Digital PCR Analysis of Maternal Plasma for Noninvasive Detection of Sickle Cell Anemia

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BACKGROUND: Cell-free fetal DNA (cffDNA) constitutes approximately 10% of the cell-free DNA in maternal plasma and is a suitable source of fetal genetic material for noninvasive prenatal diagnosis (NIPD). The objective of this study was to determine the feasibility of using digital PCR for NIPD in pregnancies at risk of sickle cell anemia.

METHODS: Minor-groove binder (MGB) TaqMan probes were designed to discriminate between wild-type hemoglobin A and mutant (hemoglobin S) alleles encoded by the *HBB* (hemoglobin, beta) gene in cffDNA isolated from maternal plasma samples obtained from pregnancies at risk of sickle cell anemia. The fractional fetal DNA concentration was assessed in male-bearing pregnancies with a digital PCR assay for the Y chromosome–specific marker DYS14. In pregnancies with a female fetus, a panel of biallelic insertion/deletion polymorphism (indel) markers was developed for the quantification of the fetal DNA fraction. We used digital real-time PCR to analyze the dosage of the variant encoding hemoglobin S relative to that encoding wild-type hemoglobin A.

RESULTS: The sickle cell genotype was correctly determined in 82% (37 of 45) of male fetuses and 75% (15 of 20) of female fetuses. Mutation status was determined correctly in 100% of the cases (25 samples) with fractional fetal DNA concentrations >7%. The panel of indels was informative in 65% of the female-bearing pregnancies.

CONCLUSIONS: Digital PCR can be used to determine the genotype of fetuses at risk for sickle cell anemia. Optimization of the fractional fetal DNA concentration is essential. More-informative indel markers are needed for this assay's comprehensive use in cases of a female fetus.

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Sickle cell anemia is caused by a recessive mutation in the *HBB*[5] (hemoglobin, beta) gene. Seventy percent of cases of sickle cell anemia in populations of African origin are caused by homozygosity for the hemoglobin S mutation *(1)*, which leads to the substitution of a valine amino acid residue for a glutamic acid residue in the *β*-globin protein. This substitution allows the mutant hemoglobin S protein to polymerize when deoxygenated *(2)*. Although the treatment for sickle cell anemia has improved, this disease is still associated with significant morbidity and reduced life expectancy worldwide *(3)*. Although the prevalence is highest in African countries, the increasing mobility of populations means the disease is becoming more frequent in Europe and the UK. Since 2003, newborn screening for sickle cell disease has been offered in the UK regardless of ethnicity *(4)*. Approximately 3 in 1000 births are affected by sickle cell disease in South East London, whereas the national average is 1 in 2000. This fact makes sickle cell disease more common than cystic fibrosis in the UK *(1)*. It is now the most common indication for invasive prenatal diagnostic testing in the UK, with approximately 440 cases reported annually *(5)*. Since 2006 we have been storing maternal plasma samples from all women who undergo invasive prenatal diagnosis at University College London Hospitals, with a view to develop noninvasive prenatal diagnosis (NIPD)[6] for a variety of genetic conditions *(6)*. We report the use of digital PCR and the relative mutation dosage (RMD) for the NIPD of sickle cell anemia.

Cell-free fetal DNA (cffDNA) in the maternal circulation is a source of fetal genetic material that offers an alternative to sampling chorionic villi or amniocytes for prenatal diagnosis (7) and avoids the risk of miscarriage associated with invasive procedures (8). Substantial technical challenges are associated with NIPD, however, because the cffDNA in early pregnancy constitutes only approximately 10% of the total circulating free DNA, the majority being maternal in origin (9). Current clinical applications for NIPD are restricted to the identification of alleles present in the fetus but not in the mother (e.g., alleles inherited from the father or arising de novo). These applications include: determining the sex in pregnancies at high risk of sex-linked genetic disorders (10); RHD (Rh blood group, D antigen) gene typing in Rh D–negative mothers (11); diagnosis of monogenic disorders, such as achondroplasia (12) or torsion dystonia (13); and the exclusion of affected status in autosomal recessive disorders, such as β thalassemia and cystic fibrosis, in which the father has a mutation different from that of the mother (14–16). Definitive diagnosis of X-linked or autosomal recessive conditions in cases in which both parents carry the same mutation is more complex, because the majority of mutant alleles in plasma will be maternal in origin. Prenatal diagnosis with cffDNA in these circumstances requires the determination of allelic ratios (or RMD) in maternal plasma rather than the presence or absence of a mutation not carried by the mother.

