

The IONA[®] Test: Development of an Automated Cell-Free DNA-Based Screening Test for Fetal Trisomies 13, 18, and 21 That Employs the Ion Proton Semiconductor Sequencing Platform

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Keywords

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Abstract

Objective: To develop a screening test for fetal trisomy 13, 18, and 21 using cell-free DNA from maternal blood with an automated workflow using the Ion Proton sequencing platform. **Methods:** An automated next-generation sequencing workflow was developed using the Ion Proton sequencing platform and software developed for straightforward bioinformatic analysis. An algorithm was developed using 239 samples to determine the likelihood of trisomy, using DNA fragment counts and a fetal fraction validity check; the results were compared with those from invasive diagnostic procedures. A further 111 samples were used to assess the tests' sensitivity (detection rate) and specificity (1 minus false-positive rate). **Results:** The 110 of a possible 111 valid samples used to verify the IONA[®] test gave 100% sensitivity and specificity, compared with invasive diagnostic procedures; one failed the fetal fraction validity check giving a sample failure rate of 0.29% across all 350 analysed samples. **Conclusion:** The data indicate that the IONA test provides a robust, accurate automated workflow suitable for use on

maternal blood samples to screen for trisomies 13, 18, and 21. The test has the potential to reduce the number of unnecessary invasive procedures performed and facilitate testing by screening laboratories.

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Introduction

Trisomy is the presence of three copies of a specific chromosome, rather than the two copies of a normal karyotype, leading to congenital abnormalities and miscarriage. However, some can lead to a live birth such as trisomy 21 (Down syndrome) [1], trisomy 18 (Edwards syndrome), and trisomy 13 (Patau syndrome) [2]. Pregnant women are currently offered screening for chromosomal abnormalities which calculate a risk score using maternal age, nuchal translucency ultrasound scan, and serum biochemical markers. Those considered high risk (≥ 150 , NHS, UK) are then given the option to proceed to invasive diagnostic testing, such as an amniocentesis or chorionic villus sampling (CVS) [3]. This enables women to make informed discussions with their health-care provider on whether or not to continue with the pregnancy.

Current screening methods have a detection rate of about 90%, and 5% false-positive rates [4], which may lead to unnecessary invasive procedures that carry a risk of miscarriage [5].

Noninvasive prenatal testing (NIPT) can be performed by analysis of cell-free DNA (cfDNA) from a maternal blood sample, as a fraction of this DNA is derived from the placenta [6]. For example, fetal chromosome copy number can be determined by comparison of sequence read counts from the chromosome of interest with counts from reference chromosomes. Chromosome 21 makes up approximately 1.3% of total autosomal DNA in the genome and a person with trisomy 21 has a 1.5-fold increase in DNA from this chromosome. A blood sample that has a 15% fetal fraction will show an increase from approximately 1.3 to 1.4% of DNA fragments that originate from chromosome 21 [7]. Hence, the assay must be highly accurate to be able to detect a small difference in genetic material from the relevant chromosome. The DNA fragments that are initially sequenced may be either from an unselected sample of the fragments in the maternal plasma, or alternatively, from a selected sample that is derived from particular chromosomes, such as chromosomes 13, 18, and 21 [7]. A further method is the analysis of single nucleotide polymorphism distributions, by targeted amplification and sequencing of polymorphic sites on selected chromosomes, to compute the risk of trisomy [6, 8, 9]. All of the above-mentioned methods outperform traditional screening techniques for trisomies 13, 18, and 21 such as the First-Trimester Screening Test (FTST). The FTST provides a risk score calculated from the results of ultrasound (fetal nuchal translucency thickness measurement), maternal biochemical serum markers (pregnancy-associated plasma protein-A, β -human chorionic gonadotropin), and maternal age prior risk, and has a detection rate of approximately 90% and a false-positive rate of 5% [10, 11].

For the unselected DNA fragment method, a large number of DNA fragments within the maternal plasma must be sampled to observe the small difference in percentage of fragments from the chromosome of interest. Next-generation sequencing (NGS) is a technology that has the capability to enumerate the circulating DNA fragments at a level that can demonstrate this difference. The initial NGS technology was massively parallel shotgun sequencing (MPSS). This uses sequence adaptors, or tags, to sequence many different fragments of DNA in parallel, using a bead-based approach. This is now the basis behind most NGS technologies [12] and is used in the IONA[®] test. The MPSS technology has been validated for the screening of fetal aneuploidies and has been shown to

provide lower false-positive rates than standard screening, with values as low as 0.5% being reported by Bianchi et al. [13]. In another study, this type of technology has given a sensitivity of 99.94% for trisomy 21 and 100% for both trisomy 18 and trisomy 13. Specificity values were 99.46% for trisomy 21, 99.24% for trisomy 18, and 100% for trisomy 13 [14].

