May 2011

QuantiTect® Multiplex PCR Handbook

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

QuantiTect Multiplex PCR NoROX Kit — with master mix free of ROX passive reference dye

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

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Contents

Kit Contents

QuantiTect Multiplex PCR Kit	(40)	(200)	(1000)
Catalog no.	204541	204543	204545
Number of 50 µl reactions	40	200	1000
2x QuantiTect 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® DNA Polymerase, QuantiTect Multiplex PCR Buffer, dNTP mix (dATP, dCTP, dGTP, and dTTP/dUTP), and ROX passive reference dye.

QuantiTect Multiplex PCR NoROX Kit	(40)	(200)	(1000)
Catalog no.	204741	204743	204745
Number of 50 µl reactions	40	200	1000
2x QuantiTect Multiplex PCR NoROX 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 DNA Polymerase, QuantiTect Multiplex PCR Buffer, and dNTP mix (dATP, dCTP, dGTP, and dTTP/dUTP).

Shipping and Storage

QuantiTect 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 QuantiTect Multiplex PCR Master Mixes can also be stored protected from light at 2–8°C for up to 6 months, depending on the expiration date, without showing any reduction in performance.

To maintain optimal performance of QuantiTect Multiplex PCR Kits for 1000 x 50 µl reactions, we recommend storing the 25 ml master mix as appropriately sized aliquots in sterile, polypropylene tubes.

Product Use Limitations

QuantiTect 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).

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

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 QuantiTect 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).

Product Description

Component	Description
HotStarTaq DNA Polymerase*†	HotStarTaq DNA Polymerase is a modified form of a recombinant 94 kDa DNA polymerase, originally isolated from *Thermus aquaticus*, cloned into *E. coli*. (Deoxynucleoside-triphosphate: DNA deoxynucleotidyltransferase, EC 2.7.7.7).
QuantiTect Multiplex PCR Buffer*†	Optimized buffer for quantitative, multiplex, real-time PCR; contains 11 mM $MgCl_2$
dNTP mix*†	Contains dATP, dCTP, dGTP, and dTTP/dUTP of ultrapure quality
Fluorescent dye*	ROX
RNase-free water	Ultrapure quality, PCR-grade

* Included in 2x QuantiTect Multiplex PCR Master Mix.

† Included in 2x QuantiTect Multiplex PCR NoROX Master Mix.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each component of QuantiTect Multiplex PCR Kits is tested against predetermined specifications to ensure consistent product quality. See the quality-control label inside the kit box or on the kit envelope for lot-specific values.

Introduction

QuantiTect Multiplex PCR Kits provide accurate real-time PCR quantification of DNA and cDNA targets in a multiplex format. Depending on the real-time cycler used, up to 5 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 65). The kits are compatible with all types of probe chemistries, providing flexibility in the choice of probes for multiplex PCR assays. High specificity and sensitivity in multiplex PCR are achieved without any time-consuming optimization steps through the use of the hot-start enzyme HotStarTaq DNA Polymerase together with a specialized PCR buffer. Gene expression analysis and other applications involving quantitative, multiplex, real-time PCR can be performed using the kits.

The kits are available in 2 formats:

- **QuantiTect 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 ROX dye for fluorescence normalization (e.g., instruments from Applied Biosystems®).
- **QuantiTect Multiplex PCR NoROX Kit**: This kit is supplied with a master mix that is free of ROX dye. The kit is intended for use with cyclers that can function without ROX dye (e.g., instruments from Bio-Rad/MJ Research, Cepheid, QIAGEN, Eppendorf, Roche, and Agilent). Running reactions without ROX dye increases multiplexing capacity and allows greater flexibility when choosing reporter dyes for probes.

2x QuantiTect Multiplex PCR Master Mixes

The components of 2x QuantiTect Multiplex PCR Master Mix include HotStarTaq DNA Polymerase, QuantiTect Multiplex PCR Buffer, and ROX passive reference dye (see descriptions below). 2x QuantiTect Multiplex PCR NoROX Master Mix contains HotStarTaq DNA Polymerase and QuantiTect 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 DNA Polymerase

HotStarTaq 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 15-minute, 95°C incubation step. The hot start enables reactions to be set up rapidly and conveniently at room temperature.

QuantiTect Multiplex PCR Buffer

QuantiTect Multiplex PCR Buffer has been specifically developed for quantitative, multiplex, real-time PCR using sequence-specific probes. In addition to various salts and additives, the buffer also contains a specially optimized 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 usually not required. The buffer also contains synthetic Factor MP, which facilitates multiplex PCR. Factor MP 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 QuantiTect 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 (formerly Stratagene). 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 can support reactions without ROX passive reference dye.

For instruments from Applied Biosystems, we recommend using the QuantiTect Multiplex PCR Kit, which provides a master mix that contains ROX dye. For instruments from all other suppliers, we recommend using the QuantiTect Multiplex PCR NoROX Kit, which contains a master mix that is free of ROX dye.

Use of uracil-N-glycosylase (UNG)

QuantiTect Multiplex PCR Kits contain dUTP, which partially replaces dTTP. QuantiTect Multiplex PCR Kits therefore allow the optional use of a uracil-N-glycosylase (UNG) pretreatment of the reaction if contamination with carried-over PCR products is suspected.

Note: UNG is not included in QuantiTect Multiplex PCR Kits and must be purchased separately.

Sequence-specific probes

QuantiTect Multiplex PCR Kits can be used with all types of probes. 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 58.

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.

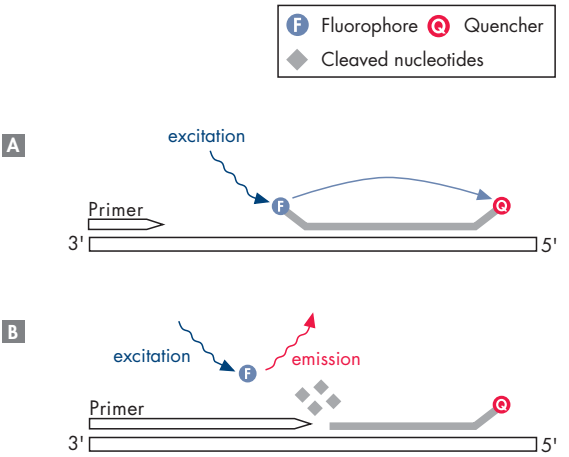

Figure 1. Principle of TaqMan probes in quantitative, real-time PCR. A Both the TaqMan probe and the PCR primers anneal to the target sequence during the PCR annealing step. The proximity of the fluorophore with the quencher prevents the fluorophore from fluorescing. B During the PCR extension step, *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 QuantiTect 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 page 65.