Digital PCR has been used successfully to detect trisomy 21 via analysis of relative chromosome dosage, which looks for overrepresentation of chromosome 21 sequences in maternal plasma compared with reference DNA sequences (17). This technique has been refined for use in diagnosing single-gene disorders and has been referred to as "relative mutation dosage" (18, 19). Lun and colleagues have described their use of digital PCR and RMD to determine allelic balance in 10 pregnancies at risk of β thalassemia in which both parents carried the same mutation in the β-globin gene (18). The same group has also described the successful application of this technique for hemophilia in 7 pregnancies at risk because the mothers were carriers of this X-linked condition (19). NIPD based on detection of allelic imbalance requires accurate measurement of the fractional fetal DNA concentration, which can be estimated only by using a DNA sequence not present in the mother. The studies reported in the literature to date have been limited to male-bearing pregnancies and have used Y-chromosome sequences for fractional cffDNA assessment. The development of an assay for assessing the fractional cffDNA content of female-bearing pregnancies is essential for allowing testing of all pregnancies at risk for sickle cell anemia. We describe the use of digital PCR and RMD for prenatal detection of sickle cell anemia in both male- and female-bearing pregnancies.

Materials and Methods

PATIENT RECRUITMENT
Blood samples were collected from women who visited the Fetal Medicine Unit at University College Hospital NHS Foundation Trust, London, for an invasive diagnostic test. Whenever possible, paternal samples were also collected. Informed consent was obtained before venipuncture, and the study was approved by the University College London Hospitals Ethics Committee A (reference 01/0095).

SAMPLE PROCESSING AND DNA EXTRACTION
Plasma was separated from 20 mL of blood (on average) by centrifugation at 1500g for 10 min. The supernatant was then transferred to fresh tubes, with care taken to ensure that the blood pellet remained intact. The plasma was then centrifuged at 16 000g for 10 min and transferred into 2-mL LoBind tubes (Eppendorf). Plasma and maternal cell pellets were stored at −80 °C immediately after processing and kept frozen until DNA extraction. The time from venipuncture to plasma separation was recorded in most cases. Plasma DNA was extracted from 5 mL plasma with the QIAamp Circulating Nucleic Acid Kit (Qiagen), with a Qiagen vacuum manifold, and in accordance with the manufacturer's instructions. Plasma DNA was then eluted into a final volume of 75 μL AVE elution buffer (in the Qiagen kit). Genomic DNA (gDNA) was extracted from 2 mL of the thawed blood pellet with the Quickgene-610L Nucleic Acid Isolation System (Fujifilm) into a final volume of 200 μL elution buffer.

QUANTITATIVE REAL-TIME PCR FOR DETERMINATION OF FETAL SEX IN CELL-FREE DNA AND ASSESSMENT OF INFORMATIVE SINGLE-NUCLEOTIDE POLYMORPHISMS
Quantitative real-time PCR (qPCR) was carried out with the ABI PRISM 7300 Real-Time PCR System (Applied Biosystems), and data were analyzed with Sequence Detection Software (version 1.4; Applied Biosystems). A TaqMan® hydrolysis assay (Applied Biosystems) for SRY (sex determining region Y) was used to detect male cffDNA, and a TaqMan hydrolysis assay for CCR5 [chemokine (C-C motif) receptor 5 (gene/pseudogene)] was used as a control to detect total cell-free DNA (cfDNA). All qPCR reactions were carried out in ABI PRISM 96-Well Optical-Reaction Plates (Applied Biosystems). We prepared 20-μL reactions with a 1× TaqMan assay, 1× TaqMan Universal PCR Master Mix with no AmpErase® uracil-N-glycosylase (UNG) (Applied Biosystems), and 5 μL of plasma DNA. Two replicates were performed per sample for

SRY detection, and 1 replicate was performed per extract for *CCR5* detection. Positive results for both replicates of the *SRY* assay were considered indicative of a male fetus; negative results for both replicates indicated a female fetus. When only 1 of the 2 replicates was positive, the assay was repeated. Two control replicates containing no template were performed for each assay to control for PCR contamination, and both replicates were required to show no amplification. In cases of a male fetus, we used the QC protocol (detailed below) for measuring the fractional fetal DNA concentration of the plasma sample. When the results of the *SRY* assay were negative (indicating a female fetus), we used informative insertion/deletion polymorphisms (indels) to measure the fractional fetal DNA concentration.

