Video Article

Digital PCR for Quantifying Circulating MicroRNAs in Acute Myocardial Infarction and Cardiovascular Disease

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Abstract

Circulating serum microRNAs (miRNAs) have shown promise as biomarkers for the cardiovascular disease and acute myocardial infarction (AMI), being released from the cardiovascular cells into the circulation. Circulating miRNAs are highly stable and can be quantified. The quantitative expression of specific miRNAs can be linked to the pathology, and some miRNAs show high tissue and disease specificity. Finding novel biomarkers for cardiovascular diseases is of importance for medical research. Quite recently, digital polymerase chain reaction (dPCR) has been invented. dPCR, combined with fluorescent hydrolysis probes, enables specific direct absolute quantification. dPCR exhibits superior technical qualities, including a low variability, high linearity, and high sensitivity compared to the quantitative polymerase chain reaction (qPCR). Thus, dPCR is a more accurate and reproducible method for directly quantifying miRNAs, particularly for the use in large multi-center cardiovascular clinical trials. In this publication, we describe how to effectively perform digital PCR in order to assess the absolute copy number in serum samples.

Video Link

The video component of this article can be found at https://www.jove.com/video/57950/

Introduction

Circulating miRNAs have been identified as promising markers for a number of diseases, including cardiovascular disease[1]. The miRNAs are small, non-coding single-stranded RNA molecules (approximately 22 nucleotides long) are involved in the post-transcriptional regulation *via* the alteration of the messenger RNA translation and influencing gene expression[2], and are released into the circulation in both physiological and pathological states. The quantitative expression of specific miRNAs can be linked to the pathology, and some miRNAs show high tissue and disease specificity[1]. In cardiovascular diseases, miRNAs have become attractive candidates as novel biomarkers because they are remarkably stable in the serum and can easily be quantified with the help of PCR methodology[3]. The potential value of miRNAs as biomarkers for myocardial infarction has been evaluated in small studies, but a validation in large cohorts is lacking[2]. For example, miR-499 is found highly expressed in the myocardial muscle, and it has been shown to be significantly increased in an AMI[4,5,6]. Further, it regulates programmed cell death (apoptosis) and the differentiation of cardiomyocytes and is thus involved in several mechanisms following an AMI[7]. Apart from some small studies reporting a superiority and incremental value of miRNAs for the diagnosis of AMI, the superiority or equality to high-sensitivity cardiac troponins has not yet been proven in large-scale studies[2,5,6,8]. More prospective studies in large cohorts are, therefore, needed to assess the potential diagnostic value of miRNAs. Additionally, methods of miRNA quantification need to be optimized and standardized using comparable protocols[9]. Standardized assays may reduce inconsistent results and may help miRNAs to become potential biomarkers for the routine clinical application, as biomarkers need to be quantified in a reproducible way to ensure their clinical applicability.

Recently, dPCR has been introduced as an end-point analysis. It partitions the sample into approximately 20,000 individual reactions[10]. The dPCR system then utilizes a mathematical Poisson statistical analysis of fluorescent signals (positive and negative reactions), enabling an absolute quantification without a standard curve[10]. When combining dPCR with fluorescent hydrolysis probes, the highly specific direct absolute quantification of miRNAs is made possible. Digital polymerase chain reaction has shown to exhibit superior technical qualities (including a decreased variability, an increased day-to-day reproducibility, a high degree of linearity, and a high sensitivity) for quantifying miRNA levels in the circulation compared to quantitative real-time PCR[10,11]. These superior technical qualities might help to mitigate current limitations on using circulating miRNAs as biomarkers and may lead to the establishment of miRNAs as biomarkers in large multi-center cardiovascular clinical trials

and as a diagnostic method in general. In a previous study, we recently applied dPCR for the absolute quantification of circulating miRNAs in patients with an AMI and were able to demonstrate superior diagnostic potential compared to the quantification of miRNAs by qPCR[12].

In this publication, we want to demonstrate that using dPCR is an accurate and reproducible method for directly quantifying circulating cardiovascular miRNAs. The absolute quantification of miRNA levels in serum, using digital PCR, shows potential for the use in large multi-center cardiovascular clinical trials. In this publication, we describe in detail how to effectively perform digital PCR and how to detect the absolute miRNA copy number in serum.

