

# Analysis of microRNA signatures using size-coded ligation-mediated PCR

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## ABSTRACT

The expression pattern and regulatory functions of microRNAs (miRNAs) are intensively investigated in various tissues, cell types and disorders. Differential miRNA expression signatures have been revealed in healthy and unhealthy tissues using high-throughput profiling methods. For further analyses of miRNA signatures in biological samples, we describe here a simple and efficient method to detect multiple miRNAs simultaneously in total RNA. The size-coded ligation-mediated polymerase chain reaction (SL-PCR) method is based on size-coded DNA probe hybridization in solution, followed by ligation, PCR amplification and gel fractionation. The new method shows quantitative and specific detection of miRNAs. We profiled miRNAs of the *let-7* family in a number of organisms, tissues and cell types and the results correspond with their incidence in the genome and reported expression levels. Finally, SL-PCR detected *let-7* expression changes in human embryonic stem cells as they differentiate to neuron and also in young and aged mice brain and bone marrow. We conclude that the method can efficiently reveal miRNA signatures in a range of biological samples.

## INTRODUCTION

MicroRNAs (miRNAs) are short regulatory RNAs found in many eukaryotes including all multicellular organisms and also in a single-cell alga (1). Recently, more than 15 000 miRNA genes from animals, plants and viruses have been registered (<http://www.mirbase.org/>) and even more have been predicted using various algorithms (2–4). MicroRNAs are first transcribed as pri-miRNAs and then processed to short hairpin structures called pre-miRNA by Drosha complex. After export to the cytoplasm, pre-miRNAs are converted to the functional mature form of 21–23-nt single-stranded miRNAs by Dicer and the RNA-induced silencing complex (RISC) and mature miRNAs regulate expression of target genes through post-transcriptional gene silencing (5–7). miRNAs are involved in multiple biological processes, including cell differentiation, organ development, life span, disease and response to environmental stresses (7).

Profiling miRNA expression is difficult due to their short length; therefore, new techniques have been developed. Comprehensive miRNA profiling using high-throughput methods including various microarray, bead-based flow cytometric platforms and deep sequencing reveals distinct expression patterns of multiple miRNAs. These patterns, which are characteristic of cells and tissues in various biological conditions, are referred to as miRNA signatures (8,9). In some cases, expression profiling of

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

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miRNA signatures is sufficient for subsequent investigations. miRNA signatures may also provide useful biomarkers for disease diagnosis (10). A number of methods have been optimized to detect expression of miRNAs individually, including northern blotting with LNA probes (11), real-time stem-loop reverse transcription (RT)-polymerase chain reaction (PCR) (12) and Padlock probes/rolling circle amplification (13). However, many current techniques require specialized and expensive equipments/reagent and some involve laborious steps or radioactive materials. Most hybridization-based approaches are limited in their ability to distinguish highly related miRNAs and in discriminating between mature miRNA and precursors, whereas most PCR-based methods show improvement in these areas. Nevertheless, there is a need for a method that can profile expression of multiple miRNAs together to analyze miRNA signatures among various samples. Also, many labs would benefit from more simple and accessible technology.

Here, we sought to develop a reliable method to profile the expression of several miRNAs simultaneously. The size-coded ligation-mediated PCR (SL-PCR) method takes advantages of liquid hybridization kinetics of two size-coded DNA probes on a target miRNA, T4 DNA ligation and PCR amplification. SL-PCR performs rather well in discriminating highly related miRNAs and detects mature miRNAs inclusive or exclusive of precursors. The procedure proves to be simple, rapid and requires only inexpensive materials and instruments. We successfully detected *let-7* miRNA expression signatures in various biological samples including differentiating embryonic stem cells and in young and aged mouse brain and bone marrow.

