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QuantiNova® SYBR® Green RT-PCR Kit Handbook

For highly sensitive, ultrafast quantitative real-time RT-PCR using SYBR Green I

Sample to Insight

QIAGEN

Contents

Kit Contents

QuantiNova SYBR Green RT-PCR Kit	**(100)**	**(500)**	**(2500)**
Catalog No.	**208152**	**208154**	**208156**
Number of reactions (20 µl/10 µl)	**100/200**	**500/1000**	**2500/5000**
2x QuantiNova SYBR Green RT-PCR Master Mix, containing QuantiNova DNA Polymerase composed of: Taq DNA Polymerase, QuantiNova Antibody, QuantiNova Guard, QuantiNova SYBR Green 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 and RNase Inhibitor	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 x1.9 ml	10 x 1.9 ml
Quick-Start Protocol QuantiNova SYBR Green RT-PCR	1	1	1
Quick-Start Protocol QuantiNova IC & Assay	1	1	1

Storage

QuantiNova SYBR Green 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 SYBR Green 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 SYBR Green RT-PCR Master Mix for long-term storage. For details, see “Adding ROX dye to the RT-PCR master mix”, page13.

Intended Use

The QuantiNova SYBR Green 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 SYBR Green RT-PCR Kit is tested against predetermined specifications to ensure consistent product quality.

Product Information

The QuantiNova SYBR Green RT-PCR Kit contains:

2x QuantiNova SYBR Green 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 2 minute, 95°C incubation step.
QuantiNova SYBR Green RT-PCR Buffer	Contains Tris-HCl, KCl, NH_4SO_4, $MgCl_2$ and additives enabling fast cycling, including Q-Bond®.
dNTP mix	Contains dATP, dCTP, dGTP and dTTP of ultrapure quality.

100x QuantiNova SYBR Green 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 50°C.
RNase Inhibitor	The RNase inhibitor is a recombinant mammalian protein that inhibits eukaryotic RNases, such as RNase A and B.

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 from Applied Biosystems®.
QuantiNova Yellow Template Dilution Buffer	Ultrapure quality, PCR-grade.
RNase-Free Water	Ultrapure quality, PCR-grade.

Introduction

The QuantiNova SYBR Green RT-PCR Kit provides accurate and rapid real-time quantification of RNA targets in an easy-to-handle format. The fluorescent dye, SYBR Green I, in the master mix enables the analysis of many different targets without having to synthesize target-specific labeled probes. 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. 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 an 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 50°C, the inhibitor is released from the reverse transcriptase and cDNA synthesis is initiated. The second phase of the hot-start is achieved using QuantiNova DNA Polymerase as a novel hot-start enzyme and QuantiNova Guard as a novel additive. These unique components further improve the stringency of the antibody-mediated hot-start.

The kit features a built-in control for visual identification of correct template addition as well as Q-Bond, an additive in the RT-PCR buffer that 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 SYBR Green RT-PCR Master Mix together with QuantiNova SYBR Green 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. There is also no need to set up the reaction on ice, and the whole reaction can be left for up to 2 h at room temperature without any loss of performance.

QuantiNova SYBR Green RT Mix

The QuantiNova SYBR Green RT Mix contains HotStarRT-Script Reverse Transcriptase for heat-mediated activation of the reverse-transcription step and an RNase inhibitor. The HotStaRT-Script Reverse Transcriptase is associated with a RT-blocker, rendering the enzyme almost inactive at ambient temperature. This allows room-temperature RT-PCR reaction setup without the risk of primer–dimer formation by the reverse transcriptase. Upon starting the RT-PCR protocol with the RT step at 50°C, the inhibitor is released from the reverse transcriptase and cDNA synthesis is initiated (Figure 1).

2x QuantiNova SYBR Green RT-PCR Master Mix

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

QuantiNova SYBR Green RT-PCR Buffer contains an optimized concentration of the fluorescent dye SYBR Green I. SYBR Green I binds all double-stranded DNA molecules, emitting a fluorescent signal upon binding. QuantiNova SYBR Green RT-PCR Master Mix can be stored at 2–8°C or –20°C without loss of SYBR Green I fluorescence activity. The

excitation and emission maxima of SYBR Green I are 494 nm and 521 nm, respectively, which makes the dye compatible with any real-time cycler.

