

QuantiFast® Probe RT-PCR Handbook

QuantiFast Probe RT-PCR Kit

QuantiFast Probe RT-PCR +ROX™ Vial Kit

For fast, quantitative, real-time, one-step RT-PCR using
sequence-specific probes



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Kit Contents

QuantiFast Probe RT-PCR Kit	(400)	(2000)
Catalog no.	204454	204456
Number of reactions (25 µl/20 µl/10 µl)	400/500/ 1000	2000/2500/ 5000
2x QuantiFast Probe RT-PCR Master Mix, containing:	3 x 1.7 ml	25 ml
■ HotStarTaq® <i>Plus</i> DNA Polymerase		
■ QuantiFast Probe RT-PCR Buffer		
■ dNTP mix (dATP, dCTP, dGTP, dTTP)		
■ ROX passive reference dye		
QuantiFast RT Mix, a mixture of the QIAGEN® products:	100 µl	0.5 ml
■ Omniscript® Reverse Transcriptase		
■ Sensiscript® Reverse Transcriptase		
RNase-Free Water	2 x 1.9 ml	20 ml
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QuantiFast Probe RT-PCR +ROX Vial Kit	(400)	(2000)
Catalog no.	204554	204556
Number of reactions (25 µl/20 µl/10 µl)	400/500/ 1000	2000/2500/ 5000
2x QuantiFast Probe RT-PCR Master Mix (w/o ROX), containing:	3 x 1.7 ml	25 ml
■ HotStarTaq <i>Plus</i> DNA Polymerase		
■ QuantiFast Probe RT-PCR Buffer		
■ dNTP mix (dATP, dCTP, dGTP, dTTP)		
QuantiFast RT Mix, a mixture of the QIAGEN products:	100 µl	0.5 ml
■ Omniscript Reverse Transcriptase		
■ Sensiscript Reverse Transcriptase		
50x ROX Dye Solution	210 µl	1.05 ml
RNase-Free Water	1.9 ml	20 ml
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Shipping and Storage

QuantiFast Probe RT-PCR Kits are shipped on dry ice. The kits should be stored immediately upon receipt at -30 to -15°C in a constant-temperature freezer and protected from light. When the kits are 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 QuantiFast Probe RT-PCR Master Mix and 2x QuantiFast Probe RT-PCR Master Mix (w/o ROX) can also be stored protected from light at $2-8^{\circ}\text{C}$ for up to 2 months without showing any reduction in performance.

To maintain optimal performance of QuantiFast Probe RT-PCR Kits for 2000 x 25 μl reactions, we recommend storing the 25 ml master mix and the 0.5 ml RT mix as appropriately sized aliquots in sterile, polypropylene tubes.

If desired, 50x ROX Dye Solution can be added to 2x QuantiFast Probe RT-PCR Master Mix (w/o ROX) for long-term storage. For details, see "Adding ROX dye to the master mix", page 12.

Product Use Limitations

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

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

Product Warranty and Satisfaction Guarantee

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

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

Technical Assistance

At QIAGEN we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in molecular biology and the use of QIAGEN products. If you have any questions or experience any difficulties regarding QuantiFast Probe RT-PCR Kits 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 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 safety data sheets (SDSs). These are available online in convenient and compact PDF format at www.qiagen.com/safety where you can find, view, and print the SDS for each QIAGEN kit and kit component.

Product Description

QuantiFast Probe RT-PCR Kit

2x QuantiFast Probe RT-PCR Master Mix contains:

HotStarTaq *Plus* DNA Polymerase: HotStarTaq *Plus* DNA Polymerase is a modified form of a recombinant 94 kDa DNA polymerase, originally isolated from *Thermus aquaticus*. HotStarTaq *Plus* DNA Polymerase is provided in an inactive state and has no enzymatic activity at ambient temperature. The enzyme is activated by a 5-minute, 95°C incubation step.

QuantiFast Probe RT-PCR Buffer: Contains Tris·Cl, KCl, $(\text{NH}_4)_2\text{SO}_4$, MgCl_2 , and additives enabling fast cycling, including Q-Bond®

dNTP mix: Contains dATP, dCTP, dGTP, and dTTP of ultrapure quality

ROX passive reference dye: Optimized concentration of fluorescent dye for normalization of fluorescent signals on all instruments from Applied Biosystems except the Applied Biosystems® 7500

QuantiFast RT Mix: Contains an optimized mixture of the QIAGEN products Omniscript Reverse Transcriptase and Sensiscript Reverse Transcriptase, which are recombinant heterodimeric enzymes expressed in *E. coli*.

RNase-free water: Ultrapure quality, PCR-grade

QuantiFast Probe RT-PCR +ROX Vial Kit

2x QuantiFast Probe RT-PCR Master Mix (w/o ROX) contains:

HotStarTaq *Plus* DNA Polymerase: HotStarTaq *Plus* DNA Polymerase is a modified form of a recombinant 94 kDa DNA polymerase, originally isolated from *Thermus aquaticus*. HotStarTaq *Plus* DNA Polymerase is provided in an inactive state and has no enzymatic activity at ambient temperature. The enzyme is activated by a 5-minute, 95°C incubation step.

QuantiFast Probe RT-PCR Buffer: Contains Tris·Cl, KCl, $(\text{NH}_4)_2\text{SO}_4$, MgCl_2 , and additives enabling fast cycling, including Q-Bond

dNTP mix: Contains dATP, dCTP, dGTP, and dTTP of ultrapure quality

QuantiFast RT Mix:	Contains an optimized mixture of the QIAGEN products Omniscript Reverse Transcriptase and Sensiscript Reverse Transcriptase, which are recombinant heterodimeric enzymes expressed in <i>E. coli</i> .
50x ROX Dye Solution:	Separate tube of passive reference dye for normalization of fluorescent signals on the Applied Biosystems 7500 and, optionally, on instruments from Agilent (formerly Stratagene); not required for instruments from Bio-Rad/MJ Research, Cepheid, Corbett Research, Eppendorf, and Roche
RNase-free water:	Ultrapure quality, PCR-grade

Quality Control

2x QuantiFast Probe RT-PCR Master Mix and 2x QuantiFast Probe RT-PCR Master Mix (w/o ROX)	(see quality-control label inside kit lid for lot-specific values)
RT-PCR sensitivity and reproducibility assay:	Sensitivity and reproducibility in real-time RT-PCR are tested in parallel 20 µl reactions containing 10-fold dilutions of nucleic acid template.
HotStarTaq Plus DNA Polymerase	(included in master mix) Efficiency and reproducibility in PCR are tested. Functional absence of exonucleases and endonucleases is tested.
QuantiFast RT Mix	Efficiency of cDNA synthesis and functional absence of RNases, exonucleases, and endonucleases are tested.
Buffers and reagents	(included in master mix)
QuantiFast Probe RT-PCR Buffer:	Conductivity and pH are tested.
RNase-free water:	Conductivity, pH, and RNase activities are tested.
50x ROX Dye Solution	Emission and excitation are tested

Introduction

Quantifast Probe RT-PCR Kits provide rapid real-time quantification of RNA targets in an easy-to-handle format. The kits are compatible with TaqMan® or Operon® dual-labeled probes and with FRET probes. High specificity and sensitivity in RT-PCR are achieved by the use of the hot-start enzyme, HotStarTaq *Plus* DNA Polymerase, together with a specialized fast RT-PCR buffer. The optimized Omniscript and Sensiscript blend for the reverse-transcription step further enhances sensitivity. Short cycling steps without loss of PCR sensitivity and efficiency are enabled by Q-Bond, a patent-pending additive in the RT-PCR buffer.

The kits have been optimized for use with any real-time cycler, including cyclers with standard ramping rates and cyclers with rapid ramping rates:

- **Quantifast Probe RT-PCR Kit** — instruments from Applied Biosystems (e.g., ABI PRISM® 7000, 7700, and 7900, Applied Biosystems 7300, and GeneAmp® 5700) except the Applied Biosystems 7500
- **Quantifast Probe RT-PCR +ROX Vial Kit** — Applied Biosystems 7500 and instruments from other suppliers, including DNA Engine Opticon®, iCycler iQ®, iQ5, MyiQ™, LightCycler®, Mastercycler® ep *realplex*, Mx3000P®, Mx3005P®, Mx4000®, Rotor-Gene® 3000, and SmartCycler® systems

This handbook contains general protocols for use with all these systems.

