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Digital Polymerase Chain Reaction for Assessment of Mutant Mitochondrial Carry-over after Nuclear Transfer for In Vitro Fertilization

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BACKGROUND: The quantification of mitochondrial DNA heteroplasmy for the diagnosis of mitochondrial disease or after mitochondrial donation, is performed mainly using next-generation sequencing strategies (NGS). Digital PCR (dPCR) has the potential to offer an accurate alternative for mutation load quantification.

METHODS: We assessed the mutation load of 23 low-input human samples at the m.11778 locus, which is associated with Leber's hereditary optic neuropathy (LHON) using 2 droplet digital PCR platforms (Stilla Naica and Bio-Rad QX200) and the standard NGS strategy. Assay validation was performed by analyzing a titration series with mutation loads ranging from 50% to 0.01%.

RESULTS: A good concordance in mutation rates was observed between both dPCR techniques and NGS. dPCR established a distinctly lower level of background noise compared to NGS. Minor alleles with mutation loads lower than 1% could still be detected, with standard deviations of the technical replicates varying between 0.07% and 0.44% mutation load. Although no significant systematic bias was observed when comparing dPCR and NGS, a minor proportional bias was detected. A slight overestimation of the minor allele was observed for the NGS data, most probably due to amplification and sequencing errors in the NGS workflow.

CONCLUSION: dPCR has proven to be an accurate tool for the quantification of mitochondrial heteroplasmy, even for samples harboring a low mutation load (<1%). In addition, this alternative technique holds multiple benefits compared to NGS (e.g., less hands-on time, more straightforward data-analysis, and a lower up-front capital investment).

Introduction

Mitochondria are organelles responsible for adenosine triphosphate (ATP) production in eukaryotic cells (1). They possess their own genome, the mitochondrial DNA (mtDNA), which is characterized by non-Mendelian inheritance, as it is exclusively transmitted through the female germline (2). The total mtDNA copy number in a single cell varies heavily among cell types, with copy numbers as high as 600 000 being reported for mature oocytes, whereas a sperm cell contains about 100 copies (3, 4). A human cell can harbor a mixture of non-identical mtDNA copies, which is referred to as 'heteroplasmy' (5). It has been observed that the level of heteroplasmy can rapidly shift within and between generations as mothers transmit a varying allele frequency to their offspring (6, 7). The exact mechanism explaining this stochastic process, called the mitochondrial bottleneck, has not yet been completely elucidated (8–10).

The mitochondrial bottleneck explains how a healthy woman can give birth to a child affected by an mtDNA disease. These neurometabolic disorders generally manifest only when a certain threshold of mutation load is exceeded, which is, for most mtDNA diseases,

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between 60% and 80% (11–13). The most frequently occurring maternally inherited mitochondrial disease is Leber's hereditary optic neuropathy (LHON) (14), with a prevalence of about 1 in 45 000 in the European population (15). The main clinical symptom is progressive loss of central vision, due to degeneration of the optic nerve and the retinal ganglion cell layer (16).

Germline nuclear transfer (NT), also known as mitochondrial donation, has gained more attention as a strategy to prevent the transmission of mtDNA disease from affected mothers to their offspring (17–19). This method involves the transfer of the nucleus from an oocyte or a zygote with mutated mtDNA to an enucleated donor oocyte or zygote with wild-type mtDNA. Multiple techniques for NT exist, such as maternal spindle transfer (ST) and early pronuclear transfer (ePNT). However, during both applications, not only the nuclear material is transferred, but also a very limited amount of cytoplasm surrounding this nuclear material, which inevitably also contains mtDNA. This phenomenon is called mtDNA carry-over, and unproportional mtDNA amplification could then lead to heteroplasmic mtDNA drift (19, 20). This necessitates the need for accurate assessment of mtDNA mutational load.

