

RT² miRNA PCR Arrays

The Complete System for Genome-Wide & Pathway Focused microRNA Analysis

Yexun (Bill) Wang, Daniel Kim, Jennifer Gibbons, Xiao Zeng, Liyan Pang, George Quellhorst

SABiosciences Corporation 6951 Executive Way, Frederick, MD 21703 USA
Phone: +1 (301) 682-9200 Fax: +1 (301) 682-7300 Web: www.SABiosciences.com
Email: support@SABiosciences.com

Abstract: As powerful regulators of gene expression, microRNA (miRNA) have been implicated in diverse biological pathways such as immune responses, development and differentiation, oncogenic pathways (like DNA repair and apoptosis), and oxidative stress responses. This paper discusses some major obstacles in miRNA real-time PCR assay development and evaluates the performance of the newest technique for the analysis of genome-wide miRNA expression: RT² miRNA PCR™ Arrays from SABiosciences. RT² miRNA PCR Arrays integrate the quantitative performance of SYBR® Green-based real-time RT-PCR and the multi-sequence profiling capabilities of a microarray. The miRNA PCR Array is a set of optimized real-time PCR assays, in 96-well or 384-well plates, for a miRNA panel as well as appropriate housekeeping assays and RNA quality controls. A complete system includes a small RNA isolation kit, optimized miRNA first strand synthesis kit, and an instrument specific master mix. This paper also presents scientific data showing that the miRNA PCR Arrays have significantly improved specificity, sensitivity and reproducibility.

Introduction

What is miRNA?

Mature microRNAs (miRNA) are small single stranded RNA molecules, typically 22 nucleotides in length. They were first identified in studies of *Caenorhabditis elegans* development in 1993 [1], but have since been found in many species of plant and animal. These RNA species are originally transcribed as a larger RNA species hundreds or thousands of bases long, termed the primary miRNA (pri-miRNA) [2]. The pri-miRNA is trimmed by the Microprocessor, comprised of a Drosha family member, in the nucleus into a roughly 70 nucleotide hairpin termed precursor miRNA (pre-miRNA), which is then exported to the cytoplasm. The RNase III enzyme, Dicer, further processes pre-miRNA into the shorter RNA duplexes now known as miRNA. The miRNA is then unwound, and one strand (the mature miRNA) is incorporated into the RNA-induced silencing complex (RISC), composed of Dicer, TRBP and Ago2. The RISC complex guides the mature miRNA to its messenger RNA (mRNA) target, typically base pairing imperfectly to its 3' untranslated region (3'-UTR). The recruitment of this complex to a target messenger RNA (mRNA) inhibits its translation and therefore the synthesis of the encoded protein, but can also induce degradation of the mRNA target. The imperfect nature of the base-pairing, formed from a 7-nucleotide "seed region" within the miRNA, allows a single miRNA to control multiple genes with homologous sequences in the target region. Over 700 miRNA genes have been identified in the human genome (<http://microrna.sanger.ac.uk/>), and each miRNA is proposed to have hundreds of mRNA targets [3]. Therefore, it is predicted that 30% of human genes are regulated by miRNA [4].

Why Study miRNA?

The completion of the Human Genome Project in 2003 confirmed the hypothesis that a large part of the genome is not translated into proteins. This so called "junk" DNA was thought to be evolution's debris with no function. We now realize that a portion of this non-coding DNA is highly relevant to the regulation of gene expression. While only about 1000 human miRNA sequences have been identified so far, with each one having the possibility of hundreds of mRNA targets, the potential effects of this class of RNA molecule is quite

Contents

Introduction.....	1
A) <i>What is miRNA?</i>	1
B) <i>Why Study miRNA</i>	1
C) <i>How is miRNA Studied?</i>	2
The Complete miRNA PCR Array System	2
A) Improved Sensitivity & Dynamic Range.....	2
B) High PCR Efficiency, Improved Specificity & Discrimination.....	3
C) High Reproducibility	4
RT ² miRNA PCR Array Application Examples.....	5
I. Cancer Research: Identifying miRNA Cancer Biomarkers	5
II. Development Research: Identifying Tissue-Specific miRNA Biomarkers.....	6
III. Signal Transduction Research: Screen the Genome for p53-Responsive miRNA.....	6
IV. miRNA Expression Analysis from Formalin-fixed Paraffin-embedded Tissue Sections	6
Summary.....	7
References.....	7

large. The importance of miRNA regulatory roles is underscored by literature evidence that miRNA function is involved in almost all biological processes, including cancer [5], stem cell regulation [6], immune function [7], neurogenesis [8], metabolism [9] and others. The expression patterns of miRNA have been exploited as a new class of biomarkers for disease phenotypes and tissue classifications [10, 11], with the potential to hold better predictive value than coding-gene based biomarkers.

How is miRNA Studied?

The first question researchers want to know is: among the hundreds of miRNAs, which ones are differentially expressed in their model system. To get this answer, microarray and reverse transcription (RT) real-time PCR are the most often used platforms. Detecting every miRNA across the entire genome in a specific and sensitive way is a very challenging technological task. Many miRNA family members and otherwise distinct miRNA species have very similar sequences, some only differing by a single nucleotide.

