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Re-evaluating Percoll Density Gradient Centrifugation as a Bovine Sperm Sexing Method: DNA-Based Assessment and Field Fertility Outcomes in Holstein–Friesian Cattle in Lowland Indonesia

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Abstract

This study re-evaluated the effectiveness of Percoll density gradient centrifugation (PDGC) for separating X- and Y-chromosome-bearing spermatozoa in Holstein–Friesian (HF) cattle. Although PDGC has long been used in Indonesia, previous evaluations mainly relied on sperm morphometry, which assumes size differences between X- and Y-bearing spermatozoa. Non-sexed (NS) semen and PDGC-derived putative X- and Y-enriched fractions (SX and SY) were produced from contemporaneous ejaculates collected from two HF bulls, with eight ejaculates per bull. Post-thaw sperm quality was evaluated using computer-assisted semen analysis (CASA), chlortetracycline (CTC) staining, the Halomax assay, imaging flow cytometry, and real-time PCR (RT-qPCR) targeting X- and Y-linked markers. A field trial involving 1,649 HF cattle inseminated with NS or SX semen was conducted to assess pregnancy rate (PR), calving rate (CvR), and female calf proportion. PDGC processing reduced progressive motility and increased immotile spermatozoa compared with NS semen. In the absence of a sham-processed control, these effects likely reflected the PDGC workflow rather than chromosomal enrichment. Capacitation status and sperm DNA fragmentation were not significantly affected by treatment, whereas acrosome reaction showed a significant overall treatment effect. Imaging flow cytometry detected statistically significant but biologically small X:Y shifts, whereas RT-qPCR showed only modest, non-significant differences; both results remained close to the physiological 50:50 ratio. Field results showed no significant differences in PR or female calf proportion between NS and SX semen, although CvR was lower in the SX group. The evaluated PDGC protocol did not achieve meaningful chromosomal enrichment or enhance female calf production.

Introduction

The application of spermatozoa sexing technology is a practical approach to accelerate genetic progress in livestock and enhance the profitability of farming operations (Neculai-Valeanu and Ariton 2021). Sperm sexing involves altering the natural 1:1 ratio of X- and Y-chromosome-bearing spermatozoa using specific techniques to achieve a desired proportion (Rahman and Pang 2020). The use of sexed semen in artificial insemination (AI) enhances the probability of obtaining offspring with the desired sex, which is advantageous for both beef and dairy production systems (Espinosa-Cervantes and Córdova-Izquierdo 2012; Quelhas et al. 2021). In Indonesia, the pregnancy rate of Holstein-Friesian (HF) cattle raised in lowland areas remains relatively low at 24–28% (Utami et al. 2026), compared to over 50% in highland areas (Syah et al. 2023; Syah et al. 2024a). This suboptimal reproductive performance limits the availability of replacement heifers. Sperm sexing technology offers a potential means to increase female calf production and support herd replacement in lowland dairy systems. In dairy cattle production systems, the use of sexed semen is anticipated to facilitate the recovery of the female cattle population after the re-emergence of foot-and-mouth disease in Indonesia in 2022, which led to an approximate 12.9% reduction in population size (BPS 2022; Syah et al. 2024b).

The most widely recognized sperm sexing method is cell sorting, which exploits differences in DNA content between X- and Y-chromosome-bearing spermatozoa (DeJarnette et al. 2009; Garner 2006). This

technique can achieve purity levels of up to approximately 90% (De Vries et al. 2008). However, it requires expensive, high-technology equipment and skilled operators, and it poses a relatively high risk of sperm damage following the separation process (Garner et al. 2013). In addition, the sperm concentration in sexed semen is generally lower than that in conventional semen (Schenk et al. 1999).

One of the sperm separation approaches commonly used in Indonesia is Percoll density gradient centrifugation (PDGC) employing a 10-layer Percoll gradient (Safa et al. 2025). Percoll is a suspension of colloidal silica particles coated with polyvinylpyrrolidone (PVP), designed to be osmotically balanced and relatively non-toxic to cells (Pertoft 2000). During spermatozoa separation, Percoll is diluted with a suitable extender to establish density gradients of defined concentrations. The application of PDGC for sperm sexing is based on the assumption that the slightly higher DNA content of X-chromosome-bearing spermatozoa may produce a density difference sufficient for fractionation from Y-chromosome-bearing spermatozoa (Machado et al. 2009). Compared with flow cytometric cell sorting, PDGC is simpler, less expensive, and does not require specialized equipment; however, its practical appeal does not necessarily indicate equivalent accuracy or reliability for chromosomal sperm sexing (Utami et al. 2025).

Several studies have reported sperm quality after separation using Percoll, including motility ranging from 44.1% to 55.82%, viability from 41.68% to 60.27%, abnormality rates between 3.64% and 7.81%, DNA integrity values reported at approximately 76.16% to 76.69%, and sperm concentrations reaching up to 122.13 million/mL (Rahmawati et al. 2025; Safa et al. 2025; Wahyudi et al. 2025). Furthermore, PDGC has been reported to produce 72–77.5% X- or Y-sperm in Ongole bulls (Kusumawati et al. 2019), 79.8–85.4% in Bali bulls (Rahmawati et al. 2025), and 77.1–80.6% in HF bulls (Yekti et al. 2023) based on morphometric analysis (Rasad et al. 2019). However, the reliability of sperm head size as a marker for X- and Y-chromosome-bearing spermatozoa remains controversial. Although Van Munster (2002) observed differences in bovine sperm dimensions, other studies using objective morphometry (Hossain et al. 2001), fluorescence *in situ* hybridization (Zavaczki et al. 2006), and atomic force microscopy (Carvalho et al. 2013) have reported limited or no reliable morphological differences between X- and Y-chromosome-bearing spermatozoa. Given these discrepancies, previous morphometry-based estimates of PDGC sexing efficiency require validation using more robust DNA-related approaches (Butler et al. 2014; Garner 2006; Srinivasa Prasad et al. 2010). Therefore, this study aimed to re-evaluate the capacity of the 10-layer PDGC protocol to enrich X- or Y-chromosome-bearing spermatozoa in HF bull semen using imaging flow cytometry and RT-qPCR marker analysis, while also assessing post-thaw sperm quality. Because laboratory evidence alone may not reflect field applicability, the study further evaluated pregnancy rate, calving among cows diagnosed pregnant at 60 days, and female calf proportion after AI using conventionally processed NS semen or the PDGC-derived putative SX fraction.

