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QuantiNova™ Reverse Transcription Kit Handbook

For cDNA synthesis with integrated removal of
genomic DNA contamination
For use in real-time two-step RT PCR

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Kit Contents

QuantiNova Reverse Transcription Kit	(10)	(50)	(200)
Catalog No.	205410	205411	205413
Number of standard reactions*	10	50	200
QuantiNova gDNA Removal Mix†	20 µl	100 µl	4 x 100 µl
QuantiNova Reverse Transcription Enzyme	10 µl	50 µl	4 x 50 µl
QuantiNova Reverse Transcription Mix‡	40 µl	200 µl	4 x 200 µl
QuantiNova Internal Control RNA (optional)	20 µl	100 µl	4 x 100 µl
RNase-Free Water	1.9 ml	1.9 ml	1.9 ml
Quick-Start Protocol QN Reverse Transcription Kit	1	1	1
Quick-Start Protocol QN IC RNA and Assay	1	1	1

*A standard reaction is 20 µl in a volume containing 10 pg to 5 µg total RNA

† Also contains RNase inhibitor

‡ Includes Mg²⁺, RT primers and dNTPs

Storage

The QuantiNova Reverse Transcription Kit is shipped on dry ice. The kit, including all reagents and buffers, should be stored immediately upon receipt at -30 to -15°C in a constant-temperature freezer.

Intended Use

The QuantiNova Reverse Transcription Kit is intended for research use. No claim or representation is intended to provide information 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.

Quality Control

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

Product information

Component	Description
QuantiNova gDNA Removal Mix	Buffer and enzyme for effective removal of genomic DNA contamination from starting RNA samples, including an RNase inhibitor.
QuantiNova Reverse Transcription Enzyme	An optimized enzyme developed for use in real-time two-step PCR
QuantiNova Reverse Transcription Mix	Buffer optimized for reverse transcription with QuantiNova Reverse Transcription Enzyme; contains an optimized combination of oligo-dT and random primers and includes Mg ²⁺ and dNTPs. It allows high cDNA yields from all regions of RNA transcripts, even from 5' regions.
QuantiNova Internal Control RNA	Synthetic transcript for monitoring successful reverse transcription
RNase-Free Water	Ultrapure quality, PCR-grade

Introduction

The QuantiNova Reverse Transcription Kit provides a fast, convenient procedure for efficient reverse transcription and effective genomic DNA removal. The kit is designed for use in real-time two-step RT-PCR, and provides high cDNA yields for sensitive quantification of even low-abundance transcripts. It also includes an internal control to monitor successful reverse transcription.

Principle and Procedure

The QuantiNova Reverse Transcription procedure takes only 20 minutes and comprises 2 main steps: removal of genomic DNA and reverse transcription (see flowchart, page 9).

Removal of genomic DNA

The purified RNA sample is optionally incubated in QuantiNova gDNA Removal Mix at 45°C for 2 minutes to effectively reduce contaminating genomic DNA. In contrast to other methods, the RNA sample is then used directly in reverse transcription.

Accurate results in real-time RT-PCR depend on the use of primers or probes designed to eliminate or minimize detection of genomic DNA. If such primers or probes are not available, then genomic DNA contamination in RNA samples must be removed.

Reverse transcription

After genomic DNA removal, the RNA sample is ready for reverse transcription using a reverse-transcription master mix prepared from QuantiNova Reverse Transcription Enzyme and QuantiNova Reverse Transcription Mix. After a 3 minute primer annealing step at 25°C, the reaction takes place at 45°C for 10 minutes and is then inactivated at 85°C.

The QuantiNova Reverse Transcription Enzyme has a high affinity for RNA and is optimized for efficient and sensitive cDNA synthesis from 10 pg to 5 µg of RNA. Its high RNA affinity, in combination with QuantiNova Reverse Transcription Mix, enables high cDNA yields even from templates with high GC-content or complex secondary structure.

The blend of primers contained in the QuantiNova Reverse Transcription Mix ensures cDNA synthesis from all regions of RNA transcripts, even from 5' regions. This allows high yields of cDNA template for real-time PCR analysis regardless of where the target region is located on the transcript.

Reverse transcription



Mix RNA, gDNA Removal Mix, QN IC RNA (optional) and RNase-free water

Incubate at 45°C for 2 min, then place on ice



Combine QuantiNova Reverse Transcription Enzyme with QuantiNova Reverse Transcription Mix, then add to template and mix

Incubate at 25°C for 3 min and at 45°C for 10 min



Incubate at 85°C for 5 min to inactivate the QuantiNova Reverse Transcription Enzyme



Add cDNA to real-time PCR mix and distribute

Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

For genomic DNA elimination and reverse transcription:

- Plastic tubes (for 20 µl reactions)
- Ice
- PCR cycler, heating block or water bath (capable of reaching 85°C)
- Vortexer
- Microcentrifuge
- Optional: gene-specific primers

For quantitative real-time PCR:

- Optimized kit for quantitative real-time PCR typically including Taq polymerase, quantitative real-time PCR buffer, primers, probe or SYBR® Green I dye and nucleotides (for details, see Appendix C, page 30).
- QIAGEN's QuantiNova PCR Kits are optimized, ready-to-run kits for highly specific and sensitive real-time PCR.

