May 2011

QuantiTect® Probe PCR Handbook

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

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QIAGEN Sample and Assay Technologies

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- Automation of sample and assay technologies

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Contents

Kit Contents

QuantiTect Probe PCR Kit	(40)	(200)*	(1000)
Catalog no.	**204341**	**204343**	**204345**
Number of 50 µl reactions	**40**	**200**	**1000**
2x QuantiTect Probe PCR Master Mix, containing: ■ HotStarTaq® DNA Polymerase ■ QuantiTect Probe PCR Buffer ■ dNTP mix, including dUTP ■ ROX™ passive reference dye ■ 8 mM $MgCl_2$	1 ml	3 x 1.7 ml	25 ml
RNase-Free Water	2 ml	2 x 2 ml	20 ml
Handbook	1	1	1

* For optional UNG treatment, we recommend the QuantiTect Probe PCR +UNG Kit (cat. no. 204363), which consists of the QuantiTect Probe PCR Kit (200) and an optimized UNG solution.

Shipping and Storage

The QuantiTect Probe PCR 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). 2x QuantiTect Probe PCR Master Mix 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 the QuantiTect Probe PCR Kit 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 Probe PCR Kit is intended for molecular biology applications. This product is not intended for the diagnosis, prevention, or treatment of a disease.

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

Product Warranty and Satisfaction Guarantee

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

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

Technical Assistance

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

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

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

Safety Information

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

24-hour emergency information

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

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

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 Probe PCR Buffer*	Contains Tris·Cl, KCl, $(NH_4)_2SO_4$, 8 mM $MgCl_2$, pH 8.7 (20°C)
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 Probe PCR Master Mix.

Quality Control

Component	Test
QuantiTect Probe PCR Master Mix†	PCR sensitivity and reproducibility assay: Sensitivity and reproducibility in real-time PCR are tested in parallel reactions containing 10-fold dilutions of nucleic acid template.
HotStarTaq DNA Polymerase‡	Efficiency and reproducibility in PCR are tested. Functional absence of exonucleases and endonucleases is tested.
QuantiTect Probe PCR Buffer‡	Conductivity and pH are tested.
RNase-free water	Conductivity, pH, and RNase activities are tested.

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

‡ Included in 2x QuantiTect Probe PCR Master Mix.

Introduction

The QuantiTect Probe PCR Kit provides accurate real-time quantification of DNA and cDNA targets in an easy-to-handle format. The kit 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 24). The kit is designed for use with all types of probes, including TaqMan® dual-labeled probes, FRET probes, and Molecular Beacons. High specificity and sensitivity in PCR are achieved by the use of the hot-start enzyme HotStarTaq DNA Polymerase together with a specialized PCR buffer. The buffer also contains ROX dye, which allows fluorescence normalization on certain cyclers.

The kit has been optimized for use with any real-time cycler, including Rotor-Gene® cyclers* and instruments from Applied Biosystems®, Bio-Rad, Cepheid, Eppendorf, Roche, and Agilent (formerly Stratagene). This handbook contains general protocols for use with cyclers from these suppliers.

2x QuantiTect Probe PCR Master Mix

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

HotStarTaq DNA Polymerase

HotStarTaq DNA Polymerase is a modified form of QIAGEN *Taq* DNA Polymerase, and 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, leading to 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 Probe PCR Buffer

QuantiTect Probe PCR Buffer is based on the unique QIAGEN PCR buffer system, and has been specifically adapted for real-time PCR using sequence-specific probes. 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 usually not required.

* To take advantage of the fast-cycling capabilities of Rotor-Gene cyclers, use optimized Rotor-Gene Kits; for details, visit www.qiagen.com/goto/Rotor-GeneKits.

Passive reference dye

For certain real-time cyclers, the presence of ROX passive reference dye in real-time PCR compensates for non-PCR–related variations in fluorescence detection. Fluorescence from ROX dye does not change during the course of real-time PCR, but provides a stable baseline to which PCR-related fluorescent signals are normalized. Thus, ROX dye compensates for differences in fluorescence detection between wells due to slight variations in reaction volume or to differences in well position.

The use of ROX dye is necessary for all instruments from Applied Biosystems and is optional for instruments from Agilent (e.g., Mx3000P®, Mx3005P®, and Mx4000®). Rotor-Gene cyclers and instruments from Bio-Rad, Cepheid, Eppendorf, and Roche do not require ROX dye. The presence of ROX dye in the master mix does not interfere with real-time PCR on any instrument, since the dye is not involved in the reaction and has an emission spectrum completely different from fluorescent dyes commonly used for probes.

