April 2005

QuantiTect® Reverse Transcription Handbook

For cDNA synthesis with integrated removal of genomic DNA contamination

For use in real-time, two-step RT-PCR

Contents

Kit Contents

QuantiTect Reverse Transcription Kit	**(50)**
Catalog no.	**205311**
Number of standard reactions*	**50**
gDNA Wipeout Buffer, 7x	100 µl
Quantiscript® Reverse Transcriptase†	50 µl
Quantiscript RT Buffer, 5x‡	200 µl
RT Primer Mix	50 µl
RNase-Free Water	1.1 ml
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* A standard reaction is 20 µl in volume with 10 pg to 1 µg total RNA.

† Also contains RNase inhibitor.

‡ Includes Mg^{2+} and dNTPs.

Shipping and Storage

The QuantiTect Reverse Transcription Kit is shipped on dry ice. The kit, including all reagents and buffers, should be stored immediately upon receipt at –20°C in a constant-temperature freezer.

Product Warranty and Satisfaction Guarantee

QIAGEN guarantees the performance of all products in the manner described in our product literature. The purchaser must determine the suitability of the product for its particular use. Should any product fail to perform satisfactorily due to any reason other than misuse, QIAGEN will replace it free of charge or refund the purchase price. We reserve the right to change, alter, or modify any product to enhance its performance and design. If a QIAGEN® product does not meet your expectations, simply call your local Technical Service Department or distributor. We will credit your account or exchange the product — as you wish.

A copy of QIAGEN terms and conditions can be obtained on request, and is also provided on the back of our invoices. If you have questions about product specifications or performance, please call QIAGEN Technical Services or your local distributor (see back cover).

Product Use Limitations

The QuantiTect Reverse Transcription Kit is developed, designed, and sold for research purposes only. It is not to be used for human diagnostic or drug purposes or to be administered to humans unless expressly cleared for that purpose by the Food and Drug Administration in the USA or the appropriate regulatory authorities in the country of use. All due care and attention should be exercised in the handling of many of the materials described in this text.

Technical Assistance

At QIAGEN we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in molecular biology and the use of QIAGEN products. If you have any questions or experience any difficulties regarding the QuantiTect Reverse Transcription Kit or QIAGEN products in general, please do not hesitate to contact us.

QIAGEN customers are a major source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at QIAGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

For technical assistance and more information please call one of the QIAGEN Technical Service Departments or local distributors (see back cover).

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs). These are available online in convenient and compact PDF format at www.qiagen.com/ts/msds.asp where you can find, view, and print the MSDS for each QIAGEN kit and kit component.

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

Quality Control

As part of the stringent QIAGEN quality assurance program, the performance of the QuantiTect Reverse Transcription Kit is monitored routinely on a lot-to-lot basis. All components are tested separately to ensure highest performance and reliability.

Product Description

The QuantiTect Reverse Transcription Kit contains:

gDNA Wipeout Buffer, 7x:	Buffer for effective elimination of genomic DNA contamination from starting RNA samples.
Quantiscript Reverse Transcriptase:	An optimized blend of enzymes developed for use in real-time, two-step PCR, comprising Quantiscript Reverse Transcriptase and an RNase inhibitor. Quantiscript Reverse Transcriptase is a unique mix of Omniscript® and Sensiscript® Reverse Transcriptases, which are recombinant heterodimeric enzymes expressed in *E. coli*. The RNase inhibitor is a 50 kDa protein that strongly inhibits RNases A, B, and C as well as human placental RNases.
Quantiscript RT Buffer, 5x:	Buffer optimized for reverse transcription with Quantiscript Reverse Transcriptase; contains dNTPs.
RT Primer Mix:	Optimized blend of oligo-dT and random primers dissolved in water. RT Primer Mix allows high cDNA yields from all regions of RNA transcripts, even from 5' regions.
RNase-Free Water:	Ultrapure quality, PCR-grade

Introduction

The QuantiTect Reverse Transcription Kit provides a fast and convenient procedure for efficient reverse transcription and effective genomic DNA elimination. The kit is dedicated for use in real-time, two-step RT-PCR, and provides high cDNA yields for sensitive quantification of even low-abundance transcripts.

Principle and procedure

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

Elimination of genomic DNA

The purified RNA sample is briefly incubated in gDNA Wipeout Buffer at 42°C for 2 minutes to effectively remove 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 eliminated.

