February 2012

QuantiFast® Probe RT-PCR Plus Handbook

For quantitative, probe-based, real-time one-step RT-PCR with integrated genomic DNA removal

QIAGEN®

Sample & Assay Technologies

QIAGEN Sample and Assay Technologies

QIAGEN is the leading provider of innovative sample and assay technologies, enabling the isolation and detection of contents of any biological sample. Our advanced, high-quality products and services ensure success from sample to result.

QIAGEN sets standards in:

- Purification of DNA, RNA, and proteins
- Nucleic acid and protein assays
- microRNA research and RNAi
- Automation of sample and assay technologies

Our mission is to enable you to achieve outstanding success and breakthroughs. For more information, visit www.qiagen.com.

Contents

Kit Contents

QuantiFast Probe RT-PCR Plus Kit	(80)	(400)
Number of 25 µl reactions	**80**	**400**
Cat. no.	**204482**	**204484**
2x QuantiFast Mix 1*	0.5 ml	2 x 1.3 ml
2x QuantiFast Mix 2 (Probe)†	0.5 ml	2 x 1.3 ml
QuantiFast RT Mix‡	100 µl	100 µl
ROX™ Dye Solution	45 µl	210 µl
High-ROX Dye Solution	45 µl	210 µl
RNase-Free Water	1.9 ml	2 x 1.9 ml
Handbook	1	1

* Contains gDNA Wipeout Buffer.

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

‡ Contains a mixture of the QIAGEN® products Omniscript® Reverse Transcriptase and Sensiscript® Reverse Transcriptase.

Shipping and Storage

The QuantiFast Probe RT-PCR Plus Kit is shipped on dry ice. The kit should be stored immediately upon receipt at –20°C in a constant-temperature freezer and protected from light. When the kit is 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). The 2x QuantiFast Mix 2 (Probe) can also be stored protected from light at 2–8°C for up to 1 month without showing any reduction in performance.

If required, ROX Dye Solution or High-ROX Dye Solution can be added to 2x QuantiFast Mix 2 (Probe) (w/o ROX) for long-term storage. For details, see "Passive reference dye", page 11.

Intended Use

The QuantiFast Probe RT-PCR Plus Kit is intended for molecular biology applications. This product is not intended for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN products to adhere to the NIH guidelines

that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of QuantiFast Probe RT-PCR Kit is tested against predetermined specifications to ensure consistent product quality.

Product Description

Component	Description
QuantiFast Mix 1	Contains gDNA Wipeout Buffer to eliminate contaminating genomic DNA
QuantiFast Mix 2 (Probe)	■ Contains HotStarTaq® *Plus* DNA Polymerase: HotStarTaq *Plus* DNA Polymerase is a modified form of a recombinant 94 kDa DNA polymerase, originally isolated from *Thermus aquaticus*. HotStarTaq *Plus* DNA Polymerase is provided in an inactive state and has no enzymatic activity at ambient temperature. The enzyme is activated by a 5-minute, 95°C incubation step. ■ QuantiFast Probe RT-PCR Plus Buffer: Contains Tris·Cl, KCl, $(NH_4)_2SO_4$, $MgCl_2$, and additives enabling fast cycling, including Q-Bond® ■ dNTP mix: Contains dATP, dCTP, dGTP, and dTTP of ultrapure quality
ROX passive reference dye*	Optimized concentration of fluorescent dye for normalization of fluorescent signals on Applied Biosystems® 7500 Real-Time PCR Systems, the ABI ViiA7, and, optionally, instruments from Agilent.
High-ROX Dye Solution	Optimized concentration of fluorescent dye for normalization of fluorescent signals on all instruments from Applied Biosystems except Applied Biosystems 7500 Real-Time PCR Systems and the ABI ViiA7.
QuantiFast RT Mix	Contains an optimized mixture of the QIAGEN products Omniscript Reverse Transcriptase and Sensiscript Reverse Transcriptase, both of which are recombinant heterodimeric enzymes expressed in *E. coli*.
RNase-free water	Ultrapure quality, PCR-grade

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

Introduction

The QuantiFast Probe RT-PCR Plus Kit provides rapid, real-time one-step RT-PCR quantification of RNA targets in a singleplex or duplex format with probe detection. To minimize the potential for false-positive signals during gene expression analysis, genomic DNA contamination is effectively eliminated through an optimized incubation step prior to real-time RT-PCR. The presence of genomic DNA, particularly in samples containing highly degraded RNA (e.g., RNA from FFPE [formalin fixed, paraffin-embedded] tissue samples), can result in false positive RT-PCR signals. After addition of the master mix for real-time RT-PCR, reverse transcription and real-time PCR takes place subsequently in the same tube (see Flowchart, page 9).

Specificity and sensitivity in one-step RT-PCR are achieved without any time-consuming optimization steps through the use of QuantiFast RT Mix and the hot-start enzyme HotStarTaq *Plus* DNA Polymerase together with a specialized RT-PCR buffer. Short cycling steps without loss of PCR sensitivity and efficiency are enabled by Q-Bond, a patent-pending additive in the RT-PCR buffer.

Genomic DNA removal with QuantiFast Mix 1

QuantiFast Mix 1 contains gDNA Wipeout Buffer which removes residual genomic DNA from RNA samples prior to the PCR amplification step.

