JSAR Innovative Technology Award

Sex Preselection in Bovine by Separation of X- and Y-Chromosome Bearing Spermatozoa

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Abstract. Flow cytometrically-sorted sperm has been involved in the production of sex preselected offspring. More than 30,000 bovine offspring have been produced using AI and other means using spermatozoa separated by flow cytometer. Flow cytometric sperm sorting based on differences in their DNA content is the best method for separation of X- and Y-chromosome bearing spermatozoa. At first, flow cytometers were modified for DNA confirmation and sorting of sperm with high resolution. The beveled insertion needle can regulate orientation of flat-shaped bull sperm heads. The forward fluorescence detector is essential for measuring the DNA content of sperm. Recently, high-speed sperm sorting with orienting nozzles has resulted in production of 90% pure X- and Y-sperm at rate of 15–20 million sperm per hour. Application of this new technique will enable conduct of more conventional technologies for both artificial insemination and cryopreservation in the bovine and in other farm animals using X- or Y-sperm.

Key words: Bovine, DNA, Flow cytometer, Sex preselection, Spermatozoa

(*J. Reprod. Dev.* 53: 27–38, 2007)

In bovine, females are essential for milk production and production of calves, while males are usually desired for beef production because they grow more efficiently. In addition to the faster genetic progress obtained through use of separated X- or Y-sperm or embryos, there are also additional advantages for the management and efficiency of livestock production. There has been great interest in sex preselection in the bovine, and there are clear economic and management advantages to be gained. There are also indirect benefits, such as management of future needs and greater control over genetic improvement.

As with the utilization of most assisted reproductive technologies, including IVF, ICSI, and ET, separated X-chromosome bearing sperm (X-sperm) or Y-chromosome bearing sperm (Y-sperm) for production of bovine with preselected sex is

dependent on economics and efficiency of use. These factors are the keys to widespread use of separated X- or Y-sperm, whether by means of IVF or AI with low numbers of sperm. At present, economics and ease of use remain goals to be attained since low-dose AI has not been perfected. Since the first report of utilizing DNA as a marker for separating X-sperm from Y-sperm [1] in which more than 50 rabbits were born of the predicted sex, an estimated 30,000 animals have been born worldwide using this method.

It is known that sex is determined by the presence or absence of the Y chromosome in mammals. Most of initial studies have been attempted to identify either X- and Y-sperm [2] or the sex of embryos [3]. In the embryo, both sexes can be produced by fertilization, and sex is determined by the chromosomes of the sperm. In the process of spermatogenesis, X-sperm and Y-sperm can be produced. Therefore, the most effective and convenient method for sex control is

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Accepted for publication: November 2, 2006

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based on the use of separated X-sperm or Y-sperm. Sex predetermined offspring using X- or Y-sperm separated using a flow cytometric cell sorter have been born from rabbits [1] and pigs [4] by surgical artificial insemination, sheep [5] and cattle [6] by intra- cytoplasmic sperm injection, and cattle [7] and pigs [8] by *in vitro* fertilization techniques. Most births have been in bovine since the technology is used commercially in that species. Production of sex preselected calves on a wide commercial basis will require additional work to optimize and standardize bovine production systems with separated X- or Y-sperm.

Separation and confirmation of X- and Y-sperm and use of flow cytometer for separation of X- and Y-sperm in the bovine are presented in this paper.

X- and Y-Sperm Separation

Plenty of research has been devoted to separation of X- and Y-sperm by assumptive differences. The physical characteristics of sperm are a potential means of distinguishing X- from Y-sperm. Burstein and Schenker [9] reported that a sex ratio of 73% males was obtained in a human sperm population treated with an albumin gradient method, which was modified from Ericsson *et al.* [10]. Clinical data compiled from various laboratories around the world suggests that about 75% of the offspring obtained from this method are male [11]. However, Brandriff *et al.* [12] subsequently reported 43% Y-sperm in samples treated with albumin gradient and 50% Y-sperm in untreated samples. Ueda and Yanagimachi [13] found an average of 48% Y-sperm in treated samples, and this was no different from the untreated control samples. Beal *et al.* [14] reported that they obtained 45% males from inseminations with semen treated with albumin gradient and 54% males from inseminations with control semen; those results were confirmed using was a flow cytometer. They concluded that treated samples were not enriched Y-sperm. Amann [15] suggested that the Ericsson's procedure might not produce a sample enriched with Y-sperm, but rather that it might differentially alter some nature of either Y- or X-sperm, such that there is greater inclination for Y-sperm to fertilize human oocytes that complete gestation.

