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QuantiTect® SYBR® Green RT-PCR Handbook

For quantitative, real-time one-step RT-PCR
using SYBR Green I

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Contents

Kit Contents

QuantiTect SYBR Green RT-PCR Kit	(200)	(1000)
Catalog no.	204243	204245
Number of 50 µl reactions	200	1000
2x QuantiTect SYBR Green RT-PCR Master Mix, containing: ■ HotStarTaq® DNA Polymerase ■ QuantiTect SYBR Green RT-PCR Buffer ■ dNTP mix, including dUTP ■ SYBR Green I ■ ROX™ passive reference dye ■ 5 mM $MgCl_2$	3 x 1.7 ml	25 ml
QuantiTect RT Mix, a mixture of the QIAGEN® products: ■ Omniscript® Reverse Transcriptase ■ Sensiscript® Reverse Transcriptase	100 µl	500 µl
RNase-Free Water	2 x 2 ml	20 ml
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Shipping and Storage

The QuantiTect SYBR Green RT-PCR Kit is shipped on dry ice. The kit should be stored immediately upon receipt at –20°C in a constant-temperature freezer and protected from light. When the kit is stored under these conditions and handled correctly, performance is guaranteed until the expiration date (see the quality-control label inside the kit box or on the kit envelope). 2x QuantiTect SYBR Green RT-PCR Master Mix can also be stored protected from light at 2–8°C for up to 6 months, depending on the expiration date, without showing any reduction in performance. However, QuantiTect RT Mix must always be stored at –20°C to guarantee performance.

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

Product Use Limitations

The QuantiTect SYBR Green RT-PCR Kit is intended for molecular biology applications. This product is not intended for the diagnosis, prevention, or treatment of disease.

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

Product Warranty and Satisfaction Guarantee

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

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

Technical Assistance

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

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

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

Safety Information

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

24-hour emergency information

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

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

Product Description

Component	Description
HotStarTaq DNA Polymerase*	HotStarTaq DNA Polymerase is a modified form of a recombinant 94 kDa DNA polymerase, originally isolated from *Thermus aquaticus,* cloned into *E. coli.* (Deoxynucleoside-triphosphate: DNA deoxynucleotidyltransferase, EC 2.7.7.7).
QuantiTect SYBR Green RT-PCR Buffer*	Contains Tris·Cl, KCl, $(NH_4)_2SO_4$, 5 mM $MgCl_2$, pH 8.7 (20°C)
dNTP mix*	Contains dATP, dCTP, dGTP, and dTTP/dUTP of ultrapure quality
Fluorescent dyes*	SYBR Green I and ROX
QuantiTect RT Mix	Contains an optimized mixture of the QIAGEN products Omniscript Reverse Transcriptase and Sensiscript Reverse Transcriptase, both of which are recombinant heterodimeric enzymes expressed in *E. coli.*
RNase-free water	Ultrapure quality, PCR-grade

* Included in 2x QuantiTect SYBR Green RT-PCR Master Mix.

Quality Control

Component	Test
QuantiTect SYBR Green RT-PCR Master Mix*	RT-PCR sensitivity and reproducibility assay: Sensitivity, reproducibility, and specificity in real-time RT-PCR are tested in parallel 50 µl reactions containing 10-fold dilutions of nucleic acid template.
HotStarTaq DNA Polymerase†	Efficiency and reproducibility in PCR are tested. Functional absence of exonucleases and endonucleases is tested.
QuantiTect SYBR Green RT-PCR Buffer†	Conductivity and pH are tested.
QuantiTect RT Mix	Efficiency of cDNA synthesis and functional absence of RNases, exonucleases, and endonucleases are tested.
RNase-free water	Conductivity, pH, and RNase activities are tested.

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

† Included in 2x QuantiTect SYBR Green RT-PCR Master Mix.

Introduction

The QuantiTect SYBR Green RT-PCR Kit provides accurate real-time quantification of RNA targets in an easy-to-handle format. The fluorescent dye SYBR Green I in the master mix enables the analysis of many different targets without having to synthesize target-specific labeled probes. High specificity and sensitivity in RT-PCR are achieved by the use of the hot-start enzyme HotStarTaq DNA Polymerase together with a specialized RT-PCR buffer. The buffer also contains ROX dye, which allows fluorescence normalization on certain cyclers. The optimized Omniscript and Sensiscript blend for the reverse-transcription step further enhances sensitivity.

