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Quantitation of sperm distribution into the fractions during a temperature controlled differential extraction procedure

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Thesis

**QUANTITATION OF SPERM DISTRIBUTION INTO THE FRACTIONS
DURING A TEMPERATURE CONTROLLED DIFFERENTIAL EXTRACTION
PROCEDURE**

by

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ABSTRACT

The typical differential extraction procedure utilized by the forensic science community to extract male deoxyribonucleic acid (DNA) from the sperm cells of the perpetrator separately from female DNA from the epithelial cells of the victim is both time-consuming and labor-intensive. This has contributed greatly to the backlog of unanalyzed sexual assault evidence collection kits (SAECK) seen in many laboratories today and has encouraged research in new methods that are more efficient and more effective in achieving better sperm DNA recovery.

The Cotton Lab has developed a Temperature-Controlled Differential Extraction (TCDE) procedure geared towards attaining better sperm recovery and better distribution of male DNA in the sperm fraction (SF) to generate a single source or distinguishable male profile. The TCDE protocol is a direct-lysis procedure that utilizes highly temperature-controlled enzymes, or enzymes that are active at or near their optimal temperatures. This procedure has been previously shown to decrease extraction time significantly and to extract samples that are suitable for downstream analysis.

This research specifically attempted to modify the TCDE procedure in the hopes of obtaining higher sperm DNA recovery and eliminating previous concerns of too much

sperm being retained by the cotton swab material. It also compared a slightly modified TCDE procedure where the material fraction (MF) and SF are kept as separate fractions (the Separate Method) and a method that results in a recombined MF and SF (Recombined Method) to see if there was a greater distribution of the total male DNA eluted into the SF. Preliminary experimentation with swabs prepared with semen was performed to help make effective modifications. Then, vaginal swabs from eight different female donors were prepared with semen to mimic forensic casework samples and extracted using the Separate and Recombined Methods for comparison of the two extraction methods.

Despite unusual epithelial cell lysis results for some samples, the quantitation of the fractions by quantitative polymerase chain reaction (qPCR) showed that for approximately half of the samples extracted using the Separate Method, a majority of total male DNA was eluted into the SF. For these samples, a single source or distinguishable male profile can be generated. However, it was also demonstrated that even with good separation, a very small proportion of the female DNA in the SF still overwhelms the male DNA that is present in much smaller amounts, particularly for the Recombined Method where there are only two fractions.

Though further experimentation is necessary, these modifications proved effective in achieving high sperm recovery in the SF and generating a distinguishable male profile when extracting samples using the Separate Method. This research has confirmed that the TCDE procedure can be faster and less labor intensive while still producing clean DNA profiles in downstream analysis, and thus has the potential to be implemented in forensic laboratories after some of the concerns are addressed.

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LIST OF ABBREVIATIONS

ADE	Acoustic differential extraction
ALS	Alternate light source
bp	Base pair
CE	Capillary electrophoresis
DI	Deionized
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EA1	Erebus Antarctica 1
E-cell	Epithelial cell
EDTA	Ethylenediaminetetraacetic acid
EF	Epithelial cell fraction
EPG	Electropherogram
LCM	Laser capture microdissection
MF	Material fraction
Mg	Magnesium
μ L	Microliter
mM	Millimolar
NaCl	Sodium chloride
nm	Nanometers
ng	Nanogram
qPCR	Quantitative polymerase chain reaction

PCR	Polymerase chain reaction
Pro K	Proteinase K
RAINN	Rape, Abuse & Incest National Network
RFU	Relative fluorescent unit
rpm	Revolutions per minute
SAECK	Sexual Assault Evidence Collection Kit
SANE	Sexual Assault Nurse Examiner
SDS	Sodium dodecyl sulfate
SF	Sperm fraction
STR	Short tandem repeat
TCDE	Temperature-Controlled Differential Extraction
U	Units
US	United States

1. INTRODUCTION

1.1 DNA

Deoxyribonucleic acid, or DNA, is the genetic material present in the nucleus of all dividing cells in the human body (1). The chain of a DNA molecule consists of a regular sugar and phosphate backbone with four different types of nitrogenous bases (adenine, guanine, cytosine, and thymine) attached to each sugar. The double stranded helix structure of DNA is partially held together by hydrogen bonding between these nitrogenous bases. These two strands fit together because of complementary pairing between the bases of each strand (adenine pairs with thymine and cytosine pairs with guanine). The precise sequence of these bases codes for the synthesis of proteins, and thus ultimately dictates how an organism develops, functions, and survives (1). Because of base pairing between strands, one strand of DNA has the ability to serve as the template for a complementary strand, yielding newly replicated double stranded DNA. This process is important because it is what allows for cell reproduction within an organism (2).

1.1.1 Genetic Inheritance through DNA

The long DNA molecule is packaged into chromosomes in the nucleus of a cell, with diploid human cells containing 23 pairs of chromosomes. Of those 23 pairs that make up the human genome, autosomal chromosomes make up 22 pairs and sex chromosomes (XX for females and XY for males) make up one pair (3). In diploid cells, both parent contributes a chromosome to each pair, resulting in an individual obtaining half of all genetic information from their mother and half from their father (2). Each gametic cell, or

a cell utilized for reproduction, is haploid, as it contains only one set of the 23 chromosomes rather than a pair. The female gamete, or the egg, is fertilized by the male gamete, or spermatozoa, to form an embryo containing a set of each chromosome. Genes are the basic units of heredity along these chromosomes that are transferred from the parents when the egg is fertilized and thus act as instructions for protein molecules that lead to a particular function or characteristic of a human (4).

1.1.2 DNA Packaging in Somatic Cells

In a somatic cell, or any normal cell that is diploid and thus not a reproductive cell (i.e. a gamete), DNA is packaged within chromosome structures in the nucleus. This packaging is conducted through the use of a series of proteins and becomes necessary due to the elongated length of DNA (each human cell contains approximately 2 meters of DNA) (5). Because DNA has a strong negative charge stemming from its phosphate backbone, small positively charged proteins called histones are utilized to compact the DNA. The electrostatic forces between these positive and negative charges provide the energy the DNA needs to wrap itself around the histones, creating a protein-DNA complex called a nucleosome. These nucleosomes consist of 146 base pairs (bp) of DNA wrapped around a complex of eight histone proteins, called a histone octamer, and are repeated throughout the structure of DNA (5)(6). Ultimately, a chromosome in a somatic cell can be defined simply as a long chain of nucleosomes that are compacted very tightly together in order to fit inside of the cells that make up humans and other organisms.

1.1.3 DNA Packaging in Spermatozoa

Spermatozoa, or the male gametes, are the male reproductive cells that carry the male's haploid genome. The structure of sperm is optimized for the purpose of fertilizing the egg of the female, and thus the sperm has adapted to be small, extremely mobile, and chemically able to fertilize the egg (7). A long flagellum tail is used to propel the sperm through the cytoplasm medium, while the strong sperm head carries the haploid DNA in the nucleus. This nucleus is further protected by an acrosome cap on the sperm head.

