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Simultaneous Prenatal cfDNA Screening of Aneuploidy, Recessive Single-Gene Conditions, and Fetomaternal Blood Compatibility

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BACKGROUND: The provision of prenatal genetic screening in 3 core clinical contexts—recessive conditions, aneuploidy, and serological incompatibility—involves multiple tests across reproductive partners. This complexity limits utilization and impairs adherence to guideline-recommended care, particularly in carrier screening, where male partners are frequently not tested when a female carrier is identified. Here, we describe the analytical validation of a fetomaternal integrated recessive, serological, and trisomy genetic screen (FIRSTGENE), a single assay that harnesses in silico fragment-length trajectory analysis to evaluate all 3 contexts simultaneously, identifying clinically relevant variants in both the mother and the fetus without requiring a paternal sample.

METHODS: FIRSTGENE screens singleton pregnancies for mutations in 20 recessive genes; RhD compatibility; aneuploidies in chromosomes 13, 18, 21, X, and Y; and 22q11.2 microdeletion. Each part of the test was individually validated using a relevant subset of plasma samples from a curated collection (478 total samples from 456 patients) and 93 cell-line mixtures digested to resemble maternal and fetal cell-free DNA.

RESULTS: FIRSTGENE demonstrated ≥98.2% sensitivity and ≥99.0% specificity for fetal alleles in recessive-disease genes in plasma and cell lines; 100% sensitivity and specificity for RhD compatibility in plasma; 100% sensitivity and ≥99.8% specificity for fetal chromosomal abnormalities in plasma; and ≥99.9% sensitivity and specificity for maternal alleles in recessive-disease genes in plasma.

CONCLUSIONS: FIRSTGENE demonstrated high analytical sensitivity and analytical specificity for each component of the assay. Its capability to generate multiple prenatal screening results from a single blood draw may improve the efficiency and accessibility of prenatal genetic screening.

Introduction

Guidelines from the American College of Obstetricians and Gynecologists and the American College of Medical Genetics and Genomics recommend offering routine prenatal and preconception screening for common chromosomal aneuploidies (1) and autosomal recessive conditions (2, 3), respectively. In addition, the American College of Obstetricians and Gynecologists acknowledges that cell-free DNA (cfDNA) screening for fetomaternal blood compatibility is acceptable for pregnant patients with Rh-D alloimmunization in response to the RhoGAM shortage in the US (4). Couples that pursue noninvasive screening for these anomalies typically undergo several separate tests that involve one or both reproductive partners: prenatal cfDNA screening for aneuploidy risk (5, 6), targeted analysis of *RHD* via polymerase chain reaction (7, 8) or next-generation sequencing (NGS) (9, 10), and carrier screening (3, 11, 12) (Fig. 1A). While preconception carrier screening and genetic counseling allow couples to consider the full range of reproductive options (2), in practice, carrier screening is often performed during pregnancy. Couples revealed by carrier screening to be at high recessive reproductive risk may pursue diagnostic testing via invasive procedures such as amniocentesis or chorionic villus sampling (13) or via noninvasive prenatal diagnosis of single genes (14). However, approximately 59% of reproductive partners do not undergo screening when their female partner has been identified as a carrier of a severe condition

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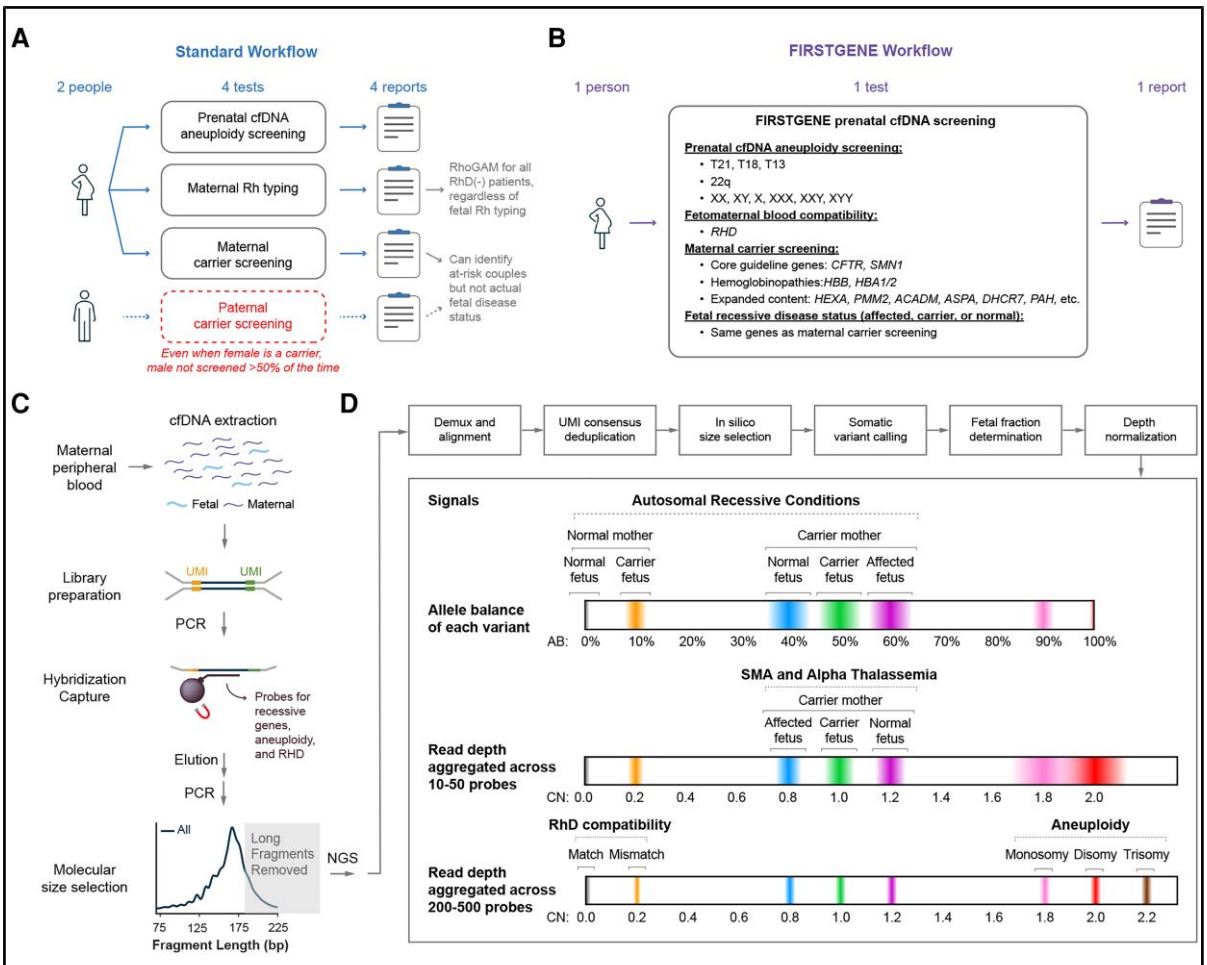


