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QuantiNova[®] Multiplex PCR Kit Handbook

For highly sensitive, ultrafast, quantitative,
multiplex real-time PCR and two-step RT-PCR
using sequence-specific probes

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Kit Contents

QuantiNova Multiplex PCR Kit	(100)	(500)	(2500)
Catalog no.	208452	208454	208456
Number of reactions	100/	500/	2500/
(20 µl/10 µl)	200	1000	5000
4x QuantiNova Multiplex PCR Master Mix, containing:	0,5 ml	2 x 1.3 ml	10 x 1.3 ml
● QuantiNova DNA Polymerase composed of: Taq DNA Polymerase, QuantiNova Antibody, and QuantiNova Guard			
● QuantiNova Multiplex PCR Buffer			
● dNTP mix (dATP, dCTP, dGTP, dTTP)			
QuantiNova Yellow Template Dilution Buffer	500 µl	500 µl	2 x 500 µl
QN ROX™ Reference Dye	250 µl	1 ml	5 x 1 ml
RNase-Free Water	1.9 ml	3 x 1.9 ml	20 x 1.9 ml
Quick-Start Protocol	1	1	1

Storage

QuantiNova Multiplex PCR Kits are shipped on dry ice. The kits should be stored immediately upon receipt at -15 to -30°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 kit label and the quality-control label inside the kit box). QuantiNova Multiplex PCR Master Mix, QuantiNova Yellow Template Dilution Buffer, and QN ROX Reference Dye (protected from light) can also be stored at 2 – 8°C for up to 12 months, depending on the expiry date.

If desired, QN ROX Reference Dye can be added to 4x QuantiNova Multiplex PCR Master Mix for long-term storage. For details, see “Adding ROX dye to the master mix”, page 11.

Intended Use

The QuantiNova Multiplex 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.

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.



CAUTION: DO NOT add bleach or acidic solutions directly to the sample preparation waste.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of QuantiNova Multiplex PCR Kit is tested against predetermined specifications to ensure consistent product quality.

Product Information

The QuantiNova Multiplex PCR Kit contains:

4x QuantiNova Multiplex PCR Master Mix

Component	Description
QuantiNova DNA Polymerase	QuantiNova DNA Polymerase is a modified form of a recombinant 94 kDa DNA polymerase, originally isolated from <i>Thermus aquaticus</i> . QuantiNova DNA Polymerase is provided in an inactive state and has no enzymatic activity at ambient temperature. The enzyme is activated by a 2-minute, 95°C incubation step.
QuantiNova Multiplex PCR Buffer	Contains Tris-HCl , KCl, NH ₄ Cl, MgCl ₂ , and additives enabling fast cycling, including Q-Bond®
dNTP mix	Contains dATP, dCTP, dGTP, and dTTP of ultrapure quality

Other components

Component	Description
QN ROX Reference Dye	Optimized concentration of fluorescent dye for normalization of fluorescent signals on all instruments from Applied Biosystems®
QuantiNova Yellow Template Dilution Buffer	Ultrapure quality, PCR-grade
RNase-Free Water	Ultrapure quality, PCR-grade

Introduction

QuantiNova Multiplex PCR Kits provide highly sensitive and rapid real-time quantification of DNA and cDNA targets in an easy-to-handle multiplex format. Depending on the real-time cyler used, up to 5 targets can be quantified simultaneously in the same well or tube. The kits can be used in real-time PCR of genomic DNA targets, and also in real-time, two-step RT-PCR of RNA targets following reverse transcription with, for example, the QuantiNova Reverse Transcription Kit (see ordering information, page 28). The kits are compatible with dual-labeled probes e.g., TaqMan® probes. High specificity and sensitivity in multiplex real-time PCR are achieved by the use of a novel hot-start enzyme, QuantiNova DNA Polymerase, together with a specialized real-time PCR buffer based on QIAGEN's proprietary multiplex PCR buffer technology. QuantiNova Guard, a novel additive, further improves the stringency of the antibody-mediated hot-start. The kits also feature a built-in control for visual identification of correct template addition. Short cycling steps without loss of PCR sensitivity and efficiency are enabled by Q-Bond, an additive in the multiplex PCR buffer.

The kits have been optimized for use with any real-time cyler. The QN ROX Reference Dye is provided in a separate tube and can be added if using a cyler that requires ROX as a passive reference dye.

