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Quick sex determination of mouse fetuses

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Abstract

We designed a rapid, simple and accurate PCR method to determine sexual identity of mouse fetuses collected on embryonic day 15. A multiplex PCR amplification was used to detect male-specific sequence (Sry) in DNA extracted from fetal livers through SDS denaturation followed by high salt extraction and precipitation. This extraction method resulted in sufficiently purified DNA in <1 h and was suitable for PCR. The DNA obtained was amplified using a robot thermal cycler for 33 cycles. The reaction was performed in 50 μl, using two sets of primers specific for Sry gene (chromosome Y) and IL3 gene (chromosome 11). Amplification duration was 1.5 h. The assessment of the results was done by electrophoresis in 3% agarose run at high voltage. The 402 bp band (Sry) obtained identifies the male fetuses and the 544 bp product (IL3) confirms the correct amplification of the template DNA. The entire procedure took <4 h. The specificity of the method was confirmed by fluorescent in situ hybridization using a specific male probe on cultured male and female neural stem cells. This method allowed the preparation and culture of pure male and female neural stem cells from fetal tissue.

Keywords: PCR; FISH; Sry; Neural stem cells; Sexual differentiation; DNA extraction; Rapid

1. Introduction

Several polymerase chain reaction (PCR) methods have been described for sexing mouse embryos (Han et al., 1993; Greenlee et al., 1998). These methods were optimized for sexing of single cells from murine embryos by nested PCR and require a full day of work before producing a result. When cultivating materials from fetuses, such as neural stem cells, individual fetuses need to be pooled. In order to prepare sex-specific cultures, it is necessary to develop a faster procedure for sexual determination.

Most sexing methods use DNA extracted by proteinase K digestion followed by organic extraction and DNA precipitation. This extraction procedure requires at least 3 h. A rapid DNA extraction method using high salt protein extraction followed by ethanol precipitation has been previously optimized (Lahiri and Schnabel, 1993; Hofstetter et al., 1997). This method allowed the preparation of purified DNA from blood samples suitable for molecular biology. By modifying their method, we were able to extract embryonic liver DNA in <1 h.

PCR detection of Y chromosome allows rapid and accurate identification of male DNA. Sex determining region protein gene (Sry) (Koopman et al., 1991; Gubbay et al., 1992) is encoded by Y chromosome and well conserved among species. However, PCR amplification is typically performed with thermal cyclers, and usually requires up to 2.5 h including heating and cooling periods. The amplification time required for PCR can be reduced by the use of a robot thermal cycler containing several heating blocks that can be programmed at different temperatures, therefore eliminating the heating and cooling periods.

We combined the use of high salt DNA extraction, a robot thermal cycler and a rapid electrophoresis to design a fast protocol for the amplification of sex-specific DNA. This new method allowed reliable sexing of fetuses in <4 h, and permitted sexing fetuses before

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processing tissues. In this report, we utilize this rapid sexing method to obtain EGF-dependent striatal neural precursor cells from exclusively female or male embryonic day 15 brains.

2. Material and methods

2.1. Source of fetuses

Mice (BALB/c males and females) were purchased from Taconic Farms (Germantown, NY). The animals were bred and the females were checked for vaginal plugs daily to establish day 1 of gestation. Pregnant females were group-housed (3–4 per cage). All animals received food and water ad libitum. On embryonic day 15, pregnant females were sacrificed by cervical dislocation and fetuses dissected and conserved on Petri dishes (Falcon, Becton-Dickinson, Franklin Lakes, NJ) filled with culture medium (see details in Section 2.5).

