Arraystar Handbook
miRStar™ PCR Array Systems

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miRStar™ Human Cancer Focus miRNA & Target mRNA PCR Array

Cat#: AS-MR-003

Instruction Manual version 1.0

Product Summary

Kit components

Catalog Number	Contents	Storage
AS-MR-003	miRStar™ Human Cancer Focus miRNA & Target mRNA PCR Array 384HC, dried down assays in 384-well plate	-20°C

Additional required materials

- Thermal cycler
- Real time qPCR instrument, compatible with 384-well format
- Arraystar™ miRNA First-Strand cDNA Synthesis Kit (Cat# AS-FS-002)
- Arraystar™ SYBR Green qPCR Master Mix (Cat# AS-MR-006-5)
- Nuclease free PCR-grade water

Introduction

Mature human microRNAs (miRNAs) are a class of single stranded, small non-coding RNAs around 22 nucleotides in length [1]. miRNAs can base pair with their target mRNAs at the complementary sites and mediate gene silencing predominantly by mRNA degradation. One mRNA transcript may have several miRNA response elements (MRE) for different miRNAs, and conversely, one miRNA may target as many as 100 different mRNAs in a networked gene regulation [2, 3]. There are over a thousand of known human miRNAs, which may target up to 60% of the human genes. miRNAs are associated with many biological processes and human diseases. In particular, some miRNAs may function either as oncogenes or tumor suppressors by targeting corresponding mRNAs. Dysregulated miRNAs can promote tumorigenesis and cancer progression [4-12]. Studying microRNAs has become an important part of cancer research.

Arraystar's miRStar™ Human Cancer Focus miRNA & Target mRNA PCR Array contains 184 critical tumor-related miRNAs and 178 well defined mRNA targets of these miRNAs. The array is a powerful tool to conveniently and quickly analyze the miRNAs most relevant to cancer. More importantly, it also simultaneously profiles the mRNA targets of the miRNAs, thereby providing insights into the interaction between the cancer-related miRNAs and their target mRNAs.

To ensure high data quality, the panel includes 8 reference sets for miRNAs and 5 reference sets for target mRNAs to better quantify and normalize the qPCR data. cDNA synthesis and PCR efficiency are evaluated by using the synthetic cel-miR-39-3p as the Spike-in RNA control. Potential genomic DNA contamination is monitored by using the genomic DNA control (GDC).

Array Layout

The cancer-associated miRNAs (colored green) and their target mRNAs (colored blue) are in the alternate rows in Well A01-O16. The control assays (circled in red) are in Well O17-O24 for miRNA and in Well P11-P24 for mRNA (Figure 1).

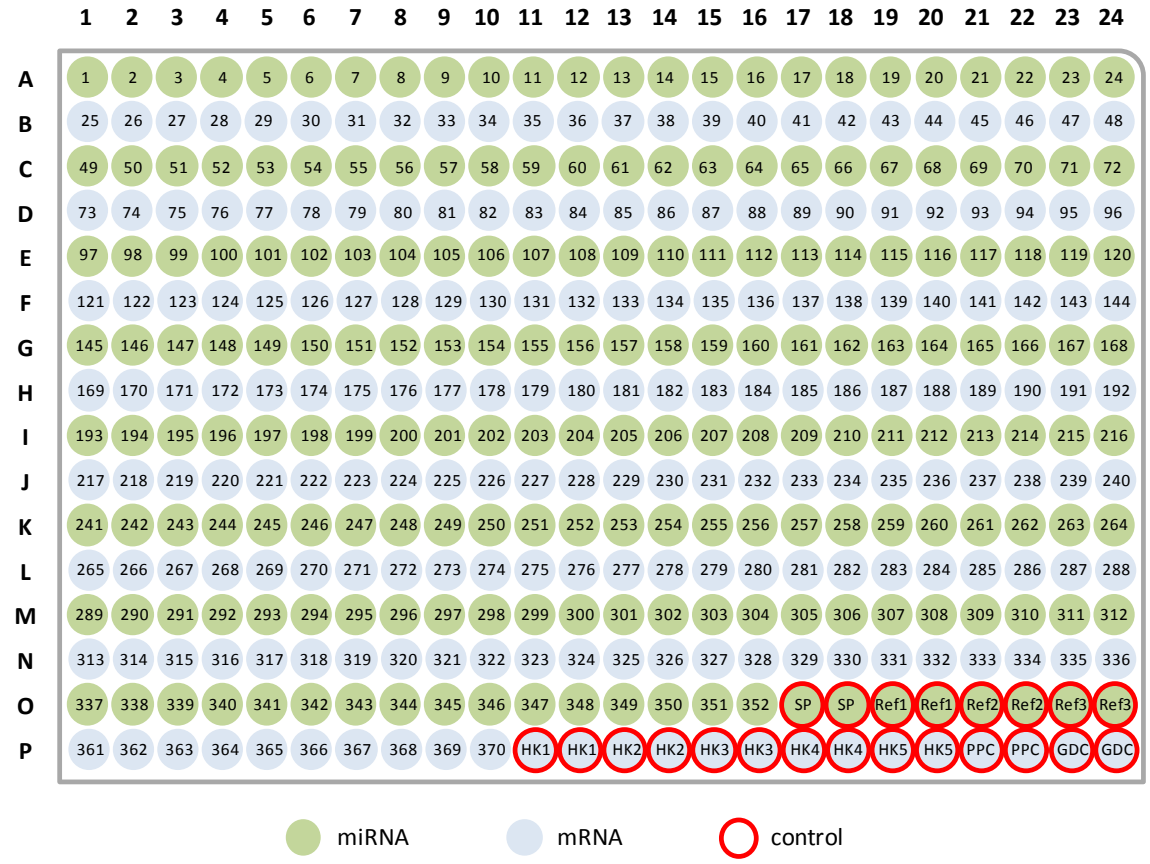

Figure 1. The array layout for miRStar Human Cancer Focus miRNA & Target mRNA PCR Array.

A1 through O16:	184 cancer-associated miRNAs and 178 representative target mRNAs.
O17 and O18:	Spike-in Control (**SP**) in duplicate, to evaluate cDNA synthesis and PCR efficiency.
O19 through O24:	Three small nuclear or small nucleolar RNAs in duplicates, RNU6-2-F (**Ref1**), SNORD43-F (**Ref2**), and SNORD95-F (**Ref3**), to normalize qPCR data for the miRNAs. Besides these genes, five housekeeping miRNAs are included as the internal quantification controls or reference genes: hsa-miR-16-5p(99), hsa-miR-191-5p(114), and has-miR-423-3p(264), hsa-miR-425-5p(290), and hsa-miR-93-5p(345).

P11 through P20: Five housekeeping genes (**HK**) in duplicates, to normalize qPCR data for the mRNA target genes.

P21 and P22: Positive PCR Control (**PPC**) in duplicate, to self-test the efficiency of the PCR by using pre-dispensed artificial DNA sequence and its PCR primer pair.

P23 and P24: Genomic DNA Control (**GDC**) in duplicate, to detect potential genomic DNA contamination with high sensitivity and specificity.

Description of Control Assays

There are five types of control assays built in the miRStar Human Cancer Focus miRNA & Target mRNA PCR Array 384HC. Each control assay is in duplicate. Their uses and meanings are explained below.