Microarray-based miRNA detection relies on the direct hybridization of miRNA in the sample to specific probes on a solid surface. Only a single hybridization event determines the detection specificity. Unlike microarrays, real-time PCR is more specific and more quantitative. Not only multiple annealing events, but also the Taq polymerase confers assay specificity. Taq polymerase extends imperfectly annealed, non-specific primer-template complexes less efficiently over specific annealing events. Additionally, real-time PCR has greater sensitivity and a much wider detection range than microarrays. In fact, most researchers choose real-time PCR as a tool to further validate their microarray data.

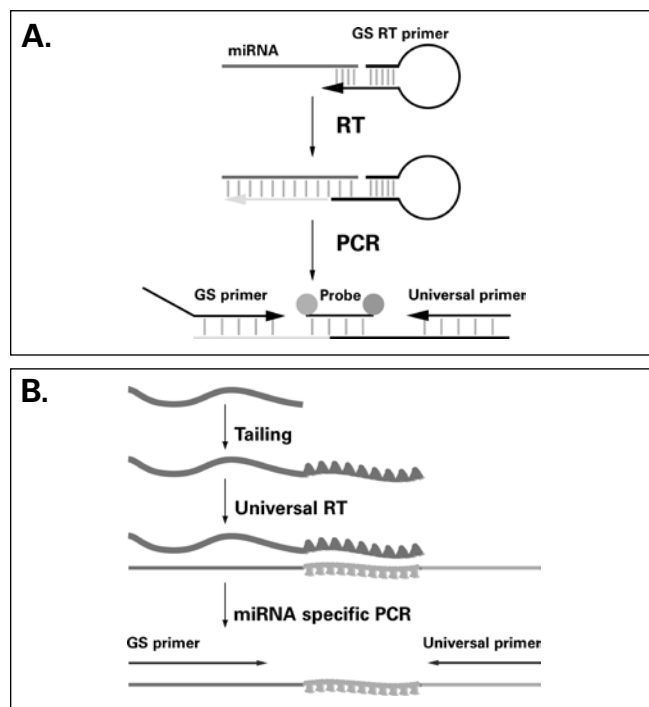


Figure 1: Strategies for miRNA RT-PCR. A) Stem-loop RT-PCR B) Universal poly-(A) tailing RT-PCR

Conventional schemes for RT real-time PCR assay design are not suitable for miRNA detection due to the short nature of the target templates. There are two main popular real-time PCR based approaches for miRNA detection (Figure 1). One uses sequence-specific stem-loop RT primers (designed to anneal to the 3'-end of a mature miRNA) to convert miRNA into cDNA [12]. Then, a forward primer (specific to the 5'-end of the miRNA), a universal reverse primer (specific for the stem-loop RT primer sequence) and a 5'-nuclease hydrolysis probe (matching part of the miRNA sequence and part of the RT primer sequence) are used in real-time PCR detection. The other approach uses RNA poly-(A) polymerase to add a poly-(A) tail to the free 3'-hydroxyl end of the mature miRNA molecule. Then, an oligo-dT-containing universal RT primer is used to convert miRNA into cDNA [13]. In the end, a miRNA-specific forward primer (utilizing the entire mature miRNA sequence) and a universal reverse primer (specific for the universal RT primer) are used in a SYBR[®] Green-based real-time PCR assay.

The limitation of the stem-loop RT-qPCR approach is that each miRNA assay requires a separate RT reaction, making the analysis of multiple miRNAs quite cumbersome. As each RT product can only be used for detecting one miRNA, it consumes more samples than needed when multiple miRNAs have to be assayed. Although the RT reaction can be multiplexed with many gene-specific stem-loop primers, performance and flexibility suffer when assaying a few hundred miRNAs together. Recently, it has been reported that miRNA variants are quite often expressed simultaneously in cells [14]. Those miRNA variants differ at 3'-ends due to variability in Dicer processing. Old gene-specific RT primer designs will not detect all of these variants. Because poly-(A) tailing RT-qPCR represents a universal approach, RT reaction efficiency should not be affected by the presence of variants. The cDNA generated by universal poly-(A) tailing RT reaction can be used to assay many different miRNAs. Archived cDNA can be conveniently stored and used for other or any new miRNAs in the future. No new RT assay design and performance is required for newly discovered miRNA species.

Although 5'-nuclease hydrolysis probe-based qPCR is generally perceived to be more specific, this advantage is limited for miRNA, given their short 20 to 30 nucleotide-long template sequences. With optimized reaction chemistry and primer design, universal poly-(A) tailing RT and SYBR[®] Green-based qPCR assays deliver similarly high specificity (see below) for miRNA with much more flexibility.

The Complete miRNA PCR Array System

The SABiosciences' RT² miRNA RT-PCR system offers a simple and scalable solution for the quantitative analysis of miRNA expression. PCR arrays allow the simultaneous detection of over 700 miRNAs, representing most functional miRNAs. Knowing and understanding the various challenges associated with miRNA detection, we came up with creative solutions in both reaction chemistry and assay design to deliver greatly improved performance over other traditional methods.