2.2. Modified rapid DNA extraction

The fetal liver was excised and ~ 1 mg was cut and transferred to a 1.5 ml microfuge tube containing 100 μl of TKM buffer (10 mM Tris pH 7.6, 10 mM KCl, 2 mM EDTA, 4 mM $MgCl_2$). The cells were separated and homogenized with a 200 μl pipette (Eppendorf, Brinkmann Instruments, Inc., Westbury, NY) using a filter-tip (Eppendorf); 400 μl of TKM buffer and 37.5 μl of SDS 10% (final concentration of SDS: 0.75%) were then added and vortexed. The tube was incubated at 55°C for 5 min to denature the proteins. Proteins were precipitated by adding 200 μl of saturated NaCl (~ 6 M), vortexing, and centrifugation for 5 min at $12\,000 \times g$. An aliquot of the supernatant (500 μl) was transferred into a fresh microfuge tube containing 900 μl of 100% ethanol to precipitate the DNA. The DNA was pelleted by centrifugation and washed once in 70% ethanol. Ethanol was removed by pipetting and the DNA pellet was dried for 3 min at room air, then dissolved in 100 μl TE (10 mM Tris HCl pH 8, 0.1 mM EDTA) for 10 min at 55°C. The samples were used immediately for PCR amplification without assessing DNA concentration. For quality control, some sample concentrations were measured using a spectrophotometer (Beckman Coulter Inc., Fullerton, CA).

2.3. Multiplex PCR amplification

In order to have an internal control of the efficiency of PCR amplification, we developed a multiplex PCR using two sets of primers amplifying the male-specific Sry gene (Koopman et al., 1991; Gubbay et al., 1992) and the autosomal IL3 gene (Miyatake et al., 1985) in the same reaction tube. This technique, using an optimized ratio of primer pairs, allowed the detection of Y-specific sequences in males, with internal control of PCR amplification for all samples.

Two microliters of dissolved DNA was used for multiplex PCR in a 50 μl reaction under mineral oil (Sigma, St. Louis, MO). Taq polymerase (0.025 U/μl, AmpliTaq®, Perkin Elmer, Norwalk, CT) diluted in manufacturer provided buffer were used with 0.2 mM each dNTP (Stratagene, La Jolla, CA), 0.2 μM male specific Sry primers (Mouse Sry locus, 8276–8295 5′-TGGGACTGGTGACAATTGTC-3′ and 8677–8658 5′-GAGTACAGGTGTGCAGCTCT-3′, synthesized by Life Technologies/Gibco-BRL, Rockville, MD) (Schiffmann et al., 1995), and 0.12 μM IL3 specific primers (mouse IL3 gene, 792–801 5′-GGGACTC-CAAGCTTCAATCA-3′ and 1335–1316 5′-TGGAG-GAGGAAGAAAAGCAA-3′, synthesized by Life Technologies/Gibco-BRL, Rockville, MD). A robot thermal cycler (RobotCycler®, Stratagene, La Jolla, CA) was used with the following program conditions: 95°C for 4.5 min followed by 33 cycles of 95°C for 35 s, 50°C for 1 min and 72°C for 1 min. PCR products were terminated with a final extension at 72°C for 5 min.

2.4. Fast electrophoresis

A 15-μl aliquot of each PCR product was loaded on a 3% agarose gel (Life Technologies/Gibco BRL, Rockville, MD) containing 0.2 μg/ml ethidium bromide (Sigma, St. Louis, MO). Electrophoresis was performed in $1 \times$ TAE (40 mM Tris–acetate, 1 mM EDTA) for 10–15 min at 11 V/cm with a fan overhead to prevent overheating, then imaged under UV transillumination with a CCD camera through a molecular imaging software (Bio-Rad, Hercules, CA).