Principle and Procedure

4x QuantiNova Multiplex PCR Master Mix

The components of 4x QuantiNova Multiplex PCR Master Mix include QuantiNova DNA Polymerase and QuantiNova Multiplex PCR Buffer. The optimized Master Mix ensures ultrafast multiplex real-time PCR amplification with high specificity and sensitivity, comparable to the corresponding singleplex reactions.

Novel, antibody-mediated, hot-start mechanism

QuantiNova DNA Polymerase is provided in an inactive state and has no enzymatic activity at ambient temperature. This prevents the formation and extension of nonspecifically annealed primers during reaction setup and the first denaturation step, leading to high PCR specificity and accurate quantification. At low temperatures, the QuantiNova DNA Polymerase is kept in an inactive state by the QuantiNova Antibody and a novel additive, QuantiNova Guard, which stabilizes the complex. This improves the stringency of the hot-start.

Within 2 minutes of raising the temperature to 95°C, QuantiNova Antibody and QuantiNova Guard are denatured and QuantiNova DNA Polymerase is activated, enabling PCR amplification (Figure 1). The hot-start enables reactions to be set up rapidly and conveniently at room temperature. Furthermore, the realtime PCR can be stored after setup at up to 30°C for up to 12 hours without impairing the performance of the subsequent reaction

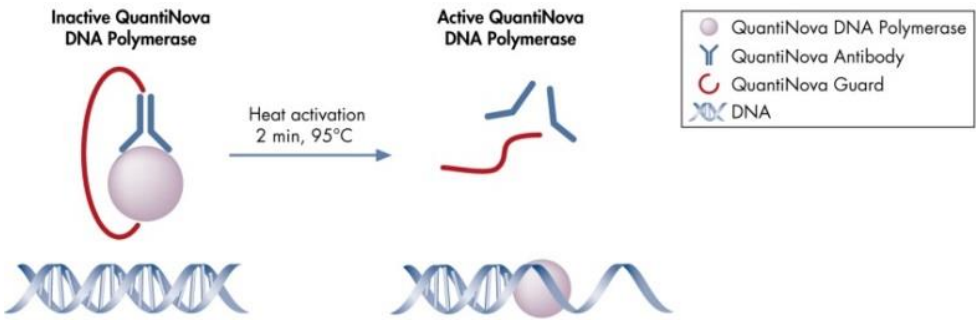


Figure 1. Principle of the novel QuantiNova hot-start mechanism. QuantiNova DNA Polymerase is kept in an inactive state by QuantiNova Antibody and QuantiNova Guard until the initial heat activation step.

Built-in visual control for correct pipetting

The master mix supplied with the QuantiNova Multiplex PCR Kit contains an inert blue dye that does not interfere with the real-time PCR, but increases visibility in the tube or well. QuantiNova Yellow Template Dilution Buffer contains an inert yellow dye. When the template nucleic acid, diluted with the QuantiNova Yellow Template Dilution Buffer, is added to the master mix, the color of the solution changes from blue to green, providing a visual indication of correct pipetting. The use of the QuantiNova Yellow Template Dilution buffer is optional.

QuantiNova Multiplex PCR Buffer

QuantiNova Multiplex PCR Buffer is specifically designed for ultrafast, multiplex, real-time PCR using sequence-specific probes. The buffer additive, Q-Bond, allows short cycling times on any real-time cycler. Q-Bond increases the affinity of the QuantiNova DNA Polymerase 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 multiplex PCR. In addition, the unique composition of the buffer supports the melting behavior of DNA, enabling short denaturation and annealing/extension times.

QuantiNova Multiplex PCR Buffer is also based on the unique QIAGEN PCR buffer system. The buffer contains a balanced combination of KCl and NH₄Cl, 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 MgCl₂ concentration, so optimization by titration of Mg²⁺ is not required.

The buffer also contains Factor MP, which facilitates multiplex PCR. This synthetic factor increases the local concentration of primers and probes at the DNA template and stabilizes specifically bound primers and probes, allowing efficient annealing and extension. The

combination of these various components of QuantiNova Multiplex PCR Buffer prevents multiple amplification reactions from affecting each other.

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 different from fluorescent dyes commonly used for probes.

