Novel digital PCR assay utilizing in-droplet methylation-sensitive digestion for estimation of fetal cfDNA from plasma

BIO-RAD

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Background

Accurate quantification of the fetal fraction in cell-free DNA (cfDNA) is crucial for obtaining reliable non-invasive prenatal screening (NIPS) results. While next-generation sequencing (NGS) is presently the predominant NIPS method, its high cost and turnaround time impede universal and equitable NIPS implementation. We present a methylation-based approach for quantifying fetal fraction via droplet digital PCR (ddPCR) and methylation-sensitive restriction enzyme (MSRE) digestion, demonstrating ddPCR as an attractive solution for fetal fraction determination.

Methods

Assays were designed to target MSRE-compatible regions showing high methylation contrast between maternal and fetal cfDNA. Fetal assays targeted sites hypermethylated in fetal cfDNA and Maternal assays targeted sites hypermethylated in maternal cfDNA; universally hypo- or hyper-methylated regions were included as controls (Fig. 1). A proof-of-concept of in-droplet MSRE-ddPCR workflow was established on the Bio-Rad QX600 platform.

Following biomarker discovery and assay design, the assays were tested against 130 clinical samples. ddPCR-derived fetal fraction values were compared against Y-chromosome concentrations (male pregnancies) and NGS-annotated values for (all pregnancies, regardless of fetal sex).

Methylation Biomarker Discovery & Semi-Validation

Placental samples were collected from normal pregnancies at early pregnancy and at term. Additionally, maternal blood plasma was collected from donors that were non-pregnant, pregnant with euploid fetuses, and pregnant with aneuploid fetuses (Table 1). gDNA or cfDNA was isolated from samples and analyzed via bisulfite sequencing to obtain methylation frequency data (Fig. 3). This sequence information informed differentially-methylated biomarker discovery.

The methylation frequency ratio of plasma samples were consistent with the status of the donor; donors with aneuploid pregnancies showed an elevated ratio for the relevant aneuploidy (Fig. 4).

Table 1. Placental and plasma samples used for methylation biomarker discovery.

DNA Source	Sample Source	Gestational Age	Fetal Fraction	Fetal Status	N
cfDNA	Non-pregnant plasma	NA	NA	NA	3
cfDNA	Pregnant plasma	Unknown	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-term placenta (fetal)	14.8+-3.2	~100%	Normal	10
gDNA	Full-term placenta (newborn)	>37	~100%	Normal	14

