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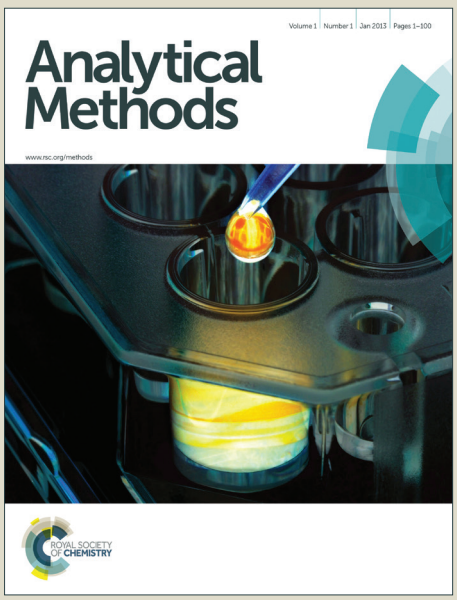

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ARTICLE

A Universal Linker-RT PCR Based Quantitative Method for the Detection of Circulating miRNAs

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Circulating miRNAs have been identified as key regulators of gene expression in many physiological and pathological processes. Real-time quantitative PCR is a conventional method that is indispensable, although many different approaches have been employed for miRNA expression profiling. We have established a universal, sensitive, highly efficient, cost effective and time-saving reverse transcription quantitative PCR for the measurement of circulating miRNA. This method involves the use of a random pre-adenylated DNA oligonucleotide linked to miRNAs followed by a universal reverse transcription and individual miRNA quantitative process. This method was optimized, and its specificity and sensitivity were evaluated. Circulating miRNAs from lung cancer patients were detected for verification. The results suggest that this random pre-adenylated DNA oligo-based miRNA quantitative method is sufficiently efficient and sensitive to detect circulating miRNA.

1. Introduction

MicroRNAs (miRNAs) are small non-coding, endogenous RNA (~22 nts) that play roles in the regulation of the post-transcriptional expression of genes involved in many basic cell processes, including cell proliferation, cell differentiation and cell apoptosis. MicroRNAs were first found in Caenorhabditis Elegans through forward genetic screens that identified lin-4[1]. Since then, miRNAs have received increasing attention from researchers and have become an emerging field of life science. In recent years, an increasing number of reports have demonstrated that miRNA expression is involved in the development and progression of cancer[2, 3]. The detection of miRNA expression is an essential step for understanding these complex cell processes.

Many technologies have been developed for and successfully applied to miRNA profiling, including northern blotting, real-time quantitative PCR (qPCR) and microarray and high-throughput sequencing technology[4-9]. However, microarray technology has a deficiency in accuracy such that less abundant miRNAs often escape detection, and high-throughput sequencing has been a significant tool not only for profiling the relative expression levels of miRNAs but also for the discovery of novel miRNAs[10-13]. High-throughput sequencing is currently considered to be the ideal means for the detecting miRNA expression. However, the expense, long cycles, complexity of sample preparation and high instrumentation requirements of this method have limited its application to studies for which this method is necessary, and many other techniques, including quantitative PCR, are needed to supplement high throughput sequencing. Quantitative PCR seems to be essential for miRNA studies due to the flexibility of this method. Quantitative real-time PCR is a classical detection method that has been widely applied due to its specificity, accuracy and reliability; thus, quantitative real-time PCR has become a "gold standard" in the genetic quantitative research. Stem-loop RT-qPCR was developed for the specific and efficient quantification of small RNAs and has become a widely used technology that is primarily applied to canonical, well-characterized small RNAs8. Several other approaches, including tail-adding reactions[14-16], the stem-loop primer-based method[17, 18] and primer extension[19, 20] have subsequently been reported for the expression detection of miRNAs. However, due to the rising interest in the expression of circulating miRNAs and their potential regulatory roles n important biological processes, an efficient, sensitive and cost-saving quantitative method for the detection of circulating miRNAs detection is still needed.

In the present study, we established a sensitive, universal, cost-effective and time-saving qRT-PCR method involving pre-adenylated DNA oligonucleotides. Because the 3' ends of miRNAs exhibit differential preferences for the nucleotide(s) of adaptor 5' end [21], two random nucleotides at the 5' end of the adapter were introduced. Furthermore, circulating miRNAs from the plasma of lung cancer patients were detected with this sensitive method for verification.

