Quantification of Circulating miRNAs by Droplet Digital PCR: Comparison of EvaGreen- and TaqMan-Based Chemistries

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Abstract

Droplet digital PCR (ddPCR) has been successfully used with TaqMan assays to assess gene expression through the quantification of mRNA and miRNA. Recently, a new ddPCR system that can also run DNA-binding dye-based assays has been developed but it has not yet been tested for miRNA. We tested and compared the feasibility of quantifying miRNA with the new QX200 Droplet Digital PCR system when used with EvaGreen dye– and TaqMan probe–based assays. RNA from plasma and serum of 28 patients with cancer and healthy persons was reverse-transcribed and quantified for two circulating miRNAs and one added exogenous miRNA, with both EvaGreen dye–based miRCURY LNA miRNA assays and TaqMan assays. Amplification and detection of target miRNAs were performed on the QX200 ddPCR system. Conditions required to run miRCURY LNA miRNA assays were optimized. The EvaGreen-based assay was precise, reproducible over a range of concentrations of four orders of magnitude, and sensitive, detecting a target miRNA at levels down to 1 copy/μL. When this assay was compared with TaqMan assays, high concordance was obtained for two endogenous miRNAs in serum and plasma (Pearson $r > 0.90$). EvaGreen dye–based and TaqMan probe–based assays can be equally used with the ddPCR system to quantify circulating miRNAs in human plasma and serum. This study establishes the basis for using EvaGreen dye–based assays on a ddPCR system for quantifying circulating miRNA biomarkers and potentially other low-abundance RNA biomarkers in human biofluids.

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Introduction

The concept of digital PCR was first introduced in 1992 to describe a PCR carried out in limiting dilution conditions and able to detect very rare targets (1, 2). For many years, this technology was poorly exploited, but the recent development of several commercial platforms has brought the technology into the research and diagnostic settings (3). In particular, the droplet digital PCR (ddPCR) system (4) is based on the partitioning of a reaction mixture into thousands of oil-dispersed, nanoliter-sized microdroplets.

ddPCR has been successfully applied in the detection of copy-number variations (5, 6) and in the diagnosis of viral and bacterial infections (7, 8), providing equivalent results to standard procedures using qPCR. Moreover, ddPCR proved to be more tolerant than qPCR to the presence of inhibitors of the amplification reaction (9). Recently, ddPCR has also been applied to the quantification of miRNAs circulating in the blood. In particular, Hindson and colleagues (10) found that, for quantifying circulating miRNAs, a prototype of the QX100 Droplet Digital PCR system (Bio-Rad Laboratories) was superior to qPCR carried out with TaqMan miRNA assays (Applied Biosystems and Life Technologies).

Now, a second-generation instrument (named QX200) has been developed that is able to detect both TaqMan probe and DNA-binding dye chemistries with comparable precision and accuracy (11). In that work, ddPCR using the recently commercialized EvaGreen DNA-binding dye was shown to be comparable with TaqMan assays for quantification of mRNA; however, miRNA was not investigated. Therefore, we compared the detection and quantification of two circulating miRNAs using TaqMan and EvaGreen chemistries on the same instrument.

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Materials and Methods

Ethics statement

The study protocol was approved by the Ethics Committee of Ferrara University Hospital. All participants provided written informed consent for the use of their samples for research purposes.

Blood samples and RNA isolation

Peripheral venous blood was collected from 18 persons with any type of cancer and from 10 healthy persons at Ferrara University Hospital. For plasma, 5 mL blood was collected in EDTA (ethylenediaminetetracetic acid) tubes (Vacuette); samples were centrifuged at 1,000 × *g* for 10 minutes to remove blood cells, and the supernatant plasma was dispensed in aliquots. For serum, 5-mL blood was collected in serum tubes (Vacuette), kept at room temperature to clot for at least 60 minutes, and then spun at 1,000 × *g* for 10 minutes; the serum was removed and dispensed in aliquots. Aliquots were stored at −80°C until use.

