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Rapid method for the isolation of mammalian sperm DNA

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Abstract

The unique DNA packaging of spermatozoa renders them resistant to DNA isolation techniques used for somatic cells, requiring alternative methods that are slow and labor intensive. Here we present a rapid method for isolating high-quality sperm DNA. Isolated human sperm cells were homogenized with 0.2 mm steel beads for 5 min at room temperature in the presence of guanidine thiocyanate lysis buffer supplemented with 50 mM tris(2-carboxyethyl)phosphine (TCEP). Our method yielded >90% high-quality DNA using 3 different commercially available silica-based spin columns. DNA yields did not differ between immediate isolation (2.84 ± 0.04 pg/cell) and isolation after 2 weeks of homogenate storage at room temperature (2.91 ± 0.13 pg/cell). DNA methylation analyses revealed similar methylation levels at both time points for three imprinted loci. Our protocol has many advantages: it is conducted at room temperature; lengthy proteinase K (ProK) digestions are eliminated; the reducing agent, TCEP, is odorless and stable at room temperature; nucleic acids are stabilized, allowing storage of homogenate; and it is adaptable for other mammalian species. Taken together, the benefits of our improved method have important implications for settings where sample processing constraints exist.

Keywords

epigenetics; sperm; methods; nucleic acids; DNA purification; spermatozoa; DNA isolation; genetics

There is a growing interest in elucidating the role of sperm genetics and epigenetics on reproductive success and the life-course trajectory of health outcomes of subsequent generations. Recent genetic studies have shown a positive association between germline de novo mutations and paternal age (1–4). Aberrations in sperm DNA methylation of imprinted genes (5–7) and epigenome-wide dysregulation (8–10) have also been reported among men with infertility issues such as low sperm count and sperm quality. Moreover, compelling

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Author Contributions

H.W., M.D.G ., G.L ., and J.R.P. contributed to the development and execution of the experiments as well as the writing and editing of the manuscript. H.W. and J.R.P. contributed to the analysis of the experiments.

Competing interests

The authors declare no competing interests.

animal data indicate that the epigenome of sperm harbors a legacy of environmental exposures that can influence offspring phenotype (11–13).

Spermatogenesis requires extensive epigenetic reprogramming during the progression from diploid spermatogonia to haploid spermatozoa and involves stage- and testis-specific gene expression and mitotic and meiotic divisions (14,15). Extensive reorganization of chromatin structure occurs where 90% and 99% of histones are replaced by protamines in humans and mice, respectively (16,17). During this protamine-histone transition, tight compaction of the sperm nucleus is achieved by the oxidation of cysteinerich residues of protamines and the subsequent formation of disulfide bridges that link protamines together (18). This nuclear compaction is necessary for sperm motility and protection of the genome from oxidation within the female reproductive tract (19). Furthermore, the protamine-bound packaging of DNA precludes transcriptional activity and has been considered a nontraditional form of epigenetic regulation unique to sperm cells (20).

Their unique DNA packaging renders spermatozoa resistant to DNA isolation techniques used for somatic cells (21,22). The development of efficient methods for isolating DNA from mammalian sperm has been a gradual process. All existing protocols use a combination of three components to gain access to sperm DNA: *(i)* detergents and/or chaotropic salts to facilitate cell lysis; *(ii)* proteinase K (ProK) to digest nuclear proteins; and *(iii)* reducing agents to break disulfide bonds between protamines. One such widely adopted method for the isolation of mammalian sperm DNA uses an ionic detergent, sodium dodecyl sulfate (SDS), ProK, and either dithiothreitol (DTT) or 2-mercaptoethanol (βME). After overnight incubation at 55°C, DNA is isolated by ethanol precipitation (9) or silica-based spin columns (11). Other popular approaches utilize guanidine salts, such as guanidinium thiocyanate (GTC), as the cell lysis reagent. GTC is a chaotropic agent that disrupts cell membrane and organelles by solubilizing individual molecules or cellular structures, including separating nucleic acids from associated proteins (23). In addition, it is able to denature proteins, inactivate nucleases, and enhance ProK activity (23,24). Bahnak et al. first reported a protocol incorporating GTC in a lysis buffer along with the ionic detergent, sarkosyl, and bME (21). However, this protocol required overnight incubations and time consuming CsCl ultra-centrifugation for DNA isolation. More recently, this protocol has been modified to include ProK in the lysis buffer, which significantly reduced incubation time to 2 h and replaced lengthy CsCl ultra-centrifugation with isopropanol precipitation of DNA, resulting in an 80% yield of sperm DNA (24,25).

