

ORIGINAL ARTICLE

Development of a new methylation-based fetal fraction estimation assay using multiplex ddPCR

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Abstract

Background: Non-invasive prenatal testing (NIPT) for fetal aneuploidies has rapidly been incorporated into clinical practice. Current NGS-based methods can reliably detect fetal aneuploidies non-invasively with fetal fraction of at least 4%. Inaccurate fetal fraction assessment can compromise the accuracy of the test as affected samples with low fetal fraction have an increased risk for misdiagnosis. Using a novel set of fetal-specific differentially methylated regions (DMRs) and methylation sensitive restriction digestion (MSRD), we developed a multiplex ddPCR assay for accurate detection of fetal fraction in maternal plasma.

Methods: We initially performed MSRD followed by methylation DNA immunoprecipitation (MeDIP) and NGS on fetal and non-pregnant female tissues to identify fetal-specific DMRs. DMRs with the highest methylation difference between the two tissues were selected for fetal fraction estimation employing MSRD and multiplex ddPCR. Chromosome Y multiplex ddPCR assay (YMM) was used as a reference standard, to develop our fetal fraction estimation model in male pregnancy samples. Additional 123 samples were tested to examine whether the model is sex dependent and/or ploidy dependent.

Results: In all, 93 DMRs were identified of which seven were selected for fetal fraction estimation. Statistical analysis resulted in the final model which included four DMRs (FFMM). High correlation with YMM-based fetal fractions was observed using 85 male pregnancies ($r = 0.86$ 95% CI: 0.80–0.91). The model was confirmed using an independent set of 53 male pregnancies.

Conclusion: By employing a set of well-characterized DMRs, we developed a SNP-, sex- and ploidy-independent methylation-based multiplex ddPCR assay for accurate fetal fraction estimation.

KEYWORDS

differentially methylated regions, fetal fraction estimation, multiplex ddPCR, non-invasive prenatal testing

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1 | INTRODUCTION

Non-invasive prenatal testing (NIPT) for fetal aneuploidies has rapidly been incorporated into clinical practice. A large number of validation studies have demonstrated high aneuploidy detection rates, leading professional organizations to recommend NIPT as a primary screening in pregnant women regardless of their risk status (Benn et al., 2015; "Committee Opinion No. 640: Cell-free DNA Screening for Fetal Aneuploidy", 2015; Gregg et al., 2016).

Since the discovery of cell-free fetal DNA (cffDNA) in the maternal circulation (Lo et al., 1997), several approaches have been developed for the detection of pregnancy-induced complications (Leung, Zhang, Lau, Chan, & Lo, 2001; Sekizawa et al., 2001) and fetal abnormalities (El Khattabi et al., 2016; Papageorgiou et al., 2011; Tong, Chiu, et al., 2010; Tong et al., 2006; Tong, Jin, et al., 2010; Tsaliki et al., 2012) while the highest impact in the NIPT field was made with the introduction of next-generation sequencing (Fan, Blumenfeld, Chitkara, Hudgins, & Quake, 2008; Koumbaris et al., 2016; Zhang et al., 2015; Zimmermann et al., 2012); although the limited amount of cffDNA in the maternal circulation still presents a challenge. cffDNA constitutes about 10%–20% of the total cell-free DNA (Chiu et al., 2011) and can range from less than 4% to more than 30% (Canick, Palomaki, Kloza, Lambert-Messerlian, & Haddow, 2013; Jiang et al., 2012). Furthermore, it has been shown that NGS-based methods can reliably detect fetal aneuploidies non-invasively with fetal fraction of at least 4% (Norton et al., 2012; Palomaki et al., 2011; Samango-Sprouse et al., 2013). Therefore, inaccurate estimation of fetal fraction can potentially compromise the sensitivity of the assay (Koumbaris et al., 2016; Straver, Oudejans, Sistermans, & Reinders, 2016; Takoudes & Hamar, 2015).

