April 2018

UCP Multiplex PCR Kit Handbook

Ultra-Clean Production master mix for multiplex hot-start PCR and microbiome applications

Sample to Insight

QIAGEN

Contents

Kit Contents

UCP Multiplex PCR Kit	**(100)**	**(500)**
Catalog no.	**206742**	**206744**
Number of preps	**100**	**500**
UCP Multiplex PCR Master Mix, 4x	500 µl	5 x 500 µl
UCP Template Tracer, 25x	200 µl	2 x 200 µl
UCP Master Mix Tracer, 125x	50 µl	50 µl
UCP Water	1.9 ml	5 x 1.9 ml
Quick Start Protocol	1	1

Storage

The UCP Multiplex PCR Kit is shipped on dry ice and should be stored immediately upon receipt at –15 to –30°C in a constant-temperature freezer. When the kit is stored under these conditions and handled correctly, performance is guaranteed until the expiration date printed on the kit label. The UCP Multiplex PCR Master Mix can also be stored at 2–8°C for up to 6 months, or the expiration date printed on the kit label. UCP (Ultra-Clean Production) reagents are depleted of nucleic acids, and appropriate measures should be taken to prevent any contamination during storage or use.

If desired, the UCP Master Mix Tracer can be added to the UCP Multiplex PCR Master Mix for long term storage. For details, see section on Adding UCP Master Mix Tracer to the UCP Master Mix (page 8 and Table 1, page 9).

Intended Use

The UCP Multiplex 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 Multiplex PCR Kits is tested against predetermined specifications to ensure consistent product quality.

Product Specifications

The UCP Multiplex PCR Kit contains:

Component	Description
UCP Multiplex PCR Master Mix, 4x	Contains UCP Multiplex PCR Buffer and additives that enable fast cycling and direct loading of the reactions onto agarose gels. Also contains DNA Polymerase and dNTP-Mix. UCP Master Mix is produced under Ultra-Clean Production standards and further depleted of potentially contaminating DNA.
UCP Master Mix Tracer, 125x	Orange dye allows tracking of master mix addition and monitoring of agarose gel loading and electrophoresis. The dye runs at approximately 50 bp on a 1% agarose gel. UCP Master Mix tracer is produced under Ultra-Clean Production standards and further depleted of potentially contaminating DNA
UCP Template Tracer, 25x	Blue dye allows tracking of template DNA addition and monitoring of agarose gel loading and electrophoresis. The dye runs at approximately 4000 bp on a 1% agarose gel. UCP Template tracer is produced under Ultra-Clean Production standards and further depleted of potentially contaminating DNA
UCP Water	Ultrapure quality, PCR-grade, depleted of potentially contaminating DNA and filled under Ultra-Clean Production standards.

Introduction

The UCP Multiplex PCR Kit provides a convenient format for highly sensitive and specific multiplex hot-start PCR using any DNA or cDNA template.

Dedicated processes are implemented to enable Ultra-Clean Production of this master mix, including depletion of potential DNA background to a minimum. Combined with superior inhibitor resistance and minimized GC bias, this makes the kit an ideal choice for microbiome applications like 16S or 18S amplification. Additional features include: visual pipetting controls, a fast cycling protocol, room-temperature stability during and after reaction setup and a 4x concentrated master mix format, allowing for higher sample input volumes.

UCP Multiplex PCR Kit components

- UCP Multiplex PCR Master Mix
  - This ready-to-use master mix reduces hands-on time and contamination risk as it already contains DNA polymerase, a sophisticated PCR buffer and dNTPs. At low temperatures, the DNA polymerase is kept in an inactive state by an antibody and a novel guard additive, which stabilizes the complex. This improves the stringency of the hot-start and prevents any enzymatic activity at ambient temperatures and until heat activation at 95°C. The 4x master mix concentration allows greater flexibility for template input volumes over common 2x concentration master mixes. The UCP Master Mix is depleted of potentially contaminating DNA and tested for fungal and bacterial nucleic acids.
- UCP Master Mix Tracer, UCP Template Tracer and UCP Water
  - The blue and orange dyes in the UCP Template Tracer and in the UCP Master Mix Tracer, respectively, allow visual tracking of pipetted samples during PCR setup to prevent errors. When template colored with the blue dye is added to orange PCR

Master Mix, the color changes to green, confirming that sample was added. The use of these tracers is optional.