While the previous methods for sperm DNA isolation have progressed over time, they still have drawbacks. First the limited stability of DTT, βME, and ProK in aqueous solutions at room temperature requires fresh preparation of lysis buffers and involves long incubations ranging from 2 h to overnight at 56°C (9,24–26). In addition, DT T and βME possess odors that may not be tolerated, especially in clinical settings. Finally, most protocols recover DNA from sperm lysate through ethanol precipitation, which increases processing time and may result in co-precipitation of proteins and/or ethanol carryover that may affect downstream applications.

Given the need for a simple and rapid protocol for sperm DNA isolation, we developed a novel approach for isolating high-quality sperm DNA. Our protocol incor porate s a 5 min me chanic al homogenization step in the presence of a guanidine-based lysis buffer and a thiol-free reducing agent, TCEP, to facilitate sperm cell lysis and dissociation of disulfide bonds without the use of ProK. Sperm lysate is then applied to silica-based columns for the isolation of high-quality DNA with > 90% yield. To further streamline our protocol, we use commercially-available reagents that are stable at room temperature. This method is likely to expedite genetic and epigenetic research of sperm in clinical settings as well as in other mammalian species.

Methods

Isolation of sperm cells

This study was approved by the Institutional Review Board at the University of Massachusetts Amherst (#2014–2337). All participants gave written informed consent and were required to have at least 48 h of abstinence prior to each donation. Five healthy male participants each donated multiple whole ejaculate samples throughout the course of the study. To remove somatic cell contamination, sperm cells were isolated using a continuous one-step 90% gradient (ART-2100 and ART-1006; Sage, Beverly, MA) per the manufacturer's protocol. Sperm pellets were washed once, re-suspended, counted on a hemocytometer using the average of eight grid areas, and visually inspected for somatic cell contamination. Isolated sperm from individuals ranged from 20 to 109 million cells.

Cell lysis

Reducing agents TCEP (final concentration: 10–50 mM) (#77720; Pierce, Rockford, IL), DTT (final concentration: 150 mM) (#V3151; Promega, Madison, WI), or βME (final concentration: 2%) were added to Buf fer RLT (#79216; Qiagen, Limburg, The Netherlands) to a final volume of 500 μL. Three different homogenization techniques were also evaluated: (*i*) sperm were pulse-vor texed in lysis buf fer for 5 min, diluted 1:1 in nuclease free water, and then incubated with ProK (final concentration: 200 μg/mL) at 56°C for 2 h; (*ii*) sperm were pulse-vortexed in lysis buffer for 5 min, and lysates were loaded onto Qiashredder columns (#79656; Qiagen) and centrifuged for 2 min at maximum speed (≥17,000 × *g*); (*iii*) sperm cells were homogenized in the presence of lysis buffer and 0.1 g of 0.2 mm stainless steel beads (#SSB02; Next Advance, Averill Park, NY) for 5 min on a Disruptor Genie (#SI-238; Scientific Industries, Bohemia, NY). To ensure equal aliquots of sperm, resuspended sperm cells after gradient isolation were vortexed for 10 s between each aliquot as previously described (27).

DNA isolation

Sperm DNA was extracted with three dif ferent commercially-available kits using modified protocols:

AllPrep DNA/RNA Mini Kit (#80204; Qiagen)—Lysates were added to spin columns and centrifuged at 10,000 × *g* for 30 s to bind DNA. Subsequent washing steps followed the manufacturer's protocol. To elute, 50 μL of Buffer EB (preheated to 70°C) was added to the

columns, which were incubated at room temperature for 3 min and then centrifuged for 1 min at ma ximum speed. This process was repeated twice for a total elution volume of 150 μL.

QIAamp DNA Mini Kit (#51304; Qiagen)—Lysates were combined with equal volumes of Buffer AL and 100% ethanol, loaded onto the spin columns, and the columns were centrifuged at 6000 × *g* for 1 min to bind DNA. Wash and elution steps followed the manufacturer's protocol, including 3 separate 200 μL elutions to maximize yield.