Analysis of mtDNA heteroplasmy for diagnosis or quality control after NT has almost exclusively been realized by targeted next-generation sequencing (NGS). This time-consuming, labor-intensive technology requires an up-front capital investment and highly trained staff. Herein, we investigate the potential of digital polymerase chain reaction (dPCR) as a suitable alternative for NGS. To perform dPCR, the assessed sample is divided over thousands of separate reaction compartments, mostly droplets or micro-chambers. Upon limiting dilution, the number of positive partitions for both alleles is counted after performing end-point PCR. Quantification of both alleles is performed based on the Poisson distribution. dPCR has already shown its capability of sensitively and precisely detecting minor alleles (21). The suitability of dPCR for mtDNA heteroplasmy assessment was evaluated by genotyping the LHON-associated m.11778 G > A mutation using two dPCR systems (Stilla Naica and Bio-Rad QX200) and NGS for 23 low-input human samples, comprising patient samples, nonpatient samples, and samples resulting from NT.

Materials and Methods

SAMPLE PROCESSING

To assess the potential of dPCR for heteroplasmy assessment, 3 types of samples were analyzed: (i) patient samples harboring a very high mutation load (samples 1–13); (ii) homoplasmic wild-type samples donated by a healthy volunteer (samples 14–16); and (iii) samples that underwent NT, thus carrying a low mutation load due to mtDNA carry-over (samples 17–23). A detailed overview of all samples can be found in Supplemental File 1. The samples were transferred into a separately labeled 200 µL PCR tube (Westburg BV) containing 10 µL of PicoPure DNA extraction buffer with proteinase K (PicoPure DNA extraction kit, Thermo Fisher Scientific Inc.). All samples were incubated at 65 °C for 3 h, centrifuged briefly and heated at 95 °C for 10 min to inactivate proteinase K. Due to the low quantity of genetic material in the samples, no extraction replicates could be performed. One extraction blank was included. No nucleic acid quality control could be performed as material was scarce. The use of human oocytes and embryos was approved by the Ghent University Hospital Ethical Committee (EC 2016/0871) and the Belgian Federal Commission for medical and scientific research on embryos in vitro (FCE-ADV_071_UZ Gent).

HETEROPLASMY ASSESSMENT USING NGS

Amplicons comprising the m.11778 locus were generated by PCR amplification using 2.5 µL undiluted DNA extraction product. Forward (5′-TTCAATCA GCCACATAGCCC) and reverse (5′-TGTGTTGTG GTAAATATGTAGAGGG) primers were added at a final concentration of 1 µmol/L each. PCR was performed in a total volume of 10 µL, containing 1× Kapa2G Robust Master Mix (Kapa Biosystems). After initial denaturation at 95 °C for 3 min, 35 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 10 s and elongation at 72 °C for 15 s were performed. A final elongation step of 1 min at 72 °C was performed. The resulting amplicons were purified using AMPure XP beads (Beckman Coulter Inc.) and quantified using the Quant-iT™ PicoGreen™ dsDNA Assay (Thermo Fisher). DNA libraries for sequencing were prepared with the Nextera XT DNA Library Preparation Kit and Nextera XT Index Kit v2 Set A and Set B (Illumina Inc.), according to the manufacturer's recommendations. After purification using AMPure XP beads, library quantification with the Sequencing Library qPCR Quantification Kit (Illumina) was performed. Three equimolar pools were prepared from the libraries, followed by three paired-end sequencing runs on a MiSeq sequencer using a MiSeq Reagent Kit v2 (500 cycles) (Illumina).

Sequencing read quality was checked with FastQC (v0.11.5; Babraham Institute). Adapter removal and quality trimming of the reads were done using cutadapt (v1.15) (22) with a phred-score threshold of 20 and removal of all read pairs containing ambiguities. The trimmed, overlapping forward and reverse reads of each pair were merged into fragments using PEAR (v0.9.11) (23) with default settings. Merged reads were mapped

on the human mitochondrion reference genome (NG_012920.1) using bowtie2 (v2.3.4) (24). Coverage depth of all alleles was determined from the alignment bam files using igvtools (v2.3.98) (25).

