Anal Bioanal Chem
DOI 10.1007/s00216-015-8737-8

RESEARCH PAPER

The application of alkaline lysis and pressure cycling technology in the differential extraction of DNA from sperm and epithelial cells recovered from cotton swabs

Deepthi V. Nori[1] · **Bruce R. McCord**[1]

Received: 16 January 2015 / Revised: 14 April 2015 / Accepted: 24 April 2015
© Springer-Verlag Berlin Heidelberg 2015

Abstract This study reports the development of a two-step protocol using pressure cycling technology (PCT) and alkaline lysis for differential extraction of DNA from mixtures of sperm and vaginal epithelial cells recovered from cotton swabs. In controlled experiments, in which equal quantities of sperm and female epithelial cells were added to cotton swabs, 5 min of pressure pulsing in the presence of 0.4 M NaOH resulted in 104±6 % recovery of female epithelial DNA present on the swab. Following the pressure treatment, exposing the swabs to a second 5-min alkaline treatment at 95 °C without pressure resulted in the selective recovery of 69 ±6 % of the sperm DNA. The recovery of the vaginal epithelia and sperm DNA was optimized by examining the effect of sodium hydroxide concentration, incubation temperature, and time. Following the alkaline lysis steps, the samples were neutralized with 2 M Tris (pH 7.5) and purified with phenol-chloroform-isoamyl alcohol to permit downstream analysis. The total processing time to remove both fractions from the swab was less than 20 min. Short tandem repeat (STR) analysis of these fractions obtained from PCT treatment and alkaline lysis generated clean profiles of female epithelial DNA and male sperm DNA for 1:1 mixtures of female and male cells and predominant male profiles for mixtures up to 5:1 female to male cells. By reducing the time and increasing the recovery of DNA from cotton swabs, this new method presents a novel and potentially useful procedure for forensic differential extractions.

Keywords Forensics · Toxicology · Nucleic acids (DNA | RNA) · Differential extraction · Short tandem repeat · PCR · Pressure cycling

Introduction

Since its discovery in 1984 by Sir Alec Jeffreys, DNA typing has become an indispensable tool in applications ranging from paternity testing and criminal investigations to the study of genetic disorders [1–3]. The application of DNA technology in the criminal justice system has resulted in an unprecedented expansion in the capabilities of forensic laboratories for the detection of violent crimes such as rape and murder. With improvements in automation, the procedure has expanded to permit determination of property crimes and misdemeanors as well. However, as the number of applications for DNA technology expands, inevitable backlogs have occurred due to increasing sample loads. Factors such as time lapse between the incident and sample recovery, exposure to external elements, and storage conditions may all result in sample degradation [4–6]. Hence, it is critical to be able to efficiently recover and reliably analyze the evidence collected.

Traditionally, cotton swabs have been used for the collection of biological samples such as body fluids, touch samples, and other trace evidence. Despite being a common tool for sample collection, sample extraction from these swabs can be challenging due to strong adherence of the sample to the matrix. Often, the bulk of a collected biological sample remains entrapped in the cotton fibers even after elution. This results in a loss of precious evidence [7]. For example, in a multi-laboratory study reported by Vuichard et al., sample

Published in the topical collection *Capillary Electrophoresis of Biomolecules* with guest editor Lisa Holland.

✉ Bruce R. McCord
mccordb@fiu.edu

[1] Department of Chemistry CP304, Florida International University, 11200 SW 8th St, Miami, FL 33199, USA

Published online: 09 June 2015

losses of 94–98 % were reported following differential extractions when compared to direct extraction [8]. Norris et al. also studied this problem and demonstrated that recovery can be improved through careful optimization of elution conditions. However, in this paper, they also noted that sperm cell recovery is poor (~40 %) from cotton swabs using conventional procedures [7].

There have been a number of attempts to develop methods to increase the efficiency of cell recovery from cotton swabs. Enzymatic methods are based on the hypothesis that digestion of cotton fibers with cellulase enzyme will improve the release of cells from the swab [9]. Alternatively, appropriate detergents or buffers may improve recovery of DNA from the swabs [7]. Different types of swabs and substrates have also been proposed to improve elution of DNA from these swabs, yet this remains an area of active research [10].

Another issue is the selectivity of the extraction of DNA. When performing differential extraction of sperm and epithelial cells, it is critical to obtain a clean, unambiguous male profile. This is done by lysing the female epithelial cells with detergent followed by centrifugation and recovery of sperm DNA using detergent with dithiothreitol (DTT) to aid in the digestion and recovery of DNA from the sperm cells [2]. Because the procedure is difficult, time-consuming, and includes multiple wash steps, its recovery is sometimes poor and user-dependent [8]. For example, in the collaborative study by Vuichard et al., it was found that there were large lab-to-lab variations in the relative amount of male-to-female DNA in the epithelial fraction, with some laboratories seeing more male DNA (30 %) while most finding more female [8]. Because of these problems, a number of alternative procedures have been attempted to permit differential detection of male and female DNA. These include laser micro-dissection, removal of female DNA through alkaline lysis and/or DNase digestion, microfluidics, and flow cytometry [11–16].

Thus, there have been a number of efforts to improve DNA recovery from swabs taken following a sexual assault, especially for situations in which sample is limited. The organic differential extraction method developed by Gill et al. and its modified versions are still being used in analyzing mixtures, but its shortcomings create the need for an efficient, quick, and reliable method that can successfully separate DNA profiles in a mixture without compromising sample recovery [17, 18].

Pressure cycling technology for sample preparation (PCT) is a novel method that employs cycles of hydrostatic pressure between ambient and high levels to induce mechanical stress on cells resulting in compromised cellular integrity [19, 20]. Application of alternating cycles of hydrostatic pressure between ambient and high levels causes disruption of biomolecular interactions. When high pressure is applied, the lipid bilayer is compressed and release of this pressure results in destabilization of the cell membrane, which leads to compromised cellular integrity and subsequent release of cellular components [19]. Cell lysis and efficient recovery of the cellular components dictate the success of downstream applications. Pressure cycling technology has been used successfully in a variety of applications in molecular biology. Smejkal et al. successfully extracted protein from *Escherichia coli* using PCT [21]. It has also been used in the extraction of biomolecules such as DNA and RNA from animal and plant tissues, insects, and microbes [20, 22, 23]. The advantage of pressure cycling is its ability to produce a highly efficient extraction and disruption of cell nuclei from a wide variety of substrates.

