August 2025

Microbial DNA dPCR Handbook

Custom dPCR Microbial Assays

dPCR Microbial DNA Detection Assays

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

Sample to Insight

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Kit Contents

dPCR Microbial DNA Detection Assay/Custom dPCR Microbial Assay	250207: DMA#####-(F/H/T/R/C/S)	250208: CMA#####-(F/H/T/R/C/S)
Catalog no.	**250207**	**250208**
No. of reactions	**200***	**150 or 400†**
Microbial DNA Detection Assay lyophilized (tube)	1	1

* 200 rxn in 40 µL (Nanoplate 26k); 666 rxn in 12 µL (Nanoplate 8.5k)

† 150/400 rxn in 40 µL (Nanoplate 26k); 500/1332 rxn in 12 µL (Nanoplate 8.5k)

dPCR instruments compatible with the assay portfolio

Type	Instruments	Nanoplate formats	Instrument cat. nos.
2 channels	QIAcuity® 1–2plex	96-well (96LV) 24-well (24HV)	911001
5 channels	QIAcuity 1–5plex QIAcuity 4–5plex QIAcuity 8–5plex	96-well (96LV) 24-well (24HV)	911021 911042 911052

dPCR Mastermixes compatible with the assay portfolio

Name	Description	Mastermix cat. nos.
QIAcuity Probe PCR Kit	Standard Mastermix for probe-based assays in nanoplates on the QIAcuity	250101 250102 250103
QIAcuity UCP Probe PCR Kit	Ultra Clean Production Mastermix for probe-based assays in nanoplates on the QIAcuity	250121 250122
QIAcuity Onestep Advanced Probe PCR Kit	One-step RT-PCR Mastermix optimized for the quantification of RNA and DNA targets in nanoplates on the QIAcuity	250131 250132
QIAcuity High Multiplex Probe PCR Kit	Allows multiplexing of up to 12 assays in one reaction on QIAcuity instruments with QIAcuity Software Suite version 3.1 or higher	250133 250134

Shipping and Storage

dPCR Microbial DNA Detection Assays and Custom dPCR Microbial Assays consist of 2 primers and 1 hydrolysis probe that are lyophilized in a single tube. The lyophilized primer–probe mix is shipped at ambient temperature and should upon receipt be stored protected from light at –30°C to –15°C in a constant-temperature freezer. Under these conditions, the assays are stable without showing any reduction in performance and quality, until the date indicated on the label. Before usage, the lyophilized primer– probe mix has to be resuspended. Before opening the tube, centrifuge the tube briefly to collect all material at the bottom of the tube. To resuspend the primer–probe mix, add the specified amount (see Table 1) of sterile, nuclease-free 10 mM TE buffer (pH 8.0) with low EDTA (0.1 mM), mix, and leave for 20 minutes to allow the primer–probe mix to completely resuspend. We do not recommend resuspension in water. The primer–probe mixes are less stable in water compared to TE buffer, and some may not resuspend easily in water. For short term storage, the resuspended dPCR Assay mix can be stored at 2–8°C. Repeated freeze-thaw cycles should be avoided by storing in aliquots.

Table 1. Assay resuspension to achieve a 20x primer–probe mix

Assay Product (cat. no.)	Amount of 10 mM TE buffer (pH 8.0) with low EDTA (0.1 mM) to be added (µL)
dPCR Microbial DNA Detection Assay 200 rxns (250207)	400
Custom dPCR Microbial Assay 150 rxns (250208)	330
Custom dPCR Microbial Assay 400 rxns (250208)	880

Intended Use

The dPCR Microbial DNA Detection Assays and the Custom dPCR Microbial Assays 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.

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**, 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 dPCR Microbial DNA Detection Assay is tested against predetermined specifications to ensure consistent product quality.

Introduction

Bacteria, fungi, viruses, and parasitic metazoa are ubiquitous in the environment and have adapted to various ecological niches, including the human body. This microbial diversity includes organisms that together bring a wide variety of harmful and beneficial properties to humans. They are part of all aspects of human life, from human health to food production. Research and developments in recent years have described the diverse physiological capabilities of microbes and the complexity of their lives as they interact with their environment. For example, the human microbiome found on and in our bodies has been estimated to include 10 times more microbes than human cells in the human body. Each microbe, in turn, can trigger or influence specific physiological processes. This makes the specific detection and monitoring of microbes an important tool for understanding their biological function, particularly in the context of infection or colonization of the human body.

DNA-based methods have proven to be effective for the detection and identification of both microbial species and microbial genes. dPCR Microbial DNA Detection Assays, together with the Custom dPCR Microbial Assays, are screening tools for rapid profiling and identification of microbial species (bacteria, fungi, viruses, and parasitic metazoa), antibiotic resistance genes, and virulence genes.

These Microbial 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 factor genes. The possibility to select each assay with any of the 6 fluorescent dyes FAM, HEX, TAMRA, ROX, Cy5, or ATTO 700 enables to combine at least 12 targets in a single multiplex dPCR reaction on the QIAcuity.

dPCR Microbial DNA Detection Assay Designs

The dPCR Microbial DNA Detection Assay portfolio encompasses a broad spectrum of target species across various application areas, including microbes associated with wastewater, infectious diseases, human pathogens, the human microbiome, multidrug resistance, sepsis, food production, and environmental samples. Assays are listed in the *dPCR Microbial DNA Detection Assays Technical Information* (**www.qiagen.com/PROM-20556**). Each assay has undergone rigorous bench verification and is ready to use for the detection of microbial species or genes. The dPCR Microbial DNA Detection Assay portfolio is continuously expanding with additional wet-lab tested assays to address emerging microbial threats and other microbial targets of broader interest.

Assays for bacterial detection primarily target ribosomal RNA genes, especially the 16S rRNA gene, and are designed using the GreenGenes database and type strain DNA sequences from NCBI. Fungal, viral, and metazoan assays focus on specific genetic regions, including ribosomal RNA and other marker genes, all sourced from NCBI. Assays for antibiotic resistance genes were developed using databases like Lahey and ARDB, while those for virulence factor genes utilize resources such as VFDB.

Custom dPCR Microbial Assay Designs

The Custom dPCR Microbial Assays are custom assay designs for detecting bacterial (16s rRNA), fungal (ITS), or viral targets. The online custom tool designs microbial probe-based assays based on sequence information taken from the NCBI sequence database. The target species can be selected by the user via NCBI taxonomy ID or species names. The automated design tool generates assay designs and provides an in-silico prediction of potential off-target taxa among species closely related to the chosen target. The custom assay design tool is not intended for designing assays specific for antibiotic resistance or virulence factor genes.

Further assay design support and custom design options

For additional questions regarding the output of Custom dPCR Microbial Assay designs, design parameters, or assay design needs not supported by the online tool—such as multiplex assays—you can contact us by clicking the "Contact us" button on the Design Tool interface. You may also explore QIAGEN's comprehensive custom design services offered through Genomics Services for more tailored solutions.

Identification of microbial taxa

Microbial DNA Detection Assays and Custom dPCR Microbial Assays identify and quantify various microbial species within a sample. By combining individual assays with up to 6 distinct fluorescently labeled probes (FAM, HEX, TAMRA, ROX, Cy5, and ATTO 700), these assays enable comprehensive profiling of the sample's microbial composition. Accurate quantification of microbial species is essential, as their presence and abundance significantly impact health and disease. With at least one control and one experimental sample, the dPCR Microbial DNA Detection Assays can (I) assess microbial composition at a specific body site, (II) monitor changes over time, (III) compare populations, and (IV) evaluate the effects of treatments. These capabilities facilitate the discovery of novel microbial biomarkers.

To perform a microbial identification experiment, a minimum of one experimental sample and one No Template Control (Microbial DNA-Free Water, cat. no. 338132) sample is necessary. The dPCR Microbial DNA Detection Assays and Custom dPCR Microbial Assays are compatible with various sample types, including isolated bacterial colonies, blood cultures, swabs, stool, and other metagenomic samples.

Identification of microbial virulence and resistance genes

dPCR Microbial DNA Detection Assays can help identify various microbial virulence and resistance genes present in a sample. By utilizing up to 6 differentially labeled probes (FAM,

HEX, TAMRA, ROX, Cy5, and ATTO 700) in combination with individual assays, these assays provide detailed profiling of antibiotic resistance and virulence gene composition. Monitoring these genes is critical not only for detecting dangerous hospital pathogens with multidrug resistance but also for advancing our understanding and control of harmful microorganisms. When paired with digital PCR, the dPCR Microbial DNA Detection Assays enable precise quantification and targeted detection of a broad array of microbial virulence and resistance genes.

