

The Beltsville Sperm Sexing Technology: High-Speed Sperm Sorting Gives Improved Sperm Output for In Vitro Fertilization and AI

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ABSTRACT: The Beltsville sperm sexing technology is currently the only effective means of altering the sex ratio of offspring in livestock. The method is based on the flow-cytometric separation of X- and Y-chromosome-bearing sperm based on X/Y DNA content difference. It is an effective means of producing progeny of predetermined sex in cattle, swine, sheep, and laboratory animals. The method involves treating sperm with a DNA-binding fluorochrome, Hoechst 33342, and flow-cytometrically sorting them into separate X and Y populations that can subsequently be used for surgical intratubal or intrauterine insemination, deep-uterine insemination, regular artificial insemination in some cases, in vitro fertilization to produce sexed embryos for transfer, and intracytoplasmic sperm injection of ova. Skewed sex ratios of 85 to 95% of one sex or the other have been repeatedly achieved in most species. The method has been used worldwide to produce several hundred morphologically

normal animal offspring of the predicted sex. It has also been validated in the laboratory using DNA reanalysis of the sorted sperm populations and by fluorescence in situ hybridization and PCR of individual sperm. We developed a new orienting nozzle that we have fitted to both conventional and high-speed cell sorters that have been modified for sperm sorting. Recently we completed the adaptation of the new orienting nozzle to a Cytomation MoFlo high-speed cell sorter modified for sperm. This adaptation of the nozzle has increased the overall production rate of sorted X and Y sperm from about .35 million/h to 5 or 6 million sperm/h (each population). Calves have been born from cows artificially inseminated using conventional technique and sexed sperm. In addition, numerous litters of pigs have been born after transfer of embryos produced from X or Y sorted sperm.

Key Words: Spermatozoa, Flow Cytometry, Cells, Artificial Insemination

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Introduction

Advancements in biotechnology are central to improving the efficiency of livestock reproduction and overall production. One current example that illustrates the importance of biotechnology development is a process that allows livestock producers to predetermine the sex of offspring from agriculturally important animals. This process is based on the separation of X- and Y-chromosome bearing spermatozoa. Sperm are separated on the basis of DNA using flow-cytometric sperm sorting. This has been effective for many species (Johnson et al., 1989; Johnson, 1995). Sex ratios have been skewed in experimental and field

studies with rabbits (Johnson et al., 1989); swine (Johnson, 1991; Rath et al., 1997); cattle (Cran et al., 1995; Seidel et al., 1997; Johnson et al., 1998); sheep (Johnson, 1995; Catt et al., 1996; Cran et al., 1997); and humans (Johnson et al., 1993; Fugger et al., 1998). The shift in the sex ratio is usually from the standard 1:1 to about 8 or 9:1 or vice versa. The sexing technology used in this research has been described (Johnson, 1995; Johnson, 1997; Johnson, 1998).

The current technology for sexing sperm was developed in the late 1980s (Johnson et al., 1989) and has been in use to the present day. This technology was developed for use on conventional-speed cell sorters that were developed in the early 1970s and

improved dramatically in 1980. There has been incremental improvement in these standard sorting systems over the past 18 yr, mostly related to advancing computer technology. Even with these improvements, only small advances were made in the sorting speed of commercial cell sorters. This has now changed with the development of commercial high-speed flow sorting systems.

Improvements in the Beltsville technology in the past 2 yr has involved two nearly simultaneous approaches: 1) The development of a new flow nozzle to more effectively orient the sperm head to the laser beam, and 2) the development of a commercial high-speed cell sorter (MoFlo; Cytomation, Inc., Fort Collins, CO), which could be modified to sort sperm, that we adapted with the improved orienting nozzle to improve sperm orientation. This paper is devoted to outlining these improvements and discussing the utility of the changes in producing offspring of the preselected sex.

