Original Paper

Fetal Diagnosis and Therapy

Fetal Diagn Ther
DOI: 10.1159/000367626

Received: August 4, 2014
Accepted after revision: August 13, 2014
Published online: September 12, 2014

Microarray-Based Cell-Free DNA Analysis Improves Noninvasive Prenatal Testing

Kara Juneau Patrick E. Bogard Stephanie Huang Morassa Mohseni
Eric T. Wang Paul Ryvkin Christopher Kingsley Craig A. Struble
Arnold Oliphant Jacob M. Zahn

Ariosa Diagnostics Inc., San Jose, Calif., USA

Key Words
Aneuploidy · Down syndrome · Fetal diagnosis · First trimester · Noninvasive prenatal diagnosis · Prenatal diagnosis · Prenatal screening · Trisomy 13 · Trisomy 18 · Trisomy 21

Abstract
Objective: To develop a microarray-based method for noninvasive prenatal testing (NIPT) and compare it with next-generation sequencing. ***Methods:*** Maternal plasma from 878 pregnant women, including 187 trisomy cases (18 trisomy 13, 37 trisomy 18, 132 trisomy 21), was evaluated for trisomy risk. Targeted chromosomes were analyzed using Digital Analysis of Selected Regions (DANSR™) assays. DANSR products were subsequently divided between two DNA quantification methods: microarrays and next-generation sequencing. For both microarray and sequencing methodologies, the Fetal-Fraction Optimized Risk of Trisomy Evaluation (FORTE™) algorithm was used to determine trisomy risk, assay variability across samples, and compute fetal fraction variability within samples. ***Results:*** NIPT using microarrays provided faster and more accurate cell-free DNA (cfDNA) measurements than sequencing. The assay variability, a measure of variance of chromosomal cfDNA counts, was lower for microarrays than for sequencing, 0.051 versus 0.099 ($p < 0.0001$). Analysis time using microarrays was faster, 7.5 versus 56 h for sequencing. Additionally, fetal fraction precision was improved 1.6-fold by assaying more polymorphic sites with microarrays ($p < 0.0001$). Microarrays correctly classified all trisomy and nontrisomy cases. ***Conclusions:*** NIPT using microarrays delivers more accurate cfDNA analysis than next-generation sequencing and can be performed in less time.

Introduction

Noninvasive prenatal testing (NIPT) using cell-free DNA (cfDNA) obtained from maternal plasma has been shown to improve the accuracy of fetal aneuploidy risk assessment over conventional serum marker screening [1, 2]. The false-positive rate for trisomy 21 using the Harmony™ Prenatal Test is lower than 0.1%, while first trimester combined screening has a 5% false-positive rate [3].

The DANSR™ targeted approach was developed to reduce the amount of sequencing required for NIPT [4]. DANSR efficiently generates relevant data by focusing as-

KARGER

E-Mail karger@karger.com
www.karger.com/fdt

Dr. Kara Juneau, PhD
Ariosa Diagnostics Inc.
5945 Optical Court
San Jose, CA 95138 (USA)
E-Mail kjuneau@ariosadx.com

say resources on chromosomes of clinical relevance. Chromosomes 13, 18, and 21 together comprise only about 8% of the human genome [5]. Targeting hundreds of DANSR assays to each of these chromosomes provides deep cfDNA analysis at a lower cost. In contrast, random whole-genome sequencing of plasma cfDNA generates data indiscriminately from regions of the genome with little clinical utility for aneuploidy screening.

Currently, all commercially available NIPT utilizes next-generation sequencing. However, microarrays also provide efficient genetic analysis, and it is increasingly common for array CGH to be used to analyze amniotic samples [6] and preimplantation embryos [7–9]. Microarray analysis allows millions of genomic locations to be studied simultaneously, with a single patient sample being evaluated on each array [6]. The difficulty is that while array CGH commonly tests pure samples of fetal DNA, from amniotic fluid and chorionic villus sampling, cfDNA typically contains only 11% fetal DNA [10, 11]. By combining the targeted DNA analysis afforded by DANSR with the greater analysis space provided by arrays, we initiated the development of a robust microarray-based NIPT.