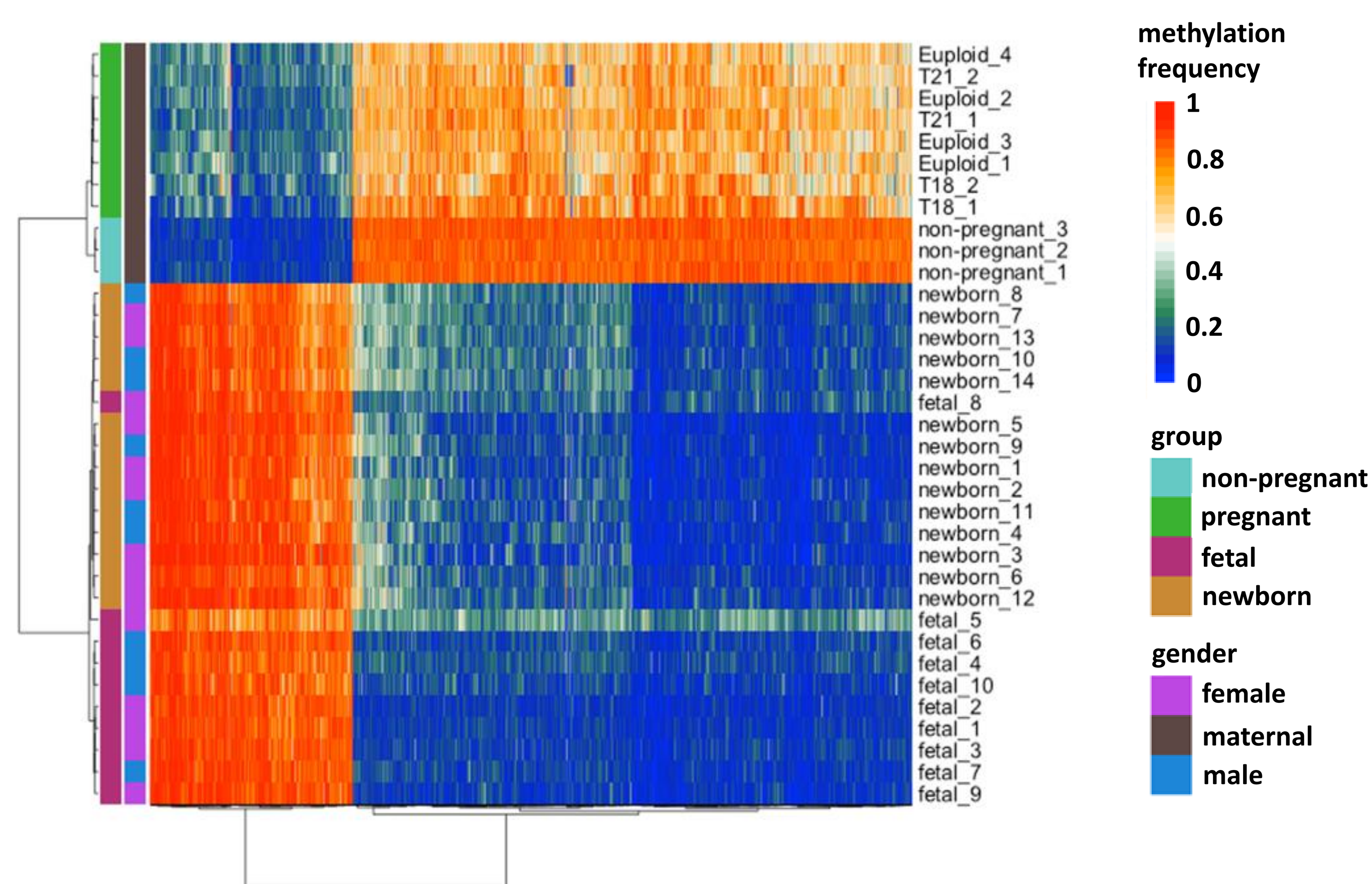

Figure 3. Heatmap of differentially methylated CpG frequencies across all samples, comparing fetal-hypermethylated and maternal-hypermethylated biomarkers.

