Prenatal Diagnosis

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Digital PCR Assay Utilizing In-Droplet Methylation-Sensitive Digestion for Estimation of Fetal cfDNA From Plasma

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ABSTRACT

Objective: Recent guidelines suggest that non-invasive prenatal screening (NIPS) should be offered to all patients with singleton and twin pregnancies. Accurate determination of fetal fraction in cell-free DNA (cfDNA) is vital for reliable NIPS outcomes. We propose a methylation-based approach using droplet digital PCR (ddPCR) and methylation-sensitive restriction enzyme (MSRE) digestion for fetal fraction quantification as an affordable and fast solution.

Method: Following biomarker discovery using early pregnancy placental genomic DNA (gDNA) and cfDNA from non-pregnant female individuals, we designed assays targeting MSRE-compatible regions based on contrasting methylation patterns between maternal and fetal cfDNA. We established a proof-of-concept ddPCR workflow on the Bio-Rad Droplet Digital PCR QX600 instrument.

Results: Testing the fetal fraction assay multiplex on 137 prospective clinical samples demonstrated high concordance with NGS results for both female and male pregnancies as well as with chromosome Y-based calculations for samples with a male fetus. Reproducibility analysis indicated lower variability compared to previously reported NGS performance.

Conclusion: This study showcases the potential of this novel, 6-color, high-multiplex methylation ddPCR panel for accurate measurement of fetal fraction in cfDNA samples. It presents opportunities to integrate such methodology as a standalone measurement to assess the quality of samples undergoing NIPS.

1 | Introduction

The landscape of prenatal screening has undergone a transformative shift with the advent of minimally invasive sampling methods involving a maternal peripheral blood draw for prenatal screening (referred to as NIPS, non-invasive prenatal screening) [1]. This innovative approach has received significant attention as a recommended standard for all pregnancies [2], redefining the paradigm of prenatal care by offering a non-invasive yet highly accurate method for assessing fetal genetic

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Summary

- What's is already known about this topic?
  - Accurate fetal fraction (FF) quantification is essential for reliable non-invasive prenatal screening (NIPS) outcomes.
  - FF quantification via next-generation sequencing (NGS) varies by algorithm, leading to inconsistent results across laboratories.
  - An unmet need for fast and accurate FF quantification is emerging for existing and new diagnostic applications.
- What does this study add?
  - A novel multiplexed ddPCR approach using methylation signatures for FF quantification.
  - Genome-wide map of methylation profiles across early pregnancy placenta and non-pregnant cell-free DNA.

information. The fundamental principle behind NIPS involves the detection and analysis of fetal cell-free DNA (cfDNA) circulating in maternal blood, providing a comprehensive view of the fetal genome without posing risks to the mother or the developing fetus [3]. While next-generation sequencing (NGS) is presently the predominant NIPS readout technology, its high cost and processing time impede universal and equitable NIPS implementation. In addition, a small number of whole-genome NIPS results come back inconclusive due to various factors, including incidental detection of maternal malignancies, causing uncertainty and anxiety among patients [4]. On the other hand, targeted approaches, such as digital PCR offer faster turnaround times, are more cost-effective and are unaffected by maternal malignancies. A critical step in the NIPS workflow lies in determining fetal fraction (FF), a value representing the proportion of fetal cfDNA in maternal plasma. This metric is critical in assessing the reliability and sensitivity of NIPS as well as non-invasive prenatal diagnosis (NIPD) [5]. The ability to accurately measure FF allows for precise interpretation of the result, ensuring optimal sensitivity in detecting genetic aberrations while minimizing false positives or negatives.

To date, several approaches for quantifying fetal fraction in maternal plasma across targeted (PCR-based) and non-targeted (whole-genome sequencing-based) methodologies have been proposed. Regardless of the readout method (targeted or genome-wide), three major approaches include: (i) quantification of Y-chromosome-specific sequences [6, 7], (ii) single-nucleotide polymorphism (SNP)-based approaches where informative (paternally inherited) SNP frequency is used to calculate fetal load [8–10], and (iii) SNP-independent approaches relying on universal differential properties (methylation, size) between fetal and maternal cfDNA populations [11, 12].

While quantifying fetal cfDNA load through chrY sequence-specific assays (approach i) can be highly accurate, this approach is only applicable to pregnancies with a male fetus and is therefore non-universal, leading to the adoption of approaches (ii) and (iii) described above. Techniques leveraging paternally inherited polymorphic nucleotides or insertion/deletion polymorphisms as markers (approach ii) rely on parental genotypes, and in some cases, their implementation is impeded due to the unavailability of paternal DNA. For targeted detection in the absence of a paternal DNA sample, pre-amplification of maternal cfDNA is often required along with pre-quantification of the maternal sample by NGS or droplet digital PCR (ddPCR) for identification of paternally inherited SNPs, increasing the cost and turnaround time of the analysis [10]. Another downside of this approach is that, regardless of paternal sample availability, a personalized assay panel must be constructed for targeted fetal fraction quantification. Non-targeted fetal fraction quantification carries additional drawbacks: aside from increased sequencing costs, it precludes early fetal fraction assessment, potentially wasting resources on samples with inadequate fetal fraction levels that could have been identified and excluded prior to fully sequencing.

To address these challenges, SNP-independent algorithms, such as fetal cfDNA quantification based on size or methylation status [11, 12] as well as nucleosome positioning [13], have been proposed (approach iii). While these approaches serve as a workaround for limited paternal sample availability, fragment size- and nucleosome positioning-based approaches are only applicable when whole genome sequencing is used as a readout. Methylation-based approaches, on the other hand, are promising for targeted applications such as digital PCR, although these methods may also be limited by target region selection and the multiplexing capability of the instrument. Therefore, an unbiased and informed selection of biomarker regions is crucial for overcoming these challenges and facilitating widespread implementation of a methylation-based digital PCR approach.

