Simple, quantitative primer-extension PCR assay for direct monitoring of microRNAs and short-interfering RNAs

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ABSTRACT

There has been a surge of interest in the biology of microRNAs and the technology of RNA interference. We describe a simple, robust, inexpensive assay for quantitative analysis of microRNAs and short-interfering RNAs. The method relies on primer extension conversion of RNA to cDNA by reverse transcription followed by quantitative, real-time PCR. Technical parameters critical to the success of the assay are presented. Measurements of microRNA levels are sensitive, with most assays allowing measurements in the femtomolar range, which corresponds to tens of copies per cell or less. The assay has a high dynamic range and provides linear readout over differences in microRNA concentrations that span 6–7 orders of magnitude. The assay is capable of discriminating between related microRNA family members that differ by subtle sequence differences. We used the method for quantitative analysis of six microRNAs across 12 tissue samples. The data confirm striking variation in the patterns of expression of these noncoding regulatory RNAs.

Keywords: primer extension; quantitative PCR; locked nucleic acid; microRNA; siRNA; SYBR green

INTRODUCTION

MicroRNAs (miRNAs) are endogenous regulators of gene expression that are expressed as precursor transcripts from genomic DNA (Bartel 2004). Short-interfering RNAs (siRNAs) are derived from double-stranded RNA molecules (Tomari and Zamore 2005). Both are processed into ~21-nucleotide (nt), single-stranded molecules that become incorporated into the RNA-induced silencing complex (RISC) (Liu et al. 2004; Song et al. 2004). RISC mediates down-regulation of gene expression through translational inhibition, transcript cleavage, or both (Tang 2005). RISC is also implicated in transcriptional silencing in the nucleus of a wide range of eukaryotes (Morris et al. 2004; Matzke and Birchler 2005).

Analytical tools for monitoring nucleic acids are an indispensable part of molecular biology. Several groups have described microarray methods for monitoring microRNA expression (Babak et al. 2004; Barad et al. 2004; Miska et al. 2004; Nelson et al. 2004; Sempere et al. 2004; Sun et al. 2004; Thomson et al. 2004). These efforts have provided a qualitative overview of microRNA expression patterns in cell lines and in normal and diseased human tissues (Babak et al. 2004; Baskerville and Bartel 2005). With gene expression microarrays, quantitative, transcript-specific PCR assays have proven to be a powerful complementary tool for validating initial observations and for extending data for transcript regions that are not addressed by probes on the array. In an analogous manner, we sought to create a quantitative assay for miRNAs that would allow us to assess absolute copy (Lagos-Quintana et al. 2003; Lim et al. 2005) numbers and a flexible assay for siRNAs that would allow us to measure any engineered interfering sequence. Several detection methods have been reported in the literature, including Northern blots (Lagos-Quintana et al. 2003; Lim et al. 2005), primer extension (Zeng and Cullen 2003), Invader Assay (Allawi et al. 2004), signal-amplifying ribozymes (Hartig et al. 2004), and mirMASA bead-based technologies (Babak et al. 2004). None of these approaches could be easily implemented in our laboratory, so we sought to construct an assay method by combining existing molecular biology techniques.

The research of Zeng and Cullen (2003) demonstrated that primer extension (PE) was a viable method for quantitative analysis of microRNA expression. Rather than using

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Article and publication are at http://www.rnajournal.org/cgi/doi/10.1261/rna.2148705.

radioactive band-shift assays, we wished to detect primer-extended products by quantitative PCR (qPCR). The short length of miRNAs and siRNAs presents a unique challenge in PCR design. Most conventional PCR primers are similar in length to interfering RNAs, implying that very short primers would be required for assay design. Locked nucleic acids (LNA) possess a 2′-O,4′-C methylene bridge in the ribose moiety of nucleotide (Petersen and Wengel 2003). The modification stabilizes the conformation of the sugar group and thereby increases the hybridization affinity of oligonucleotides that contain LNA bases. These nucleotide analogs are an integral part of PCR primers used in the assay. We also conducted a systematic survey of sequence length requirements necessary for sequence-specific PE. Oligonucleotides that were 10–12 nt in length promoted highly efficient PE cDNA synthesis from miRNA templates. Lastly, Schmittgen et al. (2004) report a qPCR method for monitoring of microRNA precursors that relies on SYBR green detection of short amplicons. We combined PE cDNA synthesis, LNA-containing short PCR primers, and SYBR green qPCR to develop the primer-extension, quantitative PCR (PE-qPCR) method described here.

