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Original Research

Detection and Quantification of Male-Specific Fetal DNA in the Serum of Pregnant Cynomolgus Monkeys (*Macaca fascicularis*)

Lubna Yasmin,[1] Jun-ichiro Takano,[1] Yasushi Nagai,[2] Junko Otsuki, [1,2,3] and Tadashi Sankai[1,*]

Because of their developmental similarities to humans, nonhuman primates are often used as a model to study fetal development for potential clinical applications in humans. The detection of fetal DNA in maternal plasma or serum offers a source of fetal genetic material for prenatal diagnosis. However, no such data have been reported for cynomolgus monkeys (*Macaca fascicularis*), an important model in biomedical research. We have developed a specific, highly sensitive PCR system for detecting and quantifying male-specific fetal DNA in pregnant cynomolgus monkeys. We used multiplex quantitative real-time PCR to analyze cell-free DNA in maternal blood serum obtained from 46 pregnant monkeys at gestational weeks 5, 12, and 22. The presence of *SRY* gene and DYS14 Y chromosomal sequences was determined in 28 monkeys with male-bearing pregnancies. According to confirmation of fetal sex at birth, the probe and primers for detecting the Y chromosomal regions at each time point revealed 100% specificity of the PCR test and no false-positive or false-negative results. Increased levels of the *SRY*-specific sequences (mean, 4706 copies/mL serum DNA; range, 1731 to 12,625) and DYS14-specific sequences (mean, 54,814 copies/mL serum DNA; range, 4175–131,250 copies) were detected at week 22. The *SRY*- and DYS14-specific probes appear to be an effective combination of markers in a multiplex PCR system. To our knowledge, this report is the first to describe the detection of cell-free DNA in cynomolgus monkeys.

Abbreviation: C_t, threshold cycle.

Analysis of cell-free circulating nucleic acids in human maternal plasma or serum has led to the development of risk-free methods for prenatal genetic diagnosis and the assessment of several fetal and maternal conditions, for example, sex determination for paternally inherited diseases, pregnancy-associated complications, sex-linked disorders for ambiguous genitalia, and embryo tracking.[1,4,12,14,18,19] Technical challenges associated with detecting fetal DNA arise due to the low concentration of fetal DNA in maternal plasma during pregnancy and the difficulty of differentiating the genetic material of the fetus from that of the mother.[5,13,20] Fetal sex determination using sequences derived from the Y chromosome only is relatively simple and has a reported accuracy rate in humans of approximately 99.0% at 7 wk of gestation and 100% after 20 wk, depending on the protocol and methods used.[3,5,17,20] In other species, researchers have used real-time PCR assays during pregnancy to predict fetal sex from cell-free DNA at an accuracy of 100%.[9,10,11] Cell-free fetal DNA in the maternal circulation represents only 3% to 6% of the total free DNA obtained from plasma throughout pregnancy; however, this percentage is variable between pregnancies.[5,13,20]

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[1]Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Tsukuba, Ibaraki, Japan, [2]Department of Obstetrics and Gynecology, Nagai Clinic, Saitama, Japan, and [3]Hanabusa Women's Clinic, Kobe, Hyogo, Japan
[*]Corresponding author. Email: t-sankai@nibio.go.jp

In clinical biomedical research, it is essential to develop animal models for human diseases to reveal their mechanisms.[16,22] Continued progress in surgical intervention and molecular medicine suggests that it may soon be possible to develop potential treatments or even cures for several fetal genetic diseases at an early stage of pregnancy.[15] Fetal developmental research during early pregnancy might be facilitated by using cell-free fetal DNA in the maternal blood rather than other methods, such as serum screening and ultrasonography. Nonhuman primates, especially macaques, are useful model animals for studying fetal development because of the similarity of the reproductive characteristics, placental structure, and developmental events between these animals and humans.[9,10] These developmental similarities highlight the importance of the study of cell-free fetal DNA in nonhuman primates and its usefulness as a marker to obtain genetic information about the fetus.