Using at least one control and one experimental sample, these assays can (I) determine the composition of resistance and virulence genes at a specific site, (II) track changes over time, (III) compare findings against established standards, and (IV) assess shifts due to antimicrobial treatments or environmental changes. These capabilities support the development of novel strategies for controlling microbial virulence and resistance.

To conduct a microbial identification experiment, at least one experimental sample and one No Template Control (Microbial DNA-Free Water, cat. no. 338132) are required. The dPCR Microbial DNA Detection Assays are compatible with various sample types, including isolated bacterial colonies, blood cultures, swabs, stool, and other metagenomic samples.

Best practices for positive identification of microbial targets in dPCR

To reliably detect a target microbial species or gene within a sample, it is essential to distinguish between positive and negative samples. While the dPCR Microbial DNA Detection Assays and Custom dPCR Microbial Assays are engineered for maximum specificity, no template controls (NTCs) may occasionally yield single positive partitions. This background signal must be considered when evaluating a sample as positive.

We recommend classifying a sample as positive for the target if the following condition is met:

- The mean number of detected copies/µL (Sample) exceeds the Limit of Blank plus 1.645 times the standard deviation of the detected copies/µL (Sample).

Notes:

- To ensure statistical significance, it is advisable to perform 3 NTC replicates and utilize the 24-well 26K nanoplate.
- For a detailed calculation method to determine a positive sample in comparison to the NTC, please refer to "Appendix A: Data Analysis ", on page 45.

Principle and Procedure

Digital PCR

The dPCR Microbial DNA Detection Assay portfolio and Custom dPCR Microbial assays are designed for nanoplate digital PCR. Digital PCR (dPCR) divides the PCR reaction into numerous partitions, randomly distributing the template across them. Fluorescence from sequence-specific DNA probes indicates amplification of the target. Utilizing Poisson statistics, the average target DNA per valid partition is calculated, and the total target DNA across all partitions in a well is determined by multiplying this average by the number of valid partitions. Absolute quantification via dPCR negates the need for standard curves to assess target DNA levels in a sample.

Assay Design

Each assay in the portfolio and from the custom design relies on endpoint PCR amplification of a species-specific genetic region or a specific microbial gene. The amplified product is detected using target-specific fluorescent hydrolysis probes, enhancing the assay's specificity. The assays are available with 6 selectable probes, FAM, HEX, TAMRA, ROX, Cy5, and ATTO 700, for multiplexing with each other or other probe-based assays. The dPCR Microbial DNA Detection Assay portfolio contains a number of PAN assays for broad specificity towards bacteria or fungi. These assays are capable of quantifying the presence of a broad spectrum of bacteria and fungi to assess the bacterial or fungal load of a sample.

Assay Multiplexing

Each assay in the catalog portfolio, including Custom dPCR Microbial Assays, can be ordered with one of 6 dyes: FAM, HEX, TAMRA, ROX, Cy5, or ATTO 700. This enables the combination of up to 12 assays in a single dPCR reaction, known as a multiplex reaction.

Multiplexing is particularly useful when multiple target organisms need to be detected and quantified in one sample or when sample quantity is limited. Several assay bundles targeting marker organisms or genes across various fields, including the human microbiome, probiotics, infectious diseases, wastewater, virulence genes, resistance genes, and food production have been wet lab- tested. For a list of wet lab- tested assay bundles, visit **www.qiagen.com/dPCRMicrobialDNADetectionAssays**

To prepare a multiplex reaction, simply combine the corresponding assays in the reaction mixture. As individual assay combinations may interfere with one another, it is necessary to experimentally test the compatibility of assays from the dPCR Microbial DNA Assay portfolio, as well as any combinations of catalog assays with Custom dPCR Microbial assays.

When combining multiple assays in multiplex reactions, several factors must be considered. While individual assay designs may be compatible in multiplex reactions, certain assays can interfere with one another, depending on the microbes present in the sample and/or the target region of the assay. Therefore, potential incompatibilities should be carefully assessed.

As a guideline for compatibility, amplicons of homologous genes should not overlap to avoid cross-reactivity between assays targeting closely related species with highly similar sequences. Additionally, when analyzing samples with a high presence of closely related species—such as environmental samples—there is an increased risk of interference between multiplexed assays due to similar target sequences. Thus, having prior knowledge of the microbial composition expected in the sample can be advantageous when planning multiplex reactions.

To ensure compatibility of the selected assays for the specific samples being analyzed, we recommend experimentally testing the combinations of assays from the dPCR Microbial DNA Assay portfolio, as well as any combinations of catalog assays with Custom dPCR Microbial assays. Using positive controls, such as synthetic templates or genomic DNAs (gDNAs), can further help validate compatibility.

Controls

For the majority of the dPCR Microbial DNA Detection assays in the catalog assay portfolio, the Microbial DNA Positive Control (cat. no. 338135) can be used as a positive control template. The Microbial DNA Positive Control is a pool of synthetic DNA templates that is supplied at 10x concentration and requires dilution with Microbial DNA-Free Water. It can be used to ensure that the experimental conditions and PCR setup are correct. For further details on handling, usage, and portfolio coverage of the Microbial DNA Positive Control, see "Appendix B: Microbial DNA Positive Control (PMCv2)" on page 50 for more information.

For targets absent in the Microbial DNA Positive Control or customized positive control templates, use the sequence information for the region of interest provided on **GeneGlobe.com** for the dPCR Microbial DNA Detection Assays or in the product sheets for the Custom dPCR Microbial Assays to order synthetic DNA templates from secondary suppliers.

A no template control (NTC) reaction is essential to verify the specificity and accuracy of the dPCR results. It helps ensure that any amplification observed is due to the target DNA. Including an NTC allows you to confidently interpret your experimental data, ensuring the results are free from false positives.

The dPCR Microbial DNA Detection Assay portfolio entails Pan assays designed to detect a broad range of bacterial or fungal species that can serve as an amplification control and/or microbial load control of the sample. Two different Pan Bacteria designs are included to increase the coverage of bacterial species detected. Furthermore, *Pan-Aspergillus/Candida* and *Pan-Aspergillus/Penicillium* detect fungal species of *Aspergillus* and *Candida*, and *Aspergillus* and *Penicillium* genera, respectively. The Pan Assays can be used to monitor proper DNA extraction of bacteria and fungi. Also, they can be used to determine the overall bacterial, fungal, *Aspergillus/Candida*, and *Aspergillus/Penicillium* load in a sample.

Workflow

The QIAcuity Digital PCR System simplifies the analysis of microbial targets through an easy, streamlined workflow (see Figure 1). Starting with a prepared DNA/RNA sample, the process involves 4 steps: (1) set up the dPCR reaction, (2) load reaction mix into nanoplate wells, (3) place nanoplate into QIAcuity and run fully automated dPCR, and (4) analyze the data using the system's software suite. This workflow delivers results in less than 2.5 hours and supports the parallel analysis of multiple microbial targets in a single reaction. The QIAcuity software also offers versatile options for data analysis, visualization, and export, enhancing the efficiency and ease of the overall process. For further information on dPCR reaction setup and data analysis see *QIAcuity Application Guide* (**www.qiagen.com/HB-2839**) and *QIAcuity User Manual* (**www.qiagen.com/HB-2717**).

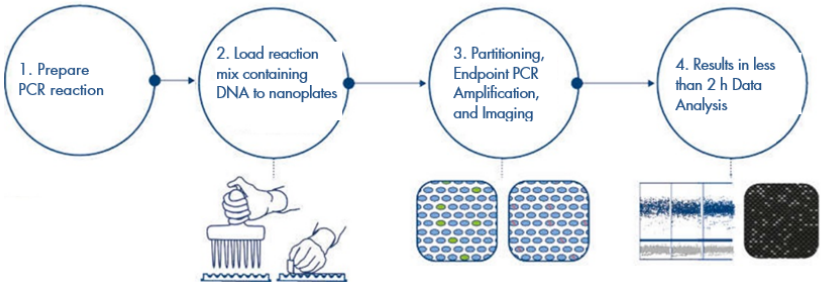

Figure 1. Overview of the microbial DNA/RNA target analysis using dPCR. The workflow consists of 4 steps: **(1)** preparing the PCR reaction by combining microbial genomic DNA/RNA with QIAcuity-ready mastermixes, **(2)** loading the reaction mix into the wells of the dPCR nanoplate and sealing it, **(3)** conducting the dPCR reaction on the QIAcuity instrument, and **(4)** analyzing the results using the QIAcuity Software Suite, with the option for manual analysis through Excel®-compatible export files.

