Cancer MicroRNA qPCR Array with QuantiMir™

Cat. # RA610A-1

User Manual

Store kit at -20°C on receipt

(ver. 1-070306)

Contents

I. Introduction and Background

A. Overview

This manual provides details and information necessary to use the QuantiMir™ RT Kit to tag and convert small non-coding RNAs into detectable and quantifiable cDNAs. The system allows for the ability to quantitate fold differences of 95 separate microRNAs between 2 separate experimental RNA samples. The array plate also includes the U6 transcript as a normalization signal. All 95 microRNAs chosen for the array have published implications with regard to potential roles in cancer, cell development and apoptosis. To ensure optimal results, please read the entire manual before using the reagents and material supplied with this kit.

B. Importance of MicroRNAs and Other Small Non-Coding RNAs

The field of non-coding RNAs has gained increasing attention in recent years, particularly due to the discovery of small interfering RNAs (siRNAs) and micro RNAs (miRNA). These RNAs are short (typically 19-24 nucleotides) single stranded moieties that regulate the expression of target genes by interacting with complementary sites within the target mRNAs and either repressing translation or eliciting target mRNA degradation. miRNAs and siRNAs are conserved groups of non-coding RNAs with very important regulatory roles.

Mature miRNAs and siRNAs are excised from stem-loop precursors, which are themselves transcribed as part of longer primary transcripts. These primary miRNAs appear to be first processed by the RNase Drosha in the nucleus, after which the precursor miRNAs are exported to the cytoplasm where the RNase Dicer further processes them. These enzymes are also involved in the generation of mature small inhibitory RNAs (siRNA) from exogenously transferred double stranded siRNA precursors.

The current, standard method for detecting and quantifying novel miRNA and siRNA molecules involves Northern blotting with hybridization. Detecting and quantitating known miRNAs can be done using pre-designed reverse priming and reverse transcription followed by primer sets built for the specific miRNA for Real-time PCR analysis. These sets require many steps and can take several hours to complete and trouble-shoot. The QuantiMir™ RT kit provides all the reagents necessary to anchor-tail and convert small, non-coding RNAs into cDNA starting from total RNA samples. Once the user performs the reactions on their RNA samples, the cDNAs are ready to use for either End-point PCR experiments or to perform Real-time qPCR analysis. MicroRNA expression signatures have become more clinically

important recently with the discovery of distinct expression patterns and fold changes observed in Normal versus Tumor RNA samples. The Cancer MicroRNA qPCR Array with QuantiMir™ enables the discovery of new MicroRNA signatures using 95 different MicroRNAs known to be involved in apoptosis, cell fate, development, and cancer from a diverse set of RNA samples.

