	Applied Biosystems (384-well block) 7900HT (384-well block), QuantStudio, ViiA 7; Bio-Rad CFX384™	384-well
F	Roche® LightCycler® 480 (96-well block)	96-well
G	Roche LightCycler 480 (384-well block)	384-well
R	QIAGEN Rotor-Gene®	Rotor-Disc 100

## Cyclers compatible with ROX or fluorescein reference dye

Master Mix	Reference Dye	Suitable real-time cyclers
Microbial qPCR Mastermix	ROX	All Applied Biosystems, Agilent (formerly Stratagene®) and QIAGEN instruments; BioRad Opticon, Opticon 2, and Chromo4; Roche LightCycler 480; Eppendorf Mastercycler ep <i>realplex</i> 2, 2S, 4, 4S
Microbial qPCR Mastermix	Fluorescein	BioRad iCycler, MyiQ and iQ5

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## Storage

Microbial DNA qPCR Arrays are shipped at ambient temperature or on blue ice packs, depending on the destination and accompanying products.

Microbial DNA qPCR Assays/Multi-Assay Kits are shipped on blue ice packs. Upon receipt, store at  $-30$  to  $-15^{\circ}\text{C}$ .

Microbial qPCR Mastermixes are shipped on blue ice packs. Upon receipt, store at  $-30$  to  $-15^{\circ}\text{C}$ .

If stored under these conditions, Microbial DNA qPCR Arrays and Microbial DNA qPCR Assays are stable for six months after receipt.

## Intended Use

Microbial DNA qPCR Arrays and Microbial DNA qPCR Assays/Multi-Assay Kits 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.

QIAcube<sup>®</sup> Connect is designed to perform fully automated purification of nucleic acids and proteins in molecular biology applications. The system is intended for use by professional users trained in molecular biological techniques and the operation of QIAcube Connect.

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# 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/safety](http://www.qiagen.com/safety) where you can find, view and print the SDS for each QIAGEN kit and kit component.

# Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of Microbial DNA qPCR Array and Microbial DNA qPCR Assay is tested against predetermined specifications to ensure consistent product quality.

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# Introduction

The human microbiome has a profound influence on human health, as it has been estimated that there are 10 times more microbes than human cells residing on and inside our bodies. For accurate characterization of the microbiome, a reliable method is needed to detect both microbial species and microbial genes. qPCR-based methods have proven to be effective for the detection and identification of both microbial species and microbial genes. Microbial DNA qPCR Arrays are screening tools for rapid profiling or identification of microbial species and antibiotic resistance genes. Each array is composed of Microbial DNA qPCR Assays that have been bench-verified for sensitivity and specificity.

In a single experiment, an entire profile of the microbial species or antibiotic resistance genes in a sample is generated.

## Microbial DNA qPCR Arrays for profiling

Microbial DNA qPCR Arrays are collections of assays that detect different microbial species that may be found at a particular body site, and can therefore be used for profiling the microbial composition of that region. Since the presence or abundance of different microbial species can exert profound influences on health and disease, relative quantification of these species is a prerequisite in order to determine microbe–disease associations. Using at least one control and one experimental sample, the Microbial DNA qPCR Array can determine the microbial composition at a particular body site and how the composition changes over time, compares to different populations or changes due to treatments. This capability creates the potential for discovery of novel microbial biomarkers.



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## Microbial DNA qPCR Arrays for identification

Microbial DNA qPCR Arrays can also screen for and identify disease-causing microbes or antibiotic resistance genes. These arrays can lead to the identification of the etiological agent of antibiotic resistance or infectious disease. To perform a microbial identification experiment, at least one experimental sample and one No Template Control (Microbial DNA-Free Water) sample are required. Using QIAGEN's data analysis software, a positive, negative or inconclusive result is determined for each sample. If the result is inconclusive, then the "Determination of Inconclusive Microbial DNA qPCR Array/Assay Results" protocol can be used to verify if the result is positive or negative. The identification arrays can be used with a variety of sample types such as isolated bacterial colonies, blood culture, swabs, stool and other metagenomic samples.

## Microbial DNA qPCR Assays/Multi-Assay Kits

Microbial DNA qPCR Assays provide a method for obtaining specific, accurate and reproducible results for an individual or related group of microbial species, antibiotic resistance genes or virulence factors. Every assay has been bench-verified and is ready-to-use for bacterial species or gene detection.

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# Principle

Each assay is based on PCR amplification of a species-specific genetic region of the relevant microbe, or a region of an individual microbial gene. The amplified product is detected using target-specific fluorescent hydrolysis probes, which helps to improve the specificity of the assay. Assays for detection of bacterial species target the 16s rRNA gene and were designed using the GreenGenes database for 16s sequences. Various databases were used for the design of assays for antibiotic resistance genes (lahey.org, ARDB, etc.) and virulence factor genes (VFDB). For relative profiling applications, host genomic DNA (if applicable) and overall bacterial load is measured. Inclusion of these assays allows the user to normalize using  $\Delta\Delta C_T$ . For identification of microbial species or microbial genes, a positive call is based on the  $\Delta C_T$  method, where a No Template Control (NTC) sample is used to normalize for assay background due to various factors.

To complete the Microbial DNA qPCR Array procedure (Figure 1), start with at least 500 ng genomic DNA isolated from a metagenomic sample or as low as 250 ng DNA from an isolated bacterial colony. Then, mix your DNA with the included ready-to-use Microbial qPCR Mastermix and aliquot the mixture into each well of the same plate containing pre-dispensed, gene-specific primer and hydrolysis probe sets.

To complete a Microbial DNA qPCR Assay/Multi-Assay Kit experiment (Figure 2), four PCR reactions are set up for each sample.  $C_T$  values from the PCR run are imported into the data analysis software, which will automatically determine if the sample is positive.

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# Microbial qPCR Mastermix HotStart DNA Polymerase

HotStart DNA Polymerase is provided in an inactive state and has no enzymatic activity at ambient temperatures, thereby preventing the formation of misprimed products and primer-dimers during reaction setup and the first denaturation step. Competition for reactants by PCR artifacts is therefore avoided, enabling high PCR specificity and accurate quantification. The enzyme is activated by a 10 minute, 95°C incubation step which enables reactions to be set up rapidly and conveniently at room temperature. The inactivation step can be easily incorporated into existing thermal cycling programs.

## Passive reference dye

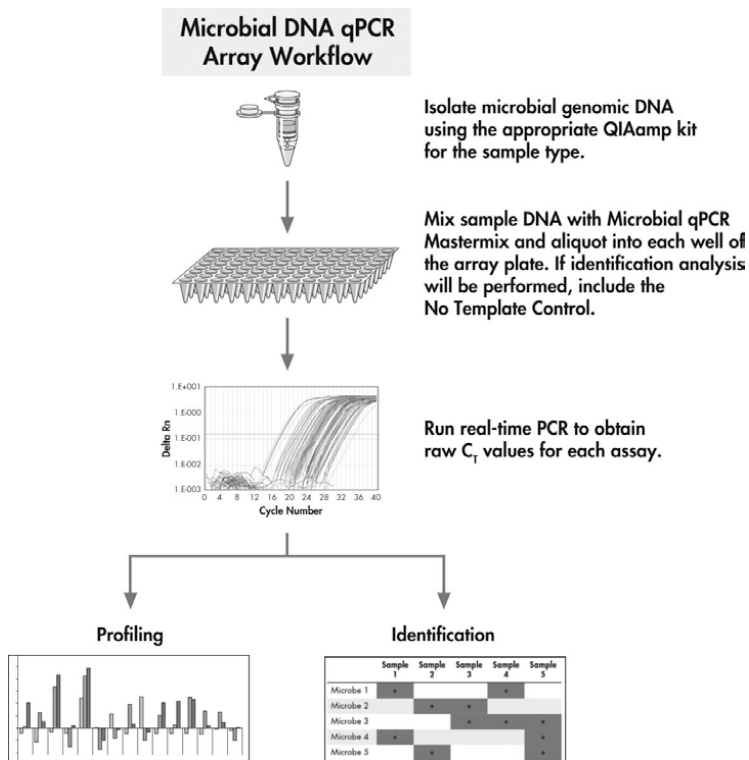
For certain real-time cyclers, the presence of a passive reference dye in real-time PCR compensates for non-PCR-related variations in fluorescence detection. Two versions of the Microbial qPCR Mastermix are available which contain either ROX passive reference dye or fluorescein passive reference dye. For a list of cyclers that can be used with each master mix, refer to the table on page 5. The presence of ROX dye does not interfere with the function of real-time PCR cyclers that do not require passive reference dye (e.g., Roche LightCycler).

## Procedure

The Microbial DNA qPCR Array procedure involves DNA purification, real-time PCR detection using Microbial DNA qPCR Arrays or Assays/Multi-Assay Kits and data analysis.

The Microbial DNA qPCR Arrays and Assays/Multi-Assay Kits provide accurate and verifiable results using a variety of sample types. Microbial DNA qPCR Arrays and Assays/Multi-Assay Kits have been tested using genomic DNA from stool, sputum, tooth plaque, isolated bacterial colony, vaginal swab and sewage samples and can be used with a variety of other sample

types. The procedure can be performed using 500 ng genomic DNA from metagenomic samples or 250 ng from an isolated bacterial colony per 96-well array (~5 ng per 25  $\mu$ l assay). Genomic DNA is added to the ready-to-use Microbial qPCR Mastermix plus Microbial DNA-Free Water and aliquoted into each well of a PCR plate, which contains pre-dispensed primers and hydrolysis probe sets. Dependent upon the application, microbial profiling or identification are completed using the data analysis software (see Appendix A).

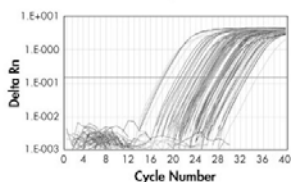


**Figure 1. Overview of the Microbial DNA qPCR Array procedure.** The simple workflow involves mixing the microbial genomic DNA from the sample of interest with ready-to-use Microbial qPCR Mastermix, aliquoting the reaction mix into each well of the array plate, performing real-time PCR and analyzing the data using the web-based data analysis software or Excel® template.

## Microbial DNA qPCR Assay/ Multi-Assay Kit Workflow



Set up 4 PCR reactions per sample that include positive and negative controls.



Run real-time PCR to obtain raw  $C_T$  values for each assay.

### Identification

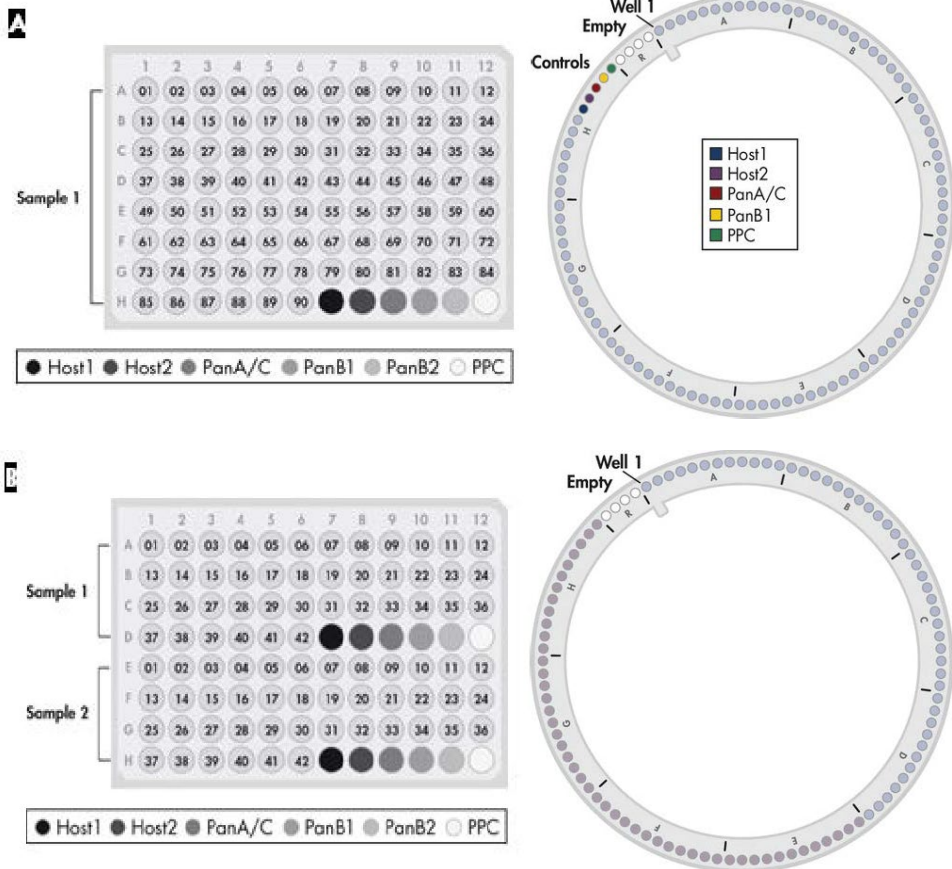
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Microbe 1	+			+	
Microbe 2		+			
Microbe 3			+	+	
Microbe 4	+				+
Microbe 5		+			+

