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Interlaboratory Comparison of Fetal Male DNA Detection from Common Maternal Plasma Samples by Real-Time PCR

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Background: Analysis of fetal DNA from maternal plasma by PCR offers great potential for noninvasive prenatal genetic diagnosis. To further evaluate this potential, we developed and validated a standard protocol to determine whether fetal DNA sequences could be reproducibly amplified and measured across multiple laboratories in a common set of specimens.
Methods: Each of five participating centers in a National Institute of Child Health and Human Development consortium collected 20 mL of peripheral blood from 20 pregnant women between 10 and 20 weeks of gestation. The plasma fraction was separated according to a common protocol, divided, and frozen in five aliquots. One aliquot was shipped to each participating laboratory, where DNA was extracted according to a standard protocol. All plasma samples (n = 100) were then analyzed blindly for the presence and quantity of total DNA (*GAPDH*) and male fetal DNA (*SRY*) by real-time PCR. Genomic DNA was isolated from female and male cells at one center, quantified, and shipped to the others to serve as calibrators for *GAPDH* and *SRY*, respectively.
Results: The amplification of known quantities of DNA was consistent among all centers. The mean quantity of male DNA amplified from maternal plasma when the fetus was male ranged from 51 to 228 genome equivalents (GE)/mL. Qualitative concordance was found overall among centers. The sensitivity of the assay for detection of male DNA when the fetus was male varied from 31% to 97% among centers. Specificity was more consistent (93–100%) with only four false-positive results obtained across the entire study.
Conclusions: All centers were able to consistently amplify frozen and shipped DNA. The PCR procedure used here is reliable and reproducible. Centers that extracted and amplified more DNA per milliliter of maternal plasma had superior sensitivities of Y chromosome sequence detection. The specificity of the assay was more consistent among centers. A robust and thoroughly optimized protocol for the extraction of DNA from maternal plasma is needed to make testing of fetal DNA in maternal plasma a clinically relevant analytical tool.
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Since the first report in 1997, cell-free fetal DNA in the maternal circulation has become a primary target for noninvasive prenatal diagnosis *(1)*. The detection of male-specific (Y chromosome) DNA sequences has been used for the assessment of X-linked disorders *(2)*, the detection of unique gene sequences such as the RhD locus to determine fetomaternal blood group incompatibility *(3)*, and the detection of dominantly inherited, paternally derived mutations for diagnoses of single gene disorders *(1)*. The quantity of fetal DNA in maternal plasma or

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serum has been used as a marker of genetic disorders and complications of pregnancy, including common trisomies *(4–7)*, preterm labor *(8)*, and preeclampsia *(9)*.

Real-time PCR amplification of Y-chromosome sequences from the plasma or serum of women pregnant with male fetuses has been used in many studies as a model for the detection and quantification of fetal DNA. Y-chromosome DNA has been detected as early as 5 weeks of gestation *(10, 11)*, before the time period in which invasive testing is typically performed. Thus, early detection of fetal DNA could have a profound effect on the way pregnancies are managed. Some investigators have reported >95% sensitivity and specificity for real-time PCR *(12, 13)*. However, these data are typically generated by a single laboratory under specific conditions and often include data from a small number of patients. In addition, confounders that could impact routine clinical application may be undetected in smaller studies. Indeed, it has been shown that methods of processing can drastically affect the amount of DNA detected by real-time PCR *(14)*. For PCR assays to become part of standard prenatal care, their accuracy and reproducibility must be improved and the underlying variables that affect performance must be better understood.

In this study, we first established a common protocol for sample preparation, DNA extraction, and PCR analysis to be used by five independent laboratories that are part of the National Institute of Child Health and Human Development (NICHD) Fetal Cell Isolation Study. To test the potential clinical utility of fetal DNA analysis, a common set of samples was then evaluated at all sites by the standardized protocol. We report here the results of the first interlaboratory evaluation of fetal DNA detection in maternal plasma from a common sample set.

