

—Technology Report—

## Sexing of Dog Sperm by Fluorescence *In Situ* Hybridization

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**Abstract.** Effective preselection of sex has been accomplished in several species of livestock and also in humans using the flow cytometric sperm sorting method. A guaranteed high sorting accuracy is a key prerequisite for the widespread use of sperm sexing. The standard validation method is flow cytometric remeasurement of the DNA content of the sexed sperm. Since this method relies on the same instrument that produced the original sperm separation, it is not truly independent. Therefore, to be able to specifically produce either male or female offspring in the dog, we developed a method of direct visualization of sex chromosomes in a single sperm using fluorescence *in situ* hybridization (FISH) as a validation method. Denaturation of canine spermatozoa by immersion in 1 M NaOH for 4 min yielded consistent hybridization results with over 97% hybridization efficiency and a good preservation of sperm morphology. There was no significant difference between the theoretical ratio (50:50) and the observed ratio of X- and Y-chromosome-bearing spermatozoa in any of the three dogs. In addition, the mean purities of flow-sorted sex chromosomes in spermatozoa of the three dogs were 90.8% for the X chromosome fraction and 89.6% for the Y chromosome fraction. This sorting was evaluated by using the dual color FISH protocol. Therefore, our results demonstrated that the FISH protocol worked reliably for both unsorted and sexed sperm samples.

**Key words:** Dog, Fluorescence *in situ* hybridization (FISH), Sexing, Sperm

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The specific production of either male or female offspring by separation of X- or Y-bearing spermatozoa and artificial insemination is a long standing goal in breeding of domestic and other animals. Sperm sexing allows for specific production of male calves for beef production and female calves for milk production. For dogs, higher percentages of female puppies are desired in breeding of guide dogs for the blind as well as companion animals. Most blind people prefer to use a female guide dog rather than a male because they are easier to manage and utilize. Male dogs are stronger and require two evacuation bags in addition to street clothes. However, female dogs require the installation of one evacuation bag only. Thus, for equal numbers of male and female trained guide dogs, there is a mismatch between the supply and demand of the required items.

Separation of X- and Y-bearing spermatozoa by flow sorting based on their DNA content is the only proven effective method for sexing viable mammalian spermatozoa [1]. Effective preselection of sex has been accomplished in several livestock [2–5] and humans [6], but not in the canine, using the flow cytometric sperm sorting method. A guaranteed high sorting accuracy by flow cytometry is a key prerequisite for the widespread use of sperm sexing. The standard validation method is flow cytometric remeasurement of the DNA content of the sexed sperm [1]. Since this method relies on the same instrument that produced the original sperm separation, it is

not truly independent. Thus, a convenient validation method that is independent of the DNA content is required for sorting spermatozoa with either X or Y chromosomes in each species. Suitable methods may include quantitative PCR and, in particular, direct visualization of sex chromosomes in a single sperm using fluorescence *in situ* hybridization (FISH). A method of sperm sexing by FISH has been developed for use in several mammalian species such as the mouse [7, 8], swine [9], bovine [10–13] and human [14–16], but not in the canine. The FISH protocol is suitable for reliable routine validation of sexed canine sperm and meets the high standards required for sperm sexing. A crucial hurdle to any protocol is adequate preparation of sperm and sufficient denaturation of the densely packed sperm DNA such that a labeled DNA probe can actually bind to its complementary target. We describe here a possible method of direct visualization of sex chromosomes in a single sperm from the dog using FISH.

When the sperm cells were treated with 1 M NaOH for 3, 4 or 5 min and hybridized with a Y chromosome probe labeled with digoxigenin, FISH signals in sperm heads were clearly detected in each experimental group regardless of the time of NaOH treatment (Fig. 1). As shown in Table 1, there was no significant difference between the theoretical ratio (50:50) and the observed ratio of Y-chromosome-bearing spermatozoa treated with 1 M NaOH for 4 min from all three dogs ( $P>0.05$ ). However, treatment with 1M NaOH for 3 min yielded a significant scarcity of spermatozoa having Y chromosome in semen of all the three dogs ( $P<0.05$ ). After the treatment of sperm cells with 1 M NaOH for 5 min, the observed ratio of Y chromosome in spermatozoa derived from two of the three dogs was not significantly different from the theoretical ratio. These results suggested that NaOH denaturation was equally simple