Novel, antibody-mediated hot-start mechanism

QuantiNova DNA Polymerase is provided in an inactive state and has no enzymatic activity at ambient or higher temperatures. The enzyme remains completely inactive during the reverse-transcription reaction and does not interfere with it. The antibody-mediated hot-start mechanism prevents the formation and extension of nonspecific RT-PCR products and primer–dimers during reaction setup, reverse transcription and the first denaturation step. Therefore, this mechanism allows higher PCR specificity and accurate quantification. 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.

After the reverse transcription and within 2 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. After setup, the RT-PCR can be stored for up to 2 hours at room temperature without impairing the performance of the subsequent reaction.

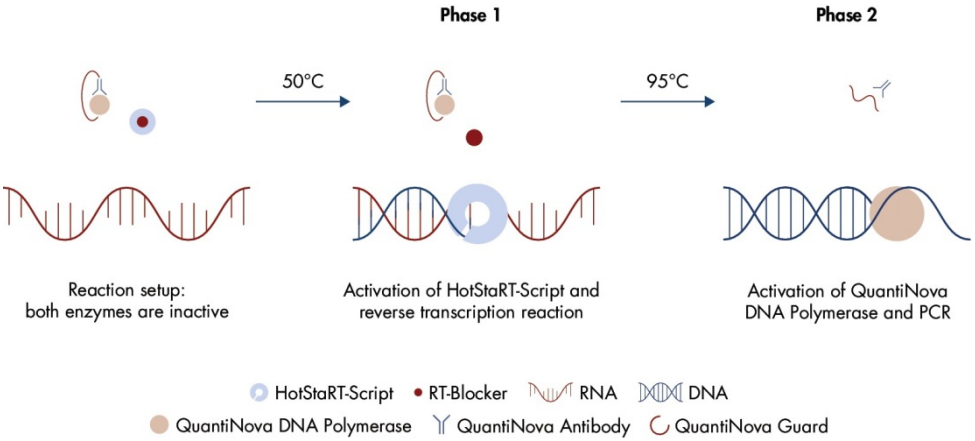

Figure 1. Principle of the novel QuantiNova two-phase hot-start mechanism. At ambient temperature the HotStaRT-Script is inhibited by the RT-Blocker and the QuantiNova DNA Polymerase is kept inactive by QuantiNova Antibody and QuantiNova Guard. At 50°C the RT is activated while the QuantiNova DNA polymerase 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 SYBR Green RT-PCR Kit contains an inert blue dye that does not interfere with the RT-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 SYBR Green RT-PCR Buffer

QuantiNova SYBR Green RT-PCR Buffer is specifically designed to facilitate both efficient reverse transcription and fast real-time PCR. 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 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 SYBR Green RT-PCR Buffer is also based on the unique QIAGEN PCR buffer system. The buffer contains a balanced combination of KCl and $(NH_4)_2SO_4$, which promotes a high ratio of specific to nonspecific primer binding during the annealing step of each PCR cycle. This creates stringent primer annealing conditions, leading to increased PCR specificity. When using this buffer, primer annealing is only marginally influenced by the $MgCl_2$ concentration, so optimization by titration of Mg^{2+} is not required.

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 room temperature 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 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 detection, use the Ctrl_QNIC_1_SG QuantiTect Primer Assay (QT02589307).

The QN IC RNA can be used optionally, and added to the experimental RNA sample. An additional control sample which only contains the QN IC RNA should also be set up. C_q shifts between the template RNA + QN IC RNA samples compared to the QN IC RNA only 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 the SYBR Green dye.

