October 2014

RT² lncRNA PreAMP cDNA Synthesis Handbook

RT² PreAMP cDNA Synthesis Kit

RT² Microfluidics qPCR Reagent System

RT² lncRNA PreAMP Primer Mix

Custom RT² lncRNA PreAMP Primer Mix

For synthesis and preamplification of cDNA from small RNA samples and RNA from formalin-fixed, paraffin-embedded samples for use with RT² lncRNA PCR Arrays or RT² lncRNA qPCR Assays

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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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Kit Contents

RT² PreAMP cDNA Synthesis Kit	**(12)**
Catalog no.	**330451**
Number of cDNA synthesis reactions	**12**
Buffer GE	24 µl
5x Buffer BC3	48 µl
cDNA Synthesis Enzyme Mix	12 µl
RNase Inhibitor	12 µl
Control P2	12 µl
Nuclease-Free Water	1 ml
RT² PreAMP PCR Mastermix	600 µl
Side Reaction Reducer	96 µl
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RT² lncRNA PreAMP Primer Mix*	**(12)**
Catalog no.	**Varies**
Number of preamplification reactions	**12**
RT² lncRNA PreAMP Primer Mix	90 µl

* Each RT² PreAMP lncRNA Primer Mix can be used only with the corresponding gene- or pathway-specific RT² lncRNA PCR Array. Verify that the lot number of the RT² lncRNA PreAMP Primer Mix is compatible with that of the RT² lncRNA PCR Array to be used.

Custom RT² lncRNA PreAMP Primer Mix*	**(48)**
Catalog no.	**Varies**
Number of preamplification reactions	**12**
Custom RT² lncRNA PreAMP Primer Mix	4 x 90 µl

* Each RT² lncRNA PreAMP Primer Mix can be used only with the corresponding gene- or pathway-specific RT² lncRNA PCR Array. Verify that the lot number of the RT² lncRNA PreAMP Primer Mix is compatible with that of the RT² lncRNA PCR Array to be used.

Custom RT² lncRNA PreAMP Primer Mix, Format H*	(2 tubes)
Catalog no.	Varies
Number of preamplification reactions	960
RT² lncRNA PreAMP Primer Mix	2 x 1.66 ml

* Each RT² lncRNA PreAMP Primer Mix can be used only with the corresponding gene- or pathway-specific RT² lncRNA PCR Array. Verify that the lot number of the RT² lncRNA PreAMP Primer Mix is compatible with that of the RT² lncRNA PCR Array to be used.

Shipping and Storage

The RT² PreAMP cDNA Synthesis Kit is shipped on dry ice or blue ice packs and must be stored at –20°C upon receipt. To ensure that RT² PreAMP PCR Mastermix does not become contaminated and to avoid repeated freezing and thawing, divide into appropriate aliquots. Store away from any sources of template DNA. RT² lncRNA PreAMP Primer Mix is shipped frozen or at ambient temperature. Store at –20°C immediately upon receipt. If entire volume will not be used at the same time, divide into aliquots and store at –20°C. Avoid repeated freezing and thawing.

Under these conditions, these components can be kept for at least 6 months from the date of receipt without any reduction in performance.

Intended Use

The RT² PreAMP cDNA Synthesis Kit and RT² lncRNA PreAMP Primer Mix 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 Quality Management System, each lot of RT^2 PreAMP cDNA Synthesis Kit and RT^2 lncRNA PreAMP Primer Mix is tested against predetermined specifications to ensure consistent product quality.

Introduction

Recent advances in tissue preparation and RNA purification procedures allow researchers to work with very small amounts of RNA such as those from small cell populations, laser capture microdissection samples, fluorescence-activated cell sorting (FACS®) samples, or fine needle aspiration biopsies. However, the low RNA yields obtained from these small samples are often insufficient for reliable gene expression analysis, even using sensitive techniques such as real-time RT-PCR.

Around the world, archives of formalin-fixed, paraffin-embedded (FFPE) tissue sections with known clinical annotations represent a valuable and extensive source of material for biomedical research. However, analysis of RNA from those samples presents many challenges. RNA in FFPE samples is likely to be fragmented and chemically modified by formaldehyde with extensive crosslinking between protein, DNA, and RNA. Some of this RNA damage is irreversible and greatly reduces the amount of template available for downstream real-time RT-PCR analysis, thus affecting performance and sensitivity.

The RT^2 PreAMP cDNA Synthesis Kit and RT^2 lncRNA PreAMP Primer Mixes are a breakthrough technology enabling expression analysis of pathway- or disease-focused lncRNAs using as little as 1 ng total RNA from fresh/frozen samples or 100 ng total RNA from FFPE samples. RT^2 PreAMP technology uses multiplex, PCR-based preamplification to provide amplification of gene-specific cDNA target templates with minimal bias. The RT^2 PreAMP cDNA Synthesis Kit and RT^2 lncRNA PreAMP Primer Mixes enable synthesis and preamplification of cDNA from total RNA samples, prior to lncRNA expression analysis using RT^2 lncRNA PCR Arrays. Each RNA sample can be used to prepare enough cDNA for gene expression analysis of up to 4 different lncRNA PCR arrays.

Principle and procedure

The workflow for the RT^2 PreAMP cDNA Synthesis Kit and RT^2 lncRNA PreAMP Primer Mixes is convenient and quick, accessible for routine use in every research laboratory. The procedure consists of 2 steps: cDNA synthesis and preamplification.

cDNA synthesis

The RT^2 PreAMP cDNA Synthesis Kit provides sufficient reagents for synthesis of cDNA from 12 RNA samples. The kit includes a built-in external RNA control template that is detected by the reverse transcription control (RTC) in RT^2 lncRNA

PCR Arrays. This control allows detection of any inhibitors of reverse transcription that could compromise the efficiency of cDNA synthesis reactions.

