Overcoming the challenges of real-time, multiplex PCR

Quantitative, multiplex real-time PCR is a powerful tool for gene expression analysis and other applications. However, it can be a challenging technique and appropriate conditions that enable robust PCR of all targets must be used to ensure successful and reliable results. Here, we explain how QuantiFast® and Rotor-Gene® Multiplex PCR Kits overcome the problems often encountered in multiplex real-time PCR and present examples using application data from QIAGEN, as well as data provided by researchers in the field. Important points to consider when starting a multiplex PCR assay are also outlined.

Advantages of multiplex real-time PCR

The amplification and detection of several cDNA or genomic DNA targets in the same reaction vessel offers many benefits:

- Reduced costs — targets are amplified together instead of separately
- Reliable results — coamplification eliminates well-to-well variability
- Conservation of precious samples — obtain more data per sample
- Increased throughput — more targets can be analyzed per run

QuantiFast Multiplex Kits enable fast and reliable quantification in real-time PCR, two-step RT-PCR, and one-step RT-PCR analysis. The innovative features of QuantiFast Multiplex PCR Kits ensure that even challenging multiplex assays work without optimization. Significantly reduced PCR run times are achieved, not only on fast cyclers with rapid ramping rates, but also on all standard cyclers (Figure 1). In addition, a specially developed master mix and a universal protocol for all cyclers ensure fast and reliable quantification of up to 4 genes in the same reaction (e.g., 1 control gene and 3 target genes). QIAGEN's Rotor-Gene Multiplex Kits include all the innovative features of QuantiFast Kits, but are dedicated for use on Rotor-Gene instruments.

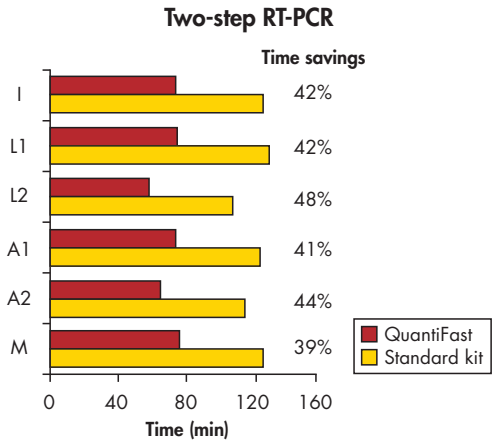
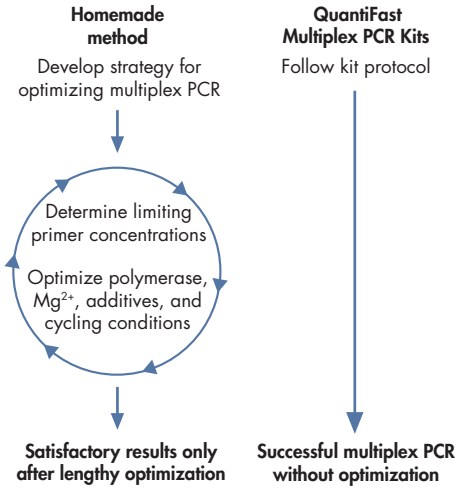


Figure 1. Significantly reduced PCR times. QuantiFast Multiplex PCR Kits reduce total PCR run time by ~50% in real-time two-step RT-PCR (40 cycles run; comparison with a standard-cycling multiplex PCR Kit). **I**: iCycler® iQ; **L1**: LightCycler® 480; **L2**: LightCycler 2.0; **A1**: ABI PRISM® 7900; **A2**: Applied Biosystems® 7500 Fast System; **M**: Mx3005P®.

Reliable multiplex PCR — without optimization

Optimizing the conditions for a multiplex real-time PCR assay can be tedious and time consuming (see flowchart). Several factors need to be considered, including the concentrations of primers, Mg^{2+}, *Taq* DNA polymerase, and dNTPs, and the composition of the PCR buffer, to ensure all multiplex reactions occur with equal efficiency to their corresponding singleplex reactions, even when the targets vary greatly in abundance. Innovative PCR buffers included in QuantiFast and Rotor-Gene Multiplex Kits ▶