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 65.

Using the correct protocol

This handbook contains several protocols. The choice of protocol depends on the real-time cycler to be used and on whether duplex, triplex, or 4plex PCR will be carried out. To select the right protocol, refer to "Selecting kits and protocols" (page 12). Certain real-time cyclers are also capable of 5plex PCR; for a protocol, contact QIAGEN Technical Services.

For background information on real-time PCR, please refer to the appendices of this handbook (pages 58–63), which contain guidelines for the following: design and handling of primers and probes; data analysis; and evaluation of multiplex assays. See also "Guidelines for real-time PCR and RT-PCR" at www.qiagen.com/resources/info, which covers a wide range of topics.

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 58). 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 58). Use RNase/DNase-free water and plastic consumables to prepare TE buffer.
- Optional: Uracil-N-glycosylase (see page 9)

Important Notes

Selecting kits and protocols

To select the correct QuantiTect Multiplex PCR Kit and protocol to use with your real-time cycler, refer to Table 1. 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 QuantiTect Multiplex PCR Kits are very limited due to its detection optics.

Table 1. Choosing the correct QuantiTect Multiplex PCR Kit and protocol for your real-time cycler

Cycler	Kit	Duplex protocol	Triplex and 4plex protocol
ABI PRISM® 7000	QuantiTect Multiplex PCR Kit	Protocol 1, page 32	Protocol 2, page 36
ABI PRISM 7700	QuantiTect Multiplex PCR Kit	Protocol 1, page 32†	
Applied Biosystems® 7300	QuantiTect Multiplex PCR Kit	Protocol 1, page 32	Protocol 2, page 36
Applied Biosystems 7500*	QuantiTect Multiplex PCR Kit	Protocol 1, page 32	Protocol 2, page 36
Applied Biosystems 7900HT	QuantiTect Multiplex PCR Kit	Protocol 1, page 32	Protocol 2, page 36
Applied Biosystems StepOne™	QuantiTect Multiplex PCR Kit	Protocol 1, page 32†	
Applied Biosystems StepOnePlus™	QuantiTect Multiplex PCR Kit	Protocol 1, page 32	Protocol 2, page 36
iCycler iQ®	QuantiTect Multiplex PCR NoROX Kit	Protocol 4, page 44	Protocol 5, page 48
LightCycler 2.0	QuantiTect Multiplex PCR NoROX Kit	Protocol 3, page 40	Protocol 3, page 40
LightCycler 480	QuantiTect Multiplex PCR NoROX Kit	Protocol 4, page 44	Protocol 5, page 48

* 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 1. Continued

Cycler	Kit	Duplex protocol	Triplex and 4plex protocol
Mastercycler® ep *realplex*	QuantiTect Multiplex PCR NoROX Kit	Protocol 4, page 44	
Mx3000P®, Mx3005P®, and Mx4000®	QuantiTect Multiplex PCR NoROX Kit	Protocol 4, page 44	Protocol 5, page 48
Rotor-Gene® 3000 and Rotor-Gene 6000	QuantiTect Multiplex PCR NoROX Kit	Protocol 4, page 44	Protocol 5, page 48
SmartCycler® II	QuantiTect Multiplex PCR NoROX Kit	Protocol 4, page 44	Protocol 5, page 48
Other*	QuantiTect Multiplex PCR NoROX Kit	Protocol 4, page 44	Protocol 5, page 48

* Refer to manufacturer's instructions for multiplex capacity.

Guidelines for effective multiplex assays

QuantiTect 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 14.
- PCR products should be as short as possible, ideally 60–150 bp. For details, see Appendix A, page 58.
- 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 58.
- Check the concentration and integrity of primers and probes before starting. For details, see Appendix A, page 58.

- 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 2). 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.

Table 2. Dyes commonly used in quantitative, multiplex, real-time PCR

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

* Emission spectra may vary depending on the buffer conditions.

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 3–15 (pages 16–30).
- 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 4plex 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 4plex analyses may only be possible with nonfluorescent quenchers, especially with instruments from Applied Biosystems.

Recommendations for instruments from Applied Biosystems

Tables 3–8 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 3. 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 QuantiTect 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 4. 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)	
4plex	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 4plex 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, a component of 2x QuantiTect 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 5. 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 QuantiTect 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 6. 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 QuantiTect 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 7. 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 QuantiTect 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 8. 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 QuantiTect 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 9 (page 21).

- 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 9 (page 21).

Table 9. 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
4plex	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 10 (page 23).

- 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 10. 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
4plex	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 Rotor-Gene 3000

The Rotor-Gene 3000 system uses a 4-channel light source and a detection filter wheel with 6 detection 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 Rotor-Gene 3000 system are given in Table 11 (page 25).

- The Rotor-Gene 3000 system has 4 preset channels that provide the best choice for multiplex assays. Each channel detects a particular reporter dye (FAM, JOE, ROX, or Cy5) and is named after the dye it detects. Other reporter dyes with similar spectra can also be detected by these channels, and do not require calibration.
- The detection filter wheel contains three 10 nm band-pass filters (only signals within a certain wavelength band can pass through) and 3 high-pass filters (only signals with a wavelength above a certain limit can pass through). With regard to the 4 preset channels, the FAM, JOE, and ROX channels each use a 10 nm band-pass filter, while the Cy5 channel uses a high-pass filter. The band-pass filters are named after the wavelength they let through followed by the unit "nm": 510 nm, 555 nm, and 610 nm. The high-pass filter is named after its wavelength limit followed by "hp": 665 hp. There are 2 additional high-pass filters (610 hp and 585 hp), which can be used to detect dyes that cannot be detected using the preset channels. However, their use may be limited in multiplex assays.
- 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 *Rotor-Gene Software Manual* for additional information on setting up detection channels and correctly setting up the instrument for multiplex analysis.

