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Performance Characteristics of a Next Generation Sequencing-Based cfDNA Assay for Common Aneuploidies in a General Risk Population

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Abstract

Introduction: BillionToOne developed and launched the UNITY Aneuploidy Screen, a Non-Invasive Prenatal Test (NIPT) that uses Next-Generation Sequencing (NGS) to detect autosomal trisomies and sex chromosome aneuploidies from cell-free DNA.

Methods: In this study, we provide an overview of the clinical performance of the UNITY Aneuploidy Screen. Neonatal and fetal outcomes were obtained from pregnancies that had the UNITY Aneuploidy Screen as part of clinical care, and performance analytics were calculated.

Results: The median turnaround time was 4 days, and more than 99% of tests had an informative fetal risk result (1.3% initial no-call rate and 0.15% no-call rate after redraw). The outcomes cohort included 1,691 pregnancies with an average maternal age of 29 years. The sensitivity for autosomal trisomies was 99.7% (95% CI: 98.3%-99.7%) and specificity was 99.9% (95% CI: 73.4%-100%). The PPV for autosomal trisomies was 90.8% (95% CI: 85.9%-95.78%) for the full cohort and 94.6% (95% CI: 89.4%-99.7%) for individuals 35 years or older.

Conclusion: These data demonstrate the UNITY Aneuploidy Screen has excellent performance and is comparable or superior to other commercially available aneuploidy screens. Furthermore, despite having a younger study sample (average age at delivery of 29 years with 80% under the age of 35 at delivery), the autosomal trisomy PPV was on par with PPVs reported in other studies with maternal ages averaging 35 years and was higher than the PPVs listed by ACOG 226 Practice Bulletin.

Keywords: Cell-Free DNA (cfDNA); Next-Generation Sequencing (NGS); Noninvasive Prenatal Testing (NIPT); Negative Predictive Value (NPV); Positive Predictive Value (PPV)

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Introduction

The discovery of fetal cell-free DNA (cfDNA) in the circulating blood of pregnant individuals over two decades ago led to the development of Noninvasive Prenatal Testing (NIPT) [1]. NIPT became commercially available in 2011 using a blood sample from a pregnant individual to analyze cfDNA and determine the risk for common fetal aneuploidies [2,3]. Chromosomal aneuploidies occur in approximately 1 in 150 pregnancies with trisomies 21, 18, and 13 being the most prevalent aneuploidies among live births. Presently, the American College of Obstetricians and Gynecologists (ACOG) recognizes that "cfDNA is the most sensitive and specific screening test for the common fetal aneuploidies" and recommends offering aneuploidy screening to all pregnant women [4].

The UNITY Aneuploidy Screen is a clinical cfDNA NIPT assay offered by BillionToOne Laboratory for a general risk pregnant population. It assesses the fetal risk for autosomal trisomies (21, 18, and 13) and sex chromosome aneuploidies (monosomy X, XXX, XXY, and XYY) as early as 10 weeks gestation. Optionally, the ordering provider can request reporting of fetal sex as well as fetal red blood cell antigen status. Fetal RhD NIPT can be used to guide administration of Rho(D) immune-globulin for RhD-negative individuals and Fetal Antigen NIPT can be used to guide surveillance of alloimmunized pregnancies; both practices that has been widely adopted in European Health Systems [5-9].

The UNITY Aneuploidy Screen uses targeted next-generation sequencing (NGS) to determine the chromosomal dosage. BillionToOne's patented Quantitative Counting Template (QCT) molecular counting technology helps to ensure robust clinical performance and quality control even at low fetal fractions. The fetal risk is determined *via* a likelihood ratio approach that uses a unified

statistical model of observed chromosome dosage and fetal fraction, which can be more robust to maternal mosaicism and/or low fetal fraction than fetal risk models that use dosage only [10]. Importantly, the model also identifies samples in which there is insufficient evidence to differentiate between an affected and unaffected fetus, in which case a backup specimen is tested. A repeat test from a backup specimen is also performed to confirm all positive cases.

To increase the accuracy of fetal genetic sex and sex chromosome aneuploidy, the UNITY Aneuploidy Screen assesses a combination of data points including the presence and fetal fraction of the Y (ffY) chromosome, detection of the paternal X chromosome, the dosage of the X chromosome, and detection of the *SRY* gene. The combination and concordance of the data obtained from multiple data points rather than relying on a single data point such as the presence or absence of Y chromosome material leads to superior performance including the ability to identify potential egg donor pregnancies, maternal organ transplants, or undetected twins.

The UNITY Aneuploidy Screen also includes an opt-in choice to detect fetal antigen status to inform pregnancy management for RhD-negative and/or alloimmunized pregnant individuals. UNITY also offers screening for the 22q11.2 copy number variant and carrier screening with reflex to single-gene NIPT for common autosomal recessive conditions. The performance of these assays has been previously published [10-13]. In this paper, we provide a comprehensive overview of the clinical performance of the UNITY Aneuploidy Screen for trisomies 13, 18, 21, and monosomy X.