Apoptotic fetal extravillous trophoblasts have been established as the main contributors of fetal cfDNA in maternal plasma [14–16]. As this cell population originates from placental tissue, we proposed that a genome-wide approach to discover regions of contrasting methylation status between fetal placenta gDNA and non-pregnant plasma cfDNA serving as maternal background could provide a candidate set of targets for a downstream methylation-based analysis of fetal fraction.

In this study, we demonstrate a proof of concept for a methylation-based approach for quantifying fetal fraction via multiplexed ddPCR combined with methylation-sensitive restriction enzyme (MSRE) digestion. By targeting multiple regions of high methylation contrast in one reaction, we demonstrate the potential for ddPCR to become an attractive solution for universal fetal fraction determination. Finally, we demonstrate the clinical performance of our approach in comparison to NGS readout using cfDNA isolated from patients in the first trimester of pregnancy.

2 | Methods

2.1 | Participants and Sample Collection

2.1.1 | Biomarker Discovery Cohort—Plasma Samples

Plasma samples used for the biomarker discovery cohort were obtained from Cerba Research and iSpecimen. Signed consent

TABLE 1 | Biomarker discovery cohort sample summary.

DNA source	Sample source	Gestational age (mean ± SD), weeks	Fetal fraction (mean ± SD), %	Fetal aneuploidy status	*N*
cfDNA	Non-pregnant plasma	NA	NA	NA	3
cfDNA	Pregnant plasma	18.8 ± 4.9	16.8 ± 3.6	Normal	4
cfDNA	Pregnant plasma	16.4 ± 0.1	12.0 ± 1.4	Trisomy 18	2
cfDNA	Pregnant plasma	13.5 ± 0.1	15.5 ± 3.5	Trisomy 21	2
gDNA	Early pregnancy placenta	14.8 ± 3.2	~100%	Normal	10

Note: Summary of the sample cohort used for methylation biomarker discovery. The table includes the source of DNA (cfDNA or gDNA) and sample type (plasma or placenta), along with the gestational age, fetal fraction, and fetal aneuploidy status where applicable. Mean values and standard deviations (mean ± SD) are reported for gestational age (in weeks) and fetal fraction (in percentage). Sample sizes (*N*) for each category are also provided. Aneuploidy status is listed as "Normal," "Trisomy 18," or "Trisomy 21" where applicable.

forms were obtained from all participants prior to sample collection. Isolated plasma was snap frozen and shipped to Bio-Rad Laboratories for cfDNA isolation and ddPCR analysis as described below.

Three plasma samples from non-pregnant female individuals were obtained from iSpecimen. Eight plasma samples from pregnant individuals (normal pregnancy, $n = 4$ and aneuploid, $n = 4$) were obtained from Cerba Research (Table 1). Inclusion criteria consisted of more than 10 weeks of gestation and no previous study enrollment along with available or pending NIPS results. Exclusion criteria for pregnant sample collection were prenatally diagnosed genetic or extracardiac abnormalities, twin gestation, growth restriction, or significant uteroplacental disease such as pre-eclampsia or maternal pre-gestational diabetes. Detailed clinical maternal and fetal data were collected.

2.1.2 | Biomarker Discovery Cohort—Placental Samples

Placental samples were collected from normal early pregnancy (GA 9–19 weeks, $n = 10$) after elective termination by mechanical evacuation (Table 1). Pregnancies were dated by the last menstrual period and were confirmed by first-trimester ultrasound. The institutional review board on human research at NYU Langone approved the study protocol. A description of all plasma and placental samples used for biomarker discovery is provided in Table 1.

2.1.3 | Biomarker Validation Cohort—Plasma Samples

Prospective plasma collections from pregnant individuals were performed at three independent collection sites (Cerba Research, NYU Langone Hospital Long Island, Columbia University in the City of New York) according to standardized procedures. Eligible participants at more than 10 weeks of gestational age satisfying the study inclusion criteria were enrolled. A total of 259 plasma aliquots were obtained from 137 pregnant individuals. Gestational age and NGS NIPS results per sample are listed in Table S1. Between 2 and 4 Streck Cell-Free DNA BCT tubes were collected per patient for subsequent plasma isolation. Blood collection tubes containing whole blood were centrifuged for 10 min at 10°C and 1600*g*. Up to 5 mL of plasma was transferred into 15 mL centrifuge tube for a subsequent centrifugation step at 3200*g* for 10 min. Isolated plasma was snap frozen and shipped to Bio-Rad Laboratories for cfDNA isolation and ddPCR analysis. Signed consent forms were obtained from all participants prior to sample collection.

2.2 | Bisulfite Library Preparation and Sequencing of Placental and Cell-Free DNA Samples

Placental library preparation and sequencing were performed at NYU Langone's Genome Technology Center. Cell-free DNA library preparation was performed at Bio-Rad Laboratories and libraries were submitted for sequencing to Novogene Inc.

