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A Study of a Method to Assess the Purity of Sorted Bovine Semen Using Rapid Single-Sperm Sexing PCR

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Abstract: Sort reanalysis using flow cytometry is the most common method for determining the purity of X or Y enriched semen. The high cost of this technique (including the required expensive, proprietary machine) limits efforts to improve the technique and to promote develop applications for the sorted semen. In this study, the sperm sex (the presence of the X or Y chromosome) was identified by both rapid PCR and flow cytometry reanalysis. The rapid PCR results showed that the percentages of X and Y sperm were 48 and 52% in unsorted semen, 92 and 8% in X-enriched semen and 17 and 83% in Y-enriched semen, respectively. Reanalysis of the DNA content of the sorted samples revealed that the X and Y sperm frequencies were 92 and 8% in X-enriched semen and 15 and 85% in Y-enriched semen, respectively. The sex ratio of unsorted semen analyzed by PCR did not significantly deviate from the expected ratio of 1:1 and there was no significant difference between the sex ratios of sorted semen samples determined by PCR and flow cytometry reanalysis. These results indicate that we have established an effective, reliable and rapid PCR method to verify the purity of sorted semen. This method should contribute greatly to the improvement of sperm sorting techniques and the development of applications for sorted semen.

Key words: Single sperm, sex typing, rapid PCR, bovine semen, cytometry, sorted semen

INTRODUCTION

The sex of an animal plays an important economic role in animal production, especially in the cattle industry (Welch and Johnson, 1999; Johnson, 2000; Checa *et al*., 2002; Habermann *et al*., 2005). For example, milking traits are only expressed in female dairy cattle and male animals grow much more quickly than females. Because the sex of the offspring is determined by the X or Y chromosome carried by the sperm, artificial insemination with X-enriched semen is favored by dairy cattle farmers and artificial insemination with Y-enriched semen is favored by breeders of breeding bulls (Seidel, 2007).

Flow cytometry has developed into a mainstream technique for sex selection in animal breeding programs (Welch and Johnson, 1999; Johnson, 2000; Garner, 2001, 2006; Habermann *et al*., 2005). The efficiency of sex control relies on the purity of the sorted semen which is validated primarily by sort reanalysis using flow cytometry (Johnson, 2000; Yan *et al*., 2006). However, this method consumes the valuable time of the sorting equipment and requires relatively large samples (at least 50,000 sperm) (Welch and Johnson, 1999; Johnson *et al*., 2005). Sort reanalysis uses the same resources as those used for the initial sorting (sperm sorter, DNA and Hoechst 33342 dye) which is another drawback of this method (Welch and Johnson, 1999). Finally, the dependence of this technique on a high-cost instrument makes purity determination feasible in only a few laboratories. To improve the sorting method and to facilitate the development of applications for sorted semen, a convenient validation method independent of the measurement of the DNA content is needed (Habermann *et al*., 2005).

To avoid the need for specialized equipment and to reduce the cost of semen purity analysis, Fluorescence *In Situ* Hybridization (FISH) has been used to determine the purity of sorted semen by determining the sexes of single

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sperm. Although, the detection efficiency of FISH has often approached 100% (Rens *et al*., 2001; Habermann *et al*., 2005) this method is both time and labor intensive. The single-sperm PCR method is more attractive than FISH (Zhang *et al*., 1992; Zhao *et al*., 2000). The cost-effectiveness in herent simplicity, reliability, built-in amplification, contamination control and wide use with other techniques make PCR a valuable technique for use in single-sperm sexing. Duplex nested PCR and primer extension preamplification-PCR with primers specific to the sex determining gene (SRY) have been commonly used and have produced accurate results (Welch *et al*., 1995; Tominaga, 2004). Especially important when the semen is particularly valuable or only available in a limited quantity, accurate determination of the purity of the sorted semen can be obtained by PCR-based methods using approximately 100-200 single sperm (Welch *et al*., 1995; Welch and Johnson, 1999). If the amplification time could be shortened, the efficiency of detection would be improved and rapid PCR could be easily adapted to the sexing of single blastomeres from embryos or cultured bovine cell lines or to meat quality testing.

