miRNome microRNA Profilers QuantiMir™

Human Cat. # RA660A-1
Mouse Cat. # RA670A-1
Rat Cat. # RA680B-1

User Manual

Store kit at -20°C on receipt

A limited-use label license covers this product. By use of this product, you accept the terms and conditions outlined in the Licensing and Warranty Statement contained in this user manual.

(ver. 5-072613)

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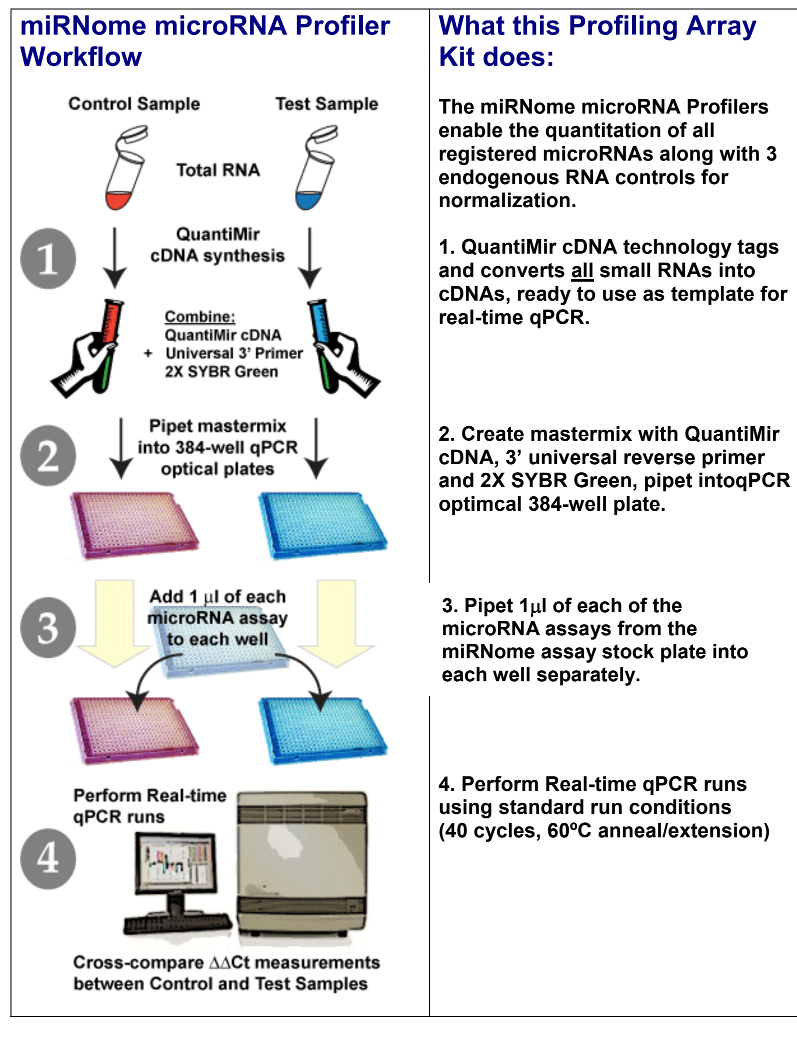
I. Introduction and Background

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 microRNAs between 20 separate experimental RNA samples. The array plate also includes three endogenous RNA assays as normalization signals. To ensure optimal results, please read the entire manual before using the reagents and material supplied with this kit.

These MicroRNA qPCR Array Panels come with all the reagents necessary to tag and convert small RNAs from 20 different total RNA samples into quantifiable cDNA using the sensitive QuantiMir™ technology. The kits include assays in pre-formatted plates for either all human, mouse or rat microRNAs with three endogenous reference RNA controls on each plate. All microRNA assays based on the Sanger miRBase microRNA database registered entries.

B. miRNome microRNA qPCR profiler workflow



What this Profiling Array Kit does:

The miRNome microRNA Profilers enable the quantitation of all registered microRNAs along with 3 endogenous RNA controls for normalization.

