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Evaluation of Cell Lysis Techniques for Direct Amplification of Sexual Assault Samples

Sarah Schellhammer
Virginia Commonwealth University

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Sarah K. Schellhammer

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Dawson Green Forensic Molecular Biology Laboratory
Virginia Commonwealth University, Richmond VA
Department of Forensic Science

Tracey Dawson Green, Ph.D.
Chair and Professor
VCU Department of Forensic Science

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Abstract

Sexual assault is one of the most common crimes committed in the United States today and analysis of evidence from these crimes can take weeks to months and even years, resulting in the "rape kit backlog". The traditional method to extract DNA from sexual assault evidentiary samples containing both male and female DNA is a differential extraction, which takes hours itself to complete. Because of the inefficient and time-consuming nature of this technique, the Dawson Green Laboratory is currently developing a microfluidic device that performs cell lysis and amplification on sexual assault samples in approximately one hour. The current lysis method used, *forensic*GEM™ Sperm, has exhibited low-level profiles, allelic drop-out, and inhibition. Thus, this research study examined a variety of different cell lysis techniques ranging from direct cell lysis to natural sperm decondensation assays to develop a method which can consistently generate high-quality profiles.

Six cell lysis methods, in addition to the currently used *forensic*GEM™ Sperm kit (i.e., control), were performed on semen swabs from ten different donors. Quantification using Investigator® Quantiplex HYres revealed a significant difference between the control method and both the SwabSolution™ and Casework Direct methods (p = 0.000116 and p = 0.0102, respectively). No significant differences were noted between the control method and NP-40 cell lysis buffer or HTF medium + glutathione + heparin (HGH). Based on a number of factors including average DNA yield, processing time, reaction volume, and statistical significance, it was determined that the best conditions to pursue for downstream analysis were the 30-minute incubation for SwabSolution™, 25µL reaction volume for Casework Direct, 0.5% strength for NP-40, and the 15-minute incubation for HGH, as well as continuing to test the alkaline and HEPES + Triton X-100 (HTX) methods.

Once quantified, samples underwent amplification with the Promega™ PowerPlex® Fusion 5C System using both a normalized assay to mimic the traditional in-tube workflow and a non-normalized assay to simulate how samples would be processed in the microdevice. Those samples processed through the traditional, in-tube assay revealed three methods trending in the right direction: Casework Direct, alkaline Lysis, and NP-40 cell lysis buffer due to their increase in peak heights compared to the control and their interlocus balance (CV of LPH:TPH) being similar to the control (CV = 0.38) or lower. As for those samples processed using a simulated chip-based approach where a specific volume of sample was amplified, Casework Direct and alkaline lysis exhibited a trend in the right direction based on their average peak heights (2797 RFU and 3572 RFU, respectively). Additionally, HGH cell lysis exhibited a promising trend with an average peak height of 3110 RFU. In addition, these three methods also exhibited a CV of LPH:TPH of that similar to the control and just above the expected CV (>0.35) with 100% STR alleles detected.

Overall, the data provided positive preliminary results for the use of alternative cell lysis techniques both for in-tube assays in the laboratory, as well as the chip-based approach for a microfluidic device. Moving forward, these cell lysis methods should be tested in conjunction with the separation assay on simulated mixtures to represent a sexual assault sample. Additionally, they should be tested on-chip to identify any issues that may arise when performing lysis in that environment. Lastly, it may be necessary to explore modifications to improve these cell lysis methods in the future.

Keywords: differential extraction, direct cell lysis, natural sperm decondensation, SwabSolution™, Casework Direct, Alkaline Lysis, NP-40 cell lysis, HEPES, Triton X-100, HTF medium, Glutathione, Heparin, quantification, STR amplification, interlocus balance

Introduction

Statement of Purpose

Bodily fluid identification has been crucial to the field of forensic science for more than 50 years; however, the first case using DNA analysis in the United States occurred in 1986. When first used, DNA analysis was merely a means of characterization, meaning a wrongful conviction could potentially be made. However, advanced studies on DNA itself over the past few decades have led to the understanding that DNA may be a means of absolute identification [1]. Further, improvements in DNA technology have enabled automation, increased sensitivity in DNA detection allowing DNA profiles to be produced from sub-nanogram amounts, the ability to multiplex samples (simultaneous amplification of several target loci), as well as >350 exonerations in the United States [2-5]. While advances in DNA testing have led to quicker (and more discriminatory) results, sexual assaults are still committed at a rate that forensic scientists are unable to keep up with. On average, there are 433,648 victims of rape and sexual assault each year in the United States, and for every ten individuals, nine of them are women [6,7]. Due to both the abundance of sexual assaults and the time required to process samples in forensic laboratories, there is a delay in the processing of such evidence samples. This delay results in what is commonly referred to as the 'rape kit backlog'.

During a sexual assault, there is a variety of physical evidence left behind by the perpetrator. Thus, it is important that the victim calls the police, goes to the hospital, and gets a physical evidence recovery kit (PERK) exam in order for the evidence to be retrieved. The PERK exam allows for the collection of any potential evidence transferred from the suspect to the victim at the time of the assault, including (but not limited to): clothing, hair, swabs from various areas of the body, and the documentation of any external injuries [8,9]. The most probative items of evidence

are generally gynecological swabs. These samples are often referred to as “mixed” samples because of the likely presence of both female and male cells after an assault; they are historically processed in forensic laboratories using a differential cell lysis technique. Differential cell lysis allows for the separation of male and female cellular components based on their varied resistance to and interaction with the reagents used [10]. Unfortunately, differential cell lysis is a time-consuming, labor intensive technique requiring many tube-to-tube transfers; even when automated, this method can take up to three hours [11]. Given the additional time needed for processing, the prevalence of sexual assault samples submitted, and the understaffing that many forensic laboratories are experiencing, a significant ‘rape kit backlog’ has persisted in many US jurisdictions.

In 2004, the President proposed a multi-million-dollar funding policy to address the issues with DNA analysis, specifically, the DNA backlog. This backlog consists of two primary groups (casework samples and convicted offender samples) and it has two perceived root causes: law enforcement never submitting/requesting DNA analysis and evidence submission awaiting analysis for long periods of time within the laboratory [12,13]. The NIJ’s most recently reported casework sample backlog (sexual assaults and homicides alone) is approximately 350,000 and the convicted offender backlog of collected, untested samples is approximately 200,000 – 300,000 as of 2017 [12]. However, even with this funding set aside, the backlog still continues to be a major problem today. While the President’s initiative also addressed the need for improvement in laboratory techniques and equipment, and some advancements have been made, the number of samples submitted to forensic labs has increased significantly, outpacing these advancements [12,14]. A potential approach to reduce this backlog is to develop a more efficient cell lysis and DNA extraction method for sexual assault samples. An ideal method would limit the tube-to-tube

transfers, eliminate wash steps, be more easily automatable, and more efficiently recover DNA from the male component. Together, these improvements could significantly reduce not only sample processing time, but also the time required for back-end DNA profile interpretation.

In the Dawson Green research laboratory at Virginia Commonwealth University, an antibody-binding assay is under development which can be used for differential separation of sexual assault samples into male and female fractions. After separation, samples are processed with the *forensic*GEM™ Sperm kit (microGEM; Dunedin, New Zealand), an enzyme-based assay that uses a mixture of thermophilic and mesophilic enzymes at various temperatures to lyse sperm cells without any purification steps or inhibitory chemicals [15]. This DNA liberation assay can be performed in a single tube, eliminating tube-to-tube transfers and ultimately minimizing the loss of DNA. Additionally, the processing time is quick and simple, and the procedure does not require reducing agents, which are known to cause inhibition during STR amplification [16]. Further, the absence of wash steps makes this approach ideal for implementation into microdevices designed for rapid sample processing. Unfortunately, preliminary results have caused some concern, as average DNA profile peak heights were lower than expected, poor interlocus balance was observed, and only partial DNA profiles were obtained when this kit was used alongside the Identifiler™ amplification kit [17]. In order to accurately identify the contributors of a sexual assault sample, it is imperative that full DNA profiles with high peak heights are yielded so that major and minor contributors can be differentiated. With the DNA liberation method currently being used (described above), this is not probable. Consequently, it would be beneficial to explore alternate cell lysis/DNA purification methods for rapid differential cell separation from sexual assault samples. Ideally, alternate methods should maintain the same efficiency in that they should not require tube-to-tube transfers or wash steps, they should not use reducing agents, should utilize

relatively small volumes (about 25 µL), and they should be simple and quick. Additionally, they should be more effective at eliminating the inhibition currently seen so that full, high-quality DNA profiles can be achieved. These methods would also ideally be non-proprietary for simplification of future microdevice licensing and commercialization efforts.

This research study will aim to develop a more efficient cell lysis method for differential processing of sexual assault samples by exploring an assortment of DNA liberation techniques including cell lysis buffer solutions and commercial direct amplification reagents, as well as a natural spermatozoa decondensation approach. Once methods are identified, each will be tested and optimized before comparison to existing and traditional methods. The best performing method(s) will then be utilized on samples that have been subjected to the antibody-mediated cell capture assay developed by the Dawson Green laboratory for separation of male (sperm) and female (non-sperm) fractions.

Laboratory Processing of Sexual Assault Samples

The majority of sexual assaults are committed by males; therefore, one of the most common forms of biological evidence encountered in sexual assault cases is semen. Semen is a fluid secreted by males that is composed of a variety of nutrients along with spermatozoa, added as semen travels through the ejaculatory ducts and mixes with the nutrients and fluids contributed from the seminal vesicles, prostate, and bulbourethral glands. When a male ejaculates, an average of 200-500 million sperm cells are released by the testes into the seminal fluid [18].

In the mid-17th century, Antony van Leeuwenhoek made significant improvements to the microscope and made it possible to identify spermatozoa [19]. With the ability to visualize sperm cells, it became possible to distinguish their morphology from other cells and understand why it is

more difficult to break open their nuclei and access their DNA. Spermatozoan structure can be broken down into three main portions: the head, midpiece, and tail. The head is composed of the nucleus, nuclear cap, and acrosome. The nucleus contains the genetic material of interest, the nuclear cap partially covers the nucleus to protect it, and the acrosome is comprised of vacuoles whose function is not yet known [20,21]. Due to the presence of a nuclear cap and acrosome protecting the nucleus (in addition to the plasma membrane), sperm cells are much tougher than somatic cells, requiring a harsher cell lysis method [22].

In addition to sperm cells, vaginal epithelial cells are also commonly encountered in sexual assault gynecological samples. Due to the nature of how these samples are deposited and the sites from which they are collected, there is generally an overwhelming amount of female DNA compared to male DNA in sexual assault evidence samples, often resulting in an imbalanced mixture DNA profile and/or a masked male DNA profile [10]. Given that approximately 3.5 picograms (pg) of DNA are contained in a single sperm cell, hundreds of sperm cells are needed to reach the optimal input DNA needed for polymerase chain reaction (PCR) for human identification (0.5-1.0 ng) [23-26]. Research has shown that a secondary DNA contributor cannot be detected if it is present at a ratio exceeding 1:10 major:minor DNA of a two-person mixture [10]. To circumvent this, a differential cell lysis is typically performed for sexual assault evidence as a way to physically separate epithelial and sperm cells based upon their different morphologies and susceptibilities to lysis reagents. Using this method, an eluted sample is first treated with proteinase K and sodium dodecyl sulfate (SDS) (mild lysis reagents), resulting in the preferential lysis of epithelial cells while preserving the intact sperm cells in solution. After centrifugation, DNA and debris from lysed epithelial cells in solution are moved to a separate tube and then sperm heads are treated with proteinase K, SDS, and dithiothreitol (DTT), a disulfide bond reducer, which

causes the sperm membrane to break and the male DNA to be released for downstream analyses [10,27,28].