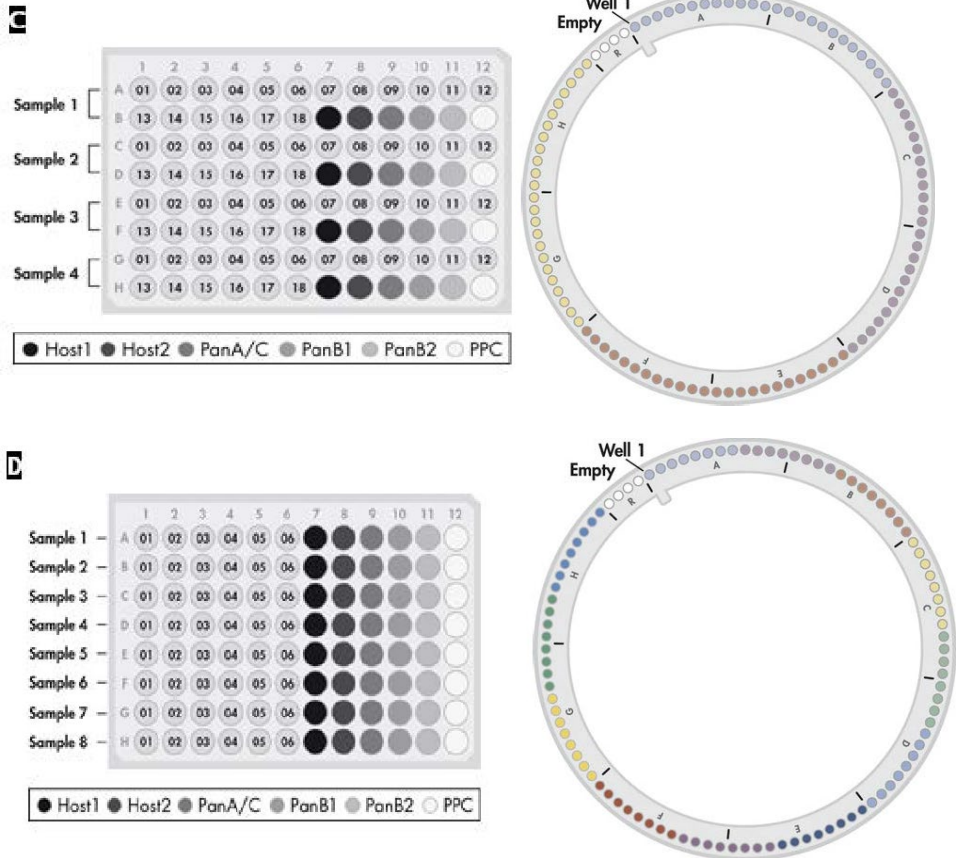
Use data analysis software to identify which species/genes are present.

**Figure 2. Overview of the Microbial DNA qPCR Assay/Multi-Assay Kit procedure.** For each sample, four separate PCR reactions are set up. These include controls for Positive PCR Control, No Template Control, Microbial DNA Positive Control and Microbial DNA qPCR Assay. Real-time PCR is performed and data is analyzed using web-based data analysis software or Excel templates.

# Microbial DNA qPCR Array plate layout

Microbial qPCR Arrays are available in 96-well plate, 100-well ring and 384-well plate formats (Figures 3 and 4).





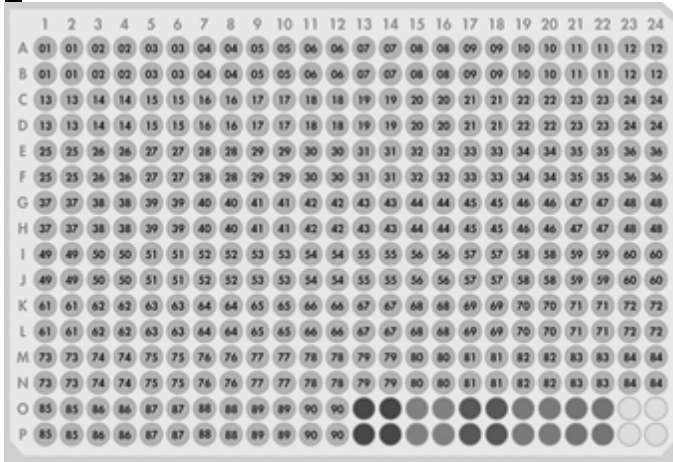
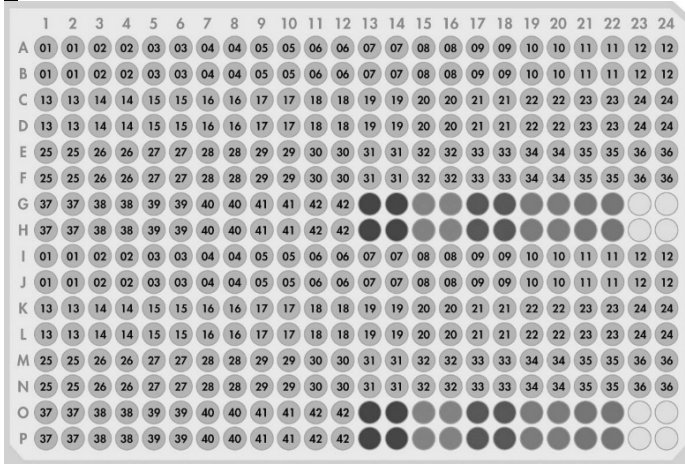
**Figure 3. Microbial DNA qPCR Array layout for plate formats A, C, D and F (96-well) or R (100-well).**

**A** Typically, wells A1–H6 of the standard 96-well format or 100-well format of the Microbial DNA qPCR Array contain assays for detection of microbes found in the same body site or disease, or antibiotic resistance genes. Control assays for Pan-Aspergillus/Candida (PanA/C), human/mouse GAPDH (Host1), human/mouse HBB1 (Host2), Pan Bacteria (PanB1, PanB2) and Positive PCR Control (PPC) are included. Depending on the specific array content, slight variations in plate layout may occur.

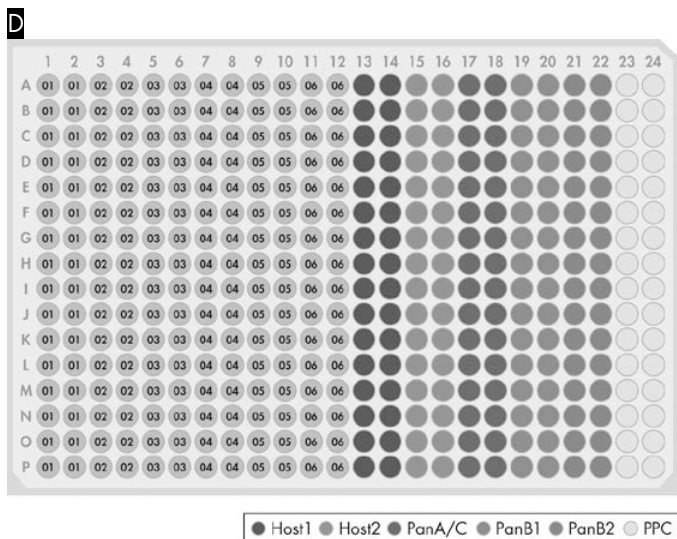
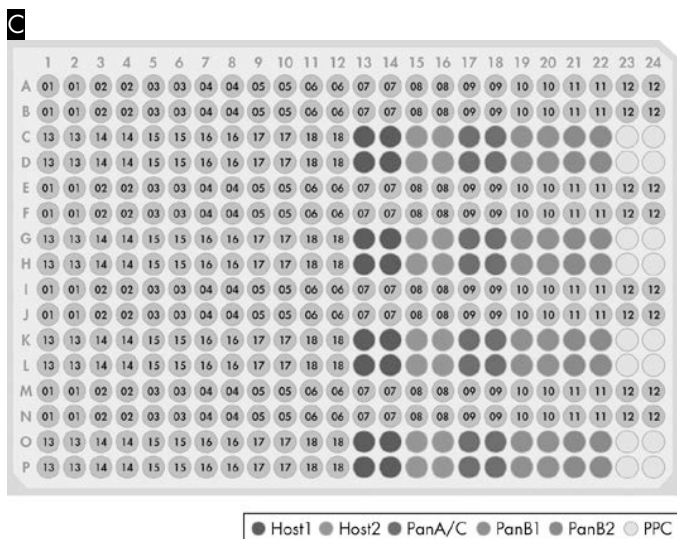
**B** An alternative 2 x 48 option enables profiling of two samples on each plate (sample 1: wells A1 to D12; sample 2: wells E1 to H12).

**C** An alternative 4 x 24 option enables profiling of four samples on each plate (sample 1: wells A1 to B12; sample 2: wells C1 to D12, sample 3: wells E1 to F12; sample 4: wells G1 to H12).

**D** An alternative 8 x 12 option enables profiling of eight samples on each plate (sample 1: wells A1 to A12; sample 2: wells B1 to B12; sample 3: wells C1 to C12; sample 4: wells D1 to D12; sample 5: wells E1 to E12; sample 6: wells F1 to F12; sample 7: wells G1 to G12; sample 8: wells H1 to H12).

**A****B**





**Figure 4. Microbial DNA qPCR Array layout for plate formats E and G (384-well).**

**A** The standard 384-well Microbial DNA qPCR Array format includes four replicates of the equivalent 96-well plate format, enabling testing of four samples. Alternative 384-well array options include: **B** an 8 x 48 option that allows profiling of eight samples on each plate, **C** a 16 x 24 option that allows profiling of 16 samples on each plate and **D** a 32 x 12 option that allows profiling of 32 samples on each plate.

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## Controls

Each array contains two Pan Bacteria assays that are designed to detect a broad range of bacterial species. Two different designs are included on each array to increase the coverage of bacterial species detected. Depending on the array content, the control Pan-*Candida/Aspergillus*, host GAPDH, and host HBB1 assays are included. Pan-*Candida/Aspergillus* detects fungal species of *Candida* and *Aspergillus* genera and can be used to monitor proper DNA extraction of fungi. Also, it can be used to determine the overall *Candida/Aspergillus* load in a sample. Host GAPDH/HBB1 assays are used to detect the presence of host (human or mouse) genomic DNA and can be used to normalize for relative microbial quantification. Each array also contains a Positive PCR Control (PPC) to test for the presence of inhibitors in the sample or the efficiency of the polymerase chain reaction itself using a pre-dispensed artificial DNA sequence and the primer set that detects it.

No Template Control and Microbial DNA Positive Control can be used to ensure that the experimental conditions and PCR setup are correct (see Appendix D for more information about Microbial DNA Positive Control).

# Automated Purification on QIAcube Instruments

Purification of microbial genomic DNA can be fully automated on the QIAcube Connect. The innovative QIAcube Connect uses advanced technology to process QIAGEN spin columns, enabling seamless integration of automated, low-throughput sample prep into your laboratory workflow. Sample preparation using the QIAcube Connect follows the same steps as the manual procedure (i.e., lyse, bind, wash and elute), enabling you to continue using QIAamp® kits for purification of high-quality microbial genomic DNA.

QIAcube instruments are preinstalled with protocols for purification of plasmid DNA, genomic DNA, RNA, viral nucleic acids and proteins, plus DNA and RNA cleanup. The range of protocols available is continually expanding and additional QIAGEN protocols can be downloaded free of charge at [www.qiagen.com/qiacubeprotocols](http://www.qiagen.com/qiacubeprotocols).



**QIAcube Connect.**

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# 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.

- Genomic DNA isolation kit (check Table 1, Page 21 for DNA purification kit recommendations)
- Real-time PCR cycler; the table on page 5 indicates the appropriate real-time cycler for each array format  
**Note:** Microbial DNA qPCR Arrays are not recommended for the Cepheid® SmartCycler® or the Roche LightCycler 2.0 due to the heat block format of these instruments.
- Multichannel pipettor
- Nuclease-free pipette tips and tubes

## Important Notes

### General precautions

For accurate and reproducible PCR array results, it is essential to avoid contamination of the assay with foreign DNA, especially PCR products from previously run plates. The most common sources of DNA contamination are the products of previous experiments.

To maintain a working environment free of DNA contamination, we recommend the following precautions:

- Wear gloves throughout the procedure. Use only fresh PCR-grade labware (tips and tubes).

- Use sterile pipette tips with filters.
- Store and extract positive materials (specimens, positive controls and amplicons) separately from all other reagents.
- 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 open any previously run and stored PCR array plate. Removing the thin-wall 8-cap strips or the adhesive film from PCR arrays releases PCR product DNA into the air where it can contaminate the results of future experiments. In the event that PCR products need to be analyzed by an independent method, ensure that any labware and bench surfaces are decontaminated.
- Do not remove the PCR array plate from its protective sealed bag until immediately before use.

**Table 1. Recommended DNA purification kits by sample type**

<b>Sample material</b>	<b>DNA purification kit</b>	<b>Catalog number</b>
Stool	QIAamp DNA Stool Mini Kit	51504
Blood	QIAamp UCP PurePathogen Blood Kit	50112
Cervical swab in transport media	QIAamp MinElute Media Kit	57414
Blood culture, bronchoalveolar lavage, carious dentine, cervical swab, isolated bacterial colony, sputum, saliva, swabs	QIAamp UCP Pathogen Mini Kit	50214

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# Protocol: Real-Time PCR Using Microbial DNA qPCR Array 96-well Formats

This protocol is for use with Microbial DNA qPCR Array formats A, C, D and F, using the 96 option array (one sample per plate), 2 x 48 option array (two samples per plate), 4 x 24 option array (four samples), and 8 x 12 option array (eight samples).

