March 2011

RT^2 miRNA PCR Array Handbook

For miRNA expression profiling

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- Nucleic acid and protein assays
- microRNA research and RNAi
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Product Use Limitations

RT^2 miRNA PCR Arrays are intended for molecular biology applications. This product is 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.

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I. Background and Introduction

MicroRNA (miRNA), first discovered in *C. elegans* in early 1990s, are a class of naturally occurring small RNA molecules, generally 17 to 30 nucleotides long, processed from much larger stem-loop structures (pri-miRNA and pre-miRNA). Mature miRNA is recognized by and guides the RNA-Induced Silencing Complex (RISC) to specific messenger RNA transcript sequences, causing either translation repression or mRNA degradation. This phenomenon adds even more complexity to the regulation of gene expression. Each miRNA can regulate multiple mRNA targets, and a single target mRNA may be regulated by multiple miRNA sequences. Researchers are currently correlating miRNA expression profiles to biological phenotypes in attempts to better understand miRNA-based regulation of gene or mRNA expression.

The RT^2 miRNA PCR Array is designed to analyze miRNA expression using real-time, reverse transcription PCR, or qRT-PCR, the most sensitive and reliable method for nucleic acid expression analysis currently available. The arrays take advantage of a SYBR® Green real-time PCR detection system designed for high performance to analyze the expression of many mature miRNA sequences simultaneously. Each 96- or 384-well array plate contains a panel of primer sets for a thoroughly researched set of 88 or 376 relevant, pathway- or disease-focused miRNA, plus four housekeeping assays and two RNA and PCR quality controls. The Genome V2.0 RT^2 miRNA PCR Arrays contain 528 (Human) or 440 (Mouse) miRNA sequences as annotated by the Sanger miRBase Release 14. (See Figure 2 for the layout of a typical PCR Array.) The specificity and single mismatch discrimination, sensitivity and dynamic range, and reproducibility of the miRNA expression analysis is guaranteed when using the complete miRNA PCR Array System.

To complete the miRNA PCR Array procedure, reverse transcribe the experimental small RNA samples into first strand cDNA, the template for the PCR, using the RT^2 miRNA First Strand Kit. (See Figure 1 for an overview of the miRNA PCR Array procedure.) Then, mix the template with one of our instrument-specific and ready-to-use RT^2 SYBR Green qPCR Mastermixes. Aliquot the mixture into each well of the same plate containing the pre-dispensed miRNA-specific assays. Perform PCR, and finally determine relative expression with your real-time instrument and the $\Delta\Delta C_t$ method. The simplicity of the RT^2 miRNA PCR Arrays makes them accessible for routine use in every research laboratory.

Benefits of the RT^2 miRNA PCR Arrays:

- **Genome-Wide Coverage:** Arrays represent either a large panel of the most relevant miRNA in the human and mouse genomes (as annotated by the Sanger miRBase Release 14) or pathway- or disease-focused panels

- **Reliable, Sensitive, Specific:** Simple real-time PCR method with optimized primer design and reaction formulation improves discrimination and sensitivity.

- **Designed for Routine Use:** Brings miRNA expression profiling to almost any lab with a real-time PCR instrument.

