



QuantiMir RT Kit

Small RNA Quantitation System

Cat. # RA420A-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. 2-070209)

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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. 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. The user simply only needs to design the forward primer which corresponds to the miRNA or siRNA of interest and provide Real-time PCR SYBR green master mix (for Real-time PCR) or typical PCR mastermix with *Taq* DNA polymerase for End-point PCR expression profiling.

Recently, previously unknown germline specific classes of miRNA-like molecules were identified in mouse testes and mouse oocytes, illustrating the need for continued and in-depth miRNA discovery efforts across a wide range of tissues. These facts taken together demonstrate an ever-increasing need for simple, robust, and sensitive methods that enable discovery and quantitation of microRNAs and their precursors.

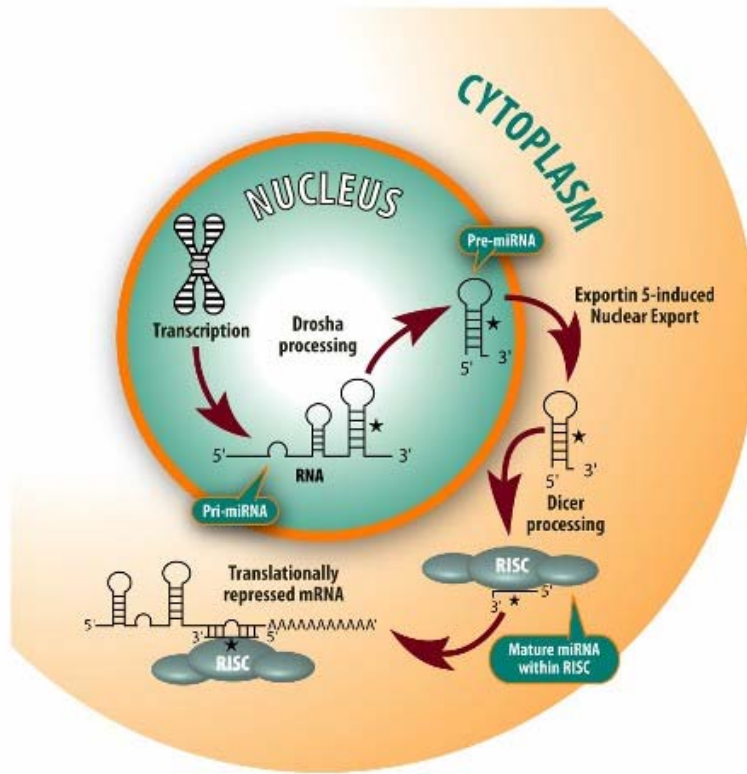


Fig. 1. Diagram of MicroRNA biogenesis, processing and function.

