Noninvasive prenatal detection and selective analysis of cell-free DNA obtained from maternal blood: evaluation for trisomy 21 and trisomy 18

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OBJECTIVE: We sought to develop a novel biochemical assay and algorithm for the prenatal evaluation of risk for fetal trisomy 21 (T21) and trisomy 18 (T18) using cell-free DNA obtained from maternal blood.

STUDY DESIGN: We assayed cell-free DNA from a training set and a blinded validation set of pregnant women, comprising 250 disomy, 72 T21, and 16 T18 pregnancies. We used digital analysis of selected regions in combination with a novel algorithm, fetal-fraction optimized risk of trisomy evaluation (FORTE), to determine trisomy risk for each subject.

RESULTS: In all, 163/171 subjects in the training set passed quality control criteria. Using a Z statistic, 35/35 T21 cases and 7/7 T18 cases had Z statistic >3 and 120/121 disomic cases had Z statistic <3. FORTE produced an individualized trisomy risk score for each subject, and correctly discriminated all T21 and T18 cases from disomic cases. All 167 subjects in the blinded validation set passed quality control and FORTE performance matched that observed in the training set correctly discriminating 36/36 T21 cases and 8/8 T18 cases from 123/123 disomic cases.

CONCLUSION: Digital analysis of selected regions and FORTE enable accurate, scalable noninvasive fetal aneuploidy detection.

Key words: aneuploidy detection, Down syndrome, noninvasive prenatal diagnostics, trisomy

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The American Congress of Obstetricians and Gynecologists (ACOG) recommends that pregnant women be offered noninvasive screening for fetal chromosomal abnormalities.[1] However, existing screening methods exhibit detection rates in the range of 90-95% and false-positive rates in the range of 3-5%.[2-6] Thus, ACOG also recommends that patients categorized by screening as high risk for fetal aneuploidy be offered invasive testing such as amniocentesis or chorionic villus sampling. Although these invasive procedures are highly accurate, they are expensive and entail a risk of miscarriage.[7,8] To address these limitations, several groups have pursued methods for noninvasive fetal aneuploidy detection.

★ EDITORS' CHOICE ★

Initial efforts, which were focused on isolation and analysis of circulating fetal cells, turned out to be challenging.[9,10] The realization that fetal nucleic acids are present in maternal blood spawned efforts to analyze cell-free DNA (cfDNA) for fetal conditions.[11-15] In the last few years, massively parallel shotgun sequencing (MPSS) has been used to quantify precisely cfDNA fragments for fetal trisomy detection.[16-19] Several groups have recently used this approach to identify fetal trisomy 21 (T21), and with less success, trisomy 18 (T18) and trisomy 13.[20-24]

The chromosomal dosage resulting from fetal aneuploidy is directly related to the fraction of fetal cfDNA. For example, a cfDNA sample containing 4% DNA from a T21 fetus should exhibit a 2% increase in the proportion of reads from chromosome 21 (chr21) as compared to a normal fetus. Distinguishing these 2 scenarios with high confidence requires a large number (>93,000) of chr21 observations.[24] Because MPSS is indiscriminate with respect to chromosomal origin, and because chr21 represents ~1.5% of the human genome, ~6.3 million uniquely mapped reads are required to ensure sufficient chr21 counts. Given typical MPSS mapping yields of ~25%, this translates to 25 million raw sequencing reads per sample. This requirement constrains the throughput, cost efficiency, and clinical utility of MPSS for aneuploidy detection. For example, a recently launched product that detects T21 via MPSS[22] has a list price of approximately $2700 per test.

Selective sequencing of relevant chromosomes can address these constraints. We recently described a novel assay, digital analysis of selected regions (DANSR), which enables highly multiplexed sequencing of selected loci from specific chromosomes of interest (Appendix).[25] We used DANSR to evaluate loci on chromosome 18 (chr18) and chr21 in a set of subject samples whose aneuploidy status

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was known at the time of analysis and demonstrated accurate aneuploidy detection.

In this study, we extend DANSR to assay simultaneously polymorphic and nonpolymorphic loci in a single reaction, enabling estimation of chromosome proportion and fetal fraction. We describe a novel analysis algorithm, the fetal-fraction optimized risk of trisomy evaluation (FORTE), which uses this information to compute the likelihood of fetal trisomy in each subject. We demonstrate the power of this approach in a blinded set of 167 pregnant women, including 36 T21 and 8 T18 pregnancies.