2.2.1 | Placental Samples Processing

Placental gDNA was isolated from snap-frozen tissue using a QIAamp Fast DNA Tissue kit (Cat#51404, Qiagen, Germantown, MD). The sex of the placentas was determined via analysis of the sex-linked chromosome genes ZFX and ZFY by real-time PCR. Primers targeting the sex chromosome genes ZFX (GenBank Acc.eNo. NG_021253, NM_003410) and ZFY (GenBank Acc.eNo. NG_008113, NM_003411) as described by Weiss and Johnston [17] were used for PCR amplification: ChrX-Y_F 5′-ATTTGTTCTAAGTCGCCATATTCTCT-3′, ChrX_R 5′-GAACACACTACT-GAGCAAAATGTATA-3′ and ChrY_R 5′-CATCTTTACAAGCTTGTAGACACACT-3′. Each DNA sample was amplified with primer sets ChrX-Y_F/ChrX_R and ChrX-Y_F/ChrY_R using LightCycler 480 SYBR Green I Master (cat# 04707516001, Roche Diagnostics, IN) on a QuantStudio3 thermal cycler (Applied Biosystems, Waltham, MA). Thermocycling conditions were set as an initial polymerase activation step for 10 min at 94°C, followed by 40 cycles of 45 s at 94°C for template denaturation, 45 s at 50°C for annealing and 30 s at 72°C for extension, followed by a melting curve ranging from 65 °C to 95 °C. All samples were positive for primers ChrX-Y_F/ChrX_R (represent of X chromosome). Samples positive with primers ChrX-Y_F/ChrX_R and ChrX-Y_F/ChrY_R (present of Y chromosome) were assigned to the male group and samples positive with primers ChrX-Y_F/ChrX_R but negative with ChrX-Y_F/ChrY_R were assigned to the female group.

Placental sample sequencing was performed at the NYU Langone's Genome Technology Center (RRID: SCR_017929). Sequencing libraries were prepared with 200 ng genomic DNA using the NEBNext Enzymatic Methyl-seq Kit (cat# E7120, New

England Biolabs, Ipswich, MA) according to the manufacturer's protocol, except 5 cycles (200 ng DNA input) of PCR amplification were performed. Libraries were sequenced with the NovaSeq 6000 Sequencing System (Illumina) on S4 flow cells in a PE150 run.

2.2.2 | Cell-Free DNA Sample Processing

Libraries from cfDNA were prepared using cell-free DNA extracted from 2 mL of plasma using the Apostle MiniMax High Efficiency Cell-Free DNA Isolation Kit (Apostle Inc.). Sequencing libraries were prepared with 5–20 ng of cfDNA using the NEBNext Enzymatic Methyl-seq Kit (cat# E7120, New England Biolabs, Ipswich, MA) according to the manufacturer's protocol Libraries were sequenced with the NovaSeq 6000 Sequencing System (Illumina) on S4 flow cells in a PE150 run at Novogene Inc.

2.3 | NGS Data Analysis

NGS data analysis was performed at Bio-Rad for both placental and cell-free DNA samples.

Methylation sequencing reads were aligned to the hg38 genome assembly using the Bismark aligner [18]. The Bismark Methylation Extractor was used to parse methylation count and frequency statistics for CpG loci across all samples. A customized python script was written to analyze methylation frequencies (methylated reads divided by the total reads) and to identify differentially methylated CpG loci between sample groups. Binomial logistic regression was performed after Haldane–Anscome correction using the python statsmodels command:

$$\text{smf.glm}('\text{methylated\_counts} + \text{unmethylated\_counts} \\ \sim \text{sample\_group}', \text{family} = \text{sm.families.Binomial}(), \text{data} = \text{df}),$$

where “sample_group” is an indicator variable that specifies fetal versus maternal samples. Only loci with $10 \leq \text{cov} \leq 45$ were considered: low coverage sites were removed due to the lack of statistical power and high coverage sites were removed as outliers caused by low-complexity or repetitive regions. Statistical significance was declared for loci with a methylation frequency difference > 50% between fetal and maternal groups and a *p*-value below 0.05 after Bonferroni correction.

Custom Python and R scripts were written to analyze and QC samples using read coverage, methylation conversion rates, and methylation frequency distributions.

Gene set enrichment analysis was performed using GSEA 4.3.3 in preranked mode [19, 20]. Genes were ranked according to the median *p*-value (following our differential methylation analysis) of the gene promoter region that we defined as the set of CpG loci within 2 kb of the transcriptional start sites.

2.4 | ddPCR Assay Design and Experimental Validation

The fetal fraction assay targets regions of high methylation contrast between maternal and fetal cfDNA. Assays were designed for use with the QX600 instrument (Bio-Rad Laboratories), a six-color ddPCR fluorescence reader. High levels of multiplexing were achieved using universal probes optimized for ddPCR, requiring only one probe per fluorescent channel. An in-house primer multiplex design engine was used to produce multiplex-compatible primer designs, incorporating methylome sequence data to define targets of interest. The target amplicon size was 70 ± 2 bp, enabling amplification for most fetal and maternal molecules in the sample. Following in silico design, the assays were iteratively developed based on empirical ddPCR performance and confirmation by amplicon sequencing analysis (Table 2).

2.4.1 | ddPCR Experimental Validation

To assess ddPCR assay performance against the reported FF NGS result, plasma samples originating from 137 unique patients were tested with the fetal fraction ddPCR assay and compared with attached NIPS NGS results provided by the collection site. The fetal fraction NGS readout for all patient samples was obtained using the MaterniT21 PLUS Core + SCA or MaterniT21 test (Labcorp, USA). For some patients, replicate plasma samples with the same collection date were tested, resulting in a total of 259 plasma samples tested with the FF ddPCR assay.