Fig. 1. Overview of FIRSTGENE. (A), Schematic of the standard workflow for prenatal care, where genetic risks for fetal aneuploidy, RhD blood type compatibility, and recessive conditions are assessed via 4 separate tests, involving both reproductive partners. Dashed lines indicate the additional logistical challenge of requiring carrier screening results on the male partner (11, 12). (B), Schematic of the FIRSTGENE workflow, which integrates multiple prenatal screening modalities into a single test using maternal blood, without the requirement for paternal screening. (C), Molecular workflow of FIRSTGENE. (D), Illustration of computational pipeline and example signals for fetal and maternal genotype deconvolution at 20% FF. The text annotation above each color gradient indicates the corresponding fetal or maternal status. For recessive single-gene screening, the colored distributions reflect allele balance (AB) values of SNVs or small INDELs. For copy number analysis, the colored distributions reflect aggregated read depth signals across 10 to 50 probes for SMA and alpha thalassemia or across 200 to 500 probes for RhD and aneuploidy. Abbreviation: UMI, unique molecular identifier.

(11, 12). This gap is particularly relevant given the wide variety of family structures in which a sperm donor may be used, or a biological male partner may not be available for testing, potentially leading to elective diagnostic testing that may not be medically necessary.

cfDNA in the plasma of a pregnant person is a blend of genomic material from both the pregnant person and the fetus (15, 16), providing the opportunity to noninvasively analyze both genomes from a single sample (17). However, simultaneously determining aneuploidy, recessive genotypes, and *RHD* status of the fetus from cfDNA is challenging: cfDNA is limited in abundance, only a fraction of it [the "fetal fraction" (FF)] is derived from placental tissue (18), and the background maternal genotype confounds the fetal signal, especially for monogenic recessive-disease analysis.

A single noninvasive screening assay does not yet exist that (*a*) simultaneously tests for aneuploidy, recessive, and fetomaternal blood compatibility conditions; (*b*) does not require a sample from the male reproductive partner; (*c*) works across the range of FF levels; and (*d*) does not require reflex testing. Most existing approaches to noninvasive fetal genotyping in multiple genes focus on dominant diseases and paternally inherited variants (19–21). These methods lack adequate handling of maternally inherited alleles and are therefore unsuitable for screening recessive conditions. More recently, whole-exome sequencing- and whole-genome sequencing-based approaches have been applied to cfDNA (22–24), but the depth of sequencing required to detect mutations can be prohibitively expensive for first-line screening (24). Further, these approaches do not produce reliable results for the nearly half of pregnancies that have FF < 15% (22, 23) and, in some cases, require a sample from both parents (23, 24). Finally, while a currently available clinical offering reports data for certain aneuploidies, recessive conditions, and fetomaternal blood compatibility (25), it runs the constituent tests as separate assays and performs fetal monogenic testing as a reflex that is executed individually for each gene in which the mother is a carrier, complicating scaling for larger recessive panels.

In this study, we introduce and describe the analytical validation of a fetomaternal integrated recessive, serological, and trisomy genetic screen (FIRSTGENE), a targeted, high-depth, NGS-based screening platform that performs allelic imbalance and depth imbalance analyses across thousands of sites at once via deep NGS to inform single nucleotide variant (SNV), insertion and deletion (INDEL), copy number variant (CNV), and aneuploidy calling (Fig. 1B). A novel "trajectory analysis"—in which in silico size selection of the NGS reads modulates each sample's FF to elucidate the fetal genotypes—undergirds all analyses in FIRSTGENE.

Materials and Methods

SAMPLE COLLECTION

One hundred one plasma samples [up to 4 tubes of maternal blood in cfDNA Blood Collection Tubes (Streck) per patient] were prospectively collected between April 2021 and June 2023 from external institutes in the United States and Ukraine. When available, an additional EDTA blood tube from the pregnant participant and fetal DNA from cultured cells obtained via amniocentesis or chorionic villus sampling were collected for orthogonal confirmatory testing. A preferred working volume of 4.8 mL plasma (minimum 4 mL) was used in a single run of the FIRSTGENE assay, and up to 3 replicates were run on a subset of samples to evaluate assay inter- and intra-batch reproducibility (Supplemental Table 1).

Four hundred four plasma samples (approximately 1.3–3.5 mL each) tested between July 2021 and July 2023 were retrospectively obtained from residual research-allowed clinical samples processed through a whole-genome sequencing-based prenatal cfDNA screen (26) run at a CLIA laboratory. Samples were selected to represent variants of interest in the validation. The number of samples was determined in consultation with a CLIA laboratory director.

Twenty-seven samples were excluded from the validation cohort, including 19 biological failures (FF < 3%, twin-like anomalies, etc.) and 8 technical errors. Based on the availability of materials for orthogonal confirmation, 478 (456 unique) plasma samples were used for fetal aneuploidy screening, 264 (244 unique) for maternal recessive disease screening, and 79 (59 unique) for fetal recessive disease and RhD compatibility assessment (see flow of participants in Supplemental Fig. 1).

CELL–LINE MIXTURE

Genomic DNA from cell lines derived from related individuals (Coriell Institute; Supplemental Table 2) was fragmented, size-selected, and mixed at defined proportions (3%, 5%, 10%, 15%, and 20%) to approximate the fragment-length profile of genuine cfDNA at various fetal fractions. Ninety-three cell-line mixtures were used for fetal recessive disease assessment.

MOLECULAR BIOLOGY WORKFLOW

In the FIRSTGENE targeted sequencing-based molecular workflow (Fig. 1C), plasma was isolated from whole blood collected in Streck tubes; cfDNA was extracted [MiniMax High Efficiency cfDNA Isolation Kit (Apostle)] and prepared into libraries using the xGen cfDNA and FFPE Library Prep Kit (IDT) with unique molecular identifiers (27) (Supplemental Fig. 2A).

Samples were multiplexed and hybridized with biotinylated 120 bp DNA probes (Twist; see Supplemental Note 1), using the xGen Hybridization Capture Core Reagents (IDT).

Sequencing libraries were size-selected to be <180 bp, minimizing long maternal fragments while slightly increasing FF (Supplemental Fig. 2B and C). Samples were sequenced to an average molecular depth of 1387 (for 4–4.8 mL of input plasma); higher depth gave diminishing returns (Supplemental Fig. 2D). See Supplemental Note 2 for details on molecular biology protocols.