Quick-gDNA MiniPrep (#D3025; Zymo, Ir vine, CA)—DNA /RNA Shield (#R110 0; Zy mo) and Quick gDNA Genomic Lysis Buffer (included in kit), were used for sperm lysis instead of Buffer RLT. Samples in the Genomic Lysis Buf fer were loaded onto the columns, while samples in DNA /RNA Shield were combined with 3 volumes of Genomic Lysis Buffer before being loaded onto spin columns. Samples were centrifuged at 10,000 × *g* for 1 min to bind. Wash and elution steps followed the manufacturer's protocol for a final elution volume of 100 μL.

DNA yields and quality were determined using the Nanodrop 2000 Spectrophotometer (#E112352; Thermo Scientific, Somerset, NJ). A total of 350 ng of genomic DNA (gDNA) was resolved on a 0.7% agarose gel at 100 V for 45 min, stained with 0.5 μg/mL ethidium bromide solution, and visualized on a BioDoc-It Imaging System (#97-0172-01; UVP, Upland, CA). Given that haploid cells are expected to contain 3 pg DNA per cell, DNA yields were calculated as the observed yield/expected yield based on cell count. The full protocol for DNA isolation is provided in the Supplementary Material.

RNA isolation

Sperm cell lysate may be partitioned for the isolation of sperm RNA by adding 1:1 ratio of Qiazol and following the protocol of Goodrich et al. (28) starting at step 18 under section 3.1.

DNA methylation analysis

DNA methylation analyses of imprinted genes were performed on Sequenom's (San Diego, CA) MassARRAY platform, which uses RNA base-specific cleavage (MassCLE AVE) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) for quantitative DNA methylation analyses of PCR-amplified bisulfite-converted DNA (29). Briefly, 7.5 ng of bisulfite converted DNA (#D5002, EZ DNA Methylation Kit; Zymo Research) was amplified with reverse primers containing a T7-promoter tag. Primers and PCR conditions are provided in Supplementary Table S1. After treatment with shrimp alkaline phosphatase to remove unincorporated dNTPs, amplification products were subjected to in vitro transcription and T-specific cleavage and were then analyzed by MALDI-TOF MS. The C/T changes introduced by bisulfite treatment are reflected as G/A changes on the T7-directed RNA transcript and result in a mass difference of 16 Da for each CpG dinucleotide. The level of methylation for each CpG unit was quantified using EpiTYPER (Sequenom, San Diego, CA) software and methylation across loci was calculated as the average methylation of individual CpG units.

Results and discussion

To improve processing time and work flow, we exploited several areas in current protocols where considerable improvements could be achieved. Because recently published methods for sperm DNA isolation relied on user-prepared GTC-based lysis buffer and ethanol precipitation, we reasoned that commercially-available GTC lysis buffers could offer an effective alternative to user-prepared lysis buffers as well as provide optimal DNA binding conditions for silica-based spin columns, thereby avoiding ethanol precipitation. Similar to the reported yield of 80% in a recent GTC-based method with ethanol precipitation (24), treatment of sperm cells with diluted Buffer RLT, 150 mM DTT, and 200 μg/mL ProK for two hours followed by DNA isolation via AllPrep DNA columns resulted in a 79% yield (2.37 ± 10 pg/cell) (Figure 1A). As an alternative, we examined the utility of TCEP, an odorless, room-temperature stable, thiol-free reducing agent primarily used for protein biochemistry. We found that 50 mM TCEP resulted in no appreciable difference in DNA yield (2.32 ± 0.09 pg/cell) compared with 150 mM DTT (Figure 1A), indicating that TCEP is a viable alternative to thiol-based reducing agents for the isolation of sperm DNA.

With Buffer RLT's high concentration of GTC (2.8–4.0 M), we also reasoned that lengthy ProK digests could be circumvented if sperm cells are efficiently lysed. Therefore, we examined the utility of different mechanical homogenization techniques. QIAshredder, a column-based mechanical homogenization system offered by Qiagen for the rapid homogenization of cells and tissues, resulted in better yield (1.51 ± 0.23 pg/ cell) compared with no homogenization (0.42 ± 0.01 pg/cell) (Figure 1B), but was less effective compared with the 80% yields achieved by ProK digestions.