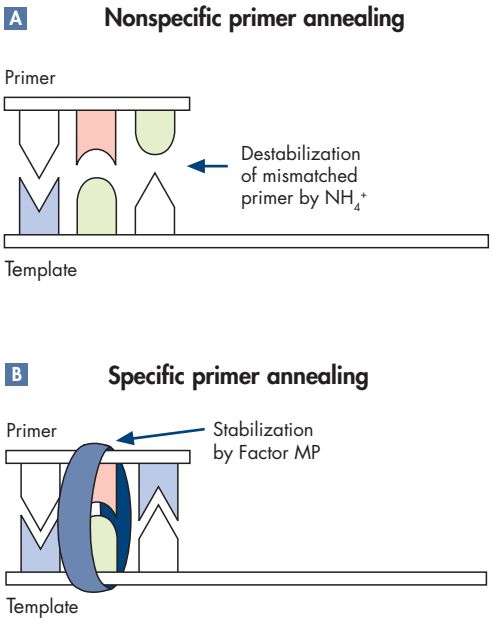


Figure 2. Unique PCR buffer. A NH_4^+ ions prevent nonspecific primers from annealing to the template. B Synthetic Factor MP, an innovative PCR additive, increases the local concentration of primers at the template. Together with K^+ and other cations, Factor MP stabilizes specifically bound primers, allowing efficient primer extension by HotStarTaq *Plus* DNA Polymerase.

provide a preoptimized solution for real-time, multiplex PCR and contain several unique features that ensure reliable multiplex results.

Unique real-time, multiplex PCR buffer

In addition to various salts and additives, QIAGEN's fast cycling PCR buffers contain a specially optimized combination of KCl and $(NH_4)_2SO_4$, which promotes a high ratio of specific to nonspecific primer binding during each PCR annealing step (Figure 2a). With this buffer, primer annealing is only marginally influenced by $MgCl_2$ concentration, so optimization by titration of Mg^{2+} is usually not required.

Accurate multiplex results and fast cycling without the need for optimization are further enabled by several exclusive features:

Multiplex without optimization

By increasing the local concentration of primers at the DNA template and stabilizing specifically bound primers, the unique synthetic additive Factor MP allows efficient primer extension by HotStarTaq® *Plus* DNA Polymerase (Figure 2b).

Fast cycling

Under standard cycling conditions, denaturation, annealing, and extension usually occur as a 3-step process, where the template is first denatured, followed by primer annealing to form a binary complex, and then polymerase binding to form a tertiary complex. QuantiFast and Rotor-Gene Multiplex PCR Master Mixes include Q-Bond®, which reduces this 3-step process to a faster 2-step process, where the template is first denatured and the tertiary complex is then formed by the simultaneous binding of primer and polymerase, allowing extension to start within seconds (Figure 3).

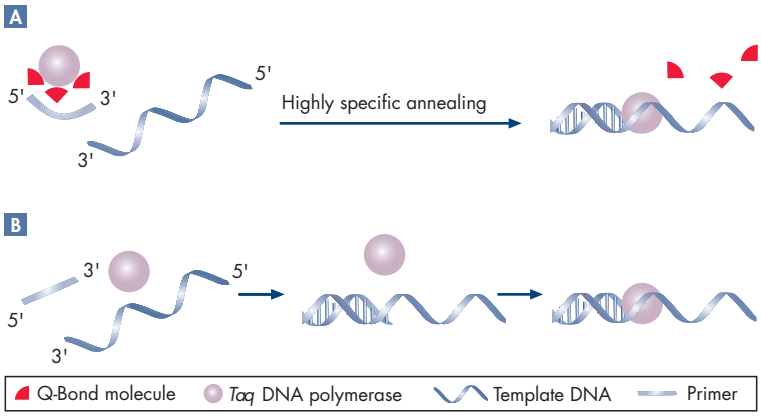


Figure 3. Fast primer annealing. A Q-Bond increases the affinity of DNA polymerase for short single-stranded DNA, reducing primer annealing time to a few seconds. In addition, the unique buffer composition supports the melting of DNA, reducing denaturation and extension times. B Without Q-Bond, the primer and polymerase bind sequentially to the template, increasing primer annealing time.

Stringent hot start for real-time multiplex PCR

HotStarTaq *Plus* DNA Polymerase is inactive at room temperature and becomes active only after incubation for 5 minutes at 95°C. This stringent hot start prevents the formation of misprimed products and primer–dimers during reaction setup and the first denaturation step. Competition for reactants by these PCR artifacts is therefore avoided.

