

## Characterization of Sperm Quality and Proteomic Profiling of Semen in Kuantan Cattle

Yendraliza <sup>1</sup>, Muhamad Rodiallah <sup>1</sup> and Jaswandi <sup>2</sup>

<sup>1</sup>Departemen of Animal Science, Faculty of Agriculture and Animal Science, Universitas Islam Negeri Sultan Syarif Kasim Riau, Pekanbaru, 28293, Riau, Indonesia

<sup>2</sup>Department of Livestock Production Technology, Faculty of Animal Science, Universitas Andalas, Padang, 25163, West Sumatera, Indonesia

\*Corresponding author: [yendraliza@uin-suska.ac.id](mailto:yendraliza@uin-suska.ac.id)

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### ABSTRACT

Cryopreservation is essential for artificial insemination and genetic conservation, yet it can impair sperm functional quality and protein integrity. Information on semen cryosurvival and protein stability in Kuantan cattle remains limited. Semen was collected from Kuantan bulls using an artificial vagina for 40 weeks. Diluent used for semen is a Tris-egg yolk extender. Semen was collected weekly from Kuantan bulls for 40 weeks using an artificial vagina and processed with a Tris-egg yolk extender. Fresh and post-thaw sperm quality was evaluated following international AI standards, including motility, viability, membrane integrity, acrosome status, and morphology. Total protein concentration and molecular weight distribution were analyzed using the Bradford method and SDS-PAGE. Fresh semen exhibited excellent quality, with progressive motility ( $84.37 \pm 6.30\%$ ), high viability ( $93.17 \pm 1.70\%$ ), and low abnormalities ( $6.83 \pm 1.70\%$ ). Post-thaw samples maintained high motility ( $88.25 \pm 7.90\%$ ) and viability ( $85.50 \pm 6.70\%$ ), demonstrating strong cryosurvival. The highest protein concentration occurred in seminal plasma ( $1.996 \text{ mg/mL}$ ), followed by plasma + extender, fresh semen, and frozen semen. SDS-PAGE identified 12 protein bands in fresh semen and seminal plasma, whereas only nine bands persisted after freezing, indicating cold-shock-related protein loss. Notably, the consistent detection of  $\sim 10 \text{ kDa}$  (Cytochrome C) and  $\sim 45 \text{ kDa}$  (PGK2) proteins across all fractions highlights their functional resilience and potential as fertility biomarkers. In conclusion, Kuantan cattle semen demonstrates excellent cryosurvival capacity and retains key molecular fertility markers, supporting its suitability for AI-based breeding and as a strategic candidate for genetic conservation.

**Keywords:** Cryopreservation, Frozen semen, Kuantan cattle, Seminal plasma protein, Proteomics.

### INTRODUCTION

Local cattle play a strategic role in Indonesia's socio-economic system, particularly in rural livelihoods, food security, and sustainable livestock development. Therefore, conservation and breeding programs for indigenous cattle must be grounded in accurate genetic, reproductive, and technological data (Agung et al. 2019; Hilmiati et al. 2024). Kuantan cattle are one of Indonesia's recognized local breeds originating from Riau Province, as formally designated in the Decree of the Minister of Agriculture No. 1052/Kpts/SR.120/10/2014. As *Bos indicus*, Kuantan cattle represent an important genetic resource and a regional flagship commodity in Kuantan Singingi Regency. Based on data from the Riau Livestock and Animal Health Service, the Kuantan cattle population has decreased by

30% in the last five years (BPS 2024). This decline is driven by multiple factors, including the scarcity of superior breeding bulls, uncontrolled slaughter, and extensive use of artificial insemination using semen from non-local breeds. Such practices increase the risk of genetic dilution and loss of unique breed characteristics (Suhardi et al. 2022). The sex ratio of Kuantan cattle is also increasingly unbalanced due to the continuous reduction in male numbers (Yendraliza et al. 2020) further limiting natural mating capacity. Artificial insemination is a practical solution to overcome bull scarcity and accelerate genetic conservation. However, the success of AI programs depends strongly on semen quality and cryosurvival performance. The development of frozen semen from Kuantan bulls therefore represents a critical initial step. Conventional semen quality parameters such as motility,