Kits are available for SYBR Green, probe or multiplex detection. For more details, visit www.qiagen.com/PCR. For Ordering Information, see page 34.

Protocol: Reverse Transcription with Genomic DNA Removal for Quantitative Real-Time PCR

Important points before starting

- The protocol is optimized for use with 10 pg to 5 µg of RNA. If using >5 µg RNA, scale up the reaction linearly to the appropriate volume as described in protocol step 2.
- Set up all reactions on ice to minimize the risk of RNA degradation. Although the 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.
- RNase inhibitor and dNTPs are already included in the kit components. Do not add additional RNase inhibitor and dNTPs.
- The QuantiNova Internal Control RNA (QN IC RNA) is an internal amplification control that is 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 (cat no. 205813) or the QuantiTect® Primer Assay for SYBR Green-based detection (cat no. QT02589307). Assays need to be ordered separately. The IC RNA provided in the QuantiNova Reverse Transcription Kit should be used undiluted.
- 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.
- A mixture of RT primers is included in the Reverse Transcription Mix. The RT primer mix is optimized to provide high cDNA yields for all RNA transcript regions.

- If gDNA removal is not desired, the removal step can be omitted, but all kit buffer components still need to be combined to enable an efficient RT reaction. To omit gDNA removal, prepare the reverse-transcription master mix by combining the Reverse Transcription Mix with the Reverse Transcription Enzyme and adding this to the gDNA Removal Mix. Finally, add RNA template and, optionally, IC RNA.
- It is recommended to set up the reactions in 200 µl PCR tubes and to use a PCR cyclor for the incubation steps.
- After reverse transcription, the reaction must be inactivated by incubation at 85°C for 5 minutes.
- The temperature steps can be conveniently set up using the cycling protocol described in Table 3.
- If working with RNA for the first time, read Appendix A, page 23.
- For details on performing real-time PCR after reverse transcription, see Appendix C, page 30. For details on appropriate controls, see Appendix D, page 32.

Procedure

1. Thaw template RNA, QN IC RNA (optional), gDNA Removal Mix and Reverse Transcription Enzyme on ice. Thaw Reverse Transcription Mix and RNase-free water at room temperature (15–25°C).

Mix each solution by flicking the tubes. Centrifuge briefly to collect residual liquid from the sides of the tubes, and then store on ice.

2. Prepare the genomic DNA removal reaction on ice according to Table 1.

Mix and then store on ice.

Note: If setting up more than one reaction, prepare a master mix of gDNA Removal Buffer and RNase-free water with a volume 10% greater than required for the total number of reactions. Distribute the appropriate volume of master mix into individual tubes, followed by each RNA sample. Keep the tubes on ice.

Note: The protocol is for use with 10 pg – 5 µg RNA. If using >5 µg RNA or a template volume exceeding 12 µl, scale up the reaction linearly while keeping the volume for the QN IC RNA constant. For example, if using 10 µg RNA, double the volumes of all reaction components except the QN IC RNA. Do not exceed a final volume of 30 µl for the gDNA removal reaction.

Table 1. Genomic DNA removal reaction components

Component	Volume/reaction
gDNA Removal Mix*	2 µl
Template RNA, up to 5 µg†	Variable
Internal Control RNA (optional)	1 µl
RNase-Free Water	Variable
Total reaction volume	15 µl

* Also contains RNase inhibitor.

† This amount corresponds to the entire amount of RNA present, including any rRNA, mRNA, viral RNA, and carrier RNA present, regardless of the primers used or cDNA analyzed.

3. Incubate for 2 min at 45°C. Then place immediately on ice.

Note: Do not incubate at 45°C for longer than 10 min.

4. Prepare the reverse-transcription master mix on ice according to Table 2.

Mix and then store on ice. The reverse-transcription master mix contains all components required for first-strand cDNA synthesis except template RNA.

Note: If setting up more than one reaction, prepare a volume 10% greater than required for the total number of reactions. Distribute the appropriate volume into individual tubes.

Note: If using >5 µg RNA, scale up the reverse-transcription reaction linearly but keep the QN IC RNA volume constant. For example, if using 10 µg RNA, double the volumes of all reverse-transcription reaction components whilst keeping the volume for the QN IC RNA constant. Do not exceed a final volume of 40 µl.