The study investigated rapid PCR as a quick and efficient method of evaluating the purity of sorted semen to provide an alternative to sorting reanalysis and to help optimize sperm sorting methods and facilitate the development of applications for sorted semen. In this study, the semen was sorted, single sperm from the sorted and unsorted semen samples were sexed with rapid PCR, the sexing results were evaluated using sort reanalysis with flow cytometry and differences between the outcomes of these two methods were statistically analyzed.

MATERIALS AND METHODS

Collection and preparation of materials: The DNA templates for the control group analyses were prepared from anticoagulant-treated blood samples (taken in the institute) from male and female Holsteins. Semen was collected from bulls used for artificial insemination at the XY Breeding Livestock Co. in Tianjin, China, according to their semen collecting protocol. The semen was either sorted or sperm were isolated for single-sperm sex typing. DNA templates were prepared from the blood samples using the alkaline lysis method. The animals were maintained on concentrate and green fodder and hay and water were provided *ad libitum*. Animal welfare and experimental procedures were strictly in accordance with the guide for the care and use of animals and the related ethical regulations of the Institute of Animal Science, Chinese Academy of Agricultural Sciences according to internationally accepted principles.

The separation of X and Y sperm

Staining of sperm: Fresh semen samples with sperm motility >70%, sperm density no $<10^9$ sperm mL^{-1} and a sperm deformation rate no >15% were used for single-sperm sorting by flow cytometry. After 0.65 mL fresh semen was diluted with 1.3 mL semen dilution in a 5.0 mL Falcon tube, 45 μL of 2 mg mL^{-1} Hoechst 33342 fluorescent dye (Invitrogen, USA) was added, a 2 mL sperm solution with a concentration of 3.0×10^8 sperm mL^{-1} was obtained. This solution was incubated at 35°C for 1 h in the dark and then 2 mL of Tyrode's Albumin Lactate Pyruvate (TALP) with 4.0% egg yolk and 0.1% red food dye (FD and C #40) was added to depigment the remaining dead sperm. Finally, the sample was filtered through a 30-Inylon mesh to exclude dead sperm and impurities.

Separation of X and Y sperm: Sperm were sorted based on the presence of the X and Y chromosomes using a 70 μm nozzle and a 150 mW UV laser. The sorting rate was 4500-6000 sperm sec^{-1} with an accuracy of 92%. The sorted sperm samples were collected in two tubes containing 2 mL of TEST yolk buffer (TES-Tris (TEST) yolk solution was prepared with 43.25 g TES, 10.265 g Tris and 10 g glucose in 1 L distilled water and with the addition of 20% egg yolk). Sperm were collected and subjected to centrifugation at 800 g for 10 min. The sperm pellet was resuspended in a 0.25 mol L^{-1} sucrose solution and kept at 4°C for later analysis.

Single sperm isolation and lysis: A 0.5% agarose solution was prepared with 0.1 g low-melting agarose and 20 mL ddH_2O. A 2 μL sperm suspension with a concentration of 1×10^7 sperm mL^{-1} was mixed with the agarose solution. When the temperature of the solution fell to 37°C, the agarose-sperm mixture was poured into a 10 cm diameter culture plate to form a gel approximately 0.25 mm thick. The agarose gel was then dried at 37°C until solid.

The congealed agarose plate was examined by inverted fluorecence microscope at 200×magnification to locate single sperm. Each single sperm was excavated using a tiny scoop, placed into a PCR tube and centrifuged at 800 g for 1 min. Then 1 μL lysis solution (500 KOH and 125 mmol L^{-1} DTT) was added to each tube and the sperm was lysed for 10 min at room temperature. Next, 2.5 μL of neutralizing buffer (900 mmol L^{-1} Tris HCl, pH 8.3) was added and the sample was immediately amplified by PCR amplification.

PCR sex typing

Primer design: Using the GenBank sequences of the bovine SRY gene (gi: 4878004) and chromosome 3 (gi: 119890289), the sex-specific primers Y12F and R and

internal standard primers C34F and R were designed, respectively with Oligo 6.0 software. The sex-specific primers (Y12F: 5'-CTA CTA GAC ATA CAC CGA GAC-3' and Y12R: 5'-CCG TGC TGC CAA TGT TAC CT-3') produce a 294 bp Y-chromosome band and the control primers (C34F: 5'-TTG CTG CTC TTG CCT TTG CTT-3' and C34R: 5'-GTC CAC CTG CCA CAA CTA AAT-3') produce a 208 bp control band. The primers were synthesized by Invitrogen (Shanghai, China).

