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Optimized Real-Time Quantitative PCR Measurement of Male Fetal DNA in Maternal Plasma

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Background: **Circulating fetal DNA (cfDNA) in maternal plasma has been measured to investigate its possible relationship with pregnancy-related disorders, including fetal trisomy 21 and preeclampsia. The circulating concentrations of single-copy fetal genes, however, are close to the detection limits of PCR methods.**

Methods: **We optimized a protocol for the real-time quantitative PCR amplification of the multicopy sequence *DYS14* on the Y-chromosome. This was compared with an established real-time PCR assay for the single-copy *SRY* gene.**

Results: **By probit regression analysis, the measurements of male DNA by the *DYS14* assay had a 10-fold lower detection limit (0.4 genome equivalents) than did measurements of *SRY*. For plasma samples from women in the first trimester of pregnancy, imprecision (CV) was 2%–22% when amplifying *DYS14* compared with 26%–140% for *SRY*.**

Conclusions: **The low copy numbers of fetal DNA in plasma of women in the first trimester of pregnancy cannot be measured precisely when targeting single-copy sequences. Better results are obtained by amplifying a sequence that is present in multiple copies per male genome.**

In 1997, Lo et al. *(1)* first described PCR detection of cell-free circulating fetal DNA (cfDNA)[3] in maternal plasma and serum. Since then, cfDNA amplification by PCR has emerged as an important method for noninvasive prenatal diagnosis. The advent of real-time PCR *(2)* soon made it possible to measure cfDNA concentrations in maternal plasma *(3)*. The current main clinical application is the determination of fetal rhesus D status in pregnant rhesus D–negative women *(4, 5)*. In addition, the determination of fetal sex by real-time quantitative PCR (qPCR) is useful for clinical assessment of pregnancies at risk for X-linked disorders *(6)*. Y-Chromosome–specific sequences have also been measured to investigate a possible correlation between cfDNA concentrations and pregnancy-associated complications such as preeclampsia, fetal trisomies, and preterm labor *(7–11)*. Although the method is applicable to only 50% of samples because it is restricted to pregnancies carrying male fetuses, its ease of use makes it a valuable research tool.

In the first and second trimesters, cfDNA concentrations are very low; the template concentration in the samples thus is close to the limit of detection (LOD) *(12)*. The precision and reproducibility of quantitative measurements can be increased by amplifying Y-specific sequences that are present in more than 1 copy per Y chromosome *(13)*. We developed an assay specific to the multicopy sequence *DYS14* and compared its performance with that of our currently used assays for the measurement of the single-copy *SRY* sequence.

Materials and Methods

Genomic reference DNA was extracted from leukocytes from males and quantified with a spectrophotometer

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[3] Nonstandard abbreviations: cfDNA, circulating fetal DNA; qPCR, quantitative PCR; LOD, limit(s) of detection; GE, genome equivalents; C_T, threshold cycle; and LOQ, limit(s) of quantification.

(NanoDrop-1000; Witec AG), and the results were converted to genome equivalents (GE) by use of a conversion factor of 6.6 pg of DNA per cell *(3)*.

The study was approved by the Cantonal Institutional Review Board of Basel, Switzerland. At the Harris Birth Right Research Center of Fetal Medicine in King's College in London, 3.5-mL tubes containing EDTA were used for collecting blood samples from pregnant women presenting for amniocentesis in the first trimester. The blood was centrifuged at 1600*g* for 10 min, the supernatant was transferred into polypropylene tubes and centrifuged at 16 000*g* for 10 min, and 900 μL of supernatant was collected in polypropylene tubes, stored at −80 °C, and sent to Basel for further processing.

