Stem Cell MicroRNA qPCR Array with QuantiMir™

Cat. # RA620A-1

User Manual

Store kit at -20°C on receipt

(ver. 1-070508)

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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.

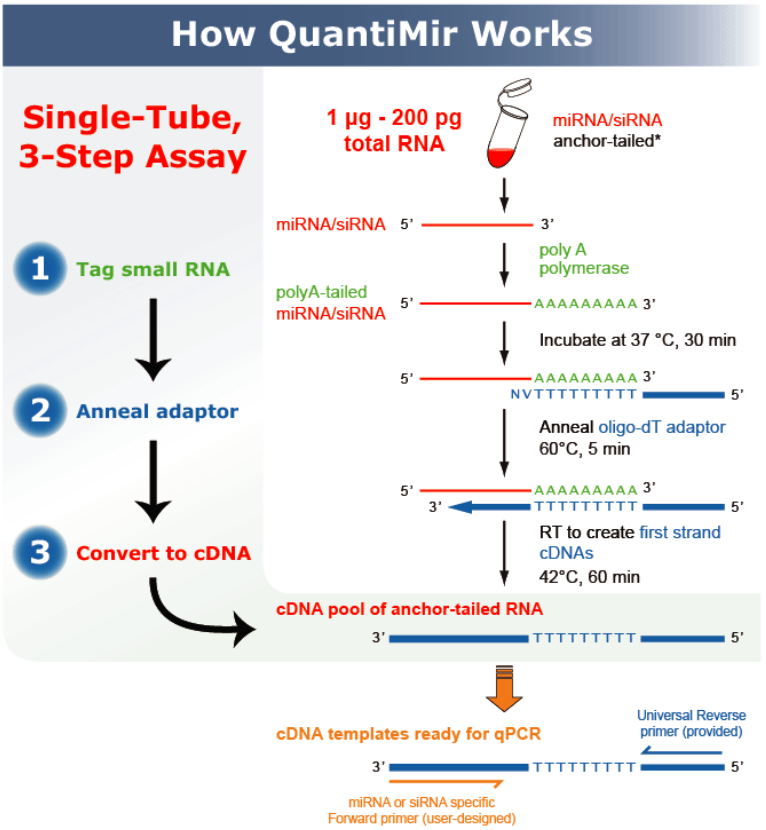
B. Importance of MicroRNAs and Other Small Non-Coding RNAs

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.

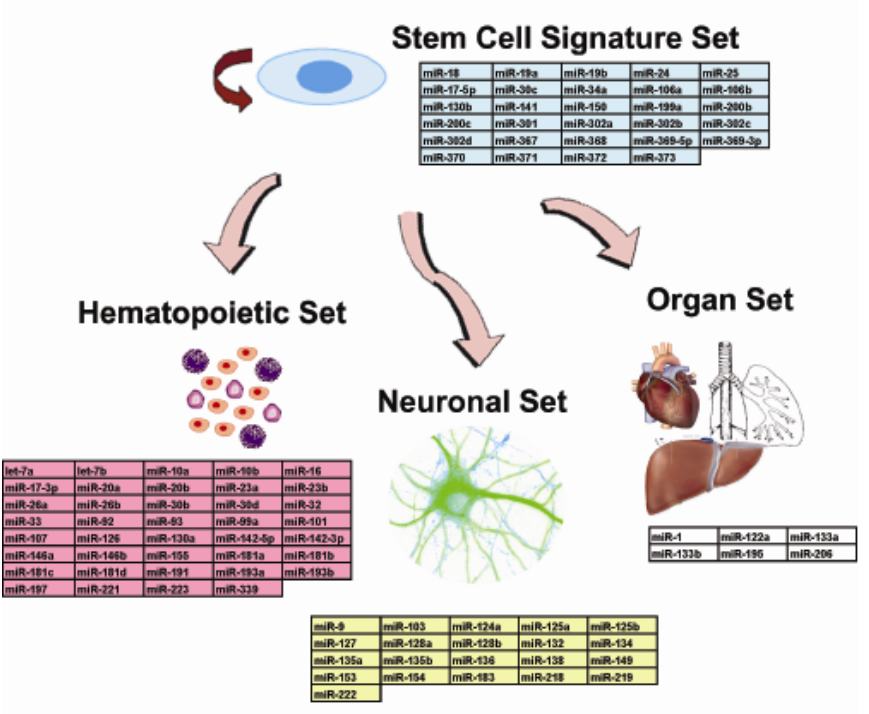
Mature miRNAs and siRNAs are excised from stem-loop precursors, which are themselves transcribed as part of longer primary transcripts. These primary miRNAs appear to be first processed by the RNase Drosha in the nucleus, after which the precursor miRNAs are exported to the cytoplasm where the RNase 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 during cellular development. The Stem Cell MicroRNA qPCR array includes primers specific for microRNAs known to be involved in stem cell self-renewal, hematopoiesis, neuronal development and differentiated tissue identification.