One-step RT-PCR

Use of 2x Quantifast Probe RT-PCR Master Mix or 2x Quantifast Probe RT-PCR Master Mix (w/o ROX) together with Quantifast RT Mix allows both reverse transcription and PCR to take place in a single tube. All reagents required for both reactions are added at the beginning, so there is no need to open the tube once the reverse-transcription reaction has been started.

The components of 2x Quantifast Probe RT-PCR Master Mix include HotStarTaq *Plus* DNA Polymerase, Quantifast Probe RT-PCR Buffer, and ROX passive reference dye (see descriptions below). 2x Quantifast Probe RT-PCR Master Mix (w/o ROX) contains HotStarTaq *Plus* DNA Polymerase and Quantifast Probe RT-PCR Buffer, but no ROX passive reference dye. Quantifast RT Mix contains an Omniscript and Sensiscript blend (see descriptions below).

Omniscript and Sensiscript

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

highly efficient and sensitive reverse transcription over a wide range of RNA template amounts.

HotStarTaq *Plus* DNA Polymerase

HotStarTaq *Plus* DNA Polymerase is a modified form of QIAGEN *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 primer–dimers during reaction setup, reverse transcription, and the first denaturation step. The enzyme is activated after the reverse-transcription step by a 5-minute, 95°C incubation step. The hot start also inactivates the reverse-transcriptase enzymes, ensuring temporal separation of reverse transcription and PCR, and allowing both steps to be performed sequentially in a single tube. In addition, the concentration of the polymerase in the master mix is optimized to allow short extension times in the combined annealing/extension step of each PCR cycle.

QuantiFast Probe RT-PCR Buffer

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

QuantiFast Probe RT-PCR Buffer is also based on the unique QIAGEN OneStep RT-PCR buffer system. The buffer contains a balanced combination of KCl and $(\text{NH}_4)_2\text{SO}_4$, which promotes a high ratio of specific to nonspecific primer binding during the annealing step of each PCR cycle. This creates stringent primer annealing conditions, leading to increased PCR specificity. When using this buffer, primer annealing is only marginally influenced by the MgCl_2 concentration, so optimization by titration of Mg^{2+} is not required.

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. ROX dye does not

interfere with real-time PCR since it is not involved in the reaction and has an emission spectrum completely different from fluorescent dyes commonly used for probes.

The use of ROX dye is necessary for instruments from Applied Biosystems and is optional for instruments from Agilent. Before starting your real-time PCR experiment, refer to Table 1 to select the correct kit for your cycler.

Table 1. Real-time cyclers compatible with QuantiFast Probe RT-PCR Kits

Real-time cycler	ROX use	Compatible kit	Protocol
ABI PRISM 7000 ABI PRISM 7700 ABI PRISM 7900 Applied Biosystems 7300 GeneAmp 5700	Yes	QuantiFast Probe RT-PCR Kit (master mix already contains ROX dye*)	Page 13
Applied Biosystems 7500	Yes	QuantiFast Probe RT-PCR +ROX Vial Kit (supplied ROX dye [†] must be added to master mix)	Page 16
Mx3000P Mx3005P Mx4000	Optional	QuantiFast Probe RT-PCR +ROX Vial Kit (supplied ROX dye [†] can be added to master mix)	Page 16
DNA Engine Opticon DNA Engine Opticon 2 iCycler iQ iQ5 MyiQ LightCycler 1.x LightCycler 2.0 LightCycler 480 Mastercycler ep <i>realplex</i> Rotor-Gene 3000 Rotor-Gene 6000 SmartCycler	No	QuantiFast Probe RT-PCR +ROX Vial Kit (supplied ROX dye should not be used)	Page 16 (dual-labeled probes) or page 19 (FRET probes)

* The ROX concentration is optimal for all instruments from Applied Biosystems except the Applied Biosystems 7500.

† The supplied ROX dye allows use of a lower concentration of ROX dye with the Applied Biosystems 7500 and instruments from Stratagene.

Adding ROX dye to the master mix

If you only use the Applied Biosystems 7500 or if you only use the Mx3000P, Mx3005P, or Mx4000 and always run reactions with ROX dye, you can add 50x ROX Dye Solution to 2x QuantiFast Probe RT-PCR Master Mix (w/o ROX) for long-term storage. Simply add the appropriate volume of ROX dye to a previously unopened tube of master mix (see Table 2), mix, and store protected from light. Master mix containing ROX dye can be stored at 2–8°C for up to 2 months, or at –20°C. Be sure to indicate on the tube of master mix that ROX dye has been added.

If you use various different cyclers (some which require ROX dye, and some which do not), store the master mix and ROX dye as separate solutions, and add ROX dye if necessary during reaction setup as described in the protocol on page 16.

Table 2. Option for users of the Applied Biosystems 7500, Mx3000P, Mx3005P, and Mx4000 — addition of ROX dye to master mix for long-term storage

Kit	Volume of ROX dye solution	Volume of 2x QuantiFast Probe RT-PCR Master Mix (w/o ROX)
QuantiFast Probe RT-PCR +ROX Vial Kit (400)	68 µl	1.7 ml
QuantiFast Probe RT-PCR +ROX Vial Kit (2000)	1 ml	25 ml

Protocol: Real-Time, One-Step RT-PCR Using Dual-Labeled Probes on Most Applied Biosystems Cyclers

This protocol is for use with the **Quantifast Probe RT-PCR Kit** and dual-labeled probes (e.g., TaqMan) on any cycler from Applied Biosystems **except the Applied Biosystems 7500**. For further information, see “Passive reference dye” on page 10.

A shortened version of this protocol for **experienced users** is available as a bench protocol to tear out (see inside cover of this handbook). This bench protocol is also part of the PDF version of the handbook, which can be downloaded at www.qiagen.com/HB/QuantifastProbeRTPCR.

Important points before starting

- Always start with the cycling conditions specified in this protocol, even if using previously established primer–probe systems.
- For the highest efficiency in real-time RT-PCR using sequence-specific probes, targets should ideally be 70–200 bp in length (for longer amplicons, refer to Appendix C, page 34).
- 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.
- Set up all reactions on ice to avoid premature cDNA synthesis.
- For 96-well block cyclers, we recommend a final reaction volume of 25 µl. For 384-well block cyclers, we strongly recommend a final reaction volume of 10 µl.
- **Always start with the Mg²⁺ concentration as provided** in 2x Quantifast Probe RT-PCR Master Mix.

Procedure

1. **Thaw 2x Quantifast Probe RT-PCR Master Mix, template RNA, primer and probe solutions, 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 3.**

Keep samples on ice while preparing the reaction mix.

Note: We strongly recommend starting with the Mg²⁺ concentration as provided in 2x Quantifast Probe RT-PCR Master Mix.

Table 3. Reaction setup

Component	Volume/reaction		Final concentration
	96-well block	384-well block	
2x QuantiFast Probe RT-PCR Master Mix	12.5 μ l	5 μ l	1x
Primer A	Variable	Variable	0.8 μ M
Primer B	Variable	Variable	0.8 μ M
Probe	Variable	Variable	0.2 μ M
QuantiFast RT Mix	0.25 μ l	0.1 μ l	
Template RNA (added at step 4)	Variable	Variable	\leq 100 ng/reaction
RNase-free water	Variable	Variable	
Total reaction volume	25 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 (\leq 100 ng/reaction) to the individual PCR vessels or wells containing the reaction mix.

5. Program your real-time cyclers according to the program outlined in Table 4.

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

Table 4. Real-time cyler conditions

Step	Time	Temperature	Ramp rate	Additional comments
Reverse transcription	10 min	50°C		
PCR initial activation step	5 min	95°C	Maximal/ fast mode	HotStarTaq <i>Plus</i> DNA Polymerase is activated by this heating step
Two-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

* If your cyler does not accept this short time, choose the shortest time acceptable (e.g., 31 s annealing/extension for the Applied Biosystems 7300).

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

Protocol: Real-Time, One-Step RT-PCR Using Dual-Labeled Probes on the Applied Biosystems 7500 and Other Cyclers

This protocol is for use with the **QuantiFast Probe RT-PCR +ROX Vial Kit** and dual-labeled probes (e.g., TaqMan) on the Applied Biosystems 7500 and on cyclers from Bio-Rad/MJ Research, Cepheid, Corbett Research, Eppendorf, Roche, and Agilent. For further information, see “Passive reference dye” on page 10.