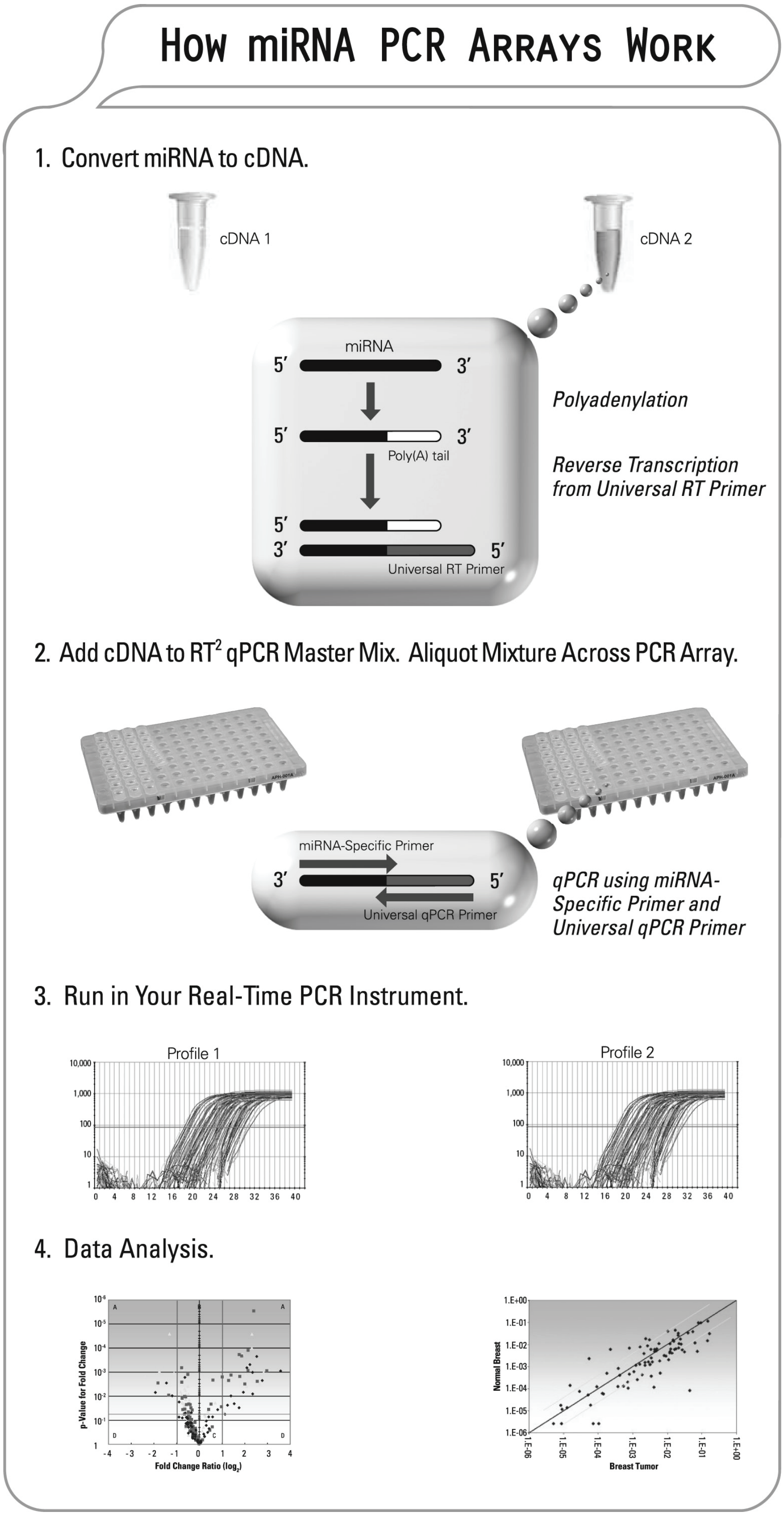

Figure 1: Overview of the RT^2 miRNA PCR Array procedure.

	1	2	3	4	5	6	7	8	9	10	11	12
A	m01	m02	m03	m04	m05	m06	m07	m08	m09	m10	m11	m12
B	m13	m14	m15	m16	m17	m18	m19	m20	m21	m22	m23	m24
C	m25	m26	m27	m28	m29	m30	m31	m32	m33	m34	m35	m36
D	m37	m38	m39	m40	m41	m42	m43	m44	m45	m46	m47	m48
E	m49	m50	m51	m52	m53	m54	m55	m56	m57	m58	m59	m60
F	m61	m62	m63	m64	m65	m66	m67	m68	m69	m70	m71	m72
G	m73	m74	m75	m76	m77	m78	m79	m80	m81	m82	m83	m84
H	m85	m86	m87	m88	HK1	HK2	HK3	HK4	RTC	RTC	PPC	PPC

Figure 2A: 96-Well Pathway-Focused and Genome RT^2 miRNA PCR Array Layout

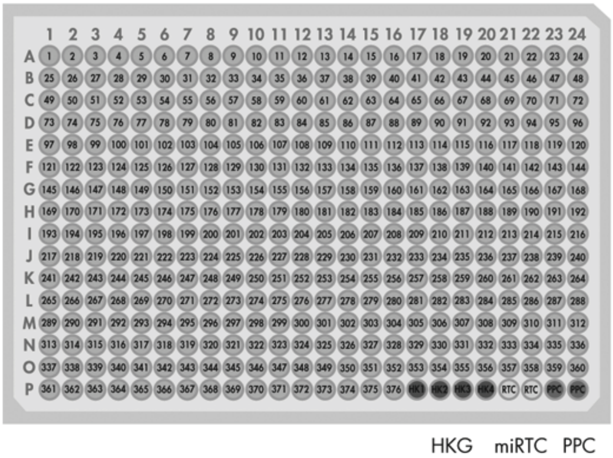

Figure 2B: 384-Well Genome (MAH-3100/MAM-3100) RT^2 miRNA PCR Array Layout

↓ 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
A	A B	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
B	C D	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
C	E F	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
D	G H	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
E	I J	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
F	K L	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
G	M N	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
H	O P	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12

