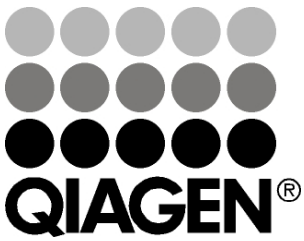
February 2012

RT² RNA QC PCR Array Handbook

For quality control analysis prior to gene expression profiling using real-time RT-PCR



Sample & Assay Technologies

QIAGEN Sample and Assay Technologies

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

QIAGEN sets standards in:

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

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Contents

Kit Contents

RT² RNA QC PCR Array Format A*	
96-well plate containing dried assays	1
Optical Thin-Wall 8-Cap Strips	12
Handbook	1

* Suitable for use with the following real-time cyclers: Applied Biosystems® models 5700, 7000, 7300, 7500, 7700, 7900HT, ViiA™ 7 (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; Stratagene® models Mx3005P®, Mx3000P®; Takara TP-800.

RT² RNA QC PCR Array Format C†	
96-well plate containing dried assays	1
Optical Adhesive Film	1
Handbook	1

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

RT² RNA QC PCR Array Format D‡	
96-well plate containing dried assays	1
Optical Thin-Wall 8-Cap Strips	12
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; Stratagene Mx4000®.

RT² RNA QC PCR Array Format E§	
384-well plate containing dried assays	1
Optical Adhesive Film	1
384EZLoad Covers	1 set of 4
Handbook	1

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

RT² RNA QC PCR Array Format F*	
96-well plate containing dried assays	1
Optical Adhesive Film	1
Handbook	1

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

RT² RNA QC PCR Array Format G†	
384-well plate containing dried assays	1
Optical Adhesive Film	1
384EZLoad Covers	1 set of 4
Handbook	1

† Suitable for use with the following real-time cycler: Roche LightCycler 480 (384-well block).

RT² RNA QC PCR Array Format R‡	
Rotor-Disc® 100 containing dried assays	1
Rotor-Disc Heat Sealing Film	1
Handbook	1

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

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

Shipping and Storage

RT² RNA QC PCR Array Formats A, C, D, E, F, G, and R are shipped at room temperature (15–25°C) or on ice, depending on the destination and accompanying products. All RT² RNA QC PCR Array Formats should be stored at –20°C upon arrival. When stored properly at –20°C, RT² RNA QC PCR Arrays are stable for up to 6 months after delivery.

Product Use Limitations

RT² RNA QC PCR Arrays are intended for molecular biology applications. These products are not intended for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Product Warranty and Satisfaction Guarantee

QIAGEN guarantees the performance of all products in the manner described in our product literature. The purchaser must determine the suitability of the product for its particular use. Should any product fail to perform satisfactorily due to any reason other than misuse, QIAGEN will replace it free of charge or refund the purchase price. We reserve the right to change, alter, or modify any product to enhance its performance and design. If a QIAGEN product does not meet your expectations, simply call your local Technical Service Department or distributor. We will credit your account or exchange the product — as you wish. Separate conditions apply to QIAGEN scientific instruments, service products, and to products shipped on dry ice. Please inquire for more information.

A copy of QIAGEN terms and conditions can be obtained on request, and is also provided on the back of our invoices. If you have questions about product specifications or performance, please call QIAGEN Technical Services or your local distributor (see back cover or visit www.qiagen.com).

Technical Assistance

At QIAGEN, we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in sample and assay technologies and the use of QIAGEN products. If you have any questions or experience any difficulties regarding RT^2 RNA QC PCR Arrays or QIAGEN products in general, please do not hesitate to contact us.

QIAGEN customers are a major source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at QIAGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

For technical assistance and more information, please see our Technical Support Center at www.qiagen.com/Support or call one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit www.qiagen.com).

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the

appropriate material safety data sheets (MSDSs). These are available online in convenient and compact PDF format at www.qiagen.com/Support/MSDS.aspx where you can find, view, and print the MSDS for each QIAGEN kit and kit component.