In the current study, we investigated the presence of cell-free fetal DNA in the maternal plasma of cynomolgus monkeys by developing and using a standardized PCR system. To this end, we selected the *SRY* (sex-determining region Y) gene and DYS14 sequences of the cynomolgus monkey to use as sex-associated markers. The Y chromosome-specific sequences in the single-copy sex determination region of *SRY* and the multicopy (thus yielding increased sensitivity) sequences of DYS14 in the *TSPY* (testis-specific protein, Y-linked) gene have had wide clinical use in humans as molecular markers for detecting and quantifying

cell-free fetal DNA.[3,7] In addition, *TSPY* has been used in bovines for detecting cell-free fetal DNA[2] and in rhesus macaques for long-term evaluation of microchimerism.[8] Given the reports of fetal sex determination in rhesus macaques[9,10] and sheep[11] by analyzing Y chromosome-specific sequences from cell-free DNA, we hypothesized that we could predict the fetal sex of cynomolgus monkeys at different stages of gestation. This information has been extremely useful in optimizing the design of experimental studies in biomedical research and in managing a nonhuman primate breeding colony.[10] Because cynomolgus and rhesus macaques are closely related members of the same genus, the current experiments are similar to a previous study.[9]

We developed an efficient 2-color multiplex PCR system to detect and quantify fetal DNA in the maternal serum of cynomolgus monkeys during pregnancy. We used 2 loci on the Y chromosome in a single PCR test to minimize the likelihood of false-positive signals. Here we report the results of detection and analysis of fetal DNA at various weeks of gestation and evaluate our PCR system for its ability to determine fetal sex from pregnant monkeys' cell-free DNA.

Materials and Methods

Blood sampling and serum separation. The IACUC of the National Institute of Biomedical Innovation approved the protocol. This research adhered to the legal requirements of Primate Society of Japan's principles for the ethical treatment of primates. The study animals (*Macaca fascicularis*) were bred and maintained at Tsukuba Primate Research Center. Monkey-rearing rooms were rectangular, and individual cages were installed on the long sides of the room. Animals were housed individually on a 12:12-h light:dark cycle. Ambient temperature in the rooms was kept at approximately 25 °C, and humidity was set at 50% to 60%. Monkeys were provided with apples in the morning, commercial chow twice daily, and water ad libitum. We followed 3 groups of monkeys: the first group (n = 11) was tested at 5 wk of gestation, the second group (n = 15) at 12 wk, and the third group (n = 15) at 22 wk. We also followed a group of 5 monkeys that were monitored continuously and tested at all 3 stages of gestation. The sex of the fetus resulting from each of the pregnancies was recorded at birth. For blood sampling, each dam was anesthetized by using ketamine hydrochloride (dose, 10 to 15 mg/kg; Ketalar, Sankyo, Tokyo, Japan). Blood (2 to 5 mL) was collected by syringe from pregnant monkeys during weeks 5, 12, and 22 of gestation. For positive and negative controls, we collected blood from male and female monkeys to extract genomic DNA. Serum samples, obtained by centrifuging the blood at 1700 × g for 15 min, were either used immediately for DNA extraction or stored at –80 °C until needed.

DNA extraction and detecting the specificity of primers. DNA was extracted from 400 µL of serum using the MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche, Mannheim, Germany). To avoid contamination, we used fully automated nucleic acid extraction system according to the manufacturer's instructions. Total blood DNA was extracted from male and female samples for the positive and negative control by using a QIAamp DNA Blood Mini/Midi Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA from serum was eluted into a final volume of 50 µL and used as a PCR template.