A shortened version of this protocol for **experienced users** is available as a bench protocol to tear out (see inside cover of this handbook). This bench protocol is also part of the PDF version of the handbook, which can be downloaded at www.qiagen.com/HB/QuantiFastProbeRTPCR.

Important points before starting

- Always start with the cycling conditions specified in this protocol, even if using previously established primer–probe systems.
- For the highest efficiency in real-time RT-PCR using sequence-specific probes, targets should ideally be 70–200 bp in length (for longer amplicons, refer to Appendix C, page 34).
- 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.
- Set up all reactions on ice to avoid premature cDNA synthesis.
- For 96-well block cyclers, we recommend a final reaction volume of 25 µl. For the Applied Biosystems 7500 Fast System and 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 Mg²⁺ concentration as provided** in 2x QuantiFast Probe RT-PCR Master Mix (w/o ROX).
- Use of the supplied ROX dye solution is necessary for the Applied Biosystems 7500 and optional for the Mx3000P, Mx3005P, and Mx4000. ROX dye solution is not required for all other cyclers.

Procedure

1. **Thaw 2x QuantiFast Probe RT-PCR Master Mix (w/o ROX), template RNA, primer and probe solutions, ROX dye solution, 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.**

Optional: If you always run reactions with ROX dye, you can add ROX dye solution to 2x QuantiFast Probe RT-PCR Master Mix (w/o ROX) for long-term storage. For details, see “Adding ROX dye to the master mix”, page 12.

2. Prepare a reaction mix according to Table 5.

Keep samples on ice while preparing the reaction mix.

Note: We strongly recommend starting with the Mg²⁺ concentration as provided in 2x QuantiFast Probe RT-PCR Master Mix (w/o ROX).

Table 5. Reaction setup

Component	Volume/reaction			Final concentration
	96-well block	Fast and capillary*	384-well block	
2x QuantiFast Probe RT-PCR Master Mix (w/o ROX)	12.5 µl	10 µl	5 µl	1x
Primer A	Variable	Variable	Variable	0.8 µM
Primer B	Variable	Variable	Variable	0.8 µM
Probe	Variable	Variable	Variable	0.2 µM
QuantiFast RT Mix	0.25 µl	0.2 µl	0.1 µl	
50x ROX Dye Solution ^{†‡}	0.5 µl	0.4 µl	– [§]	1x
Template RNA (added at step 4)	Variable	Variable	Variable	≤100 ng/reaction
RNase-free water	Variable	Variable	Variable	
Total reaction volume	25 µl	20 µl	10 µl	

* Applied Biosystems 7500 Fast System and capillary cyclers.

[†] Required for Applied Biosystems 7500 and optional for Mx3000P, Mx3005P, and Mx4000. For convenience, 2x QuantiFast Probe RT-PCR Master Mix (w/o ROX) can be premixed with 50x ROX Dye Solution (see page 12).

[‡] For cyclers which do not require ROX dye, add RNase-free water instead.

[§] A total reaction volume of 10 µl is recommended for the LightCycler 480, which does not require ROX dye.

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 (≤100 ng/reaction) to the individual PCR vessels or wells containing the reaction mix.

5. Program your real-time cycler according to the program outlined in Table 6.

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

Table 6. Real-time cycler conditions

Step	Time	Temperature	Ramp rate	Additional comments
Reverse transcription	10 min	50°C		
PCR initial activation step	5 min	95°C	Maximal/ fast mode	HotStarTaq <i>Plus</i> DNA Polymerase is activated by this heating step
Two-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

* If your cycler does not accept this short time, choose the shortest time acceptable.

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

Protocol: Real-Time, One-Step RT-PCR Using FRET Probes

This protocol is for use with the **QuantiFast Probe RT-PCR +ROX Vial Kit** and FRET probes on the LightCycler 1.x, LightCycler 2.0, and Rotor-Gene 3000. For further information, see “Passive reference dye” on page 10.

A shortened version of this protocol for **experienced users** is available as a bench protocol, which is part of the PDF version of the handbook. The PDF can be downloaded at www.qiagen.com/HB/QuantiFastProbeRTPCR.

Important points before starting

- Always start with the cycling conditions specified in this protocol, even if using previously established primer–probe systems.
- For the highest efficiency in real-time RT-PCR using sequence-specific probes, targets should ideally be 70–200 bp in length.
- 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.
- Set up all reactions on ice to avoid premature cDNA synthesis.
- For LightCycler instruments, we recommend a final reaction volume of 20 µl. For the Rotor-Gene 3000, we recommend a final reaction volume of 25 µl.
- **Always start with the Mg²⁺ concentration as provided** in 2x QuantiFast Probe RT-PCR Master Mix (w/o ROX).

Procedure

1. **Thaw 2x QuantiFast Probe RT-PCR Master Mix (w/o ROX), template RNA, primer and probe solutions, 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 7.**

Keep samples on ice while preparing the reaction mix.

Note: We strongly recommend starting with the Mg²⁺ concentration as provided in 2x QuantiFast Probe RT-PCR Master Mix (w/o ROX).

Table 7. Reaction setup

Component	Volume/reaction		Final concentration
	Rotor-Gene	LightCycler	
2x QuantiFast Probe RT-PCR Master Mix (w/o ROX)	12.5 μ l	10 μ l	1x
Primer A	Variable	Variable	0.6 μ M*
Primer B	Variable	Variable	0.6 μ M*
Probe A (donor)	Variable	Variable	0.2 μ M
Probe B (acceptor)	Variable	Variable	0.2 μ M [†]
QuantiFast RT Mix	0.25 μ l	0.2 μ l	
Template RNA (added at step 4)	Variable	Variable	\leq 100 ng/ reaction
RNase-free water	Variable	Variable	
Total reaction volume	25 μl	20 μl	

* A final primer concentration of 0.6 μ M is optimal for most applications. If it is necessary to determine the optimal concentration, perform a primer titration of 0.6 μ M to 1 μ M.

[†] In some cases, it may be advantageous to use a final concentration of 0.4 μ M Probe B.

3. Mix the reaction mix thoroughly and dispense appropriate volumes into PCR vessels.

Keep the PCR vessels on ice.

4. Add template RNA (\leq 100 ng/reaction) to the individual PCR vessels containing the reaction mix.

5. Program your real-time cycler according to the program outlined in Table 8.

Data acquisition should be performed during the annealing step.

Table 8. Real-time cycler conditions

Step	Time	Temperature	Ramp rate	Additional comments
Reverse transcription	10 min	50°C		
PCR initial activation step	5 min	95°C	Maximal/ fast mode	HotStarTaq <i>Plus</i> DNA Polymerase is activated by this heating step
Three-step cycling				
Denaturation	10 s	95°C	Maximal/ fast mode	
Annealing	15 s	50–60°C	Maximal/ fast mode	Temperature should be approx. 5–8°C below T_m of primers. Perform fluorescence data collection
Extension	15 s	72°C	Maximal/ fast mode	
Number of cycles	35–40			The number of cycles depends on the amount of template RNA

6. Place the PCR vessels in the real-time cycler and start the cycling program.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. 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 molecular biology applications (for contact information, see back cover or visit www.qiagen.com).