Materials and methods

Chemicals and reagents

Percoll (Sigma-Aldrich, St. Louis, MO, USA) was used for sperm separation through density gradient centrifugation. A Tris-aminomethane-based extender supplemented with 20% egg yolk was used for semen dilution and Percoll gradient preparation. Chlortetracycline (CTC; Sigma C4881, USA) was used for the assessment of sperm capacitation status and acrosome reaction. Sperm DNA fragmentation (SDF) was evaluated using the Halomax kit (Halotech, Spain), along with agarose, lysis solution, ethanol (70% and 97%), and FluoRed fluorochrome. Hoechst 33342 (Invitrogen, USA) and phosphate-buffered saline (PBS) were used for DNA staining during the imaging flow cytometry-based assessment of X- and Y-chromosome-bearing sperm proportions. For RT-qPCR marker analysis, total RNA was extracted using the PureLink™ RNA Mini Kit (Invitrogen™, Thermo Fisher Scientific, USA) according to the manufacturer's instructions and reverse-transcribed into cDNA using 5× iScript™ RT Supermix (Bio-Rad Laboratories, USA). Quantitative PCR amplification was performed using 2× SensiFAST™ SYBR® No-ROX Mix and gene-specific primers targeting PLP, SRY, and GAPDH. Commercially available X- and Y-chromosome-bearing sex-sorted semen produced using a cell sorter (GENEX, Shawano, WI, USA) was used as a positive control for the RT-qPCR marker-based assessment.

Animals, semen collection and experiment design

Two sexually mature HF (*Bos taurus*) bulls were used in this study and housed individually at the Singosari Artificial Insemination Center (SAIC), Malang, Indonesia. The bulls were routinely managed as semen donors and were accustomed to twice-weekly semen collection as part of the standard semen production program at the AI center. Only ejaculates with total sperm motility exceeding 70% and sperm abnormalities below 20% were included in the study. A balanced split-ejaculate design was applied. Each ejaculate was considered one biological replicate and was divided into two aliquots: one aliquot was processed as non-sexed semen (NS) according to the conventional semen, whereas the other was subjected to Percoll density gradient centrifugation (PDGC) to obtain the putative X-enriched and Y-enriched fractions, designated as SX and SY, respectively. Technical measurements from straws, microscopic fields, counted spermatozoa, imaging flow cytometry events, and RT-qPCR analysis were averaged within each ejaculate-treatment combination before statistical analysis. Thus, the experimental unit for laboratory analyses was the ejaculate, resulting in sixteen biological replicates per treatment group. It should be noted that the NS aliquot was processed according to the conventional frozen semen protocol, whereas the PDGC aliquot underwent additional gradient centrifugation, fraction recovery, washing, and re-centrifugation before cryopreservation. Therefore, comparisons of post-thaw sperm quality between NS and PDGC-derived fractions were interpreted as effects associated with the overall PDGC processing workflow rather than as isolated effects of X- or Y-chromosome-bearing sperm enrichment.

Sexing procedure

Fresh semen samples were collected from sixteen ejaculates obtained from two HF bulls, with eight ejaculates per bull. Each ejaculate was divided into two equal aliquots. One aliquot was processed as

NS, while the other underwent sperm separation using PDGC, as illustrated in Fig. 1. After separation, spermatozoa recovered from the middle and bottom fractions were operationally designated as the putative Y-enriched fraction (SY) and putative X-enriched fraction (SX), respectively, based on the conventional PDGC interpretation. Sperm separation utilized a ten-layer Percoll gradient medium (Sigma-Aldrich, St. Louis, MO, USA) with concentrations of 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, and 65%. The gradients were prepared by diluting Percoll with a Tris-aminomethane-based extender supplemented with 20% egg yolk, referred to as Tris–egg yolk extender. Each 0.5 mL layer was sequentially placed in a 15 mL centrifuge tube, from 65% at the bottom to 20% at the top, resulting in a total gradient volume of 5 mL. One mL of fresh semen, previously diluted 1:1 with Tris–egg yolk extender, was gently layered onto the gradient and centrifuged at 481 × g for 5 min. Following centrifugation, the upper 1 mL fraction was discarded according to the established SAIC PDGC workflow because this low-density layer was not used for production of the putative sex-enriched fractions. Subsequently, 2.5 mL from each of the middle and bottom fractions was transferred into new tubes for washing. Each fraction was diluted with 3 mL of Tris–egg yolk extender and centrifuged at 281 × g for 5 min. The supernatant was discarded, leaving approximately 2 mL of sperm-containing sediment. After sperm separation, NS, SX, and SY semen samples were cryopreserved according to the standard frozen semen production protocol at the SAIC. Each semen sample was diluted with Tris–egg yolk extender and gradually cooled from 37°C to 5°C. After cooling, a glycerol-containing extender was added as a cryoprotectant following the SAIC protocol. Only semen samples with sperm motility greater than 55% before freezing were processed further. The diluted semen was filled and sealed into 0.25 mL straws at a final concentration of 25 × 10^6 spermatozoa per straw. The straws were equilibrated at 5°C for 0–4 h, exposed to liquid nitrogen vapor for 12 min until the temperature reached approximately − 140°C, and then plunged into liquid nitrogen at − 196°C for storage. The frozen semen was stored in liquid nitrogen for at least 24 h before further evaluation (Wahyudi et al. 2025).

Evaluation of post-thaw sperm quality and kinematic variables

Evaluation of post-thaw sperm motility and kinematics

Post-thaw semen motility analysis was performed using the CASA system (Androscope®, Minitube GmbH, Tiefenbach, Germany). Frozen semen samples were thawed in a 37°C water bath for 30 s prior to evaluation. For slide preparation, 5 µL of thawed semen was placed on a pre-warmed microscope slide maintained at 37°C and covered with a glass coverslip. Sperm motility and kinematic parameters were assessed using the CASA system according to the protocol described by Utami et al. (2025). The evaluated parameters included individual motility (MOT), progressive motility (PMOT), straight-line distance (DSL, µm), curvilinear distance (DCL, µm), average path distance (DAP, µm), straight-line velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), average path velocity (VAP, µm/s), straightness (STR, %), linearity (LIN, %), wobble (WOB, %), amplitude of lateral head displacement (ALH, µm), and beat cross frequency (BCF, Hz). Post-thaw sperm motility and kinematic variables were evaluated using 16 biological replicates per treatment group. For each ejaculate-treatment combination, one straw was

thawed and at least five microscopic fields were recorded. Field-level measurements were averaged before statistical analysis.

