

Evaluation of Sperm Sex-Sorting Method using Flow Cytometry in Hanwoo (Korean Native Cattle)

Han-Jun Yoo¹, Kyung-Jin Lee², Yong-Seung Lee², Chang-Woo Lee⁴, Joung-Jun Park¹, Hee-Tae Cheong³, Boo-Keun Yang² and Choon-Keun Park^{2,*}

¹Developmental Biotechnology Laboratory, Myung-poomHanwoo Consulting, Hoengseong 225-807, Korea

²College of Animal Life Sciences, Kangwon National University, Chuncheon 200-701, Korea

³School of Veterinary Medicine, Kangwon National University, Chuncheon 200-701, Korea

⁴Gangwon Province Livestock Research Center, Hoengseong 225-807, Korea

ABSTRACT

This study evaluated a method of sorting X and Y chromosomes based on size using the forward angle light scatter related refractive index (FSC) of a flow cytometer. Hanwoo bulls sperm were separated to X and Y chromosomes by the parameters of FSC or Hoechst 33342 intensity. As a result, using monitor program linked flow cytometry during sorting processing, the purities were 97 ± 0.57 or $96 \pm 0.67\%$ for the X-fraction and 96 ± 0.33 or $97 \pm 1.33\%$ for the Y-fraction in the two sperm sorting methods. There were no differences in the X and Y ratios (X and Y %) between the sperm sorting methods based on FSC or DNA content. The proportions of female and male embryos used for *in vitro* fertilization and development were 66.03 ± 3.31 or $69.37 \pm 1.41\%$, and 70.56 ± 2.42 or $56.11 \pm 3.09\%$ when sperm were processed using the sex sorting method by FSC or Hoechst 33342. In conclusion, further study is needed to determine the optimum procedure and improve the nozzle to enhancing sorting accuracy or efficiency. Also, the findings of this study do not negate the possibility that the difference method of sperm sorting cannot use a UV laser beam.

(Key words : flow cytometry, sperm sorting, FSC, sheath fluid buffer, Hanwoo)

INTRODUCTION

Predetermination of sex in livestock offspring is in great demand and is of critical importance for efficient animal farming (Shim *et al.*, 2001). Sex selection is especially useful in dairy cattle due to their economic importance and the need for heifer calves for herd replacement and milk production (Maxwell *et al.*, 2004). To date, the only scientifically proven method based on the difference in DNA content between the X and Y chromosomes in mammals has been fully validated using flow cytometric sperm sorting (Johnson, 1995; Johnson and Welch 1999). However, in these studies, the preparation procedures negatively affected the viability of DNA. Sex selection via flow cytometric sorting involves staining of sperm with Hoechst 33342, which penetrates the living sperm membrane and binds to the DNA of the sperm nucleus (Choi *et al.*, 2009), in combination with ultraviolet (UV) laser treatment.

Johnson *et al.* (1989) postulated that fluorochrome dyes reduce embryonic viability by mid-gestation. This coincides with the findings of Spinaci *et al.* (2005) in boar sperm. Also,

weaker laser intensity diminishes the resolution and, indirectly, the sorting rate. There are few published reports of attempts to sort bull sperm from frozen-thawed semen (Lu *et al.*, 1999; Hollinshead *et al.*, 2004b), and the limiting factor appears to be the low resolution of the X and Y populations due to the egg yolk. Another limiting factor to the use of frozen-thawed bull sperm for sorting is poor post-thaw viability compared to that of non-sorted frozen-thawed sperm (Hollinshead *et al.*, 2004b). Also, another paper reported that the process must be carried out one cell at a time, resulting in an inherently slow system because millions or billions of sperm are needed for conventional artificial insemination (AI) (Johnson *et al.*, 1999). The current technology requires that each sperm be separately interrogated for DNA content, thus limiting the number of sorted X- or Y- sperm in cattle, sheep, swine and horses to approximately 12~20 million sperm per hour (Johnson and Welch, 1999; de Graaf *et al.*, 2007). Therefore, intensive research is required to further improve the existing technology or to identify alternatives and/or combinations with other biotechniques. On the one hand, in Korea, there are no commer-

* This work was supported by Grant No. 109007-3 from Agricultural R&D Promotion Center (ARPC), Republic of Korea.