After thawing, the samples were centrifuged at 16 000*g* for 10 min, and plasma DNA was extracted from the supernatant with the High Pure PCR Template Purification Kit (Roche). The manufacturer's instructions were amended in that we extracted 400 μL of plasma, used 40 μL of proteinase K for digestion, and eluted the DNA with 50 μL of elution buffer diluted to 200 mL/L in water. Before elution, the columns were prewarmed in a shaker at 70 °C for 5 min. Extracted DNA was stored at 4 °C.

real-time qPCR amplification

The real-time qPCR was performed in the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The 25-μL singleplex reactions were prepared on ice and contained 300 nM each primer (HPLC purified), 200 nM MGB probe (ABI), and a 1× concentration of the TaqMan Universal PCR master mixture with uracil DNA-glycosylase (AmpErase UNG; ABI). Primer and probe sequences were as follows:

SRY forward: 5′-TCC TCA AAA GAA ACC GTG CAT-3′
SRY reverse: 5′-AGA TTA ATG GTT GCT AAG GAC TGG AT-3′
SRY probe: 5′-TCC CCA CAA CCT CTT-3′
DYS14 forward: 5′-GGG CCA ATG TTG TAT CCT TCT C-3′
DYS14 reverse: 5′-GCC CAT CGG TCA CTT ACA CTT C-3′
DYS14 probe: 5′-TCT AGT GGA GAG GTG CTC-3′

The probes were labeled with 6-carboxyfluorescein (FAM; *SRY*) and VIC® (*DYS14*). Amplicon sizes were 78 and 84 bp for *SRY* and *DYS14*, respectively. Cycling conditions were 2 min at 50 °C and 10 min at 95 °C for initial denaturation of the DNA and polymerase activation, followed by 50 cycles of 1 min at 60 °C and 15 s at 95 °C. The volume of DNA added was 4 μL per replicate, which corresponded to the amount of DNA recovered from 32 μL of maternal plasma. For the single-copy sequence-targeting assay, this procedure allowed for a theoretical detection limit of 31 GE/mL. The analysis was performed with the ABI Prism 7000 SDS Software with automatic baseline setting.

During assay validation, we amplified serial 10-fold dilutions of reference genomic DNA. The logarithm of the template copy number in a reaction is inversely proportional to the cycle threshold (C_T). Thus, the linear regression can be used as a calibration curve when these 2 values are plotted against each other. The PCR efficiency was calculated from the slope of the curve by the following formula:

$$\text{Efficiency} = 10^{-(1/\text{slope})} - 1$$

To quantify the DNA from plasma samples, we used an approach similar to that of imported calibration curves *(14)*, using triplicate measurements of a single calibrator sample of known concentration (540 or 270 GE/PCR for *SRY* and 54 or 27 GE/PCR for *DYS14*, respectively; Zimmermann et al., manuscript in preparation).

For the quantification, we converted the C_T values of individual replicates into quantities and determined the mean and CVs in Excel (Microsoft). In this way, replicates without amplification were included in the calculation, which is not the case when automatic analysis is performed by the SDS software. To ensure that the reference sample of an experiment was amplified with adequate efficiency, we compared the C_T values and the final normalized fluorescence (ΔR_n) between experiments. No differences were observed.

For fetal sex determination by *SRY*, all 3 replicate amplifications of a sample had to be either positive or negative; otherwise the sample was undetermined. With the *DYS14* protocol we applied a cutoff of 1 GE/PCR to differentiate between true positives (male) and unspecific amplifications (female). Results were sent to London and compared with the sex determined by karyotype analysis.

To characterize the LOD for the 2 assays, we used probit regression analysis (predicted proportion positive; SPSS). For each assay, the proportion of positive replicate measurements per known input amount was examined. For this analysis, we used all amplifications with a known input amount of male DNA. This included all amplifications of diluted reference DNA and those of pregnancies with a female fetus. The input copy number with a 95% probability of a positive PCR result was defined as the LOD *(15)*.

Results

In a validation experiment, we amplified reference DNA samples at copy numbers of 5400, 540, and 54 GE/PCR in quadruplicates. Calibration curves were constructed by plotting the C_T as a function of the log of the template copy number:

$$SRY: C_T = -3.34 \times \log(\text{copies}) + 39.22$$

$$DYS14: C_T = -3.39 \times \log(\text{copies}) + 36.31$$

Both assays amplified with close to optimal efficiencies of 99% (*SRY*) and 97% (*DYS14*).

