June 2015

QuantiNova™ Probe RT-PCR Handbook

For highly sensitive, ultrafast, quantitative real-time RT-PCR using sequence-specific probes

Sample to Insight

QIAGEN

Contents

Kit Contents

QuantiNova Probe RT-PCR Kit	**(100)**	**(500)**	**(2500)**
Catalog No.	**208352**	**208354**	**208356**
Number of reactions (20 µl/10 µl)	**100/200**	**500/1000**	**2500/5000**
2x QuantiNova Probe RT-PCR Master Mix, containing QuantiNova DNA Polymerase composed of: Taq DNA Polymerase, QuantiNova Antibody, QuantiNova Guard, QuantiNova Probe RT-PCR Buffer, and dNTP mix (dATP, dCTP, dGTP, dTTP)	1 ml	3 x 1.7 ml	15 x 1.7 ml
100x QuantiNova RT Mix, containing: HotStaRT-Script Reverse Transcriptase, RNase Inhibitor, DNase	20 µl	100 µl	5 x 100 µl
QuantiNova Yellow Template Dilution Buffer	500 µl	500 µl	5 x 500 µl
QuantiNova Internal Control RNA	20 µl	100 µl	5 x 100 µl
QuantiNova ROX™ Reference Dye	250 µl	1 ml	5 x 1 ml
RNase-Free Water	1.9 ml	2 x 1.9 ml	10 x 1.9 ml
Quick-Start Protocol QuantiNova Probe RT-PCR	1	1	1
Quick-Start Protocol QuantiNova IC and Assay	1	1	1

Storage

QuantiNova Probe RT-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 the quality control label inside the kit box or on the kit envelope). QuantiNova Probe RT-PCR Master Mix, QuantiNova Yellow Template Dilution Buffer and QuantiNova ROX Reference Dye can also be stored protected from light at 2–8°C for up to 12 months, depending on the expiry date.

If desired, QuantiNova ROX Reference Dye can be added to 2x QuantiNova Probe RT-PCR Master Mix for long-term storage. For details, see "Adding ROX dye to the RT-PCR master mix", page 13.

Intended Use

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

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

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 Probe RT-PCR Kit is tested against predetermined specifications to ensure consistent product quality.

Product Information

The QuantiNova Probe RT-PCR Kit contains:

2x QuantiNova Probe RT-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 *Thermus aquaticus*. QuantiNova DNA Polymerase is provided in an inactive state and has no enzymatic activity at ambient temperature. The enzyme is activated by a 5 minute, 95°C incubation step.
QuantiNova Probe RT-PCR Buffer	Contains Tris•Cl, KCl, NH_4Cl, $MgCl_2$, and additives enabling fast cycling, including Q-Bond®.
dNTP mix	Contains dATP, dCTP, dGTP and dTTP of ultrapure quality.

100x QuantiNova Probe RT Mix

Component	Description
HotStarRT-Script Reverse Transcriptase	HotStarRT-Script Reverse Transcriptase is a modified form of a recombinant 77 kDa reverse transcriptase. It is provided in an inactive state and has minimal enzymatic activity at ambient temperature. The enzyme is activated during the reverse-transcription step at 45°C.
RNase Inhibitor	The RNase inhibitor is a recombinant mammalian protein that inhibits eukaryotic RNases, such as RNase A and B.
DNase	DNase, free of RNase.

Other components

Component	Description
QuantiNova Internal Control RNA	Synthetic transcript for monitoring successful reverse transcription.
QuantiNova ROX Reference Dye	Optimized concentration of fluorescent dye for normalization of fluorescent signals on all instruments requiring ROX as a reference dye.
QuantiNova Yellow Template Dilution Buffer	Ultrapure quality, PCR-grade.
RNase-Free Water	Ultrapure quality, PCR-grade.

Introduction

The QuantiNova Probe RT-PCR Kit provides highly sensitive and rapid real-time quantification of RNA targets in an easy-to-handle format. The kit can be used in real-time RT-PCR using various RNA targets such as total RNA from eukaryotes and prokaryotes, as well as poly(A)-RNA and in vitro-transcribed RNA. The kit is compatible with dual-labeled probes e.g., TaqMan® probes. High specificity and sensitivity in real-time RT-PCR are achieved by a novel two-phase hot-start procedure.