Table 11. Suitable reporter dyes — Rotor-Gene 3000

Type of assay	Channel 1 (470/510)*†	Channel 2 (530/555)*†	Channel 3 (585/610)*†	Channel 4 625/665)*†
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		ROX‡ Cy3.5‡	Cy5
4plex	6-FAM	HEX JOE VIC	ROX‡	Cy5

* The numbers in parentheses indicate the wavelengths of the excitation light source and the detection filter.

† 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.

‡ We do not recommend using channel 3 to detect Texas Red dye if channel 4 is also being used. This is because Texas Red dye is partly detected by channel 4.

Recommendations for the Rotor-Gene 6000

The Rotor-Gene 6000 system has 6 detection channels and uses a separate high-power LED as an excitation source for each channel. This provides flexibility when selecting reporter dyes for multiplex assays. However, care must be taken to select suitable combinations of reporter dyes and channels that exhibit minimal crosstalk. Suitable combinations of reporter dyes for multiplex assays using the Rotor-Gene 6000 system are given in Table 12 (page 26).

- The Rotor-Gene 6000 system has 4 preset channels that provide the best choice for multiplex assays: Green, Yellow, Orange, and Crimson. Each channel detects reporter dyes that emit light at a particular wavelength.

- 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 *Rotor-Gene Software Manual* for additional information on setting up detection channels and correctly setting up the instrument for multiplex analysis.

Table 12. Suitable reporter dyes — Rotor-Gene 6000

Type of assay	Green channel*	Yellow channel*	Orange channel*	Crimson channel*
Duplex	6-FAM	HEX VIC Yakima Yellow		
Duplex	6-FAM		ROX	
Duplex	6-FAM			Quasar® 705
Triplex	6-FAM	HEX VIC Yakima Yellow	ROX	
Triplex	6-FAM	HEX VIC Yakima Yellow		Quasar 705
Triplex	6-FAM		ROX	Quasar 705
4plex	6-FAM	HEX VIC Yakima Yellow	ROX	Quasar 705

* Use the green channel, the yellow channel, and the orange and crimson channels to detect the least abundant target, the second least abundant target, and the 2 most abundant targets, respectively.

Recommendations for the Mastercycler ep *realplex*

The Mastercycler ep *realplex* has an array of 96 individual LEDs to excite the reporter dyes in each well of a 96-well PCR plate. Each LED emits blue light at a wavelength of about 470 nm. Depending on the configuration of the Mastercycler ep *realplex*, fluorescence emitted from the excited fluorophores passes through either 2 emission filters (520 nm and 550 nm) or 4 emission filters (520 nm, 550 nm, 580 nm, and 605 nm) before being detected. Suitable combinations of reporter dyes for multiplex assays using the Mastercycler ep *realplex* are given in Table 13 (page 27). For optimal results, we recommend carrying out duplex assays.

Table 13. Suitable reporter dyes — Mastercycler ep *realplex*

Type of assay	Channel 1 (520 nm filter)*	Channel 2 (550 nm filter)*	Channel 3 (580 nm filter)*	Channel 4 (605 nm filter)*
Duplex	6-FAM	HEX JOE VIC		
Duplex	6-FAM		Bodipy TMR NED	
Duplex	6-FAM			Texas Red ROX

* Use channel 1 to detect the least abundant target and channel 2, 3, or 4 to detect the most abundant target.

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 14 (page 28).

- 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. A supplementary protocol which describes how to generate and use color compensation files for multiplex assays using TaqMan probes is available. Visit www.qiagen.com/literature, enter *PCR81* in the "Search" field, and then click "Search" to retrieve the protocol.
- Although dyes detected in channels >600 nm are not optimally excited, using the dyes recommended in Table 14 will give a detectable fluorescent signal.
- The reporter dyes shown in Table 14 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 4plex PCR, refer to Table 14 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 14. 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		
4plex	6-FAM	HEX JOE VIC	Texas Red ROX	Cy5	
4plex	6-FAM	HEX JOE VIC	Texas Red ROX		Alexa Fluor® 660 Pulsar® 650†

* Use detection channel 1, detection channel 2, and detection channels 3 and 5/6 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 channels for different excitation/emission wavelengths. The 5 detection channels cover the ranges 450–500 nm, 483–533 nm, 523–568 nm, 558–610 nm, and 615–670 nm. Suitable combinations of reporter dyes for multiplex assays using the LightCycler 480 system are given in Table 15 (page 30).

- 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.

    A supplementary protocol which describes how to generate and use color compensation files for multiplex assays using TaqMan probes is available. Visit www.qiagen.com/literature, enter *PCR82* in the "Search" field, and then click "Search" to retrieve the protocol.

Table 15. Suitable reporter dyes — LightCycler 480

Type of assay	Channel 1 (450/500)*†	Channel 2 (438/533)*†	Channel 3 (523/568)*†	Channel 4 (558/610)*†	Channel 5 (615/670)*†
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**
4plex		**6-FAM**	**HEX** JOE VIC	**Texas Red** **ROX**	**Cy5**

* The numbers in parentheses indicate the wavelengths of the excitation and emission filters. Reporter dye combinations highlighted in bold have been successfully tested by QIAGEN.

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

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 PCR on Applied Biosystems Cyclers

This protocol is intended for use with the **QuantiTect Multiplex PCR Kit** and TaqMan probes on real-time cyclers from Applied Biosystems. Using this protocol, duplex PCR is carried out in the presence of ROX passive reference dye.

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 13. 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.
- The PCR must start with an **initial incubation step of 15 min at 95°C** to activate HotStarTaq DNA Polymerase.
- **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 8 µM forward primer, 8 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 D (page 64).

Procedure

1. **Thaw 2x QuantiTect Multiplex PCR Master Mix, template DNA or cDNA, primer and probe solutions, and RNase-free water. Mix the individual solutions, and place them on ice.**

2. **Prepare a reaction mix according to Table 16 (page 34).**

   Note: We strongly recommend starting with the optimized Mg^{2+} concentration provided by 2x QuantiTect Multiplex PCR Master Mix. For only a few targets, reactions may be improved by increasing the final Mg^{2+} concentration by 0.5–1 mM.

   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 (≤ 500 ng/50 µl reaction) 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 35).**

   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.

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.

   Note: If using the Applied Biosystems 7500, it is necessary to adjust the preset threshold value to a lower value. Use a value of 0.01 as a starting point.