Following an initial feasibility study using the Ion Torrent PGM instrument, we wanted to ascertain whether the problem of the widely reported low sequencing accuracy [15–18], particularly the relatively high frequency of indels, could be overcome by appropriate bioinformatic techniques to enable this sequencing platform, with its potential advantages of cost and speed, to be harnessed in the prenatal screening application. The success of that feasibility study led to the development of the IONA test as described here, using the Ion Proton instrument. The aim was to develop a novel, completely automated, easy-to-use workflow system for the detection of fetal aneuploidies from maternal plasma.

This paper details the development of the IONA test and provides a comparison of sensitivity and specificity with reference data from invasive procedures.

Materials and Methods

Samples were selected from archived maternal plasma within the Premaitha sample collection, collected under protocol reference: 07/H0607/101. The Premaitha sample collection also includes samples kindly donated by: Great Ormond Street Hospital, collected under the RAPID study; LABCO, collected as part of the SAFE study; and the Kings College London collection. All of the samples were collected under ethically approved protocols with all subjects having given informed consent.

All samples were collected in standard CE-marked EDTA blood collection tubes and spun down at 1,600 g for 10 min to separate the plasma within 8 h of blood draw; they were then stored frozen at -80°C . Once defrosted, the samples were spun for a further 10 min at 16,000 g and the plasma decanted, to remove any cellular debris.

Development of likelihood models (a form of statistical mixture model) for trisomies 13, 18, and 21 was performed using 243 samples. Among these, 174 samples were unaffected, 36 had trisomy 21, 29 had trisomy 18, and 4 had trisomy 13. Four samples were subsequently removed from the data set, as explained in the Results section, leaving 239 samples for analysis. The samples were collected from pregnant women, identified as high risk for Down syndrome by the combined screening test, who were aged 16 and above and did not have a chromosomal abnormality. As this was a development study, cases of confined placental mosaicism or vanishing twin were excluded. Patients were only included if they were able to understand the information supplied and freely give their informed written consent.

After development, the likelihood models were tested on a further set of 112 samples. Ninety samples were unaffected, 16 had trisomy 21, 5 had trisomy 18, and 1 had trisomy 13. One sample was subsequently removed from the data set, as explained in the Results section, leaving 111 samples for analysis. All samples were verified for trisomy status by a “gold standard” invasive procedure (i.e., either amniocentesis or CVS).

The samples used for the development and the testing of the likelihood models were collected between April 2008 and June 2014. The mean maternal age was 35 years (range 19–46) and the mean gestational age at which the samples were taken was 15 weeks (range 9–35).

Workflow

Analysis was carried out at Premaitha Health Ltd, Manchester, UK. This study used the following automated workflow.

DNA Extraction

DNA was extracted from each maternal plasma sample, to isolate cfDNA, using a QIASymphony (QIAGEN, Germany) and the QIASymphony circulating DNA kit (catalogue No. 1074536) (be-spoke kit for IONA test), following the manufacturer’s instructions.

DNA Library Preparation

Each DNA eluate was used to prepare a DNA library via the following automated workflow employing a Sciclone (Perkin Elmer) robot, which performed all the steps except for the PCR and library quantification.

A DNA end repair reaction was performed to produce blunt-ended DNA fragments, which were phosphorylated at each end. An adaptor ligation reaction was then used to add an adaptor oligonucleotide to each end. The adaptor creates a unique “barcode” for the DNA fragments from each sample. This allows more than one sample to be prepared and analysed at once. The reaction was cleaned up to remove unused adaptors, and paramagnetic beads were then used to sequester the DNA from the solution. A PCR reaction, with a high-fidelity enzyme and primers that bind to the adaptor sequences, was used to amplify the DNA. On completion of the PCR, the libraries were quantified using a Lab Chip GX instrument (Perkin Elmer) before being mixed together in equal quantities to form a multiplex pool. Eight samples were mixed to form each pool. The pool mix was then cleaned up using paramagnetic beads, and refined using size selection. Size selection ensured that the DNA fragments lay within the read length range for NGS. The library pool was quantified and diluted for use in the NGS reaction. A pre-prepared sample with known trisomy status was included as a run control at the dilution step.

