OBSTETRICS

Clinical validation of a noninvasive prenatal test for genomewide detection of fetal copy number variants

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BACKGROUND: Current cell-free DNA assessment of fetal chromosomes does not analyze and report on all chromosomes. Hence, a significant proportion of fetal chromosomal abnormalities are not detectable by current noninvasive methods. Here we report the clinical validation of a novel noninvasive prenatal test (NIPT) designed to detect genomewide gains and losses of chromosomal material ≥7 Mb and losses associated with specific deletions <7 Mb.

OBJECTIVE: The objective of this study is to provide a clinical validation of the sensitivity and specificity of a novel NIPT for detection of genomewide abnormalities.

STUDY DESIGN: This retrospective, blinded study included maternal plasma collected from 1222 study subjects with pregnancies at increased risk for fetal chromosomal abnormalities that were assessed for trisomy 21 (T21), trisomy 18 (T18), trisomy 13 (T13), sex chromosome aneuploidies (SCAs), fetal sex, genomewide copy number variants (CNVs) ≥7 Mb, and select deletions <7 Mb. Performance was assessed by comparing test results with findings from G-band karyotyping, microarray data, or high coverage sequencing.

RESULTS: Clinical sensitivity within this study was determined to be 100% for T21 (95% confidence interval [CI], 94.6—100%), T18 (95% CI, 84.4—100%), T13 (95% CI, 74.7—100%), and SCAs (95% CI, 84—100%), and 97.7% for genomewide CNVs (95% CI, 86.2—99.9%). Clinical specificity within this study was determined to be 100% for T21 (95% CI, 99.6—100%), T18 (95% CI, 99.6—100%), and T13 (95% CI, 99.6—100%), and 99.9% for SCAs and CNVs (95% CI, 99.4—100% for both). Fetal sex classification had an accuracy of 99.6% (95% CI, 98.9—99.8%).

CONCLUSION: This study has demonstrated that genomewide NIPT for fetal chromosomal abnormalities can provide high resolution, sensitive, and specific detection of a wide range of subchromosomal and whole chromosomal abnormalities that were previously only detectable by invasive karyotype analysis. In some instances, this NIPT also provided additional clarification about the origin of genetic material that had not been identified by invasive karyotype analysis.

Key words: cell-free DNA, chromosomal copy number variant, genomewide, microdeletions, noninvasive prenatal testing, subchromosomal copy number variant

Introduction

Since its introduction in 2011, noninvasive prenatal tests (NIPT) have had a significant impact on prenatal care. In only 4 years, NIPT has evolved into a standard option for high-risk pregnancies.[1] Content has also evolved from exclusive trisomy 21 (T21) testing to include trisomy 18 (T18), trisomy 13 (T13), sex chromosome aneuploidies (SCAs), and select microdeletions. This standard content can be expected to detect 80-83% of chromosomal abnormalities detected by karyotyping in a general screening population,[2-4] however this leaves a gap of approximately 17-20% of alternative chromosomal/subchromosomal abnormalities not detected. Consequently, obtaining comprehensive information about the genetic makeup of the fetus requires an invasive procedure. To overcome these limitations, NIPT could be extended to cover the entire genome. However, it is challenging to maintain a very high specificity and positive predictive value (PPV) when interrogating all accessible regions in the genome.[5] In previous reports, we have overcome these technical hurdles.[6] Furthermore, a recent study by Yin et al[7] demonstrated feasibility for noninvasive genomewide detection of subchromosomal abnormalities. In this report, we have improved the assay and statistical methods to enable comprehensive genomewide detection of copy number variants (CNVs) ≥7 Mb. We present results of a large blinded clinical study of >1200 samples including >100 samples with common aneuploidies detectable by traditional NIPT and >30 samples affected by subchromosomal CNVs.

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Materials and Methods

Study design

This blinded, retrospective clinical study included samples from women considered at increased risk for fetal aneuploidy based on advanced maternal age ≥35 years, a positive serum screen, an abnormal ultrasound finding, and/or a history of aneuploidy. Archived samples were selected for inclusion in the study by an unblinded internal third party according to the requirements documented in the study plan. Samples were then blind-coded to all operators and the analysts who processed the samples. After sequencing, an automated bioinformatics analysis was performed to detect whole chromosome aneuploidies and subchromosomal CNVs. Results were compiled electronically and were reviewed by a subject matter expert who

assigned the final classification. This manual review mimics the process in the clinical laboratory, where cases are reviewed by a laboratory director before a result is signed out. Completed classification results were provided to the internal third party for determination of concordance. Analyzed samples had confirmation of positive or negative events by either G-band karyotype or microarray findings from samples collected through either chorionic villus sampling (CVS) or amniocentesis. Circulating cell-free "fetal" DNA is believed to originate largely from placental trophoblasts. Genetic differences between the fetus and the placenta can occur (eg, confined placental mosaicism), leading to discordance between NIPT results and cytogenetic studies on amniocytes or postnatally obtained samples.[8] Results from CVS by chromosomal microarray were thus considered the most accurate ground truth. Therefore, discordant results originating from amniocytes (karyotype or microarray) were resolved by sequencing at high coverage (an average of 226 million reads per sample). Sequencing depth has been shown as the limiting factor in NIPT methods, with increased depth allowing improved detection of events in samples with lower fetal fractions or improved detection of smaller events.[9] High coverage sequencing has been used in multiple studies to unambiguously identify subchromosomal events[6,10-12] and was used here as a reference for performance evaluation in discrepant amniocentesis samples.

Details of the sample demographics are described in Table 1. Indications for invasive testing are described in Table 2.

TABLE 1
Demographic and pregnancy-related data

Demographic	Median	Range
Maternal age, y (Ntotal = 1177)	36.0	17.8–47
Gestational age, wk (Ntotal = 1183)	17	8–38
Maternal weight, lb (Ntotal = 1168)	150	93–366
Procedure	Percent	(Naffected/Ntotal)
CVS	14.5	(175/1203)
Amniocentesis	85.2	(1025/1203)
Both	0.2	(3/1203)
Confirmation	Percent	(Naffected/Ntotal)
Karyotype	90.4	(1089/1205)
Microarray	5.8	(70/1205)
Both	3.8	(46/1205)

Choice of invasive procedure, choice of diagnostic test, and demographic data were not available for all 1208 samples included in study.

Naffected refers to the number of samples with the indicated procedure or confirmation; Ntotal refers to number of samples where that data were available.

CVS, chorionic villus sampling.