RESULTS AND DISCUSSION

Quantitative miRNA/siRNA assay design

The PE-qPCR assay involved two steps (Fig. 1A). In the first step, a tailed, gene-specific primer (GS primer) was used (1) to convert the RNA template into cDNA; (2) to introduce a "universal" PCR binding site to one end of the cDNA molecule; and (3) to extend the length of the cDNA to facilitate subsequent monitoring by qPCR. In the second step, the resulting primer-extended, full-length cDNA was quantified by real-time PCR using a combination of an LNA-containing, miRNA/siRNA-specific "reverse" primer (LNA-R primer) and a generic universal primer common to all assays. Amplification of this chimeric cDNA was monitored in the real-time qPCR reaction by SYBR green fluorescence.

For assay refinement, we focused on two critical parameters (Fig. 1B). First, "sensitivity" was defined as the cycle threshold (C_T) value at which an assay containing 200 pM of synthetic miRNA was detected. The lower this C_T value, the more "sensitive" was the assay. Second, "dynamic range" was defined as the C_T difference between a 200-pM template spike-in (signal) and a no-template control (background). Assays with a high dynamic range allow measurements of very low miRNA/siRNA copy numbers.

Assay optimization

The success of the PE-qPCR method was critically dependent on short, ~15-nt LNA-R primers. Two or three LNA bases were substituted into each LNA-R primer within the first 8 nt from the 5′ end of the oligonucleotide (Table 1).

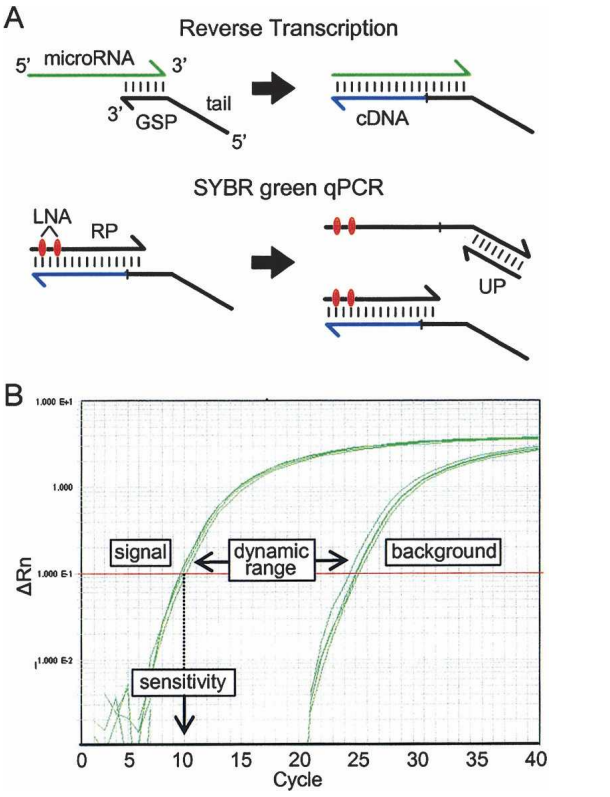

FIGURE 1. RT-qPCR assay for miRNAs and siRNAs. (*A*) The microRNA (or siRNA) template is converted to cDNA with a gene-specific primer (GSP) that includes a tail sequence. The reaction is conducted at 50°C using SuperScript III reverse transcriptase. The resulting cDNA-GSP chimera is quantified by real-time PCR. In the first cycle, the reverse primer (RP) containing LNA (red ovals) directs synthesis of the strand complementary to the cDNA. In subsequent cycles, the universal primer (UP) and LNA-R primer amplify a short sequence that can be monitored in real time by SYBR green fluorescence. (*B*) A well-behaved assay exhibits high sensitivity (low C_T) and broad dynamic range ($\Delta C_T \leq 12$) between the signal of a 1-nM input template and the no-template-control background. Quadruplicate measurements of signal and background are shown. The ΔRn shown on the *y*-axis is the change in fluorescence relative to the start of the PCR reaction.

The LNA bases were always separated by at least 1 nt, the substitutions selected were those that raised the predicted T_m by the highest amount, and the final predicted T_m of the selected primers were specified to be $\leq$55°C.

The contribution of LNA bases in 30 miRNA assays was tested empirically (Fig. 2). In almost every case, LNA substitutions increased the performance of the assays. In 8/30 cases (27%), LNA bases had little or no impact on assay characteristics (Fig. 2A), in 9/30 assays (30%), LNA bases significantly enhanced assay performance (Fig. 2B,C), and in 13/30 experiments (43%), LNA bases were absolutely required (Fig. 2D). In no case did LNA base substitutions negatively impact assay performance.

