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Validation of Fetal RHD Copy Number Calling in FirstGene, a Combined Non-Invasive Prenatal cfDNA Assay for Fetal Aneuploidy, Recessive Diseases, and Serological Screening

Ravi Patel, PhD; Juexiao Sherry Wang, PhD; Kyle Trettin, PhD; Christopher Battey, PhD; Anu Srinivasan, PhD; Janani Saikumar, PhD; Divya Kushnoor, M.Eng; Nafei Xu, MS; Summer Pierson, MS, CGC; Katie Johansen Taber, PhD; Genevieve Gould, PhD*; Dale Muzzey, PhD*

All authors are employees of Myriad Genetics, Inc., Salt Lake City, UT: *Co-senior authors

Background

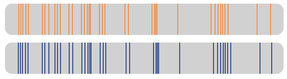
- RhD serotyping is a part of standard prenatal care to prevent the symptoms of hemolytic disease of the fetus & newborn (HDFN) caused by Rh blood type incompatibility.[1]
- In the U.S., prophylactic Rho(D) immunoglobulin is administered to all RhD-negative pregnant patients at 26–28 weeks gestation, prior to any invasive procedure, or following any potential sensitization event.[2]
- The FirstGene assay combines multiple prenatal genetic risk assessments into a single blood draw and report, including fetal full gene copy number (CN) analysis of *RHD* for RhD-negative pregnant patients.

Objective: Here we describe the analytical validation of the fetal *RHD* copy number analysis in FirstGene.

Methods

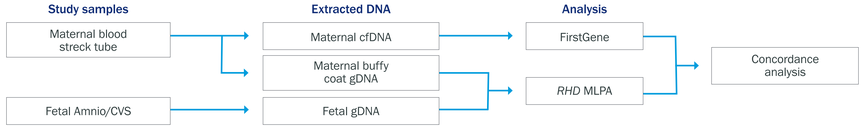
- *RHCE*, a gene homologous to *RHD*, complicates calling *RHD* copy number with short-read sequencing. Copy number and depth at differentiating bases (diffbases) are measured based on the expected depth of the sites. FirstGene utilizes over 200 diffbases for *RHD* copy number calling **(Figure 1)**.
- Validation samples:
  - 79 plasma samples from 59 pregnant patients.
  - Genomic DNA from amniotic fluid or chorionic villus sampling (CVS).
- Plasma samples were run on FirstGene, and concordance analysis was performed against MRC Holland multiplex ligation-dependent probe amplification (MLPA) run at Myriad Genetics, Inc. **(Figure 2)**.
- FirstGene utilizes a novel dynamic *in silico* insert size analysis to observe how depth signals change with fetal fraction (FF), termed "depth trajectory". Depth trajectory enables the accurate determination of maternal and fetal RHD copy number **(Figure 3)**.
- The observed fetal fraction, depth, and sample noise distributions were utilized to simulate RhD-negative and RhD-positive fetuses in a background of RhD-negative pregnant patients, enabling the assessment of sensitivity and specificity on a far larger cohort representative of fetal fraction levels in the general population **(Figure 3)**.
- Limitations of *RHD* calling:
  - *RHD* calling in FirstGene focuses on *RHD* full gene deletions, the most common molecular mechanism of the RhD-negative serotype.

Figure 1. *RHD* copy number calling approach



The grey portions represent homologous regions of the genes while the orange/blue colors represent differentiating bases. This figure is for illustrative purposes. There are many more bases that differ between the two genes and FirstGene utilizes over 200 diffbases for *RHD* copy number calling.

Figure 2. Experimental design of RHD analytical validation



Results

- 20 pregnant patients had zero copies of *RHD* (RhD-negative). FirstGene correctly identified 10/10 RhD-negative fetuses (CN=0) and 10/10 RhD-positive fetuses (CN=1), resulting in 100% sensitivity and 100% specificity **(Figure 4, 5)**.
- In 10,000 simulated samples, the expected analytical sensitivity and specificity were 100% and 100%, respectively.

