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Notes & Tips

High-throughput amplification of mature microRNAs in uncharacterized animal models using polyadenylated RNA and stem–loop reverse transcription polymerase chain reaction

Kyle K. Biggar [1], Cheng-Wei Wu [1], Kenneth B. Storey *

Institute of Biochemistry and Department of Biology, Carleton University, Ottawa, Ontario K1S 5B6, Canada

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ABSTRACT

This study makes a significant advancement on a microRNA amplification technique previously used for expression analysis and sequencing in animal models without annotated mature microRNA sequences. As research progresses into the post-genomic era of microRNA prediction and analysis, the need for a rapid and cost-effective method for microRNA amplification is critical to facilitate wide-scale analysis of microRNA expression. To facilitate this requirement, we have reoptimized the design of amplification primers and introduced a polyadenylation step to allow amplification of all mature microRNAs from a single RNA sample. Importantly, this method retains the ability to sequence reverse transcription polymerase chain reaction (RT–PCR) products, validating microRNA-specific amplification.

MicroRNAs are increasingly being shown to play important roles in regulating the gene expression response by cells and organs to environmental stress [1]. As a result of increased research interest, reliable quantification of individual mature microRNAs is necessary to understand their biological roles in a diverse array of animals. For example, research is now discovering that microRNA regulation is an important component of the natural environmental stress adaptation as seen in anoxic turtles [2,3], frozen frogs [4], and hibernating mammals [5,6], among others [7,8]. Unfortunately, all of these animals currently do not have annotated microRNA sequences, and expression analysis of microRNA transcripts requires downstream sequencing for validation. Stem–loop priming and polyadenylate tailing are individually the two most commonly used complementary DNA (cDNA)[2] synthesis methods for microRNA quantitative polymerase chain reaction (qPCR) [1–9]. To date, these two main approaches have been the primary methods to detect microRNAs by qPCR. Stem–loop amplification methods require a unique set of primers for each microRNA assay and, therefore, cannot be applied for screening of a large number of microRNAs in most research laboratories. Second, the polyadenylation method uses small linear adapting primers to detect polyadenylated microRNAs. Due to the small size of these products (limited by the design and specificity of linear adapter primers), gel electrophoresis is unable to distinguish between the specific/nonspecific amplification and PCR products cannot be sequenced for validation. Our technique combines the benefits of both methods, redesigning long stem–loop primers to incorporate a polyadenylation step, allowing high-throughput amplification and the ability to sequence and validate microRNA-specific PCR products. During the era of genome sequencing and wide-scale microRNA prediction, we have developed technical modifications that increase throughput while decreasing both time and cost of microRNA analysis in animal models that require PCR product sequencing.

Our technique makes a major advance over previous available assays because it uses both polyadenylation and stem–loop priming. The use of stem–loop priming allows for significantly longer adapter sequences that are able to retain specificity over linear primers and adapt mature microRNAs to a length that is sufficient for sequencing and amplification validation [9]. This point is crucial for studies that are validating novel microRNAs or unannotated microRNAs and require downstream validation of mature microRNA sequences. For example, our stem–loop primers have been used to evaluate expression and validate sequences by many invertebrate microRNA studies, organisms with poorly conserved microRNA sequences compared with what is available in annotation

* Corresponding author. Fax: +1 613 520 3749.
E-mail address: kenneth_storey@carleton.ca (K.B. Storey).

[1] These authors contributed equally to this article.

[2] *Abbreviations used:* cDNA, complementary DNA; qPCR, quantitative polymerase chain reaction; RT, reverse transcription; ATP, adenosine triphosphate; dNTP, deoxynucleoside triphosphate; rRNA, ribosomal RNA; Ct, cycle threshold; LoD, limit of detection.

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databases [7,8]. MicroRNA research in animal models that do not have annotated microRNAs needs techniques that allow rapid amplification while absolutely requiring the ability of sequence PCR products to validate the microRNA sequences being researched.