- **SP** (Spike-in control): An RNA spike-in control is added in the RNA sample during the first-strand cDNA synthesis (Protocol Step A.2). The SP control assay indicates the overall success and the efficiency of the reactions beginning from the adaptor ligation, cDNA synthesis to the final qPCR. Any problem(s) in these steps will result in a failed or compromised SP outcome.
- **GDC** (Genomic DNA Control): The control assay consists of PCR primers for an untranscribed genomic region. The *Ct* values should be greater than 35. A positive GDC signal indicates the array result is compromised by genomic DNA contamination.
- **PPC** (Positive PCR Control): The assay contains an artificial DNA and the PCR primer pair for its amplification. PPC indicates the amplification efficiency of the qPCR. If the *Ct* value is greater than 20, the qPCR may have low amplification efficiency problem.
- **Ref** (miRNA Reference): Three stably expressed small nuclear or small nucleolar RNA genes RNU6-2-F (**Ref1**), SNORD43-F (**Ref2**), and SNORD95-F (**Ref3**) are included in the array as the quantification references for miRNA. Additionally, five housekeeping miRNAs, namely, hsa-miR-16-5p (Well E03, #99), hsa-miR-191-5p (Well E18, #114), has-miR-423-3p (Well

K24, #264), hsa-miR-425-5p (Well M02, #290), and hsa-miR-93-5p (Well O09, #345), can also serve as the endogenous quantification or reference controls.

- **HK** (Housekeeping mRNA gene): Five human housekeeping genes β-actin, β-2 microglobulin, GAPDH, β-D-glucuronidase, and HSP90-β are included as the references (HK1, HK2, HK3, HK4 and HK5). They are used for mRNA qPCR data normalization and quantification (see Protocol Data Analysis Step C).

Arraystar has included most of the commonly used reference standards for miRNA (Ref) and mRNA (HK), which offers greater flexibility in reference selection in data analysis. All reference assays are measured in duplicates. Ideally, the abundance levels of a reference should be stable and consistent across all the sample types. In practice, one or more of the reference genes can be averaged for data analysis (Protocols Step C).

List of miRNAs and Their mRNA Targets

Control RNAs are outlined in red.

A01	let-7a-5p	B01	ABCG2	E01	miR-15a-5p	F01	DMTF1	I01	miR-25-3p	J01	FABP4	M01	miR-424-5p	N01	MAPK3
A02	let-7b-5p	B02	AGTR1	E02	miR-15b-5p	F02	DNMT3B	I02	miR-26a-5p	J02	FAS	M02	miR-425-5p	N02	MAPK7
A03	let-7b-3p	B03	AHR	E03	miR-16-5p	F03	DUSP1	I03	miR-26b-5p	J03	FGF7	M03	miR-429	N03	MAPK8
A04	let-7c	B04	AKT3	E04	miR-17-5p	F04	DUSP10	I04	miR-27a-3p	J04	FOXA2	M04	miR-451a	N04	MCL1
A05	let-7d-5p	B05	ANXA11	E05	miR-181a-5p	F05	E2F1	I05	miR-27b-3p	J05	FOXO1	M05	miR-486-5p	N05	MDM4
A06	let-7d-3p	B06	BCL2	E06	miR-181b-5p	F06	E2F2	I06	miR-28-5p	J06	FZD3	M06	miR-495-3p	N06	MET
A07	let-7e-5p	B07	BCL2L1	E07	miR-181c-5p	F07	E2F3	I07	miR-299-3p	J07	GADD45A	M07	miR-497-5p	N07	MMP1
A08	let-7f-5p	B08	BCL2L2	E08	miR-182-5p	F08	EGFL7	I08	miR-29a-3p	J08	GATA6	M08	miR-501-5p	N08	MMP10
A09	let-7g-5p	B09	BCL6	E09	miR-183-5p	F09	EGFR	I09	miR-29a-5p	J09	GLI1	M09	miR-502-3p	N09	MMP13
A10	let-7i-5p	B10	BDNF	E10	miR-184	F10	EGR2	I10	miR-29b-3p	J10	HDAC1	M10	miR-505-3p	N10	MMP16
A11	miR-1	B11	BNIP3	E11	miR-185-5p	F11	PPP1CA	I11	miR-29b-2-5p	J11	HES1	M11	miR-517a-3p	N11	MMP2
A12	miR-100-5p	B12	CAPRIN1	E12	miR-186-5p	F12	PSMD9	I12	miR-29c-3p	J12	HMGA2	M12	miR-518a-3p	N12	MMP9
A13	miR-101-3p	B13	CARD10	E13	miR-187-5p	F13	PTEN	I13	miR-30a-5p	J13	HOXA11	M13	miR-518b	N13	MTA1
A14	miR-106a-5p	B14	CASP3	E14	miR-188-5p	F14	PTGS2	I14	miR-30b-5p	J14	HOXA9	M14	miR-518c-3p	N14	MTSS1
A15	miR-106b-5p	B15	CASP9	E15	miR-18a-5p	F15	PTK2	I15	miR-30c-5p	J15	HOXB5	M15	miR-518e-3p	N15	NANOG
A16	miR-107	B16	CCL4	E16	miR-18b-5p	F16	RAB22A	I16	miR-30d-5p	J16	HOXB7	M16	miR-518f-3p	N16	NOTCH1
A17	miR-10a-5p	B17	CCND1	E17	miR-190a	F17	RAB5A	I17	miR-30e-5p	J17	HOXB8	M17	miR-519d	N17	NOTCH2
A18	miR-10b-5p	B18	CCND2	E18	miR-191-5p	F18	RASA1	I18	miR-30e-3p	J18	HOXC8	M18	miR-524-5p	N18	P2RX7
A19	miR-122-5p	B19	CCND3	E19	miR-192-5p	F19	RECK	I19	miR-31-5p	J19	HOXD10	M19	miR-532-5p	N19	PAK1
A20	miR-125a-5p	B20	CCNE1	E20	miR-193b-3p	F20	RHOA	I20	miR-32-5p	J20	HRAS	M20	miR-539-5p	N20	PARP8
A21	miR-125b-5p	B21	CCNE2	E21	miR-195-5p	F21	SERPINE1	I21	miR-320a	J21	ICAM1	M21	miR-584-5p	N21	PDCD4
A22	miR-126-3p	B22	CCNG1	E22	miR-196a-5p	F22	SGPL1	I22	miR-323a-3p	J22	IFI27	M22	miR-617	N22	PHB
A23	miR-127-3p	B23	CD276	E23	miR-196b-5p	F23	SIRT1	I23	miR-324-3p	J23	IGF1	M23	miR-629-5p	N23	PIK3R1
A24	miR-130a-3p	B24	CD34	E24	miR-197-3p	F24	SMO	I24	miR-326	J24	IGF1R	M24	miR-652-3p	N24	MYC
C01	miR-130b-3p	D01	CD40LG	G01	miR-199a-5p	H01	SNAI2	K01	miR-328	L01	IGF2	O01	miR-7-5p	P01	KLF4
C02	miR-132-3p	D02	CD44	G02	miR-19a-3p	H02	SOCS3	K02	miR-331-3p	L02	IGF2BP1	O02	miR-744-5p	P02	TGFB1
C03	miR-133a	D03	CD46	G03	miR-19b-3p	H03	SOX2	K03	miR-335-5p	L03	IKBKE	O03	miR-877-5p	P03	AR
C04	miR-133b	D04	CDC25A	G04	miR-200a-3p	H04	SOX4	K04	miR-339-5p	L04	IL1B	O04	miR-885-5p	P04	MTPN
C05	miR-134	D05	CDC27	G05	miR-200b-3p	H05	SP1	K05	miR-33a-5p	L05	IL24	O05	miR-886-3p	P05	RTL1
C06	miR-136-5p	D06	CDC34	G06	miR-202-3p	H06	SPARC	K06	miR-33b-5p	L06	IL6	O06	miR-9-5p	P06	PTPN11
C07	miR-137	D07	CDK2	G07	miR-203a	H07	TGFB2	K07	miR-340-5p	L07	IL6R	O07	miR-92a-3p	P07	BIRC5
C08	miR-139-5p	D08	CDK4	G08	miR-204-5p	H08	TGFBR2	K08	miR-342-3p	L08	ITGB1	O08	miR-92b-3p	P08	POU5F1
C09	miR-140-5p	D09	CDK6	G09	miR-205-5p	H09	THBS1	K09	miR-345-5p	L09	ITGB3	O09	miR-93-5p	P09	PPARG
C10	miR-141-3p	D10	CDKN1A	G10	miR-208a	H10	TIMP3	K10	miR-346	L10	ITGB8	O10	miR-93-3p	P10	VEGFA
C11	miR-142-5p	D11	CDKN1B	G11	miR-20a-5p	H11	TLR4	K11	miR-34c-5p	L11	JAG1	O11	miR-96-5p	P11	Actb
C12	miR-143-3p	D12	CDKN1C	G12	miR-20b-5p	H12	TNC	K12	miR-361-5p	L12	JMY	O12	miR-96-3p	P12	Actb
C13	miR-144-3p	D13	CDKN2A	G13	miR-212-3p	H13	TPPP3	K13	miR-363-3p	L13	JUN	O13	miR-98-5p	P13	B2m
C14	miR-145-5p	D14	COL1A1	G14	miR-215	H14	TRAF6	K14	miR-369-3p	L14	KIT	O14	miR-99a-5p	P14	B2m
C15	miR-146a-5p	D15	COL1A2	G15	miR-217	H15	VCAM1	K15	miR-372	L15	KRAS	O15	miR-99a-3p	P15	Gapdh
C16	miR-146b-5p	D16	CORO1A	G16	miR-218-5p	H16	WNT1	K16	miR-373-3p	L16	LAMC2	O16	miR-99b-5p	P16	Gapdh
C17	miR-147a	D17	CTBP1	G17	miR-22-3p	H17	XBP1	K17	miR-374a-5p	L17	LPL	O17	C. e miR-39	P17	Gusb
C18	miR-148a-3p	D18	CTGF	G18	miR-221-3p	H18	ZEB1	K18	miR-375	L18	LRP1	O18	C. e miR-39	P18	Gusb
C19	miR-148b-3p	D19	CTNNB1	G19	miR-222-3p	H19	ZEB2	K19	miR-379-5p	L19	MACC1	O19	RNU6-2-F	P19	Hsp90ab1
C20	miR-149-5p	D20	CYP3A4	G20	miR-223-3p	H20	ZIC3	K20	miR-382-5p	L20	MAGEA3	O20	RNU6-2-F	P20	Hsp90ab1
C21	miR-150-5p	D21	CYP7A1	G21	miR-224-5p	H21	ERBB3	K21	miR-383	L21	MAP2K1	O21	SNORD43-F	P21	PPC
C22	miR-151a-3p	D22	DICER1	G22	miR-23a-3p	H22	ESR1	K22	miR-409-3p	L22	MAPK1	O22	SNORD43-F	P22	PPC
C23	miR-152	D23	DKK1	G23	miR-23b-3p	H23	ETS1	K23	miR-422a	L23	MAPK11	O23	SNORD95-F	P23	GDC
C24	miR-155-5p	D24	DLL1	G24	miR-24-3p	H24	EZH2	K24	miR-423-3p	L24	MAPK14	O24	SNORD95-F	P24	GDC