Because the sperm cell needs to be much smaller to increase its travel speed and efficiency, the chromosomes in its haploid nucleus are even more tightly compacted in the reduced cytoplasmic volume. Thus, in mammals, sperm cells contain the most tightly compacted eukaryotic DNA, six times more tightly compacted than DNA in autosomes (8). Essentially the entire volume of the sperm nucleus is filled with DNA, necessitating that an alternative DNA packaging method be utilized. During spermiogenesis, sperm cells replace the positively charged histones that DNA uses as binding proteins with simple, even more positively charged proteins called protamines. Sperm DNA is packaged with these protamines in linear, side-by-side arrays to create an even more compact structure than the supercoiled structure of most eukaryotic DNA (8). Furthermore, the two protamines present in mammalian sperm have negatively charged phosphate groups that are removed when they bind to DNA. Following the removal of the phosphate groups, the oxidation of the cysteine residues within protamines creates extremely strong disulfide bonds, further linking the two protamines together (10). These cysteine disulfide bonds (the -SH group of one protamine to the -SH group of another) are vital to the deeply

condensed configuration of DNA in sperm cells, and thus the proper functioning of sperm (11).

1.1.3.1 The Sperm Glycocalyx

When sperm cells differentiate through the processes of meiosis and mitosis in the testis, several hundred different glycoproteins unique to male gametes become incorporated within the sperm membrane (12). This outer layer of the ejaculated human sperm, called the sperm glycocalyx, is approximately 20-60 nanometers (nm) thick and consists of these integrated glycoproteins, as well as glycolipids, glycoproteins, and carbohydrate residues that can be linked to other biomolecules (13). The purpose of this unique sperm surface is to ensure the viability of the haploid male sperm cell as it undergoes storage for long periods of time in the male tract, travels through the female reproductive system, and ultimately penetrates the female egg to fertilize it. Different modifications that can be made to the glycoconjugates that extend from the sperm glycocalyx create individuality among the sperm gametes (13). These modifications allow the sperm to not only resist any potential immune mechanisms from the female organs, but also allow for selectivity in the quality of sperm that are able to reach the female gamete (14). Because these modifications typically involve the addition of carbohydrate residues that in the end make up most of the outer surface of the sperm glycocalyx, understanding the interaction these carbohydrate residues have with their environment is important when considering how to best extract sperm from a cotton swab. While some of the interactions between the biomolecules of the sperm membrane are tightly bound, high-affinity

interactions, others are known to be weaker, transient interactions, as explored by Drisdell, *et al.* (15). Exploring ways in which those interactions can be weakened and broken to release groups of proteins from the sperm glycocalyx could be vital in helping to obtain higher male sperm DNA recovery rates from cotton swabs. For example, Schröter *et al.* found in “The glycocalyx of the sperm surface” that treating ejaculated human sperm with 0.6 M NaCl was successful in releasing a group of approximately 20 proteins of varying molecular weights (10 to 100 kDa) from the sperm glycocalyx surface (14).

1.2 Forensic DNA Analysis

The application of the technique customarily used for DNA analysis to forensic purposes has revolutionized the field of forensic science, as well as the criminal justice system (16). After the initial discovery that the human genome was comprised of some highly polymorphic DNA loci, Sir Alec Jeffreys recognized in 1984 that these loci could be detected simultaneously to positively identify an individual (17). Through the use of restriction enzymes and the southern blotting technique, flanking regions of certain genes in the human genome were determined to possess highly polymorphic short sequences of nucleotides (18). Variation was observed in the number of nucleotides per repeat and the length of the number of repeats present in short tandem repeats (STR), which are defined as polymorphic, non-coding regions of DNA with high mutation rates. Ultimately, STR testing was utilized for forensic purposes by taking advantage of these polymorphisms present in human DNA base sequences to differentiate one individual from another (16,19). The development of polymerase chain reaction (PCR) during the same time period

advanced the use of DNA analysis in forensic science by allowing for the generation of millions of copies of a particular DNA sequence. This amplification of small amounts of DNA was determined to be particularly useful for forensic samples where there may be low quantity or degraded DNA requiring analysis with increased sensitivity (20). Today, forensic DNA analysis is utilized to link suspects to a crime, to determine parentage, and even to prove the innocence of wrongly convicted individuals. The initial issues surrounding the application of DNA to the legal system, such as the risk of contamination and the lack of discriminating power of DNA, have been largely overcome, and DNA evidence has now become widely admissible in the court system (21).

1.2.1 Biological Fluid Screening

Prior to forensic DNA analysis, the biological sample must be located and collected at the crime scene. Common biological evidence that may be collected includes blood, saliva, semen, urine, feces, skin cells, and hair. Different techniques are used to locate these biological stains on various substrates, including alternate light sources (ALS) that use different types of light to cause the stain to fluoresce. Once the sample is located or collected in the form of a cutting of the stain, a swab of a particular area, etc., biological screening of the evidence is then performed with presumptive and confirmatory testing (22). Presumptive testing, which can establish the potential presence of a specific body fluid, is typically performed first, as the tests are rapid, sensitive, and cost-effective. However, presumptive testing usually lacks the desired body fluid specificity, a specificity that can be provided by further confirmatory testing of the evidence. These confirmatory

tests usually involve chemical or immunological methods that react with a particular component of the body fluid (23). Overall, biological screening helps to positively identify possible body fluids within a sample without contaminating the sample for future downstream DNA testing. This preliminary screening helps to send the best possible evidence samples to DNA processing, where proper extraction methods can be performed on the basis of the bodily fluid present.

1.2.2 Steps of Forensic DNA Analysis

Forensic DNA analysis begins with an extraction step, where cells are lysed open to release DNA from its biological source material, such as blood, semen, or saliva. The amount of DNA extracted from the source is then quantitated using quantitative PCR (qPCR). The concentrations of DNA obtained by qPCR help determine the correct input volume of the sample used for effective amplification by PCR that produces millions of copies of specific regions of DNA. The PCR products then undergo a capillary electrophoresis (CE) step, which separates STR alleles based on their size and detects them with fluorescence. Lastly, the detected STR alleles are analyzed to determine the number of repeats in a particular DNA sequence at a locus, which is the basis for generating a distinct DNA profile in the form of an electropherogram (EPG) (24).

1.3 Forensic Examination of Sexual Assault Evidence

Sexual assault is a major crime in the United States (US), referring to a range of sexual offenses that are punishable by extensive jail time. The Rape, Abuse & Incest

National Network (RAINN) reports that one in every six women in America has been the victim of either an attempted or completed rape in their lifetime (25). With such high rates of sexual assaults, it is very important to identify the perpetrator quickly. This is complicated by the fact that sexual assault evidence presents unique challenges distinct from other evidence. Generally, sexual assault evidence collected from a crime scene or from the victim contains mixtures of cells from multiple individuals, which results in mixed DNA profiles unless the cell types are able to be distinctly separated during the DNA extraction process (26,27).

Analysts attempt the challenging separation of the DNA of the perpetrator from that of the victim to obtain a clean single-source male autosomal profile whose source can be identified. This can be achieved by taking advantage of the unique tight packaging structure of sperm cells to distinctly extract and purify male sperm cells away from female epithelial cells through a technique called differential extraction (16,26). Ultimately, linkages between pieces of evidence from a crime scene to individual suspects can be established through the generation of single source autosomal male DNA profile, which is the ultimate objective based on its application to the criminal justice system. Variation in the extraction method utilized, especially in the differential extraction process, along with other procedural variation can contribute to differences in DNA recovery success rates and the level of separation achieved of the male DNA from the female DNA as seen in the profiles (26).

