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QuantiFast® Probe Assay Handbook

For fast, real-time RT-PCR using sequence-specific, hydrolysis probes in combination with QuantiFast Kits

Sample & Assay Technologies

QIAGEN Sample and Assay Technologies

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

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Kit Contents

QuantiFast Probe Assay (Box 1 of 2)	(80)	(400)
Number of 25 µl reactions	**80**	**400**
Cat. no.	**Varies**	**Varies**
20x QuantiFast Probe Assay in a single tube*	1 or 2†	1 or 2†
TE Buffer	1.9 ml	1.9 ml

* Each 20x QuantiFast Probe Assay is a mix of a forward and a reverse primer and a hydrolysis probe for a specific target. Each assay is supplied lyophilized and must be reconstituted according to the instructions in "Shipping and Storage" (page 5).

† For singleplex detection, one QuantiFast Probe Assay is provided. For duplex detection, 2 QuantiFast Probe Assays are provided.

One of the following master mix kits is supplied.

For one-step RT-PCR with singleplex or duplex detection, the following kit is supplied:

QuantiFast Probe RT-PCR Plus Kit (Box 2 of 2)	(80)	(400)
Number of 25 µl reactions	**80**	**400**
2x QuantiFast Mix 1*	0.5 ml	2 x 1.3 ml
2x QuantiFast Mix 2 (Probe)†	0.5 ml	2 x 1.3 ml
QuantiFast RT Mix‡	100 µl	100 µl
ROX™ Dye Solution	45 µl	210 µl
High-ROX Dye Solution	45 µl	210 µl
RNase-Free Water	1.9 ml	2 x 1.9 ml

* Contains gDNA Wipeout Buffer.

† Contains HotStarTaq® Plus DNA Polymerase, QuantiFast Probe RT-PCR Plus Buffer, and dNTP mix (dATP, dCTP, dGTP, and dTTP).

‡ Contains a mixture of QIAGEN® reagents, including Omniscript® Reverse Transcriptase and Sensiscript® Reverse Transcriptase.

For two-step RT-PCR with singleplex detection, the following kit is supplied:

QuantiFast Probe PCR +ROX Vial Kit (Box 2 of 2)	(80)	(400)
Number of 25 μl reactions	**80**	**400**
2x QuantiFast Probe PCR Master Mix (w/o ROX)*	1 ml	3 x 1.7 ml
ROX Dye Solution	45 μl	210 μl
High-ROX Dye Solution	45 μl	210 μl
RNase-Free Water	1.9 ml	1.9 ml

* Contains HotStarTaq Plus DNA Polymerase, QuantiFast Probe PCR Buffer, and dNTP mix (dATP, dCTP, dGTP, and dTTP).

For two-step RT-PCR with duplex detection, the following kit is supplied:

QuantiFast Multiplex PCR +R Kit (Box 2 of 2)	(80)	(400)
Number of 25 μl reactions	**80**	**400**
2x QuantiFast Multiplex PCR Master Mix (w/o ROX)†	1 ml	3 x 1.7 ml
ROX Dye Solution	45 μl	210 μl
High-ROX Dye Solution	45 μl	210 μl
RNase-Free Water	1.9 ml	1.9 ml

† Contains HotStarTaq Plus DNA Polymerase, QuantiFast Multiplex PCR Buffer, and dNTP mix (dATP, dCTP, dGTP, and dTTP).

Shipping and Storage

QuantiFast Probe Assays are shipped lyophilized at ambient temperature. Store QuantiFast Probe Assays protected from the light at –20°C, either reconstituted (see below) or lyophilized. Avoid repeated freeze–thaw cycles. When stored under these conditions and handled correctly, the reconstituted product can be kept for at least 18 months from the date of receipt without reduction in performance. Reconstitute the 20x QuantiFast Probe Assay as follows. Briefly centrifuge the vial, add TE (provided), and mix by vortexing the vial. For QuantiFast Probe Assay (80), add 110 μl TE. For QuantiFast Probe Assay (400), add 550 μl TE.

QuantiFast Kits are shipped on dry ice. They should be stored immediately upon receipt at –20°C in a constant-temperature freezer and protected from light. When 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). The 2x QuantiFast Mix 2 (Probe), QuantiFast Probe PCR Master Mix (w/o ROX), and QuantiFast Multiplex PCR Master Mix (w/o ROX) can also be stored protected from light at 2–8°C for up to 1 month without showing any reduction in performance.

