Reports

BioTechniques

An online tool for fetal fraction prediction based on direct size distribution analysis of maternal cell-free DNA

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BioTechniques 70: 00–00 (February 2020) 10.2144/btn-2020-0143

First draft submitted: 3 October 2020; Accepted for publication: 6 November 2020; Published online: 30 November 2020

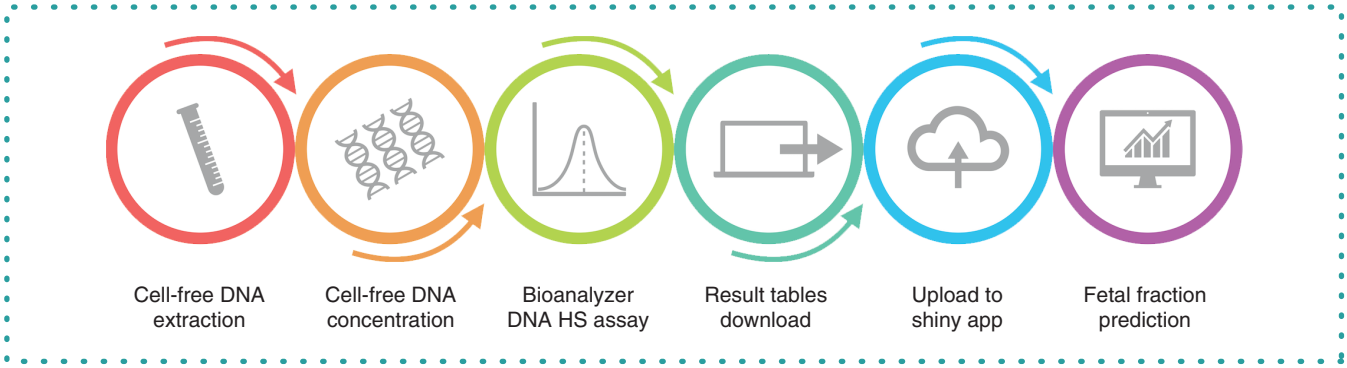
ABSTRACT

The discovery of circulating fetal DNA in the plasma of pregnant women has greatly promoted advances in noninvasive prenatal testing. Screening performance is enhanced with higher fetal fraction and analysis of samples whose fetal DNA fraction is lower than 4% are unreliable. Although current approaches to fetal fraction measurement are accurate, most of them are expensive and time consuming. Here we present a simple and cost-effective solution that provides a quick and reasonably accurate fetal fraction by directly evaluating the size distribution of circulating DNA fragments in the extracted maternal cell-free DNA. The presented approach could be useful in the presequencing stage of noninvasive prenatal testing to evaluate whether the sample is suitable for the test or a repeat blood draw is recommended.

METHOD SUMMARY

Cell-free DNA was extracted from plasma samples from pregnant women, concentrated and then analyzed using microchip-based capillary electrophoresis. Cell-free DNA from the same cohort was processed with a CE-approved kit to achieve a postsequencing fetal fraction determination. Sample records were used to investigate the correlation between electrophoresis data and the reference fetal fraction. We created a prediction model and an online tool to estimate the cell-free fetal DNA fraction starting from the major peak height and fetal fragment ratio.

GRAPHICAL ABSTRACT



KEYWORDS:

cell-free DNA • fetal fraction percentage • fetal fraction prediction model • microchip-based capillary electrophoresis • noninvasive prenatal testing

The evidence that maternal plasma includes both maternal cell-free DNA (cfDNA) and cell-free fetal DNA (cffDNA) has greatly and rapidly promoted the development of noninvasive prenatal testing (NIPT) for detecting fetal chromosomal aneuploidies [1,2] and copy number variants [3] and screening for fetal single-gene disorders [4].

The cfDNA present in the bloodstream of pregnant women is a combination of physiologically released maternal DNA and fetal DNA released through the apoptosis of trophoblast cells that occurs in all pregnancies as part of normal cell turnover events in the placenta [5]. The proportion of circulating fetal DNA fragments within the total cfDNA in maternal bloodstream is indicated as the cffDNA percentage. cffDNA has been shown to account for 10–15% of cfDNA between 10 and 20 gestational weeks [6,7]. Individual variations

in the percentage of fetal DNA are caused by several factors; for example, maternal weight [7], placenta status [8] and blood processing conditions [9].

cfDNA molecules are mainly short DNA fragments of less than 300 bp that show a fragmentation pattern resembling nuclease-cleaved nucleosomes; the distribution of molecules presents a succession of peaks, including a major 166-bp peak, a minor 143-bp peak and 10-bp periodic peaks below 143 bp [10]. The most significant difference in the size distribution between fetal and maternal DNA in maternal plasma is that fetal DNA exhibits a reduction in the 166-bp peak and an increased proportion of DNA molecules of less than 143 bp [10].