We next incorporated the novel homogenization method used for sperm RNA isolation developed by Krawetz and colleagues that utilizes 0.2 mm stainless steel beads (28). In the presence of Buffer RLT and 50 mM TCEP, homogenization with 0.2 mm stainless steel beads resulted in >90% yields (2.78 ± 0.23 pg/cell) (Figure 1B). Given the interest in obtaining sperm DNA from abnormal sperm samples (e.g., those with low sperm counts), we determined the efficiency of our method was >80% (i.e., 2.40 pg/cell) with 5×10^5 or more cells. Moreover, we tested our protocol down to 1×10^4 cells and were able to isolated sufficient DNA for bisulfite PCR of imprinted genes (data not shown).

Next, we evaluated the compatibility of the bead-based homogenization procedure with other reducing agents as well as other commerciallyavailable DNA column kits. Compared with 50 mM TCEP, we found no appreciable differences in DNA yields for 150 mM DTT and 2% βME using the same bead-based homogenization (data not shown). Furthermore, ProK digestion after homogenization did not increase DNA yield (data not shown), indicating that lengthy ProK digestions are unnecessary. Therefore, this homogenization procedure circumvented the need for time-consuming ProK incubations. Compared with the AllPrep DNA columns, QIAamp DNA and Zymo's Quick-gDNA columns resulted in slightly higher overall yields (2.70 ± 0.09 pg/cell versus 2.95 ± 0.13 pg/cell and 3.01 ± 0.28 pg/cell, respectively) (Figure 1C). Use of Zymo's Quick-gDNA kit by replacing Buffer RLT with their genomic lysis buffer (contains 4M GTC) resulted in lower A_{260}/A_{280} and A_{260}/A_{230} ratios compared with the other kits (data not shown).

We then aimed to test the stability and integrity of DNA in Buffer RLT and 50 mM TCEP after bead homogenization. Compared with immediate processing (T0), two weeks of storage (T2) at room temperature showed no decrease in temperature showed no decrease in DNA yield (2.84 ± 0.05 pg/cell versus 2.91 ± 0.13 pg/cell, respectively) (Figure 1D). Sperm genomic DNA resolved on a 0.7% agarose gel revealed high molecular weight DNA that displayed no differences in integrity between samples processed immediately (T0) or after two weeks of storage (T2) at room temperature (Figure 2). Finally, to verify the feasibility of our protocol with downstream applications, we conducted DNA methylation analyses of three imprinted loci *(SNURF, PEG10, H19)*. As expected for sperm DNA (30), differentially methylated regions (DMRs) of maternally expressed *H19* displayed high levels of methylation, while DMRs of paternally expressed genes *SNURF* and *PEG10* showed very low levels of methylation (Figure 3). Furthermore, we found no differences between samples that were processed immediately (T0) and after two weeks of storage (T2) at room temperature for DNA methylation levels of *H19* (T0: 94.1% ± 0.1% and T2: 92.2% ± 1.9%), *SNURF* (T0: 3.5% ± 0.7% and T2: 2.6% ± 1%), and *PEG10* (T0: 3.7% ± 1.8% and T2: 6.9% ± 3.2%) (Figure 3).

The use of the commercially-available GTC lysis buffers streamlined our protocol by eliminating the preparation of lysis buffers and allowing for optimal binding conditions for standard silica column–based DNA kits. We recommend Qiagen's RLT lysis buffer and columns because *(i)* DNA yields and A_{260}/A_{280} ratios were consistently greater than 90% and 1.80, respectively, and *(ii)* sperm cell lysates can be partitioned for sperm RNA purification using a previously published protocol (28). In addition to the DNA columns from the AllPrep DNA/RNA kit, we found that the QIAamp DNA Mini kit and Zymo's Quick-gDNA MiniPrep kit can also be adapted to use the RLT-based lysate with similar total yields. However, there are drawbacks to both the QIAamp and Zymo kits. The QIAamp DNA Mini Kit achieves similar total yield compared with AllPrep DNA/RNA kit, but it requires a higher elution volume for maximum yield (600 μL), thereby lowering final concentrations. This may not be desirable when there is limited starting material. While Zymo's Quick gDNA kit had the highest yield, it must be noted that the A_{260}/A_{280} and A_{260}/A_{230} ratios were consistently low, ≤1.8 and ≤1.0, respectively, suggesting low purity of DNA due to protein and/or GTC carryover.