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

Quality Control

In accordance with QIAGEN's Quality Management System, each lot of RT^2 RNA QC PCR Arrays is tested against predetermined specifications to ensure consistent product quality.

Introduction

RNA quality control using an RT^2 RNA QC PCR Array

The RT^2 RNA QC PCR Array is designed to assess the quality of 12 RNA samples simultaneously before gene expression analysis using RT^2 Profiler PCR Arrays. Use of the RT^2 RNA QC PCR Array provides complete confidence in gene expression analysis results by enabling exclusion of substandard samples prior to analysis with RT^2 Profiler PCR Arrays.

The RT^2 RNA QC PCR Array contains controls for RNA integrity, the presence of inhibitors of reverse transcription and PCR amplification, and the presence of genomic or other DNA contamination. Use of the RT^2 RNA QC PCR Array enables detection and prevention of reverse transcription inhibition, PCR amplification inhibition, genomic DNA contamination, false positive signals, and multi-peak dissociation curves. The controls included in the RT^2 RNA QC PCR Array are described below.

Housekeeping controls

Assays for 2 housekeeping genes, typically expressed at very different levels relative to one another, enable prediction of the expected threshold cycle value ranges in subsequent reactions and an estimation of RNA integrity.

Reverse-transcription control

During the reverse-transcription step, the RT^2 First Strand Kit synthesizes template from purified RNA and from a built-in external RNA control, detectable by an assay in the RT^2 RNA QC PCR Array. This reverse-transcription control tests for the presence of inhibitors of the reverse transcription reaction in the RNA samples.

Positive PCR control

The positive PCR control is a plasmid template with an artificial sequence and primers to detect it. Two controls are characterized with or without experimental template to test for the presence of PCR inhibitors in the RNA samples.

DNA contamination controls

Two methods are used to sensitively detect genomic DNA contamination. The no reverse transcription control mixes primers for a housekeeping gene with total RNA instead of template to test for genomic DNA. Another assay specifically amplifies nontranscribed genomic DNA contamination.

No template control

A control lacking experimental template serves as a no template control to test for the introduction of any DNA contamination during experimental setup. The assay in this control detects an abundant housekeeping gene.

SAMPLE	↓ 96-well →		1		2		3		4		5		6		7		8		9		10		11		12	
	384-well ↓→		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
cDNA template	A	A	HK1		HK1		HK1		HK1		HK1		HK1		HK1		HK1		HK1		HK1		HK1		HK1	
		B																								
	B	C	HK2		HK2		HK2		HK2		HK2		HK2		HK2		HK2		HK2		HK2		HK2		HK2	
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	D	G	PPC		PPC		PPC		PPC		PPC		PPC		PPC		PPC		PPC		PPC		PPC		PPC	
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	E	I	GDC		GDC		GDC		GDC		GDC		GDC		GDC		GDC		GDC		GDC		GDC		GDC	
		J																								
RNA	F	K	NRT		NRT		NRT		NRT		NRT		NRT		NRT		NRT		NRT		NRT		NRT		NRT	
		L																								
H_2O	G	M	PPC		PPC		PPC		PPC		PPC		PPC		PPC		PPC		PPC		PPC		PPC		PPC	
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	H	O	NTC		NTC		NTC		NTC		NTC		NTC		NTC		NTC		NTC		NTC		NTC		NTC	
		P																								

