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Detectability of circulating microRNAs in microRNA extracts with low purity and yield using quantitative real-time polymerase chain reaction: Supporting evidence

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Abstract

Background: Circulating microRNAs (miRNAs) are a group of noncoding RNAs with promising potential as minimal invasive biomarkers for noncommunicable diseases. However, challenges exist in the preparation of these miRNAs from peripheral blood samples for quantification purposes. The low quality of miRNA extracts presents an obstacle. Acknowledging the superior performance of quantitative real-time polymerase chain reaction (qPCR) as gold standard for gene expression analysis, we conducted this study to observe the capabilities of qPCR using the Taqman® protocol in amplifying circulating miRNAs from miRNA extracts with low purity and yield. **Methods**: miRNAs were extracted from thirty-six plasma samples that were obtained from public subjects. Four selected miRNAs were quantified using the Taqman® protocol in an integrated fluidic circuit chip that was optimized from a previous study. The amplification graph and Cq values were obtained to observe any abnormal amplification signs and expression levels, respectively. **Results**: The qualitative observation of the amplification of the four miRNAs showed no sign of abnormality, thereby indicating the successful amplification of the miRNAs without enzymatic inhibition. Furthermore, the miRNAs were quantified in high expression levels. **Conclusion**: The circulating miRNA extracts with low purity and yield were practical for the study of circulating miRNA expression based on the Taqman® protocol as the method of detection.

Keywords: amplification, circulating, isolation and purification, microRNA, quantitative real-time polymerase chain reaction

Introduction

First found in *Caenorhabditis elegans* in Victor Ambro's laboratory in 1993, microRNA (miRNA) has been extensively studied, with current research encompassing its chemical structure, biogenesis, functions, and clinical utility for diagnostic, prognostic, and therapeutic purposes.[1–3] miRNA is a class of short noncoding RNAs that mainly regulate protein expression at the post-transcriptional level in the cytoplasmic region, and

growing evidence suggests that it may also regulate gene expression at the pre-transcriptional level by binding to the gene promoters in nuclear regions.[4–6] miRNAs are found not only in intracellular environments but also in extracellular environments, such as blood and saliva.[7,8] miRNAs are transported out from cells mainly by exocytosis, and they are either encapsulated in or free from vesicles for cell–cell communication.[9]

A growing body of evidence demonstrates the promising utility of circulating miRNAs as biomarkers of noncommunicable diseases, but their pre-analytical processing and detection still suffer from several challenges.[10] Despite the good number of scientific publications about issues and improvements in certain pre-analytical parts for the study of circulating miRNAs, such as sample processing and circulating miRNA extraction, no study has reported the suitability of utilizing circulating miRNA extracts with low purity and yield in detecting miRNA using quantitative real-time polymerase chain reaction (qPCR).[11–14] qPCR is a gold standard for gene expression analysis because of its superior specificity and sensitivity in detecting diseases.[15,16] The improvement in qPCR has made this method capable of detecting DNA and RNA in low concentration samples.[17,18] Moret *et al*.[19] expressed their concern about the use of plasma samples for miRNA detection as such samples could affect qPCR efficiency through their low level, short length, and carried-over inhibitors during extraction. Nevertheless, no study has reported the hampered efficiency of qPCR by using such miRNA extracts. Moret *et al*.[19] did test the detection efficiency of circulating miRNA extracts, but they used the microarray method of detection and not qPCR. Through the revolutions in qPCR, this research tool can now compete with commercial high-throughput platforms, such as microarrays and next-generation sequencing, in terms of detecting the abundance of genes simultaneously even with a low input of starting materials.[20,21] In the present study, we showed that the detection of three circulating miRNAs in miRNA extracts with low purity and yield was possible by using qPCR. Moreover, the expression of these miRNAs was high in miRNA extracts with low purity and yield. Hence, qPCR is suitable for the study of circulating miRNA expression.

