

TECHNICAL NOTE

Criminalistics

Alternative direct-to-amplification sperm cell lysis techniques for sexual assault sample processing

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Abstract

The prevalence of sexual assault cases and increasingly sensitive DNA analysis methods have resulted in sexual assault kit backlogs in the United States. Although traditional DNA extraction and purification utilizing detergents, proteinase K, and DTT have been the primary technique for lysing sperm cell fractions from these samples, it is labor-intensive and inefficient regarding time and sperm DNA recovery – hindering the ability of forensic analysts to keep pace with evidence submissions. Thus, this study examined seven alternative sperm cell lysis techniques to develop a method that could efficiently lyse sperm and consistently generate high-quality profiles while also reducing time, labor, and cost requirements. Microscopic examination of lysates indicated only Casework Direct and alkaline techniques could lyse all spermatozoa within samples, while quantification results demonstrated all methods performed comparably to the control method of *forensicGEM™* Sperm ($p > 0.06$). Amplification with 0.25 ng DNA revealed that unpurified lysates from Casework Direct, alkaline, and NP-40 techniques produced DNA profiles with acceptable mean STR peak heights and interlocus balance, both of which were similar to or better than the control. Overall, this study demonstrated the ability of Casework Direct, alkaline, and NP-40 methods to efficiently lyse spermatozoa and provide high-quality STR profiles despite the absence of a purification step. Ultimately, based on the data reported herein, alkaline lysis is the recommended alternative sperm lysis approach given its ability to generate high-quality profiles, save time, and decrease the cost per reaction when compared to traditional sperm cell lysis methods.

KEYWORDS

alkaline, cell lysis, differential lysis, direct amplification, forensic genetics, sexual assault, spermatozoa

Highlights

- Traditional cell lysis and purification methods have high time and cost requirements.
- Casework Direct, alkaline, NP-40, and HGH methods efficiently lyse sperm.

Co-authors Sarah K. Schellhammer MS and Brittany C. Hudson MS contributed equally.

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- Alternative lysis methods herein produce semen lysates with comparable DNA yields.
- Casework Direct, alkaline, and NP-40 lysates provide high-quality STR profiles.
- Alkaline sperm lysis without purification is compatible with downstream processes.

1 | INTRODUCTION

Modern advances in DNA technology have led to more efficient processing of forensic samples with highly discriminatory results [1-4]. However, unfortunately, sexual assaults are still committed at a rate with which forensic scientists are unable to keep pace – approximately 430,000 victims of rape and sexual assault each year on average in the United States [5,6]. Due to both the abundance of sexual assaults and the time required to process their associated samples in forensic laboratories, backlogs remain a persistent issue.

The most common form of probative biological evidence encountered in sexual assault cases is semen. Semen contains spermatozoa, which are morphologically different from somatic cells; sperm cell heads possess a nuclear cap and acrosome (in addition to the plasma membrane), which protect the nucleus, as well as a midpiece and tail [7]. In addition, protamines replace 85% of the histones around sperm DNA and form many disulfide bonds that enable tight coiling, reducing the sperm cell nucleus to anywhere from 1/7-1/20th that of a somatic cell [8-11]. These differences necessitate strong reducing agents for cell lysis and subsequent access to sperm DNA. In addition to spermatozoa, vaginal epithelial cells are also commonly encountered in sexual assault samples. Due to the nature of how these samples are deposited and the sites from which they are collected, there is often an overwhelming number of (epithelial) cells from the female contributor compared to those from the male contributor in sexual assault evidence. This frequently results in an imbalanced mixture DNA profile and/or a masked male DNA profile [12]. However, the secondary DNA contributor in an STR profile is often undetectable when using traditional capillary electrophoresis if present at a level $\leq 1/10$ th that of the primary DNA contributor, a situation which is often encountered in sexual assault samples due to their intimate nature [12-14]. To circumvent this and other general issues experienced with mixture samples, a differential cell lysis is typically performed for sexual assault evidence as a way to physically separate sperm from epithelial cells. With these methods, differences in both morphology and susceptibility to lysis reagents are exploited in order to enrich the male contributor and prevent downstream complicated mixture profiles [15,16]. Given the increased sensitivity of forensic DNA kits and the fact that a single spermatozoon contains 3.3 picograms (pg) of DNA (which is half the amount within diploid epithelial cells), full DNA profiles can now be obtained from as few as 50 sperm cells [17,18]; this makes the ability to retain as many sperm cells as possible (without contaminating non-sperm cells) very crucial for male DNA profile generation.

