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Original Article

A simplified and optimal RT-qPCR strategy for extracellular miRNA profiling

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Abstract: *Introduction:* Extracellular miRNAs in the blood plasma/serum of patients as novel promising diagnostic markers for various diseases has aroused huge attention. Among various major platforms for extracellular miRNA detection, extensively used RT-qPCR is considered practical and cost-efficient in experimental investigation. However, there are also many technical challenges, hence the development of robust methodology for extracellular miRNA detection is in good need. *Methods:* Focused on the controversial procedures present in recent studies, we intend to solving these technical problems and seeking for optimal procedures for extracellular miRNA profiling using the RT-qPCR method. *Results:* We addressed our concerns during the whole detection such as various key points during blood handling, choice making during RNA isolation and miRNA RT process, practical management in global miRNAs profiling using PCR as well as data normalization and processing. Meanwhile, we described and evaluated our profiling method in detail, intended to optimize the procedures that might lead to variable results from independent experiments, and further recommend this standardized optimal procedure. *Conclusion:* Our work might provide a potent technical optimization for extracellular miRNA detection.

Keywords: Extracellular miRNA, RT-qPCR, expression profiling

Introduction

The extracellular miRNAs were surprisingly found to survive with sufficient integrity in spite of the presenting of active ribonuleases in body fluids such as blood, urine and breast milk. Hence extracellular miRNAs in the blood serum/plasma of patients has aroused huge attention for their potentials as novel diagnostic markers for various diseases [1]. They can also help diagnose fatal diseases, predict patients' outcome more precisely or even serve as monitorable drug targets. Thank to extracellular miRNA study, emerging commercial diagnostic tests of even the cancer of unknown primary and the toughest pancreatic cancer are available now [2]. It is well considered that extracellular miRNA appeared to be promising for further biomarker identification [3]. In addition, secreted extracellular miRNAs are now considered as an important form of intercellular communication besides cell junction, adhesion, autocrine, paracrine and endocrine [4].

The bright future for extracellular miRNA detection was applauded far and wide. Robust methodology was in good need. There were various major platforms for extracellular miRNAs detection, such as microarrays, quantitative RT-PCR (RT-qPCR) and next-generation sequencing, which were widely used in recent researches. MiRNA microarray has been extensively used as a relative low-cost and high-throughput method for disease-specific miRNA detection, however the specificity is not desired and the data often need to be further confirmed by RT-qPCR [5]. The high-cost next-generation sequencing (deep sequencing) is able to offer

novel miRNA sequence results for miRNA database, and hence the detected miRNAs can be global and hypotheses free [6]. However, the required RNA start quantity for miRNA microarray and next-generation sequencing was relative massive. Taking plasma as example, the amounts of blood taken from individuals could reach up to more than 10 ml each time [7, 8], which were less easily acceptable for donors. In addition, considering on financial cost and the complexity of computational data processing, sample volume for both methods could not be plentiful. Application of both methods was preferred in comparison between sample from very limited individuals from control and disease condition which makes the results easily influenced by genetic heterogeneity and medicine history of patients, unless a pool sample was prepared [9]. RT-qPCR was more practical and cost-efficient for quantification of samples with large volume [10, 11]. However, there are also many technical challenges for extracellular miRNA detection using this method. As far as we can concern, differences of procedures during plasma collection, package storage, RNA isolation, expression normalization and data handling could all lead to variable results from one study to another [12]. In spite of these barriers, RTqPCR was still widely used during all times. Researchers were dedicated to solving these problems and seeking for optimal procedures.

Focusing on the controversial procedures present in recent studies, this study took human plasma specimen as an example, and attempted to establish and recommend a simplified and optimal RT-qPCR based strategy for extracellular miRNA quantification.

Materials and methods

Blood handing

In this study, human blood samples were donated by ten individuals who went for blood routine test in secondary afflicted hospital of Xi'an Jiaotong University. All blood samples were obtained in accordance with the declaration of Helsinki guidelines and with ethics approval from both the secondary afflicted hospital and Xi'an Jiaotong University Ethics Committee. Written informed consents were obtained from all donars. Plasma sample were centrifuged from blood samples, and divided into 200 µl package in RNase-free eppendorf tubes and stored immediately at -80°C.

