dsDNA was removed from the supernatant (lane 4), which was discarded. The bead-bound dsDNA was denatured in alkaline solution (0.1 M NaOH and 1 mM EDTA) at room temperature for 15 min, and the ssDNA without biotin groups was released in the alkaline solution (lane 5). After neutralization, the ssDNA solution was combined with five volumes of Buffer PB from the MiniElute™ PCR Purification Kit (Qiagen, Valencia, CA, USA), and passed through MiniElute columns. The ssDNA was then eluted from the columns with a small volume of 10 mM Tris-HCl, pH 7.4 (lane 6). The recovery yield was 85% for this 149-nucleotide ssDNA. The whole procedure could be easily performed in 1 h either manually or by an automation workstation (e.g., Biomek® 2000; Beckman Coulter, Fullerton, CA, USA) with a robotic liquid handler, vacuum and manifold, washing unit, and a magnetic plate.

Compared to the original protocols (1–3) or other standard methods involving gel purification and ethanol precipitation (4,5), this renovated protocol saves time and effort and is cost-effective (by a dramatic reduction in the use of expensive SA-coated superparamagnetic beads), although additional materials are used. The recovery yield of this protocol is a little lower than that (approximately 95%) of the original ones (1–3) because the binding affinity of silica membrane is weak for short DNA (approximately 75% for an 88-nucleotide ssDNA; data not shown) while alcohol precipitation can effectively recover short nucleic acids from solution. When short ssDNA (60–75 nucleotides) is to be prepared with high recovery yield, instead of using silica membrane, this protocol can be modified by using a molecular sieve column (Sephadex® G-25; Amersham Biosciences, Piscataway, NJ, USA) to desalt. However, the volume of prior alkaline and acid solutions must be reduced to increase the ssDNA concentration.

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Simplex PCR assay for sex determination in mice

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Some biomedical research procedures using laboratory mice, such as the preparation of sex-specific fetal cell cultures, require the sex of fetuses and newborn pups to be determined. Although neonate mice can be sexed anatomically on the basis of the anogenital distance (AGD), 48% of newborn pups are reported to be unclassifiable using this method (1). The existing molecular methods for sexing are PCR-based assays that combine two pairs of primers together in a multiplex reaction to amplify the Y-chromosome-specific gene *Sry* and an autosomal gene—either *Il3* (chromosome 11) or *Tshb* (chromosome 3)—that serves as an internal control of PCR amplification (2,3). Here we present a novel PCR assay that uses only one pair of primers in a single reaction tube to simultaneously amplify DNA fragments from both the X- and Y-chromosomes.

Using the Sequencher™ 4.1 software (Gene Codes Corporation, Ann Arbor, MI, USA), we aligned DNA sequences of the X-chromosome-specific gene *Jarid1c* (ENSMUST00000082177) and the Y-chromosome-specific gene *Jarid1d* (ENSMUST00000055032) that we had obtained from Ensembl Mouse Genome Release 25 (www.ensembl.org/mus_musculus), knowing these genes to be homologous (4). The alignment showed that Exons 9 and 10 of *Jarid1c* have a high degree of sequence similarity with Exons 9 and 10 of *Jarid1d* (90% and 76%, respectively), with the corresponding exons of both genes having the same length (120 and 159 bp, respectively). In contrast, the intron between the two

exons (Intron 9-10) is 114 bp long in *Jarid1c* but only 85 bp long in *Jarid1d*, a difference of 29 bp. Thus, the sequences of the two genes permitted a pair of primers to be designed that we predicted would simultaneously amplify DNA fragments of 331 bp from *Jarid1c* and 302 bp from *Jarid1d* (Figure 1). For use as template DNA to test the primers, genomic DNA was extracted from embryonic day 15 (E15) fetuses and 1-day-old pups of unknown sex, using previously described methods (2,5). To obtain positive control DNA samples of known sexual identity, genomic DNA was extracted from adult males and females identified by conventional visual sexing (6). We used mice of the 129S7/SvEvBrdBkl-*Hprt*$^{b\text{-}m2}$ inbred strain (B&K Universal Ltd, Hull, Yorkshire, UK), as it provides the genetic background for many null mutations generated by gene targeting (7).

