March 2011

RT² qPCR Primer Assay Handbook

For gene expression analysis by real-time RT-PCR

Sample & Assay Technologies

QIAGEN Sample and Assay Technologies

QIAGEN is the leading provider of innovative sample and assay technologies, enabling the isolation and detection of contents of any biological sample. Our advanced, high-quality products and services ensure success from sample to result.

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- Purification of DNA, RNA, and proteins
- Nucleic acid and protein assays
- microRNA research and RNAi
- Automation of sample and assay technologies

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Contents

Kit Contents

RT² qPCR Primer Assay	**(200)**
Catalog no.	**330001**
Number of 25 µl reactions	**200**
200 µl RT² qPCR Primer Assay (10 µM) in a single tube	1 tube

Shipping and Storage

The RT² qPCR Primer Assays are shipped at ambient temperature but must be stored at –20°C upon arrival. When stored under these conditions and handled correctly, the product can be kept for at least 6 months from date of receipt without reduction in performance.

Product Use Limitations

RT² qPCR Primer Assays are intended for molecular biology applications. These products are 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.

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. Separate conditions apply to QIAGEN scientific instruments, service products, and to products shipped on dry ice. Please inquire for more information.

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 or visit www.qiagen.com).

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 sample and assay technologies and the use of QIAGEN products. If you have any questions or experience any difficulties regarding RT^2 qPCR Primer Assays 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 see our Technical Support Center at www.qiagen.com/Support or call one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit www.qiagen.com).

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/Support/MSDS.aspx 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

In accordance with QIAGEN's Quality Management System, each lot of RT^2 qPCR Primer Assays is tested against predetermined specifications to ensure consistent product quality.

Introduction

Real-time RT-PCR is a highly sensitive and reliable method for gene expression analysis for multiple applications, such as the verification of microarray data. Optimal primer design is critical for successful real-time PCR based analysis of gene expression. Carefully designed primers specifically amplify genes of interest, overcoming the challenge of eliminating nonspecific amplification due to the presence of thousands of genes in first-strand cDNA, each potentially available as a PCR template. In addition, primers that provide efficient amplification are important to ensure accurate gene expression results from the commonly used $\Delta\Delta C_T$ method, which requires a consistently high degree of amplification efficiency across all experiments. Taking advantage of an experimentally verified, proprietary computer algorithm, QIAGEN has developed high-quality, gene-specific RT^2 qPCR Primer Assays for gene expression analyses and microarray data validation.

RT^2 qPCR Primer Assays are designed for SYBR® Green based, real-time PCR detection. The primer design computer algorithm has been developed using an in vitro assay to ensure that the resulting primer sequences generate a single PCR product of the predicted size and a minimal amount of primer–dimer in 30 cycles of PCR amplification. The assay also ensures that the amplification efficiency of the primers is at least 90%. As a result, the algorithm designs highly effective primer sequences for SYBR Green based real-time PCR detection. RT^2 qPCR Primer Assays are available for every human, mouse, rat, rhesus macaque, fruit fly, and dog gene annotated by the NCBI.

Principle and procedure

For optimal performance, RT^2 qPCR Primer Assays should be used together with the RT^2 First Strand Kit for cDNA synthesis and RT^2 SYBR Green Mastermixes for PCR. These reagents have been formulated and pretested together with RT^2 qPCR Primer Assays. The RT^2 First Strand Kit includes a proprietary genomic DNA elimination step to remove any residual contamination in RNA samples before reverse transcription, thereby eliminating false positive signals. Each of the real-time instrument-specific RT^2 SYBR Green Mastermixes contains SYBR Green and an appropriate reference dye to match the instrumentation available in your laboratory. RT^2 SYBR Green Mastermixes are available for all real-time PCR instruments from QIAGEN, Applied Biosystems, Bio-Rad, Stratagene, Eppendorf, Roche, and other major suppliers.

Description of protocols

This handbook contains 2 protocols. The first protocol details cDNA synthesis by reverse transcription using purified RNA and the RT^2 First Strand Kit (page 13). This protocol should be performed prior to real-time PCR. The second protocol

describes how to perform real-time PCR using the cDNA prepared in the first protocol as template (page 15).

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.

