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UCP Probe PCR Handbook

Ultra-clean production (UCP) Master Mix kit for real-time PCR and 2-step RT-PCR using sequence-specific probes

Sample to Insight

QIAGEN

Contents

Kit Contents

UCP Probe PCR Kit	**(100)**	**(500)**
Catalog no.	**208212**	**208214**
Number of reactions (20 µl/10 µl)	**100/200**	**500/1000**
2x UCP Probe PCR Master Mix, which contains: • UCP DNA Polymerase (composed of *Taq* DNA Polymerase, Antibody, and Guard) • UCP Probe PCR Buffer • dNTP mix (dATP, dCTP, dGTP, and dTTP)	1 ml	5 x 1 ml
UCP Yellow Template Dilution Buffer	200 µl	200 µl
UCP ROX™ Reference Dye	250 µl	2 x 250 µl
RNase-free water	1.9 ml	4 x 1.9 ml
Quick-Start Protocol	1	1

Storage

The UCP Probe PCR Kit is shipped on dry ice. Store immediately upon receipt at –30 to –15°C in a constant-temperature freezer and protect from light. When stored under these conditions and handled correctly, kit performance is guaranteed until the expiration date printed on the kit label. UCP Probe PCR Master Mix, UCP Yellow Template Dilution Buffer, and UCP ROX Reference Dye can also be stored protected from light at 2–8°C for up to 12 months or the expiration date printed on the kit label. Ultra-clean production (UCP) reagents are depleted of nucleic acids, and appropriate measures should be taken to prevent any contamination during storage or use.

If desired, UCP ROX Reference Dye can be added to 2x UCP Probe PCR Master Mix for long-term storage. For details, see "Adding ROX dye to the UCP Probe PCR Master Mix".

Intended Use

The UCP Probe PCR Kit is intended for molecular biology applications. This product is 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 UCP Probe PCR Kit is tested against predetermined specifications to ensure consistent product quality.

Product Specifications

2x QuantiNova Probe RT-PCR Master Mix

Component	Description
UCP DNA Polymerase	A modified form of a recombinant 94 kDa DNA polymerase, originally isolated from *Thermus aquaticus*. UCP DNA Polymerase is provided in an inactive state and has no enzymatic activity at ambient temperature. The enzyme is activated by a 2 min 95°C incubation step.
UCP Probe PCR Buffer	Contains Tris·Cl, KCl, NH_4Cl, $MgCl_2$, and additives enabling fast cycling, including Q-Bond®.
dNTP mix	Contains dATP, dCTP, dGTP, and dTTP of ultrapure quality.

Other components

Component	Description
UCP ROX Reference Dye	Optimized concentration of fluorescent dye for normalization of fluorescent signals on all instruments requiring ROX as a reference dye. Depleted of potentially contaminating DNA and filled under UCP standards.
UCP Yellow Template Dilution Buffer	Ultrapure quality, PCR-grade product depleted of potentially contaminating DNA, and filled under UCP standards.
UCP Water	Ultrapure quality, PCR-grade product depleted of potentially contaminating DNA, and filled under UCP standards.

Introduction

The UCP Probe Kit provides highly sensitive and rapid real-time quantification of DNA and cDNA targets in an easy-to-handle format. The kit can be used for qPCR of challenging sample materials. Due to the absence of fungal and bacterial DNA background, it is perfectly suitable for bacterial and fungal detection workflows. The kit is compatible with dual-labeled probes (e.g., TaqMan® probes). The hot start is achieved using a novel hot-start enzyme and a novel Guard molecule. These unique components further improve the stringency of the antibody-mediated hot start.

In addition, the UCP Probe PCR Kit is engineered to show high inhibitor resistance against substances commonly found in sample types used in microbiome analysis workflows. Dedicated processes are implemented to enable ultra-clean production of this kit; these include depletion of potential DNA background to a minimum, improving data due to removal of bioburden which could introduce bias in microbial detection workflows.

The kit also features a built-in control for visual identification of correct template addition and an additive in the PCR buffer, which enables short cycling steps without loss of PCR sensitivity and efficiency.

The kit has been optimized for use with any real-time cycler. The UCP ROX Reference Dye is provided in a separate tube and can be added if using a cycler that requires ROX as a passive reference dye.