Table 2. Reverse-transcription reaction components

Component	Volume/reaction
Reverse Transcription Enzyme	1 μ l
Reverse Transcription Mix*	4 μ l
Template RNA	1.5 μ l
Entire genomic DNA elimination reaction (step 3)	(added at step 5)
Total reaction volume	20 μl

* Includes Mg^{2+} and dNTPs.

5. Add freshly prepared reverse-transcription master mix to each tube containing template RNA from step 3 (1.5 μ l).

Mix and then store on ice.

Note: Turn off the lid heater if using a PCR cyclor with a heated lid. The recommended temperatures in Table 3 will not cause vaporization.

Table 3. gDNA elimination and RT temperature protocol

Step	Time	Temperature	
gDNA elimination reaction	2 min	45°C	
	Pause cyclor	25°C	Remove samples, place on ice, add RT components
Reverse-transcription reaction:			After adding RT components, place samples in the cyclor again and continue
Annealing step	3 min	25°C	
Reverse-transcription step	10 min	45°C	
Inactivation of reaction	5 min	85°C	

6. Incubate for 3 min at 25°C.
7. Incubate for 10 min at 45°C.

In some rare cases (e.g., if the RT-PCR product is longer than 200 bp or if analyzing RNAs with a very high degree of secondary structure), increasing the incubation time up to 20 min may increase cDNA yields.

8. Incubate for 5 min at 85°C to inactivate the Reverse Transcriptase Enzyme.
9. Place the reverse-transcription reactions on ice and proceed directly with real-time PCR. We recommend diluting the reverse-transcription reaction 1:10 in sterile water. This enables the analysis of multiple transcripts from a single reverse-transcription reaction. For long-term storage, store the reverse-transcription reactions at –30 to –15°C (see Appendix C, page 30).

Note: QuantiNova SYBR Green PCR or QuantiNova Probe PCR Kits are recommended for real-time PCR, especially when using the QN IC RNA. For detailed information on use of the QN IC RNA and interpretation of real-time PCR results, please refer to the next section “Analysis and interpretation of Internal Control Assay results” and the *QuantiNova Internal Control RNA and Assay Quick-Start Protocol*.

Note: QuantiNova IC Assays, for the detection of QN IC RNA, need to be ordered separately as the QuantiNova IC Probe Assay (cat. no. 205813) or the Ctrl_QNIC_1_SG QuantiTect Primer Assay (cat. no. QT02589307, for SYBR Green detection) at GeneGlobe® (www.qiagen.com/geneglobe).

Analysis and interpretation of Internal Control Assay results

- The QuantiNova Internal Control RNA (QN IC RNA) is a synthetic RNA that can be optionally 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 of 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.
- After using the QuantiNova Reverse Transcription Kit, cDNA should be diluted (1:10–1:100) and an aliquot of the reaction should be used for subsequent amplification with the QuantiNova SYBR Green or Probe PCR Kits. For probe-based detection, use the appropriate volume of 10x QuantiNova IC Probe Assay (cat. no. 205813) and detection in the VIC/HEX dye channel of your real-time PCR instrument. For detection with the QuantiNova SYBR Green PCR Kit, use the appropriate volume of 10x Ctrl_QNIC_1_SG QuantiTect Primer Assay (cat. no. QT02589307).
- An additional no-template RNA control sample, which only contains the QN IC RNA, should also be processed in reverse transcription and subsequent qPCR. 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.
 1. After amplification, perform data analysis as recommended for your real-time PCR instrument.
 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.

Expected C_q value for successful RT-PCR (e.g., no inhibition of QN IC RNA)

The C_q value for the QN IC RNA in two-step RT-PCR using the QuantiNova Reverse Transcription Kit will vary depending on various parameters, such as:

- Detection format (SYBR Green or probe)
- Dilution factor of the cDNA after reverse transcription
- Volume of template cDNA added to the PCR
- Type of real-time PCR instrument
- C_q determination (threshold setting, Auto- C_q determination)

An example of typical results for the QN IC RNA:

- Dilution factor: 10
- Volume of cDNA added to PCR: 2 μ l
- Real-time instrument: Rotor-Gene Q
- C_q determination: manual threshold setting
- C_q value with QuantiNova SYBR Green PCR Kit: 21
- C_q value with QuantiNova Probe PCR Kit: 27

This example illustrates that C_q values for the QN IC RNA in two-step RT-PCR, using the QuantiNova Reverse Transcription Kit, will not be identical under all circumstances. However, the C_q values should be consistent under identical conditions; therefore, the QN IC RNA can be used to monitor successful reverse transcription and RT-PCR. A C_q difference >2 is likely to indicate sample inhibition or failure.

