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RESEARCH PAPER

Accurate quantitation of circulating cell-free mitochondrial DNA in plasma by droplet digital PCR

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Abstract To establish a method for accurate quantitation of circulating cell-free mitochondrial DNA (ccf-mtDNA) in plasma by droplet digital PCR (ddPCR), we designed a ddPCR method to determine the copy number of ccf-mtDNA by amplifying mitochondrial *ND1* (*MT-ND1*). To evaluate the sensitivity and specificity of the method, a recombinant pMD18-T plasmid containing *MT-ND1* sequences and mtDNA-deleted (ρ^0) HeLa cells were used, respectively. Subsequently, different plasma samples were prepared for ddPCR to evaluate the feasibility of detecting plasma ccf-mtDNA. In the results, the ddPCR method showed high sensitivity and specificity. When the DNA was extracted from plasma prior to ddPCR, the ccf-mtDNA copy number was higher than that measured without extraction. This difference was not due to a PCR inhibitor, such as EDTA-Na_2, an anti-coagulant in plasma, because standard EDTA-Na_2 concentration (5 mM) did not significantly inhibit ddPCR reactions. The difference might be attributable to plasma exosomal mtDNA, which was 4.21 ± 0.38 copies/μL of plasma, accounting for ~19% of plasma ccf-mtDNA. Therefore, ddPCR can quickly and reliably detect ccf-mtDNA from plasma with a prior DNA extraction step, providing for a more accurate detection of ccf-mtDNA. The direct use of plasma as a template in ddPCR is suitable for the detection of exogenous cell-free nucleic acids within plasma, but not of nucleic acids that have a vesicle-associated form, such as exosomal mtDNA.

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Keywords Circulating cell-free DNA · Mitochondrial DNA · Droplet digital PCR

Abbreviations

ccfDNA	Circulating cell-free DNA
ccf-mtDNA	Circulating cell-free mitochondrial DNA
ddPCR	Droplet digital PCR
mtDNA	Mitochondrial DNA
MT-ND1	Mitochondrial *ND1* gene
NIPT	Noninvasive prenatal testing

Introduction

The existence of circulating cell-free DNA (ccfDNA) has been known for over 60 years [1]; however, the clinical significance of ccfDNA was not recognized until 1977 [2]. Thus far, ccfDNA has been investigated as a potential biomarker in noninvasive diagnosis and prognosis, as well as disease monitoring. It was suggested that the release of ccfDNA into the bloodstream occurs from different sources, such as hematopoietic and stromal cells in normal individuals, or tumor cells in cancer patients. In addition, active secretion of DNA carried within exosomes has been demonstrated [3], but it is still unclear whether this is a relevant or negligible source of ccfDNA.

CcfDNA includes nuclear DNA (nDNA) and mitochondrial DNA (mtDNA). Distinct from nDNA, mtDNA exists as a circular, double-stranded nucleic acid with a high copy number. Variations in the copy number of circulating cell-free mtDNA (ccf-mtDNA) have been found in the plasma and serum of patients with various cancers [4–7], trauma [8, 9], diabetes [10], and acute infection [11]. Therefore, assessing the level of ccf-mtDNA could prove significant for monitoring these diseases.

Despite its potential, there are two major hindrances in clinical application of ccf-mtDNA, which are in sample processing method and in the differences of both the DNA extraction methods and quantification technology. Those also are the main hindrances in the determination of ccfDNA [12]. Many extraction methods and commercial kits can be used in ccfDNA studies at present. However, it was demonstrated that the extraction methods for the isolation of ccfDNA from plasma or serum differed in the efficiency. In the quantification technology, to date, the copy number of mtDNA has been detected only by quantitative real-time PCR (qPCR) [13], which depends on the establishment of a standard curve derived from amplification of serially diluted, target gene-containing recombinant plasmid. Plasmid copy number depended on the Cq value and calculated based on the concentration of plasmid DNA; therefore, it can be affected by the accuracy of the concentration measurements or dilution of the plasmid. Moreover, the variability of qPCR is increased, leading to further imprecision and bias at low copy numbers. Therefore, the low copy number of ccf-mtDNA demands a greater quantity of plasma in the quantification by using qPCR.

Droplet digital PCR (ddPCR) is a form of digital PCR that uses a water–oil emulsion droplet system [14], which is capable of detecting rare mutation, and performing absolute quantification of gene copy number and gene expression analysis. With the advantages of high sensitivity, precision, and reproducibility, ddPCR has been applied to noninvasive prenatal testing (NIPT), cancer research, transplantation medicine, and virology [15]. DdPCR relies on an end-point threshold to score each reaction as either positive or negative. The absolute template quantity can then be determined by Poisson statistics. Therefore, for copy number determination, ddPCR is capable of quantitating the sample directly without using a standard curve, requiring less sample input. These features indicate that ddPCR may be suitable for detecting ccf-mtDNA in plasma.