Principle and procedures

2x UCP Probe PCR Master Mix

The components of the 2x UCP Probe PCR Master Mix include UCP DNA Polymerase and UCP Probe PCR Buffer. The optimized Master Mix ensures fast real-time PCR amplification with high specificity and sensitivity. The UCP Probe PCR Buffer is also based on the unique QIAGEN

PCR buffer system. The buffer contains a balanced combination of KCl and NH_4Cl, which promotes a high ratio of specific to nonspecific primer binding during the annealing step of each PCR cycle. This creates stringent primer annealing conditions, leading to increased PCR specificity. Primer annealing is only marginally influenced by the Mg^{2+} concentration, so optimization by titration of Mg^{2+} is not required.

Antibody-mediated hot-start mechanism

UCP DNA Polymerase is provided in an inactive state and has no enzymatic activity at ambient temperature. At low temperatures, the UCP DNA Polymerase is kept in an inactive state by an Antibody and a Guard, which stabilize the complex and improve the stringency of the hot start. The antibody-mediated hot-start mechanism prevents the formation and extension of nonspecific PCR products and primer–dimers during reaction setup and the first denaturation step. Therefore, this mechanism allows higher PCR specificity and more accurate quantification.

Within 2 min of the temperature getting raised to 95°C, the Antibody and Guard are denatured and the DNA polymerase is activated, enabling PCR amplification (Figure 1). The hot start enables rapid and convenient reaction setup at room temperature (15–25°C).

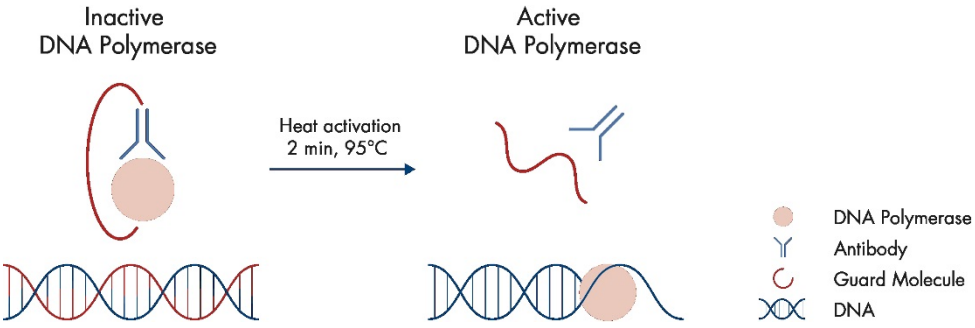

Figure 1. Principle of the novel 2-phase hot-start mechanism. At ambient temperatures, the DNA polymerase is kept inactive by the Antibody and the Guard. At 95°C, the Antibody and Guard are denatured and the DNA polymerase is activated.

Built-in visual control for correct pipetting

The Master Mix supplied with the UCP Probe PCR Kit contains an inert blue dye that increases visibility in the tube or well but does not interfere with the qPCR. UCP Yellow Template Dilution Buffer contains an inert yellow dye. When the template nucleic acid, diluted with the UCP Yellow Template Dilution Buffer, is added to the Master Mix, the color of the solution changes from blue to green, providing a visual indication of correct pipetting. The use of the UCP Yellow Template Dilution buffer is optional.

Passive reference dye

For certain real-time cyclers, the presence of ROX passive reference dye in real-time PCR compensates for non-PCR-related variations in fluorescence detection. Fluorescence from ROX dye does not change during the course of real-time PCR but provides a stable baseline to which PCR-related fluorescent signals are normalized. Thus, ROX dye compensates for differences in fluorescence detection between wells due to slight variations in reaction volume or to differences in well position. ROX dye does not interfere with real-time PCR because it is not involved in the reaction and has an emission spectrum different from fluorescent dyes commonly used for probes.

The use of ROX dye is necessary for instruments from Applied Biosystems®. The UCP Probe PCR Kit is provided with a separate tube of UCP ROX Reference Dye. It can be added to the real-time PCR if using a real-time cycler that uses ROX as a passive reference dye. ROX dye should be diluted 1:20 for a 1x reaction when using an instrument requiring a high ROX dye concentration. For instruments requiring a low ROX dye concentration, dilute the dye 1:200 for a 1x reaction. Refer to

Table 1 for details on real-time cyclers that require low or high ROX concentrations.