Total RNA including miRNA was extracted from 200 μL plasma or serum using the miRNeasy Mini Kit (cat. no. 217004; Qiagen) according to the manufacturer's supplementary protocol (12) with two minor variations. Specifically, after the sample was mixed with 1 mL QIAzol Lysis Reagent, 3 μL of a 4.16-nmol/L solution of the synthetic miRNA cel-miR-39-3p from *C. elegans* (custom synthesized by Integrated DNA Technologies) was added. Also, RNA was eluted from spin columns in 35 μL nuclease-free water.

Reverse transcription and ddPCR

For EvaGreen assays, 3 μL RNA was reverse-transcribed in a 20-μL reaction using the Universal cDNA synthesis Kit II (Exiqon) following the company's guidelines for miRNA profiling in serum and plasma. The resulting cDNA was diluted 1:50 for the miR-320a assay and 1:500 for the cel-miR-39-3p and miR-21-5p assays before amplification. PCR was performed in a 20-μL volume containing 10 μL 2X EvaGreen supermix (Bio-Rad), 8 μL diluted cDNA, and 1 or 2 μL of one of the following miRCURY LNA PCR primer sets (Exiqon): hsa-miR-320a (ID 204154), cel-miR-39-3p (ID 203952), hsa-miR-21-5p (ID 204230). Two volumes of primer solution were tested to optimize the assay. To test the sensitivity and accuracy of the assay over a range of target cDNA concentrations, a 2-fold dilution series of cel-miR-39-3p was prepared in water, starting from 3 μL of a 4.16 nmol/L solution, and reverse-transcribed in duplicate.

For TaqMan assays, two circulating human miRNAs (miR-320a and miR-21-5p) and one added control miRNA (cel-miR-39-3p) were reverse-transcribed individually from human plasma and serum samples using TaqMan miRNA Reverse Transcription kits (Life Technologies). For each sample, 5 μL RNA was reverse-transcribed in a 15-μL reaction using the standard protocol and primers specific for the three miRNAs: miR-320a (assay ID, 002277), miR-21-5p (assay ID, 000397), and cel-miR-39-3p (assay ID, 000200). Then, 1.3 μL of the resulting cDNA (undiluted for miR-320a or diluted 1:100 for miR-21-5p and cel-miR-39-3p) was prepared for amplification in a 20-μL reaction volume containing 10 μL 2X ddPCR Supermix for Probes (Bio-Rad) and 1 μL 20X TaqMan miRNA PCR primer probe set.

ddPCR workflow

Each ddPCR assay mixture (20 μL) was loaded into a disposable droplet generator cartridge (Bio-Rad). Then, 70 μL of droplet generation oil for probes (Bio-Rad) was loaded into each of the eight oil wells. The cartridge was then placed inside the QX200 droplet generator (Bio-Rad). When droplet generation was completed, the droplets were transferred to a 96-well PCR plate (Eppendorf) using a Rainin multichannel pipet. The plate was heat-sealed with foil and placed in a conventional thermal cycler. Thermal cycling conditions for EvaGreen assays were as follows: 95°C for 5 minutes, then 40 cycles of 95°C for 30 seconds and 58°C for 1 minute (ramping rate reduced to 2%), and three final steps at 4°C for 5 minutes, 90°C for 5 minutes, and a 4°C indefinite hold to enhance dye stabilization (11). Thermal cycling conditions for TaqMan assays were as follows: 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute (ramping rate reduced to 2%), and a final inactivation step at 98°C for 10 minutes. A no template control (NTC) and a negative control for each reverse transcription reaction (RT-neg) were included in every assay.

