April 2019

RT^2 lncRNA qPCR Assay Handbook

For long non-coding gene expression analysis by real-time RT-PCR

Sample to Insight

QIAGEN

Contents

Kit Contents

RT² lncRNA qPCR Assay	**(200)**
Catalog no.	**330701**
Number of 25 µl reactions	**200**
200 µl RT² lncRNA qPCR Assay (10 µM) in a single tube	1 tube

Shipping and Storage

RT² long non-coding RNA (lncRNA) qPCR 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 1 year from date of receipt without reduction in performance.

Intended Use

RT² lncRNA qPCR 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 RT^2 lncRNA qPCR Assays is tested against predetermined specifications to ensure consistent product quality.

Introduction

Long non-coding RNAs (lncRNA) are non-protein coding transcripts longer than 200 nucleotides. They are a pervasive and recently recognized class of RNAs. Though only a small portion of lncRNA has been studied, it has been demonstrated that lncRNA plays important biological functions such as transcription regulation, post-translational regulation, small RNA processing, scaffolding, and signaling transduction during cell-cell communications. Some lncRNAs are suggested to be useful as novel potential biomarkers for disease diagnosis, prognosis, and prediction of response to therapy.

Real-time RT-PCR is a highly sensitive and reliable method for gene expression analysis in multiple applications, such as the verification of RNAseq and microarray data. 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 $\Delta\Delta C_q$ 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, high-specificity RT^2 lncRNA qPCR Assays for long non-coding gene expression analyses and RNAseq/microarray data verification.

RT² lncRNA qPCR Assays are designed for SYBR® Green, real-time PCR lncRNA detection. QIAGEN's primer design computer algorithm has been refined using tens of thousands of designs and a wet lab verification process. The RT² lncRNA qPCR Assay design system further enhances this process with a complete transcriptome sequence check, based on a database generated from the authoritative GENCODE and RefSeq databases. Quality control via in vitro wet lab verification ensures each primer's sensitivity, specificity, and efficiency. RT² lncRNA qPCR Assays are available for almost the entire long non-coding gene collection annotated by the RefSeq and GENCODE (see the QIAGEN website for species and version coverage).

Principle and procedure

For optimal performance, RT² lncRNA qPCR Assays should be used together with the RT² First Strand Kit for cDNA synthesis and RT² SYBR Green Mastermixes for qPCR. These reagents have been formulated and pretested together with RT² lncRNA qPCR 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. In addition, an artificial RNA control template is also included that can be used to follow the reverse transcription efficiency across samples and monitor against RNase contamination. 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, and other major suppliers.

Description of protocols

This handbook contains two protocols. The first protocol details cDNA synthesis by reverse transcription using purified RNA and the RT^2 First Strand Kit (page 16). 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 17).

Note: Quantification cycle (C_q) represents the number of cycles needed to reach a set change fluorescence signal level. It is also called C_T or C_p.

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 (cat. no. 330401 and 330404)
- The appropriate RT² SYBR Green Mastermix for your real-time cycler. Available RT² SYBR Green Mastermixes include the following:
    - 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® LightCyler® 480 and LightCycler 480 Instrument II (96-well and 384-well), and LightCycler 96
    - RT² SYBR Green Fluor qPCR Mastermix: suitable for use with Bio-Rad models iCycler®, iQ™5, MyiQ™, MyiQ2
    - RT² SYBR Green ROX™ qPCR Mastermix: suitable for use with 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), QuantiStudio 6 Flex, QuantiStudio *7* Flex, and QuantiStudio 12K Flex (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
    - 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 pipet tips and tubes

- Optional: XpressRef Universal Total RNA to control PCR conditions is available for human (cat. no. 338112) and mouse (cat. no. 338114).
- Optional: RT^2 Reverse Transcription Control Assay (cat. no. 330001; assay PPX63340A)
- Optional: RT^2 Genomic DNA Contamination Assay (cat. no. 330011)

Important Notes

Preparing a workspace free of DNA contamination

For accurate and reproducible RT^2 lncRNA qPCR Assay 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 and 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 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 nuclease-free water. Do not use DEPC-treated water.

Monitoring reverse transcription and presence of RNase activity

Included with the RT^2 First Strand Kit is an artificial RNA that can be spiked into your sample to monitor the presence of RNase activity and the efficiency of reverse transcription from sample to sample. This artificial RNA has no homology to eukaryotic sequences and affords researchers added confidence in detecting low-expressing RNAs and optimal conversion of cDNA during the reverse transcription process.