NIPT methods based on massively parallel sequencing of cfDNA depend on the estimation of over- or under-represented chromosomes or genomic regions compared with the baseline representation of the same chromosome or region in plasma of women carrying a euploid fetus [11]. The measurement of unbalanced fetal genomes in the plasma of pregnant women is linearly correlated to the fetal DNA fraction; the higher the fraction of cffDNA present, the easier the detection of these differences [12,13]. Current NIPT practices establish a fetal fraction threshold below which the test is considered unreliable (usually 4%), because the restricted quantity of fetal DNA fragments to be assessed may lead to false negative results [14].

Many different methods have been developed for the measurement of fetal DNA fraction. The evaluation is direct in the presence of a male fetus, because loci derived from the Y chromosome are easily measurable [15]; however, these procedures are only suitable to pregnancies carrying male fetuses. Several methods for fetal fraction estimation by massively parallel sequencing have been developed, such as: maternal plasma DNA sequencing with parental genotypes, high-depth sequencing of maternal DNA, shallow-depth maternal DNA sequencing, differential methylation studies by bisulfite sequencing, and genome-wide nucleosome profiles [16–18]. However, although these strategies are generally accurate, they are expensive and time-consuming. As mentioned, the fetal fraction value is essential to ensure that the sample reaches the quality standard to assure a proper interpretation of the results; in NIPT disciplines a fast, simple and cost-effective method to measure fetal DNA fraction before sequencing is highly desirable.

In this perspective, we evaluated the possibility to use the size distribution of DNA fragments obtained from microchip-based capillary electrophoresis to predict the fetal DNA fraction, directly analyzing the extracted cfDNA instead of maternal plasma DNA sequencing libraries [19].

Our aim was to develop a simple, cheap, rapid and sex-independent fetal fraction predictor that could be useful in the presequencing stage of a NIPT test. The prediction could suggest that a sample has an adequate fetal fraction or that an insufficient level requires an additional blood draw after a few days, avoiding wasted sequencing costs.

Materials & methods

Participants

Blood samples of 93 women with singleton pregnancies were collected at the Institute for Maternal and Child Health IRCCS 'Burlo Garofolo' in Trieste, Italy. Informed consent was obtained in writing prior to blood draw and the study was approved by the institutional review board. Median gestational age was 12 + 2 weeks. A 20-ml sample of peripheral blood was collected from each woman into an EDTA tube.

Sample processing & cfDNA extraction

Plasma was separated from whole blood samples within 4 h of blood draw using two serial centrifugations: first, the blood tubes were centrifuged at 3200×*g* for 10 min, and the plasma portion was then transferred to a new microcentrifuge tube. Second, the collected plasma portions were centrifuged at 10,000×*g* for 10 min at 4°C to remove residual cells. The isolated plasma was stored at -80°C until DNA extraction.

Circulating DNA was extracted from 4.8 ml of plasma from each sample using the QIAsymphony DSP Circulating DNA Kit (Qiagen Benelux BV, Venlo, The Netherlands) according to the manufacturer's instructions, and DNA was eluted in 100 µl of elution buffer. All DNA samples were stored at -20°C prior to analysis. Sample concentrations were quantified on a Qubit Fluorometer (Thermo Fisher Scientific, MA, USA).

Library preparation & sequencing

Sequencing libraries were constructed with the CE-approved Clarigo™ kit (Agilent Technologies, CA, USA) according to the manufacturer's instructions. Library preparation workflow requires two sequential PCR steps. First, more than 4000 specific regions are selectively amplified in a targeted single multiplex PCR amplification reaction per individual using Agilent's proprietary MASTR technology. Next, a universal PCR is performed to label all amplicons with univocal combinations of molecular identifiers to link each read to the sample it originated from. Purified individually tagged libraries were diluted and equimolar amounts of samples were pooled to obtain a single 4 nM library.

Among the 93 samples of the set, 57 were sequenced on the HiScanSQ system (Illumina, CA, USA) and 36 on the MiSeq system (Illumina); in both cases, 75 cycles of single-end sequencing were used. The library sequencing load concentration and the number of samples in each pool were adapted to reach at least 2×10^6 classified reads per sample. This study was performed with the first version of the Clarigo kit.

