OPEN

*Original Research*

# Cell-Free DNA Analysis for the Determination of Fetal Red Blood Cell Antigen Genotype in Individuals With Alloimmunized Pregnancies

*Shannon Rego, MS, Olaide Ashimi Balogun, MD, Kirsten Emanuel, MS, FNP, Rachael Overcash, MD, Juan M. Gonzalez, MD, PhD, Gregory A. Denomme, PhD, Jennifer Hoskovec, MS, Haley King, MS, Ashley Wilson, MS, Julia Wynn, MS, MS, and Kenneth J. Moise Jr, MD*

**OBJECTIVE:** To evaluate the accuracy of next-generation sequencing–based quantitative cell-free DNA analysis for fetal antigen genotyping in individuals with alloimmunized pregnancies undergoing clinical testing in practices across the United States as early as 10 weeks of gestation, with the objective of identifying individuals with pregnancies at risk for hemolytic disease of the fetus and newborn and guiding management.

**METHODS:** This prospective cohort study included patients with alloimmunized pregnancies undergoing clinical fetal antigen cell-free DNA analysis between 10 0/7 and 37 0/7 weeks of gestation at 120 clinical sites. Both the pregnant person with the alloimmunized pregnancy and the neonates resulting from the pregnancies were included. The laboratory issued the cell-free DNA results prospectively as a part of clinical care. After delivery, neonatal buccal swabs collected between 0 and 270 days of life were sent to an outside independent laboratory for antigen genotyping. The outside laboratory was blinded to the fetal cell-free DNA results, and the results were compared. Concordance was reported for the fetal antigen cell-free DNA analysis for antigens to which the pregnant person was alloimmunized and for all antigens for which the pregnant person was genotype negative.

**RESULTS:** A total of 156 pregnant people who received clinically ordered cell-free DNA fetal antigen testing provided neonatal buccal swabs for genotyping after delivery. Overall, 15.4% of participants were Hispanic, 9.0% were non-Hispanic Black, 65.4% were non-Hispanic White, 4.5% were Asian, 1.3% were more than one race

See related editorial on page 433.

*From the BillionToOne, Inc, Menlo Park, and the Department of Obstetrics, Gynecology and Reproductive Sciences, University of California, San Francisco, San Francisco, California; Obstetrix Maternal-Fetal Medicine Specialists, Houston, Grifols Laboratory Solutions Inc, San Marcos, and the Department of Women's Health, Dell Medical School, University of Texas at Austin, and the Comprehensive Fetal Care Center, Dell Children's Medical Center, Austin, Texas; and the Division of Maternal-Fetal Medicine, Department of Women's and Infant's Services, MedStar Washington Hospital Center, Washington, DC.*

*The authors thank Emily Griffin, CGC, for her help organizing data; Kelly Chou and John Fang for bioinformatics support; and Oguzhan Atay, Shan Riku, and David Tsao for reviewing the manuscript. All individuals are paid employees of or contractors with BillionToOne, Inc.*

*Before submission to* Obstetrics & Gynecology, *this article was submitted to a preprint server: MedRxiv (MEDRXIV/2024/304584).*

*Anonymized individual participant data will be available. The data will include the pregnant person's alloimmunization status; gestational age; fetal fraction; fetal cell-free DNA results, including, if requested, the calibrated fetal antigen fraction; and neonate genotype results, including the specific genetic variant identified. Data will be available at the time of publication and for 5 years after. Access can be requested by contacting the author; sharing will be determined by the author and the clinical laboratory where the study was conducted. Data will be shared in a secure electronic format for replication purposes.*

*Each author has confirmed compliance with the journal's requirements for authorship.*

*Corresponding author: Kenneth J. Moise, MD, Department of Obstetrics and Gynecology, Department of Women's Health, Dell Medical School, Austin, TX; kmoise@austin.utexas.edu.*

***Financial Disclosure***

*Jennifer Hoskovec, Julia Wynn, and Shannon Rego are employees of BillionToOne, Inc and have options/equity in BillionToOne, Inc. Haley King and Ashley Wilson are paid contractors with BillionToOne, Inc. Kenneth J. Moise is a paid consultant of BillionToOne, Inc. Olaide Ashimi Balogun, Kirsten Emanuel, and Rachael Overcash received research funding from BillionToOne, Inc. Julia Wynn received National Society of Genetic Counselor National Meeting Speaker compensation. Shannon Rego received a stipend from the American College of Medical Genetics and Genomics as part of the Clingen Adult Actionability Working Group. The other authors did not report any potential conflicts of interest.*