Use of uracil-N-glycosylase (UNG)

The QuantiTect Probe PCR Kit contains dUTP, which partially replaces dTTP. The QuantiTect Probe PCR Kit therefore allows 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 the QuantiTect Probe PCR Kit and must be purchased separately. We recommend the QuantiTect Probe PCR +UNG Kit (cat. no. 204363), which consists of the QuantiTect Probe PCR Kit (200) and a UNG solution specially optimized for use with QuantiTect PCR buffers.

Sequence-specific probes

The QuantiTect Probe PCR Kit can be used with all types of probes. The 2 major types of sequence-specific probes used for real-time PCR are described briefly below. For a more detailed description of these probes and their design and for other probe types not mentioned here, see "Guidelines for real-time PCR" at www.qiagen.com/resources/info.

Dual-labeled probes

Dual-labeled probes, including 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.

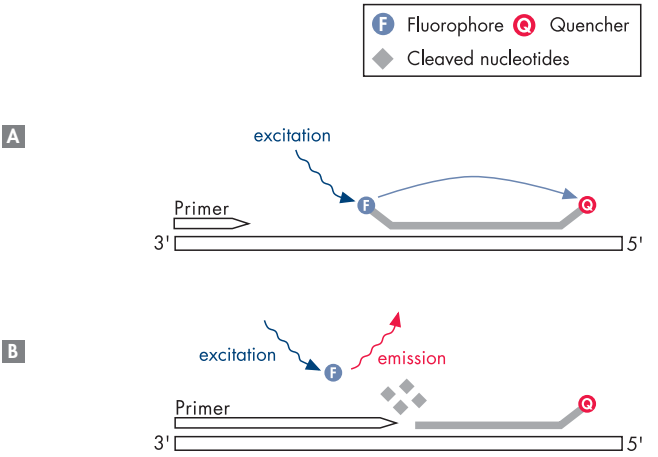

Figure 1. Schematic diagram of the principle of dual-labeled probes in quantitative, real-time PCR. A Both the dual-labeled probe and the PCR primers anneal to the target sequence during the PCR annealing step. The proximity of the fluorophore with the quencher prevents the fluorophore from fluorescing. B During the PCR extension step, *Taq* DNA Polymerase extends the primer. When the enzyme reaches the dual-labeled probe, its 5'→3' exonuclease activity cleaves the fluorophore from the probe. The fluorescent signal from the free fluorophore is measured.

FRET probes

Real-time PCR with fluorescence resonance energy transfer (FRET) probes, such as LightCycler® hybridization probes, uses 2 labeled oligonucleotide probes that bind to the PCR product in a head-to-tail fashion (Figure 2). When the 2 probes bind, their fluorophores come into close proximity, allowing energy transfer from a donor fluorophore to an acceptor fluorophore. This causes fluorescence that is proportional to the amount of PCR product. FRET probes are not cleaved during the reaction, and can bind to a target again in the next PCR cycle.

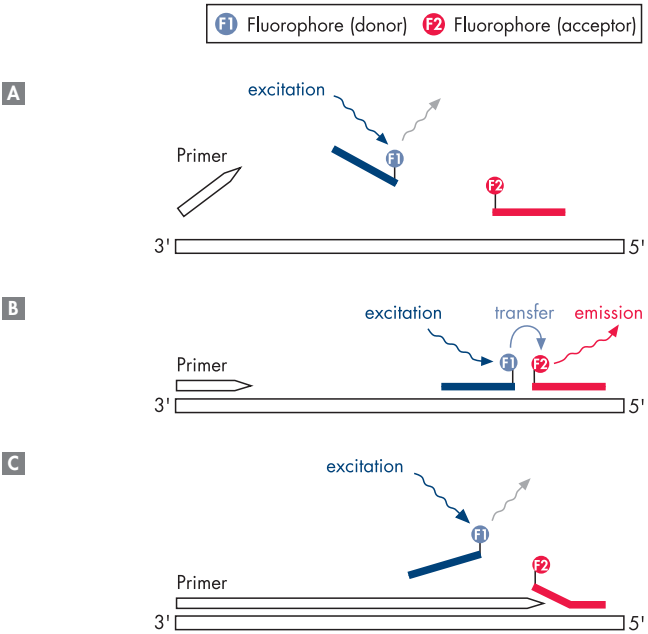

Figure 2. Principle of FRET probes in quantitative, real-time PCR. A When the FRET probes are not bound to the target sequence, no fluorescent signal from the acceptor fluorophore is detected. B During the PCR annealing step, both FRET probes hybridize to the target sequence. This brings the donor fluorophore and acceptor fluorophore into close proximity, allowing energy transfer between the fluorophores and resulting in a fluorescent signal from the acceptor fluorophore that is measured. The amount of signal is proportional to the amount of target sequence, allowing quantification of the amount of target sequence. C During the PCR extension step, the FRET probes are displaced from the target sequence and the acceptor fluorophore is no longer able to generate a fluorescent signal.