We designed primers and probes for a 2-color multiplex *Taq*-Man PCR assay according to the parameters defined by Beacon Designer 7.9 (Premiere Biosoft International, Palo Alto, CA) was synthesized and purified by Greiner Bio-One (Tokyo, Japan), using *SRY* and DYS14 sequence data from rhesus monkeys (GenBank accession nos. AC217136 and AC240711; Table 1). To evaluate this assay, we constructed standard plasmids containing the *SRY* gene and DSY14 sequences; we used these plasmids as templates to assess the primer sets, which were designed to bind to the flanking regions of the sequences (Table 1). These primers amplified the expected 122- and 95-bp target products specific to *SRY* and DYS14, respectively. A homology search of human and macaque sequences in GenBank using the DYS14 primers and probe sequences that we designed revealed 85% sequence homology with the human *TSPY* gene (accession no., pJA923) and 100% homology with the sequences of the entire Y chromosome and *TSPY* gene (accession nos., AC240711 and AB001421, respectively) in *M. mulatta* and *M. fuscata*. Conventional PCR analysis using genomic male and female DNA as templates was carried out to determine the specificity of designed primers. The total reaction volume was 25 µL. PCR with ExTaq DNA polymerase (Takara Bio, Otsu, Japan) with initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 60 to 63 °C for 30 s, and 72 °C for 30 s for extension. A final elongation step at 72 °C for 5 min was performed before maintaining the reaction at 4 °C. The amplicons were verified by 1% agarose gel electrophoresis.

Construction of a standard curve and selection of PCR conditions. To determine the detection limits of the PCR assay from a standard curve, we obtained the recombinant *SRY* gene and Y chromosome-specific sequence DYS14 by using male genomic DNA as PCR template. Amplification of 1 kb of the *SRY* gene and DYS14 was performed by using conventional PCR. Purification of the amplified product was carried out by using the NucleoSpin Gel and PCR Clean-Up gel extraction kit (Takara Bio) according to the manufacturer's instructions. The purified product was ligated into the pGEMT vector (Promega, Madison, WI) at 16 °C overnight. The ligation mixture was added to *ECOS Escherichia coli* JM109 competent cells (NIPPON GENE, Toyama, Japan). After PCR analysis, positive clones were designated as the recombinant plasmids pGEMT/SRY and pGEMT/DYS14. Sequences were confirmed by using the 3130xl Genetic Analyzer (Applied Biosystems). Plasmid DNA was extracted from bacteria by using a plasmid midi kit (Qiagen, Valencia, CA). DNA from the plasmids was prepared at a concentration of 10^{10} copies/µL in 1× Tris–EDTA buffer (pH 8.0) followed by serial 10-fold dilutions from 10^5 to 10^0 copies per reaction, which were used to generate a standard curve that enabled copy number to be determined from the threshold cycle (C_t) value obtained. This curve permitted absolute quantification of the number of *SRY* and DYS14 copies in fetal cell-free DNA from the maternal blood sample through comparison with the number of copies of plasmid DNA detected in the same reaction by using C_t values. Primer pairs and reaction conditions were optimized by using control male genomic DNA and pGEMT/SRY and pGEMT/DYS14 plasmids as a template pool before amplification of the samples.

Detection of cell-free DNA by real-time PCR. For real-time PCR analysis of cell-free fetal DNA, reaction tubes at 5 wk of pregnancy contained 100 µL, including 40 µL DNA template, 1 µL of each 20 µM primer, 0.5 µL of each 20 µM probe for *SRY* and DYS14, 50 µL of IQ Super mix (Bio-Rad Laboratories, Hercules, CA), and 5 µL of Tris–EDTA buffer or distilled water. For assays done at 12 wk (reaction volume, 50 µL), each tube contained 20

Table 1. *SRY* gene and DYS14-specific primers and probes used in the real-time PCR assay and plasmid construction. The Y chromosome-specific sequence DYS14 was extracted from AC240711 *M. mulatta* BAC clone CH250-434B12 (GenBank). *SRY* gene primers and probe were extracted from AC217136 *M. mulatta* BAC clone CH250-228N17 (GenBank)