The HotStaRT-Script Reverse Transcriptase is associated with a RT-blocker, rendering the enzyme almost inactive at ambient temperature. This allows room-temperature setup of the RT-PCR reaction without the risk of primer–dimer formation by the reverse transcriptase. When starting the RT-PCR protocol with the RT step at 45°C, the inhibitor is released from the reverse transcriptase and cDNA synthesis is initiated. Simultaneously, any contaminating genomic DNA is significantly reduced (approximately 90%) due to the presence of DNase in the RT mix. The second phase of the hot-start is achieved using a novel hot-start enzyme, QuantiNova DNA Polymerase, and a novel additive, QuantiNova Guard. These unique components further improve the stringency of the antibody-mediated hot-start.

The kit also features a built-in control for visual identification of correct template addition and an additive in the RT-PCR buffer, Q-Bond, which enables short cycling steps without loss of PCR sensitivity and efficiency.

The QuantiNova Internal Control (QN IC) RNA can be optionally used to monitor successful reverse transcription. The QuantiNova IC RNA is intended to report instrument or chemistry failures, errors in assay setup and the presence of inhibitors. Inhibitors such as phenol, ethanol, sodium dodecyl sulfate (SDS) or ethylene diaminetetraacetic acid (EDTA) may remain from the lysis and purification steps during the RNA isolation procedure.

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

Principle and Procedure

One-Step RT-PCR

Use of 2x QuantiNova RT-PCR Master Mix together with QuantiNova RT Mix allows both reverse transcription and PCR to take place in a single tube. All reagents required for both reactions are added at the beginning, so there is no need to open the tube once the reverse-transcription reaction has been started. Also, there is no need to set up the reaction on ice as the whole reaction can be left for up to 2 hours at room temperature without any loss of performance.

QuantiNova Probe RT Mix

The QuantiNova Probe RT Mix contains HotStarRT-Script Reverse Transcriptase for heat-mediated activation of the reverse-transcription step, an RNase inhibitor, and a DNase. The HotStaRT-Script Reverse Transcriptase is associated with a RT-blocker, rendering the enzyme almost inactive at ambient temperature. This allows RT-PCR reaction setup at room temperature without the risk of primer–dimer formation by the reverse transcriptase. Upon starting the RT-PCR protocol with the RT step at 45°C, the inhibitor is released from the reverse transcriptase and cDNA synthesis is initiated (Figure 1). Simultaneously, any contaminating genomic DNA is significantly reduced (approximately 90%) due to the presence of DNase in the RT mix. This minimizes the risk of potential misquantification caused by genomic DNA, particularly if exon-spanning primers cannot be used (e.g., single exon genes).

2x QuantiNova Probe RT-PCR Master Mix

The components of the 2x QuantiNova Probe RT-PCR Master Mix include QuantiNova DNA Polymerase and QuantiNova Probe RT-PCR Buffer. The optimized master mix ensures fast real-time RT-PCR amplification with high specificity and sensitivity.

Novel, antibody-mediated hot-start mechanism

QuantiNova DNA Polymerase is provided in an inactive state and has no enzymatic activity at ambient temperature. At low temperatures, the QuantiNova DNA Polymerase is kept in an inactive state by the QuantiNova Antibody and Guard, which stabilizes the complex and improves the stringency of the hot-start. The antibody-mediated hot-start mechanism prevents the formation and extension of non-specific RT-PCR products and primer–dimers during reaction setup, reverse transcription and the first denaturation step. Therefore, this mechanism allows higher PCR specificity and more accurate quantification.

After reverse transcription and within 5 minutes of raising the temperature to 95°C, the QuantiNova Antibody and Guard are denatured and the QuantiNova DNA Polymerase is activated, enabling PCR amplification (Figure 1). The hot-start enables rapid and convenient room-temperature setup and allows both steps to be performed sequentially in a single tube. Furthermore, after setup the RT-PCR can be stored for up to 2 hours at up to 30°C without impairing the performance of the subsequent reaction.