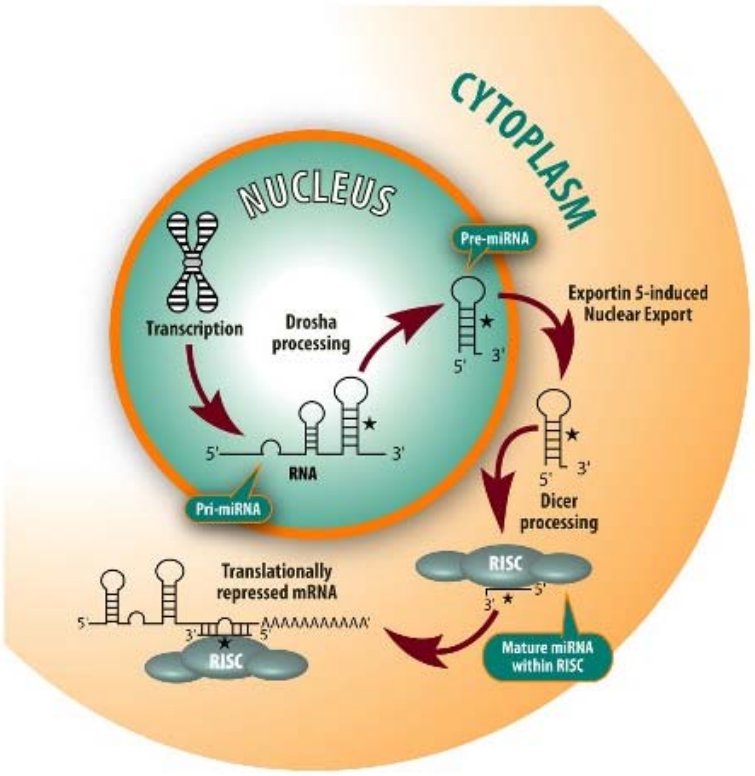
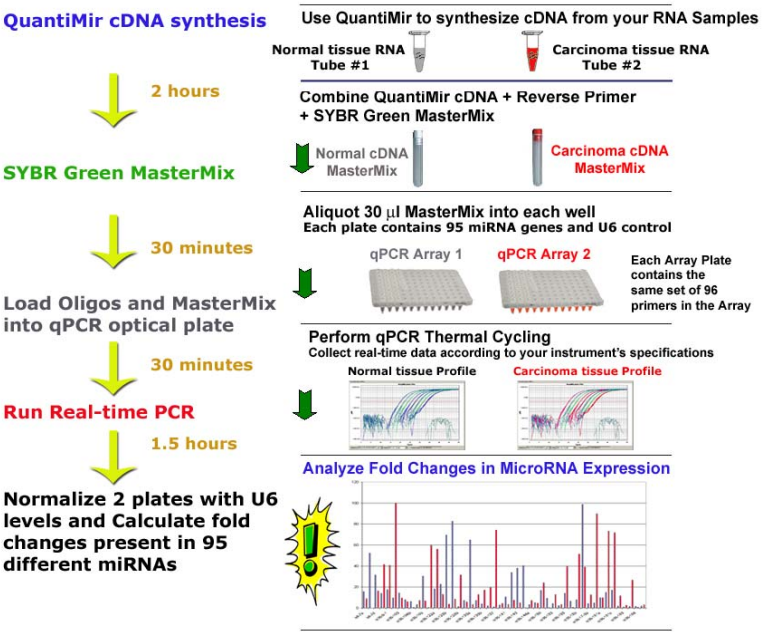


Fig. 1. Diagram of MicroRNA biogenesis, processing and function.

C. Overview of Entire Protocol

Example Cancer MicroRNA Expression Profile Setup



Discover MicroRNA Biomarkers!

Start with as little of 200 pg total RNA and convert to cDNA with the QuantiMir™ RT System. Use this cDNA as template mixed in with a SYBR® Green Mastermix plus the Universal reverse primer (included in kit). Aliquot SYBR® Green Mastermix into qPCR optical plate. Resuspend primers in Primer plate with 10µl RNase-free water, then pipet 1µl of each of the MicroRNA-specific primers from the Primer plate into the corresponding well of the qPCR plate (primer in well A1 goes into A1 in the qPCR plate, etc.). Perform Real-time PCR run and analyze fold changes in 95 different MicroRNAs after normalizing to the control U6 (well H12) in your 2 experimental sample tissues (in this example, Normal vs. Carcinoma). You can use the quantitation results MS Excel file provided on SBI's website with the kit to help you perform the normalization and fold-differences calculations with graphical analysis of your experiment if you choose.

D. List of Components

Each MicroRNA Cancer qPCR Array Kit contains the following components with enough material to perform 20 QuantiMir cDNA synthesis reactions and enough Primers in the Primer Array plate to perform 10 qPCR plates as outlined in this manual:

40 µl	5X PolyA Polymerase Buffer	10 µl Poly A Reaction (enough for 20 reactions)
10 µl	PolyA Polymerase	
20 µl	25 mM MnCl2	
30 µl	5 mM ATP	
10 µl	Oligo dT Adaptor	20 µl RT Reaction (enough for 20 reactions)
80 µl	5X Reverse Transcriptase Buffer	
20 µl	Reverse Transcriptase	
30 µl	0.1 M Dithiothreitol (DTT)	
40 µl	dNTP Mix	
600 µl	3' Universal Reverse PCR Primer	End-point or qPCR Assay (enough for 1,200 reactions)
	Array Primers, dried down in Primer plate (100 µmoles); resuspend in 10µl RNase-free Water	
1.2 ml	RNase-free Water	

The kit is shipped on blue ice and should be stored at -20°C upon arrival. Properly stored kits are stable for 1 year from the date received. The oligonucleotides for the specific MicroRNAs are dried-down in the wells of the optical qPCR plates. Resuspend in 10µl RNase-free water.

