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Comparison of Semi-Automated and Manual Differential Separation Methods for Mock Sexual Assault Samples

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Abstract

In the event of a sexual assault, separation of suspect from victim DNA is possible through differential extraction if the evidentiary mixture consists of female epithelial cells and male spermatozoa. The basis of differential extraction is the differing properties of epithelial and sperm cell membranes, specifically the comparative sturdiness of the proteins making up the sperm head containing the male DNA. Effective retrieval and separation of male and female DNA fractions is important in generating high-quality STR profiles that can be utilized in identifying suspects in a sexual assault case.

Differential extraction has been conventionally performed using Proteinase K (PK) to lyse epithelial cells, then dithiothreitol (DTT) to lyse sperm cells after the fractions were separated through centrifugation. The protocol for differential separation currently used by the FBI Laboratory is a semi-automated variation of this method using the QIAcube and EZ1 Advanced XL. SpermX is a manual differential separation method from Innogenomics that uses a novel nanofiber matrix to trap and separate sperm from nonsperm cells.

Three comparisons were performed to observe the ability of the FBI and SpermX differential extraction methods. The comparisons examined samples with different semen donors, volumes of mixture (2 uL, 10 uL, 20 uL, 50 uL), and stain materials (condoms, 100% cotton, 95% cotton/5% spandex, 100% polyester, and 100% denim). The samples' DNA quantity, degradation index, male to female ratio, proportion of mixture DNA profiles, and allele percentages were then used to observe differences between the FBI and SpermX differential extraction methods. Differences were observed in the DNA quantity and proportion of mixture profiles. A higher female fraction DNA quantity was generally observed when the FBI method was used across all comparisons, and a lower proportion of mixtures was observed for the donor comparison male fractions and the sensitivity comparison female fractions ($p<0.05$). Comparatively, the SpermX male fraction yielded higher DNA quantities for the sensitivity comparison, and a lower proportion of mixtures for the donor comparison female fractions ($p<0.05$). Overall, the DNA analysis of mock sexual assault swabs enabled the comparison of two differential separation methods, showing the FBI and SpermX methods are of comparable quality.

1. Introduction

In the event of a sexual assault, separation of suspect from victim DNA is possible through differential extraction if the evidentiary mixture consists of female epithelial cells and male spermatozoa [1]. The basis of differential extraction is the differing properties of epithelial and sperm cell membranes, specifically the comparative sturdiness of the proteins making up the sperm head containing the male DNA. Effective retrieval and separation of male and female DNA fractions is important in generating high-quality STR profiles that can be utilized in identifying suspects in a sexual assault case.

Differential extraction has conventionally been performed using Proteinase K (PK) to lyse epithelial cells, followed by dithiothreitol (DTT) to lyse sperm cells after the fractions were separated through centrifugation [1]. The protocol for differential separation currently used by the FBI Laboratory is a semi-automated variation of this method, which uses the QIAcube for separation of female and male cells, and the EZ1 Advanced XL for DNA purification.

SpermX is a manual differential separation method that uses a novel nanofiber matrix to separate sperm from epithelial cells. As a cellular mixture is washed through the SpermX device, epithelial DNA lysate will flow through while sperm cells are trapped within the matrix. Sperm DNA is released using a sperm digest buffer and extracted with the EZ1 Advanced XL [2-4].

Although the SpermX method also uses the EZ1 Advanced XL in its protocol, it does not use the automation for separation and the removal of the female fraction. If the SpermX extraction performance is comparable to the currently used FBI method, it may show utility as an alternative method in certain circumstances.

1.1. Extraction Methods

When mixtures of victim and perpetrator DNA are present in the same sample, an effort can be made to separate the two sources for downstream analysis that will aid in suspect identification. Separation of suspect from victim DNA is possible through differential extraction if the evidentiary mixture consists of female epithelial cells and male spermatozoa, such as a vaginal swab from a sexual assault victim. The basis of differential extraction is the differing properties of epithelial cell and sperm cell membranes, specifically the comparative sturdiness of the proteins making up the sperm head that contains the male DNA. The earliest differential method described was in 1985 by Gill et al. [1]. Gill stated that female cells in the mixture sample are preferentially lysed using Proteinase K (PK) as a key reagent, while male DNA is protected by the hardier structure of the sperm head. The sperm fraction is then collected through centrifugation, and the lysed epithelial DNA in solution is physically removed. The intact sperm heads are then treated with a lysis reagent, dithiothreitol (DTT). Unlike Proteinase K, DTT can break the disulfide bonds that provide cross-linking strength in sperm head proteins. After lysis of the sperm heads, the male DNA can be accessed and extracted for downstream analysis [1].

While PK and DTT are the primary reagents used in the procedure described by Gill et al. [1] (often called “conventional” differential extraction), the use of secondary reagents to enhance lysis and separation is widely practiced. Sodium dodecyl sulfate (SDS), a surfactant also used in Gill’s initial procedure, is commonly added to the reaction buffer solution to enhance the lysis of both epithelial and sperm cells [1]. SDS works by disrupting cell membranes, denaturing proteins, and deactivating cellular enzymes that may be released upon lysis when used with PK [5]. Complete separation of female from

male cells is not always successful, leading to the presence of male DNA in the female fraction or female DNA in the male fraction [2]. The elimination of this "carryover" has been a target for protocols aiming to improve differential separation quality. For instance, Garvin et. al demonstrated the ability of deoxyribonuclease I (DNase I) to degrade free DNA; when added prior to sperm lysis, persisting female DNA was degraded, resulting in a vast reduction of female DNA carryover into the male fraction [6].

In forensic laboratories, commonly used differential extraction mechanisms utilize small-scale liquid handling robots. These robots are a useful tool for automating part of the DNA analysis process and help reduce processing time and human error. The EZ1 Advanced XL is one such system which can extract DNA from up to 14 samples in a single 20-minute run. This instrument uses paramagnetic beads to bind nucleic acids in order to separate them from other contents in solution [7]. The QIAcube, also produced by QIAGEN, is another such instrument. It uses a column-based silica membrane to bind nucleic acids and wash them of debris and contaminants. While it cannot process as many samples in one run as the EZ1 Advanced XL, it can perform differential separation of male and female DNA from a single sample as well as extraction [8].

1.1.1. SpermX

SpermX, previously called SpermTrap, is a novel differential separation protocol developed by Sudhir Sinha and InnoGenomics Technologies, LLC [2, 3]. The basis of separation by a SpermX device is the use of a nanofiber filter to "entangle" intact sperm cells of a sexual assault sample while epithelial cells are lysed, eluted, and washed. The filter is made of polymer nanofibers with varying diameters of 700 nanometers or less. The small apparent pore size of 2.5 micrometers and large surface area created by the overlapping nanofibers effectively traps any intact sperm cells like a net while lysed epithelial DNA, debris, and buffer may freely flow through the nanofibers. The filter is fitted into the bottom of a specialized spin basket, which in turn fits into a SpermX tube. The tube has "lower" and "upper" locking basket positions for lysis and centrifuge steps, respectively [4]. The SpermX kit also includes caps that screw onto the basket, a specialized tool for raising the basket to the upper position from the lower position, and rack adapters to hold the SpermX and fit into a large benchtop centrifuge. The protocol also uses SpermX Epithelial and Sperm Digest Solutions, along with user-provided PK for epithelial lysis and DTT for the sperm lysis [4]. SpermX is fully automatable with the use of Hamilton AutoLys SAE Star instrumentation [9].

1.2. Common Sexual Assault Samples

A sexual assault kit (SAK) is a packaged collection of evidentiary items collected by a medical health professional from a sexual assault victim or suspect. While the collected items are dependent on the nature of the assault, the National Institute of Justice's National Best Practice for Sexual Assault Kits [10] recommends swabs (used to collect DNA evidence from the victim's or suspect's anus, hands, oral cavity, penis, scrotum, rectum, skin, or vagina), victim underwear and clothing, nail clippings, hair combings or cuttings, tampons, and condoms as items for collection [10]. Any of these items may contain a mixture of assailant(s) and victim DNA, which may then be analyzed in a laboratory setting.

Information regarding the frequency collected evidence types following a sexual assault was collected by Cross. et al in 2014, using data from 528 sexual assault cases from the state of Massachusetts. Biological swabs taken from the genital area were the most commonly collected evidence. Clothing was another commonly collected item, as 57.6% of the observed cases collected clothing, and 45.3% collected the victim's underwear worn at the time of the assault. Collection of condoms was not reported from these cases, and only 10.8% of assailants were estimated to have used condoms [11].

1.3. Objectives

This study aims to compare the currently employed FBI and the SpermX extraction methods through extraction of mock sexual assault samples utilizing different semen donors, mixture volumes, and sample media. Comparisons in extraction and separation success will be made with the extracted DNA quantity, and with DNA profile success represented by the percentage of expected male or female alleles and the proportion of mixture DNA profiles generated by each method.

2. Materials and Methods

2.1. Body fluid preparation

An epithelial solution was prepared from neat female saliva to serve as the female contribution to the sexual assault swabs. The female donor deposited saliva into a test tube, and equal parts saliva and TE $^{-4}$ buffer were combined in a microcentrifuge tube and vortexed gently. After 3 minutes of centrifugation at 3 rcf, the supernatant was removed. An equal volume of TE $^{-4}$ buffer was used to resuspend and wash the pellet of contaminants. This wash was repeated, followed by resuspension of the epithelial pellet by an identical TE^{-4} buffer volume. The result is of this procedure is a solution of epithelial cells suspended in TE buffer, in the same volume as the original neat saliva sample. Institutional Review Board (IRB#585-20) approval was granted to use human buccal and/or saliva samples with written, informed consent.

Semen (Lee Biosolutions, Maryland Heights, MO) was acquired from five donors, divided into 200uL aliquots, and stored at -20°C until use. Dilutions (1:20) were then made from thawed semen. IRB approval was not necessary for purchased specimen.

2.2. Swab Preparation

Samples were prepared in bulk prior to each separate experimental procedure to ensure consistent cell quantities on each swab. Sample preparation methods were kept consistent for all procedures. A summary of the prepared samples can be observed in Table 1.

2.2.1. Donor Comparison Swabs

Epithelial solution (25 µL) and 25 µL of 1:20 diluted semen were combined into a disposable 1.5 mL microcentrifuge tube. The mixture was collected by swirling a cotton-tipped applicator swab along the sides and bottom of the tube. Swabs were air-dried overnight under a sterile extraction hood and stored at –20°C until use.

For the donor comparison study (n=112), 20 swabs with 50 µL mixture volumes were prepared for each of the 5 semen donors, for a total of 100 experimental swabs. Control swabs were prepared in duplicate with 25 µL of epithelial solution or 25 µL of 1:20 diluted semen from each of the five semen donors, for a total of 12 swabs. All swabs were allocated equally to be extracted by either the FBI or SpermX method.

2.2.2. Sensitivity Comparison Swabs

Epithelial solution and 1:20 semen mixture swabs were prepared using the same procedure as for the donor comparison study, for total mixture volumes of 2 µL (1 µL epithelial solution and 1 µL 1:20 semen), 10 µL (5 µL epithelial solution and 5 µL 1:20 semen), 20 µL (10 µL epithelial solution and 10 µL 1:20 semen), and 50 µL (25 µL epithelial solution and 25 µL 1:20 semen).

For the sensitivity comparison study (n=96), 6 swabs were prepared for each of the 2 µL, 10 µL, 20 µL, and 50 µL volumes for two semen donors. All swabs were allocated equally to be extracted by either the FBI or SpermX method.

2.2.3. Material Comparison Swatches

Mock sexual assault swatches were prepared using 100% cotton fabric, a white 100% polyester shirt, white 95% cotton and 5% spandex underwear, 100% denim, and the inside of condoms (Trojan Twist) using 50 µL of 1:1 epithelial solution and 1:20 diluted semen mixture. [Sources of fabric, treatment/washing before use]

Two sets of material swatches were prepared using different semen donors for the body fluid mixture. In each set, six stains were prepared on each of the five garment types. The fluids were first combined in equal volumes in a microcentrifuge tube, then the mixture was applied to the fabric or condom in 50 µL volumes. After the fluid had spread on the material, the stain area was marked with sharpie dots around the outside of the stain border. All materials were air-dried under a sterile extraction hood. The fabric swatches were then stored at –20°C until extraction, while the condom samples were extracted once completely dry.

The extraction source for each sample was a swabbing of the stained material, according to the FBI DNA Casework Unit protocol. Stains were first moistened with 50 uL of reagent-grade water pipetted directly onto the stain. A cotton tipped applicator was then rubbed against the moistened area and let air dry before extraction.

For the materials comparison study (n= 60), 6 stains were prepared for each of the 100% cotton, 100% polyester, 95% cotton and 5% spandex, 100% denim, and condom materials for two semen donors. All stains were allocated equally to be extracted by either the FBI or SpermX method.

2.3. DNA Extraction

2.3.1. FBI protocol

Female fraction lysis was initiated by adding 160 µL Buffer ATL (Qiagen, Hilden, Germany), 20 µL reagent grade water, and 20 µL Proteinase K (PK) to each sample substrate in a 1.5-µL microcentrifuge tube. The samples were then incubated at 56°C for 1.5 hours with 200 rpm agitation to lyse epithelial cells. After the incubation, the sample substrate was transferred into a CoStar Spin-X centrifuge filter (Corning, Tewksbury, MA) that was placed into the original 1.5-µL tube and centrifuged at 11800 rpm for 5 minutes. The basket was then discarded, and the mixture with epithelial lysate was loaded onto the QIAcube (Qiagen) for separation of the epithelial fraction. The epithelial fraction was then loaded onto the EZ1 Advanced XL (EZ1) (Qiagen) for lysate processing and DNA extraction. The male fraction, still in the original 1.5-µL tube, was left on the QIAcube for an additional cleanup step. The male fraction tubes were removed and prepared for sperm lysis by adding 375 µL of Buffer G2 (Qiagen), 24 µL of PK, and 94 µL DTT followed by a short incubation at 70°C with 900 rpm agitation for 10 minutes. The male fraction tubes, now containing sperm lysates, were then loaded onto the EZ1 for DNA extraction. The EZ1 processing was a Large Volume protocol with elution into 50 uL of water for all extracted samples. Following extraction, the resulting female and male fractions were stored at 4°C until DNA quantification. Once quantification was complete, the samples were moved to –20°C storage until amplification.

2.3.2. SpermX Protocol

The SpermX (Innogenomics, New Orleans, LA) device consists of three parts: basket, cap, and tube (Figure 1). The basket is similar to a generic spin basket, with a nanofiber filter at the base. The cap screws onto the rim of the basket, which protects liquid from escaping the device from the top of the basket, allows easy addition of reagents to the system, and allows the basket to be transferred to a different tube with minimal contamination possibility. The basket then fits into the tube, which may lock the basket in place in two positions. When locked in the "lower position", the base of the basket is tight against the floor of the tube, and all liquid is contained within the basket's chamber without leaking into the tube or out of the device. The lower position is used during incubation periods and vortex mixing. When raised and locked in the "upper position", a space is created within the tube below the basket, which frees the bottom of the basket. While in the upper position, liquid may flow through the basket into the tube. The upper position is used during centrifugation and separation of fractions. It is notable that while in the upper position, the SpermX device is not leakproof around the rim of the tube, and mishandling may lead to loss of liquid. Transition between the upper and lower positions is aided by a special set of pliers provided by InnoGenomics. Reagents included in the InnoGenomics SpermX extraction kit include an Epithelial Digest Buffer and a Sperm Digest Solution.

To assemble the SpermX device, a SpermX basket was fully inserted into the lower position of the SpermX tube. The sample, 600 µL of Epithelial Digest Buffer, and 15 µL PK (Qiagen) was then added to the basket. The device was secured with a SpermX cap and placed in an incubation oven at 56°C for 1.5 hours, with 10 seconds of vortex mixing every half hour. After the incubation period, the baskets were raised within the SpermX tubes to the "upper position", which created an open space within the SpermX tube beneath the basket. The SpermX device was then placed into a SpermX tube rack adapter and centrifuged at 4000 rpm for 1 minute. As a result of this centrifugation, the epithelial fraction flowed through the nanofiber matrix and into the SpermX tube. After the basket was removed and placed into a new SpermX tube, the epithelial fraction from the original tube was transferred to a microcentrifuge tube for DNA extraction. The original sample substrate was then removed from the SpermX basket, and the male fraction within the nanofiber matrix was washed with 300 µL Epithelial Digest Buffer and 5 µL PK. The secondary epithelial fraction was removed after 30-minute incubation at 56°C and subsequent centrifugation with the basket in the upper position. The basket, still holding the sperm fraction in the nanofiber matrix, was placed in a 15-mL conical tube. To further purify the sperm fraction and begin the sperm digest, a series of three washes with 500 µL Sperm Digest Solution were performed, interspaced with two one-minute centrifugations at 4000 rpm. After the third addition of Sperm Digest Solution and a three-minute centrifugation at 4000 rpm, the basket was removed from the conical tube and placed into a SpermX tube. The 15 mL conical tube, now containing ~1.5 mL of liquid, was discarded. To lyse the sperm cells trapped in the nanofiber matrix, 300 µL Sperm Digest Solution, 20 µL PK, and 80 µL DTT were added to the SpermX basket. With the basket seated in the lower position, the device was incubated at 63°C for 30 minutes. Following the incubation, the basket was raised to the upper position and centrifuged at 4000 rpm for five minutes. To elute the sperm fraction, 300 µL of additional Sperm Digest solution was added to the basket and incubated at room temperature for 5 minutes. The device was centrifuged a final time at 4000 rpm for 5 minutes, after which the sperm fraction eluate was transferred to a microcentrifuge tube. The DNA in each of the female and male fractions was extracted using the EZ1, and extracts were stored at 4°C until DNA quantification. Once quantification was complete, the samples were moved to –20°C storage until amplification.

Shortly after the Donor Comparison Study was completed, a modified procedure was received from the manufacturer. Modifications to the procedure included a 45-minute sperm digestion period (previously 30 minutes), three epithelial washes and centrifugation periods of five minutes (previously two one-minute and one three-minute periods), and the retention of the evidence within the basket for the duration of the extraction. The altered procedure was implemented for the sensitivity comparison extractions.

2.4. Quantification

All samples were quantified using the Applied Biosystems™ Quantifiler™ Trio DNA Quantification Kit (Thermo Fisher Scientific). Quantification was performed using the Applied Biosystems™ 7500 Real-Time PCR System (Thermo Fisher Scientific), with thermal cycler conditions of 95°C for 2 minutes, followed by 40 cycles of 95°C for 9 seconds and 60°C for 30 seconds.

2.5. STR Amplification

Quantified samples were then amplified using the Applied Biosystems ™ GlobalFiler ™ PCR Amplification Kit (Thermo Fisher Scientific) for 24 STR loci. The reaction was performed using the Applied Biosystems ™ Proflex PCR System (Thermo Fisher Scientific). The thermal cycler conditions for amplification were an initial denaturation at 95 °C for 1 minute, 29 cycles of 94 °C for 10 seconds and 59 °C for 90 seconds, a final extension at 60 °C for 10 minutes, and a 4°C hold until the plate was transferred to storage at -20°C.

2.6. Data Analysis and Interpretation

DNA quantity, degradation index, and quantification female to male ratio were calculated using the quantification process.

After STR amplification, STR profiles were generated using the 3500xL Genetic Analyzer (Thermo Fisher Scientific). Profiles were analyzed using GeneMapper IDX v1.4 (Thermo Fisher Scientific), with an analytical threshold of 150 rfu.

The DNA profiles were analyzed using comparisons to control DNA profiles from the female and male donors. Mixture proportion was calculated by dividing the observed number of mixture profiles by the total number of profiles for each donor group. A profile was considered a mixture if at least one male-associated peak was observed in the female fraction, or if at least one female-associated peak was observed in the male fraction. Allele success was calculated by dividing the number of observed donor-attributed alleles by the total number of expected alleles in that donor's reference profile. Allele success for mixture profiles was determined by considering only the alleles not shared between the two present donors. DNA profile female to male ratio was calculated from profile data by dividing the sum of all female-assigned peak heights by the sum of all male-assigned peak heights.

Statistical testing was performed using R Studio v.4.0 (R Foundation for Statistical Computing). Testing included equal and unequal variance 2-sample t-tests and Wilcoxon Rank-Sum tests; results were considered significant with a p-value less than 0.05.

3. Results and Discussion

3.1. Donor Comparison

Donor comparison samples were separated depending on male or female fraction, male semen donor (donors 1-5), and differential extraction method. The data was then used to compare the efficacy of the FBI and SpermX differential extraction methods on mock sexual assault mixtures with varying male DNA donors. Although the DNA in the female fractions would be expected to originate from solely the female DNA donor, the female fraction data was still grouped by donor due to the potential for male DNA carryover.

