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Optimized Droplet Digital PCR Assay on Cell-Free DNA Samples for Non-Invasive Prenatal Diagnosis: Application to Beta-Thalassemia

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BACKGROUND: Thalassemias are inherited blood disorders and by far one of the most common monogenic diseases globally. Beta-thalassemia has a particularly high prevalence in Cyprus, with the IVSI-110 G>A (HBB: c.93-21G>A) pathogenic variation representing almost 79% of the total carriers. The discovery that 3% to 20% of cell-free fetal DNA (cffDNA) is present in the maternal plasma allowed the development of non-invasive prenatal diagnosis (NIPD) of monogenic diseases, like beta-thalassemia, avoiding the risks of invasive procedures. However, the development of NIPD holds major technical challenges and has not yet reached the clinical setting.

METHODS: In this study, we apply droplet digital PCR (ddPCR) coupled with the relative variant dosage approach to develop a NIPD assay for IVSI-110 G>A beta-thalassemia. We have implemented an optimization process for ddPCR to address the challenges of ddPCR assays such as inconclusive rain droplets and thus increase the sensitivity and specificity of the assay. The established protocol was evaluated on 40 maternal plasma samples with a median gestational age of 10 weeks where both parents carried the same pathogenic variation.

RESULTS: Thirty-three samples were correctly classified, 6 remained inconclusive, and 1 was misclassified. Our assay exhibited 97.06% accuracy (95% CI, 82.46–99.68), 100% sensitivity (95% CI, 76.84–100), and 95% specificity (95% CI, 75.13–99.87), demonstrating its efficiency for the non-invasive detection of both maternal and paternal alleles.

CONCLUSIONS: We have developed an efficient, simple, and cost-effective ddPCR assay for the non-invasive determination of fetal genotype in couples at risk of IVSI-110 G>A beta-thalassemia, bringing NIPD of monogenic diseases closer to the diagnostic setting.

Introduction

Thalassemias are inherited blood diseases and by far one of the most common monogenic diseases. Approximately 5% of people globally are asymptomatic carriers, resulting in an estimate of over 330 000 affected newborns annually (1–3). Beta-thalassemia has a particularly high prevalence in Cyprus with an approximate 12% carrier frequency, where the IVSI-110 G>A (HBB:c.93-21G>A) beta-globin pathogenic variation represents almost 79% of total carriers (4). Beta-thalassemia follows an autosomal recessive inheritance and around 76% of the Cypriot couples undergoing prenatal diagnosis have a pregnancy at risk of the IVSI-110 G>A pathogenic variation.

Currently, the only available prevention option is invasive prenatal diagnosis, which causes physical discomfort and carries a substantial risk of iatrogenic abortion (5,6). The discovery by Lo et al. that cell-free fetal DNA (cffDNA) is present in maternal blood circulation opened up the opportunity for the development of non-invasive approaches (7). Non-invasive prenatal diagnosis (NIPD) allows for fetal DNA analysis from a simple maternal peripheral blood sample, making it a risk-free alternative to the traditional invasive methods. Maternal plasma contains a mixture of fetal and maternal cell-free DNA (cfDNA), with the fetus comprising the minority at an average of 10% of the total cfDNA, termed fetal fraction (8,9). The cfDNA is present in short fragments,

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with the fetus-derived DNA fragments being shorter than the mother's, predominantly at 143 bp; however, the fetal genome is represented as a whole (10).

All these characteristics of cfDNA cause major technical challenges, hampering the development of NIPD applications. For this reason, NIPD applications for monogenic diseases that have reached clinical practice are limited to the detection of alleles absent from the mother, such as diseases of paternal origin (11–13). For monogenic disorders of maternal origin, highly sensitive quantitative methods are required to distinguish fetal DNA sequences against the high background of identical maternal ones. For this purpose, several groups have attempted different methods and approaches, including the relative mutation dosage (RMD) and the relative haplotype dosage (RHDO) (14–16). Next-generation sequencing (NGS) is the method of choice: a powerful, highly sensitive technique that enables the detection of small genetic variations in the genome (17–20). However, NGS is costly and requires complex bioinformatics analyses, making it less suitable for routine application.

Droplet digital PCR (ddPCR) is a PCR-based technique that enables absolute quantification of the targeted DNA molecules (21). It offers high sensitivity with lower cost compared to NGS and does not require difficult sample preparation and analyses. Its high precision enables the detection of rare target DNA sequences in the sample, making it an advantageous methodology option for the measurement of the low quantity of cffDNA in maternal plasma and a promising application for NIPD. A few researchers have exploited ddPCR for NIPD of monogenic diseases with promising results (15, 22–24). Despite the substantial progress, NIPD of monogenic diseases has yet to achieve routine clinical application. Some NIPD tests for monogenic diseases have been commercially available, but only for screening purposes with possibly unreliable results that need invasive follow-up (25).