- The blue UCP Template Tracer is provided as a 25x concentrate and should be diluted to obtain a 1x final concentration in the sample*.
- The orange UCP Master Mix Tracer is provided as a 125x concentrate and can be added directly to the master mix vial to obtain a 1x final concentration†. These tracers do not reduce sample stability or PCR performance.
- Reactions can be directly loaded onto agarose gels after cycling. Each tracer dye allows monitoring of the loading process and efficient tracking of the subsequent electrophoresis. The dyes run at approximately 50 bp (orange) and 4000 bp (blue) on a 1% agarose gel.
- Additionally, UCP PCR-Grade Water is provided. All UCP components are depleted of potentially contaminating DNA and filled under Ultra-Clean Production standards

UCP Multiplex PCR Kit procedure

The UCP Multiplex PCR Kit allows fast and easy PCR setup. Whatever the application – multiplex target detection, gene panel amplification or microbiome studies – simply mix all components together in one tube and start the thermal cycler program (see Figure 1, page 9).

Adding UCP Master Mix Tracer to the UCP Master Mix

The orange UCP Master Mix Tracer can be added directly to the UCP Multiplex PCR Master Mix for long-term storage. Since the amount of tracer added is very small (4 µl per 500 µl of UCP Master Mix), the concentration of the master mix will not be changed and the master mix can be used as indicated in the protocol (see Table 1, page 9).

* Example: add 0.2 µl of the blue UCP Template Tracer (25x) to 5 µl of sample before use. If pipetting volumes are too small to handle, the UCP Template Tracer can be pre-diluted using DNA-free water. In this example, 2 µl of 1:10 pre-diluted UCP Template Tracer could be added

† Example: Add 4 µl of the UCP Master Mix Tracer (125x) to 1 tube (500µl) of UCP Multiplex PCR Master Mix (4x). Since the amount of UCP Master Mix Tracer added is very small, the concentration of the UCP Master Mix will not be changed and the UCP Multiplex PCR Master Mix can be used as indicated in the protocol.

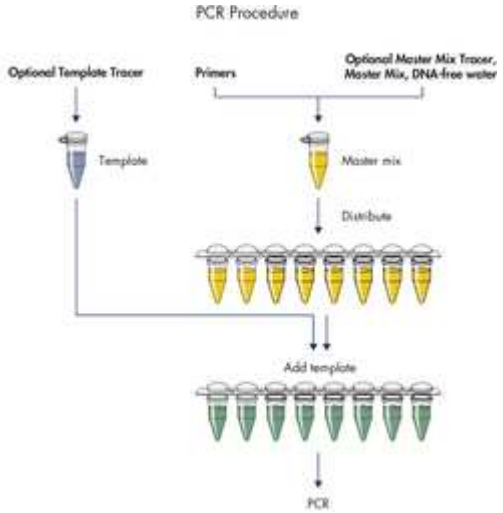

Figure 1. UCP Multiplex PCR procedure using tracer dyes.

Table 1. Addition of PCR Master Mix Tracer to the UCP Multiplex Master Mix

Volume of UCP Multiplex PCR Master Mix, 4x	Volume of UCP Master Mix Tracer
500 µl	4 µl

Special Recommendations for Microbiome Analysis

Preventing unwanted DNA contamination is a key challenge during the analysis of metagenome information using 16S or 18S sequencing. 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 40 as the background signal from the UCP Master Mix is exceptionally low.

Equipment and Reagents to Be Supplied by User

The UCP Multiplex Master Mix Kit is designed to be used with gene-specific primer sets as well as degenerate primer pools.

