September 2013

QuantiNova™ Probe PCR Handbook

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

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QIAGEN Sample and Assay Technologies

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Contents

Kit Contents

QuantiNova Probe PCR Kit	(100)	(500)	(2500)
Catalog no.	**208252**	**208254**	**208256**
Number of reactions (20 µl/10 µl)	**100/ 200**	**500/ 1000**	**2500/ 5000**
2x QuantiNova Probe PCR Master Mix, containing: ■ QuantiNova DNA Polymerase composed of: Taq DNA Polymerase, QuantiNova Antibody, and QuantiNova Guard ■ QuantiNova Probe PCR Buffer ■ dNTP mix (dATP, dCTP, dGTP, dTTP)	1 ml	3 x 1.7 ml	15 x 1.7 ml
QuantiNova Yellow Template Dilution Buffer	500 µl	500 µl	5 x 500 µl
QN ROX™ Reference Dye	250 µl	1 ml	5 x 1 ml
RNase-Free Water	1.9 ml	1.9 ml	5 x 1.9 ml
Quick-Start Protocol	1	1	1

Storage

QuantiNova Probe PCR Kits are shipped on dry ice. The kits should be stored immediately upon receipt at –15 to –30ºC 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 PCR Master Mix, QuantiNova Yellow Template Dilution Buffer, and QN ROX Reference Dye can also be stored protected from light at 2–8ºC for up to 6 months, depending on the expiry date.

If desired, QN ROX Reference Dye can be added to 2x QuantiNova Probe PCR Master Mix for long-term storage. For details, see “Adding ROX dye to the master mix”, page 10.

Intended Use

The QuantiNova Probe 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.

24-hour emergency information

Chemical emergency or accident assistance is available 24 hours a day from:

CHEMTREC

USA & Canada ▪ Tel: 1-800-424-9300

Outside USA & Canada ▪ Tel: +1-703-527-3887 (collect calls accepted)

Product Specifications

2x QuantiNova Probe PCR Master Mix contains:

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, 95ºC incubation step.
QuantiNova Probe PCR Buffer	Contains Tris·HCl , KCl, NH_4Cl, $MgCl_2$, and additives enabling fast cycling, including Q-Bond®
dNTP mix	Contains dATP, dCTP, dGTP, and dTTP of ultrapure quality
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

Quality Control

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

Component	Quality control tests
2x QuantiNova Probe PCR Master Mix	(See quality-control label inside kit lid for lot-specific values) PCR sensitivity and reproducibility assay: Sensitivity, reproducibility, and specificity in real-time PCR are tested in a duplex PCR assay in parallel 20 µl reactions containing 10-fold dilutions of nucleic acid template.
QuantiNova DNA Polymerase (included in QuantiNova Probe PCR Master Mix)	Efficiency, reproducibility, and stringency of hot-start in PCR are tested. Functional absence of exonucleases and endonucleases is tested.
Buffers and reagents (included in QuantiNova Probe PCR Master Mix)	
QuantiNova Probe PCR Buffer	Conductivity, density, pH, and ion concentrations are tested.
RNase-Free Water	Conductivity, pH, and RNase activities are tested.
QuantiNova Yellow Template Dilution Buffer	Dye concentration and RNase activities are tested.
QN ROX Reference Dye	Concentration is tested

Introduction

QuantiNova Probe PCR Kits provide highly sensitive and rapid real-time quantification of DNA and cDNA targets in an easy-to-handle format. 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 QuantiTect® Reverse Transcription Kit (see ordering information, page 30). The kits are compatible with dual-labeled probes e.g., TaqMan® probes. High specificity and sensitivity in 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 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 PCR buffer.

The kits have been optimized for use with any real-time cycler. The QN 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

2x QuantiNova Probe PCR Master Mix

The components of 2x QuantiNova Probe PCR Master Mix include QuantiNova DNA Polymerase and QuantiNova Probe PCR Buffer. The optimized Master Mix ensures ultrafast real-time 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. 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 real-time PCR can be stored after setup at up to 30°C for up to 100 hours without impairing the performance of the subsequent reaction.