The dynamic range of our method was negatively impacted by primer–dimer formation between the GS primer and the LNA-R primer (Fig. 3A). To minimize back-

TABLE 1. Primers used in this study

Target	Sequence	Comments
Universal primers		
GS primer tail sequence-UP1	CATGATCAGCTGGGCCAAGA	Standard universal primer used in this study
GS primer tail sequence-UP5	GTGTCGGTGTGTGTGTGTGTGT	Alternate universal primer used to detect miR-124
DNA templates		
miR-194	TGTAACAGCAACTCCATGTGGA	Figure 2
miR-148b	TCAGTGCATCACAGAACTTTGT	Figure 2
miR-26a	TTCAAGTAATCCAGGATAGGCT	Figure 2
miR-215	ATGACCTATGAATTGACAGAC	Figure 2
miR-21	TAGCTTATCAGACTGATGTTGA	Figure 3
miR-215	ATGACCTATGAATTGACAGAC	Figure 3
let-7a	TGAGGTAGTAGGTTGTATAGTT	Figure 4
let-7b	TGAGGTAGTAGGTTGTGTGGTT	Figure 4
let-7c	TGAGGTAGTAGGTTGTATGGTT	Figure 4
let-7d	AGAGGTAGTAGGTTGCATAGT	Figure 4
let-7e	TGAGGTAGGAGGTTGTATAGT	Figure 4
let-7f	TGAGGTAGTAGATTGTATAGTT	Figure 4
let-7g	TGAGGTAGTAGTTTGTACAGT	Figure 4
let-7i	TGAGGTAGTAGTTTGTGCTGT	Figure 4
miR-1	TGGAATGTAAAGAAGTATGTA	Figure 5
miR-24	TGGCTCAGTTCAGCAGGAACAG	Figure 5
miR-122	TGGAGTGTGACAATGGTGTTTGT	Figure 5
miR-124	TTAAGGCACGCGGTGAATGCCA	Figure 5
miR-150	TCTCCCAACCCTTGTACCAGTG	Figure 5
GS primers		
miR-194	CATGATCAGCTGGGCCAAGATCCACATGGAGT	Figure 2
miR-148b	CATGATCAGCTGGGCCAAGAACAAAGTTCTGT	Figure 2
miR-26a	CATGATCAGCTGGGCCAAGAAGCCTATCCTGG	Figure 2
miR-215	CATGATCAGCTGGGCCAAGAGTCTGTCAATTC	Figure 2
miR-21, 14 nt	CATGATCAGCTGGGCCAAGATCAACATCAGTCTG	Figure 3
miR-21, 13 nt	CATGATCAGCTGGGCCAAGATCAACATCAGTCT	Figure 3
miR-21, 12 nt	CATGATCAGCTGGGCCAAGATCAACATCAGTC	Figure 3
miR-21, 11 nt	CATGATCAGCTGGGCCAAGATCAACATCAGT	Figure 3
miR-215, 12 nt	CATGATCAGCTGGGCCAAGAGTCTGTCAATTC	Figure 3
miR-215, 10 nt	CATGATCAGCTGGGCCAAGAGTCTGTCAAT	Figure 3
miR-215, 8 nt	CATGATCAGCTGGGCCAAGAGTCTGTCA	Figure 3
miR-215, 7 nt	CATGATCAGCTGGGCCAAGAGTCTGTC	Figure 3
miR-215, 6 nt	CATGATCAGCTGGGCCAAGAGTCTGT	Figure 3
miR-215, 5 nt	CATGATCAGCTGGGCCAAGAGTCTG	Figure 3
miR-215, 4 nt	CATGATCAGCTGGGCCAAGAGTCT	Figure 3
let-7a	CATGATCAGCTGGGCCAAGAAACTATAC	Figure 4
let-7b	CATGATCAGCTGGGCCAAGAAACCACAC	Figure 4
let-7c	CATGATCAGCTGGGCCAAGAAACCATAC	Figure 4
let-7d	CATGATCAGCTGGGCCAAGAACTATGCA	Figure 4
let-7e	CATGATCAGCTGGGCCAAGAACTATACA	Figure 4
let-7f	CATGATCAGCTGGGCCAAGAAACTATAC	Figure 4
let-7g	CATGATCAGCTGGGCCAAGAACTGTACA	Figure 4
let-7i	CATGATCAGCTGGGCCAAGAACAGCACA	Figure 4
miR-1	CATGATCAGCTGGGCCAAGATACATACTTCTTT	Figure 5
miR-24	CATGATCAGCTGGGCCAAGACTGTTCCTGCTG	Figure 5
miR-122	CATGATCAGCTGGGCCAAGAACAAACACC	Figure 5
miR-124	GTGTCGGTGTGTGTGTGTGTGTTGGCATTCACCG	Figure 5
miR-150	CATGATCAGCTGGGCCAAGACACTGGTACAAG	Figure 5
LNA-R primers		LNA substitutions are preceded by a "+"
miR-194	TG+TAA+CAGCAACTCCA	Figure 2
miR-194	TGTAACAGCAACTCCA	non-LNA-containing R primer; Figure 2
miR-148b	T+CAG+TGCATCACAGAA	Figure 2
miR-148b	TCAGTGCATCACAGAA	non-LNA-containing R primer; Figure 2

