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# Determination of sex in cDNA samples from mice

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## ABSTRACT

The need to take sex into account in biomedical research is now recognized and mandated by funding institutions. In laboratory rodents, such as mice, sexing is usually performed anatomically or by genotyping using multiplex or simplex PCR techniques on genomic DNA. Here we present a simple RT-PCR-based method targeting *Kdm5c* and *Kdm5d* to determine genetic sex in mouse cDNA samples, allowing for retrospective sex determination.

## METHOD SUMMARY

A single-step PCR technique targeting *Kdm5c* (X-chromosome-specific) and *Kdm5d* (Y-chromosome-specific) genes was established to determine the chromosomal sex in cDNA samples from mouse tissues.

## KEYWORDS:

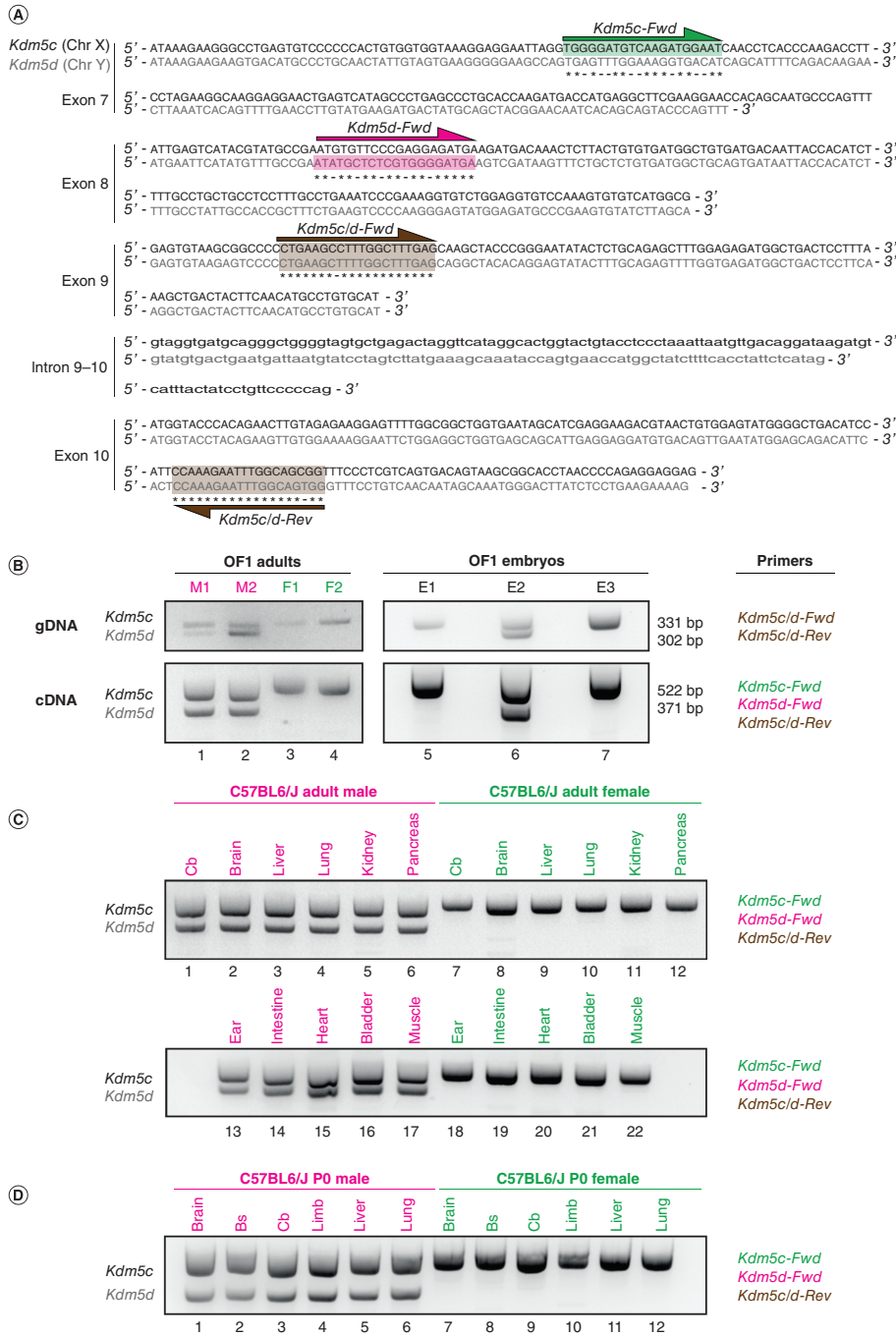
brain • cDNA • development • *Kdm5c* • *Kdm5d* • mouse • sexing • simplex PCR

