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Original Research

Clinical Performance of Cell-Free DNA for Fetal RhD Detection in RhD-Negative Pregnant Individuals in the United States

Julio F. Mateus-Nino, MD, Julia Wynn, MS, MS, Jenny Wiggins-Smith, MD, J. Brett Bryant, MD, J. Kris Citty, MD, J. Kyle Citty, MD, Samir Ahuja, MD, and Roger Newman, MD

OBJECTIVE: To evaluate the performance of a cell-free DNA (cfDNA) assay that uses next-generation sequencing with quantitative counting templates for the clinical detection of the fetal *RHD* genotype in a diverse RhD-negative pregnant population in the United States.

METHODS: This retrospective cohort study was conducted in four U.S. health care centers. The same next-generation sequencing quantitative counting template cfDNA fetal RhD assay was offered to nonalloimmunized RhD-negative pregnant individuals as part of clinical care. Rh immune globulin (RhIG) was administered at the discretion of the clinician. The sensitivity, specificity, and accuracy of the assay were calculated considering the neonatal RhD serology results.

RESULTS: A total of 401 nonalloimmunized RhD-negative pregnant individuals who received clinical care in the period from August 2020 to November 2023 were included in the analysis. The D antigen cfDNA result was 100% concordant with the neonatal serology, resulting in 100% sensitivity, 100% positive predictive value (95% CI, 98.6–100% for both), 100% specificity, and 100% negative predictive value (95% CI, 97.4–100% for both). There were 10 pregnant individuals in whom the cfDNA analysis identified a non-*RHD* gene deletion, including *RhDΨ* (n=5) and *RHD–CE–D* hybrid variants (n=5). Rh immune globulin was administered antenatally to 93.1% of pregnant individuals, with cfDNA results indicating an RhD-positive fetus compared with 75.0% of pregnant individuals with cfDNA results indicating an RhD-negative fetus, signifying that clinicians were using the cfDNA results to guide pregnancy management.

CONCLUSION: This next-generation sequencing with quantitative counting templates cfDNA analysis for detecting fetal RhD status is highly accurate with no false-positive or false-negative results in 401 racially and ethnically diverse pregnant individuals with 100% follow-up of all live births. This study and prior studies of this assay support a recommendation to offer cfDNA screening for fetal Rh status as an alternative option to prophylactic RhIG for all nonalloimmunized RhD-negative individuals, which will result in more efficient and targeted prenatal care with administration of RhIG only when medically indicated.

(Obstet Gynecol 2025;145:402–8)

DOI: 10.1097/AOG.0000000000005850

From the Atrium Health, Concord, North Carolina; BillionToOne Inc, Menlo Park, California; the Medical University of South Carolina, Charleston, South Carolina; Shannon Health, San Angelo, Texas; Unity Health Searcy, Searcy, Arkansas; Wellstar Health System, Marietta, Georgia; and University Hospital, Mentor, Ohio.

This study was sponsored by BillionToOne, Inc, and participating clinical sites received research funding from BillionToOne to conduct the study.

The authors thank Shannon Rego for her assistance with data analysis and manuscript preparation. They also thank individuals who conducted the chart extractions, including Todd Morgan, Lindsey Hendry, Arghal Ahmad, Anita K. LaMonica, Becky J. Covington, Brittany Nugent, and Gretchen Hoelscher.

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

Corresponding author: Julio F. Mateus-Nino, MD, Atrium Health, Concord, NC; Julio.MateusNino@atriumhealth.org.

Financial Disclosure

Samir Ahuja is a paid consultant from BillionToOne, Inc. Julia Wynn is an employee of BillionToOne, Inc and has stock options in the company. She received an honorarium for speaking at NSGC 2023 and 2022. J. Brett Bryant's practice uses BillionToOne for noninvasive prenatal testing for their patients. Jenny Wiggins-Smith, J. Bret Bryant, J. Kris Citty, J. Kyle Citty, and Samir Ahuja received funding for this research project from BillionToOne, Inc.