(continued)

TABLE 1. *Continued*

Target	Sequence	Comments
miR-26a	TT+CA+AGTAATCCAGGA	Figure 2
miR-26a	TTCAAGTAATCCAGGA	non-LNA-containing R primer; Figure 2
miR-215	A+T+GA+CCTATGAATTG	Figure 2
miR-215	ATGACCTATGAATTG	non-LNA-containing R primer; Figure 2
miR-21	T+AG+CT+TATCAGACTGAT	Figure 3
miR-215	A+T+GA+CCTATGAATTG	Figure 3
let-7a	T+GA+GGTAGTAGGTTG	Figure 4
let-7b	T+GA+GGTAGTAGGTTG	Figure 4
let-7c	T+GA+GGTAGTAGGTTG	Figure 4
let-7d	A+GA+GGTAGTAGGTTG	Figure 4
let-7e	T+GA+GGTAGGAGG	Figure 4
let-7f	T+GA+GGTAGTAGATTG	Figure 4
let-7g	T+GA+GGTAGTAGTTTG	Figure 4
let-7i	T+GA+GGTAGTAGTTTG	Figure 4
miR-1	TG+GAA+TG+TAAAGAAGTA	Figure 5
miR-24	TGG+CTCAGTTCAGC	Figure 5
miR-122	T+GGAGTGTGACAATGGT	Figure 5
miR-124	TTAA+GGCACGCGGTGA	Figure 5
miR-150	TCT+CC+CAACCCTTGTA	Figure 5
Precursor primers		
pre-miR-124F	GTTCACAGCGGACCTTGATT	Figure 5
pre-miR-124R	ACCGCGTGCCTTAATTGTAT	Figure 5

ground, GS primers with serial 1-nt deletions were analyzed; a representative series is shown in Figure 3A. Trimming GS primers from 14 to 11 nt had little effect on signal, while increasing dynamic range (by increasing background C_T values) substantially. These data motivated an exploration of the minimum sequence requirements for efficient PE-mediated cDNA synthesis (Fig. 3B). To our surprise, we found very little change in assay performance as GS priming sequences were reduced in length from 12 nt to 7 nt. Assay sensitivity decreased sharply, however, for priming sequences $\leq$6 nt. We currently use GS-priming sequences in the range of 10–12 nt, regardless of base composition.

To test specificity, separate assays were designed to monitor the human let-7 family of eight related sequences (Fig. 4A). GS priming sequences of 8 nt were used along with sequence-specific LNA-R primers to explore assay specificity among these related miRNAs (Fig. 4B). Every assay tested exhibited substantial target sequence specificity, and significant "cross talk" detection of other let-7 family members was encountered in only a few cases. The series of let-7 paralog-specific assays was applied to five human tissue RNA samples (Fig. 4C). Substantial expression of let-7a and let-7f and modest expression of let-7i were observed in these five tissues.

Occasionally assay designs failed, either from failures of the PCR to amplify a product or from nonspecific background that restricts the dynamic range of the assay. We encountered such failures in seven of 130 ($\sim$5%) of the miRNA/siRNA assays we created. For three of these assays, we explored substituting alternative universal priming site tails on the GS primers (Table 1). In two cases, a well-behaved assay was recovered. Taken together, these data suggest that most miRNA/siRNA sequences can be monitored using the method we describe.