Comments and suggestions

No signal, or one or more signals detected late in RT-PCR

- | | | |
|----|---|---|
| a) | Wrong cycling conditions | Always start with the optimized cycling conditions specified in the protocols. Be sure that the cycling conditions include the RT step (50°C for 10 min) and the initial step for activation of HotStarTaq <i>Plus</i> DNA Polymerase (95°C for 5 min), and the specified times for denaturation and annealing/extension. |
| b) | HotStarTaq <i>Plus</i> DNA Polymerase not activated | Ensure that the cycling program includes the HotStarTaq <i>Plus</i> DNA Polymerase activation step (5 min at 95°C) as described in the protocols. |
| c) | RT step not performed | Ensure that the cycling program includes the RT step (10 min at 50°C) as described in the protocols. |
| d) | Pipetting error or missing reagent | Check the concentrations and storage conditions of the reagents, including primers, probes, and template nucleic acid. See Appendix B, page 28, for details on evaluating the concentration of primers and probes. Repeat the RT-PCR. |
| e) | Wrong or no detection step | Ensure that fluorescence detection takes place during the combined annealing/extension step when using TaqMan probes, or during the annealing step when using FRET probes. |

Comments and suggestions

- f) Primer or probe concentration not optimal
- Use optimal primer concentrations. For TaqMan probes, use each primer at 0.8 μM . For FRET probes, use each primer at 0.6 μM (in some cases, it may be necessary to determine the optimal concentration by performing a primer titration from 0.6 μM to 1 μM).
- In most cases, a probe concentration of 0.2 μM gives satisfactory results. For FRET probes, it may be advantageous in some cases to use a final concentration of 0.4 μM for the acceptor probe.
- Check the concentrations of primers and probes by spectrophotometry (see Appendix B, page 28).
- g) Problems with starting template
- Check the concentration, storage conditions, and quality of the starting template (see Appendix A, page 27).
- If necessary, make new serial dilutions of template nucleic acid from the stock solutions. Repeat the RT-PCR using the new dilutions.
- h) Insufficient amount of starting template
- Increase the amount of template, if possible. Ensure that sufficient copies of the target nucleic acids are present in your sample.
- i) Insufficient number of cycles
- Increase the number of cycles.
- j) Reaction volume too high
- For 96-well block cyclers, we recommend a final reaction volume of 25 μl . For the Applied Biosystems 7500 Fast System and 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 .
- k) RT-PCR product too long
- For optimal results, RT-PCR products should be between 100 and 150 bp. RT-PCR products should not be outside the range of 70–200 bp. For amplicons of 200–500 bp, see Appendix C, page 34.

Comments and suggestions

- | | | |
|----|---------------------------------------|--|
| l) | Primer design not optimal | Check for RT-PCR products by gel electrophoresis. If no specific RT-PCR products are detected, review the primer design guidelines (see Appendix B, page 28). |
| m) | Probe design not optimal | If the amplification reaction was successful, there may be a problem with the probe. Review the probe design guidelines (see Appendix B, page 28). |
| n) | Wrong detection channel/filter chosen | Ensure that the correct detection channel is activated or the correct filter set is chosen for the reporter dye. |
| o) | PCR annealing temperature too high | Decrease annealing temperature in steps of 2°C. |
| p) | PCR annealing temperature too low | Increase annealing temperature in steps of 2°C. |
| q) | No detection activated | Check that fluorescence detection was activated in the cycling program. |
| r) | Probe synthesis not optimal | Check the quality of dual-labeled probes by incubation with DNase I. A correctly synthesized probe, containing both fluorophore and quencher, will show a significant increase in fluorescence after DNase I incubation. |
| s) | 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 exceed maximum recommended amounts of template. |
| b) | Template amount too low | Increase template amount, if possible. |

Increased fluorescence or C_T value for “No Template” control

- | | | |
|----|---------------------------|---|
| a) | Contamination of reagents | Discard all the components of the assay (e.g., master mix, primers, and probes). Repeat the assay using new components. |
|----|---------------------------|---|

Comments and suggestions

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|---|---|
| b) Contamination during reaction setup | Take appropriate precautions during reaction setup, such as using aerosol-barrier pipet tips. |
| c) Minimal probe degradation, leading to sliding increase in fluorescence | Check the amplification plots, and adjust the threshold settings. |

High fluorescence in “No Reverse Transcription” control

- | | |
|--|--|
| Contamination of RNA sample with genomic DNA | Design primers and/or probes that span exon-exon boundaries, so that only cDNA targets can be amplified and detected.

Alternatively, treat the RNA sample with DNase to digest the contaminating genomic DNA. |
|--|--|

Varying fluorescence intensity

- | | |
|--|--|
| a) Contamination of real-time cycler | Decontaminate the real-time cycler according to the manufacturer’s instructions. |
| b) Real-time cycler no longer calibrated | Recalibrate the real-time cycler according to the manufacturer’s instructions. |

All cycler systems:

- | | |
|---|--|
| c) Wavy curve at high template amounts for highly expressed targets | In the analysis settings, reduce the number of cycles used for background calculation (if your real-time cycler allows you to do so) or reduce the amount of template. |
| d) ΔR_n values unexpectedly too high or too low | The real-time cycler was used with the wrong QuantiFast Probe RT-PCR Kit. To choose the right kit for your cycler, refer to Table 1, page 11. |

Applied Biosystems 7000 only:

- | | |
|--|---|
| e) Uneven curves or high standard deviations | Do not use reaction volumes smaller than 25 μ l and always use optical adhesive covers to seal plates. In some cases, increasing the reaction volume to 50 μ l may improve results. |
|--|---|

LightCycler system only:

- | | |
|------------------------------------|--|
| f) RT-PCR mix not in capillary tip | Centrifuge the capillary to bring the RT-PCR mix into the capillary tip. |
|------------------------------------|--|

Comments and suggestions

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|---|--|
| g) Capillary not pushed down completely | Ensure that the capillary is completely pushed down in the LightCycler carousel. |
| h) Wrong detection channel | Make sure that the correct channel is chosen. |

Applied Biosystems 7500 only:

- | | |
|---|--|
| i) No amplification signal using "Auto Ct", "Analysis Settings", or the preset threshold value of 0.2 | The QuantiFast Probe RT-PCR Kit was used instead of the QuantiFast Probe RT-PCR +ROX Vial Kit, leading to an excessively high ROX signal. To analyze the signals, adjust the preset threshold value to a lower value. Use a value of 0.01 as a starting point. For details, see Appendix F, page 38. |
|---|--|

Mastercycler ep realplex only:

- | | |
|--|--|
| j) Late signals, high and scattering end fluorescence, or high standard deviations | Reactions were run using ROX passive reference dye. Although the cycler software provides the option of using ROX dye, we do not recommend it. Either deselect this option or, preferably, use the master mix from the QuantiFast Probe RT-PCR +ROX Vial Kit without adding ROX dye. |
|--|--|

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

Template 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, ensuring the highest-quality templates for real-time RT-PCR, including RNeasy® Kits for preparation of RNA from various sources, and Oligotex® Kits (low-throughput) and TurboCapture Kits (high-throughput) for mRNA purification. For details, visit www.qiagen.com/RNA. QIAGEN also offers automated solutions for RNA purification, including the QIAcube® for automation of spin-column preps. For details, visit www.qiagen.com/automation.

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. For accuracy, absorbance readings at 260 nm should fall between 0.15 and 1.0. An absorbance reading of 1.0 at 260 nm in a 1 cm detection path corresponds to an RNA concentration of 40 µg/ml.

Note that absorbance measurements cannot discriminate between DNA and RNA. Depending on the method used for template preparation, RNA may be contaminated with DNA, and this will result in misleadingly high A_{260} values. It is particularly important to bear this in mind when preparing standards for absolute quantification (see Appendix D, page 35).

The ratio between the absorbance values at 260 nm and 280 nm gives an estimate of the purity of RNA. To determine RNA purity, we recommend measuring absorbance in 10 mM Tris-Cl, * pH 7.5. Pure RNA has a 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 RNA is purified using QIAGEN kits, no degradation is detectable for at least 1 year under these conditions. Diluted solutions of nucleic acids (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 nucleic acids to the tube walls, which would reduce the concentration of nucleic acids in solution.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs), 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: Assay Design and Handling Primers and Probes

Important factors for successful quantitative, real-time RT-PCR include the design of optimal primer pairs and probes, the use of appropriate primer and probe concentrations, and the correct storage of primers and probes.

Assay design

Guidelines for the optimal design of primers and probes are provided in Table 9 below. It is particularly important to minimize nonspecific annealing of primers and probes. This can be achieved through careful assay design.

Table 9. General guidelines for design of primers and probes

Length	18–30 nucleotides
GC content	30–70%
T_m	TaqMan assays: <ul style="list-style-type: none">■ Use specialized design software (e.g., PrimerExpress® Software) to design primers and probes.■ T_m of all primers should be 58–62°C and within 2°C of each other.■ T_m of probes should be 8–10°C higher than the T_m of the primers.■ Avoid a guanidine at the 5' end of probes, next to the reporter, since this causes quenching.■ Avoid runs of 4 or more of the same nucleotide, especially of guanidine.■ Choose the binding strand so that the probe has more C than G bases.■ All assays should be designed using the same settings to ensure that they will work optimally under the same cycling conditions (60°C annealing/extension).