While traditional sperm cell fraction lysis methods are viable, the techniques used are inefficient, laborious, time-consuming, and often require multiple tube-to-tube transfers [15,16,19-21]. Thus, a

cell lysis method that considerably decreases processing time while reducing the risk for contamination and sample loss is needed so that labs can process samples of this nature more efficiently and effectively, potentially providing a step toward reduction of the existing sexual assault backlogs. The issues outlined above have spurred an influx of research on the use of alternate cell lysis techniques [10,21-29] – including those that may be amenable to PCR *without* subsequent purification [30,31]. Such methods encompass alkaline-based cell lysis and the use of nonyl phenoxyethoxyethanol (NP-40) cell lysis buffer, both of which are non-proprietary chemical approaches that have been reported to disrupt cell membranes from a variety of cell types, including sperm cells [27,32-36]. Alkaline lysis solutions exert a strong denaturing effect on proteins and are an efficient means of protein solubilization due to the ionization of certain amino acids. This technique utilizes sodium hydroxide (NaOH) to disrupt plasma membranes, denature nucleases, and preserve the DNA, while the subsequent addition of Tris-HCl neutralizes the lysate and enhances stability [32,33,37]. NP-40 cell lysis buffer is a mild, non-ionic detergent commonly used for DNA extraction and purification in other, non-forensic applications [38,39]. NP-40 has also been successfully used for direct amplification of crude blood samples and is believed to have potential for various sample types (including semen) [36].

Alternatively, there are proprietary, commercially available direct-to-PCR kits that can be used to lyse cells commonly associated with sexual assault samples without disrupting downstream PCR processes. For example, Promega's™ Casework Direct utilizes 1-thioglycerol in place of DTT for successful lysis of sperm cells. This kit has produced reliable profiles from a variety of forensically relevant samples and has already been implemented in forensic science for Y-screening and autosomal STR profiling [40-43]. Another proprietary reagent from Promega™ that has been investigated for direct-to-PCR amplification of semen samples is SwabSolution™. This lysis reagent requires only a single incubation step, has zero tube transfers, and has been used to reliably produce high-quality STR profiles in previous studies [31,44].

In addition to the aforementioned non-proprietary and commercial cell lysis techniques, natural sperm decondensation approaches could potentially be used to expose the DNA within semen samples. Published studies on intracytoplasmic sperm injection (ICSI) employing this approach have revealed the use of Triton X-100 (TX), glutathione (GSH), HEPES buffer, and heparin for sperm cell decondensation and the removal of acrosomal caps in such a way that resembles the natural fertilization process [45-48]. Although there has yet to be any research on the implementation of these reagents for extracting DNA from spermatozoa for forensic applications, a combination of these reagents could allow for the plasma

and acrosomal membranes to be easily removed, thereby permitting the sperm nucleus to be decondensed and the nuclear material to be quickly released.

Ultimately, the development of alternative cell lysis techniques could prove helpful in tackling the current sexual assault case backlog by saving both time and costs. In an effort to identify a faster, inexpensive, more efficient process for sperm cell lysis that could be easily implemented into the current forensic DNA workflow, several of these non-traditional cell lysis techniques were evaluated and compared to identify the best performing method based on DNA yields, quality of resulting STR profiles, as well as cost and time requirements.

2 | MATERIALS AND METHODS

2.1 | Sample Collection and Preparation

Semen samples were collected from 10 anonymous donors in accordance with the university-approved Institutional Review Board (IRB) protocol HMW20002931 and were diluted 1:10 by volume in 1X phosphate-buffered saline (PBS) (pH 7.4) (Quality Biological). Fisherbrand™ PurSwab foam swabs (Fisher Scientific) were dipped into the semen dilutions (absorbing approximately 80 μ l) and were allowed to dry overnight at room temperature; multiple dilutions and swabs were prepared per donor to accommodate all methods and replicates tested. Once dry, the swabs were cut into twelfths and stored at 4°C. Subsequent testing for all cell lysis methods utilized 1/12th of a foam swab (equivalent to approximately 0.67 μ l of neat semen) for each donor in triplicate.

2.2 | Cell Lysis and DNA Liberation

2.2.1 | *forensicGEM*™ Sperm

The *forensicGEM*™ Sperm kit (microGEM™) served as the control direct-to-amplification cell lysis method for this study. For this method, 2.0 μ l *forensicGEM*™ enzyme, 10 μ l Acrosolv, and 10 μ l 10X ORANGE+ Buffer were added to each semen sample and the reactions were brought up to 100 μ l with HyPure Molecular Biology Grade (MBG) Water (GE Healthcare Life Sciences). Reactions were placed onto the ProFlex™ PCR 2x96-well PCR system (Thermo Fisher Scientific) and incubated as follows: 52°C for 10 min, 75°C for 3 min, and 95°C for 3 min.

2.2.2 | SwabSolution™

Modified versions of the SwabSolution™ and proteinase K method described by Tobe et al. were assessed in this study to determine the appropriate incubation time for optimal lysis [31]. Samples were incubated in 23 μ l of SwabSolution™ (Promega™) and 2.0 μ l Proteinase

K (Thermo Fisher Scientific) at 70°C for either 15, 30, or 60 min [31,49]. Lysates were stored at 4°C until further processing. The best performing reaction time was used in all subsequent tests.

2.2.3 | Casework Direct System

Modified versions of the manufacturer-recommended protocol for the Casework Direct System (Promega™) were tested in an attempt to reduce the total reaction volume. Semen swab cuttings were incubated for 30 min at 70°C in either 25, 50, or 100 μ l of Casework Direct solution (Promega™) containing 0.125, 0.25, and 0.5 μ l 1-thioglycerol (Promega™), respectively [40]. After incubation, swabs were placed in a spin basket and centrifuged for five minutes at 10,000 \times g to maximize liquid recovery. Lysates were stored at 4°C until further processing. The best performing reaction condition was used in all subsequent tests.