Product Use Limitations

The QuantiFast Probe Assay, QuantiFast Probe RT-PCR Plus Kit, QuantiFast Probe PCR +ROX Vial Kit, and QuantiFast Multiplex PCR +R Kit are intended for molecular biology applications. These products are not intended for the diagnosis, prevention, or treatment of a disease.

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

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 QuantiFast Probe Assay, QuantiFast 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 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).

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of QuantiFast Probe Assay, QuantiFast Probe RT-PCR Kit, QuantiFast Probe PCR +ROX Vial Kit and QuantiFast Multiplex PCR +R Kit is tested against predetermined specifications to ensure consistent product quality.

Safety Information

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

24-hour emergency information

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

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

Introduction

QuantiFast Probe Assays are predesigned assays based on hydrolysis probe detection (also known as TaqMan® assays). Each QuantiFast Probe Assay consists of a premixed primer pair and hydrolysis probe. Two dye labels are available: FAM™ (for all genes) and MAX™ (for selected reference genes). All QuantiFast Probe Assays are labeled with IBFQ (Iowa Black™ fluorescent quencher). IBFQ is a dark quencher with a broad absorbance spectrum ranging from 420 to 620 nm.

Used in combination with QuantiFast Kits, QuantiFast Probe Assays enable rapid real-time RT-PCR quantification of cDNA or RNA targets in a singleplex format (FAM reporter dye) or duplex format (FAM and MAX reporter dyes). QuantiFast Probe Assays are available for every human, mouse, or rat gene.

QuantiFast Probe Assays are designed using a proprietary algorithm to enable amplification and detection of RNA and cDNA targets less than 100 bp in size with high efficiency and reliability. They are highly suited for use with degraded starting material such as formalin-fixed, paraffin-embedded (FFPE) analytes.

For guaranteed results in target detection, QuantiFast Probe Assays are combined with QuantiFast Kits for two-step or one-step real-time RT-PCR.

Hydrolysis probes

Hydrolysis probes (e.g., TaqMan probes) are sequence-specific oligonucleotides with a fluorophore and a quencher moiety attached (Figure 1). The fluorophore is at the 5' end of the probe, and the quencher moiety is usually located at the 3' end or internally. During the extension phase of PCR, the probe is cleaved by the 5'→3' exonuclease activity of *Taq* DNA polymerase, separating the fluorophore and the quencher moiety. This results in detectable fluorescence that is proportional to the amount of accumulated PCR product.

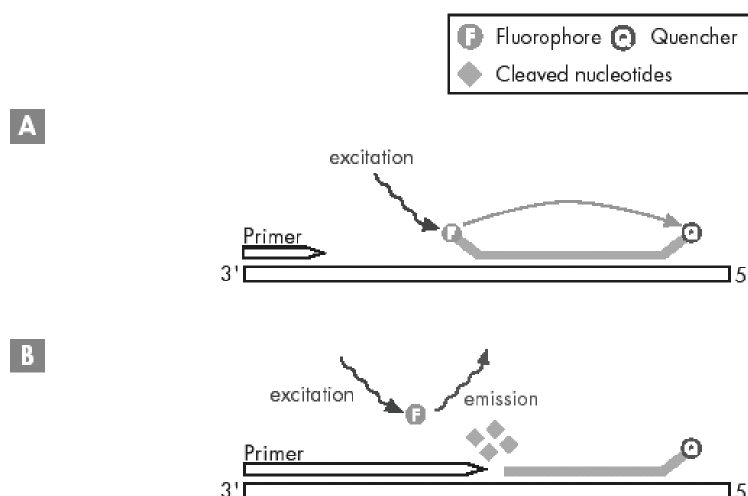

Figure 1. Principle of hydrolysis probes in quantitative, real-time PCR. **A** Both the hydrolysis probe and the PCR primers anneal to the target sequence during the PCR annealing step. The proximity of the fluorophore with the quencher prevents the fluorophore from fluorescing. **B** During the PCR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

Duplex, real-time PCR requires the simultaneous detection of different fluorescent reporter dyes (Table 1). QuantiFast Probe Assays use FAM and MAX, dyes with well separated fluorescence spectra. MAX can be used as a VIC® replacement on instruments from Applied Biosystems® without the need for an extra calibration step.

Table 1. Dyes used for QuantiFast Probe Assays

Dye	Excitation maximum (nm)	Emission maximum (nm)*
FAM	494	518
MAX	524	557

* Emission spectra may vary depending on the buffer conditions.