Name	Direction	Sequence (5′ to 3′)
FMSRY	Sense	TCC AGG AGG CAC AGA AAC TA
RMSRY	Antisense	AGA GGG ATC TGC CGG AAG
FMDYS14	Sense	GGA TGG AGT CTC TAC AGG AGG
RMDYS14	Antisense	CGC CAT TAT GTC ATC CGC TC
SRY	Probe	FAM–AGC ATC TTC GCC TTC CGA CGA GGT–BHQ1
DYS14	Probe	HEX–CCC AAA GCT ACC TGC TCG CTC TGC–BHQ1
FpSRY	Sense	TGG TTG GGC GGA GTT GAG AG
RpSRY	Antisense	GGCGGGATCACTTCTGGATG
FpDYS14	Sense	GCTCCTGGTGCCCTTTGGTC
RpDYS14	Antisense	TGTGAGGCTGACCGCACTGA

µL DNA template, 0.5 µL of each 20 µM primer, 0.25 µL of each 20 µM probe for *SRY* and DYS14, 25 µL IQ Super mix (Bio-Rad Laboratories), and 3.5 µL Tris–EDTA buffer or distilled water. At 22 wk of pregnancy, assay tubes each contained 25 µL total, comprising 2 µL DNA template, 0.25 µL of each 20 µM primer, 0.125 µL of each 20 µM probe for *SRY* and DYS14, 12.5 µL of IQ Super mix (Bio-Rad Laboratories), and 9.75 µL of Tris–EDTA buffer or distilled water.

Amplification parameters were: initial denaturation for enzyme activation at 95 °C for 3 min, followed by 55 cycles of denaturation at 95 °C for 10 s and annealing and extension at 63 °C for 30 s in an iQ5 real-time PCR detection system (Bio-Rad). Multiplex quantitative PCR was optimized with DNA from constructed plasmids, and genomic DNA from individual male and female monkeys was used for controls. Results are presented as the amount of DNA in genome-equivalent copies per mL of serum. The C_t values reflect the linear phase in the amplification curve used for quantification of the template input. All PCR tests were performed duplicate or triplicate. Samples were considered positive for *SRY* and DYS14 when results were confirmed by in least 2 tubes in duplicate or triplicate tests.

Statistical analysis. All parameters were calculated by using real-time PCR data from fetal DNA and the iQ5 optical system software (version 1.0.1384.0CR, BioRad). Results are expressed as means based on the mean C_t values. The coefficient of regression (r^2) was calculated from linearity data of the standard curve. The efficiency of each PCR primer–probe set was calculated from the standard curve by using the autoefficiency function of the iQ5 software. The Student *t* test was used to assess differences in *SRY* and DYS14 average copy number between weeks 5 and 12 and between weeks 12 and 22 of gestation. *P* values less than 0.05 were considered significant.

Results

Specificity and lower limit of detection of the PCR assay for the detection of *SRY* and DYS14. We performed conventional PCR analysis of genomic DNA from both male and female cynomolgus monkeys as a control and were successful in detecting male-specific bands. The specific primers yielded the expected 122-bp and 95-bp bands for *SRY* and DYS14, respectively, after gel electrophoresis (data not shown). Real-time PCR analysis using the selected primers and probes and control male and female genomic DNA led to effective amplification of male-specific *SRY* and DYS14 sequences only from male DNA. No amplification products were generated from genomic DNA isolated from female macaques; that is, none of the target-specific primers and probes amplified nontarget sequences (data not shown).

Standard curves were generated successfully from serially diluted pGEMT/*SRY* and pGEMT/DYS14 (Figure 1). We obtained good correlation (r^2 = 0.97 and r^2 = 0.99, respectively) between C_t values and initial DNA quantities in terms of fold change, with a range of 2×10^1 to 2×10^5 copies per reaction for a subset of *SRY* and DYS14 reactions. PCR amplification efficiencies for *SRY* and DYS14 were 104.6% and 98.6%, respectively. The male-specific gene and sequence were amplified from as few as 20 copies of pGEMT/*SRY* and pGEMT/DYS14 standard plasmid DNA per 25-µL reaction. The lower limit of detection in this 2-color multiplex assay was 20 copies per reaction, whereas little or no amplification resulted from 1 to 10 copies per reaction (Figure 1).