Materials and Methods

study design

A prospective, repeated-measures design with five replications for each individual was used to assess concordance in qualitative and quantitative PCR analysis of fetal DNA in maternal plasma. Before implementation, the study protocol, methods to ensure quality of results, and scoring were clearly defined as described below.

patient enrollment/sample collection

Institutional Review Board approval for this study was obtained by each of the five participating centers (Baylor College of Medicine, Genzyme Genetics, Tufts-New England Medical Center, University of Basel, and University of Illinois at Chicago), which are hereafter denoted A–E. After receiving informed consent, we enrolled pregnant women in their late first or second trimesters (10–20 weeks of gestation) as study participants. Mean gestational ages ranged from 13.75 to 18.13 weeks across the five sites. Prospectively, each participating center collected 20 mL of peripheral blood from 20 pregnant women (total of 100).

sample processing

Sample processing began within 24 h of blood collection. Blood was centrifuged at 800*g* for 10 min in the original collection tube. Plasma was removed, pooled if more than one tube of blood was collected, and divided into five 1-mL aliquots. The 1-mL samples were then centrifuged for 10 min at 13 500*g* to remove all residual intact cells *(14)*. Supernatant (900 μL) was removed from each aliquot and stored at −80 °C. One aliquot of each sample was shipped on dry ice to each of the four other participating centers. Thus each center performed DNA extraction and PCR analysis of the 20 samples that were collected on site and also on each of the 80 samples shipped from other centers.

dna extraction

After shipment, samples were thawed at room temperature, and 800 μL of plasma was used for DNA extraction. The QIAamp DNA Blood Mini Kit was used as specified by the manufacturer (Qiagen Inc.), with minor modifications. Reagents were increased proportionately to accommodate the 800-μL sample size. To elute DNA from the column, buffer AE prewarmed to 56 °C was used. After 50 μL of the buffer was applied to each column, the column was incubated at 56 °C for 5 min and then centrifuged at 6000*g* for 1 min. This procedure was then repeated for a final elution volume of 100 μL. Samples were stored at 4 °C pending analysis.

pcr analysis

PCR was performed with a Perkin-Elmer Applied Biosystems 7700 Sequence Detection System (Applied Biosystems). Extracted DNA was analyzed for both the *GAPDH* and *SRY* loci. The *SRY* sequence was used to measure the quantity of fetal DNA present in each sample from a patient bearing a male fetus, and the *GAPDH* sequence was used to confirm the presence and quality of DNA in each sample as well as measure the quantity of total (maternal and fetal) DNA in each sample. Primer and probe sequences were as follows:

- *SRY* forward primer: 5′-TCC TCA AAA GAA ACC GTG CAT-3′
- *SRY* reverse primer: 5′-AGA TTA ATG GTT GCT AAG GAC TGG AT-3′
- *SRY* TaqMan probe: 5′-CAC CAG CAG TAA CTC CCC ACA ACC TCT TT-3′
- *GAPDH* forward primer: 5′-CCC CAC ACA CAT GCA CTT ACC-3′
- *GAPDH* reverse primer: 5′-CCT AGT CCC AGG GCT TTG ATT-3′
- *GAPDH* TaqMan probe: 5′-AAA GAG CTA GGA AGG ACA GGC AAC TTG GC-3′

Reactions were set up in a 50-μL volume using 25 μL of PE-ABI Universal Mastermix and 5 μL of extracted DNA. Primers and probes were used at final concentrations of 300 and 200 nM, respectively, with the exception of center

D, which used primer and probe concentrations of 100 and 50 nM, respectively. Each sample was run in triplicate for both loci, and the mean of the values was used for further calculations. Each reaction plate was run simultaneously with a duplicate calibration curve of titrated DNA, which was extracted and quantified from male and female cells by center C and then shipped to all centers for use. For *GAPDH* reactions, the calibration curve consisted of eight points at a 2× serial dilution from 13 200 to 103.2 pg. For *SRY* reactions, the curve consisted of eight points between 3300 and 25.8 pg. All reaction plates were run with three wells each containing 8515 pg of genomic female DNA. This functioned as a negative control for *SRY* reactions and as a positive control for *GAPDH* reactions. Three samples with no target DNA (i.e., no-template controls) were also included on each reaction plate. Cycling conditions for all reactions consisted of a 2-min incubation at 50 °C to allow UNGErase activity, an initial denaturation step of 95 °C for 10 min, and then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All samples were analyzed blindly with respect to fetal gender. The standard factor of 6.6 pg was used to convert the data to genome equivalents (GE).

