

April 2019

RT² IncRNA PCR Array Handbook

RT² IncRNA PCR Array

RT² First Strand Kit

RT² SYBR[®] Green qPCR Mastermix

RT² SYBR Green Fluor qPCR Mastermix

RT² SYBR Green ROX[™] qPCR Mastermix

RT² SYBR Green ROX FAST Mastermix

For IncRNA expression profiling using
real-time RT-PCR

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Kit Contents

RT² IncRNA PCR Array Format A*	
96-well plate containing dried assays	2, 6, 12, or 24
Optical Thin-Wall 8-Cap Strips (12 per 96-well plate)	24, 72, 144, or 288
Handbook	1

* Suitable for use with the following real-time cyclers: Applied Biosystems® models 5700, 7000, 7300, 7500, 7700, 7900HT, ViiA™ 7 (96-well standard block); QuantStudio 6, QuantiStudio 7, and QuantiStudio 12K (Standard 96-well block); Bio-Rad® models iCycler®, iQ™5, MyiQ™, MyiQ2; Bio-Rad/MJ Research Chromo4™; Eppendorf® Mastercycler® ep *realplex* models 2, 2S, 4, 4S; Agilent (Stratagene) models Mx3005P®, Mx3000P®; Takara TP-800.

RT² IncRNA PCR Array Format C†	
96-well plate containing dried assays	2, 6, 12, or 24
Optical Adhesive Film (1 per 96-well plate)	2, 6, 12, or 24
Handbook	1

† Suitable for use with the following real-time cyclers: Applied Biosystems models 7500 (Fast block), 7900HT (96-well Fast block), StepOnePlus™, ViiA 7 (96-well Fast block), QuantStudio 6 (96-well Fast block), QuantStudio 7 (96-well Fast block), QuantStudio 12K (96-well Fast block).

RT² IncRNA PCR Array Format D‡	
96-well plate containing dried assays	2, 6, 12, or 24
Optical Thin-Wall 8-Cap Strips (12 per 96-well plate)	24, 72, 144, or 288
Handbook	1

‡ Suitable for use with the following real-time cyclers: Bio-Rad CFX96™; Bio-Rad/MJ Research models DNA Engine Opticon®, DNA Engine Opticon 2; Agilent (Stratagene) Mx4000®.

RT² IncRNA PCR Array Format E 384 (4 x 96) option^{*†}	
384-well plate containing dried assays	4
Optical Adhesive Film (1 per 384-well plate)	4
384EZLoad Covers (1 set of 4 per 384-well plate)	4 sets

* Suitable for use with the following real-time cyclers: Applied Biosystems models 7900HT (384-well block), ViiA 7 (384-well block), QuantStudio 6 (384-well block), QuantStudio 7 (384-well block), QuantStudio 12K (384-well block), Bio-Rad CFX384™.

† For a description of the 384 (4 x 96) option, see page 13.

RT² IncRNA PCR Array Format F[‡]	
96-well plate containing dried assays	2, 6, 12, or 24
Optical Thin-Wall 8-Cap Strips (12 per 96-well plate)	2, 6, 12, or 24
Handbook	1

‡ Suitable for use with the following real-time cycler: Roche® LightCycler® 480 (96-well block), Roche LightCycler 480 II (96-well block), and Roche LightCycler 96.

RT² IncRNA PCR Array Format G 384 (4 x 96) option^{§,†}	
384-well plate containing dried assays	4
Optical Adhesive Film (1 per 384-well plate)	4
384EZLoad Covers (1 set of 4 per 384-well plate)	4 sets
Handbook	1

§ Suitable for use with the following real-time cyclers: Roche® LightCycler® 480 (384-well block), Roche LightCycler 480 II (384-well block).

† For a description of the 384 (4 x 96) option, see page 13.

RT² IncRNA PCR Array Format H^{**}	
96-well plate containing assays in solution, 96 x 96 chip compatible	1
Handbook	1

** Suitable for use with the following real-time cycler: Fluidigm® BioMark™ system.

RT² IncRNA PCR Array Format R*	
Rotor-Disc® 100 containing dried assays	2, 6, 12, or 24
Rotor-Disc Heat Sealing Film (1 per Rotor-Disc 100)	2, 6, 12, or 24
Handbook	1

* Suitable for use with the following real-time cyclers: QIAGEN® Rotor-Gene® Q; Rotor-Gene 6000; other Rotor-Gene cyclers.

RT² First Strand Kit	(12)
Catalog no.	330401
Number of 20 µl reactions	12
Buffer GE	24 µl
5x Buffer BC3	48 µl
RE3 Reverse Transcriptase Mix	24 µl
Control P2	12 µl
Nuclease-Free Water	1 ml

RT² First Strand Kit	(50)
Catalog no.	330404
Number of 20 µl reactions	50
Buffer GE	100 µl
5x Buffer BC3	200 µl
RE3 Reverse Transcriptase Mix	100 µl
Control P2	50 µl
Nuclease-Free Water	1 ml

RT² SYBR Green qPCR Mastermix*	(2)	(12)	(24)	(8)	(25 ml)
Catalog no.	330500	330502	330503	330501	330509
Number of array reactions	2 x 96-well	12 x 96-well	24 x 96-well	4 x 384-well	2000 x 25 µl reactions
2x SYBR Green qPCR Mastermix, containing the following:	2 x 1.35 ml	12 x 1.35 ml	24 x 1.35 ml	8 x 1.35 ml	25 ml
<ul style="list-style-type: none"> ● HotStart DNA <i>Taq</i> Polymerase ● PCR Buffer ● dNTP mix (dATP, dCTP, dGTP, dTTP) ● SYBR Green dye 					

* 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, LightCycler 480 II (96-well and 384-well), and LightCycler 96.

RT² SYBR Green Fluor qPCR Mastermix*	(2)	(12)	(24)	(8)	(25 ml)
Catalog no.	330510	330512	330513	330511	330519
Number of array reactions	2 x 96-well	12 x 96-well	24 x 96-well	4 x 384-well	2000 x 25 µl reactions
2x SYBR Green Fluor qPCR Mastermix, containing the following:	2 x 1.35 ml	12 x 1.35 ml	24 x 1.35 ml	8 x 1.35 ml	25 ml
<ul style="list-style-type: none"> ● HotStart DNA <i>Taq</i> Polymerase ● PCR Buffer ● dNTP mix (dATP, dCTP, dGTP, dTTP) ● SYBR Green dye ● Fluorescein passive reference dye 					

* Suitable for use with the following real-time cyclers: Bio-Rad models iCycler, iQ5, MyiQ, MyiQ2.

RT² SYBR Green ROX qPCR Mastermix*	(2)	(12)	(24)	(8)	(25 ml)
Catalog no.	330520	330522	330523	330521	330529
Number of array reactions	2 x 96-well	12 x 96-well	24 x 96-well	4 x 384-well	2000 x 25 µl reactions
2x SYBR Green ROX qPCR Mastermix, containing the following:	2 x 1.35 ml	12 x 1.35 ml	24 x 1.35 ml	8 x 1.35 ml	25 ml
<ul style="list-style-type: none"> ● HotStart DNA <i>Taq</i> Polymerase ● PCR Buffer ● dNTP mix (dATP, dCTP, dGTP, dTTP) ● SYBR Green dye ● ROX passive reference dye 					

* 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 (Standard and Fast 96-well block, 384-well block), QuantStudio 7 (Standard and Fast 96-well block, 384-well block); and QuantStudio 12K (Standard and Fast 96-well block, 384-well block); Eppendorf Mastercycler ep *realplex* models 2, 2S, 4, 4S; Agilent (Stratagene) models Mx3000P, Mx3005P, Mx4000; Takara TP-800.

