Applied Biosystems

Illuminate the Mystery of Biological Dark Matter: miRNA Expression Analysis Research Tools

Introduction

First referred to as the "biological equivalent of dark matter"[1], microRNAs (miRNAs) are small, highly conserved RNA molecules that act as key regulators of development, cell proliferation, differentiation, and the cell cycle. The active, mature miRNAs are 17–24 base, single-stranded RNA molecules expressed in eukaryotic cells and are known to affect the translation and/or stability of target messenger RNAs. Each miRNA is believed to regulate multiple genes, with predictions that greater than one third of all human genes may be regulated by miRNA molecules [2].

In order to gain insight into these tiny regulators, researchers around the world are asking fundamental research questions such as: What miRNAs are expressed?", " Where and when are they expressed?", What cell processes do miRNAs regulate and what genes do miRNAs control?".

Now that many miRNA sequences are known (cataloged in the miRBase Sequence Database at **http://microrna.sanger.ac.uk**), one of the most common next steps is analysis of miRNA expression levels between different tissues, developmental stages, or disease states. miRNA expression levels can be studied using several different methods: Microarray analysis, real-time PCR, Northern blots, in situ hybridization and solution hybridization. Of these techniques, quantitative reverse transcription PCR (qRT-PCR) is the most sensitive and accurate method.

MicroRNAs can be a challenge to study because of their small size. They require specialized and dedicated tools for analysis. For qRT-PCR applications they include:

1. Effective method of miRNA isolation from samples
2. RT-PCR reagents optimized for miRNA detection
3. Assays specific to the miRNAs of interest
4. Real-time analytical instruments and reagents validated for miRNA detection protocols

Here we provide an experimental overview for quantitating specific miRNA expression levels by qRT-PCR (outlined in Figure 1). In this study, we analyzed miRNA levels from total RNA and RNA samples enriched for small RNA. These samples included both frozen or RNA*later*® solution treated mouse, brain, liver and lung tissues.

Isolate microRNA	Convert to cDNA	Quantitate microRNA Expression
*mir*Vana™ miRNA Isolation Kit	TaqMan® MicroRNA Reverse Transcription Kit	TaqMan® MicroRNA Assays TaqMan® Universal PCR Master Mix 7900HT Fast Real-time PCR System

Figure 1. miRNA Experimental Overview

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Isolate microRNA | Convert to cDNA | Quantitate microRNA Expression

1. Effective method of miRNA isolation from samples (*mir*Vana™ miRNA Isolation Kit)

A. Sample acquisition and storage

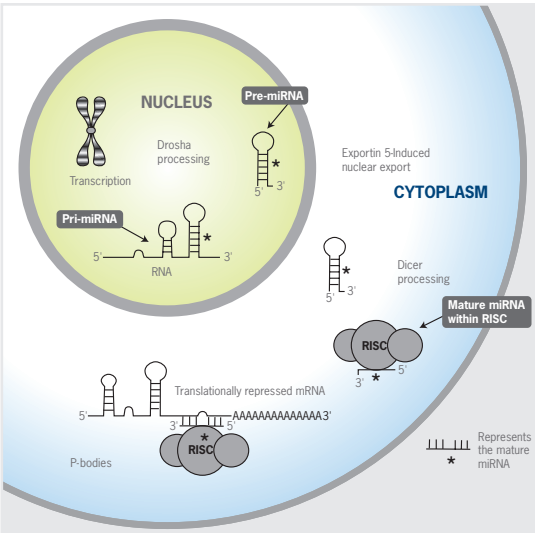
Once samples have been obtained, they should be processed immediately – tissue should be frozen (small pieces in liquid nitrogen is preferable), or placed in RNA*later* solution for storage until RNA extraction is performed. RNA*later* is an aqueous tissue storage reagent that protects RNA within intact, unfrozen samples.

In our Study, frozen mouse brain (Cat # 55004-2), liver (Cat # 55023-2), and lung (Cat # 55024-2) tissues were acquired from Pel-Freeze Biologicals. Fresh brain, liver, and lung tissues stored in RNA*later* Solution were extracted and processed from C57BL/6J male mice from The Jackson Laboratory (Cat # 1628517).