A) Improved Sensitivity & Dynamic Range:

There are multiple reasons for the superior sensitivity and dynamic

range for our miRNA assays. Key reason among these is the patent-pending chemistry of the RT² miRNA First Strand Kit that preferentially add poly-(A) tails and reverse transcribes mature miRNA, thereby decreasing non-specific background caused by other RNA species.

Poly-(A) tailing of single strand RNA is not a sequence specific process [15]. However, the poly-adenylation reaction does require the free 3'-hydroxyl groups on all miRNA molecules. RNA fragments with 3'-phosphorylation are not tailed or converted into cDNA. RNA poly-(A) polymerase is also very sensitive to template secondary structure. It works best with single stranded RNA ends, and tails double-stranded, blunt or non-protruding 3'-ends very inefficiently [15]. Most pre-miRNA molecules, having hair-pin structures, are not substrates for poly-(A) addition or reverse transcription.

While developing the miRNA qPCR system, we noticed that some RNA species (such as small nuclear RNA, tRNA and small rRNA) can be poly-adenylated and converted to cDNA by oligo-dT primers. Other RNA species with an intrinsic poly-(A) tract, like mRNA, can also be converted to cDNA. Because they all carry the universal priming sequence (from the oligo-dT RT primer), significant non-specific amplification can happen, leading to high background and making the specific analysis of mature miRNA problematic (Fig 2). To address this issue, we exploited the single-strand RNA dependence of both the poly-(A) polymerase and the reverse transcriptase. Both enzymes act on highly structured RNA very inefficiently. Thus, we developed a patent-pending buffer system, which favors the formation of RNA secondary structure during the poly-(A) tailing and reverse transcription process. In the same buffer, the very short miRNAs does not form higher order structures. As a result, our buffer conditions achieve much lower background compared to traditional poly-(A) tailing and RT chemistry. The overall improvement of sensitivity can be as much as 1000-fold (Figure 2).

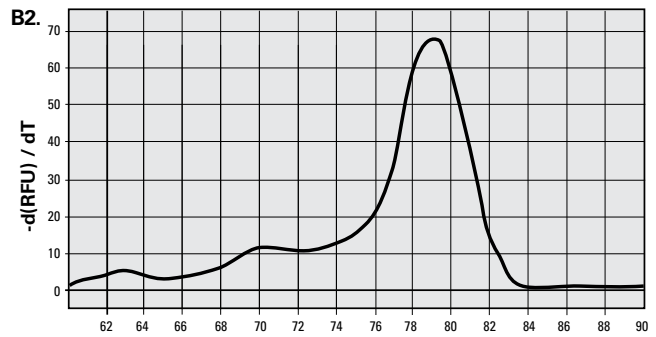
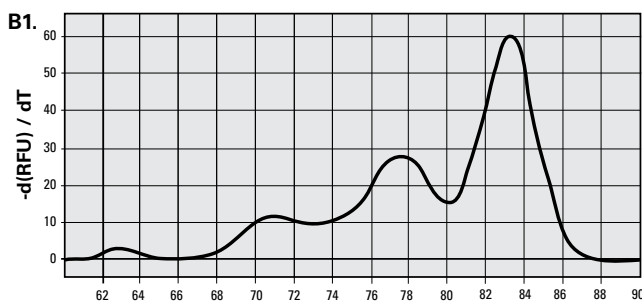
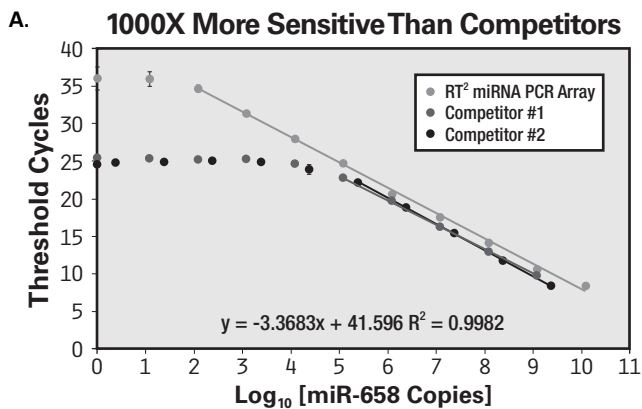


Figure 2: Superior Sensitivity & Dynamic Range with miRNA PCR Array System. A) Synthetic mir-658 was serially diluted into a constant amount (100 ng) of small RNA enriched from 293H cells lacking that sequence. Samples were analyzed with mir-658-specific RT² miRNA qPCR Assays and other commercial assays. The resulting threshold cycle values from the assays are plotted versus the log₁₀ of the number of mir-658 template copies. B) The elimination of non-specific background contributes to the observed wider dynamic range. Melt curve analysis reveals multiple peaks in another commercial assay (B1), but a single peak in the SABiosciences' assay (B2).