Evaluation of sperm capacitation and acrosome reaction

The CTC (Sigma C4881, USA) assay was used to evaluate the capacitation status and acrosomal reaction of spermatozoa (Pramudhita et al. 2025), following the protocol described by Benko et al. (Benko et al. 2024). Capacitation status and acrosome reaction were evaluated in sixteen biological replicates per treatment group. At least 200 spermatozoa were evaluated for each ejaculate-treatment combination. Based on the fluorescence distribution pattern, spermatozoa were classified into three staining categories. The F pattern was characterized by uniform fluorescence over the entire sperm head, indicating non-capacitated or freshly ejaculated spermatozoa. The B pattern showed fluorescence restricted to the acrosomal region with no fluorescence in the post-acrosomal area, indicating capacitated spermatozoa. The acrosome reaction pattern exhibited fluorescence localized in the equatorial segment, indicating acrosome-reacted spermatozoa (Mattioli et al. 1996; Maxwell and Johnson, 1997).

Evaluation of Sperm DNA Fragmentation

SDF was assessed using the Halomax kit (Halotech HT-BT40, Spain) in accordance with the method described by García-Macías et al. (2007). Sperm samples were diluted in PBS to obtain a final concentration of 15–20 × 10^6 sperm/mL. The agarose solution was initially heated in a water bath at 95–100°C for 5 min and subsequently equilibrated at 37°C for 5 min. An aliquot of 25 µL sperm suspension was gently mixed with 50 µL of liquefied agarose in an Eppendorf tube maintained at 37°C. A 2 µL droplet of the mixture was placed onto a glass slide, covered with a coverslip, and incubated on a pre-cooled glass surface (2–8°C) for 5 min. Following incubation, the coverslip was carefully removed and the preparation was immersed in lysis solution for 5 min, then rinsed thoroughly with distilled water for an additional 5 min. The samples were sequentially dehydrated using 70% and 97% ethanol and air-dried. Subsequently, a 2 µL aliquot was applied to the slide and stained with the high-sensitivity fluorochrome FluoRed (Halotech HT-RFS100, Spain). SDF was evaluated in sixteen biological replicates per treatment group. At least 200 spermatozoa per sample were evaluated using a fluorescence microscope (Olympus BX-33, Japan) at 1000× magnification. Spermatozoa exhibiting DNA fragmentation were identified by the presence of large, irregular halos of chromatin dispersion, whereas non-fragmented spermatozoa displayed compact nucleoids with small, uniform halos.

Evaluation of X/Y-chromosome-bearing Sperm Proportions and X-/Y-Linked Molecular Marker Signals after PDGC Separation

Identification of X- and Y-chromosome-bearing sperm proportions in post-thaw PDGC-derived fractions using

imaging flow cytometry

The procedure followed the method described by Thongkham et al. (2021) The proportion of X- and Y-chromosome-bearing spermatozoa was evaluated in 16 biological replicates per treatment group, represented by split ejaculates from two bulls. Technical replicate measurements were averaged within each ejaculate-treatment combination before statistical analysis. Thawed sperm samples were centrifuged at 269 × g for 8 minutes, then diluted to a concentration of 1×10^6 sperm/mL in PBS containing 1.2 µL of Hoechst 33342 (50 µg/mL). The samples were incubated at 37°C in the dark for 10 minutes before analysis. Imaging flow cytometry (FlowSight, Seattle, WA, USA) was employed using a 10 mW 405-nm laser to excite Hoechst 33342. Events were sequentially gated to exclude debris, aggregates, unfocused events, and Hoechst-negative particles before fluorescence intensity was used to discriminate putative X- and Y-chromosome-bearing sperm populations. Because X-chromosome-bearing spermatozoa contain more DNA than Y-chromosome-bearing spermatozoa, they were expected to emit stronger Hoechst fluorescence signals. The fluorescence intensity of Hoechst 33342 was visualized as histogram plots and analysed using IDEAS software version 6.2 (Amnis, Seattle, WA, USA).

Assessment of relative X- and Y-linked molecular marker signals using SYBR® Green RT-qPCR

Total RNA was extracted using the PureLink™ RNA Mini Kit and subsequently reverse-transcribed into cDNA using 5× iScript™ RT Supermix. The resulting cDNA was used as a template for molecular marker analysis to compare the relative abundance of X- and Y-linked signals among treatment groups. Because this assay was performed using RNA-derived cDNA rather than genomic DNA, it was interpreted as supportive molecular marker evidence and not as a direct genomic quantification of the X:Y sperm ratio. Quantitative PCR was performed using 2× SensiFAST™ SYBR® No-ROX Mix on a qTOWER 2.2 RT-qPCR System (Analytik Jena GmbH, Jena, Germany). For each treatment group, cDNA samples were analysed in triplicate for each target gene, including GAPDH as an internal control, PLP as an X-chromosome-associated marker, and SRY as a Y-chromosome-associated marker. The relative amplification levels of PLP and SRY were used to indicate the relative abundance of X- and Y-linked molecular marker signals, respectively. The primer sequences, amplicon sizes, and GenBank accession numbers for GAPDH, PLP, and SRY are provided in Table 1 (Parati et al. 2006).

Table 1
Specific primer pairs used for the amplification of bovine SRY, PLP, and GAPDH genes

Gene	Sequence (5′ → 3′)	Length (bp)	Accession No.
Y chromosome specific			
SRY-Forward	GAAAATAAGCACAAGAAAGTCCAGG	124	EU581861.1
SRY-Reverse	CAAAAGGAGCATCACAGCAGC		
X chromosome specific			
PLP-Forward	GGTGTGTTAGTTTCTGCTGTACAATAAATGG	96	AJ009913.1
PLP-Reverse	GATGGCAGGTGAGGGTAGGA		
Housekeeping gene			
GAPDH-Forward	GGCGCCAAGAGGGTCAT	120	NM_001034034.2
GAPDH-Reverse	GGTGGTGCAGGAGGCATT		

SRY: sex-determining region Y; PLP: proteolipid protein; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

For each sample, the three target genes were amplified in separate wells using an identical cDNA template volume of 1 µL. Each RT-qPCR reaction was followed by melting curve analysis to verify amplification specificity and to detect potential primer-dimer formation. The 20 µL PCR reaction mixture consisted of 1 µL cDNA template, 1 µL forward primer working solution, 1 µL reverse primer working solution, 7 µL distilled water, and 10 µL 2× SensiFAST™ SYBR® No-ROX Mix. Amplification was performed according to the manufacturer's optimized protocol as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 15 s, and extension at 72°C for 30 s.

