



User Manual

RT² ProfilerTM PCR Array System

Pathway-Focused Gene Expression Profiling
Using Real-Time PCR

See Purchaser Notification for limited use license and warranty information (page 3).

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Pathway-Focused Gene Expression Profiling
Using Real-Time PCR

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(For Catalog Numbers Prefixed by: PAHS, PAMM, and PARN)

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- Fax: 1-888-465-9859 (US) 301-682-7300 (outside US)
- On-line Order: www.SABiosciences.com
- E-MAIL: order@SABiosciences.com (to place an order)
support@SABiosciences.com (for technical support)

You may place orders by fax, e-mail or from our website. Each order should include the following information:

- Your contact information (name, phone, email address)
- Product name, catalog number and quantity
- Purchase order number or credit card information (Visa or MasterCard)
- Shipping address
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LIMITED PRODUCT WARRANTY

This product is intended for research purposes only and is not intended for drug or diagnostic purposes or for human use. This warranty limits our liability to replace this product in the event the product fails to perform due to any manufacturing defect. SABiosciences Corporation makes no other warranties of any kind, expressed or implied, including without limitation, warranties of merchantability or fitness for a particular purpose. SABiosciences Corporation shall not be liable for any direct, indirect, consequential or incidental damages arising out of the use, the results of use or the inability to use this product.

NOTICE TO PURCHASER

The purchase of RT²Profiler PCR Arrays includes a limited, nonexclusive license to use the kit components for research use only. This license does not grant rights to use the kit components for reproduction of any primer pair mix, to modify kit components for resale or to use RT²Profiler PCR Array to manufacture commercial products without written approval of SABiosciences Corporation. No other license, expressed, implied or by estoppels, is granted. Patent pending.

I. Background and Introduction

Real-time reverse transcription (RT) PCR is the most 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. The RT² Profiler PCR Array takes advantage of real-time PCR performance and combines it with the ability of microarrays to detect the expression of many genes simultaneously. RT² Profiler PCR Arrays are designed to analyze a panel of genes related to a disease state or biological pathway. The product is especially suitable for researchers who are more familiar with or prefer real-time PCR technology but are looking for the multi-gene profiling capabilities of a microarray.

To complete the PCR Array procedure, start by converting your experimental RNA samples into first strand cDNA, the template for the polymerase chain reaction, using our RT² First Strand Kit. (See Figure 1 for an overview of the PCR Array procedure.) Then, mix your template with one of our instrument-specific and ready-to-use RT² qPCR Master Mixes. Aliquot the mixture into each well of the same plate containing pre-dispensed gene-specific primer sets. Perform PCR, and finally, determine relative expression with your real-time instrument and the $\Delta\Delta C_t$ method.

Each array contains a panel of 96 or 384 primer sets for a thoroughly researched set of 84 or 370 relevant, pathway- or disease-focused genes, plus five housekeeping genes and three RNA and PCR quality controls. The PCR Arrays are available in either a 96-well or 384-well plate format, containing either one or four replicates, respectively, of the 96-primer set panel. (See Figure 2 for the layout of typical PCR Arrays.) The PCR Array 384HT products (catalog numbers greater than 3000) contain a 370-gene panel in a 384-well plate format. SABiosciences's qPCR Assays, Master Mixes, and first strand kit have been optimized hand-in-hand for SYBR Green real-time RT-PCR detection, providing the PCR Arrays with superior sensitivity and wide linear dynamic ranges. The simplicity of the PCR Arrays also makes them accessible for routine use in every research laboratory.

Benefits of the RT² Profiler PCR Arrays:

- ■ **Pathway Focused:** Profile the expression of a panel of genes relevant to a pathway or disease state.
- ■ **Simple and Accurate:** Simple real-time PCR procedure provides high sensitivity and wide dynamic range.
- ■ **Designed for Routine Use:** Bring expression profiling to almost any lab with a real-time PCR instrument.

Combine microarray profiling capabilities with real-time PCR performance!

Figure 2A: Layout of the 96-Well and 384-Well PCR Arrays

Wells A1 through G12 contain a real-time PCR assay for genes from the same biological pathway or the same disease state or genes that are otherwise functionally-related.

Wells H1 through H5 contain a housekeeping gene panel to normalize PCR Array data.

The product information included with each cataloged PCR Array contains a list of the pathway-focused and housekeeping genes on the array.

Well H6 contains the Genomic DNA Control (GDC). Wells H7 through H9 contain replicate Reverse Transcription Controls (RTC). Wells H10 through H12 contain replicate Positive PCR Controls (PPC).

The 384-well format of the PCR Arrays includes four replicates of the same 96-well format, in which each two-by-two set of wells (wells labeled 1 - 4 in gray above) contains the same primer set represented by the 96-well designations.

Figure 2B: Layout of the PCR Array 384HT

Wells A1 through P10 (1-370) each contain a real-time PCR assay for genes from the same biological pathway or the same disease state or genes that are otherwise functionally-related.

Wells P12 through P15 contain a housekeeping gene panel to normalize PCR Array data.

The product information included with each cataloged PCR Array contains a list of the pathway-focused and housekeeping genes on the array.

Wells P16 through P18 contain replicate Genomic DNA Controls (GDC). Wells P19 through P21 contain replicate Reverse Transcription Controls (RTC). Wells P22 through P24 contain replicate Positive PCR Controls (PPC).