Table 1. Real-time cyclers requiring high/low concentrations of ROX dye

High ROX concentration (1:20 dilution of UCP ROX Reference Dye in 1x reaction)	Low ROX concentration (1:200 dilution of UCP ROX Reference Dye in 1x reaction)
ABI PRISM® 7000	Applied Biosystems 7500
Applied Biosystems 7300	Applied Biosystems ViiA®7
Applied Biosystems 7900	Applied Biosystems QuantStudio® Systems
Applied Biosystems StepOne®	
Applied Biosystems StepOne Plus	

If desired, UCP ROX Reference Dye can be diluted with 2x Probe PCR Master Mix for long-term storage (Table 2). For details, see "Adding ROX dye to the UCP Probe PCR Master Mix".

Adding ROX dye to the UCP Probe PCR Master Mix

If only using cyclers from Applied Biosystems with the UCP Probe PCR Kit, UCP ROX Reference Dye can be added to 2x UCP Probe PCR Master Mix for long-term storage, if desired. For information on the concentration of ROX required for Applied Biosystems instruments, refer to Table 2. For reaction setups with Master Mix that already contains a high concentration of added UCP ROX Reference Dye, refer to Appendix A.

Table 2. Addition of UCP ROX Reference Dye to the Master Mix

Volume of 2x UCP Probe PCR Master Mix (without QN ROX Reference Dye)	Volume of UCP ROX Reference Dye for high ROX concentration/low ROX concentration
1 ml	100/10 µl

Special recommendations for microbiome analysis

Preventing unwanted DNA contamination is a key challenge during the analysis of low biomass microbial targets. We suggest setting up PCR reactions in a clean PCR cabinet. All surfaces should be UV decontaminated prior to working. All accessories used (e.g., pipettes, racks, and PCR disposables) should be dedicated for this particular use and should remain in the PCR cabinet. Please refer to standard publications for further recommendations.

In addition, we recommend including no-template controls (NTCs) and using as much template input and as few PCR cycles as possible. This will help prevent PCR bias. However, in case of small sample amounts or low DNA content, cycle numbers may be increased up to 45 because the background signal from the UCP Master Mix is exceptionally low.

Protocol: Singleplex and Duplex Real-Time PCR Using Dual-Labeled Probes

This protocol is for use with the UCP Probe PCR Kit and dual-labeled probes (e.g., TaqMan probes) on any cycler.

Important points before starting

- This protocol is optimized for the quantification of DNA/cDNA targets using TaqMan probes in a singleplex or duplex reaction with any real-time cycler and conditions for fluorescence normalization. ROX dye is required for various cyclers at the following concentrations:
  - **No requirement for ROX dye**: Rotor-Gene®, Bio-Rad® CFX, Roche® LightCycler® 480, and Agilent® Technologies Mx instruments
  - **Low concentration of ROX dye**: Applied Biosystems 7500, ViiA 7, and QuantStudio Real-Time PCR Systems
  - **High concentration of ROX dye**: ABI PRISM 7000, Applied Biosystems 7300 and 7900, and StepOne Real-Time PCR Systems
- UCP ROX Reference Dye is provided as a separate tube of passive reference dye for normalization of fluorescent signals on all real-time cyclers from Applied Biosystems. ROX dye should be diluted 1:20 for a 1x reaction when using an instrument requiring a high ROX dye concentration. For instruments requiring a low ROX dye concentration, dilute the dye 1:200 for a 1x reaction.

- The dye in UCP Yellow Template Dilution Buffer allows tracking of pipetted samples in the qPCR. When template is added to the blue UCP Probe PCR Master Mix, the color changes from blue to green. The use of this buffer is optional. It is provided as a 100x concentrate and should be diluted (using water or buffer) to obtain a 1x final concentration within the sample.* To generate a template dilution series (e.g., for absolute quantification or determination of PCR efficiency), dilute the 100x concentrate (using template and UCP Water) to obtain a final concentration of 1x UCP Yellow Template Dilution Buffer. The buffer does not affect sample stability or qPCR.
- For the highest efficiency in real-time qPCR using TaqMan probes, amplicons should ideally be 60–150 bp in length. However, longer amplicons (e.g., for total bacterial load analysis) might also work.
- Always start with the cycling conditions and primer concentrations specified in this protocol.
- The PCR protocol must start with an initial incubation step of 2 min at 95°C to activate the DNA polymerase.
- For ease of use, we recommend preparing a 20x primer–probe mix containing target-specific primers and probes for each target. A 20x primer–probe mix consists of 6 µM of each primer and 2 µM probe in TE buffer or UCP Water in case of microbial target amplification. Alternatively, it may be preferable to prepare the reaction mix with separate primer and probe solutions. We recommend using 1 µl of 20x primer mix per 20 µl reaction
- For 96-well block cyclers, we recommend a final reaction volume of 20 µl. For 384-well block cyclers, we recommend a final reaction volume of 10 µl.
- Always readjust the threshold value for analysis of every run.