Preamplification of cDNA for pathway-specific lncRNAs

Each RT^2 lncRNA PreAMP Primer Mix is specific for one RT^2 lncRNA PCR Array. During the amplification step, the RT^2 lncRNA PreAMP Primer Mix enables amplification of cDNA specific for the genes targeted by the RT^2 lncRNA PCR Array. A cDNA synthesis reaction from 1–100 ng total RNA from fresh/frozen samples or 100 ng – 1 µg total RNA from FFPE samples provides sufficient cDNA for amplification by 4 different RT^2 lncRNA PreAMP Primer Mixes, allowing gene expression analysis of up to 4 different lncRNA PCR arrays. Following preamplification, the Side Reaction Reducer eliminates residual primers. Preamplified cDNA is then ready for PCR array analysis using the appropriate RT^2 lncRNA PCR Array.

lncRNA expression analysis using RT^2 lncRNA PCR Arrays

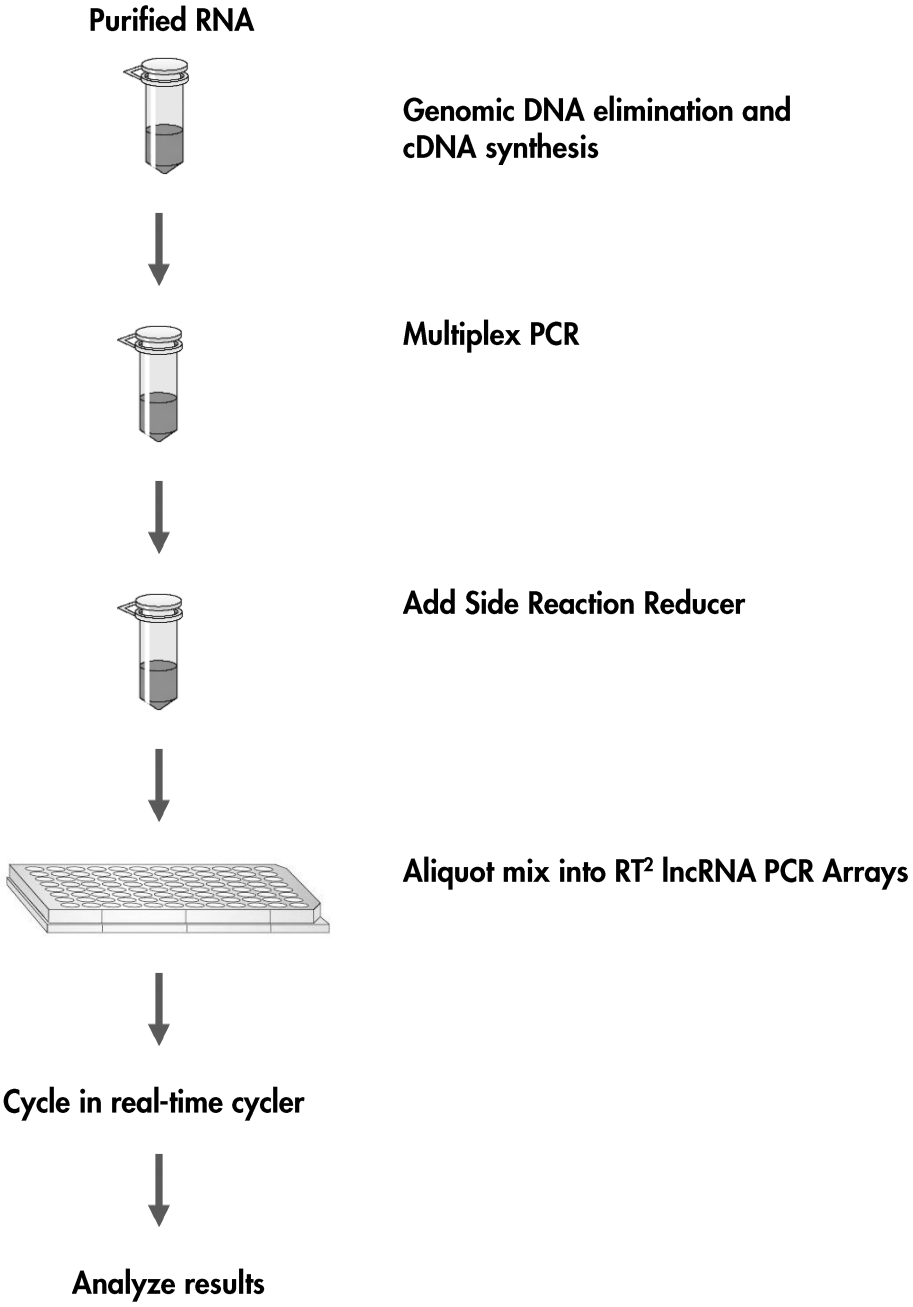
For PCR array analysis, preamplified cDNA is mixed with RT^2 SYBR® Green Mastermix. Various RT^2 SYBR Green Mastermixes are available for use with different real-time cyclers. The mixture is aliquoted into the wells of an RT^2 lncRNA PCR Array which contains predispensed gene-specific primer pairs. PCR is performed and relative expression is determined using the $\Delta\Delta C_q$ method.

Description of protocols

This handbook contains 4 protocols. The first protocol describes first-strand cDNA synthesis from your RNA samples (page 17). After completion of this protocol, cDNA samples are preamplified in the second protocol (page 19). After preamplification, samples are ready to be used for analysis with an RT^2 lncRNA PCR Array. The fourth protocol describes how these steps are performed for Format H, the array format used with the Fluidigm BioMark system. For RT^2 lncRNA PCR Array analysis, follow the instructions in the third protocol (page 21) and in the *RT^2 lncRNA PCR Array Handbook*.