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

One-step RT-PCR

Use of 2x QuantiTect SYBR Green RT-PCR Master Mix together with QuantiTect RT Mix allows both reverse transcription and PCR to take place in a single tube. All reagents required for both reactions are added at the beginning, so there is no need to open the tube once the reverse-transcription reaction has been started.

The components of 2x QuantiTect SYBR Green RT-PCR Master Mix include HotStarTaq DNA Polymerase, QuantiTect SYBR Green RT-PCR Buffer, SYBR Green I, and ROX passive reference dye (see descriptions below). QuantiTect RT Mix contains an Omniscript and Sensiscript blend (see descriptions below).

Omniscript and Sensiscript

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

HotStarTaq DNA Polymerase

HotStarTaq DNA Polymerase is a modified form of QIAGEN *Taq* DNA Polymerase, and is provided in an inactive state and has no enzymatic activity at ambient temperature. The enzyme remains completely inactive during the reverse-transcription reaction and does not interfere with it. This prevents formation of misprimed RT-PCR products and

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

primer–dimers during reaction setup, reverse transcription, and the first denaturation step. The enzyme is activated after the reverse-transcription step by a 15-minute, 95°C incubation step. The hot start also inactivates the reverse-transcription enzymes, ensuring temporal separation of reverse transcription and PCR, and allowing both steps to be performed sequentially in a single tube.

QuantiTect SYBR Green RT-PCR Buffer

QuantiTect SYBR Green RT-PCR Buffer is designed to facilitate both efficient reverse transcription and specific amplification in a one-tube format. It is based on the unique QIAGEN OneStep RT-PCR buffer system, and has been specifically adapted for SYBR Green-based real-time RT-PCR. The buffer contains a balanced combination of KCl and $(NH_4)_2SO_4$, which promotes a high ratio of specific to nonspecific primer binding during the annealing step of each PCR cycle. This creates stringent primer annealing conditions, leading to increased PCR specificity. When using this buffer, primer annealing is only marginally influenced by the $MgCl_2$ concentration, so optimization by titration of Mg^{2+} is usually not required.

SYBR Green I

2x QuantiTect SYBR Green RT-PCR Master Mix contains an optimized concentration of the fluorescent dye SYBR Green I. SYBR Green I binds all double-stranded DNA molecules, emitting a fluorescent signal on binding. 2x QuantiTect SYBR Green RT-PCR Master Mix can be stored at 2–8°C or –20°C without loss of SYBR Green I fluorescence activity. The excitation and emission maxima of SYBR Green I are at 494 nm and 521 nm, respectively, which are compatible with use on any real-time cycler.

Passive reference dye

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

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

Use of uracil-N-glycosylase (UNG)

The QuantiTect SYBR Green RT-PCR Kit contains dUTP, which partially replaces dTTP. The QuantiTect SYBR Green RT-PCR Kit therefore allows the optional use of a uracil-N-glycosylase (UNG) pretreatment of the reaction if contamination with carried-over PCR products is suspected.

However, 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 the QuantiTect SYBR Green RT-PCR Kit and must be purchased separately.

Validated, ready-to-use primer sets

For optimal results in gene expression analysis, we recommend using QuantiTect Primer Assays. These are ready-to-use primer sets that are guaranteed to provide specific and sensitive quantification when used in combination with QuantiTect SYBR Green Kits. Where possible, primer sets are designed to specifically amplify cDNA sequences derived from mRNA transcripts. This prevents amplification of contaminating genomic DNA, which would then lead to inaccurate quantification. QuantiTect Primer Assays are easily ordered online at www.qiagen.com/GeneGlobe and are available genomewide for human, mouse, rat, and many other species.

Note: If using QuantiTect Primer Assays in combination with the QuantiTect SYBR Green RT-PCR Kit, follow the protocols in the *QuantiTect Primer Assay Handbook*, which can be downloaded at www.qiagen.com/HB/PrimerAssay.