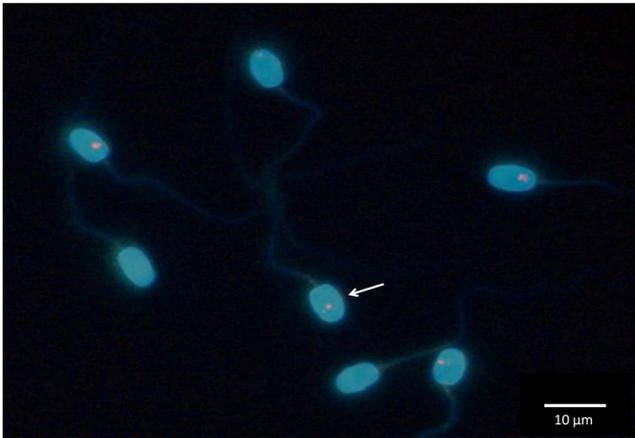
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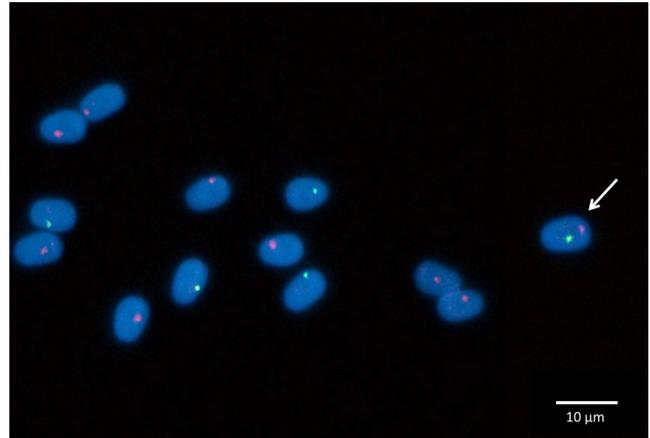
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**Fig. 1.** Canine spermatozoa decondensed with 1 M NaOH for 4 min. Y chromosomes hybridized with Cy3 labelled probes. Only sperm heads with red a signal were considered to be Y-chromosome-bearing spermatozoa (arrow). Bar=10  $\mu$ m.



**Fig. 2.** Detection of X and Y chromosomes on canine spermatozoa by dual color FISH. Sperm heads with green (SpectrumGreen) and red (Cy3) signals were considered to be X- and Y chromosome-bearing spermatozoa, respectively. A sperm head carrying both green and red signals (arrow) was considered to be aneuploid.

**Table 1.** Percentages of FISH signals for the Y chromosome in canine spermatozoa treated with 1 M NaOH for 3, 4 and 5 min

Time of NaOH treatment (min)	Dog ID	Percentage of Y chromosome	Significance (deviation from 50:50)
3	A	44.79	P<0.05
	B	45.4	P<0.05
	C	44.4	P<0.01
4	A	47.4	NS
	B	48.28	NS
	C	47.04	NS
5	A	46.81	NS
	B	45.37	P<0.05
	C	46.76	NS

In each experimental group, 500–600 spermatozoa were counted. NS: not significant ( $P>0.05$ ).

and easy to control.

The results of dual color FISH for the canine spermatozoa decondensed by 1 M NaOH for 4 min from three individuals are shown in Fig. 2 and Table 2. There was no significant difference between the theoretical ratio (50:50) and the observed ratio of X and Y chromosomes in spermatozoa of all the three dogs. The hybridization efficiency was 98–99%.

The purities of flow sorted sex chromosomes in spermatozoa ( $60\text{--}200 \times 10^4$  cells) of the three dogs ranged from 88% to 93% for the X chromosome fraction (mean 90.8%) and 86% to 93% for the Y chromosome fraction (mean 89.6%). The evaluation was done by using the dual color FISH method (Table 3). The hybridization efficiency ranged between 97–99%.