* Example: Add 0.5 µl Yellow Template Dilution Buffer to a 50 µl sample, which can be used as template in various PCR runs, regardless of the volume added to each reaction. Yellow Template Dilution Buffer can be prediluted using UCP Water. In this example, add 5 µl of 1:10 prediluted Yellow Template Dilution Buffer.

Procedure

1. Thaw 2x UCP Probe PCR Master Mix, UCP Yellow Template Dilution Buffer, template DNA or cDNA, primers, probes, UCP ROX Reference Dye (if required), and UCP Water. Mix the individual solutions.
2. Prepare a reaction mix according to Table 3. Due to the hot start of the PCR reactions, it is not necessary to keep samples on ice during reaction setup or while programming the real-time cycler.

Table 3. Reaction setup

	Volume/reaction		
Component	**96-well block**	**384-well block**	**Final concentration**
2x UCP Probe PCR Master Mix	10 µl	5 µl	1x
UCP ROX Reference Dye (Applied Biosystems cycler only)	1 µl/0.1 µl*	0.5 µl/0.05 µl*	1x
20x primer–probe mix 1†	1 µl	0.5 µl	0.3 µM forward primer 1 0.3 µM reverse primer 1 0.1 µM TaqMan Probe 1
20x primer–probe mix 2†	1 µl	0.5 µl	0.3 µM forward primer 2 0.3 µM reverse primer 2 0.1 µM TaqMan Probe 2
Template DNA (added at step 4)	Variable	Variable	≤100 ng/reaction
UCP Water	Variable	Variable	
Total reaction volume	**20 µl**	**10 µl**	

* Results in a 1:20 dilution for high ROX cyclers (i.e., ABI PRISM 7000, Applied Biosystems 7300, 7900, and StepOne Real-Time PCR Systems) and a 1:200 dilution for low ROX dye cyclers (i.e., Applied Biosystems 7500, ViiA 7, and QuantStudio Real-Time PCR Systems).

† A 20x primer–probe mix consists of 6 µM of each primer and 2 µM of each probe in UCP Water. Ideal concentrations may vary, depending on the assay used. Primers can either be premixed and added simultaneously or added separately.

3. Mix the reaction thoroughly and dispense appropriate volumes into PCR tubes or wells of a PCR plate.
4. Add template DNA or cDNA (≤100 ng per reaction) to the individual PCR tubes or wells containing the reaction mix.

5. Program the real-time cycler according to the program outlined in Table 4 or Table 5. Data acquisition should be performed during the combined annealing/extension step.

Table 4. Two-step cycling conditions

Step	Time	Temperature	Ramp rate
PCR initial heat activation	2 min	95°C	Maximal/fast mode
2-step cycling			
Denaturation	5 s	95°C	Maximal/fast mode
Combined annealing/extension			
• Singleplex	5 s*	60°C	Maximal/fast mode
• Duplex	30 s*	60°C	Maximal/fast mode
Number of cycles	40†		

* If your cycler does not accept this short time for data acquisition, use the shortest acceptable time.
† The number of cycles depends on the amount of template DNA.

Table 5. Three-step cycling conditions for longer or difficult amplicons

Step	Time	Temperature	Ramp rate
PCR initial heat activation	2 min	95°C	Maximal/fast mode
3-step cycling			
Denaturation	5 s	95°C	Maximal/fast mode
Annealing	15 s	50–60°C	Maximal/fast mode
Extension	20 s*	72°C	
Number of cycles	40†		

* If your cycler does not accept this short time for data acquisition, use the shortest acceptable time.
† The number of cycles depends on the amount of template DNA.

