Quantification of Fetal DNA by Use of Methylation-Based DNA Discrimination

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BACKGROUND: Detection of circulating cell-free fetal nucleic acids in maternal plasma has been used in non-invasive prenatal diagnostics. Most applications rely on the qualitative detection of fetal nucleic acids to determine the genetic makeup of the fetus. This method leads to an analytic dilemma, because test results from samples that do not contain fetal DNA or are contaminated with maternal cellular DNA can be misleading. We developed a multiplex approach to analyze regions that are hypermethylated in placenta relative to maternal blood to evaluate the fetal portion of circulating cell-free DNA isolated from maternal plasma.

METHODS: The assay used methylation-sensitive restriction enzymes to eliminate the maternal (unmethylated) fraction of the DNA sample. The undigested fetal DNA fraction was then coamplified in the presence of a synthetic oligonucleotide to permit competitive PCR. The amplification products were quantified by single-base extension and MALDI-TOF MS analysis.

RESULTS: Using 2 independent markers, (sex determining region Y)-box 14 (SOX14) and T-box 3 (TBX3), we measured a mean of 151 copies of fetal DNA/mL plasma and a mean fetal fraction of 0.13 in samples obtained from pregnant women. We investigated 242 DNA samples isolated from plasma from pregnant and nonpregnant women and observed an analytical sensitivity and specificity for the assay of 99% and 100%, respectively.

CONCLUSIONS: By investigating several regions in parallel, we reduced the measurement variance and enabled quantification of circulating cell-free DNA. Our results indicate that this multiplex methylation-based reaction detects and quantifies the amount of fetal DNA in a sample isolated from maternal plasma.

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Since its discovery, circulating cell-free fetal DNA (ccff DNA)3 isolated from maternal plasma (1) has drawn much attention because it provides genetic information about the fetus with a reduced risk of complications compared with invasive procedures (2). Several applications for noninvasive prenatal diagnosis (NIPD) are available for the detection of fetal sex (3, 4), rhesus D blood type (5, 6), and paternal-derived mutations (7, 8). Despite the achievements made in both qualitative and quantitative analysis of fetal DNA in maternal plasma (9), there are still limitations to this technology. Most applications rely on differences in DNA sequence between the fetal and maternal genomes. In these applications, absence of the investigated fetal sequence would correspond to noninheritance of the paternal allele. A lack of detectable signal, however, could also be due to a low fetal DNA concentration in the sample (10). Many factors can contribute to these false-negative results, but all can lead to false interpretation of the fetal DNA component. Therefore, a method that can be used to detect and quantify a universal fetal DNA marker is needed (11, 12).

Until recently the search for a universal fetal marker was largely restricted to genetic polymorphisms located on the autosomal chromosomes (13), but to ensure at least 1 informative marker a more complex test is necessary that consumes a large portion of the available DNA sample. A potential alternative to sequence-based detection of fetal DNA is to use other factors to discriminate between maternal and fetal DNA (14–16). Recently several independent studies have presented a comprehensive set of genomic regions in which fetal-specific tissue is differentially methylated compared with the corresponding maternal peripheral blood mononuclear cells (PBMCs) (17–21). This epigenetic difference can be exploited to establish an assay that targets the fetal portion of the cell-free DNA in plasma.
We developed a fetal quantitative assay (FQA) for the simultaneous quantification of both differentially methylated regions and chromosome-Y–specific sequences. The FQA also includes assays to quantify the total amount of circulating cell-free DNA and controls to measure the fraction of nondigested material. This format allows direct determination of total DNA copy numbers (maternal + fetal), fetal DNA copy numbers, and male-specific copy numbers, and consequently the ability to calculate the fraction of fetal DNA present in the plasma sample. We successfully evaluated this assay in DNA model systems, verified its specificity in plasma samples from nonpregnant women, and in the final step tested its feasibility in a large set of plasma samples from pregnant women.

