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# QuantiTect® SYBR® Green PCR Handbook

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

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Sample & Assay Technologies

# QIAGEN Sample and Assay Technologies

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# Contents

## Kit Contents

| QuantiTect SYBR Green PCR Kit | (40) | (200)* | (1000) |
|---|---|---|---|
| Catalog no. | 204141 | 204143 | 204145 |
| Number of 50 µl reactions | 40 | 200 | 1000 |
| 2x QuantiTect SYBR Green PCR Master Mix, containing:<br>■ HotStarTaq® DNA Polymerase<br>■ QuantiTect SYBR Green PCR Buffer<br>■ dNTP mix, including dUTP<br>■ SYBR Green I<br>■ ROX™ passive reference dye<br>■ 5 mM $MgCl_2$ | 1 ml | 3 x 1.7 ml | 25 ml |
| RNase-Free Water | 2 ml | 2 x 2 ml | 20 ml |
| Handbook | 1 | 1 | 1 |

* For optional UNG treatment, we recommend the QuantiTect SYBR Green PCR +UNG Kit (cat. no. 204163), which consists of the QuantiTect SYBR Green PCR Kit (200) and an optimized UNG solution.

## Shipping and Storage

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

To maintain optimal performance of the QuantiTect SYBR Green 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 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 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 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 |
| RNase-free water | Ultrapure quality, PCR-grade |

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

# Quality Control

| Component | Test |
|---|---|
| QuantiTect SYBR Green PCR Master Mix* | PCR sensitivity and reproducibility assay: Sensitivity, reproducibility, and specificity in real-time 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 PCR Buffer† | Conductivity and pH are tested. |
| RNase-free water | Conductivity, pH, and RNase activities are tested. |

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

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

# Introduction

The QuantiTect SYBR Green PCR Kit provides accurate real-time quantification of DNA and cDNA targets in an easy-to-handle format. The kit can be used in real-time PCR of genomic DNA targets, and also in real-time two-step RT-PCR of RNA targets following reverse transcription with, for example, the QuantiTect Reverse Transcription Kit (see ordering information, page 27). 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 PCR are achieved by the use of the hot-start enzyme HotStarTaq DNA Polymerase together with a specialized PCR buffer. The buffer also contains ROX dye, which allows fluorescence normalization on certain cyclers.

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

## 2x QuantiTect SYBR Green PCR Master Mix

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

### HotStarTaq DNA Polymerase

HotStarTaq DNA Polymerase is a modified form of QIAGEN *Taq* DNA Polymerase, and is provided in an inactive state and has no enzymatic activity at ambient temperature. This prevents the formation of misprimed products and primer–dimers during reaction setup and the first denaturation step, leading to high PCR specificity and accurate quantification. The enzyme is activated at the start of a reaction by a 15-minute, 95°C incubation step. The hot start enables reactions to be set up rapidly and conveniently at room temperature.

### QuantiTect SYBR Green PCR Buffer

QuantiTect SYBR Green PCR Buffer is based on the unique QIAGEN PCR buffer system, and has been specifically adapted for SYBR Green-based real-time 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.

* 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.

**SYBR Green I**

2x QuantiTect SYBR Green 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 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 PCR Kit contains dUTP, which partially replaces dTTP. The QuantiTect SYBR Green PCR Kit therefore allows the optional use of a uracil-N-glycosylase (UNG) pretreatment of the reaction if contamination with carried-over PCR products is suspected.

**Note**: UNG is not included in the QuantiTect SYBR Green PCR Kit and must be purchased separately. We recommend the QuantiTect SYBR Green PCR +UNG Kit (cat. no. 204163), which consists of the QuantiTect SYBR Green PCR Kit (200) and a UNG solution specially optimized for use with QuantiTect PCR buffers.

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

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

For reverse transcription, we recommend using the QuantiTect Reverse Transcription Kit. The kit provides a fast and convenient procedure, requiring only 20 minutes to synthesize first-strand cDNA and eliminate genomic DNA contamination. An optimized mix of oligo-dT and random primers enables cDNA synthesis from all regions of RNA transcripts, even from 5' regions of very long mRNA transcripts. cDNA yields are high, allowing sensitive detection of even low-abundance transcripts in real-time two-step RT-PCR. An alternative to the QuantiTect Reverse Transcription Kit is the FastLane® Cell cDNA Kit, which allows cDNA to be prepared directly from cultured cells without RNA purification. The FastLane Cell cDNA Kit is useful for experiments where archiving of purified RNA is not required. For ordering information for these 2 kits, see page 27.