Protocol

Workflow Overview

A miRStar Human Cancer Focus miRNA & Target mRNA PCR Array experiment consists of several major steps in a workflow shown in Figure 2.

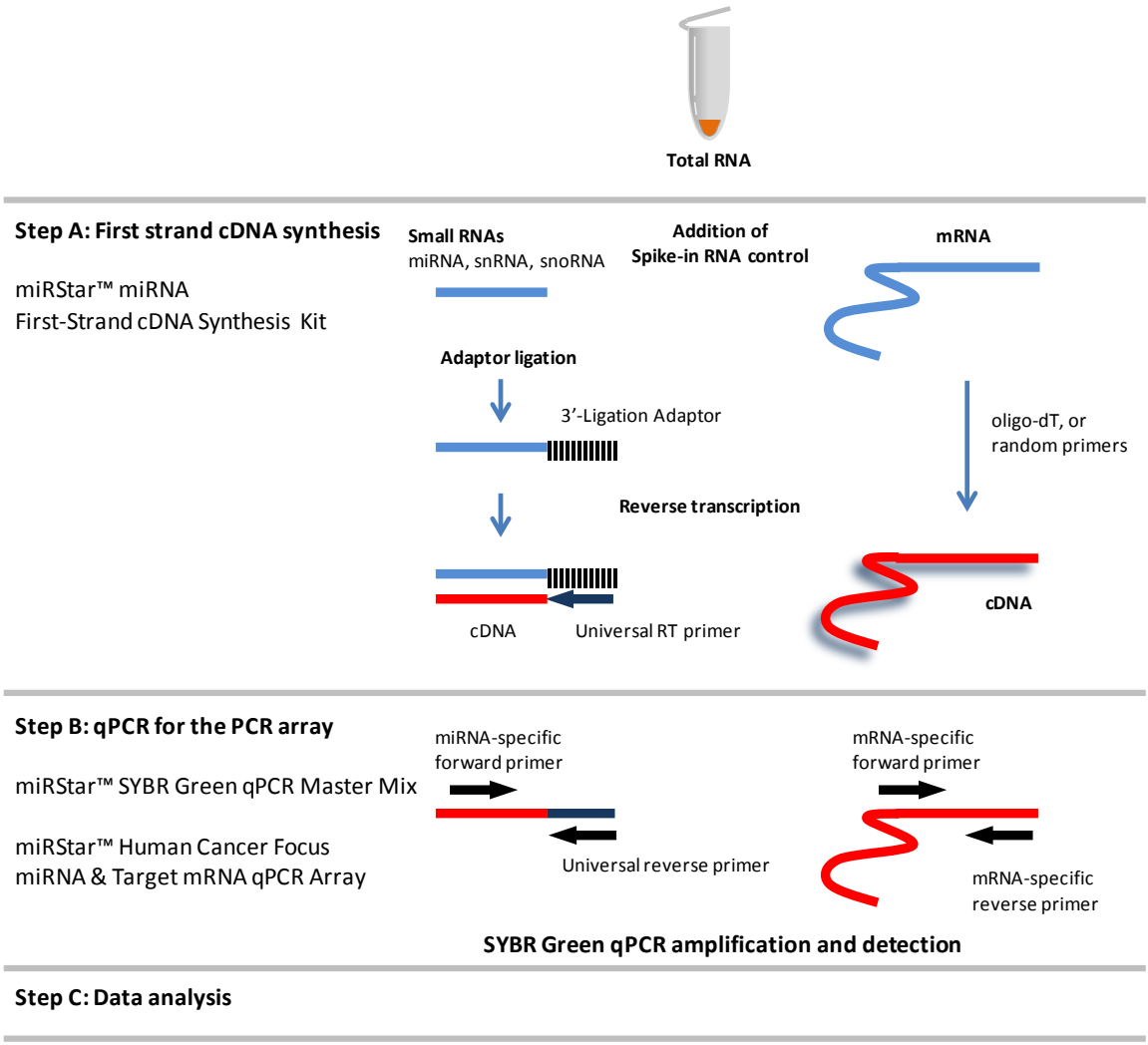

Figure 2. Workflow overview of miRStar™ Human Cancer Focus miRNA & Target mRNA PCR Array experiment.

Step A. First-strand cDNA synthesis

Total RNA samples should be extracted by a method that can recover small RNA fraction, for example, TRIzol® Reagent method.

