The importance of determining the limit of detection of non-invasive prenatal testing methods

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ABSTRACT

Objective Several non-invasive prenatal testing (NIPT) methods, which analyze circulating fetal cell-free DNA (cfDNA) in maternal plasma, suggest a fetal fraction (FF) ≥4% for a reportable result, with the assumption that fetal aneuploidies may not be detectable at lower FF. This study determined the actual limit of detection (LOD) of a massively parallel sequencing-based NIPT method and evaluated its performance in testing samples with low FF.

Method An experimental model, involving the creation of artificial plasma mixtures with a final aneuploid FF ranging from 1% to 4%, simulated samples at different proportions of fetal cfDNA. We then analyzed 7103 blood samples, from pregnant women undergoing NIPT, to assess the impact of low FF on the performance of cfDNA testing.

Results Detection of common aneuploidies in samples with an FF as low as 2% is well within the ability of this technology. Of 105 pregnancies confirmed chromosomally abnormal, 25 (23.8%) involving a 2% < FF < 4% were consistently detected. These high-risk pregnancies would have not been identified using the suggested 4% FF cut-off.

Conclusion This study underscores the importance of determining the actual LOD for each specific NIPT methodology. It may reduce the incidence of test cancelations and shorten the time required for the diagnosis of aneuploidy. © 2016 John Wiley & Sons, Ltd.

INTRODUCTION

Non-invasive prenatal testing (NIPT), based on the analysis of circulating fetal cell-free DNA (cfDNA) in maternal plasma, has become rapidly integrated into clinical practice for detection of common fetal chromosomal aneuploidies.1

The high sensitivity and specificity resulting from multiple clinical trials2–7 have resulted in many institutions routinely adopting NIPT for aneuploidy screening in high-risk pregnancies. Different NIPT methods have been developed for the analysis of cfDNA in maternal plasma using next-generation sequencing (NGS) technology, including whole genome sequencing by massively parallel sequencing (MPS)8–10 or selected chromosome targeted sequencing.11,12 These methodologies rely on the ability to detect proportional increases in cfDNA derived from the aneuploid fetus against the much larger background of euploid maternal DNA. On average, the amount of fetal cfDNA in plasma from a pregnant woman is approximately 10% when sampling is carried out between 10 and 20 gestational weeks,11–15 but there is a large variance in the fraction of fetal cfDNA between patients.

Regardless of the approach used, the reliability of cfDNA testing results depends on several factors, including the number of sequence tags counted for each chromosome (i.e., the sequencing depth)16,17 and the proportion of cfDNA in the maternal plasma, which is of fetal origin, that is, the fetal fraction (FF).

The FF is a key determinant of assay performance.18–20 In trisomic pregnancies, cfDNA derived from the extra fetal chromosome results in a higher proportion of fetal cfDNA than in disomic pregnancies. The ability to identify this small increase in the amount of fetal cfDNA of a given chromosome, and therefore to detect a chromosomal aneuploidy, is directly related to the FF. If there is sufficient fetal cfDNA in the plasma sample, then the assay can provide accurate counting of the fetal chromosome fragments. The larger the FF, the better the ability to distinguish between euploid and aneuploid pregnancies, thereby better the test performance. Instead, if the FF is too low, then a chromosomal abnormality could be masked by the overwhelming proportion of euploid maternal cfDNA, thereby increasing the risk of achieving false negative test results.16–20
Recently, Benn and Cuckle\textsuperscript{17} theoretically determined the important role of FF in combination with the sequencing depth for reliable NIPT results. A low FF can partly be compensated by higher sequencing depth, and the number of generated reads may thus overcome the statistical noise.

The majority of the NIPT approaches involve measurement of fetal cfDNA as basic quality metric required to ensure the reliability of interpretation of test results.\textsuperscript{11,12,21} Other assays do not involve FF measurement, providing accurate results without knowledge of the amount of the fetal cfDNA in a sample.\textsuperscript{10,22}

Clinical tests that rely on quantification of FF require the amount of fetal cfDNA in maternal plasma to be above a minimum level for accurate aneuploidy assessment.\textsuperscript{11–13,21,22,24} An FF of \(-4\%\) or greater is the suggested minimum threshold currently employed by these NIPT methods for a reportable result. Below this value, the cfDNA test is usually presented as a failure and no result is reported.\textsuperscript{14,17,18,25}

The 4\% cut-off was defined using statistical modeling based on the requirement for sufficient sequencing read depth as a function of FF in order to detect fetal aneuploidies.\textsuperscript{16–18,25} The assumption was that at lower FF, the small differences in circulating cfDNA between trisomic and disomic pregnancies may not be detectable\textsuperscript{13,20,21} causing false negative results or leading to inaccurate or failed test results. However, no experimental data are available on the actual limit of detection (LOD) of these NIPT approaches (i.e., the lowest FF with a detectable aneuploidy), supporting the aforementioned hypothesis. As a consequence, the use of the above cut-off value could be not necessarily appropriate for all cfDNA testing methodologies.