C. Overview of Protocol

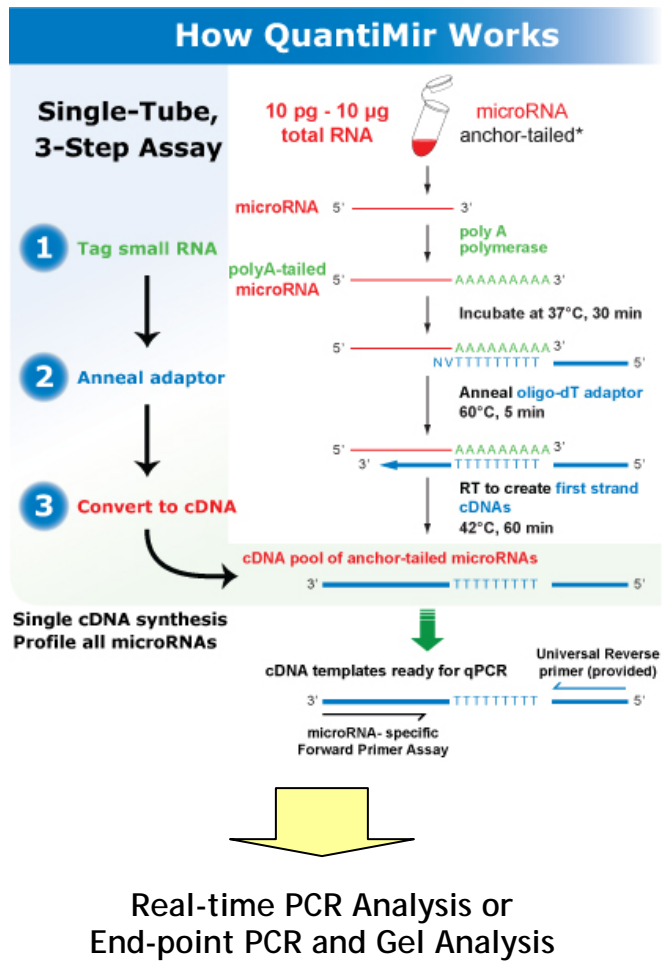


Fig. 2. Workflow schematic for a typical QuantiMir™ RT experiment.

* Trizol-purified total RNA is recommended to ensure small RNA fraction present.

D. List of Components

Each QuantiMir™ RT Kit contains the following components with enough material to perform 20 reactions 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 MnCl ₂	
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)	
50 µl	dNTP Mix	
600 µl	3' Universal Reverse PCR Primer	End-point or qPCR Assay
50 µl	5' Human U6 Control Forward Primer (10µM)	
50 µl	5' Mouse U6 Control Forward Primer (10µM)	
50 µl	5' Human/Mouse miR-16 Control Forward Primer (10µM)	
1.2 ml	RNase-free Water	

Human U6 snRNA Control Forward Primer Sequence:
 5' -CGCAAGGATGACACGCAAATTC - 3'
 Tm = 59°C, amplicon size = 84 bp

Mouse U6 snRNA Control Forward Primer Sequence:
 5' - TGGCCCCTGCGCAAGGATG - 3'
 Tm = 63°C, amplicon size = 97 bp

Human/Mouse miR-16 Control Forward Primer Sequence:
 5' - TAGCAGCACGTAAATATTGGCG - 3'
 Tm = 58.9°C, amplicon size = 68 bp

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.

E. Additional Required Materials

- 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)
- Real-time qPCR Instrument

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 RT² Real-Time™ SYBR Green / ROX PCR (Cat. #s PA-012 and PA-112) from SuperArray.

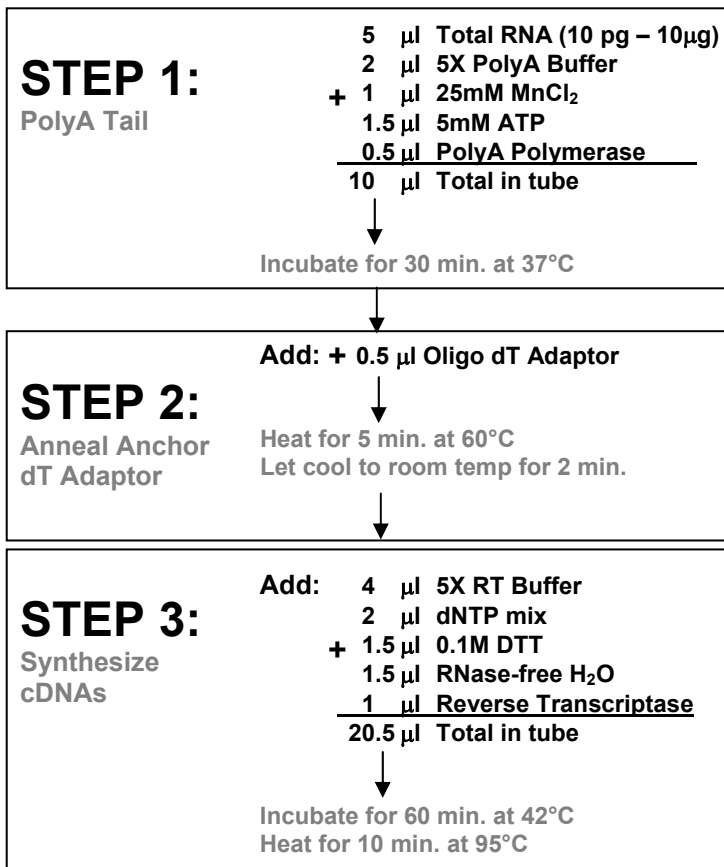
II. Protocol

A. QuantiMir™ RT Reaction Setup



It is important to start with total RNA that includes the small RNA fraction.