## MATERIALS AND METHODS

### Oligo RNAs and cellular RNA preparations

All Oligo RNAs corresponding to miRNAs were chemically synthesized and high-performance liquid chromatography (HPLC)-purified by Biomers (Ulm, Germany). Precursors of *hsa-let-7a-3*, *hsa-let-7c*, *hsa-let-7g* and *hsa-mir-98* were prepared by PCR amplification of related genes using T7 promoter containing primers listed in Supplementary Table 2 and subsequent *in vitro* transcription using T7 RNA polymerase (Fermentas, Ontario, Canada). To avoid DNA contamination, we purified RNA precursors from polyacrylamide gel. *Caenorhabditis elegans* worms mixed population cultured in M9 plates was a kind gift from Tomas Burglin (Södertörns University College, Stockholm, Sweden). Total RNA from whole organisms of *C. elegans* and *Drosophila melanogaster* as well as *Arabidopsis thaliana* leaves and all cells and tissues was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. *Arabidopsis thaliana* leaves were ground in liquid nitrogen prior to RNA extraction. Total RNA preparations from mice brain and bone marrow were a gift from Sara Soudi (Stem Cell Research Center, Tehran, Iran). Small RNA-enriched fractions were prepared using PureLink miRNA isolation kit (Invitrogen, Carlsbad, CA, USA).

### Size-coded DNA probes

All DNA probes were designed to cover almost half of an miRNA sequence. Two probes covering 5' and 3' halves of a single miRNA have similar  $T_m$  when bound to miRNAs and synthesized by Biomers (Ulm, Germany) (Supplementary Table S1). The probes that cover the 5' half of the miRNA sequence (5'-side-probe) were phosphorylated using T4 Polynucleotide Kinase according to manufacturer's instruction (Fermentas, Ontario, Canada).

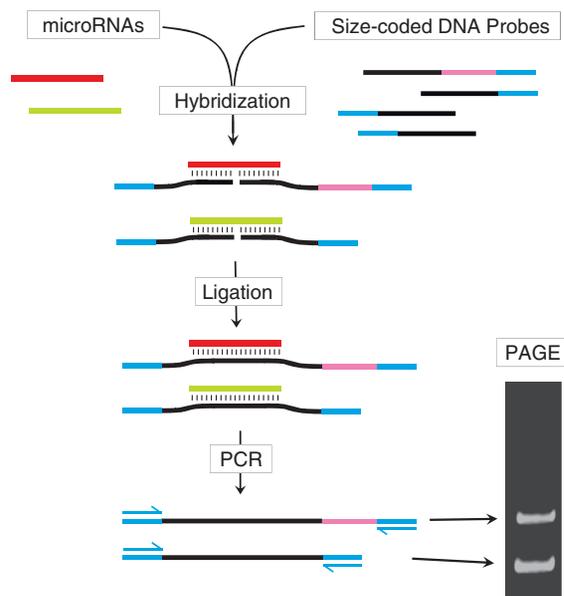
### Ligation reaction and PCR

Synthetic miRNAs (0.5 nM unless specified) or total RNA samples (100 ng) were incubated with 0.01  $\mu$ M of each probe in 10  $\mu$ l of ligation reaction at final concentrations of 50 mM Tris-HCl (pH 7.6 at 25°C), 10 mM MgCl<sub>2</sub>, 5 mM DTT, 0.1 mM spermidine, 0.1 mM ethylenediamine-tetraacetic acid (EDTA), 5  $\mu$ M ATP, 10% (W/V) PEG 4000 and 1.5 Weiss unit/ $\mu$ l T4 DNA ligase at 42°C for 2 h. Note that 0.08  $\mu$ M of *let-7*-5' side probe was used for each reaction, since the probe is common for eight *let-7* miRNAs. If pre-heating was required, RNA samples together with probes were incubated at 70°C for 5 min prior to ligation. The reactions were inactivated at 80°C for 10 min. PCR amplification was performed using universal primers: 5'-GTAAAACGACGGCCAGT-3' and 5'-CAGGAAACAGCTATGAC-3' at annealing temperature of 65.5°C for 35–40 cycles. PCR products were fractionated using 16% polyacrylamide/TBE gels electrophoresis. Gels were stained with ethidium bromide and pictured using ultraviolet (UV) trans-illuminator and charge-coupled device (CCD) camera. Bands intensities in gels were analyzed with TotalLab gel analysis software, version 1.10 (Nonlinear Dynamics, Newcastle upon Tyne, UK).

