May 2011

QuantiFast® Multiplex PCR Handbook

QuantiFast Multiplex PCR Kit — with master mix containing ROX™ passive reference dye

QuantiFast Multiplex PCR +R Kit — with separate tube of ROX passive reference dye, and master mix that does not contain ROX dye

For quantitative, multiplex, real-time PCR and two-step RT-PCR with fast cycling using sequence-specific probes

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QIAGEN Sample and Assay Technologies

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Contents

Kit Contents

QuantiFast Multiplex PCR Kit	(80)	(400)	(2000)
Catalog no.	204652	204654	204656
Number of 25 µl reactions	80	400	2000
2x QuantiFast Multiplex PCR Master Mix*	1 ml	3 x 1.7 ml	25 ml
RNase-Free Water	2 ml	2 x 2 ml	20 ml
Handbook	1	1	1

* Contains HotStarTaq® *Plus* DNA Polymerase, QuantiFast Multiplex PCR Buffer, dNTP mix (dATP, dCTP, dGTP, and dTTP), and ROX passive reference dye.

QuantiFast Multiplex PCR +R Kit	(80)	(400)	(2000)
Catalog no.	204752	204754	204756
Number of 25 µl reactions	80	400	2000
2x QuantiFast Multiplex PCR Master Mix (w/o ROX)†	1 ml	3 x 1.7 ml	25 ml
50x ROX Dye Solution	45 µl	210 µl	1.05 ml
RNase-Free Water	2 ml	2 x 2 ml	20 ml
Handbook	1	1	1

† Contains HotStarTaq *Plus* DNA Polymerase, QuantiFast Multiplex PCR Buffer, and dNTP mix (dATP, dCTP, dGTP, and dTTP).

Shipping and Storage

QuantiFast Multiplex PCR Kits are shipped on dry ice. The kits should be stored immediately upon receipt at –20°C in a constant-temperature freezer and protected from light. When the kits are stored under these conditions and handled correctly, performance is guaranteed until the expiration date (see the quality-control label inside the kit box or on the kit envelope). 2x QuantiFast Multiplex PCR Master Mixes can also be stored protected from light at 2–8°C for up to 1 month without showing any reduction in performance.

To maintain optimal performance of QuantiFast Multiplex PCR Kits for 2000 x 25 µl reactions, we recommend storing the 25 ml master mix as appropriately sized aliquots in sterile, polypropylene tubes.

If desired, 50x ROX Dye Solution can be added to 2x QuantiFast Multiplex PCR Master Mix (w/o ROX) for long-term storage. For details, see "Passive reference dye", pages 9–10.

Product Use Limitations

QuantiFast Multiplex PCR Kits are intended for molecular biology applications. These products are not intended for the diagnosis, prevention, or treatment of a disease.

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

Product Warranty and Satisfaction Guarantee

QIAGEN guarantees the performance of all products in the manner described in our product literature. The purchaser must determine the suitability of the product for its particular use. Should any product fail to perform satisfactorily due to any reason other than misuse, QIAGEN will replace it free of charge or refund the purchase price. We reserve the right to change, alter, or modify any product to enhance its performance and design. If a QIAGEN product does not meet your expectations, simply call your local Technical Service Department or distributor. We will credit your account or exchange the product — as you wish. Separate conditions apply to QIAGEN scientific instruments, service products, and to products shipped on dry ice. Please inquire for more information.

A copy of QIAGEN terms and conditions can be obtained on request, and is also provided on the back of our invoices. If you have questions about product specifications or performance, please call QIAGEN Technical Services or your local distributor (see back cover or visit www.qiagen.com).

Technical Assistance

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

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

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

Safety Information

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

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

Quality Control

Component	Test
2x QuantiFast Multiplex PCR Master Mixes*	PCR sensitivity and reproducibility assay: Sensitivity and reproducibility in real-time PCR are tested in parallel reactions containing variable amounts of DNA templates.
HotStarTaq *Plus* DNA Polymerase†	Efficiency and reproducibility in PCR are tested. Functional absence of exonucleases and endonucleases is tested.
QuantiFast Multiplex PCR Buffer†	Conductivity and pH are tested.
RNase-free water	Conductivity, pH, and RNase activities are tested.
50x ROX Dye Solution‡	Emission and excitation are tested.

* See quality-control label inside the kit box or on the kit envelope for lot-specific values.

† Included in 2x QuantiFast Multiplex PCR Master Mixes.

‡ Supplied with the QuantiFast Multiplex PCR +R Kit.

Product Description

Component	Description
HotStarTaq *Plus* DNA Polymerase*†	HotStarTaq *Plus* DNA Polymerase is a modified form of a recombinant 94 kDa DNA polymerase, originally isolated from *Thermus aquaticus*. The enzyme is provided in an inactive state and has no enzymatic activity at ambient temperature. The enzyme is activated by a 5-minute, 95°C incubation step.
QuantiFast Multiplex PCR Buffer*†	Novel PCR buffer for fast and highly sensitive quantification of DNA and cDNA targets in multiplex format; includes Factor MP to facilitate multiplex PCR
dNTP mix*†	Contains dATP, dCTP, dGTP, and dTTP of ultrapure quality
ROX passive reference dye*	Optimized concentration of fluorescent dye for normalization of fluorescent signals on all instruments from Applied Biosystems except Applied Biosystems® 7500 Real-Time PCR Systems
50x ROX Dye Solution‡	Separate tube of passive reference dye for normalization of fluorescent signals on Applied Biosystems 7500 Real-Time PCR Systems and, optionally, on instruments from Agilent (formerly Stratagene); not required for instruments from Bio-Rad/MJ Research, Cepheid, Eppendorf, and Roche
RNase-free water	Ultrapure quality, PCR-grade

* Included in 2x QuantiFast Multiplex PCR Master Mix.

† Included in 2x QuantiFast Multiplex PCR Master Mix (w/o ROX).

‡ Supplied with the QuantiFast Multiplex PCR +R Kit.

Introduction

QuantiFast Multiplex PCR Kits provide rapid real-time PCR quantification of DNA and cDNA targets in a multiplex format. Depending on the real-time cycler used, up to 4 targets can be quantified simultaneously in the same well or tube. The kits can be used in real-time PCR of genomic DNA targets, and also in real-time two-step RT-PCR of RNA targets following reverse transcription with, for example, the QuantiTect® Reverse Transcription Kit (see ordering information, page 55). High specificity and sensitivity in multiplex PCR are achieved without any time-consuming optimization steps through the use of the hot-start enzyme HotStarTaq *Plus* DNA Polymerase together with a specialized PCR buffer. Short cycling steps without loss of PCR sensitivity and efficiency are enabled by Q-Bond®, a patent-pending additive in the PCR buffer.

The kits have been optimized for use with TaqMan® probes and are available in 2 formats:

- **QuantiFast Multiplex PCR Kit**: This kit is supplied with a master mix containing ROX passive reference dye, and is optimized for use with real-time cyclers that require a high concentration of ROX dye for fluorescence normalization (e.g., instruments from Applied Biosystems, but not Applied Biosystems 7500 Real-Time PCR Systems).
- **QuantiFast Multiplex PCR +R Kit**: This kit is supplied with a master mix that is free of ROX dye, and also includes a separate solution of ROX dye which the user can add to reactions, depending on the real-time cycler used. The kit is intended for use with cyclers that require a lower concentration of ROX dye for fluorescence normalization (e.g., Applied Biosystems 7500 Real-Time PCR Systems), for use with cyclers that allow optional use of ROX dye (e.g., instruments from Agilent), and for use with cyclers that do not require ROX dye. Running reactions without ROX dye increases multiplexing capacity and allows greater flexibility when choosing reporter dyes for probes.

For users of Rotor-Gene® cyclers, the Rotor-Gene Multiplex PCR Kit is recommended. The kit is supplied with a dedicated protocol that allows even shorter cycling steps. For ordering information, see page 56.

2x QuantiFast Multiplex PCR Master Mixes

The components of 2x QuantiFast Multiplex PCR Master Mix include HotStarTaq *Plus* DNA Polymerase, QuantiFast Multiplex PCR Buffer, and ROX passive reference dye (see descriptions below). 2x QuantiFast Multiplex PCR Master Mix (w/o ROX) contains HotStarTaq *Plus* DNA Polymerase and QuantiFast Multiplex PCR Buffer, but no ROX passive reference dye. The optimized master mixes ensure that the PCR products in a multiplex reaction are amplified with the same efficiency and sensitivity as the PCR products in corresponding singleplex reactions.

HotStarTaq *Plus* DNA Polymerase

HotStarTaq *Plus* DNA Polymerase is a modified form of QIAGEN *Taq* DNA Polymerase. It is provided in an inactive state and has no enzymatic activity at ambient temperature. This prevents the formation of misprimed products and primer–dimers during reaction setup and the first denaturation step. Competition for reactants by PCR artifacts is therefore avoided, enabling high PCR specificity and accurate quantification. The enzyme is activated at the start of a reaction by a 5-minute, 95°C incubation step. The hot start enables reactions to be set up rapidly and conveniently at room temperature. In addition, the concentration of the polymerase in the master mix is optimized to allow short extension times in the combined annealing/extension step of each PCR cycle.

QuantiFast Multiplex PCR Buffer

QuantiFast Multiplex PCR Buffer is specifically developed for fast-cycling, multiplex, real-time PCR using sequence-specific probes. A novel additive in the buffer, Q-Bond, allows short cycling times on standard cyclers and on fast cyclers with rapid ramping rates. Q-Bond increases the affinity of *Taq* DNA polymerases for short single-stranded DNA, reducing the time required for primer/probe annealing to a few seconds. This allows a combined annealing/extension step of only 30 seconds in duplex PCR. In addition, the unique composition of the buffer supports the melting behavior of DNA, enabling short denaturation and annealing/extension times.

