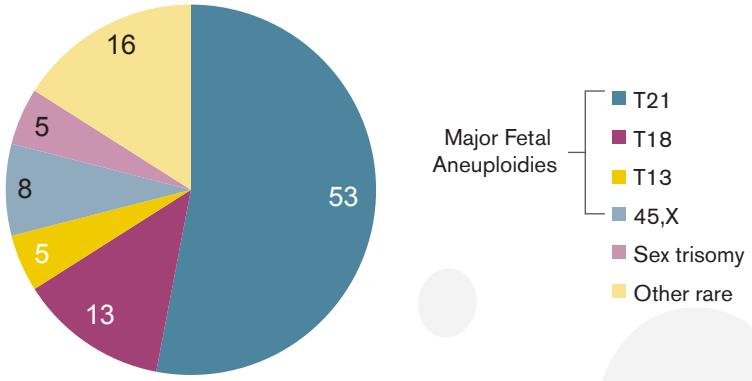
Analytical Validation of the verifi® prenatal test: Enhanced Test Performance for Detecting Trisomies 21, 18, and 13 and the Option for Classification of Sex Chromosome Status

INTRODUCTION

In March 2012, Verinata Health began offering the verifi® prenatal test to healthcare providers in the US. The validation study for the test performance was reported by Bianchi, et al. and published in *Obstetrics and Gynecology*[1]. The verifi® test detects trisomies 21, 18 and 13 from a single maternal blood sample, and is indicated for pregnant women with singleton gestation at 10+ weeks and at high-risk for fetal aneuploidy. In July 2012, the verifi® prenatal test was expanded to include the Monosomy X (MX or Turner Syndrome) Option, currently indicated for patients with fetal cystic hygroma. As shown in Figure 1, these four fetal chromosome aneuploidies detected by the verifi® test account for ~80% of the total prenatal chromosomal abnormalities.

Figure 1. Prenatal Prevalence of Chromosomal Abnormalities



Data adapted from Wellesley, D, *et al.*[2], Rare chromosome abnormalities, prevalence and prenatal diagnosis rates from population-based congenital anomaly registers in Europe. *Eur J of Hum Gen*, 11 January 2012.

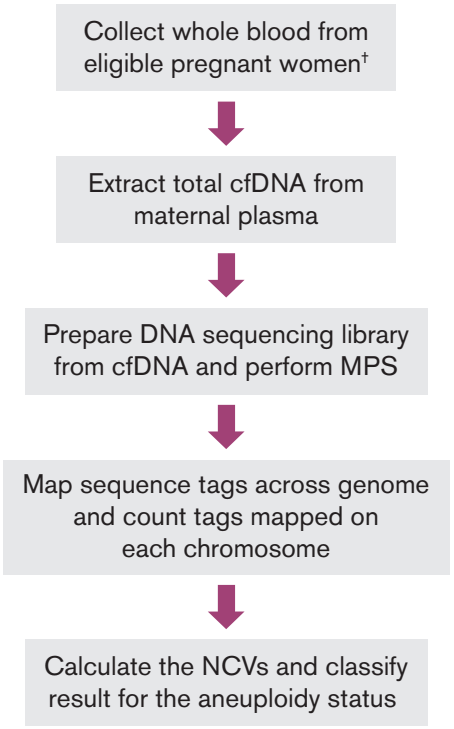
The verifi® prenatal test is provided through Verinata's CLIA certified, CAP accredited clinical laboratory. Verinata's research team continues to study and apply the latest advances in sequencing technology to further expand and improve the test performance. This paper describes the most recent results and advancements incorporated into the newly updated verifi® prenatal test, which, in addition to testing for the most common autosomal aneuploidies, also now includes the new Sex Chromosome Option. This option may be elected by the healthcare provider and patient to provide a more detailed level of information. For example, in cases where sex-linked disorders or ambiguous genitalia are of concern and/or to detect sex chromosome aneuploidies, such as monosomy X, which are frequently associated with pre- and postnatal medical complications that could benefit from early recognition.

BACKGROUND

Massively parallel sequencing (MPS) of total cell free DNA (cfDNA) extracted from maternal plasma has been proven as an accurate and reliable method to detect fetal chromosome aneuploidies[1,3,4]. Unique to Verinata's method, a Normalized Chromosome Value (NCV) is calculated for each chromosome tested. This NCV calculation removes

variation within and between sequencing runs to optimize test precision. The procedure of MPS and NCV classification is summarized in Figure 2.

Figure 2. Procedure of MPS Analysis and NCV Classification.



†A single tube of maternal blood is shipped to Verinata laboratory and processed to plasma upon receipt.