## RESULTS

### Principle of the SL-PCR method

T4 DNA ligase can repair nicks in the DNA strand of a DNA:RNA hybrid and this activity has been used in single RNA molecule detection techniques (13–15). Here, we designed a method for specific detection of several miRNAs based on this activity of T4 DNA ligase, PCR and polyacrylamide gel electrophoresis (PAGE). The method is illustrated in Figure 1. Briefly, two DNA probes are hybridized to a common target miRNA in optimized buffer and temperature conditions. Each probe pair has a defined length as a 'size code' for its target miRNA; therefore, several probe pairs designed to detect several different miRNAs can be applied within a single reaction. Subsequently, T4 DNA ligase joins adjacent hybridized probes. Next, all joined probes are amplified by PCR concurrently using a universal primer pair. An important feature of the method is that all probe pairs have the same primer sites and therefore the amplification reaction involves only a single pair of primers as in a standard PCR reaction, providing a process that is much simpler than multiplex reactions. The lengths of the attached probe pairs (and PCR amplicons) are <100 bp



**Figure 1.** Illustration of the size-coded ligation-mediated PCR (SL-PCR) method. Multiple miRNAs (here two) simultaneously are hybridized to their corresponding pair of size-coded probes in solution. Hybridized probes are then joined by T4 DNA ligase and amplified by PCR using universal primers. Next, PCR products are fractionated using high-resolution PAGE and stained with EtBr. Each band at a defined size represents a certain miRNA in the original sample.

and the size difference among them is 3 bp; therefore, bias in amplification efficiencies should be minimized. Finally, PCR products are fractionated in high-resolution PAGE. Each band indicates a specific miRNA and the band intensity is representative of the miRNA expression level. Since the band intensity also depends on the size of DNA, we adjusted the intensity values based on PCR product length using the following formula:

$$\frac{\text{Value of band intensity}}{\text{number of base pairs in PCR product}}$$

### Specific, quantitative and sensitive detection of *let-7* miRNAs

We first tested whether the new ligation and PCR-based method is specific and can distinguish very similar sequences. In fact, T4 DNA ligase has been reported to seal DNA nicks with mismatches with decreased efficiency (16,17). In DNA::RNA hybrids, the enzyme fidelity is enhanced and single-nucleotide mismatches that are located at the ligation junction are discriminated by a factor of 20- and 200-fold (18). However, for some miRNAs it is not possible to design probes where the ligation junction is located at the site of distinct nucleotides. Therefore, we also optimized the incubation temperature and time for more stringent condition and found that at 42°C for 2 h the ligation reaction is efficient and specific (data not shown). The combination of

short-sequence hybridization and improved ligation reaction appears to provide highly specific detection.

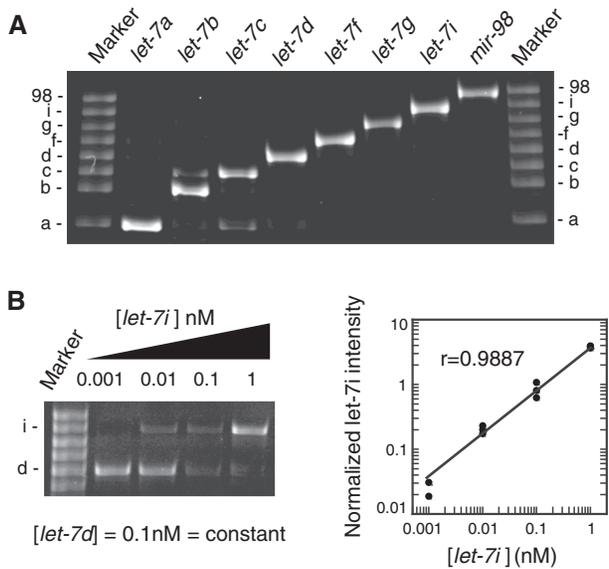
The *let-7* miRNA family in human, rat and mouse consists of 11 genes that encode eight distinct, mature miRNAs. The *let-7* miRNAs and similar *hsa-miR-98* differ by only 1–4 nt from each other. Therefore, this family provides a challenging set for testing the specificity of detection methods. Here, we used synthetic oligo RNAs corresponding to *hsa-miR-98* and *let-7* miRNAs to test specificity of SL-PCR detection. For all tested miRNAs, the probe that matches the 5' half of the miRNA sequence (5' side probe) was identical because the entire miRNA family shares the same sequence in that region. The partner probes designed for the 3' half of the miRNA sequence (3' side probe) differed in recognition sequence and contained a specific length as 'size-code' for each *let-7* member. Note that only '5' side probe' and not '3' side probes' bore 5' phosphate groups, ensuring the desired ligation direction (for probe sequences see Supplementary Table S1).