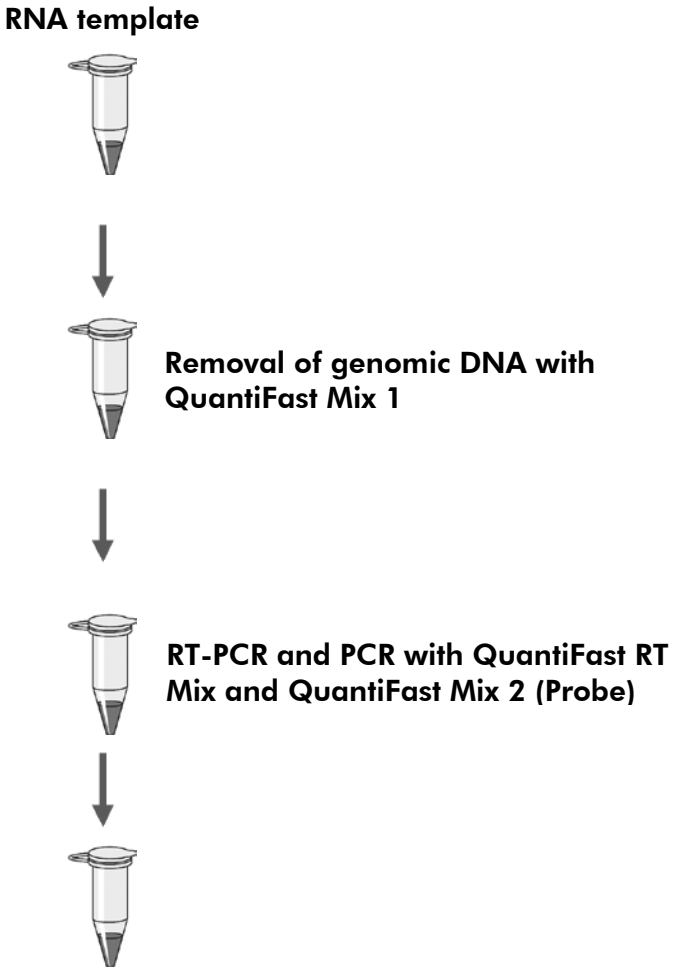
One-step RT-PCR with QuantiFast Mix 2 (Probe)

Use of a 2x QuantiFast Mix 2 (Probe) together with QuantiFast RT Mix allows both reverse transcription and PCR to take place in a single tube.

All reagents required for both reactions are added at the beginning, so there is no need to open the tube once the reverse-transcription reaction has been started.

The components of 2x QuantiFast Mix 2 (Probe) include HotStarTaq *Plus* DNA Polymerase and QuantiFast Probe RT-PCR Plus Buffer. ROX passive reference dye is added separately (see descriptions on the next few pages). The optimized master mix ensures that the RT-PCR products in a duplex reaction are amplified with the same efficiency and sensitivity as the RT-PCR products in corresponding singleplex reactions.

QuantiFast Probe RT-PCR Plus Procedure



QuantiFast RT Mix

QuantiFast RT Mix contains an optimized Omniscript and Sensiscript blend. Both enzymes exhibit a high affinity for RNA, facilitating transcription through secondary structures that may inhibit other reverse transcriptases. Omniscript is designed for reverse transcription of RNA amounts greater than 50 ng, and Sensiscript is optimized for use with very small amounts of RNA (<50 ng). This enzyme combination provides highly efficient and sensitive reverse transcription over a wide range of RNA template amounts.

HotStarTaq *Plus* DNA Polymerase

HotStarTaq *Plus* DNA Polymerase is a modified form of QIAGEN's *Taq* DNA Polymerase. It is provided in an inactive state and has no enzymatic activity at ambient temperature. The enzyme remains completely inactive during the reverse-transcription reaction and does not interfere with it. This prevents formation of misprimed RT-PCR products and primer dimers during reaction setup, reverse transcription, and the first denaturation step. Competition for reactants by RT-PCR artifacts is therefore avoided, enabling high RT-PCR specificity and accurate quantification. The enzyme is activated after the reverse transcription step by a 5-minute, 95°C incubation step. The hot start also inactivates the reverse transcriptases, ensuring temporal separation of reverse transcription and PCR, and allowing both steps to be performed sequentially in a single tube. In addition, the concentration of the polymerase in the master mix is optimized to allow short extension times in the combined annealing/extension step of each PCR cycle.

QuantiFast Probe RT-PCR Plus Buffer

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

QuantiFast Probe RT-PCR Plus Buffer is also based on the unique QIAGEN OneStep RT-PCR buffer system, and is designed to facilitate both efficient reverse transcription and specific amplification in a one-tube format. The buffer contains a balanced combination of KCl and $(NH_4)_2SO_4$, which promotes a high ratio of specific to nonspecific primer binding during the annealing step of each PCR cycle. This creates stringent primer annealing conditions, leading to increased PCR specificity. When using this buffer, primer annealing is only

marginally influenced by the $MgCl_2$ concentration, so optimization by titration of Mg^{2+} is not required. The buffer also contains Factor MP, which facilitates multiplex PCR. This synthetic factor increases the local concentration of primers and probes at the DNA template and stabilizes specifically bound primers and probes, allowing efficient annealing and extension. The combination of these various components of QuantiFast Probe RT-PCR Plus 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 duplex, real-time PCR with ROX passive reference dye on these instruments, we do not recommend using probes that have ROX or Texas Red® fluorophore as the reporter dye, since their performance in the presence of ROX dye is unpredictable. When performing reactions using probes labeled with ROX, Texas Red, or other equivalent fluorophore, use a real-time cycler that does not require ROX dye for fluorescence normalization.

The master mix supplied with the QuantiFast Probe RT-PCR Plus Kit contains no ROX dye. To adjust the reaction setup for instruments from Applied Biosystems (models 7000, 7300, 7700, 7900HT, StepOne™, and StepOnePlus™, but not Applied Biosystems 7500 Real-Time PCR Systems or ViiA7), it is necessary to add High-ROX Dye Solution to the master mix (see “Protocol: Duplex RT-PCR on Most Applied Biosystems Cyclers”, page 31).