2.2.4 | NP-40 Cell Lysis Buffer

Three different strengths of NP-40 cell lysis buffer (Thermo Fisher Scientific) were evaluated to ensure optimal cell lysis while also minimizing PCR inhibition. Semen swab cuttings were submerged in 25 μ L of either 1%, 0.75%, or 0.5% NP-40 lysis buffer (diluted in MBG water). All reactions were incubated on ice for 30 min with vortexing every 10 min, as recommended by the manufacturer protocol [50]. Swabs were transferred to spin baskets and centrifuged at 13,000 \times g for 10 min. Lysates were stored at -20°C until further processing. The best performing reaction strength was used in all subsequent tests.

2.2.5 | Alkaline Lysis

Semen-soaked swab cuttings were incubated in 16 μ l of 1X PBS and 4.0 μ l of 1 M NaOH (Thermo Fisher Scientific) at 75°C for 5 min [32,33]. Following incubation, 4.0 μ l of 1 M Tris-HCl (Invitrogen) were added to neutralize the lysate and the samples were briefly vortexed. The swabs were then transferred to a spin basket and centrifuged for 5 min at 13,000 \times g. Lysates were stored at -20°C until further processing.

2.2.6 | HEPES Buffer + Triton X-100 (HTX)

In an attempt to mimic the female body's approach for sperm cell decondensation and lysis, semen samples were subjected to two different "natural sperm decondensation" assays. The first assay utilized a stock solution of HEPES buffer (Sigma Aldrich) containing 0.04% Triton X-100 (Thermo Fisher Scientific). For this approach, semen samples were submerged in 25 μ l of HEPES/Triton X-100 (HTX) solution and vortexed for one minute [45]. Swab cuttings

were then placed in spin baskets and centrifuged for three minutes at 17,000 × g. Lysates were stored at 4°C in foil until further processing to prevent reactive-oxygen species formation [51].

2.2.7 | Modified HTF Medium + Glutathione + Heparin (HGH)

The second “natural sperm decondensation” assay consisted of a stock solution of modified HTF medium (Irvine Scientific; Santa Ana, CA) containing 10 mmol/L glutathione (Sigma Aldrich) and 46 μmol/L heparin (Sigma Aldrich) [48,52]. Semen-soaked swab cuttings were incubated in 25 μl of the HTF/glutathione/heparin (HGH) solution at 37°C for either 15, 30, or 60 min [48,52]. Lysates were stored at 4°C until further processing. The best performing reaction time was used in all subsequent tests.

2.3 | Microscopy

Prior to and following cell lysis, Kernechtrot Picroindigocarmin Stain (KPICS) was applied to each sample to visually gauge the effectiveness of each lysis method. For this, two microliters of pre- and post-lysis samples were spotted onto a microscope slide and stained with one drop of Kernechtrot stain (Serological Research Institute; Richmond, CA) and one drop of Picroindigocarmin stain (Serological Research Institute). Sperm cells were then visualized under a Micromaster microscope (Thermo Fisher Scientific) using 400x magnification. Each sperm slide was scored using a 0–4+ scale with “0” indicating that no sperm were identified, “1+” indicating there was a single spermatozoon observed in some fields, “2+” indicating 1–5 sperm were observed in most fields, “3+” indicating 5–10 sperm were observed in most fields, and “4+” meaning more than 10 sperm were observed in all fields; for each sample, 10 different fields-of-view were scored to produce a mean score.

2.4 | DNA Quantification

In order to determine the total amount of DNA obtained from each sample after each lysis method, resulting lysates were quantified using the Investigator® Quantiplex HYres kit (QIAGEN) on the Applied Biosystems® 7500 Real-Time PCR System (Thermo Fisher Scientific) following manufacturer recommendations, with modifications for half-volume reactions. This included 4.5 μl Reaction Mix FQ, 4.5 μl Primer Mix IC YQ, and 1.0 μl template DNA per sample. However, given that SwabSolution™ and Casework Direct kit components are known to inhibit real-time PCR (qPCR), an additional 2.0 μl of 5X AmpSolution™ (Promega™) were added to these lysate groups (and corresponding standards) to ensure accurate results [40,49]. Thermalcycling conditions were as follows: 95°C for three minutes, followed by 40 cycles of 95°C for 5 s and 60°C for 35 s. Data were analyzed using the Sequence Detection System (SDS)

software version 1.4 (Applied Biosystems™) with an automatic threshold and baseline for analysis of each target.

Prior to analysis of DNA quantity, qualitative metrics for amplification and component plots were assessed using criteria previously described by Hudson et al. [53] to identify any potential signs of inhibition. Total DNA yields for each sample were calculated by multiplying the appropriate target's concentration by the elution/sample volume (which differed for each method and variable tested). The mean and standard deviation for each experimental group were then calculated and compared. If a potential outlier was observed, Grubb's outlier test was performed by subtracting the mean from the suspected outlier value and dividing by the standard deviation. If the G_{test} was greater than the G_{critical} , the outlier was confirmed and removed. For the lysis methods that had multiple conditions tested (e.g., SwabSolution™, Casework Direct, NP-40, and HGH), an ANOVA was performed to compare the DNA yields of the control method (*forensicGEM™ Sperm*) to the three conditions tested of that given method ($\alpha = 0.05$). Any significant differences were further identified using a Tukey HSD test in order to establish which condition(s) to select for downstream analysis. Once the best performing conditions were identified, an ANOVA was then performed to compare all cell lysis methods to one another, and any significant differences were further identified using a Tukey HSD test.