C. Overview of the Stem Cell microRNA Collection



Track cellular differentiation using microRNA Biomarkers

Start with as little of 200 pg total RNA and convert to cDNA with the QuantiMir™ RT System for individual microRNA measurements. 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 experimental sample cells. The Excel file provided on the CD with the kit is designed 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 Stem Cell 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
10 µl	PolyA Polymerase	(enough for 20 reactions)
20 µl	25 mM MnCl2	
30 µl	5 mM ATP	
10 µl	Oligo dT Adaptor	20 µl RT Reaction
80 µl	5X Reverse Transcriptase Buffer	(enough for 20 reactions)
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
	Array Primers, dried down in Primer plate (100 µmoles); resuspend in 10µl RNase-free Water	(enough for 1,200 reactions)
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.

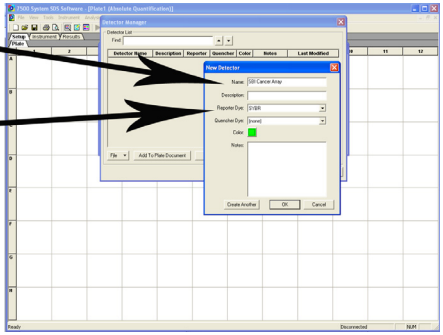
*** Use a Multichannel pipette to load the qPCR plate with MasterMix and Primers:** Pour the Mastermix into a reservoir trough and use a 8 or 12 channel pipette to load the entire 96-well qPCR plate with the Mastermix. Then load the primers from the primer plate to the qPCR plate using a separate multichannel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

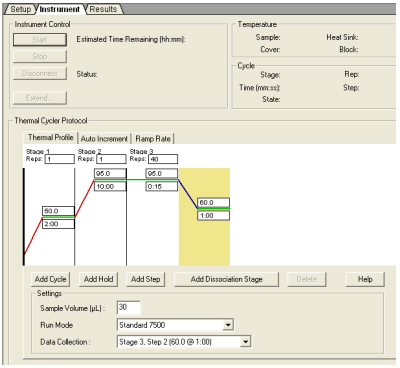


Instrument Setup:

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)



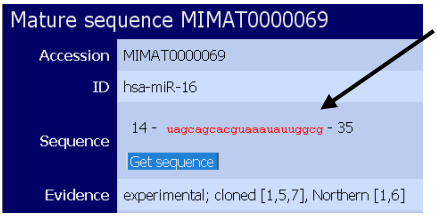
An additional recommendation is to include a melt analysis after the qPCR run to assess the Tm of the PCR amplicon to verify the specificity of the amplification reaction. Refer to the User Manual for your specific instrument to conduct the melt analysis and the data analyses of the amplification plots 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://microrna.sanger.ac.uk/sequences/search.shtml).

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:
5' – TAGCAGCACGTAAATATTGGCG – 3'
Tm= 58.9°C, 45% GC and length = 22 bases.