Detection of cell-free fetal DNA in maternal serum. For the detection of cell-free fetal DNA, DNA from the serum of 46 pregnant monkeys was tested by PCR at 5, 12, and 22 wk of gestation. PCR tests for the detection of *SRY* and DYS14 were performed in 11 pregnant monkeys at the fifth week of gestation, 15 at 12 wk, and 15 at 22 wk. We detected both the male-specific *SRY* and DYS14 sequences from cell-free DNA in 28 pregnant monkeys and thus predicted them to have male-bearing pregnancies (Table 2). We predicted that the rest of the pregnancies (n = 18) had female fetuses because there was no amplification of the target Y chromosome sequences from the maternal circulation. At all gestational stages, prediction of fetal sex from results of the real-time PCR assay was in 100% accordance with fetal sex determination at the time of delivery. In addition, 100% of the male-bearing pregnant monkeys had detectable levels of cell-free DNA specific for a male fetus.

Absolute quantification of fetal DNA in maternal serum. Quantification data for the copy number of the *SRY* gene and DYS14 sequences during pregnancy are summarized in Table 2. Isolation of DNA from 400 µL of serum consistently produced amplification of the target sequences at 5, 12, and 22 wk of pregnancy in all monkeys bearing a male fetus. The lowest amount of DNA detected in the extracted specimen was estimated to contain 1.0×10^1 DNA copies per 40 µL of the original sample. The average copy number of male fetal DNA with the *SRY* gene in the serum increased significantly at each subsequent pregnancy stage: 75.3,

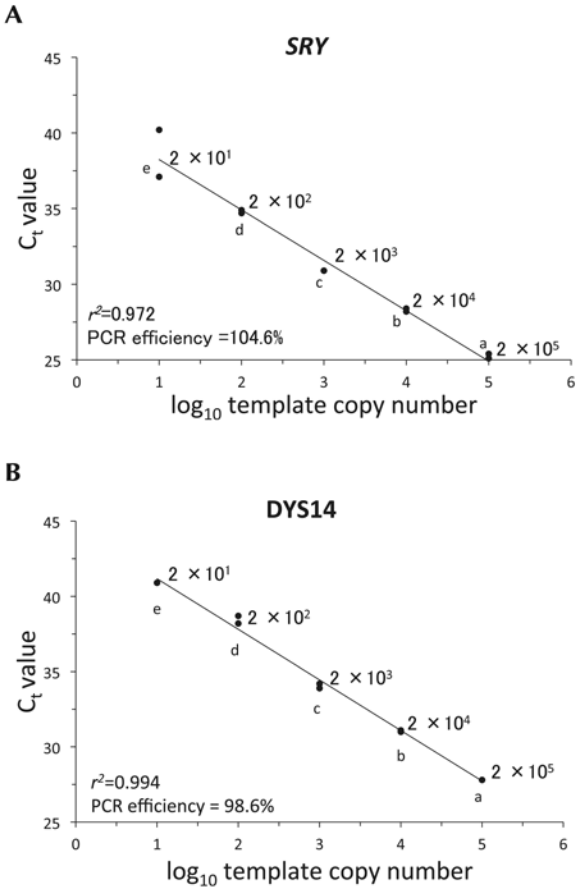

Figure 1. Standard curve and detection limits of copy number for quantification of the *SRY* gene and DYS14 sequences in the *Taq*Man multiplex PCR assay. Data represent the C_t values of 2 repetitions per test. The linear regression line was obtained by plotting the C_t values against the $\log_{10}$ of the initial quantity of the input plasmid DNA. C_t values for both markers were detected at the following template copies per reaction: 2×10^5 (a), 2×10^4 (b), 2×10^3 (c), 2×10^2 (d), and 2×10^1 (e). No C_t value was detected at 2×10^0 template copies per reaction. (A) *SRY* gene. (B) DYS14.

