Video Article

Absolute Quantification of Plasma MicroRNA Levels in Cynomolgus Monkeys, Using Quantitative Real-time Reverse Transcription PCR

Takuma Iguchi[1], Noriyo Niino[1], Satoshi Tamai[1], Ken Sakurai[1], Kazuhiko Mori[1]

[1]Medicinal Safety Research Laboratories, Daiichi Sankyo Co., Ltd.

Correspondence to: Takuma Iguchi at iguchi.takuma.kz@daiichisankyo.co.jp

URL: https://www.jove.com/video/56850
DOI: doi:10.3791/56850

Keywords: Molecular Biology, Issue 132, microRNA, biomarker, plasma, quantitative polymerase chain reaction (qPCR), absolute quantification, pre-amplification

Date Published: 2/12/2018

Citation: Iguchi, T., Niino, N., Tamai, S., Sakurai, K., Mori, K. Absolute Quantification of Plasma MicroRNA Levels in Cynomolgus Monkeys, Using Quantitative Real-time Reverse Transcription PCR. *J. Vis. Exp.* (132), e56850, doi:10.3791/56850 (2018).

Abstract

RT-qPCR is one of the most common methods to assess individual target miRNAs. MiRNAs levels are generally measured relative to a reference sample. This approach is appropriate for examining physiological changes in target gene expression levels. However, absolute quantification using better statistical analysis is preferable for a comprehensive assessment of gene expression levels. Absolute quantification is still not in common use. This report describes a protocol for measuring the absolute levels of plasma miRNA, using RT-qPCR with or without pre-amplification.

A fixed volume (200 µL) of EDTA-plasma was prepared from the blood collected from the femoral vein of conscious cynomolgus monkeys (n = 50). Total RNA was extracted using commercially available system. Plasma miRNAs were quantified by probe-based RT-qPCR assays which contains miRNA-specific forward/reverse PCR primer and probe. Standard curves for absolute quantification were generated using commercially available synthetic RNA oligonucleotides. A synthetic cel-miR-238 was used as an external control for normalization and quality assessment. The miRNAs that showed quantification cycle (Cq) values above 35 were pre-amplified prior to the qPCR step.

Among the 8 miRNAs examined, miR-122, miR-133a, and miR-192 were detectable without pre-amplification, whereas miR-1, miR-206, and miR-499a required pre-amplification because of their low expression levels. MiR-208a and miR-208b were not detectable even after pre-amplification. Sample processing efficiency was evaluated by the Cq values of the spiked cel-miR-238. In this assay method, technical variation was estimated to be less than 3-fold and the lower limit of quantification (LLOQ) was 10^2 copy/µL, for most of the examined miRNAs.

This protocol provides a better estimate of the quantity of plasma miRNAs, and allows quality assessment of corresponding data from different studies. Considering the low number of miRNAs in body fluids, pre-amplification is useful to enhance detection of poorly expressed miRNAs.

Video Link

The video component of this article can be found at https://www.jove.com/video/56850/

Introduction

An increasing number of studies have been exploring microRNAs (miRNAs) as biomarkers for the diagnosis and prognosis of cancers, or monitoring and detecting other diseases in nonclinical and clinical studies[1,2,3]. Quantitative real-time reverse transcription PCR (RT-qPCR) is one of the most common methods used to assess individual target miRNAs, because this technique is more sensitive than microarray[4] and RNA sequencing based platforms[5]. In general, miRNA expression is measured relative to a reference sample using the ΔCq method[6]. This approach is appropriate for investigating physiological changes in target gene expression levels. However, relative quantification of circulating miRNAs has limited utility because of their small quantities. In addition, technical variation makes it difficult to compare the results from different studies, because different laboratories customize the RT-qPCR experimental protocols differently, which leads to inconsistent or even contradictory results from different studies[7].

In view of the concerns mentioned above, absolute quantification might be more suitable for the assessment of the small quantities of miRNAs in body fluids. The absolute quantification method uses a standard curve generated from known concentrations of synthetic RNA oligonucleotides that are identical in sequence to the corresponding target miRNA[8]. The Health and Environmental Sciences Institute (HESI) Technical Committee on Genomics recently conducted comprehensive studies to compare the results of absolute measurements of plasma miRNAs, across multiple test sites. The results showed that using a standard protocol for the absolute quantitation of miRNAs yielded comparable results across the multiple test sites[9]. The RT-qPCR assay method described in the present study is almost identical to the HESI's standard protocol, which includes multiplexed analysis of multiple miRNA targets, and pre-amplification to aid the detection of low expression miRNAs.

In this study, a fixed volume (200 µL) of EDTA-plasma prepared from the blood collected from the femoral vein of conscious cynomolgus monkeys (n = 50) was used[10]. The following protocol describes the procedure for the preparation of plasma samples, extraction of miRNA, and RT-qPCR, including pre-amplification. More importantly, additional technical information about the protocol has been included, so that the

quantity of target miRNAs in the samples can be validated in combination with a well-qualified process. First, the standard curve of each miRNA was validated for its individual detection range, prior to its quantification in biological samples. Second, the quality of the current methodology was comprehensively evaluated by means of Cq values of an external control (cel-miR-238). Therefore, this platform yields more informative and reliable data for comparing results from different studies or laboratories.