All of the MicroRNA-specific primers for the QuantiMir™ Stem Cell 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. Stem Cell MicroRNA Array Arrangement

	1	2	3	4	5	6	7	8	9	10	11	12
A	miR-18	miR-19a	miR-19b	miR-24	miR-25	miR-17-5p	miR-30c	miR-34a	miR-106a	miR-106b	miR-130b	miR-141
B	miR-150	miR-199a	miR-200b	miR-200c	miR-301	miR-302a	miR-302b	miR-302c	miR-302d	miR-367	miR-368	miR-369-5p
C	miR-369-3p	miR-370	miR-371	miR-372	miR-373	let-7a	let-7b	miR-10a	miR-10b	miR-16	miR-17-3p	miR-20a
D	miR-20b	miR-23a	miR-23b	miR-26a	miR-26b	miR-30b	miR-30d	miR-32	miR-33	miR-92	miR-93	miR-99a
E	miR-101	miR-107	miR-126	miR-130a	miR-142-5p	miR-142-3p	miR-146a	miR-146b	miR-155	miR-181a	miR-181b	miR-181c
F	miR-181d	miR-191	miR-193a	miR-193b	miR-197	miR-221	miR-223	miR-339	miR-9	miR-103	miR-124a	miR-125a
G	miR-125b	miR-127	miR-128a	miR-128b	miR-132	miR-134	miR-135a	miR-135b	miR-136	miR-138	miR-149	miR-153
H	miR-154	miR-183	miR-218	miR-219	miR-222	miR-1	miR-122a	miR-133a	miR-133b	miR-195	miR-206	U6

Stem Cell Signature Set Neuronal Set

Hematopoietic Set Organ Set

All 95 microRNAs chosen for the array have published implications with regard to potential roles in stem cell self-renewal, hematopoiesis, neuronal development and differentiated tissue identification (see **Section V.B.**). The array plate also includes the U6 RNA as a normalization signal (well H12).

Well	miRNA	Accn	MicroRNA Sequence
A1	miR-18a	MIMAT0000072	UAAGGUGCAUCUAGUGCAGAUA
A2	miR-19a	MIMAT0000073	UGUGCAAAUCUAUGCAAAACUGA
A3	miR-19b	MIMAT0000074	UGUGCAAAUCCAUGCAAAACUGA
A4	miR-24	MIMAT0000080	UGGCUCAGUUCAGCAGGAACAG
A5	miR-25	MIMAT0000081	CAUUGCACUUGUCUCGGUCUGA
A6	miR-17-5p	MIMAT0000070	CAAAGUGCUUACAGUGCAGGUAGU
A7	miR-30c	MIMAT0000244	UGUAAACAUCCUACACUCUCAGC
A8	miR-34a	MIMAT0000255	UGGCAGUGUCUUAGCUGGUUGUU
A9	miR-106a	MIMAT0000103	AAAAGUGCUUACAGUGCAGGUAGC