PCR ANALYSIS QUALITY CONTROL AND CODING INFORMATION

Using Sequence Detection System software, we defined the baseline fluorescence as between cycle 3 and two cycles before initial amplification of the most highly concentrated calibrator or unknown point. Thresholds for determining threshold cycle values were set at 25 times the SD given by the software. Plates were considered valid when the calibration curve slope was less than −3.0 and the correlation coefficient of the calibration curve was >0.96. Plates were rejected when *GAPDH* DNA did not amplify in female controls, when *SRY* DNA did amplify in female controls, when no-template controls amplified, or when a center detected other problems with an assay. In these cases, the entire reaction was repeated. If the second run was considered valid, those results were used. Individual samples within a plate were considered invalid if there was discordance between the three wells (e.g., positive amplification in only one or two wells) that could not be explained by technical problems. In the situation of individual sample discordance, the sample was rerun. Samples were designated as male if they had at least two replicates with positive amplification in the final validated assay for that sample. All other results were considered insufficient to detect male DNA and were thus designated as female.

OUTCOMES

The primary outcome was qualitative and reflected whether fetal DNA was present, and was based on information obtained through PCR analysis. The secondary outcome was quantitative and reflected the mean amount of DNA amplified.

STATISTICAL METHODS

Descriptive statistics were generated to assess quality issues (e.g., number of plates or samples needing to be rerun, calibration curve slopes, and correlations) and coding of outcomes. All analyses were stratified by center. To assess to what extent each of the five centers obtained the same qualitative PCR results for both the *GAPDH* and *SRY* loci, we estimated the concordance among centers by use of κ statistics, which measure the degree of agreement beyond that expected by chance. Pairwise κ statistics were estimated for each pair of participating centers, based on the 100 samples analyzed. The pairwise κ statistics were subsequently combined to produce an overall estimate of agreement among centers with respect to DNA detection. To compare quantitative values (i.e., amount of DNA detected), we estimated intraclass correlation coefficients (ICC) according to the same procedure as described for κ. The ICC statistic that was chosen quantifies both the consistency and absolute agreement of DNA detection. All significance tests were two-sided and the level of significance was fixed at 0.05. Performance characteristics (i.e., sensitivity and specificity) were also estimated between PCR qualitative results and definitive fetal gender detection obtained from all sites except center C, which did not have access to fetal gender. SAS, Ver. 8.0, was used to perform all statistical analyses.

Results

Each center ran between five and seven plates for the *GAPDH* locus and between six and eight plates for the *SRY* locus, and only center D needed to rerun an entire plate, because of a PCR instrument malfunction. We evaluated PCR efficiency at each center by comparing the results obtained for the initial concentration of the standard female control DNA (*GAPDH*) supplied by center C (Table 1). For standard female DNA, the mean *GAPDH* concentration varied from 7626.71 to 8804.42 pg/reaction, in which 8515 pg was the approximate starting quantity that was distributed. The mean slopes and correlation coefficients over all plates by locus and center (Table 1) indicated that the quality and study criteria were met (i.e., all slopes less than −3.0 and all correlation coefficients >0.96).

Of the 100 plasma samples analyzed, fetal gender was known for 63 (35 male and 28 female) at the time of data analysis. All centers were able to amplify *GAPDH* from all samples except center C, which successfully analyzed only 99 samples because of extraction failure. The mean quantity of *GAPDH* detected in these samples ranged from 5939 to 12 397 GE/mL (Table 2). Individual centers were able to amplify the *SRY* sequence from between 11 and 34 of the 35 known male samples. The mean quantity of *SRY* detected in the known male samples with positive values ranged from 51 to 228 GE/mL.

Rates of correct identification by PCR analysis of known fetal gender varied from 31% to 94% among centers and was, in general, directly related to the amount

Table 1. Center-specific PCR analysis of standardized control DNA.

	Mean (SD) *GAPDH* in female controls, pg/reaction	Calibration curve for *GAPDH*[a]		Calibration curve for *SRY*[a]	
		Mean (SD) slope	Mean (SD) correlation coefficient	Mean (SD) slope	Mean (SD) correlation coefficient
A	7972 (1052)	−3.63 (0.18)	0.988 (0.007)	−3.95 (0.38)	0.974 (0.009)
B	8793 (990)	−3.62 (0.16)	0.992 (0.004)	−3.51 (0.18)	0.978 (0.011)
C	8194 (709)	−3.49 (0.05)	0.989 (0.010)	−3.48 (0.17)	0.981 (0.010)
D	8804 (646)	−3.80 (0.08)	0.994 (0.003)	−4.15 (0.15)	0.981 (0.008)
E	7627 (846)	−3.50 (0.11)	0.994 (0.004)	−3.55 (0.12)	0.984 (0.009)

[a] Study criteria: slope less than −3.0 and correlation coefficient >0.96 to be valid.

of DNA amplified (Table 2). The rank ordering of rates of male gender detection and mean *GAPDH* were identical across centers; the centers with the highest and lowest concentrations of *GAPDH* amplified had the highest and lowest sensitivities, respectively. The relationship between detection rates and mean GE of *SRY* was similar. The center with the lowest detection rate (31%) had the second-lowest mean concentration of amplified male DNA (64 GE/mL), whereas the center with the highest detection rate had the highest concentration of amplified male DNA. The specificities among centers were more consistent, varying from 93% to 100%. Male DNA was detected in only four samples from women carrying female fetuses in the entire study; a false-positive result was not obtained on any sample in more than one laboratory.

