

## GENOMICS

# Noninvasive Whole-Genome Sequencing of a Human Fetus

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Analysis of cell-free fetal DNA in maternal plasma holds promise for the development of noninvasive prenatal genetic diagnostics. Previous studies have been restricted to detection of fetal trisomies, to specific paternally inherited mutations, or to genotyping common polymorphisms using material obtained invasively, for example, through chorionic villus sampling. Here, we combine genome sequencing of two parents, genome-wide maternal haplotyping, and deep sequencing of maternal plasma DNA to noninvasively determine the genome sequence of a human fetus at 18.5 weeks of gestation. Inheritance was predicted at  $2.8 \times 10^6$  parental heterozygous sites with 98.1% accuracy. Furthermore, 39 of 44 de novo point mutations in the fetal genome were detected, albeit with limited specificity. Subsampling these data and analyzing a second family trio by the same approach indicate that parental haplotype blocks of ~300 kilo-base pairs combined with shallow sequencing of maternal plasma DNA is sufficient to substantially determine the inherited complement of a fetal genome. However, ultradeep sequencing of maternal plasma DNA is necessary for the practical detection of fetal de novo mutations genome-wide. Although technical and analytical challenges remain, we anticipate that noninvasive analysis of inherited variation and de novo mutations in fetal genomes will facilitate prenatal diagnosis of both recessive and dominant Mendelian disorders.

## INTRODUCTION

On average, ~13% of cell-free DNA isolated from maternal plasma during pregnancy is fetal in origin (1). The concentration of cell-free fetal DNA in the maternal circulation varies between individuals, increases during gestation, and is rapidly cleared postpartum (2, 3). Despite this variability, cell-free fetal DNA has been successfully targeted for noninvasive prenatal diagnosis including for development of targeted assays for single-gene disorders (4). More recently, several groups have demonstrated that shotgun, massively parallel sequencing of cell-free DNA from maternal plasma is a robust approach for noninvasively diagnosing fetal aneuploidies such as trisomy 21 (5, 6).

Ideally, it should be possible to noninvasively predict the whole-genome sequence of a fetus to high accuracy and completeness, potentially enabling the comprehensive prenatal diagnosis of Mendelian disorders and obviating the need for invasive prenatal diagnostic procedures such as chorionic villus sampling with their attendant risks. However, several key technical obstacles must be overcome for this goal to be achieved using cell-free DNA from maternal plasma. First, the sparse representation of fetal-derived sequences poses the challenge of detecting low-frequency alleles inherited from the paternal genome as well as those

arising from de novo mutations in the fetal genome. Second, maternal DNA predominates in the mother's plasma, making it difficult to assess maternally inherited variation at individual sites in the fetal genome.

Recently, Lo *et al.* showed that fetal-derived DNA is distributed sufficiently evenly in maternal plasma to support the inference of fetal genotypes, and furthermore, they demonstrated how knowledge of parental haplotypes could be leveraged to this end (7). However, their study was limited in several ways. First, the proposed method depended on the availability of parental haplotypes, but at the time of their work, no technologies existed to measure these experimentally on a genome-wide scale. Therefore, an invasive procedure, chorionic villus sampling, was used to obtain placental material for fetal genotyping. Second, parental genotypes and fetal genotypes obtained invasively were used to infer parental haplotypes. These haplotypes were then used in combination with the sequencing of DNA from maternal plasma to predict the fetal genotypes. Although necessitated by the lack of genome-wide haplotyping methods, the circularity of these inferences makes it difficult to assess how well the method would perform in practice. Third, their analysis was restricted to several hundred thousand parentally heterozygous sites of common single-nucleotide polymorphisms (SNPs) represented on a commercial genotyping array. These common SNPs are only a small fraction of the several million heterozygous sites present in each parental genome and include few of the rare variants that predominantly underlie Mendelian disorders (8). Fourth, Lo *et al.* did not ascertain de novo mutations in the fetal genome. Because de novo mutations underlie a substantial fraction of dominant genetic disorders, their detection is critical for comprehensive prenatal genetic diagnostics. Therefore, although the Lo *et al.* study demonstrated the first successful construction of a genetic map of a fetus, it required an invasive procedure and did not attempt to determine the whole-genome sequence of the fetus. We and others recently demonstrated methods for experimentally determining haplotypes for both rare and common variation

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on a genome-wide scale (9–12). Here, we set out to integrate the haplotype-resolved genome sequence of a mother, the shotgun genome sequence of a father, and the deep sequencing of cell-free DNA in maternal plasma to noninvasively predict the whole-genome sequence of a fetus.