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viability, membrane integrity, acrosome integrity, and morphology remain key indicators of fertilizing potential (Wang et al. 2018). At the molecular level, sperm function is closely regulated by protein expression and stability, particularly proteins involved in energy metabolism, motility regulation, capacitation and membrane protection (Khan et al. 2015; Xue et al. 2025). Cryopreservation is known to induce structural and biochemical alterations in sperm cells, including protein loss or redistribution caused by cold shock and oxidative stress. Proteomic studies consistently report changes in proteins associated with mitochondrial activity, cytoskeleton dynamics, and membrane integrity following freezing and thawing (Sharafi et al. 2022). In Indonesia, proteomic evaluation of seminal plasma and sperm has been reported for several local cattle breeds, including 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). These studies demonstrate that breed-specific protein profiles can serve as indicators of semen freezability and fertility. To date, no comprehensive research has simultaneously evaluated conventional semen quality parameters and cryopreservation-induced changes in sperm protein profiles in Kuantan bulls. The lack of integrated functional and proteomic data limits the development of breed-specific frozen semen standards and hinders the identification of molecular markers associated with cryosurvival and fertility. Therefore, this study aimed to evaluate semen quality and characterize the protein composition of sperm in fresh and frozen semen from Kuantan cattle, providing a scientific basis for artificial insemination programs and genetic conservation strategies.

## MATERIALS AND METHODS

### Ethical approval

This research has received approval from the Ethics Commission of LPPM UIN Suska Riau with Number: 672/Un.04/L.1/TL.01/07/2025.

### Semen collection

Semen was collected weekly for 40 weeks from two Kuantan bulls using an artificial vagina maintained at 42–45°C. The ejaculates were transported to the BIBD laboratory for macroscopic and microscopic evaluation. Only samples with a minimum progressive motility of 70% were included in the study.

### Semen diluent, equilibration and freezing

Fresh semen was diluted with tris citrate egg yolk diluent (Table 1).

**Table 1:** Composition of Tris citrate Diluent

No	Ingredients	Amount used
1.	Tris (Hydroxymethyl) Aminomethane	3.028g
2.	Citric acid	1.7g
3.	Fructose	1.25g
4.	Penicillin	0.5mL
5.	Sterptomycin	0.4mL
7.	Glycerol	6mL
8.	Egg Yolk	20mL
9.	Aquabidest	80mL

The diluents, Tris (Hydroxymethyl) Aminomethane, citric acid, and fructose, were weighed using an analytical balance, then transferred to a 250mL Erlenmeyer flask and distilled water added to reach a volume of 80mL. The mixture was homogenized using a magnetic stirrer for 15 minutes and covered with aluminum foil. This solution was referred to as a buffer solution. A total of 74mL of the buffer solution was then transferred to a 250mL beaker, 20mL of egg yolk and 6mL of glycerol were added, covered again with aluminum foil, and homogenized using a magnetic stirrer for 60 minutes. Next, 0.5mL of penicillin and 0.4mL of streptomycin were added using a syringe through the aluminum foil cover. This final solution was referred to as Tris-egg yolk diluent.

The diluent for fresh semen is calculated using the following formula:

$$\text{Diluent Volume} = \frac{\text{Volume} \times \text{Concentration} \times \text{Motility}}{\text{IB Dose} (100 \times 10^6)} - \text{Semen Volume}$$

A 100mL tris egg yolk diluent solution is then warmed in a water bath at 37°C and taken as needed based on the calculation. After the dilution process, the semen is covered with aluminum foil and prepared for the filling and sealing stage. Semen straws are printed using a straw printing machine. They are then placed in a straw sterilization cabinet for 60 seconds. Afterward, the straws are arranged on straw racks and filled and sealed. The filled straws are then placed in a cool top machine at 5°C for 4 hours (Yendraliza et al. 2025). Before the freezing stage, the straws are placed in a box filled with liquid nitrogen for 15 minutes, then completely immersed in liquid nitrogen for 24 hours. The thawing process is carried out in warm water at 37°C for 30 seconds, after which the semen is evaluated.