For Applied Biosystems 7500 Real-Time PCR Systems, the ABI ViiA7, and instruments from Agilent, ROX dye is required at a lower concentration. This is provided by the reaction, which requires the user to add the supplied ROX Dye Solution to the master mix during reaction setup (see “Protocol: Duplex RT-PCR on Rotor-Gene Instruments, the Applied Biosystems 7500, the ABI ViiA7, and Other Cyclers”, page 36).

Instruments from all other suppliers, which do not require ROX dye for fluorescence normalization, should be used with the QuantiFast Mix 2 (Probe) without addition of ROX dye solution (see “Protocol: Duplex RT-PCR on Rotor-Gene Instruments, the Applied Biosystems 7500, the ABI ViiA7, and Other Cyclers”, page 36).

Sequence-specific probes

The QuantiFast Probe RT-PCR Plus Kit can be used with all types of probes. This handbook contains optimized protocols for use with TaqMan® probes (e.g., QuantiFast Probe Assays), a major type of sequence-specific probe used in quantitative, real time PCR (see below).

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.

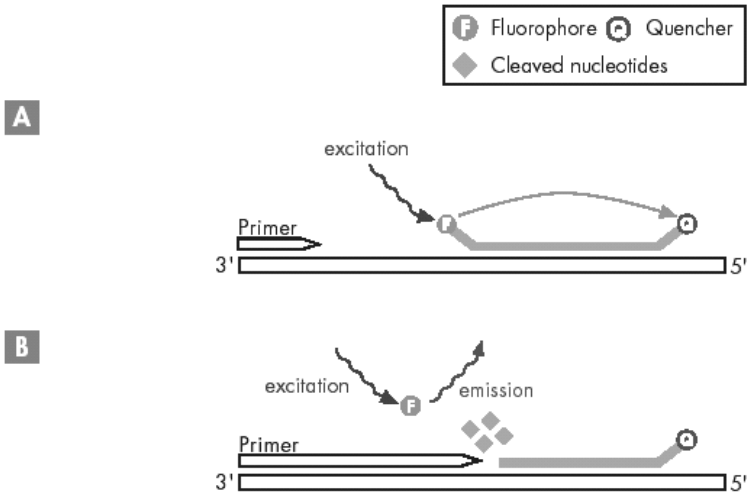


Figure 1. Principle of TaqMan probes in quantitative, real-time PCR. A Both the TaqMan probe and the PCR primers anneal to the target sequence during the PCR annealing step. The proximity of the fluorophore with the quencher prevents the fluorophore from fluorescing. B During the PCR extension step,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

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 (e.g., QuantiFast Probe Assays; see www.qiagen.com/GeneGlobe for more information). 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. 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 (e.g., Rotor-Gene® Q real-time cycler; see www.qiagen.com/RotorGeneQ for more information)
- 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. Use RNase/DNase-free water and plastic consumables to prepare TE buffer.
- Optional: QIAgility® for rapid, high-precision automated PCR setup; for details, visit www.qiagen.com/goto/QIAgility.

Important Notes

Selecting protocols

To select the correct protocol to use with your real-time cycler, refer to Table 1. In general, the following cyclers are not compatible with duplex, real-time PCR: GeneAmp® 5700, MyiQ™, and DNA Engine Opticon® (i.e., the single-color machine). The capabilities of the LightCycler® 1.x for duplex, real-time PCR using QuantiFast Probe RT-PCR Plus Kit are very limited due to its detection optics.

Table 1. Choosing the correct protocol for your real-time cycler

Cycler	Protocol
ABI PRISM® 7000	Page 31
ABI PRISM 7700	Page 31
Applied Biosystems 7300	Page 31
Applied Biosystems 7500*	Page 36
ABI ViiA7	Page 37
Applied Biosystems 7900HT	Page 31
Applied Biosystems StepOne	Page 31†
Applied Biosystems StepOnePlus	Page 31
iCycler iQ® and CFX	Page 36
LightCycler 2.0 and LightCycler 480	Page 36
Mastercycler® ep *realplex*	Page 36
Mx3000P®, Mx3005P®, and Mx4000®	Page 36
Rotor-Gene Q, Rotor-Gene 3000 and 6000	Page 36
SmartCycler® II	Page 36
Other‡	Page 36

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

† Only duplex assays are possible due to hardware limitations.

‡ Refer to manufacturer's instructions for multiplex capacity.

Guidelines for effective duplex assays

The QuantiFast Probe RT-PCR Plus Kit works with most existing probe systems that have been designed using standard design methods. However, for optimal performance of a probe system in quantitative, duplex, real-time 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 duplex assay.
- Choose compatible reporter dyes and quenchers. For details, see "Suitable combinations of reporter dyes", page 16.
- PCR products should be as short as possible, ideally 60–150 bp.
- 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]).
- Check the concentration and integrity of primers and probes before starting.
- Check the real-time cycler user manual for **correct setup of the cycler for duplex 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 duplex 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 duplex assays (e.g., amplifying each target individually and comparing the results with those for the duplex assay).

Suitable combinations of reporter dyes

Duplex, 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 duplex analysis.

Table 2. Dyes commonly used in duplex 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
MAX	524	557
Bodipy® TMR	542	574
NED™	546	575
Cy®3	552	570
TAMRA™	560	582
Cy3.5	588	604
ROX	587	607
Texas Red	596	615
Cy5	643	667

* Emission spectra may vary depending on the buffer conditions.

General recommendations

- Before starting, choose suitable combinations of reporter dyes and quenchers that are compatible with duplex 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 3–14 (pages 18–29).
- For duplex analysis, the use of nonfluorescent quenchers (e.g., Black Hole Quencher® [BHQ®] on TaqMan probes) is preferred over fluorescent quenchers (e.g., TAMRA fluorescent dye). TAMRA quencher can be used in duplex analysis if the 2 reporter dyes are 6-FAM dye and HEX, JOE, MAX, or VIC dye.

Recommendations for Rotor-Gene instruments

The Rotor-Gene Q and Rotor-Gene 6000 have up to 6 detection channels and use 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 Q and Rotor-Gene 6000 are given in Table 3 (page 18).