Rapid PCR conditions and detection: After sperm lysis and neutralization, DNA from each single sperm was amplified by rapid PCR using a Mastercycler Thermal Cycler (Eppendorf, Germany). The PCR reaction included 1.5 μL of 10X buffer, 0.6 μL of 62.5 mM Mg^{2+}, 5 μL of 1 mM dNTP, 2.25 μL of 2 μM Y12 primers, 2.25 μL of 2 μM C34 primers, 0.7 μL of 2.5 U μL^{-1} Taq enzyme and sufficient ddH_2O to reach a final volume of 25 μL. The rapid PCR program consisted of a preheating stage at 94°C for 30 sec, 29 cycling stages and a final extension stage at 72°C for 1 min. The cycling stages consisted of denaturation for 1 sec at 94°C, annealing for 1 sec at 51°C and extension for 1 sec at 72°C. The rapid PCR products were detected by 2.5% agarose gel electrophoresis.

Reanalysis of the purity of sorted semen: The tails of the sorted X and Y sperm (about 2,000,000 of each in 0.25 mL TALP) were removed by sonication (power: 60 W; frequency: 12 times min^{-1}; duration: 5 sec). Then, 1 μL of 0.5 mg mL^{-1} Hoechst 33342 was added to the suspension which was incubated at 35°C for 15 min to resaturate the sperm DNA. The settings of the flow cytometer used in the reanalysis were the same as those used in sorting (above). The sperm were subjected to flow cytometry at an event rate of 100 sperm sec^{-1} and 10,000 signals were collected. A Gaussian curve was fitted to the DNA distribution of the sorted sperm to determine the percentages of X and Y sperm.

Statistical analysis: The purity of the X and Y enriched semen samples determined by PCR analysis was compared with that determined by flow cytometry reanalysis and a Chi-squared test using SAS 8.2 software was used to determine whether the measured X:Y sperm ratio determined using single-sperm typing was significantly different from the expected ratios of unsorted semen and X and Y enriched semen.

RESULTS AND DISCUSSION

Sorting results from flow cytometry: The flow cytometry sorting results for the X and Y sperm are shown in Fig. 1. The dead sperm (R5 in Fig. 1a) which took up the red food dye were gated out from the live sperm based on their lower fluorescence intensity. Properly oriented live sperm were gated (R1 in Fig. 1a) and sorted into X sperm (right peak in Fig. 1b) and Y sperm (left peak in Fig. 1b), according to their fluorescence intensity.

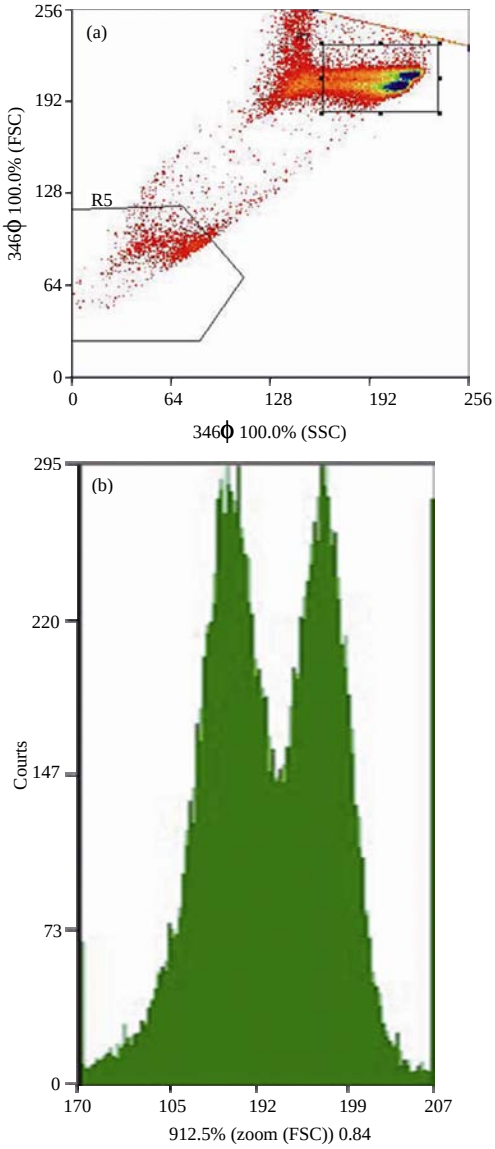

Fig. 1: Flow cytometry sorting of X and Y sperm; (a): The dot plot distribution of sperm during flow cytometry sorting. R1 represents properly oriented live sperm. R5 represents the dead sperm that took up red food dye and showed a diminished fluorescence intensity. (b): The histogram of the distribution of the DNA content of the properly oriented live sperm. The left peak is the Y sperm population and the right peak is the X sperm population