E. Additional Required Materials

- Real-time qPCR Instrument
- Instrument-specific optical qPCR plates
- Thermocycler (with heated lid)
- Thermocycler PCR tubes or plates for end-point reactions
- PCR Mastermix, including *Taq* polymerase for PCR
- 3.0-3.5% Agarose Gel in Tris-Borate EDTA (TBE) or Tris-Acetate EDTA (TAE) Buffer
- DNA Size Ladder with markers from 50 to 2,000 bp (Bio-Rad AmpliSize™ DNA Ladder; Cat. # 170-8200)
- Nuclease-free water for qPCR reactions

IMPORTANT:

- **Recommended 2X SYBR Green qPCR Mastermixes:**
  SBI has tested and recommends SYBR Green Master mix from three vendors: Power SYBR Master Mix® (Cat. #s 4368577, 4367650, 4367659, 4368706, 4368702, 4368708, 4367660) from Applied Biosystems; SYBR GreenER™ qPCR SuperMix for ABI PRISM® instrument from Invitrogen (Cat. #s 11760-100, 11760-500, and 11760-02K); and RT^2 Real-Time™ SYBR Green / ROX PCR (Cat. #s PA-012 and PA-112) from SuperArray.

II. Protocol

A. QuantiMir™ RT Reaction Setup

(for 1 RNA sample to be assayed on 1 qPCR plate)

It is important to start with total RNA that includes the small RNA fraction. RNA input can be as low as 10 ng/µl. For optimum signals, perform the following.

Dilute your RNA to ~100 ng/µl

Start: **In a thin-walled PCR tube or PCR-compatible plate well, combine:**

STEP 1: PolyA Tail

	5	µl	Total RNA (~500ng)
	2	µl	5X PolyA Buffer
+	1	µl	25mM $MnCl_2$
	1.5	µl	5mM ATP
	0.5	µl	PolyA Polymerase
	10	µl	Total in tube

Incubate for 30 min. at 37°C

STEP 2: Anneal Anchor dT Adaptor

Add: + 0.5 µl Oligo dT Adaptor

Heat for 5 min. at 60°C
Let cool to room temp for 2 min.

STEP 3: Synthesize cDNAs

Add:	4	µl	5X RT Buffer
	2	µl	dNTP mix
	1.5	µl	0.1M DTT
+	1.5	µl	RNase-free H_2O
	1	µl	Reverse Transcriptase
	20.5	µl	Total in tube

Incubate for 60 min. at 42°C
Heat for 10 min. at 95°C

Done!

*** The QuantiMir™ cDNAs can be stored at -20°C. For more sensitive applications, a single phenol:chloroform extraction with ethanol precipitation can be performed on the cDNA to remove proteins, unutilized dNTPs, and primers. Typically, this is not necessary.**

B. Real-time qPCR Reaction Setup

1. Mastermix qPCR Reaction Setup for 1 entire 96-well qPCR plate

To determine the expression profile for your miRNAs under study, mix the following for 1 entire qPCR plate:

For 1 entire plate:

	1,750	µl	**2X SYBR Green*** qPCR Mastermix buffer
+	60	µl	**Universal Reverse Primer (10 µM)**
	20	µl	**User synthesized QuantiMir™ cDNA**
	1,670	µl	**RNase-free water**
	3,500	µl	Total

Aliquot 29µl of Mastermix per well in your qPCR Plate.

* SBI has tested and recommends SYBR Green Master mix from three vendors:

1. Power SYBR Master Mix® (Cat. #s 4368577, 4367650, 4367659, 4368706, 4368702, 4368708, 4367660) from Applied Biosystems
2. SYBR GreenER™ qPCR SuperMix for ABI PRISM® instrument from Invitrogen (Cat. #s 11760-100, 11760-500, and 11760-02K)
3. RT² Real-Time™ SYBR Green / ROX PCR (Cat. #s PA-012 and PA-112) from SuperArray.

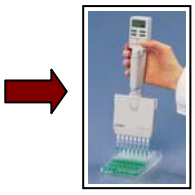
Resuspend Primers in Primer plate with 10µl RNase-free water per well before use. (the primers are dried-down in the Primer plate)

Then :

Load 1µl per well of each of the Primers from the Primer plate into your qPCR plate (well A1 into qPCR plate A1, etc.)