1.3.1 Sexual Assault Evidence Collection Kits (SAECKs)

Various different forms of sexual assault evidence can be collected in an all-encompassing forensic examination kit called a Sexual Assault Evidence Collection Kit (SAECK) that is offered to victims of sexual assault who seek medical attention. Biological and trace evidence specimens, such as vaginal swabs, rectal swabs, pubic hair combings, oral swabs, and other related items, are commonly collected in this process, along with a full health history and documentation of injuries and secretions (28). These kits are generally collected at a hospital or other professional medical center by a Sexual Assault Nurse Examiner (SANE) that has undergone special training. The SANE training program has been held in high regard across the US for its ability to improve victim outcomes and enhance evidence collection, leading to higher conviction rates in the criminal justice system (29). The use of one clear and comprehensive kit collected by a trained SANE for evidence in sexual assault crimes is invaluable to court cases and the criminal justice system.

While the kit contents vary, there are a few common substrate types that are utilized by the SANEs in their evidence collection. Common substrates encountered with sexual assault evidence in general include cotton swabs, swatches of fabric from clothing or bed sheets, and glass slides for smears of different body fluids (28,29). The substrate that a particular specimen is on can greatly affect the DNA extraction method employed for a particular piece of evidence, and ultimately, the success rate of DNA recovery from that item. Thus, it is vital to consider the substrate and possible body fluid(s) present when deliberating on the extraction method DNA used for that piece of evidence.

1.3.2 Backlog of Sexual Assault Evidence

A backlog exists within many public forensic laboratories with regards to the analysis of sexual assault evidence, which can be a very frustrating problem for the criminal justice system as a whole and particularly the victims affected by these crimes. As of 2016, it has been reported that there are hundreds of thousands SAECKs in evidence lockers of many law enforcement agencies and forensic laboratories that have yet to be tested (30). With the power DNA testing has in verifying and identifying a suspect in a sexual assault, the biological evidence from these kits can have high probative value if tested. Thus, it has become a vital concern to identify any hinderances to fast processing of sexual assault evidence and find potential solutions.

While some of the sources of this backlog have been linked to the lack of tracking and inventory for these kits, the lack of funds and resources possessed by forensic laboratories is also a contributing factor (30,31). An additional challenge that forensic laboratories grapple with in testing sexual assault evidence is how laborious and time-consuming the differential extraction process is, which must be conducted to separate the sperm DNA from the non-sperm DNA. The differential extraction process requires two lengthy incubation periods and is not easily automated (27,31). In the end, it becomes apparent that an improved differential extraction procedure that reduces the lengthy incubation times and labor-intensive manual process has immense promise for reducing the backlog of sexual assault evidence.

1.4 DNA Extraction Methods

The first step of forensic DNA analysis is extraction, which must be conducted to release DNA into solution through a lysis of both the nuclear membrane and the cellular membrane. DNA extraction isolates DNA from proteins, inhibitors and contaminants, and other cellular materials that may hinder downstream analysis by taking advantage of the molecular properties of DNA that differ from those of the undesirable cellular components. The goal of extraction is to recover the maximum amount of DNA from the sample while also removing as many PCR inhibitors as possible to generate a clean DNA profile once amplified (32). In order to achieve this end goal, forensic laboratories use many different extraction methods. The factors that influence the choice of extraction method include the sample size of the sample, the substrate type, the type of biological evidence present on the substrate, the experience of the analyst, the speed and efficiency of the procedure, and the reagents and chemicals required for the method (33).

Typical extractions used in forensic settings involve the initial lysis of the cellular and nuclear membrane of the cell to release DNA into solution, the enzymatic digestion and denaturation of proteins by disrupting their secondary and tertiary structure, and lastly the separation of the DNA from these other cellular components (33). To keep DNA in solution during extraction, commonly used lysis buffers typically contain an ionic detergent, such as 1-2% sodium dodecyl sulfate (SDS), or a non-ionic detergent, such as Triton X, to disrupt the cellular and nuclear membranes and proteins. The detergent SDS, for example, possesses a net negative charge that is adopted by proteins when they bind to the molecules of SDS. The newly acquired negative charge of the proteins then disrupts

the protein structure. A small amount of sodium chloride (NaCl) is also added to the buffer to stabilize the negatively charged DNA, while the addition of ethylenediaminetetraacetic acid (EDTA) chelates magnesium (Mg^{2+}) to prevent nucleases from degrading DNA (Mg^{2+} is a required cofactor for most of these nucleases). The addition of proteases like Proteinase K (Pro K) helps to degrade nucleases and other proteins bound to the DNA because of their ability to cut at multiple sites. With hair and sperm substrates where disulfide bonds are present, dithiothreitol (DTT) may be added to disrupt these stronger bonds.

1.4.1 Phenol-Chloroform Organic Extraction Method

Phenol-chloroform extraction, the one form of organic extraction sometimes still used in forensic laboratories, uses organic reagents to distinctly separate proteins and unwanted cellular components found in an organic layer from DNA in aqueous solution. In this technique, phenol-chloroform is added to the cell lysate that forms following the lysis of the cell membrane. The phenol denatures the proteins, which ultimately sit at the interface of the organic and aqueous layer because both the hydrophobic and hydrophilic parts of the proteins are positioned where they are most stable (32). Because DNA has a higher solubility in water than the other proteins and cellular components, it resides in the aqueous layer where it can ultimately be purified from. While the technique has been gradually phased out due to its labor intensive procedure that requires many tube transfers and the use of hazardous chemicals, it is proficient at extracting a very pure DNA product free of most inhibitors (33,34).

1.4.2 Silica-Based Extraction Methods

Silica-based extraction methods refer to methods that use silica beads or columns designed to bind DNA in certain conditions and let unwanted cellular material pass through. With these methods, the normal lysis buffer is used with the addition of chaotropic salt, which dominates hydrogen bonding with water to lessen the hydrogen bonding between water and DNA. The chaotropic salt also causes protein denaturation by disrupting the hydrogen bonding and hydrophobic interactions that contribute to protein structure. At a pH greater than 7.5 (achieved through controlling the pH of the lysis buffer), the DNA in the cell lysate binds to the silica in the beads or column while all the other inhibitors and contaminants pass through (32,33). Once the salts are removed through ethanol wash steps, a low salt buffer at an alkaline pH is washed over the silica beads or column to elute the DNA into solution. Similar to the phenol-chloroform method, the purified DNA extracted through silica-based methods is clean and robust with a high molecular weight; however, the procedure is still lengthy and requires multiple tube transfers (34).