Determination of the sex of single sperm using rapid PCR

Sex-typing results from blood DNA and single sperm: The amplification results shown in Fig. 2 shows that both a 294 bp sex-specific (Y chromosome) band and a 208 bp control band were obtained from male blood samples and only the 208 bp control band was obtained from female blood samples. The expected results were obtained from all blood samples. The electrophoresis results in Fig. 2 also show that both the sex-specific and the control bands were obtained in one single-sperm lane as for the male blood samples whereas only the control band was obtained in the other two single-sperm lanes as for the female blood samples. The amplification results of all of the samples could be clearly identified using gel electrophoresis.

Sex determination results of single sperm from unsorted semen using rapid PCR: A total of 102 single sperm isolated from unsorted semen were lysed and amplified using rapid PCR and representative amplification results are shown in Fig. 3. In total, 93 sperm were successfully typed the detailed results are shown in Table 1. The statistical analysis revealed that X sperm constituted 48% of the unsorted semen sample which did not deviate significantly from the theoretical frequency of 50%. Meanwhile, the total amplification time was shortened to 44 min and the ideal amplification results can be obtained using this rapid PCR from a single sperm (only one copy of DNA template). These results indicate that this PCR-based method was reliable for sex identification of single sperm.

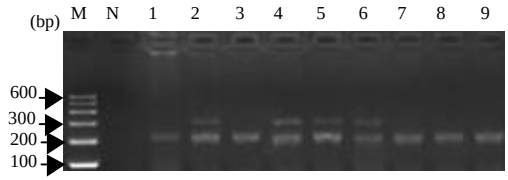

Fig. 2: Sexing results from the blood DNA templates and single sperm; Lanes 1-3: sexing results for single sperm; lanes 4-6: sexing results from bull blood samples; lanes 7-9: sexing results from cow blood samples; M: molecular marker I; N: negative control

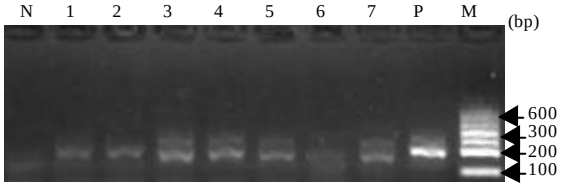

Fig. 3: Representative sex determination results from single, unsorted sperm using rapid PCR; Lanes 1-7: sexing results from single sperm; lanes 1, 2 and 6: X sperm; lanes 3, 4, 5 and 7: Y sperm; P: positive control (sexing result from bull blood DNA sample); N: negative control; M: molecular marker I

Sex typing results of single sperm from X-enriched semen using rapid PCR: A total of 143 single sperm isolated from X-enriched semen were lysed and sex identification was performed by rapid PCR; the detailed results are shown in Table 1. In total, 128 single sperm were successfully typed using rapid PCR. The statistical analysis revealed that X sperm constituted 92% of the X-enriched semen sample which did not deviate from the reanalysis frequency of 92%.

Sex typing results of single sperm from Y-enriched semen using rapid PCR: A total of 121 single sperm isolated from Y-enriched semen were lysed and amplified using rapid PCR. Representative amplification results are shown in Fig. 4 detailed results are shown in Table 1. In total, 106 single sperm were successfully typed using rapid PCR. Y sperm constituted 83% of the Y-enriched semen sample which did not deviate significantly from the reanalysis frequency of 85%.