Materials and Methods

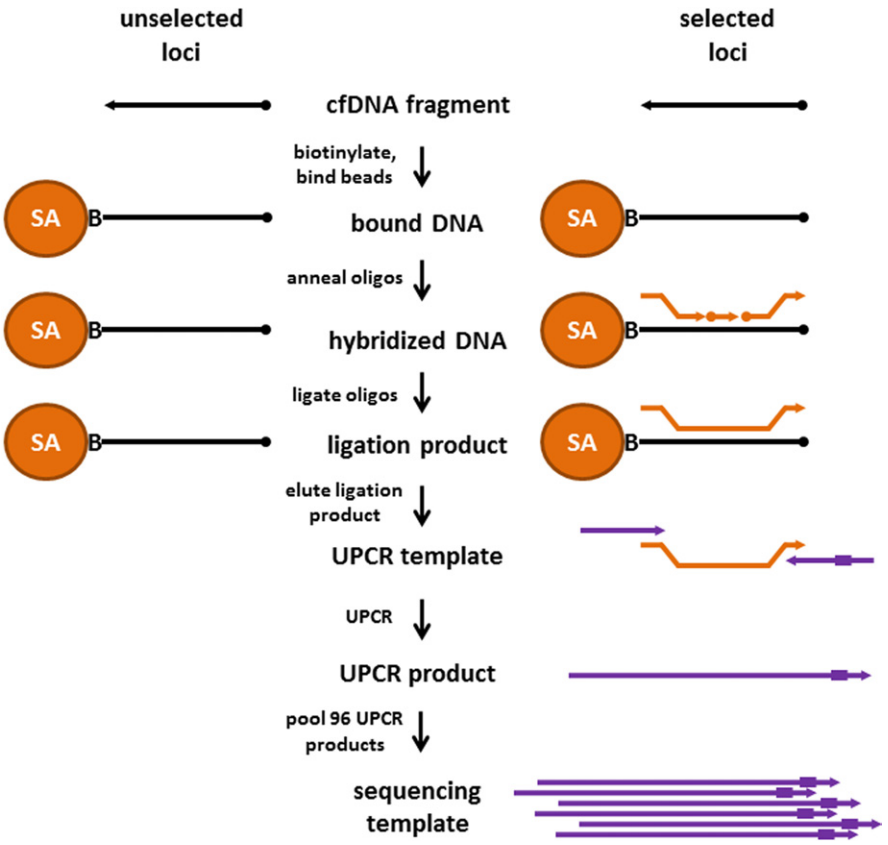
Subjects

Subjects were prospectively enrolled upon providing informed consent, under protocols approved by institutional review boards. Subjects were required to be at least 18 years of age, to be at least 10 weeks' gestational age, and to have singleton pregnancies. A subset of enrolled subjects, consisting of 250 women with disomic pregnancies, 72 with T21 pregnancies, and 16 with T18 pregnancies, was selected for inclusion in this study. The subjects were randomized into a training set consisting of 127 disomic pregnancies, 36 T21 pregnancies, and 8 T18 pregnancies, and a validation set consisting of 123 disomic pregnancies, 36 T21 pregnancies, and 8 T18 pregnancies. The trisomy status of each pregnancy was confirmed by invasive testing (fluorescent in situ hybridization and/or karyotype analysis). The trisomy status of the training set was known at the time of analysis; in the validation set, the trisomy status was kept blinded until after FORTE analysis.

DANSR assay

We designed DANSR assays against loci in the human genome as previously described.[25] To assess chromosome proportion, we designed assays against 576 nonpolymorphic loci on each of chr18 and chr21, where each assay consisted of 3 locus-specific oligonucleotides: a left oligo with a 5' universal amplification tail, a 5' phosphorylated middle oligo, and a 5' phosphorylated right oligo with a 3' universal amplification tail. To assess fetal fraction, we designed assays against a set of 192 single nucleotide polymorphism (SNP)-containing loci on chromosomes 1-12, where 2 middle oligos, differing by 1 base, were used to query each SNP. SNPs were optimized for minor allele frequency in the HapMap 3 dataset (http://hapmap.ncbi.nlm.nih.gov/. Accessed Feb. 14, 2012). Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA) and pooled together to create a single multiplexed DANSR assay pool.

FIGURE 1
Schematic of digital analysis of selected regions (DANSR) assay



DANSR process applied to unselected (*left*) vs selected (*right*) loci. *Circles* and *arrows* indicate 5'phosphate and 3'hydroxyl moieties, respectively. Cell-free DNA (cfDNA) (*black*) is first labeled with biotin (B) moiety and bound to streptavidin-coated magnetic beads (SA). Next, locus-specific DANSR oligos (*orange*) are annealed to cfDNA. When DANSR oligos hybridize to their cognate locus sequences in cfDNA, their termini form 2 nicks. Ligation of these nicks results in creation of ligation product capable of supporting amplification using universal polymerase chain reaction (UPCR) primers (*purple*). Elution of this ligation product followed by UPCR with UPCR primers containing 96 distinct 7-base sample tags (*purple box*) enables pooling and simultaneous sequencing of 96 different UPCR products on a single lane. Left and right UPCR primers contain universal tail sequences that support sequencing of locus-specific 56 bases and 7 sample-specific bases, respectively. In addition, UPCR primers contain universal tail sequences that support HiSeq (Illumina, San Diego, CA) cluster amplification.

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DANSR product was generated from each subject sample as previously described (Figure 1).[25] Briefly, 8 mL of blood per subject was collected into a cfDNA tube (Streck, Omaha, NE) and stored at room temperature for up to 3 days. Plasma was isolated from blood via double centrifugation and stored at −20°C for up to a year. cfDNA was isolated from plasma using viral nucleic acid DNA purification beads (Dynal, Grand

Island, NY), biotinylated, immobilized on MyOne C1 streptavidin beads (Dynal), and annealed with the multiplexed DANSR oligonucleotide pool. Appropriately hybridized oligonucleotides were catenated with Taq ligase, eluted from the cfDNA, and amplified using universal polymerase chain reaction primers. Polymerase chain reaction product from 96 independent samples was pooled and used as a template for cluster amplification on a single lane of a TruSeq v2 SR flow slide (Illumina, San Diego, CA). The slide was processed on an Illumina HiSeq 2000 to produce a 56-base locus-specific sequence and a 7-base sample tag sequence from an average of 1.18 million clusters/sample. Locus-specific reads were compared to expected locus sequences. An average of 1.15 million (97%) reads had <3 mismatches with expected locus sequences, resulting in an average of 854 reads/locus/sample.