Different approaches have been developed for quantification of fetal fraction in maternal plasma. The most common methods include quantification of Y-chromosome-specific sequences using PCR (Lo et al., 1998; Zimmermann, El-Sheikhah, Nicolaidis, Holzgreve, & Hahn, 2005). In order to overcome the limitation of detecting only male-bearing pregnancies, techniques have been developed to detect paternally inherited polymorphic nucleotides (SNPs) or insertion/deletion polymorphisms as markers for fetal fraction quantification (Barrett et al., 2017; Jiang et al., 2012; Zimmermann et al., 2012). Such approaches can rely heavily on the genotypes of both parents; therefore, their implementation may be limited by the fact that paternal DNA may not be available (Peng & Jiang, 2017). Algorithms such as FetalQuant and FetalQuant^{SD} have been utilized to measure the fetal DNA fraction only from maternal plasma requiring either very high read depth or maternal genotype information, respectively (Jiang et al., 2012, 2016). SNP-independent algorithms showing good correlation with Y-chromosome-based methods

What is already known about the topic?

- Despite the technological advances in NIPT, accurate fetal fraction estimation still presents a challenge.
- NGS-based methods can reliably detect fetal aneuploidies non-invasively with fetal fraction of at least 4%.
- Inaccurate estimation of fetal fraction can potentially compromise the sensitivity of the assay.

What does this study add?

- We describe a methylation-based assay, using a novel set of well-characterized differentially methylated regions, for accurate estimation of fetal fraction using multiplex ddPCR.
- Our assay can rapidly be incorporated into laboratory workflows and can provide accurate fetal fraction information prior to sample library preparation minimizing further any possibility of incorrect calls due to low fetal fraction.

have also been developed although it is unclear if their performance is suitable from low fetal fractions. Fragment size differences between fetal and maternal DNA and nucleosome positioning methods showed promising results, but are not accurate enough as a standalone test for direct clinical implementation (Kim et al., 2015; Straver et al., 2016; van Beek et al., 2017). Sex- and polymorphism-independent procedures focusing on the methylation differences between fetal and maternal DNA have also been described. However, the limited number of available fetal-specific differentially methylated regions in order to account for inter-individual methylation variability and the high number of positive reactions required for statistical robustness rendered their clinical implementation impractical (Chan et al., 2006; Hindson et al., 2011; Poon, Leung, Lau, Chow, & Lo, 2002; Zejskova, Jancuskova, Kotlabova, Doucha, & Hromadnikova, 2010).

We hereby describe a methylation-based assay, using a novel set of well-characterized differentially methylated regions, for accurate estimation of fetal fraction. Taking in consideration the limitations of PCR and methylation-based methods, we developed a new multiplex SNP-independent and sex-independent ddPCR assay for the quantification of fetal fraction. As such, the assay described herein can rapidly be incorporated into laboratory workflows and can provide accurate fetal fraction information prior to sample library preparation minimizing any possibility of incorrect calls due to low fetal fraction, therefore reducing dramatically sample library preparation and sequencing processing cost.

2 | METHODS

2.1 | Sample collection and preparation

The study was approved by the Cyprus National Bioethics Committee (EEBK/EII/2013/03, April 2013) and informed consent form was obtained from all participants. Peripheral blood samples of 10 ml were collected anonymously from women with singleton pregnancies of at least 18 years of age and 10th week of gestation into Streck cell-free DNA BCT tubes. First trimester chorionic villus samplings (CVS) were collected from collaborating centers.

A mean of 4 ml of plasma was isolated via a double centrifugation protocol of 1,000 *g* for 10 min, followed by 16,000 *g* for 10 min and cfDNA isolation from plasma was performed using the Qiasymphony DSP Circulating DNA Kit (Qiagen). DNA isolation from CVS was performed using the QIAmp DNA Mini kit. All samples were stored at -80°C prior to further processing.

2.2 | Methylation sensitive restriction digestion

Methylation sensitive restriction digestion was used to enrich fetal-specific differentially methylated regions for DMR discovery and confirmation of their methylation status on CVS and plasma samples and finally for fetal fraction estimation on pregnancy plasma samples. DNA was simultaneously digested with *HhaI* and *HpaII* for 2 hr at 37°C followed by 20 min inactivation at 80°C . Each digestion reaction consisted of 20 U of *HhaI*, 40 U of *HpaII*, 20 μl of DNA, 4 μl of 10X CutSmart buffer, and 10 μl HPLC H_2O . Two enzymatic reactions were performed for each sample, which were then pooled prior further processing.