Materials and Methods

UNITY Aneuploidy turn around time and no call rate were computed from consecutive cases completed on the current version of the assay from March 1st, 2023, through December 12th, 2023.

Fetal/neonatal outcomes were collected from pregnancies that had the UNITY Aneuploidy Screen as part of clinical care at US-based clinics. Outcomes were solicited via email, text, and phone calls to providers and patients regarding pregnancies screened from February 8th, 2021 to June 17th, 2023 with a due date prior to October 10th, 2023. Most outcomes were collected through the BTO Quality Assurance (QA) program. The BTO QA program preferentially solicits outcomes from cases where the UNITY Aneuploidy NIPT screen returned a "high-risk" fetal result. Outcomes were also collected *via* a retrospective chart review of pregnancies and resulting neonates from four collaborating institutions where the UNITY Aneuploidy Screen was part of clinical care. A minority of unsolicited outcomes were reported by the ordering provider. All samples were analyzed on the most recently validated UNITY Aneuploidy NIPT algorithm. Outcome classifications were determined using a previously published classification system [14]. All outcome determinations were made by two or more certified genetic counselors.

Statistical analysis

UNITY Aneuploidy NIPT results were categorized as "low risk" or "high risk" in accordance with our clinical reporting practices. "Low risk" results for trisomies 13, 18, and 21 and monosomy X apply to those with a post-test risk of <1 in 10,000. "High risk" results for trisomies 13, 18 and 21 and monosomy X refer to those with a post-test fetal risk of 1 in 10 or higher. A case was determined to be concordant if the UNITY Aneuploidy NIPT result was "low risk" and the fetus/neonate was unaffected or if the UNITY Aneuploidy NIPT result was "high risk" and the fetus/neonate was affected.

In the current paper, outcomes were solicited from 50% of the cases with UNITY Aneuploidy Screen "high-risk" results and 38% of the outcomes were confirmed. Preferentially obtaining high-risk screen results is a common practice when assessing the Positive Predictive Value (PPV) of screening assays for rare conditions [15,16]; however, this sampling method impacts the other performance metrics. The Negative Predictive Value (NPV), specificity, and sensitivity were computed based on cohort size and proportion of confirmed outcomes collected using a previously established method [16]. Briefly, this method computes values for the full cohort under the assumption of random sampling of outcomes within each outcome group (high risk and low risk) and the proportion of true positives and false positives outcomes obtained is representative of the full cohort [15,16]. The number of true negatives, false negatives and false positives are a function of the proportion of known outcomes for the "high-risk" and "low-risk" cases. The limits of the confidence intervals for sensitivity and specificity were computed using a published method [16] and 95% confidence intervals for PPV and NPV were computed assuming a normal distribution of data.

Results

The clinical experience of the UNITY Aneuploidy Screen across consecutive clinical cases in 2023 showed a median turnaround time of four days with 95% of results returned within 10 days and a median fetal fraction of 8.5% (mean 9.3%). An informative fetal risk result was returned for 98.7% of cases after the initial sample was submitted, and 0.15% were no-called following a second draw (Table 1). A high-risk result for an autosomal aneuploidy was returned to 0.49% of pregnancies and 0.29% of pregnancies had a high-risk result for monosomy X.

UNITY Aneuploidy Screen performance metrics were calculated for a cohort of 1,691 pregnancies with outcomes on the fetus/neonate; of those, 121 had an autosomal aneuploidy and 24 had monosomy X. The cohort had an average maternal age of 29 years (range: 15-46 years) with 80% of the sample being younger than 35 years of age at the time of delivery. The UNITY Aneuploidy Screen results and fetal/neonatal outcomes are summarized in (Table 2).

The sensitivity and specificity for the autosomal trisomies were both greater than 99%, and the PPV was 90.8% (95% CI: 85.9%-95.8%) (Table 3). When stratified by maternal age at delivery, the observed PPV was higher for individuals age 35 years and older at delivery (94.6%, 95% CI: 89.4%-99.7%) and lower for individuals younger than 35 at delivery (86.0%, 95% CI: 76.9%-95.0%). This PPV

Table 1: UNITY aneuploidy screen test characteristics on consecutive samples in 2023.

	Mean	Median	5%-95% tile
Gestational Age (weeks)	13.9	12.4	10.3-23.1
Fetal Fraction	9.30%	8.50%	3.4%-18.1%
Turn Around Time (days)	5	4	2-10

Table 2: Pregnancy outcomes for 1691 UNITY aneuploidy screened pregnancies.

	Trisomy 21	Trisomy 18	Trisomy 13	Monosomy X
True Positive	67	41	11	21
False Positive	7	1	4	29
True Negative	1616	1648	1676	1638
False Negative	1	1	0	3

Table 3: Performance metrics and confidence intervals of UNITY aneuploidy NIPT in a general risk population.