## RESULTS

We set out to predict the whole-genome sequence of a fetus in each of two mother–father–child trios (II, a first trio at 18.5 weeks of gestation; G1, a second trio at 8.2 weeks of gestation). We focus here primarily on the trio for which considerably more sequence data were generated (II) (Table 1).

In brief, the haplotype-resolved genome sequence of the mother (II-M) was determined by first performing shotgun sequencing of maternal genomic DNA from blood to 32-fold coverage (coverage = median-fold coverage of mapping reads to the reference genome after discarding duplicates). Next, by sequencing complex haploid subsets of maternal genomic DNA while preserving long-range contiguity (9), we directly phased 91.4% of  $1.9 \times 10^6$  heterozygous SNPs into long haplotype blocks [N50 of 326 kilo-base pairs (kbp)]. The shotgun genome sequence of the father (II-P) was determined by sequencing of paternal genomic DNA to 39-fold coverage, yielding  $1.8 \times 10^6$  heterozygous SNPs. However, paternal haplotypes could not be assessed because only relatively low-molecular weight DNA obtained from saliva was available. Shotgun DNA sequencing libraries were also constructed from 5 ml of maternal plasma (obtained at 18.5 weeks of gestation), and this composite of maternal and fetal genomes was sequenced to 78-fold nonduplicate coverage. The fetus was male, and fetal content in these libraries was estimated at 13% (Fig. 1A). To properly assess the accuracy of our methods for determining the fetal genome solely from samples obtained noninvasively at 18.5 weeks of gestation, we also performed shotgun genome sequencing of the child (II-C) to 40-fold coverage via cord blood DNA obtained after birth.

Our analysis comprised four parts: (i) predicting the subset of “maternal-only” heterozygous variants (homozygous in the father) transmitted to the fetus; (ii) predicting the subset of “paternal-only” heterozygous variants (homozygous in the mother) transmitted to the fetus; (iii) predicting transmission at sites heterozygous in both parents; (iv) predicting sites of de novo mutation—that is, variants occurring only in the genome of the fetus. Allelic imbalance in maternal plasma, manifesting across experimentally determined maternal haplotype blocks, was used to predict their maternal transmission (Fig. 1B). The observation (or lack thereof) of paternal alleles in shotgun libraries derived from maternal plasma was used to predict paternal transmission

(Fig. 1C). Finally, a strict analysis of alleles rarely observed in maternal plasma, but never in maternal or paternal genomic DNA, enabled the genome-wide identification of candidate de novo mutations (Fig. 1D). Fetal genotypes are trivially predicted at sites where the parents are both homozygous (for the same or different allele).