ISSN: 0029-7844/24

**or ethnicity, and 4.5% were unknown. The median gestational age at the time of testing was 16.4 weeks with a median fetal fraction of 11.1%. Concordance between cell-free DNA analysis results and neonatal genotype was determined for 465 antigen calls for the following antigens: K1 (n=143), E (124), C (60), Fy$^a$ (50), c (47), and D(RhD) (41). These 465 calls included 145 in which the fetus was antigen positive and 320 in which the fetus was antigen negative. We observed complete concordance between prenatal fetal antigen cell-free DNA analysis results and neonatal genotypes for the 465 calls, resulting in 100% sensitivity, specificity, and accuracy.**

**CONCLUSION: In a diverse multicenter cohort, cell-free DNA analysis was highly sensitive and specific for determining fetal antigen genotype as early as 10 weeks of gestation in individuals with alloimmunized pregnancies. Taken together with previously published evidence, this study supports the implementation of cell-free DNA testing to manage individuals with alloimmunized pregnancies in the United States.**

*(Obstet Gynecol 2024;144:436–43)*
*DOI: 10.1097/AOG.0000000000005692*

Hemolytic disease of the fetus or newborn is a potentially life-threatening form of anemia caused by alloimmunization.[1] The American College of Obstetricians and Gynecologists recommends testing the reproductive partner's antigen status when alloimmunization is diagnosed in pregnancy.[2] However, rates of reproductive partner screening uptake are low, and results can be misleading in the setting of nonpaternity.[3–5]

Cell-free DNA is already used as a standard of care in many European countries for determining fetal antigen status and guiding pregnancy management.[6–8] In September 2022, a cell-free DNA assay using next-generation sequencing and quantitative counting template technology for determining fetal antigen status was first offered clinically in the United States.[9] The assay improves on European assays by combining next-generation sequencing with quantitative counting template technology, which facilitates the detection and absolute quantification of variants that are more common in the diverse U.S. population.[9]

In a prior study, we performed the initial validation of next-generation sequencing–based cell-free DNA analysis with quantitative counting template technology for fetal antigen genotyping. Although the validation demonstrated 100% sensitivity and specificity of the assay on 1,061 preclinical samples and precision of 99.9% on 1,683 clinical samples, the number of clinical samples with neonatal genotype or serology outcomes was limited to 23 biobank samples from pregnant individuals and 30 pregnancies with prospectively reported results, which showed 100% concordance with ground-truth outcomes.[9] Here, we build on prior work with a prospective cohort study evaluating the concordance between fetal antigen cell-free DNA analysis results and neonatal antigen genotyping performed at an independent laboratory.

## METHODS

Participants were recruited into an IRB-approved fetal antigen patient registry (WCG IRB protocol No. 20225380). Eligible participants were identified through the quality assurance program of the clinical laboratory or by their managing clinician at collaborating clinical sites and enrolled into this prospective cohort study. Pregnant people and their neonates were eligible for inclusion in the study if 1) the patient was clinically confirmed to be alloimmunized to at least one of the following antigens: K1 (Kell), Fy$^a$ [also known as Fy(a+)], C, c, E, or D(RhD); and 2) the patient underwent fetal antigen cell-free DNA analysis in the United States between September 15, 2022, and December 15, 2023, and spoke English or Spanish. Those who agreed to participate provided written informed consent for themselves and their neonate. Participants were compensated for their participation.

Details of the fetal antigen cell-free DNA analysis have been previously published.[9] In brief, we developed and validated an approach to noninvasive prenatal testing that uses next-generation sequencing and quantitative counting templates to determine fetal antigen genotypes by analyzing cell-free DNA in plasma samples from pregnant individuals. The addition of quantitative counting templates enables the absolute quantification of detected fetal antigen molecules, which then is compared with the expected number of fetal molecules based on fetal fraction to determine the fetal genotype. The fetal genotype can then be used to predict the fetal antigen phenotype. When the predicted fetal phenotype is antigen positive for an antigen to which the pregnant person is alloimmunized, the pregnancy is at risk for hemolytic disease of the fetus and newborn. This test can be performed as early as 10 weeks of gestation to determine fetal antigen status in pregnant people who are alloimmunized to the following antigens: K1, Fy$^a$, C, c, E, or D(RhD). Results were reported clinically only for the antigens to which the patient was alloimmunized. All samples were run on the same version of the fetal antigen cell-free DNA analysis; the assay did not change during the duration of the study.

