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Simple workflow for genome and methylation analyses of ejaculated bovine spermatozoa with low sperm input

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ABSTRACT

We developed a simplified workflow of gDNA extraction from ejaculated bovine sperm using a low total number of sperm and a short time frame that yields high-quality DNA suitable for downstream methylation and genome analyses. These techniques have broad implications in human biomedical sciences and agriculture, including clinical diagnoses of infertility, the identification of single-nucleotide polymorphisms and aberrant methylation patterns that can impact fertility, lower embryo development and contribute to heritable disease. The methods described here provide a reliable, simplistic approach for analyzing both the genomic and epigenomic status of whole sperm ejaculates that can be adapted for laboratory diagnostics, clinical reproductive practice and basic research.

METHOD SUMMARY

We developed a cost-effective, user-friendly and reliable protocol (see Supplementary data) for DNA extraction from low-input samples of frozen-thawed bovine sperm, with a simple workflow for PCR amplification, bisulfite conversion and methylation analyses of individual amplicons.

KEYWORDS

bisulfite • DNA • epigenome • fertility • forensic • methylation • oligospermia • sequencing • sperm

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Genomic and epigenomic analyses of sperm DNA are increasingly necessary techniques, with broad applications that include fertility diagnostics, forensic analyses and basic research [1,2]. Additionally, male infertility is a well-recognized concern that contributes to failed pregnancies in humans and agricultural animals [3]. Alterations to the genome or DNA methylation status of sperm can impact sperm function and embryo development [4]. Recent evidence suggests that aberrant methylation of spermatozoa inhibits proper sperm function and results in lower embryo survival [5]. Importantly, both sperm number and processing time are limiting factors that can influence downstream analyses. Simplified methods for gDNA extraction of sperm followed by successful bisulfite conversion, PCR amplification and downstream sequencing are needed to improve the workflow in clinical settings and provide more rigorous analyses with minimal processing times. However, extraction of DNA from sperm presents unique challenges that differ from somatic cells, including an acrosomal barrier and protamine compaction of chromatin that often results in low DNA yield [6–8]. Some protocols have been developed to address these challenges; however, most involve prolonged incubation times, proprietary reagents and high numbers of total sperm input that may be incompatible with available material [9]. Our goal was to simplify the extraction process and to shorten the time required for DNA extraction of mammalian sperm, while maintaining sufficient yield for downstream genome and methylation analyses. We developed a commercially viable column-based protocol to meet these objectives that require minimal sperm input conducive to *in vitro* fertilization techniques and a short, single-day extraction process that is compatible for downstream genomic and epigenomic analyses (Figure 1).

Unless otherwise specified, reagents were purchased from Sigma-Aldrich (MO, USA). Primers for PCR amplification are shown in Table 1. Isolated DNA was subjected to bisulfite conversion followed by purification using the EpiTect Bisulfite Kit (cat. no. 59104) and the QIAquick PCR Purification Kit (cat. no. 28104; both Qiagen, Hilden, Germany).

Frozen sperm from two different bulls (CentralStar Cooperative, Inc., MI, USA) were pooled for experiment with analyses conducted by the addition of technical replicates. Sperm were thawed in a water bath at 37°C for 30 s and then purified using a 45:90 Percoll gradient for experiment [10]. Purified sperm were pooled from two bulls, extended to 4×10^6–4×10^2 total motile sperm by serial dilution in 400 µl volumes and placed in a Tyrode albumin lactate pyruvate medium [11]. Sperm aliquots were added to 3.6 ml of sperm wash (SW) reagent buffer (150 mM NaCl and 10 mM EDTA [pH 8.0]) in 10-ml Eppendorf tubes. Samples were vortexed for 10 s at full speed and then centrifuged at 2500×*g* for 5 min. The supernatant was removed to approximately 1.0 ml, and the remaining sperm sample was transferred to a 2.0-ml microcentrifuge and centrifuged at 17,000×*g* for 2 min to obtain a sperm pellet. All supernatants were removed, and the sperm pellet resuspended in 220 µl of sperm lysis (SL) buffer (100 mM Tris-Cl, 10 mM EDTA, 500 mM NaCl, 1% SDS). After adding 30 µl of DDT (100 mM final) and 50 µl of proteinase K (40 mg/ml), samples were incubated at 55°C for 2 h. After incubation, 500 µl of buffer E (5 M guanidium thiocyanate, 50 mM Tris-HCl [pH 8.0] and 25 mM NaCl) was added with an additional 10-min incubation period at 55°C for 10 min. Ethanol (400 µl) was added to the sperm lysate and passed through QIAamp Mini columns (Qiagen) by centrifugation at 9000 rpm for 1 min. Flow-through was discarded. A total of 500 µl of reagent WB1 (3 M guanidium thiocyanate, 100 mM Tris-HCl [pH 8.0], 20 mM EDTA and 50% EtOH) buffer was added and centrifuged again. Buffer WB2 (10 mM Tris-HCl [pH 7.4], 1 mM EDTA, 100 mM NaCl and 80% EtOH) was then added in 500 µl volumes