Cell-free DNA was extracted from 4 mL of plasma using a semi-automated workflow with the KingFisher instrument and Apostle MiniMax High Efficiency Cell-Free DNA Isolation Kit (Apostle Inc.). Operator-defined elution volume was set to 60 µL; however, the actual eluted volume post-extraction ranged between 50 and 55 µL. Following extraction, buffer exchange was performed using AMPure beads, and samples were subse-

TABLE 2 | Multiplexed ddPCR assay target summary and channel assignment.

Target	“Hypo”	“Maternal”	“Hyper”	“Control”	“ChrY”	“Fetal”	6-target total
QX600 channel	FAM	HEX	Cy5	Cy5.5	ROX	ATTO590	—
Assay plex size	6	7	7	9	10	8	47

Note: Summary of the multiplexed ddPCR assay targets and their corresponding channel assignments. The table lists each target category (“Hypo,” “Maternal,” “Hyper,” “Control,” “ChrY,” and “Fetal”) alongside the associated detection channel on the QX600 system (FAM, HEX, Cy5, Cy5.5, ROX, and ATTO590). The total number of targets for each assay plex is indicated, summing to 47 across the six target categories.

quently eluted into 45 μL of DNA Resuspension Buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0) (Teknova). Quality control (QC) was performed on 2 μL of the eluted sample using the TapeStation system, while the remaining volume was divided between Fetal Fraction determination (1 ddPCR well) and a bespoke assay (3 ddPCR wells, not discussed in this manuscript). To account for sample loss during droplet generation, 9.9 μL was dispensed per ddPCR well, of which 9 μL of DNA was taken into droplet preparation. The fetal fraction reaction included in-droplet MSRE digestion to enable discrimination between fetal and maternal cfDNA. The ddPCR reaction and amplification conditions are included in Supporting Information S1: Supplementary Methods. ddPCR was performed on a Bio-Rad QX600 system with an automated droplet generator and a C1000 thermocycler (Bio-Rad Laboratories). The total time from reaction setup to obtaining results ranged from 6 to 8 h depending on sample batch size. All ddPCR experiments were performed in accordance with the digital MIQE guidelines to ensure reproducibility, accuracy, and transparency in data reporting [21].

2.4.2 | ddPCR Analysis Pipeline

ddPCR files were visualized using QX Manager Software (v2.1) followed by applying a novel in-house-developed color calibration and automated thresholding algorithm to raw ddPCR files (Figure S1C). Clustering analysis was used to determine thresholds to classify positive and negative droplets. Fetal fraction was calculated using the algorithm provided in Supporting Information S1: Supplementary Methods.

3 | Results

3.1 | Genome-Wide Differential Methylation Map Construction

As an intial step, we assessed clustering tendencies of sequenced samples based on their genome-wide methylation frequency profiles utilizing Uniform Manifold Approximation and Projection (UMAP). Our UMAP analysis revealed distinct differences between pregnant and non-pregnant cfDNA samples compared with placenta tissue collected from early pregnancies (Figure 1A). Following this analysis, "fetal" and "maternal" hypermethylated CpG loci were determined statistically by comparing methylated and non-methylated counts across all fetal samples against those from non-pregnant female plasma ("maternal") samples using binomial logistic regression. Differentially methylated sites were identified using a bonferroni-adjusted p-value threshold of 0.05 and requiring a minimum 50% change in methylation frequency between sample groups (Figure 1B). Differential methylation analysis between early pregnancy gDNA placental and cfDNA maternal plasma samples uncovered 1,011,976 out of 21,339,684 tested CpG loci with significant methylation differences. Next, we conducted hierarchical clustering analysis on both sets of differentially methylated loci across all samples, demonstrating differential methylation profiles within placental and plasma samples in the cohort (Figure 1C). Notably, these clustering patterns also show a distinct grouping of pregnant plasma samples, consistent with the expected composition of those samples which is comprised of a mixture of fetal and maternal DNA. Our analysis did not reveal any significant differences in sample clustering patterns attributable to fetal sex or fetal aneuploidy status. Comparative analysis of differentially methylated CpG loci across all samples was used to define fetal and maternal biomarkers. "Fetal" biomarkers show hypomethylation status in non-pregnant cfDNA samples, contrasted sharply by hypermethylation status in early pregnancy placental samples. Conversely, "maternal" biomarkers show hypomethylated status in early pregnancy placenta samples, while being hypermethylated in non-pregnant cfDNA samples. The methylation frequencies observed in cfDNA from pregnant individuals show that the average distribution of both fetal and maternal biomarkers are closer together, in line with the established fact that cfDNA of pregnant individuals is a mixture of DNA derived from both fetal and maternal cells [22] (Figure 1D).

3.2 | In Silico Biomarker Validation

To further confirm the specificity of the sites identified as differentially methylated, we examined the proportion of methylated reads across all significant loci by sample and chromosome, normalized to the mean chromosomal fractions in euploid samples. Z-scores were computed for each chromosome by sample. Chromosomal z-scores revealed elevated methylation frequencies of chromosomes 18 and 21 in the trisomy 18 and trisomy 21 cfDNA samples, respectively. The increase in methylation frequency is consistent with the increase in copy number of the respective chromosomes in the context of fetal aneuploidy (Figure 2A). Maternal differential site frequencies were then analyzed using the same approach. A decrease in methylation frequency for aneuploid chromosomes is consistent with the increased proportion of fetal copies for that chromosome (Figure 2B).

In order to illustrate the implications of our findings, gene set enrichment analysis (GSEA) was performed. Sixty-five cell type signature gene sets (C8 in MSigDB [23, 24]) were marked as significant (FDR < 25%). The identified pathways are primarily associated with gene expression and chromatin accessibility in fetal or adult cell types, thereby underscoring the biological relevance of these biomarkers in distinguishing fetal from maternal cell types (Figure 2C).