The Genomic DNA Control (GDC) is an assay that specifically detects non-transcribed genomic DNA contamination with a high level of sensitivity. The Reverse Transcription Control (RTC) tests the efficiency of the RT² First Strand Kit (C-03) reaction with a primer set detecting the template synthesized from the kit's built-in external RNA control. The Positive PCR Control (PPC) tests the efficiency of the polymerase chain reaction itself using a pre-dispensed artificial DNA sequence and the primer set that detects it.

The sets of replicate control wells (GDC, RTC, and PPC) also test for inter-well, intra-plate consistency.

Custom PCR Arrays have your specified layout, and the product information literature enclosed with the array reiterates that layout and the genes included.

II. Materials Provided:

The PCR Arrays are available in six different plate formats, each tailored to a specific subset of real-time PCR instruments and associated blocks. Formats A, C, D, and F are 96-well plates, while Formats E and G are 384-well plates.

Format	For Real-Time Instruments	Plate
A	All ABI "standard" blocks: 7000, 7300, 7500, 7700, 7900 Bio-Rad iCycler, MyiQ, iQ5 Chromo4 (MJ Research)	96-well
	Eppendorf RealPlex Stratagene Mx3005p, Mx3000p	
	ABI 7500 and 7900HT FAST 96-well blocks ABI StepOnePlus™	
C	ABI 7900HT 384-well block	384-well
D	Bio-Rad CFX-96 Opticon and Opticon 2 (MJ Research)	96-well
	Stratagene Mx4000	
	Roche LightCycler 480 96-well block	
F	Roche LightCycler 480 384-well block	384-well
G	Roche LightCycler 480 384-well block	384-well

NOTE: The format of the PCR Array is indicated by the last letter of the catalog number. Be sure that you have the correct PCR Array format for your instrument before starting the experiment.

The 96-well PCR Arrays (Formats A, C, D, and F) are shipped in sets of two (2), twelve (12), or twenty-four (24), while the 384-well PCR Arrays (Formats E and G) are shipped in sets of four (4). The PCR Array 384HT is shipped in sets of two (2), twelve (12), or twenty-four (24).

Each PCR Array shipment includes the arrays and either twelve (12) optical thin-wall 8-cap strips (Formats A and D) or one (1) optical adhesive film (Formats C, E, F, and G) per array.

Each 96x4 Format 384-Well PCR Array (Formats E and G) also includes one set of four 384EZLoad™ Covers (Catalog #PA-384) for each PCR Array provided in the package.

NOTE: Each 384EZLoad™ Cover is for a Single Use ONLY.

Storage Conditions:

All components included in this kit are shipped at ambient temperature but must be stored at -20 °C upon receipt. When stored properly at -20 °C, their quality is guaranteed for 6 months.

Technical Support: 888.503.3187 (US) 301.682.9200

III. Additional Materials Required:

- A. **RNA Isolation Kit:** See Page 12 for specific recommendations.
- B. **RT² First Strand Kit** (Cat. No. C-03)
MANDATORY for a Complete and Successful Experiment
For Reverse Transcription Control Detection (RTC, Wells H7 through H9)
- C. **SABiosciences RT² qPCR Master Mix**
MANDATORY for a Complete and Successful Experiment
 Be sure to pick the correct one for the instrumentation in your laboratory.

1. 96-Well PCR Arrays

RT² SYBR Green / ROX qPCR Master Mix: Specifically designed for:

All ABI and Stratagene Instrumentation

Eppendorf Mastercycler® ep *realplex* Instruments with ROX filter set

Catalog Number Size

PA-012	For 2 RT ² Profiler PCR Arrays
PA-012-12	For 12 RT ² Profiler PCR Arrays
PA-012-24	For 24 RT ² Profiler PCR Arrays

RT² SYBR Green / Fluorescein qPCR Master Mix:

Specifically designed for BioRad iCycler®, MyiQ®, and iQ5 Instrumentation

Catalog Number Size

PA-011	For 2 RT ² Profiler PCR Arrays
PA-011-12	For 12 RT ² Profiler PCR Arrays
PA-011-24	For 24 RT ² Profiler PCR Arrays

RT² SYBR Green qPCR Master Mix:

Specifically designed for instrumentation that does not require a reference dye:

BioRad CFX96

BioRad (MJ Research) Opticon, Opticon 2, and Chromo 4

Roche LightCycler® 480 System

Eppendorf Mastercycler® ep *realplex* Instruments without ROX filter set

Catalog Number Size

PA-010	For 2 RT ² Profiler PCR Arrays
PA-010-12	For 12 RT ² Profiler PCR Arrays
PA-010-24	For 24 RT ² Profiler PCR Arrays

2. 384-Well PCR Arrays

Each 384-well PCR Array 4-pack (Formats E & G) requires four (4) of the smaller size of the correct master mix for your instrument (4 X PA-01#).

3. PCR Array 384HT

The PCR Array 384HT two (2), twelve (12), and twenty-four (24) packs (Formats E & G) require a quantity of two (2) of the correct master mixes for your instrument of the size for the corresponding 96-well PCR Array packs (2 X PA-01# or 2 X PA-01#-12 or 2 X PA-01#-24).

D. Equipment:

1. For recommendations on specific real-time instrumentation (thermal cyclers with fluorescent detection), see the list of master mixes and plate formats above.