Determination of fetal fraction by certified test

The sequences obtained from Clarigo multiple parallel sequencing were uploaded to a dedicated cloud-based software tool, Clarigo Reporter (Agilent Technologies). This tool enables a fast and consistent determination of the cfDNA-linked fetal aneuploidy status (for chromosomes 21, 18 and 13), fetal gender evaluation and fetal fraction quantification.

The fetal fraction determination is based on single nucleotide polymorphism (SNP) profiling and minor allele frequency assessment. The informative SNPs correspond to alleles where the majority of the reads covering a genetic locus (i.e., those originating from the mother's DNA) contain the same nucleotide sequence (i.e., have an AA genotype) and a smaller fraction of the reads (originating from the fetus) contain an alternative allele (i.e., have an AB genotype). The minor allele frequency of the informative SNPs (B allele from the fetus) corresponds to the fetal fraction in the sample divided by two, because only one of the two fetal alleles differs from the maternal homozygous state; thus the calculated fetal fraction is the minor allele frequency of the informative SNPs multiplied by two. Homozygous SNPs and SNPs with a minor allele frequency higher than 0.15 are not considered for fetal fraction estimation. Fetal fraction results from the Clarigo test were used as the reference standard in this study.

Microchip-based capillary electrophoresis

To increase the signal intensity during the capillary electrophoresis, 40 µl of each extracted cfDNA was concentrated using the DNA Clean & Concentrator-5 Kit (Zymo Research, CA, USA) according to the manufacturer's instructions; the DNA was eluted in 8 µl of biology-grade water.

The microchip-based capillary electrophoresis was performed on the Agilent 2100 Bioanalyzer using the High Sensitivity DNA Kit (Agilent Technologies) according to the manufacturer's instructions.

cfDNA electropherogram analyses were carried out using the Agilent 2100 expert software (Agilent Technologies). Sample baseline correction was performed using the software-implemented algorithm. The regions of interest in terms of base pairs were selected and analyzed using the DNA smear assays; we analyzed several regions and finally defined region 1 as 78–143 bp and region 2 as 163–168 bp, corresponding to the fetal enriched region and the maternal enriched region respectively. The Corr.Area value (i.e., the area under the curve within the region) was used to estimate the amount of DNA fragments within that region. Results of chip runs were exported by selecting 'Result Tables' from Electrophoresis Export Options; the CSV file generated contains sample electrophoresis information such as peaks data and examined regions data.

Data analysis & model building

For data analysis, Result tables.csv files were loaded into the RStudio (v. 1.1.463, RStudio, Inc.) environment using an in-house script. All sample records were stored in a database to investigate correlation within sample electrophoresis data and the reference fetal fraction obtained from the Clarigo test. These data were used to train a linear model that predicts the fetal DNA fraction of a cfDNA sample from its electrophoresis data. Linear models were fitted and evaluated using the R 'stats' package [20] and graphic data were produced using the R 'ggplot2' package [21].

To estimate the fetal fraction based on our model, we built an interactive web app using the R 'shiny' package [22]. A detailed protocol is given in the supplementary methods, available online.

Results & discussion

Size distribution analysis of maternal plasma cfDNA

Because plasma samples with a higher fetal DNA fraction are characterized by an increased proportion of DNA molecules shorter than 150 bp and a decreased proportion of fragments around 166 bp compared with samples with a lower fetal DNA fraction [19], and the measurement of plasma DNA size distribution can be achieved by capillary electrophoresis of sequencing libraries [19], we hypothesized that the size information could be acquired straight from microchip-based electrophoresis of the maternal extracted cfDNA and used to predict the fetal DNA fraction in maternal plasma.

We analyzed the size distribution of 93 cfDNA samples from women with singleton pregnancies using the Bioanalyzer. A typical electropherogram of a maternal extracted cfDNA obtained from the capillary electrophoresis shows the characteristic dominant peak at roughly 170 bp and a minor peak at roughly 340 bp (Figure 1). Region 1 and region 2 correspond to DNA fragment sizes of 78–143 bp and 163–168 bp, respectively. These regions were chosen based on previous reports as those showing the major difference between maternal and fetal cfDNA [19]. During the analysis we tested different combinations of size regions in relation to cffDNA length distribution. Among them, 78–143 bp and 163–168 bp gave the best-fitted model.