Most of the extraction methods in forensic biology rely on a combination of physical and chemical extraction protocols to achieve maximum yields. Achieving maximum DNA yields from a sample is especially challenging because the evidence collected from a crime scene is mostly present on a substrate, and recovery from the substrate is inefficient when the material is an absorbent. Long incubation times, contamination issues, health hazards from the use of chemicals, and the combination of treatments that are too harsh on the biomolecules of interest are some of the biggest disadvantages associated with the current extraction methods. Pressure cycling technology for sample preparation was developed to rapidly and efficiently release cellular contents from biological samples using alternate cycles of high and ambient pressure in a safe and controlled environment.

This study reports an initial study on the potential of alkaline lysis on the differential recovery of sperm DNA and epithelial DNA from a cotton swab. The alkaline lysis step was coupled with a novel method utilizing pressure cycling technology to aid in sample recovery and differential digestion of mixtures from cotton swabs. The procedure improves the speed and recovery of DNA from differential extractions by eliminating the need for long incubation and digestion times as well as reducing the number of manual wash steps.

Materials and methods

Sample preparation

Vaginal epithelial cells and semen samples were collected from volunteers in accordance with protocols approved by the Institutional Review Board (IRB) of Florida International University. The samples were suspended in 1× PBS buffer (pH 7.4) (Fisher Scientific, NJ) and diluted to approximately one million cells per milliliter. The cell count was performed using a disposable hemocytometer (INCYTO C-Chip, Covington, GA, USA) [24]. Equal volume of epithelial cells and sperm cells was added to a cotton swab and air-dried at room temperature. Post-coital samples obtained from healthy volunteers were stored at −20 °C until further use.

Since pressure cycle technology has not been previously applied to rape kits, it was necessary to determine the

efficiency of this treatment on DNA recovery. For comparison of relative efficiency, standard organic extraction was performed to determine the amount of DNA in each cell type. The quantity of DNA recovered from pressure treatment was plotted as percent recovery of DNA obtained from organic extraction. Organic extraction was performed by incubating the samples in stain extraction buffer (10 mM Tris, 100 mM NaCl, 10 mM EDTA, 2 % SDS, 39 mM DTT) and proteinase K (20 mg/ml) at 56 °C for 2–4 h followed by phenol-chloroform-isoamyl alcohol purification and ethanol precipitation.

DNA extraction

Pressure cycling technology

A Barocycler® NEP 2320 (Pressure BioSciences Inc., South Easton, MA) was used to generate cycles of high pressure and ambient pressure to apply mechanical stress on the cells and cause lysis. There are four main parameters that can be varied to achieve an optimum environment for cellular disruption. The applied pressure, exposure time, and number of cycles can all be adjusted using a programmable interface. The Barocycler® NEP2320 (Pressure BioSciences Inc., South Easton, MA) is a commercially available instrument that is equipped with a hydrostatic pressure chamber that can withstand high pressures up to 45,000 psi, and samples can be exposed to target high pressure or ambient pressure from 1 to 99 s. Cycling between high pressure and ambient pressure can be repeated up to 99 times. Both solid tissue and liquid samples can be processed using this instrument. Furthermore, connecting an external circulating water bath can control the temperature of the pressure chamber. The instrument is connected to a compressor that creates high pressure. PULSE™ tubes (Pressure BioSciences Inc., South Easton, MA) are specially designed tubes that are able to withstand high pressures. To perform pressurized extraction, swabs containing mixtures of sperm cells and vaginal epithelial cells were transferred to PULSE™ tubes and exposed to 10,000, 20,000, or 45,000 psi in the presence of a lysis buffer to determine the optimum pressure to achieve selective lysis of female cells. The efficiency of the pressure treatment in the presence of increasing number of cycles was studied by varying the cycle number between 10, 20, and 60 cycles. During each pressure cycle, the holding time of sample at ambient pressure (T1) and target pressure (T2) was 15 s each.

Alkaline lysis

Hudlow et al. developed an alkaline-based differential extraction method that, when combined with DNase digestion, generated a purified sperm fraction as determined by STR genotyping [14]. Upon applying this method and quantifying the extracted DNA, it was observed that there was a significant loss of sperm DNA. This led us to initiate a study to determine the effect of alkaline lysis on sperm cells and vaginal epithelial cells without DNase digestion at different temperatures and concentrations of sodium hydroxide.

Sodium hydroxide crystals (Fisher Scientific, NJ) were dissolved in molecular biology grade water (Fisher Scientific, NJ) to achieve concentrations of 0.2, 0.4, 0.6, 0.8, and 1 N NaOH concentration. In order to maximize DNA recovery from cotton swabs, three different incubation temperatures (75, 85, and 95 °C) and two different incubation times (2 and 5 min) were studied for each concentration of sodium hydroxide solution. A cotton swab containing an equal quantity of sperm cells and epithelial cells was suspended in 400 μL of a specific concentration of sodium hydroxide solution and exposed to either 75, 85, or 95 °C for 2 or 5 min.

Post-extraction purification

Following extraction, the samples were neutralized with 2 M Tris (pH 7.5). Then, the swabs were transferred to DNA IQ™ Spin Baskets (Promega Corp., Madison, WI) and centrifuged at 13,000 rpm for 5 min. Extracted samples were purified by adding an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) (Sigma-Aldrich, St. Louis, MO) and precipitated with 3 M sodium acetate and 95 % ethanol. Following precipitation, the pellet was washed with 70 % ethanol, air-dried, and resuspended in 1× Tris-EDTA buffer (pH 8.0) (Fisher Scientific, NJ).