2. Materials and methods

2.1 Sample collection

Nine lung cancer patients and thirteen normal controls were enrolled in this study. Peripheral blood samples (2 mL) were collected from lung cancer patients and healthy controls at the Jiangsu Province Hospital with informed consent. This project was approved by the Ethics Committee of Jiangsu Province

Hospital. Corresponding tissue samples were also collected from the lung cancer patients.

2.2 RNA extraction

Circulating RNA was isolated from 500 µl of plasma with miRNeasy Serum/Plasma Kits (Qiagen, Hilden, Germany), and total RNA was extracted from the tissue using mirVana miRNA Isolation Kits (Ambion, Life Technologies, Grand Island, NY, USA). Both of these procedures were performed according to the respective manufacturers' instructions. The qualities and quantities of the RNA were assessed using the Qubit® RNA HS Assay Kit by Qubit® 2.0 Fluorometer (Life Technologies, Grand Island, NY, USA) according to the manufacturer's protocol.

2.3 Evaluation of the ligation-based miRNA qPCR method

The sensitivity and specificity of the miRNA qPCR method were evaluated in the present study. Differential 3'-end synthetic RNA nucleotides were used to evaluate the efficiency of the random DNA adaptor ligation. The concentration of the random DNA adaptor was also optimized for different concentrations of sample RNA.

Ligation and Reverse Transcription:

For the ligation reaction, the sample RNAs were mixed with the adaptor, preheated at 70℃ for approximately 5 min, cooled on ice. Then, 1 µL of 10× buffer and 200 units of T4 RNA ligase 2 (truncated) were added to the 10 µL reaction system and incubated at 25℃ for 60 min. Standard curves with 100-fold dilutions were created for the synthetized miRNA assays to evaluate the sensitivity and efficiency of the method. After ligation, reverse transcription was performed in a 20 µL reaction containing 2 µL of the ligation product, 1 µL of 5 µM RT primer, 1 µL of 10 mM dNTP, 4 µL of 5×PrimeScript Buffer, and 200 units of PrimeScript RTase (TaKaRa, Dalian, China). The reaction was incubated at 50ºC for 60 min and then terminated by heating at 85ºC for 5 min. No-template controls (NTCs) and no-reverse transcriptase controls (-RTs) were included in all RT reactions.

For the comparison to the poly (A) method, the sample RNA was polyadenylated with a Poly (A) Polymerase Tailing Kit (Epicentre) according to the manufacturer's instructions. The RT primer for the poly (A) method and the stem-loop method was designed according to a previous study with some modifications[8, 14].

Quantitative PCR:

All quantitative PCRs of the miRNAs were performed using the SYBR Green I® Quantitative Real-Time PCR (qPCR) Assay with individual specific primers used in previous studies8. Briefly, a total volume of 20 µL containing 2 µL cDNA diluent, 10 µL 2×SYBR Premix Ex Taq (TaKaRa, Dalian, China), 1 µL universal primer (10 µM) and 1 µL specific forward primer (10 µM) was used. Dissociation curves were created for each reaction to verify the effectiveness. All reactions were run in triplicate, and the average threshold cycle and SD values were calculated.

2.4 Detection of circulating miRNA in lung cancer patients

To further verify our miRNA qPCR system, the circulating miRNAs from lung cancer samples were detected and analyzed. Plasma RNAs separated from samples from lung cancer patients and healthy control were quantified with the ligation-based method. Hsa-let-7b and hsa-miR-21 were selected for detection based on previous studies[22-24].

2.5 Data analyses

The quantifications were based on the determination of the quantification cycles (Cqs), and PCR efficiencies were calculated from the log-linear portion of the standard curves16. Comparisons between the different primer and reaction conditions were performed with two-sided Student's t-tests for paired samples. The threshold for significance was set at P-value < 0.05.