The aims of this study were firstly, to determine the LOD of an MPS-based NIPT method, in order to define the actual lower FF required to detect common fetal autosomal trisomies, using a model system to simulate samples at different proportions of fetal cfDNA. Secondly, to assess the impact of low FF on the performance of cfDNA-based maternal plasma testing for aneuploidies.

METHODS

Study design
This study was organized into three steps. The first step consisted of a retrospective analysis of 2006 frozen plasma samples, collected from pregnant women undergoing invasive prenatal diagnosis, with known fetal karyotypes and confirmed outcomes. Samples were collected between 11 and 20 weeks gestation, prior to amniocentesis or chorionic villus sampling. This cohort was used as part of the laboratory development NIPT process, with the aim of defining the boundaries of the dual-threshold classification model reported elsewhere.\textsuperscript{26–28}

In the second step, an experimental model, involving the creation of artificial plasma mixtures with a final aneuploid FF ranging from 1\% to 4\%, was designed to simulate samples at different proportions of fetal cfDNA. The aim was to determine the LOD of fetal aneuploidy by cfDNA testing using an MPS-based NIPT approach.

In the third step, a total of 7103 blood samples collected from pregnant women undergoing cfDNA testing were analyzed in order to assess the performance of the assay in samples with low FF.

Study population and sample collection
Data included in the current analysis were generated during the process of NIPT for fetal aneuploidy in the GENOMA laboratory (Rome, Italy) between September and December 2014.

The indication for testing was one or more of the following: advanced maternal age (defined as maternal age 35 years or greater), previous positive prenatal screen, fetal ultrasound abnormality, and prior pregnancy with fetal aneuploidy or parental anxiety. Women with a singleton pregnancy and a qualified blood sample were included in the study. All testing was performed on whole blood samples (10 mL) received in cfDNA BCT\textsuperscript{™} tubes (Streck, Omaha, NE, USA), collected from patients with a confirmed pregnancy greater than 10 weeks of gestation (mean 12.8 ± 2.3). Samples were received within 5 days of blood draw and accessioned with a complete test requisition form. cfDNA was extracted and processed for library preparation, sequencing, and data analysis, following the protocol described in the succeeding sections.

Ethical approval
The material used in this study was obtained with patients’ informed consent and Institutional Review Board approval from the Genoma Center.

Artificial mixture preparation
To determine the limit of detection of the MPS-based NIPT approach, we designed an experimental model closer to the real clinical practice. It involved the creation of artificial mixtures at different proportions of plasma samples derived from aneuploid male fetuses with known FF% and samples from non-pregnant women, as detailed in the Supporting Information.

Cell-free DNA was then extracted from the reconstructed plasma samples, processed for library preparation, and sequenced as described in the succeeding section.

Sample preparation, sequencing, and classification
The blood samples were first centrifuged at 1600 \(\times\) g for 10 min at 4°C to separate the plasma from peripheral blood cells. The plasma portion was then carefully transferred into a polypropylene tube and subjected to a second centrifugation at 16,000 \(\times\) g for 10 min at 4°C, in order to remove residual cells.\textsuperscript{9} cfDNA was extracted from 900 \(\mu\)L of maternal plasma using the QIAamp DNA Blood Mini Kit (Qiagen), following the manufacturer’s protocol.

Sequencing libraries were prepared using TruSeq nano Kit (Illumina) according to the manufacturer’s recommendations. Data were analyzed by SAFErtm algorithm, to calculate the NCV and determine the ploidy status of the fetus. More details are available in the Supporting Information.

Fetal fraction measurement
Fetal fraction measurement, for the artificial mixtures of plasma samples and clinical samples, was performed using the MPS tag-count data with the X chromosome ratios, in samples from pregnancies carrying male fetuses, and/or with aneuploid chromosome ratios, in samples from women.
carrying fetuses with a chromosome aneuploidy, as previously reported. More details are available in the Supporting Information.

Clinical outcomes
All patients were followed for pregnancy outcomes. Chromosomally abnormal results of cfDNA testing were confirmed performing a metaphase karyotyping after an invasive prenatal diagnostic procedure. Chromosomally normal results were confirmed by newborn physical examination.

On the basis of outcome information, results were categorized in one of the following ways: (1) ‘concordant’ if the NIPT result matched a karyotype result or birth outcome or (2) ‘discordant’ for unexplained NIPT results that do not match a karyotype from any source or birth outcome.