Results

Optimization of EvaGreen-based ddPCR for miRNA quantification

EvaGreen assays have not been tested for miRNA quantification on a ddPCR instrument. Therefore, we had to identify the optimal concentrations of miRCURY LNA primers (Exiqon) for the QX200 Droplet Digital system (Bio-Rad). Moreover, because plasma presents a complex background for PCR, we tested the assay using 10 different RNA preparations from plasma of patients with cancer and healthy persons. We quantified both a synthetic miRNA (cel-miR-39-3p) that was added to plasma at the beginning of RNA extraction and a human miRNA (miR-320a) that we knew from microarray experiments (data not shown) to be present in the samples. To avoid positive droplet saturation, target miRNAs were quantified starting from a cDNA dilution of 1:50 for the miR-320a assay and 1:500 for the cel-miR-39-3p and miR-21-5p assays. To optimize the primer concentration, we tested both the recommended volume (2 μL) and half that volume, to see which gave a better separation between positive and negative droplets; this step was motivated by the fact that EvaGreen can bind with low affinity to single-stranded DNA (13). The results of the ddPCR for cel-miR-39-3p in two plasma samples are shown in Fig. 1A and B. There was a higher degree of separation between positive and negative droplets when 1 μL of primer solution was used, with comparable results in terms of quantification (Fig. 1C). The miR-320a assay was also performed using both 2 μL and 1 μL of LNA primers