Protocol

1. Extraction of miRNA from Plasma/Serum

Note: In order to quantify miRNAs appropriately, the correct microRNA isolation from the plasma/serum is a crucial step. A key thing to keep in mind, especially because different protocols exist, is to adhere to the same workflow while processing the samples. In this protocol, miRNA is extracted from 50 µL of serum. Do not use more than 200 µL as this limit the correct extraction process.

1. Prepare serum or thaw frozen samples.
2. Add 250 µL (5 volumes) commercial lysis reagent to 50 µL of serum. Support the lysis by vortexing. Place the tube with the lysate on the bench at room temperature for 5 min.
3. Spike the lysate with 3.5 µL of spike-in control (1.6 x 10^7 copies/µL) and mix them by vortexing.
4. Add 50 µL of chloroform (*i.e.*, an equal amount as to the starting serum sample) for the phase separation. Shake the tube vigorously for 15 s. Place the tube on the bench at room temperature for 2 - 3 min.
5. For the phase separation, centrifuge the tube at 12,000 x g at 4 °C for 15 min.
   Note: The samples are now separated into an upper aqueous phase containing the RNA, a white interphase, and a lower, pink organic phase.
6. Transfer the upper aqueous phase (100 µL) containing the RNA into a new tube. Do not transfer any white interphase material as this falsifies the correct RNA count.
7. Add 150 µL (*i.e.*, 1.5x the volume of the transferred sample) of 100% ethanol. Mix the materials by pipetting them up and down. Do not centrifuge them, and quickly move on to the next step.
8. Pipette 250 µL of the sample onto a commercial spin column in a 2 mL collection tube. Close the lid. Centrifuge the column at >8,000 x g at room temperature for 15 s. Discard the flow-through.
9. Add 700 µL of commercial washing buffer number 1 to the spin column. Close the lid and centrifuge the column at >8,000 x g at room temperature for 15 s. Discard the run-through washing buffer.
10. Add 500 µL of commercial washing buffer number 2 to the spin column. Close the lid and centrifuge the column at >8, 000 x g at room temperature for 15 s. Discard the run-through washing buffer.
11. Pipette 500 µL of 80% ethanol to the spin column. Close the lid, centrifuge the column at >8, 000 x g at room temperature for 2 min. Discard the collection tube with the flow-through.
12. Transfer the spin column to a new 2 mL collection tube. Centrifuge the column at full speed with an opened lid to dry the membrane. Discard the collection tube with the flow-through.
13. Transfer the spin column into a new 1.5 mL collection tube. Elute the RNA by applying 30 µL of RNase-free water directly to the center of the membrane. Close the lid. Centrifuge the column at full speed for 1 min.
14. Store the RNA at -70 °C.
    Note: The storage of purified RNA in RNase-free water between -70 °C to -20 °C ensures no degradation of the RNA for 1 year. Following the manufacturer's protocol, the minimum amount of RNase-free water to dilute the RNA is 10 µL. Using less than 10 µL may insufficiently hydrate the membrane and reduce the total RNA yield.

2. Reverse Transcription

Note: Complementary DNA (cDNA) was synthesized using the following 15 µL reverse transcription (RT) protocol (total reaction volume).

1. Thaw the RT kit on ice. Keep the enzymes in the freezer as long as possible to prevent them from degrading and spin them down before use. Thaw RT primers on ice and spin them down before use.
2. Prepare the master mix as described in **Table 1**.
3. Combine 10 µL of master mix and 5 µL of extracted RNA per well in a 96 well-plate.
4. Centrifuge the well plate at 2,000 x g at 4 °C for 2 min.
5. Use the thermal cycle protocol described in **Table 2** for the reverse transcription.
   Note: As described below, cDNA may directly be processed in the digital PCR machine (see **Table of Materials**); alternatively, it may be stored at -20 °C.

3. Droplet Generation and Digital PCR

Note: Digital PCR was performed using the following 40 µL protocol.