Using the correct protocol

This handbook contains 2 protocols. The first protocol (page 11) is for use with most real-time cyclers, including Rotor-Gene cyclers and instruments from Applied Biosystems, Bio-Rad, Cepheid, Eppendorf, Roche, and Agilent. The second protocol (page 16) is for use with the LightCycler® 1.x and LightCycler 2.0 only.

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

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

This protocol is intended for use with most real-time cyclers, including Rotor-Gene cyclers and instruments from Applied Biosystems, Bio-Rad, Cepheid, Eppendorf, Roche, and Agilent. If using the **LightCycler 1.x or LightCycler 2.0**, follow the protocol on page 16.

Reaction volume

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

When reducing the reaction volume, remember to reduce the volume of master mix and RT mix used in the reaction: the volume of 2x QuantiTect SYBR Green RT-PCR Master Mix should always be half of the final reaction volume, and the volume of QuantiTect RT Mix should always be one-hundredth of the final reaction volume. In addition, be sure to keep the concentration of primers, template, and UNG the same as described in Table 1 (however, if using a SmartCycler system, primer concentration should be increased to 1 µM).

Important points before starting

- For the highest efficiency in real-time RT-PCR using SYBR Green I, targets should ideally be **100–150 bp in length**.
- After reverse transcription, the PCR step of the RT-PCR must start with an **initial incubation step of 15 min at 95°C** to activate HotStarTaq DNA Polymerase.
- Set up all reactions on ice to avoid premature cDNA synthesis.
- The kit has been optimized for a final reaction volume of 50 µl. If other reaction volumes are used, adjust the amounts of 2x QuantiTect SYBR Green RT-PCR Master Mix and QuantiTect RT Mix used so that the ratio between them remains constant.
- Always start with an **initial Mg^{2+} concentration of 2.5 mM** as provided in 2x QuantiTect SYBR Green RT-PCR Master Mix. However, if a higher Mg^{2+} concentration is required, use a stock solution of $MgCl_2$ for further optimization.
- Always readjust the threshold value for analysis of every run.
- 2x QuantiTect SYBR Green RT-PCR Master Mix contains dUTP, which allows the use of a uracil-N-glycosylase (UNG) pretreatment of the reaction. Only **heat-labile** UNG should be used.
- 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 using **QuantiTect Primer Assays**, please follow the protocols in the *QuantiTect Primer Assay Handbook*, which can be downloaded at www.qiagen.com/HB/PrimerAssay.
- 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 A (page 26).
- If using a **SmartCycler system**, the reverse-transcription step can be reduced to 20 min.
- If using the **Applied Biosystems 7500**, it may be necessary to adjust the preset threshold value to a lower value. For details, see Appendix B (page 27).

Procedure

1. **Thaw 2x QuantiTect SYBR Green RT-PCR Master Mix (if stored at –20°C), template RNA, primers, 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 1.**

   Keep samples on ice while preparing the reaction mix.

   If final reaction volumes other than 50 µl are used, adjust the volumes of 2x QuantiTect SYBR Green RT-PCR Master Mix and QuantiTect RT Mix used so that the ratio between them remains constant.

   Note: We strongly recommend starting with an initial Mg^{2+} concentration of 2.5 mM as provided by 2x QuantiTect SYBR Green RT-PCR Master Mix. For a few targets, reactions may be improved by using Mg^{2+} concentrations of up to 4 mM.

Table 1. Reaction setup

Component	Volume/reaction	Final concentration
2x QuantiTect SYBR Green RT-PCR Master Mix*	25 µl†	1x
Primer A	Variable	0.5 µM‡
Primer B	Variable	0.5 µM‡
QuantiTect RT Mix	0.5 µl§	
Template RNA (added at step 4)	Variable	≤500 ng/reaction
RNase-free water	Variable	
Optional: Uracil-N-glycosylase, heat-labile	Variable	1–2 units/reaction
Total reaction volume	**50 µl**	

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

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

‡ A final primer concentration of 0.5 µM is usually optimal. However, for individual determination of optimal primer concentration, a primer titration from 0.4 µM to 1 µM can be performed. SmartCycler users should use a final primer concentration of 1 µM for each primer; if necessary, a primer titration from 0.5 µM to 2 µM can be performed to determine the optimal primer concentration.