* Correspondence : E-mail : parkck@kangwon.ac.kr

cialization sperm sorting system because of the limitations on sorting speed, sorting accuracy, sperm ability and number of sorted sperm. Thus, the progress in this field has been slow.

Therefore, the objective of this study was to evaluate Hanwoo (Korean native cattle) sperm sorted into X- and Y-chromosomes by using two evaluation methods from a flow cytometry. Sperm were prepared on the basis of differences in DNA mass and FSC for flow sorting, and sexed sperm were measured for the parameters of sex ratio using PCR.

MATERIALS AND METHODS

1. Preparation of Sheath Fluid Buffer

Sheath fluid is an isotonic buffer saline solution that is pumped through the flow chamber, causing single cells to flow through the middle of the stream. Preparation of sheath fluid buffer, known as HEPES sheath flow, used in stallions (Buss, 2005) was performed from each of the reagents shown in Table 1 in a mixture with distilled water. Briefly, metal ions such as $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (Sigma-Aldrich, St. Louis, MO, USA), which were involved in chelation, dissolved in 800 ml distilled water. After completely dissolved, remaining powder reagents were added, and then the remaining liquid components were dissolved to incomplete compounds of sheath flow. The sheath flow buffer is adjusted to 1 l with distilled water and pH 7.2. The filtration was performed through 0.2 μm pore size filters. Generally, this solution was prepared as a five-fold stock solution and was stored at 4°C before use. The shelf-life

Table 1. Composition of HEPES sheath fluid buffer for sex-sorting of bull sperm using flow cytometry

Compound	Concentration (g/l)
$\text{CaCl}_2 \cdot \text{H}_2\text{O}$	0.14
KCl	0.37
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	0.1
$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	0.03
NaCl	6.54
Na-pyruvate	0.02
Lactic acid (60%)	3.51
HEPES	1.19
NaHCO_3	0.42
Penicillin G	0.058
Streptomycin sulfate	0.05

of the solution was considered to be only one week because instability of the stream during flow cytometric sperm sorting is observed after longer storage times.

2. Cryopreservation and Thawing of Sperm

For all experiments herein, Korean native cattle (Hanwoo) semen was collected using an artificial vagina and a teaser at the Hoengseong Livestock Cooperative Farm. Immediately after collection, motility and concentration of each bull sperm sample was assured using a phase-contrast microscope and a hemocytometer, respectively. Collected semen (volume: 5~15 ml; density: $2 \sim 10 \times 10^8$ ml; live ratio >75%) was diluted 1:1 (v/v) with Triladyl containing 20% egg yolk, and the semen tube was placed in a 500 ml, 35°C water jacket before being cooled to 4°C over 6 h in a refrigerator. The second dilution to a concentration of 10×10^6 sperm per ml was then performed with an aliquot of semen diluted with the same freezing extender as was used in the first dilution. At this point, sperm were loaded into 0.5 ml straws, frozen and submerged in liquid nitrogen for storage. For use, semen straws were thawed for 45 s in a 37°C water bath and dried. Semen was placed on a continuous density gradient tube to remove non-viable sperm and cryodiluent

3. Sperm Pretreatment

Percoll, a sterile colloidal silica suspension and isotonic salt gradient solution, was diluted to a 65% fraction with 10x HEPES buffer. The 65% Percoll (Nidacoll, Göteborg, Sweden) was prepared by placing 1 ml in a 1.5 ml micro tube. The semen (0.5 ml) was placed over the gradient and centrifuged at 1,700 rpm (Micro 12, Hanil Science Industrial, Korea) for 15 min. The sperm pellets were assessed for concentration and resuspended in 1 ml of HEPES sheath flow buffer. The sperm was then subjected to one more washing step in order to improve the resolution of flow cytometry for sexing.