To determine which indel markers were suitable for use for estimating the fractional fetal DNA concentration in female-bearing pregnancies, we subjected gDNA prepared from maternal samples and paternal samples (when available) for each of these maternal plasmas to qPCR with primers (Sigma-Aldrich) and hydrolysis probes (Applied Biosystems) specific for a panel of indels that we had selected for their informative potential (for primer sequences, see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol58/issue6). All assays were initially tested with serially diluted male gDNA (Promega), and calibration curves were produced to ensure similar qPCR efficiencies. We prepared 20-μL reactions with 1× TaqMan Universal PCR Master Mix with no UNG (Applied Biosystems), 900 nmol/L primer, 200 nmol/L probe, and 1.5 μL of gDNA. We carried out 2 replicates per sample. Alleles present in the father but absent in the mother were considered potentially informative. These assays were then tested with the plasma DNA samples. When paternal DNA was not available, we tested plasma DNA for the presence of all markers that were absent from maternal gDNA; any assays that showed positive results for the plasma DNA were used in a digital PCR assay to measure the fractional fetal DNA concentration.

MEASUREMENT OF THE FRACTIONAL FETAL DNA CONCENTRATION FOR MATERNAL PLASMA IN MALE PREGNANCIES (QC ASSAY)

We measured fractional fetal DNA concentrations from male pregnancies with a duplex assay that detects both a multiple-copy sequence on the Y chromosome (DYS14) and the *ZCCHC2* (zinc finger, CCHC domain containing 2) gene on chromosome 18, as previously described *(20)*. The sequences of primers and hydrolysis probes are listed in Table 1 in the online Data Supplement. In brief, we carried out digital PCR with the

12.765 Digital Array™ chip on the BioMark™ System (Fluidigm). The chip contains 12 panels with 765 chambers of 6-nL volume. One panel of each chip was used for each DNA sample, with a short duplex assay used to measure the total number of copies per milliliter for DNA (*ZCCHC2*) and fetal DNA (DYS14). Final concentrations of 900 nmol/L for each primer and 200 nmol/L for each probe were used with 2× TaqMan Gene Expression Master Mix (Applied Biosystems) in a total reaction volume of 10 μL. We ran 3.5 μL cfDNA in each panel. The following PCR conditions were used: 50 °C for 2 min, 95 °C for 10 min, and 45 cycles of 15 s at 95 °C and 1 min annealing/extension at 60 °C. The number of target molecules per panel was determined with BioMark Digital PCR Analysis software. Because the DYS14 assay detects 22–30 copies per Y chromosome, 30 copies per Y chromosome was factored into the conversion of the number of DYS14 targets to the number of Y targets. The Y-chromosome targets were then expressed as a percentage of the total DNA targets.

DIGITAL PCR USING INDEL MARKERS TO MEASURE THE FRACTIONAL FETAL DNA CONCENTRATION IN FEMALE-BEARING PREGNANCIES

To enable measurement of the fractional fetal DNA concentration with only 1 chip panel per sample, we designed multiplex TaqMan assays. We analyzed *CCR5* [6-carboxyfluorescein (FAM)-labeled probe] to determine the total number of cfDNA copies and used indel assays (VIC dye–labeled hydrolysis probes) to determine the numbers of cffDNA copies. Assays for indels S04a, S04b, S05b, and MID847 were designed with FAM-labeled hydrolysis probes. Two panels per sample were required for analysis of these assays because they could not be multiplexed with the *CCR5* assay. The fractional fetal DNA concentration was calculated as: (estimated indel copy number)/(total cfDNA copy number) × 200%.

SICKLE CELL DIGITAL PCR

We used Primer Express Software (Applied Biosystems) to design a qPCR assay to distinguish the allele for the sickle cell mutation on the *HBB* gene. Each assay contained allele-specific minor-groove binder (MGB) hydrolysis probes for the mutant allele (VIC-labeled) and the wild-type allele (FAM-labeled). Primer sequences and probes are listed in Table 1 in the online Data Supplement. Digital PCR was carried out with 12.765 Digital Array chips. Six panels of each chip were used for each DNA sample. Final concentrations of 900 nmol/L for each primer and 200 nmol/L for each probe were used with 2× TaqMan Gene Expression Master Mix (Applied Biosystems) and 4 μL cfDNA per panel. The following PCR conditions were used: 50 °C for 2

min, 95 °C for 10 min, and 50 cycles of 15 s at 95 °C and 1 min annealing/extension at 60 °C.