1.4.3 Direct Lysis Extraction Methods

With direct lysis extraction methods, a cell lysis buffer breaks open cells to release all cellular components, including DNA, into solution, where they remain for the entirety of the extraction process. Instead of removing the contaminants and inhibitors from the solution to separate them from the DNA, this technique only attempts to lessen the interactions between the proteins and DNA (33). The direct lysis can be achieved through

a number of different approaches, including heat, enzymes, and detergents. These methods, while generally effective in producing DNA that is pure enough to generate a clean DNA profile, are much cruder than the previously discussed methods because inhibitors are still present in the sample that will be amplified. However, the direct lysis methods are still very rapid, efficient, and allow for maximum DNA recovery because all cellular contents stay in solution and never leave the tube (35).

1.4.3.1 Erebus Antarctica (EA1) Protease

An enzymatic approach to extraction by direct lysis has been successful with the use of the Erebus Antarctica (EA1) protease. This protease is a strain derived from *Bacillus sp.* and has a significantly greater thermophilic nature than other metalloproteinases because of an amino acid substitution near the region that confers thermal stability. The enzyme is also activated by zinc and stabilized by calcium, two elements that can be added to lysis buffers with ease and at relatively low costs (36). The thermostable nature of EA1 creates a narrow window of enzyme activity at a high temperature. The protease is inactive at lower temperatures before reaching its optimal temperature of activity of 75°C, a temperature that is high enough for the enhancement of the proteolytic activity of the enzyme, yet well above the optimum temperature of many other enzymes and below the denaturation temperature of DNA. It is able to degrade nucleases and remove and denature proteins but becomes inactivated at a higher temperature (90°C). Thus, potentially harmful DNA-degrading nucleases are no longer active in the solution (37).

EA1 is manufactured by MicroGEM International PLC (Charlottesville, VA), which optimized a recombinant variety of the EA1 protease as a reagent called *forensicGEM™* (37). This product is marketed in a *forensicGEM™* Universal kit as an enzyme that can release DNA from a variety of forensic samples and can produce extremely high quality and high yield DNA through a rapid process performed in a single tube. It has been proven to be compatible with downstream PCR amplification (38). EA1's highly specific window of activity allows for far less product and reagent expenditure, which is cost effective (37,38). Like the other direct lysis procedures, using the *forensicGEM™* enzyme allows for the detection of much smaller amounts of DNA, since the sample does not leave the tube and no DNA is lost to washes or other procedural manipulations. However, inhibitors and contaminants are not purified out of these samples and therefore may be present in some samples, which could potentially inhibit amplification in downstream analysis.

1.5 Differential Extraction

Differential extraction refers to any extraction technique that results in the separation of two different cell types that are originally found together in a mixture. This separation technique is used for the processing of sexual assault evidence because female and male DNA ratios are typically extremely imbalanced due to the excess of female epithelial cells on samples collected directly from the victim. Without separation, the amplification of the major female component of the mixture predominates during the PCR, and the male profile may be either uninterpretable or undetected (39). This amplification

problem was solved by the development of a differential extraction procedure that allows for the separate lysis and isolation of spermatozoa and non-sperm cells, such as epithelial cells, into two distinct fractions. This differential extraction method was first reported by Gill et al. in 1985 as the “preferential lysis” of sperm nuclei separate from vaginal cellular debris (16,39). While the epithelial cells can be digested under common mild lysis conditions, the strong disulfide bonds between the protamines of sperm are resilient to traditional DNA extraction conditions. Thus, intact sperm can be separated (without any release of the male DNA) from the solution of lysed female DNA (33). The male DNA from the sperm can then be released into solution in a separate tube utilizing a reapplication of a detergent, protease, and reducing agent to break those disulfide bonds. The downside of the differential extraction process is that it is more time-consuming and labor intensive than most extraction methods performed for other types of biological evidence. Furthermore, complete separation is not always feasible, sometimes resulting in mixtures that generate profiles that are uninterpretable or have male allelic dropout. These male profiles are essential for linking a suspect to a crime of sexual assault.

1.5.1 Traditional Differential Extraction Methods

The preferential lysis extraction method developed by Gill et al. requires the initial preferential lysis of large amounts of female cells into solution by an incubation step in SDS and Pro K. The intact sperm cells, unaffected by this treatment, can then be pelleted at the bottom of the tube through a centrifugation step. The removal of the supernatant into a separate tube creates an epithelial cell fraction (EF), which consists of the female

DNA in solution. The sperm cell pellet remaining in what now can be labeled as the sperm cell fraction (SF) then undergoes a more intense chemical treatment. This includes the addition of DTT, which lyses tightly packaged sperm cell nuclei containing the male DNA (16). The differential extraction procedure yields two different fractions (the EF and the SF) which will ideally generate two different profiles, one of the victim and one of the sperm donor, once downstream DNA profiling is performed (Figure 1). While alterations have been made to this procedure over time, the majority of forensic laboratories still use a similar procedure for the differential extraction of casework samples.

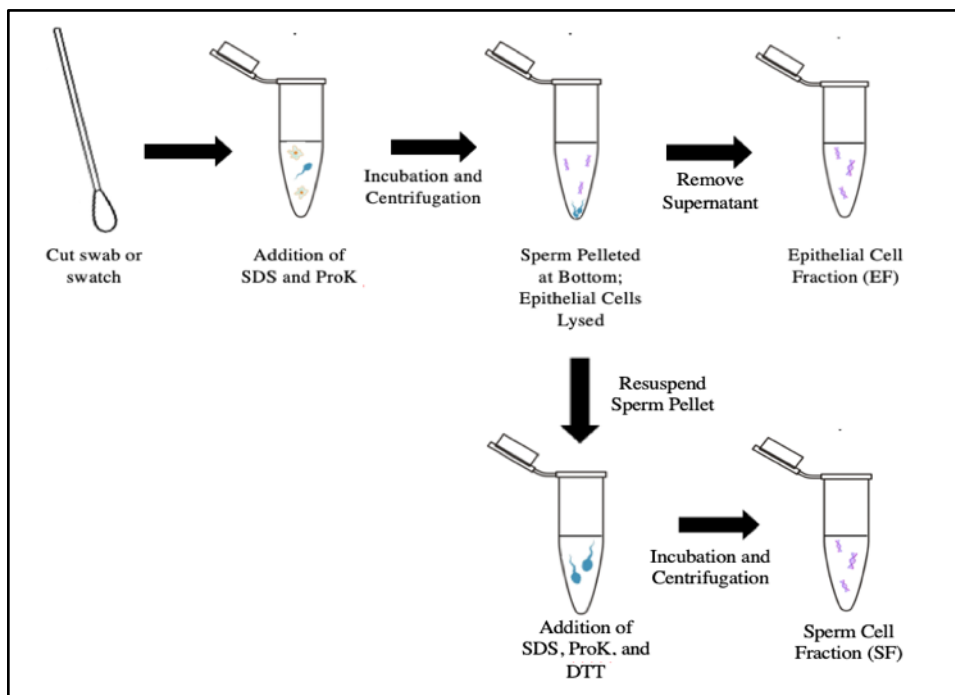


Figure 1. A diagram of the traditional differential extraction process as described by Gill et al.