RT² SYBR Green ROX FAST Mastermix*	(2)	(12)	(24)	(8)	(25 ml)
Catalog no.	330620	330622	330623	330621	330629
Number of array reactions	2 x 96-well	12 x 96-well	24 x 96-well	4 x 384-well	2000 x 25 µl reactions
2x SYBR Green ROX FAST Mastermix, containing:	2 x 1.35 ml	12 x 1.35 ml	24 x 1.35 ml	8 x 1.35 ml	25 ml
<ul style="list-style-type: none"> ● HotStart DNA <i>Taq</i> Polymerase ● PCR Buffer ● dNTP mix (dATP, dCTP, dGTP, dTTP) ● SYBR Green dye ● ROX passive reference dye 					

* Suitable for use with the Rotor-Gene Q (QIAGEN) and Rotor-Gene 6000.

Note: RT² IncRNA PCR Arrays cannot be used in the Cepheid SmartCycler® or the Roche LightCycler 2.0.

Shipping and Storage

RT² IncRNA PCR Array Formats A, C, D, E, F, G, and R are shipped at room temperature (15–25°C) or on ice or dry ice, depending on the destination and accompanying products. RT² IncRNA PCR Array Format H is shipped on ice or dry ice. All RT² IncRNA PCR Array Formats should be stored at –20°C upon arrival. When stored properly at –20°C, RT² IncRNA PCR Arrays are stable for up to 1 year after delivery.

RT² SYBR Green Mastermixes are shipped on cold packs. For long-term storage, keep tubes at –20°C. If the entire volume will not be used at once, we recommend dividing into aliquots and storing at –20°C. Avoid repeated freezing and thawing. If stored under these conditions, RT² SYBR Green Mastermixes are stable for 6 months after receipt.

The RT² First Strand Kit is shipped frozen. For long-term storage, keep the kit at –20°C. If stored under these conditions, the RT² First Strand Kit is stable for 6 months after receipt. We recommend a maximum of 6 freeze-thaw cycles.

Intended Use

The RT² IncRNA PCR Arrays, the RT² First Strand Kit, the RT² Microfluidics qPCR Reagent System, and the RT² SYBR Green Mastermixes 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 lncRNA PCR Arrays, RT² First Strand Kits, and RT² SYBR Green Mastermixes 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 roles in 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. Its wide dynamic range makes real-time RT-PCR the preferred choice for the simultaneous quantification of both rare and abundant genes in the same sample. RT² lncRNA PCR Arrays take advantage of the combination of real-time PCR performance and the ability to detect the expression of a panel of lncRNAs simultaneously. RT² lncRNA PCR Arrays are designed to analyze a panel of lncRNAs related to a disease state or biological pathway. RT² lncRNA PCR Arrays are especially suitable for researchers who are more familiar with or prefer real-time RT-PCR technology and are looking for the multiple lncRNA profiling capabilities.

RT² lncRNA PCR Arrays, RT² SYBR Green Mastermixes, and the RT² First Strand Kit have been optimized in combination for SYBR Green-based, real-time RT-PCR detection, providing the RT² lncRNA PCR Arrays with superior sensitivity and wide linear dynamic ranges. The simplicity of the RT² lncRNA PCR Arrays makes them accessible for routine use in every research laboratory.

First-strand cDNA synthesis and Mastermixes

Performance of RT² lncRNA PCR Arrays is only guaranteed when used with RT² SYBR Green Mastermixes and the RT² First Strand Kit. Therefore, the use of the complete RT² lncRNA PCR Array System is essential for obtaining accurate real-time PCR profiling results.

The chemically modified and tightly controlled HotStart DNA *Taq* Polymerase enzyme and other proprietary chemical components in RT² SYBR Green Mastermixes uniquely provide more accurate SYBR Green results by preventing the amplification of primer-dimers and other nonspecific products. They also help ensure high amplification efficiencies, even for genes that are difficult to amplify. When other sources of enzymes are tested with RT² lncRNA PCR Arrays, primer-dimers and other nonspecific products are frequently observed, leading to difficult-to-interpret SYBR Green-based, real-time PCR results. Real-time cyclers use different reference dyes to normalize their optics; therefore, be sure to use the correct Mastermix for the real-time cycler in your laboratory.

The RT² First Strand Kit includes a proprietary buffer to eliminate any residual genomic DNA contamination in RNA samples before it can be amplified into secondary products that would otherwise cause false positive signals. The reverse-transcription controls (RTC) on the RT² lncRNA PCR Array can only be evaluated with the built-in external RNA control of the RT² First Strand Kit. These controls do not yield results when used with other sources of reverse transcriptase or first strand synthesis kits. The buffer components and the magnesium concentration in the RT² First Strand Kit are also more compatible with RT² SYBR Green Mastermixes than other enzymes or kits, providing the RT² lncRNA PCR Arrays with maximum levels of sensitivity with nanogram to microgram amounts of total RNA. For very limited RNA amounts or serum/plasma samples, preamplification is also available (see the *RT² lncRNA PreAMP cDNA Synthesis Handbook*).

Principle and procedure

RT² lncRNA PCR Arrays are provided in 96-well plates, 96-well H plate for use with 96 x 96 microfluidic chips, 384-well plates, or Rotor-Discs (Figures 1-4). RT² lncRNA PCR Arrays in 96-well plates contain primer assays for 84 disease- or pathway-focused lncRNAs and 5 reference genes. In addition, 1 well contains a genomic DNA control, 3 wells contain reverse-transcription controls, and 3 wells contain positive PCR controls (Figure 1).

RT² lncRNA PCR Arrays in 384-well plates are available in a 4 x 96 format. The 384 (4 x 96) plate contains 4 replicate primer assays for each of 84 pathway- or disease-focused genes and 4 replicate primer assays for each of 5 reference genes. In addition, 4 wells contain genomic DNA controls, 12 wells contain reverse-transcription controls, and 12 wells contain positive PCR controls (Figure 2).

RT² lncRNA PCR Arrays in Rotor-Disc 100 format contain primer assays for 84 pathway- or disease-focused genes and 5 reference genes. In addition, 1 well contains a genomic DNA control, 3 wells contain reverse-transcription controls, and 3 wells contain a positive PCR control. Wells 97–100 of the Rotor-Disc 100 do not contain assays (Figure 3). During the procedure, Mastermix is added to these wells for balance, but the wells are not used for analysis.

RT² lncRNA PCR Arrays in format H contain primer assays in solution to be aliquoted onto 96 x 96 chips. These include primer assays for 84 pathway- or disease-focused genes and 5 reference genes. In addition, 1 well contains a genomic DNA control, 3 wells contain reverse-transcription controls, and 3 wells contain a positive PCR control (Figure 1).

Custom RT² lncRNA PCR Arrays contain primer assays defined by the customer. The plate layout and targeted genes are detailed in the Product Sheet provided.

Each well of the RT² lncRNA PCR Array (Formats A, C, D, E, F, G, R) contains a primer assay mixed with an inert dye (the dye is used for manufacturing quality control and does not affect assay performance or fluorescence detection).

Format H RT² lncRNA PCR Arrays do not contain the dye.

Definition of controls in RT² lncRNA PCR Arrays

Assays for 5 reference genes included in the arrays enable normalization of data. The genomic DNA control (GDC) is an assay that specifically detects nontranscribed genomic DNA contamination with a high level of sensitivity. The reverse-transcription control (RTC) is an assay that tests the efficiency of the reverse-transcription reaction performed with the RT² First Strand Kit by detecting template synthesized from the kit's built-in external RNA control. The positive PCR control (PPC) consists of a predisposed artificial DNA sequence and the assay that detects it. This control tests the efficiency of the polymerase chain reaction itself. Controls provided in replicates can be used to test for inter-well, intra-plate consistency.