4. Hoechst33342 Staining

Sperm preparation and staining were based on the method described by Johnson *et al.* (1989) in order to maintain viability through sorting and fertilization. Briefly, pretreated aliquots of fresh or frozen-thawed semen of $1 \sim 5 \times 10^7$ ml were stained with 40 μM Hoechst 33342 (Sigma-Aldrich, St. Louis, MO, USA) and were incubated for 30 min at 38°C to foster fluorochrome penetration. Finally, extra fluoro-dye, which was not bound to the adenine-thymine regions of the sperm DNA,

was eliminated via centrifugation (1,500 rpm, 5 min). Also, an unstained control samples was prepared as a reference comparison of intensity of Hoechst 33342 on flow cytometry during fluorescence compensation and sex-sort processing using FSC. Generally, samples were prepared for separation in several loading tubes because stained sperm aggregate during sperm sorting after 18~25 min.

5. Sex-sorting

We based our sorting method on a histogram of X- and Y-chromosomes that had undergone Hoechst 33342 staining to produce a dot plot of sperm cell population using reverse order. Fig. 1 shows blue or purple coloring between the R3 and R4 regions, which matched the dot plot and histogram created using FACSDiva software. This result suggests that each region may independently perform the important role in separate X- and Y-chromosome as criterion of sperm sex-sorting. Intact sperm were sorted using a flow cytometer/cell sorter (BD FACs Aria II; BD, USA). Intact, viable Hanwoo sperm were flow-cytometrically separated into X and Y populations on the basis of relative DNA contents and FSC (Fig. 2). Twenty to forty percent of living intact sperm were oriented with this process, and a sorting rate of approximately 50 to 200 sperm/sec was achieved for each population. The sperm cells were measured and sorted using a 100 μ m nozzle and a HEPES sheath fluid pressure of 20 psi. Sperm were sorted in single-cell mode into each 5 ml round tube that had been treated with 0.4% BSA solution to prevent the sperm from sticking to the wall of the tube, resulting in a final concentration of at least $1\sim5 \times 10^6$ ml per tube. The tubes were then stored at room temperature (25°C; RT) until analysis at RT. After a to-

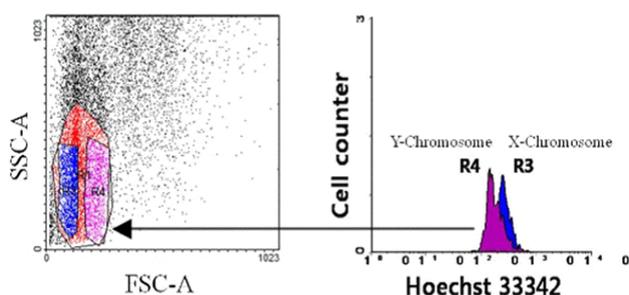


Fig. 1. R3 and R4 peaks of the histogram matched the gated region of the dot plot created by BD FACSDiva software. Independently-gated R3 and R4 regions on the dot plot coincide with the intensity peak of Hoechst 33342 on the histogram.

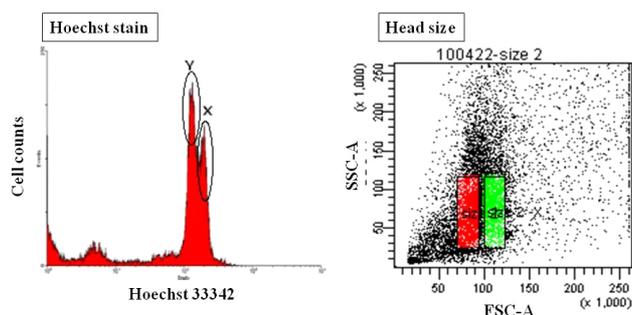


Fig. 2. Visualized histogram of X- and Y-chromosomes after Hoechst 33342 staining and a dot plot of sperm cell population. Hanwoo sperm were flow-cytometrically separated into X and Y populations based on differences in DNA content and size according to forward angle light scatter related refractive index. Twenty to forty percent of living intact sperm were oriented with this process, and sorting rates of approximately 50 to 200 sperm/sec were achieved for each population. With regard to FSC, the red and green regions indicate the X- and Y-enriched population, respectively.

tal of $1\sim5 \times 10^6$ sperm cells were collected, the sperm were concentrated via centrifugation at 1,500 rpm for 7 min. The resultant pellet was resuspended with HEPES buffer and 2% egg yolk and was processed for immediate use.

