DIFFERENTIAL EXTRACTION: A COMPARISON OF SpermX AND Sampletype i-sep DL-MB

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Abstract

Differential extraction is a modified extraction that was developed in 1985 to separate male DNA (sperm cells) from female DNA (epithelial cells) in mixed samples. This process presents challenges such as the loss of male spermatozoa cells and the potential for epithelial cell carryover. Recent advancements in DNA analysis kits have improved separation during the differential extraction process. In this project, Biotype's Sampletype i-sep® DL-MB and InnoGenomic's SpermX™ kits were compared to a variation of the Qiagen DNA Investigator Kit to determine cell separation efficiency and sensitivity. Ease of use and processing time were also taken into consideration. Mixed samples at semen concentrations of neat, 1:10, and 1:100 dilutions were created and extracted according to the kit protocols and quantified with Quantifiler Trio™. Results obtained from triplicate testing show that SpermX™ is highly effective at isolating the sperm fraction from neat and 1:10 sperm samples. SpermX™ yielded an average of 0.75 ng/µl compared to 0.22 ng/µl for neat and 0.12 ng/µl compared to 0.041 ng/µl for 1:10, when correlated with i-sep® DL-MB. Conversely, the i-sep® method outperforms SpermX™ in extracting DNA from the epithelial fraction. Notably, the DNA quantities from SpermX™ at 1:100 dilution were similar to those from i-sep® at the same dilution (0.031 ng/µl and 0.028 ng/µl), and both methods surpassed the Qiagen DNA Investigator Kit. Future applications for SpermX™ and i-sep® DL-MB would be to develop a way for the protocols to be automated or for the kits to be used in cold cases.

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Introduction

The advent of DNA identification has revolutionized the science of crime detection. This technique, when performed according to strict guidelines, is a highly reliable tool for quantifying human individuality and for exonerating innocent individuals. DNA identification was first used in 1985 for an immigration case in the United Kingdom. Dr. Alec J. Jeffreys, of the University of Leicester, was approached by a lawyer representing a Ghanian family, with the mother having lived in the UK for some time with two of her children. Her remaining child had recently arrived in Britain but had been accused of having a fake passport and was at risk of being permanently sent back to Ghana. After discussions with the lawyer, Dr. Jeffreys took a sample of blood from the mother and the boy she claimed was her son, as well, as her other children. He then extracted their DNA and compared their genetic information. The information showed that not only was the boy definitely the mother's son, but that he was related to her other children as well. As a result, the case was dropped and the boy was permitted to stay in the UK (Your Genome, 2023).

The first forensic use with DNA "fingerprinting" and semen was in 1986 when police in the UK requested Dr. Jeffreys to verify a suspect's confession that he was responsible for two rape-murders. The case begins in 1983 when 15-year-old Lynda Mann took a shortcut on her way home from babysitting instead of taking her normal route home. She did not return and her parents and neighbors spent the night searching for her. The next morning, she was found raped and strangled on a deserted footpath known locally as the Black Pad. Using forensic science techniques available at the time, police linked a semen sample taken from her body to a person with type A blood and an enzyme profile that matched only 10% of males. Three years later, in 1986, 15-year-old Dawn Ashworth left her home to visit a friend's house. Her parents expected her to return at 9:30 pm; when she failed to do so they called police to report her missing. Two

days later, her body was found in a wooded area near a footpath called Ten Pound Lane. She had been beaten, savagely raped, and strangled. The modus operandi matched that of the first attack, and semen samples revealed the same blood type. An initial suspect was Richard Buckland, 17-years-old, who came forth with knowledge of Ashworth's body and eventually admitted to the crime after questioning. In early 1987, police asked every local male between the ages of 16 and 34 to voluntarily give blood samples for DNA testing. Tests proved that Buckland had not committed the crimes and was released. Eventually in late 1987, Colin Pitchfork was revealed as the culprit. He was identified as the culprit after a female colleague reported to police that another colleague of Pitchfork's had submitted a sample on his behalf. In January 1988 he was sentenced to life imprisonment for the two murders and 10 years for raping the victims; he was also sentenced to three years for each count of sexual assault and three years for perverting the course of justice, with all sentences to run concurrently (Wambaugh, 1995).

The first person to be convicted based on DNA evidence was Robert Melias in 1987 (Belair *et al*., 1991). Robert Melias was convicted of raping 43-year-old crippled women in Bristol, UK. A semen stain was found on her clothing that was identified to his genetic profile via a blood sample (Lohr, 1987). In the same year, Tommy Lee Andrews became the first American to be convicted on DNA evidence (Panneerchelvam & Norazmi, 2003). Andrews broke into Karen Munroe's house via her daughter's bedroom window where he raped Munroe at knifepoint. Andrews was spotted by a couple near Munroe's home, which was reported to police. Police eventually spotted his car, chased him, and Andrews eventually crashed his vehicle leading to his arrest. He was convicted of rape based on DNA evidence, in which his DNA profile was identified with that of semen traces recovered from the victim (Fox, 2022). Since these cases, DNA techniques and analyses have consistently been improving.

DNA analysis follows four specific steps to obtain accurate and informative results. These are: extraction, quantification, amplification, and separation/detection. DNA is extracted from its biological source material via a lysis phase to disrupt the nuclear membrane. This allows for nuclear DNA to be obtained. The extracted DNA is then cleaned via a series of wash steps and then precipitated for further applications. DNA extraction is followed by quantification that is typically conducted with quantitative PCR (qPCR). qPCR is a molecular technique that amplifies and quantifies targeted DNA molecules to determine the amount of starting template concentration. After quantification, specific regions of DNA are targeted and amplified using the polymerase chain reaction (PCR). For this instance, multiplex PCR is used.

Multiplex PCR is an enzymatic process used to amplify multiple specific segments of DNA, producing millions to billions of copies from a very small initial sample. Multiplex PCR works by mimicking the natural process of DNA replication, but in a controlled and repeated manner using a machine called a thermal cycler. Multiplex PCR requires several key components: a template of DNA containing the target sequences, short DNA primers that define the regions to be copied, dNTPs (Deoxynucleoside triphosphates) as building blocks, a buffer solution with magnesium ions, and a heat-stable enzyme known as Taq polymerase, which synthesizes new DNA strands. The Multiplex PCR process occurs in cycles, each consisting of three main steps. First, during denaturation, the reaction is heated to around 94–98°C to separate the double-stranded DNA into single strands by breaking hydrogen bonds. Next, during annealing, the temperature is lowered to approximately 50–65°C, allowing the primers to bind to complementary sequences on each DNA strand. Finally, during extension, the temperature is raised to about 72°C, which is optimal for DNA polymerase to add nucleotides and extend the primers, forming new strands of DNA. Each cycle doubles the amount of target DNA, leading to

exponential amplification; after about 30 cycles, this results in roughly a billion copies of the desired sequence (Chimera & Dyer, 1992). Amplification kits, for example PowerPlex® Fusion 5C System, are commonly used to enable simultaneous PCR of 24 short tandem repeat (STR) markers. STRs are short, tandemly repeated, non-coding DNA sequences that consist of a repetitive unit of 1–6 base pairs. Forensic science relies on these regions because they are non-coding, highly variable (polymorphic), and are prone to frequent mutations (Fan & Chu, 2007).