NOTE: *The PCR Arrays can only be used in 96-well and 384-well real-time PCR instruments. PCR Arrays can not be used in the Cepheid SmartCycler®, the Roche LightCycler® 2.0, or the Corbett Research Rotorgene.*

2. Calibrated Multi-Channel Pipettor
3. RNase / DNase-free pipette tips and tubes

IV. Complementary Products:

A. RT² RNA QC PCR Array (Optional):

Pick the correct catalog number for your species of interest (see below) and the correct plate format for the instrument in your lab. (See table on Page 7.)

<u>Human</u> RT ² RNA QC PCR Array	(Cat. No. PAHS-999)
<u>Mouse</u> RT ² RNA QC PCR Array	(Cat. No. PAMM-999)
<u>Rat</u> RT ² RNA QC PCR Array	(Cat. No. PARN-999)

B. XpressRef™ Universal Total RNA:

Universal RNA to control PCR conditions is available from the following species:

Human XpressRef™ Universal Total RNA	(Cat. No. GA-004)
Mouse XpressRef™ Universal Total RNA	(Cat. No. GA-005)
Rat XpressRef™ Universal Total RNA	(Cat. No. GA-006)

V. Protocol:

Please read through this entire protocol before beginning your experiment. RNA samples are very sensitive to RNase digestion; therefore, wear gloves and maintain an RNase-free work area while performing this protocol.

NOTE: Master Mix and First Strand Synthesis Considerations

The performance of our RT² Profiler PCR Arrays is only guaranteed with SABiosciences RT² qPCR Master Mixes and the RT² First Strand Kit. Therefore, the use of the complete RT² Profiler PCR Array System is absolutely essential for obtaining accurate real-time PCR profiling results.

The chemically-modified and tightly controlled HotStart enzyme and other proprietary chemical components in our RT² qPCR Master Mixes uniquely provide more accurate SYBR Green results by preventing the amplification of primer dimers and other non-specific products. They also help ensure high amplification efficiencies even for those genes that are the most difficult to amplify. When we test other sources of enzymes with our PCR Arrays, we frequently see primer dimers and other non-specific products that confound SYBR Green-based real-time PCR detection. Because each instrument uses a different reference dye to normalize their optics, be sure that you use the correct master mix for the instrumentation in your laboratory.

The RT² First Strand Kit includes a proprietary buffer to eliminate any residual genomic DNA contamination in your RNA samples before it can be amplified into secondary products that would otherwise cause false positive signals. The Reverse Transcription Controls (RTC) on the 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 transcriptases or first strand synthesis kits. The buffer components and the magnesium concentration in our RT² First Strand Kit are also more compatible with our PCR master mixes than other enzymes or kits providing the PCR Arrays with maximum levels of sensitivity with ng to µg amounts of total RNA.

NOTE: Preparing a Workspace Free of DNA Contamination

For accurate and reproducible PCR Array results, it is very 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 your working environment. Please follow the recommendations below on how to set up and maintain a working environment free of DNA contamination.

1. Wear gloves throughout the procedure. Use only fresh PCR-grade reagents (H_2O) and lab ware (tips and tubes).
2. Physically separate the workspaces used for PCR setup and post-PCR processing or non-PCR operations. Decontaminate your PCR workspace and lab ware (pipettor barrels, tube racks, etc.) before each new 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.
3. Close all tubes containing PCR products once you are finished adding or removing volumes. Before discarding any lab ware (tips or tubes) containing PCR products or other DNA, treat with 10% bleach.
4. Do not remove the PCR Array plate from its protective sealed bag until immediately ready to use. Do not leave lab ware (tubes and tip boxes) exposed to the air for long periods of time.
5. Do not open any previously run and stored PCR Array plate. Removing the thin-wall 8-cap strips or the adhesive film from PCR Arrays releases PCR product DNA into the air where it will contaminate and confound the results of future real-time PCR experiments.

A. RNA Preparation 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 consistent, high-quality RNA from every experimental sample. Therefore, the sample handling and RNA isolation procedures are critical to the success of the experiment. Residual traces of proteins, salts or other contaminants will either degrade the RNA or decrease the efficiency of (if not block completely) the enzyme activities necessary for optimal reverse transcription and real-time PCR performance.

1. Recommended RNA Preparation Methods:

High quality total RNA for your real-time PCR experiment must be prepared using one of the following methods, each specific for your biological sample:

a. Cultured Cells:

Use SABiosciences's RT² qPCR-Grade RNA Isolation Kit (Catalog # PA-001) or the Qiagen RNeasy® Mini Kit (Catalog # 74104).