6. *In vitro* Embryo Production

Ovaries were collected from cows at a local slaughterhouse and transported to the laboratory. Oocytes were aspirated from antral follicles with an 18-gauge needle fixed to a 10 ml disposable syringe. The cumulus-oocyte complexes (COCs) were selected and then cultured *in vitro* maturation media at 38.5°C, 5% CO₂. Then, COCs were transferred into each 100 μ l of TCM 199 containing EGF and 10% FBS that had been previously coated with mineral oil in a polystyrene culture dish (35 \times 10 mm, Nunc, Roskilde, Denmark) and were equilibrated in 5% CO₂ in air for approximately 4 h. After 20~22 h of incubation in maturation culture, oocytes were removed from cumulus cells by treatment with 300 IU/ml of hyaluronidase and repeated pipetting. After washing with Tyrode's albumin lactate pyruvate (TALP-Fer), oocytes were transferred into a 50 μ l drop of TALP-Fer covered with mineral oil containing 0.4% (w/v) BSA (Fraction V). The dishes were stored in 38.5°C, 5% CO₂ until the sperm were used for insemination. For IVF, sex-sorted sperm were washed in TALP-SP for remove egg yolk and resuspended in 200~500 μ l TALP-SP.

A maximum of 50 μ l of sperm suspension was added to 50 μ l of the fertilization drops containing 10 oocytes. After 16 h of insemination, oocytes were removed from the fertilization medium and cultured in 100 μ l of CR1aa containing 10% FBS at 38.5°C, 5% CO₂.

7. Sex Determination Using the Polymerase Chain Reaction (PCR) Assay

To detect the S4 gene regions of 16~32 cell stage embryos produced via IVF with sex-sorted Hanwoo sperm using the above two sex-sorting methods, embryos were analyzed using genomic PCR. In brief, the zonapellucida of the embryos was removed by repetitive pipetting, and the cytoplasm of the embryos was washed three times with PBS and transferred into DNA extract tubes (AccuPower[®] Genomic DNA Extraction Kit; Bioneer, Daejeon, Korea) with 200 μ l of deionized water. Also, DNA of male and female positive controls were extracted from each hair root. DNA extraction was performed in the following steps: DNA binding with binding buffer and proteinase K for 10 min, washing with washing buffer 1, 2 at 8,000 rpm, 1 min and extract DNA by elution buffer. Extracted genomic DNA was transferred into a PCR mixture tube (AccuPower[®] PCR PreMix; Bioneer, Daejeon, Korea), then 10 pmol of each primer were added to the PCR tube, in addition to 1 U of Taq DNA polymerase, each deoxynucleosidetriphosphate at a concentration of 250 μ M, 50 mM Tris-HCl (pH 8.3), 40 mM KCl, 1.5 mM MgCl₂ and gel loading dye. The final volume was adjusted to 20 μ l with distilled water. PCR amplification was performed according to the standard protocol using the forward primer 5'-CAA GTG CTG CAG AGG ATG TGG AG -3' and the reverse primer 5'-GAG TGA GAT TTC TGG

ATC ATA TGG CTA CT -3'. The primers were designed to amplify a male-specific product (178 bp) from the repeated S4 bovine sequence (DDBJ/GenBank database accession number D16357), in addition to a product common to both sexes (145 bp) (Kageyama *et al.*, 2004). The PCR reaction conditions consisted of denaturation at 95°C for 5 min, followed by 35 amplification cycles of denaturation at 95°C for 30 sec; annealing at 54°C for 45 sec; extension at 72°C for 45 sec. An additional extension was performed at 72°C for 5 min. After amplification, a 10 μ l aliquot of the PCR reaction mixture was subjected to electrophoresis in a 2% agarose gel and 1x TAE buffer (40 mM Tris-acetate, pH 8.0, 1 mM EDTA). The gels were stained with 0.5 μ g/ml ethidium bromide, and amplified DNA bands were visualized under ultraviolet illumination.