1.5.2 Unconventional Differential Extraction Methods

Other differential extraction methods have been constructed from the procedure of Gill et al. in an attempt to eliminate many of the labor-intensive steps and speed up the procedure, to reduce E-cell carryover into the sperm fraction, to improve recovery of sperm cells from the substrate itself, and even to reduce possible contamination that may occur during the washing and centrifugation steps (27). These unconventional differential extraction methods use varying manual techniques, reagents, and enzymes and may include automated equipment to achieve better male DNA recovery and better separation efficiency for cleaner male DNA profiles.

1.5.2.1 Laser Microdissection

Laser capture microdissection (LCM) is a cell separating technique that is capable of isolating specific cell types from mixed samples, and thus is very applicable to sexual assault evidence (27). A sexual assault sample can be stained with a chemical stain that can differentiate sperm cells from other cells in the sample. The stained sample is then magnified under a microscope, where the sperm cells of interest can be clearly visualized and separated into a microcentrifuge tube using laser manipulation. The sperm cells are still morphologically intact with DNA contained in the nucleus and can thus immediately undergo an extraction procedure once dissected (40,41). LCM separates sperm cells effectively without any E-cell carryover, allowing for the successful production of distinguishable male profiles, unless sperm from multiple donors is present. This is especially important when the sample consists of very few male cells, as it ensures that

they are captured and are not overwhelmed by the excess of female epithelial cells. However, this technique has not been widely adopted by the forensic community (27,41).

1.5.2.2 Sperm Capture by Di-electrophoresis

An innovative DEPArray™ system uses di-electrophoretic cages found in a single-use microfluidic cartridge to isolate individual sperm cells from complex heterogenous samples. An array of electrodes creates a non-uniform electric field that can exert force on the polarizable cells to move the whole cell in the di-electrophoretic cages to different locations. The arrangements of the electric field can be altered using the electrodes to relocate the cell to a recovery chamber, where it can be collected for extraction at a later time (42). Similarly to the LCM technique, the di-electrophoresis method is capable of improving the specificity and sensitivity of sperm cell capture and decreasing potential inhibitors present in the samples (42). However, this new technique does not necessarily remedy the shortcoming related to the lengthiness of the procedure, as the technique still requires an abundance of time by the operator (27).

1.5.2.3 Acoustic Differential Extraction

Acoustic differential extraction (ADE) uses the forces that are generated by acoustic waves to separate sperm cells from other cellular material on the basis of their density, size, and compressibility. This acoustic extraction method was developed on a microfluidic device, which creates a closed and automated system for analysis by integrating sample preparation steps on a single device (43). Sperm cells have a very

distinct size and shape that allows for their differentiation and eventual capture isolated from the other cellular material. While the larger epithelial cells pass through the acoustic field unaffected, the acoustic waves create a great enough force to retain the sperm cells in the acoustic nodes before they are collected in a collection reservoir. Essentially, the acoustic radiation forces acting on the sperm cells must be greater than the drag forces pulling the sperm cells back along the bulk fluid flow (44). Applying this technique to mock sexual assault samples has proved fruitful, with the successful isolation of the male sperm cells and the enrichment of the sperm cell concentrations, even at a 40:1 ratio of female to male cells (45). This method is rapid and has the potential for automation but is not currently commercially available (27).

1.5.3 Recovery of Cells from Cotton Swabs

Achieving a high recovery of cells from swabs with a cotton matrix is essential when dealing with SAECKs, which rely on cotton swabs to collect biological secretions from the vagina, rectum, mouth, and other areas of the body. However, it has been demonstrated that substantial quantities of DNA are retained by the cotton fibers of swabs and subsequently lost prior further downstream DNA profiling (46). This is particularly of concern for sexual assault cases where the male sperm cells are already in a much lower quantity compared to the excess of female epithelial cells present on the swabs. If some of the male DNA is lost when the sperm cells stick to the cotton swab and is thus not recovered in the swab elution, the likelihood of obtaining a clear male profile is reduced (47,48). While it is not well-defined what causes cells, especially sperm cells, to stick to the fibers

in cotton, it has been suggested in prior research that sperm cells interact with cotton fibers through what can best be described as an adsorption process (46). Because of the suspected adhesion of the surfaces of the cells to the length of the cotton microfibrils, some research has been focused on the alteration of some extraction variables to weaken the adhesion and allow for better elution off the cotton (46).

Previous research has attempted to improve the recovery of male DNA by weakening the adsorption through the digestion of the cotton microfibrils. This can potentially be accomplished through the enzymatic digestion of the cellulose polymers of the cotton microfibrils to release intact sperm cells into solution (48). Experimentation with the differential extraction buffer has been performed to identify different components that may assist in the recovery process. For example, Voorhees, et al. showed that the digestion of cotton swab samples with *Aspergillus niger* cellulase enhances the release of sperm cells from cotton swabs by breaking attachments between the cell surfaces and the mass of the cellulose cotton fibers (48,49). Other research by Norris et al. altered the differential extraction buffer to include the anionic detergents Sarkosyl and SDS for improved cell lysis. This group observed that utilizing buffers with these anionic detergents yielded higher sperm cell recoveries (47). Any improvement in the recovery of the sperm cells during differential extraction leads to improved male profiles from the SF with less allelic dropout.

1.6 Temperature-Controlled Direct-Lysis Differential Extraction Method

The Cotton Lab at Boston University has developed a novel Temperature-Controlled Differential Extraction (TCDE) method that seeks to eliminate the challenges associated with other differential extraction methods, particularly the length and labor-intensiveness of the procedure and the low sperm DNA recovery achieved. The procedure utilizes direct-lysis enzymes that are highly temperature-controlled, meaning that the enzymes are only active near or at their optimal temperatures (Figure 2). This direct-lysis method has been proven to extract DNA from various substrate types that may be seen in sexual assault cases, including swatches and cotton swabs, helps to reduce the manual steps required, and lessens incubation times (50–54). This procedure results in both an E-cell fraction and a sperm fraction, created through separate enzymatic digestion with two enzymes, one designed for the specific purpose of lysing epithelial cells (*forensicGEM™*) and one for lysing sperm cells (*forensicGEM™ Sperm*). A material fraction (MF) is also generated. This fraction has been used experimentally to monitor the proportions of both female and male DNA retained by the cotton swab. This novel procedure also utilizes Benzonase® nuclease to digest any previously lysed DNA remaining in the MF and SF prior to sperm lysis, thus reducing any E-cell carryover into the fractions that aim to generate a male profile. Lastly, the addition of the AcroSolv protease to the *forensicGEM™ Sperm* enzyme cocktail helps to facilitate the lysis of the sperm cells and the release of male DNA from the sperm nuclei. The procedure creates lysates that do not require further purification (when inhibitor-free) and does not use reducing agents, resulting in qPCR and PCR-ready DNA for downstream STR analysis (50–54).