The amount of DNA fragments within the regions is the area under the curve within each region. A fetal fragments ratio (FFR) representing the relative proportion of fetal enriched and maternal enriched fragments was calculated for each sample as follows:

$$Fetal\ \ Fragments\ \ Ratio = \frac{Area\ \ 1(78 - 143)}{Area\ \ 2(163 - 168)}$$

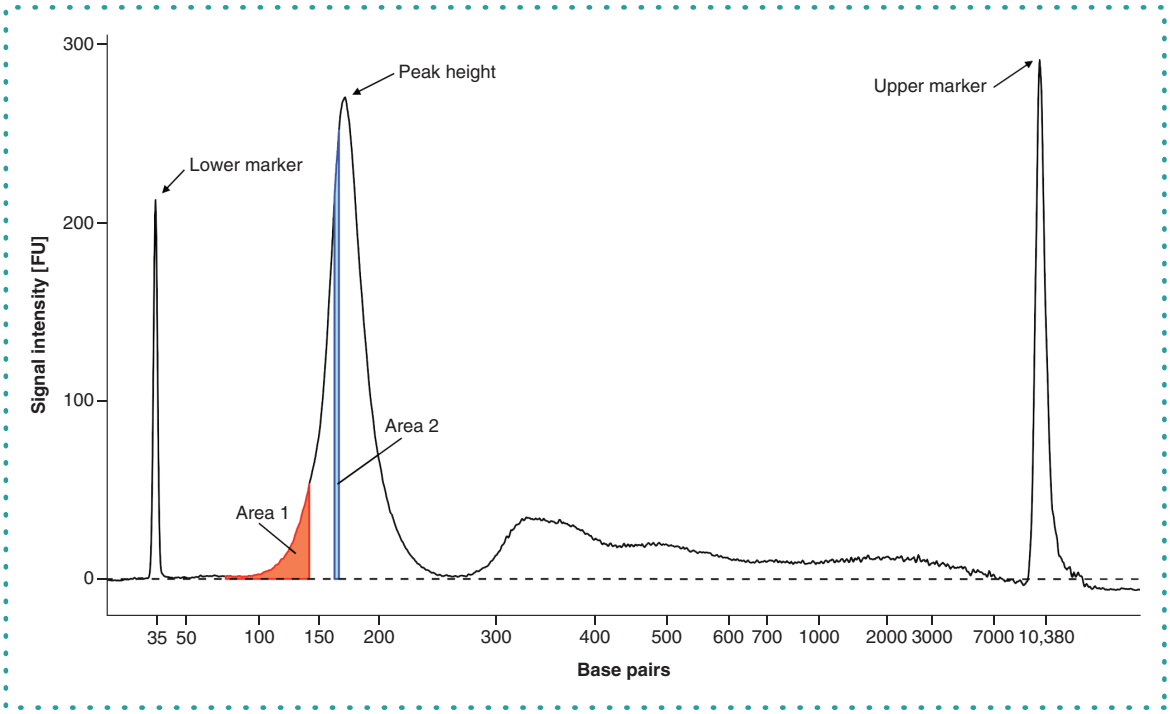


Figure 1. A typical bioanalyzer electropherogram of extracted cell-free DNA. Areas 1 and 2 correspond to cfDNA fragment sizes of 78–143 bp and 163–168 bp, respectively. The amount of DNA fragments corresponds to the highlighted area under the curve. Peak height is the maximum signal intensity value of the dominant peak.
FU: Fluorescence unit.

where Area 1 (78–143 bp) represents the amount of DNA fragments of the fetal enriched region 1 and Area 2 (163–168 bp) represents the amount of DNA fragments of the maternal enriched region 2.

Massive sequencing analysis of maternal plasma cfDNA

In parallel, the same samples underwent next-generation sequencing analysis with two platforms. The first sample set, sequenced on the HiScanSQ system, included 57 samples; the second set, sequenced on the MiSeq system, included 36 samples. For the HiScan SQ set, we obtained a median of 8.68 million total reads (interquartile range [IQR]: 6–9.89), of which a median of 7.65 million (IQR: 5.37–9.14) reads were successfully matched to the reference Clarigo amplicon set (average classified reads fraction = 90.14%). For the MiSeq set, we obtained a median of 2.97 million (IQR: 2.65–3.21) total reads, of which a median of 2.66 million (IQR: 2.25–2.89) reads were successfully matched to the reference Clarigo amplicon set (average classified reads fraction = 89.99%).