DATA ANALYSIS

All samples were prepared, all assays were carried out, and all data were analyzed blinded to the results of sickle cell testing with chorionic villus samples, which were revealed only after the analysis had been completed.

The actual number of target molecules for each allele was obtained by Poisson correction of the number of counts, according to the following equation:

$$\text{Target molecules} = -\ln[(N - P)/N] \times N,$$

where N is the total number of wells analyzed, and P is the number of positive wells for the allele being examined. We then used the sequential probability ratio test to determine whether there was a statistically significant difference between the counts of mutant and wild-type alleles in a plasma sample (18). In brief, this method tests 2 alternative hypotheses: that the mutant and wild-type alleles are in balance (i.e., the fetus is heterozygous), or that either the mutant allele (affected fetus) or the wild-type allele (unaffected fetus) is overrepresented. The allele with the higher count is designated the potentially overrepresented allele, and its proportion, P_r, among the total number of molecules (sum of the mutant and wild-type alleles) is calculated. This proportion is compared with the theoretically expected P_r to accept the null hypothesis or the alternative hypothesis. The expected P_r for the null hypothesis is 0.5, whereas the expected P_r for the alternative hypothesis is dependent on the fractional fetal DNA concentration. The theoretically expected P_r is calculated as follows:

$$P_r = (100\% + \text{fetal DNA percentage})/200.$$

Details of the calculations are included in the Supplemental Methods (see the online Data Supplement).

Any samples that remained unclassified after the first 6 panels were run with another 6 panels until a statistically significant result was obtained or the sample was depleted.

Results

MEASURING FRACTIONAL FETAL DNA CONCENTRATIONS

Digital PCR was used to measure the fractional fetal DNA concentrations of 45 maternal plasma samples from women carrying male fetuses. This analysis was carried out with a QC assay to detect the DYS14 marker on the Y chromosome [median gestational age, 13 weeks plus 1 day, i.e., 13 + 1 weeks (gestational age hereafter presented in this format); interquartile range,

11 + 5 weeks to 16 + 5 weeks; see Table 2 in the online Data Supplement]. The fractional fetal DNA concentration of the samples ranged from 3.1% to 17.8% (median, 6.9%).

Female fetuses required the use of indel markers. Twenty-four biallelic indels have previously been used to assess for the presence of cffDNA (21). Because the length of these amplicons ranged from 78 bp to 227 bp and because the mean length of the fetal component of cffDNA is 143 bp (22), we redesigned the qPCR assays to yield shorter amplicons wherever possible. Any qPCR assays that could not be designed to yield a final amplicon length <143 bp were excluded. We designed 1 additional assay after consulting the dbSNP [National Center for Biotechnology Information database of genetic variation (23)] and added it to the panel, giving a total of 14 indel markers suitable for use.

To confirm the accuracy of these assays for measuring the fractional fetal DNA concentration, we obtained 15 plasma DNA samples from women carrying male fetuses and compared the fractional fetal DNA concentrations estimated with the DYS14 QC analysis with those obtained with the indel panel. With the DYS14 assay, the fractional fetal DNA concentration in these 15 samples varied between 4.9% and 17.6% (see Table 3 in the online Data Supplement). Maternal gDNA for each sample was assessed with the panel of indels. Maternal plasma DNA was positive for the presence of at least 1 indel not seen in maternal gDNA in 12 of 15 cases (80% informative), indicating inheritance from the father. The fractional fetal DNA concentration obtained with each marker was measured by digital PCR (see Table 3 in the online Data Supplement). S05b was the most frequently informative indel in this cohort of 12 samples (informative in 6 cases), and a paired 2-tailed t-test showed no statistically significant difference ($P = 0.31$) between the fractional fetal DNA concentration obtained with this single-nucleotide polymorphism to the result obtained with the QC assay.