Table continued on next page

Table 9. Continued

	<p>For best results, use commercially available oligo-design software such as OLIGO 6 (oligo.net) or Web-based tools such as Primer3 (frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)[*] to determine primer and probe T_ms.</p> <p>Simplified formula for estimating melting temperature (T_m): $T_m = 2^{\circ}\text{C} \times (\text{number of [A+T]}) + 4^{\circ}\text{C} \times (\text{number of [G+C]})$</p> <p>Whenever possible, design primer pairs with similar T_m values.</p> <p>Optimal annealing temperatures may be above or below the estimated T_m. As a starting point, use an annealing temperature 5°C below T_m.</p>
Sequence	<ul style="list-style-type: none">■ Always check the specificity of primers by performing a BLAST[®] search (www.ncbi.nlm.nih.gov/blast). Ensure that primer sequences are unique for your template sequence.■ Ensure the length of the PCR product is less than 200 bp.■ Avoid complementarity of 2 or more bases at the 3' ends of primer pairs to minimize primer–dimer formation.■ Avoid mismatches between the 3' end of primers and the template sequence.■ Avoid runs of 3 or more Gs or Cs at the 3' end.■ Avoid a 3'-end T. Primers with a T at the 3' end have a greater tolerance of mismatch.■ Avoid complementary sequences within a primer sequence and between the primer pair.■ Commercially available computer software (e.g., OLIGO 6) or Web-based tools (e.g., Primer3) can be used for primer design. Use the software to minimize the likelihood of formation of stable primer–dimers.

Table continued on next page

^{*} Rozen, S. and Skaletsky, H.J. (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz, S. and Misener, S., eds. Bioinformatics Methods and Protocols: Methods in Molecular Biology. Totowa, NJ: Humana Press, pp. 365–386.

Table 9. Continued

<p>Special considerations for design of RT-PCR primers and probes</p>	<ul style="list-style-type: none"> ■ Design primers or probes so that one half hybridizes to the 3' end of one exon and the other half to the 5' end of the adjacent exon (see Figure 1). The primers or probes will therefore anneal to cDNA synthesized from spliced mRNAs, but not to genomic DNA, eliminating detection of contaminating DNA. ■ Alternatively, RT-PCR primers should be designed to flank a region that contains at least one intron. Products amplified from cDNA (no introns) will be smaller than those amplified from genomic DNA (containing introns). If possible, select a target with very long introns: the RNA target may then be preferentially amplified because of the higher PCR efficiency of this shorter PCR product without introns. If genomic DNA is detected (i.e., presence of amplification product in "No RT" control), treat the template RNA with RNase-free DNase. Alternatively, redesign primers to avoid amplification of genomic DNA.
--	--

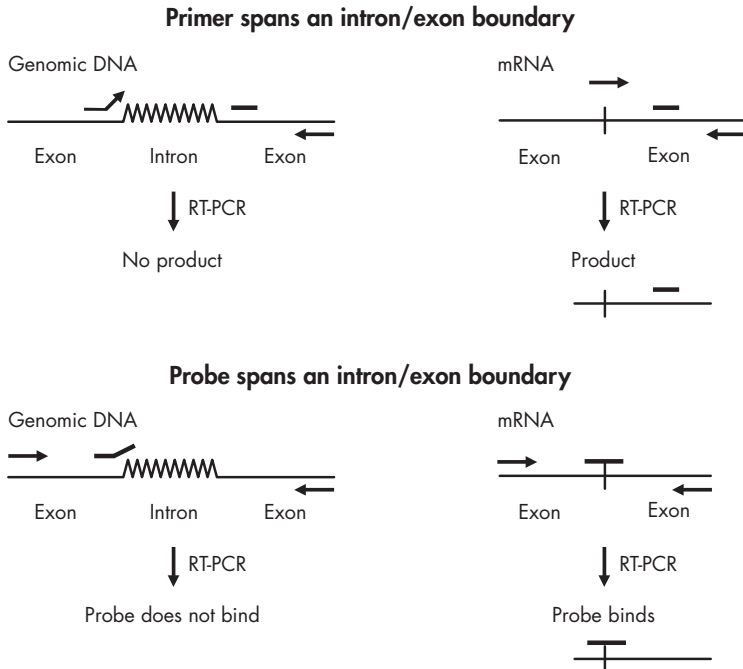


Figure 1. Primer/probe design. Primer/probe design to eliminate signals from contaminating genomic DNA.

Handling and storing primers and probes

Guidelines for handling and storing primers and probes are provided in Table 10 below. For optimal results, we recommend only combining primers of comparable quality.

Table 10. General guidelines for handling and storing primers and probes

Storage buffer	<p>Lyophilized primers and probes should be dissolved in a small volume of low-salt buffer to give a concentrated stock solution (e.g., 100 μM). We recommend using TE (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) for standard primers and probes labeled with most fluorescent dyes.</p> <p>However, probes labeled with fluorescent dyes such as Cy[®]3, Cy3.5, Cy5, and Cy5.5 should be stored in TE, pH 7.0, since they tend to degrade at higher pH.</p>
Storage	<p>Primers should be stored in TE in small aliquots at -20°C. Standard primers are stable under these conditions for at least 1 year. Fluorescently labeled probes are usually stable under these conditions for at least 6–9 months. Repeated freeze–thaw cycles should be avoided, since they may lead to degradation.</p>
Dissolving primers and probes	<p>Before opening a tube containing lyophilized primer or probe, spin the tube briefly to collect all material at the bottom of the tube. To dissolve the primer or the probe, add the required volume of TE, mix, and leave for 20 minutes to allow the primer or probe to completely dissolve. Mix again and determine the concentration by spectrophotometry as described below.</p> <p>We do not recommend dissolving primers and probes in water. They are less stable in water than in TE and some may not dissolve easily in water.</p>
Concentration	<p>Spectrophotometric conversion for primers and probes:</p> $1 A_{260} \text{ unit} = 20\text{--}30 \mu\text{g/ml}$ <p>To check primer concentration, the molar extinction coefficient (ϵ_{260}) can be used:</p> $A_{260} = \epsilon_{260} \times \text{molar concentration of primer or probe}$ <p>If the ϵ_{260} value is not given on the data sheet supplied with the primers or probes, it can be calculated from the primer sequence using the following formula:</p> $\epsilon_{260} = 0.89 \times [(A \times 15,480) + (C \times 7340) + (G \times 11,760) + (T \times 8850)]$

Table continued on next page

Table 10. Continued

	<p>Example</p> <p>Concentration of diluted primer: $1 \mu\text{M} = 1 \times 10^{-6} \text{ M}$</p> <p>Primer length: 24 nucleotides with 6 each of A, C, G, and T bases</p> <p>Calculation of expected A_{260}: $0.89 \times [(6 \times 15,480) + (6 \times 7340) + (6 \times 11,760) + (6 \times 8850)] \times (1 \times 10^{-6}) = 0.232$</p> <p>The measured A_{260} should be within $\pm 30\%$ of the theoretical value. If the measured A_{260} is very different to the theoretical value, we recommend recalculating the concentration of the primers or probes, or having the primers or probes resynthesized.</p> <p>For probes, the fluorescent dye does not significantly affect the A_{260} value.</p>
Primer and probe quality	<p>The quality of 18–30mers can be checked on a 15% denaturing polyacrylamide gel; a single band should be seen. Please contact QIAGEN Technical Services or your local distributor (see back cover) for a protocol.</p>
Probe quality	<p>The quality of the fluorescent label and the purity of TaqMan probes can be determined by comparing fluorescence before and after DNase digestion. Incubate probes with or without 5 U DNase at 37°C for 1 hour. A significant difference in fluorescence following DNase treatment should be detectable.</p>

Probe design

To design probes for quantitative PCR, follow the general guidelines for primer design (page 28). In addition, the following considerations should be followed for specific types of probes.

Since there are more constraints for probes than for PCR primers, it is recommended to design the probe first and then select primers that flank the probe region.

Dual-labeled probes

Dual-labeled probes, such as TaqMan probes, contain a fluorescent reporter and a quencher at their 5' and 3' ends, respectively. During the extension phase of PCR, the 5'→3' exonuclease activity of HotStarTaq *Plus* DNA Polymerase cleaves the fluorophore from the quencher. This results in detectable fluorescence that is proportional to the amount of accumulated PCR product.

