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Accuracy and reproducibility of fetal-fraction measurement using relative quantitation at polymorphic loci with microarray

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ABSTRACT

Objectives *Various methods of fetal-fraction measurement have been employed in conjunction with different approaches to cell-free DNA testing for fetal aneuploidy. In this study, we determined the accuracy and reproducibility of fetal-fraction measurement using polymorphic assays that are incorporated into the test design as part of the Harmony® prenatal test and evaluated whether the single nucleotide polymorphisms selected for and used in these assays can be applied broadly to all patient populations.*

Methods *Clinical maternal plasma samples were assayed using a custom microarray with Digital ANalysis of Selected Regions (DANSR) assays designed to cover non-polymorphic targets on chromosomes of interest for aneuploidy assessment (13, 18, 21, X and Y) and polymorphic targets for fetal-fraction assessment. In a consecutive series of 47 512 maternal plasma samples, fetal-fraction measurements based on polymorphic assays were compared with those from Y-sequence quantitation. Reproducibility was examined between first- and second-tube measurements for the same patient sample in 734 cases. The fraction of informative loci was calculated for 13 988 samples.*

Results *There was a strong correlation between fetal fractions determined using the polymorphic assays and using Y-chromosome sequence quantitation ($r = 0.97$). Fetal-fraction measurement between the first and second tubes was highly reproducible ($r = 0.98$). The fraction of informative loci observed in a clinical series was consistent with predictions based on assay design.*

Conclusions *The method based on relative quantitation at polymorphic loci on a microarray is accurate and reproducible for fetal-fraction estimation and is equally informative across global populations. This study provides a useful benchmark for ensuring the reliability and accuracy of fetal-fraction measurement.* © *2018 Roche Sequencing Solutions. Ultrasound in Obstetrics & Gynecology published by John Wiley & Sons Ltd on behalf of the International Society of Ultrasound in Obstetrics and Gynecology.*

INTRODUCTION

The development of methods for the analysis of circulating cell-free DNA (cfDNA) has dramatically changed prenatal screening for fetal trisomy and opened up new possibilities for other types of non-invasive genetic testing. cfDNA originates predominantly from hematopoietic cell lines[1]; however, during pregnancy, a minor proportion derives from the conceptus[2,3]. The proportion of pregnancy-derived cfDNA in maternal plasma, commonly termed fetal fraction, averages 10%, but is subject to significant variation[4,5]. For any kind of fetal cfDNA-based testing, it is reasonable to assume that a minimum amount of fetal cfDNA must be present. This is supported by theoretical models demonstrating that the ability to distinguish between fetal trisomy and disomy suffers with decreasing fetal fraction[6,7]. As a result, measurement of fetal fraction is now recognized widely as an essential quality metric[8–10].

Many laboratories measure fetal fraction, but different methodologies have necessitated different approaches. Quantitation of Y-chromosome sequences was the first method employed because this DNA can be taken as a direct representation of fetal cfDNA, but is informative only in pregnancies with a male fetus[4,11]. Analysis of single-nucleotide polymorphisms (SNPs) is more powerful because it can be applied regardless of fetal sex[12]. It is well suited to methodologies that target specific sequences

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but is difficult to apply to massively parallel shotgun sequencing approaches, as these tend to have a very shallow depth at any given polymorphic locus. Massively parallel shotgun sequencing-based methodologies have therefore relied on less direct assessment based on observed differences between fetal and maternal cfDNA fragments[13–15]. There is, however, concern about the consistency of the different approaches[16].

We have previously described the application of Digital ANalysis of Selected Regions (DANSR) to fetal trisomy screening[17,18]. This includes non-polymorphic assays for the determination of chromosome proportions and polymorphic assays leveraging SNPs for fetal-fraction determination in a single reaction[12]. The current study aimed to evaluate rigorously this method for fetal-fraction estimation using a number of parameters. First, accuracy was assessed by comparing DANSR assays with quantitation of Y-chromosome sequences. Second, reproducibility was determined in a series of paired tubes drawn from the same patient. Finally, we evaluated whether this method is equally informative across populations.