HETEROPLASMY ASSESSMENT USING dPCR

A SNP-genotyping assay was developed for the m.11778 G > A variant (GenBank accession number NC_012920.1), based on TaqMan™ minor groove binding (MGB) hydrolysis probes. Assay design was performed using the custom design tool of TaqMan™. Primer specificity was checked by NCBI Primer-BLAST. Amplification results in an amplicon of 93 nucleotides (m.11725—m.11817). Primer and probe sequences (Thermo Fisher) can be found in Table 1. A 1:30 dilution of the extracted samples in nuclease-free water was prepared. The absolute DNA concentration of these samples was too low to quantify, as some of the analyzed samples consist of extracted DNA of a single oocyte. Aliquots of 20 µL were stored at -20 °C. After optimization of sample input volumes, 2 µL of the 1:30 dilution was used as input material for both dPCR platforms. dPCR was initially performed using the droplet-based Naica System (Stilla Technologies), followed by transfer of the assay to the droplet-based Bio-Rad QX-200 (Bio-Rad Laboratories Inc). The guidelines for the Minimum Information for Publication of Quantitative Digital PCR Experiments (dMIQE2020) were adopted for experimental design, execution, data-analysis and reporting (Supplemental File 2) (26).

For the Naica system, a reaction volume of 25 µL, containing 1X Perfecta Multiplex qScript ToughMix (VWR International, Cat. No. 733-2324), 0.25 µmol/L of both probes, 0.9 µM of both primers, 0.1 µmol/L fluorescein (Merck KGaA), and 2 µL sample was loaded in the Sapphire chip (Stilla). No optimization of the primer or probe concentration could be performed as the primer probe mix was provided pre-mixed. A Naica Sapphire chip has 4 reaction cavities, and 3 chips can be analyzed in parallel. During each run, at least 1 no-template control (NTC) sample was included, containing the reaction mixture and nuclease-free water. An extensive validation of the dPCR assay was performed on the Naica platform by analyzing 3 independently prepared titration series of the synthetic mixture samples (gBlocks™, IDT) with mutation loads ranging from 50% to 0.01%. It should be noted that the amplification efficiency of these linear synthetic fragments might slightly differ from circular mtDNA, due to potential supercoiling of the mtDNA. For these validation experiments, the total input was 10 000 templates per sample. After loading of the sapphire chip in the Geode device (Stilla), the reaction volumes were divided in 25 000–30 000 droplets (0.43 ± 0.03 nL), followed by thermal cycling. After an initial denaturation of 10 min at 95 °C, amplification was realized by 40 cycles of denaturation for 30 s at 95 °C and combined annealing/elongation for 15 s at 60 °C, according to the assay manufacturer's instructions. Fluorescence readout was accomplished using the Naica Prism3 System (Stilla). A fluorescence spillover compensation matrix was determined and applied to all samples, using the CrystalMiner Software (Stilla). Partition classification was performed using ddpcRquant with default settings (27), a data-driven thresholding method based on the NTC(s) included in the dPCR run. Heteroplasmy calculation was done using the mixed models dPCR analysis application developed by Vynck et al. (28).

For the Bio-Rad QX200 system, a reaction mixture of 20 µL was prepared, using the same primers and probes at identical concentrations. The reaction mixture contained 1x dPCR™ Supermix for Probes (Bio-Rad, Cat. No. 186-3010), the input was kept at 2 µL of the 1:30 dilution of the clinical samples. After loading up to 8 samples in a DG8™ Cartridge, about 20 000 nanoliter-sized droplets per sample were generated using the QX200™ Droplet Generator (Bio-Rad). Thermal cycling was performed using the C1000 Touch™ thermal cycler, after transferring the generated droplets to a 96-well PCR-plate. The same cycling conditions were applied as used for the Naica system. A NTC was included in each run. Data were retrieved using the QuantaSoft™ Software (Bio-Rad) and compensated for fluorescence spill-over using a compensation matrix.

Table 1. Oligonucleotide sequences used for dPCR

Oligonucleotide name	Oligonucleotide sequence (5′ - 3′)
Forward primer	TGCCTAGCAAACTCAAACTACGAA
Reverse primer	GGAGTAGAGTTTGAAGTCCTTGAGA
Wild-type probe	VIC-ACTCACAGTCGCATCAT- NFQ
Mutant probe	FAM-CTCACAGTCACATCAT-NFQ

NFQ, non fluorescent quencher; SNP position is underlined.

