nrStar™ Human Canonical Conserved miRNA PCR Array

Cat#: AS-NR-005

Instruction Manual   Version 1.0

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I. Introduction

A. Overview

MicroRNAs (miRNAs) are small noncoding RNAs that regulate gene expression by translational inhibition and mRNA destabilization. Currently, thousands of miRNAs have been reported in human. However, only about one-third of the 1881 human miRBase entries are confidently supported as miRNA genes. The canonical conserved miRNAs are those evolutionarily conserved in the vertebrates and generated by the microprocessors in the canonical biogenesis pathway. They tend to be more likely biologically functional and expressed at higher levels than the novel ones with low conservation scores. Most miRNA PCR arrays on the market cover the miRNA subset selection based on the prior expression. Arraystar Canonical Conserved miRNA PCR Array aims at the canonical conserved mature miRNAs that are functionally most significant in gene regulation.
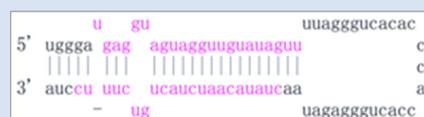
B. Canonical Mature miRNAs

Mammalian miRNAs can be divided into two broad classes, canonical and noncanonical, based on how the pri-miRNAs are processed into mature miRNAs[1, 2]. In the canonical miRNA biogenesis, there are two major pri-miRNA and pre-miRNA processing events. In the nucleus, pri-miRNAs are first processed into pre-miRNAs by the microprocessor complex comprising the RNase III enzyme Drosha, the double-stranded RNA-binding protein DGCR8, and additional proteins. In the second step in the cytoplasm, the pre-miRNAs are processed to form miRNA duplex intermediates of ~22 base pairs by RNase III enzyme Dicer [3]. Of the two strands in the duplex, the one retained at higher level is defined as the guide strand (*Mature miRNA*) and the other as the passenger strand (*Star miRNA*), when the difference between the two strands is > 2-fold. If the two strands are similar, both strands can be co-mature miRNAs[6]. The guide strand interacts with Ago in the RISC to guide mRNA targeting, whereas the passenger strand is degraded as a RISC complex substrate[4]. Some stable star miRNAs are known to be functionally active [5]. The star miRNAs have a higher nucleotide substitution rate than mature ones during metazoan evolution. Intriguingly, a > 46-fold difference exists between the mutation rate at positions 2-8 of the mature sequence and the positions 2-8 of the star sequence.

The gatekeeper of the canonical pathway is the microprocessor, which discriminates between authentic pri-miRNAs and the hairpins in other RNAs. Authentic pri-miRNAs give rise to pre-miRNA hairpins that have a 22 bp stem with a 5' phosphate and a 2-base 3' overhang, which are the ideal substrates for mammalian Dicer. Generally, the functional miRNAs generated from canonical pathway satisfy all of the criteria in the *Box 1*, including the expression of both arms, two nucleotide offsets (the result of two consecutive RNase III cuts), 5' end homogeneity, and appropriate loop length (*Box 1*) [7]. Using these criteria, the numbers of confidently

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Box 1. Consistent criteria for metazoan canonical miRNAs

- 20~26 nt expressed from both arms in a hairpin precursor.
- 2-nt offsets between processed 5p and 3p arms.
- 5'-end homogeneity of expression.
- At least 16-nt complementarity between the two arms.
- The minimum loop size is > 8 nt. The maximum is ~40 nt for single Dicer or no limit for two or more Dicer species.

For non-canonical miRNAs, their maturation can occur in the absence of Drosha or DGCR8 and bypass one or more steps in the canonical biogenesis pathway. Noncanonical miRNAs origins are diverse, including introns, snoRNAs, endogenous shRNAs and tRNAs. Most of them are both poorly conserved and lowly expressed, suggesting a lack of significant regulatory functions [7]. In contrast, canonical miRNAs have much higher expression levels and the tissues/sample specificities being either broad or specific (*Fig. 1*) [9].
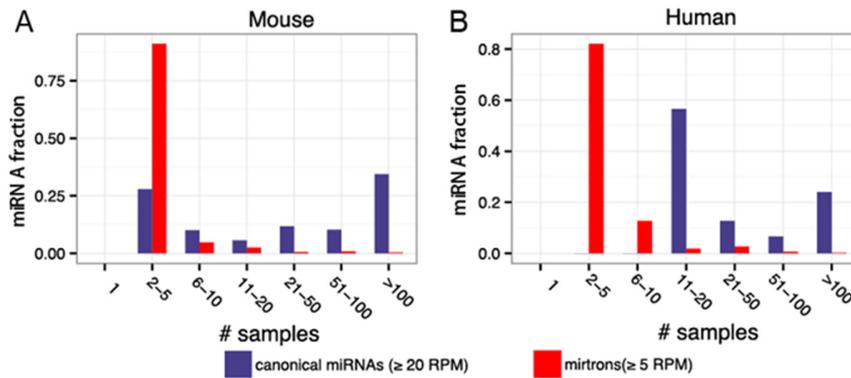

Figure 1. Distribution of expressed canonical miRNAs and mirtrons across sRNA-seq samples. A mirtron ≥ 5 RPM or canonical miRNA ≥ 20 RPM in a single sample is considered as expressed. Canonical miRNAs can display broad or specific distribution in the samples, with a peak distribution in > 100 samples, or more specific distribution (a peak of 2-5 samples in mouse or 11-20 samples in human). Mirtron expression distributions are more restricted in 2-5 samples.

C. Evolution of Canonical miRNAs

miRNAs are widely expressed in all animal and plant cells. Ancient miRNAs show a level of sequence conservation exceeding that of ribosomal DNAs and were rarely lost within most evolutionary lineages. Coupled with the continuous acquisition of miRNAs in all metazoans, miRNAs are one of the most useful classes of characters in phylogenetics to discern the interrelationships among the major lineages and the origin of vertebrate characteristics [10] (*Fig.2*) . For human, about 349 miRNA genes are broadly conserved in vertebrates[11].
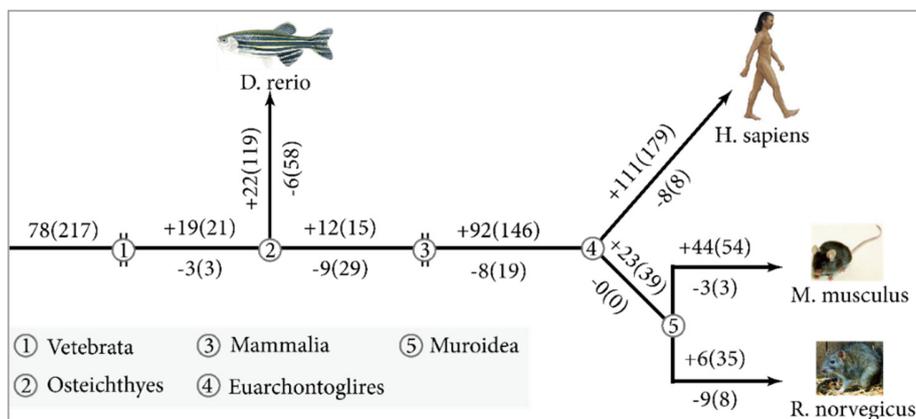
Figure 2. The evolutionary history of canonical miRNA genes across the animal kingdom. For each node (*i.e.* branching point of the evolution milestone), the numbers of miRNA families and genes (in parentheses) gained (+top) and lost (-bottom) are indicated.