### Research parameters

#### Motility

Motility was observed using a light microscope (Motic BA310®) by mixing 0.2mL of semen onto a glass slide covered with a cover slip to thin it and prevent evaporation. The sample was examined under a light microscope at 10x45 magnification. The percentage of spermatozoa motility was calculated by comparing the movement of sperm with the non-moving ones using the formula:

$$\text{Motility}(\%) = \frac{\text{Number of sperm moving forward}}{\text{Total of sperm counted}} \times 100\%$$

#### Abnormalities

The percentage of sperm abnormalities was measured by applying a drop of eosin dye to the tip of a clean slide and 0.2mL of semen using a tip and micropipette, then stirring until homogeneous. A thin smear was then prepared and dried. It was then observed under a light microscope (Motic BA310®) at 10x45 magnification. Sperm with morphological changes, such as curled tails, broken tails, and folded midsections, were counted in eight views and measured using the formula:

$$\text{Abnormality}(\%) = \frac{\text{Number of abnormal spermatozoa}}{\text{Total of spermatozoa counted}} \times 100\%$$

#### Intact Plasma Membrane (IPM)

The IPM percentage was measured by mixing 20µl of

semen with 200µL of Hypoosmotic Sweeling Test (HOST) solution, which was incubated in a water bath at 37°C for 45 minutes. The HOS-Test solution composition consisted of solution (A: 1.35g fructose + 50mL aquabidest, B: 0.735g sodium citrate + 50mL aquabidest) and solution B were mixed (SNI 2017).). After incubation, 10µL of semen was placed on a glass slide and covered with a cover glass, then observed under a light microscope (Motic BA310®) at 400x magnification. Sperm with damaged plasma membranes were characterized by straight tails, while spermatozoa with intact plasma membranes were characterized by coiled or bulging tails.

$$\text{Intact Plasma Membrane (IPM)(\%)} = \frac{\text{Number of sperm with intact plasma membrane}}{\text{Total of spermatozoa counted}} \times 100\%$$

### Intact Acrosome (IAC)

The IAC percentage is measured by mixing one drop of semen with 5 drops of formalin saline (physiological NaCl + 1% formalin) in an Eppendorf tube, leaving it for 10 seconds, and then dropping it onto a glass slide, then covering it with a cover glass. The examination is performed using a phase-contrast microscope at 400x magnification on at least 200 spermatozoa. An intact acrosome is indicated by a black head tip.

$$\text{Intact Acrosome (IAC)(\%)} = \frac{\text{Total sperm with an intact acrosome cap}}{\text{Total of spermatozoa counted}} \times 100\%$$

### Protein analysis

The sample preparation procedure for protein analysis was performed with modifications to the method developed by Jaswandi et al. (2024). Fresh semen obtained was centrifuged at a speed of 3000rpm for 45 minutes. After centrifugation, the plasma and sediment fractions were separated, then each was placed in a 2mL Eppendorf tube and stored at 4°C until used for quantitative and qualitative protein content analysis. A total of 500µL of fresh semen sediment was mixed with 1mL of PBS (Phosphate Buffer Saline) in a 1.5mL microtube. The mixture was then centrifuged at 13.000rpm for 15 minutes. The supernatant was discarded, while the spermatozoa cells were mixed with 400µL of PRO-PREPTM solution and homogenized using a vortex. In accordance with the manufacturer's procedure, spermatozoa protein extraction was performed using PRO-PREPTM solution (Protein Extraction Solution, 17081, iNtRON Biotechnology, Korea). Next, the mixture was incubated at -20°C for 15 minutes. After that, sonication was performed for 20 seconds three times, then centrifuged again at 13.000rpm (4°C) for 5 minutes. The supernatant obtained was transferred to a 1.5mL microtube for protein content measurement.

