



Quality and Kinematic Characteristics of Frozen Sexed Bali Bull Sperm: Optimizing Percoll Density Gradient Centrifugation

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Abstract | This research aims to determine and evaluate the quality, proportion and kinematic characteristics of frozen Bali bull sexed sperm using Percoll density gradient centrifugation (PDGC) method with various gradients using egg yolk tris aminomethane diluent. Semen was obtained from a Bali bull, with a confirmed individual motility of $\geq 70\%$, for this research. The data were analyzed by Analysis of Variance (ANOVA) with a Randomized Group Design in R studio (4.4.3 version). T1 = ten gradients, T2 = five gradients, and T3 = three gradients, each consisting of ten replicates. The analysis showed that gradient variation significantly affected individual motility in the bottom layer of sexed semen before freezing ($P < 0.01$). It also had a very significant impact on individual motility, concentration, and total motile sperm (TMS) in the bottom layer of frozen sexed semen ($P < 0.01$) and a significant effect on the viability of the top layer of frozen sexed semen ($P < 0.05$). Meanwhile, gradient variation did not affect kinematic characteristic parameters with CASA ($P > 0.05$). Regarding the proportion of X and Y sexed sperm, the results were: T1 = 85.40% and 79.80%; T2 = 81.20% and 77.90%; T3 = 75.10% and 79.90%. In conclusion, the five-gradients Percoll (bottom layer) can replace the ten-gradients PDGC method based on visual motility and proportion. Further research using 1 mL fresh semen with five and three gradients is recommended for improved results.

Keywords | Bali bull, Computer-assisted sperm analysis, Freezing, Percoll density gradient centrifugation, Proportion, Sexed semen

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INTRODUCTION

Artificial insemination (AI) technology is being implemented in the program to achieve self-sufficiency in meat production in Indonesia by 2026. This effective reproductive technology is known for producing superior

livestock breeds (Ministry of Agriculture, 2019). AI can be enhanced through sperm sexing technology to produce superior livestock breeds tailored to the desired sex (Utami *et al.*, 2022). This technology is being developed to enable farmers to obtain offspring that align with their gender preferences. In the beef cattle industry, there is a particular

interest in producing male calves, as they grow faster and command higher prices than female calves, thus boosting efficiency in livestock production (Joshi *et al.*, 2021). Sperm sexing technology works by separating X and Y sperm based on differences in weight, size, shape, density, motility, charge, and biochemical composition on their surfaces (Ngcobo *et al.*, 2024). The PDGC ten gradients sexing method has been used by an artificial insemination center in Indonesia to produce sexed semen using Tris aminomethane diluent. This diluent is a commonly used semen extender, often combined with egg yolk (Baharun *et al.*, 2017). Throughout the sperm sexing process, the Tris aminomethane diluent plays a crucial role in maintaining the quality of the sexed semen until subsequent processing steps are carried out (Yekti *et al.*, 2024). The PDGC sexing method is based on the principle of the difference in specific gravity between X and Y sperm. The PDGC sexing method is predicated on the principle of differing specific gravities between X and Y sperm. During this process, X sperm quickly settles and forms sediment, as it has a larger mass and size compared to Y sperm (Kusumawati *et al.*, 2017). PDGC sexing method is commonly used in Indonesia, this method is simpler to prepare than other media because the Percoll density variation is easier to make (Utami *et al.*, 2025).

In Indonesia, the PDGC sexing method employs ten gradients (20, 25, 30, 35, 40, 45, 50, 55, 60, and 65%), yielding X sperm at a proportion of 77% and Y sperm at 80.6%. The individual motility for the sexed frozen semen quality shows the bottom layer (bearing sperm X) at 44.60% and the top layer (bearing sperm Y) at 42.10% (Yekti *et al.*, 2023). However, utilizing ten gradients is deemed more challenging, as preparing multiple gradients is time-consuming. A PDGC method using seven gradients (40, 50, 60, 65, 70, 75, 80%) has been developed, with optimal results at a Percoll gradient percentage of 65-70%, achieving a proportion of X sperm at 60.75% and a motility rate of 95.86% (Promthep *et al.*, 2016). Meles *et al.* (2022) explored PDGC sexing with both three gradients (30, 60, 90%) and five gradients (30, 45, 60, 75, 90%) on Simmental bulls. For Y-bearing sperm, individual motility was 77.80% (three gradients) and 75.40% (five gradients), while for frozen Y-bearing sperm individual motility was 44.52% (three gradients) and 43.36% (five gradients). The most recent study by Yekti *et al.* (2024) investigated PDGC with ten and five gradient sexing using the diluents tris aminomethane and andromed in Belgian Blue bulls. This research reported proportions exceeding 70% for each treatment, with the best motility observed in the ten gradients using the tris aminomethane diluent. However, the findings indicated that the five gradients with tris aminomethane diluent could also be effective for the PDGC method. Given the discussion above, it is evident that research on the PDGC method with variations in gradient counts of ten, five, and

three has not been conducted comprehensively. Therefore, this study aims to evaluate the quality and kinematic characteristics of frozen sexed semen utilizing the PDGC method across varying gradient levels, leveraging a Computerized-Assisted Sperm Analyzer (CASA).

MATERIALS AND METHODS

ETHICAL APPROVAL

This study had no animal ethics issues as the semen collection process was conducted by employees of the Singosari National Artificial Insemination Center (SNAIC) with all procedures taking into account animal welfare in accordance with the production management used by SNAIC, namely the ISO 9001:2015 National Standard. Therefore, this study has received an ethical review certificate No. 67 / EC / KEPK from the Faculty of Medicine, Universitas Brawijaya.

MATERIALS

ANIMAL EXPERIMENTAL DESIGN AND SEMEN COLLECTION:

The research was conducted at the SNAIC located on Jalan BBIB, Ngujung, Toyomarto, Singosari, Malang District, East Java, as well as at the Animal Reproduction Laboratory, Faculty of Animal Science, Universitas Brawijaya. The study focused on a 7-year-old Bali bull (ID: Sabala), weighing 508 kg, with a body conditioning score (BCS) of 6 (scale of 1 to 9). Semen collection procedures followed to the standard operational protocols of SNAIC. Fresh semen was collected weekly, using an artificial vagina (AV; IMV Technologies, France). The semen was considered viable if it exhibited a mass motility quality of at least 2+, with progressive motility $\geq 70\%$ and abnormal sperm below 20%.

SEMEN PROCESSING AND EVALUATION: Fresh semen that has been collected by SNAIC was immediately examined for macroscopic tests, including volume, color, odor, pH, and consistency. Microscopic tests include mass motility, individual motility, viability, abnormality, concentration, and total motile sperm (TMS). Additionally, motility characteristics and kinetic patterns were analyzed using CASA. A morphometric assessment was performed to determine the proportion of X and Y sperm in sexed semen. The quality of sexed semen before freezing was evaluated based on individual motility, viability, and abnormality, while the post-thawed quality was assessed through individual motility, viability, abnormality, concentration, and TMS, along with motility characteristics and kinetic patterns analyzed using CASA.

Based on Figure 1 experimental design of frozen Bali bull sexed sperm using PDGC method with various gradients, analyzed the quality of fresh Bali bull semen (Table 1) and then processed it for sexing. Bali cattle belong to *Bos sondaicus* cattle breed which is native to Indonesia so the use

of Bali cattle can represent local cattle in Indonesia. The PDGC sexing process refers to [Yekti et al. \(2024\)](#), according to [Figure 2](#) and [Figure 3](#), the sexed semen was produced by Percoll density gradient centrifugation using gradients of ten, five, and three with tris aminomethane diluent. The diluent consisted of tris aminomethane combined with 20% egg yolk from SNAIC, including the following components: tris aminomethane, citric acid, lactose, raffinose, penicillin, streptomycin, distilled water, and egg yolk.

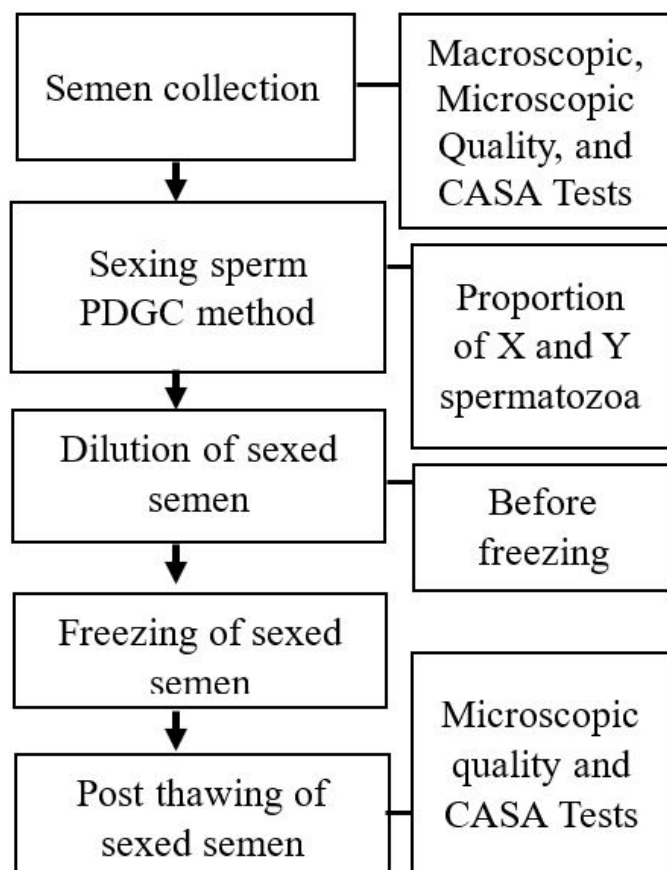


Figure 1: Experimental design of frozen bali bull sexed sperm using percoll density gradient centrifugation method with various gradients.