ISSN: 0029-7844/25

Approximately 15% of pregnant individuals in the United States are RhD negative.[1,2] Current national guidelines support the administration of prophylactic anti-D immunoglobulin (RhIG) at 28 weeks of gestation and in other circumstances in which al-

loimmunization can occur.[1] However, in 35–40% of these pregnancies, the fetus is negative for the D antigen; the pregnant person is therefore not at risk for sensitization, and RhIG is unnecessary.[2]

Prenatal cell-free DNA (cfDNA) analysis, also known as noninvasive prenatal testing, to predict fetal RhD status can be a more efficient and targeted approach to prophylactic RhIG administration in RhD-negative nonalloimmunized pregnant patients, limiting the administration of RhIG only to patients carrying an identified RhD-positive fetus. In April 2024, the American College of Obstetricians and Gynecologists stated that fetal RhD cfDNA analysis is a reasonable consideration to prioritize RhIG use and to conserve RhIG supply for practices experiencing RhIG shortages.[3,4]

The United Kingdom and several other European countries have used cfDNA analysis to guide the administration of RhIG for more than a decade.[5–11] This approach has not been adopted in the United States because of concerns about the inclusivity and accuracy of the European-based assays for the U.S. population.[1] European assays use polymerase chain reaction (PCR) technology, which is particularly affected by low concentrations of the target gene, causing high background noise, DNA amplification variability, and potential for nonspecific amplification, leading to inaccurate results particularly at early gestational ages.[12,13] Some of these assays have primers that are able to predict the presence a non-*RHD* gene deletion, genotypes more common in individuals of non-European ancestry; however, they are unable to determine fetal RhD status, resulting in a higher frequency of inconclusive results in people of non-European ancestry.[6,12,14,15] A new cfDNA assay that uses next-generation sequencing with quantitative counting template technology is currently available in the United States.[16,17] Quantitative counting template technology with next-generation sequencing allows the precise quantification of DNA at low concentrations. This assay sequences and quantifies the critical exons of the *RHD* gene, including those that distinguish it from the *RHCE* homolog gene and *RhDΨ* variant, to predict the fetal RhD status for both the *RHD* gene deletion and non–*RHD* gene deletions at a gestational age as early as 10 weeks.[17] Prior studies of this assay in alloimmunized patients demonstrated 100% concordance of the cfDNA results with the neonatal antigen genotype for red blood cell (RBC) antigens, including D; however, the clinical use of this assay has not been evaluated in a consecutive cohort of nonalloimmunized pregnant individuals.[16,17]

Given the limitations of European-based assays, particularly for the diverse U.S. population, the objective of this study was to examine the clinical accuracy of next-generation sequencing quantitative counting template cfDNA analysis for the detection of the fetal *RHD* genotype for the prediction of the fetal RhD phenotype in a nonalloimmunized RhD-negative U.S. pregnant population. This study also examined how cfDNA results were used to guide RhIG administration and how the frequency of this changed over the course of the study.

METHODS

This retrospective study was conducted at four health care institutions in the United States from August 2020 to November 2023 on nonalloimmunized RhD-negative pregnancies of 10 or more weeks of gestation that were not conceived with an egg donor or carried by a gestational surrogate with an expected delivery before May 2024 who were having the same cfDNA fetal RhD test as part of clinical care. All participating institutions and the sponsor site received IRB approval. The study was exempted from patient consent because it was a retrospective study of medical records from clinical care.

As part of standard clinical procedures for the cfDNA fetal RhD assay, the patient blood sample was collected at the clinician's office and shipped to the central testing laboratory, a Clinical Laboratory Improvement Amendments– and College of American Pathologists–accredited clinical laboratory. The methodology and algorithm of the cfDNA fetal RhD laboratory-developed test did not change during the study and have been described previously in detail.[16] Briefly, plasma is isolated from a blood sample from the pregnant individual, and cfDNA is extracted. Five amplicons across the *RHD* gene that are unique between the wild-type *RHD* gene, the *RHCE* homolog gene, and the *RHDΨ* variant are amplified and sequenced by next-generation sequencing. Common polymorphic loci used as reference loci to determine fetal fraction and expected molecular counts for the *RHD* gene are also amplified and sequenced by next-generation sequencing. For each amplicon, molecular counts are calculated with quantitative counting templates that are added to the sample before amplification. The postamplification reads-per-molecule value is used to compute the absolute detected number of preamplification molecules, and informative polymorphic loci are used to calculate the absolute expected number of molecules. The absolute detected number of preamplification molecules, divided by the absolute expected number of molecules, is the calibrated fetal