The profiles of 8 miRNAs have been included in this report as representative results from the assay method described here. These miRNAs have been proposed as potential safety biomarkers associated with tissue injury to the liver (miR-122 and miR-192), heart (miR-1, miR-208a, miR-208b, and miR-499a), and skeletal muscle (miR-133a and miR-206) in rodents and humans[3,11,12,13].

Protocol

All experiments were approved by the Institutional Animal Care and Use Committee of Daiichi Sankyo Co., Ltd.

1. Sample Preparation

1. Collect blood (at least 0.5 mL) from the femoral vein of cynomolgus monkeys into EDTA 2K-containing tubes.
   NOTE: Citrate and heparin are not acceptable because these anticoagulants inhibit subsequent PCR[14,15].
2. Place the collected samples immediately on ice and process for plasma isolation within 2 hours of collection.
3. Centrifuge the samples at 10,000 x g at 4 °C for 5 min.
4. Transfer the supernatant into a 2 mL microtube, followed by centrifugation at 16,000 x g at 4 °C for 5 min to remove cell debris and residual platelets.
   NOTE: Quantification of miRNAs can be affected significantly by platelet contamination[16].
5. Place 200 µL aliquots of the supernatant into fresh 2 mL-microtubes, and store at -80 °C until use.
   NOTE: A fixed volume of each sample is used for RNA extraction; therefore, the volume of the aliquot must be exact.

2. RNA Extraction

1. Thaw frozen samples on ice (from step 1.5).
   1. **Keep sample cold on ice during RNA extraction. Chill lysis reagent and chloroform on ice prior to use.**
2. Add 5 volumes (1000 µL) of lysis reagent, containing monophasic solution of phenol and guanidine isothiocyanate, to the sample (200 µL), and mix vigorously by vortexing for 1 min.
3. Add 5 µL of 5 nM synthetic *Caenorhabditis elegans* miRNA (Syn-cel-miR-238-3p).
4. Add 1 volume (200 µL) of chloroform, and mix vigorously by vortexing for 1 min.
5. Keep on ice for 2 to 3 min, and then centrifuge the samples at 12,000 x g at 4 °C for 15 min.
6. Transfer the aqueous phase carefully to a new microtube.
   NOTE: Do not transfer any of the organic phase (red) or interphase (white). The volume of the aqueous phase collected should be uniform to avoid handling inconsistency, which results in increased technical variation. The usual amount of aqueous phase transferred in this protocol was 650 µL.
7. Add 1.5 volumes (975 µL) of ethanol, and mix well by pipetting up and down.
8. Transfer the sample into a corresponding column and adapter, followed by vacuum drying for 3 min using vacuum manifolds. If the sample volume is more than 700 µL, repeat this step to process the remaining solution.
   NOTE: Column-based RNA isolation is compatible with vacuum and centrifugation method.
9. Add 200 µL of ethanol to the column, followed by vacuum drying for 1 min.
10. Add 800 µL of RWT Buffer to the column, followed by vacuum drying for 2 min.
11. Add 800 µL of RPE Buffer to the column, followed by vacuum drying for 2 min.
12. Repeat step 2.11.
13. Add 300 µL of ethanol to the column, followed by vacuum drying for 1 min.
14. Place the column onto a new microtube, and centrifuge at 12,000 x g at room temperature (15 to 25 °C) for 1 min.
15. Transfer the column to a new microtube, and add 50 µL of nuclease-free water.
16. Stand at room temperature (15 to 25 °C) for 3 min, and centrifuge at 8,000 × g at room temperature (15 to 25 °C) for 1 min.
17. Re-apply the eluate to the column.
18. Repeat step 2.16, and store eluate at -80 °C until use.

3. cDNA Synthesis

1. Thaw frozen samples (from step 2.18).
2. **Prepare known concentration of synthetic RNA oligonucleotides that correspond to target miRNAs.**
   1. Use synthetic RNA oligonucleotide for generating a standard curve in qPCR. Stock solution of 1 x 10^8 copy/µL concentration is prepared for storage purposes.
   2. Dilute the stock solution 10-fold to obtain 1 x 10^7 copy/µL (*Not* pre-amplified samples) or 1 x 10^5 copy/µL (pre-amplified samples) working solution as the highest concentration for constructing the standard curve. In general, a suggested range for the standard curve concentrations is 1 x 10^7 to 1 x 10^2 copy/µL (*Not* pre-amplified samples) or 1 x 10^5 to 1 x 10^0 copy/µL (pre-amplified samples).
3. Prepare multiplex RT primer pool by mixing equal volumes of 20x RT primers for target miRNAs.
   NOTE: As shown in **Figure 2**, a pool containing up to 4 target miRNAs can be made by mixing the 20x RT primers of each of the miRNAs in the pool. Cel-miR-238 (external control) must be included as one of the target miRNAs in each tube. In case of fewer than 4 target miRNAs, add equal volumes of 1/10 TE buffer instead of 20x RT primer.

4. Prepare RT reaction mix: 3 µL of RT primer pool (from step 3.3), 0.15 µL of 100 mM dNTPs with dTTP, 1 µL of reverse transcriptase (50 U/µL), 1.5 µL 10x RT buffer, 0.19 µL of RNase inhibitor (20 U/µL), and 4.16 µL of nuclease-free water.
5. Mix 10 µL of RT reaction mix with 5 µL of RNA sample (from step 3.1) or oligonucleotides (from step 3.2) by pipetting, and incubate on ice for 5 min.
6. Run reverse transcription on a thermal cycler apparatus: 16 °C for 30 min, 42 °C for 30 min, followed by a final reverse transcriptase inactivation step at 85 °C for 5 min. Store reverse transcribed samples at -80 °C until use.

