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Noninvasive prenatal diagnosis in a fetus at risk for methylmalonic acidemia

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Purpose: Prenatal diagnosis of fetal Mendelian disorders can benefit from noninvasive approaches using fetal cell-free DNA in maternal plasma. Detecting metabolic disorders before birth can result in immediate treatment postpartum in order to optimize outcome.

Methods: We developed a mathematical model and an experimental methodology to analyze the case of a fetus with a 25% risk of inheriting two known mutations in *MUT* that cause methylmalonic acidemia. To accomplish this, we measured allelic counts at the mutation sites and the fetal fraction from high minor-allele-frequency single-nucleotide polymorphism positions.

Results: By counting linked alleles, the test was able to distinguish 11 positive markers from the negative controls and thereby determine whether or not the mutations carried by the parents were inherited by the fetus. For a homozygous fetus, the *Z*-score of the mutation site was 5.97, whereas the median *Z*-score of all the linked alleles was 4.56 when all negative (heterozygous) controls had a *Z*-score <2.5.

Conclusion: The application of this methodology for diagnosing methylmalonic acidemia shows that this is a cost-effective and noninvasive approach to diagnosing known mutations related to Mendelian disorders in the fetus.

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Key Words: cell-free; diagnostics; methylmalonic; *MUT*; newborn

Noninvasive prenatal testing (NIPT) using cell-free DNA has proven to be highly sensitive and specific for the detection of fetal aneuploidy (e.g., Down syndrome).[1–4] NIPT works by analyzing circulating fetal DNA, the concentration of which comprises between 3 and 40% of the total cell-free DNA in maternal serum. Although invasive prenatal tests such as amniocentesis and chorionic villus sampling are currently the gold standard procedures for the diagnosis of fetal aneuploidy, the safety profile and early application (often in the first trimester) of NIPT have led to its use in pregnancies deemed as at risk for fetal aneuploidy on the basis of standard first- or second-trimester aneuploidy screening, previous pregnancy history, or findings suggestive of aneuploidy on prenatal ultrasound examination.[5] Invasive prenatal diagnostic tests are also currently used to detect recessive diseases in fetuses of pregnant women who are known to be carriers of Mendelian gene mutations. Therefore, NIPT for fetal monogenic diseases holds the same compelling clinical argument as for aneuploidy testing. Because of its safety profile, NIPT can be particularly useful in the third trimester, allowing for (i) diagnosis without the risk of premature labor and (ii) appropriate planning and preparation for acute perinatal and neonatal management as required.

One approach to addressing Mendelian diseases comprehensively is via whole or partial genome sequencing of cell-free fetal DNA in maternal blood.[6,7] However, because specific mutations carried by the parents are often identified before the prenatal testing of the fetus, noninvasive methods (using digital polymerase chain reaction (dPCR)) that focus on specific mutations have also been proposed. dPCR has the advantages of economy, speed, and independence from an informatics infrastructure.[8,9] Thus far, the success rate of using dPCR for monogenic diseases has not matched the high sensitivity and specificity of aneuploidy detection, which can be used successfully as early as 10 weeks. This is due to more limited circulating fetal markers: although NIPT for aneuploidy detection targets any DNA fragments from whole chromosomes, NIPT for monogenic diseases must target specific mutations. Because only 500–1,000 genomic copies of cell-free DNA exist per milliliter of blood, obtaining sufficient fetal DNA can be challenging.

This article describes a method to simultaneously measure both allelic counts in plasma for fetal mutations and the fetal fraction (the fraction of fetal content in cell-free DNA). The fetal fraction can be important for confidence estimates but has lacked a reliable method of measurement, especially in cases involving a female fetus that lacks a unique Y chromosome to target.[4,8] For pregnancies involving a female fetus, previous work has targeted point mutations, but those were informative in only 65% of studied cases.[9] Here, we developed a method using

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low-bias multiplex amplification to reliably determine a fetal fraction with multiple markers (13 were used here), regardless of fetal gender and without consuming substantial amounts of sample. In addition to directly targeting the mutation site, we also followed a set of markers in a haplotype related to the mutation in order to expand the statistical power of the test.