RhD antigen fraction for each amplicon. The second highest calibrated fetal RhD antigen fraction across the five amplicons is used as the overall calibrated fetal RhD antigen fraction. A calibrated fetal RhD antigen fraction of 0% is predicted for a D antigen–negative fetus, and a calibrated fetal RhD antigen fraction of 100% is predicted for a D antigen–positive fetus. Only amplification of exon 10 is predicted for an *RHD–CE–D* hybrid variant associated with an RhD-negative phenotype, and amplification of the 37–base pair insertion in exon 4 is expected for the *RhDΨ* variant. The calibrated fetal RhD antigen fraction values of these specific amplicons indicate whether the fetus, and only the fetus, is negative as a result of a non-*RHD* deletion genotype or whether the fetus and pregnant person are RhD negative as a result of a non-*RHD* deletion genotype.[17]

The newborn D antigen serology was completed as part of clinical care in the accepted methodology of the clinical institution, and results were collected as part of the chart review. The standard clinical method of type and screen includes forward and reverse typing, which includes mixing the RBCs from the patient with RhD antibodies and measuring hemagglutination (forward) and mixing the serum from the patients with RhD antigens and measuring hemagglutination (reverse).[18] Other abstracted data included pregnant patient demographic information, maternal age, race and ethnicity, gestational age at the time of testing, results of maternal pregnant person antigen serology, RBC antibody screening, and frequency and gestational age of antenatal and postnatal RhIG administration. The study population race and ethnicity were collected to understand how they compared with the race and ethnicity of the U.S. RhD-negative population. Data were abstracted by research personnel who were not directly informed of the cfDNA results. However, cfDNA results were in the medical records because testing was done as part of clinical care and may have been inadvertently viewed; therefore, extractors may not have been completely blinded to the prenatal results.

The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of the fetal cfDNA RhD assay were calculated by comparing the predicted fetal RhD status with neonatal RhD serology. Results were considered concordant if cfDNA reported RhD detected and the neonatal serology was RhD positive or the cfDNA reported RhD not detected and neonatal serology was RhD negative. Twin cases were classified as concordant if cfDNA results were RhD detected and neonatal serology for one or both twins was RhD positive or if cfDNA results were RhD not detected and both twins' neonatal serology was RhD negative. A competing-risk model was used to test for a relationship between fetal loss, and therefore no neonatal serology results, and the cfDNA assay.[19] A sample size of 335 cfDNA assays was selected on the basis of a conservative predicted sensitivity of 98%, reflective of reported sensitivities of European-based assays and the previously available U.S. assay, to allow the calculation of the assay analytics with a marginal error of 1.5%.[6,12,15] A two-sample *t* test was used to compare the frequency of antenatal RhIG when the cfDNA results reported RhD detected and RhD not detected for all cases. A two-sample *t* test was also used to look at the frequency of antenatal RhIG for the cfDNA RhD not detected cases during two time periods: August 2020 to December 2022, when half of the tests occurred, and January 2023 to November 2023, when the second half of the tests occurred. A supplemental meta-analysis of the assay performance was completed by combining the data from the current study with previously published studies of the same assay.[16,17] Finally, all reported false negatives of this assay received at the laboratory are described in the Appendix 1, available online at http://links.lww.com/AOG/E10. Analysis was completed in R 4.2, and 95% CIs were calculated for all metrics. This investigation met the STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) guidelines for a cohort study.

RESULTS

There were 410 nonalloimmunized RhD-negative pregnant individuals in whom the cfDNA fetal RhD assay was performed across the four clinical sites during the period studied. The race and ethnicity of the individuals were reflective of the U.S. population (Table 1) (Appendix 2, available online at http://links.lww.com/AOG/E10). Pregnancies were excluded if the medical records showed the pregnant person to be RhD positive or to have D antibodies (were alloimmunized) or the pregnant individual did not deliver at the study site. An informative cfDNA fetal RhD result was reported for all 410 pregnant individuals (no-call rate 0%, 95% CI, 0–0.9%) without needing a repeat sample. Neonatal serology results were available for the 401 pregnancies that resulted in a live birth. A total of nine pregnant individuals had a fetal loss related to multiple congenital anomalies (n=2), trisomy 21 (n=1), and unknown reasons (n=6, Fig. 1). The competing-risk model found no relationship between the cfDNA fetal RhD results and pregnancy outcome (*P*=.58, 95% CI, 0.98–1.

