



## Amplification of Porcine SRY Gene for Sex Determination\*

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**ABSTRACT :** The separation of X and Y chromosome-bearing sperm is of use in many aspects of livestock maintenance. In this study, we sought to determine the difference in DNA content between X- and Y-bearing sperm, separate sperm into X- and Y-enriched pools, and assess the efficacy of sorting. Sperm collected from Duroc and miniature pigs were stained with 20.8  $\mu$ M Hoechst 33342 and analyzed using a high-speed cell sorter. Measurement of the fluorescence intensity of stained sperm nuclei revealed that the X-bearing sperm of Duroc and miniature pigs respectively contain 2.75% and 2.88% more DNA than Y-bearing sperm. In total, 50.18% of the sperm were assigned to the X-sorted sample and 49.82% was assigned to the Y-sorted sample for Duroc pigs. For miniature pigs, the X-sorted sample represented 50.19% of the population and the Y-sorted represented 49.81% of the population. Duplex PCR was used to evaluate accuracy of sorting. A fast and reliable method for porcine sexing was developed through amplification of the sex-determining region of the Y chromosome gene (SRY). Oligonucleotide primers were designed to amplify the conserved porcine SRY high motility group (HMG) box sequence motif. We found that the primer pair designed in this study was 1.46 times more specific than previously reported primers. Thus, this study shows that the present method can be applied in porcine breeding programs to facilitate manipulation of the sex ratio of offspring and to achieve precise sexing of porcine offspring by amplification of the HMG box of the SRY gene. (**Key Words :** SRY Gene, Sex Determination, Sperm Sorting, Duplex PCR)

### INTRODUCTION

A lot of domestic animal associated technologies have been developed recently. For example, evaluation of boar sperm viability technique was developed (Byun et al., 2008). Among them, sexing skill has been getting the limelight. Predetermination of sex in livestock offspring is of critical importance for the efficient production of the world's food supply (Johnson et al., 2000). In addition, sex pre-determination of offspring also has important implications for the management and conservation of captive wildlife species (O'Brien et al., 2005).

The most widely used method of X and Y chromosome-

bearing sperm selection, commonly known as Beltsville Sperm Sexing Technology, is based on flow cytometric sorting of Hoechst 33342-labeled sperm, which is to detect differences in the total DNA content between X and Y chromosome-containing spermatozoa (Johnson et al., 2005). However, the equipment for sexing sperm is fairly complicated and expensive over \$350,000 per sperm sorter. It is also expensive to install and maintain. Skilled operators are required which results in training costs (Seidel Jr., 2007). It is well known that Hoechst 33342 could have toxic and mutagenic effects upon certain cell types (Durand and Olive, 1982), and also that u.v. light produces an increase in chromosome structural abnormalities in mouse spermatozoa (Matsuda and Tobari, 1988). Concerns have been raised that simultaneous use of both agents may affect the genetic integrity of sperm selection by flow cytometry (Ashwood-Smith, 1994; Munne, 1994; Gardiner-Garden, 1999).

To confirm sperm sorting, different techniques, such as the Quinacrine mustard staining for Y-chromosome bearing sperms identification (Ogawa et al., 1988), the Quantitative Southern Blotting (Beckett et al., 1989), eventually associated with autoradiography analysis (Lobel et al., 1993), and the capillary electrophoresis (Checa et al., 2002)

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have been developed. Nevertheless, all these kinds of approaches were often approximate and time-consuming (Parati et al., 2006).

In 1995, Sathasivam and colleagues developed a method for sexing domestic pigs by PCR amplification of the high motility group (HMG) box of the Y chromosome-specific sequences in male genomic DNA (Rubes et al., 1999) developed a method for multicolor fluorescence *in situ* hybridization with chromosomal probes. Similarly, the Chromogenic *in situ* Hybridization method was developed (Kim et al., 2007). The other method, Mara et al. (2004) developed a duplex PCR method for sex determination of ovine *in vitro*-produced embryos prior to implantation (Lu et al., 2007).

Here, we describe a similar method, which involves amplification of the HMG-box sequence of the porcine SRY gene using a novel primer pair. Using both sorted and unsorted semen, we show that this PCR based method can detect the presence of the Y chromosome with high sensitivity and accuracy. The objective of this study was to amplify porcine genomic sequences for the SRY gene along with oligonucleotide primers. This study also relates to a method of sexing porcine sperm using duplex PCR by obtained specific DNA sequence. This study will be a method for the genetic manipulation or sexing technology in domestic animals. Ultimately, this technology will be of use in the production of livestock of the desired sex, allowing for herd expansion reliable genetic replacement.