Conserved and older miRNA genes tend to be expressed at easily detectable and usually higher levels than the novel ones [*7, 12*]. As newly emerged miRNA genes likely to be deleterious or effectively neutral, many of them were lost rapidly during evolution[*13*]. A miRNA expression level strongly indicates its rate of evolution, i.e., lowly expressed miRNA genes evolved more rapidly [*14*].
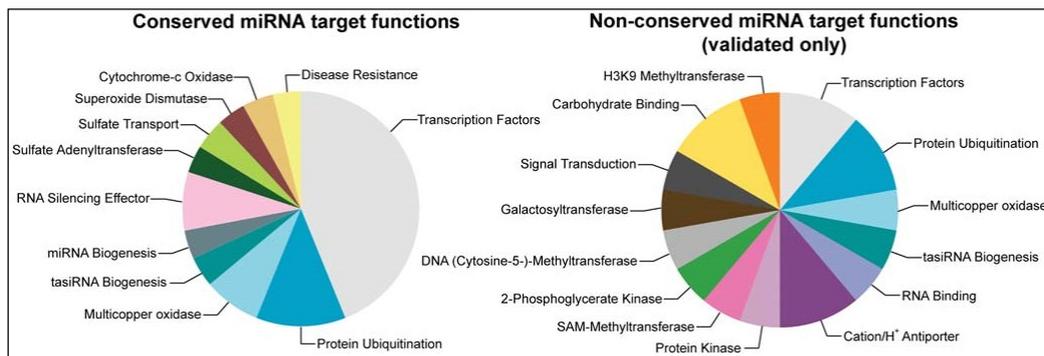

Figure 3. Enrichment of target mRNA functions for conserved (left) and nonconserved (right) miRNAs. Transcription factor functions are highly enriched in the conserved miRNA targets.

The functions of conserved miRNA target mRNAs, less diverse than that of the nonconserved miRNAs, are enriched in transcription factor functions in the bottleneck positions of regulatory networks (*Fig 3*)[*18*]. miRNA evolution is synchronously related with their target gene conservation. For instance, the targets of conserved miR-132 present considerably higher conservation scores than that of non-conserved, human-only miR-941 [*19*]. Also, the conserved cardiac miRNA target genes have maintained highly conserved cardiac functions and the early evolved cardiac miRNA target genes tend to participate in the early stages of heart development [*20*]. Furthermore, conserved miRNAs seemingly co-evolved with specific functions in particular tissues. For example, miR-155 is a well-conserved miRNA with it origin as early as *Ciona intestinalis,* an organism having developed innate and undeveloped adaptive immune system. Since then, miR-155 had been ingrained in the immune regulation and mainly expressed in the myeloid and lymphoid systems in all jawed vertebrates including human [*21*]. In short, conserved miRNAs tend to be expressed at higher levels and have more prominent roles in physiological and pathological processes (*Table 1*).

Table 1. The differences between conserved and nonconserved miRNA genes.

	Conserved miRNA genes	*Nonconserved miRNA genes*
Evolution	Conserved	Rapidly lost
Expression level	Higher	lower
Targets conservation	Higher	Poorer
Targets No.	> 300	< 75
Targets Functions	Mainly transcription factors	Diverse

D. Profiling the Canonical Conserved miRNAs

PCR Arrays are a reliable and accurate tool for analyzing the expression of a selected panel of genes. nrStar™ Human Canonical Conserved miRNA PCR Array aims to profile 372 canonical, evolutionarily conserved, functionally significant, mature miRNAs by qPCR. To eliminate the impact of precusors and isomiRs, rtStar™ First-Strand cDNA Synthesis Kit (3' and 5' adaptor) is recommended for making the miRNA qPCR library. Additionally, rtStar™ PreAMP cDNA Synthesis Kit can be used when the input sample amounts are low (down to 100 pg). With the powerful combination of the PCR array and the kits, a new level of accuracy on the miRNA expression is achieved. The expression data are analyzed and annotated with the wealth of information, such as differential expression, expressional variation of miRNA clusters, memberships in miRNA families, and evolutionary milestone nodes.

To ensure data quality, the panel includes 6 reference sets for small non-coding RNAs for qPCRdata normalization and quantification. cDNA synthesis and PCR efficiency are monitored and evaluated by the RNA Spike-in control, potential genomic DNA contamination by Genomic DNA Control (GDC), and PCR efficiency and inter-plate calibration by Positive PCR control (PPC).
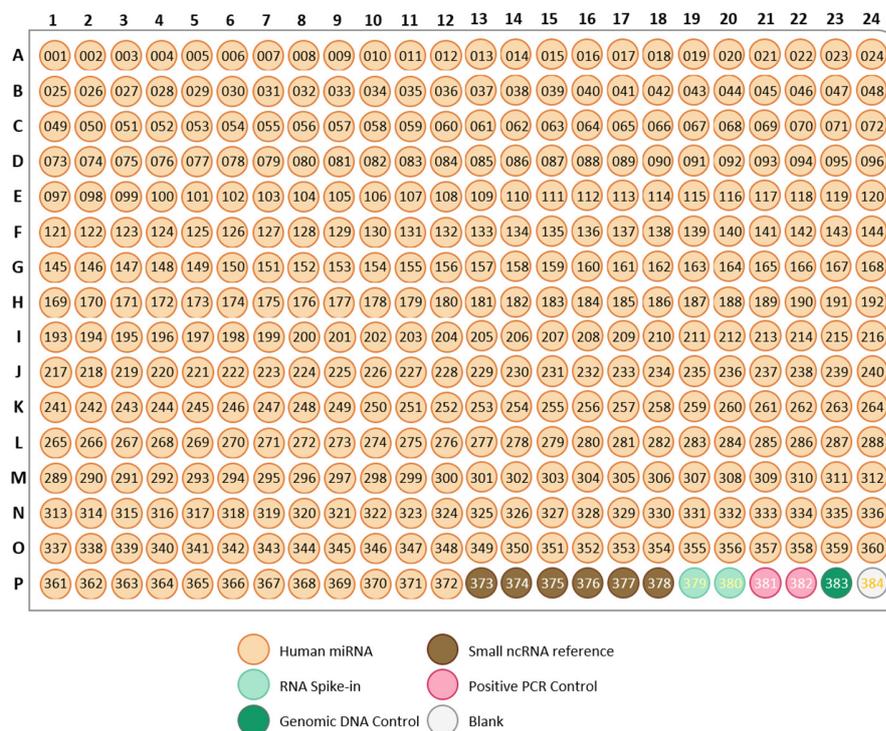
E. Product summary

Figure 4. The array layout of nrStar™ Human Canonical Conserved miRNA PCR Array.

nrStar™ Human Canonical Conserved miRNA PCR Array focuses on the 372 canonical miRNAs conserved during metazoan evolution. Our miRNA profiling system efficiently distinguishes miRNAs from their precursors and isomiRs, with additional power to measure low input miRNAs when using the pre-amplification kit.