B. Isolation of miRNA containing Total RNA: Isolation using the *mir*Vana™ miRNA Isolation Kit

Isolation of miRNA begins when total RNA that includes the small RNA fraction is isolated from the samples of interest. However, not all isolation methods retain the small RNA fraction. Therefore, it is important to use RNA isolation methods specifically adapted to retain it. The *mir*Vana miRNA Isolation Kit was developed to retain these small RNA species either in a background of total RNA or as an enriched fraction of RNA species 200 nucleotide in size or smaller. The initial organic extraction of the *mir*Vana miRNA Isolation Kit provides a robust front-end purification that removes cell debris and most DNA. Although enrichment of the small RNA fraction can increase sensitivity in many applications, for qRT-PCR detection of miRNA, total RNA is usually sufficient.

In our experiment, total RNA was isolated from the brain, liver and lung tissue of 3 different mouse specimens (Figure 3). The tissue samples were disrupted in Lysis Buffer and then Acid-Phenol:Chloroform extracted, following the *mir*Vana miRNA Isolation Kit procedure. Final extract volumes were measured in preparation for the second phase of the procedure. Total RNA was then purified by adding ethanol to the samples and passing them through a glass-fiber filter (GFF), which immobilized the RNA. The filter was washed a few times and the total RNA was eluted using a low ionic-strength solution.



miRNA Processing Pathway

(1) miRNAs are expressed in the nucleus as parts of long primary miRNA transcripts (Pri-miRNA) that have 5' caps and 3' poly(A) tails. (2) The hairpin structure that likely forms around the miRNA sequence of the pri-miRNA acts as a signal for digestion by a double-stranded (ds) ribonuclease (Drosha) to produce the precursor miRNA (Pre-miRNA). (3) Exportin-5 mediates nuclear export of pre-miRNAs. (4) A cytoplasmic dsRNA nuclease (Dicer) cleaves the pre-miRNA leaving 1–4 nt 3' overhangs. The single-stranded mature miRNA associates with a complex that is similar, if not identical, to the RNA Induced Silencing Complex (RISC). (5) The miRNA/RISC complex represses protein translation by binding to sequences in the 3' untranslated region of specific mRNAs. The exact mechanism of translation repression is still undefined.

*=mature miRNA sequence.

C. Yields and quality of RNA

Typically, yields for total RNA follow the "1/1000th rule", i.e. one can expect to get about 1 µg of RNA for every mg of tissue. This rule varies with tissue type, e.g. skin yield is much less, but most yields are within a 5-fold level. The *mir*Vana Isolation Kit provides reagents and a procedure to enrich the population of RNAs that are 200 bases and smaller, using two sequential filtrations through GFFs with different ethanol concentrations. Although generally not necessary for real-time PCR applications, small RNA enrichment results in lower background and enhanced sensitivity of small RNA detection by solution hybridization, Northern analysis, and other methods, as compared to the same assay using total RNA.

RNA yield, purity and quality are factors that are important for successful gene expression analysis. RNA yield can be measured by looking at the A_{260} reading. A reliable and inexpensive method to look at RNA quality is to run the samples on a polyacrylamide gel. In this experiment, 250 ng RNA from 1 biological replicate set was combined with 5 µL of Ambion's Gel Loading Buffer II and concentrated using a Savant SpeedVac® on medium heat to a final volume of 10 µL. Samples were then incubated for 2 minutes at 95°C and immediately placed on ice. Decade™ Marker was prepared according to protocol using Ambion's *mir*Vana™ Probe & Marker Kit (Ambion Cat.# AM1554). Samples were run on a polyacrylamide gel (Figure 4).