METHODS

Dataset

The data analyzed in this study were sourced from existing information generated as part of clinical testing of venous blood samples submitted for the Harmony® prenatal test to the College of American Pathologists-accredited and Clinical Laboratory Improvement Amendments-certified Ariosa Diagnostics laboratory in San Jose, CA, USA. Patients provided consent for the performed testing and samples were processed in accordance with all applicable laws. All data were anonymized prior to analysis. Analysis was limited to samples from singleton pregnancies of at least 10 weeks' gestation, as reported by ordering providers on the sample submission form. Samples with insufficient fetal fraction for analysis (less than 4%, as measured using DANSR assays) and samples that did not meet laboratory quality-control thresholds were excluded. Samples were included irrespective of the probability score generated for fetal aneuploidy.

Sample collection and test method

Sample preparation and analysis for the Harmony prenatal test were performed as described previously[12,18]. In brief, blood samples were collected in either cfDNA BCT tubes (Streck, Omaha, NE, USA) or cfD tubes (Roche, Pleasanton, CA, USA) and processed to yield cell-free plasma within 7 days of collection. cfDNA was purified from this plasma and DANSR products were made using non-polymorphic assays of chromosomes 13, 18, 21, X and Y and polymorphic assays of chromosomes 1–12. DANSR products were hybridized and signals measured using a custom microarray.

Fetal-fraction measurement

Fetal-fraction measurement was performed, as described previously, using 576 DANSR assays designed against polymorphic loci on chromosomes 1–12 (Figure 1)[12,18]. The assays are unbiased, cover each chromosome uniformly and target SNPs with high minor-allele frequencies in the HapMap dataset[19] to maximize the number of informative loci for fetal-fraction measurement.

Relative allele frequencies were used to compute fetal fraction with the minor percentage of the fraction assumed to be fetal. No prior genotyping of the mother, father or fetus was carried out.

The relative signal intensities of DANSR assays corresponding to non-polymorphic loci were evaluated to estimate the relative amount of each of the interrogated chromosomes. The relative signal intensities for the Y chromosome were taken as a direct representation of fetal fraction in pregnancies with a putative male fetus.

DANSR single-nucleotide polymorphism assays *vs* Y-chromosome quantitation

Fetal fraction was determined by both methods in a consecutive series of 47 512 plasma samples from singleton pregnancies with a putative male fetus. Pearson's correlation coefficient (r) was calculated to determine the correlation between methods of fetal-fraction measurement.

Fetal-fraction reproducibility

DANSR SNP assays were used to measure fetal fractionEnter your text in two tubes from 734 randomly chosen, de-identified, frozen plasma samples. Patients provided consent to have their sample de-identified and used for additional study. Over a period spanning 3 months, the first and second tubes were processed separately from one another but together with other clinical samples. Second-tube fetal-fraction information was compared with anonymized first-tube data. Pearson's correlation (r) and the median absolute deviation from the mean were calculated.

Calculation of fraction of informative loci

DANSR assays are designed to be equally informative across global populations. Figure 1 shows mean minor-allele frequencies and the predicted fraction of informative loci for different populations based on data from the 1000 Genomes Project[20].

To assess experimentally whether DANSR assays are informative for different populations, the number of informative loci was determined in 13 988 clinical samples consisting of the reproducibility dataset and other samples analyzed in the same time frame. The fraction of informative loci is calculated as the proportion of determined maternal homozygous loci that are determined to be heterozygous in the fetus.

RESULTS

The correlation between fetal fractions measured using DANSR SNP assays and relative Y-chromosome quantitation for 47 512 samples in which Y-chromosome