B) High PCR Efficiency, Improved Specificity & Discrimination:

For poly-(A) tailing RT and SYBR® Green-based qPCR of miRNA, conventional wisdom would utilize forward miRNA-specific primer sequence identical to the mature miRNA sequence [13], because they are about the same length. However, in many cases, this primer selection criterion creates various problems, such as primer dimers, low amplification efficiency, high non-specific amplification, and low discrimination among homologous miRNA. So what options are available besides abandoning such assays?

miR-99a	AACCCGUAGAUCCGAUCUUGUG
miR-100	AACCCGUAGAUCCGA AC UUGUG

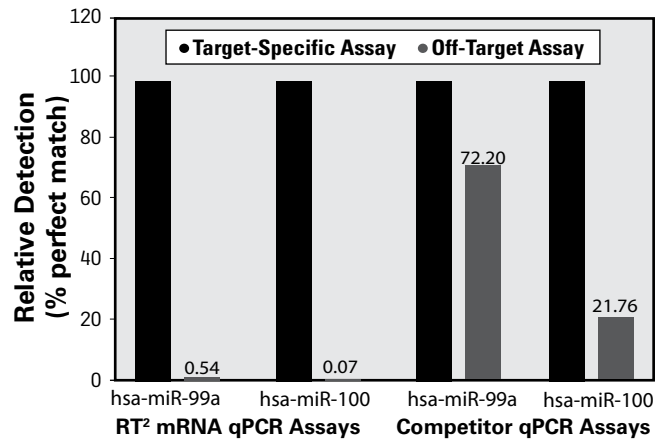


Figure 3: RT² miRNA PCR Assays Distinguish miRNA Family Members with Single Nucleotide Mismatches via a Proprietary Primer Design. Relative detection as a percent of the perfect match (100 x 2^{-ΔΔC_t}) is calculated from the C_t values of target- and off-target assays used to detect 105 copies of synthetic miRNA template. Mismatches between assay and synthetic template are shown in bold.

Many miRNA sequences only differ by one or two nucleotides. Primer annealing differences between perfectly matched and mismatched cDNA templates, as well as the negative impact of mismatch on Taq polymerase's activity, help real-time PCR achieve better sequence discrimination than microarrays. However, some primers designed for mature miRNA sequences following conventional rules cannot

achieve the level of discrimination required. We have empirically validated and adopted primer design optimization rules that can make assays more discriminative. For example, we studied the cross-reactivity of two primer assays for hsa-miR-99a and hsa-miR-100. The difference between their mature miRNA sequences is only one nucleotide (Figure 3). Our competitor's miRNA primer assays show high degree of cross-reactivity (Fig 3). In contrast our hsa-miR-99a assay has very low level of amplification from hsa-miR-100 template, and conversely the hsa-mir-100 assay does not detect hsa-mir-99a template. Similarly, other highly homologous miRNA sequences are well differentiated with our miRNA assays (Table 1).

Synthetic miRNA template	Assay Primers	Relative Detection (%Perfect Match)	miRNA Template Sequence
miR-10a	miR-10a	100.00	UACCCUGUAGAU U CCGAAUUUGUG
	miR-10b	0.98	
miR-10b	miR-10b	100.00	UACCCUGUAGAU AC CGAAUUUGUG
	miR-10a	0.01	
miR-196a	miR-196a	100.00	UAGGUAGUUUC A UGUUGUUGGG
	miR-196b	0.20	
miR-196b	miR-196b	100.00	UAGGUAGUUUC C UGUUGUUGGG
	miR-196a	1.64	

Table 1: High Specificity of miRNA PCR Array. Relative detection as a percent of the perfect match ($100 \times 2^{-\Delta\Delta C_t}$) is calculated from the C_t values of target and off-target assays used to detect 105 copies of synthetic miRNA template. The observed cross-reactivities seen between RT² miRNA qPCR Assays for different miRNA species are compared. Location of miRNA sequence mismatch is shown in bold.

Our proprietary miRNA specific primer design algorithm minimizes non-specific amplification and maximizes the discrimination of miRNA family members in SYBR[®] Green-based real-time PCR. To insure the performance of our design algorithm, we apply three stringent quality control (QC) criteria to each and every miRNA assay in wet bench tests.

(1) Each miRNA assay must have a very low background. The best test of each miRNA assay's background is to perform a mock poly-(A) tailing without the use of RNA poly-(A) polymerase followed by RT-PCR using RNA extracted from cells. Under this condition, no miRNA molecules should be converted into cDNA. Anything detected by real-time PCR indicates non-miRNA background, which compromises specific detection for the particular miRNA. Background largely comes from two sources, primer dimmers and non-specific interactions between primers and other non-miRNA derived cDNA templates. Table 2 summarizes the differences in backgrounds between our assays and one of our competitors' for multiple miRNA sequences. Both the primer design algorithm and the unique buffer system mentioned above contribute to the improved performance achieved by our assays.

ID	Poly A Minus C_t Values	
	SABiosciences	Competitor
hsa-mir-16	34.23	30.67
hsa-mir-21	No C_t	31.57
hsa-mir-99a	No C_t	31.96
hsa-mir-100	No C_t	33
hsa-mir-658	37.77	34.1
hsa-mir-769-5p	36.4	30.46

Table 2. SABiosciences First Strand Kit Shows Lower Background than the Competitor. Total RNA was reverse-transcribed without Poly-(A) Polymerase using SABiosciences' or the competitor's buffer system. The cDNA was then used in the corresponding miRNA qPCR assays. A C_t value >35 is considered not reliably detected and a negative call.