Quantification

The extracted DNA was quantified using real-time PCR assay with a commercially available kit, the Plexor® HY system (Promega Corp., Madison, WI) on Rotor-Gene 6000 (Corbett, Australia). The system can simultaneously quantify autosomal DNA and male DNA. By calculating the autosomal/Y ratio, the amount of male DNA and female DNA recovered from the mixture could be determined. The percent recovery of each cell type was determined by comparing the resultant quantity of DNA with samples containing a single cell type that have been directly extracted with proteinase K (20 mg/mL) based organic extraction. All the samples were analyzed in triplicate, and the Plexor® HY analysis was performed following the manufacturer's instructions (Plexor® HY system, Technical Manual# TM299, Promega Corp., Madison, WI).

Short tandem repeat analysis

The quality of the DNA recovered from alkaline lysis and pressure treatment was assessed by performing short tandem repeat (STR) analysis using PowerPlex® 16 HS system (Promega Corp., Madison, WI) according to manufacturer's

protocols (PowerPlex® 16 HS system, Technical Manual# TMD022, Promega Corp., Madison, WI). Quantification data was used to normalize the input DNA for STR analysis. One nanogram of sample was amplified using GeneAmp® 9700 thermal cyclers, and 1 μL of the amplified products was separated using ABI Prism™ 310 genetic analyzer (Life Technologies, Grand Island, NY). Data analysis was performed using GeneMapper® *ID* v3.2 (Life Technologies, Grand Island, NY). The percentage of male DNA was calculated using relative fluorescence units (RFU) as [(2×Y rfu)/(X rfu+Y rfu)]×100 %, and the percentage of female DNA was calculated as [(X rfu−Y rfu)/(X rfu+Y rfu)]×100 % [10].

DNA recovery from post-coital samples

A set of post-coital samples containing both sperm and vaginal epithelial cells were extracted using two different protocols: alkaline lysis coupled with pressure cycling technology and an extraction protocol used by the Broward County Sheriff's Office (BSO) crime laboratory. A comparison of STR profiles of the male and female DNA fractions extracted with these two protocols was performed to determine the applicability of alkaline lysis and pressure cycling technology in processing rape kits.

In the differential extraction protocol used by the BSO crime laboratory, BioRobot EZ1 workstation (Qiagen, Inc., Valencia, CA) is used to purify the extracted samples. This method gives better yields than standard organic differential extraction, but it employs long incubation times to recover sample from the substrate. In the first step, the 1× TNE buffer (10 mM Tris, 1 mM EDTA $Na_2 \cdot 2H_2O$, 1 M NaCl) containing 1 % SDS and proteinase K (20 mg/mL) is added to the swab and incubated in a water bath at 56 °C for 2 h with intermittent mixing at 900 rpm. The supernatant is separated to recover female DNA with EZ1 purification, and the swab is incubated at 56 °C for 2 h in 1× TNE buffer-containing proteinase K (20 mg/mL) and 1 M DTT. This fraction is purified with EZ1 trace protocol to recover sperm DNA.

Results and discussion

Effect of alkaline lysis on DNA recovery from swab

The goal of the alkaline lysis studies was to determine parameters to maximize sperm DNA recovery from a cotton swab while maintaining sufficient selectivity to enable differential lysis of mixtures. The effect of varying concentrations of NaOH at different temperatures and incubation times was studied, and it was observed that incubating the swab in 0.4 N NaOH at 95 °C for 5 min resulted in the recovery of 2.5 times more sperm DNA than epithelial DNA (Table 1). Moreover, depending on NaOH concentration and temperature, either sperm DNA or epithelial DNA exhibited relatively higher recoveries from mixtures. According to a study conducted by Hudlow et al., microscopic observations following alkaline lysis at identical conditions revealed that the sample had few remaining intact sperm cells and no epithelial cells in the mixture [14]. The quantification results from the current study indicate the lysis of both sperm and epithelial cells occurs with relatively more DNA recovered

Table 1 Amount of DNA recovered from mixtures in the presence of alkaline conditions at high temperatures. DNA recovery values represent the percent recovery of DNA in each fraction compared to the total amount of DNA extracted using organic extraction of neat samples and are expressed as the mean (n=3±standard error)

Concentration of NaOH solution (N)	Incubation temperature (°C)	Incubation time (min)	Sperm DNA recovered (%)±SE	Vaginal epithelial DNA recovered (%)±SE
0.2	95	2	60±6	75±12
0.2	95	5	98±10	78±21
0.4	95	2	131±22[a]	64±18
0.4	*95*	*5*	*99.6±1.0*	*41±2*
0.6	95	2	115±15[a]	46±4
0.6	95	5	64±11	54±8
0.8	95	2	94±7	42±5
0.8	95	5	52±12	41±3
1	95	2	71±4	33±1
1	95	5	49±7	45±5
0.2	85	2	44±13	85±14
0.2	85	5	70±8	60±4
0.4	85	2	86±5	68±17
0.4	85	5	75±10	47±9
0.6	85	2	75±10	52±10
0.6	85	5	66±6	35±3
0.8	85	2	55±6	35±9
0.8	85	5	62±8	45±5
1	85	2	81±6	53±17
1	85	5	54±2	55±9
0.2	75	2	33±6	69±7
0.2	75	5	40±6	41±3
0.4	75	2	60±9	46±6
0.4	75	5	57±1	30±8
0.6	75	2	113±6[a]	52±21
0.6	75	5	87±19	46±6
0.8	75	2	83±16	41±16
0.8	75	5	64±17	38±3
1	75	2	62±6	34±9
1	75	5	61±2	34±2

Italicized parameters represent the optimized alkaline conditions for selective sperm DNA recovery from mixtures

[a] More DNA was recovered with the experimental parameters compared to the organic extraction of a similar amount of neat semen sample

from the sperm cells. The amount of DNA recovered from epithelial cells decreased with increasing concentration of sodium hydroxide. This decrease in epithelial DNA recovery can be attributed to denaturation of DNA recovered from lysed epithelial cells following continuous exposure to alkaline conditions. The quantification data of sperm DNA is in concordance with Hudlow's observation where the conditions lyse most of the sperm cells in the mixture.