Between 0 and 270 days of life, a buccal swab was obtained from the neonate resulting from the alloimmunized pregnancy with ORAcollectDNA buccal swabs. The samples were sent to Grifols Laboratory Solutions Inc (San Marcos, Texas), which performed antigen genotyping with BGG Navigator, a polymerase chain reaction (PCR) and genomic hybridization-based genotyping test using ID CORE XTTM technology. Neonatal genotype and predicted phenotype were reported for the following antigens included in fetal antigen cell-free DNA analysis: K1, $Fy^a$, C, c, and E. For pregnancies alloimmunized to the D(RhD) antigen, neonatal genomic DNA extracted from the swabs was used to amplify exons 1–10 and their flanking regions of the *RHD* gene, along with amplification of a hybrid *RHD-CE* exon 3–intron 3 region and sequenced with BigDye Terminator 3.1 Cycle Sequencing kit to determine the *RHD* genotype and predicted RhD phenotype, including the identification of *RHD-CE-D* hybrid genotypes and the RHDΨ variant.

Concordance was determined separately for 1) only those antigens to which the pregnant person was alloimmunized and 2) all antigens for which the pregnant person was genotype negative. A pregnant person must be genotype negative for an antigen (not express the antigen) to be alloimmunized to it. Alloimmunization status does not affect the assay performance because the assay is genotype, not protein (antigen or antibody), based. Therefore, by examining all antigens for which a pregnant person was genotype negative, we were able to examine assay performance with a larger sample size of antigen calls. The investigators and Grifols Laboratory staff were blinded to neonatal and fetal analysis results, respectively, until both assays had been completed. Antigen genotypes were considered concordant when fetal antigen cell-free DNA analysis–predicted fetal phenotype (reported as antigen detected or antigen not detected) matched neonatal predicted phenotype (reported as antigen positive or antigen negative). If a pregnancy was a twin gestation, the cell-free DNA analysis results were concordant if both neonates were antigen genotype negative and the cell-free DNA analysis–predicted antigen was not detected or at least one neonate was antigen genotype positive and the cell-free DNA analysis–predicted antigen was detected.

A sample size of 200 alloimmunized cell-free DNA assays was selected on the basis of a conservative predicted sensitivity of 97% and published antigen allele frequencies to allow the calculation of the assay analytics with a type I error of up to 5% and a marginal error of 5% (span of 95% CIs). Demographic characteristics, including maternal age, gestational age, and maternal race and ethnicity, were collected from the patient's clinical test requisition form and, in addition to fetal fraction, were reported as descriptive summary statistics. The frequency of genetic variants affecting antigen status can vary depending on race and ethnicity, so we included this information in the study to show the diversity of the cohort in whom we were assessing assay performance.

In addition, sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy were calculated for the calls on antigens to which the pregnant person was alloimmunized. For the larger sample of all antigens to which the pregnant person was genotype negative, we calculated sensitivity, specificity, and accuracy. PPV and NPV were not calculated in the latter sample because PPV and NPV are affected by the prevalence of antigen-positive status and therefore are best used in a sample in which the expected rate of antigen positivity is similar to that expected in the intended use population (alloimmunized antigen calls). These statistics were calculated with medcalc.org.

This investigation met the STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) guidelines for a cohort study.

## RESULTS

Overall, 156 participants and their neonates enrolled from 120 different U.S. practices in 37 states and submitted neonatal buccal swabs (Fig. 1 and Appendix 1, available online at http://links.lww.com/AOG/D779). The cohort included four twin pregnancies. The median gestational age at the time of testing was 16.4 weeks, and the median fetal fraction was 11.1% (Table 1). Pregnancy characteristics and patient demographics of participants in the cohort were similar to those who were approached (but not enrolled; data not shown).

The 156 participants were alloimmunized to 191 antigens, and concordance was assessed for 190 alloimmunized antigen calls from 155 patients. One case had fetal cell-free DNA results but inconclusive results on neonatal *RHD* sequencing from the outside laboratory, so a concordance assessment was not possible. The most common alloimmunized antigen was E (n=53, 34.0%). Forty-six patients (29.5%) were alloimmunized to K1, 41 (26.3%) to D(RhD), 27 (17.3%) to C, 20 (12.8%) to c, and 4 (2.6%) to $Fy^a$. Thirty-four patients were alloimmunized to more than one antigen (Table 2).