- Microcentrifuge tubes or PCR strips
- PCR tubes or plates
- Tubes such as LoBind® (Eppendorf®) or MAXYMum Recovery™ (Axygen®) tubes
- Thermal cycler
- Microcentrifuge
- Vortexer
- Pipettes and pipette tips
- UV cabinet (recommended for microbiome applications)

Protocol: Multiplex PCR Using Standard or Microbial Primer Sets

Important points before starting

- The protocol has been optimized for 0.1 pg–1 µg of total DNA.
- UCP PCR Kits are designed to be used with a final primer concentration of 0.25 µM for each primer. For high-grade multiplexing approaches, the amount may need to be adapted. For ease of use, we recommend preparing a 20x primer mix containing target-specific primers. A 20x primer-mix consists of 5 µM forward primer and 5 µM reverse primer in UCP Water or DNA-free TE buffer. Alternatively, it may be preferable to prepare the reaction mix with separate primers.
- Up to 20 targets or more can successfully be discriminated by traditional gel or capillary electrophoresis. More complex multiplexing protocols and detection strategies may require adaptions. Optimal results are achieved if <15 targets ≤ 1kbp are amplified.
- DNA Polymerase contained in the UCP Master Mix requires a heat-activation step of 2 min at 95°C.
- It is not necessary to keep PCR tubes on ice as nonspecific DNA synthesis cannot occur at room temperature due to the inactive state of the Taq Polymerase.

Procedure

1. Thaw UCP Master Mix, template DNA or cDNA, primer solutions, UCP Water, UCP Template Tracer (optional) and UCP Master Mix Tracer (optional).
   Mix thoroughly before use by vortexing. For multiplex reactions, we recommend preparing a combined primer mix prior to PCR setup.
2. Prepare a reaction mix according to Table 2 (page 13). The reaction mix contains all components except the template DNA. Prepare a volume of reaction mix 10% greater than that required for the total number of reactions to be performed. It is not necessary to keep samples on ice during reaction setup or while programming the cycler.
   Note: A negative control (without template) should be included in every experiment.

Table 2. Reaction setup for the UCP Multiplex PCR Kit

Component	Volume/reaction	Final concentration
UCP Multiplex Master Mix, 4x	5 µl	1x
20x primer mix*	1 µl	0.25 µM for each primer
UCP Water	Variable	–
Optional: UCP Master Mix Tracer, 125x	0.04 µl	1x
Template DNA (added at step 4)	Variable	0.1 pg–1 µg/reaction
Total reaction volume	20 µl†	

* A 20x primer mix consists of 5 µM forward primer and 5 µM reverse primer in DNA-free TE buffer or UCP Water for each target. Primers can either be pre-mixed and added simultaneously or added separately for each target. If the concentration of the primer mix(es) differ, the respective added volume needs to be adjusted to achieve a final concentration of 0.25 µM for each primer.

† For PCR in a 384-well plate, we recommend a final reaction volume of 10 µl. Reduce pipetting volumes accordingly.

3. Mix the reaction mix gently but thoroughly, for example, by pipetting up and down a few times or by vortexing for a few seconds. Dispense appropriate volumes into PCR tubes or the wells of a PCR plate.
4. Add template DNA (1 µg–100 fg per reaction, depending on target abundance) to the individual PCR tubes. The UCP Multiplex Master Mix Kit can be used with genomic DNA, cDNA, plasmid DNA, oligonucleotides and other DNA molecules as template. If using cDNA as template, the input volume from undiluted cDNA reaction should not exceed 10% of the total PCR reaction volume.
5. Program the thermal cycler according to the manufacturer's instructions, using the conditions outlined in Tables 3 and 4 (page 14).
6. Place the PCR tubes or plates in the thermal cycler and start the PCR program.
   Note: After amplification, samples can be stored at –15 to –30°C for long term storage.
7. We have evaluated several hints and guides. See appendix A–E.

Table 3. UCP Multiplex PCR Kit cycling conditions for multiplex reactions

Step	Time	Temperature	Comments
Initial PCR activation	2 min	95°C	This heating step activates the DNA Polymerase.
3-step cycling:			
Denaturation	5 sec	95°C	Do not exceed this temperature.
Annealing	15 sec	55°C	Approximately 5°C below Tm of primers.
Extension	30 sec	72°C	For PCR products up to 1000 bp, an extension time of 30 s is sufficient. For multiplex reactions of more than 10 targets or low sample amounts, increasing extension time to 60 s/kb may be beneficial.
Number of cycles	≤40		The optimal cycle number depends on the amount of template and the abundance of the target.

Note: The protocol in Table 4 (below) is recommended for qualitative 16S/18S analysis of samples using primer panels with low complexity.