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 product, or product detected late in real-time PCR (problems occurring during reverse transcription)

- | | |
|--|---|
| a) Pipetting error or missing reagent when setting up reverse-transcription reaction | Check the pipets used for experimental setup. Mix all reagents well after thawing, and repeat the reverse-transcription reaction. |
| b) Incorrect setup of reverse-transcription reaction | Be sure to set up the reaction on ice. |
| c) Volume of reverse-transcription reaction added to the real-time PCR was too high | Adding a high volume of reverse-transcription reaction to the PCR mix may reduce amplification efficiency and the linearity of the reaction. Generally, the volume of reverse-transcription reaction added should not exceed 10% of the final PCR volume. |
| d) Temperature of reverse-transcription reaction | Reverse transcription should be carried out at 45°C. Check the temperature of your heating block or water bath. In rare cases, when analyzing RNAs with a very high degree of secondary structure, it may be advantageous to increase the temperature up to 50°C. However, temperatures >45°C will reduce the activity of the reverse transcription enzyme and therefore affect the cDNA yield. |
| e) Short incubation time | The standard reverse-transcription reaction requires a 10-min incubation. In rare cases, when analyzing RNAs with a very high degree of secondary structure or if the RT-PCR product is longer than 200 bp, it may be advantageous to increase the incubation time to 20 min. |

Comments and suggestions

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|--|--|
| f) Poor quality or incorrect amount of template RNA for reverse-transcription reaction | Check the concentration, integrity and purity of the template RNA (see Appendix B, page 26) before starting the protocol. Mix well after thawing the template RNA. Even minute amounts of RNases can affect synthesis of cDNA and sensitivity in RT-PCR, particularly with small amounts of RNA. |
| g) RNA concentration too high or too low | Reverse transcription enzyme is designed for use with 10 pg to 5 µg RNA. If using >5 µg RNA, scale up the reaction linearly to the appropriate volume. |
| h) RNA denatured | Denaturation of the template RNA is not necessary. If denaturation was performed, the integrity of the RNA may be affected. |
| i) Incubation temperature too high | Reverse transcription should be carried out at 45°C. Higher temperatures may reduce the length of cDNA products or the activity of the reverse transcription enzyme. Check the temperature of your heating block or water bath. |

No product, or product detected late in real-time PCR or only primer-dimers detected (problems occurring during real-time PCR)

- | | |
|---|---|
| a) PCR annealing time too short | Use the annealing time specified in the protocol for the real-time PCR kit you are using. |
| b) PCR extension time too short | Use the extension time specified in the protocol for the real-time PCR kit you are using. |
| c) Mg ²⁺ concentration in PCR not optimal | Always start with the Mg ²⁺ concentration recommended in the protocol for the real-time PCR kit you are using. Perform titration in 0.5 mM steps. |
| d) Pipetting error or missing reagent when setting up PCR | Check the concentrations and storage conditions of reagents, including primers and cDNA. |
| e) <i>Taq</i> DNA Polymerase not activated with a hot-start | Ensure that the cycling program includes the hot-start activation step for <i>Taq</i> DNA polymerase; for details, check the instructions supplied with the polymerase. |
| f) PCR product too long | For optimal results, PCR products should be 100–150 bp in length and should not exceed 300 bp. |
| g) Primer design for real-time PCR not optimal | Check for the presence of PCR products by gel electrophoresis or melting curve analysis. If no specific PCR products are detected, review the primer design. |
| h) Primer concentration for real-time PCR not optimal | Use the primer concentrations recommended in the protocol for the real-time PCR kit you are using. |
| i) Insufficient number of cycles | Increase the number of cycles. |
| j) PCR annealing/extension temperature too high | Decrease annealing/extension temperature in 2°C steps. |
| k) PCR annealing/extension temperature too low | Increase annealing/extension temperature in 2°C steps. |

Comments and suggestions

l) No detection activated	Check that fluorescence detection was activated in the cycling program.
m) Wrong detection step	Ensure that fluorescence detection takes place during the extension step of the PCR cycling program.
n) Real-time PCR primers/probes degraded	Check for possible degradation of primers/probes on a denaturing polyacrylamide gel.
o) Wrong dye layer/filter chosen	Ensure that the appropriate layer/filter is activated.
p) Insufficient starting template	Increase the amount of template cDNA, if possible.
q) Primer-dimers co-amplified in real-time PCR with SYBR Green I	Include an additional data acquisition step above the T_m of the primer-dimer but below the T_m of the specific product in the cycling program to avoid the detection of primer-dimers.