A10	miR-106b	MIMAT0000680	UAAAGUGCUGACAGUGCAGAU
A11	miR-130b	MIMAT0000691	CAGUGCAAUGAUGAAAGGGCAU
A12	miR-141	MIMAT0000432	UAACACUGUCUGGUAAAGAUGG
B1	miR-150	MIMAT0000451	UCUCCCAACCCUUGUACCAGUG
B2	miR-199a	MIMAT0000231	CCCAGUGUUCAGACUACCUGUUC
B3	miR-200b	MIMAT0000318	UAAUACUGCCUGGUAAUGAUGAC
B4	miR-200c	MIMAT0000617	UAAUACUGCCGGGUAAUGAUGG
B5	miR-301	MIMAT0000688	CAGUGCAAUAGUAUUGUCAAAGC
B6	miR-302a	MIMAT0000683	UAAACGUGGAUGUACUUGCUUU
B7	miR-302b	MIMAT0000714	ACUUUAACAUGGAAGUGCUUUCU
B8	miR-302c	MIMAT0000716	UUUAACAUGGGGGUACCUGCUG
B9	miR-302d	MIMAT0000718	UAAGUGCUUCCAUGUUUGAGUGU
B10	miR-367	MIMAT0000719	AAUUGCACUUUAGCAAUGGUGA
B11	miR-368	MIMAT0000720	ACAUAGAGGAAAUUCCACGUUU
B12	miR-369-5p	MIMAT0001621	AGAUCGACCGUGUUAUAUUCGC
C1	miR-369-3p	MIMAT0000721	AAUAAUACAUGGUUGAUCUUU
C2	miR-370	MIMAT0000722	GCCUGCUGGGGUGGAACCUGG
C3	miR-371	MIMAT0000723	GUGCCGCCAUCUUUUGAGUGU
C4	miR-372	MIMAT0000724	AAAGUGCUGCGACAUUUGAGCGU
C5	miR-373	MIMAT0000725	ACUCAAAAUGGGGGCGCUUUCC
C6	let-7a	MIMAT0000062	UGAGGUAGUAGGUUGUAUAGUU
C7	let-7b	MIMAT0000063	UGAGGUAGUAGGUUGUGUGGUU
C8	miR-10a	MIMAT0000253	UACCCUGUAGAUCCGAAUUUGUG
C9	miR-10b	MIMAT0000254	UACCCUGUAGAACCGAAUUUGU
C10	miR-16	MIMAT0000069	UAGCAGCACGUAAAUAUUGGCG
C11	miR-17-3p	MIMAT0000071	ACUGCAGUGAAGGCACUUGU
C12	miR-20a	MIMAT0000075	UAAAGUGCUUAUAGUGCAGGUAG
D1	miR-20b	MIMAT0001413	CAAAGUGCUCAUAGUGCAGGUAG
D2	miR-23a	MIMAT0000078	AUCACAUUGCCAGGGAUUUCC
D3	miR-23b	MIMAT0000418	AUCACAUUGCCAGGGAUUACC
D4	miR-26a	MIMAT0000082	UUCAAGUAAUCCAGGAUAGGC
D5	miR-26b	MIMAT0000083	UUCAAGUAAUUCAGGAUAGGUU
D6	miR-30b	MIMAT0000420	UGUAAACAUCCUACACUCAGCU
D7	miR-30d	MIMAT0000245	UGUAAACAUCCCCGACUGGAAG
D8	miR-32	MIMAT0000090	UAUUGCACAUUACUAAGUUGC
D9	miR-33	MIMAT0000091	GUGCAUUGUAGUUGCAUUG
D10	miR-92	MIMAT0000092	UAUUGCACUUGUCCCGGCCUG
D11	miR-93	MIMAT0000093	AAAGUGCUGUUCGUGCAGGUAG
D12	miR-99a	MIMAT0000097	AACCCGUAGAUCCGAUCUUGUG

