Non-human primates, especially macaques, are invaluable experimental animals in biomedical studies because of the close relationship between their biological nature and that of humans [11]. It is well known that their reproductive characteristics, placental structure and developmental events are very similar to those of humans [6]. This is why macaques are widely used in studies of fetal development, and the fetal influence of drugs/substances transferred through the placenta [9]. Prenatal determination of the fetal sex is required in these studies when sex differences in the fetal response to administered drugs or substances are anticipated. Detection of male fetal DNA (fDNA) in maternal blood has been developed as a non-invasive method for fetal sex determination [7] instead of amniocentesis. Since the amount of fDNA circulating in maternal blood is very low [8], a much more sensitive and accurate method is needed. Recently, real-time PCR with the TaqMan system was reported to detect male fDNA in the serum of pregnant macaques [5]. However, the TaqMan real-time PCR method requires a specific TaqMan probe and expensive real-time PCR apparatus.

In the present study, we established a nested PCR method to detect the fetal male DNA sequence of the sex determining region Y (SRY) gene in maternal plasma. This nested PCR method makes it possible to perform fetal sex determination not only with real-time PCR apparatus, but also with a regular PCR machine.

Animals: Pregnant rhesus macaques (Macaca mulatta, total number: 21, mean age: 10.8 ± 3.8 years) at the Primate Research Institute (PRI), Kyoto University, Japan were used in this study. The macaques were housed
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in individual cages or in group cages and were fed monkey chow supplemented with fresh fruits and vegetables. Experiments and animal husbandry were performed in accordance with the PRI’s institutional guide for the care and use of laboratory primates, which is based on the ‘Guide for the Care and Use of Laboratory Animals’ by the US National Research Council, 1996 [1]. All experimental procedures were approved by the institutional animal care and use committee.

Gestational days (GD) were estimated using the date of timed mating and were confirmed by changes in the plasma levels of estradiol and progesterone by ELISA (Neogen Corp., Lexington, KY, USA) in early gestation [3]. Fetal age was also confirmed by measuring the biparietal diameter with ultrasonography from 6 weeks of gestation until term [10].

**Blood sample collection and DNA extraction:** Peripheral blood (2 ml) was collected into heparin-containing syringes from pregnant rhesus macaques during gestation of 42 to 183 days. Plasma samples were obtained by centrifugation of the blood at 1,000 g for 15 min and stored at –80°C until use. DNA was extracted from 800 µl of plasma using a QIAmp DNA Blood Kit (Qiagen, Tokyo, Japan) according to the manufacturer’s instructions. DNA was eluted into a final volume of 40 µl and used as a PCR template.

**Nested PCR with real-time apparatus using SYBR Green:** Real-time PCR using SYBR Green as a product-reporting system was performed using an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA) under the conditions described previously [4]. In brief, PCR was carried out in a 10-µl reaction mixture containing 1 µl of DNA template, 5 µl of 2× SYBR Premix Ex Taq (Takara Bio Inc., Otsu, Japan), 0.2 µl of 50× ROX Reference Dye (Takara Bio Inc.), and 2 pmol each of forward and reverse primers. The thermal conditions were 95°C for 10 s and 30 cycles of (95°C for 5 s, 63°C for 30 s) with generation of a dissociation curve at the end of the assay to verify the amplification of the target gene. The standard curves from GAPDH amplicons of known concentration were used to calculate the quantity of target genes in samples [4]. Figure 1 shows the sequence of the rhesus SRY gene (Genbank accession# AF284310) and the binding sites of specific primer pairs (SRY-1 and SRY-3, forward and reverse, respectively). The outer primer pair (SRY-1-forward, SRY-1-reverse) in this study was designed to amplify the sequence of the SRY gene in the first PCR. The inner primer pair (SRY-3-forward, SRY-3-reverse), which was described previously [2], was used to amplify a 75-bp fragment inside the amplicon of the first PCR. The sequences of these primers were as follows:

**SRY-1-forward:**

5’-GATAAAGTGAAGCGACCCATGAAACGCATTCATTTGTGGTC

**SRY-1-reverse:**

5’-CATCTTCGCCTTCCGACCGGAG -3’

**SRY-3-forward:**

5’-CGATCAGGGCGCAAGATG -3’

**SRY-3-reverse:**

5’-TGTTATCCAGCTGTGTGGCT -3’

**GAPDH-forward:**

5’-CCATGGAGAAGGCTGGGG -3’

**Fig. 1. SRY gene sequence of Macaca mulatta.** Nt344 to 583 of the published SRY gene sequence (Genbank accession number AF284310) is shown. Sequences within boxes indicate the primer binding sites for the first PCR. Underlined sequences indicate the primer binding sites of the second PCR. The amplicon of the nested PCR is 75 bp.
NESTED SRY-PCR FOR FETAL SEX DETERMINATION

GAPDH-reverse:
5'-CAAAGTTGTCATGGATGACC -3'.

All PCRs were performed in triplicate for SRY sequences and in duplicate for the reference gene (GAPDH).

First PCR: The DNA samples extracted from plasma were used as the template in the first PCR, in which SRY DNA was amplified using the primer set of SRY-1. In the same PCR run, the number of copies of GAPDH fragments in each sample was assayed to estimate the DNA level in the template sample.

Second PCR: The first PCR plate was centrifuged briefly, and the PCR product of SRY-1 was collected. This first PCR product was diluted 1:100 and used as the template for the second PCR. The primer pair of SRY-3 was employed to amplify the inner sequence of the SRY-1 amplicon (Fig. 1).

Conventional nested PCR with regular PCR machine: Conventional nested PCR was performed under the same conditions as described above except that a regular PCR machine (Perkin-Elmer Inc., Waltham, MA, USA) was used. After the nested second PCR, the SRY-3 amplicon was subjected to polyacrylamide gel electrophoresis followed by staining with SYBR Green.

Sequence analysis of PCR product: PCR products were subjected to sequence analysis with an ABI Genetic Analyzer 310 (Applied Biosystems) to confirm their DNA sequences.

SRY DNA detection in pregnant macaques with male fetuses: After the first PCR using the SRY-1 primer set, all plasma DNA samples from pregnant macaques with male fetuses yielded an SRY DNA fragment by nested PCR using the SRY-3 primer set. The SRY-3 amplicon showed a symmetric dissociation curve with a peak of 81.4°C (Fig. 2A) and a single band of 75 bp in polyacrylamide gel electrophoresis (Fig. 2B). DNA sequence analysis of the nested PCR product revealed that the target sequence of SRY DNA was amplified using the nested PCR conditions (data not shown). However, plasma DNA samples from pregnant macaques with female fetuses yielded no SRY-3 amplicon by this nested PCR (Table 1). These findings indicate that the present nested PCR using primer sets SRY-1 for the first PCR

Fig. 2. Representative single PCR product obtained by nested PCR (SRY-3 primer set) of plasma DNA samples from pregnant macaques with male fetuses. A: Dissociation curves of SRY-3 fragment produced by real-time PCR. The temperature of its peak is 81.4°C. B: Polyacrylamide gel electrophoresis (PAGE) of GAPDH and SRY-3 amplicons. M: Marker (φX174/HaeIII). DNA samples from maternal plasma with male fetus (lanes 1 & 2) and with female fetus (lanes 3 & 4). PCR amplicon of GAPDH (lanes 1 & 3) and of SRY (lanes 2 & 4).
and SRY-3 for the second PCR is a reliable method for fetal sex determination.

Moreover, the PCR detection of circulating SRY DNA was possible at an early stage of gestation. The SRY-3 amplicon was detected from plasma samples obtained at early gestation of 42, 52, 64, or 65 days (Table 1).