Table 16. Reaction setup

Component	Volume*	Final concentration
2x QuantiTect Multiplex PCR Master Mix	25 µl	1x
20x primer–probe mix 1†	2.5 µl	0.4 µM forward primer 1‡ 0.4 µM reverse primer 1‡ 0.2 µM probe 1§
20x primer–probe mix 2†	2.5 µl	0.4 µM forward primer 2‡ 0.4 µM reverse primer 2‡ 0.2 µM probe 2§
RNase-free water	Variable	–
Optional: Uracil-N-glycosylase	Variable	0.5 units/reaction¶
Template DNA or cDNA (added at step 4)	Variable	≤ 500 ng/reaction
Total reaction volume	**50 µl***	**–**

* If your real-time cycler requires a final reaction volume other than 50 µl, adjust the amount of master mix and all other reaction components accordingly. If using 384-well plates on the ABI PRISM 7900, use a reaction volume of 20 µl.

† A 20x primer–probe mix for duplex PCR consists of 8 µM forward primer, 8 µM reverse primer, and 4 µM probe in TE buffer.

‡ A final primer concentration of 0.4 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.

¶ The activity of uracil-N-glycosylase may differ between suppliers. Depending on your supplier, the optimal concentration may be 0.25–1 units per 50 µl reaction.

Table 17. Cycling conditions

Step	Time	Temperature	Additional comments
Optional: UNG (carryover prevention)	2 min	50°C	UNG will eliminate any dUMP-containing PCR products resulting from carryover contamination
PCR initial activation step	**15 min**	**95°C**	HotStarTaq DNA Polymerase is activated by this heating step
2-step cycling:			**Important: Optimal performance is only assured using these cycling conditions**
Denaturation	**60 s**	**94°C**	
Annealing/extension	**60 s**	**60°C**	Combined annealing/ extension step with fluorescence data collection
Number of cycles	40–50		The number of cycles depends on the amount of template DNA or cDNA and the expression level of the target gene

Protocol 2: Triplex and 4plex PCR on Applied Biosystems Cyclers

This protocol is intended for use with the **QuantiTect Multiplex PCR Kit** and TaqMan probes on real-time cyclers from Applied Biosystems. Using this protocol, triplex or 4plex PCR is carried out in the presence of ROX passive reference dye.

Note: Due to cycler specifications, 4plex PCR is only possible on the Applied Biosystems 7500.

Important points before starting

- Always start with the **cycling conditions** and **primer concentrations** specified in this protocol. Please note that the cycling conditions and primer concentrations differ from those described in the protocol for duplex assays (page 32).
- 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 13. 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.
- The PCR must start with an **initial incubation step of 15 min at 95°C** to activate HotStarTaq DNA Polymerase.
- **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 4plex PCR consists of 4 µM forward primer, 4 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 D (page 64).

Procedure

1. **Thaw 2x QuantiTect Multiplex PCR Master Mix, template DNA or cDNA, primer and probe solutions, and RNase-free water. Mix the individual solutions, and place them on ice.**
2. **Prepare a reaction mix according to Table 18 (page 38).**

   Note: We strongly recommend starting with the optimized Mg^{2+} concentration provided by 2x QuantiTect Multiplex PCR Master Mix. For only a few targets, reactions may be improved by increasing the final Mg^{2+} concentration by 0.5–1 mM.

   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 (≤ 500 ng/50 µl reaction) 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 19 (page 39).**

   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.
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.

   Note: If using the Applied Biosystems 7500, it is necessary to adjust the preset threshold value to a lower value. Use a value of 0.01 as a starting point.

Table 18. Reaction setup

Component	Volume*	Final concentration
2x QuantiTect Multiplex PCR Master Mix	25 µl	1x
20x primer–probe mix 1†	2.5 µl	0.2 µM forward primer 1‡ 0.2 µM reverse primer 1‡ 0.2 µM probe 1§
20x primer–probe mix 2†	2.5 µl	0.2 µM forward primer 2‡ 0.2 µM reverse primer 2‡ 0.2 µM probe 2§
20x primer–probe mix 3†	2.5 µl	0.2 µM forward primer 3‡ 0.2 µM reverse primer 3‡ 0.2 µM probe 3§
Only for 4plex PCR: 20x primer–probe mix 4†	2.5 µl	0.2 µM forward primer 4‡ 0.2 µM reverse primer 4‡ 0.2 µM probe 4§
RNase-free water	Variable	–
Optional: Uracil-N-glycosylase	Variable	0.5 units/reaction¶
Template DNA or cDNA (added at step 4)	Variable	≤ 500 ng/reaction
Total reaction volume	**50 µl***	**–**

* If your real-time cycler requires a final reaction volume other than 50 µl, adjust the amount of master mix and all other reaction components accordingly. If using 384-well plates on the ABI PRISM 7900, use a reaction volume of 20 µl.

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

‡ A final primer concentration of 0.2 µM is optimal. Before adapting primer concentration, check the concentration of your primer solutions. In some cases, using other primer concentrations between 0.1 µM and 0.3 µM may further improve performance.

§ 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.

¶ The activity of uracil-N-glycosylase may differ between suppliers. Depending on your supplier, the optimal concentration may be 0.25–1 units per 50 µl reaction.

Table 19. Cycling conditions

Step	Time	Temperature	Additional comments
Optional: UNG (carryover prevention)	2 min	50°C	UNG will eliminate any dUMP-containing PCR products resulting from carryover contamination
PCR initial activation step	**15 min**	**95°C**	HotStarTaq DNA Polymerase is activated by thisheating step
2-step cycling:			**Important: Optimal performance is only assured using these cycling conditions**
Denaturation	**60 s**	**94°C**	
Annealing/extension	**90 s**	**60°C**	Combined annealing/ extension step with fluorescence data collection
Number of cycles	40–50		The number of cycles depends on the amount of template DNA or cDNA and the expression level of the target gene

Protocol 3: Multiplex PCR on the LightCycler 2.0

This protocol is intended for use with the **QuantiTect Multiplex PCR NoROX Kit** and TaqMan probes on the LightCycler 2.0. Using this protocol, duplex, triplex, or 4plex PCR is carried out without any ROX passive reference dye in the reaction.