Figure 1. RT² RNA QC PCR Array layout. Row A contains replicate assays for a highly expressed housekeeping gene, **HK1** (the ACTB gene). **Row B** contains replicate assays for a lowly expressed housekeeping gene, **HK2** (the HPRT1 gene). Expression levels of these housekeeping genes enable prediction of C_T values in future analyses and assessment of RNA integrity. **Row C** contains replicate assays for the reverse transcription control (**RTC**) which test the efficiency of the RT² First Strand Kit. These assays detect external RNA control template built in to the RT² First Strand Kit. **Rows D and G** contain replicate positive PCR controls (**PPC**) which comprise an artificial DNA sequence and the assay that detects it. Template cDNA is added to row D and water is added to row G. This tests for PCR inhibitors. **Row E** contains replicate assays for the genomic DNA control (**GDC**). These assays specifically detect nontranscribed genomic DNA contamination with a high level of sensitivity. **Row F** contains replicate assays for the no reverse transcription control (**NRT**). These assays test for genomic DNA contamination in the RNA sample by trying to amplify a housekeeping gene directly from the RNA sample. **Row H** contains replicate assays for the no template control (**NTC**). These assays test for general DNA contamination in the PCR system introduced during plate setup. During experimental setup, template cDNA and master mix are added to rows A–E; RNA and master mix are added to row F; and master mix only is added to rows G and H. The 384-well format of the RT² RNA QC PCR Array includes 4 replicates of each well of the 96-well format. Each of the 4 replicates (wells labeled 1–4 in gray) contains the same control as shown for the 96-well format.

First-strand cDNA synthesis and mastermixes

Performance of RT² RNA QC 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 PCR Array System is absolutely 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 RNA QC PCR Arrays and RT² Profiler 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 RNA QC 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 RNA QC PCR Arrays and RT² Profiler PCR Arrays with maximum levels of sensitivity with nanogram to microgram amounts of total RNA.

Each well of the RT² Profiler 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 Profiler PCR Arrays do not contain the dye.

Description of protocols

This handbook contains 2 protocols. The first protocol details cDNA synthesis by reverse transcription using purified RNA and the RT² First Strand Kit (page 17). This protocol should be performed prior to real-time PCR. The second protocol details real-time PCR performed using the cDNA prepared in the first protocol as the template (page 19).

Equipment and Reagents to Be Supplied by User

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

In addition to the RT^2 RNA QC PCR Array, the following are required:

- RT^2 First Strand Kit (cat. no. 330401)
- RT^2 SYBR Green Mastermix (for details of the appropriate RT^2 SYBR Green Mastermix for your real-time cycler, consult the *RT^2 Profiler PCR Array Handbook*)
- 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 RT^2 PreAMP Pathway Primer Mix)
- Nuclease-free pipet tips and tubes
- Optional: XpressRef Universal Total RNA to control PCR conditions is available for human (cat. no. 338112), mouse (cat. no. 338114), and rat (cat. no. 338116)
- Optional: RT^2 PCR Array Loading Reservoir (cat. no. 338162)

Important Notes

Preparing a workspace free of DNA contamination

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

- Wear gloves throughout the procedure. Use only fresh PCR-grade reagents (water) and labware (tips and tubes).
- Physically separate the workspaces used for PCR setup and post-PCR processing or non-PCR operations. Decontaminate the PCR workspace and labware (pipettor barrels, tube racks, etc.) before each use with UV light (to render any contaminating DNA ineffective in PCR through the formation of thymidine dimers) or with 10% bleach (to chemically inactivate and degrade any DNA).
- Close all tubes containing PCR products once you are finished adding or removing volumes. Before discarding any labware (tips or tubes) containing PCR products or other DNA, treat with 10% bleach.
- Do not remove the RT^2 Profiler 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 Profiler 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 total RNA for your real-time PCR experiment should be prepared using one of the methods described below, depending on the biological

sample. For optimal results, RNA samples should be suspended in RNase-free water. Do not use DEPC-treated water.

Cultured cells

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

Tissue samples

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

Formalin-fixed paraffin-embedded (FFPE) samples

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

Small samples yielding <100 ng total RNA

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

Whole blood samples

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

Total RNA isolated using a phenol-based method

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

Other biological samples

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

RNA quantification and quality control

For best results from the RT^2 Profiler 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 on page 12.