Table 4. UCP Multiplex PCR Kit cycling conditions for amplification of 16/18S sequences

Step	Time	Temperature	Comments
Initial PCR activation	2 min	95°C	This heating step activates the DNA Polymerase.
3-step cycling:			
Denaturation	10 sec	95°C	Do not exceed this temperature.
Annealing	30 sec	55°C	Approximately 5°C below Tm of primers.
Extension	15 sec	72°C	As most 16S amplicons are <500bp this is sufficient. For longer amplicons, 30 s may be beneficial.
Number of cycles	≤40		The optimal cycle number depends on the amount of template and the abundance of the target. However, for 16S, the number chosen should be as low as possible.

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
Little or no product	
a) Pipetting error or missing reagent	Check the concentrations and storage conditions of reagents, including primers. Repeat the PCR.
b) DNA polymerases not activated	Ensure that the cycling program included the DNA polymerase activation step (2 minutes at 95°C or 3 min at 93° C) as described in the cycling protocols (page 14).
c) Primer concentration not optimal or primers are degraded	A primer concentration of 0.25 µM is strongly recommended. However, if the desired results are not obtained using this concentration, repeat the PCR with different primer concentrations from 0.2–1.0 µM in 0.1 µM increments. For high-grade multiplexing applications a reduced primer concentration might be needed.
d) PCR conditions not optimal	Follow the protocol described in Appendix A (page 18).
e) Problems with starting template	Check the concentration, integrity, purity and storage conditions of the starting template (see Appendix D, page 20). If necessary, make new serial dilutions of template DNA from stock solutions. Repeat the PCR using the new dilutions.
f) Mg^{2+} concentration not optimal	Perform PCR with different final concentrations of Mg^{2+} by adding $MgCl_2$ in 0.5 mM increments to the reaction. Do not exceed 3 mM additional $MgCl_2$.
g) Insufficient number of cycles	Increase the number of cycles in increments of 5.
h) Incorrect annealing temperature or time	Decrease annealing temperature in 2°C increments. Annealing time should be between 15 and 30 seconds. Difficulties in determining the optimal annealing temperature can be overcome in many cases by performing touchdown PCR (see Appendix C, page 27).

	Comments and suggestions
i) Incorrect denaturing temperature or time	Denaturation should take place at 95°C for 5–10 seconds. Ensure that the cycling program included the DNA Polymerase activation step (2 minutes at 95°C) as described in the cycling protocols (page 14). If amplifying PCR products longer than 1 kb, decrease denaturation temperature to 93°C and increase denaturation time to 15 seconds.
j) Insufficient starting template	Increase the template amount used.
k) Primer design not optimal	Review primer design (see Appendix B, page 20). Only use gene-specific primers.
l) Amplifying long fragments	Increase the concentration of template DNA.
m) Reactions overlaid with mineral oil when using a thermal cycler with a heated lid	When using a thermal cycler with a heated lid that is switched on, do not overlay the reactions with mineral oil as this may decrease the yield of PCR products.
n) Problems with the thermal cycler	Check the power to the thermal cycler and that the thermal cycler has been programmed correctly.
o) Enzyme concentrations too low	When using UCP Master Mix, use 5 µl Master Mix per 20 µl reaction.
p) Extension time too short	Increase the extension time in increments of 10 seconds.
Product is multi-banded	
q) PCR annealing temperature is too low	Increase annealing temperature in 2°C increments. Difficulties in determining the optimal annealing temperature can be overcome in many cases by performing touchdown PCR (see Appendix E, page 27).
r) Primer concentration not optimal or primers degraded	A primer concentration of 0.25 µM is strongly recommended. However, if the desired results are not obtained using this concentration, repeat the PCR with different primer concentrations from 0.25–1.0 µM in 0.1 µM increments.
s) Primer design not optimal	Review primer design (see Appendix B, page 20). Use only target-specific primers.
t) Contamination with genomic DNA	Pretreat starting cDNA template with DNase I. Alternatively, use primers located at splice junctions of the target mRNA to avoid amplification from genomic DNA (see Appendix B, page 20).
Product is smeared	
u) Too much starting template	Check the concentration of the starting template (see Appendix A, page 20). If necessary, make new serial dilutions of template DNA from stock solutions. Repeat the PCR using the new dilutions.
v) Carry-over contamination	If negative controls (without template) show PCR products or smears, change all reagents. Use disposable pipette tips containing hydrophobic filters to minimize cross-contamination. Set up all reaction mixtures in an area separate from that used for DNA preparation or PCR product analysis.
w) Too many cycles	Reduce the number of cycles in increments of 3.