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

RT² lncRNA PreAMP cDNA Synthesis Procedure



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.

In addition to the RT² PreAMP cDNA Synthesis Kit and RT² lncRNA PreAMP Primer Mix, the following are required:

- Purified RNA samples (for recommended purification methods, see page 12)
- RT² lncRNA PCR Array
  RT² lncRNA PCR Arrays are available in different formats, to suit different real-time cyclers. For full details, see the *RT² lncRNA PCR Array Handbook*. Each RT² lncRNA PreAMP Primer Mix is specific for a particular pathway- or disease-focused RT² lncRNA PCR Array. Check the label to verify that the correct RT² lncRNA PreAMP Primer Mix is used for the appropriate RT² lncRNA PCR Array. Verify that the lot number of the RT² lncRNA PreAMP Primer Mix is compatible with that of the RT² lncRNA PCR Array to be used. We strongly recommend that RT² lncRNA PreAMP Primer Mix and the RT² lncRNA PCR Array are purchased together whenever possible. If the RT² lncRNA PreAMP Primer Mix and the RT² lncRNA PCR Array have been purchased at different times, check with QIAGEN Technical Services to ensure their compatibility.
- RT² SYBR Green Mastermix suitable for use with your real-time cycler. Available RT² SYBR Green Mastermixes 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 LightCycler 96
  - 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); QuantStudio™ 6, QuantiStudio 7, and QuantiStudio 12K (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
- Real-time PCR cycler with 0.2 ml tube heat block, heated lid, and 10–100 µl reaction capacity
- 0.2 ml tubes or 8-tube strip PCR tubes with caps
- Multichannel pipettor
- 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).

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 during real-time RT-PCR, 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 remove the RT^2 lncRNA PCR Array from its protective sealed bag until immediately before use. Do not leave labware (tubes and tip boxes) exposed to the air for long periods of time.
- Do not open any previously run and stored RT^2 lncRNA PCR Array. Removing the thin-wall 8-cap strips or the adhesive film from PCR arrays releases PCR product DNA into the air where it may affect the results of future real-time PCR experiments.

RNA preparation, quantification, and quality control

The most important prerequisite for any gene expression analysis experiment is purification of consistently 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.

Laser capture microdissected (LCM) samples

We recommend capturing LCM samples on Arcturus® CapSure® Caps and purifying RNA using the Arcturus PicoPure® RNA Isolation Kit following the manufacturer's instructions. Special care should be taken during sample preparation and microdissection to minimize RNA degradation. For example, LCM sessions longer than 30 minutes per slide may lead to precipitous loss in recovery of intact RNA. Keep the staining and dissection procedure as short as possible.

Fine needle aspiration biopsies (FNAB) and other small biological samples

We recommend use of the miRNeasy® Micro Kit (cat. no. 217084) for most small samples with a small number of cells such as fine needle aspiration biopsies, manual dissection samples, or FACS samples. Alternatively, RNeasy® Micro Kit (cat. no. 74004) can be used. In addition, the Arcturus PicoPure RNA Isolation Kit can also be used for non-LCM samples such as cell samples in suspension. Irrespective of the kit used, it is important to perform DNase treatment during the procedure.

FFPE samples

We recommend use of the miRNeasy FFPE Kit (cat. no. 217504), alternatively RNeasy FFPE Kit (cat. no. 73504) can also be used for RNA purification from FFPE 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) other than QIAGEN miRNeasy Kits should be further purified using the RNeasy Mini Kit (cat. no. 74104). It is important to perform the on-column DNase digestion step described in the *RNeasy Mini Handbook*.

RNA quantification and quality control

For optimal performance of RT^2 PreAMP procedures and best results from the RT^2 lncRNA PCR Array, all RNA samples should be resuspended in Nuclease-Free Water. Do not use DEPC-treated water for resuspension.

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

Integrity of RNA from fresh/frozen samples

If RNA samples are at least 2 ng, quality control can be performed by analyzing 200 pg of each sample on an Agilent® Bioanalyzer using an RNA 6000 Pico LabChip®. The QIAGEN QIAxcel® Advanced System can also be used. Verify that there is a sharp distinction at the small side of both the 18S and 28S ribosomal RNA (rRNA) peaks. Any smearing or shoulder on the rRNA peaks indicates that degradation has occurred in the RNA sample.

When working with small samples, the RNA yield can be extremely low. Therefore it is often impossible to confirm the quality of total RNA using any analytical means. It is essential to choose a suitable RNA purification method for the biological sample as described above, and follow the manufacturer's protocol carefully.

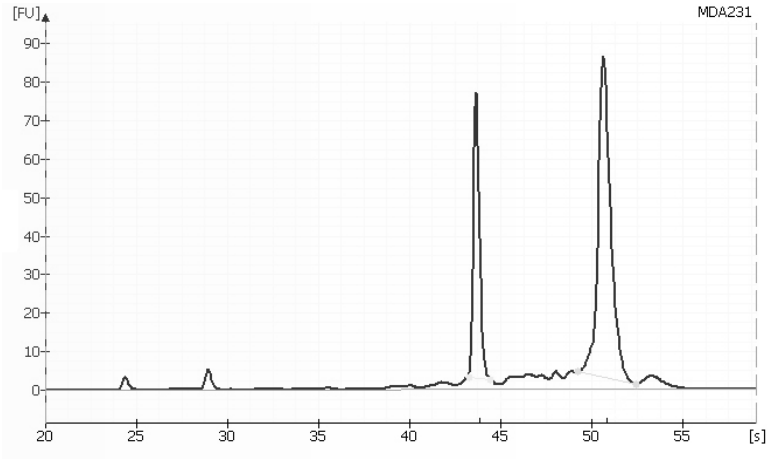


Figure 1. Ribosomal RNA band integrity is important for optimal PCR array results. An Agilent Bioanalyzer electropherogram of a high-quality total RNA preparation shows strong and sharp peaks without shoulders (especially to the left of each peak) for the 18S (left) and 28S (right) ribosomal RNA.