Fast cycling, on all real-time cyclers

The protocols provided with QuantiFast Multiplex PCR Kits have been developed by QIAGEN on a wide range of real-time cyclers. Since all reaction and cycling parameters are already established, there is no need to perform optimization steps, such as determining limiting primer concentrations or adjusting cycling conditions. This allows reliable multiplex quantification on virtually any real-time cycler (Figure 4).

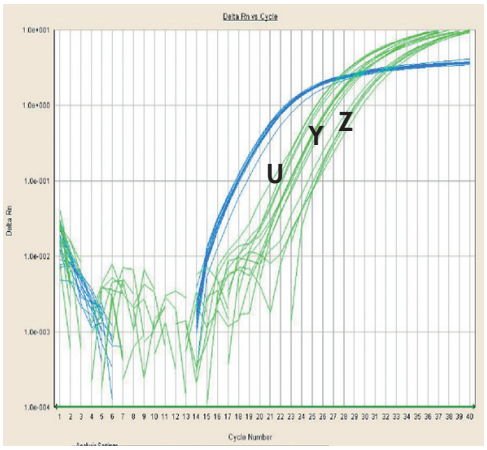
To suit the requirements of different real-time cyclers, two different kits are available. The QuantiFast Multiplex PCR Kit, which is supplied with master mix containing the ROX™ passive reference dye, is intended for cyclers that require ROX dye for fluorescence normalization (e.g., instruments from Applied Biosystems, but not Applied Biosystems 7500 Real-time PCR Systems). The QuantiFast Multiplex PCR +R Kit, which is supplied with master mix free of ROX dye, is recommended for all other cyclers, as it allows greater multiplexing through the use of probes labeled with ROX, Texas Red®, or other equivalent dyes. These cyclers include the Rotor-Gene Q, iCycler iQ, LightCycler 2.0 and LightCycler 480, Mx3000P, MX3005P, and Mx4000 systems. For a complete list of appropriate cyclers and their multiplex capacities, see the QuantiFast Multiplex PCR Handbook at www.qiagen.com/goto/qfmpcr.

Rotor-Gene Multiplex PCR Kits include all of the same features of QuantiFast Multiplex PCR Kits and have been specially designed for use with the Rotor-Gene Q and other Rotor-Gene cyclers. They provide ultrafast, highly reliable quantification through the combination of an optimized master mix and the unique Rotor-Gene cycler.

Reliable multiplex analysis over a wide linear range

QuantiFast and Rotor-Gene Multiplex PCR Kits are designed to provide quantification of targets over a wide linear range. The kits are therefore highly suited for analyzing samples containing low- and high-abundance targets. Figure 5 demonstrates that real-time, multiplex PCR results using the Rotor-Gene Multiplex PCR Kit are equivalent to results obtained in real-time, singleplex PCR over template amounts ranging from 100 ng to 1 pg. ▶

A



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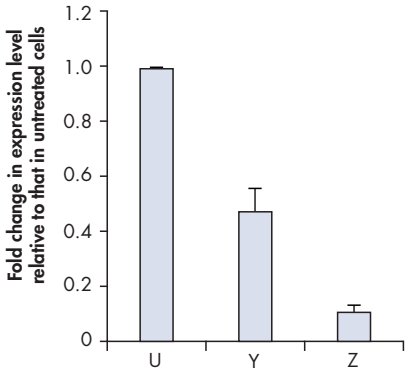
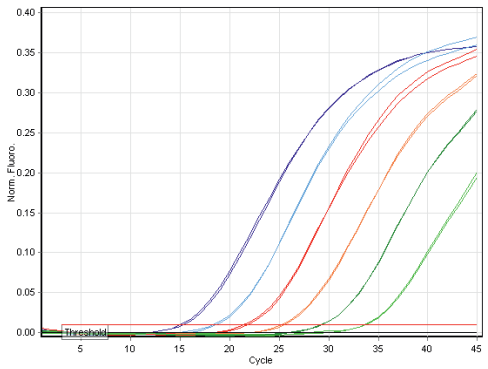