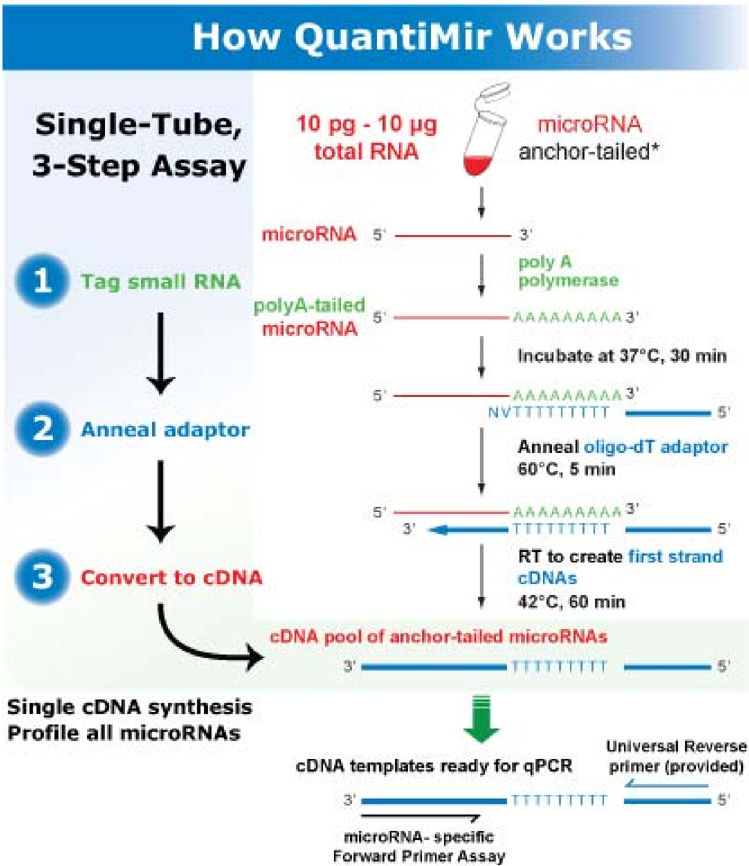
1. QuantiMir cDNA technology tags and converts <u>all</u> small RNAs into cDNAs, ready to use as template for real-time qPCR.

2. Create mastermix with QuantiMir cDNA, 3' universal reverse primer and 2X SYBR Green, pipet intoqPCR optimcal 384-well plate.

3. Pipet 1µl of each of the microRNA assays from the miRNome assay stock plate into each well separately.

4. Perform Real-time qPCR runs using standard run conditions (40 cycles, 60ºC anneal/extension)

C. How QuantiMir cDNA synthesis works

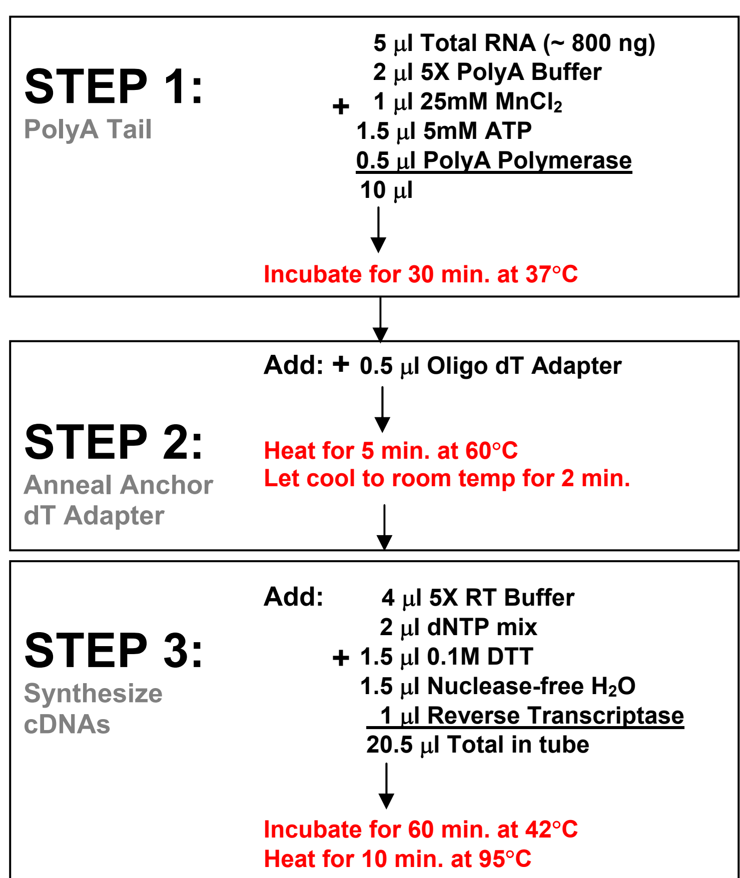


II. Protocol

A. QuantiMir RT reaction setup

(for 1 RNA sample to be assayed on multiple qPCR 384-well plate)

It is important to start with total RNA that includes the small RNA fraction. RNA input can be as low as 100 ng total. For optimum signals, perform the following.