## MATERIALS AND METHODS

### Sperm preparation for flow cytometric sorting

Duroc semen was supplied by a local AI center (Darby Genetics Inc., Anseong, Korea), and miniature pig semen was donated by the Kangwon National University. Sperm concentration was determined with a hemocytometer. The semen was washed three times in Dulbecco's Phosphate Buffered Saline (DPBS, Gibco-BRL, Grand Island, NY) at 26.16 xg for 3 min. After washing, the sperm concentration was  $9.5 \times 10^8$  cells/ml. Sperm were suspended in DPBS with 20.8  $\mu$ M Hoechst 33342 (H33342; Sigma, St. Louis, MO) and incubated at 37°C for 15 min. Hoechst 33342, which selectively binds to A-T base pairs, produces a signal that is proportional to the amount of DNA carried by a single sperm.

### Flow cytometric sperm sorting

The viable sperm-sorting procedure was performed essentially as previously described (Johnson et al., 1999). Sperm was sorted with a FACS Vantage SE (Becton Dickinson, Franklin Lakes, NJ) using DPBS (Gibco-BRL) as sheath fluid. The FL5-H sorting parameter was used, and

data were analyzed with Cell Quest (BD CellQuest Pro Software). After the X/Y sorting process, 1,340,000 sperm were collected in a conical tube and transported to the laboratory under -150°C storage conditions. The difference in the DNA content between the two populations was then calculated by defining X to be the mean fluorescence of the X-bearing sperm and Y to be the mean fluorescence of the Y-bearing sperm (Garner et al., 1983; O'Brien et al., 2005).

### Duplex polymerase chain reaction

The sorted sperm (approximately 10-15 ml, at a concentration of  $0.5 \times 10^6$  spermatozoa/ml) were washed several times in DPBS (Gibco-BRL), transferred to 0.2 ml PCR tubes containing 5  $\mu$ l distilled water. A proteinase K solution (2  $\mu$ l at 20 mg/ml, Qiagen, Chatsworth, CA, USA) was added to digest the cellular proteins and facilitate access to the DNA. The tubes were then incubated in a thermal cycler (MJ Mini™, Bio-Rad, USA) at 56°C for 15 min and then at 95°C for 15 min to inactivate the enzyme (Carlos et al., 2005).

Following the proteinase K treatment, 20  $\mu$ l of PCR reaction mixture consisting of 1 U Taq DNA polymerase, 250  $\mu$ M in each dNTP, Tris-HCl pH 9.0, 40 mM KCl, and 1.5 mM MgCl<sub>2</sub>, (Bioneer, Inc. USA) was added to the material to be amplified. For each amplification, two pairs of primers were used: one pair was specific to chromosome 1 and the other was specific to the Y chromosome (Table 1). The Y chromosome-specific primers were designed to detect the *Sus scrofa* SRY protein gene complete coding sequence (Gen-Bank Accession U49860, gi: 1330325). The sequences of the primers for Y chromosome are as follows: (5' primer) 5'-GTTGCACTTTACGGACGCAGC-3' and (3' primer) 5'-CTAGCCATTGCTCGCCATAGC-3'. The expected amplification product is 377 bp in length. The second primer pair was as follows: (5' primer) 5'-AAGTCACTCACAGCCCATGAA-3' and (3' primer) 5'-CCATGGAAGTTCCTGTATCAT-3'. We assessed the effectiveness of PCR amplification with the new Y chromosome primer pair. The amplification of this region produces a 581 bp product.

The PCR amplification was carried out in a thermal cycler (MJ Mini™, Bio-Rad, USA) under the following conditions. Complete DNA denaturation was achieved by incubation at 95°C for 45 min. Then the reactions was carried out with 35 cycles of amplification at the following temperatures: 95°C for 15 sec for DNA denaturation, 65°C for 1 min for annealing of the oligonucleotides, 72°C for 1 min for polymerization of DNA chains, and a final primer extension at 72°C for 5 min.