In this study, two DNA quantitation methods, next-generation DNA sequencing and DNA microarrays, were compared for their ability to accurately quantify DANSR products for NIPT.

Methods

Subjects

A total of 878 maternal venous blood samples were analyzed under an Institutional Review Board-approved protocol. Samples had the following trisomy classifications: 691 were disomic, 18 were trisomy 13, 37 were trisomy 18, and 132 were trisomy 21. Maternal blood samples were collected between 10 and 34 weeks' gestation from singleton pregnancies in women at least 18 years old (table 1). The trisomy classification had previously been determined for all samples tested; 486 samples were originally tested using the Harmony Prenatal Test from Ariosa Diagnostics Inc. (San Jose, Calif., USA), and 392 samples were obtained from patients who underwent invasive genetic testing or postnatal newborn examination followed by detailed genetic analysis, when trisomy was suspected.

Sample Preparation

Sample preparation and analysis are summarized in figure 1. Blood was collected in Cell-Free DNA BCT® tubes from Streck Inc. (Omaha, Nebr., USA). Plasma samples were stored at –20°C. As described previously [4, 12, 13], cfDNA was purified from each plasma sample, and DANSR products were made from 864 assays on each of chromosomes 13, 18, and 21. Greater analysis space was afforded by microarrays, which allowed the development of additional polymorphic assays to more accurately quantify fetal fraction. In this study, the total number of polymorphic assays was increased to 576, whereas 192 polymorphic assays were used in previous studies [4]. The DANSR product from each sample was divided; one portion was sequenced and the remaining portion was hybridized to a custom-manufactured DNA microarray.

Table 1. Demographic characteristics of the analysis population

Sample type	Cases	Maternal age, years	Gestational age, weeks
Euploid	691	31.6 (5.8)	14.2 (3.7)
Trisomy 13	18	34.8 (7.7)	17.4 (4.7)
Trisomy 18	37	37.5 (7.9)	18.7 (6.2)
Trisomy 21	132	35.2 (5.9)	16.8 (4.2)
Total	878	32.5 (6.1)	14.8 (4.2)

Data for maternal age and gestational weeks are reported as averages followed by the standard deviations in parentheses.

DANSR Assay Products Were Quantified Using Microarrays and Sequencing

Custom DNA microarrays from Affymetrix Inc. (Santa Clara, Calif., USA) with >100,000, 6-μm features were manufactured to specifically quantify products of the DANSR assays. Microarrays were imaged on an Affymetrix GeneTitan® Multi-Channel Instrument. Each patient sample was assayed on a single custom microarray. Microarrays were manufactured and processed in interconnected sets of 384. Next-generation sequencing data were produced on an Illumina HiSeq® 2500 (San Diego, Calif., USA). Clusters were generated on an Illumina Cluster Station using TruSeq™ Cluster Generation reagents. Assay space is limited for sequencing: when more assays are quantified, the number of sequence counts per assay declines. Because more assays were included in this study than in previous studies [4], sample multiplexing was reduced to ensure that sufficient sequencing counts were obtained for each assay. On average, 1,104 sequencing counts per assay were obtained for this analysis, which is typical for Harmony Prenatal Test sequencing.

Data Analysis

A previously published algorithm, Fetal-Fraction Optimized Risk of Trisomy Evaluation (FORTE™), was used to calculate risk scores [4, 14]. Samples with risk scores 1% or greater were classified as high risk and those with risk scores below 1% were classified as low risk. Nonpolymorphic DANSR assays on chromosomes 13, 18, and 21 were used to determine chromosome proportions and assign trisomy risks. Polymorphic DANSR assays were used to ascertain fetal fraction. Assay variability was defined as the coefficient of variation (CV) of sequence counts (sequencing) or intensities (microarrays) for a nonpolymorphic assay across samples. Fetal fraction variability was defined as the relative standard error of the measured fetal fraction. Lower assay and lower fetal fraction variability is preferred. All statistical analyses were performed using R version 3.03. The Wilcoxon paired test was used to compare CV and fetal fraction variability.