3. Results

3.1 Adenylated DNA oligo-based qPCR strategy for miRNA detection

In the present study, an effective RT-qPCR method for the quantification of circulating miRNA is proposed. As shown in Fig. 1, this scheme primarily consists of three steps: ligation of a DNA adaptor to the 3' end of the RNA, reverse transcription and real-time qPCR. First, total RNA or size-selected miRNAs were ligated to pre-adenylated DNA oligonucleotides with T4 RNA ligase. With a 3' end blocker modification, the pre-adenylated DNA adaptor links to the 3' hydroxyl of the RNA specifically in the presence of (truncated) T4 RNA ligase 2. Two random nucleotides were introduced in the 5' end of the adaptor to reduce the bias of ligation. A tailing primer was involved in the subsequent reverse transcriptions, and traditional quantitative PCR was then performed with a miRNA-specific forward primer and a universal reverse primer. All of the oligonucleotides used in this study were synthesized and purified by TaKaRa Biotechnology Co., Ltd. (Dalian, China). The DNA sequences of primers are listed in Table 1.

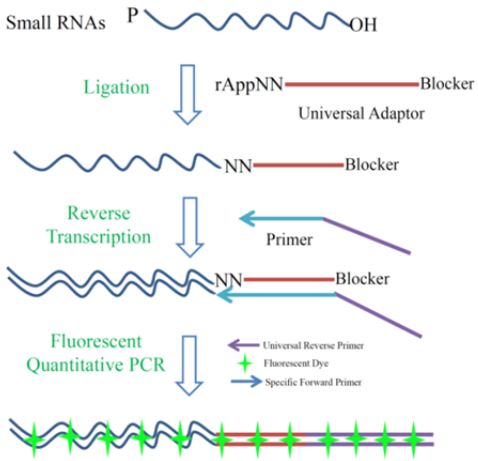


Fig. 1 Random adenylated DNA oligo-based qPCR strategy. All mature miRNAs are ligated to pre-adenylated random adapters based on this structural feature. Then, a universal tailing primer is introduced to the reverse transcription reaction. The subsequent qPCR is performed using a miRNA-specific forward primer and a universal reverse primer.

Table 1 Sequence of synthesized oligonucleotides

Name	DNA sequences (5'---3')
U-end	UGAGGUAGUAGGUUGUAUGGUU
G-end	UACCCGUAAUGUUUCCGCUGAG
A-end	AGCUGAUUUCGUCUUGGUAAUAA
C-end	UUCAGAGUUCUACAGUCCGAGAUC
DNA adaptor	rAppTGGAATTCTCGGGTGCCAAGG–NH_2
randomized adapter	rAppNNTGGAATTCTCGGGTGCCAAGG–NH_2
Universal RT primer	CTGCCCCGGGTTCCTCATTCTCTCTGCTGT AGCCTTGGCACCCG AGAATTCCA
RT primer for poly (A)	CTGCCCCGGGTTCCTCATTCTCTCTGCTGT AGTTTTTTTTTTTTVN*
qPCR universal primer	CTGCCCCGGGTTCCTCATTCT
hsa-let-7b-F	TGAGGTAGTAGGTTGTGTGGTT
hsa-miR-145-F	GTCCAGTTTTCCCAGGAATCCCT
hsa-miR-320a-F	AAAAGCTGGGTTGAGAGGGCGA
hsa-miR-16-F	TAGCAGCACGTAAATATTGG
hsa-miR-141-F	TCCCTGATACCCTTTAACCTGTGA
hsa-miR-21-F	TAGCTTATCAGACTGATGTTGA
hsa-miR-24-F	TGGCTCAGTTCAGCAGGAACAG

*V = A, G, C; N = A, T, G, C.

3.2 Adapters with random nucleotide ligations reduce bias and increase efficiency

The introduction of the adenylated DNA adaptors with two random nucleotides was thought to be the key factor that contributed to the efficiency of the quantitative PCR for miRNA detection. To evaluate whether the random nucleotides at the 5' end of adaptor reduced bias, ligation with conventional adaptors without random nucleotides was used for comparison. The Ct value obtained in the qRT-PCRs of four miRNAs mixed balanced with different nucleotide at 3'-end was measured. As seen in Fig. 2, The qRT-PCR method that employed the random nucleotide adaptors obtained uniform Ct values, and no significant differences between the four different 3'-end miRNAs were found (t-test); however, when the specific nucleotide adaptors were used in the qRT-PCR, significantly different Ct values were obtained for the A-end miRNAs compared to all others ($P<0.01$) and for the U-end compared to the C-end miRNAs ($P<0.05$). We suggested that the different 3'-end miRNAs had different ligation efficiencies when the normal adaptors were used in our qRT-PCR method.
Additionally, the results shown in Fig. 2 also demonstrated that the ligation efficiency of the random nucleotide adaptor was higher than that of the specific nucleotide adaptor.