6. Place the PCR tubes or plates in the real-time cycler and start the cycling program.

Guidelines for effective duplex assays

The UCP Probe PCR Kit works well with most existing probe systems that have been designed using standard design methods. However, for optimal performance of a probe system in quantitative duplex real-time qPCR, some considerations need to be made, including the choice of a compatible combination of reporter dyes (i.e., the fluorophores on the probes) and the quality of the primers and probes. Please read the following guidelines before starting.

- Check the functionality of each set of primers and probe in individual assays before combining the different sets in a duplex assay.
- Perform appropriate controls for evaluating the performance of your duplex assays (e.g., amplifying each target individually and comparing the results with those for the duplex assay).
- For duplex analysis, the use of nonfluorescent quenchers (e.g., Black Hole Quencher® [BHQ] on TaqMan probes) is preferred over fluorescent quenchers (e.g., TAMRA™ fluorescent dye). TAMRA quencher can be used in duplex analysis if the 2 reporter dyes are 6-FAM™ dye and HEX, JOE™, or VIC® dye.
- PCR products should be as short as possible, ideally 60–150 bp. Always use the same algorithm or software to design the primers and probes. For optimal results, only combine assays that have been designed using the same parameters (e.g., similar melting points [T_m]). For details, see Appendix B.
- Check the concentration and integrity of primers and probes before starting. For details, see Appendix B.
- Check the real-time cycler user manual for correct setup of the cycler for duplex analysis (e.g., setting up detection of 2 dyes from the same well). Be sure to activate the detector for each reporter dye used.
- Some real-time cyclers require you to perform a calibration procedure for each reporter dye. Check whether the reporter dyes you selected for your duplex assay are part of the standard set of dyes already calibrated on your cycler. If they are not, perform a

calibration procedure for each dye before using them for the first time (for details, refer to the manufacturer's instructions for your real-time cycler).

- Always start with the cycling conditions specified in the protocol.
- Optimal analysis settings (i.e., baseline settings and threshold values) for each reporter dye are a prerequisite for accurate quantification data. For details, check the literature from the manufacturer of your real-time cycler.

Suitable combinations of reporter dyes

Duplex real-time PCR requires the simultaneous detection of 2 different fluorescent reporter dyes. For accurate detection, the fluorescence spectra of the dyes should be well separated or exhibit only minimal overlap (Table 6).

Note: Please refer to the user manual or other technical documentation for your instrument to find out which reporter dyes can be used in duplex analysis.

Table 6. Dyes commonly used in multiplex real-time PCR

Dye	Excitation maximum (nm)	Emission maximum (nm)*
FAM	494	518
TET™	521	538
JOE	520	548
VIC	538	552
Yakima Yellow®	526	552
HEX	535	553
Bodipy® TMR	542	574
NED™	546	575
Cy®3	552	570
TAMRA	560	582
Cy3.5	588	604
ROX	587	607
Texas Red®	596	615
Cy5	643	667

* Emission spectra may vary depending on the buffer conditions.

Troubleshooting Guide

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

	Comments and suggestions
No signal or one or more signals detected late in PCR	
a) Incorrect cycling conditions	Always start with the optimized cycling conditions specified in the protocols. Be sure that the PCR step of your cycling conditions include the initial step for activation of the DNA polymerase (95°C for 2 min) and the specified times for denaturation and annealing/extension.
b) DNA polymerase not activated	Ensure that the cycling program includes the DNA polymerase activation step (2 min at 95°C) as described in the protocols.
c) Pipetting error or missing reagent	Check the concentrations and storage conditions of the reagents, including primers, probes, and template nucleic acid. See Appendix B for details on evaluating the concentration of primers and probes. Repeat the PCR. Use the provided UCP Yellow Template Dilution Buffer to prevent errors during reaction setup.
d) Wrong or no detection step	Ensure that fluorescence detection takes place during the combined annealing/extension step when using TaqMan probes.
e) Primer or probe concentration not optimal	Use optimal primer concentrations. For TaqMan assays, use each primer at 0.3 µM. In most cases, a probe concentration of 0.1 µM provides satisfactory results. Check the concentrations of primers and probes by spectrophotometry (see Appendix B).
f) Problems with starting template	Check the concentration, storage conditions, and quality of the starting template (see Appendix B). If necessary, make new serial dilutions of template nucleic acid from the stock solutions. Repeat the PCR using the new dilutions. Ensure that the template is free of DNase contamination to avoid degradation during reaction setup.
g) Insufficient amount of starting template	Increase the amount of template, if possible. Ensure that sufficient copies of the target nucleic acids are present in your sample.
h) Insufficient number of cycles	Increase the number of cycles.