Methods

Subject recruitment. The protocol was reviewed and approved by the IIUM Research Ethics Committee (IREC 457), Human Research Ethics Committee of Universiti Sains Malaysia (USM/JEPeM/16010032), and Medical Review and Ethics Committee (NMRR15-1976-27156). Thirty-six subjects in the Pahang and Kelantan states of Malaysia were recruited randomly. Signed consent forms were obtained from the subjects who volunteered to participate in the study. The personal details of the subjects were kept confidential, and their names were replaced with identification numbers. During the recruitment, demographic data were taken from each participant.

Plasma sample collection. Exactly 10 mL of blood was drawn from the antecubital vein of each subject by using a syringe and 21-gauge needle. The blood was collected into a blood collection tube containing ethylenediaminetetraacetic acid, kept in ice, and processed within 4 h after collection. Plasma was obtained by centrifuging the blood for 15 min at 1200 × g at room temperature and then stored in a freezer at −80 ℃ until further use.[22]

Circulating miRNA extraction. The miRNAs from the plasma samples were extracted using the NucleoSpin® miRNA plasma kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol with a few modifications by Wozniak *et al*.[23] In the miRNA extraction, the plasma was subjected to Proteinase K treatment to digest protein and thereby increase the miRNA yield. The supernatant obtained after the Proteinase K treatment was spiked-in with 10 pmol mirVana™ ath-miR-159a mimic (Thermo Fisher Scientific, Massachusetts, United States) for quality control of the miRNA isolation efficiency. Exactly 2 µg of glycogen (Roche, Basel, Switzerland) was then added as an RNA carrier to improve miRNA binding to the NucleoSpin® miRNA column. DNA digestion was performed after filtration of the supernatant to prevent the interference of co-purified DNA during the quantification of miRNAs using 50 µL of rDNase. Then, the column was washed with the washing buffers of the kit prior to elution using RNase-free water that was preheated to 56 ℃ to improve the elution efficiency. The purity and RNA yield of the miRNA extracts were measured spectrophotometrically using NanoDrop™ 1000 (Thermo Fisher Scientific, Massachusetts, United States).

Reverse transcription reaction. In reverse transcription (RT) reaction, fixed volume was used as the starting material rather than the equal quantity of miRNA as it is recommended for samples that could not be quantified spectrophotometrically, such as plasma.[24,25] In addition to the spike-in miRNA (i.e., ath-miR-159a), three circulating miRNAs previously reported to be present in peripheral blood, namely, hsa-miR-16, hsa-miR-93, and hsa-miR-191, were used for detection.[26–31]

The master mix for RT reaction was prepared by mixing 37 µL Multiscribe™ RT enzyme (50 U/µL), 37 µL 10× RT buffer, 7.4 µL dNTP mix (100 mM), 5.55 µL RNase inhibitor (20 U/µL), and 111 µL 0.156× RT primer pool (consisting of ath-miR-159a, hsa-miR-16, hsa-miR-93, and hsa-miR-191). Then, 5.35 µL of the RT master mix was mixed with 4.65 µL of samples in PCR tubes,

including 4.65 µL of nuclease-free water for no template control (NTC) for RT reaction (NTC RT). The RT reaction was performed in a ProFlex PCR System (Applied Biosystems, California, United States) with the following settings: 16 ℃ for 30 min for the first stage, followed by 42 ℃ for 30 min and 85 ℃ for 5 min. Once the reaction was completed, the RT products were diluted with nuclease-free water with a ratio of 1:4.

Preamplification reaction. The master mix for preamplification reaction was prepared by mixing 185 µL Taqman® PreAmp Master Mix (2×) and 92.5 µL 0.2× preamplification primer pool (consisting of ath-miR-159a, hsa-miR-16, hsa-miR-93, and hsa-miR-191). Then, 7.5 µL of the master mix was mixed with 2.5 µL of the diluted RT products, including the NTC RT product to make an NTC for preamplification (NTC PA), in PCR tubes. The preamplification reaction was performed in the ProFlex PCR System (Applied Biosystems, California, United States) with the following settings: 95 ℃ for 10 min for the first stage, followed by 95 ℃ for 15s, and 60 ℃ for 4 min in the second stage for 16 cycles. Once the reaction was completed, the preamplification products were diluted with nuclease-free water with a ratio of 1:5.