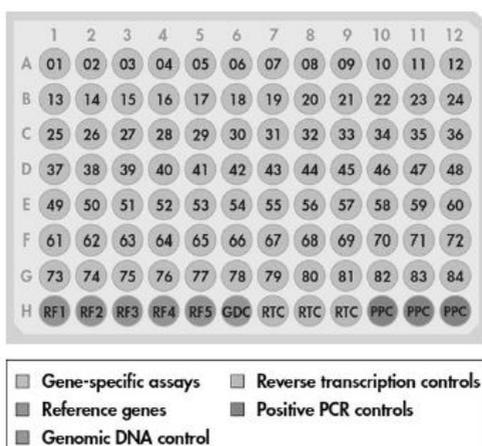


Figure 1. RT² lncRNA PCR Array Formats A, C, D, F, H layout. RT² lncRNA PCR Array Formats A, C, D, F, H: Wells A1 to G12 each contains a real-time PCR assay for a pathway/disease/functionally related gene. Wells H1 to H5 contain a reference gene panel to normalize array data (RF 1-5). Well H6 contains a genomic DNA control (GDC). Wells H7 to H9 contain replicate reverse-transcription controls (RTC). Wells H10 to H12 contain replicate positive PCR controls (PPC).

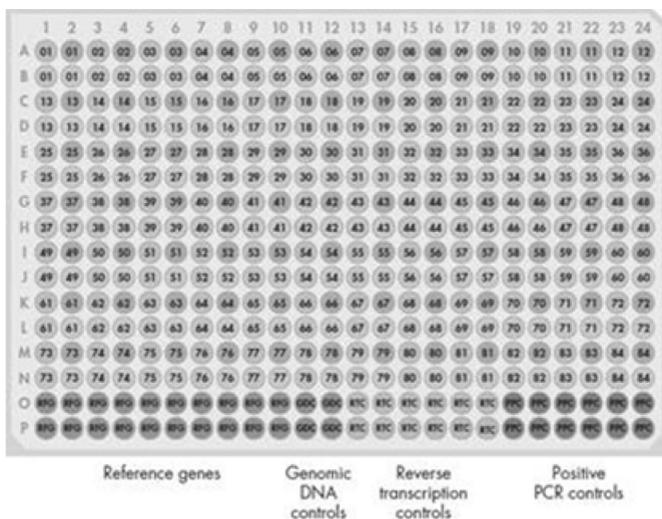


Figure 2. RT² IncRNA PCR Array Formats E, G 384 (4 x 96) option layout. RT² IncRNA PCR Arrays with the 384 (4 x 96) option include 4 replicates of the same assays as provided in the 96-well format shown in Figure 1.

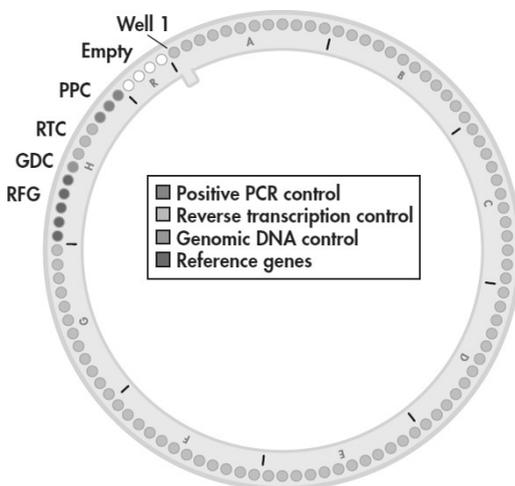


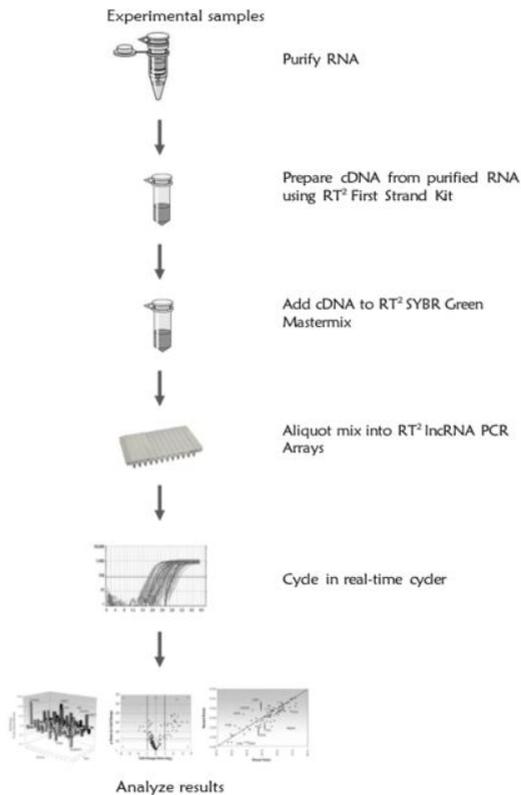
Figure 3. RT² IncRNA PCR Array Format R layout. Wells 1 to 84 each contain an IncRNA real-time PCR assay for a pathway/disease/functionally-related gene. Wells 85 to 89 contain a reference gene panel to normalize array data. Well 90 contains a genomic DNA control. Wells 91 to 93 contain replicate reverse-transcription controls. Wells 94 to 96 contain replicate PCR controls. Wells 97 to 100 are empty.

Workflow

The procedure begins with the conversion of the experimental RNA samples into first-strand cDNA using the RT² First Strand Kit. Next, the cDNA is mixed with an appropriate RT² SYBR Green Mastermix. This mixture is aliquoted into the wells of the RT² IncRNA PCR Array. PCR is performed, and finally relative expression is determined using data from the real-time cycler and the $\Delta\Delta C_q$ method.

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

Figure 4. RT² IncRNA PCR Array Procedure



RNA quality control using an RT² RNA QC PCR Array

The RT² RNA QC PCR Array is designed to assess the quality of 12 RNA samples simultaneously before gene expression analysis using RT² IncRNA PCR Arrays. Use of the RT² RNA QC PCR Array provides complete confidence in gene expression analysis results by enabling exclusion of substandard samples prior to analysis with RT² IncRNA PCR Arrays. For further details, consult the *RT² RNA QC PCR Array Handbook*.

Description of protocols

This handbook contains 4 protocols. The first protocol details cDNA synthesis by reverse transcription using purified RNA and the RT² First Strand Kit (page 26).

This protocol should be performed prior to real-time PCR.

The other 3 additional protocols detail real-time PCR performed using the cDNA prepared in the first protocol as the template. The protocol on page 29 should be used for 96-well and 384-well RT² IncRNA PCR Arrays Formats A, C, D, E, F, and G. The protocol on page 38 should be used for Rotor-Disc 100 RT² IncRNA PCR Array Format R. For users of the Fluidigm BioMark real-time PCR system and RT² IncRNA PCR Array Format H, the protocol on page 43 should be used for cDNA synthesis, preamplification, and real-time PCR.

Equipment and Reagents to Be Supplied by User

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

In addition to the RT² IncRNA PCR Array, RT² First Strand Kit, and RT² SYBR Green Mastermix, the following are required:

- Purified RNA samples
- Real-time PCR cycler
- High-quality, nuclease-free water. Do not use DEPC-treated water.
- Multichannel pipettor
- Single-channel pipettor (if using RT² IncRNA PreAMP Primer Mix)
- Nuclease-free pipette tips and tubes
- Optional: XpressRef Universal Total RNA to control PCR conditions is available for human (cat. no. 338112), mouse (cat. no. 338114), and rat (cat. no. 338116)
- Optional: RT² PCR Array Loading Reservoir (cat. no. 338162)

For users of RT² IncRNA PCR Array format H with the Fluidigm BioMark system:

- 20x DNA Binding Dye Sample Loading Reagent (Fluidigm, cat. no. 100-0038)
- 8-strip PCR 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 and tubes) containing PCR products or other DNA, treat with 10% bleach.
- Do not remove the RT² IncRNA 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 RT² IncRNA 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

High-quality RNA is essential for obtaining good real-time PCR results. The most important prerequisite for any gene expression analysis experiment is 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.

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.

Cultured cells

We recommend the miRNeasy® Mini Kit (cat. no. 217004)* for RNA purification from cultured cells. 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); 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 FFPE Kit (cat. no. 217084); 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.

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, RNeasy Lysis 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 IncRNA PCR Array, all RNA samples should also demonstrate consistent quality according to the criteria described below. In addition, as some contaminants are difficult to detect by simply looking at RNA integrity and can be missed by UV spectrophotometry, it is essential to choose an appropriate RNA purification method for your biological sample as described in pages 24-25.