- The Rotor-Gene Q and Rotor-Gene 6000 have 5 preset channels that provide the best choice for multiplex assays: Green, Yellow, Orange, Red, 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 dye channels installed on the instrument. Ensure that each reporter dye is detected by a different channel.
- Refer to the user manual supplied with your Rotor-Gene cycler for information on setting up additional dye channels and correctly setting up the instrument for multiplex analysis.

Table 3. Suitable reporter dyes — Rotor-Gene cyclers

Type of assay	Green channel*	Yellow channel*	Orange channel*	Red channel*	Crimson channel*
Duplex	6-FAM	MAX HEX VIC Yakima Yellow			
Duplex	6-FAM		ROX		
Duplex	6-FAM			Cy5	
Duplex	6-FAM				Quasar® 705

* Channels correspond to those of the Rotor-Gene Q and Rotor-Gene 6000.

Recommendations for instruments from Applied Biosystems

Tables 4–9 on the next few pages give specific recommendations for the ABI PRISM 7000, ABI PRISM 7700, Applied Biosystems 7300, Applied Biosystems 7500, ABI ViiA7, Applied Biosystems 7900HT, StepOne, and StepOnePlus.

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

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

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

‡ Filter D is for detecting ROX passive reference dye, a component of 2x QuantiFast Probe RT-PCR Plus 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 7500 and ABI ViiA7

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

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

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

‡ Filter D is for detecting ROX passive reference dye, which is supplied as a separate solution with the QuantiFast Probe RT-PCR Plus Kit. The dye can be added to reactions, or premixed with 2x QuantiFast Mix 2 (Probe).

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

Table 6. Suitable reporter dyes — Applied Biosystems 7900HT

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

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

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

‡ ROX fluorescent dye is used as passive reference and is a component of 2x QuantiFast Mix 2 (Probe).

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

Table 7. Suitable reporter dyes — Applied Biosystems StepOne¶

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

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

** Use filter 1 to detect the least abundant target.

†† ROX fluorescent dye is used as passive reference and is a component of 2x QuantiFast Mix 2 (Probe).

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

Table 8. Suitable reporter dyes — Applied Biosystems StepOnePlus

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

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

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

‡ Filter 4 is for detecting ROX passive reference dye, a component of 2x QuantiFast Mix 2 (Probe).

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

Table 9. Suitable reporter dyes — ABI PRISM 7700¶

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

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

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

†† ROX fluorescent dye is used as passive reference and is a component of 2x QuantiFast Mix 2 (Probe).

‡‡ 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 duplex 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 duplex assays using Mx3000P, Mx3005P, and Mx4000 systems are given in Table 10 (page 23).

- Before performing a duplex 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 duplex analysis.
- Different detection filter sets are available for Mx3000P, Mx3005P, and Mx4000 systems. Note that only some combinations of filter sets are compatible with duplex analysis using commonly used reporter dyes. For details, see Table 10 (page 23).

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

Type of assay	Optical path 1 (FAM filter set)†	Optical path 2 (HEX/ JOE filter set)†	Optical path 3 (ROX filter set)†	Optical path 4 (Cy5 filter set)†
Duplex	6-FAM	HEX JOE VIC MAX		
Duplex	6-FAM		Texas Red ROX	
Duplex	6-FAM			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 duplex 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 duplex assays using the iCycler iQ system are given in Table 11 (page 25).

- Before performing a duplex 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 duplex 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 duplex analysis.

Table 11. Suitable reporter dyes — iCycler iQ

Type of assay	Channel 1 (filter 490/530)*†	Channel 2 (filter 530/575)*†	Channel 3 (filter 575/620)*†	Channel 4 (filter 635/680)*†
Duplex	6-FAM	HEX JOE VIC MAX		
Duplex	6-FAM		Texas Red ROX	
Duplex	6-FAM			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 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 12 (page 26). For optimal results, we recommend carrying out duplex assays.

Table 12. 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 MAX		
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 duplex assays using the LightCycler 2.0 system are given in Table 13 (page 27).

- The LightCycler 2.0 system always detects fluorescence in all of its detection channels. Therefore, there is no need to activate/deactivate the appropriate detection channels for each duplex assay.
- Duplex 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 that describes how to generate and use color compensation files for duplex 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 13 (page 27) will give a detectable fluorescent signal.

- The reporter dyes shown in Table 13 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 PCR.
- Refer to the *LightCycler 2.0 Instrument Operator's Manual* for additional information on the detection optics and correctly setting up the instrument.

Table 13. 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 MAX			
Duplex	6-FAM		Texas Red ROX		

* 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.

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 duplex assays using the LightCycler 480 system are given in Table 14 (page 29).

- Check that each selected reporter dye is compatible with one of the detection channels installed on the instrument. Ensure that each reporter dye is detected by a different channel.
- Refer to the *LightCycler 480 Operator's Manual* for additional information on activating and deactivating detection channels and correctly setting up the instrument for duplex 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 that 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 14. Suitable reporter dyes — LightCycler 480

Type of assay	Channel 1 (450/500)*†	Channel 2 (483/533)*†	Channel 3 (523/568)*†	Channel 4 (558/610)*†	Channel 5 (615/670)*†
Duplex		**6-FAM**	**MAX** **HEX** JOE VIC		
Duplex		**6-FAM**		**Texas Red** **ROX**	
Duplex		**6-FAM**			**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. Elimination of DNA contamination by the genomic DNA removal step can be followed 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 residual contaminating DNA.

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: Duplex RT-PCR on Most Applied Biosystems Cyclers

This protocol is for use with the QuantiFast Probe RT-PCR Plus Kit and TaqMan probes on any real-time cycler from Applied Biosystems **except Applied Biosystems 7500 Real-Time PCR Systems and the ABI ViiA7**. For further information, see "Passive reference dye", page 11.