The following guidelines should be followed when designing dual-labeled probes:

- Cleavage must occur between the reporter and quencher. A reporter at the 5' end with a quencher at the 3' end gives the most room for cleavage to occur. Table 11 lists dyes commonly used for dual-labeled probes.
- The probe should be 20–30 bases in length.
- The probe–template hybrid should have a T_m that is 8–10°C higher than the T_m for the primer–template hybrids. Use commercially available oligo-design software (see Table 9, page 28) to calculate this T_m . For best results, choose a probe with a T_m of 68–70°C.
- Avoid a guanidine at the 5' end, next to the reporter, which causes quenching.
- Avoid runs of 4 or more of the same nucleotide, especially of guanidine.
- Choose the binding strand so that the probe has more C than G bases.

Table 11. Commonly used dual-labeled probes available from Operon Biotechnologies

5' fluorophore	3' quencher
6-FAM™, HEX™, TET™	TAMRA™
Fluorescein, 6-FAM, HEX, TET, Cy3, Cy5, TAMRA, ROX, Texas Red®, Oregon Green®	Black Hole Quencher®

FRET probes

PCR with fluorescence resonance energy transfer (FRET) probes, such as LightCycler hybridization probes, uses 2 labeled oligonucleotide probes that bind to the PCR product in a head-to-tail fashion. One probe is labeled with a donor fluorophore, and the other is labeled with an acceptor (or reporter) fluorophore. During the annealing step of PCR, the 2 oligonucleotides bind to the target, and their fluorophores come into close proximity, allowing energy transfer between the fluorophores. This causes fluorescence that is proportional to the amount of product.

The following guidelines should be followed when designing FRET probes for real-time quantification:

- Two probes are required: one probe has fluorescein at its 3' end (donor probe), and the other probe commonly has LightCycler-Red 640 (LC-Red 640) at its 5' end (acceptor probe). The acceptor probe also needs a phosphate at its 3' end to prevent extension during PCR.
- When annealed to the targets, the 2 fluorophores must be close to each other to allow FRET (4–25 Å). Design the probes so that the 3' end of the donor probe is within 1–5 bases of the 5' end of the acceptor probe. For initial experiments, a gap of 1 base is recommended.

- Design probes to bind close to the 3' end of the target strand, but not overlapping the primer-binding region.
- Design probes in a region with nearly equal distribution of the 4 bases.
- The probe–template hybrids should have T_m values that are 5–10°C higher than the T_m for the primer–template hybrids. Use commercially available oligo-design software (see Table 9, page 28) to calculate this T_m . Both probes should have equal T_m values.
- Avoid clusters of G and C at either end. These can cause the probe to bind too tightly to the target and interfere with amplification.
- Avoid probes that are rich in A and G bases. These can cause poor hybridization of the probe to the target.

Appendix C: Amplification of Long Amplicons

For the highest efficiency in real-time RT-PCR using sequence-specific probes, targets should ideally be 70–200 bp in length. If this is not possible and the target is 200–500 bp in length, follow the cycling conditions in Table 12 below instead of the cycling conditions given in the protocols.

Table 12. Real-time cycler conditions for real-time RT-PCR with amplicons of 200–500 bp

Step	Time	Temperature	Ramp rate	Additional comments
Reverse transcription	15 min	50°C		
PCR initial activation step	5 min	95°C	Maximal/fast mode	HotStarTaq <i>Plus</i> DNA Polymerase is activated by this heating step
Three-step cycling				
Denaturation	15 s	95°C	Maximal/fast mode	
Annealing	30 s	60°C	Maximal/fast mode	
Extension	30 s	72°C	Maximal/fast mode	Perform fluorescence data collection
Number of cycles	35–40			The number of cycles depends on the amount of template RNA

Appendix D: Quantifying Gene Expression Levels and Generating Standard Curves

This appendix provides information on quantification of target nucleic acids. Further information can be found in *Critical Factors for Successful Real-Time PCR*. To obtain a copy, contact QIAGEN Technical Services, or visit www.qiagen.com/literature/defaultbrochures.aspx to download a PDF.

Absolute and relative quantification

Target nucleic acids can be quantified using either absolute quantification or relative quantification. Absolute quantification determines the absolute amount of a target (expressed as a copy number or concentration), whereas relative quantification determines the ratio between the amount of a target and the amount of a reference nucleic acid, usually a suitable housekeeping gene. This normalized value can then be used to compare, for example, differential gene expression in different samples.

Absolute quantification

The absolute amount of a target nucleic acid is determined using external standards. The sequence of the standards is usually the same as or very similar to the target sequence, but the primer binding sites of the standards must be identical to those in the target sequence. This ensures that both the standards and the target are amplified with equivalent efficiencies, which is essential for absolute quantification. A standard curve (plot of C_T value/crossing point against log of amount of standard) is generated using different dilutions of the standard. The target and each of the standards are amplified in separate tubes. The C_T value of the target is compared with the standard curve, allowing calculation of the initial amount of the target. It is important to select an appropriate standard (see page 36).

Relative quantification

With this method, the amounts of the target genes and the reference gene within the same sample are determined, and ratios are calculated between each target gene and the reference gene. These normalized values can then be used to compare, for example, differential gene expression in different samples. The most common application of this method is analysis of gene expression or, more generally, determination of the abundance of RNA targets. The expression level of the reference gene, 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 genes and the reference gene are amplified with comparable or different efficiencies. For determination of PCR efficiency, see below.

Different amplification efficiencies

The amplification efficiencies of target and reference genes are sometimes different due to differences in primer binding sites, PCR product sequences, and PCR product sizes. If this is the case, we recommend generating several standard curves (see below), one for each target or reference gene. The standards can be, for example, total RNA prepared from a reference cell line. The amounts of the target genes and the reference gene are determined by comparing their C_T values with the corresponding standard curve. Ratios can then be calculated between each target gene and the reference gene. Since the expression of the reference gene remains the same between different samples, the ratio of the target genes to the reference gene will vary depending on the expression of the target genes (e.g., in different tissues).

Comparable amplification efficiencies

If the amplification efficiencies of the target and reference genes are the same, only the standard curve for the reference gene needs to be generated. The amounts of the target and reference genes are determined by comparing their C_T values with this standard curve.

Alternatively, the comparative or $\Delta\Delta C_T$ method can be used. This involves comparing C_T values, and does not require preparation of standard curves. This method can only be used if the amplification efficiencies of the target and reference genes are nearly equivalent.

Determination of PCR efficiency

To compare the amplification efficiencies of, for example, 2 target genes (targets A and B), prepare different dilutions each target. Amplify the different dilutions by real-time RT-PCR. Subtract the C_T values of target A from the C_T values of target B. Plot the differences in C_T values against the logarithm of amount of target. If the slope of the resulting straight line is <0.1 , the amplification efficiencies are comparable.

Generating standard curves

Standard curves can be used in both absolute and relative quantification. To generate a standard curve, at least 5 different amounts of the standard should be quantified, and the amount of unknown target should fall within the range of the standard curve. Reactions should be carried out in at least triplicate, especially when quantifying standards of low copy number.

Standards

For absolute quantification of RNA molecules (see page 35), the copy number or concentration of the nucleic acids used as standards must be known. In addition, standards should show the following features:

- Primer binding sites identical to the target to be quantified
- Sequence between primer binding sites identical or highly similar to target sequence
- Sequences upstream and downstream from the amplified sequence identical or similar to “natural” target

For quantification of RNA, we strongly recommend using RNA molecules as standards. Depending on the sequence and structure of the target and the efficiency of reverse transcription, only a proportion of the target RNA will be reverse transcribed. The DNA generated during reverse transcription serves as the template for amplification in the subsequent PCR. The use of RNA standards takes the variable efficiency of the RT reaction into account.

RNA standards can be created by cloning part or all of the transcript of interest into a standard cloning vector. The insert can be generated by RT-PCR from total RNA or mRNA, or by PCR from cDNA. The cloning vector must contain an RNA polymerase promoter such as T7, SP6, or T3. Ensure that in vitro transcription of the insert leads to generation of the sense transcript. After in vitro transcription, plasmid DNA must be removed completely with RNase-free DNase, since residual plasmid DNA will lead to errors in spectrophotometric determination of RNA concentration and will also serve as a template in the subsequent PCR. Furthermore, ensure that the RNA used as a standard does not contain any degradation products or aberrant transcripts by checking that it migrates as a single band in gel electrophoresis.