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 nuclease-free water. 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® 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 5). Any smearing of the RNA bands or shoulders on the RNA peaks indicates that degradation has occurred in the RNA sample.

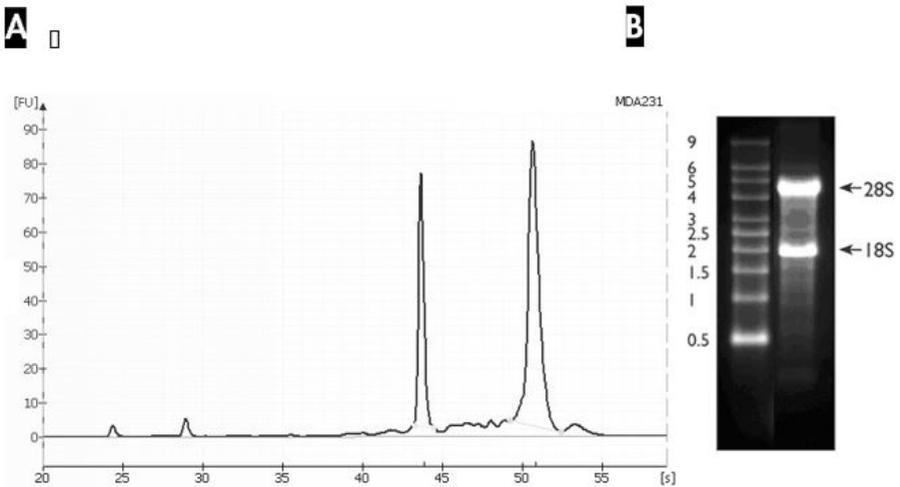


Figure 5. Ribosomal RNA integrity. **A** An Agilent Bioanalyzer electropherogram of high-quality total RNA showing strong and sharp peaks for the 18S (left) and 28S (right) ribosomal RNA. Due to high quality of the RNA, peaks do not have shoulders (especially to the left of each peak). **B** Agarose gel electrophoresis shows sharp bands (especially at the bottom of each band) for 28S and 18S ribosomal RNA.

RT² RNA QC PCR Array (optional)

The RT² RNA QC PCR Array is particularly useful for researchers who are unsure of their RNA purification technique. The RT² RNA QC PCR Array and the RT² First Strand Kit (sold separately) test for a number of RNA quality control parameters, including the following:

- High and low reference gene expression levels
- Reverse transcription and polymerase chain reaction efficiency
- Genomic and general DNA contamination

For further details, consult the *RT² RNA QC PCR Array Handbook*.

Genomic DNA contamination

Eliminating genomic DNA contamination is essential for obtaining optimal real-time gene expression profiling results using the RT² IncRNA PCR Array. The genomic DNA control in each RT² IncRNA PCR Array specifically tests for genomic DNA contamination in each sample during each run. A genomic DNA control quantification cycle value of less than 35 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 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

The RT² IncRNA PCR Array System provides results with as little as 25 ng or as much as 5 µg total RNA per array.* For small starting RNA amounts, the RT² PreAMP cDNA Synthesis Kit and RT² IncRNA PreAMP Primer Mix enable 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 IncRNA 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 and increase false negative calls.

* Using more RNA than the recommended maximum may potentially overload the restriction enzyme system.

For successful results and maximum positive call rates, we recommend that first-time users start with 1 µg total RNA for 96-well plate formats, 1.6 µg total RNA for Rotor-Disc 100 formats, and 800 ng total RNA for formats E and G [384 (4 x 96) option]. It is important to use a consistent amount of total RNA for all samples in a single experiment.

Protocol: cDNA Synthesis Using the RT² First Strand Kit

Use of the RT² First Strand Kit is critical for obtaining optimal results and for detection of the reverse transcription controls contained in the RT² IncRNA PCR Array. If using the Fluidigm BioMark real-time PCR system, refer to page 43 for the cDNA synthesis protocol and for sample/plate preparation instructions.

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 µg total RNA for 96-well plate formats, 1.6 µg total RNA for Rotor-Disc 100 formats, and 800 ng total RNA for formats E and G (384 [4 x 96]) option. Use of less than 100 ng RNA will result in a high rate of false negatives. If ≤100 ng RNA is available, refer to the *RT² IncRNA PreAMP cDNA Synthesis Handbook*. Use of more than the recommended amount of RNA may potentially overload the system.
- Do not use DEPC-treated water. Use high-quality, nuclease-free water.
- The RT² First Strand Kit is not compatible with the chemicals in the DNA-free™ Kits from Life Technologies®. If your RNA sample has been treated with DNA-free kit reagents, contact QIAGEN Technical Service.

Procedure

1. **Thaw the reagents of the RT² First Strand Kit. Briefly centrifuge (10-15 seconds) to bring the contents to the bottom of the tubes.**
2. **Prepare the genomic DNA elimination mix for each RNA sample according to Table 1. Mix gently by pipetting up and down then centrifuge briefly.**

Table 1. Genomic DNA elimination mix

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

* If using the kit for the first time, use the RNA amount recommended in “Important points before starting” above.

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.**

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
Nuclease-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 10 µl 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. **Add 91 µl Nuclease-Free Water to each reaction. Mix by pipetting up and down several times.**
8. **Place the reactions on ice and proceed with the real-time PCR protocol.**

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

For quality control analysis using the RT² RNA QC PCR Array, follow the protocol in the *RT² RNA QC PCR Array Handbook* using a 6 μ l aliquot of the diluted cDNA template.

Protocol: Real-Time PCR for RT² IncRNA PCR Arrays Formats A, C, D, E, F, and G

This protocol describes real-time PCR using RT² IncRNA PCR Arrays in combination with RT² SYBR Green Mastermixes. Use of RT² SYBR Green Mastermixes is critical to obtain accurate results from the RT² IncRNA PCR Array. If unsure of the RNA quality or purification technique, examine the RNA quality before performing this protocol using species- and cycler-specific RT² RNA QC PCR Arrays.

For RT² IncRNA PCR Array Format H, refer to the protocol on page 43.

Important points before starting

- Ensure that the RT² SYBR Green Mastermix and the RT² IncRNA PCR Array format are suitable for your real-time cycler. The format of the RT² IncRNA PCR Array is indicated by the last letter of the catalog number. An incorrect RT² IncRNA PCR Array format will not fit the real-time cycler properly and may damage the real-time cycler.
- **Do not** cut the plastic plate of the RT² IncRNA PCR Array.
- 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 IncRNA PCR Array 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.

Procedure

1. **Briefly centrifuge the RT² SYBR Green Mastermix (10–15 seconds) 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 IncRNA PCR Array format, as described in Table 3.

Table 3. PCR components mix

Array format	96-well A, C, D, F	384-well (4 x 96) option E, G
2x RT ² SYBR Green Mastermix	1350 µl	650 µl
cDNA synthesis reaction	102 µl	102 µl
Nuclease-Free Water	1248 µl	548 µl
Total volume	2700 µl	1300 µl

Note: This provides an excess volume of 300 µl (formats A, C, D, F) or 340 µl (formats E, G: 384 [4 x 96] option) to allow for pipetting errors. Perform pipetting steps as precisely as possible to ensure that each well receives the required volume.

Note: For Custom RT² IncRNA PCR Arrays, prepare a volume of PCR components mix 10% greater than required for the total number of reactions to be performed.

Note: Save the remaining 9 µl cDNA synthesis reaction at –20°C, as it may be needed to perform quality control analysis.

3. Dispense the PCR components mix into the RT² IncRNA PCR Array depending on the RT² IncRNA PCR Array format, as described below.

Note: Change pipet tips following each pipetting step to avoid cross-contamination between the wells.

Note: If using an instrument to automate this step, contact Technical Service for plate specifications.