In the same experiment, we analyzed 2-fold dilutions

Table 1. Low template dilutions of genomic DNA amplified in 2 experiments.

	Input copy number						
	5.4	**2.7**	**1.36**	**0.68**	**0.34**	**0.17**	**0.08**
Copy number, GE (CV)							
DYS14 experiment 1	3.46 (7.6%)	1.82 (40%)	1.03 (9.4%)	0.69 (50%)	0.22 (30%)	0.19 (19%)	0.13 (39%)
DYS14 experiment 2		2.4 (25%)	1.83 (30%)	0.67 (28%)	0.55 (46%)	0.39 (75%)	0.52 (9.4%)
SRY experiment 1	2.25 (60%)	1.97 (63%)	0.85 (173%)	0.10 (173%)	0.31 (173%)	0.00	0.00
SRY experiment 2		1.48 (82%)	0.23 (173%)	0.05 (173%)	1.96 (100%)	0.00	0.00
DYS14 replicates with amplification, n	4/4	8/8	8/8	8/8	8/8	7/8	8/8
SRY replicates with amplification, n	4/4	6/8	2/8	2/8	3/8	0/8	0/8

of template DNA between 5.4 and 0.08 GE/PCR with the *DYS14*- and *SRY*-specific assays. The results demonstrated the increased sensitivity and reproducibility of the *DYS14* protocol for low copy numbers (Table 1). The quantitative results obtained by the *SRY* assay deviated considerably among replicates, with CVs of quadruplicate measurements >50%. The limit for accurate quantification (LOQ) was thus clearly higher than 5 template copies; many replicates without amplification occurred at sample inputs <5 copies (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol51/issue9/).

The *DYS14*-specific protocol allowed more reproducible amplification of low target amounts. Quantification of the low-input samples was still precise, with quadruplicate CVs of 7%–50%, in contrast to those for *SRY*.

The probit regression analysis was performed with the data for the diluted DNA samples and all tested plasma samples from female pregnancies. Probit regression determined the probability of a positive PCR result for the template input amount (Fig. 1 and Table 2). The 95% LOD of the *DYS14* assay was at a DNA input of 0.35 GE (95% confidence interval, 0.22–0.96 GE), and the LOD of the *SRY* assay was at 4.19 (2.97–8.70) GE, reflecting the 10-fold higher copy number of the *DYS14* locus per genome. We propose setting the LOQ by PCR at a 5-fold higher copy number than the detection limit. Thus, the LOQ are ~2 GE for *DYS14* and ~20 GE for *SRY*.

When we performed a BLAST search with the primer and probe sequences from the original *DYS14* targeting protocol, we observed considerable homologies to sequences on chromosomes other than Y *(13, 16)*, which would lead to unspecific amplification from the maternal background DNA. It was very difficult to develop an assay without any unspecific amplification because homologies to sequences on other chromosomes could not fully be avoided. According to the BLAST search for short, nearly exact matches in the human chromosome database (http://www.ncbi.nlm.nih.gov/BLAST/), the primer pair for *DYS14* produces 9 amplicons 84 bp in length with 100% match and 1 with a single mismatch at base 6 of the forward primer per Y chromosome. The short amplicon size is necessary because the cfDNA can be very short *(17, 18)*. In addition, short amplicons are amplified with optimum efficiency, which is a prerequisite for smaller variation *(19)*.