3.1.1. Donor Comparison: DNA Quantity

DNA quantities produced by each extraction method were compared to identify whether the extracted quantities would differ depending on male DNA donor in a mock sexual assault mixture. From 50 uL mixture volumes, the FBI method produced male DNA quantities of 0.03-6.70 ng/uL and female DNA quantities of 1.10-3.83 ng/uL. In comparison, the SpermX method produced male DNA quantities of 0.01-3.27 ng/uL and female DNA quantities of 0.004-12.36 ng/uL. The means and standard deviations of the DNA quantities can be observed in Table 2.

When comparing the FBI- and SpermX-extracted female fraction quantities, the larger FBI-extracted quantities for donors 1, 3, and 4 were found to be statistically significant ($p<0.05$), as was the larger SpermX-extracted quantity for donor 5 (Figure 3). Therefore, there is some evidence that across mock sexual assault mixtures with different male DNA donors, the FBI method may extract a larger quantity of female DNA than the SpermX method. The large discrepancy between the donor 5 female DNA quantities (Figure 2) most likely originates from the saliva used in sample preparation. The consistency of fluid and distribution of buccal epithelial cells varies within amounts of saliva and among saliva collected at different times. While the epithelial cell preparation assisted in the consistency of resulting DNA quantities, the creation of different “batches” of epithelial cell solution may have been a source of inconsistency in buccal epithelial cell distribution, and therefore DNA quantity.

The male fraction quantities were likewise compared, and the larger FBI-extracted quantities for donors 1, 2, and 4 were found to be statistically significant ($p<0.05$), shown in Figure 3. There is some evidence that across mock sexual assault mixtures with different male DNA donors, the FBI method may extract a larger quantity than the SpermX method.

3.1.2. Donor Comparison: Degradation Index

Degradation indices (DI) for each extraction method were compared to identify whether DI would differ depending on the male DNA donor in a mock sexual assault mixture. The FBI method produced female fraction DI values of 0.83-1.39 and male fraction DI values of 0.60-1.07. In comparison, the SpermX method produced female fraction DI values of 0.75-1.41 and male fraction DI values of 0.49-1.07. The means and standard deviations of the degradation indices can be observed in Table 2.

When comparing the two extraction methods’ DI values, no significant differences were observed between the female fraction degradation indices, and therefore there is no evidence to suggest that extraction method may influence degradation indices for the female fractions of mock sexual assault samples with different male donors. Among the male fractions, the larger DI observed (donor 4) when the FBI method was used and when the SpermX method (donor 5) was used were found

to be significant ($p<0.05$). There is little evidence that extraction method may influence degradation indices for the male fractions of mock sexual assault samples with different male donors (Figure 4). The female fraction DI values were found to be significantly larger ($p<0.05$) than the male fraction DI values, indicating that there is evidence that independent of donor or extraction method, the female fraction DNA may be more prone to degradative or inhibitory effects.

Because the tested samples could be defined as "not degraded" according to the manufacturer's protocol [11], this experiment may not be sufficient to make a conclusion about either methods' ability to extract DNA from degraded samples. Additional studies using naturally or artificially degraded samples or sexual assault kits stored for a long period of time would be necessary to make any further conclusions.

3.1.3. Donor Comparison: Female to Male Ratio (Quantification)

The female to male (F:M) ratios as determined by quantification for each extraction method were compared to identify whether the F:M ratio would differ depending on the male donor in a mock sexual assault mixture. As the F:M ratio increases beyond a value of 1, the amount of female DNA in comparison to male DNA increases. As the F:M ratio decreases below a value of 1, the amount of male DNA in comparison to female DNA increases. A lack of F:M ratio data for a sample indicates that a female and male DNA mixture was not detected by quantification. For the female fractions, the FBI method produced F:M ratios of 3.09-43.27, and the SpermX method produced F:M ratios of 1.56-633.85. For the male fractions, no F:M ratios were determined from quantification, suggesting that all male fractions were of a single DNA source. The means and standard deviations of the F:M ratios can be observed in Table 2.

The larger F:M ratio observed when the FBI method was used for donor 1 was found to be statistically significant ($p<0.05$), as were the larger F:M ratios when the SpermX method was used for donors 3 and 5 ($p<0.05$), shown in Figure 5. No meaningful conclusions regarding the performance of either extraction method or female to male ratio could be made from this data.

3.1.4. Donor Comparison: Proportion of Mixture Profiles

The proportion of mixture profiles generated from the DNA extracted by each method were compared to determine whether the presence of different male DNA donors would influence the amount of observed mixture DNA profiles. A DNA profile was considered a mixture if it contained both female and male alleles or more alleles than the amount expected for a single male or female. The amount of persisting male or female DNA contributing to a mixture is addressed in subsequent sections 3.1.5-3.1.7. The means and standard deviations of the proportions of mixture profiles can be observed in Table 2.

Out of 196 generated profiles, 92 were mixtures and 104 were single source profiles. The female fraction produced a higher proportion of mixture profiles compared to the male fractions (female= 0.73, male= 0.20); this result is expected because of the presence of male epithelial cells or white blood cell in semen, which cannot be differentiated from female epithelial cells in a differential separation process. Male DNA in the female fraction may also be a result of unintentional premature lysis of sperm heads from age, degradation, or handling. The proportions of mixture DNA profiles separated by fraction and method may be viewed in Figure 6.

No significant differences in the two separation methods' DNA profile proportions were observed for the individual male or female fractions. However, when male or female data was pooled, larger proportions of mixture profiles ($p<0.05$) were observed in the FBI-extracted female fractions and the SpermX-extracted male fractions. Independent of DNA donor, there is some evidence to suggest that when extracting mock sexual assault mixtures, the FBI method may generate a larger proportion of mixture profiles in the female fraction, and the SpermX method may generate a larger proportion of mixture profiles in the male fraction.

3.1.5. Donor Comparison: Percentage of Successful Alleles- Female Fraction

In a female fraction DNA profile, a large percentage of female alleles indicate high profiling success. The presence of male alleles in a female fraction indicates a mixture DNA profile, which is addressed in Section 3.1.4, and a high percentage of male alleles in the female fraction may identify poor separation success. In the following discussion, the percentages of male alleles include data from mixture profiles in which only male alleles were present. The percentage of successful female alleles ranged from 88.46-100% for the FBI method and 4.35-100% for the SpermX method. For the mixture profiles observed in the female fractions, the percentage of male alleles ranged from 3.22-65.38% for the FBI method and 3.23-96.15% for the SpermX method. The means and standard deviations of the allele percentages can be observed in Table 2.

The female and male allele percentages may be observed in Figure 7. The percent of successful female alleles for the donor 3 female fraction was larger when the SpermX method was used ($p<0.05$), and no other significant differences in female allele percentage were observed. The percentage of male alleles was larger when the FBI method was used for donor 3, and larger when the SpermX method was used for donor 1 ($p<0.05$). Due to limited and contradicting significant observations, there is little evidence to suggest that one extraction method may produce a higher percentage of male or female alleles in the female fraction DNA profile of mock sexual assault mixtures.

3.1.6. Donor Comparison: Percentage of Successful Alleles- Male Fraction

In a male fraction DNA profile, a large percentage of male alleles indicates high profiling success. The presence of female alleles in a male fraction indicates a mixture DNA profile, which is addressed in Section 3.1.4. However, given the presence of a mixture DNA profile, the percentage of female alleles in the male fraction may further identify poor separation success. The female allele percentages mentioned in the following discussion include data only from mixture profiles where female alleles were present. The means and standard deviations of the allele percentages can be observed in Table 2.

The mean male and female allele percentages may be observed in Figure 8. Statistical comparisons were performed to draw conclusions about the percentages of male and female alleles in the male fractions. When comparing the extraction methods, the FBI method gave a higher male allele percentage for donor 5 ($p<0.05$), but no differences in male allele success was observed for the other donors' male fractions. No significant differences in female alleles in the male fraction were observed. There is little evidence to suggest that when extracting mock sexual assault mixtures with different male DNA donors, the percentage of successful male alleles is influenced by the extraction method used. Additionally, there is no evidence to suggest that female alleles in the male fraction is influenced by extraction method.

3.1.7. Donor Comparison: Female to Male Ratio (DNA Profile)

The DNA profile female to male (F:M) ratios were calculated by comparing the peak heights of the female and male-associated alleles. DNA profile female to male ratio was calculated from profile data by dividing the sum of all female-assigned peak heights by the sum of all male-assigned peak heights. While no F:M quantification ratio was observed for the male fractions (Section 3.1.3.), female-associated peaks were observed in some of the male fraction profiles, allowing for a DNA profile F:M ratio to be calculated. The means and standard deviations of the F:M ratios can be observed in Table 2.

When the FBI method was used, the F:M ratios determined from the DNA profiles were between 3.40-314.92 for the female fraction and 0.004-0.04 for the male fraction. When the SpermX method was used, the F:M ratios determined from the DNA profiles were between 0.67-173.24 for the female fraction and 0.006-0.22 for the male fraction. The mean female fraction DNA profile F:M ratios may be observed in Figure 9, and the male fraction F:M ratios may be observed in Figure 10.

Statistical comparisons were performed to draw conclusions about the observed differences in profile-determined F:M ratio across the donors. The larger F:M ratios observed in the female fractions when the FBI method was used for donors 1 and 2 were found to be significant ($p<0.05$), as was the larger F:M ratio when the SpermX method was used for donor 3 ($p<0.05$). Therefore, there is some evidence to suggest that the F:M ratio as determined from the DNA profile may vary depending on extraction method for the female fractions of mock sexual assault swabs. No significant differences in the F:M ratios of the male fractions were observed, giving no evidence that within the male fraction, F:M ratio is affected by extraction method. It was also observed that the female to male ratio varied across the different semen donors. A possible explanation for this is that female to male ratio may be dependent on the quantity of male DNA (Table 2); the higher quantity semen donors (donors 1, 4) generally gave low female to male ratios, while the low quantity donor (donor 2) gave much higher female to male ratios.

3.2. Sensitivity Comparison

Data for the sensitivity comparison samples were separated depending on male or female fraction, mixture volume (2 uL, 10 uL, 20 uL, 50 uL), and differential extraction method. The data groups were then used to compare the efficacy of the FBI and SpermX differential extraction methods using mock sexual assault mixtures of different volumes. Semen donors 3 and 5 were used as the male DNA donors for this comparison as they yielded moderate DNA quantities. Data was not separated according to male donor in order to achieve a larger sample size for comparison.

3.2.1. Sensitivity Comparison: DNA Quantity

DNA quantities of different mock sexual assault mixture volumes were compared to identify whether the extracted quantities would differ depending on the extraction method used. From the 2 uL mixture volumes, the FBI method produced female DNA quantities of 0.13-0.28 ng/uL and male quantities of 0.002-0.06 ng/uL, while the SpermX method produced female DNA quantities of 0.18-0.28 ng/uL and male DNA quantities of 0.02-0.06 ng/uL. When 10 uL volumes were extracted, the FBI method produced female DNA quantities of 1.39-1.69 ng/uL and male quantities of 0.09-0.33 ng/uL, while the SpermX method produced female DNA quantities of 1.21-3.39 ng/uL and male DNA quantities of 0.13-1.14 ng/uL. From the 20 uL volumes, the FBI method produced female DNA quantities of 2.08-3.52 ng/uL quantities and male DNA quantities of 0.23-0.53 ng/uL, and the SpermX method produced

female DNA quantities of 0.98-2.81 ng/uL and male quantities of 0.22-0.51 ng/uL. When 50 uL volumes were extracted, the FBI method produced female DNA quantities of 6.51-7.85 ng/uL and male quantities of 0.44-1.54 ng/uL, and the SpermX method produced female DNA quantities of 5.69-6.57 ng/uL and male quantities of 0.27-2.49 ng/uL. The means and standard deviations of the quantities can be observed in Table 3.

The tested volumes' female DNA quantities were compared to identify whether the extraction method would influence quantity (Figure 11). For the donor 3 female fractions, the larger quantity extracted using the FBI method for 10 uL volume was found to be significant ($p<0.05$). For the donor 5 female fractions, the larger quantities extracted using the FBI method for 20 uL and 50 uL volumes were each found to be significant ($p<0.05$). For the combined donor 3 and donor 5 data, the larger quantity extracted using 10 uL volume was found to be significant ($p<0.05$). Overall, there is some evidence to suggest the FBI method may extract larger quantities of female DNA from mock sexual assault samples of different volumes.

The male DNA quantities extracted by each method using tested volumes were compared to identify whether the extraction method would influence quantity (Figure 12). For the donor 3 male fractions, the larger quantities extracted using the SpermX method for 10 uL, 20 uL, and 50 uL were found to be significant ($p<0.05$). For the donor 5 male fractions, none of the observed differences between the extraction methods were found to be significant; likewise, when the donor 3 and donor 5 male fraction quantity data was combined, no significant differences between the quantities were observed. Overall, there is some evidence to suggest the SpermX method may extract larger quantities of male DNA from mock sexual assault samples of different volumes.

3.2.2. Sensitivity Comparison: Degradation Index

Degradation indices of different mock sexual assault mixture volumes identify differences between the differential extraction methods. The FBI method produced female fraction DI values of 0.71-0.98 and male fraction DI values of 0.64-0.86. In comparison, the SpermX method produced female fraction DI values of 0.72-1.00 and male fraction DI values of 0.64-0.98. The means and standard deviations of the degradation indices can be observed in Table 3.

Similar to the donor-compared samples, the observed degradation indices across all samples (0.64-0.98) indicate no degradation has occurred throughout this study (Figure 13). No significant differences in DI were observed between the extraction methods when the individual volumes for combined and individual donor data were compared. There is no evidence to suggest that among different volumes, degradation index will differ when different extraction methods are used.

3.2.3. Sensitivity Comparison: Female to Male Ratio (Quantification)

The female to male (F:M) ratios as determined by quantification for each extraction method were compared to identify whether the F:M ratio would differ depending on the male donor in a mock sexual assault mixture. For the female fractions of the different mixture volumes, the FBI method produced quantification F:M ratios of 8.76-147.68, while the SpermX method produced quantification F:M ratios of 9.27-311.70. No F:M ratios were determined from quantification for the male fractions. The means and standard deviations of the F:M ratios can be observed in Table 3, and a visualization may be observed in Figure 14.

The larger F:M ratio for 2 uL volume was found to be significant ($p<0.05$), but no other significant differences were observed. There is some evidence to suggest that when extracting mock sexual assault mixtures of different volumes, the SpermX method may produce a larger quantification F:M ratio in the female fraction.

3.2.4. Sensitivity Comparison: Proportion of Mixture DNA Profiles

The proportion of mixture DNA profiles generated from the DNA extracted from each method were compared to determine whether mixture volume would influence profile quality. The amount of persisting male or female DNA contributing to a mixture is addressed in subsequent sections 3.2.5-3.2.7. Out of 96 generated profiles, 24 were mixture profiles, and 72 had a single DNA source. Like the donor comparison samples (Section 3.1.4), the female fractions had an overall higher proportion of mixture profiles (female proportion= 0.33, male proportion= 0.17). The means and standard deviations of mixture proportions can be observed in Table 3.

The donor 3 and donor 5 data was combined in order to create a larger sample size to observe differences between the tested volumes, which can be observed in Figure 15. No significant differences were observed between the female fraction mixture proportions generated by each extraction method. For the male profiles, the SpermX method yielded a significantly larger ($p<0.05$) proportion of mixture DNA profiles; therefore, there is some evidence to suggest that the SpermX method may generate a higher proportion of mixture DNA profiles when extracting mock sexual assault samples of different volumes.

3.2.5. Sensitivity Comparison: Percentage of Successful Alleles, Female Fraction

The percentages of successful female or male alleles in the female fraction of different mock sexual assault mixture volumes were compared to identify whether the extracted quantities would differ depending on the extraction method used. The percentage of successful female alleles ranged from 4.35-100% for the SpermX method, and for the FBI method 100% of female alleles were observed for all profiles. For the mixture profiles observed in the female fractions, the percentage of male alleles ranged from 3.85-23.08% for the FBI method and 3.85-11.54% for the SpermX method. The donor 3 and donor 5 data was combined in order to create a larger sample size, and the means and standard deviations of the allele percentages can be observed in Table 3.

Statistical comparisons were performed to draw conclusions about the percentages of successful female and male alleles in the female fractions (Figure 16). No significant differences in female fraction allele success were observed between the extraction methods when the individual volumes for combined and individual donor data were compared. There is no evidence to suggest that among the female fractions of different volumes of mock sexual assault mixtures, percent allele success will differ when different extraction methods are used.

3.2.6. Sensitivity Comparison: Percentage of Successful Alleles, Male Fraction

The percentages of successful male or female alleles in the male fraction of different mock sexual assault mixture volumes were compared to identify whether the extracted quantities would differ depending on the extraction method used. The percentage of successful male alleles ranged from 2.17-100% for the FBI method and 80.43-100% for the SpermX method. For the mixture profiles observed in the female fractions, the percentage of female alleles ranged from 3.33-6.67% for the SpermX method, and no mixture profiles were observed for the FBI method. The donor 3 and donor 5 data was combined

in order to create a larger sample size, and the means and standard deviations of the allele percentages can be observed in Table 3.

Statistical comparisons were performed to draw conclusions about the percentages of male and female alleles in the male fractions (Figure 17). None of the observed differences in male allele success were found to be statistically significant for any of the tested volumes. There is no evidence to suggest that among the male fractions of different volumes of mock sexual assault mixtures, percent male allele success will differ when different extraction methods are used. Statistical analysis regarding the comparison of mixture profiles in the male fraction is included in Section 3.2.4.

3.2.7. Sensitivity Comparison: Female to Male Ratio (DNA Profile)

The female to male (F:M) ratios as determined from the DNA profiles was calculated by comparing the peak heights of the female and male-associated alleles. The DNA profile F:M ratios were then compared to identify whether the F:M ratio would differ depending on the male donor in a mock sexual assault mixture. When the FBI method was used, the DNA profile F:M ratios were between 11.01-84.97 for the female fraction. No F:M ratios could be determined from the FBI method male fractions because all profiles were of a single source. When the SpermX method was used, the F:M ratios determined from the DNA profiles were between 39.33-130.09 for the female fractions, and between 0.01-0.09 for the male fraction. The means and standard deviations of the F:M ratios can be observed in Table 3.

Statistical comparisons were performed to draw conclusions about the observed differences in the female fraction DNA profile F:M ratios across the different volumes (Figure 18). No significant differences were observed between the DNA profile F:M ratios from the extracted volumes. There is no evidence to suggest that across different volumes of mock sexual assault mixtures, the F:M ratio as determined from the DNA profile will differ between different extraction methods.

3.3. Material Comparison

Material comparison samples were separated depending on male or female fraction, stain material, and differential extraction method. The materials compared were condoms, 100% cotton (cotton), 95% cotton/5% spandex blend (cotton/spandex), 100% polyester (polyester), and 100% denim (denim). The categories of data were then used to compare the efficacy of the FBI and SpermX differential extraction methods using mock sexual assault mixtures on different stain materials. Semen donors 3 and 5 were used as the male DNA donors for this comparison as they yielded moderate DNA quantities in the previous experiments. Data was not separated according to male donor in order to achieve a larger sample size for comparison.

3.3.1. Material Comparison: DNA Quantity

DNA quantities recovered by each extraction method were compared to identify whether the quantities would differ depending on the stain material used in a mock sexual assault mixture. From swabbed 50 uL stains on condoms, the FBI method produced female DNA quantities of 2.02-3.19 ng/uL and male quantities of 0.58-2.19 ng/uL, while the SpermX method produced female quantities of 1.35-2.17 ng/uL and male quantities of 0.52-1.64 ng/uL. When the 50 uL swabbed stains on cotton were extracted, the FBI method produced female DNA quantities of 0.51-0.89 ng/uL and male quantities of 0.08-0.47 ng/uL, while the SpermX method produced female quantities of 0.18-0.91 ng/uL and male quantities of 0.02-0.09 ng/uL. When the 50 uL stains on cotton/spandex were swabbed and extracted,

the FBI method produced female DNA quantities of 0.18-0.50 ng/uL and male quantities of 0.01-0.18 ng/uL, and the SpermX method produced female quantities of 0.12-0.26 ng/uL and male quantities of 0.02-0.31 ng/uL. From swabbed 50 uL stains on polyester, the FBI method produced female DNA quantities of 0.21-0.62 ng/uL and male quantities of 0.05-0.16 ng/uL, while the SpermX method produced female quantities of 0.11-0.33 ng/uL and male quantities of 0.03-0.58 ng/uL. From swabbed 50 uL stains on denim, the FBI method produced female DNA quantities of 0.39-0.78 ng/uL and male quantities of 0.02-0.14 ng/uL, while the SpermX method produced female quantities of 0.18-0.35 ng/uL and male quantities of 0.02-0.28 ng/uL. The means and standard deviations of the quantities can be observed in Table 4.