Formats A, C, D, or F (96-well)

- Remove the RT² IncRNA PCR Array from its sealed bag.
- Optional: If the PCR components mix is in a tube, transfer to a loading reservoir, such as the RT² PCR Array Loading Reservoir (cat. no. 338162).
- Add 25 μ l PCR components mix to each well of the RT² IncRNA PCR Array using an 8-channel pipettor or a 12-channel pipettor using only 8 tips.
- Proceed to step 4.

Formats E or G 384 (4 x 96) option

Note: Each 384-well plate contains 4 replicates of 96 assays that can be used for analysis of 4 samples, with reactions for each sample separated from one another by only 1 well. The spacing between the tips of standard multichannel pipettors allows rows or columns to be skipped when adding each sample. Be sure to load each sample into the correct set of wells using Figure 6 as a guide.

- Remove the RT² IncRNA PCR Array from its sealed bag.
- Optional: If the PCR components mix is in a tube, transfer to a loading reservoir, such as the RT² PCR Array Loading Reservoir (cat. no. 338162).
- Add PCR components mix to each well of the RT² IncRNA PCR Array using an 8-channel pipettor or a 12-channel pipettor using only 8 tips, and the 384EZLoad Covers (provided) using Figure 6 as a guide.
- Place 384EZLoad Cover 1 (white) on the plate. Add 10 μ l PCR components mix for sample 1 to the open wells (odd number wells of rows A, C, E, G, I, K, M, and O). Remove and discard 384EZLoad Cover 1.
- Place 384EZLoad Cover 2 (yellow) on the plate. Add 10 μ l PCR components mix for sample 2 to the open wells (even number wells of rows A, C, E, G, I, K, M, and O). Remove and discard 384EZLoad Cover 2.

- Place 384EZLoad Cover 3 (black) on the plate. Add 10 µl PCR components mix for sample 3 to the open wells (odd number wells of rows B, D, F, H, J, L, N, and P). Remove and discard 384EZLoad Cover 3.
- Place 384EZLoad Cover 4 (red) on the plate. Add 10 µl PCR components mix for sample 4 to the open wells (even number wells of rows B, D, F, H, J, L, N, and P). Remove and discard 384EZLoad Cover 4.

Sample 1

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
B	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
C	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
D	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
E	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
F	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
G	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
H	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
I	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
J	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
K	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
L	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
M	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
N	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
O	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
P	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4

Sample 2

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
B	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
C	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
D	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
E	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
F	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
G	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
H	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
I	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
J	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
K	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
L	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
M	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
N	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
O	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
P	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4

Sample 3

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
B	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
C	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
D	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
E	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
F	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
G	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
H	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
I	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
J	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
K	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
L	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
M	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
N	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
O	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
P	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4

Sample 4

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
B	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
C	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
D	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
E	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
F	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
G	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
H	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
I	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
J	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
K	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
L	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
M	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
N	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
O	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
P	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4

Figure 6. Loading RT² IncRNA PCR Arrays Formats E or G 384 (4 x 96) option. Add 10 µl PCR components mix for each of the 4 samples into the staggered wells with the same number as indicated in the figure.

- Proceed to step 4.

Custom RT² IncRNA PCR Arrays

- Remove the RT² IncRNA PCR Array from its sealed bag.
- Optional: If the PCR components mix is in a tube, transfer to a loading reservoir, such as the RT² PCR Array Loading Reservoir (cat. no. 338162).

- Add PCR components mix to each well of the RT² IncRNA PCR Array: 25 µl per well for 96-well Custom RT² IncRNA PCR Arrays or 10 µl per well for 384-well Custom RT² IncRNA PCR Arrays.
- Proceed to step 4.

4. Tightly seal the RT² IncRNA PCR Array with Optical Thin-Wall 8-Cap Strips (Formats A and D) or Optical Adhesive Film (Formats C, E, F, and G).

Important: Users of Bio-Rad and Eppendorf real-time cyclers must ensure that the real-time cycler has been calibrated to use clear, flat optical caps with the RT² IncRNA PCR Array plates prior to initiating the run.

5. Centrifuge for 1 minute at 1000 x g at room temperature (15–25°C) to remove bubbles. Visually inspect the plate from underneath to ensure no bubbles are present in the wells.

Note: The presence of bubbles in the wells interferes with results.

6. Place the RT² IncRNA PCR Array on ice while setting up the PCR cycling program.

Note: The RT² IncRNA PCR Array containing PCR components mix may be stored at –20°C wrapped in aluminum foil for up to 1 week if desired.

7. Program the real-time cycler according to Table 4, 5, or 6, depending on the real-time cycler used. If prompted by your cycler software, select “Absolute Quantitation” to begin.

Note: For additional help with instrument setup, see our Instrument-Specific Setup Instructions and Protocol Files at RT² IncPCR Arrays Supplementary Protocols: www.qiagen.com/us/shop/pcr/primer-sets/rt2-incrna-pcr-arrays.

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

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

* Recommended for the following cyclers: Applied Biosystems models 5700, 7000, 7300, 7500, 7700, 7900HT, StepOnePlus, QuantiStudio 6, QuantiStudio 7, and QuantiStudio 12K; Bio-Rad models iCycler, iQ5, MyiQ, MyiQ2, CFX96, CFX384; Agilent (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 Instrument-Specific Setup Instructions and Protocol Files at RT² PCR Arrays Supplementary Protocols: www.qiagen.com/us/shop/pcr/primer-sets/rt2-lncrna-pcr-arrays

Table 5. Cycling conditions for Roche LightCycler 480*

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

* 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 and 1.8°C/s for the 384-well block. Refer to our Instrument-Specific Setup Instructions and Protocol Files at RT² PCR Arrays Supplementary Protocols, www.qiagen.com/us/shop/pcr/primer-sets/rt2-lncrna-pcr-arrays, for more information on other required changes to settings for Melt Curve Acquisition.

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

Cycles	Duration	Temperature	Comments
1	10 min	95°C	HotStart DNA <i>Taq</i> Polymerase is activated by this heating step.
40	15 s	95°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.
	30–40 s	55°C	
	30 s	72°C	

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

8. **Place the RT² IncRNA PCR Array in the real-time cycler. If recommended by the cycler user manual, use a compression pad with RT² IncRNA PCR Arrays sealed with optical adhesive film (formats C, E, F, and G). Start the run.**

9. **Calculate the quantitation cycle (C_q) for each well using the real-time cycler software, as described in the following steps.**

Note: If using the Roche LightCycler 480 or 96, there are 2 options for data analysis: using the second derivative max setting (in this case it will calculate the C_p) or using “Fit Points” (in this case the C_q should be defined manually).

10. **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.**

11. **Manually define the threshold by using the log view of the amplification plots. Choose a threshold value above the background signal but within the lower one-third to lower one-half of the linear phase of the amplification plot.**

Note: Ensure that the threshold values are the same across all RT² IncRNA PCR Array runs in the same analysis. The absolute position of the threshold is less critical than its consistent position across arrays. If the RNA sample is of sufficient quality, the cycling program has been carried out correctly, and threshold values have been defined correctly, the value of C_q^{PPC} should be 20 ± 2 for all arrays or samples.

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

Note: The GeneGlobe Data Analysis Center is a web resource for the analysis of real time PCR or NGS data (www.qiagen.com/shop/genes-and-pathways/data-analysis-center-overview-page). To access the center, new users can register online. Once on the

site, the data analysis software will be found under the “Analysis” tab. The “RT² Profiler PCR Array Data Analysis” spreadsheet can be found under the “Resources” tab at www.qiagen.com/us/shop/pcr/primer-sets/rt2-lncrna-pcr-arrays.

13. **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, and primer-dimer peaks are usually seen as additional peak(s) at low melting temperatures.**

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/minute (optics on).

Note: For cycler-specific melting curve analysis settings, please refer to instrument-specific setup instructions that can be found under Supplemental Protocols at www.qiagen.com/us/shop/pcr/primer-sets/rt2-lncrna-pcr-arrays.

Note: Plates 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 plate 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 RT² lncRNA PCR Array. Removing the Optical Thin-Wall 8-Cap Strips or the Optical Adhesive Film from RT² lncRNA PCR Arrays releases PCR product into the air where it may contaminate and affect the results of future real-time PCR experiments.