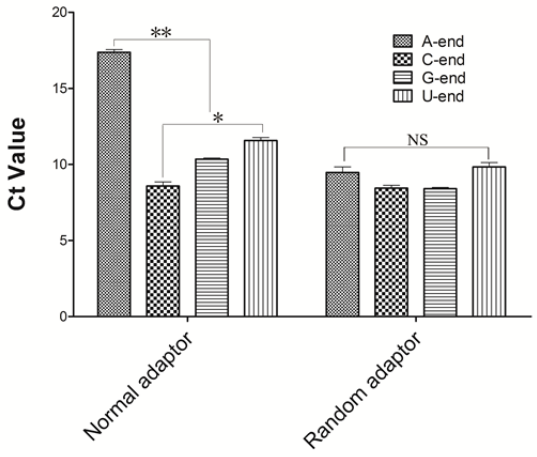


Fig.2 Comparison of the random adaptors and non-random adaptors. NS denotes non-significant differences between the different 3'-end miRNAs; *denotes significant differences ($P<0.05$), and ** denoted highly significant differences ($P<0.01$).

3.3 Optimizing adapter concentration improves ligation efficiency

The concentrations of the adaptor and the template RNAs (the latter is another key factor for RNA ligation) were also important in our proposed qRT-PCR method. Regarding the template RNA concentrations, we choose to examine two concentrations that might be representative of typical samples from tissues and plasma/serum. We used RNA template concentrations of 150 ng/μL for the tissue sample and 10 ng/μL for the plasma sample in the present study. For the higher and lower concentration templates, the adaptors were diluted from 50 μM to 1 μM and from 2.5 μM to 0.01 μM, respectively. Two miRNAs, miR-21 and miR-145, were quantified in each sample. The results of the qRT-PCR are shown in Fig. 3. The same peak points were obtained from the two miRNAs in this study. The adaptor concentration of 5 μM highly efficient for tissue sample RNA ligation, and the 0.1 μM adaptor concentration were optimal for the RNA sample from the plasma. Adaptor concentrations that were too high or too low resulted in lower ligation efficiencies.

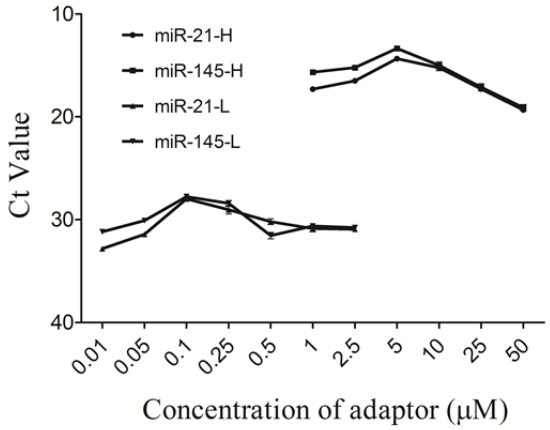


Fig.3 Optimization of the adapter concentrations for different RNA inputs. The figure shows that 5 μM was the optimal adaptor concentration for larger RNA sample inputs (150 ng), and 0.1 μM was the optimal adaptor concentration for smaller RNA sample inputs (10 ng).

Because we used total RNA to evaluate ligation efficiency in this study, it was difficult to precisely quantitate the RNA molecules, but general molecule ratio ranges could be obtained based on the sample RNA length distribution and the concentrations. The concentration of the adaptors utilized can be adjusted according to the ratio and the sample RNA input.

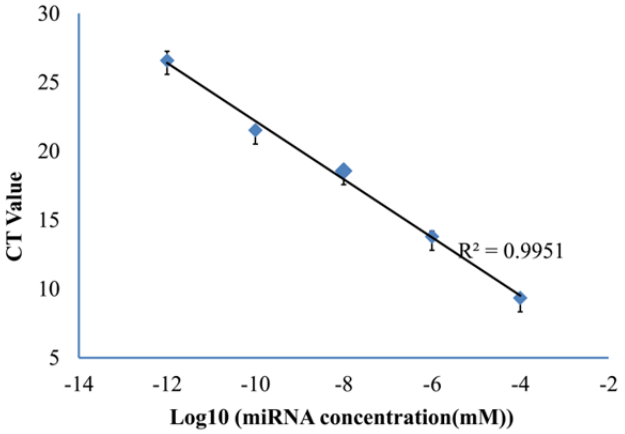
3.4 Assay validation, sensitivity and dynamic range