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, unused dNTPs and primers, typically this is not necessary.

B. Real-time qPCR Reaction Setup

Before using the miRNome kit, you will need to aliquot the stock primer plate. First dissolve the stock primers with 22 µl water. Pipette up and down a few times to dissolve the primers. Then spin down the plate to collect the water at the bottom of the wells. Wait about 20-30 minutes. Then aliquot 1 µl per well into 20 separate plates. Plates can be dried, or covered and stored at 4°C. These steps will ensure that the stock primer concentration does not change due to evaporation.

1. Mastermix qPCR Reaction Set up for ONE entire 384-well qPCR plate

To determine the expression profile for your miRNAs under study, mix the following for 1 entire 384 qPCR plate:

For 1 entire plate :

+	1150 µl	**2X SYBR Green*** qPCR Mastermix buffer
	39 µl	**Universal Reverse Primer (10µM)**
	5 µl	**User-synthesized QuantiMir cDNA**
	1090 µl	**Nuclease-free water**
	2284 µl	Total

Aliquot 5µl of Mastermix *into every well* in your 384-well qPCR Plate

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

Resuspend the MicroRNA assay Primers with 20µl water in each well before use. Spin briefly to collect contents at bottom of wells.

Then :

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

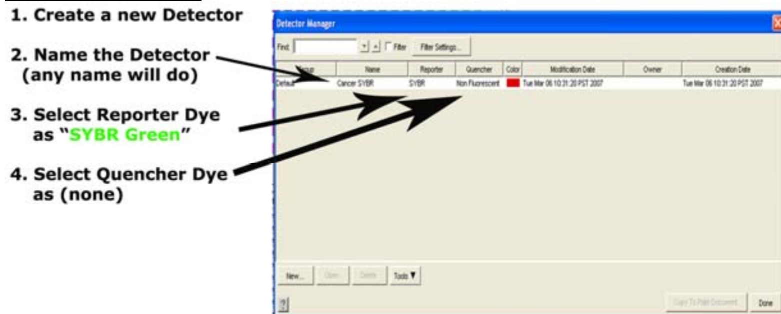
Once reagents are loaded into the wells, cover the plate with an optical adhesive cover and spin briefly in a centrifuge to bring contents to bottom of wells. Place plate in the correct orientation (well A1, upper left) into the Real-time qPCR instrument and perform analysis run.

*** 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 384-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 7900HT Real-time PCR System but can also apply to any other 384-well system. The details of the thermal cycling conditions used in testing at SBI are below. A screenshot from SBI's 7900HT Real-time instrument setup is shown below also. Default conditions are used throughout.

<u>**Create a detector**</u>:



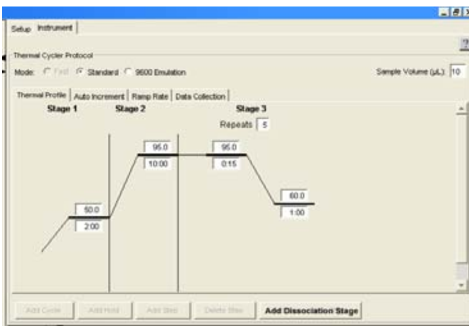
Instrument setup:

qPCR cycling and data accumulation conditions:

Standard Protocol

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

(40 cycles of Stage 3), data read at 60°C 1 min. Step.



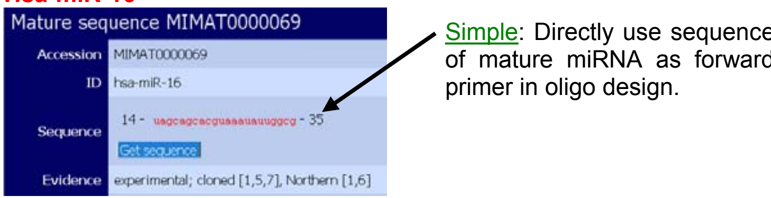
An additional recommendation is to include a **Dissociation Stage** after the qPCR run to assess the Tm of the PCR amplicon to verify the specificity of the amplification reaction. Refer to the User Manual for your specific instrument to conduct the melt analysis and the data analyses of the amplification 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.

