

miRNA qPCR ASSAYS

powered by NAWGEN

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Our miRNA qPCR Assays were developed by miRNA experts at Nawgen to improve upon previously available qPCR assays, resulting in assays that are more specific, more sensitive, and less variable.

INCLUDED

- Reverse transcription primer
- Forward and reverse primers for each miRNA
- Reagents provide enough for 250 reactions

QPCR ASSAYS FOR MIRNA EXPRESSION PROFILING

MicroRNAs (miRNAs) are a family of small non-coding RNA molecules that downregulate the expression of their gene target. miRNAs are master regulators of many important biological processes including a variety of human diseases. One major challenge in miRNA research is to accurately and conveniently determine the expression level of miRNAs in various experimental systems. As deregulated expression of miRNAs often indicates functional deregulation, screening for miRNAs with altered expression profiles is likely to provide clues for the involvements of miRNAs in disease development.

Currently, several commercial products are available for PCR-based miRNA detection and quantitation. However, there are major design limitations associated with these available assays, with inadequate considerations for both assay sensitivity and specificity. To address these technical issues, we have developed a new real-time RT-PCR method, using a bioinformatics algorithm for miRNA primer design by incorporating multiple design filters to improve miRNA assay specificity, sensitivity and homogeneity (Wang 2009). This algorithm has incorporated multiple design features that have been proved to be important in our previous studies (Wang and Seed 2003). In addition, our new algorithm also includes novel primer features that are uniquely associated with miRNA assay design.

The overall experimental strategy using these new miRNA assays is summarized in Figure 1. As the first step, multiplexed RT reaction is performed using a pool of RT primers. These RT primers anneal specifically to the target miRNAs, leading to a pool of mixed cDNA products from the miRNAs of interest. By performing a multiplexed RT reaction, the overall experimental cost is significantly reduced