NEXT–GENERATION SEQUENCING BIOINFORMATICS PIPELINE

An integrated bioinformatics pipeline (Fig. 1D) leveraged distinct signal patterns based on various combinations of maternal and fetal genotypes. In silico size-selected BAM

files were generated by partitioning reads with various insert sizes using samtools view. Eight in silico size selection bins (isss-bins, 0–190 bp, 0–175 bp, 0–168 bp, 0–165 bp, 0–160 bp, 0–155 bp, 0–150 bp, 0–145 bp) were used in the trajectory-based depth and allele balance (AB) analysis. Reads without a proper pair and unmapped reads were removed. GATK (v4.3.0.0) Mutect2 and FilterMutectCalls were used to call and filter SNVs and INDELs in each isss-bin in the covered gene regions and single nucleotide polymorphism (SNP) sites.

FF CALCULATION

FF is the genome-equivalents proportion of library molecules derived from fetal or placental cells. We estimate this quantity using a binomial mixture model on ABs of 2313 common SNP sites in each isss-bin (see Supplemental Note 3). The predicted FF was highly correlated with a chromosome Y-based measurement in male-fetus pregnancies ($R^2 = 0.997$), with an absolute error of 0.28% (Supplemental Fig. 3).

ALLELE BALANCE TRAJECTORY ANALYSIS FOR SNV AND INDEL CALLING

We used AB distributions from the common SNPs as a reference for inferring fetal and maternal genotypes in recessive-disease genes. Fetal and maternal genotype inference for SNV and INDEL variants was performed using an AB trajectory-based Gaussian mixture model. First, an AB trajectory was generated for each variant by conducting a linear fit of AB and FF values across 8 isss-bins: $E(v_i|f) = \beta_{0i} + \beta_{1i} \times f$, where f is fetal fraction, v_i is observed AB of variant i, and β_{0i} and β_{1i} are the intercept and slope of the trajectory for variant i, respectively. Next, we defined the Gaussian mixtures over 2 dimensions: trajectory slope (β_{1i}) and a trajectory-predicted AB ($E(v_i|f_{max})$) at an elevated FF (f_{max}). Filtering and edge-case handling are described in Supplemental Note 4.

SMN1, HBA1/2 DOUBLE–CIS DELETIONS, AND RHD COPY NUMBER ANALYSIS

Fetal and maternal full-gene copy-number calling involves (*a*) dynamic haplotyping analysis to determine sample-specific reliable sites, (*b*) calculating a consensus depth across multiple observations (probes) in a gene region of interest, (*c*) constructing aggregated expected depth distributions for each maternal-fetal copy number combination (hereafter denoted as M#F#, where # represents the copy number), (*d*) comparing the consensus depth to the aggregated expected depth distributions in each isss-bin, and (*e*) examining call consistency across isss-bins. The same algorithm was used for *SMN1*, *HBA1/2* double-cis deletions, and *RHD*, with different gene-specific sites defining the consensus depth. See Supplemental Note 5 for algorithmic details.

EXON–LEVEL MATERNAL CNV DETECTION

Exon-level CNVs were determined by a hidden Markov model-based method, previously described by Vysotskaia et al. (28). An isss-bin designed to enrich maternal reads (insert sizes ranging from 155 to 200 bp) was used to detect maternal CNVs in the targeted genes (excluding *SMN1*, *HBA1/2*, and *RHD*).

DEPTH–BASED MIXTURE MODEL FOR ANEUPLOIDY DETECTION

Fetal aneuploidy is detected by checking for excess or depleted read depth on a target chromosome or region and then verifying that the change in depth as a function of FF is consistent with a fetal rather than a maternal anomaly. In contrast to other depth-based aneuploidy callers that rely on comparing depth deviations to a large panel of normal samples (29), here we developed a model that compares depth deviations to a sample-specific FF estimate (Supplemental Fig. 4). This approach allows us to explicitly model mosaicism, minimizes reliance on a static background set, and enables differentiation of fetal from maternal anomalies. See Supplemental Note 6 for algorithmic details and adjustments for fetal sex, sex chromosome aneuploidy, and 22q11.2 microdeletion calling.

CONFIRMATION OF POSITIVE RESULTS IN FIRSTGENE

Maternal and fetal variant calling in the recessive-disease genes was confirmed by testing genomic DNA isolated from maternal whole blood or cultured fetal cells, respectively, with a validated targeted sequencing-based expanded carrier screen (30). Fetal aneuploidy results were confirmed by testing maternal plasma with a validated whole-genome sequencing-based prenatal cfDNA screening assay (26). Fetal origin of monosomy X (MX) calls was confirmed by amniocentesis or postnatal diagnostic assay results (microarray or karyotype), where available.

Maternal and fetal *RHD* copy number values were evaluated via multiplex ligation-dependent probe amplification using the SALSA MLPA Probemixes P401-A1, P402-A1, and P403-A1 Blood Group Genotyping Kit (MRC Holland) (31).

VALIDATION METRICS

Validation metrics—e.g., how sensitivity and specificity are calculated—are described in Supplemental Note 7. Technical replicates were processed as independent samples during validation and may exhibit slightly different FFs due to separate molecular size-selection reactions. Results from all technical replicates were presented unless otherwise specified. Analytical sensitivity and specificity were calculated using only unique samples, with one randomly selected representative from a set of technical replicates included in the calculation.