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Sample collection

In total, 1222 maternal plasma samples were previously collected using 4 investigational review board (IRB)-approved protocols with a small subset (9 samples) comprising remnant plasma samples collected from previously consented patients in accordance with the Food and Drug Administration guidance on informed consent for in vitro diagnostic devices using leftover human specimens that are not individually identifiable.[13] Samples from 2 of the protocols (Compass IRB no. 00508 and Western IRB no. 20120148) were collected from high-risk pregnant subjects prior to undergoing a confirmatory invasive procedure (1189 samples). Samples from the other 2 protocols (Compass IRB no. 00351 and Columbia University IRB no. AAAN9002) came from subjects who were enrolled in the studies after receiving the fetal karyotype and/or microarray results of a confirmatory invasive procedure (24 samples). All subjects provided written informed consent prior to undergoing any study-related procedures. A total of 5321 high-risk subjects were recruited into the 4 clinical studies indicated above at the time of sample selection. To be eligible for inclusion into this study, subjects had to have met all protocol inclusion and no exclusion criteria, have fetal outcome determined by karyotype and/or microarray, and have at least 1 plasma aliquot of $\geq$3.5 mL obtained from whole blood collected in a BCT tube (Streck, Omaha, NE). There was no sample selection preference based on high-risk indication. All subjects meeting these selection criteria with an abnormal fetal outcome as needed for the study were identified and pulled from inventory. These were then supplemented with randomly selected subjects with samples meeting the same selection criteria but with a normal karyotype to reach the total of 1222 samples for testing.

Library preparation, sequencing, and analytical methods

Libraries were prepared and quantified as described by Tynan et al.[14] To reduce noise and increase signal, sequencing depth for this analysis was increased to target 32 million reads per sample. Sequencing reads were aligned to hg19 using Bowtie 2.[15] The genome was then partitioned into 50-kbp nonoverlapping segments and the total number of reads per segment was determined, by counting the number of reads with 5' ends overlapping with a segment. Segments with high read count variability or low mapability were excluded. The 50 kbp read counts were then normalized to remove coverage and guanine/cytosine biases and other higher-order artifacts using the methods previously described in Zhao et al.[6]

The presence of fetal DNA was quantified using the regional counts of whole genome single-end sequencing data as described by Kim et al.[16]

TABLE 2
Indications for testing

Indication	Euploid, n = 1009	T21, n = 87	T18, n = 29	T13, n = 15	SCA, n = 26	CNV, n = 44	Total, n = 1210
Positive serum screening	404 (40%)	36 (41.4%)	13 (44.8%)	4 (26.7%)	3 (11.5%)	16 (36.4%)	476 (39.3%)
Maternal age >35 y	418 (41.4%)	28 (32.2%)	10 (34.5%)	4 (26.7%)	2 (7.7%)	10 (22.7%)	472 (39%)
Ultrasound abnormality	152 (15.1%)	34 (39.1%)	6 (20.7%)	10 (66.7%)	16 (61.5%)	22 (50%)	239 (19.8%)
Family history	33 (3.3%)	4 (4.6%)	1 (3.4%)	0 (0%)	1 (3.8%)	1 (2.3%)	40 (3.3%)
Not specified	85 (8.4%)	11 (12.6%)	5 (17.2%)	2 (13.3%)	4 (15.4%)	2 (4.5%)	109 (9%)

Each cell lists count (%) of indication within chromosome category. Samples may have multiple indications for testing and therefore do not sum to 100%. As 2 T18 samples also had SCA, total number of abnormalities and euploid samples sum to 1210.

CNV, copy number variant; *SCA*, sex chromosome aneuploidy; *T13*, trisomy 13; *T18*, trisomy 18; *T21*, trisomy 21.

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Genomewide detection of abnormalities

Circular binary segmentation (CBS) was used to identify CNVs throughout the entire genome by segmenting each chromosome into contiguous regions of equal copy number.[17] A segment-merging algorithm was then used to compensate for oversegmentation by CBS when the signal-to-noise ratio was low.[18] Z-scores were calculated for both CBS-identified CNVs and whole chromosome variants by comparing the signal amplitude with a reference set of samples in the same region. The measured Z-scores form part of an enhanced version of Chromosomal Aberration Decision Tree previously described in detail in Zhao et al.[6]

To further improve specificity of CNV detection, bootstrap analysis was performed as an additional measure for the confidence of the candidate CNVs. The within sample read count variability was compared to a normal population (represented by 371 euploid samples) and quantified by bootstrap confidence level. To assess within sample variability, the bootstrap resampling described below was applied to every candidate CNV.

For each identified segment within the CNV, the median shift of segment fraction from the normal level across the chromosome was calculated. This median shift was then corrected to create a read count baseline for bootstrapping. Next, a bootstrapped segment of the same segment length as the candidate CNV was randomly sampled with replacement from the baseline read counts. The median shift was then applied to this bootstrapped fragment. The segment fraction of the bootstrapped fragment was calculated as follows:

$$\text{segment fraction} = \frac{\sum \text{read counts within segment}}{\sum \text{read counts across the autosome}}$$

This process was repeated 1000 times to generate a bootstrap distribution of segment fractions for an affected population. A normal reference distribution was created based on the segment fraction of the same location as the candidate CNV in 371 euploid samples. A threshold was then calculated as the segment fraction that was at least 3.95 median absolute deviations away from the median segment fraction of the reference distribution. Lastly, the bootstrap confidence level was calculated as the proportion of bootstrap segments whose fractions had absolute z-statistics above the significance threshold.

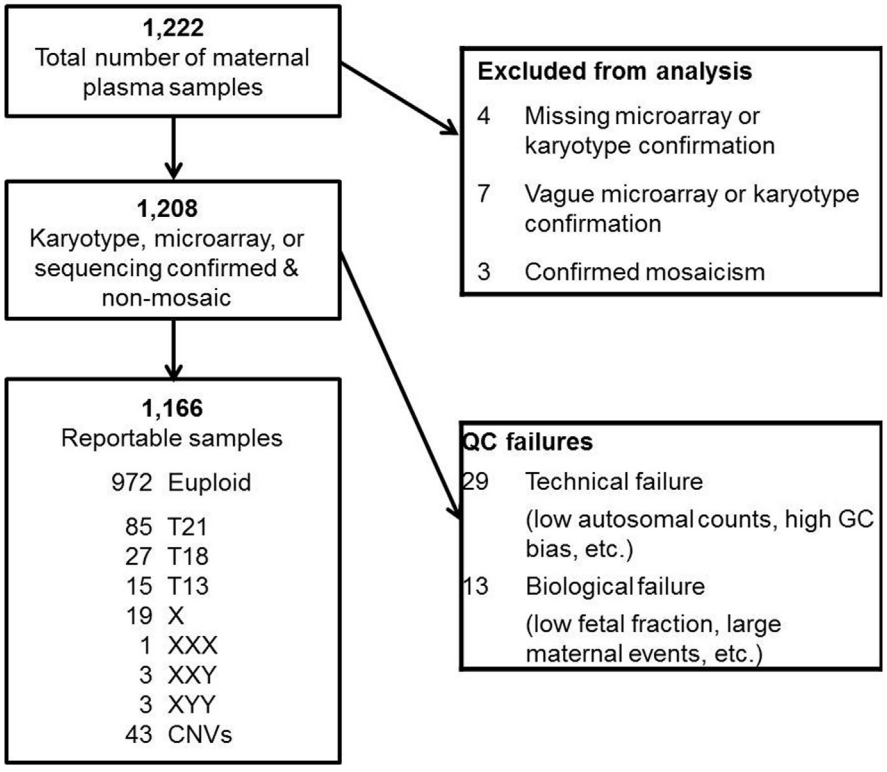
A whole chromosome or subchromosomal abnormality is detected as described in Zhao et al.[6]