Sperm protein concentration was determined using the Bradford method (Bradford 1976). The analysis procedure was performed using Bradford reagent (BR05, Eco Tech). The reagent was diluted with aquabides at a ratio of 1:4, while the standard solution was prepared by dissolving 0.563mg of Bovine Serum Albumin (BSA, Sigma) in PBS. Next, three types of solutions were prepared: blank (3000µL working solution and 50µL PBS), standard (3000µL working solution and 50µL standard solution), and sample (3000µL working solution and 50µL sample). Each mixture was incubated at room temperature for 10

minutes. Optical density (OD) measurements were then performed at a wavelength of 595nm using a spectrophotometer (Shimadzu, UV-1800). Calculate the total concentration of dissolved protein using the formula:

$$\text{Total Protein Concentration} = \frac{\Delta A1}{\Delta A2} \times c \times f$$

ΔA1: Sampel OD – OD Blanko

ΔA2: Standar OD – OD Blanko

c: Concentration standard (0.563mg/mL)

f: Sample dilution factor prior to testing

### SDS-PAGE

Sperm protein analysis was performed using the SDS-PAGE method with a sample concentration of 20µg/mL. The sample amount was determined using a Quibic fluorometer (Quibic, Invitrogen). The sample was placed in a microtube, then 10µg of buffer was added and homogenized using a vortex for 1 minute. Next, the samples were heated in a water bath at 70°C for 10 minutes, then centrifuged for 1 minute. The protein samples were then injected onto a 10% polyacrylamide gel (Q-PAGETM TGN Precast Gel, QP4210, SMOBIO® Technology, Inc., Taiwan) for 90 minutes using Tris-glycine buffer solution (Tris-Glycine Running Buffer, TGS 10 Eco Tech) at a voltage of 110 volts and a current of 110mA.

Protein bands on the gel were stained with Coomassie Blue solution (Coomassie Blue Rapid Staining Solution, E-IR-RI129) for 24 hours while gently shaking. The remaining dye was then removed by soaking the gel in ddH<sub>2</sub>O in a closed container and washing it three times. The formed protein bands were observed using Gel Doc (iBright 1500, Invitrogen, Thermo Fisher Scientific). The protein bands from the samples were then compared with the molecular marker bands (ExcellBand 3-color Broad Range Protein Marker, 10–245kDa, SMOBIO® Technology, Inc., Taiwan) to determine the molecular weight of the proteins. The SDS-PAGE visualization data were used for qualitative analysis of protein molecular weight by comparing the sample band patterns with the marker bands.

### Data Analysis

Data on semen characteristics, chemical composition, and seminal plasma protein profiles of Kuantan cattle were analyzed descriptively. Results are presented as mean values and standard deviations to describe variation among samples, following the approach recommended by Steel et al. (1991).

## RESULTS

### Quality of fresh kuantan cattle semen

The percentage of Kuantan bull sperm concentration was 1.175±400million/mL (Table 2) with total motility of 84.37±6.30% and mass movement “+++”. The abnormality rate of 6.83±1.70%. Plasma membrane integrity reached 87.54±2.56%, while acrosome integrity was recorded at 80.65±5.67%.

**Table 2:** The average quality of fresh Kuantan Cattle semen

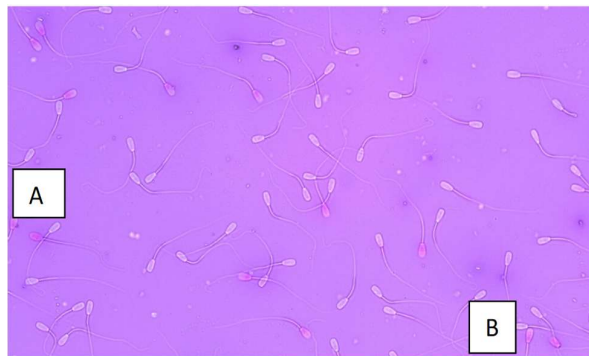
Characteristic	Average±SE
Volume (mL)	3.33±0.89
Colour	Milk white
pH	6.5±0.15
Consistency	Medium
Concentration (million/mL)	1.175±400
Mass movement	++
Motility (%)	84.37±6.30
Abnormality (%)	6.83±1.70
Viability	87.54±5.50
Percentage of intact plasma membrane (%)	87.54±2.56
Percentage of intact acrosome (%)	80.65±5.67