Expression profiles of miRNAs

One application of the PE-qPCR technique described here is quantitative analysis of miRNA expression patterns. Assay calibration was performed using the standard curve PCR method to calibrate assay data (Raymond et al. 2004). For standard curves, it was found that DNA oligonucleotides yield C_T values that are identical to or 1–2 C_T values lower than RNA oligonucleotides. The slopes of DNA and RNA standard curves are identical. Because RNA oligonucleotides are rather expensive and frequently contaminated with impurities, DNA standards were used. This practice results in highly accurate relative measurements between samples, but it may lead to an underestimation of absolute expression levels.

In Figure 5, expression profiles for six miRNAs across 12 tissues are shown. The data are presented in units of miRNA copies per 10 pg of total RNA. These units were chosen since human cell lines typically yield $\leq$10 pg of total RNA per cell. Hence the data shown are rough estimates of miRNA copies per cell. As reported previously, very high levels of striated muscle-specific expression were found for miR-1, liver expression for miR-122, and brain expression for miR-124 (Lagos-Quintana et al. 2003). Quantitative analysis reveals that these microRNAs are present at tens

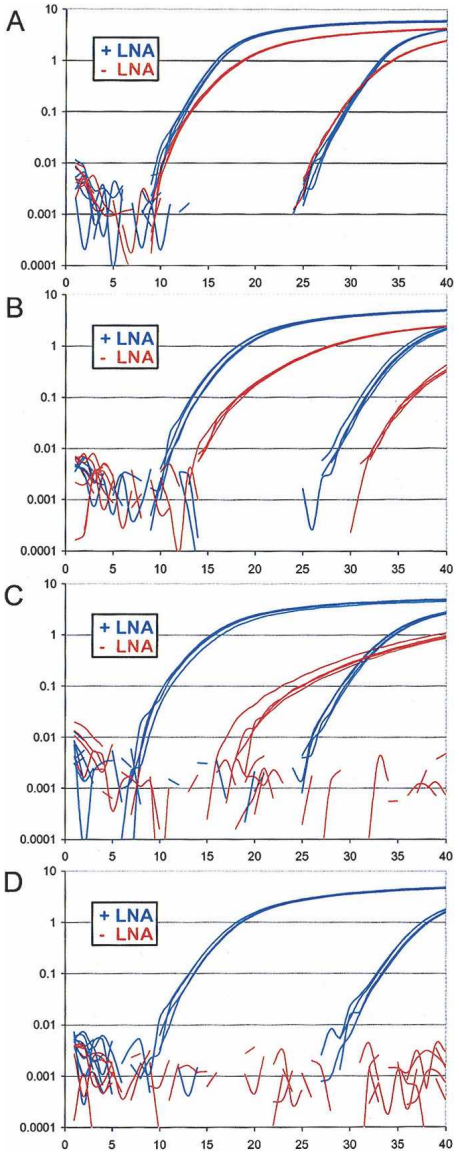

FIGURE 2. LNA base substitutions in short PCR primers contribute to the performance of the miRNA/siRNA quantitative assay. (*A*) For the miR-194 assay, the LNA substitution had a modest effect. The sensitivity and dynamic range of assays using non-LNA primers (red) versus LNA-substituted primers (blue) yielded similar results. Eight of 30 (27%) assays had this characteristic. (*B,C*) The miR-148b (*B*) and miR-26a (*C*) assays were significantly improved by LNA substitutions. The background in −LNA (*C*) fell beyond the 40 C_T limit of detection. Nine of 30 (30%) assays were significantly improved with LNA substitutions. (*D*) The miR-215 assay required LNA substitutions. Thirteen of 30 (43%) assays were LNA dependent. Quadruplicate measurements of signal and background are shown for each assay.

to hundreds of thousands of copies per cell, and are in agreement with quantitative Northern blot estimates of miR-1 and miR124 levels (Lim et al. 2005). These data underscore the fact that microRNAs are among the most abundant RNAs present in cells. Consistent with previous findings (Baskerville and Bartel 2005), miR-150 was highly expressed in the immune-related lymph node, thymus, and spleen samples, while miR-24 and miR-148b showed more constitutive levels of expression, being expressed at high and low levels, respectively, in the tissues examined.