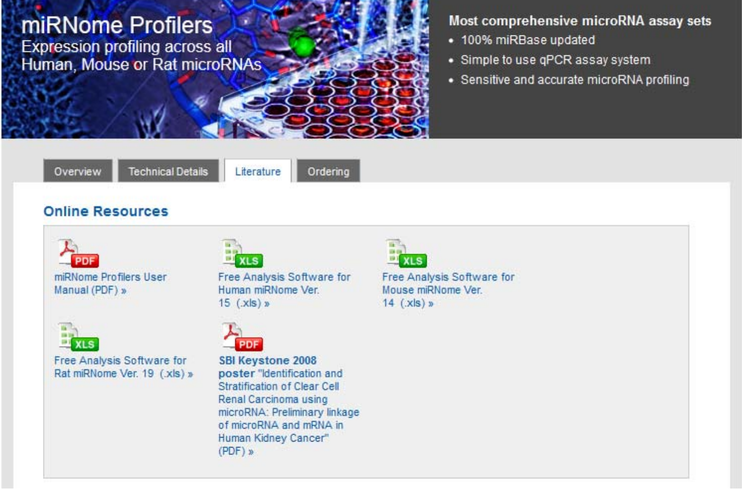
The mature miRNA sequence 5' – uagcagcacguaaauauuggcg – 3' can be simply converted to a DNA sequence and used directly as the forward primer for end-point and qPCR analysis.

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

All of the Micro-RNA-specific primers for the HT 384 microRNA Profiler were designed in this fashion.

D. miRNome MicroRNA Profiler Array arrangement

The array plate contains assays for microRNAs and also includes three endogenous reference RNAs as normalization signals. Please see the SBI website to download the qPCR array arrangement and ΔΔCT analysis software. **www.systembio.com/mirnome**


miRNome Profilers
Expression profiling across all
Human, Mouse or Rat microRNAs
Most comprehensive microRNA assay sets
• 100% miRBase updated
• Simple to use qPCR assay system
• Sensitive and accurate microRNA profiling
Overview
Technical Details
Literature
Ordering
Online Resources
miRNome Profilers User Manual (PDF) »
Free Analysis Software for Human miRNome Ver. 15 (.xls) »
Free Analysis Software for Mouse miRNome Ver. 14 (.xls) »
Free Analysis Software for Rat miRNome Ver. 19 (.xls) »
SBI Keystone 2008 poster "Identification and Stratification of Clear Cell Renal Carcinoma using microRNA: Preliminary linkage of microRNA and mRNA in Human Kidney Cancer" (PDF) »

E. List of Components

Each miRNome microRNA Profiler Kit contains the following components with enough material to perform 20 QuantiMir cDNA synthesis reactions and enough Primers in the microRNA assay array plate to perform 20 384-well qPCR plate sets as outlined in this manual:

Optimized for 6 µl qPCR reactions

Amount	Unit	Component
40	µl	5X PolyA polymerase Buffer
10	µl	PolyA polymerase
20	µl	25 mM $MnCl_2$
30	µl	5 mM ATP
10	µl	3′ Oligo dT Adaptor
80	µl	5X Reverse Transcriptase Buffer
20	µl	Reverse Transcriptase
30	µl	0.1 M Dithiothreitol (DTT)
50	µl	dNTP Mix
2400	µl	3′ Universal Reverse PCR Primer
20	µl	Each of microRNA assays + three controls
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. **Resuspend each primer assay well with 20µl water before use.**

F. Additional Required Materials

- Nuclease-free water for qPCR reactions
- 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)

IMPORTANT:

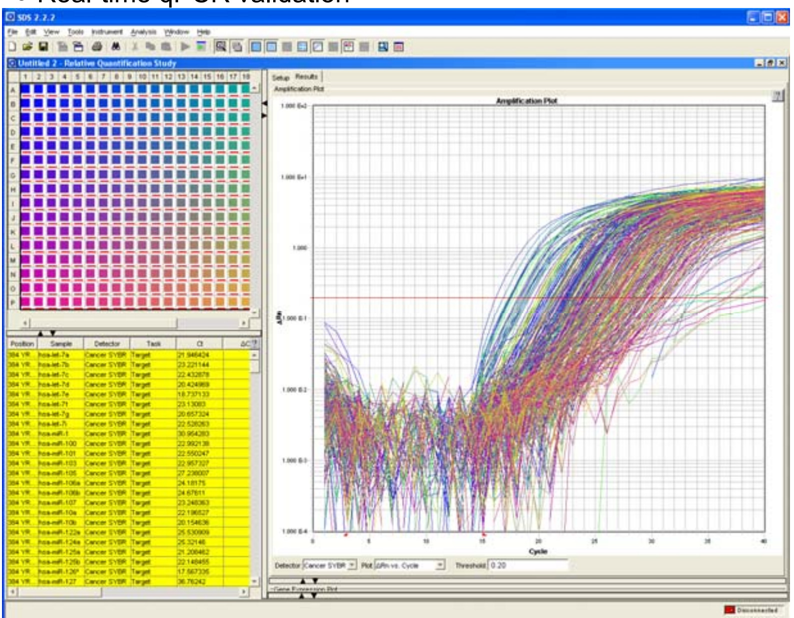
- Recommended 2X SYBR Green qPCR Mastermixes

  SBI has tested and recommends SYBR Green Master mix from three vendors: Power SYBR Master Mix® (Cat numbers 4368577, 4367650, 4367659, 4368706, 4368702, 4368708, 4367660) from Applied Biosystems; SYBR GreenER™ qPCR SuperMix for ABI PRISM® instrument from Invitrogen (Cat numbers 11760-100, 11760-500, and 11760-02K); and RT² Real-Time™ SYBR Green / ROX PCR (Cat numbers PA-012 and PA-112) from SuperArray.

III. Quality Control and Sample Data

A. miRNome Array Primer Validation Tests & endogenous Controls

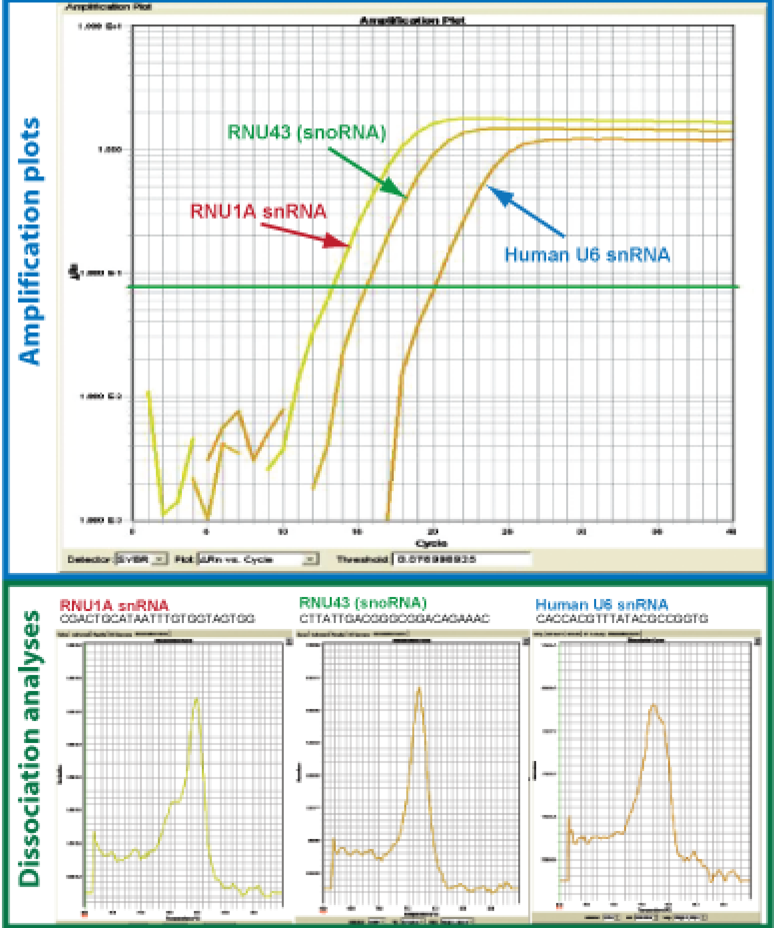
- Real-time qPCR validation



The miRNome microRNA Profiler qPCR Array plates were tested using a 500 ng RNA sample converted to cDNA using the QuantiMir RT Kit. The resulting cDNA was tested using about 1 ng cDNA per well. Shown above is the resulting Real-time amplification plot for the entire plate. The Cts ranged from 16.15 to 37.8 and the Ct value for the Human U6 assay was 26.2 in this experiment.

