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Comparison of DNA isolation methods from mammalian sperm cells and development of a new protocol

Hatice Sevim Nalkiran[1], Ihsan Nalkiran[1], Sema Yilmaz Rakici[2], Levent Tumkaya[3], Ali Irfan Guzel[1]

[1]Recep Tayyip Erdogan University, Department of Medical Biology, Rize, Turkey
[2]Recep Tayyip Erdogan University, Department of Radiation Oncology, Rize,Turkey
[3]Recep Tayyip Erdogan University, Department of Histology and Embryology, Rize, Turkey

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Abstract

Sperm DNA tightly packed with protamines makes the DNA isolation procedure from sperm cells long and laborious. Cell lysis is also a challenging step because of the di-sulfide bonds-rich membranes of the sperm cells. In this study we aimed to evaluate potential rapid DNA isolation protocols to isolate DNA from mammalian sperm cells, and develop an easy, rapid, and cost-effective protocol for sperm DNA isolation which can be used in molecular biology and diagnostics. Sperm samples were collected from seven adult rats. Our developed protocol included Proteinase K and small amount of β-mercaptoethanol (βME) for cell lysis. A modified salting-out technique was then employed to collect DNA. Alternative protocols involving InstaGene matrix and cell sonication techniques were also applied to achieve DNA isolation. Concentration of the DNA yield was measured, and the degradation of DNA was checked using agarose gel electrophoresis. The intactness of the DNA yield was assessed and validated using polymerase chain reaction (PCR) and capillary gel electrophoresis techniques. The lysis of the cells and high-quality DNA yield have only been achieved using our developed optimized protocol. To confirm the quality of DNA for assays, PCR product was synthesized for rat actin β (RActβ) gene and then analyzed using capillary gel electrophoresis. A strong peak at right m/z value for the amplicon was obtained. We described an improved protocol over the previous methods suggesting the use of combined commercial kits and long incubation times. Degradation-free DNA was produced in a relatively rapid protocol (90 minutes) using the equipment and supplies common to most research and clinical laboratories.

Keywords: Mammalian sperm cells, DNA isolation, comparison of methods, protocol development

Introduction

A tissue contains a group of specialized cells exhibiting similar structure and function. High variability is therefore present among the molecular structures of mammalian cells from different tissues. Mammalian spermatozoa have one of the most specially differentiated sub-cellular compartments. The sperm chromatin is condensed by protamines in the form of highly compact structures [1, 2]. Isolation of an intact DNA is required to investigate sperm related genetic and epigenetic alterations. Extensive research is being currently conducted in the field of male infertility using techniques that involve the structural, genetic, and epigenetic analyses of sperm DNA [3-6].

Several methods intensively used exist for the isolation of DNA from blood and other tissues. Some protocols containing phenol-chloroform for DNA isolation require intensive laboratory work and might result in inhibition of polymerase chain reaction (PCR) by the use of compounds through the extraction protocol [7, 8]. In contrast, a chelex resin-based protocol, InstaGene matrix (Bio-Rad Laboratories, USA), offers a practical and rapid method to produce high-quality DNA [9]. However, the commercial InstaGene matrix kit is mainly designed for efficient isolation of DNA from bacteria, whole blood, and cultured cells [10, 11].

The strong nuclear membrane and a structural difference in nuclear compaction between somatic cells and sperm cells complicate the use of standard DNA isolation protocols for sperm cells. These DNA isolation techniques might not be proper to be used for all tissue types and require modifications [12]. The use of proteinase K, 1,4-dithiothreitol (DTT), β-mercaptoethanol (βME), and guanidine thiocyanate, tris(2-carboxyethyl)phosphine (TCEP) later included in the solutions to improve the DNA isolation from

***Corresponding Author:** Hatice Sevim Nalkiran, Rize Recep Tayyip Erdogan University, Department of Medical Biology, Rize, Turkey.
E-mail: haticesevimtr@gmail.com

sperm cells as reported by previous studies [12-14]. Despite the availability of several methods, problems in isolating intact high-quality DNA from sperm cells in a rapid and less laborious process persist. Here, we describe an optimized protocol combining the use of βME and proteinase K digestion in a rapid protocol for DNA isolation from sperm cells. DNA is further precipitated and collected using a modified salting-out method [15].