This article details our current polyadenylated microRNA amplification technique. Compared with the previous methodology, where cDNA is generated in a microRNA-specific manner, our adaptation adds an additional polyadenylation step before cDNA synthesis, yielding a cDNA sample that can be used to measure any mature microRNAs of interest. The basis behind this procedure (Fig. 1) is to initially extend the 3′ end of the mature microRNAs through the addition of a polyadenylate tail, essentially creating microRNAs that can be reverse transcribed into cDNA using universal long stem–loop primers that contain a repeat of thymine nucleotides. A microRNA-specific forward primer is designed from the conserved 5′ microRNA end, decreasing the difficulty of designing primers based on the conservation of microRNAs between select related species. Degenerate bases could also be used for animals that might not have suitably conserved sequences. Following microRNA-specific amplification, products can be sequenced and validated to confirm microRNA sequence.

To validate the proposed modifications to our microRNA stem–loop reverse transcription (RT)–PCR protocol, we amplified five microRNAs (*iti-let-7a*, *iti-miR-125a*, *iti-miR-150*, *iti-miR-21*, and *iti-miR-378b*) from a single polyadenylated RNA sample from the brown adipose tissue of the 13-lined ground squirrel (*Ictidomys tridecemlineatus*). To illustrate the ability of our approach to measure expression levels and allow sequencing of the amplification product, we also sequenced and determined the relative expression of each microRNA between control and late torpor.

To design primers for amplification, forward microRNA primers were designed from the consensus sequence of *Homo sapiens* and *Mus musculus* mature microRNAs using Primer Designer (version 3.0, Scientific and Educational Software). The mature microRNA consensus sequences were downloaded from the microRNA Registry database (http://miRNA.sanger.ac.uk). The modified sequences used in this study were universal stem–loop RT primer 5′-CTCAC AGTACGTTGGTATCCTTGTGATGTTCGATGCCATATTGTACTGTGAGTT TTTTTTTVN-3′, forward primer 5′-ACACTCCAGCTGGGNNNNNNN NNNNN-3′, and a universal reverse primer 5′-CTCACAGTACGTTGG TATCCTTGTG-3′, where nucleotides denoted with an N indicate microRNA-specific sequence binding regions, and will vary depending on the target microRNAs, and nucleotides denoted with a V indicate either A, C, or G nucleotides.

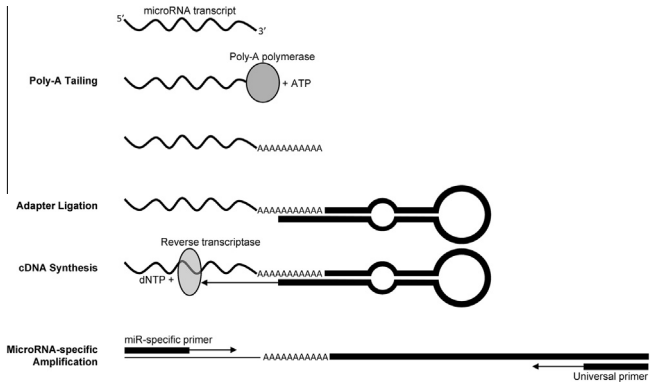


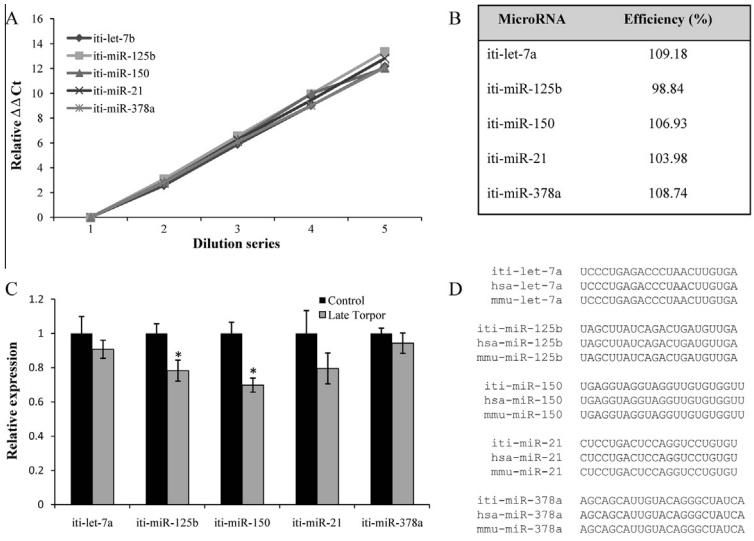
Fig.1. Depiction of the proposed polyadenylated microRNA amplification protocol using stem–loop primers. A polyadenylate tail is added to microRNAs, followed by cDNA synthesis using an adapted primer and reverse transcriptase. The resulting cDNA is ready for RT–PCR amplification with a universal PCR primer and a microRNA-specific forward primer. This unique amplification system provides ease of use and efficient cDNA synthesis for the quantification of many mature microRNAs with a high degree of sensitivity and specificity.