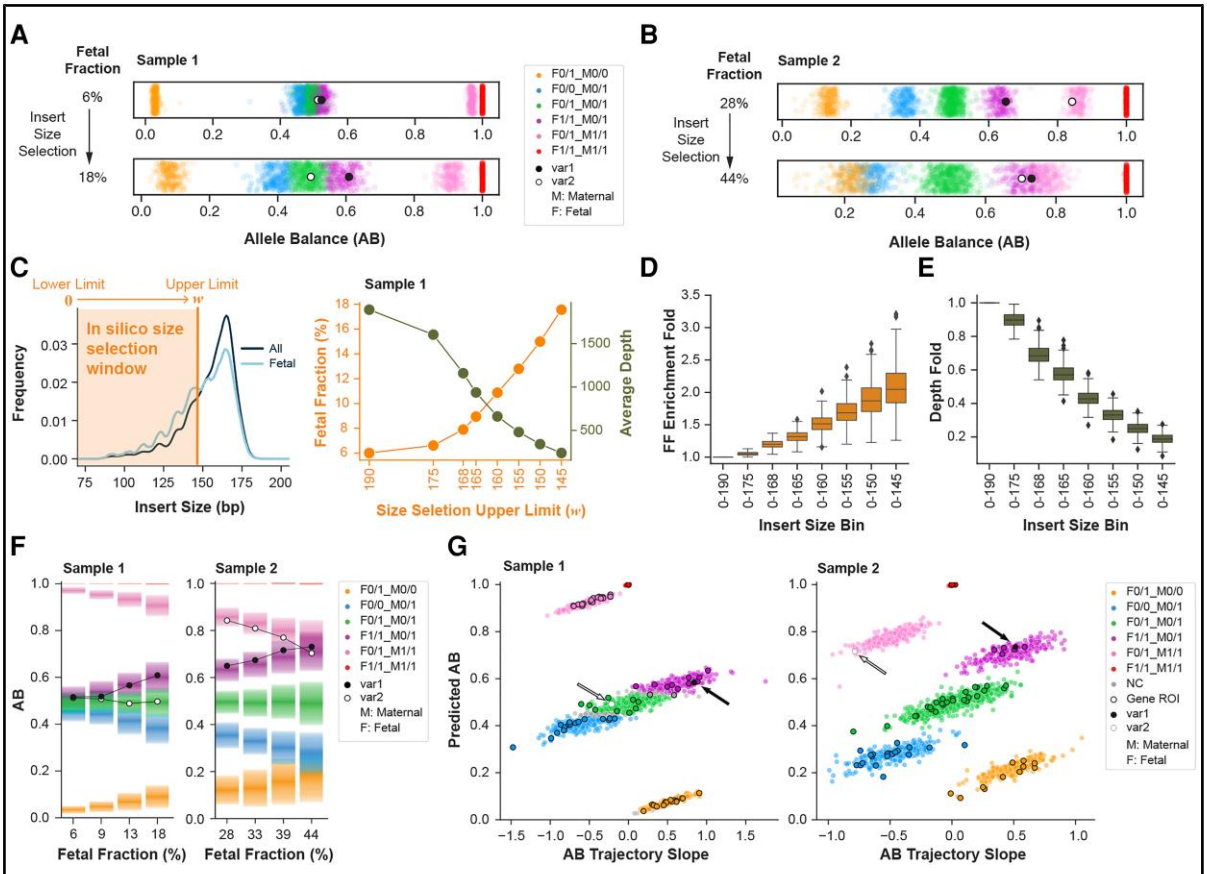
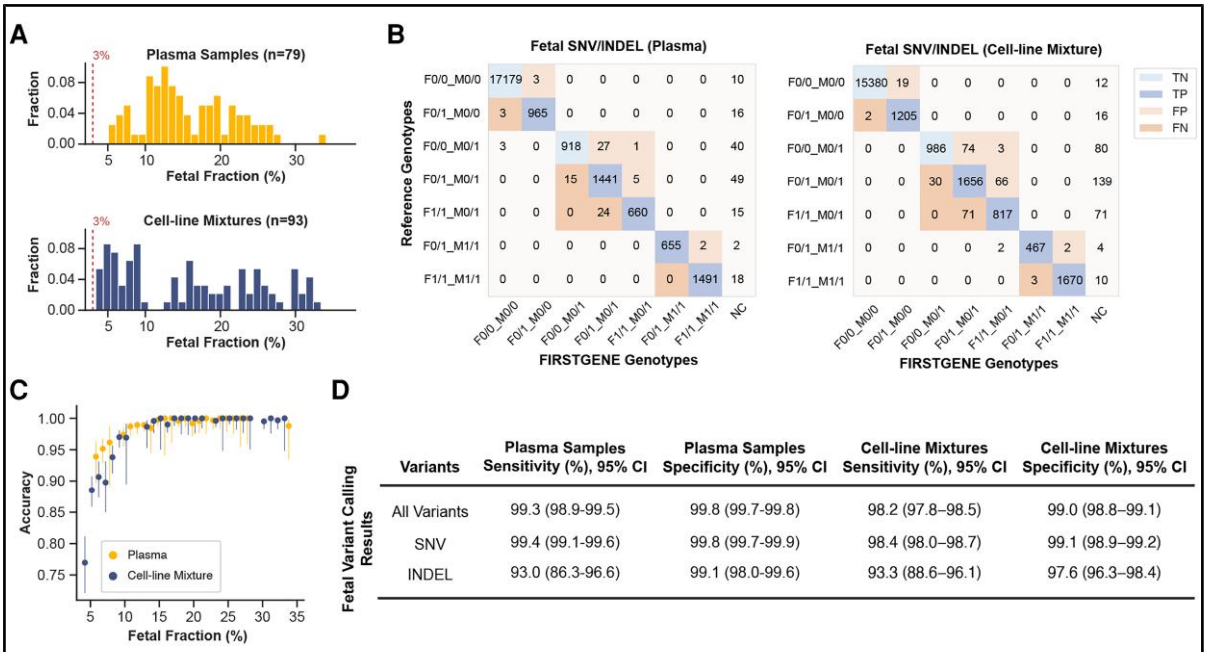


Fig. 2. Trajectory-based allele balance (AB) analysis. (A), AB values of background SNP sites before (top) and after (bottom) FF enrichment via insert size selection in a low-FF sample. Each fetal and maternal genotype combination is represented by a different color. The white and black circles depict 2 example variants with different genotypes. (B), AB values of background SNP sites before and after insert size selection in a high-FF sample. The genotypes of the example variants are initially well separated based on AB but become difficult to discriminate after insert size selection. (C), Dynamic in silico FF enrichment. Left, length distributions of fetal-derived and all fragments, with the vertical orange line representing the upper limit (w) of an insert-size sliding window. Sequencing reads are partitioned into isss-bins if their insert size falls within the shaded area (0-w bp). Right, FF and depth of each isss-bin as a function of w in example sample 1. (D and E), Fold changes of FF and depth by isss bin in 478 plasma samples. (F), Shifts in AB distributions as FF increases with in silico size selection. AB trajectories of the same variants as in (A) and (B) are illustrated by the lines connecting the black and white circles. (G), AB trajectory-based Gaussian mixture model. Each dot represents a single variant position, either within the target gene (indicated by black outlines) or at background SNP sites. Arrows highlight the locations of the same variants as in (A) and (B).