Materials and Methods

Marker Discovery
We performed marker discovery by coupling methylcytosine immunoprecipitation to CpG island microarrays (Agilent Technologies) as previously described (22). Regions were selected as differentially methylated if the adjusted P value after microarray analysis was lower than 0.001. Five differentially methylated regions [T-box 3 (TBX3)4; solute carrier family 38, member 10 (MGC15523); (sex determining region Y)-box 14 (SOX14); CDC42 effector protein (Rho GTPase binding) 1 (CDC42EP1); and sialophorin (SPN)] were selected, and differential methylation was confirmed by using Epityper analysis as previously described (23). Primer sequences are listed in Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol56/issue10. A detailed description of the marker selection and Epityper methods is described in online Supplemental Data Information 1.

Assay Design
We designed PCR primers and MALDI-TOF MS TypePLEX™ extension primers by using Sequence® AssayDesigner 4.2 software. All oligonucleotides were obtained from IDT. The FQA contained 4 types of assays for the detection of total copy numbers, fetal methylated copy numbers, chromosome-Y sequences, and controls for digestion efficiency. The oligonucleotide sequences can be found in online Supplemental Table 2.

Model System
To determine the limits of quantification of the method, we developed a model system to simulate circulating cell-free DNA samples isolated from plasma. These samples contained maternal nonmethylated DNA copies isolated from PBMCs, into which we spiked different amounts of either male or female placental DNA. We spiked the samples with amounts ranging from 0% to 40% relative to the maternal nonmethylated DNA copy numbers while we kept the total number of DNA molecules constant. An additional model was developed to be used as a series of standards when we determined the total number of amplifiable genomic copies in a sample. A subset of different DNA samples isolated from the blood of nonpregnant women was tested. Each sample was diluted to contain approximately 1000, 500, 250, 125, 63, 32, 16, 8, 4, 2, or 1 methylated copies per reaction.

Plasma Samples
For this study, whole blood samples of 10 mL were collected in EDTA tubes and shipped on wet ice. The plasma was isolated by centrifugation of the whole blood at 2500g for 10 min. To eliminate potential cellular debris, an additional centrifugation step was performed at 15 000g for 10 min. All plasma samples were processed within 6 h of the blood draw. Two sets of DNA were isolated from a total of 248 unique plasma samples. Set 1 consisted of 48 plasma samples from nonpregnant women and set 2 consisted of 200 plasma samples from pregnant women. This study was conducted according to an institutional review board–approved protocol. All study participants gave informed consent.

DNA Isolation
DNA was extracted from 200 μL of buffy coat or 0.2 g of placental tissue by using the QiaAMP Blood Minikit (Qiagen). Circulating cell-free DNA was extracted from 4 mL of plasma with the QIAamp Circulating Nucleic Acid Kit (Qiagen) and eluted in a 100-μL volume.

FQA Assay
Methylation-sensitive restriction enzymes (24) were used before PCR to digest the nonmethylated maternal fraction of circulating cell-free DNA. The remaining methylated fetal fraction could then be quantified by using an established mass spectrometry–based method. Quantification was achieved by parallel amplification of a synthetic oligonucleotide of known concentration followed by MALDI-TOF MS analysis to

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4 Human genes: TBX3, T-box 3; MGC15523, solute carrier family 38, member 10; SOX14, (sex determining region Y)-box 14; CDC42EP1, CDC42 effector protein (Rho GTPase binding) 1; SPN, sialophorin; ALB, albumin; APOE, apolipoprotein E; RNASEP, ribonuclease P; SRY, sex determining region Y; UTY, ubiquitously transcribed tetratricopeptide repeat gene, Y-linked; MGC15523, solute carrier family 38, member 10; LDHA, lactate dehydrogenase A; POP5, processing of precursor 5, ribonuclease P/MRP subunit; RASSF1A, Ras association (RhoGDS/AF-6) domain family member 1.
separate and quantify the amplification products (25). The limited number of fetal DNA copies introduced a variance in the quantitative measurement; however, the ability of our assay to multiplex and measure several regions simultaneously helped to reduce this variance (Fig. 1).