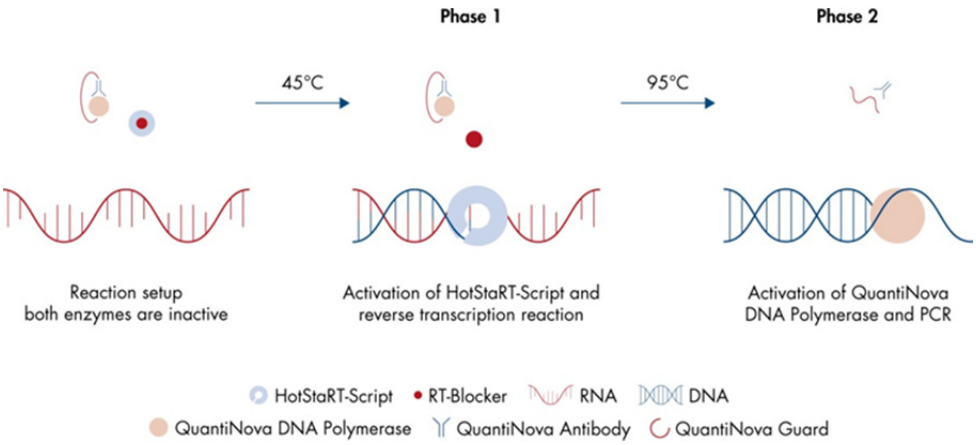

Figure 1. Principle of the novel QuantiNova two-phase hot-start mechanism. At ambient temperature the HotStaRT-Script is inhibited by the RT-Blocker and the QuantiNova DNA Polymerase is kept inactive by QuantiNova Antibody and QuantiNova Guard. At 45°C the RT is activated while the QuantiNova DNA polymerase remains inactive. At 95°C the RT enzyme is denatured and the DNA polymerase is activated.

Built-in visual control for correct pipetting

The master mix supplied with the QuantiNova Probe RT-PCR Kit contains an inert blue dye that increases visibility in the tube or well but does not interfere with the RT-PCR. 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 Probe RT-PCR Buffer

The QuantiNova Probe RT-PCR Buffer is specifically designed to facilitate both efficient reverse transcription and fast 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. In addition, the unique composition of the buffer supports the melting behavior of DNA, enabling short denaturation and annealing/extension times.

QuantiNova Probe RT-PCR Buffer is also based on the unique QIAGEN PCR buffer system. The buffer contains a balanced combination of KCl and NH_4Cl, which promotes a high ratio of specific to non-specific primer binding during the annealing step of each PCR cycle. This creates stringent primer annealing conditions, leading to increased PCR specificity. When using this buffer, primer annealing is only marginally influenced by the $MgCl_2$ concentration, and therefore optimization by titration of Mg^{2+} is not required.

The composition of the novel RT stabilizing buffer allows room-temperature RT-PCR reaction setup without the need for cooling. The reaction can be stored for up to 2 hours at up to 30°C without impairing the performance of the subsequent reaction. Although the RNase inhibitor included in the RT Mix effectively reduces the risk of RNA degradation, template RNA of high quality and purity should be used, and any contamination should be prevented to ensure reliable qRT-PCR results.

QuantiNova Internal Control RNA

The QN IC RNA is a synthetic RNA that can optionally be used to monitor successful reverse transcription. The QN IC RNA is intended to report instrument or chemistry failures, errors in assay setup and the presence of inhibitors. Inhibitors such as phenol, ethanol, sodium dodecyl sulfate (SDS) or ethylene diaminetetraacetic acid (EDTA) may remain from the lysis and purification steps during the RNA isolation procedure.

The primer and probe sequences for the detection of the QN IC RNA have been bioinformatically validated for non-homology against hundreds of eukaryotic and prokaryotic organisms. Additionally, they have been experimentally tested against a multitude of human, mouse and rat RNA samples from multiple tissues and cell lines.

The QN IC RNA is detected as a 200 bp amplicon. For probe-based detection, use the QuantiNova IC Probe Assay (cat.no. 205813). The QN IC RNA can be detected using the VIC®/HEX™ dye channel of your real-time PCR instrument and the QuantiNova Probe PCR and RT-PCR Kits.