- RT^2 First Strand Kit (cat. no. 330401)
- RT^2 SYBR Green Mastermix suitable for use with your real-time cycler. RT^2 SYBR Green Mastermixes available include:
  - RT^2 SYBR Green qPCR Mastermix: suitable for use with real-time cyclers that do not require a reference dye, including: Bio-Rad® models CFX96™, CFX384™; Bio-Rad/MJ Research models Chromo4™, DNA Engine Opticon® 2; Roche® LightCycler® 480 (96-well and 384-well)
  - RT^2 SYBR Green Fluor qPCR Mastermix: suitable for use with the following real-time cyclers: Bio-Rad models iCycler®, iQ™5, MyiQ™, MyiQ2
  - RT^2 SYBR Green ROX™ qPCR Mastermix: suitable for use with the following real-time cyclers: Applied Biosystems® models 5700, 7000, 7300, 7500 (Standard and Fast), 7700, 7900HT (Standard and Fast 96-well block, 384-well block), StepOnePlus™, ViiA™ 7 (Standard and Fast 96-well block, 384-well block); Eppendorf® Mastercycler® ep realplex models 2, 2S, 4, 4S; Stratagene® models Mx3000P®, Mx3005P®, Mx4000®; Takara TP-800
  - RT^2 SYBR Green ROX FAST Mastermix: suitable for use with the Rotor-Gene® Q and other Rotor-Gene cyclers
- Purified RNA samples
- Real-time PCR cycler
- High-quality, nuclease-free water. Do not use DEPC-treated water.
- Nuclease-free pipet tips and tubes
- Optional: XpressRef Universal Total RNA to control PCR conditions is available for human (cat. no. 338112), mouse (cat. no. 338114), and rat (cat. no. 338116).

Important Notes

Preparing a workspace free of DNA contamination

For accurate and reproducible PCR array results, it is important to avoid contamination of the assay with foreign DNA. Any DNA contamination will artificially inflate the SYBR Green signal, yielding skewed gene expression profiles and false-positive signals. The most common sources of DNA contamination are the products of previous experiments spread into the air of the working environment. To set up and maintain a working environment free of DNA contamination, follow the guidelines below.

- Wear gloves throughout the procedure. Use only fresh PCR-grade reagents (water) and labware (tips and tubes).
- Physically separate the workspaces used for PCR setup and post-PCR processing or non-PCR operations. Decontaminate the PCR workspace and labware (pipettor barrels, tube racks, etc.) before each use with UV light (to render any contaminating DNA ineffective in PCR through the formation of thymidine dimers) or with 10% bleach (to chemically inactivate and degrade any DNA).
- Close all tubes containing PCR products once you are finished adding or removing volumes. Before discarding any labware (tips or tubes) containing PCR products or other DNA, treat with 10% bleach.
- Do not leave labware (tubes and tip boxes) exposed to the air for long periods of time.

RNA preparation, quantification, and quality control

The most important prerequisite for any gene expression analysis experiment is consistent, high-quality RNA from every experimental sample. Residual traces of proteins, salts, or other contaminants may degrade the RNA or decrease the efficiency of enzyme activities necessary for optimal reverse transcription and real-time PCR performance.

Recommended RNA preparation methods

High quality total RNA for your real-time PCR experiment should be prepared using one of the methods described below, depending on the biological sample. For optimal results, RNA samples should be suspended in RNase-free water. Do not use DEPC-treated water.

Cultured cells

We recommend the RNeasy® Mini Kit (cat. no. 74104) for RNA purification from cultured cells. It is important to perform the on-column DNase digestion step

described in the *RNeasy Mini Handbook* (using the RNase-Free DNase Set [cat. no. 79254]).

Tissue samples

We recommend the RNeasy Microarray Tissue Mini Kit (cat. no. 73304) including the optional on-column DNase digestion step described in the *RNeasy Microarray Tissue Handbook* (using the RNase-Free DNase Set [cat. no. 79254]).

Formalin-fixed paraffin-embedded (FFPE) samples

We recommend the RNeasy FFPE Kit (cat. no. 73504) for RNA purification from FFPE samples.

Small samples yielding <100 ng total RNA

We recommend the RNeasy Micro Kit (cat no. 74004) for RNA purification from small samples.

Whole blood samples

We recommend the PAXgene® Blood RNA Kit (cat. no. 762174) for preparation of total RNA from whole blood samples. Alternatively, the QIAamp® RNA Blood Mini Kit (cat. no. 52304) can also be used for this purpose.

Total RNA isolated using a phenol-based method

Total RNA from any biological source material prepared using a phenol-based method (e.g., QIAzol® Lysis Reagent, TRIzol® Reagent, RNAzol® Reagent) should be further purified using the RNeasy Mini Kit. It is important to perform the on-column DNase digestion step described in the *RNeasy Mini Handbook*.

Other biological samples

Refer to the existing literature to find protocols for high-quality RNA purification from other biological samples or contact QIAGEN Technical Service.

RNA quantification and quality control

For best results from the RT^2 qPCR Primer Assays, all RNA samples should also demonstrate consistent quality according to the following criteria.