The use of ROX dye is necessary for instruments from Applied Biosystems. The QuantiNova Multiplex PCR Kit is provided with a separate tube of QN ROX Reference Dye. It can be added to the real-time PCR if using a real-time cycler that uses ROX as a passive reference dye. The QN ROX Reference Dye should be diluted 1:20 in the 1x real-time PCR sample when used on instruments requiring a high ROX concentration and 1:200 for those instruments requiring a low ROX concentration. Refer to Table 1 for details on real-time cyclers that require low or high ROX concentrations. If desired, QN ROX Reference Dye can be added to 4x QuantiNova Multiplex PCR Master Mix for long-term storage (Table 2). For details, see “Adding ROX dye to the master mix”, page 11.

Table 1. Real-time cyclers requiring high/low concentrations of ROX

High ROX concentration (1:20 dilution of QN ROX Reference Dye in 1x reaction)	Low ROX concentration (1:200 dilution of QN ROX Reference Dye in 1x reaction)
ABI PRISM® 7000	Applied Biosystems 7500
Applied Biosystems 7300	Applied Biosystems ViiA™ 7
Applied Biosystems 7900	Applied Biosystems QuantStudio® Systems
Applied Biosystems StepOne	
Applied Biosystems StepOne Plus	

Adding ROX dye to the master mix

If only Applied Biosystems cyclers will be used with the QuantiNova Multiplex PCR Kit, QN ROX Reference Dye Solution can be added to 4x QuantiNova Multiplex PCR Master Mix for long-term storage, if desired. For information on the concentration of ROX required for Applied Biosystems instruments, refer Table 1 page 11. For reaction setups with master mix that already contains a high concentration of added QuantiNova ROX Reference Dye, refer to Appendix D: Reaction Setup Using Master Mix Containing High Concentration of ROX (page 26).

Table 2. Addition of QN ROX Reference Dye to master mix

Volume of 4x QuantiNova Multiplex PCR Master Mix (w/o QN ROX Reference Dye)	Volume of QN ROX Reference Dye for high ROX concentration/low ROX concentration
0.5 ml	100/10 µl
1.3 ml	260/26 µl

cDNA synthesis for real-time, multiplex, two-step RT-PCR

If quantifying cDNA targets with QuantiNova Multiplex PCR Kits, RNA must first be reverse transcribed into cDNA. A portion of the reverse-transcription reaction is then transferred to

another tube where real-time PCR takes place. This entire process is known as real-time, two-step RT-PCR, since reverse transcription and real-time PCR are carried out in separate tubes.

For reverse transcription, we recommend using the QuantiNova Reverse Transcription Kit. The kit provides a fast and convenient procedure, requiring only 20 minutes to synthesize first-strand cDNA and eliminate genomic DNA contamination. An optimized mix of oligo-dT and random primers enables cDNA synthesis from all regions of RNA transcripts, even from 5' regions of very long mRNA transcripts. cDNA yields are high, allowing sensitive detection of even low-abundance transcripts in real-time, two-step RT-PCR. Furthermore it contains the QuantiNova Internal Control (QN IC) RNA, which can be optionally used to monitor successful reverse transcription and subsequent qPCR. The QuantiNova IC RNA is intended to report instrument or chemistry failures, errors in assay setup and the presence of inhibitors. The QN IC RNA can conveniently and precisely be co-detected in multiplex procedures as a 200 bp amplicon, using the VIC®/HEX™ dye channel of your real-time PCR instrument. For probe-based detection, use the QuantiNova IC Probe Assay (cat.no. 205813). For ordering information, see page 28.

For two-step RT-PCR, the volume of the cDNA added (from the undiluted RT reaction) should not exceed 25% of the final PCR volume.

Sequence-specific probes

The QuantiNova Multiplex PCR Kit handbook contains protocols optimized for use with TaqMan probes. Other type of probes can be used, however attention should be paid e.g. regarding data acquisition points or combination of suitable dyes and quenchers. Please follow the recommendations of your probe or instrument provider.

For more details on commonly used dyes, sequence-specific probes, and their design and handling, see Appendix A and B, page 22.

Protocol: Multiplex Real-Time PCR and Two-Step RT-PCR Using Dual-Labeled Probes

This protocol is for use with the QuantiNova Multiplex PCR Kit and dual-labeled probes (e.g., TaqMan probes) on any cycler.