Purification of microbial DNA and RNA for detection in dPCR

A successful dPCR reaction depends on the purity and integrity of the template, primers, and probes used. For the dPCR Microbial DNA Detection Assays and the Custom dPCR Microbial Assays, the template is DNA either purified from a biological sample being analyzed or cDNA synthesized from RNA, or a known amount of DNA to be used as a standard or positive control. Because PCR consists of multiple rounds of enzymatic reactions, it is more sensitive to impurities such as proteins, phenol/chloroform, salts, and EDTA than single-step enzyme-catalyzed reactions.

Purification of microbial genomic DNA from highly diverse sample material requires the use of dedicated extraction chemistries that are optimized for handling its specific contaminants. Purity of nucleic acid templates is important for dPCR because contaminants can interfere with fluorescence detection. QIAGEN® offers a complete range of nucleic acid purification systems that are dedicated for the isolation of bacterial, fungal, and viral DNA and RNA from a variety of biological samples and that provide pure, high-quality templates for PCR and RT-PCR. These include QIAprep® kits for purification of plasmid DNA, QIAamp® and DNeasy® kits for purification of genomic DNA, and AllPrep® Power kits with inhibitor removal technology. A selection of recommended purification kits is listed in Table 2. Details about QIAGEN kits for nucleic acid purification can be found at **www.qiagen.com/dna-purification/microbial-dna**

Table 2. Recommended DNA purification kits by sample type

Sample material	DNA purification kit	Catalog no.
Stool	QIAamp PowerFecal Pro DNA Kit	51804
	QIAsymphony PowerFecal Pro DNA Kit	938036
	RNeasy PowerFecal Pro Kit	78404
	AllPrep PowerFecal Pro DNA/RNA Kit	80254
Blood	QIAamp UCP PurePathogen Blood Kit	50112
	QIAamp DNA Blood Mini Kit	51104
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
	QIAamp UCP DNA Micro Kit	56204
	QIAamp DNA Microbiome Kit	51704
Plasma, serum, cell-free body fluids	QIAamp MinElute Virus Kit	57704
Wastewater	AllPrep PowerViral DNA/RNA Kit	28000-50
	RNeasy PowerFecal Pro Kit	78404

Template Input

For each single reaction in the dPCR Microbial DNA Detection workflow, start with at least 5 ng of genomic DNA isolated from a metagenomic sample or as low as 2.5 ng DNA from an isolated bacterial colony. For example, 10 ng of *Escherichia coli* gDNA corresponds to ~2,000,000 copies of a single-copy gene. This number increases for multi-copy genes and also might differ in other microbial organisms.

DNA sample digestion

Random template partitioning is essential for accurate quantification in dPCR systems. For the vast majority of QIAcuity dPCR applications, template DNA is uniformly distributed throughout

the QIAcuity Nanoplate reaction chambers. In QIAcuity reactions using PCR products, formalin-fixed, paraffin-embedded (FFPE) DNA, circulating cell-free DNA (cfDNA), or complementary DNA (cDNA) as template, a uniform distribution of PCR signal is observed. However, DNA molecules >30 kb are unevenly partitioned, which leads to over quantification of template concentration.

By adding restriction enzymes directly to the QIAcuity dPCR reaction mixes, large templates can be fragmented to smaller sizes, which results in even template distribution and accurate quantification. When adding restriction enzymes to reaction mixes, users must ensure that the enzymes do not cut within the amplicon sequence. A summary of recommended enzymes is listed in Table 3.

Table 3. List of recommended enzymes

6-Cutter restriction enzymes		4-Cutter restriction enzymes	
*EcoR*I	0.25 U/µL EcoRI-HF®, NEB® 0.025 U/µL Anza™ 11 EcoRI, TFS	*Alu*I	0.025 U/µL AluI, NEB 0.025 U/µL Anza 44 AluI, TFS
*Pvu*II	0.025 U/µL PvuII, NEB 0.025 U/µL Anza 52 PvuII, TFS	*CviQ*I	0.025 U/µL CviQI, NEB 0.025 U/µL Csp6I (CviQI), TFS
*Xba*I	0.025 U/µL Anza 12 XbaI, TFS	*Hae*III	0.025 U/µL BsuRI (HaeIII), TFS

* NEB, New England Biolabs; TFS, Thermo Fisher Scientific.

dPCR mastermixes for microbial DNA detection

The dPCR Microbial DNA Detection Assays and Custom dPCR Microbial Assays are designed to detect a variety of microbial taxa and genes, including DNA targets like 16S bacterial sequences and resistance genes, or RNA targets, such as RNA viruses. Depending on whether your sample contains DNA or RNA, you will need to use different dPCR mastermix solutions or workflows: the QIAcuity Probe PCR Kit for DNA targets, and the QIAcuity OneStep Advanced Probe Kit for RNA targets, enabling One-step RT-dPCR directly in the dPCR nanoplate.

Alternatively, RNA targets can be reverse-transcribed to cDNA and analyzed with the QIAcuity Probe PCR Kit. For ultra-clean experimental setups, the QIAcuity UCP Probe PCR Kit is recommended. For multiplexing of up to 12 targets, please use the QIAcuity High Multiplex Probe PCR Kit.

The kits work in conjunction with the QIAcuity Digital PCR System (**www.qiagen.com/qiacuity-digital-pcr-system**) and the QIAcuity Nanoplates (**www.qiagen.com/qiacuity-nanoplates-and-accessories**).

For further information please refer to *QIAcuity User Manual* (**www.qiagen.com/HB-2717**).

QIAcuity Probe PCR Kit

The dPCR Microbial DNA Detection Assay Portfolio and the Custom dPCR Microbial Assays work in conjunction with the QIAcuity Probe PCR Kit. The QIAcuity Probe PCR Kit contains a 4x concentrated, ready to use Master Mix optimized for microfluidic use in the QIAcuity Nanoplates. This special master mix enables accurate quantification of up to 5 targets having widely differing abundance in a well of the QIAcuity Nanoplate. This saves time, money, and reduces the amount of sample material needed. Moreover, the duplex or multiplex PCR data obtained is comparable with that obtained from a singleplex PCR.

QIAcuity UCP Probe PCR Kit

Contamination in the PCR reaction with low amounts of microbial DNA is a commonly recognized phenomenon. For the quantification and detection of microbes, this can be a problem because it lowers the sensitivity towards the contaminating target species. For example, *E. coli* DNA is frequently found in mastermixes due to recombinant production of polymerase in *E. coli*.

The QIAcuity UCP Probe Mastermix is developed for ultra clean experimental setups. A special depletion procedure reduces the content of potentially contaminating microbial DNA

to a level that significantly reduces the background signal and enables the detection and absolute quantification of very low abundant target species and genes. UCP Probe Mastermix is particularly recommended when analyzing low biomass samples or using generic priming strategies like PAN 16S or 18S detection, because these will amplify any potential microbial DNA contamination.

QIAcuity OneStep Advanced Probe Kit

The QIAcuity OneStep Advanced Probe PCR Kit enables sensitive quantification of RNA or RNA+DNA targets on the QIAcuity digital PCR instrument in one combined RT-PCR reaction. The OneStep chemistry allows the RT and amplification for viral RNA of interest in a single step. In the OneStep reaction, RNA and DNA targets can also be detected together, for example, viral RNA with bacterial DNA.

QIAcuity High Multiplex Probe PCR Kit

The dPCR Microbial DNA Detection Assay Portfolio and the Custom dPCR Microbial Assays also work with the QIAcuity High Multiplex Probe PCR Kit. With an optimized buffer and the highest concentration of Taq polymerase in any QIAcuity Master Mix, this mix can be used to quantify up to 12 DNA targets per reaction. This saves time, money, and reduces the amount of sample needed. For more details on higher order multiplexing, refer to the *QIAcuity High Multiplex Probe PCR Kit Handbook*.

Considerations for RNA targets

The dPCR Microbial DNA Detection Assay portfolio and the Custom dPCR Microbial Assays offer a number of viral RNA targets that must be transcribed into cDNA prior to dPCR analysis. This can be done either in a Two-step or One-step workflow (see Figure 2).