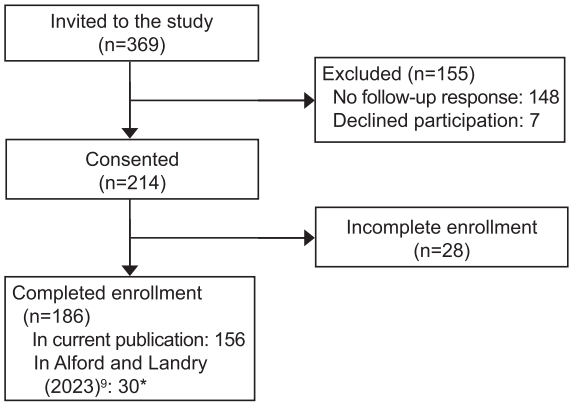


**Fig. 1.** Flow diagram of patient enrollment. *One hundred percent concordance was also demonstrated.

*Rego. Cell-Free DNA for Fetal Antigen Genotyping. Obstet Gynecol 2024.*

Overall, 91 fetal antigen results (47.6%) were reported clinically as antigen detected, meaning the fetal antigen genotype predicted an antigen-positive phenotype, and 100 (52.4%) were antigen not detected, meaning the fetal antigen genotype predicted an antigen-negative phenotype (Appendix 2a, available online at http://links.lww.com/AOG/D779).

Concordance for the 190 fetal antigen calls for antigens to which the patients were alloimmunized was 190 of 190 (100%) (Table 3). Sensitivity, specificity, PPV, NPV, and accuracy are reported in Table 3. The total number of available assays was fewer than recommended by the a priori power calculation; however, the desired level of statistical power was still achieved with the margins of error less than 5% for all analyses.

In addition, concordance was 100% for 465 fetal antigen calls on antigens to which the pregnant person was genotype negative (Table 4). These 465 calls include 190 for antigens to which the pregnant person was alloimmunized, as well as an additional 275 calls on antigens to which the pregnant person was not alloimmunized but for which they were genotype negative (and therefore able to become alloimmunized to the antigen; Appendix 3 [available online at http://links.lww.com/AOG/D779] and Table 4). The 465 antigen calls were for the following antigens: K1 (n=143), E (n=124), C (60), $Fy^a$ (n=50), c (n=47), and D(RhD) (n=41) (Appendix 2b, available online at http://links.lww.com/AOG/D779). Sensitivity, specificity, and accuracy are reported in Table 4. There was one fetus for which cell-free DNA analysis correctly called D(RhD) negative because of an *RHDΨ* variant, which was confirmed by postnatal *RHD* sequencing showing compound heterozygosity for the *RHD* gene deletion and *RHDΨ* variant in the neonate.

There were two cases in which the cell-free DNA analysis reported "not detected" for the C antigen and the neonatal genotyping revealed the hybrid *RHD-CE-D* allele *RHD*DIIIa-ceVS.03(4–7)-RHCE*ce*, present in about 0.3% of the U.S. population and associated with an extremely weak C phenotype.[10,11] Individuals with this weak C phenotype have been documented to produce anti-C antibodies when exposed to C antigen.[10] Therefore the cell-free DNA "not detected" call is likely an appropriate determination that the fetus is not at risk for hemolytic disease of the fetus and newborn. However, there is no literature to confirm this, so these cases were excluded from the concordance calculation (Table 4).

**Table 1. Demographics and Pregnancy Characteristics of the Participants (N=156)**

| Characteristic | Value |
|---|---|
| Self-reported race and ethnicity | |
| Asian | 7 (4.5) |
| Black | 14 (9.0) |
| Latina or Hispanic | 24 (15.4) |
| More than 1 race or ethnicity or both | 2 (1.3) |
| Unknown | 7 (4.5) |
| White | 102 (65.4) |
| Maternal age at estimated due date (y) | 31 (18–44) |
| Gestational age at time of fetal antigen testing (wk) | 16.4 (10.0–37.0) |
| Fetal fraction (%) | 11.1 (2.4–32.2) |
| Trimester of fetal antigen testing | |
| 1st | 52 (33.3) |
| 2nd | 77 (49.4) |
| 3rd | 27 (17.3) |

Data are n (%) or median (range).