2.3 | DMR discovery

Extracted DNA from two CVS samples and one female non-pregnant plasma sample were subjected to blunt ending and sequencing adapter ligation as previously described (Koumbaris et al., 2016). Following methylation sensitive restriction digestion, MeDIP was performed (Borgel, Guibert, & Weber, 2012) and a unique barcode was assigned to all samples. Adaptors from the sequencing reads were removed with cutadapt v1.2 and subsequently aligned using BWA (Li & Durbin, 2009). Duplicate reads were removed from the resulting BAM files using Picard. Following sequencing output normalization, each sample's read depth was calculated for each CpG dinucleotide excluding CpG sites overlapping with repetitive elements and LCRs. The distribution of the normalized read difference between CVS and plasma was

then calculated. Sites that lay beyond the 90th quantile of the distribution were used as candidate sites for DMR selection. Subsequently, candidate CpG sites located less than 50bp away were merged to form larger cohesive DMRs. In order to minimize the presence of maternal (background) methylation, only DMRs which did not exhibit any aligned reads in the plasma samples were selected. Final DMR selection criteria included the presence of at least two restriction sites of *HpaII* and/or *HhaI* restriction enzymes (NEB) and exclusion of copy number variants (MacDonald, Ziman, Yuen, Feuk, & Scherer, 2014).

2.4 | DMR screening

For the purposes of this study, fetal-specific DMR selection was performed only on chromosome 1. Primers for the selected DMRs were designed with amplicon size ranging from 70 to 110bp. Primer amplification efficiency was tested on control genomic DNA with a pre-determined concentration (known concentration of 25 ng/ μl). Following ddPCR amplification of each primer set, the concentration of the control genomic DNA was calculated. Failure to obtain the expected concentration resulted in the exclusion of the primer from downstream experiments. DMRs with satisfactory amplification efficiency were initially screened using digested pooled DNA obtained from non-pregnant female plasma samples (Sera Laboratories International). DMRs with less than 3% methylation enrichment in the pool non-pregnant cfDNA were subsequently confirmed in three normal CVS, three Trisomy 21 CVS, and three individual non-pregnant female plasma samples following restriction digestion.

Methylation levels for each DMR were calculated in two individual simplex Evagreen-based ddPCR reactions on QX200 Droplet Digital System (Bio-Rad) using primers targeting the DMR under investigation and a reference/digestion control region on chromosome 1 (REF1) that did not include restriction sites for *HhaI* and *HpaII*. This region was used for calculation of total DNA concentration. DMRs with less than 3% methylation enrichment in non-pregnant plasma samples and higher than 15% in CVS were selected for multiplexing.

2.5 | Multiplex design

2.5.1 | Fetal fraction multiplex mix

Selected DMRs were initially multiplexed in a two-channel octaplex ddPCR reaction using probes labeled with FAM and/or HEX fluorophores (Integrated DNA Technologies, Leuven, Belgium). In total, seven DMRs in addition to the

control region (REF1) were combined in the multiplex mix at different ratios of FAM and HEX fluorophores in order to achieve distinct cluster populations for each DMR. Thus, we were able to analyze and validate each DMR in the multiplex reaction separately using their amplification efficiency on genomic DNA (0.125 ng/ul; Table 1). Followed by statistical analysis, four out of seven DMRs which showed the best performance for fetal fraction estimation were combined in a multiplex ddPCR assay, termed as fetal fraction multiplex mix (FFMM).

2.5.2 | Chromosome Y multiplex mix

As reference standard for fetal fraction estimation, a two-channel chromosome Y multiplex mix (YMM) was developed. Seven primer/probe sets targeting nine regions on chromosome Y and one set of primer/probe targeting

chromosome 1 (REF1) were equimolarly added in a single multiplex mix (Table 2).

2.6 | Droplet digital PCR

Each multiplex ddPCR reaction mix consisted of 9.6 ul digested DNA (plasma or CVS), 12 ul of 2X ddPCR Master Mix, 1.2 ul primer/probe mix at final concentrations of 900 nM and 250 nM, respectively, in a final volume of 24 ul. All multiplex ddPCR reactions were performed in triplicate using the following conditions: initial denaturation at 95°C for 10 min, followed by 45 cycles at 95°C for 30 s, 60°C for 60 s and final extension at 98°C for 10 min. The droplet generation was performed on QX200 AutoDG droplet digital system (BioRad), and fluorescent signal was measured and analyzed using the QX200 droplet reader. Replicates with less than 10,000 droplets were excluded from analysis.