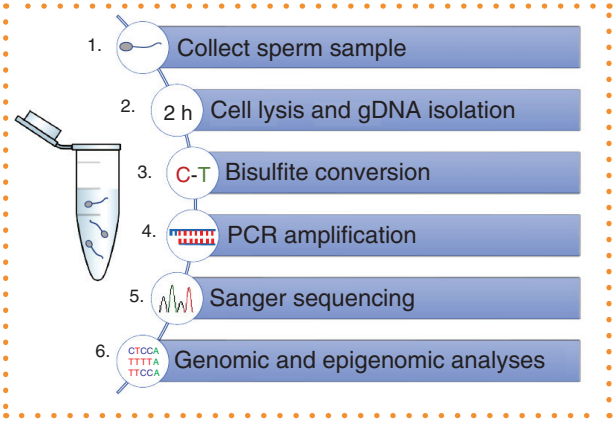

Figure 1. Workflow for gDNA extraction of mammalian sperm for genomic and epigenomic analyses of whole sperm ejaculates.

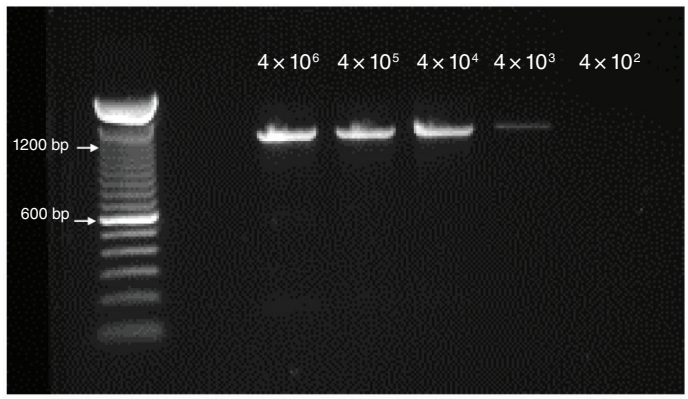

Figure 2. Sensitivity assay of gDNA extraction from whole sperm. PCR product amplification of the *POU5F1* locus of pooled bull sperm following serial dilution of 4 × 10^6–4 × 10^2 total motile sperm (n = pooled bull sperm from two bulls).

and centrifuged for an additional 1 min at 9000 rpm. Buffer WB2 (750 µl) was added to the column and centrifuged at 14,000 rpm for 3 min. Columns were transferred to a new collection tube for an additional 1 min of centrifugation at maximum speed. For DNA elution, columns were transferred to clean 2.0-ml collection tubes and eluted twice in 23 µl of RNAse-free H_2O and quantified by NanoDrop (Table 2).

The assay sensitivity of gDNA isolation from whole bull sperm was tested by amplification of a 1242-base pair (bp) region of exon 2 from the *POU5F1* locus using a single reaction in 40-µl volumes consisting of 20 µl of GoTaq Hot Start Green Master Mix (2×; cat. no. M5123; Promega, WI, USA) with the addition of 0.8 µl (4 pM) of forward and reverse primer each and 18.4 µl of template (up to 50 ng) and water. For samples with DNA isolation below limitations of NanoDrop detection, 18.4 µl of presumed DNA elution was used. PCR conditions were as follows: 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 30 s and a final extension of 72°C for 7 min. After amplification, 5 µl of PCR product was loaded onto a 0.9% agarose gel containing EtBr (0.5 µg/ml) and 0.5× TBE running buffer (40 mM Tris-Cl, 45 mM boric acid and 1 mM EDTA) and run at 95 V for 1 h (Figure 2).