## DISCUSSION

This multisite, prospective U.S. cohort study demonstrates that next-generation sequencing–based cell-free DNA analysis using quantitative counting template technology is a highly accurate approach for determining fetal antigen genotypes and predicted phenotypes for individuals with alloimmunized pregnancies. Concordance between fetal antigen genotype as determined by cell-free DNA analysis and neonatal antigen genotype as determined by an outside laboratory was 100% for all 190 calls on antigens to which the pregnant person was alloimmunized. Concordance was also 100% when the antigen calls were expanded to include all 465 antigens for which the

**Table 2. Summary of Alloimmunized Antigens Among Pregnant Individuals and Concordance Between Neonatal Antigen Genotyping and Cell-Free DNA Results (n=155 Participants With 190 Alloimmunized Antigen Calls)***

| Alloimmunized Antigen(s) | No. of Patients Tested | No. (%) of Patients With Concordant Fetal Antigen Cell-Free DNA and Neonatal Antigen Genotyping Results |
|---|---|---|
| E | 39 | 39 (100) |
| K | 43 | 43 (100) |
| C, D | 18 | 18 (100) |
| D | 19 | 19 (100) |
| C | 10 | 10 (100) |
| E, c | 9 | 9 (100) |
| C | 7 | 7 (100) |
| $Fy^a$ | 3 | 3 (100) |
| E, D | 3 | 3 (100) |
| $Fy^a$, E | 1 | 1 (100) |
| K, c | 1 | 1 (100) |
| E, K | 1 | 1 (100) |
| $Fy^a$, C, K | 1 | 1 (100) |

* One of the 156 enrolled participants was excluded from the analysis of alloimmunized antigen calls because of an inconclusive neonatal genotyping result for the D antigen, the antigen to which the patient was alloimmunized.

pregnant person was genotype negative, resulting in a calculated assay sensitivity and specificity of 100%.

The addition of quantitative counting templates to next-generation sequencing–based cell-free DNA technology enables the absolute quantification of detected fetal antigen molecules. By comparing the detected number of fetal antigen cell-free DNA molecules with the expected number of molecules based on the fetal fraction, the assay ensures high sensitivity and specificity for the determination of fetal genotype for early gestational ages and low fetal fractions.[9] An ongoing phase III trial (ClinicalTrials.gov NCT05912517) of the prevention of hemolytic disease of the fetus and newborn enrolls at 13–16 weeks of gestation, highlighting the importance of an assay that is accurate at early gestational ages (eg, before 15 weeks).[12] This unique approach of this assay results in high sensitivity and specificity independently of fetal fraction or gestational age.[9]

A prior study demonstrated that fetal antigen cell-free DNA analysis with next-generation sequencing and quantitative counting template technology provided informative results for 99.9% of patients, and the current study has shown 100% accuracy.[9] The real-time PCR-based approach that has long been used in clinical practice in several countries outside the United States has higher rates of no results or inconclusive results (1.0–14.3%).[13] European-based assays using real-time PCR have not been adopted in the United States because of concerns about performance–both accuracy and inclusivity–for the diverse U.S. population.[14] The real-time PCR-based assays cannot precisely measure the depth of amplification and therefore cannot quantify the *RHD* gene molecules. Many rely on the assumption that the pregnant individual is RhD negative as a result of an *RHD* gene deletion, and therefore, if the control gene indicates the presence of fetal DNA, it is concluded that any *RHD* gene amplified is of fetal origin; if no *RHD* gene is amplified, it is concluded that the fetus is RhD negative. These assumptions can result in false-positive or inconclusive results when a non–*RHD*-gene-deletion genotype is present, as well as false-negative results at early gestational ages when fetal fractions are lower.[7,15] The next-generation sequencing–based multiexon sequencing cell-free DNA analysis for fetal antigen genotyping evaluated in this study uses quantitative counting template technology to detect and quantify *RHD*-negative genotypes, including the common *RHD* gene deletion and variants such as *RHDΨ* and the *RHD-CE-D* hybrid genes, which are present in up to 50% of RhD-negative Black Americans.[16] As a result, this assay has higher call rates (99.9% in a prior study) than European-based assays and a median turnaround time of 7 days.[9]

The recommended approach for determining fetal antigen genotype in individuals with alloimmunized pregnancies in the United States has relied on reproductive partner antigen testing, with amniocentesis indicated when fetal genotype cannot be assumed from paternal antigen status.[2] However, amniocentesis carries the risk of fetal loss, worsening alloimmunization, and low uptake.[17–20] Subsequently, the fetal