Multiple peaks in melting temperature analysis/multiple PCR products

a) Reaction set up at room temperature	To avoid non-specific primer annealing, set up the real-time PCR in cooled reaction vessels and/or use a Taq DNA polymerase which requires a hot-start. The use of the QuantiNova SYBR Green and Probe PCR Kits enables reaction setup at room temperature due to the stringent PCR hot-start.
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High fluorescence in "No RT" control reactions

a) Contamination with genomic DNA	Check that the genomic DNA elimination step with gDNA Removal Mix was performed correctly. Check the temperature of your heating block or water bath and the concentration of the reaction components. When purifying RNA, we recommend using RNeasy Plus Kits or RNeasy Plus Universal Kits, which remove genomic DNA contamination (see page 34 for Ordering Information).
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No linearity in ratio of C_q value/crossing point to log of the template amount

a) Template amount too high	Do not exceed maximum recommended amounts of template cDNA. For details, see the protocol for the real-time PCR kit you are using.
b) Template amount too low	Increase amount of template RNA, if possible.

High fluorescence in "No Template" control

a) Contamination of reagents	Discard reaction components and repeat with new reagents.
b) Contamination during reaction setup	Take appropriate safety precautions (e.g., use filter tips).

Varying fluorescence intensity

a) Real-time cyclers contaminated	Decontaminate the real-time cycler according to the supplier's instructions.
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Comments and suggestions

- | | |
|---|---|
| b) Real-time cyclers no longer calibrated | Recalibrate the real-time cyclers according to the supplier's instructions. |
|---|---|

Appendix A: General Remarks on Handling RNA

Handling RNA

Ribonucleases (RNases) are very stable and active enzymes that generally do not require cofactors to function. Since RNases are difficult to inactivate and even minute amounts are sufficient to degrade RNA, do not use any plasticware or glassware without first eliminating possible RNase contamination. Although the QuantiNova Reverse Transcription Kit contains an RNase inhibitor, we still recommend that care should be taken to avoid inadvertently introducing RNases into the RNA sample during or after the purification procedure. In order to create and maintain an RNase-free environment, the following precautions must be taken during pretreatment and use of disposable and non-disposable vessels and solutions while working with RNA.

General handling

Proper microbiological, aseptic technique should always be used when working with RNA. Hands and dust particles may carry bacteria and molds and are the most common sources of RNase contamination. Always wear latex or vinyl gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin or from dusty laboratory equipment. Change gloves frequently and keep tubes closed whenever possible.

Disposable plasticware

The use of sterile, disposable polypropylene tubes is recommended throughout the procedure. These tubes are generally RNase-free and do not require pretreatment to inactivate RNases.

Non-disposable plasticware

Non-disposable plasticware should be treated before use to ensure that it is RNase-free. Plasticware should be thoroughly rinsed with 0.1 M NaOH,* 1 mM EDTA* followed by RNase-free water. Alternatively, chloroform-resistant plasticware can be rinsed with chloroform* to inactivate RNases.

Glassware

Glassware should be treated before use to ensure that it is RNase-free. Glassware used for RNA work should be cleaned with a detergent,* thoroughly rinsed, and oven baked at 240°C for 4 hours or more (overnight, if more convenient) before use. Autoclaving alone will not fully inactivate many RNases. Alternatively, glassware can be treated with DEPC* (diethyl pyrocarbonate). Fill glassware with 0.1% DEPC (0.1% in water), allow to stand overnight (12 hours) at 37°C, and then autoclave or heat to 100°C for 15 minutes to eliminate residual DEPC.

Solutions

Solutions (water and other solutions) should be treated with 0.1% DEPC. DEPC is a strong, but not absolute, inhibitor of RNases. It is commonly used at a concentration of 0.1% to inactivate RNases on glass or plasticware or to create RNase-free solutions and water. DEPC inactivates RNases by covalent modification. Add 0.1 ml DEPC to 100 ml of the solution to be treated and shake vigorously to bring the DEPC into solution. Let the solution incubate for 12 hours at 37°C. Autoclave for 15 minutes to remove any trace of DEPC. DEPC will react with primary amines and cannot be used directly to treat Tris* buffers. DEPC is highly unstable in the presence of Tris buffers and decomposes rapidly into ethanol and CO₂. When preparing Tris buffers, treat water with DEPC first, and then dissolve Tris to make the appropriate buffer. Trace amounts of DEPC will modify purine residues in RNA by carbethoxylation. Carbethoxylated RNA is translated with very low efficiency in cell-free

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate data sheets (SDSs), available from the product supplier.

systems. However, its ability to form DNA:RNA or RNA:RNA hybrids is not seriously affected unless a large fraction of the purine residues have been modified. Residual DEPC must always be eliminated from solutions or vessels by autoclaving or heating to 100°C for 15 minutes.