For bisulfite conversion of isolated gDNA, 50 ng (up to 20 µl) of extracted DNA was placed with 85 µl of bisulfite reaction mixture and 35 µl of DNA protect buffer in a 200-µl PCR tube. Tubes were mixed by inversion, briefly centrifuged and placed in a thermocycler to carry out cytosine conversion using the following manufacture-specified reaction: 95°C for 5 min, 60°C for 25 min, 95°C for 5 min, 60°C for 85 min, 95°C for 5 min and 60°C for 175 min. Cleanup and isolation of bisulfite-converted DNA was continued according to kit-specified guidelines, with final elutions performed in 23 µl of RNAse-free H_2O. Quantification of converted DNA was measured by NanoDrop, using RNA (Factor 40) settings that resemble converted DNA. Converted DNA was held at 10°C overnight or stored at -20°C, until quantification and further processing (Table 2).

Bisulfite-converted DNA was used for PCR amplification and Sanger sequencing to analyze the methylation pattern of the bovine *NRF1* locus (Figure 3A). *NRF1* primers

Table 1. Primer sequences used for PCR amplification of bull sperm.

Primer		Sense		Length (nt)
***NRF1* Primer sequences (NCBI: AC_000180.1)**				
Bisulfite:	Exon 1	F	GAGAAGTAAAGGTTATTTTAAAGG	
		R	TAAAACACTCACCTCAAAAC	558
Bisulfite seq:	Exon 1	F	AGGGAAATGTGAATGTAGGGAGA	
***POU5F1* Primer sequences (NCBI: AC_000180.1)**				
	Exon 2	F	CGTGTGTTTGTGAATGTGCG	
		R	GGAAAGAAATGGGCAGGCAA	1242

seq: Sequence.

Table 2. Quality and quantity of sperm DNA after isolation of genomic, bisulfite-converted and PCR-amplified DNA from pooled bull sperm (n = 6 samples).

	gDNA		Bisulfite-converted DNA	
Sample	Input (number of motile sperm)	Output (ng)	Output (ng)	PCR output (ng)
1	4 × 10^6	1616	762	789
2	4 × 10^6	1698	1540	1090
3	4 × 10^6	1316	1018	1010
4	4 × 10^6	832	480	1068
5	4 × 10^6	1962	1418	537
6	4 × 10^6	866	620	876