Table 2. Mutation load of all samples as determined by NGS and both dPCR platforms.

Sample code	Sample type	Mutation load (NGS) (%)	Mutation load (dPCR, Naica) (%)	Mutation load (dPCR, Bio-Rad) (%)
1	Patient	99.09	99.87	99.93
2	Patient	99.13	99.16	99.39
3	Patient	99.16	99.91	99.86
4	Patient	99.05	99.92	99.90
5	Patient	99.14	99.88	99.87
6	Patient	99.06	99.80	99.55
7	Patient	99.03	99.87	99.30
8	Patient	99.02	99.85	99.33
9	Patient	99.10	99.86	99.84
10	Patient	98.89	99.64	99.75
11	Patient	99.01	99.84	99.92
12	Patient	98.90	99.84	99.92
13	Patient	99.03	99.87	99.89
14	Healthy volunteer	0.30	0.00	0.17
15	Healthy volunteer	0.28	0.00	0.00
16	Healthy volunteer	0.25	0.00	0.00
17	Nuclear transfer	2.21	2.28	2.19
18	Nuclear transfer	1.92	1.66	0.49
19	Nuclear transfer	1.16	1.08	0.99
20	Nuclear transfer	1.54	0.98	0.99
21	Nuclear transfer	1.18	0.78	0.88
22	Nuclear transfer	2.97	3.21	1.92
23	Nuclear transfer	0.96	0.97	0.23

Data analysis was performed with ddpcRquant as described above.

Results

MTDNA HETEROPLASMY ASSESSMENT USING NG

In total, 3 732 323 Illumina sequencing reads passed quality control and were included for genotyping, resulting in an average coverage of 162 275 reads per sample. Table 2 shows the genotyping results of the examined samples. To estimate the noise floor caused by PCR and sequencing errors around the SNP of interest, detected frequencies of the 3 minor alleles between positions 11 700 and 11 860 were plotted, originating from either true minor alleles or noise. Examples of these plots are shown in Fig. 1 for a nonpatient sample (sample 14) and a sample subjected to NT (sample 23). A variant was considered reliably detected when its allele fraction is at least twice as high as the average of the second highest allele fraction at any other position in the amplicon, corresponding to a limit of detection (LOD) of 0.68%. This noise is created by sequencing errors that are accumulated during the several steps of an NGS protocol, including library preparation, sequencing, and read alignment (29).

VALIDATION OF THE M.11778 HYDROLYSIS PROBE-BASED ASSAY ON DPCR

To assess the linearity, the accuracy, and the LOD of the hydrolysis probe-based assay, 3 titration series of 10 mixture samples with mutation loads ranging from 50% to 0.01% were analyzed using the Naica system. Supplemental Fig. 1 shows the observed and expected mutation loads. Three false negative results were obtained for input mutation loads lower than 0.1%. Therefore, the limit of detection is set at 0.1%, corresponding to a theoretical input of 10 molecules. A clear linear relationship is detected between expected and observed mutation loads for mutation loads as low as 0.5%. A greater variability was observed in the titration series compared to the replicates of clinical samples, due to the random pipetting error