Concentration and purity determined by UV spectrophotometry

The concentration and purity of RNA should be determined by measuring the absorbance in a spectrophotometer. Prepare dilutions and measure absorbance

in 10 mM Tris·Cl,* pH 8.0. The spectral properties of nucleic acids are highly dependent on pH. An absorbance reading of 1.0 at 260 nm in a 1 cm detection path corresponds to an RNA concentration of 40 µg/ml.

- A_{260}:A_{230} ratio should be greater than 1.7
- A_{260}:A_{280} ratio should be 1.8 to 2.0
- Concentration determined by A_{260} should be >40 µg/ml

Ribosomal RNA band integrity

Run an aliquot of each RNA sample on a denaturing agarose gel or the Agilent® Bioanalyzer using an RNA 6000 Nano LabChip®. Verify that there are sharp bands/peaks present for both the 18S and 28S ribosomal RNAs (Figure 1). Any smearing of the RNA bands or shoulders on the RNA peaks indicate that degradation has occurred in the RNA sample.

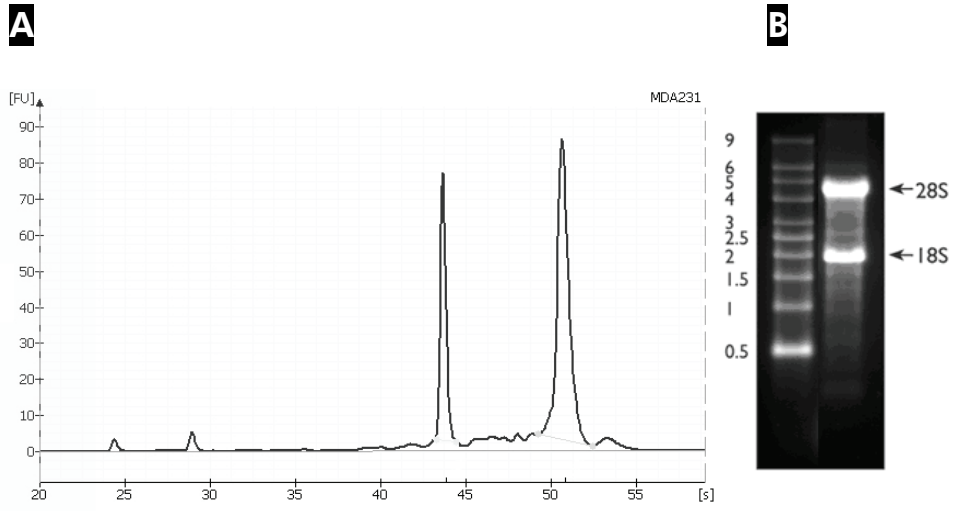


Figure 1. Ribosomal RNA integrity. **A** Agilent Bioanalyzer electropherogram of high-quality total RNA showing sharp peaks for the 18S (left) and 28S (right) ribosomal RNA. Due to high quality of the RNA, peaks do not have shoulders (especially to the left of each peak). **B** Agarose gel electrophoresis shows sharp bands (especially at the bottom of each band) for 28S and 18S ribosomal RNA.

Genomic DNA contamination

Eliminating genomic DNA contamination is essential for obtaining optimal real-time gene expression results using RT² qPCR Primer Assays. Use of a no reverse transcription (NRT) control, in which reverse transcriptase is replaced with water in the cDNA synthesis reaction, is the most accurate way to detect DNA contamination. If the difference in C_T values between the NRT control and a complete reaction for the same gene of interest is greater than 6, then any DNA

* 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), available from the product supplier.

contamination will not affect the reliability of the relative gene expression analysis.

To remove any residual contamination from your RNA samples, we strongly recommend RNA purification using the RNeasy Mini Kit including the optional on-column DNase digestion step, followed by cDNA synthesis using the RT^2 First Strand Kit. If required, individual, species-specific RT^2 qPCR Primer gDNA Controls are available.

Starting RNA amounts

RT^2 qPCR Primer Assays provide results with as little as 25 ng or as much as 5 µg total RNA per cDNA synthesis reaction. For smaller starting RNA amounts, the RT^2 PreAMP cDNA Synthesis Kit (cat. no. 330451) enables gene expression analysis from as little as 1 ng total RNA or 100 ng RNA from FFPE samples by preamplifying first strand cDNA. This allows gene expression analysis from samples such as fine needle biopsy samples, laser captured microdissection samples, stem cell clusters or embryoid bodies, FACS® generated cells, or FFPE samples. For more details, see the *RT^2 PreAMP cDNA Synthesis Handbook*.