Note: The protocol is can be easily adapted for singleplex RT-PCR by using one primer-probe mix.

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

Things to do before starting

- For ease of use, we recommend preparing for each of your targets a 20x primer–probe mix containing target-specific primers and probe. A 20x primer–probe mix for duplex RT-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 are using QuantiFast Probe Assays, please reconstitute with TE buffer according to the instructions in the product sheet. After reconstitution, QuantiFast Probe Assays (20x) can be used in the following protocol.

Procedure

1. **Thaw 2x QuantiFast Mix 1, 2x QuantiFast Mix 2 (Probe), High-ROX Dye Solution, template RNA, primer and probe solutions, and RNase-free water. Mix the individual solutions. QuantiFast RT Mix should be taken from –20°C immediately before use, and returned to storage at –20°C immediately after use.**
2. **Prepare a reaction mix for removal of genomic DNA according to Table 15.**

Table 15. Reaction setup for genomic DNA removal

Component	Volume/reaction	Final concentration
2x QuantiFast Mix 1	6.25 µl	1x
Template RNA (added at step 4)	Variable	≤100 ng/reaction
RNase-free water	Variable	
Total reaction volume	**13 µl***	

* Related to a final volume of 25 µl during QuantiFast real-time PCR.

3. **Mix the reaction mixture thoroughly, and dispense appropriate volumes into PCR tubes or the wells of a PCR plate.**

   Note: Do not keep the PCR vessels or plates on ice.
4. **Add template RNA (≤100 ng) to the individual PCR tubes or wells and incubate for 5 min at room temperature (15–25°C).**

   Note: The incubation step can be prolonged up to 15 min.
5. **Prepare a QuantiFast reaction mix according to Table 16 (page 33). If you are using QuantiFast Probe Assays, prepare a QuantiFast reaction mix according to Table 17 (page 34).**

Table 16. Setup of QuantiFast reaction mix

Component	Volume/reaction	Final concentration
2x QuantiFast Mix 2 (Probe)	6.25 µl	1x
20x primer-probe mix 1†	1.25 µl	0.4 µM forward primer 1‡ 0.4 µM reverse primer 1‡ 0.2 µM probe 1§
20x primer-probe mix 2†	1.25 µl	0.4 µM forward primer 2‡ 0.4 µM reverse primer 2‡ 0.2 µM probe 2§
High-ROX Dye Solution	0.5 µl	
QuantiFast RT Mix	0.25 µl	
RNase-free water	2.5 µl	
Total reaction volume	**12 µl***	

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

† A 20x primer–probe mix for duplex RT-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.

Table 17. Setup of QuantiFast reaction mix with QuantiFast Probe Assays

Component	Volume/reaction	Final concentration
2x QuantiFast Mix 2 (Probe)	6.25 µl	1x
20x QuantiFast Probe Assay (FAM)	1.25 µl	1x
20x QuantiFast Probe Assay (MAX)	1.25 µl	1x
High-ROX Dye Solution	0.5 µl	
QuantiFast RT Mix	0.25 µl	
RNase-free water	2.5 µl	
Total reaction volume	**12 µl***	

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

† A 20x primer–probe mix for duplex RT-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.

6. **Mix the QuantiFast reaction mixture thoroughly and dispense appropriate volumes into PCR vessels or plates containing the genomic DNA removal reaction.**

   Note: If the transfer to the real-time cycler needs more than 5 min, store the plates on ice.

7. **Program the real-time cycler according to Table 18 (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.

8. **Place the PCR tubes or plate in the real-time cycler, and start the cycling program.**
9. **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 18. Cycling conditions

Step	Time	Temperature	Additional comments
Reverse transcription	**20 min**	**50°C**	RNA is reverse transcribed into cDNA
PCR initial activation step	**5 min**	**95°C**	HotStarTaq *Plus* DNA Polymerase is activated by this heating step
2-step cycling			**Important: Optimal performance is only assured using these cycling conditions**
Denaturation	**15 s**	**95°C**	
Annealing/extension	**30 s**	**60°C**	Combined annealing/ extension step with fluorescence data collection
Number of cycles	40–45		The number of cycles depends on the amount of template RNA and the expression level of the target gene.

Protocol: Duplex RT-PCR on Rotor-Gene Instruments, the Applied Biosystems 7500, the ABI ViiA7, and Other Cyclers

This protocol is for use with the QuantiFast Probe RT-PCR Plus Kit and TaqMan probes on Rotor-Gene Instruments, Applied Biosystems 7500 Real-Time PCR Systems, the ABI ViiA7, and on real-time cyclers from Bio-Rad/MJ Research, Cepheid, Eppendorf, Roche, and Agilent. For further information, see "Passive reference dye", page 11.

Note: The protocol can be easily adapted for singleplex RT PCR by using one primer-probe mix.

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 duplex assay.
- Read "Guidelines for effective duplex assays", page 15. Check whether your real-time cycler is compatible with the chosen combination of reporter dyes.
- If using an already established duplex real-time RT-PCR assay, use the previously established primer and probe concentrations in combination with the cycling conditions specified in this protocol. It is not necessary to determine primer limiting concentrations again.
- **Optimal analysis settings are a prerequisite for accurate quantification data.** For data analysis, you should always readjust the analysis settings (i.e., baseline settings and threshold values) for analysis of every reporter dye channel in every run.
- If using the **LightCycler 2.0** or **LightCycler 480**, be sure to create a color compensation file. For details, download QIAGEN Supplementary Protocol PCR81 (for the LightCycler 2.0) or PCR82 (for the LightCycler 480) 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 RT-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 are using QuantiFast Probe Assays, please reconstitute with TE buffer according to the instructions in the product sheet. After reconstitution, QuantiFast Probe Assays (20x) can be used in the following protocol.