We compared the 2 assays by analyzing 12 maternal plasma samples of unknown fetal sex (gestational age between 11 + 6 and 13 + 5 weeks). Karyotype analysis of invasively obtained material showed that 6 samples were from male and 6 from female pregnancies. Each plasma sample was extracted in duplicate, and each DNA extraction was analyzed individually, a total of 24 DNA analyses. With the *SRY*-specific protocol, we were able to determine 8 of 12 male and 11 of 12 female DNA extracts correctly; the other 5 were inconclusive (Fig. 2). The

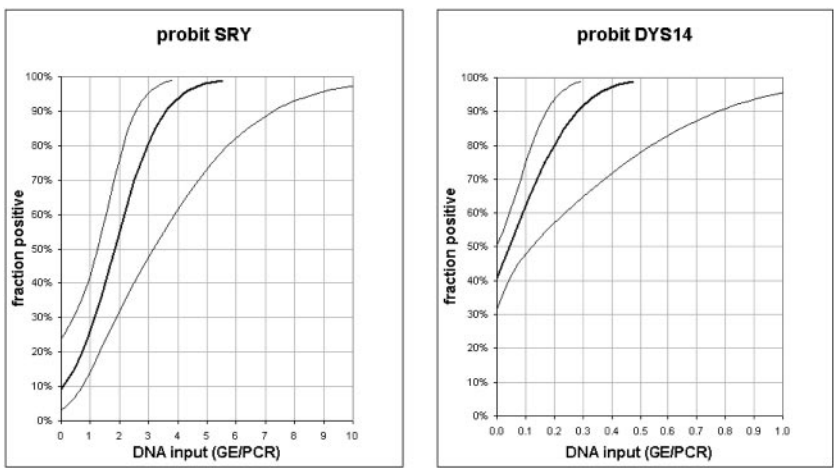

Fig. 1. Predicted proportion of positive PCRs plotted vs the input copy number for the *SRY* and *DYS14* real-time PCR tests.

The data points represent the measurements of genomic DNA and female pregnancy plasma samples. The *outer lines* indicate the 95% confidence intervals.

Table 2. Predicted proportion of positive PCRs vs the input copy number of genomic DNA for the *SRY* and *DYS14* real-time PCR tests.

	Input GE (95% CI)[a]	
Probit	*SRY*-specific assay	*DYS14*-specific assay
1%	−1.83 (−6.82 to −0.63)	−0.39 (−1.16 to −0.22)
10%	0.08 (−1.72 to 0.72)	−0.19 (−0.61 to −0.10)
20%	0.73 (−0.33 to 1.33)	−0.11 (−0.38 to −0.05)
30%	1.16 (0.43–1.90)	−0.05 (−0.21 to −0.01)
40%	1.51 (0.91–2.51)	0.00 (−0.09 to 0.04)
50%	1.84 (1.25–3.17)	0.04 (0.00–0.12)
60%	2.16 (1.53–3.88)	0.09 (0.05–0.24)
70%	2.51 (1.81–4.69)	0.14 (0.08–0.37)
80%	2.95 (2.12–5.71)	0.20 (0.12–0.54)
90%	3.59 (2.57–7.26)	0.28 (0.17–0.77)
95%	4.19 (2.97–8.70)	0.35 (0.22–0.96)
99%	5.51 (3.82–11.9)	0.47 (0.29–1.33)

[a] 95% CI, 95% confidence interval.

amplifications with the *SRY* assay were very specific: only 2 of 36 replicates from female pregnancies showed amplification at high cycle numbers (corresponding to 0.8 and 1.8 GE). With the *DYS14* protocol, we observed 13 false amplifications. Because we were aware of the possible unspecific hybridization to maternal DNA sequences, we had set a cutoff at 1.0 GE to discern between true and false positives. When this precaution was taken, none of the replicates from pregnancies with a female fetus was assessed to be male (Table 2 in the online Data Supplement). All extracts from pregnancies with male fetuses were amplified, and results were well above this cutoff value. Using this precaution, we were able to correctly determine the fetal sex for all 24 DNA extractions by *DYS14*-specific real-time PCR.

