Research Article

Integrated Baseline Analysis of Semen Quality and Sperm Protein Profile in Kuantan Bulls During Cryopreservation

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Abstract | This study aimed to integrate conventional semen quality parameters with sperm protein profile characteristics of Kuantan bulls during cryopreservation and to explore their functional associations. Semen was collected from Kuantan bulls using an artificial vagina and processed into frozen semen using a Tris–egg yolk extender. Evaluations were conducted at the pre-freezing and post-thawing stages. Sperm quality parameters included motility, abnormality, viability, plasma membrane integrity (PMI), and acrosome integrity (AI). Protein analysis included total protein concentration determined by the Bradford method and protein band profiling using SDS-PAGE. Data were analyzed using a t-test and Pearson correlation at a significance level of $p < 0.05$. Cryopreservation resulted in a significant increase in sperm abnormality and a decrease in PMI and AI ($p < 0.05$), while motility and viability remained within acceptable standards for artificial insemination. Total protein concentration and the number of protein bands decreased after freezing, particularly in medium- and high-molecular-weight proteins. Total protein concentration showed significant positive correlations with motility, PMI, and AI ($p < 0.05$), whereas protein band loss was negatively associated with PMI. In conclusion, semen from Kuantan bulls maintained acceptable post-thaw quality despite structural and molecular alterations. Cryopreservation reduced protein concentration and altered protein profiles, which were associated with key functional sperm parameters. These findings provide preliminary baseline data for future proteomic and fertility-related studies.

Keywords | Kuantan bull, Motility, Plasma membrane integrity, SDS-PAGE, Seminal plasma protein, Viability

Received | January 26, 2026; **Accepted** | May 12, 2026; **Published** | June 17, 2026
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Citation | Yendraliza, Rodiallah M, Jaswandi, Akmal K (2026). Integrated baseline analysis of semen quality and sperm protein profile in Kuantan bulls during cryopreservation. J. Anim. Health Prod. 14(3): 830-839.
DOI | https://dx.doi.org/10.17582/journal.jahp/2026/14.3.830.839
ISSN (Online) | 2308-2801

INTRODUCTION

Local cattle play an essential role in Indonesia's socio-economic system; therefore, conservation and breeding programs for Indonesian cattle must be carefully designed based on genetic potential and available information (Agung *et al*., 2019). Kuantan cattle are one of Indonesia's local cattle breeds originating from Riau Province, as stipulated in the Decree of the Minister of Agriculture No. 1052/Kpts/SR.120/10/2014. Kuantan cattle (*Bos indicus*) represent an important Indonesian genetic resource, particularly in the Kuantan Singingi region of Riau. According to data from the Riau Provincial Livestock and Animal Health Service, the population of Kuantan cattle has declined by approximately 30% over the past five years (BPS, 2024). This decline is attributed to several factors,

including the limited availability of high-quality breeding bulls and the extensive use of artificial insemination with semen from exotic breeds, which poses a risk of eroding the genetic characteristics of Kuantan cattle (Suhardi *et al.*, 2022). Currently, the sex ratio in Kuantan cattle populations is imbalanced, primarily due to the continuous reduction in the number of breeding bulls over the years (Yendraliza *et al.*, 2020). To overcome bull scarcity, artificial insemination (AI) is required. The first step is the development of frozen semen from Kuantan bulls, which must be preceded by comprehensive semen quality evaluation. Wang *et al.* (2018) reported that sperm motility, viability, acrosome integrity, capacitation, and acrosome reaction are influenced by the expression of specific proteins. These proteins play critical roles in fertilization processes, including maintaining and protecting sperm motility (Khan *et al.*, 2015). Proteomic analyses have revealed significant changes in protein abundance and distribution following cryopreservation, affecting key proteins involved in energy metabolism, motility, and membrane stability. Several studies on semen quality and protein profiles in local Indonesian cattle have been conducted in Bali cattle (Iskandar *et al.*, 2023), Madura cattle (Azizah *et al.*, 2023), Pesisir cattle (Ananda *et al.*, 2025) and Donggala cattle (Baharun *et al.*, 2025). However, studies focusing on semen quality and sperm protein profiles in Kuantan bulls remain very limited. Therefore, this study was designed as a preliminary baseline investigation to integrate sperm functional parameters with protein profile characteristics during cryopreservation in Kuantan bulls. This study provides foundational molecular information to support artificial insemination programs and future advanced proteomic investigations for the conservation of this indigenous cattle breed.

MATERIALS AND METHODS

Study period and location

Semen collection from Kuantan bulls was conducted at the Riau Provincial Artificial Insemination Center from August to November 2025. Biomolecular analyses were performed at the Technology Laboratory, Universitas Andalas, Padang. The overall research workflow is illustrated in Figure 1.