To each ligation reaction, only one synthetic miRNA and a mixture of all probes for all miRNAs was added. PCR amplification and PAGE fractionation showed a band of the expected size for each miRNA, with little or no cross-hybridization (Figure 2A). For accurate determination of PCR band length, a high-resolution DNA size marker (3 bp) was made and used in PAGE analysis. The result indicates that the SL-PCR method is able to distinguish very closely related miRNAs that are similar in sequence with reasonable specificity. In the lanes for *let-7b* and *let-7c*, however, a faint non-specific band was detected probably because of wobble base pairing of miRNAs *let-7b* and *let-7c* to the *let-7c*-3' side probe and *let-7a*-3' side probe, respectively. Moreover, we tested our method on the *hsa-mir-302* family of highly related miRNAs (Supplementary Figure S1).

We next examined whether the SL-PCR method is quantitative and sensitive, using serial 10-fold dilutions of a synthetic oligo RNA corresponding to the mature form of *hsa-let-7i*. To provide an internal control of known concentration, a constant amount of a second oligo RNA corresponding to *hsa-let-7d* was included (Figure 2B). To normalize the variable miRNA (*let-7i*) band intensity, the measured value was divided by the value obtained from the control miRNA (*let-7d*). Normalized band intensity was plotted against the initial concentration of variable miRNA (*let-7i*) from three independent experiments (Figure 2B). A line fitted to the data shows a linear correlation ( $r = 0.9887$ ) over three orders of magnitude (from 0.001 up to 1 nM of variable miRNA), indicating that the SL-PCR is quantitative. In addition, this result shows that as little as 0.001 nM of the oligo RNA was detectable under these optimized conditions. Therefore, the new approach appears to be applicable for specific, quantitative and sensitive detection of miRNA expression over a broad concentration range.

### Optional detection of miRNA precursors

miRNA precursors are not always processed into mature sequences, suggesting that miRNAs can be regulated

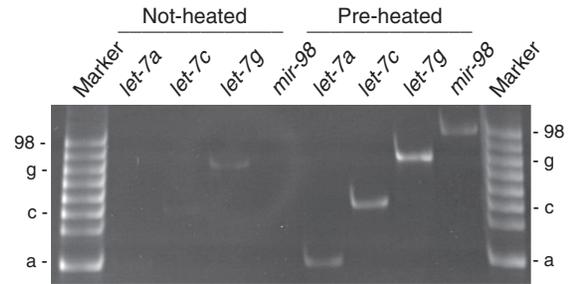


**Figure 2.** Specific, quantitative and sensitive detection of *let-7* miRNAs using SL-PCR. (A) Synthetic *let-7* miRNAs were separately subjected to detection reactions containing all probe pairs. A DNA size marker shows the position of the PCR band for each *let-7* miRNA. Each band of the size marker is denoted by the corresponding *let-7* lettered suffix and also '98' for *hsa-miR-98*. (B) Left, synthetic *let-7i* and *let-7d* was applied to detection reactions together, where the first miRNA was added in serial tenfold dilutions and the latter was constant, providing an internal control for normalization. Right, normalized band intensity values of the variable miRNA (*let-7i*) were plotted against initial concentrations of *let-7i* in a standard curve.

post-transcriptionally (19). Consequently, the levels of the precursors and their mature forms are not necessarily correlated. To understand the function of miRNAs it is necessary to discriminate mature from precursor forms. However, miRNA precursor transcripts naturally share sequences with their mature products and this creates a challenge for discrimination. To test whether SL-PCR can detect miRNA precursors, *in vitro* transcribed *hsa-let-7a-3*, *hsa-let-7c*, *hsa-let-7g* and *hsa-mir-98* precursors were subjected to SL-PCR detection. The results show that miRNA precursors produce negligible signals in PAGE analyses (Figure 3). This is probably because probes fail to hybridize to precursors due to sequestration of target sequence in the hairpin structure. Next, we examined whether pre-heat treatment can overcome the possible sequestration issue to detect precursors. Accordingly, miRNA precursors together with probes were incubated at 70°C for 5 min before the ligation reaction. SL-PCR could detect miRNA precursors with the latter setting (Figure 3). Therefore, SL-PCR is able to include or exclude miRNA precursors from detection using temperature adjustments and this flexibility may be desirable in many functional studies of miRNA.