4. Preamplification (Optional)

NOTE: The miRNAs that show Cq values above 35 or more in subsequent qPCR are pre-amplified.

1. Thaw frozen samples (from step 3.6).
2. Make 10-fold serial dilutions of RT product from synthetic RNA oligonucleotides; 1 x 10^5 to 1 x 10^0 copy/µL for generating standard curve.
3. Prepare multiplex assay primer pool by mixing equal volumes (5 µL) of 20x assay primers for target miRNAs with the final concentration of each assay primer being diluted 200-fold by TE buffer in a final volume of 1,000 µL.
   NOTE: The assay primers whose target miRNAs can be detectable in subsequent qPCR without pre-amplification, and those for cel-miR-238 (external control) are not included in primer pool.
4. Prepare pre-amplification reaction mix: 12.5 µL of 2x ready-to-use preamplification reagent, 3.75 µL of assay primer pool (from step 4.3), and 6.25 µL of nuclease-free water.
5. Mix 22.5 µL of pre-amplification reaction mix with 2.5 µL of reverse transcribed sample (from step 4.1) or oligonucleotides (from step 4.2) by pipetting, and incubate on ice for 5 min.
6. Run reaction on a thermal cycler apparatus: 95 °C for 10 min; followed by 12 cycles of 95 °C for 15 s and 60 °C for 4 min. Store pre-amplified samples at -80 °C until use.

5. Quantitative Real-time PCR (qPCR)

1. Thaw frozen samples (from step 3.6 or step 4.6).
2. Dilute the samples 5-fold with sterile water.
3. Prepare 10-fold serial dilutions of the RT product derived from synthetic RNA oligonucleotides; 1 x 10^7 to 1 x 10^2 copy/µL (*Not* pre-amplified samples) or 1 x 10^5 to 1 x 10^0 copy/µL (pre-amplified samples) for generating standard curves.
4. Prepare qPCR reaction mix: 10 µL of 2x ready-to-use amplification reagent, 1 µL of 20x assay reagent, containing forward/reverse PCR primer and probe corresponding to target miRNA, and 7 µL of nuclease-free water.
5. Transfer 18 µL from the qPCR reaction mix to the fast optical 96-well reaction plates, and add 2 µL of the diluted samples (from step 5.2 or step 5.3) into the wells.
   NOTE: Samples and standards for qPCR are set up in duplicates.
6. Seal the plate with adhesive film, and centrifuge briefly.
7. Run reaction on a real-time thermal cycler: 95 °C for 20 s, followed by 45 cycles of 95 °C for 1 s and 60 °C for 20 s.

6. Data Analysis

1. Compute the raw copy number of each sample using data analysis software that works with corresponding real-time thermal cycler.
   NOTE: Threshold line is set manually to "1.0" in all plates in the study, to confirm the reproducibility by comparison with their Cq vales. Cut-off level is set at Cq >40 cycles.
2. Calculate the average of the raw copy number of each sample from duplicates.
3. Calculate the correction factor by dividing a copy number of cel-miR-238 in each sample by the average of copy number of cel-miR-238 from all samples in a corresponding tube.
   NOTE: As shown in **Figure 2**, each tube contains up to 4 target miRNAs including cel-miR-238 (external control). Therefore, correction factor is calculated for each tube using corresponding cel-miR-238 value.
4. Calculate the adjusted copy number by dividing the averaged raw copy number of each sample (from Step 6.2) by the correction factor (from Step 6.3) for each sample.
5. Calculate the absolute copy number by multiplying the adjusted raw copy number of each sample (from Step 6.4) by the the dilution factor for each sample.
   NOTE: The dilution factor is "5" in this protocol, which is derived from Step 5.2.
6. Calculate the efficiencies of PCR amplification from the slope of the plot of the Cq values for each serial dilution against log cDNA concentration.
   NOTE: Formula for calculating the efficiency; E = $(10^{(-1/slope)} -1) \times 100$ %.
7. Calculate the technical variation using the Cq values of cel-miR-238 from all samples using the formula E = (2 x amplification efficacy)$^{\Delta Ct(Max(Cq)-Min(Cq))}$.
   NOTE: Steps 6.5 and 6.7 are used for quality assessment of the procedure.

Representative Results

Workflow of miRNA assay by RT-qPCR and quality assessment

Figure 1 shows the workflow of miRNA assay from blood samples using qPCR[10]. The quality of the experiments can be verified by including cel-miR-238 as an external control. This will reveal technical variations in RNA extraction and subsequent RT-qPCR processes. In this study, the mean ± SD of the Cq values computed from 50 samples was 21.0 ± 0.4 (**Table 1**). If the cel-miR-238 was diluted because of the pre-amplification step, the Cq value was 24.2 ± 0.4 (**Table 1**). In the assay method described here, the extent of technical variation was estimated to be less than 3-fold based on the difference in the Cq values. The degree of variation stayed consistent even when additional pre-amplification step had to be included. These values are compatible with those for other samples from different animal species, such as mice, rats, and humans performed in this laboratory (data not shown).