While this method can significantly reduce the occurrence of sexual assault mixture DNA profiles, differential extraction is a laborious, time-consuming procedure that requires multiple tube-to-tube transfers and typically must be completed manually [11]. This method is also dependent on the successful elution of sperm from the substrate prior to lysis. Typically, only ~10-40% of sperm cells are eluted from common forensic substrates [29]. Additionally, it has been reported that 60-90% of male DNA is lost using the traditional differential lysis and DNA extraction technique [30]. Given the fairly low recovery rates, alongside the high potential for sample loss due to the tube-to-tube transfers with traditional methods, low-level, poor-quality male DNA profiles may be prevalent. Furthermore, the successful development of a male autosomal STR profile is highly dependent on the circumstances of the case, the suspect's semen characteristics, and the time elapsed between the alleged assault and collection of swabs/evidence [10,31]. Circumstances of the crime such as time, place, the occurrence of ejaculation, and condom usage are important factors when determining the best method for testing. Although sperm have a lifespan of up to 12 days (depending on environmental conditions), the optimal time to retrieve a gynecological swab is within 48 hours post-coitus. Sperm heads and tails will begin to degrade once inside the vagina due to the acidic environment and immune responses, ultimately destroying the cell and the nuclear material inside [32]. Moreover, some men are azoospermatic (i.e. the absence of sperm in semen), may have undergone a vasectomy (a preventative measure to keep sperm from entering the semen), or may have low sperm counts. In these cases, performing a traditional differential extraction may compound existing problems, rendering the DNA analysis even less likely to produce a sufficient DNA profile [33].

Because traditional differential lysis and DNA extraction techniques are reliant upon a variety of factors, are time consuming and laborious, and are inefficient, they may not be the best approach for processing sexual assault samples in a timely manner. An extraction method that significantly decreases processing time, as well as reduces the risk for contamination and sample loss is necessary in order to process samples of this nature more efficiently and effectively. A number of methods and modifications to the traditional differential extraction process have been explored with the aim of improving resulting STR profiles. One study experimented with slight modifications to the traditional manual differential extraction. First, the addition of a second mild lysis step using stain extraction buffer (SEB) and proteinase K at the beginning of the process was reported to result in an improved male:female DNA ratio in the sperm fraction by three- to six-fold [34]. The second modification was the relocation (tube transfer) of the resuspended sperm pellet (in PBS) for the subsequent lysis and washes. With this modification, the male:female DNA ratio in the sperm fraction was improved by four- to 90-fold [34]. Some have explored adding nuclease to the sperm fraction to digest the remaining epithelial cells, which resulted in single-source male DNA profiles [35]. Although these methods demonstrate improvements, they still employ the same basic time-consuming process and require similar wash steps and tube transfers.

The differential lysis and extraction process can be performed robotically, as a way to decrease the analyst's hands-on time, increase the number of samples processed, and reduce contamination. One such study compared the results obtained from an automated differential extraction on a QIAcube liquid handling robot (QIAGEN; Hilden, Germany) to a traditional manual process and concluded the automated method was more effective at separating non-sperm cells from sperm cells, as the automated method reduced the number of mixtures observed during STR analysis [36]. Microdevices have also been recently explored as a faster, more efficient way

to process sexual assault samples. The chemistry utilized in the VCU microdevice allows for the male and female DNA fractions to be separated using an antibody-binding assay for sperm cell capture, allowing only the female epithelial cells to be moved into a separate chamber, resulting in a male-only DNA fraction. This eliminates the need for mixture interpretation since male and female cell contributions are separated prior to lysis. This device allows for STR loci to be amplified in approximately 45 minutes and for the generation of CE-ready amplicons in less than two hours [37]. Unfortunately, as noted earlier, only partial DNA profiles were yielded, average DNA profile peak heights were low, and interlocus imbalance was observed. Further, the current extraction chemistry used (*forensic*GEM™ Sperm kit) is a commercial product which further complicates the licensing and commercialization processes.

Novel DNA Liberation Techniques

Cell Lysis Solutions

Cell lysis, the process used to extract nucleic acids or proteins from inside a cell, is the disruption and destruction of the cell membrane, which in turn allows for the release of cellular material [38]. There are a variety of cell lysis solutions currently used throughout fields of biomedical research and testing, some proprietary and some not, each with their own advantages and disadvantages. One such approach is the use of alkaline lysis solutions, which exert a strong denaturing effect on proteins and are an efficient means of protein solubilization due to the ionization of certain amino acids. When a sample is incubated in an alkaline solution, the solution disrupts the plasma membranes, denatures nucleases, and preserves the DNA as it is relatively stable in an alkaline environment [39-41]. Alkaline lysis involves an initial incubation of cells in sodium hydroxide (NaOH) for various amounts of time and at various temperatures, depending on

the nature of the sample, in order to achieve successful cell lysis. This is followed by the addition of Tris-HCl to obtain a neutral pH. Incubation of the sample in distilled water prior to adding the NaOH may improve results, although some report better results when this step is omitted [39-41]. Regardless, analysis of the reported data show that this method is sufficient for DNA extraction, rendering the genomic DNA accessible as a template for PCR. This process is quick, inexpensive, and performed in a single tube (thus reducing the risk of contamination and sample loss) [39-41]. Others have reported on the usefulness of aptamers for separation of sperm cells from epithelial cells using a lysis buffer, comprised of a synthetic compound (e.g. EDTA), detergents commonly used for extraction (e.g. Triton X-100 and Proteinase K), and a reducing agent (e.g. DTT). The combination of these reagents proved to be an efficient way to isolate and purify sperm cells without the female fraction interfering and was demonstrated to be comparable to the traditional differential extraction [28].

Another non-proprietary lysis buffer that can be purchased or easily formulated in the laboratory is nonyl phenoxypolyethoxylethanol, or NP-40 [42]. NP-40 is a non-ionic detergent commonly used for DNA extraction and purification. This buffer is mild and therefore usually preferred over harsher detergents given it is gentler to the DNA and will likely not result in degradation. Additionally, NP-40 lysis buffer has been used to prepare cell extracts shown to be suitable for analysis with a variety of techniques, including antibody binding [43]. When used for DNA extraction, NP-40 lysis buffer reduces the risk for contamination by reducing the c-Raf detection in nuclear extracts [44]. The use of a cocktail solution consisting of NP-40 lysis buffer in combination with L-carnitine, D-(+)-trehalose, and heparin has also been reported to enhance PCR. It has been shown that this formulation of NP-40 improves DNA sequencing by stimulating DNA polymerase activity at high temperatures and reducing the frequency of nonspecific binding

[45,46]. It was determined this cocktail solution could provide a reliable enrichment of PCR amplification compared to that of other PCR additives. This cocktail has been successfully used to complete direct amplification on crude blood samples and it is believed to have potential for various sample types, including semen stains [45]. The research described used a 0.8% NP-40 lysis buffer, however it has been reported that 0.1 – 1.0% NP-40 is safe to use through cell lysis and amplification [44-48].

Direct Cell Lysis/Amplification

The typical forensic workflow for DNA analysis consists of cell lysis and DNA extraction, quantification, PCR amplification of short tandem repeats (STR), and separation/analysis of STR amplicons using capillary electrophoresis (CE). It is a very laborious, time-consuming process with risk of sample loss and contamination. Direct PCR, an alternative to the standard workflow, is the amplification of a sample without prior DNA extraction/purification or quantification [49]. The approach was described in the 1990s; however, it was first used on forensic samples in 2010. This technique has gained interest in the forensic community due to its increased sensitivity and reduction in processing time, allowing for DNA profiles to be obtained from more low-level and challenged DNA samples [50,51]. Direct amplification eliminates the extraction and quantification steps in the traditional workflow, minimizes the risk of sample contamination, reduces time and expenses, and results in the reduction of DNA loss [49-52]. However, because the PCR DNA input is not standardized, the resulting reactions may contain an excessive amount of template DNA, resulting in unwanted effects such as increased stutter artifacts, high baseline levels, and incomplete adenylation by *Taq* polymerase [53]. There are several commercially available kits

currently used to perform direct PCR, two of which will be addressed in this research: the PowerPlex® ESX 16/17 Kit (Promega; Madison, WI) and the Casework Direct Kit (Promega).

The PowerPlex® ESX 16/17 kit is a sample processing kit utilized for direct PCR, primarily for buccal swabs or reference samples, using SwabSolution™ as its lysis buffer. One study tested fabric cuttings using this kit and concluded that more STR alleles at higher RFU values were obtained compared to samples extracted with QIAamp DNA Micro kit [51]. Another study tested the use of direct PCR on a variety of semen dilutions, first creating simulated sexual assault samples with a short differential enrichment process in order improve separation of the male and female components. This process consisted of combining the sample with SwabSolution™ and proteinase K, followed by a 15-60 minute incubation. After incubation, the supernatant was transferred for direct PCR and the sperm pellet was washed twice with the SwabSolution™ before being transferred to direct PCR. They concluded that even though the differential protocol added a short amount of time, it enhanced the male profile significantly, even in samples with an excess of female cells. Additionally, full profiles were obtained with as low as a 1:80 dilution and a high level of dropout not observed until a 1:160 dilution. Lastly, they noted that as the semen dilutions increased, the resulting peak heights decreased, as would be expected [50]. Given these conclusions, it is evident that the SwabSolution™ reagent, has the ability to provide full male DNA profiles from semen without significantly increasing the amount of time needed to obtain the results.

The Casework Direct kit is another cell lysis kit used to rapidly process a broad range of evidentiary samples [54]. This kit, unlike the PowerPlex® ESX kits, does not require a differential enrichment protocol. It contains both an extraction buffer and a reducing agent (1-thioglycerol), which in combination produce a DNA lysate that is ready for amplification within about 35

minutes. Since this kit is able to extract the DNA present without any wash steps from a sperm pellet, it has been recommended for use particularly with sexual assault samples [55,56]. A recent validation study determined the Casework Direct kit provides reliable results from a range of samples commonly encountered in forensic cases, including sexual assault samples, as well as samples collected on a variety of substrates. Most importantly, this research displayed that the Casework Direct Kit produces reliable and reproducible results from a range of bodily fluids at different volumes, including mixtures of those fluids [56]. Another study compared the Casework Direct kit to the Maxwell 16 System DNA IQ Casework Pro kit (Promega). In this study, allele dropout was observed in higher levels than expected in both kits and it was suggested that 0.125 ng of DNA was needed to obtain results reliable enough to consistently call the correct STR alleles [55]. In another study, mock case samples were prepared and tested using this kit for both Y-screening and autosomal STR profiling. Full Y-STR profiles and full autosomal STR profiles (except those that were inhibited due to the substrate sampled) were observed [56,57]. It is evident this kit produces amplification-ready lysates from low DNA input and will result in high quality male STR profiles. The reliability of results from low-level input DNA, along with no wash steps, is an important feature of this kit, specifically for sexual assault samples containing a limited amount of the suspect's DNA [56]. This reliability, along with its efficiencies, makes this a preferred method for testing samples of this nature.

It is imperative to note, for both of the direct cell lysis methods described above, a proprietary 5X AmpSolution™ reagent (Promega) is required if quantification and amplification with certain kits are to be performed, as the absence of this reagent can result in the inhibition of real-time and end-point PCR [58,59].

Natural Spermatozoa Decondensation

When spermatozoa enter the vagina, they either continue to the ovary for fertilization of the egg or they are quickly degraded by the acidic environment, chemicals, and/or immune response. Oocytes, or immature egg cells, inherently possess factors that aid in fertilization and the development of embryos. One of these factors was found to be protein disulfide isomerase A3 (PDIA3), which is necessary for sperm decondensation. Sperm decondensation is essential for fertilizing the oocyte and ultimately, producing an embryo. In a study conducted to confirm the relevance of this factor, some oocytes were injected with DTT and others with PDIA3 [60]. DTT is a disulfide bond reducer commonly used; however, it is also a qPCR inhibitor as it blocks signal detection through fluorescence quenching [61]. There were no significant differences in sperm decondensation rates observed between DTT-treated and PDIA3-treated oocytes, supporting the idea that PDIA3 catalyzes disulfide bond reduction [60].

As stated above, sperm decondensation is essential for fertilizing the oocyte and producing an embryo. PDIA3 is a factor present on the oocyte to assist in the fertilization; however, more than just PDIA3 is needed for sperm decondensation. In order for the sperm nucleus to decondense and fertilize the oocyte, the plasma membrane and acrosomal cap must also be removed to allow for the chromatin to exit the sperm nucleus and enter the oocyte. Several studies have been implemented with the use of intracytoplasmic sperm injection (ICSI) for oocyte fertilization. With this type of application, both the sperm chromatin and acrosome are introduced into the oocyte. ICSI has been reported successful, but at low efficiency; therefore, the effects of acrosomal enzymes have been examined, and it was observed that contents of the acrosome can be harmful to oocytes [62,63]. The removal of the membrane and acrosome not only accelerates oocyte activation and reduces oocyte death in the development of embryos, but it also allows access to

the sperm nucleus for the purposes of this research [63]. With the intention of removing the acrosomal membrane, plasma membrane-disrupting agents were tested and evaluated. Lysolecithin (LL), a hydrolysis product of membrane phospholipids, and Triton X-100 (TX), a detergent commonly used in cell lysis, have both been tested in independent studies. One study demonstrated a 52% increase of acrosomal membrane removal with the use of TX and a 65% increase when using LL [62]. Others have supported these findings, observing the removal of both the plasma and acrosomal membranes when using these methods [63]. Of the two methods, LL is the preferred disrupting agent as it is a hydrolysis product of phospholipase A and is therefore not foreign to sperm cells [62,63]. This method, in-tube, requires use of HEPES buffer to maintain proper conditions. HEPES buffer has a phototoxic effect, which means it produces hydrogen peroxide when exposed to ambient light [64,65]. Due to this effect, it is essential to keep samples in darkness to obtain the highest quality results since hydrogen peroxide degrades DNA [64-66].

