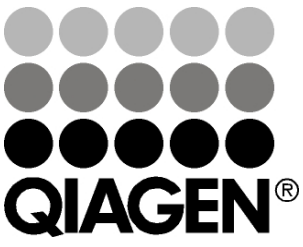
July 2011

QuantiTect® Primer Assay Handbook

For genomewide, ready-to-use real-time RT-PCR assays using SYBR® Green detection



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:

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- Nucleic acid and protein assays
- microRNA research and RNAi
- Automation of sample and assay technologies

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Kit Contents

QuantiTect Primer Assay	**(200)**
Catalog no.	**Varies***
Number of 50 µl reactions	**200**
Number of 25 µl reactions	**400**
10x QuantiTect Primer Assay† in a single tube	1

* Visit www.qiagen.com/GeneGlobe to select and order a QuantiTect Primer Assay.

† Each 10x QuantiTect Primer Assay contains a mix of forward and reverse primers for a specific target. Each assay is supplied lyophilized and must be reconstituted according to the instructions in "Shipping and Storage" (page 5).

Shipping and Storage

QuantiTect Primer Assays are shipped lyophilized at ambient temperature. Store them at –20°C, either lyophilized or reconstituted (see next paragraph). Avoid repeated freeze–thaw cycles. When stored under these conditions and handled correctly, the product can be kept for at least 18 months from date of receipt without reduction in performance.

To reconstitute a tube of 10x QuantiTect Primer Assay, briefly centrifuge the tube, add 1.1 ml TE, pH 8.0,* and mix by vortexing the tube 4–6 times; if necessary, gently warm the tube to help the primers dissolve. We recommend freezing the reconstituted primers in aliquots in order to avoid repeated freezing and thawing.

Product Use Limitations

QuantiTect Primer Assays are intended for molecular biology applications. These products are not intended for the diagnosis, prevention, or treatment of a disease.

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

* For details, see "Equipment and Reagents to Be Supplied by User", page 12.

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 QuantiTect Primer Assays 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

Introduction

QuantiTect Primer Assays allow accurate real-time quantification of target RNA using SYBR Green-based detection on any real-time cycler. The assays have been optimized to provide guaranteed performance when used in combination with one of the following kits, enabling maximum sensitivity and a wide dynamic range:

- **Rotor-Gene® SYBR Green PCR Kit** — for fast two-step RT-PCR on Rotor-Gene cyclers
- **QuantiFast® SYBR Green PCR Kit** — for fast two-step RT-PCR
- **QuantiTect SYBR Green PCR Kit** — for two-step RT-PCR
- **Rotor-Gene SYBR Green RT-PCR Kit** — for fast one-step RT-PCR on Rotor-Gene cyclers
- **QuantiFast SYBR Green RT-PCR Kit** — for fast one-step RT-PCR
- **QuantiTect SYBR Green RT-PCR Kit** — for one-step RT-PCR

QuantiTect Primer Assays can also be used in standard RT-PCR applications, such as gel-based detection of PCR products.

QuantiTect Primer Assays are available for all genes from human, mouse, rat, and many other species. Simply visit www.qiagen.com/GeneGlobe, and select and order the assay for your gene. In addition, other gene-specific products, such as siRNAs, expression vectors, and miRNA mimics, inhibitors, and assays, can also be ordered.

Principle and procedure

QuantiTect Primer Assays enable fast and affordable RNA analysis, and eliminate tedious primer design and assay optimization steps. Each assay consists of specific forward and reverse primers that are derived from gene sequences contained in the NCBI Reference Sequence database (www.ncbi.nlm.nih.gov/RefSeq). Where possible, primers are designed to cross exon/exon boundaries in order to prevent coamplification of genomic DNA, which may compromise assay specificity and dynamic range (see Figure 1). QuantiTect Primer Assays are bioinformatically validated, and they detect RNA only, provided that no pseudogenes with high cDNA similarity exist or that the transcript is not derived from a single-exon gene.

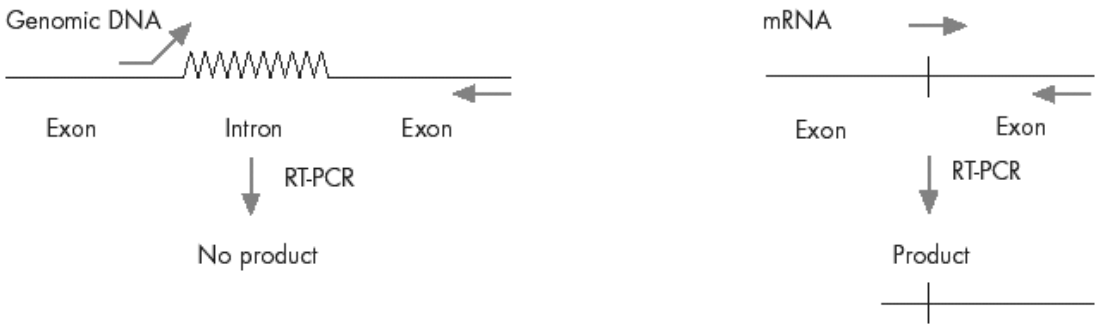

Figure 1. Primer spans an exon/exon boundary. QuantiTect Primer Assays are designed to eliminate amplification of contaminating genomic DNA.

QuantiTect Primer Assays have been optimized for use with any real-time cycler, including Rotor-Gene cyclers and all 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. Performance is guaranteed since QIAGEN has tested QuantiTect Primer Assays on a wide range of real-time cyclers using Rotor-Gene SYBR Green Kits, QuantiFast SYBR Green Kits, and QuantiTect SYBR Green Kits.

Rotor-Gene SYBR Green Kits

Rotor-Gene SYBR Green Kits are ready-to-use, optimized master mixes that provide fast results in SYBR Green-based real-time RT-PCR on Rotor-Gene cyclers. The combination of kits and cycler enables PCR run times of as short as 39 minutes. Kits are available for two-step RT-PCR (Rotor-Gene SYBR Green PCR Kit) and one-step RT-PCR (Rotor-Gene SYBR Green RT-PCR Kit).

Fast PCR run times are achieved through the combination of high-speed data acquisition and a specially developed fast PCR buffer. The components of this buffer, which include the patent-pending additive Q-Bond®, enables a denaturation step of only 5 seconds and a combined annealing/extension step of just 10 seconds. The buffer also contains HotStarTaq® *Plus* DNA Polymerase, which requires only 5 minutes at 95°C for activation. For one-step RT-PCR, the optimized Rotor-Gene RT Mix enables cDNA synthesis in just 10 minutes.

For more details about Rotor-Gene SYBR Green Kits, refer to the *Rotor-Gene SYBR Green Handbook*.

QuantiFast SYBR Green Kits

QuantiFast SYBR Green Kits are ready-to-use, optimized master mixes that provide time savings of up to 60% in SYBR Green-based real-time RT-PCR. Fast and specific results are achieved not only on fast cyclers with rapid ramping rates, but also on all standard cyclers. Kits are available for two-step RT-PCR (QuantiFast SYBR Green PCR Kit) and one-step RT-PCR (QuantiFast SYBR Green RT-PCR Kit).

Fast PCR run times are achieved through a specially developed fast PCR buffer. The components of this buffer, which include the patent-pending additive Q-Bond, significantly reduce denaturation, annealing, and extension times. The buffer also contains HotStarTaq *Plus* DNA Polymerase, which requires only 5 minutes at 95°C for activation. For one-step RT-PCR, the optimized QuantiFast RT Mix enables cDNA synthesis in just 10 minutes.

For more details about QuantiFast SYBR Green Kits, refer to the *QuantiFast SYBR Green PCR Handbook* or *QuantiFast SYBR Green RT-PCR Handbook*.

QuantiTect SYBR Green Kits

QuantiTect SYBR Green Kits are ready-to-use, optimized master mixes for SYBR Green-based real-time RT-PCR. Kits are available for two-step RT-PCR (QuantiTect SYBR Green PCR Kit) and one-step RT-PCR (QuantiTect SYBR Green RT-PCR Kit). The master mixes contain dUTP, which partially replaces dTTP. The master mixes therefore allow the optional use of a uracil-N-glycosylase (UNG) pretreatment of the reaction, if contamination with carried-over PCR products is suspected.

For one-step RT-PCR, only **heat-labile** UNG can be used, since UNG isolated from *E. coli* is stable at elevated temperatures and will destroy any cDNA synthesized during the RT step at 50°C. Heat-labile UNG is active only at the very beginning of the RT step and will eliminate any dUMP-containing RT-PCR products resulting from carryover contamination. After a few minutes, the heat-labile UNG will have lost any activity and therefore cannot interfere with cDNA synthesis.

Note: UNG is not included in QuantiTect SYBR Green Kits and must be purchased separately. For two-step RT-PCR, we recommend purchasing the QuantiTect SYBR Green PCR +UNG Kit, which is comprised of the QuantiTect SYBR Green PCR Kit and a specially developed UNG solution.

For more details about QuantiTect SYBR Green Kits, refer to the *QuantiTect SYBR Green PCR Handbook* or *QuantiTect SYBR Green RT-PCR Handbook*.

Selecting protocols

To easily find protocols for your real-time cycler, please refer to Table 1.

Table 1. Protocols for real-time RT-PCR using QuantiTect Primer Assays

		Protocols for		
Procedure	**Kit**	**Rotor-Gene***	**Block cyclers†**	**Capillary cyclers‡**
Fast two-step RT-PCR	Rotor-Gene SYBR Green PCR Kit	Page 14	–	–
Fast two-step RT-PCR	QuantiFast SYBR Green PCR Kit	–	Page 17	Page 17
Two-step RT-PCR§	QuantiTect SYBR Green PCR Kit¶	Page 20	Page 20	Page 23
Fast one-step RT-PCR	Rotor-Gene SYBR Green RT-PCR Kit	Page 26	–	–
Fast one-step RT-PCR	QuantiFast SYBR Green RT-PCR Kit	–	Page 29	Page 29
One-step RT-PCR§	QuantiTect SYBR Green RT-PCR Kit	Page 32	Page 32	Page 35

* Includes the Rotor-Gene Q, Rotor-Gene 3000, and Rotor-Gene 6000.

† Includes instruments from Applied Biosystems, Bio-Rad, Cepheid, Eppendorf, Roche (LightCycler® 480), and Agilent.

‡ Includes the LightCycler 1.x and LightCycler 2.0 (Roche).

§ Optional UNG pretreatment possible.

¶ If performing optional UNG pretreatment, use the QuantiTect SYBR Green PCR +UNG Kit.

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.

For reconstitution of 10x QuantiTect Primer Assays

TE, pH 8.0 contains 10 mM Tris·Cl and 1 mM EDTA. To prepare 100 ml TE, pH 8.0, mix the following stock solutions:

- 1 ml of 1 M Tris·Cl, pH 8.0 (autoclaved)
- 0.2 ml of 0.5 M EDTA, pH 8.0 (autoclaved)
- 98.8 ml of distilled water

Alternatively, ready-made TE can be purchased from chemicals suppliers.