The QN IC RNA can be used optionally, and added to the experimental RNA sample. Additionally, a no-template RNA control containing only QN IC RNA, should be set up. C_q shifts between the template RNA+QN IC RNA samples compared to the QN IC RNA only samples, and between the different template RNA containing samples, indicate inhibition in the RT-PCR.

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 Probe RT-PCR Kit is provided with a separate tube of QuantiNova 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. ROX dye should be diluted 1:20 for a 1x reaction when using an instrument requiring a high ROX dye concentration. For instruments requiring a low ROX dye concentration, dilute the dye 1:200 for a 1x reaction. Refer to Table 1 for details on real-time cyclers that require low or high ROX concentrations. If desired, QuantiNova ROX Reference Dye can be diluted with 2x QuantiNova Probe RT-PCR Master Mix for long-term storage (Table 2). For details, see “Adding ROX dye to the RT-PCR master mix”, page 13.

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 ViiA7™
Applied Biosystems 7900	Applied Biosystems QuantStudio® Systems
Applied Biosystems StepOne™	
Applied Biosystems StepOne Plus	

Adding ROX dye to the RT-PCR master mix

If only using cyclers from Applied Biosystems with the QuantiNova Probe RT-PCR Kit, QuantiNova ROX Reference Dye can be added to 2x QuantiNova Probe RT-PCR Master Mix for long-term storage, if desired. For information on the concentration of ROX required for Applied Biosystems instruments, refer to Table 1. For reaction setups with master mix that already contains a high concentration of added QuantiNova ROX Reference Dye, refer to Appendix A, page 25.

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

Volume of 2x QuantiNova Probe RT-PCR Master Mix (without QN ROX Reference Dye)	Volume of QN ROX Reference Dye for high ROX concentration/low ROX concentration
1 ml	100/10 µl
1.7 ml	170/17 µl

Protocol: Singleplex and Duplex Real-Time RT-PCR Using Dual-Labeled Probes

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

Important points before starting

- This protocol is optimized for quantification of RNA targets using TaqMan probes in a singleplex or duplex reaction with any real-time cycler and conditions for fluorescence normalization. ROX dye is required for various cyclers at the following concentrations:
  No requirement for ROX dye: Rotor-Gene®, Bio-Rad® CFX, Roche® LightCycler® 480 and Agilent® Technologies Mx instruments.

  Low concentration of ROX dye: Applied Biosystems 7500, ViiA7 and QuantStudio Real-Time PCR Systems.

  High concentration of ROX dye: ABI PRISM 7000, Applied Biosystems 7300, 7900 and StepOne Real-Time PCR Systems.
- QuantiNova ROX Reference Dye is provided as a separate tube of passive reference dye for normalization of fluorescent signals on all real-time cyclers from Applied Biosystems. ROX dye should be diluted 1:20 for a 1x reaction when using an instrument requiring a high ROX dye concentration. For instruments requiring a low ROX dye concentration, dilute the dye 1:200 for a 1x reaction.
- The QuantiNova Probe RT Mix contains HotStarRT-Script Reverse Transcriptase for heat-mediated activation of the reverse-transcription step, a DNase for removing more than 90% of residual gDNA in the RNA preparation and an RNase inhibitor.

  Note: Although the included RNase inhibitor effectively reduces the risk of RNA degradation, template RNA of high quality and purity should be used, and any contamination should be prevented to ensure reliable qRT-PCR results.