To further evaluate this qRT-PCR method for miRNA detection, we focused on three critical criteria: dynamic range, sensitivity and specificity. Initially, we used a synthetic let-7b to evaluate

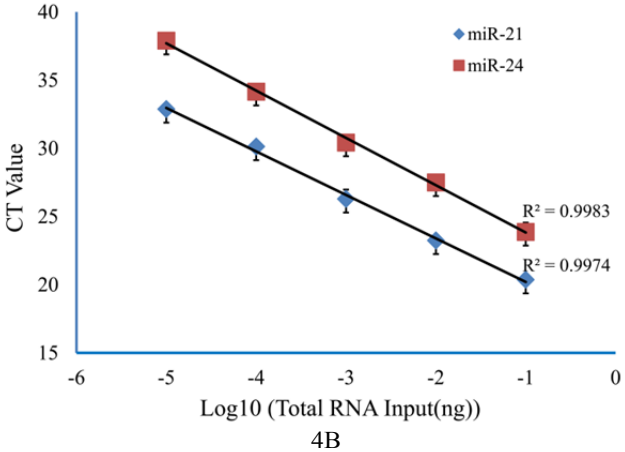
the sensitivity and dynamic range of the proposed miRNA quantitation scheme.

The synthetic miRNA were diluted from 20 μM to 2 fM in the ligation reaction. The results exhibited excellent linearity over 8 orders of magnitude between the log of the target input and the CT value (R2=0.9951), which demonstrates that this assay has a dynamic range of at least 8 logs and is capable of detecting concentrations of synthetic miRNA as low as 2 fM (Fig. 4A). The dissociation curve exhibited one peak in the PCR amplification, which demonstrated the specificity of the reaction (Fig. s1).

To further validate the performance of this approach, we used total RNA from tissue as an input and detected miR-21 and miR-24. All of the assays exhibited strongly linearity between the log of the total RNA input and the threshold cycle (Ct) value (Fig. 4B). All of the correlation coefficients were greater than 0.99. The dissociation curve also exhibited a single peak (data not shown), which indicates that this qRT-PCR method can detect miRNA expression in complex backgrounds with high sensitivity and a wide dynamic range.



4A



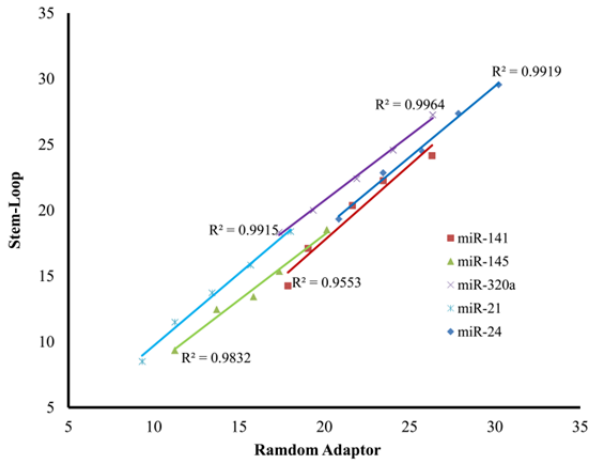
4B

Fig.4 Dynamic range and sensitivity of the synthetic miRNA assay. (A) Standard curve for the synthetic miRNA (R2=0.9951). The target inputs ranged from 20 μM to 2 fM. (B) The total RNA inputs from the lung cancer tissues ranged from 10^{-1} ng to 10^{-5} ng per reaction. The horizontal axis represents the log of the total RNA input. MiR-21 and miR-24 were detected.

