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# Establishment of plasma microRNA detection method by using taqman probe based quantitative reverse transcription PCR

Y-N. Wang[1,2*], L. Yu[3*], X-S. Zhao[4], W. Zhang[5], J. Wan[1,6 # ✉] and B. Yu[2,5 # ✉]

[1] Shenzhen Key Laboratory for Neuronal Structural Biology, Biomedical Research Institute, Shenzhen Peking University - The Hong Kong University of Science and Technology Medical Center, Shenzhen 518036, Guangdong Province, China.
[2] Department of Dermatology, Peking University Shenzhen Hospital, Shenzhen 518036, Guangdong Province, China.
[3] Department of gynaecology and obstetrics, Peking University Shenzhen Hospital, Shenzhen 518036, Guangdong Province, China.
[4] Peking University People's Hospital, Peking University Institute of Hematology, Collaborative Innovation Center of Hematology, Peking University, No. 11 Xizhimen South Street, Beijing, China.
[5] Shenzhen Key Laboratory for Translational Medicine of Dermatology, Biomedical Research Institute, Shenzhen Peking University - The Hong Kong University of Science and Technology Medical Center, Shenzhen 518036, Guangdong Province, China.
[6] Division of Life Science, The Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong, China.

**Corresponding author:** Dr. Jun Wan, Shenzhen Key Laboratory for Neuronal Structural Biology, Biomedical Research Institute, Shenzhen Peking University - The Hong Kong University of Science and Technology Medical Center, Shenzhen, Guangdong Province, China. E-mail: wanj@ust.hk
Dr. Bo Yu, Shenzhen Key Laboratory for Translational Medicine of Dermatology, Biomedical Research Institute, Shenzhen Peking University - The Hong Kong University of Science and Technology Medical Center; Department of Dermatology, Peking University Shenzhen Hospital, Shenzhen 518036, Guangdong Province, China. E-mail: yubomd@hotmail.com

[*] These authors contributed equally to this work.
[#] These authors contributed equally to this work.

**Abstract**

MicroRNAs (miRNAs) are a kind of short non-coding RNAs that regulate gene expression at the post-transcriptional level. Recently, many studies have found that circulating miRNAs have the potential to sever as diagnostic biomarkers for many diseases. However, the methods for the quantification of circulating miRNAs still need more adjustment. In this study, we tried to establish a reliable method to quantify the plasma miRNAs. We used quantitative real-time PCR with taqman probes to detect the plasma miR-153 level. Three controls were used in this study, including two external miRNAs control from *C. elegans* miRNAs (cel-miR-54 and cel-miR-238) and one internal control (hsa-miR-486). All of these controls were stable in plasma and the cel-miR-238/cel-miR-54/hsa-miR-486 combination could improve the normalization process. The expression level of the target miRNA, human plasma miR-153, could be quantified accurately with taqman probes .The assay has high accuracy, high sensitivity and a large dynamic range from 100 copies to $10^{13}$ copies in the PCR reaction. Our study provided a standardized quantification method for plasma miRNAs which might be used as biomarker in many diseases research.

***Key words:*** *C. elegans* microRNA (Cel-miR), MicroRNA-153 (miR-153), MicroRNA-486 (miR-486), Plasma microRNA, Quantitative reverse transcription-PCR (qRT-PCR), Taqman probe.

## Introduction

MicroRNAs (miRNAs), a class of naturally occurring small non-coding RNAs of 18–25 nucleotides in length, can regulate downstream gene expression at the posttranscriptional level. They degrade or block translation of target mRNAs by binding to the 3′ untranslated region (3'UTR) of them (1,2). Previous studies have suggested that miRNAs are present in human plasma in a high stable form (3-6) and can serve as novel and noninvasive biomarkers for a number of diseases such as cancer (7-13) and acute graft-versus-host disease (14,15) .