The concordance of fetal DNA detection among centers is shown in Table 3. The overall κ value for all sites combined was 0.48 ($P<0.05$), indicating statistically significant agreement among centers beyond that expected by chance. There was a stronger association ($\kappa>0.61$; $P<0.05$) between sites A and C, A and D, and C and D. These three sites (A, C, and D) had the highest mean concentrations of amplified DNA for both *GAPDH* and *SRY* (Table 2), and the best results with respect to performance characteristics (i.e., sensitivity and specificity of male gender detection). With regard to the concordance of total DNA detection (*GAPDH*), the overall κ value for all sites combined as well as between any two sites was 1.00.

The concordance assessments for quantity of total DNA and fetal DNA detected between sites, expressed in terms of ICC, are shown in Tables 4 and 5, respectively. The ICC statistic signifies both consistency (i.e., agreement in terms of ordering) and reliability (i.e., agreement in absolute value). Entries in Tables 4 and 5 that are expressed in bold indicate statistical significance ($P<0.05$) with respect to quantitative concordance between centers (e.g., A and C, B and C, B and E, C and D, and C and E all have significant concordance with respect to *SRY* detection). A large but statistically insignificant ICC statistic (e.g., A and D and A and E for *SRY* detection) implies good consistency but poor reliability (i.e., high variability in absolute assessments).

Discussion

The discovery of nucleic acids of fetal origin circulating in the maternal plasma and their continuing characterization has raised the possibility of their use in noninvasive prenatal diagnosis. However, the eventual clinical application of circulating DNA technology will require thorough identification and understanding of the factors that may affect its performance in different laboratories. Through the processing of a common set of plasma samples by five laboratories using a standardized protocol for DNA extraction and real-time PCR amplification, along with the blinded analysis of the raw data, we have addressed some of the issues required to translate this technology into clinical utility. Our results indicate that each of five sites could reproduce the PCRs and amplify previously extracted DNA of known quantity that had been frozen and shipped. However, not all centers were able to obtain this level of performance on maternal plasma samples that required DNA extraction to be performed. Differences in sensitivity among laboratories correlated strongly with the amounts of total and fetal DNA detected, suggesting that the extraction procedure was the most likely factor confounding the results.

Some factors that may influence technical performance have been avoided or eliminated in this study. Each center used the same type of PCR instrument and reagents, and data generated from the common DNA calibrators were comparable among centers, suggesting that the instru-

Table 2. Center-specific characteristics of fetal DNA detection (35 known male and 28 known female fetuses).

Center	Males detected, n	Mean (SD) *SRY*, GE/mL	Detection rate, %	Mean (SD) *GAPDH*, GE/mL	False positives, n	Specificity, %
A	33	122 (88)	94	11 451 (36 803)	0	100
B	11	64 (38)	31	5939 (17 927)	0	100
C	27	154 (442)	77	9415 (31 491)	2	93
D	34	228 (145)	97	12 397 (34 587)	1	96
E	15	51 (27)	43	8337 (26 402)	1	96

Table 3. Concordance of fetal DNA detection among centers: κ statistics.[a]

Center	A	B	C	D	E
A					
B	0.39				
C	**0.69**[b]	0.32			
D	**0.86**	0.33	**0.64**		
E	0.51	0.26	0.37	0.43	

[a] Overall κ = 0.48. κ = 0, poor; 0.01–0.20, slight; 0.21–0.40, fair; 0.41–0.60, moderate; 0.61–0.80, substantial; 0.81–1.00, high.
[b] **Bold** indicates significant agreement ($P < 0.05$).