For very small RNA samples (as little as 1 ng), we recommend carrying out whole transcriptome amplification using the QuantiTect Whole Transcriptome Kit, which provides high yields of up to 40 µg cDNA for unlimited real-time PCR analysis. The kit contains all the necessary reagents for reverse transcription followed by cDNA ligation and amplification of all cDNA targets. The relative abundance of each transcript is preserved after whole transcriptome amplification, ensuring reliable gene expression analysis. For ordering information, see page 27.

## Validated, ready-to-use primer sets

If performing real-time two-step RT-PCR, 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 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 15) is for use with the LightCycler® 1.x and LightCycler 2.0 only.

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

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

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

### Reaction volume

A reaction volume of 50 µl should be used with most real-time cyclers. However, the reaction volume 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 used in the reaction: the volume of 2x QuantiTect SYBR Green PCR Master Mix should always be half 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.

### Important points before starting

- For the highest efficiency in real-time PCR using SYBR Green I, targets should ideally be **100–150 bp in length**.
- The PCR must start with an **initial incubation step of 15 min at 95°C** to activate HotStarTaq DNA Polymerase.
- Always start with an **initial $Mg^{2+}$ concentration of 2.5 mM** as provided in 2x QuantiTect SYBR Green PCR Master Mix.
- Always readjust the threshold value for analysis of every run.
- 2x QuantiTect SYBR Green PCR Master Mix contains dUTP, which allows the use of a uracil-N-glycosylase (UNG) pretreatment of the reaction.
- 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 24).
- 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 25).

**Procedure**

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

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

   **Note**: We strongly recommend starting with an initial $Mg^{2+}$ concentration of 2.5 mM as provided by 2x QuantiTect SYBR Green PCR Master Mix. For a few targets, reactions may be improved by using $Mg^{2+}$ concentrations of up to 5 mM.

**Table 1. Reaction setup**

| Component | Volume/reaction | Final concentration |
|---|---|---|
| 2x QuantiTect SYBR Green PCR Master Mix* | 25 µl† | 1x |
| Primer A | Variable | 0.3 µM‡ |
| Primer B | Variable | 0.3 µM‡ |
| Template DNA or cDNA (added at step 4) | Variable | ≤500 ng/reaction |
| RNase-free water | Variable | |
| Optional: Uracil-N-glycosylase | Variable | 0.5 units/reaction |
| **Total reaction volume** | **50 µl** | |

* Provides a final concentration of 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 0.3 µM is usually optimal. However, for individual determination of optimal primer concentration, a primer titration from 0.2 µM to 1 µM can be performed. SmartCycler users should use a final primer concentration of 0.5 µM for each primer; if necessary, a primer titration from 0.5 µM to 1 µM can be performed to determine the optimal primer concentration.

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

   For two-step RT-PCR, the volume of the cDNA added (from the undiluted RT reaction) should not exceed 10% of the final PCR volume.

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

   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 14).

**Table 2. Real-time cycler conditions**

| Step | Time | Temperature | Additional comments |
|---|---|---|---|
| **Optional: UNG** (carryover prevention) | 2 min | 50°C | UNG will eliminate any dUMP-containing PCR products resulting from carryover contamination |
| **PCR initial activation step** | **15 min** | **95°C** | HotStarTaq DNA Polymerase is activated by this heating step |
| **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 DNA |

* SmartCycler users can reduce 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 25.

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

   We strongly recommend performing this analysis routinely to verify the specificity and identity of 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 a PCR product depends on buffer composition and salt concentration. $T_m$ values obtained when using the QuantiTect SYBR Green 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 13). 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 PCR product(s) by agarose gel electrophoresis.**

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

This protocol is intended for use with the LightCycler 1.x and LightCycler 2.0 only. 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. Pay particular attention to Table 4 (page 17) and step 7 (page 18).
- For the highest efficiency in real-time PCR using SYBR Green I, targets should ideally be **100–150 bp in length**.
- The PCR must start with an **initial incubation step of 15 min at 95°C** to activate HotStarTaq DNA Polymerase.
- Always start with **an initial $Mg^{2+}$ concentration of 2.5 mM** as provided 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.
- 2x QuantiTect SYBR Green PCR Master Mix contains dUTP, which allows the use of a uracil-N-glycosylase (UNG) pretreatment of the reaction.
- 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 PCR Master Mix (if stored at –20°C), template DNA or cDNA, primers, and RNase-free water. Mix the individual solutions.**
2. **Prepare a reaction mix according to Table 3.**

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

   **Note**: We strongly recommend starting with an initial $Mg^{2+}$ concentration of 2.5 mM as provided by 2x QuantiTect SYBR Green PCR Master Mix. For a few targets, reactions may be improved by using $Mg^{2+}$ concentrations of up to 5 mM.

