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Further Improvement in Quantifying Male Fetal DNA in Maternal Plasma

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BACKGROUND: Cell-free fetal DNA (cffDNA) in maternal plasma can be clinically useful for detecting prenatal disorders and pregnancy monitoring. More sensitive, specific, and quantitative detection of cffDNA in maternal plasma may expand the clinical utility of such measurements.

METHODS: We developed a quantitative real-time PCR (qPCR) assay [Y chromosome repetitive sequence (YRS) assay] based on a highly repetitive short sequence specific for the Y chromosome. Both standard qPCR and digital qPCR were performed to compare the sensitivity and specificity of this new assay against already established male DNA–specific assays.

RESULTS: The YRS assay was at least 10-fold more sensitive than the currently most sensitive DYS14 assay. The YRS assay was able to detect 0.5 genome equivalents (GE) per PCR reaction when fetal DNA was present at 0.2% of the total DNA. The background noise for the YRS assay was much lower than for the DYS14 assay in analyses of plasma samples from pregnancies with female fetuses.

CONCLUSIONS: The YRS assay is a substantial improvement for quantifying rare male fetal DNA in maternal plasma. The higher sensitivity and specificity may expand the clinical and research utility of cffDNA.

The detection of male-specific DNA in the plasma of pregnant women demonstrated the presence of cell-free fetal DNA (cffDNA)[3] in the maternal circulation *(1)*. The *SRY*[4] (sex-determining region Y) gene located on chromosome Y *(2)* was initially used in the quantitative real-time PCR (qPCR) design for detecting male fetus–specific DNA in maternal plasma. The amplicon length was reduced from 137 bp *(2)* to 78 bp *(3)* to improve sensitivity. Further improvement was made by designing a qPCR assay for the repetitive DYS14 sequence located in the *TSPY* (testis-specific protein, Y-linked) gene region (hereafter referred to as the DYS assay), which produced an approximately 10-fold increase in sensitivity *(3)*. Given the low relative and absolute concentrations of fetal DNA in maternal plasma, particularly early in gestation, a more sensitive assay with high specificity would be valuable.

Women with euploidy pregnancies who visited the KK Women's and Children's Hospital, Singapore, were recruited. Informed consent was obtained under the ethics approval obtained from the SingHealth CRIB Committee. We collected 10 mL of peripheral blood from each participant into tubes containing EDTA. The gestational ages of the pregnancies are listed in Table 1 and in Table 1 of the Data Supplement that accompanies the online version of this Brief Communication at http://www.clinchem.org/content/vol58/issue2. Details of the protocols for blood sample processing and DNA extraction are also provided in the online Data Supplement. DNA was extracted from 200 μL plasma prepared from samples obtained from women with first- and second-trimester pregnancies. For digital PCR, 1.6 mL of plasma from third-trimester pregnancies was used so that sufficient amounts of DNA were available for quantitative comparisons. DNA samples were eluted with 50 μL of DNase- and RNase-free water (Sigma-Aldrich) and stored at −80 °C.

Highly repetitive sequences within the Y chromosome were identified with the aid of an online tool [http://protegeno.uv.es/; *(4)*]. The most repetitive 14-nucleotide sequences were selected and mapped to the human genome hg19 assembly. For designing qPCR assays, the mapped genomic regions were extended further to 200 bp. PCR primers (YRS-F and YRS-R) and a TaqMan probe (YRS-P) were designed to specifically target these sequences located on the Y chromosome (see the online Data Supplement for the sequences of the primers and probe). The specificities of the primers and probe were verified by cloning and sequencing PCR products of samples from both males and females. The TaqMan probe does not recognize

[3] Nonstandard abbreviations: cffDNA, cell-free fetal DNA; qPCR, quantitative real-time PCR; DYS, repetitive DYS14 sequence located within the *TSPY* (testis-specific protein, Y-linked) gene region (assay); YRS, Y chromosome repetitive sequence (assay); GE, genome equivalents; C_q, quantification cycle of the PCR reaction.

[4] Human genes: *SRY*, sex-determining region Y; *TSPY*, testis-specific protein, Y-linked; *NQO1*, NAD(P)H dehydrogenase, quinone 1.

Table 1. Male-DNA quantification in the maternal plasma of pregnancies with female and male fetuses by the YRS, DYS, and *SRY* assays.