Figure 2C: 384-Well Pathway-Focused and Genome V2.0 RT^2 miRNA PCR Array Layout

Figure 2: Layout of the RT^2 miRNA PCR Arrays

Layout of the RT^2 miRNA PCR Arrays

- MicroRNA Sequence Specific Assays contained within each RT^2 miRNA PCR Array include one universal primer and one gene-specific primer for each miRNA sequence.

- RT^2 miRNA PCR Arrays are provided to assess the expression of 88 pathway-focused miRNAs or the complete miRNA Genome (528 Human miRNA sequences / 440 Mouse miRNA sequences) as annotated by the Sanger miRBase Release 14.

Control Elements
The product specification sheet included with your cataloged RT^2 miRNA PCR Array contains a list of the pathway-focused and housekeeping genes on the array.

- **Housekeeping Assay Panel**

The small nuclear RNA housekeeping assay panel (HK1 – HK4), including:
SNORD 48, 47, and 44 as well as U6, normalizes qPCR Array data.

  - Wells H5 to H8 of the 96-Well & 384-well (4x96 format) RT^2 miRNA PCR Arrays
  - Wells P17 to P20 of the 384-well RT^2 miRNA PCR Arrays (MAH-3100/MAM-3100)

- **Reverse Transcription Control (RTC)**

Duplicate Reverse Transcription Controls (RTC) test the efficiency of the RT^2 miRNA First Strand Kit (cat. no. 331401) reaction with a primer set detecting the template synthesized from the kit's built-in miRNA External RNA Control (ERC).

  - Wells H9 and H10 of the 96-Well & 384-well (4x96 format) RT^2 miRNA PCR Arrays
  - Wells P21 and P22 of the 384-well RT^2 miRNA PCR Arrays

- **Positive PCR Control (PPC)**

Duplicate Positive PCR Controls (PPC) to test the efficiency of the polymerase chain reaction itself using a pre-dispensed artificial DNA sequence and the primer set that detects it.

  - Wells H11 and H12 of the 96-Well & 384-well (4x96 format) RT^2 miRNA PCR Arrays
  - Wells P23 and P24 of the 384-well RT^2 miRNA PCR Arrays

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

The 384-well format of the pathway-focused RT^2 miRNA PCR Arrays includes four replicates of the same 96-well format, in which each two-by-two set of wells (gray-labeled wells 1 – 4 in Figure 2C) contains the same primer set represented by the corresponding 96-well designations (black-labeled).

Custom RT^2 miRNA PCR Arrays have your specified layout, and the product information enclosed with the array reiterates your layout and the genes included.

II. Materials Provided:

NOTE: *The format of the RT^2 miRNA 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.*

Format	For Real-Time Instruments	Plate
A	ABI "**standard**" blocks: 5700, 7000, 7300, 7500, 7700, 7900HT (96-block) Bio-Rad: iCycler®, iQ™5, MyiQ™, Chromo4™ (MJ Research) Eppendorf: MasterCycler® ep RealPlex Stratagene: Mx3005P®, Mx3000P® Takara: TP-800	96-well
C	ABI: 7500 **FAST** block, 7900HT **FAST** block, StepOnePlus™	96-well
D	Bio-Rad: CFX96™, Opticon® and Opticon 2 (MJ Research) Stratagene: Mx4000®	96-well
E	ABI: 7900HT (384-well block) Bio-Rad: CFX384™	384-well
F	Roche: LightCycler® 480 96-well block	96-well
G	Roche: LightCycler 480 384-well block	384-well

A. 96-Well Formatted Arrays

1. **Pathway-Focused Arrays** (Cat. Nos. MA#-001, MA#-102, & MA#-103 in formats A, C, D, & F)
   These RT^2 miRNA PCR Arrays each represent 88 miRNA sequences, and are shipped in sets of two (2), twelve (12), or twenty-four (24) identical arrays.

2. **Genome V2.0 Array Set** (Cat. Nos. MAH-200 & MAM-200 in formats A, C, D, & F)
   These six-array (Human) and five-array (Mouse) sets represent a combined total of 528 (Human) / 440 (Mouse) miRNA sequences, and are shipped as a duplicate set of the six (Human) or five (Mouse) different arrays.