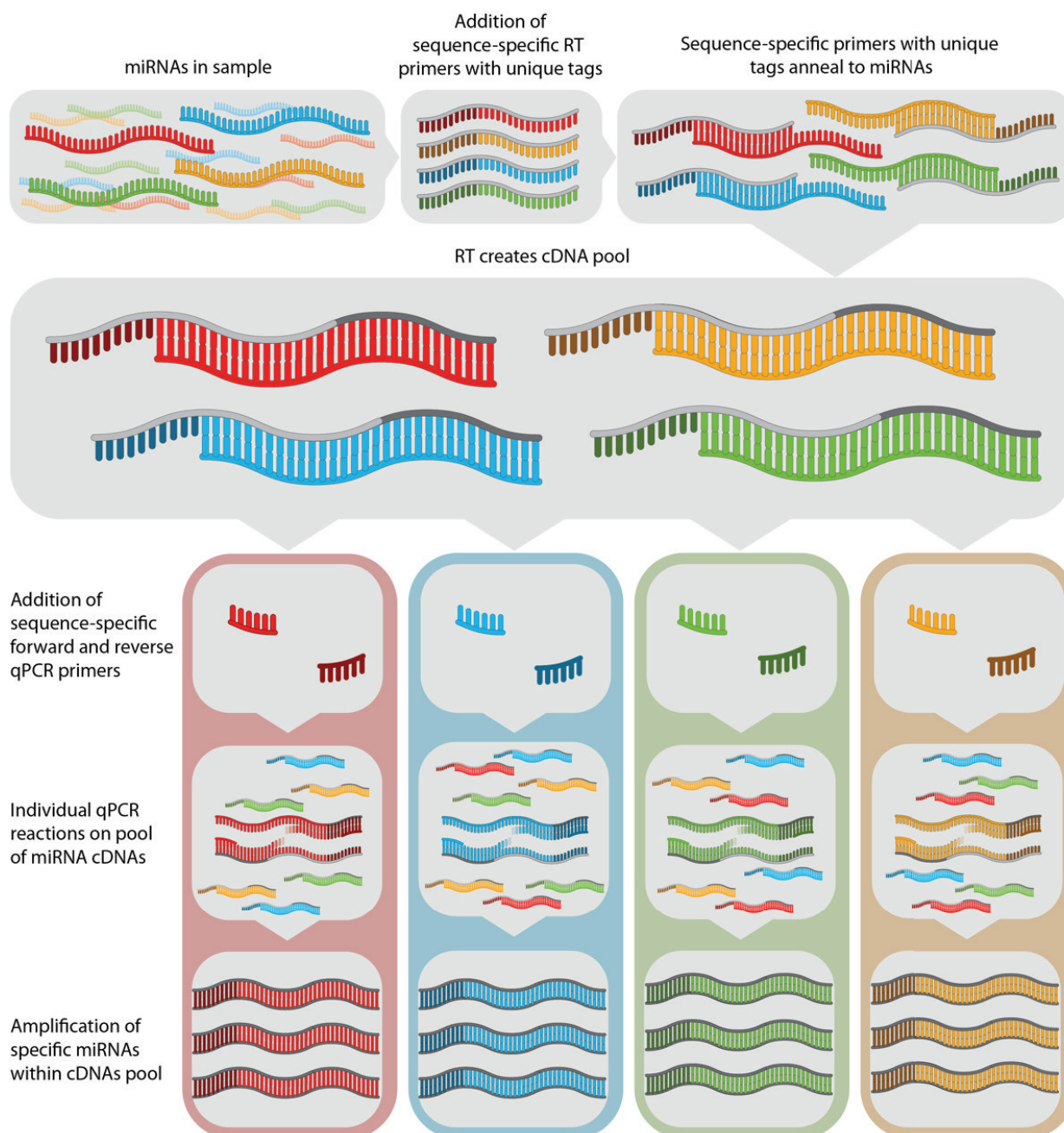


Figure 1. The experimental strategy for a new miRNA expression profiling platform with real-time RT-PCR. Multiplexed RT is performed with a pool of miRNA-specific RT primers. These RT primers contain unique tag sequences at the 3'-end. Real-time PCR is then performed with the cDNA templates generated from the multiplexed RT reaction. The PCR reverse primer specifically anneals to the 3'-end of the cDNA template and the PCR forward primer specifically anneals to the tag sequence in the RT primer.

since only one RT reaction is needed for each RNA sample. After the RT reaction, separate real-time PCR assays are performed to quantify the expression of individual miRNAs. Each PCR is performed with two primers that are uniquely associated with the miRNA of interest, one with miRNA-specific sequence and the other from the unique tag sequence in the RT primer. Thus, these primers are not likely to cross-react to primers from other miRNA assays even though multiplexed RT reactions are performed. Many bioinformatics parameters have been included in the design algorithm to avoid primer cross-reactivity, potential primer dimer and secondary structure formation as well as to ensure uniform PCR assay properties, such as primer T_m , length, GC content, and the amplicon length. In this way, all miRNA assays can be performed together under the same experimental condition.

EXPERIMENTAL VALIDATION OF THE MIRNA ASSAYS

Nawgen miRNA qPCR assays have been extensively validated experimentally. Specifically, we have experimentally validated all functional miRNAs in humans and mice. The sensitivity of the miRNA assays was evaluated with standard curve analysis, which indicates that our assays can sensitively and reliably quantify as few as only several miRNA copies (Figure 2A). Further specificity analysis indicated that our miRNA assays can reliably detect a single nucleotide variation in the miRNA sequence, which represents a much higher specificity than miRNA microarrays (Figure 2B). To test the assay reproducibility on clinical tissues, we have compared