The Percoll gradient percentages (Sigma-Aldrich, St. Louis, MO, USA) used included ten gradients (20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%), five gradients (20%, 30%, 40%, 50%, 60%), and three gradients (20%, 40%, 60%). Each gradient was arranged in its respective test tube (Pyrex) from highest to lowest density, with the volumes allocated at T1 (0.5 mL), T2 (1 mL), and T3 (1.5 mL). The fresh semen meeting the required standards was then diluted with the diluent at a 1:1 ratio for this experiment, specifically using 0.5 mL of semen and 0.5 mL of diluent. Centrifugation was carried out at 451 G for five minutes for separation, followed by washing at 286 G for five minutes in each layer, the top layer (bearing Y sperm) and the bottom layer (bearing X sperm). The experiment included three treatments, each with ten replications, as follows:

- T1: ten Percoll gradients with tris aminomethane diluent + 20% egg yolk
- T2: five Percoll gradients with tris aminomethane diluent + 20% egg yolk
- T3: three Percoll gradients with tris aminomethane diluent + 20% egg yolk

Table 1: Average quality of fresh semen from bali bull.

Parameters	Mean ± SD
Macroscopic	
Volume (ml)	8.44 ± 2.31
Color	Milky white
pH	6.5 ± 0.13
Odor	Specific
Consistency	Dilute
Microscopic	
Mass motility	++
Individual motility (%)	83.22 ± 4.50
Viability (%)	88.46 ± 3.24
Abnormality (%)	5.33 ± 0.78
Concentration (10 ⁶ /ml)	903.50 ± 371.62
TMS (10 ⁶ /ml)	745.79 ± 294.65
CASA	
Motility (%)	88.27 ± 4.27
Progressive (%)	73.48 ± 3.63
DCL (µm)	71.87 ± 6.19
DSL (µm)	46.34 ± 4.78
DAP (µm)	51.54 ± 5.50
VCL (µm/s)	185.39 ± 11.54
VSL (µm/s)	120.27 ± 7.76
VAP (µm/s)	132.77 ± 7.56
STR (%)	90.11 ± 1.97
LIN (%)	67.36 ± 3.35
WOB (%)	73.92 ± 3.35
ALH (µm)	5.62 ± 0.77
BCF (Hz)	37.21 ± 4.58

Sexed semen that has been tested were processed for freezing according to the SNAIC frozen semen production procedure. This involves adding VA1 (at a ratio of 1:1) in a water bath (Advantage, USA) set at 37°C. The mixture was then placed in a cool tub (IMV, France) to facilitate a temperature drop to 5°C, followed by 18-22 hours of cooling, after which VA2 was introduced. VB (Tris aminomethane + 13% glycerol) was added and then tested before freezing with the condition that sperm motility >55%. Filling, sealing, and racking straw in the cool tub (glycerol equilibration 0-4 hours) and first freezing with liquid nitrogen vapor for 12 minutes to reach a temperature of -140°C, after which a second freezing with liquid nitrogen is carried

out to reach a temperature of -196°C for 24 hours. Frozen sexed semen straws were thawed at 37°C for 30 seconds (National Standards, 2024).

is calculated by multiplying the progressive motility of the semen by the total concentration of the sperm and calculation of TMS according to Yekti *et al.* (2024).

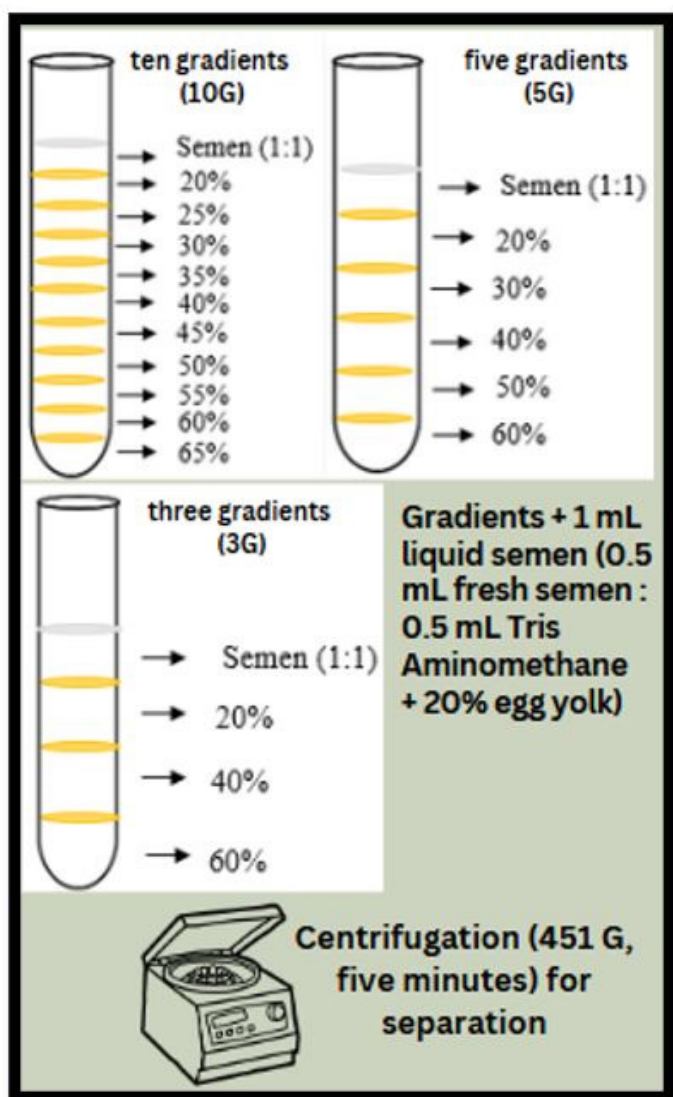


Figure 2: Percoll density gradient centrifugation method with various gradients.

MEASURED PARAMETERS

Sperm individual motility (%): was assessed using $10\ \mu\text{L}$ of sample observed under a binocular light microscope (Olympus CX-23, Japan) at a magnification of $400\times$. Individual motility is determined as the average across five fields of view (National Standards, 2017).

Viability and abnormality (%): based on Susilawati *et al.* (2022), a smear was prepared by homogenizing $10\ \mu\text{L}$ of semen with $10\ \mu\text{L}$ of eosin nigrosin on an object glass (On-eLab, China) and examined at a magnification of $400\times$. The calculations for viability and abnormality are based on Yekti *et al.* (2024).

Total motile sperm (TMS): based on Susilawati *et al.* (2022), TMS represents the number of motile sperm and

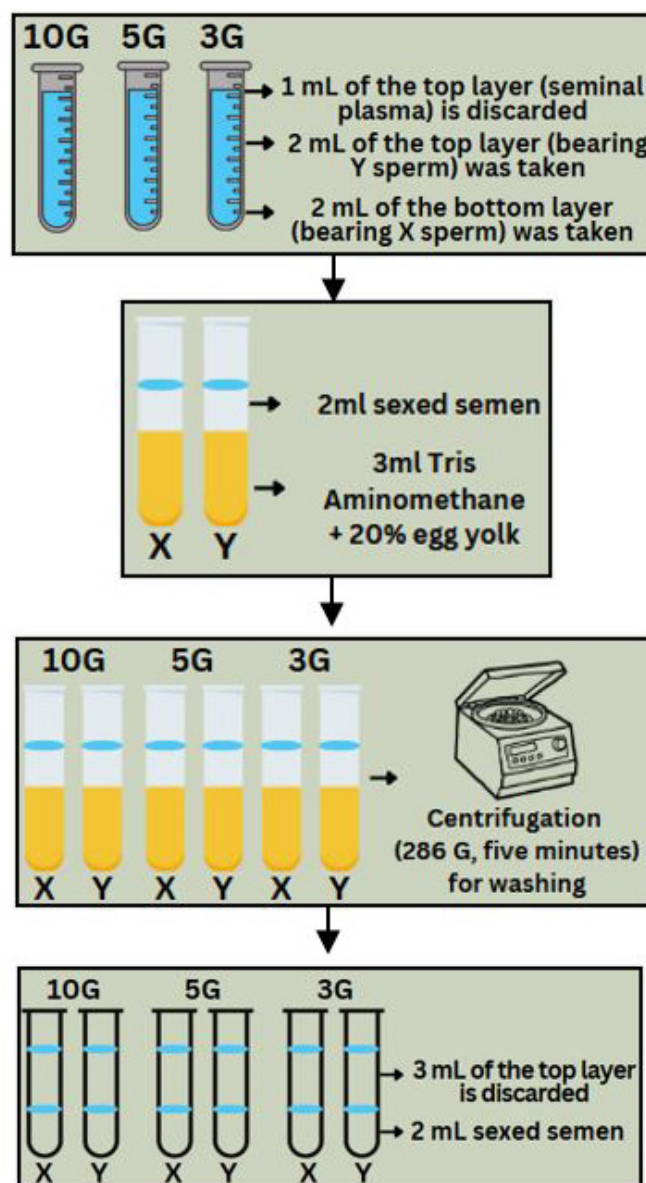


Figure 3: Process after first centrifugation of frozen bali bull sexed sperm using percoll density gradient centrifugation method with various gradients.