#### ***let-7* signatures in cellular, tissue and whole-organism samples**

To test the method on various biological samples, we compared SL-PCR patterns generated from unfractionated total RNA preparation and small RNA enriched fractions



**Figure 3.** Optional detection of miRNA precursors using SL-PCR method. *In vitro* transcribed precursors of miRNAs *hsa-let-7a-3*, *-let-7c*, *-let-7g* and *hsa-mir-98* with or without pre-heating at 70°C for 5 min were analyzed by SL-PCR.

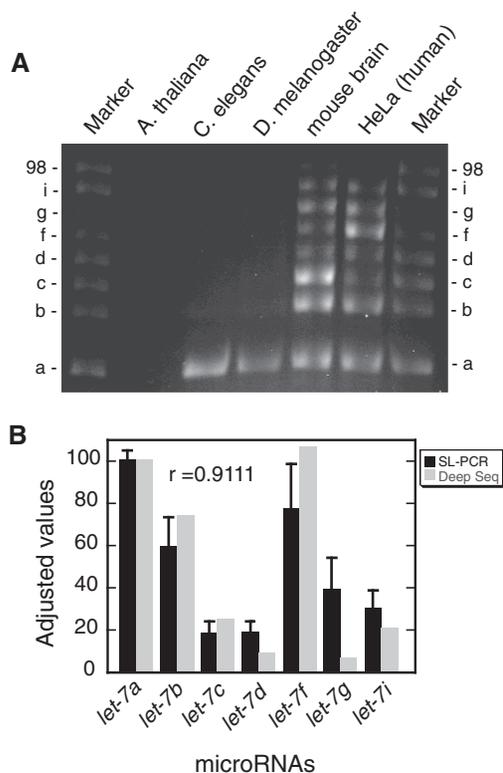
of total RNA. We found no difference in specificity and efficiency of detection between the two types of RNA preparations (data not shown) and, thereafter, in all subsequent experiments we were able to use unfractionated total RNA.

To test specificity in the biological samples, we used a phylogenic approach by exploiting the fact that the presence of *let-7* miRNAs differs among species. Using the probe set designed for human *let-7* miRNAs, we analyzed RNA samples from the worm *C. elegans*, the fly *D. melanogaster* and the plant *A. thaliana* and also, mouse brain and HeLa cells. *Caenorhabditis elegans* and *D. melanogaster* are known to have only one *let-7* miRNA that shares its sequence with *hsa-let-7a*, and *A. thaliana* lacks the *let-7* family. Therefore, these species provide natural controls for a specificity test in the presence of a complex pool of RNA. As expected, only a single band in the place of *let-7a* was observed for *C. elegans* and *D. melanogaster* samples, and no band was detected for *A. thaliana* using SL-PCR method. Detection of *let-7* miRNAs expression in HeLa cells and mouse brain revealed a *let-7* signature for each sample (Figure 4A).

We next asked whether the miRNA signatures obtained by SL-PCR are comparable with other methods. To this end, the results of the SL-PCR method were compared with those of a small RNA library deep sequencing project (9) (see also report of miRNA expression for *hsa\_Cervix-HeLa-adh* data set at <http://www.mirz.unibas.ch/cloningprofiles/>). In this project, miRNAs from various samples were gel fractionated, cloned and sequenced. The occurrence of miRNA sequence reads determines the level of expression. Values for HeLa cell *let-7* miRNAs obtained from both methods are presented in a bar chart (Figure 4B). Note that the values from each technique were adjusted to the value of *let-7a* set at 100. The comparison shows that the HeLa cells *let-7* signatures achieved using SL-PCR correlates with deep sequencing results ( $r = 0.9111$ ). Therefore, the new method can profile miRNAs in total RNA of various biological samples with high specificity and accuracy and it performs in agreement with existing method.