Results

DYNAMIC INSERT SIZE ANALYSIS DISAMBIGUATES FETAL AND MATERNAL GENOTYPES

FF varies widely between patient samples (typical range: 2%–35%) (18) and is unknown prior to sequencing. While cfDNA size selection can effectively increase FF (26), it also reduces unique molecular depth, leading to increased AB variability (Fig. 2A and B). These factors together make it challenging to define a universal FF enrichment strategy applicable to all samples. We developed a dynamic in silico FF enrichment method (Fig. 2C) and applied it to 478 plasma samples and 93 cell-line mixtures. We observed a monotonic increase



Variants	Plasma Samples Sensitivity (%), 95% CI	Plasma Samples Specificity (%), 95% CI	Cell-line Mixtures Sensitivity (%), 95% CI	Cell-line Mixtures Specificity (%), 95% CI
All Variants	99.3 (98.9-99.5)	99.8 (99.7-99.8)	98.2 (97.8–98.5)	99.0 (98.8–99.1)
SNV	99.4 (99.1-99.6)	99.8 (99.7-99.9)	98.4 (98.0–98.7)	99.1 (98.9–99.2)
INDEL	93.0 (86.3-96.6)	99.1 (98.0-99.6)	93.3 (88.6–96.1)	97.6 (96.3–98.4)

Fig. 3. Analytical validation of fetal SNV and INDEL calling in autosomal-recessive-disease genes. (A), FF distributions of plasma and cell-line mixture samples for fetal variant-calling validation (with a bin size of 1%). The dotted line is at 3% FF, the threshold below which a sample is set to fail due to low FF. (B), Confusion matrices of fetal SNV and INDEL calling in 17 genes. Genotypes violating Mendelian inheritance patterns, such as F1/1_M0/0 or F0/0_M1/1, cannot be detected by the assay. Fetal genotypes at sites at which the mother is correctly identified as homozygous for the reference allele, heterozygous, or homozygous for the alternative allele are highlighted, with different colors representing true negative (TN), true positive (TP), false positive (FP), and false negative (FN). The right-most column represents no-calls (NC) in FIRSTGENE. (C), Comparison of fetal genotype prediction accuracy between plasma and cell-line mixture samples by FF. Samples with similar FFs are grouped into bins based on the nearest integer FF values. Error bars represent the 95% CI. A small jitter is applied to the x-axis for better visualization. (D), Analytical sensitivity and specificity for fetal variant calling by sample and variant types.

of FF and a corresponding decrease in molecular depth across isss-bins (Fig. 2D and E, Supplemental Figs. 5 and 6).

We refer to the functional relationship between AB and FF as the "AB trajectory" (Fig. 2F) and applied a Gaussian mixture model to differentiate fetal and maternal genotypes. Plotting the AB trajectory slope vs trajectory-predicted AB allowed the gaussian mixture model to differentiate genotype clusters that overlap when considering only the raw ABs (Fig. 2G).

PERFORMANCE CHARACTERIZATION AND VALIDATION OF FETAL SNV AND INDEL CALLING IN RECESSIVE–DISEASE GENES

We validated the model's fetal SNV and INDEL calling performance across 17 recessive conditions using 79 plasma samples and 93 contrived cell-line mixtures; observed FF was 5.6% to 33.6% in plasma samples and 3.9% to 33.3% in cell-line mixtures (Fig. 3A).

Across all plasma samples, FIRSTGENE achieved an aggregated fetal genotype-prediction accuracy of 97.7% at maternal heterozygous sites and 99.7% at maternal homozygous sites (Fig. 3B, left panel; see Supplemental Fig. 7A and B for separate concordance matrices of SNVs and INDELs; see Supplemental Fig. 8 for performance by FF). Despite the low overall number of observed discordances, 90% of the incorrect calls and 83% of the no-calls were at sites where the pregnant person was heterozygous, as expected because of maximal maternal AB interference at those sites. None of the no-calls or incorrect calls involved a variant with a pathogenic classification. Samples flagged by quality metrics indicative of low performance at maternal heterozygous sites are rerun during routine clinical use of the assay to minimize potential clinical false positives or false negatives.

Among the 93 cell-line mixtures (Fig. 3B, right panel; Supplemental Fig. 7C and D), all 15 cystic fibrosis-affected contrived fetuses—either homozygous for the common ΔF508 variant or compound heterozygous for ΔF508 and a second pathogenic variant—received a screening result that would suggest follow-up with diagnostic testing (Supplemental Fig. 9). Performance in cell-line mixtures may provide a conservative estimate of assay performance, as fetal genotype-prediction accuracy in cell-line mixtures was lower than that in plasma samples when FF was 6% to 9% (assay performance below 5% FF in plasma samples remains to be established, as such samples are rare after molecular size selection) (Fig. 3C).

The overall analytical sensitivity and specificity of fetal variant calling were 99.3% and 99.8% for plasma samples and 98.2% and 99.0% for cell-line mixtures, respectively (Fig. 3D). These data show that FIRSTGENE provides accurate fetal genotype-prediction results on a multigene recessive-disease panel across a wide range of FFs, with improved performance in samples with FF < 15%, where previous studies have faced challenges (22, 23).

FETAL COPY NUMBER ANALYSIS IN CHALLENGING GENES

The remaining 2 of the 19 recessive conditions—spinal muscular atrophy (SMA) and Hb Bart syndrome—as well as fetomaternal RhD compatibility assessment, require fetal and maternal copy number analysis of *SMN1*, *HBA1/2*, and *RHD* genes, respectively. In these genes, short cfDNA sequencing reads often cannot be aligned unambiguously because of high homology with other genomic regions (32–34).

Using an ensemble approach among unique and "reliable" sites (described in the Methods and Supplemental Note 5), homology interference and depth variability were significantly reduced (Fig. 4A). We found that target region-specific depth trajectories can further improve calling accuracy. Figure 4B (left) shows depth trajectories of a plasma sample with 12.8% FF. In the 2 bins with the lowest FF (2 leftmost points), the consensus signal (black) falls between the green and blue distributions, indicating an inconclusive call. However, by expanding the trajectory, it becomes evident that the consensus is confidently within the green distribution (fetus copy number 1).

VALIDATION OF FETAL COPY NUMBER ANALYSIS

FIRSTGENE is designed to report fetal SMA status when the pregnant patient is a carrier. Among the 79 plasma samples analyzed, one was from an SMA carrier whose fetus was tested for SMA status using both cultured amniocytes and FIRSTGENE; both assays called fetal copy number 1 (Fig. 4B, left). In addition, 25 cell-line mixtures were created from mother-child pairs where the mothers were SMA carriers and the children were either noncarriers, carriers, or affected. Depth trajectories of cell-line mixtures resemble data from genuine cfDNA (Fig. 4B, right). Four affected, 9 carrier, and 9 noncarrier contrived fetuses were called correctly, and the remaining 3 (all at a 3% mixing ratio) could not be called confidently (Fig. 4C). Two of the low-confidence calls fell between carrier and affected, leading to a borderline high-risk classification, while the third fell between carrier and noncarrier, resulting in a borderline low-risk classification (Supplemental Fig. 10). During clinical use of this assay, borderline low-risk results are rerun and resolved before release whenever possible. In contrast, borderline high-risk results may be reported without a rerun—making clear that they are borderline high-risk—to ensure that the patient has sufficient time to pursue diagnostic testing; however, the patient may choose to provide a redraw at a later gestational age for another assessment at a higher FF.