The Mastermix contents can be scaled up or down depending upon on your experimental needs. If you want to perform the reactions in triplicate, scale up the QuantiMir reactions by 3-fold and add 3X the RNA input. Or, simply follow the above recipe three times for each of the qPCR plates you want to run as replicates. Once reagents are loaded into the wells, cover the plate with an optical adhesive cover and spin briefly in a centrifuge to bring contents to bottom of wells. Place plate in the correct orientation (well A1, upper left) into the Real-time qPCR instrument and perform analysis run.

*** Use a Multichannel pipette to load the qPCR plate with MasterMix and Primers:** Pour the Mastermix into a reservoir trough and use a 8 or 12 channel pipette to load the entire 96-well qPCR plate with the Mastermix. Then load the primers from the primer plate to the qPCR plate using a separate multichannel pipette.

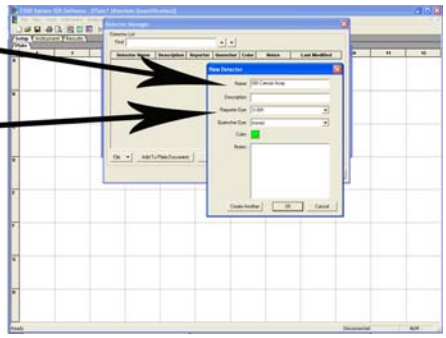


2. Real-time qPCR Instrument Parameters

Follow the guidelines as detailed for your specific Real-time instrumentation. The following parameters tested by SBI were performed on an Applied Biosystems 7300 Real-time PCR System but can also apply to an ABI 7500 or an ABI 7900 96-well system. The details of the thermal cycling conditions used in testing at SBI are below. A screenshot from SBI's ABI7300 Real-time instrument setup is shown below also. Default conditions are used throughout.

Create a detector:

1. Create a new Detector
2. Name the Detector (any name will do)
3. Select Reporter Dye as "SYBR"
4. Select Quencher Dye as (none)
5. Highlight all wells and select this new detector to measure the signals

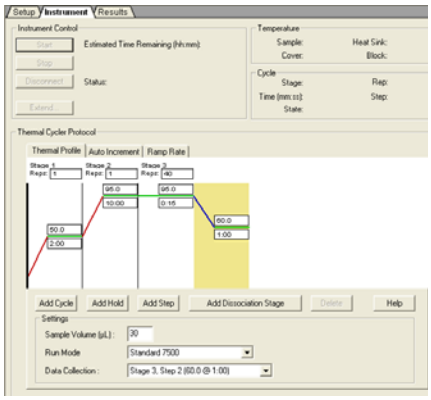


Instrument Setup:

qPCR cycling and data accumulation conditions:

1. 50°C 2 min.
2. 95°C 10 min.
3. 95°C 15 sec.
4. 60°C 1 min.

(40 cycles of steps 3 and 4), data read at 60°C 15 sec. Step (gold rectangle)



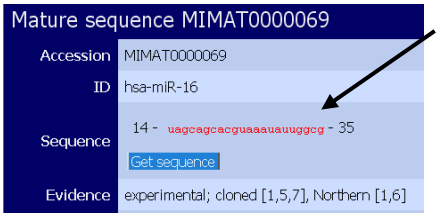
An additional recommendation is to include a melt analysis after the qPCR run to assess the Tm of the PCR amplicon to verify the specificity of the amplification reaction. Refer to the User Manual for your specific instrument to conduct the melt analysis and the data analyses of the amplification plots and Cycle Threshold (Ct) calculations. In general, Cycle thresholds should be set within the exponential phase of the amplification plots with software automatic baseline settings.

C. How the miRNA-Specific Primers are Designed for Detection and Quantitation in the Array

MicroRNAs typically range in size from 19 – 24 nt. We recommend using the exact sequence of the miRNA or siRNA being studied when designing the forward primer. If the miRNA under study is known and documented, using the miRBase database can be an easy starting point:
(http://www.mirbase.org).

An example of the known and documented miRNA, Human miR-16, is shown below.

Hsa-miR-16



Simple: Directly use sequence of mature miRNA as forward primer in oligo design.