Flow cytometry reanalysis of DNA content to determine the purity of the sorted sperm: Flow cytometry reanalysis of DNA to determine the purity of the X or Y enriched

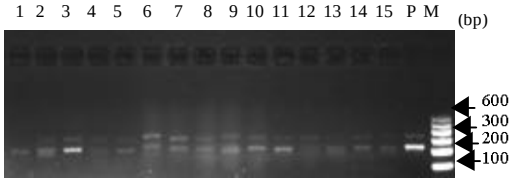

Fig. 4: Representative sex typing results from single sperm from Y-enriched semen using rapid PCR; Lanes 1-15: sexing results from single sperm; lane 1: X sperm; lanes 2-15: Y sperm; P: positive control; M: molecular marker I

Table 1: The statistical results of the X sperm frequency in unsorted, X-enriched and Y-enriched semen using rapid PCR and sort reanalysis

Samples	No. of sperm excavated	No. of X sperm	No. of Y sperm	No. of undetected sperm	Fraction of X sperm	p value	Fraction of Y sperm	p-value	Fraction of X sperm by reanalysis
Unsorted sperm	102	45	48	9	0.48	0.84	0.52	0.84	0.50
Sorted X sperm	143	118	10	15	0.92	1.00	0.08		0.92
Sorted Y sperm	121	18	88	15	0.17		0.83	0.66	0.85

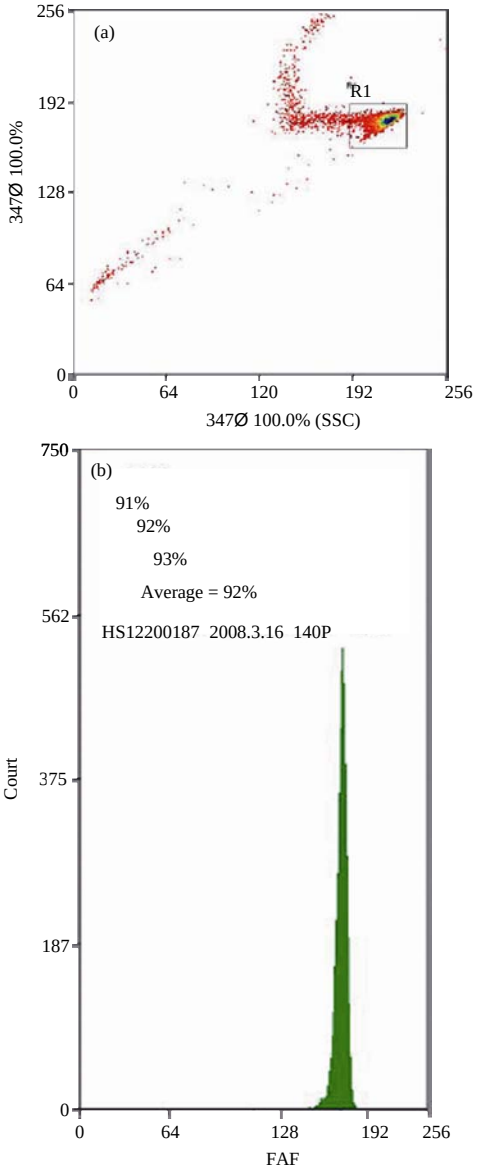

Fig. 5: Flow cytometry reanalysis of DNA content to determine the purity of the X-enriched sperm; (a): A dot plot with the properly oriented sperm nuclei in R1 gated for reanalysis; (b): A histogram of DNA content showing a major peak on the right (X sperm) and a minor shoulder on the left (Y sperm) in the X-enriched sample