Currently, mature miRNAs can be detected by many methods such as northern blot (16), microarray (17), in situ hybridization (18), deep sequencing (19) and real-time quantitative PCR (20,21). Among these methods, both the northern blotting and microarray are low sensitivity and require large amounts of RNA sample. In situ hybridization is a semiquantitative, inefficient and low-throughput method. Deep sequencing technology is relatively high cost and time consuming, which seems difficult to be applied in clinical practice at present. Recent attempts to measure miRNAs with isothermal methods have met with some success, though they are labor intensive (22,23). To date, real-time PCR is one of the most sensitive and specific methods widely used for efficient detection of miRNAs in different tissues and cells. However, a method described well to detect the small amounts of miRNAs in plasma and an effective normalization strategy for technical variations in the entire procedure are still in infancy, though several studies have reported the detection of miRNAs in serum by qRT-PCR (10,24).

We found that circulating miR-153 in plasma may have a diagnostic role for acute graft-versus-host disease (aGVHD) after allogeneic hematopoietic cell transplantation (allo-HSCT) (unpublished data), so it was needed to establish a method for the accurate quantification of miR-153. In our previous study, more than forty miRNAs in plasma were screened from 300 healthy donors and 200 patients with autoimmune diseases

**Table 1.** The Primers of target genes.

| Gene name | | Primer sequence |
|---|---|---|
| Universal reverse primer | | GTGTCGTGGAGTCGGCAA |
| hsa-miR-153 | Forward | GCGGTTGCATAGTCACAAAAGT |
| | Probe | 5'-FAM-AGTCGGCAATTGCACTGGATACGAC-TAM-'3 |
| cel-miR-238 | Forward | AGCCTTTGTACTCCGATGC |
| | Probe | 5'-FAM-CACTGGATACGACTCTGAATGGCA-TAM-'3 |
| cel-miR-54 | Forward | GCAGACTCGTACCCGTAA |
| | Probe | 5'-FAM-CACTGGATACGACCTCGGATTATG-TAM-'3 |
| hsa-miR-486 | Forward | ACCGTCCTGTACTGAGCT |
| | Probe | 5'-FAM-AGTCGGCAATTGCACTGGATACGAC-TAM-'3 |

(unpublished data). MiR-486 was found to have a relatively high and stable expression level in plasma.

In this study, we discussed the relevant issues of reference control selection, data normalization and absolute quantification by standard curves. We established an approach with high accuracy, high sensitivity and wide dynamic range, which was based on real-time PCR with taqman probes, to determine the copy numbers of the miRNAs in plasma.

## Materials and methods

### *Plasma samples*

A total of 350 human plasma samples from 70 patients, who underwent allo-HSCT from Sep 2012 to Jun 2013, were obtained from Peking University Institute of Hematology. All patients provided informed consents. This study was approved by the institutional review board at the Peking University Institute of Hematology (Protocol number 2013-42). All samples from patients were collected in EDTA tubes.

### *Probe and Primers*

The taqman probes and all the primers (Table 1) are designed by primer6.0 and purchased from Invitrogen.

### *RNA Isolation*

Plasma RNA was isolated from 200 µl plasma of individuals with Trizol LS reagent (Sigma). All steps to isolate RNA are mentioned in the manufacturer's protocol. To normalize the technical variability of the plasma RNA extraction, 10 µl synthetic C.elegans miRNAs (cel-miR-54 and cel-miR-238, 0.02 ng/µl, Invitrogen) were added to each sample after the initial denaturation of plasma. Finally, RNA was dissolved in 30 L of RNase-free water.

### *Reverse transcription*

For reverse transcription (RT) of mature miRNAs, 5 µl total RNA or each dilution of synthetic human mature miR-153 standards was initially heated at 70°C in the presence of 250 nM of stem-loop reverse primers for 5 min, snapped and chilled on ice, and reverse transcribed in a total of 20 µl RT reaction comprised of 5.5 µl nuclease-free water, 4 µl 5×Buffer, 2 µl $MgCl_2$, 0.5 µl Ribonuclease Inhibitor (20units/µl), 1 µl 10mM nucleotide mix, 1 µl Reverse transcriptase (20 units/µl). The reverse transcription reaction was carried out at 25°C for 5min, 42°C for 60min, and then 70°C for 15 min.