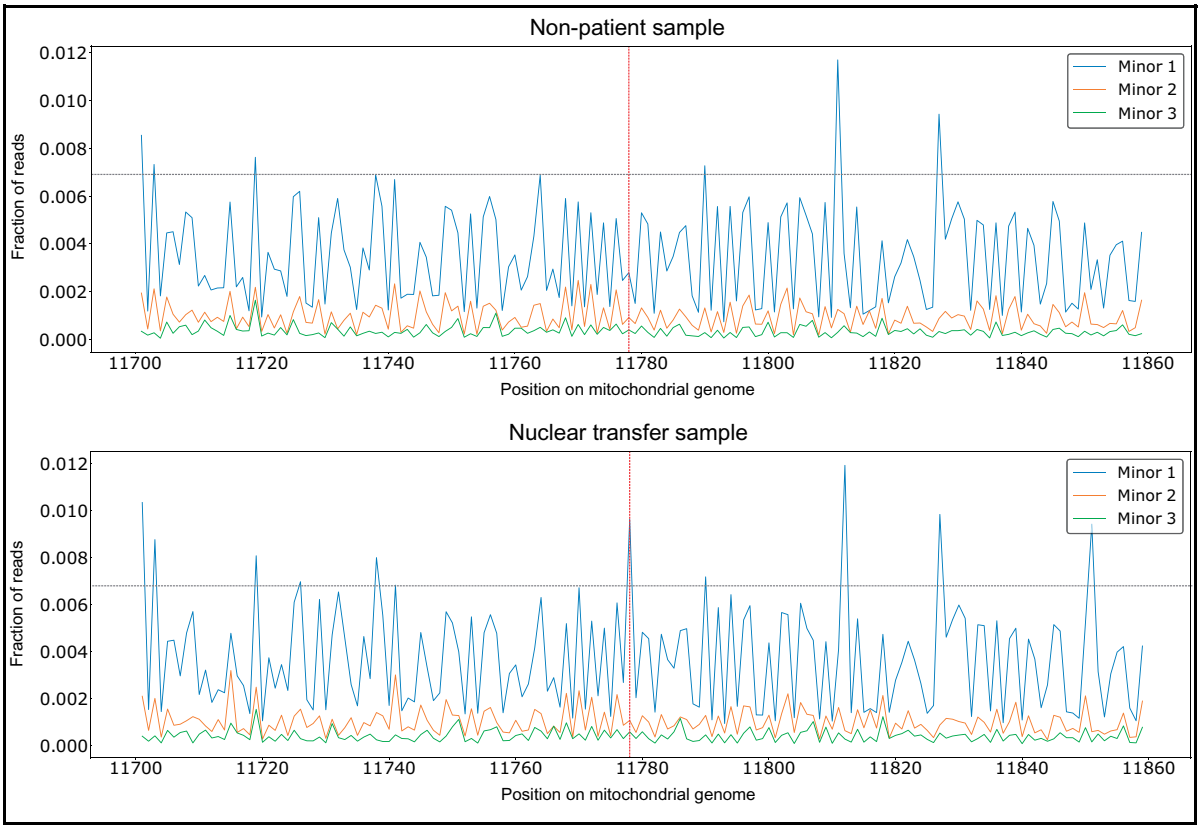

Fig. 1. Frequencies of the 3 minor alleles as detected using NGS between positions 11700 and 11860 of the mitochondrial genome. The vertical dotted line indicates the SNP of interest, the horizontal dotted line indicates the 0.68% threshold.

associated to the construction of these independently prepared titration series.

MTDNA HETEROPLASMY ASSESSMENT USING dPCR

Figure 2 shows a representative 2 D-plot of a patient sample, a nonpatient sample, a sample that underwent NT, and a NTC obtained using the Naica platform. Quantification of both genotypes enabled mutation load calculation; these results can be found in Table 2. Supplemental Table 1 shows the number of analyzed partitions for all samples, the number of positive partitions per probe, the mutation load, and the 95% confidence interval. This confidence interval is calculated based on Poisson statistics and does not account for sampling and pipetting variations. To assess the between-run variability, 3 technical replicates were obtained for 6 samples. The obtained data can be found in Supplemental Table 3, along with the calculated standard deviations and coefficients of variation. The standard deviations range between 0.07% and 0.44% mutation load.

The average difference in mutation load, comparing NGS and Naica dPCR, was 0.34%, which is not deemed to be clinically significant. No significant systematic bias was observed, as can be deduced from the Bland-Altman plot shown in Fig. 3. The 95% confidence interval around the mean difference comprises zero. Similar results were obtained for all 23 clinical samples using the Bio-Rad QX200 platform (Table 2). A Bland-Altman plot comparing both dPCR systems in Supplemental Fig. 2 showed that the average difference between both dPCR techniques was 0.17% mutation load. No systematic or proportional bias could be observed. For only 2 samples, the difference between both measurements exceeded 1%. Some of the NTCs included in the runs on both platforms showed one or multiple false positive partitions, as shown in Supplemental Table 4 and Fig. 3. Most of these partitions were positive for the wild-type allele, indicating a minor contamination.