Experimental design

This study employed a descriptive-comparative experimental design focusing on two cryopreservation stages pre-freezing and post-thawing. Due to the limited availability of Kuantan breeding bulls, this study was conducted using only two individuals. Repeated semen collections were treated as technical replicates to assess within-individual variability. Therefore, the findings should be interpreted as preliminary baseline data.

Semen collection

Semen was collected using an artificial vagina at 42–45°C once weekly for eight weeks from two Kuantan bulls. Semen evaluation included macroscopic and microscopic assessments. Only ejaculates with a minimum motility of 70% were used in this study.

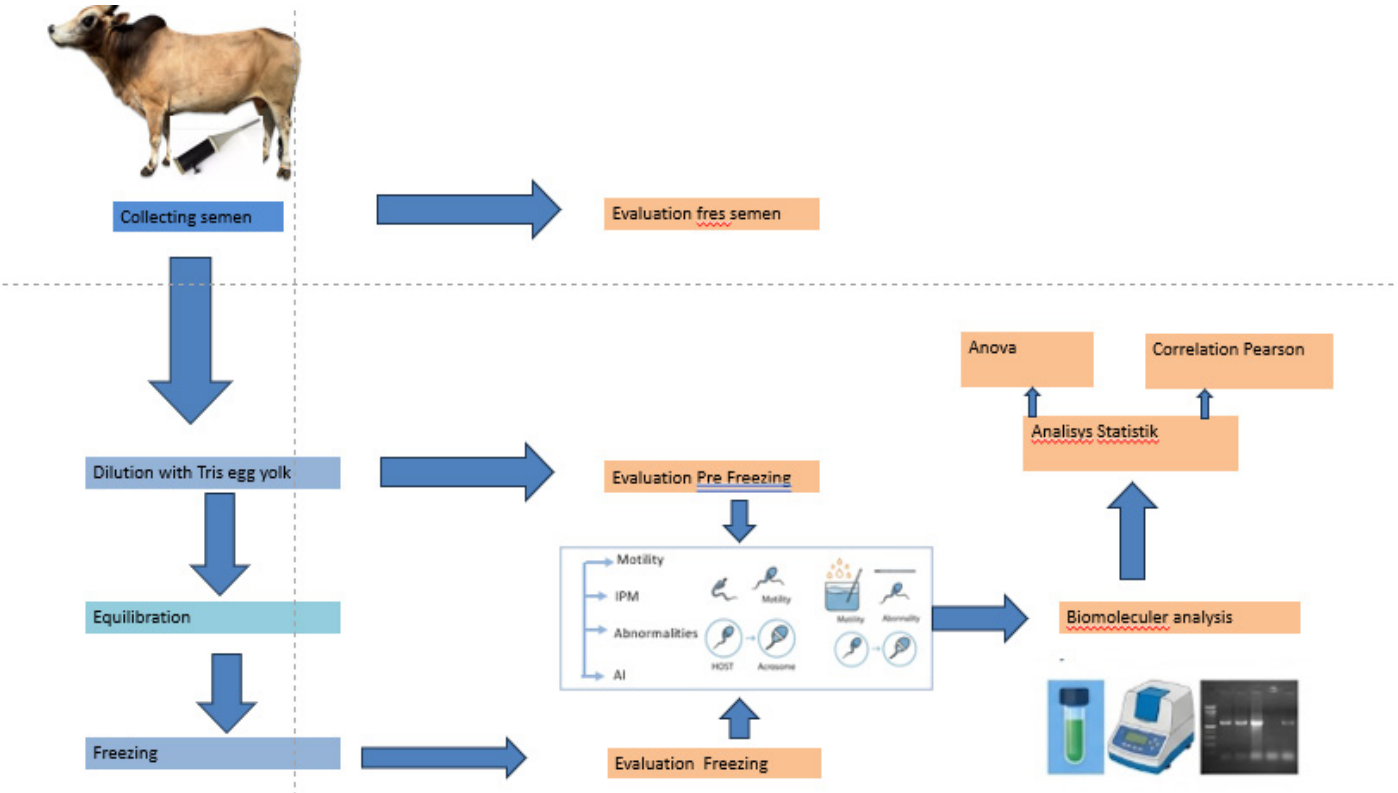

Figure 1: Research workflow for Kuantan bull semen quality analysis.

Semen extender, equilibration, and freezing

Fresh semen was diluted using a Tris–egg yolk citrate extender (Table 1). Tris (hydroxymethyl) aminomethane, citric acid, and fructose were weighed using an analytical balance and transferred into a 250 mL Erlenmeyer flask, followed by the addition of distilled water to a final volume of 80 mL. The mixture was homogenized using a magnetic stirrer for 15 minutes and covered with aluminum foil to obtain the buffer solution. A total of 74 mL of buffer solution was transferred into a 250 mL beaker, mixed with 20 mL egg yolk and 6 mL glycerol, covered again with aluminum foil, and homogenized for 60 minutes. Subsequently, 0.5 mL penicillin and 0.4 mL streptomycin were added using a syringe through the foil cover. This final solution was referred to as the Tris–egg yolk extender.