For two-step RT-PCR protocols

- QuantiTect Primer Assay (visit www.qiagen.com/GeneGlobe to select and order the assay for your gene)
- Rotor-Gene SYBR Green PCR Kit, QuantiFast SYBR Green PCR Kit, or QuantiTect SYBR Green PCR Kit (see ordering information, page 60); if performing UNG pretreatment, use the QuantiTect SYBR Green PCR +UNG Kit (for more information, see page 10 and Appendix D, page 53)
- Buffer, dNTPs, primers, RNase-inhibitor, reverse transcriptase, and RNase-free water for reverse-transcription reactions. We recommend using the QuantiTect Reverse Transcription Kit or the FastLane® Cell cDNA Kit. For more information, see "Important Notes", page 13.

For one-step RT-PCR protocols

- QuantiTect Primer Assay (visit www.qiagen.com/GeneGlobe to select and order the assay for your gene)
- Rotor-Gene SYBR Green RT-PCR Kit, QuantiFast SYBR Green RT-PCR Kit, or QuantiTect SYBR Green RT-PCR Kit (see ordering information, page 60)
- Ice or cooling block (reactions must be set up on ice in order to avoid premature cDNA synthesis)
- Optional (for QuantiTect SYBR Green RT-PCR Kit only): **heat-labile** uracil-N-glycosylase. For more information, see page 10 and Appendix D, page 53.

Important Notes

Reverse transcription

When using QuantiTect Primer Assays for two-step RT-PCR, the RNA must first be reverse transcribed into cDNA. Either total RNA or messenger RNA (mRNA) can be used as template. For optimal results, we strongly recommend using one of the following kits:

- FastLane Cell cDNA Kit — for preparation of cDNA directly from cultured cells in only 45 minutes
- QuantiTect Reverse Transcription Kit — for synthesis of cDNA from purified RNA in only 20 minutes

Both kits are dedicated for use in real-time two-step RT-PCR, and provide:

- High cDNA yields for sensitive detection of even low-abundance transcripts
- Elimination of genomic DNA contamination
- cDNA synthesis from a wide range of RNA amounts (10 pg to 1 µg RNA)
- cDNA synthesis from difficult templates, such as those with high GC-content or complex secondary structure
- cDNA synthesis from all regions of RNA transcripts

For ordering information, see page 60. If using other methods for reverse transcription, follow the guidelines below.

Primers

We strongly recommend using a **mixture of random nonamers (final concentration of 10 µM) and oligo-dT (final concentration of 1 µM) as primers** in the RT step. This guarantees optimal results for all QuantiTect Primer Assays. No special preincubation step (as sometimes performed for random hexamers) is required.

Starting template

The efficiency of the reverse-transcription reaction is highly dependent on the quality and quantity of the starting RNA template. It is important to use intact RNA as starting template. Even trace amounts of contaminating RNases in the RNA sample can cause RNA cleavage, resulting in shortened and incomplete cDNA products. Chemical impurities, such as protein, poly-anions (e.g., heparin), salts, EDTA, ethanol, and phenol, can affect the activity and processivity of the reverse transcriptase. To ensure reproducible and efficient reverse transcription, it is important to determine the quality and quantity of the starting RNA. For details, see Appendix A, page 47.

Protocol: Two-Step RT-PCR (Fast Protocol) for Rotor-Gene Cyclers

This protocol is for use with the Rotor-Gene SYBR Green PCR Kit on the Rotor-Gene Q, Rotor-Gene 3000, or Rotor-Gene 6000.

Important points before starting

- The Rotor-Gene SYBR Green PCR Kit has been developed for use in a **2-step cycling** protocol, with a denaturation step at 95°C and a combined annealing/extension step at 60°C.
- The PCR must start with an **initial incubation step of 5 minutes at 95°C** to activate HotStarTaq *Plus* DNA Polymerase (included in 2x Rotor-Gene SYBR Green PCR Master Mix).
- We recommend a final reaction volume of 25 µl.
- **Always start with the Mg^{2+} concentration as provided** in 2x Rotor-Gene SYBR Green PCR Master Mix.

Things to do before starting

- If using the QuantiTect Primer Assay for the first time, be sure to reconstitute it before use according to the instructions in "Shipping and Storage", page 5.

Procedure

1. **Thaw 2x Rotor-Gene SYBR Green PCR Master Mix (if stored at –20°C), 10x QuantiTect Primer Assay, template cDNA, and RNase-free water. Mix the individual solutions.**
2. **Prepare a reaction mix according to Table 2.**

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

   Note: We strongly recommend starting with the Mg^{2+} concentration as provided in 2x Rotor-Gene SYBR Green PCR Master Mix.

   Note: The volume of the cDNA added (from the undiluted RT reaction) should not exceed 10% of the final PCR volume.

Table 2. Reaction setup for fast two-step RT-PCR on Rotor-Gene cyclers

Component	Volume/reaction	Final concentration
2x Rotor-Gene SYBR Green PCR Master Mix	12.5 µl	1x
10x QuantiTect Primer Assay	2.5 µl	1x
Template cDNA (added at step 4)	Variable	≤100 ng/ reaction
RNase-free water	Variable	–
Total volume	**25 µl**	**–**

3. **Mix the reaction mix thoroughly, and dispense appropriate volumes into PCR tubes.**
4. **Add template cDNA (≤100 ng/reaction) to the individual PCR tubes containing the reaction mix.**
5. **Program the Rotor-Gene cycler according to Table 3.**

   Data acquisition should be performed during the combined annealing/ extension step.

Table 3. Cycling conditions for fast two-step RT-PCR on Rotor-Gene cyclers

Step	Time	Temperature	Additional comments
PCR initial activation step	**5 min**	**95°C**	This step activates HotStarTaq *Plus* DNA Polymerase
2-step cycling:			
Denaturation	5 s	95°C	
Combined annealing/ extension	10 s	60°C	Perform fluorescence data collection
Number of cycles	35–40		The number of cycles depends on the amount of template cDNA and the abundance of the target

6. **Place the PCR tubes in the Rotor-Gene cycler, and start the cycling program.**

   Optional: Melting curve analysis of the PCR product(s) may be performed to verify their specificity and identity. Melting curve analysis is an analysis step built into the software of Rotor-Gene cyclers. Please follow the instructions supplied with the instrument.

   Note: The T_m of a PCR product depends on buffer composition and salt concentration. T_m values obtained when using Rotor-Gene SYBR Green PCR reagents may differ from those obtained using other reagents.

A step-by-step guide to software setup for Rotor-Gene cyclers is available: visit www.qiagen.com/literature/protocols and search for protocol PCR106

Protocol: Two-Step RT-PCR (Fast Protocol)

This protocol is for use with the QuantiFast SYBR Green PCR Kit and any real-time cycler.

Important points before starting

- The QuantiFast SYBR Green PCR Kit has been developed for use in a **2-step cycling** protocol, with a denaturation step at 95°C and a combined annealing/extension step at 60°C.
- The PCR must start with an **initial incubation step of 5 minutes at 95°C** to activate HotStarTaq *Plus* DNA Polymerase (included in 2x QuantiFast SYBR Green PCR Master Mix).
- For 96-well block cyclers, we recommend a final reaction volume of 25 µl. For capillary cyclers, we recommend a final reaction volume of 20 µl. For 384-well block cyclers, we strongly recommend a final reaction volume of 10 µl.
- **Always start with the Mg^{2+} concentration as provided** in 2x QuantiFast SYBR Green PCR Master Mix.

Things to do before starting

- If using the QuantiTect Primer Assay for the first time, be sure to reconstitute it before use according to the instructions in "Shipping and Storage", page 5.
- **If using the iCycler iQ®, iQ5, or MyiQ™**: Well factors must be collected at the beginning of each experiment. Well factors are used to compensate for any system or pipetting nonuniformity. For details, refer to the user manual supplied with the instrument or Appendix E (page 54).

Procedure

1. **Thaw 2x QuantiFast SYBR Green PCR Master Mix (if stored at –20°C), 10x QuantiTect Primer Assay, template cDNA, and RNase-free water. Mix the individual solutions.**
2. **Prepare a reaction mix according to Table 4.**

   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 the Mg^{2+} concentration as provided in 2x QuantiFast SYBR Green PCR Master Mix.

   Note: The volume of the cDNA added (from the undiluted RT reaction) should not exceed 10% of the final PCR volume.

Table 4. Reaction setup for fast two-step RT-PCR

Component	Volume/reaction			Final concentration
	96-well block	Capillary cycler	384-well block	
2x QuantiFast SYBR Green PCR Master Mix	12.5 µl	10 µl	5 µl	1x
10x QuantiTect Primer Assay	2.5 µl	2 µl	1 µl	1x
Template cDNA (added at step 4)	Variable	Variable	Variable	≤100 ng/ reaction
RNase-free water	Variable	Variable	Variable	–
Total volume	**25 µl**	**20 µl**	**10 µl**	**–**

3. **Mix the reaction mix thoroughly, and dispense appropriate volumes into PCR vessels or plates.**
4. **Add template cDNA (≤100 ng/reaction) to the individual PCR vessels or wells containing the reaction mix.**
5. **Program the real-time cycler according to Table 5.**

   Data acquisition should be performed during the combined annealing/ extension step.

Table 5. Cycling conditions for fast two-step RT-PCR

Step	Time	Temperature	Ramp rate	Additional comments
PCR initial activation step	**5 min**	**95°C**	Maximal/ fast mode	This step activates HotStarTaq *Plus* DNA Polymerase
2-step cycling:				
Denaturation	10 s	95°C	Maximal/ fast mode	
Combined annealing/ extension	30 s	60°C	Maximal/ fast mode	Perform fluorescence data collection
Number of cycles	35–40			The number of cycles depends on the amount of template cDNA and the abundance of the target

6. **Place the PCR vessels or plates in the real-time cycler, and start the cycling program.**

   Optional: Melting curve analysis of the PCR product(s) may be performed to verify their specificity and identity. Melting curve analysis is an analysis step built into the software of real-time cyclers. Please follow the instructions provided by the supplier.

   Note: The T_m of a PCR product depends on buffer composition and salt concentration. T_m values obtained when using QuantiFast SYBR Green PCR reagents may differ from those obtained using other reagents.

A step-by-step guide to software setup for your cycler can be found at www.qiagen.com/fastPCR

Protocol: Two-Step RT-PCR (Standard Protocol)

This protocol is for use with the QuantiTect SYBR Green PCR Kit or QuantiTect SYBR Green PCR +UNG Kit on real-time cyclers from Applied Biosystems, Bio-Rad, Cepheid, QIAGEN, Eppendorf, Roche (LightCycler 480), and Agilent. **For two-step RT-PCR using the QuantiTect SYBR Green PCR Kit or QuantiTect SYBR Green PCR +UNG Kit on capillary cyclers (e.g., LightCycler 1.x and LightCycler 2.0)**, follow the protocol on page 23.