Table 1. Participant Demographics and Pregnancy Characteristics (N=401)

Characteristic	Value
Gestation size	
Singleton	400 (99.8)
Twin	1 (0.2)
Maternal race and ethnicity	
Asian	3 (0.8)
Black	31 (7.8)
Hispanic	68 (17.1)
More than 1 race	3 (0.8)
White	293 (73.6)
Unknown	3
Maternal age (y)	28 (17–45)
Fetal fraction (%)	8.2 (1.6–28.9)
Trimester at testing (wk)	13.82 (10–34)
1st	317 (79)
2nd	80 (20)
3rd	4 (1)

Data are n (%) or mean (range) unless otherwise specified.

04). Furthermore, the medical record review did not identify a relationship between the pregnancy outcomes and the pregnant person's RhD status. These cases were excluded from the analysis of the assay performance.

Of the 401 pregnant individuals with neonatal serology, 140 (34.9%) were RhD negative and 261 (65.1%) were RhD positive (Fig. 1). The predicted fetal D antigen cfDNA result was 100% concordant with the neonatal serology results, resulting in 100% sensitivity (95% CI, 98.6–100%), 100% specificity (95% CI, 97.4–100%), 100% PPV (95% CI, 98.6–100%), 100% NPV (95% CI, 97.4–100%), and 100% accuracy (95% CI, 99.1–100%, Table 2). When the data from the current study and two prior studies were combined, the assay still had 100% performance (Appendices 1 and 3, available online at http://links.lww.com/AOG/E10). The cfDNA assay also correctly identified the fetal D antigen phenotype in 10 cases of non-*RHD* gene deletions. The predicted fetal D phenotype was concordant with the postnatal D serology phenotype for all of these cases. In five cases, the *RhDΨ* variant was identified in three patients who identified as Black and two patients who identified as Hispanic (Appendix 4, available online at http://links.lww.com/AOG/E10). The *RHD–CE–D* hybrid was identified in five other pregnant individuals, including three who identified as White, Non-Hispanic, one who identified as Black, and one who identified as more than one race. In three cases, cfDNA detected the *RHD–CE–D* hybrid variant in the fetus but not in the pregnant individual, whereas, in two cases, the variant was present in the pregnant individual (Appendix 4, http://links.lww.com/AOG/E10).

Of the 616 total doses of RhIG administered in the sample, 364 doses (59.1%) were administered antenatally, including in 16 individuals (n=6 with cfDNA RhD not detected results, n=10 with cfDNA RhD detected results) who received two antenatal RhIG doses (Table 3). The frequency of antenatal RhIG administration was significantly higher in pregnant individuals with the fetal RhD detected results than in those with fetal RhD not detected results (93.1% vs 75.0%, $P<.001$). Of the 140 pregnant individuals with fetal RhD not detected results, 33 did not receive RhIG at any point during care; the other 107 received 111 RhIG doses (Table 3). When examined by the study site, the difference in RhIG administration based on cfDNA results was significant at three sites (one site had no difference and one site contributed only one case). At one site, no RhIG was administered (antenatally or postnatally) to pregnant individuals with cfDNA RhD not detected results. In addition, there was a significant reduction over time in RhIG administration in pregnant individuals with

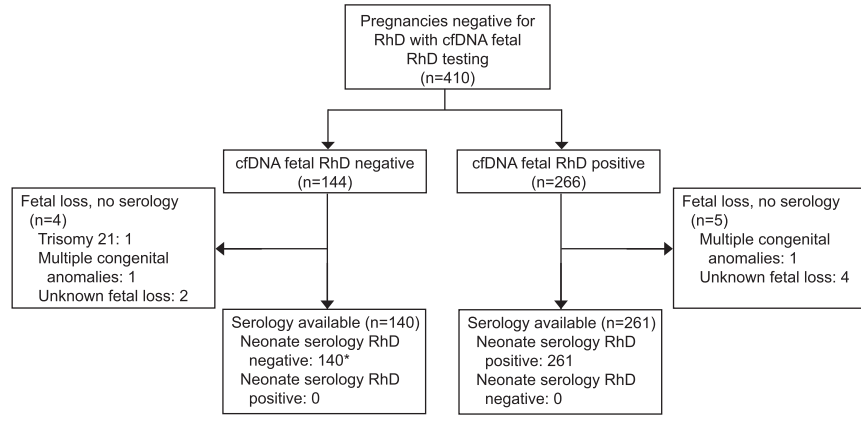

Fig. 1. Flow diagram of pregnant individuals excluded and included in the study. *Includes 10 patients who had a non-RHD gene deletion as identified by the cell-free DNA (cfDNA) analysis (five who have the *RHD–CE–D* variant and five who have the RhDψ variant).