Analysis of nonpolymorphic loci for chromosome proportion

Sequence counts were normalized by systematically removing sample and assay biases. Sequence counts follow a log normal distribution, so biases were estimated using median polish on log-transformed counts.[25-27] A chr21 proportion metric was then computed for each sample as the mean of counts for selected chr21 loci divided by the sum of the mean of counts for selected chr21 loci and the mean of counts for all 576 chr18 loci. A chr18 proportion metric was similarly calculated for each sample. A standard Z test of proportions was used to compute Z statistics:

$$Z_j = \frac{p_j - p_o}{\sqrt{\frac{p_o(1-p_0)}{n_j}}} \qquad (1)$$

where p_j is the observed proportion for a given chromosome of interest in a given sample j, p_0 is the expected proportion for the given test chromosome calculated as the median p_j, and n_j is the denominator of the proportion metric.

Z statistic standardization was performed using iterative censoring on each lane of 96 samples. At each iteration, the samples falling outside of 3 median absolute deviations were removed. After 10 iterations, mean and SD were calculated using only the uncensored samples. All samples were then standardized against this mean and SD. The Kolmogorov-Smirnov test[28] and Shapiro-Wilk test[29] were used to establish the normality of the uncensored samples' Z statistics.

Locus selection using training samples

Sequence count data from the training samples were first normalized as described above and previously.[25-27] These samples were subsequently analyzed to select 384 of the 576 loci on chr21 and chr18 best able to discriminate T21 and T18 from normal samples. The 384 loci on each chromosome exhibiting the greatest residual difference between normal and trisomy samples were identified using Z statistics derived from individual loci for the test chromosome and all 576 loci for the comparison chromosome.

Analysis of polymorphic loci for fetal fraction

Informative polymorphic loci were defined as loci where fetal alleles differ from maternal alleles. Because DANSR exhibits allele specificities >99%, informative loci were readily identified when the fetal allele proportion of a locus was measured to be between 1-20%. A maximum likelihood estimate using the binomial distribution was employed to determine the most likely fetal fraction based upon measurements from several informative loci. The results correlate well (R^2 >0.99) with the weighted average approach presented by Chu and colleagues.[30]

Aneuploidy detection using FORTE

The FORTE algorithm estimates the risk of aneuploidy using an odds ratio comparing a model assuming a disomic fetal chromosome and a model assuming a trisomic fetal chromosome. Let $x_j = p_j - p_0$ be the difference of the observed proportion p_j for sample j and the estimated reference proportion p_0. FORTE computes:

$$\frac{P(x_j|T)}{P(x_j|D)}, \qquad (2)$$

where T is the trisomic model and D is the disomic model. The disomic model D is a normal distribution with mean 0 and a sample specific SD estimated by Monte Carlo simulations described below. The trisomic model T is also a normal distribution with mean 0, by transforming x_j to $x_j = p_j - \hat{p}_j$, the difference between the observed proportion and a fetal fraction adjusted reference proportion defined by $\hat{p}_j$

$$\hat{p}_j = \frac{(1 + 0.5f_j)p_0}{((1 + 0.5f_j)p_0) + (1 - p_0)} \qquad (3)$$

where f_j is the fetal fraction for sample j. This adjustment accounts for the expected increased representation of a trisomic fetal chromosome.

Monte Carlo simulations are used to estimate sample-specific SD for disomic and trisomic models of proportion differences. Observed proportions for each sample can be simulated by nonparametric bootstrap sampling of loci and calculating means, or parametric sampling from a normal distribution using the mean and SE estimates for each chromosome from the observed nonpolymorphic locus counts. Similarly, the reference proportion p_0 and fetal fraction f_j can be simulated by nonparametric sampling of samples and polymorphic loci respectively, or chosen from normal distributions using their mean and SE estimates to account for measurement variances. Parametric sampling was used in this study. Simulations were executed 100,000 times, and proportion differences were computed for each execution to construct the distributions. Based on the results of these simulations in the training set, normal distributions were found to be good models of disomy and trisomy.

The final FORTE risk score is defined as

$$\frac{P(x_j|T)P(T)}{P(x_j|D)P(D)}, \qquad (4)$$

where $P(T)/P(D)$ is the prior risk of trisomy vs disomy. The prior risk was taken from well-established tables capturing the risk of trisomy associated with the subject's maternal and gestational age.[31]

TABLE
Sample characteristics

Cohort	Status	Subjects, n	Maternal age, y				Gestational age, wk			
			Average	SD	Minimum	Maximum	Average	SD	Minimum	Maximum
Training	Normal	127	34.4	6.3	18	44	17.1	4.3	10.3	32.4
	T18	8	37.7	6.1	27	44	18.4	4.4	13.0	25.9
	T21	36	34.2	6.6	18	44	18.9	4.4	11.0	33.0
	Total	171	34.5	6.3	18	44	17.6	4.4	10.3	33.0
Validation	Normal	123	33.1	7.1	18	51	18.3	3.5	11.0	30.4
	T18	8	36.6	6.5	25	43	15.7	3.3	11.7	21.0
	T21	36	34.1	7.1	18	46	20.1	5.1	12.3	36.1
	Total	167	33.5	7.1	18	51	18.6	4.0	11.0	36.1

T18, trisomy 18; *T21,* trisomy 21.