Quantitative real-time polymerase chain reaction. A Dynamic Array™ integrated fluidic circuit (IFC) chip (Fluidigm, California, United States) was used for the qPCR assays. Before preparing the qPCR assay, control line fluid was injected into the accumulators on the IFC chip, followed by loading into the IFC controller (Fluidigm, California, United States). The "Prime" script was subsequently run to pump the fluid into the microfluidic channels of the IFC chip.

For the qPCR procedure, two 96-well plates were used as sample and assay plates to prepare the master mix preparation. For the sample plate, a master mix of premixed samples was prepared for 37 reactions by mixing 92.5 µL of Taqman® Universal Master Mix (2×) with 9.25 µL of GE sample loading reagent in a 1.5 mL tube. Then, 4.4 µL of the master mix was mixed with 3.6 µL of the diluted preamp products, including the NTC PA product to make an NTC for PCR (NTC PCR), in each well of the sample plate. For the assay plate, the assay master mix was prepared for triplicate reactions by mixing 7.5 µL of Taqman® Assay real-time primer (20×) with 7.5 µL of assay loading reagent in a qPCR tube for each primer.

In using the IFC chip, either the sample inlets or the assay inlets need to be replicated. Once the samples and assays are pumped from their inlets into a reaction chamber using the IFC controller (Fluidigm, California, United States), replication occurs in the reaction chamber. In the current study, the assays were replicated thrice. Exactly 5 µL of the sample master mix from the sample plate was dispensed in the sample inlets on the chip. Then, 5 µL of the assay master mix was dispensed in the assay inlets in triplicate. The chip was subsequently loaded into the IFC controller (Fluidigm, California, United States), and the "Load Mix" script was run to pump the samples and assays to the reaction chamber. The chip was then loaded into a Biomark™ HD system (Fluidigm, California, United States) for qPCR based on the default protocol.

Data collection and calculation. The graphs of amplification curves were obtained by importing the raw data from the Biomark™ HD system (Fluidigm, California, United States) to the Fluidigm Real-Time PCR Analysis software (Fluidigm, California, United States). The same software was also used to calculate Cq under auto-threshold and auto-baseline settings prior to further analyses. The mean Cq, standard deviation (SD), and coefficient of variation (CV) were calculated using Microsoft Excel.

Results

Table 1 summarizes the demographic data of the subjects. The mean age was 50.4 ± 15.3.

Table 1. Descriptive demographic data of subjects

Variables	Frequency
Sex	
Male	26
Female	10
Race	
Malay	28
Chinese	7
Aborigine	1

Purity and yield of plasma miRNA extracts. The spectrophotometric results (Table 2) showed the presence of miRNAs with low purity and yield in the miRNA extracts. As observed in the absorbance graph in Figure 1, an apparent high peak emerged around a wavelength of 230 nm, and it indicated the high concentration of contaminants that absorbed light at such wavelength. Meanwhile, the peaks at 280 nm were not as apparent as the peak at 230 nm. Although all miRNA extracts showed low values for the 260/230 and 260/280 ratios, the trends reflected in the graph were consistent across the samples.

Amplification curves of ath-miR-159, hsa-miR-16, hsa-miR-93, and hsa-miR-191. The amplification curves of ath-miR-159a, hsa-miR-16, hsa-miR-93, and hsa-miR-191 in all the plasma miRNA extracts showed the expected normal sigmoid property. Moreover, no abnormality in the shape of the amplification curves was observed (Figure 2). No hook effect-like shape, an abnormal shape for a qPCR amplification curve, was

observed in the amplification curves, thereby indicating the successful amplification of miRNAs in the experiment. These results indicated that the circulating miRNA extracts were of good quality and could be used for qPCR applications.