MATERIALS AND METHODS

Sample extraction and processing

Maternal blood was collected into EDTA-coated tubes during pregnancy. The sample came from a third-trimester pregnant woman who had a previous child with a homozygous knockout *MUT* mutation on exon 2 (NM_000255.3:c.322C>T, p.R108C, rs121918257).[10] Maternal blood was centrifuged at 1,600*g* for 10 min at 4 °C, and 8 ml of plasma supernatant was removed carefully without disturbing the buffy coat. The plasma was centrifuged again at 16,000*g* for 10 min at 4 °C to remove any residual contaminating cells. Cell-free DNA was eluted from plasma using a QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) without the manufacturer's RNA carrier. The plasma was divided into three portions: 15% was used for direct dPCR for allele counting and DNA quantification, another 15% was used for fetal fraction determination, and 50% was used for allelic counting via the haplotype. The remaining portion was held in reverse and ultimately not used.

Pure DNA from the cellular portion of maternal blood and fetal cord blood was extracted with a QIAamp Mini Blood Kit (Qiagen). Both types of DNA were sheared with Covaris S220 using the recommended settings for 1.5-kbp fragments. dPCR was performed to confirm genotypes using the same primer/probes. For negative controls, the maternal blood was used as a mock sample for a maternal heterozygous, fetus heterozygous genotype.

Direct counting of mutation site

A Taqman primer/probe pair targeted the *MUT* c.322C>T mutation on chromosome 6 and could differentiate between genotypes: ACGTGGACCATATCCTACCATGTAT (primer 1), TTGCTTTCTTCCACAGTACTAAAACCA (primer 2), FAM-ATACTGGCAGATGGTC (mutant probe), and VIC-ACT GGCGGATGGTC (wild-type probe). The primer/probe pair was validated using pure maternal DNA to ensure proper separation of VIC and FAM populations after dPCR. A temperature gradient was used in conjunction with dPCR to select the optimal temperature for primer/probe function.

After droplets were generated from plasma DNA (QX100 Droplet Generator, Biorad, Hercules, CA) and PCR was performed on all droplets, a fluorescent droplet reader (QX100 Droplet Reader, Biorad) read the FAM and VIC signals of the probes in each droplet (see **Supplementary Figure S3** online). We treated positive droplets as binomial random variables to estimate the true counts based on the total number of droplets and number of positive droplets for each fluorophore. This approach allowed for quantification of the plasma DNA (48,000 haploid equivalents from 8 ml of plasma) as well as the separate counts of each allele. To calculate the amount of initial DNA, we assumed a uniform droplet volume of 0.91 nl designated by the manufacturer. This allowed calculations that do not require the knowledge of the dead volume, which is about 30% but can also be variable.

Fetal fraction determination

For fetal fraction, Taqman primer and probes (ABI, Foster City, CA; sequences not known) were chosen for regions on database of Single-Nucleotide Polymorphisms (dbSNP) locations targeted to high minor-allele-frequency positions (see **Supplementary Table S1** online).

Maternal blood was genotyped to determine positions in the mother that were homozygous. The corresponding primers and probes for those positions were pooled together and used to preamplify a portion of plasma DNA using the Taqman PreAmp reaction mix (ABI). The preamplified reaction was diluted 2× and distributed into individual reactions for all positions on the droplet dPCR. For all positions, a minor allele fraction was calculated by taking the smaller fraction of the two counted alleles. Substantial deviation from zero signifies useful positions where the fetus is heterozygous rather than homozygous and carries a paternally derived allele that is different from that in the mother. Doubling the minor allele fraction at useful positions gives the fetal fraction because the minor allele represents only the paternal-specific allele from the fetus and not the maternal-specific allele.

Indirect counting of mutation via haplotype

To obtain allelic counts of known positions in the haplotype,[10] the approach we used was similar to that for fetal fraction determination. Thus, a multiplex amplification of the plasma DNA was followed by individual targeting of positions by dPCR using