### *Real-Time Quantitative PCR*

After two-fold-diluted 3 times, 2 µl RT product was combined with 8µl PCR assay reagents [SYBR Green I based real-time PCR: 0.2µl Taq, 2µl forward primer (1µM), 2µl reverse primer (1µM), 0.05µl SYBR Green I, 1.2 µl $MgCl_2$ (25 mM), 0.2 µl dNTPs (10 mM), 1 µl 10×PCR buffer, and 1.35 µl water; Taqman based real-time PCR: 0.2 µl Taq DNA polymerase, 3 µl primer mix (prepared by10 µl forward primer (10 µM), 10µl reverse primer (10 µM), 10 µl taqman probe (10 µM), 120 µl water), 1.2 µl $MgCl_2$ (25 mM), 0.2 µl dNTPs (10 mM), 1 µl 10×PCR buffer, and 2.4 µl water]. Real-time PCR was carried out on a CFX96™ Real-Time PCR detection system (BioRad) at 95°C for 5 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. All reactions were performed in triplicate.

### *Generation of standard curves*

The standard curve was generated by synthetic miR-153 (RIBOBIO, China) and was carried out in parallel with qRT-PCR of plasma samples. Standard curves were prepared by ten-fold serial dilutions of synthetic hsa-miR-153 from $10^2$ copies to $10^{13}$ copies. The levels of the synthetic miRNAs were assessed by qRT-PCR assay. The resulting Ct values were plotted versus the $\log_{10}$ of the amount of the synthetic miRNAs.

### *Evaluation of reference genes stability*

The geNorm (25)and NormFinder (26) were used to determine the expression stability of the target reference genes. The NormFinder software calculates the average stability for a reference gene. The geNorm software calculates the expression stability value M of candidate references and determines the optimal number of reference genes for accurate normalization. In both programs, the lower stability values means the higher stably expressed genes.

### *Calculation and normalization of experimental qRT-PCR data*

The $y = a\log_{10}(x) + b$ equation derived from standard curve was used to calculate absolute copy numbers of miR-153 input into the RT reaction. $x$ represents the absolute miR-153 copy number while $y$ represents the Ct value of each plasma sample in the RT reaction. The copy number per microliter of miR-153 was calculated with the equation. The data was normalized with minor modification as previously described. Briefly, for each plasma sample, an average Ct value was generated by the Ct values acquired from the two spiked-in *C.*

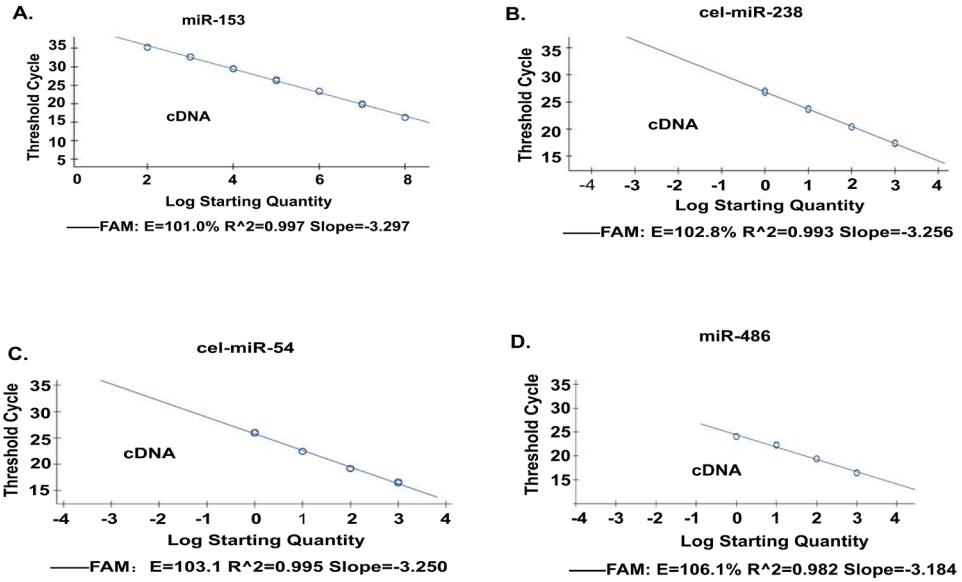


**Figure 1. The melt curves of the genes used in this study.** A-D. The cDNA of the candidate genes were amplified by real-time PCR assay. Melt curves of the genes are shown.