Protocol: Real-Time PCR for RT² IncRNA PCR Arrays Format R

Important points before starting

- Ensure that RT² IncRNA PCR Array Format R and RT² SYBR Green ROX FAST Mastermix are used with a Rotor-Gene cycler. The format of the RT² IncRNA PCR Array is indicated by the last letter of the catalog number.
- 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 IncRNA PCR Array 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.

Procedure

1. **Briefly centrifuge the RT² SYBR Green ROX FAST Mastermix, water, and cDNA synthesis reaction (10–15 seconds) 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 components mix

Array format:	Rotor-Disc 100
2x RT ² SYBR Green ROX FAST Mastermix	1 150 µl
cDNA synthesis reaction	102 µl
Nuclease-Free Water	1 048 µl
Total volume	2300 µl

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

Note: Save the remaining 9 µl cDNA synthesis reaction at –20°C, as it may be needed to perform quality control analysis using the RT² RNA QC PCR Array.

- Carefully remove the RT² IncRNA PCR Array from its sealed bag. Slide the array into the Rotor-Disc 100 Loading Block using the tab at position A1 and the tube guide holes.**
- Add 20 µl PCR components mix to each well of the RT² IncRNA PCR Array. Proceed to step 5.**

Note: Change pipet tips following each pipetting step to avoid cross contamination between the wells.

Note: PCR components mix can be dispensed manually or using the QIAgility® (www.qiagen.com/goto/QIAgility). For the QIAgility program for dispensing PCR components mix to the Rotor-Disc 100, please contact QIAGEN technical support.

Note: Although wells 97-100 do not contain assays, it is essential to add PCR components mix for optimized balancing of the RT² IncRNA PCR Array.

- Carefully seal the RT² IncRNA PCR Array with Rotor-Disc Heat-Sealing Film using the Rotor-Disc Heat Sealer.**

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

Note: The RT² IncRNA PCR Array containing PCR components mix may be stored at –20°C wrapped in aluminum foil for up to 1 week if desired.

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

Note: For additional help with instrument setup, see our instrument-specific setup instructions that can be found under Supplemental Protocols at www.qiagen.com/us/shop/pcr/primer-sets/rt2-lncrna-pcr-arrays .

Table 8. Cycling conditions for Rotor-Gene cyclers

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

7. Insert the RT² lncRNA PCR Array 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*.

8. Calculate the quantification cycle (C_q) 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 lncRNA PCR Array runs in the same analysis.

9. **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 IncRNA PCR Array runs in the same analysis. The absolute position of the threshold is less critical than its consistent position across arrays. If the RNA sample is of sufficient quality, the cycling program has been carried out correctly, and threshold values have been defined correctly, the value of C_q^{PPC} should be 14 ± 2 for all arrays or samples.

10. **Export the C_q values for all wells to a blank Excel spreadsheet. Data analysis can then be conducted at QIAGEN'S GeneGlobe Data Analysis Center using software-based 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 (www.qiagen.com/shop/genes-and-pathways/data-analysis-center-overview-page). To access the center, new users can register online. Once on the site, the data analysis software will be found under the "Analysis" tab. The "RT² Profiler PCR Array Data Analysis" spreadsheet can be found under the "Resources" tab at www.qiagen.com/us/shop/pcr/primer-sets/rt2-incrna-pcr-arrays.

11. **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 instrument-specific setup instructions that can be found under Supplemental Protocols at www.qiagen.com/us/shop/pcr/primer-sets/rt2-incrna-pcr-arrays for more information.

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 ($15\text{--}25^{\circ}\text{C}$), 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 RT² lncRNA PCR Array. Removing the film from RT² lncRNA PCR Arrays releases PCR product into the air, where it may contaminate and affect the results of future real-time PCR experiments.

Protocol: cDNA Synthesis and Real-time PCR for RT² IncRNA 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 carried out with the RT² IncRNA PreAMP Primer Mix Format H. Finally, real-time PCR is performed using RT² IncRNA PCR Array Format H in combination with Microfluidics qPCR Master Mix (contains EvaGreen®).

Considerations regarding amount of RNA

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 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 (RT Mastermix). 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

* If performing the experiment for the first time, we recommend 1 µg RNA.

- 3. Incubate the genomic DNA elimination mix for 5 minutes at 37°C, then place immediately on ice for at least 1 minute.**
- 4. Add 6 µl BC4 Solution to each well and 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 minutes, 95°C for 5 minutes, 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.**

If you wish to store the reactions, transfer them to a –20°C freezer.

Preamplification using RT² IncRNA PreAMP Primer Mix Format H

- 7. Thaw RT² IncRNA 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*
RT ² IncRNA PreAMP Primer Mix	3 μ l	330 μ l
RT ² PreAMP PCR Mastermix (PA-30)	5 μ l	550 μ l
Total volume	8 μl	880 μl

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

9. **Pipet 8 μ l preamplification mix into each well of an empty 96-well plate.**
10. **Add 2 μ l 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 <i>Taq</i> Polymerase is activated by this heating step.
14	15 s	95°C	
	2 min	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 μ l Side Reaction Reducer to each well. Mix gently by pipetting up and down and spin briefly.**
15. **Incubate at 37°C for 15 minutes followed by heat inactivation at 95°C for 5 minutes.**
16. **Add 44 μ l Nuclease-Free Water to each well.**

This is a 5-fold dilution (11 μ l preamplification mix + 44 μ l 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 .

Sample mix preparation

18. Prepare a sample mix according to Table 12.

Table 12. Sample mix

Component	Volume for one reaction	Volume for a BioMark 96.96/48.48 Dynamic Array™*
2x Microfluidics qPCR Master Mix	3 μ l	330 μ l/165 μ l
20x DNA Binding Dye Sample Loading Reagent (Fluidigm, cat. no. 100-0388)	0.3 μ l	33 μ l/16.5 μ l
1x Low EDTA-TE buffer (0.1 mM EDTA)	0.7 μ l	77 μ l/38.5 μ l
Total volume	4.0 μ l	440 μ l/220 μ l

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

19. Pipette 53 μ l (96.96 Dynamic Array) or 26 μ l (48.48 Dynamic Array) sample mix into each tube of an 8-strip PCR tube.

20. Using an 8-channel pipettor, transfer 4 μ l sample mix into each well of an empty 96-well plate (for the 48.48 Dynamic Array, use only half of the 96-well plate).

21. Add 2 μ l of each preamplified sample from step 17 to a well of the 96-well plate containing the sample mix.

Note: Preamplified sample can be transferred using an 8-channel pipettor.

22. Cover the plate with plate sealer. Mix and spin briefly.

23. Label the template as "sample."

Assay mix preparation

24. Remove the RT² IncRNA PCR Array Format H from –20°C. Thaw for 10 minutes at room temperature (15–25°C). Briefly vortex and spin the plate to bring the contents to the bottom of the wells.
25. Mark the caps of the RT² IncRNA PCR Array so that they can be replaced in the original order. Remove the caps.
26. Pipet 45 µl 2x Assay Loading Reagent (provided by Fluidigm) into each tube of an 8-strip PCR tube.
27. Transfer 3 µl 2x Assay Loading Reagent from the 8-strip tube into each well of an empty 96-well plate.

This step can be performed using an 8-channel pipettor.

Note: For a 96.96 Dynamic Array, all 96 assays can be used at one time. When using a 48.48 Dynamic Array, only 48 assays can be used at once. A second 48.48 Dynamic Array must be run to utilize all 96 assays.
28. Transfer 3 µl from each well of the RT² IncRNA PCR Array to the corresponding well of the 96-well plate from step 27.
29. Cover the plate with a plate sealer. Mix by vortexing and spin briefly.
30. Label this plate as “assay.”

Priming and loading the Fluidigm BioMark HD Dynamic Array

31. Peel the blue protective film from the underside of the BioMark chip. Place the BioMark Dynamic Array into the IFC Controller.
32. Prime the Dynamic Array using standard Fluidigm protocols.