Table 1: Composition of Tris–citrate extender.

No.	Ingredients	Amount
1.	Tris (hydroxymethyl) aminomethane	3.028 g
2.	Citric acid	1.7 g
3.	Fructose	1.25 g
4.	Penicillin	0.5 mL
5.	Streptomycin	0.4 mL
7.	Glycerol	6 mL
8.	Egg yolk	20 ml
9.	Distilled water	80 ml

The dilution volume was calculated based on semen volume. The extender was pre-warmed in a water bath at 37°C before use. After dilution, semen was loaded into straws using a straw-filling machine, sterilized for 60 seconds, sealed, and equilibrated at 5°C for 4 hours (Yendraliza *et al.*, 2025). Prior to freezing, straws were exposed to liquid nitrogen vapor for 15 minutes and then immersed in liquid nitrogen for 24 hours. Thawing was performed at 37°C for 30 seconds prior to evaluation.

Parameters

Motility

Motility was evaluated as total motility (%) using CASA. Detailed kinematic parameters (VSL, VAP, VCL) were not included and are acknowledged as a limitation. A 0.2 mL semen sample was placed on a glass slide, covered with a coverslip, and examined under a light microscope at 10×45 magnification. Motility percentage was calculated by comparing motile and non-motile spermatozoa.

Abnormality

Sperm abnormality was assessed by mixing a drop of eosin stain with 0.2 mL semen, followed by smear preparation and air-drying. Observations were performed under a light microscope (Motic BA310®) at 10×45 magnification. Morphological abnormalities such as bent tails, broken tails, and folded midpieces were counted in eight microscopic fields.

Plasma membrane integrity (PMI)

PMI was assessed using the Hypoosmotic Swelling Test (HOST). A total of 20 µL semen was mixed with 200 µL HOST solution and incubated at 37°C for 45 minutes. After incubation, samples were examined under a light microscope (Motic BA310®) at 400× magnification. Sperm with curled or swollen tails were classified as having intact plasma membranes.

Acrosome integrity

Acrosome integrity was evaluated by mixing one drop of semen with five drops of saline formalin solution (physiological NaCl + 1% formalin). Observations were performed using a phase-contrast microscope at 400× magnification, evaluating at least 200 spermatozoa. Intact acrosomes were indicated by a dark-stained apical region.

Total protein concentration analysis

Sample preparation for protein analysis was performed with modifications to the method developed Jaswandi *et al.* (2024). Fresh semen samples were centrifuged at 3000 rpm for 45 min. After centrifugation, the plasma and sediment fractions were separated, each transferred into 2 mL Eppendorf tubes, and stored at 4 °C until further use for quantitative and qualitative protein analyses. A total of 500 µL of fresh semen sediment was mixed with 1 mL of phosphate-buffered saline (PBS) in a 1.5 mL microtube and subsequently centrifuged at 13,000 rpm for 15 min. The supernatant was discarded, and the spermatozoa pellet was resuspended in 400 µL of PRO-PREP™ solution and homogenized using a vortex mixer. In accordance with the manufacturer's protocol, sperm protein extraction was carried out using PRO-PREP™ Protein Extraction Solution (Protein Extraction Solution, 17081, iNtRON Biotechnology, Korea). The mixture was then incubated at –20 °C for 15 min. Subsequently, the samples were sonicated three times for 20 s each and centrifuged again at 13,000 rpm for 5 min at 4 °C. The resulting supernatant was transferred into a 1.5 mL microtube for protein content determination. Sperm protein concentration was determined using the Bradford method (Bradford, 1976). The analysis was performed using Bradford reagent (BR05, Eco Tech), which was diluted with distilled water at a ratio of 1:4. The standard solution was prepared by dissolving 0.563 mg bovine serum albumin (BSA; Sigma) in PBS. Three types of solutions were prepared: A blank (3000 µL working reagent + 50 µL PBS), a standard (3000 µL working reagent + 50 µL standard solution), and a sample (3000 µL working reagent + 50 µL sample). Each mixture was incubated at room temperature for 10 min. Optical density (OD) was then measured at a wavelength of 595

nm using a spectrophotometer (Shimadzu UV-1800).