Discussion

Conventional Cell Sorting Using a Beveled Needle for Orienting Sperm

Early development research using the conventional speed sorter (Epics V and 753; Beckman-Coulter, Miami, FL) with system pressures of 844 g/cm² has provided the basis for our research progress since 1982. Using these systems we were able to prove that the beveled needle and forward optical detector would enhance the ability of the system to differentiate between X- and Y-chromosome bearing sperm (Johnson and Pinkel, 1986). This led to the sorting of sperm heads (Johnson et al., 1987). Although the sperm were dead because the tails had been removed through sonication, the sperm were "alive" from a DNA standpoint and were capable of forming pronuclei in hamster eggs (Johnson and Clarke, 1988). Sorting sperm heads into the X and Y populations was relatively straightforward since the analysis of sperm for sex ratio (Johnson and Pinkel, 1986) based on DNA could be done quite efficiently. Orientation of sperm heads is not problematic since there are no tails to contend with. However, sorting living sperm is different in that the tails remain attached; the sperm also remain motile and create a motion that is harder to control. However, this problem was solved with our report (Johnson et al., 1989) that living sperm completely intact were capable of fertilization to produce offspring.

A key component of the early work, both for sorting dead, tailless sperm and for living sperm, was the ability to orient the sperm as they pass the laser beam using a beveled needle to widen and flatten the sample core stream and positioning the sperm head in such a way that the broad surface of the sperm head was perpendicular to the laser beam (Figure 1). Twenty to thirty percent of living intact sperm could be oriented with this process and a sort rate of approximately 80 to 100 sperm/s or 350,000/h of each population (Johnson et al., 1989) was achieved. The lower percentage of orientation of living sperm was due to the presence of the tail and the motility.

Development of the Orienting Nozzle for Improved Sexed Sperm Production

The characteristic most in need of improvement was to increase the percentage of sperm that could be oriented efficiently by improving the beveled needle or redesigning the nozzle in such a way as to bring the orientation forces lower in the nozzle and closer to the laser beam (Figure 2). This was accomplished by redesigning the interior shape of the nozzle into a double ellipse (Rens et al., 1998). This change improved the efficiency of orientation from the 25% to 60 to 70%, two to three times that of the original orientation ability. This improved the throughput of the existing standard speed sorter from the 350,000 sperm/h to about 800,000 sperm/h of each population, a significant improvement (Rens et al., 1999).

Development of High-Speed Cell Sorting

In 1985, Peters and colleagues described the first experimental high-speed sorter (HiSS; Peters et al., 1985). This unit was built at the Lawrence Livermore National Laboratories, Livermore, CA, and was capable of processing and sorting cells at the rate of 20,000/s. This high-speed sorter was characterized by higher pressures (200 psi) and faster throughput than is the case for conventional-speed sorters (844 g/cm² and 8,000/s). We tested intact bull sperm on this high-speed system in 1987 (L. A. Johnson and D. Pinkel, unpublished data). The sperm that we tested on the system were dead after sorting, which we attributed to the high system pressure.

The second and improved high-speed system was also built at Livermore by van den Engh and Stokdijk (1989). This was an improved system in several ways, notably for the potential for application to sperm because the pressure associated with the operation of the system was reduced (from 14,078 g/cm² on the original HiSS to 1,408 to 7,038 g/cm² on this later