Though cotton swabs have been traditionally used to collect evidentiary material, recovery of biological samples is poor from this matrix. Many methods have been reported previously to improve cell recovery from cotton swabs, but they involve 2–4 h incubation time [8, 9, 17]. In cases where there are mixtures present, all the cellular materials must be eluted prior to the extraction step before differential lysis can take place. Our initial results demonstrated that by incubating the swab at high temperature in alkaline conditions, almost all of the cellular DNA can be extracted from the substrate in as little as 5 min. Furthermore, differential extraction of epithelial and sperm cells can be performed directly off of the swab without prior elution by adjusting the concentration of base and the incubation parameters.

The optimized parameters for single tube alkaline differential extraction method developed by Hudlow et al. utilizes 0.1 N NaOH treatment at 95 °C for 2 min for non-sperm lysis, addition of DNase digestion to remove non-sperm DNA followed by 1 N NaOH treatment at 75 °C for 2 min for sperm cell lysis. The quantification results from the current study indicate that more than 50 % of the sperm DNA is recovered at 95 °C with 2 min incubation in 0.2 N NaOH. Since non-sperm cells account for <15 % of the sperm concentration in healthy individuals [25, 26], our data suggests that the optimized epithelial cell lysis conditions suggested by Hudlow et al. may also cause premature sperm cell lysis. This observation led to the study to determine conditions that will permit selective digestion and maximized recovery of sperm DNA from cotton substrates.

The results from alkaline lysis of sperm cells and epithelial cells show that at any given temperature, maximum DNA recovery from sperm cells is produced at 0.4–0.6 N NaOH, whereas 0.2 N NaOH produces optimal recovery of epithelial DNA. The best selectivity and reproducibility for the removal of sperm cells from the swabs were achieved by increasing the incubation time to 5 min in the presence of 0.4 N NaOH. The removal of sperm was further improved by increasing the temperature to 95 °C, with the result that 99.6± 1.0 % of the sperm DNA was recovered from controlled mixtures. Lower temperatures and lower concentrations of base were more conducive for the selective recovery of DNA from female epithelial cells. Overall results are show in Table 1.

Optimization of PCT parameters

Although the results from the alkaline lysis studies showed that 0.4 N NaOH gave the best recovery and selectivity for sperm DNA from mixtures, there was still some DNA recovery from epithelial cell lysis that resulted in a mixed profile. Epithelial cell lysis during sperm DNA recovery was minimized by introducing a pressure-based extraction step before incubating the swab at high temperature. In order to do this, it was important to determine the effect of pressure treatment on the swabs in the presence of 0.4 N NaOH. The swabs were exposed to 0.4 N NaOH during PCT treatment based on the observations that continued exposure to alkaline conditions at 0.4 N NaOH further reduced female DNA, possibly due to denaturation of DNA released from lysed epithelial cells, which may further contain non-sperm DNA carryover into the sperm fraction. This is especially important during extraction of mixtures with high ratios of female epithelial cells to male sperm cells. The use of a lower concentration of sodium hydroxide did not efficiently lyse the female epithelial cells when the mixture contained more than five times the amount of epithelial cells compared to sperm cells. Initial studies to determine the optimum alkaline conditions during PCT extractions indicated that increasing the concentration of sodium hydroxide to more than 0.4 N NaOH during PCT treatment affected the overall sperm DNA recovery. Therefore, pressure treatment in the presence of 0.4 N NaOH was determined to be the optimum parameter to lyse epithelial cells and minimize female DNA carryover without affecting sperm DNA recovery.

Our results indicated that 104±6 % recovery of epithelial DNA occurred at 20,000 psi with a minimum of 10 cycles of pressure in the presence of 0.4 N NaOH (Table 2). Furthermore, this mild pressure treatment did not have a significant impact on sperm cell lysis, thus enabling the development of a two-step differential extraction protocol. Another benefit of this treatment was that compared to the current extraction

Table 2 Effect of pressure treatment on DNA recovery from mixtures in the presence of 0.4 N NaOH solution. DNA recovery values represent the percent recovery of DNA in each fraction compared to the total amount of DNA extracted using organic extraction of neat samples and are expressed as the mean (n=3±standard error)

Pressure (psi)	Number of cycles	Sperm DNA recovered (%)±SE	Vaginal epithelial DNA recovered (%)±SE
10,000	10	8±1	93±3
20,000	10	25±13	104±6
20,000	20	17±3	110±20
20,000	60	43±9	46±13
45,000	20	16±3	58±5
45,000	60	26±6	54±3

methods which can take up to 2–4 h to remove cells from a swab, the total time required to remove epithelial DNA from the swab using the pressure cycling procedure under alkaline conditions was only 5 min. It should be noted that semen also contains non-spermatogenic cells such as epithelial and inflammatory cell types that may be susceptible to digestion under same conditions as vaginal epithelial cells. Thus, the presence of male DNA in the epithelial fraction may also be due to the presence of the non-sperm cells in the sample [27].

Development of a two-step protocol for differential extraction

To achieve complete separation of both cell fractions and obtain a clean DNA profile, a two-step method was developed in which swabs were first placed in 0.4 N NaOH and exposed to pressure cycling for 5 min at ambient temperature to lyse and remove the epithelial DNA. The cells remaining on the swab (sperm fraction) were then subjected to alkaline lysis at 95 °C for 5 min.