(2) Each miRNA assay must have high amplification efficiency. Within the same chemistry, differences in primer annealing, estimated by primer melting temperature (T_m), often cause differences in real time PCR efficiency. The limited miRNA sequence space does not always allow the selection of miRNA primers with the best T_m calculation. Moreover, theoretical T_m calculations do not always agree with empirical measurements. Rather than relying solely on T_m , we evaluate each primer's amplification efficiency using the standard curve method. Specifically, a pool of synthetic cDNA templates for all queried miRNA sequences is prepared. Then, PCR efficiency for each miRNA-specific primer is calculated from experiments using serial dilutions of this pool. All validated primers should have close to 100% efficiencies.

(3) Each miRNA assay must generate a single PCR product as judged by the melt curve analysis. As discussed earlier, sub-optimized miRNA assays may have non-specific amplifications, as indicated by the presence of multiple peaks or a peak with an incorrect T_m in a melt curve analysis. We test all of our pre-designed miRNA primers in PCR with enriched small RNA from four different cell lines separately following poly-(A) tailing and RT reactions. Good primer assays yield single dissociation peaks at the same temperature (T_m) in all four cell samples.

All of the above criteria should be applied to any SYBR[®] Green-based miRNA assay designs. We have used our expertise to provide high-quality PCR assays so researchers do not have to do the development work themselves. In our experience, we find that about 47% assays failed our three QC tests initially. For many miRNA, additional rounds of primer design optimization improve PCR efficiency, decrease background, and increase specificity. For others, we are still developing solutions in order to offer those assays in the future.

C) High Reproducibility

One of the challenges for the miRNA PCR Array system is the amplification of every relevant, pathway- or disease-focused miRNA during the same PCR run. Due to the consistent QC criteria for every assay during our wet-bench validation process, the same uniform PCR conditions achieve the high level of sensitivity and specificity expected of real-time PCR.

Our optimized reaction chemistry insures a high degree of

reproducibility from the miRNA PCR Array System. To demonstrate this concept, one end-user characterized the same human RNA sample in technical duplicates with two separate RT² miRNA First Strand Kits and RT² miRNA PCR Arrays. The raw threshold cycle values for the entire array's gene panel were then compared between the replicates. The resulting scatter plot comparison yields the predicted ideal straight line with a slope close to 1.0 and a correlation coefficient of 0.99 (Figure 4). The highly reproducible nature of the raw data means that results can be reliably compared from run-to-run, plate-to-plate, and sample-to-sample. Any observed differences in expression can therefore be attributed to the biology under study and not to any variability in the technology itself.

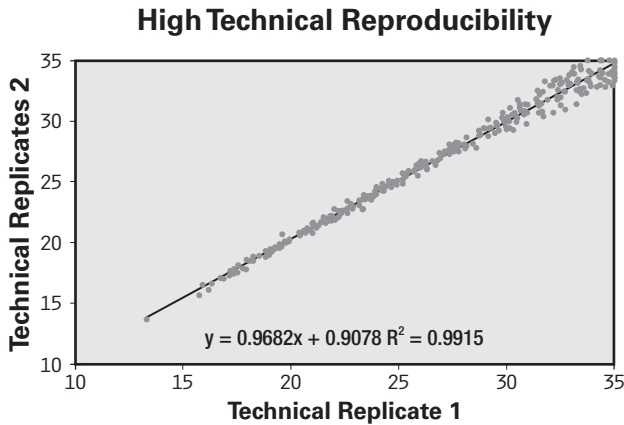


Figure 4: The High Reproducibility of the RT² miRNA PCR Arrays. Duplicate samples of small RNA enriched from human brain tissue (Ambion, 200 ng) were characterized using the RT² Human Genome miRNA PCR array on an ABI 7900HT. Raw C_t values greater than 35 or reported as “not determined” were first changed to 35. The values from the replicate arrays were plotted against each other, and then the data was fit to a straight line.

RT² miRNA PCR Array Application Examples

The regulated expression of miRNA adds another layer of control to an already complex gene regulatory network. One miRNA can regulate multiple mRNA targets, and one target mRNA may be regulated by multiple miRNA sequences. Therefore, the role that any given miRNA sequence plays has yet to be completely defined. Correlating miRNA expression profiles to biological phenotypes adds to our understanding of miRNA-based gene regulation.

Analyzing a representative set of sequences with the Genome or miFinder arrays discovers novel functional roles for each miRNA. Focused miRNA panels, like those on the Cancer or Cell Differentiation & Development arrays, screen miRNA biomarkers known to be most important to their specific model system. The representative results below will illustrate examples of experiments that may be performed with the RT² miRNA PCR Array System.

I. Cancer Research: Identifying miRNA Cancer Biomarkers

One currently burgeoning application of miRNA expression profiling is the identification, screening, and validation of cancer biomarkers. Because miRNAs have been shown to regulate genes in multiple oncogenic pathways, it is reasonable to assume that some of the miRNAs themselves may be dysregulated in tumors and cancer cells. Screening for potential biomarkers can be performed efficiently with RT² miRNA PCR Arrays by quantitatively analyzing a large number of miRNAs simultaneously. To exemplify the ease that interpretable results can be obtained, we chose to compare miRNA expression levels between a colon tumor and the corresponding matched adjacent normal tissue (Figure 5). The RT² Human Cancer miRNA PCR Array identifies potential colon cancer biomarkers, as shown by the miRNAs that are up- or down-regulated in this experiment.