	Comments and suggestions
i) Reaction volume too high	For 96-well block cyclers, we recommend a final reaction volume of 20 µl. For 384-well block cyclers, we recommend a final reaction volume of 10 µl.
j) qPCR product too long	For optimal results, PCR products should be between 60 and 150 bp. PCR products should not exceed 300 bp.
k) Primer design not optimal	Check for PCR products by gel electrophoresis. If no specific PCR products are detected, review the primer design guidelines (see Appendix B).
l) Probe design not optimal	If the amplification reaction was successful, there may be a problem with the probe. Review the probe design guidelines (see Appendix B).
m) Wrong detection channel/ filter chosen	Ensure that the correct detection channel is activated or that the correct filter set is chosen for the reporter dye.
n) No detection activated	Check that fluorescence detection was activated in the cycling program.
o) Probe synthesis not optimal	Check the quality of dual-labeled probes by incubation with DNase I. A correctly synthesized probe, containing both fluorophore and quencher, will show a significant increase in fluorescence after DNase I incubation.
p) Primers degraded	Check for possible degradation of primers on a denaturing polyacrylamide gel.
Increased fluorescence or C_q value for "No Template" control	
a) Contamination of reagents	Discard all the components of the assay (e.g., Master Mix, primers, and probes). Repeat the assay using new components.
b) Contamination during reaction setup	Take appropriate precautions during reaction setup, such as using aerosol-barrier pipette tips.
c) Minimal probe degradation, leading to sliding increase in fluorescence	Check the amplification plots and adjust the threshold settings.
Varying fluorescence intensity	
a) Contamination of real-time cycler	Decontaminate the real-time cycler according to the manufacturer's instructions.
b) Real-time cycler no longer calibrated	Recalibrate the real-time cycler according to the manufacturer's instructions.
All cycler systems	
Wavy curve at high template amounts for highly expressed targets	In the analysis settings, reduce the number of cycles used for background calculation (if your real-time cycler allows you to do so) or reduce the amount of template.
Applied Biosystems instruments only	
ΔRn values unexpectedly too high or too low	The concentration of the UCP ROX Reference Dye is wrong. To choose the right ROX concentration for your cycler, refer to Table 1.

Appendix A: Reaction Setup Using Master Mix Containing a High Concentration of ROX

Note: This appendix is only relevant for a reaction setup using a Master Mix containing a high concentration of ROX that has been added according to Table 2. When using a Master Mix containing a low concentration of ROX, the volume of ROX added is negligible and the standard reaction setup as described in Table 3 should be used.

Table 7. Reaction setup

	Volume/reaction		
Component	**96-well block**	**384-well block**	**Final concentration**
2x UCP Probe PCR Master Mix*	11 µl	5.5 µl	1x
20x primer–probe mix 1	1 µl	0.5 µl	0.3 µM forward primer 1 0.3 µM reverse primer 1 0.1–2 µM TaqMan probe 1
20x primer–probe mix 2†	1 µl	0.5 µl	0.3 µM forward primer 2 0.3 µM reverse primer 2 0.1 µM TaqMan probe 2
Template DNA (added at step 4)	Variable	Variable	10 fg – 100 ng/reaction
UCP Water	Variable	Variable	
Total reaction volume	**20 µl**	**10 µl**	

* Contains a 1:20 dilution for high ROX instruments (i.e., ABI PRISM 7000, Applied Biosystems 7300, 7900, and StepOne Real-Time PCR Systems).

† A 20x primer–probe mix consists of 6 µM of each primer and 2 µM of each probe in UCP Water. Ideal concentrations may vary, depending on the assay used. Primers can either be premixed and added simultaneously or added separately.

Appendix B: Assay Design and Handling Primers and Probes

Important factors for successful quantitative singleplex and duplex real-time RT-PCR include the design of optimal primer pairs and probes, the use of appropriate primer and probe concentrations, and the correct storage of primers and probes.

Assay design

Guidelines for the optimal design of primers and probes are given below. It is particularly important to minimize nonspecific annealing of primers and probes. This can be achieved through careful assay design.