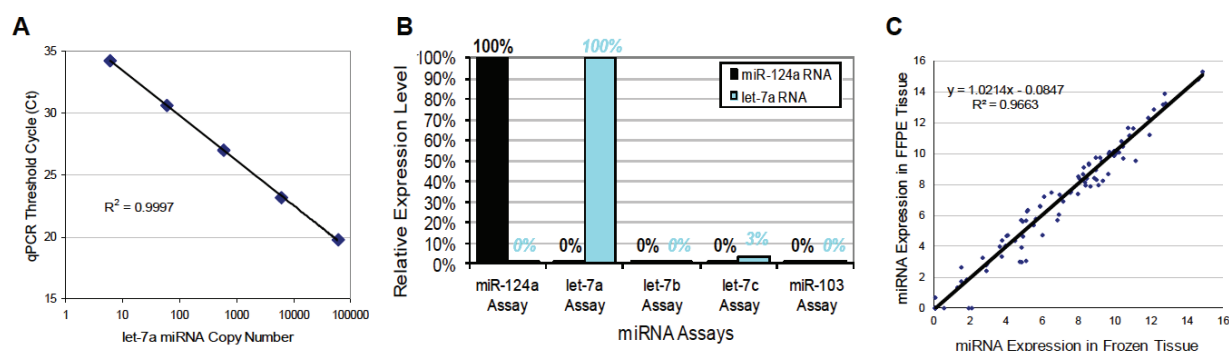


Figure 2. Quality metrics to evaluate the sensitivity, specificity and reproducibility of the miRNA assays. (A) Standard curve analysis to evaluate the dynamic range and sensitivity of the miRNA assays. (B) Experimental validation of the miRNA assay specificity. let-7c and let-7b miRNAs are different from let-7a by one and two nucleotides, respectively. Despite the small sequence variations, synthetic let-7a was not detected by the assay designed for let-7b and there was only 3% cross-reactivity to synthetic let-7a using the let-7c assay. (C) Evaluation of the reproducibility of the miRNA assays. miRNA expression profiles were determined in both frozen and FFPE tumor tissues from the same patients using our miRNA assays. Each data point in the graph represents one individual miRNA.

the profiles of archived formalin-fixed paraffin-embedded (FFPE) tissues and frozen tissues from the same c patients. As shown in Figure 2C, miRNA expression profile in the FFPE tissue was very similar to that in the frozen tissue, indicating high reproducibility of the miRNA assays when applied to challenging clinical specimens.

As an example of our miRNA profiling studies, we have recently profiled 368 cancer-related miRNAs in two hundred FFPE tumor tissues. First, One master RT reaction was performed with a pool of 368 RT primers, followed by separate real-time PCR for each miRNA. Most of the 368 miRNAs had detectable expression in FFPE cancer tissues with these new assays (Figure 3A). In contrast, none of the 368 miRNAs was detected in total RNA extracted from *E. Coli*, which does not contain any miRNA and thus served as negative control (Figure 3B).

COMPARISON TO TAQMAN ASSAYS

We also compared Nawgen assays with TaqMan-based miRNA assays. TaqMan assays are commercially available and are widely used for analyzing the expression of miRNAs. Both TaqMan assays and Nawgen assays are based on real-time RT-PCR, but the miRNA assay design strategies are drastically different. For this comparative