2.5. Neural stem cells isolation and culture

During the sex determination of each embryo by PCR, the striatum was dissected (Reynolds et al., 1992), kept as whole tissue in Petri dishes (Falcon, Becton–Dickinson) filled with culture media (see below) and placed at 37°C in a humidified incubator with 5% CO_2/95% atmospheric air. Once the sex was determined by PCR, the striatum was mechanically dissociated with a fire-polished Pasteur pipette into a single cell suspension and pooled into separate male and female samples. The cells were then centrifuged at $350 \times g$ for 10 min and resuspended at a concentration of 75 000 viable cells/ml culture media with 20 ng/ml EGF (Collaborative Research, Bedford, MA) in a 75-cm^2 flask (Falcon, Becton–Dickinson). The cells were kept in a 37°C humidified incubator with 5% CO_2/95% atmospheric air in serum-free culture media: DMEM/F12 (1:1) including $NaHCO_3$ (28.6 mM), Hepes buffer (5

mM), and a mixture of insulin (23 μg/ml), transferrin (92 μg/ml), progesterone (20 nM), putrescine (55 μM), and selenium (27.5 nM) (Bottenstein and Sato, 1979; Reynolds et al., 1992). The cultures were fed every 48 h with 5 ml fresh medium and passaged into new 75-cm^2 flask at 75 000 viable cells/ml in fresh medium every 7 days.

2.6. Fluorescent in situ hybridization (FISH)

The accuracy of male/female identification by PCR was tested by FISH. Neural stem cells were kept in culture for two passages, harvested, washed in PBS, and fixed in Carnoy (anhydrous methanol 75%, glacial acetic acid 25%). FISH was performed on cells cytospined on glass slides as described previously (Nilsson et al., 1996). Shortly, the method consists of proteinase K digestion (0.2 μg/ml for 1.5 min at 37°), overnight incubation of slides with a digoxigenin-labeled Y specific probe (Weier et al., 1994) at 42° in formamide, washing (three times in 50% formamide then two times in 2 × SSC all at 45°), then detection of the probe by monoclonal antibody coupled to rhodamine. Slides were counterstained with DAPI to reveal the intact nucleus, then coverslipped and examined under fluorescent microscope (Axioplan2, Carl Zeiss, Thornwood, NY).

3. Results

3.1. DNA extraction

The DNA extraction using high salt protein precipitation gave good quality DNA with minimal RNA contamination and complete protein decontamination. In order to evaluate average DNA quality, 27 samples were analyzed by spectrophotometer. The ratio of UV absorbency at 260–280 nm averaged 2.00 ± 0.01 and the concentration of dissolved DNA averaged 339 ± 29 mg/ml. The range of DNA concentration was 157–769 mg/ml. Electrophoresis evaluation of the size of the genomic DNA obtained showed an average size over 12 kb.

3.2. Multiplex PCR amplification

PCR using a set of primers specific for the Sry male-specific primers pair generated a 402 bp length band in male derived DNA that was absent in female-derived DNA. Amplification of the IL3 gene produced a band of 544 bp length in both male and female derived DNA (Fig. 1). The ratio of IL3 to Sry primer to be used in this multiplex PCR was optimized to 0.6 in order to produce a Sry-specific band more intense than the IL3-specific band in the male samples. This allowed clear-cut differences between male and female DNA. Furthermore, variable amounts of template DNA from 100 to 800 ng were amplified and produced the expected bands.

PCR amplification of unidentified fetal DNA samples led to observable bands (Fig. 1). For some samples, two bands were observed: one 544 bp product representing the IL3 gene and one 402 bp band representing the Y-specific Sry gene. These are the presumptive male fetuses. In other samples, only the IL3-associated 544 bp band was observed. These are the presumptive female fetuses. The male control presented two bands matching those seen at 544 and 402 bp as seen in some of the fetal samples.

In order to verify the assumption that our primers were not amplifying human sequences, we prepared human DNA from peripheral blood leukocytes and used 400 ng for PCR amplification. Both male and female DNA showed absent specific signal at 402 and 544 bp positions with a few non-specific bands of low intensity over 1 kb of size (Fig. 2).