Reactions having a total volume of 25 µL were carried out inside 0.2-mL 8-well tube strips (Becton Dickinson, Mississauga, ON, Canada), with each PCR consisting of 17.7 µL nuclease-free water (Ambion, Austin, TX, USA), 2.5 µL 10× reaction buffer [500 mM KCl, 100 mM Tris-HCl (pH 9.0 at 25ºC), 1% Triton® X-100], 1.6 mM $MgCl_2$, 0.2 mM each of dATP, dTTP, dCTP, and dGTP (Invitrogen Canada, Burlington, ON, Canada), 0.5 µM each primer (synthesized by MWG Biotech, High Point, NC, USA), 1.5 U *Taq* DNA polymerase (Promega, Madison, WI, USA), and 100 ng genomic DNA. Reactions were incubated at 94°C for 5 min, followed by 35 cycles of 94°C for 20 s, 54°C for 1 min, and 72°C for 40 s, followed by 72°C for 10 min in a Primus 96 Plus thermal cycler (MWG Biotech). Following thermal cycling, 15 µL from each PCR product were mixed with 5 µL 6× loading dye solution (Fermentas Life Sciences, Burlington, ON, Canada) before being loaded onto a 2% (w/v) agarose gel (Onbio, Richmond Hill, ON, Canada) next to 0.5 µg 100-bp DNA ladder (GeneRuler™; Fermentas Life Sciences) and electrophoresed in 1× TAE buffer (40 mM Tris-acetate, 2 mM EDTA) at 8 V/cm for 45 min.

After the gel had been stained with ethidium bromide (BDH, Toronto, ON, Canada), UV light transillumination revealed single bands of 331 bp generated from some DNA samples, which were presumptive females, and two bands of 302 and 331 bp generated from the remaining samples, which were presumptive males (Figure 2A). Consistent with the predicted action of the primers, each of the female controls had a single band of 331 bp (Figure 2A, lane 2), while each of the male controls had two bands of 302 and 331 bp (Figure 2A, lane 9), which matched those seen in the fetal (Figure 2A, lanes 3–5) and newborn (Figure 2A, lanes 6–8) samples. The sex of each mouse assayed using this technique was confirmed by both of the existing multiplex PCR assays (2,3) and, when possible, by conventional visual sexing at 3 weeks of age (6). To assess the effectiveness of the simplex PCR assay on other mouse strains, the primers were used on genomic DNA from male mice of the classical inbred strains 129P3/J, BALB/cByJ, CBA/CaJ, DBA/2J, and FVB/NJ, the *Mus musculus molossinus* strain JF1/Ms, and the *Mus spretus* strain SPRET/EiJ, which had been obtained from the Mouse DNA Resource of The Jackson Laboratory (Bar Harbor, ME, USA) (Figure 2B).

Despite the primer sequence mismatches with *Jarid1c*, the signal intensities among male samples of the 331-bp band representing the X-specific *Jarid1c* gene and the 302-bp band representing the Y-specific *Jarid1c* gene were approximately