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Results

Sample characteristics

The Table describes the clinical characteristics and demographics of the patients whose samples were analyzed in this study. The mean maternal age of the disomic, T21, and T18 subjects was 34, 34, and 37 years, respectively. The mean gestational age of the disomic, T21, and T18 subjects was 17.7, 19.6, and 17.0 weeks. The mean maternal ages of the disomic, T21, and T18 subjects were not significantly different between training and validation sets (all t test $P > .05$). Similarly, the mean gestational ages of the disomic, T21, and T18 subjects were not significantly different between training and validation sets (all t test $P > .05$).

Chromosome proportion Z statistics in training set

To select loci to be used for aneuploidy detection, we evaluated a set of subjects whose aneuploidy status was known. This training set consisted of 127 normal, 36 T21, and 8 T18 pregnancies. Six normal, 1 T18, and 1 T21 samples (8/171, or 5%) did not meet quality control (QC) criteria (low count, fetal fraction <3%, and/or evidence from SNPs of a nonsingleton pregnancy) and were removed from the data set. We computed chromosome proportion Z statistics in the remaining samples for chr18 and chr21 (Figure 2). In all, 120/121 (99.2%) disomic samples had Z statistics <3; 1 disomic sample had a chr21 Z statistic of 3.5. In all, 35/35 (100%) T21 and 7/7 (100%) T18 samples had chromosome proportion Z statistics >3. Thus, using Z statistic analysis, DANSR exhibited 99.2% specificity and 100% sensitivity for T21, and 100% specificity and 100% sensitivity for T18.

Fetal fraction in training set

A principal determinant of the chromosome proportion response to aneuploidy is the fraction of fetal DNA in the sample. To measure fetal fraction reliably, we incorporated 192 DANSR assays targeting SNPs into our multiplex assay pool. By measuring fetal fraction and chromosome proportion in the same reaction we ensure estimates of fetal fraction from polymorphic assays closely represent fetal fraction in the nonpolymorphic assays used to assess chromosome proportion. Fetal fraction exhibited a strong correlation (R^2 >0.90) with the chromosome proportion Z statistic in trisomic pregnancies (Figure 2).

Importantly, the Z statistic was not responsive to fetal fraction in normal pregnancies (Figure 2), reflecting a major limitation of the Z statistic metric: samples with low Z statistic values arise from both euploid samples and aneuploid samples with modest fetal fraction. We reasoned that a metric that was responsive to fetal fraction in euploid as well as aneuploid pregnancies would be preferable. We therefore developed FORTE, which uses fetal fraction information to: (1) define expected chromosome proportions for trisomic vs disomic test chromosomes, and (2) compute the odds that a sample belongs to one or the other group.

Analysis of training set using FORTE

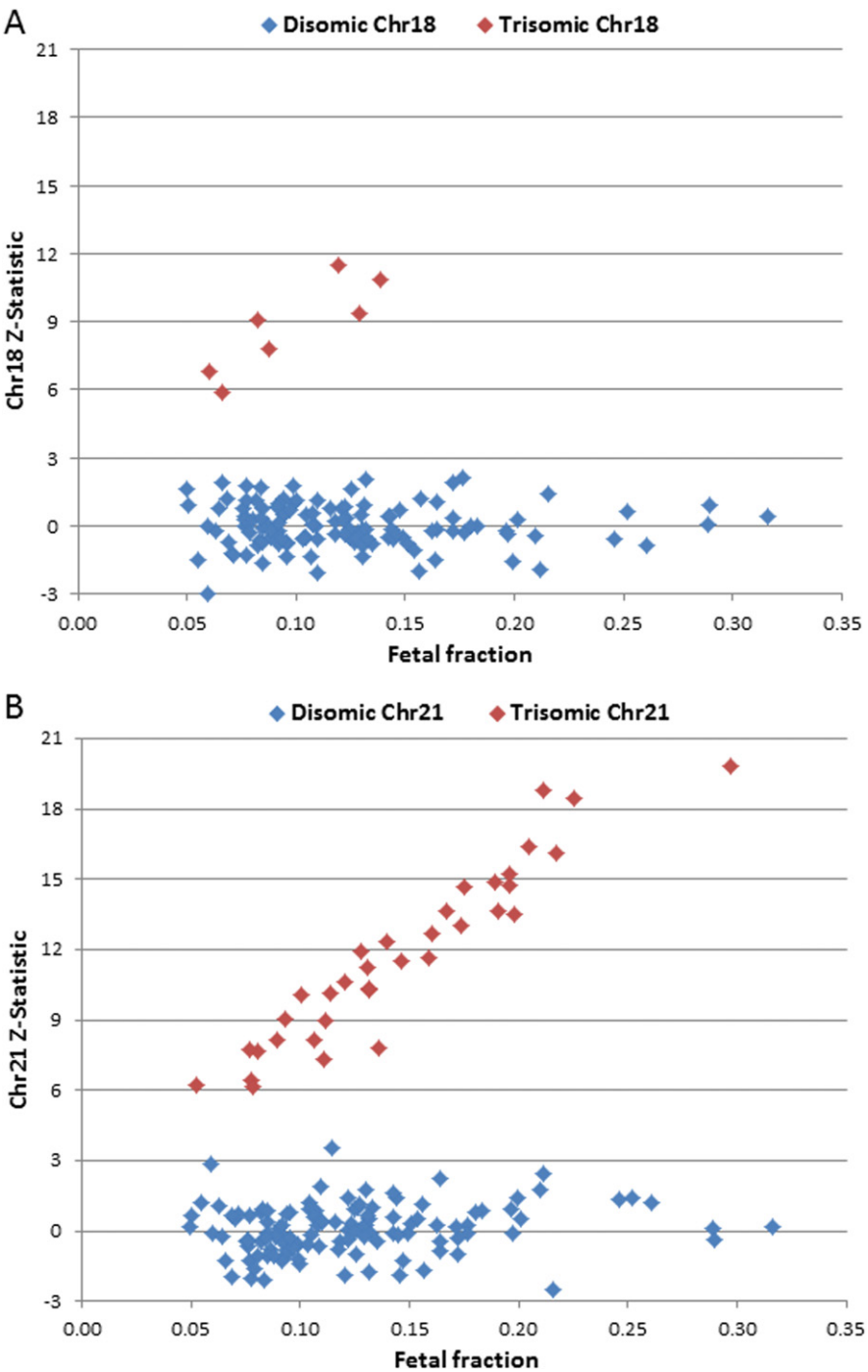
We used FORTE to compute the odds of trisomy vs disomy of chr18 and chr21 in each sample within the training set (Figure 3). As expected, the FORTE odds demonstrated a response to fetal fraction in both trisomic and disomic samples, and the response magnitude was approximately equivalent in the 2 groups. FORTE correctly discriminated all euploid from aneuploid samples, and the difference between the lowest aneuploid odds and the euploid odds was $>10^{12}$. All aneuploidy samples had odds $>10^{10}$.