§ If using a total reaction volume other than 50 µl, calculate the volume of RT mix required using this formula: Volume of RT mix (µl) = 0.01 x [Total reaction volume (µl)].

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 (≤500 ng/reaction) to the individual PCR tubes or wells containing the reaction mix.**

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

5. **Program your real-time cycler according to the program outlined in Table 2.**

   Data acquisition should be performed during the extension step. After performing melting curve analysis (see step 7, page 14), an additional data acquisition step for further runs with the same target can be integrated (for details, see steps 8 and 9, page 15).

Table 2. Real-time cycler conditions

Step	Time	Temperature	Additional comments
Reverse transcription*	**30 min**	**50°C**	
PCR initial activation step	**15 min**	**95°C**	HotStarTaq DNA Polymerase is activated by this heating step
3 (4)-step cycling:			
Denaturation*	15 s	94°C	
Annealing	30 s	50–60°C	Approximately 5–8°C below T_m of primers
Extension	30 s	72°C	Perform fluorescence data collection, unless an additional data acquisition step has been integrated
Optional: Data acquisition	15 s	x°C	T_m dimer < x < T_m product: see steps 8 and 9 for details
Number of cycles	35–45		The number of cycles depends on the amount of template RNA and transcript abundance

* SmartCycler users can reduce reverse-transcription time to 20 min and denaturation time to 1 s to take advantage of cycling capacities.

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. For details, see Appendix B, page 27.

7. **Perform a melting curve analysis of the RT-PCR product(s).**

   We strongly recommend performing this analysis routinely to verify the specificity and identity of RT-PCR products. Melting curve analysis is an analysis step built into the software of real-time cyclers. Please follow the instructions provided by the supplier. Generally, melting curve data between 65°C and 95°C should be acquired.

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

   Depending on primer design and copy number of target, primer–dimers may occur. These can be distinguished from the specific product through their lower melting point.

8. **Optional: Repeat the previous run, including an additional data acquisition step.**

   To suppress fluorescence readings caused by the generation of primer–dimers, an additional data acquisition step can be added to the 3-step cycling protocol (see Table 2, page 14). The temperature should be above the T_m of primer–dimers but approximately 3°C below the T_m of the specific PCR product. This method can increase the dynamic range and reliability of quantification by several orders of magnitude if primer–dimers are coamplified.

9. **Optional: Check the specificity of RT-PCR product(s) by agarose gel electrophoresis.**

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

This protocol is intended for use with the LightCycler 1.x and LightCycler 2.0 only. For all other cyclers, follow the protocol on page 11.

Important points before starting

- Always start with the cycling conditions specified in this protocol, even if using previously established primer–template systems.
- For the highest efficiency in real-time RT-PCR using SYBR Green I, targets should ideally be **100–150 bp in length**.
- After reverse transcription, the PCR step of the RT-PCR must start with an **initial incubation step of 15 min at 95°C** to activate HotStarTaq DNA Polymerase.
- Set up all reactions in cooled capillaries to avoid premature cDNA synthesis.
- The kit has been optimized for a final reaction volume of 20 µl. If other reaction volumes are used, adjust the amounts of 2x QuantiTect SYBR Green RT-PCR Master Mix and QuantiTect RT Mix used so that the ratio between them remains constant.
- Always start with an **initial Mg^{2+} concentration of 2.5 mM** as provided in 2x QuantiTect SYBR Green RT-PCR Master Mix.
- Always readjust the noise band for analysis of every run if using the "fit-point" method for data analysis.
- 2x QuantiTect SYBR Green RT-PCR Master Mix contains dUTP, which allows the use of a uracil-N-glycosylase (UNG) pretreatment of the reaction. Only **heat-labile** UNG should be used.
- If using **QuantiTect Primer Assays**, please follow the protocols in the *QuantiTect Primer Assay Handbook*, which can be downloaded at www.qiagen.com/HB/PrimerAssay.