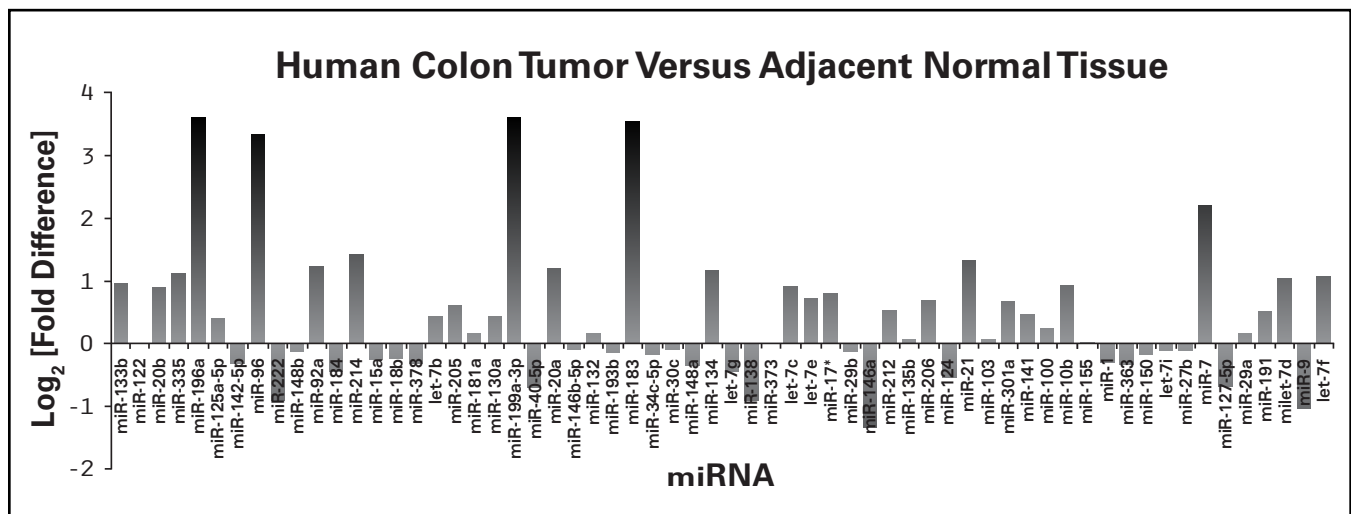


Figure 5: RT² Human Cancer miRNA PCR Array identifies potential colon cancer biomarkers. Small RNA isolated from human colon tumor and matched adjacent normal tissue (Biochain) was characterized with PCR Arrays containing assays specific for 88 cancer-related human miRNA sequences. Fold-differences are calculated from raw C_t values normalized to a panel of housekeeping snRNA genes.

II. Development Research: Identifying Tissue-Specific miRNA Biomarkers

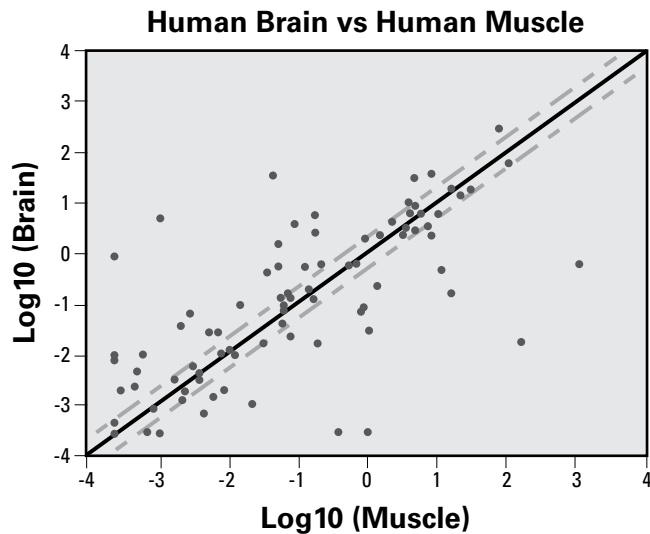


Figure 6: RT² Human Differentiation miRNA PCR Array Identifies Tissue Specific miRNAs. Small RNA enriched from human brain and muscle tissue total RNA (200 ng, Ambion) were characterized with PCR Arrays containing assays for 88 differentiation-related miRNA sequences. Normalized miRNA sequence expression levels for both tissues are calculated and plotted against each other. Symbols outside the dotted lines represent miRNA with at least a 2-fold expression difference between the respective tissues.

MicroRNA research began and continues to expand in the developmental research field. Our miRNA RT-PCR array easily screens for potentially important miRNAs in your tissue of interest. As an example, we compared miRNA expression in two different tissues, muscle and brain (Figure 6). The RT² Human Cell Differentiation & Development miRNA PCR Array identifies 26 brain- and 20 muscle-specific miRNA, as defined by greater than a 2-fold enrichment in these respective tissues.