	Trisomy 21 (CI)	Trisomy 18 (CI)	Trisomy 13 (CI)	T21, T18, T13 (CI)	Monosomy X (CI)
Sensitivity[a,b]	99.7% (98.5%-99.7%)	99.5% (97.6%-99.5%)	100% (100%-100%)	99.7% (98.3%-99.7%)	97.3% (87.5%-98.7%)
Specificity [a,b]	99.7% (83.5%-100%)	>99.9% (90.2%-100%)	>99.9% (96.1%-100%)	99.9% (73.4%-100%)	99.9% (87.2%-100%)
PPV[c]	90.5% (83.9%-97.2%)	97.6% (93%-100%)	73.3% (50.9%-95.7%)	90.8% (85.9%-95.8%)	42% (28.3%-55.7%)
NPV[a,c]	>99.9% (99.8%-100%)	>99.9% (99.8%-100%)	>99.9% (100%-100%)	>99.9% (99.7%-100%)	>99.9% (100%-100%)

[a]Calculated based on sample size proportion of confirmed outcomes [16]
[b]Confidence intervals were computed according to previously published methods [16]
[c]95% confidence intervals were computed assuming data was normally distributed [15]

pattern is expected as the prevalence of autosomal trisomies increases with age and PPV is a function of disease prevalence. The PPV for monosomy X was 42.0% (95% CI: 28.3%-55.7%) (Table 3).

Discussion

In this study, we examined the performance of the UNITY Aneuploidy Screen, a sequencing-based NIPT analysis for the detection of common aneuploidies. These data demonstrate the UNITY Aneuploidy Screen has excellent performance for common autosomal aneuploidies and monosomy X. Importantly, the performance is comparable or superior to other commercially available aneuploidy assays with different technologies including SNP-based and shotgun sequencing [15-17].

An important distinction of the UNITY Aneuploidy Screen is the use of BillionToOne's proprietary QCT molecular counting technology to assess sample quality. Other assays rely on fetal fraction to determine quality of the sample. Fetal fraction is not a complete assessment of the quantity of molecules from the chromosomes of interest; rather, it is a measure of the total proportion of any cfDNA of fetal origin. UNITY Aneuploidy Screen uses QCT technology to quantify the molecules of the chromosomes of interest and assesses if it is proportional to the fetal fraction to ensure accurate fetal risk assessment, reducing false negative calls; particularly at low fetal fractions. Finally, the UNITY Aneuploidy Screen utilizes an intermediate likelihood ratio (neither high risk nor low risk) in which the results are internally reflexed to a backup sample, while still keeping the no-call rate minimal.

Similar to other commercially available assays, the UNITY Aneuploidy Screen returns a maximum quantitative risk of 9 in 10 for autosomal aneuploidies. The observed PPV of 90.8% in this sample was consistent with the reported quantitative risk. Furthermore, the PPV remained comparable to those reported by other publications despite the study sample having a younger average age (average age at delivery of 29 years with 80% younger than 35 years old at delivery) than those of other publications where the average maternal age ranged from 34 to 35 years and the PPV for autosomal trisomies ranged from 83.5% to 89.1% [15-17]. PPV is a product of the test sensitivity and prevalence of the condition, therefore as disease prevalence increases the PPV of a test increases. The risk for autosomal aneuploidies increases with maternal age so the prevalence of these conditions increases. As a result, the PPV of the same aneuploidy NIPT assay will be higher when studied in a sample of older pregnant individuals as compared to a sample of younger individuals. In the current study, the PPV was 94.6% for individuals 35 years or older at delivery and 86% for individuals younger than 35 years at delivery. Finally, when examined by individual trisomy, the PPVs remained consistent with published reports and well above ACOG-reported performance analytics for aneuploidy NIPT assays [4].

The UNITY Aneuploidy Screen also performed similarly or superiorly to other aneuploidy NIPT assays for the detection of monosomy X. In this study, the PPV for monosomy X was 42%. Other publications report PPVs ranging from 12.5% to 69% [18-20]. These differences reflect the variable assay designs; the UNITY Aneuploidy Screen does not employ a no call threshold for monosomy X and therefore is most comparable to other laboratories that also do not include a no call range for monosomy X [18]. Finally, through the use of QCT molecular counting technology to compare the chromosome dosage and fetal fraction, UNITY Aneuploidy Screen can identify potential maternal chromosome abnormalities, for example, maternal monosomy X mosaicism, which is a well-documented phenomenon [21].

Conclusion

In summary, the UNITY aneuploidy screen shows comparable or superior performance to other commercially available aneuploidy NIPT assays. The UNITY aneuploidy screen is available for standard aneuploidies of 21, 18, and 13 and sex chromosome aneuploidies (monosomy X, XXX, XXY, XYY) with the option to learn fetal sex, screen for 22q11.2 deletion syndrome, and fetal RhD and other fetal red blood cell antigen status. This assay is available for both single and twin gestations and for twins can report zygosity. Additionally, UNITY is the only NIPT assay that includes carrier screening with single-gene NIPT for the ACOG recommended autosomal recessive conditions including cystic fibrosis, spinal muscular atrophy, alpha thalassemia, and beta hemoglobinopathies.

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