Several investigators have attempted to use antibodies against the H-Y antigen [16], which

might be localized on the plasma membrane over the head and middle piece of the sperm [17]. Zavos [18] also used monoclonal H-Y antibody in separation with an immunofiltration column. Theoretically, however, the amount of H-Y antigen in X- and Y-sperm is considered to be similar. There is data [19, 20] showing that human sperm can be successfully separated into two distinct populations by free-flow electrophoresis, which is based on differences in surface charge. However, there may be combined effects of surface charge and swimming characteristics when applied to separation of motile sperm. Amann [15] claimed that the difference in surface charge between X- and Y-sperm could not be explained by modification after exposure to epididymal fluid and/or seminal plasma.

Iizuka [21] reported that female babies were born after artificial insemination with semen collected from the bottom fraction of eight layers of Percoll gradient. Upreti *et al.* [22] were unable to isolate bovine X- and Y-sperm after centrifugation when using a continuous Percoll gradient method. Hamano and Uchiyama [23] confirmed by chromosome analysis of sperm penetrated into zona-free hamster oocytes that there is no difference in the X- and Y-sperm ratio of the bull semen collected from bottom fraction separated by 8 fractions of Percoll density gradient centrifugation. As described above, there has been little reliable evidence to support the claims made for various physical, morphological, or non-DNA based separation procedures. The majority of attempts using these methods have ended in failure [24].

Flow Cytometer Modification and X- and Y-Sperm Separation

In mammals [25], it is known that there are differences in the DNA content of X- and Y-sperm because the X chromosome is larger than the Y chromosome; in the bull, there is a 3 to 4% difference [26] as shown in Fig. 1. It has been reported that there is a difference in the DNA content of X- and Y-sperm nuclei from European breeds (*Bos taurus*) and animals originating in Asia (*Bos indicus*) based on the differing sizes of the Y-chromosomes of these two species. Subtle X-Y differences, however, have also been observed in

Fig. 1. Karyotyping of chromosomes in bull spermatozoa. The total length of chromosomes of bovine X-chromosome bearing spermatozoa is about 3.8% greater than that of Y-chromosome bearing spermatozoa.

the sperm nuclei within a species; sperm from Jersey bulls exhibited a greater X-Y difference than those from Holstein or Hereford bulls [26, 27]. X-sperm has more DNA content than Y-sperm, and the DNA difference between X- and Y-sperm varies among species; it ranges from about 2.8% in the humans to 12.5% in the creeping vole [28, 29]. In farm animals, the DNA difference between both sperms ranges from 3.5% to 4.2% [30]. Thus, the only scientifically validated and measurable difference between X- and Y-sperm is DNA content. This fact was initially reported by Gledhill *et al.* [31] who analyzed X- and Y-sperm by flow cytometry. Flow cytometry provides the ability to measure cellular DNA and the distribution of DNA. Based on this principle, flow cytometer has been used for immunological analysis and separation of somatic cells. Theoretically, flow cytometry can distinguish X-sperm from Y-sperm. Pinkel *et al.* [32] suggested that DNA

analysis to differentiate X- from Y-sperm is possible using an epi-illumination flow cytometer. Flow cytometry has successfully been applied to the differentiation of DNA in X- and Y-sperm [32–34]. Hoechst 33342 can bind to the adenine-thymine region of nucleic acids. The fluorescent signals represent both the orientation and amount of DNA content in a single sperm. Sorting was carried out by setting a sort window on 0 degree fluorescent signals out of each peak. The deflected droplets of either X- or Y-sperm fall into a collection tube. Thus, sorted sperm can be fractionated. Recently, Johnson *et al.* [35, 36] recommended that 1 μ l of food coloring, FD&C #40, be mixed into sperm suspension stained with Hoechst 33342 in order to stain dead sperm. Live sperm membranes are highly permeable to Hoechst 33342 when incubated at 35 C [34], whereas the food coloring penetrates the membranes of dead sperm and quenches the intensity of fluorescence, thus resulting in eliminating of dead sperm from the viable sperm population [35–37]. In general, propidium iodide is also used for the same purpose but has the drawback of being an intercalating dye.