The concentration of sperm (million/straw) was determined using a Neubauer counting chamber (Marienfeld, Germany) as described by Susilawati *et al.* (2022): 1) A microtube (Onemed, Indonesia) was prepared by adding $990\ \mu\text{L}$ of 3% NaCl, followed by the addition of $10\ \mu\text{L}$ of sexed semen, which was carefully transferred using a micropipette (Socorex, Germany). The exterior of the yellow tip was wiped with a tissue before inserting it into the 3% NaCl solution within the microtube. 2) The mixture was then homogenized by manually moving the microtube in a figure-eight pattern for 2 minutes. 3) After homogenization, $10\ \mu\text{L}$ of the semen and saline mixture was placed into the Neubauer counting chamber (Marienfeld, Germany).

4) Sperm were observed under a binocular light microscope (Olympus CX 23, Japan). Sperm counts were conducted in five chamber boxes (top right and left, bottom right and left, and center) following the calculation method outlined by Mahendra *et al.* (2018).

Identification of sperm aims to determine the proportion of X and Y chromosome sperm using a morphometric test. This test involves measuring the length and width (area) of the sperm head. Sperm categorized as X has a head area larger than average, while Y sperm exhibit a smaller head area than the average (Solihati *et al.*, 2023). The stages of identifying X and Y sperm are as follows: 1. A drop of 10 μ l of sexed semen is taken and mixed with 10 μ l of eosin-nigrosin on a glass slide (OneLab, China). 2. The samples are observed under a trinocular microscope (Olympus CX-33, Japan) at a magnification of 400 $\times$. The length and width of the heads of 100 sperm are measured using LCmicro software. For comparison, the dimensions of 1000 fresh semen sperm were measured to obtain a natural X:Y proportion of 50:50, with the average head area of fresh semen serving as the benchmark for identifying sexed semen sperm (Yekti *et al.*, 2024). The formula for proportion calculation is based on Yekti *et al.* (2024).

Observation of Motility Characters using CASA: The procedure for testing motility characteristics with the CASA system from Hamilton Thorne (France), specifically the IVOS-II (IVOS = Integrated Visual Optical System), is conducted in accordance with the Laboratory Work Instruction Guidelines of the SNAIC. The steps are as follows: 1) Turn on the machine by pressing the ON button, then open the IVOS II application and allow the system to warm up to a temperature between 36 $^{\circ}$ C and 38 $^{\circ}$ C. 2) Prepare the sample for testing using a micropipette (Socorex, Germany) to take 4 μ l of the sample. Place this sample onto a glass slide (OneLab, China) and cover it with a cover slip (Herma). 3) Press the load button to initiate the sample entry process and use the jog in and jog out buttons to adjust the field of view position. 4) Set the live configuration, ensuring that the machine accurately detects both the head and tail of the sperm. 5) Adjust the focus using the focus setting button, and then click auto capture to capture the field of view automatically across five observations. 6) Review the results of the motility testing, including kinematic parameters, in the results tab. Parameters Measured Using CASA: Motility (%); Progressive (%); Distance Straight Line (DSL, μ m); Distance Curvilinear (DCL, μ m); Distance Average Path (DAP, μ m); Velocity Straight Line (VSL, μ m/s); Curvilinear Velocity (VCL, μ m/s); Velocity Average Path (VAP, μ m/s); Straightness (STR, %); Linearity (LIN, %); Wobble (WOB, %); Amplitude of Lateral Head (ALH, μ m); and Beat Cross Frequency (BCF, Hz). The advantage of CASA technology is to analyze the quality of sperm by looking at motility characteristics quick-

ly using the help of computer devices. The tools used for CASA are standardized tools for evaluating sperm motility characters and kinetic patterns so that more accurate and objective data can be obtained (Susilawati *et al.*, 2022). It was also mentioned by Ehlers *et al.* (2011) that the analysis of sperm motility using CASA will get kinetic observations with the results obtained will be accurate and objective. However, according to Mortimer *et al.* (2015) the CASA instrument has the limitation of not being able to analyze flagellum beats directly, so it relies on tracking the movement of the sperm head. In extreme situations, Brownian motion of a stationary object similar in size and appearance to the sperm head can be misinterpreted as sperm motility. This makes it difficult to define non-progressive sperm motility, which requires the CASA test field of view to be free of debris.

DATA ANALYSIS

The research utilized a Randomized Group Design (RGD) featuring three treatments and ten replications. Each 1 mL sample of bull semen with different quality is used as a repeat. Grouping based on the quality of each 1 mL of semen. Previously, the data had been tested for normality using Shapiro-Wilk and Barlett's test for data homogeneity in R studio 4.4.3. The quality of the samples before freezing was evaluated, followed by continued testing using the Duncan multiple range test, with statistical analyses conducted through the R Studio 4.4.3 application. Additionally, the individual motility of samples before freezing and sexed frozen semen, concentration, and proportion of sexed frozen semen, were analyzed using Chi-Square tests.

RESULTS AND DISCUSSION

QUALITY OF FRESH SEMEN FROM BALI BULL

The quality of fresh semen serves as the primary indicator for assessing its suitability for subsequent processing, including dilution, freezing, or sexing. This quality is evaluated through both macroscopic and microscopic examinations. Research findings regarding the average macroscopic and microscopic evaluations of fresh semen from Bali bulls are presented in Table 1.

Based on Table 1, it is known that the average semen volume is 8.44 ± 2.31 mL with a range of 5.8-12 mL. In comparison, the semen volume results for Bali bulls in our study are consistent with those reported by Setyani *et al.* (2017), where the average semen volume was recorded at 7.79 mL, within a range of 4.8 to 12 mL. The viability of fresh semen from Bali bulls in this study was $88.46 \pm 3.24\%$, which is like the findings of Brilliant *et al.* (2021), indicating an average viability of $87.63 \pm 1.85\%$ in older cattle (7-8 years of age). The average result of mass motility was good (++), and the average of individual motility was $83.22 \pm 4.50\%$. The motility characteristics of fresh semen from Bali bulls

in this study were notably higher, potentially due to the lower progressive motility of 68.5% reported by [Haryani et al. \(2016\)](#). Thus, the semen is deemed suitable for further processing, meeting the [National Standards \(2021\)](#), which stipulate that fresh semen for processing should have a motility of at least $\geq 70\%$ and a maximum abnormality rate of 20%.

THE IMPACT OF VARIOUS GRADIENTS IN PDGC SEXING ON THE QUALITY OF SEXED SEMEN FROM BALI BULL BEFORE FREEZING

Based on [Table 2](#), gradient variation gave a highly significant difference in the individual motility of sexed semen before freezing in the bottom layer ($P < 0.01$). T1 showed the highest results and T2 showed results that were not different from T3 in the lower layer individual motility parameter, respectively T1 ($61.68 \pm 8.63\%^a$); T2 ($56.34 \pm 8.71\%^b$); and T3 ($57.20 \pm 10.27\%^b$). The gradient variation had no difference on individual motility in the top layer, as well as viability and abnormality of the sexed semen before freezing in both the top and bottom layers ($P > 0.05$). T1 (10 Percoll gradients) showed the highest results compared to other treatments in both layers with individual motility (48.78 ± 9.64 ; $61.68 \pm 8.63\%$), viability (53.88 ± 10.64 ; $65.65 \pm 10.15\%$), and lowest results for abnormality (6.96 ± 2.15 ; $4.22 \pm 1.54\%$). Group analysis revealed a highly significant difference in individual motility of sexed semen before freezing for both layers, as well as in the viability of sexed semen in the top layer ($P < 0.01$), with significant differences also present in the bottom layer ($P < 0.05$). Additionally, a significant difference was noted in the abnormality of sexed semen before freezing in the bottom layer ($P < 0.05$), whereas no significant differences were observed in the top layer ($P > 0.05$).

Table 2: Average quality of sexed semen before freezing from bali bull.