**FQA REACTION**

Methylation-based DNA discrimination was performed by using 25 μL of eluted DNA per reaction. For this study all samples were run in triplicate. All reagents and apparatus were obtained from Sequenom, unless stated otherwise. Digestion of plasma DNA was performed for 60 min at 41 °C by the addition of 10 μL of a mixture containing 3.5× PCR Buffer (Sequenom no. 1738), 2.22 mmol/L MgCl₂, 10 U HhaI (New England Biolabs), 10 U HpaII (New England Biolabs), and 10 U ExoI (New England Biolabs). The exonuclease was added to eliminate single-stranded DNA that would escape digestion and lead to overestimation of the fetal fraction. After the restriction was complete the enzymes were inactivated and the DNA denatured by heating the mixture for 10 min at 98 °C. The nondigested DNA was PCR amplified by the addition of 15 μL of a PCR mixture containing 1× PCR Buffer (Sequenom no. 1738), 0.125 mmol dNTPs, 5 U Fast Start Polymerase (Roche), 0.1 μmol/L PCR primers, and competitors. A list of all PCR primers, competitor oligonucleotides, and amounts used can be found in online Supplemental Table 2. PCR was initiated by a 5-min incubation at 98 °C, followed by 45 cycles (30 s at 95 °C, 30 s at 64 °C, and 30 s at 72 °C), and the PCR was ended with a 3-min incubation at 72 °C. After the PCR, 5 μL of a mix containing 1 mAnson unit of protease (Qiagen) was added to degrade peptides present in DNA extracted from plasma samples. Protease treatment was performed for 30 min at 55 °C and ended with a inactivation step for 5 min at 95 °C. To minimize post-PCR–induced variance, each PCR was analyzed in quadruplicate; 20 μL of the PCR reaction was split into 4 parallel reactions of 5 μL and dephosphorylated for 40 min at 37 °C by the addition of 2 μL of a mixture containing 0.5 U shrimp alkaline phosphatase and 5 U RNase I (New England Biolabs) in 0.85× shrimp alkaline phosphatase buffer (Sequenom no. 10055). The RNase was added to degrade the large amounts of carrier RNA that were added in the DNA isolation and would interfere with the MALDI-TOF analysis. The reaction was ended with a 5-min incubation at 85 °C to inactivate the enzymes. Single-base extension was performed by addition of 2 μL single-base extension reaction mix to the dephosphorylated primary PCR reaction mix, and included extend primer (see on-

![Fig. 1. Outline of the FQA procedure.](image-url)
line Supplemental Table 2), TypePLEX Extend mix, 0.222 ¥ TypePLEX buffer, and 0.04 U TypePLEX enzyme. We performed 200 cycles of single-base extension using the following program: 94 °C for 30 s, followed by 20 cycles of 94 °C for 5 s followed by 5 repetitions of 52 °C for 5 s and 72 °C for 5 s. After desalting the mixtures by the addition of 6 mg Clean Resin, we transferred 15 nL of each TypePLEX extend mixture to a SpectroCHIP® II-G384 and recorded mass spectra using a MassARRAY system. Spectra were acquired by using Sequenom SpectroAcquire software. The software parameters were set to acquire 20 shots from each of 9 raster positions. The resulting mass spectra were summed, and peak detection and intensity analysis were performed by using Sequenom Typer 4 software.