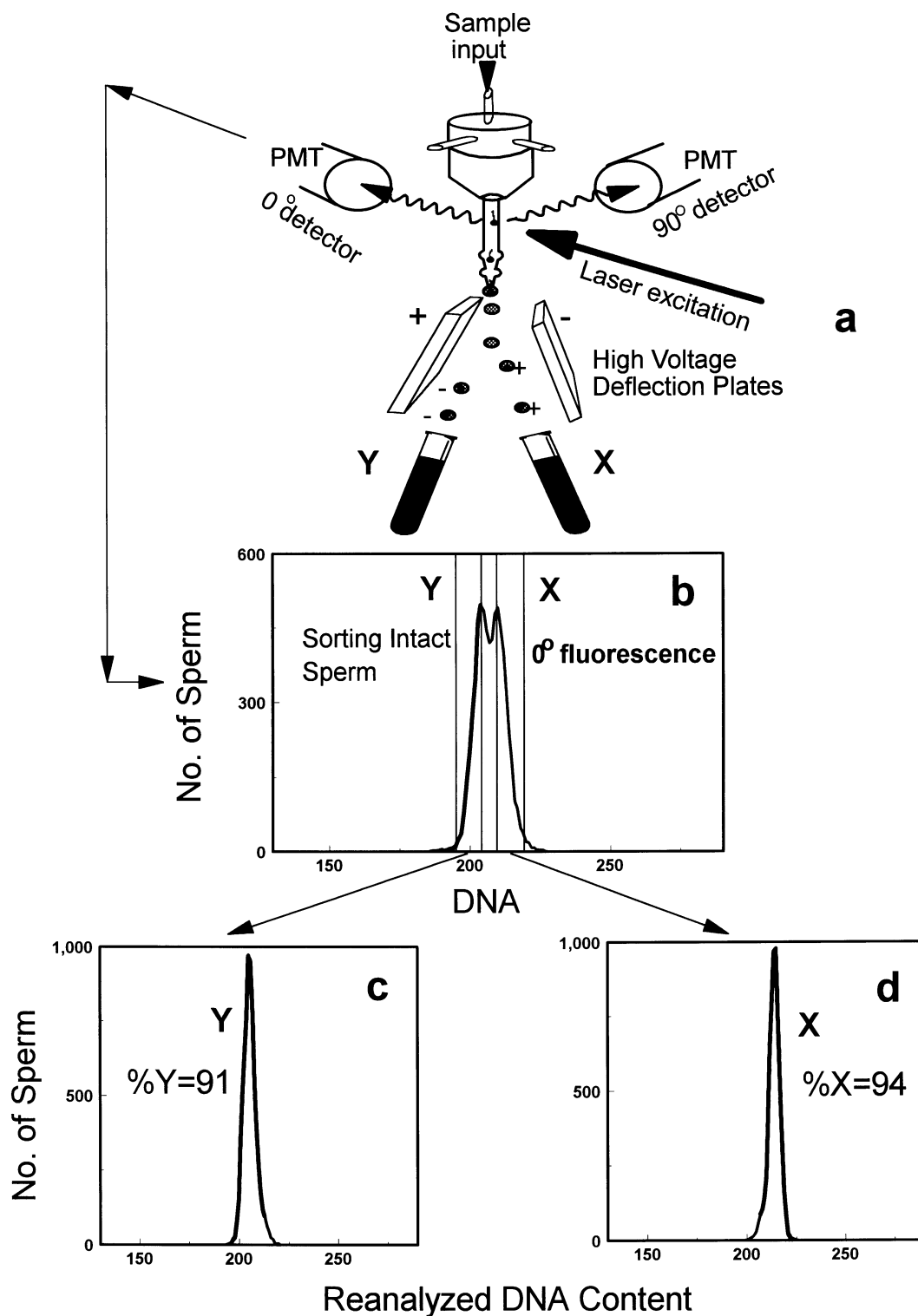
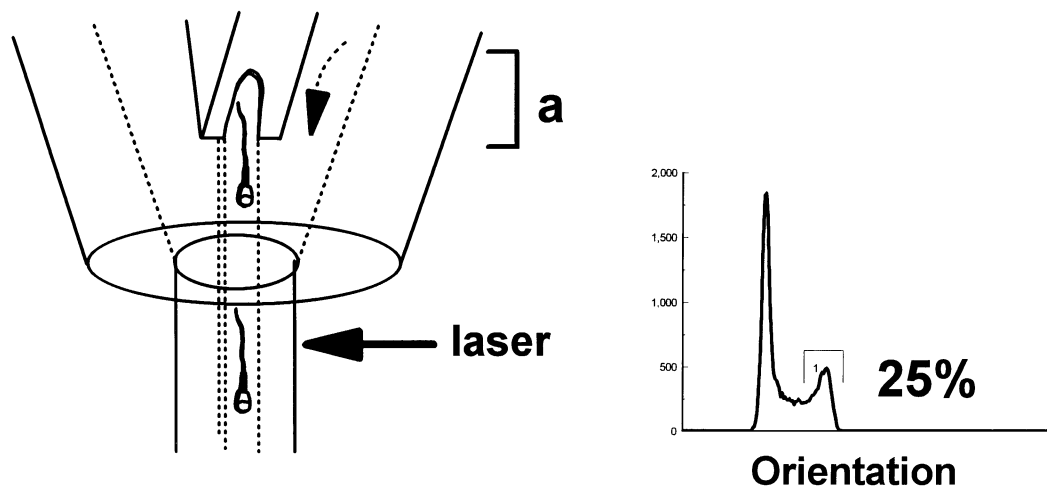