Figure 3. *RHD* depth trajectory differentiates maternal and fetal copy number

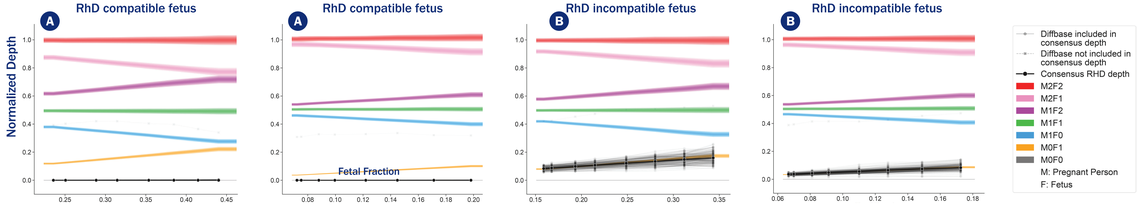


Figure 3: The plots show the depth shifts as FF increases with *in silico* size selection, where the solid black line is consensus depth, grey lines are the depths from individual diffbases. The colored distributions represent the aggregated expected depth distributions at different fetal-maternal copy number combinations as a function of FF (**A,** left two plots). Example depth trajectory plots of *RHD* copy number calling in samples with RhD compatible fetuses (zero copies of *RHD* in both the mother and the fetus). **B,** (right two plots) Example depth trajectory plots of *RHD* copy number calling in samples with RhD incompatible fetuses (zero copies of *RHD* in the mother and one copy in the fetus).

Figure 4. Examples of real and simulated *RHD* results

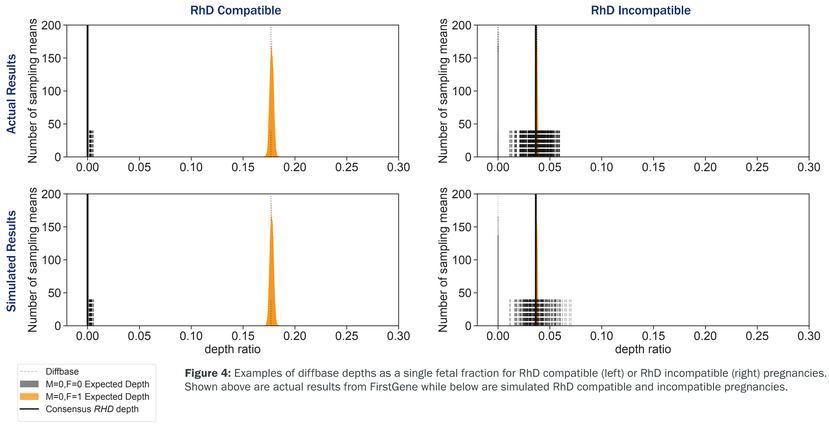
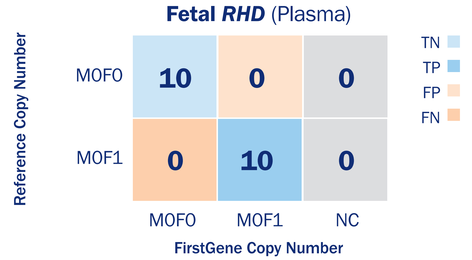


Figure 4: Examples of diffbase depths as a single fetal fraction for RhD compatible (left) or RhD incompatible (right) pregnancies. Shown above are actual results from FirstGene while below are simulated RhD compatible and incompatible pregnancies.

Figure 5. *RHD* fetal copy number calling performance



Analysis Method	Sensitivity (%) 95% CI	Specificity (%) 95% CI
Plasma	100 (72.25-100)	100 (72.25-100)
Simulation	100 (99.62-100)	100 (99.62-100)

Conclusions

- The FirstGene assay accurately determined fetal *RHD* copy number for RhD-negative pregnant patients.
- This screening approach may help prevent unnecessary treatment and intensive pregnancy monitoring for HDFN.

References: 1. Zipursky, A. The universal prevention of Rh immunization. *Clin Obstet Gynecol.* 14(3):869-84 (1971). **2.** Yoham AL, Casadesus D. Rho(D) Immune Globulin. [Updated 2023 May 22]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2024 Jan. **3.** Saramago P, 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. Southampton (UK): NIHR Journals Library; 2018 Mar. (*Health Technology Assessment*, No. 22.13.) Chapter 1, Background.

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