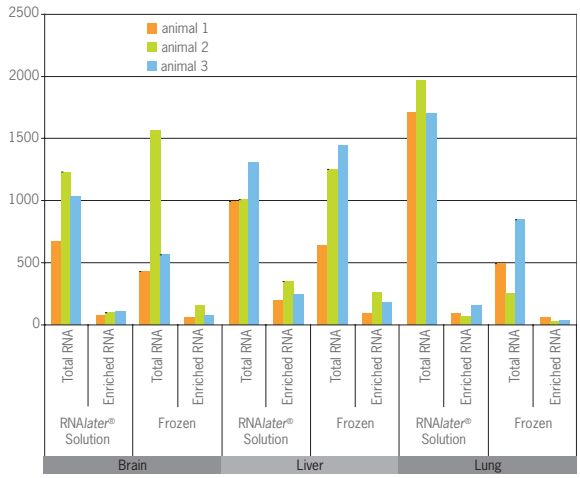


Figure 3. Efficient Recovery of MicroRNAs Total RNA was isolated from brain, liver and lung tissue using the *mir*Vana™ miRNA Isolation Kit. Typically, one can expect to get about 1 µg RNA for every mg of tissue. The *mir*Vana Isolation Kit also provides reagents and a procedure to enrich the population of RNAs that are 200 bases and smaller. Since tRNA and other small functional RNAs comprise 5-20% of the total RNA population, the gross recovery of enriched RNA by A_{260} will only be about a tenth of that of total, but the amount of miRNA present will be the same.

Figure 3 demonstrates efficient recovery of total and enriched miRNA from tissues of 3 different animals using the *mir*Vana Isolation Kit. About 1 µg of total RNA was recovered in each animal for all tissue types. Since tRNA and other small functional RNAs comprise 5–20% of the total RNA population, the gross recovery of enriched RNA by A_{260} was about a tenth that of total, but the amount of miRNA present was about the same. Figure 4 illustrates the yield of the varying RNA sizes run on a polyacrylamide gel, where losses in yield are due to losing the high molecular weight RNA species that are embedded in the gel at the origin. Here also the enriched RNA is about a tenth that of the total RNA.

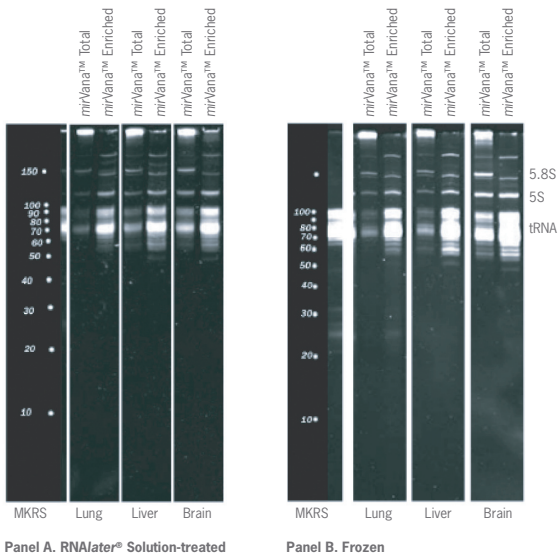


Figure 4. RNA Yield in Frozen Versus RNA*later*® Solution-Treated Samples. The greatly enriched presence of tRNA (~70 nt) is apparent, as equal amounts of RNA were loaded. The RNA*later*® solution-treated samples (A) provide equivalent samples in terms of banding patterns when compared to frozen samples (B). The enrichment procedure is not totally size-dependent, but also enriches for some small RNAs preferentially (perhaps due to structural qualities). The mass of large RNAs (trapped in the gel origin) are greatly reduced. The lower molecular weight bands seen in the lung samples are occasionally seen in this sample type and could be degradation products. Each sample was run on a 7 M urea/15% polyacrylamide gel with 1 µL unlabeled Decade™ Markers (MKRS; Ambion; Cat #AM7778). Prior to sample loading, gels were run at 300 V for 10 min, and the wells were flushed with 1X TBE Buffer. The gel was run at 200 V until exit of the bromophenol blue dye front from the gel. Gels were stained for 30 minutes with a 1:10,000 dilution of SYBR® Gold Dye and photographed using Alphaimager v5.5 software. Sample loading was standardized according to ng RNA loaded.

Isolate microRNA | **Convert to cDNA** | Quantitate microRNA Expression

2. Optimized RT-PCR reagents for miRNA detection (TaqMan® MicroRNA Reverse Transcription Kit)

Gene expression studies require high quality cDNA. The TaqMan® MicroRNA Reverse Transcription Kit delivers a high level of performance for accuracy, precision and quality of cDNA. The kit provides the necessary components for optimal performance of TaqMan MicroRNA Assays. Components of this kit are used with the Reverse Transcription (RT) primer provided with the specific TaqMan MicroRNA Assay to convert miRNA to cDNA.