Figure 1. Sperm are flow cytometrically analyzed (a) for their DNA content by collecting fluorescence information that is proportionate to the DNA content from the flattened face of the sperm head. This information is collected in the 0° (a modification) fluorescence detector. The 90° fluorescence detector is used to determine how the sperm is oriented. Sort windows (b) around the dimmer Y chromosome-bearing sperm and the brighter X chromosome-bearing sperm are used to determine which sperm are collected. Validation of the sorted sperm is done by reanalyzing some of the sperm from both collected fractions, Y (c) and X (d).

Beveled Needle



New Orienting Nozzle

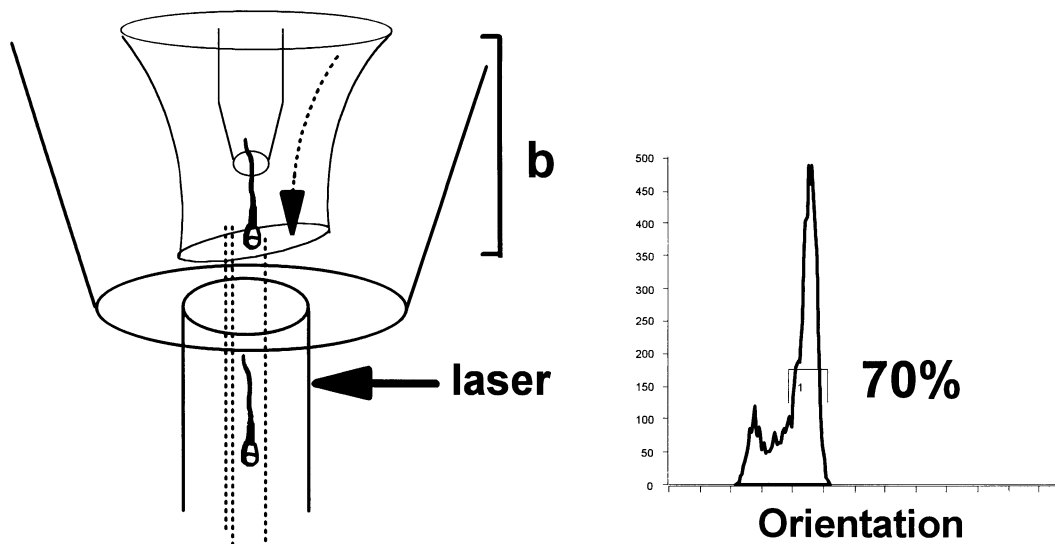


Figure 2. Top schematic shows the orientation forces produced by using a beveled sample injection needle. (a) Note the beveled needle. The orientation forces are further from the analysis point (laser beam), making it more difficult to maintain orientation efficiency. The bottom schematic shows the newer orienting nozzle with its elliptically tapered interior (b). In this latter case, the orientation forces are being applied lower in the nozzle, nearer the laser intercept and analysis point. Consequently, a higher percentage of sperm are properly oriented during laser excitation (60 to 70% vs 20 to 30%).

version). In addition, the throughput rate that the system was capable of handling was increased to 200,000 cells/s based on a parallel-processing data acquisition system. This design was licensed by Cytomation and developed for commercial production with the trade name MoFlo. It came on the market in 1996.