After determination of RNA concentration by spectrophotometry, the copy number of standard RNA molecules can be calculated using the following formula:

$$(X \text{ g}/\mu\text{l RNA} / [\text{transcript length in nucleotides} \times 340]) \times 6.022 \times 10^{23} = Y \text{ molecules}/\mu\text{l}$$

Example

Transcript length: 500 nucleotides

Concentration: 30 ng/ μl = 30×10^{-9} g/ μl

Calculation: $(30 \times 10^{-9} \text{ g}/\mu\text{l} / [500 \times 340]) \times 6.022 \times 10^{23} = 1.1 \times 10^{11}$ molecules/ μl

An alternative to the use of in vitro transcripts as RNA standards is the use of a defined RNA preparation (e.g., from a cell line or virus preparation), for which the absolute concentration of the target has already been determined.

Appendix E: 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.

RT control

All RT-PCR experiments should include a negative control to test for contaminating DNA. However, detection of this contamination can be eliminated by using suitable primers or probes (see Table 9, page 28). If it is not possible to use such primers or probes, DNA contamination can be detected by performing a control reaction in which no reverse transcription is possible. The control RT reaction contains all components including template RNA, except for the reverse transcriptase enzyme. Reverse transcription therefore cannot take place. When an aliquot of this control is used as a template in PCR, 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 to test only for presence or absence of the target, but does not yield detailed quantitative information. An internal, positive control (detected with a second, differently labeled probe) can be used to test for the presence of PCR inhibitors. Ensure that the positive control contains at least the minimum amount of DNA required for accurate detection.

Appendix F: Data Analysis

When carrying out data analysis, follow the recommendations provided by the manufacturer of your real-time cycler. Fundamental guidelines for data analysis and some important considerations are given below.

General considerations for data analysis

Real-time PCR data are produced as sigmoidal-shaped amplification plots (when using a linear scale), in which fluorescence is plotted against the number of cycles (Figure 2, page 39).

- The threshold cycle (C_T value) serves as a tool for calculation of the starting template amount in each sample. This is the cycle in which there is the first detectable significant increase in fluorescence.
- The optimal threshold setting depends on the reaction chemistries used for PCR. Therefore, an optimal threshold setting established for another kit may not be suitable for QuantiFast Probe RT-PCR Kits, and may need to be adjusted.

- The method for determination of C_T values differs depending on the real-time cycler used. Check the handbook or the software help file for your real-time cycler for details on threshold settings.
- Whenever possible, select the option for automatic calculation of threshold and baseline for your real-time cycler. **However, note that the default values for data analysis in the cycler software will not always provide the most accurate results.**
- Most real-time cyclers contain a function that determines the noise level in early cycles, where there is no detectable increase in fluorescence due to PCR products (usually referred to as the baseline settings). Adjust the settings for this function.
- For real-time PCR assays, the analysis settings need to be adjusted for each of the reporter dyes used.

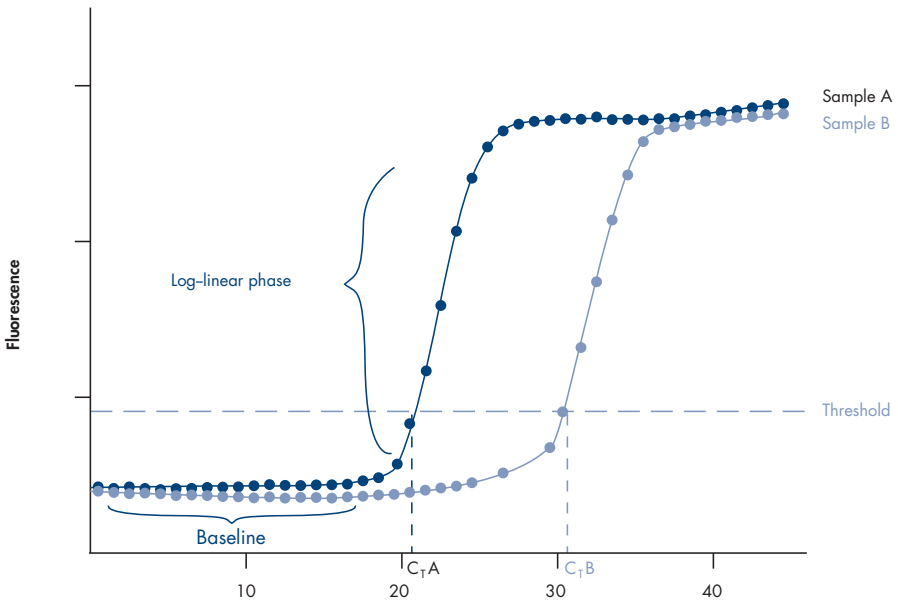


Figure 2. Typical amplification plot. Amplification plots showing increases in fluorescence from 2 samples (A and B). Sample A contains a higher amount of starting template than sample B.

Applied Biosystems instruments

Before performing data analysis on Applied Biosystems instruments, read the important points below. For further details, refer to the handbook, supplementary literature, or software help file for the instrument being used.

ABI PRISM 7900

The following points only apply to SDS software version 2.1 or higher. If you work with a version earlier than 2.1, we recommend updating your SDS software to the most recent version.

- The analysis settings for the baseline and threshold need to be set. These settings are specific for each of the reporter dyes and must be individually set for each reporter dye present on the plate.
- Details on data analysis are available in the SDS 2.1 online help (from the "Help" menu, select "SDS Online Help" and enter a search term).
- Select the option for automatic calculation of threshold and baseline for each reporter dye.
- If you observe wells with a C_T value that strongly deviates from those of replicate wells when using the automatic analysis settings, you should record the positions of these unusual wells and reanalyze the plate. Reanalysis needs to be done for each reporter dye present on the plate.
- If you observe problems using the automatic calculation option, you may configure the analysis settings manually. **Note that the default values for the analysis settings entered in the software will not always provide the most accurate results.**

ABI PRISM 7700

The following points only apply to SDS software version 1.7 or higher. If you work with a version earlier than 1.7, we recommend updating your SDS software to the most recent version.

- Check baseline and threshold settings.
- Analyze the plate. You may wish to save this setup in a separate file.
- Export the C_T values for the assays if you want to perform data analysis (e.g., using a spreadsheet program).
- **Note that the default values for the analysis settings entered in the software will not always provide the most accurate results.**

ABI PRISM 7000

The following points only apply to software version 1.1 or higher. If you work with a version earlier than 1.1, we recommend updating your SDS software to the most recent version.

- The analysis settings for the baseline and threshold need to be set. These settings are specific for each of the reporter dyes and must be individually set for each reporter dye present on the plate.

- Details on data analysis are provided in the online help (from the “Help” menu, select “Contents and Index” and enter a search term).
- Select the option for automatic calculation of threshold and baseline for each reporter dye.
- If you observe wells with a C_T value that strongly deviates from those of replicate wells, you should record the positions of these unusual wells and reanalyze the plate. Reanalysis needs to be done for each reporter dye present on the plate.
- If you observe problems using the automatic option, you may configure the analysis settings manually. **Note that the default values for the analysis settings entered in the software will not always provide the most accurate results.**

Applied Biosystems 7300

The following points only apply to software version 1.22 or higher. If you work with a version earlier than 1.22, we recommend updating your SDS software to the most recent version.

- The analysis settings for the baseline and threshold need to be set. These settings are specific for each of the reporter dyes and must be individually set for each reporter dye present on the plate.
- Details on data analysis are provided in the online help (from the “Help” menu, select “Contents and Index” and enter a search term).
- Select the option for automatic calculation of threshold and baseline for each reporter dye.
- If you observe wells with a C_T value that strongly deviates from those of replicate wells, you should record the positions of these unusual wells and reanalyze the plate. Reanalysis needs to be done for each reporter dye present on the plate.
- If you observe problems using the automatic option, you may configure the analysis settings manually. **Note that the default values for the analysis settings entered in the software will not always provide the most accurate results.**

Applied Biosystems 7500

The following points only apply to software version 1.22 or higher. If you work with a version earlier than 1.22, we recommend updating your SDS software to the most recent version.