- The dye in QuantiNova Yellow Template Dilution Buffer allows tracking of pipetted samples in the qRT-PCR. When template is added to the blue QuantiNova Probe RT-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 or buffer) 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 or buffer) to obtain a final concentration of 1x QuantiNova Yellow Template Dilution Buffer. The buffer does not affect sample stability or qRT-PCR.
- For the highest efficiency in real-time RT-PCR using TaqMan probes, amplicons should ideally be 60–150 bp in length.
- Always start with the cycling conditions and primer concentrations specified in this protocol.
- The PCR section of the RT-PCR protocol must start with an initial incubation step of 5 min at 95°C to activate the QuantiNova DNA Polymerase.
- 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 16 µM forward primer, 16 µM reverse primer and 4 µM probe in TE buffer. Alternatively, it may be preferable to prepare the reaction mix with separate primer and probe solutions.
- The QuantiNova Internal Control RNA (QN IC RNA) is an internal amplification control used to test successful reverse transcription/amplification. It is intended to report instrument or chemistry failures, errors in assay setup and the presence of inhibitors. It is detected as a 200 bp internal control (IC) in the yellow channel on the Rotor-Gene Q or in the VIC/HEX dye channel on other real-time PCR instruments, using the QuantiNova IC Probe Assay. The QuantiNova IC Probe Assay needs to be ordered separately (cat. no. 20581). Before use, add 180 µl (or 900 µl) of RNase-free water to 20 µl (or 100 µl) of QN IC RNA provided in the kit and mix thoroughly by vortexing.
- The QN IC RNA (optional) is added to the experimental RNA sample. An additional no-template RNA control sample, which only contains the QN IC RNA, should also be set up. C_q shifts >2 between the template RNA+QN IC RNA compared to the QN IC RNA

only samples, and between the different template RNA containing samples, indicate inhibition of the RT-PCR.

- 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.
- Always readjust the threshold value for analysis of every run.

Procedure

1. Thaw 2x QuantiNova Probe RT-PCR Master Mix, QuantiNova Yellow Template Dilution Buffer, template RNA, QN IC RNA (optional), primers, probes, QN ROX Reference Dye (if required) and RNase-Free Water. Thawing of the QuantiNova RT Mix is not required. Mix the individual solutions.

   Prepare a reaction mix according to Table 3.

2. Due to the two-phase hot-start of both the RT and the PCR reactions, it is not necessary to keep samples on ice during reaction setup or while programming the real-time cycler.

Table 3. Reaction setup

	Volume/reaction		
Component	**96-well block**	**384-well block**	**Final concentration**
2x QuantiNova Probe RT-PCR Master Mix	10 µl	5 µl	1 x
QN ROX Reference Dye (Applied Biosystems cycler only)	1 µl/0. 1 µl*	0.5 µl/0.05 µl*	1 x
QN Probe RT-Mix	0.2 µl	0.1 µl	1 x
20x primer-probe mix 1	1 µl	0.5 µl	0.8 µM forward primer 0.8 µM reverse primer 0.2 µM TaqMan Probe
20x primer–probe mix 2† (or QuantiNova Probe IC Assay†)	1 µl	0.5 µl	0.8 µM forward primer 0.8 µM reverse primer 0.2 µM TaqMan Probe
QN IC RNA (optional)	1 µl	1 µl	1 x
Template RNA (added at step 4)	Variable	Variable	≤400 ng/ reaction
RNase-Free Water	Variable	Variable	
Total reaction volume	**20 µl**	**10 µl**	

* Results in a 1:20 dilution for high ROX cyclers (i.e., ABI PRISM 7000, Applied Biosystems 7300, 7900 and StepOne Real-Time PCR Systems) and a 1:200 dilution for low ROX dye cyclers (i.e., Applied Biosystems 7500, ViiA7 and QuantStudio Real-Time PCR Systems).

† If using the QN IC RNA to monitor RT-PCR amplification, please add 2 µl of the 10x QuantiNova Probe IC Assay.

3. Mix the reaction thoroughly and dispense appropriate volumes into PCR tubes or wells of a PCR plate.
4. Add template RNA (≤400 ng – 100 fg per reaction, depending on target transcript abundance) to the individual PCR tubes or wells containing the reaction mix.
5. Program the real-time cycler according to the program outlined in Table 4.

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

Table 4. Real-time cycler conditions

Step	Time	Temperature	Ramp rate	Additional comments
Reverse transcription	10 min	45°C	Maximal/fast mode	HotStaRT-Script Reverse Transcriptase is activated, residual gDNA is removed
PCR initial activation step	5 min	95°C	Maximal/fast mode	QuantiNova DNA Polymerase is activated
Two-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 RNA

* 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.

Analysis and interpretation of Internal Control Assay results

To analyze the QN IC RNA with the QuantiNova Probe RT-PCR Kit, add the appropriate volume of 10x QuantiNova IC Probe Assay (cat. no. 205813) to the sample. Signal detection is performed on the filter/channel for HEX/VIC of your real-time PCR instrument.

