miProfile™ miRNA PCR Arrays (384-Well)

For high-throughput profiling of miRNA expression

User Manual

GeneCopoeia, Inc.
9620 Medical Center Drive, #101
Rockville, MD 20850
USA

301-762-0888

support@genecopoeia.com

www.genecopoeia.com

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USER MANUAL

miProfile™ miRNA PCR Array

I. Introduction

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression at the post-transcriptional level. Usually 21-23 nucleotides in length, miRNAs are important modulators in cellular pathways and are highly conserved in eukaryotic organisms. Irregularities in miRNA-regulated gene expression have been found to be associated with cancers, cardiovascular disorders and a variety of other diseases.

The miProfile miRNA PCR Arrays are designed for profiling the expressions of pre-defined or customized sets of miRNAs in various tissues or cells. The resulting differential expressions of profiled miRNAs help researchers to identify those miRNAs that are of biological significance and importance relevant to their research. Each 384-well plate contains up to 360 pairs of PCR primers (forward: miRNA-specific primer; reverse: universal primer), which have been pre-validated and deposited in designated wells. Each plate also has 24 wells that contain different types of controls for monitoring the efficiency of the entire experimental process: from reverse transcription to qPCR reaction.

The All-in-One™ miRNA First-Strand cDNA Synthesis Kits for miRNA qPCR array (QP017, QP018) and qPCR Mix Kits (QP001, QP002, QP004) are the recommended RT-PCR reagents for use with the miProfile miRNA qPCR arrays. These reagents have been optimized to produce high sensitivity, efficiency, and specificity. The All-in-One reverse transcriptase mix contains a novel and optimized blend of polyA polymerase and reverse transcriptase in a buffer that allows high activities and maximal performances of both enzymes. In such reactions, the polyA polymerase adds poly-A tails to mature miRNAs to generate polyA miRNAs. In the same reaction, m-MLV RTase and a unique oligo dT adaptor primer (compatible with the PCR universal reverse primer pre-deposited in the miRNA plates) reverse-transcribe the polyA miRNAs.

The All-in-One qPCR Mix containing SYBR® Green is used to specifically detect the reverse-transcribed miRNA with the miRNA-specific forward primer and PCR universal reverse primer which are pre-deposited in the miRNA plates. Similar reagents from third-party vendors may be compatible for use. However, their uses are not supported.

Using a universal real-time PCR condition, one can easily profile and analyze the miRNA expression in a high-throughput fashion.

Small RNA is recommended as the input RNA to increase the specificity of detection, although use of total RNA can achieve similar results.

Key advantages

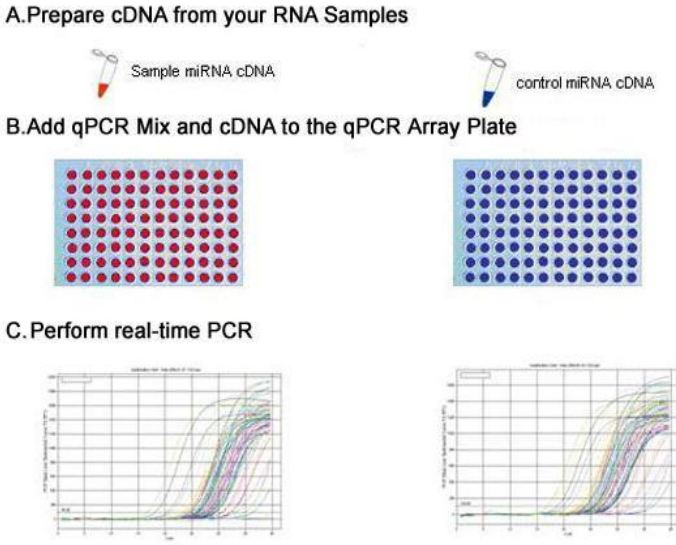
- **Genome-wide coverage, pre-arranged groups, or customized groups**
  Largest genome-wide miRNA coverage
  Cancer-related groups
  Customized miRNA arrays for focused study
- **Robust performance**