Cq data of ath-miR-159, hsa-miR-16, hsa-miR-93, and hsa-miR-191. The Cq data of the four miRNAs in the plasma miRNA extracts are presented in Table 3 and Figure 3. The qPCR results showed a high expression of all the tested miRNAs in all the plasma miRNA extracts. Moreover, the SD for all miRNAs was not too large to show outliers that might indicate interference during the reaction. Table 3 shows that the CV of ath-miR-159a was lower than those of hsa-miR-16, hsa-miR-93, and hsa-miR-191. The spike-in of ath-miR-159a into the samples was conducted consistently across the samples.

Table 2. Purity and miRNA yield values of plasma samples for validation

Sample	260/230 ratio	260/280 ratio	miRNA yield (ng/µL)
CP5	0.17	0.95	3.2
CP9	0.14	0.94	4.8
CP17	0.18	0.86	4.3
CP18	0.10	0.54	2.7
CP19	0.13	0.75	4.5
CP21	0.15	0.75	2.9
CP22	0.21	0.94	6.3
CP23	0.15	0.78	8.8
CP24	0.16	0.79	2.0
CP25	0.14	0.93	5.3
CP29	0.10	0.58	1.1
CP30	0.07	0.87	1.8
CP31	0.19	0.83	4.5
CP32	0.20	1.01	8.6
CP35	0.20	1.11	6.3
CP37	0.19	1.06	7.9
CP40	0.22	1.08	9.5
CP43	0.12	0.61	2.6
CP49	0.14	0.69	2.2
CP50	0.13	0.65	2.1
CP52	0.18	0.80	3.4
CP51	0.12	0.64	2.2
CP53	0.02	0.54	0.9
CP57	0.12	0.68	5.6
CP58	0.17	0.81	5.3
CP59	0.13	0.85	4.8
CP60	0.13	0.71	3.1
CP64	0.13	0.67	2.9
CP66	0.17	0.88	5.6
CP74	0.18	1.22	3.2
CK7	0.23	0.97	8.0
CK9	0.08	0.77	2.3
CK10	0.15	0.83	2.0
CK11	0.13	0.72	2.4
CK12	0.18	0.72	4.8
CK18	0.15	0.72	4.3

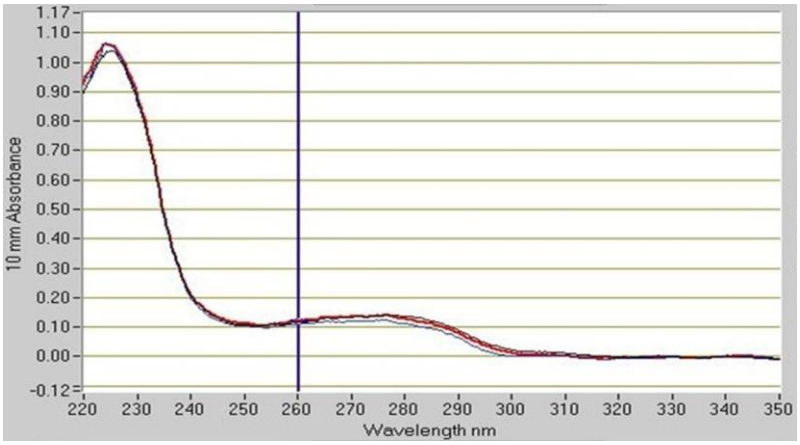

Figure 1. Example of absorbance graph produced by NanoDrop™ 1000 for miRNA extracts from plasma samples in the current study