Kits supplied with QuantiFast Probe Assays

QuantiFast Probe Assays are supplied with a different QuantiFast Kit depending on the type of PCR to be performed:

- For one-step RT-PCR with singleplex detection: QuantiFast Probe RT-PCR Plus Kit
- For one-step RT-PCR with duplex detection: QuantiFast Probe RT-PCR Plus Kit
- For two-step RT-PCR with singleplex detection: QuantiFast Probe PCR +ROX Vial Kit
- For two-step RT-PCR with duplex detection: QuantiFast Multiplex PCR +R Kit

One-step RT-PCR with singleplex or duplex detection

For singleplex or duplex one-step RT-PCR, QuantiFast Probe Assays are used in combination with the QuantiFast Probe RT-PCR Plus Kit for the detection of RNA. This kit offers an integrated genomic DNA removal step that effectively eliminates genomic DNA contamination to avoid false-positive signals. The presence of genomic DNA, particularly in samples containing highly degraded RNA (e.g., RNA from FFPE tissue samples), frequently results in false positive RT-PCR signals. After addition of the master mix, reverse transcription and real-time PCR take place in the same tube.

Two-step RT-PCR with singleplex or duplex detection

For singleplex or duplex two-step RT-PCR, QuantiFast Probe Assays are used in combination with the QuantiFast Probe PCR +ROX Vial Kit or QuantiFast Multiplex PCR +R Kit, respectively. QuantiFast Probe Assays, used together with QuantiFast Kits, provide reliable and highly sensitive detection of cDNA targets and eliminate tedious design and optimization steps. For cDNA synthesis, the QuantiTect® Reverse Transcription Kit is recommended. The QuantiTect Reverse Transcription Kit provides an easy and fast cDNA synthesis procedure and includes a genomic DNA elimination step to avoid false-positive signals.

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.

The use of ROX dye is necessary for instruments from Applied Biosystems and is optional for instruments from Agilent (formerly Stratagene). The master mix supplied with the QuantiFast Probe RT-PCR Plus Kit, QuantiFast Probe PCR +ROX Vial Kit, and QuantiFast Multiplex PCR +R Kit does not contain ROX dye. In order to adjust the reaction for instruments from Applied Biosystems, it is

necessary to add High-ROX Dye Solution (models 7000, 7300, 7700, 7900HT, StepOne™, and StepOnePlus™) or ROX Dye Solution (model 7500) to the master mix.

Instruments from all other suppliers, which do not require ROX dye for fluorescence normalization, should be used with the 2x QuantiFast Master Mix 2 (Probe), QuantiFast Probe PCR Master Mix (w/o ROX), or QuantiFast Multiplex PCR Master Mix (w/o ROX) without addition of ROX dye solution.

Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

- Nuclease-free (RNase/DNase-free) consumables. Special care should be taken to avoid nuclease contamination of PCR reagents and consumables.
- Real-time PCR thermal cycler (e.g., Rotor-Gene® Q real-time cycler; see www.qiagen.com/RotorGeneQ for more information)
- PCR tubes or plates (use thin-walled PCR tubes or plates recommended by the manufacturer of your real-time cycler)
- Optional: QIAgility® for rapid, high-precision automated PCR setup; for details, visit www.qiagen.com/goto/QIAgility.

Important Notes

Selecting protocols

To select the correct protocol, refer to Table 2. In general, the following cyclers are not compatible with duplex, real-time PCR using QuantiFast Probe Assays: GeneAmp® 5700, MyiQ™, DNA Engine Opticon® (i.e., the single-color machine), and LightCycler® 1.x.

Table 2. Choosing the correct protocol

Technique	Protocol
Two-step RT-PCR with singleplex detection	Page 15
Two-step RT-PCR with duplex detection	Page 18
One-step RT-PCR with singleplex or duplex detection	Page 21

Controls

No template control (NTC)

All quantification experiments should include an NTC, containing all the components of the reaction except for the template. This enables detection of contamination.

No RT control

All RT-PCR experiments should include a negative control to test for contaminating DNA. Elimination of DNA contamination by the genomic DNA removal step can be followed by performing a control reaction in which no reverse transcription is possible. The control “no RT reaction” contains all components including template RNA, except for the reverse transcriptase. Reverse transcription therefore cannot take place. When an aliquot of this control is used as a template in PCR, the only template available would be residual contaminating DNA. In one-step RT-PCR, the “no RT control” is performed by replacing the reverse transcriptase with a corresponding volume of water.