Figure 4. Reliable relative quantification. Cell line X was treated with one of two compounds (Y or Z) or left untreated (U). RNA was purified and duplex, real-time one-step RT-PCR was carried out on the Applied Biosystems 7500 Fast System using the QuantiFast Multiplex RT-PCR +R Kit and TaqMan® Gene Expression Assays for myogenin and GAPDH. For each treatment, 5 independent experiments were performed. A Changes in myogenin expression were detected with high accuracy and reproducibility (green curves). The expression of the housekeeping gene GAPDH was similar in all experiments (blue curves), allowing normalization of myogenin expression levels using the $\Delta\Delta C_T$ method of relative quantification. B The fold changes in normalized myogenin expression level relative to that in untreated cells were consistent between experiments, as indicated by the small error bars.

(Data kindly provided by Angelika Meyer, Novartis Pharma AG, Basel, Switzerland.)

A



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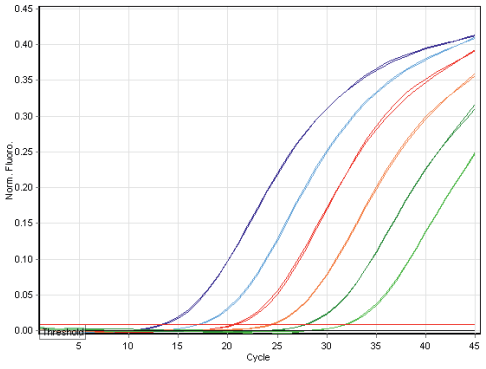


C

C_T values

	C_T values (IL8)		C_T values (ACTB)	
Template	**Duplex**	**Singleplex**	**Duplex**	**Singleplex**
100 ng	15.17	15.70	14.78	14.25
10 ng	18.57	19.14	18.20	17.23
1 ng	21.51	22.65	21.77	21.26
100 pg	25.42	26.18	25.50	24.98
10 pg	28.96	29.41	29.01	28.48
1 pg	33.76	34.75	32.88	32.36
NTC	45.00	45.00	45.00	45.00

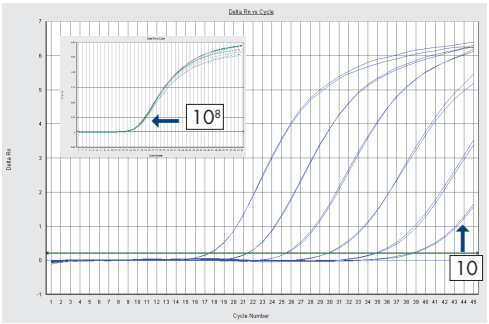
Figure 5. Reliable duplex PCR. Duplex, real-time two-step RT-PCR was carried on the Rotor-Gene Q using the Rotor-Gene Multiplex PCR Kit and self-designed TaqMan assays for A IL8 (interleukin 8) and B ACTB (β-actin). Analysis of tenfold dilutions of leukocyte cDNA template from 100 ng to 1 pg provided high PCR efficiencies of around 95%. C The C_T values were comparable with those achieved in control singleplex reactions, demonstrating the reliability of the duplex assay.

Sensitive detection of as little as 10 copies of template

Compared to a kit from another supplier, the QuantiFast Multiplex PCR +R Kit provided higher sensitivity and specificity, even when detecting as few as 10 copies of each target (Figure 6).

A

QuantiFast Multiplex PCR +R Kit (fast-cycling mode)



B

Kit from Supplier A_{II} (standard-cycling mode)

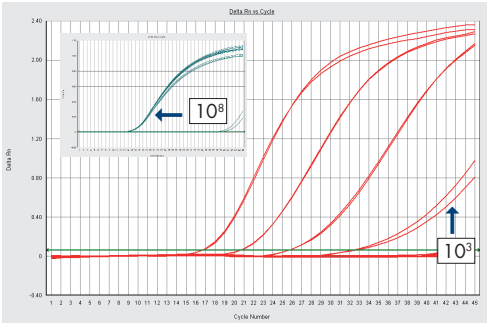


Figure 6. Sensitive duplex PCR, down to 10 copies of template. Duplicate reactions were run on the Applied Biosystems 7500 Fast System using a DNA template mix providing 10^8 copies of β-actin (data shown in insets) and 10^6 to 10 copies of RPS27A (a ribosomal protein). The A QuantiFast Multiplex PCR +R Kit showed clearly higher sensitivity than the B duplex PCR kit from Supplier A_{II}, enabling the cycler in fast-cycling mode to detect 10 copies of target and quantify over 6 log dilutions of template.