Detectable range from standard curve for target miRNA

No pre-amplification of the samples

The reverse transcribed (RT) product from synthetic RNA oligonucleotides at concentrations of 1 x 10^7 copy/µL was serially diluted prior to subsequent qPCR. These series were used for generating standard curve which ensured consistent coverage of all biological samples that were detectable without pre-amplification step. The Cq values of the standard sample at 1 x 10^2 copy/µL concentration were generally close to the cut off levels for each target miRNA. Therefore, the detection limit was estimated to be 1 x 10^2 copy/µL (**Figure 3**).

Pre-amplified samples

To determine the detectable range of samples requiring pre-amplification, two different series of diluted samples were compared, as illustrated in **Figure 4**. For generating standard curve A, serial dilutions of the RT product prior to pre-amplification step were prepared. Then the series of pre-amplified standards were used for subsequent qPCR. For generating standard curve B, the first dilution was made of pre-amplified samples to a concentration of 1 x 10^5 copy/µL. These standard curves showed apparently different detection limits (**Figure 5**). Although standard curve B showed linear range of increase down to 1 copy/µL, standard curve A could not be used for specific amplicons at concentrations less than 10^2 or 10^3 copy/µL (**Figure 5**). This result suggested that extremely small quantities of miRNAs cannot be assessed even with pre-amplification step. In addition, the pre-amplified samples should be interpreted carefully when the Cq values are >30, because the detection limits of the pre-amplified samples were close to this value. Therefore, as a rule, standard curve B was used in the method described here, after determining the detectable range, only in order to avoid using insufficient number of standard plots.

Consequently, the lower limit of quantification (LLOQ) was 10^2 copy/µL for most of the miRNAs (**Figure 3** and **Figure 5**). Only miR-1 showed a higher LLOQ (10^3 copy/µL) (**Figure 5**). The average amplification efficiency was approximately 90%, with the correlation coefficient (R^2) of the standard curves ranging from 0.998 to 1.000. The hallmarks of an accurate RT-qPCR assay are considered to be a linear R^2 equal to or greater than 0.98 and a PCR amplification efficiency of 90 to 110%[17]. Therefore, the quality of the standard curves in the assay was verified.

Effects of Pre-amplification for small quantities of miRNAs

The miRNAs that showed Cq values above 35 or higher in subsequent qPCR were pre-amplified. This procedure enhanced the detection of small quantities of miRNAs. For example, the Cq values (Mean ± SD) of non-pre-amplified miR-206 was 37.9 ± 1.9, while those of pre-amplified miR-206 decreased to 27.0 ± 2.2. Significant differences between duplicate measurement pairs were observed in non-pre-amplified samples at high Cq values (**Figure 6**). In contrast, pre-amplified samples showed almost equal values in duplicates (**Figure 6**). These results indicated that pre-amplification would be effective for providing more accurate and reliable data in cases of small quantities of samples.

Profiling of plasma miRNAs in cynomolgus monkeys

Using the established assay platform, plasma levels of 8 miRNAs (miR-1, miR-122, miR-133a, miR-192, miR-206, miR-208a, miR-208b, and miR-499a) from 50 cynomolgus monkeys were analyzed (**Figure 7**)[10]. In this assay, miR-122, miR-133a, and miR-192 were detectable without pre-amplification, whereas miR-1, miR-206, and miR-499a required pre-amplification because of their low expression levels. However, neither miR-208a nor miR-208b was detectable even with pre-amplification. The data were not normally distributed; therefore, a logarithmic transformation was performed to calculate their mean and standard deviations. Among the miRNAs examined, miR-122 showed the highest mean plasma level (5.71 x 10^4 copy/µL), with a small dynamic range (20-fold). In contrast, large dynamic ranges were observed for miR-1 (581-fold), miR-133a (971-fold), and miR-206 (426-fold).

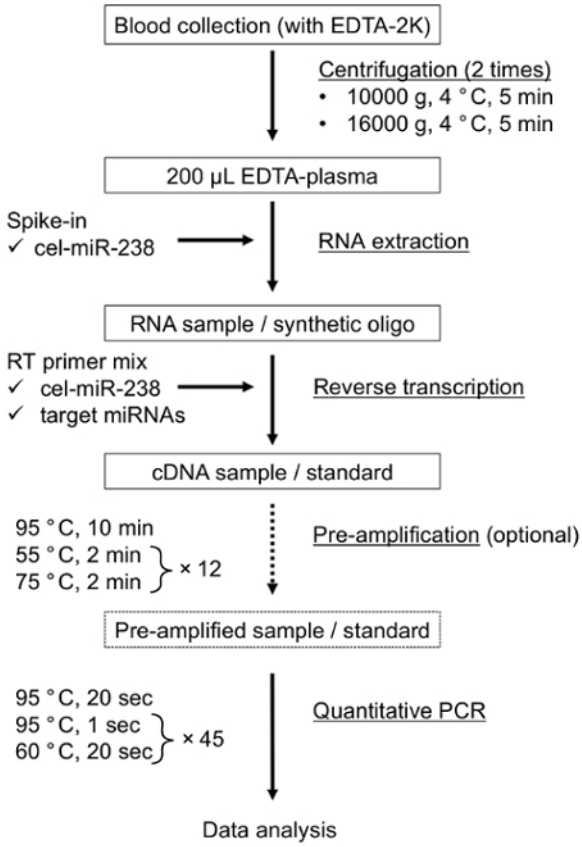

Figure 1: Schematic workflow of miRNA assay by RT-qPCR. Please click here to view a larger version of this figure.