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



*The QuantiMir™ cDNAs can be stored at -20°C.

Done!

How much cDNA template per qPCR reaction?

Starting RNA	QuantiMir cDNA per qPCR reaction
10pg -100 ng	0.5ul to 1ul
100ng – 1 μ g	Dilute 1: 100
> 1 μ g	Dilute 1: 1000

B. Primer Design Considerations for QuantiMir™ cDNA Detection and Quantitation

The user needs only to design the forward “sense” orientation primer to perform the end-point or qPCR reactions. 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

Mature sequence MIMAT0000069	
Accession	MIMAT0000069
ID	hsa-miR-16
Sequence	14 - uagcagcagcguaaaauuggcg - 35
	Get sequence
Evidence	experimental; cloned [1,5,7], Northern [1,6]

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

The mature miRNA sequence 5' – **uagcagcagcguaaaauuggcg** – 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.

If the user is developing a new assay for a novel miRNA, follow the guidelines of the example above. Design the primer to have a Tm of at least 50°C and to have a length of at least 18 bases. If the primer being designed for the miRNA to be studied has a Tm below 50°C, lower the annealing temperature in your cycling conditions for end-point and qPCR instrument settings. An example of successfully using this approach is demonstrated in **Figure 5**.

C. End-point PCR Reactions

The following PCR reaction conditions are recommended:

	1 μ l	QuantiMir™ cDNA (diluted, see p.# 7)
	0.5 μ l	Universal Reverse Primer (10 μ M)
+	1 μ l	miRNA-specific Forward Primer (10 μ M, user designed)
	2.5 μ l	10X PCR Buffer with 2.5 mM MgCl ₂
	1 μ l	10 mM dNTPs mix
	1 μ l	Taq DNA PCR polymerase
	20 μ l	RNase-free water
	25 μ l	Total

Prepare reaction in a suitable PCR tube or plate and thermocycle as follows:

Heat denature at 95°C, 10 min.

Heat denature at 95°C, 15 sec.] 30 cycles
Anneal Primers at 60°C, 1 min.]

Hold at 15°C (optional)

Prepare and pour a 3.5% agarose (1X TAE or 1X TBE) gel with a suitable stain (Ethidium Bromide, etc.). Add 2.5 μ l of 10X Loading dye, mix, and load 10 μ l into a well of the gel. Also run a suitable DNA size marker (50-2,000 bp) along with your samples. Take care to only electrophorese your gels for 10-15 minutes and then visualize. An example of end-point PCR primer tests and gel electrophoresis is shown in **Figure 3**.

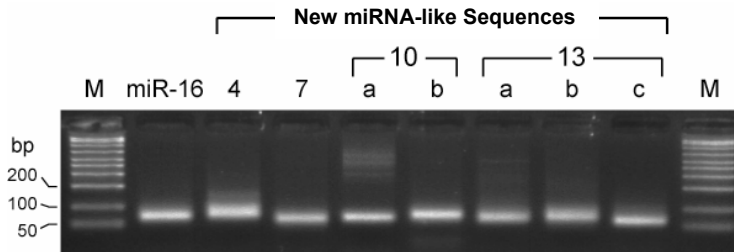


Fig. 3. QuantiMir™ end-point PCR for miR-16 and seven newly identified miRNA-like sequences by SBI. Numbers on the top of the gel correspond to cDNA clone number. Letters indicate specific sequence found in corresponding clone. Include a DNA size ladder with markers in the range of 50-2,000 bp (e.g., Bio-Rad AmpliSize™ DNA Ladder, Cat. # 170-8200). M denotes DNA Marker lanes.

* The size range of the PCR products typically ranges between 50 and 100 bp. The adaptor segment is 46 bp with the miRNA sequence comprising the remaining PCR product size.

D. Real-time qPCR Reactions using QuantiMir™ cDNAs

For end-point PCR reactions, please refer to the **Section C** above, “End-point PCR Reactions using QuantiMir™ cDNAs”.