Since publication of the clinical validation results, Verinata's research team has analyzed and implemented several changes to the testing procedure that yield enhanced test performance. These changes include:

- Incorporating new DNA sequencing chemistry
- Further optimizing NCV calculations through increased counting statistics
- Expanding verifi® test results to include sex chromosome status with six possible classifications
- Re-defining classification terminology for chromosomes 21, 18, and 13 to include "Aneuploidy Suspected (Borderline Value)" to more accurately reflect results that are suggestive of aneuploidy and warrant further clinical evaluation

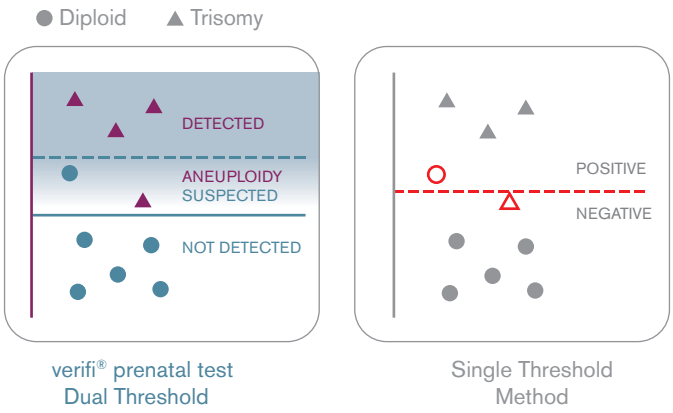
Incorporating New DNA Sequencing Chemistry

The performance of fetal aneuploidy testing by MPS is largely dependent on the counting statistics dictated by the capacity of the sequencing method[5]. Utilizing the latest sequencing technology improves counting statistics and thus leads to better test precision and performance. In this study, the number of sequence tags produced by the updated sequencing chemistry (TruSeq v3.0) is **2.4 times greater**[6] than the number generated in the original clinical validation study[1] using the older chemistry (TruSeq v2.5). This improvement in counting statistics provides further optimization of the classification algorithm and enhanced performance for testing chromosomes 21, 18, and 13 as shown below. An additional benefit is the ability to more accurately classify the status of sex chromosomes.

Dual-Threshold Detection for Chromosomes 21, 18, and 13

The classification algorithm implemented in this study provides greater clarity on borderline results that can occur with all sequencing-based testing methods. The "Aneuploidy Suspected (Borderline Value)" is designed to minimize the percentage of cases that give borderline results yet provide useful information. This result designation gives an indication to the provider and patient that chances of a false positive are higher than in the "Aneuploidy Detected" zone. As with any cfDNA test for fetal aneuploidy, the confirmation via an invasive procedure is recommended for 'positive' results (either "Aneuploid Detected" or "Aneuploidy Suspected") from the verifi® prenatal test. A comparison between the verifi® test classification and a conventional single threshold method is show in Figure 3.

Figure 3. Comparison between the dual-threshold verifi® prenatal test and the conventional single threshold method.



METHODS

DNA Sequencing and NCV Calculation

Eligible samples from singleton pregnancies that had previously been analyzed in the MELISSA study[1] were used to validate the performance of the verifi® prenatal test with the new test enhancements described above. Samples were available for each category of analysis as follows: chr 21 (n = 500), chr 18 (n = 501), chr 13 (n = 501), and sex chromosomes (n=508, including 10 samples with sex chromosome aneuploidies). DNA sequencing libraries were prepared as previously described[1]. MPS was performed on the Illumina Hi-Seq 2000 instrument using TruSeqTM v3.0 sequencing chemistry. Sequence tags from MPS were mapped to the human genome and NCVs were calculated for chromosomes 21, 18, 13, X, and Y using an improved algorithm that involved a new training set and calculation of new optimal reference chromosome denominators.

Classification of Aneuploidy Status

Under the new test conditions, the classification scheme for aneuploidy status of chromosomes 21, 18, and 13 are "Aneuploidy Detected", "No Aneuploidy Detected", and "Aneuploidy Suspected (Borderline Value)".

Sex chromosome results are classified into one of six discrete categories: XX, XY, MX, XXX, XXY, and XYY. The classification scheme for sex chromosome status does not employ the "Aneuploidy Suspected (Borderline Value)" category.

RESULTS

Chromosomes 21, 18, and 13

Since the precision of NCV calculations are proportional to the number of unique chromosome sites mapped by sequence tags[5], the incorporation of both new sequencing chemistry and an improved analysis algorithm has resulted in improved test performance. In Table 1 below, **both "Aneuploidy Detected" and "Aneuploidy Suspected**

(Borderline Value)" results were considered 'positive' results for performance calculation. For chromosome 21 there was one false positive result, for chromosome 18 there were two false positives and one false negative, and for chromosome 13 there were two false negatives. These results from analytical validation are comparable to (or better than) the performance data of the MELISSA study published previously[1].

Table 1. verifi® prenatal test Performance for Chromosomes 21, 18, and 13.