STR alleles are interpreted relative to PCR amplification artifacts following separation by size using capillary electrophoresis and data analysis software. Capillary electrophoresis is a method for separating and identifying fragments of fluorescently labeled DNA by passing them through very thin polymer-filled capillaries. These capillaries apply a specific voltage to the DNA fragments which allow them to migrate through the capillary array. The fragments are separated by size, with the smallest fragments moving fastest. As the fluorescently labeled fragments move through the capillaries, they pass a detection window. Within the detection window is a laser that excites the fluorescent dyes. The emitted light is spectrally separated and recorded by a CCD (charge-coupled device) camera. The CCD camera records the fluorescent signature of each fragment, as well as the elapsed time for the fragment to migrate through the polymer. The raw data is sent to the collection software in the form of a peak and is then ready for analysis (Promega, 2007). These techniques and technologies are what helped make DNA analysis the gold standard in forensic science.

A problem still occurring today arises with a technique in DNA analysis called differential extraction. Issues during this process may include the loss of crucial male spermatozoa cells or incomplete separation. Recent advancements in DNA analysis supply kits have helped improve these problems. These kits include: Differex, Erase, forensicGEM,

MACSprep, i-sep DL-MB, and SpermX. However, only minimal experiments have compared them. A direct comparison between traditional extraction methods and these kits does not yield a clear winner. Also, enhanced workflows, such as direct PCR and Rapid DNA, have the potential to streamline processing of forensic evidence items, including those commonly submitted in sexual assault kits, but the FBI Quality Assurance System guidelines restrict CODIS-approved labs from implementing these methods for forensic samples (Harrel & Homes, 2022).

Differential Extraction

There are approximately 433,000 victims of rape and sexual assault in the US every year (Charlie Health Editorial Team, 2025). However, only 5% of cases involve DNA extraction (Forr *et al*., 2017). Once a sexual assault or rape has been reported, a sexual assault evidence recovery kit is utilized. This kit collects epithelial cells and possible sperm cells from the vaginal, oral, and anal cavities of the victim. The best technique for extracting DNA from these types of samples is a differential extraction. Differential extraction is a modified extraction method that was developed in 1985 to separate male DNA (sperm cells) from female DNA (epithelial cells) in mixed samples. The process involves a two-phase lysis followed by a preferred extraction method.

First, sodium dodecyl sulfate (SDS), ethylenediaminetetraacetic acid (EDTA), and proteinase K are added to separate out the female fraction. SDS works as a detergent to break down cell membranes and release DNA; EDTA is used to prevent DNA from degrading; and proteinase K is used to break down proteins. The sample is centrifuged to remove intact sperm and buffer containing the DNA from lysed epithelial cells from the solid matrix. The sample is then transferred to a spin basket where it is centrifuged again. The centrifugation pellets the sperm and cell debris. The solution containing the epithelial DNA is removed, but because of the

loose nature of the pellet, some of the solution, containing epithelial DNA, remains. To obtain sperm free of epithelial DNA, the sperm cells are treated with the same chemicals as before, plus dithiothreitol (DTT) (to break the disulfide bonds in the sperm cell membrane), and the male fraction is isolated after re-centrifugation (Figure 1). A benefit of differential extraction is that it allows the separation of male and female DNA prior to PCR, resulting in cleaner profiles and removing the need for mixture analysis. However, it only works for males who contribute spermatozoa and will not work for vasectomized or azoospermic males who lack sperm cells (Gill *et al*., 1985).

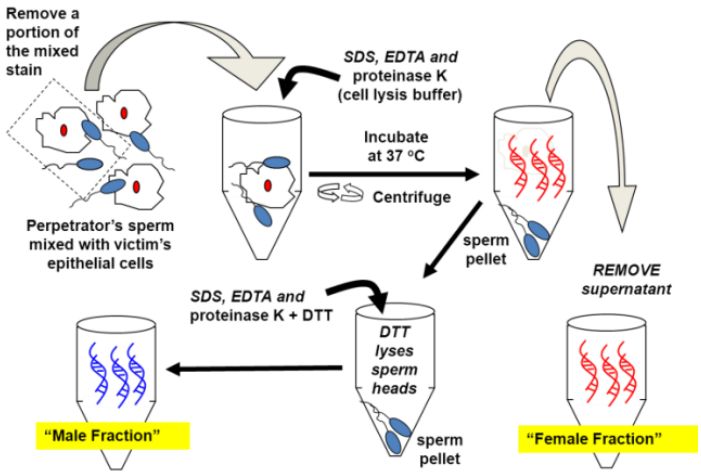

Figure 1: Visual Representation of How the Differential Extraction Process is Executed Starting with Reagents Added to the Mixed Sample and Finishing with an Isolated Male Fraction (Butler, 2012)

Even though differential extraction is the best method to separate sperm cells from epithelial cells, it isn't perfect. The first issue is that differential extraction can fail with very small and degraded samples or when the semen fraction is reduced, leading to loss of sperm DNA (Gusmão *et al.*, 2008). The second problem is that cell separation can result in the loss of 94-98% of the male sperm DNA component (Vuichard *et al.*, 2011). This is a problem because the male profile is often the most important part to analyze, since it is associated with the suspect. The third obstacle is that this process involves time-consuming, labor-intensive steps of

selective cell lysis, centrifugation, and separation into female and male cell fractions, which can take up to 8 hours. However, to combat this time-consuming process, automation has been implemented for some of the processes. One such kit that has implemented automation is the Differex kit (Clark *et al.,* 2021). The last dilemma is that this process can still result in a mixed profile, which is time-consuming during the analysis phase. These predicaments are why this field still needs to improve. The purpose of this study is to conduct a comparison experiment to determine the cell separation efficiency and sensitivity of the i-sep DL-MB and SpermX kits, which may ultimately improve the sperm yield in differential extractions.

Recent Differential Extraction Kits

Several automated and semi-automated techniques have adopted the fundamentals of conventional differential extraction techniques. However, lengthy incubation, several manual steps, and carryover of non-sperm material into the sperm fraction are some of the major limitations of this technique. An early method to combat these issues was conducted by Horsman *et al.* (2005) using a microfluidic device. This separation utilized the differential physical properties of the cells, which resulted in the epithelial cells settling to the bottom of the inlet reservoir and subsequently adhering to the glass substrate. This allowed the sperm cells to be separated from the epithelial cells.