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

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

For reverse transcription, we recommend using the QuantiTect Reverse Transcription Kit. The kit provides a fast and convenient procedure, requiring only 20 minutes to synthesize first-strand cDNA and eliminate genomic DNA contamination. An optimized mix of oligo-dT and random primers enables cDNA synthesis from all regions of RNA transcripts, even from 5' regions of very long mRNA transcripts. cDNA yields are high, allowing sensitive detection of even low-abundance transcripts in real-time two-step RT-PCR. An alternative to the QuantiTect Reverse Transcription Kit is the FastLane® Cell cDNA Kit, which allows cDNA to be prepared directly from cultured cells without RNA purification. The FastLane Cell cDNA Kit is useful for experiments where archiving of purified RNA is not required. For ordering information for these 2 kits, see pages 24–25.

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 24.

Using the correct protocol

This handbook contains 2 protocols. The first protocol (page 12) is for use with most real-time cyclers, including Rotor-Gene cyclers and instruments from Applied Biosystems, Bio-Rad, Cepheid, Eppendorf, Roche, and Agilent. The second protocol (page 15) is for use with the LightCycler 1.x and LightCycler 2.0 only.

For background information on real-time PCR, please refer to "Guidelines for real-time PCR" at www.qiagen.com/resources/info, which contains guidelines on template preparation, primer and probe design, controls, data analysis, and other topics.

Protocol: Real-Time PCR and Two-Step RT-PCR Using Applied Biosystems Cyclers and Other Cyclers

This protocol is intended for use with dual-labeled probes (e.g., TaqMan) and most real-time cyclers, including Rotor-Gene cyclers and instruments from Applied Biosystems, Bio-Rad, Cepheid, Eppendorf, Roche, and Agilent. If using the **LightCycler 1.x or LightCycler 2.0**, follow the protocol on page 15.

Reaction volume

A reaction volume of 50 µl should be used with most real-time cyclers. However, the reaction volume must be reduced to 25 µl if using the **Applied Biosystems 7500 Fast System** or a **SmartCycler® system**, or to 10 µl if using a **LightCycler 480**.

When reducing the reaction volume, remember to reduce the volume of master mix used in the reaction: the volume of 2x QuantiTect Probe PCR Master Mix should always be half of the final reaction volume. In addition, be sure to keep the concentration of primers, probe, template, and UNG the same as described in Table 1.

Important points before starting

- Always start with the cycling conditions specified in this protocol, even if using previously established primer–probe systems.
- For the highest efficiency in real-time PCR using sequence-specific probes, targets should ideally be **100–150 bp in length**.
- The PCR must start with an **initial incubation step of 15 min at 95°C** to activate HotStarTaq DNA Polymerase.
- Always readjust the threshold value for analysis of every run.
- The kit has been optimized for a final reaction volume of 50 µl. If other reaction volumes are used, the amount of 2x QuantiTect Probe PCR Master Mix must be adjusted accordingly.
- 2x QuantiTect Probe PCR Master Mix contains dUTP, which allows the use of a uracil-N-glycosylase (UNG) pretreatment of the reaction.
- 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.

Procedure

1. **Thaw 2x QuantiTect Probe PCR Master Mix (if stored at –20°C), template DNA or cDNA, primer and probe solutions, and RNase-free water. Mix the individual solutions.**

2. **Prepare a reaction mix according to Table 1.**

   Due to the hot start, it is not necessary to keep samples on ice during reaction setup or while programming the real-time cycler.

   Note: We strongly recommend starting with an initial Mg^{2+} concentration of 4 mM as provided by 2x QuantiTect Probe PCR Master Mix. For a few targets, reactions may be improved by using Mg^{2+} concentrations of up to 6 mM.