Total RNA was isolated from brown adipose tissue of control and late torpid 13-lined ground squirrels using Trizol (Invitrogen). Polyadenylated microRNA was created using a polymerase tailing kit (Epi-Bio, cat. no. PAP5104H). Reactions were prepared with 1 μl of 10× polyadenylate polymerase buffer, 1 μl of adenosine triphosphate (ATP, 10 mM), 0.5 μl of *Escherichia coli* poly(A) polymerase (2 U,) and 3 μg of total RNA to a final volume of 10 μl with RNase-free water. Reactions were incubated at 37 °C for 30 min, followed by 95 °C for 5 min to terminate adenylation, and were transferred directly to ice. RT–PCR was performed as described previously [9]. A 10.0-μl aliquot of polyadenylated RNA (3.0 μg) from the previous step was incubated with 5.0 μl of 250 nM universal stem–loop RT primer. The reaction was heated at 95 °C for 5 min to denature the RNA and then incubated for 5 min at 60 °C to anneal the stem–loop primer. After cooling on ice for 1 min, the remaining reagents (4 μl of 5× first-strand buffer, 2 μl of 0.1 M dithiothreitol [DTT], 1 μl of deoxynucleoside triphosphate [dNTP] mixture containing 25 mM of each nucleotide, and 1 μl of mouse Maloney leukemia virus [M-MLV] reverse transcriptase) were added. The reaction proceeded for 30 min at 16 °C, followed by 30 min at 42 °C and 85 °C for 5 min. Following reverse transcription, the RT product was serial diluted and stored at −20 °C. Real-time PCR was performed on a Bio-Rad MyiQ2 Detection System (Bio-Rad, cat. no. 170-9790). The 10-μl RT–qPCR included 2.5 μl of diluted RT product, 5 μl of SsoFast EvaGreen Supermix (Bio-Rad, cat. no. 172-5201), 1.25 μl of 12.5 μM forward primer (*iti-let-7a:* ACACTCCAGCTGGGTGAGGTAGTAGGTTG; *iti-miR-125b:* ACACTCCAGCTGGGTCCCTGAGACCCTAAC; *iti-miR-150:* ACACTCC AGCTGGGTCTCCCAACCCTTGTA; *iti-miR-21:* ACACTCCAGCTGGGT AGCTTATCAGACTGA; *iti-miR-378a:* ACACTCCAGCTGGGTTTGTT CGTTCGGCTC; 5S rRNA: ACCGGTTCTCGTCCGATCACCGAAGT), and 1.25 μl of 12.5 μM reverse primer (5′-CTCACAGTACGTTGGTAT CCTTGTG-3′). Reactions were incubated in a 96-well plate at 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. A melting curve analysis was performed for each microRNA analyzed.

Amplified microRNAs were normalized against expression of 5S ribosomal RNA (rRNA) from the same RNA sample. Each cycle threshold (Ct) was normalized to the Ct of the endogenous control. The comparative ΔΔCt method was used to calculate relative quantification of gene expression.