Application of High-Speed Sorting to Sperm

We acquired one of the early MoFlo high-speed systems (1996) and it was modified in our laboratory for sperm analysis and sorting by the installation of a forward-angle fluorescence collection lens with a corresponding photomultiplier tube and beveled needle (based on Johnson and Pinkel, 1986; Welch et al., 1998). We found that using the beveled needle orientation system the percentage of oriented sperm was about 30%, a figure similar to our conventional sorting system mentioned earlier. However, processing speed was much improved, as was overall throughput. We found that 1 to 2 million sperm (each population) could be sorted in an hour's time using this configuration or about four or five times that obtainable with the original Epics system.

Orienting Nozzle and High-Speed Sorting for Sperm Sorting

Adapting the orienting nozzle (Rens et al., 1998) to the high-speed sorter required some minor modification of the instrument. However, the results of this adaptation were dramatic. The combination of a higher percentage of sperm being oriented with the orienting nozzle (70%) and the greater capability of the high-speed system to sort and acquire data resulted in a 10- to 15-fold improvement in throughput attained with the old Epics systems using the beveled needle (Johnson and Pinkel, 1986). Average sorting speed for the orienting nozzle adapted to the high-speed sorter is 5 to 6 million sperm sorted 1 h in each (X/Y) direction (Figure 3).

Sperm Preparation and X/Y Collection Changes Required for High-Speed Sorting

Conventional cell sorting for cells other than sperm generally requires samples in the range of 5×10^6 /mL. However, sperm must be kept in as high a concentration as possible due to the inherent dilution effect. Our protocols for sorting the sperm of most species involves the staining of 15 million sperm with Hoechst 33342 (Johnson et al., 1989). Stain concentration is about 7

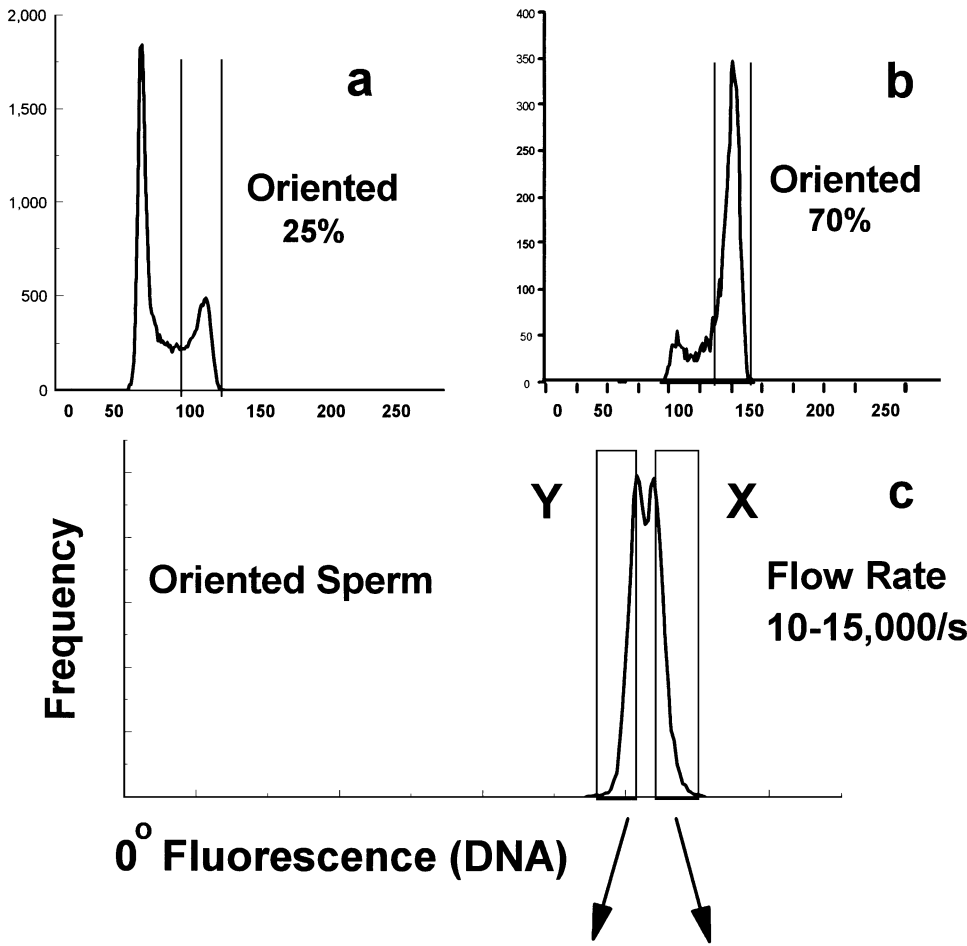
μ M. Stain concentration requires optimization because concentration is critical for uniform staining.