The mature miRNA sequence 5' – uagcagcacguaaauauuggcg – 3' can be simply converted to a DNA sequence and used directly as the forward primer for end-point and qPCR analysis.

Forward primer for hsa-miR-16 (included in kit):
5' – TAGCAGCACGTAAATATTGGCG – 3'
Tm= 58.9°C, 45% GC and length = 22 bases.

All of the MicroRNA-specific primers for the QuantiMir™ Cancer qPCR Array were designed in this fashion. For the MicroRNA family members, degenerate primers were designed to detect the MicroRNA family members as listed in the Array plate arrangement (**Section II.D.**).

D. Cancer MicroRNA Array Arrangement

Plate Array Arrangement

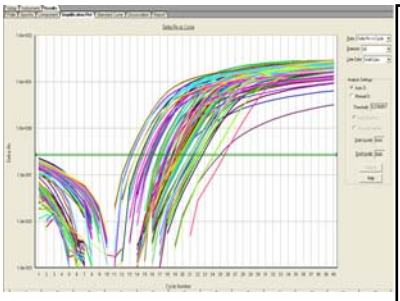
	1	2	3	4	5	6	7	8	9	10	11	12
A	let-7-family	miR-7	miR-92a	miR-93	miR-9	miR-101	miR-103	miR-106a	miR-106b	miR-107	miR-10b	miR-1
B	miR-122	miR-125a-5p	miR-125b	miR-126	miR-128	miR-132	miR-133a	miR-134	miR-135b	miR-136	miR-137	miR-140
C	miR-141	miR-142-3p	miR-143	miR-145	miR-146a	miR-149	miR-150	miR-151	miR-153	miR-154	miR-155	miR-15a
D	miR-15b	miR-16	miR-17*	miR-17	miR-181a	miR-181b	miR-181c	miR-181d	miR-183	miR-185	miR-186	miR-188-5p
E	miR-18a	miR-190	miR-191	miR-192	miR-194	miR-195	miR-196a	miR-197	miR-198	miR-199a+b	miR-30b	miR-19a+b
F	miR-95	miR-20a	miR-200a	miR-200b	miR-200c	miR-202	miR-203	miR-204	miR-205	miR-206	miR-21	miR-210
G	miR-214	miR-215	miR-372	miR-373	miR-218	miR-219	miR-22	miR-488	miR-221	miR-222	miR-223	miR-224
H	miR-23a	miR-24	miR-25	miR-26a	miR-26b	miR-27a+b	miR-30c	miR-29a+b+c	miR-30a*	miR-30a	miR-296	U6 snRNA

All 95 microRNAs chosen for the array have published implications with regard to potential roles in cancer, cell development and apoptosis (see **Section V.B.**). The array plate also includes the U6 transcript as a normalization signal (well H12). See SBI's website for access to these details.
http://www.systembio.com/index.php?id=microrna-research_expression-profiling_oncomir-collection#product_29_tab_1_2

III. Quality Control and Sample Data

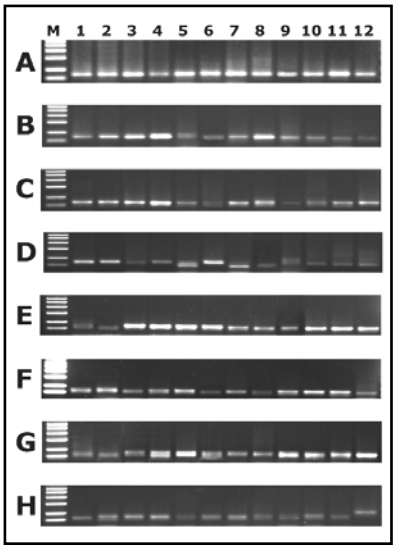
A. Cancer qPCR Array Primer Validation Tests

1. Real-time qPCR Validation



The Cancer qPCR Array plate was tested using a pool of 18 Normal and 7 Tumor RNA samples converted to cDNA using the QuantiMir RT Kit. The resulting cDNA was tested using 0.5 µl per well. Shown at left is the resulting Real-time amplification plot for the entire plate. The Cts ranged from 13.93 to 25.50, reflecting over a 4-log fold expression detection range. The experiment was performed as detailed in Section II.E. Quantitative signals were observed for all wells in the array.