Parameters	Treatments					
	Top Layer (Mean)			Bottom Layer (Mean)		
	T1	T2	T3	T1	T2	T3
Motility (%)	48.78±9.64	48.42±8.02	48.36±7.73	61.68±8.63 ^b	56.34±8.71 ^a	57.2±10.27 ^a
Viability (%)	53.88±10.64	52.23±8.40	53.57±8.82	65.65±10.15	63.08±11.64	60.81±11.04
Abnormality (%)	6.96 ± 2.15	7.9 ± 2.77	7.29 ± 2.51	4.22±1.54	4.4 ± 1.46	5.3 ± 2.77

T1: (10 percoll gradients); T2: (5 percoll gradients); T3: (3 percoll gradients); ^{a,b} Uncommon superscript in the same row indicates a very significant difference ($P < 0.01$).

In PDGC sexing, the decrease in individual semen motility is caused by the centrifugation process, which damages the sperm plasma membrane ([Safa et al., 2025](#)). Following this, the sexed semen undergoes a freezing process after being

diluted. It has been observed that semen quality can decline by 40-50% during the cryopreservation process ([Susilawati et al., 2022](#)). Consequently, it is essential to ensure that the liquid semen possesses high quality prior to freezing. For effective cryopreservation, liquid semen should have a minimum individual motility of 55% ([To'aloh et al., 2023](#)). The individual motility before freezing the top layer is lower than the bottom layer. According to [Susilawati et al. \(2014\)](#), the individual motility before freezing in the top layer was 49.5% and the bottom layer was 56.5%. T1 has the highest quality of individual motility. This can be attributed to the higher viscosity encountered at five gradients compared to ten gradients, and the increased viscosity at three gradients relative to five gradients. During the sexing process, sperm tend to lose more energy in higher viscosity gradients, resulting in lower individual motility observed in the five and three gradient ([Simbolon et al., 2024](#)). Chi-square analysis revealed that all treatments met the expected 55% motility threshold in the bottom layer. However, the top layer did not meet this benchmark. It is suspected that this discrepancy is due to a higher presence of abnormal and immotile sperm, many of which exhibited damaged membranes. Such conditions negatively impact sperm metabolism and their ability to generate energy ([Susilawati et al., 2017](#)). Consequently, during the cooling process of sexed semen in the top layer before freezing, individual motility is likely to decline. According to [Dasrul et al. \(2013\)](#), sperm metabolism is influenced by the energy metabolic capacity of the environment, temperature, and components of the extracellular medium. Both endogenous and exogenous energy limitations can adversely affect sperm motility.

The viability of sexed semen before freezing the top layer is lower than the bottom layer. According to [Susilawati et al. \(2014\)](#), sexed semen before freezing the PDGC method, the viability of the top layer is 61.65% and the bottom layer is 69.92%. The decrease in the viability of sexed semen at the before freezing is due to the cooling process of sperm. The occurrence of cold shock during this cooling phase increases the production of reactive oxygen species (ROS), leading to damage to the sperm cell membrane ([Meles et al. 2022](#)). Such damage disrupts both metabolic and physiological processes, ultimately resulting in decreased sperm motility and increased mortality. The differences in the average abnormalities observed in semen sexing, particularly before the freezing stage, can be linked to multiple factors, including the separation and cooling processes. [Kusumawati et al. \(2017\)](#) also noted that the sexing, cooling, and freezing processes contribute to an increase in sperm abnormalities. This is primarily due to the damage sustained by the sperm cell membrane during the sexing process and subsequent cooling and freezing, which result from cold shock and the resulting osmotic pressure imbalances during metabolism. Additionally, the presence of toxic substances from dead sperm or oxidized diluents during storage can

lead to elevated levels of free radicals, further compromising the integrity of the sperm plasma membrane. These changes ultimately affect cellular water and ion concentrations, potentially causing damage to the acrosome and the tail of the sperm, which are particularly vulnerable during smearing procedures (Mahfud *et al.*, 2019).

Table 3: Average quality of sexed semen post thawing from bali bull.

Parameters	Treatments					
	Top Layer (Mean)			Bottom Layer (Mean)		
	T1	T2	T3	T1	T2	T3
Motility (%)	38.37± 8.33	46.92± 11.84	35.60± 13.72	54.12± 10.76	52.02± 15.40	47.72± 10.67
Progressive (%)	26.98± 6.72	31.13± 10.34	22.44± 8.07	40.30± 8.39	34.39± 9.86	33.04± 13.36
Individual Motility (%)	30.08± 6.12	30.40± 7.36	30.96± 8.77	43.72± 3.52 ^b	41.14± 3.05 ^{ab}	35.58± 6.85 ^a
Viability (%)	33.13± 10.34 ^{ab}	37.16± 8.21 ^b	28.67± 6.06 ^a	41.68± 9.32	42.63± 10.69	36.81± 8.45
Abnormality (%)	6.62± 2.00	7.80± 3.61	6.73 ± 2.50	3.78± 1.22	4.41± 1.78	5.08± 1.53
Concentration (million/straw)	19.94± 9.04	17.19± 6.96	16.81± 6.78	20.31± 7.52 ^b	19.22± 7.02 ^{ab}	15.81± 4.05 ^a
TMS (million/ straw)	6.37± 3.78	5.57 ± 3.10	5.3 ± 2.83	8.82± 3.06 ^b	7.95± 2.94 ^b	5.64± 1.85 ^a

T1: (10 percoll gradients); **T2:** (5 percoll gradients); **T3:** (3 percoll gradients). ^{a-b}Uncommon superscript in the same row indicates a very significant difference (P<0.01) and significant difference (P<0.05).

THE IMPACT OF VARIOUS GRADIENTS IN PDGC SEXING ON THE QUALITY OF SEXED SEMEN FROM BALI BULL POST-THAWING

Based on Table 3, variations in gradient significantly affect the individual motility, concentration, and total motile sperm (TMS) in the bottom layer (P<0.01). T1 showed the highest results in terms of individual motility, concentration, and TMS in the bottom layer, but did not differ from T2, respectively individual motility (T1 43.72 ± 3.52%^b; T2 (41.14 ± 3.05%^{ab}); T3 (35.58 ± 6.85%^a), concentration T1 (20.31 ± 7.52×106/straw^b); T2 (19.22 ± 7.02×106/straw^{ab}); T3 (15.81 ± 4.05×106/straw^a), and TMS T1 (8.82 ± 3.06×106/straw^b); T2 (7.95 ± 2.94×106/straw^b); T3 (5.64 ± 1.85×106/straw^a). In contrast, gradient variation significantly influenced the viability in the top layer (P<0.05). T2 showed the highest results in top layer viability parameters, but not different from T1, respectively T1 (33.13 ± 10.34%^{ab}); T2 (37.16 ± 8.21%^b); and T3 (28.67 ± 6.06%^a). Nevertheless, gradient variation did not affect motility and progressive in both layers, individual motility, abnormality, concentration, and TMS in the top layer, nor did it impact the viability and abnormality in the bottom layer (P>0.05).

Moreover, there was a very significant difference in concentration across both layers and in TMS in the bottom layer based on grouping (P<0.01). This discrepancy is attributed to variations in the quality of the fresh semen concentration in each replication. Based on group analysis, a significant difference was noted in the abnormalities of the bottom layer (P<0.05). However, there were no significant differences in individual motility, viability, or abnormalities of the bottom layer, as well as individual motility and viability of the top layer (P>0.05).

The very difference is thought to be due to differences in the distance and viscosity of the gradient penetrated by sperm during separation. T1, with a lower viscosity and a distance of 0.5 mL per gradient, makes it easier for sperm to penetrate the gradient layer, to minimize membrane damage and a decrease in the quality of sperm until the freezing process. The post thawing process causes sperm cells to experience temperature changes and extreme osmolarity which results in damage to the lipid membrane structure resulting in decreased motility or movement of sperm (Meles *et al.*, 2022). Based on Chi-Square, the individual motility of post-thawing stage T1 and T2 bottom layer sexed semen has met the expected value, which is higher than the expected value of 40% (National Standards, 2024). The bottom layer of T3 and top layer (T1, T2, T3) did not meet the expected value of 40%. However, only T1 met the expected value of progressive motility, testing with CASA, of more than 40%. Based on Chi-Square, the sperm concentration of frozen sexed semen has not met the National Standards (2024) for frozen semen of at least 25 million/straw. The study results were lower than those of Mardi *et al.* (2020), namely frozen sexing semen SGDP ten gradients method after post-thawing in the top layer of concentration as much as 25.23×106/straw. This is thought to be due to using fresh semen in the study as much as 1 mL. The initial volume of fresh semen for the sexing process in the research of Mardi *et al.* (2020) with a volume of 1.5 mL and 2 mL has a higher concentration of 25.73×106/straw and 26.18×106/straw. Meanwhile, the study only used 0.5 mL fresh semen with the addition of 0.5 mL diluent (1:1).

Separation by centrifugation causes the separation of sperm with seminal plasma and membrane damage which causes a decrease in the quality of sperm. Following Diliyana *et al.* (2014) that the release of seminal plasma will decrease membrane protection so that the sperm membrane becomes unstable. Damage to the sperm membrane structure increases intracellular calcium levels in sperm cells, which results in a decrease in viability, motility, and membrane integrity (Mahfud *et al.*, 2019). The sperm abnormalities in the bottom layer were less than in the top layer in post-thawing sexed semen.