Plasma RNA isolation and miRNA reverse-transcription (RT) process

Plasma at volume of 200 µl was combined with 750 µl Tri-reagent® BD (MRC Cat.TB126, USA) and 20 µl 5M HAc (also RNase-free) for sample denaturizing. Plasma sample is much viscous, hence the denaturizing was performed by mechanistically vortexing for at least 1 min until the plasma component was eventually invisible. 5 µl 50 pM single-stranded synthetic *Caenorhabditis elegans* originated cel-miR-39 (GenePharma, China) molecule was added as an internal control at a 1.25 pM concentration presenting in undetermined plasma. Incorporation of foreign cel-miR-39 should be done after the plasma was fully denatured using Tri-reagent BD. Because the synthetic RNAs added into the plasma will be immediately and easily degraded. RNA was extracted as convention, using chloroform and isopropanol, 75% ethanol and finally dissolved in 8 µl RNase free water. Extracted total RNA was evaluated for concentration and purity using a UV-Vis spectrophotometer (NanoDrop 2000c, Thermo, USA) before use to make sure decent quantities and quality of plasma RNA could be obtained.

RT process was accomplished using universal miRNA one-step RT kit (TakaRa, Japan) according to manufacturer's instruction. In a system with total volume of 20 µl, 6 µl total RNA solution was mixed with 10 µl 2×RT buffer, 2 µl 0.1% BSA and 2 µl RT enzyme. Following condition was used for reaction: 37°C for 60 min, followed with 85°C for 5 s.

qPCR

RT products were generally diluted by 5 times, and 2 µl cDNA template was used for qPCR. Only for the detection of miRNA in plasma and blood pellet at the same time, cDNA was diluted by 100 times for an optimal amplification of both templates. qPCR reaction was performed on a SYBR Green system (using Fast Start universial SYBR Green master dye, Roche, USA) on iQ™5 (Bio-rad, USA) realtime PCR platform. Specificity of the reaction was based on sequence specific forward primers (miRNA specific primers, with identical sequence of 20-22 nucleotide mature miRNAs), while the unvier-

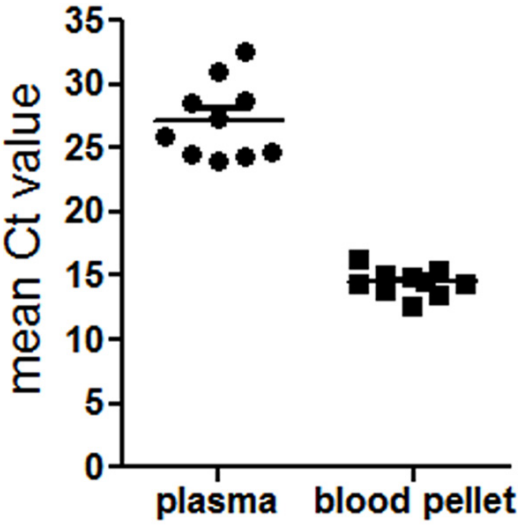


Figure 1. Comparison of endogenous miRNA in blood pellet and plasma from the same individuals MiR-21 was detected by using RT-qPCR in blood pellet and plasma from the same individuals (n=10), bar represent SEM.

sial reverse primers were provided in RT kit. The forward primer for cel-miR-39 (Accession Number: MIMAT0000010) was 5'-TCACCGGGTGTAAATCAGCTTG-3'. The forward primer for miR-21 (full name as hsa-miR-21-5p, Accession Number: MIMAT0000076) was 5'-TAGCTTATCAGACTGATGTTGA-3'. PCR reaction was started at 95°C for 10 min (for hot start enzyme initiation), going on for 40 cycles of 95°C for 5 s, 60°C for 30 s and followed with the melt curve. A weighted average cDNA dilution solution from each sample from all groups in the study was chosen as a QC between real time PCR batches which was present in each batch.