3. **Genome Array Set** (Cat. Nos. MAH-100 & MAM-100 in formats A, C, D, & F)
   These four-array sets represent a combined total of 352 miRNA sequences, and are shipped as a duplicate set of the four different arrays.

B. 384-Well Formatted Arrays:

1. **Pathway-Focused Arrays** (Cat. Nos. MA#-001, MA#-102, & MA#-103 in formats E & G)
   These RT^2 miRNA PCR Arrays each represent four replicate sets of 88 miRNA sequences, and are shipped as a set of four (4) identical arrays.

2. **Genome V2.0 Array Set** (Cat. Nos. MAH-200 & MAM-200 in formats E & G)
   These RT^2 miRNA PCR Arrays each represent 528 (Human) or 440 (Mouse) miRNA sequences, and are shipped in sets of six (6) different arrays for use with 4 samples.
3. **Genome Array Set** (Cat. Nos. MAH-3100 & MAM-3100 in formats E & G)
   These RT^2 miRNA PCR Arrays each represent 376 miRNA sequences, and are shipped in sets of two (2), twelve (12), or twenty-four (24) identical arrays.

C. Numbers of Plates and Optical Cap Strips or Optical Adhesive Film:
Each RT^2 miRNA PCR Array shipment includes the array plates 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 plate.

D. 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.

III. Additional Materials Required:

A. miRNeasy Mini Kit (Cat. No. 217004)

B. RT^2 miRNA First Strand Kit (Cat. No. 331401)
MANDATORY for a Complete and Successful Experiment
The universal primer in each assay is only compatible with the sequence added to the cDNA template by the primer in this kit.

C. RT^2 SYBR Green qPCR Mastermix
MANDATORY for a Complete and Successful Experiment
Be sure to pick the correct one for the instrumentation in your laboratory.

1. 96-Well RT^2 miRNA PCR Arrays

RT^2 SYBR Green ROX™ qPCR Mastermix: Specifically designed for:
All ABI and Stratagene Instrumentation
Eppendorf Mastercycler® ep *realplex* Instruments with ROX filter set

Catalog Number	Size
330520	For 2 RT^2 miRNA PCR Arrays
330522	For 12 RT^2 miRNA PCR Arrays
330523	For 24 RT^2 miRNA PCR Arrays

RT^2 SYBR Green Fluor qPCR Mastermix:
Specifically designed for BioRad iCycler, MyiQ, and iQ5 Instrumentation

Catalog Number	Size
330510	For 2 RT^2 miRNA PCR Arrays
330512	For 12 RT^2 miRNA PCR Arrays
330513	For 24 RT^2 miRNA PCR Arrays

RT^2 SYBR Green qPCR Mastermix:
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
330500	For 2 RT^2 miRNA PCR Arrays
330502	For 12 RT^2 miRNA PCR Arrays
330503	For 24 RT^2 miRNA PCR Arrays

2. **384-Well PCR Arrays: 96 x 4 Format**

RT^2 SYBR Green ROX qPCR Mastermix: Specifically designed for:
All ABI and Stratagene Instrumentation

Catalog Number	**Size**
330521	For 4 384-well RT^2 miRNA PCR Arrays

RT^2 SYBR Green Fluor qPCR Mastermix:
Specifically designed for instrumentation that requires the Fluorescein Reference Dye

Catalog Number	**Size**
330511	For 4 384-well RT^2 miRNA PCR Arrays

RT^2 SYBR Green qPCR Mastermix:
Specifically designed for instrumentation that does not require a reference dye:
BioRad CFX384
Roche LightCycler 480 System

Catalog Number	**Size**
330501	For 4 384-well RT^2 miRNA PCR Arrays

3. **RT^2 miRNA PCR Arrays (MAH-3100/MAM-3100)**

The RT^2 miRNA PCR Arrays (MAH-3100/MAM-3100) available in 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 330500, 330510, or 330520; 2 x 330502, 330512, or 330522; 2 x 330503, 330513, or 330523).

D. Equipment:

NOTE: *The RT^2 miRNA PCR Arrays are* ***NOT*** *recommended for the Cepheid SmartCycler®, the Roche LightCycler 2.0, or the Corbett Research/QIAGEN® Rotor-Gene®.*

1. For recommendations on specific real-time instrumentation (thermal cyclers with fluorescent detection), see the list of master mixes and plate formats above.
2. Calibrated Multi-Channel Pipettor
3. RNase / DNase-free pipette tips and tubes

IV. 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 Reverse Transcription Kit Considerations

The performance of our RT^2 miRNA PCR Arrays is only guaranteed with our RT^2 SYBR Green qPCR Mastermixes and the RT^2 miRNA First Strand Kit. Therefore, the use of the complete miRNA qPCR Array System is absolutely essential for obtaining any real-time PCR profiling results.