Table 4: Average Quality of sexed semen post thawing from bali bull with CASA.

Parameters	Treatments					
	Top Layer (Mean)			Bottom Layer (Mean)		
	T1	T2	T3	T1	T2	T3
DCL (μm)	64.23± 11.16	55.06± 15.38	56.77± 16.44	67.99 ± 15.16	62.40 ± 12.28	61.51 ± 11.81
DSL (μm)	29.66± 3.48	26.69± 5.80	24.96± 5.90	30.51 ± 3.57	29.11 ± 3.05	27.38 ± 5.41
DAP (μm)	35.53± 4.44	32.45± 6.57	30.9 ± 7.82	37.36 ± 5.85	35.26 ± 4.24	33.61 ± 5.89
VCL (μm/s)	154.54 ± 31.12	136.53 ± 32.64	135.28 ± 36.45	157.72 ± 31.27	146.94 ± 33.23	147.57 ± 25.85
VSL (μm/s)	69.95± 8.33	62.32± 11.06	60.28± 12.90	71.23 ± 6.42	68.46 ± 7.84	65.64 ± 12.20
VAP (μm/s)	85.78± 13.62	76.42 ± 13.57	74.55 ± 17.56	87.23 ± 11.37	83.27 ± 12.38	80.97 ± 12.97
STR (%)	81.94± 4.89	81.94 ± 5.89	82.61 ± 6.51	81.96 ± 4.83	82.48 ± 6.07	81.4 ± 5.35
LIN (%)	48.61 ± 6.22	50.61 ± 8.73	51.41 ± 9.92	47.98 ± 6.59	50.87 ± 8.44	48.46 ± 7.32
WOB (%)	58.18 ± 4.15	59.96 ± 6.43	60.49 ± 6.99	57.51 ± 4.65	60.15 ± 6.30	57.91 ± 5.18
ALH (μm)	7.77 ± 1.91	6.76 ± 1.85	7.21 ± 2.29	7.95 ± 1.72	7.31 ± 2.14	7.29 ± 1.59
BCF (Hz)	26.7 ± 4.62	26.91 ± 5.35	27.62 ± 11.53	24.77 ± 4.44	25.03 ± 4.88	25.92 ± 4.41

T1: (10 percoll gradients); **T2:** (5 percoll gradients); **T3:** (3 percoll gradients); The distance traveled by sperm is obtained from the distance traveled by sperm in one second on each track, namely DCL (Distance Curve-Line), DSL (Distance Straight Line), and DAP (Distance Average Path); The velocity parameter in the character of sperm motility using CASA is the speed of sperm, which consists of three parameters: VCL (Velocity Curvilinear), VSL (Velocity Straight Linear), and VAP (Velocity Average Pathway); The percentage parameters STR (Straightness), LIN (Linearity), and WOB (Wobble) in sperm motility characters using CASA are derived from velocity parameters. STR is the ratio between straight and average trajectories (VSL/VAP×100). LIN is the ratio between straight and curved paths (VSL/VCL×100). WOB expresses the relationship between average and curved paths (VAP/VCL×100); The ALH (Amplitude of Lateral Head) is the average width of sperm head oscillations and BCF (Beat Cross Frequency) is the frequency of sperm head crossing the average path in both directions.

According to [Susilawati et al. \(2017\)](#) frozen semen sexing method SGDP ten gradient shows the results of damage or leakage of the sperm membrane and the disconnection of the sperm head with the tail. There are many sperm whose membranes leak, especially in the top layer, more dead sperm are found with the discovery of debris resulting

from damage to the sperm membrane. The average TMS of the top layer sexed semen is lower than the bottom layer. This is because the amount of concentration and motility of the top layer of sexed semen is lower than the bottom layer so that it will affect the TMS of the sexed semen. In the top layer during the centrifugation process, there are many Y sperm that will quickly run out of energy because of their faster motility than X sperm which are found in the bottom layer. Therefore, TMS decreases in the top layer because many sperm populations drop to the bottom layer ([Yekti et al., 2023](#)) and many sperm die when debris is found due to damage to the sperm membrane ([Susilawati et al., 2017](#)).

Based on [Table 4](#) the results of the analysis of parameters on the motility character of sperm both in the top and bottom layers under different gradient treatments, showed no significant differences in the values of DCL, DSL, DAP, VCL, VSL, VAP, STR, LIN, WOB, ALH, and BCF for sexed frozen semen ($P>0.05$). By group, there was a significant difference in DCL and DAP of the bottom layer ($P<0.05$), but no difference in DCL, DSL, and DAP of the top and bottom layers and DSL of the bottom layer ($P>0.05$). By group, there was a very significant difference in VCL of the top layer ($P<0.01$) and a significant difference in VCL of the bottom layer as well as VAP of the top layer ($P<0.05$). However, by group, there was no difference in the VSL of the top layer and the VAP of the bottom layer ($P>0.05$). Meanwhile, based on the group, there were highly significant differences in STR of the top and bottom layers and LIN of the bottom layer ($P<0.01$) and significant differences in LIN of the top layer and WOB of the top and bottom layers ($P<0.05$). Meanwhile, by group, there were very significant differences in ALH of top and bottom layers and BCF of bottom layers ($P<0.01$) and no significant differences in BCF of top layers ($P>0.05$).

The distance parameter in sperm motility, as measured by CASA, includes three components: DCL, DSL, and DAP. [Syarifuddin et al. \(2018\)](#) stated that the distance parameter or the distance traveled by sperm is obtained from the distance traveled by sperm in one second on each track, namely the curved track (DCL), the straight track (DSL), and the average track in the groove (DAP). The distance traveled by the movement of sperm is the ability of sperm to move so that it will support the success of sperm in fertilization ([Raafi et al., 2021](#)). The different gradients in SGDP sexing did not affect the values of distance parameters, namely DCL, DSL, and DAP of sexed frozen semen in both the top and bottom layers. This indicates that the three treatments can maintain semen quality. According to [Putranti \(2018\)](#) DCL is the distance traveled by the curved line of the sperm tail, DSL is the distance traveled by the straight line of the sperm tail, and DAP is the average distance traveled by the sperm line. Based on [Table 5](#) shows

that the higher the DSL value, the higher the DAP value. A high DCL value means that the sperm move faster, which is also correlated with the VCL value because velocity is positively correlated with distance, which means that the higher the VCL value, the higher the DCL value (Maulana and Said, 2019).

Table 5: The proportion of sperm x and y in sexed semen from bali bull.

Layers	Sperm	T1 (%)	T2 (%)	T3 (%)
Top	Y	79.80	77.90	79.90
	X	20.20	22.10	20.10
Bottom	Y	14.60	18.80	24.90
	X	85.40 ^a	81.20 ^{ab}	75.10 ^b

The velocity parameter in the character of sperm motility using CASA is the speed of sperm, which consists of three parameters: VCL, VSL, and VAP. VCL is the curved speed in the actual path of sperm for one second, VSL is the speed of sperm in a straight-line distance from the beginning to the end of the sperm path for one second, and VAP is the average path speed of sperm for 1 second (Kanno *et al.*, 2023). This indicates that the three treatments can maintain semen quality. However, the values of each treatment are quite different, T1 showed the highest values for VCL, VSL, and VAP, suggesting that a ten-gradient system better preserves the velocity of sexed frozen semen compared to T2 and T3. This is thought to be because the ten gradients feature a shorter gradient distance and lower Percoll density than the five and three-gradient systems. Consequently, sperm experience less membrane damage, which positively impacts motility quality and, thus, the velocity parameters of sexed frozen semen. This observation aligns with Yekti *et al.* (2022), who reported that centrifugation, osmotic shifts, and contact with Percoll media can compromise sperm membrane integrity and intracellular components. VCL <20 $\mu\text{m/s}$ is considered immotile sperm, VCL 20-60 $\mu\text{m/s}$ is considered slow, VCL 60-110 $\mu\text{m/s}$ is considered medium, and VCL >110 $\mu\text{m/s}$ is considered fast (Novo *et al.*, 2021). Based on VCL in each treatment, sperm move quickly. Velocity assessment with VAP > 25 $\mu\text{m/s}$ indicates the progressive motility of sperm (Raafi *et al.*, 2021). This shows that each treatment produces sperm with progressive movement. Sperm to be able to penetrate the ovum is to have VCL >70 $\mu\text{m/s}$, VAP, and VSL >45 $\mu\text{m/s}$ (Arif *et al.*, 2020). Tris aminomethane diluent is thought to minimize the deterioration of frozen sexed semen quality. According to Ratnawati *et al.* (2017) during cold storage semen with tris aminomethane diluent has a higher VCL value, which means the vigor of sperm movement is more substantial than with skim milk diluent and CEP-2. The treatment of different gradients showed that the value of VCL, VSL, and VAP is good to make sperm have good progressive motility.