While two different semen donors were used in the samples, the data for each donor was also combined to achieve a larger sample size for comparison. No significant differences were observed when the data for the different donors were considered separately, but when the donor 3 and donor 5 data was combined to achieve a larger sample size, significant differences were observed. For the female fractions, the larger quantities extracted when the FBI method was used for condoms, polyester, and denim were found to be significant ($p<0.05$) , shown in Figure 19. There is some evidence to suggest that across different stain materials with mock sexual assault mixtures, the FBI method may extract larger quantities of female fraction DNA. For the male fractions, the larger quantity extracted when the FBI method was used for cotton was found to be significant ($p<0.05$), shown in Figure 20; this suggests that the FBI method may extract larger quantities of male fraction DNA from mock sexual assault mixtures on cotton.

3.3.2. Material Comparison: Degradation Index

Degradation indices (DI) of the extracted samples were compared to identify whether DI would differ depending on the stain material of a mock sexual assault mixture stain. From stains on condoms, the FBI method produced female fraction DI values of 0.84-1.05 and male fraction DI values of 0.63-0.81, while the SpermX method produced female fraction DI values of 0.74-0.96 and male fraction DI values of 0.63-0.78. From stains on cotton, the FBI method produced female fraction DI values of 0.86-0.98 and male fraction DI values of 0.62-0.79, while the SpermX method produced female fraction DI values of 0.71-0.94 and male fraction DI values of 0.52-0.76. From stains on cotton/spandex, the FBI method produced female fraction values of 0.78-1.00 and male fraction values of 0.54-0.73, while the SpermX method produced female fraction values of 0.70-0.86 and male fraction values of 0.71-0.74. From stains on polyester, the FBI method produced female fraction DI values of 0.71-0.98 and male fraction values of 0.65-0.88, while the SpermX method produced female fraction DI values of 0.77-0.91 and male fraction values of 0.79-0.86. From stains on denim, the FBI method produced female fraction DI values of 0.88-1.07 and male fraction values of 0.55-0.83, while the SpermX method produced female fraction values of 0.84-0.98 and male fraction values of 0.56-0.70. The means and standard deviations of the degradation indices can be observed in Table 4.

The data for both semen donors was combined to achieve a larger sample size to observe differences in mean degradation index (Figure 21). As with the donor and sensitivity comparison samples, the observed degradation indices across all material comparison samples (0.54-1.07) indicate little to no degradation or inhibition has occurred across any material stains. Statistical comparisons were performed to draw conclusions about the differences in degradation index. For the female fractions, the larger DI value observed when the FBI method was used for the cotton/spandex blend was found to be statistically significant ($p<0.05$); therefore, there is some evidence to suggest that when the

FBI method is used to extract DNA from mock sexual assault stains on cotton/spandex blend fabric, a higher degradation index value will be observed. Additionally, the cotton male fraction extracted by the FBI method had a significantly larger DI value ($p<0.05$).

As with the donor comparison and volume comparison samples, these samples can be considered undegraded, giving little weight to the conclusions regarding cotton/spandex and cotton. Therefore, the natural or artificial degradation of samples would be required to further investigate the abilities of either extraction method on mock sexual assault samples using different materials.

3.3.3. Material Comparison: Female to Male Ratio (Quantification)

The female to male (F:M) ratios as determined by quantification for each extraction method were compared to identify whether the F:M ratio would differ depending on the male donor in a mock sexual assault mixture. For the female fractions, the FBI method produced F:M ratios of 2.35-112.65, and the SpermX method produced F:M ratios of 3.64-956.03. For the male fractions, no F:M ratios were determined because there was no male DNA detected during quantification. The means and standard deviations of the F:M ratios can be observed in Table 4.

Statistical comparisons were performed to draw conclusions about the larger female fraction quantification F:M ratios observed for the SpermX-extracted volumes (Figure 22). None of the differences were found to be statistically significant, and therefore there is no evidence to suggest that when extracting mock sexual assault mixtures on different materials, the F:M ratio as determined by quantification may differ depending on the extraction method used.

3.3.4. Material Comparison: Proportion of Mixture DNA Profiles

The proportion of mixture DNA profiles generated from the DNA extracted via each method were compared to determine whether mixture volume would influence profile quality. The amount of persisting male or female DNA contributing to a mixture is addressed in subsequent sections 3.3.5-3.3.7. Out of 119 generated profiles, 45 were mixture profiles, and 74 had a single DNA source. The female fractions demonstrated a higher overall proportion of mixture profiles (female proportion= 0.52, male proportion= 0.24). The means and standard deviations of the mixture proportions can be observed in Table 4.

The donor 3 and donor 5 data was combined in order to create a larger sample size to observe differences between the tested volumes (Figure 23). No statistically significant differences were observed between the FBI - and SpermX-extracted mixture proportions for any male or female fractions. There is no evidence to suggest that either method may generate a higher proportion of mixture DNA profiles when extracting mock sexual assault stains on different materials.

3.3.5. Material Comparison: Percentage of Successful Alleles, Female Fraction

The percentages of successful female or male alleles in the female fraction of mock sexual assault stains from different materials were compared to identify whether the extracted quantities would differ depending on the extraction method used. Across all tested materials, the percentage of successful female alleles ranged from 96.30-100% for the FBI method, and 86.96-100% for the SpermX method. For the mixture profiles observed in the female fractions, the percentage of male alleles ranged from 3.23-88.46% for the FBI method, and 3.85-73.08% for the SpermX method. The means and standard deviations of the allele percentages can be observed in Table 4.

The donor 3 and donor 5 data was combined in order to create a larger sample size. No significant differences in female or male allele success were observed between the extraction methods (Figure 24). Therefore, there is no evidence to suggest that among the female fractions of mock sexual assault stains on different materials, percent allele success will differ when different extraction methods are used. Overall, both methods showed high profiling success when the female alleles were observed. One female fraction profile was not analyzed due to contamination from a DNA profile that did not match the female source or any of the male sources.

3.3.6. Material Comparison: Percentage of Successful Alleles, Male Fraction

The percentages of successful male or female alleles in the male fraction of mock sexual assault mixtures on different materials were compared to identify whether the extraction method influenced profiling success. The percentage of successful male alleles in the male fraction ranged from 39.13-100% for the FBI method and 8.7-100% for the SpermX method. For the mixture profiles observed in the male fractions, the percentage of female alleles ranged from 3.33-6.67% for the FBI method, and 3.33-13.33% for the SpermX method. The means and standard deviations of the allele percentages can be observed in Table 4.

The donor 3 and donor 5 data was combined in order to create a larger sample size. Statistical comparisons were performed to draw conclusions about the percentages of successful male and female alleles in the male fractions (Figure 25). The larger percentage of successful male alleles observed when the FBI method was used for condoms was found to be significant ($p<0.05$). This gives evidence to suggest that when the FBI method is used, higher male allele success may be observed from mock sexual assault mixtures on condoms. There were no other significant differences between the FBI method and SpermX method allele success percentages in the male fraction for mock sexual assault mixtures on different materials.

As with the female fractions, both methods showed high male allele profiling success. Of the 29 male fraction profiles generated by the DNA extracted using the FBI method, four profiles did not yield 100% of the expected male alleles. The male fraction counterpart to the contaminated female profile was eliminated from consideration.

3.3.7. Material Comparison: Female to Male Ratio (DNA Profile)

The female to male (F:M) ratios as determined from the DNA profiles was calculated by comparing the peak heights of the female and male-associated alleles. The DNA profile F:M ratios were then compared to identify whether the F:M ratio would differ depending on the male donor in a mock sexual assault mixture. When the FBI method was used, the DNA profile F:M ratios ranged from 2.94-1203.86 for the female fraction and 0.005-0.1 for the male fraction. When the SpermX method was used, the F:M ratios were between 3.73-116.23 for the female fraction and 0.005-0.025 for the male fraction. The means and standard deviations of the F:M ratios can be observed in Table 4.

Differences in DNA profile F:M ratio were measured between the different materials. No significant differences were observed across the materials for either the male or female fractions (Figure 26). There is no evidence to suggest that across different volumes of mock sexual assault mixture, the DNA profile F:M ratio will differ between extraction methods.

4. Conclusions

Two differential extraction methods, the currently utilized FBI method and the novel nanofiber matrix SpermX method, were compared in their ability to extract mock sexual assault samples of different male donors, volumes, and stain materials.

For many of the tested metrics, the extraction methods were comparable. No conclusions could be drawn from the donor, sensitivity, or material comparisons regarding degradation index, the percentage of successful female alleles across female and male fractions, the percentage of successful male alleles in female fractions, or the DNA profile female to male ratios. Overall, a high-quality DNA profile may be expected as a product of each extraction method, with some differences between the methods.

The metrics that exhibited significant differences between the extraction methods were DNA quantity and the proportion of mixture profiles. Across the donor, sensitivity, and materials tests, a larger DNA quantity was generally observed when the FBI method was used for both the female and male fractions. The FBI method also exhibited a lower proportion of mixture DNA profiles, and therefore a higher separation success, for the donor comparison male fraction data and the sensitivity comparison female fraction data. These conclusions suggest that the FBI differential extraction method is effective in extracting large quantities of DNA and separating male and female cells from mock sexual assault samples. During this study, the secondary epithelial fraction and epithelial washes of the SpermX protocol were discarded, but if a SpermX user wishes to increase their female fraction yield, extraction of these secondary fractions is possible.

There is evidence to suggest that the SpermX method is effective in the separation of male and female cells and the generation of a DNA profile. The SpermX method demonstrated a larger quantification female to male ratio from the sensitivity comparison, a lower proportion of mixture profiles for the donor comparison, and a larger percentage of successful male alleles for the materials comparison. Each of these conclusions are indicative of successful separation and DNA profiling when the SpermX differential extraction method is used to extract mock sexual assault samples.

Finally, the ease of procedure may also be addressed when comparing the FBI and SpermX differential methods. While both methods include some automation, the FBI method is markedly more hands-off than the SpermX method and allows the user to complete other tasks during lysis incubations or automated steps. The SpermX method, while including some hands-off steps, does require manual handling of the devices, and includes the time-consuming epithelial wash series. Automation of additional portions of the SpermX procedure is possible with Hamilton products, but such instrumentation was not available for this study. The design of the SpermX device is simple and user friendly, and additional accessories included in the SpermX kit such as the wrench and tube holders assist in the extraction process.

The lack of conclusions regarding the influence of degraded DNA in these comparisons is a topic that deserves to be more thoroughly explored. Sexual assault kit backlogs have caused SAKs to remain in storage for extended periods of time, and it has not been addressed how the SAKs' DNA evidence may have degraded due to the time between collection and analysis. Understanding how degradation occurs in sexual assault samples and the identification of an extraction method best suited for compromised samples may benefit those affected by the SAK backlog. To do this, a reliable method of artificially or naturally degrading mock sexual assault samples would need to be established, and comparisons between potential differential extraction methods would need to be made.

References

[1] Gill, P., Jeffreys, A. J., & Werrett, D. J. (1985). Forensic application of DNA 'fingerprints'. *Nature, 318*(6046), 577-579. doi:10.1038/318577a0

[2] Sinha, S. (2016). U.S. Patent No. US 2016/0222375 A1. Washington, DC: U.S. Patent and Trademark Office.

[3] Spermx™. (2021, January 08). Retrieved from https://innogenomics.com/products/sperm-x/

[4] InnoGenomics. (2020, October 7). *SpermXTM Differential Extraction User Guide*.

[5] Hilz, H., Wiegers, U., & Adamietz, P. (1975). Stimulation of Proteinase K action by denaturing agents: Application to the isolation of nucleic acids and the degradation of 'Masked' Proteins. *European Journal of Biochemistry, 56*(1), 103-108. doi:10.1111/j.1432-1033.1975.tb02211.x

[6] Garvin, A. M., Bottinelli, M., Gola, M., Conti, A., & Soldati, G. (2009). DNA Preparation from Sexual Assault Cases by Selective Degradation of Contaminating DNA from the Victim. *Journal of Forensic Sciences, 54*(6), 1297-1303. doi:10.1111/j.1556-4029.2009.01180.x

[7] QIAGEN (2017). *EZ1 Advanced XL User Manual*. Hilden, Germany.

[8] QIAGEN (2020). *QIAcube Connect User Manual.* Hilden, Germany.

[9] Sinha, S. K., Ph.D. (2019). Highly Efficient Sperm Cell Separation from Sexual Assault Samples for DNA Analysis Using Novel Polymer Filter Technology. (United States, Department of Justice, National Institute of Justice).

[10] United States, Department of Justice, Office of Justice Programs. National Best Practices for Sexual Assault Kits: A Multidisciplinary Approach.

[11] Cross, T. P., Ph.D, Alderden, M., Ph.D., Wagner, A., M.A., Sampson, L., M.S.W., Peters, B., M.S., Spencer, M., M.A., & Lounsbury, K., M.A. (2014). Forensic Evidence and Criminal Justice Outcomes in a Statewide Sample of Sexual Assault Cases (United States, Department of Justice, National Institute of Justice).

[11] Applied Biosystems. (2017). Quantifiler™ HP and Trio DNA Quantification Kits User Guide.

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- B. Jaffe, S. Rink, C. Guedot (2019) “Red-Headed Flea Beetle: Basic Biology, Density Feeding Patterns, and Spatial Distribution”. 2019 Cranberry School Proceedings.

Presentations:

- S. Rink, K. Elwick, J.M. Robertson. “Comparison of Semi-Automated and Manual Differential Separation Methods for Mock Sexual Assault Swabs”, American Academy of Forensic Sciences 2021 Annual Meeting, Young Scientists Forum Poster Session (February 2021).
- S. Rink, Jaffe B. “Consideration of Time, Temperature, and Host Plant in the Rearing of Red-Headed Flea Beetles”, University of Wisconsin-Madison Undergraduate Research Symposium, Madison, WI (April 2019).

Appendices

Table 1: Sample Preparation										
	Female DNA		Male DNA			Mixtures				
Comparison	Donor	Volume	Donor	Dilution	Volume	Material	Total Volume or Material	Extracted with FBI Method	Extracted with SpermX Method	Total
Female Control	SR	25 uL				Swab	25 uL	1	1	2
Male Control			1	1:20	25 uL	Swab	25 uL	1	1	2
			2	1:20	25 uL	Swab	25 uL	1	1	2
			3	1:20	25 uL	Swab	25 uL	1	1	2
			4	1:20	25 uL	Swab	25 uL	1	1	2
			5	1:20	25 uL	Swab	25 uL	1	1	2
Donors	SR	25 uL	1	1:20	25 uL	Swab	50 uL	10	10	20
			2	1:20	25 uL	Swab	50 uL	10	10	20
			3	1:20	25 uL	Swab	50 uL	10	10	20
			4	1:20	25 uL	Swab	50 uL	10	10	20
			5	1:20	25 uL	Swab	50 uL	10	10	20
							TOTAL	50	50	100
Sensitivity	SR	1 uL	3	1:20	1 uL	Swab	2 uL	3	3	6
		5 uL			5 uL	Swab	10 uL	3	3	6
		10 uL			10 uL	Swab	20 uL	3	3	6
		25 uL			25 uL	Swab	50 uL	3	3	6
		1 uL	5	1:20	1 uL	Swab	2 uL	3	3	6
		5 uL			5 uL	Swab	10 uL	3	3	6
		10 uL			10 uL	Swab	20 uL	3	3	6
		25 uL			25 uL	Swab	50 uL	3	3	6
							TOTAL	24	24	48
Materials	SR	25 uL	3	1:20	25 uL	100% cotton	50 uL	3	3	6
						100% polyester	50 uL	3	3	6
						95% cotton, 5% spandex	50 uL	3	3	6
						10% denim	50 uL	3	3	6
						condom	50 uL	3	3	6
			5	1:20	25 uL	100% cotton	50 uL	3	3	6
						100% polyester	50 uL	3	3	6
						95% cotton, 5% spandex	50 uL	3	3	6
						10% denim	50 uL	3	3	6
						condom	50 uL	3	3	6
							TOTAL	30	30	60

Table 2: Donor Comparison Summary															
Fraction	Method	Semen Donor	Mean Quantity (ng/uL)	Quantity SD (ng/uL)	Mean DI	DI SD	Proportion of Mixture DNA Profiles	Mean Quant F:M Ratio	Quant F:M Ratio SD	Mean Female Alleles	Female Alleles SD	Mean Male Alleles	Male Alleles SD	Mean DNA Profile F:M Ratio	DNA Profile F:M Ratio SD
Female	FBI	1	3.308	0.354	0.945	0.120	0.9	5.230	0.690	100	0	18.222	9.821	15.596	8.540
		2	2.413	1.023	0.984	0.101	0.8	16.120	6.350	99.565	1.376	7.661	4.202	98.244	98.266
		3	2.990	0.318	1.031	0.104	1	4.090	1.600	97.037	1.562	52.692	13.081	5.356	3.023
		4	2.500	0.449	1.096	0.138	0.9	6.350	0.830	96.488	3.378	18.930	15.543	19.047	16.244
		5	1.966	0.368	1.137	0.131	0.6	31.380	6.380	98.681	1.778	5.914	5.168	59.004	22.068
	SpermX	1	2.532	0.759	0.897	0.161	1	3.940	1.620	100	0	39.600	19.906	11.324	3.871
		2	1.586	0.926	1.020	0.138	0.4	19.970	8.610	90.358	25.785	11.290	6.177	28.408	19.252
		3	1.937	0.336	1.028	0.161	1	8.470	1.370	100	0	30.000	24.176	13.617	6.494
		4	1.965	0.405	0.998	0.148	0.7	5.520	1.970	90.586	8.876	10.053	4.121	21.307	10.318
		5	6.197	4.717	1.045	0.141	0.1	225.500	218.670	90.218	30.179	3.226		173.240	
Male	FBI	1	5.321	1.572	0.737	0.035	0					95.218	14.376		
		2	0.145	0.050	0.713	0.048	0.2			2.941	0	83.888	14.085	0.019	0.011
		3	1.489	0.385	0.834	0.069	0.2			3.704	0	97.257	4.830	<0.001	<0.001
		4	3.625	0.601	0.936	0.079	0.1			3.846	-	97.609	2.799	0.011	
		5	0.395	0.115	0.736	0.065	0					96.740	2.759		
	SpermX	1	2.015	0.843	0.716	0.092	0.2			3.704	0	90.319	22.693	0.005	<0.001
		2	0.041	0.017	0.733	0.213	0.4			6.618	2.816	87.853	9.601	0.023	0.010
		3	1.228	0.452	0.904	0.103	0.2			3.704	0	96.840	7.464	0.008	0.002
		4	1.138	0.131	0.742	0.063	0.2			15.385	16.318	97.673	2.940	0.116	0.151
		5	0.428	0.095	0.802	0.047	0.4			11.667	8.819	89.028	11.212	0.037	0.032

Table 3: Sensitivity Comparison Summary															
Fraction	Method	Mixture Volume	Mean Quantity (ng/uL)	Quantity SD (ng/uL)	Mean DI	DI SD	Proportion of Mixture DNA Profiles	Mean Quant F:M Ratio	Quant F:M Ratio SD	Mean Female Alleles	Female Alleles SD	Mean Male Alleles	Male Alleles SD	Mean DNA Profile F:M Ratio	DNA Profile F:M Ratio
Female	FBI	2	0.210	0.059	0.770	0.049	0.50	35.555	30.264	100	0	11.538	6.662	55.160	27.769
		10	1.514	0.117	0.853	0.048	0.17	52.539	47.156	100	0	3.846		82.315	
		20	2.741	0.582	0.851	0.063	0.50	59.953	53.917	100	0	11.538	3.846	38.521	9.314
		50	6.801	0.903	0.912	0.054	0.50	64.931	62.164	100	0	11.538	10.176	43.477	29.566
	SpermX	2	0.226	0.046	0.830	0.096	0.17	91.495	49.128	99.638	0.886	3.846		125.388	
		10	1.210	0.171	0.892	0.070	0.33	88.950	76.781	99.638	0.886	7.692	5.439	68.800	41.676
		20	2.776	0.394	0.845	0.071	0.17	109.091	106.468	83.652	38.862	11.538		40.996	
		50	6.192	0.329	0.875	0.037	0.33	142.488	141.630	98.100	2.560	3.846	0	126.066	5.699
Male	FBI	2	0.033	0.021	0.781	0.033	0					81.523	38.933		
		10	0.204	0.103	0.753	0.088	0					98.190	1.637		
		20	0.396	0.139	0.807	0.047	0					94.928	3.277		
		50	0.932	0.506	0.770	0.046	0					97.825	2.383		
	SpermX	2	0.031	0.015	0.713	0.069	0.67			5.926	3.099	97.746	1.762	0.033	0.036
		10	0.297	0.168	0.860	0.114	0.33			13.333	4.714	95.455	3.291	0.044	0.011
		20	0.680	0.466	0.834	0.069	0.17			3.333		95.633	2.746	0.010	
		50	1.374	0.950	0.803	0.081	0.17			6.667		92.577	9.492	0.022	