Thirty-one gDNA samples from women carrying female fetuses were interrogated with the panel of 14 indel markers. Analysis of cfDNA in samples negative for at least 1 indel in maternal gDNA identified a minimum of 1 informative marker in 20 (65%) of the cases. Digital PCR subsequently estimated the fractional fetal DNA concentration for these 20 cases (median gestational age, 13 + 4 weeks; interquartile range, 11 + 3 weeks to 15 + 3 weeks) to range from 1.8% to 11.5% (median, 4.4%; see Table 4 in the online Data Supplement).

RMD ANALYSIS

Counts per panel for the wild-type and mutant alleles were converted to estimated targets per panel, and the
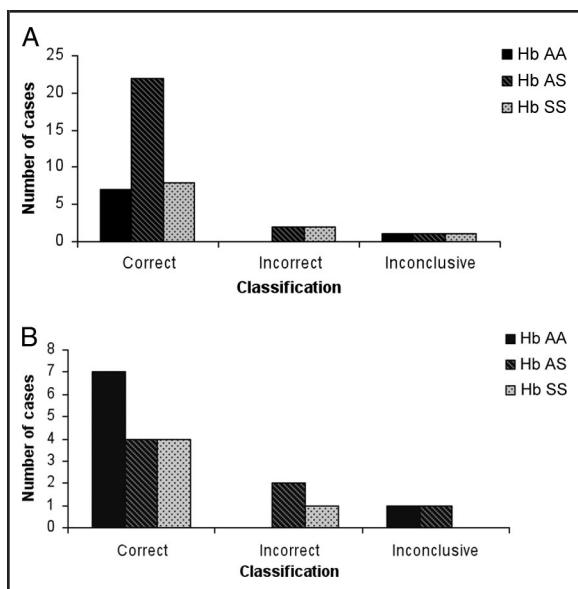
Fig. 1. Genotype for each classification for male-bearing (A) and female-bearing (B) pregnancies.

The number of samples with a particular genotype is shown for correct, incorrect, and inconclusive classifications.
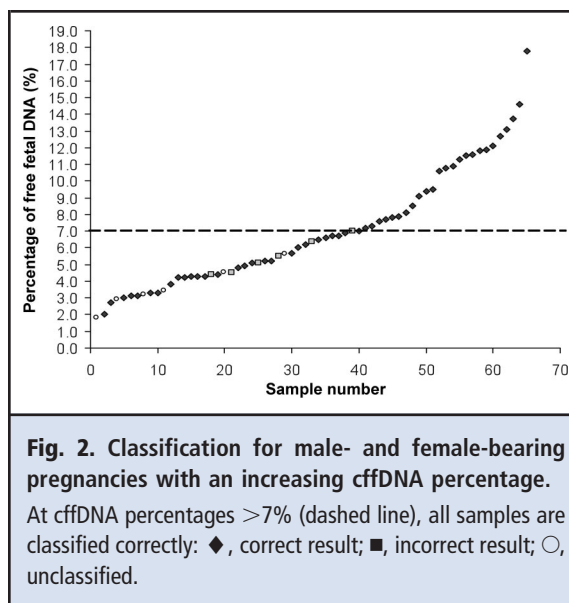

Fig. 2. Classification for male- and female-bearing pregnancies with an increasing cffDNA percentage.

At cffDNA percentages >7% (dashed line), all samples are classified correctly: ◆, correct result; ■, incorrect result; ○, unclassified.

sequential probability ratio test was used to determine a predicted classification for the sample, which was then compared with the results obtained by analyzing chorionic villi. Of the male samples, 37 of 45 were correctly classified (82%, Fig. 1A; see Table 2 in the online Data Supplement), 3 samples were unclassified because no sample remained for repeat testing, and 5 samples were incorrectly classified.

Fifteen (75%) of 20 samples from female-bearing pregnancies were correctly classified, 2 samples were classified incorrectly, and 3 samples remained unclassified after all of the sample had been used (Fig. 1B; see Table 4 in the online Data Supplement). This result is similar to that obtained with the male fetuses.

Overall, for the 25 samples with fractional fetal DNA concentrations >7%, the fetal genotype was correctly determined in all cases (100% accuracy, Fig. 2).