				Male DNA, GE/mL plasma				
Sample ID	Fetal sex	Gestation, weeks	Total DNA, GE/mL plasma	YRS assay	DYS assay	*SRY* assay	Fetal DNA by YRS assay, %	Fetal DNA by DYS assay, %
P10	F	13 5/7	577	0.416	1.03	0	0.0722	0.178
P11	F	9 1/7	161	0	0.962	0	0	0.599
P12	F	12 6/7	141	0	0.621	0	0	0.440
P13	F	12	130	0	0	0	0	0
P14	F	17 5/7	196	0.0183	0	0	0.00937	0
P15	F	17 5/7	104	0	0	0	0	0
P16	F	19 3/7	138	0	0.818	0	0	0.593
P17	F	16 6/7	135	0	0	0	0	0
P18	F	13	324	0.0297	0	0	0.00917	0
P19	F	12 6/7	481	0	0	0	0	0
P20	F	13	127	0	0	0	0	0
P21	M	18	132	15.9	9.27	11.1	12.0	7.03
P22	M	14 4/7	303	22.2	13.7	26.1	7.35	4.51
P23	M	19	136	11.9	29.5	0	8.76	21.7
P24	M	16 6/7	155	11.3	9.26	0	7.31	5.98
P25	M	16 4/7	204	13.1	12.5	0	6.44	6.15
P26	M	16 4/7	229	32.2	15.5	0	14.0	6.78
P27	M	15 4/7	360	13.3	17.7	0	3.69	4.92
P28	M	17 3/7	272	17.2	19.4	23.2	6.32	7.12
P29	M	13 1/7	109	10.5	13.6	0	9.66	12.5
P30	M	16 4/7	213	28.5	16.7	31.1	13.4	7.85

nonspecific PCR products from DNA samples from females. This male-specific qPCR assay is termed the "Y chromosome repetitive sequence" (YRS) assay.

The qPCR assays were performed in the ABI 7500 Real-Time PCR System (Applied Biosystems). All assays were 20-μL reactions. The details of the PCR reaction components, primers, probes, and thermocycling conditions are provided in the online Data Supplement. We amplified a male genomic DNA sample with the new YRS assay and 2 previously described assays, for DYS and for *SRY (3)*. Male genomic DNA was amplified in duplicate with 303, 30.3, 3.03, 1.01, and 0.34 genome equivalents (GE) per PCR reaction. The PCR conditions for the DYS and *SRY* assays were exactly as described previously *(3)*, except that the ABI 7500 Real-Time PCR System was used in the present study. The calibration curves (Fig. 1) for the 3 qPCR assays were: $y = -3.358x + 28.33$ C_q (YRS assay), $y = -3.402x + 31.65$ C_q (DYS assay), and $y = -3.393x + 35.70$ C_q (*SRY* assay), where y is C_q (the quantification cycle of the PCR reaction) and x is log(GE). PCR efficiency was calculated as: Efficiency $= 10^{-1/\text{Slope}} - 1$.

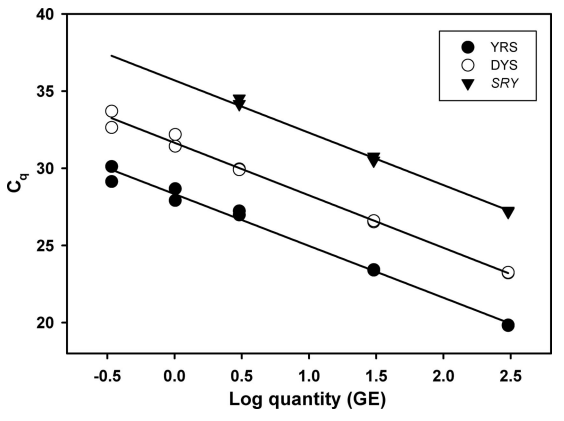

Fig. 1. Calibration curves for the YRS, DYS, and SRY assays.

The equations describing the YRS, DYS, and *SRY* calibration curves are, respectively: $y = -3.358x + 28.33$ C_q ($r^2 = 0.990$), $y = -3.402x + 31.65$ C_q ($r^2 = 0.993$), and $y = -3.393x + 35.70$ C_q ($r^2 = 0.997$). C_q corresponds to y in these equations; log(GE) corresponds to x.