Procedure

1. **Thaw 2x QuantiFast Mix 1, 2x QuantiFast Mix 2 (Probe), ROX Dye Solution, template RNA, primer and probe solutions, and RNase-free water. Mix the individual solutions. QuantiFast RT Mix should be taken from –20°C immediately before use, always kept on ice, and returned to storage at –20°C immediately after use.**
2. **Prepare a reaction mix for removal of genomic DNA according to Table 19.**

Table 19. Reaction setup for genomic DNA removal

Component	Volume/reaction	Final concentration
2x QuantiFast Mix 1	6.25 µl	1x
Template RNA (added at step 4)	Variable	≤ 100 ng/reaction
RNase-free water	Variable	
Total reaction volume	**13 µl***	

* If your real-time cycler requires a final reaction volume other than 25 µl, adjust the amount of master mix and all other reaction components accordingly.

3. **Mix the reaction mixture thoroughly, and dispense appropriate volumes into PCR tubes, PCR capillaries, or the wells of a PCR plate.**

   Note: Do not keep the PCR vessels or plates on ice.
4. **Add template RNA (≤100 ng) to the individual PCR tubes, PCR capillaries, or wells and incubate for 5 min at room temperature (15–25°C).**

   Note: The incubation step can be prolonged up to 15 min.
5. **Prepare a QuantiFast reaction mix according to Table 20 (page 38). If you are using QuantiFast Probe Assays, prepare a QuantiFast reaction mix according to Table 21 (page 39).**

Table 20. Setup of QuantiFast reaction mix

Component	Volume/reaction	Final concentration
2x QuantiFast Mix 2 (Probe)	6.25 µl	1x
20x primer-probe mix 1[†]	1.25 µl	0.4 µM forward primer 1[‡] 0.4 µM reverse primer 1[‡] 0.2 µM probe 1[§]
20x primer-probe mix 2[†]	1.25 µl	0.4 µM forward primer 2[‡] 0.4 µM reverse primer 2[‡] 0.2 µM probe 2[§]
ROX Dye Solution[¶]	0.5 µl	
QuantiFast RT Mix	0.25 µl	
RNase-free water	2.5 µl	
Total reaction volume	**12 µl***	

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

† A 20x primer–probe mix for duplex RT-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.

¶ For cyclers that do not require ROX dye, add RNase-free water instead.

Table 21. Setup of QuantiFast reaction mix with QuantiFast Probe Assays

Component	Volume/reaction	Final concentration
2x QuantiFast Mix 2 (Probe)	6.25 µl	1x
20x QuantiFast Probe Assay (FAM)	1.25 µl	1x
20x QuantiFast Probe Assay (MAX)	1.25 µl	1x
ROX Dye Solution[¶]	0.5 µl	
QuantiFast RT Mix	0.25 µl	
RNase-free water	2.5 µl	
Total reaction volume	**12 µl***	

* If your real-time cycler requires a final reaction volume other than 25 µl, adjust the amount of master mix and all other reaction components accordingly.

† A 20x primer–probe mix for duplex RT-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.

¶ For cyclers that do not require ROX dye, add RNase-free water instead.

6. Mix the QuantiFast reaction mixture thoroughly and dispense appropriate volumes into PCR vessels or plates containing the genomic DNA removal reaction.

Note: If the transfer to the real-time cycler needs more than 5 min, store the plates on ice.

7. **Program the real-time cycler according to Table 22 (below).**

   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.

8. **Place the PCR tubes, capillaries, or plate in the real-time cycler, and start the cycling program.**

9. **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. Cycling conditions

Step	Time	Temperature	Additional comments
Reverse transcription	**20 min**	**50°C**	RNA is reverse transcribed into cDNA
PCR initial activation step	**5 min**	**95°C**	HotStarTaq *Plus* DNA Polymerase is activated by this heating step
2-step cycling			**Important: Optimal performance is only assured using these cycling conditions**
Denaturation	**15 s**	**95°C**	
Annealing/extension	**30 s**	**60°C**	Combined annealing/ extension step with fluorescence data collection
Number of cycles	40–45		The number of cycles depends on the amount of template RNA 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 *Plus* DNA Polymerase (95°C for 5 min), and the specified times for denaturation and annealing/extension.
b) HotStarTaq *Plus* DNA Polymerase not activated	Ensure that the cycling program includes the HotStarTaq *Plus* DNA Polymerase activation step (5 min at 95°C) as described in the protocols.
c) Pipetting error or missing reagent	Check the concentrations and storage conditions of the reagents, including primers, probes, and template nucleic acid. Repeat the RT-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	In most cases, a primer concentration of 0.4 µM gives satisfactory results on all real-time cyclers. Before adapting primer concentration, check the concentration of your primer solutions. 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. If using commercial probe-based assays (e.g., TaqMan Gene Expression Assays), the final concentration in reactions should be 1x, as recommended by the supplier.
f) Mg^{2+} concentration not optimal	The Mg^{2+} concentration in QuantiFast Mix 2 (Probe) is already optimized. Increasing the final Mg^{2+} concentration by 0.5–1 mM may improve results.
g) Problems with starting template	Check the concentration, storage conditions, and quality of the starting template. If necessary, make new serial dilutions of template nucleic acid from the stock solutions. Repeat the RT-PCR using the new dilutions. Ensure that all reagents, buffers, and solutions used for purification and dilution of template nucleic acids are free of nucleases.
h) Insufficient amount of starting template	Increase the amount of template if possible. Ensure that sufficient copies of the target nucleic acids are present in your sample.
i) Insufficient number of cycles	Increase the number of cycles.
j) Probe design not optimal	If the amplification reaction was successful, there may be a problem with the probe. Review the probe design guidelines.