High quality cDNA synthesis is vital for the following qPCR performance. We highly recommend Arraystar miRNA First-Strand cDNA Synthesis Kit (Cat# AS-FS-002), which is fully compatible with and is specifically optimized for miRStar™ Human Cancer Focus miRNA & Target mRNA PCR Array. Please refer to the Instruction Manual of the Kit for its use.

1. Dilute the 3’ Ligation Adapter from the Kit with RNase-free water. The dilution factor is 1/10 for 10 - 500 ng or 1/3 for 0.5 - 2 µg of the starting total RNA. Use the same amount of total RNA for each sample in the experiment.
2. Set up the adaptor ligation reaction in a 200 µL PCR tube using the following components for each sample:

4.5 µL	Total RNA in nuclease-free water
1.0 µL	diluted 3’ Ligation Adapter
1.0 µL	RNA Spike-in
6.5 µL	total volume

3. Incubate in a thermal cycler at 70°C for 2min; chill on ice immediately.
4. Add the following reagents and mix well. The final volume will be 10 µL.

2.0 µL	5× Ligase Reaction Mix
1.0 µL	RNA ligase
0.5 µL	RNase Inhibitor
10.0 µL	final volume

5. Incubate at 22°C for 60 min, 72°C for 2 min, and on ice for 2 min.
6. For reverse transcription, add 1 µL Universal RT Primer Mix, mix gently.
7. Incubate at 65°C for 2 min; place on ice for at least 2 min.
8. Prepare Reverse Transcription Master Mix and add 10 µL to each sample above.

8.5 µL	RT Reaction Master Mix
0.5 µL	RNase Inhibitor
1.0 µL	MMLV Reverse Transcriptase
10.0 µL	total volume per sample

9. Incubate at 42°C for 60 min; inactivate the reaction at 85°C for 5 min.

Step B. Perform qPCR for the PCR array

1. Dilute the cDNA in nuclease free water. If Arraystar miRNA First-Strand cDNA Synthesis Kit (Cat# AS-FS-002) is used for the cDNA synthesis with 10 ng - 2.0 µg total RNA sample as the starting material, dilute the cDNA product 1/80 in water. The diluted material is used as the qPCR template.
2. Use Arraystar SYBR Green Real-Time Quantitative PCR Master Mix to prepare qPCR Master Mix for each sample per qPCR well. There are total 384 reactions in a 384-well qPCR array plate, 192 wells for miRNA and 192 wells for mRNA (Figure 1 and Table 1). Add some extra reactions as needed by the liquid handling operation. Multiply this number with the individual amounts of the components in the table below and prepare a qPCR Mix.

5.0 µL	SYBR Green Master Mix

1.5 µL	Diluted cDNA template
3.5 µL	ddH_2O
10.0 µL	total volume/well

3. Dispense 10 µl of the Mix uniformly to each well on the qPCR array plate.
4. Run the qPCR using the following program. Consult the instructions for the instrument for details.

Cycles	Temperature	Time
1	95 ℃	10 minutes
40	95 ℃	10 seconds
	55-65 ℃	1 minute
Melting curve analysis		

Step C. Data analysis

1. Calculate the Δ*Ct* for each miRNA or mRNA

$$\Delta Ct_{\text{miRNA}} = Ct_{\text{miRNA}} - \text{average}\,(Ct_{\text{Ref\_miRNA}})$$

$$\Delta Ct_{\text{mRNA}} = Ct_{\text{mRNA}} - \text{average}\,(Ct_{\text{HK\_mRNA}})$$

where $Ct_{\text{Ref\_miRNA}}$ are the values taken from one or more duplicates of the miRNA references (**Ref**) in Well O19 through O24; $Ct_{\text{Ref\_mRNA}}$ are the values taken from one or more duplicates of the designated housekeeping (**HK**) mRNA references in Well P11 through P20 (Table 1).

If no particular reference gene(s) are designated as the quantification reference, all the Refs or HKs can be averaged and used in the above formula, but only if the

difference between the averaged values is less than 1 cycle when comparing the two groups.

2. Calculate the $\Delta\Delta Ct$ between two samples or groups for a gene:

$$\Delta\Delta Ct = \Delta Ct_{\text{sample2}} - \Delta Ct_{\text{sample1}}, \text{ or}$$

$$\Delta\Delta Ct = \Delta Ct_{\text{group2}} - \Delta Ct_{\text{group1}}$$

Where sample1 or group1 is the control and sample2 or group2 is the experimental.

3. Calculate the fold change from group 1 to group 2 for a gene as:

$$\textit{fold change} = 2^{-\Delta\Delta Ct}$$

OPTIONAL: If the fold-change is greater than 1, the result may be reported as a fold up-regulation. If the fold-change is less than 1, the negative reciprocal may be reported as a fold down-regulation.

miRStar™ Human Cancer Focus miRNA PCR Array

Cat#: AS-MR-001

Instruction Manual version 1.0

Product Summary

Kit components

Catalog Number	Contents	Storage
AS-MR-001	miRStar™ Human Cancer Focus miRNA PCR Array, dried down assays in 384-well plate	-20°C

Description

Arraystar's miRStar™ Human Cancer Focus microRNA PCR Array contains two identical sets of 184 miRNAs most relevant to cancer. Two individual samples, for example tumor vs. peritumoral or biological replicates, can be profiled in parallel. To ensure high data quality, the panel includes 8 miRNA reference sets to better quantify and normalize the qPCR data. cDNA synthesis and PCR efficiency are evaluated by using the synthetic cel-miR-39-3p as the Spike-in RNA control. The array is a powerful tool to conveniently and quickly analyze the expression levels of miRNAs most relevant to cancers, which is valuable for cancer biology research and cancer biomarker discovery.

Array Layout

The cancer-associated miRNAs for each sample are in the alternate rows (shaded in light or darker colors for Sample 1 and Sample 2). The control assays are circled in red.

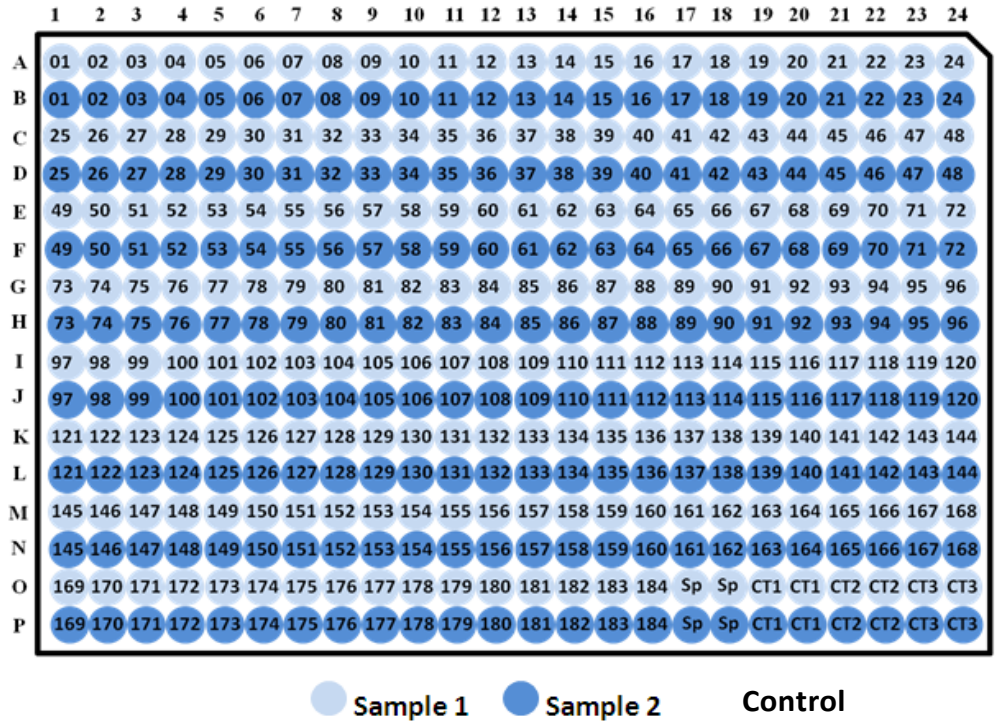

Figure 1. The array layout for miRStar Human Cancer Focus miRNA PCR Array.