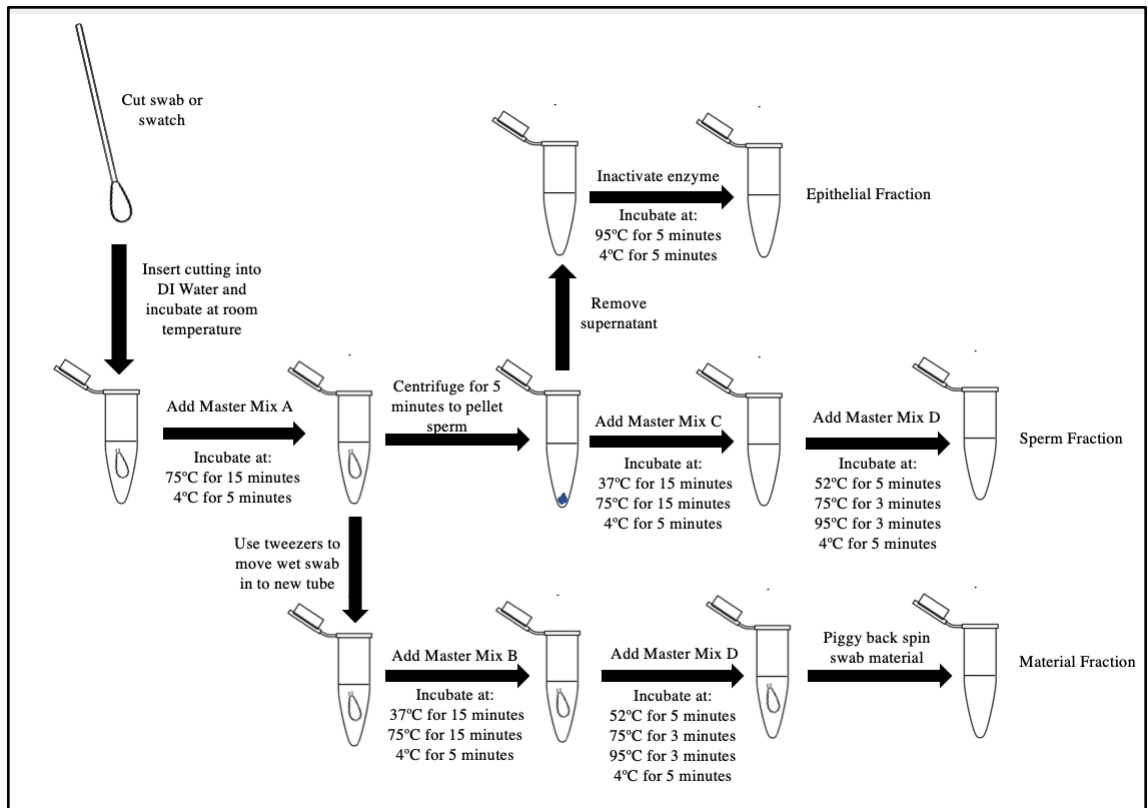


Figure 2. A diagram of the TCDE protocol developed by the Cotton Lab as of 2021 prior to modifications

1.6.1 *forensicGEM*[™] Sperm Enzyme Cocktail

A different enzyme cocktail of EA1 and other enzymes is used in the *forensicGEM*[™] Sperm kit also manufactured by MicroGEM International PLC (Charlottesville, VA). The *forensicGEM*[™] Sperm enzyme mixture has both thermophilic and mesophilic enzymes that work at low temperatures to quickly degrade the sperm cell proteins protecting the DNA of the sperm nucleus (55).

1.6.2 Benzonase[®] Nuclease

Because the extensive E-cell carryover into the sperm fraction has consistently been a problem in differential extraction methods, the TCDE procedure incorporates a nuclease

that is capable of digesting any female DNA already extracted from the epithelial cells that remains in solution of the MF and SF. The Benzonase[®] nuclease (Sigma-Aldrich, St. Louis, MO) has been shown to be effective in degrading E-cell DNA and reducing E-cell carryover (51). The Benzonase[®] nuclease is an endonuclease derived from the bacteria *Serratia marsecens* that has the ability to degrade DNA and RNA in any form when it is active. Its temperature of optimal activity is 37°C, which is much lower than that of the EA1 protease also added at this step. This allows for the degradation of any female DNA remaining in solution first at 37°C. Once the EA1 protease gets activated at 75°C, the EA1 protease is able to digest the leftover Benzonase[®] (before being deactivated itself at 95°C) because EA1 is non-specific and has multiple cut sites on the Benzonase[®] nuclease (56). The Benzonase[®] nuclease has no residual function after its digestion by EA1, making it the ideal candidate for the selective degradation of nucleases.

1.6.3 AcroSolv

Acrosolv is a component of the *forensicGEM*[™] Sperm kit that aids in the lysis of sperm cells and their nuclei to release male DNA into solution. It consists of a proprietary mixture of reagents delivered as a lyophilized powder. The temperature for the optimal activity of Acrosolv is 52°C. When used in combination with the *forensicGEM*[™] Sperm enzyme cocktail, the EA1 protease can digest the AcroSolv at its optimal activity at 75°C before it is inactivated itself at a higher temperature (55). These characteristics of the specific enzymes in the *forensicGEM*[™] Sperm kit are exploited by this novel temperature-controlled differential extraction method because it allows for the preferential lysis of

sperm cells and epithelial cells on the basis of the different optimal temperatures of the enzymes. Because all of the enzymes (both proteases and nucleases) can be deactivated in solution by digestion prior to PCR, no additional wash steps to purify the samples are required. This procedure ideally diminishes the chance of losing DNA.

1.6.4 Previous Findings in the Cotton Lab

Due to the variability observed in the number of female epithelial cells collected in sexual assault samples, the necessary amount of EA1 protease or the necessary final concentration of EA1 protease needed becomes impossible to predict. With the potential hazard of too little enzyme being present in solution with a high amount of female epithelial cells, it is possible that the EA1 protease does not lyse all the female epithelial cells. In terms of a differential extraction procedure, this would be troublesome as it leaves female epithelial cells that have not been lysed to be carried into the sperm fraction in the pellet (57).

Previous studies conducted by the Cotton Lab and by Sinha et al. revealed that there is great variation in where the male DNA was distributed during the separation process of the developed procedure (58). Other students found that the majority of the male DNA was left on the swab and thus released into the MF rather than the SF. This is not ideal because great variability in the amount of female DNA that is released into the MF was also revealed, and any decent amount of female DNA has a tendency to overwhelm the male DNA in the MF because of its abundance. Therefore, the material fraction profiles tended to have a major female profile at times or a female profile with similar allelic peak heights to the male profile. This is especially not desired in these cases where the majority

of the male DNA is eluting in MF because it is then likely that no fraction can generate a major male profile with high allelic peak heights. In the cases where the sperm was released majorly in the sperm fractions of the samples, these fractions also tended to be mixtures with female DNA, and with the lower recovery rates, the male profile tended to have allelic dropout or was not distinct from the female profile.

1.7 Aim of the Research

This research attempts to improve the release of sperm cells from a cotton swab, which is the primary substrate used to collect sexual assault evidence. Additionally, this project aims to improve the distribution of male DNA between the various fractions of the TCDE procedure by increasing the amount the male DNA released into the sperm fraction. The research also compares a modified procedure with the recombination of the material and sperm fractions of mock-casework samples to the normal TCDE procedure to investigate which achieves better separation and male DNA recovery.