8. Statistical Analysis

Statistical analysis was performed with analysis of variance (ANOVA) using SAS (version 9.1, SAS Institute Inc., Cary, NC, USA). Differences among sperm analysis mean values from the sperm sorting method results were processed using Duncan's multiple range tests. Significance was defined at a level of $p < 0.05$.

RESULTS

To demonstrate sorting accuracy, we identified embryos produced through *in vitro* fertilization using sexed semen (Fig. 3). As shown in Table 2, there was a correlation between sorting accuracy and sex ratio. Using a monitoring program linked flow cytometry during sorting processing, purities were found to be 97 ± 0.57 or $96 \pm 0.67\%$ for the X-fraction and 96 ± 0.33

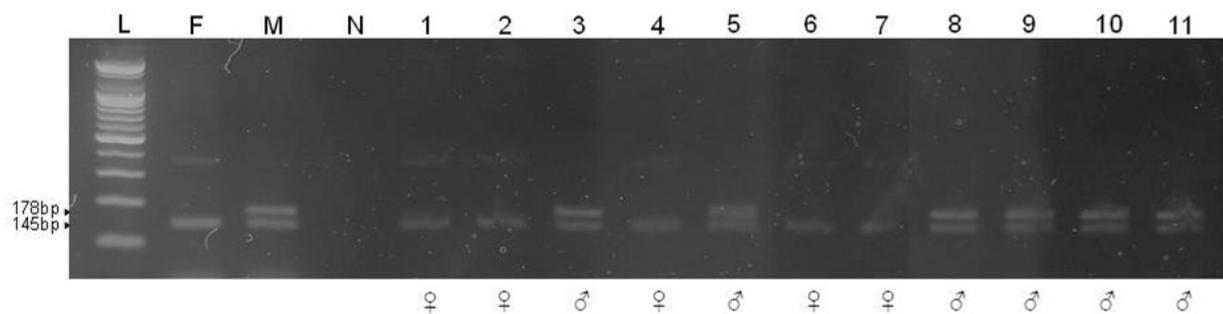


Fig. 3. Embryo sexing after PCR with the S4BF/R primer set to determine the efficiency of the sex-sorting method based on FSC and DNA content. The primers were designed to amplify a male-specific product (178 bp) in addition to a product common to both sexes (145 bp) from the repeated sequence, named S4 (DDBJ/GenBank database accession number D16357). L: 100 bp DNA ladder, F: positive control of female oocyte, M: positive control of male sperm, N: negative control, 1~11: bovine embryos.

or $97 \pm 1.33\%$ for the Y-fraction by two sperm sorting methods using parameters FSC or Hoechst 33342. Repetition of the experiment using sperm obtained from different bulls resulted in similar histograms and purities for both X- and Y-fractions. No differences in the X or Y ratio (X or Y%) were found between sperm sorting method based on differences in DNA content or FSC (Table 2). The X and Y ratios of the embryos by PCR were $66.03 \pm 3.31\%$ and $70.56 \pm 2.42\%$, respectively, when sorted according to FSC using flow cytometry. In the other sorting method using Hoechst 33342, the results were demonstrated $69.37 \pm 1.41\%$ for the X-ratio and $56.11 \pm 3.09\%$ for the Y-ratio. On the other hand, all sorted sperm showed very low fertility (Table 2).