The percentage parameters STR, LIN, and WOB in sperm motility characters using CASA are derived from velocity parameters, namely VCL, VSL, and VAP. STR is the ratio between straight and average trajectories ($\text{VSL}/\text{VAP} \times 100$). LIN is the ratio between straight and curved paths ($\text{VSL}/\text{VCL} \times 100$). WOB expresses the relationship between average and curved paths ($\text{VAP}/\text{VCL} \times 100$) (Baeta *et al.*, 2019). The treatment of different gradients in Percoll density gradient centrifugation sexing does not affect the percentage of STR, LIN, and WOB of sexed frozen semen. LIN is the linear progression rate calculated as the VSL/VCL ratio in percentage, STR is the VSL/VAP ratio in percentage to measure the compactness of the track, and WOB is a measurement of the oscillation of the actual path against the average path of the sperm (Valverde *et al.*, 2020). The percentage of STR and LIN is used to identify the pattern of sperm movement, to identify the presence of hyperactive sperm, namely sperm moving quickly and strongly but not progressive and linear (Susilawati *et al.*, 2018). The percentage of LIN in sperm can indicate the direction of movement or straightness of sperm swimming (El-Bahrawy, 2017). Based on Table 4 it can be seen that the percentage of STR and LIN of the three treatments has an average percentage of >80% and >45%, respectively. Utami *et al.* (2025) stated that sperm are considered to move linearly if they show a percentage of STR > 50% and LIN > 35%. According to Setiyono *et al.* (2020) sperm swim linearly if the percentage of LIN > 35% and swim non-linearly if the percentage of LIN <30%. This shows that both treatments can move linearly or straight ahead with a percentage of STR > 80% and LIN > 45%.

The amplitude of the Lateral Head (ALH) is the average width of sperm head oscillations and Beat Cross Frequency (BCF) is the frequency of sperm head crossing the average path in both directions (Missio *et al.*, 2018). According to Amann and Waberski (2014) explained that the centroid of deviation from the average trajectory is called the amplitude of lateral head displacement (ALH) and the intersection of curved trajectories that intersect the average trajectory, and the number of such intersections is called the beat cross frequency (BCF). ALH and BCF are used in CASA analysis to evaluate various aspects of sperm motility, such as speed, movement pattern, and wobble frequency (Bravo *et al.*, 2011), which are also characteristics of sperm wobble (Lu *et al.*, 2014). According to Marquez and Suarez (2007), ALH >7 μm indicate that sperm are hyperactive. The results showed ALH >7 μm , thus indicating hyperactive sperm, except at T2 on the top layer. The higher the ALH value, marked capacitation in sperm, the biochemical changes that increase its ability to fertilize an eggs (Utami *et al.*, 2025). Some statements of sperm hyperactivity are as follows: the category of hyperactive sperm with VCL > 150 $\mu\text{m/s}$; LIN < 50% and ALH > 7 μm (Susilawati *et al.*, 2018). BCF value is a parameter that can identify changes in flagellar

motion patterns (Afriani *et al.*, 2024). The higher the BCF value, the more stable the sperm movement is thought to be (Ratnawati *et al.*, 2020). $BCF > 20$ Hz means sperm have good fertility (Belala *et al.*, 2019). This study shows the results of the appropriate BCF value, so it is concluded that the three treatments have sperm with good fertility.

IMPACT OF DIFFERENT GRADIENTS IN PDGC SEXING ON THE PROPORTION OF X AND Y SPERM IN SEXED SEMEN FROM BALI BULL

Results of sperm proportion of Bali bull semen in Table 5 based on Chi-square analysis, the proportion of X and Y sperm in each treatment all met the expected value of $> 70\%$. This indicates that each treatment can separate X and Y sperm from their natural proportions. This happens because X and Y sperm have different characteristics. The principle of the PDGC sexing method is the difference in sperm weight; X sperm are heavier than Y sperm because they have a larger head size. Therefore, X sperm will settle to the bottom layer faster during the centrifugation process than Y sperm. Identification of X and Y sperm using morphometric test based on sperm head area. According to Solihati *et al.* (2023) the head area of X sperm is larger than Y sperm because it contains more DNA. The average DNA size difference between X and Y chromosome sperm in cattle is about 4%, with little difference between breeds (Vishwanath and Moreno, 2018). The results of identifying the length and width (area) of the head of 1000 fresh semen sperm obtained the average head area of male Bali bull sperm, which is $37.58 \mu\text{m}$. The results of the proportion of X and Y chromosome sperm in fresh semen were close to the natural proportion, which obtained 49% X chromosome and 51% Y chromosome. When morphometric testing with sperm head area, if the sperm head area is greater than or equal to the average head area of fresh semen sperm, it is identified as X sperm. If the sperm head area is smaller than the average, it is identified as Y sperm (Yekti *et al.*, 2024). As in Figure 4, sperm A has a head area of $34.47 \mu\text{m}$, so it is identified as Y sperm, while sperm B has a head area of $42.43 \mu\text{m}$, so it is identified as X sperm.

The proportion of the results of the study was higher than Yekti *et al.* (2023), PDGC ten gradient semen sexing produced a proportion of X sperm 77.1% and Y sperm 80.6%. The results of the study were also not much different from Yekti *et al.* (2024), the proportion of X and Y sperm in ten gradients PDGC sexing semen was 74.20% and 84.10%, respectively, and in five gradients, 83.10% and 85.10%. Some factors that can affect the proportion of sperm in the PDGC sexing method include molecular weight density gradient, centrifugation time, and speed (Yekti *et al.*, 2024). The highest proportion of X sperm was T1 85.40%, but not different from T2 81.20% and different from T3 75.10% ($P > 0.01$). This suggest that the difference in gradient affects

the separated X sperm, the higher the percoll density, the less X sperm are able to penetrate the gradient. The highest proportion of Y sperm was found in T3 79.90%, but not different from the proportions of T1 79.80% and T2 77.90% ($P > 0.05$), presumably because the Percoll density in the T3 gradient was the highest. This happens because the size of the sperm Y is smaller, so it is more difficult to penetrate the higher Percoll density gradient so that many sperm Y is stuck in the top layer. The proportion of X and Y sperm in the study showed separate results because the proportion value was $> 70\%$. If the percentage proportion is higher, it means that the more X and Y sperm are separated. In the research of Susilawati *et al.* (2023) PDGC sexed semen produced a proportion of 80.79% Y-sperm, the proportion of bull calves born from crossbred Ongole cattles inseminated using the Y-sexing semen with a double dose of 78.95%. This suggest that the higher the percentage of separated sperm, the higher the chance of the birth of offspring according to the desired sex.

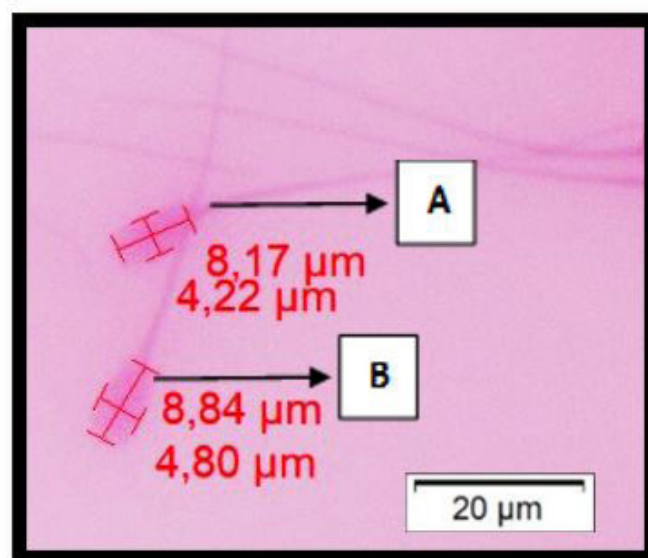


Figure 4: Identification of X and Y semen sperm sexing by morphometric test. **Description:** A: Y Chromosome Sperm; B: X Chromosome Sperm.

CONCLUSIONS AND RECOMMENDATIONS

In conclusion, the gradient variation used significantly affected sperm quality, but no significant effect on CASA parameters. Liquid sexing semen that passed the SNI requirements for liquid semen to be frozen were all treatments in the bottom layer. Meanwhile, frozen sexing semen that passes the requirements of SNI frozen semen is the bottom layer of gradient 10 with progressive motility parameters with CASA 40.30%. The proportion of all treatments was more than the expected value of 70%. These findings indicate that five gradients with tris aminometh-

ane diluent + 20% egg yolk bottom layer can be used for the PDGC sperm sexing method based on visual motility and proportion. So, further research needs to be done with five and three gradients using 1 mL fresh semen research material to get better results.

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NOVELTY STATEMENT

The novelty of this study is in the establishment of Percoll three and five gradients sexed semen through the application of the PDGC method and using tris aminomethane diluent, which has not been extensively explored in the context of semen processing. The results demonstrate that this methodology retains a high quality of sexed frozen semen as assessed by kinematic characteristics measured via Computer-Assisted Sperm Analysis (CASA), with a yield proportion exceeding 70%. This discovery represents a noteworthy advancement in reproductive and livestock technology, providing a more efficient and effective approach for enhancing both the quality and quantity of sperm sex determination outcomes.