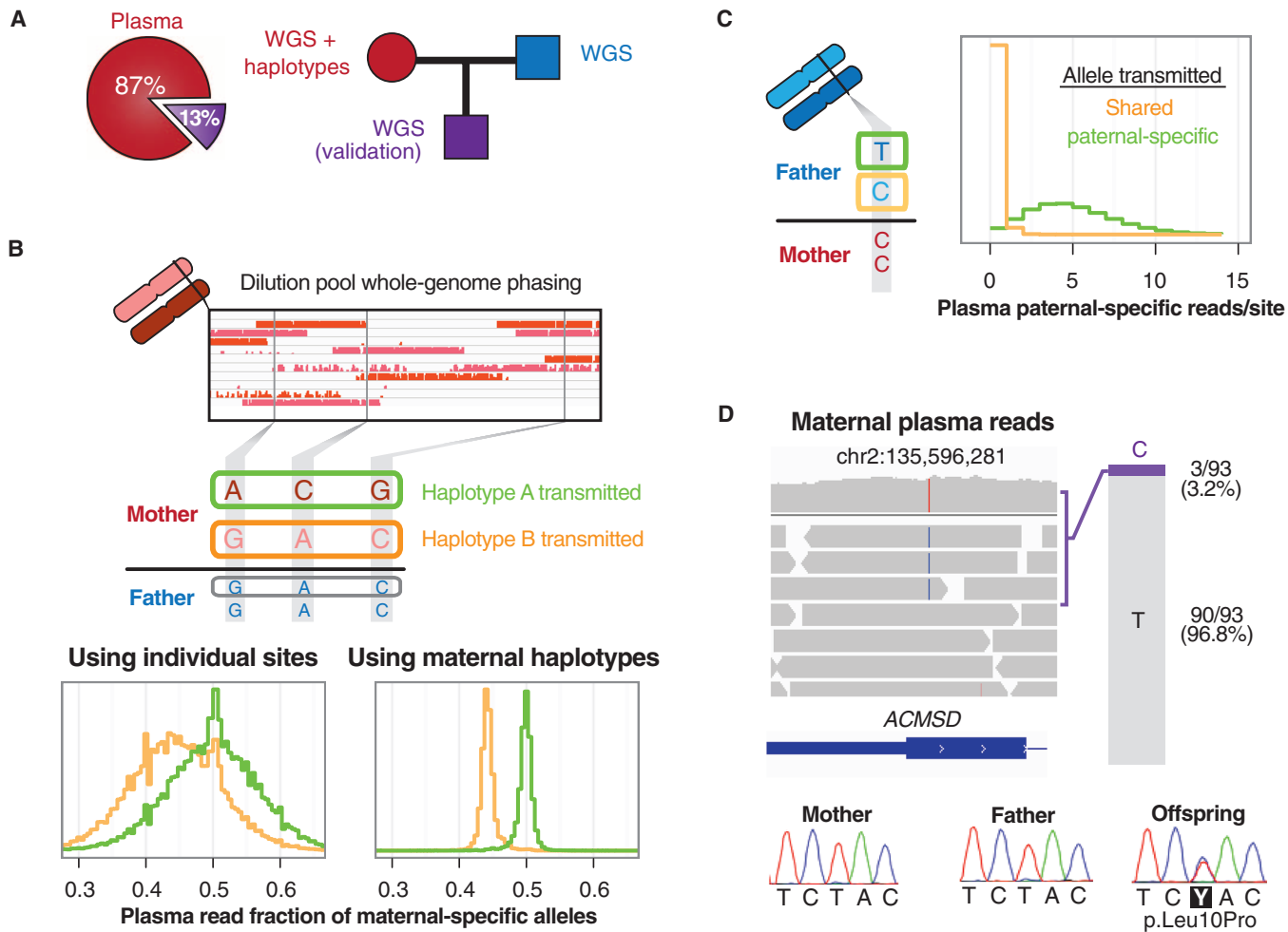
We first sought to predict transmission at maternal-only heterozygous sites. Given the fetal-derived proportion of ~13% in cell-free DNA, the maternal-specific allele is expected in 50% of reads aligned to such a site if it is transmitted versus 43.5% if the allele shared with the father is transmitted. However, even with 78-fold coverage of the maternal plasma “genome,” the variability of sampling is such that site-by-site prediction results in only 64.4% accuracy (Fig. 2). We therefore examined allelic imbalance across blocks of maternally heterozygous sites defined by haplotype-resolved genome sequencing of the mother (Fig. 1B). As anticipated given the haplotype assembly N50 of 326 kbp, the overwhelming majority of experimentally defined maternal haplotype blocks were wholly transmitted, with partial inheritance in a small minority of blocks (0.6%,  $n = 72$ ) corresponding to switch errors from haplotype assembly and to sites of recombination. We developed a hidden Markov model (HMM) to identify likely switch sites and thus more accurately infer the inherited alleles at maternally heterozygous sites (Figs. 3 and 4 and Supplementary Materials). With the use of this model, accuracy of the inferred inherited alleles at  $1.1 \times 10^6$  phased, maternal-only heterozygous sites increased from 98.6 to 99.3% (Table 2). Remaining errors were concentrated among the shortest maternal haplotype blocks (fig. S1), which provide less power to detect allelic imbalance in plasma DNA data compared with long blocks. Among the top 95% of sites ranked by haplotype block length, prediction accuracy rose to 99.7%, suggesting that remaining inaccuracies can be mitigated by improvements in haplotyping.