Cultured cells

We recommend the miRNeasy® Mini Kit (cat. no. 217004) for RNA purification from cultured cells. Alternatively, the RNeasy® Mini Kit (cat. no. 74104) can also be used for this purpose. It is important to perform the on-column DNase digestion step described in the *miRNeasy Mini Handbook* (using the RNase-Free DNase Set [cat. no. 79254]).

Tissue samples

We recommend the PAXgene Tissue miRNA Kit (alternatively, the RNeasy Microarray Tissue Mini Kit, cat. no. 73304, can also be used) 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 miRNeasy FFPE Kit (cat. no. 217504); alternatively, the RNeasy FFPE Kit (cat. no. 73504) can also be used for RNA purification from FFPE samples.

Small samples yielding <100 ng total RNA

We recommend the miRNeasy Micro Kit (cat. no. 217084); alternatively, the RNeasy Micro Kit (cat. no. 74004) can also be used for RNA purification from small samples.

Whole blood samples

We recommend the PAXgene® Blood miRNA Kit (cat. no. 763134) for preparation of total RNA from whole blood samples. Alternatively, the PAXgene Blood RNA Kit (cat. no. 762174) and QIAamp® RNA Blood Mini Kit (cat. no. 52304) can also be used for this purpose.

Serum/Plasma samples

We recommend the miRNeasy Serum/Plasma Kit (cat. no. 217184) or exoRNeasy Serum/Plasma Maxi Kit (cat. no. 77064) for preparation of total RNA from serum/plasma samples.

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 miRNeasy Mini Kit. It is important to perform the on-column DNase digestion step described in the *miRNeasy 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 lncRNA qPCR 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 dependent 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® or the QIAxcel Advanced System using the QIAxcel RNA QC Kit v2.0 (929104). 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 indicates that degradation has occurred in the RNA sample.

* 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.

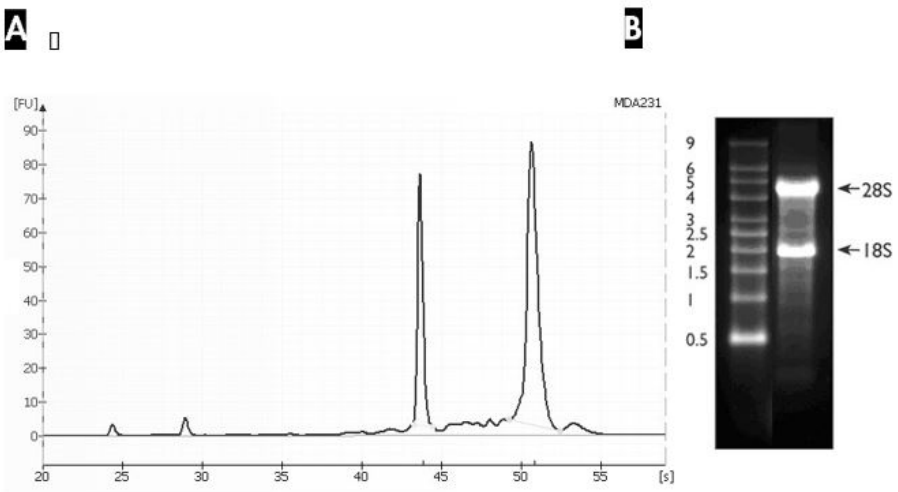

Figure 1. Ribosomal RNA integrity. A An Agilent Bioanalyzer electropherogram of high-quality total RNA showing strong and sharp peaks for the 18S (left) and 28S (right) ribosomal RNA. Due to high quality of the RNA, peaks do not have shoulders (especially to the left of each peak). B Agarose gel electrophoresis 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 profiling results using the RT² lncRNA qPCR 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_q values between the NRT control and a complete reaction for the same gene of interest (GOI) 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 miRNeasy 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 (cat. no. 330011).

Starting RNA amounts

The RT² lncRNA qPCR 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 lncRNA expression analysis from as little as 1 ng total RNA or 100 ng RNA from FFPE samples by preamplifying first strand cDNA. This allows lncRNA 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 lncRNA 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 1 g to 2 g total RNA. We recommend using a consistent amount of total RNA for all samples in a single experiment to facilitate the selection of stable normalization genes during data analysis.