For details, consult the Fluidigm 96.96 or 48.48 Real-Time PCR Workflow Reference Guide.

33. Using an 8-channel pipettor, aliquot 5 μ l from each well of the “sample” plate into appropriate sample inlets on the BioMark Dynamic Array (loading wells on the right side of the chip).
34. Using an 8-channel pipettor, aliquot 5 μ l from each well of the “assay” plate into appropriate assay inlets on the BioMark Dynamic Array (loading wells on the left side of the chip).
35. Using the IFC Controller HX (96.96 Dynamic Arrays) or the IFC Controller MX (48.48 Dynamic Arrays), run the Load Mix (136x) Script for 96.96 IFCs or the Load Mix (113x) Script for 48.48 IFCs.
36. Remove the BioMark Dynamic Array from the IFC Controller.
37. Remove any dust particles from the BioMark Chip surface.

Running the BioMark Dynamic Array IFC

38. Double-click the “Data Collection Software” icon to launch the software.”
39. Click “Start a New Run,” place the chip into the reader, and click “Load.”
40. Verify chip barcode and chip type, choose project settings (if applicable), and click “Next.”
41. Chip run file: Select “New” or “Predefined,” browse to a file location for data storage, and click “Next.”
42. For “Application, Reference, Probes,” select the following:
 - a) “Application Type: Gene Expression”
 - b) “Passive Reference: ROX”
 - c) “Select Assay: single probe”
 - d) “Select probe type: EvaGreen.”Click “Next.”
43. Click “Browse” to find thermal protocol file “GE 96 x 96 Standard v1.pcl.” or “GE 48 x 48 Standard v1.pcl.”

Note: Make sure that you use a 96.96-specific protocol or 48.48-specific protocol depending on the Dynamic Array type.

44. Change the thermal protocol file to the conditions in Table 13. Thermal cycling protocols can be found under Supplementary protocols at www.qiagen.com/us/shop/pcr/primer-sets/rt2-lncrna-pcr-arrays.

Table 13. Cycling conditions for Fluidigm BioMark 96.96 Dynamic Array IFCs

Cycles	Duration	Temperature	Comments
1	120 s	50°C	Thermal mix*
1	1800 s	70°C	(only for 96.96 Dynamic Array IFC, Thermal Mix not needed for 48.48 Dynamic Array IFC)
1	600 s	25°C	
1	600 s	95°C	HotStart DNA <i>Taq</i> Polymerase is activated by this heating step.
40	15 s	94°C	
	60 s	60°C	Perform fluorescence data collection.

Ramp rate: Slow 1°C/s.

* If you are using a 96.96 Dynamic Array IFC, add a Thermal Mix segment by checking the box. The Thermal Mix is a step that helps the assay and sample chambers diffuse better on the small chambers on the 96.96 Dynamic Array. You do not need a Thermal Mix if you are using any kind of chip other than the 96.96 Dynamic Array IFC.

45. Confirm that “Auto Exposure” is selected, then click “Next.”

46. Verify the Dynamic Array run information and click “Start Run.”

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 www.qiagen.com/shop/pcr/primer-sets/rt2-profiler-pcr-arrays. 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 genomic DNA contamination

- | | |
|--|---|
| a) DNase digestion not performed | We strongly recommend performing the on-column DNase digestion step when purifying RNA using the RNeasy Mini Kit. |
| b) RT ² First Strand Kit not used | We strongly recommend using the RT ² First Strand Kit for cDNA synthesis. This kit includes a genomic DNA elimination step. |
| c) Reagents, tips, or tubes contaminated | See “Preparing a workspace free of DNA contamination.” If using the RT ² RNA QC PCR Array, the no template control (NTC) indicates the level of DNA contamination in the experimental setup. |
| d) Genomic DNA difficult to remove | Fold-changes in gene expression may still be obtained. However, it is very important to verify any results for individual genes by a separate, more rigorous real-time PCR analysis that includes a “minus RT” control. |

Inefficient reverse transcription

- | | |
|------------------|---|
| Poor quality RNA | Check the $A_{260}:A_{280}$ and $A_{260}:A_{230}$ ratios of RNA samples. Be sure to perform the dilutions for spectrophotometry in RNase-free Tris pH 8.0. If necessary, repurify RNA using a spin-column method such as the RNeasy Mini Kit. |
|------------------|---|

Poor PCR amplification efficiency

- a) Real-time cyler sensitivity Real-time cyclers vary in their level of sensitivity. If an average C_q^{PPC} value of 20 ± 2 is difficult to obtain from the positive PCR control (PPC), the observed average C_q^{PPC} value should be acceptable as long as it does not vary by more than 2 cycles between RT² IncRNA PCR Arrays.
- b) Cycling program incorrect Be sure that the initial heat activation step at 95°C was lengthened to 10 minutes from the shorter time in the default program. Be sure that all other cycle parameters also have been correctly entered according to the recommendations in the protocol.
- c) Poor quality RNA Check the $A_{260}:A_{280}$ and $A_{260}:A_{230}$ ratios of RNA samples. Be sure to perform the dilutions for spectrophotometry in RNase-free Tris pH 8.0. If necessary, repurify RNA using a spin-column method such as the RNeasy Mini Kit.

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

Data analysis can then be conducted at QIAGEN'S GeneGlobe Data Analysis Center using software-based or spreadsheet-based tools. C_q data can be entered and the tools will automatically perform quantification using the $\Delta\Delta C_q$ method as described below. The results will be presented in a tabular format, a scatter plot, a three-dimensional profile, and a volcano plot (when replicates are included).

Note: The GeneGlobe Data Analysis Center is a web resource for the analysis of real-time PCR or NGS data (www.qiagen.com/shop/genes-and-pathways/data-analysis-center-overview-page). To access the center, new users can register online. Once on the site, the data analysis software will be found under "Analysis." The RT² Profiler PCR Array Data Analysis spreadsheets can be found under "Product Resources" at www.qiagen.com/us/shop/pcr/primer-sets/rt2-profiler-pcr-arrays.

Data analysis using the $\Delta\Delta C_q$ method for 96-well and 384-well formats

The following procedure describes data analysis for formats A, C, D, E, F, and G. For data analysis using format H with 96 x 96 microfluidic chips, visit QIAGEN's GeneGlobe Data Analysis Center.

A1. Change all C_q values reported as greater than 35 or as N/A (not detected) to 35.

At this point, any C_q value equal to 35 is considered a negative call.

A2. Examine the C_T values of the genomic DNA control wells (GDC) as follows:

Note: If the RT² PreAMP cDNA Synthesis Kit was used for preamplification, consult the *RT² IncRNA PreAMP cDNA Synthesis Handbook* for the correct control values.

- Calculate C_q^{GDC} .
- If the value is greater than 33, 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 33, genomic DNA contamination is evident. See the "Troubleshooting Guide."

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

Note: If RT² PreAMP cDNA Synthesis Kit was used for preamplification, consult the *RT² IncRNA PreAMP cDNA Synthesis Handbook* for the correct control values.

- Calculate $\Delta C_q = \text{AVG } C_q^{\text{RTC}} - \text{AVG } C_q^{\text{PPC}}$.
- If this value is less than 5, then no inhibition of the reverse-transcription reaction is apparent. No action is needed.
- If this value is greater than 5, there is evidence of impurities that may have inhibited the reverse transcription reaction. See the "Troubleshooting Guide."

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

- Calculate ΔC_q^{PPC} .
- The average C_q^{PPC} value should be 20 ± 2 on each RT² IncRNA PCR Array and should not vary by more than 2 cycles between RT² IncRNA 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."

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). Use the formula:

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

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 IncRNA 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. Calculate the $\Delta\Delta C_q$ for each gene across 2 RT² IncRNA PCR Arrays (or group of samples). Use 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. Calculate the fold change for each gene from group 1 to group 2 as $2^{(-\Delta\Delta C_q)}$.

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.