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 13. 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.
- The PCR must start with an **initial incubation step of 15 min at 95°C** to activate HotStarTaq DNA Polymerase.
- **Optimal analysis settings are a prerequisite for accurate quantification data.** For data analysis, you should always check the results of the automatic analysis function of every reporter dye channel in every run. In the unlikely event of the automatic analysis function failing to provide optimal results, use the fit points method for data analysis.
- Be sure to create a color compensation file. For details, download QIAGEN Supplementary Protocol PCR81 at www.qiagen.com/literature.

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 consists of 4 µM forward primer, 4 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 D (page 64).

Procedure

1. **Thaw 2x QuantiTect Multiplex PCR NoROX Master Mix, template DNA or cDNA, primer and probe solutions, and RNase-free water. Mix the individual solutions, and place them on ice.**
2. **Prepare a reaction mix according to Table 20 (page 42).**

   Note: We strongly recommend starting with the optimized Mg^{2+} concentration provided by 2x QuantiTect Multiplex PCR NoROX Master Mix. For only a few targets, reactions may be improved by increasing the final Mg^{2+} concentration by 0.5–1 mM.

   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 capillaries.**
4. **Add template DNA or cDNA (≤ 200 ng/20 µl reaction) to the individual PCR capillaries.**

   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 43).**
6. **Place the PCR capillaries in the real-time cycler, and start the cycling program.**
7. **Perform data analysis.**

   Check whether the automatic analysis function provides optimal results for all probes. In the unlikely event of the automatic analysis function failing to provide optimal results, use the fit points method for data analysis. Optimal analysis settings are a prerequisite for accurate quantification data.

Table 20. Reaction setup

Component	Volume*	Final concentration
2x QuantiTect Multiplex PCR NoROX Master Mix	10 µl	1x
20x primer–probe mix 1†	1 µl	0.2 µM forward primer 1‡ 0.2 µM reverse primer 1‡ 0.2 µM probe 1§
20x primer–probe mix 2†	1 µl	0.2 µM forward primer 2‡ 0.2 µM reverse primer 2‡ 0.2 µM probe 2§
Only for triplex and 4plex PCR: 20x primer–probe mix 3†	1 µl	0.2 µM forward primer 3‡ 0.2 µM reverse primer 3‡ 0.2 µM probe 3§
Only for 4plex PCR: 20x primer–probe mix 4†	1 µl	0.2 µM forward primer 4‡ 0.2 µM reverse primer 4‡ 0.2 µM probe 4§
RNase-free water	Variable	–
Optional: Uracil-N-glycosylase	Variable	0.2 units/reaction¶
Template DNA or cDNA (added at step 4)	Variable	≤ 200 ng/reaction
Total reaction volume	**20 µl***	**–**

* If using capillaries for 100 µl reaction volumes, adjust the amount of master mix and all other reaction components accordingly.

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

‡ A final primer concentration of 0.2 µM is optimal. Before adapting primer concentration, check the concentration of your primer solutions. In some cases, using other primer concentrations between 0.1 µM and 0.3 µM may further improve performance.

§ 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.

¶ The activity of uracil-N-glycosylase may differ between suppliers. Depending on your supplier, the optimal concentration may be 0.1–0.4 units per 20 µl reaction.

Table 21. Cycling conditions

Step	Time	Temperature	Ramp	Additional comments
Optional: UNG (carryover prevention)	2 min	50°C	20°C/s	UNG will eliminate any dUMP-containing PCR products resulting from carryover contamination
PCR initial activation step	**15 min**	**95°C**	20°C/s	HotStarTaq DNA Polymerase is activated by this heating step
2-step cycling:				**Important: Optimal performance is only assured using these cycling conditions**
Denaturation	**60 s**	**94°C**	20°C/s	
Annealing/extension				
Duplex PCR: Triplex PCR: 4plex PCR:	**60 s** **90 s** **90 s**	**60°C** **60°C** **60°C**	20°C/s 20°C/s 20°C/s	Combined annealing/ extension step with fluorescence data collection
Number of cycles	40–50			The number of cycles depends on the amount of template DNA or cDNA and the expression level of the target gene

Protocol 4: Duplex PCR on Other Cyclers

This protocol is intended for use with the **QuantiTect Multiplex PCR NoROX Kit** and TaqMan probes on real-time cyclers from Bio-Rad/MJ Research, Cepheid, QIAGEN, Eppendorf, and Agilent, and on the LightCycler 480. Using this protocol, duplex PCR is carried out without any ROX passive reference dye in the reaction.

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 13. 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.
- The PCR must start with an **initial incubation step of 15 min at 95°C** to activate HotStarTaq DNA Polymerase.
- **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 480**, be sure to create a color compensation file. For details, download QIAGEN Supplementary Protocol PCR82 at www.qiagen.com/literature.

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 8 µM forward primer, 8 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 D (page 64).

Procedure

1. **Thaw 2x QuantiTect Multiplex PCR NoROX Master Mix, template DNA or cDNA, primer and probe solutions, and RNase-free water. Mix the individual solutions, and place them on ice.**
2. **Prepare a reaction mix according to Table 22 (page 46).**

   Note: We strongly recommend starting with the optimized Mg^{2+} concentration provided by 2x QuantiTect Multiplex PCR NoROX Master Mix. For only a few targets, reactions may be improved by increasing the final Mg^{2+} concentration by 0.5–1 mM.

   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 (≤ 500 ng/50 µl reaction) 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 23 (page 47).**

   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.
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.

Table 22. Reaction setup

Component	Volume*	Final concentration
2x QuantiTect Multiplex PCR NoROX Master Mix	25 µl	1x
20x primer–probe mix 1†	2.5 µl	0.4 µM forward primer 1‡ 0.4 µM reverse primer 1‡ 0.2 µM probe 1§
20x primer–probe mix 2†	2.5 µl	0.4 µM forward primer 2‡ 0.4 µM reverse primer 2‡ 0.2 µM probe 2§
RNase-free water	Variable	–
Optional: Uracil-N-glycosylase	Variable	0.5 units/reaction¶
Template DNA or cDNA (added at step 4)	Variable	≤ 500 ng/reaction
Total reaction volume	**50 µl***	**–**

* If your real-time cycler requires a final reaction volume other than 50 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.

† A 20x primer–probe mix for duplex PCR consists of 8 µM forward primer, 8 µM reverse primer, and 4 µM probe in TE buffer.

‡ A final primer concentration of 0.4 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.