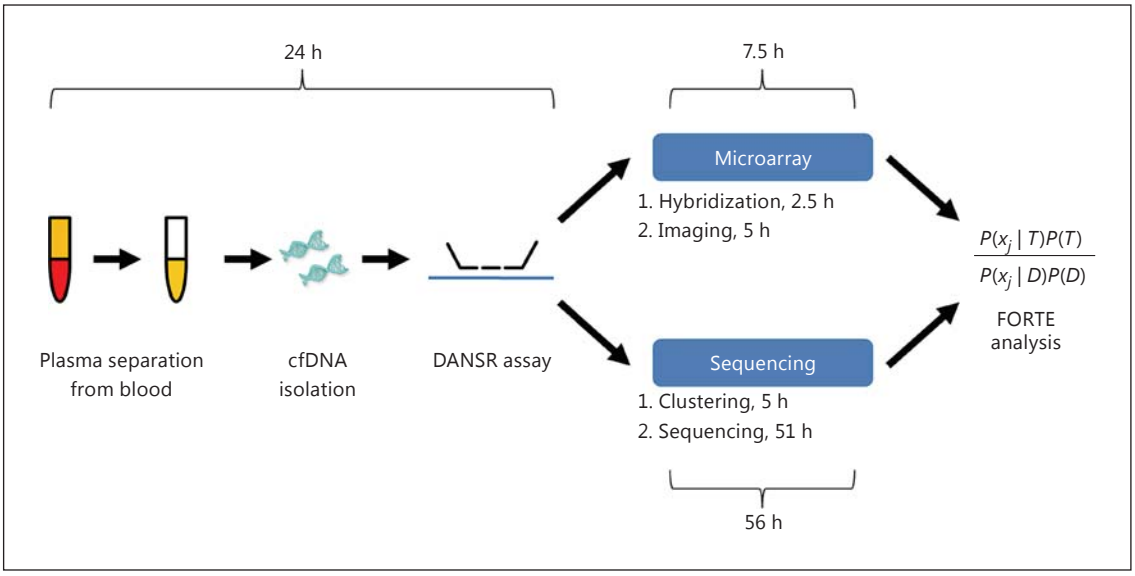


Fig. 1. Study design. DANSR products, produced from cfDNA, were divided between two DNA quantitation methodologies prior to FORTE risk assessment. Whole blood was centrifuged to separate plasma from cells. cfDNA was purified from plasma and used in the DANSR assay. Selected and amplified DANSR products were divided and quantified using either microarray or sequencing methodologies. All DANSR detection data were analyzed using the FORTE algorithm.

Results

Correlation between Microarray-Based Risk Scores and Trisomy Risk Classification

In this study, 878 plasma samples were assayed for trisomy risk using both microarrays and sequencing. The average maternal age of all samples was 32.5 years old with a standard deviation of 6.1 years. The average gestational age was 14.8 weeks with a standard deviation of 4.2 weeks. Additional data, specific for each trisomy and euploid class, are provided in table 1.

All of the samples had been previously classified for trisomy risk status (18 cases classified as trisomy 13, 37 as trisomy 18, 132 as trisomy 21, and 691 as euploid). There was complete concordance between microarray trisomy risk classification and previously determined trisomy risk classification (fig. 2). These data demonstrate that trisomy risk scores were accurately obtained from microarrays.