The need to take sex into account in biomedical research is now recognized and mandated by funding institutions [1]. It is essential for better reproducibility and interpretation of results, accurate conclusions and addressing scientific questions for the benefit of all humankind. Determination of sex in adult rodents is classically performed anatomically by measuring anogenital distance to distinguish between male and female mice, a shorter distance being visible/attributed for females [2]. While this technique has proven to be highly accurate in adult mice, it is not as efficient in fetuses and neonates [3,4]. In young mice, many alternative observations can be used to determine the sex, such as the presence of teats (females, starting between 8 and 10 days of life) [3,4], the externalization of testes (males, starting between 19 and 23 days) [4] or the presence of perineal pigmentation in darkly colored male mice (absent in darkly colored female mice) [3]. However, determination of sex via anatomical observations in fetuses and neonates requires a good conservation of anatomical features and is prone to errors of observation. An alternative is the use of genomic DNA (gDNA) and PCR genotyping. Multiplex PCR has been used to amplify the male sex-determining factor gene *Sry* [5] together with an autosomal gene as an internal control for the PCR [6,7]. Simplex PCR reactions have also been reported targeting either the pseudoautosomal genes *Sry/Xlr* [8], *Rbm31x/Rbm31y* [9], or the *Kdm5c* and *Kdm5d* genes ([10]; formerly known as *Jarid1c* and *Jarid1d*, respectively). However, gDNA might not always be accessible, either because only RNA extracts have been collected or because sexing was not planned initially. To overcome these issues and allow retrospective sexing of samples, we developed a PCR amplification assay on cDNA with a triad of primers in a single reaction. This assay simultaneously amplifies sequences from the X- and Y-specific *Kdm5c* and *Kdm5d* genes coding for histone lysine demethylases [11,12] using a common primer and thus is more reliable than multiplex amplification of *Xist* with another gene. Both *Kdm5c* and *Kdm5d* are broadly expressed in most tissues (with the exception of spleen for *Kdm5d*) and as early as E2–4 in mouse embryos (Gene Expression Database, Mouse Genome Informatics [13]).

Exons 9 and 10 of the X-chromosome-specific gene *Kdm5c* (ENSMUST00000082177) and the Y-chromosome-specific gene *Kdm5d* (ENSMUST00000055032) are highly similar (90 and 76%, respectively [14]), but the intron separating these two exons is 29 bp shorter in *Kdm5d*. This difference was used by Clapcote *et al.* to design a PCR allowing determination of sex using gDNA (primers *Kdm5c/d-Fwd* and *Kdm5c/d-Rev*; Figure 1A & Table 1) [10]. To design a PCR that would enable determination of sex on cDNA samples, we analyzed the similarities and differences in the coding sequences of *Kdm5c* and *Kdm5d*. Using the Ensembl Mouse Genome Release 39 [15], we retrieved the sequences of exons 7–10 of both genes. Using the Primer3Plus website [16], we designed a PCR consisting of two forward primers – one specific for *Kdm5c* (in exon 7) and the other for *Kdm5d* (in exon 8) – and the common reverse primer for *Kdm5c* and *Kdm5d* used by Clapcote *et al.* (Figure 1A & Table 1) [10]. This amplification reaction was expected to result in the synthesis of two PCR products of different sizes: 522 bp for *Kdm5c* and 371 bp for *Kdm5d* cDNA, corresponding to products from X and Y chromosomes, respectively (Figure 1A).