SDS-PAGE sperm protein analysis

Sperm protein band analysis was performed using the SDS-PAGE method with a sample concentration of 20 µg/mL. Sample quantity was determined using a Qubit fluorometer (Qubit, Invitrogen). Samples were placed in microtubes, mixed with 10 µg of loading buffer, and homogenized by vortexing for 1 min. The samples were then heated in a water bath at 70 °C for 10 min, followed by centrifugation for 1 min. Protein samples were subsequently loaded onto a 10% polyacrylamide gel (Q-PAGE™ TGN Precast Gel, QP4210, SMOBIO® Technology, Inc., Taiwan) and electrophoresed for 90 min using Tris–glycine running buffer (TGS 10, Eco Tech) at 110 V and 110 mA. Protein bands were stained with Coomassie Blue solution (Coomassie Blue Rapid Staining Solution, E-IR-RI129) for 24 h under gentle agitation. Excess stain was removed by immersing the gel in ddH_2O in a closed container and washing it three times. Pita protein yang terbentuk diamati menggunakan Gel Doc (iBright 1500, Invitrogen, Thermo Fisher Scientific). The resulting protein bands were visualized using a Gel Doc system (iBright 1500, Invitrogen, Thermo Fisher Scientific). Sample protein bands were compared with a molecular weight marker (ExcellBand 3-color Broad Range Protein Marker, 10–245 kDa, SMOBIO® Technology, Inc., Taiwan) to determine protein molecular weights. SDS-PAGE visualization data were used for qualitative analysis of protein molecular weight by comparing sample banding patterns with the marker. The parameters assessed using SDS-PAGE were the number of protein bands and the distribution of protein bands based on molecular weight (kDa). Protein bands were classified into three groups: low-molecular-weight proteins (<25 kDa), medium-molecular-weight proteins (25–100 kDa), and high-molecular-weight proteins (>100 kDa).

Data analysis

Data were analyzed using a Completely Randomized Design with two cryopreservation stages (pre-freezing and post-thawing). Semen collections served as replicates. Differences were tested using a t-test, and relationships between sperm functional and biomolecular parameters were analyzed using Pearson correlation at $p < 0.05$. Results are presented as mean ± standard deviation.

RESULTS AND DISCUSSION

Fresh semen quality of kuantan bulls

A summary of the characteristics of fresh semen from Kuantan bulls, including ejaculate volume, sperm concentration, motility, viability, and the incidence of morphological abnormalities, is presented in Table 2. These sperm characteristics play a crucial role in determining male gamete fertility.

Table 2: Average quality of fresh Kuantan bull semen.

Characteristic	Average ±SE
Volume (mL)	3.33 0.89
Colour	Milky white
pH	6.5 ± 0.15
Consistency	currently
Concentration (million/mL)	1.175 ±4.00
Mass movement	++

Note: Results are presented as mean ± standard deviation.

The average ejaculate volume of fresh Kuantan bull semen was 3.33 ± 0.89 mL, with a milky white color, moderate consistency, and a pH of 6.5 ± 0.15 (Table 2). These volume and pH values fall within the physiological range reported for tropical bull semen and are compatible with sperm motility and membrane stability during the initial handling period prior to dilution. Macroscopic evaluation (volume, color, consistency, and pH) is useful as an initial screening tool, as marked deviations in these parameters often indicate contamination, technical errors during collection, or physiological stress in bulls, which may subsequently affect microscopic parameters such as motility and viability (Bollwein and Malama, 2023). The sperm concentration of Kuantan bulls was 1.175 ± 0.4 million/mL (Table 2), with total motility of 84.37 ± 6.30% and mass movement scored as "++", indicating a dense and highly active spermatozoa population (Azevedo *et al.*, 2024). The abnormality rate of 6.83 ± 1.70% was classified as low and consistent with semen from healthy bulls; abnormality levels below 10% are generally considered acceptable for use in artificial insemination programs following appropriate dose adjustment (Standar Nasional Indonesia (SNI), 2017). Plasma membrane integrity (87.54 ± 2.56%) and acrosome integrity (80.65 ± 5.67%) further confirmed that the majority of spermatozoa retained essential organelles required for fertilization processes, including capacitation, the acrosome reaction, and oocyte penetration, indicating adequate freezability and fertilizing capacity of the semen (Zoca *et al.*, 2023). Fresh semen exhibited high motility and viability due to minimal oxidative stress and limited structural damage to the sperm membrane. Sperm membrane stability is closely associated with the ability of spermatozoa to maintain mitochondrial energy production and sustained motility activity (Giaretta *et al.*, 2025).

Based on Table 3, there was an increase in sperm abnormalities, a decrease in intact plasma membrane parameters, and acrosome integrity. This decline represents a common effect of the cryopreservation process, which

induces osmotic pressure changes and protein denaturation within the spermatozoa membrane (Westfalewicz *et al.*, 2021).

Table 3: Average sperm quality pre-freezing and post-thawing in Kuantan cattle.