In this experiment, an RNA mass equivalent of 5.13 µg of tissue was added to a final RT reaction volume of 15 µL. RT was performed in 384 well format using Applied Biosystems TaqMan miRNA RT Kit Protocol.

Isolate microRNA | Convert to cDNA | **Quantitate microRNA Expression**

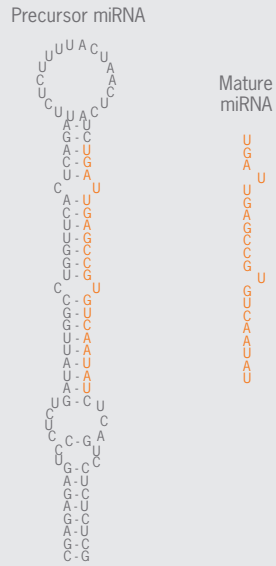
3. Assays specific to the miRNAs of interest (TaqMan® MicroRNA Assays)

Until now, small RNAs like microRNAs (miRNAs) could not be analyzed by traditional PCR. TaqMan MicroRNA Assays have been validated to amplify specific small RNAs, enabling accurate and specific quantitation of miRNA expression levels. A large collection of pre-designed, off-the-shelf TaqMan MicroRNA Assays for human, mouse, rat, *A. thaliania*, *C. elegans*, and *D. melanogaster* are available from Applied Biosystems. Each one is functionally validated, convenient and easy to use.

In our study, the following TaqMan MicroRNA Assays and controls were used: hsa-miR-1, hsa-miR-24, hsa-miR-16, hsa-miR-133a, hsa-miR-145, RNU6B (U6 Control), and RNU19 (U19 Control). The miRNAs to which these assays were designed have been shown to exhibit differential expression patterns in cancerous tissues as compared to normal tissues and may play a role in oncogenesis [2–4].

TaqMan® miRNA Mechanism

Panel A. TaqMan® MicroRNA Assay



Panel B. Precursor and Mature miRNA

Figure 5. TaqMan® MicroRNA Assay Mechanism. (A) A simple, two-step mechanism brings the advantages of real-time PCR to miRNA quantitation. The basis of TaqMan MicroRNA Assays is a target-specific stem-loop structure, reverse-transcription primer. Its innovative design overcomes a fundamental problem in miRNA quantification: the short length of mature miRNAs (~22 nt) prohibits conventional design of a random-primed RT step followed by a specific real-time assay. (B) The stem-loop accomplishes two goals : (1) specificity for only mature miRNA target, and (2) formation of a RT primer/mature miRNA –chimera, extending the 5' end of the miRNA. The resulting longer RT amplicon presents a template amenable to standard real-time PCR, using TaqMan assays.

4. Real-Time analytical instruments and reagents validated for miRNA detection protocols (Applied Biosystems 7900HT Fast Real-time instrument, TaqMan® Universal PCR Master Mix)

Applied Biosystems Real-Time PCR Systems make real-time PCR more accessible than ever before by providing powerful solutions to fit the needs of any laboratory. By taking advantage of gold-standard TaqMan reagent-based technology with universal thermal cycling conditions, TaqMan Universal PCR Master Mix is the ideal reagent solution for TaqMan assays using DNA or cDNA as the target. Both have been validated for use with TaqMan MicroRNA Assays.

In our experiment, real-time PCR was performed by adding 1.34 µL (a 458 ng tissue equivalent) of each completed RT reaction to a target TaqMan MicroRNA Assay reaction using TaqMan Universal PCR Master Mix (final reaction volume equal 20 µL). Samples were tested in triplicate and run on the Applied Biosystems 7900HT Fast Real-Time PCR system (Figure 6). Assay results were collected and analyzed using SDS 2.2.2 software.

Figure 6. Applied Biosystems 7900HT Fast Real-Time PCR System.

How Does a Real-Time PCR Assay Work?