Recently, advanced cell separation techniques have shown great promise for separating sperm cells from mixtures based on size, shape, composition, and membrane structure, as well as the antigens present on sperm membranes (Dash, 2024). Some examples are: Differex, Erase, forensicGEM, MACSprep, Dielectrophoresis (DEP) array, ADE (Acoustic Differential Extraction), FACS (Fluorescence-Activated Cell Sorting), LCM (Laser Capture Microdissection), i-sep DL-MB, and SpermX. These kits are either for sperm enrichment, cell

sorting, or for conducting experiments in one tube. No differential extraction step is needed for the all-in-one tube kits.

i-sep DL-MB

i-sep DL-MB is a multi-tube kit that uses a filter column to increase the concentration of DNA in solution while eliminating a portion of the fluid from the sample. This is done by retaining intact sperm heads, while the contaminants and lysis solutions are washed away from the sample. This kit is similar to a traditional differential extraction in that it uses a buffer solution, proteinase K, and DTT. The difference is that the buffer solution is preheated to 56°C rather than used at room temperature (21°C). The i-sep DL-MB kit works by placing a swab or stained material into the filter column. Buffer solution and proteinase K are added to the sample in the filter column. The sample is then incubated and centrifuged briefly at high rpm. This step is repeated to ensure all epithelial DNA has been obtained and removed from the spermatozoa. The last step is to lyse the sperm cells using buffer, proteinase K, and DTT, incubate the solution, and centrifuge (Figure 2). The two portions will then undergo purification, amplification, and separation/detection (Biotype, 2021).

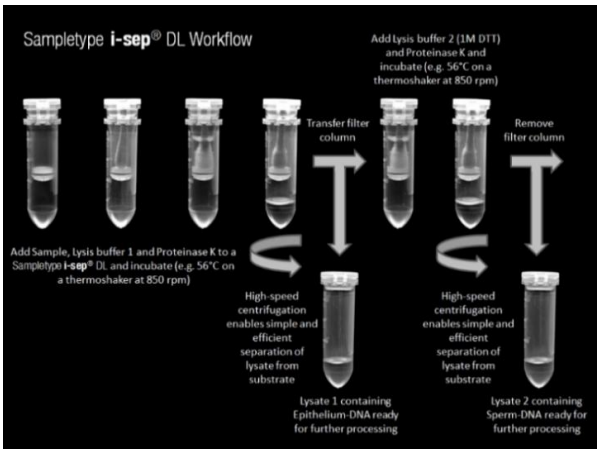

Figure 2: Visual Representation of How the i-sep DL-MB Process is Executed Starting with the Initial Sample and Ending with an Epithelial and Sperm Fraction (Biotype, 2021)

SpermX

SpermX is an all-in-one differential extraction kit. SpermX is a matrix-based system that requires low manipulation of the sample, produces a high yield of sperm DNA, and reduces epithelial cell carryover. This kit works by first conducting an epithelial cell digest. The sample is placed inside the provided tube along with the epithelial digest buffer. The tube is incubated, then centrifuged to trap the sperm cells in the matrix while the epithelial cells flow through. The epithelial fraction is then removed. A second epithelial digest is performed on the sperm fraction to reduce epithelial carryover. The tube is then centrifuged, and the liquid portion is removed. Three washes with a sperm digest solution are conducted to remove any residual chemicals or epithelial cells. This liquid is removed before the sperm digest buffer is added. After the addition of the sperm digest buffer, the tube is incubated and centrifuged. Once completed, the sperm digestion solution is added, the tube is centrifuged, and the sperm cells are collected (Figure 3). The two isolated samples can be purified and amplified, then subjected to separation/detection (InnoGenomics, 2020).

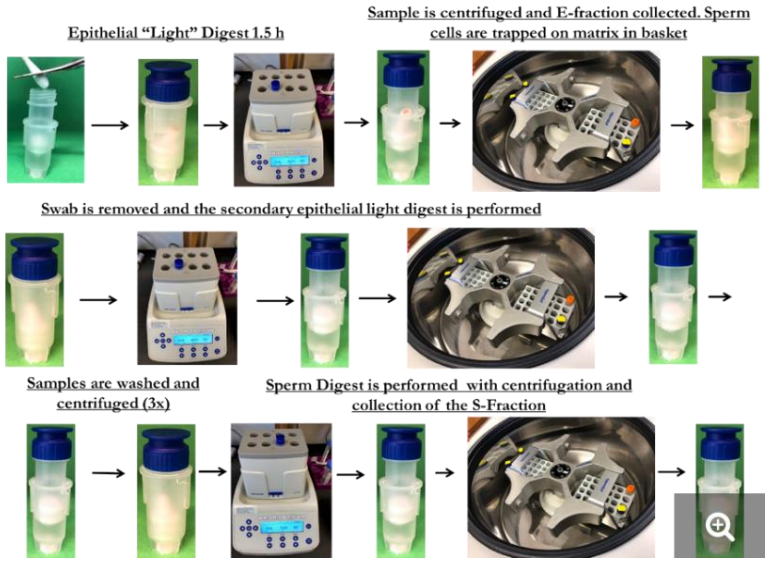

Figure 3: Visual Representation of How the SpermX Process is Executed, Following the Sample Through Each Incubation, Centrifugation, and Sample/Tube Manipulation (InnoGenomics, 2020)

Published Research

In a validation study conducted by Bogas *et al.* (2017), they tested the ability of Sampletype's i-sep® DL-MB extraction system to separate epithelial from sperm cells, followed by purification with Prepfiler. Their methodology consisted of making mock casework samples, in triplicate, containing mixtures of blood + sperm and saliva + sperm in different proportions (1:10, 1:1 and 10:1) totaling 42 samples. Bogas *et al.* (2017) also used 46 samples collected from sexual-assault victims. All samples were extracted with i-sep® DL-MB and purified with the Prepfiler forensic DNA extraction kit. Results showed that almost every mock sample subject to differential extraction resulted in an isolated male profile or a mixture with the male profile in majority. From the 46 sexual-assault samples, 21 samples resulted in an isolated male profile or with few extra alleles in minority and 5 had a mixture profile with the suspect's profile in majority. Overall, 60% of the samples resulted in an isolated profile or with the male contribution in majority.

In a comparative study by White *et al.* (2022), the extraction performance was compared between the i-sep DL-MB column and a conventional pellet-based differential separation method. Semen, buccal, and vaginal swabs were collected from volunteers. The semen samples were then diluted in a series of nine dilutions (0.5, 0.1, 0.05, 0.01, 0.005, 0.001, 0.0005, 0.0001, and 0.00005 µl). The buccal swabs were used to create cell mixtures. The vaginal swabs and semen dilutions were used to create mock sexual assault samples. The protocols for each kit were followed using the created samples. Results showed that the i-sep method recovered more DNA at all semen dilutions tested than the pellet differential method, with up to a six-fold improvement in DNA quantification values. Results also showed that the pellet method failed to

recover 15–88% of the sperm fraction DNA, while the i-sep method recovered >99% in the initial extraction.

In a validation study conducted by Sinha *et al.* (2022), they evaluated the performance of SpermX under conditions of sensitivity, stability, precision (reproducibility and repeatability), mixtures, and a method comparison with traditional differential extraction. Sensitivity studies were performed to determine the range over which the SpermX matrix membrane can capture sperm cells, ranging from a high of over 2.78 million cells (9159 ng) to a low of 50 cells (0.17 ng). The SpermX method recovered more than 100% relative to the theoretical expectation for the first four samples, and 69% and 55% for the lower sample volumes. All semen volumes yielded sufficient target DNA to produce full DNA profiles. Stability studies were performed to test various substrates (carpet, cotton, denim, polyester, and silk) that may be encountered in sexual assault cases from clothing and undergarments. Assessment of inhibition and degradation yielded normal ranges, indicating that SpermX removed any inhibitors and that the DNA was not degraded across all substrates. All substrates resulted in full male profiles of good quality, meaning good intra-locus (peak height ratio of alleles within a locus) and inter-locus (peak heights across loci in the same dye channel) balance, except for the carpet profile. The precision study showed that SpermX gave reproducible results.