The chemically-modified and tightly controlled HotStart enzyme and other proprietary chemical components in our RT^2 SYBR Green qPCR Mastermixes provide more accurate SYBR Green results by preventing the amplification of primer dimers and other non-specific products. They also help insure high amplification efficiencies. When we test other sources of enzymes with our RT^2 miRNA 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 universal primer in every assay of the RT^2 miRNA PCR Arrays is specific only for the unique and proprietary sequence incorporated into the cDNA by the universal RT primer in the RT^2 miRNA First Strand Kit. The RT^2 miRNA PCR Arrays cannot detect cDNA generated from miRNA using other sources of miRNA first strand synthesis or miRNA reverse transcription kits. Similarly, cDNA generated from the RT^2 miRNA First Strand Kit cannot be used with other sources of real-time qPCR assays for miRNA.

The Reverse Transcription Controls (RTC) on the RT^2 miRNA PCR Array can only be evaluated using the RT^2 miRNA First Strand Kit and its built-in miRNA External RNA Control (ERC). These RTC assays do not yield results when used with other sources of reverse transcriptases or first strand synthesis kits.

The RT^2 miRNA First Strand Kit also includes a proprietary buffer to preferentially reverse transcribe miRNA into cDNA. The buffer components and the magnesium concentration in our RT^2 miRNA First Strand Kit are also more compatible with our PCR master mixes than other enzymes or kits providing the RT^2 miRNA PCR Arrays with maximum levels of sensitivity with ng amounts of small 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_20) 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 <u>ESSENTIAL</u> for obtaining good real-time PCR results.

The most important prerequisite for any miRNA expression analysis experiment is consistent, high-quality small 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.

Although other real-time PCR-based miRNA detection methods call for the use of total RNA that contains miRNA as the starting input material, we highly recommend starting with small RNA (< 200 nucleotides) to reduce background noise for optimal sensitivity.

1. **Recommended RNA Preparation Methods:**

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

Cultured Cells OR Tissue Samples: Use the miRNeasy Mini Kit (cat. no. 217004).

a. Extract RNA from the tissue using the QIAzol® Lysis Reagent included in the miRNeasy Mini Kit. Be sure to use a sufficient amount of QIAzol Lysis Reagent. During homogenization, add a volume of reagent at least ten times greater than the tissue volume.
b. Then, further clean up and enrich the small RNA following the protocol provided in the miRNeasy Mini Handbook.

Whole Blood Samples:
Remove red blood cells (RBC) from whole blood samples using a density gradient centrifugation medium (for example, Lymphoprep, Greiner Bio-One, Catalog # 1031966). Isolate small RNA from the white blood cell fraction as described for cultured cells above.

Previously Isolated Total RNA:
If you have already prepared total RNA from <u>any</u> biological source material, be sure that it used a phenol-based method (such as TRIzol®, RNAzol, etc.). Then, enrich for small RNA using the miRNeasy Mini Kit (cat. no. 217004).

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, or alternatively in RNase-free 10 mM Tris buffer pH 8.0. 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) A_{260}:A_{230} ratio should be greater than 1.7.
ii) A_{260}:A_{280} ratio should be greater than 2.0.
iii) Concentration by A_{260} should be greater than 10 μg / ml small RNA

b. RNA Quality & Integrity

Characterize ~ 10 ng of the small RNA on an Agilent® Bioanalyzer using an RNA 6000 Nano LabChip®. The RNA should contain a single sharp peak at a low molecular weight with no smearing and no additional peaks at higher molecular weights.

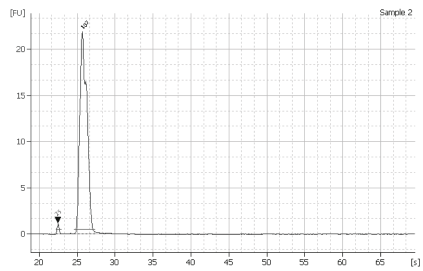

Figure 3: Good Small RNA Band Integrity Is Important for Best Results from the miRNA qPCR Array. Displayed is an Agilent Bioanalyzer electropherogram of a high-quality small RNA preparation showing a sharp low molecular weight peak without a shoulder.