Vaginal swabs and semen samples collected from volunteers were suspended in 1× PBS buffer (pH 7.4) (Fisher Scientific, NJ) and diluted to approximately one million cells per milliliter. Approximately, 60 μL of each cell type was added to a cotton swab and air-dried at room temperature. To perform the two-step extraction procedure, the swab was transferred to a PULSE tube containing 800 μL of 0.4 N NaOH solution and exposed to 20,000 psi pressure for 10–20 cycles. Following pressure treatment, the sample was immediately neutralized with 57.6 μL of 2 M Tris (pH 7.5) and the swab was transferred to a spin basket placed in 2.0 mL tube and centrifuged at 13,000 rpm for 5 min. DNA was purified with phenol-chloroform-isoamyl alcohol (25:24:1) followed by ethanol precipitation (epithelial fraction). The swab contained in the spin basket was transferred to a 1.5-mL tube containing 400 μL of 0.4 N NaOH solution, and the sample (sperm fraction) was incubated at 95 °C for 5 min. Following incubation, the sample was neutralized with 28.8 μL of 2 M Tris (pH 7.5) and the swab was transferred to a spin basket for centrifugation at 13,000 rpm for 5 min. The swab was discarded along with the spin basket, and DNA from the sperm fraction was purified using phenol-chloroform-isoamyl alcohol and ethanol precipitation (Fig. 1). An equal volume of phenol-chloroform-isoamyl alcohol (25:24:1 *v*/*v*) (Sigma-Aldrich, St. Louis, MO) and 80 μL of 3 M sodium acetate solution was added to the extracted samples. After vortexing for 10 s, the samples were centrifuged at 13,000 rpm for 10 min and the aqueous layer was transferred to a 1.5-mL micro-centrifuge tube. Double the volume of absolute ethanol and 40 μL of 3 M sodium acetate solution were added to the aqueous phase followed by gentle agitation and overnight incubation at 4 °C (or 1–2 h incubation at −20 °C). The samples were centrifuged at 13,000 rpm for 10 min, and the DNA pellet was washed with 1 mL of 70 % ethanol. After air-drying, the pellet was resuspended in water or 1× Tris-EDTA (TE) buffer (Sigma-Aldrich, St. Louis, MO) and incubated in a water bath at 56 °C for 15 min.

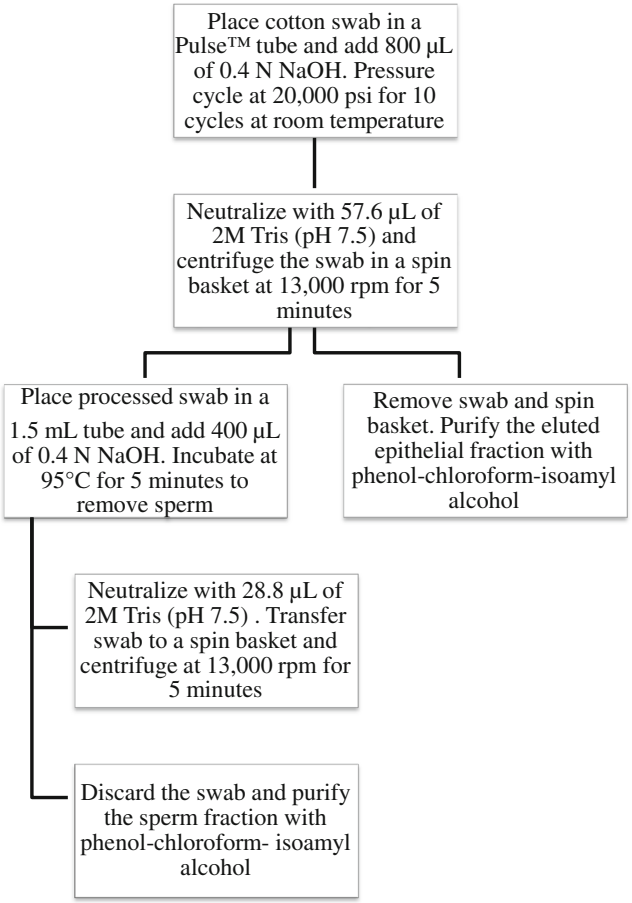
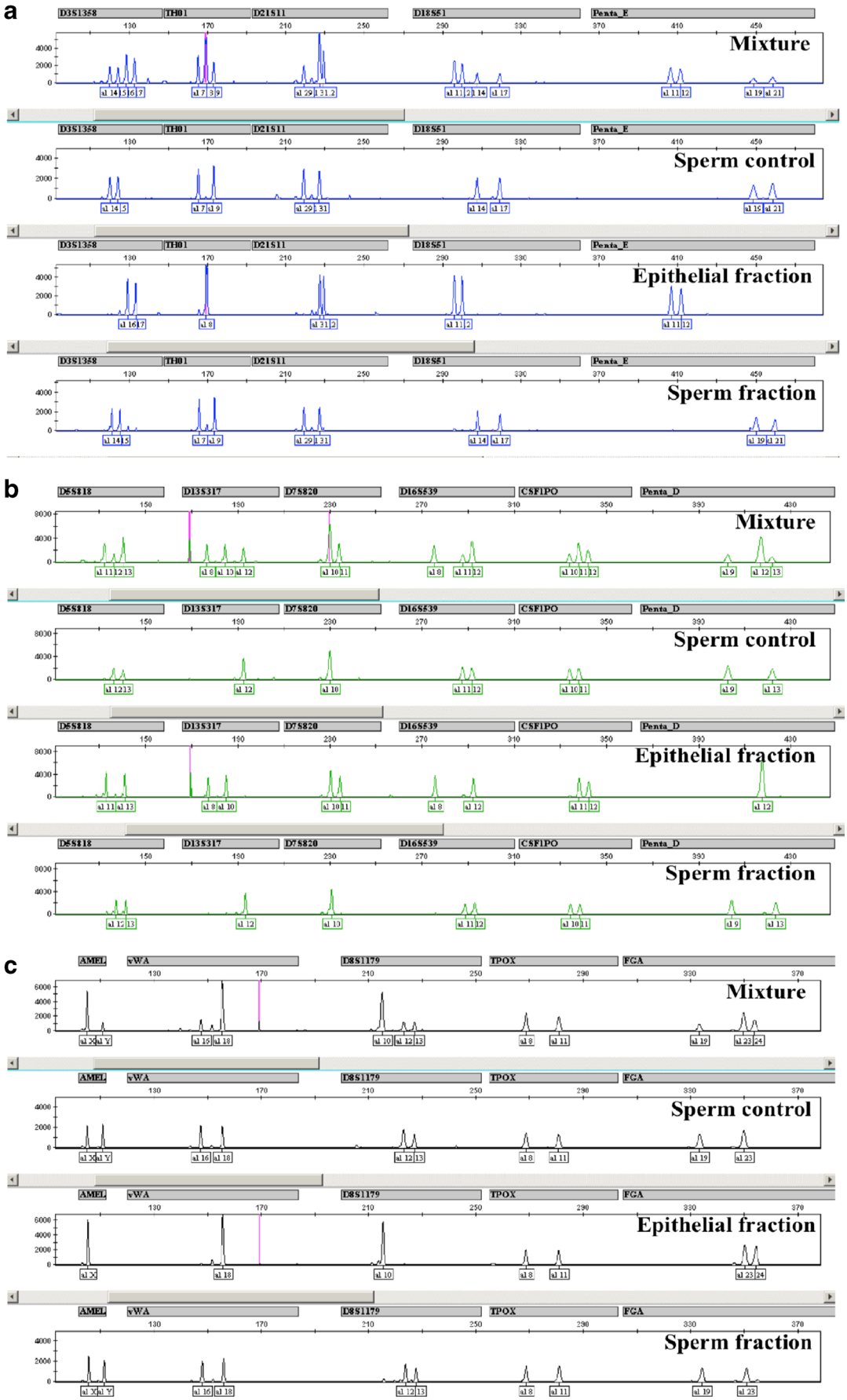