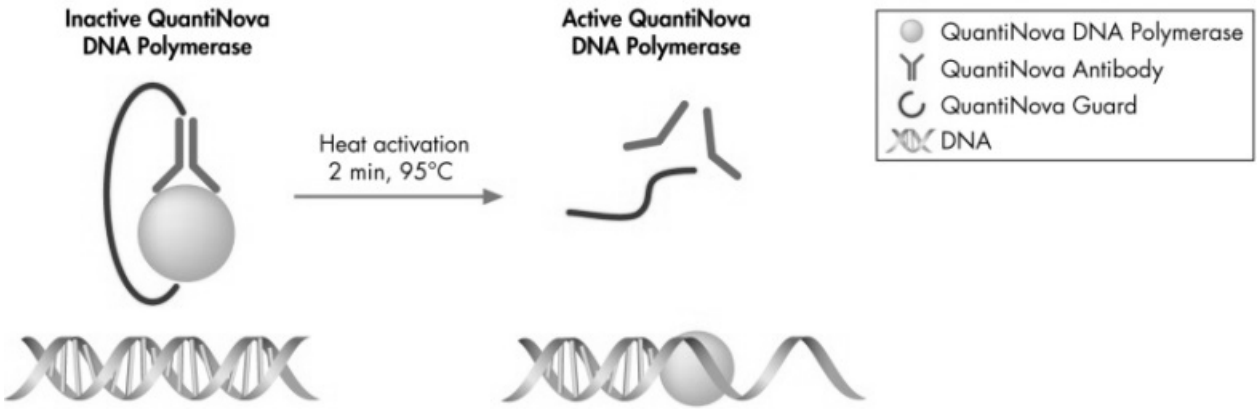

Figure 1. Principle of the novel QuantiNova hot-start mechanism. QuantiNova DNA Polymerase is kept in an inactive state by QuantiNova Antibody and QuantiNova Guard until the initial heat activation step.

Built-in visual control for correct pipetting

The master mix supplied with the QuantiNova Probe 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 Probe PCR Buffer

QuantiNova Probe PCR Buffer is specifically designed for ultrafast, 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 5 seconds. In addition, the unique composition of the buffer supports the melting behavior of DNA, enabling short denaturation and annealing/extension times.

QuantiNova Probe 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 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.

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 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 2x QuantiNova Probe PCR Master Mix for long-term storage (Table 2). For details, see “Adding ROX dye to the master mix”, page 10.

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 12K Flex
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 Probe PCR Kit, QN ROX Reference Dye Solution can be added to 2x QuantiNova Probe 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 10. For reaction setups with master mix that already contains high

concentration of added QN ROX Reference Dye, refer to Appendix A: Reaction Setup Using Master Mix Containing High Concentration of ROX.

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

Volume of 2x QuantiNova Probe PCR Master Mix (w/o 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

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

If quantifying cDNA targets with QuantiNova Probe 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 QuantiTect 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. For ordering information, see page 30.

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

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

Important points before starting

- Always start with the cycling conditions specified in this protocol, even if using previously established primer–probe systems.
- For the highest efficiency in real-time PCR using sequence-specific probes, targets should ideally be 60–150 bp in length. The PCR must start with an initial incubation step of 2 minutes at 95ºC to activate 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 dye in QuantiNova Yellow Template Dilution Buffer allows tracking of pipetted samples in the qPCR. When added to the blue QuantiNova Probe PCR Master Mix, the color changes from blue to green, indicating the successful inclusion of template. The use of this buffer is optional. It is provided as 100x concentrate and should be diluted (using water or Tris buffer) to obtain a final concentration of 1x within samples. To generate a template dilution series (e.g., for absolute quantification or determination of PCR efficiency), dilute the 100x concentrate (using water or Tris buffer) to obtain a final concentration of 1x QuantiNova Yellow Template Dilution Buffer. The buffer does not affect the sample stability and qPCR.

Procedure

1. **Thaw 2x QuantiNova Probe 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.**
2. **Prepare a reaction mix according to Table 3.**

   Due to the hot-start, it is not necessary to keep samples on ice during reaction setup or while programming the real-time cycler.

