Benchmarks

Single-step PCR-based genetic sex determination of rat tissues and cells

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The advent of genome editing strategies has expanded the range of animal models available for gene manipulation and renewed research interest in the rat. Gender is a key variable for in vivo gene function analyses. Here, we present a simple PCR-based method to determine genetic sex in the rat.

Historically, the rat has been an important animal model for biomedical research in the field of reproductive physiology, including the dissection of mechanisms controlling hemochorial placentation (1). Over the past few decades, the absence of effective tools for manipulating the rat genome limited its utility for in vivo experimentation. However, recent advances in genome editing strategies have expanded the utility of under-utilized animal models for in vivo mechanistic analyses and have rekindled interest in the rat (2).

Recognition of gender-specific phenotypes has drawn the attention of researchers world-wide, and gender has emerged as an important variable in pregnancy research (3). Sexual dimorphism exists in fetal and placental development, adaptations to environmental challenges, and vulnerability to pregnancy-related disease states (4–9). Postnatally, the sex of a rat can be determined by visual inspection of the external genitalia. External landmarks (distance between the anus and the external genitalia) can be used to distinguish male versus female pups within

a few days after birth. There are also sex chromosome-specific behaviors in certain cell culture lines, including trophoblast stem (TS) cells (10), necessitating biochemical/molecular approaches for sex determination.

Traditionally, sex chromosome status has been biochemically assessed using PCR amplification of the Y chromosome-specific Sry gene (11,12), but this approach has inherent difficulties due to the absence of an internal control for the female genome. Several different single-step PCR-based methods have been established to identify the sex chromosome status of mice utilizing primer sets designed to amplify specific genes on the X and Y chromosomes, yielding distinct amplicons (13–15). However, no such protocols have been reported for genetic sex determination in rats.

To address this deficit, we developed and validated a single-step PCR method using three primers to identify the genetic sex of rat tissues and cells. Our method is a modification and improvement of the protocol described by Clapcote and Roder for mice (13).

The mouse procedure is based on the amplification of X chromosome (*Kdm5c*) and Y chromosome (*Kdm5d*) specific genes, which possess significant sequence identity (13). Two common primers are used to amplify mouse *Kdm5c* and *Kdm5d* from genomic DNA, generating amplicons differing by 29 bp. For the rat, we have identified a common reverse primer for the sex chromosome-specific genes (NC_005120.4 for *Kdm5c* and NC_024475.1 for *Kdm5d*) and two unique forward primers, each recognizing a distinct sequence in *Kdm5c* or *Kdm5d* (Figure 1). The PCR reaction generates 250 bp (Y chromosome-specific) and 692 bp (X chromosome-specific) amplicons, which can be easily resolved by agarose gel electrophoresis.

Initially, genomic DNA was isolated from rat cell lines (2×10^6 cells to 5×10^7 cells per cell line), a mixture of rat fetal tissues at gestation day (gd) 14.5 and gd 18.5, and tail-tip biopsies from adult outbred and inbred rats. The rat cell lines used in the analyses included PC-12 [CRL-1721; American Tissue Culture Collection (ATCC), Manassas, VA], MMQ (CRL-10609; ATCC), NR8383 (CRL-2192; ATCC), R2C (CCL-97; ATCC), Rcho-1 (16), NB2 (17), RNK16 (18), and TS cells (19). DNA was isolated according to the instructions provided in the Extract-N-Amp tissue PCR kit (XNAT2; Sigma-Aldrich, St. Louis, MO). Each primer (Figure 1) was diluted to 10 μ M using nuclease-free water. The primer cocktail was prepared by mixing equal volumes of each of the three primers. A PCR reaction volume of 20 μ l was prepared in 200- μ l 8-well tube strips (T-3035–1; BioExpress, Kaysville, UT). Each PCR reaction contained 15.35 μ l nuclease-free water (10977–015; Invitrogen; San Diego, CA), 2 μ l 10 $\times$ DreamTaq green buffer (K1081; Thermo Fisher Scientific, Pittsburgh, PA), 0.4 μ l each of 10 nM dNTP (R0192; Thermo Fisher Scientific), 0.25 μ l DreamTaq DNA polymerase (EP0711; Thermo Fisher Scientific), 1 μ l primer cocktail, and 1 μ l genomic DNA template. Amplification was performed

METHOD SUMMARY

A single-step PCR technique was established to determine the chromosomal sex of rat tissues and cells. Sex determination utilized detection of an X-chromosome-specific gene (*Kdm5c*) and an Y-chromosome-specific gene (*Kdm5d*).

Figure 1. Locations of primers for genes *Kdm5c* (X chromosome, NC_005120.4) and *Kdm5d* (Y chromosome, NC_024475.1) used in rat sex determination. The 19-nucleotide reverse primer (R) for *Kdm5c* has a single-nucleotide mismatch (underlined) for *Kdm5d* at nucleotide 532649 of the Y chromosome. Unique forward primers F1 and F2 correspond to *Kdm5c* on the X chromosome and *Kdm5d* on the Y chromosome, respectively.

with an initial denaturation step at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 42 s, with a final extension step of 7 min at 72°C using an Applied Biosystems thermocycler (Model No. 2720; Foster City, CA). PCR products (10 µl each) were resolved by electrophoresis at 110 V for 20 min in 1% agarose gels containing 0.02% ethidium bromide. UV-light imaging was performed in a Gel Doc XR+ instrument (Bio-Rad, Hercules, CA). Representative images from sex determination tests are shown in Figure 2. X chromosome-specific and Y chromosome-specific amplicons could be easily resolved by electrophoresis. Our results demonstrate that this PCR-based procedure for determining sex chromosome status in rat tissues and cells is simple and highly reproducible.

Author contributions

P.D. and M.J.S. designed the research, analyzed data, and wrote the paper. P.D. performed the research.

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Competing interests

The authors declare no competing interests.

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Figure 2. Distinct PCR products corresponding to X and Y chromosome-specific genes. (A) L: Thermo Fisher Scientific GeneRuler 100-bp Plus DNA Ladder; Lane 1: male trophoblast stem (TS) cells; Lane 2: female TS cells; Lane 3: gestation day (gd) 14.5 male fetus; Lane 4: gd 14.5 female fetus; Lane 5: gd 18.5 male fetus; Lane 6: gd 18.5 female fetus; Lane 7: adult male Holtzman Sprague Dawley (HSD) rat; Lane 8: adult female HSD rat; Lane 9: adult male Brown Norway (BN) rat; Lane 10: adult female BN rat. (B) L: GeneRuler 100-bp Plus DNA Ladder; Lane 1: PC-12 (adrenal medulla) cell line; Lane 2: MMQ (pituitary) cell line; Lane 3: Rcho-1 (trophoblast) cell line; Lane 4: NR8383 (lung macrophage) cell line; Lane 5: NB2 (T cell) cell line; Lane 6: RNK16 (natural killer cell) cell line; Lane 7: R2C (Leydig cell) cell line; Lane 8: adult female HSD rat; Lane 9: adult male HSD rat.

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