You must perform the recommended on-column DNase treatment step.

b. Tissue Samples:

- i. First, extract RNA from the tissue using the TRIzol® protocol (Invitrogen, Catalog # 15596-026). Be sure to use a sufficient amount of TRIzol® reagent. During homogenization, add a volume of reagent at least ten times greater than the tissue volume.
- ii. Then after the ethanol precipitation step, further clean up the RNA using SABiosciences's RT² qPCR-Grade RNA Isolation Kit (Catalog # PA-001) or the Qiagen RNeasy® Mini Kit (Catalog # 74104). You must perform the recommended on-column DNase treatment step.

c. Whole Blood Samples:

- i. Before RNA preparation, red blood cells (RBC) must be removed from whole blood samples using a density gradient centrifugation medium (for example, Lymphoprep, Greiner Bio-One, Catalog # 1031966).
- ii. The white blood cell fraction is then used for RNA isolation with SABiosciences's RT² qPCR-Grade RNA Isolation Kit (Catalog # PA-001) or the Qiagen RNeasy® Mini Kit (Catalog # 74104). You must perform the recommended on-column DNase treatment step.
- iii. Alternatively, the PAXgene Blood RNA Kit (Qiagen, Catalog # 762164) can also be used to prepare total RNA from whole blood samples.

d. Total RNA Isolated Using a Phenol-Based Method:

If you have already prepared total RNA from any biological source material using a phenol-based method (such as TRIzol®, RNAZol, etc.), you must clean up the RNA with SABiosciences's RT² qPCR-Grade RNA Isolation Kit (Catalog # PA-001) or the Qiagen RNeasy® Mini Kit (Catalog # 74104).

You must perform the recommended on-column DNase treatment step.

e. For Other Biological Samples:

Refer to the existing literature to find isolation protocols for high-quality RNA from other biological samples or contact one of our Technical Support representatives.

For best results from the PCR Array, all RNA samples should be suspended in the RNase-free water provided with the RNA Isolation kit. DO NOT use DEPC-treated water!

2. RNA Quality Control:

For best results from the PCR Array, all RNA samples should also demonstrate consistent quality according to the following criteria:

a. RNA Concentration and Purity by UV Spectrophotometry

NOTE: Prepare dilutions and measure absorbance in 10 mM Tris, pH 8.0 buffer. The spectral properties of nucleic acids are highly dependent on pH.

- i) A₂₆₀:A₂₃₀ ratio should be greater than 1.7.
- ii) A₂₆₀:A₂₈₀ ratio should be greater than 2.0.
- iii) Concentration by A₂₆₀ should be greater than 4 µg / ml total RNA

b. Ribosomal RNA band integrity

Electrophorese a fraction of each RNA sample on a denaturing agarose gel or on an Agilent BioAnalyzer using an RNA 6000 Nano LabChip® and verify that there is a sharp distinction at the small side of both the 18S and 28S ribosomal RNA (rRNA) bands or peaks. Any smearing or shoulder to the rRNA bands or peaks indicates that degradation has occurred in the RNA sample.

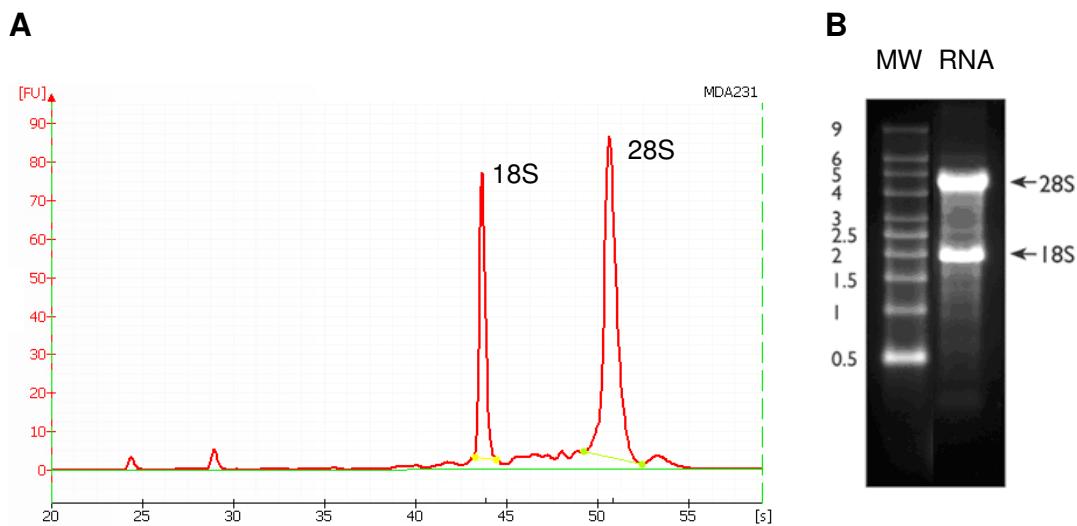


Figure 3: Good Ribosomal RNA Band Integrity Is Important for Optimal PCR Array Results.

Panel A displays an Agilent BioAnalyzer electropherogram of a high-quality total RNA preparation showing sharp peaks without shoulders (especially to the left of each peak) for the 18S and 28S ribosomal RNA (left to right). Panel B, right-hand lane, displays an analysis of the same high-quality total RNA preparation by agarose gel electrophoresis demonstrating sharp bands (especially at the bottom of each band) for the 28S and 18S ribosomal RNA (top to bottom).

Because some contaminants are difficult to detect by simply looking at RNA integrity and can be missed by UV spectrophotometry, it is essential to choose the proper RNA isolation method for your biological sample as described above.

c. The RT² RNA QC PCR Array (Optional):

The RT² RNA QC PCR Array and the RT² First Strand Kit (each sold separately) test for a number of RNA quality control parameters including:

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

The RNA QC PCR Arrays are particularly useful for researchers who are unsure of their RNA isolation technique. Follow the recommendations for the use and interpretation of the RT² RNA QC PCR Array found in its User Manual.