	Comments and suggestions
k) Wrong detection channel/filter chosen	Ensure that the correct detection channel is activated or the correct filter set is chosen for each reporter dye. Check whether the chosen combination of reporter dyes is compatible with the selected detection channels or filter sets.
l) Fluorescence crosstalk	Check that the reporter dyes used in your assay are suitable for duplex analysis on your instrument. Run appropriate controls to estimate potential crosstalk effects.

Differences in C_T values or in PCR efficiencies between a duplex RT-PCR assay and the corresponding singleplex RT-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 *Plus* DNA Polymerase (95°C for 5 min), and the specified times for denaturation and annealing/extension.
b) Analysis settings (e.g., threshold and baseline settings) not optimal	Check the analysis settings (threshold and baseline settings) for each reporter dye. Repeat analysis using optimal settings for each reporter dye.
c) Imprecise spectral separation of reporter dyes	Since duplex RT-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 duplex 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 duplex 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	When signals are coming up at very early C_T values, adjust the analysis settings accordingly.
b) Template amount too low	Increase template amount if possible. Note that detection of very low starting copy numbers may not be in the linear range of a standard curve.
Increased fluorescence or C_T value for "No Template" control	
a) Contamination of reagents	Discard all the components of the duplex assay (e.g., master mix, primers, and probes). Repeat the duplex assay using new components.
b) 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	
a) Incomplete genomic DNA removal	In rare cases a C_T <40 is observed. Prolong the incubation step after adding RNA from 5 min up to 15 min (step 4 of the protocols). Make sure that the reagents used for the incubation at step 4 are not cooled on ice.
b) Sequence dependent effects	In rare cases a C_T <40 is observed although a prolonged incubation step of up to 15 min after adding RNA (step 4 of the protocols) has been performed.
Varying fluorescence intensity	
a) Contamination of real-time cycler	Decontaminate the real-time cycler according to the manufacturer's instructions.
b) Real-time cycler no longer calibrated	Recalibrate the real-time cycler according to the manufacturer's instructions.
c) Wavy curve at high template amounts for highly expressed targets	In the analysis settings, reduce the number of cycles used for background calculation (if your real-time cycler allows you to do so) or reduce the amount of template.

	Comments and suggestions
d) **ABI PRISM 7000**: Uneven curves or high standard deviations	Do not use reaction volumes smaller than 25 µl, and always use optical adhesive covers to seal plates. In some cases, increasing the reaction volume to 50 µl may improve results.

Appendix A: Assay Design and Handling Primers and Probes

Important factors for success in quantitative, duplex, 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.

For predesigned primer-probe assays for your targets, visit www.qiagen.com/GeneGlobe.

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 duplex assays, we recommend preparing 20x primer–probe mixes, each containing 2 primers and 1 probe for a particular target at the suggested concentrations (see protocols).

Dissolving primers and probes

Before opening a tube containing lyophilized primer or probe, centrifuge the tube briefly to collect all material at the bottom of the tube. To dissolve the primer or the probe, add the required volume of sterile, nuclease-free TE buffer, 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:

$\varepsilon_{260} = 0.89 \times [(A \times 15{,}480) + (C \times 7340) + (G \times 11{,}760) + (T \times 8850)]$

Example

Concentration of diluted primer: 1 µM = 1×10^{-6} M

Primer length: 24 nucleotides with 6 each of A, C, G, and T bases

Calculation of expected A_{260}: $0.89 \times [(6 \times 15{,}480) + (6 \times 7340) + (6 \times 11{,}760) + (6 \times 8850)] \times (1 \times 10^{-6}) = 0.232$

The measured A_{260} should be within ±30% of the theoretical value. If the measured A_{260} is very different to the theoretical value, we recommend recalculating the concentration of the primers or probes, or having the primers or probes resynthesized.

For probes, the fluorescent dye does not significantly affect the A_{260} value.

Primer and probe quality

The quality of 18–30mers can be checked on a 15% denaturing polyacrylamide gel;* a single band should be seen. Please contact QIAGEN Technical Services or your local distributor for a protocol.

Probe quality

The quality of the fluorescent label and the purity of TaqMan probes can be determined by comparing fluorescence before and after DNase digestion. Incubate probes with or without 5 units DNase* at 37°C for 1 hour. A significant difference in fluorescence following DNase treatment should be detectable.

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

Appendix B: 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 duplex 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 50).

- 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 QuantiFast Probe RT-PCR Plus Test 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 16).