  Sensitive – Detect miRNAs from as little as 10 pg of input small RNA or 20 pg of total RNA
  Specific – Capable of distinguishing miRNAs with single nucleotide mismatches. Each primer set has been experimentally validated for specific amplification
  Broad linearity – Allow miRNAs at different expression levels to be detected simultaneously

Reproducible -High reproducibility (R^2> 0.99) for inter-array and intra-array replicates

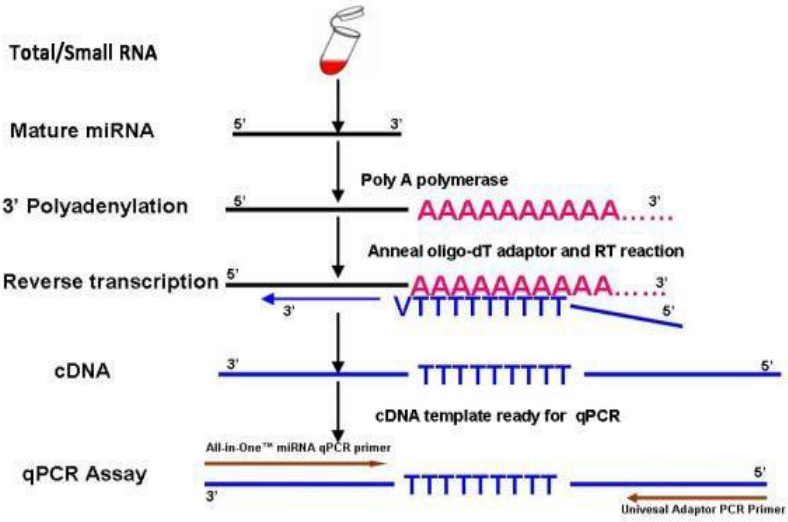
- **Validated miRNA primers**
  Each miRNA primer is designed using a proprietary algorithm and has been experimentally validated

Protocol overview



(A)

qPCR Array Catalog#:		HmiRWG-07								
No.	Accession#	miRNA ID	Homo Brain Assay Data(Sample)			Homo Liver Assay Data(Control)			Fold Change	Ttest
			Ct Value-1	Ct Value-2	Ct Value-3	Ct Value-1	Ct Value-2	Ct Value-3	2^-ΔΔCt	P_Value
1	MIMAT0004982	hsa-miR-939	26.27	26.00	26.32	29.79	30.06	30.28	112.69	1.93E-06
2	MIMAT0004951	hsa-miR-887	27.16	27.15	27.30	30.84	30.78	30.95	98.51	3.97E-06
3	MIMAT0004804	hsa-miR-615-5p	32.75	31.99	32.55	33.93	34.02	33.87	22.32	9.52E-05
4	MIMAT0004696	hsa-miR-323-5p	20.32	20.35	20.82	27.69	27.81	27.59	1154.03	6.04E-06
5	MIMAT0003324	hsa-miR-661	27.08	27.25	27.12	31.49	31.89	31.05	156.93	6.38E-05
6	MIMAT0004983	hsa-miR-940	18.95	19.02	18.89	19.12	18.95	19.02	8.27	1.52E-04
7	MIMAT0003306	hsa-miR-636	20.40	20.39	20.45	24.97	24.78	25.11	182.60	1.49E-06
8	MIMAT0005867	hsa-miR-663b	27.41	27.39	27.42	22.80	22.48	22.59	0.28	1.81E-03
9	MIMAT0002178	hsa-miR-487a	23.93	23.94	24.01	31.83	31.56	31.78	1703.48	9.72E-07
10	MIMAT0003180	hsa-miR-487b	21.42	22.01	21.89	28.77	28.89	28.59	987.94	1.18E-05



(B)

Figure 1. miRNA PCR array experiment work flow (A) and miRNA RT-PCR mechanism (B)