III. Signal Transduction Research: Screen the Genome for p53-Responsive miRNA

Cancer and cell differentiation are the two research fields that have most widely adopted the concept of miRNA expression profiling and therefore high value miRNA candidates can be easily grouped together. Scientists working in other biomedical research fields still need to discover which miRNA sequences regulate genes involved in the biological or disease processes that they study or simply their single gene of interest. For example, if a protein decreases its apparent level of expression on a Western blot but maintains its level of expression at the RNA level by RT-PCR, then a post-transcriptional mechanism of gene regulation, like miRNA, is most likely involved. In fact, any researcher who is interested in understanding how their genes of interest are regulated should consider miRNA expression profiling. Fortunately, unlike gene expression profiling, the relative small size of the mammalian miRNA genome allows a 96-well or 384-well PCR plate to simultaneously profile up to a quarter or even a half of the genome on the miFinder or Genome RT² miRNA PCR Arrays.

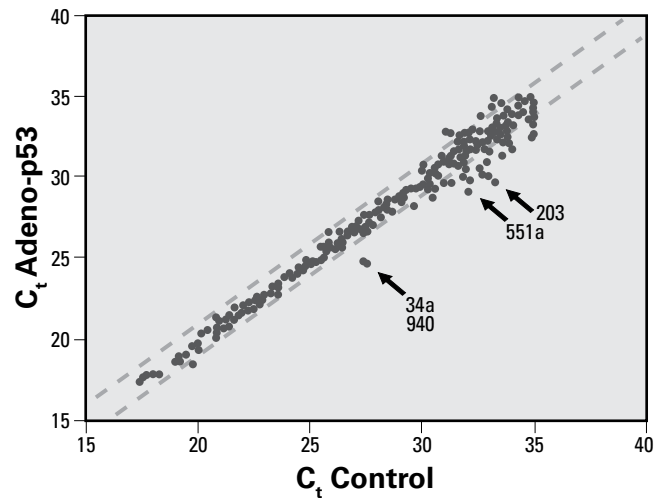


Figure 7: RT² Human Genome miRNA PCR Array Identifies Potential p53 Responsive miRNAs. PC3 cells were transduced with adenovirus either encoding p53 or containing an empty vector. After 48 h, small RNA was characterized with PCR Arrays containing 376 human miRNA-specific assays. The scatter plot shows the differences in miRNA expression upon p53 over-expression. Symbols represent miRNA with greater than two fold changes in the expression.

To illustrate this idea, we profiled expression change for a large portion the human miRNA genome (376 sequences) upon overexpression of the p53 oncogene using Human Genome RT² miRNA PCR Arrays. The RT² Human Genome miRNA PCR Array identifies known and novel miRNA sequences responsive to p53 over-expression (Figure 7). The array identifies the well-known p53 targets, mir-34a and mir-34c [16], as well as a dozen novel candidate targets including mir-203, mir-551a, mir-940, mir-614, and others. The results of this experiment interestingly suggest yet another layer of complexity to gene expression regulation. The transcription factor, as gene expression regulator, also regulates the expression of miRNA, a new class of gene expression regulators at the post-transcriptional level. More importantly, this experiment also demonstrates how any area of biomedical research can easily contribute to a better understanding not only of the role of miRNA in their model system of interest, but also of miRNA function in general, all with a very simple experiment using the RT² miRNA PCR Arrays.

IV. miRNA Expression Analysis from Formalin-fixed Paraffin-embedded Tissue Sections

Formalin-fixed paraffin embedded (FFPE) samples represent an invaluable source of research materials providing access to well documented links between molecular and clinical information. It has been suggested that miRNA, being small in size, are less prone to degradation during the fixation and storage process. Thus, we studied whether our miRNA RT-PCR array system can be used to profile miRNA expression from FFPE samples. As an example, total RNA was extracted from one 20 μ m section of a two-year old human colon FFPE block using the RT² FFPE RNA Extraction Kit. One microgram extracted RNA was used with RT² miRNA First Strand Kit and Human Cancer miRNA PCR Array protocol. The results (Fig. 8) indicate that the majority of miRNAs from FFPE samples are well preserved and can be used as the template for our miRNA RT-PCR array system. This opens up the opportunity of carrying out miRNA biomarker analysis in the vast amount of archived samples.