Table 4: Materials Comparison Summary															
Fraction	Method	Stain Material	Mean Quantity (ng/uL)	Quantity SD (ng/uL)	Mean DI	DI SD	Proportion of Mixture DNA Profiles	Mean Quant F:M Ratio	Quant F:M Ratio SD	Mean Female Alleles	Female Alleles SD	Mean Male Alleles	Male Alleles SD	Mean DNA Profile F:M Ratio	DNA Profile F:M Ratio SD
Female	FBI	Condom	2.685	0.530	0.937	0.072	0.83	17.020	15.311	100	0	30.397	24.036	256.450	530.175
		Cotton	0.695	0.141	0.913	0.045	0.50	38.684	32.987	99.383	1.512	28.205	16.013	15.563	13.535
		Cot Span	0.267	0.131	0.901	0.072	0.50	25.285	23.246	100	0	64.103	32.711	4.643	1.934
		Polyester	0.303	0.160	0.816	0.093	0.67	36.852	26.374	99.383	1.512	18.921	14.047	44.235	26.461
		Denim	0.497	0.148	0.955	0.076	0.50	49.699	52.255	99.638	0.886	42.308	23.077	6.720	4.956
	SpermX	Condom	1.738	0.305	0.851	0.086	0.50	39.934	38.277	100	0	25.641	9.679	14.176	3.448
		Cotton	0.458	0.268	0.823	0.093	0.50	57.617	61.970	100	0	5.128	2.221	88.134	35.904
		Cot Span	0.222	0.050	0.812	0.055	0.50	72.441	85.145	98.658	2.089	24.359	4.441	12.663	8.961
		Polyester	0.161	0.087	0.845	0.066	0.17	95.012	75.701	99.277	1.121	11.538		55.908	
		Denim	0.252	0.070	0.893	0.054	0.50	217.001	370.800	96.592	5.572	50.000	20.352	4.600	0.997
Male	FBI	Condom	1.228	0.733	0.725	0.062	0.17			6.667		100	0		
		Cotton	0.266	0.194	0.730	0.063	0.17			6.667		89.855	24.850	0.053	
		Cot Span	0.081	0.065	0.686	0.076	0.33			3.519	0.262	100	0	0.053	
		Polyester	0.116	0.061	0.743	0.095	0.00					98.614	2.108	0.010	
		Denim	0.056	0.046	0.690	0.105	0.33			3.704	0	99.638	0.886	0.006	0.001
	SpermX	Condom	1.061	0.571	0.694	0.055	0.00					98.553	1.121		
		Cotton	0.056	0.034	0.619	0.097	0.33			3.333	0	100	0	0.007	0.001
		Cot Span	0.153	0.137	0.728	0.009	0.33			8.333	2.357	99.638	0.886		
		Polyester	0.218	0.225	0.814	0.035	0.50			6.667	0	100	0	0.006	0.001
		Denim	0.149	0.131	0.646	0.052	0.17			13.333		84.422	37.106	0.020	0.003

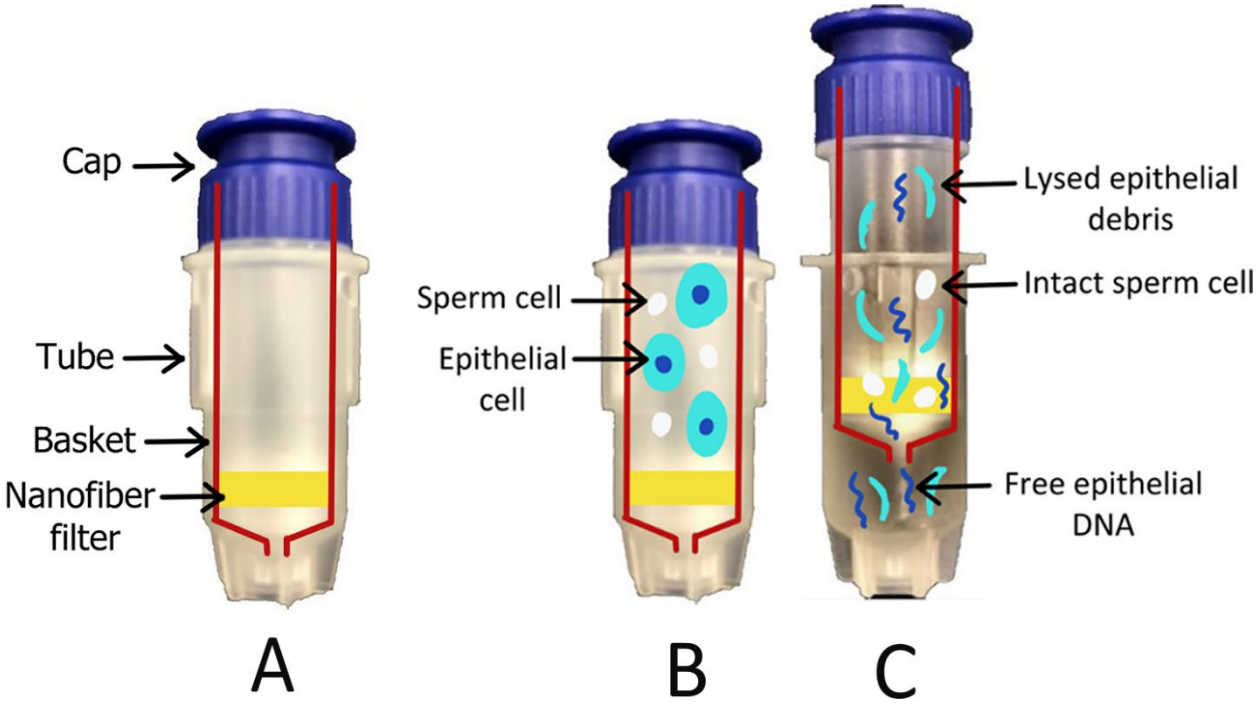
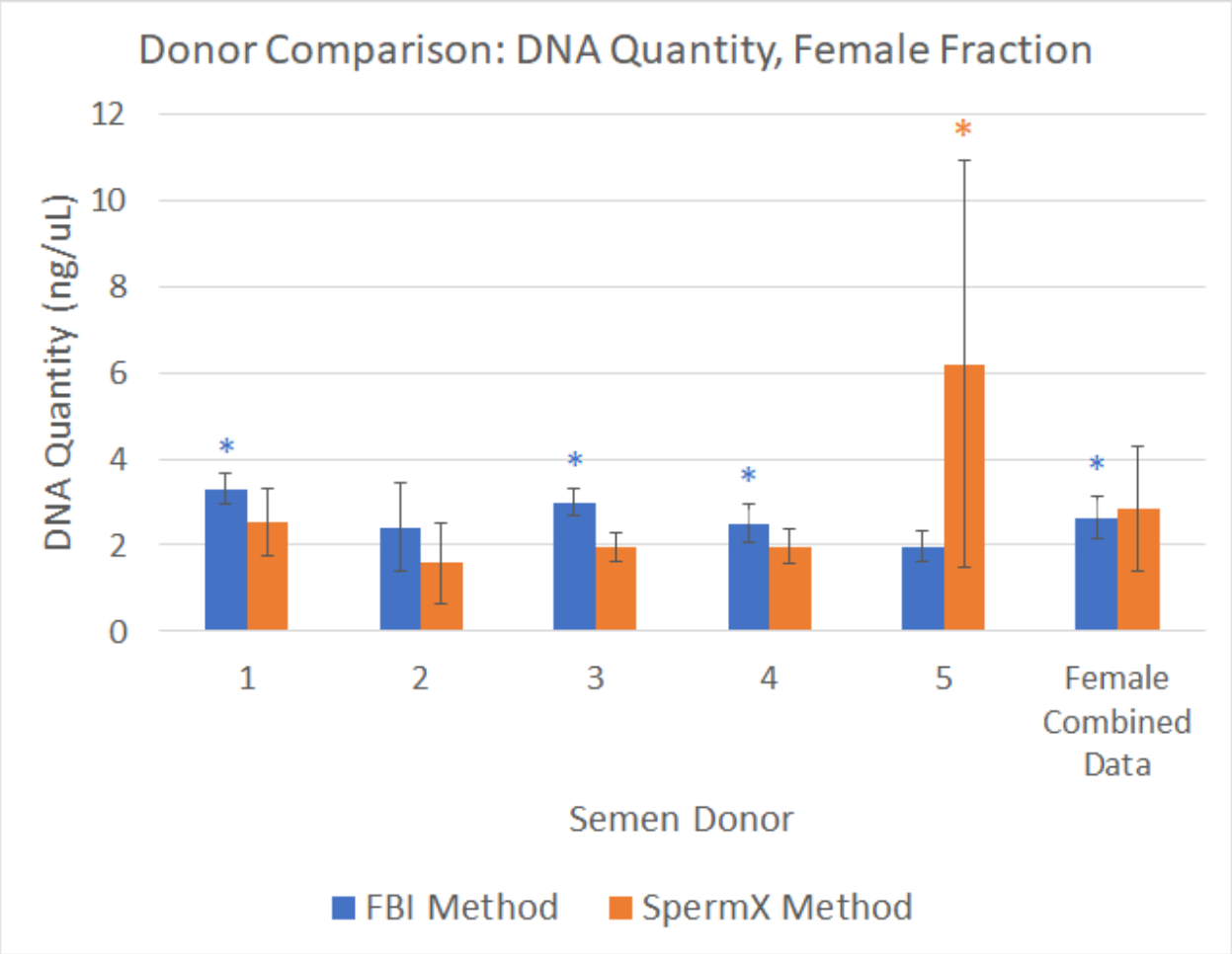


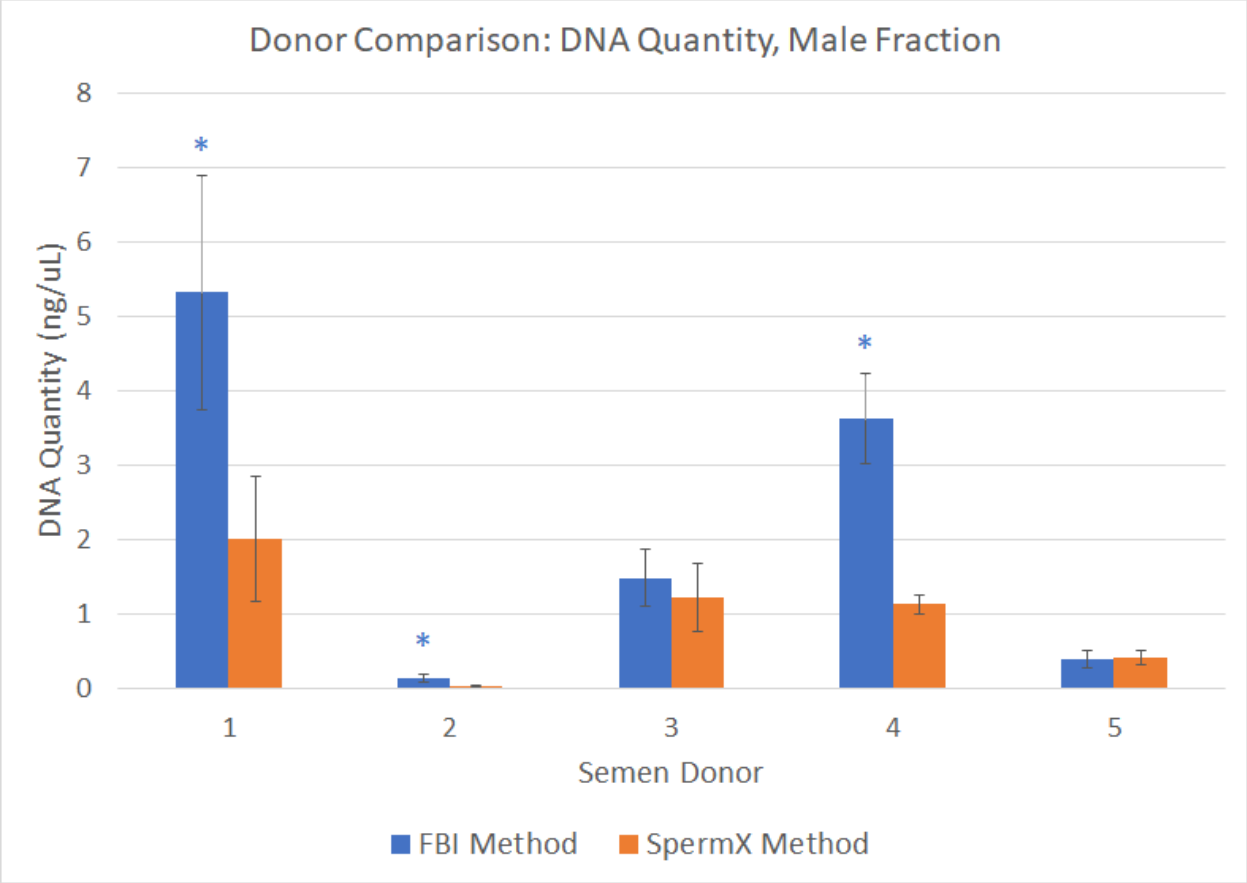
Figure 1: The SpermX Device. The components of the SpermX device are shown (A) with the cap, tube, basket, and nanofiber filter labelled. The basket and filter are outlighted and highlighted for clarity. The device is also shown in the lower (B) and upper (C) positions, with illustrated epithelial and sperm cells. While in the lower position, the elution pore is tight against the bottom of the tube, making the system liquid tight. The lower position is used during lysis steps, where all desired liquid is retained in the basket chamber. While in the upper position, the basket is raised, creating a cavity between the basket and the tube. The upper position is used during centrifugation, where liquid containing lysate may flow through the nanofiber filter out of the basket and into the tube. Any unlysed sperm heads will remain trapped in the nanofiber filter.

Figure 2: Donor Comparison: DNA Quantity, Female Fraction. The mean female fraction DNA quantities in nanograms per microliter (ng/uL) for each semen donor are represented by blue or orange bars when



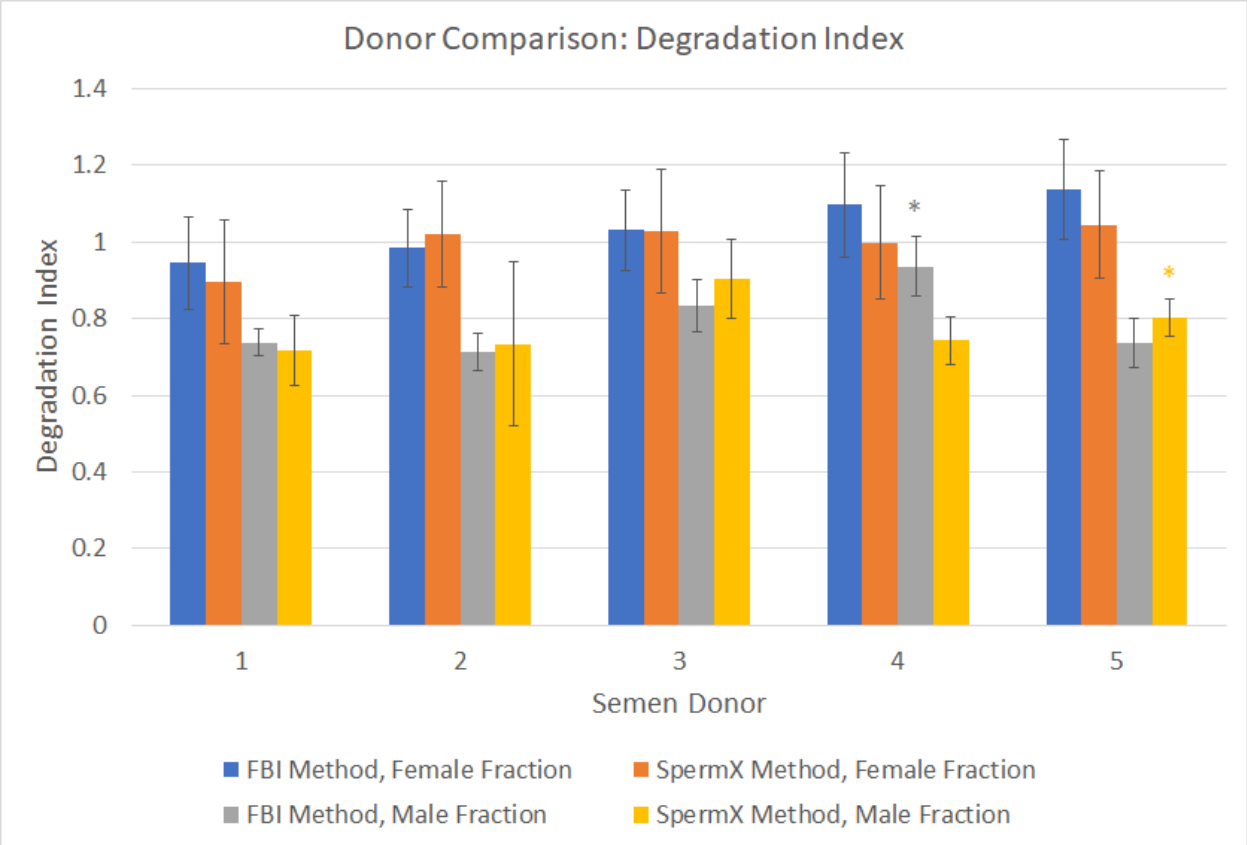
the FBI or SpermX methods were used, respectively. The error bars represent the standard deviation of the data, and an asterisk (*) above a bar for one extraction method indicates a statistical significance ($p<0.05$).

Figure 3: Donor Comparison: DNA Quantity, Male Fraction. The mean male fraction DNA quantities in



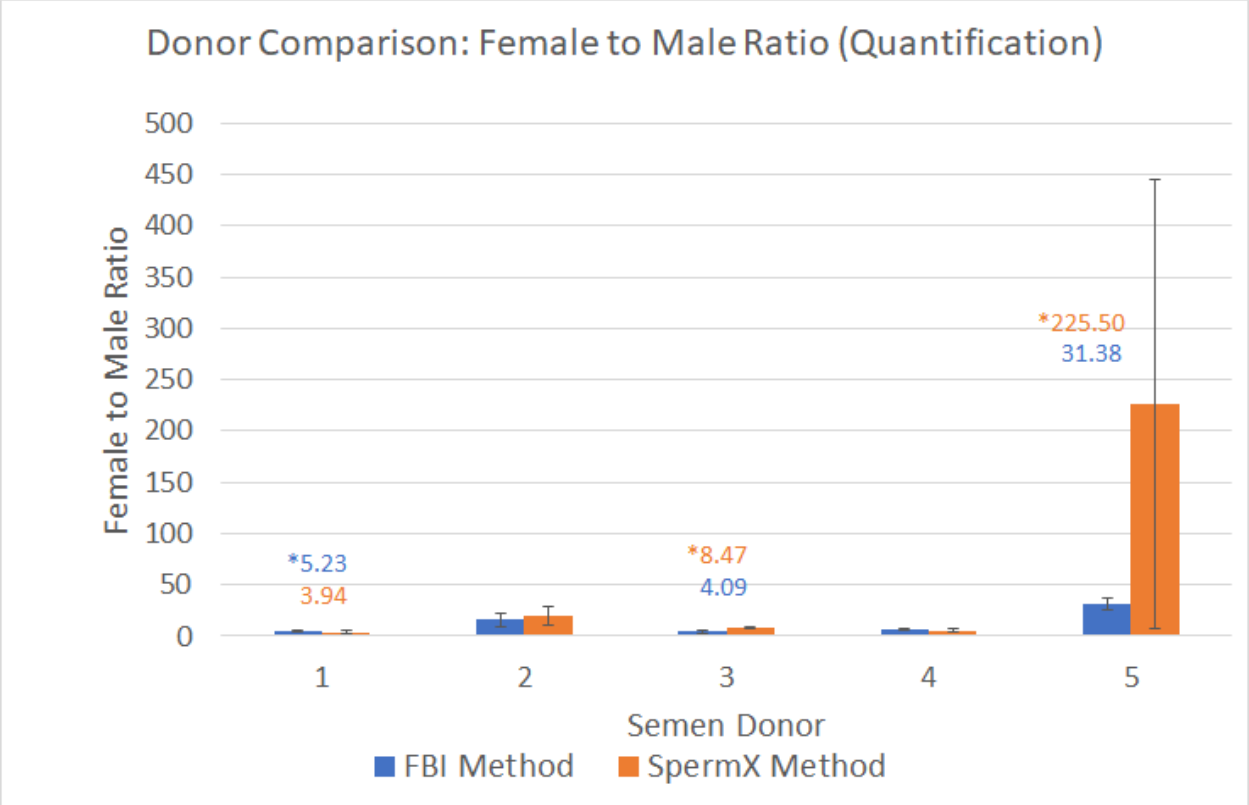
nanograms per microliter (ng/uL) for each semen donor are represented by blue or orange bars when the FBI or SpermX methods were used, respectively. The error bars represent the standard deviation of the data, and an asterisk (*) above a bar for one extraction method indicates a statistical significance ($p<0.05$).