Appendix B: Preparation, Storage, Quantification, and Determination of Quality of RNA

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.

Preparation of RNA

Reverse transcriptases are used *in vitro* for first-strand cDNA synthesis with RNA as the starting template. The efficiency of the reaction is highly dependent on the quality and quantity of the starting RNA template.

It is important to have intact RNA as starting template. Even trace amounts of contaminating RNases in the RNA sample can cause RNA cleavage, resulting in shortened cDNA products. Chemical impurities, such as protein, poly-anions (e.g., heparin), salts, EDTA, ethanol and phenol, can affect the activity and processivity of the reverse transcriptase. To ensure reproducible and efficient reverse transcription, it is important to determine the quality and quantity of the starting RNA (see below).

For best results, we recommend starting with RNA purified using silica-gel-membrane technology. For Ordering Information, see page 34.

Storage of RNA

Purified RNA may be stored at -20°C or -70°C in water. Under these conditions, no degradation of RNA is detectable after 1 year.

Quantification of RNA

The concentration of RNA should be determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer. To ensure significance, readings should be greater than 0.15. An absorbance of 1 unit at 260 nm corresponds to 44 μg of RNA per ml ($A_{260}=1 \rightarrow 44 \mu\text{g/ml}$). This relation is valid only for measurements at a neutral pH. Therefore, if it is necessary to dilute the RNA sample in a buffer with neutral pH. As discussed below (see "Purity of RNA", page 28), the ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity.

When measuring RNA samples, be certain that cuvettes are RNase-free especially if the RNA is to be recovered after spectrophotometry. This can be accomplished by washing cuvettes with 0.1 M NaOH,* 1 mM EDTA* followed by washing with RNase-free water (see "Solutions", page 24). Use the buffer in which the RNA is diluted to zero the spectrophotometer.

An example of the calculation involved in RNA quantification is shown below:

Volume of RNA sample = 100 μl

Dilution = 20 μl of RNA sample + 180 μl distilled water (1/10 dilution)

Measure absorbance of diluted sample in a 0.2 ml cuvette (RNase-free): $A_{260} = 0.2$

Concentration of RNA sample = 44 $\mu\text{g/ml}$ \times A_{260} \times dilution factor
= 44 $\mu\text{g/ml}$ \times 0.2 \times 10
= 88 $\mu\text{g/ml}$

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate data sheets (SDSs), available from the product supplier.

Total amount = concentration x volume of sample in ml
= 88 µg/ml x 0.1 ml
= 8.8 µg of RNA

Purity of RNA

The ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV, such as protein. However, the A_{260}/A_{280} ratio is influenced considerably by pH. Since water is not buffered, the pH and the resulting A_{260}/A_{280} ratio can vary greatly. Lower pH results in a lower A_{260}/A_{280} ratio and reduced sensitivity to protein contamination.* For accurate values, we recommend measuring absorbance in 10 mM Tris•Cl, pH 7.5.† Pure RNA has an A_{260}/A_{280} ratio of 1.9–2.1‡ in 10 mM Tris•Cl, pH 7.5. Always be sure to calibrate the spectrophotometer with the same solution.

For determination of RNA concentration, however, we still recommend dilution of the sample in a buffer with neutral pH since the relationship between absorbance and concentration (A_{260} reading of 1 = 44 µg/ml RNA) is based on an extinction coefficient calculated for RNA at neutral pH (see “Quantification of RNA”, page 27).

Integrity of RNA

The integrity and size distribution of total RNA can be checked by denaturing agarose gel electrophoresis and ethidium bromide* staining or by using the QIAxcel® system or Agilent® 2100 Bioanalyzer. The respective ribosomal RNAs should appear as sharp bands or peaks. The apparent ratio of 28S rRNA to 18S rRNA should be approximately 2:1. If the ribosomal bands or peaks of a specific sample are not sharp, but appear as a smear towards smaller

* Wilfinger, W.W., Mackey, M., and Chomczynski, P. (1997) Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. *BioTechniques* 22, 474.

† When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

‡ Values up to 2.3 are routinely obtained for pure RNA (in 10 mM Tris•Cl, pH 7.5) with some spectrophotometers.

sized RNAs, it is likely that the sample suffered major degradation either before or during RNA purification. The QIAxcel and Agilent 2100 Bioanalyzer also provide an RNA Integrity Score (RIS) or RNA Integrity Number (RIN) as useful measures of RNA integrity. Ideally, the RIS / RIN should be close to 10, but in many cases (particularly with tissue samples), RNA quality is greatly influenced by how well the original sample was preserved.