## Important points before starting

- Before beginning the procedure, read “Important Notes”.
- If the arrays are used for microbial profiling, skip step 2. If the arrays are used for microbial identification, setup of No Template Control PCR reaction (step 2) is required for data analysis software.
- Use supplied Microbial DNA-Free Water for setting up PCR reactions.
- Limit the number of times each tube of Microbial DNA-Free Water and Microbial qPCR Mastermix is opened to three times. This will help to prevent contamination of the Microbial DNA-Free Water and Microbial qPCR Mastermix.
- It is essential to start with high-quality DNA. For recommended genomic DNA preparation methods, refer to Table 1.
- For best results, all DNA samples should be resuspended in DNase-free water or, alternatively, in DNase-free 10 mM Tris buffer, pH 8.0.
- Ensure that you are using the correct master mix for your real-time instrument before beginning this procedure. For a list of cyclers that can be used with each master mix, refer to the table on page 5.
- PCR array plates should only be used in the compatible real-time PCR cycler listed in the table on page 5. The PCR array plates will not fit properly into incompatible real-time PCR cyclers and may cause damage to the cyclers.

- 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.
- For best results, use an 8-channel pipettor to load the PCR array. Alternatively, use eight tips of a 12-channel pipettor.
- Change pipette tips following each addition of master mix to the PCR array to avoid cross-contamination between the wells or PCR.
- To compensate for any potential environmental contamination, it is required to run at least one No Template Control (NTC) sample for the identification arrays and use  $\Delta C_T$  for the data analysis.

### Things to do before starting

- Determine DNA concentration and purity by preparing dilutions and measuring absorbance in 10 mM Tris, pH 8.0 buffer. For best results, the A260/A280 ratio should be greater than 1.8.
- Thaw genomic DNA and Microbial qPCR Mastermix on ice (4 °C). After thawing, mix gently by repeated pipetting or quick vortex, then quick spin.

### Procedure

1. Prepare a reaction mix according to Table 2.

For each sample, the minimum amount of microbial genomic DNA is listed in Table 3. For possible detection of low-abundance microbial species, at least 10x the minimum amount of genomic DNA can be used.

Add Microbial DNA-Free Water so that final volume per sample equals total volume per sample.

**Table 2. Reaction mix preparation for 96-well formats**

<b>Array Format</b>	<b>48-assay</b>	<b>96-assay</b>	<b>24-assay</b>	<b>12-assay</b>
<b>No. of samples</b>	<b>2</b>	<b>1</b>	<b>4</b>	<b>8</b>
Microbial qPCR Mastermix	680 µl	1275 µl	340 µl	170 µl
Microbial Genomic DNA	250 ng	500 ng	125 ng	65 ng
Microbial DNA-Free Water	varies	varies	varies	varies
Total volume per sample <sup>†</sup>	1360 µl	2550 µl	680 µl	340 µl

\* Minimum DNA amount for each sample.

<sup>†</sup> Provides an excess volume of 150 µl (96-assay array), 160 µl (2 x 48 option array), 80 µl (4 x 24 option array) and 40 µl (12 x 8 option array). Care should be taken when adding the reaction mix to the Microbial DNA qPCR Array to ensure each well receives the required 25 µl volume.

**Table 3. Minimum DNA amounts for sample types**

<b>Array Format</b>	<b>96-assay</b>	<b>48-assay</b>	<b>24-assay</b>	<b>12-assay</b>
Metagenomic sample	500 ng	250 ng	125 ng	65 ng
Isolated colony	250 ng	125 ng	65 ng	35 ng

- If performing experiments for identification, set up reactions for No Template Control (NTC) as detailed in Table 4.

**Table 4. Minimum DNA amounts for sample types**

<b>Array Format</b>	<b>96-assay</b>	<b>48-assay</b>	<b>24-assay</b>	<b>12-assay</b>
Microbial qPCR Mastermix	1275 µl	680 µl	340 µl	170 µl
Microbial DNA-Free Water	1275 µl	680 µl	340 µl	170 µl
Total volume per sample*	2550 µl	1360 µl	680 µl	340 µl

\* Provides an excess volume of 150 µl (96-assay array), 160 µl (2 x 48 option array), 80 µl (4 x 24 option array), and 40 µl (12 x 8 option array). Care should be taken when adding the reaction mix to the Microbial DNA qPCR Array to ensure each well receives the required 25 µl volume.

- Carefully remove the Microbial DNA qPCR Array from its sealed bag.
- Dispense reaction mix into an RT<sup>2</sup> PCR Array Loading Reservoir (ordered separately; cat. no. 338162).  
Use of the RT<sup>2</sup> PCR Array Loading Reservoir is recommended to assist in loading.



## 5. Microbial DNA qPCR Arrays for profiling

Add reaction mix to the Microbial DNA qPCR Array as follows:

- For a 96-assay array (1 sample): Add 25  $\mu$ l of sample to each well of another whole plate.
- For a 2 x 48-assay array (2 samples): Add 25  $\mu$ l reaction mix for sample 1 reaction into each well of rows A, B, C and D. Add 25  $\mu$ l reaction mix for sample 2 into each well of rows E, F, G and H.
- For a 4 x 24-assay array (4 samples): Add 25  $\mu$ l reaction mix of sample 1 reaction into each well of rows A and B. Add 25  $\mu$ l reaction mix for sample 2 into each well of rows C and D. Add 25  $\mu$ l reaction mix for sample 3 into each well of rows E and F. Add 25  $\mu$ l reaction mix for sample 4 into each well of rows G and H.
- For an 8 x 12-assay array (8 samples): Add 25  $\mu$ l reaction of sample 1 reaction into each well of row A. Add 25  $\mu$ l reaction mix for sample 2 into each well of row B. Add 25  $\mu$ l reaction mix for sample 3 into each well of row C. Add 25  $\mu$ l reaction mix for sample 4 into each well of row D. Add 25  $\mu$ l reaction mix for sample 5 into each well of row E. Add 25  $\mu$ l reaction mix for sample 6 into each well of row F. Add 25  $\mu$ l reaction mix for sample 7 into each well of row G. Add 25  $\mu$ l reaction mix for sample 8 into each well of row H.

### Microbial DNA qPCR Arrays for identification

Add reaction mix to the Microbial DNA qPCR Array as follows:

- For 96-assay array (1 sample): Add 25  $\mu$ l of NTC reaction to each well of one whole plate. Add 25  $\mu$ l of sample to each well of another whole plate.
- For 2 x 48-assay array (2 samples): Add 25  $\mu$ l reaction mix for NTC reaction into each well of rows A, B, C and D. Add 25  $\mu$ l reaction mix for sample 1 into each well of rows E, F, G and H.
- For 4 x 24-assay array (4 samples): Add 25  $\mu$ l reaction mix of NTC reaction into each well of rows A and B. Add 25  $\mu$ l reaction mix for sample 1 into each well of rows C and D. Add 25  $\mu$ l reaction mix for sample 2 into each well of rows E and F. Add 25  $\mu$ l reaction mix for sample 3 into each well of rows G and H.

- For 8 x 12-assay array (8 samples): Add 25 µl reaction of NTC reaction into each well of row A. Add 25 µl reaction mix for sample 1 into each well of row B. Add 25 µl reaction mix for sample 2 into each well of row C. Add 25 µl reaction mix for sample 3 into each well of row D. Add 25 µl reaction mix for sample 4 into each well of row E. Add 25 µl reaction mix for sample 5 into each well of row F. Add 25 µl reaction mix for sample 6 into each well of row G. Add 25 µl reaction mix for sample 7 into each well of row H.
6. Tightly seal the Microbial DNA qPCR Array with the optical thin-wall 8-cap strips (A and D formats) or the optical adhesive film (C and F formats).
- Note:** Ensure that no bubbles remain in any of the wells of the array. To remove bubbles, tap the plate gently on the bench top and centrifuge the plate at 1000 rpm for 1 minute.
7. Program the PCR cycler as described in Table 5.
- The PCR array plate should be placed on ice until the PCR cycler is set up. Arrays that are not processed immediately may be stored wrapped in aluminum foil at –30 to –15°C for up to one week.

**Table 5. Cycling conditions**

Step	Time	Temperature	Number of cycles
Initial PCR activation step	10 min	95°C	1
<b>2-step cycling:</b>			
Denaturation	15 s	95°C	40 <sup>†</sup>
Annealing and extension*	2 min <sup>†</sup>	60°C	

\* If applicable to the PCR instrument, change ramp rate to 1°C/s.

<sup>†</sup> Detect and record FAM fluorescence from every well during the annealing/extension step of each cycle.

<sup>‡</sup> On Roche LightCycler 480, perform 45 cycles.

8. Place one plate in the real-time thermal cycler. Use a compression pad with the optical film-sealed plate formats (C and F formats) if recommended in the cycler’s user manual. Start the run.

9. Calculate the threshold cycle ( $C_T$ ) for each well using the cycler’s software. For best results with the Microbial DNA qPCR Arrays, we recommend manually setting the baseline and threshold values. See Table 6 for recommended settings for selected real-time cyclers. These settings should be used when data analysis is for identification.

**Table 6. Recommended values for threshold and baseline settings**

Instrument	Baseline setting	Threshold setting
Applied Biosystems 7900 HT	8–20 cycles	0.2
Applied Biosystems 7500	8–20 cycles	0.2
Agilent Mx3000P and Mx3005P	Varies	0.1
Roche LightCycler	Abs. quant./2nd derivative max.	

To define the baseline value, use the linear view of the amplification plots and set the cycler to use the readings from cycle 5 up to 2 cycles before the earliest visible amplification, usually around cycle number 15, but not more than cycle number 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.

**Note:** Ensure the baseline and threshold settings are the same across all PCR array runs in the same analysis. If the DNA sample quality has been adequately controlled and the cycling program has been executed correctly, then the  $C_T$  value for the control sample PPC should be  $22 \pm 2$  (except for Roche LightCycler 480, where  $C_T = 22.75 \pm 2$ ) across all arrays or samples. If not, consult the “Troubleshooting Guide”.

10. Multi-Assay Kit Data Analysis Template Excel Software (refer to Appendix A).
11. If any results are inconclusive, then follow “Protocol: Determination of Inconclusive Microbial DNA qPCR Array/Assay Results”.

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# Protocol: Real-Time PCR Using Microbial DNA qPCR Array 384-well Formats

This protocol is for use with Microbial DNA qPCR Array formats E and G, using the 4 x 96 option array (four samples per plate), the 8 x 48 option array (eight samples per plate), the 16 x 24 option array (16 samples per plate), or the 32 x 12 option array (32 samples per plate).

## Important points before starting

- Before beginning the procedure, read “Important Notes”.
- If the arrays are used for microbial profiling, skip step 2. If the arrays are used for microbial identification, setup of No Template Control PCR reaction (step 2) is required for data analysis software.
- Use supplied Microbial DNA-Free Water for setting up PCR reactions.
- Limit the number of times each tube of Microbial DNA-Free Water and Microbial qPCR Mastermix is opened to three times. This will help to prevent contamination of the Microbial DNA-Free Water and Microbial qPCR Mastermix.
- It is essential to start with high-quality DNA. For recommended genomic DNA preparation methods, refer to Table 1.
- For best results, all DNA samples should be resuspended in DNase-free water or, alternatively, in DNase-free 10 mM Tris buffer, pH 8.0.
- Ensure that you are using the correct master mix for your real-time instrument before beginning this procedure. For a list of cyclers that can be used with each master mix, refer to the table on page 5.
- PCR array plates should only be used in the compatible real-time PCR cycler listed in the table on page 5. The PCR array plates will not fit properly into incompatible real-time PCR cyclers and may cause damage to the cyclers.
- 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.

- For best results, use a 12-channel pipettor to load the PCR array.
- Change pipette tips following each addition of master mix to the PCR array to avoid cross-contamination between the wells or PCR.
- To compensate for any potential environmental contamination, it is required to run at least one No Template Control (NTC) sample for the identification arrays and use  $\Delta C_T$  for the data analysis.

### Things to do before starting

- Determine DNA concentration and purity by preparing dilutions and measuring absorbance in 10 mM Tris, pH 8.0 buffer. For best results, the concentration measured at A260 should be greater than 10 ng/ml DNA, and the A260/A280 ratio should be greater than 1.8.
- Thaw genomic DNA and Microbial qPCR Mastermix on ice (4°C). After thawing, mix gently by repeated pipetting or quick vortex, then quick spin.

### Procedure

#### 12. Prepare a reaction mix according to Table 7.

For each sample, the minimum amount of microbial genomic DNA is listed in Table 8. To increase the possibility of detecting low-abundance microbial species, at least 10x the minimum amount of genomic DNA can be used.

Add Microbial DNA-Free Water so that the final volume per sample equals the total volume per sample.

**Table 7. Reaction mix preparation for 384-well formats**

Array Format No. of samples	96-assay 4	48-assay 8	24-assay 16	12-assay 32
Microbial qPCR Mastermix	510 $\mu$ l	272 $\mu$ l	136 $\mu$ l	68 $\mu$ l
Microbial Genomic DNA*	200 ng	100 ng	50 ng	25 ng
Microbial DNA-Free Water	varies	varies	varies	varies
Total volume per sample	1020 $\mu$ l	544 $\mu$ l	272 $\mu$ l	136 $\mu$ l

\* Minimum DNA amount for each sample

† Provides an excess volume of 60  $\mu$ l (4 x 96-assay array), 64  $\mu$ l (8 x 48 option array), 32  $\mu$ l (16 x 24 option array) and 16  $\mu$ l (32 x 12 option array). Care should be taken when adding the reaction mix to the Microbial DNA qPCR Array to ensure that each well receives the required 10  $\mu$ l volume.