We performed simulations to characterize how the accuracy of haplotype-based fetal genotype inference depended on haplotype block length, maternal plasma sequencing depth, and the fraction of fetal-derived DNA. To mimic the effect of less successful phasing, we split the maternal haplotype blocks into smaller fragments to create a series of assemblies with decreasing contiguity. We then subsampled a range of sequencing depths from the pool of observed alleles in maternal plasma and predicted the maternally contributed allele at each site as above (Fig. 5A). The results suggest that inference of the inherited allele is robust either to decreasing sequencing depth of maternal plasma or to shorter haplotype blocks, but not both. For example, using only 10% of the plasma sequence data (median depth =  $8x$ ) in conjunction with full-length haplotype blocks, we successfully predicted inheritance at 94.9% of maternal-only heterozygous sites. We achieved nearly identical accuracy (94.8%) at these sites when highly fragmented haplotype blocks (N50 = 50 kbp) were used with the full set of plasma sequences. We next simulated decreased proportions of fetal DNA in the maternal plasma by spiking in additional depth of both maternal alleles at each site and subsampling from these pools, effectively diluting away the signal of allelic imbalance used as a signature of inheritance (Fig. 5B). Again, we found the accuracy of the model to be robust to either lower fetal DNA concentrations or shorter haplotype blocks, but not both.

We next sought to predict transmission at paternal-only heterozygous sites. At these sites, when the father transmits the shared allele, the paternal-specific allele should be entirely absent among the fetal-derived sequences. If instead the paternal-specific allele is transmitted, it will on average constitute half the fetal-derived reads within the maternal plasma genome (about five reads given 78-fold coverage

**Table 1.** Summary of sequencing. Individuals sequenced, type of starting material, and final fold coverage of the reference genome after discarding PCR or optical duplicate reads.

Individual	Sample	Depth of coverage
Mother (II-M)	Plasma (5 ml, gestational age 18.5 weeks)	78
	Whole blood (<1 ml)	32
Father (II-P)	Saliva	39
Offspring (II-C)	Cord blood at delivery	40

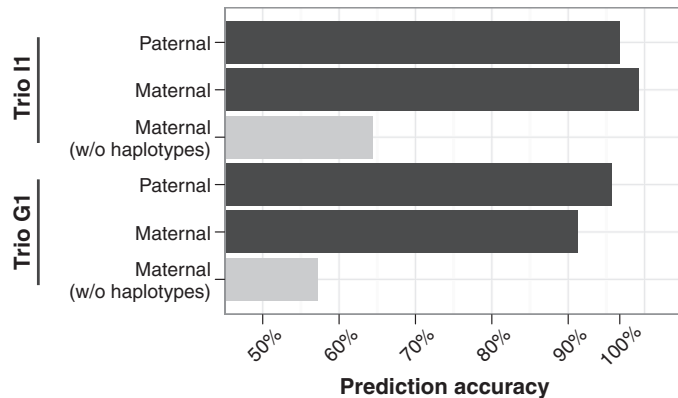


**Fig. 1.** Experimental approach. **(A)** Sequenced individuals in a family trio. Maternal plasma DNA sequences were ~13% fetal-derived on the basis of read depth at chromosome Y and alleles specific to each parent. WGS, whole-genome shotgun. **(B)** Inheritance of maternally heterozygous alleles inferred using long haplotype blocks. Among plasma DNA sequences, maternal-specific alleles are more abundant when transmitted (expected, 50% versus 43.5%), but there is substantial overlap between the distributions of allele frequencies when considering sites in isolation (left histogram: yellow, shared allele transmitted; green, maternal-specific allele transmitted). Taking average allele balances across haplotype blocks (right histogram) provides much greater separation, permitting more accurate inference of maternally transmitted alleles. **(C)** Histogram of fractional read depth

among plasma data at paternal-specific heterozygous sites. In the overwhelming majority of cases when the allele specific to the father was not detected, the opposite allele had been transmitted (96.8%,  $n = 561,552$ ). **(D)** De novo missense mutation in the gene *ACMSD* detected in 3 of 93 maternal plasma reads and later validated by PCR and resequencing. The mutation, which is not observed in dbSNP nor among coding exons sequenced from >4000 individuals as part of the National Heart, Lung, and Blood Institute Exome Sequencing Project (<http://evs.gs.washington.edu>), creates a leucine-to-proline substitution at a site conserved across all aligned mammalian genomes (University of California, Santa Cruz, Genome Browser) in a gene implicated in Parkinson’s disease by genome-wide association studies (25).

assuming 13% fetal content). To assess these, we performed a site-by-site log-odds test; this amounted to taking the observation of one or more reads matching the paternal-specific allele at a given site as evidence of its transmission and, conversely, the lack of such observations as evidence of nontransmission (Fig. 1C). In contrast to maternal-only heterozygous sites, this simple site-by-site model was sufficient to correctly predict inheritance at  $1.1 \times 10^6$  paternal-only heterozygous sites with 96.8% accuracy (Table 2). We anticipate that accuracy could likely be improved by deeper sequence coverage of the maternal plasma DNA (fig. S2) or, alternatively, by taking a haplotype-based approach if high-molecular weight genomic DNA from the father is available