From the Clarigo Reporter fetal trisomy call analysis, 84 samples were negative for the presence of a trisomy (for chromosome 21, 18 or 13) in the fetal genome, 5 samples were not automatically called (three for trisomy 21, one for trisomy 18 and one for trisomy 13) and 4 samples had a fetal fraction below 4%. The median fetal fraction obtained from the Clarigo test was 8% (IQR: 6.8–9.9) and the mean fetal fraction was 8.3% (range: 2–16.8%).

Correlation between sequencing-determined fetal DNA fraction & data from electrophoresis of plasma cfDNA

In order to develop a rapid and sex-independent fetal fraction predictor that could be useful in the presequencing stage of NIPT, we examined the correlation between the FFR and the fetal DNA fraction as calculated from the Clarigo Reporter. We found a positive correlation between the FFR and the cffDNA% (Pearson r = 0.77; 95% CI: 0.68–0.85; $p < 0.0001$) and then we fitted a linear model to evaluate the relationship (adjusted $R^2 = 0.5972$; $p < 0.0001$; Root Mean Square Error [RMSE]: 1.681; Figure 2).

We further explored whether other information from the microchip-based electrophoresis would have been significant as additional independent variable. Using the principle of parsimony, we found that the dominant peak height (Figure 1) variable did lead to a significantly improved fit (analysis of variance, p = 0.0016) over the model that took into account only cffDNA% and FFR; moreover, the linear model based on peak height and FFR better represents the relationship between these values and the cffDNA% (adjusted $R^2 = 0.6355$; $p < 0.0001$; RMSE: 1.589).

The fetal DNA fraction of the 93 samples was predicted using the obtained regression equation as follows:

$$cffDNA\% = 0.007 + (10.92 \times Fetal\ \ Fragments\ \ Ratio) + (0.0087 \times Peak\ \ Height)$$

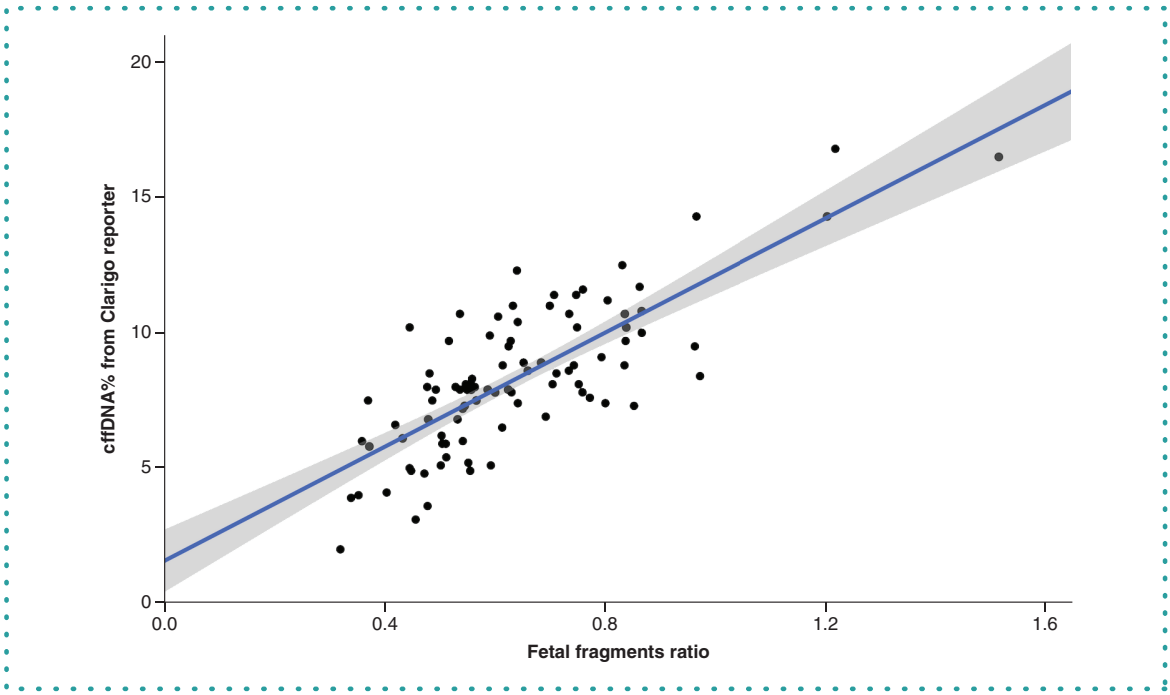


Figure 2. Correlation between fetal fragments ratio from electropherogram and cffDNA percentage calculated from the Clarigo Reporter. The blue line is the fitted linear model to data. The gray shadow represents the range in which the true regression line lies in the 95% CI. Each dot represents a single sample.