**Table 8. Reaction mix preparation for 384-well formats**

Array Format	96-assay	48-assay	24-assay	12-assay
Metagenomic sample	200 ng	100 ng	50 ng	25 ng
Isolated colony	100 ng	50 ng	25 ng	15 ng

13. If performing experiments for identification, set up reactions for No Template Control (NTC) as described in Table 9.

**Table 9. Reaction mix preparation for 384-well formats**

Array Format	96-assay	48-assay	24-assay	12-assay
Microbial qPCR Mastermix	510 $\mu$ l	272 $\mu$ l	136 $\mu$ l	68 $\mu$ l
Microbial DNA-Free Water	510 $\mu$ l	272 $\mu$ l	136 $\mu$ l	68 $\mu$ l
Total volume per sample*	1020 $\mu$ l	544 $\mu$ l	272 $\mu$ l	136 $\mu$ l

\* Provides an excess volume of 60  $\mu$ l (4 x 96-assay array), 64  $\mu$ l (8 x 48 option array), 32  $\mu$ l (16 x 24 option array) and 16  $\mu$ l (32 x 12 option array). Care should be taken when adding the reaction mix to the Microbial DNA qPCR Array to ensure that each well receives the required 10  $\mu$ l volume.

14. Carefully remove the Microbial DNA qPCR Array from its sealed bag.
15. Dispense reaction mix into an RT<sup>2</sup> PCR Array Loading Reservoir (ordered separately; cat. no. 338162).  
Use of the RT<sup>2</sup> PCR Array Loading Reservoir is recommended to assist in loading.
16. Add reaction mix to the Microbial DNA qPCR Array as follows using 384EZLoad Covers (see Figure 5):
- Note:** The spacing between the tips of standard multi-channel pipettors will enable you to skip rows or columns when adding each sample.

**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

**B**

	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



	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



	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 Microbial DNA qPCR Array formats E or G, 4 x 96 option array, 8 x 48 option array, 16 x 24 option array or 32 x 12 option array.** For the 4 x 96 option array: Add 10 µl reaction mix from each numbered sample into the staggered wells with the same number as indicated in the figure. For the 8 x 48 option array: use cover 1 to add 10 µl reaction mix from samples 1 and 2; use cover 2 to add 10 µl reaction mix from samples 3 and 4; use cover 3 to add 10 µl reaction mix from samples 5 and 6; use cover 4 to add 10 µl reaction mix from samples 7 and 8. For the 16 x 24 option array: use cover 1 to add 10 µl reaction mix from samples 1, 2, 3 and 4; use cover 2 to add 10 µl reaction mix from samples 5, 6, 7 and 8; use cover 3 to add 10 µl reaction mix from samples 9, 10, 11 and 12; use cover 4 to add 10 µl reaction mix from samples 13, 14, 15 and 16. For the 32 x 12 option array: use cover 1 to add 10 µl reaction mix from samples 1, 2, 3, 5, 6, 7 and 8; use cover 2 to add 10 µl reaction mix from samples 9, 10, 11, 12, 13, 14, 15 and 16; use cover 3 to add 10 µl reaction mix from samples 17, 18, 19, 20, 21, 22, 23 and 24; use cover 4 to add 10 µl reaction mix from samples 25, 26, 27, 28, 29, 30, 31 and 32. **A** cover 1; **B** cover 2; **C** cover 2; **D** cover 4.



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## Microbial DNA qPCR Arrays for profiling

Place cover 1 (white) on the plate.

For 4 x 96 option array (4 samples): add 10  $\mu$ l 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.

For 8 x 48 option array (8 samples): add 10  $\mu$ l sample 1 reaction mix to the open wells of rows A, C, E and G; add 10  $\mu$ l sample 2 reaction mix to the open wells of rows I, K, M and O. Remove and discard the cover.

For 16 x 24 option array (16 samples): add 10  $\mu$ l sample 1 reaction mix to the open wells of rows A and C; add 10  $\mu$ l sample 2 reaction mix to the open wells of rows E and G; add 10  $\mu$ l sample 3 reaction mix to the open wells of rows I and K; add 10  $\mu$ l sample 4 reaction mix to the open wells of rows M and O. Remove and discard the cover.

For 32 x 12 option array (32 samples): add 10  $\mu$ l sample 1 reaction mix to the open wells of row A; add 10  $\mu$ l sample 2 reaction mix to the open wells of row C; add 10  $\mu$ l sample 3 reaction mix to the open wells of row E; add 10  $\mu$ l sample 4 reaction mix to the open wells of row G; add 10  $\mu$ l sample 5 reaction mix to the open wells of row I; add 10  $\mu$ l sample 6 reaction mix to the open wells of row K; add 10  $\mu$ l sample 7 reaction mix to the open wells of row M; add 10  $\mu$ l sample 8 reaction mix to the open wells of row O. Remove and discard the cover.

Place cover 2 (yellow) on the plate.

For 4 x 96 option array (4 samples): add 10  $\mu$ l sample 2 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.

For 8 x 48 option array (8 samples): add 10  $\mu$ l sample 3 reaction mix to the open wells of rows A, C, E and G; add 10  $\mu$ l sample 4 reaction mix to the open wells of rows I, K, M and O. Remove and discard the cover.

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For 16 x 24 option array (16 samples): add 10  $\mu$ l sample 5 reaction mix to the open wells of rows A and C; add 10  $\mu$ l sample 6 reaction mix to the open wells of rows E and G; add 10  $\mu$ l sample 7 reaction mix to the open wells of rows I and K; add 10  $\mu$ l sample 8 reaction mix to the open wells of rows M and O. Remove and discard the cover.

For 32 x 12 option array (32 samples): add 10  $\mu$ l sample 9 reaction mix to the open wells of row A; add 10  $\mu$ l sample 10 reaction mix to the open wells of row C; add 10  $\mu$ l sample 11 reaction mix to the open wells of row E; add 10  $\mu$ l sample 12 reaction mix to the open wells of row G; add 10  $\mu$ l sample 13 reaction mix to the open wells of row I; add 10  $\mu$ l sample 14 reaction mix to the open wells of row K; add 10  $\mu$ l sample 15 reaction mix to the open wells of row M; add 10  $\mu$ l sample 16 reaction mix to the open wells of row O. Remove and discard the cover.

Place cover 3 (black) on the plate.

For 4 x 96 option array (4 samples): add 10  $\mu$ l 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.

For 8 x 48 option array (8 samples): add 10  $\mu$ l sample 5 reaction mix to the open wells of rows B, D, F and H; add 10  $\mu$ l sample 6 reaction mix to the open wells of rows J, L, N and P. Remove and discard the cover.

For 16 x 24 option array (16 samples): add 10  $\mu$ l sample 9 reaction mix to the open wells of rows B and D; add 9  $\mu$ l sample 10 reaction mix to the open wells of rows F and H; add 10  $\mu$ l sample 11 reaction mix to the open wells of rows J and L; add 10  $\mu$ l sample 12 reaction mix to the open wells of rows N and P. Remove and discard the cover.

For 32 x 12 option array (32 samples): add 10  $\mu$ l sample 17 reaction mix to the open wells of row B; add 10  $\mu$ l sample 18 reaction mix to the open wells of row D; add 10  $\mu$ l sample 19 reaction mix to the open wells of row F; add 10  $\mu$ l sample 20 reaction mix to the open wells of row H; add 10  $\mu$ l sample 21 reaction mix to the open wells of row J; add

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10  $\mu$ l sample 22 reaction mix to the open wells of row L; add 10  $\mu$ l sample 23 reaction mix to the open wells of row N; add 10  $\mu$ l sample 24 reaction mix to the open wells of row P. Remove and discard the cover.

Place cover 4 (red) on the plate.

For 4 x 96 option array (4 samples): add 10  $\mu$ l sample 4 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.

For 8 x 48 option array (8 samples): add 10  $\mu$ l sample 7 reaction mix to the open wells of rows B, D, F and H; add 10  $\mu$ l sample 8 reaction mix to the open wells of rows J, L, N and P. Remove and discard the cover.

For 16 x 24 option array (16 samples): add 10  $\mu$ l sample 13 reaction mix to the open wells of rows B and D; add 10  $\mu$ l sample 14 reaction mix to the open wells of rows F and H; add 10  $\mu$ l sample 15 reaction mix to the open wells of rows J and L; add 10  $\mu$ l sample 16 reaction mix to the open wells of rows N and P. Remove and discard the cover.

For 32 x 12 option array (32 samples): add 10  $\mu$ l sample 25 reaction mix to the open wells of row B; add 10  $\mu$ l sample 26 reaction mix to the open wells of row D; add 10  $\mu$ l sample 27 reaction mix to the open wells of row F; add 10  $\mu$ l sample 28 reaction mix to the open wells of row H; add 10  $\mu$ l sample 29 reaction mix to the open wells of row J; add 10  $\mu$ l sample 30 reaction mix to the open wells of row L; add 10  $\mu$ l sample 31 reaction mix to the open wells of row N; and add 10  $\mu$ l sample 32 reaction mix to the open wells of row P. Remove and discard the cover.

## Microbial DNA qPCR Arrays for identification

Place cover 1 (white) on the plate.

For 4 x 96 option array (4 samples): add 10  $\mu$ l NTC 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.

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For 8 x 48 option array (8 samples): add 10  $\mu$ l NTC reaction mix to the open wells of rows A, C, E and G; add 10  $\mu$ l sample 1 reaction mix to the open wells of rows I, K, M and O. Remove and discard the cover.

For 16 x 24 option array (16 samples): add 10  $\mu$ l NTC reaction mix to the open wells of rows A and C; add 10  $\mu$ l sample 1 reaction mix to the open wells of rows E and G; add 10  $\mu$ l sample 2 reaction mix to the open wells of rows I and K; add 10  $\mu$ l sample 3 reaction mix to the open wells of rows M and O. Remove and discard the cover.

For 32 x 12 option array (32 samples): add 10  $\mu$ l NTC reaction mix to the open wells of row A; add 10  $\mu$ l sample 1 reaction mix to the open wells of row C; add 10  $\mu$ l sample 2 reaction mix to the open wells of row E; add 10  $\mu$ l sample 3 reaction mix to the open wells of row G; add 10  $\mu$ l sample 4 reaction mix to the open wells of row I; add 10  $\mu$ l sample 5 reaction mix to the open wells of row K; add 10  $\mu$ l sample 6 reaction mix to the open wells of row M; add 10  $\mu$ l sample 7 reaction mix to the open wells of row O. Remove and discard the cover.

Place cover 2 (yellow) on the plate.

For 4 x 96 option array (4 samples): add 10  $\mu$ l 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.

For 8 x 48 option array (8 samples): add 10  $\mu$ l sample 2 reaction mix to the open wells of rows A, C, E and G; add 10  $\mu$ l sample 3 reaction mix to the open wells of rows I, K, M and O. Remove and discard the cover.

For 16 x 24 option array (16 samples): add 10  $\mu$ l sample 4 reaction mix to the open wells of rows A and C; add 10  $\mu$ l sample 5 reaction mix to the open wells of rows E and G; add 10  $\mu$ l sample 6 reaction mix to the open wells of rows I and K; add 10  $\mu$ l sample 7 reaction mix to the open wells of rows M and O. Remove and discard the cover.

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For 32 x 12 option array (32 samples): add 10  $\mu$ l sample 8 reaction mix to the open wells of row A; add 10  $\mu$ l sample 9 reaction mix to the open wells of row C; add 10  $\mu$ l sample 10 reaction mix to the open wells of row E; add 10  $\mu$ l sample 11 reaction mix to the open wells of row G; add 10  $\mu$ l sample 12 reaction mix to the open wells of row I; add 10  $\mu$ l sample 13 reaction mix to the open wells of row K; add 10  $\mu$ l sample 14 reaction mix to the open wells of row M; add 10  $\mu$ l sample 15 reaction mix to the open wells of row O. Remove and discard the cover.

Place cover 3 (black) on the plate.

For 4 x 96 option array (4 samples): add 10  $\mu$ l sample 2 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.

For 8 x 48 option array (8 samples): add 10  $\mu$ l sample 4 reaction mix to the open wells of rows B, D, F and H; add 10  $\mu$ l sample 5 reaction mix to the open wells of rows J, L, N and P. Remove and discard the cover.

For 16 x 24 option array (16 samples): add 10  $\mu$ l sample 8 reaction mix to the open wells of rows B and D; add 10  $\mu$ l sample 9 reaction mix to the open wells of rows F and H; add 10  $\mu$ l sample 10 reaction mix to the open wells of rows J and L; add 10  $\mu$ l sample 11 reaction mix to the open wells of rows N and P. Remove and discard the cover.

For 32 x 12 option array (32 samples): add 10  $\mu$ l sample 16 reaction mix to the open wells of row B; add 10  $\mu$ l sample 17 reaction mix to the open wells of row D; add 10  $\mu$ l sample 18 reaction mix to the open wells of row F; add 10  $\mu$ l sample 19 reaction mix to the open wells of row H; add 10  $\mu$ l sample 20 reaction mix to the open wells of row J; add 10  $\mu$ l sample 21 reaction mix to the open wells of row L; add 10  $\mu$ l sample 22 reaction mix to the open wells of row N; add 10  $\mu$ l sample 23 reaction mix to the open wells of row P. Remove and discard the cover.

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Place cover 4 (red) on the plate.