In this study, we applied ddPCR technology to quantify plasma ccf-mtDNA copy number by amplifying human mitochondrial *ND1* (*MT-ND1*). To evaluate the sensitivity and specificity of the method, a recombinant pMD18-T plasmid with *MT-ND1* and DNA from mtDNA-deleted (ρ^0) HeLa cells were used as the templates, respectively. Considering the clinical application, we attempted to utilize plasma directly, instead of the samples with DNA extraction on the basis of accurate detection of mtDNA by ddPCR. Therefore, we designed three different experimental modules to evaluate the accuracy and feasibility of utilizing plasma directly in ddPCR reactions, which was significant for clinical detection. Subsequently, we investigated the detection of plasma exosomal mtDNA with very low copy numbers, which also was a valuable attempt for the studies of exosomes and clinical application.

Materials and methods

Construction of recombinant plasmid

The *MT-ND1* gene was amplified from human mitochondrial DNA by PCR using the following primers: forward 5′ CAGCCGCTATTAAAGGTTCG 3′, reverse 5′ AGAGTGCGTCATATGTTGTTC 3′, which were synthesized by Genscript Corporation (Nanjing, China). With these PCR primers, the fragment length of the amplified product was 1041 bp [nt3017-4057 in *Homo sapiens* mtDNA (GenBank No. NC_012920.1)].

The reaction volume of 20 μL contained 10 μL of 2 × PCR Buffer, 1.5 μL of 25 mM $MgCl_2$, 2.0 μL of a dNTP mixture (25 mM each), 0.1 μL of Ex Taq (5 U/μL) (TaKaRa, Japan), 0.2 μL of each 10 μM primer, 4.0 μL of H_2O, and 2.0 μL of DNA template. PCR was performed on a Bio-Rad S1000™ Thermal Cycler (Bio-Rad, USA). The PCR reaction conditions were as follows: 1 cycle at 95 °C for 5 min and 35 cycles at 95 °C for 30 s, 56 °C for 45 s, 72 °C for 1 min, and finally 1 cycle at 72 °C for 6 min. Subsequently, *MT-ND1* was cloned into the pMD18-T vector by using a pMD™18-T Vector Cloning Kit (TaKaRa, Japan), and the ligation product was transformed into *Escherichia coli* DH5α.

Determination of plasmid copy number

Plasmids were extracted from *E. coli* DH5α with the MiniBEST Plasmid Purification Kit (TaKaRa, Japan), and the concentration was measured on a NanoDrop2000 (Thermo Scientific, USA). The plasmid copy number was calculated as follows:

Formula 1: $MW_{DNA}(\text{daltons}) = (\text{pMD18}-\text{T vector} + \text{insert}) \times 660\ (\text{daltons/bp}) = (2692\ \text{bp} + 1041\ \text{bp}) \times 660\ (\text{daltons/bp}) = 2.46 \times 10^6(\text{daltons})$

Formula 2: $CN_{DNA}(\text{copies}/\mu\text{L}) = C_{DNA}(\text{ng}/\mu\text{L}) \times 6.02 \times 10^{14}/MW_{DNA} = C_{DNA}(\text{ng}/\mu\text{L}) \times 6.02 \times 10^{14}/2.46 \times 10^6 = C_{DNA}(\text{ng}/\mu\text{L}) \times 2.45 \times 10^8$

(CN_{DNA} Plasmid copy number
C_{DNA} Plasmid DNA concentration
MW_{DNA} Plasmid molecular weight)

Then, the plasmids were serially diluted to 10^4, 10^3, 10^2, 10, and 1 copies/μL as the templates.

Quantification of mtDNA copy number by qPCR

The copy number of mtDNA was quantified using qPCR by amplification of the *MT-ND1* gene using the following primers: forward 5′ CCCTAAAACCCGCCACATCT 3′, reverse 5′ GAGCGATGGTGAGAGCTAAGGT 3′; fragment length was 69 bp (*Homo sapiens* mtDNA nt3485-3553). The fluorescent probe used was 5′ (FAM) CCATCACCCTCTAC

ATCACCGCCC (TAMRA) 3′. The probe and primers were synthesized by Genscript Corporation (Nanjing, China).

The qPCR reaction volume of 20 μL contained 10 μL of 2 × HS qPCR Master Mix (TaKaRa, Japan), 0.5 μL of each 10 μM primer, 0.5 μL of the 10 μM probe, 6.5 μL of H_2O, and 2.0 μL of DNA template, which was 1 to 10^4 copies/μL of the recombinant pMD18-T plasmid with the *MT-ND1* insert.

PCR was performed on a Bio-Rad CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA). The reaction conditions were as follows: 1 cycle at 95 °C for 2 min, 40 cycles at 95 °C for 10 s, and 60 °C for 1 min.

ddPCR

The primers and probe utilized in the ddPCR analysis were listed previously, though the quencher was changed to BHQ1. The ddPCR reaction mixture volume of 20 μL contained 10 μL of 2 × Supermix (no dUTP), 1.8 μL of each 10 μM primer, 1 μL of the 10 μM probe, 2 μL of DNA template and/or plasma, and 3.4 μL of H_2O. 1 to 10^4 copies/μL of recombinant plasmids were used for testing the sensitivity. Specificity of the mtDNA assay was determined by using DNA from the ρ^0 HeLa cells that lacks mtDNA. As a negative control, 2 μL of H_2O was added separately to the ddPCR reaction instead of template DNA.