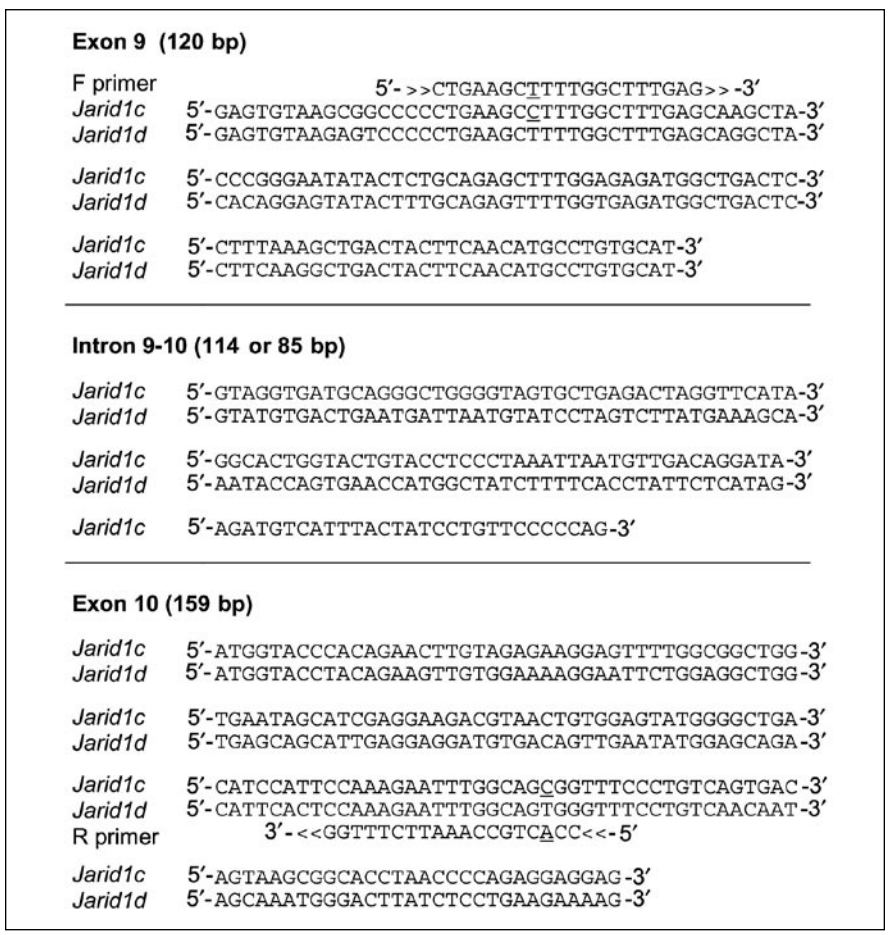

Figure 1. Alignment of PCR primers with exons 9 and 10 of *Jarid1c* and *Jarid1d*. The forward primer (5′-CTGAAGCTTTTGGCTTTGAG-3′) corresponds exactly with nucleotides 45, 210, 135 to 45, 210, 154 of the Y-chromosome and has a single nucleotide mismatch (underlined) with nucleotides 142, 135, 895 to 142, 135, 914 of the X-chromosome. The reverse primer (5′-CCACTGCCAAATTCTTTGG-3′) is the exact reverse complement of nucleotides 45, 210, 418 to 45, 210, 436 of the Y-chromosome and has a single nucleotide mismatch (underlined) with nucleotides 142, 136, 207 to 142, 136, 225 of the X-chromosome.

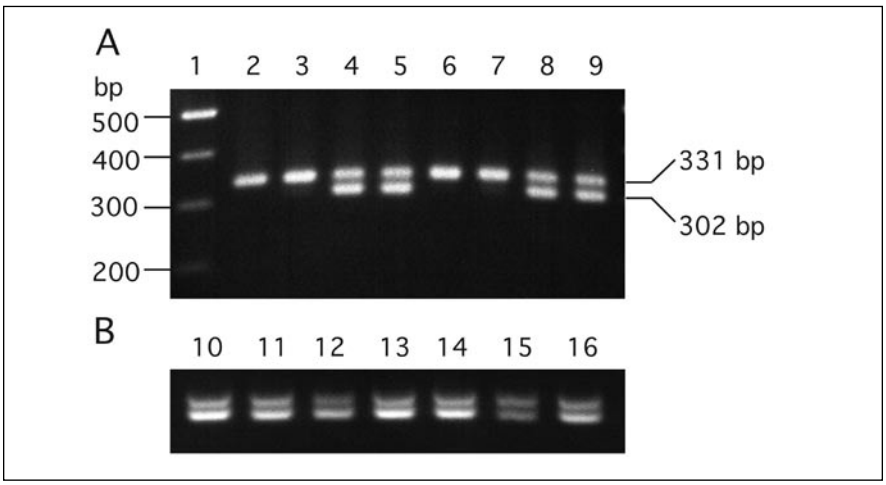

Figure 2. PCR products obtained by simultaneous amplification of X- and Y-chromosome-specific genes. (A) Lane 1, 100-bp DNA ladder (200-bp, 16.5 ng; 300-bp, 24.5 ng; 400-bp, 33.0 ng; 500-bp, 82.0 ng); lane 2, female adult positive control; lane 3, female E15 fetus; lanes 4 and 5, male E15 fetus; lanes 6 and 7, female 1-day-old pup; lane 8, male 1-day-old pup; lane 9, male adult positive control. All mice are 129S7/SvEvBrdBkl-*Hprt*$^{b\text{-}m2}$. The sizes of the DNA ladder fragments and PCR products are indicated on the sides of the figure. (B) Lane 10, male 129P3/J; lane 11, male BALB/cByJ; lane 12, male CBA/CaJ; lane 13, male DBA/2J; lane 14, male FVB/NJ; lane 15, male JF1/Ms (*Mus musculus molossinus*); lane 16, male SPRET/EiJ (*Mus spretus*).

equal. In addition, there was little difference between male and female samples in signal intensity of the 331-bp band, even though males have one fewer copy of the *Jarid1c* gene than females.

Although other methods for sex determination in murine fetuses and pups have been described (2,3), such multiplex reactions have the disadvantage, in comparison with simplex reactions, that their efficiency is heavily dependent on factors like the relative concentration of the primers, the concentration of the PCR buffer, the balance between the $MgCl_2$ and deoxyribonucleotide triphosphate concentrations, and the cycling conditions (8). The simplex PCR assay presented here avoids such potential problems and permits a saving of PCR costs and preparation time. Although single cells from mouse preimplantation embryos were not included in the present study, the assay also has potential utility in embryo sexing, the current method for which uses six pairs of primers in a multiplex nested PCR (9). In conclusion, the PCR assay presented here is a rapid, simple, and accurate method to distinguish fetuses and newborn pups bearing two X-chromosomes from those bearing one X- and one Y-chromosome. We are presently using the assay to monitor sex-specific effects of gene mutations in utero.

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The authors declare no competing interests.

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