Performance data

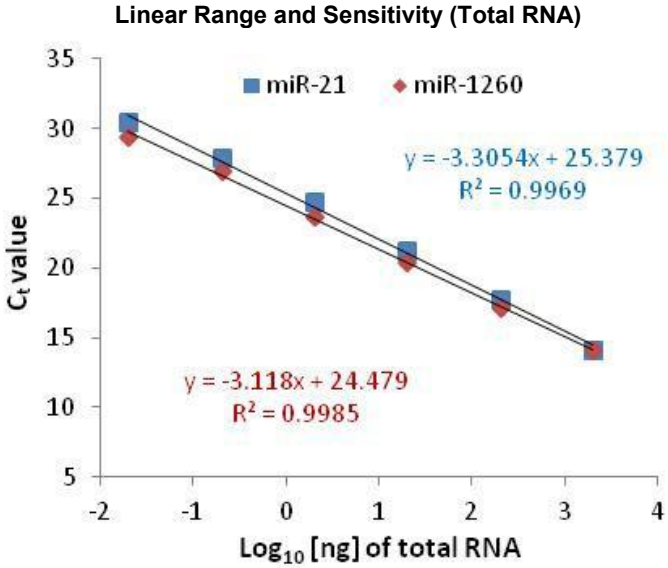


Figure 2. Broad linear range and high sensitivity
Starting with serially diluted amounts of human colon cancer total RNA, miR-21 and miR-1260 were detected using All-in-One™ miRNA qRT-PCR Detection Kit. The resulting Ct values were plotted against the log10 of the amounts of input total RNA. The data demonstrated a broad linear dynamic range from 20pg to 2 µg of input total RNA as well as high sensitivity. This allows the detection of miRNAs at varying expression levels, including low expressers.

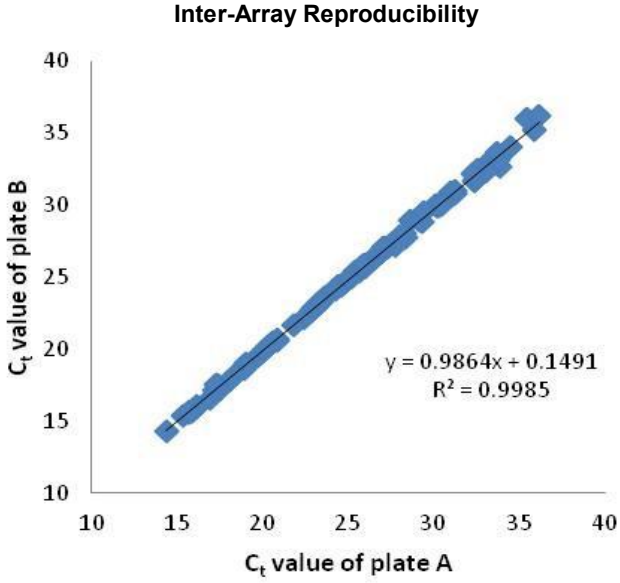
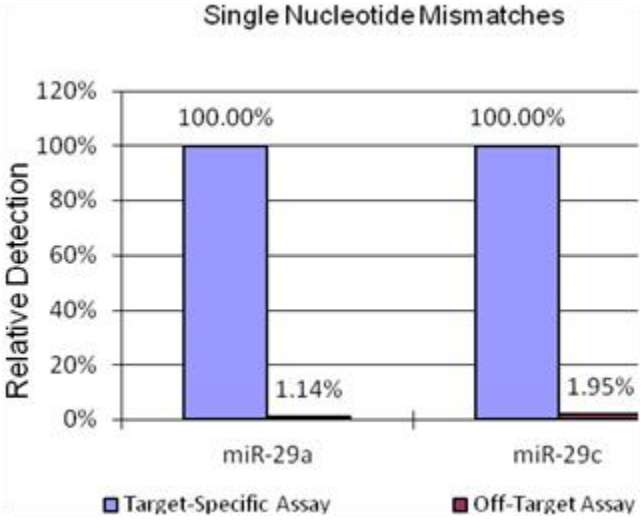


Figure 3. High inter-array reproducibility
Two miProfile PCR array replicates (plate A and B) were analyzed using human total RNA (10-tissue mix) on the Bio-Rad iQ5. The Ct values of the replicate plates were plotted against each other. R2 > 0.99 were observed for high inter-array reproducibility. R2 > 0.99 is also observed for intra-array reproducibility (data not shown).