Results

The study comprised a total of 1222 maternal plasma samples. After unblinding, 11 samples were excluded because they had no or insufficient karyotype or microarray information (Figure 1) and 3 samples were excluded because of confirmed mosaicism.

Of the remaining 1208 samples, 42 were flagged as nonreportable using quality criteria that had been established prior to analysis, leaving 1166 reportable samples for analyses comprising 13% (n = 153) of samples with common whole chromosome 21, 18, 13, X, or Y aneuploidies and 3.6% (n = 43) of samples with subchromosomal CNVs or rarer whole chromosome aneuploidies. Technical failure criteria included but were not limited to: low library concentration, low raw autosomal counts, high GC bias, poor normalization, and high bin variability (Figure 1). Biological failure criteria included low fetal fraction (<4%) and large maternal CNV events. The most common reason for failure was low fetal fraction (n = 11). During review of the data, 1 sample was signed out as T18 (and was included in the analyzed cohort), even though it did not meet the autosomal count minimum. This sample had sufficient counts for the determination of standard aneuploidies, but not sufficient counts for the detection of subchromosomal CNVs. The 42 nonreportable samples showed no evidence of enrichment for whole chromosome/subchromosomal positive samples (5 positive of 42 nonreportable samples vs 204 positive of 1166 reportable samples). Details of nonreportable and excluded samples are provided in Supplemental Table 1. Concordant with previous studies, the overall reportable rate on first aliquots of maternal plasma was 96.5%. The overall no-call rate per patient is expected to be approximately 1% when a second aliquot is available, based on published clinical laboratory experience.[19]

FIGURE 1
Flow chart showing sample exclusions and reasons for nonreportable samples



One trisomy 18 (T18) sample was also XXY sample and another T18 sample was also X sample. For samples with discordant results for sequencing vs karyotype or microarray derived from testing of amniocytes, uniplex sequencing was used for confirmation.

CNV, copy number variant; *QC*, quality control; *T13*, trisomy 13; *T21*, trisomy 21.

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T21, T18, and T13 detection

Among the 1166 reportable samples, there were 85 T21 samples, 27 T18 samples, and 15 T13 samples (Figure 1). All euploid, T21, T18, and T13 samples (determined by invasive diagnostic procedures) were classified correctly by NIPT. One sample was classified by NIPT as T21 but had a normal (46, XX) karyotype by amniocentesis (Supplemental Table 2). This discrepancy was adjudicated through high coverage uniplex sequencing (typically with >180 million reads) according to our study plan (Supplemental Figure 1, D). The sample showed 16.3% fetal fraction, but the gain of genetic material from chromosome 21 was concordant with 6.5% fetal fraction. This is suggestive of confined placental mosaicism, a relatively common cause of discordant results between amniocyte results and CVS or placentally derived cell-free DNA (cfDNA) analysis.[8] Table 3 summarizes the performance for T21, T18, and T13. For T21, the sensitivity was 100% (95% confidence interval [CI], 94.6–100%) and the specificity was 100% (95% CI, 99.6–100%). For T18, the sensitivity was 100% (95% CI, 84.4–100%) and the specificity was 100% (95% CI, 99.6–100%). For T13, the sensitivity was 100% (95% CI, 74.7–100%) and the specificity was 100% (95% CI, 99.6–100%).

TABLE 3
Clinical performance for indicated abnormalities and fetal sex

Abnormality	Concordant positive	Discordant positive	Concordant negative	Discordant negative	Sensitivity (95% CI)	Specificity (95% CI)
T21	85	0	1081	0	100% (94.6–100%)	100% (99.6–100%)
T18	27	0	1139	0	100% (84.4–100%)	100% (99.6–100%)
T13	15	0	1151	0	100% (74.7–100%)	100% (99.6–100%)
SCA	26	1	1117	0	100% (84.0–100%)	99.9% (99.4–100%)
CNVs[a]	42	1	1122	1	97.7% (86.2–99.9%)	99.9% (99.4–100%)

Analyte	Concordant male	Discordant male	Concordant female	Discordant female	Accuracy (95% CI)
Fetal sex	583	4	578	1	99.6% (98.9–99.8%)

CI, confidence interval; *CNVs*, copy number variants; *SCA*, sex chromosome aneuploidy; *T13*, trisomy 13; *T18*, trisomy 18; *T21*, trisomy 21.

[a] Includes 8 samples with detected whole chromosome trisomies, and 35 samples with subchromosomal CNVs.