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. First-time users are recommended to start with 1–2 µg total RNA.
- **Do not use DEPC-treated water**. Use high-quality, nuclease-free water.
- The RT² First Strand Kit is not compatible with the chemicals in DNA-free™ DNA Removal 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 Strand Kit (10–15 seconds) 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. To perform a no-template control, omit the RNA and substitute with 10 µl Nuclease-Free Water.**

Table 1. Genomic DNA elimination mix

Component	Amount
RNA	25 ng – 5 µg
Buffer GE	2 µl
Nuclease-Free Water	Variable
Total volume	**10 µl**

3. **Incubate the genomic DNA elimination mix for 5 minutes at 42°C, then place immediately on ice for at least 1 minute.**
4. **Prepare the reverse-transcription mix according to Table 2. To prepare an NRT control, replace the RE3 Reverse Transcriptase Mix with the appropriate volume of Nuclease-Free Water.**

Table 2. Reverse-transcription mix

Component	Volume for 1 reaction	Volume for 6 reactions	Volume for 24 reactions
5x Buffer BC3	4 µl	24 µl	96 µl
Control P2	1 µl	6 µl	24 µl
RE3 Reverse Transcriptase Mix	2 µl	12 µl	48 µl
Nuclease-Free Water	3 µl	18 µl	72 µl
Total volume	**10 µl**	**60 µl**	**240 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 37°C for exactly 60 minutes, then immediately stop the reaction by incubating at 95°C for 5 minutes.**
7. **Place the reactions on ice and add 91 µl Nuclease-Free Water to each reaction. Mix by pipetting up and down several times.**
8. **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 RT² RNA QC PCR Array, follow the protocol in the *RT² RNA QC PCR Array Handbook* using a 6 µl aliquot of the diluted cDNA template.

Protocol: Real-Time PCR Using RT^2 lncRNA qPCR Assays and RT^2 SYBR Green Mastermixes

Important points before starting

- Ensure that the RT^2 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 the wells/tubes when pipetting.
- Do not use DEPC-treated water. Use high-quality, nuclease-free water.
- If precipitates are present in the master mix tubes, warm the reagents at 42°C for 1 minute 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 1 µl diluted cDNA template. To monitor variability between assays, prepare triplicate reactions for each template.
- For every experimental sample, prepare reactions for every GOI and for a set of reference genes (REF) to normalize the raw data. Choose REF(s) known to not change their expression under the experimental conditions. Refer to Table 3 for recommended REFs.

Table 3. List of recommended reference genes

Species	Gene symbol	Description	Species	Gene symbol	Description
Human	ACTB	Actin, beta	Mouse	Actb	Actin, beta
Human	B2M	Beta-2-microglobulin	Mouse	B2m	Beta-2-microglobulin
Human	GAPDH	Gluceraldehyde-3-phophate dehydrogenase	Mouse	Gapdh	Gluceraldehyde-3-phophate dehydrogenase
Human	GUSB	Glucuronidase, beta	Mouse	Gusb	Glucuronidase, beta
Human	HPRT1	Hypoxanthine phosphoribosyl-transferase 1	Mouse	Hsp90ab1	Heat shock protein 90 alpha (cytosolic), class B member I
Human	HSP90ABI	Heat shock protein 90kDa alpha (cytosolic), class B member I	Mouse	Ldha	Lactate dehydrogenase A
Human	LDHA	Lactate dehydrogenase A	Mouse	Pgk 1	Phosphoglycerate kinase I
Human	NONO	Non-POU domain containing, octamer-binding	Mouse	Ppih	Peptidyl prolyl isomerase H
Human	PGKI	Phosphoglycerate kinase I	Mouse	Rn18s	18S ribosomal RNA
Human	PPIA	Peptidylprolyl isomerase A (cyclophilin A)	Mouse	Sdha	Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)
Human	PPIH	Peptidylprolyl isomerase H (cyclophilin H)	Mouse	Tbp	TATA box binding protein
Human	RPLP0	Ribosomal protein, large, P0	Mouse	Tfrc	Transferrin receptor
Human	RPLPI	Ribosomal protein, large, P1	Mouse	Ubc	Ubiquitin C
Human	SDHA	Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	Mouse	Snora73b	Small nucleolar RNA, H/ACA box 73b

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Species	Gene symbol	Description	Species	Gene symbol	Description
Human	TBP	TATA box binding protein			
Human	TFRC	Transferrin receptor (p90, CD71)			
Human	SNORA73A	Small nucleolar RNA, H/ACA box 73A			