(A)

hsa-miR-29a	UAGCACCAUCUGAAAUCGGUUA
hsa-miR-29c	UAGCACCAUUUGAAAUCGGUUA

(B)

Figure 4. Specificity of miRNA detection
miRNA miR-29a and miR-29c with one single nucleotide mismatch (B) can be distinguished. Relative detection, defined as a percentage of the perfect match (100% x 2 - ΔCt), was calculated using the Ct values of on-target and off-target assays, which were performed to detect miRNA plasmid DNA templates using All-in-One miRNA qRT-PCR Detection Kits (A).

II. Product, Array Layout and Array Format Options

Catalog miProfile miRNA qPCR arrays

To see the complete list, please visit https://www.genecopoeia.com/product/mirna-qpcr-arrays/

Array format options

GeneCopoeia provides three 384-well qPCR array formats (F, G, and H) suitable for use with the following real-time cyclers.

Important note: Upon receiving, please check to make sure that the correct array format was ordered to ensure the compatibility with your qPCR instrument.

Plate format	Instrument provider	qPCR instrument model
F (384-well)	Applied Biosystems	7900HT (384-well block), ViiA™ 7 (384-well block)
G (384-well)	Bio-Rad Laboratories	Bio-Rad CFX384™ 384-well
H (384-well)	Roche Applied Science	Roche LightCycler® 480 (384-well block)

Catalog Array layout

	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	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
B	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
C	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72
D	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96
E	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120
F	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144
G	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168
H	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192
I	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216
J	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240
K	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255	256	257	258	259	260	261	262	263	264
L	265	266	267	268	269	270	271	272	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288
M	289	290	291	292	293	294	295	296	297	298	299	300	301	302	303	304	305	306	307	308	309	310	311	312
N	313	314	315	316	317	318	319	320	321	322	323	324	325	326	327	328	329	330	331	332	333	334	335	336
O	337	338	339	340	341	342	343	344	345	346	347	348	349	350	351	352	353	354	355	356	357	358	359	360
P	GDC	GDC	GDC	GDC	HK1	HK1	HK2	HK2	HK3	HK3	HK4	HK4	HK5	HK5	HK6	HK6	RT	RT	RT	RT	PCR	PCR	PCR	PCR

Figure 5. Illustration of miProfile miRNA qPCR array layout 384-well plate)

- **miRNA primer pairs:** Wells 1-360 are designated wells for pre-deposited miRNA primer pairs.
- **NC:** Negative controls, which only have the pre-deposited reverse universal primers
- **HK1-6:** Six pre-deposited housekeeping snRNAs (HK1-6) primer pairs, which can be used as endogenous positive controls as well as for array normalization.
- **RT:** Spike-in reverse transcription controls, which can be used to monitor the efficiency of the RT reactions. These pre-deposited primer pairs specifically amplify the cDNA template reversed transcribed from the spike-in exogenous RNA in the sample.
- **PCR:** Positive PCR controls, which are used to verify the PCR efficiency by amplifying the pre-deposited DNA template with its specific pre-deposited primer pairs.

RNA extraction RT-PCR reagents required (sold separately)

Cat. No.	Products	Quantity/set	Shipping and storage condition
QP020	RNAzol® RT RNA Isolation Reagent	50 ml	Shipped at room temperature. Stable for at least two years when stored at room temperature.
QP017 **QP018**	All-in-One miRNA first-strand cDNA synthesis kit For miRNA qPCR array	20 reactions 60 reactions	Shipped with dry ice. Store at –20°C (Stable for at least 12 months).Alternatively, store at –80°C in aliquots. Avoid repeated freezing/ thawing.
QP001 **QP002** **QP004**	All-in-One qPCR mix	200 reactions 600 reactions 4000reactions	Shipped with dry ice. Store at –20°C (Stable for at least 12 months).Alternatively, store at –80°C in aliquots. Avoid repeated freezing/ thawing.