Material and Methods

Sample Collection

Sperm samples (60 µl) were collected from seven adult Sprague-Dawley rats numbered from 1 to 7 (R1, R2, R3, R4, R5, R6, R7). Sperm samples were harvested postmortem from the ductus deferens. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed (Local Ethics Committee for Animal Experiments; Faculty of Medicine, Recep Tayyip Erdogan University Decision No: 2014/21). All procedures performed in the studies involving animals were following the ethical standards of the institution or practice at which the studies were conducted.

InstaGene Matrix Method

30 µl of sperm samples were washed with sterile double distilled water (ddH_2O) and centrifuged at 4500 rpm for 5 min. The supernatants were carefully removed except for approximately 25-30 µl. 200 µl of InstaGene matrix (Bio-rad Laboratories, Hercules, CA, US) was added to the pellet and incubated at 56 °C for 30 min. InstaGene matrix was re-suspended to obtain a homogenous solution and transferred using a wide-bore pipette tip. Following incubation, the cell suspension was vortexed at high speed for 10 sec and placed on a heat block at 99 °C for 8 min. Cell disruption was not achieved and the incubation was extended to 20 min. Because the lysis of the cells was still not achieved at this step, the further steps in this protocol did not proceed.

Cell Sonication Followed by Salting-out Method

Sperm samples (60 µl) were washed with sterile ddH_2O and centrifuged at 4500 rpm for 5 min, and then the supernatant was removed. 750 µl of tris-EDTA-9 (TE-9) buffer (500 mM Tris-HCl pH 9.0, 20 mM EDTA, 10 mM NaCl) was added to the tube and the cell suspension on ice was sonicated with 10 cycles (10 sec on, 30 sec off). 150 µl of 6 M NaCl solution was added, shaken vigorously, and centrifuged at high speed for 10 min. The pellet includes the proteins removed from the mixture, the supernatant was transferred into a clean tube and the centrifuge at high speed repeated once more for 5 min. The supernatant was again transferred into a clean tube and 100 % ethanol (800 µl) was added into the tube, then the solution was inverted several times to achieve the condensation of DNA. The mixture was centrifuged at high speed for 5 min and the supernatant was removed before adding 70 % ethanol to the pellet. The mixture was centrifuged at high speed for 5 min and this step was repeated once more. The supernatant was removed, and the pellet was left for drying for 10 min at room temperature. The pellet was re-suspended in a sterile ddH2O and incubated at 70 °C for 20 min, then stored at 4 °C for downstream applications.

Developed Protocol

Sperm samples (60 µl) were washed with 750 µl of 75 mM NaCl, 25 mM EDTA buffer and centrifuged at 3500 rpm for 5 min. Supernatants were removed and a lysis buffer composed of 250 µl of TE-9 buffer (500 mM Tris-HCL pH 9.0, 20 mM EDTA, 10 mM NaCl), 80 µl of 10 % sodium dodecyl sulfate (SDS) and 60 µl of Proteinase K [Sigma-Aldrich, Steinheim, Germany]) was added on pellets. The mixtures were incubated at 56 °C for 30 min in a shaking heat block. Following incubation, 10 µl of βME (Sigma-Aldrich, Steinheim, Germany) was added to the tubes and mixed by vortexing for 10 sec then incubated at 56 °C for 10 min in a shaking heat block. 150 µl of 6 M NaCl solution was added, shaken vigorously, and centrifuged at high speed for 10 min. The pellet includes the proteins removed from the mixture, the supernatant was transferred into a clean tube and the centrifuge at high speed repeated once more for 5 min. The supernatant was again transferred into a clean tube and 100 % ethanol (800 µl) was added into the tube, then the solution was inverted several times to achieve the condensation of DNA. The mixture was centrifuged at high speed for 5 min and the supernatant was removed before adding 70 % (800 µl) ethanol on the pellet. The mixture was centrifuged at high speed for 5 mins and the last step was repeated once more. The supernatant was removed, and the pellet was left for drying for 10 min at room temperature. The pellet was re-suspended in a sterile ddH_2O and incubated at 70 °C for 20 min, then stored at 4 °C for downstream applications (Figure 1).