Field trial

A total of 1,649 HF cattle were sourced from 783 smallholder farmers in Malang Regency, East Java, Indonesia, during a field fertility evaluation conducted from November 2023 to December 2024. The animals were allocated into two AI groups based on semen type: the NS group (n = 442) and the SX semen group (n = 1,207). Farmers conducted estrus detection twice daily, in the morning and afternoon, and reported animals exhibiting behavioural and physiological signs of estrus to local field officers. Certified inseminators performed AI between 08:00 and 16:00. Pregnancy status was assessed 60 days after AI via rectal palpation. Animals that failed to maintain pregnancy were recorded, and after calving, the sex of the newborn calves was recorded. The field fertility parameters included pregnancy rate (PR), calving among cows diagnosed pregnant at 60 days (CvR), and female calf proportion. Because

allocation was performed under field conditions, potential farm-level, cow-level, inseminator-level, seasonal, and semen-batch confounders should be considered when interpreting the fertility outcomes.

Statistical analysis

Statistical analyses were performed using R software (version 4.4.1; R Core Team, Vienna, Austria). For laboratory data, each ejaculate was considered the experimental unit. Because each ejaculate was divided into matched NS, putative SX, and putative SY fractions, treatment effects were analysed using mixed-effects models with treatment as a fixed effect, bull as a fixed blocking factor, and ejaculate nested within bull as a random effect. Bull was treated as a fixed blocking factor because only two bulls were included. Technical replicate measurements from straws, microscopic fields, counted spermatozoa, imaging flow cytometry events, and RT-qPCR wells were averaged or aggregated within each ejaculate-treatment combination before analysis. Continuous variables, including CASA motility and kinematic parameters, were analysed using linear mixed-effects models. Proportion-based outcomes, including capacitation status, acrosome reaction, SDF, and X-chromosome-bearing sperm proportion determined by imaging flow cytometry, were analysed using binomial generalized linear mixed-effects models when count data were available, or after logit transformation when only proportional values were available. Pairwise comparisons were performed using estimated marginal means with Tukey adjustment. For the field fertility evaluation, pregnancy rate, calving among cows diagnosed pregnant at 60 days, and female calf proportion were analysed as binomial outcomes using logistic regression or chi-square tests when only aggregate data were available. Because complete cow-level and farm-level covariates were not available for all inseminations, field outcomes were interpreted as observational fertility results rather than definitive causal evidence. Statistical significance was declared at $p < 0.05$.

Results

Sperm Motility and Kinematics

The post-thaw motility and kinematic characteristics of conventionally processed NS semen and PDGC-processed putative SX and SY fractions are presented in Table 2. Treatment significantly influenced several motility variables, including progressive motility, fast motility, slow motility, local motility, and immotile spermatozoa ($p < 0.01$). Because the SX and SY fractions underwent additional gradient centrifugation, fraction recovery, washing, and re-centrifugation, these differences were interpreted as effects associated with the overall PDGC processing workflow rather than direct impacts of X- or Y-chromosome-bearing spermatozoa. Progressive motility was highest in NS semen (55.25 ± 1.28%), lower in SX semen (47.18 ± 1.28%), and intermediate in SY semen (51.34 ± 1.28%), with no significant difference between SY and either NS or SX. Fast motility was reduced in SX semen (32.05 ± 1.09%) compared to NS (36.00 ± 1.09%) and SY semen (36.82 ± 1.09%). Slow motility was greater in NS semen (16.57 ± 0.68%) than in SX (12.55 ± 0.68%) and SY semen (11.84 ± 0.68%). Local motility was significantly lower in SY semen (9.33 ± 0.93%) than in NS (16.11 ± 0.93%) and SX semen (15.27 ± 0.93%).

Conversely, the proportion of immotile spermatozoa was lower in NS semen (28.65 ± 1.41%) than in SX (37.57 ± 1.41%) and SY semen (39.34 ± 1.41%). Circle motility was not significantly affected by treatment ($P = 0.89$).

Treatment significantly influenced several sperm kinematic variables, including VCL, VAP, DCL, and ALH. VCL was lower in NS semen (121.54 ± 5.00 μm/s) compared to SX (135.71 ± 5.00 μm/s) and SY semen (138.81 ± 5.00 μm/s). VAP differed among treatments ($P = 0.04$), with SY semen exhibiting a higher value than NS semen, and SX semen displaying an intermediate value. DCL was also higher in SY semen (35.72 ± 0.99 μm) than in NS semen (32.31 ± 0.99 μm), with SX semen again showing an intermediate value (33.53 ± 0.99 μm). Similarly, ALH was greater in SX (3.26 ± 0.13 μm) and SY semen (3.37 ± 0.13 μm) than in NS semen (2.84 ± 0.13 μm). In contrast, VSL, DSL, DAP, BCF, HAC, LIN, and STR were not significantly affected by treatment ($p > 0.05$). The bull effect was not significant for any motility or kinematic variable evaluated ($p > 0.05$).

Table 2
Post-thaw motility and kinematic variables of conventionally processed non-sexed semen and PDGC-processed putative sperm fractions from Holstein–Friesian bulls

Parameter	NS	SX	SY	*P-value*[1]	*P-value*[2]
PM (%)	55.25 ± 1.28	47.18 ± 1.28	51.34 ± 1.28	< 0.01	0.56
FM (%)	36.00 ± 1.09	32.05 ± 1.09	36.82 ± 1.09	< 0.01	0.98
SM (%)	16.57 ± 0.68	12.55 ± 0.68	11.84 ± 0.68	< 0.01	0.22
CM (%)	2.69 ± 0.17	2.59 ± 0.17	2.69 ± 0.17	0.89	0.93
LM (%)	16.11 ± 0.93	15.27 ± 0.93	9.33 ± 0.93	< 0.01	0.34
Immotile (%)	28.65 ± 1.41	37.57 ± 1.41	39.34 ± 1.41	< 0.01	0.27
VCL (μm/s)	121.54 ± 5.00	135.71 ± 5.00	138.81 ± 5.00	< 0.01	0.10
VSL (μm/s)	49.00 ± 1.82	52.60 ± 1.82	53.30 ± 1.82	0.21	0.37
VAP (μm/s)	56.71 ± 2.07	62.75 ± 2.07	63.69 ± 2.07	0.04	0.07
DCL (μm)	32.31 ± 0.99	33.53 ± 0.99	35.72 ± 0.99	< 0.01	0.12
DSL (μm)	12.26 ± 0.42	11.33 ± 0.42	12.50 ± 0.42	0.12	0.54
DAP (μm)	14.68 ± 0.43	14.64 ± 0.43	15.72 ± 0.43	0.12	0.11
ALH (μm)	2.84 ± 0.13	3.26 ± 0.13	3.37 ± 0.13	< 0.01	0.09
BCF (Hz)	8.96 ± 0.28	8.41 ± 0.28	8.48 ± 0.28	0.22	0.53
HAC	0.37 ± 0.02	0.40 ± 0.02	0.38 ± 0.02	0.52	0.16
LIN (%)	0.29 ± 0.02	0.30 ± 0.02	0.28 ± 0.02	0.47	0.14
STR (%)	0.50 ± 0.05	0.55 ± 0.05	0.50 ± 0.05	0.55	0.19