The PCR reaction conditions were as follows: 1 cycle at 95 °C for 10 min, 40 cycles at 95 °C for 30 s, 60 °C for 1 min, and 98 °C for 10 min. Droplet reading was performed on a Bio-Rad QX200 droplet digital PCR system (Bio-Rad, USA), and analysis was performed with Bio-Rad QuantaSoft software version 1.3.2.

Subjects

To apply the ddPCR method on plasma samples, 38 healthy volunteers, 20 males and 18 females, aged from 20–30 years, were recruited from the Medical Center in the First Affiliated Hospital of Wenzhou Medical University of Zhejiang Province in China. The study was approved by the hospital's Ethics Committee, and methodologies conformed to the standards set by the Declaration of Helsinki.

Plasma cell-free DNA extraction

Blood samples were collected from the healthy volunteers into EDTA-Na_2-containing tubes, and the plasma was separated. Plasma DNA was extracted from 250 μL of plasma using a Plasma Cell-Free DNA Isolation Kit (Maibo Biotech, China), which uses a magnetic bead method.

Briefly, 250 μL of plasma was incubated with 20 μL of 20 mg/mL Proteinase K at 37 °C for 15 min; then, 300 μL of lysis buffer, 5 μL of acryl carrier, and 20 μL of magnetic beads were added, followed by mixing for 30 min. The tube was then loaded on the magnetic separator (Sigma, USA) for 1 min to capture the beads, and the supernatant was discarded. Next, 550 μL of washing buffer was used to wash the beads three times. Finally, the DNA was eluted from the beads into 50 μL of elution buffer.

Evaluation of plasma ccf-mtDNA detection by ddPCR

To evaluate the potential of utilizing plasma directly as the source of ddPCR templates, plasma preconditioning was performed first. Briefly, the plasma samples from 10 subjects were centrifuged at 1600*g* for 10 min; then, the supernatant from each sample was divided into two aliquots. One of the aliquots was centrifuged at 16,000*g* for 10 min (method 1), and the other was ultrafiltered through a 0.22-μm filter (Millipore, USA, method 2).

Further, to confirm the accuracy of the results obtained when plasma was utilized as the template source in ddPCR reactions, we set three experimental modules.

Module 1: EDTA-Na_2 is a commonly used anticoagulant, and it is also an inhibitor of PCR. In the clinical lab, the standard concentration of EDTA-Na_2 as an anticoagulant in plasma is 5 mM. Therefore, to evaluate the effects of EDTA-Na_2 on ddPCR, 50, 100, 150, 200, and 250 mM EDTA-Na_2 was added separately into recombinant plasmid samples (100 copies/μL) at a ratio of 1:9 and diluted to a final EDTA-Na_2 concentration of 5, 10, 15, 20, or 25 mM.

Module 2: To evaluate the effects of plasma samples on ddPCR, known copy numbers of plasmid, 12.41, 83.45, 318.15, or 1050.20 copies/μL (Log_{10}: 1.09, 1.92, 2.50, 3.02 copies/μL), as determined previously by ddPCR, were added separately into plasma samples from 10 subjects at a ratio of 1:1, as group 1, 2, 3, and 4, respectively. Then, these four groups of plasma samples and the original plasma samples were detected simultaneously by ddPCR. The different copy number values were calculated according to Formula 3 and compared with that of the added plasmid.

Formula 3: $\mathrm{Log}[\mathrm{Calculated}CN_{mtDNA}] = \mathrm{Log}[CN_{mtDNA}\mathrm{in}(\mathrm{plasma} + \mathrm{added\ plasmid}) - CN\mathrm{mtDNA}\mathrm{in}(\mathrm{plasma} + H_2O)]$

CN_{mtDNA} mtDNA copy number

Module 3: To evaluate the feasibility of plasma as the template in ddPCR, plasma samples from the same subjects, with or without DNA extraction, were analyzed by ddPCR and compared.

Exosomal isolation, identification, and DNA extraction

Plasma samples from 18 healthy volunteers were collected and preconditioned using method 1 (centrifugation at 1600*g* for 10 min and 16,000*g* for an additional 10 min) described above and then ultrafiltered with a 0.22-μm filter (Millipore, USA). Subsequently, 250 μL of pretreated plasma was incubated with 2.5 μL of 500 U/mL thrombin (SBI, USA) at 37 °C for 5 min and then centrifuged at 10,000*g* for 5 min. Next, ExoQuick exosome precipitation solution (SBI, USA) was added at a ratio of 4:1 and incubated overnight at 4 °C, after which, the solution was centrifuged at 1500*g* for 30 min, and the supernatant was discarded. The precipitate was centrifuged again for 5 min, and any remaining supernatant was removed. To obtain pure exosomes, the precipitate was resuspended in 200 μL of PBS. Subsequently, 50 μL of ExoQuick exosome precipitation solution was added to isolate exosomes a second time, with final resuspension in 250 μL PBS.

To identify the exosomal fraction, exosomes were observed with a transmission electron microscope (TEM) by negative staining. Exosome-specific markers Tsg101 and CD9 were analyzed by Western blotting.

Before the extraction of exosomal DNA, one unit of DNase I was added to the isolated exosomal precipitates resuspended in PBS, and the reaction mixtures were incubated at 37 °C for 1 h to digest extra-exosomal DNA. Then, DNA was extracted with the Plasma Cell-Free DNA Extraction kit by the magnetic bead method as described before. Finally, DNA was eluted into 50 μL elution buffer.