3.3 | ddPCR Validation of Methylation Biomarker Panel

Following biomarker discovery, a highly multiplexed 6-channel ddPCR-based FF assay (Table 2) was designed using a subset of the biomarkers. Two of the channels were designed around biomarkers that were (A) hypermethylated in fetal cell types and hypomethylated in adult cell types ("Fetal" channel) and (B) hypermethylated in adult cell types and hypomethylated in fetal cell types ("Maternal" channel). These channels quantified the amounts of fetal and maternal cfDNA in the maternal blood sample. Two additional channels were designed around biomarkers that were (A) hypermethylated in both fetal and adult cell types ("Hyper") and (B) hypomethylated in both cell types

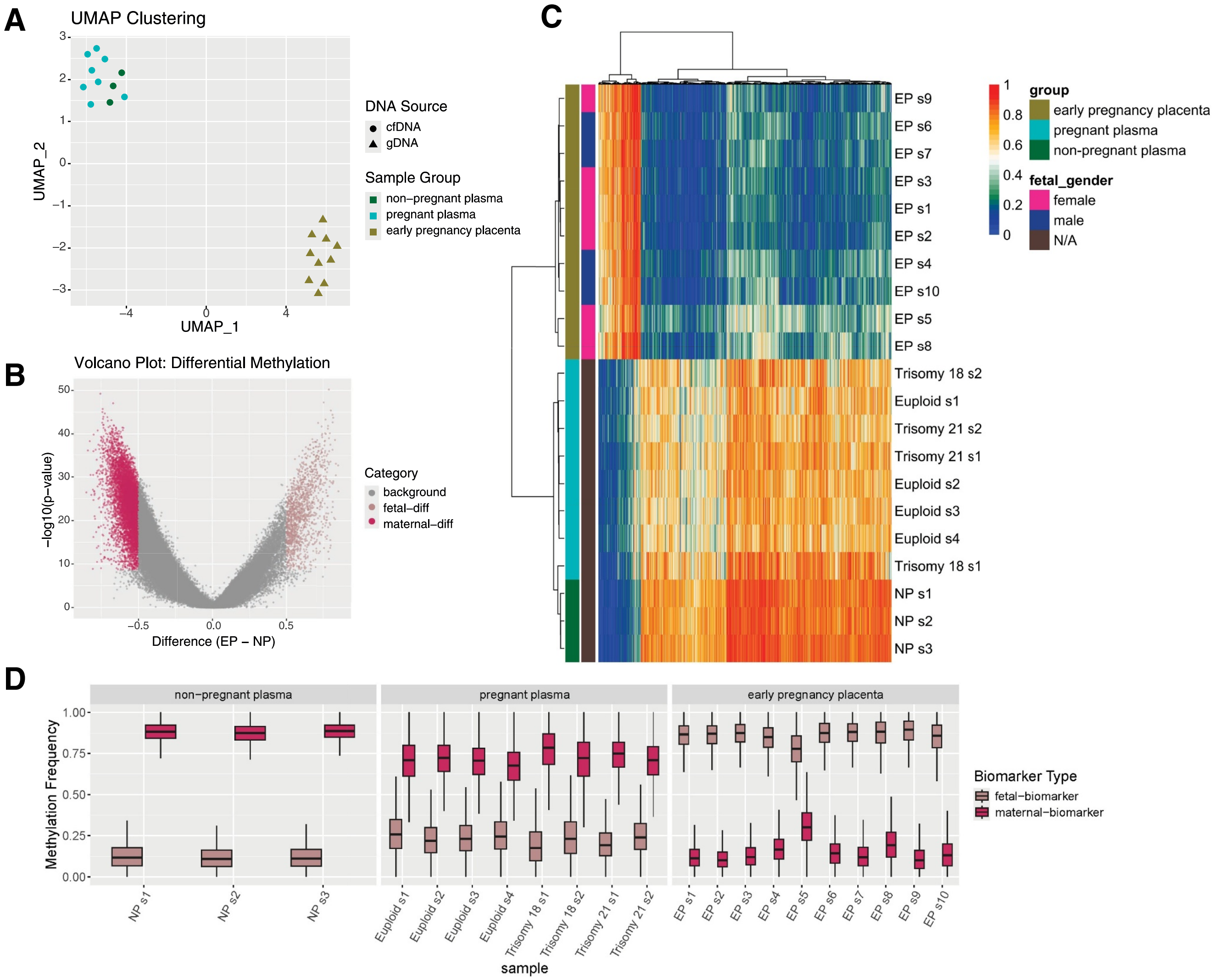

FIGURE 1 | Discovery of fetal and maternal biomarkers using whole genome methylation sequencing of placenta tissue and non-pregnant maternal cfDNA. (A) Genome-wide methylation frequencies reveal similarities between samples of the same group. UMAP projections of samples based on whole genome methylation frequency profiles separate samples by DNA source (gDNA and cfDNA) and sample source (placenta vs. plasma). (B) Significant differentially methylated CpG loci were identified distinguishing early pregnancy (EP) placenta tissue samples from non-pregnant (NP) female cfDNA samples. A Volcano plot was made using 2500 randomly selected CpG loci, sites were colored if the average methylation frequency difference between sample groups was > 0.5 or less than −0.5 and the Bonferroni adjusted *p*-value was < 0.05. The colored points refer to significantly differentially methylated sites, where "fetal-diff" loci are significantly hypermethylated in placenta tissue compared to non-pregnant cfDNA, and vice versa for "maternal-diff" sites. (C) Differentially methylated loci distinguish sample origins. A heatmap was made using 2500 randomly selected CpG loci. The non-pregnant plasma samples (NP), pregnant plasma samples, and early pregnancy (EP) placenta sample groups and fetal genders were annotated by color on the left. Individual sample identifiers (S#) were included on the right with each sample group. A color heat scale was used to represent the methylation frequency of CpG sites, blue for hypo-methylated and red for hyper-methylated status. (D) Biomarkers were selected from differentially methylated sites and labeled as fetal if they were hypermethylated (≥ 0.8) in early pregnancy placenta and hypomethylated (≤ 0.2) in non-pregnant samples and vice versa for maternal. Boxplots of methylation frequency distributions for both fetal biomarkers and maternal biomarkers were made for all samples and separated into panels for each sample group.