Microarray Data Decrease the Assay Variability by Approximately 2-Fold

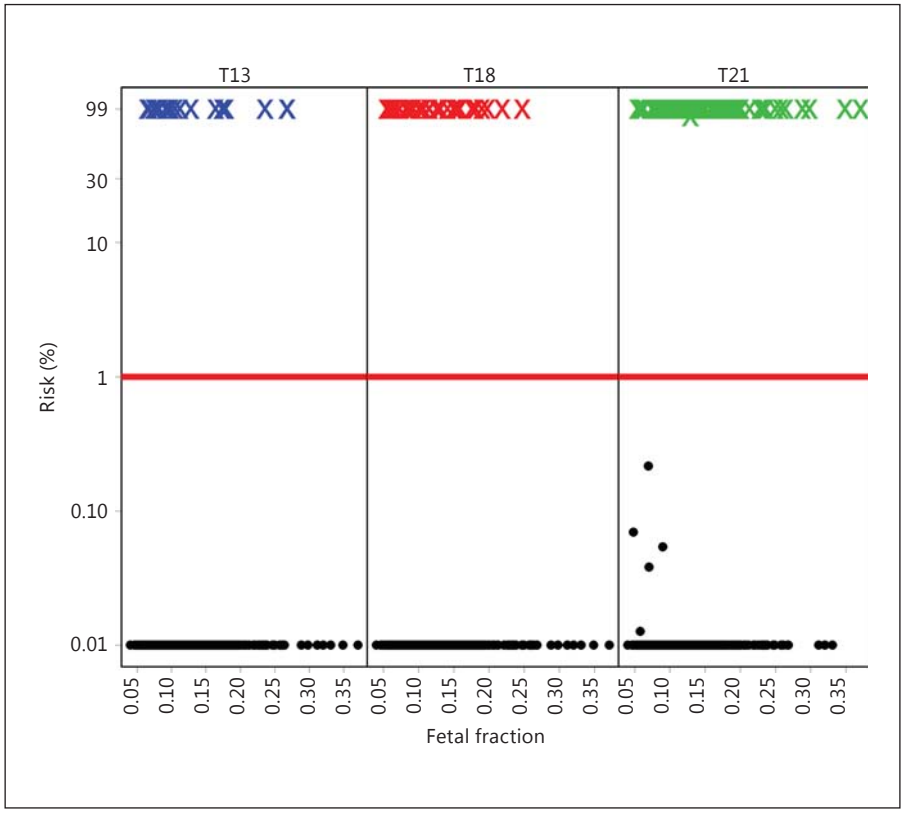
Assay variability, as measured by the variance of chromosomal cfDNA counts, was lower for microarrays than sequencing. The median assay variability for microarray detection showed a nearly 2-fold improvement over next-generation sequencing (0.051 vs. 0.099; $p < 0.0001$; fig. 3).

Microarrays Provide Lower Fetal Fraction Variability

A core component of the Harmony Prenatal Test is the FORTE algorithm, which leverages fetal fraction measurements to generate accurate trisomy risk scores. In this study, where the DANSR products were split between sequencing and microarrays, the FORTE-calculated fetal fractions were highly reproducible ($R^2 > 0.99$). Because the space available on the arrays for quantifying assays was not limiting, more polymorphic assays were included here than in previous sequence-based studies (576 vs. 192) [4]. Including more polymorphic assays in the array-based NIPT provided more precise fetal fraction estimates; the median relative standard error for microarrays was 0.013 compared to 0.021 for previous sequencing studies ($p < 0.0001$).

Data Acquisition Time Decreased Using Microarrays

The analysis of DANSR products is shortened by 2 days using microarrays. Microarray hybridization and imaging required 7.5 h. In contrast, cluster generation and sequencing for next-generation sequencing required 56 h (fig. 1). The microarray imager used in this study (GeneTitan Multi-Channel Instrument) imaged >75 microarrays per machine hour. In contrast, even when samples were multiplexed in groups of 96 samples per lane, the throughput was only 15 samples per machine hour on the HiSeq 2500 sequencing system.



Color version available online

Fig. 2. Microarray analysis correctly classified all trisomy (X) and nontrisomy (●) cases. Data for trisomy 13 (T13), trisomy 18 (T18), and trisomy 21 (T21) are plotted adjacent to each other. Microarray-based risk scores are plotted on the y-axis. Fetal fraction is plotted on the x-axis of each plot. Samples with risk scores ≥1% (cutoff; red line, color refers to the online version only) were classified as high risk and those <1% were classified as low risk.