Procedure

1. **Thaw 2x QuantiTect SYBR Green RT-PCR Master Mix (if stored at –20°C), template RNA, primers, 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 3.**

   Keep capillaries cooled while preparing the reaction mix.

   If final reaction volumes other than 20 µl are used, adjust the volumes of 2x QuantiTect SYBR Green RT-PCR Master Mix and QuantiTect RT Mix used so that the ratio between them remains constant.

   Note: We strongly recommend starting with an initial Mg^{2+} concentration of 2.5 mM as provided by 2x QuantiTect SYBR Green RT-PCR Master Mix. For a few targets, reactions may be improved by using Mg^{2+} concentrations of up to 4 mM.

Table 3. Reaction setup

Component	Volume/reaction	Final concentration
2x QuantiTect SYBR Green RT-PCR Master Mix*	10 µl	1x
Primer A	Variable	1 µM†
Primer B	Variable	1 µM†
QuantiTect RT Mix	0.2 µl	
Template RNA (added at step 4)	Variable	≤ 500 ng/reaction
RNase-free water	Variable	
Optional: Uracil-N-glycosylase, heat-labile	Variable	1–2 units/reaction
Total reaction volume	**20 µl**	

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

† A final primer concentration of 1 µM is usually optimal. However, for individual determination of optimal primer concentration, a primer titration from 0.5 µM to 2 µM can be performed.

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

   Keep the PCR capillaries cooled.

4. **Add template RNA (≤ 500 ng/reaction) to the individual PCR capillaries containing the reaction mix.**

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

5. **Program the LightCycler according to the program outlined in Table 4. Set fluorescence gains as described in Table 5 (for LightCycler software versions earlier than 3.5).**

   Data acquisition should be performed during the extension step. After performing melting curve analysis (see step 7, page 19), an additional data acquisition step for further runs with the same target can be integrated (for details, see steps 8 and 9, page 19).

Table 4. Real-time cycler conditions

Step	Time	Temperature	Ramp rate	Additional comments
Reverse transcription	**20 min**	**50°C**	**20°C/s**	
PCR initial activation step	**15 min**	**95°C**	**20°C/s**	HotStarTaq DNA Polymerase is activated by this heating step
3 (4)-step cycling:				
Denaturation	15 s	94°C	20°C/s	
Annealing	20–30 s	50–60°C	20°C/s	Approximately 5–8°C below T_m of primers
Extension	30 s	72°C	2°C/s	Perform fluorescence data collection, unless an additional data acquisition step has been integrated. Extension time depends on product length. Allow 5 s per 100 bp, with a minimum extension time of 10 s.
Optional: Data acquisition	5 s	x°C	20°C/s	T_m dimer < x < T_m product: see steps 8 and 9 for details
Number of cycles	35–55			The number of cycles depends on the amount of template RNA and transcript abundance

Table 5. Fluorescence parameters

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

Display mode: 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.

6. **Place the PCR capillaries in the LightCycler, and start the cycling program.**

   Ensure that the temperature is set to 50°C during the "seek sample" process.

7. **Perform a melting curve analysis of the RT-PCR product(s).**

   We strongly recommend performing this analysis routinely to verify the specificity and identity of RT-PCR products. Melting curve analysis is an analysis step built into the LightCycler software. Please follow the instructions provided by the supplier. Generally, melting curve data between 65°C and 95°C should be acquired.

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

   Depending on primer design and copy number of target, primer–dimers may occur. These can be distinguished from the specific product through their lower melting point.

8. **Optional: Repeat the previous run, including an additional data acquisition step.**

   To suppress fluorescence readings caused by the generation of primer–dimers, an additional data acquisition step can be added to the 3-step cycling protocol (see Table 4). The temperature should be above the T_m of primer–dimers but approximately 3°C below the T_m of the specific PCR product. This method can increase the dynamic range and reliability of quantification by several orders of magnitude if primer–dimers are coamplified.