Important points before starting

- The PCR must start with an **initial incubation step of 15 minutes at 95°C** to activate HotStarTaq DNA Polymerase (included in 2x QuantiTect SYBR Green PCR Master Mix).
- Always readjust the threshold value for analysis of every run.
- When using the ABI PRISM® 7000, we strongly recommend using optical adhesive covers to seal PCR plates. Do not use final reaction volumes of less than 25 µl when using this instrument.

Things to do before starting

- If using the QuantiTect Primer Assay for the first time, be sure to reconstitute it before use according to the instructions in "Shipping and Storage", page 5.
- **If using SmartCycler® systems**: Use a final reaction volume of 25 µl, with 12.5 µl of 2x QuantiTect SYBR Green PCR Master Mix and 2.5 µl of 10x QuantiTect Primer Assay.
- **If using the iCycler iQ, iQ5, or MyiQ**: Well factors must be collected at the beginning of each experiment. Well factors are used to compensate for any system or pipetting nonuniformity. For details, refer to the user manual supplied with the instrument or Appendix E (page 54).

Procedure

1. **Thaw 2x QuantiTect SYBR Green PCR Master Mix (if stored at –20°C), 10x QuantiTect Primer Assay, template cDNA, and RNase-free water. Mix the individual solutions.**
2. **Prepare a reaction mix according to Table 6.**

   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: No optimization of the Mg^{2+} concentration is required. The final Mg^{2+} concentration of 2.5 mM provided by 2x QuantiTect SYBR Green PCR Master Mix gives optimal results.

   Note: The volume of the cDNA added (from the undiluted RT reaction) should not exceed 10% of the final PCR volume.

Table 6. Reaction setup for two-step RT-PCR

Component	Volume/reaction	Final concentration
2x QuantiTect SYBR Green PCR Master Mix*	25 µl	1x
10x QuantiTect Primer Assay	5 µl	1x
Template cDNA (added at step 4)	Variable	≤100 ng/reaction
Optional: Uracil-N-glycosylase†	0.5 µl	0.5 units/reaction
RNase-free water	Variable	–
Total volume	**50 µl‡**	**–**

* Provides a final concentration of 2.5 mM $MgCl_2$.

† Supplied with the QuantiTect SYBR Green PCR +UNG Kit.

‡ If using a total volume other than 50 µl, adjust the amounts of the master mix and the primer assay so that their final concentrations remain 1x, but continue to use 0.5 units of UNG and ≤100 ng of template cDNA.

3. **Mix the reaction mix thoroughly, and dispense appropriate volumes into PCR tubes or plates.**
4. **Add template cDNA (≤100 ng/reaction) to the individual PCR tubes or wells containing the reaction mix.**
5. **Program the real-time cycler according to Table 7.**

   Data acquisition should be performed during the extension step.

Table 7. Cycling conditions for two-step RT-PCR

Step	Time	Temperature	Additional comments
UNG (optional) 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**	This step activates HotStarTaq DNA Polymerase
3-step cycling:			
Denaturation*	15 s	94°C	
Annealing	30 s	55°C	
Extension†	30 s	72°C	Perform fluorescence data collection
Number of cycles	35–40 cycles		The number of cycles depends on the amount of template cDNA and abundance of the target

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

† Due to software requirements, the fluorescence detection step must be at least 30 s with the ABI PRISM 7000, or 34 s with the Applied Biosystems® 7300 and 7500.

6. Place the PCR tubes or plates in the real-time cycler, and start the cycling program.

If using the Applied Biosystems 7500, we recommend adjusting the default "Manual Ct" threshold value of 0.2 to a lower value (e.g., 0.02) in order to analyze the data properly.

Optional: Melting curve analysis of the PCR product(s) may be performed to verify their specificity and identity. Melting curve analysis is an analysis step built into the software of real-time cyclers. Please follow the instructions provided by the supplier.

Note: The T_m of a PCR product depends on buffer composition and salt concentration. T_m values obtained when using QuantiTect SYBR Green PCR reagents may differ from those obtained using other reagents.

Protocol: Two-Step RT-PCR (Standard Protocol) for Capillary Cyclers

This protocol is for use with the QuantiTect SYBR Green PCR Kit or QuantiTect SYBR Green PCR +UNG Kit on capillary cyclers (e.g., LightCycler 1.x and LightCycler 2.0). For two-step RT-PCR using the QuantiTect SYBR Green PCR Kit or QuantiTect SYBR Green PCR +UNG Kit on other real-time cyclers (including the LightCycler 480), follow the protocol on page 20.

Important points before starting

- The PCR must start with an **initial incubation step of 15 minutes at 95°C** to activate HotStarTaq DNA Polymerase (included in 2x QuantiTect SYBR Green PCR Master Mix).
- Always readjust the noise band for analysis of every run if using the "fit-point" method for data analysis.

Things to do before starting

- If using the QuantiTect Primer Assay for the first time, be sure to reconstitute it before use according to the instructions in "Shipping and Storage", page 5.

Procedure

1. **Thaw 2x QuantiTect SYBR Green PCR Master Mix (if stored at –20°C), 10x QuantiTect Primer Assay, template cDNA, and RNase-free water. Mix the individual solutions.**
2. **Prepare a reaction mix according to Table 8.**

   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: No optimization of the Mg^{2+} concentration is required. The final Mg^{2+} concentration of 2.5 mM provided by 2x QuantiTect SYBR Green PCR Master Mix gives optimal results.

   Note: The volume of the cDNA added (from the undiluted RT reaction) should not exceed 10% of the final PCR volume.

Table 8. Reaction setup for two-step RT-PCR using capillary cyclers

Component	Volume/reaction	Final concentration
2x QuantiTect SYBR Green PCR Master Mix*	10 µl	1x
10x QuantiTect Primer Assay	2 µl	1x
Template cDNA (added at step 4)	Variable	≤100 ng/reaction
Optional: Uracil-N-glycosylase†	0.5 µl	0.5 units/reaction
RNase-free water	Variable	–
Total volume	**20 µl‡**	**–**

* Provides a final concentration of 2.5 mM $MgCl_2$.

† Supplied with the QuantiTect SYBR Green PCR +UNG Kit.

‡ If using 100 µl capillaries instead of 20 µl capillaries, increase the amounts of the master mix and the primer assay by 5-fold, but continue to use 0.5 units of UNG and ≤100 ng of template cDNA.

3. **Mix the reaction mix thoroughly, and dispense appropriate volumes into PCR capillaries.**
4. **Add template cDNA (≤100 ng/reaction) to the individual PCR capillaries containing the reaction mix.**
5. **Program the real-time cycler according to Table 9. For LightCycler software earlier than version 3.5, set fluorescence gains as described in Table 10.**

   Data acquisition should be performed during the extension step.
6. **Place the PCR capillaries in the real-time cycler, and start the cycling program.**

   Optional: Melting curve analysis of the PCR product(s) may be performed to verify their specificity and identity. Melting curve analysis is an analysis step built into the software of real-time cyclers. Please follow the instructions provided by the supplier.

   Note: The T_m of a PCR product depends on buffer composition and salt concentration. T_m values obtained when using QuantiTect SYBR Green PCR reagents may differ from those obtained using other reagents.

Table 9. Cycling conditions for two-step RT-PCR using capillary cyclers

Step	Time	Temperature	Ramp	Additional comments
UNG (optional) 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**	This step activates HotStarTaq DNA Polymerase
3-step cycling:				
Denaturation	15 s	94°C	2°C/s	
Annealing	20 s	55°C	2°C/s	
Extension	20 s	72°C	2°C/s	Perform fluorescence data collection
Number of cycles	35–40 cycles			The number of cycles depends on the amount of template cDNA and abundance of the target

Table 10. Fluorescence parameters for the LightCycler 1.x

Fluorimeter gain	Value
Channel 1 (F1)	15
Channel 2 (F2)	10
Channel 3 (F3)	10

Display mode (LightCycler 1.x): fluorescence channel 1/1 (F1/1)

LightCycler software versions 3.5 or later automatically adapt the fluorimeter gains for the fluorescence channels. No user-defined setting is required.

Display mode (LightCycler 2.0): channel settings 530/610

Protocol: One-Step RT-PCR (Fast Protocol) for Rotor-Gene Cyclers

This protocol is for use with the Rotor-Gene SYBR Green RT-PCR Kit on the Rotor-Gene Q, Rotor-Gene 3000, or Rotor-Gene 6000.

Important points before starting

- The Rotor-Gene SYBR Green RT-PCR Kit has been developed for use in a **2-step cycling** protocol, with a denaturation step at 95°C and a combined annealing/extension step at 60°C.
- After reverse transcription, the PCR step of the RT-PCR must start with an **initial incubation step of 5 minutes at 95°C** to activate HotStarTaq *Plus* DNA Polymerase (included in 2x Rotor-Gene SYBR Green RT-PCR Master Mix).
- **Set up all reactions on ice** to avoid premature cDNA synthesis and the formation of primer–dimers by Rotor-Gene RT Mix.
- We recommend a final reaction volume of 25 µl.
- **Always start with the Mg^{2+} concentration as provided** in 2x Rotor-Gene SYBR Green RT-PCR Master Mix.

Things to do before starting

- If using the QuantiTect Primer Assay for the first time, be sure to reconstitute it before use according to the instructions in "Shipping and Storage", page 5.

Procedure

1. **Thaw 2x Rotor-Gene SYBR Green RT-PCR Master Mix (if stored at –20°C), 10x QuantiTect Primer Assay, template RNA, and RNase-free water. Mix the individual solutions, and place them on ice. Rotor-Gene 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 according to Table 11.**

   Keep samples on ice while preparing the reaction mix.

   Note: We strongly recommend starting with the Mg^{2+} concentration as provided in 2x Rotor-Gene SYBR Green RT-PCR Master Mix.

Table 11. Reaction setup for fast one-step RT-PCR on Rotor-Gene cyclers

Component	Volume/reaction	Final concentration
2x Rotor-Gene SYBR Green RT-PCR Master Mix	12.5 µl	1x
10x QuantiTect Primer Assay	2.5 µl	1x
Rotor-Gene RT Mix	0.25 µl	–
Template RNA (added at step 4)	Variable	≤10 ng/reaction
RNase-free water	Variable	–
Total volume	**25 µl**	**–**

3. **Mix the reaction mix thoroughly, and dispense appropriate volumes into PCR tubes.**

   Keep the PCR tubes on ice.

4. **Add template RNA (≤10 ng/reaction) to the individual PCR tubes containing the reaction mix.**
5. **Program the Rotor-Gene cycler according to Table 12. Keep the samples on ice until the Rotor-Gene cycler is programmed.**

   Data acquisition should be performed during the combined annealing/ extension step.