In this study, we have exploited the use of ddPCR to develop and optimize a NIPD assay for the detection of the most common Cypriot beta-thalassemia pathogenic variation, the IVSI-110 G>A, in cfDNA to cover the largest portion of pregnancies at risk of beta-thalassemia in Cyprus. For the determination of fetal genotype, the relative variant dosage (RVD) approach was exploited based on allelic ratios and *Z*-score calculations. The evaluation of the assay was performed on 40 maternal plasma samples from couples at risk for beta-thalassemia, with both parents carrying the same pathogenic variation.

Materials and Methods

SAMPLE COLLECTION AND ETHICS STATEMENTS

Approximately 9 mL of peripheral blood samples was collected from 40 pregnant women with risk of IVSI-110 G>A beta-thalassemia in their newborn. Samples were collected using standard EDTA-containing blood collection tubes (n = 33) or Cell-Free DNA BCT® tubes (Streck) (n = 7) between the 8th and 13th week of gestation (median = 10). Only singleton pregnancies were selected. Peripheral blood samples were also collected from the pregnant woman's partner, the couple's parents, and from individuals of all 3 genotypes with respect to the IVSI-110 G>A pathogenic variation, using standard EDTA-containing tubes.

The study was approved by the Cyprus National Bioethics Committee (EEBK/ΕΠ/2018/51) and all participants provided informed written consent. All procedures were conducted in agreement with the Declaration of Helsinki (26).

PLASMA SEPARATION

Plasma was separated from EDTA or Streck blood collection tubes from all pregnant women and 3 IVSI-110 G>A carriers by centrifugation at 2500*g* for 10 min. The plasma was always separated within 2 h of blood collection, to prevent maternal cell lysis and maternal DNA release. The supernatant was centrifuged at 17 000*g* for 40 min, to completely remove platelets and precipitates. The resulting supernatant was collected into single-use 1 mL aliquots and stored at −20°C until cfDNA extraction or −80°C for longer storage.

DNA EXTRACTION

Cell-free DNA was extracted from 2 mL plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen) and eluted in 65 μL buffer AVE, according to the manufacturer's instructions. Genomic DNA (gDNA) was extracted using Puregene Blood Core Kit C (Qiagen) or MagCore Genomic DNA Whole Blood Kit (RBC Bioscience) according to the manufacturers' instructions.

SONICATION

Nine gDNA samples (7 × GA, 1 × GG, 1 × AA) were sheared into smaller fragments to mimic the fetal cfDNA fragments in maternal plasma to be used in ddPCR optimization experiments. The samples were sonicated using a Bioruptor Pico sonication system (Diagenode). We applied 40 cycles with the settings 3 s on/30 s off per cycle, resulting in the fragments of 168–177 bp being optimal for our purposes, as determined through repetitive series of cycle trials. To see the fragment sizes after sonication, the Agilent 2200 TapeStation system was used with the High Sensitivity D1000 ScreenTape and Reagents (Agilent Technologies).

DDPCR

A ddPCR assay was developed to detect the IVSI-110 G>A pathogenic variation. Primers were designed to yield amplicons of less than 100 bp length (93 bp) because of the small length of cfDNA in maternal plasma. FAM (fluorescein amidite) and HEX (hexachloro-fluorescein) fluorescently labelled probes have been designed to detect the wild-type (G) and mutant (A) alleles, respectively (Supplemental Table 1). All Supplemental Tables and Figures can be found in the online Data Supplement.

The ddPCR experiments were performed using the QX200 AutoDG droplet digital PCR system (Bio-Rad Laboratories) following the manufacturer's protocol with minor modifications, as described in the online Data Supplement. All cfDNA samples were analyzed in 6 replicates. Each ddPCR run included 4 maternal cfDNA samples; therefore, samples were analyzed in different ddPCR runs to demonstrate repeatability.

Through the QuantaSoft™ analysis software (version 1.7.4.0917), thresholds were set manually to assign droplets as positives or negatives. Any cases for which a well exhibited fewer than 10 000 total droplets (i.e., due to error in droplet generation) or unusual 1D and 2D amplitude plots were excluded from subsequent analysis.

RVD APPROACH AND ANALYSIS STRATEGY

The results elucidated from QuantaSoft™ software were analyzed to determine the fetal genotype. To do that, the RVD approach was exploited in combination with the calculation of allelic ratios and *Z*-scores. RVD is based on the principle of the RMD approach, which focuses on the calculation of allelic ratios when the mother is heterozygous. In case of a heterozygous fetus, the two alleles are in complete balance so the allelic ratio is expected to be 1. In the case of a homozygous fetus, the allelic ratio is expected to skew to the overrepresented allele, therefore, be >1 or <1 (14). The allelic ratio of each sample was considered to be the mean value of all its 6 replicates, which is automatically calculated by the QuantaSoft™ software.

The calculation of *Z*-scores was also exploited with the RVD approach to determine the fetal genotypes, following a similar principle as Perlado et al.(15). More specifically, *Z*-score was calculated using the concentration of the two alleles, as seen below:

$$Z\text{-}score = \frac{G - A}{\sqrt{G + A}}$$

where G and A are the concentrations of the FAM-labelled G-allele and HEX-labelled A-allele, respectively, as elucidated from the software (copies/μL).