1. qPCR Reaction Setup

To determine the expression profile for your miRNA under study, mix the following per well:

For Single well determination:

	15	μl	2X SYBR Green qPCR Mastermix buffer
+	1	μl	User-designed Forward Primer (10 μM)
	0.5	μl	Universal Reverse Primer (10 μM)
	1	μl	Diluted QuantiMir™ cDNA (see p.# 7)
	12.5	μl	RNase-free water
	<hr/>		
	30	μl	Total / well

The Mastermix contents can be scaled up or down depending upon on your experimental needs. Once reagents are loaded into the wells, cover the plate with the 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.

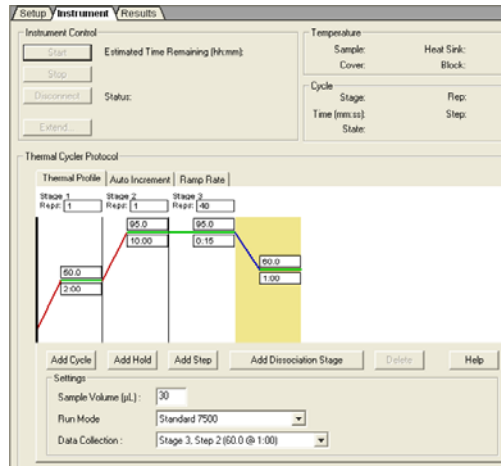
2. Real-time qPCR Instrument Parameters

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 thermocycling conditions used in testing at SBI are shown below. A screenshot from SBI’s instrument set up is shown on the right. Default conditions are used throughout except for those cases where the Forward Primer’s Tm is below 55°C, then the annealing temperature in Step 2 of Stage 3 is lowered from 60°C to 50°C.

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.

III. Quality Control and Sample Data

A. 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).

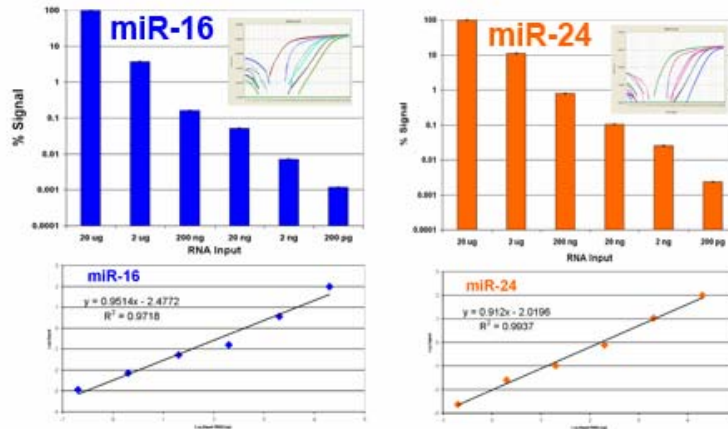


Fig. 4. Real-time qPCR data for Human miR-16 and Human miR-24.

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.

B. 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

Mature sequence MIMAT0003389	
Accession	MIMAT0003389
ID	hsa-miR-542-3p
Sequence	53 - ugugacagauugauaacugaaa - 74
	Get sequence
Evidence	experimental; cloned [1]

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.