For 4 x 96 option array (4 samples): add 10  $\mu$ l 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.

For 8 x 48 option array (8 samples): add 10  $\mu$ l sample 6 reaction mix to the open wells of rows B, D, F and H; add 10  $\mu$ l sample 7 reaction mix to the open wells of rows J, L, N and P. Remove and discard the cover.

For 16 x 24 option array (16 samples): add 10  $\mu$ l sample 12 reaction mix to the open wells of rows B and D; add 10  $\mu$ l sample 13 reaction mix to the open wells of rows F and H; add 10  $\mu$ l sample 14 reaction mix to the open wells of rows J and L; add 10  $\mu$ l sample 15 reaction mix to the open wells of rows N and P. Remove and discard the cover.

For 32 x 12 option array (32 samples): add 10  $\mu$ l sample 24 reaction mix to the open wells of row B; add 10  $\mu$ l sample 25 reaction mix to the open wells of row D; add 10  $\mu$ l sample 26 reaction mix to the open wells of row F; add 10  $\mu$ l sample 27 reaction mix to the open wells of row H; add 10  $\mu$ l sample 28 reaction mix to the open wells of row J; add 10  $\mu$ l sample 29 reaction mix to the open wells of row L; add 10  $\mu$ l sample 30 reaction mix to the open wells of row N; add 10  $\mu$ l sample 31 reaction mix to the open wells of row P. Remove and discard the cover.

17. Tightly seal the Microbial DNA qPCR Array with the optical adhesive film.

**Note:** Ensure that no bubbles remain in any of the wells of the array. To remove bubbles, tap the plate gently on the bench top and centrifuge the plate at 2000 rpm for 2 minutes.

18. Program the PCR cycler as described in Table 10. The PCR array plate should be placed on ice until the PCR cycler is set up. Arrays that are not processed immediately may be stored wrapped in aluminum foil at  $-30$  to  $-15^{\circ}\text{C}$  for up to one week.

**Table 10. Cycling conditions**

Step	Time	Temperature	Number of Cycles
Initial PCR activation step	10 min	95°C	1
<b>2-step cycling:</b>			
Denaturation	15 s	95°C	40 <sup>†</sup>
Annealing and extension*	2 min <sup>†</sup>	60°C	

\* If applicable to the PCR instrument, change ramp rate to 1°C/sec.

<sup>†</sup> Detect and record FAM fluorescence from every well during the annealing/extension step of each cycle.

<sup>‡</sup> On Roche LightCycler 480, perform 45 cycles.

19. Place one plate in the real-time thermal cycler. Use a compression pad with the optical film-sealed plate formats (C and F formats) if recommended in the cycler's user manual. Start the run.
20. Calculate the threshold cycle ( $C_T$ ) for each well using the cycler's software. For best results with the Microbial DNA qPCR Arrays, we recommend manually setting the baseline and threshold values. See Table 11 for recommended settings for selected real-time cyclers. These settings should be used when data analysis is for identification.

**Table 11. Recommended values for threshold and baseline settings**

Instrument	Baseline setting	Threshold setting
Applied Biosystems 7900 HT	8–20 cycles	0.2
Applied Biosystems 7500	8–20 cycles	0.2
Agilent Mx3000P and Mx3005P	Varies	0.1
<b>Roche LightCycler</b>		Abs. quant./2nd derivative max.

To define the baseline value, use the linear view of the amplification plots and set the cycler to use the readings from cycle 5 up to 2 cycles before the earliest visible amplification, usually around cycle number 15, but not more than cycle number 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.

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**Note:** Ensure the baseline and threshold settings are the same across all PCR array runs in the same analysis. If the DNA sample quality has been adequately controlled and the cycling program has been executed correctly, then the  $C_T$  value for the control sample PPC should be  $22 \pm 2$  (except for Roche LightCycler 480, where  $C_T = 22.75 \pm 2$ ) across all arrays or samples. If not, consult the "Troubleshooting Guide".

21. Export the resulting threshold cycle values for all wells to a blank Excel spreadsheet for data analysis for use with Microbial DNA qPCR Array/Multi-Assay Kit Data Analysis Template Excel Software (refer to Appendix A).
22. If any results are inconclusive, then follow the "Protocol: Determination of Inconclusive Microbial DNA qPCR Array/Assay Results".



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# Protocol: Real-Time PCR Using Microbial DNA qPCR Array Rotor-Gene Q Format

This protocol is for use with Microbial DNA qPCR Array formats R, using the 96 option array (one sample per Rotor-Disc 100), 2 x 48 option array (two samples per Rotor-Disc 100), 4 x 24 option array (four samples), and 8 x 12 option array (eight samples).

## Important points before starting

- Before beginning the procedure, read “Important Notes”.
- If the arrays are used for microbial profiling, skip step 2. If the arrays are used for microbial identification, setup of No Template Control PCR reaction (step 2) is required for data analysis software.
- Use supplied Microbial DNA-Free Water for setting up PCR reactions.
- Limit the number of times each tube of Microbial DNA-Free Water and Microbial qPCR Mastermix is opened to three times. This will help to prevent contamination of the Microbial DNA-Free Water and Microbial qPCR Mastermix.
- It is essential to start with high-quality DNA. For recommended genomic DNA preparation methods, refer to Table 1.
- For best results, all DNA samples should be resuspended in DNase-free water or, alternatively, in DNase-free 10 mM Tris buffer, pH 8.0.
- Ensure that you are using the correct master mix for your real-time instrument before beginning this procedure. For a list of cyclers that can be used with each master mix, refer to the table on page 5.
- PCR array rings should only be used in compatible Rotor-Gene Q PCR cyclers. The PCR rings will not fit properly into incompatible real-time PCR cyclers and may cause damage to the cyclers.

- 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.
- For best results, use a repeating pipettor to load the PCR ring.
- To compensate for any potential environmental contamination, it is required to run at least one No Template Control (NTC) sample for the identification arrays and use  $\Delta C_T$  for the data analysis.

### Things to do before starting

- Determine DNA concentration and purity by preparing dilutions and measuring absorbance in 10 mM Tris, pH 8.0 buffer. For best results, the A260/A280 ratio should be greater than 1.8.
- Thaw genomic DNA and Microbial qPCR Mastermix on ice (4 °C). After thawing, mix gently by repeated pipetting or quick vortex, then quick spin.

### Procedure

1. Prepare a reaction mix according to Table 12.

For each sample, the minimum amount of microbial genomic DNA is listed in Table 13. For possible detection of low-abundance microbial species, at least 10x the minimum amount of genomic DNA can be used.

Add Microbial DNA-Free Water so that final volume per sample equals total volume per sample.

**Table 12. Reaction mix preparation for 100-well ring formats**

<b>Array Format</b>	<b>96-assay</b>	<b>48-assay</b>	<b>24-assay</b>	<b>12-assay</b>
<b>No. of samples</b>	<b>1</b>	<b>2</b>	<b>4</b>	<b>8</b>
Microbial qPCR Mastermix	1020 µl	545 µl	275 µl	140 µl
Microbial Genomic DNA*	400 ng	225 ng	110 ng	55 ng
Microbial DNA-Free Water	varies	varies	varies	varies
<b>Total volume per sample<sup>†</sup></b>	<b>2040 µl</b>	<b>1090 µl</b>	<b>550 µl</b>	<b>280 µl</b>

\* Minimum DNA amount for each sample.

<sup>†</sup> Provides excess volume. Care should be taken when adding the reaction mix to the Microbial DNA qPCR Array to ensure each well receives the required 20 µl volume.

**Table 13. Minimum DNA amounts for sample types**

<b>Array Format</b>	<b>96-assay</b>	<b>48-assay</b>	<b>24-assay</b>	<b>12-assay</b>
Metagenomic sample	400 ng	225 ng	110 ng	55 ng
Isolated colony	200 ng	110 ng	55 ng	30 g

2. If performing experiments for identification, set up reactions for No Template Control (NTC) as detailed in Table 14.

**Table 14. No Template Control reaction setup for 96-well plates**

<b>Array Format</b>	<b>96-assay</b>	<b>48-assay</b>	<b>24-assay</b>	<b>12-assay</b>
Microbial qPCR Mastermix	1020 µl	545 µl	275 µl	140 µl
Microbial DNA-Free Water	1020 µl	545 µl	275 µl	140 µl
<b>Total volume per sample*</b>	<b>2040 µl</b>	<b>1090 µl</b>	<b>550 µl</b>	<b>280 µl</b>

\* Provides excess volume. Care should be taken when adding the reaction mix to the Microbial DNA qPCR Array to ensure each well receives the required 20 µl volume.

3. Carefully remove the Microbial DNA qPCR Array from its sealed bag. Place the array into the Rotor-Disc 100 Loading Block using the tab at position A01 and the tube guide holes.
4. Dispense reaction mix into an RT2 PCR Array Loading Reservoir (ordered separately; cat. no. 338162).

Use of the RT2 PCR Array Loading Reservoir is recommended to assist in loading.

## 5. Microbial DNA qPCR Arrays for profiling

Add reaction mix to the Microbial DNA qPCR Array as follows:

- For a 96-assay array (1 sample): Add 20 µl of sample to each well starting from position A01.
- For a 2 x 48-assay array (2 samples): Add 20 µl reaction mix for sample 1 reaction into each well corresponding to rows A, B, C and D. Add 20 µl reaction mix for sample 2 into each well corresponding to rows E, F, G and H.
- For a 4 x 24-assay array (4 samples): Add 20 µl reaction mix of sample 1 reaction into each well of rows A and B. Add 20 µl reaction mix for sample 2 into each well of rows C and D. Add 20 µl reaction mix for sample 3 into each well of rows E and F. Add 20 µl reaction mix for sample 4 into each well of rows G and H.
- For an 8 x 12-assay array (8 samples): Add 20 µl reaction of sample 1 reaction into each well of row A. Add 20 µl reaction mix for sample 2 into each well of row B. Add 20 µl reaction mix for sample 3 into each well of row C. Add 20 µl reaction mix for sample 4 into each well of row D. Add 20 µl reaction mix for sample 5 into each well of row E. Add 20 µl reaction mix for sample 6 into each well of row F. Add 20 µl reaction mix for sample 7 into each well of row G. Add 20 µl reaction mix for sample 8 into each well of row H.

## Microbial DNA qPCR Arrays for identification

Add reaction mix to the Microbial DNA qPCR Array as follows:

- For 96-assay array (1 sample): Add 20 µl of NTC reaction to each well starting from position A01. Add 20 µl of sample to each well of another ring.
- For 2 x 48-assay array (2 samples): Add 20 µl reaction mix for NTC reaction into each well corresponding to rows A, B, C and D. Add 20 µl reaction mix for sample 1 into each well corresponding to rows E, F, G and H.
- Reaction mix can be dispensed manually or using the QIAgility ([www.qiagen.com/goto/QIAgility](http://www.qiagen.com/goto/QIAgility)).

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

- For 4 x 24-assay array (4 samples): Add 20 µl reaction mix of NTC reaction into each well of rows A and B. Add 20 µl reaction mix for sample 1 into each well of rows C and D. Add 20 µl reaction mix for sample 2 into each well of rows E and F. Add 20 µl reaction mix for sample 3 into each well of rows G and H.
  - For 8 x 12-assay array (8 samples): Add 20 µl reaction of NTC reaction into each well of row A. Add 20 µl reaction mix for sample 1 into each well of row B. Add 20 µl reaction mix for sample 2 into each well of row C. Add 20 µl reaction mix for sample 3 into each well of row D. Add 20 µl reaction mix for sample 4 into each well of row E. Add 20 µl reaction mix for sample 5 into each well of row F. Add 20 µl reaction mix for sample 6 into each well of row G. Add 20 µl reaction mix for sample 7 into each well of row H.
6. Tightly seal the Microbial DNA qPCR Array with Rotor-Disc Heat-Sealing Film using the Rotor-Disc Heat Sealer.
  7. Program the PCR cycler as described in Table 15.

The PCR array should be placed on ice until the PCR cycler is set up. Arrays that are not processed immediately may be stored wrapped in aluminum foil at -30 to -15°C for up to one week.

**Table 15. Cycling conditions**

Step	Time	Temperature	Number of cycles
Initial PCR activation step	10 min	95°C	1
<b>2-step cycling:</b>			
Denaturation	15 s	95°C	40
Annealing and extension*	2 min*	60°C	

\* Detect and record FAM fluorescence from every well during the annealing/extension step of each cycle.

- 7a. 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*.

7b. Calculate the threshold cycle ( $C_T$ ) for each well using the cycler’s software. For best results with the Microbial DNA qPCR Arrays, we recommend manually setting the baseline and threshold values. See Table 16 for recommended settings for use with Rotor-Gene Q Series Software version 2.3.1. These settings should be used when data analysis is for identification.

**Table 16. Recommended values for threshold and baseline settings**

Parameter	Setting
Dynamic Tube	On
Slope Correct	On
Ignore First	5
Take off Adj.	15/20
Threshold	0.02
Eliminate Cycles Before	10

To define the baseline value, use the linear view of the amplification plots and set the cycler to use the readings from cycle 5 up to 2 cycles before the earliest visible amplification, usually around cycle number 15, but not more than cycle number 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.

**Note:** Ensure the baseline and threshold settings are the same across all PCR array runs in the same analysis. If the DNA sample quality has been adequately controlled and the cycling program has been executed correctly, then the  $C_T$  value for the control sample PPC should be  $20 \pm 2$  across all arrays or samples. If not, consult the “Troubleshooting Guide”.