Statistical analysis
All statistical analyses were carried out in Microsoft Excel® software. Statistical analysis between the incidence of aneuploidy in the different groups was performed using a chi-square ($\chi^2$) test. Statistical significance was set at $p < 0.05$. Point estimates for sensitivity and specificity along with exact 95% confidence intervals (CI) using the Clopper–Pearson method were computed for each chromosome analyzed.

RESULTS

Retrospective analysis (training set data)
A total of 2006 plasma samples were analyzed in the training cohort, 6 (0.3%) of which had a quality metrics failure and 2000 (99.7%) were with a conclusive result. The clinical characteristics of the patients included are summarized in Table SII.

The results from 1969 samples with a normal fetal karyotype showed a normalized chromosome value (NCV) ranging between −3.5 and 3.55. Of the remaining 31 aneuploid samples, 25 with a T21 karyotype showed an NCV ranging between 3.18 and 33.4 samples with a T18 karyotype had NCVs ranging between 6.5 and 11.8, while 2 samples with a T13 karyotype showed an NCV of 7.7 and 24.7. The NCVs obtained from the training set samples are shown in Figure S1.

The NCVs of euploid samples were lower than 3 in 99.8% of the samples. Instead, aneuploid fetuses showed NCV >4 in 87% of the samples analyzed. Four (12.9%) aneuploid and four (0.2%) euploid samples had $3 < \text{NCV} < 4$. Therefore, based on the dual-threshold classification model reported in previous studies, NCV boundaries used in this study were the following: NCV >4 for an aneuploidy call and NCV <3 for an euploid call. A zone for borderline values between aneuploidy and euploidy calls, involving samples with $3 < \text{NCV} < 4$, was also included. Results in this range were termed ‘unclassified’ and reported as ‘suspected aneuploidy’. These boundary values were adopted for the classification of the results for the clinical samples.

Determination of the limit of detection
To determine the LOD, a set of 156 samples from reconstructed experiments was analyzed (Table SII).

The FF from each reconstructed sample was measured using both the X chromosome and aneuploid chromosome ratios. As shown in Figure S2, the FF values obtained with the two methods resulted strongly correlated.

The NCV values for T21, T18, and T13 obtained from each reconstructed sample were plotted versus FF% (Figures 1–3), measured with the X chromosome ratios. The lower FF with a detectable aneuploid call was determined, based on the NCV boundaries defined after testing samples of the training set. As shown in Figures 1–3, the lower FF with a detected aneuploidy was 2% for chromosome 21 and 1.5% for chromosomes 18 and 13. Based on the aforementioned findings, the LOD of the MPS-based method was established at a level of 2% FF, the lower value allowing the detection of all trisomies. This cut-off value was used for cfDNA testing in clinical samples.

Clinical samples data analysis
A total of 7103 pregnant women were enrolled in the study. The patient demographic characteristics, type of invasive procedure, and indication for testing are summarized in Table 1. The mean age of the pregnant women was 36.4 ± 4.7 years (range, 24–54 years), and gestational age ranged from 10 weeks to 30 weeks (mean 12.8 weeks ± 2.3 days).

Of the 7103 samples received, 21 (0.3%) were excluded from the analysis because of a quality metrics failure (Supporting Information). The remaining 7082 (99.7%) samples were with
a call; 3628/7082 (51.2%) of the fetuses were male and 3454/7082 (48.8%) were female (Table 2).

In the clinical samples, the FF was determined using both the X chromosome and aneuploid chromosome ratios. The FF was not measured in pregnancies with a female fetus. Among the samples with a result, 79 (1.1%) from pregnancies with a male fetus were canceled because fetal cfDNA resulted below the LOD (FF < 2%) value (Table 2). A blood redraw was requested for these samples. It is expected that a similar amount of samples from pregnancies with a female fetus involved an FF below the LOD. These samples were not identified because the protocol does not allow FF determination in such pregnancies. Although it may represent a limitation of the NIPT approach used, the performance of the cfDNA test was not affected.

Normalized chromosome values obtained in the test set for chromosomes 21, 18, and 13 are shown in Figure 4. One hundred and five out of 107 aneuploid results were confirmed by metaphase karyotyping following amniocentesis. Two samples, one T21 and one T18, had a false positive result. No false negative cases have been reported.