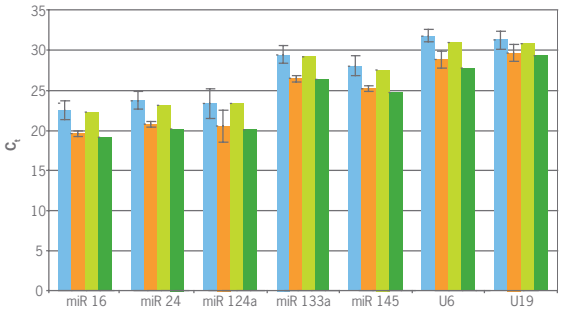
A real-time PCR assay is a tool that can be used to quantitate differential gene expression. This type of assay measures (quantitates) the amount of a nucleic acid target during each amplification cycle of the PCR. The target may be DNA, cDNA, or RNA. In the initial cycles of PCR, there is little change in fluorescence signal. This defines the baseline for the amplification plot. An increase in fluorescence above the baseline indicates the detection of accumulated PCR product. A fixed fluorescence threshold can be set above the baseline. The parameter C_t (threshold cycle) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold.

Relative quantitation might be used to measure gene expression in response to a chemical (drug). The level of gene expression of a particular gene of interest in a chemically treated sample would be compared relative to the level of gene expression in an untreated sample. Another method of looking at gene expression using qRT-PCR is to look at absolute quantitation and look at standard curves made using a dilution curve.

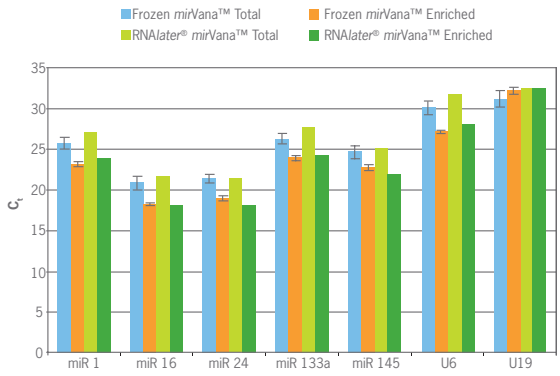
Results and Conclusions

Figure 7 shows a ~3.3 C_t difference between miRNA levels in the total RNA samples and the samples enriched for miRNA, which indicates about a tenfold enrichment. This is consistent with our expectations given that the data were normalized to input mass of RNA. Of note, the differential miRNA expression level trends between the different tissues were similar in both sample types. This experiment, as well as others done in our labs, demonstrates that miRNA enrichment preserves miRNA differential expression patterns as compared to total RNA. Although we find that total RNA isolated with the *mir*Vana miRNA Isolation Kit yields more than sufficient signal with TaqMan MicroRNA Assays, other techniques such as microarray and Northern analysis require this enrichment to yield sufficient signal for analysis.

Figure 7 also indicates that the frozen and RNA*later* solution treated samples yielded Cts that were roughly equivalent. This experiment demonstrates that there is no significant difference in miRNA expression profiles from frozen and RNA*later* Solution-treated tissues when RNA is isolated using the *mir*Vana miRNA Isolation Kit.



Panel A. miRNA Target Summary for Frozen Brain Tissue versus RNA*later*® Solution stored Brain Tissue



Panel B. miRNA Target Summary for Frozen Lung Tissue versus RNA*later*® Solution stored Lung Tissue

Figure 7. Real-time PCR Results in Frozen Versus RNA*later*® Solution-Treated Sample. The frozen and RNA*later* solution-treated samples are roughly equivalent, and the enriched samples show ~3.3 C_t's increase in signal, consistent with about a tenfold enrichment. U6 and U19 are TaqMan® MicroRNA Assay Controls, which have been designed to aid in relative quantitation.

The Next Step: Functional Analysis

To date, the functional roles of only a handful of miRNAs have been determined. These roles have been shown to encompass a wide range of biological functions ranging from developmental timing (e.g. *lin-4* in *C. elegans*), to apoptosis (e.g. *bantam* in *Drosophila*), to tissue growth (e.g. *JAW* in plants) [2,3]. These new findings suggest that the functions of miRNAs are even more varied and include a role in asymmetric gene expression [5]. Specific miRNA function can be examined by up and down regulating specific miRNA levels to study endogenous or reporter gene regulation and phenotypic response. microRNA functional analysis can be performed by using synthetic miRNA mimics (Pre-miR™ miRNA Precursors; Ambion) and miRNA inhibitors (Anti-miR™ miRNA Inhibitors; Ambion) which are now available for most known miRNAs.