NS, conventionally processed non-sexed semen; SX, putative X-enriched fraction recovered after PDGC; SY, putative Y-enriched fraction recovered after PDGC; PM: progressive motility; FM: fast motility; SM: slow motility; CM: circular motility; LM: local motility; VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; DCL: curvilinear distance; DSL: straight-line distance; DAP: average path distance; ALH: amplitude of lateral head displacement; BCF: beat cross frequency; HAC: head angle change; LIN: linearity; STR: straightness. Different letters indicate significant differences among treatments at $P < 0.05$. P-value1 indicates the treatment effect. P-value2 indicates the bull effect.

Spermatozoa capacitation and acrosome reaction

Based on the post-thaw evaluation of sperm capacitation status, the NS group tended to show a higher proportion of non-capacitated spermatozoa while maintaining a favourable proportion of capacitated spermatozoa compared with the SX and SY groups. However, mixed-model analysis indicated that the

proportions of capacitated and non-capacitated spermatozoa did not differ significantly among treatment groups ($p > 0.05$). Similarly, the bull effect was not significant for capacitated spermatozoa ($P = 0.34$) or non-capacitated spermatozoa ($P = 0.60$). Acrosome-reacted spermatozoa showed a significant overall treatment effect ($p < 0.01$), however, pairwise comparisons indicated that NS did not differ significantly from either PDGC-derived fraction. The bull effect on acrosome-reacted spermatozoa was also not significant ($P = 0.31$).

Sperm DNA Fragmentation

Post-thaw SDF results in NS, SX, and SY groups are shown in Table 3. SDF was not significantly affected by treatment ($P = 0.0716$) or bull effect ($P = 0.9999$), although the SX group showed a numerically higher value than the NS and SY groups.

Table 3
Post-thaw capacitation status, acrosome reaction, and sperm DNA fragmentation in conventionally processed non-sexed semen and PDGC-processed putative sperm fractions

Parameter	NS	SX	SY	*P-value*[1]	*P-value*[2]
Capacitated spermatozoa (%)	26.39 ± 2.26	28.41 ± 6.25	27.96 ± 4.46	0.52	0.34
Non-capacitated spermatozoa (%)	71.26 ± 2.72	70.03 ± 6.14	68.25 ± 3.32	0.15	0.60
Acrosome-reacted spermatozoa (%)	3.47 ± 1.23	2.68 ± 1.03	4.92 ± 1.69	< 0.01	0.31
SDF (%)	3.55 ± 0.32	4.37 ± 0.39	3.27 ± 0.29	0.07	0.99

NS, conventionally processed non-sexed semen; SX, putative X-enriched fraction recovered after PDGC; SY, putative Y-enriched fraction recovered after PDGC; SDF, sperm DNA fragmentation.

Values for capacitation status, acrosome reaction, and SDF are presented as model-estimated means ± SE; totals may not equal exactly 100% because of rounding and independent model estimation. P-value1 indicates the treatment effect. P-value2 indicates the bull effect.

X- and Y-chromosome-bearing spermatozoa proportions by imaging flow cytometry

The proportions of X- and Y-chromosome-bearing spermatozoa in post-thaw NS, SX, and SY samples, as determined by flow cytometry using Hoechst 33342 DNA staining, are shown in Fig. 2. Mixed-model analysis showed that treatment significantly affected the proportions of both X- and Y-chromosome-bearing spermatozoa ($P = 0.0023$), whereas the bull effect was not significant ($P = 0.7454$). The proportion of Y-chromosome-bearing spermatozoa was lower in NS (50.98 ± 0.40%) than in SX (52.90 ± 0.40%) and SY (52.67 ± 0.40%), while the proportion of X-chromosome-bearing spermatozoa was higher in NS (49.02 ± 0.40%) than in SX (47.10 ± 0.40%) and SY (47.33 ± 0.40%). However, despite these

statistically significant differences, all groups remained close to the expected physiological X ratio of approximately 50:50.

Relative abundance of X- and Y-linked molecular marker signals by RT-qPCR

RT-qPCR analysis was conducted to assess the relative abundance of X- and Y-chromosome-associated molecular marker signals by measuring the fold relative expression of PLP and SRY, as shown in Fig. 3. The NS group served as the baseline control, whereas sorted-X and sorted-Y semen samples were used as positive controls. In the NS group, PLP and SRY marker signals were relatively balanced, consistent with the expected non-enriched pattern. The SX fraction showed a higher relative PLP signal than SRY signal, whereas the SY fraction showed a slightly higher relative SRY signal than PLP signal. However, the differences in mean fold relative expression between the SX and SY groups were not statistically significant for either the Y-specific marker (SRY; $P = 0.640$) or the X-specific marker (PLP; $P = 0.525$). In contrast, the positive controls showed clear marker-specific predominance. Sorted-X semen displayed a strong predominance of PLP, whereas sorted-Y semen exhibited a strong predominance of SRY. Overall, the PDGC-derived putative fractions showed only modest, non-significant shifts in X- and Y-linked marker abundance, whereas the sorted semen controls confirmed that the RT-qPCR marker approach could detect marked differences in X- and Y-associated molecular signals. Because this assay used RNA-derived cDNA, the findings should not be interpreted as direct genomic quantification of X- and Y-chromosome-bearing sperm proportions.