	Comments and suggestions
x) Primer concentration not optimal or primers degraded	A primer concentration of 0.25 µM is strongly recommended. However, if the desired results are not obtained using this concentration, repeat the PCR with different primer concentrations from 0.25–1.0 µM in 0.1 µM increments. In particular, when performing highly sensitive PCR, check for possible degradation of the primers on a denaturing polyacrylamide gel.
y) Primer design not optimal	Review primer design (see Appendix B, page 20). Use only target-specific primers.
Contamination of 16S PCR	
z) Contamination of NTCs for 16S PCRs	PCR amplification of16S sequences are especially prone to contamination through operator or environmental causes. To reduce contamination risk, please follow the process recommendations outlined in the Special Recommendations for Microbiome Analysis (page 10) and in standard publications. In case of positive NTCs start with replacing single components, e.g., water or primers. In general, we recommend aliquoting reagents and using dedicated pipettes and disposables.

Appendix A: Starting Template

Both the quality and the quantity of nucleic acids used as template affect PCR, particularly the sensitivity and efficiency of amplification.

Quality of starting template

Since PCR consists of multiple rounds of enzymatic reactions, it is more sensitive to impurities such as proteins, phenol/chloroform, salts, ethanol, EDTA and other chemical solvents compared to single-step enzyme-catalyzed processes. QIAGEN offers a complete range of nucleic acid preparation systems, ensuring the highest-quality templates for PCR. Examples include the QIAprep® system for rapid plasmid purification, and the QIAamp® and DNeasy® systems for rapid purification of genomic DNA and viral nucleic acids. Other kits are designed for microbiome research or have undergone Ultra-clean production procedures (see Ordering Information, page 33). For more information about QIAprep, QIAamp and DNeasy products, contact please contact QIAGEN Technical Support at **support.qiagen.com**.

Quantity of starting template

The efficiency with which primers anneal to templates is an important factor in PCR. Due to the thermodynamic nature of the reaction, the primer:template ratio strongly influences the specificity and efficiency of PCR and should be optimized empirically. If too little template is used, primers may not be able to find their complementary sequences. Too much template may lead to an increase in mispriming events. As an initial guide, spectrophotometric and molar conversion values for different nucleic acid templates are listed in Tables 5 and 6 (page 19).

Table 5. Spectrophotometric conversions for nucleic acid templates

1 A_{260} unit*	Concentration (µg/ml)
Double-stranded DNA	50
Single-stranded DNA	33
Single-stranded RNA	40

*Absorbance at 260 nm = 1.

Table 6. Molar conversions for nucleic acid templates

Nucleic acid	Size	pmol/µg	Molecules/µg
1 kb DNA	1000 bp	1.52	9.1×10^{11}
pUC19 DNA	2686 bp	0.57	3.4×10^{11}
pTZ18R DNA	2870 bp	0.54	3.2×10^{11}
pBluescript II DNA	2961 bp	0.52	3.1×10^{11}
Lambda DNA	48,502 bp	0.03	1.8×10^{10}
Average mRNA	1930 bp	1.67	1.0×10^{12}
Genomic DNA	**Size**	**pmol/µg**	**Molecules/µg**
Escherichia coli	4.7×10^{6}*	3.0×10^{-4}	1.8×10^{8}†
Drosophila melanogaster	1.4×10^{8}*	1.1×10^{-5}	6.6×10^{5}†
Mus musculus (mouse)	2.7×10^{9}*	5.7×10^{-7}	3.4×10^{5}†
Homo sapiens (human)	3.3×10^{9}*	4.7×10^{-7}	2.8×10^{5}†

*Base pairs in haploid genome.
† For single-copy genes.