Next-Generation Sequencing

NGS was performed using an automated workflow on the OT2 and Ion Proton (Life Technologies, CA, USA). A clonal amplification reaction was performed on the Ion Chef to produce single-stranded copies of each DNA fragment with a barcode adaptor. The fragments were then sequenced by synthesis of the complementary strand in an MPSS approach.

Data Analysis

Data analysis was performed under the proprietary Bioinformatics Data Platform development software environment. Execu-

tion of the main IONA bioinformatics pipeline hosted in this environment proceeded as follows: for a sequencing run of 8 samples, multiplexed sequence reads were retrieved from the sequencing platform in the form of an unmapped BAM file. The multiplexed assembly of reads was initially subject to a barcode classification step, in which barcoded 5’ adaptors were identified and matched against a predefined set, in order to split the multiplex into reads against individual samples. Further processing then took place for each sample.

Following early quality filtering steps to remove a small number of very short reads (i.e., ≤ 8 bp) and trim the leading 10 bp from the 5’ end of those remaining, fragments were mapped to the “hg19” human reference genome. To overcome the potential problem of a high indel sequencing error rate with this semiconductor sequencing platform, a processing pipeline incorporating a gap-tolerant read alignment module was devised. Post-filtering of alignment results was then carried out to remove duplicate reads that arose in the PCR stages of the test workflow. These were determined as reads whose 5’ ends mapped to the reference at the same position as for any other read.

Fragments determined to have aligned uniquely in the genome reference were then binned by autosome, with the resulting counts subject to a calibration step to correct sequencing coverage bias correlated to GC content.

Finally, the resulting fragment count data were used as the input to a set of mixture models, which incorporate distributions of expected values under both trisomy-affected and unaffected hypotheses for trisomy 13, 18, and 21 tests. Each model generated a test likelihood ratio to quantify the relative likelihood that the DNA fragment count data supported a trisomy-affected or unaffected hypothesis for each trisomy under consideration.

The bioinformatics development pipeline also performed validity checks, as follows. Following the generation of per-autosome fragment counts, the run validity check took place. This validity check first identified fragments derived from sequencing the run control, and then compared the proportion of counts from these fragments which aligned to chromosome 21 against a reference range previously determined as part of a specification-setting study. If the proportion met the reference criteria, the run validity check passed.

Additional validity checks also took place for each sample. These first ensured that the aligned fragment count was sufficient for the likelihood mixture model to be used, and secondly that the proportion of cfDNA in the sample that was of fetal origin (i.e., “fetal fraction”) was sufficient for a result to be reported; this utilised a dynamic check which also incorporated information on sequencing count density individually for each sample, with specifications determined from likelihood model fits to training data with known fetal fraction and trisomy status.

Results

Assay Development

Initially, 243 samples from women in a high-risk group were used for developing and refining the model. Four samples were removed from the data set; 3 samples had no “gold standard” reference result from an invasive di-

Table 1. Combined trisomy data – 100% sensitivity and 100% specificity was observed when the IONA[®] test was compared with the reference method (amniocentesis or chorionic villus sampling) for 110 samples

IONA [®] test result	Reference method		
	unaffected	trisomy	total
Unaffected	89	0	89
Trisomy 21	0	16	16
Trisomy 18	0	4	4
Trisomy 13	0	1	1
Total	89	21	110

Table 2. Sensitivity and specificity of the IONA[®] test, compared with the reference method (amniocentesis or chorionic villus sampling)

	Sensitivity, % (95% confidence interval)	Specificity, % (95% confidence interval)
Trisomy 21	100 (79.4–100)	100 (96.2–100)
Trisomy 18	100 (39.8–100)	100 (96.6–100)
Trisomy 13	100 (2.5–100)	100 (96.7–100)
Overall	100 (83.9–100)	100 (95.9–100)

agnostic procedure available for comparison, and 1 sample was found to have originated from a breast cancer patient [19]. Presence of cancer is an exclusion criterion for the IONA test, since the presence of cancer can confound results by shedding cell-free tumor DNA into plasma. With these exclusions, data from 239 samples were included in the development of the likelihood model. Of these samples, 173 were unaffected, 36 had trisomy 21, 26 had trisomy 18, and 4 had trisomy 13. There was clear separation between trisomy and unaffected cases, and each trisomy case was negative for the other trisomies. One sample failed the minimum fragment count validity check. This gave a failure rate of 0.42%.