We analyzed 62 fetuses using this method and found an important variability of the male/female ratio among different litters. This is shown in Table 1: each

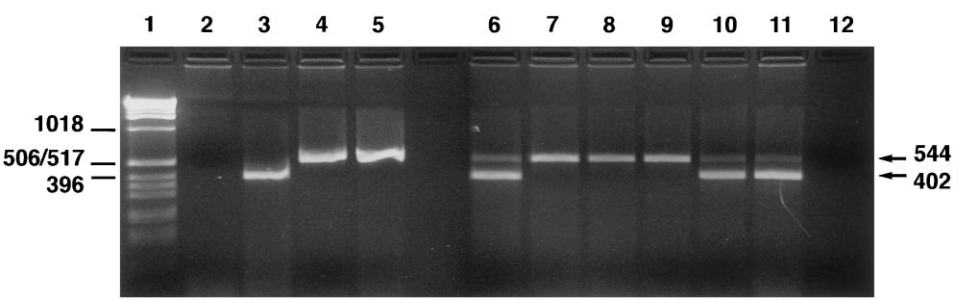

Fig. 1. PCR amplification of fetal liver DNA. Lane 1: 1 kb marker (DNA molecular weight marker X, 0.07–12.2 kb, Roche Molecular Biochemicals, Indianapolis, IN). Lanes 2–3: respectively, female and male DNA amplified with Sry male-specific primers. Lanes 4–5: respectively, female and male DNA amplified with IL3 primers. Lanes 6–12: fetal liver DNA amplified with both set of primers (Sry and IL3) by multiplex PCR; lane 11: male control; lane 12: reagent control (no DNA). The sizes of marker fragments and specific PCR products are indicated on the sides of the figure.

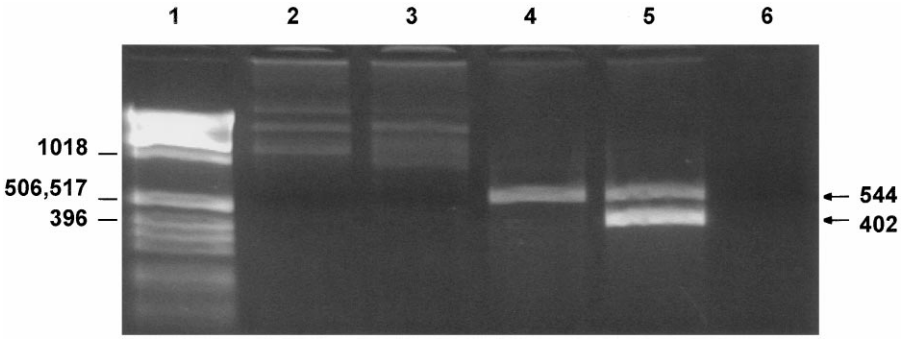

Fig. 2. PCR amplification of human DNA for contamination control using Sry and IL3 primers in a multiplex reaction. Lane 1: 1 kb marker (DNA molecular weight marker X, 0.07–12.2 kb, Roche Molecular Biochemicals, Indianapolis, IN). Lanes 2–3: respectively female and male human DNA. Lanes 4–5: respectively, female and male murine DNA. Lane 6: reagent control (no DNA).

litter is numbered A to J, the number of fetuses and the result of sexing by PCR is shown. Rarely the PCR amplification led to no amplification of either IL3 or Sry genes; the sex of the fetuses was called undetermined and samples were discarded. These samples were found to contain no DNA by densitometry and were ignored for pooled male and female cultures.

3.3. Fluorescent in situ hybridization

Fetal neural stem cells were isolated from striatum and cultured as separate male or female pooled cells from two to six fetuses. After two passages, cells were harvested and analyzed by FISH for male Y-specific sequence. The cells pooled from PCR-identified female fetuses (counterstained with DAPI) show no male specific signal (rhodamine) whereas the cells pooled from male fetuses show a small fluorescent signal in every cell (rhodamine) identifying the Y-chromosome (Fig. 3). We performed a thorough counting of three separate male and female mixed neural stem cell cultures. We found 300/300 Y-chromosome specific fluorescent signal in the male cultures and 0/300 in the female cultures.