The optimal amount of starting material depends on the relative abundance of the transcripts of interest. Lower abundance transcripts require more RNA; higher abundance transcripts require less RNA. Greater amounts of input total RNA yield a greater number of positive calls (i.e., genes expressed in the linear dynamic range of the method). Lower amounts of input total RNA yield a smaller number of positive calls.

For successful results, we recommend that first-time users start with 0.5 µg to 1 µg of total RNA. It is important to use a consistent amount of total RNA for all reactions in a single experiment.

Protocol: cDNA Synthesis Using the RT² First Strand Kit

Important points before starting

- Use the same amount of total RNA for reverse transcription of each sample to be analyzed. First-time users are recommended to start with 0.5–1 µg total RNA. Use of less than 100 ng RNA will result in a high rate of false negatives.
- Do not use DEPC-treated water. Use high-quality, nuclease-free water.
- The RT² First Stand Kit is not compatible with the chemicals in DNA-free™ kits from Ambion. If your RNA sample has been treated with DNA-free reagents, contact QIAGEN Technical Service.

Procedure

1. **Briefly centrifuge the reagents of the RT² First Stand Kit (10–15 s) to bring the contents to the bottom of the tubes.**
2. **Prepare the genomic DNA elimination mix for each RNA sample in a sterile PCR tube according to Table 1. Mix gently by pipetting up and down and then centrifuge briefly.**

Table 1. Genomic DNA elimination mix

Component	Amount
RNA	25 ng–5 µg
Buffer GE	2 µl
RNase-free water	Variable
Total volume	**10 µl**

3. **Incubate the genomic DNA elimination mix for 5 min at 42°C, then place immediately on ice for at least 1 min.**

4. **Prepare the reverse-transcription mix according to Table 2.**

Table 2. Reverse-transcription mix

Component	Volume for 1 reaction	Volume for 2 reactions	Volume for 4 reactions
5x Buffer BC3	4 µl	8 µl	16 µl
Control P2	1 µl	2 µl	4 µl
RE3 Reverse Transcriptase Mix	2 µl	4 µl	8 µl
RNase-free water	3 µl	6 µl	12 µl
Total volume	**10 µl**	**20 µl**	**40 µl**

5. **Add 10 µl reverse-transcription mix to each tube containing genomic DNA elimination mix. Mix gently by pipetting up and down.**
6. **Incubate at 42°C for exactly 15 min. Then immediately stop the reaction by incubating at 95°C for 5 min.**
7. **Add 91 µl RNase-free water to each reaction. Mix by pipetting up and down several times.**
8. **Place the reactions on ice and proceed with the real-time PCR protocol.**

   If you wish to store the reactions prior to real-time PCR, transfer them to a –20°C freezer.

   For quality control analysis using the RT^2 RNA QC PCR Array, follow the protocol in the *RT^2 RNA QC PCR Array Handbook* using a 6 µl aliquot of the diluted cDNA template.

Protocol: Real-Time PCR Using RT² qPCR Primer Assays and RT² SYBR Green Mastermixes

Important points before starting

- Ensure that the RT² SYBR Green Mastermix is suitable for your real-time cycler (see page 8).
- For accuracy and precision, ensure that micropipettors are calibrated before beginning the protocol. Be sure not to introduce bubbles into wells/tubes when pipetting.
- Do not use DEPC-treated water. Use high-quality, nuclease-free water.
- If precipitates are present in the Mastermix tubes, warm the reagents at 42°C for 1 min and vortex briefly to dissolve. Repeat if necessary.
- To ensure that each experimental sample yields a reliably detectable C_T value in real-time PCR, we recommend using undiluted cDNA template and a 1:10 dilution of cDNA template in separate reactions. In addition, prepare either duplicate or triplicate reactions for each template at each concentration.
- For every experimental sample, prepare reactions for every gene of interest and for a single housekeeping gene or a set of housekeeping genes to normalize the raw data. Choose housekeeping gene(s) known to not change their expression under the experimental conditions.
- Prepare a positive control reaction using template known to represent the genes of interest, such as template generated from XpressRef Universal Total RNA.
- To control for DNA contamination introduced during reaction setup, prepare a no template control (NTC) reaction replacing template with water.
- To control for genomic DNA contamination, perform one assay for each gene of interest and each housekeeping gene using an equivalent volume of product from the no reverse transcription (NRT) reaction performed for each RNA sample.
- Optional: Generate a standard curve for each gene of interest and housekeeping gene(s). To generate a standard curve, prepare a 5-point series of 5- or 10-fold dilutions in duplicate using a template known to represent the genes of interest, such as template generated from XpressRef Universal Total RNA.