**DATA ANALYSIS**

For each assay, we calculated the fraction of each individual DNA analyte and synthetic competitor by dividing the intensity of the representative allele by the sum of both allele intensities: fraction (DNA analyte) = Intensity(DNA signal)/[Intensity(DNA signal) + Intensity(Competitor signal)]. For quantification of the total number of molecules present in the reaction, the DNA-specific analyte for each amplicon was divided by the competitor-specific analyte to give a ratio. We could then determine the total number of DNA molecules by multiplying the ratio by the number of added competitor molecules. The number of fetal DNA copies in each sample was calculated by using the mean of the Y-chromosome–specific markers for male fetuses and the mean of the methylated fraction for all samples. Because the total amount of nucleic acid present in a sample is a sum of maternal and fetal nucleic acids, the fetal contribution can be considered to be a fraction of the larger, background maternal contribution. Therefore, the chromosome-Y–derived fetal fraction \[k_{(\text{chr} \ Y)}\] of the total nucleic acid present in the sample is equal to the equation: \[k_{(\text{chr} \ Y)} = 2 \times R_{(\text{chr} \ Y)}/R_{(\text{Total})}\]; where \(R\) is the ratio between the DNA analyte and the competitor analyte multiplied by the number of competitor oligonucleotides added. All copy numbers below 20, i.e., those for which the fraction of DNA allele was below 0.1, were considered as background. A similar calculation for the fetal fraction was performed by using the methylation-specific markers. In contrast to Y-chromosome–specific markers, these markers were from diploid targets. Thus, the fetal fraction \[k_{(\text{Meth})}\] can be determined by using the equation: \[k_{(\text{Meth})} = R_{(\text{Meth})}/R_{(\text{Total})}\], with the assumption that the marker regions are fully methylated.

**RESULTS**

**MARKER DISCOVERY**

Using methyl-CpG immunoprecipitation coupled with high-resolution oligonucleotide microarray analysis, we analyzed 8 sample pairs derived from placental tissue and maternal buffy coat. A subset of these regions was confirmed by using Sequenom® EpiTYPER® technology. For the FQA we selected 5 candidate regions, CDC42EP1, MGC15523, SOX14, SPN, and TBX3, which showed increased placental methylation and no methylation in maternal PBMCs (see online Supplemental Fig. 1).

**ASSAY SETUP**

To enable quantification we used a competitive PCR approach. This method is well established and was recently used for validation of DNA copy number variants (26). This method allows for simultaneous measurement of both high total copy numbers and low fetal copy numbers, because each assay is independently amplified. In this approach we used placental methylation as the discriminating factor to identify fetal-derived DNA, and relied on methylation-sensitive restriction enzymes to digest the maternal portion of the DNA sample. In our assay format, incomplete digestion of maternal DNA would lead to an overestimation of the fetal fraction. Hence, it was important to design an amplicon that minimized incomplete digestion of target DNA. For optimal digestion efficiency we used 2 methylation-sensitive restriction enzymes and selected only target regions that contained at least 2 restriction sites.

Because this approach required a double-stranded DNA template, if a portion of the maternal-derived DNA was single stranded, these sequences would escape digestion and lead to overestimation of the fetal fraction. Therefore, to protect against overestimating the fetal DNA component, we performed a simultaneous exonuclease/digestion reaction to remove any single-stranded DNA present in the sample. The efficiency of the restriction reaction was monitored by the introduction of 2 restriction controls into the multiplex. These assays targeted 2 regions known to be unmethylated in both maternal blood and placental tissue. The FQA was designed to contain 4 different assay types including: (a) assays for quantification of the total DNA (maternal and fetal), (b) assays for quantification of chromosome-Y copy numbers, (c) assays for quantification of fetal methylated DNA, and (d) control assays to estimate the digestion efficiency. The resulting multiplex contained 13 assays, 3 for total DNA quantification [albumin (ALB), apolipoprotein E (APOE), and ribonuclease P (RNASEP)], 3 for quantification of chromosome Y [2 sex determining region Y
and ubiquitously transcribed tetratricopeptide repeat gene, Y-linked (UTY)], 5 for quantification of differentially methylated fetal DNA [CDC42EP1; solute carrier family 38, member 10 (MGC15523); SOX14; SPN; and TBX3], and 2 assays as restriction controls [lactate dehydrogenase A (LDHA) and processing of precursor 5, ribonuclease P/MRP subunit (POP5)].