1370.6, and 4705.7 copies per milliliter serum at 5, 12, and 22 wk, respectively ($P < 0.001$ between 5 and 12 wk; $P < 0.05$ between 12 and 22 wk; Table 2). A higher average copy number of DYS14 was detected in the same serum sample as that for *SRY*: 1997.5, 22,718.5, and 54,814.3 copies per milliliter serum ($P < 0.005$ between 5 and 12 wk; no difference between 12 and 22 wk; Table 2).

Comparison of the mean copy numbers of *SRY* and DYS14 at different pregnancy stages in individual monkeys. The analysis for copy number variations of the *SRY* gene and DYS14 sequence were confirmed by the results in 3 of the 5 monkeys tested (nos. 1310107066, 1219302022, 1310006041, 1219408142, and 1219505076) throughout pregnancy (Figure 2) Maternal serum from 1310107066, 1219302022, and 1310006041 contained male-specific DNA detected by PCR, with male fetal sex confirmed at birth; the other 2 monkeys (1219408142 and 1219505076) both carried female fetuses (Table 2). Although interindividual variation in copy number was high for both *SRY* and DYS14 (Table 2 and Figure 2), *SRY* data differed significantly ($P < 0.001$) between the 3 male compared with 2 female offspring (Table 2 and Figure 2). Because of high interindividual variation, average DYS14 copy number in all randomly selected monkeys did not differ between 12 and 22 weeks of gestation, however this parameter did differ ($P < 0.001$) among the subset of monkeys that was monitored throughout pregnancy (Table 2 and Figure 2).

Discussion

The successful detection and quantification of *SRY* and DYS14 sequences in the maternal serum of pregnant cynomolgus monkeys revealed the presence of cell-free fetal DNA in sufficiently high quantities to support analysis. Therefore, the selected primers and probe combinations were effective in differentiating male from female fetal DNA and in detecting male-specific DNA in a laboratory setting.

Our results support the hypothesis that cell-free fetal DNA is reliable for predicting the sex of the fetus at 5, 12, and 22 wk of pregnancy in cynomolgus monkeys. The results obtained from the PCR test using DNA from maternal serum were 100% consistent with observations of the newborns after delivery. We evaluated 2 loci of the Y chromosome, and we were able to demonstrate 100% test specificity and 92% to 100% PCR amplification efficiency for fetal DNA in maternal serum and to determine fetal sex reliably at as early as the fifth week of gestation. Despite disagreement regarding the reliability and diagnostic accuracy of sex determined by fetal DNA in humans at an early stage of pregnancy, cell-free fetal DNA has been detected in nonhuman primates at 20 to 50 d of gestation.[9,10,17] Regardless, we should repeat PCR tests used to detect fetal DNA at an early stage of pregnancy. Using a larger volume of plasma or serum and a highly sensitive molecular technique like our real-time or digital PCR system might help to minimize difficulties in determining fetal DNA in early pregnancy.

Real-time PCR analysis is a highly sensitive method for quantifying genomic DNA. The male-specific *SRY* and DYS14 sequences have been used effectively to track prenatal DNA in human maternal serum samples by using PCR assays.[3,23] The presence of fetal DNA in maternal serum or plasma has been described previously in humans and other species.[7,9] In the current study, we quantified the male-specific genomic cell-free DNA from maternal serum and obtained results that are consistent with others' observations.[9,13] We found a higher copy number of *SRY* in cell-free DNA than did a previous study.[9] The reason might be the sampling stage, because the previous study used a range of gestational days; in contrast, we focused specifically on the gestational time points of weeks 5, 12, and 22. In addition, the DNA extraction method might play a role in the number of copies of cell-free DNA that can be detected. Similar to findings from other studies and by using *SRY* and DYS14 as markers, we confirmed significantly increasing numbers of copies of fetal DNA as pregnancy advanced, suggesting the increased release of placental trophoblast-derived apoptotic bodies to the maternal circulation as the fetus and placenta develop.[3,6]