#### ***let-7* signatures as cell-type-specific biomarkers**

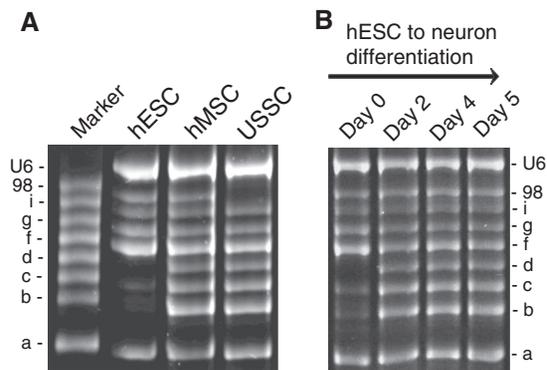
miRNAs are expressed in a cell- or tissue-specific manner (20). Changes in cell identity following differentiation or



**Figure 4.** *let-7* signatures in cellular, tissue and whole -organism samples as revealed by SL-PCR. (A) Total RNA preparations from various biological sources were subjected to SL-PCR in the presence of probes for human *let-7* miRNAs and *hsa-miR-98*. (B) Comparison of *let-7* signatures of HeLa cells derived from SL-PCR (black bars) and deep sequencing results (grey bars) with miRNA levels from both techniques given as values relative to 100 for *let-7a*. Values obtained from SL-PCR are mean  $\pm$  SD of three independent experiments.

cancer progression are associated with changes in miRNA expression patterns (21,22). To test the potential for SL-PCR to display differences in miRNA signatures of various cell types we detected the *let-7* signatures in three types of stem cells: human embryonic stem cell (hESC), unrestricted somatic stem cells (USSCs) and human mesenchymal stem cells (hMSCs) (Figure 5A). The new detection method revealed that each type of stem cell has a distinct *let-7* signature. Furthermore, *let-7* expression patterns were investigated in differentiating hESC to neuron, driven by retinoic acid. As expected, patterns of *let-7* signatures changed during differentiation (Figure 5B). The changes in *let-7* expressions occurred during the early days of differentiation, suggesting important and potentially causative role for these miRNAs in cell identity determination. In all reactions, we included probes for detection of U6 snRNA to provide a small RNA internal control. Collectively, the results demonstrated that SL-PCR can reveal differences and changes in miRNA signature patterns among various cell types and can be used as biomarker to disclose the cell identity.

Then, we measured the fold changes of the *let-7* miRNA expression 4 days after differentiation of hESCs to



**Figure 5.** *let-7* signatures in stem cells and during differentiation to neurons. (A) *let-7* signatures of human embryonic stem cell (hESC), human mesenchymal stem cells (hMSC) and unrestricted somatic stem cells (USSC). 'U6' stands for human U6 snRNA (B) *let-7* signatures during differentiation of hESC to neurons, driven by retinoic acid treatment in 5 days.

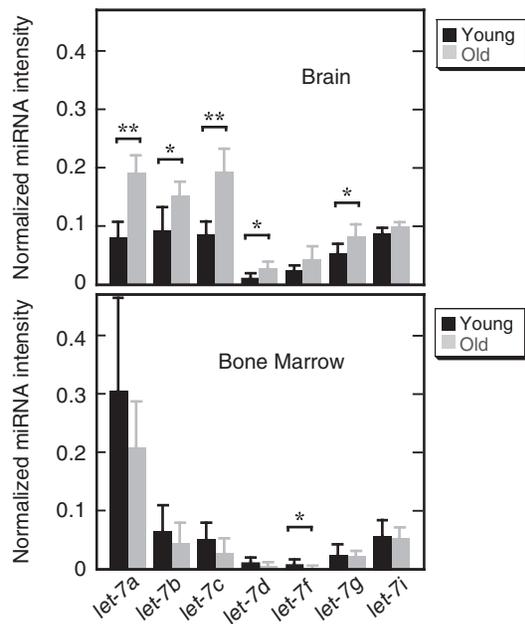
neurons using SL-PCR and compared it with data obtained from stem-loop quantitative RT-PCR (Supplementary Figure S2). Results again confirmed that SL-PCR is comparable with qRT-PCR in quantifying the expression fold changes between two samples ( $r = 0.964$ ).

#### Quantification of the *let-7* miRNA expressions in aged murine brain and bone marrow

To test the ability of SL-PCR to quantify changes in miRNA expression, we aimed to determine possible variations in *let-7* levels during the process of aging. Aging can be associated with changes in miRNA expression patterns of cells, for instance, central nervous system (CNS) progenitor and stem cells (23). For this analysis, we used total RNA preparations from whole brain or bone marrow of five 3-month-old and five 2-year-old mice. As an internal control, we used probes to detect U6 snRNA. Values for miRNAs were normalized using those for U6 snRNA. Analysis of miRNA signatures showed increases in the expression of *let-7* miRNAs in aged brain, and changes in the levels of several miRNAs were statistically significant. However, older bone marrow signatures did not display significant changes in levels of *let-7* miRNAs expression (except for *let-7f*) compared to younger tissues (Figure 6). The results support the ability of the new detection technique in quantification of the *let-7* miRNA expression levels in mice tissues.