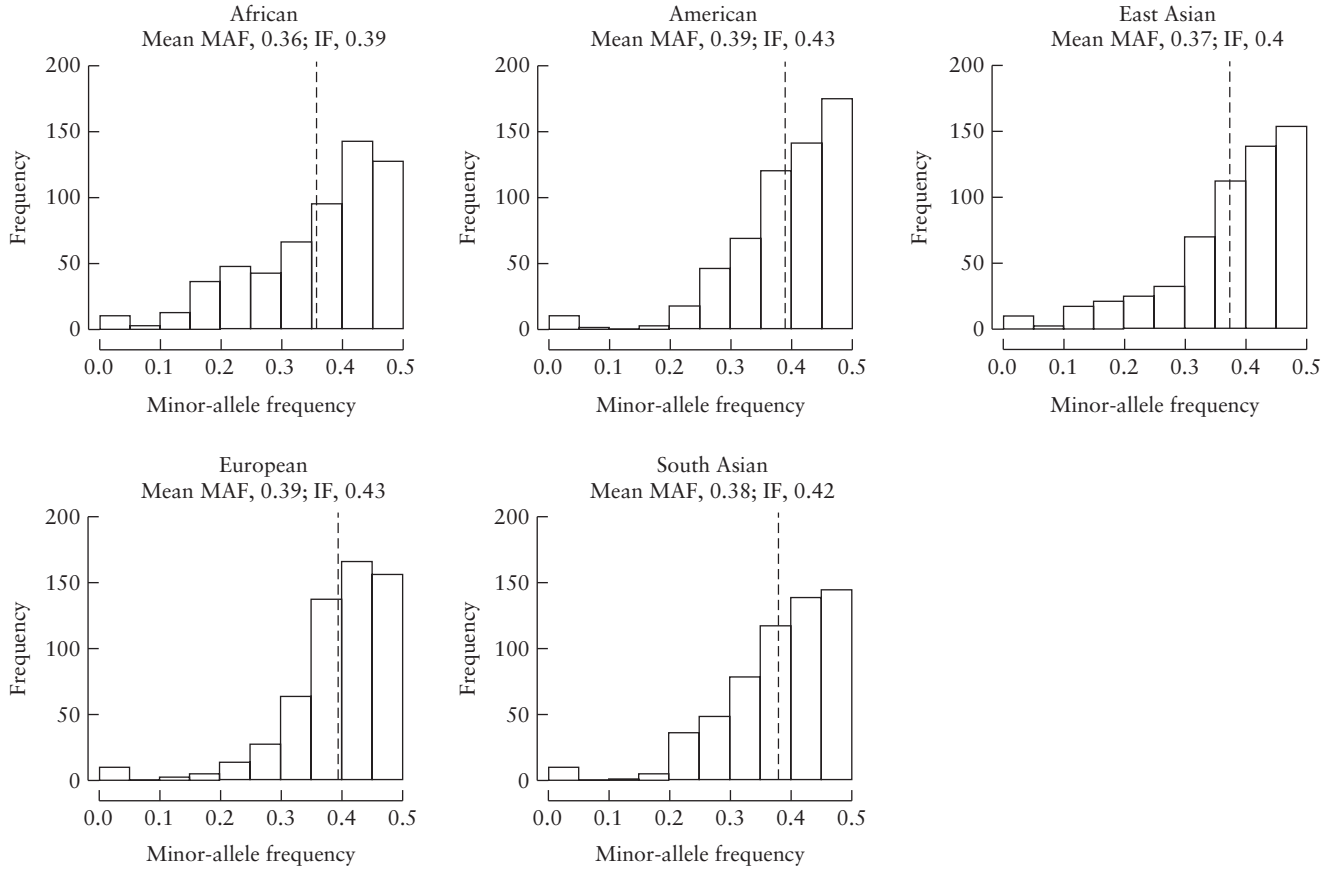

Figure 1 Distribution of minor-allele frequencies (MAFs) of Harmony® prenatal test polymorphic assays across five major populations in the 1000 Genomes Project, with mean MAF (– – –) and expected informative fraction (IF) provided for each population.

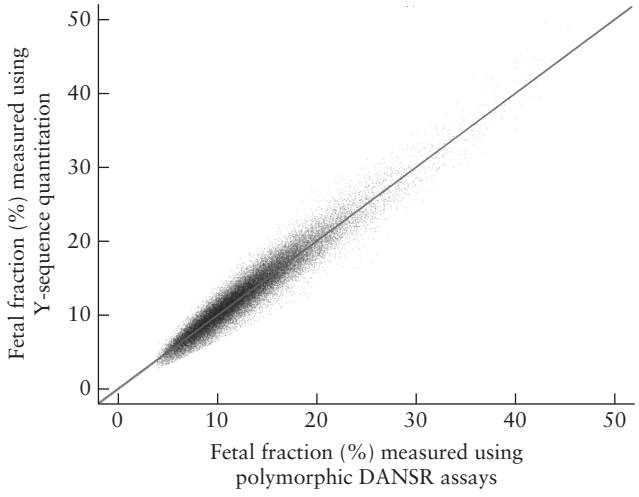

Figure 2 Correlation between fetal fraction determined using polymorphic Digital ANalysis of Selected Regions (DANSR) assays and that determined using Y-sequence quantitation ($r = 0.97$) in 50 000 samples from women with singleton pregnancy and putative male fetus. Identity and best-fit lines (——) are superimposed.