Discussion

We have shown that digital PCR can be used for NIPD of sickle cell anemia. Combining the results for male and female fetuses, we obtain a correct classification in 52 (80%) of 65 cases. At a fractional fetal DNA concentration >7%, we achieved 100% accuracy. This result supports previous publications showing that assays of samples with higher percentages of fetal DNA are more likely to yield correct results (18). For optimizing

cffDNA concentrations, centrifuging a sample immediately after blood draw is preferable. When the time to processing is going to be >8 h, the use of tubes containing a material that stabilizes maternal cells is helpful (20, 24). We began collecting samples in 2006, but we did not fully appreciate the importance of prompt processing until late in 2009. In the present study, we collected 23 of the 65 samples collected before this time, and 14 of these samples had fractional fetal DNA percentages <7%. Ten cases had a time to processing >8 h. These samples included those from 5 males, 4 of whom had fractional fetal DNA concentrations of <7%, and 2 of these samples yielded discordant results. All 5 of the samples from female-bearing pregnancies with a long time to processing had fractional fetal DNA concentrations <7%, and the results were unclassifiable in 2 cases. Thus, optimizing sample preparation and accurately recording preparation data may improve the cffDNA fraction and improve results. Of all samples with processing times ≥8 h, 6 samples were correctly classified despite their low cffDNA percentages, so sample processing time is clearly not the only factor to be considered in improving the test for clinical use.

Other factors that may influence the measured fractional fetal DNA concentration include the choice of amplicons used for quantification in the digital PCR assays. Our DYS14 assay is based on the observation that the primers detect 25–30 copies per genome equivalent (20); therefore, we used a copy number of 30 to calculate target number so as not to overestimate fetal DNA quantity. This adjustment may lead to small errors if there are actually fewer copies of the DYS14 se-

quence. DYS14 has been suggested not to be suitable for quantifying cffDNA, owing to the interindividual variation in copy number (25); however, the increased sensitivity of the DYS14 assay over such single-copy genes as *SRY* has been found to yield more accurate quantification of fetal DNA (26, 27). A comparison of the fractional fetal DNA concentration detected with our *DYS14* assay and a previously published assay for *ZFX* (zinc finger protein, X-linked) and *ZFY* (zinc finger protein, Y-linked) (9) shows that the DYS14 assay actually yields a statistically significantly higher proportion of the cfDNA as fetal than the *ZFX/ZFY* assay ($P = 0.0041$; see Table 5 in the online Data Supplement). This result may be because the DYS14 amplicon is half the length of the *ZFX/ZFY* amplicons, given a previous finding that the use of short amplicons improves the detection of cffDNA copies (28). The amplicons for the indels are all longer than the amplicons produced in the DYS14 assay, which may explain why plasma DNA from women carrying female fetuses appears to have a lower fractional fetal DNA concentration (median of 4.4% compared with a median of 6.9% for pregnancies involving a male fetus). The marker yielding the highest fetal DNA percentages (MID847) also has the shortest amplicon of the indel assays. Work is ongoing to design a series of indels with even shorter amplicons. Additionally, a larger cohort of samples is being used to compare the results obtained with DYS14 (QC), *ZFX/ZFY*, and *SRY* amplicons for detecting male DNA sequences with the results obtained for the shorter indel amplicons and for next-generation sequencing of fetal identifiers, because accurate measurement of cffDNA concentration is critical to NIPD with RMD. The recent release of the MiSeq next-generation sequencing platform (Illumina) will allow fractional fetal DNA concentration data to be obtained relatively easily via targeted sequencing of a small number of fetal-specific amplicons containing single-nucleotide polymorphisms.

Recent work by Palomaki et al. (29) has shown that the fractional fetal DNA concentration decreases significantly with increasing maternal weight, owing to the increased blood volume diluting the fetal DNA. The mean fractional fetal DNA concentration was 17.8% in a woman weighing 100 pounds, but it was reduced to 7.3% in individuals weighing 250 pounds. The cffDNA percentage was also significantly associated with ethnicity. Information regarding maternal weight is not available for all our samples, but it is a possible cause of the low mean percentage of fetal DNA.

In summary, we have extended the use of RMD for single-gene disorders to include the prenatal diagnosis of sickle cell anemia, and, for the first time, we have applied this approach to the detection of an autosomal recessive disease in female fetuses. These results further demonstrate the potential use of cffDNA for safer prenatal diagnosis, but to make this approach applicable to all pregnancies at risk of sickle cell anemia or to extend it to other autosomal recessive disorders, we need further optimization of cffDNA collection and extraction, as well as a larger panel of informative indel markers for use in screening female-bearing pregnancies.

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