2. End-point PCR Validation



The Cancer qPCR Array plate was tested using a pool of 18 Normal and 7 Tumor RNA samples converted to cDNA using the QuantiMir RT Kit. The resulting cDNA was tested using 0.5 μl per well. Shown at left is the resulting End-point PCR analysis with equal amount of each PCR reaction loaded. The PCR products were separated on a 3.5% agarose gel and imaged. The array locations are identical to the well labels for the specific MicroRNA primer being tested. Various band intensities reflect the spectrum of expression levels observed for the particular MicroRNA detected in the cDNA sample pool.

B. Sensitivity Tests

The QuantiMir™ cDNAs were synthesized using decreasing amounts of total starting RNA input from a pool of Human Brain, Heart, Kidney, Placenta, and Testes RNAs. Real-time quantitative qPCR assays were performed with Forward primers specific for Human miR-16 and Human miR-24 (For procedure, see **Section II.D.1,** Protocol: Real-time qPCR).

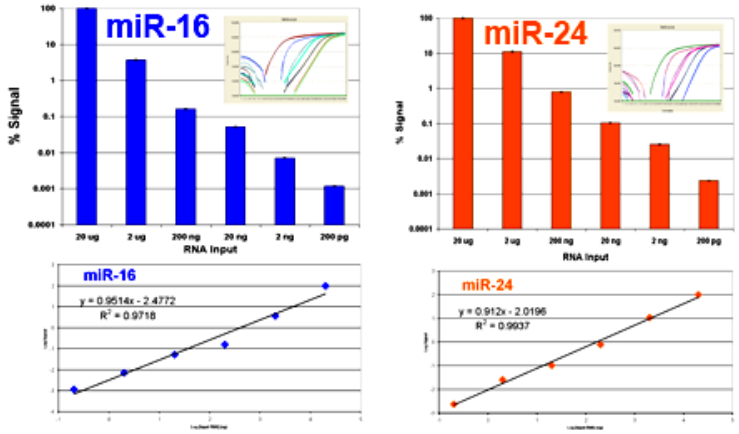
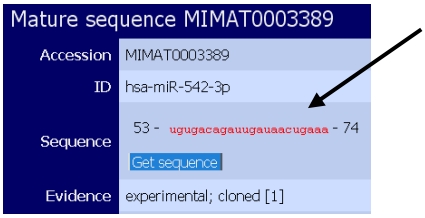


Fig. 2. Real-time qPCR data for Human miR-16 and Human miR-24. Real-time qPCR amplification plots are shown in the upper inset. Cycle threshold (Ct) values were determined using the software automatic baseline and Ct settings. The Bar graph depicts the relative %Signal per RNA input amount for the microRNA. The graph below shows the linear regression analysis with a R^2 value of 0.971 for miR-16 and 0.993 for miR-24. Both microRNAs are readily detectable down to 200 pg of total starting RNA input.

C. Specificity Tests

To assess the specificity and proper orientation of the miRNA array, oligonucleotide primers are synthesized both in the "sense" and the "antisense" orientation. An example for the known, documented miRNA miR-542-3p is detailed below.

Hsa-miR-542-3p



Sequence of mature miRNA as forward primer in "sense" oligo design, and then designed in the "antisense" oligo as control.

The mature miRNA sequence 5' – ugugacagauugauaacugaaa – 3' can be converted to a DNA sequence along with designing its complement, or "antisense" primer sequence.

Forward "sense" primer for hsa-miR-542-3p:
5' – TGTGACAGATTGATAACTGAAA – 3'

Forward "antisense" primer for hsa-miR-542-3p:
5' – TTTCAGTTATCAATCTGTCACA – 3'
Tm= 49.6°C, 32% GC and length = 22 bases.