Table 12. Cycling conditions for fast one-step RT-PCR on Rotor-Gene cyclers

Step	Time	Temperature	Additional comments
Reverse transcription	**10 min**	**55°C**	
PCR initial activation step	**5 min**	**95°C**	This step activates HotStarTaq *Plus* DNA Polymerase, and also ends reverse transcription and denatures template cDNA
2-step cycling:			
Denaturation	5 s	95°C	
Combined annealing/ extension	10 s	60°C	Perform fluorescence data collection
Number of cycles	35–40		The number of cycles depends on the amount of template RNA and abundance of the target

6. **Place the PCR tubes in the Rotor-Gene cycler, and start the cycling program.**

   Optional: Melting curve analysis of the RT-PCR product(s) may be performed to verify their specificity and identity. Melting curve analysis is an analysis step built into the software of Rotor-Gene cyclers. Please follow the instructions supplied with the instrument.

   Note: The T_m of an RT-PCR product depends on buffer composition and salt concentration. T_m values obtained when using Rotor-Gene SYBR Green RT-PCR reagents may differ from those obtained using other reagents.

A step-by-step guide to software setup for Rotor-Gene cyclers is available: visit www.qiagen.com/literature/protocols and search for protocol PCR107

Protocol: One-Step RT-PCR (Fast Protocol)

This protocol is for use with the QuantiFast SYBR Green RT-PCR Kit and any real-time cycler.

Important points before starting

- The QuantiFast SYBR Green RT-PCR Kit has been developed for use in a **2-step cycling** protocol, with a denaturation step at 95°C and a combined annealing/extension step at 60°C.
- After reverse transcription, the PCR step of the RT-PCR must start with an **initial incubation step of 5 minutes at 95°C** to activate HotStarTaq *Plus* DNA Polymerase (included in 2x QuantiFast SYBR Green RT-PCR Master Mix).
- **Set up all reactions on ice** to avoid premature cDNA synthesis and the formation of primer–dimers by QuantiFast RT Mix.
- For 96-well block cyclers, we recommend a final reaction volume of 25 µl. For capillary cyclers, we recommend a final reaction volume of 20 µl. For 384-well block cyclers, we strongly recommend a final reaction volume of 10 µl.
- **Always start with the Mg^{2+} concentration as provided** in 2x QuantiFast SYBR Green RT-PCR Master Mix.

Things to do before starting

- If using the QuantiTect Primer Assay for the first time, be sure to reconstitute it before use according to the instructions in "Shipping and Storage", page 5.
- **If using the iCycler iQ, iQ5, or MyiQ**: Well factors must be collected at the beginning of each experiment. Well factors are used to compensate for any system or pipetting nonuniformity. For details, refer to the user manual supplied with the instrument or Appendix E (page 54).

Procedure

1. **Thaw 2x QuantiFast SYBR Green RT-PCR Master Mix (if stored at –20°C), 10x QuantiTect Primer Assay, template RNA, and RNase-free water. Mix the individual solutions, and place them on ice. 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 according to Table 13.**

   Keep samples on ice while preparing the reaction mix.

   Note: We strongly recommend starting with the Mg^{2+} concentration as provided in 2x QuantiFast SYBR Green RT-PCR Master Mix.

Table 13. Reaction setup for fast one-step RT-PCR

Component	Volume/reaction			Final concentration
	96-well block	Capillary cycler	384-well block	
2x QuantiFast SYBR Green RT-PCR Master Mix	12.5 µl	10 µl	5 µl	1x
10x QuantiTect Primer Assay	2.5 µl	2 µl	1 µl	1x
QuantiFast RT Mix	0.25 µl	0.2 µl	0.1 µl	–
Template RNA (added at step 4)	Variable	Variable	Variable	≤10 ng/ reaction
RNase-free water	Variable	Variable	Variable	–
Total volume	**25 µl**	**20 µl**	**10 µl**	**–**

3. **Mix the reaction mix thoroughly, and dispense appropriate volumes into PCR vessels or plates.**

   Keep the PCR vessels or plates on ice.

4. **Add template RNA (≤10 ng/reaction) to the individual PCR vessels or wells containing the reaction mix.**

5. **Program the real-time cycler according to Table 14. Keep the samples on ice until the real-time cycler is programmed.**

   Data acquisition should be performed during the combined annealing/ extension step.

Table 14. Cycling conditions for fast one-step RT-PCR

Step	Time	Temperature	Ramp rate	Additional comments
Reverse transcription	**10 min**	**50°C**		
PCR initial activation step	**5 min**	**95°C**	Maximal/ fast mode	This step activates HotStarTaq *Plus* DNA Polymerase, and also ends reverse transcription and denatures template cDNA
2-step cycling:				
Denaturation	10 s	95°C	Maximal/ fast mode	
Combined annealing/ extension	30 s	60°C	Maximal/ fast mode	Perform fluorescence data collection
Number of cycles	35–40			The number of cycles depends on the amount of template RNA and abundance of the target

6. **Place the PCR vessels or plates in the real-time cycler, and start the cycling program.**

   Optional: Melting curve analysis of the RT-PCR product(s) may be performed to verify their specificity and identity. Melting curve analysis is an analysis step built into the software of real-time cyclers. Please follow the instructions provided by the supplier.

   Note: The T_m of an RT-PCR product depends on buffer composition and salt concentration. T_m values obtained when using QuantiFast SYBR Green RT-PCR reagents may differ from those obtained using other reagents.

A step-by-step guide to software setup for your cycler can be found at www.qiagen.com/fastPCR

Protocol: One-Step RT-PCR (Standard Protocol)

This protocol is for use with the QuantiTect SYBR Green RT-PCR Kit and real-time cyclers from Applied Biosystems, Bio-Rad, Cepheid, QIAGEN, Eppendorf, Roche (LightCycler 480), and Agilent. **For one-step RT-PCR using the QuantiTect SYBR Green RT-PCR Kit on capillary cyclers (e.g., LightCycler 1.x and LightCycler 2.0)**, follow the protocol on page 35.

Important points before starting

- After reverse transcription, the PCR step of the RT-PCR must start with an **initial incubation step of 15 minutes at 95°C** to activate HotStarTaq DNA Polymerase (included in 2x QuantiTect SYBR Green RT-PCR Master Mix).
- **Set up all reactions on ice** to avoid premature cDNA synthesis and the formation of primer–dimers by QuantiTect RT Mix.
- Always readjust the threshold value for analysis of every run.
- When using the ABI PRISM 7000, we strongly recommend using optical adhesive covers to seal PCR plates. Do not use final reaction volumes of less than 25 µl when using this instrument.
- If performing optional UNG pretreatment (see page 53), be sure to use **heat-labile** UNG.

Things to do before starting

- If using the QuantiTect Primer Assay for the first time, be sure to reconstitute it before use according to the instructions in "Shipping and Storage", page 5.
- **If using SmartCycler systems**: Use a final reaction volume of 25 µl, with 12.5 µl of 2x QuantiTect SYBR Green RT-PCR Master Mix, 2.5 µl of 10x QuantiTect Primer Assay, and 0.25 µl QuantiTect RT Mix. In addition, the reverse-transcription step can be reduced to 20 minutes.
- **If using the iCycler iQ, iQ5, or MyiQ**: Well factors must be collected at the beginning of each experiment. Well factors are used to compensate for any system or pipetting nonuniformity. For details, refer to the user manual supplied with the instrument or Appendix E (page 54).

Procedure

1. **Thaw 2x QuantiTect SYBR Green RT-PCR Master Mix (if stored at –20°C), 10x QuantiTect Primer Assay, template RNA, and RNase-free water. Mix the individual solutions, and place them on ice. QuantiTect 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 according to Table 15.**

   Keep samples on ice while preparing the reaction mix.

   Note: No optimization of the Mg^{2+} concentration is required. The final Mg^{2+} concentration of 2.5 mM provided by 2x QuantiTect SYBR Green RT-PCR Master Mix gives optimal results.

Table 15. Reaction setup for one-step PCR

Component	Volume/reaction	Final concentration
2x QuantiTect SYBR Green RT-PCR Master Mix*	25 µl	1x
10x QuantiTect Primer Assay	5 µl	1x
QuantiTect RT Mix	0.5 µl	0.5 µl/reaction
Template RNA (added at step 4)	Variable	≤10 ng/reaction
Optional: Uracil-N-glycosylase, heat-labile	Variable	1–2 units/reaction
RNase-free water	Variable	–
Total volume	**50 µl†**	**–**

* Provides a final concentration of 2.5 mM $MgCl_2$.

† If using a total volume other than 50 µl, adjust the amounts of the master mix, the primer assay, and the RT mix so that the ratio between them remains constant (e.g., if the total volume is 25 µl, use 12.5 µl master mix, 2.5 µl primer assay, and 0.25 µl RT mix). However, continue to use 1–2 units of UNG and ≤10 ng of template RNA.

3. **Mix the reaction mix thoroughly, and dispense appropriate volumes into PCR tubes or plates.**

   Keep the PCR tubes or plates on ice.

4. **Add template RNA (≤10 ng/reaction) to the individual PCR tubes or wells containing the reaction mix.**

   For the optional UNG treatment, leave samples for at least 10 min on ice.

5. **Program the real-time cycler according to Table 16. Keep the samples on ice until the real-time cycler is programmed.**

   Data acquisition should be performed during the extension step.

Table 16. Cycling conditions for one-step PCR

Step	Time	Temperature	Additional comments
Reverse transcription	**30 min**	**50°C**	
PCR initial activation step	**15 min**	**95°C**	This step activates HotStarTaq DNA Polymerase, and also ends reverse transcription and denatures template cDNA
3-step cycling:			
Denaturation*	15 s	94°C	
Annealing	30 s	55°C	
Extension†	30 s	72°C	Perform fluorescence data collection
Number of cycles	35–40 cycles		The number of cycles depends on the amount of template RNA and abundance of the target

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

† Due to software requirements, the fluorescence detection step must be at least 30 s with the ABI PRISM 7000, or 34 s with the Applied Biosystems 7300 and 7500.

6. **Place the PCR tubes or plates in the real-time cycler, and start the cycling program.**

   If using the Applied Biosystems 7500, we recommend adjusting the default "Manual Ct" threshold value of 0.2 to a lower value (e.g., 0.02) in order to analyze the data properly.

   Optional: Melting curve analysis of the RT-PCR product(s) may be performed to verify their specificity and identity. Melting curve analysis is an analysis step built into the software of real-time cyclers. Please follow the instructions provided by the supplier.

   Note: The T_m of an RT-PCR product depends on buffer composition and salt concentration. T_m values obtained when using QuantiTect SYBR Green RT-PCR reagents may differ from those obtained using other reagents.

Protocol: One-Step RT-PCR (Standard Protocol) for Capillary Cyclers

This protocol is for use with the QuantiTect SYBR Green RT-PCR Kit and capillary cyclers (e.g., LightCycler 1.x and LightCycler 2.0). For one-step RT-PCR using the QuantiTect SYBR Green RT-PCR Kit on other real-time cyclers (including the LightCycler 480), follow the protocol on page 32.