Reverse transcription

After genomic DNA elimination, the RNA sample is ready for reverse transcription using a master mix prepared from Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, and RT Primer Mix. The entire reaction takes place at 42°C and is then inactivated at 95°C. In contrast to other methods, additional steps for RNA denaturation, primer annealing, and RNase H digestion are not necessary.

Quantiscript Reverse Transcriptase has a high affinity for RNA and is optimized for efficient and sensitive cDNA synthesis from 10 pg to 1 µg of RNA. This high RNA affinity, in combination with Quantiscript RT Buffer, enables high cDNA yields, even from templates with high GC-content or complex secondary structure.

RT Primer 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.

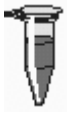
Quantiscript Reverse Transcription Procedure

Mix RNA,
gDNA Wipeout Buffer,
and RNase-free water

Incubate at 42°C for 2 min

Add Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, and RT Primer Mix, and mix

Incubate at 42°C for 15 min



Incubate at 95°C for 3 min to inactivate Quantiscript Reverse Transcriptase

Add cDNA to real-time PCR mix and distribute

Quantitative, real-time PCR

Enzymatic activities of reverse transcriptase

Reverse transcriptase enzymes are generally derived from RNA-containing retroviruses such as avian myeloblastosis virus (AMV), Moloney murine leukemia virus (MMLV), or human immunodeficiency virus (HIV). Quantiscript Reverse Transcriptase is from a new source.

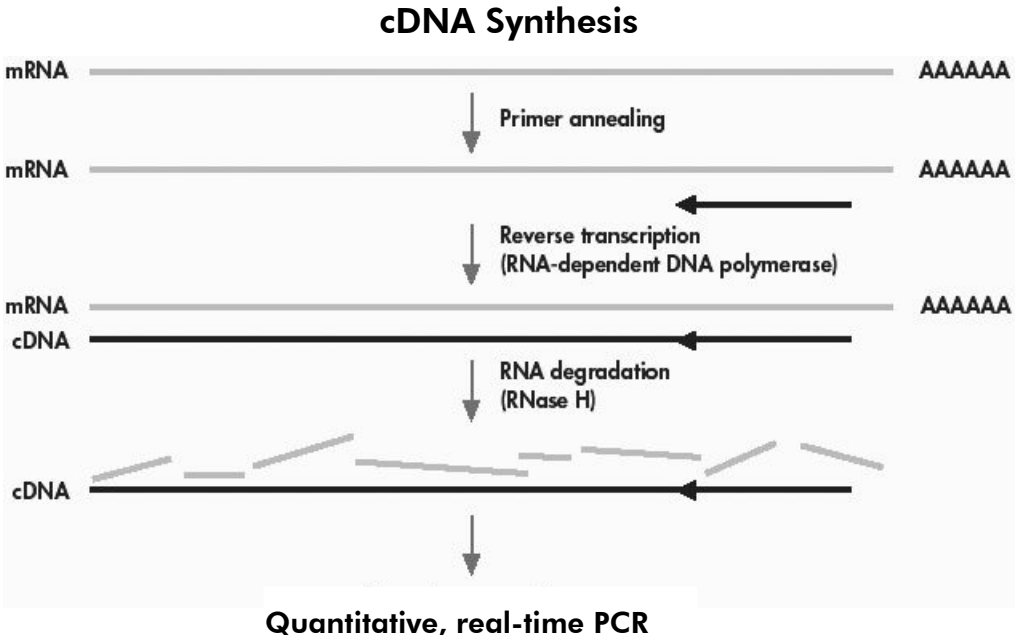


Figure 1 Quantiscript Reverse Transcriptase in first-strand cDNA synthesis.

In general, reverse transcriptase is a multifunctional enzyme with 3 distinct enzymatic activities: an RNA-dependent DNA polymerase, a hybrid-dependent exoribonuclease (RNase H), and a DNA-dependent DNA polymerase. In vivo, the combination of these 3 activities allows transcription of the single-stranded RNA genome into double-stranded DNA for retroviral infection. For reverse transcription in vitro (Figure 1), the first 2 activities are utilized to produce single-stranded cDNA:

- **RNA-dependent DNA-polymerase activity** (reverse transcription) transcribes cDNA from an RNA template. This activity of Quantiscript Reverse Transcriptase allows synthesis of cDNA for use in quantitative, real-time PCR.
- **RNase H activity** of Quantiscript Reverse Transcriptase specifically degrades only the RNA in RNA:DNA hybrids. Therefore, this RNase H activity affects RNA hybridized to cDNA, but has no effect on pure RNA. A separate RNA degradation step using RNase H enzyme is not necessary prior to real-time PCR. Furthermore, the Quantiscript RNase H activity, acting during reverse transcription, may improve the sensitivity of subsequent real-time PCR.