QuantiFast Multiplex PCR Buffer is also based on the unique QIAGEN PCR buffer system. The buffer contains a balanced combination of KCl and $(NH_4)_2SO_4$, 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. When using this buffer, primer annealing is only marginally influenced by the $MgCl_2$ concentration, so optimization by titration of Mg^{2+} is not required. The buffer also contains Factor MP, which facilitates multiplex PCR. This synthetic factor increases the local concentration of primers and probes at the DNA template and stabilizes specifically bound primers and probes, allowing efficient annealing and extension. The combination of these various components of QuantiFast Multiplex PCR Buffer prevents multiple amplification reactions from affecting each other.

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. However, when performing multiplex, real-time PCR with these instruments, the presence of ROX passive reference dye will limit their multiplexing capability.

The use of ROX dye is necessary for instruments from Applied Biosystems and is optional for instruments from Agilent. When performing multiplex, real-time PCR with ROX passive reference dye on these instruments, we do not recommend using probes that have ROX or Texas Red® fluorophore as the reporter dye, since their performance in the presence of ROX dye is unpredictable. When performing reactions using probes labeled with ROX, Texas Red, or other equivalent fluorophore, use a real-time cycler that does not require ROX dye for fluorescence normalization.

The master mix supplied with the QuantiFast Multiplex PCR Kit contains ROX dye at a concentration that is optimal for instruments from Applied Biosystems (models 7000, 7300, 7700, 7900HT, StepOne™, and StepOnePlus™, but not Applied Biosystems 7500 Real-Time PCR Systems).

For Applied Biosystems 7500 Real-Time PCR Systems and instruments from Agilent, ROX dye is required at a lower concentration. This is provided by the QuantiFast Multiplex PCR +R Kit, which requires the user to add the supplied ROX dye solution to the master mix during reaction setup. If desired, ROX dye can be premixed with an entire tube of master mix in a 1:25 ratio (see Table 1). When stored protected from light, the premixed solution is stable at 2–8°C for up to 1 month or at –20°C until the expiration date. Remember to indicate on the tube that ROX dye has been added.

Table 1. Addition of ROX dye to master mix for long-term storage*

Kit	Volume of ROX dye solution	Volume of 2x QuantiFast Multiplex PCR Master Mix (w/o ROX)
QuantiFast Multiplex PCR +R Kit (80)	40 µl	1 ml
QuantiFast Multiplex PCR +R Kit (400)	68 µl	1.7 ml
QuantiFast Multiplex PCR +R Kit (2000)	1 ml	25 ml

* Option for users of the Applied Biosystems 7500, Mx3000P®, Mx3005P®, and Mx4000®.

Instruments from all other suppliers, which do not require ROX dye for fluorescence normalization, should be used with the QuantiFast Multiplex PCR +R Kit, which provides master mix that does not contain ROX dye.

Sequence-specific probes

QuantiFast Multiplex PCR Kits can be used with all types of probe. This handbook contains optimized protocols for use with TaqMan probes, a major type of sequence-specific probe used in quantitative, real-time PCR (see below). For more details on sequence-specific probes, and their design and handling, see Appendix A, page 50.

TaqMan probes

TaqMan probes are sequence-specific oligonucleotides with a fluorophore and a quencher moiety attached (Figure 1). The fluorophore is at the 5' end of the probe, and the quencher moiety is usually located at the 3' end or internally. During the extension phase of PCR, the probe is cleaved by the 5'→3' exonuclease activity of *Taq* DNA polymerase, separating the fluorophore and the quencher moiety. This results in detectable fluorescence that is proportional to the amount of accumulated PCR product.

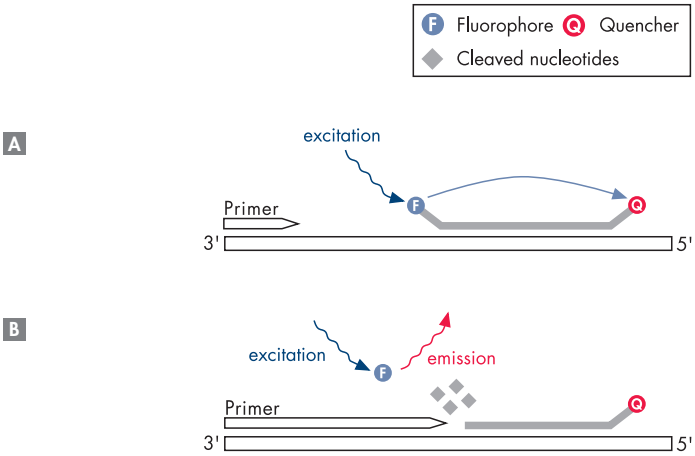

Figure 1. Principle of TaqMan probes in quantitative, real-time PCR. A Both the TaqMan probe and the PCR primers anneal to the target sequence during the PCR annealing step. The proximity of the quencher to the fluorophore strongly reduces the fluorescence emitted by the fluorophore. B During the PCR extension step, *Taq* DNA polymerase extends the primer. When the enzyme reaches the TaqMan probe, its 5'→3' exonuclease activity cleaves the fluorophore from the probe. The fluorescent signal from the free fluorophore is measured. This signal is proportional to the amount of accumulated PCR product.

cDNA synthesis for real-time two-step RT-PCR

If quantifying cDNA targets with QuantiFast Multiplex PCR Kits, RNA must first be reverse transcribed into cDNA. A portion of the reverse-transcription reaction is then transferred to another tube where real-time PCR takes place. This entire process is known as real-time two-step RT-PCR, since reverse transcription and real-time PCR are carried out in separate tubes.

For reverse transcription, we recommend using the QuantiTect Reverse Transcription Kit. The kit provides a fast and convenient procedure, requiring only 20 minutes to synthesize first-strand cDNA and eliminate genomic DNA contamination. An optimized mix of oligo-dT and random primers enables cDNA synthesis from all regions of RNA transcripts, even from 5' regions of very long mRNA transcripts. cDNA yields are high, allowing sensitive detection of even low-abundance transcripts in real-time two-step RT-PCR. An alternative to the QuantiTect Reverse Transcription Kit is the FastLane® Cell cDNA Kit, which allows cDNA to be prepared directly from cultured cells without RNA purification. The FastLane Cell cDNA Kit is useful for experiments where archiving of purified RNA is not required. For ordering information for these 2 kits, see pages 55–56.

For very small RNA samples (as little as 1 ng), we recommend carrying out whole transcriptome amplification using the QuantiTect Whole Transcriptome Kit, which provides high yields of up to 40 µg cDNA for unlimited real-time PCR analysis. The kit contains all the necessary reagents for reverse transcription followed by cDNA ligation and amplification of all cDNA targets. The relative abundance of each transcript is preserved after whole transcriptome amplification, ensuring reliable gene expression analysis. For ordering information, see page 56.

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 material safety data sheets (MSDSs), available from the product supplier.

- Primers and probes from an established oligonucleotide manufacturer. Primers should be of standard quality, and probes should be HPLC purified. Lyophilized primers and probes should be dissolved in TE buffer to provide a stock solution of 100 µM; concentration should be checked by spectrophotometry (for details, see Appendix A, page 50). Primer and probe stock solutions should be stored in aliquots at –20°C. Probe stock solutions should be protected from exposure to light.
- Nuclease-free (RNase/DNase-free) consumables. Special care should be taken to avoid nuclease contamination of PCR reagents and consumables.
- Real-time PCR thermal cycler
- PCR tubes or plates (use thin-walled PCR tubes or plates recommended by the manufacturer of your real-time cycler)
- Optional: Trizma® base and EDTA for preparing TE buffer for storing primers and probes (see Appendix A, page 50). Use RNase/DNase-free water and plastic consumables to prepare TE buffer.
- Optional: QIAgility® for rapid, high-precision automated PCR setup; for details, visit www.qiagen.com/goto/QIAgility. Please note that the number of reactions will be 10–20% lower than indicated on the kit packaging to guarantee process safety and optimal performance.

Important Notes

Selecting kits and protocols

To select the correct QuantiFast Multiplex PCR Kit and protocol to use with your real-time cycler, refer to Table 2. In general, the following cyclers are not compatible with multiplex, real-time PCR: GeneAmp® 5700, MyiQ™, and DNA Engine Opticon® (i.e., the single-color machine). The capabilities of the LightCycler® 1.x for multiplex, real-time PCR using QuantiFast Multiplex PCR Kits are very limited due to its detection optics. For users of Rotor-Gene cyclers, we recommend the Rotor-Gene Multiplex PCR Kit, which provides even shorter cycling steps (see page 56 for ordering information).

