Use of targeted sequencing of SNPs to achieve highly accurate non-invasive detection of fetal aneuploidy of chromosomes 13, 18, 21, and sex chromosomes.

Brynn Levy[1], Zachary Demko[2], Sallie McAdoo[2], Bernhard Zimmermann[2], Matthew Hill[2], Matthew Rabinowitz[2]

[1]Department of Pathology and Cell Biology, Columbia University, New York, NY [2]Natera Inc., San Carlos, CA.

Abstract

Objective: To non-invasively detect all whole chromosome abnormalities on chromosomes 13, 18, 21, X and Y, in the fetus through analysis of cell-free fetal DNA in maternal blood.

Study Design: 763 maternal plasma samples (including 673 euploid and 90 aneuploid samples) were collected from patients at ≥9 weeks of gestation under institutional review board (IRB) approved protocols and analyzed using Natera's Panorama NIPT test. 351 samples were externally blinded,* and the remainder were internally blinded where the number and identity of aneuploidies were not known. 138 of the samples had a paternal genetic sample available, which was included in the analysis. Aneuploid samples included Trisomy 21, Trisomy 18, Trisomy 13, and Monosomy X. Cell-free DNA was isolated, amplified using multiplex PCR targeting 19,488 SNP loci covering chromosomes 13, 18, 21, X, and Y, and sequenced. Sequencing data was analyzed using the informatics enhanced Next-generation Aneuploidy Testing Using SNPs (NATUS). The algorithm uses Bayesian statistics to analyze multiple copy number hypotheses and performs a Maximum Likelihood Estimation (MLE) over the hypotheses. The method determines a calculated accuracy for each call, and does not require a reference chromosome.

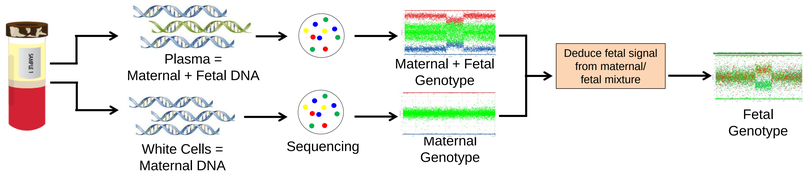
Results: 718 samples passed the quality control thresholds and were called at all five chromosomes with one wrong call (3,594/3,595 correct calls) and a positive predictive value of 100%. The mean calculated accuracy of calls in samples that passed the QC threshold was >99.8%. Samples that failed to meet this threshold (45 / 763; 5.9%) were typically of poor DNA quality. Identification of these samples enables a quick redraw and retesting of the patient rather than increasing the risk of false negatives/positives. One false negative and no false positives were observed in this cohort.

Conclusion: The Panorama™ test using the NATUS algorithm detected chromosomally abnormal fetuses from a maternal blood sample at chromosomes 13, 18, 21, X, and Y with sensitivity of 100% at autosomes and 92% at the sex chromosomes and specificity of 100% overall. NATUS also calculated an accuracy for each call, demonstrating similar accuracy across all chromosomes tested, permitting maternal blood draws early in the pregnancy while maintaining a very low error rate.

*Samples provided by Kypros Nicolaides and Harbinder Brar.