Statistical analysis

Statistical analysis was performed by SPSS 17.0. All copy numbers were converted to Log_{10} (copy numbers) per microliter of samples and expressed as the mean with standard deviation. Linear regression was applied to analyze the relationship between the qPCR and ddPCR results and between the results for plasma, with or without prior DNA extraction. The Mann–Whitney rank test was used for data with an abnormal distribution; a P value of < 0.05 was considered statistically significant.

Results

Copy number determination of recombinant plasmids by qPCR and ddPCR

The *MT-ND1* gene (1041 bp) was amplified by PCR (Fig. 1a), cloned into the pMD18-T vector, and verified by sequencing (Fig. 1b).

By utilizing specific primers and a probe (as shown in Fig. 1b), qPCR and ddPCR were performed to detect the *MT-ND1* sequences of the recombinant plasmid from 1 to 10^4 copies/μL, as shown in Fig. 2a–c, and a linear correlation was seen between the two methods (Fig. 2d). However, when the plasmid copy number was 10 and 1 copies/μL, the Cq value was as much as 31.4 and 34.4, respectively (Fig. 2b). This discrepancy indicated that, at low copy number, qPCR did not detect the plasmid accurately, but ddPCR did detect it quantitatively (Fig. 2c). Additionally, DNA from ρ^0 HeLa cells was not amplified, confirming the specificity of the ddPCR detection method (Fig. 2e).

Preconditioned plasma mtDNA copy number detection by ddPCR

To determine the effect of different preconditioning methods on the analysis, we compared mtDNA copy number detection from plasma after two preconditioning methods. There was no significant difference between the two methods in detecting mtDNA copy number from 50 μL plasma DNA. With method 1, there were 157.50 ± 24.16 (Log_{10}: 2.17 ± 0.06) copies/μL, and with method 2, we detected 168.83 ± 31.95 (Log_{10}: 2.19 ± 0.09) mtDNA copies/μL (Fig. 3a).

Detection of plasma ccf-mtDNA copy number by ddPCR

In the *module 1* experiments, plasmid samples with different final concentrations (5, 10, 15, 20, and 25 mM) of EDTA-Na_2 were used to evaluate the potential inhibitory effect of EDTA-Na_2 on ddPCR. The results indicated that EDTA-Na_2 did not inhibit amplification by ddPCR until the concentration reached 20 mM or higher, well over the standard of 5 mM (Fig. 3c). In the experiments of *module 2*, the initial and calculated plasmid copy number difference was similar in all groups except group 1, which had a low plasmid copy number (Log_{10}: 1.090 copies/μL) (Fig. 3d). The results from *module 3* experiments indicated that the amount of mtDNA was higher in plasma samples with extraction than those without extraction (Fig. 3b).

Detection of plasma exosomal mtDNA by ddPCR

After purification of exosomes, we observed 30–100 nm diameter microvesicles by TEM (Fig. 4a). Additionally, the exosome-specific markers Tsg101 and CD9 were detected by Western blotting, as shown in Fig. 4b.

In these validated exosome samples from 18 subjects, the mean copy number of exosomal mtDNA was 4.21 ± 0.38 (Log_{10}: 0.59 ± 0.04) copies/μL plasma. For determining the proportion of exosomal mtDNA in plasma ccf-mtDNA, the plasma ccf-mtDNA copy number in the samples was detected simultaneously (Fig. 4c). The results demonstrated that the mean copy number of plasma ccf-mtDNA was 23.52 ± 2.14 (Log_{10}: 1.34 ± 0.04) copies/μL, and the proportion of exosomal mtDNA in plasma ccf-mtDNA was 0.193 ± 0.018.