Clinical research can present its own set of unique challenges. In studies where semen samples are processed at an in vitro fertilization laboratory, organic thiols may not be permissible due to their strong sulfur odor. TCEP irreversibly reduces disulfides and is reported to be more effective at reducing disulfides and is reported to be more effective at reducing disulfides than DTT below pH 8.0 (31), which represents the typical pH of GTC lysis buffers (e.g., Qiagen RLT is pH 7.0). Moreover, while DTT and βME readily undergo atmospheric oxidation, TCEP is resistant to oxidation, allowing for room temperature storage in aqueous solutions. Our results show no appreciable difference in DNA yield between TCEP and thiol reductants and support previous data that TCEP is equally as effective as DTT at lysing sperm cells and may provide an effective substitute for organic thiols (27). Taken together, TCEP offers an odor-free and room-temperature stable

alternative for the reduction of disulfide bonds, thereby making it our preferred reducing agent for DNA isolation of sperm cells.

The workflow of our protocol presents several practical advantages when isolating sperm DNA. First, our protocol provides flexibility, such that the stability of DNA after homogenization allows for the convenient storage of lysates for future downstream DNA isolation. Secondly, our protocol is streamlined by incorporating commercially available reagents that are stable at room temperature and eliminating the need for lengthy ProK digestions. These first two points have important implications, especially in regard to research in clinical settings where technician time may be limited. Moreover, the potential for lysate storage prior to DNA isolation and the use of commercially available reagents minimizes potential batch effects when conducting large epidemiologic studies. Our protocol is also amenable to partitioning lysate for RNA purification from the same sample. For example, given 500 μL of lysate that contains 3×10^7 sperm, 100 μL of lysate is expected to provide, at 90% yield, approximately 16 μg of DNA with our protocol. The remaining 400 μL of lysate can then be used for the isolation of RNA, which is present in extremely low quantities in sperm (50 fg/cell) (28). By incorporating the sperm RNA isolation method of Goodrich et al. (28), our theoretical example is estimated to provide 1.2 mg of RNA. These yields provide ample nucleic acids for many downstream applications, including next-generation sequencing techniques. Additionally, our protocol is optimized for silica-based spin columns, thereby avoiding ethanol precipitation procedures. Lastly, our protocol can be extended for isolation of sperm DNA in other mammalian species. For example, our method is equally effective for isolating sperm DNA from mice (data not shown).

In conclusion, our optimized 5 min room-temperature homogenization protocol results in >90% yield of high-quality sperm DNA by utilizing steel beads to facilitate sperm cell lysis, in concert with the odorless reducing agent TCEP to dissociate disulfide bonds without the use of ProK. After homogenization, in lieu of lengthy ethanol precipitation, DNA can be extracted by user-preferred silica-based spin columns for a total processing time of 15–20 min. Our protocol also stabilizes nucleic acids, allowing for optional storage of homogenate for DNA isolation at a later date. A schematic of the DNA isolation method is shown in Figure 4, and a full protocol can be found in the Supplementary Material. Our improved method has important practical advantages for research in clinical settings where sample processing constraints likely exist.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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METHOD SUMMARY

Our optimized protocol for isolation of sperm DNA utilizes bead-based homogenization to facilitate sperm cell lysis in concert with an odorless reducing agent, tris(2-carboxyethyl)phosphine (TCEP), to dissociate disulfide bonds without the use of proteinase K (ProK). The procedure is conducted at room temperature, and the nucleic acids are sufficiently stabilized to allow storage of homogenate for future DNA isolation. After the homogenization step, DNA can be extracted by silica-based spin columns for a total processing time of 15–20 min.