Another sperm decondensation approach tested is the combination of glutathione (GSH) and heparin in HTF modified medium (Irvine Scientific; Santa Ana, CA), which uses a buffering system to maintain optimal conditions. GSH is a naturally occurring disulfide bond reducer within the female genital tract, while heparin destabilizes the plasma membrane. There are heparin receptors on specific areas of the sperm plasma membrane that bind to heparin and lead to the destabilization of the plasma membrane, allowing GSH to interact with and decondense the sperm nucleus [67,68]. Additionally, heparan sulfate, an analogue of heparin, can also be used as a decondensing agent [68,69].

In addition to the biological compounds mentioned above, the use of glutathione-S-transferase omega 2 (GSTO2) has been demonstrated to accelerate nuclear decondensation of spermatozoa. On the head of a sperm cell there is the perinuclear theca (PT), which can be divided

into the subacrosomal layer (SAL) and the postacrosomal sheath (PAS). The PAS houses proteins essential for fertilization and is also the region where nuclear decondensation begins. The PT houses the PAS along with GSTO2; therefore, it is proposed that spermatozoa utilize this enzyme during sperm head decompartmentalization, accelerating nuclear decondensation. One research group expressed the significance of GSTO2 in sperm nuclear decondensation as a delay was observed when GSTO2 was inhibited [70]. If GSTO2 were combined with one of the approaches mentioned above, it could allow for nuclear decondensation to be achieved in even less time, yielding a more efficient process.

Although there has yet to be any research on the implementation of these reagents for extracting DNA from spermatozoa for forensic purposes, the combination of PDIA3 and GSH-LL/TX or PDIA3 and GSH-heparin could allow for the plasma and acrosomal membranes to be removed, thereby permitting the sperm nucleus to be decondensed and the nuclear material to be released. The addition of GSTO2 to any of the aforementioned combinations could possibly further enhance nuclear decondensation, thereby making sperm DNA extraction more efficient by decreased incubation and overall sample processing times.

The goals of this work included identifying commercial and non-proprietary techniques for direct cell lysis of sexual assault samples, in addition to identifying any natural sperm decondensation approaches that may be effective on samples of this nature. Additionally, the goals of this work included evaluating, optimizing, and comparing each of these techniques in hopes of meeting the desired results to provide a cheaper, quicker, and more efficient cell lysis technique for forensically relevant samples that is not only easily implemented into the current forensic DNA workflow, but also transferrable to a microfluidic device.

Materials and Methods

Sample Collection and Preparation

Ten semen samples were collected from anonymous donors in accordance with the university-approved Institutional Review Board (IRB) protocol HMW20002931 and were diluted by volume 1:10 in phosphate buffered saline (PBS) (pH 7.4) (Quality Biological; Gaithersburg, MD). Foam swabs were dipped into the 1:10 semen dilutions where they absorbed approximately 80 µL of the total 100 µL and were then left at room temperature to dry overnight. Once dry, the swabs were cut into twelfths and stored at 4°C. Subsequent testing for all cell lysis methods utilized $1/12^{th}$ of a foam swab for each donor in triplicate. All samples were stored at 4°C until downstream analyses were performed, unless otherwise stated.

Cell Lysis and DNA Liberation

forensicGEM™ Sperm

The *forensic*GEM™ Sperm kit (microGEM™; Charlottesville, VA) served as the control cell lysis method, as it is currently the method used for microchip-mediated direct cell lysis in the Dawson Green lab. This approach was followed for the same semen samples used with all previously discussed lysis techniques and results were compared. For this method, 2.0 µL *forensic*GEM™ enzyme, 10 µL Acrosolv, and 10 µL 10x Orange+ Buffer were added to each sample and the reactions were brought up to 100 µL with HyPure Molecular Biology Grade (MBG) Water (GE Healthcare Life Sciences; Marlborough, MA). Reactions were placed onto the Proflex™ PCR Dual 96-well PCR system (Thermo Fisher Scientific) and incubated as follows: 52°C for ten minutes, 75°C for three minutes, and 95°C for three minutes.

SwabSolution™ Direct Lysis

First, 1/12th of a 1:10 semen swab and 1/12th of a buccal swab were incubated in 1.0 mL of SwabSolution™ (Promega; Madison, WI) for 30 minutes at 70°C, per manufacturer recommendation. Test samples were incubated in 23 µL of SwabSolution™ and 2.0 µL Proteinase K (Thermo Fisher Scientific; Waltham, MA) in triplicate. One set of reactions were incubated at 70°C for either 15, 30, or 60 minutes.

Casework Direct Kit

Modified versions of the manufacturer recommended protocol for the Casework Direct System (Promega) were tested in an attempt to reduce the total reaction volume. The diluted semen samples were incubated in 25, 50, and 100 µL of Casework Direct solution (Promega) containing 0.125 µL, 0.25 µL, and 0.5 µL 1-thioglycercol (Promega), respectively for 30 minutes at 70°C. After incubation, swabs were placed in a spin basket and centrifuged for five minutes at 10,000 x g to maximize liquid recovery.

NP-40 Cell Lysis

Three different strengths of NP-40 cell lysis buffer (VWR; Radnor, PA) were evaluated to ensure optimal cell lysis while also minimizing PCR inhibition. The first set of diluted semen samples was submerged in 25 µL of 1% NP-40 lysis buffer. The second set of samples was submerged in 18.75 µL of 1% NP-40 lysis buffer and 6.25 µL of HyPure MBG Water (GE Healthcare Life Sciences) making the final NP-40 concentration 0.75%. The third set of samples was submerged in 12.5 µL 1% NP-40 cell lysis buffer and 12.5 µL of HyPure MBG Water (final NP-40 concentration of 0.5%). All reactions were incubated on ice for 30 minutes with vortexing

every ten minutes. Swabs were transferred to spin baskets and centrifuged at 13,000 x g for ten minutes. Lysates were stored at -20°C until further processing.

Alkaline Lysis

Semen-soaked swabs were incubated in 16 µL of PBS and 4.0 µL of 1M NaOH (Thermo Fisher Scientific), at 75°C for five minutes. Succeeding incubation, 4.0 µL of 1M Tris-HCl (Invitrogen; Carlsbad, CA) was added to the samples, which were briefly vortexed. The swabs were then transferred to a spin basket and centrifuged for five minutes at 13,000 x g. Lysates were stored at -20°C until further processing.

Natural Sperm Decondensation Approaches

HEPES Buffer + Triton-X (HTX)

In an attempt to mimic the female body's approach to sperm cell decondensation and lysis, semen samples were subjected to two different "natural sperm decondensation" assays. The first assay utilized HEPES buffer and Triton X-100. A stock solution of HEPES (Sigma Aldrich; St. Louis, MO) containing 0.04% Triton X-100 (Thermo Fisher Scientific) was prepared. All semen samples were submerged in 25 µL of stock solution and vortexed for one minute. Swabs were then placed in spin baskets and centrifuged for three minutes at 17,000 x g. Lysates were stored in foil (to protect from light) at 4°C until further processing.

HTF Modified Buffer + Glutathione + Heparin (HGH)

A second modified natural sperm decondensation assay was tested using components considered to be naturally occurring within the vagina. A stock solution of modified HTF medium

(Irvine Scientific) was prepared with 10 mmol/L glutathione (Sigma Aldrich) and 46 µmol/L heparin (Sigma Aldrich). Three sets of semen-soaked swabs were prepared, and each were incubated in 25 µL of the stock solution at 37°C for either 15, 30, or 60 minutes.

Microscopy

Prior to and following cell lysis, KPICS staining was performed on each sample to gauge the effectiveness of each lysis method. For this a total of 2.0 µL of sample was spotted on a microscope slide, allowed to dry, fixed with Sprayfix® Cytology Fixative (Leica Biosystems; Wetzlar, Germany) and dried for five minutes, stained with one drop of Kernechtrot stain (Serological Research Institute; Richmond, CA) for 15 minutes, rinsed off with water, stained with one drop of Picroindigocarmine stain (Serological Research Institute) for 20 seconds, and then gently rinsed off with water. After staining, 20 µL of HyPure MBG Water (GE Healthcare Life Sciences) was added to the stain and a cover slide was placed on top. The Kernechtrot stain dyed the sperm nuclei red and the Picroindigocarmine stain dyed the tails green. Sperm cells were then visualized under a Micromaster microscope (Thermo Fisher Scientific) using 400x magnification. The sperm slides were scored using a 0-4+ scale (as described in Table 1) by averaging across ten different fields-of-view for each sample slide.

DNA Quantification

In order to determine the total amount of DNA in the samples after each lysis method, all resulting lysates were quantified using the Investigator® Quantiplex HYres kit (QIAGEN; Hilden, Germany) 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. These recommendations were followed for sample sets that were lysed using the NP-40, alkaline, *forensic*GEM™ Sperm, and natural sperm decondensation methods. However, the reagents used in the SwabSolution™ and Casework Direct kits can inhibit qPCR and thus require 5X AmpSolution™ (Promega) to be added during quantification to ensure accurate results. For these samples (and corresponding DNA standards), each reaction included 4.5 µL Reaction Mix FQ, 4.5 µL Primer Mix IC YQ, 2.0 µL 5X Amp Solution, and 1.0 µL template DNA.

For all runs, standards were quantified in duplicate. Thermalcycling conditions were as follows: 95°C for three minutes followed by 40 cycles [95°C for 5 minutes; 60°C for 35 seconds]. Quantification data was analyzed using the Sequence Detection System (SDS) software version 1.4 (Applied Biosystems™; Foster City, CA) and an automatic threshold and baseline for analysis of each targets. Total DNA yields were calculated by multiplying the appropriate target's concentration by the elution/sample volume (which differed for each method and variable tested). The averages and standard deviations for each donor were calculated to compare each cell lysis method and to determine how much DNA to amplify and inject for separation and detection in downstream analyses. If an outlier was observed, the 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 this data, an ANOVA was performed to compare the DNA yields of the control method (*forensic*GEM™ Sperm) to the three conditions tested for SwabSolution, Casework Direct, NP-40 cell lysis, and HTF + Glutathione + Heparin natural decondensation assay ($\alpha = 0.05$). If the ANOVA resulted in a significant difference, a Tukey HSD test was performed to identify where these significant

differences appeared in order to establish which condition(s) to move forward with for downstream analysis.

Additionally, amplification plots were examined for a sigmoidal shape consisting of exponential, linear, and plateau phases, with the plots crossing the cycle threshold (C_q) during the exponential phase; the C_q for samples typically falls between 20 and 30, while the acceptable C_q for the internal positive control (IPC) is 27 ± 2 cycles. Multicomponent plots were examined for any deviation from the expected fluorescent signals of the three amplification targets, which should remain flat prior to exponential growth of the PCR product at around 22 cycles. Furthermore, the passive reference dye signal was examined for any deviation from a flat, consistent curve throughout the entirety of the assay.

STR Amplification

All samples were amplified using the Promega™ PowerPlex® Fusion 5C System. Amplification was performed using the following parameters on the Proflex Dual 96-well PCR System: 96°C for one minute, 30 cycles [94°C for ten seconds; 59°C for one minute; 72°C for 30 seconds], and a 60°C hold for 45 minutes. Each sample was amplified using both an in-tube and a simulated chip-based approach, in which target DNA input or specific volumes were achieved, respectively.

Amplification for the in-tube assay included a normalized amount of input DNA across all samples; 0.25 ng of template DNA input for each sample analyzed. To achieve this standard input, each sample was diluted to 0.1 ng/µL and 2.5 µL was added to the reaction tube. For each sample reaction, 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 was added to the

template DNA. If samples yielded DNA concentrations below the target DNA amount, they were vacuum centrifuged to a specific volume that would result in the desired concentration (determined using excel).