¶ The activity of uracil-N-glycosylase may differ between suppliers. Depending on your supplier, the optimal concentration may be 0.25–1 units per 50 µl reaction.

Table 23. Cycling conditions

Step	Time	Temperature	Additional comments
Optional: UNG (carryover prevention)	2 min	50°C	UNG will eliminate any dUMP-containing PCR products resulting from carryover contamination
PCR initial activation step	**15 min**	**95°C**	HotStarTaq DNA Polymerase is activated by this heating step
2-step cycling:			**Important: Optimal performance is only assured using these cycling conditions**
Denaturation	**60 s**	**94°C**	
Annealing/extension	**60 s**	**60°C**	Combined annealing/ extension step with fluorescence data collection
Number of cycles	40–50		The number of cycles depends on the amount of template DNA or cDNA and the expression level of the target gene

Protocol 5: Triplex and 4plex PCR on Other Cyclers

This protocol is intended for use with the **QuantiTect Multiplex PCR NoROX Kit** and TaqMan probes on real-time cyclers from Bio-Rad/MJ Research, Cepheid, QIAGEN, Eppendorf, and Agilent, and on the LightCycler 480. Using this protocol, triplex or 4plex PCR is carried out without any ROX passive reference dye in the reaction.

Important points before starting

- Always start with the **cycling conditions** and **primer concentrations** specified in this protocol. Please note that the cycling conditions and primer concentrations differ from those described in the protocol for duplex assays (page 44).
- 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 13. 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.
- The PCR must start with an **initial incubation step of 15 min at 95°C** to activate HotStarTaq DNA Polymerase.
- **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 480**, be sure to create a color compensation file. For details, download QIAGEN Supplementary Protocol PCR82 at www.qiagen.com/literature.

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 4plex PCR consists of 4 µM forward primer, 4 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 D (page 64).

Procedure

1. **Thaw 2x QuantiTect Multiplex PCR NoROX Master Mix, template DNA or cDNA, primer and probe solutions, and RNase-free water. Mix the individual solutions, and place them on ice.**
2. **Prepare a reaction mix according to Table 24 (page 50).**

   Note: We strongly recommend starting with the optimized Mg^{2+} concentration provided by 2x QuantiTect Multiplex PCR NoROX Master Mix. For only a few targets, reactions may be improved by increasing the final Mg^{2+} concentration by 0.5–1 mM.

   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 (≤ 500 ng/50 µl reaction) 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 25 (page 51).**

   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.
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.

Table 24. Reaction setup

Component	Volume*	Final concentration
2x QuantiTect Multiplex PCR NoROX Master Mix	25 µl	1x
20x primer–probe mix 1†	2.5 µl	0.2 µM forward primer 1‡ 0.2 µM reverse primer 1‡ 0.2 µM probe 1§
20x primer–probe mix 2†	2.5 µl	0.2 µM forward primer 2‡ 0.2 µM reverse primer 2‡ 0.2 µM probe 2§
20x primer–probe mix 3†	2.5 µl	0.2 µM forward primer 3‡ 0.2 µM reverse primer 3‡ 0.2 µM probe 3§
Only for 4plex PCR: 20x primer–probe mix 4†	2.5 µl	0.2 µM forward primer 4‡ 0.2 µM reverse primer 4‡ 0.2 µM probe 4§
RNase-free water	Variable	–
Optional: Uracil-N-glycosylase	Variable	0.5 units/reaction¶
Template DNA or cDNA (added at step 4)	Variable	≤ 500 ng/reaction
Total reaction volume	**50 µl***	**–**

* If your real-time cycler requires a final reaction volume other than 50 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.

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

‡ A final primer concentration of 0.2 µM is optimal. Before adapting primer concentration, check the concentration of your primer solutions. In some cases, using other primer concentrations between 0.1 µM and 0.3 µM may further improve performance.

§ 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.

¶ The activity of uracil-N-glycosylase may differ between suppliers. Depending on your supplier, the optimal concentration may be 0.25–1 units per 50 µl reaction.

Table 25. Cycling conditions

Step	Time	Temperature	Additional comments
Optional: UNG (carryover prevention)	2 min	50°C	UNG will eliminate any dUMP-containing PCR products resulting from carryover contamination
PCR initial activation step	**15 min**	**95°C**	HotStarTaq DNA Polymerase is activated by this heating step
2-step cycling:			**Important: Optimal performance is only assured using these cycling conditions**
Denaturation	**60 s**	**94°C**	
Annealing/extension	**90 s**	**60°C**	Combined annealing/ extension step with fluorescence data collection
Number of cycles	40–50		The number of cycles depends on the amount of template DNA or cDNA and the expression level of the target gene

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 DNA Polymerase (95°C for 15 min), and the specified times for denaturation and annealing/extension.
b)	HotStarTaq DNA Polymerase not activated	Ensure that the cycling program includes the HotStarTaq DNA Polymerase activation step (15 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 58, for details on evaluating the concentration of primers and probes. Repeat the PCR.
d)	Wrong or no detection step	Ensure that fluorescence detection takes place during the combined annealing/ extension step when using TaqMan probes.

		Comments and suggestions
e)	Primer or probe concentration not optimal	Use optimal primer concentrations. For duplex PCR on all real-time block cyclers, use each primer at 0.4 µM. For triplex or 4plex PCR on all real-time block cyclers, use each primer at 0.2 µM. For multiplex PCR using TaqMan probes on the LightCycler 2.0 system, use a primer concentration of 0.2 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 58).
f)	Mg^{2+} concentration not optimal	The Mg^{2+} concentration in 2x QuantiTect Multiplex PCR Master Mixes is already optimized, giving a final Mg^{2+} concentration of 5.5 mM in multiplex PCR. For a few targets, 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 template. If necessary, make new serial dilutions of template nucleic acid from the stock solutions. Repeat the PCR using the new dilutions.
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 58).

		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.
Differences in C_T values or in PCR efficiencies between a multiplex PCR assay and the corresponding singleplex PCR 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 DNA Polymerase (95°C for 15 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 PCR uses 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. **ABI PRISM 7700**: Perform analysis with and without spectral compensation. **LightCycler 2.0**: In some instances, the shape of the amplification plots for singleplex and multiplex reactions may differ due to the color compensation algorithms.