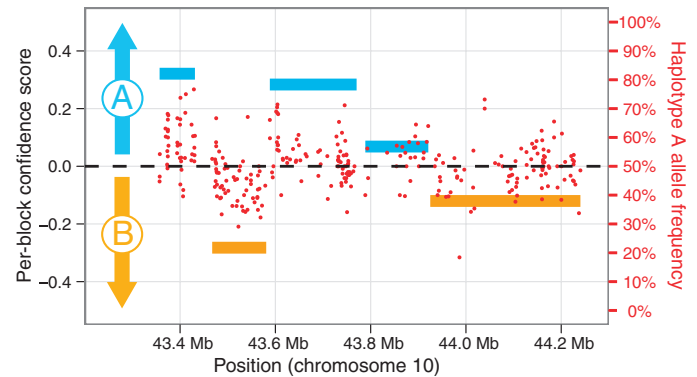
We next considered transmission at sites heterozygous in both parents. We predicted maternal transmission at such shared sites phased using neighboring maternal-only heterozygous sites in the same haplotype block. This yielded predictions at 576,242 of 631,721 (91.2%) of shared heterozygous sites with an estimated accuracy of 98.7% (Table 2). Although we did not predict paternal transmission at these sites, we anticipate that analogous to the case of maternal transmission, this could be done with high accuracy given paternal haplotypes. We note that shared heterozygous sites primarily correspond to common alleles (fig. S3), which are less likely to contribute to Mendelian disorders in nonconsanguineous populations



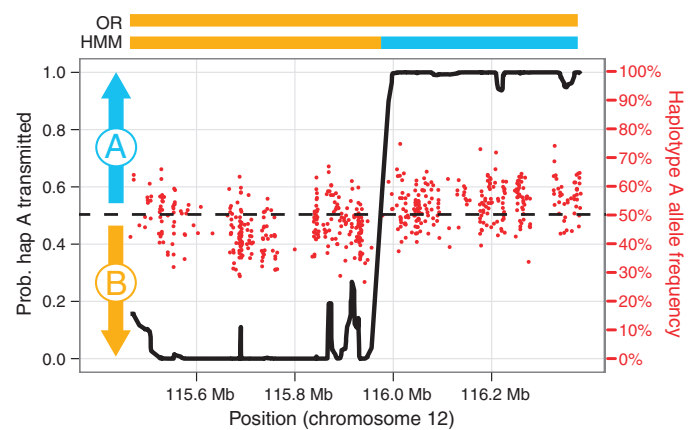
**Fig. 2.** Accuracy of fetal genotype inference from maternal plasma DNA sequencing. Accuracy is shown for paternal-only heterozygous sites and for phased maternal-only heterozygous sites, either using maternal phase information (black) or instead predicting inheritance on a site-by-site basis (gray).

De novo mutations in the fetal genome are expected to appear within the maternal plasma DNA sequences as “rare alleles” (Fig. 1D), similar to transmitted paternal-specific alleles. However, the detection of de novo mutations poses a much greater challenge: Unlike the  $1.8 \times 10^6$  paternally heterozygous sites defined by sequencing the father (of which ~50% are transmitted), the search space for de novo sites is effectively the full genome, throughout which there may be only ~60 sites given a prior mutation rate estimate of  $\sim 1 \times 10^{-8}$  (13). Indeed, whole-genome sequencing of the offspring (I1-C) revealed only 44 high-confidence point mutations (“true de novo sites”; table S1). Taking all positions in the genome at which at least one plasma-derived read had a high-quality mismatch to the reference sequence, and excluding variants present in the parental whole-genome sequencing data, we found  $2.5 \times 10^7$  candidate de novo sites, including 39 of the 44 true de novo sites. At baseline, this corresponds to sensitivity of 88.6% with a signal-to-noise ratio of 1-to- $6.4 \times 10^5$ .