● Endogenous control assays

- 500 ng total RNA used for QuantiMir™ cDNA synthesis
- 0.01 µl cDNA used per 6 µl qPCR reaction, 333 nM Assay+170 nM 3' Reverse
- ABI 7900HT Instrument, MCLab HotSYBR® Green Reagent



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.

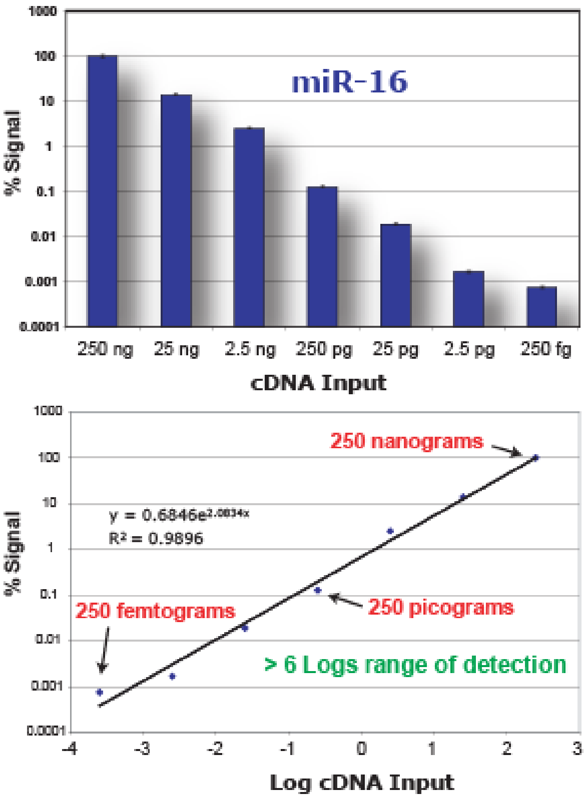


Fig 4. Real-time qPCR data for Human miR-16.
Real-time qPCR amplification plots are shown in the upper inset. Cycle threshold (Ct) values were determined using the software automatic baseline and Ct settings. The Bar graph depicts the relative %Signal per RNA input amount for the microRNA. The graph below shows the linear regression analysis with a R^2 value of 0.989 for miR-16.

C. Specificity Tests

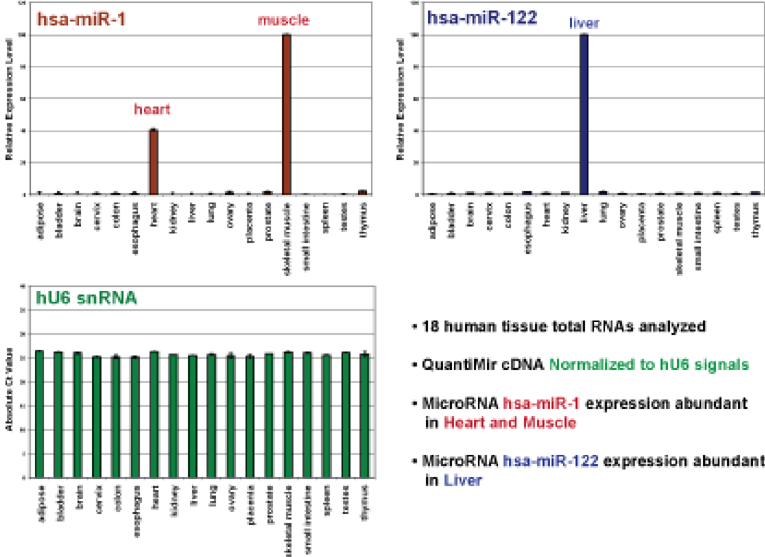


Fig 5. The QuantiMir cDNA sets were synthesized from 18 separate normal Human tissues and tested with 2 primers specific for 2 known miRNA molecules: miR-1 (heart and skeletal muscle-specific) and miR-122a (abundant in liver). The corresponding expression bar graphs are shown in **Fig. 5**. **above.**

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.