Table 3. Reaction setup

Component	Volume/reaction		Final concentration
	96-well block	384-well block	
2x QuantiNova Probe 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
Primer A	Variable	Variable	0.4 µM
Primer B	Variable	Variable	0.4 µM
Probe	Variable	Variable	0.2 µM
Template DNA or cDNA (added at step 4)	Variable	Variable	≤100 ng/ reaction
RNase-Free water	Variable	Variable	
Total reaction volume	**20 µl**	**10 µl**	

* Use a 1:20 dilution for high ROX instruments (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 and ViiA7™ Real-Time PCR Systems).

3. **Mix the reaction mix thoroughly and dispense appropriate volumes into PCR vessels or plates.**
4. **Add template gDNA or cDNA (≤100 ng/reaction) to the individual PCR vessels or wells containing the reaction mix.**

   For two-step RT-PCR, the volume of the cDNA added (from the undiluted RT reaction) should not exceed 10% of the final PCR volume.
5. **Program your 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
PCR initial activation step	2 min	95ºC	Maximal/ fast mode	QuantiNova DNA Polymerase is activated by this heating step
Two-step cycling				
Denaturation	5 s	95ºC	Maximal/ fast mode	
Combined annealing/ extension	5 s*	60ºC	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 vessels or plates in the real-time cycler and start the cycling program.

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

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

Guidelines for effective duplex assays

The QuantiNova Probe 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 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 nonfluorescent 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 2 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.

Important points before starting

- Always start with the cycling conditions specified in this protocol, even if using previously established primer–probe systems.
- For the highest efficiency in duplex real-time PCR using sequence-specific probes, targets should ideally be 60–150 bp in length. The PCR must start with an initial incubation step of 2 minutes at 95ºC to activate QuantiNova DNA Polymerase.
- 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.
- For ease of use, we recommend preparing a 20x primer–probe mix for both of your targets, containing target-specific primers and probe. A 20x primer–probe mix consists of 8 µM forward primer, 8 µM reverse primer, and 4 µM probe in TE buffer. Alternatively, it is also possible to prepare the reaction mix with separate primer and probe solutions.
- The dye in QuantiNova Yellow Template Dilution Buffer allows tracking of pipetted samples in the qPCR. When added to the blue QuantiNova Probe PCR Master Mix, the color changes from blue to green, indicating the successful inclusion of template. The use of this buffer is optional. It is provided as 100x concentrate and should be diluted (using water or Tris buffer) to obtain a final concentration of 1x within samples. To generate a template dilution series (e.g., for absolute quantification or determination of PCR efficiency), dilute the 100x concentrate (using water or Tris buffer) to obtain a final concentration of 1x QuantiNova Yellow Template Dilution Buffer. The buffer does not affect the sample stability and qPCR.

Procedure

1. **Thaw 2x QuantiNova Probe 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.**
2. **Prepare a reaction mix according to Table 6.**

   Due to the hot-start, it is not necessary to keep samples on ice during reaction setup or while programming the real-time cycler.
3. **Mix the reaction mix thoroughly and dispense appropriate volumes into PCR vessels or plates.**
4. **Add template DNA or cDNA (≤100 ng/reaction) to the individual PCR vessels or wells containing the reaction mix.**

   For two-step RT-PCR, the volume of the cDNA added (from the undiluted RT reaction) should not exceed 10% of the final PCR volume.
5. **Program your real-time cycler according to the program outlined in Table 7.**

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

Table 6. Reaction setup

Component	Volume/reaction		Final concentration
	96-well block	384-well block	
2x QuantiNova Probe PCR Master Mix	10 µl	5 µl	1x
QN ROX Reference Dye (Applied Biosystems instruments only)	1 µl/0.1 µl*	0.5 µl/0.05 µl*	1x
20x primer–probe Mix 1	1 µl	0.5 µl	0.4 µM forward primer 1 0.4 µM reverse primer 1 0.2 µM TaqMan probe 1
20x primer–probe Mix 2	1 µl	0.5 µl	0.4 µM forward primer 1 0.4 µM reverse primer 1 0.2 µM TaqMan probe 1
Template DNA or cDNA (added at step 4)	Variable	Variable	≤100 ng/ reaction
RNase-Free water	Variable	Variable	
Total reaction volume	**20 µl**	**10 µl**	

*Use a 1:20 dilution for high ROX instruments (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 and ViiA7 Real-Time PCR Systems).