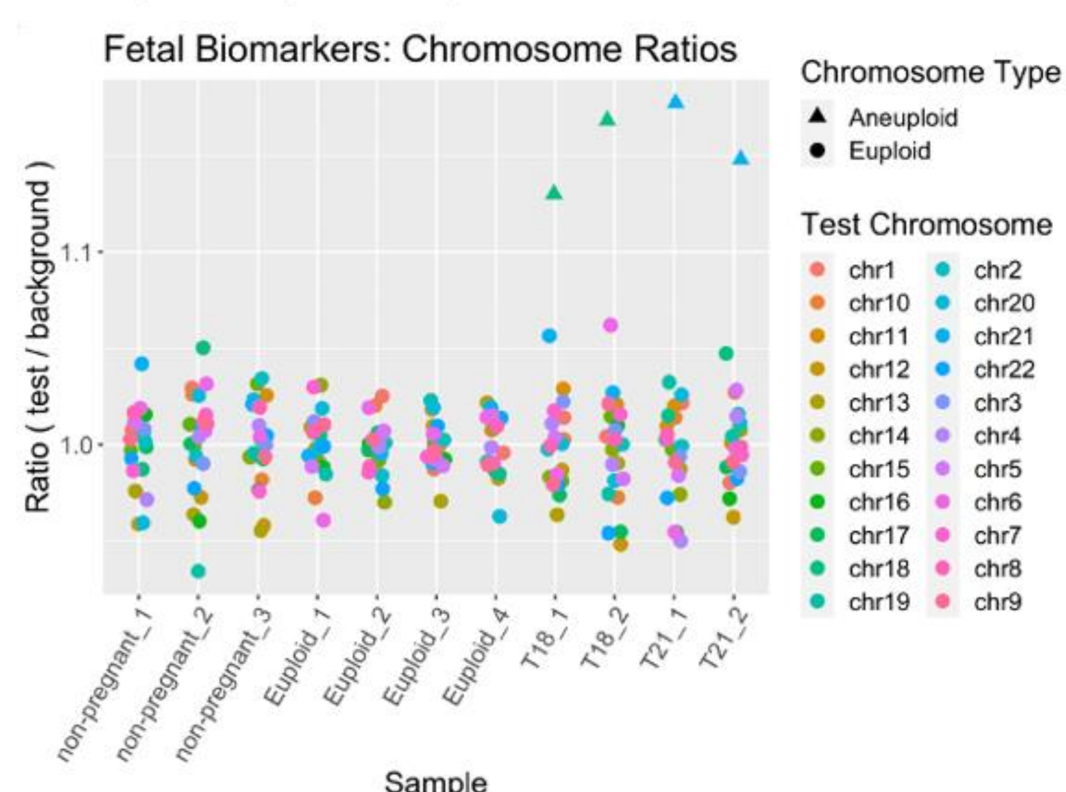

Figure 4. Methylation frequency ratio for cfDNA from plasma samples, comparing chromosome response of non-pregnant and euploid pregnant donors against aneuploid pregnant donors.

Distinguishing Fetal from Maternal cell-free DNA via ddPCR

- Methylation-sensitive restriction enzymes (MSREs) enable ddPCR to visualize differences in fetal and maternal DNA methylation (Fig. 1).
- MSRE digestion is performed in-droplet, with no disruption to the ddPCR workflow