Positive control

In some cases it may be necessary to include a positive control, containing a known concentration or copy number of template. Positive controls can be absolute standards or known positive samples.

Absolute standards include commercially available standards and in-lab standards, such as a plasmid containing cloned sequences. Absolute standards are used at a known copy number and provide quantitative information.

A positive sample is usually a substitute for an absolute standard and is used only to test for presence or absence of the target.

Protocol: Two-Step RT-PCR with Singleplex Detection

This protocol is for use with the QuantiFast Probe PCR +ROX Vial Kit and one QuantiFast Probe Assay.

Important points before starting

- Always use the **cycling conditions** and **QuantiFast Probe Assay concentrations** specified in this protocol.
- **Optimal analysis settings are a prerequisite for accurate quantification data.** For data analysis, you should always readjust the analysis settings (i.e., baseline settings and threshold values) for each QuantiFast Probe Assay used.
- For cDNA synthesis, the QuantiTect Reverse Transcription Kit is highly recommended. This kit provides highly efficient, integrated genomic DNA elimination.

Thing to do before starting

- Reconstitute the 20x QuantiFast Probe Assay as follows. Briefly centrifuge the vial, add TE (provided), and mix by vortexing the vial. For QuantiFast Probe Assay (80), add 110 µl TE. For QuantiFast Probe Assay (400), add 550 µl TE.

Procedure

1. **Thaw 2x QuantiFast Probe PCR Master Mix (w/o ROX), template cDNA, and RNase-free water. Mix the individual solutions.**

   If using an Applied Biosystems cycler, also thaw ROX Dye Solution or High-ROX Dye Solution. For details of which ROX Dye Solution to use, see page 10.

2. **Prepare a reaction mix according to Table 3.**

Table 3. Reaction setup for two-step RT-PCR with singleplex detection

Component	Volume/reaction (96-well plate, Rotor-Disc™ 72, Rotor-Disc 100)	Volume/reaction (384-well plate)	Final concentration
2x QuantiFast Probe PCR Master Mix (w/o ROX)	12.5 µl	5 µl	1x
20x QuantiFast Probe Assay	1.25 µl	0.5 µl	1x
Optional: ROX Dye Solution or High-ROX Dye Solution*	0.5 µl	0.2 µl	
RNase-free water	Variable	Variable	
Template cDNA	Variable	Variable	≤100 ng/reaction
Total reaction volume	**25 µl**†	**10 µl**†	

* ROX Dye Solution or High-ROX Dye Solution should be used with real-time cyclers that require ROX, see page 10 for more information.

† If your real-time cycler requires a final reaction volume other than 25 µl or 10 µl, adjust the amount of master mix and all other reaction components accordingly.

3. **Mix the reaction mix thoroughly and dispense appropriate volumes into PCR vessels or plates.**
4. **Program the real-time cycler according to Table 4.**

Table 4. Cycling conditions for two-step RT-PCR with singleplex detection

Step	Time	Temperature	Additional comments
PCR initial activation step	**3 min**	**95°C**	HotStarTaq Plus DNA Polymerase is activated by this heating step
2-step cycling:			**Important: Optimal performance is only assured using these cycling conditions**
Denaturation	**3 s**	**95°C**	
Annealing/extension	**30 s**	**60°C**	Combined annealing/ extension step with fluorescence data collection
Number of cycles	40–45		The number of cycles depends on the amount of template cDNA and the expression level of the target gene.

5. **Place the PCR tubes, plate, or Rotor-Disc in the real-time cycler, and start the cycling program.**
6. **Perform data analysis.**

   Before performing data analysis, select the analysis settings for each QuantiFast Probe Assay (i.e., baseline settings and threshold values). Optimal analysis settings are a prerequisite for accurate quantification data.

Protocol: Two-Step RT-PCR with Duplex Detection

This protocol is for use with the QuantiFast Multiplex PCR +R Kit and 2 QuantiFast Probe Assays.

Important points before starting

- Always use the **cycling conditions** and **QuantiFast Probe Assay concentrations** specified in this protocol.
- **Optimal analysis settings are a prerequisite for accurate quantification data.** For data analysis, you should always readjust the analysis settings (i.e., baseline settings and threshold values) for each reporter dye channel in every run.
- For cDNA synthesis, the QuantiTect Reverse Transcription Kit is highly recommended. This kit provides highly efficient, integrated genomic DNA elimination.