AUTHOR'S CONTRIBUTIONS

Nanda Ayu Rahmawati: Conceptualization, drafting the original manuscript, collecting data, analyze statistics, drafting and revisions.

Aulia Puspita Anugra Yekti: Conceptualization, supervision.

Aditiya Wahyudi: Collecting data.

Putri Utami and Habib Asshidiq Syah: Conceptualization, drafting and revisions.

Fardha Ad Durrun Nafis: Collecting data.

Sri Wahjuningsih, Achadiyah Rachmawati, Nurul Isnaini and Trinil Susilawati: Conceptualization, supervision, and review the final manuscript.

Each author has reviewed and approved the manuscript's published form.

CONFLICT OF INTEREST

The author declares that there is no conflict of interest with stakeholders related to the material written in this manuscript.

REFERENCES

Afriani T, Udin Z, Wahyudi D, Asyraf M (2024). Testing motility

parameters of post-thawing pesisir bulls (*Bos indicus*) semen with and without sexing. Int. J. Vet. Sci., 13(4): 453–457.

Amann RP, Waberski D (2014). Computer-assisted sperm analysis (CASA): capabilities and potential developments. Theriogenology, 81: 5–17. <https://doi.org/10.1016/j.theriogenology.2013.09.004>

Arif AA, Maulana T, Kaiin EM, Purwantara B, Arifiantini RI, Memili E (2020). Comparative analysis of various step-dilution techniques on the quality of frozen Limousin bull semen. Vet. World, 13(11): 2422–2428. <https://doi.org/10.14202/vetworld.2020.2422-2428>

Baeta CA, Martos SG, Jime'nez SM, Santolaria P, Ya'niz J, Palaci'n I, Casao A, Pe'rez JAC, Blanco TM, Pe RP (2019). Open CASA: a new open-source and scalable tool for sperm quality analysis. PLOS Comput. Biol., 15(1): 1–18. <https://doi.org/10.1371/journal.pcbi.1006691>

Baharun A, Arifiantini RI, Yusuf TL (2017). Freezing capability of Pasundan Bull sperm using tris egg yolk, tris-soy, and Andromed® diluents. J. Kedok. Hewan, 11(1): 45–49. <https://doi.org/10.21157/j.ked.hewan.v11i1.5810>

Belala R, Amirat LB, Martinot A, Thorin C, Michaud S, Desherces S, Youngs CR, Bencharif D (2019). A comparison of liquid and lyophilized egg yolk plasma to low density lipoproteins for freezing of canine spermatozoa. Reprod. Domest. Anim., 1–8. doi: 10.1111/rda.13476

Bravo JA, Montanero J, Calero R, Roy TJ (2011). Identification of sperm subpopulations with defined motility characteristics in ejaculates from Ile de France rams. Anim. Reprod. Sci., 129(1–2): 22–29. <https://doi.org/10.1016/j.anireprosci.2011.10.005>

Brillianti FF, Sianto P, Rahardjo D, Sardjito T, Suprayogi TW, Triana IN (2021). Kualitas semen sapi pejantan berdasarkan umur, suhu, dan kelembaban di taman ternak Pendidikan Universitas Airlangga. Ovoozoa, 10(3): 81–89. <https://doi.org/10.20473/ovz.v10i3.2021.81-89>

Dasrul, Yaman MA, Zulfan (2013). Pemisahan spermatozoa berkromosom X dan Y Kambing Boer dan aplikasinya melalui inseminasi buatan untuk mendapatkan jenis kelamin anak sesuai harapan. Agripet, 13(1): 6–15. <https://doi.org/10.17969/agripet.v13i1.546>

Diliyana DF, Susilawati T, Rahayu S (2014). Keutuhan membran spermatozoa disekuensing sentrifugasi gradien densitas percoll berpengencer Andromed dan CEP-2 yang ditambahkan kuning telur. J. Vet., 15(1): 23–30.

Ehlers J, Behr M, Bollwein H, Beyerbach M, Waberski D (2011). Standardization of computer-assisted semen analysis using an e-learning application. Theriogenology, 76(3): 448–454. <https://doi.org/10.1016/j.theriogenology.2011.02.021>

El-Bahrawy KA (2017). The influence of caffeine supplementation and concerted utilization of enzymatic and mechanical semen liquefaction on freezability of dromedary camel sperms. Int. J. Vet. Med., 5: 121–127. <https://doi.org/10.1016/j.ijvsm.2017.09.005>

Haryani R, Toleng AL, Sonjaya H, Yusuf M (2016). Characteristic of Bali Bulls sperms assessed using computerized assisted semen analysis (casa). Int. J. Sci. Basic Appl. Res., 28(2): 161–168.

Joshi H, Mathur M, Mohanty AK, Kumar S, Kaushik JK, Mohanty TK, Kumar D, Singh SK, Bhushan V, Parashar A,

- Bhardwaj P, Yata VK (2021). Semen sexing in bovine: current status and the need to develop alternative techniques. *Anim. Reprod. Update*, 1(1): 17–31. <https://doi.org/10.48165/aru.2021.1203>
- Kanno C, Sakamoto KQ, Kang SS, Yanagawa Y, Katagiri S, Nagano M (2023). The difference in subpopulation structures in sex-sorted and non-sorted semen by discriminant analysis of bull sperm motility data collected by a computer-assisted sperm analysis system. *Andrologia*, 9944344: 1–7. <https://doi.org/10.1155/2023/9944344>
- Kusumawati ED, Isnaini N, Yekti APA, Luthfi M, Affandhy L, Pamungkas D, Kuswati, Ridhowi A, Sudarwati H, Susilawati T, Rahayu S (2017). The quality of sexed semen on Filial Ongole bull using percoll density gradient centrifugation method. *J. Microbiol. Biotechnol. Environ. Sci.*, 19(1): 189 – 199.
- Lu JC, Huang YF, Lu NQ (2014). Computer-aided sperm analysis: past, present and future. *Andrologia*, 46: 329–338. <https://doi.org/10.1111/and.12093>
- Mahendra HC, Samsudewa D, Ondho YS (2018). Evaluation of semen quality of buffalo frozen semen produced by artificial insemination center. *J. Indon. Trop. Anim. Agric.*, 43(1): 26–34. <https://doi.org/10.14710/jitaa.43.1.26-34>
- Mahfud A, Isnaini N, Yekti APA, Kuswati, Susilawati T (2019). Kualitas spermatozoa post thawing semen beku spermatozoa Y hasil sexing pada sapi Limousin. *J. Trop. Anim. Prod.*, 20(1): 1–7. <https://doi.org/10.21776/ub.jtapro.2019.020.01.1>
- Mardi I, Yekti APA, Kuswati, Luthfi M, Susilawati T (2020). Kualitas semen beku sexing sapi Peranakan Ongole menggunakan volume semen awal yang berbeda. *J. Ilmu Teknol. Peternak. Trop.*, 7(3): 238–346. <https://doi.org/10.33772/jitro.v7i3.12203>
- Marquez B, Suarez SS (2007). Bovine sperm hyperactivation is promoted by alkaline stimulated Ca²⁺ influx. *Bio Reprod.*, 76(4): 660–665. <https://doi.org/10.1095/biolreprod.106.055038>
- Maulana T, Said (2019). Kinematics motility of frozen-thawed X and Y sperm of Sumba Ongole Bull. *IOP Conf. Ser. Earth Environ. Sci.*, 387(1): 1–4. <https://doi.org/10.1088/1755-1315/387/1/012030>
- Meles DK, Mustofa I, Hariadi M, Wurlina, Susilowati S, Amaliya A, Suparto, Rimayanti (2022). The Enriched Y-bearing sperm combined with delayed fixed-time artificial insemination for obtaining male Simmental crossbred offspring. *Vet. World*, 15: 102–109. <https://doi.org/10.14202/vetworld.2022.102-109>
- Ministry of Agriculture (2019). Program Upsus Siwab Kementan Dorong Peningkatan Populasi Sapi/Kerbau Secara Signifikan. <https://ditjenpkh.pertanian.go.id/berita/896-program-upsiw-siwab-kementan-dorong-peningkatan-populasi-sapi-kerbau-secara-signifikan>. Accessed on May 5, 2024.
- Missio D, Folchini NP, Leivas FG, Pavin CIIUM, Pinto HF, Cibin FWS, Brum DS (2018). Reduction in Percoll volume increases recovery rate of sex-sorted semen of bulls without affecting sperm quality and early embryonic development. *Anim. Reprod. Sci.*, 192: 146–153. <https://doi.org/10.1016/j.anireprosci.2018.03.002>
- Mortimer ST, Horst GVD, Mortimer D (2015). The future of computer-aided sperm analysis. *Asian J. Androl.*, 17: 545–553. <https://doi.org/10.4103/1008-682X.154312>
- National Standards (2017). Semen Beku – Bagian 1: Sapi. Badan Standardisasi Nasional. SNI 4869-1:2017. BSN Jakarta.
- National Standards (2021). Semen Beku – Bagian 1: Sapi. Badan Standardisasi Nasional. SNI 4869-1:2021. BSN Jakarta.
- National Standards (2024). Semen Beku – Bagian 1: Sapi. Badan Standardisasi Nasional. SNI 4869-1:2024. BSN Jakarta.
- Ngcobo JN, Nedambale TL, Sithole SM, Mtileni B, Mpofu TJ, Ramukhithi FV, Chokoe TC, Nephawe KA (2024). A systematic review on the prospects of X- and Y-sexed semen in ruminant livestock: implications for conservation, a South African perspective. *Front. Vet. Sci.*, 11: 1–14. <https://doi.org/10.3389/fvets.2024.1384768>
- Novo AF, Lopez SS, Masa CB, Mozas P, Mercado E, Caceres E, Garrafa A, Martin JVG, Villalobos NP, Oliet A, Astiz S, Garnelo SSP (2021). Effect of extender, storage time and temperature on kinetic parameters (CASA) on bull semen samples. *Biology*, 10(805): 1–17. <https://doi.org/10.3390/biology10080806>
- Prasetyowati MH, Pradista LA, Widyas N, Prastowo S (2021). Estimation of most probable producing ability of Bali cattle semen quality. *ICLiTE-1*. <https://doi.org/10.1088/1755-1315/902/1/012008>
- Promthep K, Satitmanwiwat S, Kitiyanant N, Tantiwattanakul P, Jirajaroenrat K, Sitthigripong R, Singhapol C (2016). Practical use of Percoll density gradient centrifugation on sperm sex determination in commercial dairy farm in Thailand. *Indian J. Anim. Res.*, 50(3): 310–313. <https://doi.org/10.18805/ijar.8427>
- Putranti OD (2018). Pengaruh kaferin terhadap lengkung ekor spermatozoa epididimis Sapi Bali. *Agrisaintifika J. Ilmu-Ilmu Pertanian*, 2(2): 143–145. <https://doi.org/10.32585/ags.v2i2.265>
- Raafi M, Yusuf M, Toleng AL, Diansyah AM, Surahman, Sahiruddin (2021). Movement patterns of sperms at different bull breeds using computer-assisted sperm analysis (CASA). *IOP Conf. Ser. Earth Environ. Sci.*, 104(4): 978–986. <https://doi.org/10.1088/1755-1315/788/1/012137>
- Ratnawati D, Luthfi M, Pamungkas D, Affandhy L (2020). Motility characterization of albumin sexed spermatozoa in two different diluents and additional antioxidant. *J. Indon. Trop. Anim. Agric.*, 45(4): 277–286. <https://doi.org/10.14710/jitaa.45.4.277-286>
- Ratnawati D, Isnaini N, Susilawati T (2017). Pemanfaatan CASA dalam observasi motilitas spermatozoa semen cair sapi madura dalam pengencer berbeda. *J. Ilmu-Ilmu Peternakan*, 27(1): 80–95. doi: 10.21776/ub.jiip.2017.027.01.07
- Safa F, Yekti APA, Utami P, Syah HA, Febrianto N, Rachmawati A, Yuswati A, Amaliya A, Sulistyowati D, Susilawati T (2025). Sperm quality, kinematics, membrane integrity, and DNA fragmentation of frozen sexed semen in Holstein-Friesian bulls. *Trop. Anim. Sci. J.*, 48(3): 199–210. <https://doi.org/10.5398/tasj.2025.48.3.199>
- Setiyono A, Setiadi MA, Kaiin EM, Karja NWK (2020). Pola gerakan spermatozoa sapi setelah diinkubasi dalam media fertilisasi dengan imbuhan heparin dan/atau kafein. *J. Vet.*, 21(3): 458–469.
- Setyani NMP, Sarini NO, Oka IGL (2017). Heterogenitas kuantitas dan kualitas semen Sapi Bali pejantan di Unit Pelaksana Teknis Balai Inseminasi Buatan Daerah Baturiti,