The mixture portion of the study showed that 84% of female DNA was recovered and 60–67% of male DNA was obtained. This portion also showed that the vast majority of male DNA recovered was isolated in the sperm fraction, indicating no carryover. Lastly, in the comparison study between SpermX and the traditional differential extraction method, SpermX recovered an average of over 6-fold more male DNA in the sperm fraction than the traditional differential extraction method. Keeping the swab in the SpermX device during sperm digestion

yielded more sperm DNA. Removal of the swab in the conventional differential extraction in this study resulted in a significant loss of sperm DNA. It was also shown that, when SpermX was used, all samples contained more male DNA than female DNA in the electropherogram data (Sinha *et al.*, 2022).

After the validation study, InnoGenomics ran an interlaboratory mixture study. The study consisted of eight swabs, in triplicate, with different female-to-male ratios using the SpermX platform and standard differential extraction. The results showed that SpermX produced higher male DNA yields relative to the theoretical amount of DNA placed on the swabs. On average, SpermX recovered 1.5 to 10 times more DNA than expected at normal sperm concentrations. At low sperm concentrations, SpermX yielded 20 to 1,533 times more recovered DNA (Taylor, 2022).

A year later, Sgueglia *et al*. (2023) conducted an interlaboratory study consisting of the same team as Sinha *et al.* (2022), by comparing SpermX™ to standard differential extraction protocols. Mock samples with known ratios of female epithelial cells and sperm cells were created using saliva from a female donor and semen from a male donor. There were three laboratories involved in this study, each processing eight triplicate swab sets. The swabs were in concentrations of 100:1, 1000:1 (x2), 750:1, 600:1, 1200:1, 4545:1, and 18,182:1. All three laboratories performed the SpermX protocol and used the reagents provided in the kit. For the standard differential extraction protocol, Lab 1 used their validated lab protocol, Lab 2 followed the Qiagen EZ1® DNA Investigator® protocol, and Lab 3 used a modification of the Qiagen EZ1® DNA Investigator® protocol. The modifications were to the epithelial digest, incubation times, and sperm pellet washes. For DNA quantification, Lab 1 used the InnoQuant® HY kit on a QuantStudio™ 5 Real-Time PCR System, Lab 2 used the Quantifiler™ Trio Quantification Kit

on a 7500 Real-Time PCR System, and Lab 3 used the Quantifiler™ Trio Quantification Kit on a QuantStudio™ 5 Real-Time PCR System. For STR analysis, Lab 1 used the PowerPlex® Fusion kit and an Applied Biosystems 3130. Labs 2 and 3 used GlobalFiler™ and an Applied Biosystems 3500. The results from the interlaboratory study showed that SpermX recovered seven-fold more male DNA than standard differential extraction methods. Also, SpermX consistently provided CODIS up-loadable DNA profiles, even with as few as 25 sperm cells, whereas standard differential extraction methods failed to produce usable results.

Current research has determined that differential extraction is a modified extraction method used to separate male DNA (sperm cells) from female DNA (epithelial cells) in mixed samples. The problems that occur during this process are loss of the male spermatozoa cells or epithelial cell carry-over. To combat these problems, recently developed kits such as i-sep DL-MB and SpermX are used. The SpermX method resulted in an average of over 6-fold more male DNA recovered in the sperm fraction than with traditional differential extraction, and 60–67% of male DNA was obtained in mixed samples. i-sep DL-MB was able to recover more DNA than the traditional differential extraction method. Also, the i-sep method recovered >99% of the sperm fraction during the initial extraction. These results show that the new differential extraction methods are superior to the traditional method. However, there is still room for improvement due to the recurring loss of spermatozoa.

Material and Methods

Sample Preparation

Sperm samples were obtained from a human male donor via Innovative Research. The sperm samples were stored at -20°C until they were ready for experimentation. Twenty-seven half-buccal swabs, 9 for each differential extraction method for triplicate purposes, were obtained, via cotton swabs, from a female donor for epithelial cells. To test the sensitivity of each method, semen concentrations at neat, 1:10, and 1:100 dilutions were created. One set of dilutions was made for all kits tested, at the same time, to eliminate differences in the dilutions. Ten µl of each semen dilution was added to a female buccal swab to create a mixed sample to simulate a sexual assault mixture sample.

i-sep DL-MB Method

Two lysis buffer solutions were prepared for this protocol. Lysis buffer 1 contains 480 µl of Sampletype i-sep® MB buffer with 20 µl of proteinase K. Lysis buffer 2 contains 460 µl of Sampletype i-sep® MB buffer, 20 µl of proteinase K, and 20 µl of DTT. Each of these were preheated to 56°C. For epithelial cells lysis, half cuttings of each mixed sample were placed into a Sampletype i-sep® DL Filter Column inside the Sampletype i-sep® Collection Tube. Five hundred µl of lysis buffer 1 was added to the tube, incubated for 30 minutes at 56°C, and then centrifuged for 2 minutes at 7,000 rpm. The filter column was transferred to a new collection tube, and the flow through was retained for DNA extraction. These steps were repeated except the flow through was discarded. For sperm cell lysis, 500 µl of lysis buffer 2 was added to the filter column, incubated for 60 minutes at 56°C, and centrifuged for 2 minutes at 7,000 rpm. The

filter column was discarded and the flow through was retained for DNA purification (Biotype, 2021).

SpermX Method

A half cutting of a mixed sample swab was added to the inner tube of an assembled SpermX device. Five hundred eighty-five µL of Epithelial Digest Solution and 30 µL of proteinase K was added to the inner tube, vortexed, and incubated for 90 minutes at 56°C, vortexing every 30 minutes. The tubes were centrifuged at 4000 rpm for 5 minutes in the raised position. The resulting flow-through was collected as the first epithelial fraction (E1). The inner tube was moved to a new outer tube and 600 µL of Epithelial Digest Solution and 15 µL of Pro K was added to the inner tube. They were then centrifuged for 90 seconds at 4000 rpm. The samples were incubated for 30 minutes at 56°C, vortexing every 15 minutes, and centrifuged for 5 minutes at 4000 rpm in the raised position. The flow-through was collected as the secondary epithelial fraction (E2). The SpermX inner tube was placed in a 15 mL conical tube, washed with 500 µL of Sperm Digest Solution, and centrifuged at 4000 rpm for 5 minutes. This step was repeated 3 times, discarding the entire 1.5 mL of wash solution after completion. The SpermX inner tube was moved to a new outer tube. A master mix containing 2700 uL of Sperm Digest Solution, 220 uL of proteinase K, and 730 uL of DTT was created for the sperm cell lysis. 400 µL of the master mix was added to the inner tube and centrifuged for 90 seconds at 4000 rpm. The SpermX device was incubated for 45 minutes at 63° C, vortexing every 15 minutes, and centrifuged for 5 minutes at 4000 rpm. The flow through was collected as the sperm fraction. The sperm fraction was retained for DNA purification (InnoGenomics, 2020).