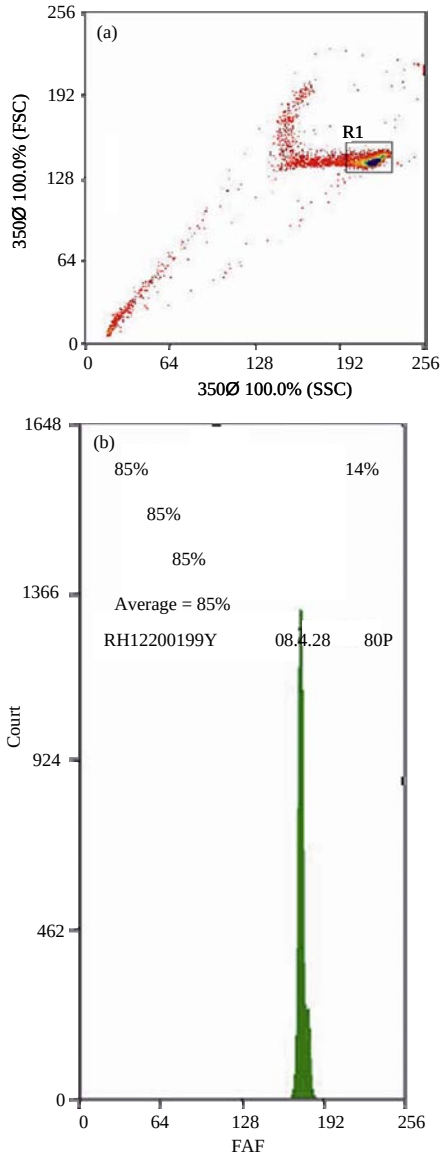

Fig. 6: Flow cytometry reanalysis of DNA content to determine the purity of the Y-enriched sperm. (a): A dot plot with the properly oriented sperm nuclei in R1 gated for reanalysis; (b): A histogram of DNA content showing a major peak on the left (Y sperm) and a minor shoulder on the right (X sperm) in the Y-enriched sample

semen was repeated 3 times. The results revealed that there was a single major peak for the X-sorted and Y-sorted semen samples.

The average purity of X sperm in the X-enriched samples was 92% (Fig. 5) whereas the average purity of Y sperm in Y-enriched samples was 85% (Fig. 6).

Difficulty of sperm lysis: To successfully transfer the genetic information to the next generation, sperm develop tougher cell structures than do somatic cells making sperm more difficult to lyse. Therefore, a double lysis technique was used in previous studies. The sperm were lysed by strong reducing agents followed by the addition of alkaline solution. Finally, a neutralizing solution is added and the DNA is amplified by PCR (Cui, 1997). In

this study, the traditional lysis method was improved and better PCR results were obtained using a one-step lysis method that took only approximately 10 min. The failed detection of a few samples was most likely due to the loss of the sperm during isolation. The size of the agarose gel significantly influenced the concentration of the lysis solution which was diluted by large gels, resulting in failure to lyse the sperm.

Reducing the transition time between temperature and the cycle times improves single-sperm PCR: Due to the limitations associated with lower DNA quantities and the influence of the extreme pH of the lysis solution on PCR, multiple rounds of amplification by primer extension preamplification-PCR (Zhang *et al*., 1992; Zhao *et al*., 2000) or nested PCR (Welch *et al*., 1995; Cui, 1997) have been used to amplify sperm DNA. Although, ideal results could be obtained the goal of fast detection has not yet been realized and the risk of contamination is higher with these procedures. Moreover, the interactions among multiple primers in duplex PCR are stronger than those in a simple PCR system (Liguori *et al*., 2007). In this study, there were more complex factors that intensified the interactions between primers such as the small amount of template DNA (one sperm) and the stronger lysis solution used to overcome tough sperm membranes.

To overcome the shortfalls of PCR for the determination of the purity of sexed semen, the use of quantitative real-time PCR has been explored (Parati *et al*., 2006). The DNA template was prepared from sexed semen and a special quantitative fluorescence PCR instrument was used. There was an inevitable deviation from the actual samples due to the loss of template during DNA preparation; costs and labor were also increased. The amplification efficiency and specificity however, were increased when the transitions between temperatures and the periods of denaturation, annealing and extension were shortened (Wittwer *et al*., 1990; Cha *et al*., 1992; Eggerding, 1995). In the study, each cycle step was shortened to 1 sec. As a result, the total amplification time was shortened to 44 min and the efficiency and specificity were improved (Welch *et al*., 1995; Welch and Johnson, 1999). Additionally, the study used a random sample from a large number of sperm and the statistical results in Table 1 indicate that only a small number of sperm were lost. Therefore, the test results are more accurate and reliable than those of other groups.

CONCLUSION

Despite being limited by the low amount of DNA template and strongly alkaline conditions, one-step lysis and rapid PCR for single-sperm sexing were successfully used in this study. The overall length of lysis and amplification was reduced to <1 h and the cost was also greatly reduced. More importantly, the determination of the purity of the sorted semen did not require the use of expensive, proprietary equipment and could be performed in an ordinary laboratory. As the use of sorted semen becomes more popular in animal production, this robust method will be helpful to the improvement of the previous sorting methods and the exploration of the new sorting techniques and will promote the use of X and Y sorted semen in animal husbandry. It would be worthwhile for X and Y enriched semen production companies to integrate single-sperm sexing methods into conventional flow-sorting procedures. The single-sperm sex PCR method presented herein should contribute to the improvement of detection methods in many fields such as forensic medicine, clinical medicine and sex identification.