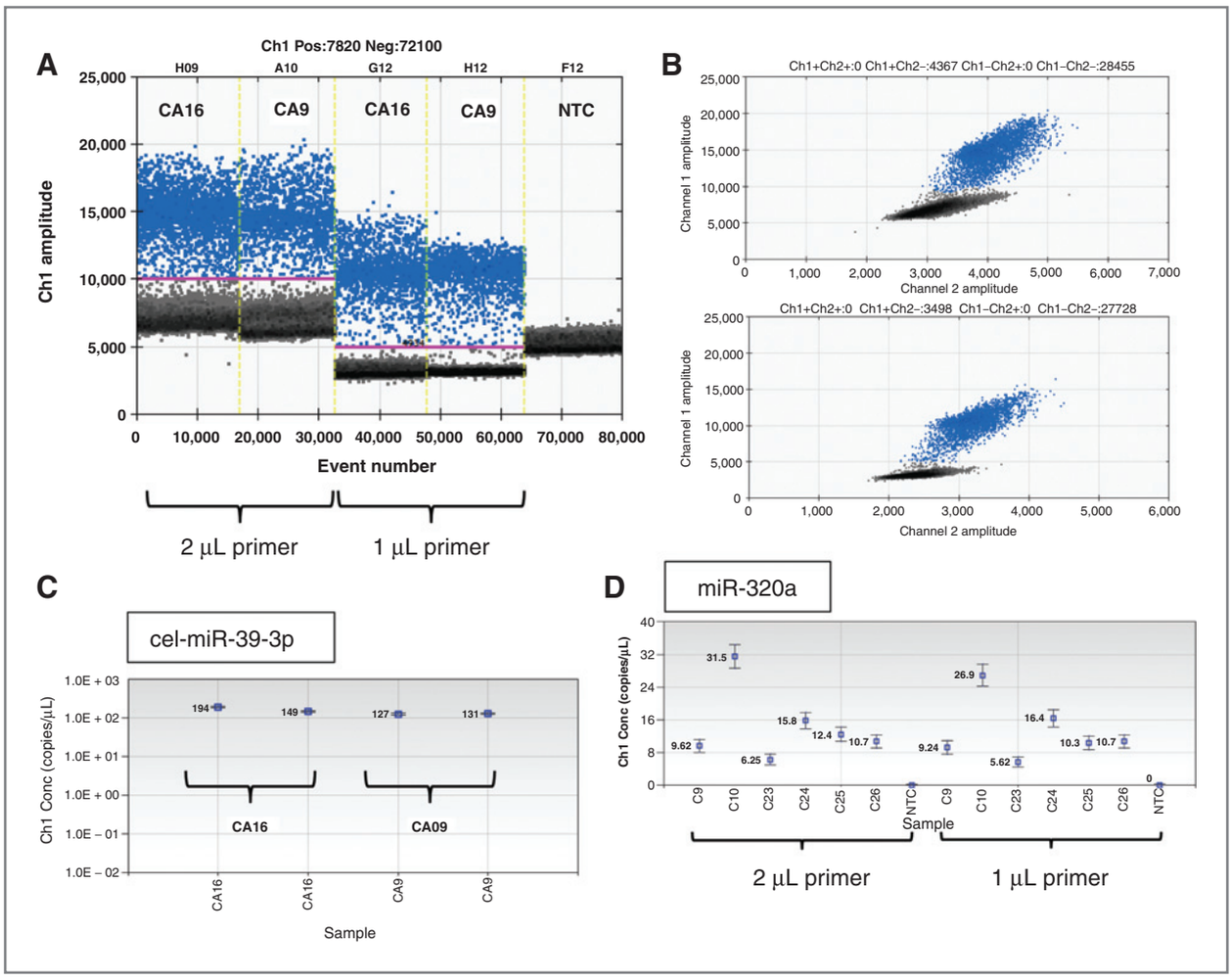

Figure 1. Primer optimization. Effect of LNA primer concentration on the amplitude of positive (blue) and negative (black) droplets in EvaGreen-based ddPCR. Results are presented as copies per microliter in the amplification reaction. A, reducing the volume of primers from 2 µL to 1 µL gives a better performance in terms of droplet separation and reduces the spread of negative droplets, seen for two samples, CA16 and CA9, in the cel-miR-39-3p assay; no positive droplets were obtained for the NTC. B, bi-dimensional droplet plots for the cel-miR-39-3p assay with 2 µL (top) or 1 µL (bottom) of LNA primers. C, reducing the volume of primers for cel-miR-39-3p did not change the quantification results; from the left, normalized copies/µL of CA16 sample with 2 or 1 µL of primer solution and of CA9 sample with 2 or 1 µL of primers. D, EvaGreen quantification of miR-320a in 6 samples using 2 µL LNA primer (left) or 1 µL LNA primer (right). Reducing the amount of primer did not change the quantification results. Error bars, Poisson 95% confidence interval.

and cDNA from 6 samples (Fig. 1D). We obtained almost identical results so we decided to use 1 µL of primer solution in subsequent experiments.

Precision and sensitivity of EvaGreen-based ddPCR

To calculate the within-run and overall precision of EvaGreen-based ddPCR assays, we ran the cel-miR-39-3p assay on 10 RNA samples from plasma in triplicate and we repeated the analysis on 3 days (Fig. 2A). The mean within-run coefficient of variation (CV) was 5.1% (range, 0.7%–10.7%) and the mean overall CV was 13.4% (range, 7.3%–20.4%; Supplementary Table S1).

Then, working in duplicate, we reverse-transcribed the RNA from 8 plasma samples and amplified cel-miR-39-3p (which had been added before RNA extraction) using the EvaGreen-based assay (Fig. 2B). Quantification of this miRNA in the two independent series displayed high concordance (Pearson $r = 0.96$), although we observed a high variability in cel-miR-39-3p recovery. To assess the sensitivity and accuracy of EvaGreen ddPCR at different target cDNA concentrations, we ran the assay in duplicate on serial dilutions of cel-miR-39-3p (10–10,000 copies/µL) in water (Fig. 2C). We found an extremely high concordance between the two series, and the responses were highly linear over four orders of magnitude. Moreover, the assay was able to detect cel-miR-39-3p down to the lower limit of 1 copy/µL.

Comparison of EvaGreen and TaqMan ddPCR for miRNA quantification

To determine if the EvaGreen-based assay gives comparable results to the TaqMan assay on the QX200 ddPCR