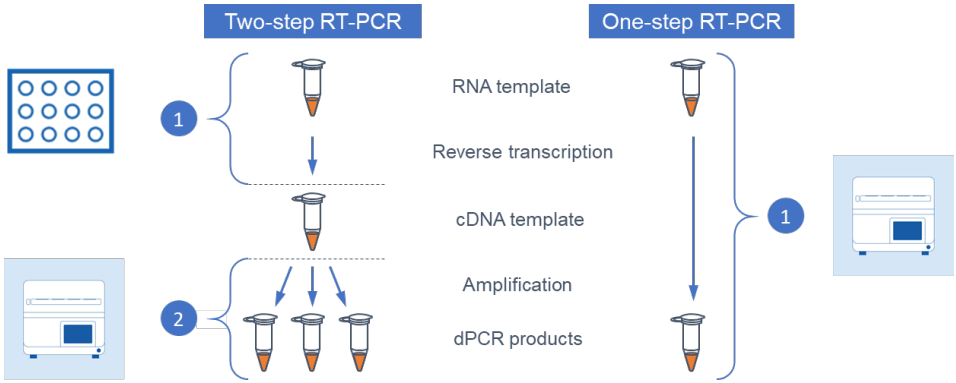

Figure 2. Workflows of Two-step and One-step RT-PCRs using RNA templates. Target RNA Detection via cDNA synthesis can be done following a Two-step or a One-step workflow. In the Two-step workflow, cDNA synthesis occurs in a separate step. The resulting cDNA is diluted and used as a template in a separate step, the dPCR reaction which is done in the well of the dPCR nanoplate. In the One-step procedure cDNA synthesis and dPCR amplification are both done in a single well of the dPCR nanoplate.

Two-step Workflow

In the Two-step workflow, cDNA synthesis and dPCR amplification are separated. To this end, the extracted RNA has to be reverse-transcribed into cDNA in a separate reaction prior to usage in dPCR. After reverse transcription, the resulting cDNA is diluted and used as a template in a second step, the dPCR reaction, which is done in the wells of the dPCR nanoplate (Figure 2). For cDNA synthesis, the use of the QuantiTect Reverse Transcription Kit is recommended. Please follow the instructions provided in the *QuantiTect Reverse Transcription Kit Handbook*(**www.qiagen.com/HB-0189**).

One-step Workflow

In the One-step workflow, both the cDNA synthesis and dPCR amplification occur in the well of the dPCR nanoplate (Figure 2). Because the Mastermix has to contain both RT enzyme and

Taq polymerase, One-step workflows require the use of a mastermix other than the QIAcuity Probe Mastermix.

The QIAcuity One-step Advanced Probe Kit is the recommended One-step solution for the quantification of RNA and DNA targets with hydrolysis probes in a singleplex or multiplex (up to 5 targets) reaction. It is optimized for nanoplate digital PCR on the QIAcuity and prevents uncontrolled RT activity.

General considerations

The number of cDNA templates is calculated using Poisson statistics based upon the total number of positive partitions. In extreme cases, that is:

- **Samples with low number of cDNA transcripts**: transcript abundances might vary from the true abundance as a rare target transcript could be lost in the dead volume or as the rare target transcript might not be correctly represented in the fraction of the original cDNA that was used for quantification.
- **Samples with high number of target transcripts**: with increasing number of target transcripts being randomly distributed over the partitions, a partition could contain multiple target transcripts. In extreme cases, when all partitions are positive, it will no longer be possible to conduct any quantification.

In case the amount of target RNAs of interest is unknown and not expected to be low, we recommend to use cDNA dilutions of 1:10 and 1:100 as input template for dPCR.

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 or RNA isolation kit
- Digital PCR Instrument
- QIAcuity dPCR Nanoplates
- Multichannel pipettor
- Nuclease-free pipette tips and tubes

Important Notes

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

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 dPCR setup and post-dPCR processing operations. Decontaminate your dPCR workspace and labware (pipettes, tube racks, etc.) with UV light before each new use to render any contaminated DNA ineffective in dPCR 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 dPCR nanoplate. Removing the sealer foil from dPCR nanoplate releases dPCR product DNA into the air where it can contaminate the results of future experiments. In the event of contamination ensure that any affected labware and bench surfaces are decontaminated.
- Do not remove the dPCR nanoplate from its protective sealed bag until immediately before use.

Protocol: Detection of Pathogen DNA Targets Using dPCR Microbial DNA Detection Assays or Custom dPCR Microbial Assays

This protocol is optimized for the detection and quantification of specific microbial DNA targets using the dPCR Microbial DNA Detection Assays listed in Appendix H (cat. no. 250207) or Custom dPCR Microbial Assays (cat. no. 250208) with the QIAcuity® Probe PCR Kit (cat. nos. 250101, 250102, and 250103), the QIAcuity UCP Probe PCR Kit (cat. nos. 250121 and 250122), or QIAcuity High Multiplex Probe PCR Kit (cat. nos. 250133 and 250134) in 24-well 26K nanoplates and 96-well 8.5K nanoplates (cat. nos. 250001, 250011, 250021, and 250031).

Important points before starting

- Use supplied UCP PCR Water for setting up dPCR reactions.
- Limit the number of times each tube of UCP PCR Water and QIAcuity Probe Mastermix is opened to 3 times. This will help to prevent contamination.
- It is essential to start with high-quality DNA. For recommended nucleic acid preparation methods, refer to “Purification of microbial DNA for detection in dPCR” , on page 18.
- dPCR Microbial DNA Detection Assays and Custom dPCR Microbial Assays come as a 20x primer–probe mix lyophilized in a single tube and have to be resuspended prior to usage. For further dilution and storage information please refer to "Shipping and Storage" on page 6.
- 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.

- Pipetting accuracy and precision affect the consistency of results. Be sure that all pipettes and instruments have been checked and calibrated according to the manufacturer's recommendations. Also make sure that no bubbles are introduced into the wells of the QIAcuity Nanoplate during pipetting.
- To compensate for any potential environmental contamination, it is required to run at least one No Template Control (NTC) sample for the identification assays.
- The QIAcuity High Multiplex Probe PCR Kit requires the QIAcuity Software Suite version 3.0 or higher. For amplitude-based multiplexing, the QIAcuity Software Suite version 3.1 or higher is required.

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/µL DNA, the A_{260}/A_{280} ratio should be greater than 1.8.
- Thaw genomic DNA and QIAcuity Probe 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. Thaw the QIAcuity (UCP/High Multiplex) Probe PCR Mastermix, template DNA or cDNA, 20x primer–probe mix, and UCP PCR water. Vigorously mix the QIAcuity (UCP/High Multiplex) Probe PCR Mastermix and the individual solutions. Centrifuge briefly to collect liquids at the bottom of the tubes.

2. Prepare a reaction mix for the number of reactions needed according to Table 4, Table 5, or Table 6. Due to the hot-start, it is not necessary to keep samples on ice during reaction setup or while programming the QIAcuity instrument.
3. Vortex the reaction mix.
4. Dispense appropriate volumes of the reaction mix, which contains all components except the template, into the wells of a standard PCR plate. Then, add template DNA or cDNA into each well that contains the reaction mix.
    - **Note**: The appropriate amount of reaction mix and template DNA depends on various parameters. Please refer to the *QIAcuity User Manual Extension: Application Guide* for details.
    - **Note**: For Two-step RT-PCR, the volume of the cDNA added (from the undiluted reverse-transcription reaction) should not exceed 15% of the final PCR volume.
5. Transfer the content of each well from the standard PCR plate to the wells of the nanoplate.
6. Seal the nanoplate properly using the QIAcuity Nanoplate Seal provided in the QIAcuity Nanoplate Kits.

    Note: For exact sealing procedure, please see the *QIAcuity User Manual*.
7. If a restriction enzyme for DNA digestion has been included in the reaction, leave the plate for 10 min at ambient temperature.

Table 4. Reaction setup using QIAcuity Probe PCR MM or QIAcuity UCP Probe PCR MM

	Volume/reaction		
Component	**Nanoplate 8.5k (24-well, 96-well)**	**Nanoplate 26k (24-well, 8-well)**	**Final concentration**
4x QIAcuity (UCP) Probe PCR Mastermix	3 µL	10 µL	1x
20x primer–probe mix 1*	0.6 µL†	2 µL†	0.6 µM forward primer 0.6 µM reverse primer 0.2 µM probe
20x primer–probe mix 2, 3, 4, 5* (for multiplex)	0.6 µL†	2 µL†	0.6 µM forward primer 0.6 µM reverse primer 0.2 µM probe
Restriction Enzyme (optional)	Up to 1 µL	Up to 1 µL	0.025–0.25 U/µL
UCP PCR water	Variable	Variable	
Template DNA or cDNA (added at step 4)	Variable‡	Variable‡	
Total reaction volume	**12 µL**	**40 µL**	

* For respective dye recommendation for the probe and available channels on the QIAcuity, please see the *QIAcuity User Manual* or the *QIAcuity User Manual Extension: QIAcuity Application Guide*.

† Volume might vary, depending on concentration of the primer/probe mix used.

‡ Appropriate template amount depends on various parameters. Please see the *QIAcuity User Manual Extension: QIAcuity Application Guide* for details.