1. Thaw the commercial dPCR mix, the prepared cDNA, and the PCR primers on ice. Centrifuge them shortly before use.
2. Prepare the master mix as described in **Table 3.**
3. Add 1.33 µL/well of the RT product and centrifuge it briefly.
4. Pipette 20 µL of the sample into each well of the 8-well cassette (medium row).

Note: If the 8-well cassette is not completely filled, fill up the remaining wells with non-template controls (NTCs) that omit cDNA (*i.e.*, the RT product produced with water instead of extracted RNA) or dPCR commercial buffer. The NTC serves as a contamination control. Water must not be used in the remaining wells, because that will impair the quality of the droplet formation.
5. Pipette 70 µL of the droplet generation oil for the probes into the oil wells (bottom row) of the 8-well cassette.
6. Place a gasket on top of the 8-well cassette and place the cassette in the droplet generator. Close the droplet generator. Wait until the 3 indicator lights are solid green.
Note: Droplets are now being generated.
7. Carefully and slowly transfer 40 µL of the droplet-formed sample (top row) into separate wells of a 96-well plate by pipetting.
8. After completing the droplet formation on the samples, seal the PCR plate with a pierceable dPCR foil at 180 °C for 4 s.
9. Place the foil-sealed PCR-plate in the cycler and cycle it as described in **Table 4,** at a ramp rate of 2.5 °C/s.

4. Droplet Reading and Analyzing

1. Transfer the plate from the thermal cycler to the base of the plate holder. Tighten the PCR plate by placing the top of the plate holder onto the PCR plate.
2. Start the commercial dPCR software.
3. Enter the sample name, the name of the experiment, and the target in the setup. Select the absolute quantification.
4. Start the droplet reading by clicking **RUN**. Select **FAM/HEX** or **FAM/VIC** as detection chemistry when working with hydrolysis probes.
Note: The commercial dPCR software will then auto-analyze the data.
5. Check the droplet numbers in the results table to ensure a correct droplet generation.
6. Select all wells with the same quantified miRNA and click **ANALYZE**. Use the 2D blot to mark positive droplets and manually correct the auto-analyzed data (**Figure 1**).

5. Correct Calculations on the Sample

1. Normalize the sample by multiplying the sample by the normalization factor (NF).
NF = Median [*C. elegans* miR-39 measurements]$_{\text{all samples}}$/[*C. elegans* miR-39]$_{\text{given sample}}$
2. Calculate the final miRNA serum concentration, adjusting for dilution factors (DF).
$[\text{miRNA}]_{\text{final}} = [\text{miRNA}]_{\text{raw value}} \times DF_{RT} \times DF_{dPCR} \times DF_{Ex}$
Where:
$[\text{miRNA}]_{\text{raw value}}$ = the miRNA quantified by commercial dPCR software;
DF_{RT} = the dilution factor of the template in reverse transcription;
DF_{dPCR} = the dilution factor of the template in dPCR;
DF_{Ex} = the dilution factor in the extraction.

6. Synthetic Oligonucleotide Dilution Series

1. Thaw lyophilized synthetic oligonucleotide on ice. Briefly centrifuge it.
2. Dilute the lyophilized synthetic oligonucleotide in nuclease-free water to a final concentration of 10 pmol/µL. Briefly centrifuge it.
3. Dilute it in nuclease-free water as described in **Table 5**. Centrifuge the dilution briefly between each dilution step at 8,000 x g for 10 s.
4. Continue with the reverse transcription as described in step 2 and analyze the samples with digital PCR as described in step 3 and step 4.

Representative Results

Digital PCR combined with fluorescent hydrolysis probes enables researchers to directly quantify the absolute amounts of specific miRNAs in copies/µL. As the sample in dPCR is partitioned in approximately 20,000 individual PCR reactions, dPCR does not require technical replicates[10]. The dPCR system utilizes a mathematical Poisson statistical analysis of fluorescent signals (differing between positive and negative reactions) enabling an absolute quantification without the need for a standard curve[10]. In order to correctly calculate the concentrations, an acceptable droplet count is needed (>15,000). No template controls ensure the exclusion of considerable nonspecific amplification. All samples included in the analysis are processed using the same volume, method, and PCR protocol to strengthen the validity of the obtained results. The obtained concentrations in dPCR are normalized using a median normalization procedure across all samples analyzed with the synthetic spiked-in *C. elegans* miR-39[13]. Normalizing the data to the measured amount of spiked-in *C. elegans* miR-39 corrects for the sample-to-sample variation in the RNA extraction efficiency and serves as a PCR reaction control, further adding to the validity of the results. There is no established gold standard for normalizing serum miRNAs; however, exogenous controls such as the spiked-in *C. elegans* miR-39 are an elegant solution for normalization procedures, as endogenous miRNAs are often altered in various disease states[9].