We applied a series of increasingly stringent filters (fig. S4) intended to remove sites prone to sequencing or mapping artifacts. We first removed alleles found in at least one read among any other individual sequenced in this study, known polymorphisms from dbSNP (release 135), and sites adjacent to 1- to 3-mer repeats, reducing the number of candidate de novo sites to  $1.8 \times 10^7$ . We next filtered out sites with insufficient evidence (fewer than two independent reads supporting the variant allele, or variant base qualities summing to less than 105) as well as those with excessive reads supporting the variant allele (uncorrected  $P < 0.05$ , per-site one-sided binomial test using fetal-derived fraction of 13%), cutting the total number of candidate sites to 3884, including 17 true de novo sites. This candidate set is substantially depleted for sites of systematic error and is instead likely dominated by errors originating during polymerase chain reaction (PCR), because even a single round of amplification with a proofreading DNA polymerase with an error rate of  $1 \times 10^{-7}$  would introduce hundreds of false-positive candidate sites. Notably, this ~2800-fold improvement in signal-to-noise ratio reduced the candidate set to a size that is an order of magnitude fewer than the number of candidate de novo sites requiring validation in a previous study involving pure genomic DNA from parent-child trios within a nuclear family (14). In a clinical setting, validation efforts would be targeted to those sites considered most likely to be pathogenic. For example, only 33 of the 3884 candidate sites (0.84%) are predicted to alter



**Fig. 3.** HMM-based predictions correctly predict maternally transmitted alleles across ~1 Mbp on chromosome 10 despite site-to-site variability of allelic representation among maternal plasma DNA sequences (red).



**Fig. 4.** HMM-based detection of recombination events and haplotype assembly switch errors. A maternal haplotype block of 917 kbp on chromosome 12q is shown, with red points representing the frequency of haplotype A alleles among plasma reads and the black line indicating the posterior probability of transmission for haplotype A computed by the HMM at each site. A block-wide odds ratio (OR) test predicts transmission of the entire haplotype B, resulting in incorrect prediction at 272 of 587 sites (46.3%). The HMM predicts a switch between chromosomal coordinates 115,955,900 and 115,978,082, and predicts transmission of haplotype B alleles from the centromeric end of the block to the switch point, and haplotype A alleles thereafter, resulting in correct predictions at all 587 sites. All three overlapping informative clones support the given maternal phasing of the SNPs adjacent to the switch site, suggesting that the switch predicted by the HMM results from a maternal recombination event rather than an error of haplotype assembly.

protein sequence, and only a subset of these are in genes associated with Mendelian disorders.

## DISCUSSION

We have demonstrated noninvasive prediction of the whole-genome sequence of a human fetus through the combination of haplotype-resolved genome sequencing (9) of a mother, shotgun genome sequencing of a father, and deep sequencing of maternal plasma DNA. Notably, the types and quantities of materials used were consistent with those routinely collected in a clinical setting (Table 1). To replicate



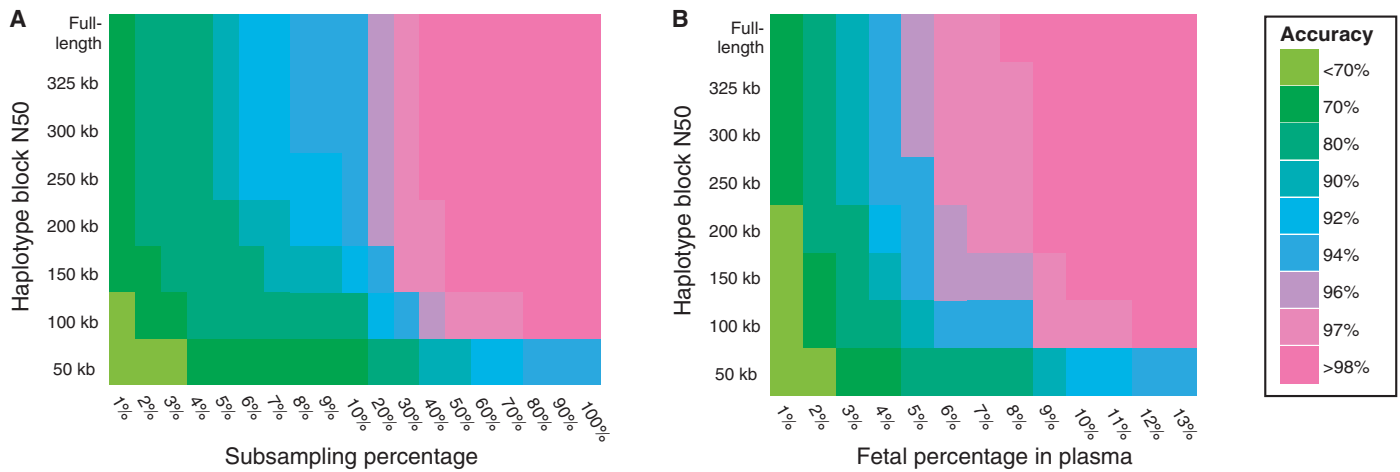
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**Table 2.** Accuracy of fetal genome inference. Number of sites and accuracy of fetal genotype inference from maternal plasma sequencing (percentage of transmitted alleles correct out of all predicted) by parental genotype and phasing status. Sites later determined by trio sequencing (including the off-