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 RNase-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®. Verify that there are sharp bands/peaks present for both the 18S and 28S ribosomal RNAs (Figure 2). Any smearing of the RNA bands or shoulders on the RNA peaks indicates that degradation has occurred in the RNA sample. For reliable data from RT^2 Profiler PCR Arrays, an RNA Integrity Number (RIN) of 7 or higher is recommended. Consistent RIN values across multiple samples within each experiment are desirable for reliable quality data comparisons.

Samples with degraded RNA, such as from FFPE blocks/slides, with RINs less than 7, may be of sufficient quality, but the final PCR data must be checked for quality.

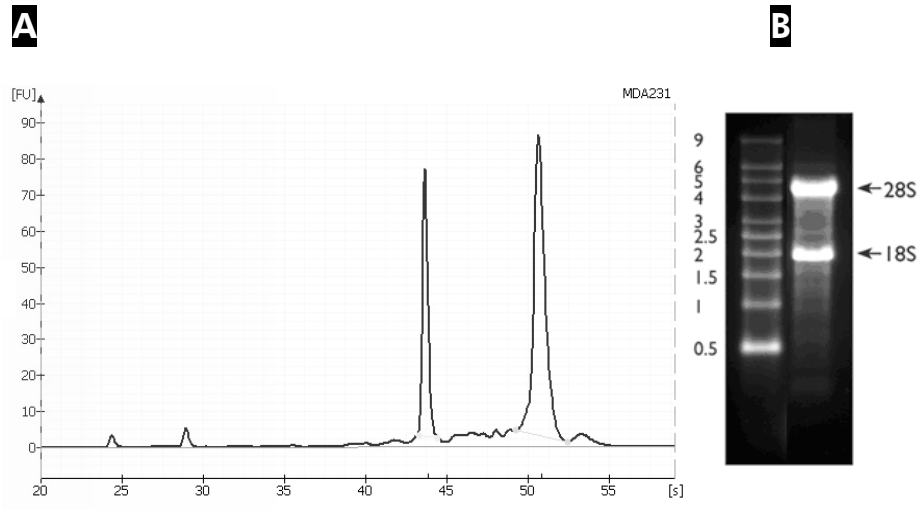

Figure 2. Ribosomal RNA integrity. **A** Agilent Bioanalyzer electropherogram of high-quality total RNA showing sharp peaks for the 18S (left) and 28S (right) ribosomal RNA. Due to high quality of the RNA, peaks do not have shoulders (especially to the left of each peak). **B** Agarose gel electrophoresis shows sharp bands (especially at the bottom of each band) for 28S and 18S ribosomal RNA.

Genomic DNA contamination

Eliminating genomic DNA contamination is essential for obtaining optimal real-time gene expression profiling results using the RT^2 Profiler PCR Array. The genomic DNA control in each RT^2 Profiler PCR Array specifically tests for genomic DNA contamination in each sample during each run. A genomic DNA control threshold 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 RT^2 First Strand Kit. If required, individual, species-specific RT^2 qPCR Primer gDNA Controls are available.

Starting RNA amounts

The RT^2 Profiler PCR Array System provides results with as little as 1 ng or as much as 5 µg total RNA per array*. For smaller starting RNA amounts, the RT^2 PreAMP cDNA Synthesis Kit and RT^2 PreAMP Pathway 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 *RT^2 PreAMP cDNA Synthesis Handbook*.

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

For successful results and maximum positive call rates, we recommend that first-time users start with 0.5 µg total RNA for 96-well plate formats, 0.8 µg total RNA for Rotor-Disc 100 formats, 400 ng total RNA if using RT^2 Profiler PCR Array format E and G 384 (4 x 96) option, or 1 µg total RNA if using RT^2 Profiler PCR Array format E and G 384 HT option. It is important to use a consistent amount of total RNA for all samples in a single experiment.

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

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 RNA QC PCR Array.