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 material safety data sheets (MSDSs), available from the product supplier.

For genomic DNA elimination and reverse transcription:

- Plastic tubes (for 20 µl reactions)
- Ice
- Heating block or water bath (capable of reaching 95°C)
- Vortexer
- Microcentrifuge
- Optional: gene-specific primers

For quantitative, real-time PCR:

- Optimized kit for quantitative, real-time PCR, which includes *Taq* polymerase, quantitative, real-time PCR buffer, primers, probe or SYBR® Green I dye, and nucleotides (for details, see Appendix C, page 24).
- QIAGEN offers the following ready-to-run solutions for quantitative, real-time PCR using SYBR Green I dye:
  - QuantiTect SYBR Green PCR Kit — preoptimized master mix
  - QuantiTect Primer Assays — functionally validated primer sets

  QIAGEN also offers the following ready-to-run solutions for quantitative, real-time PCR using sequence-specific probes:
  - QuantiTect Probe PCR Kit — preoptimized master mix for single PCR
  - QuantiTect Multiplex PCR Kits — preoptimized master mix for multiplex PCR
  - QuantiTect Gene Expression Assays — functionally validated primer–probe sets
  - QuantiTect Custom Assays — custom-designed primer–probe sets
  - QuantiTect Endogenous Control Assays — functionally validated primer–probe sets for housekeeping genes for use in multiplex PCR

  For more details, visit www.qiagen.com . For ordering information, see page 27.

Protocol: Reverse Transcription with Elimination of Genomic DNA for Quantitative, Real-Time PCR

Important points before starting

- The protocol is optimized for use with 10 pg to 1 µg of RNA. If using >1 µg RNA, scale up the reaction linearly to the appropriate volume.
- Set up all reactions on ice to minimize the risk of RNA degradation.
- **RNase inhibitor and dNTPs are already included in the kit components.** Do not add additional RNase inhibitor and dNTPs.
- RT Primer Mix (supplied) or gene-specific primers (not supplied) should be used. RT Primer Mix is optimized to provide high cDNA yields for all regions of RNA transcripts.
- **For convenience, premix RT Primer Mix and 5x Quantiscript RT Buffer in a 1:4 ratio if RT Primer Mix will be used routinely for reverse transcription.** This premix is stable when stored at –20°C.
- Separate denaturation and annealing steps are not necessary before starting the reverse-transcription reaction.
- **After reverse transcription, the reaction must be inactivated by incubation at 95°C for 3 minutes.**
- If working with RNA for the first time, read Appendix A, page 19.
- For details on performing real-time PCR after reverse transcription, see Appendix C, page 24. For details on appropriate controls, see Appendix D, page 25.
- **FastLane Cell cDNA Kit users:** If you have purchased the QuantiTect Reverse Transcription Kit in order to perform additional reverse-transcription reactions with the FastLane Cell cDNA Kit, follow the protocol in the *FastLane Cell cDNA Handbook*. Do not follow the protocol in the *QuantiTect Reverse Transcription Handbook*.

Procedure

1. **Thaw template RNA on ice. Thaw gDNA Wipeout Buffer, Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, RT Primer 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 elimination reaction on ice according to Table 1.**

   Mix and then store on ice.

   Note: If setting up more than one reaction, prepare a volume of master mix 10% greater than that required for the total number of reactions to be performed. Then 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 to 1 µg RNA. If using >1 µg RNA, scale up the reaction linearly. For example, if using 2 µg RNA, double the volumes of all reaction components for a final 28 µl reaction volume.

Table 1. Genomic DNA Elimination Reaction Components

Component	Volume/reaction	Final concentration
gDNA Wipeout Buffer, 7x	2 µl	1x
Template RNA	Variable (up to 1 µg*)	
RNase-free water	Variable	
Total volume	**14 µl**	**–**

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

3. **Incubate for 2 min at 42°C. Then place immediately on ice.**

   Note: Do not exceed an incubation time of 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 of master mix 10% greater than that required for the total number of reactions to be performed.