3. Amount Considerations:

The miRNA PCR Array System yields relative gene expression profiles with as little as 50 ng or as much as 400 ng of small RNA per array. However, the optimal amount of starting material depends upon the relative abundance of the sequences of interest. Lower abundance sequences require more RNA; higher abundance sequences require less RNA. Greater amounts of input RNA yield a greater number of positive calls; that is, sequences expressed in the linear dynamic range of the method. Lower amounts of input RNA yield a smaller number of positive calls and increase false negative calls.

The use of the RT^2 miRNA First Strand Kit maximizes the number of positive calls at low amounts of RNA over other sources of miRNA reverse transcriptase and first strand synthesis kits. For successful results and maximum positive call rates, we recommend that first time users try starting with 100 ng of small RNA. It is also important to use a consistent amount of RNA for each sample to be characterized and compared.

B. RT^2 miRNA First Strand Kit

NOTE: *RNA samples must meet the standards of integrity and purity from protein and organics contamination described on the previous two pages.*
NOTE: *Thaw 5x miRNA RT Buffer 2 on ice, briefly vortex the tube to mix the contents well, and centrifuge to the bottom of the tube before each use.*

1. Recommended Amounts of RNA Starting Material
   a. Although the reaction accommodates 50 to 400 ng of small RNA (0.5 to 4.0 µg of total RNA), first time users are recommended to start with following amounts of RNA depending on the cataloged product used:

Arrays or Sets	Catalog #	Formats	RNA
384-well Pathway-Focused	MAH-001, MAM-001 MAH-102, MAM-102 MAH-103, MAM-103	E, G	50 ng small (0.5 µg total)
96-well Pathway-Focused	MAH-001, MAM-001 MAH-102, MAM-102 MAH-103, MAM-103	A, C, D, F	100 ng small (1.0 µg total)
384-well Genome	MAH-3100, MAM-3100	E,G	200 ng small (2.0 µg total)
96-well Genome 96-well Genome V2.0	MAH-100, MAM-100 MAH-200, MAM-200	A, C, D, F	400 ng small (4.0 µg total)
384-well Genome V2.0	MAH-200, MAM-200	E,G	300 ng small (3.0 µg total)

   b. Use the same amount of RNA in this reaction for every sample.
   c. Lower amounts of small RNA than 50 ng will increase the false negative rate of the miRNA Array method.
   d. Do not use more than 400 ng of small RNA (4 µg of total RNA) per reaction to avoid expending reagents and inhibiting the reaction.

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

Small RNA	50 ng to 400 ng (or 0.5 – 4 µg total RNA)
miRNA RT Primer & ERC Mix	1.0 µL
5x miRNA RT Buffer 2	2.0 µL
miRNA RT Enzyme Mix	1.0 µL
miRNA Nucleotide Mix	1.0 µL
RNase-free **H_2O** to a final volume of	10.0 µL

3. Mix the contents gently with a pipettor followed by brief centrifugation.

4. Incubate at 37°C for 2 hours.

5. Heat at 95°C for 5 minutes to degrade the RNA & to inactivate the reverse transcriptase.

6. Chill on ice for at least one minute and add 90 µl of RNase-free H_2O to each 10-µl of cDNA synthesis reaction. Mix well.

7. Hold the finished First Strand cDNA Synthesis Reaction on ice until the next step or store overnight at -20°C.

C. Performing Real-Time PCR:

NOTE: *The use of RT^2 SYBR Green qPCR Mastermixes is critical for obtaining the most accurate results from the RT^2 miRNA PCR Arrays. Be sure to use the correct master mix for your instrument before continuing with this protocol (See Pages 10 and 11).*

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 8).*

NOTE: *The accuracy and precision of your pipetting determines 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.*

1. **Experimental Cocktail Preparation:**

For 96-well Arrays, use the following table:

Mix the following components in a 15-ml tube or a multi-channel reservoir:

PCR Arrays & Plate Format:	96-well Pathway-Focused Arrays Catalog Numbers 001, 102, 103 Formats A, C, D, & F	96-well Genome Array Sets MAH-100, MAM-100 Formats A, C, D, & F	96-well Genome Array Sets MAH-200 Formats A, C, D, & F	96-well Genome Array Sets MAM-200 Formats A, C, D, & F
2X RT^2 SYBR Green qPCR Mastermix	1275 µL	5.0 mL	7.5 mL	6.25 mL
Diluted first strand reaction	100 µL	0.1 mL	0.1 mL	0.1 mL
ddH_2O	1175 µL	4.9 mL	7.4 mL	6.15 mL
Total volume	2550 µL	10.0 mL	15.0 mL	12.5 mL