Table 2. Choosing the correct QuantiFast Multiplex PCR Kit and protocol for your real-time cycler

Cycler	Kit	Duplex protocol	Triplex and 4-plex protocol
ABI PRISM® 7000	QuantiFast Multiplex PCR Kit	Protocol 1, page 31	Protocol 2, page 34
ABI PRISM 7700	QuantiFast Multiplex PCR Kit	Protocol 1, page 31†	
Applied Biosystems 7300	QuantiFast Multiplex PCR Kit	Protocol 1, page 31	Protocol 2, page 34
Applied Biosystems 7500*	QuantiFast Multiplex PCR +R Kit	Protocol 3, page 38	Protocol 4, page 42
Applied Biosystems 7900HT	QuantiFast Multiplex PCR Kit	Protocol 1, page 31	Protocol 2, page 34
Applied Biosystems StepOne	QuantiFast Multiplex PCR Kit	Protocol 1, page 31†	
Applied Biosystems StepOnePlus	QuantiFast Multiplex PCR Kit	Protocol 1, page 31	Protocol 2, page 34
iCycler iQ®	QuantiFast Multiplex PCR +R Kit	Protocol 3, page 38	Protocol 4, page 42
LightCycler 2.0 and LightCycler 480	QuantiFast Multiplex PCR +R Kit	Protocol 3, page 38	Protocol 4, page 42

* Includes the Applied Biosystems 7500 Fast Real-Time PCR System.

† Only duplex assays are possible due to hardware limitations.

Table continues on next page.

Table 2. Continued

Cycler	Kit	Duplex protocol	Triplex and 4-plex protocol
Mx3000P, Mx3005P, and Mx4000	QuantiFast Multiplex PCR +R Kit	Protocol 3, page 38	Protocol 4, page 42
SmartCycler® II	QuantiFast Multiplex PCR +R Kit	Protocol 3, page 38	Protocol 4, page 42
Other*	QuantiFast Multiplex PCR +R Kit	Protocol 3, page 38	Protocol 4, page 42

* Refer to manufacturer's instructions for multiplex capacity.

Guidelines for effective multiplex assays

QuantiFast Multiplex PCR Kits work with most existing probe systems that have been designed using standard design methods. However, for optimal performance of a probe system in quantitative, multiplex, real-time PCR, 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 multiplex assay.
- Choose compatible reporter dyes and quenchers. For details, see "Suitable combinations of reporter dyes", page 16.
- PCR products should be as short as possible, ideally 60–150 bp. For details, see Appendix A, page 50.
- 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 A, page 50.
- Check the concentration and integrity of primers and probes before starting. For details, see Appendix A, page 50.
- Check the real-time cycler user manual for **correct setup of the cycler for multiplex analysis** (e.g., setting up detection of multiple 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 multiplex assay are part of the standard set of dyes already calibrated on your instrument. 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** you are following.
- 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.
- Perform appropriate controls for evaluating the performance of your multiplex assays (e.g., amplifying each target individually and comparing the results with those for the multiplex assay).

Suitable combinations of reporter dyes

Multiplex, real-time PCR requires the simultaneous detection of different fluorescent reporter dyes (Table 3). For accurate detection, the fluorescence spectra of the dyes should be well separated or exhibit only minimal overlap. **Please read the general recommendations and instrument-specific recommendations on the next few pages before starting**.

Note: If there are no specific recommendations below for your real-time cycler, please refer to the user manual or other technical documentation for your instrument to find out which reporter dyes can be used in multiplex analysis.

General recommendations

- Before starting, choose suitable combinations of reporter dyes and quenchers that are compatible with multiplex analysis using the detection optics of your real-time cycler. Order the probes from an established oligonucleotide manufacturer.
- For optimal results, follow the recommended combinations of dyes shown in Tables 4–13 (pages 18–29).
- 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.
- **For triplex and 4-plex analyses, we strongly recommend using nonfluorescent quenchers.** Due to the detection optics of more recent real-time cyclers and the possible combinations of reporter dyes, triplex and 4-plex analyses may only be possible with nonfluorescent quenchers, especially with instruments from Applied Biosystems.

Table 3. Dyes commonly used in multiplex, real-time PCR

Dye	Excitation maximum (nm)	Emission maximum (nm)*
ATTO 390	390	479
AMCA-X	353	442
Marina Blue®	362	459
FAM	494	518
TET™	521	538
JOE	520	548
VIC	538	552
Yakima Yellow®	526	552
HEX	535	553
CAL Fluor® Orange 560	538	559
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
Tye 665	645	665
Cy5	643	667
Quasar® 705	690	705

* Emission spectra may vary depending on the buffer conditions.

Recommendations for instruments from Applied Biosystems

Tables 4–9 on the next few pages give specific recommendations for the ABI PRISM 7000, ABI PRISM 7700, Applied Biosystems 7300, Applied Biosystems 7500, Applied Biosystems 7900HT, StepOne, and StepOnePlus.

Table 4. Suitable reporter dyes — ABI PRISM 7000 and Applied Biosystems 7300

Type of assay	Filter A*	Filter B*	Filter C*	Filter D†
Duplex	6-FAM	HEX‡ JOE VIC		ROX (passive reference)
Duplex	6-FAM		Bodipy TMR‡ NED	ROX (passive reference)
Triplex§	6-FAM	HEX‡ JOE VIC	Bodipy TMR‡ NED	ROX (passive reference)

* Use filter A, filter B, and filter C to detect the least abundant target, the second least abundant target, and the most abundant target, respectively.

† Filter D is for detecting ROX passive reference dye, a component of 2x QuantiFast Multiplex PCR Master Mix.

‡ Before using HEX or Bodipy TMR dye, a pure dye calibration of the real-time cycler using this dye must be performed. See the manufacturer's manual for details. Supplementary protocols for pure dye calibration of major real-time cyclers are available from QIAGEN Technical Services.

§ Each probe in the triplex assay must contain a nonfluorescent quencher.

Table 5. Suitable reporter dyes — Applied Biosystems 7500

Type of assay	Filter A*	Filter B*	Filter C*†	Filter D‡	Filter E*
Duplex	6-FAM	HEX§ JOE VIC		ROX (passive reference)	
Duplex	6-FAM		Bodipy TMR§ NED	ROX (passive reference)	
Triplex	6-FAM	HEX§ JOE VIC	Bodipy TMR§ NED	ROX (passive reference)	
4-plex	6-FAM	HEX§ JOE VIC	Bodipy TMR§ NED	ROX (passive reference)	Cy5

* Use filter A, filter B, and filters C and E to detect the least abundant target, the second least abundant target, and the 2 most abundant targets, respectively.

† When using filter C in duplex, triplex, or 4-plex PCR, all probes in the reaction must be labeled with a nonfluorescent quencher instead of a fluorescent quencher such as TAMRA dye.

‡ Filter D is for detecting ROX passive reference dye, which is supplied as a separate solution with the QuantiFast Multiplex PCR +ROX Vial Kit. The dye can be added to reactions, or premixed with 2x QuantiFast Multiplex PCR Master Mix (w/o ROX).

§ Before using HEX or Bodipy TMR dye, a pure dye calibration of the real-time cycler using this dye must be performed. See the manufacturer's manual for details. Supplementary protocols for pure dye calibration of major real-time cyclers are available from QIAGEN Technical Services.

Table 6. Suitable reporter dyes — Applied Biosystems 7900HT

Type of assay	Target 1*	Target 2*	Target 3*†	Passive reference‡
Duplex	6-FAM	HEX§ JOE VIC		ROX
Duplex	6-FAM		Bodipy TMR§ NED	ROX
Triplex	6-FAM	HEX§ JOE VIC	Bodipy TMR§ NED	ROX

* Target 1, target 2, and target 3 correspond to the least abundant target, the second least abundant target, and the most abundant target, respectively.

† When using a Bodipy TMR or NED labeled probe in duplex or triplex PCR, all probes in the reaction must be labeled with a nonfluorescent quencher instead of a fluorescent quencher such as TAMRA dye.

‡ ROX fluorescent dye is used as passive reference and is a component of 2x QuantiFast Multiplex PCR Master Mix.

§ Before using HEX or Bodipy TMR dye, a pure dye calibration of the real-time cycler using this dye must be performed. See the manufacturer's manual for details. Supplementary protocols for pure dye calibration of major real-time cyclers are available from QIAGEN Technical Services.

Table 7. Suitable reporter dyes — Applied Biosystems StepOne*

Type of assay	Filter 1†	Filter 2†	Passive reference‡
Duplex	6-FAM	HEX§ JOE VIC	ROX

* This real-time cycler is only designed for duplex analysis with the standard calibration.

† Use filter 1 to detect the least abundant target.

‡ ROX fluorescent dye is used as passive reference and is a component of 2x QuantiFast Multiplex PCR Master Mix.

§ Before using HEX dye, a pure dye calibration of the real-time cycler using this dye must be performed. See the manufacturer's manual for details. Supplementary protocols for pure dye calibration of major real-time cyclers are available from QIAGEN Technical Services.

Table 8. Suitable reporter dyes — Applied Biosystems StepOnePlus

Type of assay	Filter 1*	Filter 2*	Filter 3*†	Filter 4‡
Duplex	6-FAM	HEX§ JOE VIC		ROX (passive reference)
Duplex	6-FAM		Bodipy TMR§ NED	ROX (passive reference)
Triplex	6-FAM	HEX§ JOE VIC	Bodipy TMR§ NED	ROX (passive reference)

* Use filter 1, filter 2, and filter 3 to detect the least abundant target, the second least abundant target, and the most abundant target, respectively.

† When using filter 3 in duplex or triplex PCR, all probes in the reaction must be labeled with a nonfluorescent quencher instead of a fluorescent quencher such as TAMRA dye.

‡ Filter 4 is for detecting ROX passive reference dye, a component of 2x QuantiFast Multiplex PCR Master Mix.

§ Before using HEX or Bodipy TMR dye, a pure dye calibration of the real-time cycler using this dye must be performed. See the manufacturer's manual for details. Supplementary protocols for pure dye calibration of major real-time cyclers are available from QIAGEN Technical Services.