Note: The protocol is for use with 10 pg to 1 µg RNA. If using >1 µg RNA, scale up the reaction linearly. For example, if using 2 µg RNA, double the volumes of all reaction components for a final 40 µl reaction volume.

Table 2. Reverse-Transcription Reaction Components

Component	Volume/reaction	Final concentration
Reverse-transcription master mix		
Quantiscript Reverse Transcriptase*	1 µl	
Quantiscript RT Buffer, 5x†‡	4 µl	1x
RT Primer Mix‡	1 µl	
Template RNA		
Entire genomic DNA elimination reaction (step 3)	14 µl (add at step 5)	
Total volume	**20 µl**	**–**

* Also contains RNase inhibitor.

† Includes Mg^{2+} and dNTPs.

‡ For convenience, premix RT Primer Mix and 5x Quantiscript RT Buffer in a 1:4 ratio if RT Primer Mix will be used routinely for reverse transcription. This premix is stable when stored at –20°C. Use 5 µl of the premix per 20 µl reaction.

5. **Add template RNA from step 3 (14 µl) to each tube containing reverse-transcription master mix.**

   Mix and then store on ice.

6. **Incubate for 15 min at 42°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 30 min may increase cDNA yields.

7. **Incubate for 3 min at 95°C to inactivate Quantiscript Reverse Transcriptase.**

8. **Add an aliquot of each finished reverse-transcription reaction to real-time PCR mix (see Appendix C, page 24).**

   Store reverse-transcription reactions on ice and proceed directly with real-time PCR, or for long-term storage, store reverse-transcription reactions at –20°C. For real-time PCR, we recommend using QuantiTect Kits and Assays (see page 10).

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocol in this handbook or molecular biology applications (see back cover for contact information).

	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 42°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 >42°C will reduce the activity of Quantiscript Reverse Transcriptase and therefore affect the cDNA yield.
e) Short incubation time	The standard reverse-transcription reaction requires a 15-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 30 min.

	Comments and suggestions
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 21) 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	Quantiscript Reverse Transcriptase is designed for use with 10 pg to 1 µg RNA. If using >1 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) Incorrect concentration or degradation of primers for reverse-transcription reaction	If using a gene-specific primer for reverse transcription, check the concentration and integrity of the primer. If necessary, perform reverse transcription with different primer concentrations or use the supplied RT Primer Mix. If using RT Primer Mix, be sure to use 1 µl of RT Primer Mix in a 20 µl reaction.
j) Incubation temperature too high	Reverse transcription should be carried out at 42°C. Higher temperatures may reduce the length of cDNA products or the activity of Quantiscript Reverse Transcriptase. Check the temperature of your heating block or water bath.
FastLane Cell cDNA Kit users	
k) Wrong protocol followed	If using the QuantiTect Reverse Transcription Kit to perform additional reverse-transcription reactions with the FastLane Cell cDNA Kit, follow the protocol in the *FastLane Cell cDNA Handbook*.

	Comments and suggestions
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) *Taq* DNA Polymerase not activated with a hot start	Ensure that the cycling program includes the hot start activation step for *Taq* 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 temperature too high	Decrease annealing temperature in 3°C steps.
k) PCR annealing temperature too low	Increase annealing temperature in 2°C steps.
l) No detection activated	Check that fluorescence detection was activated in the cycling program.

	Comments and suggestions
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 coamplified in real-time PCR with SYBR Green I	Include an additional data acquisition step in the cycling program to avoid the detection of primer–dimers.
r) Detection temperature too high in optional data acquisition step for real-time PCR with SYBR Green I	Ensure that the detection temperature is at least 3°C lower than the T_m of the specific product. When establishing a new primer-template system, always perform a 3-step cycling reaction first, without the optional fourth step.
Multiple peaks in melting temperature analysis/multiple PCR products	
Reaction set up at room temperature	To avoid nonspecific primer annealing, set up the real-time PCR in cooled reaction vessels and/or use a *Taq* DNA polymerase which requires a hot start.
High fluorescence in "No RT" control reactions	
Contamination with genomic DNA	Check that the genomic DNA elimination step with gDNA Wipeout Buffer was performed correctly: check the temperature of your heating block or water bath and the concentration of the reaction components.