The results obtained showed that 100% of the samples with a normal fetal karyotype had an NCV ranging between -3 and +2.99. All aneuploid samples displayed an NCV > 3. Specifically, samples with T21 (n = 77) showed an NCV ranging between 3.18 and 33.3, samples with T18 (n = 21) had NCVs ranging between 5.2 and 27, and samples with T13 (n = 9) showed an NCV between 4.36 and 24.7. Ten samples, classified as suspected aneuploidy (3 < NCV < 4), were confirmed with an aneuploid karyotype. The two false positive samples, T21 and T18, had an NCV of 5.2 and 5.03, respectively (Figure 4).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>n</th>
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<tbody>
<tr>
<td>No. of eligible patients</td>
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<tr>
<td>Maternal age (year)</td>
<td>36.4±4.7</td>
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<tr>
<td>Min–max</td>
<td>24–54</td>
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<tr>
<td>Gestational age at sample collection – week</td>
<td>12.8±2.3</td>
</tr>
<tr>
<td>Min–max</td>
<td>10–30</td>
</tr>
<tr>
<td>Follow-up by invasive prenatal diagnosis – no./total no. (%)</td>
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</tr>
<tr>
<td>CVS</td>
<td>22</td>
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<tr>
<td>Aneamniocentesis</td>
<td>85</td>
</tr>
<tr>
<td>Indication for NIPT</td>
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<td>Parental anxiety</td>
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<td>Advanced maternal age onlya</td>
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<tr>
<td>Positive prenatal screen</td>
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</tr>
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</tr>
<tr>
<td>Prior pregnancy with fetal aneuploidy</td>
<td>95</td>
</tr>
<tr>
<td>More than one indication</td>
<td>1789</td>
</tr>
</tbody>
</table>

CVS, chorionic villus sampling; NIPT, non-invasive prenatal testing.
aAge >35 years.
Sensitivity of the cfDNA testing for T21, T18, and T13 was 100%. Specificity was 99.99% for T21 and T18, and 100% for T13 (Table 3).

Performance of the massively parallel sequencing-based protocol on samples with low fetal fraction

To assess the impact of low FF on the performance of cfDNA-based testing for aneuploidies, samples were categorized on the basis of FF%, estimated using the aneuploid chromosome ratios. Figure 5A shows the graphical distribution of the 107 aneuploid samples according to the FF%. One hundred and five pregnancies, rated as chromosomally abnormal, were confirmed as true positives, 25 (23.8%) of which involved a 2% < FF < 4%. Seventeen samples were rated as T21, three with T18 and five with T13 (Table 2 and Figure 5B). These high-risk pregnancies would have not been identified if the common 4% FF cut-off had been used. Within the group of samples with a 2% < FF < 4%, two were false positives, one with T21 and the other with T18 (Table 2 and Figure 5B). In a separate analysis, FF was measured using the X chromosome ratios, and the values obtained for each sample were compared with those resulting from the use of the aneuploid chromosome ratios. As shown in Table SIII, the mean FF calculated with the two methods was not significantly different.

In addition, we plotted the values of the FF obtained using both the X chromosome and aneuploid chromosome ratios. These independent measurements values strongly correlated, demonstrating that the two methods may either be used for FF determination (Figure S3).

To calculate the incidence of aneuploidy in the group with an FF < 4% compared with those with FF ≥ 4%, we limited the analysis to male fetuses only. Among the 3628 samples from male fetuses, 3318 (91.5%) had an FF ≥ 4% and 310 (8.5%) had FF < 4%, 231 (6.4%) of which were with a 2% < FF < 4% and 79 (2.2%) with FF < 2% (Figure S4A and Table 4). For samples with FF < 2%, not conclusive result because of low FF was reported, and a blood redraw was requested. After reanalysis, all the earlier samples were classified as euploid, and the results were confirmed by newborn physical examination. The incidence of aneuploidy in samples with an FF ≥ 4% was 1.1% (36/3318) and 6.9% in samples with a 2% < FF < 4% (16/231) (Figure S4B and Table 4). An increased (sixfold) incidence of aneuploidy (6.9% vs 1.1%, p < 0.001) was noticed in samples with 2% < FF < 4% versus samples with FF ≥ 4% (Figure S4B).

**DISCUSSION**

The results of this study demonstrate that the minimum FF level required for accurate aneuploidy assessment should be related to the actual LOD of each specific NIPT approach used and not necessarily fixed at 4% for all cfDNA testing methodologies.

In fact, the MPS-based NIPT protocol used here allowed the detection of chromosomal aneuploidies at FFs as low as 2%. This was demonstrated in the experimental model for the detection of the related LOD and confirmed by the 25 chromosomally abnormal pregnancies involving a 2% < FF < 4%, which were consistently detected. These high-risk pregnancies would have not been identified if the 4% FF cut-off had been used.

The results obtained indicate that testing samples with a low amount (<4%) of fetal cfDNA is well within the ability of this technology and does not affect the accuracy of cfDNA-based aneuploidy screening assay. Specifically, no false negative results occurred in samples with low fetal cfDNA fractions nor was the sensitivity and specificity of cfDNA testing to detect fetal aneuploidy influenced.
A major advantage of using an FF cut-off adjusted to the LOD of the specific NIPT approach is related to the potential of decreasing the incidence of test cancelations and, consequently, lowering the redraw rate.