Amplification curves from the current study

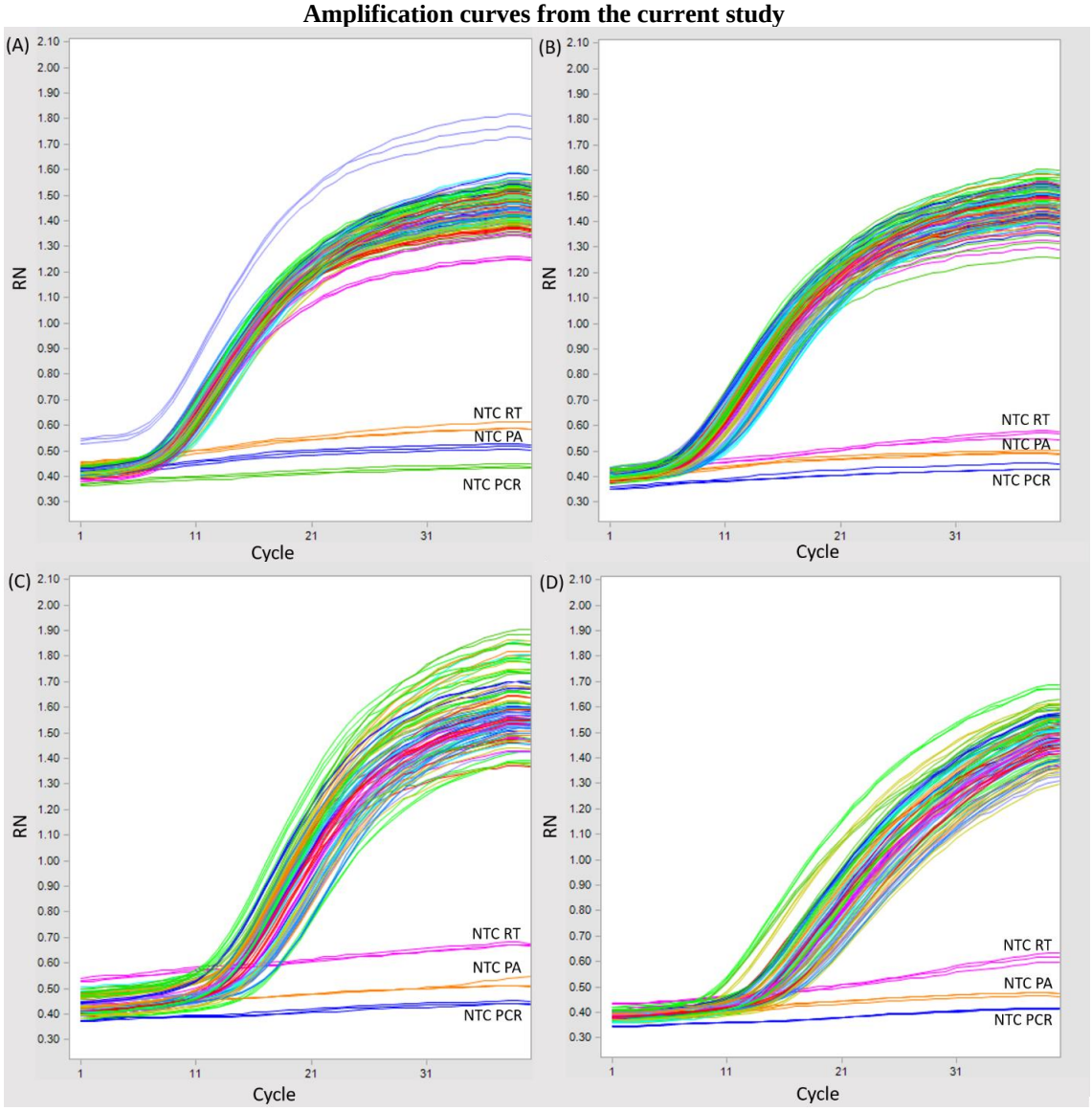

Figure 2. Amplification curves of (A) ath-miR-159a, (B) hsa-miR-16, (C) hsa-miR-93, and (D) hsa-miR-191

Table 3. Cq data for ath-miR-159a, hsa-miR-16, hsa-miR-93, and hsa-miR-191

miRNAs	Cq (Mean ± SD)	CV
ath-miR-159a	6.765 ± 0.656	0.097
hsa-miR-16	6.739 ± 1.077	0.160
hsa-miR-93	12.045 ± 1.490	0.124
hsa-miR-191	12.981 ± 1.951	0.150