- Tabanan. J. Trop. Anim. Sci., 5(1): 91–104.
- Simbolon CNA, Pramudhita AD, Rizqianti RA, Yekti APA, Isnaini N, Susilawati T (2024). Quality and proportion of spermatozoa in the Percoll density gradient centrifugation sexing method at different gradients using AndroMed® Diluent. ICIAS. BIO Web Conf. 88, 00042 (2024). <https://doi.org/10.1051/bioconf/20248800042>
- Solihati N, Rasad SD, Hilmia N, Irianti SA (2023). Identifikasi sperma pembawa kromosom X dan Y secara morfometri sebagai dasar aplikasi sexing semen sapi pasundan. Nukleus Peternak, 10(2): 44–51. <https://doi.org/10.35508/nukleus.v10i2.13323>
- Susilawati T (2014). Sexing spermatozoa hasil penelitian laboratorium dan aplikasi pada sapi dan kambing. Malang: UB Press. ISBN: 978-602-203711-8
- Susilawati T, Bustari WO, Crisara IPB, Kuswati, Huda AN, Satria AT, Yekti APA (2023). Sex ratio of calves resulted from artificial insemination implementation using sexed semen with percoll gradient density centrifugation method in ongole crossbred cows. Indian J. Anim. Res., 57(7): 841–844.
- Susilawati T, Kusumawati ED, Isnaini N, Yekti APA, Sudarwati H, Ridhowi A (2017). Effect of sexing process using percoll density gradient centrifugation and frozen on motility and damage to spermatozoa membran of Filial Ongole. Adv. Health Sci. Res., 5(1), 227–231.
- Susilawati T, Rahayu S, Udrayana S, Sudarwati H, Nugroho E (2014). Effect of different centrifugation duration on Simmental Bull sperm quality and membran status after sexing, cooling, and freezing processes. Am. Eur. J. Sustain. Agric., 8(7): 28–34.
- Susilawati T, Ratnawati D, Isnaini N, Kuswati, Yekti APA (2018). Character of liquid semen motility in various diluents on Balinese cattle during cold storage. Asian Jr. of Microbiol. Biotech. Env. Sc., 20(1): 166–172.
- Susilawati T, Suyadi, Ihsan N, Wahjuningsih S, Isnaini N, Rachmawati A, Yekti APA, Utami P (2022). Manajemen reproduksi dan inseminasi buatan. Malang: UB Press. ISBN: 978-623-296-624-6.
- Syarifuddin NA, Toleng AL, Rahardja DP, Ismartoyo (2018). Analisis semen berbasis komputerisasi (CASA) untuk memprediksi fertilitas sperma Sapi Bali. Pros. Sem. Nas. Lingk. Lahan Basah, 3(1): 80–85.
- To'aloh N, Mubarakati NJ, Jayanti GE (2023). Analisis motilitas spermatozoa sebelum dan sesudah pembekuan pada sapi Peranakan Ongole (*Bos indicus*) di BBIB Singosari Malang. J. Compr. Sci., 2(5): 1031–1038. <https://doi.org/10.59188/jcs.v2i5.315>
- Utami P, Hanif MK, Yekti APA, Prafitri R, Huda AN, Kuswati, Kusmartono, Susilawati T (2022). Evaluation the success of artificial insemination using frozen sexed semen based on different estrus characters. Jurnal Agripet, 22(2): 190–196. <https://doi.org/10.17969/agripet.v22i2.23423>
- Utami P, Yekti APA, Simbolon CNA, Syah HA, Amaliya A, Siswoyo TA, Isnaini N, Susilawati T (2025). Analysis of kinetic parameters of sexed Holstein-Friesian bull spermatozoa using Percoll density gradient centrifugation with computer-assisted sperm analysis. Vet. World, 18(2): 287–295. <https://doi.org/10.14202/vetworld.2025.287-295>
- Valverde A, Barquero V, Soler C (2020). The application of computer-assisted semen analysis (CASA) technology to optimise semen evaluation. A review. J. Anim. Feed Sci., 29(3): 189–198. <https://doi.org/10.22358/jafs/127691/2020>
- Vishnawat R, Moreno JF (2018). Review: Semen sexing—current state of the art with emphasis on bovine species. Animal, 12(S1): 85–96. <https://doi.org/10.1017/S1751731118000496>
- Yekti APA, Arif AA, Utami P, Syah HA, Pramudhita AD, Rizqiantin RA, Susilawati T (2024). Quality and proportion of X and Y sperm after sexing process using Percoll density gradient centrifugation method on different gradients and diluents in Belgian Blue cross bull. Adv. Anim. Vet. Sci., 12(11): 2211–2220. <https://doi.org/10.17582/journal.aavs/2024/12.11.2211.2220>
- Yekti APA, Mustofa R, Lutfi M (2022). Relationship of motility and acrosome reaction on sexing sperm in Ongole crossbred bull. E3S Web Conf., 335(42): 00042. <https://doi.org/10.1051/e3sconf/202233500042>
- Yekti APA, Rahayu S, Ciptadi G, Susilawati T (2023). The quality and proportion of spermatozoa X and Y in sexed frozen semen separated with Percoll density gradient centrifugation method on Friesian Holstein Bull. Adv. Anim. Vet. Sci., 11(3): 371–378. <https://doi.org/10.17582/journal.aavs/2023/11.3.371.378>