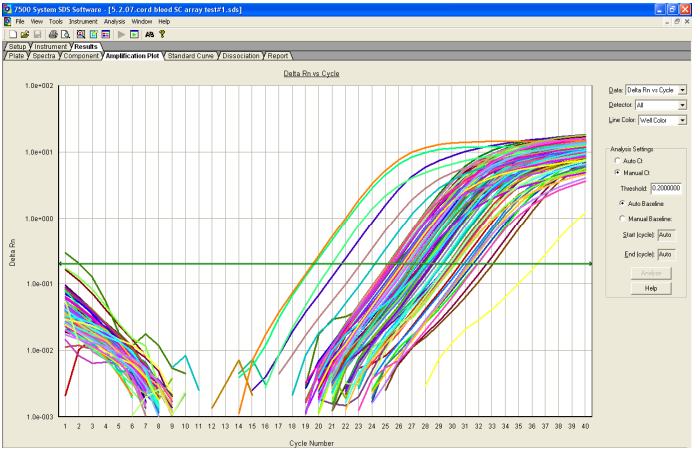
Well	miRNA	Accn	MicroRNA Sequence
E1	miR-101	MIMAT0000099	UACAGUACUGUGAUAACUGAAG
E2	miR-107	MIMAT0000104	AGCAGCAUUGUACAGGGCUAUCA
E3	miR-126	MIMAT0000444	CAUUAUUACUUUUGGUACGCG
E4	miR-130a	MIMAT0000425	CAGUGCAAUGUUAAAAGGGCAU
E5	miR-142-5p	MIMAT0000433	CAUAAAGUAGAAAGCACUAC
E6	miR-142-3p	MIMAT0000434	UGUAGUGUUUCCUACUUUAUGGA
E7	miR-146a	MIMAT0000449	UGAGAACUGAAUUCCAUGGGUU
E8	miR-146b	MIMAT0002809	UGAGAACUGAAUUCCAUAGGCU
E9	miR-155	MIMAT0000646	UUAAUGCUAAUCGUGAUAGGGG
E10	miR-181a	MIMAT0000256	AACAUUCAACGCUGUCGGUGAGU
E11	miR-181b	MIMAT0000257	AACAUUCAUUGCUGUCGGUGGG
E12	miR-181c	MIMAT0000258	AACAUUCAACCUGUCGGUGAGU
F1	miR-181d	MIMAT0002821	AACAUUCAUUGUUGUCGGUGGGUU
F2	miR-191	MIMAT0000440	CAACGGAAUCCCAAAAGCAGCU
F3	miR-193a	MIMAT0000459	AACUGGCCUACAAAGUCCCAG
F4	miR-193b	MIMAT0002819	AACUGGCCCUCAAAGUCCCGCUUU
F5	miR-197	MIMAT0000227	UUCACCACCUUCUCCACCCAGC
F6	miR-221	MIMAT0000278	AGCUACAUUGUCUGCUGGGUUUC
F7	miR-223	MIMAT0000280	UGUCAGUUUGUCAAAUACCCC
F8	miR-339	MIMAT0000764	UCCCUGUCCUCCAGGAGCUCA
F9	miR-9	MIMAT0000441	UCUUUGGUUAUCUAGCUGUAUGA
F10	miR-103	MIMAT0000101	AGCAGCAUUGUACAGGGCUAUGA
F11	miR-124a	MIMAT0000422	UUAAGGCACGCGGUGAAUGCCA
F12	miR-125a	MIMAT0000443	UCCCUGAGACCCUUUAACCUGUG
G1	miR-125b	MIMAT0000423	UCCCUGAGACCCUAACUUGUGA
G2	miR-127	MIMAT0000446	UCGGAUCCGUCUGAGCUUGGCU
G3	miR-128a	MIMAT0000424	UCACAGUGAACCGGUCUCUUUU
G4	miR-128b	MIMAT0000676	UCACAGUGAACCGGUCUCUUUC
G5	miR-132	MIMAT0000426	UAACAGUCUACAGCCAUGGUCG
G6	miR-134	MIMAT0000447	UGUGACUGGUUGACCAGAGGG
G7	miR-135a	MIMAT0000428	UAUGGCUUUUUAUUCCUAUGUGA
G8	miR-135b	MIMAT0000758	UAUGGCUUUUCAUUCCUAUGUG
G9	miR-136	MIMAT0000448	ACUCCAUUUGUUUUGAUGAUGGA
G10	miR-138	MIMAT0000430	AGCUGGUGUUGUGAAUC
G11	miR-149	MIMAT0000450	UCUGGCUCCGUGUCUUCACUCC
G12	miR-153	MIMAT0000439	UUGCAUAGUCACAAAAGUGA
H1	miR-154	MIMAT0000452	UAGGUUAUCCGUGUUGCCUUCG
H2	miR-183	MIMAT0000261	UAUGGCACUGGUAGAAUUCACUG
H3	miR-218	MIMAT0000275	UUGUGCUUGAUCUAACCAUGU
H4	miR-219	MIMAT0000276	UGAUUGUCCAAACGCAAUUCU
H5	miR-222	MIMAT0000279	AGCUACAUCUGGCUACUGGGUCUC
H6	miR-1	MIMAT0000416	UGGAAUGUAAAGAAGUAUGUA
H7	miR-122a	MIMAT0000421	UGGAGUGUGACAAUGGUGUUUGU
H8	miR-133a	MIMAT0000427	UUGGUCCCCUUCAACCAGCUGU
H9	miR-133b	MIMAT0000770	UUGGUCCCCUUCAACCAGCUA
H10	miR-195	MIMAT0000461	UAGCAGCACAGAAAUAUUGGC
H11	miR-206	MIMAT0000462	UGGAAUGUAAGGAAGUGUGUGG
H12	U6	NCBI: X07425.1	caccacguuuauacgccggug