Parameters	Pre-freezing	Post thawing
Motility (%)	84.37 6.30	88.25 ±7.90
Abnormality (%)	6.83 ± 1.70[b]	9.72 ±6.7[a]
Viability (%)	87.54 ± 5.50	85.5 ±6.7
Plasma membrane integrity (%)	87.54 ± 2.56 [a]	78.32 ±4.7 [b]
Acrosome integrity (%)	80.65 ± 5.67 [a]	78.25 ±9.0 [b]

Note: [a,b] Different superscripts in the same row show significant differences at p<0.05. Results are presented as mean standard deviation.

Motility

The motility percentage of Kuantan bull spermatozoa 24 h after freezing with a 3 h equilibration period reached 88.25 ± 7.90%. This value indicates that Kuantan bull spermatozoa exhibit a strong ability to withstand the freezing process. The maintenance of post-thaw motility suggests that tail structure, mitochondrial integrity, and energy supply were not significantly compromised during cryopreservation (Yanez-ortiz *et al.*, 2022). In addition, the high motility observed reflects the ability of spermatozoa to adapt well to the use of a Tris–egg yolk extender. According to the Indonesian National Standard (BSN), thawed semen evaluated at 37 °C for 30 s should exhibit a minimum motility of 40%; therefore, the results of this study demonstrate excellent post-thaw motility quality. The motility values obtained in this study were higher than those reported for several Indonesian local cattle breeds, such as Aceh cattle (52.58–55.27%) (Sophian *et al.*, 2025) and Pesisir cattle (70,22%) (Ananda *et al.*, 2025). These differences may be attributed to variations in extender composition and breed-specific characteristics (Tamargo *et al.*, 2024).

Viability

The percentage of sperm viability in Kuantan bulls after post-thawing did not differ significantly from pre-freezing viability ($p > 0.05$) (Table 3). This finding is likely associated with the high motility percentage observed. Sperm viability is an indicator of sustained metabolic activity and ion transport, which are highly dependent on plasma membrane stability and the presence of antioxidant systems (Evans *et al.*, 2020). The viability values obtained in this study were higher than those reported for several Indonesian local cattle breeds, including Aceh cattle (Dwitya *et al.*, 2019), Madura cattle (Rosyada *et al.*, 2023), and Pesisir cattle (Ramadhan *et al.*, 2024). Viability is one of the key indicators of semen quality because it directly reflects spermatozoa survival capacity (Kudratullah *et al.*, 2024). Differences in viability values are primarily attributed to variations in individual motility levels. High viability indicates that spermatozoa possess intact plasma membranes, which is essential for maintaining cellular function and fertilizing ability (Manjunath, 2012). Although post-thaw motility values remained high, the slight difference compared to pre-freeze values likely reflects variations in CASA detection sensitivity rather than a true biological improvement. Cryopreservation generally causes structural and metabolic stress that can reduce sperm motility.

Abnormality

Sperm abnormalities increased from the fresh condition (6.83 ± 1.70%) to 9.72 ± 6.70% after thawing (Table 3); however, this level remained within the acceptable range for frozen semen used in artificial insemination. Raheja *et al.* (2018) reported that an increase in sperm abnormalities after freezing is a common phenomenon associated with the processes of dilution, equilibration, freezing, and thawing. The post-freezing increase in abnormalities is generally attributed to structural damage caused by osmotic stress, ice crystal formation, and lipid phase transitions during the freeze–thaw cycle, particularly affecting the tail and midpiece, which are the most vulnerable regions (Rivera-concha *et al.*, 2024). According to the Standar Nasional Indonesia (SNI) (2017) for frozen semen requirements, semen may be diluted, frozen, and used for artificial insemination if the abnormality rate is below 20%. The abnormality percentage observed in Kuantan bulls differed from that reported in several Indonesian local cattle breeds, such as Pesisir cattle (9.72% vs. 5.90%) (Ananda *et al.*, 2025) and Bali cattle (9.72% vs 4.96%) (Mappanganro *et al.*, 2025). Differences in animal genotype and extender composition contribute to variations in abnormality rates among these local cattle breeds (Akhtar *et al.*, 2022).

Plasma membrane integrity

The intact plasma membrane (IPM) status of Kuantan bull spermatozoa after thawing differed significantly from the pre-freezing intact plasma membrane percentage (Table 3). Nevertheless, the IPM percentage of Kuantan bull semen remained above the minimum requirement for frozen semen established by the Indonesian National Standard (SNI). Kumar *et al.* (2015) reported that the plasma membrane of cells contains carbohydrates bound to lipids (glycolipids) or proteins (glycoproteins), collectively referred to as the cell coat or glycocalyx. The lipid composition of the sperm plasma membrane, particularly the presence of polyunsaturated fatty acids (PUFAs), plays a critical role in maintaining sperm physiology and cellular integrity (Giaretta *et al.*, 2025). However, the high PUFA content also renders spermatozoa more susceptible to damage by reactive oxygen species (ROS), which may