Important points before starting

- After reverse transcription, the PCR step of the RT-PCR must start with an **initial incubation step of 15 minutes at 95°C** to activate HotStarTaq DNA Polymerase (included in 2x QuantiTect SYBR Green RT-PCR Master Mix).
- **Set up all reactions in cooled capillaries** to avoid premature cDNA synthesis and the formation of primer–dimers by QuantiTect RT Mix.
- Always readjust the noise band for analysis of every run if using the "fit-point" method for data analysis.
- If performing optional UNG pretreatment (see page 53), be sure to use **heat-labile** UNG.

Things to do before starting

- If using the QuantiTect Primer Assay for the first time, be sure to reconstitute it before use according to the instructions in "Shipping and Storage", page 5.

Procedure

1. **Thaw 2x QuantiTect SYBR Green RT-PCR Master Mix (if stored at –20°C), 10x QuantiTect Primer Assay, template RNA, and RNase-free water. Mix the individual solutions, and place them on ice. QuantiTect 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 according to Table 17.**

   Keep the PCR capillaries cooled while preparing the reaction mix.

   Note: No optimization of the Mg^{2+} concentration is required. The final Mg^{2+} concentration of 2.5 mM provided by 2x QuantiTect SYBR Green RT-PCR Master Mix gives optimal results.

Table 17. Reaction setup for one-step RT-PCR using capillary cyclers

Component	Volume/reaction	Final concentration
2x QuantiTect SYBR Green RT-PCR Master Mix*	10 µl	1x
10x QuantiTect Primer Assay	2 µl	1x
QuantiTect RT Mix	0.2 µl	0.2 µl/reaction
Template RNA (added at step 4)	Variable	≤10 ng/reaction
Optional: Uracil-N-glycosylase, heat-labile	Variable	1–2 units/reaction
RNase-free water	Variable	–
Total volume	**20 µl†**	**–**

* Provides a final concentration of 2.5 mM $MgCl_2$.

† If using 100 µl capillaries instead of 20 µl capillaries, increase the amounts of the master mix, the primer assay, and the RT mix by 5-fold, but continue to use 1–2 units of UNG and ≤10 ng of template RNA.

3. **Mix the reaction mix thoroughly, and dispense appropriate volumes into PCR capillaries.**

   Keep the PCR capillaries cooled.

4. **Add template RNA (≤10 ng/reaction) to the individual PCR capillaries containing the reaction mix.**

   For the optional UNG treatment, leave samples for at least 10 min in the cooled PCR capillaries.

5. **Program the real-time cycler according to Table 18. For LightCycler software earlier than version 3.5, set fluorescence gains as described in Table 19. Keep the PCR capillaries cooled until the real-time cycler is programmed.**

   Data acquisition should be performed during the extension step.

Table 18. Cycling conditions for one-step RT-PCR using capillary cyclers

Step	Time	Temperature	Ramp	Additional comments
Reverse transcription	**20 min**	**50°C**	**20°C/s**	
PCR initial activation step	**15 min**	**95°C**	**20°C/s**	This step activates HotStarTaq DNA Polymerase, and also ends reverse transcription and denatures template cDNA
3-step cycling:				
Denaturation	15 s	94°C	2°C/s	
Annealing	20 s	55°C	2°C/s	
Extension	20 s	72°C	2°C/s	Perform fluorescence data collection
Number of cycles	35–40 cycles			The number of cycles depends on the amount of template RNA and abundance of the target

Table 19. Fluorescence parameters for the LightCycler 1.x

Fluorimeter gain	Value
Channel 1 (F1)	15
Channel 2 (F2)	10
Channel 3 (F3)	10

Display mode (LightCycler 1.x): fluorescence channel 1/1 (F1/1)

LightCycler software versions 3.5 or later automatically adapt the fluorimeter gains for the fluorescence channels. No user-defined setting is required.

Display mode (LightCycler 2.0): channel settings 530/610

6. **Place the PCR capillaries in the real-time cycler, and start the cycling program.**

   If using the LightCycler 1.x and 2.0, ensure that the temperature is set to 50°C during the "seek sample" process.

   Optional: Melting curve analysis of the RT-PCR product(s) may be performed to verify their specificity and identity. Melting curve analysis is an analysis step built into the software of the real-time cycler. Please follow the instructions provided by the supplier.

   Note: The T_m of an RT-PCR product depends on buffer composition and salt concentration. T_m values obtained when using QuantiTect SYBR Green RT-PCR reagents may differ from those obtained using other reagents.

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, or only primer–dimers detected	
a) PCR annealing time too short	**Fast protocols for Rotor-Gene cyclers (pages 14 and 26):** Be sure to use a combined annealing/extension time of 10 s. **Fast protocols (pages 17 and 29)**: Be sure to use a combined annealing/extension time of 30 s. **Standard protocols (pages 20 and 32)**: Be sure to use an annealing time of 30 s. **Standard protocols for capillary cyclers (pages 23 and 35)**: Be sure to use an annealing time of 20 s.
b) Extension time too short	**Fast protocols for Rotor-Gene cyclers (pages 14 and 26):** Be sure to use a combined annealing/extension time of 10 s. **Fast protocols (pages 17 and 29)**: Be sure to use a combined annealing/extension time of 30 s. **Standard protocols (pages 20 and 32)**: Be sure to use an extension time of 30 s. For the Applied Biosystems 7300 or 7500, the extension time should be 34 s. **Standard protocols for capillary cyclers (pages 23 and 35)**: Be sure to use an extension time of 20 s.
c) Pipetting error or missing reagent	Check the concentrations and storage conditions of the reagents, including primers and RNA. Repeat the RT-PCR.

	Comments and suggestions
d) Hot-start DNA polymerase not activated	**Fast protocols for Rotor-Gene cyclers (pages 14 and 26)**: Be sure to activate HotStarTaq *Plus* DNA Polymerase at the start of the cycling program (5 min at 95°C). **Fast protocols (pages 17 and 29)**: Be sure to activate HotStarTaq *Plus* DNA Polymerase at the start of the cycling program (5 min at 95°C). **Standard protocols (pages 20, 23, 32, and 35)**: Be sure to activate HotStarTaq DNA Polymerase at the start of the cycling program (15 min at 95°C).
e) Hot-start DNA polymerase activated too early (in one-step RT-PCR protocols)	**Fast protocol for Rotor-Gene cyclers (page 26)**: Be sure that the reverse-transcription reaction (10 min at 55°C) has finished before activating HotStarTaq *Plus* DNA Polymerase. **Fast protocol (page 29)**: Be sure that the reverse-transcription reaction (10 min at 50°C) has finished before activating HotStarTaq *Plus* DNA Polymerase. **Standard protocol (page 32)**: Be sure that the reverse-transcription reaction (30 min at 50°C) has finished before activating HotStarTaq DNA Polymerase. For SmartCycler systems, reverse transcription can be carried out for 20 min at 50°C. **Standard protocol for capillary cyclers (page 35)**: Be sure that the reverse-transcription reaction (20 min at 50°C) has finished before activating HotStarTaq DNA Polymerase.
f) Incorrect temperature for RT reaction (in one-step RT-PCR protocols)	**Fast protocol for Rotor-Gene cyclers (page 26)**: We recommend performing the RT reaction at 55°C. **Fast and standard protocols (pages 29 and 32)**: We recommend performing the RT reaction at 50°C.

	Comments and suggestions
g) Incorrect volume of RT Mix (in one-step RT-PCR protocols)	**Fast protocol for Rotor-Gene cyclers (page 26)**: Be sure to use 0.25 µl Rotor-Gene RT Mix per 25 µl reaction. **Fast protocol (page 29)**: Be sure to use the volume of QuantiFast RT Mix specified for your cycler (see Table 13). **Standard protocol (page 32)**: Be sure to use 0.5 µl QuantiTect RT Mix per 50 µl reaction. For SmartCycler systems, use 0.25 µl QuantiTect RT Mix per 25 µl reaction. **Standard protocol for capillary cyclers (page 35)**: Be sure to use 0.2 µl QuantiTect RT Mix per 20 µl reaction.
h) Incorrect ratio of RT Mix to 2x RT-PCR Master Mix (in one-step RT-PCR protocols)	The ratio of RT Mix to 2x RT-PCR Master Mix must always be 1:50 (e.g., 0.1 µl RT Mix and 5 µl 2x RT-PCR Master Mix).
i) Target gene not expressed in the cell line or tissue studied	Find out whether the gene of interest is expressed in the cell line or tissue being studied. Go to www.ncbi.nlm.nih.gov, select the "UniGene" database, enter the name of your gene, and click "Go". A list of genes will appear. Click on the appropriate gene, and in the next page which appears, click on "Expression Profile" to view the expression of the gene in different tissues.
j) Problems with RNA	Check the concentration, integrity, purity, and storage conditions of the RNA (see Appendix A, page 47). If necessary, make new serial dilutions of RNA from stock solutions. Repeat the RT-PCR using the new dilutions.
k) Insufficient RNA	Increase the amount of RNA used in RT-PCR, if possible.
l) Insufficient number of cycles	Increase the number of cycles.

	Comments and suggestions
m) PCR annealing temperature too high	**Fast protocols for Rotor-Gene cyclers (pages 14 and 26)**: Be sure to perform the combined annealing/extension step at 60°C. **Fast protocols (pages 17 and 29)**: Be sure to perform the combined annealing/extension step at 60°C. **Standard protocols (pages 20, 23, 32, and 35)**: Be sure to use an annealing temperature of 55°C.
n) No detection activated	Check that fluorescence detection was activated in the cycling program.
o) Wrong detection step	**Fast protocols for Rotor-Gene cyclers (pages 14 and 26)**: Ensure that fluorescence detection takes place during the combined annealing/extension step of each PCR cycle. **Fast protocols (pages 17 and 29)**: Ensure that fluorescence detection takes place during the combined annealing/extension step of each PCR cycle. **Standard protocols (pages 20, 23, 32, and 35)**: Ensure that fluorescence detection takes place during the extension step of each PCR cycle.
p) QuantiTect Primer Assays degraded due to repeated freeze–thaw cycles	Check for possible degradation of QuantiTect Primer Assays on a denaturing polyacrylamide gel. For details, see the protocol at www.qiagen.com/literature/protocols/pdf/PCR03.pdf. We recommend using 20 µl of reconstituted QuantiTect Primer Assay.
Applied Biosystems, Bio-Rad, QIAGEN, and Agilent systems only:	
q) Wrong detection channel/filter chosen	Ensure that the correct detection channel is activated or the correct filter set is chosen for SYBR Green I.

Applied Biosystems 7300 and 7500 only:

r) No automatic detection of amplification signals when using QuantiTect Kits	Adjust the default "Manual Ct" threshold value of 0.2 to a lower value (e.g., 0.02) and reanalyze the data.