	Comments and suggestions
No linearity in ratio of C_T 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 cycler contaminated	Decontaminate the real-time cycler according to the supplier's instructions.
b) Real-time cycler no longer calibrated	Recalibrate the real-time cycler 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 QuantiTect Reverse Transcription Kit contains 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 nondisposable 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.

Nondisposable plasticware

Nondisposable 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 (see "Solutions", page 20). Alternatively, chloroform-resistant plasticware can be rinsed with chloroform* to inactivate RNases.

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

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 CO_2. 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 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.

Note: QIAGEN solutions, such as Quantiscript RT Buffer and RNase-free water, are guaranteed RNase-free without using DEPC treatment and are therefore free of any DEPC contamination.

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

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

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 27.

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 40 µg of RNA per ml ($A_{260}=1 \rightarrow 40$ µg/ml). This relation is valid only for measurements in water. Therefore, if it is necessary to dilute the RNA sample, this should be done in water. As discussed below (see "Purity of RNA", page 22), 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 20). Use the solution in which the RNA is diluted to zero the spectrophotometer.

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

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	= 40 µg/ml x A_{260} x dilution factor
	= 40 µg/ml x 0.2 x 10
	= 80 µg/ml
Total amount	= concentration x volume of sample in ml
	= 80 µg/ml x 0.1 ml
	= 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 water since the relationship between absorbance and concentration (A_{260} reading of 1 = 40 µg/ml RNA) is based on an extinction coefficient calculated for RNA in water (see "Quantification of RNA", page 21).

* 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 material data sheets (MSDSs), 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.

Integrity of RNA

The integrity and size distribution of total RNA can be checked by denaturing agarose gel electrophoresis and ethidium bromide* staining. The respective ribosomal bands (Table 3) should appear as sharp bands on the stained gel. 28S ribosomal RNA bands should be present at approximately twice the amounts of the 18S RNA band. If the ribosomal bands in a given lane are not sharp, but appear as a smear towards smaller sized RNAs, it is likely that the RNA sample suffered major degradation during preparation.

Table 3. Size of Ribosomal RNAs (rRNA) from Various Sources

Source	rRNA	Size (kb)
Mouse	18S	1.9
	28S	4.7
Human	18S	1.9
	28S	5.0

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

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, gene-specific 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 threshold cycle or the crossing point. For reliable results in quantitative, real-time PCR of cDNA generated using the QuantiTect Reverse Transcription Kit, we recommend using QuantiTect Kits and Assays (see page 10 for more information).

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 2 separate reaction tubes. With the QuantiTect Reverse Transcription Kit, RT Primer Mix (supplied) or gene-specific primers (not supplied) can be used to synthesize cDNA for quantitative, real-time, two-step RT-PCR. In addition, cDNA can be stored for later analysis.

C1. Carry out reverse transcription according to the protocol on page 11, using the QuantiTect Reverse Transcription Kit and 10 pg to 1 µg RNA.

C2. Add an aliquot of each finished reverse-transcription reaction to real-time PCR mix.

Note: No more than 1/10 of the final PCR volume should derive from the finished reverse-transcription reaction. For example, for a 50 µl PCR assay, use ≤5 µl of the finished reverse-transcription reaction.

C3. Carry out real-time PCR as recommended by the supplier.

We recommend using QuantiTect Kits and Assays (see page 10).

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

RT control

With the QuantiTect 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 Quantiscript Reverse Transcriptase. 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 2).