3.4. Timing

Timing of each step of the procedure was evaluated. Dissection of fetal livers, then brains was done by one operator, while the second operator was analyzing seven to 16 samples at a time. Fetal dissection and DNA extraction took ≈ 1 h to obtain dissolved DNA. The PCR amplification procedure including preparation of DNA samples and master mix took <2 h. The rapid electrophoresis including loading of samples took <30 min. The entire procedure from fetal dissection to the observation of bands on agarose gel took <4 h.

4. Discussion

This PCR technique was developed to rapidly and accurately determine the sex of mouse fetuses. Its originality resides in the fact that all steps of the PCR procedure were optimized with regards to timing in order to perform it in <4 h. As compared with other sexing methods, it is faster by at least 2 h (Han et al., 1993; Greenlee et al., 1998). The gains were made at several steps: DNA extraction, PCR and electrophoresis. The extraction of liver DNA using SDS denaturation followed by high salt extraction and ethanol precipitation is inexpensive and produced pure DNA in <1 h. The amount retrieved was small; 34 ± 3 μg on average, but sufficient for PCR. In addition, variability of the recovery was small, obviating the need for evaluating the DNA concentration prior to amplification. In other procedures, the use of proteinase K and/or phenol extraction requested more time and additional DNA quantitation prior to PCR. The use of a robot thermal cycler limited the amplification time to 1 h and 35 min. This represents more than a 30% gain as compared to the classical single heating block thermal

Table 1
Summary of sex determination by PCR. Percentages are shown in parentheses

Litter	Fetuses (no.)	Males (%)	Females (%)	Undetermined (%)
A	5	2 (40)	3 (60)	0 (0)
B	6	2 (33)	3 (50)	1 (17)
C	5	3 (60)	2 (40)	0 (0)
D	2	2 (100)	0 (0)	0 (0)
E	8	5 (63)	3 (38)	0 (0)
F	10	0 (0)	9 (90)	1 (10)
G	6	3 (50)	3 (50)	0 (0)
H	7	2 (29)	4 (57)	1 (14)
I	4	1 (25)	3 (75)	0 (0)
J	9	3 (33)	6 (67)	0 (0)
Total	62	23 (37)	36 (58)	3 (5)