LightCycler 1.x only:

s) Chosen fluorescence gains are too low	When using software earlier than version 3.5, ensure that fluorescence gain for channel 1 is set to "15".

Multiple peaks in melting temperature analysis/multiple RT-PCR products

a) Reaction set up at room temperature (in one-step RT-PCR protocols)	Set up the RT-PCR in cooled reaction vessels to avoid premature cDNA synthesis from nonspecific primer annealing.
b) Primer concentration too high	10x QuantiTect Primer Assay was reconstituted using less than the volume of TE, pH 8.0 recommended (page 5).
c) Starting conditions for reverse-transcription reaction incorrect (in one-step RT-PCR protocols)	Ensure that the RT-PCR program is started immediately after transfer of samples to the real-time cycler.
d) Mg^{2+} concentration not optimal	Do not adjust the final Mg^{2+} concentration provided by the 2x PCR Master Mix or 2x RT-PCR Master Mix.
e) PCR annealing temperature too low	**Fast protocols for Rotor-Gene cyclers (pages 14 and 26)**: Be sure to perform the combined annealing/extension step at 60°C. **Fast protocols (pages 17 and 29)**: Be sure to perform the combined annealing/extension step at 60°C. **Standard protocols (pages 20, 23, 32, and 35)**: Be sure to use an annealing temperature of 55°C.

	Comments and suggestions
f) Contamination with genomic DNA	Pretreat RNA samples with DNase I. We recommend using the QIAGEN RNase-Free DNase Set (see ordering information, page 60). If performing real-time two-step RT-PCR, synthesize cDNA using the QuantiTect Reverse Transcription Kit, which provides integrated removal of genomic DNA contamination.
g) Reverse-transcription reaction temperature too low	A reverse-transcription reaction temperature of 55°C is recommended for Rotor-Gene Kits. For QuantiFast and QuantiTect Kits, the recommended temperature is 50°C. However, if this does not yield satisfactory results, the reaction temperature may be increased up to 55°C.
h) Primers degraded	Check for possible degradation of primers on a denaturing polyacrylamide gel. For details, see the protocol at www.qiagen.com/literature/protocols/pdf/PCR03.pdf. We recommend using 20 µl of reconstituted QuantiTect Primer Assay.
No linearity in ratio of C_T value/crossing point to log of the template amount	
a) Template amount too high	Do not use more than 100 ng cDNA for two-step RT-PCR or more than 10 ng RNA for one-step RT-PCR.
b) Template amount too low	Increase the amount of template nucleic acid, if possible.

	Comments and suggestions
c) Incorrect volume of RT Mix (in one-step RT-PCR protocols)	**Fast protocol for Rotor-Gene cyclers (page 26)**: Be sure to use 0.25 µl Rotor-Gene RT Mix per 25 µl reaction. **Fast protocol (page 29)**: Be sure to use the volume of QuantiFast RT Mix specified for your cycler (see Table 13). **Standard protocol (page 32)**: Be sure to use 0.5 µl QuantiTect RT Mix per 50 µl reaction. For SmartCycler systems, use 0.25 µl QuantiTect RT Mix per 25 µl reaction. **Standard protocol for capillary cyclers (page 35)**: Be sure to use 0.2 µl QuantiTect RT Mix per 20 µl reaction.
LightCycler 1.x and 2.0 only:	
d) Incorrect ramp setting when using QuantiTect Kits	Use a ramp setting of 2°C/s for all cycling steps.
High fluorescence in "No Template" control	
a) Contamination of reagents	Discard reaction components and repeat with new reagents.
b) Contamination during reaction setup	Take appropriate safety precautions (e.g., use filter tips). **QuantiTect SYBR Green Kits only**: Use uracil-N-glycosylase to prevent carryover from previous reactions. For one-step RT-PCR, be sure to use **heat-labile** uracil-N-glycosylase.
High fluorescence in "No RT" control reactions	
Contaminating genomic DNA in RNA preparation	DNase digest RNA samples. If performing real-time two-step RT-PCR, synthesize cDNA using the QuantiTect Reverse Transcription Kit, which provides integrated removal of genomic DNA contamination.
Varying fluorescence intensity	
a) Real-time cycler contaminated	Decontaminate the real-time cycler according to the supplier's instructions.

	Comments and suggestions
b) Real-time cycler no longer calibrated	Recalibrate the real-time cycler according to the supplier's instructions.

Applied Biosystems, Bio-Rad, and Agilent systems only:

c) Wavy curve at high template amounts	Reduce the number of cycles used for baseline calculation.

LightCycler 1.x and 2.0 only:

d) PCR mix not in capillary tip	Centrifuge the capillary to bring the PCR mix into the capillary tip.
e) Capillary not pushed down completely	Ensure that the capillary is pushed down completely in the LightCycler carousel.
f) Wrong detection channel	**LightCycler**: Make sure that channel 1 is chosen **LightCycler 2.0**: Make sure that channel setting 530 is chosen

Run does not start (Bio-Rad® iQ systems only)

External well factors need to be collected	Remove the experimental reaction plate from the Bio-Rad iQ system, replace it with an external well factor plate, and collect well factors (see Appendix E, page 54). After well factor collection, return the experimental reaction plate to the Bio-Rad iQ system, and start the run. If the experimental reaction plate is for a one-step RT-PCR experiment, keep it on ice during well factor collection. This prevents premature cDNA synthesis.

Appendix A: Preparation, Quantification, Determination of Quality, and Storage of RNA

RNA preparation and quality

Since PCR consists of multiple rounds of enzymatic reactions, it is more sensitive to impurities such as proteins, phenol/chloroform, salts, and EDTA than single-step enzyme-catalyzed reactions. Purity of nucleic acid templates is particularly important for real-time PCR, since contaminants can interfere with fluorescence detection. QIAGEN offers a complete range of RNA purification systems that provide the highest-quality templates for real-time RT-PCR:

- PAXgene® Blood RNA system — for collection and stabilization of whole blood samples and subsequent RNA purification
- QIAamp® RNA Blood Mini Kit — for purification of RNA from whole blood
- RNeasy® Protect Kits — for stabilization of RNA in cells, tissues, animal blood, saliva, and bacteria, and subsequent RNA purification
- RNeasy Kits — for purification of RNA from cells, tissues, yeast, and plants
- Oligotex® and TurboCapture® Kits — for purification of mRNA from cells and tissues
- QIAcube® — for fully automated sample preparation using QIAGEN spin-column kits
- QIAsymphony® SP — for automated purification of RNA from up to 96 samples
- QIAxcel® — for automated analysis of RNA quantity and quality

If working with cultured cells, the FastLane Cell cDNA Kit provides a high-speed procedure for preparing cDNA directly from cells without any RNA purification.

For more information about these products, visit www.qiagen.com, or contact your local QIAGEN office to request the brochure *High-Performance RNA for Gene Expression Analysis*.

Determining concentration and purity of RNA

The concentration of RNA should be determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer. The sample should be diluted in 10 mM Tris·Cl, pH 7.0,* since the relationship between absorbance and concentration (A_{260} reading of 1 = 44 µg/ml RNA) is based on an extinction

* 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.

coefficient calculated for RNA in a buffer with neutral pH. To ensure significance, readings should fall between 0.15 and 1.0.

Note that absorbance measurements cannot discriminate between DNA and RNA. Depending on the method used for RNA preparation, RNA may be contaminated with DNA, and this will result in misleadingly high A_{260} values.

The ratio between the absorbance values at 260 nm and 280 nm gives an estimate of RNA purity. To determine RNA purity, we recommend measuring absorbance in 10 mM Tris·Cl, pH 7.5.* Pure RNA has an A_{260}/A_{280} ratio of 1.9–2.1.† Lower ratios indicate the presence of contaminants such as proteins.

Storage of RNA

Purified RNA should be stored at –20°C or –70°C in RNase-free water. When purified using QIAGEN systems, no degradation is detectable for at least 1 year under these conditions. Diluted solutions of RNA (e.g., dilution series used as standards) should be stored in aliquots and thawed once only. We recommend storage of aliquots in siliconized tubes where possible. This avoids adsorption of the RNA to the tube walls, which would reduce the concentration of RNA in solution.

DNA contamination in RNA samples purified from different tissues

Depending on the type of tissue used as starting material for RNA purification, a fluorescent signal may also be generated in "no RT" control reactions. When using QuantiTect Primer Assays, pseudogenes that are almost identical to the gene transcript sequence and also genes lacking introns may generate a signal from contaminating genomic DNA in late PCR cycles (see assay specifications). When RNA is purified from tissues such as spleen or thymus without performing DNase I digestion, a large amount of contaminating genomic DNA may be present. We strongly recommend using the QuantiTect Reverse Transcription Kit for reverse transcription, as the kit eliminates genomic DNA contamination in starting RNA samples. Alternatively, RNA can be purified from cells and tissues using RNeasy Plus Kits, which include gDNA Eliminator columns. For other RNeasy Kits, a DNase I digestion step (e.g., using the QIAGEN RNase-Free DNase Set, cat. no. 79254) can be performed during RNA purification.

* 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.

† Values up to 2.3 are routinely obtained for pure RNA (in 10 mM Tris·Cl, pH 7.5) with some spectrophotometers.

Appendix B: Determination of PCR Efficiency and Quantification of Gene Expression Levels

Number of cycles

Cycling programs usually consist of up to 35–55 cycles. When using QuantiTect Primer Assays in combination with Rotor-Gene SYBR Green Kits, QuantiFast SYBR Green Kits, or QuantiTect SYBR Green Kits, 35–40 cycles are sufficient, regardless of the number of copies of starting template and the type of instrument used for real-time PCR.

Relative quantification

Gene expression levels can be normalized to the amount of RNA in the reaction. However, this relies on accurate determination of RNA concentration (see Appendix A, page 47). Relative quantification determines the ratio between the amount of target and a reference molecule that is in all samples. In this approach, the normalized value can be used to compare, for example, differential gene expression in different tissues. The expression level of the reference molecule, such as a housekeeping gene, must not vary under different experimental conditions, or in different states of the same tissue (e.g., "disease" versus "normal" samples). The level is therefore used as a reference value for quantification. The quantification procedure differs depending on whether the target nucleic acid and the reference molecule are amplified with comparable efficiencies. For determination of PCR efficiency, see page 51. QuantiTect Primer Assays are tested for high amplification efficiency. However, since amplification efficiencies are dependent on a number of factors (e.g., instrumentation, variation in pipetting and handling, presence of inhibitors in the sample, and the effect of RT reaction volume on PCR efficiency), we recommend quantifying target and reference amounts by using standard curves for both genes (see next paragraph).