For 384-well Arrays, use the following table:

Mix the following components in a 15-ml tube or a multi-channel reservoir:

PCR Arrays & Plate Format:	384-well Pathway-Focused Arrays Catalog Numbers 001, 102, 103 Formats E & G	384-well Genome Array MAH-3100, MAM-3100 Formats E & G	384-well Genome Array Sets MAH-200 Formats E & G	384-well Genome Array Sets MAM-200 Formats E & G
2X RT^2 SYBR Green qPCR Mastermix	550 µL	2.0 mL	3.0 mL	2.5 mL
Diluted first strand reaction	100 µL	0.1 mL	0.1 mL	0.1 mL
ddH$_2$O	450 µL	1.9 mL	2.9 mL	2.4 mL
Total volume	1100 µL	4.0 mL	6.0 mL	5.0 mL

NOTE: *This recipe provides an excess volume of* ONLY *150 to 600 µL per array plate or array set. Very carefully add the cocktail to the RT^2 miRNA PCR Array precisely as described below to insure that each well receives the required volume.*

2. **Loading the PCR Arrays:**

   a. **CAREFULLY** remove the needed RT^2 miRNA PCR Arrays from their sealed bags:

96-Well Pathway-Focused Arrays & 384-well Genome Arrays: One *plate* per sample
96-Well Genome Arrays: One *set of six plates (Human) or five plates (Mouse)* per sample
384-Well Pathway-Focused Arrays: One plate for *four* samples
384-Well Genome V2.0 Arrays: Six (Human) or Five (Mouse) plates for *four* samples

   b. Add the appropriate volume of the correct Experimental Cocktail to each corresponding well of the PCR Arrays – 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.*

96-Well Pathway-Focused Arrays & 384-well Genome Arrays:
Add 25 µL of the same cocktail to each well of a 96-well array.
Add 10 µL of the same cocktail to each well of a 384-well array.
96-Well Genome Arrays:
Add 25 µL of the same cocktail to each well of all four arrays in the set.
384-Well Pathway-Focused & 384-well Genome V2.0 Arrays:
Add 10 µL each sample's cocktail to the appropriate sets of wells of the array.

NOTE: *Each 384-well pathway-focused 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.*