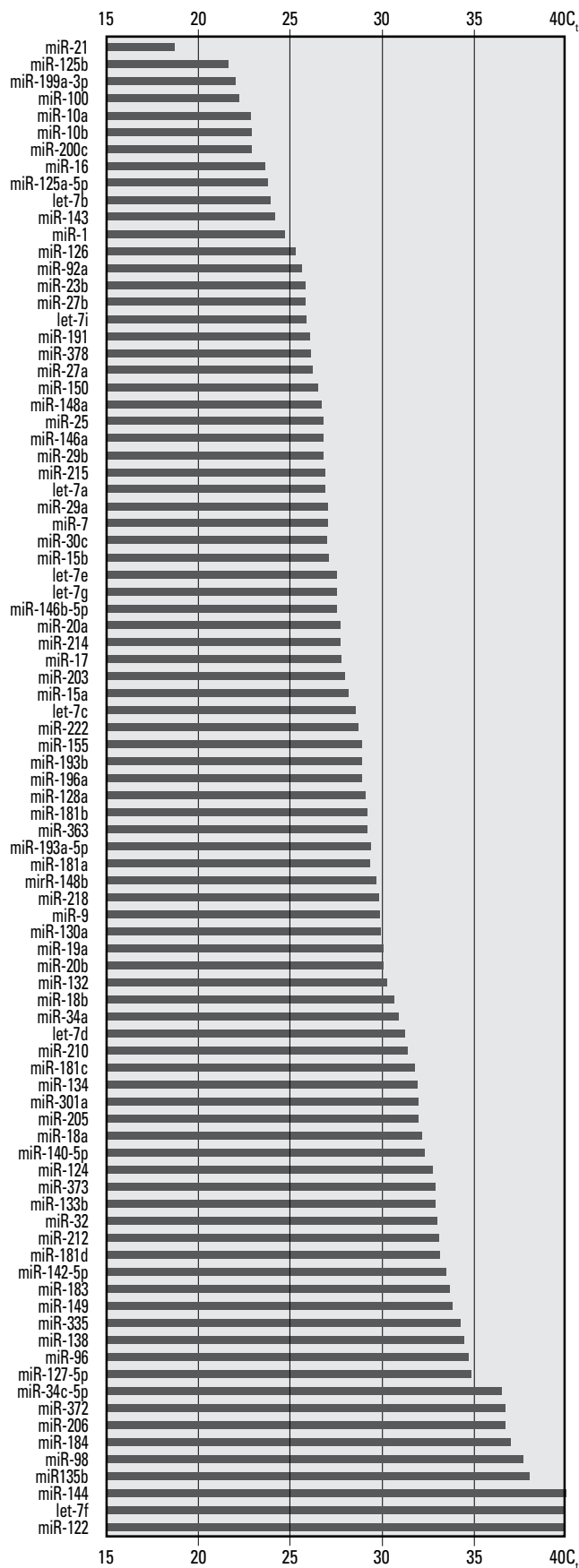


Figure 8: Expression rank order of 88 cancer related miRNAs in RNA extracted from one 20mm section of a 2-year old normal human colon FFPE block using the RT² FFPE RNA Extraction Kit.

Summary

The shortness and homology of miRNA sequences present significant challenges for their accurate detection. We have developed patent-pending chemistry and a proprietary primer design algorithm. Together with a rigorous validation process, these technical advances result in the high performance miRNA assays, which form the foundation of the RT² miRNA PCR Array system. The flexibility, simplicity, and convenience of standard SYBR Green PCR detection methodology make the miRNA PCR Array system accessible for routine use in any research laboratory. The arrays feature a pathway-focused, genome-wide or even customizable miRNA content design, while demonstrating the sensitive, specific and reproducible performance expected from real-time PCR. As a result, the RT² miRNA PCR Array System is ideally suited to allow every laboratory to combine the performance of real-time PCR with the profiling capabilities of a microarray for miRNA expression profiling.

Reference

1. Lee, R.C., R.L. Feinbaum, and V. Ambros, The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell*, 1993. 75(5): p. 843-54.
2. Bushati, N. and S.M. Cohen, microRNA functions. *Annu Rev Cell Dev Biol*, 2007. 23: p. 175-205.
3. Lim, L.P., et al., Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature*, 2005. 433(7027): p. 769-73.
4. Ross, J.S., J.A. Carlson, and G. Brock, miRNA: the new gene silencer. *Am J Clin Pathol*, 2007. 128(5): p. 830-6.
5. Visone, R. and C.M. Croce, MiRNAs and cancer. *Am J Pathol*, 2009. 174(4): p. 1131-8.
6. Gangaraju, V.K. and H. Lin, MicroRNAs: key regulators of stem cells. *Nat Rev Mol Cell Biol*, 2009. 10(2): p. 116-25.
7. Xiao, C. and K. Rajewsky, MicroRNA control in the immune system: basic principles. *Cell*, 2009. 136(1): p. 26-36.
8. Shen, Q. and S. Temple, Fine control: microRNA regulation of adult neurogenesis. *Nat Neurosci*, 2009. 12(4): p. 369-70.
9. Tavintharan, S., et al., Riboregulators and metabolic disorders: getting closer towards understanding the pathogenesis of diabetes mellitus? *Curr Mol Med*, 2009. 9(3): p. 281-6.
10. Bartels, C.L. and G.J. Tsongalis, MicroRNAs: novel biomarkers for human cancer. *Clin Chem*, 2009. 55(4): p. 623-31.
11. Rosenfeld, N., et al., MicroRNAs accurately identify cancer tissue origin. *Nat Biotechnol*, 2008. 26(4): p. 462-9.
12. Chen, C., et al., Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res*, 2005. 33(20): p. e179.
13. Shi, R. and V.L. Chiang, Facile means for quantifying microRNA expression by real-time PCR. *Biotechniques*, 2005. 39(4): p. 519-25.
14. Morin, R.D., et al., Application of massively parallel sequencing to microRNA profiling and discovery in human embryonic stem cells. *Genome Res*, 2008. 18(4): p. 610-21.
15. Martin, G. and W. Keller, Tailing and 3'-end labeling of RNA with yeast poly(A) polymerase and various nucleotides. *RNA*, 1998. 4(2): p. 226-30.
16. He, L., et al., A microRNA component of the p53 tumour suppressor network. *Nature*, 2007. 447(7148): p. 1130-4.