spring) to have poor genotype quality scores or genotypes that violated Mendelian inheritance were discarded for the purpose of evaluating accuracy (14,000 maternal-only, 32,233 paternal-only, and 480 shared heterozygous sites, or 1.5% of all sites). ND, not determined.

Individual	Site	Other parental genotype	Number of sites	Accuracy (%)
Mother (I1-M)	Heterozygous, phased	Homozygous	1,064,255	99.3
		Heterozygous	576,242	98.7*
	Heterozygous, not phased	All	121,425	ND
Father (I1-P)	Heterozygous	Homozygous	1,134,192	96.8
		Heterozygous	631,721	ND

\*Among biparentally heterozygous sites, accuracy was assessed only where the offspring was homozygous (48.8%,  $n = 631,721$ ), allowing the “true” transmitted alleles to be unambiguously inferred from trio genotypes.



**Fig. 5.** Simulation of effects of reduced coverage, haplotype length, and fetal DNA concentration on fetal genotype inference accuracy, defined as the percentage of sites at which the inherited allele was correctly identified out of all

sites where prediction was attempted. (A and B) Heat maps of accuracy after in silico fragmentation of haplotype blocks and (A) shallower sequencing of maternal plasma or (B) reduced fetal concentration among plasma sequences.

these results, we repeated the full experiment for a second trio (G1) from which maternal plasma was collected earlier in the pregnancy, at 8.2 weeks after conception (tables S2 and S3). Both the overall sequencing depth and the fetal-derived proportion were each lower relative to the first trio (by 28 and 51%, respectively), resulting in an average of fewer than four fetal-derived reads per site. Nevertheless, we achieved 95.7% accuracy for prediction of inheritance at maternal-only sites, consistent with accuracy obtained under simulation with data from the first trio (Fig. 5). These results underscore the importance of specific technical parameters in determining performance, namely, the length and completeness of haplotype-resolved sequencing of parental DNA, and the depth and complexity of sequencing libraries derived from low starting masses of plasma-derived DNA (less than 5 ng for both I1 and G1 in our study).

There remain several key avenues for improvement. First, although we predicted inheritance at  $2.8 \times 10^6$  heterozygous sites with high accuracy (98.2% overall), there were  $7.5 \times 10^5$  sites for which we did not attempt prediction (Table 2). These include  $6.3 \times 10^5$  shared sites heterozygous in both parents for which we could not assess paternal transmission and  $1.2 \times 10^5$  maternal-only heterozygous sites that were not included in our haplotype assembly. The shared sites are in principle accessible but require haplotype-resolved (rather than solely shotgun)

sequencing of paternal DNA, which was not possible here with either the I1 or the G1 trio owing to unavailability of high-molecular weight DNA from each father. The unphased maternal sites are also in principle accessible but require improvements to haplotyping technology to enable phasing of SNPs residing within blocks of relatively low heterozygosity as well as within segmental duplications. More generally, despite recent innovations from our group and others (9–12), there remains a critical need for genome-wide haplotyping protocols that are at once robust, scalable, and comprehensive. Significant reductions in cost, along with standardization and automation, will be necessary for compatibility with large-scale clinical application.

Second, although we were successful in detecting nearly 90% of de novo single-nucleotide mutations by deep sequencing of maternal plasma DNA, this was with very low specificity. The application of a series of filters resulted in a ~2800-fold gain in specificity at a ~2-fold cost in terms of sensitivity. However, there is clearly room for improvement if we are to enable the sensitive and specific prenatal detection of potentially pathogenic de novo point mutations at a genome-wide scale, a goal that will likely require deeper than 78-fold coverage of the maternal plasma genome (15) in combination with targeted validation of potentially pathogenic candidate de novo mutations

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