Estimates of number of RT-PCR reactions required for EACH SAMPLE

Array format	Numbers of plates	Numbers of RT reactions	Numbers of PCR reactions
384-well plate	1	2	450
	3	6	1350
	5	10	2250
	6	12	2700
	10	20	4500

Other materials required but not provided

Small/total RNA extraction kit (i.e. RNAzol® RT)
DNase/RNase free tips, PCR reaction tubes, 1.5 ml microcentrifuge tubes
5 ml and 10 ml graduated pipettes, beakers, flasks, and cylinders
10 µl to 1,000 µl adjustable single channel micropipettes with disposable tips
5 µl to 20 µl adjustable multichannel micropipette, disposable tips, and reservoir
qPCR instrument, compatible with miRNA qPCR arrays ordered

III. Preparation

Important notes

1. Before use, remove any condensation that has accumulated on the plate sealing surface and centrifuge plates briefly to collect the contents to the bottom of the plate wells.
2. Strictly follow the standard procedures for PCR to avoid nucleic acid contamination and non-specific amplifications.
3. Read the instructions thoroughly before attempting to perform the procedures.

Estimates of RNA and number of RT-PCR reactions required for EACH SAMPLE

Array format	Number of plates	Total RNA recommended	Number of RT reactions	Number of qPCR reactions
384-well plate	1	2-4 ug	2	450
	3	6-12 ug	6	1350
	5	10-20 ug	10	2250
	6	12-24 ug	12	2700
	10	20-40 ug	20	4500

RNA quantification and quality control

1. Dilute the RNA sample with the RNase-free water and measure the absorbance at 260 nm and 280 nm. A260/280 should be greater than 1.8.
2. Use the formula A260 × dilution × 40 = µg RNA/mL to determine the RNA concentration.
3. Check the RNA integrity by agarose electrophoresis.

IV. Procedure

First-strand cDNA synthesis

Note: High-quality cDNA is a prerequisite for accurate detection of miRNA expression. GeneCopoeia's All-in-One miRNA First-Strand cDNA Synthesis Kit is required for small cDNA synthesis.

1. Thaw the reagents in All-in-One miRNA First-Strand cDNA Synthesis Kit, mix by gently flicking the tube, briefly centrifuge to bring the contents to the bottom of the tubes and then place them on ice.
2. Prepare miRNA polyA polymerase (PAP) and reverse transcriptase (RT) reaction mix. Add the following reagents to the ice-chilled RNase-free reaction tubes to a final total volume of 25 µl.

Component	Volume[a]	Quantity
Small RNA		0.5~1.0 µg[b]
2.5U/µl PolyA Polymerase	1 µl	2.5 U
RTase mix	1 µl	
5×PAP/RT buffer	5 µl	1×
Spike-in RT control	1 µl	
ddH2O(RNase/DNase free)	to final 25µl	

a. cDNA product from a standard miRNA reverse transcription reaction (25 µl) should be enough for 2 plates of 96-Well reactions. Prepare at least 10 standard miRNA reverse transcription reactions for the 19 plates of whole genome miRNA PCR Arrays.
b. To increase the rate of positive detection, an input of 0.5~1.0µg of small RNA is recommended for the standard miRNA reverse transcription reaction (25 µl).

3. Perform reverse transcription reaction:
Mix the prepared reaction mix gently by pipetting up and down. Incubate at 37℃ for 60 minutes. Terminate the reaction by incubating at 85℃ for 5 minutes. After the incubation, dilute the cDNA products 10 times by adding 225µl of sterile water to each RT reaction and use it for the subsequent qPCR reactions. The diluted cDNA can be stored at -20℃ for several weeks.

qPCR reaction

Note: Be sure the miProfile miRNA PCR Array plate is compatible with your qPCR instrument before beginning this protocol.

1. Thaw the reagents of All-in-One miRNA qPCR Mix Kit. Invert the tubes to mix gently but thoroughly. Briefly centrifuge to bring the contents to the bottom of the tubes and then place them on ice. Remove any condensation that has accumulated on the plate sealing surface and centrifuge briefly to collect the contents to the bottom of the plate wells. Carefully remove sealing film before use 384-Well-qPCR.
2. Prepare qPCR solution on ice

Components	1 well	N well[a]
2×All-in-One qPCR Mix	10µl	11µl × N
miRNA cDNA（10 times dilution）	1µl	1.1µl × N
50 X Rox Reference Dye[b]	0.4µl	0.44µl× N
ddH2O		
■ Not using Rox Reference Dye	9 µl	9.9 µl× N
■ Using Rox Reference Dye	8.6 µl	9.5 µl× N
Final Volume	20µl	22µl× N

a. miProfile miRNA PCR Array is used to detect multiple miRNAs simultaneously in the same sample. Ensure sufficient mix is available by preparing enough for the number of reactions to be used with a 10% additional volume for pipetting loss.
b. 50×Rox Reference Dye is added only for qPCR instruments that require ROX for calibration.