2.5 | STR Amplification

All samples in this study were amplified using the Promega™ PowerPlex® Fusion 5C System with a template DNA input of 0.25 ng and following manufacturer recommendations, but with half-volume reactions. Thus, each reaction consisted of 2.5 μl sample (at 0.1 ng/μl), 2.5 μl PowerPlex® Fusion 5X Master Mix, 2.5 μl PowerPlex® Fusion 5X Primer Pair Mix, and 5.0 μl amplification-grade water. Amplification was carried out using the ProFlex™ 2x96-well PCR System and the following parameters: 96°C for 1 min, 30 cycles (94°C for 10 s; 59°C for 1 min; 72°C for 30 s), and a 60°C hold for 45 min.

2.6 | Capillary Electrophoresis and Data Analysis

STR amplicons were separated on an ABI® 3130 Genetic Analyzer (Thermo Fisher Scientific) using Data Collection software v3.1 (Thermo Fisher Scientific). One microliter of an amplified sample or allelic ladder was added to 0.3 μl WEN ILS 500 (Promega™) and 9.7 μl Hi-Di™ Formamide (Thermo Fisher Scientific), except for samples lysed using the SwabSolution™ and Casework Direct methods (for which 0.5 μl of each sample was added to 0.3 μl WEN ILS 500 and 10.2 μl Hi-Di™ Formamide). Injection parameters followed manufacturer recommendations and included a 36 cm capillary array (Thermo Fisher Scientific), POP-4® polymer (Thermo Fisher Scientific), and a 3 kV 5 s injection. Results were analyzed with GeneMapper™ software v4.1 (Thermo Fisher

Scientific) following manufacturer recommendations and an analytical threshold of 100 RFU [54].

Profiles were qualitatively and quantitatively evaluated for any signs of inhibition (e.g., interlocus balance and allelic dropout). Mean peak heights were calculated by finding the average peak height of all observed STR alleles across all sample profiles obtained for each lysis method. To account for homozygosity, the peak heights for homozygous alleles were halved to represent each of the assumed two copies of the allele at that locus. Based on our laboratory's internal validation for PowerPlex® Fusion 5C, the ideal mean peak height for the target input was expected to be ~1645 RFU (B. Hudson, personal communication, Nov. 2020). The coefficient of variation (CV) for locus peak height: total peak height (LPH:TPH) ratios for each locus of the entire DNA profile was calculated to estimate interlocus balance, excluding the Amelogenin and DYS391 loci (since one is not an STR locus and the other is a single-copy locus, respectively). The CV was calculated by first determining the LPH:TPH ratio by dividing the sum of a locus's peak height by the sum of the peak heights at all loci; this was repeated for each locus. The standard deviation of all LPH:TPH ratios was then calculated and divided by the mean of those ratios to calculate the CV. Based on the available literature, ideal CV values when using this method are ≤ 0.35 , which signifies the peak heights at any given locus vary no more than 35% from peak heights at other loci within the same DNA profile [55]. For the mean peak heights, the mean percentage of STR alleles detected, and the mean CV of LPH:TPH, an ANOVA was performed to compare the results of the control method to the six additional lysis methods evaluated ($\alpha = 0.05$). If the ANOVA resulted in a $p < 0.05$, a Tukey HSD test was performed to identify where the significant differences occurred.

3 | RESULTS AND DISCUSSION

3.1 | Method Optimization

Based on quantification data, sample processing times, and assay volumes, the 30-minute incubation time for SwabSolution™, the 25 μ l reaction volume for Casework Direct, the 0.5% NP-40 cell lysis buffer concentration, and the 15-min incubation time for HGH cell lysis were selected as the best conditions for each method (data not shown). Thus, these conditions were used for all downstream analyses.

3.2 | Microscopy

Based upon microscopic visualization of the lysates, the Casework Direct and alkaline lysis techniques were the only methods that resulted in the complete lysis of sperm cells (i.e., mean score of "0"). Samples lysed with NP-40 and HTX methods resulted in a mean score of "3+," while samples lysed using *forensicGEM*™ Sperm resulted in a mean score of "1+" (data not shown). Additionally, as

expected, those samples lysed with SwabSolution™ and HGH methods exhibited a decrease in the number of sperm visualized as the incubation time increased (data not shown).

3.3 | DNA Quantification

A significant difference in DNA yields was observed across all cell lysis methods tested ($p = 0.0037$). Although the *forensicGEM*™ Sperm, alkaline, and HGH cell lysis groups produced the highest mean DNA yields, subsequent statistical analysis with Tukey HSD revealed that only the differences between the HTX and the HGH cell lysis groups were significant. HTX cell lysis samples produced DNA yields that were as much as 13% lower than those produced by HGH cell lysis ($p = 0.0377$, Figure 1). No other significant differences in DNA yields were revealed between any other experimental groups ($p > 0.06$, Figure 1).