2. MATERIALS AND METHODS

2.1 Preliminary Sperm Recovery Experiments

In an attempt to better understand the sperm distribution throughout the different fractions of the TCDE protocol and to test methods that may improve sperm recovery from the cotton swabs, preliminary experiments were conducted with the application of just sperm cells. In these experiments, dilutions of semen were applied to sterile cotton swabs by putting a volume of a semen dilution in a microcentrifuge tube and placing the cotton swab upright to absorb the liquid, before letting the swabs air-dry. Two different commercial semen donor aliquots frozen at -20°C , were used to prepare 1:50, 1:100, and 1:500 dilutions of semen in deionized (DI) water, which were applied to sterile cotton swabs in a volume of 10 μL to produce swabs with varying amounts of DNA. The cotton swabs were dried overnight after the application of the semen in a fume hood before being placed back in their original packaging.

For these preliminary experiments, the procedure of the previously developed TCDE protocol as of 2021 was followed, with slight modifications (54). For the sperm experiments that were testing only the efficiency of the AcroSolv and *forensicGEM*TM Sperm digestion where there were no epithelial cells present, the initial E-cell lysis and Benzonase[®] nuclease steps were not needed. Instead, the initial one hour incubation in DI water was performed before proceeding to the AcroSolv and *forensicGEM*TM Sperm digestion step. The supernatant that is created after the sperm is pelleted at the bottom of the tube in these investigations was also digested with AcroSolv and *forensicGEM*TM Sperm. This was conducted in order to test for any amount of male DNA being released

into the EF during E-cell lysis to better understand the sperm distribution among the three different fractions as well as improve overall recovery into the fractions where a single-source male profile can be generated.

2.1.1 Modifications to the TCDE Protocol for the Preliminary Experiments

Because previous research by the Cotton Lab had considered the potential carryover of epithelial cells to the MF due to the liquid retained by the swab, a piggyback spin was performed after E-cell lysis. Rather than moving the swab saturated in liquid to the MF tube, a piggyback spin was performed by transferring the wet swab to a spin column in a 2.0 μ L tube. The swab was spun down at 14000 revolutions per minute (rpm) for three minutes to release the liquid from the swab into the tube. The dry swab was then removed to the MF tube. The tube containing the liquid was then vortexed to resuspend any possible sperm pellet that may have formed at the bottom of the tube, and the liquid was returned to the original 0.5 mL tube prior to the formation of the sperm pellet.

The amount of liquid retained by the swab was approximately 130 to 150 μ L. Subsequently, 150 μ L of 1X RED+ Buffer was added to the MF tubes to keep the swab covered in liquid during future digestions and keep the concentrations of the components similar to those used in previous process development.

Also, to ensure that the entire sperm pellet was retained in the SF and not accidentally pipetted up when the supernatant was removed, another modification of the procedure was to leave approximately 30 μ L of liquid over the sperm pellet rather than the 20 μ L that the procedure called for.

2.1.2 Amount of Male DNA Used

The amounts of male DNA in the dilutions of semen varied from approximately 5 ng to approximately 250 ng, which was calculated from the quantitation of direct AcroSolv and *forensicGEM*[™] Sperm digestions of the semen dilutions. These values were similar to the amounts observed in other mock sexual assault swabs, such as those used in a research paper by Sinha et al., which prepared swabs with volumes of semen containing DNA amounts ranging from 5 to 50 ng (58).

2.2 Sample Preparation for Mock Casework Samples

Swabs were prepared to simulate casework samples that are seen in SAECKs. These swabs were used for the testing of the TDCE method with the various modifications mentioned above. Because previous experiments had shown that a proportion of the male DNA was not released from the swab and thus remained in MF, rather than the SF, the mock casework swabs underwent two different modified TCDE protocols for comparison. In one method, the MF and SF were kept as separate fractions as was previously described (referred to here as the Separate Method). In the other method used for comparison, the MF and SF were recombined into one tube after the E-cell supernatant was removed (referred to here as the Recombined Method) in the hopes of recovering a larger amount of male DNA in one fraction. With this method, the aim was to obtain cleaner male profiles due to the larger amount of male DNA that would ideally be found in the one fraction resulting from the combination of the MF and SF.

2.2.1 Sample Collection of Vaginal Swabs

To most closely replicate sexual assault evidence that would be seen in casework, sterile cotton swabs were used as the substrate of choice for all experiments. Eight anonymous donors provided four vaginal swabs using four individually packaged sterile cotton swabs in a sample collection kit. The instructions requested that the swabs were airdried before being placing the swabs back in their original paper packaging and returning the sample collection kit to the laboratory. To avoid potential contamination, donors were requested not to collect swabs if they were currently menstruating or if they had had sexual intercourse within the past week. All swabs were collected from donors following a protocol approved by the Boston University School of Medicine Institutional Review Board.

2.2.2 Mock Post-Coital Swabs

Each of the eight donors provided four vaginal swabs. Two swabs from each individual were used for each extraction method, the Separate Method and the Recombined Method (16 total swabs for each method). Semen dilutions containing approximately 10 ng or 50 ng of male DNA were prepared. The volumes of semen applied to the swab were calculated based on concentration values obtained through qPCR, with an average concentration of approximately 9.5 ng/ μ L for a 1:10 dilution digested with the normal *forensicGEM*TM Sperm and AcroSolv procedure. The semen was diluted with DI water, and 30 μ L aliquots were soaked up by the already prepared vaginal swabs to absorb proportionally over the entire swab. Two swabs from each individual were prepared with

dilutions containing 10 ng of single-source male sperm DNA, and two swabs were prepared with dilutions containing 50 ng of the same single-source male semen DNA (Figure 3). One swab of each sperm DNA amount was extracted with each method. The swabs from each individual were labeled 1-8 for their corresponding donor, and A-D depending on the mass of DNA applied to the swab and the extraction procedure used (**A** for 10 ng Swabs with MF + SF Separate Method, **B** for 50 ng with MF + SF Separate Method, **C** for 10 ng with MF + SF Recombined Method, and **D** for 50 ng with MF + SF Recombined Method). The swabs were then dried overnight before being repackaged in their original envelopes.