**Post-Thaw semen quality of kuantan cattle**

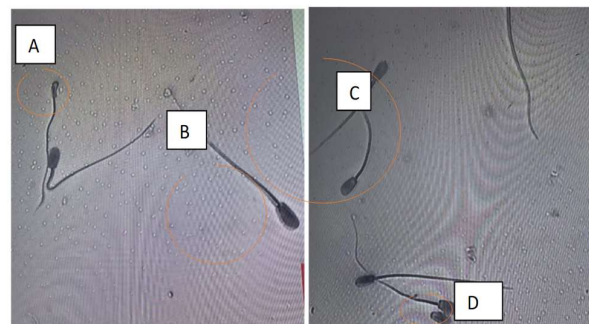
Post-thaw sperm quality parameters are presented in Table 3. Progressive motility reached 88.25±7.90%, while sperm viability was 85.50±6.70%. As illustrated in Fig. 1, the proportion of live spermatozoa exceeded that of dead cells. Sperm abnormalities increased from 6.83±1.70% in fresh semen to 9.72±6.70% after thawing, as shown in Fig. 2. Plasma membrane integrity, assessed by the hypo-osmotic swelling test, reached 78.32% (Fig. 3) and the percentage of intact acrosomes was 78.25%, as depicted in Fig. 4.

**Table 3:** Average of quality sperm post-thaw of Kuantan cattle

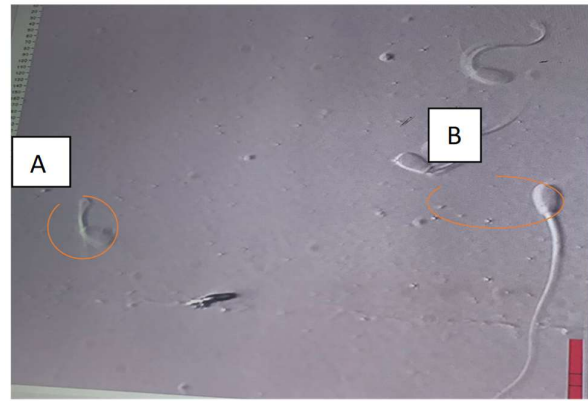
Characteristic	Mean+SE
Motility (%)	88.25±7.90
Abnormality (%)	9.72±6.7
Viability (%)	85.5±6.7
Percentage of intact plasma membrane (%)	78.32±4.7
Percentage of intact acrosome (%)	78.25±9.0



**Fig. 1:** Viability of Kuantan cattle sperm after freezing A. Live sperm, B. Dead sperm.



**Fig. 2:** Sperm Abnormalities A. Small head, B and C. Normal sperm, D. Broken tail.



**Fig. 3:** Intact Plasma Membrane of Sperm. A. Intact plasma membrane B. Damaged plasma membrane.



**Fig. 4:** Kuantan Cattle Sperm Intact Acrosome.

**Protein concentration and protein profile of kuantan cattle semen**

Total protein concentrations of Kuantan cattle semen fractions are shown in Table 4. The highest protein concentration was observed in seminal plasma (1.996 mg/mL), followed by plasma + diluent (1.77mg/mL), fresh semen (1.34mg/mL), and frozen semen (1.219mg/mL).

**Table 4:** Total Protein in Kuantan Bull Semen

Semen of Kuantan Cattle	Total Protein (mg/mL)
Fresh semen	1.34
Frozen semen	1.219
Semen plasma	1.996
Plasma and diluent	1.77

SDS-PAGE analysis revealed 12 protein bands in fresh semen, seminal plasma, and plasma + diluent fractions, whereas frozen semen showed only 9 protein bands. The distribution of protein bands based on molecular weight is presented in Table 5 and Fig. 5. The results of the analysis using the 1D-SDS-PAGE method show that fresh semen, semen plasma, and plasma + diluent have a total of 12 protein bands, indicating higher protein diversity compared to frozen semen, which only shows 9 bands. The loss of several protein bands in frozen semen, particularly in the 20–25kDa and 140–180kDa ranges.