TABLE 1 Multiplex mix information designed for fetal fraction estimation using ddPCR followed by methylation sensitive restriction digestion

Name		Sequence	FAM:HEX ratio	Chromosomal Location
DMR 1 ^a	Forward	CGTTAAGGTAATGAACGGCG	1:1	1p34.2
	Reverse	CCAGACCCGCAGAAAGTG		
	Probe	TCGAAAGTTCAGCGCCCC		
DMR 2 ^a	Forward	CTTCCAGCCAAGCGCTG	2:1	1p34.1
	Reverse	GTGATGCAAATCCGCTCC		
	Probe	CCTCGCTTTACGGAAAGAACGC		
DMR 3	Forward	ACACGTCCCCACCTATTTGG	1:1	1p32.3
	Reverse	TGTGGGGAGGAGAAGTGACA		
	Probe	TCTTCTCCGGGACCTGAGGT		
DMR 4 ^a	Forward	AGCCTCCCCTTTCTGTCT	1:1	1p21.2
	Reverse	GGTGCGTGTCTTCTGTGATT		
	Probe	AGGAGCGTGCACAGGTCTT		
DMR 5 ^a	Forward	GAAGGAAAGGAGCTTAGGCG	FAM	1p32.1
	Reverse	CGCAACCATCGAAGTTCAATC		
	Probe	CCTGGACGGAGCTGAGACAAT		
DMR 6	Forward	AGGAGAACGCTGAGGTCG	FAM	1p32.2
	Reverse	GCGGACTACCTTAGTGGCAC		
	Probe	CAACTGCAGCTCGCGCT		
DMR 7	Forward	GCCGCTTCAGTAGCACAA	FAM	1p21.2
	Reverse	AGCCCGTGGCCTTAAATAGGA		
	Probe	CTCAAACCGCGCATCTCTGGC		
REF 1 ^a	Forward	GTTGTGATGTTCTTAAGGCAGA	HEX	1p31.1
	Reverse	AATTGGGATTTCCACAGGAG		
	Probe	TCTTCATAAAAAGGAAAGTAATGGCA		

^aMarkers selected for the final fetal fraction estimation model (FFMM).

TABLE 2 Multiplex mix information designed for fetal fraction estimation using chromosome Y multiplex mix (YMM)

Name		Sequence	Location
CHRY1	Forward	CAGTGTATTTGTGGAAATGCCT	Yq11.21
	Reverse	CTAACTTTTCCAGACAGCAGC	
	Probe	ACTGTGTAGTGATAAAGACCTGCT	
CHRY2	Forward	ACTTCTAGTTTCCTGCTTTAGC	Yp11.31
	Reverse	CCAACTGGTTTAATTTGATGGG	
	Probe	GTGCAAATTTTATGAAGTCTTGGCA	
CHRY3	Forward	GACCTGCCCCATCTCCAT	Yq11.221
	Reverse	AGGTTGGATGGAAGATGAAGT	
	Probe	GTCACAACGACAGTCATCATTCT	
CHRY4	Forward	CCTCCTTTGAATACTTATTTACGATT	Yq11.221
	Reverse	ACTATGTTTTGCAACCTTTGTT	
	Probe	AGTCAAGTTATATGAGTATGTTCAACC	
CHRY5	Forward	TGTCATCAACATGGGAAGCA	Yq11.221 (two loci)
	Reverse	TATCTCCCTGAGCAGCAACTA	
	Probe	TGG TGA GATCTC TGA GGT CT	
CHRY6	Forward	CTCATCACCTGAATTTATGTCTATTT	Yq11.222 (two Loci)
	Reverse	GCTGGGTTTGTCTTTAGGT	
	Probe	AAA GAC ACTTGT GGG CCT GT	
CHRY7	Forward	CGCTTAACATAGCAGAAGCA	Yp11.31
	Reverse	AGTTTCGAACTCTGGCACCT	
	Probe	TGTCGCACTCTCCTTGTTTTT	
REF1	Forward	GTTGTGATGTTCTTAAGGCAGA	1p31.1
	Reverse	AATTGGGATTTCCACAGGAG	
	Probe	TCTTCATAAAAAGGAAAGTAATGGCA	