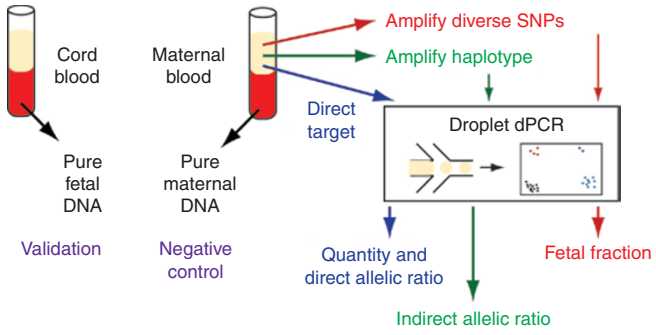
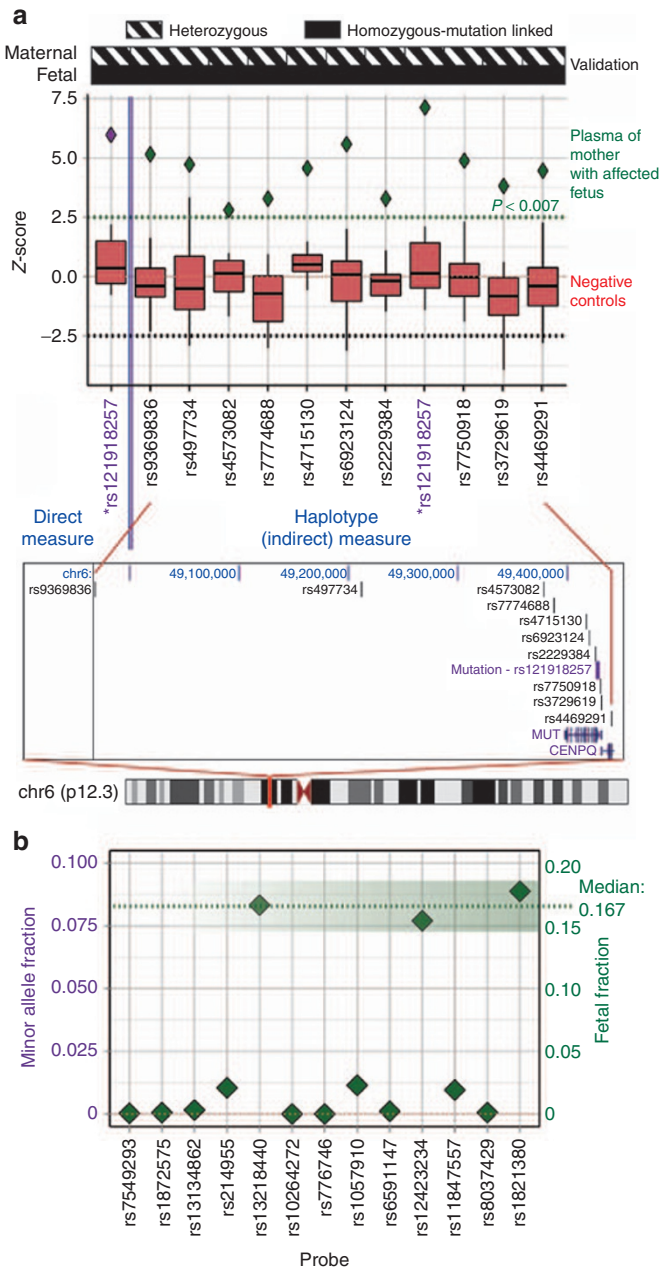


Figure 1 Schematic representation of the methodology. Both maternal blood and cell portions of cord blood (taken at birth) verify the genotype of all probes used for mutation status, fetal fraction, and haplotype determination. Plasma from the pregnant mother was split into three portions. Direct targeting of the c.322C>T (p.R108C) mutation was conducted with digital polymerase chain reaction (dPCR) to count the alleles in plasma directly and to provide a quantitative measure for the absolute amount of DNA in plasma (7,200 molecules used). Diverse single-nucleotide polymorphism (SNPs) (13) were targeted in a multiplex amplification to determine the fetal fraction (7,200 molecules used). Finally, a separate multiplex amplification of 11 targeted single-nucleotide variations for a haplotype linked to c.322C>T was performed on 24,000 molecules of input.

Taqman genotyping primers/probes. Once individual counts were received, we used the quantification of original DNA to normalize the counts to the estimated original number of target molecules for both wild-type and mutant alleles.

Analysis

Data were extracted from a Digital PCR Droplet Reader using QuantaSoft (Biorad). All calculations were performed in Microsoft Excel or with R Studio. From the original counts of positive droplets, we determined the estimated amount of original molecules. The volume of each droplet was assumed to be 0.91 nl. With the estimated original counts, the lowest total counts were taken as the value to downsample all counts to in relative proportions so that the *Z*-score could be properly compared.



RESULTS

To demonstrate this noninvasive test, first, we directly counted the number of mutant and wild-type alleles for the mutation site using dPCR and a Taqman primer/probe targeting two different fluorophores to each allele (**Figure 1**). Because we used only one genetic locus, we wanted to determine when we could confidently call the fetal genotype ($P < 0.007$ or Z-score >2.5) and when our method had insufficient fetal material.