With the *DYS14* protocol, quantification of the pregnancies with male fetuses was very precise: the CV ranged from 2% to 22% in the 12 triplicate measurements, and cfDNA was determined to be between 6.8 and 17.6 GE/reaction. With the *SRY* protocol, the deviations were much greater (CVs between 26% and 140%), and there were 6 negative replicates. For duplicate extractions, the mean (range) CVs were 17 (14–28)% for *DYS14* and 74 (63–103)% for *SRY*. These results indicate that the *SRY* plasma DNA measurements were below the LOQ and that the *SRY* protocol, therefore, is only semiquantitative. Surprisingly, the DNA concentrations determined by the *DYS14* protocol were 2.5-fold higher than those determined by the *SRY* protocol (range, 1.4- to 4.3-fold).

To investigate the discrepancy between the copy numbers determined by both assays, we amplified genomic DNA dilutions in triplicate with *DYS14* and *SRY*. With the results from these amplifications we constructed new calibration curves and quantified 4 plasma DNA samples from male pregnancies. For 3 samples, the same copy number was determined by both assays, and in 1 sample the *SRY* result was 3 times lower (3.7 vs 11.1 GE per PCR; Table 3 in the online Data Supplement). The old and new calibration curves generated for the *DYS14* protocol were very similar (Fig. 3A). In contrast, the *SRY* calibration curves clearly diverged: The mean C_Ts for the calibrator samples with copy numbers of 5400 and 540 GE were within 0.1 cycles; the C_T of one 54-GE replicate in experiment B was much higher and had a pronounced effect on the slope (Fig. 3B). This higher C_T value of 1 amplification in experiment B produced a difference of more than 1 cycle at the intercept. Consequently, using calibration curve B instead of curve A would give 2-fold greater template numbers in reactions with <10 GE and lead to similar copy number determinations by *SRY* and *DYS14* in the plasma samples. Measurements of low copy numbers can be skewed by random variability, as seen in the comparison of the 2 *SRY* calibration curves, confirming that copy numbers for the plasma samples as provided by the *SRY* protocol can be considered as only semiquantitative estimates.

The specificity of the *DYS14* protocol for sex determination was tested on an additional 34 maternal plasma samples obtained in a blinded manner at a fetal gesta-

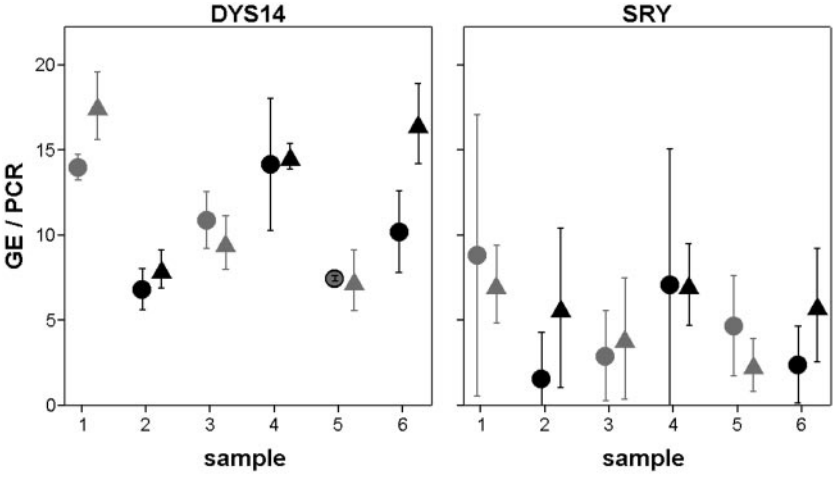

Fig. 2. Plasma samples from 6 male pregnancies extracted in duplicate and analyzed by the *DYS14*- and *SRY*-specific assays.

Symbols are the mean and the *error bars* are the CV of triplicate measurements.