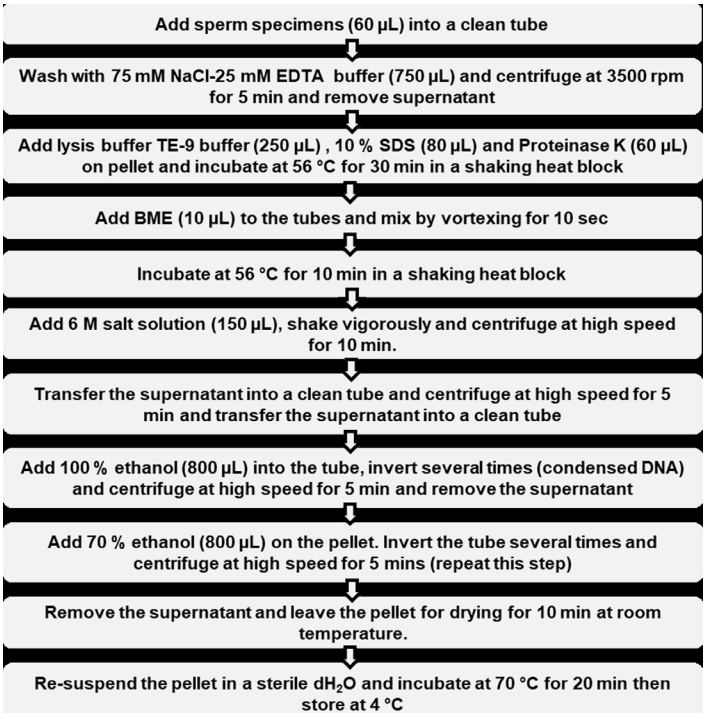

Figure 1. Flowchart for the developed DNA isolation protocol

Agarose Gel Electrophoresis and DNA Quantification

1 % agarose gel was prepared in 0.5 X Tris/Borate/EDTA (TBE) buffer by adding 0.1 µl 5 mg/ml ethidium bromide (EtBr) (Sigma-Aldrich, Steinheim, Germany). 2 µl of genomic DNA samples were loaded on the gel and run at 100 V for 30 min, then the gel visualized and photographed using Gel Documentation system (DNR Bio-

Imaging System Ltd., Jerusalem, Israel). DNA quantification was performed by measuring absorbance at 260 nm using a Multiskan GO microplate spectrophotometer (Thermo Scientific, Waltham, MA, USA). The purity of DNA was determined by measuring the absorbance of the sample at 280 nm for protein concentration and 260 nm for DNA concentration. A DNA sample has an A260/A280 in the range of 1.7 -2.0 was considered pure.

PCR Amplification

The final volume of 25 µl reaction included 25 ng of genomic DNA, PCR buffer containing 1.5 mM $MgCl_2$ (Qiagen, Germany), 0.2 mM of each dNTP (Boehringer Mannheim GmbH, Mannheim, Germany), 2.5 IU of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA), 0.5 µl for each of the forward and reverse primers (10 mM) and ddH_2O. One of the primers of each pair was labeled with a fluorescent tag carboxyfluorescein (FAM) to the 5'-end. The primer set for rat actin β (RActβ) gene; forward (5'-GTGTTGTCCCTGTATGCC-3') and reverse (5'-GTGGTGGTGAAGCTGTAG-3') was used to produce 192 base pair (bp) long amplicons. Amplifications were performed on a Gene Amp PCR Systems 9700 (Applied Biosystems, Foster City, CA, USA) with the following program: 95 °C for 4 min, 30 cycles of 95 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min, followed by 72 °C final extensions for 5 min.