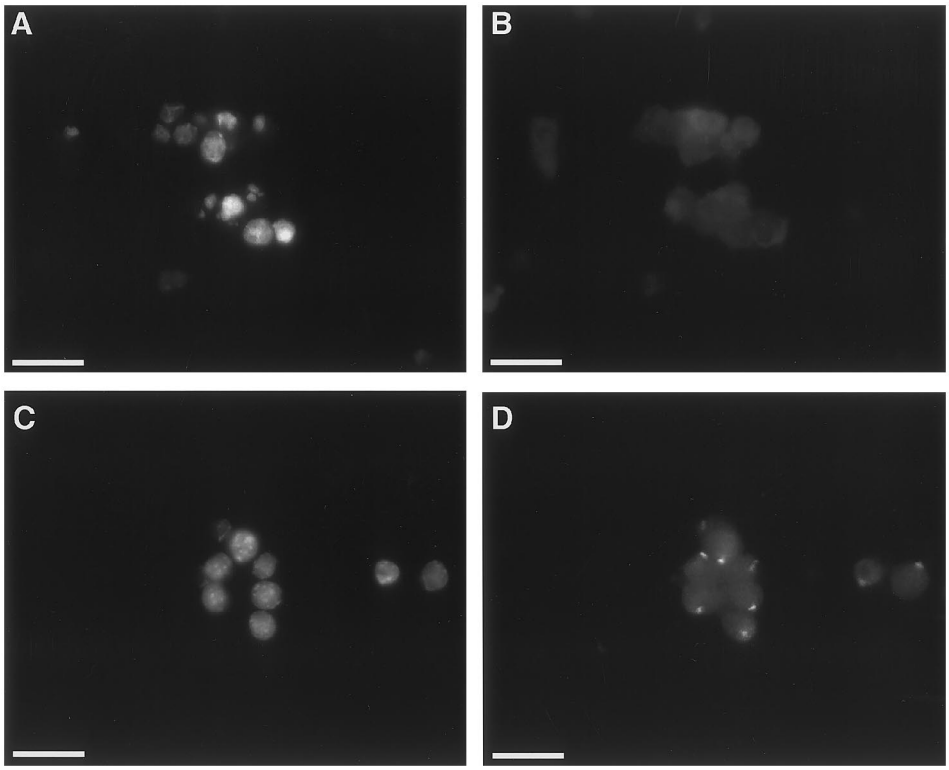

Fig. 3. FISH for male-specific sequences on cultured neural stem cells previously separated into male and female pooled cultures by PCR. Female cells were examined under DAPI filter showing nuclear integrity (A) and with rhodamine filter showing a lack of male-specific signal (B). Male cells are shown with DAPI filter (C) and rhodamine (D); each spot represents the Y-specific sequence hybridized with digoxigenin-conjugated probe and stained with a rhodamine-labeled antibody.

cycler, which needs considerable additional cooling and re-heating time between the different cycling temperatures. We included an internal control for amplification of every sample using a multiplex PCR with two sets of primers for IL3 gene and for male-specific Sry gene. The IL3 gene was chosen for its location on an autosomal gene, chromosome 11 (Ihle et al., 1987), thus giving the same PCR signal intensity in male and female (two alleles). Primers were designed to give products with small length difference, thus allowing easy identification by electrophoresis. Despite similarities between mouse and human sequences, these primers do not align to human IL3 and Sry sequences by computerized BLAST analysis (Altschul et al., 1990; BLAST 2.0 is available at http://www.ncbi.nlm.nih.gov/BLAST/), thus eliminating the risk of human DNA contamination. Amplification of human DNA confirmed the absence of the specific bands (Fig. 2). A ratio of 0.6 was used between IL3 and Sry primers. This gave a competitive advantage to Sry product, thus eliminating the risk of false negative and producing a lighter IL3 product in male samples than in female (Fig. 1). Finally, we optimized the DNA electrophoresis process allowing separation of the 544 bp product from the 402 bp product in 10–15 min. The analysis of individual litters showed substantial variability in the male/female ratio (Table 1). This observation emphasizes the importance of sex determination even in mixed embryonic cultures.

This quick sex determination of mouse fetuses was used to selectively culture pure male and female fresh neural stem cells excised from the brain striatum of fetuses at embryonic day 15. The specificity of the method could be confirmed by FISH for male-specific sequences performed on cultured neural stem cells.

Despite its attractive design, the use of PCR to identify male-specific sequences has the ever-present risk of contamination of female DNA by either adjacent male sample or previously amplified material. In order to minimize this risk, we used filtertips for liver homogenization and for PCR mix preparation. In addition, we tested the effect of contamination of female DNA by 5% male DNA and found that despite the presence of a weak male-specific band, the ratio between IL3 and Sry band was in favor of IL3. This inversion of the band ratio permitted the clear detection of male/female contamination. In a few samples the DNA pellet was lost during extraction procedure and thus gave no band by multiplex PCR. The IL3 band was found to be an important internal control and permitted to eliminate sex-undetermined fetuses prior to male and female pooling. The use of another Y-specific marker in the multiplex reaction, such as ZFY,

may be useful in single cell amplification to confirm the male identification, but was not required in presence of high DNA quantity available from fetal liver.

We designed a simple and accurate PCR method for rapid sexing of fetuses. This technique is applicable to any project requiring a separate male and female embryonic tissue culture. We are presently applying the procedure to culture of neural stem cells isolated from striatum.

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