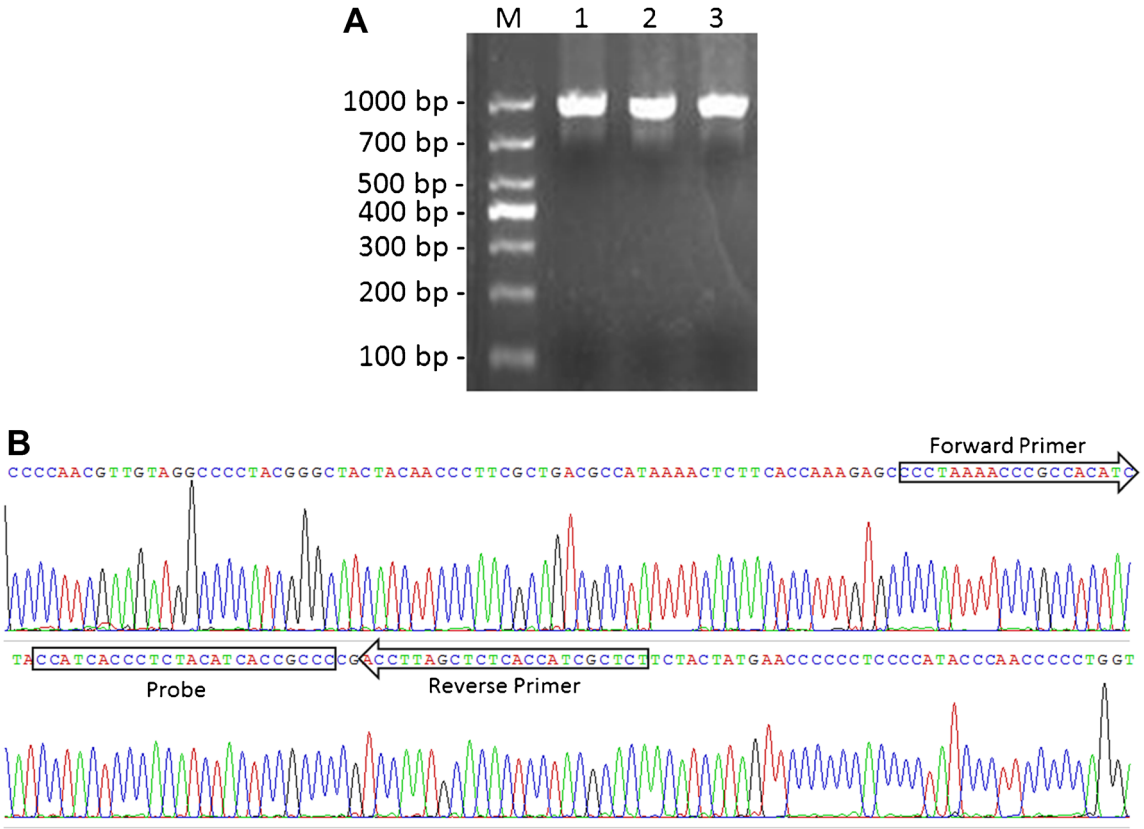

Fig. 1 Verification of the recombinant plasmid with the *MT-ND1* gene insert. **A** Agarose gel electrophoresis of *MT-ND1* PCR products, *lines 1–3* are the PCR products from three different *E. coli* colonies separately. **B** Partial sequence of the *MT-ND1* insert DNA, np3485-3553 (69 bp), which corresponds to the primers and probe sequence for detecting mtDNA copy number by qPCR and ddPCR

Discussion

To date, the use of ddPCR in mtDNA analysis has predominantly focused on the detection of deletions and rare heteroplasmic mutations [16, 17], with few studies focused on ccf-mtDNA levels [18]. Compared with the detection of mutations, ccf-mtDNA copy number quantification showed easier in technology. Moreover, ccf-mtDNA has higher copy numbers than nuclear genes in the cells, which means the mild variations of the mtDNA copy number may denote the changes in the body efficiently.

However, the quantitative determination process of ccf-mtDNA still lacks more convenient and precise methods in DNA extraction and detection. QPCR-based absolute quantification is the most commonly used in ccf-mtDNA quantitative method, which needs the establishment of a standard curve as well as the accuracy and stability of qPCR decreased in the detection of low copy numbers. Compared with traditional qPCR, ddPCR is more convenient, allowing direct quantitation without a standard curve, therefore alleviating the need for reference material required. ddPCR as well demonstrated high sensitivity and precision even in analyzing the trace nuclear acid which is very significant for clinical analysis.

Considering these advantages of ddPCR, in this study, we established a ddPCR method to detect ccf-mtDNA copy number directly from plasma and showed that ddPCR could even detect exosomal mtDNA in very low copy numbers, relevant to disease monitoring and prognosis.

To evaluate the validity of the ddPCR method, the sensitivity and specificity were tested first in our present study. A total of $1\sim10^4$ copies/μL of MT-ND1-pMD18 recombinant plasmids were detected by qPCR and ddPCR separately for testing the sensitivity. The results showed that ddPCR was capable of detecting 1 copy/μL of template, beyond the threshold of accurate detection by qPCR (Fig. 2a–d) [19], demonstrating high sensitivity and accuracy for the ddPCR method of detecting mtDNA. Specificity was proved by the data that DNA from the ρ^0 cells was undetectable by ddPCR (Fig. 2e). Therefore, in further experiments, we attempted to detect plasma ccf-mtDNA from healthy volunteers by using the ddPCR method.

Plasma is a complex biological fluid containing a variety of proteins, ions, nutrients, hormones, and so on, at least some of which can inhibit amplification by PCR. Thus, plasma DNA is extracted routinely prior to analysis by PCR. However, considering the high inhibitor tolerance of ddPCR [20], we performed ddPCR utilizing plasma without DNA extraction,