High-speed sorting required a change in these protocols in order to accommodate the faster throughput of the system. Our staining and sperm preparation is currently optimized at 75 to 150×10^6 sperm/mL. Generally we prepare 2 mL of sperm with the appropriate amount of stain and extender to give 200 to 300×10^6 sperm from which to draw. Generally, this is used for sorting over about a 2-h period.

Collection of the sorted sperm is about the same for high-speed sorting as it is for the conventional system except that 12- to 15-mL collection tubes are used for high speed and 1.6-mL microfuge tubes are used for the conventional sorter. Tubes are soaked in BSA solution to overcome the sticking of sperm to the wall of the tube. Test-yolk extender (Johnson et al., 1989) has been increased from 50 μ L to .2 mL added to the tube before sorting. The purpose of the test-yolk buffer in the collection tube is to provide a concentrated environment for the sorted sperm to swim into. This assists in maintaining viability during the sorting process. Centrifugation of the collected sorted sample is done and the sperm pellet is diluted according to its use.

Utilization of Sexed Sperm from Conventional-Speed Sperm Sorting

Numerous methods of sexed sperm delivery have been used to produce offspring in various species using both the conventional sorting system and the high-speed sorting system. Johnson (1995) gives a summary of the fertility results to that date. The summary includes data from the birth of sexed offspring from rabbits, swine via surgical insemination, of cattle through in vitro fertilization, and embryo transfer. Since those results were published, additional data were collected from the combination of in vitro fertilization (IVF) and sexed sperm in which two litters of pigs were born that were 100% female (Rath et al., 1997). These results represented the first offspring born after using sorted sperm in combination with IVF in the pig. With respect to cattle, a collaborative study was conducted in which bull semen was collected in Pennsylvania (Atlantic Breeders Cooperative, Lancaster) and transported to Beltsville, MD, by auto, where it was prepared and sorted into separate populations of X and Y sperm. Following the sorting process, it was shipped in an Equitainer to Fort Collins, CO, and inseminated via deep-uterine insemination into heifers that had been synchronized. Approximately 200,000 sperm were used per insemination. From these inseminations, 17 calves were



Orientation	20-30%	60-70%
Resulting Sort Rate	↓ ↓	↓ ↓
Sperm / s	500	1,500
Millions / h	1.75	4-5

Figure 3. This figure shows a comparison of using the beveled needle to orient and sort sperm (a) and the orienting nozzle (b). The histogram in (c) represents the analytical histogram on which the sorting is done, showed a threefold increase in the sorted sperm. The bottom panel illustrates the impact of changes in orientation rate and percentage.

Table 1. Farrowing results from gilts receiving embryos produced from sexed sperm sorted at Beltsville using orienting nozzle and high-speed sorting

Treatment:	Farrowing no.	No. of piglets		Litter size, (n)	Predicted sex, % ^a	Actual
		Male	Female			
High-speed sorting/orienting nozzle						
Sorted for X ^b	6	1	33	5.8	93	97% X
Control ^b	3	12	11	7.8	50	48% X
Sorted for X ^c	5	1	23	4.8	88	97% X
Sorted for Y ^c	3	9	0	3.0	86	100% Y

^aBy reanalysis of sorted sperm for DNA content.