Appendix B: Primer Design, Concentration and Storage

Designing Multiplex Primers

When designing primers for multiplex PCR, note the following points:

- Avoid complementarity of 2 or 3 bases at the 3' ends of primer pairs to reduce primer-dimer formation
- Avoid mismatches between the 3' end of the primer and the target template sequence
- Avoid runs of 3 or more G and/or C bases at the 3' end
- Avoid complementary sequences within primers and between primer pairs
- Ensure primers are unique to your template sequence. Check similarity to other known sequences with BLAST (**www.ncbi.nlm.nih.gov/blast/Blast.cgi**).
- Commercially available computer software (e.g., OLIGO 6) or web-based tools such as Primer3 (**frodo.wi.mit.edu/primer3/**) can be used for primer design.

Annealing temperatures

If necessary, perform a gradient PCR to determine the optimal annealing temperature. Otherwise, use the recommendations in Table 7.

Table 7. Recommended annealing temperatures for multiplex PCR

Lowest primer T_m	Annealing temperature
<60°C	Perform gradient PCR over the range of 48–60°C
60-66°C	53-63°C

Distinguishing individual PCR products

Depending on the method of detection, primers should be chosen so that the corresponding PCR products can be easily distinguished from one another (e.g., through size differences), by using primers labeled with different fluorescent dyes or by other appropriate detection procedures.

Successful PCRs

Prerequisites for successful PCR include the design of optimal primer pairs, the use of appropriate primer concentrations and the correct storage of primer solutions. Some general guidelines are given in Table 8 (below)8.

Table 8. Guidelines for designing, handling and storing primers

Feature	Description
Length	18–30 nucleotides
G/C content	40–60%
*T*m:	Simplified formula for estimating melting temperature (T_m) T_m = 2°C x (A+T) + 4°C x (G+C) Whenever possible, design primer pairs with similar T_m values. Optimal PCR annealing temperatures may be above or below the estimated T_m. As a starting point, use an annealing temperature 5°C below T_m . Functionality and specificity of all primer pairs should be checked in individual reactions before combining them in a multiplex PCR assay.
Location	If detecting mRNA after conversion into cDNA, design primers so that one half of the primer hybridizes to the 3' end of one exon and the other half to the 5' end of the adjacent exon (see Figure 2A, page 233). Primers will anneal to cDNA synthesized from spliced mRNAs, but not to genomic DNA. Thus, amplification of contaminating DNA is eliminated. Alternatively, PCR primers should be designed to flank a region that contains at least one intron (see Figure 2B, page 23). Products amplified from cDNA (no introns) will be smaller than those amplified from genomic DNA (containing introns). Size difference in products is used to detect the presence of contaminating DNA.

	If only the mRNA sequence is known, choose primer annealing sites that are at least 300–400 bp apart. It is likely that fragments of this size from eukaryotic DNA contain splice junctions. As explained in the previous point and Figure 2B (page 23), such primers can be used to detect DNA contamination.
Sequence	Avoid complementarity of two or more bases at the 3' ends of primer pairs to reduce primer–dimer formation. Avoid mismatches between the 3' end of the primer and the target-template sequence. Avoid runs of 3 or more G or C nucleotides at the 3' end. Avoid a 3'- end T. Primers with a T at the 3' end have a greater tolerance of mismatch. Avoid complementary sequences within a primer sequence and between the primers of a primer pair. Commercially available computer software can be used for primer design.
Concentration	Spectrophotometric conversion for primers: 1 A_{260} unit ≡ 20–30 µg/ml Molar conversions: **Primer length** / **pmol/µg** / **12.5 pmol (0.25 µM in 25 µl)** / **10 pmol (0.25 µM in 20 µl)** 18mer / 168 / 37 ng / 30 ng 20mer / 152 / 42 ng / 34 ng 25mer / 121 / 52 ng / 41 ng 30mer / 101 / 62 ng / 50 ng Use 0.25–1.0 µM of each primer in PCR. For most applications, a primer concentration of 0.25 µM will be optimal.
Storage	Lyophilized primers should be dissolved in a small volume of TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0) to make a concentrated stock solution. Prepare small aliquots of working solutions containing10 pmol/µl to avoid repeated thawing and freezing. For microbiome studies dissolving in DNA-free water should be considered to prevent DNA intake into the TE buffer used. Store all primer solutions at –15 to –30°C.