Important points before starting

- This protocol is optimized for quantification of gDNA or cDNA targets in a multiplex format (up to 5-plex), using TaqMan® probes with any real-time cycler and condition for fluorescence normalization. ROX™ dye is required for various cyclers (see page 11).
- Check the functionality of each set of primers and probe in individual assays before combining the different sets in a multiplex assay.
- Perform appropriate controls for evaluating the performance of your multiplex assays (e.g., amplifying each target individually and comparing the results with those for the multiplex assay).
- Before performing multiplex analyses, choose suitable combinations of reporter dyes and quenchers that are compatible with multiplex analysis using the detection optics of your real-time cycler. We recommend using dual-labeled probes with non-fluorescent quenchers.
- Some realtime cyclers require you to perform a calibration procedure for each reporter dye. Check whether the reporter dyes you selected for your multiplex assay are part of the standard set of dyes already calibrated on your cycler. If they are not, perform a calibration procedure for each dye before using them for the first time (for details, refer to the manufacturer's instructions for your realtime cycler).
- For information on suitable combinations of dyes for multiplex PCR using the Rotor-Gene Q see Appendix A, page 22.

- Optimal analysis settings (i.e. baseline settings and threshold values) for each reporter dye are a prerequisite for accurate quantification data. For details, check the literature from the manufacturer of your real-time cycler.
- For the highest efficiency in realtime PCR using TaqMan probes, amplicons should ideally be 60–150 bp in length.
- Check the concentration and integrity of primers and probes before starting. For details, see Appendix B, page 23.
- For ease of use, we recommend preparing a 20x primer–probe mix containing target-specific primers and probes for each target. A 20x primer–probe mix consists of 8 μM forward primer, 8 μM reverse primer and 5 μM probe in TE buffer. Alternatively, it may be preferable to prepare the reaction mix with separate primer and probe solutions.
- Always start with the cycling conditions and primer concentrations specified in this protocol.
- The PCR must start with an initial incubation step of 2 min at 95°C to activate the QuantiNova DNA Polymerase.
- For 96-well block cyclers, we recommend a final reaction volume of 20 μl . For 384-well block cyclers, we recommend a final reaction volume of 10 μl .
- The reference dye in QuantiNova Yellow Template Dilution Buffer allows tracking of pipetted samples in the qPCR. When template is added to the blue QuantiNova Multiplex PCR Master Mix, the color changes from blue to green. The use of this buffer is optional. It is provided as a 100x concentrate and should be diluted (using water) to obtain a 1x final concentration within the sample*. To generate a template dilution series (e.g., for absolute quantification or determination of PCR efficiency), dilute the 100x concentrate (using template and water) to obtain a final concentration of 1x QuantiNova Yellow Template Dilution Buffer. The buffer does not affect sample stability and qPCR results.

(* Example: add 0.5 μl Yellow Template Dilution Buffer to 50 μl sample, which can be used as template in various PCR runs, regardless of the volume added to each reaction set up. If pipetting volumes are too small to handle, Yellow Template Dilution Buffer can

be pre-diluted using sterile water. In this example 5µl of 1:10 pre-diluted Yellow Template Dilution Buffer could be added.)

Procedure

1. Thaw 4x QuantiNova Multiplex PCR Master Mix, QuantiNova Yellow Template Dilution Buffer, template gDNA or cDNA, primers, probes, QN ROX Reference Dye (if required), and RNase-free water. Mix the individual solutions.

The 4x QuantiNova Multiplex PCR Master Mix appears as a turbid emulsion, which is normal, and should be mixed on a vortexer.

2. Prepare a reaction mix according to Table 3.

Due to the hotstart, it is not necessary to keep samples on ice during reaction setup or while programming the realtime cyclers.

Table 3. Reaction setup

Component	Volume/reaction		Final concentration
	96-well block, Rotor-Gene	384-well block	
4x QuantiNova Multiplex PCR Master Mix	5 µl	2,5 µl	1 x
QN ROX Reference Dye (AB instruments only)	1 µl/0.1 µl*	0.5 µl/0.05 µl*	1 x
20x primer-probe mix† (for each of up to 5 targets)	1 µl	0,5 µl	0.4 µM forward Primer 0.4 µM reverse Primer 0,25 µM TaqMan Probe
Template DNA or cDNA (added at step 4)	Variable	Variable	≤800 ng/reaction
RNase-Free water	Variable	1 Variable	
Total reaction volume	20 µl	10 µl	

* Results in a 1:20 dilution for high ROX instruments (i.e., ABI PRISM 7000, Applied Biosystems 7300, 7900, and StepOne™ RealTime PCR Systems), and a 1:200 dilution for low-ROX dye cyclers (i.e., Applied Biosystems 7500 and

, ViiA7™ and QuantStudio Real-Time PCR Systems) in the final 1x reaction.