Procedure

1. **Briefly centrifuge the RT² SYBR Green Mastermix, RT² qPCR Primer Assay, and cDNA synthesis reaction (10–15 s) to bring the contents to the bottom of the tubes.**

   Note: As the RT² SYBR Green Mastermix contains HotStart DNA *Taq* Polymerase that is active only after heat activation, reactions can be prepared at room temperature (15–25°C).

2. **Prepare the PCR components mix in a 5 ml tube, as described in Table 3.**

Table 3. PCR components mix for one reaction

Component	Volume
RT² SYBR Green Mastermix	12.5 µl
cDNA synthesis reaction	1 µl
RT² qPCR Primer Assay (10 µM stock)	1 µl
RNase-free water	10.5 µl
Total volume	**25 µl**

Note: If performing multiple reactions, prepare a mix containing RT² SYBR Green Mastermix, RT² qPCR Primer Assay, and RNase-free water by scaling up the volumes shown in Table 3. Prepare 10% more mix than is required to allow for pipetting errors (i.e., for 96 reactions, prepare enough PCR components mix for 106 reactions). Add the mix to the cDNA synthesis reactions using a repeat pipet.

3. **Briefly centrifuge the PCR components mix and place the tube(s) into the real-time cycler.**

   If using plates instead of tubes, centrifuge the plate for 1 min at 1000 *g* to remove bubbles.

4. **Program the real-time cycler according to Table 4, 5, or 6, depending on the real-time cycler used. Run the program.**

   Note: For additional help with instrument setup, see our Instrument-Specific Setup Instructions and Protocol Files at: www.SABiosciences.com/pcrarrayprotocolfiles.php.

Table 4. Cycling conditions* for Applied Biosystems, Bio-Rad,† Stratagene, and Eppendorf‡ cyclers

Cycles	Duration	Temperature	Comments
1	10 min	95°C	HotStart DNA *Taq* Polymerase is activated by this heating step.
40	15 s	95°C	
	1 min	60°C	Perform fluorescence data collection.

* Recommended for the following cyclers: Applied Biosystems models 5700, 7000, 7300, 7500, 7700, 7900HT, StepOnePlus, ViiA 7; Bio-Rad models iCycler, iQ5, MyiQ, MyiQ2, CFX96, CFX384; Stratagene models Mx3000P, Mx3005P, Mx4000P; Eppendorf Mastercycler ep realplex models 2, 2S, 4, 4S.

† For Bio-Rad models CFX96 and CFX384: adjust the ramp rate to 1°C/s.

‡ For Eppendorf Mastercyler ep realplex models 2, 2S, 4, and 4S: for the Silver Thermoblock, adjust the ramp rate to 26%; for the Aluminum Thermoblock, adjust the ramp rate to 35%. Refer to the Instrument Setup Guide at www.SABiosciences.com/pcrarrayprotocolfiles.php for detailed setup instructions.

Table 5. Cycling conditions for Roche cyclers§

Cycles	Duration	Temperature	Comments
1	10 min	95°C	HotStart DNA *Taq* Polymerase is activated by this heating step.
45	15 s	95°C	
	1 min	60°C	Perform fluorescence data collection.

§ Recommended for the Roche LightCycler 480. If using a Roche LightCycler 480, adjust the ramp rate to 1°C/s. Refer to the "Instrument Setup Guide" at www.SABiosciences.com/pcrarrayprotocolfiles.php for more information on other required changes to settings for Melt Curve Acquisition.

Table 6. Cycling conditions for Bio-Rad and Takara cyclers and all other cyclers*

Cycles	Duration	Temperature	Comments
1	10 min	95°C	HotStart DNA *Taq* Polymerase is activated by this heating step.
40	15 s	95°C	
	30–40 s	55°C	Perform fluorescence data collection. Different cyclers need different lengths of time to detect the fluorescent signal. Choose the appropriate time for the annealing step (55°C) for your cycler.
	30 s	72°C	

* Recommended for the following cyclers: Bio-Rad/MJ Research models Chromo4, DNA Engine Opticon, DNA Engine Opticon 2; Takara TP-800; all other cyclers.

5. **Recommended: Perform dissociation (melting) curve analysis to verify PCR specificity. Run a melting curve program and generate a first derivative dissociation curve for each well using the real-time cycler software. A single peak should appear in each reaction at temperatures greater than 80°C.**

   Note: If your instrument does not have a default melting curve program, run the following program instead: 95°C, 1 min; 65°C, 2 min (optics off); 65°C to 95°C at 2°C/min (optics on).

   Note: For cycler-specific melting curve analysis settings, please refer to the Instrument Setup Guide for your cycler at www.SABiosciences.com/pcrarrayprotocolfiles.php.