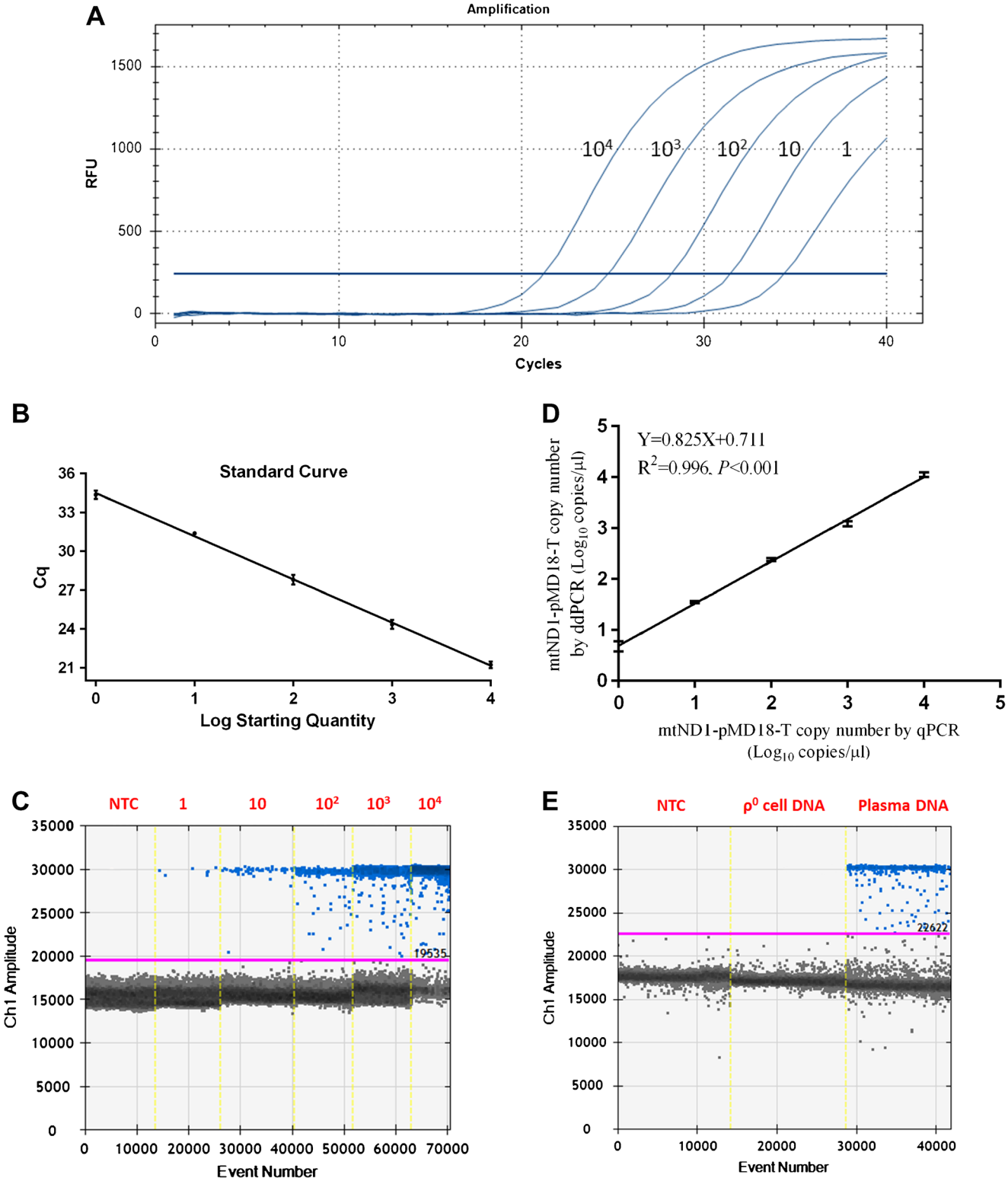

Fig. 2 Quantification of the mtDNA copy number by qPCR and ddPCR. **A**, **B** Amplification and standard curves of the recombinant plasmids by qPCR. **C** 1-D plots of the ddPCR assays with recombinant plasmids as templates. *NTC*: negative control. *Blue dots* represent positive droplets containing a DNA template, *black dots* represent negative droplets without a DNA template, and the *pink line* is the threshold line. **D** Linear correlation between the mtDNA copy numbers by qPCR and ddPCR. **E** 1-D plots of ddPCR assays for the negative control, DNA from ρ^0 cell line, and extracted plasma DNA