- **Optional:** 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 GOI and each REF 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 GOI and REF. 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 for real-time PCR detection

1. **Briefly centrifuge the RT² SYBR Green Mastermix, RT² lncRNA qPCR Assay, and cDNA synthesis reaction (10–15 seconds) 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 inactivation, reactions can be prepared at room temperature (15–25°C).
2. **Prepare the PCR components in a nuclease-free tube as described in Table 4.**

Table 4. PCR components per reaction

Component	Volume for 1 reaction	Volume for 3 reactions
Nuclease-Free Water	10.5 µl	34.7 µl
RT² SYBR Green Mastermix	12.5 µl	41.3 µl
cDNA	1 µl	3.3 µl
RT² lncRNA qPCR Assay (10 µM stock)	1 µl	3.3 µl
Total volume	**25 µl**	**82.6 µl (25 µl/well)**

Note: If performing multiple reactions, prepare a mix containing RT² SYBR Green Mastermix, cDNA synthesis reaction, and Nuclease-Free Water by scaling up the volumes shown in Table 4. We recommend preparing 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 RT² lncRNA qPCR Assay solutions 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 minute at 1000 x *g* to remove bubbles.

4. **Program the real-time cycler according to Table 5, 6, or 7, depending on the real-time cycler used. Run the program.**

   Note: For additional help with instrument set-up, see our setup protocol for your cycler at **www.qiagen.com/products/catalog/assay-technologies/real-time-pcr-and-rt-pcr-reagents/rt2-profiler-pcr-arrays#resources**.

Table 5. Cycling conditions for Applied Biosystems, Eppendorf Mastercycler ep realplex, and Bio-Rad CFX96 and CFX384 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, StepOnePlus, ViiA 7, QuantiStudio 6 Flex, QuantiStudio 7 Flex, and QuantiStudio 12K Flex; 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 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 the setup protocol at **www.qiagen.com/products/catalog/assay-technologies/real-time-pcr-and-rt-pcr-reagents/rt2-profiler-pcr-arrays#resources** for detailed setup instructions.

Table 6. 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.

§ Recommended for the Roche LightCycler 480. If using a Roche LightCycler 480 II, adjust the ramp rate to 1.5°C/s for the 96-well block and 1.8°C/s for the 384-well block. Refer to the setup protocol for your cycler at **www.qiagen.com/products/catalog/assay-technologies/real-time-pcr-and-rt-pcr-reagents/rt2-profiler-pcr-arrays#resources** for more information on other required changes to settings for Melt Curve Acquisition.

Table 7. Cycling conditions for Bio-Rad MJ Research models Chromo4, DNA Engine Opticon, DNA Engine Opticon 2; Takara TP-800; 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 cyders: Bio-Rad/MJ Research models Chromo4, DNA Engine Opticon, DNA Engine Opticon 2; Takara TP-800; all other cyders.

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 minute; 65°C, 2 minutes (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 setup protocol for your cycler at **www.qiagen.com/products/catalog/assay-technologies/real-time-pcr-and-rt-pcr-reagents/rt2-profiler-pcr-arrays#resources.**

   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 RT² lncRNA qPCR 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.qiagen.com/FAQ/FAQList.aspx**. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and/or protocols in this handbook or sample and assay technologies (for contact information, visit **www.qiagen.com**).

		Comments and suggestions
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_q values between the NRT control and a complete reaction for the same GOI 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 transcripts	The human transcriptome is much larger than has been previously appreciated, especially following the discovery of non-coding RNAs. Even when using the most advanced transcriptome database available, many alternative transcripts or unknown non-coding transcripts exist. RT² lncRNA qPCR Assays cover the transcripts in the assay list and detect the sum of their expression. Primer design cannot account for unannotated 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_q values are too high (>33 or not detectable)

a)	Experimental error	Use a template known to contain the GOI as a positive control to check the PCR reagents and experimental procedure.
b)	Poor RNA quality	Use more input RNA for reverse transcription, especially if the lower end of the recommended range had been used previously.
c)	Insufficient template	Use a larger volume of template per reaction, but do not use more than 2.5 µl of undiluted cDNA template per 25 µl reaction. Use the same volume of template in each reaction.
d)	Specific transcript not covered by default assay	High or undetectable C_q values will result if the target lncRNA transcript is not covered by the default assay, or if the transcript is exogenously expressed from a vector, plasmid, or other construct that contains a specific transcript of an lncRNA gene and the lncRNA assay does not cover that transcript. Refer to the covered target transcripts list on the Product Sheet provided with the RT² lncRNA qPCR Assay.