The analyzed serum samples were acquired before a percutaneous coronary intervention (PCI), 8 h after a PCI, and 16 h after a PCI, from patients with an acute ST-segment elevation myocardial infarction (STEMI). STEMI patients exhibit severe ischemia and thus qualify for the evaluation of new ischemia biomarkers. To evaluate the kinetics of miR-499 release and the potential use of miR-499 as a biomarker for ischemia in cardiovascular disease, miR-499 was analyzed with dPCR across all patients for the three different time points[14].

Figure 1 demonstrates the selection of positive droplets giving the final miRNA concentration calculated by the software. The fraction of positives in a sample determines the concentration of copies/µL as calculated by the commercial dPCR software. The results can also be visualized in a 2D plot and the positives can be manually marked with circles. Concentrations are only considered if they are above the nonspecific amplification of the NTCs.

Figure 2 shows the linearity and goodness-of-fit of a synthetic miRNA oligonucleotide hsa-miR-499-5p dilution series in duplicates. An 8-step dilution series was performed from 2,500 copies/µL to 0 copies/µL. The calculated expected copies/µL as described in **Table 5** assume 100% RT

and digital PCR efficiency. In this setup, digital PCR demonstrates a high degree of linearity (r^2 = 0.99). The limit of detection (LoD = 0.12) and the limit of quantification (LoQ = 0.23) are also presented in **Figure 2**, showing that even a low miRNA expression can be detected successfully. Thus, dPCR can be used to quantify even low serum levels of circulating miRNAs. The limit of detection (LoD) and the limit of quantification (LoQ) are calculated following the approach of Forootan *et al.*[15] and are defined as LoD = LoB + 1.645 x $\sigma_{\text{low concentration sample}}$, where the limit of blank (LoB) is calculated as LoB = $\text{mean}_{\text{blank}}$ + 1.645 x σ_{blank}. The LoQ is then estimated running replicate standard curves[15]. To ensure the reproducible quantification of miRNAs, only miRNA concentrations that lie above both the LoD and the LoQ are considered as a valid exact value.

Figure 3 shows representative results of miR-499 levels of patients with an ST-elevation myocardial infarction (STEMI), *n* = 16 at each time point. Samples were taken before a percutaneous intervention (PCI) (*t* = 0), 8 h after a PCI (*t* = 8), and 16 h after a PCI (*t* = 16), and the miR-499 levels were compared to miR-499 levels of patients with stable coronary artery disease (CAD, *n* = 20). The main patient characteristics can be found in **Table 6**. After the initial release of miR-499 into the serum in the first 8 h following a myocardial infarction, miR-499 levels decrease again after 16 h. A trend in increase can already be seen at the beginning of the STEMI (*t* = 0), and a significant increase of miR-499 levels compared to patients with CAD can be seen 8 h after the STEMI (*t* = 8, *p* <0.01) when comparing miR-499 levels to miR-499 levels of stable CAD patients. The receiver operating curves (ROC) show the possible utility of miR-499 as a novel biomarker to differentiate between patients with CAD and patients with a STEMI [the area under the curve (AUC) = 0.62 for samples taken before a percutaneous intervention (PCI), AUC = 0.75 for samples taken 8 h after a PCI, and AUC = 0.78 for samples taken 16 h after a PCI compared to serum levels of miR-499 in patients with CAD].