Alpha-thalassemia calling in FIRSTGENE is designed to detect double-cis loss of *HBA1/2* in the fetus, the most common mechanism of severe fetal disease (35), when the pregnant individual is a carrier. Fetal performance was assessed using 5 cell-line mixtures in which the contrived fetus had 2 double-cis deletions, with each of the 2 deletions having different ancestry-specific breakpoints (Supplemental Fig. 11). Four cell-line mixtures were called correctly, and 1 (at a 3% mixing ratio) could not be called confidently and was classified as borderline high-risk (Fig. 4C).

RhD incompatibility detection in FIRSTGENE is focused on detecting the presence of *RHD* in the fetus when the pregnant individual has 2 full-gene deletions of *RHD*. Twenty out of the 79 plasma samples had RhD disease-relevant genotypes (10 with compatible RhD status and 10 with incompatible RhD status). All samples were called correctly compared to an orthogonal multiplex ligation-dependent probe amplification assay, resulting in 100% sensitivity and specificity in plasma samples (Fig. 4C, Supplemental Fig. 12).

To estimate the sensitivity and specificity of fetal copy number calling on a larger number of plasma samples, we simulated over 10 000 samples across a range of observed depth and FFs. Simulation supported 100% sensitivity and specificity for *RHD* copy-number calling. In a simulated cohort of carrier mothers, simulations showed 99.2% sensitivity and 96.1% specificity for fetal *SMN1* calling and 99.8% sensitivity and 99.7% specificity for fetal *HBA1/2* double-cis deletion calling (Fig. 4D; see Supplemental Note 8).

VALIDATION OF MATERNAL CARRIER SCREENING FROM CFDNA

FIRSTGENE simultaneously predicts maternal and fetal genotypes. To validate its performance in maternal

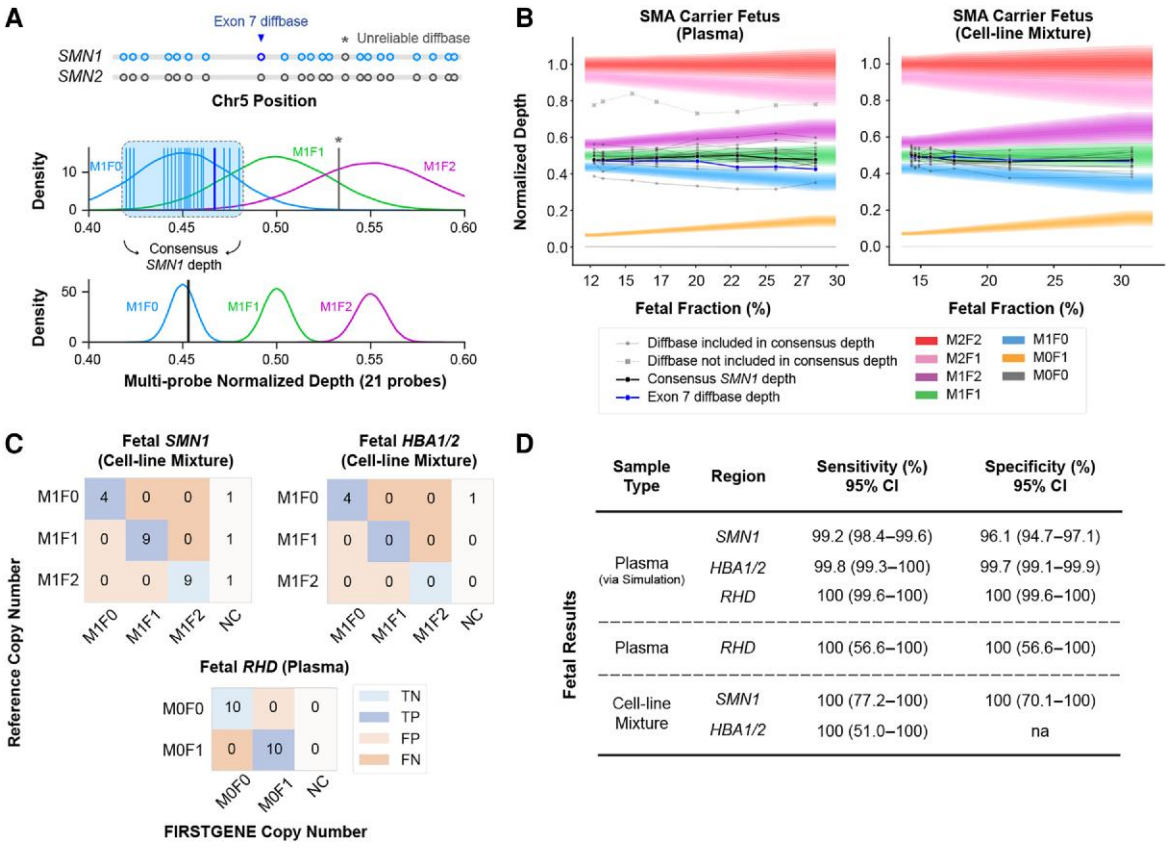


Fig. 4. Development and validation for copy-number analysis in challenging genes. (A), SMN1 copy number calling based on a single probe vs multiple probes. The top panel shows 22 sequence differences (diffbases) between SMN1 and SMN2, including the exon 7 diffbase that defines their functional distinction. The middle panel displays normalized depth of the exon 7 diffbase (thick, dark-blue vertical line in dashed box) and additional diffbases (thin, light-blue lines in dashed box) relative to the single-probe expected depth distributions when the mother is copy number 1; asterisk denotes an unreliable diffbase identified in the sample. The bottom panel shows the consensus depth (thick, black vertical line) and the aggregated expected depth distributions. While the exon 7 diffbase alone produces an ambiguous fetal copy number result, the aggregated approach provides an unambiguous result. (B), Example depth trajectories of SMN1 copy number calling in plasma and cell-line mixture samples with SMA carrier fetuses. The plots show the depth shifts as FF increases with in silico size selection. The colored distributions represent the aggregated expected depth distributions at different fetal-maternal copy number combinations. (C), Confusion matrices summarizing fetal SMN1 and HBA1/2 double-cis deletion copy number calling performance in cell-line mixture samples and fetal RHD copy number calling performance in plasma samples. (D), Analytical sensitivity and specificity of fetal copy number calls by region and sample type.