3. Genomic DNA Contamination:

Eliminating genomic DNA contamination is essential for obtaining optimal real-time gene expression profiling results using the PCR Array. The Genomic DNA Control in each PCR Array specifically tests for genomic DNA contamination in each sample during each run. A GDC threshold cycle value less than 35 indicates the presence of a detectable amount of genomic DNA contamination that should be addressed.

We highly recommend performing the on-column DNase treatment step in the RT² qPCR-Grade RNA Isolation Kit (PA-001) or the Qiagen RNeasy® Mini Kit (Catalog # 74104) followed by using the RT² First Strand Kit (C-03) to remove any and all residual contamination from your RNA samples.

4. Amount Considerations:

The PCR Array System yields results with as little as 25 ng or as much as 5 µg total RNA per array. However, the optimal amount of starting material depends on the relative abundance of the transcripts of interest. Lower abundance transcripts require more RNA; higher abundance transcripts require less RNA. Greater amounts of input total RNA yield a greater number of positive calls; that is, genes expressed in the linear dynamic range of the method. Lower amounts of input total RNA yield a smaller number of positive calls and increase false negative calls.

The use of the RT² First Strand Kit (C-03) maximizes the number of positive calls at low amounts (25 ng) of total RNA over other sources of reverse transcriptase and first strand synthesis kits. For successful results and maximum positive call rates, we recommend that first time users try starting with anywhere from 0.5 µg to 1.0 µg of total RNA. It is also important to use a consistent amount of total RNA for all samples in a single experiment to be characterized and compared.

B. RT² First Strand Kit (C-03)

NOTE: The use of SABiosciences's RT² First Strand Kit (Cat. No. C-03) is critical for detecting the Reverse Transcription Controls (RTC, Wells H7-H9) and for obtaining the best results from the PCR Array. For more information on the importance of this kit, refer to the notes found on Pages 10 and 14.

NOTE: RNA samples must meet the standards of integrity and purity from protein, organics, and genomic DNA contamination described on the previous two pages.

1. Genomic DNA Elimination Mixture:

- For each RNA sample, combine the following in a sterile PCR tube:

Total RNA	25.0 ng to 5.0 µg
GE (5X gDNA Elimination Buffer)	2.0 µl
RNase-free H ₂ O to a final volume of ...	10.0 µl

NOTE: Use the same amount of total RNA in this reaction for every sample. First time users are recommended to start with 0.5 or 1.0 µg of total RNA for 96-well plate formats or with 0.2 to 0.5 µg of total RNA for 384-well plate formats. Lower amounts of total RNA than 100 ng will dramatically increase the false negative rate of the PCR Array method.

- Mix the contents gently with a pipettor followed by brief centrifugation.
- Incubate at 42 °C for 5 min.
- Chill on ice immediately for at least one minute.

2. Prepare the RT Cocktail:

RT Cocktail	1 reaction	2 reactions	4 reactions
BC3 (5X RT Buffer 3)	4 µl	8 µl	16 µl
P2 (Primer & External Control Mix)	1 µl	2 µl	4 µl
RE3 (RT Enzyme Mix 3)	2 µl	4 µl	8 µl
RNase-free H ₂ O	3 µl	6 µl	12 µl
Final Volume	10 µl	20 µl	40 µl

3. First Strand cDNA Synthesis Reaction:

- Add 10 µl of RT Cocktail to each 10-µl Genomic DNA Elimination Mixture.
- Mix well but gently with a pipettor.
- Incubate at 42 °C for exactly 15 min and then immediately stop the reaction by heating at 95 °C for 5 minutes.
- Add 91 µl of ddH₂O to each 20-µl of cDNA synthesis reaction. Mix well.
- Hold the finished First Strand cDNA Synthesis Reaction on ice until the next step or store overnight at -20 °C.

4. RNA Quality Control Check (Optional):

If desired, proceed to characterize a small aliquot (6 µl) of the diluted cDNA template on the correct species-specific and instrument-specific RT² RNA QC PCR Array following the instructions provided in its User Manual. Save the remainder at -20 °C.

C. Performing Real-Time PCR:

NOTE: The use of SABiosciences's RT² qPCR Master Mixes is critical for obtaining the most accurate results from the PCR Array. Be sure to use the correct master mix for your instrument before continuing with this protocol (See Pages 8 and 10).

NOTE: An incorrectly chosen PCR Array plate format will not properly fit into your real-time PCR instrument, and its use will damage the instrument. Be sure you have the correct PCR Array format for your instrument before continuing with this protocol (See Page 7).

NOTE: The accuracy and precision of your pipetting determine the consistency of your results. Be sure that all of your micro-pipettors are calibrated before beginning this procedure. Also, make sure to not introduce any bubbles into the wells of the PCR Array.

NOTE: If unsure of your RNA quality or isolation technique, examine the quality of your RNA before this step using SABiosciences's species- and instrument-specific RT² RNA QC PCR Arrays (See Page 14).

1. Experimental Cocktail Preparation:

Mix the following components in a 5-ml tube or a multi-channel reservoir:			
Plate Format: Plate Format Designation:	96-well A, C, D, & F	384-well E & G	384HT E & G
2X SABiosciences RT ² qPCR Master Mix	1275 µl	550 µl	2000 µl
Diluted First Strand cDNA Synthesis Reaction	102 µl	102 µl	102 µl
ddH ₂ O	1173 µl	448 µl	1898 µl
Total Volume	2550 µl	1100 µl	4000 µl

NOTE: This recipe provides an excess volume of ONLY ~ 140 µl. Very carefully add the cocktail to the PCR Array precisely as described below to insure that each well receives the required volume.