1. After amplification, perform data analysis as recommended for your real-time PCR instrument. The C_q value for the QN IC RNA in the QuantiNova Probe RT-PCR Kit depends on the real-time PCR instrument used and can be expected within a C_q range of 23–25.
2. Compare C_q values between the QN IC RNA only and samples containing QN IC RNA plus template RNA. Consistent C_q values indicate successful RT-PCR and reliable results. A C_q difference >2 is likely to indicate inhibition or sample failure.

3. If a shifted C_q of >2 appears, indicating inhibition or failure of a specific sample, we recommend the following:

   a. Check equipment for accurate performance and repeat sample/experiment to rule out pipetting or handling errors.

   b. Dilute the affected template RNA using RNase-free water before repeating the experiment. This dilutes inhibitors present in the sample.

   c. Consider repeating the RNA extraction and avoid contamination or carry-over of inhibitors (e.g., use an appropriate RNeasy® Kit). Alternatively, the RNeasy MinElute Cleanup Kit (cat. no. 74204) can be used to remove potential inhibitors and concentrate the RNA template.

Guidelines for effective duplex assays

The QuantiNova Probe RT-PCR Kit works well with most existing probe systems that have been designed using standard design methods. However, for optimal performance of a probe system in quantitative duplex real-time RT-PCR, some considerations need to be made, including the choice of a compatible combination of reporter dyes (i.e., the fluorophores on the probes) and the quality of the primers and probes. Please read the following guidelines before starting.

- Check the functionality of each set of primers and probe in individual assays before combining the different sets in a duplex assay.
- Perform appropriate controls for evaluating the performance of your duplex assays (e.g., amplifying each target individually and comparing the results with those for the duplex assay).
- For duplex analysis, the use of non-fluorescent quenchers (e.g., Black Hole Quencher® [BHQ] on TaqMan probes) is preferred over fluorescent quenchers (e.g., TAMRA™ fluorescent dye). TAMRA quencher can be used in duplex analysis if the two reporter dyes are 6-FAM™ dye and HEX, JOE™ or VIC dye.

- PCR products should be as short as possible, ideally 60–150 bp. Always use the same algorithm or software to design the primers and probes. For optimal results, only combine assays that have been designed using the same parameters (e.g., similar melting points [T_m]). For details, see Appendix B, page 26).
- Check the concentration and integrity of primers and probes before starting. For details, see Appendix B, page 26.
- Check the real-time cycler user manual for correct setup of the cycler for duplex analysis (e.g., setting up detection of two dyes from the same well). Be sure to activate the detector for each reporter dye used.
- Some real-time cyclers require you to perform a calibration procedure for each reporter dye. Check whether the reporter dyes you selected for your duplex 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 real-time cycler).
- Always start with the cycling conditions specified in the protocol.
- 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.

Suitable combinations of reporter dyes

Duplex real-time PCR requires the simultaneous detection of two different fluorescent reporter dyes. For accurate detection, the fluorescence spectra of the dyes should be well separated or exhibit only minimal overlap (Table 5).

Note: Please refer to the user manual or other technical documentation for your instrument to find out which reporter dyes can be used in duplex analysis.

Table 5. 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.

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/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 PCR step of your cycling conditions include the initial step for activation of QuantiNova DNA Polymerase (95°C for 5 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 (5 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: A, page 26, 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 0.8 µM. In most cases, a probe concentration of 0.2 µM provides satisfactory results. Check the concentrations of primers and probes by spectrophotometry (see Appendix B, page 26).
f) Problems with starting template	Check the concentration, storage conditions and quality of the starting template (see Appendix B, page 26). If necessary, make new serial dilutions of template nucleic acid from the stock solutions. Repeat the PCR using the new dilutions. Ensure template is free of RNAse contamination to avoid degradation during reaction set up, even if RNAse inhibitor is present in the RT Mix.