("Hypo"). These two channels acted as references for MSRE digestion and adjusted the FF calculation to account for incomplete digestion. Finally, the remaining two channels were designed around methylation insensitive regions to quantify total nucleic acid content ("Control") and the Y-chromosome in the case of pregnancies with a male fetus ("chrY") (Table 2, Figure S1A,B). A total of 259 plasma aliquots from 137 pregnant individuals were analyzed by ddPCR in this assessment. Mean gestational age at the time of sampling for the validation cohort was 12.4 weeks (SD: 4.7 weeks). An average of 20,850 accepted droplets (SD: 1103) were analyzed per sample. The ddPCR data generated for each sample along with clinical sample information are provided in Table S1.

The assay performance was first evaluated by comparing the methylation-based ddPCR readout with the chrY-based ddPCR readout (Figure 3A). The correlation ($R = 0.64$, $p =$ 3e-7, $n = 67$) between these orthogonal readouts in male samples confirmed

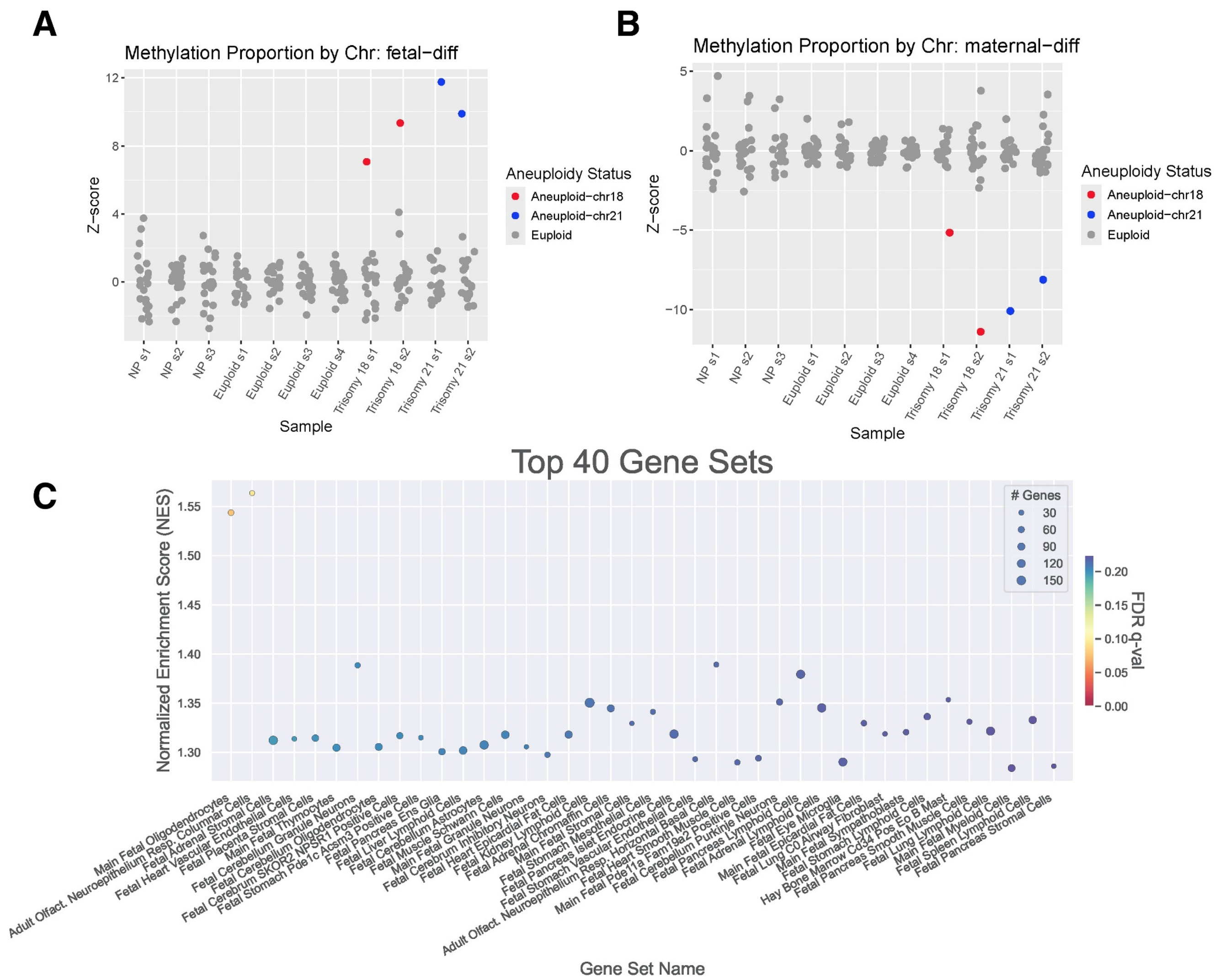

FIGURE 2 | In silico validation of differential methylation analysis. (A) Chromosomal *z*-scores showed elevated methylation frequencies at fetal-specific differential sites (fetal-diff) on chromosomes 18 and 21 in the trisomy 18 (T18) and trisomy 21 (T21) cfDNA samples, respectively. *Z*-score distributions are displayed for each chromosome in every sample, representing the proportion of CpG base sequencing counts methylated at fetal-diff sites, normalized to the distributions observed in four euploid pregnancies. Chromosomes 18 and 21 are highlighted in the respective trisomy 18 and 21 samples. NP corresponds to non-pregnant cfDNA. (B) Conversely, chromosomal *z*-scores revealed decreased methylation frequencies at maternal-specific differential sites (maternal-diff) on chromosomes 18 and 21 in the trisomy 18 and trisomy 21 samples, respectively. The *z*-score distribution plot for maternal-diff sites was normalized as described in (A). (C) Cell types relevant to early fetal development were identified by gene set enrichment analysis (GSEA). GSEA was performed on all genes containing significantly differentially methylated fetal or maternal biomarkers at their promoter regions (± 2 kb from the transcription start site). Genes were ranked by the median *p*-value of CpGs within the promoter region.

the utility of the selected biomarkers for cell-free DNA analysis in pregnant individuals. Based on this, we developed an algorithm that integrates methylation-based and chrY-based ddPCR FF estimates into a unified readout.

To further validate the approach, we assessed the correlation between the ddPCR-based FF results and those obtained from NGS-based methods (Figure 3B). The correlation ($R = 0.70$, $p = 5\text{e-}18$, $n = 113$) observed demonstrates the effectiveness of targeting methylation biomarkers for accurate fetal fraction quantification.