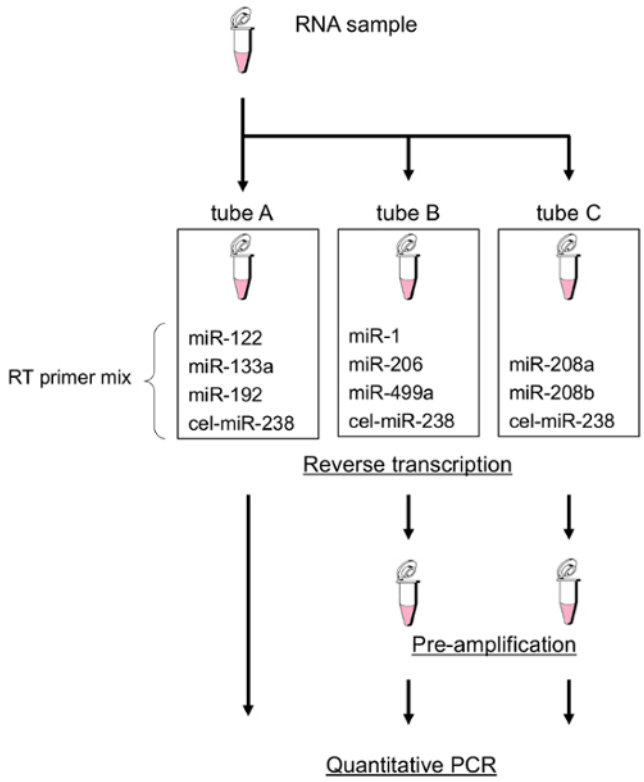

Figure 2: Schematic workflow of reverse transcription of miRNA assay. A pool containing up to 4 target miRNAs can be made by mixing the 20x RT primers of each of the miRNAs in the pool. Cel-miR-238 (external control) must be included as one of the target miRNAs in each tube. Please click here to view a larger version of this figure.

	miR-238 data analysis	
	Without Pre-amplification	**With Pre-amplification**
N	50	50
Mean	21.0	24.2
SD	0.4	0.4
Max Cq	21.8	25.3
Min Cq	20.3	23.5
Median	21.0	24.2
Max-Min	1.3	1.8
Amplification efficacy	89	89
Variation	2.4	2.8

Table 1: Quality assessment using quantification cycle (Cq) values of cel-miR-238. Technical variations were evaluated from the Cq values of miR-238 in each assay. Fifty samples were divided into two groups corresponding to with (Right) or without (Left) the pre-amplification step. Variation was calculated using the formula E = (2 x amplification efficacy)$^{\Delta Ct(Max(Cq)-Min(Cq))}$. The amplification efficacy was calculated from the slope of the standard curve. The samples requiring pre-amplification step were diluted during the pre-amplification step (miR-238 itself was not pre-amplified). This figure has been modified from our report[10].

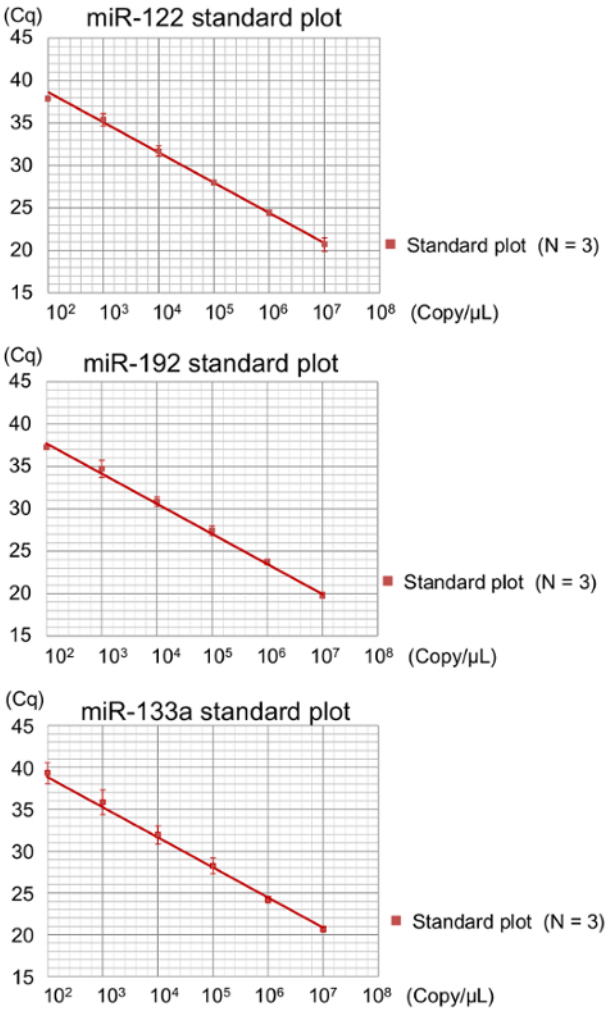

Figure 3: Plot of standard curves for non-pre-amplified samples. Standard curves (N = 3, duplicate) were generated by plotting the log concentration versus Cq. Linear regression analysis was computed to determine the slope, which corresponds to the amplification efficiency. Standard curves for miR-122 (Upper), miR-192 (Middle), and miR-133a (Lower) showed linear relationship between Cq and concentration at the tested range. In the standard curves, error bars represent one standard deviation from three independent assays. Please click here to view a larger version of this figure.