A potential issue with NIPT as a universal method of screening for aneuploidies is the possibility of obtaining a test failure, which is primarily related to the FF present in maternal plasma. This is relevant to comparisons with conventional screening, which rarely reports failure to obtain a result.

Overall, the chance of test failures ranges from approximately 1% to 8% and varies, in part, by the sequencing depth and whether the laboratory measures FF and requires a minimum concentration.2,11,13,23,24,26,27,29,30

In this study, 8.5% of the samples involving an FF < 4% would have been reported as failed tests if using the suggested minimum threshold currently employed for a reportable result, compared with 2.2% of test failures that would have occurred if using the 2% cut-off related to the actual LOD of the approach. Although repeat sampling can be performed after a failed test result because of insufficient fetal fraction on the first draw, this adds additional time to the screening process and may delay the diagnosis of aneuploidy, thereby potentially limiting reproductive options, especially because only about 50% to 60% of repeat screens have been reported to provide a result.3,15,23,24,31

An increased incidence of fetal aneuploidy was noticed in samples that failed to provide a reportable result from cfDNA testing because of low FF.23,30 Norton et al.30 recently reported aneuploidy in 4.7% of pregnancies that did not yield a cfDNA result due to FF < 4%, compared with 0.4% of those that did, indicating a tenfold increase in aneuploidy incidence. Similar data have also been reported by Pergament et al.,23 indicating that samples with low FF are four times more likely to be aneuploidy. In our study, a sixfold increased incidence of aneuploidy (6.9% vs 1.1%, p < 0.001) was observed in samples with 2% < FF < 4% versus samples with FF ≥ 4%. The aforementioned data confirm the finding that an FF < 4% is associated with a higher risk of aneuploidy and underscores the importance of testing samples with a very low amount of fetal cfDNA, using NIPT methods with a demonstrated accuracy at low FF.

These findings also raise the question of how pregnancies should be managed in such instances.3–5,32 Any decision should be considered with regard to gestational age at testing and the presence of other indications, such as abnormal ultrasound findings or maternal serum screening markers and patient preferences. Prior to redrawing, given the increased risk and considering that a repeat cfDNA screen will be successful in almost half of cases, the option of invasive testing could be

### Table 3 Performance of the massively parallel sequencing-based NIPT protocol

<table>
<thead>
<tr>
<th></th>
<th>Trisomy 21 (n=7082)</th>
<th>Trisomy 18 (n=7082)</th>
<th>Trisomy 13 (n=7082)</th>
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<tbody>
<tr>
<td>True positive – no</td>
<td>76</td>
<td>20</td>
<td>9</td>
</tr>
<tr>
<td>False positive – no</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>True negative – no</td>
<td>7005</td>
<td>7061</td>
<td>7073</td>
</tr>
<tr>
<td>False negative – no</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sensitivity (95% CI [%])</td>
<td>100.00 (95.26–100.00)</td>
<td>100.00 (83.16–100.00)</td>
<td>100.00 (66.37–100.00)</td>
</tr>
<tr>
<td>Specificity (95% CI [%])</td>
<td>99.99 (99.92–100.00)</td>
<td>99.99 (99.92–100.00)</td>
<td>100.00 (99.95–100.00)</td>
</tr>
<tr>
<td>Positive predictive value (95% CI [%])</td>
<td>98.70 (92.98–99.97)</td>
<td>95.24 (76.18–99.88)</td>
<td>100.00 (66.37–100.00)</td>
</tr>
<tr>
<td>Negative predictive value (95% CI [%])</td>
<td>100.00 (99.95–100.00)</td>
<td>100.00 (99.95–100.00)</td>
<td>100.00 (99.95–100.00)</td>
</tr>
</tbody>
</table>

CI, confidence interval; NIPT, noninvasive prenatal testing.
offered to women with no results on cfDNA testing. Another option would be the use of an NIPT method with a low LOD and/or to repeat the test at greater depth of sequencing.17

It is worth noting that the performance reported for most NIPT approaches is based on the requirement for a minimal amount of fetal cfDNA to be present for testing. The finding of an increased incidence of fetal aneuploidy in samples with FF < 4% strongly suggests that ‘no result’ cases should be taken into account when calculating test performance. If not detected aneuploidies from pregnancies that had no results on cfDNA testing were included, it would lower the detection rate of cfDNA testing, thereby providing more truthful test performance calculations.30

## CONCLUSION

In conclusion, this study underscores the importance of determining the actual LOD for each specific NIPT methodology, rather than using a theoretically determined fixed FF cut-off for all cfDNA testing methodologies. It may reduce the incidence of test cancelations and shorten the time required for the diagnosis of aneuploidy by invasive prenatal testing.

