April 2020

RT^2 qPCR Primer Assay Handbook

For gene expression analysis
by real-time RT-PCR

Sample to Insight

QIAGEN

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Kit Contents

RT² qPCR Primer Assay	**(200)**
Catalog no.	**330001**
No. of reactions	**200**
200 µl RT² qPCR Primer Assay (10 µM)	1 tube

Shipping and Storage

The RT² qPCR Primer Assays are shipped at ambient temperature but must be stored at –30 to –15°C upon arrival. Under these conditions, the components are stable for 1 year without showing any reduction in performance and quality, unless otherwise indicated on the label.

Intended Use

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.

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 RT² 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 RT² qPCR Primer Assays for gene expression analyses and microarray data validation.

Principle and procedure

For optimal performance, RT² qPCR Primer Assays should be used together with the RT² First Strand Kit for cDNA synthesis and RT² SYBR® Green Mastermixes for PCR. These reagents have been formulated and pretested together with RT² qPCR Primer Assays. The RT² 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 RT² SYBR® Green Mastermixes contains SYBR® Green and an appropriate reference dye to match the instrumentation available in your laboratory. RT² SYBR® Green Mastermixes are available for all real-time PCR instruments from QIAGEN, Applied Biosystems®, Bio-Rad®, Stratagene®, Eppendorf®, Roche®, Agilent®, and other major suppliers.

Description of protocols

This handbook contains 3 protocols:

- "Protocol: cDNA Synthesis Using the RT² First Strand Kit" details cDNA synthesis by reverse transcription using purified RNA and the RT² First Strand Kit (page 13). This protocol should be performed prior to real-time PCR.
- "Protocol: Real-Time PCR Using RT2 qPCR Primer Assays and RT2 SYBR® Green Mastermixes" describes how to perform real-time PCR using the cDNA prepared in the first protocol as template (page 15).
- "Protocol: Real-Time PCR for RT2 qPCR Primer Assays with Rotor-Gene Q" describes how to use RT² qPCR Primer Assays on a Rotor-Gene® Q instrument (page 20).

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.

- RT² First Strand Kit (12) or RT² First Strand Kit (50) (cat. no. 330401 or 330404)
- RT² SYBR® Green Mastermix suitable for use with your real-time cycler. RT² SYBR® Green Mastermixes available include:
  - RT² 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), and QIAquant™ (96- and 384-well)
  - RT² SYBR® Green Fluor qPCR Mastermix, suitable for use with the following real-time cyclers: Bio-Rad models iCycler®, iQ™5, MyiQ™, MyiQ2
  - RT² 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® TP800
  - RT² 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 pipette tips and tubes

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*, **www.qiagen.com/HB-0435** (using the RNase-Free DNase Set [cat. no. 79254]).

Tissue samples

We recommend the RNeasy Plus Universal Mini Kit (50) (cat. no. 73404), including the optional on-column DNase digestion step described in the *RNeasy Plus Universal Handbook*, **www.qiagen.com/HB-0391**.

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

The QIAamp® RNA Blood Mini Kit (cat. no. 52304) and the RNeasy Protect Animal Blood Kit (cat. no. 73224; for animal blood) 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 RT² 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.

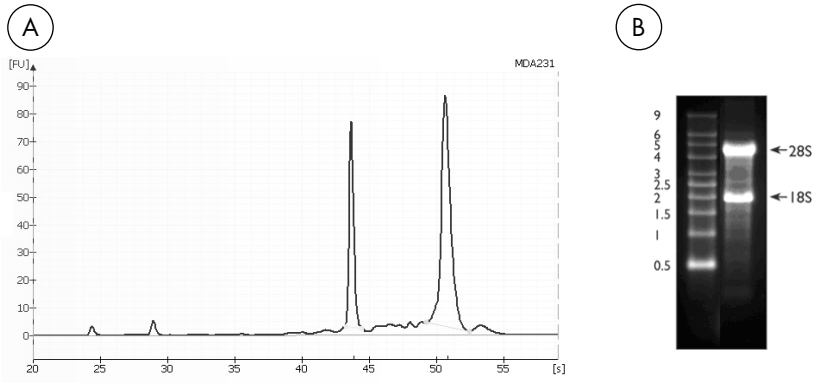

Figure 1. Ribosomal RNA integrity. A: Agilent Bioanalyzer electropherogram of high-quality total RNA showing sharp peaks for the 18S (left) and 28S (right) ribosomal RNA. Due to high quality of the RNA, peaks do not have shoulders (especially to the left of each peak). **B**: Agarose gel electrophoresis 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 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 RT² First Strand Kit. If required, individual, species-specific RT² qPCR Primer gDNA Controls are available.