The PCR efficiencies for the YRS, DYS, and *SRY* assays were 98.5%, 96.8%, and 97.1%, respectively. The PCR efficiencies for the DYS and *SRY* assays are nearly optimal and comparable to those of previously published data *(3)*. The intercept C_q at 1 GE for the YRS assay was 3.32 C_q and 7.37 C_q lower than the C_q values for the DYS and *SRY* assays, respectively. These results suggest that the YRS assay is more sensitive, because all 3 assays were close to optimal.

We next used digital PCR to quantitatively assess the copy number difference between the YRS and DYS assays for 4 third-trimester plasma samples from pregnancies carrying male fetuses. Digital PCR was performed with the BioMark System (Fluidigm). The concentration of each PCR component was the same as in the regular qPCR assays described above. As shown in Table 1 and Fig. 1 in the online Data Supplement, the YRS assay detected 10.0- to 15.9-fold higher copy numbers than the DYS assay, a result in line with a C_q difference of 3.32 between the YRS and DYS qPCR assays.

We further tested the specificity of the YRS assay by means of a regular qPCR. No false-positive result was detected for the YRS assay for female genomic DNA at concentrations of 2 ng per PCR reaction (or 303 GE per PCR reaction). We also performed digital PCR reactions for the YRS assay with 5 third-trimester plasma samples from pregnancies with female fetuses. As shown in Fig. 2 in the online Data Supplement, no false-positive result was detected for 3 of the samples. One of 765 reactions in the fourth sample was positive, and 2 of 765 reactions in the fifth sample were positive. To assess the specificity and sensitivity of the YRS assay simultaneously, we performed a serial dilution of male DNA at different absolute concentrations (2.5, 1, and 0.5 GE per PCR reaction) and relative concentrations (1%, 0.5%, and 0.2% of the total DNA). The YRS assay was able to quantify male DNA at a 0.2% relative concentration and an absolute copy number of 0.5 GE (see Fig. 3 in the online Data Supplement). The DYS assay was able to achieve the same sensitivity, although C_q values were consistently higher than for the YRS assay. Not surprisingly, the *SRY* assay did not achieve robust detection at these extremely low concentrations because this assay targets a single-copy genomic region.

We also performed a preliminary assessment of the YRS assay's clinical utility with 21 plasma samples from pregnant women (11 with female fetuses and 10 with male fetuses). The mean gestational ages were 14.4 weeks (range, 9 1/7 weeks to 19 3/7 weeks) and 16.4 weeks (range, 13 1/7 weeks to 19 weeks) for pregnancies with female and male fetuses, respectively (Table 1).

We quantified total cell-free DNA (including both maternal and fetal DNA) with a qPCR assay that targets *NQO1* (NAD(P)H dehydrogenase, quinone 1), a single-copy region on chromosome 16. The mean total cell-free DNA concentrations were 228 GE/mL plasma (range, 104–577 GE/mL plasma) and 211 GE/mL plasma (range, 109–360 GE/mL plasma) for pregnancies with female and male fetuses, respectively (Table 1). All samples were analyzed in duplicate with the *SRY*, DYS, and YRS assays. A sample was deemed male positive when both duplicates were positive.

For the 11 pregnancies with female fetuses, all of the results with the *SRY* assay were negative. The DYS assay was weakly positive for 4 of the samples (0.0124, 0.0164, 0.0192, and 0.0206 GE per PCR reaction, or 0.621, 0.818, 0.962, and 1.03 GE/mL plasma). The YRS assay was weakly positive for 3 of the samples (0.000367, 0.000595, and 0.00833 GE per PCR reaction, or 0.0183, 0.0297, and 0.416 GE/mL plasma). When relative fetal DNA percentages were calculated, the values for the 4 samples positive in the DYS assay were 0.178%, 0.440%, 0.593%, and 0.599%, and the 3 samples positive in the YRS assay were 0.00917%, 0.00937%, and 0.0722%. Weak false-positive signals for the DYS assay have previously been reported *(3)*. The YRS assay showed some even weaker false-positive signals. The highest apparent male-DNA signal from the 11 maternal plasma samples from pregnancies with female fetuses was 0.416 GE/mL plasma (or 0.0722% fetal DNA), compared with 10.5–32.2 GE/mL plasma (or 3.69%–14.0% fetal DNA) for 10 maternal plasma samples from pregnancies with male fetuses (Table 1; see Table 2 and Fig. 4 in the online Data Supplement).