Table 1. Reaction setup

Component	Volume/reaction	Final concentration
2x QuantiTect Probe PCR Master Mix*	25 µl†	1x
Primer A	Variable	0.4 µM‡
Primer B	Variable	0.4 µM‡
Probe	Variable	0.1–0.2 µM
Template DNA or cDNA (added at step 4)	Variable	≤ 500 ng/reaction
RNase-free water	Variable	
Optional: Uracil-N-glycosylase	Variable	0.5 units/reaction
Total reaction volume	**50 µl**	

* Provides a final concentration of 4 mM $MgCl_2$.

† If using a total reaction volume other than 50 µl, calculate the volume of 2x master mix required using this formula: Volume of 2x master mix (µl) = 0.5 x [Total reaction volume (µl)]

‡ A final primer concentration of 0.4 µM is optimal for most applications. However, for individual determination of optimal primer concentration, a primer titration from 0.4 µM to 1 µM can be performed. SmartCycler users should use a final primer concentration of 0.5 µM for each primer; if necessary, a primer titration from 0.5 µM to 1 µM can be performed to determine the optimal primer concentration.

3. **Mix the reaction mix thoroughly, and dispense appropriate volumes into PCR tubes or plates.**
4. **Add template DNA or cDNA (≤ 500 ng/reaction) to the individual PCR tubes or wells containing the reaction mix.**

   For two-step RT-PCR, the volume of the cDNA added (from the undiluted RT reaction) should not exceed 10% of the final PCR volume.
5. **Program your real-time cycler according to the program outlined in Table 2.**
6. **Place the PCR tubes or plates in the real-time cycler, and start the cycling program.**

Table 2. Real-time cycler 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:			
Denaturation*	15 s	94°C	
Combined annealing/ extension	60 s	60°C	Perform fluorescence data collection
Number of cycles	35–45		The number of cycles depends on the amount of template DNA

* SmartCycler users can reduce denaturation time to 1 s to take advantage of cycling capacities.

Protocol: Real-Time PCR and Two-Step RT-PCR Using the LightCycler 1.x and 2.0

This protocol is intended for use with the LightCycler 1.x and LightCycler 2.0 only, and is suitable for FRET probes and dual-labeled probes (e.g., TaqMan). For all other cyclers, follow the protocol on page 12.

Important points before starting

- Always start with the cycling conditions specified in this protocol, even if using previously established primer–probe systems.
- For the highest efficiency in real-time PCR using sequence-specific probes, targets should ideally be **100–150 bp in length**.
- The PCR must start with an **initial incubation step of 15 min at 95°C** to activate HotStarTaq DNA Polymerase.
- We recommend using the "second derivative maximum" method for data analysis.
- Always readjust the noise band for analysis of every run if using the "fit-point" method for data analysis.
- 2x QuantiTect Probe PCR Master Mix contains dUTP, which allows the use of a uracil-N-glycosylase (UNG) pretreatment of the reaction.

Procedure

1. **Thaw 2x QuantiTect Probe PCR Master Mix (if stored at –20°C), template DNA or cDNA, primer and probe solutions, and RNase-free water. Mix the individual solutions.**
2. **Prepare a reaction mix according to Table 3.**

   Due to the hot start, it is not necessary to keep samples on ice during reaction setup or while programming the real-time cycler.

   Note: We strongly recommend starting with an initial Mg^{2+} concentration of 4 mM as provided by 2x QuantiTect Probe PCR Master Mix. For a few targets, reactions may be improved by using Mg^{2+} concentrations of up to 6 mM.

Table 3. Reaction setup

Component	Volume/reaction	Final concentration
2x QuantiTect Probe PCR Master Mix*	10 µl	1x
Primer A	Variable	0.5 µM†
Primer B	Variable	0.5 µM†
Probe	Variable	0.1–0.2 µM
Template DNA or cDNA (added at step 4)	Variable	≤1 µg/reaction
RNase-free water	Variable	
Optional: Uracil-N-glycosylase	Variable	0.5 units/reaction
Total reaction volume	**20 µl**	

* Provides a final concentration of 4 mM $MgCl_2$.

† A final primer concentration of 0.5 µM is optimal for most applications. However, for individual determination of optimal primer concentration, a primer titration from 0.5 µM to 1 µM can be performed.