Table 9. Suitable reporter dyes — ABI PRISM 7700*

Type of assay	Target 1†	Target 2†	Passive reference‡
Duplex	6-FAM	HEX§ JOE VIC	ROX

* This real-time cycler is only designed for duplex analysis with the standard calibration.

† Target 1 and target 2 correspond to the least abundant target and the second least abundant target, respectively.

‡ ROX fluorescent dye is used as passive reference and is a component of 2x QuantiFast Multiplex PCR Master Mix.

§ Before using HEX dye, a pure dye calibration of the real-time cycler using this dye must be performed. See the manufacturer's manual for details. Supplementary protocols for pure dye calibration of major real-time cyclers are available from QIAGEN Technical Services.

Recommendations for Mx3000P, Mx3005P, and Mx4000 systems

Mx3000P, Mx3005P, and Mx4000 systems allow the use of different combinations of excitation and emission filters. This provides flexibility when selecting reporter dyes for multiplex assays. However, care must be taken to select suitable combinations of reporter dyes and filters that exhibit minimal crosstalk. Suitable combinations of reporter dyes for multiplex assays using Mx3000P, Mx3005P, and Mx4000 systems are given in Table 10 (page 23).

- Before performing a multiplex assay on the Mx3000P, Mx3005P, or Mx4000 system:
  - Check which reporter dyes can be detected with the sets of excitation and emission filters installed on your instrument.

    View the installed filter sets on your instrument as follows. Make sure the computer is connected to the instrument, and start the instrument software. Click the "Options" menu and select "Optics Configuration" to open the "Optics Configuration" dialog box. Click the "Dye Assignment" tab: the 4 filter sets displayed correspond to the filter sets installed on your instrument.

    View the dyes assigned to the installed filter sets as follows. In the "Dye Assignment" tab, click "Additional Dye Information" to open the "Dye Information" dialog box. Select "Detected dyes" to display the filter sets installed on your instrument and the defined dyes that are compatible with them.
  - Ensure that each reporter dye is detected by a different filter set in a distinct optical path.
- Refer to the *Mx4000 Multiplex Quantitative PCR System Instruction Manual*, the *Mx3005P Real-Time PCR System Instruction Manual*, or the *Mx3000P Real-Time PCR System On-line Help Manual* for additional information on the detection optics and correctly setting up the instrument for multiplex analysis.
- Different detection filter sets are available for Mx3000P, Mx3005P, and Mx4000 systems. Note that only some combinations of filter sets are compatible with multiplex analysis using commonly used reporter dyes. For details, see Table 10 (page 23).

Table 10. Suitable reporter dyes — Mx3000P, Mx3005P, and Mx4000*

Type of assay	Optical path 1 (FAM filter set)†	Optical path 2 (HEX/JOE filter set)†	Optical path 3 (ROX filter set)†	Optical path 4 (Cy5 filter set)†
Duplex	6-FAM	HEX JOE VIC		
Duplex	6-FAM		Texas Red ROX	
Duplex	6-FAM			Cy5
Triplex	6-FAM	HEX JOE VIC	Texas Red ROX	
Triplex	6-FAM	HEX JOE VIC		Cy5
Triplex	6-FAM		Texas Red ROX	Cy5
4-plex	6-FAM	HEX JOE VIC	ROX	Cy5

* The Mx3005P has 5 detection channels. This table shows the recommended dyes for use with four of the channels.

† Use optical path 1, optical path 2, and optical paths 3 and 4 to detect the least abundant target, the second least abundant target, and the 2 most abundant targets, respectively.

Recommendations for the iCycler iQ system

The iCycler iQ system is capable of using different combinations of excitation and emission filters. This provides flexibility when selecting reporter dyes for multiplex assays. However, care must be taken to select suitable combinations of reporter dyes and filters that exhibit minimal crosstalk. Suitable combinations of reporter dyes for multiplex assays using the iCycler iQ system are given in Table 11 (page 25).

- Before performing a multiplex assay on the iCycler iQ system:
  - Check that a filter set for each selected reporter dye is installed on the instrument. Ensure that each reporter dye is detected by a different filter set.
  - Calibrate each selected reporter dye on the instrument using a pure dye. The iCycler iQ Dye Calibrator Solution Kit (Bio-Rad, cat. no. 170-8792) may be used. Pure dye calibration data are used to separate the total fluorescence signal into the individual dyes. Pure dye calibration data are stored in the file **RME.ini**, which is stored at **C:\Program Files\Bio-Rad\iCycler\Ini**.
- Recalibrating the instrument (i.e., overwriting **RME.ini**) is required when changing the reaction volume, when switching from using caps to optical tape (or vice versa), or when adding new dyes for use in multiplex assays. If desired, the current **RME.ini** file can be archived before it is overwritten.
- The iCycler iQ system requires the collection of well factors before each run. If your sample plate does not contain the same dyes at the same concentrations in all wells, external well factors must be used. Collecting external well factors can be done using iCycler iQ External Well Factor Solution (Bio-Rad, cat. no. 170-8794).
- Refer to the *iCycler iQ Real-Time PCR Detection System Instruction Manual* for additional information on filter wheel setup, external well factors, selection and calibration of dyes, and correctly setting up the instrument for multiplex analysis.

Table 11. Suitable reporter dyes — iCycler iQ

Type of assay	Channel 1 (filter 490/530)*†	Channel 2 (filter 530/575)*†	Channel 3 (filter 575/620)*†	Channel 4 (filter 635/680)*†
Duplex	6-FAM	HEX JOE VIC		
Duplex	6-FAM		Texas Red ROX	
Duplex	6-FAM			Cy5
Triplex	6-FAM	HEX JOE VIC	Texas Red ROX	
Triplex	6-FAM	HEX JOE VIC		Cy5
Triplex	6-FAM		Texas Red ROX	Cy5
4-plex	6-FAM	HEX JOE VIC	Texas Red ROX	Cy5

* The numbers indicate the excitation/emission wavelengths of the detection filter set.

† Use channel 1, channel 2, and channels 3 and 4 to detect the least abundant target, the second least abundant target, and the 2 most abundant targets, respectively.

Recommendations for the LightCycler 2.0 system

The LightCycler 2.0 system has a LED light source that emits blue light of 470 nm and 6 band path detection filters. The available detection channels are 530 nm, 560 nm, 610 nm, 640 nm, 670 nm, and 705 nm. Suitable combinations of reporter dyes for multiplex assays using the LightCycler 2.0 system are given in Table 12 (page 27).

- The LightCycler 2.0 system always detects fluorescence in all of its detection channels. Therefore, there is no need to activate/deactivate the appropriate detection channels for each multiplex assay.
- Multiplex analysis on the LightCycler 2.0 system requires the generation of a color compensation file to separate the fluorescent signals and eliminate crosstalk between the individual detection channels. For details, see Appendix C, page 54.
- Although dyes detected in channels >600 nm are not optimally excited, using the dyes recommended in Table 12 will give a detectable fluorescent signal.
- The reporter dyes shown in Table 12 have been tested by QIAGEN and give reasonable fluorescent signals on the LightCycler 2.0 system. We do not recommend using other reporter dyes. Other dyes that can be potentially detected in channels >600 nm (i.e., detection channels 4, 5, and 6) may be poorly excited by the LightCycler 2.0 system, resulting in poor fluorescent signals.
- Although there are 6 detection channels, we recommend only using detection channels 1 (530 nm), 2 (560 nm), and 3 (610 nm) for duplex and triplex PCR. If performing 4-plex PCR, refer to Table 12 for possible combinations of dyes and their corresponding detection channels.
- Refer to the *LightCycler 2.0 Instrument Operator's Manual* for additional information on the detection optics and correctly setting up the instrument.

Table 12. Suitable reporter dyes — LightCycler 2.0

Type of assay	Detection channel 1 (530 nm filter)*	Detection channel 2 (560 nm filter)*	Detection channel 3 (610 nm filter)*	Detection channel 5 (670 nm filter)*	Detection channel 6 (705 nm filter)*
Duplex	6-FAM	HEX JOE VIC			
Duplex	6-FAM		Texas Red ROX		
Triplex	6-FAM	HEX JOE VIC	Texas Red ROX		
4-plex	6-FAM	HEX JOE VIC	Texas Red ROX	Cy5	
4-plex	6-FAM	HEX JOE VIC	Texas Red ROX		Alexa Fluor® 660 Pulsar® 650†

* Detection channel 1, detection channel 2, and detection channels 3 and 5/6 should be used to detect the least abundant target, the second least abundant target, and the 2 most abundant targets, respectively.

† This dye exhibits a broad emission peak. The greatest signal can be detected using the 705 nm channel. Probes labeled with Pulsar 650 dye are available from Biosearch Technologies (www.biosearchtech.com).

Recommendations for the LightCycler 480 system

The LightCycler 480 system uses a xenon lamp as its light source and has 5 excitation filters and 6 emission filters, allowing the setup of 5 detection channels suitable for multiplex analysis. Suitable combinations of reporter dyes for multiplex assays using the LightCycler 480 system are given in Table 13 (page 29).