		Comments and suggestions
No linearity in ratio of C_T value/crossing point to log of the template amount		
a)	Template amount too high	Do not exceed maximum recommended amounts of template. If you need to use a large volume of reverse-transcription reaction as template, determine the maximum acceptable volume for the multiplex assay being carried out. We recommend using the QuantiTect Reverse Transcription Kit, since any inhibitory effect of using a large volume of reverse-transcription reaction in PCR (>15% of PCR volume) will be much less compared with other reverse transcriptases.
b)	Template amount too low	Increase template amount, if possible.
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)	Contamination during reaction setup	Take appropriate precautions during reaction setup, such as using aerosol-barrier pipet tips, and using UNG to prevent carryover from previous reactions.
c)	Minimal probe degradation, leading to sliding increase in fluorescence	Check the amplification plots, and adjust the threshold settings.
High fluorescence in "No Reverse Transcription" control		
	Contamination of RNA sample with genomic DNA	Design primers and/or probes that span exon-exon boundaries, so that only cDNA targets can be amplified and detected. Perform reverse transcription with the QuantiTect Reverse Transcription Kit, which provides cDNA synthesis with integrated genomic DNA removal. Alternatively, treat the RNA sample with DNase to digest the contaminating genomic DNA.

		Comments and suggestions
Varying fluorescence intensity		
a)	Contamination of real-time cycler	Decontaminate the real-time cycler according to the manufacturer's instructions.
b)	Real-time cycler no longer calibrated	Recalibrate the real-time cycler according to the manufacturer's instructions.
All cycler systems:		
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.
ABI PRISM 7000 only:		
d)	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.
Applied Biosystems 7500 only:		
e)	No amplification signal using preset threshold value of 0.2	Adjust the preset threshold value to a lower value. Use a value of 0.01 as a starting point.
LightCycler 2.0 only:		
f)	Unexpected fluorescence signal in one or more detection channels	Use a color compensation file to correct for crosstalk between detection channels. For details, download supplementary protocol PCR81 at www.qiagen.com/literature.
g)	Sample not recognized by instrument	Samples should give a detectable fluorescence signal to enable recognition of sample-containing capillaries by the instrument. When using channels 3–6 to detect samples containing only probes with reporter dyes with an emission maximum of >600 nm (e.g., when performing control singleplex reactions), there may not be sufficient fluorescence for detection. To allow sample recognition, add to each of the affected samples either 10 nM fluorescein dye or an unrelated probe labeled with 6-FAM dye at 0.2 µM.

LightCycler 480 only:

h)	Unexpected fluorescence signal in one or more detection channels	Use a color compensation file to correct for crosstalk between detection channels. For details, download supplementary protocol PCR82 at www.qiagen.com/literature.

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 detected.

* 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: Data Analysis

When carrying out data analysis, follow the recommendations provided by the manufacturer of your real-time cycler. Fundamental guidelines for data analysis and some important considerations are given below. Further information can be found in *Critical Factors for Successful Real-Time PCR*. To obtain a copy, contact QIAGEN Technical Services, or visit www.qiagen.com/literature/brochures to download a PDF.

General considerations for multiplex data analysis

Real-time PCR data are produced as sigmoidal-shaped amplification plots (when using a linear scale), in which fluorescence is plotted against the number of cycles (Figure 2, page 62).

- The threshold cycle (C_T value) serves as a tool for calculation of the starting template amount in each sample. This is the cycle in which there is the first detectable significant increase in fluorescence.
- The optimal threshold setting depends on the reaction chemistries used for PCR. Therefore, an optimal threshold setting established for another kit may not be suitable for the QuantiTect Multiplex Kit you are using, and may need to be adjusted.
- The method for determination of C_T values differs depending on the real-time cycler used. Check the handbook or the software help file for your real-time cycler for details on threshold settings.
- Most real-time cyclers contain a function that determines the noise level in early cycles, where there is no detectable increase in fluorescence due to PCR products (usually referred to as the baseline settings). Adjust the settings for this function.
- For multiplex assays, the analysis settings need to be adjusted for each of the reporter dyes used.
- Depending on your real-time cycler, low levels of signal crosstalk, even between apparently well separated reporter dyes, may influence multiplex results in rare cases. In most cases, low levels of crosstalk can be overcome by optimal analysis settings. If this is not successful, repeat the multiplex assay using the optimal combination of reporter dyes recommended for your real-time cycler (see "Suitable combinations of reporter dyes", page 14).

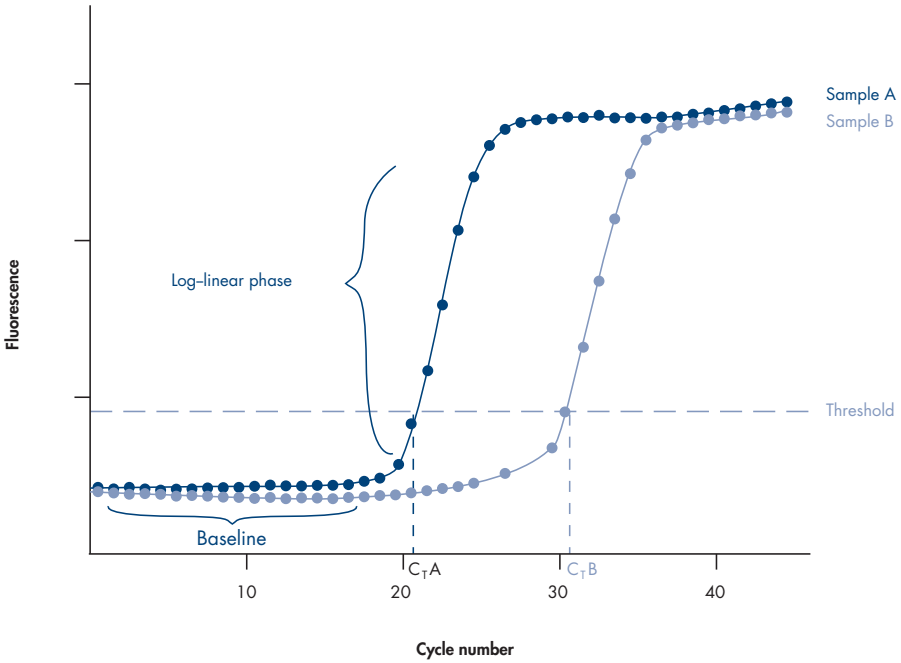

Figure 2. Typical amplification plot. Amplification plots showing increases in fluorescence from 2 samples (Sample A and Sample B).