C_q 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. Dilute the template first if <1 µl is needed. 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 lncRNA qPCR Assay is being used to verify a knockout mouse model where only a portion of the endogenous gene is replaced, and the RT² lncRNA qPCR Assay is not located in the replaced sequence of the resulting lncRNA transcript. Ask for custom RT² lncRNA qPCR Assays for this purpose.

No template control (NTC) shows a C_q value <33 cycles

a)	DNA contamination of reagents, tips, and tubes	See "Preparing a workspace free of DNA contamination."
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.

Appendix A: Data Analysis

$\Delta\Delta C_q$ method

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

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

For example:

Control:	C_q (GOI) = 24.25	C_q (REF) = 16.49
Experimental:	C_q (GOI) = 19.17	C_q (REF) = 16.36

For each sample, calculate the difference between the C_q values (ΔC_q) for each GOI and the REF or the average C_q value of the set of REFs.

For example:

ΔC_q (control) = C_q (GOI) – C_q (REF) = 24.25 – 16.49 = 7.76

ΔC_q (experimental) = C_q (GOI) – C_q (REF) = 19.17 – 16.36 = 2.81

For each pair-wise set of samples to be compared, calculate the difference in ΔC_q values ($\Delta\Delta C_q$) for the genes of interest between the 2 samples.

For example:

$\Delta\Delta C_q$ = ΔC_q (experimental) – ΔC_q (control) = 2.81 – 7.76 = –4.95

Calculate the fold-change in gene expression. Due to high levels of amplification efficiency using RT² lncRNA qPCR Assays, the fold-change in gene expression is equal to $2^{-\Delta\Delta Cq}$.

For example:

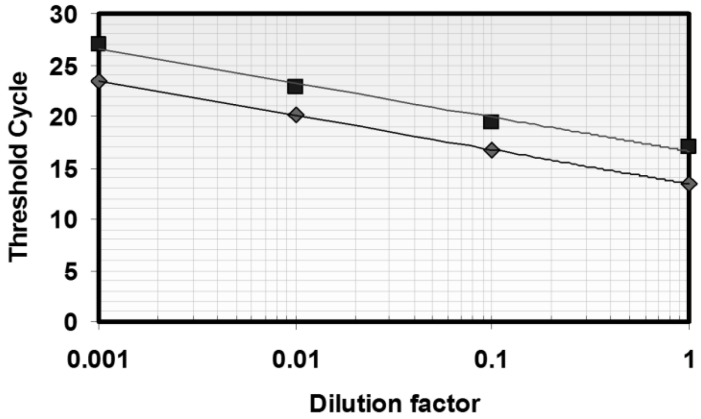
Fold change = $2^{(-\Delta\Delta Cq)} = 2^{(-[-4.95])} = 2^{(4.95)} = 30.9$

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 quantification cycle (C_q) 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 GOI and for each REF. 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:



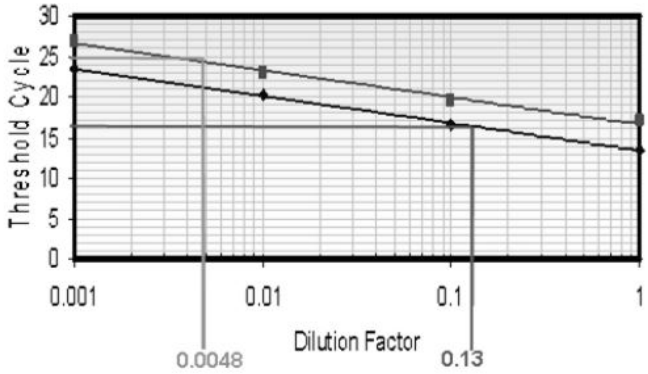
GOI: $C_q = -3.302\text{Log}(L) + 16.6$

REF: $C_q = -3.351\text{Log}(L) + 13.4$

For every reaction containing template synthesized from experimental samples, use the C_q value and the appropriate standard curve (based on the gene-specific RT² lncRNA qPCR Assay used in the reaction) to calculate the relative expression level of each gene of interest