T_m of primers for TaqMan assays

- Use specialized design software (e.g., Primer Express® Software) to design primers and probes.
- T_m of all primers should be 58–62°C and within 2°C of each other.
- T_m of probes should be 8–10°C higher than the T_m of the primers.
- Avoid a guanidine at the 5' end of probes, next to the reporter, because this causes quenching.
- Avoid runs of 4 or more of the same nucleotide, especially of guanidine.
- Choose the binding strand so that the probe has more C than G bases.
- All assays should be designed using the same settings to ensure that they will work optimally under the same cycling conditions (60°C annealing/extension).

Primer sequence

- Length: 18–30 nucleotides
- GC content: 30–70%
- Always check the specificity of primers by performing a BLAST® search (**www.ncbi.nlm.nih.gov/blast**). Ensure that primer sequences are unique for your template sequence.
- Check that primers and probes are not complementary to each other.
- Try to avoid highly repetitive sequences.
- Avoid complementarity of 2 or 3 bases at the 3' ends of primer pairs to minimize primer-dimer formation.
- Avoid mismatches between the 3' end of primers and the template sequence.
- Avoid runs of 3 or more Gs and/or Cs at the 3' end. Avoid complementary sequences within a primer sequence and between the primer pair.

Product size

Ensure that the length of RT-PCR products is 60–150 bp. Some longer amplicons may amplify efficiently in duplex RT-PCR, with minimal optimization.

Handling and storing primers and probes

Guidelines for handling and storing primers and probes are given in Table 8. For optimal results, we recommend only combining primers of comparable quality.

Table 8. Guidelines for handling and storing primers and probes

	Description
Storage buffer	Lyophilized primers should be dissolved in a small volume of low-salt buffer to give a concentrated stock solution (e.g., 100 µM). We recommend using TE (10 mM Tris·Cl, 1 mM EDTA, pH 8.0) for standard primers and probes labeled with most fluorescent dyes. For applications requiring ultraclean settings, oligonucleotides may be dissolved in UCP Water. However, probes labeled with fluorescent dyes such as Cy3, Cy3.5, Cy5, and Cy5.5 should be stored in TE buffer, pH 7.0, because they tend to degrade at higher pH.
Storage	Primers should be stored in sterile, nuclease-free TE buffer in small aliquots at –20°C. Standard primers are stable under these conditions for at least 1 year. Fluorescently labeled probes are usually stable under these conditions for at least 6–9 months. Repeated freeze-thaw cycles should be avoided, because they may lead to degradation. For applications requiring ultraclean settings, oligonucleotides may be dissolved in UCP Water. For easy and reproducible handling of primer–probe sets used in duplex assays, we recommend preparing 20x primer–probe mixes, each containing 2 primers and 1 probe for a particular target at the suggested concentrations (see protocols).
Dissolving primers and probes	Before opening a tube containing lyophilized primer or probe, centrifuge the tube briefly to collect all material at the bottom of the tube. To dissolve the primer or the probe, add the required volume of sterile, nuclease-free TE buffer, and then mix and leave for 20 min to allow the primer or probe to completely dissolve. Mix again and determine the concentration by spectrophotometry as described below.
Concentration	Spectrophotometric conversion for primers and probes: 1 A_{260} unit = 20–30 µg/ml To check primer concentration, the molar extinction coefficient (ε_{260}) can be used: A_{260} = ε_{260} x molar concentration of primer or probe If the ε_{260} value is not given on the data sheet supplied with the primers or probes, it can be calculated from the primer sequence using the following formula: ε_{260} = 0.89 x [(A x 15,480) + (C x 7340) + (G x 11,760) + (T x 8850)] Example: Concentration of diluted primer: 1 µM = 1 x 10^{-6} M Primer length: 24 nucleotides with 6 each of A, C, G, and T bases Calculation of expected A_{260}: 0.89 x [(6 x 15,480) + (6 x 7340) + (6 x 11,760) + (6 x 8850)] x (1 x 10^{-6}) = 0.232 The measured A_{260} should be within ±30% of the theoretical value. If the measured A_{260} is very different to the theoretical value, we recommend recalculating the concentration of the primers or probes or having the primers or probes resynthesized. For probes, the fluorescent dye does not significantly affect the A_{260} value.
Primer quality	The quality of 18–30mers can be checked on a 15% denaturing polyacrylamide gel; a single band should be seen. Please contact QIAGEN Technical Services for a protocol.
Probe quality	The quality of the fluorescent label and the purity of TaqMan probes can be determined by comparing fluorescence before and after DNase digestion. Incubate probes with or without 5 units DNase at 37°C for 1 hour. A significant difference in fluorescence following DNase treatment should be detectable.