Discussion

In our study, the mutation loads achieved by both dPCR techniques correspond well to those acquired by NGS. No significant systematic bias was observed

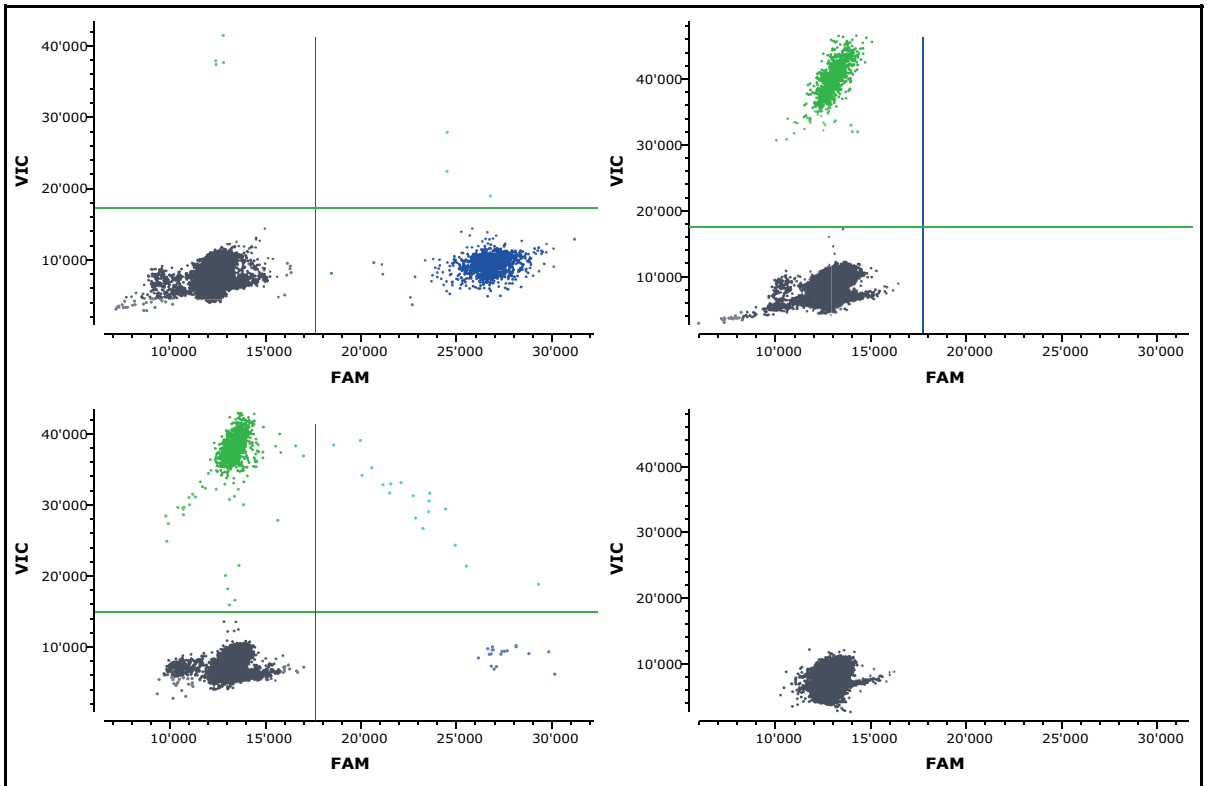

Fig. 2. 2 D-plots of 4 dPCR experiments on the Naica platform. The VIC-channel corresponds to the wild-type probe, the FAM-channel to the mutant probe. Upper left: heteroplasmic patient sample; upper right: homoplasmic wild-type sample; lower left: heteroplasmic sample after nuclear transfer; lower right: no-template control.

between the 2 technologies. However, a proportional bias was observed. Using NGS, the minor allele frequency of nearly all heteroplasmic samples was overestimated when compared to the results obtained by dPCR. Most probably, this minor discrepancy can be attributed to sequence errors introduced in the NGS experiments during PCR-amplification, bridge amplification, and sequencing-by-synthesis. Moreover, amplification bias might occur during PCR, altering the minor allele frequency.