Appendix C: Quantitative Real-Time Two-Step RT-PCR

For the quantification of RNA transcripts, quantitative real-time RT-PCR is the most sensitive and reliable method. Real-time RT-PCR begins with the reverse transcription of RNA into cDNA, and is followed by PCR amplification of the cDNA. RNA is transcribed into single-stranded cDNA using random primers or oligo-dT primers that specifically hybridize to the poly A tail of mRNAs. The quantity of cDNA is determined during the exponential phase of PCR by the detection of fluorescence signals that exceed a certain threshold. Fluorescence signals are generated by fluorophores incorporated into the PCR product (e.g., in assays using SYBR Green I dye) or by fluorophores which are coupled to short oligonucleotide probes (i.e., in probe-based assays). In real-time RT-PCR, the level of RNA transcripts is calculated from the number of the PCR cycle at which the threshold is exceeded. This cycle is called the quantification cycle, threshold cycle or the crossing point. For reliable results in quantitative real-time PCR of cDNA generated using the QuantiNova Reverse Transcription Kit, we recommend using a QuantiNova Kit in combination with the internal control. In quantitative real-time two-step RT-PCR, cDNA is first synthesized by reverse transcription. An aliquot of the finished reverse-transcription reaction is then used for PCR. Reverse transcription and PCR are performed sequentially in two separate reaction tubes. In addition, cDNA can be stored for later analysis.

1. Carry out reverse transcription according to the protocol on page 11, using the QuantiNova Reverse Transcription Kit and 10 pg – 5 µg RNA.

-
2. Add an aliquot of each finished reverse-transcription reaction to real-time PCR mix. Before doing so, we recommend diluting the reverse-transcription reaction 1:10 in sterile water. This enables the analysis of multiple transcripts from a single reverse-transcription reaction.

Note: No more than 1/10 of the final PCR volume should derive from the finished reverse-transcription reaction. For example, for a 20 μ l PCR assay, use ≤ 2 μ l of the finished reverse-transcription reaction.

3. Carry out real-time PCR as recommended by the supplier. We recommend using a Rotor-Gene or QuantiNova Kit.

Appendix D: Recommended Controls for Quantitative Real-Time RT-PCR

No RT control

With the QuantiNova Reverse Transcription Kit, genomic DNA is efficiently removed in a single step. However, all reverse-transcription experiments should include a negative control to test for contaminating genomic DNA. Genomic DNA contamination can be detected by performing a control reaction in which no reverse transcription is possible. This control contains all components including template RNA except for the reverse transcription enzyme. Reverse transcription therefore cannot take place and the only template available is contaminating genomic DNA. In rare cases in which genomic DNA is still amplified, detection of contaminating DNA can be eliminated with specially designed primers or probes (Figure 1).

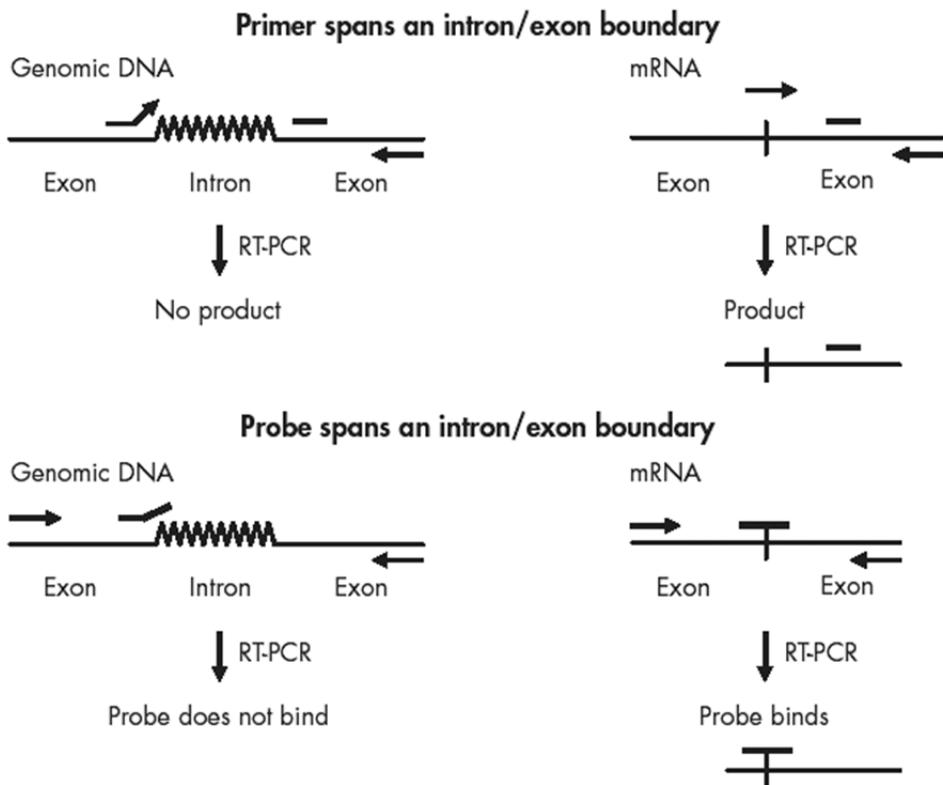


Figure 1. Primer/probe design. Primer/probe design to eliminate signals from contaminating genomic DNA.