Amplification for the simulated chip-based assay utilized a standard volume of input DNA for each sample group (based on the average DNA concentration across the group); thus, DNA inputs were not normalized. Amplification reactions for samples from the HTX and NP-40 (seven donors) cell lysis methods included 2.0 µL undiluted sample lysate, 2.5 µL PowerPlex® Fusion 5x Master Mix, 2.5 µL PowerPlex® Fusion 5x Primer Pair Mix, and 5.5 µL amplification-grade water. Reactions for samples from the *forensic*GEM™ Sperm, SwabSolution™, and Casework Direct cell lysis methods included 1.0 µL sample lysate, 2.5 µL PowerPlex® Fusion 5x Master Mix, 2.5 µL PowerPlex® Fusion 5x Primer Pair Mix, and 6.5 µL amplification-grade water. Lastly, samples from the NP-40 (three donors), alkaline, and HGH cell lysis methods were amplified using 0.5 µL sample lysate, 2.5 µL PowerPlex® Fusion 5x Master Mix, 2.5 µL PowerPlex® Fusion 5x Primer Pair Mix, and 7.0 µL amplification-grade water.

Capillary Electrophoresis and Data Analysis

Following amplification, samples were separated on an ABI 3130 Genetic Analyzer® (Thermo Fisher Scientific). For samples analyzed using the in-tube amplification approach, 1.0 µL of amplified product from each sample was added to 0.3 µL WEN ILS 500 (Promega™) and 9.7 µL Hi-Di Formamide (Thermo Fisher Scientific), with the exception of samples lysed using the SwabSolution™ and Casework Direct kits (for which 0.5 µL of each sample analyzed was added to 0.3 µL WEN ILS 500 and 10.2 µL Hi-Di Formamide). For those samples analyzed using the chip-based amplification approach, 1.0 µL of amplified product from samples subjected to the

*forensic*GEM™ Sperm and HTX lysis was added to 0.3 µL WEN ILS 500 and 9.7 µL Hi-Di Formamide. For those samples analyzed using the chip-based amplification approach and subjected to the SwabSolution™, Casework Direct, NP-40, alkaline, and HGH cell lysis methods, 0.5 µL of amplified sample was added to 0.3 µL WEN ILS 500 and 10.2 µL Hi-Di Formamide. For typing, 1.0 µL of allelic ladder was added to 0.3 µL WEN ILS 500 and 9.7 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. Succeeding separation, results were analyzed with GeneMapper™ v4.1 following manufacturer recommendations and using an analytical threshold of 100 RFU.

Profiles were qualitatively and quantitatively evaluated for any signs of inhibition (e.g., interlocus balance, allelic dropout) and pull-up. Average peak heights were calculated finding the mean peak height of all STR alleles across an individual sample profile. To account for homozygosity, the peak heights for homozygous alleles were halved to represent the two copies of the allele at that locus. Based on our laboratory's internal validation for the PowerPlex® Fusion 5C kit, the validated average peak height for the target input indicated is ~1645 RFUs. Interlocus balance was calculated using the coefficient of variation (CV) for locus peak height:total peak height (LPH:TPH) ratios for each locus of the entire DNA profile, excluding the Amelogenin and DYS391 loci. By calculating interlocus balance using this method, one can determine the variation in peak height for each locus in comparison to the average peak height across the entire profile. The ideal CV is ≤ 0.35, which means that the peak heights at a given locus vary no more than 35% from peak heights at other loci in the DNA profile [71]. Lastly, to evaluate pull-up, the number of loci containing pull-up peaks for each sample was noted and the average number of loci exhibiting pull-up was calculated for each lysis method. For the average peak heights, the average percentage

of STR alleles detected, and average CV, an ANOVA was performed to compare the results of the control method to the six additional methods tested ($\alpha = 0.05$). If the ANOVA resulted in a $p < 0.05$, a Tukey HSD test was performed identify where these significant differences appeared.

Results and Discussion

Preliminary Evaluation

KPICS Microscopy

In an attempt to visualize the effectiveness of the methods tested, KPICS staining and microscopy was performed on one sample from each donor after cell lysis for each method evaluated. Ideally, complete sperm cell lysis would result from each method. Those samples evaluated with the control method, *forensic*GEM™ Sperm, did result in the complete lysis of sperm cells in 60% of the samples with minimal cells being visible in the other 40% (Figure 1). Interestingly, the Casework Direct and alkaline cell lysis techniques were the only methods to result in complete sperm lysis across all donors (Figure 1). As for samples subjected to the NP-40 cell lysis buffer, there was no difference in the amount of sperm cells lysed regardless of the lysis buffer strength; a large number of sperm cells was visualized across donors for all three strengths tested with a median score of 3+ (Figure 1). Similarly, cell lysis using HEPES + Triton X-100 resulted in the visualization of a large number of sperm cells across all donors with a median score of 3+ (Figure 1). When cell lysis was achieved using SwabSolution™, the total amount of cells successfully lysed differed across donors and incubation times. Samples incubated for only 15 minutes contained a large number of sperm cells across donors with a median score of 3+. However, sperm counts continuously decreased as the incubation time increased, ultimately resulting in a

median score of 0 (complete lysis) at 60 minutes (Figure 2). The same trend was also observed for those samples lysed using the natural sperm decondensation assay, HGH (Figure 2).

Preliminary Testing

In an effort to establish which conditions for each lysis method would be optimal to test with downstream analyses, a preliminary study was performed. An ANOVA comparing the three time intervals tested for SwabSolution™ and the control, *forensic*GEM™ Sperm, revealed a significant difference in DNA yields obtained from semen samples ($p = 0.000116$). A Tukey HSD revealed that all three SwabSolution™ time intervals (15 minutes, 30 minutes, and 60 minutes) were significantly lower than the control, by as much as 86% ($p = 0.0004$, 0.0005, and 0.001, respectively) (Figure 3A). Similarly, the three volumes tested for Casework Direct also resulted in a significant difference in DNA yields obtained from semen samples ($p = 0.0102$). The Tukey HSD revealed significant decreases for the 25 µL and 50 µL lysis volumes of Casework Direct when compared to the control condition, by as much as 75% ($p = 0.011$ and 0.034, respectively) (Figure 3B). Additionally, ANOVAs were performed comparing the control to the three strengths of NP-40 cell lysis buffer and to the three time intervals tested for the HGH natural decondensation assay; however, contrary to the previously discussed methods, no significant differences in DNA yields from semen samples were revealed ($p = 0.15$ and 0.97, respectively) (Figures 3C and 3D, respectively).

In order to identify which conditions to use in future experiments, a number of additional factors for each method were evaluated in addition to DNA yields, including assay processing time and reaction volumes. Ideally, a condition that would provide a large enough DNA yield on average for both the in-tube and simulated chip-based approach would be identified. A large

enough DNA yield means being between 0.25 ng and the maximum input accepted without producing excessive artifacts. Additionally, the control method, *forensic*GEM™ Sperm, takes ~20 minutes to process a single semen sample; therefore, ideally, any new methods selected would require a similar or less amount of time. Lastly, volume is an important factor to consider for adaptation to a microchip environment, which is a goal. The current sexual assault microdevice, as designed, can hold up to ~25 µL of reagents in the sample lysis chambers. Therefore, ideally, the volume of cell lysis reagents for the selected methods should not exceed this amount.

While the SwabSolution™ lysis method revealed a significant lowering of DNA yields versus the control for all time intervals tested, no significant differences were identified between the three incubation times themselves (Figure 3A). Consequently, the 30-minute incubation was selected as the condition to move forward for this lysis method. This incubation time makes the method only 15 minutes longer than the currently used method and the 30 minute incubation time generated appropriate DNA yields for both the in-tube and chip-based approaches when semen samples were analyzed. Despite significantly lower DNA yields when the 25 µL Casework Direct method was compared to the control, this condition was selected for additional downstream work. No significant increases were gained when volume was increased to 100 µL ($p = 0.314$). Further, the semen samples analyzed with 25 µL resulted in reasonably high yields (Figure 3B**)** and this volume is most suitable for integration onto the microdevice. Because no significant differences in DNA yield were observed between the control and the variable conditions tested for NP-40 or the HGH cell lysis techniques, other factors were considered for these methods. The 0.5% NP-40 lysis buffer strength was selected for downstream use as it produced the highest yields and utilized the smallest amount of NP-40 cell lysis buffer. This approach would minimize any potential STR inhibition (as 1% is at the highest safe level reported for use with amplification) while still

providing enough NP-40 to allow for the enhancement of PCR (Figure 3C) [44-48]. Lastly, the 15-minute incubation for the HGH method was identified as the most suitable condition for further testing as it maintained the same quick processing time as the control method and generated the highest DNA yields, on average, for that method when semen samples were tested (Figure 3D).

DNA Quantification

Quantification results for all samples showed the expected sigmoidal curves for each target dye, as well as a characteristic flat curve for the passive reference dye (ROX). The amplification plots for all semen samples tested revealed a characteristic plot 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. However, under certain lysis conditions, the internal PCR control (IPC) plots crossed the threshold at a later cycle than expected ($C_q \geq 29$) which is consistent with inhibition; this often results in the underestimation of DNA concentration [72]. This phenomenon occurred most notably in those semen samples lysed with SwabSolution™ and Casework Direct. This was anticipated, as the addition of the 5X AmpSolution™ produced a lower efficiency reaction by inherently diluting the other reaction and primer mix components. Additionally, this IPC delay occurred in 20% of the donors lysed with alkaline cell lysis and 70% of the donors lysed using the HTX and HGH cell lysis methods. Because the IPC was not delayed in every sample, it is likely due to donor differences or a different lot for reaction components. The first set of semen samples for all cell lysis methods (n = 3) were quantified using the same lot of the quantification kit. Only SwabSolution™ and Casework Direct resulted in delayed IPCs when this lot was utilized. When the additional seven semen donors were processed, there were too many reactions to process with a single kit; therefore, two separate kits

were utilized. Delayed IPCs were observed only in the alkaline, HTX, and HGH samples which were all processed using the same lot; different from the lot used for those samples lysed with *forensic*GEM™ Sperm, SwabSolution™, Casework Direct, and NP-40 cell lysis. It is unknown as to why a newer kit would have produced more inhibition.

Comparative Study - In-Tube Assay

DNA yields were compared across all seven cell lysis methods. An ANOVA comparing all methods revealed a significant difference in DNA yields obtained from semen samples (p = 0.0037). A Tukey HSD revealed that DNA yields produced by HTX cell lysis were significantly lower than those produced by HGH cell lysis, by as much as 13% (p = 0.0377, Figure 4). No other significant differences in DNA yields from semen samples were revealed ($p > 0.06$).

Quantification produces estimated DNA concentrations for each sample, but is not always highly accurate, especially when inhibition is present; thus, STR analysis was pursued in order to obtain a truer representation of how effectively each cell lysis methods performed. Furthermore, DNA profiles are the endpoint for DNA analysis; they, not DNA quantity, are the basis of proving whether an individual is innocent or guilty – so this was an important perspective to gain. For the in-tube assay, target DNA inputs were normalized across all samples to 0.25 ng of input DNA for amplification. Subsequent STR profiles were analyzed across all donors tested and compared to each other and the control method (*forensic*GEM™ Sperm).

The goal was to identify the best performing method, or that which was most likely to achieve 100% STR allele detection and to generate DNA profiles of equal or higher quality than the control method (*forensic*GEM™ Sperm). Surprisingly, no method examined herein generated 100% STR allele detection across all samples; however, the HTX cell lysis profiles displayed a

significant reduction in the percentage of STR alleles detected compared to all other cell lysis methods, by as much as 48% ($p < 0.00005$, Figure 5). All other methods tested produced semen sample STR profiles with over 90% of expected STR alleles detected and these values were not significantly different from one another (Figure 5).

The internal validation of the PowerPlex® Fusion 5C STR kit performed in our laboratory demonstrated an average STR allele peak height of 1645 RFU from reference buccal samples when 0.25 ng of template DNA was input into the amplification reaction. It is important to have peak heights at or above the validation value and within the dynamic range of the instrument (up to ~5500 RFU) because with a mixture, minor contributors are more likely to be detected with higher peak heights and lower peak heights can complicate the overall mixture interpretation and lead to inconclusive results. Therefore, for each cell lysis method tested herein, the goal was to achieve STR allele peak heights consistent with those observed in the validation and to identify any methods that offered significant gains in peak height when compared to the control method (*forensic*GEM™ Sperm). The Casework Direct, alkaline, and NP-40 cell lysis methods all generated average STR allele peak heights from semen samples that were slightly higher than those obtained from samples processed using the control method (*forensic*GEM™ Sperm) ($p > 0.1$, Figure 6). Further, the alkaline and NP-40 cell lysis groups had more samples reach or exceed the expected average STR allele peak height than all other methods tested, including the control (Figure 6). Significant decreases in mean allele peak height were observed from the HTX cell lysis samples when compared to the alkaline lysis ($p = 0.0009$), Casework Direct cell lysis ($p = 0.0108$), and NP-40 cell lysis groups ($p = 0.0029$). Additionally, peak heights from those samples processed using the HGH cell lysis method were significantly lower than those processed with the alkaline lysis method ($p = 0.0216$).