Using NGS, the observed mutation load of the nonpatient samples ranged between 0.25% and 0.30%. Although strategies exist to reduce the background noise of NGS data (e.g., the use of unique molecular identifiers), these approaches are not widely adopted in clinical practice yet. The sequencing mediated background noise was omitted using dPCR. Moreover, sequence errors introduced by the polymerase during the first cycles of the amplification step only affected the partition in which it arose. This reduction in background noise was demonstrated by our data, showing no positive signal at all for the mutant allele in nonpatient samples, using the Naica platform. Analysis on the Bio-Rad platform resulted in a single positive partition for the mutant allele for one nonpatient sample, corresponding to a mutation load of 0.17%. Besides that, multiple NTCs showed positive partitions, mainly for the wild-type allele. This could either be attributed to the high abundancy of the mitochondrial genomes in a single cell, which increases, for example, the chance of reagent contamination with wild-type mitochondrial DNA, or could be attributed to the amplification artifacts which have been described in digital PCR (27, 30). The low number of false positive partitions for the wild-type allele in the healthy volunteer samples indicates the absence of cross-reactivity of the assay. A decidedly higher number of positive partitions was observed for the NTCs included in the Bio-Rad experiments. This might be due to the use of other reagents, another lab environment, and another instrument user. As these positive partitions were mainly wild-type positive, only limited effects on the detection of minor mutant alleles were expected. For the assay to be introduced in the clinic, testing should comply to Good Laboratory Practices and Good Clinical Practices to avoid this issue, which is also the case for the currently used NGS-workflow.