The tubes containing the PCR product were once again incubated in the thermal cycler at 94°C for 5 min for

**Table 1.** Primers used for PCR sex determination of boar sperm

Marker	Primer sequence	Product size (bp)	Chromosome
pCh1 (F)	5'-GTTGCACTTTCACGGACGCAGC-3'	244	X and Y
pCh1 (R)	5'-CTAGCCCATTGCTCGCCATAGC-3'		
pSRY (F)	5'-AAGTCACTCACAGCCCATGAA-3'	581	Y
pSRY (R)	5'-CCATGGAAGTTCCTGTATCAT-3'		
pChY (F)	5'-AATCCACCATACTCATGGACC-3'	377	Y
pChY (R)	5'-TTTCTCCTGTATCCTCCTGC-3'		
mSRY (F)	5'-CATGACCACCACCACCACCAA-3'	441	Y
mSRY (R)	5'-TCATGAGACTGCCAACCACAG-3'		

Porcine chromosome 1 specific (pCh1) primers were used to notify presence of porcine chromosome (Jantsch, 1989; Rubes et al., 1999; Parrilla et al., 2003). Porcine SRY (pSRY) primer sequences were designed on the basis of the *Sus scrofa* SRY protein gene complete cds. Porcine chromosome Y specific (pChY) primers were polymerized for testifying sorting efficiency (McGraw et al., 1988; Rubes et al., 1999; Parrilla et al., 2003). *Mus musculus* SRY (mSRY) primers were used for testifying species specificity (McClive and Sinclair, 2001).

complete DNA denaturation and then subjected to 50 cycles of amplification under the following conditions: 93°C for 30 sec, 50°C for 15 sec, and 72°C for 1 min (Carlos et al., 2005). We then performed gel electrophoresis (1% agarose, TAE buffer), and by comparing the relative band intensity we were able to quantify the sorting efficiency. Gels were analyzed using the Image J 1.38 program (NIH, USA).

#### Experimental design

In Experiment 1, the difference in DNA content between X- and Y-bearing spermatozoa was determined and sorted as follows: i) Duroc, ii) Miniature pig. In Experiment 2, the accuracy of sorting was assessed as follows: i) pCh1 with pSRY primer, ii) pSRY with pChY primer, iii) pCh1 primer, iv) pSRY primer, v) pChY primer, vi) mSRY primer.

## RESULTS

The histogram illustrates the separation of the X and Y populations from the original population of histogram in Figure 1. The area between the two sort windows represents sperm that are mixed (X and Y) in varying percentages and which were subsequently discarded. Sperm carrying the smaller Y chromosome, and therefore less total DNA, comprise the population with lower fluorescence (left), and similarly, the larger X chromosome-bearing sperm comprise the population with higher fluorescence. The percent differences in DNA content between X and Y-bearing spermatozoa were similar in miniature pig and Duroc (Table 2). X-enriched (X-sort) samples represented 50.18% and 50.19% of the population, and Y-enriched (Y-sort) represented 49.82% and 49.81% of the population for Duroc and miniature pig, respectively (Table 3).

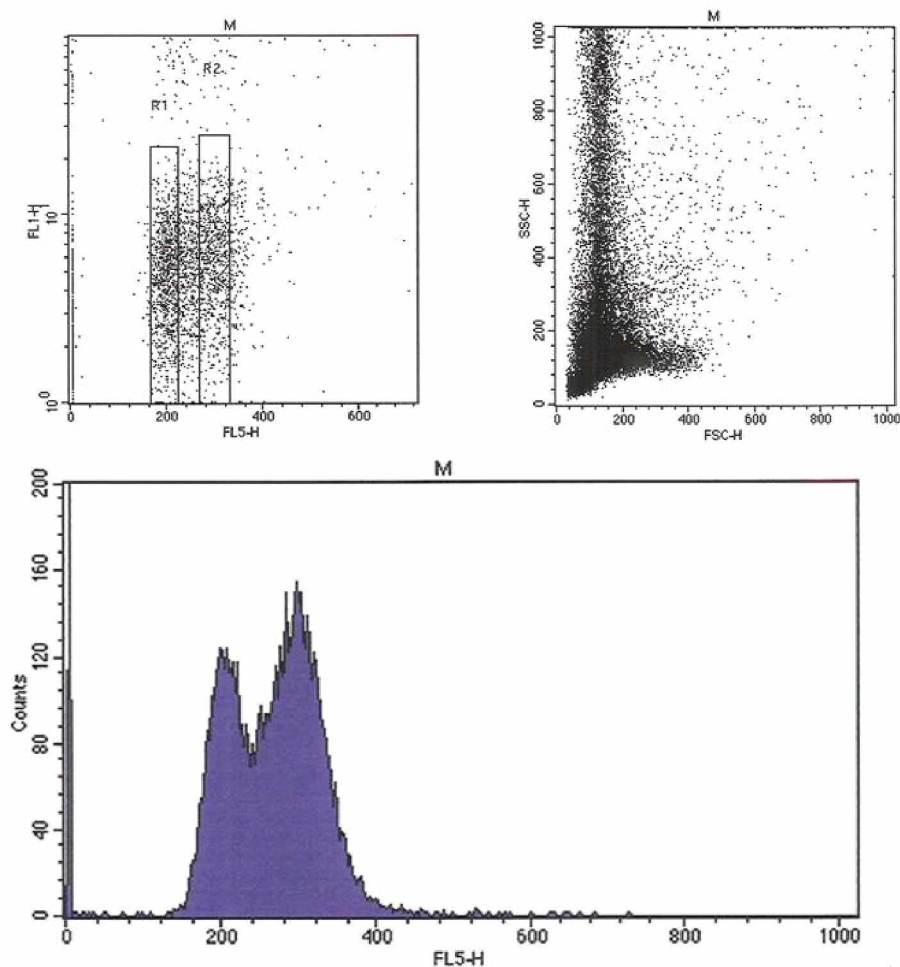
We next evaluated the specificity and efficiency of the new PCR primer pair (Figure 2). Control reactions for all primers combinations were carried out using the same