Additionally, quantification results for all samples showed qPCR component plots with the expected sigmoidal curves for each target dye, as well as a characteristic flat curve for the passive reference dye. The amplification plots revealed a characteristic trend with samples crossing the threshold during the exponential phase, where those samples with higher DNA concentrations crossed the threshold at an earlier cycle than those with lower DNA concentrations, as expected. However, under certain lysis conditions, the internal PCR control (IPC) curves crossed the threshold at a later cycle than expected ($C_q \geq 29$), which is consistent with inhibition and often results in the underestimation of DNA concentration [56–58]. This phenomenon occurred most notably in those semen samples lysed with SwabSolution™ and Casework Direct, which was anticipated given that the addition of the 5X AmpSolution™ generated a lower efficiency reaction by inherently diluting the other master mix components. Additionally, 20% of the donor samples processed with alkaline cell lysis and 70% of the donor samples lysed using the HTX and HGH methods displayed delayed IPC values; however, because

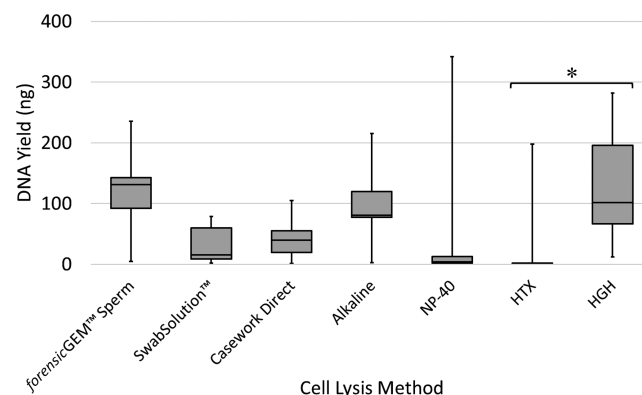


FIGURE 1 Mean DNA yields obtained from real-time PCR (qPCR) comparing *forensicGEM*™ Sperm ($n = 9$) to six alternative cell lysis methods ($n = 10$). Significant decreases in DNA yield were observed between HTX cell lysis and HGH cell lysis groups ($p < 0.05$). No other significant differences were observed

the IPC was not delayed in every sample and was only noted in the non-proprietary experimental groups when a new quantification kit lot was utilized, these unexpected observations could be due to donor differences or lot-to-lot variation among the reaction components.

3.4 | STR Analysis

STR profiles were analyzed and compared across all donors for each lysis method, with the goal of identifying the best performing technique(s) – that which was most likely to achieve full STR profiles and generate STR profiles of equal or higher quality than the control method (*forensicGEM™ Sperm*). The HTX cell lysis sample profiles displayed a significant reduction in the percentage of STR alleles detected compared to all other lysis methods by as much as 48% ($p < 0.00005$, Figure 2). No significant differences were observed between any of the other alternative methods and the control, and all other lysis methods produced profiles with >90% of expected STR alleles detected (Figure 2).

In addition to profile completeness, STR allele peak heights for each lysis method tested were expected to be consistent with those previously observed in the laboratory's internal validation (B. Hudson, personal communication, Nov. 2020) and within the dynamic range of the instrument (up to ~8200 RFU) [59], as well as at or above those observed from the control method (*forensicGEM™ Sperm*). Although not significant, the Casework Direct, alkaline, and NP-40 cell lysis methods generated mean STR allele peak heights that were slightly higher than those obtained from samples processed using *forensicGEM™ Sperm* ($p > 0.1$, Figure 3). Further, the alkaline and NP-40 cell lysis groups had more samples reach or exceed the expected mean STR allele peak height than all other methods tested (Figure 3). Significant decreases in mean peak height were observed with HTX samples when compared to alkaline ($p = 0.0009$), Casework Direct ($p = 0.0108$), and NP-40 cell lysis groups ($p = 0.0029$). Additionally,

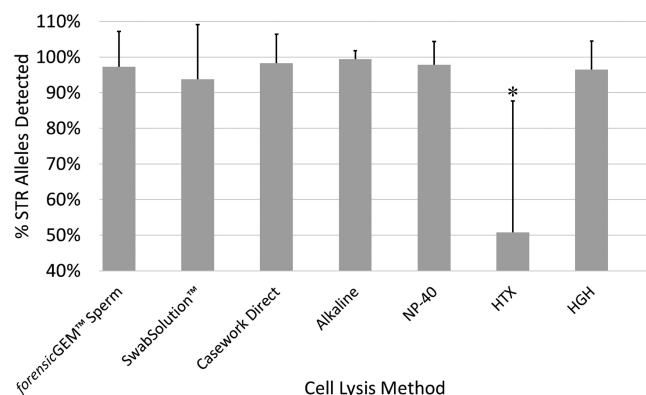


FIGURE 2 Percentage of STR alleles detected for samples processed with each cell lysis method ($n = 10$). HTX cell lysis produced significantly fewer STR alleles than all other cell lysis methods ($p < 0.00005$), but no other significant differences were observed