Successful X- and Y-sperm separation must take into account the relative susceptibilities of gametes to staining, laser exposure, high dilution, elevation pressure, and resistance to several changes of media composition during the sorting process [38]. Fluorescence signals are emitted when Hoechst 33342-stained sperm are illuminated with the 351 and 364 nm lines of an argon laser, thereby providing an accurate measurement of the DNA content of X- and Y-chromosome bearing sperm for sorting [2, 30, 39, 40]. For sperm sorted with illumination but without staining, DNA damage increased by 2.0%, while the DNA damage of sperm sorted with staining but no illumination increased by another 1.5%. The potential DNA damage of sperm sorted with both staining and laser illumination increased by 1.5% [27]. After Hoechst-stained and UV-laser irradiated sperm nuclei were microinjected into hamster eggs, the number of DNA breaks and triradial and quadriradial exchanges between the chromosomes that developed increased slightly. In a recent study, no genotoxic effects of sorting sperm with Hoechst 33342 were found in the cells of resulting pigs [41].

McNutt and Johnson [42] demonstrated the direct damage of laser power on mammalian

Fig. 2. Modification of a beveled injection needle. The ribbon-shaped fluid stream produced by the beveled insertion needle easily maintains the sperm head orientation. For high resolution sperm DNA content, fluorescent signals should be collected from the oriented sperm head. The beveled needle was modified to provide hydrostatic pressure, resulting in laminar flow. Laminar flow provides increased fluidic pressure so that the flattened oval shape of the bull sperm head is oriented in the laminar stream.

embryo development. Johnson *et al.* [36] examined laser output powers of 25 and 125 mW during high-speed sorting and showed that 125 mW did not affect the subsequent fertilization rate or embryo development in pigs.

At that time, sorting of sperm was accomplished only in sperm heads [43] and has subsequently been developed as a successful method of producing viable sperm by flow cytometry [1]. It, however, appears to be difficult to distinguish the small differences in fluorescent intensity between X- and Y-sperm populations, because the fluorescent signal intensity can be affected more by morphology and orientation of the sperm to the laser excitation than by DNA content. Since the shape of the sperm head is flat, sperm cannot maintain their orientation in the ordinary fluid stream.

Garner summarized a comparison of the dimensions of the sperm heads of some mammals [27]. The effectiveness of utilizing DNA content differences between X- and Y-chromosome carrying sperm depends not only on relative DNA differences, but also on the ability to precisely orient these gametes at the time of measurement in the flow cytometer. Mammalian sperm with flattened, oval heads tend to be more readily oriented in a sperm sorter using hydrodynamics than gametes possessing more rounded or angular

shaped heads. Garner suggests that the easiest sperm to separate in a flow cytometer would be from the bull because the area of the head profile is $34.5 \mu\text{m}^2$ and the X-Y difference in DNA content is 3.8%, resulting in the highest sorting index of 131 [27]. Species differences exist in the rates by which living sperm can be stained with Hoechst 33342. These uptake differences are small, but the highest resolution of DNA content can only be obtained by optimizing the sperm staining procedure for each species or even individual males within a species [44].

Misorientation of the sperm head causes high refraction of fluorescent emissions. Johnson and Pinkel [33] modified a commercially acquired flow cytometer to sort sperm heads. A ribbon-shaped fluid stream produced using a beveled insertion needle easily maintained the sperm head orientation [31, 32] (Fig. 2). The emitted fluorescence from the edge of the sperm head was brighter than from the flat side. Because the edge fluorescence is used to characterize the orientation of sperm, a 90 degree fluorescence detector could gather the edge emissions of the sperm head (Fig. 3). For high resolution sperm DNA content, fluorescent signals should be collected from the oriented sperm head. An additional forward fluorescence detector is essential for measuring fluorescent emissions from the flat side of the

Fig. 3. Modified EPICS 753 flow cytometer for X- and Y-sperm separation. The system is configured with two optical detectors. In the actual sorting system, sperm that fit the proper criteria for the amount of DNA they contain are enclosed in droplets that fall through an electrostatic field. The sperm are then drawn to their respective tubes depending on the charge and subsequently fall into the appropriate collection tube.

sperm head. A modified flow cytometer could collect the emitted fluorescent signals from both the edge and flat side of a bull sperm head [22]. In each sample, about 65–95% of the sperm heads were properly oriented, whereas 20–40% of them were properly oriented to the laser beam, fell into the orientation gates and were observed by the 0 degree detector (Fig. 4). These modifications contributed to establishment of the current sperm separation technology by commercial flow cytometer (Fig. 3).