Validation of FORTE aneuploidy analysis on blinded set

To test the performance of the DANSR/FORTE assay in an independent set of subjects, we assayed a blinded validation set consisting of 123 normal, 36 T21, and 8 T18 pregnancies. All samples passed QC criteria and were assigned FORTE odds scores for chr18 and chr21 (Figure 4). As above, FORTE correctly discriminated all trisomy from disomy subjects. The difference between the lowest aneuploid odds and the highest euploid odds was $10^{3.9}$. All 36 T21 and 8 T18 samples had trisomy odds $>10^{2.67}$ (>99.8% risk of trisomy).

Current prenatal aneuploidy screening tests employ varying risk thresholds,

FIGURE 2
Training set Z statistics vs fetal fraction



Chromosome proportion Z statistic is plotted for **A,** chromosome 18 (chr18) or **B,** chromosome 21 (chr21) vs fraction of fetal DNA for each training set subject. Disomic subjects are represented as *blue diamonds*, trisomic subjects as *red*. Z statistic of trisomic subjects increases with increasing fetal fraction. By contrast, Z statistic of disomic subjects is not responsive to fetal fraction. Thus, when evaluating disomic subjects, Z statistic does not reflect increase in certainty expected to result from increased fetal fraction.

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but generally risks of 1 in 100 (10^{-2}) to 1 in 300 ($10^{-2.5}$) are referred to invasive testing.[3,32] If this threshold range were applied to the FORTE odds for the blinded set, it would yield 99.2% specificity and 100% sensitivity for each chromosome. This compares favorably with current screening methods, which can entail 5% false-positive and 10% false-negative rates.[3,5] Moreover, because the minimum difference between the euploid and aneuploid subjects' FORTE odds was almost 4 orders of magnitude for T21 and 14 orders of magnitude for T18, a variety of thresholds produce perfect sensitivity and specificity.

COMMENT

Principal findings of this study

This study demonstrates the analytical performance of DANSR and FORTE in detecting fetal T21 and T18 in pregnant women of at least 10 weeks' gestational age. DANSR refers to the biochemical assay that involves directed analysis of cfDNA. FORTE refers to the algorithm that provides an individualized risk score for T21 and T18 taking into account age-related risks and fetal fraction of the sample. The combination of DANSR and FORTE correctly identified all 36 cases of T21 and 8 cases of T18 as having >99% risk for each trisomy in a blinded analysis. There was at least 1000-fold magnitude separation in the risk score between trisomic and disomic samples.

Difference between DANSR and MPSS assay

By generating sequencing template from chromosome-specific assays and by producing high mapping rates, DANSR permits aneuploidy detection using ~1 million raw reads per subject, enabling analysis of 96 subjects per sequencing lane. By contrast, MPSS evaluates the entire genome, and requires ~25 million raw reads per subject, which limits sequencing throughput to 4-6 samples per lane. Thus, DANSR enjoys a substantive advantage over MPSS in sequencing cost and throughput.

DANSR enables genotyping of individual polymorphic loci that is not possible using current MPSS approaches. DANSR allowed us to develop an integrated assay

to assess polymorphic as well as nonpolymorphic loci, thereby permitting simultaneous determination of fetal fraction and chromosome proportion. We used fetal fraction information by imposing a QC requirement that each sample have at least 3% fetal DNA, thereby avoiding low confidence calls arising from low proportions of fetal DNA. In addition, we developed the FORTE algorithm to produce a fetal fraction-dependent risk score indicating the odds of a sample being trisomic vs disomic.