volume of unsorted porcine semen for each reaction. The results confirm that the new pSRY primers (Figure 2a, lane E) were approximately 1.46 times more specific for porcine DNA than conventional pChY primers (Figure 2a, lane F; Rubes et al., 1999; Parrilla et al., 2003). *Mus musculus* SRY (mSRY) primers (lane G; Peter and Andrew, 2001) were used for PCR sex determination of boar sperm, but these primers failed to amplify the SRY gene from boar sperm DNA.

PCR for sex determination in sorted Duroc boar sperm is shown in Figure 2b. The PCR products amplified from the first peak (R1) were loaded in lanes B and C, and the products from the second peak (R2) were loaded in lanes D and E. The presence of a single band indicates the presence of porcine-specific chromosome 1 in the sperm (B and D lane). In contrast, the presence of two bands indicates that the population contains male sperm (C lane). Therefore, these results indicate that the first peak was Y-bearing sperm. Other lane (E lane) was no amplification, and it just showed that non-Y sperm, but we could analogize second peak indicated X-bearing sperm through FACS results (Park et al., 2001; Carlos et al., 2005; Whyte et al., 2007).

## DISCUSSION

We have described a accurate PCR-based method to determine the presence of male-specific sequences in porcine DNA. We have verified the fidelity of this method on X/Y-sorted semen samples. We find that this method is sensitive enough to determine the presence of the SRY gene in samples containing as few as eight sperm cells. This level of sensitivity would allow for the application of this method in the sexing of germ-cell derivatives (Notarianni et al., 1992; Condon et al., 2007), embryos (Sohn et al., 2002; Kelly et al., 2003; Saravanan et al., 2003; Chen et al., 2007; Huang et al., 2007), embryonic cell lines (Veerhuis et al., 1994; Taketo et al., 2005), and fetus.



**Figure 1.** Flow cytometric Dotplot and Histogram (Miniature pig). R1 channel indicated first peak and R2 indicated second peak. Fluorescence light (FL) of first peak on the histogram was 268 nm, and second peak was 352 nm. To assess FACS sorting, duplex PCR was conducted. It was verified that first peak (R1) was Y-bearing sperm and second peak (R2) was X-bearing sperm.