9. **Optional: Check the specificity of the RT-PCR product(s) by agarose gel electrophoresis.**

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 RT-PCR, or only primer–dimers detected		
a)	Annealing time too short	Use the recommended annealing time. **LightCycler 1.x and 2.0**: Annealing time is 20–30 s. **All other cyclers**: Annealing time is 30 s.
b)	Extension time too short	Always use the extension times specified in the protocols. **LightCycler 1.x and 2.0**: Extension time should be 5 s per 100 bp of RT-PCR product, with a minimum extension time of 10 s. **All other cyclers**: Extension time should be 30 s for RT-PCR products up to 500 bp.
c)	Mg^{2+} concentration not optimal	Always start with the Mg^{2+} concentration provided in 2x QuantiTect SYBR Green RT-PCR Master Mix (2.5 mM final concentration). For a few targets, an increase up to 4 mM Mg^{2+} may be helpful. Perform the titration in 0.5 mM steps.
d)	Pipetting error or missing reagent	Check the concentrations and storage conditions of the reagents, including primers and template RNA.* Repeat the RT-PCR.
e)	HotStarTaq DNA Polymerase not activated	Ensure that the cycling program includes the HotStarTaq DNA Polymerase activation step (15 min at 95°C) as described in the protocols.

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

		Comments and suggestions
f)	HotStarTaq DNA Polymerase activated too early	Check the cycling program. Ensure that the reverse-transcription reaction is complete before activating HotStarTaq DNA Polymerase. **LightCycler 1.x and 2.0**: 20 min at 50°C **All other cyclers**: 30 min at 50°C
g)	Incorrect temperature for RT reaction	We recommend performing the RT reaction at 50°C. However, if this temperature does not yield satisfactory results, the temperature can be adjusted between 48°C and 55°C.
h)	Incorrect amount of QuantiTect RT Mix	Use the correct amount of QuantiTect RT Mix. **LightCycler 480**: 0.1 µl RT Mix per reaction **LightCycler 1.x and 2.0**: 0.2 µl RT Mix per reaction **SmartCycler and Applied Biosystems 7500 Fast System**: 0.25 µl RT Mix per reaction **All other cyclers**: 0.5 µl RT Mix per reaction
i)	Incorrect ratio of QuantiTect RT Mix to QuantiTect SYBR Green RT-PCR Master Mix	If not using the standard reaction volumes, ensure that the volume of QuantiTect RT Mix is changed proportionately so that the ratio of QuantiTect RT Mix to QuantiTect SYBR Green RT-PCR Master Mix remains the same.
j)	RT-PCR product too long	For optimal results, RT-PCR products should be between 100 and 150 bp. RT-PCR products should not exceed 500 bp.
k)	Primer design not optimal	Check for RT-PCR products by melting curve analysis* or gel electrophoresis. If no specific RT-PCR products are detected, review the primer design guidelines.* Alternatively, use QuantiTect Primer Assays, which are predesigned primer sets for real-time RT-PCR (see ordering information, page 28).
l)	Primer concentration not optimal	Use optimal primer concentrations. **LightCycler 1.x and 2.0 and SmartCycler**: 1 µM each primer **All other cyclers**: 0.5 µM each primer Check the concentration of primers by spectrophotometry.*

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

		Comments and suggestions
m)	Problems with starting template	Check the concentration, storage conditions, and quality of the starting template RNA.* If necessary, make new serial dilutions of template RNA from the stock solutions. Repeat the RT-PCR using the new dilutions.
n)	Insufficient amount of starting template	Increase the amount of template, if possible. Ensure that sufficient copies of the target RNA are present in your sample.
o)	Insufficient number of cycles	Increase the number of cycles.
p)	Annealing temperature too high	Decrease annealing temperature in 3°C steps.
q)	No detection activated	Check that fluorescence detection was activated in the cycling program.
r)	Wrong detection step	Ensure that fluorescence detection takes place during the extension step of the cycling program.
s)	Primers degraded	Check for possible degradation of primers on a denaturing polyacrylamide gel.
t)	Transcript not expressed	Repeat the RT-PCR and include a positive control to make sure the absence of RT-PCR product was not due to problems with reverse transcription, amplification, and detection.*
u)	Heat-labile UNG not used	When performing optional UNG pretreatment before starting RT-PCR, be sure to use **heat-labile** UNG. UNG from *E. coli* is stable at elevated temperatures and will destroy any cDNA synthesized during reverse transcription at 50°C.
v)	**Optional data acquisition step only**: Detection temperature too high	Ensure that the detection temperature is at least 3°C lower than the T_m of the specific product. When establishing a new primer–template system, always perform a 3-step cycling reaction first, without the optional data acquisition step (step 8 of the protocols).