Capillary Gel Electrophoresis

Capillary gel electrophoresis was performed using The ABI Prism 310 Genetic analyzer (Applied Biosystems, Foster City, CA, USA). Samples included PCR product (1.5 µl), Hi-Di Formamide (Applied Biosystems, California, USA) (20 µl) internal size standard and (0.5 µl) (GeneScan-500 LIZ size standard (Applied Biosystems, Warrington, UK), the samples were denatured for 2 min at 96 °C, snap-cooled and injected at 15 kV for 5 sec into a 36-cm capillary containing POP-4 polymer (Applied Biosystems, Foster City, CA, USA). Electrophoresis was performed at 15 kV for 32 min at 60 °C. Peak heights were analyzed using the ABI GeneScan analysis software (Applied Biosystems, Foster City, CA, USA).

Statistical Analysis

Grubb's Test (Graphpad QuickCalcs, online calculator for scientists at https://www.graphpad.com/quickcalcs/, GraphPad Software, San Diego, CA, USA) was used as a preliminary test to detect the significant outliers from the rest of the samples.

Results

InstaGene Matrix and cell sonication followed by salting-out methods were applied to isolate DNA from sperm cells. The lysis of the sperm cells was not achieved by the InstaGene matrix method. Following the incubation step at 99 °C, it was observed that sperm cells in the solution were still visible as white floating clumps. Although the incubation time was found to be inadequate and extended to 20 min, there was no improvement in the lysis of the cells. Therefore, the protocol was ended at this step. The cell sonication followed by the salting-out method resulted in high-quality DNA. However, further PCR reactions showed no amplicons produced using these DNA samples. DNA yield of the samples ranged from 22 to 60.24 µg/ml. On the assessment of purity for the samples, we found that all samples were within the range of 1.7-2.0 except R1 which has an absorbance at 260 (A260)/absorbance at 280 (A280) ratio above 2.0 (Table 1) Statistical analysis of the results for A260/A280 ratios showed R1 sample to be furthest from the rest but not a statistically significant outlier ($p > 0.05$).

Table 1. Amount of genomic DNA isolated from the rat sperm samples

Samples	DNA Concentration (µg/ml)	A260/280 Ratio
R1	22.00	2.27
R2	31.04	1.86
R3	42.40	1.72
R4	48.24	1.87
R5	60.24	1.90
R6	29.04	1.96
R7	32.32	1.98

Abbreviations: R1-R7: Samples from the rat numbered from 1 to 7, A260: Absorbance at 260 nm, A280: Absorbance at 280 nm

Here, we described an optimized protocol that was improved over the previous protocols suggesting the use of combined commercial kits, sonication technique, and long incubation times. Isolated DNA samples were loaded on 1 % agarose gel and intact DNA samples were observed (Figure 2).

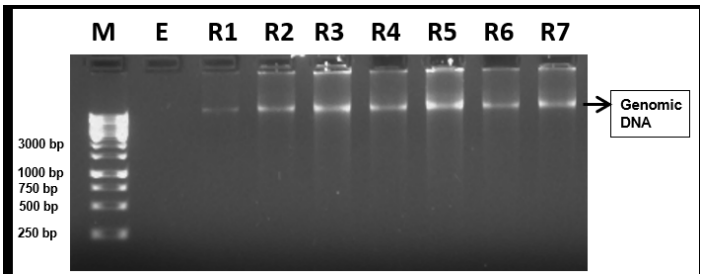

Figure 2. Agarose gel electrophoresis of genomic DNA isolated from rat sperm samples using the developed protocol. Abbreviations: M: marker DNA, E: empty well, R1-R7: rat numbers.