- Check that each selected reporter dye is compatible with one of the detection channels installed on the instrument. Ensure that each reporter dye is detected by a different channel.
- Refer to the *LightCycler 480 Operator's Manual* for additional information on activating and deactivating detection channels and correctly setting up the instrument for multiplex analysis.
- Make sure to select suitable combinations of reporter dyes and filters that exhibit minimal crosstalk. There are 2 options for avoiding crosstalk on the LightCycler 480 system:
  - Dyes can be used that have widely separated emission spectra (e.g., FAM and Cy5). However, it is still recommended to determine the degree of crosstalk for these assays.
  - Alternatively, the LightCycler 480 system can use a color compensation file that contains information to correct crosstalk between different detection channels. Check that each selected reporter dye is compatible with one of the detection channels installed on the instrument. Ensure that each reporter dye is detected by a different channel

Table 13. Suitable reporter dyes — LightCycler 480

Type of assay	Channel 1 (450/500)*†	Channel 2 (483/533)*†	Channel 3 (523/568)*†	Channel 4 (558/610)*†	Channel 5 (615/670)*†
Duplex	Cyan 500	6-FAM			
Duplex		6-FAM	HEX‡		
Duplex		6-FAM		Texas Red ROX	
Duplex		6-FAM			Cy5
Triplex	Cyan 500	6-FAM	HEX‡		
Triplex	Cyan 500	6-FAM		Texas Red ROX	
Triplex	Cyan 500	6-FAM			Cy5
Triplex		6-FAM	HEX‡	Texas Red ROX	
Triplex		6-FAM	HEX‡		Cy5
Triplex		6-FAM		Texas Red ROX	Cy5
4-plex	Cyan 500	6-FAM	HEX‡	Texas Red ROX	
4-plex	Cyan 500	6-FAM	HEX‡		Cy5
4-plex	Cyan 500	6-FAM		Texas Red ROX	Cy5
4-plex		6-FAM	HEX‡	Texas Red ROX	Cy5

* The numbers in parentheses indicate the wavelengths of the excitation and emission filters on the first-generation LightCycler 480. Newer versions of the LightCycler 480 have slightly different filters installed: please check the user manual supplied with your instrument. All reporter dye combinations shown have been successfully tested by QIAGEN on the first-generation LightCycler 480 only.

† Use channel 2, channel 3, and channels 1, 4 and 5 to detect the least abundant target, the second least abundant target, and the most abundant targets, respectively.

‡ JOE or VIC dye (not tested by QIAGEN) can be used instead of HEX dye.

Controls

No template control (NTC)

All quantification experiments should include an NTC, containing all the components of the reaction except for the template. This enables detection of contamination.

No RT control

All RT-PCR experiments should include a negative control to test for contaminating DNA. However, detection of this contamination can be eliminated by using primers or probes that avoid amplification and detection of genomic DNA sequences. If it is not possible to use such primers or probes, DNA contamination can be detected by performing a control reaction in which no reverse transcription is possible. The control "no RT reaction" contains all components including template RNA, except for the reverse transcriptase. Reverse transcription therefore cannot take place. When an aliquot of this control is used as a template in PCR, the only template available would be contaminating DNA.

Alternatively, DNA in the sample can be removed by digestion with DNase before RT-PCR amplification.

Positive control

In some cases it may be necessary to include a positive control, containing a known concentration or copy number of template. Positive controls can be absolute standards or known positive samples.

Absolute standards include commercially available standards and in-lab standards, such as a plasmid containing cloned sequences. Absolute standards are used at a known copy number and provide quantitative information.

A positive sample is usually a substitute for an absolute standard and is used only to test for presence or absence of the target.

Protocol 1: Duplex, Real-Time PCR Using TaqMan Probes on Most Applied Biosystems Cyclers

This protocol is for use with the **QuantiFast Multiplex PCR Kit** and TaqMan probes on any real-time cycler from Applied Biosystems **except Applied Biosystems 7500 Real-Time PCR Systems**. For further information, see "Passive reference dye", pages 9–10.

Important points before starting

- Always start with the **cycling conditions** and **primer concentrations** specified in this protocol.
- We strongly recommend testing the performance of primer–probe sets in individual assays before combining them in a multiplex assay.
- Read "Guidelines for effective multiplex assays", page 15. Check whether your real-time cycler is compatible with the chosen combination of reporter dyes.
- If using an already established duplex real-time PCR assay, use the previously established primer and probe concentrations in combination with the cycling conditions specified in this protocol. It is not necessary to determine primer limiting concentrations again.
- **Optimal analysis settings are a prerequisite for accurate quantification data.** For data analysis, you should always readjust the analysis settings (i.e., baseline settings and threshold values) for analysis of every reporter dye channel in every run.

Things to do before starting

- For ease of use, we recommend preparing for each of your targets a 20x primer–probe mix containing target-specific primers and probe. A 20x primer–probe mix for duplex PCR consists of 10 µM forward primer, 10 µM reverse primer, and 4 µM probe in TE buffer. Alternatively, it may be preferable to prepare the reaction mix with separate primer and probe solutions. If you commonly set up reactions this way, see Appendix B (page 53).

Procedure

1. **Thaw 2x QuantiFast Multiplex PCR Master Mix, primer and probe solutions, RNase-free water, and template DNA or cDNA. Mix the individual solutions, and place on ice.**
2. **Prepare a reaction mix according to Table 14 (page 32).**

   Note: We strongly recommend starting with the optimized Mg^{2+} concentration provided by 2x QuantiFast Multiplex PCR Master Mix.

Note: Due to the hot start, it is not necessary to keep samples on ice during reaction setup nor while programming the real-time cycler.

Table 14. Reaction setup

Component	Volume*	Final concentration
2x QuantiFast Multiplex PCR Master Mix	12.5 µl	1x
20x primer–probe mix 1†	1.25 µl	0.5 µM forward primer 1‡ 0.5 µM reverse primer 1‡ 0.2 µM probe 1§
20x primer–probe mix 2†	1.25 µl	0.5 µM forward primer 2‡ 0.5 µM reverse primer 2‡ 0.2 µM probe 2§
RNase-free water	Variable	–
Template DNA or cDNA (added at step 4)	Variable	≤100 ng/reaction
Total reaction volume	**25 µl***	**–**

* If your real-time cycler requires a final reaction volume other than 25 µl, adjust the amount of master mix and all other reaction components accordingly. If using 384-well plates on the Applied Biosystems 7900HT, use a reaction volume of 10 µl.

† A 20x primer–probe mix for duplex PCR consists of 10 µM forward primer, 10 µM reverse primer, and 4 µM probe in TE buffer.

‡ A final primer concentration of 0.5 µM is optimal. Before adapting primer concentration, verify the concentration of your primer solutions.

§ A final probe concentration of 0.2 µM gives satisfactory results in most cases. Depending on the synthesis quality and purification method used, the optimal concentration may be between 0.1 µM and 0.4 µM.

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

   Note: For two-step RT-PCR, the volume of cDNA (from the undiluted RT reaction) added as template should not exceed 10% of the final PCR volume.
5. **Program the real-time cycler according to Table 15 (page 33).**

   Note: Check the real-time cycler's user manual for correct instrument setup for multiplex analysis (e.g., setting up detection of multiple dyes from the same well). Be sure to activate the detector for each reporter dye used. Depending on your instrument, it may also be necessary to perform a calibration procedure for each of the reporter dyes before they are used for the first time.

Table 15. Cycling conditions

Step	Time	Temperature	Additional comments
Initial PCR activation step	**5 min**	**95°C**	HotStarTaq *Plus* DNA Polymerase is activated by this heating step
2-step cycling			**Important: Optimal performance is only assured using these cycling conditions**
Denaturation	**30 s**	**95°C**	
Annealing/extension	**30 s**	**60°C**	Combined annealing/ extension step with fluorescence data collection
Number of cycles	40–45		The number of cycles depends on the amount of template DNA or cDNA and the expression level of the target gene

6. **Place the PCR tubes or plate in the real-time cycler, and start the cycling program.**
7. **Perform data analysis.**

   Before performing data analysis, select the analysis settings for each probe (i.e., baseline settings and threshold values). Optimal analysis settings are a prerequisite for accurate quantification data.

Protocol 2: Triplex and 4-plex, Real-Time PCR Using TaqMan Probes on Most Applied Biosystems Cyclers

This protocol is for use with the **QuantiFast Multiplex PCR Kit** and TaqMan probes on any real-time cycler from Applied Biosystems **except Applied Biosystems 7500 Real-Time PCR Systems**. For further information, see "Passive reference dye", pages 9–10.

Important points before starting

- Always start with the **cycling conditions** and **primer concentrations** specified in this protocol. Please note that the cycling conditions differ from those described in the protocol for duplex assays (page 31).
- We strongly recommend testing the performance of primer–probe sets in individual assays before combining them in a multiplex assay.
- Read "Guidelines for effective multiplex assays", page 15. Check whether your real-time cycler is compatible with the chosen combination of reporter dyes.
- If using an already established multiplex real-time PCR assay, use the previously established primer and probe concentrations in combination with the cycling conditions specified in this protocol. It is not necessary to determine primer limiting concentrations again.
- **Optimal analysis settings are a prerequisite for accurate quantification data.** For data analysis, you should always readjust the analysis settings (i.e., baseline settings and threshold values) for analysis of every reporter dye channel in every run.

Things to do before starting

- For ease of use, we recommend preparing for each of your targets a 20x primer–probe mix containing target-specific primers and probe. A 20x primer–probe mix for triplex and 4-plex PCR consists of 10 µM forward primer, 10 µM reverse primer, and 4 µM probe in TE buffer. Alternatively, it may be preferable to prepare the reaction mix with separate primer and probe solutions. If you commonly set up reactions this way, see Appendix B (page 53).

Procedure

1. **Thaw 2x QuantiFast Multiplex PCR Master Mix, primer and probe solutions, RNase-free water, and template DNA or cDNA. Mix the individual solutions, and place on ice.**
2. **Prepare a reaction mix according to Table 16 (page 36).**

   Note: We strongly recommend starting with the optimized Mg^{2+} concentration provided by 2x QuantiFast Multiplex PCR Master Mix.