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 0.5 µg total RNA for 96-well plate formats, 0.8 µg total RNA for Rotor-Disc 100 formats, 400 ng total RNA for format E and G 384 (4 x 96) option RT² Profiler PCR Arrays, or 1 µg total RNA for format E and G 384 HT option RT² Profiler PCR Arrays. Use of less than 100 ng RNA will result in a high rate of false negatives. Using more then the recommended total RNA may potentially overload the system.
- **Do not** use DEPC-treated water. Use high-quality, nuclease-free water.
- The RT² First Stand Kit is not compatible with the chemicals in DNA-free™ kits from Ambion. If your RNA sample has been treated with DNA-free reagents, contact QIAGEN Technical Service.

Procedure

1. **Thaw the reagents of the RT² First Strand Kit. Briefly centrifuge (10–15 s) 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 and then centrifuge briefly.**

Table 1. Genomic DNA elimination mix

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

* 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 min at 42°C, then place immediately on ice for at least 1 min.**
4. **Prepare the reverse-transcription mix according to Table 2.**

Table 2. Reverse-transcription mix

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

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

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

Protocol: Real-Time PCR for RT² RNA QC PCR Arrays

Important points before starting

- Ensure that the RT² SYBR Green Mastermix and the RT² RNA QC PCR Array format are suitable for your real-time cycler (see page 4). The format of the RT² RNA QC PCR Array is indicated by the last letter of the catalog number. An incorrect RT² RNA QC 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 RNA QC 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 RNA QC PCR Array when pipetting.
- **Do not** use DEPC-treated water. Use high-quality, nuclease-free water.
- If precipitates are present in the Mastermix tubes, warm the reagents at 42°C for 1 min and vortex briefly to dissolve. Repeat if necessary.

Procedure

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

   Note: As the RT² SYBR Green 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 3 separate PCR components mixes for each RNA sample in 1.5 ml microcentrifuge tubes as described in Tables 3–5.**

   Note: cDNA template must be synthesized using the RT² First Strand Kit to ensure correct analysis of the reverse transcription control.

   Note: Refer to page 9 to view the RT² RNA QC PCR Array layout.

Table 3. PCR components mix 1*

Components	Volume for formats A, D, C, F	Volume for formats E, G
2x RT^2 SYBR Green Mastermix	75 µl	60 µl
cDNA synthesis reaction	6 µl	4.8 µl
RNase-free water	69 µl	55.2 µl
Total volume	**150 µl**	**120 µl**

* For rows A to E on 96-well formats or rows A to J on the 384-well formats.

Table 4. PCR components mix 2†

Components	Volume for formats A, D, C, F	Volume for formats E, G
2x RT^2 SYBR Green Mastermix	13 µl	13 µl
1/100 dilution of input total RNA‡	1 µl	1 µl
RNase-free water	13 µl	13 µl
Total volume	**27 µl**	**27 µl**

† For row F on 96-well formats or rows K and L on 384-well formats.

‡ Amount of RNA used in the cDNA synthesis reaction.

Table 5. PCR components mix 3§

Components	Volume for formats A, D, C, F	Volume for formats E, G
2x RT^2 SYBR Green Mastermix	30 µl	25 µl
RNase-free water	30 µl	25 µl
Total volume	**60 µl**	**50 µl**

§ For rows G and H on 96-well formats or rows M to P on 384-well formats.

3. Dispense the PCR components mixes into the RT^2 RNA QC PCR Array depending on the RT^2 RNA QC PCR Array format, as described below.

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

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

- Carefully remove the RT² RNA QC PCR Array from its sealed bag.
- Add 25 µl PCR components mix 1 to rows A, B, C, D, and E.
- Add 25 µl PCR components mix 2 to row F.
- Add 25 µl PCR components mix 3 to rows G and H.
- Proceed to step 4.