Methods

The Panorama Test™ Methodology



The Next-generation Aneuploidy Test Using SNPs (NATUS) Algorithm

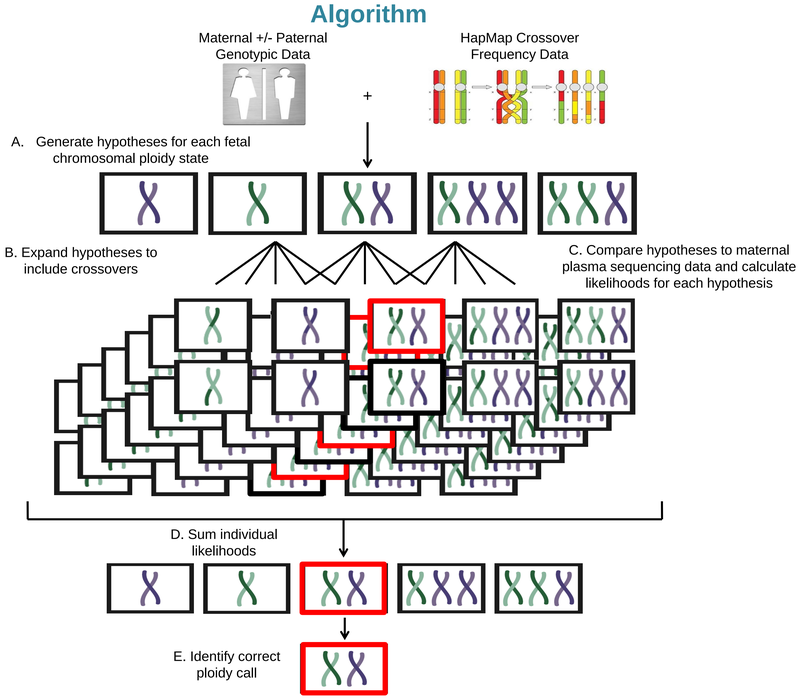


Figure 1: The Panorama™ Non-Invasive Prenatal Test (NIPT)/NATUS Method. The NATUS algorithm considers parental genotypes, HapMap crossover frequency data, and possible fetal chromosome copy number to calculate expected allele distributions for a large number of hypothetical possible fetal genotypes and ploidy states (A-B). It then calculates a likelihood for each hypothesis by comparing the various predicted allele distributions to the actual allelic distributions measured in the cfDNA sample (C), sums the likelihoods for each hypothesis corresponding to the three ploidy states (monosomy, disomy, or trisomy) based on the sequencing data (D), and calls the ploidy state with maximum likelihood as the actual ploidy state, also giving the fetal fraction in the Maximum Likelihood optimization (E).

Graphical Representation of NATUS Results

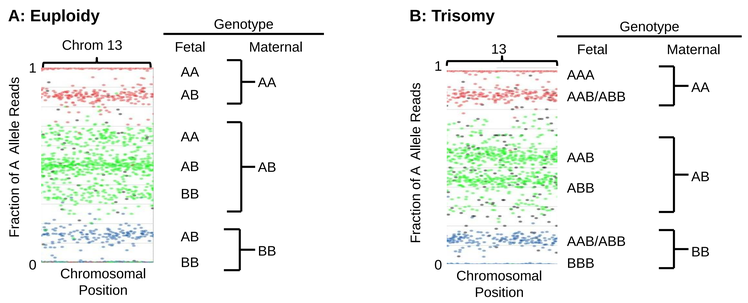


Figure 2: The SNP data is presented here in a simplified fashion as plots depicting the ratios of the two most likely alleles, arbitrarily labeled as A and B. Note that this is not how the algorithm makes ploidy calls, but is one method for visualizing the data. X-axis: Individual SNPs located on the target chromosome. Y-axis: [A allele reads] / [Total allele reads]. Fetal and maternal genotypes are indicated to the right. Each spot represents the sum of maternal and fetal cfDNA allele reads. Red and blue SNPs represent homozygous maternal AA and BB alleles, respectively; green SNPs represent heterozygous maternal AB alleles. **A.** The typical pattern depicting euploidy on an autosomal chromosome. The center trio of green clusters and presence of red and blue peripheral clusters indicate the presence of two chromosomes. **B.** The typical pattern depicting trisomy. This plot depicts a single trisomic chromosome 13. The peripheral clusters are largely unaffected, however, the center trio of clusters has condensed into a duo of clusters. Together, this indicates the presence of three chromosomes.