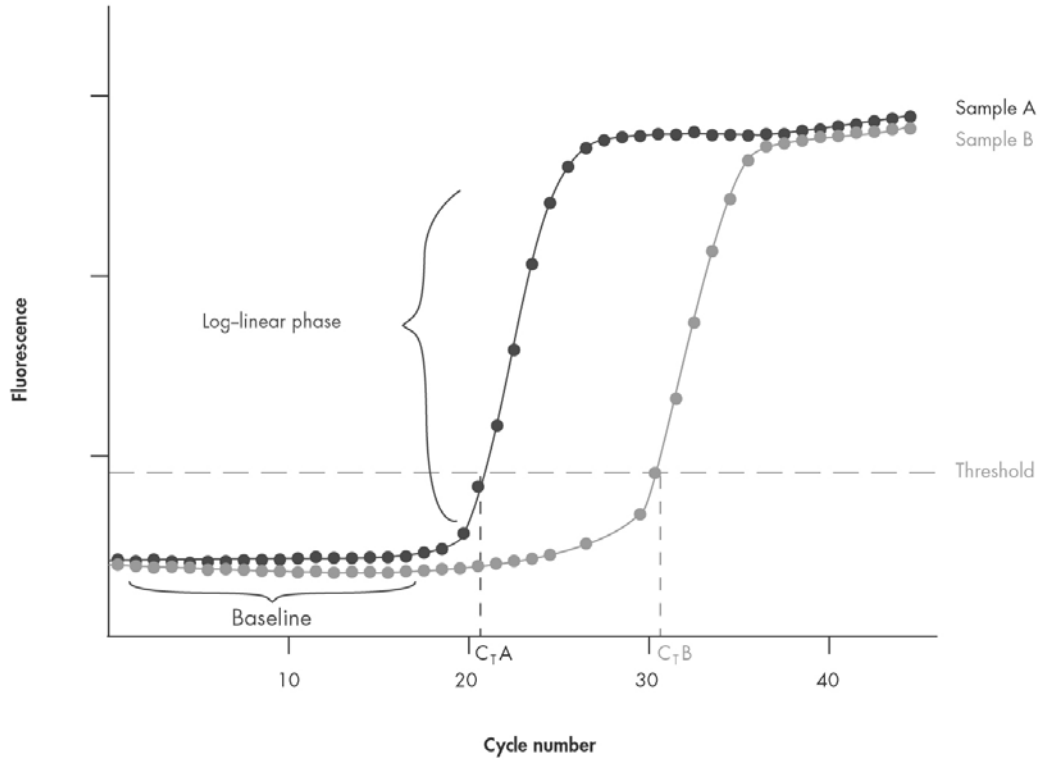


Figure 2. Typical amplification plot. Amplification plots showing increases in fluorescence from 2 samples (Sample A and Sample B).

Appendix C: Evaluating the Quality of Quantitative, Real-Time, Duplex 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 duplex assay.
- Compare the performance of the duplex 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 duplex 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. In vitro transcripts or purified mRNA can be used as template if performing duplex, 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 49, and the user manual supplied with your real-time cycler.

Note: When running singleplex and duplex 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)".

References

QIAGEN maintains a large, up-to-date online database of scientific publications utilizing QIAGEN products. Comprehensive search options allow you to find the articles you need, either by a simple keyword search or by specifying the application, research area, title, etc.

For a complete list of references, visit the QIAGEN Reference Database online at www.qiagen.com/RefDB/search.asp or contact QIAGEN Technical Services or your local distributor.

Ordering Information

Product	Contents	Cat. no.
QuantiFast Probe RT-PCR Plus Kit (80)	For 80 x 25 µl reactions: 0.5 ml 2x QuantiFast Mix 1, 0.5 ml 2x QuantiFast Mix 2 (Probe), 100 µl QuantiFast RT Mix, 210 µl ROX Dye Solution, 210 µl High-ROX Dye Solution, 2 ml RNase-Free Water	204482
QuantiFast Probe RT-PCR Plus Kit (400)	For 400 x 25 µl reactions: 2 x 1.3 ml 2x QuantiFast Mix 1, 2 x 1.3 ml 2x QuantiFast Mix 2 (Probe), 100 µl QuantiFast RT Mix, 210 µl ROX Dye Solution, 210 µl High-ROX Dye Solution, 2 ml RNase-Free Water	204484
Related products		
QuantiFast Probe Assays*	For 80 x 25 µl reactions: dual-labeled, probe-based, predesigned 20x lyophilized assays; includes master mix and reagents for real-time one-step or two-step RT-PCR	Varies
QuantiFast Probe Assays*	For 400 x 25 µl reactions: dual-labeled, probe-based, predesigned 20x lyophilized assays; includes master mix and reagents for real-time one-step or two-step RT-PCR	Varies
For purification of total RNA from formalin-fixed, paraffin-embedded tissue sections		
RNeasy® FFPE Kit (50)	For 50 minipreps: 50 RNeasy MinElute® Spin Columns, 50 gDNA Eliminator Mini Spin Columns, Collection Tubes, RNase-Free Reagents and Buffers	73504

* Primer/probe sets for singleplex or duplex PCR labeled with FAM or MAX. Visit www.qiagen.com/GeneGlobe to search for and order this product.

Product	Contents	Cat. no.
For purification of microRNA and total RNA from formalin-fixed, paraffin-embedded tissue sections		
miRNeasy FFPE Kit (50)	For 50 minipreps: 50 RNeasy MinElute Spin Columns, 50 gDNA Eliminator Spin Columns, Collection Tubes, Proteinase K, RNase-Free Reagents and Buffers	217504
For simultaneous purification of DNA and RNA from formalin-fixed, paraffin-embedded tissue sections		
AllPrep® DNA/RNA FFPE Kit (50)	For 50 minipreps: 50 AllPrep DNA Spin Columns, 50 RNeasy Mini Spin Columns, Collection Tubes, RNase-Free Water and Buffers	80234

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.

Notes

www.qiagen.com

Australia ■ techservice-au@qiagen.com

Austria ■ techservice-at@qiagen.com

Belgium ■ techservice-bnl@qiagen.com

Brazil ■ suportetecnico.brasil@qiagen.com

Canada ■ techservice-ca@qiagen.com

China ■ techservice-cn@qiagen.com

Denmark ■ techservice-nordic@qiagen.com

Finland ■ techservice-nordic@qiagen.com

France ■ techservice-fr@qiagen.com

Germany ■ techservice-de@qiagen.com

Hong Kong ■ techservice-hk@qiagen.com

India ■ techservice-india@qiagen.com

Ireland ■ techservice-uk@qiagen.com

Italy ■ techservice-it@qiagen.com

Japan ■ techservice-jp@qiagen.com

Korea (South) ■ techservice-kr@qiagen.com

Luxembourg ■ techservice-bnl@qiagen.com

Mexico ■ techservice-mx@qiagen.com

The Netherlands ■ techservice-bnl@qiagen.com

Norway ■ techservice-nordic@qiagen.com

Singapore ■ techservice-sg@qiagen.com

Sweden ■ techservice-nordic@qiagen.com

Switzerland ■ techservice-ch@qiagen.com

UK ■ techservice-uk@qiagen.com

USA ■ techservice-us@qiagen.com

QIAGEN

1071013 02/2012

Sample & Assay Technologies