		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 recommend a final reaction volume of 10 µl.
j)	RT-PCR product too long	For optimal results, RT-PCR products should be between 60 and 150 bp. RT-PCR products should not exceed 300 bp.
k)	Primer design not optimal	Check for RT-PCR products by gel electrophoresis. If no specific RT-PCR products are detected, review the primer design guidelines (see Appendix B, page 26).
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 26).
m)	Wrong detection channel/filter chosen	Ensure that the correct detection channel is activated or the correct filter set is chosen for the reporter dye.
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)	Incorrect temperature for RT reaction	We recommend performing the RT reaction at 45°C. However, if this temperature does not yield satisfactory results, the temperature can be adjusted between 42°C and 50°C.
r)	Incorrect ratio of QuantiNova RT Mix to QuantiNova Probe RT-PCR Master Mix	If not using the standard reaction volumes, ensure that the volume of QuantiNova RT Mix is changed proportionately so that the ratio of QuantiNova RT Mix to QuantiNova Probe RT-PCR Master Mix remains the same.
Increased fluorescence or C_q 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.
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.

		Comments and suggestions
Varying fluorescence intensity		
a)	Contamination of real-time cycler	Decontaminate the real-time cycler according to the manufacturer's instructions.
b)	Real-time cycler no longer calibrated	Recalibrate the real-time cycler according to the manufacturer's instructions.
All cycler systems:		
a)	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.
Applied Biosystems instruments only:		
a)	ΔRn 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 13.

Appendix A: 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 Table 2. When using a master mix containing a low concentration of ROX, the volume of ROX added is negligible and the standard reaction setup as described in Table 3 should be used.

Table 6. Reaction setup

	Volume/reaction		
Component	**96-well block**	**384-well block**	**Final concentration**
2x QuantiNova Probe RT-PCR Master Mix*	11 µl	5.5 µl	1x
QN Probe RT-Mix	0.2 µl	0.1 µl	1x
20x primer-probe mix 1	1 µl	0.5 µl	0.8 µM forward primer 0.8 µM reverse primer 0.2 µM TaqMan probe
20x primer–probe mix 2† (or QuantiNova Probe IC Assay†)	1 µl	0.5 µl	0.8 µM forward primer 0.8 µM reverse primer 0.2 µM TaqMan probe
QN IC RNA (optional)	1 µl	1 µl	1x
Template RNA (added at step 4)	Variable	Variable	≤400 ng/ reaction
RNase-Free Water	Variable	Variable	
Total reaction volume	**20 µl**	**10 µl**	

* Contains a 1:20 dilution for high ROX instruments (i.e., ABI PRISM 7000, Applied Biosystems 7300, 7900 and StepOne Real-Time PCR Systems).

† If using the QN IC RNA to monitor RT-PCR amplification, please add 2 µl of the 10x QuantiNova Probe IC Assay.

Appendix B: Assay Design and Handling Primers and Probes

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

Assay design

Guidelines for the optimal design of primers and probes are given below. It is particularly important to minimize non-specific 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) 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).

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 RT-PCR products is 60–150 bp. Some longer amplicons may amplify efficiently in duplex RT-PCR, with minimal optimization.

Handling and storing primers and probes

Guidelines for handling and storing primers and probes are given in Table 7 below. For optimal results, we recommend only combining primers of comparable quality.

Table 7. Guidelines for handling and storing primers and probes

	Description
Storage buffer	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. 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.
Storage	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. For easy and reproducible handling of primer–probe sets used in duplex 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).
Dissolving primers and probes	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. 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.

	Description
Concentration	Spectrophotometric conversion for primers and probes: 1 A_{260} unit = 20–30 µg/ml To check primer concentration, the molar extinction coefficient (ε_{260}) can be used: A_{260} = ε_{260} x molar concentration of primer or probe 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: ε_{260} = 0.89 x [(A x 15,480) + (C x 7340) + (G x 11,760) + (T x 8850)] Example: Concentration of diluted primer: 1 µM = 1 x 10^{-6} M Primer length: 24 nucleotides with 6 each of A, C, G, and T bases Calculation of expected A_{260}: 0.89 x [(6 x 15,480) + (6 x 7340) + (6 x 11,760) + (6 x 8850)] x (1 x 10^{-6}) = 0.232 The measured A_{260} should be within +/– 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. For probes, the fluorescent dye does not significantly affect the A_{260} value.
Primer quality	The quality of 18–30mers can be checked on a 15% denaturing polyacrylamide gel; a single band should be seen. Please contact QIAGEN Technical Services 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.