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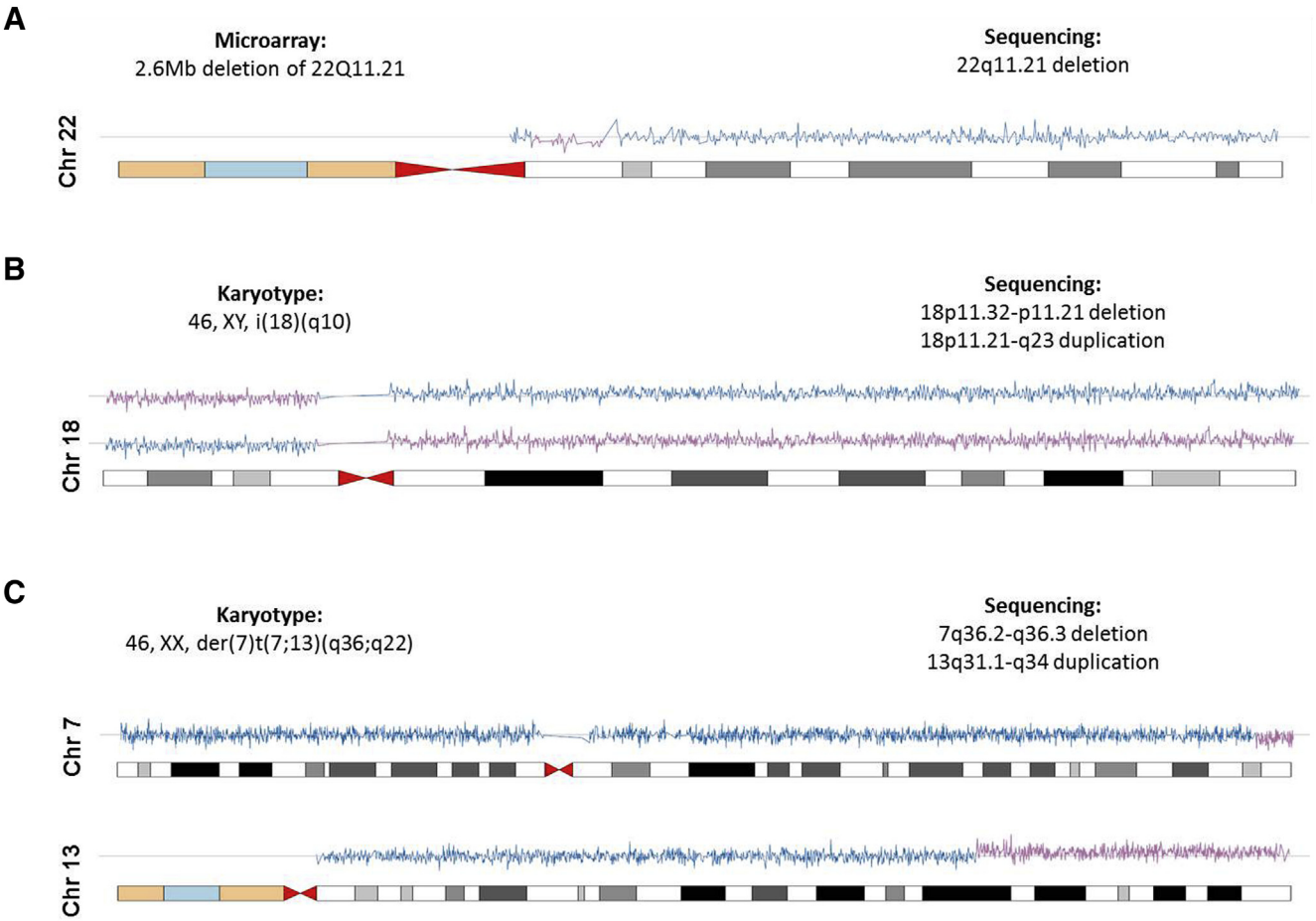
FIGURE 2
Examples of copy number variants detected by sequencing



A, Chromosome (Chr) 22 ideogram showing sequencing-based detection of 22q11.21 deletion (DiGeorge syndrome) that was confirmed by karyotype and microarray. **B**, Chr 18 ideogram for sample with karyotype 46, XY, i(18)(q10). **C**, Chr 7 and 13 ideograms for sample with karyotype 46, XX, der(7)t(7;13)(q36;q22).

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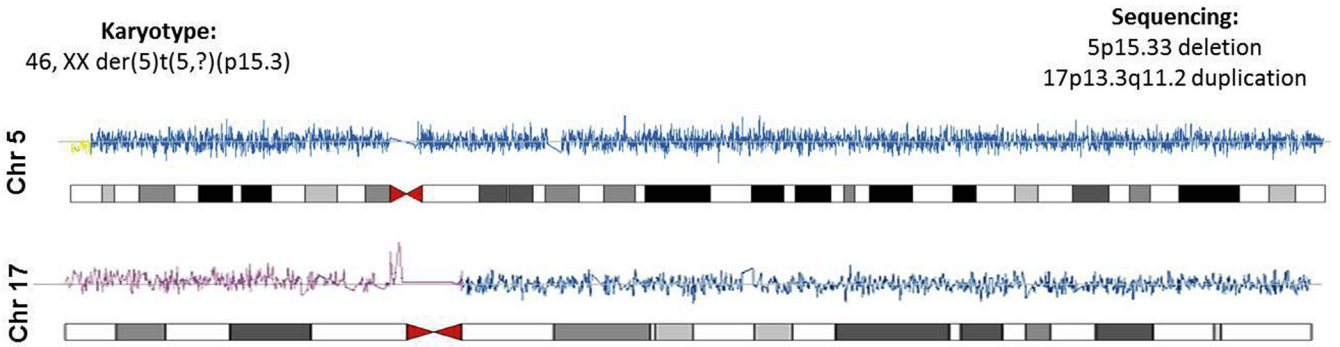
SCA detection

In the 1166 samples reportable for all chromosomal abnormalities, there were 21 samples flagged as nonreportable specifically for SCA classification based on thresholds for the chromosome X Z-score and chromosome Y Z-score as described by Mazloom et al.[20] There was also 1 additional sample with an apparent maternal XXX that was flagged as nonreportable for SCA because the maternal event distorted the sex chromosomal representation to a degree that fetal events could not be classified. Among the remaining 1144 samples reportable for SCAs, there were 7 discordant positives that were classified as normal (46, XX) by karyotype and as XO by sequencing at 6-plex (Supplemental Table 2). In all discordant cases, the karyotype had been obtained from amniocyte samples. This phenomenon is well described and may be attributed to varying levels of placental or maternal mosaicism.[21] Uniplex sequencing confirmed 6 of the 7 XO samples. The seventh sample had a nonreportable result at uniplex coverage, hence the existing amniocentesis result was used as truth resulting in 1 false-positive assignment. Overall, the sensitivity for SCA was 100% (95% CI, 84.0−100%) with a specificity of 99.9% (95% CI, 99.4−100%) (Table 3).

Genomewide detection of CNVs

The test was also designed to detect CNVs ≥7 Mb (including whole chromosome abnormalities other than T13, T18, T21, and SCAs) as well as select microdeletions <7 Mb.[5] Among the 1166 samples reportable for subchromosomal abnormalities, there were 43 samples that had positive results for a variety of CNV aberrations: Wolf-Hirschhorn syndrome deletions, DiGeorge syndrome deletions, Prader-Willi/Angelman syndrome deletions, cri du chat syndrome deletions, and a variety of ≥7-Mb CNVs including both subchromosomal CNVs and whole chromosome trisomies. Several

FIGURE 3
Clarification of karyotype findings by sequencing of maternal plasma



Normal regions (blue). Deletions and duplications that are detected with high bootstrap confidence levels (>0.99) (red). Low bootstrap confidence events (yellow). Chromosome (Chr) 5 and 17 ideograms are shown for sample with karyotype of 46, XX, der(5)t(5;?)(p15.3;?). Unidentified duplication on karyotype is from Chr 17.

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examples of NIPT-detected CNVs confirmed by microarray or karyotype are shown in Figure 2.