DISCUSSTION

It has been reported that the sorting processes accelerate the maturation of sorted frozen-thawed ram sperm, reducing their fertilizing lifespan (Hollinshead *et al.*, 2003; Hollinshead *et al.*, 2004a). Also, several groups (Cran *et al.*, 1993; Lu *et al.*, 1999) have reported similar cleavage rates but reduced blastocyst development following fertilization *in vitro* with sorted bovine sperm. Additionally, lower pregnancy rates after fertilization with sex-sorted compared to non-sorted sperm have been reported in a number of studies (Johnson, 1995; Seidel *et al.*, 1999; Hollinshead *et al.*, 2002; Seidel and Garner, 2002). Capacitation-like changes have been observed after the sorting of both ram and boar sperm (Catt *et al.*, 1997; Maxwell *et al.*, 1998; Maxwell and Johnson, 1999), similar to those observed

after freezing and thawing of ram sperm (Gillan *et al.*, 1997), reducing their fertilizing lifespan *in vivo* (Watson, 1995). The present study showed a low fertility of sorted sperm in the range of 22~37% (Table 2), which may be caused by factors that influence fertility or inadaptability of the IVF protocol. Sorted sperm had significantly decreased fertility or showed no significant difference between sorting methods based on DNA content and FSC. Meanwhile, it was reported that the addition of 10% seminal plasma to the staining medium helped prevent undesirable membrane changes when added to the collection medium in boar (Maxwell and Johnson, 1997).

The presence of egg yolk is the main cause of poor resolution in sperm samples due to its opaque appearance; therefore, it should be removed prior to sorting in order to achieve adequate resolution during flow cytometric sorting. Hollinshead *et al.* (2004b) found that density gradient separation was a more effective method for removing egg yolk compared to standard washing in order to gain better resolution. A large proportion of orientated sperm may lead to faster sorting with no loss of accuracy. Also, this method allows for a higher concentration of sorted sperm and has the benefit of reducing the time for collecting sorted sperm in a highly diluted state.

In this study, the overall flow rate was approximately 1,500~2,500 intact sperm/sec. The intact X- and Y-bearing sperm fractions were sorted simultaneously from the population of input sperm at rates of 200~400 X-bearing sperm/sec and 300~500 Y-bearing sperm/sec, respectively, under the low flow rate condition used in the Hoechst 33342 and FSC parameters. In the high flow rate condition, sperm were sorted at a rate of

Table 2. The proportions of male and female embryos rates at 48 h after IVF with sex-sorted sperm in Hanwoo cattle

Sperm group	No. of oocytes	Cleavage rate (%)	Sorting efficiency ¹⁾ (%)	Percentage of males	Percentage of females
FSC-x ²⁾	120	22.49 ± 0.46^c	97 ± 0.57	33.97 ± 3.31	66.03 ± 3.31
FSC-y ³⁾	121	27.17 ± 1.42^b	96 ± 0.33	70.56 ± 2.42	29.44 ± 2.42
H-x ⁴⁾	115	30.93 ± 1.27^b	96 ± 0.67	30.63 ± 1.41	69.37 ± 1.41
H-y ⁵⁾	121	37.53 ± 1.24^a	97 ± 1.33	56.11 ± 3.09	43.89 ± 3.09

(Mean \pm SEM, $p < 0.05$)

¹⁾ This parameter indicates correctly separated ratio of sperm in flow cytometry.

²⁾ FSC-x: Sorted X sperm without near UV laser beam in flow cytometry.

³⁾ FSC-y: Sorted Y sperm without near UV laser beam in flow cytometry.

⁴⁾ H-x: Sorted X sperm using differences in DNA content by Hoechst 33342.

⁵⁾ H-y: Sorted Y sperm using differences in DNA content by Hoechst 33342.