† A 20x primer–probe mix consists of 8 µM forward primer, 8 µM reverse primer, and 5 µM probe in TE buffer for each target. Primers and probes can either be pre-mixed and added simultaneously, or primer–probe mixes for each target can be added separately. If concentration of primer–probe mix(es) differ, the respective added volume needs to be adjusted, to achieve a final concentration of 0.4 µM for each primer and 0.25 µM for each probe.

3. Mix the reaction mix thoroughly and dispense appropriate volumes into PCR tubes or wells of a PCR plate.
4. Add template gDNA or cDNA (≤800 ng/reaction) to the individual PCR tubes or wells containing the reaction mix.

Note: For two-step RT-PCR, the volume of the cDNA added (from the undiluted RT reaction) should not exceed 25% of the final PCR volume.

5. Program your realtime cycler according to the program outlined in Table 4.

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

Table 4. Cycling conditions

Step	Time	Temperature	Ramp rate	Additional comments
PCR initial activation step	2 min	95°C	Maximal/ fast mode	QuantiNova DNA Polymerase is heat-activated
2-step cycling				
Denaturation	5 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 DNA

* If your cycler does not accept this short time for data acquisition, choose the shortest acceptable time (e.g., 31 s annealing/extension for the ABI PRISM 7000 or Applied Biosystems 7300).

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

7. Perform data analysis.

Note: Before performing data analysis, select the analysis settings for each probe (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: <https://www.qiagen.com/support/>. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information or protocols in this handbook or sample and assay technologies (for contact information visit www.qiagen.com).

Comments and suggestions

No signal or one or more signals detected late in PCR

- | | |
|--|---|
| a) Incorrect 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 QuantiNova DNA Polymerase (95°C for 2 min), and the specified times for denaturation and annealing/extension. |
| b) QuantiNova DNA Polymerase not activated | Ensure that the cycling program includes the QuantiNova DNA Polymerase activation step (2 min at 95°C) as described in the protocols. |
| c) Pipetting error or missing reagent | Check the concentrations and storage conditions of the reagents, including primers, probes, and template nucleic acid. See Appendix B: Assay Design and Handling Primers and Probes, page 23, for details on evaluating the concentration of primers and probes. Repeat the PCR. Use the provided QuantiNova Yellow Template Dilution Buffer to prevent errors during reaction setup. |
| d) Wrong or no detection step | Ensure that fluorescence detection takes place during the combined annealing/extension step when using TaqMan probes. |
| e) Primer or probe concentration not optimal | Use optimal primer concentrations. For TaqMan assays, use each primer at 0.4 µM. In most cases, a probe concentration of 0.25 µM provides satisfactory results. Check the concentrations of primers and probes by spectrophotometry (see Appendix B, page 23). |
| 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 PCR using the new dilutions. |

Comments and suggestions

g) 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.
h) Insufficient number of cycles	Increase the number of cycles.
i) Reaction volume too high	For 96-well block 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.
j) PCR product too long	For optimal results, PCR products should be between 60 and 150 bp.
k) Primer design not optimal	Check for PCR products by gel electrophoresis. If no specific PCR products are detected review the primer design guidelines (see Appendix B, page 23).
l) 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 23).
m) 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.
n) No detection activated	Check that fluorescence detection was activated in the cycling program.
o) 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.
p) Primers degraded	Check for possible degradation of primers on a denaturing polyacrylamide gel.
q) RT-PCR only: Volumes of RT reaction added were too high	High volumes of RT reaction added to the PCR may reduce amplification efficiency. Generally, the volume of undiluted reverse-transcription reaction added should not exceed 25% of the final PCR volume. If you need to use a large volume of reverse-transcription reaction as template, determine the maximum acceptable volume for the assay being carried out.
r) Fluorescence crosstalk	Check that the reporter dyes used in your assay are suitable for multiplex analysis on your instrument. Run appropriate controls to estimate potential crosstalk effects.