In order to test our protocol, we compared results obtained using PCR genotyping by the Clapcote *et al.* protocol [10] on gDNA samples with the results obtained using our protocol on cDNA samples. For this, we obtained gDNA and total RNA samples from adult male

# Benchmark



**Figure 1.** *Kdm5c* and *Kdm5d* can be used for mouse sexing using cDNA. (A) Alignment of sequences of *Kdm5c* and *Kdm5d* spanning exons 7–10 and intron 9/10. Primers are highlighted on the sequences: *Kdm5c-Fwd* (green) and *Kdm5d-Fwd* (pink) are specific to *Kdm5c* (X chromosome) and *Kdm5d* (Y chromosome), respectively; while *Kdm5c/d-Fwd* and *Kdm5d-Rev* (brown) bind to both *Kdm5c* and *Kdm5d* [10]. Conservation between *Kdm5c* and *Kdm5d* nucleotide sequences at the positions of the primers are highlighted, \*: marks identical base pairs. (B) PCR products obtained by simultaneous amplification of *Kdm5c* and *Kdm5d* sequences from gDNA samples using the previously published protocol [10] or from cDNA using our new protocol. M1–2 (lanes 1–2) and F1–2 (lanes 3–4) are male and female OF1 mice, respectively ( $n = 4$ , two female and two male). E1–E3 (lanes 5–7) are E15–E15.5 embryos from OF1 strain ( $n = 7$ ). For gDNA PCR, two bands at 331 and 302 bp are detected in samples from male mice, while only the 331-bp band is detected in female samples. For cDNA PCR, two bands at 522 and 371 bp are detected in samples from male mice, while only the 522-bp band is detected in female samples. (C & D) PCR products from cDNA obtained by simultaneous amplification of *Kdm5c* and *Kdm5d* in samples from different organs from C57BL6/J male (pink) and female (green) (C) adult and (D) P0 ( $n = 8$ ) mice: (C) cerebellum (cb), brain, liver, lung, kidney and adrenal glands (kidney), pancreas, ear, small intestine (intestine), heart, bladder and muscle; (D) cerebellum (cb), whole cerebrum (brain), brain stem (bs), whole liver (liver), lungs (lung) and the whole left front limb (limb). In all the organs tested, two bands at 522 and 371 bp are observed in samples from male mice (C: lanes 1–6, 13–17; D: lanes 1–6), while only the 522-bp band is observed in samples from female mice (C: lanes 7–12, 18–22; D: lanes 7–12).

Primer	Sequence	Ref.
Kdm5c/d-Fwd	5'-CTGAAGCTTTTGGCTTTGAG-3'	[10]
Kdm5c/d-Rev	5'-CCACTGCCAAATCCTTTGG-3'	[10]
Kdm5c-Fwd	5'-TGGGGATGTCAAGATGGAAT-3'	
Kdm5d-Fwd	5'-ATATGCTCTCGTGGGGATGA-3'	

The Kdm5c/d-Rev primer is used either with the Kdm5c/d-Fwd primer for gDNA sexing [10], or with Kdm5c-Fwd and Kdm5d-Fwd for cDNA sexing.

	Disruption	Homogenization
<b>E15–E15.5</b>		
Full head	18G needle	26G
P0		
Limb	Stirrer	23G
Liver, lung	18G needle	23G
Brain, brain stem, cerebellum	18G needle	25G
<b>Adults</b>		
Bladder, ear, heart, quadriceps, small intestine	Stirrer	23G
Brain, cerebellum	18G needle	26G
Kidney,† liver,† lung, pancreas†	18G needle	23G

† Organs were loaded in the syringe first to ensure a better tissue disruption. Stirrer homogenization was made in a Potter-Elvehjem, PTFE Pestle glass tube and using an ES overhead stirrer from VELP Scientifica at 1300 r.p.m.