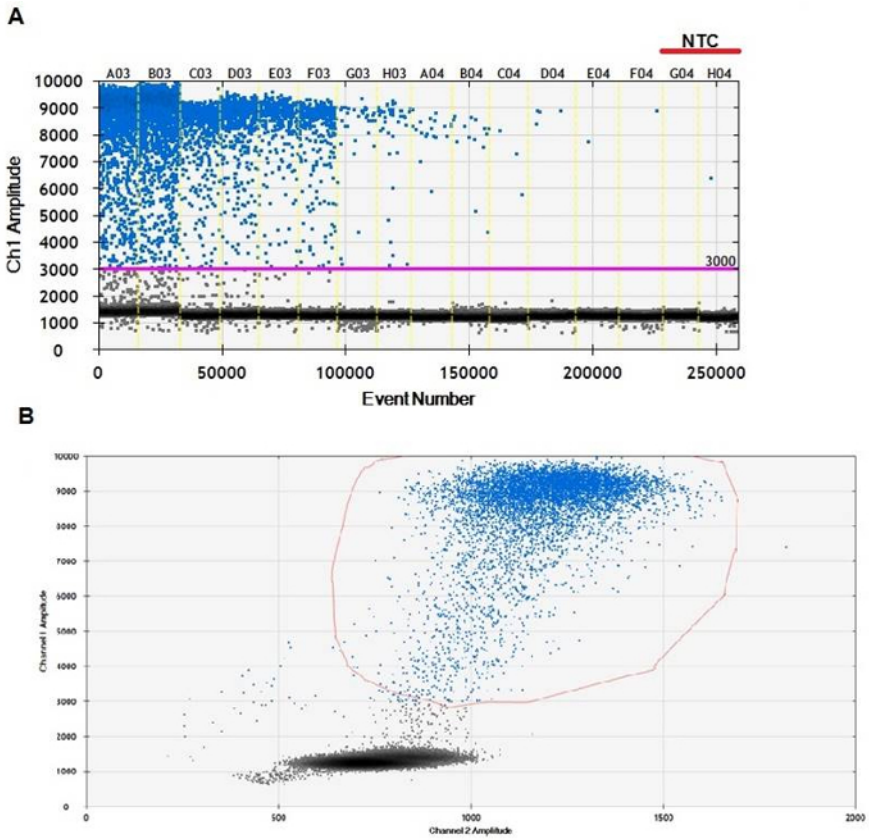

Figure 1: Selection of positive droplets in a dilution series performed in duplicates. (**A**) The threshold is set manually above the negative droplets. (**B**) The results are visualized in a 2D blot, the positives selected by circling. Please click here to view a larger version of this figure.