■ Kit Contents

Table 2   List of human Canonical Conserved miRNAs and the Controls

A01	let-7a-5p	C17	miR-122-5p	F09	miR-200a-3p	I01	miR-375-3p	K17	miR-508-3p	N09	miR-887-3p
A02	let-7b-5p	C18	miR-125a-5p	F10	miR-200b-3p	I02	miR-376a-3p	K18	miR-510-5p	N10	miR-887-5p
A03	let-7c-5p	C19	miR-125b-5p	F11	miR-200c-3p	I03	miR-376b-3p	K19	miR-511-3p	N11	miR-888-5p
A04	let-7d-5p	C20	miR-126-3p	F12	miR-202-3p	I04	miR-376c-3p	K20	miR-511-5p	N12	miR-889-3p
A05	let-7e-5p	C21	miR-126-5p	F13	miR-202-5p	I05	miR-377-3p	K21	miR-513b-5p	N13	miR-890
A06	let-7f-5p	C22	miR-127-3p	F14	miR-203a-3p	I06	miR-377-5p	K22	miR-514a-3p	N14	miR-892a
A07	let-7g-5p	C23	miR-128-3p	F15	miR-204-5p	I07	miR-378a-3p	K23	miR-514b-5p	N15	miR-892b
A08	let-7i-5p	C24	miR-129-5p	F16	miR-205-5p	I08	miR-379-5p	K24	miR-516b-5p	N16	miR-892c-3p
A09	miR-1-3p	D01	miR-130a-3p	F17	miR-206	I09	miR-380-3p	L01	miR-518b	N17	miR-934
A10	miR-7-5p	D02	miR-130b-3p	F18	miR-208a-3p	I10	miR-380-5p	L02	miR-518c-3p	N18	miR-935
A11	miR-9-5p	D03	miR-132-3p	F19	miR-208b-3p	I11	miR-381-3p	L03	miR-518d-5p	N19	miR-944
A12	miR-10a-5p	D04	miR-133a-3p	F20	miR-210-3p	I12	miR-382-3p	L04	miR-518f-3p	N20	miR-1180-3p
A13	miR-10b-5p	D05	miR-133b	F21	miR-211-5p	I13	miR-382-5p	L05	miR-519c-3p	N21	miR-1185-1-3p
A14	miR-15a-5p	D06	miR-134-5p	F22	miR-212-5p	I14	miR-383-5p	L06	miR-520a-3p	N22	miR-1185-2-3p
A15	miR-15b-5p	D07	miR-135a-5p	F23	miR-214-3p	I15	miR-409-3p	L07	miR-520d-3p	N23	miR-1185-5p
A16	miR-16-5p	D08	miR-135b-5p	F24	miR-215-5p	I16	miR-410-3p	L08	miR-522-3p	N24	miR-1197
A17	miR-17-5p	D09	miR-136-3p	G01	miR-216b-5p	I17	miR-411-5p	L09	miR-523-3p	O01	miR-1247-5p
A18	miR-18a-5p	D10	miR-137-3p	G02	miR-217-5p	I18	miR-412-5p	L10	miR-524-3p	O02	miR-1249-3p
A19	miR-18b-5p	D11	miR-138-5p	G03	miR-218-5p	I19	miR-421	L11	miR-524-5p	O03	miR-1251-5p
A20	miR-19a-3p	D12	miR-139-5p	G04	miR-219a-2-3p	I20	miR-423-3p	L12	miR-525-3p	O04	miR-1264
A21	miR-19b-3p	D13	miR-140-3p	G05	miR-219a-5p	I21	miR-423-5p	L13	miR-525-5p	O05	miR-1271-5p
A22	miR-20a-5p	D14	miR-141-3p	G06	miR-221-3p	I22	miR-424-5p	L14	miR-532-5p	O06	miR-1277-5p
A23	miR-20b-5p	D15	miR-142-3p	G07	miR-222-3p	I23	miR-425-5p	L15	miR-539-3p	O07	miR-1283
A24	miR-21-5p	D16	miR-142-5p	G08	miR-223-3p	I24	miR-429	L16	miR-541-5p	O08	miR-1298-5p
B01	miR-22-3p	D17	miR-143-3p	G09	miR-224-5p	J01	miR-431-5p	L17	miR-542-3p	O09	miR-1301-3p
B02	miR-23a-3p	D18	miR-144-5p	G10	miR-296-5p	J02	miR-432-5p	L18	miR-543	O10	miR-1307-3p
B03	miR-23b-3p	D19	miR-145-5p	G11	miR-299-3p	J03	miR-433-3p	L19	miR-545-5p	O11	miR-1307-5p
B04	miR-24-3p	D20	miR-146a-5p	G12	miR-299-5p	J04	miR-448	L20	miR-551a	O12	miR-1323
B05	miR-25-3p	D21	miR-146b-5p	G13	miR-301a-3p	J05	miR-449a	L21	miR-551b-3p	O13	miR-1343-3p
B06	miR-26a-5p	D22	miR-147b-3p	G14	miR-301b-3p	J06	miR-449b-5p	L22	miR-574-3p	O14	miR-1911-5p
B07	miR-26b-5p	D23	miR-148a-3p	G15	miR-302a-3p	J07	miR-449c-5p	L23	miR-576-5p	O15	miR-1912-3p
B08	miR-27a-3p	D24	miR-148b-3p	G16	miR-302b-3p	J08	miR-450a-5p	L24	miR-577	O16	miR-2114-3p
B09	miR-27b-3p	E01	miR-149-5p	G17	miR-302c-3p	J09	miR-450b-5p	M01	miR-579-5p	O17	miR-2114-5p
B10	miR-28-3p	E02	miR-150-5p	G18	miR-302d-3p	J10	miR-451a	M02	miR-582-3p	O18	miR-2355-3p
B11	miR-29a-3p	E03	miR-151a-3p	G19	miR-323a-3p	J11	miR-452-5p	M03	miR-584-5p	O19	miR-2681-3p
B12	miR-29b-3p	E04	miR-151a-5p	G20	miR-323b-3p	J12	miR-454-3p	M04	miR-589-5p	O20	miR-2681-5p
B13	miR-29c-3p	E05	miR-152-3p	G21	miR-324-5p	J13	miR-455-3p	M05	miR-590-3p	O21	miR-3059-5p
B14	miR-30a-5p	E06	miR-153-3p	G22	miR-326	J14	miR-455-5p	M06	miR-597-3p	O22	miR-3085-3p