Traditional Method

The traditional differential method was conducted using a variation of the Qiagen DNA Investigator Kit. Thirty µl of proteinase K and 570 µl of G2 Buffer were added to 2 ml microcentrifuge tubes and pulse-vortexed for 10 seconds to mix the solutions with the sample. The microcentrifuge tubes were placed in an incubator and incubated at 56°C for 1 hour. The samples were vortexed every 10 minutes. The tubes were briefly centrifuged to remove any droplets on the lid once incubation was complete. The solutions were then transferred to new tubes containing a spin basket. The tubes were centrifuged for 5 minutes at full speed, after which all the supernatants were transferred to new tubes. The pellets at the bottom were resuspended in 500 µl of G2 Buffer, pulse-vortexed for 10 seconds, centrifuged for 5 minutes at full speed, and all the supernatant was discarded. This process was repeated twice. Four hundred µl of G2 Buffer, 25 µl of proteinase K, and 100 µl of 1M DTT were added to the pellets and pulse-vortexed for 10 seconds. The tubes were placed in an incubator and incubated at 70°C for 20 minutes. The samples were vortexed every 3 minutes. The microcentrifuge tubes were briefly centrifuged to remove any droplets on the lid. The sperm fraction was retained for DNA purification.

DNA Purification

DNA purification for all samples, from each method, was conducted using the QIAamp® DNA Mini kit. The flow throughs from each method were transferred to a QIAamp MinElute column. To the QIAamp MinElute column, 500 µl of Buffer AW1 was added, followed by centrifuging at 8000 rpm for 1 minute. The columns were transferred to another 2 ml tube and the previous tube was discarded. Five hundred µl of Buffer AW2 was added to the columns, followed by centrifuging at 8000 rpm for 3 minutes. The columns were transferred to another 2

ml tube and the previous tube was discarded. 40 µl of Buffer ATE was added to the center of the membrane and incubated at room temperature for 1 minute, then centrifuged at full speed for 1 minute. The flow throughs were collected and stored at -20°C until ready for DNA quantification (Qiagen, 2020).

DNA Quantification

DNA quantification was conducted using the Quantifiler™ Trio DNA Quantification Kit. All reagents were thawed completely and vortexed for 3-5 seconds. The standards for this protocol were made first and added to the wells on the plate. The creation of the serial dilution standards followed Table 1. Each serial dilution was pipetted into their corresponding tube labeled 1-5. Quantifiler™ THP DNA Dilution Buffer was vortexed for 3-5 seconds and then pipetted into each tube (10 µl for tube 1 and 90 µl for tubes 2-5). Quantifiler™ THP DNA Standard was vortexed for 3-5 seconds, 10 µl added to tube 1, and then pipette mixed. Ten µl from each tube was pipetted into the next until all standards were created.

Table 1: Serial Dilution Quantities for Quantifiler™ Trio DNA Quantification Kit (ThermoFisher, 2018)

Standard	Concentration (ng/µL)	Example volumes	Dilution factor
Std. 1	50.000	10 µL [100 ng/µL stock] + 10 µL Quantifiler™ THP DNA dilution buffer	2×
Std. 2	5.000	10 µL [Std. 1] + 90 µL Quantifiler™ THP DNA dilution buffer	10×
Std. 3	0.500	10 µL [Std. 2] + 90 µL Quantifiler™ THP DNA dilution buffer	10×
Std. 4	0.050	10 µL [Std. 3] + 90 µL Quantifiler™ THP DNA dilution buffer	10×
Std. 5	0.005	10 µL [Std. 4] + 90 µL Quantifiler™ THP DNA dilution buffer	10×

A master mix containing 216 µl of Quantifiler Trio Primer Mix (8 µl for each sample) and 270 µl of Quantifiler™ THP PCR Reaction Mix (10 µl for each sample) was created. Both reagents were thawed completely and vortexed for 3-5 seconds before the master mix was

created. Eighteen µL of the master mix was pipetted into each reaction well. Two µl of each sample, 2 µl of each standard, and 2 µl of nuclease free water (negative control) were added to their corresponding wells. The plate was sealed and centrifuged at 3,000 rpm for 20 seconds to remove any bubbles. The plate was run in the Quant Studio 5 PCR instrument (ThermoFisher, 2018).

Amplification

Amplification was conducted using the PowerPlex® Fusion 5C System kit. All reagents were thawed completely, vortexed for 15 seconds and briefly centrifuged. An amplification master mix containing 67.5 µl of PowerPlex® Fusion 5X Master Mix (2.5 µl for each sample), and 67.5 µl of PowerPlex® Fusion 5X Primer Pair Mix (2.5 µl for each sample) was created. This mixture was vortexed for 10 seconds. Five µl of amplification master mix was pipetted into each reaction well. Seven point five µl of each DNA sample was pipetted into their corresponding wells. For the positive control, 7.5 µl of 2800 M Control DNA (diluted to 0.5 ng/µl) was added to a well containing 5 µl of amplification master mix. For the negative control, 7.5 µl of amplification grade water was added to a well containing 5 µl of amplification master mix. The plate was sealed and centrifuged at 3,000 rpm for 20 seconds to remove any bubbles. The plate was then placed in an Applied Biosystems 9700 thermal cycler with the protocol: 96°C for one minute, 94°C for ten seconds, 59°C for one minute, 72°C for 30 seconds, repeating for 27 cycles. This was followed by 60°C for 20 min and then a 4°C soak. These steps were conducted to amplify the DNA (Promega, 2020).

Separation and Detection

Separation and detection were conducted via the Applied Biosystems Genetic Analyzer 3500. Before being placed in the Genetic Analyzer 3500, all 27 samples needed to be prepared for separation/detection. A master mix containing 14 µl of internal lane standard (WEN) and 257 µl of formamide was created. The master mix was vortexed for 15 seconds. Ten µl of the master mix and 1 µl of amplified sample was pipetted into each corresponding well. One µl ladder was added to 10 µl of master mix to one well in each injection column. The plate was sealed and centrifuged at 3,000 rpm for 20 seconds to remove any bubbles. The plate was then denatured at 95°C for 3 minutes, and then immediately chilled on crushed ice. The plate was placed in a plate base, covered with a plate retainer, loaded into a pre-calibrated Applied Biosystems 3500 Genetic Analyzer, and a run was conducted.

Results

For each method, the main focus was to determine how much DNA was extracted from the sperm fraction of each sample based on the kit used. This was determined using quantification data from the Quantifiler™ Trio DNA Quantification Kit. A subjective measurement was also taken into consideration based on ease of use and processing time for each method.