No template control (NTC)

All real-time PCR quantification experiments should include an NTC containing all the components of the reaction except for the template. This enables detection of carryover contamination from previous experiments.

Ordering Information

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 Reverse Transcription Kit (50)	For 50 x 20 µl reactions: 100 µl 8x gDNA removal Mix, 50 µl Reverse Transcription Enzyme, 200 µl Reverse Transcription Mix (containing RT primers), 100 µl Internal Control, 1.9 ml RNase-Free Water	205411
QuantiNova Reverse Transcription Kit (200)	For 200 x 20 µl reactions: 4 x 100 µl 8x gDNA removal Mix, 4 x 50 µl Reverse Transcription Enzyme, 4 x 200 µl Reverse Transcription Mix (containing RT primers), 4 x 100 µl Internal Control, 4 x 1.9 ml RNase-Free Water	205413
QuantiNova IC Probe Assay (200)	For 200 x 20 µl reactions: 400 µl primer/probe mix (10x), detecting IC RNA	205813
QuantiNova IC SYBR Green Assay	For 500 x 20 µl reactions: QuantiTect Primer Assay for SYBR based detection of IC RNA, available via GeneGlobe	QT 02589307

Product	Contents	Cat. no.
QuantiNova SYBR Green PCR Kit (100)	For 100 x 20 µl reactions: 1 ml 2x QuantiNova SYBR Green PCR Master Mix, 500 µl QuantiNova Yellow Template Dilution Buffer, 250 µl QN ROX Reference Dye, 1.9 ml Water	208052
QuantiNova Probe PCR Kit (100)	For 100 x 20 µl reactions: 1 ml 2x QuantiNova Probe PCR Master Mix , 250 µl QN ROX Reference Dye, 500 µl QuantiNova Yellow Template Dilution Buffer, 1.9 ml Water	208252
QuantiNova 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
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
RNeasy MinElute Cleanup Kit (50)	50 RNeasy MinElute Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free Reagents and Buffers	74204

Product	Contents	Cat. no.
Rotor-Gene Multiplex PCR Kit – for ultrafast multiplex real-time PCR and two-step RT-PCR on the Rotor-Gene Q		
Rotor-Gene Multiplex PCR Kit (400)*	For 400 x 25 µl reactions: 3 x 1.7 ml 2x Master Mix, 2 x 2 ml RNase-Free Water	204774
QuantiFast Multiplex PCR Kits – for fast multiplex real-time PCR and two-step RT-PCR		
For all instruments from Applied Biosystems® except the Applied Biosystems 7500		
QuantiFast Multiplex PCR Kit (400)*	For 400 x 25 µl reactions: 3 x 1.7 ml 2x Master Mix (with ROX dye), 2 x 2 ml RNase-Free Water	204654
For the Applied Biosystems 7500 and instruments from other suppliers		
QuantiFast Multiplex PCR +R Kit (400)*	For 400 x 25 µl reactions: 3 x 1.7 ml 2x Master Mix (without ROX dye), 210 µl ROX Dye Solution, 2 x 2 ml RNase-Free Water	204754
Instruments		
Rotor-Gene Q 2plex HRM Platform	Real-time PCR cyclers and High Resolution Melt analyzer with 2 channels (green, yellow), HRM channel, laptop computer, software, accessories: 1-year warranty on parts and labor. Installation and training not included	9001560

Product	Contents	Cat. no.
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
Related products		
RNeasy Plus Kits – for purification of total RNA from cells and tissues using gDNA Eliminator columns or plates		
RNeasy Plus Micro Kit (50)	For 50 micropreps: RNeasy MinElute® Spin Columns, gDNA Eliminator Mini Spin Columns, Collection Tubes, Carrier RNA, RNase-Free Reagents and Buffers	74034
RNeasy Plus Mini Kit (50)	For 50 minipreps : RNeasy Mini Spin Columns, gDNA Eliminator Mini Spin Columns, Collection Tubes, RNase-Free Reagents and Buffers	74134
RNeasy Plus 96 Kit (12)	For 12 x 96 preps: gDNA Eliminator 96 Plates, RNeasy 96 Plates, Elution Microtubes CL, Caps, S-Blocks, AirPore Tape Sheets, RNase-Free Water and Buffers	74192

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