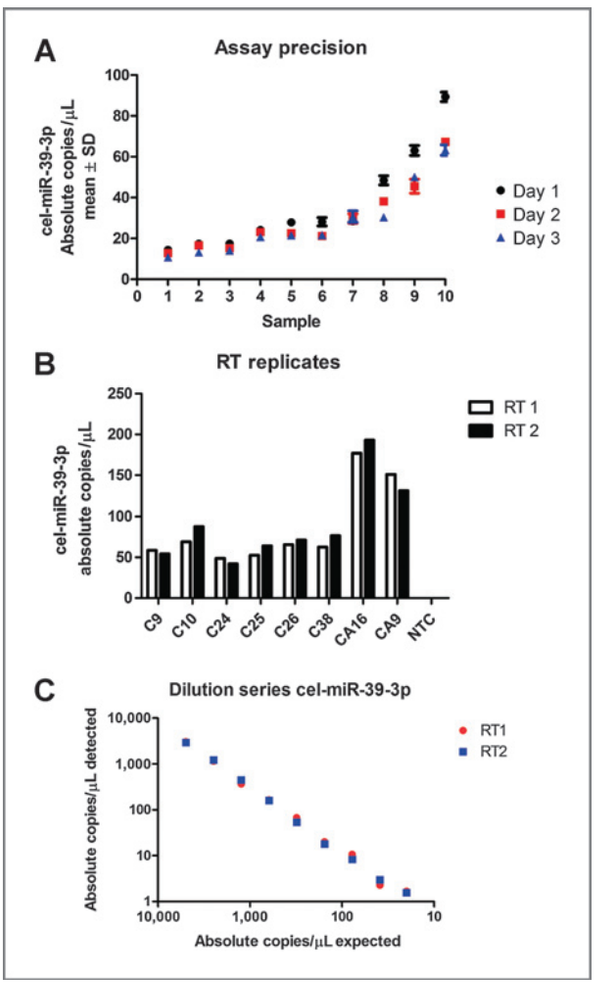

Figure 2. Precision and performance of EvaGreen ddPCR assay. A, cel-miR-39-3p was quantified in triplicate on 3 days in a panel of 10 RNA preparations from plasma to which cel-miR-39-3p had been added. Each dot, average and SD of one day. Day replicates are represented by different colors. Coefficients of variation are reported in Supplementary Table S1. B, replicate analyses of cel-miR-39-3p assay over two independent RT reactions from eight RNA samples and an NTC. Pearson correlation of RT replicates was 0.96. C, quantification of cel-miR-39-3p in a water matrix by EvaGreen ddPCR shows linearity across the dynamic range of miRNA concentrations. Two independent RT reactions and dilution series were performed starting from a known amount of cel-miR-39-3p. Linearity was maintained across four orders of magnitude. The lower limit of detection was 1 copy/μL. Results are presented as copies per microliter of the amplification reaction mixture.

system, we quantified the synthetic cel-miR-39-3p (added as internal control) and the naturally occurring human circulating miR-320a in 10 RNA preparations from plasma. For TaqMan assays, the two miRNAs were reverse-transcribed using miRNA-specific primers and the resulting cDNA was used for amplification diluted 100-fold for cel-miR-39-3p and undiluted for miR-320a. To run the EvaGreen-based assay, the RNA samples were reverse-transcribed using the Exiqon Universal cDNA Synthesis Kit and the resulting cDNA was diluted 500-fold for cel-miR-39-3p and 50-fold for miR-320a.

The quantification, expressed as absolute copies per microliter plasma, was highly concordant between the two assays for both miRNAs (Fig. 3A and B). Pearson correlation coefficients between TaqMan and EvaGreen-based assays were $r = 0.917$ for cel-miR-39-3p and $r = 0.986$ for miR-320a. The concentration of the endogenous miR-320a ranged from 1,500 to 23,000 copies/μL of plasma (Fig. 3B). Finally, we compared the two assays for their ability to quantify another naturally occurring miRNA (miR-21-5p) in serum, where concentrations of endogenous miRNAs are lower than in plasma. This work showed that, in 16 RNA preparations from serum, miR-