Field trial

PR, CvR, and female calf proportion following AI using NS and PDGC-derived putative SX semen are presented in Table 4. A total of 95 cows (21.59%) inseminated with NS semen and 297 cows (24.61%) inseminated with SX semen became pregnant. The PR did not differ significantly between the NS and SX groups ($P = 0.188$). Among the pregnant cows, 87.37% of those inseminated with NS semen and 75.42% of those inseminated with SX semen maintained pregnancy until calving, resulting in a significantly higher CvR in the NS group ($P = 0.014$). However, the proportion of female calves born from cows inseminated with NS semen (42.17%) and SX semen (42.41%) did not differ significantly ($P = 0.970$). Because field-level confounders were not fully controlled, the lower CvR observed in the SX group should be interpreted cautiously and should not be attributed solely to PDGC processing.

Table 4
Pregnancy outcome and female calf proportion following artificial insemination with conventionally processed non-sexed semen and the PDGC-derived putative X-enriched fraction in Holstein–Friesian cattle raised in lowland areas of Indonesia

Parameters	**NS**	**PDGC-derived putative SX fraction (SX)**	***P-value***
n	442	1,207	-
PR (%)	21.59 (95/442)	24.61 (297/1,207)	0.19
CvR (%)	87.37 (83/95)	75.42 (224/297)	0.01
Female calf proportion (%)	42.16 (35/83)	42.41 (95/224)	0.97

NS, conventionally processed non-sexed semen; SX, putative X-enriched fraction recovered after PDGC; PR, pregnancy rate; CvR, calving among cows diagnosed pregnant at 60 days.

*Female calf proportion refers to the percentage of female calves among calves born.

Discussion

This study re-evaluated the effectiveness of PDGC as a bovine sperm sexing method using DNA-content-based imaging flow cytometry, RT-qPCR marker analysis, and field fertility outcomes. This re-evaluation is important because PDGC has long been used in Indonesia as a practical and low-cost sperm separation method (Safa et al. 2025), and several previous cattle studies reported apparent enrichment of X- or Y-chromosome-bearing spermatozoa based mainly on sperm head morphometry (Rahmawati et al. 2025; Yekti et al. 2023; Yekti et al.2024). The present findings indicate that the evaluated 10-layer PDGC protocol did not produce biologically meaningful chromosomal enrichment in HF bull semen. Although imaging flow cytometry detected small statistical shifts in X- and Y-chromosome-bearing sperm proportions, all treatment groups remained close to the physiological 50:50 ratio, and RT-qPCR marker analysis showed only modest, non-significant differences in PLP and SRY signals. These findings suggest that earlier morphometry-based estimates may have overestimated the capacity of PDGC to separate X- and Y-chromosome-bearing spermatozoa.

The decline in progressive motility and the increase in immotile spermatozoa observed in the PDGC-derived fractions are likely related to the additional processing steps required for gradient separation, including centrifugation, migration through Percoll layers, fraction recovery, washing, re-centrifugation, and additional handling time. However, because a sham-processed control was not included, the present study cannot distinguish the specific effects of Percoll gradient exposure from those of centrifugation, washing, recovery, and handling. Therefore, differences in post-thaw sperm quality between NS semen and PDGC-derived fractions should be interpreted as responses associated with the overall PDGC processing workflow rather than as direct effects of X- or Y-chromosome-bearing sperm enrichment. Future studies should include a sham-processed control that undergoes equivalent dilution, centrifugation, washing, and handling without Percoll gradient fractionation.

The PDGC-derived fractions showed limited changes in selected functional endpoints, including capacitation status and SDF; however, these results should be interpreted cautiously because no sham-processed control was included. A key finding of this study was that statistically detectable differences in X- and Y-chromosome-bearing sperm proportions did not translate into biologically meaningful enrichment. The imaging flow cytometry findings were consistent with the RT-qPCR marker analysis (Paitoon et al. 2024; Sringarm et al. 2022; Thongkham et al. 2021), although the latter should be interpreted as relative X- and Y-linked marker abundance rather than a direct genomic measurement of sperm sex ratio (Maleki et al. 2013; Sringarm et al. 2022). The sorted-X and sorted-Y controls confirmed that the RT-qPCR marker approach could detect marked differences in PLP and SRY signals, but the PDGC-derived fractions showed only modest, non-significant shifts. Collectively, these findings support the interpretation that the evaluated PDGC protocol failed to achieve substantial chromosomal enrichment under the conditions tested.

The failure of Percoll to separate X- and Y-chromosome-bearing spermatozoa may be due to the small difference in DNA content between the two sperm populations in cattle. This difference is only about 3.8%. Such a minor disparity limits the resolution possible with density-gradient separation (Wolf et al. 2008). The mechanism behind Percoll-mediated sperm selection remains incompletely understood (Kobayashi et al. 2004). Its effectiveness seems to depend on technical factors such as gradient volume, centrifugation force, and centrifugation time. Under certain conditions, centrifugation may even result in both X- and Y-chromosome-bearing spermatozoa being recovered together in the final pellet (Buranaamnuay et al. 2015). This prevents meaningful chromosomal enrichment. Taken together, these findings suggest that Percoll is more likely to select spermatozoa with favourable functional characteristics than to provide a reliable or consistent method for sperm sexing.

The study was subsequently extended to a field fertility evaluation. The objective was to determine whether use of the PDGC-derived putative SX fraction affected fertility outcomes and female calf production under field conditions. The field results showed no significant improvement in PR and no increase in female calf proportion after AI with the SX fraction compared with NS semen. This finding is consistent with the laboratory evidence showing that PDGC did not meaningfully enrich X-chromosome-bearing spermatozoa. Although CvR was significantly lower in the SX group, this result should be interpreted cautiously because field-level, cow-level, farm-level, inseminator-level, seasonal, and semen-batch confounders were not fully controlled. Therefore, the lower CvR cannot be attributed solely to PDGC-derived semen. Nevertheless, this observation indicates that pregnancy maintenance after AI with PDGC-processed semen warrants further investigation under better-controlled field conditions (Purnawan et al. 2025).

This study has several limitations. First, the laboratory experiment included semen from only two HF bulls; therefore, the findings should be interpreted within the context of the tested bulls and the specific 10-layer PDGC protocol used. Second, a sham-processed control was not included, preventing separation of the effects of Percoll gradient exposure from those of centrifugation, washing, fraction recovery, and additional handling. Third, the RT-qPCR assay was based on RNA-derived cDNA and should

therefore be interpreted as a relative marker-abundance analysis rather than a direct genomic quantification of X- and Y-chromosome-bearing sperm proportions. Fourth, although the field evaluation included a large number of inseminations, cow-level, farm-level, inseminator-level, seasonal, and semen-batch confounders were not fully controlled. Consequently, the field outcomes should be interpreted as supportive observational fertility results rather than definitive causal evidence. These limitations highlight the need for future studies involving a larger number of bulls, appropriate sham-processing controls, genomic DNA-based validation, and more rigorously controlled field fertility designs.