*elegans* miRNAs and has-miR-486.The median of the average Ct values obtained from all of the samples to be compared was next calculated. A Normalization Factor was then calculated for each sample based on the following formula:
Normalization Factor=1/[2ˆ(Median Control Ctvalue)-(Control Average Ct value of the given sample).

### *Statistical Analyses*

Data are presented as mean ± standard error of mean. Student's t test was utilized to determine statistical significance. P values lower than 0.05 were considered statistically significant. All statistical analysis was performed using SPSS 17.0 software.

## Results

### *Specificity and sensitivity of the TaqMan assay*

To determine the sensitivity of the taqman assay, serial dilutions of cDNA were used to generate a standard curve. A linear relationship between the input cDNA and the CT values with regression coefficient ($R^2$) for the target genes was obtained. The efficiency of amplification was range from 101.0% to 106.1% indicating a higher sensitivity of the amplification (Fig. 1A-D). SYBR-Green based real-time PCR was used to determine the specificity of the primers and probes used in this study. The melting curves of the genes showed a single peak at Tm values 82°C, 81.5°C, 82°C and 82°C for miR-153, cel-miR-238, cel-miR-54 and has-miR-486, respectively (Fig. 2A-D).

### *Stability of reference controls*

It is generally accepted that reference genes should be stably expressed. To validate the presumed stable expression of a given control gene, we used the geNorm and NormFinder to determine the expression stability of each reference gene. In both programs, the lowest stability values characterize the most stably expressed genes and allow for ranking the genes according to

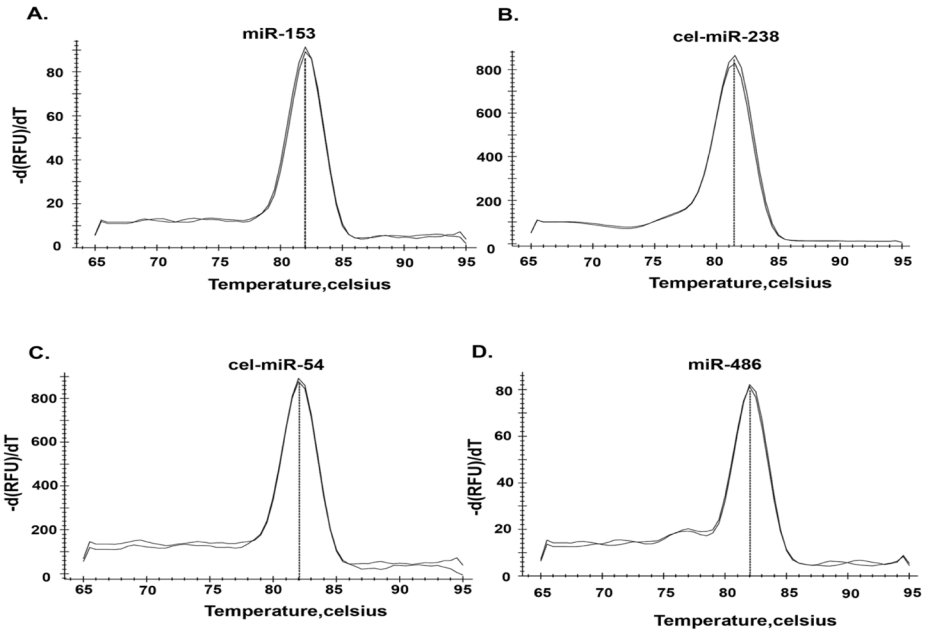


**Figure 2. The parameters of the genes used in this study.** A-D. The cDNA of the genes were amplified by real-time PCR assay. The parameters of the genes are shown. Standard curves are plotted as Ct versus Log (starting template per real-time PCR assay).

their expression stability. Real-time PCR data included results from 100 human plasma samples. Using the NormFinder algorithm, we found that the combination of cel-miR-238/cel-miR-54/hsa-miR-486 was the most stable gene control (Stability value=0.210±0.054) and hsa-miR-486 was the least stable gene control (Stability value=0.816±0.086). The combination of cel-miR-238/cel-miR-54 (Stability value =0.260±0.050) showed a second stable gene control. cel-miR-54 (Stability value =0.539±0.052) and cel-miR-238 (Stability value =0.468±0.050) showed a similar stability value(Fig.3A). Because M values of all candidate reference genes in the geNorm were between 1.23 and 1.37, lower than the default limit of 1.5, we found that all candidate reference genes showed high expression stability. The cel-miR-238/cel-miR-54/hsa-miR-486 combination could improve the normalization process, though a different ranking order of the candidate reference genes was observed with cel-miR-238 and cel-miR-54 as the most stable gene (M=1.23) followed by cel-miR-238/cel-miR-54 (M=1.21), cel-miR-238/cel-miR-54/ hsa-miR-486 (M=1.26) , miR-486 (M=1.37) (Fig. 3B).