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Table 2. Concordance of the Cell-Free DNA Fetal RhD Assay and Neonatal D Antigen Serology and Assay Performance Metrics

	Neonatal Serology		
cfDNA	RhD−	RhD+	% (95%CI)
RhD not detected	140	0	
RhD detected	0	261	
Sensitivity			100 (98.6–100)
Specificity			100 (97.4–100)
PPV			100 (98.6–100)
NPV			100 (97.4–100)
Accuracy			100 (99.1–100)

PPV, positive predictive value; NPV, negative predictive value.

cfDNA fetal RHD not detected results, with 86.2% of the first 69 individuals receiving RhIG compared with 68.7% for next 69 individuals (P=.015).

DISCUSSION

We demonstrated that cfDNA analysis through next-generation sequencing with quantitative counting template for the detection of fetal RhD status is highly accurate in a diverse U.S. clinical population of non-alloimmunized, RhD-negative pregnant individuals. The test performance demonstrated sensitivity, specificity, PPV, NPV, and accuracy of 100%. It is important to note that the cfDNA RhD assay was informative in all cases, with a 0% no-call rate, as early as 10 weeks of gestation. Furthermore, the assay correctly identified fetal RhD phenotype in the presence of non-*RHD* gene deletions. We also observed clinicians using fetal cfDNA RhD results to guide administration of RhIG. Antenatal RhIG was not administered in 33 pregnant individuals on the basis of fetal cfDNA RhD results, without any adverse clinical outcomes.

Table 3. Antenatal and Postnatal Rh Immune Globulin Administration*

	Neonatal Status†	
	RhD−	RhD+
Antenatal RhIG		
No	33 (23.6)	11 (4.2)
Yes	105 (75.0)	243 (93.1)
Unknown	2 (1.4)	7 (2.7)
Postnatal RhIG		
No	134 (95.7)	7 (2.7)
Yes	1 (0.7)	251 (96.2)
Unknown	5 (3.6)	3 (1.1)

RhIG, Rh immune globulin.
Data are n (%).
* A total of 616 doses of RhIG were administered antenatally or postnatally across the 401 patients. There were two patients with RhD-positive neonates for whom it was unknown whether RhIG was administered. All other patients with RhD-positive neonates received at least one dose of RhIG. There were six patients with cfDNA RhD not detected results and 10 patients with cfDNA RhD detected results who received two doses of antenatal RhIG. There were 33 patients with cfDNA RhD not detected results who did not receive RhIG at any time point.
† Fetal and neonatal RhD status is the same because all fetal cfDNA results were concordant with neonatal serology.

The results of this study were consistent with prior studies of the performance of this assay. In the first study of the assay, the cfDNA results were concordant with known genotype for all 455 assays using preclinical samples (parent–child genomic DNA denucleated, sheered to the size of cfDNA, and mixed at a concentration proportional to represent a pregnant person and fetal cfDNA ratios) and the neonatal serology for 21 biobanked pregnant person plasma samples.[16] In a study of 186 alloimmunized pregnant individuals, including 41 who were alloimmunized to D, the fetal cfDNA-detected antigen genotype was 100% concordant with the neonatal antigen genotype.[17]