In conclusion, the evaluated 10-layer PDGC protocol did not produce biologically meaningful enrichment of X- or Y-chromosome-bearing spermatozoa and did not increase the proportion of female calves following field insemination. Therefore, the practical effectiveness of this PDGC protocol as a sperm sexing method should be critically reconsidered and further validated under more rigorous experimental conditions.

Declarations

Animal ethics and consent to participate

All procedures involving animal experimentation in this study were approved by the Ethics Committee of the Institute of Biosciences, Universitas Brawijaya (Ethical Clearance No. 47/EC/KEPK/02/2024).

Competing interests

The authors declare no conflict of interest.

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Data availability

The dataset supporting the findings of this study is publicly available in Zenodo at https://doi.org/10.5281/zenodo.20612161.

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Figures

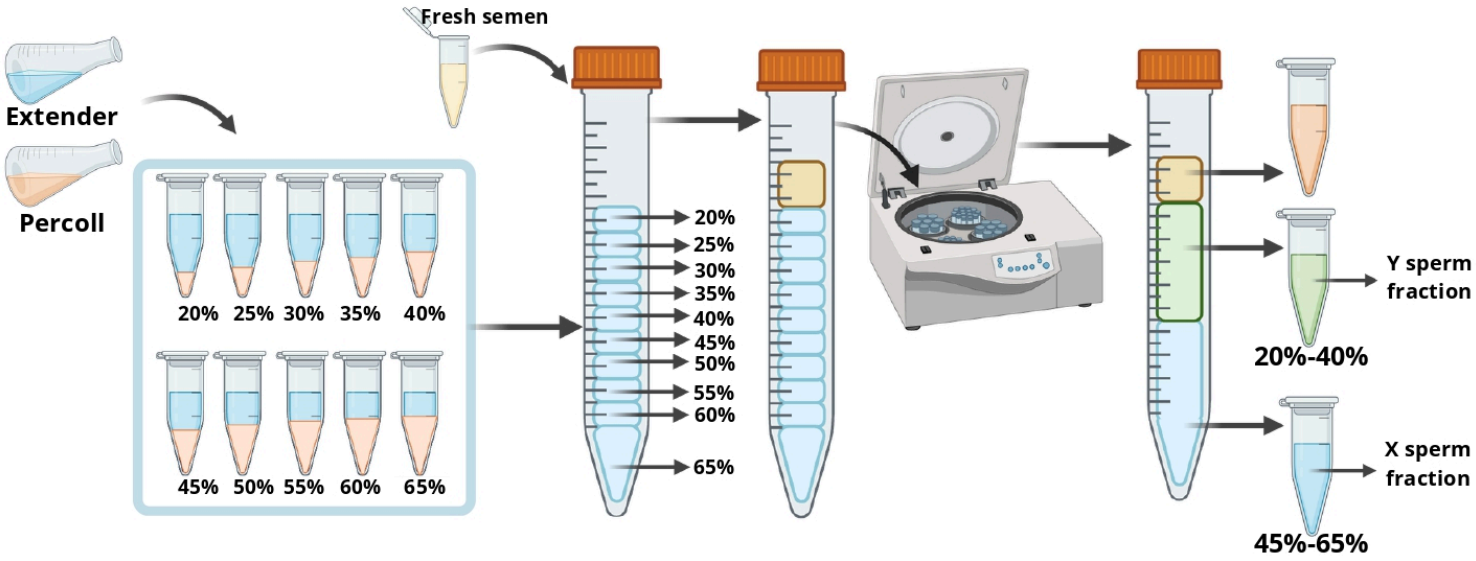


Figure 1

Schematic illustration of spermatozoa separation using a Percoll density gradient centrifugation.