Increased fluorescence or Cq 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.
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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

- | | | |
|----|--|--|
| a) | 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.

Perform reverse transcription with the QuantiNova Reverse Transcription Kit, which provides cDNA synthesis with integrated genomic DNA removal. Alternatively, treat the RNA sample with DNase to digest the contaminating genomic DNA. |
|----|--|--|

Varying fluorescence intensity

- | | | |
|----|---------------------------------------|--|
| a) | Contamination of realtime cyclers | Decontaminate the realtime cyclers according to the manufacturer’s instructions. |
| b) | Realtime cyclers no longer calibrated | Recalibrate the realtime cyclers according to the manufacturer’s instructions. |

All cyclers 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 realtime cycler allows you to do so) or reduce the amount of template. |
|----|--|---|

Applied Biosystems instruments only:

- | | | |
|----|--|---|
| d) | ΔR_n values unexpectedly too high or too low | The concentration of the QN ROX Reference Dye is wrong. To choose the right ROX concentration for your cycler, refer to Table 1, page 11. |
|----|--|---|

Differences in C_q values or in PCR efficiencies between a multiplex assay and the corresponding singleplex 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 QuantiNova DNA Polymerase (95°C for 2 min), 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. |

Comments and suggestions

- | | |
|---|---|
| c) Imprecise spectral separation of reporter dyes | Since multiplex assays use 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 _q values of up to 5% between the multiplex assay and the corresponding singleplex assays; this can usually be avoided by using optimal threshold settings. If using the ABI PRISM 7700, perform analysis with and without spectral compensation. |
|---|---|

No linearity in ratio of C_q value/crossing point to log of the template amount

- | | |
|-----------------------------|---|
| a) Template amount too high | When signals are coming up at very early C _q 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. |

Appendix A: Suitable combinations of reporter dyes on the Rotor-Gene Q instrument

Multiplex, realtime PCR requires the simultaneous detection of up to five different fluorescent reporter dyes. For accurate detection, the fluorescence spectra of the dyes should be well separated or exhibit only minimal overlap (Table 5).

For up to 4-plex analysis we recommend using the core channels: Green, Yellow, Orange and Red. If a higher multiplex degree (5-plex, 6-plex) is performed, extend the spectral range to blue channel and/or crimson channel. These channels require less frequently used fluorophors which will not be detected on all commonly used real-time PCR instruments.

Note: If using other realtime PCR instruments, please refer to the user manual or other technical documentation for your instrument to find out which reporter dyes can be used in multiplex analyses.

Table 5. Dyes commonly used in multiplex real-time PCR on RotorGene-Q

Channel	Excitation (nm)	Detection (nm)*	Examples of fluorophores detected
Blue	365±20	460±20	Marina Blue®, Edans Bothell Blue, Alexa Fluor® 350, AMCA-X, ATTO 390
Green	470±10	510±5	FAM®, Alexa Fluor 488
Yellow	530±5	557±5	JOE™, VIC®, HEX™, TET™, CAL Fluor® Gold 540, Yakima Yellow®
Orange	585±5	610±5	ROX, CAL Fluor Red 610, Cy®3.5, Texas Red®, Alexa Fluor 568
Red	625±10	660±10	Cy5, Quasar® 670, LightCycler® Red640, Alexa Fluor 633
Crimson	680±5	712 high pass	Quasar 705, LightCycler Red705, Alexa Fluor 680

* Emission spectra may vary depending on the buffer conditions.

Appendix B: Assay Design and Handling Primers and Probes

Important factors for success in quantitative, multiplex, real-time 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 given below. It is particularly important to minimize nonspecific annealing of primers and probes. This can be achieved through careful assay design.

T_m of primers for TaqMan assays

- Use specialized design software (e.g., Primer Express® Software, Primer-BLAST®) 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).
- Primers and probes should be supplied from an established oligonucleotide manufacturer. Primers should be of standard quality, and probes should be HPLC purified. Lyophilized primers and probes should be dissolved in TE buffer to provide a stock solution of 100 µM; concentration should be checked by spectrophotometry (see

Table 6. Primer and probe stock solutions and primer-probe mixes should be stored in aliquots at -20°C . Probe stock solutions should be protected from exposure to light.