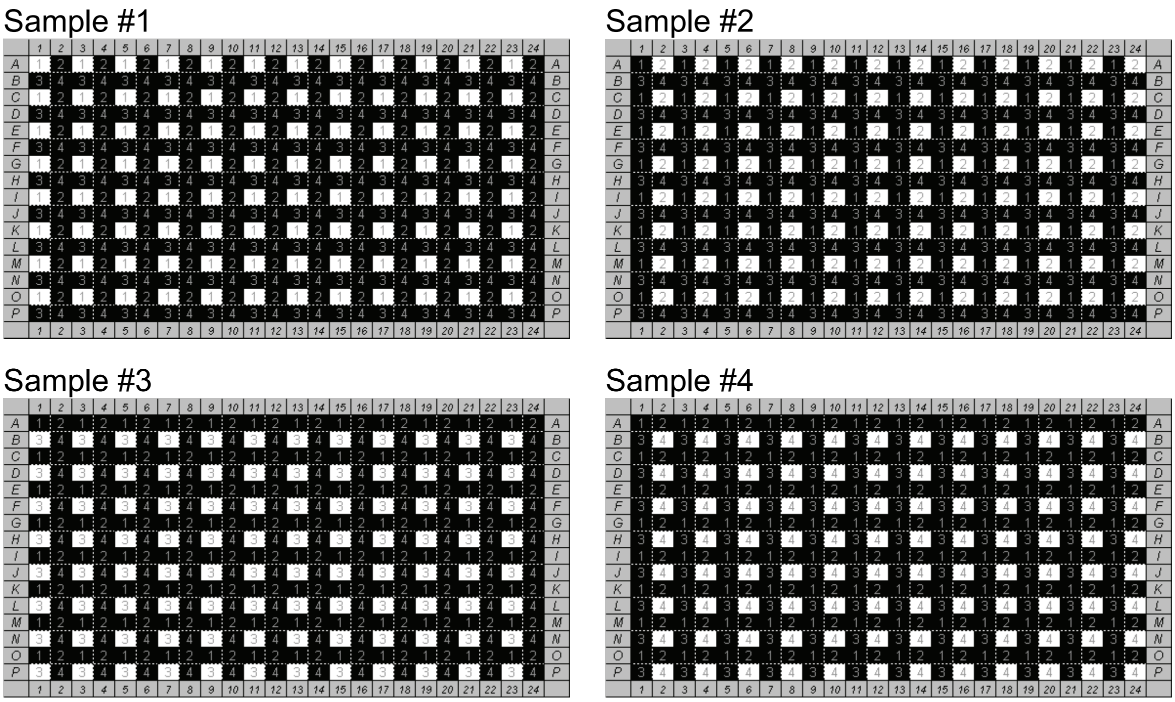

Figure 4: To load a 384-well format PCR Array, add 10 µl of the Experimental Cocktail from each numbered sample into the staggered wells with the same number as indicated in the figure.

3. **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. This recommendation is particularly useful for the set of four 96-well Genome PCR Arrays.*

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: http://www.sabiosciences.com/mirnapcrarrayprotocolfiles.php*

Cycles	Duration	Temperature
1	10 minutes[1]	95°C
40	15 seconds	95°C
	30 to 40 seconds[2,3]	60°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 (60 °C) for your instrument.

e. Calculate the threshold cycle (C_t) for each well using the instrument's software.

   i. We highly recommend letting your instrument automatically define the Baseline values but manually setting the 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 half to one-third 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.

   v. Export the resulting threshold cycle values for all wells to a blank Excel spreadsheet for use with our Data Analysis Template Excel file.

4. **Recommended Quality Control:**

   a. 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 70 °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. Reverse Transcription Control (RTC):

      Any impurities in your RNA sample that affect the reverse transcription of the miRNA External RNA Control (ERC) built-into the RT^2 miRNA First Strand Kit also affect the reverse transcription of your miRNA sequences of interest.

      Calculate ΔC_t = AVG C_t^{RTC} – AVG C_t^{PPC}.
      If this value is less than 5, then no inhibition is apparent.

      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 Guide.

   b. 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 miRNA sequences of interest.

      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.

      Larger differences in average C_t^{PPC} values between samples indicate the presence of different amounts of PCR amplification inhibitors in the different samples and that all of the RNA samples require further purification.

      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 Guide.

3. Calculate the ΔC_t for each pathway-focused gene in each plate.
   $\Delta C_t = C_t^{MOI} - C_t^{AVG\ HKG}$

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 sequences 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 small 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:

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

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:

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

The complete calculation is as follows:

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

E. Appendix:

This additional protocol provides instructions for people who have run MAH-100 or MAM-100 in the past and wanted to extend their study to new MAH-005 & MAH-006 or MAM-005 arrays.

1. Recommended Amounts of RNA Starting Material

Arrays or Sets	Catalog Numbers	Formats	RNA
96-well Array	MAH-005, MAH-006 MAM-105	A, C, D, F	100 ng small (1.0 µg total)

   a. Use the same amount of RNA in this reaction for every sample.
   b. Lower amounts of small RNA than 50 ng will increase the false negative rate of the miRNA Array method.
   c. Do not use more than 400 ng of small RNA (4 µg of total RNA) per reaction to avoid expending reagents and inhibiting the reaction.

2. Continue with Steps **B.2** to **B.7**

3. Experimental Cocktail Preparation for each sample

2x RT^2 SYBR Green qPCR Mastermix	1275	µL
Diluted first strand reaction	100	µL
ddH_2O	1175	µL
Total volume	2550	µL

4. Continue with array loading and real-time PCR detection in **C.2** and **C.3**

V. Troubleshooting and FAQs

A. Troubleshooting:

1. Evidence of Poor Reverse Transcription Efficiency:

The value of (AVG C_t^{RTC} – AVG C_t^{PPC}) is greater than 5.

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 the miRNeasy Mini Kit (cat. no. 217004).

2. Evidence of Poor PCR Amplification Efficiency:

The average C_t^{PPC} value varies by more than two (2) across the PCR Arrays being compared and/or is greater than 22.

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 for “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 PCR 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 RT^2 miRNA PCR Array?

Assuming the use of good, consistent experimental technique, real-time PCR methods such as the PCR Array provide highly 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.

Ordering Information

Product	Contents	Cat. no.
RT^2 miRNA PCR Array	Array of assays for miRNA quantification	Varies

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at www.qiagen.com or can be requested from QIAGEN Technical Services or your local distributor.

Notes

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