Ordering Information

Product	Contents	Cat. no.
QuantiNova Probe RT-PCR Kit (100)	For 100 x 20 µl reactions: 1 ml 2x QuantiNova Probe RT-PCR Master Mix, 500 µl QuantiNova Yellow Template Dilution Buffer, 250 µl QN ROX Reference Dye, 20 µl 100x QN RT Mix, 20 µl QN IC RNA, 1.9 ml RNase-Free Water	208352
QuantiNova Probe RT-PCR Kit (500)	For 500 x 20 µl reactions: 3 x 1.7 ml 2x Master Mix , 500 µl QuantiNova Yellow Template Dilution Buffer, 1 ml QN ROX Reference Dye, 100 µl 100x QN RT Mix, 100 µl QN IC RNA, 1.9 ml RNase-Free Water	208354
QuantiNova Probe RT-PCR Kit (2500)	For 2500 x 20 µl reactions: 15 x 1.7 ml 2x Master Mix, 5 x 500 µl QuantiNova Yellow Template Dilution Buffer, 5 x 1 ml QN ROX Reference Dye, 5 x 100 µl 100x QN RT Mix, 5 x 100 µl QN IC RNA, 5 x 1.9 ml RNase-Free Water	208356
QuantiNova IC Probe Assay (200)	For 200 x 20 µl reactions: 400 µl primer/probe mix (10x), detecting IC RNA	205813
QuantiNova Reverse Transcription Kit (10)	For 10 x 20 µl reactions: 20 µl 8x gDNA removal Mix, 10 µl Reverse Transcription Enzyme, 40 µl Reverse Transcription Mix (containing RT primers), 20 µl Internal Control, 1.9 ml RNase-Free Water	205410

Product	Contents	Cat. no.
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
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
RNeasy MinElute Cleanup Kit (50)	50 RNeasy MinElute Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-Free Reagents and Buffers	74204
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: RNeasy Mini Spin Columns, gDNA Eliminator Solution, Collection Tubes, RNase-Free Water 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 2plex HRM Platform	Real-time PCR cycler and High Resolution Melt analyzer with 2 channels (green, yellow) plus HRM channel, laptop computer, software, accessories: includes 1-year warranty on parts and labor, installation and training not included	9001560
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

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.

Visit **www.qiagen.com/geneXpression** to find out more about standardized solutions for gene expression analysis – from RNA preparation to real-time RT-PCR

Limited License Agreement for QuantiNova Probe RT-PCR Kit

Use of this product signifies the agreement of any purchaser or user of the product to the following terms:

1. The product may be used solely in accordance with the protocols provided with the product and this handbook and for use with components contained in the kit only. QIAGEN grants no license under any of its intellectual property to use or incorporate the enclosed components of this kit with any components not included within this kit except as described in the protocols provided with the product, this handbook, and additional protocols available at www.qiagen.com. Some of these additional protocols have been provided by QIAGEN users for QIAGEN users. These protocols have not been thoroughly tested or optimized by QIAGEN. QIAGEN neither guarantees them nor warrants that they do not infringe the rights of third-parties.
2. Other than expressly stated licenses, QIAGEN makes no warranty that this kit and/or its use(s) do not infringe the rights of third-parties.
3. This kit and its components are licensed for one-time use and may not be reused, refurbished, or resold.
4. QIAGEN specifically disclaims any other licenses, expressed or implied other than those expressly stated.
5. The purchaser and user of the kit agree not to take or permit anyone else to take any steps that could lead to or facilitate any acts prohibited above. QIAGEN may enforce the prohibitions of this Limited License Agreement in any Court, and shall recover all its investigative and Court costs, including attorney fees, in any action to enforce this Limited License Agreement or any of its intellectual property rights relating to the kit and/or its components.

For updated license terms, see **www.qiagen.com**.

Notes

Notes

www.qiagen.com

Technical Support

www.support.qiagen.com

1090921 HB-1956-001 06/2015