2.7 | Data analysis

Analysis was performed using R version 3.3.2 on a Linux platform. The training and validation of the suggested fetal fraction quantification model was performed in multiple steps. Initially, fetal fraction estimates obtained from YMM were compared to DYS-qPCR assay ($n = 47$), a gold standard assay used for fetal fraction estimation on male pregnancy samples (Kyriakou et al., 2013). Next, YMM was used to train a fetal fraction estimation model using the initial octaplex mix on a training set of male pregnancies ($n = 85$). A multiple linear regression model was fitted to the data followed by a step-wise model selection that led to the final model (FFMM). To further test/validate our findings of the estimated model, the fetal fraction was quantified in an independent set of male pregnancies using both YMM and FFMM ($n = 53$). Furthermore, additional testing of the assay was performed using FFMM on an additional independent set of pregnancy plasma samples, including both male and female pregnancy samples, consisting of both normal (euploid; $n = 109$) and trisomy 21 (aneuploid) samples ($n = 14$). Furthermore, our fetal

fraction estimation model was tested on non-pregnant plasma samples.

3 | RESULTS

3.1 | DMR discovery and screening

Two CVS samples and one non-pregnant female plasma sample were subjected to methylation sensitive restriction digestion followed by MeDIP and NGS. Reads passing quality filters were aligned to the human reference genome GRCh37/hg19. In total, CVS 433.7 and 416.7 million reads were aligned for CVS samples and 410.2 million reads for the plasma sample. Hundreds of genome-wide fetal-specific DMRs were identified between fetal and maternal tissue samples, however for the purposes of this study, we focused on the identification of DMRs on chromosome 1. In total, 93 DMRs were selected, with size ranging from 50 to 759 bp. From these, 43 DMRs, which satisfied all selection criteria were screened using ddPCR (see Section 2.3). Specifically, 151 ($n = 151$) primer sets targeting the selected DMRs

(DMR-amplicon) were designed and tested on genomic DNA for ddPCR amplification efficiency. Subsequently, the methylation status of each DMR amplicon was investigated on pool plasma obtained from non-pregnant women using methylation restriction digestion followed by ddPCR. In total, out of the 151 DMR amplicons, 59 and 54 DMR amplicons were excluded from further testing due to inefficient ddPCR amplification and high methylation percentage (>3%) on pool plasma, respectively. The methylation status of the remaining 38 DMR-amplicons was confirmed on three normal CVS, three trisomy 21 CVS, and three individual non-pregnant plasma samples. Finally, seven DMRs that showed highest and consistent methylation difference between CVS (normal and trisomy 21) and individual plasma samples were used in a multiplex reaction mix along with a reference marker targeting an unmethylated region (Figures S1 and S2).

3.2 | Fetal fraction estimation model

As a first step, fetal fraction estimates obtained from YMM and conventional qPCR-based DYS assay were obtained and compared in 47 male pregnancy samples. A high degree of correlation was observed between YMM and DYS ($r = 0.83$ with 95% CI: 0.71–0.9; Figure 1). This allowed us to subsequently use YMM as a reference to train a fetal fraction estimation model using the initial octaplex ddPCR on an independent set of 85 male pregnancy samples. A multiple linear regression model was fitted to the data and a step-wise model selection was applied resulting in the final model with our covariates/markers (FFMM; Table S1; Venables & Ripley, 2002). All coefficient estimates of the resulting model had $p < .024$. A high correlation of the model estimates with YMM-based fetal fractions was observed ($r = 0.86$; 95% CI: 0.80–0.91; Figure 2a).

In order to test our findings on an independent dataset, we applied our estimated model on 53 additional male pregnancy samples. A strong correlation of the methylation-based fetal fraction assay with the corresponding YMM values was confirmed ($r = 0.84$, 0.74–0.91; Figure 2b). Furthermore, the performance of the trained model ($n = 85$) was statistically not different from the performance on the testing set ($n = 53$) ($p = 0.7022$; Wilcoxon test).