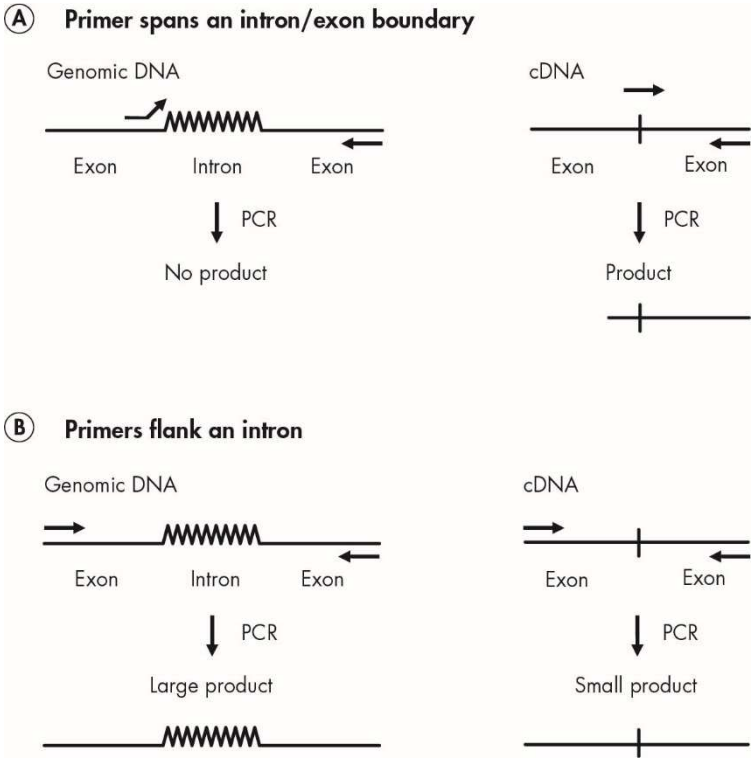

Figure 2. Designing primers for PCR. Primer design to (A) eliminate or (B) detect amplification from contaminating genomic DNA.

Degenerate PCR primers

Occasionally, the exact nucleotide sequence of the target-template DNA will not be known, for instance, when it has been deduced from an amino acid sequence or when a family of closely related sequences is to be amplified. To amplify such templates using PCR, degenerate primers can be used. These are actually mixtures of several primers whose sequences differ at the positions that correspond to the uncertainties in the template sequence.

Hot-start PCR, as enabled by the UCP PCR Kit, often improves amplification specificity in PCR using degenerate primers by reducing the formation of nonspecific PCR products and primer–dimers. Table 9 (below) gives recommendations to optimize PCR using degenerate primers. To help determine the best location for degenerate primers, Table 10 (page 25) lists the codon redundancy of each amino acid.

Table 9. Guidelines for the design and use of degenerate primers

<table>
<tr><th></th><th>Description</th></tr>
<tr><td>Sequence</td><td>Avoid degeneracy in the 3 nucleotides at the 3' end. If possible, use Met- or Trp-encoding triplets at the 3' end.

To increase primer–template binding efficiency, reduce degeneracy by allowing mismatches between the primer and template, especially towards the 5' end (but not at the 3' end).

Try to design primers with less than 4-fold degeneracy at any given position.</td></tr>
<tr><td>PCR conditions</td><td>When optimizing two-step PCR using degenerate primers, modify PCR conditions in the following order.</td></tr>
<tr><td>Primer concentration</td><td>First try a primer concentration of 0.25 µM. If this primer concentration results in poor PCR amplification, increase the primer concentration in increments of 0.2 µM until satisfactory results are obtained.</td></tr>
<tr><td>Template concentration</td><td>Increase starting template amount (up to 1 µg).</td></tr>
<tr><td>Annealing temperature</td><td>Reduce annealing temperature in steps of 2°C.</td></tr>
<tr><td>Concentration</td><td>Spectrophotometric conversion for primers: 1 A_{260} unit ≡ 20–30 µg/ml.