Traditional Method

The epithelial fraction for the traditional method was omitted, after discussion, due to irrelevance. In the raw data for the traditional method, the highest DNA value was obtained from a neat sperm sample, followed by 1:10, then 1:100 (Figure 5 and Table 2). The traditional method harnessed an average of 0.010 ng/µl for neat, 0.005 ng/µl for 1:10, and 0.00015 ng/µl for

1:100 (Figure 9). Among the three methods, the traditional method had the second longest processing time, taking approximately 5 hours. It is important to note that this method was brand new to the laboratory and not an established extraction method; therefore, it was on an even playing field as a new, unvalidated method to the lab.

SpermX and i-sep DL-MB Method

In the raw data for the SpermX method (Figure 6 and Table 3) and the i-sep DL-MB method (Figure 7 and Table 4), the most harnessed DNA was when a neat sperm sample was provided, followed by 1:10, then 1:100. This trend, for both methods, can be seen as a gradual decline. This is due to the concentration of sperm cells available caused by a serial dilution. Also, in the data for the epithelial fraction, there is no clear difference in the quantification values as the conditions were not manipulated due to the epithelial fraction not being the main focus of the study. When comparing the average quantification results between SpermX™ and i-sep® DL-MB, SpermX™ showed a higher affinity for isolating the sperm fraction. This is also shown in Figure 9 by observing the average quantification values for neat and 1:10. SpermX™ yielded an average of 0.75 ng/µl compared to 0.22 ng/µl for neat and 0.12 ng/µl compared to 0.041 ng/µl for 1:10. However, at a dilution of 1:100, the DNA quantities from SpermX™ were similar to those from i-sep® DL-MB (0.031 ng/µl and 0.028 ng/µl (Figure 8)). When comparing SpermX™ and i-sep® DL-MB to the modified Qiagen DNA Investigator kit method, both kits outperformed at every dilution.

In terms of ease of use and processing time, Biotype's Sampletype i-sep® DL-MB was the most user-friendly, easiest to incorporate into existing laboratory workflows, and had a shorter execution time than SpermX™. However, i-sep® DL-MB did not come without its shortcomings. i-sep® DL-MB tubes are unable to be incubated in a heat block and the buffer

must be heated to 56°C prior to use. InnoGenomic's SpermX™ required a specialized centrifuge insert, supplied with the kit due to the custom tube size, and took approximately 6 hours to process—the longest among the three methods.

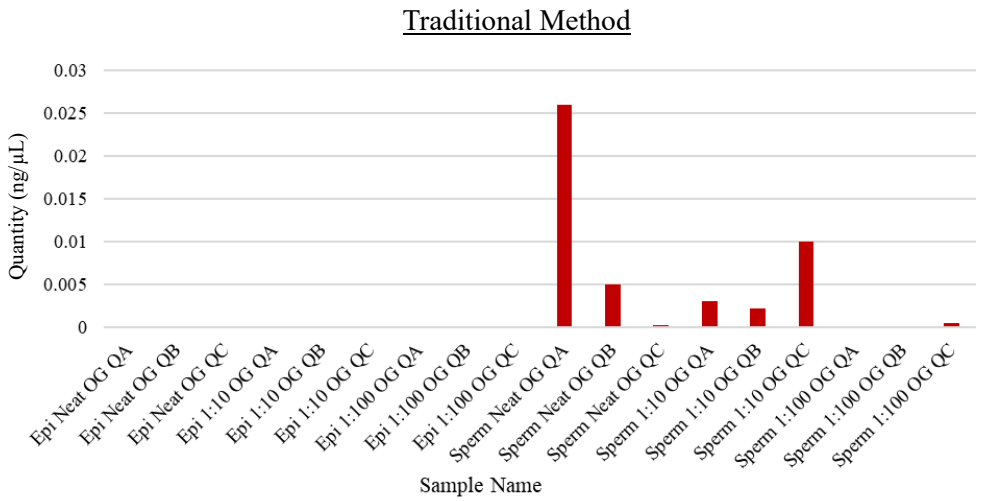

Figure 5: Graphical Representation of Traditional Method Quantification Data

Table 2: Table of the Traditional Method Quantification Data in ng/µL

Epi Neat OG QA	0.00
Epi Neat OG QB	0.00
Epi Neat OG QC	0.00
Epi 1:10 OG QA	0.00
Epi 1:10 OG QB	0.00
Epi 1:10 OG QC	0.00
Epi 1:100 OG QA	0.00
Epi 1:100 OG QB	0.00
Epi 1:100 OG QC	0.00
Sperm Neat OG QA	0.026
Sperm Neat OG QB	0.005
Sperm Neat OG QC	0.00023
Sperm 1:10 OG QA	0.003
Sperm 1:10 OG QB	0.0022
Sperm 1:10 OG QC	0.010
Sperm 1:100 OG QA	0.00
Sperm 1:100 OG QB	0.00
Sperm 1:100 OG QC	0.00044

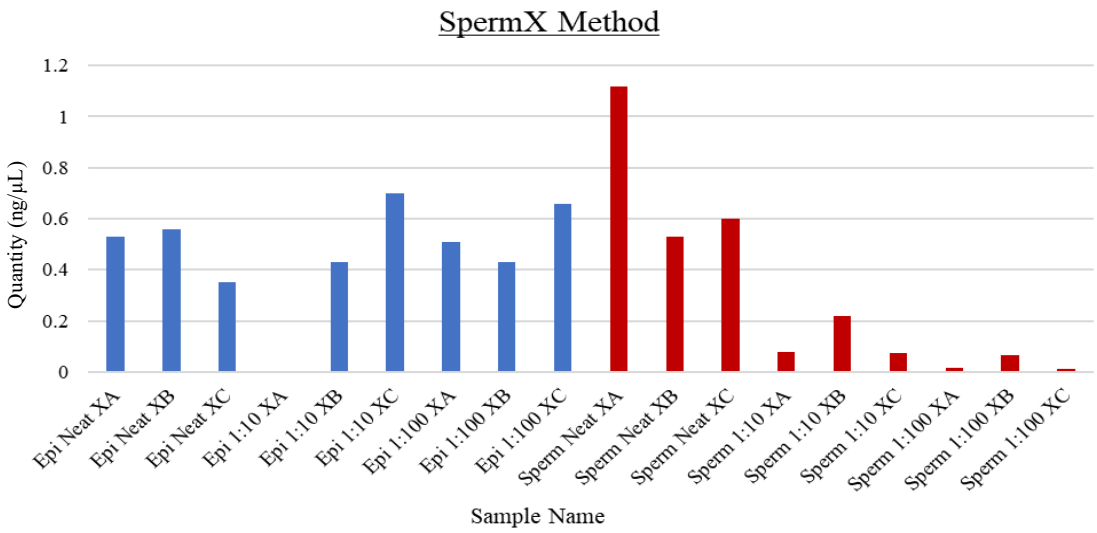

Figure 6: Graphical Representation of SpermX Method Quantification Data

Table 3: Table of the SpermX Method Quantification Data in ng/µL

Epi Neat XA	0.53
Epi Neat XB	0.56
Epi Neat XC	0.35
Epi 1:10 XA	Inconclusive
Epi 1:10 XB	0.43
Epi 1:10 XC	0.70
Epi 1:100 XA	0.51
Epi 1:100 XB	0.43
Epi 1:100 XC	0.66
Sperm Neat XA	1.12
Sperm Neat XB	0.53
Sperm Neat XC	0.60
Sperm 1:10 XA	0.078
Sperm 1:10 XB	0.22
Sperm 1:10 XC	0.075
Sperm 1:100 XA	0.014
Sperm 1:100 XB	0.067
Sperm 1:100 XC	0.013