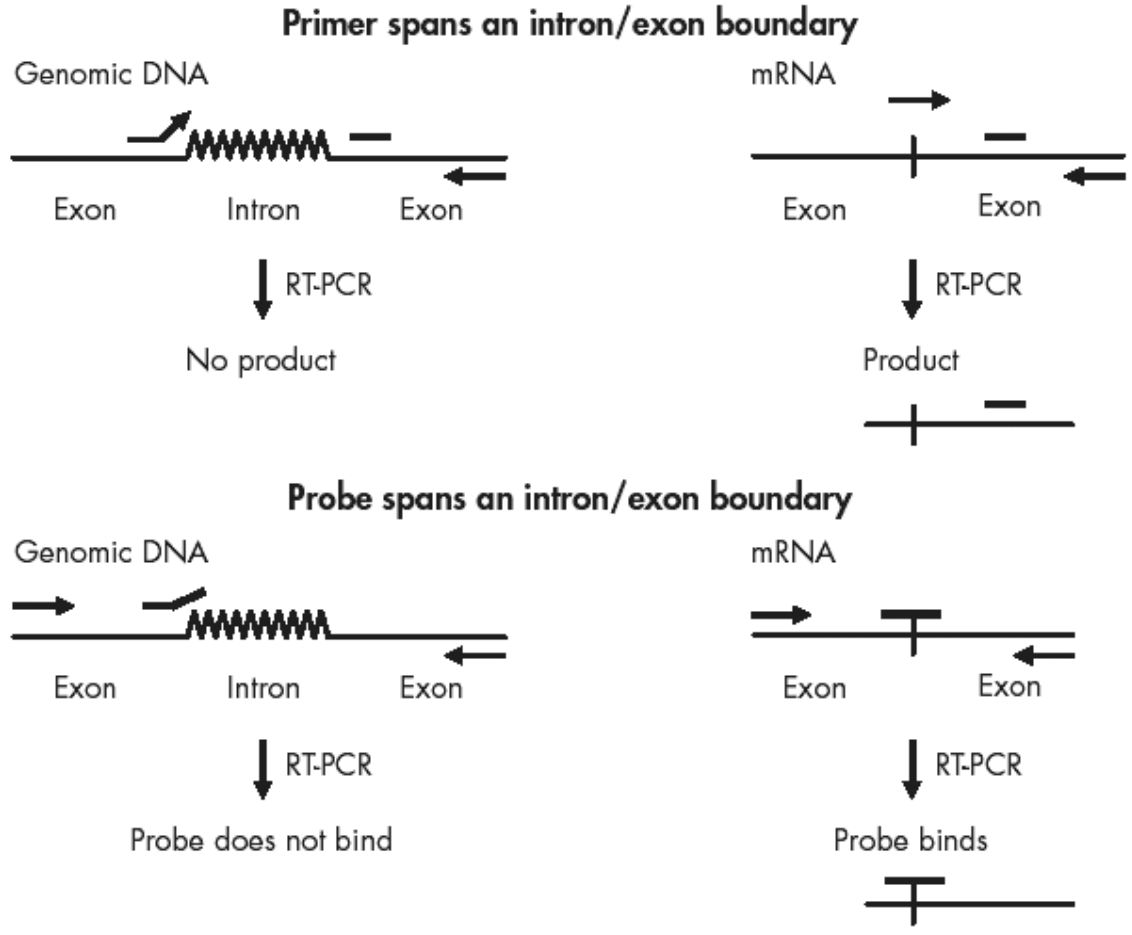


Figure 2 Primer/probe design to eliminate signals from contaminating genomic DNA.

Positive control

In some cases, it may be necessary to include a positive control containing a known concentration of template. This is usually a substitute for absolute standards and is used only to test for presence or absence of the target, but does not yield detailed quantitative information. Ensure that the positive control contains at least the minimum amount of RNA required for accurate detection.

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.

References

QIAGEN maintains a large, up-to-date online database of scientific publications utilizing QIAGEN products. Comprehensive search options allow you to find the articles you need, either by a simple keyword search or by specifying the application, research area, title, etc.

For a complete list of references, visit the QIAGEN Reference Database online at www.qiagen.com/RefDB/search.asp or contact QIAGEN Technical Services or your local distributor.

Ordering Information

Product	Contents	Cat. no.
QuantiTect Reverse Transcription Kit (50)	For 50 x 20 µl reactions: gDNA Wipeout Buffer, Quantiscript Reverse Transcriptase (contains RNase inhibitor), Quantiscript RT Buffer (contains dNTPs), RT Primer Mix, and RNase-Free Water	205311
Accessories		
QuantiTect SYBR Green PCR Kit — for quantitative, real-time PCR and two-step RT-PCR using SYBR Green I		
QuantiTect SYBR Green PCR Kit (200)*	For 200 x 50 µl reactions: 3 x 1.7 ml QuantiTect SYBR Green PCR Master Mix (providing a final concentration of 2.5 mM $MgCl_2$), 2 x 2 ml RNase-Free Water	204143
QuantiTect Primer Assays — for use in quantitative, real-time RT-PCR with SYBR Green detection		
QuantiTect Primer Assay (200)†	For 200 x 50 µl reactions (for use in a 96-well plate or single tubes) or 500 x 20 µl reactions (for use in a 384-well plate or single capillaries): 10x QuantiTect Primer Assay (lyophilized)	Varies
QuantiTect Probe PCR Kit — for quantitative, real-time PCR and two-step RT-PCR using sequence-specific probes		
QuantiTect Probe PCR Kit (200)*	For 200 x 50 µl reactions: 3 x 1.7 ml QuantiTect Probe PCR Master Mix (providing a final concentration of 4 mM $MgCl_2$), 2 x 2 ml RNase-Free Water	204343

* Larger kit size available; for details, visit www.qiagen.com .