Table 5. Preparing reaction mix for QIAcuity High Multiplex Probe PCR MM for detecting up to 6 targets per reaction (one target per channel in up to 6 channels)

	Volume/reaction		
Component	**Nanoplate 8.5k (24-well, 96-well)**	**Nanoplate 26k (24-well, 8-well)**	**Final concentration**
4x QIAcuity High Multiplex Probe PCR Master Mix	3 µL	10 µL	1x
20x primer–probe mix 1–6*	0.6 µL	2 µL	0.6 µM forward primer 0.6 µM reverse primer 0.2 µM probe
Restriction Enzyme (optional)	Up to 1 µL	Up to 1 µL	0.025–0.25 U/µL
RNase-Free Water	Variable	Variable	–
Template DNA or cDNA (added at step 4)†	Variable	Variable	–
Total reaction volume	**12 µL**	**40 µL**	

* For dye recommendations, see the *QIAcuity User Manual* or the *QIAcuity User Manual Extension: QIAcuity Application Guide*.

† Appropriate template amount depends on various parameters.

Table 6. Preparing reaction mix for QIAcuity High Multiplex Probe PCR MM for detecting up to 12 targets per reaction with amplitude-based multiplexing

	Volume/reaction		
Component	**Nanoplate 8.5k (24-well, 96-well)**	**Nanoplate 26k (24-well, 8-well)**	**Final concentration**
4x QIAcuity High Multiplex Probe PCR Master Mix	3 µL	10 µL	1x
20x Primer–probe mix 1–12* (for multiplex)	Variable	Variable	Variable †
Restriction Enzyme (optional)	Up to 1 µL	Up to 1 µL	0.025–0.25 U/µL
RNase-Free Water	Variable	Variable	–
Template DNA or cDNA (added at step 4)‡	Variable	Variable	–
Total reaction volume	**12 µL**	**40 µL**	

* For dye recommendations, see the *QIAcuity User Manual* or the *QIAcuity User Manual Extension: QIAcuity Application Guide*. Refer to the *QIAcuity® High Multiplex Probe PCR Kit Handbook* for guidance on selecting microbial assays that are suitable for amplitude-based multiplexing.

† For reactions that employ amplitude-based multiplexing, variable assay concentrations are required. Refer to the *QIAcuity High Multiplex Probe PCR Kit Handbook* for more details.

‡ Appropriate template amount depends on various parameters.

Thermal cycling conditions

1. Program the cycler of the QIAcuity instrument according to Table 7, next page. Imaging settings should be set to the default parameters.

   Note: When using the QIAcuity High Multiplex Probe PCR Kit, refer to the *QIAcuity® High Multiplex Probe PCR Kit Handbook* for guidance on imaging settings.

2. Place the nanoplate into the QIAcuity instrument and start the dPCR program.

Table 7. Thermal cycling conditions for QIAcuity Probe MM, QIAcuity UCP Probe MM, and QIAcuity High Multiplex Probe PCR MM

Step	Time	Temperature (°C)
PCR initial heat activation	2 min	95
Two-step cycling (40 cycles)		
Denaturation	15 s	95
Combined annealing/extension	60 s	58*

* Temperature during annealing/extension and number of cycles might vary depending on assay type.

Protocol: Detection of Pathogen RNA Targets Using the QIAcuity OneStep Advanced Probe Kit

This protocol is optimized for the quantification of RNA and DNA targets using the QIAcuity OneStep Advanced Probe Kit with hydrolysis probes in a singleplex or multiplex (up to 5 targets) reaction using QIAGEN's QIAcuity instruments for digital PCR.

The QIAcuity OneStep Advanced Probe Kit should be stored immediately upon receipt at –30°C to –15°C in a constant-temperature freezer and protected from light. Under these conditions, the components are stable for 12 months without showing any reduction in performance and quality, unless otherwise indicated on the label.

Important points before starting

- Refer to the *QIAcuity User Manual* (**www.qiagen.com/HB-2717**) and *QIAcuity User Manual Extension: QIAcuity Application Guide* (**www.qiagen.com/HB-2839**) for guidance on assay design and experimental setup for the QIAcuity platform.
- The QIAcuity OneStep Advanced Probe Kit has been specially formulated with a hot-start RT enzyme, allowing users to assemble reactions at ambient temperature and to run up to four and eight plates in parallel on the QIAcuity Four and QIAcuity Eight instrument, respectively.
- The optional Enhancer GC is recommended for use with all ABI TaqMan assays, amplicons >150 nt in length, GC rich amplicons, and RNA targets containing challenging secondary structures.

Procedure

Reaction mix setup

1. Place the 100x Advanced Reverse Transcription Mix on ice. Thaw the 4x QIAcuity OneStep Advanced Probe Master Mix, template RNA, primers, probes, Enhancer GC, and RNase-Free Water. Vigorously mix the QIAcuity OneStep Advanced Probe Master Mix and the individual solutions. Centrifuge the tubes briefly to settle the liquids.
2. Prepare a master mix according to Table 8, next page.

Table 8. Preparing the QIAcuity One-step Advanced RT-dPCR reaction mix

	Volume/reaction		
Component	**Nanoplate 8.5k (96-well, 24-well)**	**Nanoplate 26k (24-well, 8-well)**	**Final concentration**
4x One-step Advanced Probe Master Mix	3 µL	10 µL	1x
100x OneStep Advanced RT Mix (Reverse Transcription)	0.12	0.4 µL‡	1x
20x primer–probe mix 1*	0.6 µL‡	2 µL‡	0.6 µM forward primer 0.6 µM reverse primer 0.2 µM probe
20x primer–probe mix 2, 3, 4, 5* (for multiplex)	0.6 µL‡	2 µL‡	0.6 µM forward primer 0.6 µM reverse primer 0.2 µM probe
Enhancer GC† (optional)	1.5 µL	5 µL	
RNase-Free Water	Variable	Variable	
Template RNA (added at step 5)‡	Variable	Variable	
Total reaction volume	**12 µL**	**40 µL**	

* For dye recommendation for the probe and available channels on the QIAcuity, see the *QIAcuity User Manual* or the *QIAcuity User Manual Extension: QIAcuity Application Guide*.

†Enhancer GC is recommended for use with all ABI TaqMan assays, amplicons >150 nt in length, GC rich amplicons, and RNA targets containing challenging secondary structures.

‡ Appropriate template amount depends on various parameters. Please see the *QIAcuity User Manual Extension: QIAcuity Application Guide* for details.

3. Vortex the reaction to mix well. Dispense appropriate volumes of the reaction mix into the wells of a standard 96-well PCR pre-plate.

   Note: The pre-plate may be assembled at ambient temperature.

4. Add template RNA to wells containing the reaction mix. Thoroughly mix the template RNA with the template RNA by pipetting up and down.

One-step RT-dPCR protocol for all QIAcuity instruments

1. Transfer the contents of each well to the wells of a Nanoplate.
2. Seal the Nanoplate properly using the QIAcuity Nanoplate Seal provided in the QIAcuity Nanoplate Kits.
3. Place the Nanoplate into the QIAcuity instrument and start the dPCR program (Table 9).

Table 9. QIAcuity RT-dPCR cycling program

Step	Time	Temperature (°C)
Reverse Transcription	40 min	50
RT enzyme inactivation	2 min	95
Two-step cycling (40 cycles)		
Denaturation	5 s	95
Combined annealing/extension	30 s	58*

* Temperature during annealing/extension and number of cycles might vary depending on assay type.

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 in our Technical Support Center: **www.qiagen.com/FAQ/FAQList.aspx**. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information or protocols in this handbook (for contact information, visit **support.qiagen.com**).

	Comments and suggestions
Weak or no signal with Microbial DNA Positive Control (PMCv2)	
a) The selected fluorescence channel for dPCR data analysis does not comply with the protocol	For data analysis, select one of the 5 channels, green, yellow, orange, red, or crimson, depending on the probe dye used.
b) Incorrect programming of the QIAcuity dPCR instrument	Compare the temperature profile with the protocol. See the cycling conditions in Table 5 or Table 9. Refer to the *QIAcuity User Manual*.
c) PCR extension time too short	Use the extension time specified in the protocol.
d) 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 dPCR Microbial DNA Detection Assays, and provide a complete and efficient workflow for microbial DNA testing.
e) Incorrect configuration of the PCR	Ensure that reactions were set up according to the reaction mix preparation tables on Table 4. Repeat the dPCR run if necessary.
f) The storage conditions for one or more kit components did not comply with the instructions given in "Shipping and 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 dPCR Microbial DNA Detection Assay has expired	Check the storage conditions and the expiration date of the reagents and use a new kit if necessary.
h) Insufficient starting template	Increase the amount of template genomic DNA.
i) Assay template not entailed in PMCv2	Check if assay is covered by the PMCv2 Table 12. Repeat dPCR run with positive control gDNA or synthetic templates, if necessary.