SNV, INDEL, and copy number calling across 19 recessive conditions, we assayed 264 plasma samples (FF range: 4.0%-36.2%, Supplemental Fig. 13A).

Despite plasma cfDNA being a mixture of maternal- and fetal-derived DNA, FIRSTGENE maternal genotype-prediction accuracy remained high across the full range of FFs (99.5%-100% accuracy across FFs of 4% to over 30%) (Supplemental Fig. 13B and C). All 16 SMA carriers and 10 *HBA1/2* double-cis deletion carriers, 13 exon-level deletions, and 4 duplications in recessive-disease genes were correctly identified (Supplemental Fig. 13D and E). In summary, the overall sensitivity and specificity for maternal variant calling in recessive-disease genes were ≥99.9% (Supplemental

Fig. 13F), comparable to existing carrier screening assays performed on genomic DNA samples extracted from maternal whole blood (30).

FETAL ANEUPLOIDY AND SEX CHROMOSOME COPY NUMBER ANALYSIS AND VALIDATION

For aneuploidy calling, shifts in depth distributions as a function of FF are used to construct depth trajectories. These trajectories help determine whether the observed depth changes are consistent with a fetal rather than maternal anomaly, as a fetal-derived signal should strengthen with increasing FF (Fig. 5A and B).

We validated FIRSTGENE fetal aneuploidy and sex chromosome copy number calling using 478 plasma samples (FF range: 3.2%-45%, Fig. 5C). Depth trajectories identified one sample with maternal 22q11.2 microdeletion and 4 samples with maternal mosaic MX in FIRSTGENE (Fig. 5D, Supplemental Fig. 14). Clinical confirmation of fetal results was available for 2 of the 4 MX samples, both of which supported wild-type XX karyotypes in the fetus, whereas the orthogonal assay classified them as fetal MX. No patients with a call of maternal MX mosaicism had maternal diagnostic testing done, so these cases could reflect maternal mosaicism or confined placental mosaicism.

All samples with a fetal aneuploidy or microdeletion call in the orthogonal assay and that passed technical quality metrics in FIRSTGENE (n = 98) were called positive for an anomaly (Fig. 5E). The cohort included 57 samples with a fetal trisomy of chromosome 13, 18, or 21; 35 with a fetal sex chromosome anomaly (one sample called fetal sex XYY in the orthogonal assay and XXY in FIRSTGENE), and 6 with a 22q11.2 microdeletion. All 22q11.2 microdeletion samples tested had depth profiles consistent with the common "AD" deletion (36). Three samples with fetal trisomy 13 failed technical quality metrics due to the combination of low FF and low depth. These sample failures reflected a combination of biological factors (T13 positive pregnancies have lower FF; Supplemental Fig. 15) and low plasma input volume (Supplemental Fig. 16) that is unique to the validation study but not a factor in an operational setting where a minimum of 4 mL of plasma is required.

Across 478 samples, there was one false-positive autosomal aneuploidy call (a mosaic monosomy 18), one false-positive sex chromosome aneuploidy call (XXY), 2 sex call discrepancies, and no false-positive 22q11.2 microdeletion calls (Fig. 5E). Specificity across chr13/18/21 was 99.9% per chromosome and 99.8% per sample. Specificity for sex chromosome aneuploidy was 99.8% (Fig. 5F). Analytical sensitivity and specificity for the individual sex chromosome aneuploidies are shown in Supplemental Fig. 17. These results demonstrate that FIRSTGENE aneuploidy calling using depth signals from a targeted-sequencing assay achieved comparable performance to that of existing assays designed solely for aneuploidy detection (26, 37).

Discussion

Here we describe the development and validation of a novel prenatal cfDNA screen that simultaneously performs fetomaternal testing for recessive conditions, aneuploidy, and serological incompatibility using only the blood from a pregnant individual. Using samples starting from 10 weeks of gestation and across a wide range of FFs (Supplemental Fig. 18), we found that the validation of each constituent part of FIRSTGENE had analytical sensitivity ≥98.2% and analytical specificity ≥99.0% (Supplemental Table 3).

Recent advances in the prenatal treatment of single-gene recessive disorders such as Hb Bart syndrome (38) and SMA (39) have made the development of early and accurate fetal genotype determination increasingly important. FIRSTGENE may expand access to these emerging treatments in 2 ways: (*a*) it can identify a high-risk fetus even if the father is unknown or unavailable, and (*b*) as an integrated assay without reflex testing, it delivers fetal disease results typically within 7 to 10 days instead of multiple weeks (40), enabling earlier diagnostic confirmation and allowing families sufficient time to initiate these novel therapeutic interventions.

Securing plasma from pregnancies with fetuses affected with recessive conditions was difficult due to the rarity of the conditions as well as their ability to go undetected in early pregnancy. Thus, assessment of clinically relevant pathogenic recessive variants relied primarily on cell lines. To deepen the statistical evaluation of fetal-recessive-disease status detection overall, we expanded our analysis to measure performance on variants regardless of their pathogenicity. Nevertheless, further work is needed to establish FIRSTGENE performance on recessive conditions in a large clinical cohort, particularly in samples with FF ≤ 5%, which are present but underrepresented in FIRSTGENE testing because of the molecular size selection performed before sequencing. Another limitation of the validation study is that many of the aneuploidy-positive samples came from residual plasma leftover after other testing and thus had lower plasma volume than desired for FIRSTGENE. In clinical use, with higher plasma volume, more cfDNA should make aneuploidy calling clearer and more confident. Future work will be needed to support dizygotic twin or surrogacy samples, which currently appear as QC failures in the assay, as their genotype combination patterns differ from those of singleton pregnancies. Lastly, given the general population