Fig. 1 Flowchart depicting the protocol for differential extraction of mixtures using alkaline lysis and pressure cycling technology

Short tandem repeat (STR) analysis of both epithelial and sperm fractions revealed that the male and female components were successfully separated from the mixture (Fig. 2). Comparative analysis of genotypes of mixture and sperm control with both the extracted fractions showed that this two-step protocol resulted in a clean male DNA profile that is identical to the profile generated with organic extraction of neat semen sample.

Fig. 2 Powerplex® 16 HS products of mixture, sperm control, epithelial fraction (post-PCT purified fraction), and sperm fraction (post-alkaline lysis treatment). **a** Carboxy-tetramethylrhodamine (TMR)-labeled loci. **b** Fluorescein (FL)-labeled loci. **c** 6-Carboxy-4′, 5′-dichloro-2′, 7′-dimethoxy-fluorescein (JOE)-labeled. Electropherogram shows that the DNA profile obtained from sperm fraction is identical to sperm control at all loci


a
Mixture
Sperm control
Epithelial fraction
Sperm fraction
b
Mixture
Sperm control
Epithelial fraction
Sperm fraction
c
Mixture
Sperm control
Epithelial fraction
Sperm fraction

Sexual assault samples obtained from real crime scenes often result in a DNA profile that has such a large signal from the female victim that the male signal is washed out. In order to determine if the current method can lead to sperm fraction enrichment in the presence of an overwhelming amount of vaginal epithelial cells, the method was applied to samples containing increasing amount of epithelial cells with a maximum of 150,000 epithelial cells for a mixture containing 50 times more female cells compared to male cells. Male autosomal STR profile was obtained with samples containing up to ten times more female epithelial cells. Allelic peak heights from male and female contributors were divided by the total peak height at the respective locus. A total of seven loci with no shared alleles between the male and female DNA profiles were selected to calculate the percent contribution of male and female DNA in the sperm fraction.

Our results indicate that a complete male autosomal STR profile can be obtained with samples containing up to ten times more female epithelial cells in the mixture. STR loci that are heterozygous for both male and female profile were chosen to calculate the percent contribution to the final DNA profile generated following extraction by alkaline lysis and pressure cycling technology. In the presence of a comparable amount of female cells, a clean male profile was obtained but a mixed profile was seen with increasing amounts of female cells. In the presence of double the amount of female cells, the final sperm fraction obtained with alkaline lysis and pressure cycling technology contained four times more male DNA compared to female DNA, resulting in a predominantly male DNA profile. Male allelic dropout was observed when the sample was overwhelmed with almost 150,000 female cells compared to 3000 male cells (Fig. 3).

Using this method, a clean male DNA profile was generated from mixtures containing a comparable amount of sperm cells and vaginal epithelial cells. A mixed profile was obtained in instances where an imbalance of mixtures favoring female component is observed. The loss of male alleles was observed when the sample was overwhelmed with 50 times more female cells, but a complete male profile was obtained with mixtures containing low levels of sperm cells, indicating that in contrast to previous reports that observed almost 90 % sperm cell loss from cotton swabs due to inefficient recoveries, this method proved to be more effective in recovering sample from substrates [9].

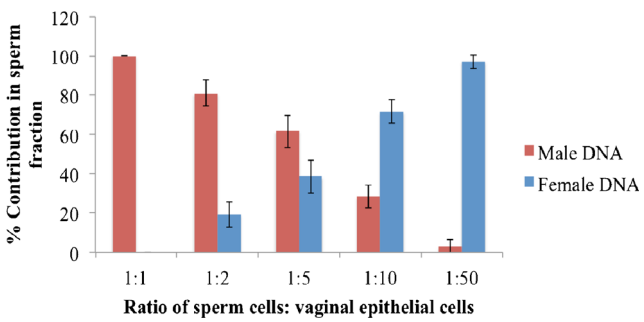


Fig. 3 The effect of alkaline lysis on pressure cycling technology on mixtures containing an imbalance of vaginal epithelial cells and sperm cells. A complete male autosomal profile is observed with samples containing up to ten times more vaginal epithelial cells (n=3±standard error)

Alkaline lysis and pressure cycling technology were further applied to post-coital swabs to determine its ability to selectively recover male DNA from real samples. To do this, a small set of post-coital samples were compared using alkaline lysis and pressure cycling with the same samples prepared using a differential extraction technique as performed at the BSO crime laboratory . The results indicated that when sufficient male DNA is present, the genotypes of female and male fractions obtained with alkaline lysis and pressure-based extraction were identical to those obtained with the BSO protocol (Fig. 4); however, as demonstrated in Fig. 3, for samples in which a large excess of female DNA is present, the procedure recovers less male DNA.