MODEL SYSTEM AND VERIFICATION OF DYNAMIC RANGE
To evaluate the performance and dynamic range of our multiplexed assay we designed a model system. Here we used DNA derived from maternal PBMCs to represent the maternal fraction and DNA isolated from placenta tissue to represent the fetal component. We obtained sample pairs with matching material from 8 women carrying a male fetus. First we evaluated the ability to quantify varying amounts of total DNA. Quantification that uses competitive PCR and MALDI-TOF MS relies on the relative evaluation of a target signal against a reference signal. Various published reports have indicated that the total amount of circulating cell-free DNA is around 2000 copies/mL of plasma and the fraction of fetal DNA is between 3% and 20% (27–29). Consequently we chose 3000 copies of single-stranded competitor DNA for the estimation of total DNA and 300 copies of competitor DNA for the estimation of fetal DNA. This system could quantify copy numbers between 450 and 7500 copies of total DNA per reaction and 45–750 copies of fetal copies (see online Supplemental Fig. 2). On the basis of these results we introduced additional quality criteria: Each sample must contain a minimum of at least 450 but no more than 7500 amplifiable copies per reaction and have digestion efficiency above 99% to be analyzed. Samples containing copy numbers outside of these ranges can be reanalyzed by adjusting the amount of input DNA. We tested the ability to quantify a fraction of methylated DNA in the presence of unmethylated DNA. We used 2000 copies of the DNA that represented maternal DNA and added 0%, 2.5%, 5%, 10%, 20%, and 40% of the DNA that represented fetal DNA. Each individual dilution series was measured with a mean $r^2$ of 0.99 (least squares method) (Fig. 2A). We observed a high correlation between each of the 5 methylation markers (mean $r > 0.98$, Pearson correlation) (see online Supplemental Fig. 3). The quantification of Y chromosomal markers is a well-accepted standard for measuring fetal DNA (30). Therefore we used 8 sample pairs that had male placental DNA to compare the copy numbers obtained by using either the Y chromosomal markers or the methylation-based markers. In Fig. 2B, the model system showed correlation between the methylation-based quantification and the established method for quantification by using chromosome-Y–specific sequences ($r = 0.98, P < 0.001$, Pearson correlation).

Fig. 2. (A), For copy number quantification, a model system was created that contained a constant number of maternal nonmethylated DNA with varying amounts of spiked-in male placental methylated DNA. We used approximately 400, 200, 100, 50, 25, or 0 methylated placental copies per reaction. Each measurement was obtained by taking the mean DNA/competitor ratio obtained from the methylation sensitive assays. Each symbol represents 1 sample pair. Obs., observed; Exp., experimental. (B), Correlation between methylation markers and chromosome Y. The copy numbers of placental DNA spiked into maternal nonmethylated DNA in varying amounts was calculated by using the ratios obtained from the methylation assays and the Y-chromosome markers compared to the total copy-number assays.
To investigate the analytical sensitivity and specificity of this method as well as the identification of sporadic methylation in clinical samples, we analyzed 48 plasma samples obtained from nonpregnant women. Three samples were excluded because they did not contain any DNA, and each of the remaining 45 samples passed the previously defined criteria for analysis. We measured a mean of 1134 (range 453–3526) genomic copies/mL plasma. Compared with genomic DNA obtained from maternal PBMCs, 3 assays, CDC42EP1, MGC15523, and SPN, had to be excluded from additional analysis because they showed aberrant methylation in the majority of the samples. These results indicate that paired maternal PBMCs and placental tissue is not an optimal system of identification of fetal-specific methylation markers. For the remaining 2 markers, SOX14 and TBX3, we measured copy numbers per milliliter of plasma that were below the quantitative range [mean (SD) 7 (5.5) copy numbers/mL; range 0–26 copy numbers/mL]. Although quantification within this range is associated with a larger measurement variation, the data showed that SOX14 and TBX3 are not methylated in maternal plasma.