It is essential to have little variation in peak heights across loci, meaning peak heights between loci are relatively similar. Interlocus balance is important for two reasons: 1) to assure that all allele peaks are adequately above the analytical threshold because if some heights drop, alleles may be lost altogether and 2) mixture profile deconvolution relies on the assumption that balance is obtained and consistent across the entire profile. With this, it is easier to distinguish peaks from one individual versus another; if there is variation from locus to locus, that can become very difficult. The CV of LPH:TPH was used to assess the interlocus allele peak height variability. The optimal CV for interlocus balance has been described as 0.35 or lower [71]; thus, the goal for this work was to achieve and compare each to cell lysis method to the control method (*forensic*GEM™ Sperm). All lysis methods performed comparably to the control method when interlocus balance was examined except HTX cell lysis, where a significant increase was revealed ($p < 0.00005$, Figure 7). While not significantly different, it should be noted that the samples processed with the alkaline lysis method produced a mean CV lower than that of the control group and lower than the optimal CV of 0.35 ($p = 0.99$, Figure 7).

Overall, when STR profiles from semen samples processed with each of seven cell lysis methods were evaluated using the in-tube approach, no method significantly or consistently outperformed the control method (*forensic*GEM™ Sperm) across each metric examined. However, three of the alternative cell lysis methods tested exhibited positive improvements in *some* metrics examined when compared to the control method. Casework Direct, alkaline lysis, and NP-40 cell lysis buffer all resulted in similar or higher percentages of STR allele detection, higher mean STR allele peak heights (some higher than expected based on the internal validation), and a comparable or lower LPH:TPH CV than that of the control (Figure 8). Notably, semen 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 drop out (Figure 9). A summary of the quantitative data compiled in this study is provided in Table 2. Based on these results, Casework Direct, alkaline lysis, and NP-40 are identified as the sperm cell lysis methods that may serve as the most suitable alternatives to the currently used method, *forensic*GEM™ Sperm, when an in-tube, manual approach is desired.

Comparative Study – Microchip-Based Approach

Currently available microdevices, including the sexual assault microdevice designed in the Dawson Green laboratory at VCU, omit DNA quantitation from the integrated on-chip workflow. Instead, in these devices, a specific, standard volume of sample lysate is metered into the PCR amplification chamber; once designed, this volume is not alterable from sample to sample. Thus, for this project, the appropriate metered volume for simulated chip-based amplification was determined for each sperm cell lysis method tested based on the average DNA yields obtained for each. By using a standardized volume of lysate for STR amplification of all samples within a group, the microchip process was effectively simulated. As with the in-tube assay testing detailed above, the goal for each sperm cell lysis method tested was to achieve 100% STR allele detection and to generate DNA profiles of equal or higher quality than the control method (*forensic*GEM™ Sperm). When a standard "metered" volume was used (regardless of quantification) for each cell lysis method tested, only two methods resulted in 100% STR allele detection across all samples: Casework Direct and HGH cell lysis (Figure 10). Again, semen samples processed using the HTX cell lysis method produced significantly fewer detectable STR alleles when compared to all other methods tested (p <0.00005); for this group, just over 50% of expected STR alleles were detected (Figure 10). All other methods produced STR profiles with more than 95% of STR alleles detected,

and none were significantly different than observed in the control group (*forensic*GEM Sperm™). Interestingly, the percentage of STR alleles detected when DNA input was not normalized were similar or higher than those obtained when DNA input was standardized. Further, variation in the percentage of STR alleles detected for each group tested was similar, regardless of whether DNA input was normalized (in-tube approach) or not (chip-based approach). This supports the suggestion that the quantification step of the forensic DNA workflow may not be needed for rapid, high volume testing of *certain* forensic evidence samples, as their DNA yields are predictable and standardized enough to produce consistent, viable results without it.

As noted above, when examining STR data, it is important to carefully evaluate profile quality, in addition to the number of alleles detected. Thus, for each cell lysis method tested using the microchip-based approached, the goal was again to achieve STR allele peak heights consistent with the height of those observed in the validation; further, methods that offered significant gains in peak height when compared to the control method (*forensic*GEM™ Sperm) should be identified as alternatives for microdevice integration. Alkaline lysis was the only method to generate average STR allele peak heights greater than that of the control method (*forensic*GEM™ Sperm); however, Casework Direct, NP-40 cell lysis buffer, and HGH cell lysis methods all generated STR mean peak heights similar to that of the control group ($p > 0.7$, Figure 11). All methods tested, with the exception of HTX cell lysis, achieved peak heights greater than the average STR allele mean peak height reported in the laboratory's internal validation (Figure 11). As with the in-tube approach reported above, the HTX cell lysis method was again found to produce significantly lower mean STR peak heights than most other methods tested ($p < 0.05$ for each).

As previously discussed, having little variation in peak heights across all loci is important, as it assures that all allele peaks are above the analytical threshold and so that mixture

deconvolution can be accurately performed. In this microchip simulation study, the CV of LPH:TPH was again used to assess peak height variability (i.e., interlocus balance) between loci, with a goal of 0.35 or lower [71]. While no method achieved a mean CV of 0.35 or lower, all sperm cell lysis methods performed comparably to the control method (*forensic*GEM™ Sperm), except for the HTX cell lysis group, where a significant increase in CV was observed ($p < 0.00005$, Figure 12). The slight increase in CV above the ideal level is not surprising given that the total amount of input DNA in these samples is variable (a standard volume is used, regardless of quantification values). Furthermore, the template DNA amounts in many of these samples is likely higher than the ideal target amount, which results in higher overall peak heights and exacerbated preferential amplification of smaller DNA fragments, which produces more variability across each profile, negatively impacting the CV [71,73].

Although it has been shown both internally and in the literature that spectral failure (ie. allelic "pull-up") is commonly seen when the PowerPlex® Fusion 5C kit is used [71,73], it was important to carefully assess this artifact with the microchip simulated sample sets, given their increased mean allele peak heights. HTX cell lysis resulted in the least amount of pull-up (Table 3); however, this was expected as it exhibited the lowest peak heights, and most allelic dropout. As for the other six sperm cell lysis methods tested, the number of loci exhibiting pull-up correlates directly to the average STR allele peak heights, as expected. For example, the samples processed using the alkaline cell lysis method had the highest average STR allele peak height (3572.18 RFU) and the largest number of loci exhibiting pull-up (9.21), while SwabSolution™ and NP-40 lysis samples produced the lowest average STR allele peak heights (2373.07 and 2349.5 RFU, respectively) along with the lowest average number of loci displaying pull-up (3.79 and 3.73, respectively). Although pull-up does create problems with profile interpretation, it is a known,

common artifact associated with the PowerPlex® Fusion 5C kit, which was used in this study. Fortunately, these artifacts can be mitigated in a microchip-based assay by simply lowering the volume of cell lysate metered into the PCR amplification chambers.

Overall, when STR profiles from semen samples processed with each of seven cell lysis methods were evaluated using the microchip-based approach, no method significantly or consistently outperformed the control method (*forensic*GEM™ Sperm) across each metric examined. This is consistent with the results observed for the in-tube assay, as described above. However, four of the alternative sperm cell lysis methods tested exhibited positive improvements in *some* metrics examined when compared to the control method. Casework Direct, alkaline lysis, NP-40 cell lysis buffer, and HGH cell lysis methods all resulted in higher percentages of STR allele detection, similar mean allele peak heights (all higher than expected based on the internal validation), and a comparable LPH:TPH CV to that of the control (Figure 13). Notably, semen samples processed using the HTX cell lysis method again consistently performed more poorly than all other sample groups, resulting in lower peak heights, poor interlocus balance, and substantial allelic drop out (Figure 14). A summary of the quantitative data compiled in this microchip-based approach study is provided in Table 4. Based on these results, Casework Direct, alkaline lysis, NP-40, and HGH are identified as the sperm cell lysis methods that may serve as the most suitable alternatives to the currently used method, *forensic*GEM™ Sperm, when integration into a microdevice is desired.

Because a microdevice offers a fundamentally different environment and approach from that of a manual process using a microcentrifuge tube, there are additional factors that must be considered prior to integration, including time required for processing (both hands-on and total processing time). The hands-on time for a traditional differential cell lysis followed by a standard

Qiagen® DNA extraction is ~90 minutes, while the total processing time is ~180 minutes (assuming an average sample set of 20) (Table 5**)** [74]. This is a manual, time-consuming process. Furthermore, this method includes proprietary components, is the most expensive per reaction (versus those methods compared in this study) and presents a number of challenges when attempting to integrate onto a multi-step microfluidic device (e.g., requires large volumes, multiple wash steps, and the difficulty of silica filtration) (Table 5) [75,76]. The cell lysis method currently used in our laboratory's sexual assault microdevice (*forensic*GEM™ Sperm) offers a fast alternative that is easily automatable, however, it is the second most expensive of those tested during this research and also contains proprietary components. All alternative methods that were explored in this study have an approximate cost of less than one dollar per reaction and only requires 30 – 50 minutes of hands-on time and 40 – 85 minutes of total processing time (Table 5). For these, the cost savings helps offset the increase in time required. Additionally, of the alternative sperm cell lysis methods tested in this study, only NP-40 cell lysis buffer and HTX cell lysis demonstrated potential issues with microchip integration. Finally, it should be noted that of the sperm cell lysis methods examined herein, only the SwabSolution™ and Casework Direct methods include proprietary components, which could complicate future attempts to license the method or resulting microdevice (Table 5). Based on this, and the quantitative data discussed above, Casework Direct, alkaline lysis, and the HGH cell lysis methods are recommended for further exploration as the most suitable methods for microdevice integration.

Conclusions

Currently, the Dawson Green laboratory at VCU is working to develop a microfluidic device that can rapidly process sexual assault samples through cell separation, cell lysis, and PCR

amplification without manual intervention. This microdevice design includes a front-end module for antibody-based, bead-mediated cell separation assay, which is followed by individual chambers used for side-by-side processing of the bound and unbound fractions through cell lysis, metering, and PCR amplification. Unfortunately, the currently used cell lysis method (*forensic*GEM™ Sperm) has consistently resulted in low-level STR profiles, frequent STR allelic dropout, and PCR inhibition [17]. Consequently, this study aimed to identify an alternative cell lysis method that can be used with sperm-containing sexual assault samples in combination with the currently used cell separation chemistry in both a manual, in-tube assay as well as an automated microchip-based assay. Further, identification of a cell lysis method that could be easily integrated into the existing sexual assault microdevice hardware platform that contains few-to-no proprietary components would be ideal. Altogether, if successful, this work will provide faster, cheaper alternatives to traditional differential cell lysis techniques, that could significantly decrease sample processing times and reduce the risk of contamination and sample loss.

Based on the results from this research, the sperm cell lysis methods deemed most suitable for a manual (in-tube) workflow are the Casework Direct, alkaline lysis, and NP-40 cell lysis buffer methods. While each of these performed comparably in our study, NP-40 comes with the lowest price per reaction, while the alkaline lysis method offers a slight edge in hands-on and total sample processing time. Similarly, when the microchip-based approach was tested, these same methods, along with the HGH natural decondensation method, provided acceptable results that were comparable to the currently used sperm cell lysis method (*forensic*GEM™ Sperm). However, the NP-40 lysis technique requires an ~-20°C incubation, which is difficult to achieve with the current microdevice platform. Additionally, the Casework Direct solution is more costly and includes proprietary components, which add challenges to the licensure process. Consequently, if looking

to integrate a sperm cell lysis method onto an automated microdevice method, one should most seriously consider either the HGH natural decondensation method or the alkaline lysis method – each offers a low-cost, non-proprietary method that consistently produces acceptable results. If looking for a single sperm cell lysis method that could be utilized as a validated manual method with the option to easily transition to an automated, microdevice-based method, the alkaline lysis procedure may be the best option given its advantages with both approaches.