#01 through #184	184 cancer-associated miRNAs.
O17 and O18 P17 and P18	Spike-in Control (**SP**) in duplicate, to evaluate cDNA synthesis and PCR efficiency.
O19 through O24 P19 through P24	Three small nuclear or small nucleolar RNAs in duplicates, RNU6-2-F (**CT1**), SNORD43-F (**CT2**), and SNORD95-F (**CT3**), to normalize qPCR data for the miRNAs.
#51, #66, #144, #146, #177	Five housekeeping miRNAs as the internal quantification controls or reference genes: hsa-miR-16-5p(#51, Well E03 and F03), hsa-miR-191-5p(#66, Well E18 and F18), has-miR-423-3p(#144, Well K24 and L24),

hsa-miR-425-5p(#146, Well M02 and N02), and hsa-miR-93-5p(#177, Well O09 and P09).

Description of Control Assays

There are three types of control assays built in the miRStar Human Cancer Focus miRNA PCR Array 384HC. Each control assay is in duplicate. Their uses and meanings are explained below.

- **SP** (Spike-in control): An RNA spike-in control is added in the RNA sample during the first-strand cDNA synthesis (Protocol Step A2). The SP control assay indicates the overall success and the efficiency of the reactions beginning from the adaptor ligation, cDNA synthesis to the final qPCR. Any problem(s) in these steps will result in a failed or compromised SP outcome.
- **CT** (miRNA Control Reference): Three stably expressed small nuclear or small nucleolar RNA genes RNU6-2-F (**CT1**), SNORD43-F (**CT2**), and SNORD95-F (**CT3**) are included in the array as the quantification references for miRNA. Additionally,
- **Housekeeping miRNA genes**: Five housekeeping miRNAs, namely, hsa-miR-16-5p(#51, Well E03 and F03), hsa-miR-191-5p(#66, Well E18 and F18), has-miR-423-3p(#144, Well K24 and L24), hsa-miR-425-5p(#146, Well M02 and N02), and hsa-miR-93-5p(#177, Well O09 and P09), can also serve as the internal quantification or reference controls.

List of miRNAs and controls

Control RNAs are outlined in red.

A01	let-7a-5p	B01	let-7a-5p	E01	miR-15a-5p	F01	miR-15a-5p	I01	miR-25-3p	J01	miR-25-3p	M01	miR-424-5p	N01	miR-424-5p
A02	let-7b-5p	B02	let-7b-5p	E02	miR-15b-5p	F02	miR-15b-5p	I02	miR-26a-5p	J02	miR-26a-5p	M02	miR-425-5p	N02	miR-425-5p
A03	let-7b-3p	B03	let-7b-3p	E03	miR-16-5p	F03	miR-16-5p	I03	miR-26b-5p	J03	miR-26b-5p	M03	miR-429	N03	miR-429
A04	let-7c	B04	let-7c	E04	miR-17-5p	F04	miR-17-5p	I04	miR-27a-3p	J04	miR-27a-3p	M04	miR-451a	N04	miR-451a
A05	let-7d-5p	B05	let-7d-5p	E05	miR-181a-5p	F05	miR-181a-5p	I05	miR-27b-3p	J05	miR-27b-3p	M05	miR-486-5p	N05	miR-486-5p
A06	let-7d-3p	B06	let-7d-3p	E06	miR-181b-5p	F06	miR-181b-5p	I06	miR-28-5p	J06	miR-28-5p	M06	miR-495-3p	N06	miR-495-3p
A07	let-7e-5p	B07	let-7e-5p	E07	miR-181c-5p	F07	miR-181c-5p	I07	miR-299-3p	J07	miR-299-3p	M07	miR-497-5p	N07	miR-497-5p
A08	let-7f-5p	B08	let-7f-5p	E08	miR-182-5p	F08	miR-182-5p	I08	miR-29a-3p	J08	miR-29a-3p	M08	miR-501-5p	N08	miR-501-5p
A09	let-7g-5p	B09	let-7g-5p	E09	miR-183-5p	F09	miR-183-5p	I09	miR-29a-5p	J09	miR-29a-5p	M09	miR-502-3p	N09	miR-502-3p
A10	let-7i-5p	B10	let-7i-5p	E10	miR-184	F10	miR-184	I10	miR-29b-3p	J10	miR-29b-3p	M10	miR-505-3p	N10	miR-505-3p
A11	miR-1	B11	miR-1	E11	miR-185-5p	F11	miR-185-5p	I11	miR-29b-2-5p	J11	miR-29b-2-5p	M11	miR-517a-3p	N11	miR-517a-3p
A12	miR-100-5p	B12	miR-100-5p	E12	miR-186-5p	F12	miR-186-5p	I12	miR-29c-3p	J12	miR-29c-3p	M12	miR-518a-3p	N12	miR-518a-3p
A13	miR-101-3p	B13	miR-101-3p	E13	miR-187-5p	F13	miR-187-5p	I13	miR-30a-5p	J13	miR-30a-5p	M13	miR-518b	N13	miR-518b
A14	miR-106a-5p	B14	miR-106a-5p	E14	miR-188-5p	F14	miR-188-5p	I14	miR-30b-5p	J14	miR-30b-5p	M14	miR-518c-3p	N14	miR-518c-3p
A15	miR-106b-5p	B15	miR-106b-5p	E15	miR-18a-5p	F15	miR-18a-5p	I15	miR-30c-5p	J15	miR-30c-5p	M15	miR-518e-3p	N15	miR-518e-3p