### *Dynamic range of the absolute miRNA assay*

The dynamic range of the absolute miR-153 quantification assay was evaluated with synthetic miR-153. Synthetic miR-153 was diluted from $10^2$ copies to $10^{13}$ copies and then was assessed by qRT-PCR assay. The Ct values were plotted versus the $\log_{10}$ of the amount of the synthetic miRNAs. The results showed that the Ct values correlated well with the $\log_{10}$ values of target input ($R^2$=0.979), demonstrating that the assay has a large dynamic range from 100 copies to $10^{13}$ copies in the PCR reaction (Fig. 4A-B).

## Discussion

Recent studies have revealed that miRNAs play important roles in the regulation of various diseases. Increasing publications have shown that circulating miRNAs can serve as novel, noninvasive biomarkers. Thus, a rapid, accurate, sensitive and high-throughput method for the detection of mature miRNAs is highly desirable. In this study, we established a approach for absolute quantification of plasma miRNAs expression based on real-time PCR with taqman probes. Our data showed that the method had a high specificity and sensitivity with a dynamic range from 100 copies/μl to $10^{13}$ copies/μl.

Human plasma and serum are popular clinical biospecimens. They have proteins of very high concentration and should be diluted by denaturing solutions

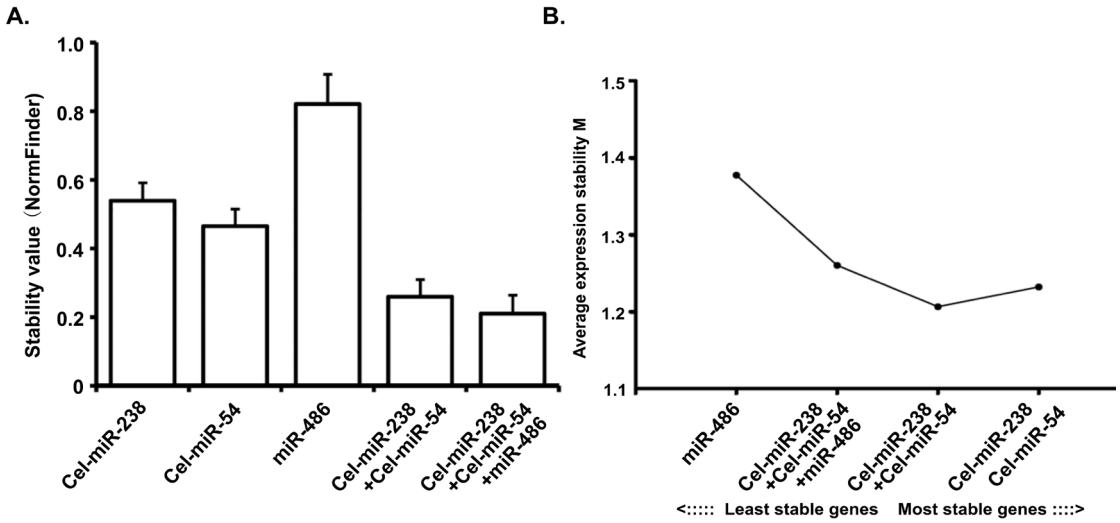


**Figure 3. Expression stability values of the reference genes.** A. The reference genes expression stability calculated by NormFinder and the best combinations of the reference genes. B. The reference genes expression stability calculated by GeNorm.