Formats E or G (384-well)

- Carefully remove the RT² RNA QC PCR Array from its sealed bag.
- Add 10 µl PCR components mix 1 to rows A, B, C, D, E, F, G, H, I, and J.
- Add 10 µl PCR components mix 2 to row K and L.
- Add 10 µl PCR components mix 3 to rows M, N, O, and P.
- Proceed to step 4.

Formats R (Rotor-Disc 100)

- Contact Technical Services for assistance.

4. **Carefully, tightly seal the RT² RNA QC 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 RT² RNA QC PCR Array plates prior to initiating the run.

5. **Centrifuge for 1 min at 1000 *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 RNA QC PCR Array on ice while setting up the PCR cycling program.**

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

7. **Program the real-time cycler according to Table 6, 7, or 8, 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: www.SABiosciences.com/pcrarrayprotocolfiles.php.

Table 6. Cycling conditions* for Applied Biosystems, Bio-Rad,† Stratagene, and Eppendorf‡ cyclers

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

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

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

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

Table 7. Cycling conditions for Roche LightCycler 480*

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

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

Table 8. Cycling conditions for Bio-Rad and Takara cyclers and all other cyclers†

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

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

8. **Place the RT² RNA QC PCR Array in the real-time cycler. If recommended by the cycler user manual, use a compression pad with RT² RNA QC PCR Arrays sealed with Optical Adhesive Film (formats C, E, F, and G). Start the run.**
9. **Calculate the threshold cycle (C_T) for each well using the real-time cycler software, as described in the following steps.**

   Note: If using the Roche LightCycler 480, there are 2 options for data analysis: using the second derivate max setting (in this case there is no need to calculate the C_T) or using "Fit Points" (in this case the C_T should be defined manually as described in step 11).
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. The threshold value should be in the lower half of the linear phase.**

    Note: Ensure that the threshold values are the same across all RT² RNA QC 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_T^{PPC} should be 20 ± 2 for all arrays or samples.

12. **Export the C_T values for all wells to a blank Excel® spreadsheet for use with the RT² RNA QC PCR Array Data Analysis Excel template or Web-based software.**

    Note: Excel-based PCR Array Data Analysis Templates are available at www.SABiosciences.com/pcrarraydataanalysis.php. Web-based PCR Array Data Analysis Software is available at www.SABiosciences.com/pcrarraydataanalysis.php.

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 at temperatures greater than 80°C.**

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

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

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

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: www.SABiosciences.com/support_faq.php?target=PCR. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit www.qiagen.com).

	Comments and suggestions
Presence of 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", page 12. The no template control (NTC) in the RT^2 RNA QC PCR Array 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 validate 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.

Comments and suggestions	
Poor PCR amplification efficiency	
a) Real-time cycler sensitivity	Real-time cyclers vary in their level of sensitivity. From the positive PCR control (PPC), if an average C_T^{PPC} value of 20 ± 2 is difficult to obtain, the observed average C_T^{PPC} value should be acceptable as long as it does not vary by more than 2 cycles between RT^2 Profiler 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: Data Analysis Using the RT² RNA QC PCR Array

To perform analysis of data from the RT² RNA QC PCR Array, visit the PCR Array Data Analysis Web portal at www.SABiosciences.com/pcrarraydataanalysis.php. Download the Excel-based RT² RNA QC PCR Array Data Analysis template by clicking on the "RT² RNA QC PCR Array Template" link. Save the Excel file to your computer, open the file in Excel, and follow the instructions provided in the "Instructions" worksheet.

If using a 384-well format (E or G), download the "384-Well Format E Data Analysis Patch" to dissect a 384-well dataset into the correct four sets of 96 genes for each of the four samples.

The RT² RNA QC PCR Array Data Analysis template automatically performs data analysis of threshold cycle data from a real-time instrument, as described below.

Data analysis of the RT² RNA QC PCR Array

Change all C_T values reported as greater than 35 or as N/A (not detected) to 35. At this point, any C_T value equal to 35 is considered a negative call.