Different amplification efficiencies

An RNA standard should be used for gene expression studies, since this takes into account the variable efficiency of reverse transcription. The RNA standard (e.g., total RNA prepared from a reference cell line or tissue) is used as a reference for all experiments. Two standard curves, one for the target gene and one for the reference molecule (such as a housekeeping gene), are prepared by accurately diluting the reference sample in 5-fold or 10-fold dilutions. Since the resulting standard curves may not be completely parallel, the differences in C_T values of the target and reference will not be constant when the amounts of target and reference are varied. The amounts of reference and target are calculated using the C_T values obtained from the sample of interest and the

corresponding standard curve. The ratio of the resulting amounts of target and reference in the sample of interest can then be determined.

Guidelines for relative quantification with different amplification efficiencies of target and reference

- Choose an appropriate endogenous reference gene (e.g., β2-microglobulin or peptidylprolyl isomerase A) whose expression level does not change under the experimental conditions or between different tissues.
- Prepare a dilution series (e.g., 5-fold or 10-fold dilutions) of a cDNA or RNA control sample to construct standard curves for target and reference.
- Perform real-time PCR/RT-PCR.
- Determine C_T values for the standards and samples of interest.
- Construct standard curves for both target and reference by plotting C_T values (Y-axis) against the log of template amount or dilution (X-axis).
- Calculate amount of target and reference in samples of interest using C_T value and corresponding standard curve.
- To calculate the normalized amount of target, divide the amount of target by the amount of reference (if replicate reactions were performed, use average value).
- Set the normalized target amount of one sample to 1 (calibrator sample). Compare relative expression level of target gene in samples of interest by dividing the normalized target amounts by the value of the calibrator.

Comparable amplification efficiencies

If the amplification efficiencies of target and reference are comparable, gene expression levels can be determined by 2 methods. A single standard curve can be generated for the reference sequence only. Unknown amounts of target and reference in each sample are calculated by comparing the resulting C_T values with the standard curve for the reference sequence. Before using this method, make sure that the amplification efficiencies of both target and reference are comparable (see page 51).

Guidelines for relative quantification with comparable amplification efficiencies of target and reference

- Choose an appropriate endogenous reference gene (e.g., β2-microglobulin or peptidylprolyl isomerase A) whose expression level does not change under the experimental conditions or between different tissues.

- Prepare a dilution series (e.g., 5-fold or 10-fold dilutions) of a cDNA or RNA control sample to construct a standard curve for the reference.
- Perform real-time PCR/RT-PCR.
- Determine C_T values for the standards and samples of interest.
- Construct a standard curve for the reference by plotting C_T values (Y-axis) against the log of template amount or dilution (X-axis).
- Calculate amount of target and reference in samples of interest using C_T value and standard curve.
- To calculate the normalized amount of target, divide the amount of target by the amount of reference (if replicate reactions were performed, use average value).
- Set the normalized target amount of one sample to 1 (calibrator sample). Compare relative expression level of target gene in samples of interest by dividing the normalized target amounts by the value of the calibrator.

An alternative approach is the comparative method ($\Delta\Delta C_T$ method), which relies on comparing differences in C_T values. Preparation of standard curves is only needed to determine the amplification efficiencies in an initial experiment, but not in each subsequent real-time PCR. However, this method can only be used if amplification efficiencies of target and reference sequence are nearly equivalent and close to 1. For more detailed information, refer to Livak, K.J. and Schmittgen, T.D (1).

Determination of PCR efficiency

Amplification efficiency should be determined by performing real-time PCR with a dilution series of nucleic acid that contains the sequence of interest. The C_T values obtained are used for construction of a standard curve. The amplification efficiency (E) for the sequence of interest can be calculated according to the following equation:

$E = 10^{(-1/S)} - 1$ (S is the slope of the standard curve)

Comparison of PCR efficiencies

To compare the amplification efficiencies of 2 target sequences, prepare a dilution series for each target (targets A and B). Amplify each dilution series by two-step or one-step RT-PCR. Subtract the C_T values/crossing points of target A from the C_T values of target B. Plot the differences in C_T values/crossing points against the logarithm of the template amount. If the slope of the resulting straight line is <0.1, amplification efficiencies are comparable.

Generating standard curves

To generate a standard curve, at least 5 different concentrations of the standard should be measured, and the amount of unknown target should fall within the range tested. Reactions should be carried out in triplicate when using cyclers from Applied Biosystems, Bio-Rad, QIAGEN, Cepheid, Eppendorf, Roche (LightCycler 480), and Agilent, or in duplicate when using LightCycler capillary cyclers. When using very low amounts of template, reactions should be carried out in quadruplicate for cyclers from Applied Biosystems, Bio-Rad, QIAGEN, Eppendorf, Roche (LightCycler 480), and Agilent, or in triplicate for LightCycler capillary cyclers and SmartCycler systems.

Appendix C: 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, since detection of genomic DNA cannot be completely eliminated for some genes. This is due to the presence of pseudogenes with identical or almost identical nucleotide sequences and also the presence of genes lacking introns. DNA contamination can be detected by performing a control reaction in which no reverse transcription takes place. When performing two-step RT-PCR, a control "no RT" reaction should be included, which contains all components including template RNA, except for the reverse transcriptase enzyme. An aliquot of this reaction is then used as a template in PCR. With one-step RT-PCR, the reverse transcriptase (i.e., Rotor-Gene RT Mix, QuantiFast RT Mix, or QuantiTect RT Mix) is omitted during the setup of the RT-PCR reaction; all other components (e.g., template RNA) are included. In both cases, reverse transcription cannot take place and the only template available is contaminating DNA.

Positive control

In some cases, it may be necessary to include a positive control containing a known concentration of template. This is usually a substitute for absolute standards and is used only to test for presence or absence of the target, but does not yield detailed quantitative information. Ensure that the positive control contains at least the minimum amount of RNA required for accurate detection.

Appendix D: Use of UNG in PCR

The following advice applies only onto QuantiTect SYBR Green Kits (master mixes contain dUTP). Do not follow this advice if using Rotor-Gene SYBR Green Kits or QuantiFast SYBR Green Kits (master mixes do not contain dUTP).

Prevention of contamination using UNG

PCR products from previous PCR runs are a major source of PCR contamination, known as carryover contamination. This problem can be reduced by enzymatic destruction of contaminants.

To enable this step to be performed, dTTP in the QuantiTect SYBR Green PCR or RT-PCR Master Mix is partially substituted by dUTP. In addition, the enzyme uracil-N-glycosylase* must be added to the PCR in an appropriate concentration (see protocols on pages 20, 23, 32, and 35). An incubation step of 50°C for 2 minutes is added to the cycling program (for two-step RT-PCR) before activation of HotStarTaq DNA Polymerase. During this incubation step, UNG removes uracil from dUMP incorporated into any contaminating molecules, leaving apyrimidinic sites. During the activation step of HotStarTaq DNA Polymerase (15 minutes at 95°C), the UNG is inactivated, and contaminating molecules are destroyed by cleavage at the abasic sites. During subsequent cycling, only target nucleic acid and not contaminating nucleic acid from previous reactions will be amplified.

If UNG treatment is carried out using heat-stable UNG from *E. coli*, the annealing temperature should not be below 55°C, and PCR products should be refrigerated immediately after the run, because heat-stable UNG maintains a residual activity following the incubation step at 95°C for 15 minutes. However, only heat-labile UNG can be used when performing one-step RT-PCR with the QuantiTect SYBR Green RT-PCR Kit. Since UNG isolated from *E. coli* is heat-stable at elevated temperatures, it will destroy any cDNA synthesized during the RT step at 50°C. Heat-labile UNG is active only at the very beginning of the RT step and removes uracil from dUMP incorporated into any contaminating molecules. After a few minutes, the heat-labile UNG will have lost any activity and therefore cannot interfere with cDNA synthesis. During the RT reaction and the activation step of HotStarTaq DNA Polymerase, the contaminating molecules are destroyed by cleavage of the abasic sites.

* For two-step RT-PCR, we recommend using the QuantiTect SYBR Green PCR +UNG Kit, which is comprised of the QuantiTect SYBR Green PCR Kit and a specially developed UNG solution.

Appendix E: Collecting Well Factors on Bio-Rad iQ Cyclers

Bio-Rad iQ cyclers (e.g., iCycler iQ, iQ5, and MyiQ) need to collect well factors at the start of each real-time PCR experiment to compensate for any excitation or pipetting nonuniformity. When performing SYBR Green-based real-time PCR, **dynamic well factors** cannot be collected from the experimental plate unless the PCR master mix has been spiked with fluorescein, an additional fluorophore. This is because SYBR Green fluoresces insufficiently in the initial PCR step, where there is insufficient double-stranded DNA to bind SYBR Green and allow fluorescence. Alternatively, **external well factors** can be collected from an external well factor plate containing only fluorescein solution. In our experience, collecting external well factors is a more reliable and convenient alternative to collecting dynamic well factors when using QuantiFast SYBR Green Kits and QuantiTect SYBR Green Kits on Bio-Rad iQ cyclers.

If using a QuantiFast SYBR Green Kit or QuantiTect SYBR Green Kit on a Bio-Rad iQ cycler, follow the procedure below to prepare and run an external well factor plate.

Procedure

E1. Dilute 10x External Well Factor Solution (Bio-Rad, cat. no. 170-8794; contains fluorescein) to a 1x concentration with distilled water.

E2. Distribute the diluted solution into the wells of a PCR plate, and seal with optically clear sealing film.

The volume of diluted solution per well depends on the real-time PCR volume. For example, if the PCR volume will be 50 µl, then distribute 50 µl of diluted solution per well.

E3. Briefly centrifuge the external well factor plate, place it into the Bio-Rad iQ cycler, and close the lid.

E4. Select the SYBR Green thermal protocol and plate setup files, and click "Run with selected Protocol".

E5. In the "RunPrep" screen, select *External Plate* as "Well Factor", and click "Begin Run".

The Bio-Rad iQ cycler automatically inserts a 3-cycle protocol, **External.tmo** in front of your thermal protocol to collect optical data.

E6. After well factors are calculated, the Bio-Rad iQ cycler pauses. Replace the external well factor plate with your experimental plate. Click "Continue Running Protocol" to start your experiment.

Note: Once the external well factor plate is prepared, it can be reused several times (over 250 times) until the Bio-Rad iQ cycler indicates that the fluorophore intensity is insufficient to calculate well factors. Store the

external well factor plate at –20°C between experiments, and thaw and centrifuge it before use. Be sure to protect the plate from exposure to light when not in use.

Appendix F: Data Analysis

For data analysis, follow the recommendations provided by the manufacturer of the detection system. Data are produced as sigmoidal-shaped amplification plots, in which the number of cycles is plotted against fluorescence (when using a linear scale). The threshold cycle (C_T) 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 increase in fluorescence. Determination of C_T values is carried out slightly differently on different kinds of cycler.