B15	miR-30b-5p	E07	miR-154-3p	G23	miR-329-3p	J15	miR-483-5p	M07	miR-598-3p	O23	miR-3140-3p
B16	miR-30c-5p	E08	miR-154-5p	G24	miR-330-3p	J16	miR-485-5p	M08	miR-605-3p	O24	miR-3145-3p
B17	miR-30d-5p	E09	miR-155-5p	H01	miR-331-3p	J17	miR-486-5p	M09	miR-624-5p	P01	miR-3146
B18	miR-30e-5p	E10	miR-181a-5p	H02	miR-335-5p	J18	miR-487a-5p	M10	miR-628-5p	P02	miR-3173-5p
B19	miR-31-5p	E11	miR-181b-5p	H03	miR-337-3p	J19	miR-487b-3p	M11	miR-651-5p	P03	miR-3200-3p
B20	miR-32-5p	E12	miR-181c-5p	H04	miR-338-3p	J20	miR-488-3p	M12	miR-652-3p	P04	miR-3613-5p
B21	miR-33a-5p	E13	miR-181d-5p	H05	miR-339-3p	J21	miR-488-5p	M13	miR-654-3p	P05	miR-3617-5p
B22	miR-33b-5p	E14	miR-182-5p	H06	miR-339-5p	J22	miR-489-3p	M14	miR-656-3p	P06	miR-3934-5p
B23	miR-34a-5p	E15	miR-183-5p	H07	miR-340-5p	J23	miR-490-3p	M15	miR-660-5p	P07	miR-4524a-3p
B24	miR-34b-5p	E16	miR-184	H08	miR-342-3p	J24	miR-491-5p	M16	miR-671-3p	P08	miR-4677-3p
C01	miR-34c-5p	E17	miR-185-5p	H09	miR-345-5p	K01	miR-493-5p	M17	miR-671-5p	P09	miR-4766-3p
C02	miR-92a-3p	E18	miR-186-5p	H10	miR-346	K02	miR-494-3p	M18	miR-675-3p	P10	miR-4766-5p
C03	miR-92b-3p	E19	miR-187-3p	H11	miR-361-3p	K03	miR-495-3p	M19	miR-676-3p	P11	miR-6529-5p
C04	miR-93-5p	E20	miR-188-5p	H12	miR-361-5p	K04	miR-496	M20	miR-708-5p	P12	miR-9851-3p
C05	miR-96-5p	E21	miR-190a-5p	H13	miR-362-5p	K05	miR-497-5p	M21	miR-744-5p	P13	RNU6
C06	miR-98-5p	E22	miR-191-5p	H14	miR-363-3p	K06	miR-498-5p	M22	miR-758-3p	P14	RNU6
C07	miR-99a-5p	E23	miR-192-5p	H15	miR-365a-3p	K07	miR-499a-5p	M23	miR-760	P15	SNORD43
C08	miR-99b-5p	E24	miR-193a-5p	H16	miR-369-3p	K08	miR-500a-3p	M24	miR-769-5p	P16	SNORD43
C09	miR-100-5p	F01	miR-193b-3p	H17	miR-369-5p	K09	miR-500b-5p	N01	miR-770-5p	P17	SNORD95
C10	miR-101-3p	F02	miR-193b-5p	H18	miR-370-3p	K10	miR-501-3p	N02	miR-802	P18	SNORD95
C11	miR-103a-3p	F03	miR-194-5p	H19	miR-371a-5p	K11	miR-502-3p	N03	miR-873-3p	P19	RNA Spike-in
C12	miR-105-5p	F04	miR-195-5p	H20	miR-372-3p	K12	miR-503-5p	N04	miR-873-5p	P20	RNA Spike-in
C13	miR-106a-5p	F05	miR-196a-5p	H21	miR-373-3p	K13	miR-504-5p	N05	miR-874-3p	P21	PPC
C14	miR-106b-3p	F06	miR-196b-5p	H22	miR-374a-3p	K14	miR-506-3p	N06	miR-875-5p	P22	PPC
C15	miR-106b-5p	F07	miR-197-3p	H23	miR-374a-5p	K15	miR-506-5p	N07	miR-876-3p	P23	GDC
C16	miR-107	F08	miR-199a/b-3p	H24	miR-374b-5p	K16	miR-507	N08	miR-885-5p	P24	Blank

■ Description of the control assays

nrStar™ Human Canonical Conserved miRNA PCR Array includes a series of external and internal controls for effective calibration and normalization of sample and qPCR variabilities as described below.

- **Ref** (small ncRNA Control Reference; Internal Controls): Three stably expressed small ncRNA genes RNU6 (Ref 1), SNORD43 (Ref 2), and SNORD95 (Ref 3) can be used as the quantification references for miRNA. Multiple common reference genes are selected by a stringent bioinformatic algorithm, which provides the flexibility of choosing the most valid reference gene(s) for your sample types for the qPCR normalization.
- **RNA Spike-In** (External Control): One External RNA Spike-In Mix is added in the RNA sample prior to the first strand cDNA synthesis. The RNA Spike-In control assay indicates the overall success and the efficiency of the reaction beginning from the cDNA synthesis to the final qPCR. Any problem(s) in these steps will result in a failed or compromised RNA Spike-In outcome. RNA spike-in assay results are compared across samples. Outliers or failed reactions are identified and can be excluded from further data analysis.
- **PPC** (Positive PCR control): 2 replicates of one artificial DNA template along with the PCR primers to indicate the qPCR amplification efficiency. A *Ct* value > 25 is an indication of low qPCR amplification efficiency. More importantly, PPCs are used as the inter-plate calibrator (IPC) between PCR plate runs

to correct for a common *Ct* value, thereby reducing run-to-run variance. Inter-plate calibration (IPC) is automatically calculated with the data analysis software tool avaliable on our website (www.arraystar.com).

- **GDC** (Genomic DNA Control): The PCR primers for an untranscribed genomic region to test genomic DNA contamination in Non-RT control sample or in RNA samples that have not been reverse transcribed. The Ct values should be greater than 35 to indicate no contamination.

■ Shipping and Storage

nrStar™ Human Canonical Conserved miRNA PCR Array is shipped at ambient temperature, on ice, or on dry ice depending on the destination and accompanying products in the package. Store at -20°C upon receipt. The contents are stable for at least 6 months.
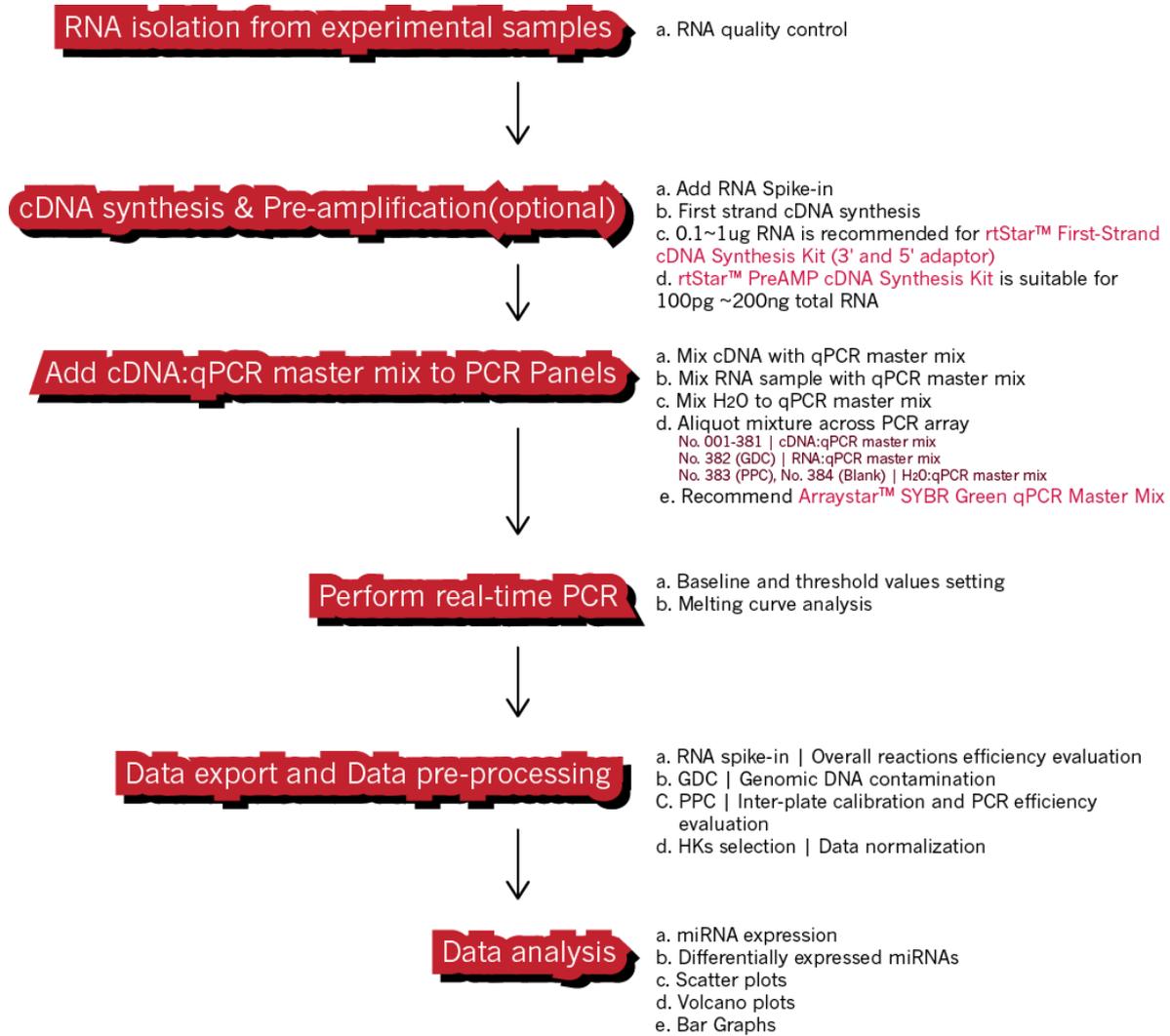
■ Additional Required Equipment