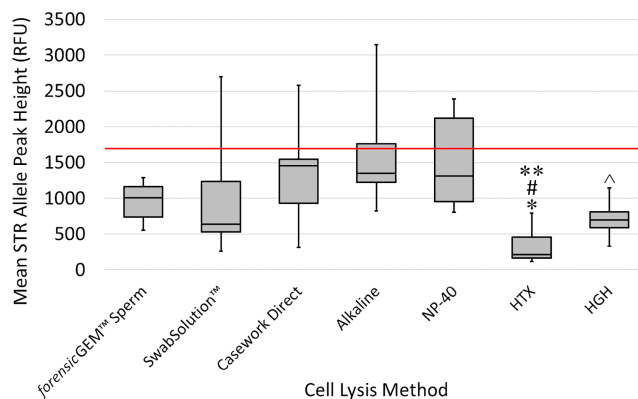


FIGURE 3 Mean STR allele peak heights observed for samples processed with each of the seven cell lysis methods ($n = 10$). Lysates from HTX cell lysis demonstrated profiles with significantly lower peak heights compared to lysates from alkaline ($*p = 0.0009$), Casework Direct ($\#p = 0.0108$), and NP-40 ($**p = 0.0029$) cell lysis methods. Additionally, STR profiles of samples lysed with HGH exhibited significantly lower peak heights than those from samples processed with alkaline cell lysis ($^{\wedge}p = 0.0216$). Although there were no significant differences when comparing mean peak height in STR profiles of samples lysed with all alternative methods compared to the control group, STR profiles from samples lysed with Casework Direct, alkaline, and NP-40 methods exhibited mean peak heights that were slightly higher. The red line throughout represents the expected mean peak height as reported in our laboratory's internal validation of the PowerPlex® Fusion 5C kit (B. Hudson, personal communication, Nov. 2020) [Color figure can be viewed at wileyonlinelibrary.com]

mean peak heights from those samples processed using the HGH method were significantly lower than those processed with alkaline lysis ($p = 0.0216$). It is important to note that, overall, peak heights were likely lower than the validated mean due to differences in sample preparation; all DNA samples amplified for the internal validation were those that had been purified after cell lysis.

Minimal variation in STR allele peak heights across all loci within a single STR profile (i.e., interlocus balance) is essential, as mixture profile deconvolution relies on the assumption that balance is obtained and is consistent across the entire sample profile. Adequate interlocus balance assures that all allele peaks are sufficiently above the analytical threshold so that allelic dropout is avoided and true homozygosity can be confidently determined, and it enables analysts to distinguish peaks from one contributor versus another. All lysis methods explored in this study exhibited comparable interlocus balance to the control method except the HTX cell lysis group, which displayed a significant increase in CV (i.e., worsened interlocus balance) versus all other methods ($p < 0.00005$, Figure 4). While not significantly different, it should be noted that samples processed with the alkaline lysis method produced a mean CV lower than that of the control group and lower than the optimal value of 0.35 ($p = 0.99$, Figure 4).

Overall, when STR profiles from semen samples processed with each cell lysis method were evaluated, no method significantly or consistently outperformed the control method (*forensicGEM™*

Sperm) across every metric examined; however, three of the alternative cell lysis methods exhibited positive improvements in some metrics when compared to the control (Table 1), making them the most suitable alternative sperm lysis methods tested. Lysates from Casework Direct, alkaline lysis, and NP-40 cell lysis buffer techniques all exhibited similar or higher percentages of STR allele detection, slightly higher mean STR allele peak heights (some higher than expected based on our internal validation), and comparable or better interlocus balance (Figure 5). Notably, samples processed using the HTX cell lysis method consistently performed more poorly than all other sample groups, resulting in lower peak heights, poor interlocus balance, and substantial allelic dropout (Figure 6). It should be noted that, although not shown here, the same semen lysates were also amplified using a specific input volume rather than a targeted DNA amount to evaluate situations in which quantification may not be performed (e.g., direct amplification and microfluidic devices). When STR profiles from each lysis method were evaluated using this approach, results were consistent with those obtained in the studies reported herein when a specific DNA input of 0.25 ng was amplified,

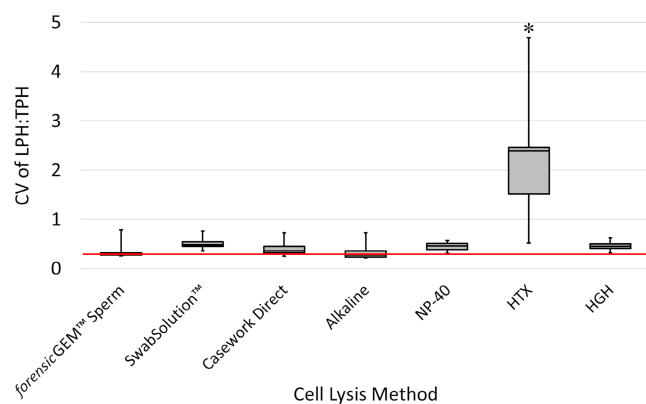


FIGURE 4 Mean interlocus balance (CV of LPH:TPH) for samples processed with the seven alternative lysis methods ($n = 10$). The optimal CV (≤ 0.35) is represented by the red line [55]. STR profiles of samples lysed with the HTX method exhibited significantly higher CV (i.e., worse interlocus balance) than all other lysis methods tested ($p < 0.00005$). STR profiles from samples lysed using all other methods exhibited CV below or slightly above 0.35, which indicates a relatively balanced DNA profile [Color figure can be viewed at wileyonlinelibrary.com]

with lysates from the HGH method also exhibiting high-quality results (data not shown).