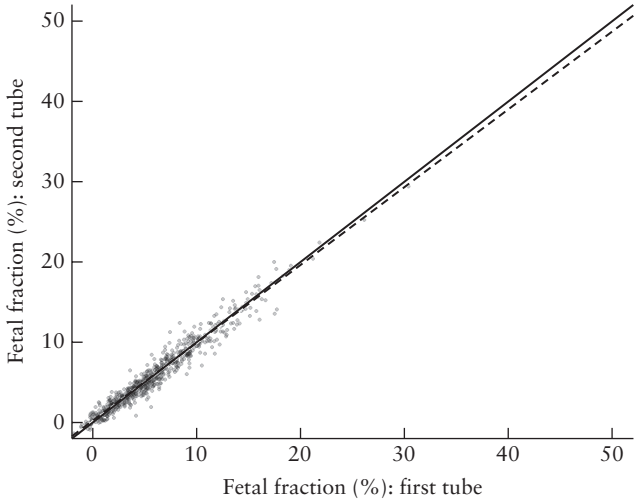

Figure 3 Correlation between first- and second-tube fetal-fraction measurements ($r = 0.98$) in 734 patient samples from women with singleton pregnancy. Identity (——) and best-fit (– – –) lines are shown.

sequences were present is shown in Figure 2. There was a strong correlation between the two methods ($r = 0.97$).

Figure 3 depicts the reproducibility of fetal-fraction measurement for 734 patient samples in two tubes drawn at the same time. The correlation (r) between measurements was 0.98, with a median absolute deviation from the mean of 0.26%.

The samples received by the Ariosa Diagnostics laboratory originate from more than 100 countries and territories across five continents and are therefore representative of a range of global racial diversity. In

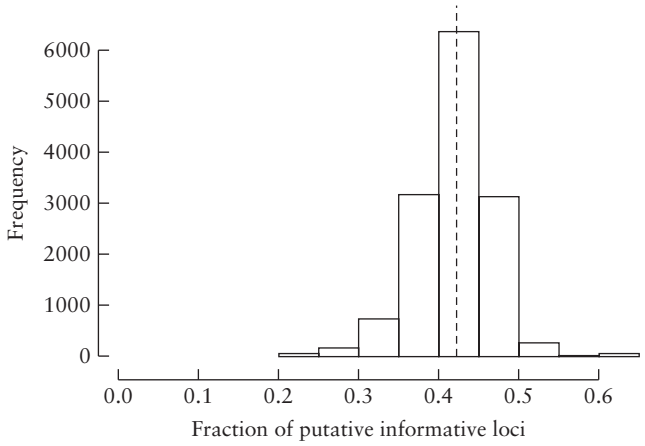

Figure 4 Distribution of observed fraction of putative informative loci in 13 988 samples from women with singleton pregnancy, showing mean informative fraction (0.42 ± 0.041, $---$), which is consistent with underlying mean minor-allele frequency in Digital ANalysis of Selected Regions (DANSR) polymorphic assays across worldwide populations (0.38).

13 988 clinical samples, the median number of observed fractions of informative loci associated with homozygous maternal genotype was 0.42 (SD, 0.041) (Figure 4), which is consistent with a mean worldwide minor-allele frequency of 38%.

DISCUSSION

Despite the increasing recognition of fetal fraction as an important quality metric in cfDNA testing for fetal trisomy, there has been little focus on the variety of methods employed and the need to ensure their reliability. Underestimating fetal fraction may cause valid samples to be rejected unnecessarily. Overestimating fetal fraction may lead to samples with insufficient fetal cfDNA being tested and a risk of false-negative results[6,7]. This may not be easily discernible in clinical studies designed solely to evaluate test performance characteristics, such as sensitivity and specificity, due to the number of affected pregnancies studied at lower fetal fractions, but can be addressed by direct investigation.