**DNA SAMPLES FROM 200 PREGNANT WOMEN**

To estimate the biological variance of differentially methylated regions, we used a set of plasma samples isolated from 200 pregnant women. Three samples were omitted from analysis because they did not contain any DNA; most likely the DNA was lost during the extraction procedure. An advantage with the FQA is that samples containing no DNA can easily be identified owing to the sole amplification of the competitor oligonucleotides. All of the remaining 197 samples passed QC with regard to total copy numbers within the quantitative range and a digestion efficiency of at least 99%. We observed a mean of 1245 amplifiable copies/mL plasma (range 487 to 4926) and measured a mean of 151 copies of fetal-derived DNA per mL plasma and a mean fetal fraction of 0.132 for all samples (Fig. 3A). We observed an increasing amount of fetal copy numbers with longer gestation, with means of 102, 114, and 163 copies/mL for samples obtained from 8-, 9-, and 10-week pregnancies, respectively. No significant difference was observed between male and female pregnancies (nonpaired t-test: P value >0.05) (Fig. 3B). We investigated the correlation between the fetal DNA fraction obtained from either methylation or chromosome Y markers in the samples obtained from male pregnancies (Fig. 3C). In this large sample cohort we confirmed the correlation between the methylation-based quantification and the established method for quantification by using chromosome-Y–specific sequences (ρ = 0.85, P < 0.001, Pearson correlation). Using the sample set consisting of 197 samples from pregnant women and 45 samples from nonpregnant women, we achieved analytical sensitivity and specificity of 99% and 100% (Fig. 3D). Together these data strongly demonstrate that the presented FQA assay can serve as a sex- and polymorphism-independent method for the quantification of ccff DNA in a plasma samples from pregnant women.

**Discussion**

In this study we present the first multiplex methylation-based approach for sex- and polymorphism-independent quantification of ccff DNA isolated from maternal plasma. For a similar method that uses differential methylation of Ras association (RalGDS/AF-6) domain family member 1 (RASSF1A), as well as markers for SRY and ALB, 3 real-time PCR reactions are necessary. Compared to DNA derived from the cellular compartment of a blood draw, cell-free DNA from plasma is several orders of magnitude less abundant. In particular, the fetal fraction, which constitutes only around 15% of all circulating cell-free DNA, is limited. This situation poses 2 main challenges for a fetal DNA quantification assay. Ideally, the majority of fetal DNA is maintained for the downstream analytical assay and not used for the quantification process itself. Also, because of the low copy numbers that have to be detected, it is desirable to have redundant measurements, which will increase the confidence in the results. Our multiplexed assay format addresses both of these challenges. All necessary measurements and the digestion controls are run in a single reaction, and because mass spectrometry allows for high multiplexing, each of these measurements can be performed in duplicate. Our results show that methylation-based quantification of ccff DNA is a feasible method that can overcome the sex-based limitations of the Y chromosomal markers. However, in this study we present only an analysis of the technical feasibility of a multiplex assay for fetal DNA quantification. Therefore the results should be interpreted with caution because a number of questions must be addressed before this assay concept can be implemented for routine clinical use. In particular, the biological stability of the methylation markers has to be further investigated. A perfect methylation marker has to fulfill specific requirements. First, to minimize maternal influence it should exhibit a large difference in methylation between maternal and fetal DNA, in which the maternal-derived DNA is not methylated and the fetal DNA is fully methylated. Second, the methylation difference must be stable, and therefore not change between individuals across populations or during gestation. Although a vast amount of
knowledge is available for genetic markers, for epigenetic markers such as DNA methylation, it is still unclear if these assays can provide the necessary stability. In this study we found 2 markers that allowed quantification of fetal DNA in 197 DNA samples isolated from plasma. ccff DNA isolated from plasma samples from pregnant women is still a scarce resource. Results of published studies indicate that the mean number of DNA molecules found in 1 mL of plasma is around 2000, with a fetal component of around 15%...
(27, 29, 31). Both of these values are subject to a large spread across individuals, and therefore it is desirable to minimize the DNA consumption of an assay that controls for the presence of fetal DNA. New methods for DNA extraction from plasma allow for higher input volumes of up to 4 mL, and consequently maximize the amount of DNA available for the analytical reaction.

In conclusion, the field of noninvasive prenatal diagnostic using cfDNA is still in its infancy. Some assays, like those for the detection of the RhD gene, are currently finding their way into clinical practice. In the future we will also see more quantitative applications such as aneuploidy detection through other assays or instrumentation. All of these new tests rely on a control reaction able to quantify the presence of cfDNA in the plasma sample (32).

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References

Fetal Quantifier Assay