Thing to do before starting

- Reconstitute the 20x QuantiFast Probe Assay as follows. Briefly centrifuge the vial, add TE (provided), and mix by vortexing the vial. For QuantiFast Probe Assay (80), add 110 µl TE. For QuantiFast Probe Assay (400), add 550 µl TE.

Procedure

1. **Thaw 2x QuantiFast Multiplex PCR Master Mix (w/o ROX), template cDNA, and RNase-free water. Mix the individual solutions.**

   If using an Applied Biosystems cycler, also thaw ROX Dye Solution or High-ROX Dye Solution. For details of which ROX Dye Solution to use, see page 10.

2. **Prepare a reaction mix according to Table 5.**

Table 5. Reaction setup for two-step RT-PCR with duplex detection

Component	Volume/reaction (96-well plate, Rotor-Disc 72, Rotor-Disc 100)	Volume/reaction (384-well plate)	Final concentration
2x QuantiFast Multiplex PCR Master Mix (w/o ROX)	12.5 μl	5 μl	1x
20x QuantiFast Probe Assay (FAM)	1.25 μl	0.5 μl	1x
20x QuantiFast Probe Assay (MAX)	1.25 μl	0.5 μl	1x
Optional: ROX Dye Solution or High-ROX Dye Solution*	0.5 μl	0.2 μl	
RNase-free water	Variable	Variable	
Template cDNA	Variable	Variable	≤100 ng/reaction
Total reaction volume	**25 μl**†	**10 μl**†	

* ROX Dye Solution or High-ROX Dye Solution should be used with real-time cyclers that require ROX, see page 10 for more information.

† If your real-time cycler requires a final reaction volume other than 25 μl or 10 μl, adjust the amount of master mix and all other reaction components accordingly.

3. **Mix the reaction mix thoroughly and dispense appropriate volumes into PCR vessels or plates.**
4. **Program the real-time cycler according to Table 6.**

Table 6. Cycling conditions for two-step RT-PCR with duplex detection

Step	Time	Temperature	Additional comments
PCR initial activation step	**5 min**	**95°C**	HotStarTaq Plus DNA Polymerase is activated by this heating step
2-step cycling			**Important: Optimal performance is only assured using these cycling conditions**
Denaturation	**30 s**	**95°C**	
Annealing/extension	**30 s**	**60°C**	Combined annealing/ extension step with fluorescence data collection
Number of cycles	40–45		The number of cycles depends on the amount of template cDNA and the expression level of the target gene.

5. **Place the PCR tubes, plate, or Rotor-Disc in the real-time cycler, and start the cycling program.**
6. **Perform data analysis.**

   Before performing data analysis, select the analysis settings for each QuantiFast Probe Assay (i.e., baseline settings and threshold values). Optimal analysis settings are a prerequisite for accurate quantification data.

Protocol: One-Step RT-PCR with Singleplex or Duplex Detection

This protocol is for use with the QuantiFast Probe RT-PCR Plus Kit and either one (singleplex detection) or 2 (duplex detection) QuantiFast Probe Assays.

Important points before starting

- Always use the **cycling conditions** and **QuantiFast Probe Assay concentrations** specified in this protocol.
- **Optimal analysis settings are a prerequisite for accurate quantification data.** For data analysis, you should always readjust the analysis settings (i.e., baseline settings and threshold values) for each reporter dye channel in every run.

Thing to do before starting

- Reconstitute the 20x QuantiFast Probe Assay as follows. Briefly centrifuge the vial, add TE (provided), and mix by vortexing the vial. For QuantiFast Probe Assay (80), add 110 µl TE. For QuantiFast Probe Assay (400), add 550 µl TE.

Procedure

1. **Thaw 2x QuantiFast Mix 1, 2x QuantiFast Mix 2 (Probe), template RNA, QuantiFast Probe Assays, and RNase-free water. Mix the individual solutions.**

   If using an Applied Biosystems cycler, also thaw ROX Dye Solution or High-ROX Dye Solution. For details of which ROX Dye Solution to use, see page 10.