Finally, we evaluated the robustness and reproducibility of the ddPCR assay by analyzing replicate blood draws. As shown in Figure 3C, the assay demonstrated excellent reproducibility ($R = 0.95$, $p = 4\text{e-}48$, $n = 126$).

4 | Discussion

Our study aimed to demonstrate the feasibility of a targeted amplification-based approach for fetal fraction quantification in maternal plasma using ddPCR. To achieve this, we carried out fetal- and maternal-specific methylation biomarker discovery, multiplex ddPCR assay design, optimization and validation on a set of 259 plasma aliquots obtained from 137 pregnant individuals.

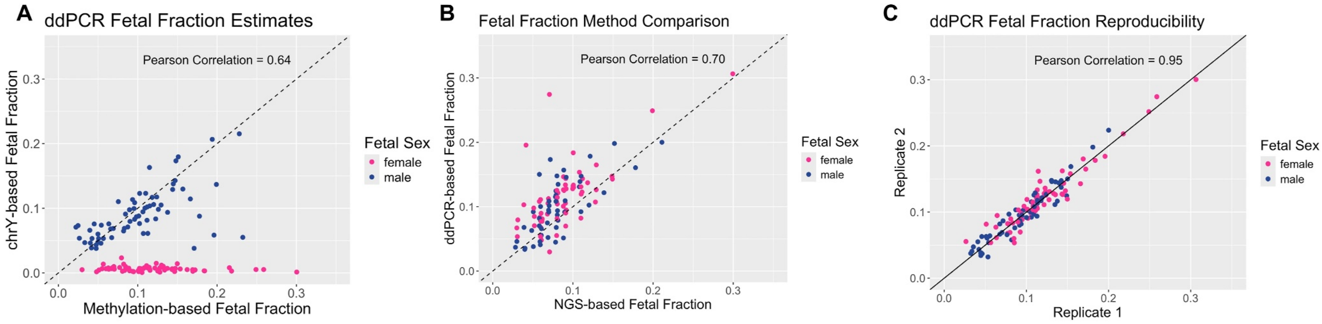

FIGURE 3 | Fetal fraction ddPCR assay performance with cfDNA samples. (A) ChrY-based fetal fraction estimates are consistent with methylation-based fetal fraction estimates for male fetal samples using ddPCR. The scatterplot compares fetal fraction estimations from clinical samples using multiplex ddPCR assays targeting chrY and methylation biomarkers. The Pearson correlation coefficient ($R = 0.64$, p = 3e-7, $n = 67$) was calculated for chrY and methylation values from male samples. (B) Combined ddPCR fetal fraction readouts aligned with NGS-based methods. The combined ddPCR readout included chrY and methylation-based measurement for male fetal samples, and methylation-based measurement for female samples. The Pearson correlation coefficient ($R = 0.70$, p = 5e-18, $n = 113$) was calculated using both male and female samples. (C) Evaluation of duplicate blood draws demonstrates the reproducibility of the ddPCR-based assay. Fetal fraction values from the methylation-based ddPCR assay were compared by analyzing measurements from independently extracted duplicate blood samples and run on different plates. The Pearson correlation coefficient ($R = 0.95$, p = 4e-48, $n = 126$) was calculated using both male and female samples.

Differential methylation analysis between early pregnancy gDNA placental and cfDNA maternal plasma samples uncovered 1,011,976 out of 21,339,684 tested CpG loci with significant methylation differences. Subsequent methylation frequency analysis at these sites allowed us to correctly detect aneuploid chromosomes in a small cohort of aneuploid samples. Therefore, we propose that the placenta provides a robust representation of the fetal state across thousands of methylation-sensitive genomic sites. Importantly, we verified that the majority of the biomarkers that differ between fetal and maternal cohorts did not significantly vary as a function of fetal sex. This uniformity is an important feature that may simplify implementation of the assay in clinical settings and ensures reliable performance across diverse patient populations and allows for fetal fraction quantification in both male and female fetus pregnancy scenarios.