For expression normalization, cel-miR-39 was used as internal control. This equal amount of spiked-in RNA control added in the same volume of plasma could not only normalize the efficiency of RNA purification and RT, but also be used as a stable reference control for qPCR. Amplification stability of this reference control was evaluated using a standard curve. In this evaluation, cel-miR-39 from 6 gradients of twofold dilution of pooled cDNA sample which went through RNA isolation, RT process, a 5 times dilution and eventually the qPCR detection at the same time with identical treatment was amplified. Theoretically, original concentration of cel-miR-39 distributed in plasma was 1.25 pM. Hence, the standard curve represented the approximation of 1.25, 0.625, 0.3125, 0.156, 0.078, 0.039 pM cel-miR-39 amplification. For those studies, in which the expression fold change of extracellular miRNAs alone was sufficient for researchers' concern, we can simply use the ΔΔCt method [13] and set the average data for control group as 1 to calculate the fold change of extracellular miRNA expression.

Statistics

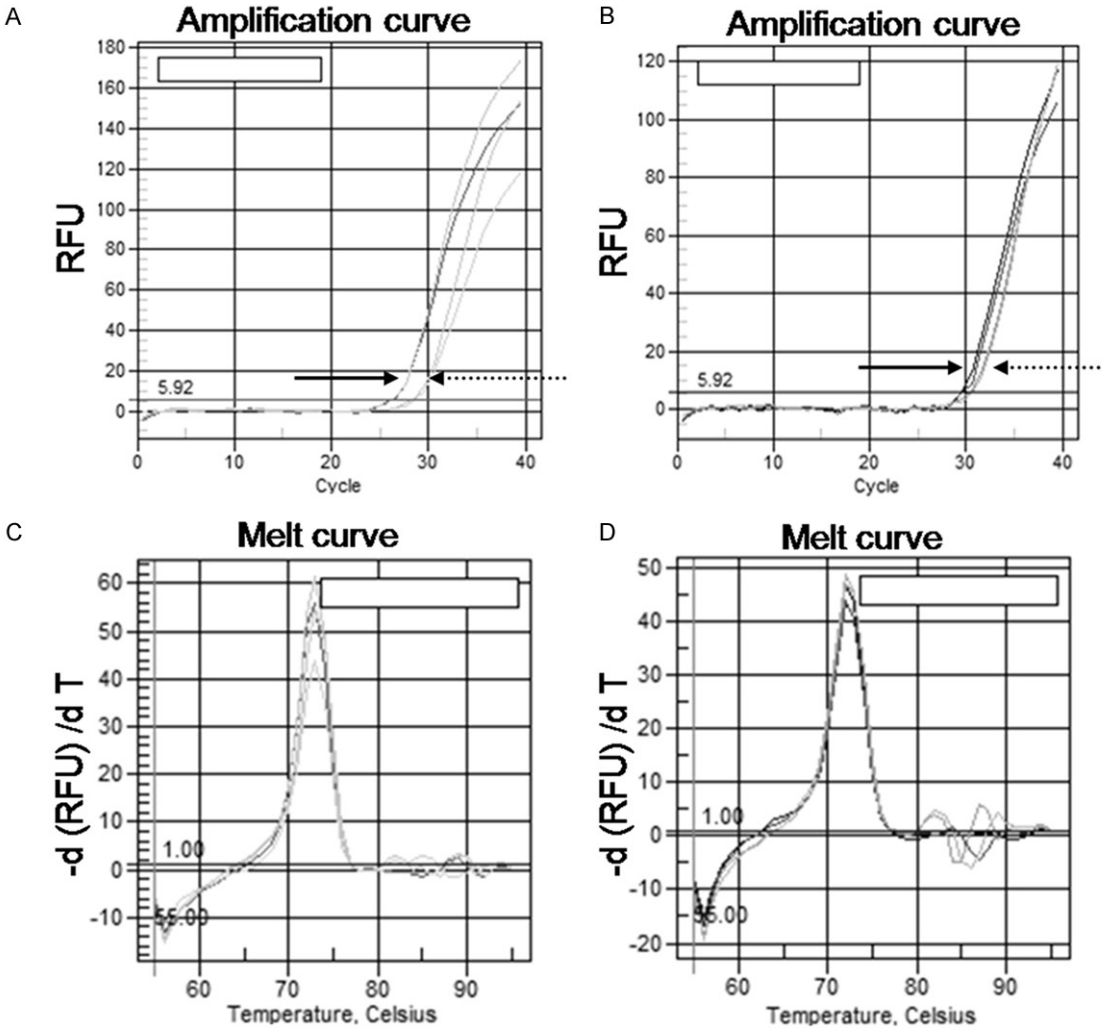
Data was presented as mean ± SEM, and coefficient of variation (CV) was used for variation description. Pearson correlation was used for analysis of standard curve.