Overall, the sensitivity for detection of whole chromosome and subchromosomal abnormalities other than T13, T18, T21, and SCAs was 97.7% (95% CI, 86.2−99.9%) and the specificity was 99.9% (95% CI, 99.4−100%) (Table 3). One case was clearly mosaic for T22 by both standard coverage and uniplex sequencing (Supplemental Table 2 and Supplemental Figure 1, E), but was classified as normal by microarray analysis. Because the invasive diagnosis came from microarray analysis of cells derived from CVS, our study design considered this as the gold standard, and this outcome was considered a discordant positive. Another case showed no gain or loss of genetic material with both standard and uniplex sequencing for a sample that had a 46, XX, der(12)t(12;19)(p13.1;q13.1) karyotype (Supplemental Figure 2, B). This outcome was considered as a discordant negative given that the invasive procedure was CVS. A set of 7 samples were classified as full chromosomal trisomies, and because the karyotype or microarray results were derived from amniocytes in these cases, uniplex sequencing was performed for adjudication per the study design. The trisomy finding was confirmed in each case by uniplex sequencing, and these findings were considered as concordant positives (Supplemental Figure 1).

In addition to the high accuracy demonstrated in the results, in some cases NIPT could also provide clarification about the origin of extra genetic material when the G-banding pattern was not sufficiently clear. In 1 case, the amniocyte karyotype finding, 46, XX, der(5)t(5;?)(p15.3;?), indicated a deletion on chromosome 5 and a duplication of unknown origin (Figure 3). NIPT identified an 18.6-Mb duplication representing the entire short arm of chromosome 17, indicating this fetal tissue possessed a trisomy of chromosome 17p that was not clarified by standard karyotype. Trisomy 17p is associated with developmental delay, growth retardation, hypotonia, digital abnormalities, congenital heart defects, and distinctive facial features.[22] Additional cases are shown in Supplemental Figure 3.

Comment

Couples and families who are seeking prenatal information about the genetic health of their baby have multiple options today, ranging from noninvasive tests that screen for select chromosomal abnormalities to invasive procedures including karyotype and microarray testing that can deliver the most comprehensive genomic assessment. Current NIPT methods provide information about a limited set of conditions that typically include T21, T18, T13, and SCAs. In this study we expand testing to the entire genome. The results demonstrate that high sensitivity and specificity were maintained while adding genomewide clinically relevant content.

Most NIPT tests suffer from the problem of multiple hypothesis testing; when multiple regions of the genome are tested independently, false-positive rates become additive.[23] This can result in unacceptably high false-positive rates and lead to maternal anxiety and additional unnecessary clinical testing. Methods that target individual regions of the genome cannot overcome these limitations because they are part of the design of the test.[24] We have shown that these limitations can be overcome by using a genomewide approach, as described here and previously.[6] In this study we demonstrate that the test can provide excellent performance for CNVs $\geq$7 Mb and select microdeletions <7 Mb across the genome.

This study does have limitations. Although we analyze 2- to 6-fold more samples with subchromosomal CNVs than previous studies,[6,11,24] the total number of affected samples is still relatively small compared to studies validating performance for detection of common whole chromosome

aneuploidies.[25] It remains challenging to statistically validate detection of rare individual chromosomal variations. As such we utilize samples with CNVs of varying size and location throughout the genome with varying frequency of individual occurrence. This study design with its high positivity rate allows only estimation of PPV. As sensitivity and specificity values have proven stable in previous studies when applied to the real-world samples, PPV can be considered a function of incidence rate. Incidence estimates of larger karyotype-detectable genomewide subchromosomal CNVs vary but are typically considered between 1 in 200 and 1 in 400.[26] Modeled PPVs for these incidence rates would therefore be between 68-83% (Supplemental Figure 4). Due to the high sensitivity observed, negative predictive values are expected to be well >99.9% when incidence rates are <1 in 200. As our study utilized only 1 aliquot for analysis, our reportable rate was 96.5%, with an expected reportable rate of ~99% with availability of 2 aliquots.[19] Of the 11 samples not reported due to the biological effect of low fetal fraction, all had normal karyotype result.

Another challenge commonly observed in novel technologies with increased performance is the compilation of samples with appropriate ground truth. In our study we face the technical challenge of comparing sequencing vs G-band karyotype or microarray, as well as the biological aspect of cfDNA vs amniocyte or CVS. We have used a method that prioritizes CVS over amniocentesis because of the placental origin of cfDNA.[8] Factors that can lead to discordant results include confined placental mosaicism, maternal mosaicism, vanishing twins, undetected tumors, and technical errors. Consequently our method of evaluation has the potential to overestimate performance with regard to fetal outcome. However, knowledge about the genetic health of the placenta might in itself be advantageous when guiding the pregnancy, for example in the case of intrauterine growth restriction. Prospective studies using the method described here with follow-up of birth outcome will further enhance understanding of clinical effectiveness.

An interesting observation in this study was the sometimes subjective nature of karyotyping. When G-banding is used as an analytical method, the optical resolution is occasionally not sufficient to determine the origin of additional genetic material, making a clinical interpretation more difficult. These ambiguities are eliminated when using NIPT because the test requires sequencing alignment to the genome before DNA gains and losses are determined. However, G-banding will, for the foreseeable future, be the superior methodology to determine copy number neutral structural changes.

In pregnancies that can benefit from additional information, this test provides more clinically relevant results than previous NIPT options. However, its role as a follow-up test to abnormal ultrasound findings or as a general population screen will likely be debated for the foreseeable future. It has been argued that the incidence of clinically relevant subchromosomal CNVs could be as high as 1.7% in a general population,[26] and early microarray data[27] indicate that as many as 30% of subchromosomal CNVs could be >7 Mb. In consequence, the incidence for these large CNVs may be around 0.5% in the general population, substantially higher than the incidence of T21.