Degradation-free DNA was produced in a rapid protocol using a small amount of βME. Isolated DNA (A260/A280 ratio of 1.7 - 2.2) was later used to amplify the RActβ gene and the 192 bp amplicon was analyzed by capillary gel electrophoresis (Figure 3).

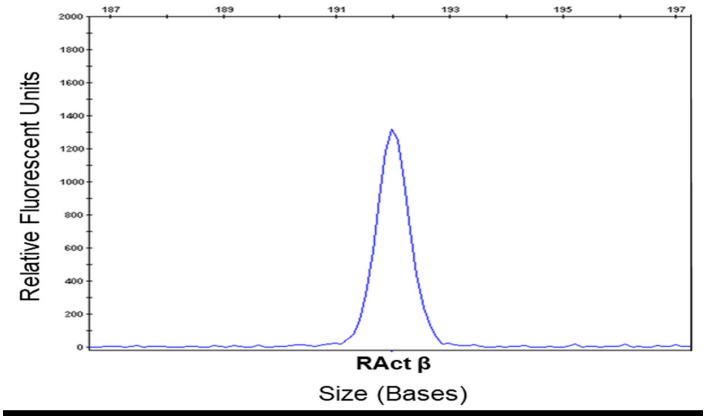

Figure 3. A representative electropherogram of 192 bp *RActβ* gene amplicon analyzed by capillary gel electrophoresis

A strong peak with a correct m/z value for the amplicon and a high abundance of the product was detected.

Discussion

This study is an attempt to establish an optimized and cost-effective protocol for isolating DNA from mammalian sperm cells. To the best of our knowledge, this type of comparison between methods for isolation of DNA from mammalian sperm specimens has not been performed before. Qiagen provides a user-developed non-optimized method by combining QIAamp DNA Mini Kit and QIAamp DNA Blood Mini kit protocols for DNA isolation from sperm cells. However, combining the consumables of two commercially available kits for one protocol might not be feasible.

The first method assessed was the InstaGene matrix method that we partially modified by increasing the incubation time to 20 min at 99 °C to achieve the lysis of the cells. However, at the end of the extended incubation time cell clumps were still present. Then, we thought a cell sonication step could solve the problem and applied a cell sonication followed by a salting-out protocol. Cell lysis was successfully achieved, and high-quality DNA was obtained using the sonication method. DNA samples were further tested by a multiplex PCR method and produced no amplicons. This could be due to the fragmentation of genomic DNA caused by sonication. Although the sonication technique is widely used for cell lysis in protein extraction protocols, it might not be a suitable method for the isolation of intact genomic DNA. Several studies previously showed the use of the sonication technique in DNA shearing methodology [16, 17]. However, it could still be possible to develop a sonication program that causes rupture of the cell and nuclear membrane while keeping the genomic DNA intact. The developed protocol resulted in a high DNA yield; however, this method did not include any RNase treatment. Therefore, the obtained A260/280 ratio of 2.27 (above 2.0) for the R1 sample as shown in Table 1 could be due to the RNA contamination. This developed protocol might be further upgraded with the addition of an RNase treatment step during the lysis.

Conclusion

Here, we report an optimized protocol that produces an intact genomic DNA from sperm cells using the combination of Proteinase K and βME. Although DTT is a more effective reducing agent than βME which has a strong odor as a drawback, DTT becomes unstable in solution and needs to be prepared freshly before the isolation. In conclusion, considering the drawbacks of the phenol-chloroform technique and other tested methods; our optimized βME-containing modified salting-out method can be used as a rapid and effective DNA isolation protocol. This protocol offers an alternative simple method that facilitates the DNA isolation process from mammalian sperm cells. Isolated genomic DNA samples from sperm cells were successfully amplified using multiplex PCR reactions and further analyzed by capillary gel electrophoresis.

Conflict of interests

The authors declare that they have no competing interests.

Financial Disclosure

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Ethical approval

Local Ethics Committee for Animal Experiments; Faculty of Medicine, Recep Tayyip Erdogan University Decision No: 2014/21

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