700 X-bearing sperm/sec and 900 Y-bearing sperm/sec using FSC method or 300 X-bearing sperm/sec and 400 Y-bearing sperm/sec using the differences of DNA content method, demonstrating an 89 to 93% efficiency rate. However, these results did not consider the sex ratio from individual bull sperm characteristics. In general, the conventional sperm sorting technology produces 12 to 20×10^6 sperm/h for X or Y sperm in cattle, sheep, swine and horses (Rath and Johnson, 2008). These differences of sort efficiency are induced from property of flow cytometry, which developed MoFlo[®] suitable sperm sorting or developed form of analyzer combine with sorter function. Present study used the FACsAria II model for sperm sorting because it is commonly used in many institutions in various research sectors of Korea. However, FACs Aria model has a limitation for sorting of sperm cells, because it has inadaptable nozzle structure induced an error in sperm orientation and read the sperm cells for sorting procedure during sperm flow for hydrodynamic focusing. Therefore, it produced error of miss matched for sorting accuracy between monitor program linked flow cytometry and sex ratio of embryo produced in IVF using sexed semen by PCR.

As a general rule, for optimal results when sorting large or fragile cells, a larger nozzle size and lower pressure should be used. To increase throughput and yield when sorting smaller or less fragile cells, a smaller nozzle size and higher pressure can be utilized. However, these conditions negatively influenced the sperm in Hanwoo when the nozzle size was changed from 100 μm to 70 μm due to a low flow rate. Consequently, the sorted sperm immediately died after sorting via flow cytometry.

Several factors prevent the widespread application of this methodology at present. First, there is a limitation on the number of sperm that can be sorted in a reasonable period of time for use in standard *in vitro* fertilization and artificial insemination. A second limitation is the high embryo mortality presumed to be related to sperm damage, unknown factors or inappropriate IVF protocols. Finally, sperm sex-sorting method has a considerably lower sorting efficiency related to the cost of using flow cytometry.

In conclusion, this study suggests that sperm sorting technology cannot use a UV laser beam. Also, consideration should be given to the fact that this is the first time that a method of this kind has been used in Hanwoo. However, further study is must needed to determine the optimal procedures and improve the nozzle to enhance sorting accuracy or efficiency.

ACKNOWLEDGEMENT

The authors are grateful to researcher Joung-Hee Park, Biotechnology Analysis Lab, Central Laboratory Kangwon National University, Korea and gratefully acknowledges the Hoengseong Livestock Cooperative and Institute of Animal Resources of Kangwon National University for providing the semen samples and analysis.

REFERENCES

- Buss H. 2005. Improvement of the freeze ability of sex-sorted stallion spermatozoa. *Anim. Reprod. Sci.* 89:315-318.
- Catt SL, Sakkas D, Bizzaro D, Bianchi PG, Maxwell WMC and Evans G. 1997. Hoechst staining and exposure to UV laser during flow cytometric sorting does not affect the frequency of detected endogenous DNA nicks in abnormal and normal human spermatozoa. *Mol. Hum. Reprod.* 3:821-825.
- Choi SG, Bae MS, Lee, Kim OS, Kim BK, Yang JH, Jeon CE, Kim HH, Hwang YJ, Lee ES and Kim DY. 2009. Amplification of porcine SRY gene for sex determination. *Asian-Aust. J. Anim. Sci.* 22:1107-1112.
- Cran DG, Johnson LA, Miller NG, Cochrane D and Polge C. 1993. Production of bovine calves following separation of X- and Y-chromosome bearing sperm and *in vitro* fertilisation. *Vet. Rec.* 132:40-41.
- Gillan L, Evans G and Maxwell WM. 1997. Capacitation status and fertility of fresh and frozen-thawed ram spermatozoa. *Reprod. Fertil. Dev.* 9:481-487.
- de Graaf SP, Evans G, Maxwell WM, Downing JA and O'Brien JK. 2007. Successful low dose insemination of flow cytometrically sorted ram spermatozoa in sheep. *Reprod. Domest. Anim.* 42:648-653.
- Hollinshead FK, O'Brien JK, Maxwell WM and Evans G. 2002. Production of lambs of predetermined sex after the insemination of ewes with low numbers of frozen-thawed sorted X- or Y-chromosome-bearing spermatozoa. *Reprod. Fertil. Dev.* 14:503-508.
- Hollinshead FK, Gillan L, O'Brien JK, Evans G and Maxwell WM. 2003. *In vitro* and *in vivo* assessment of functional capacity of flow cytometrically sorted ram spermatozoa after freezing and thawing. *Reprod. Fertil. Dev.* 15:351-359.
- Hollinshead FK, O'Brien JK, Gillan L, Meyers M, Maxwell WM and Evans G. 2004a. Liquid storage of flow cytome-