3. **Mix the reaction mix thoroughly, and dispense appropriate volumes into PCR capillaries.**
4. **Add template DNA or cDNA (≤1 µg/reaction) to the individual PCR capillaries containing the reaction mix.**

   For two-step RT-PCR, the volume of the cDNA added (from the undiluted RT reaction) should not exceed 10% of the final PCR volume.
5. **Program the LightCycler according to the program outlined in Table 4 (FRET probes) or Table 5 (TaqMan and other dual-labeled probes). Set the channels according to Table 6.**
6. **Place the PCR capillaries in the LightCycler, and start the cycling program.**

Table 4. Real-time cycler conditions for FRET probes

Step	Time	Temperature	Ramp rate	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
3-step cycling:				
Denaturation	0 s	95°C	20°C/s	
Annealing	30 s	50–60°C	20°C/s	Approximately 5–8°C below T_m of primers. Perform fluorescence data collection.
Extension	30 s	72°C	2°C/s	
Number of cycles	35–55			The number of cycles depends on the amount of template DNA

Table 5. Real-time cycler conditions for TaqMan and other dual-labeled probes

Step	Time	Temperature	Ramp rate	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:				
Denaturation	0 s	95°C	20°C/s	
Combined annealing/ extension	60 s	60°C	20°C/s	Perform fluorescence data collection
Number of cycles	35–55			The number of cycles depends on the amount of template DNA

Table 6. Channel settings during run (Display Mode) and for data analysis

	Detection channel	Display mode	Channel settings for data analysis
Dual-labeled probes (FAM™)	F1	F1/1	F1/F2
Hybridization probe (LC®-Red 640)	F2	F2/1	F2/F1
Hybridization probe (LC-Red 705)	F3	F3/1	F3/F1

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 product, or product detected late in PCR		
a)	Annealing step (FRET probes and Molecular Beacons) or annealing/extension step (dual-labeled probes) too short	Always use the annealing time or annealing/extension time specified in the protocol. In some cases, increasing the time in steps of 10 s can improve results, especially with the LightCycler 1.x and 2.0.
b)	Extension time too short (FRET probes and Molecular Beacons)	Always use the extension time specified in the protocol. In some cases, increasing the time in steps of 10 s can improve results, especially with the LightCycler 1.x and 2.0.
c)	Wrong detection step	Ensure that fluorescence detection takes place during the annealing step when using FRET probes and Molecular Beacons, or during the combined annealing/extension step when using dual-labeled probes.
d)	Pipetting error or missing reagent	Check the concentrations and storage conditions of the reagents, including primers, probe, and template nucleic acid.* Repeat the PCR.
e)	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.
f)	PCR product too long	For optimal results, PCR products should be between 100 and 150 bp. PCR products should not be outside the range of 60–300 bp.

* For details, refer to "Guidelines for real-time PCR" at www.qiagen.com/resources/info.

		Comments and suggestions
g)	Primer design not optimal	Check for PCR products by gel electrophoresis. If no specific PCR products are detected, review the primer design guidelines.*
h)	Primer concentration not optimal	Use optimal primer concentrations. **SmartCycler and LightCycler 1.x and 2.0**: 0.5 µM each primer. **All other cyclers**: 0.4 µM each primer. In some cases, increasing the primer concentration up to 1 µM can improve results. Check the concentrations of primers by spectrophotometry.*
i)	Mg^{2+} concentration not optimal	Always start with the Mg^{2+} concentration provided in 2x QuantiTect Probe PCR Master Mix (4 mM final concentration). For a few targets, an increase up to 6 mM Mg^{2+} may be helpful. Perform the titration in 0.5 mM steps.
j)	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.
k)	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.
l)	Insufficient number of cycles	Increase the number of cycles.
m)	Probe design not optimal	If the amplification reaction was successful, there may be a problem with the probe. Review the probe design guidelines.* If using Molecular Beacons, visit www.molecular-beacons.org for details on probe design.

* For details, refer to "Guidelines for real-time PCR" at www.qiagen.com/resources/info.

		Comments and suggestions
n)	Annealing temperature too high	Decrease annealing temperature in steps of 2°C.
o)	Annealing temperature too low	Increase annealing temperature in steps of 2°C.
p)	No detection activated	Check that fluorescence detection was activated in the cycling program.
q)	Probe synthesis not optimal	Check the quality of dual-labeled probes or Molecular Beacons by incubation with DNase I. A correctly synthesized probe, containing both fluorophore and quencher, will show a significant increase in fluorescence after DNase I incubation.
r)	Primers degraded	Check for possible degradation of primers on a denaturing polyacrylamide gel.
s)	**RT-PCR only**: Volumes of RT reaction added were too high	High volumes of RT reaction added to the PCR may reduce amplification efficiency and the linearity of the reaction. Generally, the volume of undiluted RT reaction added should not exceed 10% of the final PCR volume.
t)	**RT-PCR only:** Transcript not expressed	Repeat the RT-PCR and include a positive control to make sure the absence of RT-PCR product was not due to problems with amplification and detection.*
Real-time cyclers other than the LightCycler 1.x and 2.0:		
u)	Wrong detection channel/filter chosen	Ensure that the correct detection channel is activated or the correct filter set is chosen for the reporter dye.
LightCycler 1.x and 2.0 only:		
v)	Wrong detection channel chosen	Ensure that the correct detection channel is chosen (e.g., F1 for FAM labeled TaqMan probes or Molecular Beacons; F2 for LC-Red 640 labeled FRET probes; and F3 for LC-Red 705 labeled FRET probes).