To investigate whether our developed fetal fraction estimation method is gender and/or ploidy independent, additional 109 euploid pregnancies (51 females and 58 males) and 14 trisomy 21 pregnancies (4 females and 10 males) were examined. Since the model is trained and tested on male pregnancy samples, we compared the fetal fraction estimates of the female and abnormal samples with those of the euploid male pregnancy samples. The performance

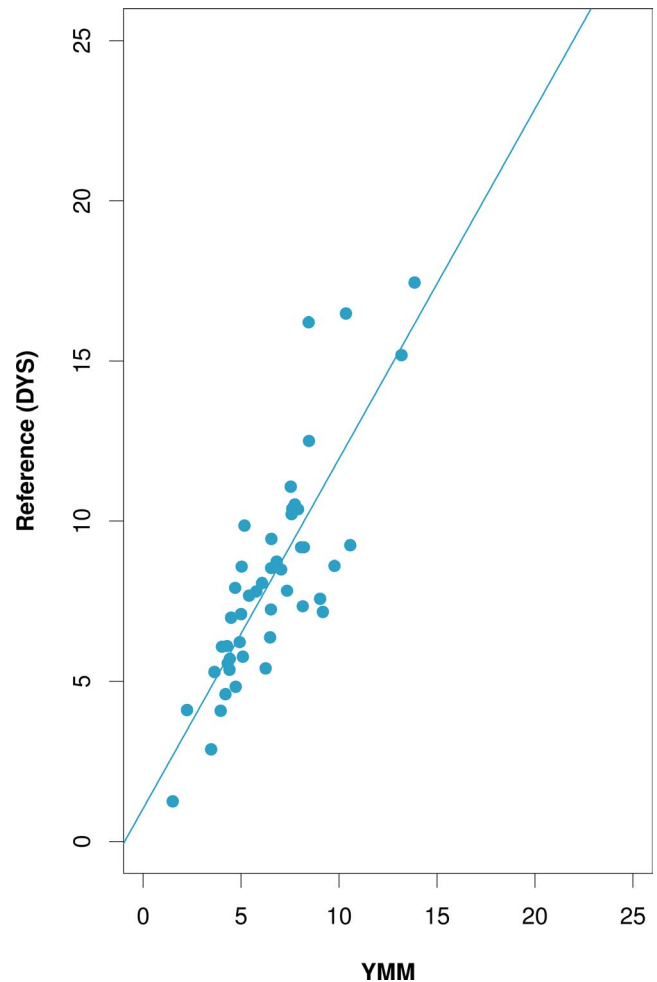
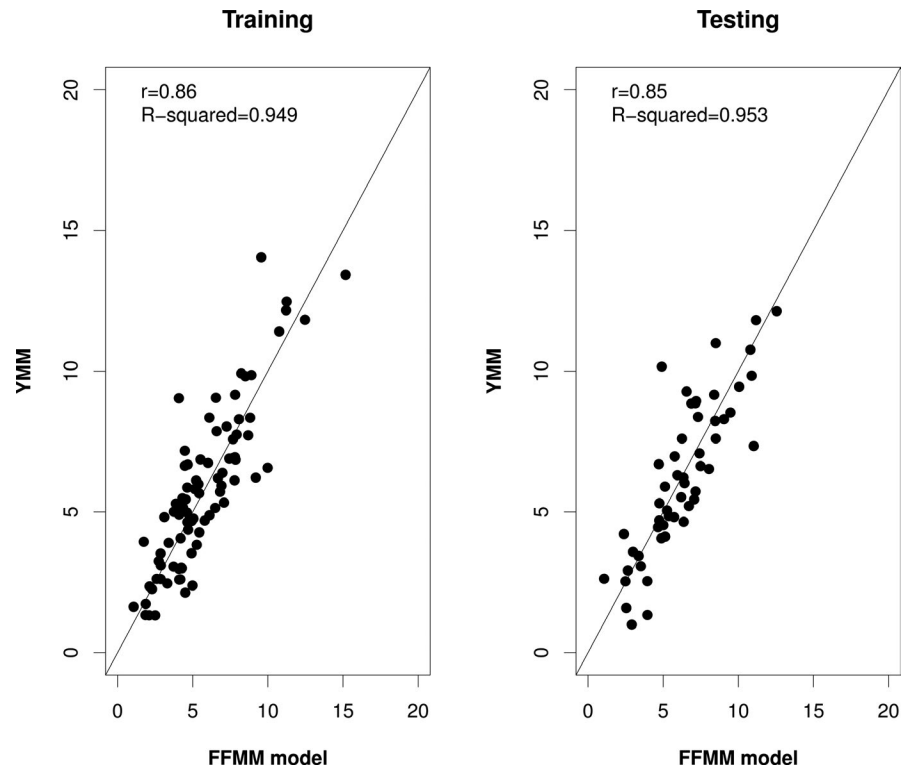