Z-score was calculated for each replicate; the final *Z*-score of the sample was the mean *Z*-score value of all its 6 replicates.

FETAL GENOTYPING

To determine the fetal genotype, standard thresholds of allelic ratios and *Z*-scores were established. To do this, we elucidated the ratios and *Z*-scores of the 30 maternal plasma samples first analyzed and categorized them in the 3 possible fetal genotypes. Then, we calculated the mean values (ratio and *Z*-score) for each fetal genotype and added one standard deviation (±1 SD) to establish the thresholds for each genotype. Four categories were defined: homozygote GG, heterozygote GA, homozygote AA (affected), and grey zone (inconclusive) (Fig. 1). These thresholds were then applied to the remaining 10 maternal plasma samples. Based on ratio and *Z*-score values of the sample, the RVD approach was applied to determine fetal genotype. The NIPD results were then compared with the invasive prenatal diagnostic results of the respective chorionic-villi samples (CVS) previously performed in our lab.

Results

OPTIMIZATION PROCESS

Optimization of the ddPCR assay was a major part of the study. Previous trials on our assay's development revealed that ratios elucidated from both gDNA and cfDNA heterozygote-only samples were not optimal; the two alleles were not in complete balance. They also revealed the presence of droplets, named rain droplets, that had an intermediate fluorescence level that could not be assigned clearly to the negative or positive droplet clusters. The presence of rain droplets made setting an accurate threshold difficult, leading to inaccurate calculation of allele copy numbers and subsequently allelic ratios and *Z*-scores. The ultimate aim of the optimization process was to have maximal and clear separation between positive and negative droplet clusters with as few rain droplets as possible.

Optimization steps entailed gradient ddPCR with temperatures from 51 to 61°C, overnight incubation of the ddPCR plate at 4°C after the completion of PCR amplification (before reading), and increasing the primer final concentrations from 450 nmol/L to 900 nmol/L. Different parameters on the PCR thermal profile were subjected to change, such as decreasing the ramp rate of each PCR step from 2°C/s to 1°C/s, increasing the denaturation time from 30 s to 1 min, and increasing the number of PCR cycles to 45 and 50 (Fig. 2). Details on the optimization process and the results are described in the online Data Supplement (Supplemental Figs. 1–7).

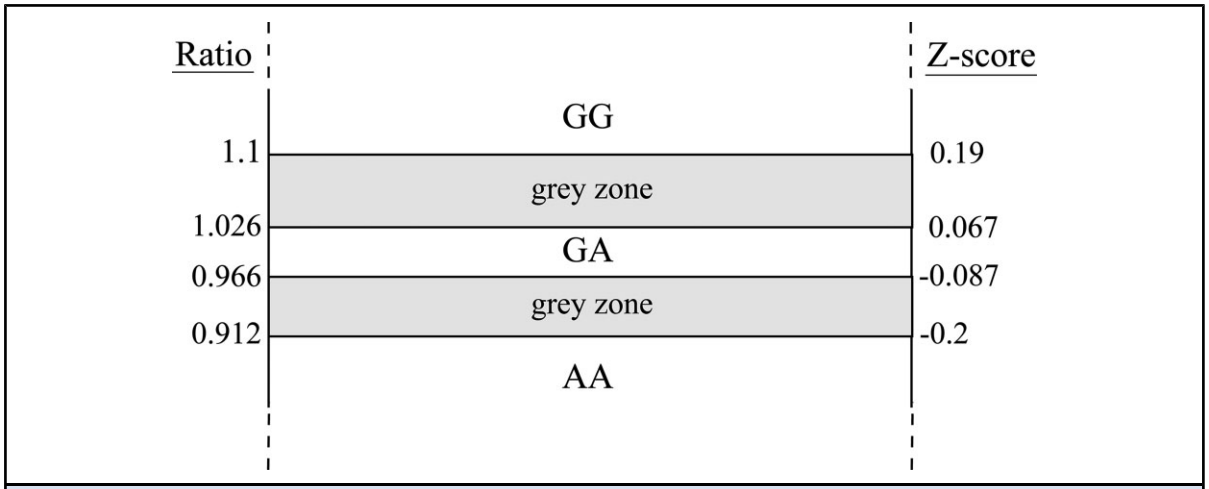

Fig. 1. Established thresholds of allelic ratios and *Z*-scores to classify fetal genotype of the sample. Four categories were defined for the RVD approach to classify the fetal genotype. The gray zone is the space between the thresholds of the 3 genotypes that lead to an inconclusive test result.

Our optimization strategy was based on saving the valuable maternal plasma samples (Fig. 2). It involved initial trials of condition alterations with the use of 7 heterozygote fetal-like fragmented gDNA samples (Supplemental Table 2), diluted to a similar concentration of cfDNA (0.5 ng/μL). Then, the optimal conditions were tested on 3 heterozygous cfDNA plasma samples (IVSI-110 G>A carriers), and the final optimized assay was evaluated on maternal cfDNA plasma samples. In summary, the final optimized protocol included: introduction of 4°C overnight plate incubation, 55°C annealing/extension temperature, 450 nmol/L primer final concentration, 1 min denaturation, 2°C/s ramp rate, and 45 cycles. Primer concentrations and ramp rate remained as in the original protocol, as the altered conditions (900 nmol/L and 1°C/s, respectively) were not beneficial.