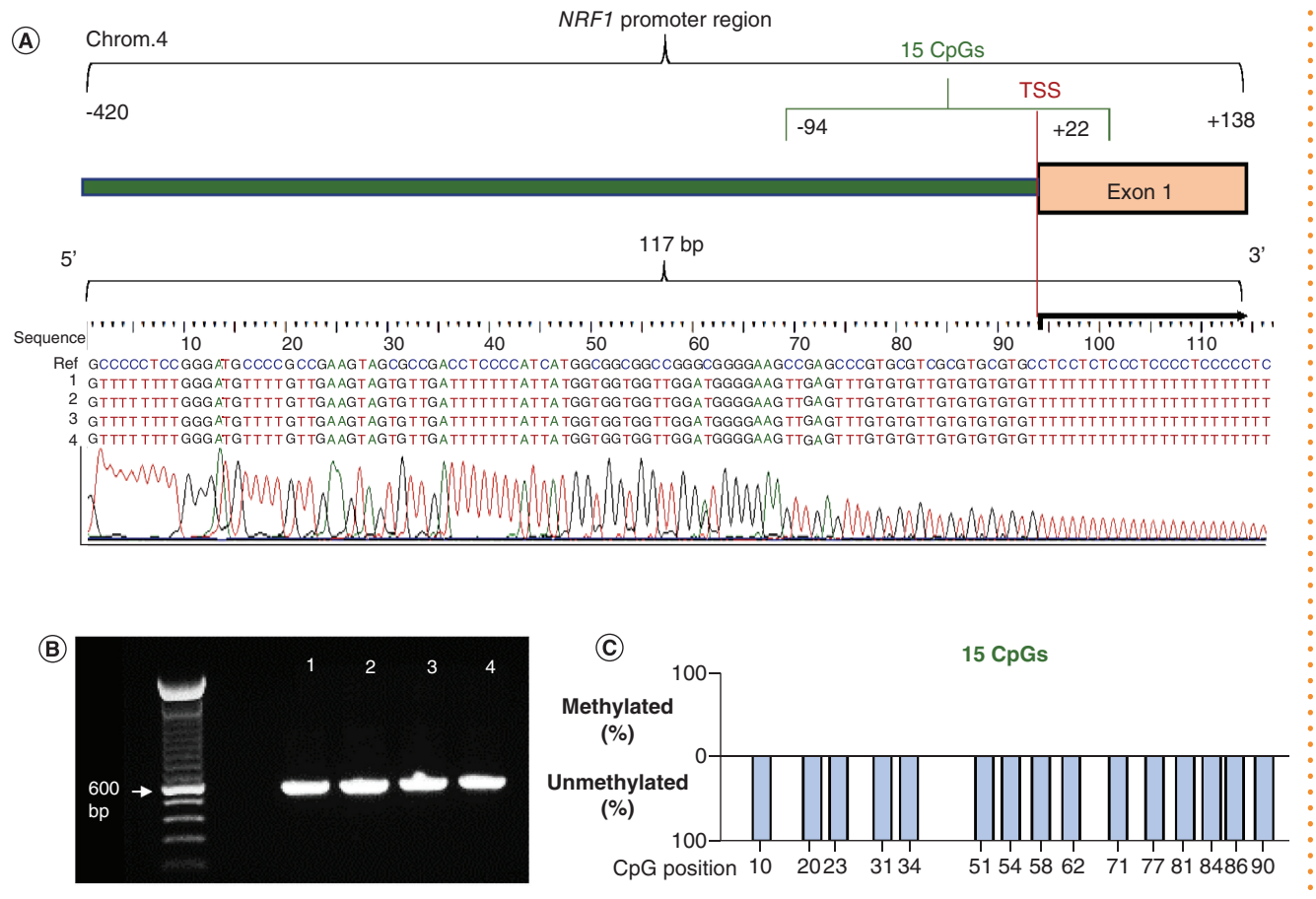

Figure 3. PCR amplification, Sanger sequencing and conversion efficiency of bisulfite-converted sperm DNA. (A) Promoter and TSS of the bovine NRF1 promoter depicting a 117-bp amplicon of four independent samples of Sanger-sequenced bisulfite-converted bull sperm aligned to a reference sequence with representative electropherogram. (B) PCR products from the *NRF1* promoter region of bisulfite-treated bull sperm visualized by gel electrophoresis after two rounds of amplification from four independent samples of pooled bull sperm. (C) Methylation status and conversion efficiency of 15 CpG islands from a 117-bp amplicon of the *NRF1* gene. (n = pooled sperm from two bulls for each independent replicate).
bp: Base pair; TSS: Transcriptional start site.

were designed using Meth Primer software (Table 1). Conditions for the first round of PCR were performed in 20-µl reactions consisting of 10 µl of GoTaq Hot Start Green Master Mix (2×; cat. no. M5123; Promega) with the addition of 0.4 µl (4 pM) of forward and reverse primer, 50 ng of template and H_2O up to 9.2 µl. PCR conditions were as follows: 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 59°C for 30 s, 72°C for 45 s and a final extension of 72°C for 7 min. A second PCR reaction was performed following the same conditions with 5 µl of PCR product from the first reaction and water. Success of amplification was determined by gel electrophoresis as previously indicated. Single-band amplicons were identified (Figure 3B), and the remaining PCR product was purified using the QIAquick PCR Purification kit, following manufacturer specifications. For final elution, 15 µl of nuclease-free H_2O was added to the center of the column, centrifuged and repeated. DNA was quantified by NanoDrop. A minimum of 40 ng of DNA from samples with a 260-/280-nm purity absorbance ratio of 1.8–1.9 was submitted for Sanger sequencing. Sequencing data were analyzed using BioEdit software (Ibis Therapeutics, CA, USA). Bisulfite conversion efficiency and CpG methylation status were determined by QUMA software (Figure 3A–C) [12].

The methods described here for evaluation of genomic and epigenomic profiles of mammalian sperm require low sperm input, are cost effective and eliminate overnight DNA extraction. This workflow is beneficial for use in clinical settings in which a limited sperm number from males may require increased assay sensitivity and processing time. Isolation of DNA from samples with 4×10^3 motile sperm using highly optimized buffer conditions was effective and efficient for downstream application (Figures 2 & 3). Recovery rates of genomic DNA and bisulfite-converted DNA were highly consistent (Table 2) and resulted in successful PCR amplification (Figure 3). Bisulfite treatment of isolated DNA resulted in high conversion efficiency (>98%; Figure 3). Finally, Sanger sequencing is also compatible with these methods for reliable genomic and epigenomic evaluation of selected amplicons (Figure 3). These techniques describe improved methodology for isolation and methylation analyses of ejaculated mammalian sperm DNA that can be used independently or as a continuous workflow adapted to suit experimental, clinical or laboratory needs. ▶

AUTHOR CONTRIBUTIONS

BW Daigneault designed, performed experiments and prepared the manuscript. SK Rajput optimized reagents and provided technical guidance and support. GW Smith provided support and supervised manuscript preparation.

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