disrupt acrosomal integrity and reduce motility. The intact plasma membrane percentage of Kuantan bull spermatozoa was higher than that reported for Aceh cattle by Sophian *et al*. (2025). Differences in the proportion of spermatozoa with intact membranes may be associated with variations in motility and viability values. Membrane integrity plays a crucial role in maintaining optimal sperm motility. An intact plasma membrane helps preserve normal flagellar structure and function, thereby supporting efficient and progressive sperm movement. Sperm fertility is largely determined by plasma membrane integrity, as this structure is essential for key physiological processes such as capacitation, the acrosome reaction, and interaction with the zona pellucida during fertilization (Sharafi *et al*., 2022).

Acrosome integrity

The percentage of intact acrosomal caps in Kuantan bull spermatozoa decreased after thawing from 80.65 ± 5.67% to 78.25% (Table 3). Acrosomal integrity is closely associated with an intact sperm plasma membrane. The sperm membrane functions as a protective barrier for the acrosome, with cholesterol being one of its major components. A reduction in cholesterol content leads to decreased membrane stability. Irregularities in sperm head morphology may serve as indicators of plasma membrane damage, which can potentially impair acrosomal structure and function (Bollwein and Malama, 2023). The acrosomal cap integrity of Kuantan bull spermatozoa was higher than that reported for Madura cattle (Azizah *et al*., 2023). Variations in acrosomal integrity percentages are influenced by differences in plasma membrane integrity. In line with Younus *et al*. (2024), the sperm head contains an acrosome with a double-membrane structure located between the plasma membrane and the anterior portion of the nucleus. The decline in the proportion of spermatozoa with intact acrosomes and plasma membranes after thawing is a common phenomenon, as the plasma membrane and acrosome are among the structures most sensitive to osmotic stress and lipid peroxidation during the freeze–thaw process (Manjunath, 2012).

Protein analysis of kuantan bull semen

The protein analysis of Kuantan bull semen is presented in Table 4. The protein profile results shown in Table 4 indicate that total protein concentration, the number of protein bands, seminal plasma proteins, and medium- and high-molecular-weight proteins decreased after freezing. The protein profile analysis presented in this study reflects a baseline qualitative characterization of sperm and seminal plasma proteins in Kuantan bulls, aimed at identifying general patterns of protein stability during cryopreservation rather than specific protein identification. This pattern is consistent with previous findings demonstrating that cryopreservation frequently leads to a reduction in seminal plasma protein levels due to cell membrane damage induced by cryoprotectants (Singh *et al*., 2016; Akhtar *et al*., 2022).

Table 4: Analysis of protein concentration, number of protein bands, and molecular weight distribution of Kuantan bull semen before and after freezing.

Parameters	Pre freezing	Post thawing
Total protein concentration (mg/mL)	1.34 [a]	1.219 [b]
Total number of protein bands	12[a]	9[b]
Seminal plasma (%)	1.996 [a]	1.77 [b]
Protein molecular weight distribution		
Low molecular weight (<25 kDa)	Same	same
Medium molecular weight (25–100 kDa)	++ [a]	+[b]
High molecular weight (>100 kDa)	++ [a]	+[b]

Note: [a,b] Different superscripts in the same row show significant differences at $p<0.05$. Results are presented as mean ± standard deviation.

The number of protein bands in Kuantan bull semen decreased from 12 to 9 bands after freezing. This reduction reflects the high sensitivity of sperm proteins to temperature changes. The higher protein concentration in seminal plasma compared with whole semen indicates that most proteins originate from accessory gland secretions, which function as mediators of spermatozoa–environment interactions. These findings are supported by Alyethodi *et al*. (2022) who reported a positive correlation between seminal plasma protein levels and the ability of semen to maintain membrane integrity after freezing. The decrease in the number of protein bands was mainly associated with changes in medium- and high-molecular-weight proteins (Table 2). High-molecular-weight proteins generally play important roles in membrane structure. Their reduction is attributed to thermal and osmotic stress during the cryopreservation process. According to Sikder *et al*. (2025), functional protein degradation may occur as a result of cold shock and exposure to reactive oxygen species (ROS) during cryopreservation.

Relationship between protein profile and sperm quality

The relationship between protein analysis and sperm quality is illustrated in Figure 2. Protein concentration showed a positive association with sperm function, whereas the number of protein bands exhibited a negative relationship with sperm quality parameters, particularly sperm motility, plasma membrane integrity, and sperm abnormalities.