- 7c. Export the resulting threshold cycle values for all wells to a blank Excel spreadsheet for data analysis for use with Microbial DNA qPCR Array/Multi-Assay Kit Data Analysis Template Excel Software (refer to Appendix A).
- 7d. If any results are inconclusive, then follow “Protocol: Determination of Inconclusive Microbial DNA qPCR Array/Assay Results”.

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# Protocol: Real-Time PCR Using Microbial DNA qPCR Assay/Multi-Assay Kits

This protocol is for use with Microbial DNA qPCR Assays/Multi-Assay Kits.

## Important points before starting

- Before beginning the procedure, read “Important Notes”.
- Use supplied Microbial DNA-Free Water for setting up PCR reactions.
- Limit the number of times each tube of Microbial DNA-Free Water and Microbial qPCR Mastermix is opened to three times. This will help to prevent contamination of the Microbial DNA-Free Water and Microbial qPCR Mastermix.
- It is essential to start with high-quality DNA. For recommended genomic DNA preparation methods, refer to Table 1.
- For best results, all DNA samples should be resuspended in DNase-free water or, alternatively, in DNase-free 10 mM Tris buffer, pH 8.0.
- Ensure that you are using the correct master mix for your real-time instrument before beginning this procedure. For a list of cyclers that can be used with each master mix, refer to the table on page 5.
- 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.
- To compensate for any potential environmental contamination, it is required to run at least one No Template Control (NTC) sample for the identification arrays and use  $\Delta C_T$  for the data analysis.

## Things to do before starting

- Determine DNA concentration and purity by preparing dilutions and measuring absorbance in 10 mM Tris, pH 8.0 buffer. For best results, the concentration measured at  $A_{260}$  should be greater than 10 ng/ml DNA, the  $A_{260}/A_{280}$  ratio should be greater than 1.8.

- Thaw genomic DNA and Microbial qPCR Mastermix on ice (4°C). After thawing, mix gently by repeated pipetting or quick vortex, then quick spin.
- Dilute 10x Microbial DNA Positive Control to 1x with Microbial DNA-Free Water.

## Procedure

1. Prepare four reaction mixes according to Table 17 for PPC and the specific Microbial DNA qPCR Assay with Microbial DNA Positive Control, Negative Template Control and sample. For a 25 µl reaction volume, a minimum of 5 ng per PCR assay is recommended. For each sample, one PPC reaction is required. For each Microbial DNA qPCR Assay, No Template Control (NTC), Microbial DNA Positive Control and sample reaction is required.

**Table 17a. Reaction List**

Reaction number	Assay	Sample
1	PPC	Sample
2	Microbial DNA qPCR Assay	Microbial DNA-Free Water (NTC)
3	Microbial DNA qPCR Assay	Microbial DNA Positive Control
4	Microbial DNA qPCR Assay	Sample

**Table 17b. Preparation of reaction mix**

Component	Per well*
Microbial qPCR Mastermix	12.5 µl
Microbial DNA qPCR Assay	1 µl
Genomic DNA sample <sup>†</sup>	5 ng
Microbial DNA-Free Water	varies
<b>Total volume per sample<sup>‡</sup></b>	<b>25 µl</b>

\* For Rotor-Gene, Gene-Disc 100 well rings or standard 0.2 mL PCR style tubes with 36- well rotor must be used.

<sup>†</sup> For NTC, add Microbial DNA-Free Water instead of genomic DNA.

<sup>‡</sup> If setting up more than one reaction, prepare a reaction mix 10% greater than that required for the total number of reactions to be performed. For Rotor-Gene, not necessary to prepare 10% greater volume reaction mix.



2. Dispense 25  $\mu$ l reaction mixes into PCR wells. For Rotor-Gene only, add 20  $\mu$ l reaction mix per well. Follow product specification sheet for proper loading sequence for use with data analysis software.
3. Tightly seal the PCR plate with optical thin-wall 8-cap strips, optical adhesive film or with Rotor Disc Heat-Sealing Film using the Rotor-Disc Heat Sealer. Note: Ensure that no bubbles remain in any of the PCR wells. To remove bubbles, tap the PCR plate or tube gently on the bench top and centrifuge at 1000 rpm for 1 minute.
4. Program the PCR cyclers as described in Table 18. Reactions should be placed on ice until the PCR cycler is set up. Assays that are not processed immediately may be stored wrapped in aluminum foil at  $-30$  to  $-15^{\circ}\text{C}$  for up to one week.

**Table 18. Cycling conditions**

Step	Time	Temperature	Number of Cycles
Initial PCR activation step	10 min	$95^{\circ}\text{C}$	1
<b>2-step cycling:</b>			
Denaturation	15 s	$95^{\circ}\text{C}$	40 <sup>†</sup>
Annealing and extension*	2 min <sup>†</sup>	$60^{\circ}\text{C}$	

\* If applicable to the PCR instrument, change ramp rate to  $1^{\circ}\text{C}/\text{sec}$ .

<sup>†</sup> Detect and record FAM fluorescence from every well during the annealing/extension step of each cycle.

<sup>‡</sup> On Roche LightCycler 480, perform 45 cycles.

5. Place PCR tubes/plate into the real-time thermal cycler or insert the Rotor-Disc into the Rotor-Disc 100 Rotor and secure with the Rotor-Disc 100 Locking Ring. Use a compression pad with the optical film-sealed plate formats if recommended in the cycler's user manual. For Rotor-Gene, tightly seal the Rotor-Disc 100 with Rotor Disc Heat-Sealing Film using the Rotor-Disc Heat Sealer. Start the run.
6. Calculate the threshold cycle ( $C_T$ ) for each well using the cycler's software. For best results with the Microbial DNA qPCR Arrays, we recommend manually setting the baseline and threshold values. See Table 19 for recommended settings for selected real-time cyclers. These settings should be used when data analysis is for identification.

**Table 19a. Recommended values for threshold and baseline settings**

Instrument	Baseline setting	Threshold setting
Applied Biosystems 7900 HT	8–20 cycles	0.2
Applied Biosystems 7500	8–20 cycles	0.2
Agilent Mx3000P and Mx3005P	varies	0.1
Roche LightCycler	Abs. quant./2nd derivative max.	

**Table 19b. Rotor-Gene recommended values for threshold and baseline settings**

Parameter	Setting
Dynamic Tube	on
Slope Correct	on
Ignore First	5
Take off Adj.	15/20
Threshold	0.02
Eliminate Cycles before	10

To define the baseline value, use the linear view of the amplification plots and set the cyclers to use the readings from cycle 5 up to 2 cycles before the earliest visible amplification, usually around cycle number 15, but not more than cycle number 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.

**Note:** Ensure that the baseline and threshold settings are the same across all PCR array runs in the same analysis. If the DNA sample quality has been adequately controlled and the cycling program has been executed correctly, then the  $C_T$  value for the control sample PPC should be  $22 \pm 2$  (except for Roche LightCycler 480, where  $C_T = 22.75 \pm 2$  and Rotor-Gene, where  $C_T = 20 \pm 2$ ) across all arrays or samples. If not, consult the “Troubleshooting Guide”.

- Export the resulting threshold cycle values for all wells to a blank Excel spreadsheet for data analysis for use with Microbial DNA qPCR Array /Multi-Assay Kit Data Analysis Template Excel Software (refer to Appendix A).
- If any results are inconclusive, then follow “Protocol: Determination of Inconclusive Microbial DNA qPCR Array/Assay Results”.

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# Protocol: Determination of Inconclusive Microbial DNA qPCR Array/Assay Results

This protocol is for determination of inconclusive results that were obtained with Microbial DNA qPCR Arrays or Assays following analysis with the Microbial DNA Data Analysis Excel sheets.

## Important points before starting

- Before beginning the procedure, read “Important Notes”.
- Use supplied Microbial DNA-Free Water for setting up PCR reactions.
- Limit the number of times each tube of Microbial DNA-Free Water and Microbial qPCR Mastermix is opened to three times. This will help to prevent contamination of the Microbial DNA-Free Water and Microbial qPCR Mastermix.
- It is essential to start with high-quality DNA. For recommended genomic DNA preparation methods, refer to Table 1.
- For best results, all DNA samples should be resuspended in DNase-free water or, alternatively, in DNase-free 10 mM Tris buffer, pH 8.0.
- Ensure that you are using the correct master mix for your real-time instrument before beginning this procedure. For a list of cyclers that can be used with each master mix, refer to the table on page 5.
- 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.

## Things to do before starting

- Determine DNA concentration and purity by preparing dilutions and measuring absorbance in 10 mM Tris, pH 8.0 buffer. For best results, the concentration measured at  $A_{260}$  should be greater than 10 ng/ml DNA, and the  $A_{260}/A_{280}$  ratio should be greater than 1.8.
- Thaw genomic DNA and Microbial qPCR Mastermix on ice (4°C). After thawing, mix gently by repeated pipetting or quick vortex.

- Dilute 10x Microbial DNA Positive Control to 1x with Microbial DNA-Free Water.
- Prepare sample DNA at two concentrations of 1x and 10x. For example, 1x = 5 ng/μl, 10x = 50 ng/μl.

## Procedure

1. Prepare 6 reaction mixes according to Table 20 for PPC with sample (reaction 3) and 10 times the sample amount (reaction 4), the specific Microbial DNA qPCR Assay with Microbial DNA Positive Control (reaction 2), No Template Control (NTC) (reaction 1), sample (reaction 5) and 10 times the amount of sample (reaction 6).

**Table 20a. Reaction List**

Reaction number	Assay	Sample
1	Microbial DNA qPCR Assay	Microbial DNA-Free Water (NTC)
2	Microbial DNA qPCR Assay	Microbial DNA Positive Control
3	PPC	Sample
4	PPC	10x sample input*
5	Microbial DNA qPCR Assay	Sample
6	Microbial DNA qPCR Assay	10x sample input*

\* Add 10 times more sample; i.e., if 5 ng DNA is added for sample, then add 50 ng DNA for 10x sample input.

**Table 20b. Preparation of reaction mix**

Array format	96-well
Microbial qPCR Mastermix	12.5 μl
Microbial DNA qPCR Assay	1 μl
Genomic DNA sample*	Refer to Table 20a
Microbial DNA-Free Water	Variable
Total volume per sample <sup>†</sup>	25 μl

\* For NTC, add Microbial DNA-Free Water instead of genomic DNA.

<sup>†</sup> If setting up more than one reaction, prepare a reaction mix 10% greater than that required for the total number of reactions to be performed. For Rotor-Gene, not necessary to prepare 10% greater volume reaction mix.

2. Dispense reaction mixes into PCR wells. Follow Appendix F o for proper loading sequence for use with data analysis software.

2a. Tightly seal the PCR plate with optical thin-wall 8-cap strips or optical adhesive film.

**Note:** Ensure that no bubbles remain in any of the PCR wells. To remove bubbles, tap the PCR plate or tube gently on the bench top and centrifuge at 1000 rpm for 1 minute.

2b. b. Program the PCR cycler as described in Table 21.

Reactions should be placed on ice until the PCR cycler is set up.

Assays that are not processed immediately may be stored wrapped in aluminum foil at  $-20^{\circ}\text{C}$  for up to one week.

**Table 21. Cycling conditions**

Step	Time	Temperature	Number of cycles
Initial PCR activation step	10 min	$95^{\circ}\text{C}$	1
<b>2-step cycling:</b>			
Denaturation	15 s	$95^{\circ}\text{C}$	40 <sup>‡</sup>
Annealing and extension*	2 min <sup>†</sup>	$60^{\circ}\text{C}$	

\* If applicable to the PCR instrument, change ramp rate to  $1^{\circ}\text{C}/\text{s}$ .

† Detect and record FAM fluorescence from every well during the annealing/extension step of each cycle.

‡ On Roche LightCycler 480, perform 45 cycles.

2c. Place PCR tubes/plate into the real-time thermal cycler. Use a compression pad with the optical film-sealed plate formats if recommended in the cycler’s user manual. Start the run.

2d. Calculate the threshold cycle ( $C_T$ ) for each well using the cycler’s software.

For best results with the Microbial DNA qPCR Arrays, we recommend manually setting the baseline and threshold values. See Table 22 for recommended settings for selected real-time cyclers. These settings should be used when data analysis for identification is performed.

**Table 22. Recommended values for threshold and baseline settings**

Instrument	Baseline setting	Threshold setting
Applied Biosystems 7900 HT	8–20 cycles	0.2
Applied Biosystems 7500	8–20 cycles	0.2
Agilent Mx3000P and Mx3005P	Varies	0.1
Roche LightCycler	Abs. quant./2nd derivative max.	

To define the baseline value, use the linear view of the amplification plots and set the cycler to use the readings from cycle 5 up to 2 cycles before the earliest visible amplification, usually around cycle number 15, but not more than cycle number 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.

**Note:** Ensure the baseline and threshold settings are the same across all PCR array runs in the same analysis. If the DNA sample quality has been adequately controlled and the cycling program has been executed correctly, then the  $C_T$  value for the control sample PPC should be  $22 \pm 2$  (except for Roche LightCycler 480, where  $C_T = 22.75 \pm 2$ ) across all arrays or samples. If not, consult the “Troubleshooting Guide”.

- 2e. Export the resulting threshold cycle values for all wells to a blank Excel spreadsheet for use with Microbial DNA qPCR Array/Multi-Assay Kit Data Analysis Template Excel Software (refer to Appendix A).