^bSperm sorted and pigs born at Beltsville, Long et al. (1998).

^cSperm sorted at Beltsville; embryos produced and pigs born at University of Missouri, Abeydeera et al. (1998).

born, and 14 were of the desired sex at birth (Seidel et al., 1997). This work represented the first offspring from sexed sperm inseminated via a deep-uterine technique.

Fertility of Sexed Sperm Produced From High-Speed Sperm Sorting With Orienting Nozzle

High-speed sperm sorting using the orienting nozzle has been used to produce the first offspring in cattle using conventional AI. With high-speed sorting producing about 5×10^6 sperm/h, cows were bred in the conventional manner; that is, the sperm were placed directly into the body of the uterus. The advantage of AI in this way is that no special training is needed to conduct the insemination, as is the case with deep-uterine AI. Although no titers in terms of number of sperm needed was conducted, it is likely that numbers could be reduced even further. A preliminary report of this work has been published (Johnson et al., 1998).

Two studies were also conducted using sexed boar sperm produced by high-speed sorting for IVF in swine (Table 1). Our initial studies using IVF and sexed sperm, mentioned above, used oocytes matured in vivo for fertilization by sexed sperm (Rath et al., 1997). However, in two studies conducted with the high-speed sorter, we used oocytes matured in vitro for fertilization under IVF conditions. One study was conducted at Beltsville and produced offspring in nine litters. The control litters (n = 3) gave 52% male and 48% female offspring. Six litters produced from sows in which sexed embryos had been transferred at the four-cell stage gave 34 total pigs, one of which was male. The offspring were 97% female (Long et al., 1998). In a second study, conducted in collaboration with the University of Missouri, eight litters were born. Three litters from Y-sorted sperm (100% male)

and five litters from X-sorted sperm (97% female). In this study, sperm were sorted at Beltsville and shipped by air and used for IVF at the University of Missouri (Abeydeera et al., 1998).

Alternative Method of Sexing Semen Based on Surface Protein

It is well understood that a method to sex semen on a large scale, at least on a scale that could be applied to semen production practice at an AI center, would be advantageous. The method most often referred to in this context is that of isolating a protein from the surface of the X- or Y-bearing sperm that is chromosome specific and thus sex specific. The theory goes that if one is able to isolate such a marker, then an antibody could be developed to attach to that X- or Y-bearing population of sperm. The assumption is that the use of affinity chromatography or magnetic beads would provide a large-scale separation process for separating X- from Y-bearing sperm and be readily adaptable to AI center use. In order to determine if such a surface protein exists on boar sperm, a study was conducted in collaboration with colleagues in The Netherlands. Boar sperm was sorted for X or Y populations at purities of about 90%. The sorted sperm were flown to The Netherlands, where the proteins were isolated and characterized. Approximately 1,000 proteins were mapped on the sperm surface. However, no difference could be detected between the proteins isolated from X sperm vs Y sperm. These results would lead one to believe that no sex-specific protein exists on the surface of the sperm (Hendricksen et al., 1996).

Implications

Sex preselection by flow-cytometric cell sorting is currently the only proven method that skews the sex

ratio of mammals. The Beltsville technology will produce 5 to 6 × 10⁶ sperm/h at 85 to 95% purity for X (female producing) or Y (male producing) sperm in most livestock species. One limitation of cell-sorting technology is that the process must be carried out one cell at a time. This makes the systems inherently slow, because millions (for cattle) and billions of sperm (for swine) are needed for conventional AI. Other than DNA, no other difference is known to exist between X and Y sperm that would allow their separation while maintaining viability. Improvements in the sperm-sorting technology have increased the throughput of sperm by 10 to 15 times. This high-speed technology using a special orienting nozzle opens the way for a much broader use of the method in terms of AI. Further improvements encompassing a dedicated sperm-sorting instrument would likely simplify the process and make it even more applicable. Continued progress in the development of greater efficiency in sorting sperm into X and Y sperm populations offers great encouragement that practical application of the sperm-sexing technology will occur within a few years.

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