Starting RNA amounts

RT² 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 RT² 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 *RT² PreAMP cDNA Synthesis Handbook*, **www.qiagen.com/HB-0502**.

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.

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, for up to 100 individual qPCR assays. An amount of 1 ng to 10 ng of total RNA per individual qPCR reaction is recommended.
- **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. If necessary, add appropriate volume of RNase-free water to each reaction, depending on the input level, estimation of the gene expression, and the number of assays. Mix by pipetting up and down several times.

8. Place the reactions on ice and proceed with the real-time PCR protocol.

9. 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 RT² RNA QC PCR Array, follow the protocol in the *RT² RNA QC PCR Array Handbook*, **www.qiagen.com/HB-0796**, 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 "Equipment and Reagents to Be Supplied by User", page 7).
- 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 reliable detectable C_T value in real-time PCR, we recommend using up to 1 µl of the undiluted cDNA template for each 25 µl qPCR reaction to get enough input (1–10 ng total RNA input per 25 µl reaction is the preferred starting amount if no optimization test result is available). To calculate the experimental variability, prepare at least 3 qPCR reactions for each qPCR assay.
- 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 not to change their expression under the experimental conditions.
- Prepare a positive control reaction using template known to represent the genes of interest.
- 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 1 assay for each gene of interest and each housekeeping gene using an equivalent volume of product from the 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.

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 indicated 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 pipette.

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 x *g* to remove bubbles.

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

   Note: Additional instrument-specific setup instructions can be found in **geneglobe.qiagen.com/product-groups/rt2-qpcr-primer-assays**, **Product Resources** > **Supplementary Protocols**.

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 1 min	95°C 60°C	Perform fluorescence data collection.

* Recommended for the following cyclers: Applied Biosystems models 5700, 7000, 7300, 7500, 7700, 7900HT, 7900 HT FAST 384-well block, StepOnePlus, QuantStudio™ 384-well block, ViiA 7 384-well block; Bio-Rad models iCycler, iQ 5, 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 Mastercycler 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 instrument-specific setup instructions that can be found in **geneglobe.qiagen.com/product-groups/rt2-qpcr-primer-assays**, under **Product Resources** > **Supplementary Protocols** for more information.

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 1 min	95°C 60°C	Perform fluorescence data collection. Adjust the ramp rate to 1°C/s.

* Recommended for the Roche LightCycler 480 96- and 384-well blocks. If using a Roche LightCycler 480 II, adjust the ramp rate to 1.8°C/s. Refer to the instrument-specific setup instructions that can be found in **geneglobe.qiagen.com/product-groups/rt2-qpcr-primer-assays**, under **Product Resources** > **Supplementary Protocols** 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	Perform fluorescence data collection.
	30–40 s	55°C	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 TP800; all other cyclers.

5. Calculate the threshold cycle (C_T) for each well using the real-time cycler software, as described in the following steps.

6. Define the baseline by choosing the automated baseline option if the cycler has the adaptive baseline function. If the cycler does not have the adaptive baseline function, set the baseline manually. To set the baseline manually, use the linear view of the amplification plots to determine the earliest visible amplification. Set the cycler to use the readings from cycle number 2 through 2 cycles before the earliest visible amplification, but no more than cycle 15. The earliest amplification will usually be visible between cycles 14 and 18.

7. Manually define the threshold by using the log view of the amplification plots. Choose a threshold value above the background signal. The threshold value should be in the lower half of the linear phase of the amplification plot.

   Note: Ensure that the threshold values are the same across all RT² qPCR Primer Assays. The absolute position of the threshold is less critical than its consistent position across arrays.

8. Export the C_T values for all wells to a blank Excel® spreadsheet. Data analysis can then be conducted at QIAGEN'S GeneGlobe Data Analysis Center using a software-based tool or with a spreadsheet-based tool that can be downloaded from QIAGEN website.

   Note: The GeneGlobe Data Analysis Center is a web resource for the analysis of real time PCR or NGS data (**geneglobe.qiagen.com/analyze**). To access the center, new users must register online. Once on the site, the data analysis software can be found under the **Analysis** tab.

9. **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.