III. Quality Control and Sample Data

A. Stem Cell qPCR Array Primer Validation Tests

1. Real-time qPCR Validation

The Stem Cell qPCR Array plate was tested using **Human cord blood CD34+ stem cell RNA** samples converted to cDNA using the QuantiMir RT Kit. The resulting cDNA was tested using 0.2 µl per well. Shown at left is the resulting Real-time amplification plot for the entire plate. The Cts ranged from 19.47 to 36.30, reflecting approximately a 5-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.



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 in the QuantiMir™ RT Kit User Manual).

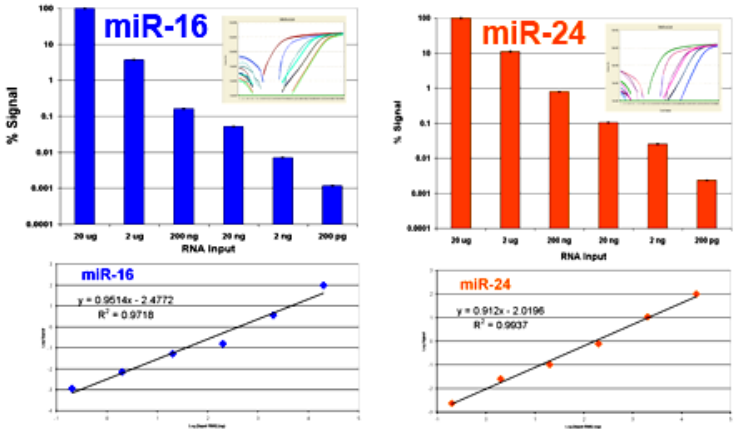
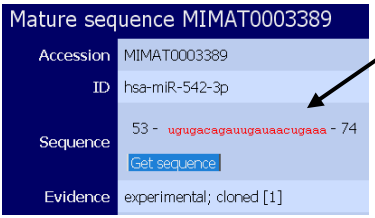


Fig. 2. Real-time qPCR data for Human miR-16 and Human miR-24. Real-time qPCR amplification plots are shown in the upper inset. Cycle threshold (Ct) values were determined using the software automatic baseline and Ct settings. The Bar graph depicts the relative %Signal per RNA input amount for the microRNA. 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.

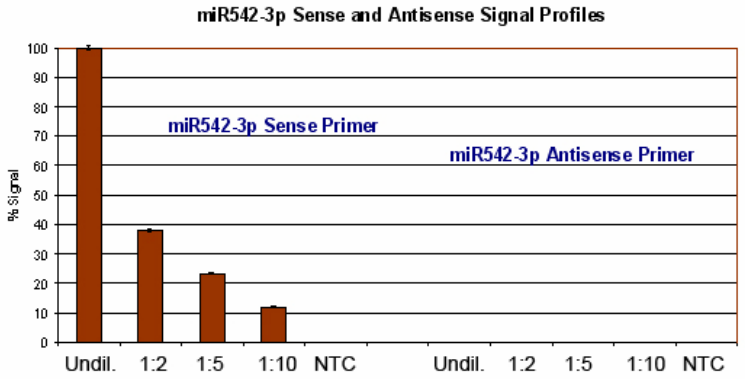


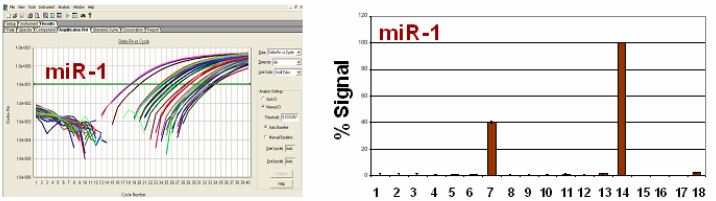
Fig. 3. Sense and antisense test of the QuantiMir™ cDNA. Dilutions of the QuantiMir™ cDNA template as well as no template controls (NTC) were tested with either sense or antisense orientation for the Human miR-542-3p molecule. Quantitative results are observed for the "sense" orientation of miR-542-3p. 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.

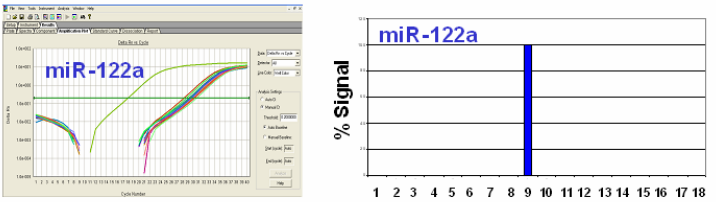


Fig. 4. Real-time qPCR data using primers specific for Human miR-1 (*Panel a.*) and for miR-122a (*Panel b.*). The amplification plots are shown on the left with the resulting expression profile bar graphs based on Ct values is shown on the right. The default qPCR cycling conditions were used with an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