Primer sequence

- Length: 18–30 nucleotides.
- GC content: 30–70%.
- 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.
- Check that primers and probes are not complementary to each other.
- Try to avoid highly repetitive sequences.
- Avoid complementarity of 2 or 3 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 and/or Cs at the 3' end. Avoid complementary sequences within a primer sequence and between the primer pair.

Product size

Ensure that the length of PCR products is 60–150 bp. Some longer amplicons may amplify efficiently in multiplex PCR, with minimal optimization.

Handling and storing primers and probes

Guidelines for handling and storing primers and probes are given in Table 6 below. For optimal results, we recommend only combining primers of comparable quality.

Table 6. Guidelines for handling and storing primers and probes

Storage buffer	<p>Lyophilized primers 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 Cy3, Cy3.5, Cy5, and Cy5.5 should be stored in TE buffer, pH 7.0, since they tend to degrade at higher pH.</p>
Storage	<p>Primers should be stored in sterile, nuclease-free TE buffer 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> <p>For easy and reproducible handling of primer–probe sets used in multiplex assays, we recommend preparing 20x primer–probe mixes, each containing 2 primers and 1 probe for a particular target at the suggested concentrations (see protocols).</p>
Dissolving primers and probes	<p>Before opening a tube containing lyophilized primer or probe, centrifuge 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 sterile, nuclease-free TE buffer, 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 buffer and some may not dissolve easily in water.</p>
Concentration	<p>Spectrophotometric conversion for primers and probes:</p> <p>1 A_{260} unit = 20–30 $\mu\text{g}/\text{ml}$</p> <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> $\epsilon_{260} = 0.89 \times [(A \times 15,480) + (C \times 7340) + (G \times 11,760) + (T \times 8850)]$ <p>Example</p> <p>Concentration of diluted primer: 1 $\mu\text{M} = 1 \times 10^{-6}$ 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 quality	The quality of 18–30mers can be checked on a 15% denaturing polyacrylamide gel or by capillary gel electrophoresis; a single band should be seen. Please contact QIAGEN Technical Services or your local distributor for a protocol.
Probe quality	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 units DNase at 37°C for 1 hour. A significant difference in fluorescence following DNase treatment should be detectable.

Appendix C: Generating Color Compensation Files on LightCycler Systems

The LightCycler 2.0 system has detection channels that allow detection of multiple reporter dyes in the same capillary. However, even when reporter dyes with well separated emission spectra are used, each reporter dye will be detected by more than one detection channel. Therefore, multiplex assay results will be inaccurate unless a correction is made. This is achieved by using a color compensation file, which contains information that corrects the crosstalk between the detection channels.

The LightCycler 480 system can also use a color compensation file to correct the crosstalk between detection channels. However, if the multiplex assay uses reporter dyes with widely separated emission spectra (e.g., FAM and Cy5), it may not be necessary to use a color compensation file.

Color compensation files can be generated before or after carrying out a multiplex assay and can be stored for later use. For detailed information, please refer to the manual of the corresponding instrument.

Appendix D: Reaction Setup Using Master Mix Containing High Concentration of ROX

Note: This appendix is only relevant for a reaction setup using a master mix containing a high concentration of ROX that has been added according to Table 2. When using a master mix containing low concentration of ROX, the volume of ROX added is negligible and the standard reaction setup should be used as described in Table 3.

Table 7. Reaction setup

Component	Volume/reaction		Final concentration
	96-well block, Rotor-Gene	384-well block	
4x QuantiNova Multiplex PCR Master Mix (containing high ROX)	6 µl	3 µl	1 x
20x primer-probe mix† (for each of up to 5 targets)	1 µl	0,5 µl	0.4 µM forward Primer 0.4 µM reverse Primer 0,25 µM TaqMan Probe
Template DNA or cDNA (added at step 4)	Variable	Variable	≤800 ng/ reaction
RNase-Free water	Variable	Variable	
Total reaction volume	20 µl	10 µl	

† A 20x primer-probe mix consists of 8 µM forward primer, 8 µM reverse primer, and 5 µM probe in TE buffer for each target. Primers and probes can either be premixed and added simultaneously, or primerprobe mixes for each target can be added separately. If concentration of primer-probe mix(es) differ, the respective added volume needs to be adjusted, to achieve a final concentration of 0.4 µM for each primer and 0.25 µM for each probe.