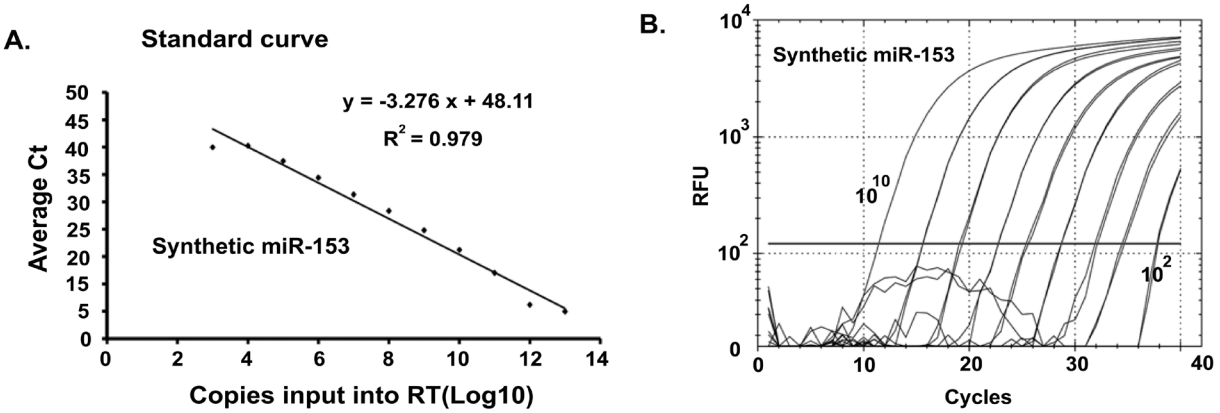


**Figure 4. Dynamic range and sensitivity of the TaqMan miR-153 assay.** A. Standard curve for miR-153. Standard curve were generated by a serial dilution of synthetic miR-153. The dilution series samples were run on the same plate as experimental samples. B. Real-time PCR amplification plots for the synthetic miR-153.

before the process of RNA extraction. Previous studies have shown that Ambion mirVana PARIS (27) and the Qiagen miRNeasy kit (28) are effective to generate a better RNA yield. Here we used the Trizol LS reagent to extract the plasma miRNA and found that this method was as effective as previous ones. Moreover, it was simpler and more cost-effective than commercial kits.

Several variabilies in the entire experiment need to be normalized, such as the amount of starting material, the yield of plasma RNA, and the efficiency of reverse transcription as well as amplification. Some strategies have been applied to normalize these variations. Currently, an effective normalization strategy is used with the *C. elegans* spiked-in control miRNAs (29,30). However, the extraction efficiency of spiked-in controls and plasma miRNAs are different, because the spiked-in controls are not able to form a miRNA-protein complex to protect them from being degraded. Thus spiked-in controls cannot normalize the variations from plasma RNA extraction. Alternative strategies are to use endogenous controls such as RNU6B and miR-16 (7,31). However, miR-16 expression in plasma seems to be inconsistent (11,32),while it remains unknown whether miR-16 could be used in aGVHD serum. Our previous studies have shown that miR-486 exhibits a high level and constant expression in plasma (unpublished data). We used the geNorm and Normfinder to identify suitable reference genes for qRT-PCR analysis of serum miRNA from patients after allo-HSCT. We found that miR-486 showed high expression stability and the combination of the spiked-in controls with miR-486 could improve the normalization process.

It is understandable that an optimal qRT-PCR assay is necessary for accurate determination of the level of expression of miRNAs in plasma. An optimal qRT-PCR assay should have high specificity, sensitivity and a wide dynamic range. In this study, we optimized the specificity of the primers by SYBR-Green based real-time PCR, and then determined the sensitivity of the taqman probes by standard curves generated with a serial dilution of cDNA. Finally, we evaluated the dynamic range of the assay by a standard curve generated with the synthetic miR-153. The assay for plasma miRNAs detection has a higher sensitivity and specificity and is capable of detecting as few as 100 copies of miRNA in plasma. Moreover, we substituted Promega probe qRT-PCR master mix for the reaction buffer prepared by ourselves, and found little difference in the effect between them. As the commercial master mix is easy to use, it may shorten the clinical detection time.

More and More publications have shown that circulating miRNAs can serve as novel, noninvasive biomarkers for a number of diseases. However, the methods to detect the minute amounts of miRNAs in plasma in the entire procedure have to be standardized before any of the novel miRNA biomarkers is used clinically. Here, the method we described for quantification of miRNA from human plasma samples should provide a practical solution for these studies.

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