Fetal-fraction measurement using relative competitive quantitation at polymorphic loci with microarray allows for a single laboratory workflow to perform simultaneously fetal-fraction measurement and sequence quantitation for trisomy assessment in the same assay under the same conditions. The current study represents a large-scale assessment of polymorphic assays, as used to measure fetal fraction as part of the Harmony prenatal test. The accuracy of fetal-fraction measurement was demonstrated by comparison with measuring Y-sequence signal intensities in a cohort of over 47 000 clinical samples. The fetal-fraction calculations are also consistent with little variation between paired samples of two tubes drawn from the same patient at the same time. The correlation of fetal-fraction measurement between the two tubes was high, with an average deviation of $\pm 0.26\%$. In practical terms, fetal-fraction measurements in samples with a theoretical absolute fetal fraction of 7% would be expected to vary on average by between only 6.74% and 7.26%. Finally, the SNPs chosen are informative across global populations, an important requirement given the rapid uptake of cfDNA screening worldwide.

Until now, most published studies addressing fetal-fraction estimation have been limited to proof-of-concept studies[13–15,21,22]. Many of these have centered on the generation of models to correlate fetal fraction with the observed differences between maternal and fetal cfDNA, such as differential methylation at specific regions[13], distribution of cfDNA fragment size[14], patterns of DNA sequence representation[15], nucleosome positioning[21] and preferred sequence end sites[22]. The differences are likely to be the consequence of differences in chromatin structure and transcriptional processes in maternal leukocytes compared with placental trophoblasts; however, the underlying biology is yet to be elucidated fully[22]. Although some are being used clinically[15], these models are complex, with tens of thousands of historical parameters to scale the sequencing count from each chromosome bin and there is considerable overlap between maternal and fetal cfDNA in the studied attributes. Furthermore, the stability of these differences may be a potential concern for use in fetal-fraction estimation. The possible influence of factors, such as gestational age and maternal elements, has not to our knowledge been evaluated comprehensively in larger series. In one recently published study, van Beek *et al.*[23] directly compared six methods in a series of 375 pregnancies with a male fetus and found that methods based on binned autosomal read counts[15] and nucleosome positioning[21] did not perform reliably as compared with Y-based methods[23].

A challenge for the evaluation of any assay that estimates fetal fraction is the establishment of a reference against which to compare the calculated proportions. Quantitation via Y-chromosome sequences is therefore considered to be a gold standard with the least potential for confounding factors and was applied in the current study to a large cohort of real pregnancy samples with male fetuses that were the same processed samples and data sources as those used for the assessment of trisomy. This study was also constrained by the use of a clinical laboratory platform that limits the reporting of fetal fraction to samples meeting a minimum requirement of 4% fetal fraction. We were not able to determine the lowest reasonable threshold for fetal-fraction measurement.

Just as different methods for trisomy screening are well validated before clinical implementation, so should be assay designs for fetal-fraction measurement. This study provides a useful benchmark for ensuring reliability. Evaluation would include demonstration of both accuracy and reproducibility. Moreover, ensuring that the used method performs well across all pregnancies is warranted.