A method of direct visualization of sex chromosomes in individual canine sperm using FISH was established in this study. In recent years, several groups have worked on the development of FISH for spermatozoa in mammals (mouse [7, 8], swine [9], bovine [10–13],

humans [14–16]). A crucial hurdle to any protocol is adequate preparation of sperm and sufficient denaturation of the densely packed sperm DNA such that the labelled DNA probe can actually bind to its complementary target [13]. However, if the nuclei are swollen to more than twice their original size, the signal from one chromosome may split and appear as two or more signals, causing the spermatozoa to be falsely scored as disomic [17]. Since the stability of mammalian sperm nuclei differs markedly among species [18], it is necessary to determine a suitable condition of sperm nuclear decondensation in each species. For example, decondensation protocols that have been used successfully on humans do not work well for bovine spermatozoa [10]. Our protocol for canine spermatozoa established in this study was based on treating spermatozoa with NaOH, which was effective for FISH in bovine [13] and humans [15] and for the primed *in situ* labelling method [19]. As shown in Figs. 1 and 2 and Tables 1 and 2, when canine spermatozoa were denatured by immersion in 1 M NaOH for 4 min, consistent hybridization results with over 97% hybridization efficiency and good preservation sperm morphology were observed. In both single and dual staining, there were no significant difference between the theoretical ratio (50:50) and the observed ratio of X- and Y-chromosome-bearing spermatozoa of all three dogs. Sperm susceptibility to the decondensation process is dependent on the content of chromatin disulphide bridges in the sperm nuclei [20], which is variable between species and also between individuals of the same species [17]. Successful FISH in spermatozoa has been reported in several mammals including humans. In those studies, it has been reported that the treatment of spermatozoa with dithiothreitol (DTT) and lithium diiodosalicylate by microwaving was effective for decondensation of the sperm nuclei in mice [8] and humans [16]. DTT with sodium dodecyl sulfate (SDS) was useful for bovine [12] and stallion [21] spermatozoa. In our preliminary experiment, however, these treatments for denaturation of sperm nuclei did not work well for canine spermatozoa. When canine spermatozoa were microwaved for decondensation and subsequent hybridization with a probe, a fluorescence signal was not detected in

**Table 2.** Percentages of X and Y chromosomes in canine spermatozoa verified by dual color FISH

Dog ID	X	Y	With X and Y signals	Without signals	Significance (deviation from 50:50)
A	50.24	48.89	0.12	0.87	NS
B	50.14	48.89	0.28	0.28	NS
C	47.79	50.09	0	2.12	NS

In each experimental group, 500–600 spermatozoa were counted. NS: not significant ( $P>0.05$ ).

**Table 3.** Percentages of canine spermatozoa sorted for the X and Y chromosomes by flow cytometry and verified by dual color FISH

Dog ID	Sorted spermatozoa for X chromosome				Sorted spermatozoa for Y chromosome			
	X	Y	With X and Y signals	Without signals	X	Y	With X and Y signals	Without signals
A	91.38	7.26	0	1.37	7.1	90.36	0	2.54
B	87.68	11.32	0	1	13.18	85.9	0	0.92
C	93.39	6.03	0	0.59	5.35	92.55	0	2.1

In each experimental group, 500–600 spermatozoa were counted.

40% of spermatozoa. In addition, the treatment of canine sperm with various DTT and SDS concentrations and exposure times resulted in an undetectable signal after hybridization. On the other hand, brief exposure of spermatozoa with 1M NaOH for decondensation of sperm nuclei effectively worked for canine spermatozoa in this study. Since this method for decondensation of sperm nuclei is quite simple, it might be highly reproducible.