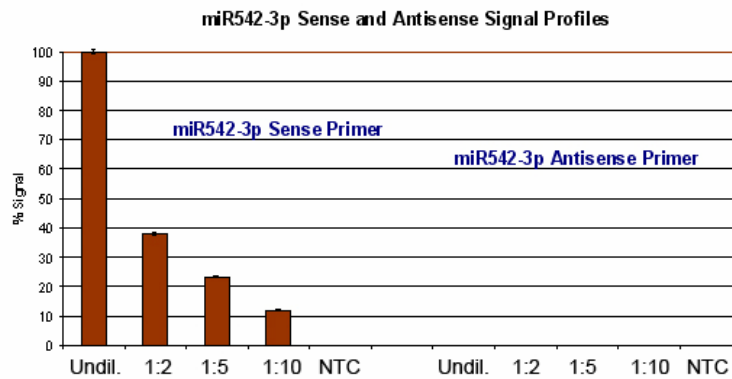


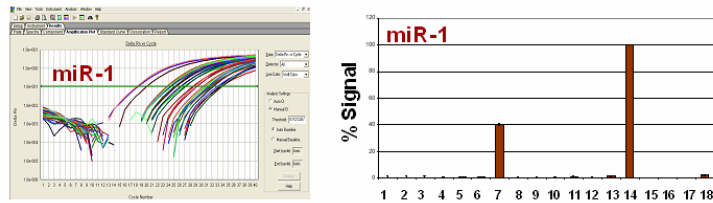
Fig. 5. Sense and antisense test of the QuantiMir™ cDNA. Dilutions of the QuantiMir™ cDNA template as well as no template controls (NTC) were tested with either sense or antisense orientation for the Human miR-542-3p molecule. Quantitative results are observed for the “sense” orientation of miR-542-3p. No signals are observed in the “antisense” or no template controls. The annealing temperature for the qPCR cycling conditions was lowered to 50°C.

C. Sample Data

1. Tissue Expression Pattern Determinations using the QuantiMir™ Kit

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 6, panels a and b.**

a.



b.

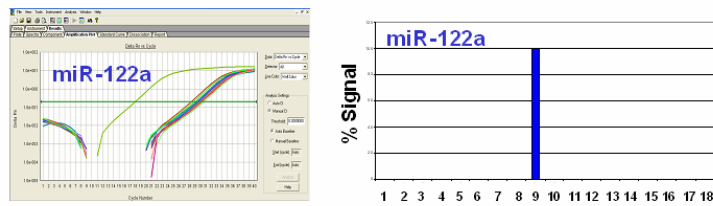


Fig. 6. Real-time qPCR data using primers specific for Human miR-1 (Panel a.) and for miR-122a (Panel b.). The amplification plots are shown on the left with the resulting expression profile bar graphs based on Ct values is shown on the right. The default qPCR cycling conditions were used with an annealing 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 using to detect the corresponding microRNA species in the tissues detailed in the expression graph below (**Figure 7**). 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.

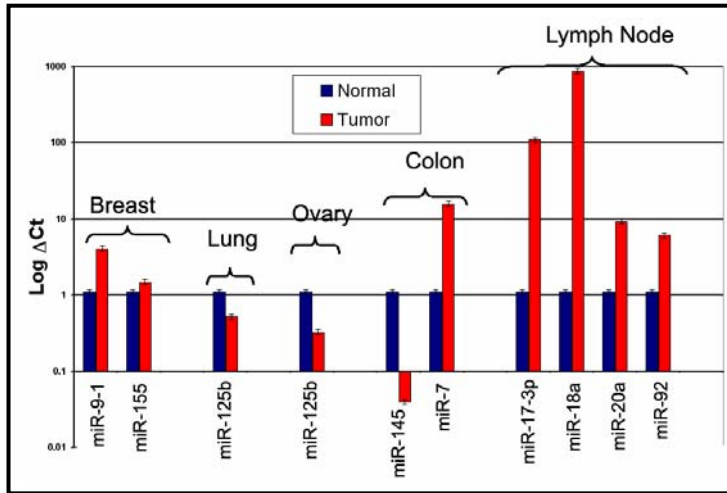


Fig. 7. Quantitative analysis of MicroRNA expression in tumor and normal tissue samples. The Bar graph data are grouped by tissue type with normal tissues in blue bars and tumor tissues in red bars. The specific MicroRNAs being detected are listed below the bar graphs. The expression levels are normalized to U6 snRNA transcript levels to control for RNA input. The MicroRNA expression levels are depicted as Δ Ct vales (Y axis). Real-time assays were performed as described in this manual.

3. Detection and Quantitation of siRNA Production from Transduced shRNA Expression Constructs

The QuantiMir™ cDNAs were synthesized from Human T-Rex 293 cells transduced with equivalent amounts of Lentivirus containing the SBI shRNA expression constructs for the p53 shRNA driven by the H1 promoter with Tet repressor elements (p53.H1.Tx2L) or without Tet responsive elements (p53.H1) present in the promoters. The T-Rex 293 cell line constitutively expresses the Tet repressor protein. The predicted structure of the p53 shRNA and the oligonucleotide primer designed (underlined) to detect the mature siRNA (boxed in the structure) are shown below in **Figure 8a**. After transduction, the cells were left untreated (- DOX) or treated with 1µg/ml Doxycycline (+ DOX) for 48 hours to alleviate the Tet repressor effects. The quantitative PCR results normalized to the U6 signals are shown in the bar graphs below in **Figure 8b**.