* 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.

Integrity of RNA from FFPE samples

RNA from FFPE tissue samples is expected to show some degree of degradation. Run an aliquot of each RNA sample on a denaturing agarose gel, or alternatively, characterize the samples using an RNA 6000 Nano Chip on an Agilent Bioanalyzer. Assess the extent of RNA degradation. The usual length of FFPE RNA fragments is expected to be 100–1000 bases.

Genomic DNA contamination

Eliminating genomic DNA contamination is essential for obtaining optimal real-time gene expression profiling results using RT^2 lncRNA PCR Arrays. The genomic DNA control in each RT^2 lncRNA PCR Array specifically tests for genomic DNA contamination in each sample during each run. A genomic DNA control quantification cycle value of less than 30 after preamplification indicates the presence of a detectable amount of genomic DNA contamination that should be addressed.

To remove any residual contamination from your RNA samples, we recommend RNA purification using the RNeasy Mini Kit including the optional on-column DNase digestion step, or using the RNeasy FFPE Kit.

Starting RNA amounts

Quantifying total RNA from very small samples using UV spectrophotometry is often impossible. Instead, estimate the total RNA amount by assuming that each eukaryotic cell contains an average of ~10 pg of total RNA or less. Note that the yield of RNA varies widely from cell type to cell type.

The RT^2 PreAMP cDNA Synthesis Kit and RT^2 lncRNA PreAMP Primer Mix generate sufficient template for gene expression analysis using RT^2 lncRNA PCR Arrays with as little as 1–100 ng total RNA (from fresh/frozen samples) or 100 ng – 1 µg total RNA (from FFPE samples) input into each first-strand cDNA synthesis reaction prior to preamplification. Each cDNA synthesis reaction allows the user to perform as many as 4 preamplification reactions and 4 RT^2 lncRNA PCR Array analyses. However, 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; that is, genes expressed in the linear dynamic range of the method. Lower amounts of input total RNA yield a smaller number of positive calls and increase false negative calls.

The RT^2 PreAMP cDNA Synthesis Kit and RT^2 lncRNA PreAMP Primer Mix maximize the number of positive calls at low amounts of total RNA. For successful results and maximum positive call rates, we recommend that first-time users start by using 10–50 ng total RNA from fresh/frozen samples or 500 ng – 1 µg total RNA from FFPE samples. It is also important to use a consistent amount of total RNA for all samples to be characterized and compared in a single experiment.

Protocol: First Strand cDNA Synthesis

Important points before starting

- RNA samples are very sensitive to RNase digestion; therefore, wear gloves and maintain an RNase-free work area while performing this protocol.
- The RT^2 PreAMP cDNA Synthesis Kit is not compatible with the chemicals in DNA-free™ kits from Ambion. If your RNA sample has been treated with DNA-free kit reagents, contact QIAGEN Technical Services.

Procedure

1. **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 for one sample (RNA from fresh/frozen sample)	Amount for one sample (RNA from FFPE sample)
RNA	1–100 ng	100 ng – 1 µg
Buffer GE	2 µl	2 µl
Nuclease-Free Water	Variable	Variable
Total volume	**10 µl**	**10 µl**

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

3. **Prepare the reverse-transcription mix according to Table 2.**
   For multiple reactions, scale up the volumes shown in Table 2 accordingly.

Table 2. Reverse-transcription mix

Component	Volume for 1 reaction
5x Buffer BC3	4 µl
Control P2	1 µl
cDNA Synthesis Enzyme Mix	1 µl
RNase Inhibitor	1 µl
Nuclease-Free Water	3 µl
Total volume	**10 µl**

4. **Add 10 µl reverse-transcription mix to each tube containing 10 µl genomic DNA elimination mix. Mix gently by pipetting up and down. Centrifuge briefly to remove any air bubbles and collect all the liquid at the bottom of the tube.**
5. **Incubate at 37°C for exactly 60 min. Then immediately stop the reaction by incubating at 95°C for 5 min.**
6. **Place the reactions on ice and proceed with the preamplification protocol.**
   If you wish to store the reactions overnight prior to real-time PCR, transfer to a –20°C freezer. Longer storage times are not recommended.

Protocol: Preamplification of cDNA Target Templates

Important point before starting

- Each RT² lncRNA PreAMP Primer Mix is specific for a particular pathway- or disease-focused RT² lncRNA PCR Array. Check the label to verify that the correct pathway-specific RT² lncRNA PreAMP Primer Mix is used for the RT² lncRNA PCR Array. Verify that the lot number of the RT² lncRNA PreAMP Primer Mix is compatible with that of the RT² lncRNA PCR Array to be used. If the RT² lncRNA PreAMP Primer Mix and the RT² lncRNA PCR Array have been purchased at different times, check with QIAGEN Technical Services to ensure their compatibility.

Procedure

1. **Thaw the RT² PreAMP PCR Mastermix and RT² lncRNA PreAMP Primer Mix at room temperature. If precipitates are visible, warm the reagents at 42°C for 1 min and vortex briefly to dissolve. Repeat if necessary.**
2. **Prepare the preamplification mix according to Table 3.**
   For multiple reactions, scale up the volumes shown in Table 3 accordingly.