The number of copies of the DYS14 sequences that we detected was higher than that of the *SRY* gene in the genome. This finding might be due to the presence of a multicopy DYS14 locus in the genome of monkeys, the number of which varies between subjects.[7] Interindividual variation in the copy number of DYS14 has not yet been determined in nonhuman primates, and further detail studies are necessary. The lack of information and incomplete sequence data for the Y-specific

Table 2. Confirmation and quantification by 2-color multiplex PCR analysis of fetal cell-free Y chromosome sequences (*SRY* and DYS14) in the serum of pregnant monkeys

		Absolute quantification (no. of copies/mL serum)		
Week of gestation	Animal ID	***SRY***	DYS14	Phenotype of newborn
5	1219105063	111	3656	male
	1219202035	31	1078	male
	1219302022[a]	48	859	male
	1219403039	ND	ND	female
	1219407112	ND	ND	female
	1219408142[a]	ND	ND	female
	1219505076[a]	ND	ND	female
	1219608115	170	1,416	male
	1219612175	ND	ND	female
	1310006041[a]	32	341	male
	1310010102	43	1231	male
	1310107066[a]	112	591	male
	1310209114	102	1063	male
	1310703030	100	3900	male
	1319709070	35	3087	male
	1319908059	44	4750	male
	Overall mean ± 1 SD	75.3 ± 45.9	1997.5 ± 1542.6	
12	1210006038	ND	ND	female
	1210010060	ND	ND	female
	1210012077	417	10,656	male
	1219302022[a]	834	11,813	male
	1219408142[a]	ND	ND	female
	1219505062	1343	23,125	male
	1219505076[a]	ND	ND	female
	1219706059	ND	ND	female
	1219809032	3,531	15,938	male
	1219909061	ND	ND	female
	1310005037	ND	ND	female
	1310006041[a]	1239	14,621	male
	1310107066[a]	472	15,938	male
	1310204058	ND	ND	female
	1310209116	ND	ND	female
	1310402034	917	40,313	male
	1310605063	1153	13,531	male
	1310605067	1084	11,875	male
	1310608109	ND	ND	female
	1319810050	2716	69,375	male
	Overall mean ± 1 SD	1370.6 ± 990.8[b]	22,718.5 ± 18,590.6[c]	
22	1219302022[a]	1731	69,375	male
	1219408142[a]	ND	ND	female
	1219505076[a]	ND	ND	female
	1310003012	ND	ND	female
	1310005037	ND	ND	female
	1310006041[a]	2394	37,530	male
	1310103027	ND	ND	female
	1310107066[a]	2288	131,250	male
	1310203041	12,625	68,750	male
	1310204058	ND	ND	female
	1310205070	3687	17,438	male

Table 2. Continued

Week of gestation	Animal ID	Absolute quantification (no. of copies/mL serum)		Phenotype of newborn
		SRY	DYS14	
	1310209115	ND	ND	female
	1310409112	ND	ND	female
	1310506075	10,875	32,250	male
	1310507077	3988	37,625	male
	1319706035	2993	20,375	male
	1319708060	3463	129,375	male
	1319802003	3013	4175	male
	1319907039	ND	ND	female
	1319911092	ND	ND	female
	Overall mean ± 1 SD	4705.7 ± 3796.3	54,814.3 ± 44,794.2	

ND, none detected

Data are expressed as the mean number of copies of 3 replicate reactions according to the mean C_t value.

[a]Pregnant macaque (n = 5) used for the detection and quantification of fetal cell-free Y chromosome sequences (*SRY* and DYS14) during subsequent fetal development.

[b]Value significantly different from that at 5 wk ($P < 0.001$) and 22 wk ($P < 0.05$).

[c]Value significantly ($P < 0.05$) different from that at 5 wk.