To test the theoretical feasibility of counting limited alleles as well as to provide a framework for the analysis, we developed a model that assumes two independent Poisson distributions representing the measured molecules or "counts" of mutant and normal alleles (see **Supplementary Materials and Supplementary Figure S1** online). From this, we derived **Supplementary Equation S1** online, which defines a theoretical Z-score defined by the difference in counts between the two different alleles. A heterozygous or unaffected fetus will have a Z-score averaging zero, whereas a homozygous fetus will have a Z-score significantly elevated from zero. The equation shows that fetal fraction is proportional to the Z-score, whereas DNA input is proportional to the square root of the Z-score. On the basis of statistical limitations (see **Supplementary Figure S2** online), mutant and normal allelic counts that are nearly equal can be due to a fetus that is either (i) heterozygous and unaffected by disease or (ii) homozygous but has an insufficient fetal fraction or DNA quantity and affected. Distinguishing between these two possibilities is critical in avoiding false negatives and achieving a reliable fetal genotype.

Using our model framework, allelic counts, fetal fraction, and DNA quantity are collected (**Figure 1**) and entered into **Supplementary Equation S2** online. We estimated the fetal fraction via a set of multiplex amplified Taqman assays that target diverse single-nucleotide variations to seek positions where the mother is homozygous and the fetus is heterozygous. The final fetal fraction was measured by three relevant positions

Figure 2 Noninvasive test results. (**a**) (Top panel) Distinguishing an affected (homozygous) fetus from mock unaffected fetuses (negative controls) by a calculated empirical Z-score (see **Supplementary Equation S2** online) based on allelic count differences of each separate position. Measurement of the alleles on the mutation site directly (leftmost column) and by a multiplex amplification of 10 additional positions (right) that are linked to the mutation through a known 1.7-Mbp haplotype. (Bottom panel) Location of mutations and haplotype positions relative to the *MUT* gene and chromosome 6. (**b**) Determination of fetal fraction by tallying the allelic counts of a panel of blindly queried single-nucleotide variations that are diversely represented in the human population. By finding single-nucleotide polymorphism (SNP) positions at which the mother is homozygous and the fetus is heterozygous (i.e., AA/AG), fetal fraction can be calculated to be double the fractional count of the alternative allele in the fetus (i.e., two times the fraction count of G). To be almost guaranteed multiple useful positions that meet the criteria for fetal fraction determination, we screened the maternal cell portion against 32 SNP positions. Positions that were homozygous and had high probe quality tallied 13. These corresponding 13 probes were pooled for a multiplex polymerase chain reaction of the plasma DNA (7,200 input molecules per position). Calculation of a minor allele fraction, which is the smaller fraction of the two counted alleles and half of the fetal fraction, helped to determine which of the 13 positions were useful. Three positions, rs13218440, rs12423234, and rs1821380, had fetal fractions of 16.7, 15.4, and 17.8%, respectively.

with calculated fractions of 15.4, 16.7, and 17.8% (**Figure 2b**). Using **Supplementary Equation S1** online and the empirically determined fetal fraction, the predicted average *Z*-score for an affected (homozygous) fetus is then 16.7% × 1,146/sqrt(1,146) = 5.65. Using **Supplementary Equation S2** online and the direct allelic counts, the *Z*-score determined by the diagnostic test was 5.97 (5.7% difference; **Figure 2a**, left column). Because the *Z*-score calculated from the test is significantly different from zero and closely matches the predicted *Z*-score for a homozygous fetus, the result of the test is that the fetus is homozygous for the mutation.

For the second approach, we sought to effectively extend the number of counts available to us by simultaneously amplifying several single-nucleotide variations linked to the mutation. This was possible given a reported 1.7-Mbp haplotype associated with the c.322C>T mutation.[10] We made primers for and multiplex-amplified 11 haplotype-linked sites, including the original mutation site, to effectively increase our sample counts by an order of magnitude. All unknown samples were downsampled to 1,146 counts so that their normalized *Z*-score could be appropriately compared. This is akin to physically measuring only the first 1,146 positive counts. The median *Z*-score for all sites was 4.56 (19.3% difference from prediction), with a range of 2.8–7.8 (all scores >99% confident that fetus was not heterozygous; **Figure 2a**, right, **Supplementary Table S1** online). To ensure model and test validity, we used the mother's lymphocytes as a negative control set because it was precisely 50/50 for the two alleles. The negative controls were consistently under an expected *Z*-score of 2.5, which demonstrates the viability of the model as well as the low bias in the multiplex amplification. The noninvasive result was confirmed with cord blood from the fetus after birth.