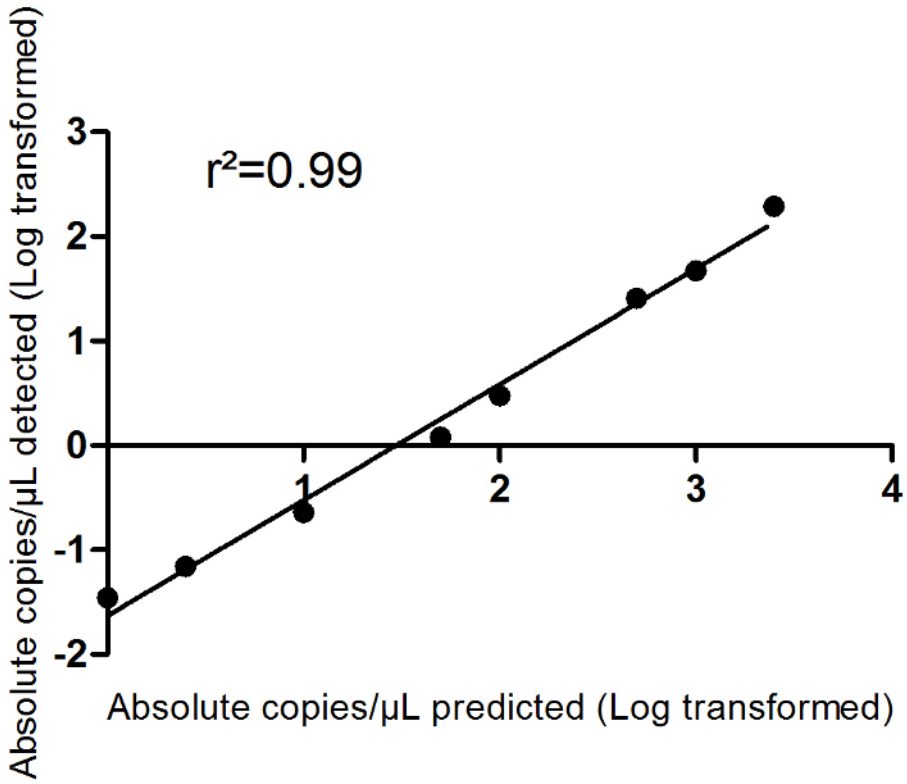

Figure 2: Synthetic miR-499 oligonucleotide dilution series. (**A**) The measurements were performed in duplicate. The data are presented log transformed as absolute copies per µL. Linear regressions and goodness-of-fit (r^2-value) are shown. The data are presented as mean ± SEM. (**B**) This panel shows the limit of detection (LoD) and the limit of quantification (LoQ). Please click here to view a larger version of this figure.

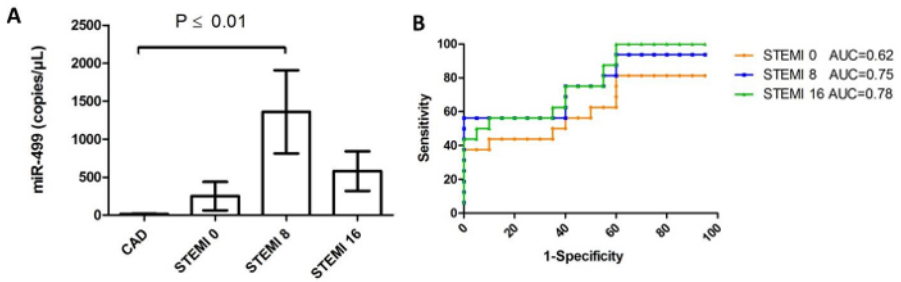

Figure 3: Quantification of circulating serum miR-499-5p in patients with ST-elevation myocardial infarction (*n* = 16) before percutaneous coronary intervention (PCI), 8 h after PCI, and 16 h after PCI, compared to patients with stable coronary artery disease (*n* = 20). (**A**) The circulating serum miR-499-5p is represented as mean with the standard error of the mean with the corresponding *p*-value calculated by one-way ANOVA followed by a Bonferroni *post hoc* test (p <0.05 was considered as statistically significant). (**B**) A receiver operating characteristic curve (ROC) analysis is performed on the data from panel **A**. The ROC analysis demonstrates the sensitivity and specificity of the biomarker. Further, the corresponding area under the curve (AUC) values are shown. Please click here to view a larger version of this figure.

sample amount	
n = 1	**/well [µl]**
nuclease-free water	4.16
10x Reverse Transcription buffer	1.5
100nM dNTP	0.15
RNAse inhibitor	0.19
specific RT Primer	3
Commercial Reverse Transcriptase **Enzyme** 50 U/ul	1
Master-Mix total	10

Table 1: Reverse Transcription Reagents Mix.

Temperature	Time
16 °C	30 mins
42 °C	30 mins
85 °C	5 mins
4 °C	∞

Table 2: Thermal Cycling Conditions for Reverse Transcription.

sample amount	
n = 1	**/well [µl]**
nuclease-free water	7.67
commercial dPCR mix	10
20x specific hydrolysis primer/probe	1
Master-Mix total	18.67

Table 3: Digital PCR Reagent Mix.

Temperature	Time
95 °C	10 mins
94 °C	30 secs (x40)
60 °C	1 min
98 °C	10 mins
4 °C	∞

Table 4: Thermal Cycling Conditions for digital PCR.

Tube	Transferred synthetic oligonucleotide miR-499 (µL)	Diluent (µL)	miRNA (copies/µL)	Expected Copies/µL in RT	Expected Copies/µL in dPCR
Original			6.022×10^{12}		
1	10	990	6.022×10^{10}		
2	10	990	6.022×10^{8}		
3	10	990	6.022×10^{6}		
4	18.7	981.3	1.128×10^{5}	37600	**2500**
5	40	60	4.511×10^{4}	15040	**1000**
6	50	50	2.256×10^{4}	7520	**500**
7	40	160	4.511×10^{3}	1504	**100**
8	50	50	2.256×10^{3}	752	**50**
9	40	160	4.511×10^{2}	150.4	**10**
10	50	150	1.128×10^{2}	37.6	**2.5**
11	0	50	0	0	**0**

Table 5: Synthetic dilution series for miR-499.