In principle, the assays that are described have the potential to detect both mature and precursor forms of microRNAs. To test this, the precursor of miR-124 was generated by in vitro transcription. We used pre-miR-124 specific PCR primers in a method similar to that described by Schmittgen et al. (2004) to generate standard curve data of the synthetic precursor species and to measure the levels of precursor present in human tissue total RNAs (Fig. 5, inset). Consistent with the pattern of mature miR-124 expression, the precursor species was only detected in brain, and the data indicate that the steady-state level of precursor species is ~3% that of the mature form. These data compare favorably with the qualitative results of Northern blots (Lagos-Quintana et al. 2003). The precursor form of miR-124 was poorly detected by the mature micro-

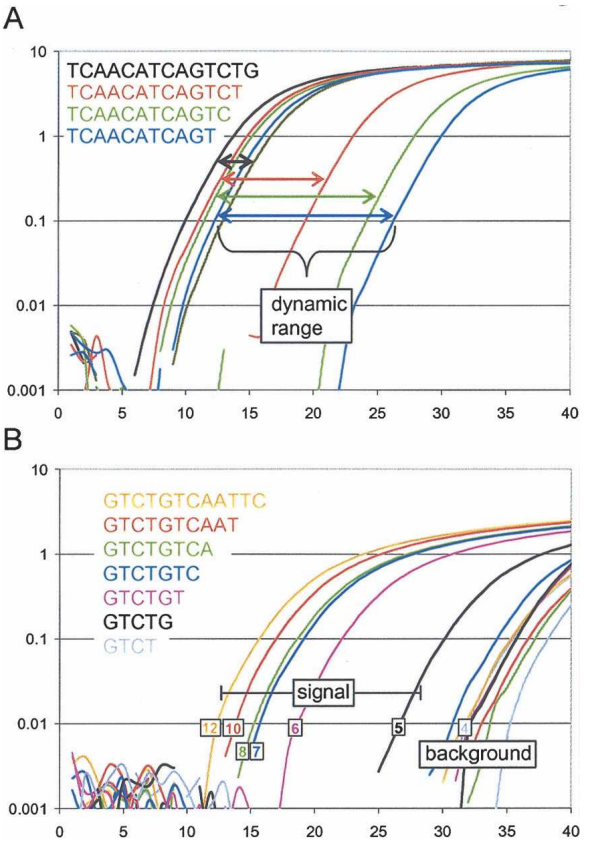

FIGURE 3. Influence of the gene-specific primer length on dynamic range and sequence-specific detection of miRNAs. (*A*) Reduction of GS priming sequences in 1-nt steps from 14 nt to 11 nt yielded substantial improvement in the dynamic range of the miR-21 assay by increasing the background C_T values. (*B*) Trimming of miR-215 priming sequences from 12 to 7 nt had little impact on assay performance. Further reductions substantially reduced miRNA specific signal. The assay was run in a background of total Hela RNA (which lacks endogenous miR-215; Babak et al. 2004; data not shown). The traces shown in *A* and *B* are the average of quadruplicate runs for a single experiment.