NOTE: If you did not perform RNA quality control with a RT² RNA QC PCR Array, save the remaining 9 µl of the cDNA synthesis reaction at -20 °C in case you need to perform one later for troubleshooting purposes.

2. Loading the 96-Well PCR Array Formats A, C, D, or F:

- CAREFULLY remove the PCR Array from its sealed bag.
- Add 25 µl of the Experimental Cocktail to each well of the PCR Array, preferably from a reservoir with an eight-channel pipettor (or a twelve-channel pipettor but only using eight tips).

NOTE: Change pipet tips following each addition to avoid any cross-contamination between the wells or reactions.

- Skip the next page and proceed to "Performing Real-Time PCR Detection" below.

3. Loading the 384-Well PCR Array Formats E or G:

NOTE: Each 384-well plate characterizes four samples in separate sets of 96-wells staggered from one another by only one well. The spacing between the tips of standard multi-channel pipettors will allow you to properly skip rows or columns when adding each sample. Be sure to load each sample into the correct set of wells. Use Figure 4 as a guide.

- a. **CAREFULLY** remove the PCR Array from its sealed bag.
 - b. Load sample cocktails to appropriate wells of the PCR Array, preferably from a reservoir with an eight- channel pipettor (or a twelve-channel pipettor but only using eight tips), using the provided 384EZLoad™ Covers (Catalog #PA-384) and the figure below as a guide.
- a. Place Cover #1 (white) on the plate. Add 10 µL of Sample 1 cocktail to the open wells. (Odd number wells of rows A, C, E, G, I, K, M & O). Remove & discard the cover.
 - b. Place Cover #2 (yellow) on the plate. Add 10 µL of Sample 2 cocktail to the open wells. (Even number wells of rows A, C, E, G, I, K, M & O). Remove & discard the cover.
 - c. Place Cover #3 (black) on the plate. Add 10 µL of Sample 3 cocktail to the open wells. (Odd number wells of rows B, D, F, H, J, L, N & P). Remove & discard the cover.
 - d. Place Cover #4 (red) on the plate. Add 10 µL of Sample 4 cocktail to the open wells. (Even number wells of rows B, D, F, H, J, L, N & P). Remove & discard the cover.

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	2	3	2	3	2	3	2	3	2	3	2	3	2	3	2	3	2	3	2	3	2	3	2	3
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
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24

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
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24

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
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24

Sample #4

	1	2	3	4	5	6	
--	---	---	---	---	---	---	--

4. Loading the PCR Array 384HT

- a. **CAREFULLY** remove the PCR Array from its sealed bag.
- b. Add 10 µl of the Experimental Cocktail to each well of the PCR Array, preferably from a reservoir with an eight-channel pipettor (or a twelve-channel pipettor but only using eight tips).

NOTE: *Change pipet tips following each addition to avoid any cross-contamination between the wells or reactions.*

5. Performing Real-Time PCR Detection:

NOTE: *Be sure to follow the manufacturer's instructions for the proper operation and maintenance of your real-time instrument.*

- a. **CAREFULLY** but tightly seal the PCR Array with the optical thin-wall 8-cap strips (Formats A and D) or with the optical adhesive film (Formats C, E, F, and G).

NOTE: *Be sure that no bubbles appear in any of the wells of the PCR Array. To remove bubbles, tap the plate gently on the bench top or centrifuge the plate briefly.*

- b. Place the plate on ice while setting up the PCR cycling program below.
- c. Place one plate in your real-time thermal cycler. If recommended by your instrument's user manual, use a compression pad with the optical film-sealed plate formats.

NOTE: *PCR Arrays containing experimental cocktail may be stored at -20 °C wrapped in aluminum foil for up to one week until ready to run.*

- d. Enter and run the appropriate program for your real-time instrument (below). If prompted by your instrument 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*

Use **Program #1**, a two-step cycling program, for all of the following instrumentation:

All ABI Instruments (7000, 7300, 7500, 7700 and 7900HT)
 BioRad iCycler®, MyiQ cycler, and iQ5 real-time PCR detection systems
 All Stratagene Instruments (Mx3000p, Mx3005p, and Mx4000p)
 Eppendorf Mastercycler® ep realplex and Roche LightCycler® 480

Cycles	Duration	Temperature
1	10 minutes ¹	95 °C
40	15 seconds 1 minute ²	95 °C 60 °C

Use **Program #2**, a three-step cycling program, for all other instruments:

For example, the: BioRad CFX96
BioRad Opticon, Opticon 2, and Chromo 4 (MJ Research)

Cycles	Duration	Temperature
1	10 minutes ¹	95 °C
40	15 seconds	95 °C
	30 to 40 seconds ^{2,3}	55 °C
	30 seconds	72 °C

¹ The 10-minute step at 95 °C is required to activate the HotStart DNA polymerase.

² Detect and record SYBR® Green fluorescence from every well during the annealing step of each cycle.

³ Different instruments need different lengths of time to detect the fluorescent signal. Choose the appropriate time for the annealing step (55 °C) for your instrument.