Results

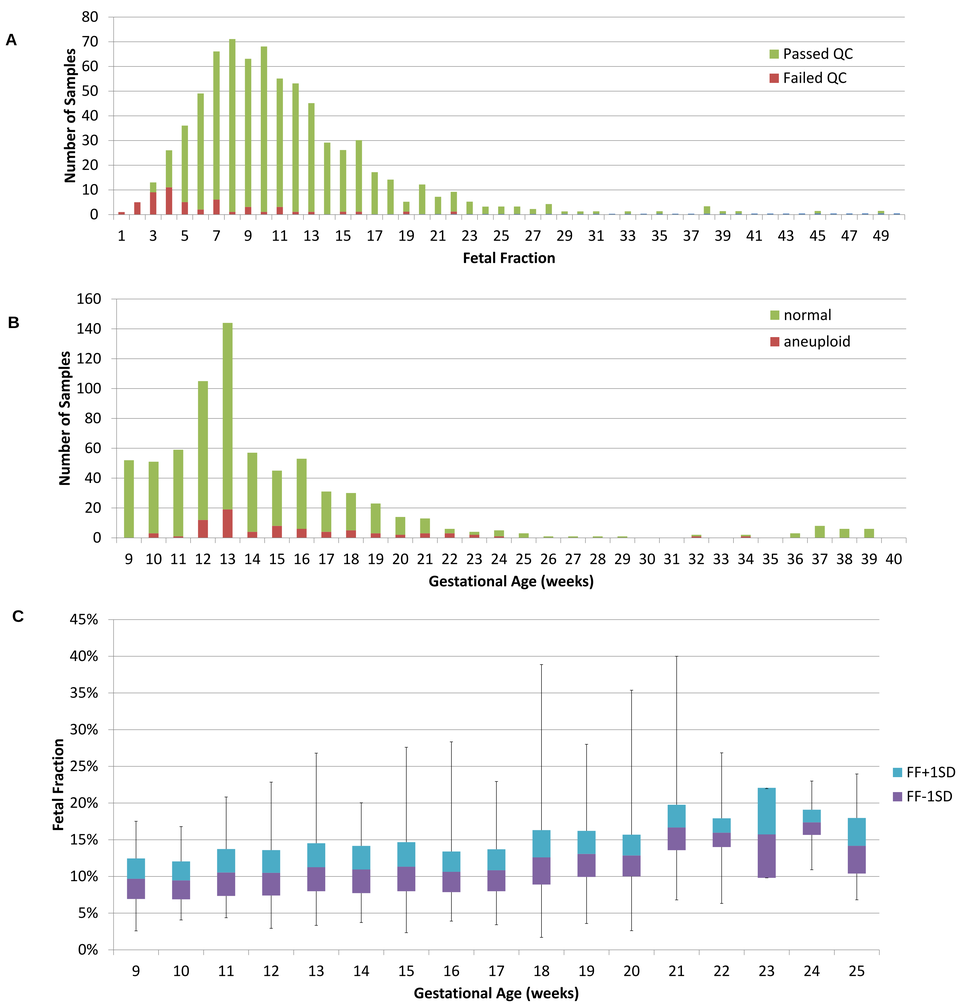


Figure 3: The NATUS method, samples, and performance. **A.** Histogram of samples in this cohort stratified by fetal fraction. Samples that passed quality control are indicated in green and samples that failed quality control are indicated in red. Average gestational age = 15.0 weeks. **B.** Histogram of the samples in this cohort stratified by gestational age. Average fetal fraction = 11.7%. Truth was verified on all samples through amniocentesis, CVS, cord blood, or child saliva or buccal samples. **C.** Box plot of mean fetal fraction. Minimum and maximum are indicated by horizontal black lines at the upper and lower limits of the vertical black lines, boxes signify one-standard-deviation above (blue) or below (purple) the mean, which is indicated where the colors meet.