- Thermal cycler
- Real time qPCR instrument, compatible with the standard 384-well plate format

■ Additional Required Reagents

- rtStar™ First-Strand cDNA Synthesis Kit (3' and 5' adaptor) (Cat# AS-FS-003)
- Arraystar SYBR® Green qPCR Master Mix(ROX+) (AS-MR-006-5)
- Nuclease-free PCR-grade water

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F. Protocol overview

RNA isolation from experimental samples
a. RNA quality control

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cDNA synthesis & Pre-amplification(optional)
a. Add RNA Spike-in
b. First strand cDNA synthesis
c. 0.1~1ug RNA is recommended for rtStar™ First-Strand cDNA Synthesis Kit (3' and 5' adaptor)
d. rtStar™ PreAMP cDNA Synthesis Kit is suitable for 100pg ~200ng total RNA

↓

Add cDNA:qPCR master mix to PCR Panels
a. Mix cDNA with qPCR master mix
b. Mix RNA sample with qPCR master mix
c. Mix H2O to qPCR master mix
d. Aliquot mixture across PCR array
   No. 001-381 | cDNA:qPCR master mix
   No. 382 (GDC) | RNA:qPCR master mix
   No. 383 (PPC), No. 384 (Blank) | H2O:qPCR master mix
e. Recommend Arraystar™ SYBR Green qPCR Master Mix

↓

Perform real-time PCR
a. Baseline and threshold values setting
b. Melting curve analysis

↓

Data export and Data pre-processing
a. RNA spike-in | Overall reactions efficiency evaluation
b. GDC | Genomic DNA contamination
C. PPC | Inter-plate calibration and PCR efficiency evaluation
d. HKs selection | Data normalization

↓

Data analysis
a. miRNA expression
b. Differentially expressed miRNAs
c. Scatter plots
d. Volcano plots
e. Bar Graphs

II. Protocol

IMPORTANT: Please read through this entire protocol before your first experiment. Prepare a workspace and use lab wares free of DNA, RNA, and nuclease contamination. Wear gloves while performing this protocol.

A. RNA sample preparation and quality control

For best results, RNA samples must meet the QC standards of integrity and purity from protein, organics, genomic DNA contamination, or excessive RNA degradation. You can measure the RNA concentration and purity by UV spectrophotometry.

- A260:A230 ratio greater than 1.7.
- A260:A280 ratio between 1.8 and 2.0.
- Total RNA concentration greater than 40 ng/μL

If the RNA sample amount is > 2 ng, you should check the RNA integrity with an Agilent® Bioanalyzer. A RIN > 7 is preferred.

Eliminating genomic DNA contamination is essential for accurate qPCR, which is particularly important for genes at low expression levels. The Genomic DNA Control (GDC) is designed to detect potential genomic DNA contamination. A Ct < 35 indicates a positive genomic DNA contamination that may compromise the qPCR results.

B. First-strand cDNA synthesis

Use the same amount of total RNA in this reaction for every sample. High quality cDNA synthesis is vital for the following qPCR performance. Because of the unique properties of miRNA, we highly recommend using rtStar™ First-Strand cDNA Synthesis Kit (3' and 5' adaptor) (Cat# AS-FS-003) for the miRNA first strand cDNA synthesis. The kit efficiently extends the cDNA length from both ends of the miRNA and increases the discriminating power for isomiRs and homologous family members. If the starting RNA is < 200 ng, rtStar™ PreAMP cDNA Synthesis Kit (Cat# AS-FS-006) is recommended for the pre-amplification and increased cDNA synthesis.

■ **Ligate the 3′ Adaptor**

> **NOTE:** Before use,

- Reconstitute RNA Spike-in by adding 80 μL Nuclease-free water.

- Reconstitute Reverse Transcription Primer by adding 60 μL Nuclease-free water.

- For low total RNA amounts at 100 ng, reduce the use of 3' Adaptor by diluting 1:2 in Nuclease-free Water.

1. Add the reagents in a RNase-free 200 μL PCR tube in the same order as in the table below:

Nuclease-free Water	variable
Input RNA	1 to 7.3 µL
3' Adaptor	1 µL
RNA Spike-in	0.5 µL
Total volume	8.8 µL

2. Incubate the above mix at 70°C for 2 min. Transfer tube to ice.

3. Add the following components:

3' Ligation Reaction Buffer	7.2 µL
3' Ligation Enzyme Mix	1 µL
RNase Inhibitor	1 µL
Total volume	18 µL

4. Incubate at 25°C for 1 hr in a thermal cycler.

NOTE: To improve the ligation efficiency for end-methylated RNA such as piRNA, longer incubation at lower temperature (*e.g.*16°C for 18 hrs) may be used. However, some concatemerization products may occur.

■ Hybridize the Reverse Transcription Primer

The excess of the unligated 3' Adaptor is hybridized with Reverse Transcription Primer to prevent adaptor-dimer formation. The double stranded DNA duplexes are not a substrate for T4 RNA Ligase 1 and will not ligate to 5' Adaptor in the 5' Adaptor Ligation step.

NOTE: For low total RNA amounts at 100 ng, reduce the use of Reverse Transcription Primer by diluting 1:2 in Nuclease-free Water

5. Add the following reagents to the tube from Step 4 and mix well:

Nuclease-free Water	1 µl
Reverse Transcription Primer	1 µl
Total volume now should be	20 µl

6. Heat samples at 75°C for 5 min. Transfer to 37°C for 15 min, followed by 25°C for 15 min.

■ **Ligate the 5' Adaptor**

NOTE: For low total RNA amounts at ~100 ng, reduce the use of 5' Adaptor by diluting 1:2 in Nuclease-free Water. Store unused 5' Adaptor at -80°C.

7. Aliquot a 5' Adaptor volume (μL) equal to 1.1× (number of samples) in a Nuclease-free 200 μl PCR tube.

8. Denature the adaptor at 70°C for 2 min and then immediately chill on ice. Use the denatured 5' Adaptor within 30 minutes.

9. Add the following components to the Reverse Transcription Primer and 3' Adaptor ligation hybridization product from Step 6 and mix well:

5´ Adaptor(denatured)	1 μL
10 mM ATP	2.5 μL
5´ Ligation Reaction Buffer	0.5 μL
5´ Ligation Enzyme Mix	1 μL
Total volume	25 μL

10. Incubate at 25°C for 1 hr in a thermal cycler. The product is the two-tailed RNA.

■ **Perform Reverse Transcription**

11. Mix the components in a RNase-free 200 μl PCR tube:

Two-tailed RNA	25 μL
First-Strand Synthesis Reaction Buffer	8 μL
0.1 M DTT	3 μL
2.5 mM dNTP Mix	2 μL
RNase Inhibitor	1 μL
Reverse Transcriptase	1 μL
Total volume	40 μL

12. Incubate at 45°C for 60 min followed by heat inactivation at 70°C for 15 min, then chill on ice.

C. Pre-amplification for Low Input Samples (Optional)

NOTE:

- The regents for cDNA synthesis and pre-amplification are provided in rtStar™ PreAMP cDNA Synthesis Kit.
- To reconstitute the PreAMP Primers, add 50 μL Nuclease-free water to the PreAMP Primers tube and mix well.