Cell-free DNA has been used for more than a decade in the United Kingdom and European countries to guide the administration of antenatal RhIG.[5–11] However, European assays use PCR technology, which relies on the assumption that a reference sequence of another gene amplifies at the same rate as the gene of interest, resulting in imprecise measurements and requiring larger starting quantities of cfDNA, thereby requiring a later gestational age at the time of testing and resulting in a higher no-call or inconclusive rates, particularly in individuals of non-European ancestry with non-*RHD* gene deletions.[12] Although some of these assays are able to identify non-*RHD* gene deletions, because of challenges of PCR technology to quantify DNA molecules of the target sequence at low concentrations, they are not able to determine the fetal RhD status in the presence of the non-*RHD* gene deletion, particularly when the pregnant person has the variant, therefore issuing an inconclusive result with a recommendation to administer RhIG.[5–12] In addition, the use of European-based assays for the U.S. population may be logistically complicated and costly. In contrast, the assay examined in this study is next-generation sequencing based with quantitative counting template technology that allows the detection and quantification of critical allogeneic exons of the *RHD* gene.[16,17] This includes the detection of 37–base pair insertion associated with the *RhDΨ* variant and differentiation between the *RHD* gene and the homolog *RHCE* gene with detection of *RHD–CE–D* hybrid variants.

Quantification with the quantitative counting template technology enables accurate fetal RhD prediction at low fetal fractions (early gestational age) with detection of fetal RhD phenotype in the setting of non-*RHD* gene deletions in both the pregnant person and fetus. In this study, the assay predicted fetal RhD status in 10 pregnant individuals with non-*RHD* gene deletions. In other assays, this would have resulted in a 2.5% overall no-call rate and a 10.1% no-call rate among Black and Hispanic individuals in the study sample. Additionally, in this study and prior publications, the assay call rate and performance were unaffected by fetal fraction.[16] Overall, this assay based on next-generation sequencing and quantitative counting template technology is an optimal test for the demographically diverse U.S. pregnant population.

This study showed a lower frequency of antenatal RhIG administration in patients with RhD not detected cfDNA results compared with patients with RhD detected cfDNA results and a greater reduction in the frequency of RhIG administration over the course of the study. Overall, the average number of RhIG doses of 1.54 per pregnancy was lower than in a prior report in a U.S. population that found 1.80 doses per RhD-negative pregnancy.[20] Together, these results indicate that clinicians used the assay to guide administration of RhIG and increased the use of the results to guide care over time.[3,4]

A challenge to cfDNA fetal RhD testing to guide pregnancy management in the United States was the availability of a sensitive, cost-effective assay for the population. Previous evaluations of other cfDNA fetal RhD assays for the U.S. population have been shown to be both clinically and economically inferior to a prophylactic RhIG protocol.[20–23] This was related to a high rate of inconclusive results, leading to unnecessary RhIG administration, lower sensitivity resulting in the potential for an increased frequency of sensitization, and high cost. The current assay has a greater than 99% call rate and sensitivity. It is run on a cost-effective next-generation sequencing–based platform, indicating its potential to have much higher utility and to be more cost effective than prior assays studied. However, a formal U.S.-based health economics study that considers the potential for an ongoing or recurring RhIG shortage may be beneficial.

The strengths of this study include the composition of a sample of consecutive pregnancies from diverse U.S.-based clinics, including more than 25% of individuals who identified as non-White, and neonatal serology available for all live births. There were 10 pregnant individuals in whom a non-*RHD* gene deletion genotype with a predicted RhD-negative phenotype was detected with cfDNA, showing the potential of this assay to detect fetal RhD phenotype in the setting of non-*RHD* gene deletions, which are more common in individuals of non-European ancestry.[14,15] We also acknowledge the limitations of our study. Although the assay correctly determined the fetal RhD status for non-*RHD* gene deletions, it is a modest sample size. In addition, as with many prenatal cfDNA assays, this assay is not indicated for pregnant individuals with a history of a bone marrow transplantation or solid organ transplantation. In these circumstances, the assay is limited because of the potential presence of cfDNA originating from neither the fetus nor the pregnant individual. The assay is also not currently indicated for pregnant individuals with three or more gestations, and the present study does not reflect the performance of the assay for twin pregnancies.

In conclusion, these data demonstrate the excellent sensitivity and specificity of this quantitative cfDNA analysis through next-generation sequencing with quantitative counting template technology for detecting fetal RhD status in a diverse U.S. population. These data and the data previously published support offering this assay to nonalloimmunized RhD-negative pregnant individuals in the context of appropriate counseling, including risks, benefits, and limitations of the test.[16,17] This implementation will result in conservation of RhIG by use only in pregnancies in which it is medically necessary.

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PEER REVIEW HISTORY

Received September 16, 2024. Received in revised form November 11, 2024. Accepted November 21, 2024. Peer reviews and author correspondence are available at http://links.lww.com/AOG/E11.