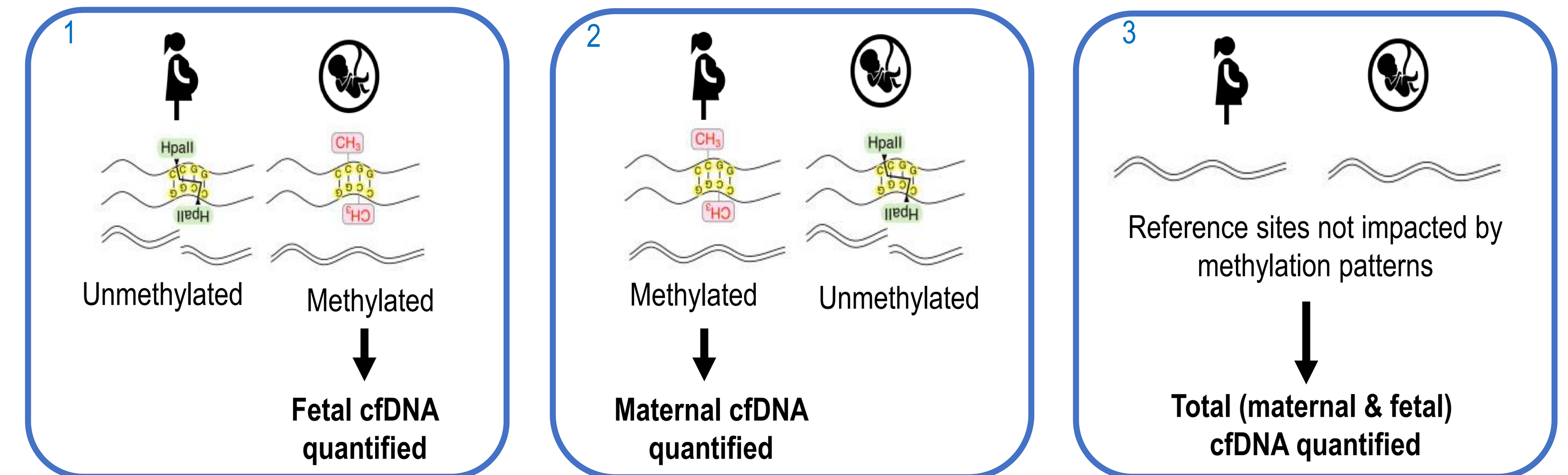

Figure 1. Fetal and maternal cfDNA are quantified simultaneously in the same ddPCR reaction. 1) Hypermethylated fetal cfDNA is quantified after MSRE digestion of hypomethylated maternal cfDNA. 2) Maternal cfDNA is similarly quantified. 3) Total cfDNA is quantified from non-digested regions.

- Molecular beacon probes were used to reduce the number of probes required for high-plex assay sets.
- A universal sequence in the forward primer allows for one molecular beacon design to work for all assays detected in a single fluorescence channel (Fig. 2).

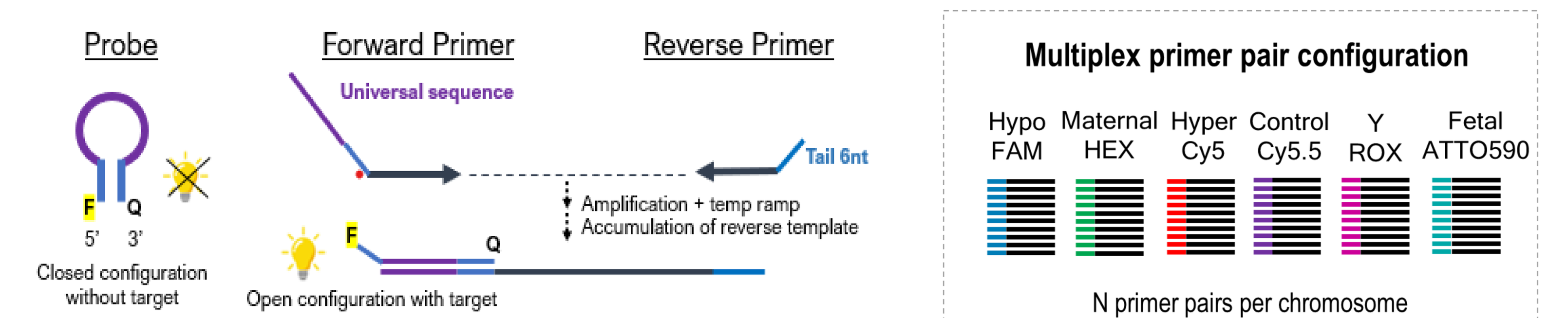

Figure 2. Molecular beacons use a hairpin structure to quench the fluorophore until an amplicon anneals to the hairpin loop, causing the structure to open and emit a signal. The universal sequence on the forward primer allows for multiple assays within a group (e.g. Hypo FAM) to utilize a signal molecular beacon design.

The fetal fraction measurement assay consists of 47 assays, grouped by function and fluorescence channel (Table 2). In addition to methylation-based measurement, the assay also quantifies fetal fraction for male pregnancies via the Y chromosome. Only 6 molecular beacon probes are needed.

Table 2. The fluorescence channel, plex size, and function of each of the 6 groups of the fetal fraction measurement assay.

Assay	Fetal	Maternal	Hypo	Hyper	Control	Y
Channel	ATTO590	HEX	FAM	Cy5	Cy5.5	ROX
Plex Size	8	7	6	7	9	10
Function	Quantifies Fetal cfDNA	Quantifies Maternal cfDNA	Digestion Reference	Digestion Reference	Quantifies total DNA	Quantifies Y chromosome

Clinical Fetal Fraction Assay Performance

The multiplex assay for fetal fraction was evaluated using 111 clinical plasma samples from individuals with euploid pregnancies. Quantification of fetal fraction using methylation-based method showed strong agreement with NGS-reported results (Fig. 5, left), as well as the chromosome Y-based calculation for male samples. Analysis of reproducibility between different runs indicated less variability compared to prior reports for NGS data (median SD 0.61% versus 1.3-3.4%) (Fig. 5, right).