Because the ability to tackle the sexual assault kit backlog is impacted by more than just STR profile quality, one must consider additional factors prior to the implementation of new lysis techniques (such as the time required for hands-on and overall processing, as well as cost); therefore, these factors were also examined. Assuming a sample size of 20, the estimated hands-on time for the sperm lysis portion of a traditional differential cell lysis and purification (e.g., a standard Qiagen® DNA extraction) is ~90 min, while the total processing time is ~180 minutes (Table 2) [60]. Not only is this a manual, time-consuming process, but it also includes proprietary components, is the most expensive per reaction (compared to alternative methods in this study), and presents a number of challenges when attempting to integrate onto an automated platform (Table 2) [61,62]. While the *forensicGEM™* Sperm kit offers a fast alternative that is easily automatable, it is the second most expensive method of those tested during this study and it contains proprietary components. Of the additional alternative lysis approaches examined herein, SwabSolution™ and Casework Direct also include proprietary components, which inherently makes them more expensive to implement in forensic laboratories (Table 2). Alternatively, all other methods that were explored in this study have an approximate cost of less than one dollar per reaction; they also only require 30–50 min of hands-on time and 40–85 minutes of total processing time ($n = 20$) (Table 2), which is advantageous over traditional methods. Finally, the NP-40 and HTX cell lysis techniques tested herein demonstrated other issues that could limit their widespread adoption. The NP-40 lysis buffer technique requires an on-ice ($\sim -4^{\circ}\text{C}$) incubation that can be difficult to achieve in an automated, miniaturized format, while cell lysis using HTX solution without downstream DNA purification resulted in inhibited STR profiles to a level that may substantially complicate profile interpretation.

Ultimately, based on the DNA quantity and STR profile quality obtained in these studies, as well as the time and cost considerations, the alkaline lysis method is proposed as the best alternative sperm cell lysis technique for sexual assault samples after the traditional, more “gentle” epithelial cell lysis and cell separation steps have been performed. This technique could also be used for direct amplification of semen, which may be needed for microdevice-based, rapid

Alternative Lysis Method	Peak Height (RFU)	Interlocus Balance (CV of LPH:TPH)	STR Alleles Detected
<i>forensicGEM™</i> Sperm	941.98 ± 385.18	0.386 ± 0.24	97%
SwabSolution™	999.15 ± 958.35	0.499 ± 0.21	94%
Casework Direct	1388.04 ± 807.84	0.389 ± 0.14	98%
Alkaline Lysis	1590.64 ± 817.17	0.326 ± 0.17	99%
NP-40 Lysis Buffer	1538.06 ± 787.84	0.454 ± 0.14	98%
Natural Decondensation – HTX	406.61 ± 305.33	1.861 ± 1.32	51%
Natural Decondensation – HGH	705.00 ± 364.23	0.457 ± 0.11	97%

TABLE 1 Summary of quantitative STR data for samples lysed with each alternative method ($n = 10$)

FIGURE 5 The green channel of representative electropherograms displaying samples lysed using the *forensicGEM™ Sperm* (A), Casework Direct (B), alkaline (C), and NP-40 (D) methods. An increase in mean STR allele peak heights, as well as similar interlocus balance, was observed for samples lysed with the alternative methods compared to the control (*forensicGEM™ Sperm*) [Color figure can be viewed at wileyonlinelibrary.com]