   Note: Reactions can be stored at –20°C wrapped in aluminum foil and melting curve analysis performed at a later time. When ready to perform melting curve analysis, warm the tube to room temperature (15–25°C), place it in the real-time cycler, and run the melting curve analysis program.

6. **Optional: Agarose gel electrophoresis analysis can be performed if necessary for troubleshooting purposes.**

   No more than one band should be visible in each lane. The RT^2 qPCR Primer Assay Product Sheet details the expected size of the PCR product.

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.SABiosciences.com/support_faq.php?target=PCR. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit www.qiagen.com).

	Comments and suggestions
Presence of multiple PCR products (bands on a gel or dissociation peaks)	
a) Genomic DNA contamination	Use a no reverse transcription (NRT) control, in which reverse transcriptase is replaced with water in the cDNA synthesis reaction, to detect DNA contamination. If the difference in C_T values between the NRT control and a complete reaction for the same gene of interest is greater than 6, then any DNA contamination will not affect the reliability of the relative gene expression analysis. We strongly recommend performing the on-column DNase digestion step when purifying RNA using the RNeasy Mini Kit. We strongly recommend using the RT^2 First Strand Kit for cDNA synthesis. This kit includes a genomic DNA elimination step.
b) Presence of undiscovered alternative transcripts	Approximately 45% of all human genes are predicted to have alternative transcripts, yet variants for only 9% of human genes have been annotated by the NCBI. RT^2 qPCR Primer Assays for genes with known variants amplify sequence common to all transcripts and detect the sum of their expression. Primer design cannot account for genes with unannotated transcripts.
c) Presence of primer–dimers	Verify the presence of primer–dimers by agarose gel electrophoresis (primer–dimmers are <50 bp in size). Use the appropriate RT^2 SYBR Green Mastermix to prevent the appearance of primer–dimers.

	Comments and suggestions
C_T values are too high (>35 or not detectable)	
a) Experimental error	Use a template known to contain the gene of interest as a positive control to check the PCR reagents and experimental procedure.
b) Poor RNA quality	Be sure to perform all recommended quality control checks on the RNA sample. Poor quality RNA can inhibit enzyme activity during reverse transcription generating an insufficient amount of template during the cDNA synthesis reaction.
c) Insufficient template	Use more input RNA for reverse transcription, especially if the lower end of the recommended range had been used previously. Use a larger volume of template per reaction, but do not use more than 2.5 µl of template per 25 µl reaction. Use the same volume of template in each reaction.
d) Nonendogenous transcript	High or undetectable C_T values will result if the target gene is exogenously expressed from a vector, plasmid, or other construct that only contains the open reading frame and the RT^2 qPCR Primer Assay is located in the 3' or 5' untranslated region (UTR). Refer to the reference positions on the Product Sheet provided with the RT^2 qPCR Primer Assay.
C_T values are too low (<12)	
Too much template	Use less input RNA for cDNA synthesis, especially if the higher end of the recommended range had been used previously. Use a smaller volume of template per reaction, but do not use less than 1 µl of template per 25 µl reaction. Use the same volume of template in each reaction.
Expression is seen when it is not expected	
a) Genomic or exogenous DNA contamination	Perform and interpret appropriate negative control reactions (NRT and NTC controls).

	Comments and suggestions
b) Knockout experiment	Expression may be detected if the RT^2 qPCR Primer Assay is being used to validate a knockout mouse model where only a portion of the endogenous gene is replaced, and the RT^2 qPCR Primer Assay is not located in the replaced sequence of the resulting mRNA transcript. Do not use RT^2 qPCR Primer Assays for this purpose.
No template control (NTC) shows a C_T value <35 cycles	
a) DNA contamination of reagents, tips, and tubes	See "Preparing a workspace free of DNA contamination", page 9.
b) Presence of primer–dimers	Verify the presence of primer–dimers by agarose gel electrophoresis (primer–dimmers are <50 bp in size). Use the appropriate RT^2 SYBR Green Mastermix to prevent the appearance of primer–dimers.

Appendix A: Data Analysis

$\Delta\Delta C_T$ method

The $\Delta\Delta C_T$ method is recommended for data analysis. Perform the $\Delta\Delta C_T$ method as described below.

In separate reactions, determine the C_T value for the housekeeping gene(s) (HKG) and for the genes of interest (GOI) in each sample. Only use C_T values less than 35. Only compare C_T values determined using the same amount of template.

For example:

Control: $C_T(GOI) = 24.25$ $C_T(HKG) = 16.49$

Experimental: $C_T(GOI) = 19.17$ $C_T(HKG) = 16.36$

For each sample, calculate the difference between the C_T values (ΔC_T) for each gene of interest and the housekeeping gene or the average C_T value of the set of housekeeping genes.