A significant improvement in needle modification was reported in 1998 by Rens *et al.* [45]. The tip of the flow cell was remolded to provide 2–3 times the orientation capability of the needle system. This novel nozzle resulted in a greater orienting percentage [35]. Rens *et al.* [45]

and Johnson *et al.* [35] suggested that hydrodynamic forces affected the duration of orientation force. An elliptical shape was molded into the interior nozzle tip just above the exit orifice. The orienting tip of the nozzle brings the orienting action much closer to the laser beam detection system, thus increasing orienting efficiency.

Efficiency of sorting for viable sperm is necessary for artificial insemination using sorted X- and Y-sperm in farm animals. For this purpose, improvement of the efficiency of sperm orientation is essential. All flow cytometers for sperm sorting in use worldwide at present have incorporated the orienting tip design [36]. The modified flow cytometer and cell sorter is essential for attaining separate populations of X- and Y-chromosome

Fig. 4. Schematic diagram of 256 selected channel histograms. Upper 2 histograms from the 90 degree detector show the gating window, selecting those sperm within their edges toward the 90 degree detector, and their flat faces toward the propagation of the laser beam and the 0 degree detector (upper left). The histogram shows the output from the signals captured by the 0 degree detector from the sperm falling within the gate window of the 90 degree detector. The DNA content of intact, living sperm (bottom left) does not resolve as well as sperm nuclei (bottom right) because their tails affect the sperm orientation.

bearing living sperm [2] on a repeatable basis. The survival of sperm after flow-sorting is extremely important because a considerable investment is made in the subject gametes. When higher proportions of sperm after sorting retain their fertility after storage, either cryopreserved and thawed or as fluid semen, fewer sperm need to be sorted for each dose. Although sorting results in elimination of dead and damaged sperm, those cells surviving the sperm sorting process tend to degenerate faster than unsorted control gametes from bulls and boars [46].

Recently, Johnson *et al.* [35, 36] developed a commercial high-speed sorter and stated that it can sort up to 2 million sperm per hour with a standard beveled needle and 6 million sperm per hour with a

orienting nozzle. There are three manufacturers offering high-speed sorters that can be adapted for sperm sorting if modified as described above [36]. Johnson [47] demonstrated the adaptation of the following three high-speed sorters as follows offered manufacturers: the MoFlo, Altra, and Facs Vantage SE. The MoFlo has been used with boar sperm in Beltsville, MD, USA, since 1996. The MoFlo is capable of sorting approximately 15–20 million sperm per hour [36]. Similar results have been obtained with the MoFlo in Germany and Australia since 2000.

X- and Y-Sperm Confirmation

A convenient, precise, and accurate procedure to identify X- and Y-sperm is essential for their successful separation. After penetration into zona-free hamster oocytes, sperm chromatin undergo decondensation; this provides a set of chromosomes suitable for karyotyping [13, 48, 49].

Highly accurate identification of individual bull Y-sperm by amplification of the bovine Y specific DNA fragment (Fig. 5) has been enabled as a result of the development of molecular biology and polymerase chain reaction (PCR) technologies. This method should be used for monitoring the separation of Y-sperm from X-sperm, especially when data on individual sperms can be obtained. Welch *et al.* [50] applied PCR to analysis of single X- and Y-sperm previously sorted into 48-well plates and found that each plate with single sperm could be determined within less than 2 hr with 96% accuracy. PCR is also effective for identifying the sex of single embryos after *in vitro* fertilization with separated sperm. However, the labor required to conduct PCR analysis of a single sperm or embryo likely prohibits its use in routine monitoring of sex identification.