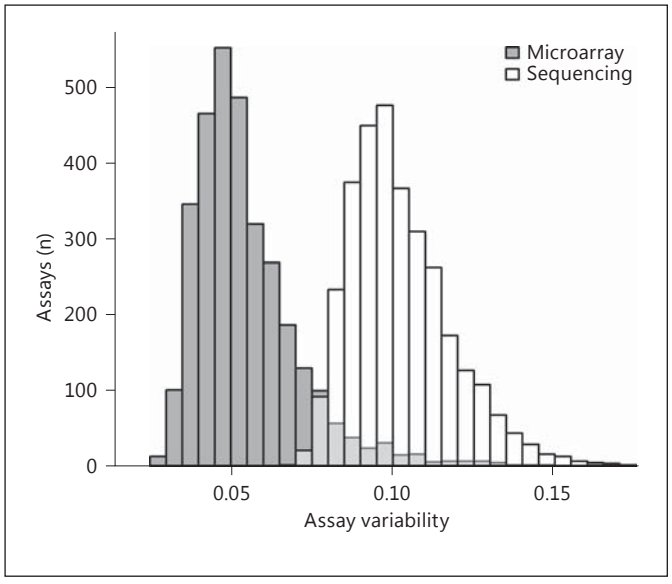


Fig. 3. Distribution of assay variability across samples for microarrays and sequencing. The bars of the histogram show the number of DANSR assays that share a specific range of assay variability. Where the two populations of data overlap, the bars are light gray. The microarray-quantified DANSR products have significantly lower assay variability. Lower assay variability is better.

Discussion

Decreased Variability and Increased Assay Space

The data in this study show that two key sources of data variability are significantly improved for microarrays compared to next-generation sequencing: (1) the variation of nonpolymorphic assays across samples (assay variability) and (2) variation of the measured fetal fraction using polymorphic assays (fetal fraction variability). Lower assay variability was shown to be an advantage of using microarray analysis, while lower fetal fraction variability was achieved by including more polymorphic assays in the DANSR process. Each source of variability plays a role in accurately assessing aneuploidy; lowering assay variability will allow aneuploidy changes to be measured in samples having a smaller fetal fraction, and lowering fetal fraction variation will provide more precise fetal fraction measurements. Clinically, these improvements could decrease the gestational age at which NIPT is conducted, as well as improve the report rates for samples with a lower fetal fraction due to higher maternal weight or early gestational age [11].

Improved Turnaround Time with Microarrays

Although next-generation sequencing technologies show great potential for research applications, they currently involve complex and costly hardware, reagent assemblages, and software systems. In contrast, DNA microarray systems are mature technologies that are widely used in high-throughput clinical laboratories. Microarray imaging is a rapid process, and the turnaround time for sample quantitation is reduced to less than a minute per sample. Faster data acquisition allows for greater sample throughput, which provides lower capital costs when microarray analysis is used.

Both microarray and sequencing technologies continue to improve. Some sequencing systems have accelerated sequencing modes that could decrease the time differential observed between microarrays and sequencing. However, in these modes, as the speed of sequencing increases, the capacity decreases and the cost per sample rises. In comparison, both time and money are saved by quantifying DNA using microarrays.

Microarray Analysis Does Not Require Sample Multiplexing

Sequence-based analysis leverages sample multiplexing in order to achieve economically efficient use of available sequence capacity. However, if the concentration of each sample is not normalized, a single sample can consume a disproportionate number of sequence reads in a flow cell, reducing the reads available for determining trisomy risk in the remaining samples. Sample normalization requires laborious custom dilutions of input DNA. Yet even when efforts are made to equalize sample input, as was reported in a recent study, a 4-fold variation in the median reads per sample was observed for a 12-plex reaction [15]. Microarray-based NIPT approaches require no sample multiplexing. Instead, each sample is hybridized individually to a single microarray. Processing throughput is enhanced by physically connecting 384 microarrays onto a single multi-microarray plate for convenient high-throughput handling. Because each sample is processed individually and sample normalization is not required, time is saved and cost is reduced.