**Table 3. Concordance Between Fetal Antigen Cell-Free DNA Genotyping Results for Alloimmunized Antigens and Neonatal Genotyping**

| | Neonatal Antigen Positive | Neonatal Antigen Negative | Total | % (95% CI) |
|---|---|---|---|---|
| cfDNA fetal antigen detected | 90 | 0 | 90 | |
| cfDNA fetal antigen not detected | 0 | 100 | 100 | |
| Total | 90 | 100 | 190 | |
| Sensitivity | | | | 100 (96.0–100) |
| Specificity | | | | 100 (96.4–100) |
| PPV | | | | 100 (96.0–100) |
| NPV | | | | 100 (96.4–100) |
| Accuracy | | | | 100 (98.1–100) |

cfDNA, cell-free DNA; PPV, positive predictive value; NPV, negative predictive value.
Data are n unless otherwise specified.

antigen status remains unknown in many pregnancies, and the pregnant individual is then monitored with serial antibody titers and Doppler ultrasound of peak systolic velocity in the middle cerebral artery by specialty clinicians, a process that is time intensive and burdensome. In addition, maternal antibody titers are nonspecific and can rise even when the fetus is antigen negative,[21] and Doppler ultrasound of peak systolic velocity in the middle cerebral artery has a reported false-positive rate of 12%.[22] When Doppler ultrasound of peak systolic velocity in the middle cerebral artery suggests fetal anemia, it can lead to unnecessary and potentially risky invasive procedures such as cordocentesis and intrauterine transfusions.[23] Although these approaches to monitoring individuals with pregnancies at risk for hemolytic disease of the fetus and newborn have limitations, they remain important tools for guiding management when the fetus is antigen positive. Recently, the American College of Obstetricians and Gynecologists released a practice guideline update stating that cell-free DNA is a reasonable alternative tool for fetal RhD testing in alloimmunized patients who decline amniocentesis; similarly, for those alloimmunized to non-D red blood cell antigens, the update stated that "cfDNA [cell-free DNA] may be considered for pregnant patients declining amniocentesis, after weighing cost, access, and the encouraging-yet-limited data supporting its use."[24] This study provides additional data supporting the use of cell-free DNA for determining fetal antigen status for antigens, including the RhD antigen and non-D antigens. When used for the purpose of determining fetal antigen status, cell-free DNA analysis can mitigate the need for unnecessary monitoring in settings where the fetus is not at risk of hemolytic disease of the fetus and newborn because of an antigen-negative genotype.

A limitation of the assay is that it does not assess fetal antigen status for all antigens known to cause hemolytic disease; rather, it is designed to determine antigen genotype for those most often implicated in clinically significant fetal anemia.[1] The assay also is not designed for use in pregnant people who have had organ or bone marrow transplantations or recent blood transfusions, whose pregnancy included a vanishing twin, or who have a known malignancy.

In addition to the accuracy and logistical burden of the options for managing individuals with alloimmunized pregnancies, cost is another important consideration. One study addressed the health economics of cell-free DNA for the management of individuals with alloimmunized pregnancies and found a nearly

**Table 4. Concordance Between Fetal Antigen Cell-Free DNA Results for All Antigens for Which the Pregnant Person Was Genotype Negative**

| | Neonatal Antigen Positive | Neonatal Antigen Negative | Total | % (95% CI) |
|---|---|---|---|---|
| cfDNA fetal antigen detected | 145 | 0 | 145 | |
| cfDNA fetal antigen not detected | 0 | 320 | 320 | |
| Total | 145 | 320 | 465 | |
| Sensitivity | | | | 100 (97.5–100) |
| Specificity | | | | 100 (98.9–100) |

cfDNA, cell-free DNA.
Data are n unless otherwise specified.

$8,000 ($7,903) reduction in the cost of care when individuals with alloimmunized pregnancies were managed with cell-free DNA fetal genotyping compared with usual care.[25,26] The sensitivity and specificity of our next-generation sequencing–based assay with quantitative counting template technology are higher than those used in the model assumptions for the previously published cost analysis (assumed 99.7% sensitivity and 96.1% specificity); therefore, the cost savings are expected to be even greater with the implementation of our new assay.