Results and discussion

Key points during blood handling

Firstly, for plasma preparation, choosing anticoagulant was of great importance. We once used heparin-containing drawing tubes for blood collection in the pilot test. Both extracellular miRNA and the spiked-in cel-miR-39 (added after plasma denaturizing) were completely undetectable by qPCR. While, both the RNA quantity and quality of total RNA (indicated by A_{260}, A_{260}/A_{280} ratio) was comparably decent. Heparin might interference with RT-qPCR system and lead to this disastrous consequence. In 2012, some researchers systematically evaluated the various anticoagulants in extracellular miRNA quantification, and also found heparin could significantly affect serum miRNA quantitation, while others such as sodium fluoride/potassium oxalate, and sodium citrate turned out as better choice [14]. Hence, from then on, we chose sodium citrate, and found it compatible with qPCR system and did not find such problems.

Secondly, hemocytolysis which might influence miRNA quantification is suggested be avoided [11] by careful observation, and once the hemachrome was found spread in separated plasma, this sample should be excluded for further detection. In addition, blood cell debris pellet contamination was strictly prevented during procedure of plasma supernatant transferring by leaving a sufficient volume of plasma during pipetting and avoiding any touching of the pipette tip from the plasma-blood cell interface. This is of great importance because the expression level of intracellular miRNA was significantly higher by hundreds or even thousands times of extracellular miRNAs in the



E

Isolation reagents	total RNA mass (μg)	A_{260}/A_{280} ratio	melting temperature	mean ± SEM (CV%) of Ct value	
				cel-miR-39	miR-21
Tri-reagent® BD (MRC)	4.52	1.77	72℃	26.37±0.005 (0.027%)	29.66±0.050 (0.24%)
Trizol® (Invitrogen)	2.18	1.70	72℃	28.66±0.075 (0.37%)	30.54±0.100 (0.46%)

CV: coefficient of variation

Figure 2. Comparison of isolation capacity of Tri-reagent® BD and Trizol® using the same pooled plasma sample from 10 individuals The amplification curve of spiked-in cel-miR-39 (A) and endogenous miR-21 (B), solid arrow: using Tri-reagent® BD, dotted arrow: using Trizol® Melt curve of cel-miR-39 (C) and endogenous miR-21 (D). (E) Diagram summarizing the differences of products at quantity and quality of total RNA mass, amplification Ct values and melting temperatures using both reagents.

same individual, and hence this means any pellet contamination could lead to disturbing of results. Other researchers suggest caution in interpretation of disease-specific results as they may reflect a blood cell-based phenomenon caused by hemocytolysis or blood cell contamination [15]. From **Figure 1**, we can see there is an averaged Ct value gap of more than

Table 1. Plasma RNA yield isolated from 10 individuals

Sample	Total RNA yield (μg)	RNA purity (A_{260}/A_{280})	Estimated miRNA mass (ng)
No.1	3.821	1.93	0.38
No.2	3.238	1.86	0.32
No.3	3.295	1.93	0.33
No.4	4.191	1.50	0.42
No.5	4.447	1.53	0.44
No.6	1.773	1.70	0.18
No.7	1.763	1.69	0.18
No.8	2.083	1.66	0.21
No.9	3.011	1.79	0.30
No.10	2.829	1.77	0.28
Average	3.045±0.302	1.74±0.05	0.30±0.03

12 cycles even for the same miRNA (using miR-21 as an example) from plasma and pellet origin of 10 individuals. Hence, the influence of hemocytolysis in extracellular miRNA detection is huge, because the miRNA was much more enriched in blood pellet compared with plasma from the same individual.