Concluding remarks

The current study presents a novel technique to extract biological fluids from cotton swabs with high recovery using pressure cycling combined with alkaline lysis and has further demonstrated its potential for differential extraction. With a total of time of less than 20 min to remove both sperm and epithelial cells from spiked swabs, this new process is quick and efficient. Current methods for removal of cellular debris from swabs require an incubation time of 2–4 h, which does not include the differential DNA extraction. By using sodium hydroxide solutions for cell lysis and phenol-chloroform-isoamyl alcohol to purify the extracted sample, we have demonstrated that by using simple solvents available in every lab, sperm and epithelial fractions could be successfully separated in a rapid and efficient manner. Despite the promising results, this study requires further exploration to minimize female DNA carryover observed in samples overwhelmed with high ratios of epithelial-to-sperm cells. The new method employs a short 5-min incubation time to lyse female cells that is not sufficient when the sample has a large excess of female epithelial cells. Thus, at present, laboratories using this procedure would need to monitor the male-to-female ratio of their samples by real-time PCR prior to using this fast protocol. Overall, short extraction times, high yields, inexpensive reagents, and semi-automated platform make alkaline lysis-based pressure cycling technology a promising candidate for extracting DNA from forensic evidentiary materials including rape kits.

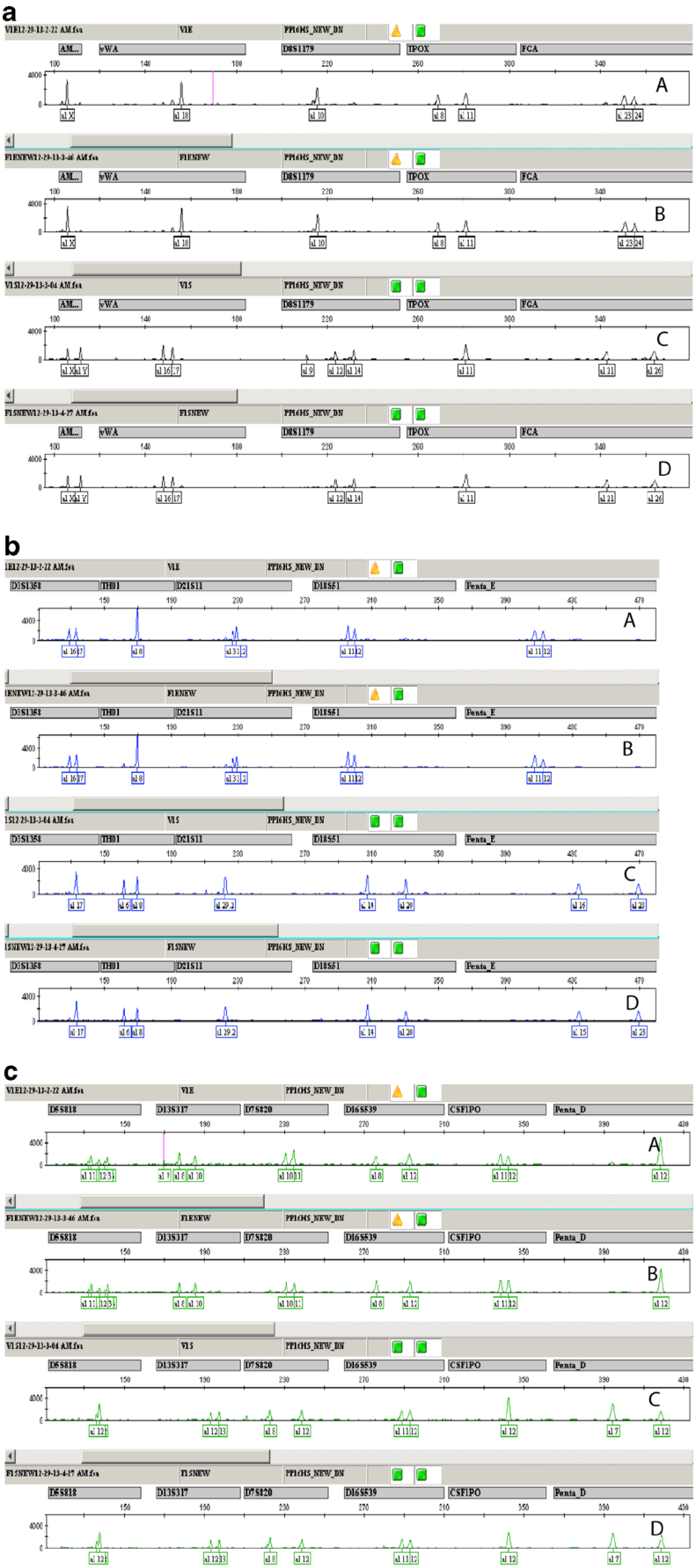


Fig. 4 STR profiles of sperm and epithelial fractions extracted from a post-coital swab. One swab was processed using the BSO crime laboratory method, and another swab from the same volunteer was processed using alkaline lysis and pressure cycling technology. A small portion of the swab was used for extraction and all the extractions were performed in triplicate. **A** Sperm fraction recovered using BSO protocol. **B** Sperm fraction recovered using alkaline lysis and pressure cycling technology. **C** Epithelial fraction recovered using BSO protocol. **D** Epithelial fraction recovered using alkaline lysis and pressure cycling technology

Acknowledgments Major support for this research was provided by award # *2011-NE-BX-K550* from the National Institute of Justice. Points of view in the document are those of the authors and do not necessarily represent the official view of the U.S. Department of Justice. We are thankful to Nathan Lawrence from Pressure Biosciences Inc. and George Duncan from the Broward Sheriff's Office (BSO) crime lab for their technical expertise and the generous support of this research. We are also thankful to Margie Phipps, Vanessa Martinez, and Pero Dimsoski for the technical support.

Conflict of Interest The authors have declared no conflict of interest.