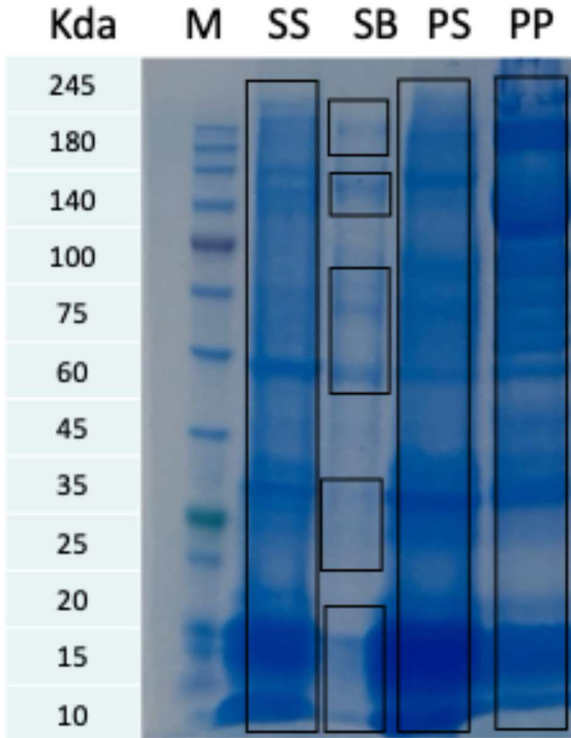
**DISCUSSION**

**Fresh semen quality of kuantan cattle**

Mass movement “++” in fresh Kuantan cattle semen indicating a dense and active spermatozoa population

**Table 5:** Distribution of Kuantan Cattle Semen Protein Bands Based on Molecular Weight

Semen of Kuantan cattle	Molecular Weight (kDa)												Number of Protein Bands
	10	15	20	25	35	45	60	75	100	140	180	245	
Fresh semen	+	+	+	+	+	+	+	+	+	+	+	+	12
Frozen semen	+	+	+	-	+	+	+	+	-	+	-	+	9
Semen plasma	+	+	+	+	+	+	+	+	+	+	+	+	12
Plasma and diluent	+	+	+	+	+	+	+	+	+	+	+	+	12



**Fig. 5:** 1D-SDS-Page of Kuantan Bull Semen. M: Marker, SS: Fresh Semen, SB: Frozen Semen, PS: Semen Plasma, PP: Plasma and Diluent.

(Azevedo et al. 2024). The macroscopic attributes of fresh Kuantan cattle semen were within the physiological limits commonly reported for tropical bulls, indicating favorable conditions for sperm motility and membrane stability during the initial handling phase (Baharun et al. 2023). Macroscopic assessment functions as a critical preliminary screening tool, as abnormalities at this level frequently indicate stress exposure, contamination, or procedural errors during collection that can compromise subsequent microscopic quality (Bollwein and Malama 2023). Elevated sperm concentration and motility, accompanied by a low proportion of morphological abnormalities, reflect efficient spermatogenesis and optimal testicular function. An abnormality rate below 10% remains acceptable for artificial insemination programs when appropriate dose adjustments are applied (Standar Nasional Indonesia (SNI) 2017). High levels of plasma membrane and acrosome integrity demonstrate that the majority of spermatozoa preserved structural components required for capacitation, acrosome reaction, and successful fertilization (Zoca et al. 2023). The high motility and viability observed in fresh semen are attributable to limited oxidative stress and minimal membrane damage. Membrane stability plays a central role in sustaining mitochondrial function, which

directly supports energy production and motility in spermatozoa (Giaretta et al. 2025).