While the results of this study are very promising, there are modifications to these sperm cell "direct" lysis methods that could be explored prior to selection and implementation. For example, it may be possible that Casework Direct will be viable with shorter incubation times in an effort to reduce overall processing times. Additionally, although HGH – mediated sperm cell lysis alone resulted in elevated STR allele peak heights with a moderately low CV, the addition of the GSTO2 additive could function as a way to accelerate nuclear decondensation [70]. Lastly, the working pH of the HEPES buffer in the HTX cell lysis method utilized herein should be closely examined in advance of adaptation. A previous study was that used HEPES buffer prior to capillary electrophoresis analysis suggested that the utilization of HEPES buffer at the proper pH (6.0) is critical in order to achieve the desired outcomes. This publication indicated the use of a HEPES buffer at a concentration of 4.766 g/L [77]; the HEPES buffer used in our study had a pH of 5.5 ± 0.5 and a concentration of 238 g/L. Although the HEPES buffer used in our study was at the approximate pH recommended, the concentration (taken from the reagent bottle recommended by Seita et al.) was 50-fold higher, leading to a higher ion concentration. These charged ions could interfere with capillary electrophoresis analysis by competing with the DNA for injection. This could explain the poor STR profiles obtained from the semen samples in this study which used

HTX as the cell lysis method. Thus, before ruling this method out, it may be beneficial to test the HTX method using a lower concentration of HEPES buffer.

While some modifications may be prudent to explore, the work described in this report provides several clear alternative approaches to direct sperm cell lysis for in-tube and automated microdevice sample processing. Each recommended method has advantages and disadvantages that must be weighed by individual laboratories and users who seek an alternate method for rapid, efficient sperm cell lysis. A single best method (alkaline lysis) has been suggested as an approach that would most easily transition between manual and automated processes. Going forward, one or more of these recommended direct cell lysis chemistries will be integrated and further tested in the sexual assault microdevice environment.

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Appendix

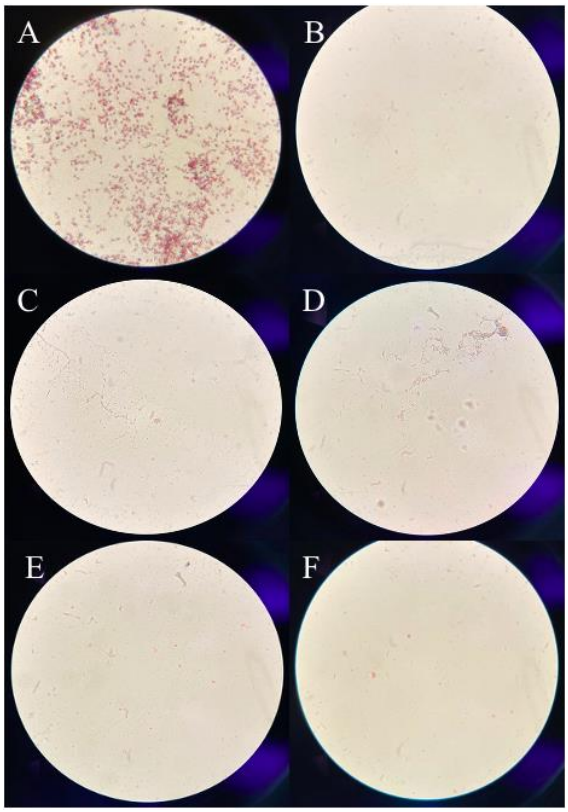

Figure 1. Microscopic images captured of sperm cells at 400X magnification after KPICS staining, subsequent to cell lysis. (A) is an untreated positive control (1:10 semen dilution). Treatment methods include (B) *forensic*GEM™ Sperm which had a median score of 1+, (C) Casework Direct and (D) alkaline lysis, which both resulted in the complete lysis of sperm cells across all donors, (E) NP-40 cell lysis, resulting in a median score of 3+, and (F) HEPES + Triton-X cell lysis, resulting in a median score of 3+.

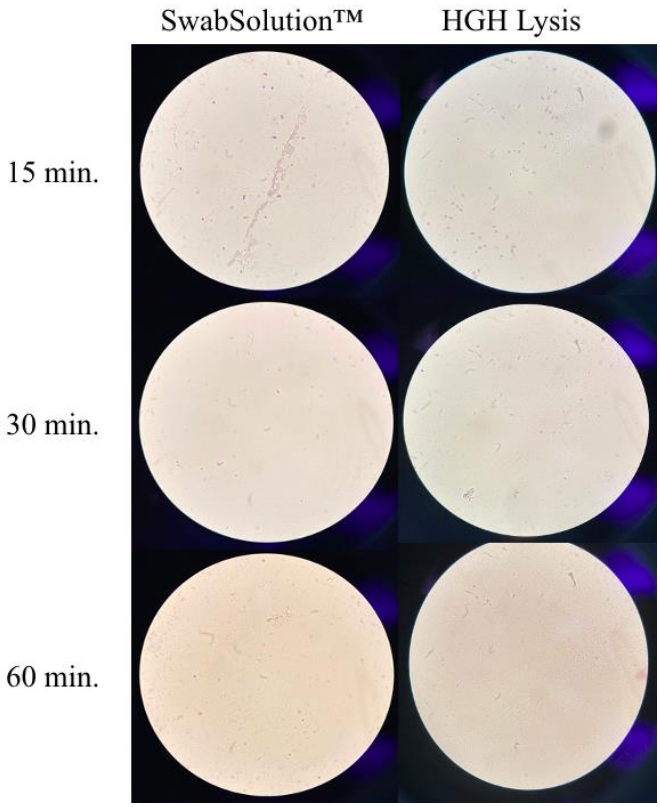

Figure 2. Microscopic images captured of sperm cells at 400X magnification after KPICS staining, subsequent to cell lysis. A representative image is displayed for the SwabSolution™ and HGH methods for each of the three time intervals tested. For both methods, the number of intact cells that remained decreased as the incubation time increased.

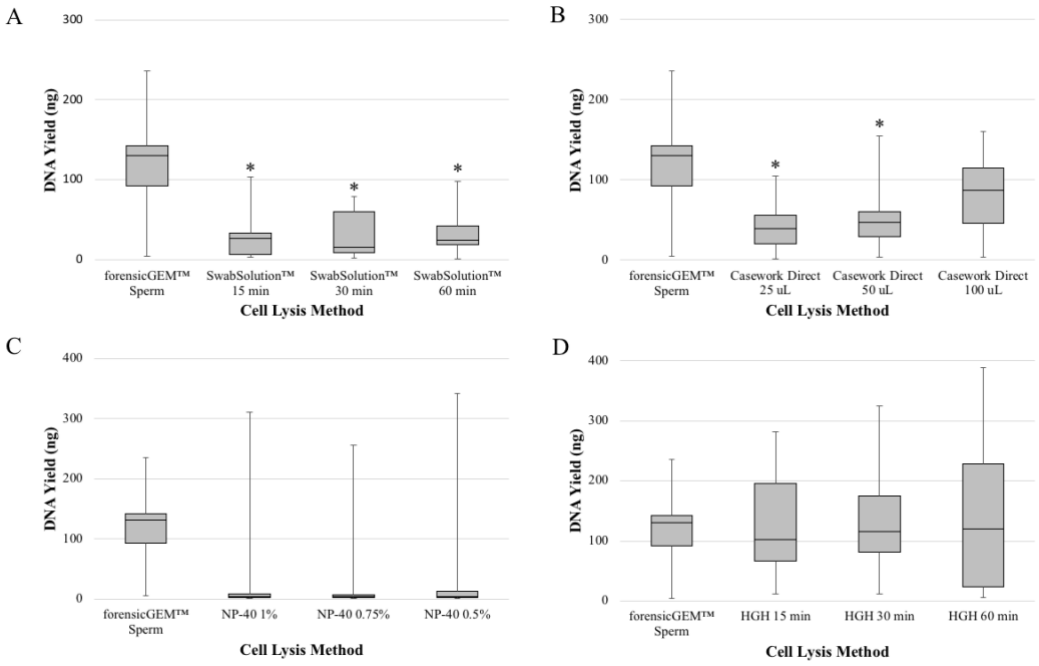

Figure 3. Mean DNA yields obtained from real-time qPCR comparing *forensic*GEM™ Sperm (n = 9) to four alternative lysis methods (n = 10). Significant decreases were observed between the control and all three time conditions for SwabSolution™ (A, $p < 0.001$). Significant decreases were observed between the control and the 25 uL and 50 uL conditions of Casework Direct (B, $p < 0.05$). These significant differences are represented by "*". No significant differences were observed between the control group and NP-40 (C) or HGH cell lysis (D). The boxes represent the upper and lower quartiles, so that it spans the interquartile range, with the median represented by the middle line. The whiskers represent the variability outside the upper and lower quartiles.

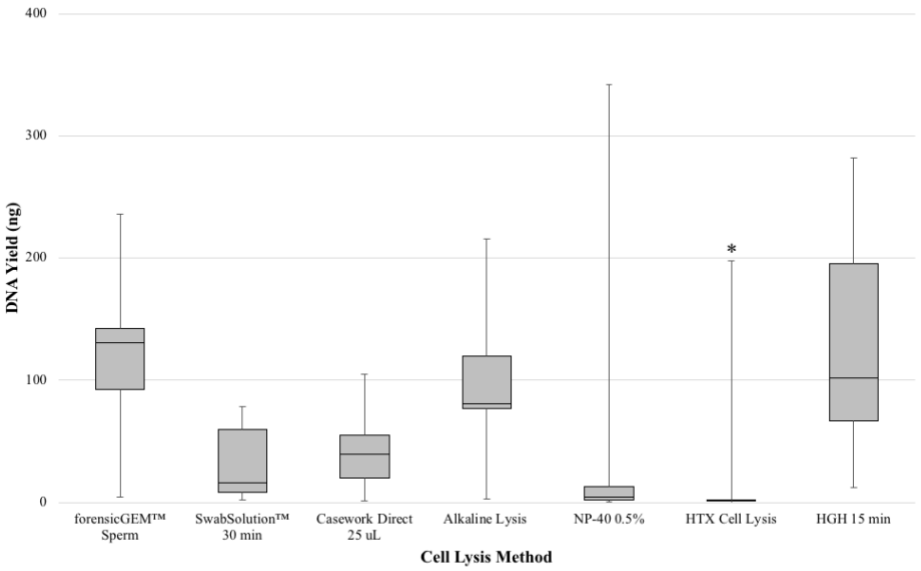

Figure 4. Mean DNA yields obtained from real-time qPCR comparing *forensic*GEM™ Sperm (n = 9) to seven alternative cell lysis methods (n = 10). Significant decreases were observed between HTX cell lysis and the 15 minute condition of HGH cell lysis ($p < 0.05$). No other significant differences were revealed. The boxes represent the upper and lower quartiles, so that it spans the interquartile range, with the median represented by the middle line. The whiskers represent the variability outside the upper and lower quartiles.

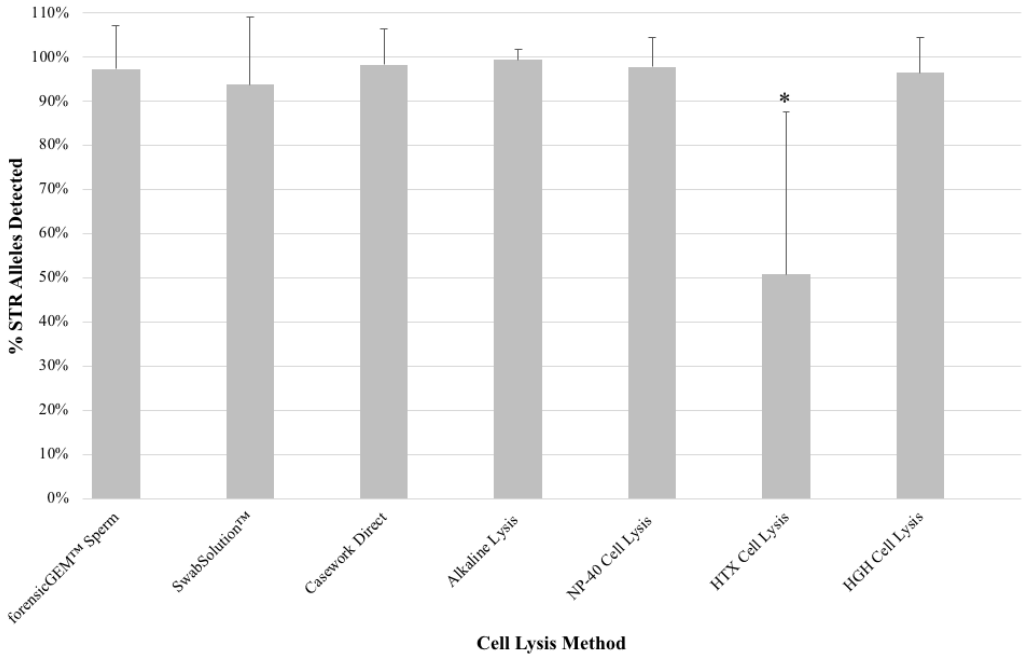

Figure 5. STR alleles detected for each cell lysis method processed with the in-tube assay (n = 10). HTX cell lysis produced significantly fewer STR alleles than all other cell lysis methods ($p < 0.00005$), while no difference was observed between the control and all other groups.