**Table 3. Reaction setup**

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

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

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

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

   For two-step RT-PCR, the volume of the cDNA added (from the undiluted RT reaction) should not exceed 10% of the final PCR volume.
5. **Program the LightCycler according to the program outlined in Table 4. Set fluorescence gains as described in Table 5 (for LightCycler software versions earlier than 3.5).**

**Table 4. Real-time cycler conditions**

| Step | Time | Temperature | Ramp rate | Additional comments |
|---|---|---|---|---|
| **Optional: UNG** (carryover prevention) | 2 min | 50°C | 20°C/s | UNG will eliminate any dUMP-containing PCR products resulting from carryover contamination |
| **PCR initial activation step** | **15 min** | **95°C** | **20°C/s** | HotStarTaq DNA Polymerase is activated by this heating step |
| **3 (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 | 10–30 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 DNA |

**Table 5. Fluorescence parameters**

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

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

**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.**

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

   We strongly recommend performing this analysis routinely to verify the specificity and identity of 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 a PCR product depends on buffer composition and salt concentration. $T_m$ values obtained when using the QuantiTect SYBR Green 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 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 PCR, or only primer–dimers detected** | | |
| a) | Annealing time too short | Use the recommended annealing time.<br>**LightCycler 1.x and 2.0**: Annealing time is 20–30 s.<br>**All other cyclers**: Annealing time is 30 s. |
| b) | Extension time too short | Always use the extension times specified in the protocols.<br>**LightCycler 1.x and 2.0**: Extension time should be 5 s per 100 bp of PCR product, with a minimum extension time of 10 s.<br>**All other cyclers**: Extension time should be 30 s for 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 PCR Master Mix (2.5 mM final concentration). For a few targets, an increase up to 5 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 nucleic acids.* Repeat the PCR. |
| e) | HotStarTaq DNA Polymerase not activated | Ensure that the cycling program includes the HotStarTaq DNA Polymerase activation step (15 min at 95°C) as described in the protocols. |
| f) | PCR product too long | For optimal results, PCR products should be between 100 and 150 bp. PCR products should not exceed 500 bp. |

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

| | | Comments and suggestions |
|---|---|---|
| g) | Annealing temperature too high | Decrease annealing temperature in 3°C steps. |
| h) | Primer design not optimal | Check for PCR products by melting curve analysis* or gel electrophoresis. If no specific 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 26). |
| i) | Primer concentration not optimal | Use optimal primer concentrations.<br>**SmartCycler and LightCycler 1.x and 2.0**: 0.5 µM each primer.<br>**All other cyclers**: 0.3 µM each primer. |
| j) | Problems with starting template | Check the concentration, storage conditions, and quality of the starting template.*<br>If necessary, make new serial dilutions of template nucleic acids from the stock solutions. Repeat the PCR using the new dilutions. |
| k) | Insufficient amount of starting template | Increase the amount of template, if possible. Ensure that sufficient copies of the target nucleic acids are present in your sample. |
| l) | Insufficient number of cycles | Increase the number of cycles. |
| m) | No detection activated | Check that fluorescence detection was activated in the cycling program. |
| n) | Wrong detection step | Ensure that fluorescence detection takes place during the extension step of the cycling program. |
| o) | UNG treatment combined with low annealing temperature | If annealing temperatures below 55°C are necessary for successful PCR, the optional UNG treatment should be performed using heat-labile UNG only. |
| p) | Primers degraded | Check for possible degradation of primers on a denaturing polyacrylamide gel. |
| q) | **RT-PCR only**: Volumes of RT reaction added were too high | High volumes of RT reaction added to the PCR may reduce amplification efficiency and the linearity of the reaction. Generally, the volume of undiluted RT reaction added should not exceed 10% of the final PCR volume. |

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

| | | Comments and suggestions |
|---|---|---|
| r) | **RT-PCR only**: Transcript not expressed | Repeat the RT-PCR and include a positive control to make sure the absence of RT-PCR product was not due to problems with amplification and detection.* |
| s) | **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). |
| ***Real-time cyclers other than the LightCycler 1.x and 2.0:*** | | |
| t) | 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:*** | | |
| u) | 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 PCR products** | | |
| a) | $Mg^{2+}$ concentration not optimal | Always start with the $Mg^{2+}$ concentration provided in 2x QuantiTect SYBR Green PCR Master Mix (2.5 mM final concentration). For a few targets, an increase up to 5 mM $Mg^{2+}$ may be helpful. Perform titration in 0.5 mM steps. |
| b) | Annealing temperature too low | Increase annealing temperature in increments of 2°C. |
| c) | 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 26). |
| d) | PCR product too long | For optimal results, PCR products should be between 100 and 150 bp. PCR products should not exceed 500 bp. |