Ordering Information

Product	Contents	Cat. no.
UCP Probe PCR Kit (100)	For 100 x 20 µl reactions: 1 ml 2x UCP Probe PCR Master Mix, 200 µl UCP Yellow Template Dilution Buffer, 250 µl UCP ROX Reference Dye, 4x 1.9 ml UCP Water	208212
UCP Probe PCR Kit (500)	For 500 x 20 µl reactions: 5 x 1 ml 2x UCP Probe PCR Master Mix, 200 µl UCP Yellow Template Dilution Buffer, 0.5 ml UCP ROX Reference Dye, 1.9 ml UCP Water	208214
UCP Probe 16S/18S Quant Kit (100)	For 100 x 20 µl reactions: 1x Microbial DNA Standard, 1x 16S/18S DNA Probe Assay, 1 ml 2x UCP Probe PCR Master Mix, 200 µl UCP Yellow Template Dilution Buffer, 250 µl UCP ROX Reference Dye, 4x 1.9 ml UCP Water	208282
UCP SYBR® Green PCR Kit — for highly sensitive, specific, and ultrafast SYBR® Green–based real-time PCR		
UCP SYBR® Green PCR Kit (100)	For 100 x 20 µl reactions: 1 ml 2x UCP SYBR® Green PCR Master Mix, 200 µl UCP Yellow Template Dilution Buffer, 250 µl UCP ROX Reference Dye, 1.9 ml RNase-free water	208012
UCP SYBR® Green PCR Kit (500)	For 500 x 20 µl reactions: 5 x 1 ml 2x UCP SYBR® Green PCR Master Mix, 200 µl UCP Yellow Template Dilution Buffer, 0.5 ml UCP ROX Reference Dye, 4x 1.9 ml RNase-free water	208014

Product	Contents	Cat. no.
UCP SYBR® Green 16S Quant Kit (100)	For 100 x 20 µl reactions: 1x Microbial DNA Standard, 1x 16S DNA SYBR® Green Assay, 1 ml 2x UCP SYBR® Green PCR Master Mix, 200 µl UCP Yellow Template Dilution Buffer, 250 µl UCP ROX Reference Dye, 1.9 ml RNase-free water	208082
UCP Probe 16S/18S Quant Kit (100)	For 100 x 20 µl reactions: 1x Microbial DNA Standard, 1x 16S/18S DNA Probe Assay, 1 ml 2x UCP Probe PCR Master Mix, 200 µl UCP Yellow Template Dilution Buffer, 250 µl UCP ROX Reference Dye, 4x 1.9 ml UCP Water	208282
Related products		
UCP HiFidelity PCR Kit (100)	For 100 × 25 µl PCR reactions: ultra-clean production Master Mix for high-fidelity hot-start PCR and microbiome applications	202742
UCP HiFidelity PCR Kit (500)	For 500 × 25 µl PCR reactions: ultra-clean production Master Mix for high-fidelity hot-start PCR and microbiome applications	202744
UCP Multiplex PCR Kit (100)	For 100 × 20 µl PCR reactions: ultra-clean production Master Mix for multiplex hot-start PCR and microbiome applications	206742
UCP Multiplex PCR Kit (500)	For 500 × 20 µl PCR reactions: ultra-clean production Master Mix for multiplex hot-start PCR and microbiome applications	206744
DNeasy® PowerSoil® Pro Kit (50)	For 50 preps: isolation of microbial genomic DNA from all soil types	47014
DNeasy PowerSoil Pro Kit (250)	For 250 preps: isolation of microbial genomic DNA from all soil types	47016

Product	Contents	Cat. no.
QIAamp® UCP DNA Micro Kit (50)	For 50 preps: ultra-clean DNA purification from small volume sample volumes	56204
QIAamp UCP Pathogen Mini Kit (50)	For 50 preps: microbial DNA purification from whole blood, swabs, cultures, and body fluids	50214
QIAgility® System HEPA/UV (incl. PC)	Robotic workstation for automated PCR setup (with UV light and HEPA filter), notebook, computer and QIAgility software: includes installation and training, 1-year warranty on parts and labor.	9001532

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Document Revision History

Date	Changes
07/2019	Initial release

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