# 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](http://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/or protocols in this handbook or sample and assay technologies (for contact information, visit [www.qiagen.com](http://www.qiagen.com)).

## Comments and suggestions

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### No signal with Microbial DNA Positive Control

- |   |  |
|---|--|
| a) The selected fluorescence channel for PCR data analysis does not comply with the protocol                      | For data analysis, select the green channel (FAM) for the samples.   |
| b) Incorrect programming of the real-time PCR instrument  | Compare the temperature profile with the protocol. See the cycling conditions in Table 5, Table 10, Table 18 or Table 21. Refer to the manufacturer's manual of the cycler to be used. |
| c) PCR extension time too short   | Use the extension time specified in the protocol.  |
| d) Wrong detection step   | Ensure that fluorescence detection takes place during the extension step of the PCR cycling program.   |
| e) Incorrect configuration of the PCR   | Ensure that reactions were set up according to the reaction mix preparation tables on Table 2, Table 7, Table 12, and Table 17b. Repeat the PCR run, if necessary.                     |
| f) The storage conditions for one or more kit components did not comply with the instructions given in "Storage". | Check the storage conditions and the expiration date (see the kit label) of the reagents and use a new kit, if necessary.  |
| g) The Microbial DNA qPCR Array/ Assay/Multi-Assay Kit has expired  | Check the storage conditions and the expiration date (Microbial DNA qPCR Array/Assay/Multi-Assay Kit) of the reagents and use a new kit, if necessary.                                 |
| h) Insufficient starting template   | Increase the amount of template genomic DNA.   |

## Comments and suggestions

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### Weak or no signal for Positive PCR Control (PPC)

- |   |  |
|---|--|
| a) The PCR conditions do not comply with the protocol   | Check that PCR conditions match the cycling conditions in Table 5, Table 10, Table 15, Table 18, or Table 21. Repeat the PCR with corrected settings, if necessary.  |
| b) PCR was inhibited  | Use the recommended DNA isolation method and closely follow the manufacturer's instructions. QIAGEN offers dedicated sample preparation kits developed to complement Microbial qPCR Arrays/Assays/Multi-Assay Kits, and provide a complete and efficient workflow for microbial DNA testing. |
| c) The storage conditions for one or more components did not comply with the instructions given in "Storage". | Check the storage conditions and the expiration date (Microbial DNA qPCR Array/Assay/Multi-Assay Kit) of the reagents and use a new kit, if necessary.   |
| d) The Microbial DNA qPCR Array/ Assay/Multi-Assay Kit has expired  | Check the storage conditions and the expiration date (Microbial DNA qPCR Array/Assay/Multi-Assay Kit) of the reagents and use a new kit, if necessary.   |

### Signals present for the negative control template (NTC $C_T < 35$ )

- |   |  |
|---|--|
| a) Contamination occurred during PCR setup  | Repeat the PCR with new reagents.<br>If possible, seal the PCR array / close the PCR tubes directly after addition of the sample to be tested.<br>Make sure to pipet the positive controls last.<br>Make sure that work space and instruments are decontaminated at regular intervals. |
| b) Contamination occurred during extraction | Repeat the extraction and PCR of the sample to be tested using new reagents.<br>Make sure that work space and instruments are decontaminated at regular intervals.   |

### The average $C_T^{PPC}$ value varies by more than 2 across the Microbial DNA qPCR Arrays being compared and is less than 24

- |                                   |  |
|-----------------------------------|--|
| a) Cycler sensitivity levels vary | If the average $C_T^{PPC}$ value of $22 \pm 2$ is difficult to obtain for your cycler, the observed average $C_T^{PPC}$ value should be acceptable as long as it does not vary by more than 2 cycles between the Microbial DNA qPCR Arrays being compared. |
|-----------------------------------|--|



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## Comments and suggestions

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### Varying fluorescence intensity

- |  |  |
|--|--|
| a) Real-time cyclers contaminated        | Decontaminate the real-time cycler according to the supplier's instructions. |
| b) Real-time cycler no longer calibrated | Recalibrate the real-time cycler according to the supplier's instructions.   |

### The NTC for Pan Bacteria Assay 1 or Pan Bacteria Assay 2 has $C_T < 35$

- |  |  |
|--|--|
| a) Pan Bacteria Assay 1 and Pan Bacteria Assay 2 may detect residual <i>E. coli</i> genomic DNA found in the Microbial qPCR Mastermix. | If the $\Delta C_T (C_{T,NTC} - C_{T,Sample}) > 3$ , then the sample is positive for Pan Bacteria Assay 1 or Pan Bacteria Assay 2. This is automatically calculated in the data analysis software under the QC report tab. |
|--|--|

# Appendix A: Data Analysis

Free data analysis software for Microbial DNA qPCR Arrays is available at <http://www.qiagen.com/Products/Catalog/Assay-Technologies/Real-Time-PCR-and-RT-PCR-Reagents/Microbial-DNA-qPCR-Array>.

## Principle for Microbial DNA qPCR Array and Microbial DNA qPCR Assays Data Analysis

For Microbial DNA qPCR Arrays, there are two data analysis methods depending on the application. For relative profiling or comparing two different populations (i.e. healthy versus disease, time 0 versus time 30 days), the  $\Delta\Delta C_T$  method is recommended. For identification of microbial species or microbial genes, the  $\Delta C_T$  method is used. The  $\Delta C_T$  method allows for higher sensitivity of detection by measuring the background of the assay. For both methods, the  $C_T$  value for a qPCR assay is inversely correlated with the abundance of the microbial species or gene in the sample.

### Microbial qPCR Profiling Arrays ( $\Delta\Delta C_T$ method)

The  $\Delta\Delta C_T$  method is used for the relative profiling or comparison between two populations. To account for different starting amounts of DNA in a sample, separate reference assays are used to normalize sample input. Depending on the type of array and application, host genomic DNA (human or mouse) or total bacterial genomic DNA can be used for normalization. As an example, for the Vaginal Flora Microbial DNA qPCR Array, GAPDH and HBB1 are used to measure the amount of host (human or mouse) genomic DNA in the sample. The  $C_T$  values of the two reference assays are averaged and this value is used for the  $\Delta C_T$  calculation for the sample.

$$\Delta C_T = C_T^{\text{Assay}} - C_T^{\text{AVG REF}}$$

The  $\Delta\Delta C$  is then calculated for each assay across the Microbial DNA qPCR Array.

$\Delta\Delta C_T = \Delta C_{T\text{group } 2} - \Delta C_{T\text{group } 1}$  where group 1 is the control sample or group of control samples and group 2 is the experimental sample or group of experimental samples.

Fold increase or decrease in abundance is calculated based on the formula:

$$2^{\Delta\Delta C_T} = 2^{\Delta C_{T\text{group } 2} - \Delta C_{T\text{group } 1}}$$

At least a 5 to 10-fold increase or decrease in relative abundance may be considered significant.

In addition, the positive PCR control (PPC) must have a  $C_T = 22 \pm 2$  (except for Roche LightCycler 480, where  $C_T = 22.75 \pm 2$ ). If the PPC does not fall into this range, then the threshold may be changed so that  $PPC = 22 \pm 2$ . Alternatively, the sample can be diluted and the PCR repeated.

### Microbial qPCR Identification Arrays ( $\Delta C_T$ method)

The  $\Delta C_T$  method allows for higher sensitivity of detection by measuring the background of the assay. The assay background and/or low levels of contamination due to environmental factors can be corrected for by performing a PCR reaction using No Template Control (NTC) as the sample. NTC is used to establish the lower  $C_T$  value for positive calls and the upper  $C_T$  value for negative calls.

Lower  $C_T$  value (positive call) = NTC – 6

Upper  $C_T$  value (negative call) = NTC – 3

For example, if NTC = 39.5, then:

Lower  $C_T$  value (positive call) = NTC – 6 = 39.5 – 6 = 33.5

Upper  $C_T$  value (negative call) = NTC – 3 = 39.5 – 3 = 36.5

If the assay  $C_T$  is between the lower  $C_T$  value and the upper  $C_T$  value, then the result is inconclusive and the “Protocol: Determination of Inconclusive Microbial DNA qPCR Array/Assay Results” should be followed.

## Data analysis for Determination of Inconclusive Microbial DNA qPCR Array/Assay

To determine the positive or negative status of an inconclusive Microbial DNA qPCR Array/Assay result, six PCR reactions are performed for each inconclusive test. The criteria for a valid PCR run are as follows:

Reaction number	Assay	Sample	C <sub>T</sub> value
1	Microbial DNA qPCR Assay	Microbial DNA-Free Water (NTC)	C <sub>T</sub> > 35*
2	Microbial DNA qPCR Assay	Microbial DNA Positive Control	C <sub>T</sub> < 34
3	PPC	Sample	C <sub>T</sub> = 22 ± 2 <sup>†</sup>
4	PPC	10x sample input	C <sub>T</sub> = 22 ± 2 <sup>†</sup>

\* RGQ only (C<sub>T</sub>>32)

<sup>†</sup> RGQ only (C<sub>T</sub>=20 ± 2)

If the run satisfies the criteria, then data analysis for the inconclusive result is as follows:

Test	C <sub>T</sub> value	Interpretation
Microbial DNA qPCR Assay	C <sub>T</sub> <sup>1</sup>	
Microbial DNA qPCR Assay (10x sample)	C <sub>T</sub> <sup>2</sup>	
ΔC <sub>T</sub> (C <sub>T</sub> <sup>1</sup> – C <sub>T</sub> <sup>2</sup> )	≥ 2.3	Positive
ΔC <sub>T</sub> (C <sub>T</sub> <sup>1</sup> – C <sub>T</sub> <sup>2</sup> )	< 2.3	Inconclusive

# Appendix B: Identification of SHV variants using SHV Antibiotic Resistance Genes qPCR Multi-Assay Kit

SHV, a gene that provides resistance against beta-lactam antibiotics, has more than 140 known variants defined by single nucleotide polymorphisms (SNPs) that modulate its resistance against different antibiotics. This is the principle for data analysis to identify which SHV variant is present in a sample. To set up PCR, follow Protocol: Real-Time PCR Using the Microbial DNA qPCR Assay/Multi-Assay Kits. For each sample, set up the following eight PCR reactions, according to the product sheet, for proper loading sequence for use with data analysis software.

Microbial DNA qPCR Assay
PPC
SHV
SHV(156G)
SHV(156D)
SHV(238G240E)
SHV(238S240K)
SHV(238S240E)
SHV(238G240K)

SHV detects all SHV variants, while the other assays detect the SNP that encodes the specified amino acid. Data analysis uses the  $\Delta C_T$  method to identify which SHV variant is present.

## $\Delta C_T$ method

The  $\Delta C_T$  method uses the calculated  $\Delta C_T$  as follows:  $C_T^{NTC} - C_T^{SHV}$  to determine if SHV is present (positive), absent (negative) or inconclusive.

When using the recommended baseline and threshold settings,  $\Delta C_T \geq 6$  is a positive call,  $\Delta C_T < 3$  is a negative call and  $3 \leq \Delta C_T < 6$  is inconclusive. If an inconclusive result occurs, then follow "Protocol: Determination of Inconclusive Microbial DNA qPCR Array/Assay Results."

To determine which SHV variant is present, the  $\Delta C_T$  is calculated for each variant as follows:  $C_T^{\text{SHV}} - C_T^{\text{SHV}}$  variant. For each SNP at the same site, the SHV variant with the lowest  $\Delta C_T$  is identified as the variant present. An example is in Table 23. One exception is if the  $\Delta C_T$  of two or more variants  $< 3$ , than an inconclusive call is made. This could be due to more than one variant present in the sample. An example is in Table 24.

**Table 23. Example data for determining SHV variants**

Microbial DNA qPCR Assay	$C_T$	$\Delta C_T$
SHV	30.1	–
SHV(156G)	30.5	0.4
SHV(156D)	40	9.9
SHV(238G240E)	30.4	0.3
SHV(238S240K)	40	9.9
SHV(238S240E)	39	8
SHV(238G240K)	39.5	9.4

The sample would be positive for SHV(156G) since the  $\Delta C_T$  of SHV(156G), 0.4, is less than the  $\Delta C_T$  of SHV(156D), 9.9. Also, the sample would be positive for SHV(238G240E), since its  $\Delta C_T$  of 0.3 is the lowest of the four SHV238/240 variants.

**Table 24. Example data for determining SHV variants**

Microbial DNA qPCR Assay	C <sub>T</sub>	ΔC <sub>T</sub>
SHV	30.1	–
SHV(156G)	30.5	0.4
SHV(156D)	40	9.9
SHV(238G240E)	30.4	0.3
SHV(238S240K)	40	9.9
SHV(238S240E)	39	8
SHV(238G240K)	39.5	9.4

The sample would be questionable for SHV(238G240E) and SHV(238S240K) since both of their  $\Delta C_T < 3$ .

# Appendix C: Identification of Methicillin-Resistant *Staphylococcus aureus* using MRSA Identification Microbial DNA qPCR Multi-Assay Kit

This data analysis protocol describes how to identify the presence of Methicillin-Resistant *Staphylococcus aureus* (MRSA) in a sample. To set up PCR, follow Protocol: Real-Time PCR Using the Microbial DNA qPCR Assay/Multi-Assay Kits. For each sample, setup the following five PCR reactions, according to the product sheet, for the proper loading sequence for use with data analysis software.