Table 3. Preamplification mix

Component	Amount for one sample
RT² PreAMP PCR Mastermix	12.5 µl
RT² lncRNA PreAMP Primer Mix	7.5 µl
Total volume	**20 µl**

3. **Pipet 5 µl cDNA synthesis reaction (from step 6, page 18) into a 0.2 ml PCR tube. Then add 20 µl preamplification mix.**
4. **Mix gently by pipetting up and down. Spin briefly to remove any air bubbles and collect all the liquid at the bottom of the tube.**

5. **Program the real-time cycler according to Table 4 or Table 5. Place the tubes in the real-time cycler and start the program.**

Table 4. Cycling conditions for preamplification of cDNA from fresh/frozen samples

Cycles	Duration	Temperature	Comments
1	10 min	95°C	HotStart DNA *Taq* Polymerase is activated by this heating step.
12	15 s	95°C	
	2 min	60°C	
Hold		4°C	

Table 5. Cycling conditions for preamplification of cDNA from FFPE samples

Cycles	Duration	Temperature	Comments
1	10 min	95°C	HotStart DNA *Taq* Polymerase is activated by this heating step.
8	15 s	95°C	
	2 min	60°C	
Hold		4°C	

6. **When cycling is finished, take the tubes from the real-time cycler and place on ice.**
7. **Add 2 µl Side Reaction Reducer to each preamplified reaction. Mix gently by pipetting up and down. Spin the tubes briefly to remove any air bubbles and collect all the liquid at the bottom of the tube.**
8. **Incubate at 37°C for 15 min followed by heat inactivation at 95°C for 5 min.**
9. **Immediately add 84 µl nuclease-free water. Mix well.**
10. **Place on ice prior to real-time PCR, or store overnight at –20°C.**

Protocol: Real-Time PCR Using RT² lncRNA PCR Arrays

Important points before starting

- Ensure that the RT² SYBR Green Mastermix and the RT² lncRNA PCR Array format are suitable for your real-time cycler. An incorrect RT² lncRNA PCR Array format will not fit the real-time cycler properly and may damage the real-time cycler. For more details, see the *RT² lncRNA PCR Array Handbook*.
- For accuracy and precision, ensure that micropipettors are calibrated before beginning the protocol. Be sure not to introduce bubbles into the wells of the RT² lncRNA PCR Array when pipetting.

Procedure

1. **Briefly centrifuge the RT² SYBR Green Mastermix (10–15 s) to bring the contents to the bottom of the tube.**

   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 or a loading reservoir depending on the RT² lncRNA PCR Array format, as described in Table 6.**

Table 6. PCR components mix

Array format:	96-well A, C, D, F	384-well E, G	Rotor-Disc® 100 R
2x RT² SYBR Green Mastermix	1275 µl	550 µl	1150 µl
Preamplification reaction (from step 10, page 19)	102 µl	102 µl	102 µl
Nuclease-Free Water	1173 µl	448 µl	1048 µl
Total volume	**2550 µl**	**1100 µl**	**2300 µl**

Note: This provides an excess volume of ~140–300 µl to allow for pipetting errors. Perform pipetting steps as precisely as possible to ensure that each well receives the required volume.

3. **Continue from the corresponding step 3 onwards of the "Real-Time PCR for RT² lncRNA PCR Arrays" protocol in the *RT² lncRNA PCR Array Handbook*.**

Protocol: cDNA Synthesis and Real-Time PCR for RT² lncRNA PCR Arrays Format H

This protocol is for users of the Fluidigm BioMark HD System. In this protocol, cDNA synthesis is performed using the RT² Microfluidics qPCR Reagent System.

Next, preamplification is performed using the RT² lncRNA PreAMP Primer Mix Format H. Finally, real-time PCR is performed using RT² lncRNA PCR Array Format H in combination with Microfluidics qPCR Master Mix (contains EvaGreen®). For more information, consult the *RT² lncRNA PCR Array Handbook*.

Considerations of RNA amount to be used

The RT² Microfluidics qPCR System yields results with as little as 10 ng or as much as 1 µg total RNA per well reaction. However, 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 RNA yield a greater number of positive calls; that is, genes expressed in the linear dynamic range of the method.

Important: Use a consistent amount of total RNA for all samples in a single experiment to be characterized and compared.

Procedure

cDNA synthesis using the RT² Microfluidics qPCR Reagent System

1. **Thaw Buffer GE2 and BC4 Solution (reverse transcription master mix). Mix each solution by flicking the tubes. Centrifuge briefly to collect residual liquid from the sides of the tubes and then store on ice.**
2. **Prepare the genomic DNA elimination mix for each RNA sample in one well of a 96-well plate according to Table 9.**

Table 9. Genomic DNA elimination mix

Component	Amount for each well
RNA	10 ng-1 µg
Buffer GE2	6 µl
Nuclease-Free Water	Variable
Total volume	**14 µl**

3. **Incubate the genomic DNA elimination mix for 5 min at 37°C, then place immediately on ice for at least 1 min.**
4. **Add 6 µl BC4 Solution to each well, mix by carefully pipetting up and down (can be done with a multi-channel pipettor). Centrifuge briefly to collect residual liquid from the sides of the tubes.**
5. **Program a thermal cycler for a single cycle as follows: 37°C for 60 min, 95°C for 5 min, 4°C hold. Place the 96-well plate in the cycler and run the program.** This is the reverse transcription step.
6. **Place the reactions on ice and proceed with the preamplification protocol.**
   Note: If you wish to store the reactions, transfer them to a –20°C freezer.