	Comments and suggestions
Signals present for the negative control template (NTC copies/µL > 0)	
a) Contamination occurred during PCR setup	Repeat the PCR with new reagents. If possible, seal the PCR array/close the PCR tubes directly after addition of the sample to be tested. Make sure to pipet the positive controls last. Make sure that workspace 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. Make sure that workspace and instruments are decontaminated at regular intervals.
c) Pan Bacteria Assays and Pan Fungi Assays may detect residual bacterial and fungal genomic DNA found in the QIAcuity Probe PCR Mastermix.	If the mean number of positive partitions$^{(Sample)}$ > (the mean number of positive partitions$^{(NTC)}$ + 2.5x standard deviation of the number of positive partitions $^{(NTC)}$), then the sample is positive for the Pan Bacteria or the Pan Fungi Assays.

References

1. Armbruster, D.A., Pry, T. (2008) Limit of Blank, Limit of Detection and Limit of Quantitation. *Clin. Biochem.* Rev. 29, 49.
2. Bustin, S.A., Benes, V., Garson, J.A., Hellemans, 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.
3. Burd, E.M. (2010) Validation of laboratory-developed molecular assays for infectious diseases. *Clin. Microbiol.* Rev. 23, 550.

Glossary – Custom dPCR Microbial Assay Design Tool

Different parameters describing the specific Custom dPCR Microbial Assay are provided in the *Custom dPCR Microbial Assay Product Data Sheet* generated specifically for the designed assay. Definitions of these parameters are listed below.

Parameter	Description
Product No.	Catalog number of product line. 250208 fixed for all assays generated using the Custom assay design tool for Microbial dPCR Assays.
Product Group	Product line this assay belongs to. "Custom dPCR Microbial Assay" fixed for all assays generated using the Custom assay design tool for Microbial dPCR Assays.
Anchor sequence	Sequence from domain region (Bacteria 16s, Fungi ITS, Viruses) used to design the assay. Optional field limited to minimum 200 bp to maximum 5000 bp. It is recommended to add a sequence by the user if a dedicated sequence should be considered for the assay design job. It is not sufficient to add a sequence ID. More than 90% of the anchor sequence should be a valid DNA code, e.g., A, T, C, G. The quality of the entered anchor sequence is in the responsibility of the user. The provided anchor sequence will not be aligned to target taxon sequences. However, during ranking and quality checking of assay candidates, assay candidates will be filtered out if primers and probe do not align to other sequences within target taxon based on a set of tntblast criteria. If no assay candidate passes the ranking and quality check, a notification is provided but no assay output. If no anchor sequence is defined by the user, the design tool will assign the first reference sequence, in the target taxon, as anchor sequence. If the first reference sequence exceeds 5000 bp as it is often the case for viral sequences, the first 5000 bp will be used for assay design. Commonly, bacteria 16S and fungi ITS sequences do not exceed 5000 bp.
Product Name	Name defined for the target during submission of the design request. Name length is limited to 30 letters.

Parameter	Description
Catalog No. / GGID	The unique catalog no. and GeneGlobe ID (GGID) identifies the exact design of the Custom dPCR Microbial Assay. It is assigned by the system and must be used for reordering. We strongly recommend that it is mentioned in the method section of scientific publications. This will allow fellow scientists to order the exact same dPCR Microbial Assay and reproduce the reported results.
Fluorophore	Fluorophore of probe chosen by the user.
Amplicon Length	Number of nucleotides that are in the amplicon of this assay.
Amplicon Region	Prolonged amplicon sequence allowing creation of a synthetic positive control for this assay.
Restriction Enzymes compatible with amplicon	Recommended restriction enzymes to prevent digest within the amplicon of this assay.
Contents	1 tube containing pre-mixed primers and probe for a specific target [150 or 400] × 40 µL reactions (QIAcuity Nanoplate 26k) or [500 or 1332] × 12 µL reactions (QIAcuity Nanoplate 8.5k).
Domain region	Either Bacteria 16S, Fungi ITS, or Viruses domain that is used for the assay design process.
Number of sequences in the domain region	Total number of sequences within the domain used for the assay design. Either 216856 (Bacteria 16S), 2964221(Viruses), or 16365 (Fungi ITS).
Target Taxon Name	NCBI taxonomy name of target taxon the assay is designed for. Defined when creating design request.
Target Taxon ID	NCBI taxonomy ID of the target the assay is designed for. Defined when creating design request.
Target Taxon Rank	Taxonomic rank in NCBI database of taxon the assay is designed for.
Parent Taxon Name	Name of the parent node in the NCBI taxonomy tree.
Parent Taxon ID	NCBI taxonomy ID of the parent node in NCBI taxonomy tree.
Number of sequences in the target taxon	Count of the number of sequences for the target taxon in the NCBI database. The assay is designed to target sequences of the target taxon.

Parameter	Description
Number of sequences in the parent taxon	Count of the number of sequences for the parent taxon in the NCBI database. The assay is designed to avoid targeting sequences of the parent taxon except those of the target taxon.
Design rank	Ranking of the assay according to the in-silico design algorithm. 1 – best, ranking order decreasing.
Count of hits in target taxon	Number of sequences in target taxon that are covered by this assay (hits). Alignment of primers and probe of the assay is based on a set of tntblast criteria which are based on empirical evidence. The assay is designed to be sensitive for the detection of the sequence(s) in the target taxon while balancing the optimal discrimination from all other sequences in the parent taxon and the rest of the sequences in the domain region. The more hits are achieved on the target taxon, the better is the resulting performance of the assay. The number of hits is limited to number of sequences in the target taxon.
Coverage (%)	Percentage of sequences within the target taxon that the primers and probe of the assay align to. The alignment is based on a set of tntblast criteria, which are based on empirical evidence. Less than 100% possible if major deviations between the sequences of the target taxon occur. Calculation: Coverage (%) = (Count of hits in target taxon) / (Number of sequences in target taxon, expressed in percentage).
Count of hits in domain region	Number of sequences in domain region that are covered by this assay (hits) but are not part of the target taxon. Alignment of primers and probe of this assay is based on a set of tntblast criteria, which are based on empirical evidence. The less hits are achieved in the domain region (outside of the target taxon) the higher the specificity of the assay for the target taxon. The number of hits is limited to the total number of sequences in the domain region subtracted by the number of sequences in the target taxon.
Off-target within domain region (%)	Percentage of sequences within the domain region that the primers and probe of the assay align to but are not part of the target taxon. Alignment is based on a set of tntblast criteria, which is based on empirical evidence. Calculation: Off-target within domain region (%) = (Count of hits in domain region) / (Number of sequences in domain region, expressed in percentage).

Parameter	Description
Representative on targets	List of NCBI sequence IDs within target taxon that the primers and probe of the assay align to based on a set of tntblast criteria, which are based on empirical evidence. Number of listed NCBI sequence IDs equals *count of hits in target taxon*.
Representative off-targets	List of NCBI sequence IDs within domain region the primers and probe of the assay align to but are not part of the target taxon. Alignment based on a set of tntblast criteria, which are based on empirical evidence. Number of listed NCBI sequence IDs equals *count of hits in domain region*.
Count of missed targets	Number of sequences in target taxon that are not covered by the assay. Alignment of primers and probe of the assay based on a set of tntblast criteria, which are based on empirical evidence. The lower the number of missed targets, the better are the expected performance characteristics of the assay. The number is limited to the number of sequences in the target taxon. Calculation: Count of expected missed targets = (Number of sequences in target taxon) – (Count of hits in target taxon)
Off Target Hit Accessions	Accession numbers of the non-target sequences predicted in silico. These hits indicate a possible cross-reactivity of the assay with these sequences.
Taxonomy Parent Accessions	Accession numbers of sequences from closely related species of the target species used for in-silico prediction of assay cross-reactivity.

Important: All taxonomy-related terms and classifications used are derived from the National Center for Biotechnology Information (NCBI) database.

Appendix A: Data Analysis

Principle for dPCR Microbial DNA Detection Assays and Custom dPCR Microbial Assay Data Analysis

In digital PCR, detection and quantification of target DNA molecules is based on copies/µL values obtained from individual PCR reactions in nanoscale volumes called partitions. Individual partitions are generated by splitting a PCR reaction over which the target molecules are randomly distributed. After partitioning, some partitions will contain no copy of the target molecule, some will contain one copy of the target molecule, and some others will contain more than one copy of the target molecule. Since the target molecules are randomly distributed, the Poisson distribution can be used to calculate the average number of copies of the target molecule per partition. The total number of copies of the target molecule in all valid partitions of a well is calculated by multiplying the average number of copies of the target molecule per partition by the number of valid partitions. The QIAcuity Software Suite calculates the target DNA molecule concentration in the reaction in copies/µL by referring to the known volume of a valid partition.