Characteristic	All	Stable CAD Patients (n=20)	STEMI Patients (n= 24)	p-value
Age	64.7 ± 11.9	66.7 ± 13.1	62.2 ± 9.9	0.2810
Males	77.8%	65%	93.8%	0.0392
DM	33.3%	40%	25%	0.3428
HTN	61.1%	60%	62.5%	0.8785
Dyslipidemia	38.9%	65%	6.3%	0.0003
Smoker	47.2%	55%	37.5%	0.3796
Family history	25%	35%	12.5%	0.1213
Overweight	58.3%	55%	62.5%	0.6501
Serum Creatinine (mg/dL)	0.94 (0.83 - 1)	0.98 (0.82 - 1.01)	0.93 (0.83 - 1)	0.7255
CK Peak (U/l)	161 (102 - 611)	126 (81 - 161)	492 (228 - 3208)	0.0004
cTnT Peak (ng/L)	322.5 (23.8 -4533)	18 (7 - 25.3)	2492 (240 - 5586)	0.0002
LVEF%	45% (40% - 50%)	45% (32.5% - 55%)	45% (45% - 50%)	0.9596
Values are presented as mean ± SD; median value (25th to 75th percentile range) or %				
DM: Diabetes mellitus				
HTN: Hypertension				
CK: Creatine kinase				
cTnT: Cardiac troponin T				
LVEF: Left ventricular ejection fraction				

Table 6: Main patient characteristics.

Discussion

Digital PCR is a relatively novel end-point method of PCR that allows the direct absolute quantification of nucleic acids within a sample. The method possesses particular advantages, including a decreased variability, an increased day-to-day reproducibility, and a superior sensitivity[11,12]. Further, due to the partitioning of the sample into approximately 20,000 single reactions and endpoint analyses, dPCR is more robust to interfering substances in PCR compared to quantitative RT-PCR[16]. These qualities in dPCR make it an attractive alternative to quantitative RT-PCR as a diagnostic tool for quantification. As circulating miRNAs are often present at low serum concentrations, so far, scientists have been challenged to appropriately quantify miRNAs by PCR[9]. On the other hand, dPCR can appropriately quantify even a low miRNA expression in serum, mitigating the problems observed in low count miRNA quantification[17]. The ability of dPCR to directly give the counts/µL, even in a very low miRNA expression, thus makes it an attractive diagnostic tool for the cardiovascular research community in miRNA biomarker studies. As the concentration given in counts/µL can be multiplied by the dilution factor used in extraction, RT-reaction, and dPCR, it is possible to achieve the exact number of copies in 1 µL of serum. The workflow presented here can be performed in up to 96 samples on a plate, hence providing a tool for large cardiovascular studies. Although dPCR exhibits several advantages over quantitative RT-PCR, it is not yet routinely applied for the quantification of miRNAs in cardiovascular trials. Further, standardized data normalization procedures are lacking.

In this approach, we demonstrate that dPCR, combined with fluorescent hydrolysis probes, enables the specific direct absolute quantification of circulating miRNAs linked to cardiovascular disease. With this report, we aimed to both demonstrate an optimized protocol for miRNA detection *via* dPCR and confirm the advantages of dPCR over quantitative RT-PCR.

We confirmed the good linearity dPCR exhibits and the low limits of the detection and quantification of dPCR for quantifying miRNAs. By spiking the sample with a synthetic oligonucleotide, the normalization of the sample is possible, adjusting for extraction efficiency and sample-to-sample variation.

In conclusion, digital PCR, as it exhibits superiority in both technical proficiency and diagnostic potential, is the best current method for miRNA quantification and may further be used for large multi-center cardiovascular miRNA biomarker studies.

Disclosures

The authors have nothing to disclose.

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