In summary, we have shown that NIPT using DNA microarrays improves accuracy over next-generation sequencing. Microarray analysis is a promising approach that provides both higher accuracy and faster turnaround time.

Acknowledgements

We would like to gratefully recognize the informatics and experimental contributions of Wade Barrett, Matthew Citarella, Kevin Lee, John Lienhart, Lindsay McBride, Nairi Pezeshkian, and Morgan Quail.

References

1 Gil MM, Akolekar R, Quezada MS, Bregant B, Nicolaides KH: Analysis of cell-free DNA in maternal blood in screening for aneuploidies: meta-analysis. Fetal Diagn Ther 2014;35:156–173.

2 Gil MM, Quezada MS, Bregant B, Syngelaki A, Nicolaides KH: Cell-free DNA analysis for trisomy risk assessment in first-trimester twin pregnancies. Fetal Diagn Ther 2014;35:204–211.

3 Nicolaides KH, Syngelaki A, Ashoor G, Birdir C, Touzet G: Noninvasive prenatal testing for fetal trisomies in a routinely screened first-trimester population. Am J Obstet Gynecol 2012;207:374.e1–6.

4 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–e9.

5 Kent WJ, Sugnet CW, Furey TS, Roskin KM, Pringle TH, Zahler AM, et al: The Human Genome Browser at UCSC. Genome Res 2002;12:996–1006.

6 Wapner RJ, Martin CL, Levy B, Ballif BC, Eng CM, Zachary JM, et al: Chromosomal microarray versus karyotyping for prenatal diagnosis. N Engl J Med 2012;367:2175–2184.

7 Colls P, Escudero T, Fischer J, Cekleniak NA, Ben-Ozer S, Meyer B, et al: Validation of array comparative genome hybridization for diagnosis of translocations in preimplantation human embryos. Reprod Biomed Online 2012;24:621–629.

8 Handyside AH, Montag M, Magli MC, Repping S, Harper J, Schmutzler A, et al: Multiple meiotic errors caused by predivision of chromatids in women of advanced maternal age undergoing in vitro fertilisation. Eur J Hum Genet 2012;20:742–747.

9 Capalbo A, Bono S, Spizzichino L, Biricik A, Baldi M, Colamaria S, et al: Sequential comprehensive chromosome analysis on polar bodies, blastomeres and trophoblast: insights into female meiotic errors and chromosomal segregation in the preimplantation window of embryo development. Hum Reprod 2013;28:509–518.

10 Ashoor G, Poon L, Syngelaki A, Mosimann B, Nicolaides KH: Fetal fraction in maternal plasma cell-free DNA at 11–13 weeks' gestation: effect of maternal and fetal factors. Fetal Diagn Ther 2012;31:237–243.

11 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.

12 Sparks AB, Wang ET, Struble CA, Barrett W, Stokowski R, McBride C, et al: Selective analysis of cell-free DNA in maternal blood for evaluation of fetal trisomy. Prenat Diagn 2012;32:3–9.

13 Hidestrand M, Stokowski R, Song K, Oliphant A, Deavers J, Goetsch M, et al: Influence of temperature during transportation on cell-free DNA analysis. Fetal Diagn Ther 2012;31:122–128.

14 Norton ME, Brar H, Weiss J, Karimi A, Laurent LC, Caughey AB, et al: Non-Invasive Chromosomal Evaluation (NICE) Study: results of a multicenter prospective cohort study for detection of fetal trisomy 21 and trisomy 18. Am J Obstet Gynecol 2012;207:137.e1–e8.

15 Jensen TJ, Zwiefelhofer T, Tim RC, Džakula Ž, Kim SK, Mazloom AR, et al: High-throughput massively parallel sequencing for fetal aneuploidy detection from maternal plasma. PLoS One 2013;8:e57381.