Preamplification using RT² PreAMP Primer Mix Format H

7. **Thaw RT² PreAMP Primer Mix and RT² PreAMP PCR Mastermix (PA-30) on ice. Mix each solution by flicking the tubes. Centrifuge briefly to collect residual liquid from the sides of the tubes and then store on ice.**
8. **Prepare preamplification mix according to Table 10.**

Table 10. Preamplification mix

Component	Amount for one sample	Amount for 96 wells*
RT² PreAMP Primer Mix	3 µl	330 µl
RT² PreAMP PCR Mastermix (PA-30)	5 µl	550 µl
Total volume	**8 µl**	**880 µl**

* These volumes provide 15% more mix than is required to allow for pipetting errors.

9. **Pipet 8 µl preamplification mix into each well of an empty 96-well plate.**
10. **Add 2 µl first-strand cDNA from each well of the 96-well plate in step 6 to each well of the 96-well plate in step 9 using an 8-channel pipettor.** The remaining first-strand cDNA can be stored for use in future experiments.
11. **Mix by carefully pipetting up and down and spin briefly.**
12. **Program the real-time cycler according to Table 11. Place the 96-well plate in the real-time cycler and start the program.**

Table 11. Cycling conditions for preamplification

Cycles	Duration	Temperature	Comments
1	10 min	95°C	HotStart DNA Taq Polymerase is activated by this heating step
14	15 s 2 min	95°C 60°C	
Hold		4°C	

13. **When cycling is finished, take the plate from the real-time cycler and place on ice.**
14. **Add 1 µl Side Reaction Reducer to each well. Mix gently by pipetting up and down and spin briefly.**
15. **Incubate at 37°C for 15 min followed by heat inactivation at 95°C for 5 min.**
16. **Add 44 µl Nuclease-Free Water to each well.** This is a 5-fold dilution (11 µl preamplification mix + 44 µl water). This dilution can be optimized if desired. Undiluted cDNA can be used for qPCR if needed.
17. **Place on ice prior to real-time PCR, or store at –20°C.**

For more details on running your sample in the Fluidigm BioMark, please consult the *RT² lncRNA PCR Array Handbook*, protocol "cDNA Synthesis and Real-Time PCR for RT² lncRNA PCR Arrays Format H."

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 protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit www.qiagen.com).

	Comments and suggestions
High C_q values for several genes including reference genes	
a) RNA input was less than recommended lower limit of 1 ng	Increase amount of input RNA.
b) Poor quality RNA	Check the concentration and quality of RNA samples as described in "Important Notes" (page 12).
Genomic DNA contamination	
a) DNase treatment not performed	Perform DNase treatment during RNA purification. If purifying RNA using the RNeasy Mini Kit, perform the DNase treatment step. If genomic DNA contamination is difficult to remove, fold-changes in gene expression may still be obtained. However, it will then be very important to verify any results for individual genes by a separate, more rigorous real-time PCR analysis that includes a "minus RT" control.
b) DNA contamination of reagents or labware	Follow the guidelines for preparing a workspace free of DNA contamination described in "Important Notes" (page 12).
Poor reverse transcription efficiency	
Poor quality RNA	If possible, recheck the A_{260}:A_{280} and A_{260}:A_{230} ratios of RNA samples in RNase-free Tris pH 8.0 buffer. If necessary, repurify RNA samples with a spin-column based cleanup method, such as the RNeasy Mini Kit.

Comments and suggestions	
Poor PCR amplification efficiency	
a) Sensitivity of real-time cycler	Different real-time cyclers have different levels of sensitivity. If an average C_q^{PPC} value of 20 ± 2 is difficult to obtain for your cycler, the observed average C_q^{PPC} value should be acceptable as long as it does not vary by more than 2 cycles between PCR arrays being compared.
b) Incorrect cycling parameters	Ensure that the initial heat activation step at 95°C was lengthened to 10 minutes from the shorter time in the default program. Ensure that all other cycle parameters were correctly entered according to the recommendations in the *RT² lncRNA PCR Array Handbook*.
c) Poor quality RNA	If possible, recheck the A_{260}:A_{280} and A_{260}:A_{230} ratios of RNA samples in RNase-free Tris pH 8.0 buffer. If necessary, repurify RNA samples with a spin-column based cleanup method, such as the RNeasy Mini Kit.

References

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

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

Appendix A: Data Analysis Using the $\Delta\Delta C_q$ Method

Visit the free PCR Array Data Analysis Web Portal at www.qiagen.com/products/genes and pathways/data-analysis-center-overview-page. At the PCR Array Data Analysis Web Portal, C_q data can be entered and the Web-based software will automatically perform quantification using the $\Delta\Delta C_q$ method as described below and interpretation of the control wells. The PCR Array Data Analysis Web Portal presents results in a tabular format, a scatter plot, a three-dimensional profile, and a volcano plot (when replicates are included).

Note: Check the "RT² PreAMP cDNA Synthesis Kit" box in the "Readout" page.

Data analysis using the $\Delta\Delta C_q$ method

A1. Consider all C_q values reported as greater than or equal to 35 as negative calls.

Assays showing C_q values greater than 35 with preamplified samples represent genes with an expression level that is too low to be reliably quantified. Consider removing these data points from the rest of the results.

A2. Process the C_q values of the genomic DNA control wells (GDC) as follows.

- Calculate C_q^{GDC}.
- If the value is greater than 30, the level of genomic DNA contamination is too low to affect gene expression profiling results. No action is needed.
- If the value is less than 30, genomic DNA contamination is evident. See the "Troubleshooting Guide", page 25.

A3. Process the C_q values of the reverse transcription control (RTC) using the values for the positive PCR control (PPC), as follows.