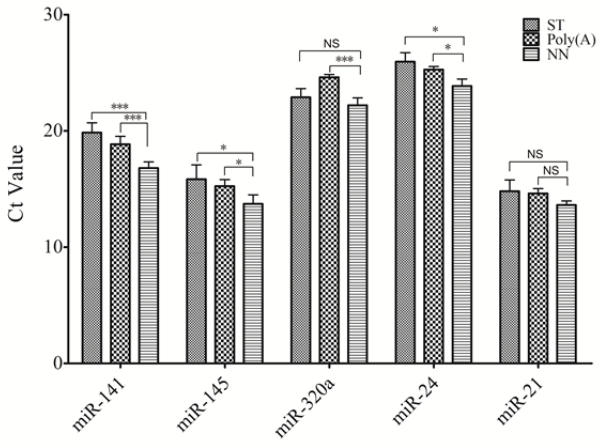
3.5 Comparison to the stem-loop and poly (A) methods

The stem-loop RT and poly (A) methods are widely accepted technologies for miRNA expression profiling, and these methods stand out as notable landmarks in the progression of miRNA PCR quantitation. To further validate the applicability of our proposed qRT-PCR method, we compared the random pre-adenylated DNA oligo-tagged approach with these two miRNA qPCR methods (Fig. 5). The results are illustrated in Fig. 5A and show that our proposed method produced good correlations with the results of the stem-loop method, which indicates that our approach is highly accurate in the detection of miRNA expression.

The efficiencies and sensitivities of the three methods were also compared. As illustrated in Fig. 5B, the Ct values produced by our method were significantly lower than those of the stem-loop method for miR-141, miR-145 and miR-24 ($P<0.05$, F-test). Comparisons of our method to the to the poly (A) method revealed significant differences for all miRNAs measured with the exception of miR-21 ($P<0.05$, F-test), which might indicate that the efficiency of our pre-adenylated oligo-tagged method for miRNA quantitation was superior.



5A



5B

Fig.5 Comparisons to the stem-loop and poly (A) methods. (A) The Ct values from the random pre-adenylated oligo-tagged miRNA quantitative method and the stem-loop-based RT-qPCR method exhibited strong correlations across different miRNAs. (B) Detailed comparisons of the Ct values produced by the

stem-loop method, the poly (A) method and our method for five different miRNAs. All Ct values were presented as the mean and the SEM of three measurements. NS denotes non-significant differences between two methods, ***denotes significant differences (P<0.001, F-test), and *denotes significant differences (P<0.05, F-test).

3.6 Detection of circulating miRNAs in lung cancer

Research on circulating miRNA as biomarkers has received increasing attention in recent years. As a detection tool, quantitative PCR has played a crucial role in miRNA biomarker studies. To test whether this system can be used to detect the circulating miRNA, small regulatory RNA molecules in the peripheral blood were assayed. We generated an assay for circulating cell-free miRNAs in plasma from lung cancer patients. The let-7 miRNA family is composed of global genetic regulators that control lung cancer oncogene expression by binding to the 3' untranslated regions of their target mRNAs. Many reports have demonstrated that the let-7 family is down-regulated in multiple cancers, including lung cancer. In contrast to the let-7 family, serum miR-21 is overexpressed in lung cancer patients compared to healthy controls. We detected let-7b and miR-21 in the plasma of nine lung cancer patients and thirteen healthy controls (Fig. 6). MiR-16 was used as an internal control miRNA. As shown in Fig. 6, significantly different expressions of circulating miRNAs were identified between the lung cancer patients and controls (P<0.001, Student's t test). These results are consistent with those of previous studies, which indicate that our proposed qRT-PCR method is capable of detecting low concentration circulating miRNAs in human plasma.

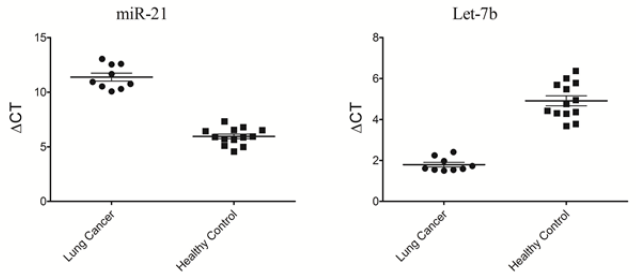


Fig.6 Circulating miRNA expression levels in the plasma of lung cancer patients and healthy controls

4. Discussion

In the present study, a universal linker-RT PCR-based miRNAs quantitative method was proposed and evaluated. The presence of random pre-adenylated DNA adaptors with the miRNAs in the ligation step and a subsequent universal reverse transcription step provided greater efficiency and thus better sensitivity than the poly(A) and stem-loop methods in the qPCR assaying of miRNAs.