Data analysis using the $\Delta\Delta C_q$ method for Rotor-Disc formats

A1. Change all C_q values reported as greater than 33 or as N/A (not detected) to 33.

At this point, any C_q value equal to 33 is considered a negative call.

A2. Examine the C_q values of the genomic DNA control wells (GDC), as follows:

- Calculate C_q^{GDC} .
- If the value is greater than 33, 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 33, genomic DNA contamination is evident. See the "Troubleshooting Guide."

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

- Calculate $\Delta C_q = \text{AVG } C_q^{\text{RTC}} - \text{AVG } C_q^{\text{PPC}}$.
- If this value is less than 5, then no inhibition of the reverse-transcription reaction is apparent. No action is needed.
- If the value is greater than 5, there is evidence of impurities that may have inhibited the reverse transcription reaction. See the "Troubleshooting Guide."

A4. Examine the C_q values of the positive PCR control wells (PPC), as follows:

- Calculate the C_q^{PPC} .
- The average C_q^{PPC} value should be 14 ± 2 one each RT². The average PCR Array should not vary by more than 2 cycles between RT² IncRNA 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 16 for all samples may indicate a problem with the cycling conditions or may be simply indicative of the relative sensitivity of the instrument. See the “Troubleshooting Guide.”

A5. Calculate the ΔC_q for each pathway-focused gene in each Rotor-Disc using the C_q values for the gene of interest (GOI) and the reference genes used for normalization (REF). Use 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 IncRNA 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 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. Calculate $\Delta\Delta C_q$ for each gene across 2 RT² IncRNA PCR Arrays (or groups of samples). Use the formula $\Delta\Delta C_q = \Delta C_q(\text{group 2}) - \Delta C_q(\text{group 1})$, 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. Calculate the fold-change for each gene from group 1 to group 2 as $2^{(-\Delta\Delta C_q)}$.

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 $\Delta\Delta C_T$ data analysis method

Due to the inverse proportional relationship between the quantification cycle (C_q) 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:

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

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.

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

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.

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

The complete calculation is as follows:

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

Appendix B: Protocol for Reference Genes RT² PCR Arrays

This protocol is for cDNA synthesis and real-time PCR using Reference Genes RT² PCR Arrays (cat. no. 330231 PAHS-000, 330231 PAMM-000, and 330231 PARN-000).

Procedure

cDNA synthesis using the RT² First Strand Kit

Perform a reverse-transcription reaction for each sample to be characterized on the array, including one sample representing your biological or experimental control. Follow the protocol on page 26.

Real-time PCR

Follow the protocol on page 30, with the following changes to the preparation of the PCR components mix and dispensing of the PCR components mix into the Reference Gene RT² PCR Array.

1. **Prepare the PCR components mix in a 5-ml tube or a loading reservoir as described in Table 14.**

Table 14. PCR components mix

Component	Volume for 96-well format	Volume for 384-well format	Volume for Rotor-Disc format
2x RT ² SYBR Green Mastermix	337.5 µl	135 µl	270 µl
cDNA synthesis reaction	27 µl	12 µl	27 µl
Nuclease-free water	310.5 µl	123 µl	243 µl
Total volume	675 µl	270 µl	540 µl
Volume per well	25 µl	10 µl	20 µl

2. Dispense the PCR components mix into the RT² Reference PCR Array.

Note: Organize sample loading onto the arrays very carefully, making sure to characterize each sample in duplicate and to include a replicate of the control sample on each plate. For example, up to 4 samples can be characterized in duplicate on a single array or duplicate determinations may be made on 2 separate arrays for larger number of samples.

		Reference genes											
		1	2	3	4	5	6	7	8	9	10	11	12
Samples	A	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
	B	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
	C	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
	D	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
	E	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
	F	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
	G	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
	H	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12

Figure 7. Layout of the reference genes RT² PCR Arrays in 96-well plates.

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

3. For each sample, average the duplicate determinations of the C_q values from each sample for each reference gene.

-
4. For each reference gene, calculate the ΔC_q (the difference between the C_q value of the gene in each experimental sample and the C_q value of the gene in the control sample).
 5. Choose the reference genes with the smallest ΔC_q value across the samples of interest to normalize the results of future RT-PCR experiments for input total RNA loading. More than one reference gene may be chosen for analyses. Simply monitor the expression of all of these reference genes and use their average C_q value as the normalization factor for each sample.

Ordering Information

Product	Contents	Cat. no.
RT ² lncRNA PCR Array	Arrays of assays for disease, pathway, or functionally related genes; available in 96-well, 384-well, and Rotor-Disc100 formats	330721
RT ² RNA QC PCR Array	Array for quality control analysis prior to experiments using RT ² lncRNA PCR Arrays; available in 96-well, 384-well, and Rotor-Disc 100 formats	330291
RT ² First Strand Kit (12)	For 12 x 20 l 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
RT ² First Strand Kit (50)	For 50 x 20 µl first strand cDNA synthesis reactions; Buffer GE (100 µl), 5x Buffer BC3 (200 µl), RE3 Reverse Transcriptase Mix (100 µl), Control P2 (50 µl), Nuclease-Free Water (1 ml)	330404
RT ² 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; 2x 1.35 ml Mastermix	330500
RT ² 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; 2x 1.35 ml Mastermix	330510

RT ² 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 1.35 ml Mastermix	330520
RT ² 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 1.35 ml Mastermix	330620
Related products		
Human XpressRef Universal Total RNA	2 tubes each containing 100 g human RNA at 1 mg/ml	338112
Mouse XpressRef Universal Total RNA	2 tubes each containing 100 g mouse RNA at 1 mg/ml	338114
RT ² PreAMP cDNA Synthesis Kit (12)	For 12 x 20 µl first-strand cDNA synthesis reactions: Buffer GE, Buffer BC3, RE3 Reverse Transcriptase Mix, RNase Inhibitor, Control P2, Nuclease-free water; for 48 x 25 µl preamplification reactions: RT ² PreAMP PCR Mastermix (600 µl); Side Reaction Reducer (96 µl)	330451
RT ² IncRNA PreAMP Primer Mix	Pathway-focused IncRNA primer mixes for use with the RT ² PreAMP cDNA Synthesis Kit	330741
Custom RT ² IncRNA PreAMP Primer Mix	Customer defined IncRNA primer mixes for use with the RT ² PreAMP cDNA Synthesis Kit	330751
RT ² Microfluidics qPCR Reagent System	For 96 x 20 µl first strand cDNA synthesis reactions; 1 tube Buffer GE2 (750 µl/tube), 1 tube BC4 Reverse Transcriptase Mix (750 µl/tube); RT ²	330431

	PreAMP PCR Mastermix (600 µl); Side Reaction Reducer (96 µl); 2x Microfluidics EvaGreen qPCR Master Mix (1650 µl)	
miRNeasy Mini Kit (50)*	For 50 preps: 50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), QIAzol Lysis Reagent, RNase-Free Reagents and Buffers	217004
RNase-Free DNase Set (50)	For DNase digestion during RNA purification: 1500 units RNase-free DNase I, RNase-free Buffer RDD, and Nuclease-free water for 50 RNA minipreps	79254
miRNeasy Micro Kit (50)	For 50 total RNA preps: 50 RNeasy MinElute Spin Columns, Collection Tubes (1.5 ml and 2 ml), QIAzol Lysis Reagent, RNase-Free Reagents and Buffers	217084
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
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

* Larger kit sizes available; please inquire.

Product	Contents	Cat. no.
RNeasy Microarray Tissue Mini Kit (50)	RNeasy Mini Spin Columns, Collection Tubes, QIAzol Lysis Reagent, RNase-free reagents and buffers	73304
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
RT ² PCR Array Loading Reservoir	12 x 5 ml capacity, irradiation-sterilized loading reservoirs	338162
384EZLoad Covers	Pack of 4 color-coded covers for loading 384-well plates	338125

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Handbook Revision History

Document	Changes	Date
HB-1877-001	Initial release	October 2014
HB-1877-002	Updated template. Updated data analysis procedure.	April 2019

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