In summary, this clinical study provides validation for an approach that extends the clinical validity of cfDNA testing, now providing detection of T21, T18, T13, SCAs, fetal sex, and genomewide detection of subchromosomal and whole chromosomal abnormalities. Overall, subchromosomal abnormalities and aneuploidies other than T13, T18, T21, and SCAs were detected with a combined clinical sensitivity and specificity of 97.7% (95% CI, 86.2–99.9%) and 99.9% (95% CI, 99.4–100%), respectively. This enables comprehensive noninvasive chromosomal assessment that was previously available only by karyotype, and in some cases, may clarify cryptic findings otherwise identifiable only by microarray. ■

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SUPPLEMENTAL TABLE 1
Breakdown of excluded and nonreported samples

Reason	SampleID	Fetal fraction	Karyotype/microarray outcome
Exclusions			
Missing invasive confirmation	53063	0.130	Normal
	77320	0.079	Normal
	53863	0.068	Normal
	RAS0027851	0.065	Other: T16 by NIPT. SCA 46-XY
Mosaicism	54172	0.093	Normal karyotype; low level mosaic T21 by FISH
	77168	0.247	Other: 46,XX,dup(1)(q21q32) - mosaic
	70600	0.094	Other: 47,XX,+i(12)(p10)[13]/46,XX[2].nuc ish (ETV6x4,AML 1/20[25/25] - tetrasomy 12p mosaicism
Vague karyotype result	72938	0.093	Other: 46,XY,var(18)(q11.2)
	71894	0.114	Other: 46XX, 22qvar
	54681	0.081	Other: 47, XX, +mar
	72288	0.148	Other: 47, XX, +mar
	54039	0.098	Other: 47, XX, +mar.ish idic(15;15)(q13;q13)(D15Z1++, SNRPN++)
	89063	0.096	Other: 47, XX, +ESAC considered normal female
	73189	0.089	Other: 47, XY, +ESAC
Biological exclusions			
Fetal fraction	55348	0.040	Normal
	53720	0.036	Normal
	54528	0.039	Normal
	89588	0.036	Normal
	89473	0.032	Normal
	77411	0.019	Normal
	77188	0.028	Normal
	89311	0.037	Normal
	53415	0.027	Normal
	53801	0.036	Normal
	53103	0.037	Normal
Maternal event	55197	0.065	Normal
	45424	0.149	T21
Technical exclusions			
Low aligned counts	77478	0.079	Normal
High variability	77065	0.087	T18
	77066	0.092	T21
	77068	0.083	Normal
	77042	0.109	Normal
CADET	77340	0.131	Normal
	55462	0.078	Normal
	89583	0.082	Normal

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SUPPLEMENTAL TABLE 1
Breakdown of excluded and nonreported samples *(continued)*

Reason	SampleID	Fetal fraction	Karyotype/microarray outcome
	77040	0.058	Normal
GC warning	93693	0.112	Normal
	77392	0.091	Normal
	89526	0.105	Normal
	93679	0.074	Normal
	89152	0.076	Normal
	53815	0.077	Normal
	73161	0.161	Normal
	73017	0.158	Normal
	71006	0.072	Other: 46xx, add(18)(q21.3)
Laboratory director review	77075	0.104	Normal
	54320	0.048	Normal
	54212	0.147	Normal
	77067	0.072	Normal
	53061	0.043	Normal
	89359	0.064	Normal
	89149	0.094	Normal
	53420	0.075	Normal
	55112	0.092	Other: 48,XXX,+18
Library failure	77347	0.148	Normal
	54227	0.112	Normal
Nonreportable for sex chromosome aneuploidy	89529	0.106	Normal
	77445	0.232	Normal
	55123	0.084	Normal
	89185	0.068	Normal
	89607	0.076	Normal
	53057	0.057	Normal
	55444	0.041	Normal
	54458	0.215	Normal
	55220	0.068	Normal
	93678	0.145	Normal
	70268	0.094	Normal
	53109	0.087	Normal
	55454	0.047	Normal
	55064	0.099	Normal
	77285	0.120	Normal
	55260	0.154	Normal
	77157	0.042	Other: 46,XX,der(18)(qter-q11.3-qter). Additional material added to 18p

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SUPPLEMENTAL TABLE 1

Breakdown of excluded and nonreported samples *(continued)*

Reason	SampleID	Fetal fraction	Karyotype/microarray outcome
	73164	0.085	Other: abnormal female karyotype with chromosome 13q deletion
	45364	0.102	T18
	89582	0.099	T18
	77476	0.108	T21
	89220	0.130	T21

CADET, Chromosomal Aberration Decision Tree; *ESAC*, extra structurally abnormal chromosome; *FISH*, fluorescent in situ hybridization; *NIPT*, noninvasive prenatal test; *SCA*, sex chromosome aneuploidy; *SNRPN*, small nuclear ribonucleoprotein polypeptide N; *T18*, trisomy 18; *T21*, trisomy 21.

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SUPPLEMENTAL TABLE 2
Uniplex sequencing results for discordant samples

	Invasive test results			Noninvasive test results	
Category	Test type	Sample type	Finding	6-plex	1-plex
T21	Karyotype	Amniocentesis	Normal	T21	T21
SCA	Karyotype	Amniocentesis	Normal	X	X
	Karyotype	Amniocentesis	Normal	X	X
	Karyotype and microarray	Amniocentesis	Normal	X	NR for SCA
	Karyotype	Amniocentesis	Normal	X	X
	Karyotype	Amniocentesis	Normal	X	X
	Karyotype	Amniocentesis	Normal	X	X
	Karyotype	Amniocentesis	Normal	X	X
CNVs	Karyotype	CVS	46, XX, der(12)t(12;19)(p13.1;q13.1) mat	Normal	Normal
	Karyotype	Amniocentesis	46, XX,der(18)(qter->q11.2::p11.3->qter)	Normal	Normal
	Karyotype	Amniocentesis	46, XY, add(20)(q11.2)	Normal	Normal
	Karyotype	Amniocentesis	46, XY, add(18)(p11.3)	Normal	Normal
	Karyotype	Amniocentesis	Normal	9p24.3p13.1 duplication	9p24.3p13.1 duplication
	Karyotype	Amniocentesis	Normal	chr1p36.33p36.22 deletion, chr17q25.1q25.3 duplication	chr1p36.33p36.22 deletion, chr17q25.1q25.3 duplication
	Karyotype	Amniocentesis	Normal	T16	T16
	Microarray	Amniocentesis	Normal	T14	T14
	Karyotype	Amniocentesis	Normal	T8	T8
	Karyotype	Amniocentesis	Normal	T22	T22
	Karyotype	Amniocentesis	Normal	T16	T16
	Microarray	CVS	Normal	T22	T22
	Karyotype	Amniocentesis	Normal	T16	T16
	Microarray	Amniocentesis	Normal	T8	T8

Samples with results at 6-plex that were discordant with microarray or karyotype findings were resequenced at higher coverage for confirmation.

CNVs, copy number variants; *CVS*, chorionic villus sampling; *SCA*, sex chromosome aneuploidy; *T*, trisomy.