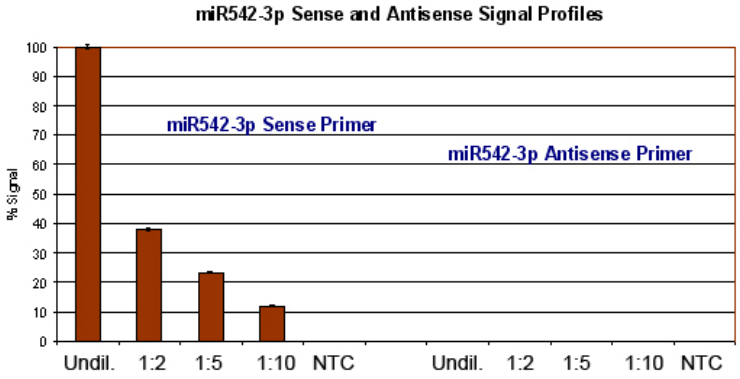


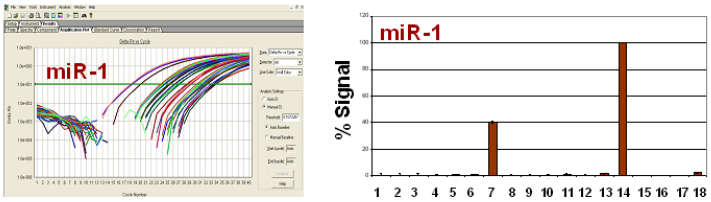
Fig. 3. Sense and antisense test of the QuantiMir™ cDNA. Dilutions of the QuantiMir™ cDNA template as well as no template controls (NTC) were tested with either sense or antisense orientation for the Human miR-542-3p molecule. Quantitative results are observed for the "sense" orientation of miR-542-3p. No signals are observed in the "antisense" or no template controls. The annealing temperature for the qPCR cycling conditions was lowered to 50°C.

D. Sample Data

1. Tissue Expression Pattern Determinations using the QuantiMir™ Kit on Normal Human Tissues

The QuantiMir™ cDNA sets were synthesized from 18 separate normal Human tissues and tested with 2 primers specific for 2 known miRNA molecules: miR-1 (heart and skeletal muscle-specific) and miR-122a (abundant in liver). The amplification plots and corresponding expression bar graphs are shown in **Figure 4**, **panels a and b.**

a.



b.

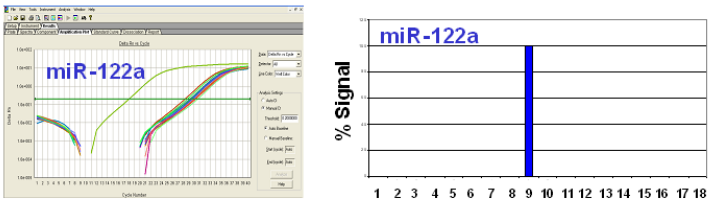


Fig. 4. Real-time qPCR data using primers specific for Human miR-1 (*Panel a.*) and for miR-122a (*Panel b.*). The amplification plots are shown on the left with the resulting expression profile bar graphs based on Ct values is shown on the right. The default qPCR cycling conditions were used with an annealing temperature of 60°C in Step 2 of Stage 3.

These two known miRNAs, miR-1 and mir-122a, have very specific tissue expression patterns. Real-time qPCR data confirmed that miR-1 is restricted to skeletal muscle and heart. The sensitivity of the assays also reveals very low but detectable signals in additional tissues. miR-122a is known to be highly abundant in liver.

2. Analysis of Tumor and Normal Tissue MicroRNA Expression Levels using the QuantiMir™ Kit and Real-time qPCR

The QuantiMir™ cDNAs were synthesized from both Normal and Tumor Breast, Lung, Ovary, Colon, and Lymph node RNAs. MicroRNA forward primers specific for miR-9-1, miR-155, miR-125, miR-145, miR-7, miR-17-3p, miR-18a, miR-20a and miR-92 were used to detect the corresponding microRNA species in the tissues detailed in the expression graph below (**Figure 5**). The signals were normalized to expression levels of the U6 snRNA transcript. Fold increases and decreases in Normal vs. Tumor tissues are graphed below and are consistent with published findings for the particular microRNA in the specific tumor type.