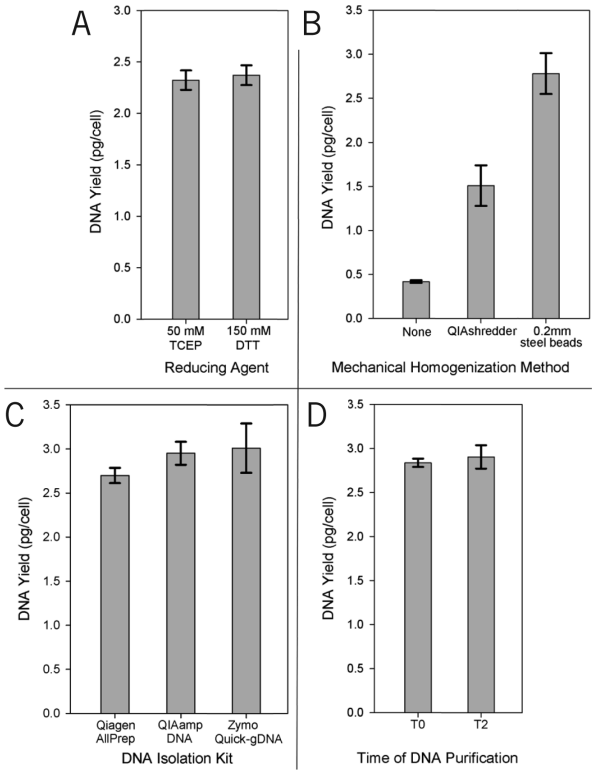

Figure 1. Utility of tris(2-carboxyethyl)phosphine (TCEP) as a reducing agent for the isolation of sperm DNA

(A) Mean (±SD) DNA yield (pg/cell) of sperm cells treated with Buffer RLT, either 50 mM TCEP or 150 mM DTT, and proteinase K (ProK) for two hours at 56°C. DNA was isolated from sperm cell lysate via AllPrep DNA columns. (B) Mean (±SD) DNA yield (pg/cell) of sperm cells treated with Buffer RLT (50 mM TCEP) and the following homogenization methods: none; Qiashredder (as directed); or 0.1 g of 0.2 mm stainless steel beads. DNA was isolated from sperm cell lysate via AllPrep DNA columns. (C) Mean (±SD) DNA yield (pg/cell) of sperm cells purified with Qiagen AllPrep, QIAmp Mini, or Zymo Quick-gDNA spin columns. Sperm cells were homogenized by 0.2 mm stainless steel beads in either Buffer RLT (AllPrep and DNA Mini) or Zymo gDNA lysis buffer (Quick gDNA), both supplemented with 50 mM TCEP. (D) Mean (±SD) DNA yield (pg/cell) of sperm cells homogenized by 0.2 mm stainless steel beads in the presence of Buffer RLT and 50 mM TCEP. DNA was isolated from sperm cell lysate via AllPrep spin columns processed immediately (T0) and after 2 weeks storage (T2) at room temperature (22°C).

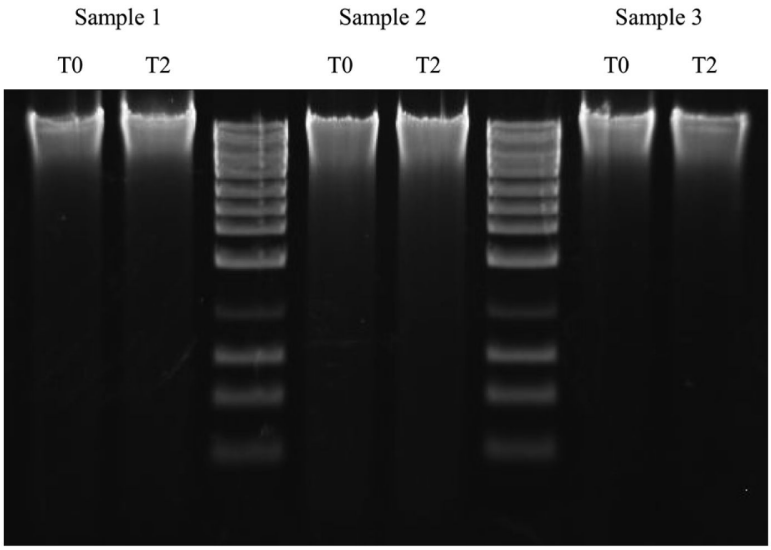

Figure 2. Electrophoresis of sperm DNA

Sperm DNA isolated from 3 individuals immediately (T0) and after 2 weeks of storage at room temperature (T2) on a 0.7% agarose gel.