Panorama Test™ Performance using NATUS Algorithm

	Sensitivity*	Specificity*
T21:	47/47 100% (CI: 92.5-100%)	672/672 100% (CI: 99.5-100%)
T18:	15/15 100% (CI:78.2-100%)	704/704 100% (CI: 99.5-100%)
T13:	7/7 100% (CI: 59.0-100%)	712/712 100% (CI: 99.5-100%)
45,X:	11/12 92% (CI: 61.5-99.8%)	707/707 100% (CI: 99.5-100%)

Note that this methodology identified Klinefelter (47,XXY) and 47,XYY in a previous dataset with perfect sensitivity and specificity, however, this sample cohort was not analyzed for and did not include any sex chromosome trisomies.[1-3] Three samples known to be mosaic (1 x T18, 2 x 45,X) were analyzed separately and excluded from this cohort. Two of three were called aneuploid, and one was called normal.

*We thank Professor Kypros Nicolaides and Dr. Harbinder Brar for providing samples.

Advantages of Redraws

Overall, 45 / 763 (5.9%) samples did not pass NATUS's quality control thresholds, and could have had a redraw requested had they been clinical samples. The NATUS QC thresholds are significantly more sophisticated than other reported methods, and include not only the number of targeted reads and fetal fraction, but also metrics indicative of the amount of input DNA, the over/underabundance of haplotype blocks due to consanguinity or multiple gestations, uninformative SNPs, possible contamination, and non-optimal molecular biology performance, giving NATUS unprecedented insight into the DNA composition of each sample.

The QC metrics are in place to ensure high predictive power of the algorithm's output. Samples not meeting set thresholds are not issued a result as a means to keep the false positive and false negative rate low. This is in contrast to methods that report a result for samples whose parameters lie in regions of higher false positive or lower detection rates. For example, most current commercial methods consider samples with a fetal fraction below 4% as having insufficient fetal DNA to make a call. However, samples having between 4% and 8% fetal fraction contain a marginally sufficient amount of data; only the NATUS algorithm can differentiate between samples that have enough data to make a high confidence call, and those which do not. Other methods are more likely to make a wrong call in this region.

The majority of patients whose samples did not pass QC will be encouraged to give a second sample; greater than 90% of these redraws are expected to give a result.[1] A small proportion of these cases would not be recommended for redraw: for example, those cases with multiple gestations and high consanguinity.

Conclusions

Panorama/NATUS-targeted analysis of SNPs represents a novel method for non-invasive prenatal aneuploidy testing. Here, the NATUS method identified chromosome copy number at chromosomes 13, 18, 21, X, and Y, detecting T13, T18, T21 with 100% sensitivity, 45,X with 92% sensitivity, and 100% specificity for all samples that passed the quality test. The method also detects 47,XXY, 47,XYY, and triploidy (data not shown) and is expected to be able to detect sub-chromosomal abnormalities. This method also obviates issues with amplification variability caused by GC bias, and generates a more powerful sample-specific calculated accuracy for samples with low fetal fractions of cfDNA . Together, this holds promise for a non-invasive screening test with unparalleled accuracy and scope, close to the ceiling of accuracy defined by mosaicism.

References

1. Zimmermann B, Hill M, Gemelos G, *et al.* Non-invasive prenatal aneuploidy testing of chromosomes 13, 18, 21, X, and Y using targeted sequencing of polymorphic loci. Prenat Diag. doi: 10.1002/pd.3993.
2. Savage M. Merging into Clinical Practice: Updates in Non-invasive Prenatal Testing. Abstract 304, 31st NSGC Annual Education Conference, October 26, 2012 in Boston, MA.
3. Rabinowitz M, Gemelos G, Hill M, *et al.* Non-Invasive Prenatal Aneuploidy Testing of Chromosomes 13, 18, 21, X, and Y Using Targeted Sequencing of Polymorphic Loci. American Society of Human Genetics Meeting, November 6-10, 2012, in San Francisco, CA.