Efficient detection of targets varying greatly in abundance

The unique components of the Rotor-Gene Multiplex PCR Master Mix ensure targets with different levels of abundance are all amplified in the same tube with the same high efficiency, enabling reliable relative quantification (Figure 7).

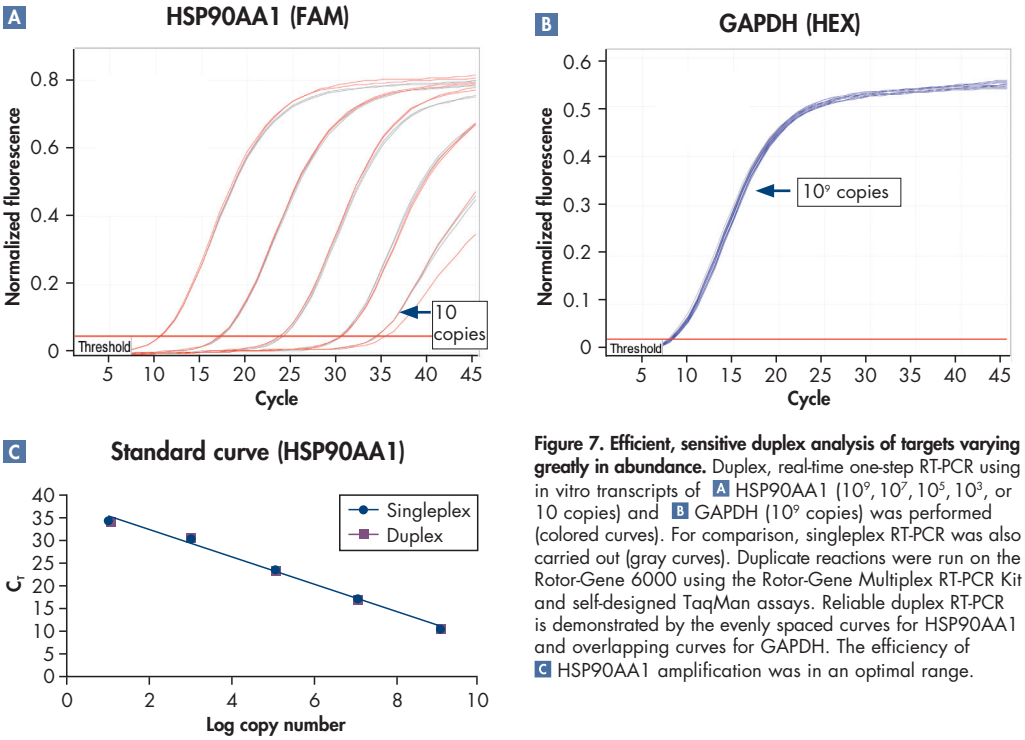


Figure 7. Efficient, sensitive duplex analysis of targets varying greatly in abundance. Duplex, real-time one-step RT-PCR using in vitro transcripts of A HSP90AA1 (10^9, 10^7, 10^5, 10^3, or 10 copies) and B GAPDH (10^9 copies) was performed (colored curves). For comparison, singleplex RT-PCR was also carried out (gray curves). Duplicate reactions were run on the Rotor-Gene 6000 using the Rotor-Gene Multiplex RT-PCR Kit and self-designed TaqMan assays. Reliable duplex RT-PCR is demonstrated by the evenly spaced curves for HSP90AA1 and overlapping curves for GAPDH. The efficiency of C HSP90AA1 amplification was in an optimal range.

Precise discrimination of small differences in template amount in singleplex and duplex reactions

QuantiFast Multiplex PCR Kits generate highly linear reactions, where the C_T values obtained in a multiplex reaction are similar to those obtained with the corresponding singleplex PCR assays. The kits ensure accurate quantification of targets, even with only twofold differences in template amount (Figure 8).