**Effect of cryopreservation on sperm quality**

Post-thaw assessment revealed a decline in plasma membrane and acrosome integrity relative to fresh semen, a well-documented effect of cryopreservation. Freezing and thawing impose osmotic stress, trigger lipid phase transitions, and promote protein denaturation, which collectively disrupt sperm membrane architecture (Westfalewicz et al. 2021). Despite these alterations, post-thaw motility remained high and exceeded national quality standards for frozen semen. This finding indicates strong cryotolerance of Kuantan cattle spermatozoa and good compatibility with the Tris-egg yolk extender. The post-thaw motility observed was higher than that reported in several Indonesian local breeds, including Aceh cattle (52.58–55.27%) (Sophian et al. 2025) and Pesisir cattle (70.22%) (Ananda et al. 2025). Superior motility after thawing further supports the presence of breed-specific variation in sperm cryosurvival capacity (Tamargo et al. 2024).

High post-thaw viability reflects sustained metabolic activity and preserved membrane functionality, both of which are essential for sperm longevity and fertilization potential. Inter-breed variation in viability is influenced by intrinsic sperm resistance, extender composition, and freezing protocols (Pieper et al. 2023). The viability value recorded in Kuantan cattle exceeded those reported for Aceh cattle (Dwitya et al. 2019), Madura cattle (Rosyada et al. 2023) and Pesisir cattle (Ramadhan et al. 2024). Sperm viability is a key determinant of semen quality, as it directly represents the proportion of functionally competent spermatozoa (Kudratullah et al. 2024).

An increase in sperm abnormalities following cryopreservation is a predictable outcome of dilution, equilibration, freezing, and thawing procedures (Singh et al. 2016). Structural defects predominantly affect the tail and midpiece due to ice crystal formation and osmotic imbalance (Rivera-concha et al. 2024). However, the abnormality percentage observed in this study remained within acceptable limits for artificial insemination (Raheja et al. 2018). Abnormality rates in Kuantan cattle differed from those reported in other Indonesian local 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). Such variation is attributable to differences in genetic background, semen extenders, and cryopreservation protocols (Akhtar et al. 2022).

Plasma membrane integrity is fundamental for maintaining sperm motility, ion regulation, capacitation, and acrosome reaction (Umirbaeva et al. 2024). The relatively high proportion of intact membranes observed indicates effective protection against cryoinjury (Rimayanti et al. 2022). Nevertheless, the abundance of

polyunsaturated fatty acids in sperm membranes increases vulnerability to oxidative stress, which may account for partial membrane disruption after freezing (Giaretta et al. 2025). The intact membrane percentage in Kuantan cattle spermatozoa was higher than that reported in Aceh cattle by Sophian et al. (2025). Differences in membrane integrity likely contribute to observed variations in motility and viability. Sperm fertilizing capacity largely depends on plasma membrane integrity because this structure regulates critical physiological events, including capacitation, acrosome reaction, and interaction with the zona pellucida (Sharafi et al. 2022).

Acrosome integrity is closely associated with plasma membrane stability. Cholesterol depletion and oxidative damage during cryopreservation can destabilize the acrosomal structure and impair its function (Manjunath 2012). Kuantan cattle exhibited a higher proportion of intact acrosomes than that reported for Madura cattle (Azizah et al. 2023). This variation is likely linked to differences in plasma membrane integrity. Representative micrographs of intact acrosome in Kuantan cattle spermatozoa are presented in Fig. 4. Consistent with Younus et al. (2024), the sperm head contains an acrosome with a double-layered structure positioned between the plasma membrane and the anterior region of the nucleus, which is essential for oocyte penetration and fertilization.

#### Seminal plasma proteins and cryopreservation effects

The higher protein concentration observed in seminal plasma compared with whole semen confirms that the majority of bioactive, fertility-related proteins originate from accessory sex gland secretions (Abah et al. 2023). The findings of this study are evident from the protein band measuring approximately 10kDa that consistently appears in all fractions, which is suspected to be Cytochrome C, an important protein involved in regulating acrosome reactions and sperm capacitation, as reported in the *Bos indicus* × *Bos taurus* crossbreeding by (Castelló-Ruiz et al. 2025).