- trically sorted ram spermatozoa. *Theriogenology* 62:587-605.
- Hollinshead FK, O'Brien JK, Maxwell WM and Evans G. 2004b. Assessment of *in vitro* sperm characteristics after flow cytometric sorting of frozen-thawed bull spermatozoa. *Theriogenology* 62:958-968.
- Johnson LA, Flook JP and Hawk HW. 1989. Sex preselection in rabbits: Live births from X and Y sperm separated by DNA and cell sorting. *Biol. Reprod.* 41: 199-203.
- Johnson LA. 1995. Sex preselection by flow cytometric separation of X and Y chromosome-bearing sperm based on DNA difference: a review. *Reprod. Fertil. Dev.* 7:893-903.
- Johnson LA and Welch GR. 1999. Sex preselection: high-speed flow cytometric sorting of X and Y sperm for maximum efficiency. *Theriogenology* 52:1323-1341.
- Johnson LA, Welch GR and Rens W. 1999. The Beltsville sperm sexing technology: high-speed sperm sorting gives improved sperm output for *in vitro* fertilization and AI. *J. Anim. Sci.* 77:213-220.
- Kageyama S, Yoshida I, Kawakura K and Chikuni K. 2004. A novel repeated sequence located on the bovine Y chromosome: Its application to rapid and precise embryo sexing by PCR. *J. Vet. Med. Sci.* 66:509-514.
- Lu KH, Cran DG and Seidel GE Jr. 1999. *In vitro* fertilization with flow-cytometrically- sorted bovine sperm. *Theriogenology* 52:1393-1405.
- Maxwell WM and Johnson LA. 1997. Chlorotetracycline analysis of boar spermatozoa after incubation, flow cytometric sorting, cooling, or cryopreservation. *Mol. Reprod. Dev.* 46:408-418.
- Maxwell WM, Long CR, Johnson LA, Dobrinsky JR and Welch GR. 1998. The relationship between membrane status and fertility of boar spermatozoa after flow cytometric sorting in the presence or absence of seminal plasma. *Reprod. Fertil. Dev.* 10:433-440.
- Maxwell WM and Johnson LA. 1999. Physiology of spermatozoa at high dilution rates: the influence of seminal plasma. *Theriogenology* 52:1353-1362.
- Maxwell WM, Evans G, Hollinshead FK, Bathgate R, De Graaf SP, Eriksson BM, Gillan L, Morton KM and O'Brien JK. 2004. Integration of sperm sexing technology into the ART toolbox. *Anim. Reprod. Sci.* 82-83:79-95.
- Rath D and Johnson LA. 2008. Application and commercialization of flow cytometrically sex-sorted semen. *Reprod. Dom. Anim.* 43(suppl. 2):338-346.
- Seidel GE Jr, Schenk JL, Herickhoff LA, Doyle SP, Brink Z, Green RD and Cran DG. 1999. Insemination of heifers with sexed sperm. *Theriogenology* 52:1407-1420.
- Seidel GE Jr and Garner DL. 2002. Current status of sexing mammalian spermatozoa. *Reproduction* 124:733-743.
- Shim DY, Yoo SJ, Kang HS, Yu JM, Lee CK and Kang SG. 2001. Discontinuous percoll gradients enrich X-bearing porcine sperms and female. *Dev. Reprod.* 5:47-52.
- Spinaci M, De Ambrogi M, Volpe S, Galeati G, Tamanini C and Seren E. 2005. Effect of staining and sorting on boar sperm membrane integrity, mitochondrial activity and *in vitro* blastocyst development. *Theriogenology* 64:191-201.
- Watson PF. 1995. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. *Reprod. Fertil. Dev.* 7:871-891.