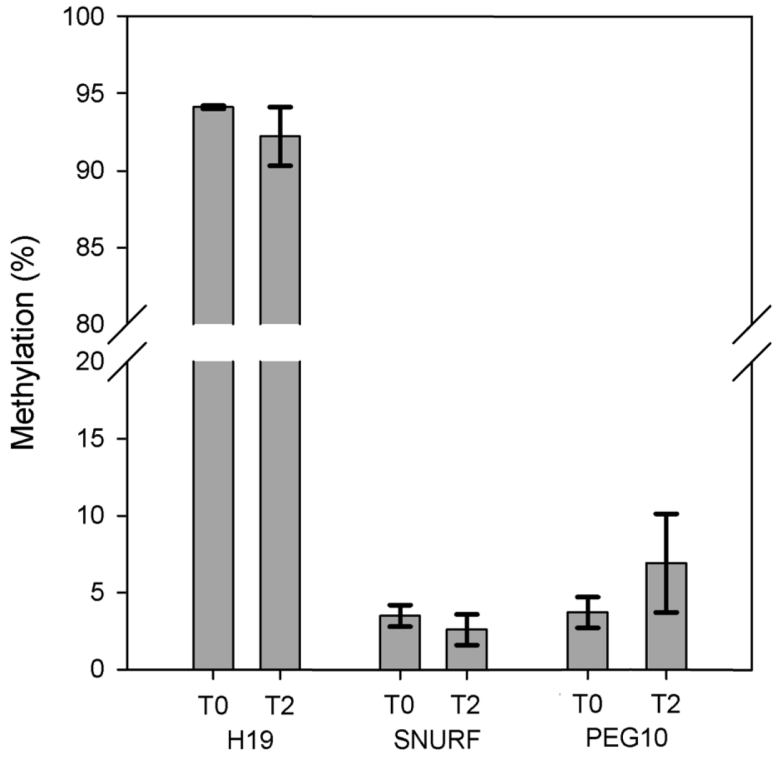

Figure 3. DNA methylation of three imprinted loci

Mean (±SD) percentage DNA methylation of CpG sites within imprinted regions of *SNURF*, *PEG10*, and *H19* using DNA isolated from sperm lysate immediately (T0) and after 2 weeks of storage at room temperature (T2).

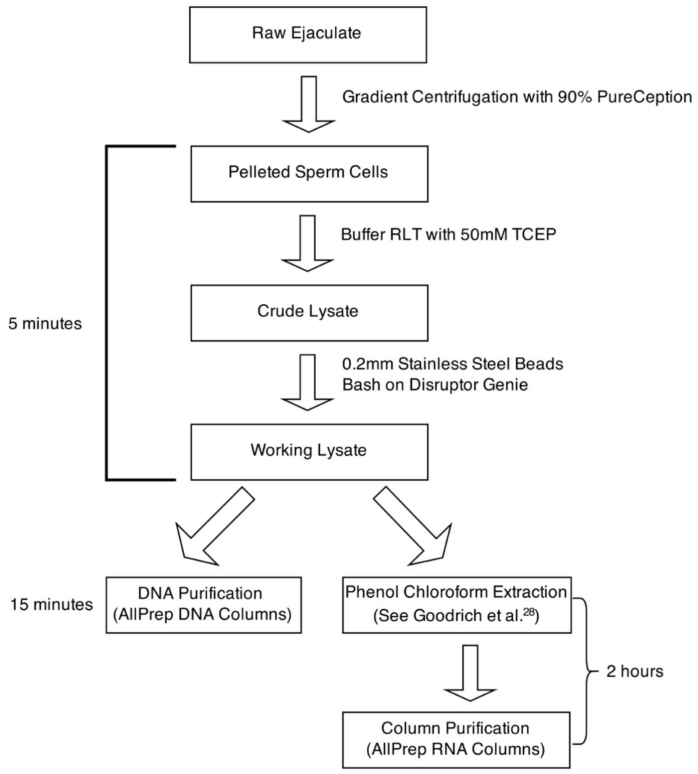

Figure 4. Schematic of sperm DNA isolation workflow

Sperm cells are isolated from ejaculate via gradient centrifugation with 90% PureCeption. Pelleted sperm cells are then homogenized with Buffer RLT, 50 mM tris(2-carboxyethyl)phosphine (TCEP), and 0.1 g of 0.2 mm stainless steel beads on a Disruptor Genie for 5 min. This produces a working lysate that is ready for DNA and RNA isolation.