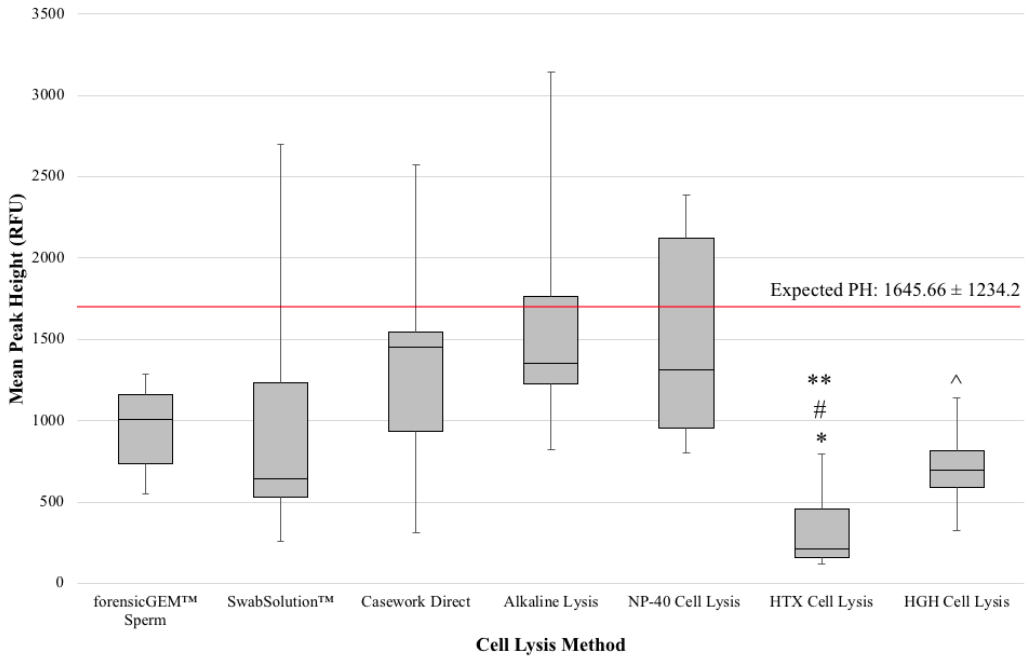

Figure 6. Mean STR allele peak heights observed for each of the seven cell lysis methods for samples processed with the in-tube assay (n = 10). HTX cell lysis produced significantly lower peak heights compared to alkaline cell lysis (*p = 0.0009), Casework Direct cell lysis (#p = 0.0108), and NP-40 cell lysis (**p = 0.0029). Additionally, HGH produced significantly lower peak heights than alkaline cell lysis (^p = 0.0216). The peak heights observed from the SwabSolution™, Casework Direct, alkaline lysis, NP-40 cell lysis, HTX lysis, and HGH cell lysis methods were not significantly different than the control group; however, Casework Direct, alkaline lysis, and NP-40 cell lysis produced mean peak heights that were slightly higher than the control group. The red line throughout represents the expected mean peak height as reported in the internal validation of the PowerPlex® Fusion 5C kit. The boxes represent the upper and lower quartiles, so that it spans the interquartile range, with the median represented by the middle line. The whiskers represent the variability outside the upper and lower quartiles.

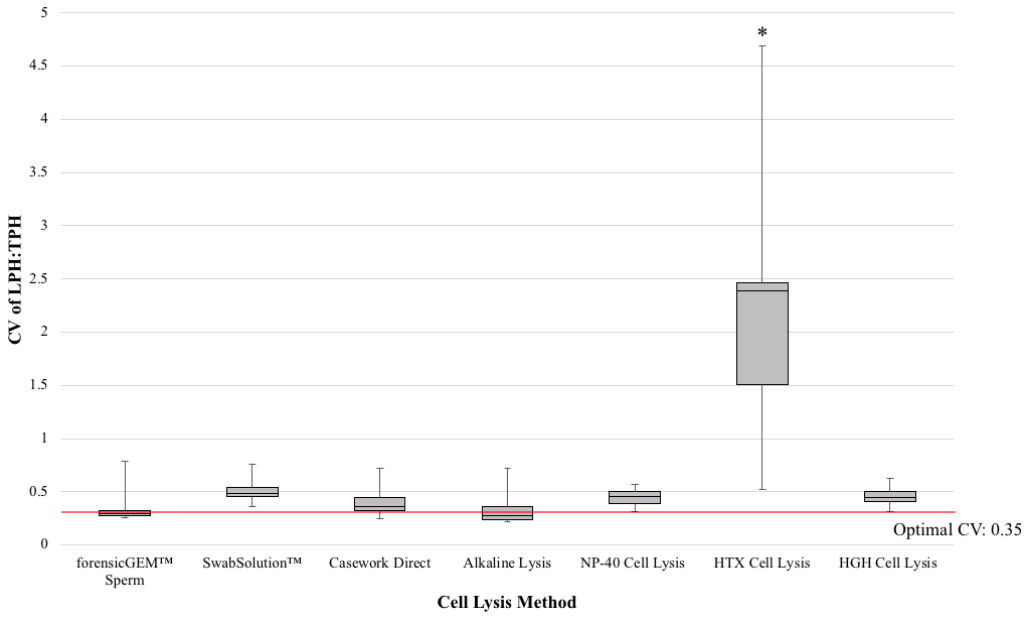

Figure 7. The coefficient of variation of LPH:TPH for the seven lysis methods processed with the in-tube assay (n = 10). The optimal CV according to Connon et al, is represented by the red line [71]. HTX cell lysis was observed to have a significantly higher CV than all other lysis methods ($p < 0.00005$). All other methods had a CV below or slightly above the optimal CV which indicates a relatively balanced interlocus profile. The boxes represent the upper and lower quartiles, so that it spans the interquartile range, with the median represented by the middle line. The whiskers represent the variability outside the upper and lower quartiles.

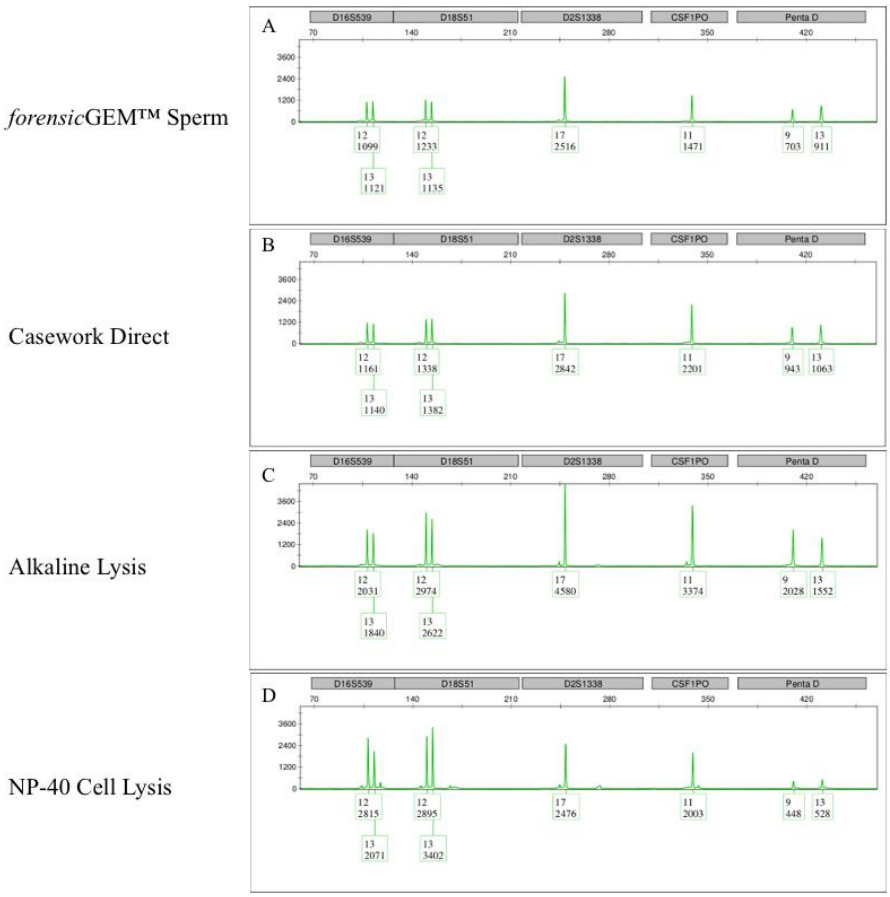

Figure 8. The green channel of a representative electropherogram displaying the control method (A)), Casework Direct (B), alkaline lysis (C), and NP-40 cell lysis (D) methods processed using the in-tube assay. This illustrates the increase in STR allele peak heights in the alternative methods compared to the control, as well as the similar interlocus balance seen between these methods.

Figure 9. The green channel of representative electropherograms comparing the control method (A) to HTX cell lysis (B). Samples lysed with HTX exhibited allelic drop-out, low peak heights, and ski-slope, resulting in the poor-quality profile using the in-tube assay.

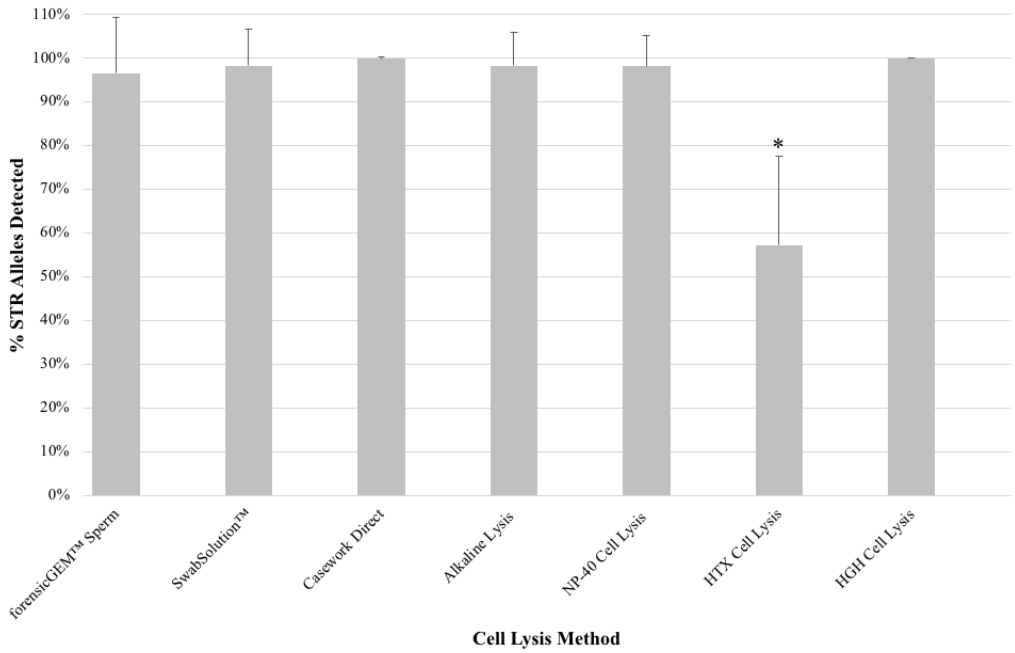

Figure 10. STR alleles detected for each cell lysis method processed though the chip-based approach (n = 10). HTX cell lysis produced significantly fewer STR alleles than all other cell lysis methods ($p < 0.00005$), while no difference was observed between the control and all other groups.