- The optimal number of pre-amplification cycles depends on the starting template amount and relative abundance of the genes. 10~12 cycles are recommended for 100 pg of input total RNA.

13. Mix the components in a RNase-free 200 μL PCR tube:

PreAMP PCR SuperMix (2×)	10 μL
PreAMP Primers	1 μL
cDNA	9 μL
Total volume	20 μL

14. Perform PCR amplification in a thermal cycler as follows:

Cycles	Temperature	Time
1	94°C	30 sec
8~12	94°C	15 sec
	62°C	30 sec
	70°C	15 sec
1	70°C	5 min
Hold	4°C	

15. Add following reagents to the tube from Step 14 and mix well:

PreAMP Primer Remover	0.25 μL
PreAMP Primer Remover Buffer	2.5 μL
Nuclease-free Water	2.25 μL
Total volume	25 μL

16. Incubate at 37°C for 30 min followed by heat inactivation at 80°C for 15 min.
17. Immediately add 75 μL nuclease-free water. Mix well.
18. Place on ice prior to real-time PCR, or store overnight at –20 °C.

OPTIONAL: The RNA Spike-in qPCR Primer Mix contains the PCR primers for the RNA Spike-in. Add 100 μL Nuclease-free Water to reconstitute the primers. Use 1 μL primer in a 10 μL qPCR reaction.

D. Perform qPCR for the PCR array

NOTE: The fellow protocol is set up for one sample. For samples, the reagent volumes should be multiplied accordingly.

1. Dilute the cDNA in Nuclease-free water. The dilution factor is empirically 1:80, if the cDNA is produced from a 1.5 µg input RNA amount using the rtStar™ First-Strand cDNA Synthesis Kit (3' and 5' adaptor) (Cat#AS-FS-003). Mix well and spin down. The prepared diluted cDNA will be used in the plate wells for miRNA assays, Internal Control References, and Spike-in External Controls.

2. For GDC Controls, combine 1.5 µL NRT (no RT) sample or 1.5 µL RNA sample, 7.5 µL SYBR Green Master Mix, and 6 µL Nuclease-free water. Mix well and spin down.

3. For Blank Controls, combine 20 µL SYBR Green Master Mix and 20 µL Nuclease-free water. Mix well and spin down.

4. Use Arraystar SYBR Green qPCR Master Mix to prepare the cocktail. There are a total of 384 wells of PCR reactions. Some extra amount is included for experimental handling. Prepare the qPCR mix according to the following table.

SYBR Green Master Mix	2,010 µL
Diluted cDNA template	1,600 µL
ddH$_2$O	390 µL
total volume	4,000 µL

5. Load the cocktail to the 384–well PCR Array plate.

   a. CAREFULLY remove the plate seal from the PCR Array;

   b. Add 10 µL of the cocktail from STEP 4 to each PCR Array plate well (except wells P21-P24);

   c. Add10 µL GDC Mixture aliquot from STEP 2 to the well P23.

   d. Add 10 µL Blank Mixture aliquot from STEP 3 to the wells P21, P22 and P24.

   e. CAREFULLY but tightly seal the PCR Array plate with the optical adhesive cover. Be sure that no bubbles appear in any of the wells. To remove bubbles, tap the plate gently on the bench top or centrifuge the plate briefly.

   f. Keep the plate on ice while setting up the PCR program below.

6. Run the qPCR

Cycles	Temperature	Time
1	95°C	10 min
40	95°C	10 sec
	60°C	1 min
Melt curve analysis		

E. Data pre-processing and data analysis

Pre-process the qPCR run data according to the qPCR instrument manufacturer's instructions. If using baseline and threshold method for Ct calculations, such as with ABI 7900HT, the optimal baseline and threshold by manual setting should be applied consistently across all assays on the plate. This is preferred over the software automatic settings for better reliability and accuracy.

Inspect the melt curve analysis on the post-PCR products to verify the PCR amplification specificity. If the melt curve has multiple peaks or poor peak morphology deviated from that of the known single amplicon product, it may indicate non-specific off-target amplification or primer dimer formation, which can compromise the quantification accuracy. In such a case, gel electrophoresis or Bioanalyzer fragment analysis can verify whether or not the PCR product is a single correct size amplicon DNA with the primer dimers.

Export the raw Ct values to Excel. Free, easy-to-use data analysis software is available online, please refer to www.arraystar.com for detailed instructions. The data analysis procedures include:

■ Data pre-processing

1. Set all Ct values ≥ 35 or N/A (not detected) to 35. From this point on, any Ct value equal to 35 is considered a negative result.

2. Examine the Ct values of the Genomic DNA Control (GDC) wells. If the value is greater than 35, no genomic DNA contamination is detected. If the value is less than 35, genomic DNA contamination is evident and the result may be compromised.

3. Before the data analysis, the RNA spike-in wells are compared. Outlier samples (Ct >25) may be identified and considered for exclusion in the further data analysis.

4. Inter-plate calibration (IPC) can be performed using the PPC assay replicates either with the data analysis software or manually. For each plate, verify if the replicates have a *Ct* standard deviation ≤ 0.5. If not, exclude the outlier that causes the large deviation. Calculate the average of the replicates for each plate (*plate_n*) and the overall average IPC for all plates (*overall*). The calibration factor for each plate is calculated as the difference between the plate average and the overall average:

$$calibration\ factor = IPC(plate\_n) - IPC(overall)$$

The *Ct* value is corrected with the calibration factor as

$$Ct_{RNA} = Ct_{RNA}(Raw\ value, plate\_n) - IPC(plate\_n) + IPC(overall)$$
$$or$$
$$Ct_{RNA} = Ct_{RNA}(Raw\ value, plate\_n) - calibration\ factor$$

For example:

	Plate 1	Plate 2	Plate 3
hsa-let-7c-5p	20.39	20.76	20.26
IPC plate average	16.70	16.70	16.40
IPC overall average	16.60	16.60	16.60
Calibration factor	0.10	0.10	-0.20
hsa-let-7c-5p (calibrated)	20.29	20.66	20.46

5. Calculate the ΔCt for each miRNA in the plate.

$$\Delta Ct_{RNA} = Ct_{RNA} - average(Ct_{Refs})$$

Where average (Ct_{Refs}) is the average of the Ct values derived from the multiple reference genes. Three most stably expressed small ncRNA Control References were selected from abroad range of samples by our stringent algorithm that evaluates the optimal properties and the number for endogenous controls.

■ Data analysis

1. Calculate the $\Delta\Delta Ct$ for each miRNA

$$\Delta\Delta Ct = \Delta Ct(sample\ 1) - \Delta Ct(sample\ 2), \text{ between samples}$$
$$\text{or}$$
$$\Delta\Delta Ct = \Delta Ct(group\ 1) - \Delta Ct(group\ 2), \text{ between groups}$$

2. Calculate the fold changes for each gene from sample 1 to sample 2 or group 1 to group 2 as following:

$$Fold\ Change = 2^{-\Delta\Delta Ct}$$

NOTE: By convention, if a fold change is greater than 1, the result is reported as a fold up-regulation. If a fold change is less than 1, its negative inverse (-1/FC) is reported as a fold down-regulation.

3. When comparing profiling differences between two groups (such as disease versus control) with biological replicates, the statistical significance of the difference can be estimated as p-value by t-test. RNAs having fold changes $\geq$ 2 and p-values $\leq$ 0.05 are selected as the significantly differentially expressed RNAs.

NOTE: Fold change is related to biological effect size. Ranking by fold change is preferred over p-value. qPCR as commonly used in confirmation has a limit of quantification of 0.5 ΔCt, which is equivalent to approximately 2-fold change.