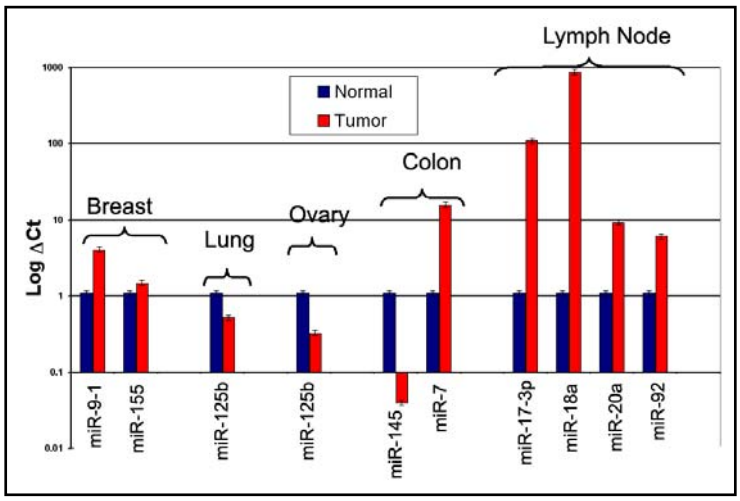


Fig. 5. Quantitative analysis of MicroRNA expression in tumor and normal tissue samples. The Bar graph data are grouped by tissue type with normal tissues in blue bars and tumor tissues in red bars. The specific MicroRNAs being detected are listed below the bar graphs. The expression levels are normalized to U6 snRNA transcript levels to control for RNA input. The MicroRNA expression levels are depicted as ΔCt vales (Y axis). Real-time assays were performed as described in **Section II.D.2** of this manual.

IV. Troubleshooting

Problem	Possible Solution
Too much background in qPCR signals	Use much less cDNA in the SYBR Green Mastermix.
No qPCR signals	1. Did you select SYBR Green as the Detector's Reporter Dye? 2. Did the U6 control work? 3. Use more cDNA in Mastermix. 4. Check Mastermix contents and try a subset with U6 as a positive control. 5. Also try lowering the Annealing Temperature to 50ºC.
How do I select the Threshold level for Ct analysis?	Typically, place the threshold setting in the upper third of the exponential phase of the amplification curve. Also, see the User Manual for your specific instrument or contact their technical support team for guidance.

V. References

A. General References

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VI. Appendix

A. Related Products

- **QuantiMir™ RT Kit** (Cat. # RA420A-1)
  Complete reagent kit for anchor-tagging small RNAs and converting them to quantifiable cDNA. Kit contains enough reagents for 20 RT reactions and can generate hundreds of qPCR templates. A universal reverse adaptor primer and positive control primers for Human U6 snRNA and Human miR-16 are also included with the kit.

- **Global MicroRNA Amplification Kit** (Cat. # RA400A-1)
  Simple amplification kit allows cDNA amplification for qRT-PCR and microarray studies from as little as 50 ng of starting total RNA.

- **Full Spectrum™ Complete Transcriptome RNA Amplification Kit** (Cat. # RA101A-1)
  The Full Spectrum RNA Amplification Kit provides an inexpensive method to amplify reverse transcribed RNA in a sequence independent, unbiased, and uniform manner with better representation of 5' end of mRNA sequences. This approach maintains the relative levels of each transcript in the starting mRNA samples—even when using starting amounts of RNA as low as 5ng or when using heavily degraded RNA.

- **Full Spectrum™ MultiStart Primers for T7 IVT**
  (Cat. # RA300A-2)
  Extract more data from your RNA than currently available primers in nearly all commercially-available T7 IVT kits using Full Spectrum™ technology. Just replace the existing T7 primer with the Full Spectrum™ primers. Compatible with Affymetrix GeneChip® hybridization.

B. Technical Support

For more information about SBI products and to download manuals in PDF format, please visit our web site:

http://www.systembio.com

For additional information or technical assistance, please call or email us at:

System Biosciences (SBI)
1616 North Shoreline Blvd.
Mountain View, CA 94043

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Technical Support: tech@systembio.com
Ordering Information: orders@systembio.com

VII. Licensing and Warranty Statement

Limited Use License

Use of the Cancer MicroRNA qPCR Array Kit wth QuantiMir™ (*i.e.*, the "Product") is subject to the following terms and conditions. If the terms and conditions are not acceptable, return all components of the Product to System Biosciences (SBI) within 7 calendar days. Purchase and use of any part of the Product constitutes acceptance of the above terms.

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SBI has pending patent applications related to the Product. For information concerning licenses for commercial use, contact SBI.

Limited Warranty

SBI warrants that the Product meets the specifications described in the accompanying Product Analysis Certificate. If it is proven to the satisfaction of SBI that the Product fails to meet these specifications, SBI will replace the Product or 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 SBI within 30 days of receipt of the Product.

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