   Note: Due to the hot start, it is not necessary to keep samples on ice during reaction setup nor while programming the real-time cycler.
3. **Mix the reaction mix thoroughly, and dispense appropriate volumes into PCR tubes or the wells of a PCR plate.**
4. **Add template DNA or cDNA (≤100 ng) to the individual PCR tubes or wells.**

   Note: For two-step RT-PCR, the volume of cDNA (from the undiluted RT reaction) added as template should not exceed 10% of the final PCR volume.
5. **Program the real-time cycler according to Table 17 (page 37).**

   Note: Check the real-time cycler's user manual for correct instrument setup for multiplex analysis (e.g., setting up detection of multiple dyes from the same well). Be sure to activate the detector for each reporter dye used. Depending on your instrument, it may also be necessary to perform a calibration procedure for each of the reporter dyes before they are used for the first time.

Table 16. Reaction setup

Component	Volume*	Final concentration
2x QuantiFast Multiplex PCR Master Mix	12.5 µl	1x
20x primer–probe mix 1†	1.25 µl	0.5 µM forward primer 1‡ 0.5 µM reverse primer 1‡ 0.2 µM probe 1§
20x primer–probe mix 2†	1.25 µl	0.5 µM forward primer 2‡ 0.5 µM reverse primer 2‡ 0.2 µM probe 2§
20x primer–probe mix 3†	1.25 µl	0.5 µM forward primer 3‡ 0.5 µM reverse primer 3‡ 0.2 µM probe 3§
Only for 4-plex PCR: 20x primer–probe mix 4†	1.25 µl	0.5 µM forward primer 4‡ 0.5 µM reverse primer 4‡ 0.2 µM probe 4§
RNase-free water	Variable	–
Template DNA or cDNA (added at step 4)	Variable	≤100 ng/reaction
Total reaction volume	**25 µl***	**–**

* If your real-time cycler requires a final reaction volume other than 25 µl, adjust the amount of master mix and all other reaction components accordingly. If using 384-well plates on the Applied Biosystems 7900HT, use a reaction volume of 10 µl.

† A 20x primer–probe mix for triplex and 4-plex PCR consists of 10 µM forward primer, 10 µM reverse primer, and 4 µM probe in TE buffer.

‡ A final primer concentration of 0.5 µM is optimal. Before adapting primer concentration, check the concentration of your primer solutions.

§ A final probe concentration of 0.2 µM gives satisfactory results in most cases. Depending on the synthesis quality and purification method used, the optimal concentration may be between 0.1 µM and 0.4 µM.

Table 17. Cycling conditions

Step	Time	Temperature	Additional comments
Initial PCR activation step	**5 min**	**95°C**	HotStarTaq *Plus* DNA Polymerase is activated by this heating step
2-step cycling			**Important: Optimal performance is only assured using these cycling conditions***
Denaturation	**45 s**	**95°C**	
Annealing/extension	**45 s**	**60°C**	Combined annealing/ extension step with fluorescence data collection
Number of cycles	40–45		The number of cycles depends on the amount of template DNA or cDNA and the expression level of the target gene

* Depending on the assay, the times for the denaturation and annealing/extension steps may be reduced to 30 seconds each. For initial experiments, we always recommend to start with the cycling conditions specified in this table.

6. **Place the PCR tubes or plate in the real-time cycler, and start the cycling program.**
7. **Perform data analysis.**

   Before performing data analysis, select the analysis settings for each probe (i.e., baseline settings and threshold values). Optimal analysis settings are a prerequisite for accurate quantification data.

Protocol 3: Duplex, Real-Time PCR Using TaqMan Probes on the Applied Biosystems 7500 and Other Cyclers

This protocol is for use with the **QuantiFast Multiplex PCR +R Kit** and TaqMan probes on Applied Biosystems 7500 Real-Time PCR Systems and on real-time cyclers from Bio-Rad/MJ Research, Cepheid, Eppendorf, Roche, and Agilent. For further information, see "Passive reference dye", pages 9–10.

Important points before starting

- Always start with the **cycling conditions** and **primer concentrations** specified in this protocol.
- We strongly recommend testing the performance of primer–probe sets in individual assays before combining them in a multiplex assay.
- Read "Guidelines for effective multiplex assays", page 15. Check whether your real-time cycler is compatible with the chosen combination of reporter dyes.
- If using an already established duplex real-time PCR assay, use the previously established primer and probe concentrations in combination with the cycling conditions specified in this protocol. It is not necessary to determine primer limiting concentrations again.
- **Optimal analysis settings are a prerequisite for accurate quantification data.** For data analysis, you should always readjust the analysis settings (i.e., baseline settings and threshold values) for analysis of every reporter dye channel in every run.
- If using the **LightCycler 2.0** or **LightCycler 480**, be sure to create a color compensation file. For details, see Appendix C (page 54). If using reporter dyes with widely separated emission spectra on the LightCycler 480, a color compensation file may not be necessary.

Things to do before starting

- For ease of use, we recommend preparing for each of your targets a 20x primer–probe mix containing target-specific primers and probe. A 20x primer–probe mix for duplex PCR consists of 10 µM forward primer, 10 µM reverse primer, and 4 µM probe in TE buffer. Alternatively, it may be preferable to prepare the reaction mix with separate primer and probe solutions. If you commonly set up reactions this way, see Appendix B (page 53).

Procedure

1. **Thaw 2x QuantiFast Multiplex PCR Master Mix (w/o ROX), 50x ROX Dye Solution, primer and probe solutions, RNase-free water, and template DNA or cDNA. Mix the individual solutions, and place on ice.**
2. **Prepare a reaction mix according to Table 18 (page 40).**

   Note: We strongly recommend starting with the optimized Mg^{2+} concentration provided by 2x QuantiFast Multiplex PCR Master Mix (w/o ROX).

   Note: Due to the hot start, it is not necessary to keep samples on ice during reaction setup nor while programming the real-time cycler.
3. **Mix the reaction mix thoroughly, and dispense appropriate volumes into PCR tubes, PCR capillaries, or the wells of a PCR plate.**
4. **Add template DNA or cDNA (≤100 ng) to the individual PCR tubes, capillaries, or wells.**

   Note: For two-step RT-PCR, the volume of cDNA (from the undiluted RT reaction) added as template should not exceed 10% of the final PCR volume.
5. **Program the real-time cycler according to Table 19 (page 41).**

   Note: Check the real-time cycler's user manual for correct instrument setup for multiplex analysis (e.g., setting up detection of multiple dyes from the same well). Be sure to activate the detector for each reporter dye used. Depending on your instrument, it may also be necessary to perform a calibration procedure for each of the reporter dyes before they are used for the first time.

Table 18. Reaction setup

Component	Volume/reaction*		Final concentration
	96-well block	Fast or capillary†	
2x QuantiFast Multiplex PCR Master Mix (w/o ROX)	12.5 µl	10 µl	1x
50x ROX Dye Solution‡	0.5 µl	0.4 µl	1x
20x primer–probe mix 1§	1.25 µl	1 µl	0.5 µM forward primer 1¶ 0.5 µM reverse primer 1¶ 0.2 µM probe 1**
20x primer–probe mix 2§	1.25 µl	1 µl	0.5 µM forward primer 2¶ 0.5 µM reverse primer 2¶ 0.2 µM probe 2**
RNase-free water	Variable	Variable	–
Template DNA or cDNA (added at step 4)	Variable	Variable	≤100 ng/reaction
Total reaction volume	**25 µl***	**20 µl***	**–**

* If your real-time cycler requires a final reaction volume other than 25 µl or 20 µl, adjust the amount of master mix and all other reaction components accordingly. If using 384-well plates on the LightCycler 480, use a reaction volume of 10 µl.

† Refers to the Applied Biosystems 7500 Fast Real-Time PCR System and capillary cyclers, such as the LightCycler 2.0.

‡ For cyclers which do not require ROX dye, add RNase-free water instead.

§ A 20x primer–probe mix for duplex PCR consists of 10 µM forward primer, 10 µM reverse primer, and 4 µM probe in TE buffer.

¶ A final primer concentration of 0.5 µM is optimal. Before adapting primer concentration, check the concentration of your primer solutions.

** A final probe concentration of 0.2 µM gives satisfactory results in most cases. Depending on the synthesis quality and purification method used, the optimal concentration may be between 0.1 µM and 0.4 µM.

Table 19. Cycling conditions

Step	Time	Temperature	Additional comments
Initial PCR activation step	**5 min**	**95°C**	HotStarTaq *Plus* DNA Polymerase is activated by this heating step
2-step cycling			**Important: Optimal performance is only assured using these cycling conditions**
Denaturation	**30 s**	**95°C**	
Annealing/extension	**30 s**	**60°C**	Combined annealing/ extension step with fluorescence data collection
Number of cycles	40–45		The number of cycles depends on the amount of template DNA or cDNA and the expression level of the target gene

6. **Place the PCR tubes, capillaries, or plate in the real-time cycler, and start the cycling program.**
7. **Perform data analysis.**

   Before performing data analysis, select the analysis settings for each probe (i.e., baseline settings and threshold values). Optimal analysis settings are a prerequisite for accurate quantification data.