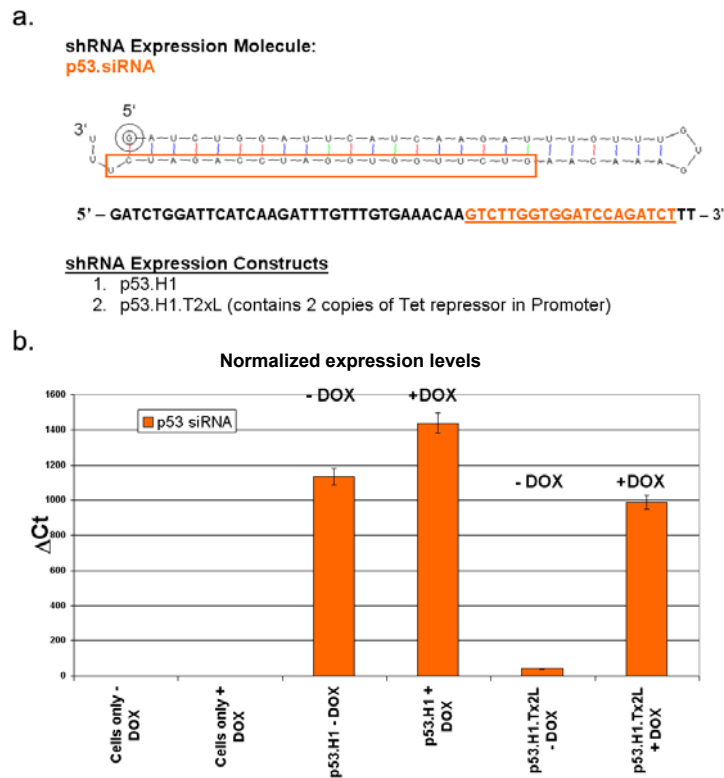


Fig. 8. Detection of mature siRNA expression from cell culture experiments.

Troubleshooting

Problem	Possible Solution
No PCR bands or qPCR signals using user-designed Forward Primer with QuantiMir™ cDNA templates.	Alter thermocycle conditions—lower annealing temperature. Include the U6 and/or miR-16 control Forward primers in separate reactions as PCR controls.
Too many PCR bands using user-designed Forward Primer with QuantiMir™ cDNA templates.	Alter thermocycle conditions—elevate annealing temperature. Include the U6 control Forward primer in a separate reaction as PCR control.
Signals in qPCR negative controls.	Dilute your QuantiMir cDNA by 1;100 and try again.
Correct size band and larger PCR band(s) observed in end-point PCR tests.	Potential precursor pri-miRNA or pre-miRNA molecules are being amplified.

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IV. Appendix

A. Related Products

- **Cancer microRNA qPCR Array** (Cat. # RA610A-1)
Pre-formatted 96-well plate containing assays for 95 oncomirs and U6 control. 10 complete profiles plus QuantiMir kit included.
- **Stem Cell microRNA qPCR Array** (Cat. # RA620A-1)
Pre-formatted 96-well plate containing assays for 95 different microRNA involved in stem cell self-renewal, hematopoiesis, neural development and tissue specificity. Array also contains U6 control. 10 complete profiles plus QuantiMir kit included.
- **miRNome Profilers** (Cat. # RA660A-1 [Human],
Cat. # RA670A-1 [Mouse])
Profile all known microRNAs (including minor star* forms) for Human or Mouse. Sanger miRBase 100% updated, 20 complete profiles with QuantiMir kit included.
- **miR-SnaREs** (Cat. # RA7xxA-1 – RA8xxA-1)
Epitope-tagged microRNA processing factors to enrich samples for microRNAs and other small RNAs. Human and Mouse Argonautes, Pasha and Dicer constructs available.
- **Lenti-miRs** (Cat. # PMIRHxxA-1)
Over express microRNA precursors using lentivectors.
- **miRZips** (Cat. # MZIPxxA-1)
Permanent microRNA knockdown using RNAi lentivectors with anti-sense microRNAs..
- **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

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

V. Licensing and Warranty Statement

Limited Use License

Use of the QuantiMir™ RT Kit (*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.

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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