The most commonly used qPCR approaches in miRNA assays are the stem-loop methods. Stem-loop RT primers consist of several (typically six) miRNA-specific bases and a stem-loop secondary structure, which provides better specificity than other methods. We obtained similar specificity in the detection of different miRNAs from human tissue samples with our strategy (Fig. 5A). However, the strength of the annealing of the stem-loop primer to the miRNA molecules might be insufficient when only six bases that are complementary to the miRNA template are used. This insufficiency might result in non-uniform efficiency and a decrease in sensitivity, which, in this study, appeared as larger Ct values in analyses of several miRNAs (Fig. 5B). Furthermore, the miRNA assay method proposed in this study involves a high-throughput RT reaction and thus provides great simplicity in the miRNA assay.

The poly (A) method is another commonly used miRNA qPCR assay approach that involves an oligo(dT) sequence followed by two degenerate nucleotides. While this method enables reverse transcription of all polyadenylated RNAs, including mRNA and other RNAs of any length (e.g., miRNAs), this promiscuity might reduce the specificity for miRNA profiling and further impair the sensitivity. As shown in Fig. 5B, the efficiency of this method was lower than that of our method.

The universal linker-RT qPCR approach employed in this study added a universal adaptor using the T4 RNA ligase 2 (truncated)[25]. As is well known, compared to long-chain RNAs with complex secondary structures, short fragment RNAs will achieve higher efficiency ligation[26, 27]. The mature miRNAs were only 18-25 nt in length; therefore, the target miRNAs will be ligated effectively and enriched in the first step. Pre-adenylated DNA adaptors with 3´-end blocking groups enable effective ligation with the T4 RNA ligase 2 (truncated) in the absence of ATP, which might prevent the self-circularization and self-ligation of the adaptor and target RNAs and thus prevent the formation of undesired ligation products (Fig. s1). As demonstrated in the results section, the ligation bias was reduced, and the efficiency was improved by the introduction of two random oligonucleotides in the adaptor (Fig. 2). The specific RT primer in the subsequent RT reactions provided both greater binding strength and thermodynamic stability between the RT primer and linked miRNA template and consequently enhanced the RT efficiency, which in turn, further improved both the sensitivity and the specificity of the miRNA assay. In this study, we demonstrated that the hsa-let-7b was successfully detected at concentrations as low as 2 fM and that has-miR-21 and has-miR-24 were successfully detected in as little as 10^{-5} ng of total RNA from lung cancer tissue samples (Fig. 4A and Fig. 4B).

The enhanced sensitivity of this ligation-based method will prove invaluable for the quantification of small amounts of miRNAs in biological samples, such as serum, plasma and other body fluids. Circulating miRNAs are highly stable and correlated with physiological and pathological events[28, 29], which makes these molecules promising biomarkers for disease diagnoses and prognoses. However, the limited quantities of miRNA in the serum/plasma demand a sensitive assay. The analysis of serum miRNA expression with microarrays is challenging because large RNA samples are needed. However, the ligation-based method explicated in this study is sufficiently sensitive for the analyses of circulating miRNAs. We successfully detected miRNAs from the plasma of humans with lung cancer using this ligation-based qPCR method.

5. Conclusions

Despite the current availability of many different miRNA qRT-PCR approaches, the demand for highly sensitive, efficient, time-saving and cost-effective miRNA qPCR methods is great, and such methods have gained increasing amounts of attention in miRNA research. Our ligation-based method has superior sensitivity, specificity and efficiency in the quantification of

miRNAs and thus provides a powerful tool for identifying tissue-specific, disease-specific and circulating miRNA biomarkers.

Conflict of Interest: The authors have declared that no conflict interests exist.

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Notes and references

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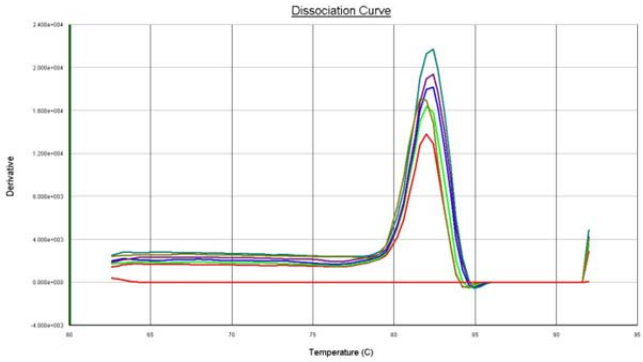

Fig.s1 Dissociation curve for the synthetic miRNA assay. The average melting temperature was 81.5℃.