Choice making during RNA isolation and miRNA RT process

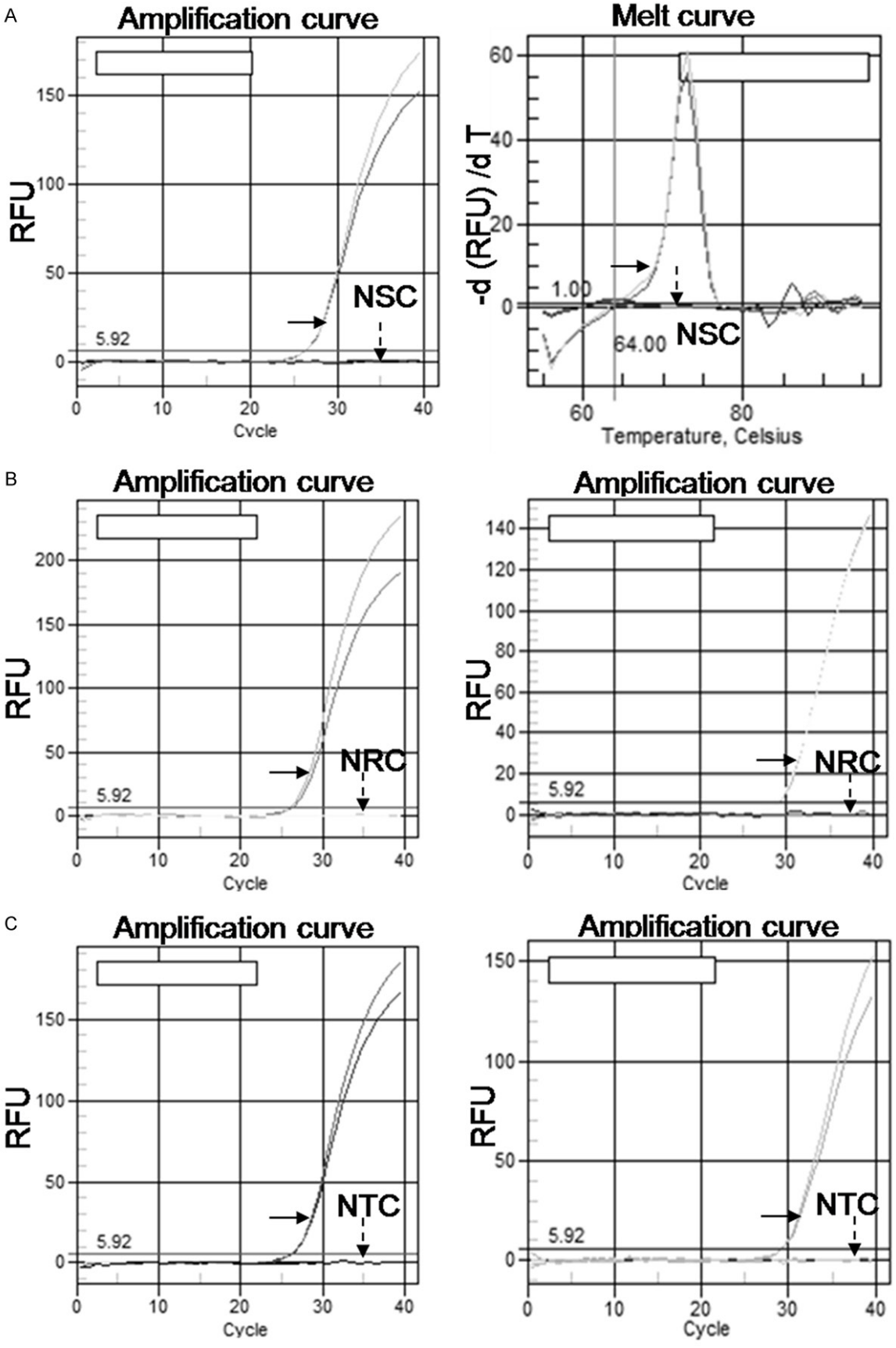
Plenty of researchers chose conditional isolation reagent with acid phase separation for plasma RNA isolation, such as Trizol® (Invitrogen, USA) and Tri-reagent® BD (MRC, USA) modified for blood sample [9, 11]. The isolation capacity of Tri-reagent® BD was compared with Trizol® (Invitrogen) using the same pooled plasma sample from 10 individuals (**Figure 2**). The amplification curves of cel-miR-39 and exogenous miR-21 show slight amplification delay when using Trizol®. Melt curves of spiked-in cel-miR-39 and exogenous miR-21 using both reagents show almost identically sharp peak signal using both reagents. The results showed that using of Tri-reagent® BD could lead to more and purer plasma total RNA mass as well as less Ct value and CV of data from RT-qPCR outputs under the same threshold for both the spiked-in cel-miR-39 and endogenous target miRNA (such as a miR-21 in this case).