Following the bioinformatic validity checks and GC correction as described in Methods, the data provided a proportion of total fragment counts aligning with the chromosomes of interest, 21, 18, and 13. The sample data were used to optimise the likelihood algorithm, guiding the modelling of the frequency distributions of count proportions of each of the three chromosomes for both affected and unaffected cases, with the procedure targeted to achieve high specificity and sensitivity when refer-

enced against cytogenetic analyses for all three chromosomes.

The developed model was then tested on a further, independent set of samples to demonstrate that it had been optimised successfully and to provide an initial estimation of the performance for the IONA test.

Assay Testing

One hundred and twelve samples were used to assess the IONA test performance characteristics against data from cytogenetic analyses. One sample was removed from the data set due to inconsistencies and abnormalities in the karyotype data (i.e., severe hypoplastic heart, suspected other chromosomal abnormality based on phenotype at birth), leaving 111 samples for analysis. Of the 111 samples utilised for data analysis, one of the trisomy 18 samples failed the fetal fraction validity check, resulting in a sample failure rate of 0.90%. Table 1 shows the numbers of samples that were concordant between the IONA test and the diagnostic reference result from amniocentesis or CVS. All 110 valid results from the IONA test were observed to be concordant with the cytogenetic diagnostic test result. The observed assay sensitivity and specificity values in this small sample were 100% for all conditions in the three chromosomes of interest and are presented in Table 2, together with confidence intervals [20, 21].

The results are also presented in Figure 1 in an alternative format that represents the dynamic nature of the analysis from raw fragment count information. For each trisomy test, the plot shows the base-10 logarithm of likelihood ratio for each sample, with an implicit likelihood ratio cut-off value of 1:1 shown as a dotted line. Samples that fell to the right of the cut-off line (likelihood ratio greater than 1:1) were considered to present a result of trisomy with the IONA test, whilst those to the left (likelihood ratio less than or equal to 1:1) were considered to present a result of unaffected. In the plot for trisomy 21, 2 samples were very close to the cut-off. These would have had low fetal fraction, but the in-built validity check passed and as such the results were valid.

When the two parts of this study were combined across the 350 samples, 348 samples presented valid results, giving a failure rate for the assay of 0.29%.

The automated workflow developed, the IONA test, allowed the complete process from plasma to likelihood ratio to be completed with a turnaround of 3 days as follows:

Day 1

1. Plasma separation and cfDNA extraction (3.5 h)

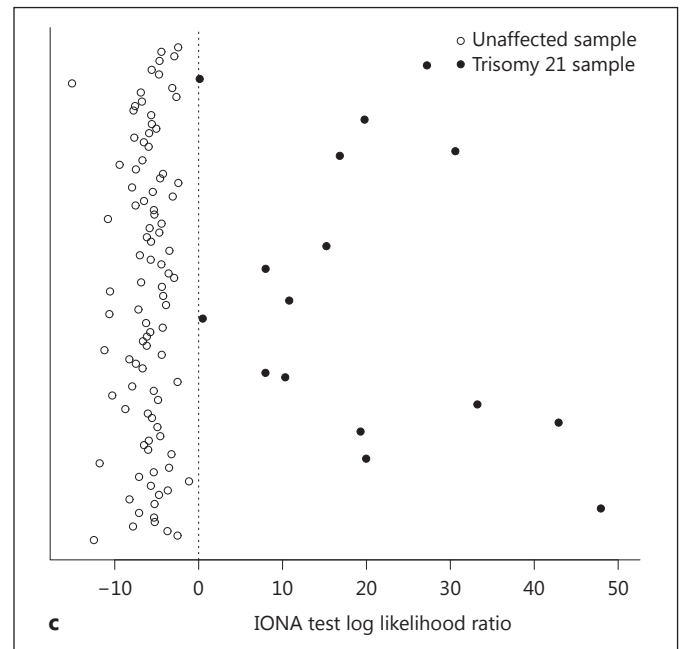
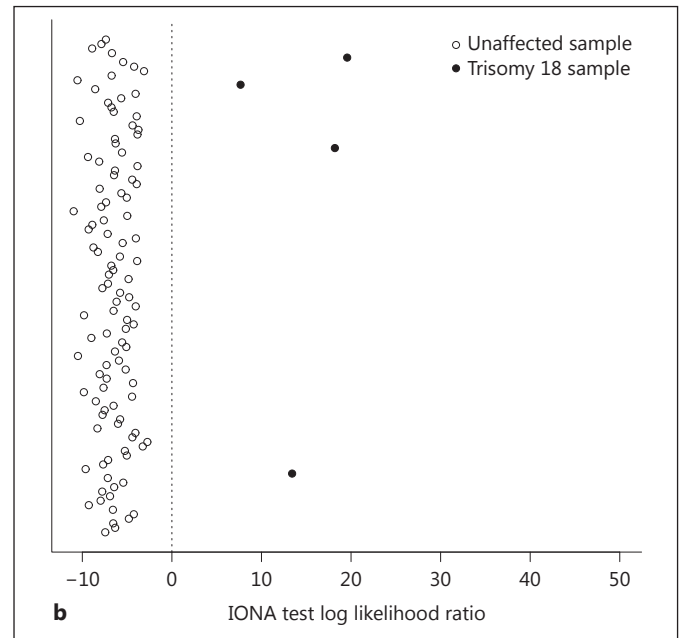
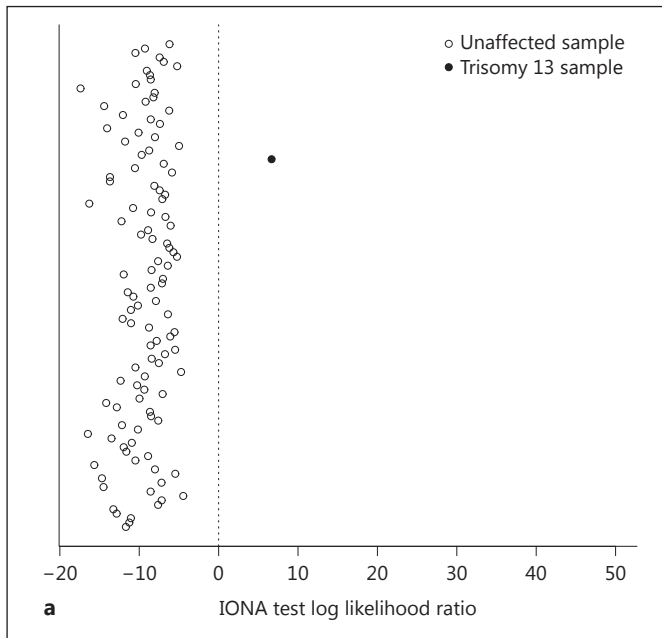


Fig. 1. Chromosome 13, 18, and 21 likelihood ratio analysis. Samples to the left of the vertical dashed line are unaffected samples. Those to the right of the dashed line are samples that show trisomy.

2. Automated library construction (8 h)
3. Automated emulsion PCR (library amplification and enrichment (13.5 h running overnight)
Day 2
4. Library DNA sequencing (5 h)
5. Automated data analysis (3 h)

This results in a processing time of 33 h for up to 32 samples in multiples of 8 for optimal throughput [22].

Discussion

The IONA test is a novel complete automated, easy-to-use workflow system for the detection of fetal aneuploidies (trisomy 13, 18, and 21) from maternal plasma. It utilises the advantages of NGS on an Ion Proton sequencing platform for a high level of sampling of the cfDNA fragments. The test can be fully automated and provides

both an analysis protocol and software to determine the likelihood of trisomy 21, trisomy 18, and trisomy 13. The protocol involves the extraction of both maternal and fetal/placental DNA fragments from a maternal plasma sample. A DNA library is then prepared using “barcoded” adaptors, which are ligated to the DNA fragments. The use of this barcode system allows a pool of libraries from different samples to be produced and analysed together. The DNA library is sequenced using a whole-genome sequencing strategy. Analysis of sequencing data utilises a proprietary algorithm to determine the likelihood of trisomy of chromosomes 13, 18, or 21.

An analytical assessment of sensitivity and specificity was performed by applying a cut-off value to the sample likelihood ratio and comparing the result to the invasive diagnostic test result.