References

1. Baird M, Balazs I, Giusti A, Miyazaki L, Nicholas L, Wexler K, Kanter E, Glassberg J, Allen F, Rubinstein P, Sussman L (1986) Allele frequency distribution of two highly polymorphic DNA sequences in three ethnic groups and its application to the determination of paternity. Am J Hum Genet 39(4):489–501
2. Gill P, Jeffreys AJ, Werrett DJ (1985) Forensic application of DNA "fingerprints". Nature 318(12):577–579
3. Jeffreys AJ, Wilson V, Thein SW (1984) Hypervariable 'minisatellite' regions in human DNA. Nature 314:67–73
4. Burger J, Hummel S, Herrmann B, Henke W (1999) DNA preservation: a microsatellite-DNA study on ancient skeletal remains. Electrophoresis 20(8):1722–1728
5. Fondevila M, Phillips C, Naverán N, Cerezo M, Rodríguez A, Calvo R, Fernández LM, Carracedo Á, Lareu MV (2008) Challenging DNA: assessment of a range of genotyping approaches for highly degraded forensic samples. Forensic Sci Int Genet 1(1): 26–28
6. Raymond JJ, van Oorschot RA, Gunn PR, Walsh SJ, Roux C (2009) Trace evidence characteristics of DNA: a preliminary investigation of the persistence of DNA at crime scenes. Forensic Sci Int Genet 4(1):26–33
7. Norris JV, Manning K, Linke SJ, Ferrance JP, Landers JP (2007) Expedited, chemically enhanced sperm cell recovery from cotton swabs for rape kit analysis. J Forensic Sci 52(4):800–805
8. Vuichard S, Borer U, Bottinelli M, Cossu C, Malik N, Meier V, Gehrig C, Sulzer A, Morerod M, Castella V (2011) Differential DNA extraction of challenging simulated sexual assault samples: a Swiss collaborative study. Investig Genet 2(1):11
9. Voorhees JC, Ferrance JP, Landers JP (2006) Enhanced elution of sperm from cotton swabs via enzymatic digestion for rape kit analysis. J Forensic Sci 51(3):574–579
10. Benschop CCG, Wiebosch DC, Kloosterman AD, Sijen T (2010) Post-coital vaginal sampling with nylon flocked swabs improves DNA typing. Forensic Sci Int Genet 4:115–121
11. Elliot K, Hill DS, Lambert C, Burroughes TR, Gill P (2003) Use of laser microdissection greatly improves the recovery of DNA from sperm on microscope slides. Forensic Sci Int 137(1):28–36
12. Garvin AM, Fischer A, Schnee-Griese J, Jelinski A, Bottinelli M, Soldati G, Tubio M, Castella V, Monney N, Malik N, Madrid M (2012) Isolating DNA from sexual assault cases: a comparison of standard methods with a nuclease-based approach. Investig Genet. doi:10.1186/2041-2223-3-25
13. Horsman KM, Barker SLR, Ferrance JP, Forrest KA, Koen KA, Landers JP (2005) Separation of sperm and epithelial cells in a microfabricated device: potential application to forensic analysis of sexual assault evidence. Anal Chem 77(3):742–749
14. Hudlow WR, Buoncristiani MR (2012) Development of a rapid, 96-well alkaline based differential DNA extraction method for sexual assault evidence. Forensic Sci Int Genet 6(1):1–16
15. Schoell WM, Klintschar M, Mirhashemi R, Pertl B (1999) Separation of sperm and vaginal cells with flow cytometry for DNA typing after sexual assault. Obstet Gynecol 94(4): 623–627
16. Schoell WM, Klintschar M, Mirhashemi R, Strunk D, Giuliani A, Bogensberger G, Pertl B (1999) Separation of sperm and vaginal cells based on ploidy, MHC class I-, CD45-, and cytokeratin expression for enhancement of DNA typing after sexual assault. Cytometry 36(4):319–323
17. Weigand P, Schurenkamp M, Schutte U (1992) DNA extraction from mixtures of body fluid using mild preferential lysis. Int J Legal Med 104(6):359–360
18. Yoshida K, Sekiguchi K, Mizuno N, Kasai K, Sakai I, Sata H, Seta S (1995) The modified method of two-step differential extraction of sperm and vaginal epithelial cell DNA from vaginal fluid mixed with semen. Forensic Sci Int 72(1):25–33
19. Gross V, Carlson G, Kwan AT, Smejkal G, Freeman E, Ivanov AR, Lazarev A (2008) Tissue fractionation by hydrostatic pressure cycling technology: the unified sample preparation technique for systems biology studies. J Biomol Tech 19:189–199
20. Tao F, Lawrence NP, Miller WW, Li C, Tuzmen P, Behnke J, Nakhai B, Kakita A, Christian T, Reed D, Manak MM, Schumacher RT (2003) Biological sample preparation system using pressure cycling technology (PCT). Adv High Press Biosci Biotechnol II:413–417
21. Smejkal GB, Robinson MH, Lawrence NP, Tao F, Saravis CA, Schumacher RT (2006) Increased protein yields from Escherichia coli using pressure-cycling technology. J Biomol Tech 17(2):173–175
22. Bradley DW, Hess RA, Tao F, Sciaba-Lentz L, Remaley AT, Laugharn JA Jr, Manak M (2000) Pressure cycling technology: a novel approach to virus inactivation in plasma. Transfusion 40: 193–200
23. Garrett PE, Tao F, Lawrence N, Ji J, Schumacher RT, Manak MM (2002) Tired of the same old grind in the new genomics and proteomics era? TARGETS 1:156–162
24. Kirkman-Brown J, Björndahl L (2009) Evaluation of a disposable plastic Neubauer counting chamber for semen analysis. Fertil Steril 91(2):627–631
25. Fedder J, Askjaer SA, Hjort T (1993) Nonspermatozoal cells in semen: relationship to other semen parameters and fertility status of the couple. Arch Androl 31:95–103
26. Patil PS, Humbarwadi RS, Patil AD, Gune AR (2013) Immature germ cells in semen correlation with total sperm count and sperm motility. J Cytol 30(3):185–189
27. Johanisson E, Campana A, Luthi R, de Agostini A (2000) Evaluation of 'round cells' in semen analysis: a comparative study. Hum Reprod Update 6(4):404–412