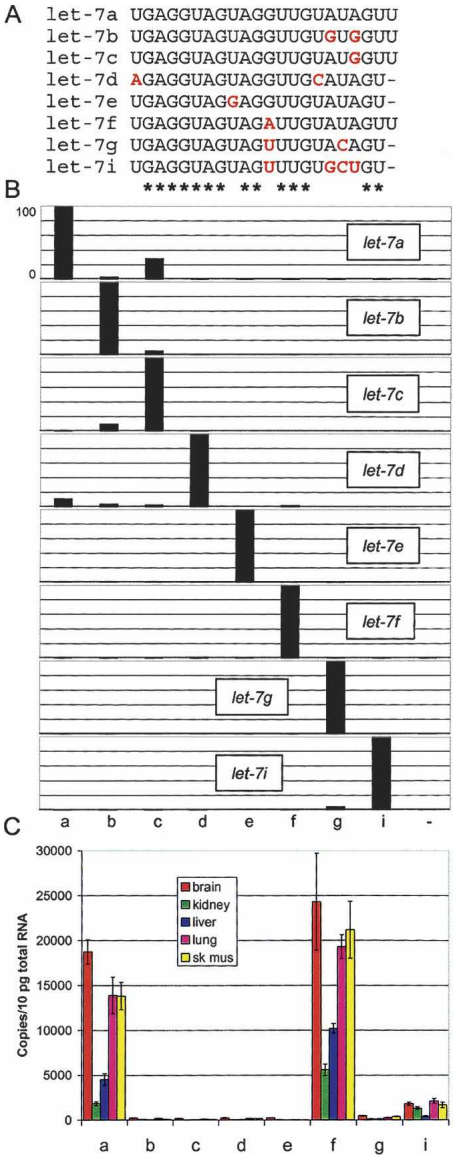

FIGURE 4. Specificity of human let-7 assays. (*A*) Multiple alignment of the eight known human let-7 sequences. Bases that differ from consensus are highlighted in red. (*B*) A microRNA assay designed for each individual let-7 sequence was run against a panel of 1 nM DNA oligonucleotide spike-ins of specific let-7 sequences as indicated on the *x*-axis. The dash indicates a no-template control. The maximum signal in each assay was normalized to 100, and the remaining values were calculated assuming an amplification efficiency of $2^{(Ct)}$. Data were plotted on a linear scale. (*C*) Quantification of let-7 microRNAs in five human tissues. Standard curves of the eight related sequences were used to calibrate the assays, and the tissue data were regressed to these standard curves. Error bars reflect one standard deviation among quadruplicate qPCR measurements.

RNA assay for miR-124. Equivalent molar amounts of mature and precursor miR-124 species were measured using primers specific for mature miR-124. The precursor molecule was detected ~2000-fold less efficiently than the mature form. Similar data were observed for two precursor species of miR-24, pre-miR-24–1 and pre-miR-24-2 (data not shown). Taken together, these data suggest that the microRNA PE-qPCR method we describe preferentially detects mature microRNAs.

CONCLUSIONS

We have created a simple, robust, inexpensive tool for monitoring of individual miRNAs and siRNAs. We anticipate this tool will find use for monitoring temporal, spatial, and pathological patterns of miRNA expression in human tissues and in samples derived from experimentally tractable organisms. Moreover, the method can be applied to engineered siRNAs, suggesting it will be a useful tool for investigating RNA interference in model systems.

MATERIALS AND METHODS

All of the oligonucleotides described here (Table 1) were purchased from Proligo LCC. MicroRNA precursor molecules were produced by in vitro transcription using the MessageMuter shRNA production kit from Epicentre. SuperScript III reverse transcription kits (InVitrogen Corp.) were used to convert miRNAs and siRNAs into cDNAs. For reverse transcription, 6 μL of RT master mix (2 μL of water, 2 μL 5× buffer, 0.5 μL of 0.1 M DTT, 0.5 μL of 10 mM dNTPs (InVitrogen), 0.5 μL of RNAse OUT (InVitrogen), and 0.5 μL of SuperScript III enzyme) were combined with 2 μL of 0.5 μM GS primer and 2 μL of template in a 96-well plate. For detection of precursor microRNA species, siRNA duplexes or shRNA hairpins, the GS primer, and template were premixed, heated at 85°C for 2 min, snap-chilled on ice, and RT premix was added. This step was found to be unnecessary for optimal detection of microRNAs. Hairpin duplexes containing an siRNA duplex joined by a 4-nt or 9-nt loop sequence were detected at 3–6 C_T higher values than siRNA duplexes or single-stranded synthetic templates even with an initial denaturation step (data not shown). The 10 μL RT reaction was incubated at 50°C for 30 min, 85°C for 5 min, cooled to room temperature, and diluted 10-fold with 90 μL of TE (10 mM Tris at pH 7.6, 0.1 mM EDTA). Standard curve dilutions of synthetic oligonucleotides ranging from 10 nM to 10 fM were performed in TE that contained 100 ng/μL of total yeast RNA (Ambion, Inc.). For quantitative analysis of samples, 0.5 μg of First Choice total RNA (Ambion, Inc.) was assayed per 10 μL RT reaction.

Following reverse transcription, quadruplicate measurements of 2 μL of cDNA were made in 10 μL final reaction volumes by qPCR in a 384-well optical PCR plate using a 7900 HT PCR instrument (Applied Biosystems). SYBR green PCR mix contained 5 μL of 2× SYBR green PCR master mix (Applied Biosystems), 1.4 μL of water, 0.8 μL of 10 μM universal primer, 0.8 μL of 10 μM LNA-R primer, and 2 μL of sample. qPCR was performed using the manufacturer's recommended conditions and dissociation curves were typically generated post-run for analysis of amplicon species (data not shown). Following PCR, the results table was exported to Excel (Microsoft Corp.), standard curves were generated, and quantitative analysis for samples was regressed from the raw data.