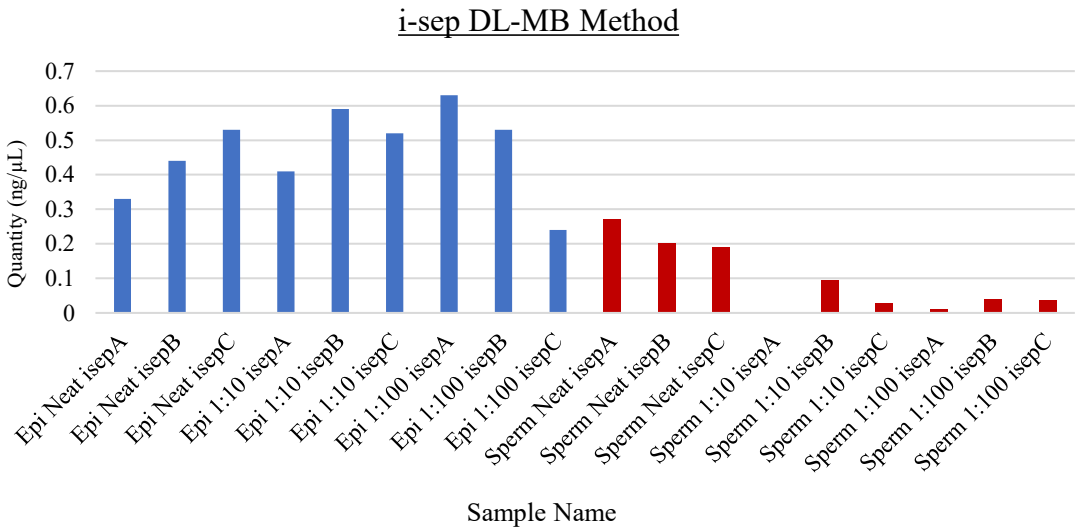

Figure 7: Graphical Representation of i-sep DL-MB Method Quantification Data

Table 4: Table of the isep DL-MB Quantification Data in ng/µL

Epi Neat isepA	0.33
Epi Neat isepB	0.44
Epi Neat isepC	0.53
Epi 1:10 isepA	0.41
Epi 1:10 isepB	0.59
Epi 1:10 isepC	0.52
Epi 1:100 isepA	0.63
Epi 1:100 isepB	0.53
Epi 1:100 isepC	0.24
Sperm Neat isepA	0.27
Sperm Neat isepB	0.20
Sperm Neat isepC	0.19
Sperm 1:10 isepA	0.00043
Sperm 1:10 isepB	0.095
Sperm 1:10 isepC	0.027
Sperm 1:100 isepA	0.009
Sperm 1:100 isepB	0.038
Sperm 1:100 isepC	0.037

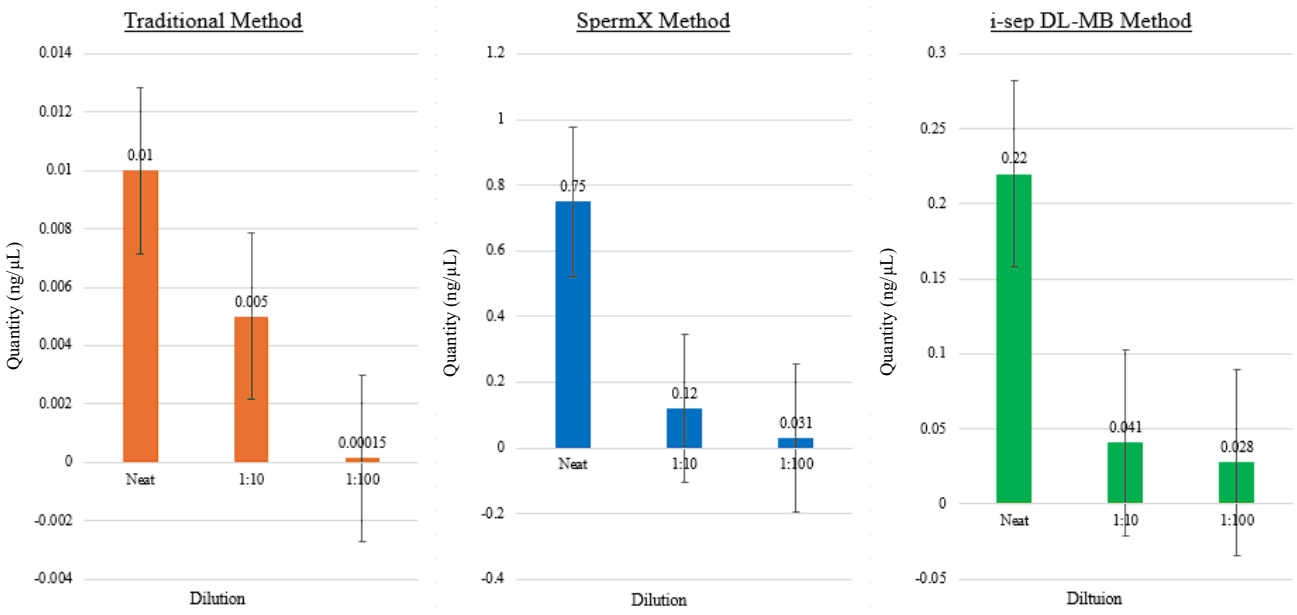

Figure 8: Graphs of the Average Comparison of Sperm Fractions for Each Method ± 1 Standard Deviation

Electropherograms

Electropherograms were generated to spot check that the appropriate male and female data was obtained and that there was no contamination within the samples. The electropherogram data was consistent with the female and male profiles in the study. The electropherogram data was not utilized for anything further.

Statistics

A one-factor ANOVA followed by a Tukey's pairwise multiple comparison test was conducted to compare the quantification results between all three methods. A one-factor ANOVA test was used due to there being more than three or more means being compared. A Tukey's pairwise multiple comparison test was conducted to test the differences among the sample means

for significance. The assumption of equal variance not assumed was tested, and the results indicated a significant difference between all three methods, $p = .03$, F-value = 4.093, 95% *CI*. The main significant difference is shown when comparing SpermX to the Traditional method, $p = .026$. When comparing i-sep DL-MB to SpermX (p = .151) and to the Traditional method ($p = .672$), there was not a significant difference (Figure 9). Therefore, we reject the null hypothesis, indicating that there is sufficient evidence to conclude that the mean quantification data differs significantly between the differential extraction kits.