A16	miR-107	B16	miR-107	E16	miR-18b-5p	F16	miR-18b-5p	I16	miR-30d-5p	J16	miR-30d-5p	M16	miR-518f-3p	N16	miR-518f-3p
A17	miR-10a-5p	B17	miR-10a-5p	E17	miR-190a	F17	miR-190a	I17	miR-30e-5p	J17	miR-30e-5p	M17	miR-519d	N17	miR-519d
A18	miR-10b-5p	B18	miR-10b-5p	E18	miR-191-5p	F18	miR-191-5p	I18	miR-30e-3p	J18	miR-30e-3p	M18	miR-524-5p	N18	miR-524-5p
A19	miR-122-5p	B19	miR-122-5p	E19	miR-192-5p	F19	miR-192-5p	I19	miR-31-5p	J19	miR-31-5p	M19	miR-532-5p	N19	miR-532-5p
A20	miR-125a-5p	B20	miR-125a-5p	E20	miR-193b-3p	F20	miR-193b-3p	I20	miR-32-5p	J20	miR-32-5p	M20	miR-539-5p	N20	miR-539-5p
A21	miR-125b-5p	B21	miR-125b-5p	E21	miR-195-5p	F21	miR-195-5p	I21	miR-320a	J21	miR-320a	M21	miR-584-5p	N21	miR-584-5p
A22	miR-126-3p	B22	miR-126-3p	E22	miR-196a-5p	F22	miR-196a-5p	I22	miR-323a-3p	J22	miR-323a-3p	M22	miR-617	N22	miR-617
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A24	miR-130a-3p	B24	miR-130a-3p	E24	miR-197-3p	F24	miR-197-3p	I24	miR-326	J24	miR-326	M24	miR-652-3p	N24	miR-652-3p
C01	miR-130b-3p	D01	miR-130b-3p	G01	miR-199a-5p	H01	miR-199a-5p	K01	miR-328	L01	miR-328	O01	miR-7-5p	P01	miR-7-5p
C02	miR-132-3p	D02	miR-132-3p	G02	miR-19a-3p	H02	miR-19a-3p	K02	miR-331-3p	L02	miR-331-3p	O02	miR-744-5p	P02	miR-744-5p
C03	miR-133a	D03	miR-133a	G03	miR-19b-3p	H03	miR-19b-3p	K03	miR-335-5p	L03	miR-335-5p	O03	miR-877-5p	P03	miR-877-5p
C04	miR-133b	D04	miR-133b	G04	miR-200a-3p	H04	miR-200a-3p	K04	miR-339-5p	L04	miR-339-5p	O04	miR-885-5p	P04	miR-885-5p
C05	miR-134	D05	miR-134	G05	miR-200b-3p	H05	miR-200b-3p	K05	miR-33a-5p	L05	miR-33a-5p	O05	miR-886-3p	P05	miR-886-3p
C06	miR-136-5p	D06	miR-136-5p	G06	miR-202-3p	H06	miR-202-3p	K06	miR-33b-5p	L06	miR-33b-5p	O06	miR-9-5p	P06	miR-9-5p
C07	miR-137	D07	miR-137	G07	miR-203a	H07	miR-203a	K07	miR-340-5p	L07	miR-340-5p	O07	miR-92a-3p	P07	miR-92a-3p
C08	miR-139-5p	D08	miR-139-5p	G08	miR-204-5p	H08	miR-204-5p	K08	miR-342-3p	L08	miR-342-3p	O08	miR-92b-3p	P08	miR-92b-3p
C09	miR-140-5p	D09	miR-140-5p	G09	miR-205-5p	H09	miR-205-5p	K09	miR-345-5p	L09	miR-345-5p	O09	miR-93-5p	P09	miR-93-5p
C10	miR-141-3p	D10	miR-141-3p	G10	miR-208a	H10	miR-208a	K10	miR-346	L10	miR-346	O10	miR-93-3p	P10	miR-93-3p
C11	miR-142-5p	D11	miR-142-5p	G11	miR-20a-5p	H11	miR-20a-5p	K11	miR-34c-5p	L11	miR-34c-5p	O11	miR-96-5p	P11	miR-96-5p
C12	miR-143-3p	D12	miR-143-3p	G12	miR-20b-5p	H12	miR-20b-5p	K12	miR-361-5p	L12	miR-361-5p	O12	miR-96-3p	P12	miR-96-3p
C13	miR-144-3p	D13	miR-144-3p	G13	miR-212-3p	H13	miR-212-3p	K13	miR-363-3p	L13	miR-363-3p	O13	miR-98-5p	P13	miR-98-5p
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C15	miR-146a-5p	D15	miR-146a-5p	G15	miR-217	H15	miR-217	K15	miR-372	L15	miR-372	O15	miR-99a-3p	P15	miR-99a-3p
C16	miR-146b-5p	D16	miR-146b-5p	G16	miR-218-5p	H16	miR-218-5p	K16	miR-373-3p	L16	miR-373-3p	O16	miR-99b-5p	P16	miR-99b-5p
C17	miR-147a	D17	miR-147a	G17	miR-22-3p	H17	miR-22-3p	K17	miR-374a-5p	L17	miR-374a-5p	O17	C. e miR-39	P17	C. e miR-39
C18	miR-148a-3p	D18	miR-148a-3p	G18	miR-221-3p	H18	miR-221-3p	K18	miR-375	L18	miR-375	O18	C. e miR-39	P18	C. e miR-39
C19	miR-148b-3p	D19	miR-148b-3p	G19	miR-222-3p	H19	miR-222-3p	K19	miR-379-5p	L19	miR-379-5p	O19	RNU6-2-F	P19	RNU6-2-F
C20	miR-149-5p	D20	miR-149-5p	G20	miR-223-3p	H20	miR-223-3p	K20	miR-382-5p	L20	miR-382-5p	O20	RNU6-2-F	P20	RNU6-2-F
C21	miR-150-5p	D21	miR-150-5p	G21	miR-224-5p	H21	miR-224-5p	K21	miR-383	L21	miR-383	O21	SNORD43-F	P21	SNORD43-F
C22	miR-151a-3p	D22	miR-151a-3p	G22	miR-23a-3p	H22	miR-23a-3p	K22	miR-409-3p	L22	miR-409-3p	O22	SNORD43-F	P22	SNORD43-F
C23	miR-152	D23	miR-152	G23	miR-23b-3p	H23	miR-23b-3p	K23	miR-422a	L23	miR-422a	O23	SNORD95-F	P23	SNORD95-F
C24	miR-155-5p	D24	miR-155-5p	G24	miR-24-3p	H24	miR-24-3p	K24	miR-423-3p	L24	miR-423-3p	O24	SNORD95-F	P24	SNORD95-F