The use of ROX dye is necessary for instruments from Applied Biosystems. The QuantiNova SYBR Green RT-PCR Kit is provided with a separate tube of QuantiNova ROX Reference Dye. It can be added to the real-time RT-PCR if using a real-time cycler that uses ROX as a passive reference dye. ROX dye has to be used as a 20x concentrate when using an instrument requiring a high ROX dye concentration. For instruments requiring a low ROX dye concentration, the dye has to be used as a 200x concentrate. 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 SYBR Green RT-PCR Master Mix for long-term storage (Table 2). For details, see "Adding ROX dye to the master mix", page 13.

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

High ROX concentration (QN ROX Reference Dye to be used at a 20x dilution)	Low ROX concentration (QN ROX Reference Dye to be used at a 200x dilution)
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 SYBR Green RT-PCR Kit, QuantiNova ROX Reference Dye can be added to 2x QuantiNova SYBR Green RT-PCR Master Mix for long-term storage, if desired (Table 2). 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: Reaction Setup Using Master Mix Containing High Concentration of ROX (page 22).

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 µl /10 µl
1.7 ml	170 µl /17 µl

Protocol: Real-Time RT-PCR

This protocol is for use with the QuantiNova SYBR Green RT-PCR Kit on any cycler.

Important points before starting

- 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 used as a 20x concentrate when using an instrument requiring a high ROX dye concentration. For instruments requiring a low ROX dye concentration, use the dye as a 200x concentrate. Pre-dilution of ROX dye is not required.
- The QuantiNova SYBR Green RT Mix contains HotStarRT-Script Reverse Transcriptase for heat-mediated activation of the reverse-transcription step 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 SYBR Green 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 and qRT-PCR.
- For the highest efficiency in real-time RT-PCR, PCR products 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 2 min at 95°C to activate the QuantiNova DNA Polymerase.
- For ease of use, we recommend preparing a 10x primer mix containing target-specific primers for the target. A 10x primer mix consists of 5 µM forward primer and 5 µM reverse primer in TE buffer. Alternatively, it may be preferable to prepare the reaction mix with separate primer 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) using the use the Ctrl_QNIC_1_SG QuantiTect Primer Assay (QT02589307), which needs to be ordered separately. 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.
- To test for potential inhibition in the experimental template RNA, the QN IC RNA has to be added to the experimental RNA sample. An additional control sample which only contains the QN IC RNA should also be set up. Cq shifts >2 between the experimental template RNA+QN IC RNA compared to the QN IC RNA only samples indicate inhibition of the RT-PCR.

  This table displays the recommended use of the QN IC RNA:

	Test Assay (experiment)	**Internal Control (optional)**	
		Test sample	**Control sample**
Template RNA to be used:	Experimental RNA sample only	Experimental RNA sample + QN IC RNA	QN IC RNA only
Primers used to be used:	Experimental Assay primers	Ctrl_QNIC_1_SG QuantiTect Primer Assay	Ctrl_QNIC_1_SG QuantiTect Primer Assay

- 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 SYBR Green RT-PCR Master Mix, QuantiNova Yellow Template Dilution Buffer, template RNA, QN IC RNA (optional), primers, 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 SYBR Green RT-PCR Master Mix	10 µl	5 µl	1x
QN ROX Reference Dye (Applied Biosystems cycler only)	1 µl/0. 1 µl*	0.5 µl/0.05 µl*	1x
QN SYBR Green RT-Mix	0.2 µl	0.1 µl	1x
10x primer mix (or QuantiTect IC Primer Assay†)	2 µl	1 µl	0.5 µM forward primer 0.5 µM reverse primer
QN IC RNA (optional)	1 µl	1 µl	1x
Template RNA (added at step 4)	Variable	Variable	≤200 ng/ reaction
RNase-Free Water	Variable	Variable	
Total reaction volume	**20 µl**	**10 µl**	

* To be used as a 20x concentrate for high ROX cyclers (i.e., ABI PRISM 7000, Applied Biosystems 7300, 7900, and StepOne Real-Time PCR Systems) and as a 200x concentrate 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, add 2 µl (for 96-well) or 1µl (for 384-well) of the 10x Ctrl_QNIC_1_SG QuantiTect Primer Assay.