4. Other analyses such as scatter plots, volcano plots, list of differentially expressed genes and bar graph of expression differences for the miRNAs are performed and included in the standard analysis package.

III. Quality Control and Sample Data

A. nrStar™ Human Canonical Conserved miRNA PCR Array

■ Real-time qPCR Validation

The performance of nrStar™ Human Canonical Conserved miRNA PCR Array was tested using a cohort of pancreatic carcinoma and para-carcinoma tissues. The extracted RNA samples were converted to cDNA using rtStar™ First-Strand cDNA Synthesis Kit (3' and 5' adaptor) (Cat# AS-FS-003). The cDNAs were profiled using the PCR array according to aforementioned protocol without modification. The real-time amplification curves are plotted for the entire plate data points for the pancreatic carcinoma RNA (left) and para-carcinoma RNA (right).
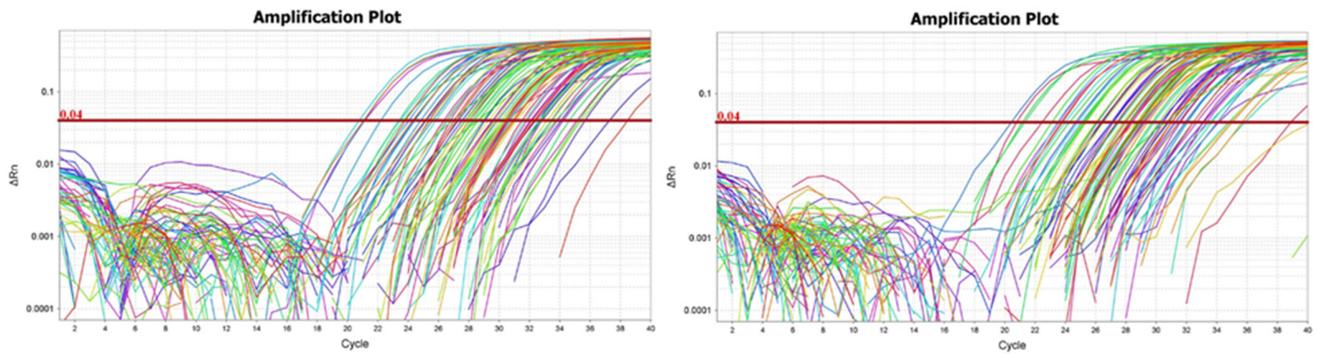
■ Sensitivity Test

The decreasing input amounts of total RNAs from a human glioblastoma cell line were converted to cDNAs and profiled by the PCR arrays. The Ct values were determined using the software default automatic baseline and Ct settings. hsa-let-7b-5p and hsa-miR-100-5p transcripts were detected at Ct values of 25.2 and 27.0 respectively, with the input RNA amount as low as 50 ng.
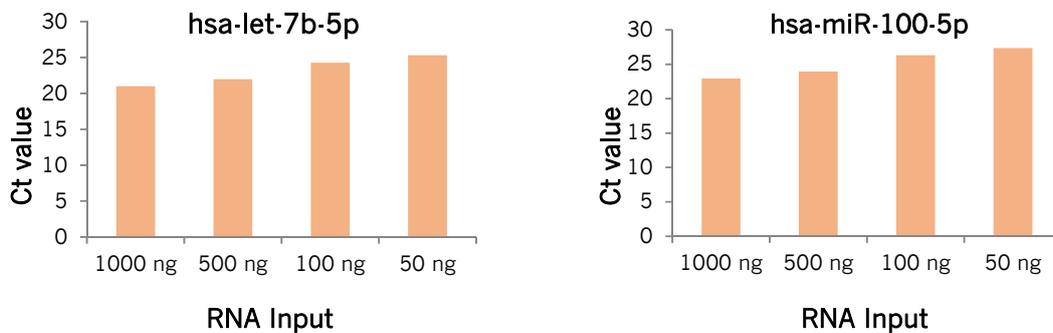
■ **Reproducibility Test**

Two independent runs of nrStar™ Human Canonical Conserved miRNA PCR Array were conducted by two different scientists A and B at two different times using RNAs from two different cell lines. The results demonstrate a high degree of reproducibility with correlation $R^2 > 0.99$.
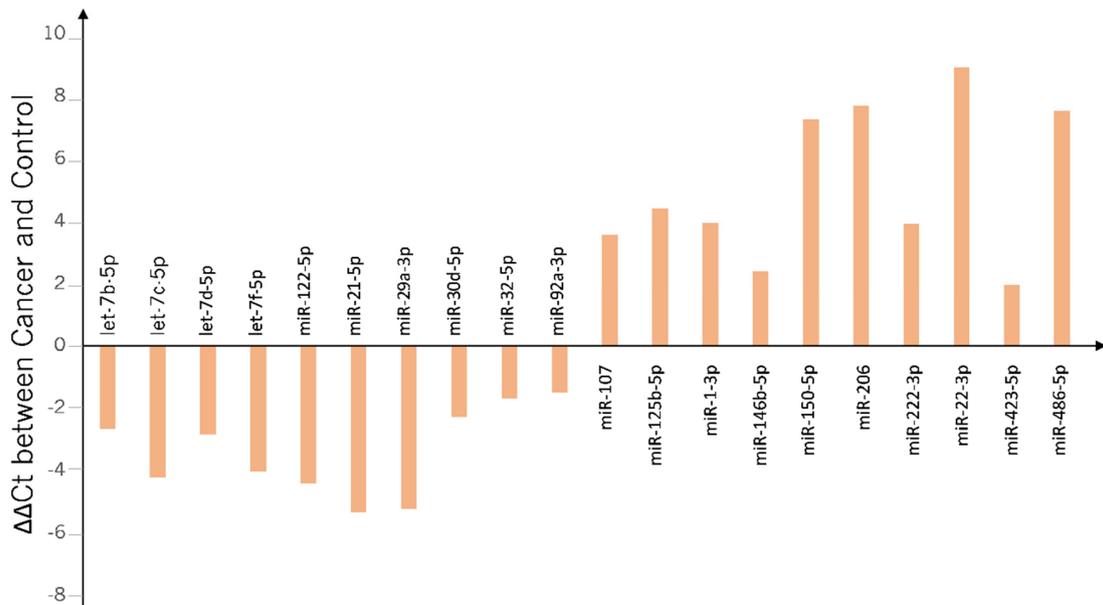