This prospective study demonstrated the accuracy of cell-free DNA with next-generation sequencing and quantitative counting template technology for fetal antigen genotyping in a diverse U.S. sample of alloimmunized pregnant individuals, including twin pregnancies. It is important to note that fetal cell-free DNA results were reported prospectively as part of the clinical care of the pregnancy without knowledge of the neonatal genotype. The laboratory performing cell-free DNA genotyping and the different laboratory performing neonatal genotyping were blinded to each other's results.

This study has limitations. Although the study enrolled participants from 120 clinical practices across the United States, including representation from individuals who identified as Asian, Black, Hispanic, White, and more than one race or ethnicity, participants identifying as White were overrepresented compared with the U.S. population. However, the no-call rate for this assay did not differ for different races and ethnicities. We did not reach our calculated sample size; however, we were still able to achieve our desired level of statistical power with margins of error less than 5%. Although the cost-effectiveness of cell-free DNA for fetal antigen genotyping has been previously demonstrated,[25] we did not perform an economic analysis as part of this study.

This study demonstrated the accuracy of an next-generation sequencing–based cell-free DNA analysis assay with quantitative counting template technology for the detection of fetal antigen status in a large, diverse U.S.-based cohort. With 100% accuracy and no need for paternal testing or invasive procedures, this assay will result in more alloimmunized individuals receiving informative results about fetal risk compared with the traditional approach, which is limited by the uptake of paternal testing and amniocentesis as well as nonpaternity.[3,4,17] Clinical implementation of fetal antigen cell-free DNA analysis for the management of individuals with alloimmunized pregnancies will remove these barriers. Cell-free DNA can also streamline clinical management and improve equitable access to care. Taken together with previously published evidence, this study supports the implementation of cell-free DNA testing to manage individuals with alloimmunized pregnancies in the United States.

## REFERENCES


1. Delaney M, Matthews DC. Hemolytic disease of the fetus and newborn: managing the mother, fetus, and newborn. Hematol Am Soc Hematol Educ Program 2015;2015:146–51. doi: 10.1182/asheducation-2015.1.146
2. Management of alloimmunization during pregnancy. ACOG Practice Bulletin No. 192. American College of Obstetricians and Gynecologists. Obstet Gynecol 2018;131:e82–90. doi: 10.1097/AOG.0000000000002528
3. Giles ChoatesM, Stevens BK, Wagner C, Murphy L, Singletary CN, Wittman AT. It takes two: uptake of carrier screening among male reproductive partners. Prenat Diagn 2020;40:311–16. doi: 10.1002/pd.5588
4. Macintyre S, Sooman A. Non-paternity and prenatal genetic screening. Lancet 1991;338:869–71. doi: 10.1016/0140-6736(91)91513-t
5. Nguyen MT, Mazza G, Nguyen BT. The completion of indicated paternal prenatal genetic and carrier testing at a public hospital in Los Angeles, California. Genet Med Open 2023;1:100831. doi: 10.1016/j.gimo.2023.100831
6. Gutensohn K, Müller SP, Thomann K, Stein W, Suren A, Körtge-Jung S Diagnostic accuracy of noninvasive polymerase chain reaction testing for the determination of fetal rhesus C, c and E status in early pregnancy. BJOG 2010;117:722–29. doi: 10.1111/j.1471-0528.2010.02518.x
7. Scheffer PG, van der Schoot CE, Page-Christiaens GCML, de Haas M. Noninvasive fetal blood group genotyping of rhesus D, c, E and of K in alloimmunised pregnant women: evaluation of a 7-year clinical experience. BJOG 2011;118:1340–48. doi: 10.1111/j.1471-0528.2011.03028.x
8. Royal College of Obstetricians & Gynaecologists. The management of women with red cell antibodies during pregnancy: green-top guideline No. 65. Accessed February 20, 2024. https://rcog.org.uk/media/oykp1rtg/rbc_gtg65.pdf
9. Alford B, Landry BP, Hou S, Bower X, Bueno AM, Chen C. Validation of a non-invasive prenatal test for fetal RhD, C, c, E, K and Fy$^a$ antigens. Sci Rep 2023;13:12786. doi: 10.1038/s41598-023-39283-3
10. Moulds JM, Noumsi GT, Billingsley KL. A comparison of methods for the detection of the r'(s) haplotype. Transfusion 2015;55:1418–22. doi: 10.1111/trf.12956
11. Chou ST, Evans P, Vege S, Coleman SL, Friedman DF, Keller M. RH genotype matching for transfusion support in sickle cell disease. Blood 2018;132:1198–207. doi: 10.1182/blood-2018-05-851360
12. ClinicalTrials.gov. A study to evaluate the safety, efficacy, pharmacokinetics and pharmacodynamics of m281 administered to pregnant women at high risk for early onset severe hemolytic disease of the fetus and newborn (HDFN).Accessed March 23, 2024. https://clinicaltrials.gov/study/NCT03842189
13. Yang H, Llewellyn A, Walker R, Harden M, Saramago P, Griffin S, et al. High-throughput, non-invasive prenatal testing for fetal rhesus D status in RhD-negative women: a systematic review and meta-analysis. BMC Med 2019;17:37. doi: 10.1186/s12916-019-1254-4
14. Prevention of Rh D alloimmunization. Practice Bulletin No. 181. American College of Obstetricians and Gynecologists. Obstet Gynecol 2017;130:e57–70. doi: 10.1097/AOG.0000000000002232