Protocol

Workflow Overview

A miRStar Human Cancer Focus miRNA PCR Array experiment consists of several major steps in a workflow shown in Figure 2.

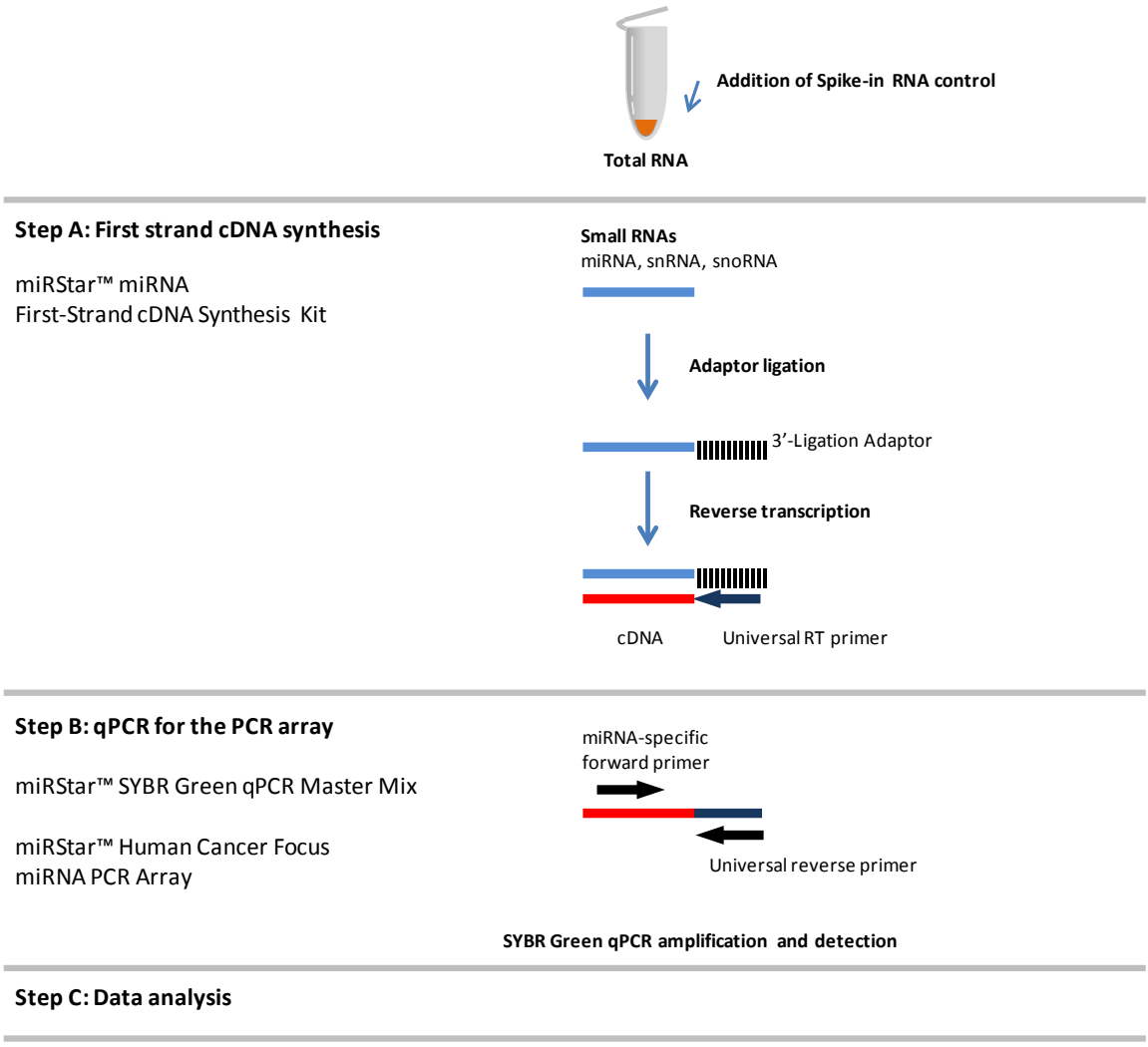

Figure 2. Workflow overview of miRStar™ Human Cancer Focus miRNA PCR Array experiment.

Step A. First-strand cDNA synthesis

Total RNA samples should be extracted by a method that can recover small RNA fraction, for example, TRIzol® Reagent method.

High quality cDNA synthesis is vital for the following qPCR performance. We highly recommend Arraystar miRNA First-Strand cDNA Synthesis Kit (Cat# AS-FS-002), which is fully compatible with and is specifically optimized for miRStar Human Cancer Focus miRNA PCR Array. Please refer to the Instruction Manual of the Kit for its use.

1. Dilute the 3' Ligation Adapter from the Kit with RNase-free water. The dilution factor is 1/10 for 10 - 500 ng or 1/3 for 0.5 - 2 μg of the starting total RNA. Use the same amount of total RNA for each sample in the experiment.
2. Set up the adaptor ligation reaction in a 200 μL PCR tube using the following components for each sample:

4.5 μL	Total RNA in nuclease-free water
1.0 μL	diluted 3' Ligation Adapter
1.0 μL	RNA Spike-in
6.5 μL	total volume

3. Incubate in a thermal cycler at 70°C for 2min; chill on ice immediately.
4. Add the following reagents and mix well. The final volume will be 10 μL.

2.0 μL	5×Ligase Reaction Mix
1.0 μL	RNA ligase
0.5 μL	RNase Inhibitor
10.0 μL	final volume

5. Incubate at 22°C for 60 min; 72°C for 2 min; and on ice for 2 min.

6. For reverse transcription, add 1 µL Universal RT Primer Mix, mix gently.
7. Incubate at 65°C for 2 min; place on ice for at least 2 min.
8. Prepare Reverse Transcription Master Mix and add 10 µL to each sample above.

8.5 µL	RT Reaction Master Mix
0.5 µL	RNase Inhibitor
1.0 µL	MMLV Reverse Transcriptase
10.0 µL	total volume per sample

9. Incubate at 42°C for 60 min; inactivate the reaction at 85°C for 5 min.

Step B. Perform qPCR for the PCR array

1. Dilute the cDNA in nuclease free water. If Arraystar miRNA First-Strand cDNA Synthesis Kit (Cat# AS-FS-002) is used for the cDNA synthesis with 10 ng - 2.0 µg total RNA sample as the starting material, dilute the cDNA product 1/80 in water. The diluted material is used as the qPCR template.
2. Use Arraystar SYBR Green Real-Time Quantitative PCR Master Mix to prepare qPCR Master Mix for each sample per qPCR well. There are total 384 reactions in a 384-well qPCR array plate, 192 wells for miRNA and 192 wells for mRNA (Figure 1). Add some extra reactions as needed by the liquid handling operation. Multiply this number with the individual amounts of the components in the table below and prepare a qPCR Mix.

5.0 µL	SYBR Green Master Mix

1.5 µL	Diluted cDNA template
3.5 µL	ddH_2O
10.0 µL	total volume per well

3. Dispense 10 µl of the Mix uniformly to each well on the qPCR array plate.
4. Run the qPCR using the following program. Consult the instructions for the instrument for details.

Cycles	Temperature	Time
1	95 ℃	10 minutes
40	95 ℃	10 seconds
	55-65 ℃	1 minute
Melting curve analysis		

Step C. Data analysis

1. Calculate the Δ*Ct* for each miRNA:

$$\Delta Ct_{\text{miRNA}} = Ct_{\text{miRNA}} - \text{average}\,(Ct_{\text{control}})$$

Where Ct_{control} are the values taken from one or more duplicates of the miRNA references (**CT**).

If no particular reference gene(s) are designated as the quantification reference, all the CTs can be averaged and used in the above formula, but only if the difference between the averaged values is less than 1 cycle when comparing the two groups.

2. Calculate the $\Delta\Delta Ct$ between two samples or groups for a gene:

$$\Delta\Delta Ct = \Delta Ct_{\text{sample2}} - \Delta Ct_{\text{sample1}}, \text{ or}$$

$$\Delta\Delta Ct = \Delta Ct_{\text{group2}} - \Delta Ct_{\text{group1}}$$

Where sample1 or group1 is the control and sample2 or group2 is the experimental.

3. Calculate the fold change from group 1 to group 2 for a gene as:

$$\textit{fold change} = 2^{-\Delta\Delta Ct}$$

OPTIONAL: If the fold-change is greater than 1, the result may be reported as a fold up-regulation. If the fold-change is less than 1, the negative reciprocal may be reported as a fold down-regulation.

miRStar™ miRNA First-Strand cDNA Synthesis Kit

Cat#: AS-FS-002

Instruction Manual version 1.0

Product Summary

Kit components

Components	Amount (×12)	Storage
3’ Ligation Adapter	12 µL	-20℃
RNA Ligase	12 µL	-20℃
5×Ligase Reaction Mix	50 µL	-20℃
RNase Inhibitor	12 µL	-20℃
Universal RT primer Mix	12 µL	-20℃
MMLV Reverse Transcriptase	12 µL	-20℃
Universal Reverse PCR Primer	200 µL	-20℃
RT Reaction Master Mix	150 µL	-20℃
RNA spike-in	12 µL	-20℃
RNA spike-in qPCR Primer Mix	24 µL	-20℃
RNase-free water	1 mL	-20℃

Additional required materials

- RNase-free 200µL PCR tubes
- Pipettors and tips
- Microcentrifuge for 200µL tubes
- Thermal cycler
- qPCR reaction reagents (SYBR Green)

Product description

The miRStar™ miRNA First-Strand cDNA Synthesis Kit is designed to create cDNA libraries from microRNAs and other small RNAs for qPCR detection. The method is based on ligation of a 5'-adenylated/3'-blocked oligonucleotide adapter (Adenylated 3' Ligation Adapter) to the 3' ends of the small RNAs, which provides the universal binding site for the reverse transcription (RT) primer.

The protocol uses a single-tube format for ligation, reverse transcription, and subsequent dilution of the cDNA library with 10 ng ~ 2.0 µg starting total RNA (Fig 1.). The Spike-in RNA can be used for monitoring the cDNA synthesis efficiency and as a reference for qPCR data comparison. Additional Universal qPCR Primer is available for order separately.