Chromosome	Samples Analyzed	Sensitivity	95% CI	Specificity	95% CI
21	500	>99.9% (90/90)	96.0 –100.0	99.8% (409/410)	98.7 –100.0
18	501	97.4% (37/38)	86.2 – 99.9	99.6% (461/463)	98.5 – 100.0
13	501	87.5% (14/16)	61.7 – 98.5	>99.9% (485/485)	99.2 – 100.0

The new classification category "Aneuploidy Suspected (Borderline Value)" is introduced to highlight borderline results where a false positive result is more likely to occur. Both affected and unaffected cases may occur in this zone. In the "Aneuploidy Suspected (Borderline Value)" zone in this study, there were 3 subjects (0.6%) for chr 21 (two trisomy 21 and one euploid), 2 subjects (0.4%) for chr 18 (one trisomy 18 and one euploid), and only one subject with trisomy 13 for chr 13 (0.2%). For results that occur in this zone and in the "Aneuploidy Detected" zone, it is recommended that clinical correlation with ultrasound findings and other screening tests be considered, and if definitive diagnosis is desired, chorionic villous sampling or amniocentesis is recommended.

Sex Chromosomes

The test performance for sex chromosome classifications are shown in Table 2. In total, 508 subjects were analyzed for sex chromosome classification. In addition to sensitivity and specificity for the classifications of 'XX' and 'XY', a single determination of accuracy is also provided. The accuracy measure used here represents overall percent agreement, and it is calculated as the sum total of true positives plus true negatives divided by the total subjects tested.

Table 2. verifi® prenatal test Performance for Sex Chromosome Classifications

Sex Chromosome Classification	Number Analyzed*	Sensitivity	95% CI	Specificity	95% CI	Accuracy	95% CI
XX	508	97.6% (243/249)	94.8–99.1	99.2% (257/259)	97.2–99.9	98.4%	96.9–99.3
XY	508	99.1% (227/229)	96.9–99.9	98.9% (276/279)	96.9–99.8	99.0%	97.7–99.7
MX	508	95.0% (19/20)	75.1–99.9	99.0% (483/488)	97.6–99.7	N/A	N/A

Of significance in this version of the test, **fetal cystic hygroma is *not* a requirement for classification of monosomy X.** The performance statistics of MX shown in Table 2 have been updated to reflect the results when all patients are tested for sex chromosome classification. In the event when MX is detected, further clinical evaluation including consideration for invasive prenatal procedure is recommended for confirmation.

The newly updated verifi® test also includes identification of three other sex chromosome aneuploidies, namely XXX, XXY, and XYY. Due to the small number of test subjects bearing these sex chromosome aneuploidies that were available for study (XXX (n=4), XXY (n=3), and XYY (n=3)), the results are given for these determinations without calculating formal performance values. Three of 4 subjects with XXX karyotype, 2 of 3 subjects with XXY karyotype, and 3 of 3 subjects with XYY karyotype were correctly classified. In practice, if the classifications of these

rare sex chromosome aneuploidies are reported, genetic counseling and clinical correlation with ultrasound findings and other screening tests is indicated. If definitive diagnosis is desired, chorionic villous sampling or amniocentesis is recommended.

The current verifi® test does not distinctly identify each cell line present if sex chromosome mosaicism is present. The occurrence of sex chromosome mosaicism is extremely low (0.27% for maternal age > 35 years or 0.17% for maternal age < 35 years[7]). In this study, seven subjects with mixed sex chromosome mosaicism (e.g., 45,X/46,XY; 45,X/46,XX; and 45,X/47,XXX) were excluded from the performance analysis. When tested, the results for such samples fall into one of the six defined sex classification zones (e.g., 45,X/46,XY falls into the 'XY' zone and the monosomy X component is masked). This information should to be taken into consideration by the provider when electing sex chromosome testing by this method and pre-test genetic counseling is recommended.

CONCLUSIONS

The verifi® prenatal test employs the latest sequencing technology with advanced data analysis to yield results for chromosomes 21, 18, and 13 as well as sex chromosomes to provide greater information and value for health care providers and patients. Utilizing massively parallel sequencing, the verifi® test leverages whole genome sequencing to expand the test menu.

The verifi® test is the only non-invasive prenatal test method to include a separate borderline result for the classification of chromosomes 21, 18, and 13. An "Aneuploidy Suspected (Borderline Value)" result alerts the clinician that the sample either represents an aneuploidy sample with lower fetal fraction that did not reach the "Aneuploidy Detected" zone, or a case at the extreme end of the normal distribution for diploids. As shown in this study, sensitivity for trisomy 21 detection was extremely high (>99.9%), and the one false positive that did occur (0.2%) was designated as "Aneuploidy Suspected (Borderline Value)". Because results in this zone suggest possible aneuploidy, further clinical evaluation including invasive prenatal procedure is warranted for confirmation.

The new sex chromosome analysis option for the verifi® test introduces the ability to classify sex chromosomes into six categories and includes the detection of sex chromosome aneuploidies such as monosomy X, which can be associated with prenatal and perinatal complications. Evidence of fetal cystic hygroma by ultrasound is no longer required to test for monosomy X, although it remains relevant to consider the verifi® test in this setting due to the relatively high association of fetal aneuploidies with cystic hygroma. Overall, it expands the options to obtain more complete prenatal information through noninvasive means than previously possible and with a high degree of accuracy.

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