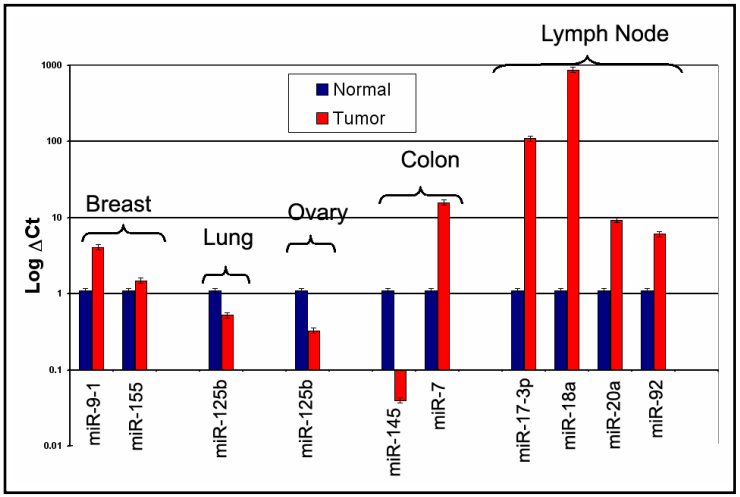


Fig. 5. Quantitative analysis of MicroRNA expression in tumor and normal tissue samples. The Bar graph data are grouped by tissue type with normal tissues in blue bars and tumor tissues in red bars. The specific MicroRNAs being detected are listed below the bar graphs. The expression levels are normalized 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 the QuantiMir™ RT Kit User 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.

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VI. Appendix

A. Related Products

- **Cancer MicroRNA qPCR Array with QuantiMir** (Cat. # RA610A-1)
  The Cancer MicroRNA qPCR Array enables the simultaneous quantitation of 95 MicroRNAs known to be involved in Cancer. The technology utilizes the QuantiMir RT Kit which tags and converts all small RNAs into cDNA, ready to use as template for real-time qPCR. A universal reverse adaptor primer and positive control primers for Human U6 snRNA are inculded in the kit.

- **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.

- **miRANDA™ qPCR-Ready miRNA Tissue Expression Array Kit** (Cat. # RA600A-1)
  18 Human individual normal tissue miRNA cDNAs arrayed into a qPCR optical plate (4 complete sets of the 18 tissues, 72 individual reactions). A universal reverse adaptor primer and a positive control forward primer (U6 snRNA) are also included with the kit.

- **miRANDA™ Universal miRNA cDNA template** (Cat. # RA601A-1)
  Pool of all 18 Human miRNA cDNAs (enough for 20 50 μl-reactions), a universal reverse adaptor primer, and a positive control forward primer (U6 snRNA)

- **MicroRNA Discovery™ Kit** (Cat. # RA410A-1)
  Rapid identification of new MicroRNAs and MicroRNA-like molecules. Amplification and cloning can be initiated in a single day (3 steps, 1 day.) The alternative method takes approximately 1 week (9 steps.)

- **Pre-Made MicroRNA-Enriched cDNAs** (Cat. # RA500A-1 – RA509A-1)
  Tissue-specific amplified cDNA generated by SBI using the MicroRNA Discovery™ Kit can be used for cloning microRNA.

- **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 5 ng 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:

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1616 North Shoreline Blvd.
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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 Stem Cell 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.

Purchase of the product does not grant any rights or license for use other than those explicitly listed in this Licensing and Warranty Statement. Use of the Product for any use other than described expressly herein may be covered by patents or subject to rights other than those mentioned. SBI disclaims any and all responsibility for injury or damage which may be caused by the failure of the buyer or any other person to use the Product in accordance with the terms and conditions outlined herein.

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.

SBI's liability is expressly limited to replacement of Product or a refund limited to the actual purchase price. SBI's liability does not extend to any damages arising from use or improper use of the Product, or losses associated with the use of additional materials or reagents. This limited warranty is the sole and exclusive warranty. SBI does not provide any other warranties of any kind, expressed or implied, including the merchantability or fitness of the Product for a particular purpose.

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