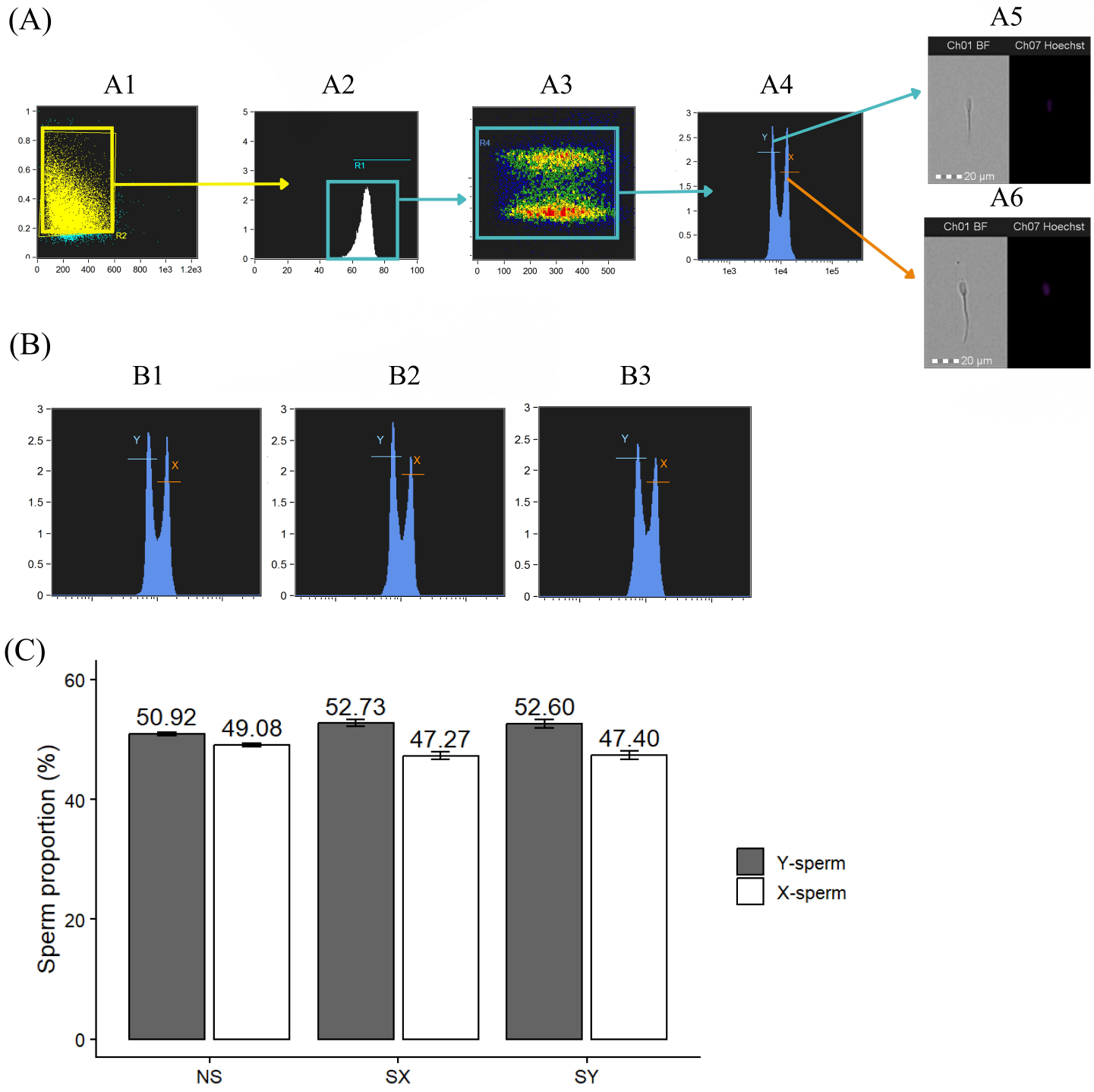


Figure 2

Discrimination of X- and Y-chromosome-bearing spermatozoa in post-thaw Holstein–Friesian bull semen following Percoll density gradient centrifugation using imaging flow cytometry. (A) Sequential gating strategy used for sperm classification: (A1) sperm events were selected while debris, aggregates, and unsuitable events were excluded; (A2) focused spermatozoa were gated to ensure accurate image-based analysis; (A3) Hoechst 33342-positive spermatozoa were selected for fluorescence-based discrimination; and (A4) the fluorescence-intensity histogram showed two distinct peaks corresponding

to the putative Y- and X-chromosome-bearing sperm populations. Representative bright-field and Hoechst fluorescence images are shown for spermatozoa classified as putative Y-bearing (A5) and X-bearing (A6). (B) Representative Hoechst 33342 fluorescence histograms for non-sexed semen (B1), the putative X-enriched fraction (B2), and the putative Y-enriched fraction (B3). The lower- and higher-fluorescence peaks represent putative Y- and X-chromosome-bearing spermatozoa, respectively. (C) Proportions of putative Y- and X-chromosome-bearing spermatozoa in each treatment group. Values are presented as least-squares means ± SE.

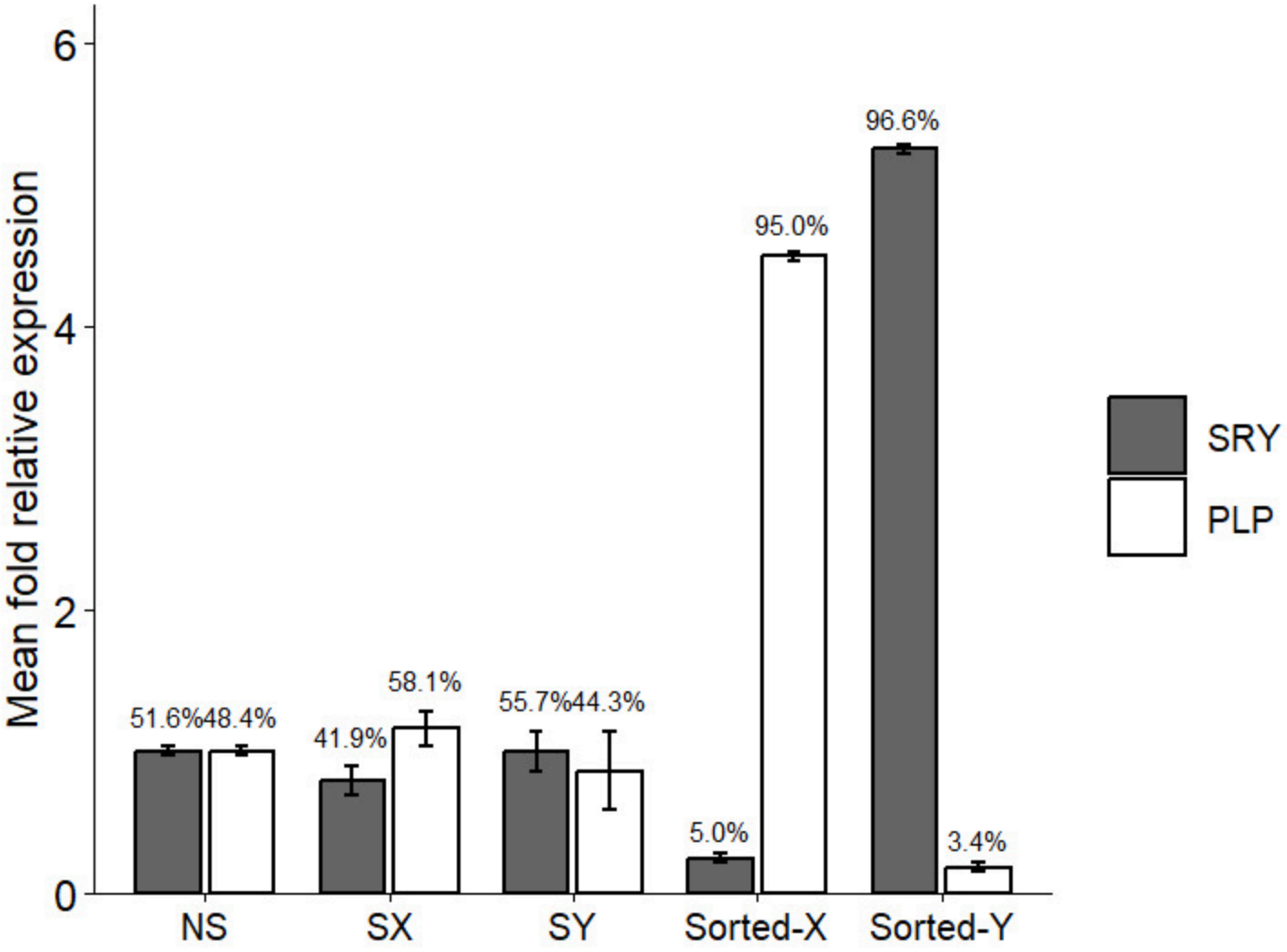


Figure 3

Relative abundance of PLP (X-chromosome marker) and SRY (Y-chromosome marker) genes in post-thaw Holstein-Friesian bull semen fractions evaluated using RT-qPCR.