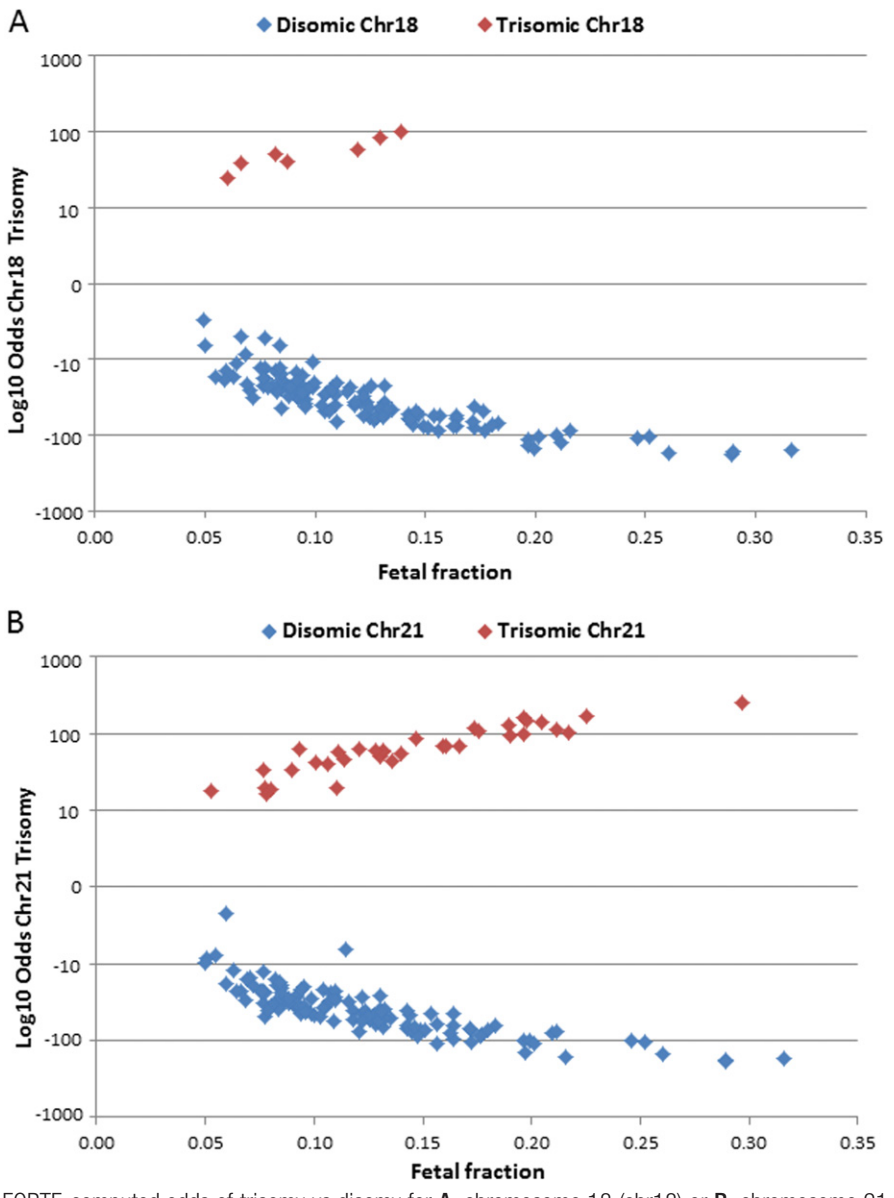
Purpose of the FORTE algorithm

FORTE is a novel algorithm that incorporates multiple risk factors to generate an individualized odds score for trisomy. While the result format of FORTE is similar to that of current prenatal screening results, the combination of DANSR and FORTE yields greatly improved performance. FORTE analysis differs from chromosome proportion Z statistic analysis in several important respects. First, because we process 96 samples in a single batch/lane, FORTE uses the observed variances within and between samples in a lane, rather than estimating variance based upon information obtained from a previously analyzed reference data set. Thus, FORTE is less susceptible to process drift and does not require external reference samples or normalizing adjustments based upon historical information.

Second, FORTE is responsive to fetal fraction in both the trisomic and disomic state, whereas Z statistic is only responsive to fetal fraction in the trisomic state. As a consequence, FORTE produces overall better separation of trisomic vs disomic samples. Moreover, because samples with low fetal fraction yield odds with lower magnitudes in both disomic and trisomic samples, FORTE communicates a more accurate understanding of the confidence with which a call is being made in disomic samples as well as trisomic samples.

Third, because the risk of aneuploidy varies significantly with maternal and gestational age, and because incorporating these risks is standard practice in reporting screening results,[5,33] FORTE is designed to accommodate incorporation of age-related risks. Specifically, because both the risk computed from DANSR and the age-related risk reflect a subject's odds of trisomy vs disomy, these risk components are readily combined. It would also be possible to incorporate other risk components such as prior pregnancy with trisomy fetus and other prenatal testing results. By contrast, the Z statistic reflects the likelihood that a sample is disomic, and therefore is not readily combined with

FIGURE 3
Training set FORTE odds vs fetal fraction



FORTE-computed odds of trisomy vs disomy for **A,** chromosome 18 (chr18) or **B,** chromosome 21 (chr21) is plotted vs fraction of fetal DNA for each training set subject. Disomic subjects are represented as *blue diamonds*, trisomic subjects as *red*. FORTE-computed odds of trisomy increases with increasing fetal fraction among trisomic subjects. Similarly, FORTE-computed odds of trisomy decreases with increasing fetal fraction among disomic subjects. Thus, when evaluating disomic and trisomic subjects, FORTE metric reflects increase in certainty resulting from increased fetal fraction.