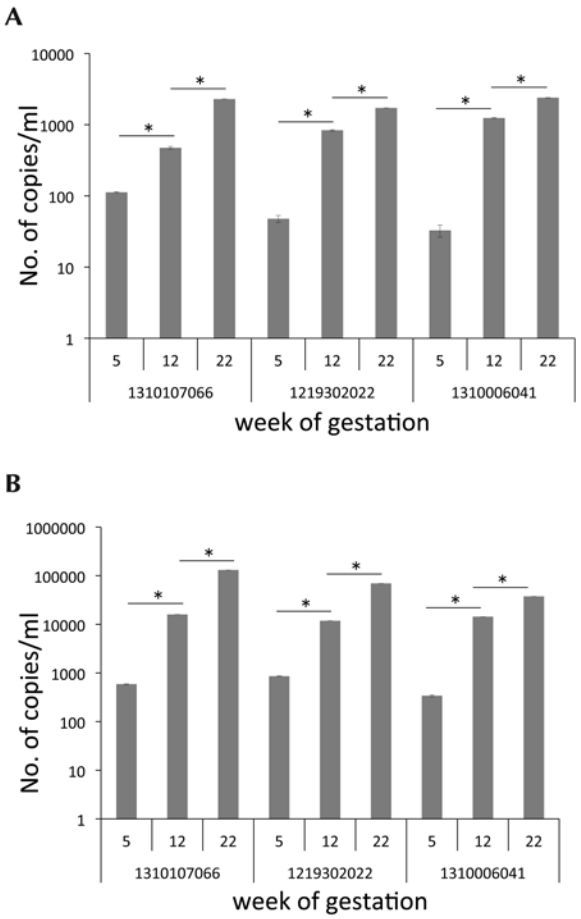

Figure 2. Comparison of the mean copy numbers of Y chromosome sequences (*SRY* and DYS14) in the maternal circulation of 5 pregnant monkeys continuously monitored during the 3 pregnancy stages. The real-time PCR quantification of copies of (A) *SRY* and (B) DYS14 sequences (mean from triplicate tests) were plotted against the time course of pregnancy. An increasing number of cell-free DNA copies in individual male-bearing pregnant monkeys (n = 3) were detected at 5, 12, and 22 wk of gestation. (A) *SRY* gene detection. (B) DYS14 detection. For both markers, the copy numbers in each animal were significantly different between weeks 5 and 12 and between weeks 12 and 22 (*, $P < 0.001$).

TSPY gene in nonhuman primates emphasize the difficulty in identifying an appropriate probe.[21] In humans, the copy number for DYS14 ranges from 50 to 200 copies per Y chromosome.[7] DYS14 reportedly is not an optimal marker for use in human quantification studies.[7]

We obtained no false-negative or false-positive results in the current study, highlighting the detection accuracy of this PCR method. By using specific probes, we were able to obtain a result in every case; indeed, targeting only a single gene in the Y chromosome might not provide an accurate result.[9] The fact that no false-negative results were found can be attributed to our use of a large quantity of DNA in the reaction. As another group has reported, contamination and false-negative results affect the reliability of the test.[17] A thorough understanding of the physiology of cell-free DNA in nonhuman primates, a large panel of biomarkers, and experiments related to fetal cell tracking could be helpful in many aspects related to the prenatal diagnosis and to increase the reliability of this test. Therefore, more studies need to be conducted regarding the availability of fetal DNA in both blood cells and plasma, and we have shown that nonhuman primates may be an important models to use. Although the application of this methodology has thus far been limited to sex determination in nonhuman primates, the recent advances toward genome editing research in nonhuman primate models of human genetic diseases may prompt investigators to adapt this method to monitor fetal genetic information at an early stage of pregnancy during the generation of strains with targeted gene modifications.

Our results confirm that our multiplex PCR technique, a noninvasive and effective method for detecting 2 target sequences from the *SRY* gene and DYS14 locus in a single reaction well by using cell-free fetal DNA in maternal blood, can be used successfully to determine fetal sex in cynomolgus monkeys. Furthermore, the results of the absolute quantification analysis demonstrated higher copy numbers of DYS14 compared with *SRY*, with a trend toward increased availability of fetal DNA in the maternal circulation during the later stages of pregnancy.

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