   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: 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.

   Note: For cycler-specific melting curve analysis settings, please refer to the instrument-specific setup instructions in **geneglobe.qiagen.com/product-groups/rt2-qpcr-primer-assays**, under **Product Resources** > **Supplementary Protocols** for more information.

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

    No more than 1 band should be visible in each lane. The RT² qPCR Primer Assay Product Sheet details the expected size of the PCR product.

Protocol: Real-Time PCR for RT^2 qPCR Primer Assays with Rotor-Gene Q

Important points before starting

- For accuracy and precision, ensure that micropipettors are calibrated before beginning the protocol. Be sure not to introduce bubbles into the wells of the Rotor-Disc® 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.
- To control for DNA contamination introduced during reaction setup, prepare an NTC reaction replacing template with water.
- To control for genomic DNA contamination, perform 1 assay for each gene of interest and each housekeeping gene using an equivalent volume of product from the 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.

Procedure

1. Briefly centrifuge the RT² SYBR® Green ROX FAST Mastermix, water, and cDNA synthesis reaction (10–15 s) to bring the contents to the bottom of the tubes.

   Note: As the RT² SYBR® Green ROX FAST 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 7.

Table 7. PCR component mix for 1 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 7. 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 pipette.

3. Briefly centrifuge the PCR components mix.

4. Slide an empty Rotor-Disc 100 into the Rotor-Disc 100 Loading Block using the tab at position A1 and the tube guide holes.

5. Dispense 20 µl PCR component mix into the individual Rotor-Disc wells.

6. Carefully, tightly seal the disc with the Rotor-Disc Heat-Sealing Film using the Rotor-Disc Heat Sealer.

   For detailed instructions, see the *Rotor-Gene Q User Manual*.

7. Program the real-time cycler according to Table 8.

   Note: Additional instrument setup instructions can be found in **geneglobe.qiagen.com/product-groups/rt2-qpcr-primer-assays**, under **Product Resources** > **Supplementary Protocols**.

Table 8. Cycling conditions for Rotor-Gene cyclers

Cycles	Duration	Temperature	Comments
1	10 min	95°C	HotStart DNA Taq polymerase is activated by this heating step.
40	15 s 30 s	95°C 60°C	Perform fluorescence data collection.

8. Insert the disc into the Rotor-Disc 100 Rotor and secure with the Rotor-Disc 100 Locking Ring. Start the run. For detailed instructions, see the *Rotor-Gene Q User Manual*.

9. Calculate the C_T for each well using the real-time cycler software. To define the baseline, select **Dynamic Tube** (default analysis setting) to ensure that the average background of each well is determined just before amplification commences.

   Optional: Select **Ignore First**. Fluorescent signal from the initial cycles may not be representative of the remainder of the run. Thus, better results may be achieved if the initial cycles are ignored. Up to 5 cycles can be ignored.

Optional: Select **Noise Slope Correction**. Selection of this option can improve data whose baseline (initial cycles) is noticeably sloped. Noise slope correction improves the data when raw data backgrounds are observed to slope upward or downward before the takeoff point (C_T).

Note: Ensure that the settings are the same across all RT² qPCR Primer Assay runs in the same analysis.

10. Manually define the threshold by using the log view of the amplification plots. Choose a threshold value above the background signal. The threshold value should be in the lower half of the linear phase of the amplification plot.

    Note: Ensure that the threshold values are the same across all RT² qPCR Primer Assays. The absolute position of the threshold is less critical than its consistent position across arrays.

11. Export the C_T values for all wells to a blank Excel spreadsheet. Data analysis can then be conducted at QIAGEN'S GeneGlobe Data Analysis Center using a software-based tool or with a spreadsheet-based tool that can be downloaded from the QIAGEN website.

    Note: The GeneGlobe Data Analysis Center is a web resource for the analysis of real time PCR or NGS data (**geneglobe.qiagen.com/analyze**). To access the center, new users must register online. Once on the site, the data analysis software can be found under the **Analysis** tab. The RT² qPCR Assay Data Analysis spreadsheets can be found in **geneglobe.qiagen.com/product-groups/rt2-qpcr-primer-assays**, under **Product Resources** > **Performance Data**.

12. **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.

    Note: Melting curve analysis can be added during creation of the Rotor-Gene Q PCR program.

Note: For Rotor-Gene Q melting curve analysis settings, refer to the instructions that can be found in **geneglobe.qiagen.com/product-groups/rt2-qpcr-primer-assays**, under **Product Resources** > **Supplementary Protocols**.