The results of the Pearson correlation analysis (Figure 2) demonstrated a positive relationship between total

protein concentration and sperm motility ($r > 0.60$; $p < 0.05$). Higher total protein concentrations were associated with improved sperm motility. This relationship can be explained by the role of seminal plasma and sperm proteins in energy provision during glycolysis. Manjunath (2012) reported that sperm proteins contribute to membrane and flagellar stability and provide protection against oxidative stress. This statement is consistent with the present findings, in which total protein concentration showed a very strong positive correlation with plasma membrane integrity ($r > 0.70$; $p < 0.01$) (Figure 2). Westfalewicz *et al.* (2021) reported that membrane stability is maintained by lipid- and cholesterol-binding proteins. As shown in Table 4, the decrease in total protein concentration after freezing was consistent with the reduction in sperm plasma membrane integrity observed after freezing in Table 3. Cryopreservation-induced protein loss observed in this study is likely associated with membrane destabilization and oxidative stress. Sperm membranes contain high levels of polyunsaturated fatty acids, making them highly susceptible to lipid peroxidation. Protein degradation during freezing may disrupt membrane-associated proteins responsible for ion transport, structural integrity, and energy metabolism. Pearson correlation analysis between sperm protein concentration and acrosomal integrity revealed a significant positive relationship ($p < 0.05$) (Figure 2). These results suggest that seminal proteins indirectly maintain acrosomal structure through plasma membrane stability, as the plasma membrane serves as a protective barrier for the acrosome. Consequently, protein degradation simultaneously affects both structures. Negative correlations were observed between the number of protein bands and sperm motility, plasma membrane integrity, and sperm abnormalities after freezing (Figure 2).

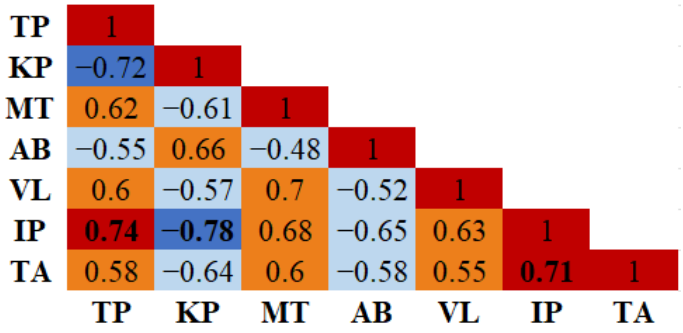

Figure 2: Pearson correlation test results for biomolecular analysis with sperm function.
Note: TP= Total Protein Concentration, KP= Protein Band Loss, MT= Motility, AB= Abnormality, VL= Viability, IP= Intact Plasma Membrane Integrity, TA= Acrosome intact. Strong positive correlation, Strong correlation, strong negative correlation, negative correlation.

A very strong negative correlation was detected between protein band loss and plasma membrane integrity ($p < 0.01$; $r < -0.75$). These results confirm that the plasma membrane is a primary target of damage resulting from protein degradation during cryopreservation. The loss of several protein bands in frozen semen, particularly within the 20–25 kDa and 140–180 kDa ranges (Figure 2), is most likely caused by the degradation of functional proteins due to cold shock and exposure to reactive oxygen species (ROS) during cryopreservation (Sikder *et al.*, 2025). This phenomenon is common and is frequently associated with damage to proteins involved in sperm motility, zona pellucida binding, and immunoregulatory functions (Willforss *et al.*, 2021). This pattern differs from that observed in Pesisir cattle, which exhibit only eight protein bands in fresh semen and six protein bands after thawing (Ananda *et al.*, 2025).