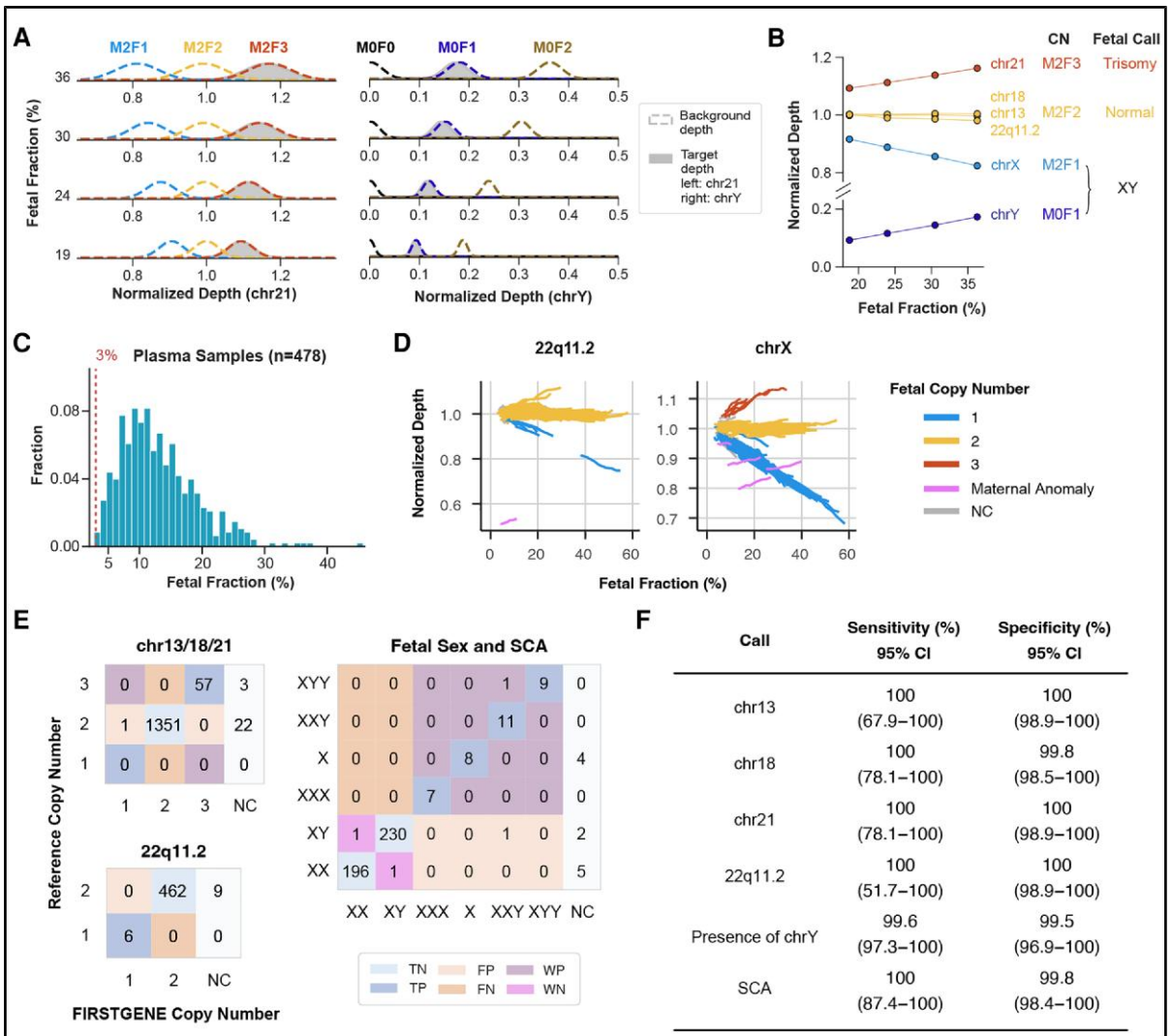


Fig. 5. Development and validation of fetal aneuploidy detection. (A), Shifts in normalized depth distributions on chr21 (left) and on chrY (right) as FF increases with in silico size selection. Dashed lines show background depth distribution of each expected copy number combination, and the grey shaded area shows the observed depth distribution of a targeted chromosome. (B), Depth trajectories of targeted regions for the same sample shown in (A). Each dot represents the median normalized depth across a targeted chromosome or region. (C), FF distribution of plasma samples for fetal aneuploidy calling validation. (D), Line plots showing the change in normalized depth as a function of FF for all validation samples, colored by the fetal copy number call. Samples with a maternal anomaly identified by depth trajectory analysis are shown in violet. (E). Confusion matrices of validation study results, comparing calls from FIRSTGENE and an orthogonal assay. The calls on axes denote the fetal copy number of the targeted chromosome. (F), Table of analytical sensitivity and specificity by call. Abbreviations: WP, wrong positive, WN, wrong negative.

frequencies of the background SNPs included in the panel (30%-70%) and their distribution across 9 chromosomes, consanguineous pregnancies are still expected to form seven distinct AB clusters depicted in Fig. 2G, except for extreme multigenerational consanguinity. However, while the assay may theoretically be applicable to consanguineous pregnancies, we have not yet established its performance in these cases.

For pregnant individuals who have had prior testing to establish that they are RHD-positive and not carriers for the screened recessive conditions, the current version of FIRSTGENE may not have substantial utility beyond a standard chromosomal-aneuploidy prenatal cfDNA screen. However, that utility is expected to expand over time because FIRSTGENE is an extensible platform that can accommodate additional content as guidelines evolve to include other genes, chromosomal abnormalities, and serological-compatibility markers. Additionally, testing for dominant and de novo variants is also readily possible, as these variants behave comparably to paternally inherited recessive alleles already tested in FIRSTGENE. As with screening for fetal chromosomal anomalies, screening for de novo variants would provide utility in all pregnancies. As sequencing costs continue to drop, we expect the scope and utility of FIRSTGENE to grow.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Ethics Declaration: Study protocols were approved by Advarra (Pro00061077 and Pro00034627), WCG (20140563), and Reproduction Genetics Clinic "Victoria" LLC (20001501/37) institutional review boards. The study complied with applicable regulatory standards. Informed consent was obtained from all participants from external institutions. For residual clinical samples collected through our CLIA laboratory, informed consent was waived, as these specimens were de-identified and approved for research use, and the study involved no diagnostic or therapeutic intervention or direct patient contact.

Data Availability: All data supporting the findings of this study are available within the article and its supplemental files. Additional data can be provided upon reasonable request, at the discretion of the corresponding authors. Requests for proprietary materials or information may be fulfilled under an NDA and/or MTA, once approved by the Myriad Genetics legal department.

Nonstandard Abbreviations: cfDNA, cell-free DNA; NGS, next-generation sequencing; FF, fetal fraction; FIRSTGENE, fetomaternal integrated recessive, serological, and trisomy genetic screen; SNV, single nucleotide variant; INDEL, insertion and deletion; CNV, copy number variant; SNP, single nucleotide polymorphism; AB, allele balance; MX, monosomy X; SMA, spinal muscular atrophy.

Human Genes: *RHD*, Rh blood group D antigen; *SMN1*, survival of motor neuron 1, telomeric; *HBA1/2*, hemoglobin subunit alpha 1 and hemoglobin subunit alpha 2.

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