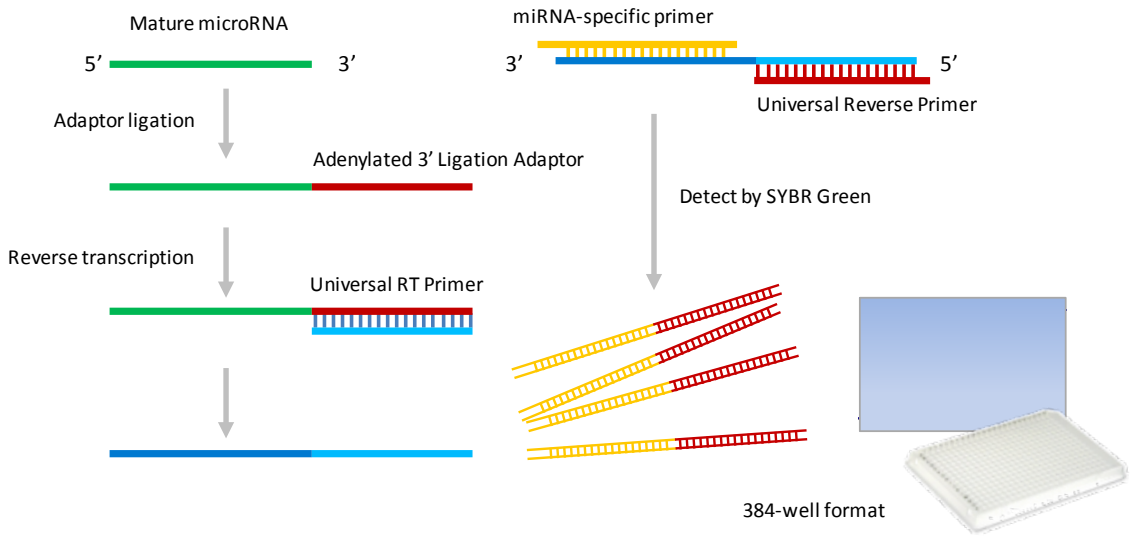

Figure 1. Workflow of miRStar™ miRNA First-Strand cDNA Synthesis.

Protocol

IMPORTANT: Total RNA samples should be extracted by a method that recovers small RNAs. Many silica filter-based RNA extraction kits do not recover RNAs less than ~100 bases.

Step A. 3'-Adapter ligation to the miRNA

1. Dilute the 3' Ligation Adapter supplied in the Kit with RNase-free water at 1/10 for 10 ~ 500 ng total RNA, or at 1/3 for 500 ng ~ 2 µg total RNA.
2. Mix the following components in a 200µL PCR tube for each sample.

4.5 µL	Total RNA in nuclease-free water
1.0 µL	diluted 3' Ligation Adapter
1.0 µL	RNA Spike-in
6.5 µL	total volume

3. Incubate the mix in a thermal cycler at 70°C for 2min; chill on ice immediately.
4. Add the following components.

2.0 µL	5×Ligase Reaction Mix
1.0 µL	RNA ligase
0.5 µL	RNase Inhibitor
10.0 µL final volume	

5. Incubate at 22°C for 60 min, 72°C for 2min, and chill on ice for 2 min.

Step B. First-Strand cDNA synthesis

1. Add 1 µL Universal RT Primer Mix to the tube from step 5, mix gently.
2. Incubate at 65°C for 2 min. Place on ice for at least 2 min.
3. Prepare Reverse Transcription Master Mix and add 10 µL to each sample above.

8.5 µL	RT Reaction Master Mix
0.5 µL	RNase Inhibitor
1.0 µL	MMLV Reverse Transcriptase
10 .0 µL	total volume per sample

4. Incubate at 42°C for 60 min; inactivate the reaction at 85°C for 5 min.
5. Add 80 µl of RNase-free water to 20 µL of the cDNA synthesis reaction (5-fold dilution). This is the miRNA cDNA library.

Step C. miRNA cDNA library validation

1. The generated miRNA cDNA library can be verified by running a qPCR on the RNA Spike-in control. Mix the following reagents:

2.0 µL	RNA spike-in qPCR Primer Mix
2.0 µL	miRNA cDNA library
5.0 µL	qPCR mix (SYBR® Green)
1.0 µL	nuclease-free water
10.0 µL	total volume

2. Run the qPCR cycles.

Cycles	Temperature	Time
1	95 ℃	10 minutes
40	95 ℃	10 seconds
	55-65 ℃	1 minute
Melting curve analysis		

3. A *Ct* value less than 30 for the RNA spike-in indicates a successful miRNA cDNA synthesis.

miRStar™ SYBR® Green Real-Time qPCR Master Mix

Cat#: AS-MR-006-5

Instruction Manual version 1.0

Product Summary

Kit components

Catalog Number	Contents	Size	Storage
AS-MR-006-5-01	2× SYBR® Green qPCR Master Mix (ROX+)	5 mL	-20°C
AS-MR-006-5-02	RNase-free Water	5 mL	-20°C

Product Description

SYBR® Green real-time quantitative PCR Master Mix is a highly optimized reaction mix containing all the components, including hot start Taq DNA polymerase, SYBR® Green I fluorescent dye, $MgCl_2$, dNTPs and stabilizers. You only need to add your template and primers to complete the qPCR reactions. The Master Mix is supplied as a 2× concentrate. The Master Mix has excellent performance properties (Fig. 1).

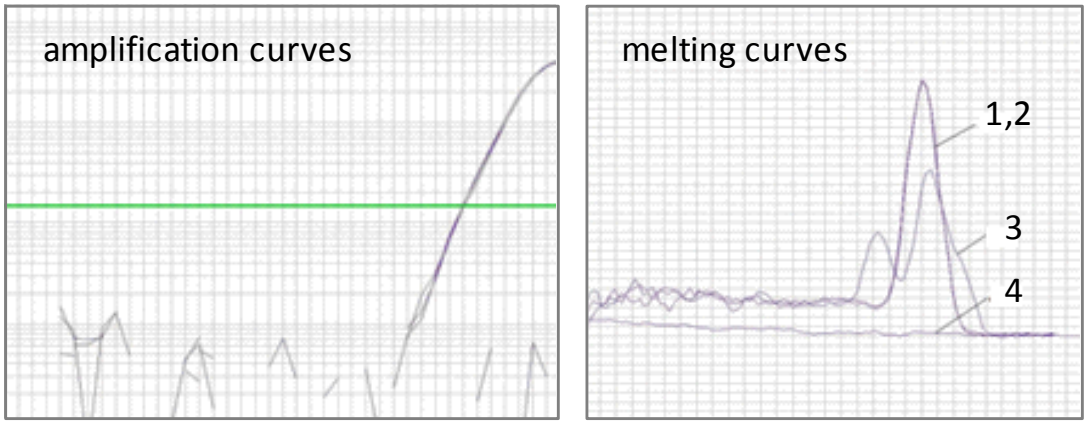

Figure 1. Highly specific amplification with miRStar™ Master Mix. In the post-amplification melting curve analysis, the PCR products by Arraystar Master Mix had single peak dissociation profiles (1 and 2), whereas the PCR products by master mix from competitor A (3 and 4) produced multiple dissociation peaks.

Protocol

1. Mix the following components with the Master Mix. For use with miRStar™ PCR Arrays, the Forward and Reverse qPCR primers are already included in the plate.

5.0 µL	qPCR mix (SYBR® Green)
__ µL	Forward primer
__ µL	Reverse primer
__ µL	Template
__ µL	PCR-grade water
10.0 µL	total volume

2. Run the qPCR using the following thermo cycling condition.

Cycles	Temperature	Time
1	95 ℃	10 minutes
40	95 ℃	10 seconds
	55-65 ℃	1 minute
Melting curve analysis		

Safety precautions

SYBR Green dye binds DNA with high affinity. Its safety profile is still being established. This product and its components should be handled only by persons trained in laboratory techniques. It is advisable that suitable protective clothing, such as laboratory overalls, safety glasses and gloves be worn. Avoid contact with skin or eyes. In case of contact, wash immediately with water.

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