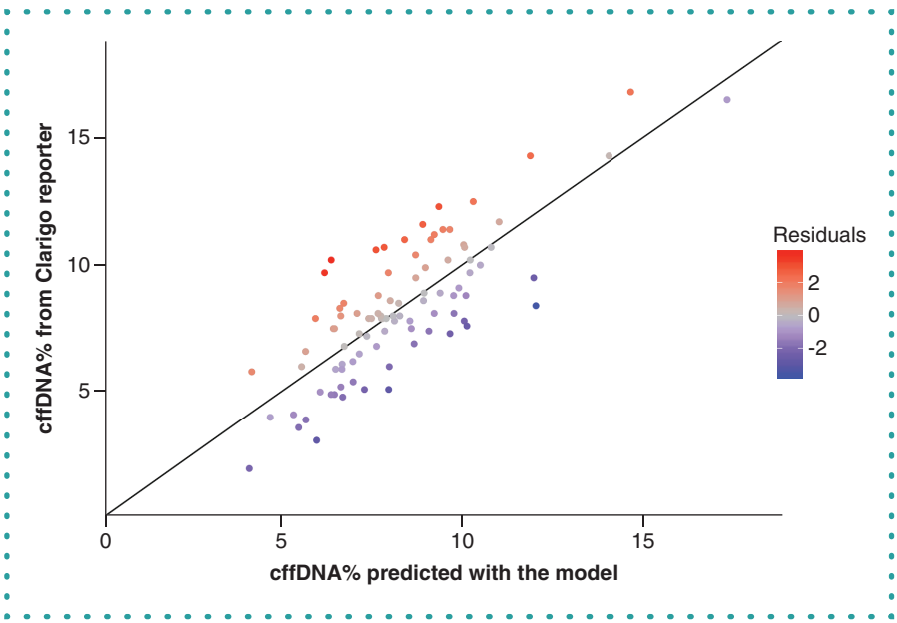


Figure 3. Plot of the predicted cffDNA percentage against the calculated percentage from the Clarigo Reporter. The prediction for the 93 samples was made using FFR and peak height. The line of intercept 0 and slope 1 represent the optimal situation in which predicted and calculated cffDNA% are equal. The residuals are reported in a color scale to evaluate their distribution.
FFR: Fetal fragment ratio.

The mean absolute difference between the predicted values and the Clarigo calculated values (mean of absolute residuals) was 1.29 (range: 0.01–3.81; Figure 3). To assess the predictive performance of the model on unseen data, we set a k-fold cross-validation (k: 10, repeats: 1) to the sample set (R^2: 0.6818; RMSE: 1.668). All sample information is listed in Supplementary Table 1.

Ⓐ

Stratification ff<=4%		Reference	
		Lower 4%	Greater 4%
Prediction	Lower 4%	0	0
	Greater 4%	5	88

Stratification ff <=7%		Reference	
		Lower 7%	Greater 7%
Prediction	Lower 7%	20	8
	Greater 7%	6	59

Stratification ff<=4%		*Stratification ff<=7%*	
Accuracy	0.95	*Accuracy*	0.85
Sensitivity	0	*Sensitivity*	0.77
Specificity	1	*Specificity*	0.88
Balanced accuracy	0.5	*Balanced accuracy*	0.82

Ⓑ

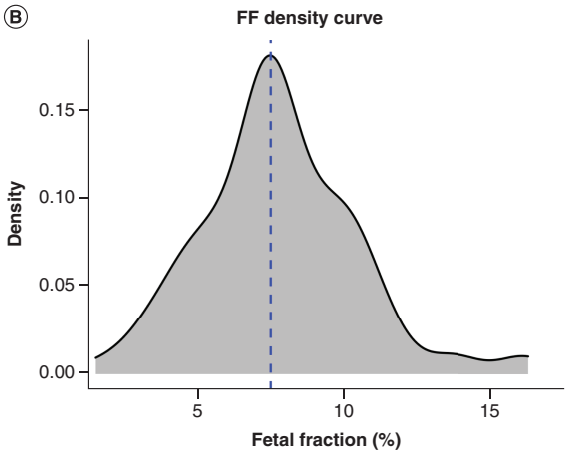


Figure 4. Predictor performance. (A) Confusion matrices and performance metrics table of stratified predicted samples. (B) density plot samples cffDNA%.
FF: Fetal fraction.