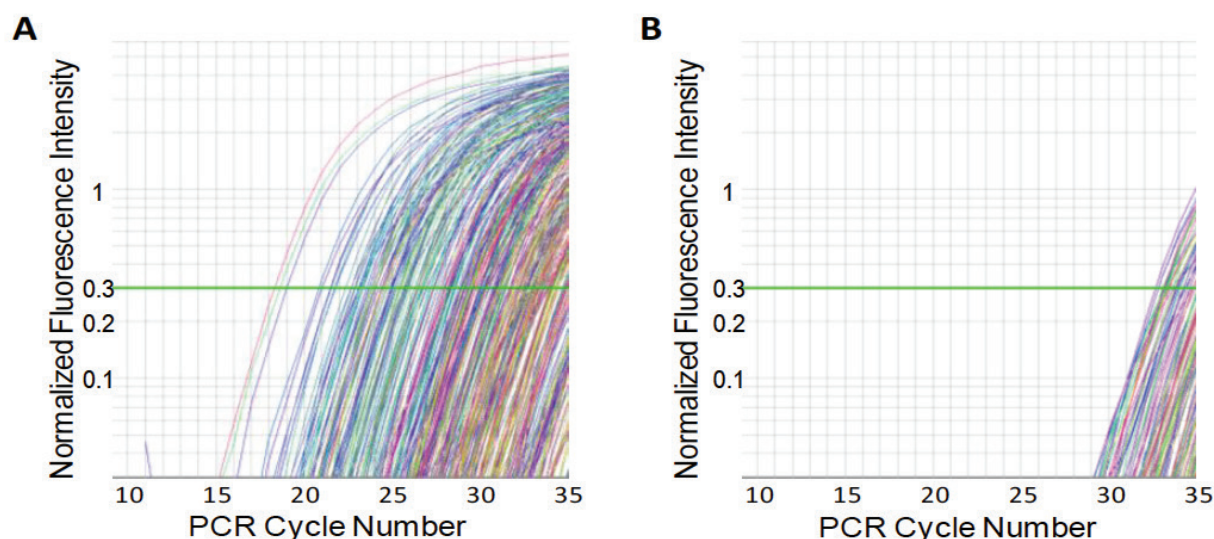


Figure 3. Real-time RT-PCR validation of 368 miRNA assays. Each line in the plot represents the amplification curve for one miRNA. The green horizontal bar is used to determine the threshold PCR cycle (Ct) for each miRNA. The Ct value is defined as the minimum number PCR cycle required for detection of miRNA expression. Detectable miRNA expression is defined as Ct <33. (A) Real-time RT-PCR was performed with the total RNA extracted from a FFPE cervical tumor. Most miRNAs had detectable expression as revealed by the Ct values. (B) Real-time RT-PCR was performed with the total RNA from *E. coli*. None of the miRNAs had detectable expression.

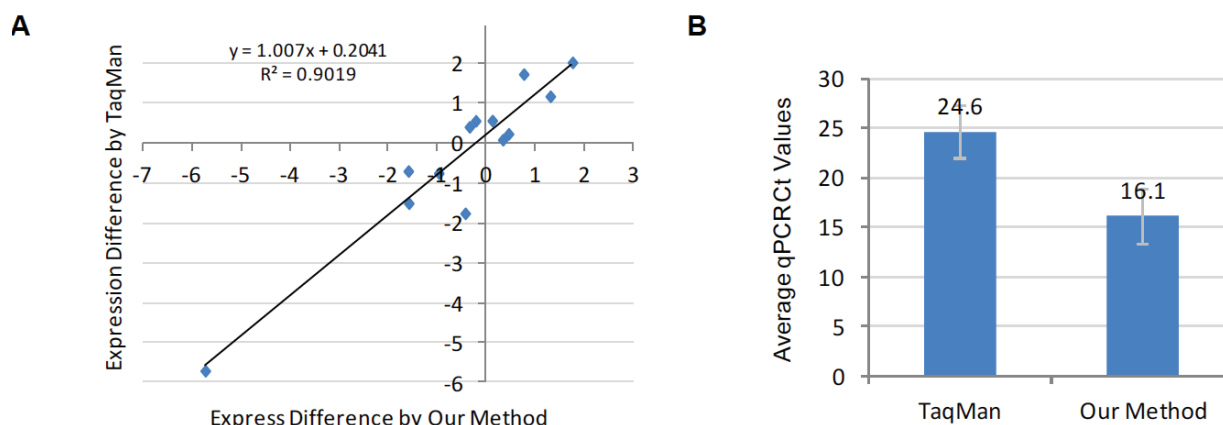


Figure 4. Performance comparison of TaqMan and Nawgen Assays. TaqMan assays and Nawgen assays were compared for profiling the expression differences of 13 selected miRNAs between natural killer (NK) cells and T cells. The same amount of input RNA was used in both methods. (A) Correlation of the methods for differential expression analysis. Each data point in the graph represents the expression difference (represented by log2 fold change) of one specific miRNA between NK cells and T cells. (B) Comparison of detection sensitivity. Raw threshold cycle (Ct) values of all miRNAs detected by each method were averaged and presented. The Ct value is defined as the minimum number PCR cycle required for detection of miRNA expression. On average, Nawgen assays were 362 ($2^{(24.6-16.1)}$) times more sensitive than TaqMan assays at detecting miRNA expression.

analysis, we determined the expression differences of selected miRNAs between natural killer (NK) cells and T cells, using both TaqMan and Nawgen assays. As shown in Figure 4A, the profiling results from the two methods were highly correlated ($r=0.95$), and identified the same set of differentially expressed miRNAs between the two cell types. However, Nawgen assays were much more sensitive than TaqMan assays at detecting miRNA expression (over 300-fold as shown in Figure 4B). Thus, Nawgen assays can be applied even when only a small amount of RNA is available (which is usually the case for clinical tissue analysis).

In summary, Nawgen miRNA qPCR assays have robust performance for profiling miRNA expression in a variety of biological samples including challenging clinical specimens.

REFERENCES

1. Wang, X. 2009. A PCR-based platform for microRNA expression profiling studies. *RNA* 15: 716-723.
2. Wang, X., and Seed, B. 2003. A PCR primer bank for quantitative gene expression analysis. *Nucleic Acids Res* 31: e154.