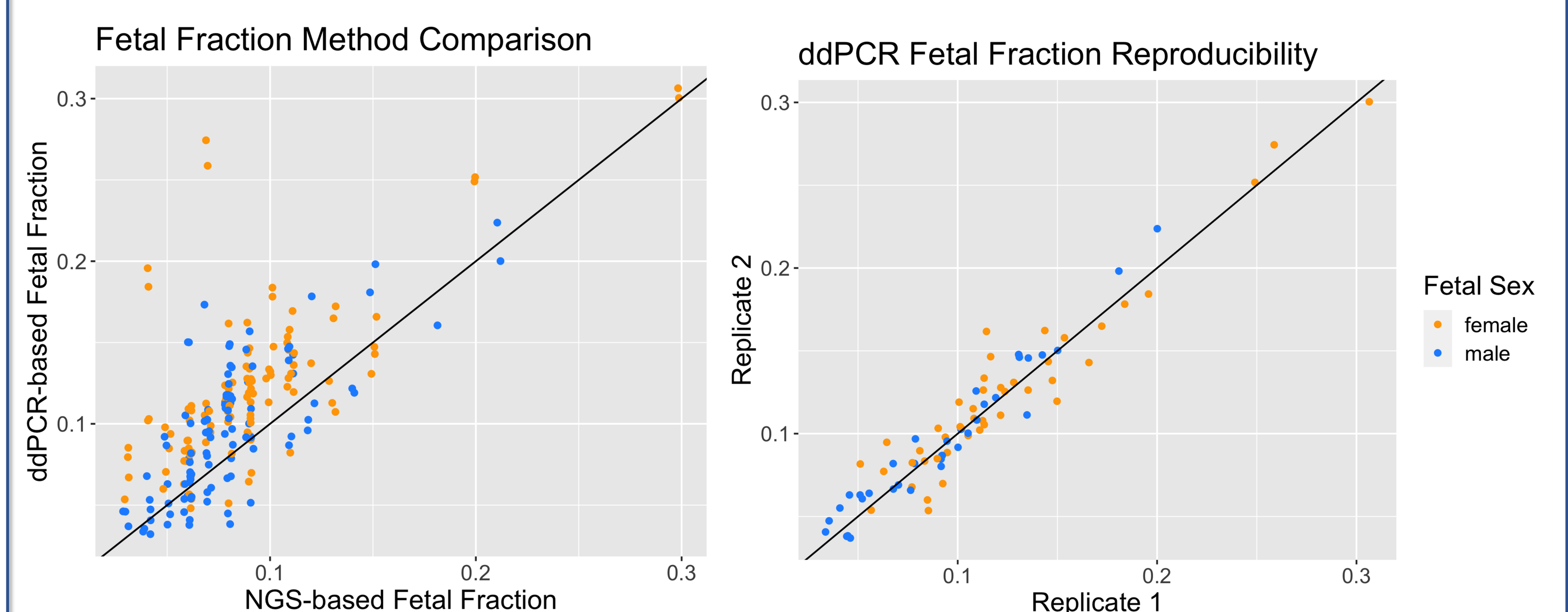

Figure 5. (Left) Comparison of fetal fraction measurements made with ddPCR against those made with NGS. (Right) Reproducibility of ddPCR fetal fraction measurements, obtained by separate analysis of paired plasma samples from the same blood draws.

Conclusions

Droplet digital PCR obtains accurate fetal fraction readouts in a more time- and cost-effective manner compared to NGS. Integrating methylation-based biomarker identification paves the way for compelling alternatives in a wide array of diagnostic applications.

References

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