The RNA yield of plasma isolated from 10 individuals was also monitored, the isolated total RNA mass and purity evaluated by A_{260}/A_{280} was described in **Table 1**. It was reported that miRNA represents approximate 0.01% of total RNA mass, hence the calculated miRNA yield from each 200 μl plasma package was about 0.30±0.03 ng, in accordance with the reported yield less than 1-10 ng miRNA mass in plasma isolation [9].

Practical management in global miRNAs profiling using PCR

It is well known that, qPCR can perform expression profiling in a high-throughput manner. For extracellular miRNA profiling, the efficiency during plasma RNA isolation, RT process and qPCR were all diverse from batch to batch, hence separate batch of experimental performance or independent analysis of biological replicate data both can lead to unrepeatable results (showing as various raw Ct value for individual miRNA) [16]. While facing the hundreds of plasma samples, we have to admit that the isolation of sound plasma RNA at the same time was not available in practice. Although application of samples in a RT reaction was much simpler than qPCR, but the reaction takes place at 37°C, which means delay of time during sample handling could possibly lead to the huge diversity in RT thermokinetics. Hence, even the 384-well system during qPCR can never be enough for the data normalization of miRNA profiling, and these challenges remains unresolved. So far, various absolute extracellular miRNA strategies employed the standard curves using RT production from fold dilution of synthetic target miRNA with known copy number. These protocols were similar, mostly derived from a original article in 2008 [17] which is detailed described, considerably precise and with strong feasibility, yet contain an unavoidable flaw that even though they can go through RT and PCR process together, the standard samples share too little in common with these samples to be measured, and the diversity in purity and background of ion concentration between the plasma miRNA and synthetic miRNA is huge. Hence, the diverse efficiency during plasma RNA isolation, RT process and qPCR from batch to batch can easily lead to unrepeatable results in separate batch of experimental performance or independent analysis of biological replicate data.

In attempt to solve these problems, we specially modified the 96-well based RT-qPCR strategy, and design the protocol for multi-batch test.


A
Amplification curve
RFU
NSC
5.92
Cycle
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1.00
64.00
NSC
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Figure 3. Controls in RT-qPCR demonstrating the specificity of this method. A. Control test for internal spike-in cel-miR-39 incorporation. Amplification Curve (left) and melt curve (right) of cel-miR-39 with (solid arrow) or without (dotted arrow) spike-in cel-miR-39 incorporation in the beginning. B. Control test for RT process. PCR amplification curve of cel-miR-39 (left) and miR-21 (right) with (solid arrow) or without (dotted arrow) RT enzyme. C. Control test of realtime PCR process. PCR amplification curve of cel-miR-39 (left) and miR-21 (right) with (solid arrow) or without (dotted arrow) template cDNA.