#### DISCUSSION

To meet the challenging requirements for miRNA expression detection, the SL-PCR method enables specific, sensitive, quantitative and simultaneous detection of multiple miRNAs in total RNA from a variety of biological sources. Although developed for miRNA, possibly with minor adjustments, the general method could be adapted to detect other classes of small RNAs, including small nucleolar RNAs (snoRNAs) and Piwi-associated RNAs (piRNAs).



**Figure 6.** *let-7* signatures in young and aged murine brain and bone marrow. *let-7* miRNAs levels from tissues of five young (black bars) or five old (grey bars) mice were quantified by SL-PCR and normalized using expression levels of U6 snRNA. The data represent the mean  $\pm$  SD of four repeats, \* $P < 0.05$  and \*\* $P < 0.01$ . Two-tailed *t*-test was used to examine statistical significance.

It has been reported that individual miRNAs can have variation or modification at the 3' end of the molecule. The functional impact of 3' end variation is not known, however, it is unlikely that miRNA function is restricted to a single variant (9). Plant miRNAs provide examples of 3' end modification, where methylation occurs on the last nucleotides. In these cases, detection methods that rely on defined 3' end composition are not applicable, and this includes certain RT-PCR-based approaches. The detection strategy described for the SL-PCR method is independent of end composition and, therefore, can reliably detect all miRNA types that are variable or modified at the terminal nucleotide.

We applied SL-PCR to detect members of the *let-7* family in purified condition and in cellular total RNA to generate signatures for various cell types. The *let-7* miRNAs are considered to be difficult to distinguish because of sequence similarities. Although SL-PCR successfully distinguished microRNAs that differ by only 1 nt such as *let7-f* and *let7-a*, however, the method could not fully discriminate between miRNAs with wobble base pairing. Therefore, the specificity of SL-PCR for small RNAs with a single mismatch probably is affected by the composition of the mismatched nucleotide.

In our detection method, gel staining with ethidium bromide produced signal intensities that reflect both the abundance and length of PCR amplicons. Here, we adjusted the band intensity values based on PCR amplicon size. In future studies, the amplicon length effect can be removed using fluorescent-labeled PCR primers. Fluorescent primers will also help to reduce the

background and improve sensitivity. Although the SL-PCR method proved to be sensitive enough to detect few pico-molars of miRNAs in the purified condition ( $\sim 10^5$  copies per microliter), however, it cannot detect very low copy number miRNAs with current optimization.

Furthermore in this study, we used U6 snRNA as the internal control; however, U6 is highly expressed compared to the *let-7* miRNAs. It is recommended to use internal controls that show the expression levels similar to that of the test miRNAs. An appropriate internal control should also have robustness in expression levels across the various tissues/cells.

Regarding biological impact of miRNA profiling, many studies have investigated the association between changes in the levels of some miRNAs and aging. Indeed, miRNA *lin-4* has a role in *C. elegans* life span (24). In addition, during *C. elegans* aging a number of other miRNAs are modulated and among them *let-7* is downregulated (25). However, it is not clear to what extent the expression of miRNAs is changed during aging of mammals. A global approach to profile miRNAs in mice lungs showed no significant difference between young and old tissues (26), whereas another study reported increased expression of *let-7b* in aged mice CNS progenitor and stem cells (23). In an application of SL-PCR to the complex case of aging tissues, our method provided quantitative detection of multiple *let-7* miRNAs in aged mice brain and bone marrow. Our finding suggests an association between *let-7* expression and age of mice brain.

Finally, expression signatures of miRNAs have been proposed as potential biomarkers for prognosis and diagnosis of cancers and other human diseases (10). Employing SL-PCR to detect disease-specific miRNA signatures will provide a convenient protocol for pathology. Collectively, this study demonstrates that the SL-PCR method has the features needed to become a preferred strategy for research and diagnostics applications that focus on detection of multiple miRNAs signatures.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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*Conflict of interest statement.* None declared.

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