Cq values of ath-miR-159a, hsa-miR-16, hsa-miR-93, and hsa-miR-191

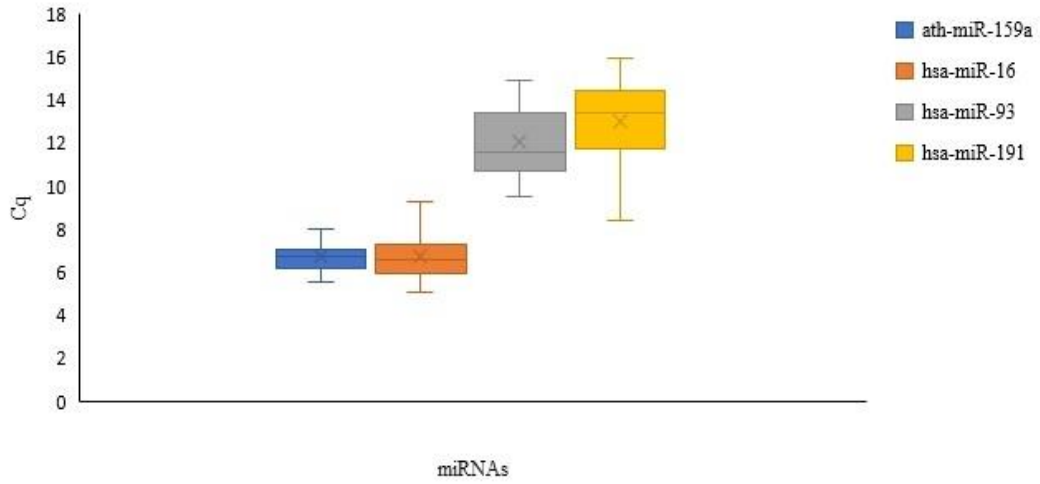

Figure 3. Box plots for ath-miR-159a, hsa-miR-16, hsa-miR-93, and hsa-miR-191. Cq: quantification cycle

Discussion

Low purity plasma RNA extracts are a cause for concern because blood samples, especially those drawn from patients, are precious. Not all patients are willing to donate blood while sick. Moreover, blood withdrawal from elderly patients is not as easy as that from younger patients. Given the low miRNA level in peripheral blood, such blood samples may become highly contaminated with organic and inorganic materials, as determined by spectrophotometry measurement, after extraction. Hence, samples are discarded as they are presumed not suitable for some laboratory procedures. Owing to the superior performance of qPCR as a gold standard for gene expression analysis, we were interested to observe the capability of qPCR in detecting miRNA in miRNA extracts with low purity and yield.

In the current study, all miRNA extracts from the plasma samples showed miRNAs with low purity and yield. The 260/230 and 260/280 ratios were extremely low, and they commonly indicate the presence of contaminants in higher amounts than the miRNAs.[32,33] Generally, this type of RNA extracts is unacceptable for several downstream applications, such as qPCR, microarray, and sequencing; hence, they were excluded from the study. The contaminants in RNA extracts, mainly organic substances, may be carried from samples or extraction reagents. These contaminants may cause RNA degradation, as well as mechanistic and enzymatic inhibition during RT or PCR steps, and thereby hamper qPCR.[34–36] Among the recognized organic PCR contaminants that are commonly present in DNA or RNA extracts from blood samples are salts; phenol; ethanol; and variety of proteins, such as hemoglobin and immunoglobulin G, which may originate from samples or extraction reagents.[36,37] Apart from the inhibitory nature of certain substances on PCR, some substances (e.g., sodium hydroxide and ethylenediaminetetraacetic acid [EDTA]) only cause the inhibitory effect when they reach certain concentrations.[38] Generally, these PCR inhibitors interfere with the reaction by targeting DNA polymerase, nucleic acids, and fluorophores.[34–36]