For example:

ΔC_T (control) = C_T (GOI) – C_T (HKG) = 24.25 – 16.49 = 7.76

ΔC_T (experimental) = C_T (GOI) – C_T (HKG) = 19.17 – 16.36 = 2.81

For each pair-wise set of samples to be compared, calculate the difference in ΔC_T values ($\Delta\Delta C_T$) for the genes of interest between the two samples.

For example:

$\Delta\Delta C_T = \Delta C_T$ (experimental) – ΔC_T (control) = 2.81 – 7.76 = –4.95

Calculate the fold-change in gene expression. Due to high levels of amplification efficiency using RT^2 qPCR Primer Assays, the fold-change in gene expression is equal to $2^{(-\Delta\Delta CT)}$.

For example:

Fold change = $2^{(-\Delta\Delta CT)} = 2^{(-(-4.95))} = 2^{(4.95)} = 30.9$

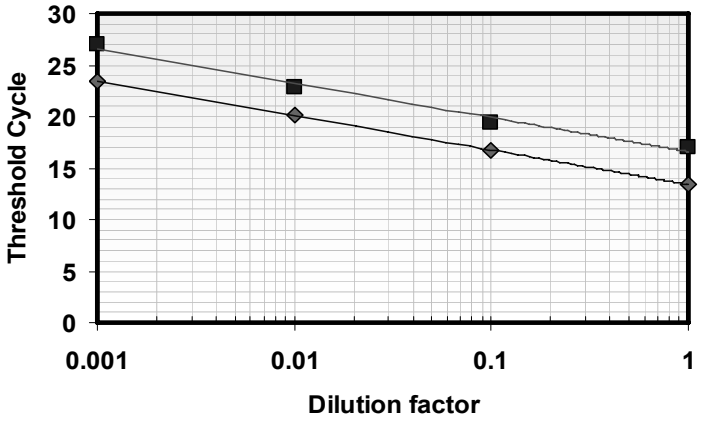
Standard curve method

The standard curve method is an alternative method for data analysis. Perform the standard curve method as described below.

Use the real-time cycler software to determine the threshold cycle value for each reaction. To generate a standard curve, plot the threshold cycle (C_T) for each standard curve reaction (y-axis) against the template dilution factor used in those reactions (x-axis, log-scale). Plot a standard curve for each gene of interest (GOI) and for each housekeeping gene (HKG). Fit the data to an

equation defining a straight line. The dilution factor in the standard curve is directly related to the relative expression level (L) of its gene.

For example:

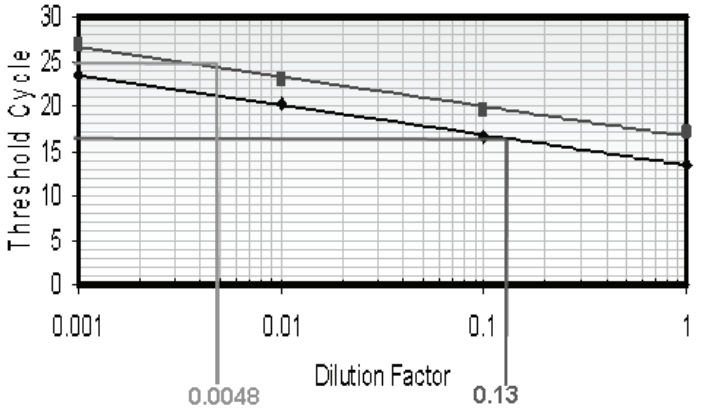


GOI: $C_T = -3.302\text{Log}(L) + 16.6$

HKG: $C_T = -3.351\text{Log}(L) + 13.4$

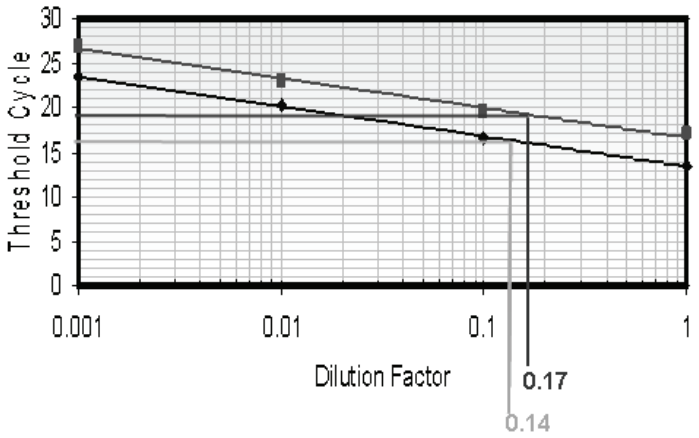
For every reaction containing template synthesized from experimental samples, use the C_T value and the appropriate standard curve (based on the gene-specific RT^2 qPCR Primer Assay used in the reaction) to calculate the relative expression level of each gene of interest (L(GOI)) and the relative expression level of each housekeeping gene (L(HKG)) in each sample. Be sure that the C_T values fall within the linear range of the appropriate standard curve.