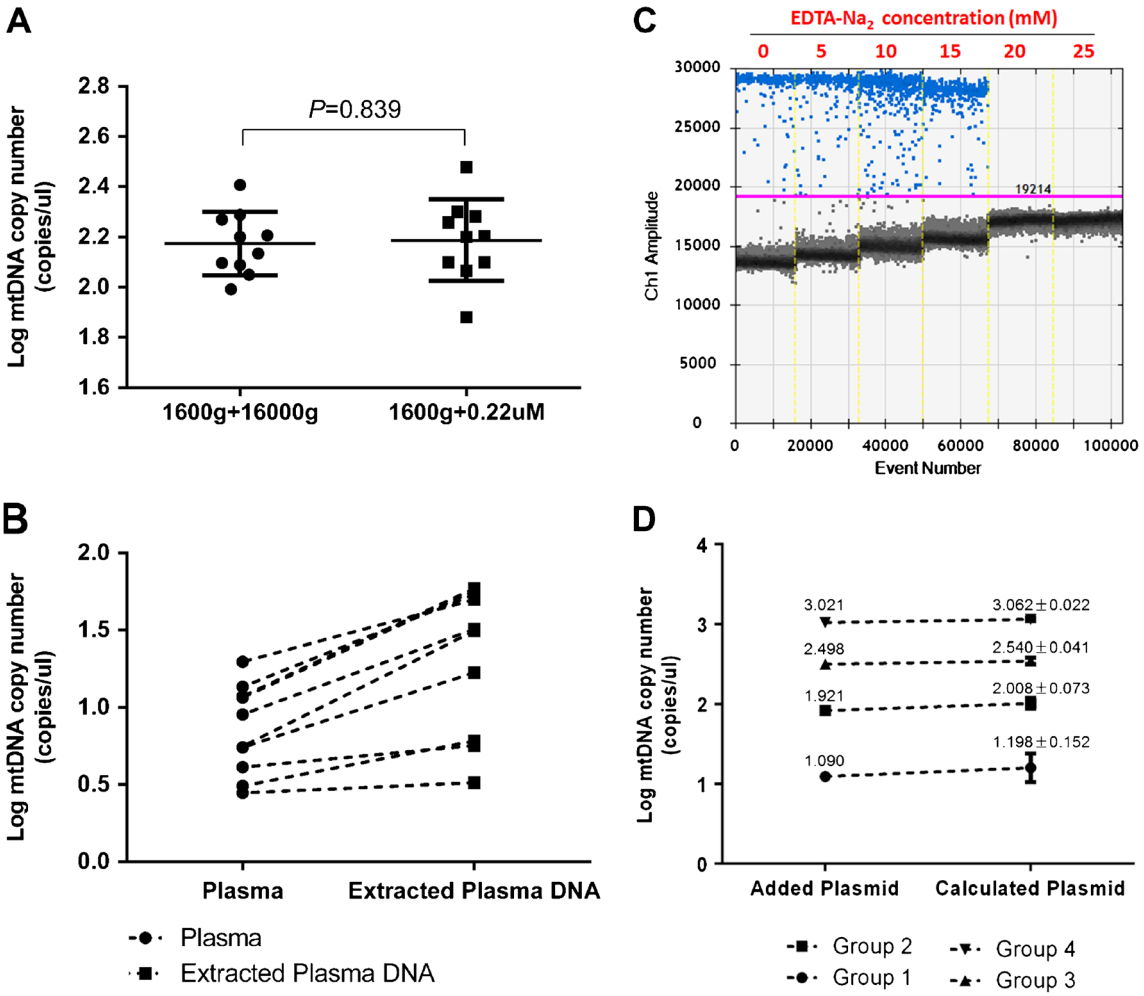

Fig. 3 mtDNA copy number of plasma samples and extracted plasma DNA detected by ddPCR. **A** mtDNA copy number of plasma with two preconditioning methods. **B** mtDNA copy number of plasma samples with or without DNA extraction from the same subjects ($n = 10$). **C** 1-D plot of plasmids with different concentration of EDTA-Na_2. **D** mtDNA copy number of added plasmid, experimental and calculated plasmid values for the plasma sample

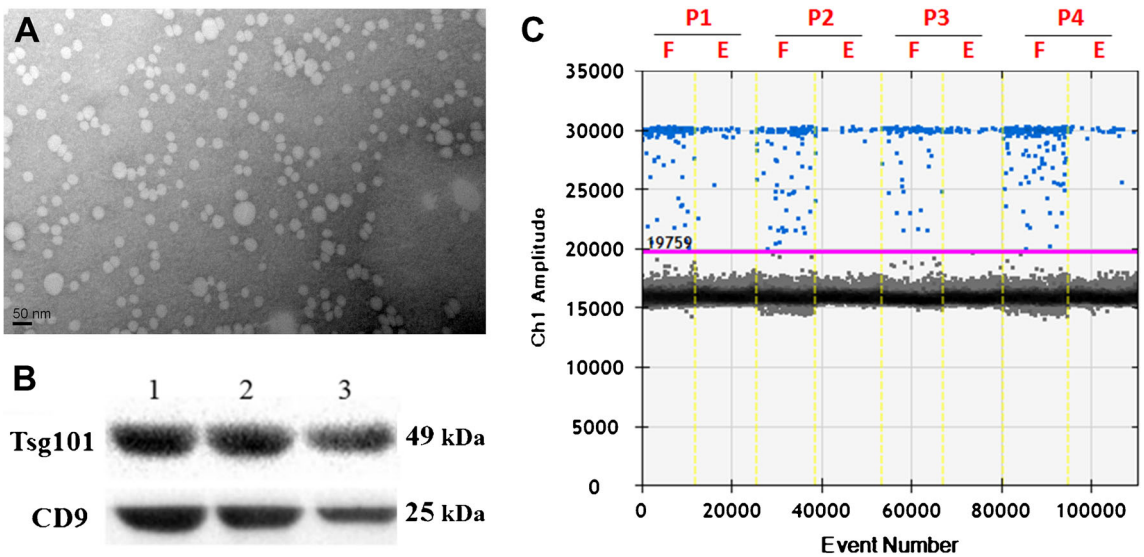

Fig. 4 Plasma ccf-mtDNA and exosomal mtDNA detected by ddPCR. **A** Plasma exosomes observed by TEM. **B** exosome-specific marker, Tsg101, CD63, and CD9 analyzed by Western blotting; *lanes 1–4* represent plasma exosomes from four healthy volunteers. **C** 1-D plot of extracted plasma ccf-mtDNA and exosomal mtDNA. *P*: plasma, *F*: free mtDNA, *E*: exosomal mtDNA

directly as a source of template. To remove residual cells or large vesicles that might affect the formation of 250-μm-diameter droplets in ddPCR, plasma was preconditioned first through centrifugation or by ultrafiltration (method 1 or method 2). The results indicated that there were no significant differences between the data of the two methods (Fig. 3a). Though DNA extraction process might lead the loss of DNA, our data showed that the mtDNA copy numbers obtained by using plasma directly in the ddPCR reaction were lower than the copy numbers obtained by using extracted plasma DNA (Fig. 3b), suggesting factors in the plasma samples might affect the results, and the common PCR inhibitors or other underlying factors were considered first.