EVALUATION ON MATERNAL PLASMA SAMPLES

The established optimized ddPCR assay was evaluated for determination of fetal genotype via the RVD approach. The evaluation was performed on 40 maternal plasma samples from at-risk pregnancies where all mothers were heterozygotes for the IVSI-110 G>A pathogenic variation. Ratios and *Z*-scores were calculated for each sample and, based on the RVD approach, were classified into one of the 4 fetal genotype categories. The NIPD results were compared to the ones obtained through invasive diagnostic analysis of the corresponding CVS sample of the fetus.

Optimization of the developed assay was apparent, as we achieved better separation between positive and negative droplet clusters with only a few rain droplets (Fig. 3). The presence of that small number of rain droplets did not affect the calculation of allele copy numbers, as the use of 3 different thresholds (low, intermediate, and high) resulted in similar allelic ratios (Table 1). For this reason, the optimized ddPCR assay resulted in accurate threshold settings and, consequently, optimal allelic ratios and *Z*-scores. Initially, fetal genotype was successfully determined in 31 samples, with the fetal genotypes being consistent with the ones assigned through invasive prenatal diagnosis (Table 2). Eight samples were inconclusive (grey zone) so they had to be repeated. Of the 8 samples, 7 had enough plasma left for a repeat ddPCR run; 2 of these repeat analyses resulted in the successful determination of fetal genotype, however, the rest remained inconclusive. Only one sample of the 40 was incorrectly classified; however, upon repeat analysis to investigate the reason, this sample was correctly classified. We left this sample as "incorrectly classified" in the overall results, because it would not have been picked up in a real case scenario. Therefore, 33 samples were correctly classified, 6 remained inconclusive, and 1 was misclassified (Fig. 4). Overall, our assay exhibited 97.06% accuracy (95% CI, 82.46–99.68), 100% sensitivity (95% CI, 76.84–100), and 95% specificity (95% CI, 75.13–99.87) for the determination of fetal genotype accounting for both maternally and paternally inherited fetal alleles.

Discussion

Prenatal diagnosis of monogenic diseases, such as beta-thalassemia, is currently dependent on invasive