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

| | | Comments and suggestions |
|---|---|---|
| e) | 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. |
| f) | Primers degraded | Check for possible degradation of primers on a denaturing polyacrylamide gel. |
| g) | **RT-PCR only**: 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 26).<br>Perform reverse transcription with the QuantiTect Reverse Transcription Kit, which provides cDNA synthesis with integrated genomic DNA removal. Alternatively, treat the RNA sample with DNase to digest the contaminating genomic DNA. |
| **No linearity in ratio of $C_T$ value/crossing point to log of the template amount** | | |
| a) | Template amount too high | Do not exceed the maximum recommended amount of template.<br>**LightCycler 1.x and 2.0**: Do not use more than 1 µg genomic DNA template.<br>**All other cyclers**: Do not use more than 500 ng genomic DNA template. |
| 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) | **RT-PCR only**: Volumes of RT reaction added were too high | High volumes of RT reaction added to the PCR may reduce amplification efficiency and the linearity of the reaction. Generally, the volume of undiluted RT reaction added should not exceed 10% of the final PCR volume. |
| **High fluorescence in "No Template" control** | | |
| a) | Contamination of reagents | Discard reaction components and repeat PCR with new reagents. |

| | | Comments and suggestions |
|---|---|---|
| b) | Contamination during reaction setup | Take appropriate safety precautions (e.g., use filter tips).<br>Use uracil-N-glycosylase to prevent carryover from previous reactions. |
| **High fluorescence in "No Reverse Transcription" control (RT-PCR only)** | | |
| | 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 26).<br>Perform reverse transcription with the QuantiTect Reverse Transcription Kit, which provides cDNA synthesis with integrated genomic DNA removal. Alternatively, treat the RNA sample with DNase to digest the contaminating genomic DNA. |
| **Varying fluorescence intensity** | | |
| a) | 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) | 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 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 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 iCycler iQ system 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 system 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.

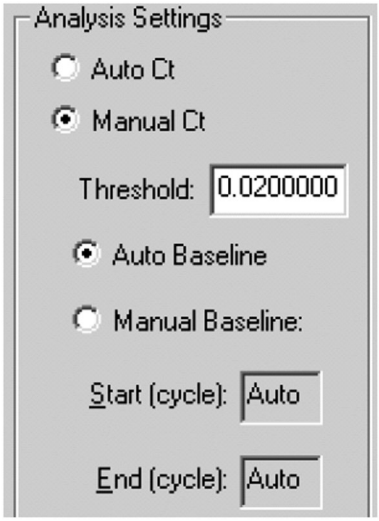


**Figure 1. Adjusting the analysis settings for the Applied Biosystems 7500.** Select "Manual Ct" and "Auto Baseline".

# Ordering Information

| Product | Contents | Cat. no. |
|---|---|---|
| QuantiTect SYBR Green 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 |
| **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 |
| **QuantiTect Reverse Transcription Kit — for fast cDNA synthesis for sensitive real-time two-step RT-PCR** | | |
| QuantiTect Reverse Transcription Kit (50) | For 50 x 20 µl reactions: gDNA Wipeout Buffer, Quantiscript® Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix, and RNase-Free Water | 205311 |
| QuantiTect Reverse Transcription Kit (200) | For 200 x 20 µl reactions: gDNA Wipeout Buffer, Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix, and RNase-Free Water | 205313 |
| **QuantiTect Whole Transcriptome Kit — for unlimited real-time PCR analysis from precious RNA samples** | | |
| QuantiTect Whole Transcriptome Kit (25) | For 25 x 50 µl reactions: T-Script Enzyme and Buffer; Ligation Enzymes, Reagent, and Buffer; and REPLI-g® DNA Polymerase and Buffer | 207043 |

# Ordering Information

| Product | Contents | Cat. no. |
|---|---|---|
| **FastLane Cell cDNA Kit — for high-speed preparation of cDNA without RNA purification for use in real-time RT-PCR** | | |
| FastLane Cell cDNA Kit (50) | Buffer FCW, Buffer FCP, and components for 50 x 20 µl reverse-transcription reactions (gDNA Wipeout Buffer, Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix, and RNase-Free Water) | 215011 |
| **Related products** | | |
| **QuantiTect SYBR Green RT-PCR Kit — for quantitative, 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 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 |

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## Notes

## Notes

## Notes

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