15. Saramago P, Yang H, Llewellyn A, Walker R, Harden M, Palmer S, et al. High-throughput non-invasive prenatal testing for fetal rhesus D status in RhD-negative women not known to be sensitised to the RhD antigen: a systematic review and economic evaluation. Health Technol Assess 2018;22:1–172. doi: 10.3310/hta22130

16. Singleton BK, Green CA, Avent ND, Martin PG, Smart E, Daka A. The presence of an RHD pseudogene containing a 37 base pair duplication and a nonsense mutation in Africans with the Rh D-negative blood group phenotype. Blood 2000; 95:12–8. doi: 10.1182/blood.V95.1.12

17. Morgan T, Tan CD, Della-Torre M, Jackson-Bey T, DiGiovanni L, Enakpene CA. Determinant of prenatal diagnostic testing among women with increased risk of fetal aneuploidy and genetic disorders. Am J Perinatol 2024;41:470–7. doi: 10.1055/a-1692-0309

18. Murray JC, Karp LE, Williamson RA, Cheng EY, Luthy DA. Rh isoimmunization related to amniocentesis. Am J Med Genet 1983;16:527–34. doi: 10.1002/ajmg.1320160411

19. Sarkar P, Bergman K, Fisk NM, Glover V. Maternal anxiety at amniocentesis and plasma cortisol. Prenat Diagn 2006;26: 505–9. doi: 10.1002/pd.1444

20. Akolekar R, Beta J, Picciarelli G, Ogilvie C, D'Antonio F. Procedure-related risk of miscarriage following amniocentesis and chorionic villus sampling: a systematic review and meta-analysis. Ultrasound Obstet Gynecol 2015;45:16–26. doi: 10. 1002/uog.14636

21. Moise KJ. D alloimmunization in pregnancy: management. Accessed February 20, 2024. https://uptodate.com/contents/d-alloimmunization-in-pregnancy-management?source=mostViewed_widget

22. Mari G, Deter RL, Carpenter RL, Rahman F, Zimmerman R, Moise KJ Jr, et al. Noninvasive diagnosis by Doppler ultrasonography of fetal anemia due to maternal red-cell alloimmunization: Collaborative Group for Doppler Assessment of the Blood Velocity in Anemic Fetuses. N Engl J Med 2000;342: 9–14. doi: 10.1056/NEJM200001063420102

23. Abels E, Adkins BD, Cedeno K,Booth GS, Allen ES, Stephens LD, et al. Assessing recommendations for determining fetal risk in alloimmunized pregnancies in the United States: is it time to update a decades-old practice? Transfus Med Rev 2023;38: 150810. doi: 10.1016/j.tmrv.2023.150810

24. The American College of Obstetricians and Gynecologists. Paternal and fetal genotyping in the management of alloimmunization in pregnancy. Obstet Gynecol 2024;144:e47–e49. doi: 10.1097/AOG.0000000000005630.

25. Gajic-Veljanoski O, Li C, Schaink AK, Guo J, Shehata N, Charames GS, et al. Cost-effectiveness of noninvasive fetal RhD blood group genotyping in nonalloimmunized and alloimmunized pregnancies. Transfusion 2022;62:1089–102. doi: 10. 1111/trf.16826

26. Ontario Health (Quality). Noninvasive fetal RhD blood group genotyping: a health technology assessment. Ont Health Technol Assess Ser 2020;20:1–160.10.123

## PEER REVIEW HISTORY

Received March 26, 2024. Received in revised form May 21, 2024. Accepted May 23, 2024. Peer reviews and author correspondence are available at http://links.lww.com/AOG/D780.