The absorbance graphs produced by NanoDrop™ 1000 from the miRNA extracts in this study were consistent with the graph by NanoDrop from a study by Moret *et al*.[19], who assessed the quality of plasma miRNA extracts from several miRNA extraction kits and indirectly validated the quality of circulating miRNA extracts. The apparent high peak at the 230 nm wavelength in the current study indicated the high concentration of contaminants that absorbed light at such wavelength, such as proteins, guanidine salt, EDTA, carbohydrates, lipids, and phenols.[33,39] The possible contaminants in the plasma miRNA extracts could have mainly originated from the sampling procedure, in which EDTA was used as the anti-

coagulant; and from the miRNA isolation procedure, in which glycogen and guanidine were used as the RNA carrier and in miRNA isolation reagents, respectively. However, protein contamination was supposed to be a minor issue in the study as the Proteinase K digestion treatment was implemented in the miRNA isolation procedure to reduce contamination.

The consistent trends reflected in the graphs across the samples made us speculate that they may be due to the nature of the samples. Furthermore, the results of this work are consistent with those of Moret *et al*.[19]. The generally low concentration of RNAs in peripheral blood, including miRNAs, as shown in the spectrometry results, could explain the purity of our miRNA extracts being as low as the purity of RNAs measured by NanoDrop™ 1000 using the ratio of nucleic acids to possible contaminants.[40–42] Therefore, a low numerator (260 for RNA) and a high denominator (230 and 280 for possible contaminants) would mathematically yield low 260/230 and 260/280 ratios. This result was in agreement with that of El-Khoury *et al*.[25], who reported that a low RNA concentration explains the low 260/230 and 260/280 ratios in low yield RNA extracts. Previous research has emphasized the inappropriateness of quantifying extracts with low nucleic acid concentration using spectrophotometry.[25,43,44] Furthermore, the miRNA isolation kit used in our work has been reported to result in better miRNA isolation performance than other commercial miRNA isolation kits.[45,46] The merit of such miRNA isolation kit is indisputable, and the isolated miRNAs are of good quality and applicable to downstream procedures. Hence, the suitability of miRNA extracts with low purity for qPCR procedures is worth assessing before considering the rejection of samples.

A previous study by El-Khoury *et al*.[25] reported the usability of miRNA extracts with low purity and yield for qPCR applications using quantitative analysis. Different from the previous study, the current study attempted to share the results qualitatively by observing the shapes of the qPCR amplification curves of the specific miRNAs in our plasma miRNA extracts. Relative to Cq values, which were considered in the work of El-Khoury *et al*.[25], amplification curves facilitate the direct assessment of the efficiency of qPCR based on their abnormalities. The current study demonstrated the capability of qPCR in efficiently amplifying and detecting ath-miR-159a, hsa-miR-16, hsa-miR-93, and hsa-miR-191 in miRNA extracts with low purity and yield from plasma samples on the basis of the normal sigmoid curves with no abnormal shapes, such as a hook effect-like shape. To the best of our knowledge, the hook effect-like shape (black-colored sigmoid curve in Figure 4) is among the reported abnormalities in the shapes of amplification curves, other than the absence of amplification signals.[47] This phenomenon occurs at the late cycle of PCR, in which

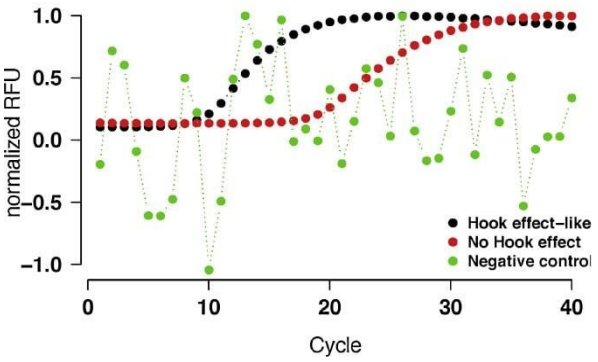

Figure 4. Hook effect-like shape of qPCR amplification curve.[49] RFU: relative fluorescence unit

the detection probes compete with the amplicons because of the increased concentration of the amplicon's nature at the late cycle and the consequent reduction in fluorescent signal detection.[47,48] This abnormal shape is an indicator of a failed experiment and can be subjected for exclusion from the study.[47] As shown in Figure 2, no hook effect-like shape was observed in the amplification curves of this study, thus indicating the successful amplification of miRNAs in the experiment.