   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 genomic DNA removal reaction mix according to Table 7.**

Table 7. Reaction setup for genomic DNA removal

Component	Volume/reaction (96-well plate, Rotor-Disc 72, Rotor-Disc 100)	Volume/reaction (384-well plate)	Final concentration
2x QuantiFast Master Mix 1	6.25 µl	2.5 µl	1x
Template RNA (added at step 4)	Variable	Variable	≤100 ng/reaction
RNase-free water	Variable	Variable	
Total reaction volume	**13 µl***	**5 µl†**	

* This will be added to 12 µl QuantiFast reaction mix in step 6 to make a final reaction volume of 25 µl. If your real-time cycler requires a final reaction volume other than 25 µl, adjust the amount of master mix and all other reaction components accordingly.

† This will be added to 5 µl QuantiFast reaction mix in step 6 to make a final reaction volume of 10 µl. If your real-time cycler requires a final reaction volume other than 10 µl, adjust the amount of master mix and all other reaction components accordingly.

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

   Note: Do not keep the PCR vessels or plates on ice.

4. **Add template RNA (≤100 ng) to the individual PCR vessels or plates and incubate for 5 min at room temperature.**

   Note: The incubation step can be prolonged up to 15 min.

5. **Prepare an RT-PCR reaction mix according to Table 8.**

Table 8. Reaction setup for one-step RT-PCR with singleplex or duplex detection

Component	Volume/reaction (96-well plate, Rotor-Disc 72, Rotor-Disc 100)	Volume/reaction (384-well plate)	Final concentration
2x QuantiFast Master Mix 2 (Probe)	6.25 µl	2.5 µl	1x
20x QuantiFast Probe Assay (FAM)*	1.25 µl	0.5 µl	1x
20x QuantiFast Probe Assay (MAX)*	1.25 µl	0.5 µl	1x
Optional: ROX Dye Solution or High-ROX Dye Solution†	0.5 µl	0.2 µl	
QuantiFast RT Mix	0.25 µl	0.1 µl	
RNase-free water	Variable	Variable	
Total reaction volume	**12 µl**‡	**5 µl**‡	

* Two QuantiFast Probe Assays are used for duplex detection; a single QuantiFast Probe Assay is used for singleplex detection.

† ROX Dye Solution or High-ROX Dye Solution should be used with real-time cyclers that require ROX, see page 10 for more information.

‡ If your real-time cycler requires a final reaction volume other than 25 µl or 10 µl, adjust the amount of master mix and all other reaction components accordingly.

6. **Mix the RT-PCR reaction mix thoroughly and dispense appropriate volumes into PCR vessels or plates containing the genomic DNA removal reaction from step 4.**

   Note: If the transfer to the real-time cycler needs more than 5 min, store the plates on ice.

7. **Program the real-time cycler according to Table 9.**

   Note: Check the real-time cycler's user manual for correct instrument setup for multiplex analysis (e.g., setting up detection of multiple dyes from the same well). Be sure to activate the detector for each reporter dye used. Place the PCR tubes or plate in the real-time cycler, and start the cycling program.

Table 9. Cycling conditions for one-step RT-PCR with singleplex or duplex detection

Step	Time	Temperature	Additional comments
Reverse transcription	**20 min**	**50°C**	RNA is reverse transcribed into cDNA
PCR initial activation step	**5 min**	**95°C**	HotStarTaq Plus DNA Polymerase is activated by this heating step
2-step cycling			**Important: Optimal performance is only assured using these cycling conditions**
Denaturation	**15 s**	**95°C**	
Annealing/extension	**30 s**	**60°C**	Combined annealing/ extension step with fluorescence data collection
Number of cycles	40–45		The number of cycles depends on the amount of template cDNA and the expression level of the target gene.

8. **Place the PCR tubes, plate, or Rotor-Disc in the real-time cycler, and start the cycling program.**

9. **Perform data analysis.**

   Before performing data analysis, select the analysis settings for each QuantiFast Probe Assay (i.e., baseline settings and threshold values). Optimal analysis settings are a prerequisite for accurate quantification data.

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 signal, or one or more signals detected late in PCR	
a) Wrong cycling conditions	Always start with the optimized cycling conditions specified in the protocols. Be sure that the cycling conditions include the initial step for activation of HotStarTaq Plus DNA Polymerase (95°C for 3 min or 5 min, depending on the protocol), and the specified times for denaturation and annealing/extension.
b) HotStarTaq Plus DNA Polymerase not activated	Ensure that the cycling program includes the HotStarTaq Plus DNA Polymerase activation step (3 min or 5 min at 95°C, depending on the protocol) as described in the protocols.
c) Pipetting error or missing reagent	Check the concentrations and storage conditions of the reagents, including template nucleic acid. Repeat the RT-PCR.
d) Wrong or no detection step	Ensure that fluorescence detection takes place during the combined annealing/extension step.
e) Mg^{2+} concentration not optimal	The Mg^{2+} concentration in QuantiFast Mixes is already optimized. Increasing the final Mg^{2+} concentration by 0.5–1 mM may improve results.
f) Problems with starting template	Check the concentration, storage conditions, and quality of the starting template. If necessary, make new serial dilutions of template nucleic acid from the stock solutions. Repeat the RT-PCR using the new dilutions. Ensure that all reagents, buffers, and solutions used for purification and dilution of template nucleic acids are free of nucleases.