Recently, new technologies have been developed in molecular biology for DNA hybridization and fluorescent labeling. Of these, fluorescence in situ hybridization (FISH) is a more efficient and convenient method for detection of specific DNA and RNA in tissues and cells within a short period. This procedure with probes for identifying the Y- and X- chromosome gives us an opportunity to use it as an independent technique for validation of separated sperm populations [51, 52]. Considering that a large number of samples are monitored in

Fig. 5. Agarose gel electrophoresis of flow sorted bull sperm amplified by PCR (left) and fluorescence *in situ* hybridization of sorted bull sperm with higher fluorescent intensity (right). Y-sperm samples amplified in the presence of Y-specific and bovine common primers (left). Nuclei with Y-chromosome hybridization are recognized as spots of fluorescein label (right).

separation of X- and Y-sperm by flow cytometer, FISH could be an efficient technique because of the potential to examine 200 or more sperm per sample (Fig. 5). Rens *et al.* [53] developed a new protocol for sexing bovine X- and Y-sperm with X- and Y-chromosome paints. They developed sex chromosome-specific paints obtained by degenerate oligonucleotide-primed-PCR (DOP-PCR) [54] amplification from a yak cell line. Application of these paint to a FISH protocol for bull sperm resulted in clear identification of more than 90% of sperm. When the fluorescence-labeled X- and Y-specific paints were tested in samples of bull sperm sorted using the Beltsville sperm sexing technology [35], the results indicated that the FISH procedure is robust, reproducible, and simple with high efficiency for evaluation of the sex of sorted sperm [53].

Production of Sex Preselected Offspring with Flow Sorted X- and Y-Sperm

The fertility of X- and Y-sperm sorted by flow cytometer has been confirmed in the rabbit [1], swine [4], and bovine [7, 58]. Many offspring have successfully been produced from sperm sorted by flow cytometer followed by surgical insemination [3, 4], IVF [7, 8, 58], and ICSI [5, 6]. Before development of high-speed sorting technology [35, 36], the primary limiting factor for use of sperm

sorted by flow cytometer in artificial insemination is that only small numbers of sperm were available in a given time period [59]; this should be overcome because good motility and a large number of sorted sperm are required for calf production.

ICSI can be done with single sperm heads without motility and seems to be an efficient method for sorted sperm (Fig. 4). In farm animals, the production rate of offspring derived from ICSI oocytes with flow-sorted sperm is lower than that from IVF [5–7]. In sheep, Catt *et al.* [5] obtained cleaved embryos at rate of 46% 6 days after ICSI with sorted ram sperm and produced one male offspring from transfer of 28 embryos. Goto *et al.* [60] produced bovine blastocysts at rate of 0.4% using ICSI with unsorted bull sperm heads. Although the fertilization rate for ICSI in oocytes was lower than that for IVF, their results were comparable to previous reports using ICSI with sorted sperm [4, 42] and unsorted sperm heads [60]. In cattle, there is currently only one report [6] of calf production by transfer of blastocysts derived from oocytes injected with flow cytometrically-sorted Y-sperm heads; eight males were produced from 10 calves using flow cytometrically-sorted Y-sperm heads (Fig. 6). In that report, the authors stated that the calf production rate after transfer of sex preselected embryos was low but comparable to those in previous studies using ICSI and IVF with sorted sperm (Tables 1, 2).

For ordinary AI in cattle, at least 10 million sperm

Fig. 6. Japanese black male calves resulting from intracytoplasmic sperm injection into *in vitro*-matured bovine oocytes with sperm nuclei separated for Y-chromosome bearing populations. *In vitro* cultured and developed embryos were transferred (one embryo per recipient) to the uteri of Holstein cows.

Table 1. Effects of maturation time on developmental ability of embryos produced with ICSI using flow sorted sperm nuclei

Maturation (h)	No. of oocytes	No. of 2-cell (%)	No. of blastocysts (%)
22	21	3 (14.3)	1 (4.8)
24	909	424 (46.6)	63 (6.9)
26	1266	610 (48.2)	44 (3.5)

Table 2. Production of sex preselected calves with ICSI using sorted Y- sperm nuclei