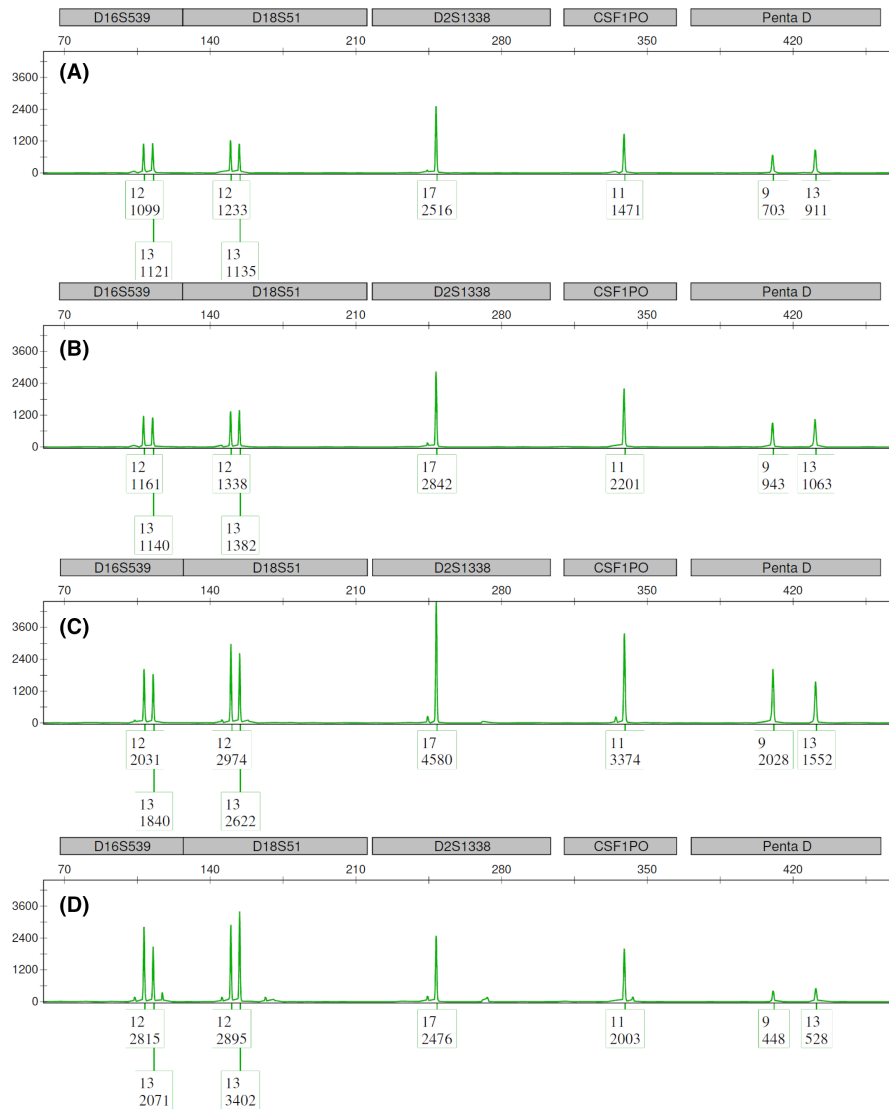
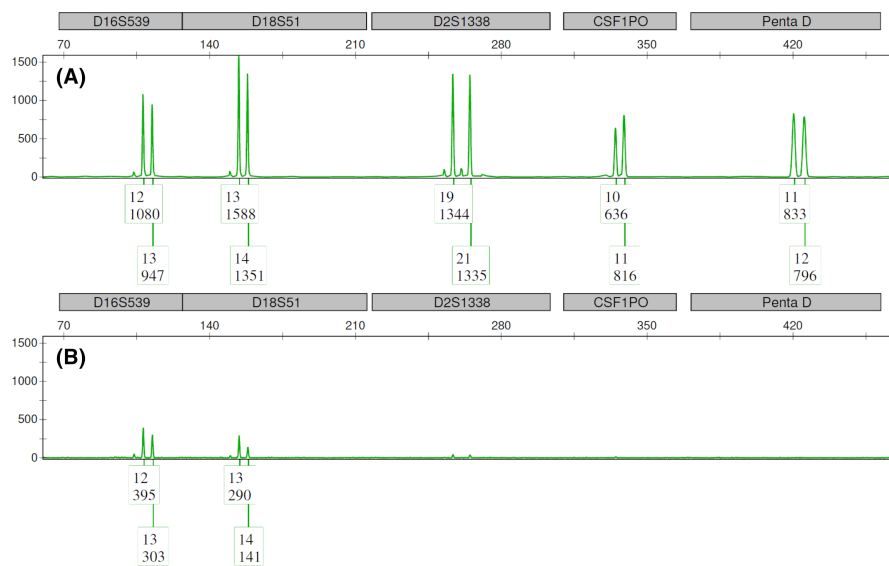


FIGURE 6 The green channel of representative electropherograms comparing samples lysed with the control method of *forensicGEM™ Sperm* (A) to those lysed using HTX (B). Samples lysed with HTX exhibited allelic dropout, diminished peak heights, and ski-slope [Color figure can be viewed at wileyonlinelibrary.com]



casework processing. Further, it should be noted that preliminary evaluation of alkaline lysis with vaginal swab eluates and mock sexual assault samples indicated its ability to lyse epithelial cells as well

as sperm cells (data not shown); therefore, it could potentially be used for lysis and direct amplification of numerous sample types. Overall, this method offers a quick, low-cost, non-proprietary option

TABLE 2 Summary of additional factors to consider when identifying alternative lysis method(s) for future implementation

Alternative Lysis Method	Cost (per reaction) ^a	Hands-on Time (mins) ^b	Total Processing Time (mins) ^b	Ownership	Potential Issue(s) with Automation
Standard Qiagen	\$5.60	90	180	Proprietary	Large volumes, wash steps, silica filtration
<i>forensicGEM</i> TM Sperm	\$4.30	30	50	Proprietary	None
SwabSolution TM	\$0.144	45	75	Proprietary	None
Casework Direct	\$0.625	50	85	Proprietary	None
Alkaline Lysis	\$0.321	45	55	Non-Proprietary	None
NP-40 Lysis Buffer	\$0.007	50	85	Non-Proprietary	Incubation on ice
Natural Decondensation – HTX	\$0.018	30	40	Non-Proprietary	CE inhibition
Natural Decondensation – HGH	\$0.031	30	50	Non-Proprietary	None

^aAll costs are approximations based on current available pricing for VCU.

^bAll times are approximations ($n = 20$) based on internally validated protocols.

that consistently produces high-quality STR typing results without the requirement of lysate purification.

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