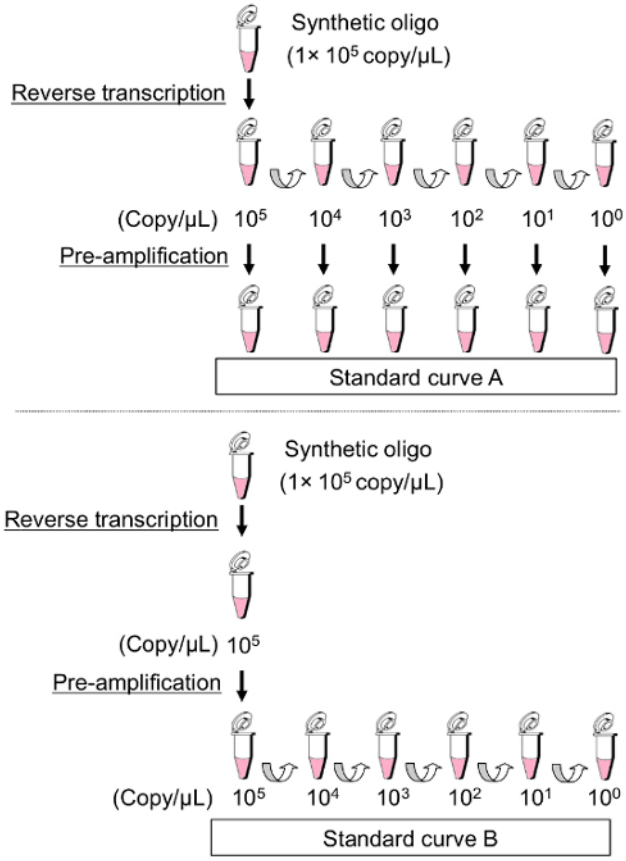

Figure 4: Procedure to generate standard curves for pre-amplified samples. (**Standard curve A)** A representative concentration of synthetic oligo (1 x 10^5 copy/μL) was reverse transcribed, followed by preparing 10-fold serial dilution series of the standard sample. These pre-amplified standards were used for subsequent qPCR. (**Standard curve B)** A representative concentration of synthetic oligo (1 x 10^5 copy/μL) was reverse transcribed and pre-amplified. Then, a 10-fold serial dilution series of standard samples were prepared prior to subsequent qPCR. Please click here to view a larger version of this figure.

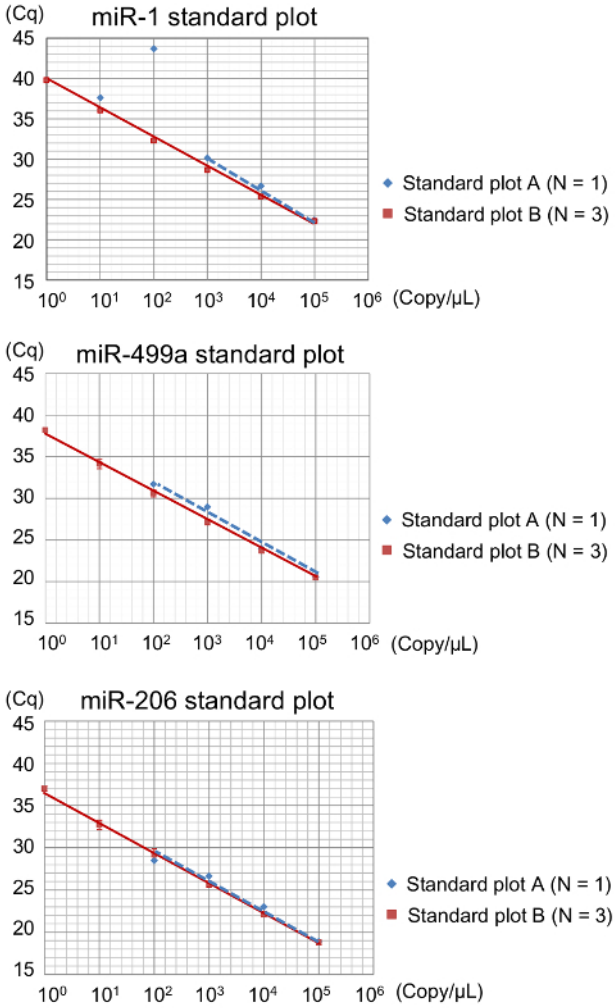

Figure 5: Plot of standard curves A and B for pre-amplified samples. Standard curve A (n = 1, duplicate) and Standard curve B (n = 3, duplicate) were generated by plotting the log concentration versus Cq. Linear regression analysis was computed to determine the slope, which corresponds to the amplification efficiency. **(Upper)** Standard curve A (*dotted line*) showed apparently non-specific amplicon at 1 x 10^2 copy/μL, whereas standard curve B (*solid line*) showed linear relationship between Cq and concentration at the tested range of miR-1.**(Middle** and **Lower)** Standard curve A (*dotted line*) showed no amplicon at concentrations of 1 x 10^2 copy/μL or lower, whereas standard curve B (*solid line*) showed linear relationship between Cq and concentration at the tested range of miR-499a and miR-206. In the standard curve B, error bars represent one standard deviation from three independent assays. Please click here to view a larger version of this figure.