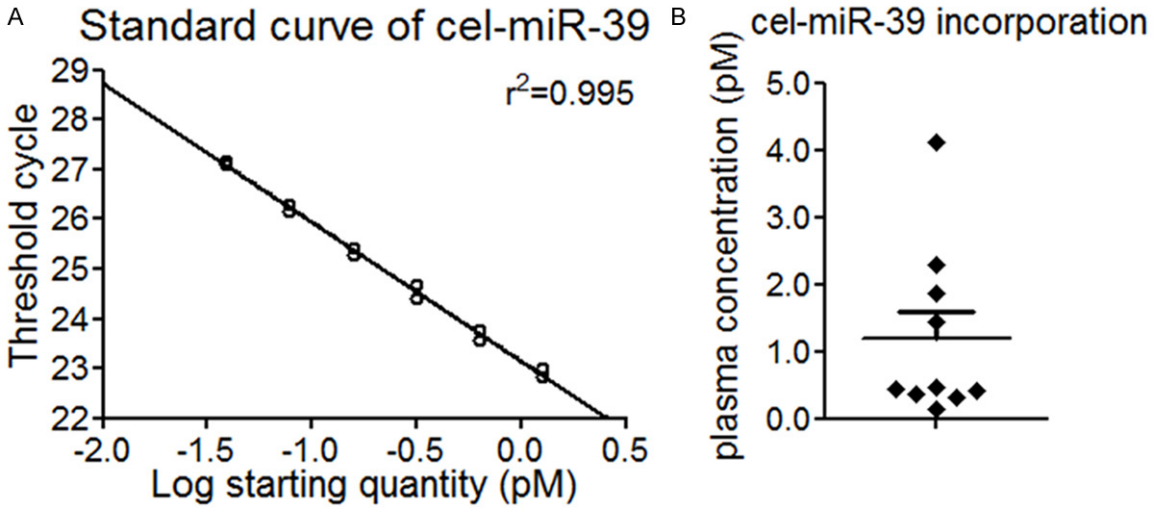

Figure 4. Evaluation of absolute calculation based on standard curves. A. The representative standard curve of cel-miR-39 from three qPCR batches Plasma concentration was transformed into log value. B. Automatically calculated concentration of cel-miR-39 in plasma from ten individuals according to above-mentioned standard curve.

Every 30 samples went through RNA isolation (while triplicates were applied in PCR assay), RT process together which takes a medium sampling skill for operators in a bio lab. In disease specific miRNA profiling, we further recommended that the unknown samples are load with equal ratio in each group (such as 15 controls: 15 patients). This method can quantify plasma miRNA from 30 individuals at the same time. For samples more than 30, multiple runs of RT-qPCR was needed as well as the strict QC for minimizing batch effect. Hence, an averaged QC sample was prepared by mixing 1 μl diluted cDNA template from each sample, and was detected during each PCR running, which was used as reference data for correction of the miRNA quantity among various batches. Also, the raw Ct value of QC sample can be used as an auto-check for any extreme variation in amplification of single PCR batch to minimize the batch-effect.

We evaluated the specificity and reliability of this strategy. Firstly, Serveral controls were set in RT-qPCR essay and the data demonstrated the specificity of this RT-qPCR methodology was decent. In a non-spike-in control (NSC), the amplification signal was undetectable with a disorganized melt curve (**Figure 3A**). In a non-RT control (NRC), the signals from foreign cel-miR-39 or endogenous miRNA (miR-21) were both undetectable (**Figure 3B**). In non-template control (NTC), the signal were absent for miRNAs detected, which excluded the presence non-specific signals such as amplification caused by the contamination from genomic DNA (**Figure 3C**).

Standard curves between different batches were monitored using r^2 of each batch in which the credibility of standard curves was accepted only when r^2 value is above 0.980. Because this strategy was based on the assumption that the theoretical concentration cel-miR-39 in the weighted average plasma in each batch is exactly 1.25 pM, our method is actually better adequate for profiling study with relative large samples, which improved the accuracy of results. In this methodology paper, we simply evaluated the linearity of the standard curves from 3 qPCR runs in a small sample consisted of ten plasma samples (**Figure 4**). The results showed that in the presence of 1.25 to 0.039 pM plasma cel-miR-39 (the log value of -0.969

to -1.409), we observed a fine linearity (r^2= 0.993, 0.997 and 0.995 for these 3 batches, significant correlation of P<0.001 for all of these batches). And we further chose the representative standard curve to automatically generate the plasma concentration of spiked-in cel-miR-39 in our ten plasma samples, and the absolute concentration we calculated was 1.19±0.40 pM, equal to the amount of 95.2±32% of theological value. All above-mentioned data demonstrated the specificity and reliability of this method.

Data normalization and processing

Spiked in cel-miR-39 as a miRNA not present in original human system was used for normalization because it can correct any possible aspects of technical variation during RNA isolation, RT and qPCR, hence is more powerful than any other molecules as an internal control in the extracellular miRNA RT-qPCR methodology. Signal detected after 35 cycles of realtime amplification was rejected for valid analyzing. In addition, any sample with extreme deviation of expression which exceeded 32 (2^5) fold change compared with the QC sample was excluded for further analyzing.

In conclusion, we described and evaluated a simplified and optimal RT-qPCR strategy in every specific detail for daily performance in bio labs, intended to optimize the procedures that might lead to variable results from independent experiments, and further recommended this standardized optimal procedure.

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Disclosure of conflict of interest

None.

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