Fundamental Research Questions

As a unique class of small RNA molecules, miRNAs require special tools for accurate and sensitive analysis. Applied Biosystems scientists (now including Ambion) have developed a portfolio of products that provide a complete solution to accelerate identification and characterization of this class of tiny RNA regulators (Figure 1). These tools will help scientists answer fundamental miRNA research questions such as: which miRNAs are expressed, where and when are they expressed, what cell processes do miRNAs regulate, and which genes do miRNAs control (Figure 8). The relationship between mRNA and protein expression can now be easily studied, and this may lead to a new segment of targets for diagnostics and therapeutics.

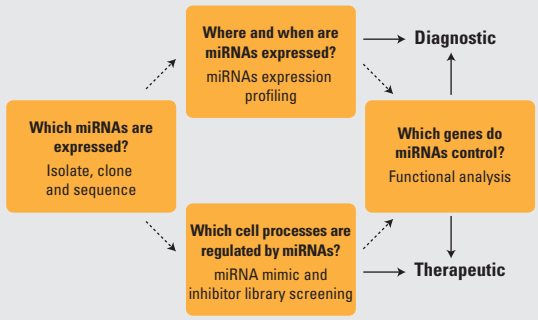


Figure 8. miRNA Fundamental Research Questions

A Complete Solution

Applied Biosystems (including Ambion) offers a complete suite of reagents, instruments and protocols dedicated to the investigation of miRNAs. In this study, we have shown that together, the *mir*Vana miRNA Isolation Kit, TaqMan MicroRNA RT Kit, TaqMan MicroRNA Assays, and Applied Biosystems 7900HT Fast Real-Time PCR System provide a validated, reliable and ready-to-use approach for quantitation of microRNA levels from a variety of sample types. We envision that this validated miRNA expression profiling platform will accelerate discovery of the many biological roles of miRNAs in cells, and more importantly, their roles in human diseases.

For more information about the entire suite of microRNA analysis tools from Applied Biosystems, visit the miRNA Resource at **www.ambion.com/miRNA**.

References

1. Lewis BP, Burge CB, Bartel DP (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets, *Cell* **120(1):**15–20.
2. Esquela-Kerscher A, Slack FJ (2006) Oncomirs - microRNAs with a role in cancer, *Nat Rev Cancer* **6(4):**259-269.
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Scientific Contributors

Rick Conrad, Yvonne Potuceck, and Emily Zeringer, Ambion.

ORDERING INFORMATION

Description	Quantity	Part Number
RNA Isolation		
RNA*later*® Solution	100 mL	AM7020
RNA*later*® Solution	500 mL	AM7021
RNA*later*® Solution	250 mL	AM7024
RNA*later*® Solution	50 x 1.5 mL	AM7022
RNA*later*® Solution	20 x 5 mL	AM7023
*mir*Vana™ miRNA Isolation Kit	up to 40 purifications	AM1560
*mir*Vana™ Probe & Marker Kit	30 rxns +10 Marker rxns	AM1554
Gel Loading Buffer II (Denaturing PAGE)	1.4 mL	AM8546G
qRT-PCR		
TaqMan® Gene Expression Assays and Controls		
hsa-miR-1	150 rxns	4373161
hsa-miR-24	150 rxns	4373072
hsa-miR-16	150 rxns	4373121
hsa-miR-133a	150 rxns	4373142
hsa-miR-145	150 rxns	4373133
RNU6B	150 rxns	4373381
RNU19	150 rxns	4373378
TaqMan® MicroRNA Reverse Transcription Kit	1000 rxns	4366597
TaqMan® Universal PCR Master Mix, No AmpErase® UNG	200 rxns	4324018
Applied Biosystems 7900HT Fast Real-Time PCR System with Fast 96-Well Block Module	1 instrument	4351405
miRNA		
Pre-miR™ miRNA Precursor Molecule	5 nmol	AM17100
Anti-miR™ miRNA Inhibitor	5 nmol	AM17000

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