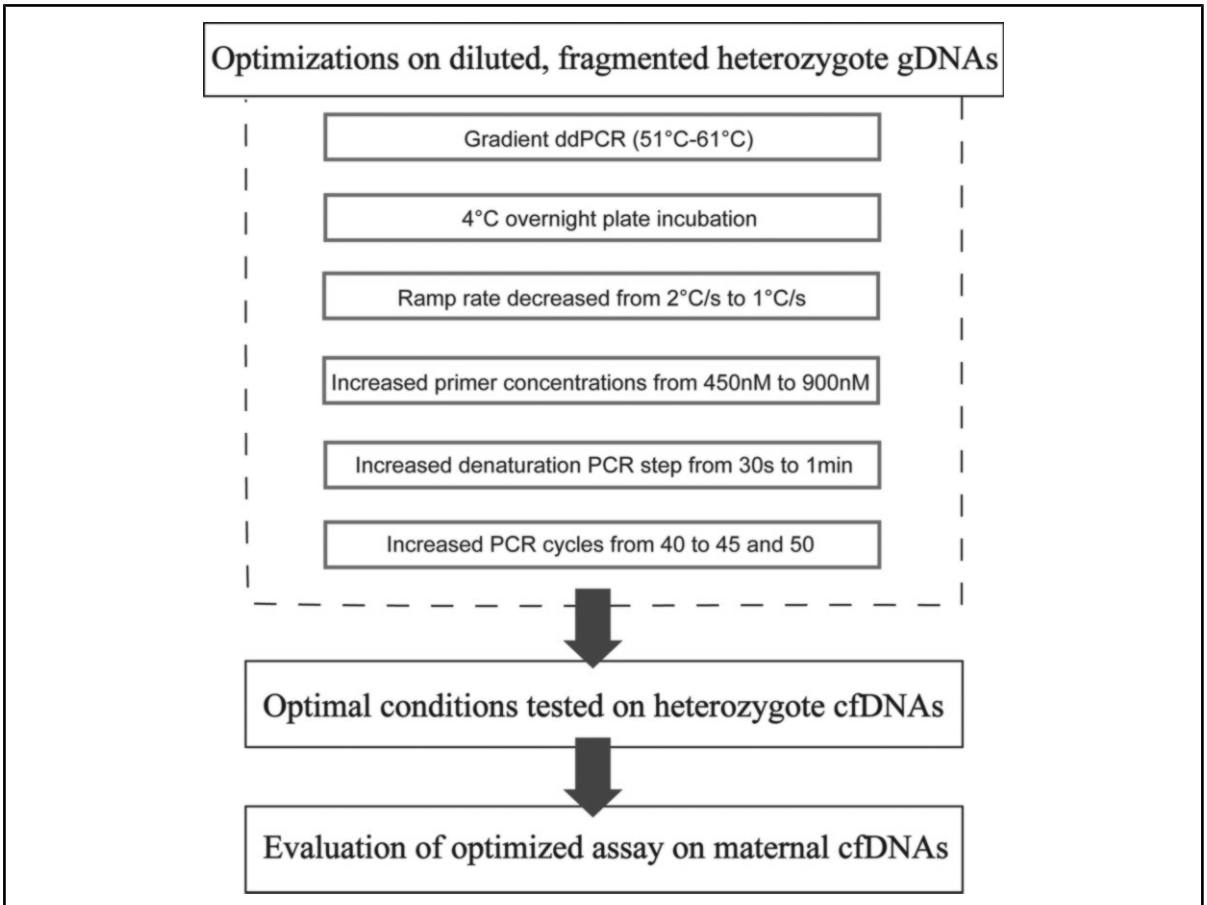

Fig. 2. ddPCR assay optimization workflow. The workflow indicates the strategy followed to preserve the valuable maternal cfDNA samples, along with the altered conditions of our optimization process. For detailed description of the optimizations, refer to the online Data Supplement (Supplemental Figs. 1–7).

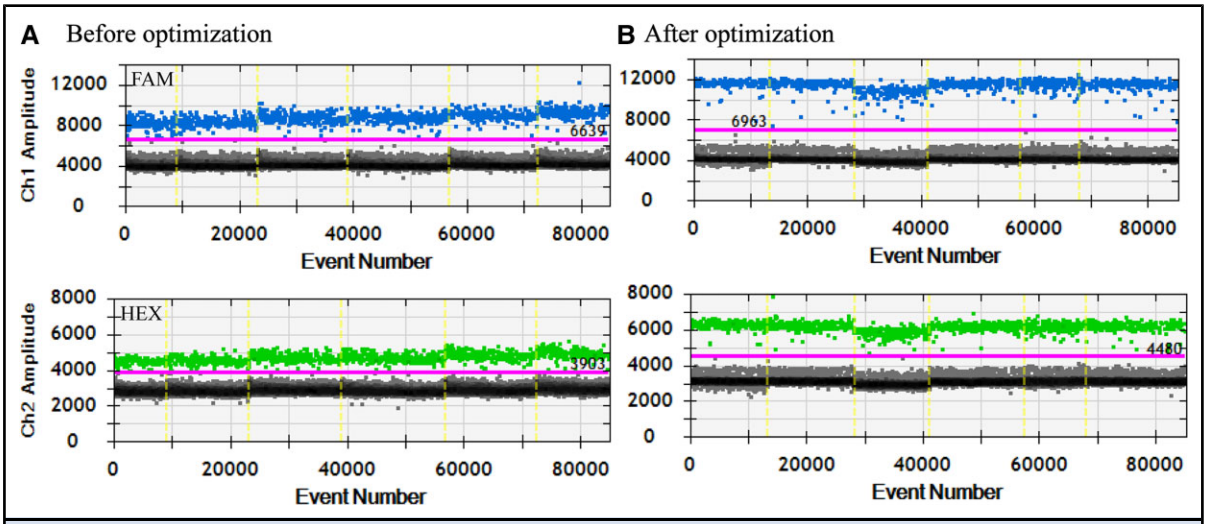

Fig. 3. FAM and HEX amplitudes of sample cfDNA 266 before (A) and after (B) the optimization process.

Table 1. Allelic ratio results from 3 different threshold settings of 6 maternal plasma samples.

Sample	Threshold[a]	Allelic ratio (G:A)
cfDNA 223	High	0.99
	Intermediate	1
	Low	1
cfDNA 157	High	0.89
	Intermediate	0.89
	Low	0.9
cfDNA 221	High	1.32
	Intermediate	1.33
	Low	1.33
cfDNA 235	High	0.99
	Intermediate	0.99
	Low	1
cfDNA 161	High	0.78
	Intermediate	0.79
	Low	0.79
cfDNA 184	High	1.24
	Intermediate	1.24
	Low	1.24

[a]Refer to Supplemental Fig. 8 for an example of the 3 threshold settings.

procedures. Over the last few years, many studies have reported successful development of NIPD methods (15–20,22,27–29); however, most of these studies were based on the determination of only the paternally inherited fetal allele (30–35), leading to the need for a method that can determine both parental alleles and, thus, be applied to all pregnancies. In the case of beta-thalassemia, several groups accomplished this goal by taking advantage of the improved analytical sensitivity of NGS and ddPCR (15, 17–20, 22). Despite these advances, NIPD of monogenic diseases has not yet reached the clinical diagnostics setting, with the exception of some that are available only for screening purposes with the need for invasive follow-up (25).

In this current study, we have applied ddPCR coupled with the RVD approach for the development of a NIPD assay that detects both parental alleles making it applicable to all pregnancies at risk of IVSI-110 G>A beta-thalassemia pathogenic variation. After a series of optimizations, a ddPCR protocol was established that can successfully determine fetal genotype from cfDNA samples of IVSI-110 G>A carrier mothers.