Table 5. Concordance in amount of fetal DNA detection among centers: ICC.[a]

Center	A	B	C	D	E
A					
B	0.23				
C	**0.32**[b]	**0.00**			
D	0.67	0.11	**0.35**		
E	0.55	**0.46**	**0.11**	0.35	

[a] Overall ICC = 0.095.
[b] **Bold** indicates significant agreement ($P < 0.05$).

ment's performance was consistent in all laboratories. It is possible that instruments from other manufacturers may perform differently, and a future comparison of different systems would be useful. In addition, it is likely that analytic interpretation of raw PCR data may vary among different laboratories. However, this was not a concern in the present study because all raw data were sent directly to the statistical analysis center and analyzed in a consistent, unbiased manner to accurately calculate the amount of DNA present. Nevertheless, it is important to establish consistent guidelines for these analyses, such as the selection of a threshold for determining the presence of a particular DNA sequence.

Other factors that may affect performance but could be not eliminated from consideration in this study were shown to have little or no influence on the PCR results. For example, it is known that the amount of cell-free fetal DNA in the maternal circulation increases with gestational age, peaking at the time of delivery *(2)*. However, all centers processed a common set of samples of various gestational ages, and there was no apparent trend between the sensitivity and specificity of fetal DNA detection and gestational age. Delay in processing after blood drawing can also be a concern, especially when samples are shipped long distances. However, because all samples were processed within 24 h and it has been shown that the detection of fetal DNA is consistent for at least 24 h *(15)*, this should not have been a concern in the present study.

Of the factors shown to influence the detection of the DNA sequence of fetal origin, most notable was the effect of DNA extraction on sensitivity. The center with the highest sensitivity (center D) had the highest mean rate of detection of both *SRY* and *GAPDH* sequences, whereas the center with the lowest sensitivity (center B) had the second lowest and lowest mean rate of detection of *SRY* and *GAPDH* sequences, respectively. This implies that the efficiency of DNA extraction correlates directly with the ability to detect fetal DNA sequences. This conclusion is further supported by the consistent results achieved among all centers when DNA was previously extracted and quantified by a single source, suggesting that real-time PCR performed at all of the centers did provide reasonable approximations of the amount of DNA present in each reaction. Although a standardized protocol was used, there are likely other factors confounding its reproducibility. These may include individual laboratory techniques and familiarity with the extraction protocol. One center (center D) used PCR primers and probes at concentrations that were lower than the other sites. Although this center had the highest sensitivity of *SRY* detection, center A obtained similar results using the higher primer/probe concentrations. Therefore, this difference likely did not have a significant effect on outcome, although this variable should be considered for further studies of PCR optimization. These concerns can potentially be eliminated by a more in-depth standardized DNA extraction procedure, as well as increased experience with the protocol. The centers with lower sensitivities are addressing these issues and are incorporating adjustments to their extraction procedures. Indeed, center B, by incorporation of a shaking heat block and vacuum manifold during the extraction process, now has achieved 97% sensitivity while maintaining 100% specificity on >100 additional maternal plasma specimens subjected to blinded analysis using the described approach.

Table 4. Concordance in amount of total DNA detection among centers: ICC.[a]

Center	A	B	C	D	E
A					
B	**0.59**				
C	0.94	**0.71**			
D	**0.82**	0.83	0.88		
E	0.86	**0.57**	**0.92**	0.70	

[a] Overall ICC = 0.79.
[b] **Bold** indicates significant agreement ($P < 0.05$).

The two sites with the highest sensitivities in gender detection also observed the highest quantities of DNA detected (centers A and D). In addition, the ICCs representing these two sites were high, indicating similarity in differentiation (i.e., agreement in terms of ordering) of fetal DNA quantities detected. However, the ICC between these sites was not statistically significant, implying poor calibration (i.e., agreement in absolute value) in the amount of fetal DNA detected. Nevertheless, there was

statistically significant qualitative agreement over all centers as indicated by the κ statistic. Similar qualitative and quantitative agreement was found with respect to total DNA detection (*GAPDH*). Our findings suggest that although qualitative agreement is attainable, it may not be possible to reliably compare absolute quantitative values of fetal DNA in maternal plasma between sites even with a highly standardized protocol. Therefore, alternative statistical approaches, such as the development of site-specific analyses using multiples of the median, should be considered *(16)*.

In conclusion, the common protocol presented here is a robust PCR assay for the detection and quantification of fetal DNA sequences in maternal plasma. Variables that were found to be critical for accurate analysis have been identified, allowing adjustments to be incorporated to minimize or eliminate these concerns. This should facilitate development of an optimized protocol that could provide a robust, clinically relevant platform for future applications.

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