	Comments and suggestions
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) Wrong detection channel/filter chosen	Ensure that the correct detection channel is activated or the correct filter set is chosen for each reporter dye. Check whether the chosen combination of reporter dyes is compatible with the selected detection channels or filter sets.

Differences in C_T values or in PCR efficiencies between a duplex RT-PCR assay and the corresponding singleplex RT-PCR assays

a) Wrong cycling conditions	Always start with the optimized cycling conditions specified in the protocols. Be sure that the cycling conditions include the initial step for activation of HotStarTaq Plus DNA Polymerase (95°C for 3 min or 5 min, depending on the protocol), and the specified times for denaturation and annealing/extension.
b) Analysis settings (e.g., threshold and baseline settings) not optimal	Check the analysis settings (threshold and baseline settings) for each reporter dye. Repeat analysis using optimal settings for each reporter dye.
c) Imprecise spectral separation of reporter dyes	Since duplex RT-PCR uses multiple probes, each with a fluorescent dye, the increased fluorescent background may affect the shape of the amplification plots obtained with some real-time cyclers. This may lead to differences in C_T values of up to 5% between the duplex assay and the corresponding singleplex assays; this can usually be avoided by using optimal threshold settings. **ABI PRISM® 7700**: Perform analysis with and without spectral compensation. **LightCycler 2.0**: In some instances, the shape of the amplification plots for singleplex and duplex reactions may differ due to the color compensation algorithms.

	Comments and suggestions
No linearity in ratio of C_T value/crossing point to log of the template amount	
a) Template amount too high	When signals are coming up at very early C_T values, adjust the analysis settings accordingly.
b) Template amount too low	Increase template amount if possible. Note that detection of very low starting copy numbers may not be in the linear range of a standard curve.
Increased fluorescence or C_T value for "No Template" control	
a) Contamination of reagents	Discard all the components of the duplex assay (e.g., master mix, assays). Repeat the duplex assay using new components.
b) 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	
a) Incomplete genomic DNA removal	In rare cases a C_T <40 is observed. Prolong the incubation step after adding RNA from 5 min up to 15 min (step 4 of the protocol for one-step RT-PCR). Make sure that the reagents used for the incubation at step 4 are not cooled on ice. For cDNA synthesis in two-step RT-PCR, use the QuantiTect Reverse Transcription Kit for cDNA synthesis. This kit includes a genomic DNA removal step.
b) Sequence dependent effects	In rare cases a C_T <40 is observed although a prolonged incubation step of up to 15 min after adding RNA (step 4 of the protocols) has been performed.
Varying fluorescence intensity	
a) Contamination of real-time cycler	Decontaminate the real-time cycler according to the manufacturer's instructions.
b) Real-time cycler no longer calibrated	Recalibrate the real-time cycler according to the manufacturer's instructions.

	Comments and suggestions
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) **ABI PRISM 7000**: Uneven curves or high standard deviations	Do not use reaction volumes smaller than 25 µl, and always use optical adhesive covers to seal plates. In some cases, increasing the reaction volume to 50 µl may improve results.

Appendix A: 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. 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/brochures to download a PDF.

General considerations for duplex 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 30).

- 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 the QuantiFast Kit you are using, 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.
- 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 multiplex assays, the analysis settings need to be adjusted for each of the reporter dyes used.
- Depending on your real-time cycler, low levels of signal crosstalk, even between apparently well separated reporter dyes, may influence multiplex results in rare cases. In most cases, low levels of crosstalk can be overcome by optimal analysis settings.