3. Mix the reaction thoroughly and dispense appropriate volumes into PCR tubes or wells of a PCR plate.
4. Add template RNA (≤200 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	50°C	Maximal/fast mode	HotStaRT-Script Reverse Transcriptase is activated
PCR initial activation step	2 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	10 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
Melting curve analysis §				

* If your cycler does not accept this short time for data acquisition, choose the shortest acceptable time.
† This temperature should also be used for QuantiTect Primer Assays and for all primer sets with a T_m well below 60°C.
‡ The number of cycles depends on the amount of template DNA.
§ Melting curve analysis is an analysis step built into the software of real-time cyclers. To perform the analysis, follow instructions provided by the supplier.

6. Place the PCR tubes or plates in the real-time cycler and start the cycling program.
7. Perform melting curve analysis of the PCR product(s).

   We strongly recommend routinely performing this analysis, which is built into the software of real-time cyclers, to verify the specificity and identity of PCR products.

   Optional: Check the specificity of PCR product(s) by agarose gel electrophoresis.

Analysis and interpretation of Internal Control Assay results

To analyze the QN IC RNA with the QuantiNova SYBR Green RT-PCR Kit, add the appropriate volume of 10x Ctrl_QNIC_1_SG QuantiTect Primer Assay (QT02589307) to the sample.

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 SYBR Green RT-PCR Kit depends on the real-time PCR instrument used and can be expected within a C_q range of 16–19.
2. Compare C_q values between the QN IC RNA only and samples containing QN IC RNA plus experimental 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.

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 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 and template nucleic acid. See Appendix B, page 23, for details on evaluating the concentration of primers. 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.
e)	Primer concentration not optimal	Use optimal primer concentrations (each primer at 0.5 µM). Check the concentrations of primers by spectrophotometry (see Appendix B, page 23).
f)	Problems with starting template	Check the concentration, storage conditions and quality of the starting template (see Appendix B, page 23). If necessary, make new serial dilutions of template nucleic acid from the stock solutions. Repeat the PCR using the new dilutions.
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.

		Comments and suggestions
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 200 bp.
k)	Primer design not optimal	Check for PCR products by melting curve analysis or gel electrophoresis. If no specific PCR products are detected review the primer design guidelines (see Appendix B, page 23). Alternatively, use QuantiTect Primer Assays, which are predesigned primer sets for real-time RT-PCR (see ordering information, page 26).
l)	Wrong detection channel/filter chosen	Ensure that the correct detection channel is activated or the correct filter set is chosen for the reporter dye.
m)	No detection activated	Check that fluorescence detection was activated in the cycling program.
n)	Primers degraded	Check for possible degradation of primers on a denaturing polyacrylamide gel.
o)	Incorrect temperature for RT reaction	We recommend performing the RT reaction at 50°C. However, if this temperature does not yield satisfactory results, the temperature can be adjusted to between 42°C and 50°C.
p)	Incorrect ratio of QuantiNova RT Mix to QuantiNova SYBR Green RT-PCR Master Mix	High volumes of RT reaction added to the PCR may reduce amplification efficiency. 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 SYBR Green RT-PCR Master Mix remains the same.
Primer dimers and/or nonspecific PCR products		
a)	Mg^{2+} concentration adjusted	Do not adjust the Mg^{2+} concentration in 2x QuantiNova SYBR Green RT-PCR Master Mix.
b)	Primer design not optimal	Check for PCR products by melting curve analysis or gel electrophoresis. If no specific PCR products are detected review the primer design guidelines (see Appendix B, page 23). Alternatively, use QuantiTect Primer Assays, which are predesigned primer sets for real time RT-PCR (see ordering information, page 26).
c)	PCR product too long	For optimal results, PCR products should be between 60 and 150 bp. PCR products should not exceed 200 bp.
d)	Primers degraded	Check for possible degradation of primers on a denaturing polyacrylamide gel.