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

Real-time cyclers other than the LightCycler 1.x and 2.0:

w)	Wrong detection channel/filter chosen	Ensure that the correct detection channel is activated or the correct filter set is chosen for SYBR Green I.

LightCycler 1.x and 2.0 only:

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

Primer–dimers and/or nonspecific RT-PCR products

a)	Reaction set up at room temperature	Set up the RT-PCR in cooled reaction vessels to avoid premature cDNA synthesis from nonspecific primer annealing.
b)	Starting conditions for reverse-transcription reaction incorrect	Ensure that the RT-PCR program is started immediately after transfer of samples to the real-time cycler.
c)	Mg^{2+} concentration not optimal	Always start with the Mg^{2+} concentration provided in 2x QuantiTect SYBR Green RT-PCR Master Mix (2.5 mM final concentration). For a few targets, an increase up to 4 mM Mg^{2+} may be helpful. Perform titration in 0.5 mM steps.
d)	Annealing temperature too low	Increase annealing temperature in increments of 2°C.
e)	Primer design not optimal	Review primer design.* If redesigning the primers is not possible, include an additional data acquisition step at a temperature above the T_m of primer–dimers (see step 5 in the protocols). Alternatively, use QuantiTect Primer Assays, which are predesigned primer sets for real-time RT-PCR (see ordering information, page 28).
f)	Contamination of RNA sample with genomic DNA	Design primers that span exon–exon boundaries, so that only cDNA targets can be amplified and detected. Alternatively, use QuantiTect Primer Assays, which are predesigned primer sets that avoid amplification of genomic DNA where possible (see ordering information, page 28). Alternatively, treat the RNA sample with DNase to digest the contaminating genomic DNA.

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

		Comments and suggestions
g)	RT-PCR product too long	For optimal results, RT-PCR products should be between 100 and 150 bp. RT-PCR products should not exceed 500 bp.
h)	Primer–dimers coamplified	Include an additional data acquisition step in the cycling program as indicated in the protocols (step 8) to avoid the detection of primer–dimers.
i)	Reverse-transcription reaction temperature too low	A reverse-transcription reaction temperature of 50°C is recommended. However, if this does not yield satisfactory results, the reaction temperature may be increased up to 55°C.
j)	Primers degraded	Check for possible degradation of primers on a denaturing polyacrylamide gel.
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 500 ng RNA template per reaction.
b)	Template amount too low	Increase the amount of template, if possible.
c)	Primer–dimers coamplified	Include an additional data aquisition step in the cycling program as indicated in the protocols (step 8) to avoid the detection of primer–dimers.
d)	Incorrect amount of QuantiTect RT Mix	Use the correct amount of QuantiTect RT Mix. **LightCycler 480**: 0.1 µl RT Mix per reaction **LightCycler 1.x and 2.0**: 0.2 µl RT Mix per reaction **SmartCycler and Applied Biosystems 7500 Fast System**: 0.25 µl RT Mix per reaction **All other cyclers**: 0.5 µl RT Mix per reaction
High fluorescence in "No Template" control		
a)	Contamination of reagents	Discard reaction components and repeat RT-PCR with new reagents.
b)	Contamination during reaction setup	Take appropriate safety precautions (e.g., use filter tips). Use heat-labile uracil-N-glycosylase to prevent carryover from previous reactions.

High fluorescence in "No Reverse Transcription" control

Contaminating genomic DNA in RNA sample	Design primers that span exon–exon boundaries, so that only cDNA targets can be amplified and detected. Alternatively, use QuantiTect Primer Assays, which are predesigned primer sets that avoid amplification of genomic DNA where possible (see ordering information, page 28). Alternatively, treat the RNA sample with DNase to digest the contaminating genomic DNA.

Varying fluorescence intensity

a)	Real-time cycler contaminated	Decontaminate the real-time cycler according to the supplier's instructions.
b)	Real-time cycler no longer calibrated	Recalibrate the real-time cycler according to the supplier's instructions.