Molar conversions:

<table>
<tr><th>Primer length</th><th>pmol/µg</th><th>12.5 pmol (0.25 µM in 25 µl)</th><th>10 pmol (0.25 µM in 20 µl)</th></tr>
<tr><td>18mer</td><td>168</td><td>37 ng</td><td>30 ng</td></tr>
<tr><td>20mer</td><td>152</td><td>42 ng</td><td>34 ng</td></tr>
<tr><td>25mer</td><td>121</td><td>52 ng</td><td>41 ng</td></tr>
<tr><td>30mer</td><td>101</td><td>62 ng</td><td>50 ng</td></tr>
</table>

Use 0.25–1.0 µM of each primer in PCR. For most applications, a primer concentration of 0.25 µM will be optimal.</td></tr>
</table>

Table 10. Codon redundancy

Amino acid	Number of codons
Met, Trp	1
Cys, Asp, Glu, Phe, His, Lys, Asn, Gln, Tyr	2
Ile	3
Ala, Gly, Pro, Thr, Val	4
Leu, Arg, Ser	6

Appendix C: Sensitive PCR Assays

PCR can be performed to amplify and detect just a single copy of a nucleic acid sequence. However, amplification of such a low number of target sequences is often limited by the generation of nonspecific PCR products and primer–dimers. The combination of DNA Polymerase and PCR Buffer in the UCP Master Mix increases specificity both at the start of and during PCR. Thus, the UCP Master Mix is well suited to such challenging and highly sensitive PCR assays.

Touchdown PCR

Touchdown PCR uses a cycling program with varying annealing temperatures. It is a useful method to increase the specificity of PCR. The annealing temperature in the initial PCR cycle should be 5–10°C above the T_m of the primers. In subsequent cycles, the annealing temperature is decreased in steps of 1–2°C per cycle until a temperature is reached that is equal to, or 2–5°C below, the T_m of the primers. Touchdown PCR enhances the specificity of the initial primer–template duplex formation and hence the specificity of the final PCR product. To program your thermal cycler for touchdown PCR, refer to the manufacturer's instructions.

Appendix D: Purification of PCR Products

After amplification, the PCR sample contains a complex mixture of specific PCR products and residual reaction components such as primers, unincorporated nucleotides, enzymes, salts, mineral oil and possibly nonspecific amplification products. Before the specific PCR products can be used in subsequent experiments, it is often necessary to remove these contaminants. The QIAquick® and MinElute® systems offer a quick and easy method for purifying the final PCR products. For more information about QIAquick or MinElute products, please visit **www.qiagen.com**.

Appendix E: Controlling Contamination

General control of nucleic acid contamination

It is extremely important to include at least one negative control in every round of PCR. This control contains no added template nucleic acid, which allows detection of possible contamination of the reaction components.

General physical precautions

Separate the working areas for setting up PCR amplifications and RNA and DNA handling, including the addition of starting template, PCR product analysis or plasmid preparation. Ideally, use separate rooms.

Use a separate set of pipettes for the PCR Master Mix. Use of pipette tips with hydrophobic filters is strongly recommended.

Prepare and freeze small aliquots of primer solutions. Use of DNA-free water is strongly recommended.

In case of contamination, laboratory benches, apparatus and pipettes can be decontaminated by cleaning them with 10% (v/v) commercial bleach solution. Afterwards, the benches and pipettes should be rinsed with distilled water.

For 16S or 18S PCR amplification reactions, we recommend setting up in UV cabinets. All surfaces should be UV decontaminated prior to working. All used accessories, for example, 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.

General chemical precautions

PCR stock solutions can also be decontaminated using UV light. However, this method is laborious, and its efficiency is difficult to control and cannot be guaranteed. We recommend storing solutions in small aliquots and using fresh aliquots for each PCR.

Ordering Information

Product	Contents	Cat. no.
UCP Multiplex PCR Kit (100)	For 100 x 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 x 20 µl PCR reactions: Ultra-Clean Production master mix for multiplex hot-start PCR and microbiome applications	206744
Related products		
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
QIAamp UCP DNA Micro Kit (50)	For 50 preps: Ultraclean DNA purification from small sample volumes	56204
QIAamp UCP Pathogen Mini Kit (50)	For 50 preps: Microbial DNA purification from whole blood, swabs, cultures and body fluids	50214
RNeasy® UCP Micro Kit (50)	For 50 preps: Purification of up to 45 µg total RNA from small or low biomass samples	73934
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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1109017 HB-2538-001 04/2018