Table 7. Real-time cycler conditions

Step	Time	Temperature	Ramp rate	Additional comments
PCR initial activation step	2 min	95ºC	Maximal/ fast mode	QuantiNova DNA Polymerase is activated by this heating step
Two-step cycling				
Denaturation	5 s	95ºC	Maximal/ fast mode	
Combined annealing/ extension	30 s*	60ºC	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 time acceptable (e.g., 31 s annealing/extension for the ABI PRISM 7000 or Applied Biosystems 7300).

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

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 or protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit www.qiagen.com).

	Comments and suggestions
No signal or one or more signals detected late in PCR	
a) 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 (95ºC 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 95ºC) 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 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 0.4 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).

		Comments and suggestions
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.
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 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)	PCR annealing temperature too high	Decrease annealing temperature in steps of 2ºC.
o)	PCR annealing temperature too low	Increase annealing temperature in steps of 2ºC.
p)	No detection activated	Check that fluorescence detection was activated in the cycling program.

	Comments and suggestions
q) 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.
r) Primers degraded	Check for possible degradation of primers on a denaturing polyacrylamide gel.
s) 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 10% 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.
Increased fluorescence or C_T 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.
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 QuantiTect 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 real-time cycler	Decontaminate the real-time cycler according to the manufacturer's instructions.

	Comments and suggestions
b) Real-time cycler no longer calibrated	Recalibrate the real-time cycler according to the manufacturer's instructions.
All cycler 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 real-time cycler allows you to do so) or reduce the amount of template.
Applied Biosystems instruments only:	
d) 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 10.

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. When using a master mix containing low concentration of ROX, the volume of ROX added is negligible and the standard reaction setup as described in Table 3 for singleplex reactions and Table 6 for duplex reactions should be used.

Table 8. Reaction setup for singleplex reactions

	Volume/reaction		
Component	**96-well block**	**384-well block**	**Final concentration**
2x QuantiNova Probe PCR Master Mix (containing high ROX)	11 µl	5.5 µl	1x
Primer A	Variable	Variable	0.4 µM
Primer B	Variable	Variable	0.4 µM
Probe	Variable	Variable	0.2 µM
Template DNA or cDNA	Variable	Variable	≤100 ng/ reaction
RNase-Free water	Variable	Variable	
Total reaction volume	**20 µl**	**10 µl**	

Table 9. Reaction setup for duplex reactions

	Volume/reaction		
Component	**96-well block**	**384-well block**	**Final concentration**
2x QuantiNova Probe PCR Master Mix (containing high ROX)	11 µl	5.5 µl	1x
20x primer–probe Mix 1	1 µl	0.5 µl	0.4 µM forward primer 1 0.4 µM reverse primer 1 0.2 µM TaqMan pProbe 1
20x primer–probe Mix 2	1 µl	0.5 µl	0.4 µM forward primer 1 0.4 µM reverse primer 1 0.2 µM TaqMan pProbe 1
Template DNA or cDNA	Variable	Variable	≤100 ng/ reaction
RNase-Free water	Variable	Variable	
Total reaction volume	**20 µl**	**10 µl**	

Appendix B: Assay Design and Handling Primers and Probes

Important factors for success in quantitative, singleplex, and duplex, 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) to design primers and probes.
- T_m of all primers should be 58–62ºC and within 2ºC of each other.
- T_m of probes should be 8–10ºC 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 (60ºC 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 PCR products is 60–150 bp. Some longer amplicons may amplify efficiently in duplex PCR, with minimal optimization.

Handling and storing primers and probes

Guidelines for handling and storing primers and probes are given in Table 10 below. For optimal results, we recommend only combining primers of comparable quality.

Table 10. Guidelines for handling and storing primers and probes

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 –20ºC. 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).

Table continued on next page

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.
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.

Table continued on next page

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 or your local distributor (see back cover) 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 37ºC for 1 hour. A significant difference in fluorescence following DNase treatment should be detectable.