When canine spermatozoa were sorted with a flow cytometer under similar conditions for bovine spermatozoa, as shown in Table 3, sexed spermatozoa were successfully obtained with a high sorting accuracy (approximately 90%). The effectiveness of utilizing DNA content differences between the X and Y chromosome in spermatozoa depends not only relative DNA differences, but also on the ability to precisely orient these gametes at the time of measurement by the flow cytometer [22]. Sperm cells with flattened and oval heads tend to be more readily oriented in a sperm sorter than those gametes possessing more rounded or angular-shaped heads [22]. Although differences in DNA content between X- and Y-chromosome-bearing spermatozoa vary in mammals, the X-Y difference in DNA content is similar between the bovine (3.8%) and canine (3.9%), and the sperms of both species have flattened, oval-shaped heads. However, sperm head size differs between them. Namely, the length, width and area of the more flattened side of the sperm head were 9.1  $\mu\text{m}$ , 4.7  $\mu\text{m}$  and 34.5  $\mu\text{m}^2$  in the bull and 7.0  $\mu\text{m}$ , 3.5  $\mu\text{m}$  and 20.9  $\mu\text{m}^2$  in the dog, respectively [22]. A sperm sorting index is an approximation of the ability to sort spermatozoa by flow cytometry. The index is calculated by multiplying the head profile area ( $\mu\text{m}^2$ ) by the X-Y sperm DNA difference (%) as proposed by Garner [22]. The sorting index has been found to be 131 in the bull and 82 in the dog [22]. This simple approximation indicates that the attributes of canine sperm make them 1.6 times more difficult to sort than sperm from bulls. However, a more restricted threshold setting could achieve a higher accuracy (over 90%) in the dog, although reduced numbers of sperm would pass through the system.

There were sperm heads with both X- and Y-chromosome-positive signals (two-signal sperm) in dual color FISH (Fig. 2 and Table 2). These diploid sperm cells were detected with a frequency of 0.13% in unsorted spermatozoa in dogs (Table 2). The percentages of the diploid spermatozoa in dogs were higher than those reported in the bull (0.03%) [10] and ram (0.03%) [23] and similar to those in the human (0.16%) [24], horse (0.10%) [25], and water buffalo (0.14%) [23]. On the other hand, diploid cells were not detected by dual color FISH in both the X and Y fractions of sorted canine spermatozoa (Table 3). These results indicated that the sorting procedure with a flow cytometer selected essentially normal haploid spermatozoa. The FISH protocol worked reliably in both unsorted and sexed sperm samples (Tables 2 and 3). The hybridization efficiency was very close to 100%. Specific production of either male or female offspring with higher accuracy might be possible by combining separation of X- and Y-bearing spermatozoa with validation by FISH in dogs.

## Methods

### *Animals and semen preparation*

Male Labrador retrievers ( $n=3$ ) in our breeding colony were used in this study. The second fraction of ejaculates was collected from dogs by manual stimulation. Five hundred microliters of ejaculate was placed into an Eppendorf tube (1.7 ml, 17401, Sorenson Bioscience, Salt Lake City, UT, USA), and then centrifuged for 5 min at 3,000 rpm. The supernatant was removed, and 1 ml of 0.01 M PBS (Phosphate Buffered Saline; 0.35 g/l  $\text{NaH}_2\text{PO}_4$ , 1.28 g/l  $\text{Na}_2\text{HPO}_4$ , 8.00 g/l NaCl, Wako, Tokyo, Japan) was added. The suspension obtained was thoroughly mixed using a pipette and subsequently centrifuged for 5 min at 3,000 rpm. The supernatant was removed, and the pellet was resuspended with 1 ml of 0.01 M PBS. The washing procedures were repeated twice. The sperm suspension with 200  $\mu\text{l}$  of 0.01 M PBS was fixed by addition of 400  $\mu\text{l}$  of 3:1 methanol:acetic acid fixative and left at room temperature for 10 min. Then, the tube containing sperm

suspension was filled with the fixative, and then centrifuged for 5 min at 3,000 rpm. This procedure was repeated four times. The fixed sperm pellet was resuspended with the fixative. The sperm concentration was adjusted to  $5 \times 10^6$  cells/ml, and 10  $\mu$ l of the suspension was then placed on a defatted glass slide. These preparations were dried at room temperature. The concentration of sperm on the slide was examined using a light microscope (ALPHAPHOT-2 YS2, Nikon, Tokyo, Japan). When the sperm density was unsatisfactory or the sample was contaminated with dust, the suspension was diluted, concentrated or washed with the fixative and centrifuged to get an appropriate concentration. Preparations were dehydrated on a hot plate at 70 C for 60 min (hardening).