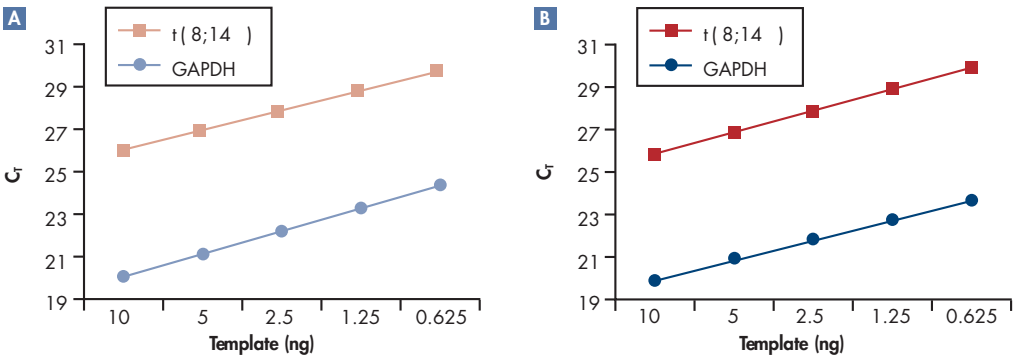


Figure 8. Linear C_T values over twofold decreases in template on the Applied Biosystems 7500 Fast System. Duplex and singleplex PCR were carried out using the QuantiFast Multiplex PCR +R Kit and assays for the t(8;14) chromosomal translocation and for GAPDH. Quadruplicate reactions were run using genomic DNA from the Ramos cell line as template (twofold dilutions from 10 ng to 0.625 ng). C_T values increased linearly by 1 C_T value with decrease in template dilution for both the A singleplex and B duplex reactions, demonstrating the ability of the kit to precisely discriminate between small differences in template amount.

Important considerations for real-time, multiplex PCR

The preoptimized master mix supplied with QuantiFast and Rotor-Gene Multiplex PCR Kits makes real-time, multiplex PCR simple. However, certain general considerations are required before starting a multiplex, real-time PCR experiment. For researchers new to this technology, these considerations are summarized below. More details can be found in the *Critical Factors for Successful Real-Time PCR* brochure.

Designing primers and probes

- Try to keep the size of the amplicons as small as possible, ideally 60–150 bp.
- Follow the guidelines for good assay design:
  - Use specialized design software to design primers and probes.
  - All assays should be designed using the same settings to ensure that they will work optimally under the same cycling conditions.
  - Check the specificity of your primers by performing a BLAST® search (www.ncbi.nlm.nih.gov/blast). Ensure that primer sequences are unique for your template sequence.
  - For two-step RT-PCR assays in which detection of genomic DNA must be avoided, design primers or probes so that one half anneals to the 3' end of one exon and the other half to the 5' end of the adjacent exon.

For convenience in real-time, multiplex PCR, we recommend using QuantiFast Probe Assays, which are predesigned, genome-wide, dual-labeled probe-based assays that can be ordered from GeneGlobe® (www.qiagen.com/geneglobe).

Table 1. Dyes commonly used in multiplex, real-time PCR

Dye	Excitation maximum (nm)	Emission maximum (nm)*
FAM™	494	518
TET™	521	538
JOE™	520	548
VIC®	538	552
Yakima Yellow™	526	552
HEX™	535	553
Bodipy TMR	542	574
NED™	546	575
Cy®3	552	570
TAMRA™	560	582
Cy3.5	588	604
ROX	587	607
Texas Red	596	615
Cy5	643	667

* Emission spectra may vary depending on the buffer conditions.

Handling and storing primers and probes

- Primers and probes should be purchased from an established oligonucleotide manufacturer.
- Upon receipt, resuspend the lyophilized primers and probes and check their concentrations by spectrophotometry.
- Lyophilized primers and probes should be dissolved in TE buffer to provide a stock solution of 100 µM. Prepare small aliquots to avoid repeated freezing and thawing.

Selecting appropriate reporter dyes and quenchers for the probes

- For accurate detection of the different targets in a real-time, multiplex PCR assay, it is essential that the sequence-specific probes are labeled with reporter dyes whose fluorescence spectra are well separated or exhibit only minimal overlap. See Table 1 for reporter dyes commonly used in real-time, multiplex PCR.