- e. Calculate the threshold cycle (C_t) for each well using the instrument's software.
 - i. We highly recommend manually setting the Baseline and Threshold Values.
 - ii. To define the Baseline, use the Linear View of the amplification plots and set the instrument to use the readings from cycle number two (2) through two (2) cycle values before the earliest visible amplification, usually around cycle number ten (10) but no more than 15.
 - iii. To define the Threshold Value, use the Log View of the amplification plots and place it above the background signal but within the lower one-third to lower one-half of the linear phase of the amplification plot.
 - iv. **IMPORTANT:** Ensure that the thresholds are the same across all 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 quality has been adequately controlled, the cycling program has been executed properly, and the thresholds have been defined correctly, then the value of C_t^{PPC} should be 20 ± 2 across all of your arrays or samples. If not, see the Troubleshooting and FAQ section.
 - v. Export the resulting threshold cycle values for all wells to a blank Excel spreadsheet for use with our Data Analysis Template Excel file.

5. Recommended Quality Control:

Dissociation (Melting) Curve:

Run a melting curve program immediately after the above cycling program, and generate a first derivative dissociation curve for each well in the entire plate using your instrument's software. No more than one peak should appear in each reaction at temperatures greater than 80 °C. 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).

If you decide not to obtain the dissociation curve immediately, save the plates wrapped in aluminum foil at – 20 °C as is, in case you need to perform this operation at a later point in time for troubleshooting purposes. When ready, simply warm the plate to room temperature, place it into your real-time instrument, and run the melting program described above.

NOTE: *Be sure to visually inspect the plate after the run for any signs of evaporation from any of the wells. If evaporation is observed, make a note of which wells so that you may qualify your data analysis appropriately.*

NOTE: *DO NOT open any previously run and stored PCR Array plate. Removing the thin-wall 8-cap strips or the adhesive film from PCR Arrays releases PCR product DNA into the air where it will contaminate and confound the results of future real-time PCR experiments. See also the Note on “Preparing a Workspace Free of DNA Contamination”.*

D. Data Analysis: $\Delta\Delta C_t$ Method

NOTE: PCR Array Data Analysis Web Portal

Access our free PCR Array Data Analysis Web Portal from the following address:
<http://www.SABiosciences.com/pcrarraydataanalysis.php>

The PCR Array Data Analysis Web Portal automatically performs the following calculations and interpretation of the control wells upon including threshold cycle data from a real-time instrument. The PCR Array Data Analysis Web Portal presents the results in a tabular format, a scatter plot, a three-dimensional profile, and a volcano plot (when replicates are included).

1. 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.
2. Examine the threshold cycle values of the control wells.
 - a. Genomic DNA Control (GDC):
 - i. Calculate C_t^{GDC} .
 - ii. If the value is greater than 35, then the level of genomic DNA contamination is too low to affect gene expression profiling results. No action is needed.
 - iii. If the value is less than 35, then genomic DNA contamination is evident. See the Troubleshooting and FAQ section.
 - b. Reverse Transcription Control (RTC):
Any impurities in your RNA sample that affect the reverse transcription of the RT² First Strand Kit's built-in external RNA control also affect the reverse transcription of your messages of interest.
 - i. Calculate $\Delta C_t = \text{AVG } C_t^{\text{RTC}} - \text{AVG } C_t^{\text{PPC}}$.
 - ii. If this value is less than 5, then no inhibition is apparent.
 - iii. If this value is greater than 5, then evidence of impurities that inhibited the reverse transcription phase of the procedure is evident. See the Troubleshooting and FAQ section.

c. Positive PCR Control (PPC):

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

- i. The average C_t^{PPC} value should be 20 ± 2 on each PCR Array and should not vary by more than two cycles between PCR Arrays being compared.
- ii. Larger differences in average C_t^{PPC} values between samples indicate the presence of different amounts of PCR amplification inhibitors in each sample and that all of the RNA samples require further purification.
- iii. An average value of C_t^{PPC} that is consistently greater than 22 for all of your samples may indicate a problem with the cycling conditions or may simply be indicative of the relative sensitivity of your instrument. See the Troubleshooting and FAQ section.

3. Calculate the ΔC_t for each pathway-focused gene in each plate.

$$\Delta C_t = C_t^{\text{GOI}} - C_t^{\text{AVG HKG}}$$

NOTE: Choosing the right normalization factor

The expression level of the housekeeping genes chosen for normalization in the $\Delta\Delta C_t$ method must not be influenced by your experimental conditions. If one or more such genes have been previously identified by independent means and if the PCR Array reproduces those results, use the average of their C_t values in the equation above. If an appropriate housekeeping gene has not been previously identified, use the average C_t value of all housekeeping genes. Or, simply use zero (0) in the place of the average of HK genes' C_t for each group to be compared, and rely on the consistency in the quantity and quality of your original input total RNA across your groups to effectively normalize your results.

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

5. Calculate the $\Delta\Delta C_t$ for each gene across two PCR Arrays (or groups).

$$\Delta\Delta C_t = \Delta C_t (\text{group 2}) - \Delta C_t (\text{group 1})$$

Where group 1 is the control and group 2 is the experimental

6. Calculate the fold-change for each gene from group 1 to group 2 as $2^{-\Delta\Delta C_t}$.

OPTIONAL: If the fold-change is greater than 1, then the result may be reported as a fold up-regulation. If the fold-change is less than 1, then the negative inverse of the result may be reported as a fold down-regulation. The fold-change ratios may also be reported as is.