Figure 4: Donor Comparison: Degradation Index. The mean degradation indices (DI) are represented by the colored bars. The blue and gray bars represent the female and male fractions of the FBI -extracted



samples, while the orange and yellow bars represent the female and male fractions of the SpermX-extracted samples. The error bars represent the standard deviation of the data, and an asterisk (*) above a bar for one extraction method indicates a statistical significance ($p<0.05$).

Figure 5: Donor Comparison, Female to Male Ratio (Quantification), Female Fraction. The mean female



to male (F:M) ratios determined by quantification for the female fraction are represented by blue bars for the FBI method and orange bars for the SpermX method. The error bars represent the standard deviation of the data, and an asterisk (*) indicates a statistical significance ($p<0.05$).

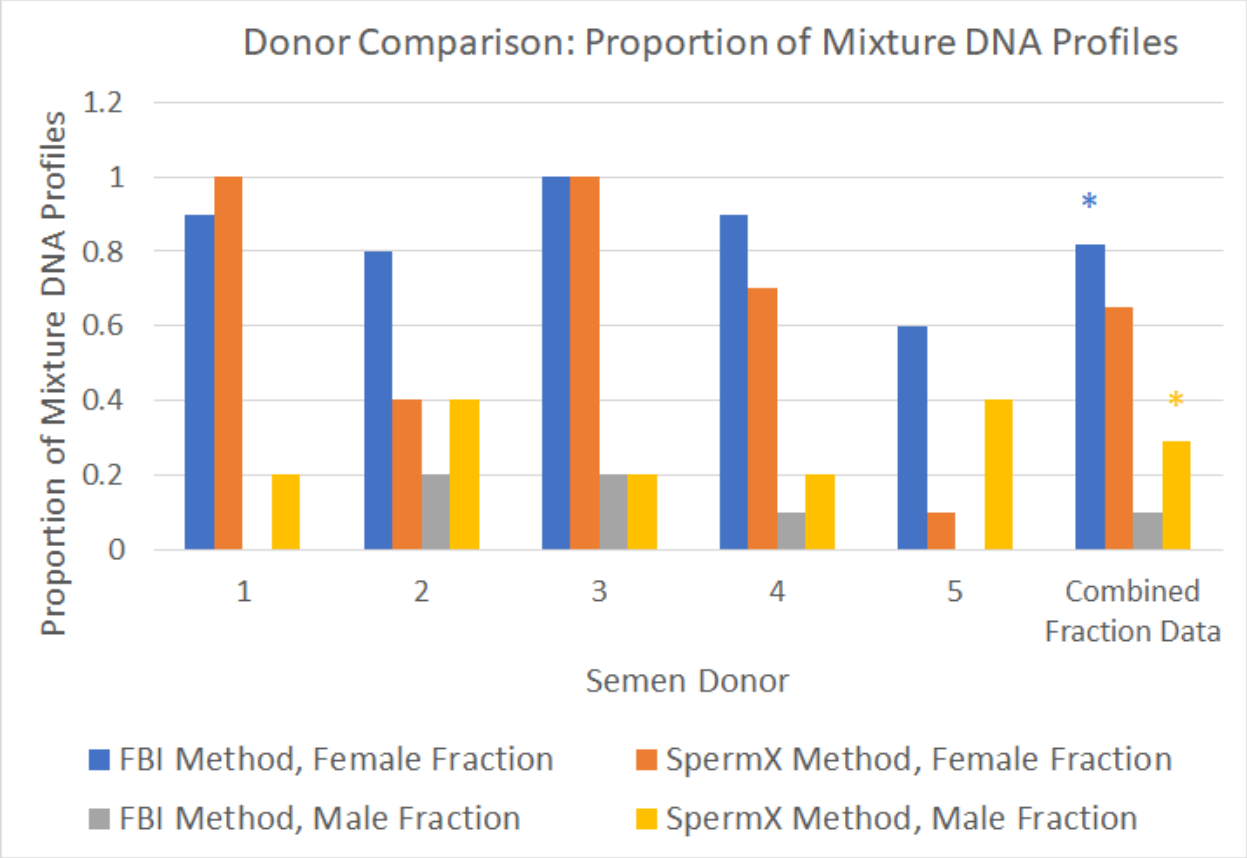


Figure 6: Donor Comparison: Proportion of Mixture DNA Profiles. The proportion of mixture DNA profiles are represented by blue or gray bars for the FBI method female or male fractions, or orange or yellow bars for the SpermX female or male fractions. Comparisons were made between the FBI and SpermX female fractions, and between the FBI and SpermX male fractions. The absence of a bar indicates only single-source profiles were observed. An asterisk (*) indicates a statistical significance ($p<0.05$).

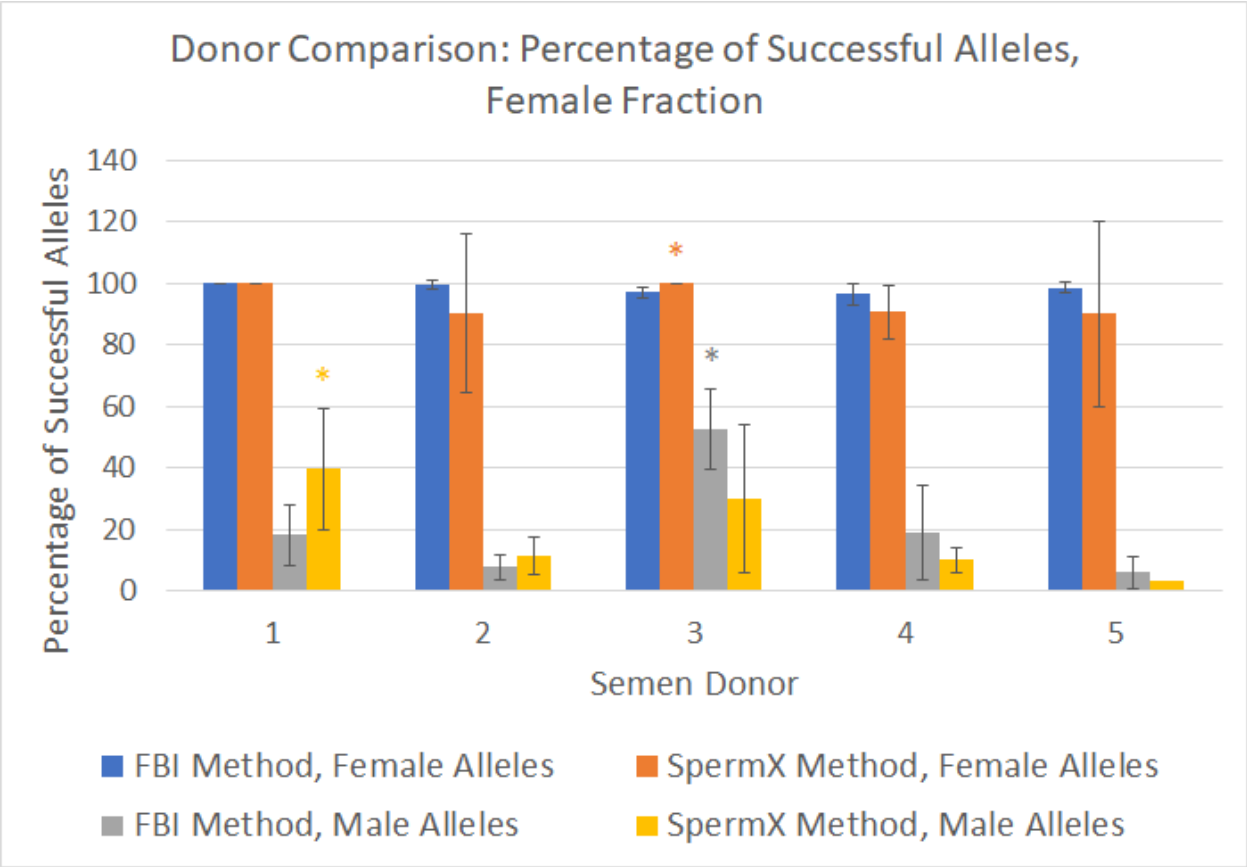


Figure 7: Donor Comparison: Percentage of Successful Alleles, Female Fraction. The mean percentage of alleles in the female fractions are represented by blue or gray bars for the FBI method female or male alleles, or orange or yellow bars for the SpermX female or male alleles. The error bars represent the standard deviation of the data, and an asterisk (*) above a bar for one extraction method indicates a statistical significance ($p<0.05$). The male allele percentages only represent the observed mixture profiles.

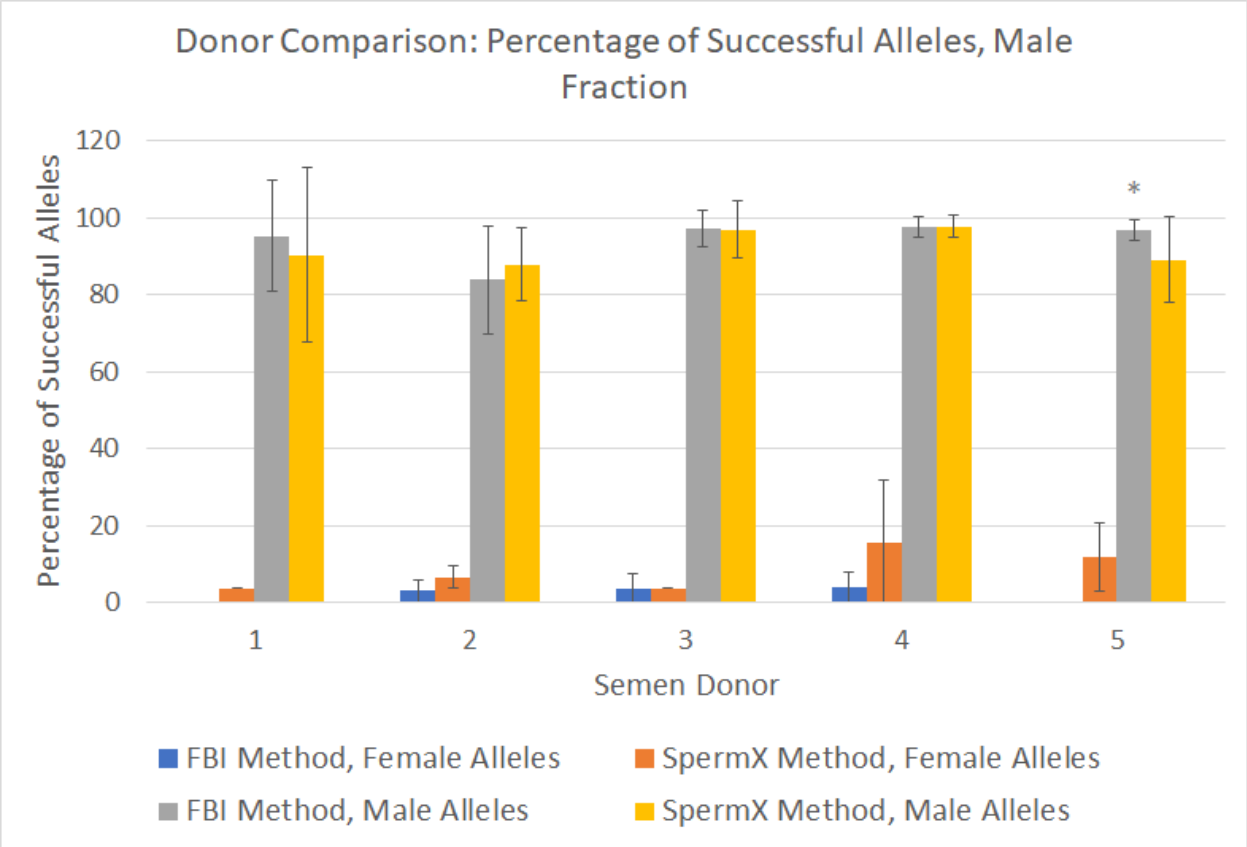


Figure 8: Donor Comparison: Percentage of Successful Alleles, Male Fraction. The mean percentage of alleles in the male fractions are represented by blue or gray bars for the FBI method female or male alleles, or orange or yellow bars for the SpermX female or male alleles. The error bars represent the standard deviation of the data, and an asterisk (*) above a bar for one extraction method indicates a statistical significance ($p<0.05$).

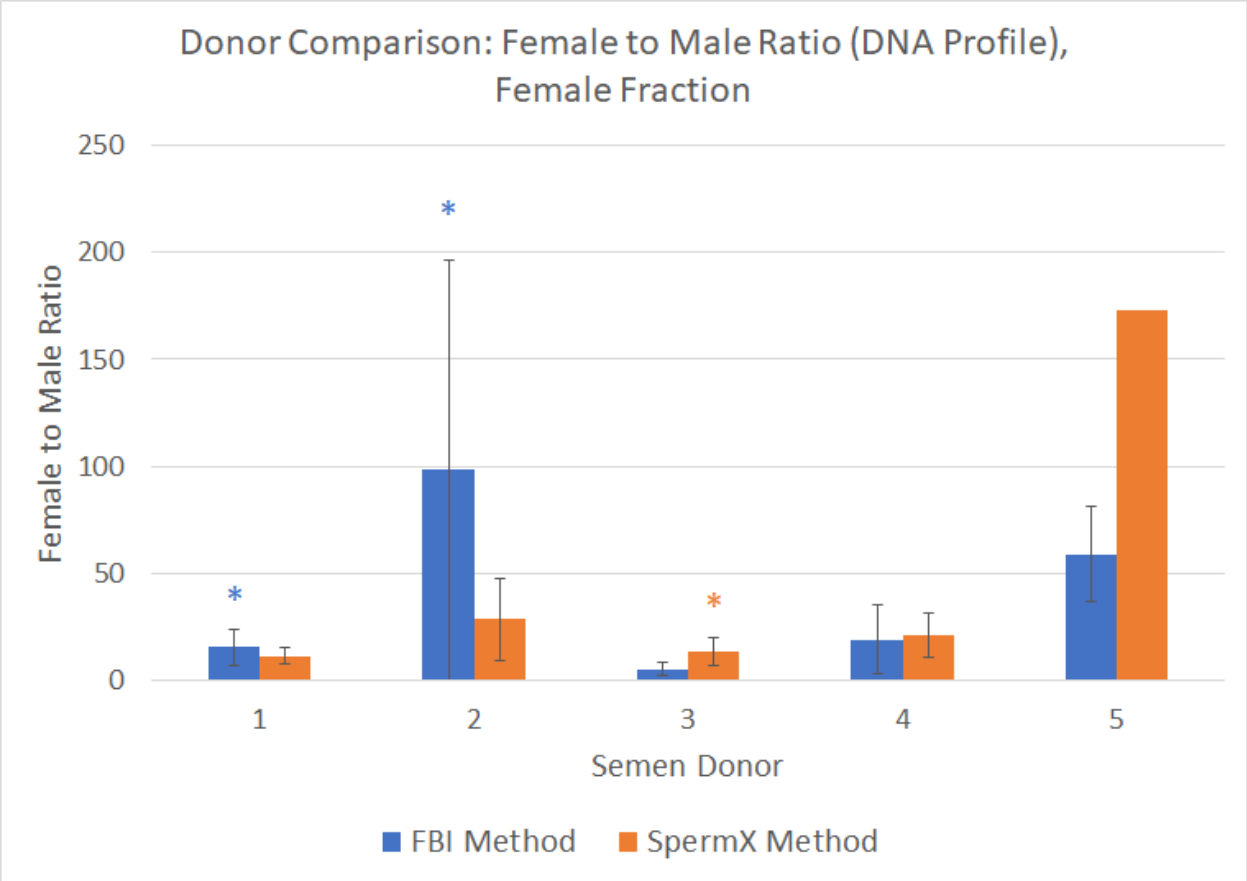


Figure 9: Donor Comparison: Female to Male Ratio (DNA Profile), Female Fraction. The mean DNA profile female to male (F:M) ratios of the female fractions are represented by blue bar for the FBI method, or an orange bar for the SpermX method. The error bars represent the standard deviation of the data, and an asterisk (*) above a bar for one extraction method indicates a statistical significance ($p<0.05$).

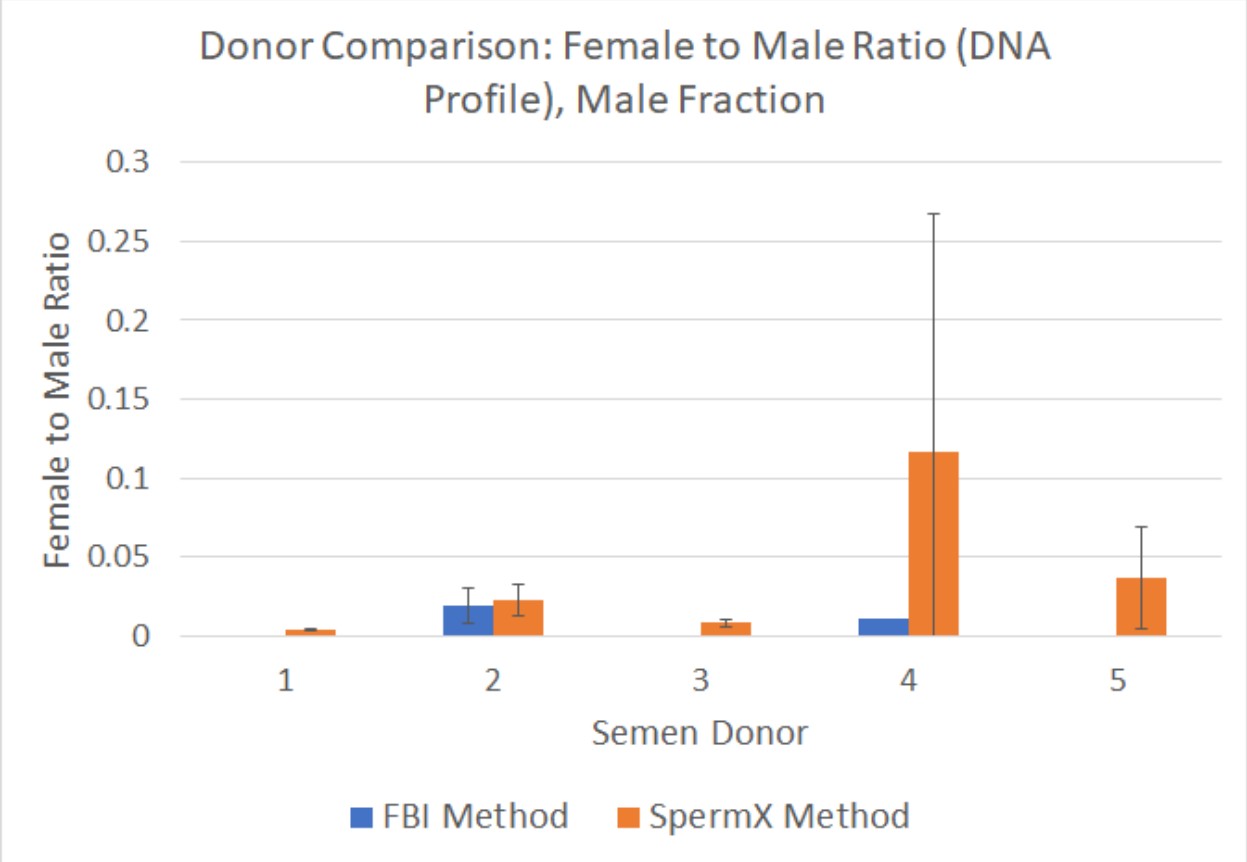


Figure 10: Donor Comparison: Female to Male Ratio (DNA Profile), Male Fraction. The mean DNA profile female to male (F:M) ratios from the male fractions are represented by a blue bar for the FBI method, or an orange bar for the SpermX method. The error bars represent the standard deviation of the data, and an asterisk (*) above a bar for one extraction method indicates a statistical significance ($p<0.05$).