EDTA-Na_2 is commonly used as an anticoagulant in clinical labs. However, it can also act as a PCR inhibitor by virtue of its ability to chelate Mg^{2+} ions, and it is possible that residual EDTA-Na_2 may be present in plasma samples. Dingle et al. [20] showed that ddPCR was more tolerant to inhibition caused by SDS and heparin than qPCR. To evaluate the effect of EDTA-Na_2 on ddPCR, the MT-ND1-pMD18 plasmids were quantitated in the presence of various concentrations of EDTA-Na_2 in our study. The data showed that DNA amplification by ddPCR was not inhibited below 20 mM of EDTA-Na_2 in the detection of 100 copies/μL of plasmid (Fig. 3c). This is higher than the concentration that is typically used clinically, suggesting that ddPCR can tolerate the conventional dose of EDTA-Na_2 without affecting DNA copy number quantification. The data also revealed that this PCR inhibitor was not the main factor leading to the difference in detection of mtDNA from plasma with and without DNA extraction.

Further, in the experiments of *Module 2*, the MT-ND1-pMD18 plasmids at a known copy number were added to plasma samples. From the collected data, the calculated copy number difference (according to Formula 3) was consistent with the copy number of the added plasmid (Fig. 3d), which indicated that the composition of plasma has little effect on the linearity of the ddPCR reactions. On the other point of view, it suggested that ddPCR might be allowed to detect exogenous nucleic acids in plasma, such as microbial DNA. It is significant to monitor infectious diseases in a timely and cost-effective manner because DNA extraction is not required.

Considering that plasma ccfDNA might be found in free form and a vesicle-associated form [21], the vesicle-associated mtDNA in plasma was evaluated next in our study. Exosomes [22–24] are one type of extracellular vesicles, 30–100 nm in diameter, assembled in the cytoplasm, and released into the extracellular environment upon fusion with the plasma membrane [25]. Exosomes contain proteins, RNA, and DNA, including mtDNA [26, 27], which could contribute to the overall level of ccf-mtDNA in plasma. The copy number of exosomal mtDNA was considered previously to be very low [28]. In our study, it was possible to detect low copy number mtDNA by ddPCR, indicating that ddPCR has the advantage over conventional qPCR in detecting trace amounts of DNA because of its high sensitivity. Our results demonstrated that the proportion of exosomal mtDNA in plasma ccf-mtDNA was 0.193 ± 0.018, and thus, it was an important component of ccf-mtDNA, more prevalent than described in a previous study [28]. Reasons for this difference might be the application of different protocols for isolating exosomes [29] and/or the use of different methods for detecting mtDNA.

Consequently, when using plasma samples directly without a DNA extraction step, mtDNA may not be released from exosomes completely at the denaturing temperature used in PCR reaction (such as 95 °C) and hence will be undetectable in ddPCR. Likewise, this could partly explain the higher level of mtDNA in plasma with prior DNA extraction compared to plasma without prior extraction.

In our study, we considered several common potential impacts and evaluate the effectiveness of the ddPCR method comprehensively, which may be valuable in future clinical application. Due to its high sensitivity, ddPCR method may be especially applicable on analyzing rapid increase of ccf-mtDNA and monitoring the disease, such as the early stage of transplantation [30], or some chronic disease, like diabetes, cancers, and aging, which demonstrated higher level of ccf-mtDNA, relevant to the chronic inflammation and development of the diseases [10, 31]. Ccf-mtDNA results from cellular apoptosis and/or necrosis [32]. Given that severe mitochondrial damage can lead to cell death by necrosis or activation of the apoptotic signaling pathway, increased ccf-mtDNA might be associated with mitochondrial dysfunction or oxidative stress. Therefore, the quantitative assessment of ccf-mtDNA by ddPCR may also allow sensitive evaluation of mitochondrial damage. Additionally, the clinical value of detecting exosomal mtDNA should be taken into account. Increased plasma exosomal mtDNA may be correlated with the release of exosomes, which has been observed under some conditions, such as oxidative stress and myocardial infarction [33, 34].

Conclusion

In summary, ddPCR can give the initial copy number directly, independent on the Cq value, and without establishment of standard curve. Therefore, ddPCR was proven to be simpler and more sensitive than qPCR and suitable for quantitatively detecting plasma ccf-mtDNA, even plasma exosomal mtDNA, which is present in low quantity. In addition, ddPCR was not affected by inhibitors, such as EDTA-Na_2. Plasma was not suitable for use directly in ddPCR for the detection of nucleic acids with a vesicle-associated form, such as exosomal mtDNA. As such, it was more feasible to perform a DNA extraction on plasma and then use the extracted DNA in the ddPCR reaction. However, plasma without extraction could be used directly in ddPCR for detecting exogenous nucleic acids.

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Compliance with Ethical Standards We state that all blood samples were collected from the individual participants, who were informed and voluntarily participated in this research work.

Conflict of interest All authors declare that they have no conflicts of interest to this work.

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