**Table 2.** Determination of difference in DNA content

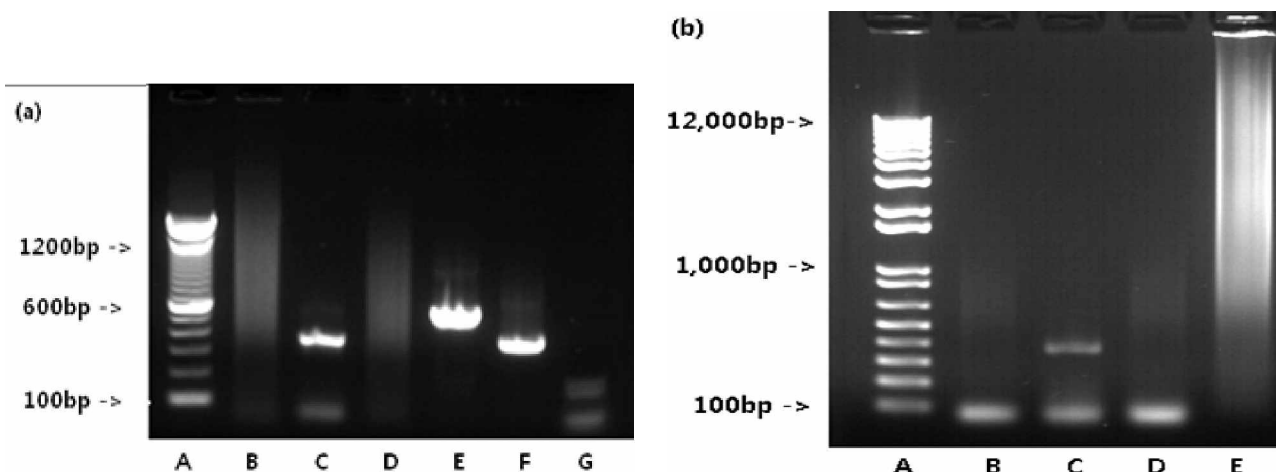
Sperm nuclei sample	Fluorescence intensity on FL5-H channel (nm)	Difference (%) in DNA content between X and Y chromosome-bearing sperm nuclei
Duroc	Y = 266.34	2.75
	X = 331.18	
Miniature pig	Y = 263.38	2.88
	X = 352.02	

Difference in DNA content (percentage separation of the fluorescent peaks representing the two populations) was then calculated using the formula: Difference =  $100 \times ((X-Y)/0.5(X+Y))$ . The percentage difference in DNA content between X and Y-bearing spermatozoa was higher for Miniature pig compared to the Duroc.

**Table 3.** Flow cytometric X- and Y- population

Strain	Peak	Sex	Cell number	Ratio (%)
Duroc	R1	Y	1,108,000	49.82
	R2	X	1,116,000	50.18
Miniature pig	R1	Y	267,000	49.81
	R2	X	269,000	50.19

Y-sorted sperms were 49.82 and 49.81%, and X-sort sperms were 50.18 and 50.19% for Duroc and Miniature pig. Y-bearing sperms of total sperms were expected 0.5 ratios. As summarized results, X-bearing and Y-bearing sperms in semen from Duroc and Miniature pig were agreed with the ratio.



**Figure 2.** PCR for sex determination in sorted boar sperm. (a), non-sorted sperm DNA amplification. pSRY primers (E lane) were around 1.46 times more efficient porcine DNA than conventional pChY primers (F lane). To assess primer working efficiency, bands were automatically selected and the peak area was calculated using Image J 1.38 x program. pSRY primer (E lane) area was arranged in 8131 pixels, and pChY primer (F lane) area was in 5573 pixels. mSRY primers (G lane) were indistinct. (b), sorted sperm DNA amplification. First peak (R1) was loaded to B-C lanes, and second peak (R2) was loaded to D-E lanes. These results indicated that R1 was Y-bearing sperm and R2 was X-bearing sperm.

This method differs from that of Thomassen and Poulsen (1993) in that sequences amplified by the PCR primers are within the HMG box of the sex-determining gene itself, rather than in DNA from the heterochromatic long arm of the Y chromosome. The present method also differs from that of Sathasivam et al. (1995), which proved to produce an inferior rate of sex prediction.

In the approach by Mara et al. (2004), the sex of *in vitro* produced ovine embryos was determined by duplex PCR. It took two sets of primers. However, it did not amplify the HMG box domain as we have in the present study. In a study by Lu et al. (2007), the amplification of the HMG box of the bovine SRY gene was achieved for the first time. However, that study did not address sexing of other species. Through optimization of the primer sequences and the cycle conditions for the PCR, we have produced a method that is reliable.

In conclusion, we show that the present method, which depends on PCR amplification of the HMG box of the porcine SRY gene, can be used for reliable sexing of porcine offspring. This technique requires a little amount of DNA, saves time, be more efficient and higher exactitude. It also makes avoid getting false-negative result. This result allows for the detection of specific nucleic acid sequences, is an ideal method for quantitatively and qualitatively assessing the purity of sorted sperm samples. This method is also a sensitive technique for molecular diagnosis of chromosomes on a few cells and could be used in both biopsied blastomeres and embryos. Further experiments will likely improve the efficiency of sexing technologies and to allow for the production of live piglets of the desired sex.

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