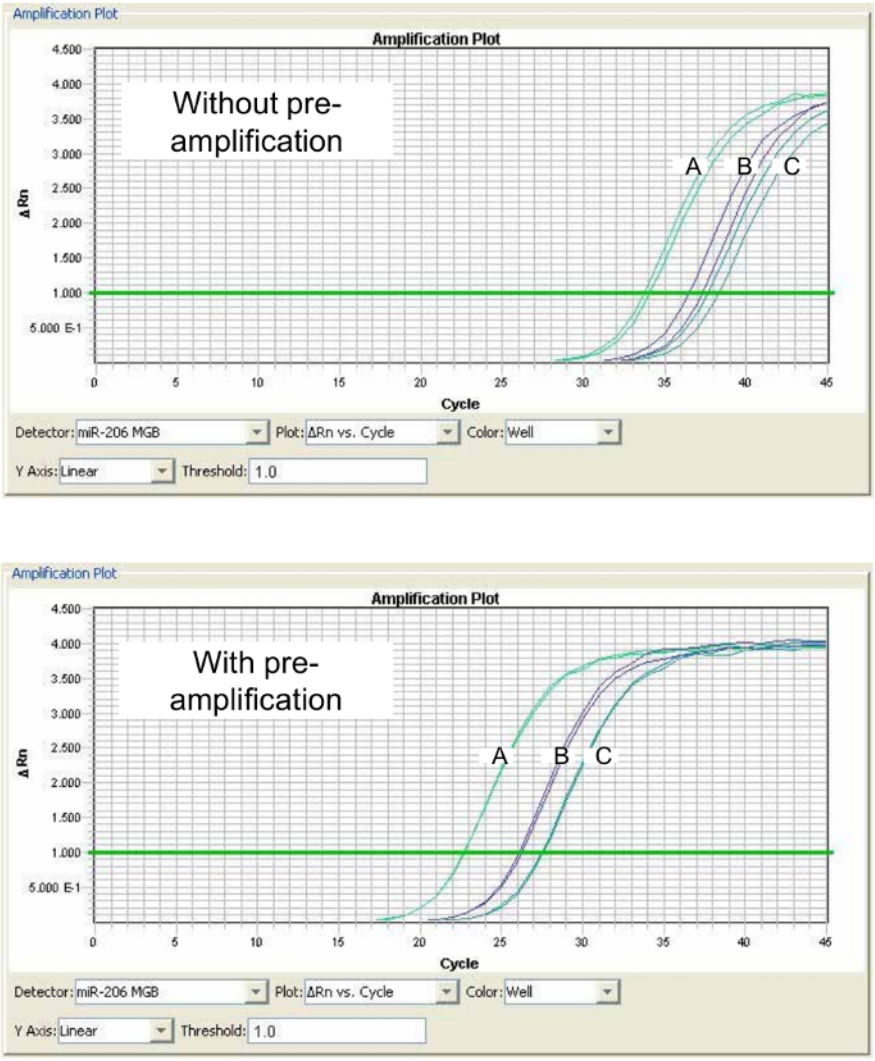

Figure 6: Amplification plot for non-pre-amplified and pre-amplified samples. (Upper) Amplification plot of miR-206 in representative samples (A, B, C) without (Upper), or with pre-amplification (Lower). Measurements were set up in duplicate. There were differences between the two samples set up as duplicates in non-pre-amplified samples, especially in higher Cq samples (A and B). Please click here to view a larger version of this figure.