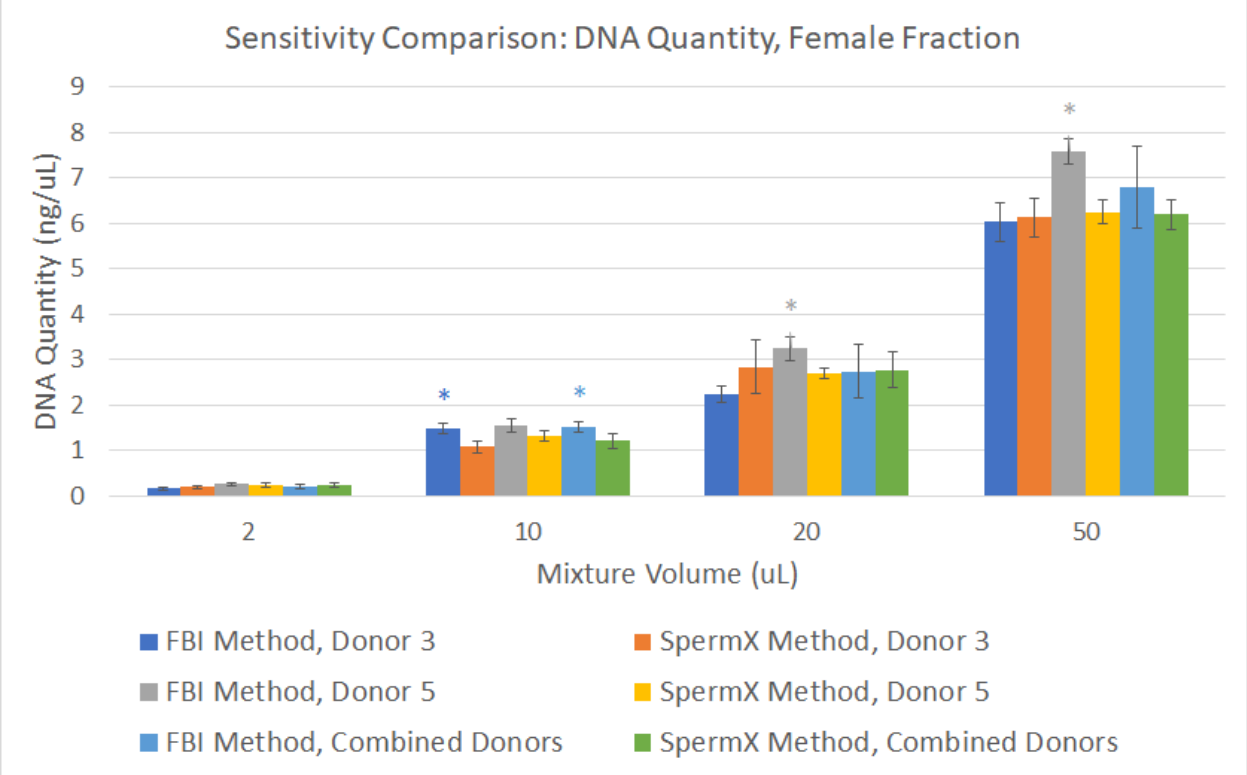


Figure 11: Sensitivity Comparison: DNA Quantity, Female Fraction. The mean female fraction DNA quantities in nanograms per microliter (ng/uL) for mixture volume in microliters (uL) are represented by the colored bars. The FBI method is represented by dark blue, gray, and light blue, further representing the respective donor 3, donor 5, and combined donor data. The SpermX method is represented by orange, yellow, and green bars, further representing respective donor 3, donor 5, and combined donor data. The error bars represent the standard deviation of the data, and an asterisk (*) above a bar for one extraction method indicates a statistical significance ($p<0.05$).

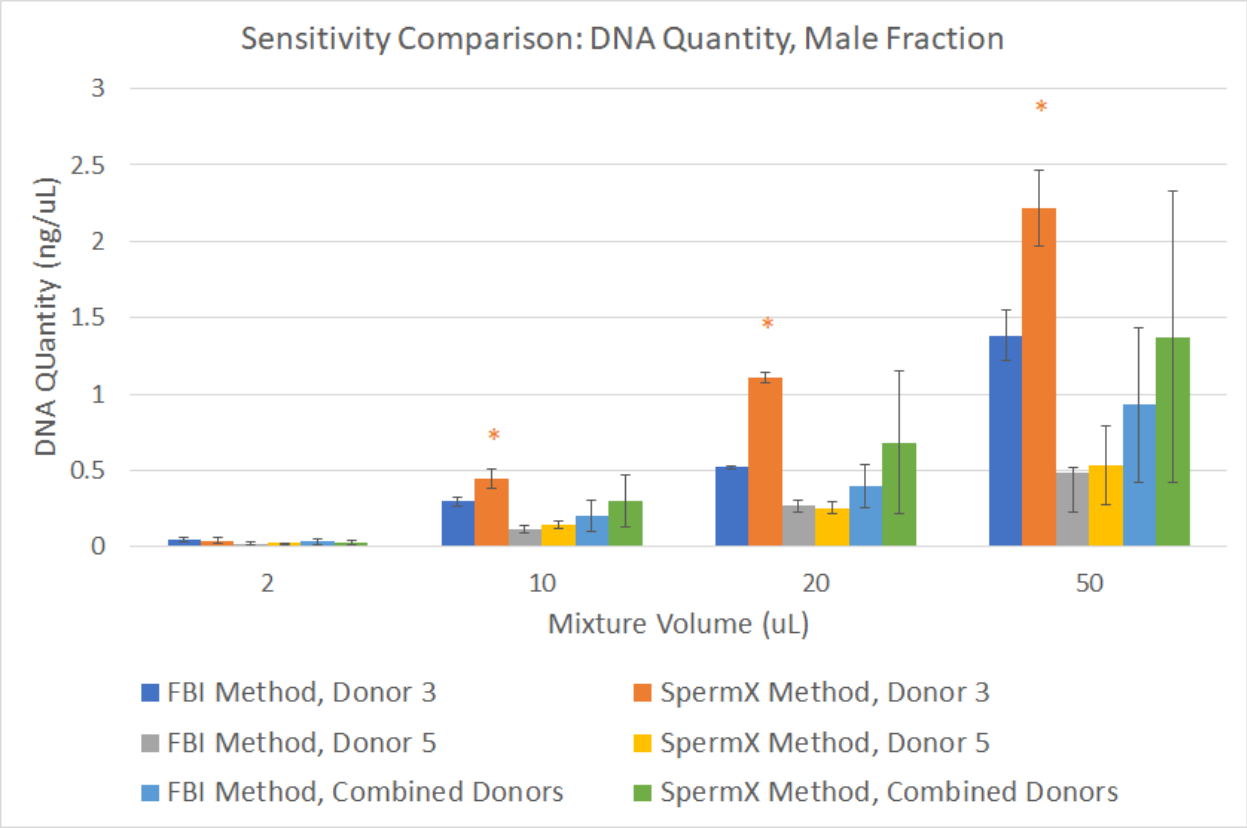


Figure 12: Sensitivity Comparison: DNA Quantity, Male Fraction. The mean male fraction DNA quantities in nanograms per microliter (ng/uL) for mixture volume in microliters (uL) are represented by the colored bars. The FBI method is represented by dark blue, gray, and light blue, further representing the respective donor 3, donor 5, and combined donor data. The SpermX method is represented by orange, yellow, and green bars, further representing respective donor 3, donor 5, and combined donor data. The error bars represent the standard deviation of the data, and an asterisk (*) above a bar for one extraction method indicates a statistical significance ($p<0.05$).

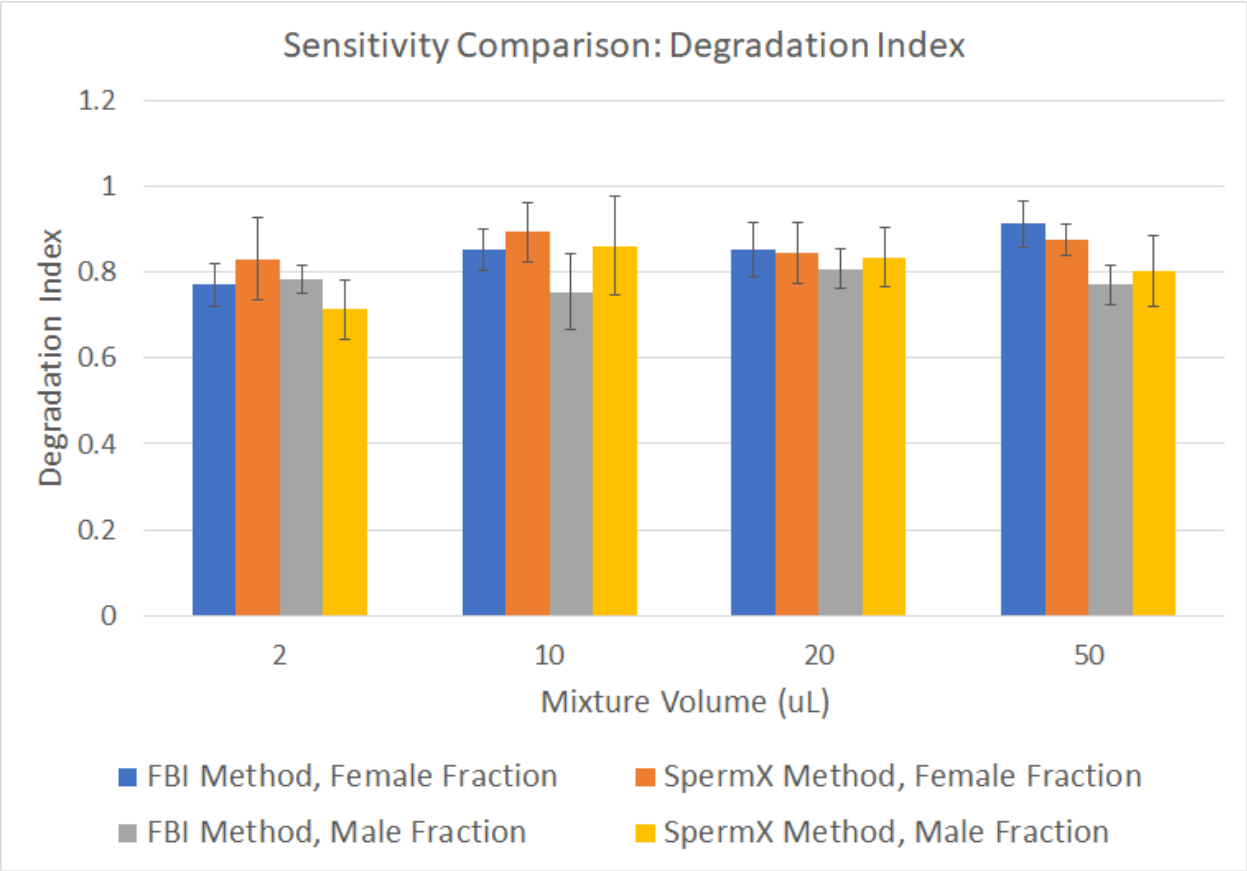


Figure 13: Sensitivity Comparison: Degradation Index. The mean degradation indices (DI) are represented by the colored bars. The blue and gray bars represent the female and male fractions of the FBI-extracted samples, while the orange and yellow bars represent the female and male fractions of the SpermX-extracted samples. The error bars represent the standard deviation of the data, and an asterisk (*) above a bar for one extraction method indicates a statistical significance ($p<0.05$).

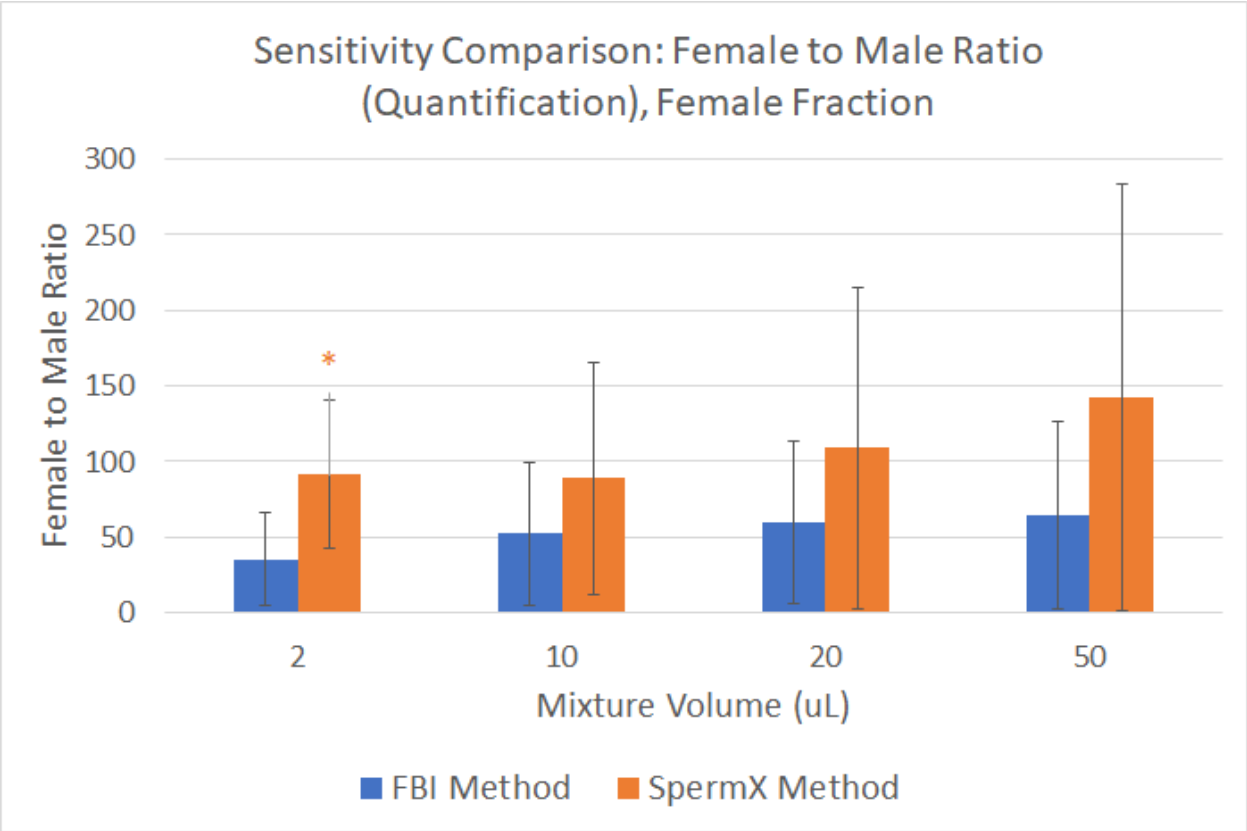


Figure 14: Sensitivity Comparison, Female to Male Ratio (Quantification), Female Fraction. The mean female to male (F:M) ratios determined by quantification for the female fraction are represented by blue bars for the FBI method and orange bars for the SpermX method. The error bars represent the standard deviation of the data, and an asterisk (*) above a bar for one extraction method indicates a statistical significance ($p<0.05$).

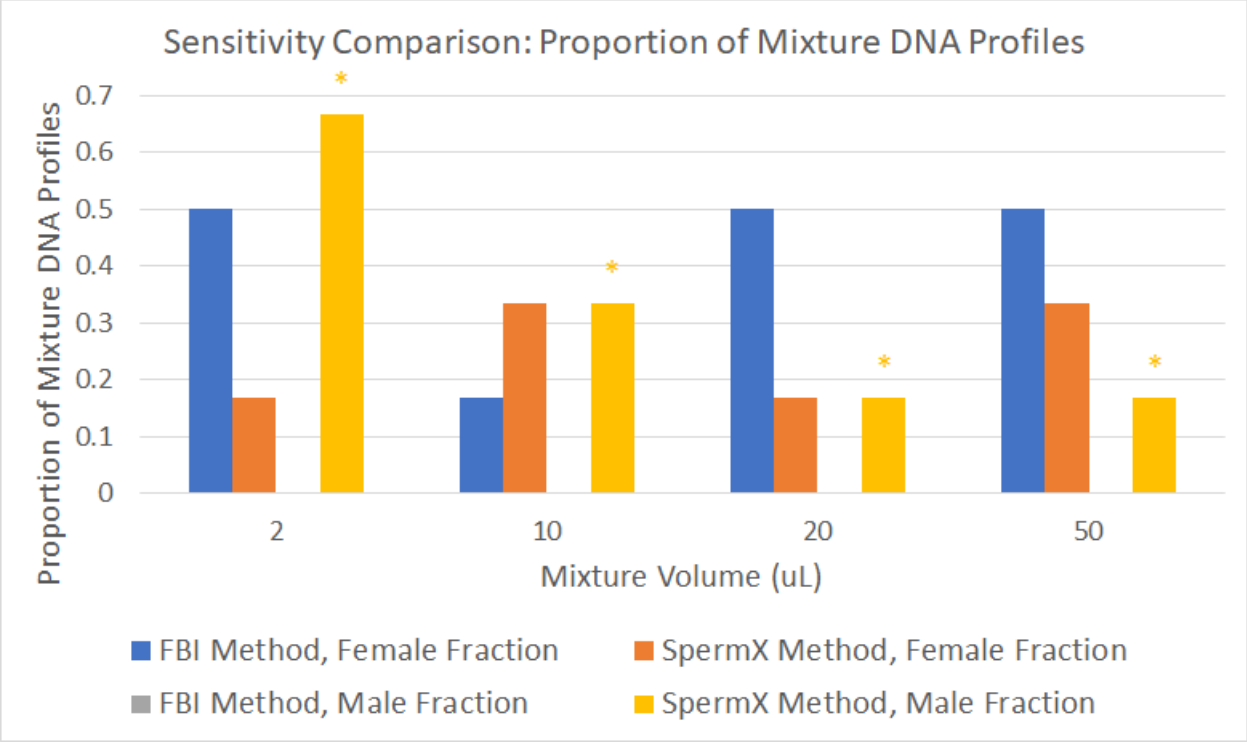


Figure 15: Sensitivity Comparison: Proportion of Mixture DNA Profiles. The proportion of mixture DNA profiles are represented by blue or gray bars for the FBI method female or male fractions, and orange or yellow bars for the SpermX female or male fractions. Statistical significance is indicated with an asterisk (*).

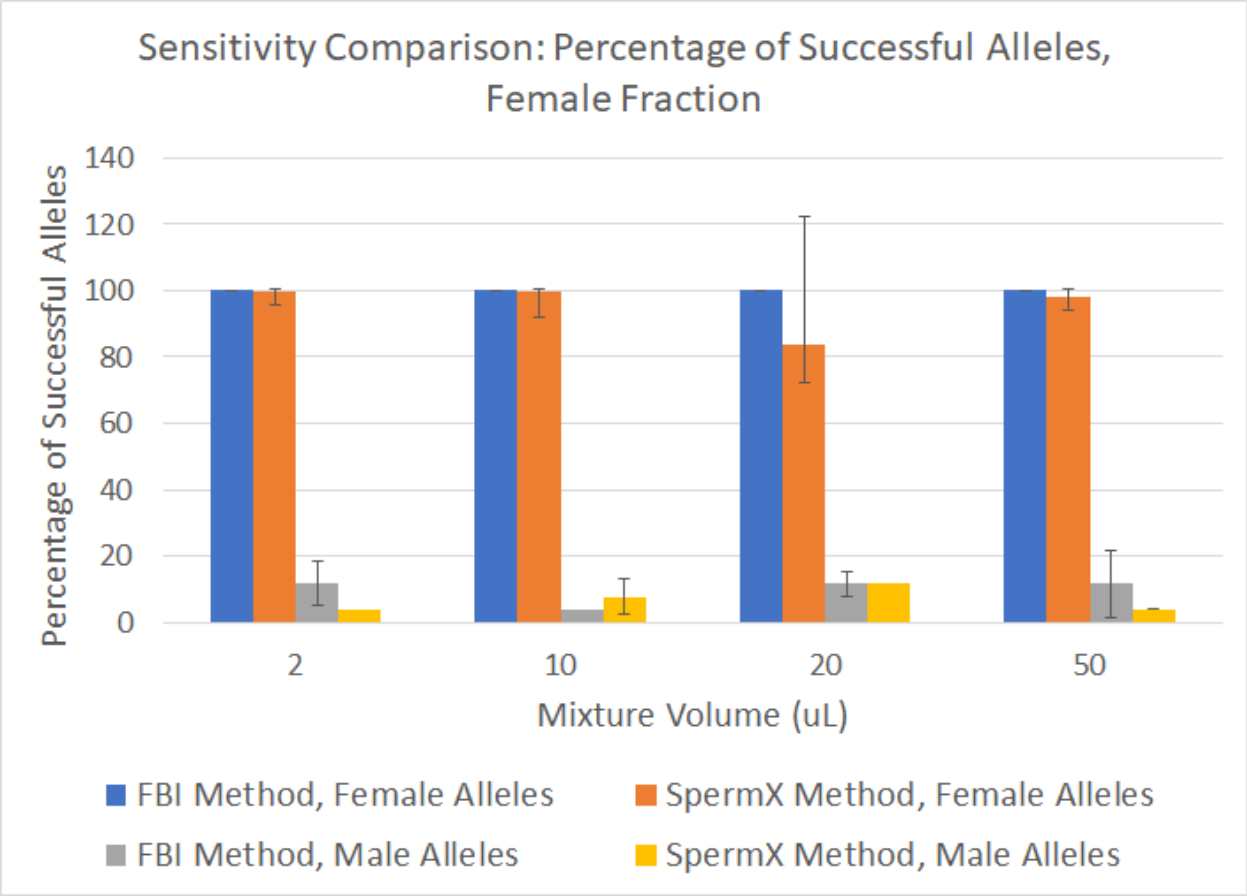


Figure 16: Sensitivity Comparison: Percentage of Successful Alleles, Female Fraction. The mean percentage of alleles in the female alleles are represented by blue or gray bars for the FBI method female or male alleles, and orange or yellow bars for the SpermX female or male fractions. The error bars represent the standard deviation of the data, and an asterisk (*) above a bar for one extraction method indicates a statistical significance ($p<0.05$).