Protocol 4: Triplex and 4-plex, Real-Time PCR Using TaqMan Probes on the Applied Biosystems 7500 and Other Cyclers

This protocol is for use with the **QuantiFast Multiplex PCR +R Kit** and TaqMan probes on Applied Biosystems 7500 Real-Time PCR Systems and on real-time cyclers from Bio-Rad/MJ Research, Cepheid, Eppendorf, Roche, and Agilent. For further information, see "Passive reference dye", pages 9–10.

Important points before starting

- Always start with the **cycling conditions** and **primer concentrations** specified in this protocol. Please note that the cycling conditions differ from those described in the protocol for duplex assays (page 38).
- We strongly recommend testing the performance of primer–probe sets in individual assays before combining them in a multiplex assay.
- Read "Guidelines for effective multiplex assays", page 15. Check whether your real-time cycler is compatible with the chosen combination of reporter dyes.
- If using an already established multiplex real-time PCR assay, use the previously established primer and probe concentrations in combination with the cycling conditions specified in this protocol. It is not necessary to determine primer limiting concentrations again.
- **Optimal analysis settings are a prerequisite for accurate quantification data**. For data analysis, you should always readjust the analysis settings (i.e., baseline settings and threshold values) for analysis of every reporter dye channel in every run.
- If using the **LightCycler 2.0** or **LightCycler 480**, be sure to create a color compensation file. For details, see Appendix C (page 54). If using reporter dyes with widely separated emission spectra on the LightCycler 480, a color compensation file may not be necessary.

Things to do before starting

- For ease of use, we recommend preparing for each of your targets a 20x primer–probe mix containing target-specific primers and probe. A 20x primer–probe mix for triplex and 4-plex PCR consists of 10 µM forward primer, 10 µM reverse primer, and 4 µM probe in TE buffer. Alternatively, it may be preferable to prepare the reaction mix with separate primer and probe solutions. If you commonly set up reactions this way, see Appendix B (page 53).

Procedure

1. **Thaw 2x QuantiFast Multiplex PCR Master Mix (w/o ROX), 50x ROX Dye Solution, primer and probe solutions, RNase-free water, and template DNA or cDNA. Mix the individual solutions, and place on ice.**
2. **Prepare a reaction mix according to Table 20 (page 44).**

   Note: We strongly recommend starting with the optimized Mg^{2+} concentration provided by 2x QuantiFast Multiplex PCR Master Mix (w/o ROX).

   Note: Due to the hot start, it is not necessary to keep samples on ice during reaction setup nor while programming the real-time cycler.
3. **Mix the reaction mix thoroughly, and dispense appropriate volumes into PCR tubes, PCR capillaries, or the wells of a PCR plate.**
4. **Add template DNA or cDNA (≤100 ng) to the individual PCR tubes, capillaries, or wells.**

   Note: For two-step RT-PCR, the volume of cDNA (from the undiluted RT reaction) added as template should not exceed 10% of the final PCR volume.
5. **Program the real-time cycler according to Table 21 (page 45).**

   Note: Check the real-time cycler's user manual for correct instrument setup for multiplex analysis (e.g., setting up detection of multiple dyes from the same well). Be sure to activate the detector for each reporter dye used. Depending on your instrument, it may also be necessary to perform a calibration procedure for each of the reporter dyes before they are used for the first time.

Table 20. Reaction setup

Component	Volume/reaction* 96-well block	Volume/reaction* Fast or capillary†	Final concentration
2x QuantiFast Multiplex PCR Master Mix (w/o ROX)	12.5 µl	10 µl	1x
50x ROX Dye Solution‡	0.5 µl	0.4 µl	1x
20x primer–probe mix 1§	1.25 µl	1 µl	0.5 µM forward primer 1¶ 0.5 µM reverse primer 1¶ 0.2 µM probe 1**
20x primer–probe mix 2§	1.25 µl	1 µl	0.5 µM forward primer 2¶ 0.5 µM reverse primer 2¶ 0.2 µM probe 2**
20x primer–probe mix 3§	1.25 µl	1 µl	0.5 µM forward primer 3¶ 0.5 µM reverse primer 3¶ 0.2 µM probe 3**
Only for 4-plex PCR: 20x primer–probe mix 4§	1.25 µl	1 µl	0.5 µM forward primer 4¶ 0.5 µM reverse primer 4¶ 0.2 µM probe 4**
RNase-free water	Variable	Variable	–
Template DNA or cDNA (added at step 4)	Variable	Variable	≤100 ng/reaction
Total reaction volume	**25 µl***	**20 µl***	**–**

* If your real-time cycler requires a final reaction volume other than 25 µl or 20 µl, adjust the amount of master mix and all other reaction components accordingly. If using 384-well plates on the LightCycler 480, use a reaction volume of 10 µl.

† Refers to the Applied Biosystems 7500 Fast Real-Time PCR System and capillary cyclers, such as the LightCycler 2.0.

‡ For cyclers which do not require ROX dye, add RNase-free water instead.

§ A 20x primer–probe mix for triplex and 4-plex PCR consists of 10 µM forward primer, 10 µM reverse primer, and 4 µM probe in TE buffer.

¶ A final primer concentration of 0.5 µM is optimal. Before adapting primer concentration, check the concentration of your primer solutions.

**A final probe concentration of 0.2 µM gives satisfactory results in most cases. Depending on the synthesis quality and purification method used, the optimal concentration may be between 0.1 µM and 0.4 µM.

Table 21. Cycling conditions

Step	Time	Temperature	Additional comments
Initial PCR activation step	**5 min**	**95°C**	HotStarTaq *Plus* DNA Polymerase is activated by this heating step
2-step cycling			**Important: Optimal performance is only assured using these cycling conditions**
Denaturation	**45 s**	**95°C**	
Annealing/extension	**45 s**	**60°C**	Combined annealing/ extension step with fluorescence data collection
Number of cycles	40–45		The number of cycles depends on the amount of template DNA or cDNA and the expression level of the target gene

6. **Place the PCR tubes, capillaries, or plate in the real-time cycler, and start the cycling program.**
7. **Perform data analysis.**

   Before performing data analysis, select the analysis settings for each probe (i.e., baseline settings and threshold values). Optimal analysis settings are a prerequisite for accurate quantification data.

Troubleshooting Guide

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

		Comments and suggestions
No signal, or one or more signals detected late in PCR		
a)	Wrong cycling conditions	Always start with the optimized cycling conditions specified in the protocols. Be sure that the cycling conditions include the initial step for activation of HotStarTaq *Plus* DNA Polymerase (95°C for 5 min), and the specified times for denaturation and annealing/extension.
b)	HotStarTaq *Plus* DNA Polymerase not activated	Ensure that the cycling program includes the HotStarTaq *Plus* DNA Polymerase activation step (5 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 A, page 50, for details on evaluating the concentration of primers and probes. Repeat the assay.
d)	Wrong or no detection step	Ensure that fluorescence detection takes place during the during the combined annealing/extension step when using TaqMan probes.

		Comments and suggestions
e)	Primer or probe concentration not optimal	In most cases, a primer concentration of 0.5 µM gives satisfactory results on all real-time cyclers. Depending on the real-time PCR assay, results may be improved by adjusting primer concentration within the range of 0.3–0.6 µM. In most cases, a probe concentration of 0.2 µM gives satisfactory results. Depending on the quality of your probe, results may be improved by adjusting probe concentration within the range of 0.1–0.4 µM. Check the concentrations of primers and probes by spectrophotometry (see Appendix A, page 50).
f)	Mg^{2+} concentration not optimal	The Mg^{2+} concentration in 2x QuantiFast Multiplex PCR Master Mixes is already optimized. Increasing the final Mg^{2+} concentration by 0.5–1 mM may improve results.
g)	Problems with starting template	Check the concentration, storage conditions, and quality of the starting nucleic acids. If necessary, make new serial dilutions of template nucleic acid from the stock solutions. Repeat the assay using the new dilutions. Ensure that all reagents, buffers, and solutions used for purification and dilution of template nucleic acids are free of nucleases.
h)	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.
i)	Insufficient number of cycles	Increase the number of cycles.
j)	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 A, page 50).

		Comments and suggestions
k)	Wrong detection channel/ filter chosen	Ensure that the correct detection channel is activated or the correct filter set is chosen for each reporter dye. Check whether the chosen combination of reporter dyes is compatible with the selected detection channels or filter sets.
l)	Fluorescence crosstalk	Check that the reporter dyes used in your assay are suitable for multiplex analysis on your instrument. Run appropriate controls to estimate potential crosstalk effects.
Differences in C_T values or in PCR efficiencies between a multiplex assay and the corresponding singleplex assays		
a)	Wrong cycling conditions	Always start with the optimized cycling conditions specified in the protocols. Be sure that the cycling conditions include the initial step for activation of HotStarTaq *Plus* DNA Polymerase (95°C for 5 min), and the specified times for denaturation and annealing/extension.
b)	Analysis settings (e.g., threshold and baseline settings) not optimal	Check the analysis settings (threshold and baseline settings) for each reporter dye. Repeat analysis using optimal settings for each reporter dye.
c)	Imprecise spectral separation of reporter dyes	Since multiplex assays use multiple probes, each with a fluorescent dye, the increased fluorescent background may affect the shape of the amplification plots obtained with some real-time cyclers. This may lead to differences in C_T values of up to 5% between the multiplex assay and the corresponding singleplex assays; this can usually be avoided by using optimal threshold settings. If using the ABI PRISM 7700, perform analysis with and without spectral compensation.
No linearity in ratio of C_T value/crossing point to log of the template amount		
a)	Template amount too high	When signals are coming up at very early C_T values, adjust the analysis settings accordingly.