We were able to show the reliability and robustness of the IONA test, with a failure rate of <1%. A low failure rate is important in clinical scenarios, allowing for improved patient and provider acceptance. We were able to achieve this failure rate using a low volume of sample input (2 mL). This allows the possibility of a second sampling run on a 10-mL maternal sample, should that be required.

The IONA test was observed to be 100% sensitive and specific, with 95% confidence limits on sensitivity and specificity of 84–100% and 96–100%, respectively, for the three trisomies combined in this small study, when compared with reference analyses (i.e., amniocentesis and CVS). Although 100% sensitivity and specificity was shown for trisomy 13 and 18, the 95% confidence limits were wide due to the small number of these samples available for inclusion in this small study (see Table 2). This high level of sensitivity and specificity is comparable to existing NIPT cfDNA screening tests [23] and superior to standard screening methods such as the combined and quadruple tests. Further, large scale, studies are required to determine the performance characteristics of the test more precisely.

The automated nature of the workflow enables a 3-day turnaround, from plasma to result, to be achieved. The clinical implication of the low failure rate and fast turnaround time is that more samples will give an accurate result on the first attempt.

The algorithms and features included in the internal bioinformatics development pipeline were subsequently transferred to the IONA software, which has been developed as medical-grade software for external use in accordance with the IEC 62304 and IEC 62,366 standards. Software features, such as the validity checks for fetal fraction

and minimum fragment count, provide the test with robustness and lower the chance of false results. For example, the invalid trisomy 18 sample reported above with a low fetal fraction would have provided a false-negative result without the fetal fraction check.

Previous work by Liao et al. [14] and Jeon et al. [18] has tested the applicability of the Ion Proton platform for NIPT. A review of that work concluded that further studies were needed to determine if this platform is indeed suitable [24]. The test documented here demonstrates the suitability of the sequencing platform when integrated into the complete IONA test workflow comprising instruments and analysis software.

The use of a likelihood ratio, as opposed to a *z* score threshold or comparable method, leads to more informative output and facilitates Bayesian combination with other factors, such as maternal age background risk, results from the combined test, or other a priori results. Wright et al. [25] have indicated that the ability to combine results of the DNA-based test with maternal age and the first-trimester combined test could be of huge benefit. Their study indicated that when the fetal fraction was $\geq 9\%$, a cfDNA test combined with maternal age detected almost all cases of trisomy 21. When the fetal fraction was less than 9%, a cfDNA test can be improved by combining it with fetal nuchal translucency thickness, serum-free β -human chorionic gonadotropin, and pregnancy-associated plasma protein-A data (the first-trimester combined test). The IONA test takes into account fetal fraction and maternal age and can be used in addition with other data, as described above. This meets the requirements for a valuable and practical cfDNA test. Two blinded clinical evaluation studies have been published in which background risk associated with maternal age has been used as the a priori. A total of 679 samples included 580 euploidy, 78 trisomy 21, 14 trisomy 18, and 7 trisomy 13 samples [22, 26]. The false-positive rates were both 0% and detection rate 100%. One sample was rejected as low fetal fraction after failing the fetal fraction validity check.

Conclusion

This type of cfDNA screening test using maternal blood for the screening for chromosomal abnormalities, such as Down syndrome, Edwards syndrome, and Patau syndrome, provides a fast, safe method for screening as a consequence of the very low false-positive rate compared to the current screening tests (i.e., FTST) and could prove a beneficial addition when implemented in a clinical care

pathway. This could be either as a reflex screen or a contingent screening strategy. In the reflex screening strategy, 2 blood samples are collected simultaneously for the FTST and if the woman is assessed as high risk, the second sample is automatically used for cfDNA testing. Only one final result is then reported to the woman. In the contingent screening strategy, a prior high-risk FTST result would lead to the women being offered a cfDNA test at a different time point. The accuracy, improved safety, and speed of cfDNA testing should produce a positive clinical impact by significantly reducing the number of unneces-

sary invasive procedures and associated miscarriages, stress, and anxiety of expectant women. The IONA test has been developed to provide a simple-to-use, CE IVD-marked, automated workflow comprising instruments and analysis software to allow it to be adopted by standard screening laboratories.

Disclosure Statement

The authors have no conflicts of interest to disclose.

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