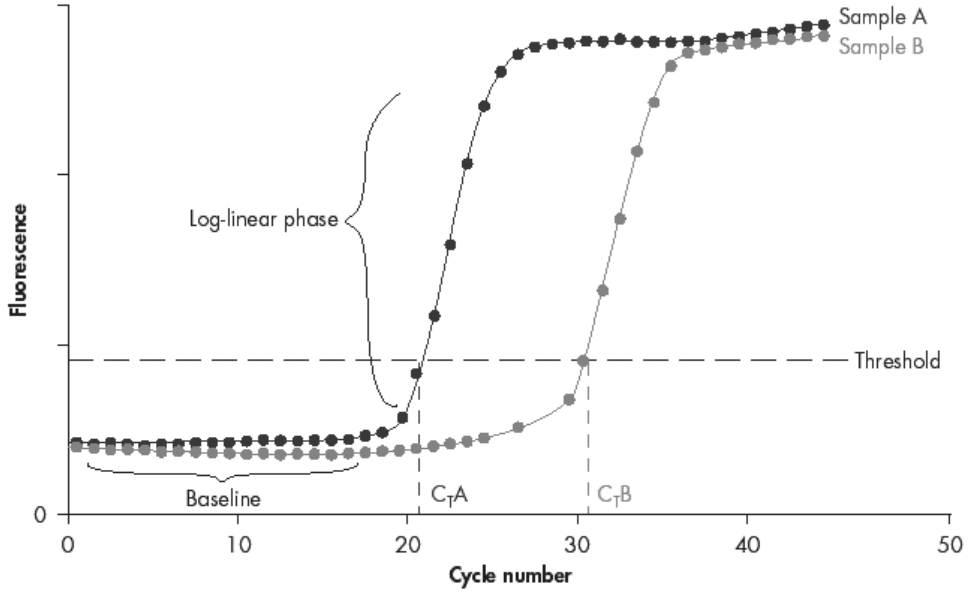

Figure 2. Typical amplification plot. Amplification plots showing increases in fluorescence from 2 samples (A and B). Sample A contains a higher amount of starting template than sample B.

Applied Biosystems instruments

Baseline: The baseline is the noise level in early cycles, typically measured between cycles 3 and 15, where there is no detectable increase in fluorescence due to PCR products. The number of cycles used to calculate the baseline can be changed and should be reduced if high template amounts are used (see Troubleshooting Guide, page 39). The baseline is subtracted from the fluorescence obtained from PCR products.

Threshold: The threshold is adjusted to a value above the baseline, but must be located in the log-linear range of the PCR and never in the plateau phase. When using the ABI PRISM 7700, begin by using the preset threshold, but check whether this is appropriate and adjust it if necessary. When using the GeneAmp® 5700, the user must select a suitable starting threshold and adjust this further if necessary. Setting of the threshold should be done using a

logarithmic amplification plot so that the log-linear range of the curve can be identified easily.

Threshold cycle (C_T): This is the cycle at which the amplification plot crosses the threshold (i.e., at which there is the first detectable increase in fluorescence).

LightCycler instruments

There are 2 different methods of calculating crossing points: the fit-point and the second derivative maximum method.

Fit-point method: The principle of this method is the same as that used for the Applied Biosystems instruments. Use the "arithmetic" mode of baseline adjustment when analyzing data obtained with SYBR Green I based assays.

Noise band: The noise band must be set according to the threshold in the log-linear phase of PCR.

Fit points: These are defined reading points in the log-linear phase, used for calculation of a straight line that represents the linear portion of the amplification plot. The number of fit points can be changed by the user.

Crossing point: This is the cycle at which the straight line (calculated using fit points) crosses the noise band.

Second derivative maximum method: The point at which the maximal increase of fluorescence within the log-linear phase takes place is calculated by determining the second derivative maxima of the amplification curves. The software calculates at which cycle number this point is reached. It is not necessary to set a noise band.

Standard curves

Standard samples with known template amounts are defined in the "sample setup" view. The results from all wells defined as standards are used following the run for the generation of a standard curve. The C_T values or crossing points are plotted against the log of the template amount, resulting in a straight line. C_T values for these samples and the standard curve are then used to calculate the amount of starting template in experimental samples.

Experiment report

The experiment report is a summary of the PCR results. At the end of experiments, sample names, template amounts, C_T values or crossing points, and standard deviations are listed.

Melting curves

A melting curve analysis of PCR product(s) may be optionally performed to verify their specificity and identity. Melting curve analysis is an analysis step built into the software of real-time cyclers. Please follow instructions provided by the supplier.

All cyclers except for the ABI PRISM 7700 (with sequence detection software earlier than 1.7) can calculate a melting curve.

To carry out melting curve analysis, the temperature is increased very slowly from a low temperature (e.g., 65°C) to a high temperature (e.g., 95°C). At low temperatures, all PCR products are double stranded, so SYBR Green I dye binds to them and fluorescence is high, whereas at high temperatures, PCR products are denatured, resulting in rapid decreases in fluorescence.

The fluorescence is measured continuously as the temperature is increased and plotted against temperature. A curve is produced, because fluorescence decreases slightly through the lower end of the temperature range, but decreases much more rapidly at higher temperatures as the melting temperatures of nonspecific and specific PCR products are reached. The detection systems calculate the first derivatives of the curves, resulting in curves with peaks at the respective T_ms. Curves with peaks at a T_m lower than that of the specific PCR product indicate the formation of primer–dimers, while diverse peaks with different T_ms or plateaus indicate production of nonspecific products or a smear.

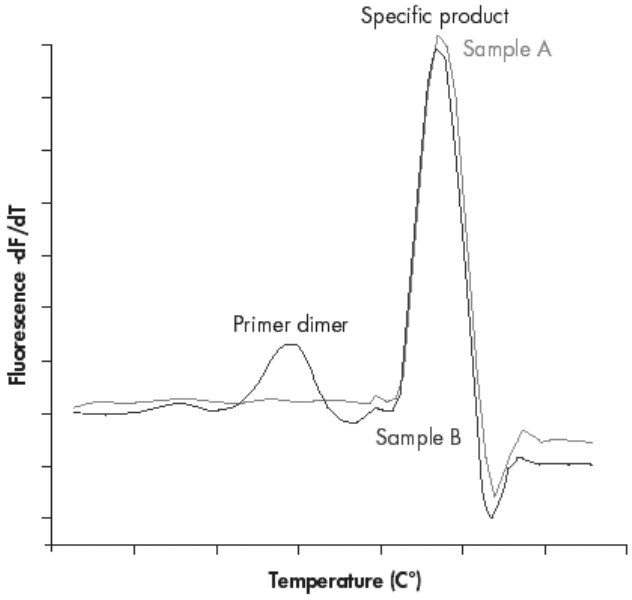

Figure 3. Melting curve analysis. Melting curve analysis of 2 samples (A and B). Sample A yields only 1 peak, resulting from the specific amplification product (primer–dimers not coamplified). Sample B shows a peak from the specific product and a peak at a lower temperature from amplification of primer–dimers.

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.

Cited references

1. Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}_{T}$ method. Methods 25, 402.

Ordering Information

Product	Contents	Cat. no.
QuantiTect Primer Assay (200)*	For 200 x 50 µl reactions or 400 x 25 µl reactions: 10x QuantiTect Primer Assay (lyophilized) supplied in single tube	Varies
Accessories		
Rotor-Gene SYBR Green PCR Kit — for fast real-time PCR and two-step RT-PCR using SYBR Green I on Rotor-Gene cyclers		
Rotor-Gene SYBR Green PCR Kit (400)†	For 400 x 25 µl reactions: 3 x 1.7 ml 2x Rotor-Gene SYBR Green PCR Master Mix, 2 x 2 ml RNase-Free Water	204074
QuantiFast SYBR Green PCR Kit — for fast real-time PCR and two-step RT-PCR using SYBR Green I		
QuantiFast SYBR Green PCR Kit (400)†	For 400 x 25 µl reactions: 3 x 1.7 ml 2x QuantiFast SYBR Green PCR Master Mix (contains ROX dye), 2 x 2 ml RNase-Free Water	204054
QuantiTect SYBR Green PCR Kit — for real-time PCR and two-step RT-PCR using SYBR Green I		
QuantiTect SYBR Green PCR Kit (200)†	For 200 x 50 µl reactions: 3 x 1.7 ml 2x QuantiTect SYBR Green PCR Master Mix (contains ROX dye), 2 x 2 ml RNase-Free Water	204143
QuantiTect SYBR Green PCR +UNG Kit — for real-time PCR and two-step RT-PCR using SYBR Green I with UNG pretreatment		
QuantiTect SYBR Green PCR +UNG Kit (200)	For 200 x 50 µl reactions: 3 x 1.7 ml 2x QuantiTect SYBR Green PCR Master Mix (contains ROX dye), 100 µl UNG, 2 x 2 ml RNase-Free Water	204163

* Visit www.qiagen.com/GeneGlobe to select and order the assay for your gene.

† Trial-size kit and larger kit also available; for details, visit www.qiagen.com.

Product	Contents	Cat. no.
Rotor-Gene SYBR Green RT-PCR Kit — for fast real-time one-step RT-PCR using SYBR Green I on Rotor-Gene cyclers		
Rotor-Gene SYBR Green RT-PCR Kit (400)*	For 400 x 25 µl reactions: 3 x 1.7 ml 2x Rotor-Gene SYBR Green RT-PCR Master Mix, 100 µl Rotor-Gene RT Mix, 2 x 2 ml RNase-Free Water	204174
QuantiFast SYBR Green RT-PCR Kit — for fast real-time one-step RT-PCR using SYBR Green I		
QuantiFast SYBR Green RT-PCR Kit (400)*	For 400 x 25 µl reactions: 3 x 1.7 ml 2x QuantiFast SYBR Green RT-PCR Master Mix (contains ROX dye), 100 µl QuantiFast RT Mix, 2 x 2 ml RNase-Free Water	204154
QuantiTect SYBR Green RT-PCR Kit — for real-time one-step RT-PCR using SYBR Green I		
QuantiTect SYBR Green RT-PCR Kit (200)*	For 200 x 50 µl reactions: 3 x 1.7 ml 2x QuantiTect SYBR Green RT-PCR Master Mix (contains ROX dye), 100 µl QuantiTect RT Mix, 2 x 2 ml RNase-Free Water	204243
FastLane Cell cDNA Kit — for high-speed preparation of cDNA without RNA purification for 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
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

* Larger kit also available; for details, visit www.qiagen.com.

† Trial-size kit and larger kit also available; for details, visit www.qiagen.com.

Product	Contents	Cat. no.
RNeasy Plus Mini Kit — for purification of total RNA from cultured cells and tissues using gDNA Eliminator columns		
RNeasy Plus Mini Kit (50)	For 50 RNA minipreps: 50 RNeasy Mini Spin Columns, 50 gDNA Eliminator Mini Spin Columns, Collection Tubes, RNase-Free Reagents and Buffers	74134
RNase-Free DNase Set — for DNase digestion during RNA purification		
RNase-Free DNase Set (50)	For 50 RNA minipreps, 25 midipreps, or 17 maxipreps: 1500 units RNase-Free DNase I, RNase-Free Buffer RDD, and RNase-Free Water	79254

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

Notes

Notes

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