FIGURE 1 Correlation of fetal fractions on male pregnancies between chr Y ddPCR assay (YMM) and reference standard. Scatter plot analysis of fetal fraction estimates for 47 male samples showed high correlation between YMM and qPCR-based DYS assay ($r = 0.83$; 95% CI: 0.71–0.9) that was treated as reference standard. Solid line represents fitted values from linear regression models. These findings allowed us to proceed to the next phase of our analysis, that is, use YMM to train a fetal fraction estimation model using our selected panel of markers

between male ($n = 58$) and female ($n = 51$) pregnancy samples did not differ ($p = .97$; Wilcoxon test; Figure S3). The fetal fraction estimation accuracy was further assessed on 14 trisomy 21 samples showing comparable performance to the euploid male samples ($p = .13$; Wilcoxon test; Figure S3).

The maternal background (noise) level at very low fetal fractions was assessed using eight non-pregnant samples. The estimated fetal fractions ranged from 0.2% to 1.09% (Figure S3). The analytical sensitivity to detect pregnancy is estimated to be greater than 99% (97%–99.9%) with 100% analytical specificity (63%–100%). The estimated threshold that discriminates pregnancy from assay noise (non-pregnant samples) is 1.355%.

FIGURE 2 Correlation of fetal fraction estimation between the training/testing model and YMM. Scatter plot analysis of fetal fraction estimates obtained from our model (FFMM) versus YMM on male pregnancy samples. Fetal fraction estimation model using methylation sensitive restriction digestion followed by multiplex ddPCR shows high correlation with YMM ($r = 0.86$; 95% CI: 0.80–0.91) on a training set of 85 male pregnancy samples (a). Findings were confirmed on additional 53 male pregnancy samples using our estimation model ($r = 0.84$; 95% CI: 0.74–0.91) (b). The performance of the trained model ($n = 85$) on an independent set of samples (validation set of size $n = 53$) is statistically not different ($p = .7022$). Solid line represents the $x = y$ line



4 | DISCUSSION

In this study, we present the development and validation of a multiplex assay using methylation sensitive restriction digestion and a robust set of novel fetal-specific differentially methylated regions for accurate detection of fetal fraction in maternal plasma. For the development of the assay, we employed digital PCR, a powerful tool used for several clinical applications due to its high sensitivity, simplicity, and precision as it allows quantification from single molecule amplification.

Previous studies have focused on the methylation differences between fetal and maternal DNA for quantification of fetal DNA in the maternal plasma (Chan et al., 2006; Nygren et al., 2010). Inherent biological limitations of methylation-based assays such as biological methylation stability of the tested markers should be considered prior to their clinical implementation (Nygren et al., 2010). Furthermore, other technologies that is, qPCR-based assays are characterized by technical limitations such as fluctuations in the amplification efficiency, or the need of different standards for construction of calibration curves (Lun et al., 2008). In addition, ddPCR quantification assays require a high number of positive PCR reactions for statistical robustness for the absolute and accurate quantification of cfDNA due to its limited abundance in maternal plasma (Fan & Quake, 2007). In our efforts to overcome the aforementioned limitations, we initially employed methylation sensitive restriction digestion followed by MeDIP and NGS for the identification of fetal-specific

hypermethylated regions. Hundreds of fetal-specific DMRs were identified genome-wide; however, DMR selection process was focused on chromosome 1 as this chromosome has rarely been implicated in first trimester chromosomal abnormalities (excluding 1p36 deletion critical region). Further DMR selection criteria were implemented taking into account the highest methylation difference between fetal (CVS) and maternal tissues (non-pregnant female plasma) and methylation stability. Finally, seven fetal-specific DMRs and one reference marker with no restriction sites used as reference/digestion control were combined in a two-channel fluorophore octaplex reaction for fetal fraction quantification. Multiple linear regression models fitted to the data resulted in a final model consisting of four DMRs in addition to the reference marker. Thus, targeting robust DMRs in a single multiplex reaction, we simultaneously overcame limitations regarding inter-individual methylation variability and substantially increased the number of positive droplets required for accurate fetal fraction estimation. Additionally, optimal digestion efficiency and removal of maternally derived background were ensured by the incorporation of at least two restriction sites within the tested DMRs.