- The analysis settings for the baseline and threshold need to be set. These settings are specific for each of the reporter dyes and must be individually set for each reporter dye present on the plate.
- Select the option for automatic calculation of threshold and baseline for each reporter dye.
- If you observe wells with a C_T value that strongly deviates from those of replicate wells, you should record the positions of these unusual wells and reanalyze the plate. Reanalysis needs to be done for each reporter dye present on the plate.
- If you observe problems using the automatic option, you may configure the analysis settings manually. **Note that the default values for the analysis settings entered in the software will not always provide the most accurate results.**

Important: When using the QuantiFast Probe RT-PCR Kit (which uses a high concentration of ROX dye) with 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 3). Use a value of 0.01 as a starting point.

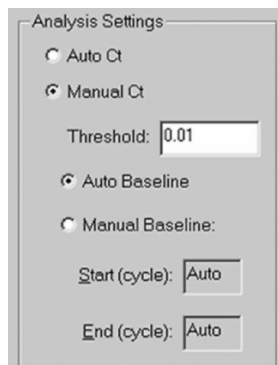


Figure 3. Analysis settings for the Applied Biosystems 7500. Adjusting the analysis settings for the Applied Biosystems 7500. Select “Manual Ct” and “Auto Baseline” for each reporter dye.

Adjust the preset threshold to a value in the range of 0.01, either by entering a value in the dialog field or by moving the threshold bar using the mouse. After adjusting the threshold, click the “Analyze” button to reanalyze the data.

- Details on data analysis are provided in the online help (from the “Help” menu, select “Contents and Index” and enter a search term).

- To be able to use the function for automatic threshold calculation on the Applied Biosystems 7500, we recommend using the QuantiFast Probe RT-PCR +ROX Vial Kit, which allows you to adjust the ROX concentration.

LightCycler system

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 proportional mode of baseline adjustment when analyzing data obtained with single-color FRET probes (only one pair of FRET probes is used) or dual-labeled probes. Use the arithmetic mode for experiments with 2 pairs of FRET probes, where the acceptor probes are labeled with different fluorophores.

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

Fit points: These are a defined number of 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 s 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.

Ordering Information

Product	Contents	Cat. no.
QuantiFast Probe RT-PCR Kit (400)	For 400 x 25 µl reactions: 3 x 1.7 ml 2x Master Mix (contains ROX dye), 100 µl RT Mix, 2 x 1.9 ml RNase-Free Water	204454
QuantiFast Probe RT-PCR Kit (2000)	For 2000 x 25 µl reactions: 25 ml 2x Master Mix (contains ROX dye), 0.5 ml RT Mix, 20 ml RNase-Free Water	204456
QuantiFast Probe RT-PCR +ROX Vial Kit (400)	For 400 x 25 µl reactions: 3 x 1.7 ml 2x Master Mix (without ROX dye), 210 µl ROX Dye Solution, 100 µl RT Mix, 1.9 ml RNase-Free Water	204554
QuantiFast Probe RT-PCR +ROX Vial Kit (2000)	For 2000 x 25 µl reactions: 25 ml 2x Master Mix (without ROX dye), 1.05 ml ROX Dye Solution, 0.5 ml RT Mix, 20 ml RNase-Free Water	204556

Related products

QuantiFast Probe PCR Kits — for fast, quantitative, real-time PCR and two-step RT-PCR using sequence-specific probes

For all instruments from Applied Biosystems except the Applied Biosystems 7500:

QuantiFast Probe PCR Kit (80)	For 80 x 25 µl reactions: 1 ml 2x Master Mix (contains ROX dye), 1.9 ml RNase-Free Water	204252
QuantiFast Probe PCR Kit (400)	For 400 x 25 µl reactions: 3 x 1.7 ml 2x Master Mix (contains ROX dye), 2 x 1.9 ml RNase-Free Water	204254
QuantiFast Probe PCR Kit (2000)	For 2000 x 25 µl reactions: 25 ml 2x Master Mix (contains ROX dye), 20 ml RNase-Free Water	204256

Ordering Information

Product	Contents	Cat. no.
For the Applied Biosystems 7500 and instruments from Bio-Rad/MJ Research, Cepheid, Corbett Research, Eppendorf, Roche, and Agilent:		
QuantiFast Probe PCR +ROX Vial Kit (80)	For 80 x 25 µl reactions: 1 ml 2x Master Mix (without ROX dye), 45 µl ROX Dye Solution, 1.9 ml RNase-Free Water	204352
QuantiFast Probe PCR +ROX Vial Kit (400)	For 400 x 25 µl reactions: 3 x 1.7 ml 2x Master Mix (without ROX dye), 210 µl ROX Dye Solution, 2 x 1.9 ml RNase-Free Water	204354
QuantiFast Probe PCR +ROX Vial Kit (2000)	For 2000 x 25 µl reactions: 25 ml 2x Master Mix (without ROX dye), 1.05 ml ROX Dye Solution, 20 ml RNase-Free Water	204356
QuantiFast SYBR® Green RT-PCR Kit — for fast, quantitative, 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 Master Mix (contains ROX dye), 100 µl RT Mix, 2 x 1.9 ml RNase-Free Water	204154
QuantiFast SYBR Green RT-PCR Kit (2000)	For 2000 x 25 µl reactions: 25 ml 2x Master Mix (contains ROX dye), 0.5 ml RT Mix, 20 ml RNase-Free Water	204156
QuantiFast SYBR Green PCR Kit — for fast, quantitative, real-time PCR and two-step RT-PCR using SYBR Green I		
QuantiFast SYBR Green PCR Kit (80)	For 80 x 25 µl reactions: 1 ml 2x Master Mix (contains ROX dye), 1.9 ml RNase-Free Water	204052
QuantiFast SYBR Green PCR Kit (400)	For 400 x 25 µl reactions: 3 x 1.7 ml 2x Master Mix (contains ROX dye), 2 x 1.9 ml RNase-Free Water	204054
QuantiFast SYBR Green PCR Kit (2000)	For 2000 x 25 µl reactions: 25 ml 2x Master Mix (contains ROX dye), 20 ml RNase-Free Water	204056

Ordering Information

Product	Contents	Cat. no.
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 or 400 x 25 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 reverse-transcription reactions: gDNA Wipeout Buffer, Quantiscript® Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix, and RNase-Free Water	205311
QuantiTect Whole Transcriptome Kit — for unlimited real-time PCR analysis from precious RNA samples		
QuantiTect Whole Transcriptome Kit (25)*	For 25 x 50 µl whole-transcriptome-amplification reactions: T-Script Enzyme and Buffer, Ligation Enzymes, Reagent, and Buffer, and REPLI-g® DNA Polymerase and Buffer	207043
RNeasy Mini Kit — for purification of total RNA from cells, tissues, and yeast, and for RNA cleanup		
RNeasy Mini Kit (50)†	50 RNeasy Mini Spin Columns, Collection Tubes, RNase-Free Reagents and Buffers	74104
RNeasy Plus Mini Kit — for purification of total RNA from cells and tissues using gDNA Eliminator columns		
RNeasy Plus Mini Kit (50)	50 RNeasy Mini Spin Columns, 50 gDNA Eliminator Mini Spin Columns, Collection Tubes, RNase-Free Reagents and Buffers	74134

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www.qiagen.com

Australia = techservice-au@qiagen.com

Austria = techservice-at@qiagen.com

Belgium = techservice-bnl@qiagen.com

Brazil = suportetecnico.brasil@qiagen.com

Canada = techservice-ca@qiagen.com

China = techservice-cn@qiagen.com

Denmark = techservice-nordic@qiagen.com

Finland = techservice-nordic@qiagen.com

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Ireland = techservice-uk@qiagen.com

Italy = techservice-it@qiagen.com

Japan = techservice-jp@qiagen.com

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

Luxembourg = techservice-bnl@qiagen.com

Mexico = techservice-mx@qiagen.com

The Netherlands = techservice-bnl@qiagen.com

Norway = techservice-nordic@qiagen.com

Singapore = techservice-sg@qiagen.com

Sweden = techservice-nordic@qiagen.com

Switzerland = techservice-ch@qiagen.com

UK = techservice-uk@qiagen.com

USA = techservice-us@qiagen.com