**Limit of detection of SRY in plasma DNA samples:** Since the concentration of DNA extracted from plasma samples is too low to measure photometrically, we do not know the amount of DNA in the template sample for the 1st PCR. To avoid false-negative results due to insufficient DNA, we employed GAPDH as a guide DNA for the 1st PCR. As shown in Fig. 3, there appears to be a threshold in the copy numbers of the GAPDH amplicon, in which the SRY amplicon of the second PCR was detected or undetected. The mean copy numbers of the GAPDH amplicon were calculated to be 22,513 for positive and 838 for false-negative samples, respectively. From these data, the critical point appears to depend on the efficiency of DNA extraction from the maternal plasma samples, and the DNA level of the template should be in a range corresponding to no less than 20,000 copies of the GAPDH amplicon.

**Rapid clearance of SRY DNA from maternal plasma after delivery:** To examine the clearance of fetal SRY DNA circulating in maternal blood, blood samples were obtained at term, on the day of delivery and after delivery, and subjected to SRY DNA detection. Typical results regarding the time course of circulating SRY DNA detected in maternal blood are shown in Fig. 4. No SRY

### Table 1. Results of SRY-PCR and fetal sex

<table>
<thead>
<tr>
<th>Gestation day</th>
<th>SRY</th>
<th>Fetal sex</th>
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<tr>
<td>42</td>
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</tr>
<tr>
<td>161</td>
<td>–</td>
<td>female</td>
</tr>
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</table>

*Gestation day at blood sampling. **Detection of SRY by nested real-time PCR. +: positive, -: negative. **Phenotype of the newborn.
DNA was detected in maternal blood 6 or 24 h after delivery, indicating rapid clearance of the fetal SRY DNA from maternal circulation within 6 h after delivery.

**SRY DNA detection with regular PCR machine:** The present method enabled fetal sex determination by a nested PCR using a regular PCR machine. Among three annealing conditions, 63, 61, and 59°C, annealing at 63°C was effective for the first PCR, while there was no difference among the three annealing conditions for the second PCR (Fig. 5). The resulting nested PCR product gave a single band of 75 bp in PAGE, indicating that the specific SRY-3 amplicon was also amplified by this conventional nested PCR with a regular PCR machine.

Since Lo et al. reported the presence of fetal DNA (fDNA) in maternal plasma and serum [7], it has been used for sex determination and/or diagnosis of several fetal disorders in humans [2]. In non-human primates, fDNA in maternal plasma/serum is also an invaluable tool for determining fetal sex by non-invasive analysis coupled with PCR. Fetal sex determination is required for some biomedical studies using monkey fetuses, in which sex differences in the response to administered drug(s) or material(s) are anticipated. In a previous study, prenatal sex determination was performed to detect fetal male SRY DNA in macaque maternal serum/plasma by means of real-time PCR using the TaqMan system [5]. That method, however, needs a specific TaqMan probe and expensive apparatus to perform real-time PCR. Here we have described a method for SRY DNA detection in macaque maternal plasma by the nested PCR method, which can be performed by either real-time PCR using the SYBR Green system or conventional PCR with a regular PCR machine.

In the present study of 21 pregnant macaques, SRY DNA circulating in maternal blood was detected in all females pregnant with male fetuses, but not in any case of pregnancy with a female fetus. Moreover, PCR detection of the circulating SRY DNA was possible at an early stage (42 days of gestation). These results indicate that the current nested PCR method is highly reliable and sensitive for determining fetal sex even in the early stage of pregnancy. We employed this nested PCR method to determine fetal sex in studies of nanomaterial effects upon male or female fetus.

As the fDNA circulating in maternal blood has mostly been of concern to applied science researchers for various prenatal diagnoses, only a few studies have addressed the biological nature and tissue origin [12]. In our macaques, circulating fetal SRY DNA decreased to an undetectable level just 6 h after parturition, indicating rapid clearance from maternal blood. This observation may also indicate placental origin of the cell-free fDNA circulating in maternal plasma.
In conclusion, a nested PCR method was established for sensitive fetal sex determination in macaque monkeys. This method can be applied not only to real-time PCR using the SYBR Green system but also to conventional PCR with a regular PCR machine.

Acknowledgments

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