The limit of detection (LoD) was defined as the last dilution in which polyadenylated microRNA was both reverse transcribed and detected in all replicates and 100% reproducibility can be guaranteed [10]. The LoD of the assay was determined using the detectable amplification of a synthesized 6-mer polyadenylated hsa-miR-150-5p oligonucleotide (5′-UCAAAUGCUCAGACUCCUGUGGU AAAAAA-3′) over seven 1:10 dilutions with four replicates of each dilution. All PCR products were sequenced using a shortened version of the universal reverse primer (5′-CTCACAGTACGTTGG-3′) by DNA Landmarks (Saint-Jean-sur-Richelieu, Quebec, Canada).

Results demonstrate that this technique is able to amplify several mature microRNAs from a single RNA sample with high amplification efficiency, allowing expression analysis as well as sequencing and validation of amplification products. The LoD was found to be 1 fmol of RNA per PCR. All microRNAs were determined to have linear amplification over a range of cDNA dilutions (Fig. 2A) and amplification efficiencies between 99 and 109% (Fig. 2B). In response to late torpor, *iti-miR-125b* was found to decrease to 78 ± 6% of control values (Student's t test, $P < 0.05$). No significant changes were found in the expression of *iti-let-7a*, *iti-miR-150*, *iti-miR21*, or *iti-miR-378a* in response to late torpor when compared with control levels. To validate the specific amplification of mature microRNAs from *I. tridecemlineatus*, we sequenced the PCR products using a shortened universal primer as described previously [9]. The PCR products (~100 bp) were



MicroRNA	Efficiency (%)
iti-let-7a	109.18
iti-miR-125b	98.84
iti-miR-150	106.93
iti-miR-21	103.98
iti-miR-378a	108.74

Fig.2. Mature microRNA amplification, expression, and sequencing from the brown adipose tissue of *I. tridecemlineatus*. (A) Relative change in the critical threshold (Ct) of amplified microRNAs from a 10-fold dilution series. (B) Efficiency of microRNA amplification protocol. (C) Histogram showing normalized mature microRNA expression levels for control and late torpor conditions. Data are means ± standard errors ($n = 4$ independent trials on tissue from different animals). An asterisk (*) indicates significant difference from the corresponding control ($P < 0.05$). (D) Mature microRNA sequences from *I. tridecemlineatus* (iti) with respective sequences of *H. sapiens* (hsa) and *M. musculus* (mmu).

confirmed to encode the *iti-let-7a*, *iti-miR-125b*, *iti-miR-150*, *iti-miR21*, and *iti-miR-378a* mature sequences. Fig. 2D shows the mature microRNA sequences obtained from the polyadenylated RNA of *I. tridecemlineatus* (iti) aligned with the sequences for *H. sapiens* (hsa) and *M. musculus* (mmu). All mature microRNA sequences from *I. tridecemlineatus* displayed 100% conservation among the aligned species. This result is not surprising given the high degree of mature microRNA conservation between vertebrates [11].

In this study, we have presented a new technique for the sequencing and high-throughput quantification of mature microRNAs in animal models that do not have verified annotation of microRNA sequences available. We presented critical modifications to a widely used microRNA amplification protocol that allows mature microRNA amplification and sequencing in animal models using stem–loop amplification primers. Unfortunately, the previous technique required that microRNA-specific cDNA be used for each amplification reaction, increasing the time, cost, and sample required for analysis. As research progresses into the post-genomic era of microRNA analysis, the need for a rapid and cost-effective method for microRNA amplification is increasing to facilitate wide-scale analysis of microRNA expression. To facilitate this, we presented and characterized new modifications that (i) allow amplification of all mature microRNAs from a single RNA sample, (ii) decrease time and cost of amplification reagents needed for analysis, and (iii) reduce the sample requirement for amplification. This method was used to successfully sequence and quantify the expression of five mature microRNAs from the 13-lined ground squirrel, *I. tridecemlineatus*.

Although the use of stem–loop RT primers for the detection and quantification of mature microRNAs has been demonstrated in previous reports, this is the first study to apply several modifications to allow successful expansion of the existing protocol to achieve high-throughput capabilities while retaining the ability to validate amplification products in unannotated animals. Similar to the original method, our protocol is extremely simple, allows the researcher to sequence PCR products and validate microRNA-specific amplification, and provides the opportunity to add to the current body of knowledge of mature microRNA annotation.

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