- Calculate ΔC_q = AVG C_q^{RTC} – AVG C_q^{PPC}.
- If this value is less than 7, then no inhibition of the reverse-transcription reaction is apparent. No action is needed.
- If this value is greater than 7, there is evidence of impurities that may have inhibited the reverse transcription reaction. See the "Troubleshooting Guide", page 25.

A4. Process the C_q values of the positive PCR control wells (PPC) as follows.

- Calculate the average C_q^{PPC}.

- The average C_q^{PPC} value should be 20 ± 2 on each RT² lncRNA PCR Array and should not vary by more than 2 cycles between RT² lncRNA PCR Arrays being compared.
- Larger differences in average C_q^{PPC} values between samples indicate the presence of PCR amplification inhibitors. This means that the RNA samples require further purification.
- An average value of C_q^{PPC} that is consistently greater than 22 for all samples may indicate a problem with the cycling conditions or may simply be indicative of the relative sensitivity of your instrument. See the "Troubleshooting Guide", page 25.

A5. Calculate the ΔC_q for each pathway-focused gene in each plate using the C_q values for the gene of interest (GOI) and the reference genes used for normalization (REF) using the formula:

$$\Delta C_q = C_q^{GOI} - C_q^{AVG\ REF}$$

Note: The expression level of the reference genes chosen for normalization must not be influenced by the experimental conditions. If one or more such reference genes have been previously identified by independent means and if the RT² lncRNA PCR Array reproduces previous results, use the average of their C_q values in the equation above. If an appropriate reference gene has not been previously identified, use the average C_q value of all reference genes. Alternatively, use zero in the place of the average C_q^{REF} for each group to be compared, and rely on the consistency in the quantity and quality of the original input total RNA to effectively normalize the results.

A6. When biological and/or technical replicates are performed, calculate the average ΔC_q value of each gene (each well) across those replicate arrays for each treatment group.

A7. The $\Delta\Delta C_q$ for each gene across 2 RT² lncRNA PCR Arrays (or groups of samples) is calculated using the formula:
$\Delta\Delta C_q = \Delta C_q$ (group 2) – ΔC_q (group 1) where group 1 is the control sample or group of control samples and group 2 is the experimental sample or group of experimental samples.

A8. The fold-change for each gene from group 1 to group 2 is calculated as $2^{(-\Delta\Delta Cq)}$.

Note: If the fold-change is greater than 1, the result may be reported as a fold upregulation. If the fold-change is less than 1, the negative inverse of the result may be reported as a fold downregulation. Fold-change ratio

calculation will not be reliable when raw C_q values from both groups are greater than 35.

Detailed mathematical explanation of $\Delta\Delta C_q$ data analysis method

Due to the inverse proportional relationship between the quantification cycle (Cq) and the original gene expression level, and the doubling of the amount of product with every cycle, the original expression level (L) for each gene of interest is expressed as:

$$L = 2^{-Cq}$$

To normalize the expression level of a gene of interest (GOI) to a reference gene (REF), the expression levels of the 2 genes are divided:

$$\frac{2^{-Cq(GOI)}}{2^{-Cq(HKG)}} = 2^{-[Cq(GOI) - Cq(HKG)]} = 2^{-\Delta Cq}$$

To determine fold change in gene expression, the normalized expression of the GOI in the experimental sample is divided by the normalized expression of the same GOI in the control sample:

$$\frac{2^{-\Delta Cq(expt)}}{2^{-\Delta Cq(control)}} = 2^{-\Delta\Delta Cq} \qquad \text{Where } \Delta Cq \text{ is equal to } \Delta Cq(expt) - \Delta Cq(control)$$

The complete calculation is as follows:

$$\frac{\frac{2^{-\Delta Cq(GOI)}expt}{2^{-\Delta Cq(HKG)}expt}}{\frac{2^{-\Delta Cq(GOI)}control}{2^{-\Delta Cq(HKG)}control}} = \frac{2^{-[\Delta Cq(GOI) - \Delta Cq(HKG)]}expt}{2^{-[\Delta Cq(GOI) - \Delta Cq(HKG)]}control} = \frac{2^{-\Delta Cq(expt)}}{2^{-\Delta Cq(control)}} = 2^{-\Delta\Delta Cq}$$

Appendix B: Preamplification Prior to Real-Time PCR Using RT² lncRNA qPCR Assays

This protocol is for preamplification and real-time PCR when using RT² lncRNA qPCR Assays for low-throughput gene expression analysis of up to 10 genes instead of high-throughput RT² lncRNA PCR Arrays. In this protocol, RT² lncRNA qPCR Assays are used for preamplification instead of an RT² lncRNA PreAMP Primer Mix.

Procedure

B1. Perform cDNA synthesis as described in the protocol on page 17.

B2. Dilute RT² lncRNA qPCR Assay(s) for up to 10 genes of interest as described in Table 7.

Table 7. RT² lncRNA qPCR Assay mix

Number of genes for expression analysis	RT² lncRNA qPCR Assay (10 µM)	Nuclease-Free Water
1 gene	1 assay x 40 µl = 40 µl	960 µl
2 genes	2 assays x 40 µl each = 80 µl	920 µl
3 genes	3 assays x 40 µl each = 120 µl	880 µl
4 genes	4 assays x 40 µl each = 160 µl	840 µl
5 genes	5 assays x 40 µl each = 200 µl	800 µl
6 genes	6 assays x 40 µl each = 240 µl	760 µl
7 genes	7 assays x 40 µl each = 280 µl	720 µl
8 genes	8 assays x 40 µl each = 320 µl	680 µl
9 genes	9 assays x 40 µl each = 360 µl	640 µl
10 genes	10 assays x 40 µl each = 400 µl	600 µl

B3. Thaw the RT² PreAMP PCR Mastermix at room temperature. If precipitates are visible, warm the reagents at 42°C for 1 min and vortex briefly to dissolve. Repeat if necessary.