The Clarigo fetal fraction determination is based on SNP profiling. This approach represents a direct and accurate method to measure the cffDNA fraction and is generally considered one of the gold standards [17]. We found that the FFR correlates well with the cffDNA% values obtained from the Clarigo test. Moreover, the prediction model based on peak height and FFR well represents the relationship between these variables and the cffDNA%.

Performance evaluation

To assess the performance of the predictor in terms of classification metrics, we stratified the samples in accordance with the reference cffDNA% and the predicted cffDNA% into 'lower than 4%' and 'greater than 4%' using a cutoff value of fetal fraction percentage $\leq$4%. As seen in Figure 4A, the predictor correctly classified all 88 samples with a cffDNA% of $>$4% but failed to classify the five samples with a cffDNA% of $<$4%. The predicted fetal fractions of these five samples were slightly higher than the cutoff (4.13, 5.98, 5.49, 5.69 and 4.70%). Given that a limitation of our study is the lack of samples with a fetal fraction percentage lower than 4%, to validate the proof of concept of our predicting method we tested a stratification cutoff of 7% (Figure 4B). The predictor correctly classified 59 samples with a cffDNA% higher than 7% and 20 samples with a cffDNA% lower than 7% and achieved a more reliable balanced accuracy compared with the previous cutoff (Figure 4A).

Interactive web app for the prediction of cffDNA percentage

To easily predict the cffDNA% from the Bioanalyzer electrophoresis analysis, we built a Shiny-based app that uses our model to fit the data (http://tools.cbm.fvg.it/Shiny_cfDNApredictor/).

Briefly, the app allows the upload of a Result tables.csv file (Supplementary Table 2) from the 2100 Expert software; the file is then parsed and sample electrophoresis data are used to predict the fetal DNA fraction (see Supplementary Methods). The app returns a table that shows the electropherogram information for each sample, the predicted cffDNA% and the bounds of the 95% prediction interval.

We have developed a simple method to predict the cffDNA fraction using information from the microchip-based electrophoresis of the maternal extracted cfDNA. The already outlined principle of predicting the cffDNA percentage from the size distribution of maternal plasma DNA by capillary electrophoresis relies on the analysis of maternal sequencing libraries [19]. In this study, we showed that this principle is suitable if applied to the direct analysis of maternal extracted cfDNA.

The percentage of cffDNA in maternal plasma is a key factor affecting the accuracy of NIPT; additionally, its evaluation is an essential quality control step to guarantee a proper interpretation of the results [23].

Currently, there are various accurate strategies for measuring the fetal DNA fraction in maternal plasma [16–18]. With the exception of the Y-chromosome based approaches [15], these methods are applicable for pregnancies of either gender. However, most of them require a DNA sequencing process.

In conclusion, the approach presented here is applicable to all pregnancies and does not involve expensive and time-consuming analysis. Its purpose is to be useful in the presequencing stage of NIPT, where the cffDNA% value prediction would support the operator in the decision whether the sample is suitable for the next-generation sequencing test or if a further blood draw should be recommended according to the increase of cffDNA during gestation and following the different clinical procedures in place.

Future perspective

Fetal DNA fraction evaluation based on microchip electrophoresis analysis of maternal extracted cfDNA is a simple and cost-effective solution that provides a quick and reasonably accurate estimate. This or similar methods could be easily implemented in the near future for routine procedures in screening and diagnostic laboratories.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2020-0143

Author contributions

L Bedon was responsible for sample preparation, data collection, data analysis and manuscript writing and revision. J Vuch performed sample preparation and data collection. S Dal Monego performed data collection and analysis. G Meroni was responsible for experimental design, manuscript writing and revision. V Pecile and D Licastro conceptualized the method. D Licastro was responsible for supervision, experimental design, data analysis and manuscript writing and approval.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Financial & competing interests disclosure

This research was funded by Friuli Venezia Giulia region POR FESR 2014–2020 program, project no. 24054/2016 entitled: '*NIPT per Aneuploidie Tracciabili e Informatizzate nel Friuli Venezia Giulia (NATI in FVG)*'. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

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References

1 Porreco RP, Garite TJ, Maurel K *et al.* Noninvasive prenatal screening for fetal trisomies 21, 18, 13 and the common sex chromosome aneuploidies from maternal blood using massively parallel genomic sequencing of DNA. *Am. J. Obstet. Gynecol.* 211(4), 365 e361–e312 (2014).

2 Zhang B, Lu BY, Yu B *et al.* Noninvasive prenatal screening for fetal common sex chromosome aneuploidies from maternal blood. *J. Int. Med. Res.* 45(2), 621–630 (2017).