* For details, refer to "Guidelines for real-time PCR" at www.qiagen.com/resources/info.

		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. **LightCycler 1.x and 2.0**: Do not use more than 1 µg template. **All other cyclers**: Do not use more than 500 ng template.
b)	Template amount too low	Increase template amount, if possible.
c)	**RT-PCR only**: Volumes of RT reaction added were too high	High volumes of RT reaction added to the PCR may reduce amplification efficiency. Generally, the volume of undiluted reverse-transcription reaction added should not exceed 10% of the final PCR volume.
High fluorescence in "No Template" control		
a)	Contamination of reagents	Discard all the components of the assay (e.g., master mix, primers, and probes). Repeat the assay using new components.
b)	Contamination during reaction setup	Take appropriate precautions during reaction setup, such as using aerosol-barrier pipet tips. Use uracil-N-glycosylase to prevent carryover from previous reactions.
High fluorescence in "No Reverse Transcription" control (RT-PCR only)		
	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.
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.

		Comments and suggestions
All cycler systems:		
c)	Wavy curve at high template amounts	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. The halogen bulb is too old. Replace the bulb every 3 months (or after a maximum of 2000 live hours).
LightCycler 1.x and 2.0 only:		
e)	PCR mix not in capillary tip	Centrifuge the capillary to bring the PCR mix into the capillary tip.
f)	Capillary not pushed down completely	Ensure that the capillary is completely pushed down in the LightCycler carousel.
g)	Wrong detection channel	Make sure that the correct channel is chosen.

Ordering Information

Product	Contents	Cat. no.
QuantiTect Probe PCR Kit (40)	For 40 x 50 µl reactions: 1 ml 2x Master Mix, 2 ml RNase-Free Water	204341
QuantiTect Probe PCR Kit (200)	For 200 x 50 µl reactions: 3 x 1.7 ml 2x Master Mix, 2 x 2 ml RNase-Free Water	204343
QuantiTect Probe PCR Kit (1000)	For 1000 x 50 µl reactions: 25 ml 2x Master Mix, 20 ml RNase-Free Water	204345
QuantiTect Probe PCR +UNG Kit (200)	For 200 x 50 µl reactions: 3 x 1.7 ml 2x Master Mix, 100 µl UNG Solution, 2 x 2 ml RNase-Free Water	204363
Accessories		
QuantiTect Reverse Transcription Kit — for fast cDNA synthesis for sensitive real-time two-step RT-PCR		
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 Reverse Transcription Kit (200)	For 200 x 20 µl reactions: gDNA Wipeout Buffer, Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix, and RNase-Free Water	205313
QuantiTect Whole Transcriptome Kit — for unlimited real-time PCR analysis from precious RNA samples		
QuantiTect Whole Transcriptome Kit (25)	For 25 x 50 µl reactions: T-Script Enzyme and Buffer; Ligation Enzymes, Reagent, and Buffer; and REPLI-g® DNA Polymerase and Buffer	207043

Ordering Information

Product	Contents	Cat. no.
FastLane Cell cDNA Kit — for high-speed preparation of cDNA without RNA purification for use in real-time RT-PCR		
FastLane Cell cDNA Kit (50)	Buffer FCW, Buffer FCP, and components for 50 x 20 µl reverse-transcription reactions (gDNA Wipeout Buffer, Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix, and RNase-Free Water)	215011
Related products		
QuantiTect Probe RT-PCR Kit — for quantitative, real-time one-step RT-PCR using sequence-specific probes		
QuantiTect Probe RT-PCR Kit (200)	For 200 x 50 µl reactions: 3 x 1.7 ml 2x Master Mix, 100 µl RT Mix, 2 x 2 ml RNase-Free Water	204443
QuantiTect Probe RT-PCR Kit (1000)	For 1000 x 50 µl reactions: 25 ml 2x Master Mix, 0.5 ml RT Mix, 20 ml RNase-Free Water	204445

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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.

Notes

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