ACKNOWLEDGMENTS

We thank Alex Amiet of Proligo LLC for helpful discussions about LNA-containing PCR primers. Peter Linsley and colleagues pro-

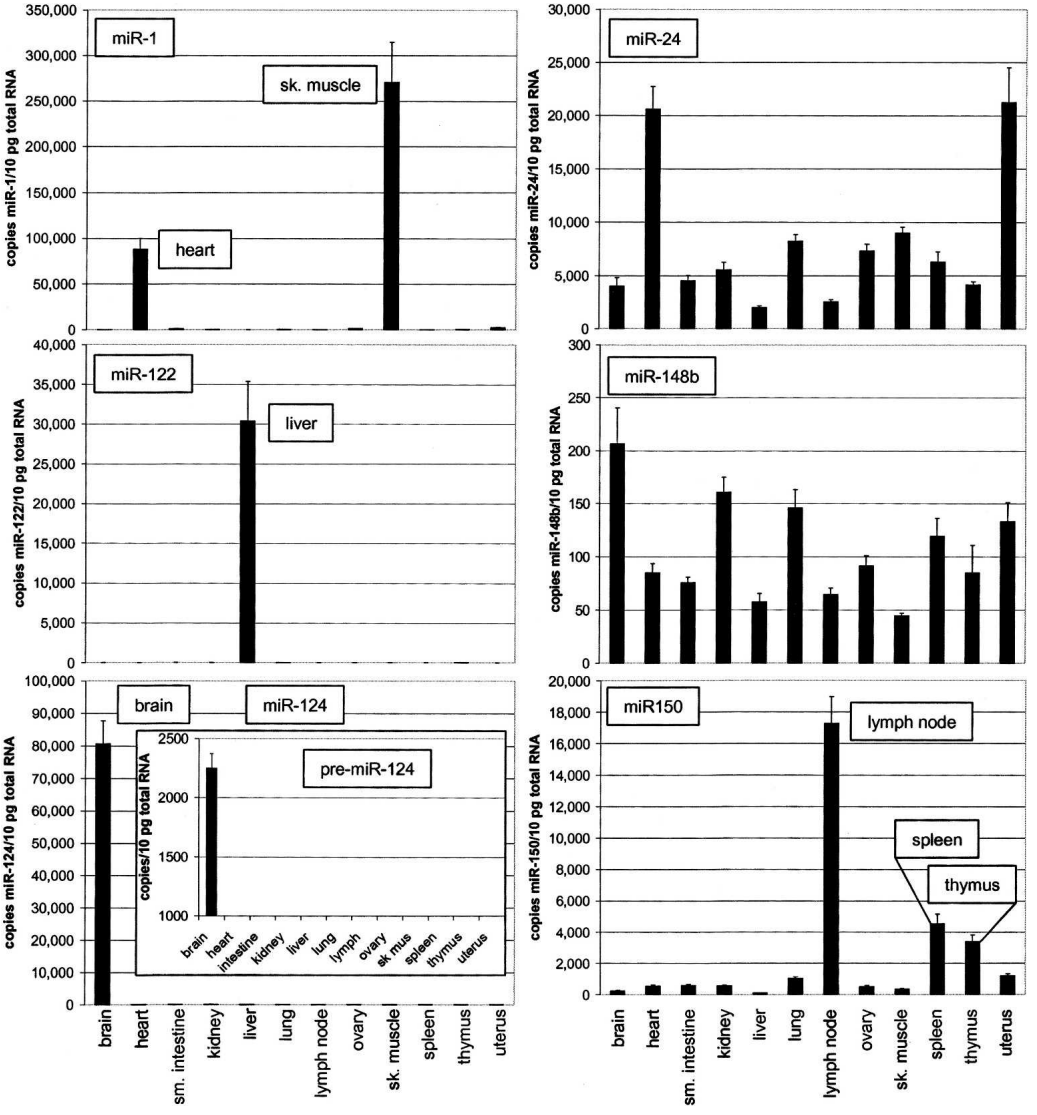

FIGURE 5. Expression profiles of six microRNAs across a panel of total RNA from 12 tissues. MicroRNA assays were calibrated with standard curves that ranged in 10-fold increments from 10 nM to 10 fM. This concentration range corresponds to molecular copy numbers of 240,000 down to 2.4 per 10 pg total RNA. Each tissue assay contained 0.5 μg of Ambion First Choice total RNA. Error bars correspond to one standard deviation of quadruplicate qPCR measurements. The *y*-axis copy number scale was adjusted to accommodate the maximum value observed in each assay. The *inset* in the miR-124 panel shows the measurement of miR-124 hairpin precursor using synthetic, in vitro transcribed precursor as a standard and PCR primers specific to the precursor molecule.

vided critical feedback during the development of this technology. Finally, thanks to John Castle and Chris Armour for fruitful discussions and comments on the manuscript.

Received June 28, 2005; accepted August 29, 2005.

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