† Visit www.qiagen.com/geneglobe to search for and order a QuantiTect Primer Assay.

Product	Contents	Cat. no.
QuantiTect Multiplex PCR Kits — for quantitative, real-time, multiplex PCR of cDNA and genomic DNA targets using sequence-specific probes		
QuantiTect Multiplex PCR Kit (200)*	For 200 x 50 µl reactions: 3 x 1.7 ml QuantiTect Multiplex PCR Master Mix (contains ROX dye), 2 x 2 ml RNase-Free Water	204543
QuantiTect Multiplex PCR NoROX Kit (200)*	For 200 x 50 µl reactions: 3 x 1.7 ml QuantiTect Multiplex PCR NoROX Master Mix (contains no ROX dye), 2 x 2 ml RNase-Free Water	204743
QuantiTect Gene Expression Assays — for gene expression analysis using functionally validated, quantitative, real-time RT-PCR assays		
QuantiTect Gene Expression Assay (100)†	For 100 x 50 µl reactions (for use in a 96-well plate or single tubes) or 250 x 20 µl reactions (for use in a 384-well plate or single capillaries): 0.5 ml 10x QuantiTect Assay Mix (dyes available: FAM)	Varies
QuantiTect Custom Assays — for gene expression analysis using custom-designed, quantitative, real-time RT-PCR assays		
QuantiTect Custom Assay (100)‡	For 100 x 50 µl reactions (for use in a 96-well plate or single tubes) or 250 x 20 µl reactions (for use in a 384-well plate or single capillaries): 0.25 ml 20x Primer Mix, 0.25 ml 20x QuantiProbe™ (dyes available: FAM, Yakima Yellow™, TET)	–

* Larger kit size available; for details, visit www.qiagen.com .

† Visit www.qiagen.com/goto/assays to view the full range of QuantiTect Gene Expression Assays.

‡ Visit www.qiagen.com/goto/assays to design your own QuantiTect Custom Assays. QuantiTect Custom Assays are available in larger sizes. Yakima Yellow dye can substitute for VIC® or HEX dye.

Product	Contents	Cat. no.
QuantiTect Endogenous Assays — for gene expression analysis of endogenous control genes using functionally validated, quantitative, real-time RT-PCR assays		
QuantiTect Endogenous Control Assay (100)*	For 100 x 50 µl reactions (for use in a 96-well plate or single tubes) or 250 x 20 µl reactions (for use in a 384-well plate or single capillaries): 0.5 ml 10x QuantiTect Assay Mix (dyes available: Yakima Yellow)	Varies
Related products		
RNeasy® Kits† — for purification of up to 100 µg, 1 mg, or 6 mg total RNA from animal cells or tissues, yeast, or bacteria		
RNeasy Mini Kit (50)‡	50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-Free Reagents and Buffers	74104
RNeasy Midi Kit (10)‡	10 RNeasy Midi Spin Columns, Collection Tubes (15 ml), RNase-Free Reagents and Buffers	75142
RNeasy Maxi Kit (12)	12 RNeasy Maxi Spin Columns, Collection Tubes (50 ml), RNase-Free Reagents and Buffers	75162
FastLane Cell cDNA Kit — for high-speed preparation of cDNA without RNA purification for real-time RT-PCR		
FastLane Cell cDNA Kit (50)	Buffer FCW, Buffer FCP, and components for 50 x 20 µl reverse-transcription reactions (gDNA Wipeout Buffer, Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix, and RNase-Free Water)	215011

* Visit www.qiagen.com/goto/assays to view the full range of QuantiTect Endogenous Control Assays. Yakima Yellow dye can substitute for VIC or HEX dye.

† Other QIAGEN products for RNA stabilization and purification are available (e.g., for blood samples, for difficult-to-lyse tissues, for high-throught purification, and for automated purification). For details, see the brochure *High-Performance RNA for Gene Expression Analysis*, available at www.qiagen.com/literature/brochures/RNA_Bro.aspx .

‡ Larger kit size available; for details, visit www.qiagen.com .

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