Our training model was constructed based on the orthogonal assay comparison between Chromosome Y (YMM) and restriction digestion multiplex assay. For both assays, ddPCR was used for quantification with similar starting amount of DNA in order to eliminate possible PCR biases. Strong evidence that YMM was acceptable to be used as a comparison standard for the training model came from the high correlation

results between YMM-ddPCR assay and DYS-qPCR, a gold standard assay used in the field for male fetus quantification.

The statistical model presented herein attains high correlation with YMM for male pregnancy samples and similar performance in both male and female samples. An examination of 14 trisomy 21 samples also showed evidence that the ploidy status of the sample does not affect our fetal fraction estimates. Furthermore, the results on the performance of the assay on female non-pregnant samples showed that traces of background methylation were detectable ranging from 0% to 1.1%. DMR selection for the development of the assay was performed based on the highest difference between fetal and maternal methylation as well as methylation stability in CVS and plasma. However, low level methylation (<3%) was also detected on non-pregnant female plasma samples (Figure S1). This is in agreement with previous studies that showed that, despite the fetal hypermethylation status of well-characterized DMRs, low maternal methylation was detected (Ioannides et al., 2014; Keravnou et al., 2016). Nevertheless, our fetal fraction estimation model adjusts for the presence of maternal background methylation by regressing methylation levels with YMM. Additional experiments using male, female, euploid, and aneuploid pregnancy samples as well as non-pregnant controls are required for a more accurate determination of our model's analytical specificity and sensitivity before the clinical implementation of the assay.

In the field of NIPT, fetal fraction estimation is one of the most critical variables which is currently assessed using NGS data. However, on average 2%–6% of NIPT samples is sent for blood redraw at later gestational age due to low fetal fraction estimation. This increases substantially the cost per sample considering the high sequencing consumable and reagent cost. Integration of an accurate, inexpensive, and simple assay, as the one described herein, as a first-tier screening for fetal fraction estimation prior to sequencing, will reduce dramatically both the sample library preparation and sequencing costs and minimize further the possibility of incorrect calls due to low fetal fraction estimation.

Increased levels of cfDNA in the maternal circulation have also been associated with maternal pathological complications and preterm birth (Monte, 2011; Sekizawa et al., 2001). Studies have shown that prior to the development of clinical symptoms in preeclampsia, one of the most frequent causes of maternal and fetal morbidity and mortality, cfDNA was significantly higher in affected pregnancies than in controls (Leung et al., 2001; Levine et al., 2004; Monte, 2011). Thus, accurate and affordable tests for identifying and monitoring presymptomatic pregnancy complications are in need. Preliminary results presented in this study show potential applicability of our assay in the detection of pregnancy-associated diseases; nevertheless, its clinical performance should be evaluated in a large cohort of affected and non-affected pregnancies.

5 | CONCLUSION

We developed an NGS, SNP, and sex-independent assay for the accurate estimation of fetal fraction in maternal plasma. Taking in consideration the high methylation differences between fetal and maternal DNA and methylation stability, we selected and confirmed a set of four DMRs in a multiplex ddPCR assay following methylation sensitive restriction digestion. In this way, we increased the number of positive targets providing statistical robustness to our data overcoming technical and biological limitations. As such, the assay described herein can rapidly be incorporated into laboratory workflows and can provide accurate fetal fraction information prior to sample library preparation minimizing further any possibility of incorrect calls due to low fetal fraction, therefore reducing dramatically sample library preparation and sequencing processing cost.

CONFLICT OF INTEREST

The authors, Marios Ioannides, Achilleas Achilleos, Skevi Kyriakou, Elena Kypri, Charalambos Loizides, Kyriakos Tsangaras, Louiza Constantinou, George Koumbaris, and Philippos C Patsalis are employed by NIPD Genetics.

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 may be found online in the Supporting Information section.

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