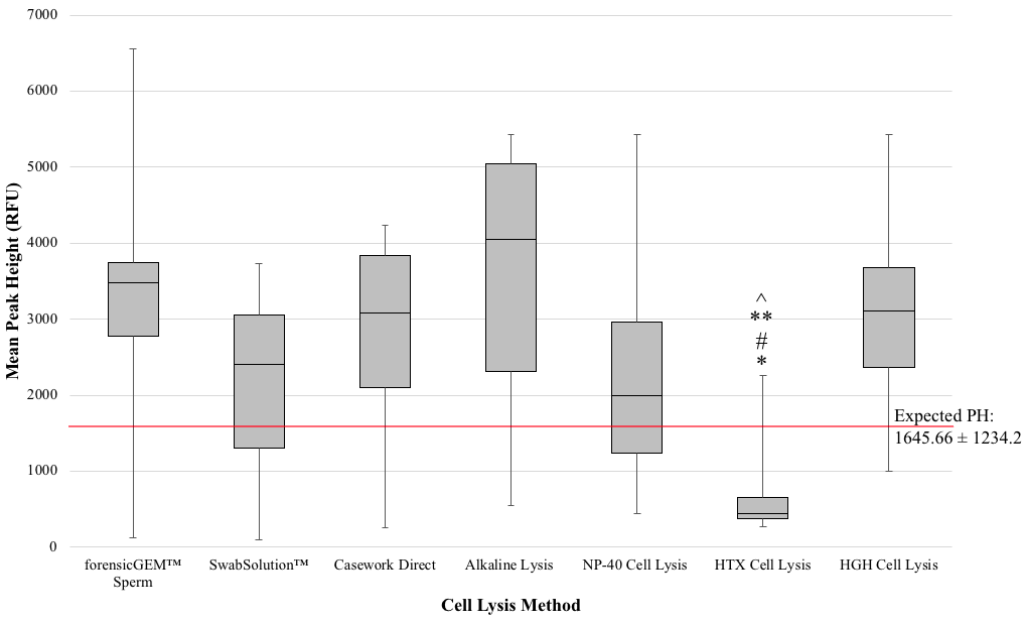

Figure 11. Mean STR allele peak heights observed for each of the seven cell lysis methods for samples processed with the chip-based approach (n = 10). HTX cell lysis produced significantly lower peak heights compared to alkaline cell lysis (*p = 0.0009), Casework Direct cell lysis (#p = 0.0470), *forensic*GEM™ Sperm cell lysis (**p = 0.0055) and HGH cell lysis (^p = 0.0144). The peak heights observed from the SwabSolution™, Casework Direct, alkaline lysis, NP-40 cell lysis, and HGH cell lysis methods were not significantly different than the control group; however, Casework Direct, alkaline lysis, and HGH cell lysis produced mean peak heights that were very similar or slightly high than the control group. The red line throughout represents the expected mean peak height according to internal validation of the PowerPlex® Fusion 5C kit. The boxes represent the upper and lower quartiles, so that it spans the interquartile range, with the median represented by the middle line. The whiskers represent the variability outside the upper and lower quartiles.

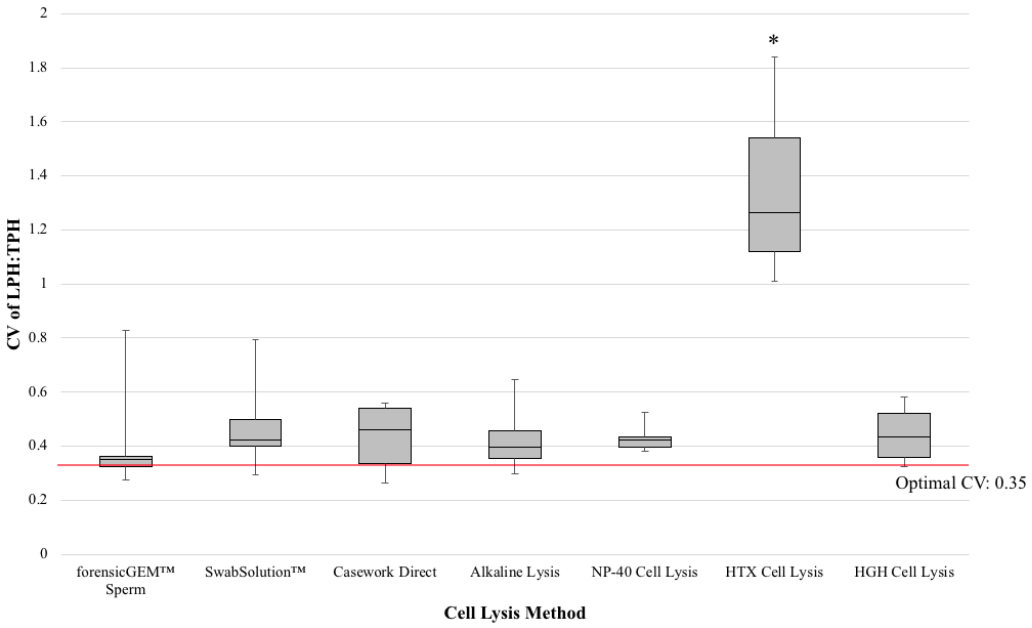

Figure 12. The coefficient of variation of LPH:TPH for the seven lysis methods processed with the chip-based approach (n = 10). The optimal CV according to Connon et al, is represented by the red line [71]. HTX cell lysis was observed to have a significantly higher CV than all other lysis methods ($p < 0.00005$). All other methods had a CV slightly above the optimal CV which indicates a relatively balanced interlocus profile. The boxes represent the upper and lower quartiles, so that it spans the interquartile range, with the median represented by the middle line. The whiskers represent the variability outside the upper and lower quartiles.

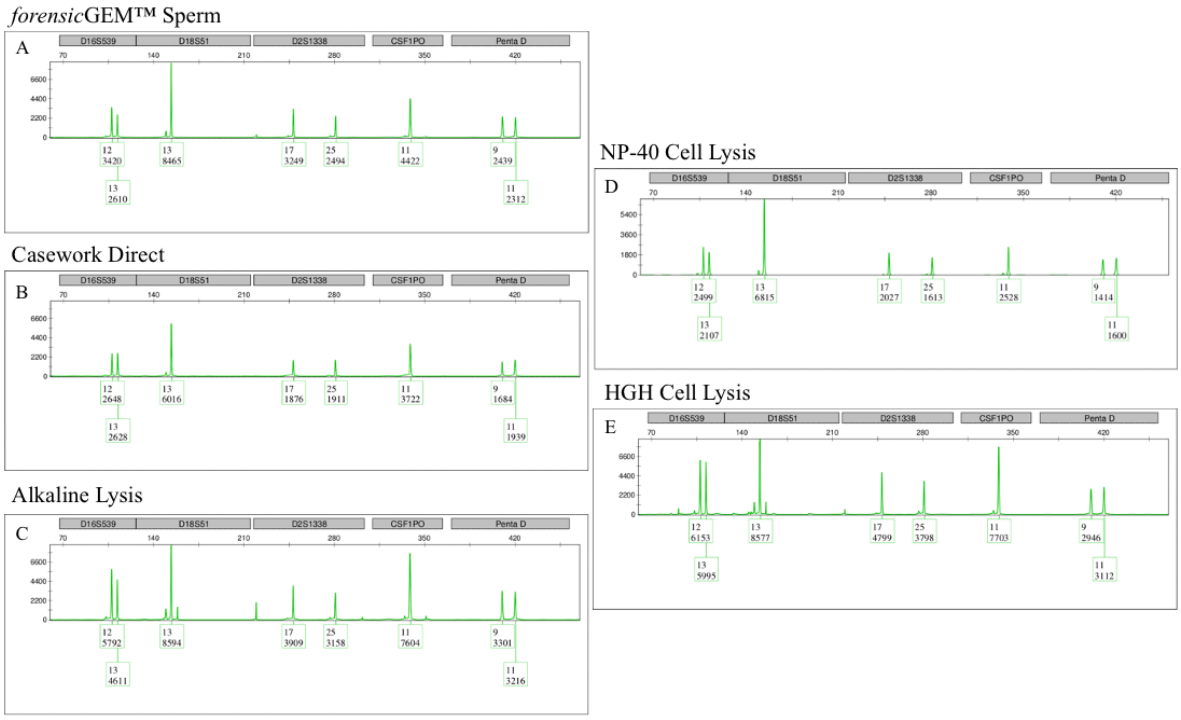

Figure 13. The green channel of a representative electropherogram displaying the control method (A), Casework Direct (B), alkaline lysis (C), NP-40 cell lysis (D), and HGH cell lysis (E) methods processed using the chip-based approach. This illustrates the (non-significant) increase in STR allele peak heights in the alternative methods compared to the control, as well as the similar interlocus balance seen between these methods.

Figure 14. The green channel of representative electropherograms comparing the control method (A) to HTX cell lysis (B). Samples lysed with HTX exhibited allelic drop-out, low-level peaks, and ski-slope, resulting in the poor-quality profile using the chip-based approach.

Table 1. Scoring method used for KPICS evaluation.

0 or None	No sperm per field
Few	Few sperm per slide (approximate number recorded where possible)
1+	1 sperm seen in some fields, difficult to find
2+	1-5 sperm seen in most fields, easy to find
3+	5-10 sperm seen in most fields
4+	More than 10 sperm in every field

Table 2. Summary of quantitative data for each cell lysis method processed using the in-tube assay.

Sample Condition (In-tube assay)	Peak Height (RFU)	Interlocus Balance (CV of LPH:TPH)	% STR Alleles Detected
***forensic*GEM™ 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	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 ± 364.23	0.457 ± 0.11	97%

The three alternative methods that performed similarly or better than the control method are highlighted by the red box.

Table 3. Mean observed pull-up across all lysis methods processed using the chip-based approach.

Sample Condition (Chip-Based Assay)	# of Loci Exhibiting Pull-Up
*forensic*GEM™ Sperm	6.65 ± 5.78
SwabSolution™	3.79 ± 5.29
Casework Direct	5.18 ± 4.68
Alkaline Lysis	9.21 ± 6.10
NP-40	3.73 ± 5.50
HEPES + Triton X-100	0.875 ± 1.51
HTF + Glutathione + Heparin	6.22 ± 5.25

Table 4. Summary of quantitative data for each cell lysis method processed using the chip-based approach.

Sample Condition (Chip-based assay)	Peak Height (RFU)	Interlocus Balance (CV of LPH:TPH)	% STR Alleles Detected
***forensic*GEM™ Sperm**	3369.07 ± 2041.9	0.406 ± 0.19	97%
SwabSolution™	2373.07 ± 1670.3	0.457 ± 0.13	98%
Casework Direct	2797.23 ± 1414.9	0.436 ± 0.13	100%
Alkaline Lysis	3572.18 ± 2015.5	0.42 ± 0.16	98%
NP-40	2349.5 ± 1863.1	0.429 ± 0.08	98%
Natural Decondensation – HTX	769.83 ± 705.6	1.349 ± 0.46	57%
Natural Decondensation – HGH	3110.27 ± 1662.8	0.442 ± 0.11	100%

The three alternative methods that performed similarly or better than the control method are highlighted by the red box.

Table 5. Summary of additional factors to consider when identifying which method(s) to recommend for future implementation.

Method	Cost (per reaction)*	Hands-on Time (mins)*	Total Processing Time (mins)*	Ownership	Issue(s) with Chip Integration
Standard Qiagen	$5.60	90	180	Proprietary	Large volumes, wash steps, silica filtration
***forensic*GEM™ Sperm**	$4.30	30	50	Proprietary	None
SwabSolution™	$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	$0.007	50	85	Non-Proprietary	Incubation on ice
HEPES + Triton X-100	$0.018	30	40	Non-Proprietary	CE inhibition
HTF + Glutathione + Heparin	$0.031	30	50	Non-Proprietary	None

* All costs and times are approximations
The red boxes highlight those methods that performed similarly or better than the control method between the in-tube and chip-based assays.

Vita

Sarah Kathleen Schellhammer was born and raised in Winchester, Virginia. Her love for forensic science began her freshman year of high school and has continuously grown since attending Virginia Commonwealth University (VCU) in Richmond, VA. She received her Bachelor's of Science in Forensic Science with a concentration in Forensic Biology in addition to dual minors in biology and chemistry, with Magna Cum Laude honors from VCU in May 2019. She is currently pursuing her Master's of Science in Forensic Science with a concentration in Forensic Biology, anticipating graduation in May 2021 with Magna Cum Laude honors from VCU. Since beginning her journey within the graduate program at VCU, Sarah has worked as a Graduate Research Assistant in the Dawson Green Forensic Molecular Biology Laboratory, located in the Department of Forensic Science. In addition to taking on this role, Sarah had the opportunity to attend the 73rd Annual American Academy of Forensic Sciences Conference in February 2021 as a student affiliate where she presented one of her research projects. As for academic accomplishments, Sarah made the Dean's List every semester as an undergraduate and graduate student and received the VA Merit Award.