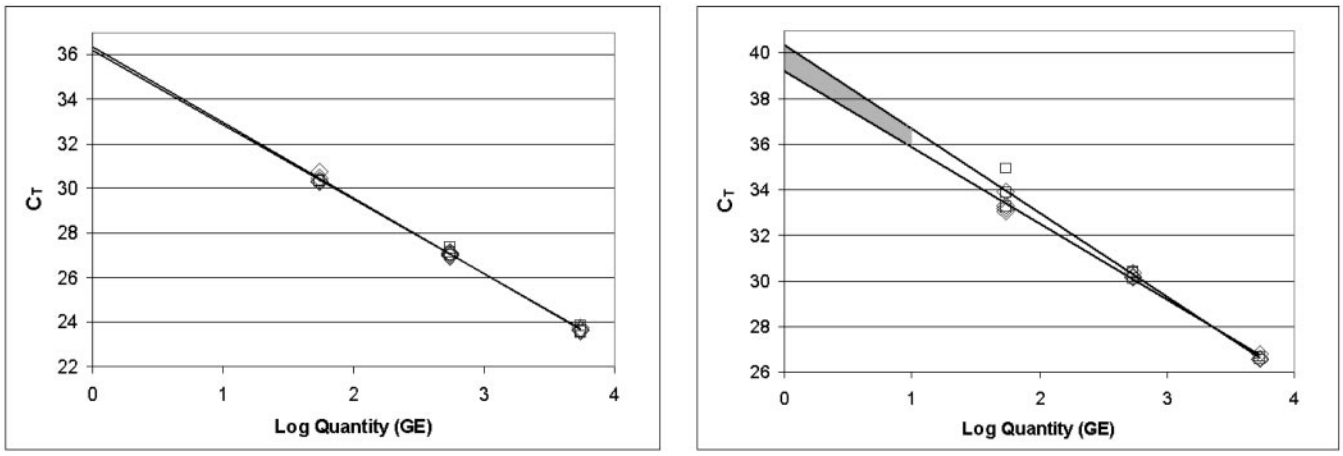

Fig. 3. Calibration curves A and B for *DYS14* (*left*) and *SRY* (*right*).

The equations describing the *DYS14* curves are: curve A, $y = -3.39 + 36.31$ ($R^2 = 0.9985$); curve B, $y = -3.34 + 36.18$ ($R^2 = 0.9978$). Equations for the *SRY* curves are: curve A, $y = -3.34 + 39.22$ ($R^2 = 0.9934$); curve B, $y = -3.68 + 40.39$ ($R^2 = 0.9816$). In the *SRY* plot (*right*), the *shaded area* indicates measurements of samples containing <10 GE, where the discrepancy between curves A and B is 2-fold.

tional age of 11 + 6 to 13 + 6 weeks. Again the fetal sex was correctly determined for all samples.

With the new assay we quantified 25 maternal plasma DNA samples from women with male fetuses (Table 4 in the online Data Supplement). Copy numbers were 3.4–31.5 GE/PCR with CVs of 2%–27%. In 33 extractions from women with female fetuses, we observed amplification curves in 36 of 99 reactions. All of these amplification curves were indicative of <1 GE/PCR, which was the cutoff to identify unspecific amplification. Our *DYS14*-specific assay thus achieved 100% specificity and sensitivity for sex determination in 58 DNA samples from maternal plasma (46 different pregnancies).

In the 17 pregnancies with male fetuses of normal karyotype with gestational ages from 11 + 6 to 13 + 6 weeks (mean, 12 + 6.4 weeks), the mean cfDNA content was 426 GE/mL of plasma [range, 108–983 GE/mL of plasma; mean CV = 14% (range, 2%–22%); Table 4 in the online Data Supplement]. Fetuses in the sample cohort had Down syndrome in 2 cases; in 1 of these cases the maternal cfDNA was 333 GE at 13 weeks, and in the other it was 750 GE at 12 weeks of pregnancy.

Discussion

Increased concentrations of cfDNA have been reported in certain pregnancy-associated disorders, such as fetal trisomy 21, preterm labor, and preeclampsia *(20)*. The measurement of male cfDNA is considered to be a simple procedure, but results differ significantly among sites even with use of the identical sample and calibrator material, implementation of the same DNA extraction and real-time qPCR procedures, and standardized and centralized data analysis *(21)*.