DISCUSSION

Methylmalonic acidemia is typically included in newborn screening programs and is known to cause severe neonatal morbidity. Immediate diagnosis and management, including precise intervention, typically with low protein intake, glucose-containing fluids, and ammonia-scavenging agents, are critical to preventing irreversible end organ damage related to metabolic acidosis and hyperammonemia. A noninvasive test for methylmalonic acidemia and other metabolic disorders may be useful at any point during the pregnancy.

We have demonstrated two noninvasive detection methods, one direct and one using genetically linked markers, to augment the DNA count by more than an order of magnitude in order to maintain diagnostic power even in the setting of low fetal fractions or blood volumes. Each target was practically priced at ~ $3 per marker assay (~15,000 droplets each) without the need for informatics infrastructure and could deliver the result within a day. On the basis of our model, a direct counting approach, useful when there are no linked markers, can potentially be used in any trimester as long as there is a sufficient amount of fetal DNA present in maternal serum. On the basis of previously published NIPT cohort studies, not all maternal samples will contain enough circulating fetal DNA for direct analysis, in which case the only viable approach is the linkage method described here.[9,11] A major advantage of the approach presented herein is that it provides useful information regarding the fetal fraction and DNA quantity, thereby allowing the test to at least declare the result as indeterminate, rather than yielding a false-negative result. Compound heterozygous scenarios can also be addressed with these methods (see **Supplementary Materials** online). Integrating these tools may allow for NIPT of fetal genotype with diagnostic reliability across a wide range of Mendelian diseases.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/gim

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DISCLOSURE

S.R.Q. is a founder and shareholder of Fluidigm Corporation and Helicos BioSciences. S.R.Q. is a shareholder of Verinata Health, which was acquired by Illumina. The other authors declare no conflict of interest.

REFERENCES

1. Bianchi DW, Platt LD, Goldberg JD, Abuhamad AZ, Sehnert AJ, Rava RP. Genome-wide fetal aneuploidy detection by maternal plasma DNA sequencing. *Obstet Gynecol* 2012;119:890–901.
2. Palomaki GE, Kloza EM, Lambert-Messerlian GM, et al. DNA sequencing of maternal plasma to detect Down syndrome: an international clinical validation study. *Genet Med* 2011;13:913–920.
3. Palomaki GE, Deciu C, Kloza EM, et al. DNA sequencing of maternal plasma reliably identifies trisomy 18 and trisomy 13 as well as Down syndrome: an international collaborative study. *Genet Med* 2012;14:296–305.
4. Fan HC, Blumenfeld YJ, Chitkara U, Hudgins L, Quake SR. Noninvasive diagnosis of fetal aneuploidy by shotgun sequencing DNA from maternal blood. *Proc Natl Acad Sci USA* 2008;105:16266–16271.
5. American College of Obstetricians and Gynecologists Committee on Genetics. Committee Opinion No. 545: Noninvasive prenatal testing for fetal aneuploidy. *Obstet Gynecol* 2012;120:1532–1534.
6. Lam K-WG, Jiang P, Liao GJW, et al. Noninvasive prenatal diagnosis of monogenic diseases by targeted massively parallel sequencing of maternal plasma: application to β thalassemia. *Clin Chem* 2012;58:1467–1475.
7. Fan HC, Gu W, Wang J, Blumenfeld YJ, El-Sayed YY, Quake SR. Non-invasive prenatal measurement of the fetal genome. *Nature* 2012;487:320–324.
8. Tsui NB, Kadir RA, Chan KC, et al. Noninvasive prenatal diagnosis of hemophilia by microfluidics digital PCR analysis of maternal plasma DNA. *Blood* 2011;117:3684–3691.
9. Barrett AN, McDonnell TC, Chan KC, Chitty LS. Digital PCR analysis of maternal plasma for noninvasive detection of sickle cell anemia. *Clin Chem* 2012;58:1026–1032.
10. Worgan LC, Niles K, Tirone JC, et al. Spectrum of mutations in mut methylmalonic acidemia and identification of a common Hispanic mutation and haplotype. *Hum Mutat* 2006;27:31–43.
11. Lench N, Barrett A, Fielding S, et al. The clinical implementation of non-invasive prenatal diagnosis for single-gene disorders: challenges and progress made. *Prenat Diagn* 2013;33:555–562.