Housekeeping gene expression

Gene expression analysis of 2 housekeeping genes expressed at a low or a high level provides an estimate of the range of threshold cycle values to be expected on subsequent PCR array analyses. Detectable threshold cycles for both of these genes also provide some confidence that RNA is intact. The expression level of the 2 housekeeping genes may or may not vary between your samples, providing a starting point toward defining a normalization factor for subsequent RT² Profiler PCR Array analyses.

Reverse-transcription control (RTC)

Any impurities in the RNA sample that affect the reverse transcription of the RT² First Strand Kit built-in external RNA control may also affect the reverse transcription of your mRNA of interest.

Calculate $\Delta C_T = C_T^C - C_T^G$ (or $C_T^{RTC} - C_T^{PPC + H2O}$).

If this value is less than 5, then no inhibition is apparent. If this value is greater than 5, then evidence exists of impurities that inhibit reverse transcription. If inhibitors of reverse transcription are evident, double-check the A_{260}:A_{280} and A_{260}:A_{230} ratios of your RNA samples ensuring that dilutions for spectrophotometry are made using RNase-free Tris pH 8.0 buffer. If necessary, repurify RNA samples with a spin-column–based clean up method, such as the RNeasy Mini Kit.

Positive PCR control (PPC)

Any impurities in your RNA sample that affect the PCR amplification of the positive control may also affect the PCR amplification for your mRNA of interest.

Calculate C_T^G (or $C_T^{PPC + H2O}$).

This value should be 20 ± 2 in each sample and should not vary by more than 2 between RNA samples being compared.

Different instruments have varying levels of sensitivity. If a C_T^G value of 20 ± 2 is difficult to obtain for your instrument, the observed C_T^G value should be acceptable as long as it does not vary by more than 2 cycles between the samples being compared.

Calculate $\Delta C_T = C_T^D - C_T^G$ (or $C_T^{PPC + cDNA} - C_T^{PPC + H2O}$).

If this value is less than 3, then no inhibition is apparent. If this value is greater than 3, then evidence exists of impurities that inhibit PCR. Large differences in C_T^D (or $C_T^{PPC + cDNA}$) values between samples indicate the presence of different amounts of PCR amplification inhibitors in the different samples. If PCR inhibitors are evident, double-check the A_{260}:A_{280} and A_{260}:A_{230} ratios of all of your RNA samples and be sure to perform the dilutions for spectrophotometry in RNase-free Tris pH 8.0 buffer. If necessary, repurify all of your RNA samples with a spin-column–based clean up method, such as the RNeasy Mini Kit, including the optional on-column DNase treatment.

Genomic DNA contamination

If the value of C_T^F (or C_T^{NRT}) is equal to 35 (or originally equal to or greater than 35 or N/A), then no genomic DNA contamination is apparent. If the value of C_T^F (or C_T^{NRT}) is less than 35, then some level of genomic DNA contamination is evident. Continue with the next calculation.

Calculate C_T^E (or C_T^{GDC}).

If the value is greater than 35, then the level of genomic DNA contamination will not affect the gene expression profiling results. No action is needed. If the value is less than 35, then genomic DNA contamination is evident and will affect the gene expression profiling results. See "Troubleshooting Guide", page 25.

General DNA contamination:

Observe the value of the no template control (NTC), C_T^H or C_T^{NTC}. If this value is equal to 35 (originally 35 or greater or N/A), then there is no evidence of general DNA contamination. If this value is less than 35, then there is evidence of general DNA contamination. See "Preparing a Workspace Free of DNA Contamination", page 12.

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 reference database online at www.SABiosciences.com/support_publication.php#pcrarray or contact QIAGEN Technical Services or your local distributor.