		Comments and suggestions
b)	Template amount too low	Increase template amount if possible. Note that detection of very low starting copy numbers may not be in the linear range of a standard curve.
Increased fluorescence or C_T value for "No Template" control		
a)	Contamination of reagents	Discard all the components of the multiplex assay (e.g., master mix, primers, and probes). Repeat the multiplex assay using new components.
b)	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.
c)	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.
d)	**ABI PRISM 7000**: Uneven curves or high standard deviations	Do not use reaction volumes smaller than 25 µl, and always use optical adhesive covers to seal plates. In some cases, increasing the reaction volume to 50 µl may improve results.

Appendix A: Assay Design and Handling Primers and Probes

Important factors for success in quantitative, multiplex, real-time 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, since 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 PCR products is 60–150 bp. Some longer amplicons may amplify efficiently in multiplex PCR, with minimal optimization.

Handling and storing primers and probes

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

Storage buffer

Lyophilized primers and probes 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 buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0) for standard primers and probes labeled with most fluorescent dyes.

However, probes labeled with fluorescent dyes such as Cy3, Cy3.5, Cy5, and Cy5.5 should be stored in TE buffer, pH 7.0, since 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, since they may lead to degradation.

For easy and reproducible handling of primer–probe sets used in multiplex 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, mix, and leave for 20 minutes to allow the primer or probe to completely dissolve. Mix again and determine the concentration by spectrophotometry as described below.

We do not recommend dissolving primers and probes in water. They are less stable in water than in TE buffer and some may not dissolve easily in water.

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:

$A_{260} = \varepsilon_{260}$ 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 and probe 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 or your local distributor 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.

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

Appendix B: Customized Pipetting Scheme for Separate Primer and Probe Solutions

For ease of use, we recommend preparing for each of your targets a 20x primer–probe mix containing target-specific primers and probe. However, in some cases, it may be preferable to prepare the reaction mix with separate primer and probe solutions. If you commonly set up reactions this way, it may be helpful to copy and fill in Table 22 with the calculated volumes of each primer to use.

Table 22. Preparing reaction mix for multiplex PCR using separate primer and probe solutions

	Volume per reaction		
Component*	**25 µl**	**20 µl**	**Other: _____ µl**
2x QuantiFast Multiplex PCR Master Mix	12.5 µl	10 µl	_____ µl
50x ROX Dye Solution	☐ 0 µl ☐ 0.5 µl	☐ 0 µl ☐ 0.4 µl	_____ µl
Forward primer 1 (0.5 µM)	_____ µl	_____ µl	_____ µl
Reverse primer 1 (0.5 µM)	_____ µl	_____ µl	_____ µl
Probe 1 (0.2 µM)	_____ µl	_____ µl	_____ µl
Forward primer 2 (0.5 µM)	_____ µl	_____ µl	_____ µl
Reverse primer 2 (0.5 µM)	_____ µl	_____ µl	_____ µl
Probe 2 (0.2 µM)	_____ µl	_____ µl	_____ µl
Forward primer 3 (0.5 µM)†	_____ µl	_____ µl	_____ µl
Reverse primer 3 (0.5 µM)†	_____ µl	_____ µl	_____ µl
Probe 3 (0.2 µM)†	_____ µl	_____ µl	_____ µl
Forward primer 4 (0.5 µM)‡	_____ µl	_____ µl	_____ µl
Reverse primer 4 (0.5 µM)‡	_____ µl	_____ µl	_____ µl
Probe 4 (0.2 µM)‡	_____ µl	_____ µl	_____ µl
RNase-free water	_____ µl	_____ µl	_____ µl
Template DNA or cDNA (added at step 4)	_____ µl	_____ µl	_____ µl
Total reaction volume	**25 µl**	**20 µl**	**_____ µl**

* The concentrations of primers and probe shown in this column represent their final concentrations in the reaction, not the concentrations of the stock solutions.

† For triplex and 4-plex PCR only.

‡ For 4-plex PCR only.

Appendix C: Generating Color Compensation Files on LightCycler Systems

The LightCycler 2.0 system has detection channels that allow detection of multiple reporter dyes in the same capillary. However, even when reporter dyes with well separated emission spectra are used, each reporter dye will be detected by more than one detection channel. Therefore, multiplex assay results will be inaccurate unless a correction is made. This is achieved by using a color compensation file, which contains information that corrects the crosstalk between the detection channels.

The LightCycler 480 system can also use a color compensation file to correct the crosstalk between detection channels. However, if the multiplex assay uses reporter dyes with widely separated emission spectra (e.g., FAM and Cy5), it may not be necessary to use a color compensation file.

Color compensation files can be generated before or after carrying out a multiplex assay and be stored for later use. Each color compensation file is specific for a set of fluorescently labeled probes used in a multiplex assay. It may be necessary to generate a new color compensation file if a new lot of fluorescently labeled probes is used.

When working with color compensation files, the following steps are required:

- Preparing samples, each containing QuantiFast Multiplex PCR Master Mix (w/o ROX) and one of the reporter dyes to be used in a multiplex assay.
- Performing a color compensation experiment. Fluorescence data are collected and used to generate a color compensation file containing information for correcting crosstalk between detection channels.
- Applying the color compensation file during a multiplex assay or afterwards when performing data analysis.

Supplementary protocols, which describe how to generate color compensation files for duplex, triplex, and 4-plex assays using TaqMan probes is available at www.qiagen.com/literature. In the "Search" field, enter either *PCR81* (protocol for LightCycler 2.0) or *PCR82* (protocol for LightCycler 480), and then click "Search" to retrieve the protocol for your cycler.

Ordering Information

Product	Contents	Cat. no.
QuantiFast Multiplex PCR Kit (80)	For 80 x 25 µl reactions: 1 ml 2x Master Mix (contains ROX dye), 2 ml RNase-Free Water	204652
QuantiFast Multiplex PCR Kit (400)	For 400 x 25 µl reactions: 3 x 1.7 ml 2x Master Mix (contains ROX dye), 2 x 2 ml RNase-Free Water	204654
QuantiFast Multiplex PCR Kit (2000)	For 2000 x 25 µl reactions: 25 ml 2x Master Mix (contains ROX dye), 20 ml RNase-Free Water	204656
QuantiFast Multiplex PCR +R Kit (80)	For 80 x 25 µl reactions: 1 ml 2x Master Mix (without ROX dye), 45 µl ROX Dye Solution, 2 ml RNase-Free Water	204752
QuantiFast Multiplex PCR +R Kit (400)	For 400 x 25 µl reactions: 3 x 1.7 ml 2x Master Mix (without ROX dye), 210 µl ROX Dye Solution, 2 x 2 ml RNase-Free Water	204754
QuantiFast Multiplex PCR +R Kit (2000)	For 2000 x 25 µl reactions: 25 ml 2x Master Mix (without ROX dye), 1.05 ml ROX Dye Solution, 20 ml RNase-Free Water	204756
Accessories		
QuantiTect Reverse Transcription Kit — for fast cDNA synthesis for sensitive real-time two-step RT-PCR		
QuantiTect Reverse Transcription Kit (10)	For 10 x 20 µl reactions: gDNA Wipeout Buffer, Quantiscript® Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix, and RNase-Free Water	205310
QuantiTect Reverse Transcription Kit (50)*	For 50 x 20 µl reactions: gDNA Wipeout Buffer, Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix, and RNase-Free Water	205311

* Larger kit size available; please inquire.

Ordering Information

Product	Contents	Cat. no.
QuantiTect Whole Transcriptome Kit — for unlimited real-time PCR analysis from precious RNA samples		
QuantiTect Whole Transcriptome Kit (25)*	For 25 x 50 µl reactions: T-Script® Enzyme and Buffer; Ligation Enzymes, Reagent, and Buffer; and REPLI-g® DNA Polymerase and Buffer	207043
FastLane Cell cDNA Kit — for high-speed preparation of cDNA without RNA purification for use in real-time RT-PCR		
FastLane Cell cDNA Kit (50)	Buffer FCW, Buffer FCP, and components for 50 x 20 µl reverse-transcription reactions	215011
Related products†		
Rotor-Gene Multiplex PCR Kit — for ultrafast multiplex real-time PCR and two-step RT PCR on Rotor-Gene cyclers		
Rotor-Gene Multiplex PCR Kit (80)	For 80 x 25 µl reactions: 1 ml 2x Master Mix, 2 ml RNase-Free Water	204772
Rotor-Gene Multiplex PCR Kit (400)	For 400 x 25 µl reactions: 3 x 1.7 ml 2x Master Mix, 2 x 2 ml RNase-Free Water	204774
DNeasy® Blood & Tissue Kit — for purification of total DNA from animal blood and tissues, and from cells, yeast, bacteria, or viruses		
DNeasy Blood & Tissue Kit (50)	For 50 minipreps: 50 DNeasy Mini Spin Columns, Proteinase K, Buffers, Collection Tubes	69504
RNeasy® Plus Mini Kit — for purification of total RNA from cultured cells and tissues using gDNA Eliminator columns		
RNeasy Plus Mini Kit (50)	For 50 minipreps: RNeasy Mini Spin Columns, gDNA Eliminator Spin Columns, Collection Tubes, RNase-Free Water and Buffers	74134

* Larger kit size available; please inquire.

† To find the right QIAGEN nucleic acid purification kit for your experiments, visit www.qiagen.com/ProductFinder.

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To find out more about the complete range of QuantiFast Kits, visit www.qiagen.com/fastPCR. Kits are available for fast-cycling in PCR, two-step RT-PCR, or one-step RT-PCR with SYBR® Green or probe detection.

Notes

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