Through the initial trials of the development, we observed the presence of rain droplets that did not allow for the clear separation of positive from negative droplets, leading to difficulties setting the threshold that separates the two droplet populations. Rain droplets might result from damaged droplets (36), non-specific amplification (37), or irregular droplet size (38). As NIPD requires highly sensitive and specific assays to distinguish the subtle change around a heterozygote's ratio, our approach was to begin optimizing our assay. To minimize the presence of rain droplets, bypass any amplification errors and cross-reactivity, and maximize droplet separation, we opted to improve the assay's performance by altering various parameters in the ddPCR workflow, PCR conditions, and reagents.

Optimization of the assay was successful; we managed to increase the separation of positive and negative droplets and reduce the presence of rain droplets. The existence of a low number of rain droplets did not affect the accurate calculation of allele copy numbers, as wide variation in the threshold setting gave similar ratios and thus the same fetal genotype. This finding is of great importance, as manually setting the threshold would not affect the accuracy of results in the clinical setting. Using the RVD approach, fetal genotype was successfully determined in 33 of the 40 analyzed cfDNA maternal plasma samples, excluding the second run of the false-positive sample, showing concordance with the results obtained from the corresponding CVS samples previously tested for routine prenatal diagnostic purposes. Eight samples were inconclusive in the first run; 7 of them were repeated in a second ddPCR run and 1 was not, due to lack of plasma sample. Two of the repeated samples were resolved, leading to the correct fetal genotype, while the rest remained inconclusive. Only one false-positive result was observed, identifying an unaffected fetus as an affected one. To investigate whether this discordance was due to contamination of the sample or low specificity of the assay, we opted for a repeat ddPCR experiment, which resulted in determination of the correct fetal genotype. Therefore, the false-positive result may be attributed to possible contamination of the sample that could happen during all stages of sample handling, such as plasma separation, cfDNA extraction, or ddPCR plate preparation. This, however, would not be picked up in a real case scenario, where the sample would not be repeated, so it remained as "misclassified" in our results.

The present study is the first study based on NIPD assay development that has implemented an optimization process for ddPCR. Optimizing an assay increases its sensitivity and specificity, which is crucial when using such challenging materials as cfDNA. Here, we propose an optimization workflow for ddPCR assays for the purposes of NIPD; starting the optimization using diluted and fragmented heterozygote gDNA samples that resemble the nature of cfDNA, moving on to using

Table 2. Determined fetal genotypes of 40 maternal plasma samples.[a]

Fetal genotype (CVS)	Sample	EDTA/ Streck	Gestation week	Ratio, G:A	*Z*-score	NIPD (cfDNA)	Ratio, G:A	*Z*-score	NIPD (repeat)
GG	cfDNA 218	EDTA	11 + 3	1.13	0.293	GG			
	cfDNA 206	EDTA	10 + 5	1.14	0.294	GG			
	cfDNA 184	EDTA	10 + 4	1.24	0.54	GG			
	cfDNA 256	EDTA	11 + 5	1.26	0.664	GG			
	cfDNA 234	EDTA	8 + 6	1.09	0.161	inconclusive[b]	1.06	0.108	inconclusive
	cfDNA 233	EDTA	10 + 3	0.9	−0.213	AA[b]	1.24	0.475	GG
	cfDNA 214	EDTA	9 + 3	1.14	0.223	GG			
	cfDNA 221	EDTA	10 + 0	1.33	0.594	GG			
	cfDNA 226	EDTA	9 + 3	0.94	−0.134	inconclusive[b]	1.09	0.207	inconclusive
	cfDNA 242	EDTA	11 + 1	1.15	0.369	GG			
	cfDNA 211	EDTA	8 + 5	1.03	0.07	inconclusive[b]	1.05	0.097	inconclusive
GA	cfDNA 212	EDTA	8 + 2	0.99	−0.016	GA			
	cfDNA 210	EDTA	9 + 4	0.95	−0.148	inconclusive[b]	1.01	0.03	GA
	cfDNA 269	EDTA	10 + 5	1.01	0.014	GA			
	cfDNA 272	EDTA	12 + 2	1	−0.023	GA			
	cfDNA 223	EDTA	12 + 3	1	−0.042	GA			
	cfDNA 222	EDTA	11 + 0	1.05	0.139	inconclusive[b]	1.05	0.136	inconclusive
	cfDNA 227	EDTA	8 + 1	0.97	−0.046	GA			
	cfDNA 235	EDTA	9 + 5	1	0.001	GA			
	cfDNA 239	EDTA	10 + 4	1	0.002	GA			
	cfDNA 232	EDTA	8 + 3	0.94	−0.132	inconclusive[b]	0.97	-0.061	GA
	cfDNA 343	Streck	12 + 4	1.07	0.167	inconclusive[b]	1.05	0.105	inconclusive
	cfDNA 358	Streck	12 + 2	1.02	0.066	GA			
	cfDNA 360	Streck	11 + 4	1.02	0.049	GA			
	cfDNA 255	EDTA	9 + 0	0.98	−0.063	GA			
AA	cfDNA 217	EDTA	11 + 1	0.83	−0.51	AA			
	cfDNA 215	EDTA	9 + 1	0.82	−0.496	AA			
	cfDNA 266	EDTA	11 + 1	0.86	−0.485	AA			
	cfDNA 237	EDTA	13 + 0	0.89	−0.226	AA			
	cfDNA 201	EDTA	8 + 5	0.91	−0.277	AA			
	cfDNA 185	EDTA	9 + 2	0.87	−0.254	AA			
	cfDNA 144	EDTA	9 + 3	0.92	−0.149	inconclusive	Not available	Not available	Not available
	cfDNA 120	EDTA	8 + 5	0.89	−0.214	AA			
	cfDNA 157	EDTA	9 + 0	0.9	−0.214	AA			
	cfDNA 224	EDTA	9 + 0	0.8	−0.302	AA			
	cfDNA 161	EDTA	9 + 6	0.79	−0.427	AA			
	cfDNA 338	Streck	12 + 5	0.85	−0.361	AA			
	cfDNA 339	Streck	12 + 5	0.65	−0.955	AA			
	cfDNA 359	Streck	10 + 5	0.83	−0.419	AA			
	cfDNA 355	Streck	13 + 0	0.77	−0.545	AA			