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REFERENCES

1. Snyder MW, Kircher M, Hill AJ, Daza RM, Shendure J. Cell-free DNA comprises an in vivo nucleosome footprint that informs its tissues-of-origin. *Cell* 2016; **164**: 57–68.
2. Lui YYN, Chik KW, Chiu RW, Ho CY, Lam CW, Lo YM. Predominant hematopoietic origin of cell-free DNA in plasma and serum after sex-mismatched bone marrow transplantation. *Clin Chem* 2002; **48**: 421–427.
3. Alberry M, Maddocks D, Jones M, Abdel Hadi M, Abdel-Fattah S, Avent N, Soothill PW. Free fetal DNA in maternal plasma in anembryonic pregnancies: confirmation that the origin is the trophoblast. *Prenat Diagn* 2007; **27**: 415–418.
4. 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* 2008; **54**: 1664–1672.
5. Wang E, Batey A, Struble C, Musci T, Song K, Oliphant A. Gestational age and maternal weight effects on fetal cell-free DNA in maternal plasma. *Prenat Diagn* 2013; **33**: 662–666.
6. 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* 2013; **33**: 667–674.
7. Benn P, Cuckle H. Theoretical performance of non-invasive prenatal testing for chromosome imbalances using counting of cell-free DNA fragments in maternal plasma. *Prenat Diagn* 2014; **34**: 778–783.
8. Benn P, Borrell A, Chiu RW, Cuckle H, Dugoff L, Faas B, Gross S, Huang T, Johnson J, Maymon R, Norton M, Odibo A, Schielen P, Spencer K, Wright D, Yaron Y. Position statement from the Chromosome Abnormality Screening Committee on behalf of the Board of the International Society for Prenatal Diagnosis. *Prenat Diagn* 2015; **35**: 725–734.
9. Committee on Genetics Society for Maternal–Fetal Medicine. Committee Opinion No. 640: Cell-free DNA screening for fetal aneuploidy. *Obstet Gynecol* 2015; **126**: e31–e37.
10. Gregg AR, Skotko BG, Benkendorf JL, Monaghan KG, Bajaj K, Best RG, Klugman S, Watson MS. Noninvasive prenatal screening for fetal aneuploidy, 2016 update: a position statement of the American College of Medical Genetics and Genomics. *Genet Med* 2016; **18**: 1056–1065.
11. Lo YM, Tein MS, Lau TK, Haines CJ, Leung TN, Poon PM, Wainscott JS, Johnson PJ, Chang AM, Hjelm NM. Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. *Am J Hum Genet* 1998; **62**: 768–775.
12. Sparks AB, Struble CA, Wang ET, Song K, Oliphant A. Noninvasive prenatal detection and selective analysis of cell-free DNA obtained from maternal blood: evaluation for trisomy 21 and trisomy 18. *Am J Obstet Gynecol* 2012; **206**: 319.e1–9.
13. Nygren AOH, Dean J, Jensen TJ, Kruse S, Kwong W, van den Boom D, Ehric M. Quantification of fetal DNA by use of methylation-based DNA discrimination. *Clin Chem* 2010; **56**: 1627–1635.
14. Yu SC, Chan KC, Zheng YW, Jiang P, Liao GJ, Sun H, Akolekar R, Leung TY, Go AT, van Vugt JM, Minekawa R, Oudejans CB, Nicolaides KH, Chiu RW, Lo YM. Size-based molecular diagnostics using plasma DNA for noninvasive prenatal testing. *Proc Natl Acad Sci USA* 2014; **111**: 4–10.
15. Kim SK, Hannum G, Geis J, Tynan J, Hogg G, Zhao C, Jensen TJ, Mazloom AR, Oeth P, Ehrich M, van den Boom D, Deciu C. Determination of fetal DNA fraction from the plasma of pregnant women using sequence read counts. *Prenat Diagn* 2015; **35**: 810–815.
16. Wataganara T, Bui TH, Choy KW, Leung TY. Debates on fetal fraction measurement and DNA-based noninvasive prenatal screening: time for standardisation? *BJOG* 2016; **123**: 31–35.
17. Sparks AB, Wang ET, Struble CA, Barrett W, Stokowski R, McBride C, Zahn J, Lee K, Shen N, Doshi J, Sun M, Garrison J, Sandler J, Hollemon D, Pattee P, Tomita-Mitchell A, Mitchell M, Stuelpnagel J, Song K, Oliphant A. Selective analysis of cell-free DNA in maternal blood for evaluation of fetal trisomy. *Prenat Diagn* 2012; **32**: 3–9.
18. Juneau K, Bogard PE, Huang S, Mohseni M, Wang ET, Ryvkin P, Kingsley C, Struble CA, Oliphant A, Zahn JM. Microarray-based cell-free DNA analysis improves noninvasive prenatal testing. *Fetal Diagn Ther* 2014; **36**: 282–286.
19. HapMap dataset. Archived at: ftp://ftp.ncbi.nlm.nih.gov/hapmap/
20. 1000 Genomes Project, http://www.internationalgenome.org/
21. Straver R, Oudejans CB, Sistermans EA, Reinders MJT. Calculating the fetal fraction for noninvasive prenatal testing based on genome-wide nucleosome profiles. *Prenat Diagn* 2016; **36**: 614–621.
22. Chan KC, Jiang P, Sun K, Cheng YK, Tong YK, Cheng SH, Wong AI, Hudecova I, Leung TY, Chiu RW, Lo YM. Second generation noninvasive fetal genome analysis reveals de novo mutations, single-base parental inheritance, and preferred DNA ends. *Proc Natl Acad Sci USA* 2016; **113**: E8159–E8168.
23. van Beek DM, Straver R, Weiss MM, Boon EMJ, Huijsdens-van Amsterdam K, Oudejans CBM, Reinders MJT, Sistermans EA. Comparing methods for fetal fraction determination and quality control of NIPT samples. *Prenat Diagn* 2017; **37**: 769–773.