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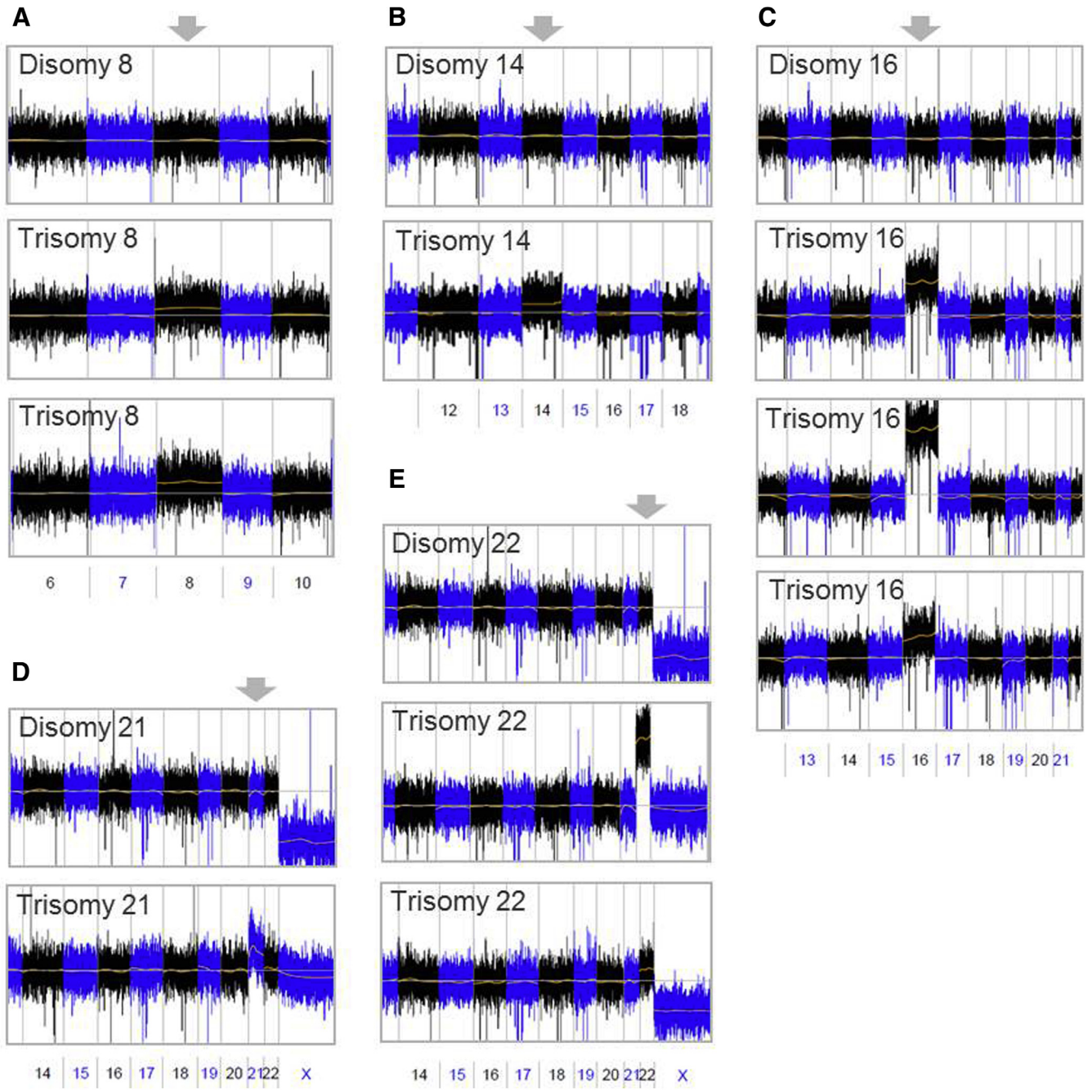
SUPPLEMENTAL FIGURE 1
Analysis of disomy and trisomy



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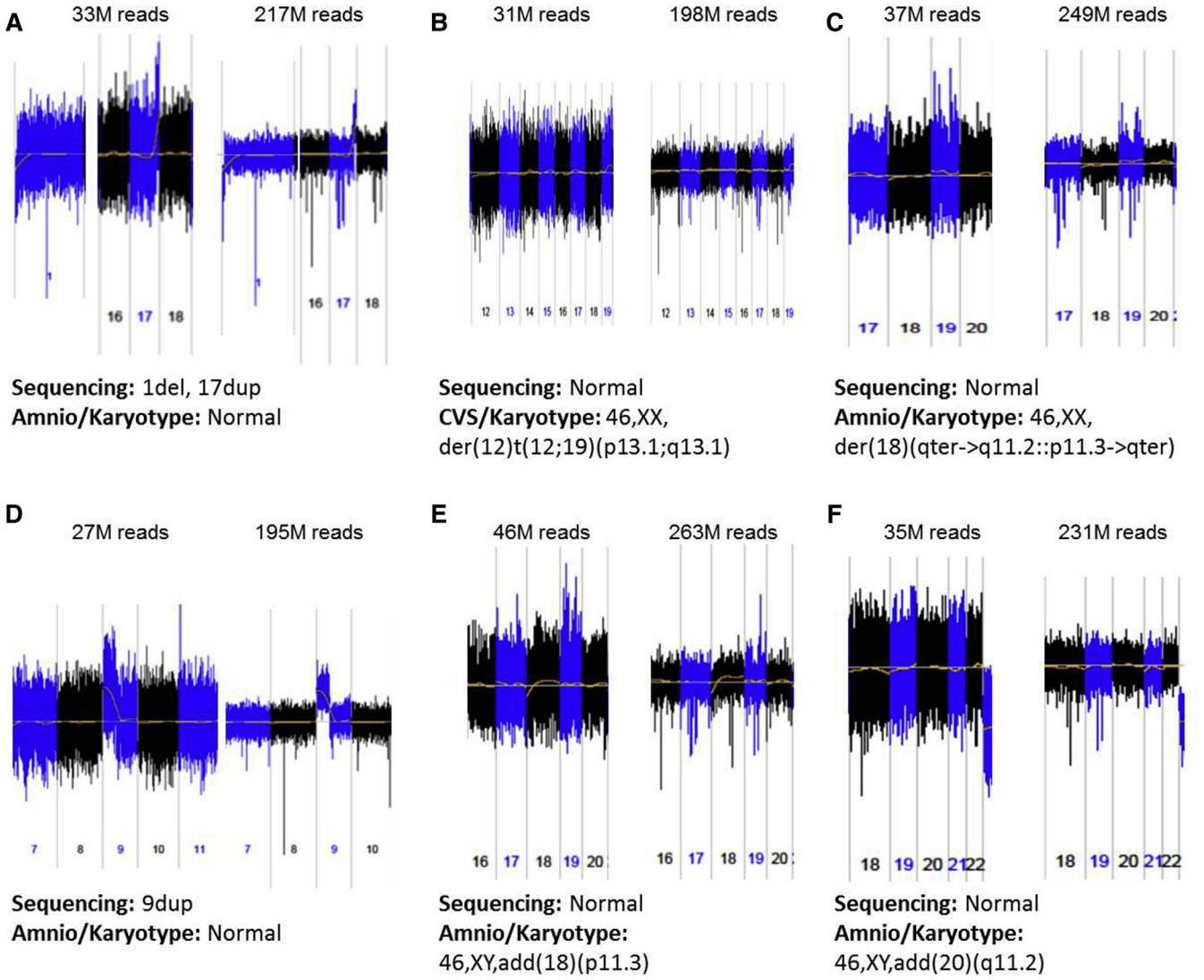
SUPPLEMENTAL FIGURE 2
Analysis of sequencing and karyotypes