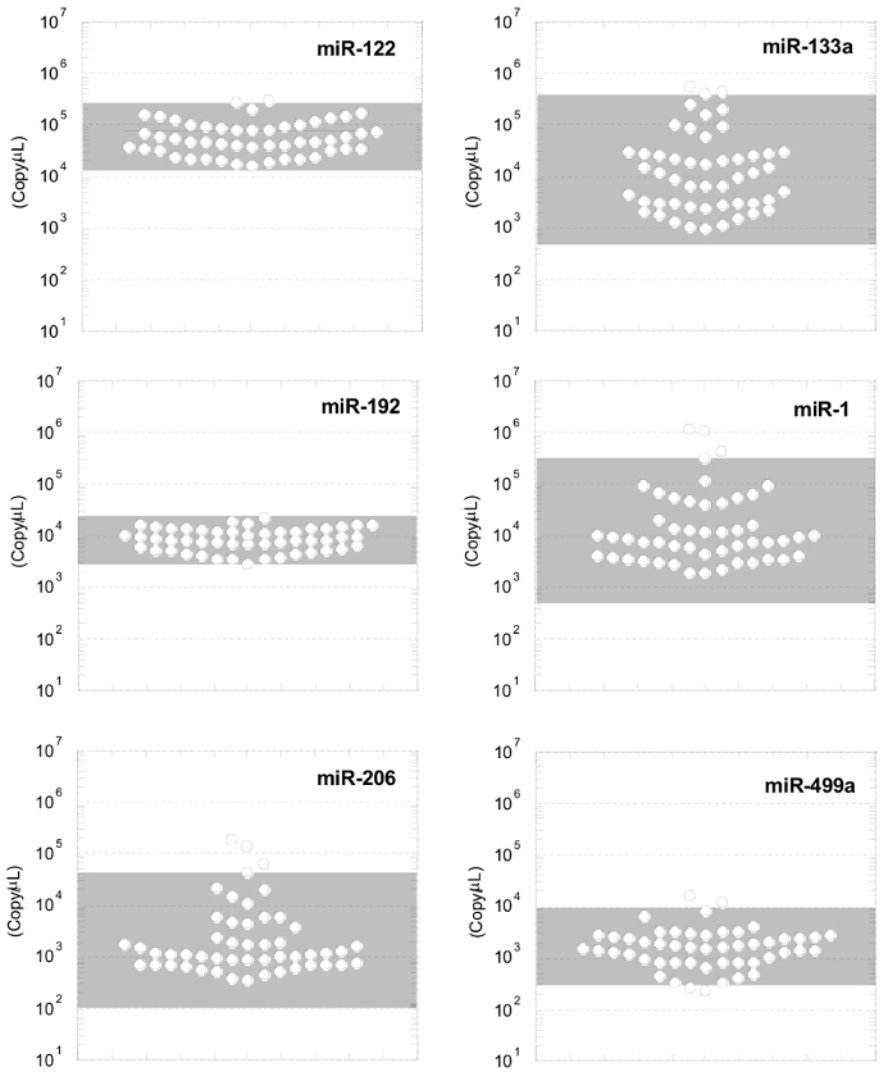

Figure 7: Absolute value of plasma microRNAs (miRNAs) in cynomolgus monkeys. The expression levels of the miRNAs are represented by dot plots. The mean, and the value for two standard deviations below and above the mean (shown in gray box), were determined after a logarithmic transformation. This figure has been modified from our report[10]. Please click here to view a larger version of this figure.

Discussion

Our comprehensive assessment provided a more rigorous statistical analysis of the extent of the dynamic range, which clearly indicated that the magnitude of variation between individual samples was extremely different among the miRNAs tested. Although these variations may be attributable to their small quantities in body fluids, it should be noted that these data reflect not only biological variations, but also technical variations. Most of the technical variation can be assessed by means of Cq values of the external control (cel-miR-238), which is used in other assay platforms[18]. Since there have been no standardized internal controls in miRNA analysis, the information on technical variation should be included in the analysis, for determining the quality of the assay[19]. Technical variation was estimated to be less than 3-fold in the method described in this report. Although it is not possible to determine the quality level for this procedure because of the lack of comparable technical information in other research, including such information contributes to improving the reliability and ensures compatibility between different studies.

A number of recent papers have reported that various pre-analytical variables such as sample handling, storage conditions, and storage duration before processing can impact the reliability and reproducibility of circulating miRNA measurements[20,21]. Although this study did not take into consideration the effects of pre-analytical variables, the following steps were followed carefully during sample preparation to minimize variations. First, plasma was processed as quickly as possible (within 2 hours post-collection) to minimize the effect of the stability of miRNAs in plasma[8,22]. Although miRNAs are considered to be stable at room temperature (15 to 25 °C), it is still unclear whether the interval between blood collection and processing of plasma or serum affects miRNA levels. Second, each plasma sample was visually inspected to eliminate analytical bias stemming from contamination with hemolyzed red blood cells, which represents one of the confounding factors that might affect the levels of circulating miRNAs[23].

Pre-amplification is useful to improve the detection of extremely small quantities of miRNAs in biofluids without introducing a detection bias[24]. In the present study, the Cq values of the standard curves constructed using pre-amplified synthetic oligonucleotides showed high reproducibility across the assays. This allowed absolute quantitation using pre-amplified samples, which enabled the detection of small quantities of miRNAs. On the other hand, low copy number standards (1 x 10^2 or 10^3 copy/µL) do not show specific amplicons, in combination with pre-amplification. Mestdagh *et al.*[24] reported that measurement of low copy number miRNAs showed higher variation after pre-amplification procedure, compared to moderate or high copy number miRNAs, due to the low efficiency of reverse transcription, which could be the result of miRNA sequence characteristics. Therefore, the detection range of standard curves for each miRNA must be validated prior to quantification of biological samples, especially for pre-amplified miRNAs, to eliminate false-positive results. More importantly, it must be noted that the absolute levels of differently processed miRNAs such as pre-amplified and non-pre-amplified samples cannot be directly compared because the quantity of pre-amplified sample is not specified in each miRNA.

This report describes a procedure for the determination of absolute miRNA levels in plasma samples using RT-qPCR. Some miRNAs were not detected even after pre-amplification. To detect such low expression miRNAs, a different approach may have to be used. Although using a larger sample quantity may circumvent this problem as a simple solution, large quantities of sample are not always available. To date, technological advances have enabled the development of various platforms for miRNA profiling[25,26]. Droplet digital PCR (ddPCR) has been developed recently and offers an alternative method to conventional RT-qPCR for absolute quantification. This innovative technology has been reported to be a precise, reproducible, and sensitive method that can detect a target miRNA at levels of 1 copy/µL[27,28]. If ddPCR can detect a low concentration of miRNAs without a pre-amplification step, it would be a great advantage because the levels of multiple miRNAs can be compared to each other. Changes in extraction procedures and subsequent RT-qPCR, molecular platform, and reagents used will inevitably yield variable results. However, these variations can be resolved if transparent results of external controls and absolute levels for individual miRNAs are provided. Appropriate quality assessment of the method is the key to improving the discernment of biological changes in miRNA profiling studies.

Disclosures

The author has no conflict of interest to disclose.

Acknowledgements

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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