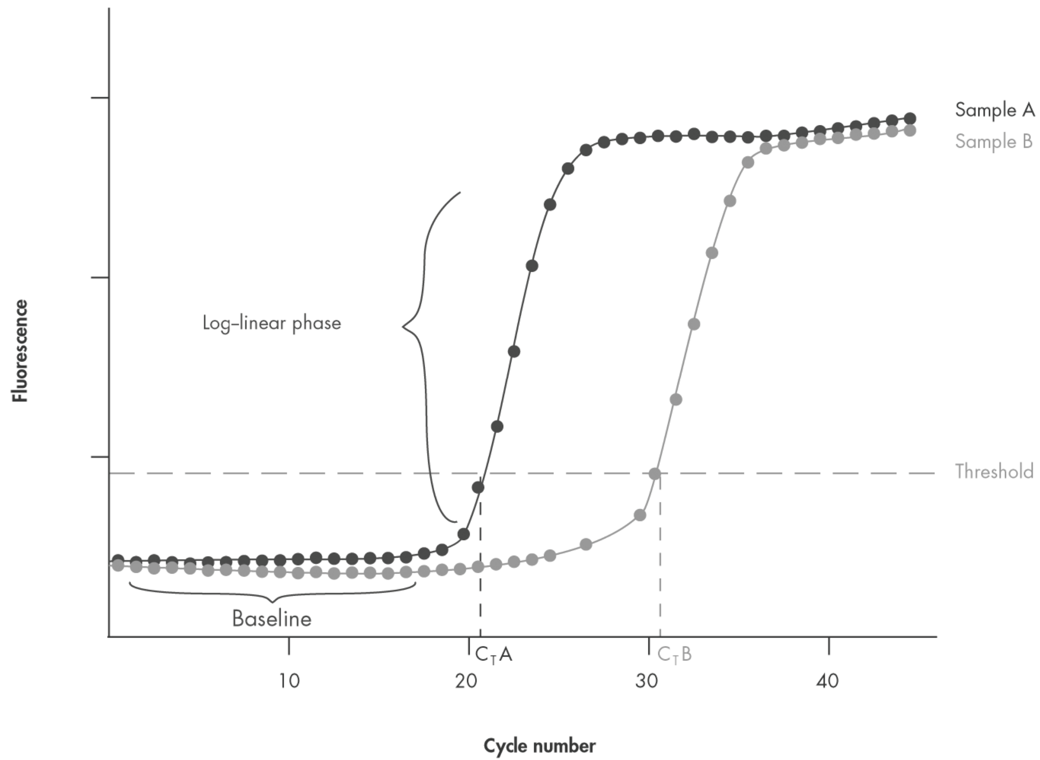

Figure 2. Typical amplification plot. Amplification plots showing increases in fluorescence from 2 samples (Sample A and Sample B).

References

QIAGEN maintains a large, up-to-date online database of scientific publications utilizing QIAGEN products. Comprehensive search options allow you to find the articles you need, either by a simple keyword search or by specifying the application, research area, title, etc.

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Ordering Information

Product	Contents	Cat. no.
QuantiFast Probe Assays (80)*	For 80 x 25 μl reactions: 20x QuantiFast Probe Assay (primers and hydrolysis probe), QuantiFast Master Mix Reagents for two-step or one-step RT-PCR	Varies
QuantiFast Probe Assays (400)*	For 400 x 25 μl reactions: 20x QuantiFast Probe Assay (primers and hydrolysis probe), QuantiFast Master Mix Reagents for two-step or one-step RT-PCR	Varies
For fast cDNA synthesis including genomic DNA removal		
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
For purification of total RNA from formalin-fixed, paraffin-embedded tissue sections		
RNeasy FFPE Kit (50)	For 50 preps: 50 RNeasy MinElute Spin Columns, Collection Tubes, Proteinase K, RNase-Free DNase I, DNase Booster Buffer, RNase-Free Buffers, RNase-Free Water	73504
For purification of microRNA and total RNA from formalin-fixed, paraffin-embedded tissue sections		
miRNeasy FFPE Kit (50)	For 50 preps: 50 RNeasy MinElute Spin Columns, Collection Tubes, Proteinase K, RNase-Free DNase I, DNase Booster Buffer, RNase-Free Buffers, RNase-Free Water	217504

* Visit www.qiagen.com/GeneGlobe to search for and order this product.

† Various kit sizes available; please inquire.

Product	Contents	Cat. no.
For simultaneous purification of DNA and RNA from formalin-fixed, paraffin-embedded tissue sections		
Allprep DNA/RNA FFPE Kit (50)	For 50 preps: 50 RNeasy MinElute Spin Columns, 50 QIAamp MinElute Spin Columns, Collection Tubes, RNase-Free Reagents, and Buffers	80234

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