The current study also reported the quantitative data of Cq values for each circulating miRNA. Overall, the Cq data showed that the circulating miRNAs measured in this study were high. The higher CV of the spike-in control, which was ath-miR-159a, in comparison with that of the circulating miRNAs indicated that the experiment was conducted well and that the qPCR reaction was efficient. The higher SD for hsa-miR-16, hsa-miR-93, and hsa-miR-191 in comparison with that for ath-miR-159a was due to the heterogeneous expression of the three miRNAs as endogenous miRNAs in human samples. The heterogeneity in human population samples and the release of miRNAs into peripheral blood were described by Wang *et al*.[49] and Sohel.[10]

Among the factors that contributed to the successful detection of circulating miRNAs in miRNA extracts with low purity and yield in this study was the use of the Taqman® protocol as a specific and sensitive detection method.[45,50] The design features of this protocol include the use of the stem-loop primer in the RT step, the addition of a preamplification step to pre-amplify RT products, and the use of a hydrolysis probe as detection chemistry during the qPCR step.[50,51] The stem-loop RT primer used in the RT step was demonstrated to be capable of discriminating the precursor and mature miRNAs and the related miRNAs that may differ by one nucleotide.[52,53] This factor was deemed to have contributed to the specificity of the Taqman® protocol.[53] The stem-loop RT primer was also highly sensitive in quantifying

low-abundance miRNAs.[52] The application of the preamplification step in detecting low-abundance miRNAs has been proven to increase the sensitivity of detection without introducing any systemic bias in the analysis of miRNA expression.[54] The preamplification step has also been optimized with an IFC chip to increase the sensitivity of detection for low-abundance miRNAs; hence, the step is considered suitable for circulating miRNA detection.[45,55] Meanwhile, the use of a hydrolysis probe, that is, the Taqman® probe, also contributes to the successful detection of low-abundance miRNAs due because of the sensitivity and specificity of the Taqman® probe in detecting DNA and RNA.[56–58] This detection probe has gained popularity among researchers because of its specificity in annealing to specific targets and its applicability to multiplex assays.[59,60] Considering the aforementioned body of evidence, we suggest that the Taqman® protocol is helpful in the detection of circulating miRNAs in miRNA extracts with low purity and yield.

We successfully demonstrated that with the use of the Taqman® protocol, circulating miRNAs can be amplified in miRNA extracts with low purity and yield without any enzymatic inhibition. The study employed an IFC chip as the microfluidic channel platform and used a small reaction volume of assay to test the qPCR amplification. Indirectly, the present study also showed that the circulating miRNAs in miRNA extracts with low purity and yield could be detected with high expression using a small reaction volume of qPCR assay that is commonly used in high-throughput platforms, provided that the Taqman® protocol is used. Regarding the quantity of RNA materials for RT reaction, a fixed volume of miRNA extracts was recommended.[24,25] Thus, miRNA extracts with low purity and yield are suitable for high-throughput platforms that use a small volume of qPCR assay and a high volume of single qPCR assay.

Conclusion

The evidence provided in this study builds a new perspective on the detection of circulating miRNAs in extracts with low purity and yield using qPCR. The unnecessary rejection of circulating miRNA extracts from any study because of their unsatisfactory purity and inadequate RNA yield could be avoided with consideration of how precious blood samples are. However, the current study employed one commercial miRNA extraction kit only. The test on several commonly used extraction kits in circulating miRNA research should generate a relatively generalized result on the application of qPCR for miRNA extracts with low purity and yield from different extraction kits. As no standardized miRNA extraction method for biofluid samples has been developed, the findings of our work are expected to aid, at least partly, in improving commercially available miRNA extraction methods and kits.

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Conflict of Interest Statement

The authors declare no conflict of interest.

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