Ordering Information

Product	Contents	Cat. no.
QuantiNova Multiplex PCR Kit (100)	For 100 x 20 µl reactions: 0,5 ml 4x QuantiNova Multiplex PCR Master Mix, 500 µl QuantiNova Yellow Template Dilution Buffer, 250 µl QN ROX Reference Dye, 1.9 ml RNase-Free Water	208452
QuantiNova Multiplex PCR Kit (500)	For 500 x 20 µl reactions: 2 x 1.3 ml 4x QuantiNova Multiplex PCR Master Mix, 500 µl QuantiNova Yellow Template Dilution Buffer, 1 ml QN ROX Reference Dye, 3 x 1.9 ml RNase-Free Water	208454
QuantiNova Multiplex PCR Kit (2500)	For 2500 x 20 µl reactions: 10 x 1.3 ml 4x QuantiNova Multiplex PCR Master Mix, 2 x 500 µl QuantiNova Yellow Template Dilution Buffer, 5 x 1 ml QN ROX Reference Dye, 20 x 1.9 ml RNase-Free Water	208456
QuantiNova IC Probe Assay (200)	For 200 x 20 µl reactions: 400 µl primer/probe mix (10x), detecting IC RNA	205813
QuantiNova Probe PCR Kit (100)*	For 100 x 20 µl reactions: 1 ml 2x QuantiNova Probe PCR Master Mix , 250 µl QN ROX Reference Dye, 500 µl QuantiNova Yellow Template Dilution Buffer, 1.9 ml Water	208252
QuantiNova SYBR Green PCR Kit (100)*	For 100 x 20 µl reactions: 1 ml 2x QuantiNova SYBR Green PCR Master Mix , 250 µl QN ROX Reference Dye, 500 µl QuantiNova Yellow Template Dilution Buffer, 1.9 ml Water	208052

Product	Contents	Cat. no.
QuantiNova Reverse Transcription Kit —cDNA synthesis with integrated gDNA removal for two-step qRT PCR		
QuantiNova Reverse Transcription Kit (10)	For 10 x 20 µl reactions: QuantiNova gDNA Removal Mix, QuantiNova Reverse Transcription Enzyme, QuantiNova Reverse Transcription Mix, QuantiNova Internal Control RNA, and RNase-Free Water	205410
QuantiNova Reverse Transcription Kit (50)	For 50 x 20 µl reactions: QuantiNova gDNA Removal Mix, QuantiNova Reverse Transcription Enzyme, QuantiNova Reverse Transcription Mix, QuantiNova Internal Control RNA, and RNase-Free Water	205411
QuantiNova Reverse Transcription Kit (200)	For 200 x 20 µl reactions: QuantiNova gDNA Removal Mix, QuantiNova Reverse Transcription Enzyme, QuantiNova Reverse Transcription Mix, QuantiNova Internal Control RNA, and RNase-Free Water	205413
RNeasy Mini Kit – for purification of total RNA from animal cells, animal tissues and yeast, and for RNA cleanup		
RNeasy Mini Kit (50)*	For 50 RNA minipreps: 50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free Reagents and Buffers	74104
RNeasy Plus Universal Mini Kit (50)	For 50 RNA minipreps: RNeasy Mini Spin Columns, gDNA Eliminator Solution, Collection Tubes, RNase-Free Water and Buffers	73404

* Other kit sizes and formats available; please inquire.

Product	Contents	Cat. no.
AllPrep® DNA/RNA Mini Kit — for simultaneous purification of genomic DNA and total RNA from the same cell or tissue sample		
AllPrep DNA/RNA Mini Kit (50)	50 AllPrep DNA Mini Spin Columns, 50 RNeasy Mini Spin Columns, Collection Tubes, RNase-Free Reagents and Buffers	80204
Instruments		
Rotor-Gene® Q 6plex Platform	Real-time PCR instrument with 6 channels (blue, green, yellow, orange, red, crimson), including laptop computer, software, accessories.	9001590
QIAgility® System HEPA/UV (incl. PC)	Robotic workstation for automated PCR setup (with UV light and HEPA filter), notebook computer and QIAgility Software: includes installation and training, 1-year warranty on parts and labor	9001532

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