3. Mix the qPCR solution thoroughly and centrifuge briefly. Accurately transfer exactly 20 µl reaction mix to each well. Change tips after each transfer to avoid cross-contamination.
4. Tightly seal the qPCR reaction plate with a new sealing film, ensure that the film seals smoothly to prevent refraction of light. Centrifuge briefly to remove bubbles.
5. Run qPCR. The following three-step PCR program is recommended for running qPCR.

Cycles	Steps	Temperature	Duration	Detection
1	Initial denaturation	95℃	10min [a]	No
	Denaturation	95℃	10sec.	No
40	Annealing	60℃[b]	20 sec.	No
	Extension	72℃[c]	10 sec.	Yes

a. The DNA polymerase used in the 2X All-in-One qPCR Mix is a special chemically modified hot-start enzyme. The indicated initial denaturation is sufficient to activate the enzyme.
b. The annealing temperatures of the cross-linked primers in All-in-One qPCR Primer Array are designed and optimized. For comparing the miRNAs with single nucleotide difference, a higher annealing temperature (65℃) might be necessary.
c. The extension time indicated above is suitable for Bio-Rad's iQ5 real-time PCR instrument. Adjust the time duration according to the documentation provided with your instrument.

When using SYBR Green dye to monitor the qPCR reaction, a melting curve analysis should be performed immediately after qPCR cycling.

Temperature range	Heating rate	Constant temperature	Detection
66℃～95℃	0.5℃/unit time	6sec./unit time	Yes

V. Data Analysis

1. Define the baseline

   The baseline is the noise level in early cycles. Each real-time PCR instrument has algorithms to perform the baseline-setting. This may be a fixed number of cycles for all samples or adaptive for each sample, depending on the type of instrument that is being used. If the lowest Ct is less than the upper limit of the baseline setting, then the baseline should be manually adjusted. Use the "Linear View" of the amplification plot to determine the earliest visible amplification, and then set the baseline from cycle 2 to two cycles before the earliest visible amplification. Normally it is between 2 to 10 cycles. Do not use cycles greater than 15.

   Ensure that baseline settings are the same across all PCR runs in the same analysis to allow comparison of results.

2. Set threshold

   Correct placement of the threshold is the next crucial step in data analysis. To adjust the threshold properly, set the threshold value within the exponential phase of all amplification plots when viewed using the logarithmic scale for the y axis. Generally, the expression level of each reference gene should be higher than most other genes.

3. Obtain the Ct or Cp values

   The Ct is defined as the cycle when sample fluorescence exceeds a chosen threshold above background fluorescence. This is also known as the Cp or crossing point.

4. Export the data. Most qPCR instruments provide a function for exporting Ct or Cp values to Excel.

5. Analyze the qPCR results using the ΔΔCT method of relative quantification and interpretation of the control wells.

6. All Ct values reported as greater than 35 or as N/A (not detected) are considered as not detectable.

QC

1. Examined amplification and melting status of each gene using the qPCR instrument software. Each reference gene, RTC and PPC should exhibit only one melting peak per reaction.

2. Examined CT values of the positive PCR control wells (PCR). If the RNA sample is of high quality, the cycling program has been correctly run, and the thresholds have been correctly defined, the value of Ct of PCR should be **20±2** across all arrays or samples.

3. Examined CT values of the positive RT control wells (RT). If the RNA sample is of high quality, the cycling program has been correctly run, and the thresholds have been correctly defined, the value of Ct of RT should be **20±3** across all arrays or samples.

Data analysis

Analyze the qPCR result with GeneCopoeia's online Data Analysis System (free), which is available at http://www.genecopoeia.com/product/qpcr/analyse/

This Data Analysis System uses the $\Delta\Delta C_t$ method to perform fold-change analysis or simple statistical analysis of the expression level (C_t or Cp values) for each gene.