#### Decondensation

For decondensation of sperm nuclei, the specimen was treated with 1 M NaOH for 3, 4 or 5 min at room temperature, rinsed with PBS for 2 min, and then washed with distilled water several times. The specimen was dehydrated by running it through an ethanol concentration series (70% for 5 min and 100% for 30 min). After air-drying, the specimen was placed on a hot plate at 70 C for 60 min.

#### FISH

For a single staining of the Y-chromosome of canine sperm, 5  $\mu$ l of Y chromosome probe labeled with digoxigenin and salmon sperm DNA, canine suppression DNA, and 50% formamide/10% dextran sulfate/2 x SSC mixture (DOY-10, Chromosome Science Laboratory, Sapporo, Japan) was added to the specimen, and then covered with a cover glass. The preparation was transferred onto a hot plate set at 70 C for 5 min. The edges of the preparation were sealed with rubber cement (Sagisaka, Tokyo, Japan). Hybridization was carried out in a moist chamber at 37 C overnight. After hybridization, preparations were soaked in 2xSSC buffer (WAKO). Thereafter, the cover glass was removed, and the specimen was washed several times with 50% formamide in 2xSSC at 37 C for 20 min and with 1xSSC alone at room temperature for 15 min. Hybridization of the digoxigenin-labelled Y probe was visualized with anti-digoxigenin Cy3 (Chromosome Science laboratory). The slides were counterstained with DAPI (4', 6-diamino-2-phenylindole, dihydrochloride, D1306, Invitrogen, Carlsbad, CA, USA) and mounted with antifade solution (AFM-01, Chromosome Science Laboratory).

For double staining of X and Y chromosome in canine spermatozoa, sex chromosome probes X-labelled with SpectrumGreen and Y-labelled with Cy3 (DXY-10, Chromosome Science Laboratory) were used. The decondensation condition of sperm nuclei was selected according to the results of a single staining experiment. The FISH procedures were similar to those described above.

#### Fluorescence microscopy and quantitative evaluation

Sperm samples were analyzed using a fluorescence microscope (Eclipse 80i, Nikon) equipped with DAPI (UV-2A, excitation 380–420 nm, absorption 450 nm), SpectrumGreen (B2-A, excitation 450–490 nm, absorption 520 nm) and Cy3 (G-2A, excitation 510–560 nm, absorption 590 nm) filters (Nikon). Digital images were acquired by a Peltier-cooled CCD camera (DS-5MC-L1, Nikon) using a software for digital photo editing (Paint.NET v3.5.10). FISH signals were analyzed visually using an ocular grid by counting random microscope fields

and scoring 500–600 spermatozoa per sample. Spermatozoa were scored only if they were intact and non-overlapped, had a clearly defined border and had not decondensed to more than twice the size of a non-decondensed sperm head, which could produce large and sometimes fragmented FISH signals [17].

#### Flow cytometric sperm sorting

Semen samples were collected as described above, and seminal plasma was removed by centrifugation at 2000 rpm for 15 min. The sorted spermatozoa were obtained following the general procedure described by Schenk *et al.* [26, 27]. Briefly, Spermatozoa were diluted with TALP [26] at a sperm concentration of  $2 \times 10^8$  cells/ml, and then stained with Hoechst 33342 (81.0–113.4  $\mu$ M, H3570, Invitrogen) and incubated for 35–40 min at 35 C. An equal volume of TALP containing 2.67% of purified egg yolk and 0.002% of food red (FD & C #40, CAS 25856-17-6, Invitrogen) was added to the sperm suspension [26]. The stained spermatozoa were sorted with a flow cytometer (MoFlo-SX, DakoCytomation, Fort Collins, CO, USA) operating at 40 PSI and with a laser power of 175 mW. Control samples consisted of unsorted spermatozoa from the same dogs.

All experiments were carried out in accordance with the guidelines for the care and use of animals approved by Obihiro University of Agriculture and Veterinary Medicine.

#### Statistical analysis

A chi-squared test was used to investigate the deviation from the expected ratio of 50:50 (X:Y). Differences were considered significant at a level of  $P < 0.05$ .

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