[a]Following the elucidated ratio and *Z*-score thresholds (Fig. 1) of the first ddPCR run, 31 samples were correctly diagnosed, 8 were inconclusive, and 1 was misclassified.

[b]Seven inconclusive samples and the misclassified sample were repeated in a new ddPCR run, where 2 of the inconclusive samples and the misclassified sample resulted in correct diagnosis.

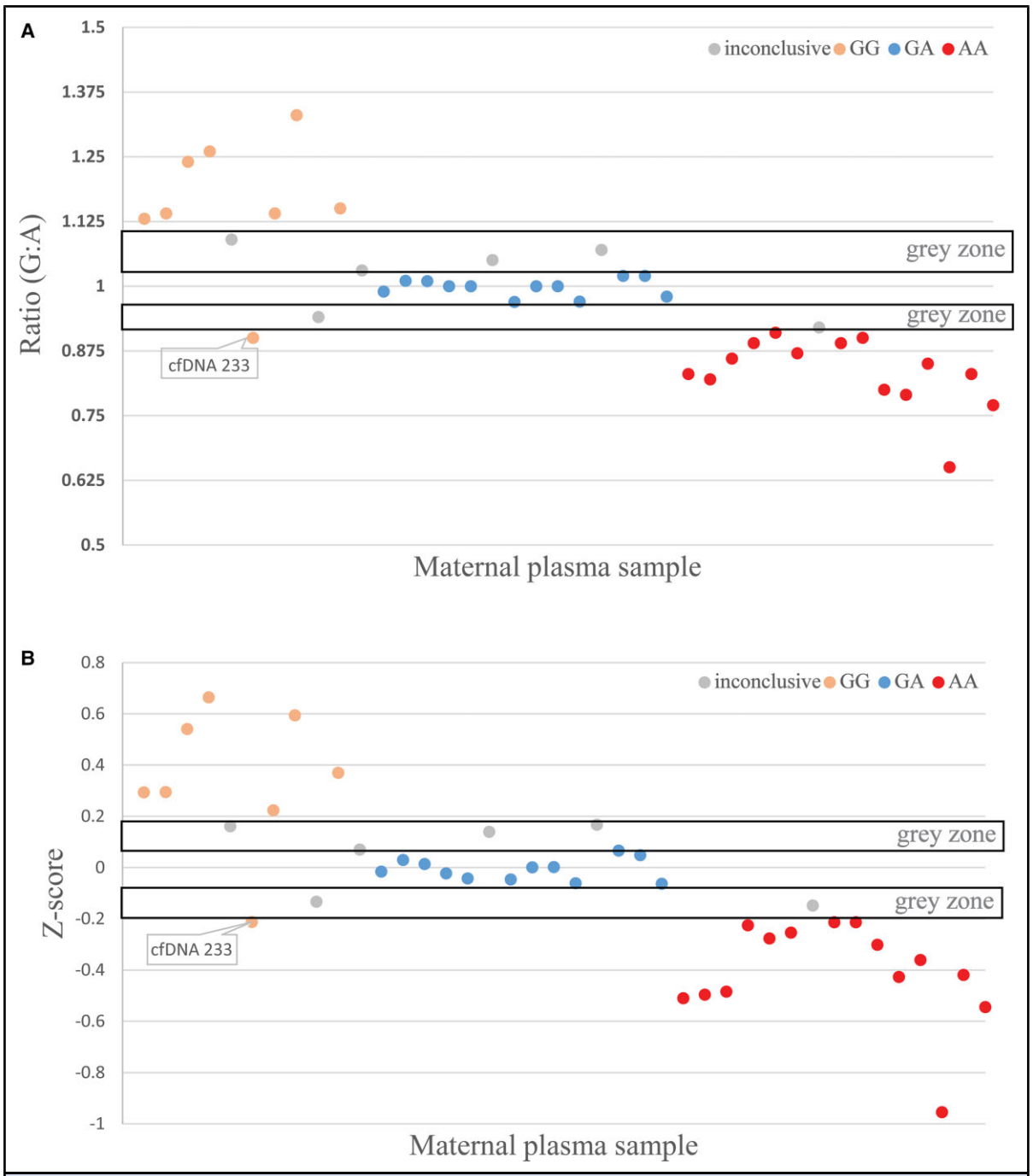

Fig. 4. Schematic representation of the ratios (A) and *Z*-scores (B) of the 40 maternal plasma samples, classified according to the RVD approach. The one false-positive sample, cfDNA 233, is indicated.