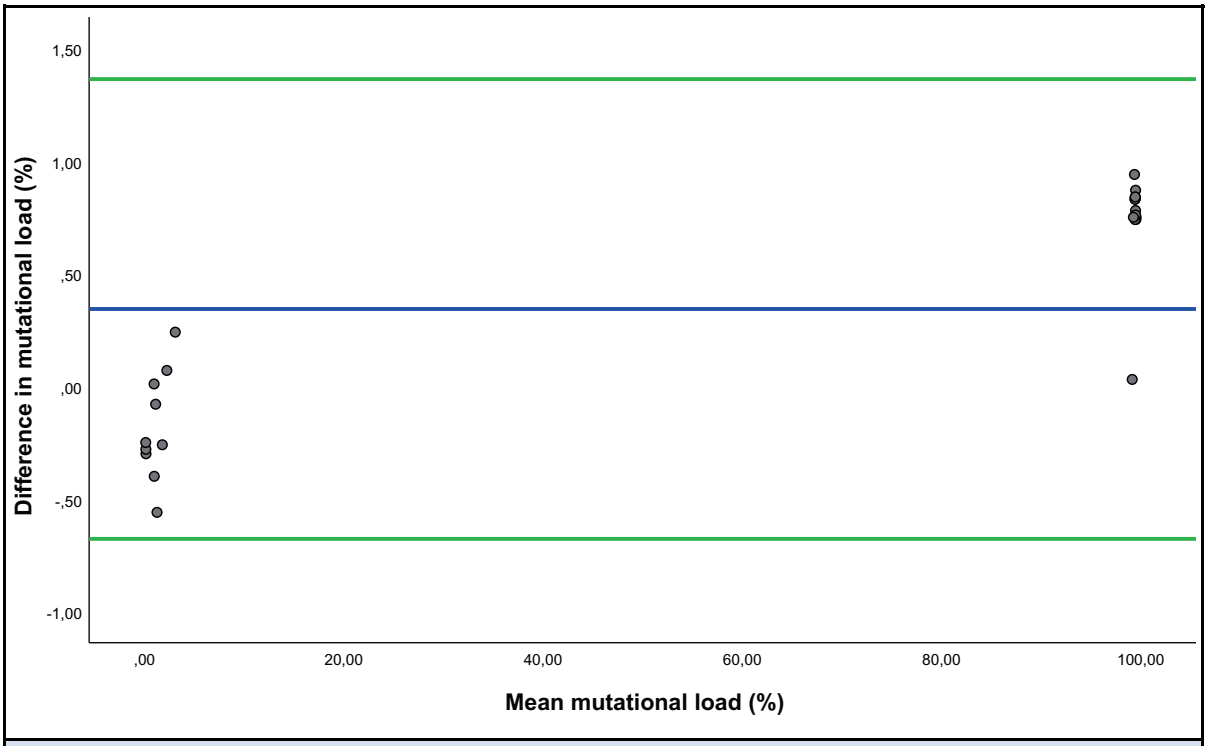

Fig. 3. Bland-Altman plot comparing Naica dPCR and NGS for mtDNA heteroplasmy assessment. The x-axis shows the mean mutation load, the y-axis shows the difference between the two measurement techniques. The black reference line represents the average difference between the measurement techniques, the grey reference lines represent the 95% CI around this difference.

dPCR has proven to be ideally suited for detecting and quantifying very low mutation loads (21, 31). The sample with the lowest mutation load, sample 19, was correctly genotyped as heteroplasmic by both dPCR platforms. Moreover, the near-homoplasmic patient samples were all genotyped positive for the wild-type allele. However, for some of these samples, the number of partitions positive for the wild-type allele was comparable to the NTCs, indicating that we cannot exclude that these wild-type counts were false positives. Lastly, results of the titration series showed that the hydrolysis probe-based assay established a sufficiently low LOD for the clinical samples assessed in this research. The capability of reliably quantifying samples characterized by very extreme allelic ratios can be explained by the limiting dilution step, during which these allelic ratios are moderated. Most of the generated partitions contain either 0, 1, or 2 templates, resulting in balanced allelic ratios on the partition level.

The exploitation of dPCR for clinical diagnosis of mitochondrial diseases instead of NGS would connote a range of benefits. A considerably higher cost is involved with NGS, including the up-front cost of a sequencer and the multitude of reagents needed for amplification, purification, library preparation, quantification, and sequencing of the sample itself. Moreover, these procedures are more time-consuming than performing a dPCR run, and they require more highly trained staff. Setting up a dPCR run is arguably more straightforward, as this only requires basic laboratory skills. A final important factor to consider is the complexity of data analysis. An extensive bioinformatics pipeline is required for data nalysis of NGS data, whereas dPCR data can be readily analyzed using the manufacturer's software. This again speeds up the entire process considerably, which is important for diagnostic applications.

Conclusions

In this study, heteroplasmy assessment of the m.11778 G > A mutation using a hydrolysis probe-based assay on 2 dPCR systems was compared to NGS. The dPCR results showed good concordance with the NGS data. Although a limited, clinically irrelevant, proportional bias could be observed, no significant systematic bias was present. dPCR proved to be at least as sensitive as NGS and thus seems suited for targeted heteroplasmy quantification for diagnosis or quality control after NT.

Moreover, the dPCR results demonstrated less noise compared to NGS: dPCR results showed almost no positive signal at all for the mutant allele in nonpatient samples while NGS results displayed a mutation load of approximately 0.3%. dPCR requires lower capital costs compared to NGS, and entails less hands-on time, less overall time, and a more straightforward data analysis. dPCR might therefore become a preferred method for heteroplasmy quantification of known mtDNA mutations.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Nonstandard Abbreviations: ATP, adenosine triphosphate; dPCR, digital polymerase chain reaction; ePNT, early pronuclear transfer; LHON, Leber's hereditary optic neuropathy; LOD, limit of detection; mDNA, mitochondrial DNA; MGB, minor groove binding; NGS, next generation sequencing; NT, nuclear transfer; NTC, no template control; SNP, single nucleotide polymorphism; ST, maternal spindle transfer

Human Gene: Mitochondrially encoded NADH, ubiquinone oxidoreductase core subunit 4 (*MT-ND4*)

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