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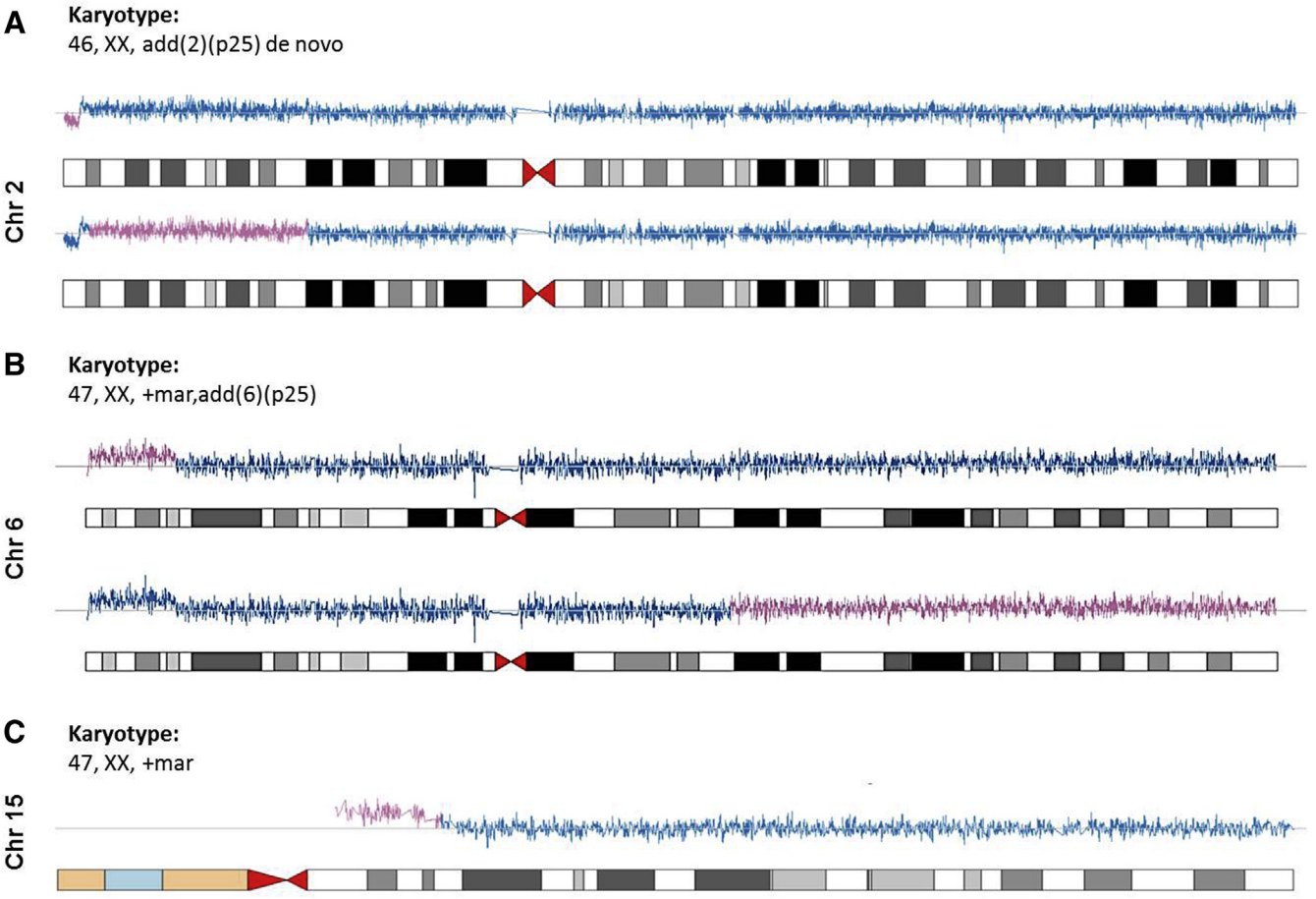
SUPPLEMENTAL FIGURE 3
Analysis of karyotypes



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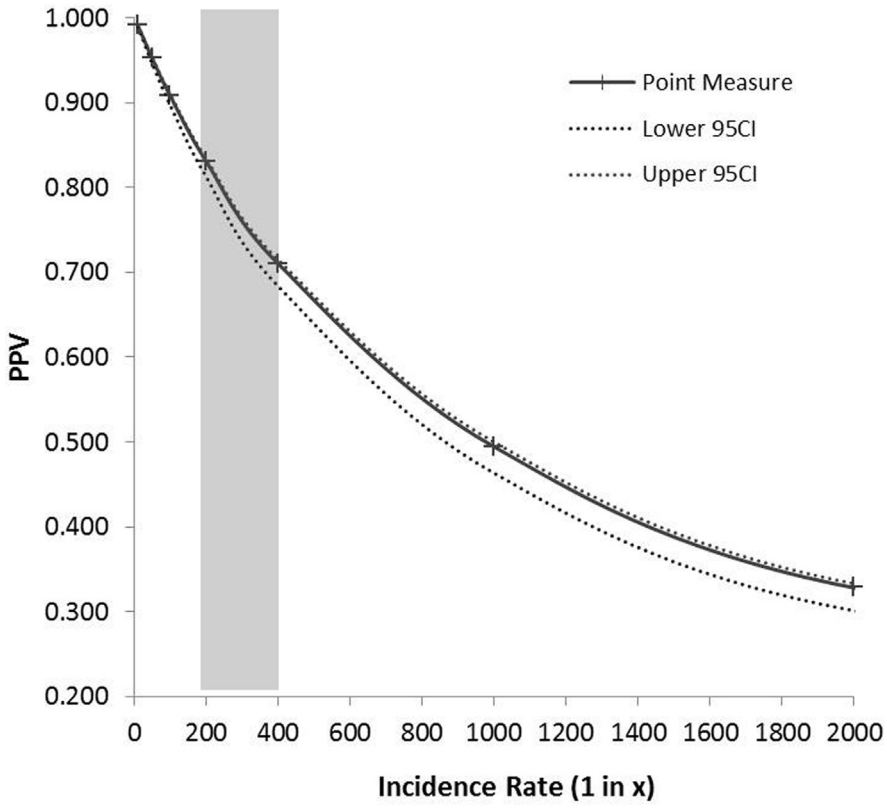
SUPPLEMENTAL FIGURE 4
Incidence rate



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