To validate our approach, we prospectively collected plasma samples from pregnant individuals and benchmarked the performance of the methylation-based FF assay we developed against chrY ddPCR readout as well as the NGS value obtained from the collection sites. Following ddPCR analysis, we observed high concordance with chrY-based ddPCR readout as well as good agreement with NGS-based methods. We demonstrated that the methylation-sensitive ddPCR assay yields fetal fraction estimates comparable to orthogonal NGS readout, albeit the influence of disparate sampling times in clinical samples potentially contributes to the observed variance in fetal fraction estimates between the two approaches.

Our study highlights the excellent reproducibility of the ddPCR fetal fraction assays, with high consistency across replicates ($R = 0.95$, p = 4e-48, $n = 126$), potentially outperforming some NGS approaches in terms of measurement stability [25]. This reproducibility is critical for clinical implementation, ensuring that results are reliable and can be replicated across different laboratories and settings.

At the same time, our study has several limitations. Firstly, our biomarker discovery cohort included limited numbers of placental samples as well as non-pregnant cfDNA controls. Although our validation cohort included samples from various backgrounds, it may not encompass the full range of diversity. Therefore, independent validation will be required to be performed by laboratories interested in implementing this approach.

Another limitation is the inherent Poisson noise associated with ddPCR. One contributing factor is the variability in the number of DNA fragments available in plasma following restriction digestion. Uniform amplification of both fetal and maternal cfDNA molecules is also a potential source of noise. Fetal specific cfDNA molecules are on average smaller (~142 bp) than maternal cfDNA molecules (~172 bp) [3]. Differences in fragmentation patterns can affect the accessibility of primers to all molecules. Additionally, the dynamic nature of DNA methylation presents challenges; whereas our current design targets multiple fetal and maternal biomarkers, so future higher multiplexed iterations could benefit from incorporating more biomarkers to mitigate genetic and temporal variability and improve averaging across these biomarker sets.

Beyond fetal fraction estimation for NIPS purposes, the technologies developed in this study position ddPCR as a promising tool for other reproductive health applications such as single gene disorder diagnosis [26] and prenatal invasive diagnostic testing. In addition, emerging applications of prenatal non-invasive whole genome and exome sequencing could benefit from implementing ddPCR fetal fraction testing as an upstream quality control step prior to sequencing. This approach is particularly useful in higher-risk cases for sample failure, such as pregnancies in obese women or samples collected before 10 weeks of gestation [27], where fetal fraction tends to be lower, making it an effective tool for informed decision-making. At the same time, by incorporating this step for every sample, laboratories could determine the sequencing coverage required for each sample to achieve the desired sensitivity for fetal variant detection while reducing sequencing cost [28]. A similar approach can be further developed for invasive applications with the objective to detect maternal cell contamination in CVS or amniotic fluid. Beyond reproductive health applications, in-droplet MSRE digestion coupled with ddPCR offers the competitive advantage in the fields of oncology and epigenetics.

While this study focuses on fetal fraction estimation through direct quantification of fetal cfDNA, this same methodology can potentially be applied for the direct detection of aneuploidies. Fetal fraction estimation is an important QC step for NIPS as the chromosome ratio signal is diluted at lower fetal fractions. Direct amplification of fetal molecules would also result in an increased signal for aneuploidy detection. These approaches potentially provide greater democratization of NIPS by lowering sample input requirements, widening the window for testing earlier gestational ages, and reducing the reliance on high-cost sequencing.

Overall, the versatility of methylation-based approaches and the flexibility of ddPCR assay design strategies underscore the broader implications of our findings and the potential for ddPCR to enhance various aspects of prenatal diagnostics. Finally, the simplicity of the ddPCR workflow, compared to more complex NGS methods, offers significant advantages in terms of cost, time, and ease of integration into existing laboratory practices.

5 | Conclusion

In summary, our study provides a comprehensive map of methylation biomarkers that distinguish fetal placental methylation patterns from the maternal background. It demonstrates the feasibility and effectiveness of a novel multiplexed methylation-based ddPCR approach for fetal fraction quantification in maternal plasma. By identifying and validating fetal and maternal methylation biomarkers, we developed an accurate, cost-effective, and efficient method. This method holds promise not only for non-invasive prenatal screening and diagnosis but also as a standalone quality control assay to assess sample quality prior to using it in downstream workflows. The technologies and findings presented here pave the way for broader adoption of ddPCR in prenatal care, ultimately improving equitable accessibility and outcomes for pregnant individuals.

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Ethics Statement

For ethics approval, placental samples from the biomarker discovery cohort were collected under a protocol approved by the NYU Langone Hospital Long Island Institutional Review Board (study #i18-01692). Prospective plasma collections for ddPCR validation were approved by the NYU Langone Hospital Long Island (study# i21-01262 approved on 12/22/2021) and Columbia University in the City of New York (study# IRB-AAAT9076 approved on 03/09/2022) Institutional Review Boards.

Consent

Written informed consent was obtained from all participants before plasma sample collection in accordance with ethical guidelines and approved institutional protocols.

Conflicts of Interest

Richard Dannebaum, Olga Mikhaylichenko, David Siegel, Monica Herrera, Eric Hall, Severine Margeridon, Kristin Loomis, Thea Riel, Madhumita Ramesh, Maria Gencoglu, Nathan Hendel, Anthony Henriquez, Chenyu Li, Nyari Dzvova and Raymond-John Abayan are current or past employees of Bio-Rad Laboratories Inc.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.