For dPCR Microbial DNA Detection Assays and Custom dPCR Microbial Assays, there are 2 data analysis methods depending on the application. For relative profiling or comparing 2 different populations (e.g., healthy versus diseased, time 0 versus time 30 days), the normalization method is recommended. For identification of microbial species or microbial genes, the NTC method is used. The NTC method allows for higher sensitivity of detection by measuring the background of the assay. For both methods, the copies/µL value for a dPCR assay is positively correlated with the abundance of the microbial species or gene in the sample.

Microbial dPCR Profiling Assays (normalization method)

The normalization method is used for the relative profiling or comparison between 2 populations. To account for different starting amounts of DNA in a sample, separate reference assays are used to normalize the sample input. Depending on the type of assay and application, total bacterial or fungal genomic DNA in combination with the Pan Bacteria and Pan Fungi assays can be used for normalization against the bacterial background of the sample. Alternatively, any established assay, for example targeting the host genomic DNA in the sample, can be used.

To calculate the normalized copies/µL values of each sample, the copies/µL values of the replicates for each reference and target assay are averaged. The normalized copies/µL value of the target molecule is obtained by dividing the average copies/µL value of the target molecule by the average copies/µL value of the reference assay. With the normalized copies/µL values the fold increase or decrease in abundance is calculated based on the formula:

$$\text{Fold change} = \log_2\left(\frac{\text{normalized cp}/\mu\text{L}_{\text{Sample 1}}}{\text{normalized cp}/\mu\text{L}_{\text{Sample 2}}}\right)$$

As an example for the calculation of abundance fold changes between samples using reference genes for normalization, Table 10 depicts dPCR analysis results for 2 samples analyzed in a duplex reaction with a target of interest-specific assay and a reference assay. Each Sample was analyzed in triplicates.

Sample 1:

Average cp/µL target Sample 1 = (68 + 73 + 69)/3 = 70 cp/µL

Average cp/µL reference Sample 1 = (172 + 165 + 164)/3 = 167 cp/µL

Normalized abundance target Sample 1 = 70/167 = 0.419

Sample 2:

Average cp/µL target Sample 2 = (111 + 105 + 114)/3 = 110 cp/µL

Average cp/µL reference Sample 2 = (79 + 84 + 86)/3 = 83 cp/µL

Normalized abundance target Sample 2 = 110/83 = 1.325

Foldchange in Sample 2 compared to Sample 1:

$FC_{Sample2} = \log_2(1.325/0.419) = 1.661$

Table 10. Example dPCR data for 2 samples analyzed with a target and a reference assay

Nanoplate Well	Sample/NTC/Control	Reaction Mix	Channel	Concentration (copies/µL)
A1	S1-replicate1	target assay	GREEN	68
A1	S1-replicate1	ref assay	YELLOW	172
A2	S1-replicate2	target assay	GREEN	73
A2	S1-replicate2	ref assay	YELLOW	165
A3	S1-replicate3	target assay	GREEN	69
A3	S1-replicate3	ref assay	YELLOW	164
B1	S1-replicate1	target assay	GREEN	111
B1	S1-replicate1	ref assay	YELLOW	79
B2	S1-replicate2	target assay	GREEN	105
B2	S1-replicate2	ref assay	YELLOW	84
B3	S1-replicate3	target assay	GREEN	114
B3	S1-replicate3	ref assay	YELLOW	86

Second level analyzes of foldchanges with abundances normalized against reference assays can be done in the QIAcuity Software Suite using the Gene Expression Plugin. Therein, one can select single and multiple reference genes for normalization as well as analyze singleplex and multiplex approaches. For further details refer to the gene expression chapter in the *QIAcuity User Manual Extension: QIAcuity Application Guide* (**www.qiagen.com/HB-2839**).

Microbial dPCR Identification Assays (NTC method)

The NTC method allows for higher sensitivity of detection by measuring the background of the assay expressed as the limit of blank (LOB). The assay background and/or low levels of contamination due to environmental factors can be corrected for by performing a dPCR reaction using No Template Control (NTC) as the sample. NTC is used to establish a threshold for the number of positive partitions above which a target in the sample can be considered as positively detected. This threshold is called the limit of detection (LOD) and can vary between assays and sample types. As an example, Table 11 shows the number of detected copies/µL in 4 NTC and 3 Sample replicates. The LOD is calculated as follows:

Average number of measured copies/µL in NTC ($mean_{NTC}$) = (0 + 0.055 + 0)/3 = 0.018

Standard deviation of measured copies/µL in NTC (SD_{NTC}) = 0.026

Limit of Blank (LOB) = $mean_{NTC}$ + 1.645*(SD_{NTC}) = 0.018 + 1.645*0.026 = 0.061

Average number of measured copies/µL in Sample ($mean_{Sample}$) = (0.268 + 0.386 + 0.377)/3 = 0.344

Standard deviation of measured copies/µL in Sample (SD_{Sample}) = 0.054

Limit of Detection (LOD) = LOB +1.645*(SD_{Sample}) = 0.061 + 1.645*0.054 = 0.149 copies/µL

LOD in total copies per reaction (8.5K nanoplate) = LOD*12 = 0.149*12 = 1.79 copies

LOD in total copies per reaction (26K nanoplate) = LOD*40 = 0.149*40 = 5.96 copies

For this particular example, the target can be considered as present because the on average measured copies/µL value ($mean_{Sample}$) is > 0.149.

For cases of ($mean_{Sample}$) < LOD the sample should be considered absent for the target.

Table 11. Example of detected positive partitions for 3 NTC and 3 Sample replicates.

Replicate	Measured copies/µL
NTC Replicate 1	0
NTC Replicate 2	0.055
NTC Replicate 3	0
Sample Replicate 1	0.268
Sample Replicate 2	0.386
Sample Replicate 3	0.377

For further details on the Limit of Detection (LOD), the Limit of Blank (LOB), please refer to the literatures listed in References.

Appendix B: Microbial DNA Positive Control (PMCv2)

The Microbial DNA Positive Control can be used to ensure that the experimental conditions and PCR setup are correct. It 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 with EB buffer or Tris·HCl as follows:

- 5 µL of 10x Microbial DNA Positive Control + 45 µL EB buffer or Tris·Cl.

  Note: 1x Microbial DNA Positive Control can be stored at –20°C. Avoid repeated freeze/thaw cycles of 1x Microbial DNA Positive Control.

- 1 µL of the 1x Microbial DNA Positive Control is contains approximately 2000 target copies. The recommended amount to use when setting up an assay is as follows:
  - **8.5k 96-well nanoplate**: 0.6 µL of 1x Microbial DNA Positive Control per 12 µL reaction.
  - **26k 24-well nanoplate**: 2 µL of 1x Microbial DNA Positive Control per 40 µL reaction.

Under proper assay setup and PCR conditions, the Microbial DNA Positive Control will yield 10–500 copies/µL reaction.

Portfolio Coverage

The Microbial DNA Positive Control PMCv2 (cat. no. 338135) does not contain templates for all assays of the dPCR Microbial DNA Assay portfolio. Table 12 lists all assays currently not covered by the Microbial DNA Positive Control.

Table 12. Assays not entailed in the Microbial DNA Positive Control PMCv2

GeneGlobe cat. no.	Assay name	Target region	Taxonomy/target type
DMA00346	*Pan Aspergillus/Candida*	28S ribosomal RNA gene	Fungi
DMA00347	Pan Bacteria 1	16S ribosomal RNA gene	Bacteria
DMA00348	Pan Bacteria 3	16S ribosomal RNA gene	Bacteria
DMA00404	*Blautia coccoides*	16S ribosomal RNA gene	Bacteria
DMA00413	*Mobiluncus spp.*	16S ribosomal RNA gene	Bacteria
DMA00476	*Sarocladium strictum*	28S ribosomal RNA gene	Fungi
DMA00477	*Aspergillus terreus* (2)	Cis-aconitate decarboxylase (cadA) gene	Fungi
DMA00479	*Fusarium solani*	Beta tubulin gene	Fungi
DMA00480	*Neoscytalidium dimidiatum*	Translation elongation factor 1 alpha gene	Fungi
DMA00481	*Alternaria alternata*	Environmental Microbes; Infectious Diseases	Fungi
DMA00482	*Aspergillus versicolor*	Mitochondrial small ribosomal RNA gene	Fungi
DMA00483	*Curvularia lunata*	Glyceraldehyde 3-phosphate dehydrogenase gene	Fungi
DMA00484	*Scopulariopsis brevicaulis*	18S ribosomal RNA gene	Fungi
DMA00485	Human astrovirus1	ORF1b polyprotein gene	Viruses
DMA00486	*Sapovirus sp.*	Polyprotein gene	Viruses
DMA00487	*Enterovirus*	Polyprotein gene	Viruses
DMA00488	*Human rotavirus A*	Outer capsid protein (VP4) gene	Viruses
DMA00489	*Aspergillus terreus (1)*	Internal Transcribed Spacer ribosomal RNA	Fungi