Cyclers from Applied Biosystems, Bio-Rad, and Agilent 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)	RT-PCR mix not in capillary tip	Centrifuge the capillary to bring the RT-PCR mix into the capillary tip.
e)	Capillary not pushed down completely	Ensure that the capillary is completely pushed down in the LightCycler carousel.
f)	**LightCycler 1.x only**: Wrong detection channel	Make sure that Channel 1 is chosen.

Appendix A: 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 QuantiTect SYBR Green Kits on Bio-Rad iQ cyclers.

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

Procedure

A1. Dilute 10x External Well Factor Solution (Bio-Rad, cat. no. 170-8794; contains fluorescein) to a 1x concentration with distilled water.

A2. 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.

A3. Briefly centrifuge the external well factor plate, place it into the Bio-Rad iQ cycler, and close the lid.

A4. Select the SYBR Green thermal protocol and plate setup files, and click "Run with selected Protocol".

A5. 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.

A6. 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 B: Analysis Settings for the Applied Biosystems 7500

When using the Applied Biosystems 7500, the function for automatic threshold calculation may not detect the amplification signal and result in an error message. This can be overcome by choosing manual threshold calculation and adjusting the preset threshold value to a lower value (Figure 1). Use a value of 0.02 as a starting point.

Adjust the preset threshold to a value in the range of 0.02, either by entering a value in the dialog field or by moving the threshold bar in the log-linear phase of the amplification plot using the mouse. After adjusting the threshold, click the "Analyze" button to reanalyze the data.

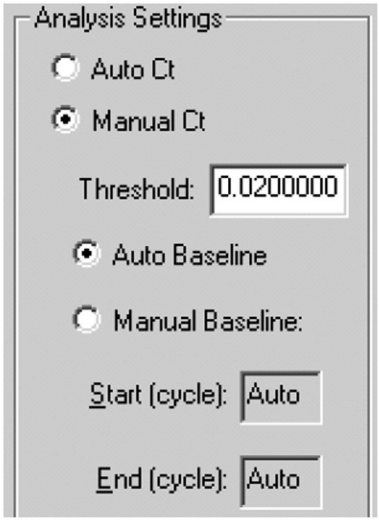

Figure 1. Adjusting the analysis settings for the Applied Biosystems 7500. Select "Manual Ct" and "Auto Baseline".

Ordering Information

Product	Contents	Cat. no.
QuantiTect SYBR Green RT-PCR Kit (200)	For 200 x 50 µl reactions: 3 x 1.7 ml 2x Master Mix, 100 µl RT Mix, 2 x 2 ml RNase-Free Water	204243
QuantiTect SYBR Green RT-PCR Kit (1000)	For 1000 x 50 µl reactions: 25 ml 2x Master Mix, 0.5 ml RT Mix, 20 ml RNase-Free Water	204245
Accessories		
QuantiTect Primer Assays — for use in real-time RT-PCR with SYBR Green detection (search for and order assays at www.qiagen.com/GeneGlobe)		
QuantiTect Primer Assay (200)	For 200 x 50 µl reactions: 10x QuantiTect Primer Assay (lyophilized)	Varies
Related products		
QuantiTect SYBR Green PCR Kit — for quantitative, real-time PCR and two-step RT-PCR using SYBR Green I		
QuantiTect SYBR Green PCR Kit (40)	For 40 x 50 µl reactions: 1 ml 2x Master Mix, 2 ml RNase-Free Water	204141
QuantiTect SYBR Green PCR Kit (200)	For 200 x 50 µl reactions: 3 x 1.7 ml 2x Master Mix, 2 x 2 ml RNase-Free Water	204143
QuantiTect SYBR Green PCR Kit (1000)	For 1000 x 50 µl reactions: 25 ml 2x Master Mix, 20 ml RNase-Free Water	204145
QuantiTect SYBR Green PCR +UNG Kit (200)	For 200 x 50 µl reactions: 3 x 1.7 ml 2x Master Mix, 100 µl UNG Solution, 2 x 2 ml RNase-Free Water	204163

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

Notes

Notes

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