Note: Rotor-Discs 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 plate to room temperature (15–25°C), place it in the real-time cycler, and run the melting curve analysis program.

Note: Visually inspect the Rotor-Disc after the run for any signs of evaporation from any of the wells. If evaporation is observed, note which wells are affected, as this may affect the results of data analysis.

Note: Do not open any previously processed PCR plates. Removing the film from PCR plates releases PCR product into the air where it may contaminate and affect the results of future real-time PCR experiments.

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 in our Technical Support Center: **www.qiagen.com/FAQ/FAQList.aspx**. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information or protocols in this handbook (for contact information, visit **support.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 RT² 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, but variants for only 9% of human genes have been annotated by the NCBI. RT² qPCR Primer Assays for genes with known variants amplify a sequence common to all transcripts and detect the sum of their expression. Primer design cannot account for genes with nonannotated transcripts.
c)	Presence of primer–dimers	Verify the presence of primer–dimers by agarose gel electrophoresis (primer–dimers are <50 bp in size). Use the appropriate RT² SYBR® Green Mastermix to prevent the appearance of primer–dimers.
C_T values are too high (>35 or undetectable)		
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.

		Comments and suggestions
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 RT² 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 RT² 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).
b)	Knockout experiment	Expression may be detected if the RT² qPCR Primer Assay is being used to validate a knockout mouse model where only a portion of the endogenous gene is replaced, and the RT² qPCR Primer Assay is not located in the replaced sequence of the resulting mRNA transcript. Do not use RT² 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 8.
b)	Presence of primer–dimers	Verify the presence of primer–dimers by agarose gel electrophoresis (primer–dimers are <50 bp in size). Use the appropriate RT² SYBR® Green Mastermix to prevent the appearance of primer–dimers.

Ordering Information

Product	Contents	Cat. no.
RT² qPCR Primer Assay (200)	For 200 reactions; mix of two 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

* Larger kit sizes available; please inquire.

Product	Contents	Cat. no.
Related Products		
RT² Profiler PCR Array	Arrays of assays for disease, pathway or functionally related genes; available in 96-well, 384-well and Rotor-Disc 100 format	Varies
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
Human XpressRef Universal Total RNA	2 tubes, each containing 100 µg human RNA at 1 mg/ml	338112
Mouse XpressRef Universal Total RNA	2 tubes, each containing 100 µg mouse RNA at 1 mg/ml	338114
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

* Larger kit sizes available; please inquire.

Product	Contents	Cat. no.
RNeasy Plus Universal Mini Kit (50)	For 50 RNA minipreps: RNeasy Mini Spin Columns, gDNA Eliminator Solution, collection tubes, RNase-free water and buffers	73404
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

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at **www.qiagen.com** or can be requested from QIAGEN Technical Services or your local distributor.

Document Revision History

Date	Changes
04/2020	Corrected column 4 header in Table 2, from "Volume for 3 reactions" to "Volume for 4 reactions". Updated URLs to the GeneGlobe Data Analysis Center and the RT2 qPCR Assay Data Analysis product page. Added QIAquant to recommended real-time cyclers for use with RT² SYBR® Green qPCR Mastermix. Removed the recommendation of PAXgene Blood RNA Kit for preparing RNA from whole blood samples, because it is an IVD product, and added recommendation of RNeasy Protect Animal Blood Kit, cat. no. 73224, for animal blood samples. Replaced recommendation for using RNeasy Microarray Tissue Mini Kit (cat. no. 73304) for preparing RNA from tissue samples, and replaced it with RNeasy Plus Universal Mini Kit (cat. no. 73404). In step 3, page 16, corrected description of centrifuge speed, from "1000 g" to "1000 x *g*". In note below Table 7, removed cross-reference to Table 1 and replaced it with cross-reference to Table 7. Removed references to XpressRef Universal Total RNA as control RNA to prevent user confusion, because it cannot be used for experimental control but only for troubleshooting. Removed recommendation for first-time users to start with 0.5–1 µg of total RNA, because this recommendation only applies to the RT2 Assay Kit. Deleted reference to analysis spreadsheet for primer assays; GeneGlobe serves this function. Despecified temperature at which a single peak should appear in step 9, page 19, because it is not necessarily applicable to all primers. Modified recommendations on dilution of 1 µl RT sample.

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