Ordering Information

Product	Contents	Cat. no.
QuantiNova Probe PCR Kit (100)	For 100 x 25 µl reactions: 1 ml 2x QuantiNova Probe PCR Master Mix, 500 µl QuantiNova Yellow Template Dilution Buffer, 250 µl QN ROX Reference Dye, 1.9 ml RNase-Free Water	208252
QuantiNova Probe PCR Kit (500)	For 500 x 25 µl reactions: 3 x 1.7 ml 2x Master Mix (contains ROX dye), 500 µl QuantiNova Yellow Template Dilution Buffer, 1 ml QN ROX Reference Dye, 1.9 ml RNase-Free Water	208254
QuantiNova Probe PCR Kit (2500)	For 2500 x 25 µl reactions: 15 x 1.7 ml 2x Master Mix (contains ROX dye), 5 x 500 µl QuantiNova Yellow Template Dilution Buffer, 5 X 1 ml QN ROX Reference Dye, 5 x 1.9 ml RNase-Free Water	208256
Accessories		
QuantiTect Reverse Transcription Kit — for fast cDNA synthesis for sensitive real-time two-step RT-PCR		
QuantiTect Reverse Transcription Kit (50)	For 50 x 20 µl reactions: gDNA Wipeout Buffer, Quantiscript® Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix, and RNase-Free Water	205311
QuantiTect Reverse Transcription Kit (200)	For 200 x 20 µl reactions: gDNA Wipeout Buffer, Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix, and RNase-Free Water	205313
QuantiFast SYBR® Green PCR Kit — for fast, quantitative, real-time PCR and two-step RT-PCR using SYBR Green I		
QuantiFast SYBR Green PCR Kit (80)	For 80 x 25 µl reactions: 1 ml 2x Master Mix (contains ROX dye), 1.9 ml RNase-Free Water	204052

Product	Contents	Cat. no.
QuantiFast SYBR Green PCR Kit (400)	For 400 x 25 µl reactions: 3 x 1.7 ml 2x Master Mix (contains ROX dye), 2 x 1.9 ml RNase-Free Water	204054
QuantiFast SYBR Green PCR Kit (2000)	For 2000 x 25 µl reactions: 25 ml 2x Master Mix (contains ROX dye), 20 ml RNase-Free Water	204056
QuantiTect Primer Assays — for use in real-time RT-PCR with SYBR Green detection (search for and order assays at www.qiagen.com/GeneGlobe)		
QuantiTect Primer Assay (200)	For 200 x 50 µl reactions or 400 x 25 µl reactions: 10x QuantiTect Primer Assay (lyophilized)	Varies
DNeasy Blood & Tissue Kit — for purification of total DNA from animal blood and tissues, and from cells, yeast, bacteria, or viruses		
DNeasy Blood & Tissue Kit (50)*	50 DNeasy Mini Spin Columns, Proteinase K, Buffers, Collection Tubes (2 ml)	69504
RNeasy® Mini Kit — for purification of total RNA from animal cells, animal tissues, and yeast, and for RNA cleanup		
RNeasy Mini Kit (50)*	50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-Free Reagents and Buffers	74104
RNeasy Plus Mini Kit — for purification of total RNA from animal cells and tissues using gDNA Eliminator columns		
RNeasy Plus Mini Kit (50)	50 RNeasy Mini Spin Columns, 50 gDNA Eliminator Mini Spin Columns, Collection Tubes, RNase-Free Reagents and Buffers	74134
Oligotex® Direct mRNA Mini Kit — for purification of poly A+ mRNA directly from animal cells or tissues		
Oligotex Direct mRNA Mini Kit (12)*	For 12 mRNA minipreps: 420 µl Oligotex Suspension, Small Spin Columns, Collection Tubes (1.5 ml), RNase-Free Reagents and Buffers	72022

Product	Contents	Cat. no.
TurboCapture 96 mRNA Kit — for rapid and easy mRNA purification from cultured cells in 96-well format		
TurboCapture® 96 mRNA Kit (1)*	1 x TurboCapture 96 mRNA Plate, and RNase-Free Buffers	72250
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

* Other kit sizes and formats available; please inquire.

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Notes

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