These proteins play essential roles in antioxidant defense, regulation of capacitation, and mediation of sperm interactions within the female reproductive tract (Tamargo et al. 2024). The disappearance of several protein bands in frozen semen, particularly within the 20–25kDa and 140–180kDa ranges, is most likely attributable to functional protein degradation induced by cold shock and increased exposure to reactive oxygen species during cryopreservation (Sikder et al. 2025). This pattern is commonly associated with damage to proteins involved in sperm motility, zona pellucida binding, and immunomodulatory functions (Willforss et al. 2021).

Compared with Kuantan cattle, Pesisir cattle exhibit a simpler protein profile, with only eight bands detected in fresh semen and six bands after thawing (Ananda et al. 2025). The reduction in total protein concentration and the loss of specific protein bands following freezing in Kuantan cattle further indicate protein degradation or leakage caused by membrane disruption and oxidative stress. The absence of bands in the 20–25kDa and 140–180kDa intervals suggests impairment of proteins associated with motility regulation and sperm–zona pellucida interaction (Willforss et al. 2021).

In contrast, the consistent detection of protein bands at

approximately 10kDa and 45kDa across all semen fractions indicates high structural and functional stability. The ~10kDa band is presumed to represent cytochrome C, a protein implicated in capacitation and regulation of acrosome-related processes (Nagata et al. 2019). The ~45kDa band corresponds to phosphoglycerate kinase 2 (PGK2a) critical glycolytic enzyme that supports ATP generation and sperm motility (Danshina et al. 2010). This 45kDa band is referred to as Phosphoglycerate Kinase 2 (PGK2) (Zahn et al. 1996). The persistence of these proteins across treatments highlights their potential as reliable fertility biomarkers in Kuantan cattle.

Additionally, the presence of a ~60kDa band identified as T-complex protein 1 (TCP1) suggests activation of cellular stress responses during spermatogenesis (Alyethodi et al. 2022). TCP1 functions as a molecular chaperone that facilitates protein folding and maintains cytoskeletal integrity and is commonly upregulated under stress conditions (Gómez-Guzmán et al. 2024). The predominance of protein bands exceeding 100kDa in most seminal plasma fractions further supports the presence of high-molecular-weight proteins, which are generally involved in immune regulation, cell adhesion, and gamete interactions within the female reproductive tract (Mughtaromah et al. 2012).

Overall, the proteome profile of Kuantan bull semen shows similarities with the proteomic patterns of local cattle that are adapted to the tropical climate. However, the loss of several critical bands in frozen semen indicates the need to optimize cryoprotectant formulations and protection strategies for seminal plasma. The dominance of functional proteins related to fertility (such as Cytochrome C and PGK2) and indications of spermatogenesis stress (TCP1) open up opportunities for further targeted proteomics-based research to determine fertility biomarkers and frozen semen formulations based on Kuantan cattle plasma in line with a modern proteomics-based genetic conservation approach.

#### Conclusion

Kuantan cattle exhibited excellent semen quality in both fresh and post-thaw conditions, with motility, viability, plasma membrane integrity, and acrosome integrity meeting or exceeding the minimum requirements for artificial insemination under SNI standards. Cryopreservation resulted in a measurable reduction in total protein content and protein band diversity, indicating structural sensitivity to freezing stress, although overall functional semen quality remained acceptable. The persistence of key fertility-associated protein bands across semen fractions supports the strong metabolic competence of Kuantan cattle sperm. These findings confirm the suitability of Kuantan cattle semen for frozen semen production and reinforce its potential role in artificial insemination programs and genetic conservation efforts.

#### DECLARATIONS

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**Conflict of Interest:** The author declares no conflict of interest

**Data Availability:** All data generated during this study are reported in the article.

**Ethics Statement:** All research procedures were carried out with minimal stress on the animals to ensure optimal animal welfare.

**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. All authors participated in reviewing and editing the manuscript and approved the final version for submission.

**Generative AI Statement:** The authors declare that no Gen AI/DeepSeek was used in the writing/creation of this manuscript.

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