We determined the LOD for 2 real-time PCR assays by assessing by probit analysis the template amount with a 95% probability of a positive PCR amplification *(22, 23)*. The LOQ was set 5-fold higher than the LOD. The quantitative limitations of the novel *DYS14* assay were ~2 GE per reaction compared with 20 GE for the assay targeting the single-copy gene *SRY*, which is less reliable because PCR data are less reproducible with very low template numbers *(24)*. Likewise, the CV for quantitative determinations from such low template reactions can be >100%. Accurate determination of 2-fold differences for an individual sample would be possible only with a high number of replicate reactions *(25)*. In this context it should be noted that the CVs from C_T values do not reflect the precision of the measurements as C_T values are an exponential function of the copy number. For example, 1% of the C_T at 36 cycles is 28% of the copy number. A CV of 1% based on the C_T thus gives the wrong impression of outstanding precision.

The determined LOQ agree with the assessment that reproducibility is reduced in samples with 10 or fewer copies *(26)* and with our opinion that for reliable quantification by real-time PCR, the CV of copy numbers should not exceed 25%. Consequently, previous study data express only trends for increases in cfDNA concentrations in pregnancy-related disorders.

We also showed that in absolute quantification, differences between experiments can be caused by the calibration curve. The use of calibration curves is widely accepted, but our simple example revealed a major problem when results from different experiments are compared. Results from consecutive experiments can have major differences caused solely by random variations of single reactions affecting the slopes of the calibration curves. Thus, if a new curve is used in every experiment, the comparability of the results is reduced, whereas the measurement of a single calibrator sample with a known concentration and the use of a fixed slope allow better interexperimental consistency. For this strategy, experiments must have equal amplification efficiencies. This can be ensured by comparing the C_Ts of the reference sample:

very similar C_Ts confirm that efficiencies are also similar. Furthermore, small nonrandom differences in efficiencies between experiments are accounted for by differences in the C_T of the reference sample. In our opinion, reference reactions (like samples) need to contain at least 50 target sequences for quantification, and variability in replicate amplifications should be minimal.

Finally, because absolute quantification by real-time PCR is always performed relative to a calibrator, results are only an approximation of the true values because DNA results obtained when densitometry *(27)* or Pico Green is used to quantify the calibrator are not precise enough to permit truly absolute quantification, regardless of the kind of material used for the calibrators (genomic DNA, plasmids, or PCR products). Consequently, unless a widely accepted standard is implemented, multiples of the mean will have to be used *(10)*.

In low-template reactions, several factors can lead to less efficient amplification and underestimation of the copy number *(26)*. Unspecific amplification of the high background of maternal DNA or of artifacts, for example from primer dimerization, can cause PCR competition and decreased efficiency or even amplification failure *(28)*.

Although our assay was not designed for determination of fetal sex, we showed in our blind analysis of 58 plasma extractions that false male interpretations can be excluded by selecting a cutoff at 1.0 GE/PCR. By this approach, the susceptibility of the assay to unspecific amplification was overcome, and the samples from pregnancies with a male fetus were clearly distinct from unspecific amplification. This approach offers an advantage over the single-copy targeting assay, in which the unspecific signals appear at the same point as the truly male samples and cannot be distinguished. The presented approach has a higher sensitivity; therefore, fetal sex determination is more robust and may be possible at even earlier stages of gestation than reported with single-copy locus-specific assays *(12)*.

In summary, thoroughly optimized and evaluated protocols for DNA extraction and quantification need to be established for the quantification of cfDNA. We present an approach that addresses the major hindrance in the generation of precise data: the scarce and short nature of the material to be quantified. With the measurement of Y-specific multicopy sequences by real-time qPCR, our assay had 10-fold better detection and quantification limits compared with a single-copy sequence-targeting protocol. This approach will be useful for the assessment of cfDNA as a possible marker of pregnancy-associated disorders and in the evaluation of sample preparation.

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