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REFERENCES

Cha, R.S., H. Zarbl, P. Keohavong and W.G. Thilly, 1992. Mismatch amplification mutation assay (MAMA): Application to the c-H-ras gene. PCR Methods Appl., 2: 14-20.

Checa, M.L., S. Dunner and J. Canon, 2002. Prediction of X and Y chromosome content in bovine sperm by using DNA pools through capillary electrophoresis. Theriogenology, 58: 1579-1586.

Cui, K.H., 1997. Size differences between human X and Y spermatozoa and prefertilization diagnosis. Mol. Hum. Reprod., 3: 61-67.

Eggerding, F.A., 1995. A one-step coupled amplification and oligonucleotide ligation procedure for multiplex genetic typing. PCR Methods Appl., 4: 337-345.

Garner, D.L., 2001. Sex-sorting mammalian sperm: Concept to application in animals. J. Androl., 22: 519-526.

Garner, D.L., 2006. Flow cytometric sexing of mammalian sperm. Theriogenology, 65: 943-957.

Habermann, F.A., A.Winter, I. Olsaker, P. Reichert and R. Fries, 2005. Validation of sperm sexing in the cattle (*Bos taurus*) by dual colour fluorescence in situ hybridization. J. Anim. Breed. Genet., 122: 22-27.

Johnson, L.A., 2000. Sexing mammalian sperm for production of offspring: The state-of-the-art. Anim. Reprod. Sci., 60-61: 93-107.

Johnson, L.A., D. Rath, J.M. Vazquez, W.M.C. Maxwekk and J.R. Dobrinsky, 2005. Preselection of sex of offspring in swine for production: Current status of the process and its application. Theriogenology, 63: 615-624.

Liguori, G., A. Lucariello, G. Colella, A. de Luca and P. Marinelli, 2007. Rapid identification of Candida species in oral rinse solutions by PCR. J. Clin. Pathol., 60: 1035-1039.

Parati, K., G. Bongioni, R. Aleandri and A. Galli, 2006. Sex ratio determination in bovine semen: A new approach by quantitative real time PCR. Theriogenology, 66: 2202-2209.

Rens, W., F. Yang, G. Welch, S. Revell and P.C. O'Brien *et al*., 2001. An X-Y paint set and sperm FISH protocol that can be used for validation of cattle sperm separation procedures. Reproduction, 121: 541-546.

Seidel, Jr., G.E., 2007. Overview of sexing sperm. Theriogenology, 68: 443-446.

Tominaga, K., 2004. Cryopreservation and sexing of *in vivo*- and *in vitro*-produced bovine embryos for their practical use. J. Reprod. Dev., 50: 29-38.

Welch, G.R. and L.A. Johnson, 1999. Sex preselection: Laboratory validation of the sperm sex ratio of flow sorted X- and Y-sperm by sort reanalysis for DNA. Theriogenology, 52: 1343-1352.

Welch, G.R., G.C. Waldbieser, R.J. Wall and L.A. Johnson, 1995. Flow cytometric sperm sorting and PCR to confirm separation of X- and Y-chromosome bearing bovine sperm. Anim. Biotechnol., 6: 131-139.

Wittwer, C.T., G.C. Fillmore and D.J. Garling, 1990. Minimizing the time required for DNA amplification by efficient heat transfer to small samples. Anal. Biochem., 186: 328-331.

Yan, J., H.L. Feng, Z.J. Chen, J. Hu, X. Gao and Y.Qin, 2006. Influence of swim-up time on the ratio of X- and Y-bearing sperm. Eur. J. Obstetrics Gynecol. Reprod. Biol., 129: 150-154.

Zhang, L., X. Cui, K. Schmitt, R. Hubert, W. Navidi and N. Arnheim, 1992. Whole genome amplification from a single cell: Implications for genetic analysis. Proc. Natl. Acad. Sci. USA., 89: 5847-5851.

Zhao, S., K. Li, M. Yu and Z. Peng, 2000. Ordering three microsatellites on porcine chromosome 12 by single sperm typing. Anim. Biotechnol., 11: 45-49.