NOTE: Detailed Mathematical Explanation of $\Delta\Delta C_t$ Data Analysis Method

Due to the inverse proportional relationship between the threshold cycle (C_t) 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:

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

To normalize the expression level of a gene of interest (GOI) to a housekeeping gene (HKG), the expression levels of the two genes are divided:

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

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:

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

The complete calculation is as follows:

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

VI. Troubleshooting and FAQs

A. Troubleshooting:

1. Removal of Genomic DNA Contamination:

You must perform the on-column DNase treatment step included in the protocol of SABiosciences's RT² qPCR-Grade RNA Isolation Kit (PA-001) or Qiagen's RNeasy® Mini Kit (Catalog # 74104). You must also then use SABiosciences's RT² First Strand Kit (C-03) with its genomic DNA elimination step.

If the genomic DNA contamination proves difficult to remove, fold-changes in gene expression may still be obtained. However, it will then be very important to validate any results for individual genes by a separate more rigorous real-time PCR analysis that includes a "minus RT" control.

Apparent genomic DNA contamination may also indicate evidence of more general DNA contamination of other reagents, tips, and tubes. See the Note about Preparing a Workspace Free of DNA Contamination at the beginning of the protocol in this User Manual. The No Template Control (NTC) in the RT² RNA QC PCR Array provides a sense of how well your technique minimizes the introduction of general DNA contamination into your assay system.

2. Improving Poor Reverse Transcription Efficiency:

Double-check the A260:A280 and A260:A230 ratios of your RNA samples and be sure to perform the dilutions for spectrophotometry in RNase-free Tris pH 8.0 buffer. If necessary, re-purify your RNA samples with a spin-column based clean up method, such as SABiosciences's RT² qPCR-Grade RNA Isolation Kit (PA-001).

3. Improving Poor PCR Amplification Efficiency:

Different instruments have different levels of sensitivity. If an average C_t^{PPC} value of 20 ± 2 is difficult to obtain for your instrument, the observed average C_t^{PPC} value should be acceptable as long as it does not vary by more than two cycles between PCR Arrays being compared.

Be sure that the initial heat activation step at 95 °C had been 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 this User Manual. Also, double check the quality of your RNA as described in "Evidence of Poor Reverse Transcription Efficiency" above.

B. Frequently Asked Questions:

1. Will pipetting error affect the PCR Array results?

The passive reference dyes in the master mixes, such as ROX and Fluorescein, are used by the real-time PCR systems to normalize variation from well to well. Therefore, these systems tolerate volume variations caused by pipetting error and evaporation.

2. How can I prevent the evaporation of reaction volume from the wells?

Be sure to carefully and completely seal the PCR Array with the optical thin-wall 8-cap strips or the optical adhesive film before placing it into your thermal cycler. Also, be sure to use a compression pad with the plate formats using the optical film seal (Formats C, E, F and G) as directed by the manufacturer of your real-time PCR instrument.

3. How reliable are the results from the RT² Profiler PCR Array?

Assuming the use of good, consistent experimental technique, real-time PCR methods such as the PCR Array provide very reproducible results. To insure the reliability of your results and to reliably detect smaller fold changes in gene expression from the PCR Array, the performance of replicate determinations (such as biological triplicates) is highly recommended. The Data Analysis Template available from our website for the PCR Array uses your replicate PCR Array data to calculate t-test p values and to generate a "Volcano Plot" illustrating the statistically significant fold-changes in gene expression.

If you have additional questions, please check our website (www.SABiosciences.com) for a more complete listing of Frequently Asked Questions (FAQs), or call our Technical Support Representatives at 1-888-503-3187 or 301-682-9200.

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Appendix: Modified Protocol for Housekeeping Gene PCR Arrays

B. First Strand cDNA Synthesis

Perform a first strand cDNA synthesis reaction for each sample to be characterized on the array including one sample representing your biological or experimental control.

C. Perform Real-Time PCR:

1. Experimental Cocktail Preparation:

Mix the following components in a 1-ml tube or a multi-channel reservoir:	
2X SABiosciences RT ² qPCR Master Mix	337.5 µl
Diluted first strand cDNA synthesis reaction	27 µl
ddH ₂ O	310.5 µl
Total volume	675 µl

2. Adding samples to PCR Array:

NOTE: Organize your 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 four samples can be characterized in duplicate on a single array or duplicate determinations may be made on two separate arrays for larger numbers of samples.

Samples	Housekeeping Genes											
	1	2	3	4	5	6	7	8	9	10	11	12
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 5: Layout of the Housekeeping Genes PCR Arrays.

D. Data Analysis by the ΔC_t Method

- For each sample, average the duplicate determinations of the C_t values from each sample for each housekeeping gene.

2. For each housekeeping gene, calculate the ΔC_t , or, in other words, the difference between the gene's C_t value in each experimental sample and the same gene's C_t value in the control sample.
3. Choose the housekeeping genes with the smallest ΔC_t value across the samples of interest to normalize the results of your future RT-PCR experiments for input total RNA loading. More than one housekeeping gene may be chosen for your analyses. Simply monitor the expression of all of these housekeeping genes, and use their average C_t value as the normalization factor for each sample.

RT² Profiler™ PCR Array User Manual

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