cfDNA samples of heterozygotes, and finally evaluating the assay's conditions on cfDNA from maternal plasma samples. This strategy aims to preserve the valuable maternal plasma samples.

Our study is also the first NIPD study with high success rate using samples from such early gestational ages. The purpose of a NIPD assay is to provide a diagnosis at the earliest possible gestational age so the couple can make an informed choice for the continuation of the pregnancy. The present NIPD assay has achieved a highly successful diagnostic rate of 97.06% using samples with median gestational age of 10 (8 to 13) weeks, which is earlier than the

conventional CVS prenatal diagnosis. It is important to note that samples were successfully diagnosed as early as the eighth week of gestation. We have managed to achieve this high accuracy by analyzing low volumes of plasma samples (2 mL) compared to previous studies (19,22,24,27,39). This is crucial as only a single 9 mL-tube of peripheral blood is needed, allowing the performance of an additional ddPCR experiment in case the first run is inconclusive. More importantly, no differences were observed in the ddPCR assay results between the 2 blood collection tubes used. This could be attributed to the fact that plasma was separated early enough, <2 h from collection, to prevent maternal DNA release into the EDTA tube.

Our results show that ddPCR is an excellent methodology option for NIPD purposes. It offers highly quantitative analysis of cfDNA, essential for the calculation of allelic ratios and detection of any imbalances that would indicate a homozygous fetus. It is a technique with fast turnaround time and simple workflow with no complex bioinformatics analyses, compared to the commonly used NGS, making it more suitable and easily implemented in the diagnostic setting. The main limitation for developing a ddPCR assay is the requirement for the variation to be previously known, and the ability to detect a single variation per assay. Multiplexing options are available with ddPCR; however, this would complicate the development of the assay and possibly lower sensitivity for each variation, something that would be critical for NIPD assays.

In our assay, we have observed a few samples where fetal genotype could not be determined. Inconclusive results were the major limitation of our assay, as 6 out of 40 samples (15%) remained inconclusive. This could be attributed to a low fetal fraction of the sample that was insufficient to shift the allelic ratio; however, since we have not developed a way to calculate it yet, a low fetal fraction cannot be excluded. This poses another limitation to our approach, as confirmation of successful isolation of fetal cfDNA or estimation of fetal fraction would improve confidence in our results. Therefore, to improve confidence and minimize any potential bias, we used 6 replicates for each sample. In the cases of a homozygous fetus we are confident in the reliability of the results, since the shift in allelic ratio confirms the presence of fetal DNA. The limitation lies in our heterozygous results, where the presence of fetal cfDNA cannot be confirmed, as an absence of it would also lead to a heterozygous result. Despite the limitations, the validity of our assay is confirmed by the retrospective analysis of CVS samples.

To overcome these limitations, an assay for the calculation of fetal fraction should be developed to ensure fetal fraction integrity. Moreover, fetal fragments could be preamplified or enriched, to ensure sufficient fetal cfDNA is present in the sample. To avoid any misclassified cases, samples could be analyzed in 2 separate ddPCR experiments for confirmation of results; in case of discordance, another run could be performed or an invasive analysis could be conducted. In the few situations of inconclusive results, the couple could opt for invasive prenatal diagnosis. Additionally, our developed ddPCR assay could be combined with another methodology, such as NGS, and merged into one pipeline for a comprehensive NIPD strategy to determine fetal genotype. One methodology would be used to confirm the other's results, leading to increased diagnostic accuracy and introduction of NIPD into clinical practice.

In summary, the present study developed an efficient, simple, and cost-effective ddPCR assay for the non-invasive determination of fetal genotype in couples at risk of IVSI-110 G>A beta-thalassemia, applicable to all pregnancies with an IVSI-110 G>A carrier mother. The assay exhibited 97% accuracy, 100% sensitivity, and 95% specificity in the analysis of 40 maternal plasma samples with 10 weeks median gestational age, avoiding the need for invasive prenatal testing of the fetus and bringing NIPD closer to the clinical setting.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Nonstandard Abbreviations: cffDNA, cell-free fetal DNA; NIPD, non-invasive prenatal diagnosis; ddPCR, droplet digital PCR; cfDNA, cell-free DNA; NGS, next-generation sequencing; RVD, relative variant dosage; gDNA, genomic DNA; CVS, chorionic-villi sample.

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