Tissue tested:

1	adipose	7	heart	13	prostate
2	bladder	8	kidney	14	skeletal muscle
3	brain	9	liver	15	small intestine
4	cervix	10	lung	16	spleen
5	colon	11	ovary	17	testes
6	esophagus	12	placenta	18	thymus

D. Sample Data

1. 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 using to detect the corresponding microRNA species in the tissues detailed in the expression graph below (**Fig. 6.**). 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.

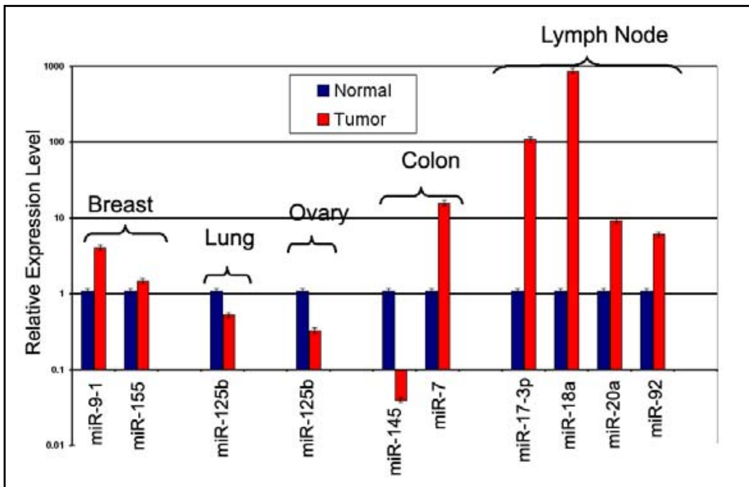


Fig. 6. Quantitative analysis of MicroRNA expression in tumor and normal tissue samples. The Bar graph data are grouped by tissue type with normal tissues in blue bars and tumor tissues in red bars. The specific MicroRNAs being detected are listed below the bar graphs. The expression levels are normalized to U6 snRNA transcript levels to control for RNA input. The MicroRNA expression levels are depicted as ∆Ct vales (Y axis). Real-time assays were performed as described in **Section II D** of this manual. Fold changes for these specific microRNAs and the particular tumor type are documented in the literature and verified using the QuantiMir RT kit and the selected miRNA primers from the Cancer Array shown above. See MicroRNA and Cancer References in **Section VI**.

IV. Troubleshooting

Problem	Possible Solution
Too much background in qPCR signals	Use much less cDNA in the SYBR Green Mastermix.
No qPCR signals	Did you select SYBR Green as the Detector's Reporter Dye? Did the controls work? Use more cDNA in Mastermix. Check Mastermix contents and try a subset with the controls as a positive control. 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 phone their technical support team for guidance.

V. General References

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VI. MicroRNA and Cancer References

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MicroRNA expression profiles classify human cancers.

Nature. 2005 Jun 9;435(7043):745-6. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR, Golub TR.

● B-cell Leukemia:

MicroRNA profiling reveals distinct signatures in B cell chronic lymphocytic leukemias.
Proc Natl Acad Sci U S A. 2004 Aug 10;101(32):11755-60. Epub 2004 Jul 29.
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VII. Related Products

- **QuantiMir RT Kit** (Cat. #RA420A-1) Complete reagent kit to anchor-tag small RNAs and convert them to quantifiable cDNA. Kit contains enough reagents for 20 RT reactions and can generate hundreds of qPCR templates. A universal reverse adaptor primer (10 µM) and positive control primers for Human U6 snRNA (10 µM) and Human miR-16 (10 µM) are also included with the kit.

- **Lenti-miR microRNA precursor clone collection.** Lentivector-based microRNA over expression clones, also available in pooled lentiviral library (cat# PMIRHxxxPA-1)

- **miRZips™ Anti-microRNA constructs** (Cat. # MZIPxxx-PA-1) Permanently knockdown endogenous micrRNAs to validate Targets and discover new phenotypes

- **Small RNA Amplification and Cloning 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™ Global mRNA 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.

VIII. 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

Phone: (650) 968-2200
(888) 266-5066 (Toll Free)

Fax: (650) 968-2277

E-mail:
General Information: info@systembio.com
Technical Support: tech@systembio.com
Ordering Information: orders@systembio.com

IX. Licensing and Warranty Statement

Limited Use License

Use of the miRNome microRNA Profilers with 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.

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.

SBI is committed to providing our customers with high-quality products. If you should have any questions or concerns about any SBI products, please contact us at (888) 266-5066.

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