Microbial DNA qPCR Assay
PPC
<i>Staphylococcus aureus</i>
<i>mecA</i>
<i>lukF</i>
<i>spa</i>

Use the following algorithm to determine if the sample contains No *Staphylococcus aureus* (No Staph), Methicillin-Sensitive *Staphylococcus aureus* (Methicillin-Sensitive SA), Hospital-Acquired Methicillin-Resistant *Staphylococcus aureus* (HA-Methicillin Resistant SA) or Community-Acquired Methicillin-Resistant *Staphylococcus aureus* (CA-Methicillin Resistant SA).

**Table 25. Determination of methicillin-resistant *Staphylococcus aureus***

	No Staph	Methicillin Sensitive SA	HA-Methicillin Resistant SA	CA-Methicillin Resistant SA
16S rRNA	–	+	+	+
<i>mecA</i>	+ or –	–	+	+
<i>PVL</i>	–	+ or –	–	+
<i>spa</i>	–	+	+	+



## Appendix D: Microbial DNA Positive Control

Microbial DNA Positive Control can be used to ensure that the experimental conditions and PCR setup are correct.

### Microbial DNA Positive Control

Microbial DNA Positive Control is a mixture of synthetic oligonucleotides that is supplied at a 10x concentration. The synthetic oligonucleotides are targets for the primer/hydrolysis probes. To use the Microbial DNA Positive Control, dilute to 1x using EpiTect® Control DNA (catalog number 59568) + EB buffer or Tris-HCl as follows:

- 5  $\mu$ l of 10x Microbial DNA Positive Control + 10  $\mu$ l of EpiTect Control DNA + 35  $\mu$ l EB buffer or Tris-Cl.

**Note:** 1x Microbial DNA Positive Control diluted with EpiTect Control DNA can be stored at  $-20^{\circ}\text{C}$ . Avoid repeated freeze/thaw cycles of 1x Microbial DNA Positive Control.

The 1x Microbial DNA Positive Control is equal to approximately 2000 target copy numbers. The recommended amount to use when setting up an assay is as follows:

- 96-well plate: 1  $\mu$ l of 1x Microbial DNA Positive Control per 25  $\mu$ l reaction
- 384-well plate: 0.4  $\mu$ l of 1x Microbial DNA Positive Control per 10  $\mu$ l reaction
- Rotor-Disc 100: 0.8  $\mu$ l of 1x Microbial DNA Positive Control per 20  $\mu$ l reaction

Under proper assay setup and PCR conditions, the Microbial DNA Positive Control will yield a  $C_T < 34$ .

**Note:** The Microbial DNA Positive Control does not contain targets for Pan Bacteria 1, Pan Bacteria 2 or Pan Bacteria 3 assays. To determine if these assays are functioning, use samples that are known to have bacterial genomic DNA (i.e., isolated bacterial colony, metagenomic samples, etc.) as a positive control.

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## Appendix E: Microbial DNA qPCR Assay Sensitivity

For each Microbial DNA qPCR Assay, the sensitivity was determined by calculating the Lower Limit of Quantification (LLOQ) using synthetic templates as targets for the qPCR assays. On a standard curve for gene copy number versus  $C_T$  value, the LLOQ is the lowest concentration that falls onto the linear portion of the standard curve. To determine the LLOQ, a standard curve was prepared by making 10-fold dilutions from 1,000,000 to 1 target copy number per PCR reaction.

Since the assays were tested using synthetic templates, LLOQ represents the number of gene copies that the assay detects. If an individual microorganism has one copy of an rRNA gene, antibiotic resistance gene or virulence factor gene, then the LLOQ would be equal to the number of bacterial or fungal cells. However, if an individual microorganism harbors more than one copy of these genes, the number of bacterial or fungal cells that can be quantitatively detected would be less than the LLOQ.

The LLOQ for each assay is listed in the product sheet.

Alternatively, Limit of Detection (LOD) may be required. To determine LOD, please consult the following:

1. Bustin, S.A., Benes, V., Garson, J.A., Hellems, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., and Wittwer, C.T. (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* **55**, 611.
2. Burd, E.M. (2010) Validation of laboratory-developed molecular assays for infectious diseases. *Clin. Microbiol. Rev.* **23**, 550.

# Appendix F: Plate Layout for Determination of Inconclusive Microbial DNA qPCR Array/Assay Results Protocol

In order to use the data analysis software, it is recommended to load a 96-well PCR plate in the following configuration.

**Table 26. Determination of inconclusive Microbial DNA qPCR Array / Assay results (six PCR reactions).**

Well	Species / gene	Template
A1	Microbial DNA qPCR Assay	NTC
A2	Microbial DNA qPCR Assay	Microbial DNA Positive Control
A3	PPC	Sample 1
A4	PPC	10x sample 1 input
A5	Microbial DNA qPCR Assay	Sample 1
A6	Microbial DNA qPCR Assay	10x Sample 1 input
A7	Microbial DNA qPCR Assay	NTC
A8	Microbial DNA qPCR Assay	Microbial DNA Positive Control
A9	PPC	Sample 2
A10	PPC	10x sample 2 input
A11	Microbial DNA qPCR Assay	Sample 2
A12	Microbial DNA qPCR Assay	10x sample 2 input
B1	Microbial DNA qPCR Assay	NTC
B2	Microbial DNA qPCR Assay	Microbial DNA Positive Control
B3	PPC	Sample 3
B4	PPC	10x sample 3 input
B5	Microbial DNA qPCR Assay	Sample 3
B6	Microbial DNA qPCR Assay	10x sample 3 input

continued on next page

**Table 27 (continued from previous page)**

<b>Well</b>	<b>Species / gene</b>	<b>Template</b>
B7	Microbial DNA qPCR Assay	NTC
B8	Microbial DNA qPCR Assay	Microbial DNA Positive Control
B9	PPC	Sample 4
B10	PPC	10x sample 4 input
B11	Microbial DNA qPCR Assay	Sample 4
B12	Microbial DNA qPCR Assay	10x sample 4 input
C1	Microbial DNA qPCR Assay	NTC
C2	Microbial DNA qPCR Assay	Microbial DNA Positive Control
C3	PPC	Sample 5
C4	PPC	10x sample 5 input
C5	Microbial DNA qPCR Assay	Sample 5
C6	Microbial DNA qPCR Assay	10x sample 5 input
C7	Microbial DNA qPCR Assay	NTC
C8	Microbial DNA qPCR Assay	Microbial DNA Positive Control
C9	PPC	Sample 6
C10	PPC	10x sample 6 input
C11	Microbial DNA qPCR Assay	Sample 6
C12	Microbial DNA qPCR Assay	10x sample 6 input
D1	Microbial DNA qPCR Assay	NTC
D2	Microbial DNA qPCR Assay	Microbial DNA Positive Control
D3	PPC	Sample 7
D4	PPC	10x sample 7 input
D5	Microbial DNA qPCR Assay	Sample 7
D6	Microbial DNA qPCR Assay	10x sample 7 input
D7	Microbial DNA qPCR Assay	NTC
D8	Microbial DNA qPCR Assay	Microbial DNA Positive Control
D9	PPC	Sample 8
D10	PPC	10x sample 8 input

continued on next page

**Table 27 (continued from previous page)**

<b>Well</b>	<b>Species / gene</b>	<b>Template</b>
D11	Microbial DNA qPCR Assay	Sample 8
D12	Microbial DNA qPCR Assay	10x sample 8 input
E1	Microbial DNA qPCR Assay	NTC
E2	Microbial DNA qPCR Assay	Microbial DNA Positive Control
E3	PPC	Sample 9
E4	PPC	10x sample 9 input
E5	Microbial DNA qPCR Assay	Sample 9
E6	Microbial DNA qPCR Assay	10x sample 9 input
E7	Microbial DNA qPCR Assay	NTC
E8	Microbial DNA qPCR Assay	Microbial DNA Positive Control
E9	PPC	Sample 10
E10	PPC	10x sample 10 input
E11	Microbial DNA qPCR Assay	Sample 10
E12	Microbial DNA qPCR Assay	10x sample 10 input
F1	Microbial DNA qPCR Assay	NTC
F2	Microbial DNA qPCR Assay	Microbial DNA Positive Control
F3	PPC	Sample 11
F4	PPC	10x sample 11 input
F5	Microbial DNA qPCR Assay	Sample 11
F6	Microbial DNA qPCR Assay	10x sample 11 input
F7	Microbial DNA qPCR Assay	NTC
F8	Microbial DNA qPCR Assay	Microbial DNA Positive Control
F9	PPC	Sample 12
F10	PPC	10x sample 12 input
F11	Microbial DNA qPCR Assay	Sample 12
F12	Microbial DNA qPCR Assay	10x sample 12 input
G1	Microbial DNA qPCR Assay	NTC
G2	Microbial DNA qPCR Assay	Microbial DNA Positive Control

**continued on next page**

**Table 27 (continued from previous page)**

<b>Well</b>	<b>Species / gene</b>	<b>Template</b>
G3	PPC	Sample 13
G4	PPC	10x sample 13 input
G5	Microbial DNA qPCR Assay	Sample 13
G6	Microbial DNA qPCR Assay	10x sample 13 input
G7	Microbial DNA qPCR Assay	NTC
G8	Microbial DNA qPCR Assay	Microbial DNA Positive Control
G9	PPC	Sample 14
G10	PPC	10x sample 14 input
G11	Microbial DNA qPCR Assay	Sample 14
G12	Microbial DNA qPCR Assay	10x sample 14 input
H1	Microbial DNA qPCR Assay	NTC
H2	Microbial DNA qPCR Assay	Microbial DNA Positive Control
H3	PPC	Sample 15
H4	PPC	10x sample 15 input
H5	Microbial DNA qPCR Assay	Sample 15
H6	Microbial DNA qPCR Assay	10x sample 15 input
H7	Microbial DNA qPCR Assay	NTC
H8	Microbial DNA qPCR Assay	Microbial DNA Positive Control
H9	PPC	Sample 16
H10	PPC	10x sample 16 input
H11	Microbial DNA qPCR Assay	Sample 16
H12	Microbial DNA qPCR Assay	10x sample 16 input

# Ordering Information

<b>Product</b>	<b>Contents</b>	<b>Cat. no.</b>
Microbial DNA qPCR Array	Array plate and master mix for detection of microbial species or genes	330261
Custom Microbial DNA qPCR Array	Array plates and master mix for detection of user-defined set of microbial species or genes	330161
Microbial DNA qPCR Assays	One 100 µl tube Microbial DNA qPCR Assay, one 1.35 ml tube Microbial qPCR Mastermix	330025
Microbial DNA qPCR Assay Kits	One 20 µl tube of Microbial DNA qPCR Assay, one tube Positive PCR Control (20 µl), one tube Microbial DNA Positive Control (50 µl), one tube Microbial DNA-Free Water (1.35 ml) and one tube Microbial qPCR Mastermix (1.35 ml)	330033
Microbial DNA qPCR Multi-Assay Kits	One 20 µl tube for each Microbial DNA qPCR Assay, one tube Positive PCR Control (20 µl), one tube Microbial DNA Positive Control (50 µl), one or two tubes Microbial DNA-Free Water (1.35 ml each), one or two tubes Microbial qPCR Mastermix (1.35 ml each)	330043

<b>Product</b>	<b>Contents</b>	<b>Cat. no.</b>
<b>Accessories</b>		
Microbial DNA-Free Water	Twelve tubes of 1.35 ml each	338132
Supplemental Microbial qPCR Mastermix ROX	Two tubes of 1.35 ml each	330530
Supplemental Microbial qPCR Mastermix Fluor	Two tubes of 1.35 ml each	330540
Microbial DNA Positive Control	One 50 µl tube at 10x concentration	338134
<b>Related products</b>		
QIAamp DNA Stool Mini Kit	For 50 DNA preps: 50 QIAamp Mini Spin Columns, QIAGEN Proteinase K, InhibitEX tablets, Buffers, Collection Tubes (2 ml)	51504
QIAamp UCP PurePathogen Blood Kit	For 10 preps, includes: QIAamp UCP Mini columns, QIAGEN Proteinase K, Tube Extenders (20 ml), Buffers, VacConnectors, Pathogen Lysis Tubes and Collection Tubes (2 ml)	50112
QIAamp MinElute Media Kit	For 50 minipreps: 50 QIAamp MinElute Columns, QIAGEN Proteinase K, Carrier RNA, Buffers, Extension Tubes (3 ml), Collection Tubes (1.5 ml)	57414
QIAamp UCP Pathogen Mini Kit	50 QIAamp UCP Mini Columns, Collection Tubes (2 ml), Tube Extenders (20 ml), Elution Tubes, VacConnectors, Buffers and Proteinase K	50214



Product	Contents	Cat. no.
<b>QIAcube Connect – for fully automated nucleic acid extraction with QIAGEN spin-column kits</b>	QIAcube Connect <sup>†</sup> Instrument, connectivity package, 1-year warranty on parts and labor	Inquire

<sup>†</sup> All QIAcube Connect instruments are provided with a region-specific connectivity package, including tablet and equipment necessary to connect to the local network. Further, QIAGEN offers comprehensive instrument service products, including service agreements, installation, introductory training and preventive subscription. Contact your local sales representative to learn about your options.

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at [www.qiagen.com](http://www.qiagen.com) or can be requested from QIAGEN Technical Services or your local distributor.

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## Notes

# Handbook Revision History

Date	Changes
January 2020	Changes to criteria for a valid PCR run for RGQ on page 61; QIAcube Connect changes; clarification on temperature ranges; Removed handbook from Kit Contents; layout updates.

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