Para-carcinoma tissue

$R^2 = 0.9969$

Pancreatic carcinoma $R^2 = 0.9945$
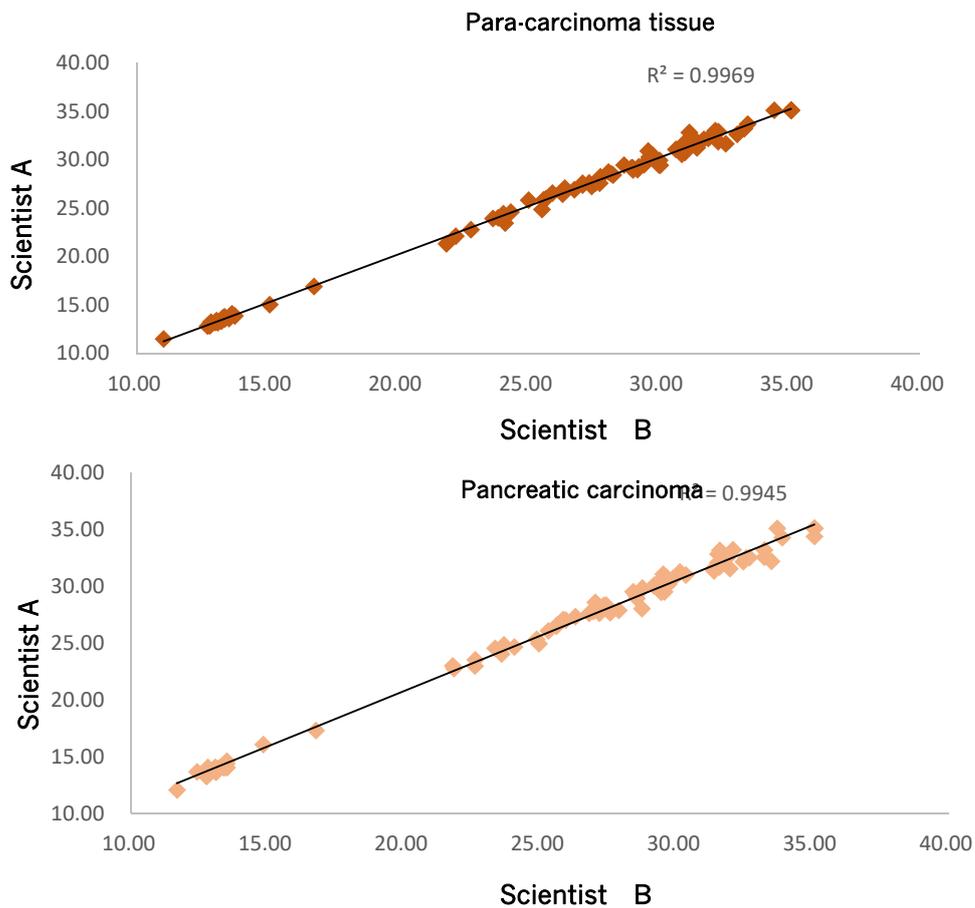
■ **Specificity Test**

The melt curves were analyzed for the amplification products of let-7a-5p, let-7c-5p, let-7e-5p, let-7f-5p,miR-10a-5p,miR-24-3p,miR-34a-5p and miR-92a-3p, all of which had single sharp melting peaks. The results demonstrate the high amplification specificities for the transcripts with the assays on the array.
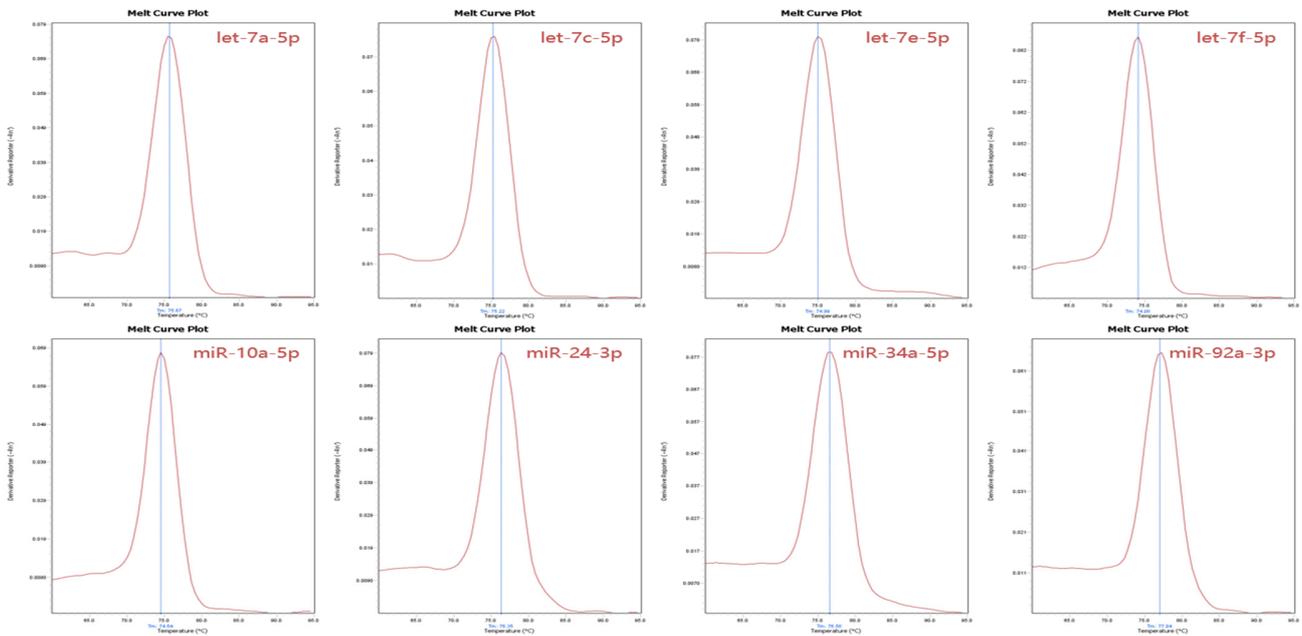
B. Example data: Analysis of human miRNA levels

The sample data were generated from the RNAs extracted from pancreatic carcinoma and para-carcinoma tissues. The normalization was carried out using the average of the Internal Control Reference genes. The $\Delta\Delta Ct$ between pancreatic carcinoma and para-carcinoma tissues are graphed in the bar chart below.

IV. Troubleshooting

Problem	Possible solution
qPCR background too high	• Reduce the amount of cDNA used in the SYBR Green Master Mix.
No qPCR signals	• Inspect if the Internal Controls have a valid qPCR signal • Set SYBR Green as the Detector's Reporter Dye • Use more cDNA in the Master Mix • Lower the annealing temperature in Protocol STEP C.6 from 60°C to 50°C.
Baseline and threshold settings	• Follow the instructions of the qPCR system manufacturer. • Contact their technical support as necessary.

V. References

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VI. Technical Support

For additional information, manual download or technical assistance, please visit our website at www.arraystar.com, or contact us at:

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Fax: 240-238-9860
Email: support@arraystar.com

VII. Terms and Conditions

By purchasing and using any part of the nrStar™ Human Canonical Conserved miRNA PCR Array, you agree to accept the following terms and conditions.

■ Product Use Limitation

Except as otherwise agreed in writing, all products are sold to end-users for research purposes only, and not for human or animal therapeutic or diagnostic use. We do not submit our products for regulatory review by any

government body or other organization for clinical, therapeutic or diagnostic use. You are solely responsible for the way you use the products in compliance with applicable laws, regulations, and governmental policies.

The purchase of Product does not grant any right to use such Product in the practice of any methods covered by Arraystar intellectual property rights. You may not perform compositional, structural, functional or other analysis of our products, or undertake deconvolution or reverse engineering with respect to our products.

■ Product Warranty

Arraystar warrants that the Product will meet the specifications stated on the technical data sheet for that product, and agrees to replace the product free of charge if the product does not conform to the specifications. Notice for non-conformity and request for replacement must be given within 30 days of receipt of Products. In consideration of the above warranty by Arraystar, the buyer agrees to and accepts the following conditions:

That the buyer's sole remedy shall be to obtain replacement of the product from Arraystar; and Arraystar Inc. shall not be responsible for replacing Product that has been improperly stored, handled, or used by buyer or End-User. Arraystar, its Agencies and Representatives disclaim liability of any kind whatsoever, including, without limitation, liability for quality, performance, merchantability and fitness for a particular purpose arising out of the use, or inability to use the product. In no event shall Arraystar be liable for claims for any other damages, whether direct, incidental, foreseeable, consequential, or special (including but not limited to loss of use, revenue or profit), whether based upon warranty, contract, tort (including negligence) or strict liability arising in connection with the sale or the failure of products to perform in accordance with the stated specifications.

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