### WHAT’S ALREADY KNOWN ABOUT THIS TOPIC?

- Several non-invasive prenatal testing (NIPT) methods that rely on quantification of fetal cell-free DNA (cfDNA) suggest a fetal fraction (FF) of 4% or greater for a reportable result.
- The 4% FF cutoff was theoretically determined, with the assumption that fetal aneuploidies may not be detectable at lower FF.

### WHAT DOES THIS STUDY ADD?

- This study demonstrates that the minimum FF level necessary for accurate aneuploidy assessment should be related to the actual limit of detection (LOD) of each specific NIPT approach used and not necessarily fixed at 4% for all cfDNA testing methodologies.
- The determination of the LOD is advisable for any NIPT method, so that the appropriate FF cutoff value can be used.

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**Figure 5** Fetal fraction (FF) distribution in clinical samples with aneuploidy. Graphical distribution of male and female aneuploid fetuses according to the FF%, calculated using the aneuploid chromosome ratios. (A) All aneuploidy samples and (B) samples with a 2% < FF < 4%. Diamonds (●) display samples resulted T13, squares (●) display samples rated T18, and triangles (●) display samples rated T21. All the cases were confirmed by metaphase karyotyping following invasive prenatal diagnosis. The two samples with discordant results (red symbol), T21 and T18, had an FF of 2.39% and 3.24%, respectively.

**Table 4** Performance of cfDNA testing according to the FF

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>FF &lt; 2%</th>
<th>2% &lt; FF &lt; 4%</th>
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<tr>
<td>No. of patients analyzed</td>
<td>3628</td>
<td>79</td>
<td>231</td>
<td>3318</td>
</tr>
<tr>
<td>Gestational age – weekb</td>
<td>12.9 ± 2.4</td>
<td>12.8 ± 2.8</td>
<td>12.4 ± 2.2</td>
<td>13.0 ± 4.0</td>
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<td>Samples with a call – no.</td>
<td>3628</td>
<td>79</td>
<td>231</td>
<td>3318</td>
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<tr>
<td>No. of aneuploid samples (%)</td>
<td>52 (1.4)</td>
<td>0</td>
<td>16 (6.9)</td>
<td>36 (1.1)</td>
</tr>
<tr>
<td>T21</td>
<td>41</td>
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<td>12</td>
<td>29</td>
</tr>
<tr>
<td>T18</td>
<td>7</td>
<td>0</td>
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<tr>
<td>T13</td>
<td>4</td>
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cfDNA, cell-free DNA; FF, fetal fraction.

*Male samples.

*Mean ± standard deviation.
REFERENCES


SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher’s web site.
THE IMPORTANCE OF DETERMINING THE LIMIT OF DETECTION OF NON INVASIVE PRENATAL TESTING METHODS

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Supplementary Material
Sequencing and classification of the results

Sequencing libraries were prepared using TruSeq nano Kit (Illumina) according to the manufacturer’s recommendations. Samples were indexed during library preparation and 15 samples were pooled for multiplex sequencing using a HiSeq2500 (Illumina), in rapid run mode with 36 bp single-end reads. An average of 16 millions ± 1.6 millions of reads (range 8.5x10^6 - 34x10^6), unambiguously mapping to a single genomic location, were counted as tags and compared to values obtained from a reference euploid human genome.

The raw output from each run was analyzed by SAFeR™, an Illumina proprietary algorithm. After demultiplexing of the samples, the algorithm performs the alignment of the reads to the human genome (assembly hg19, obtained from the UCSC database) using BowTie short read aligner, defining a statistical measure named “Normalized Chromosome Value” (NCV) for the chromosomes 13, 18, 21, X and Y. The use of NCV minimizes the intra- and inter-run sequencing variation during aneuploidy classification.

NCV is based on the ratio of normalized coverage on test and reference chromosomes and represents the standard deviations difference from average diploid Ratio.

For each sample (k) and chromosome (i) of interest, NCV_{ki} was calculated with the equation 1:

\[
NCV_{ki} = \frac{R_{ki} - \bar{R}_{ui}}{\sigma_{ui}}
\]

Where \(\bar{R}_{ui}\) is the SD obtained from a set of samples with only euploid (unaffected, \(u\)) chromosomes. The mean ratio (\(R_{ui}\)) for euploid chromosome values were calculated from same euploid samples. The ratio (\(R_{ki}\)) values for chromosome 21, 18, 13, X and Y were individually determined for each sample.

Classification of NIPT results were based on the dual-threshold classification model defined elsewhere1-4. For autosomal aneuploidy calls, samples with a NCV of 4.0 or greater for chromosome 21, 18, or 13,
were classified as “affected” (i.e. aneuploidy detected for that chromosome); samples with a NCV less than 3.0 were classified as “unaffected” (i.e. aneuploidy not detected for that chromosome). All the samples with a NCV between 3 and 4 were called “unclassified” (i.e. aneuploidy suspected for that chromosome)\(^2,3\). For classification of the fetal sex, a sequential application of NCV for both X and Y chromosomes was used. Specifically, a fetus was classified as follow \(^2\):

- Female if, for chromosome X: -2.5 < NCV < 2.5 and, for chromosome Y, NCV value was < 2.5.
- Male if, for chromosome X, NCV was < -4 and, for chromosome Y, NCV was > 33

If the chromosome X and Y normalized chromosome values did not fit into any of the above criteria, the sample was scored as unclassified for sex.