For the 10 maternal plasma samples from pregnancies with male fetuses, only 4 samples were positive in the *SRY* assay, whereas all samples were positive in the DYS and YRS assays (Table 1).

The potential of cffDNA for noninvasive prenatal diagnosis and pregnancy monitoring remains to be fully realized. The detection and quantification of male-specific cffDNA, although applicable to only half of pregnancies, is technically the least challenging assay. Male-specific cffDNA-quantification assays can serve as initial proof-of-concept studies for evaluating potential correlations between cffDNA concentrations in maternal plasma and a variety of fetal and maternal conditions, such as X-linked disorders *(5, 6)*, trisomy 21 *(7, 8)*, preeclampsia *(9–11)*, and fetal growth restriction *(12, 13)*.

The YRS assay achieved a sensitivity at least 10-fold higher than the currently most sensitive DYS assay while substantially reducing the background noise. The YRS assay thus is likely to have greater utility for quantifying cffDNA at very low concentrations when the absolute copy numbers of *SRY* and DYS14 sequences are insufficient and fluctuate stochastically. The improvements produced with the YRS assay may facilitate

future research into the clinical applications of measuring cffDNA in maternal plasma.

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References

1. Lo YM, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW, Wainscoat JS. Presence of fetal DNA in maternal plasma and serum. Lancet 1997;350:485–7.
2. Lo YM, Tein MS, Lau TK, Haines CJ, Leung TN, Poon PM, et al. Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. Am J Hum Genet 1998;62:768–75.
3. Zimmermann B, El-Sheikhah A, Nicolaides K, Holzgreve W, Hahn S. Optimized real-time quantitative PCR measurement of male fetal DNA in maternal plasma. Clin Chem 2005;51:1598–604.
4. Arnau V, Gallach M, Marin I. Fast comparison of DNA sequences by oligonucleotide profiling. BMC Res Notes 2008;1:5.
5. Rijnders RJ, van der Schoot CE, Bossers B, de Vroede MA, Christiaens GC. Fetal sex determination from maternal plasma in pregnancies at risk for congenital adrenal hyperplasia. Obstet Gynecol 2001;98:374–8.
6. Costa JM, Benachi A, Gautier E. New strategy for prenatal diagnosis of X-linked disorders. N Engl J Med 2002;346:1502.
7. Lo YM, Lau TK, Zhang J, Leung TN, Chang AM, Hjelm NM, et al. Increased fetal DNA concentrations in the plasma of pregnant women carrying fetuses with trisomy 21. Clin Chem 1999;45:1747–51.
8. Zhong XY, Burk MR, Troeger C, Jackson LR, Holzgreve W, Hahn S. Fetal DNA in maternal plasma is elevated in pregnancies with aneuploid fetuses. Prenat Diagn 2000;20:795–8.
9. Lo YM, Leung TN, Tein MS, Sargent IL, Zhang J, Lau TK, et al. Quantitative abnormalities of fetal DNA in maternal serum in preeclampsia. Clin Chem 1999;45:184–8.
10. Hahn S, Rusterholz C, Hosli I, Lapaire O. Cell-free nucleic acids as potential markers for preeclampsia. Placenta 2011;32(Suppl):S17–20.
11. Sifakis S, Zaravinos A, Maiz N, Spandidos DA, Nicolaides KH. First-trimester maternal plasma cell-free fetal DNA and preeclampsia. Am J Obstet Gynecol 2009;201:472.e1–7.
12. Sekizawa A, Jimbo M, Saito H, Iwasaki M, Matsuoka R, Okai T, Farina A. Cell-free fetal DNA in the plasma of pregnant women with severe fetal growth restriction. Am J Obstet Gynecol 2003;188:480–4.
13. Alberry MS, Maddocks DG, Hadi MA, Metawi H, Hunt LP, Abdel-Fattah SA, et al. Quantification of cell free fetal DNA in maternal plasma in normal pregnancies and in pregnancies with placental dysfunction. Am J Obstet Gynecol 2009;200:98.e1–6.

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