Table 12. Assays not entailed in the Microbial DNA Positive Control PMCv2 (continued)

GeneGlobe cat. no.	Assay name	Target region	Taxonomy/target type
DMA00490	*Oxalobacter formigenes*	16S ribosomal RNA gene	Bacteria
DMA00491	*Methanobrevibacter smithii*	RNA polymerase subunit B-like (rpoB) gene	Bacteria
DMA00595	aatA	Bacterial outer membrane protein (aatA) gene	Virulence
DMA00694	*Cronobacter spp.*	Macromolecular synthesis (MMS) operon	Bacteria
DMA00695	*Listeria spp.*	Invasion associated protein p60 (iap) gene	Bacteria
DMA00696	*Escherichia coli* (stx1)	Shiga toxin 1 subunit A (stx1A) gene	Bacteria
DMA00697	*Escherichia coli* (stx1)	Shiga toxin 2 subunit A (stx2A) gene	Bacteria
DMA00698	*Escherichia coli* (eae)	*E. coli* attaching and effacing (eae) gene	Bacteria
DMA00699	*Escherichia coli* (rfbE)	GDP-perosamine synthase RFbE/PerA gene	Bacteria
DMA00704	*Salmonella spp.* (2)	Invasion protein (invA) gene	Bacteria
DMA00705	*Legionella spp.*	16S ribosomal RNA gene	Bacteria
DMA00706	*Legionella pneumophila* (2)	Macrophage infectivity potentiator (mip) surface protein gene	Bacteria
DMA00707	*Listeria grayi*	Invasion associated protein p60 (iap) gene	Bacteria
DMA00710	Human corona virus SARS-CoV-2 N1	Conserved region 1 (N1) ribonucleoprotein gene	Viruses
DMA00711	Human corona virus SARS-CoV-2 N2	Conserved region 2 (N2) ribonucleoprotein gene	Viruses

Table 12. Assays not entailed in the Microbial DNA Positive Control PMCv2 (continued)

GeneGlobe cat. no.	Assay name	Target region	Taxonomy/target type
DMA00712	Influenza A (2)	Matrix protein 1 gene (M1)	Viruses
DMA00713	Influenza A (3)	Matrix protein 1 gene (M1)	Viruses
DMA00714	Influenza B (2)	Nuclear export protein gene (NEP)	Viruses
DMA00715	Respiratory syncytial virus (2)	Matrix protein gene (M)	Viruses
DMA00716 and all others thereafter are not included in the PMCv2			

To determine if the assays that are not present in the PMCv2 do work, use samples that are known to have bacterial and fungal genomic DNA (isolated bacterial or fungal colony, metagenomic samples, etc.) or viral target DNA as a positive control.

Ordering Information

Product	Contents	Cat. no.
dPCR Microbial DNA Detection Assays	One tube with lyophilized dPCR Microbial DNA Detection Assay	250207
Custom dPCR Microbial Assays	One tube with lyophilized Custom dPCR Microbial Assay	250208
Accessories		
QIAcuity Probe PCR Kit (1 mL)	1 tube of 1 mL 4x concentrated QIAcuity Probe Mastermix, 2 tubes of 1 mL water each.	250101
QIAcuity Probe PCR Kit (5 mL)	5 tubes of 1 mL 4x concentrated QIAcuity Probe Mastermix each, 8 tubes of 1 mL water each.	250102
QIAcuity Probe PCR Kit (25mL)	5 tubes of 5 mL 4x concentrated QIAcuity Probe Mastermix each, 4 tubes of 20 mL water each.	250103
QIAcuity UCP Probe PCR Kit (1 mL)	1 tube of 1 mL 4x concentrated QIAcuity UCP Probe Mastermix, 2 tubes of 1.9 mL UCP water each.	250121
QIAcuity UCP Probe PCR Kit (5mL)	5 tubes of 1 mL 4x concentrated QIAcuity UCP Probe Mastermix each, 8 tubes of 1.9 mL UCP water each.	250122
QIAcuity OneStep Advanced Probe Kit (1 mL)	1 tube of 1 mL 4x concentrated OneStep Advanced Probe Master Mix, 1 tube of 45 µL 100x OneStep RT Mix, 1 tube of 1 mL Enhancer GC, 1 tube of 20 µL QN Internal Control RNA, 2 tubes of 1.9 mL RNase-free water each; for 100 reactions in Nanoplate 26K and 333 reactions in Nanoplate 8.5K	250131
QIAcuity High Multiplex Probe PCR Kit (1 mL)	1 mL 4x concentrated QIAcuity HighMultiplex Probe PCR Mastermix, 2x 1.9 mL RNase-free Water, QN Internal Control DNA dPCR	250133
QIAcuity High Multiplex Probe PCR Kit (5 mL)	5 x 1 mL 4x concentrated QIAcuity High Multiplex Probe PCR Mastermix, 8x 1.9 mL RNase-free Water, QN Internal Control DNA dPCR	250134

Product	Contents	Cat. no.
QIAcuity OneStep Advanced Probe Kit (5 mL)	5 tube of 1 mL 4x concentrated OneStep Advanced Probe Master Mix each, 5 tube of 45 µL 100x OneStep RT Mix each, 5 tube of 1.00 mL Enhancer GC each, 1 tube of 20 µL QN Internal Control RNA, 8 tubes of 1.9 mL RNase-free water each; for 500 reactions in Nanoplate 26K and 1666 reactions in Nanoplate 8.5K	250132
Microbial DNA-Free Water	12 tubes of 1.35 mL each	338132
10x Microbial DNA Positive Control V2 (PMCv2)	One tube of 50 µL mix of synthetic DNA templates	338135
Nanoplate 8-well 26K	Microfluidic digital PCR plates for 8 samples with up to 26,000 partitions each	250031
Nanoplate 24-well 26K	Microfluidic digital PCR plates for 24 samples with up to 26,000 partitions each	250001, 250002
Nanoplate 24-well 8.5K	Microfluidic digital PCR plates for 24 samples with up to 8500 partitions each	250011
Nanoplate 96-well 8.5K	Microfluidic digital PCR plates for 96 samples with up to 8500 partitions each	250021
Nanoplate Seals	11 top seals for the nanoplates	250099
QIAcuity 1 2-channel	One-plate digital PCR instrument for detecting up to 2 fluorescent dyes	911001
QIAcuity 1 5-channel	One-plate digital PCR instrument for detecting up to 5 fluorescent dyes	911021
QIAcuity 4 5-channel	Four-plate digital PCR instrument for detecting up to 5 fluorescent dyes	911042
QIAcuity 8 5-channel	Eight-plate digital PCR instrument for detecting up to 5 fluorescent dyes	911052
Related Products		
QuantiTect Rev. Transcription Kit (50)	For 50 x 20 µL reactions: 100 µL 7x gDNA Wipeout Buffer, 50 µL Quantiscript® Reverse Transcriptase, 200 µL 5x Quantiscript RT Buffer, 50 µL RT Primer Mix, 1.9 mL RNase-free Water	205311

Product	Contents	Cat. no.
QIAamp Fast DNA Stool Mini Kit	For 50 preps, includes QIAamp Mini Spin Columns, QIAGEN Proteinase K, InhibitEX Buffer, wash and elution buffers, Collection Tubes (2 mL)	51604
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
QIAcube Connect — for fully automated nucleic acid extraction with QIAGEN spin-column kits		
QIAcube® Connect*	QIAcube Connect* Instrument, connectivity package, 1-year warranty on parts and labor	Inquire

*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.

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Document Revision History

Date	Changes
April 2022	Initial revision
August 2022	Updated Shipping and Storage section Inserted reference links to **www.qiagen.com** Updated QIAcuity UCP Probe Mastermix section to add information about contamination in PCR reactions Updated references to *QIAcuity User Manual Extension to QIAcuity User Manual Extension: QIAcuity Application Guide* Updated Appendix A: Data Analysis to revise LOD calculation Updated Ordering Information section
May 2025	Updated all sections by adding Custom dPCR Microbial Assays Updated all sections by adding QIAcuity High Multiplex Probe PCR Kit and the new dye ATTO 700 Updated the "Limited License Agreement" section Added the "Glossary" section
August 2025	Updated “Table 8. QIAcuity RT-dPCR cycling program”; corrected Combined annealing/extension temperature from 60°C to 58°C. Added Table “Assay resuspension to achieve a 20x primer–probe mix” in the “Shipping and Storage” section.

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