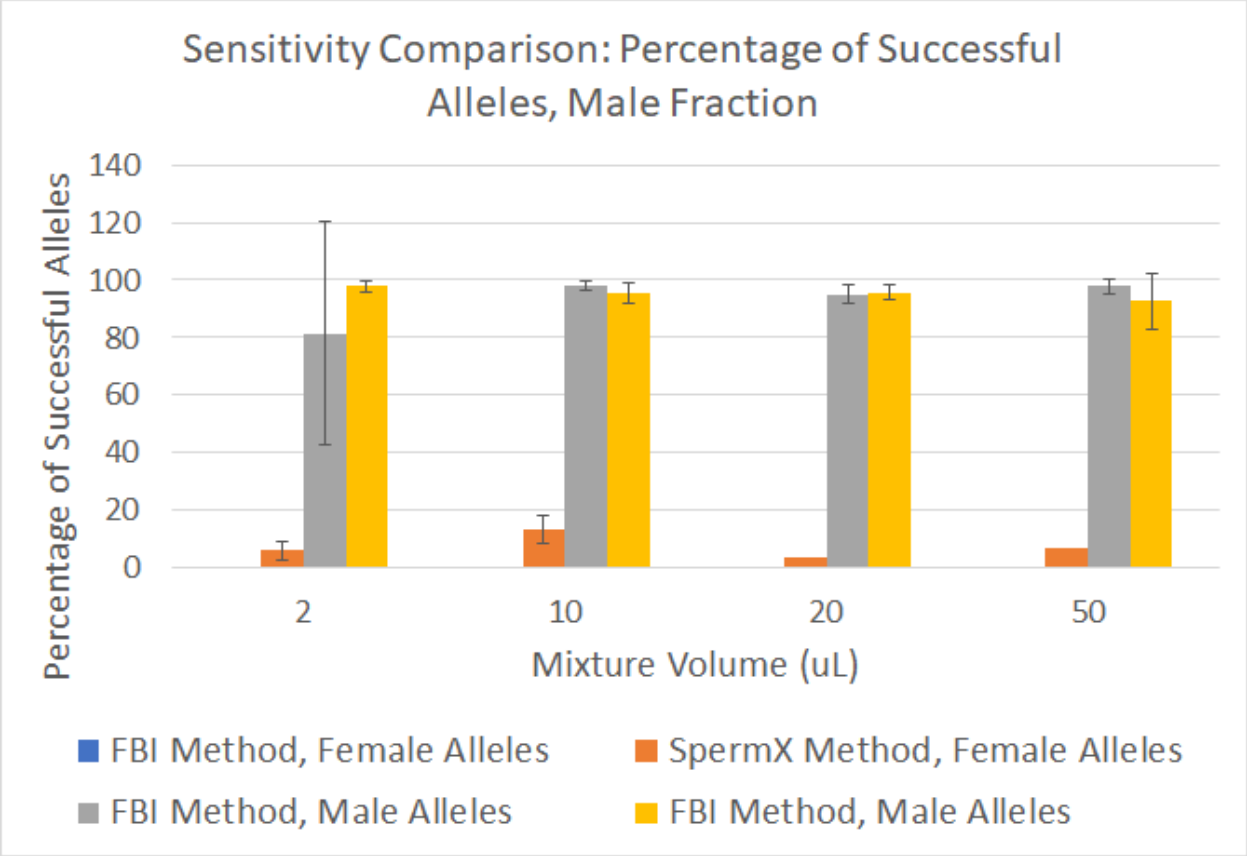


Figure 17: Sensitivity Comparison: Percentage of Successful Alleles, Male Fraction. The mean percentage of alleles in the male alleles are represented by blue or gray bars for the FBI method female or male alleles, and orange or yellow bars for the SpermX female or male fractions. The error bars represent the standard deviation of the data, and an asterisk (*) above a bar for one extraction method indicates a statistical significance ($p<0.05$).

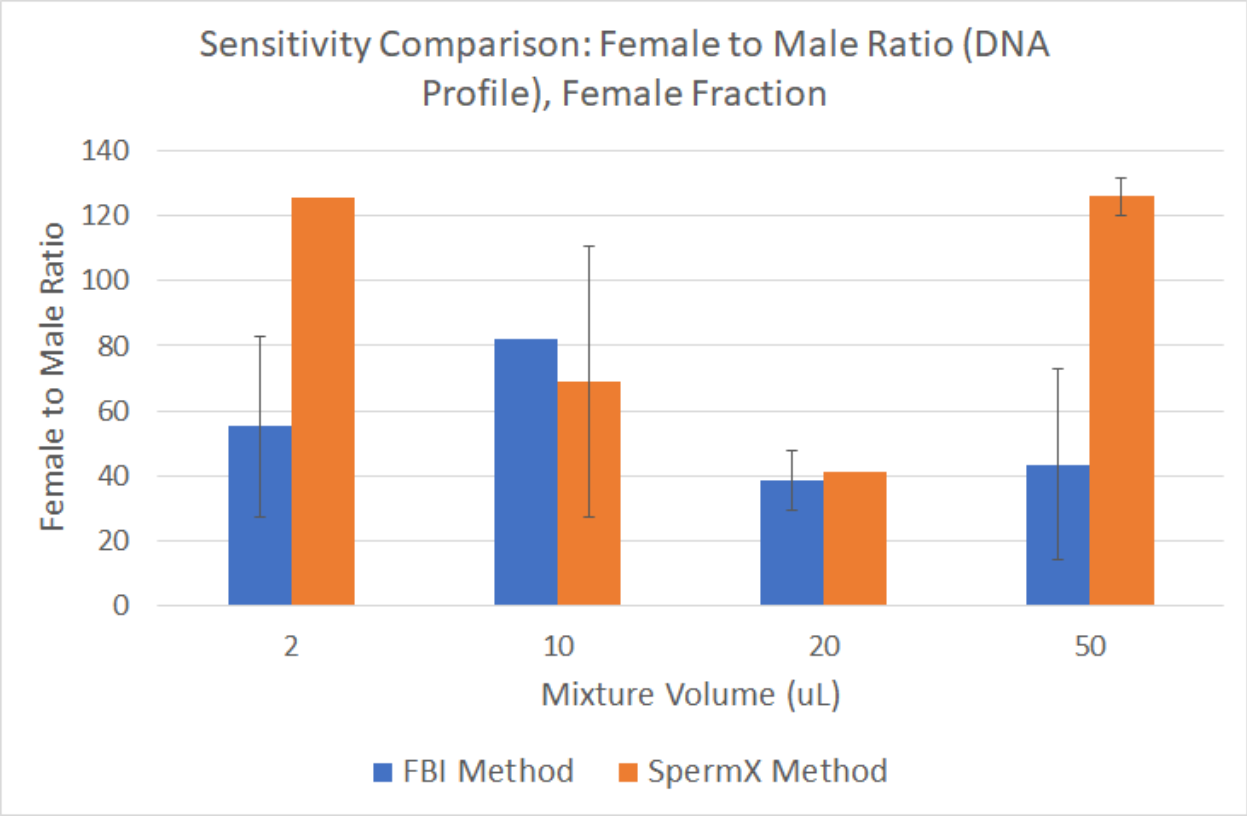


Figure 18: Sensitivity Comparison: Female to Male Ratio (DNA Profile), Female Fraction. The mean DNA profile female to male (F:M) ratios from the female fractions are represented by a blue bar for the FBI method, or an orange bar for the SpermX method. The error bars represent the standard deviation of the data, and an asterisk (*) above a bar for one extraction method indicates a statistical significance ($p<0.05$).

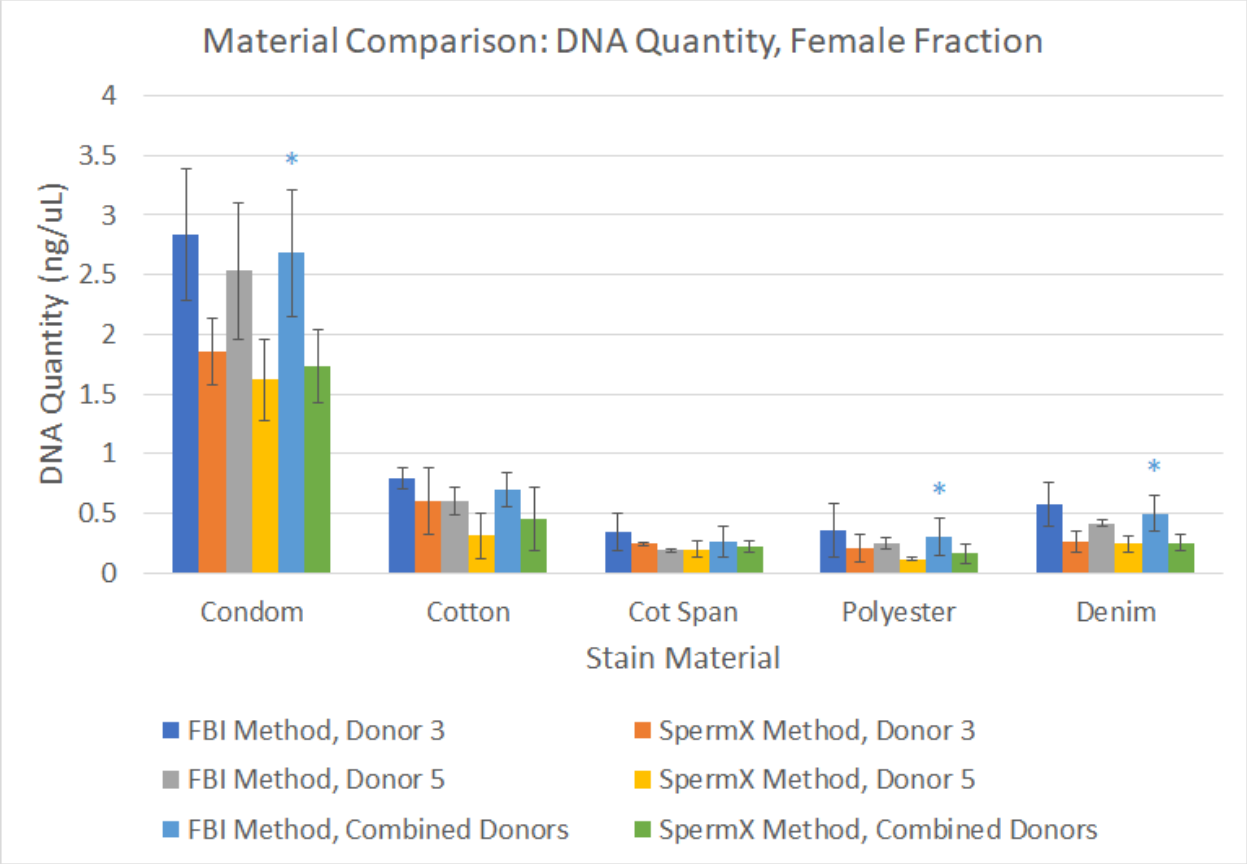


Figure 19: Material Comparison: DNA Quantity, Female Fraction. The mean female fraction DNA quantities in nanograms per microliter (ng/uL) for each stain material are represented by the colored bars. The FBI method is represented by dark blue, gray, and light blue, further representing the respective donor 3, donor 5, and combined donor data. In the figure, the stain material Cot Span is an abbreviation for the 95% cotton/ 5% spandex blend. The SpermX method is represented by orange, yellow, and green bars, further representing respective donor 3, donor 5, and combined donor data. The error bars represent the standard deviation of the data, and an asterisk (*) above a bar for one extraction method indicates a statistical significance ($p<0.05$).

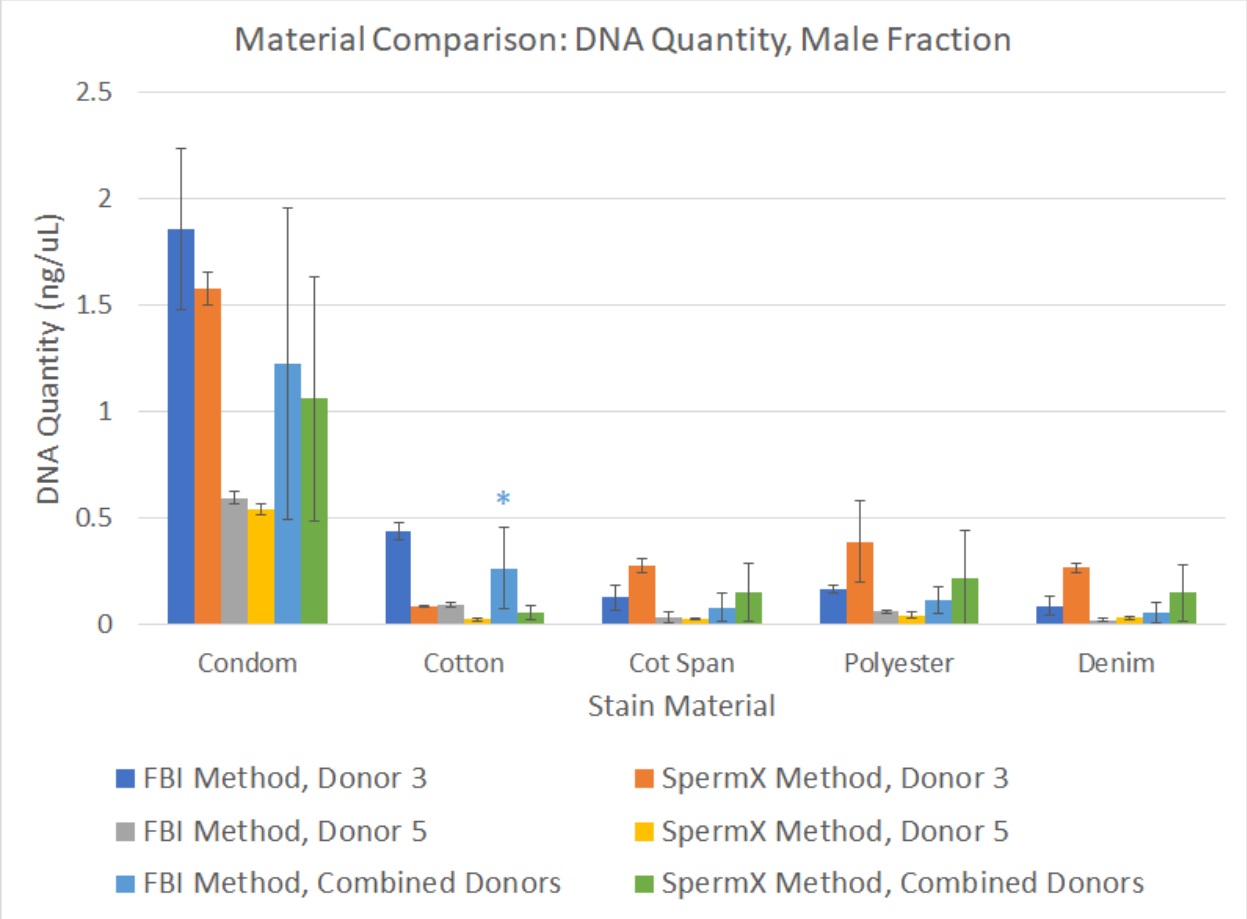


Figure 20: Material Comparison: DNA Quantity, Male Fraction. The mean male fraction DNA quantities in nanograms per microliter (ng/uL) for each stain material are represented by the colored bars. The FBI method is represented by dark blue, gray, and light blue, further representing the respective donor 3, donor 5, and combined donor data. In the figure, the stain material Cot Span is an abbreviation for the 95% cotton/ 5% spandex blend. The SpermX method is represented by orange, yellow, and green bars, further representing respective donor 3, donor 5, and combined donor data. The error bars represent the standard deviation of the data, and an asterisk (*) above a bar for one extraction method indicates a statistical significance ($p<0.05$).

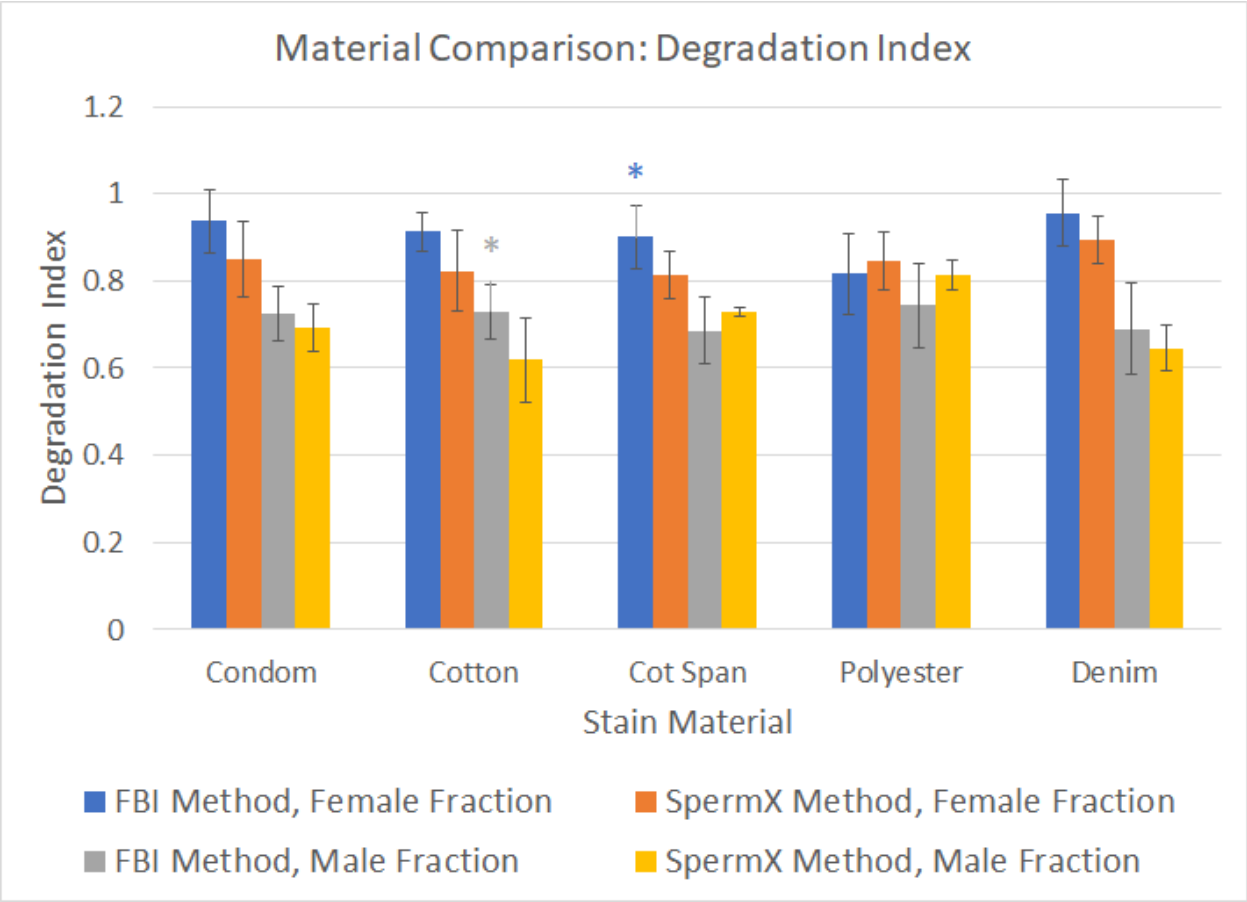


Figure 21: Material Comparison: Degradation Index. The mean degradation indices (DI, no unit) are represented by the colored bars. The blue and gray bars represent the female and male fractions of the FBI method-extracted samples, while the orange and yellow bars represent the female and male fractions of the SpermX-extracted samples. In the figure, the stain material Cot Span is an abbreviation for the 95% cotton/ 5% spandex blend. The error bars represent the standard deviation of the data, and an asterisk (*) above a bar for one extraction method indicates a statistical significance ($p<0.05$).