Appendix C: Evaluating the Quality of Quantitative, Real-Time, Multiplex PCR Assays

The following guidelines may be helpful in evaluating the quality of your newly established or previously established assays.

- Test the performance of each new assay as individual reactions before combining them in a new multiplex assay.
- Compare the performance of the multiplex assay with the corresponding singleplex assays. Assay performance can be tested by, for example, assaying serial dilutions of a sample containing the target nucleic acids. In addition, the dynamic range of the multiplex assay can be tested by, for example, making several dilutions of one target nucleic acid and keeping the concentration of the other target nucleic acid constant. As template, target nucleic acids cloned in a plasmid or prepared as a PCR product can be used if carrying out multiplex, real-time PCR. Alternatively, in vitro transcripts or purified mRNA can be used as template if performing multiplex, real-time RT-PCR.
- It is useful to construct a standard curve for each primer–probe set showing a range of template amounts plotted against the corresponding C_T values. A standard curve can be used to evaluate the linear range and the PCR efficiency of the assay.

Note: Optimal analysis settings are a prerequisite for accurate quantification data. For details, refer to Appendix B, page 61, and the user manual supplied with your real-time cycler.

Note: When running singleplex and multiplex assays on the same plate on the ABI PRISM 7700, check the guidelines for data analysis, since a special analysis procedure may be required. For details, visit www.qiagen.com/resources/info, and in the section "Guidelines for real-time PCR and RT-PCR", refer to "Data Analysis (for Users of QuantiFast Multiplex Kits and QuantiTect Multiplex Kits)".

Appendix D: 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 26 with the calculated volumes of each primer to use.

Table 26. Preparing reaction mix for multiplex PCR using separate primer and probe solutions

	Volume per reaction		
Component*	**50 µl**	**20 µl**	**Other: _____ µl**
2x QuantiTect Multiplex PCR Master Mix	25 µl	10 µl	_____ µl
Forward primer 1 (0.2 µM)†	_____ µl	_____ µl	_____ µl
Reverse primer 1 (0.2 µM)†	_____ µl	_____ µl	_____ µl
Probe 1 (0.2 µM)	_____ µl	_____ µl	_____ µl
Forward primer 2 (0.2 µM)†	_____ µl	_____ µl	_____ µl
Reverse primer 2 (0.2 µM)†	_____ µl	_____ µl	_____ µl
Probe 2 (0.2 µM)	_____ µl	_____ µl	_____ µl
Forward primer 3 (0.2 µM)‡	_____ µl	_____ µl	_____ µl
Reverse primer 3 (0.2 µM)‡	_____ µl	_____ µl	_____ µl
Probe 3 (0.2 µM)‡	_____ µl	_____ µl	_____ µl
Forward primer 4 (0.2 µM)§	_____ µl	_____ µl	_____ µl
Reverse primer 4 (0.2 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	**50 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.

† With the exception of the LightCycler 2.0, primer concentration should be 0.4 µM when performing duplex PCR. For duplex PCR reaction setup, see Table 16 (page 34), Table 20 (page 42), and Table 22 (page 46).

‡ For triplex and 4plex PCR only.

§ For 4plex PCR only.

Ordering Information

Product	Contents	Cat. no.
QuantiTect Multiplex PCR Kit (40)	For 40 x 50 µl reactions: 1 ml 2x Master Mix (with ROX dye), 2 ml RNase-Free Water	204541
QuantiTect Multiplex PCR Kit (200)	For 200 x 50 µl reactions: 3 x 1.7 ml 2x Master Mix (with ROX dye), 2 x 2 ml RNase-Free Water	204543
QuantiTect Multiplex PCR Kit (1000)	For 1000 x 50 µl reactions: 25 ml 2x Master Mix (with ROX dye), 20 ml RNase-Free Water	204545
QuantiTect Multiplex PCR NoROX Kit (40)	For 40 x 50 µl reactions: 1 ml 2x Master Mix (without ROX dye), 2 ml RNase-Free Water	204741
QuantiTect Multiplex PCR NoROX Kit (200)	For 200 x 50 µl reactions: 3 x 1.7 ml 2x Master Mix (without ROX dye), 2 x 2 ml RNase-Free Water	204743
QuantiTect Multiplex PCR NoROX Kit (1000)	For 1000 x 50 µl reactions: 25 ml 2x Master Mix (without ROX dye), 20 ml RNase-Free Water	204745
Accessories		
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
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 (50)	Buffer FCW, Buffer FCP, and components for 50 x 20 µl reverse-transcription reactions	215011

* Trial-size kit available; please inquire.

† Larger kit size available; please inquire.

Ordering Information

Product	Contents	Cat. no.
Related products		
QuantiTect Multiplex RT-PCR Kits — for gene expression analysis by quantitative, multiplex, real-time one-step RT-PCR		
Recommended for instruments from Applied Biosystems:		
QuantiTect Multiplex RT-PCR Kit (200)*	For 200 x 50 µl reactions: 3 x 1.7 ml 2x Master Mix (with ROX dye), 100 µl RT Mix, 2 x 2 ml RNase-Free Water	204643
Recommended for instruments from other suppliers:		
QuantiTect Multiplex RT-PCR NR Kit (200)*	For 200 x 50 µl reactions: 3 x 1.7 ml 2x Master Mix (without ROX dye), 100 µl RT Mix, 2 x 2 ml RNase-Free Water	204843
QuantiFast® Multiplex PCR Kits — for quantitative, multiplex real-time PCR and two-step RT-PCR with fast cycling		
For all instruments from Applied Biosystems except the Applied Biosystems 7500		
QuantiFast Multiplex PCR Kit (400)*†	For 400 x 25 µl reactions: 3 x 1.7 ml 2x Master Mix (with ROX dye), 2 x 2 ml RNase-Free Water	204654
For the Applied Biosystems 7500 and instruments from other suppliers		
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

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

QIAGEN offers a wide range of products for DNA and RNA purification and real-time PCR analysis — to find the right products for your needs, visit www.qiagen.com/ProductFinder.

* Larger kit size available; please inquire.

† Trial-size kit available; please inqure.

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