- Choose suitable combinations of reporter dyes and quenchers that are compatible with multiplex analysis using the detection optics of your real-time cycler (for details, refer to the instrument user manual). For details on reporter dyes tested and recommended by QIAGEN, refer to the handbooks supplied with QuantiFast and Rotor-Gene Multiplex PCR Kits.
- Use nonfluorescent quenchers (e.g., use BHQ® instead of TAMRA). Triplex and 4-plex analysis may only be possible using nonfluorescent quenchers.

Evaluating the performance of a real-time, multiplex PCR assay

- Check the functionality of each set of primers and probe in individual PCR assays before combining the different sets in a multiplex PCR assay.
- Compare the performance of the multiplex PCR assay with the corresponding singleplex PCR assays. Assay performance can be tested by, for example, assaying serial dilutions of a sample containing the target nucleic acids. In addition, the dynamic range of the multiplex assay can be tested by, for example, making several dilutions of one target nucleic acid and keeping the concentration of the other target nucleic acid constant; as template, target nucleic acids cloned in a plasmid or prepared as a PCR product can be used.
- Check the multiplex PCR assay for linearity by performing reactions with tenfold dilutions of template, and check whether the C_T values obtained are similar to those obtained with the corresponding single PCR assays. A standard curve can be used to evaluate the linear range and the PCR efficiency of the assay.

Programming the real-time cycler

- Be sure to activate the filters or detectors for the reporter dyes used in the multiplex PCR assay. For details on setting up your real-time cycler for multiplex PCR analysis, refer to the instrument user manual.
- Follow the optimized protocols in the handbooks. It is important to follow the specified cycling conditions, even for assays where the cycling conditions have already been established using a different kit or reagent.

Analyzing data from a real-time, multiplex PCR assay

- Optimal analysis settings (i.e., baseline settings and threshold values) for each reporter dye are a prerequisite for accurate quantification data:
  - ☐ Adjust the analysis settings for every reporter dye channel in every run. It is important to analyze the data for each channel separately.
  - ☐ Note that the default analysis settings provided by the instrument software may not provide accurate results and may need to be adjusted.
  - ☐ Save the multiplex reactions after amplification so that the PCR products can be checked on a gel if required.

Ordering Information

Product	Contents	Cat. no.
QuantiFast Multiplex PCR Kit (400)*	For 400 x 25 µl reactions: 3 x 1.7 ml 2x QuantiFast Multiplex PCR Master Mix (with ROX dye), 2 x 2 ml RNase-Free Water	204654
QuantiFast Multiplex PCR +R Kit (400)*	For 400 x 25 µl reactions: 3 x 1.7 ml 2x QuantiFast Multiplex PCR Master Mix (without ROX dye), 210 µl ROX Dye Solution, 2 x 2 ml RNase-Free Water	204754
QuantiFast Multiplex RT-PCR Kit (400)*	For 400 x 25 µl reactions: 3 x 1.7 ml 2x QuantiFast Multiplex RT-PCR Master Mix (with ROX dye), 100 µl QuantiFast RT Mix, 2 x 2 ml RNase-Free Water	204854
QuantiFast Multiplex RT-PCR +R Kit (400)*	For 400 x 25 µl reactions: 3 x 1.7 ml 2x QuantiFast Multiplex RT-PCR Master Mix (without ROX dye), 100 µl QuantiFast RT Mix, 210 µl ROX Dye Solution, 2 x 2 ml RNase-Free Water	204954
QuantiFast Probe Assay (400)*	For 400 x 25 µl reactions: dual-labeled, probe-based, predesigned 20x lyophilized assays; includes master mix and reagents for real-time one-step or two-step RT-PCR	Varies
Rotor-Gene Multiplex PCR Kit (400)*	For 400 x 25 µl reactions: 3 x 1.7 ml 2x Rotor-Gene Multiplex PCR Master Mix, 2 x 2 ml RNase-Free Water	204774
Rotor-Gene Multiplex RT-PCR Kit (400)*	For 400 x 25 µl reactions: 3 x 1.7 ml 2x Rotor-Gene Multiplex RT-PCR Master Mix, 100 µl Rotor-Gene RT Mix, 2 x 2 ml RNase-Free Water	204974

* Additional kit sizes available.

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at www.qiagen.com or can be requested from QIAGEN Technical Services or your local distributor.

Find out more at www.qiagen.com/goto/qfmpcr.

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