Ordering Information

Product	Contents	Cat. no.
RT² RNA QC PCR Array	Array for quality control analysis prior to experiments using RT² Profiler PCR Arrays; available in 96-well, 384-well, and Rotor-Disc 100 formats	330291
RT² Profiler PCR Array	Arrays of assays for disease, pathway, or functionally related genes; available in 96-well, 384-well, and Rotor-Disc 100 formats	330231
RT² First Strand Kit (12)	For 12 x 20 µl first strand cDNA synthesis reactions; Buffer GE (30 µl), Buffer BC3 (60 µl), RE3 Reverse Transcriptase Mix (28 µl), Control P2 (18 µl), RNase-Free Water (1 ml)	330401
RT² SYBR Green qPCR Mastermix (2)*	For 2 x 96 assays in 96-well plates; suitable for use with real-time cyclers that do not require a reference dye; 2 x 1.25 ml Mastermix	330500
RT² SYBR Green Fluor qPCR Mastermix (2)*	For 2 x 96 assays in 96-well plates; suitable for use with real-time cyclers that use fluorescein reference dye; 2 x 1.25 ml Mastermix	330510
RT² SYBR Green ROX™ qPCR Mastermix (2)*	For 2 x 96 assays in 96-well plates; suitable for use with real-time cyclers that use ROX reference dye; 2 x 1.25 ml Mastermix	330520
RT² SYBR Green ROX FAST Mastermix (2)*	For 2 x 96 assays in 96-well plates; suitable for use with real-time cyclers that use ROX reference dye, including the Rotor-Gene Q and Rotor-Gene 6000; 2 x 1.25 ml Mastermix	330620
Related products		
Human XpressRef Universal Total RNA	2 tubes each containing 100 µg human RNA at 1 mg/ml	338112

* Larger kit sizes available; please inquire.

Product	Contents	Cat. no.
Mouse XpressRef Universal Total RNA	2 tubes each containing 100 µg mouse RNA at 1 mg/ml	338114
Rat XpressRef Universal Total RNA	2 tubes each containing 100 µg rat RNA at 1 mg/ml	338116
RT² 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, RNase-Free Water; for 48 x 25 µl preamplification reactions: RT² PreAMP PCR Mastermix (600 µl); Side Reaction Reducer (96 µl)	330451
RT² PreAMP Pathway Primer Mix	Pathway-focused primer mixes for use with the RT² PreAMP cDNA Synthesis Kit	330241
RT² HT First Strand Kit (96)	For 96 x 20 µl first strand cDNA synthesis reactions; Buffer GE2 (750 µl), 2 x 5 ml Reagent Reservoirs, BC4 Reverse Transcriptase Mix (750 µl), 96-Well Plate, 2 Foil Adhesive Sealing Films, Compression Mat	330411
RNeasy Mini Kit (50)*	50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free reagents and buffers	74104
RNeasy FFPE Kit (50)	50 RNeasy MinElute Spin Columns, Collection Tubes, Proteinase K, RNase-Free DNase I, DNase Booster Buffer, RNase-free buffers, RNase-Free Water	73504
PAXgene Blood RNA Kit (50)	50 PAXgene Spin Columns, 50 PAXgene Shredder Spin Columns, Processing Tubes, RNase-Free DNase I, RNase-free reagents and buffers; To be used in conjunction with PAXgene Blood RNA Tubes	762174
RNeasy Microarray Tissue Mini Kit (50)	RNeasy Mini Spin Columns, Collection Tubes, QIAzol Lysis Reagent, RNase-free reagents and buffers	73304

* Larger kit sizes available; please inquire.

Product	Contents	Cat. no.
RNeasy Micro Kit (50)	50 RNeasy MinElute Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free DNase I, Carrier RNA, RNase-free reagents and buffers	74004
QIAamp RNA Blood Mini Kit (50)	50 QIAamp Mini Spin Columns, 50 QIAshredder Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free reagents and buffers	52304
RT² 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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Notes

Notes

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1071310 02/2012

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