**Quality control criteria**

Samples that failed quality control (QC) metrics and did not return a result (no-call) were scored as “Quality Metrics Failure”. These samples did not pass one or more of the following QC parameters:

1) Presence of cell-free DNA (average size of 160 bp), as determined by electrophoresis of the DNA extracted from plasma samples using Tape Station (Agilent);

2) Minimum library concentration required, as measured with Qubit high sensitivity assay (Thermo-Fisher): 10nM;

3) Minimum number of unique sequence sites (i.e., sequence tags identified with unique sites in the genome) required: 8,000,000 (8M).

**Fetal Fraction measurement**

FF measurement was performed using the MPS tag-count data with the X chromosome ratios, in samples from pregnancies carrying male fetuses, and/or with aneuploid chromosome ratios, in samples from
women carrying fetuses with a chromosome aneuploidy, as previously reported. These approaches allow determination of the fetal fraction using MPS tag counting data:

- from the aneuploid chromosome in aneuploidy pregnancies, using the following equation:

\[
FF = 2x \left| \frac{R_{Ai}}{R_{ui}} - 1 \right|
\]

where \( R_{Ai} \) is the ratio for an affected (noneuploid) chromosome \( i \) and \( R_{ui} \) is the ratio for unaffected (euploid) chromosome \( i \).

- from X chromosome in male samples, using the following equation:

\[
FF = 2x \left| 1 - \frac{R_{Ax}}{R_{ux}} \right|
\]

where \( R_{Ax} \) is the ratio for the X chromosome in a male sample (one copy of X chromosome) and \( R_{ux} \) is the ratio for the X chromosome in female samples (2 copy of X chromosome).

The above method cannot be used as a FF for pregnancies carrying female euploid fetuses, unless slightly deeper sequencing (i.e. more tags per sample) is applied to identify genome regions that have clinically benign copy number differences between the mother and fetus.

**Artificial mixture preparation**

To determine the limit of detection of the MPS-based NIPT approach, we designed an experimental model closer to the real clinical practice. It involved the creation of artificial mixtures at different proportions of plasma samples deriving from aneuploid male fetuses with known FF% and samples form a non-pregnant women, to create artificial plasma mixtures with a final aneuploid FF ranging from 1% to 4% (Supplementary Table I). Aneuploid plasma samples were obtained from pregnant women undergoing invasive prenatal diagnosis, with fetal karyotype determined by metaphase cytogenetic analysis.
In total, 156 samples were used for the reconstruction experiments. Fifty-two plasma samples with aneuploid cell-free fetal DNA with a known FF % were paired with 52 samples from non-pregnant women. Reconstructed aneuploid samples were: 56 with Trisomy T21, 24 with T18, and 24 with T13. Plasma samples from 26 non pregnant women (0% aneuploid FF) and 26 aneuploid male fetuses were also included.

cfDNA was then extracted from the reconstructed plasma samples, processed for library preparation and sequenced as described in Materials and Methods section.

REFERENCES

Chr 13 euploid
T13
Chr 18 euploid
T18
Chr 21 Euploid
T21
Chr 13 euploid
T13
Chr 18 Euploid
T18
Chr 21 Euploid
T21
Supplementary Figure 1. NCVs distribution in the training set.
Graphical distribution of 2000 samples included in the training set, according to NCV for chromosomes 13, 18 and 21. (A) Total data set. (B) Samples with 2<NCV<4. Diamonds display samples rated euploid (Ê) or aneuploid (f) for chromosome (Chr) 13, squares display samples rated euploid (¡) or aneuploid ( ) for Chr 18, and triangles display samples rated euploid (”) or aneuploid (²) for Chr 21. All aneuploid samples displayed a NCV >3. Four euploid samples were with had 3<NCV<4 (grey zone). The 2 samples with T13 karyotypes have a NCV of 7.7 and 24.7. The 4 samples with T18 karyotype have a NCV ranging from 6.5 to 11.8. The 25 samples with T21 karyotype have a NCV ranging from 3.18 to 33.
A

\[ y = 0.8557x + 0.0055 \]

\[ R^2 = 0.941 \]

B

\[ y = 0.9916x + 0.0035 \]

\[ R^2 = 0.9609 \]
Supplementary Figure 2. Comparison of Fetal Fraction determination using both the X chromosome and the aneuploid chromosome ratios in reconstructed samples.