FORTE, fetal-fraction optimized risk of trisomy evaluation.

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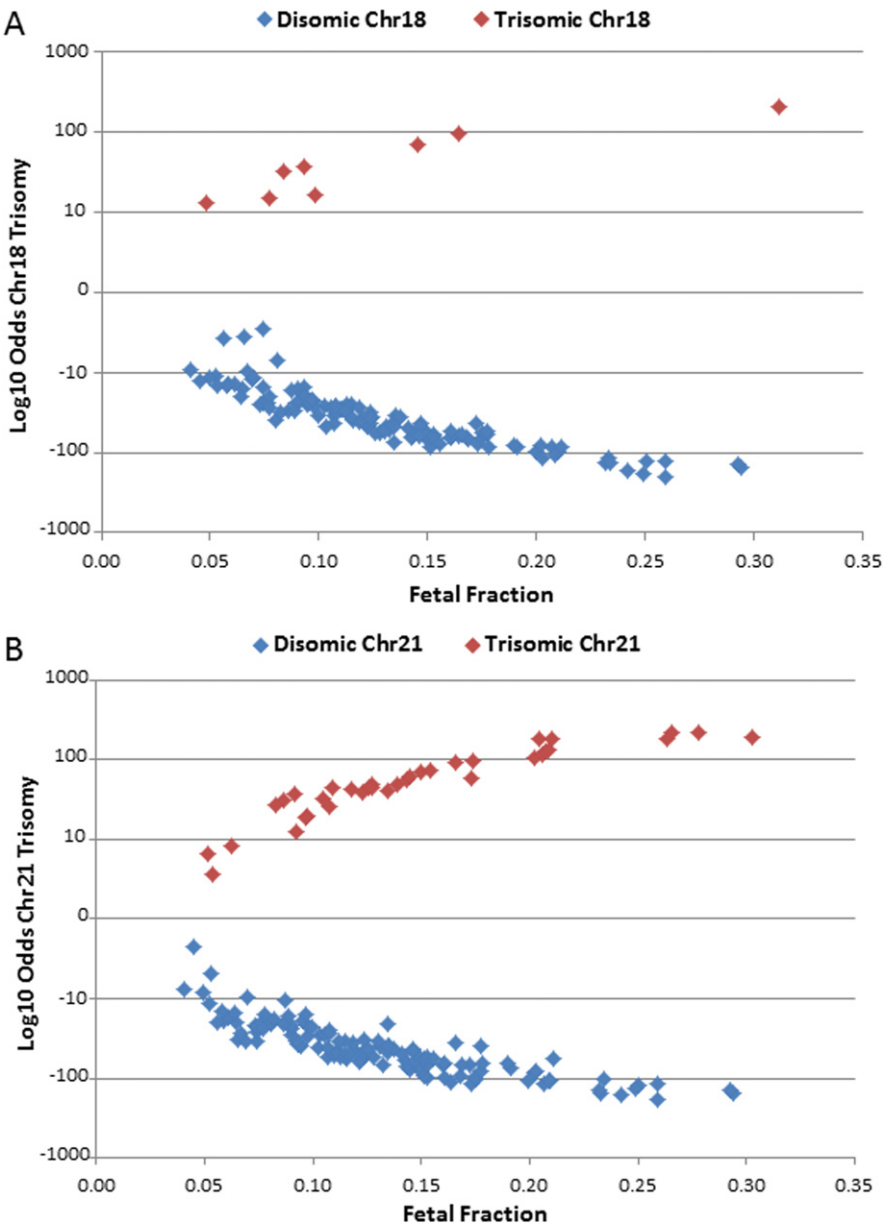
FIGURE 4
Validation set FORTE risk vs fetal fraction



FORTE-computed odds of trisomy vs disomy for **A,** chromosome 18 (chr18) or **B,** chromosome 21 (chr21) is plotted vs fraction of fetal DNA for each blinded validation set subject. Disomic subjects are represented as *blue diamonds*, trisomic subjects as *red*. As in training set, FORTE-computed odds of trisomy increases with increasing fetal fraction among trisomic subjects and decreases with increasing fetal fraction among disomic samples. Thus, FORTE metric reflects increase in certainty resulting from increased fetal fraction in both disomic and trisomic samples.

FORTE, fetal-fraction optimized risk of trisomy evaluation.

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age-related risks of trisomy vs disomy. One consequence of this deficiency is that the Z statistic will exhibit different performance depending upon a subject's age. For example, an 18-year-old subject at 12 weeks' gestation and with a Z statistic of 3 is ~38 times more likely to be a false positive than a 44-year-old subject at 12 weeks' gestation and with the same score.[31]

Importance of fetal fraction

A principal determinant of the accuracy of any cfDNA analysis method is the fraction of fetal cfDNA in the sample. The higher the fraction of fetal cfDNA, the greater the difference in the number of cfDNA fragments originating from trisomic vs disomic chromosomes and hence the easier it is to detect trisomy. The FORTE algorithm explicitly accounts for fetal fraction in calculating trisomy risk.

Limitations of the study

Whereas this study involved blinded analysis of a number of subjects, the total number of T21 and T18 subjects was modest and therefore larger studies are warranted. This study was also limited to women considered high risk for fetal trisomy, as all women went on to invasive testing. Some have questioned whether the performance of trisomy detection using cfDNA would vary between clinically high-risk and low-risk pregnancies.[34] Additional studies comparing fetal fraction between low- and average-risk pregnancies would address this matter.