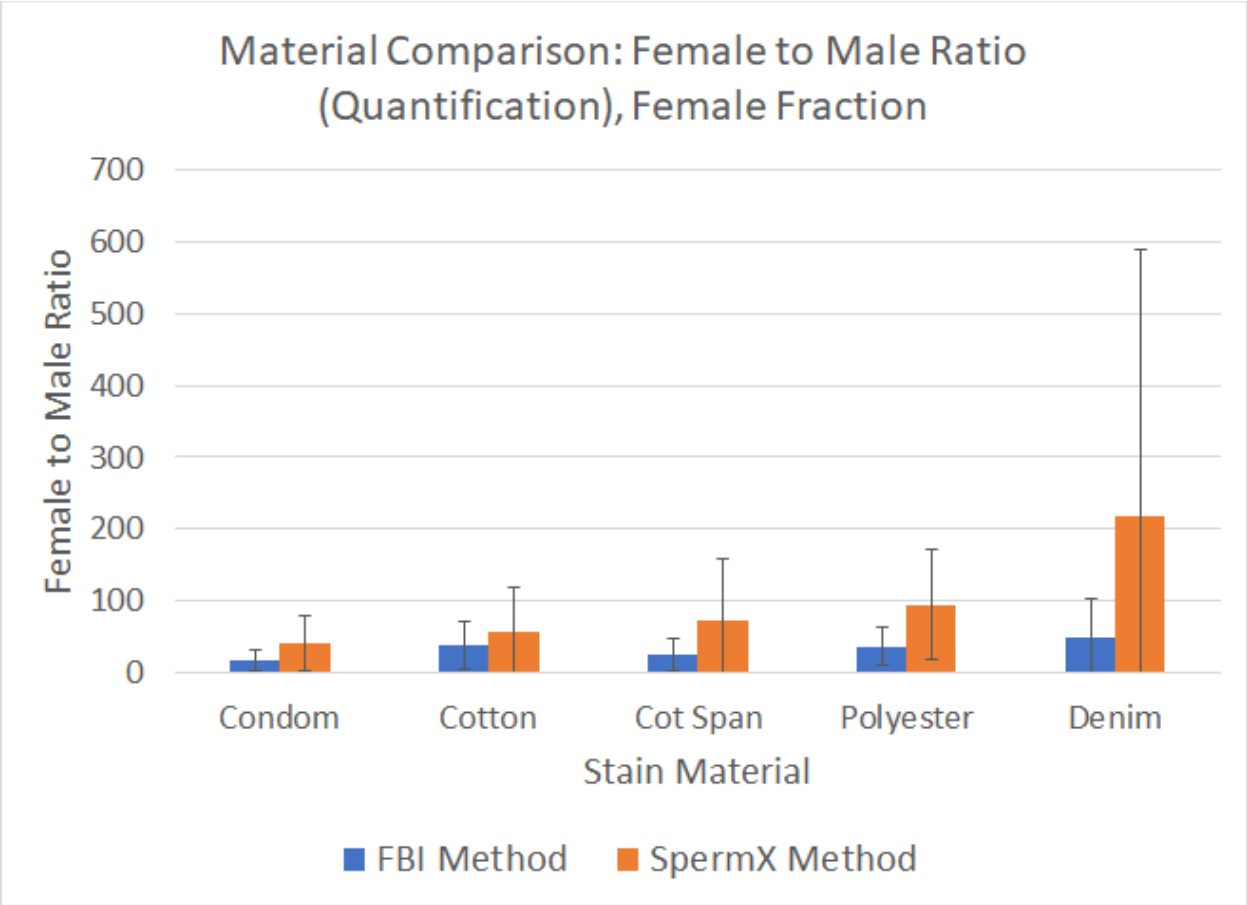


Figure 22: Materials Comparison, Female to Male Ratio (Quantification), Female Fraction. The mean female to male (F:M) ratios determined by quantification for the female fraction are represented by blue bars for the FBI method and orange bars for the SpermX method. In the figure, the stain material Cot Span is an abbreviation for the 95% cotton/ 5% spandex blend. The error bars represent the standard deviation of the data, and an asterisk (*) above a bar for one extraction method indicates a statistical significance ($p<0.05$).

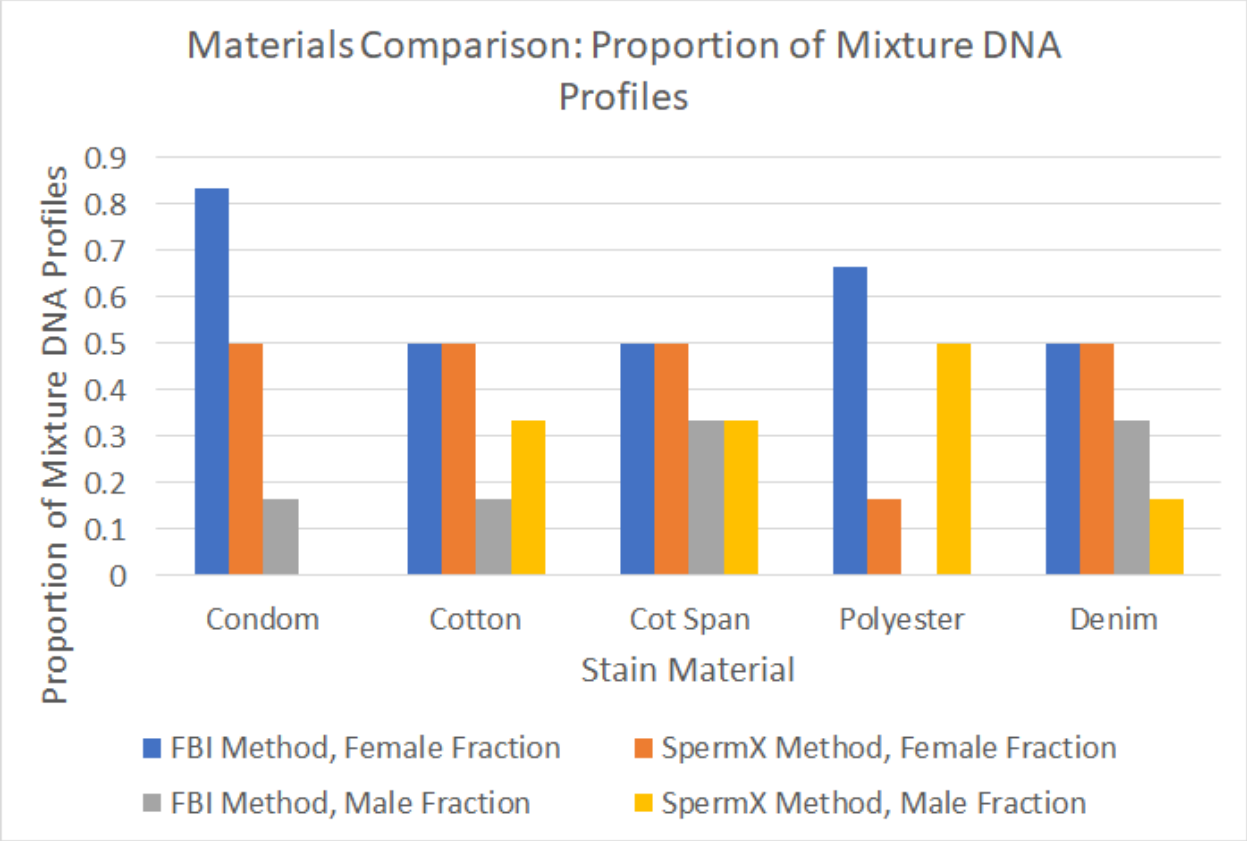


Figure 23: Material Comparison: Proportion of Mixture DNA Profiles. The proportion of mixture DNA profiles are represented by blue or gray bars for the FBI method female or male fractions, or orange or yellow bars for the SpermX female or male fractions. In the figure, the stain material Cot Span is an abbreviation for the 95% cotton/ 5% spandex blend. The absence of a bar indicates only single-source profiles were observed.

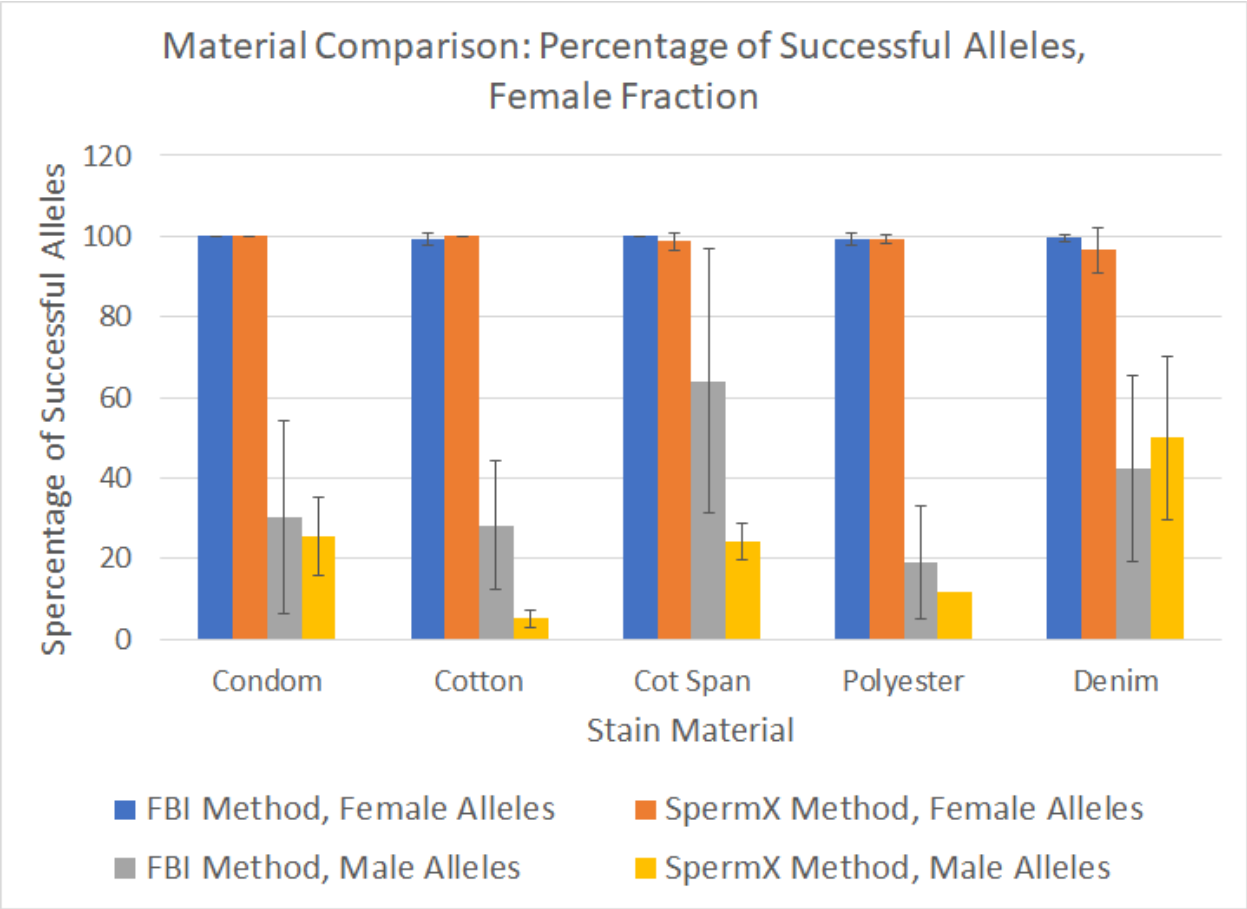


Figure 24: Material Comparison: Percentage of Successful Alleles, Female Fraction. The mean percentage of alleles in the female fractions are represented by blue or gray bars for the FBI method female or male alleles, or orange or yellow bars for the SpermX female or male alleles. In the figure, the stain material Cot Span is an abbreviation for the 95% cotton/ 5% spandex blend. The error bars represent the standard deviation of the data, and an asterisk (*) above a bar for one extraction method indicates a statistical significance ($p<0.05$).

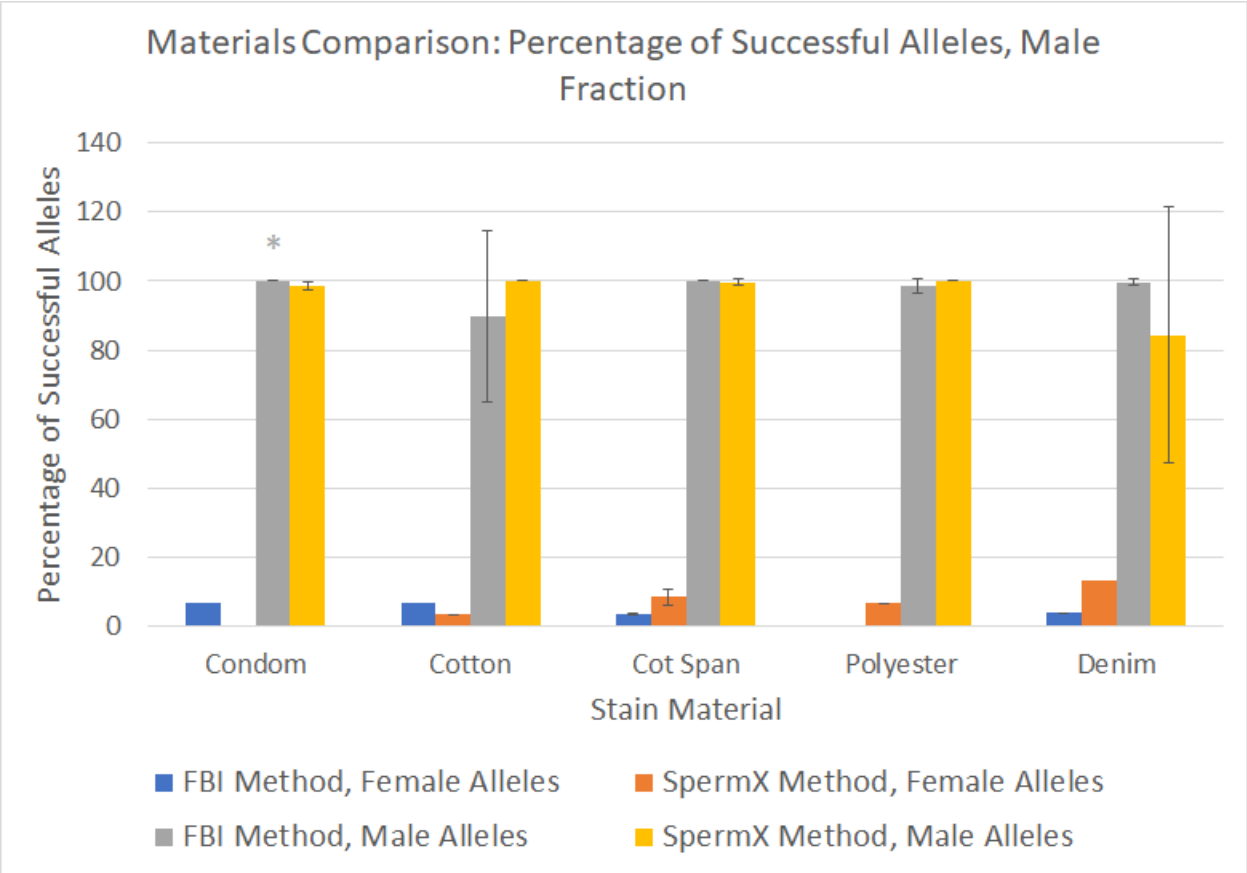


Figure 25: Material Comparison: Percentage of Successful Alleles, Male Fraction. The mean percentage of alleles in the male fractions are represented by blue or gray bars for the FBI method female or male alleles, or orange or yellow bars for the SpermX female or male alleles. In the figure, the stain material Cot Span is an abbreviation for the 95% cotton/ 5% spandex blend. The error bars represent the standard deviation of the data, and an asterisk (*) above a bar for one extraction method indicates a statistical significance ($p<0.05$).

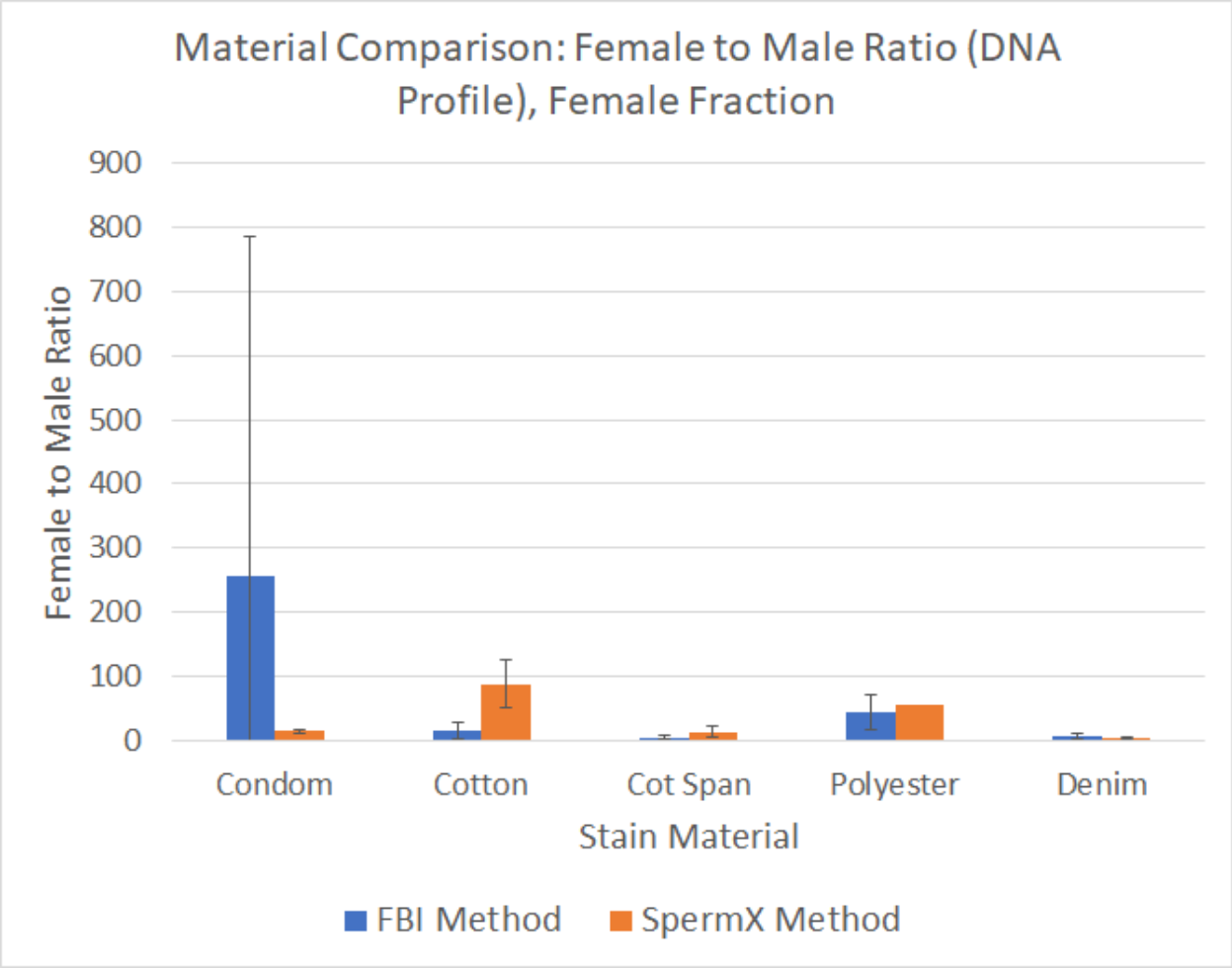


Figure 26: Material Comparison: DNA Profile Female to Male Ratio, Female Fraction. The mean DNA profile female to male (F:M) ratios from the female fractions are represented by blue bar for the FBI method, or orange bars for the SpermX method. In the figure, the stain material Cot Span is an abbreviation for the 95% cotton/ 5% spandex blend. The error bars represent the standard deviation of the data, and an asterisk (*) above a bar for one extraction method indicate a statistical significance ($p<0.05$).