B4. Prepare preamplification mix according to Table 8.

For multiple reactions, scale up the volumes shown in Table 8 accordingly.

Table 8. Preamplification mix

Component	Amount for one sample
RT² PreAMP PCR Mastermix	12.5 µl
RT² lncRNA qPCR Assay mix	7.5 µl
Total volume	**20 µl**

B5. Pipet 5 µl cDNA synthesis reaction (from step 6, page 18) into a 0.2 ml PCR tube. Then add 20 µl preamplification mix.

B6. Mix gently by pipetting up and down. Spin briefly to remove any air bubbles and collect all the liquid to the bottom of the tube.

B7. Program the real-time cycler according to Table 9. Place the tubes in the real-time cycler and start the program.

Table 9. Cycling conditions for preamplification

Cycles	Duration	Temperature	Comments
1	10 min	95°C	HotStart DNA *Taq* Polymerase is activated by this heating step.
8	15 s	95°C	
	2 min	60°C	
Hold		4°C	

B8. When cycling is finished, take the tubes from the real-time cycler and place on ice.

B9. Add 2 µl Side Reaction Reducer to each preamplified reaction. Mix gently by pipetting up and down. Spin the tubes briefly to remove any air bubbles and collect all the liquid at the bottom of the tube.

B10. Incubate at 37°C for 15 min followed by heat inactivation at 95°C for 5 min.

B11. Immediately add 28 µl Nuclease-Free Water. Mix well.

B12. Place on ice prior to real-time PCR, or store overnight at –20°C.

B13. Perform real-time RT-PCR using RT² lncRNA qPCR Assays as described in the *RT² lncRNA qPCR Assay Handbook* using 1 µl preamplification reaction as template.

Appendix C: Bench Protocol for First-Strand cDNA Synthesis, Preamplification, and Real-Time PCR

Note: Before using this bench protocol, you should be completely familiar with the safety information and detailed protocols in this handbook and the *RT² lncRNA PCR Array Handbook*.

Important point before starting

- Ensure that the pathway and lot number of the RT² lncRNA PreAMP Primer Mix are compatible with those of the RT² lncRNA PCR Array.

Procedure

C1. Add 2 µl Buffer GE to 8 µl RNA (1–100 ng for RNA from fresh/frozen sample; 100 ng – 1 µg for RNA from FFPE sample). Incubate at 42°C for 5 min and immediately chill on ice.

C2. Prepare the RT mix as shown in Table 10.

Table 10. Reverse transcription mix

Component	Volume for 1 reaction
5x Buffer BC3	4 µl
Control P2	1 µl
cDNA Enzyme Synthesis Mix	1 µl
RNase Inhibitor	1 µl
Nuclease-Free Water	3 µl

C3. Add 10 µl RT mix to 10 µl Buffer GE–RNA from step 1. Incubate at 37°C for 60 min and heat at 95°C for 5 min. Place on ice or store at –20°C until use.

C4. Mix the components from Table 11 in a PCR tube.

Table 11. Preamplification mix

Component	Amount for one sample
RT² PreAMP PCR Mastermix	12.5 µl
RT² lncRNA PreAMP Primer Mix	7.5 µl
cDNA from step C3	5 µl

C5. For cDNA from fresh/frozen samples, cycle in a real-time cycler as follows: 95°C, 10 min; 12 cycles of (95°C for 15 sec; 60°C for 2 min); 4°C hold. For cDNA from FFPE samples, cycle in a real-time cycler as follows: 95°C, 10 min; 8 cycles of (95°C for 15 sec; 60°C for 2 min); 4°C hold.

C6. Add 2 µl Side Reaction Reducer, incubate at 37°C for 15 min followed by heat inactivation at 95°C for 5 min.

C7. Add 84 µl Nuclease-Free Water. Place on ice or store at –20°C until use.

C8. Prepare the mix in Table 12 for use with a 96-well RT² lncRNA PCR Array.

Table 12. RT² lncRNA PCR Array mix

Component	Volume
2x RT² SYBR Green Mastermix	1275 µl
Preamplification reaction from step C7	102 µl
Nuclease-Free Water	1173 µl

C9. Add 25 µl of the mix to each well of the PCR array. Cycle as follows: 95°C, 10 min; 40 cycles of (95°C for 15 sec; 60°C for 60 sec).

C10. Program the cycler to detect and record the SYBR Green signal during the annealing step of each cycle.

Ordering Information

Product	Contents	Cat. no.
RT² PreAMP cDNA Synthesis Kit (12)	For 12 x 20 µl first strand cDNA synthesis reactions: Buffer GE, 5x Buffer BC3, cDNA Synthesis Enzyme Mix, RNase Inhibitor, Control P2, Nuclease-Free Water; for 48 x 25 µl preamplification reactions: RT² PreAMP PCR Mastermix (600 µl); Side Reaction Reducer (96 µl)	330451
RT² lncRNA PreAMP Primer Mix	For 12 preamplification reactions: 90 µl primer mix for a specific RT² lncRNA PCR Array	Varies
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 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

Product	Contents	Cat. no.
Related products		
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
RNeasy Mini Kit (50)*	50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free reagents and buffers	74104
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
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
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
RT² PCR Array Loading Reservoir	12 x 5 ml capacity, irradiation-sterilized loading reservoirs	338162

* Larger kit sizes available; please inquire.

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.

Notes

Notes

Limited License Agreement for RT² PreAMP cDNA Synthesis Kit

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