Future developments

Because DANSR enables directed analysis of specific genomic regions, DANSR could possibly be used to evaluate genetic conditions besides trisomy, such as subchromosomal conditions (eg, microdeletions). Because the FORTE algorithm can incorporate multiple clinical risk factors, incorporation of additional risk information, including that from ultrasound, warrants investigation. Technical improvements in DNA sequencing continue to be made at a rapid pace. Current cfDNA analysis turnaround times of 7-10 days are thus likely to be reduced as further technical advances are made.[22]

Clinical implications

The clinical application of noninvasive testing via cfDNA warrants consideration. Whereas the term "noninvasive prenatal diagnosis" has been used extensively in the literature, it is premature to regard these tests as diagnostic. A recently completed large-scale validation study using MPSS demonstrated a 98.6% detection rate for T21, raising the question of whether current cfDNA testing is

more appropriate as a higher performance screen or as a diagnostic test.[22]

To date, studies of cfDNA analysis have been focused on detection of common fetal trisomies. Other prenatal screening modalities such as ultrasound are likely to continue to play an important role in conjunction with cfDNA analysis since T21 and T18 represent a subset of fetal anomalies.[35] Should noninvasive testing via cfDNA become affordable and widely accessible, the myriad of prenatal screening and testing options today may evolve to a simpler model in which cfDNA analysis and ultrasound become a new standard for all pregnant women. ■

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Appendix
Glossary of technical terms

- **Cell-free DNA (cfDNA)**, DNA that has been isolated from the plasma portion of maternal blood.
- **Digital analysis of selected regions (DANSR)**, a process of analyzing the counts from assays targeted against selected genomic regions.
- **HapMap**, an international project to catalog human genome single nucleo-

tide polymorphisms, http://hapmap.ncbi.nlm.nih.gov/.
- **Locus**, a targeted region of the genome.
- **Massively parallel shot-gun sequencing (MPSS),** a method that randomly analyzes cfDNA fragments for fetal aneuploidy detection.
- **Nonpolymorphic locus**, a locus without known variation.
- **Oligo**, **oligonucleotides**, short synthesized sequences of DNA that are used in combination to create assays.
- **Polymorphic locus**, a locus that includes a known single nucleotide polymorphism.
- **(Sequencing) count**, the number of reads identified as coming from (mapped to) a specific locus or assay.
- **(Sequencing) read**, a single DNA sequence as determined by a sequencer.

Overview of sequencing

The Illumina HiSeq 2000 DNA sequencing system has been used to generate data for both the MPSS approaches presented in other papers and for the approach used in this paper. The basic unit of sequencing on the system is a lane that is capable of producing approximately 100 million clusters. Each of these clusters is sequenced and the resulting raw sequences are called a "read." Using a system of encoding with sequence sample tags these reads can be apportioned to several or even 100 different samples. In MPSS, random fragments from the cfDNA of several samples are used to make the raw sequence reads on a lane. These sequences are then compared to the expected sample tags and the human genome and mapped to a chromosome of origin. In digital analysis of selected regions, loci to be sequenced are selected by a set of oligonucleotide assays specific for the chromosomes and regions of interest. The products of these assays from 96 samples are then pooled on a sequencing lane, converted to raw reads, and compared to the expected 96 sample tags and sequences of the loci that have been selected by the assays. Errors in sequencing can be observed as mismatches between the observed raw sequences and the expected sequences during comparison. Typically 97% of the digital analysis of selected regions raw reads map to expected sequences with <3 mismatches.

Statistical terminology

- **Chromosome proportion**, the estimated fraction of reads originating on a specific chromosome. Fetal-fraction optimized risk of trisomy evaluation estimates this fraction as the mean count on the specified chromosome divided by the sum of mean counts on all measured chromosomes.
- **Disomic model**, a statistical model of chromosome proportion assuming the fetus is disomic (has 2 copies of the chromosome).
- **Fetal fraction**, the estimated fraction of cfDNA in a sample that originated in the fetus.
- **Fetal-fraction optimized risk of trisomy evaluation (FORTE)**, a process of using fetal fraction of cfDNA and results from sequencing cfDNA to generating a risk score.
- **Informative polymorphic locus**, a polymorphic locus where the mother has a homozygous genotype and the fetus has a heterozygous genotype.
- **Log normal**, a statistical distribution of data. The data, after log transformation, follow a normal distribution.
- **Mean count**, the average count of loci located on a specific chromosome.
- **Median polish**, an iterative algorithm for estimating the parameters of a linear model. Similar to estimating parameters in an analysis of variance, replacing mean estimates with medians for robustness to outlying measurements.
- **Monte Carlo simulation**, a computational technique used to numerically estimate values when mathematical analysis is intractable. The term "Monte Carlo" implies that appropriately computed random values are used by simulations to generate estimates.
- **Risk score,** the relative likelihood of the disomic model compared to the trisomic model, as is used in current prenatal screening.
- **Trisomic model**, a statistical model of chromosome proportion assuming the fetus is trisomic (has 3 copies of the chromosome) and the sample has the estimated fetal fraction of cfDNA.
- **Z statistic**, a number indicating how far an observation deviates from the average in a population. The unit of a Z statistic is the number of SD.