No. of embryos transferred	No. of recipients transferred	No. of calves delivered (%)	Sex of calves	
			Male (%)	Female (%)
48	48	10 (20.8)	8 (80)	2 (20)

per trial are necessary for successful pregnancy. Fifteen years ago, it was difficult to separate 10 million X- or Y-motile sperm by the flow sorting method in one day. As the technique of sorting by flow cytometry has progressed as mentioned previously, the number of sorted sperm has increased. Seidel *et al.* [61] demonstrated AI in cows using sorted bull sperm (0.1–0.2 million sperm/ 0.1 ml) by depositing the sperm deep into the uterine horn and obtained sex-predetermined offspring or fetuses. Cran *et al.* [62] inseminated sheep with a small amount of sorted sperm using the laparoscopic intrauterine procedure and produced offspring, although the rate of offspring production was low. Recently, insemination by a deep uterine procedure with low dose of X- or Y-sperm has been achieved by Seidel *et al.* [63] in cattle; a total of 0.2 million sperm per dose was sufficient to produce pregnancies resulting in 17 calves. The average percentage of females from sorted X-sperm was 82%. This technique provides a useful semen-delivery avenue needed to bring flow sorted semen to producers. In IVF of porcine

oocytes, a study using separated sperm after high-speed sorting demonstrated the effectiveness of using the sorted sperm to produce sex preselected offspring [64]. High-speed sorting of bull sperm can provide separated X- or Y-sperm for AI in the conventional manner. Testing of high-speed sorter pressures showed that there was no difference in the fertilization rates among the of 1.76, 2.81, and 4.22 kg/cm² pressures [65]. In cattle, Johnson *et al.* [35] demonstrated AI directly into the body of the uterus with 2 to 3 million motile sperm flow sorted within one hour that resulted in pregnancies and calves with the predicted sex. Thus, high-speed sorting offers the opportunity to perform conventional AI with sorted sperm as well as deep-uterine insemination [36]. Cryopreservation of sorted sperm extends their utility in many species. Johnson [36] suggested that use of frozen sorted semen in addition to fresh semen adds an important commercial dimension to the process, since frozen semen is highly important to widespread use of this sorting technology in farm animal production. In cattle, Seidel *et al.* [66, 67]

demonstrated the effectiveness of frozen, sorted spermatozoa. Johnson *et al.* [58] applied standard freezing technology to bull sorted sperm; they froze and thawed flow cytometrically-sorted bull sperm. The resulting average sperm motility and acrosomal integrity was 30% and 40%, respectively. One to 2 million sperm per 0.5 ml straw could be available after sorting [58].

Introduction of X- or Y-sorted sperm to the bovine AI industry can be approached in several ways. There would likely be great demand for X- or Y-sperm sorting at present if it was efficacious and not too costly. X- or Y-sorted sperm should increase the efficiency of meat and milk production and create new opportunities for AI to take market share away from natural breeding of the bovine. There are two possible broad objectives in introduction of X- or Y-sorted sperm for bovine AI. They are profit and satisfying the needs of the owners of bovine. At present, more than 100,000,000 doses of bovine semen are sold annually worldwide. It is likely that fertility will not be compromised greatly as the X- and Y-sorted semen product improves over the next few years. However, fertility will likely vary widely, just as it currently does with synchronized estrus and conventional bovine AI.

Conclusion

Commercialization to other species will follow improvements to the techniques of sorting X- and Y-sperm and as experience is gained in the bovine.

Morrell and Dresser [68] reported subsequent development of offspring derived from sorted sperm. Observation of two generations of reproduction in pigs and rabbits showed that none of the offspring had any morphological abnormalities.

X- and Y-sperm sorting technology was first implemented nearly 20 years after the principle of selecting sperm by their DNA content was first established. At this time, more than 30,000 calves have been born from sperm sorted for sex-chromosome differences based on DNA content. Most of these calves were produced in the United States and United Kingdom, with lesser numbers produced in several other countries. Currently, sexed bovine sperm can be purchased from companies in the United Kingdom and some regions of the United States, while licensing or commercialization of this technology for cattle is in various phases of development in several other countries. Successful cryopreservation of X- and Y-sperm was a major factor that facilitated commercialization of bovine sperm sexing [66]. Although, X- and Y-sorted bovine sperm tends to degenerate faster than unsorted control gametes, cryopreservation overcomes this problem by allowing sexed bovine sperm to be stored indefinitely so that they can be transported and used almost anywhere in the world.

Thus, X- and Y-sperm separation using a high-speed sorter could be the basic method for more efficient and commercial techniques of sex preselection in the bovine.

Acknowledgments

The author wishes to thank the Japanese Society of Animal Reproduction for conferring a JSAR Innovative Technology Award 2006 on this study. The author thanks Dr. J. Masaki and Dr. L. A. Johnson for invaluable suggestions. Finally, the author thanks the staff of the Livestock Improvement Association of Japan and especially the staff of the Maebashi Institute.

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