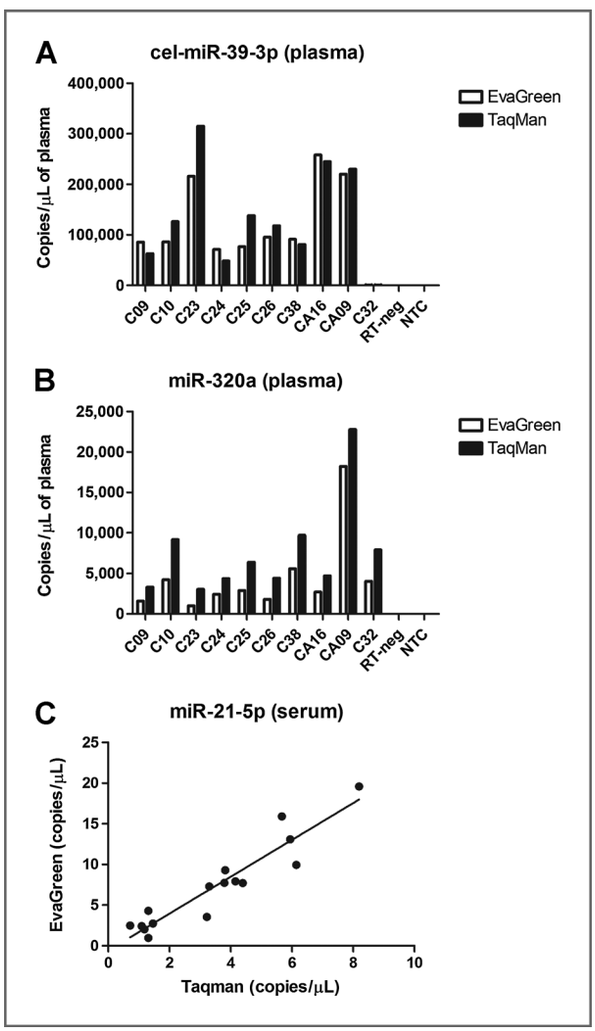

Figure 3. Concordance of miRNA quantification by TaqMan and EvaGreen ddPCR. A, concentrations of exogenous cel-miR-39-3p added to plasma obtained with TaqMan- (black) and EvaGreen–based (white) assays. Results, presented as absolute copies per microliter plasma, were highly concordant for 10 RNA preparations (Pearson $r = 0.917$). B, concentrations of endogenous human miR-320a in plasma using the same assays show high concordance (Pearson $r = 0.986$). C, quantification of miR-21-5p in 16 serum samples using the same assays gives values that are highly concordant (Pearson $r = 0.92$). These results are presented as absolute copies per microliter of amplification reaction.

21-5p was indeed present at low concentrations (Fig. 3C). In this case, the EvaGreen-based assay gave approximately 2-fold higher concentrations in the amplification reaction solution (range, 0.96–19.6 copies/μL) than did the TaqMan assay (range, 1.3–8.2 copies/μL), but there was a high concordance between the two methodologies (Pearson $r = 0.92$).

Discussion

This study demonstrates that two commercial miRNA assays, TaqMan (Life Technologies) and miRCURY LNA (Exiqon), can be successfully used to quantify specific miRNAs in human biofluid. Indeed, although based on different detection chemistries (e.g., TaqMan probes and DNA-binding EvaGreen dye), the assays provided comparable results on the QX200 ddPCR system.

When miRNA assays are performed for diagnostic purposes, TaqMan and miRCURY LNA assays are considered highly reliable because of their sensitivity and precision. It was therefore relevant to understand whether both assays could be run on a ddPCR system. Our results demonstrate that both miRNA quantification methodologies—whether based on probes or DNA-binding dye detection chemistries—can be performed using a digital PCR approach.

Circulating miRNAs are present in blood at extremely low concentrations (14), so they are difficult to quantify with other techniques such as microarray and RNA sequencing. Moreover, the amount of RNA that can be extracted from plasma and serum samples is low. In this context, it is of great importance to be able to quantify any desired miRNA using individual assays and ddPCR technology, without having to do miRNA-specific reverse transcription (as for TaqMan assays). Therefore, a universal cDNA system, like that developed by Exiqon, paired with a specific PCR assay makes the EvaGreen-based ddPCR assay attractive. With this assay, we can combine the advantages provided by digital PCR technologies with the specificities inherent to the use of LNA primers, thus expanding the spectrum of applications of ddPCR technology in the biomarker field.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M. Negrini, M. Ferracin
Development of methodology: E. Miotto, M. Ferracin
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E. Miotto, L. Lupini, E. Callegari
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E. Miotto, E. Saccenti, L. Lupini, M. Ferracin
Writing, review, and/or revision of the manuscript: E. Saccenti, L. Lupini, M. Ferracin
Study supervision: M. Negrini, M. Ferracin

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