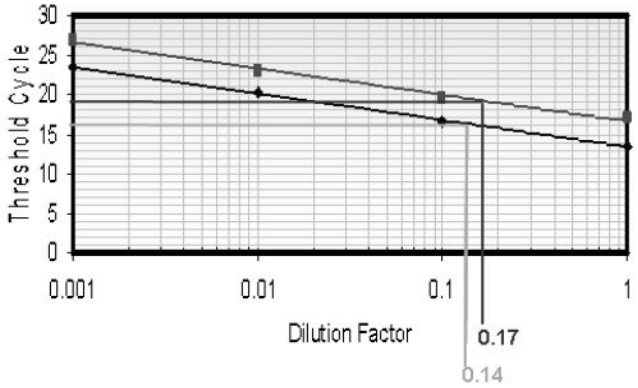
(L[GOI]) and the relative expression level of each reference gene (L[REF]) in each sample. Be sure that the C_q values fall within the linear range of the appropriate standard curve.

For example:



Control: C_q(GOI) = 24.25 C_q(REF) = 16.49

L(GOI) = 0.0048 L(REF) = 0.13



Experimental: C_q(GOI) = 19.17 C_q(REF) = 16.36

L(GOI) = 0.17 L (REF) = 0.14

Normalize the expression level of the genes of interest by dividing their relative expression level by the relative expression level of the REF or the average relative expression level of a set of REFs. 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 GOI between 2 different samples, calculate the ratio of its normalized expression levels (determined from the same PCR conditions) between those samples.

For example:

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

Ordering Information

Product	Contents	Cat. no.
RT² lncRNA qPCR Array (200)	For 200 reactions; Mix of 2 gene-specific primers provided in solution (200 µl), with 10 µM of each primer	Varies
RT² Reverse Transcription Control Assay	Primer for 200 qPCR reactions	330001 (assay# PPX63340A)
RT² qPCR Primer gDNA Controls	Primer for 200 qPCR reactions	330011
Custom RT² lncRNA PCR Array	Array for profiling a user-defined panel of lncRNAs	330731
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), Nuclease-Free Water (1 ml)	330401
RT² First Strand Kit (50)	For 50 x 20 µl first strand cDNA synthesis reactions; Buffer GE (100 µl), 5xBuffer BC3 (200 µl), RE3 Reverse Transcriptase Mix (100 µl), Control P2 (50 µl), Nuclease-Free Water (1 ml)	330404
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

* Larger kit sizes available; please inquire.

Product	Contents	Cat. no.
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		
RT² lncRNA 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 lncRNA 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

* Larger kit sizes available; please inquire.

† Search **www.geneglobe.com**.

Product	Contents	Cat. no.
miRNeasy Mini Kit (50)	50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), QIAzol Lysis Reagent, RNase-Free Reagents and Buffers	217004
RNeasy Mini Kit (50)*	50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free Reagents and Buffers	74104
miRNeasy FFPE Kit (50)	50 RNeasy MinElute Spin Columns, Collection Tubes, Proteinase K, RNase-Free DNase I, DNase Booster Buffer, RNase-Free Buffers, Nuclease-free water	217504
RNeasy FFPE Kit (50)	50 RNeasy MinElute Spin Columns, Collection Tubes, Proteinase K, RNase-Free DNase I, DNase Booster Buffer, RNase-free buffers, Nuclease-free water	73504
miRNeasy Serum/Plasma Kit (50)	50 RNeasy MinElute Spin Columns, Collection Tubes (1.5 ml and 2 ml), QIAzol Lysis Reagent, Ce-miR-39_1 miScript Primer Assay, RNase-free Reagents and Buffers	217184
PAXgene Blood miRNA Kit (50)	PAXgene Spin Columns, PAXgene Shredder Spin Columns, Processing Tubes, Microcentrifuge Tubes, RNase-Free DNase, RNase-Free Reagents and Buffers; to be used in conjunction with PAXgene Blood RNA Tubes	763134
PAXgene Blood RNA Kit (50)	50 PAXgene Spin Columns, 50 PAXgene Shredder Spin Columns, Processing Tubes, Microcentrifuge 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
miRNeasy Micro Kit (50)	50 RNeasy MinElute Spin Columns, Collection Tubes (1.5 ml and 2 ml), QIAzol Lysis Reagent, RNase-Free Reagents and Buffers	217084
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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Notes

Handbook Revision History

Document	Changes	Date
HB-1878-001	Initial release	October 2014
HB-1878-002	Updated template. Updated data analysis procedure.	April 2019