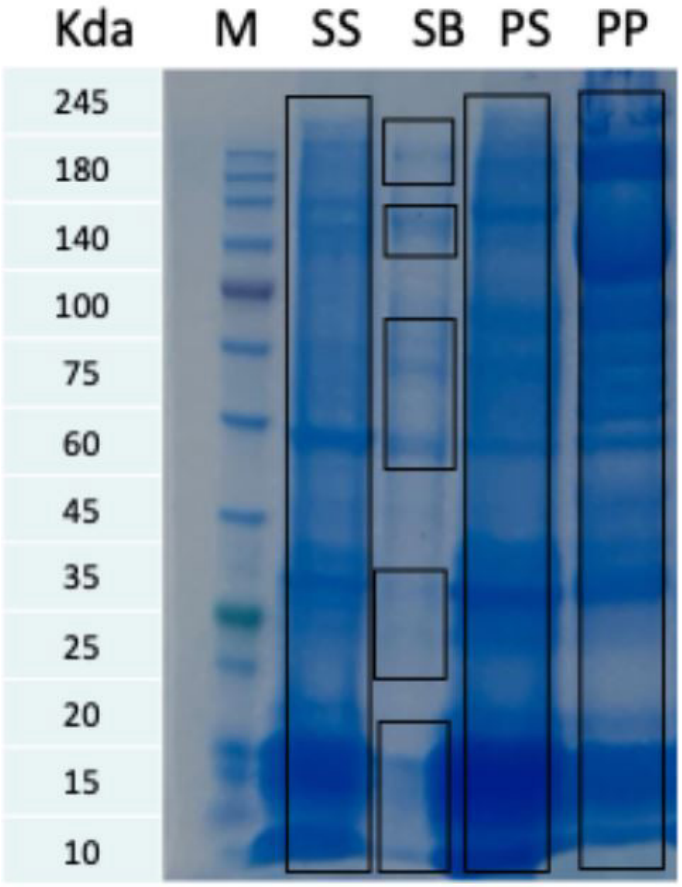

Figure 3: 1D SDS-PAGE of Kuantan bull semen.
Note: M: Marker; SS: Fresh semen;, SB: Frozen semen, PS: Seminal plasma, PP: Plasma and extender.

In contrast, Kuantan cattle retained protein bands of approximately 10 kDa, 60 kDa, and >100 kDa after freezing (Figure 3). Castelló-Ruiz *et al.* (2025) reported that the 10 kDa protein corresponds to cytochrome C, an important protein involved in regulating the acrosome reaction and sperm capacitation. The dominance of protein bands with molecular weights >100 kDa further supports the presence of macromolecular proteins that generally

function in immune mechanisms, cell adhesion, and gamete interactions within the female reproductive tract (Muchtaromah *et al*., 2012). Interestingly, the presence of the 60 kDa protein band (T-complex protein 1 subunit 5/ TCP1) suggests the potential occurrence of unstable spermatogenesis (Zahn *et al*., 1996). This is further supported by the disappearance of the 45 kDa protein band after freezing, identified as phosphoglycerate kinase 2 (PGK2), a key enzyme in the glycolytic pathway that is closely associated with sperm motility (Danshina *et al*., 2010). The presence of the 45 kDa (PGK2) band in fresh semen confirms that the glycolytic pathway was functioning optimally. Because glycolysis generates ATP, which is essential for sperm movement, this finding directly indicates high metabolic activity and good sperm motility. Previous studies have shown that under stress conditions, such as heat treatment, this chaperonin complex can translocate from the cytoplasm to the cytoskeleton, highlighting its potential role in stabilizing cellular structures (Gómez-Guzmán *et al*., 2024). The loss of several key protein bands in frozen semen highlights the need for optimization of cryoprotectant formulations and protective strategies for seminal plasma on Kuantan bull.

This study has several limitations. The small number of bulls limits generalization. SDS-PAGE provides only qualitative protein profiling without specific identification. The absence of detailed CASA kinematic parameters restricts deeper motility analysis. Future studies should include larger sample sizes and advanced proteomic approaches.

CONCLUSION

Kuantan bull semen exhibited good quality in both fresh and post-thaw conditions, with motility, viability, membrane integrity, and acrosomal integrity remaining above the minimum thresholds required for successful artificial insemination according to Indonesian National Standard (SNI) criteria. Cryopreservation of Kuantan bull semen resulted in a reduction in total protein concentration and the number of protein bands, particularly medium- and high-molecular-weight proteins. Total protein concentration in Kuantan bull semen was positively correlated with sperm motility, plasma membrane integrity, and acrosomal integrity, whereas protein band loss showed a strong negative correlation with plasma membrane integrity. These findings underscore the critical role of seminal proteins in maintaining spermatozoa function during cryopreservation.

ACKNOWLEDGEMENTS

This research was funded by the Rector of Sultan Syarif Kasim State Islamic University Riau through the Institute for Research and Community Service (LPPM) in the National Strategic Study Research Scheme with Decree (SK) 614 of 2025. We would like to thank Dewi Sartika, Ibna Hayati (research assistant), and Mr. Sam from the UPT. cattle breeding center (IBT), Tenayan Raya, for their contributions.

NOVELTY STATEMENT

The molecular reproductive baseline established in this study provides a valuable scientific reference for the identification of fertility-associated biomarkers, the optimization of semen cryopreservation protocols, and the development of genetic conservation strategies for Kuantan cattle. Furthermore, these findings offer a foundation for future advanced proteomic investigations and the enhancement of artificial insemination programs aimed at preserving this endangered indigenous breed.

AUTHOR'S CONTRIBUTION

Y contributed to the conceptualization and supervision of the research. M was responsible for methodology development, data collection, and laboratory analysis. J performed data analysis, manuscript writing, and result interpretation. K was responsible for data collection, and laboratory analysis. All authors participated in reviewing and editing the manuscript and approved the final version for submission

Ethical approval

This study received ethical approval from the Ethics Committee of LPPM UIN Sultan Syarif Kasim Riau (Approval No. 672/Un.04/L.1/TL.01/07/2025). All experimental procedures were conducted with minimal animal stress to ensure optimal animal welfare.

Generative AI and AI assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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