For example:



Control: C_T(GOI) = 24.25 C_T(HKG) = 16.49

L(GOI) = 0.0048 L(HKG) = 0.13



Experimental: C_T(GOI) = 19.17 C_T(HKG) = 16.36

L(GOI) = 0.17 L (HKG) = 0.14

Normalize the expression level of the genes of interest by dividing their relative expression level by the relative expression level of the housekeeping gene or the average relative expression level of a set of housekeeping genes. Be sure to use relative expression levels for all genes determined from the same experimental sample and under the same PCR conditions, specifically the initial template concentration (whether undiluted or diluted 1:10).

To determine the fold-change in expression for each gene of interest between 2 different samples, calculate the ratio of its normalized expression levels (determined from the same PCR conditions) between those samples.

For example:

$$\text{Fold change} = \frac{\dfrac{\text{Experimental L (GOI)}}{\text{Experimental L (HKG)}}}{\dfrac{\text{Control L (GOI)}}{\text{Control L (HKG)}}} = \frac{\dfrac{0.17}{0.14}}{\dfrac{0.0048}{0.13}} = 32.9$$

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.

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Product	Contents	Cat. no.
RT² qPCR Primer Assay (200)	For 200 reactions; Mix of 2 gene-specific primers provided in solution (200 µl); 10 µM each primer	Varies
RT² First Strand Kit (12)	For 12 x 20 µl first strand cDNA synthesis reactions; Buffer GE (30 µl), Buffer BC3 (60 µl), RE3 Reverse Transcriptase Mix (28 µl), Control P2 (18 µl), RNase-Free Water (1 ml)	330401
RT² SYBR Green qPCR Mastermix (2)*	For 2 x 96 assays in 96-well plates; suitable for use with real-time cyclers that do not require a reference dye; 2 x 1.25 ml Mastermix	330500
RT² SYBR Green Fluor qPCR Mastermix (2)*	For 2 x 96 assays in 96-well plates; suitable for use with real-time cyclers that use fluorescein reference dye; 2 x 1.25 ml Mastermix	330510
RT² SYBR Green ROX qPCR Mastermix (2)*	For 2 x 96 assays in 96-well plates; suitable for use with real-time cyclers that use ROX reference dye; 2 x 1.25 ml Mastermix	330520
RT² SYBR Green ROX FAST Mastermix (2)*	For 2 x 96 assays in 96-well plates; suitable for use with real-time cyclers that use ROX reference dye, including the Rotor-Gene Q and Rotor-Gene 6000; 2 x 1.25 ml Mastermix	330620
Related products		
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RT² RNA QC PCR Array	Array for quality control analysis prior to experiments using RT² Profiler PCR Arrays; available in 96-well, 384-well, and Rotor-Disc 100 formats	Varies

* Larger kit sizes available; please inquire.

Product	Contents	Cat. no.
Human XpressRef Universal Total RNA	2 tubes each containing 100 µg human RNA at 1 mg/ml	338112
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Rat XpressRef Universal Total RNA	2 tubes each containing 100 µg rat RNA at 1 mg/ml	338116
RNeasy Mini Kit (50)*	50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free reagents and buffers	74104
RNeasy FFPE Kit (50)	50 RNeasy MinElute Spin Columns, Collection Tubes, Proteinase K, RNase-Free DNase I, DNase Booster Buffer, RNase-free buffers, RNase-Free Water	73504
PAXgene Blood RNA Kit (50)	50 PAXgene Spin Columns, 50 PAXgene Shredder Spin Columns, Processing Tubes, RNase-Free DNase I, RNase-free reagents and buffers. To be used in conjunction with PAXgene Blood RNA Tubes	762174
RNeasy Microarray Tissue Mini Kit (50)	RNeasy Mini Spin Columns, Collection Tubes, QIAzol Lysis Reagent, RNase-free reagents and buffers	73304
RNeasy Micro Kit (50)	50 RNeasy MinElute Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free DNase I, Carrier RNA, RNase-free reagents and buffers	74004
QIAamp RNA Blood Mini Kit (50)	50 QIAamp Mini Spin Columns, 50 QIAshredder Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free reagents and buffers	52304

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