		Comments and suggestions
e)	Contamination of RNA sample with genomic DNA	Design primers that span exon-exon boundaries, so that only cDNA targets can be amplified and detected. Alternatively, use QuantiTect Primer Assays, which are predesigned primer sets that avoid amplification of genomic DNA where possible (see ordering information, page 26). Treat the RNA sample with DNase to digest the contaminating genomic DNA.
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 and primers). Repeat the assay using new components.
b)	Contamination during reaction setup	Take appropriate precautions during reaction setup, such as using aerosol-barrier pipet tips.
High fluorescence in "No Reverse Transcription" control		
a)	Contamination of RNA sample with genomic DNA	Design primers that span exon-exon boundaries so that only cDNA targets can be amplified and detected. Treat the RNA sample with DNase to digest the contaminating genomic DNA.
Varying fluorescence intensity		
a)	Contamination of real-time cycler	Decontaminate the real-time cycler according to the manufacturer's instructions.
b)	Real-time cycler no longer calibrated	Recalibrate the real-time cycler according to the manufacturer's instructions.
All cycler systems:		
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	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. Check the used ROX concentration.

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 to 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 5. Reaction setup

	Volume/reaction		
Component	**96-well block**	**384-well block**	**Final concentration**
2x QuantiNova SYBR Green RT-PCR Master Mix*	11 µl	5.5 µl	1x
QN SYBR Green RT-Mix	0.2 µl	0.1 µl	1x
10x primer mix (or QuantiTect IC Primer Assay†)	2 µl	1 µl	0.5 µM forward primer 0.5 µM reverse primer
QN IC RNA (optional)	1 µl	1 µl	1x
Template RNA (added at step 4)	Variable	Variable	≤200 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, add 2 µl (for 96-well) or 1µl (for 384-well) of the 10x Ctrl_QNIC_1_SG QuantiTect Primer Assay.

Appendix B: Assay Design and Handling Primers and SYBR Greens

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

Assay design

For guaranteed results in gene expression analysis experiments, we recommend using QuantiTect Primer Assays (see ordering information, page 26). If designing your own primers, please follow the guidelines for the optimal design of primers given below.

Since fluorescence from SYBR Green I increases strongly upon binding of the dye to any double-stranded DNA, it is particularly important to minimize nonspecific primer annealing by careful primer design

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.
- 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 with minimal optimization.

Handling and storing primers

Guidelines for handling and storing primers 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

	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 TrisHCl, 1 mM EDTA, pH 8.0) for standard primers.
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. Repeated freeze–thaw cycles should be avoided, since they may lead to degradation.
Dissolving primers and probes	Before opening a tube containing lyophilized primer, centrifuge the tube briefly to collect all material at the bottom of the tube. To dissolve the primer, add the required volume of sterile, nuclease-free TE buffer, mix, and leave for 20 minutes to allow the primer to completely dissolve. Mix again and determine the concentration by spectrophotometry as described below. We do not recommend dissolving primers in water. They are less stable in water than in TE buffer and some may not dissolve easily in water.
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: $A_{260} = \varepsilon_{260}$ 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: $\varepsilon_{260} = 0.89 \times [(A \times 15{,}480) + (C \times 7340) + (G \times 11{,}760) + (T \times 8850)]$ 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 A260 should be within +/– 30% of the theoretical value. If the measured A260 is very different to the theoretical value, we recommend recalculating the concentration of the primers or having the primers resynthesized.
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.

Ordering Information

Product	Contents	Cat. no.
QuantiNova SYBR Green RT-PCR Kit (100)	For 100 x 20 µl reactions: 1 ml 2x QuantiNova SYBR Green 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	208152
QuantiNova SYBR Green 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, 2 x 1.9 ml RNase-Free Water	208154
QuantiNova SYBR Green 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, 10 x 1.9 ml RNase-Free Water	208156
QuantiNova IC SYBR Green Assay (Ctrl_QNIC_1_SG QuantiTect Primer Assay)	For 500 x 20 µl reactions: QuantiTect® Primer Assay for SYBR based detection of IC RNA, available via GeneGlobe®	QT02589 307
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 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
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	208252
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	208352
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

* Other kit sizes and formats available; please inquire.

Product	Contents	Cat. no.
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
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

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Notes

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Technical assistance

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