3 Hu H, Wang L, Wu J *et al.* Noninvasive prenatal testing for chromosome aneuploidies and subchromosomal microdeletions/microduplications in a cohort of 8141 single pregnancies. *Hum. Genomics* 13(1), 14 (2019).

4 Yin X, Du Y, Zhang H *et al.* Identification of a *de novo* fetal variant in osteogenesis imperfecta by targeted sequencing-based noninvasive prenatal testing. *J. Hum. Genet.* 63(11), 1129–1137 (2018).

5 Faas BH, De Ligt J, Janssen I *et al.* Non-invasive prenatal diagnosis of fetal aneuploidies using massively parallel sequencing-by-ligation and evidence that cell-free fetal DNA in the maternal plasma originates from cytotrophoblastic cells. *Expert Opin. Biol. Ther.* 12(Suppl. 1), S19–S26 (2012).

6 Palomaki GE, Kloza EM, Lambert-Messerlian GM *et al.* DNA sequencing of maternal plasma to detect Down syndrome: an international clinical validation study. *Genet. Med.* 13(11), 913–920 (2011).

7 Ashoor G, Syngelaki A, Poon LC, Rezende JC, Nicolaides KH. Fetal fraction in maternal plasma cell-free DNA at 11–13 weeks' gestation: relation to maternal and fetal characteristics. *Ultrasound Obstet. Gynecol.* 41(1), 26–32 (2013).

8 Dugoff L, Barberio A, Whittaker PG, Schwartz N, Sehdev H, Bastek JA. Cell-free DNA fetal fraction and preterm birth. *Am. J. Obstet. Gynecol.* 215(2), e231–e237 (2016).

9 Meddeb R, Pisareva E, Thierry AR. Guidelines for the preanalytical conditions for analyzing circulating cell-free DNA. *Clin. Chem.* 65(5), 623–633 (2019).

10 Lo YM, Chan KC, Sun H *et al.* Maternal plasma DNA sequencing reveals the genome-wide genetic and mutational profile of the fetus. *Sci. Transl. Med.* 2(61), 61ra91 (2010).

11 Chitty LS, Lo YM. Noninvasive prenatal screening for genetic diseases using massively parallel sequencing of maternal plasma DNA. *Cold Spring Harb. Perspect. Med.* 5(9), a023085 (2015).

12 Takoudes T, Hamar B. Performance of non-invasive prenatal testing when fetal cell-free DNA is absent. *Ultrasound Obstet. Gynecol.* 45(1), 112 (2015).

13 Canick JA, Palomaki GE, Kloza EM, Lambert-Messerlian GM, Haddow JE. The impact of maternal plasma DNA fetal fraction on next generation sequencing tests for common fetal aneuploidies. *Prenat. Diagn.* 33(7), 667–674 (2013).

14 Suzumori N, Sekizawa A, Takeda E *et al.* Classification of factors involved in nonreportable results of noninvasive prenatal testing (NIPT) and prediction of success rate of second NIPT. *Prenat. Diagn.* 39(2), 100–106 (2019).

15 Lun FM, Chiu RW, Chan KC, Leung TY, Lau TK, Lo YM. Microfluidics digital PCR reveals a higher than expected fraction of fetal DNA in maternal plasma. *Clin. Chem.* 54(10), 1664–1672 (2008).

16 Van Beek DM, Straver R, Weiss MM *et al.* Comparing methods for fetal fraction determination and quality control of NIPT samples. *Prenat. Diagn.* 37(8), 769–773 (2017).

17 Peng XL, Jiang P. Bioinformatics approaches for fetal DNA fraction estimation in noninvasive prenatal testing. *Int. J. Mol. Sci.* 18(2), 453 (2017).

18 Hestand MS, Bessem M, Van Rijn P *et al.* Fetal fraction evaluation in non-invasive prenatal screening (NIPS). *Eur. J. Hum. Genet.* 27(2), 198–202 (2019).

19 Yu SC, Chan KC, Zheng YW *et al.* Size-based molecular diagnostics using plasma DNA for noninvasive prenatal testing. *Proc. Natl Acad. Sci. USA* 111(23), 8583–8588 (2014).

20 Team RC. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria (2020).

21 Wickham H. *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag, NY, USA (2009).

22 Chang W, Cheng J, Allaire J, Xie Y, McPherson J. shiny: Web Application Framework for R. (2020).

23 Hui L, Bianchi DW. Fetal fraction and noninvasive prenatal testing: what clinicians need to know. *Prenat. Diagn.* 40(2), 155–163 (2020).