FF for trisomy 21 samples (A), trisomy 18 samples (B), and trisomy 13 samples (C). The y-axis shows the %FF obtained using aneuploid chromosome ratio, and x-axis shows the %FF obtained using X chromosome ratio.
$y = 0.8864x + 0.0058$

$R^2 = 0.9014$
Supplementary Figure 3. Comparison of Fetal Fraction determination using both the X chromosome and the aneuploid chromosome ratios in clinical samples.

Fetal fraction for trisomy 21 samples (A), trisomy 18 samples (B), and trisomy 13 samples (C). The y-axis shows the %FF obtained with the X chromosome ratios and x-axis shows the %FF obtained with aneuploid chromosome ratios.

\[ y = 0.841x + 0.0167 \]
\[ R^2 = 0.9034 \]

\[ y = 0.9525x + 0.0085 \]
\[ R^2 = 0.9464 \]
Supplementary Figure 4. Fetal Fraction distribution in male samples

A) Histogram of the relative frequency of euploid and aneuploid samples according to the FF %. (B) Incidence of aneuploidy in male samples according to the different FF %. *** p<0.001. Statistical significant increased incidence of aneuploidy in samples with a 2%<FF<4% vs. samples with FF>4%.

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**Supplementary Table I.** Reconstructed samples analyzed for LOD determination

<table>
<thead>
<tr>
<th>FF</th>
<th>T 13</th>
<th>T 18</th>
<th>T 21</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of samples</td>
<td>No. of samples</td>
<td>No. of samples</td>
</tr>
<tr>
<td>0%</td>
<td>6</td>
<td>6</td>
<td>14</td>
</tr>
<tr>
<td>1%</td>
<td>6</td>
<td>6</td>
<td>14</td>
</tr>
<tr>
<td>2%</td>
<td>6</td>
<td>6</td>
<td>14</td>
</tr>
<tr>
<td>3%</td>
<td>6</td>
<td>6</td>
<td>14</td>
</tr>
<tr>
<td>4%</td>
<td>6</td>
<td>6</td>
<td>14</td>
</tr>
<tr>
<td>5-13%</td>
<td>6</td>
<td>6</td>
<td>14</td>
</tr>
<tr>
<td>Total</td>
<td>36</td>
<td>36</td>
<td>84</td>
</tr>
<tr>
<td>Characteristics</td>
<td>Training set</td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------------------------------------------------</td>
<td>--------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>No. of eligible patients</strong></td>
<td>2006</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Maternal age-yr</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>- Mean ±SD</td>
<td>36.9±4.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Min-max</td>
<td>26-45</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Gestational age at sample collection -wk</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Mean ±SD</td>
<td>12.9±2.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Min-max</td>
<td>11-22</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>No. of patients with a conclusive results</strong></td>
<td>2000</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>No. of sample with test failure (%)</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6 (0.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>No. of aneuploid samples (%)</strong></td>
<td>31 (1.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Trisomy 21</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Trisomy 18</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Trisomy 13</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Indication for NIPT</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Parental Anxiety</td>
<td>230</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Advanced maternal age only&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1000</td>
<td></td>
<td></td>
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<tr>
<td>- Positive prenatal screen</td>
<td>110</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Fetal ultrasound abnormality</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Prior pregnancy with fetal aneuploidy</td>
<td>30</td>
<td></td>
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<tr>
<td>- More than one indication</td>
<td>610</td>
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</tr>
</tbody>
</table>

<sup>a</sup> Quality metrics failure;

<sup>b</sup> age >35y
Supplementary Table III. Fetal Fraction from maternal plasma samples in pregnant women carrying fetuses with different karyotypes.

<table>
<thead>
<tr>
<th>Fetal karyotype</th>
<th>No. of samples (All)</th>
<th>FF from aneuploid chromosome ratioa</th>
<th>No. of samples (males)</th>
<th>FF from X chromosome ratioa</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Euploid Males</td>
<td>3576</td>
<td>NA</td>
<td>3576</td>
<td>0.093 (0.04)</td>
<td>NA</td>
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<tr>
<td>Trisomy 21</td>
<td>77</td>
<td>0.088 (0.05)</td>
<td>41</td>
<td>0.099 (0.05)</td>
<td>0.518</td>
</tr>
<tr>
<td>Trisomy 18</td>
<td>21</td>
<td>0.05 (0.03)</td>
<td>7</td>
<td>0.04 (0.03)</td>
<td>0.137</td>
</tr>
<tr>
<td>Trisomy 13</td>
<td>9</td>
<td>0.038 (0.03)</td>
<td>4</td>
<td>0.064 (0.04)</td>
<td>0.146</td>
</tr>
</tbody>
</table>

a FF data are presented as the mean (SD).

NA: not applicable;