and female OF1 mice (Oncins France 1 strain, 9–13 weeks old; Charles River Laboratories, MA, USA) identified by conventional visual sexing [2]. All animal protocols were approved by the Comité Regional d'Éthique en Expérimentation Animale (no. 00057.01). Animals were housed in authorized facilities of the CIRB (#D75 05 12, Center for Interdisciplinary Research in Biology, Collège de France, Paris, France). Samples were not coded after collection and randomization not implemented for adult samples, as this study was a method development study rather than a hypothesis-testing study, and risks of bias were low. The number of animals tested using our assay allowed us to determine the quality of our sexing method across ages. gDNA was extracted from tail biopsies (1 mm length) taken from animals euthanized after isoflurane inhalation (adult) or cold anesthesia. Biopsies were digested in lysis buffer (Tris-HCl 50 mM, ethylenediaminetetraacetic acid pH 8 100 mM, SDS 0.5%) for 6 h. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, CA, USA) after disrupting and homogenizing the samples with needle and syringe (Table 2). Total RNA (50 ng) was reverse-transcribed using the SuperScript™ IV VIL0™ Master Mix (Thermo Fisher Scientific, MA, USA) in a total volume of 10 µl. PCR reactions were performed with the 5× FIREPol® Master Mix with 12.5 mM of MgCl<sub>2</sub> (Solis BioDyne, Tartu, Estonia) with the primers at 0.25 µM final concentration in a total volume of 20 or 10 µl for gDNA and cDNA extracts, respectively. 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 [10]. The PCR products were resolved by electrophoresis using a 3% or a 2% (w/v) agarose gel (for the gDNA and cDNA PCRs, respectively) and visualized after staining with GelRed® nucleic acid stain 10,000× (Sigma, MO, USA). The results obtained using samples from adult mice were consistent with the visual sexing for both PCR on gDNA and our new protocol using cDNA from cerebellum (Figure 1B). The same strategy was then used to perform determination of sex on samples from E15.5 OF1 embryos. The results obtained by cDNA PCR were again consistent with the gDNA PCR results (Figure 1B, upper panel) and revealed that the sexes of the embryos were: one male (two bands of 522 and 371 bp; Figure 1B, lower panel, lane 2) and two females (one band of 522 bp; Figure 1B, lower panel, lanes 1 and 3).

We then wondered whether our PCR protocol would allow sexing from cDNA samples taken from various organs and developmental stages. Therefore we obtained samples from various organs from C57BL6/J adult and P0 mice, both male and female, and from various regions of the brain (the cerebellum, left front lobe of the cerebrum, brainstem), liver, lung lobe, left kidney and adrenal gland, tail of the pancreas, left ear, small intestine, heart, bladder and left quadriceps muscle (Table 2). The PCR on cDNA samples revealed two bands at 522 and 371 bp in every organ tested from male mice (Figure 1C, lanes 1–6 and 13–17), while a single band at 522 bp was detected by PCR in all the organs tested from the female mice (Figure 1C, lanes 7–12 and 18–22) and for both adult and P0 samples. This demonstrates that the cDNA PCR can be used even with an amount of cDNA as low as 50 ng.

While methods exist for determination of sex in mice using gDNA [6,7], ours is a simple, accurate and rapid PCR method to differentiate between mice carrying two X chromosomes and those carrying one X and one Y, and allows retrospective determination of sex in cDNA

samples obtained from various organs and various developmental stages. This method allows sexing of samples even when gDNA or biopsies are no longer available.

## Author contributions

C Bosso-Lefèvre designed the study, performed the experiments and wrote the first draft of the article. B Urrieta-Chávez performed the experiments and revised the manuscript. F Selimi provided the resources and funding, and wrote and revised the manuscript.

## Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

## Ethical conduct of research

All animal protocols were approved by the Comité Regional d'Éthique en Expérimentation Animale (no. 00057.01), animals were provided by Charles River Laboratories (MA, USA) and housed in authorized facilities of the CIRB (#D75 05 12, Center for Interdisciplinary Research in Biology, Collège de France, Paris, France).

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