1. Download and read the "Primer Array Date Analysis Operation Guide" before performing analysis.

2. Import the C_t or Cp values into the corresponding data analysis template form (*Sample Data.xls* and *Control Data.xls*). Upload the template form and choose the correct reference and analysis factors. **Note:** The reference factor chosen for qPCR Primer Array for normalization with the $\Delta\Delta C_t$ method must not be influenced by the experimental design. Therefore use one or more factors that have been previously verified experimentally. A single value or an average of the C_t values for the reference factor can be used for normalization.

3. Perform the specified analysis. When a test is repeated at least three times, statistical results (p value) are provided. The analysis results allow genes of interest to be simply and rapidly selected for further study.

VI. Appendix

ΔΔCt data analysis method

$\Delta\Delta C_t$ data analysis, a relative quantitative analysis technique, is the most simple and direct method for gene expression analyses. The method requires stable expression from a reference gene to normalize the variation introduced by each step, including sample collection, RNA isolation, reverse transcription and amplification. Typically housekeeping genes are used as reference genes.

In qPCR, as in any amplification-based technique, the number of amplification products (N) is calculated as follows:

$N = N0 \times (1+E)^{Ct}$

N0: number of template molecules
C_t: threshold cycle
E: amplification efficiency

When the amplification efficiency E is 100%, the number of template molecules in pre-amplification mix is calculated as follows:

$N0 = N \times 2^{-Ct}$

To analyze the change in expression level for the gene of interest in multiple samples using the $\Delta\Delta C_t$ method, the amount of the amplification template from different samples is normalized by dividing the expression level of the gene of interest (x) with the reference factor (r) as follows:

$Nrel = N0x/N0r = N \times 2^{-Ctx} / N \times 2^{-Ctr} = 2^{-(Ctx - Ctr)} = 2^{-\Delta Ct}$

The change in normalized expression levels of the gene of interest (x) between experimental sample (sample 1) and the control sample (sample 2) is as follows:

$Nrel_1/Nrel_2 = 2^{-\Delta Ct1}/2^{-\Delta Ct2} = 2^{-(\Delta Ct1 - \Delta Ct2)} = 2^{-\Delta\Delta Ct}$

The value of $2^{-\Delta\Delta Ct}$ is the change in expression level of the gene of interest between different samples.

VII. Limited Use License and Warranty

Limited use license

Following terms and conditions apply to use of miProfile miRNA qPCR Arrays (the Products). If the terms and conditions are not acceptable, the Product in its entirety must be returned to GeneCopoeia within 5 calendar days. A limited End-User license is granted to the purchaser of the Product. The Product shall be used by the purchaser for internal research purposes only. The Product is expressly not designed, intended, or warranted for use in humans or for therapeutic or diagnostic use. The Product must not be resold, repackaged or modified for resale, or used to manufacture commercial products or deliver information obtained in service without prior written consent from GeneCopoeia. This Product should be used in accordance with the NIH guidelines developed for recombinant DNA and genetic research. Use of any part of the Product constitutes acceptance of the above terms.

Limited warranty

GeneCopoeia warrants that the Product meets the specifications described in the accompanying Product Datasheet. If it is proven to the satisfaction of GeneCopoeia that the Product fails to meet these specifications, GeneCopoeia will replace the Product. In the event a replacement cannot be provided, GeneCopoeia will provide the purchaser with a refund. This limited warranty shall not extend to anyone other than the original purchaser of the Product. Notice of nonconforming products must be made to GeneCopoeia within 30 days of receipt of the Product. GeneCopoeia's liability is expressly limited to replacement of Product or a refund limited to the actual purchase price. GeneCopoeia's liability does not extend to any damages arising from use or improper use of the Product, or losses associated with the use of additional materials or reagents. This limited warranty is the sole and exclusive warranty. GeneCopoeia does not provide any other warranties of any kind, expressed or implied, including the merchantability or fitness of the Product for a particular purpose.

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9620 Medical Center Drive, Suite 101
Rockville, MD 20850
+1 (301) 762-0888
support@genecopoeia.com

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