ANOVA

Value

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.416	2	.208	4.093	.030
Within Groups	1.219	24	.051		
Total	1.635	26			

Multiple Comparisons

Dependent Variable: Value

Tukey HSD

(I) Method	(J) Method	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
Tranditonal Method	SpemX	-.29668111*	.10623345	.026	-.5619765	-.0313857
	i-sep DL-MB	-.09106222	.10623345	.672	-.3563576	.1742332
SpemX	Tranditonal Method	.29668111*	.10623345	.026	.0313857	.5619765
	i-sep DL-MB	.20561889	.10623345	.151	-.0596765	.4709143
i-sep DL-MB	Tranditonal Method	.09106222	.10623345	.672	-.1742332	.3563576
	SpemX	-.20561889	.10623345	.151	-.4709143	.0596765

*. The mean difference is significant at the 0.05 level.

Figure 9: ANOVA and Tukey's Test Results

Discussion

This study undertook a multi-method approach to determine if recent advancements in differential extraction kits made a significant difference over the current method used in the field. The multi-method approach employed various techniques to determine cell separation efficiency and kit sensitivity. The multi-method approach involved comparing Sperm X, i-sep DL-MB, and

a Traditional Method, that is a variation on the Qiagen DNA Investigator Kit, to each other. The data generated provided insights into the method that yielded the highest DNA recovery rate, thus providing a guideline for future practices. A subjective measurement was also taken into consideration based on ease of use and processing time for each method.

The main method to determine which approach provided the best results for cell separation efficiency and kit sensitivity was DNA quantification using the Quantifiler™ Trio DNA Quantification Kit. When reviewing quantification results from the three methods, the SpermX method produced the best results. The SpermX method gave an average of 3 times more (0.75 ng/µl compared to 0.22 ng/µl for neat and 0.12 ng/µl compared to 0.041 ng/µl for 1:10) male DNA recovery in the neat and 1:10 sperm fractions compared to the i-sep DL-MB differential extraction method. However, at a dilution of 1:100, the DNA quantities from SpermX™ were similar to those from i-sep® DL-MB (0.031 ng/µl and 0.028 ng/µl). Although SpermX™ demonstrated higher sperm DNA recovery at neat and 1:10 dilutions, differences between SpermX™ and i-sep® DL-MB diminished at the 1:100 dilution. This convergence likely reflects the inherent limitations of differential extraction when working with extremely low sperm cell quantities. From a forensic perspective, this finding suggests that while SpermX™ provides a clear advantage in moderate to high sperm loads, both kits may approach similar detection thresholds under low-template conditions.

The superior performance of the SpermX™ method may be attributed to its matrix-based capture system, which physically retains sperm cells during epithelial digestion and wash steps. Unlike pellet-based or column-based methods, this approach minimizes sperm loss caused by repeated transfers and centrifugation. Additionally, extended incubation times and multiple wash

steps likely enhanced differential lysis efficiency while reducing epithelial cell carryover, contributing to the observed increase in sperm DNA recovery.

In terms of ease of use and processing time, Biotype's Sampletype i-sep® DL-MB was the most user-friendly, easiest to incorporate into existing laboratory workflows, and had a shorter execution time than SpermX. In terms of user-friendly, Biotype's Sampletype i-sep® DL-MB tubes and spin-baskets were simple to remove and transfer between tubes. InnoGenomic's SpermX™ required a specific tool, provided with the kit, to raise and twist the insert into the proper position. To incorporate either of these kits into existing laboratory workflow, i-sep DL-MB would be the simplest due to the kit having a normal tube size. SpermX has a uniquely large tube size which required incubation in Lab Armor™ Beads instead of a heat block. Taking into consideration the execution time for each kit, Biotype's Sampletype i-sep® DL-MB was between 2.5-3 hours, while InnoGenomic's SpermX was double that, roughly 6 hours, which was also the longest of the three methods.

In examining the statistical significance pertaining to the comparison of the kits, there was a significant difference between all three methods, $p = .03$. The main significant difference is shown when comparing SpermX to the Traditional method, $p = .026$. When comparing i-sep DL-MB to SpermX ($p = .151$) and to the Traditional method ($p = .672$), there was not a significant difference. This shows that, based on quantification data, Innogenomic's SpermX™ is superior in cell separation and obtention when compared to the Traditional method. However, it cannot be statistically proven that it is superior to Biotype's Sampletype i-sep® DL-MB.

Limitations

While executing each method's protocols, some unusual instances were encountered. Biotype's i-sep® DL-MB tubes are unable to be incubated in a heat block and the buffer must be heated to 56°C prior to use. This required the use of an incubator in order to complete these specifications. InnoGenomic's SpermX™ required a specialized centrifuge insert, supplied with the kit due to the custom tube size. A large benchtop centrifuge with interchangeable adapters was also needed in order to conduct the protocol.

This study utilized semen and epithelial cells from single donors, which limits the ability to account for inter-individual biological variability, such as differences in sperm count or epithelial cell shedding. Additionally, the use of controlled mock samples does not fully replicate the degradation, inhibition, and environmental exposure encountered in actual forensic casework. These factors may influence extraction efficiency and should be considered when extrapolating results to real-world scenarios.

Forensic Benefit

These new differential DNA extraction kits are significantly improving forensic science by making the process faster, more accurate, and more reliable. Improved chemical formulations and extraction techniques produce cleaner separations of mixed DNA samples, resulting in clearer genetic profiles with less contamination from non-target cells. These kits also enhance DNA recovery, even from degraded or very small samples, which increases the likelihood of obtaining usable evidence. Additionally, safer, closed-system designs reduce contamination risks

and improve consistency across different labs. Altogether, these advancements strengthen the accuracy and reliability of forensic evidence, ultimately supporting more effective investigations.

Conclusion

The main purpose of this study was to evaluate whether recently developed differential extraction kits provide improved sperm cell separation efficiency and sensitivity compared to a commonly used traditional differential extraction method. The results of this study demonstrate that both InnoGenomic's SpermX™ and Biotype's Sampletype i-sep® DL-MB outperform the traditional method in sperm DNA recovery across all semen concentrations tested, supporting the hypothesis that newer technologies offer meaningful improvements in forensic DNA processing. However, it cannot be statistically shown that SpermX is better than i-sep® DL-MB. In order for laboratories to incorporate this kit into their workflow, accommodations would have to be made due to the uniqueness of the kit. With this being the case, this method could be used as an alternative if their original method does not produce desired results. During the execution and completion of this thesis, Gentueri Inc. has developed an insert that is developed from the SpermX kit. Their product is called the SpermX™ GenSpin™ and claims to be able to harness more sperm DNA than the original method (Nagy, 2025). A future test for this experiment would be to conduct it again with this added product to see if more sperm DNA could be obtained. From a laboratory implementation standpoint, the choice between SpermX™ and i-sep® DL-MB may depend on operational priorities rather than DNA yield alone. Laboratories processing large case backlogs or time-sensitive evidence may favor i-sep® DL-MB due to its shorter processing time and compatibility with standard laboratory equipment. Conversely, SpermX™ may be better suited for challenging samples, cold cases, or samples with limited sperm content where maximizing male DNA recovery is critical. Also, since this study had a small sample size,

conducting this experiment over with a larger sample size may show more significant results between the kits. Another possible future test for a study like this would be to repeat it with cuttings taken from different types of materials that have a mixed sample. Another suggestion that may aid in the obtaining of more DNA from the sperm fraction may be using different types of DNA purification methods or developing a way for the method to be automated.

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