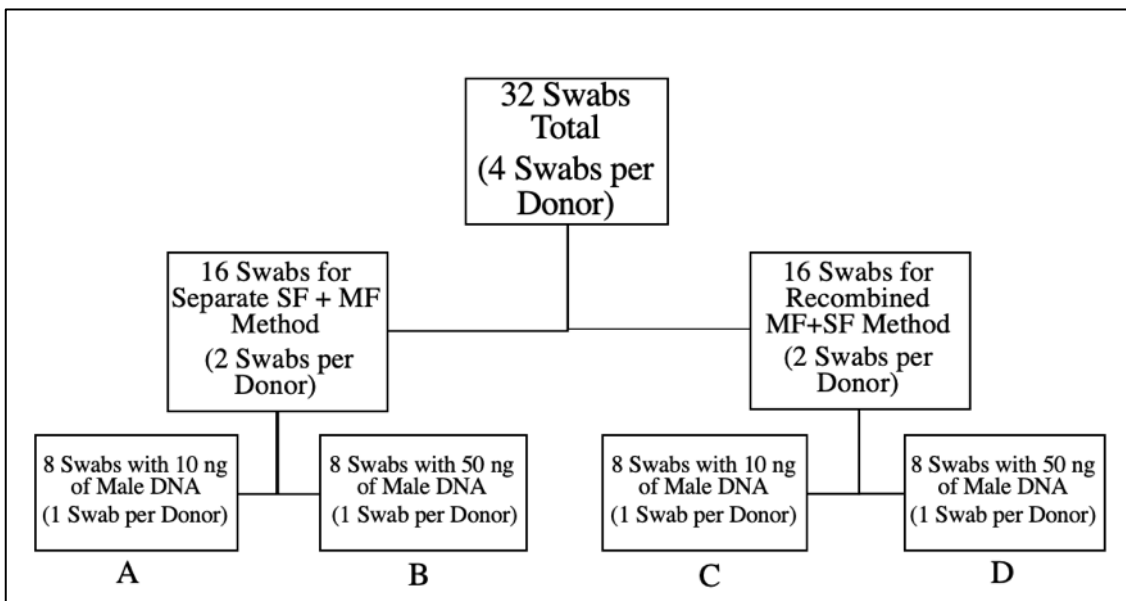


Figure 3. A diagram of the distribution of swabs from each sample collection kit, split by method and then by amount of DNA added (10 ng and 50 ng).

Half swabs were used throughout the mock casework procedures because in casework, portions of the samples must be retained for possible testing by the defense.

These swabs were cut using a sterile scalpel into half portions lengthwise and sterile tweezers were used to remove each half and place it in its own tube.

2.3 Reagent Components

The TCDE procedure requires an initial preparation of some dilutions of enzymes and reagents in buffering solutions. The EA1 protease cocktail that makes up *forensicGEM*[™] (MicroGEM International PLC, Charlottesville, VA) was used in its undiluted form, as well as in a 1:10 dilution with 1X BLUE buffer, a proprietary buffer solution to help with the cell lysis. The *forensicGEM*[™] Sperm enzyme (MicroGEM International PLC, Charlottesville, VA) was used in its undiluted form as well when combined in a sperm digest of 10X RED+ Buffer and undiluted Acrosolv. The sperm and material fractions utilize RED+ Buffer in this sperm cocktail rather than the ORANGE+ Buffer that is normally provided with the *forensicGEM*[™] Sperm kit. Previous research showed that samples with high concentrations of ORANGE+ Buffer demonstrated PCR inhibition when using the GlobalFiler[®] PCR Amplification Kit. No PCR inhibition has occurred when RED+ buffer is used in the master mixes (53).

Benzonase[®] nuclease (Sigma-Aldrich, St. Louis, MO) was purchased at a concentration of 25 units (U), and a 1:100 dilution of Benzonase[®] in 2X Benzonase[®] buffer was created, resulting in an enzyme concentration of 2.5 U/microliter (μ L) in 2X Benzonase[®] buffer. The 2X Benzonase[®] buffer was prepared with the following concentrations of its different components: 40 millimolar (mM) Tris HCl (pH 8.0), 4 mM MgCl₂, and 40 mM NaCl. It was prepared in a total volume of 100 mL by combining 4

mL of 1M Tris stock (pH 8.0), 0.0381 g of MgCl₂, and 0.2338 g of NaCl and bringing the final volume up to 100 mL with DI water.

2.4 Master-Mix Preparation for Preliminary Experiments

For the preliminary experiments, the Master Mixes were prepared as was stated in the most updated TCDE protocol without any modifications. Four Master Mixes (labeled A through D), as described by the TCDE protocol updated in 2021, were prepared in 1.5 μ L microcentrifuge tubes for speed, convenience, and to reduce errors in pipetting and potential contamination. The Master Mixes had the following volumes of each component per sample (the amounts are number of samples +2 to account for losing volume while pipetting). Master-Mix A for E-cell lysis, which used *forensicGEM*TM from the *forensicGEM*TM Universal kit, was prepared using 50 μ L of 10X BLUE buffer and 3 μ L of *forensicGEM*TM per sample. Master-Mix B, which contains a Benzonase[®] and *forensicGEM*TM Cocktail of specific volumes for the MF, was prepared using 2.5 μ L of 1:10 *forensicGEM*TM in 1X BLUE buffer, 10 μ L of 2.5U/ μ L Benzonase[®] in 2X Benzonase[®] buffer, and 20 μ L of 2X Benzonase buffer per sample. Master-Mix C, which contains Benzonase[®] and *forensicGEM*TM Cocktail with specific volumes for the SF, was prepared using 2.5 μ L of 1:10 *forensicGEM*TM in 1X BLUE buffer and 10 μ L of 2.5U/ μ L Benzonase[®] in 2X Benzonase[®] buffer. Master-Mix D, which contains the AcroSolv and *forensicGEM*TM Sperm mixture for both the MF and SF, was prepared using 20 μ L 10X RED+ Buffer, 40 μ L AcroSolv, and 8 μ L of *forensicGEM*TM Sperm per sample. From

Master-Mix D, 44 μL was added to the MF and 17 μL was added to the SF. Table 1 outlines the Master-Mixes and the volumes of the reagents used per sample.

Table 1. A list of the components of each Master-Mix (A, B, C, D)

<i>Master-Mix</i>	<i>Components per Sample</i>
A	50 μL of 10X BLUE buffer 3 μL of <i>forensicGEM</i> TM
B	2.5 μL of 1:10 <i>forensicGEM</i> TM in 1X BLUE buffer 10 μL of 2.5U/ μL Benzonase [®] in 2X Benzonase [®] buffer 20 μL of 2X Benzonase [®] buffer
C	2.5 μL of 1:10 <i>forensicGEM</i> TM in 1X BLUE buffer 10 μL of 2.5U/ μL Benzonase [®] in 2X Benzonase [®] buffer
D	20 μL of 10X RED+ buffer 40 μL of AcroSolv 8 μL of <i>forensicGEM</i> TM Sperm

2.4.1 Modifications to the Master-Mixes for Separate MF and SF Method

In order to keep the same concentrations and amounts of enzyme in each fraction after modifications were made to the TCDE protocol, such as the addition of the piggyback spin, it became necessary to alter some of the Master-Mixes. The Master-Mixes also had to be adjusted for the Recombined Method being tested for comparison with one recombined MF and SF.

As discussed in Results section 3.2, preliminary experiments showed that an initial incubation for one hour in 1X BLUE buffer produced a greater recovery of male DNA when compared to an incubation in DI water. Thus, to keep the buffer concentration at 1X BLUE buffer for the later steps of the procedure, Master-Mix A was changed to use 50 μL

of 10X BLUE buffer and 447 μL of DI water, which was now added to the tube prior to the initial one hour incubation step. After the one hour incubation, 3 μL of *forensicGEM*[™] was then added directly to each sample prior to incubation in the thermal cycler for E-cell lysis. Master-Mix A and the procedure of adding the *forensicGEM*[™] was equivalent when extraction was performed for either of the methods being compared.

Master-Mix B used for the separate MF and SF fractions contains the components necessary for nuclease treatment, which was a Benzonase[®] and *forensicGEM*[™] cocktail, but for just the MF. A 1:13 dilution of *forensicGEM*[™] in 10X BLUE buffer was prepared, with 1 μL of *forensicGEM*[™] and 12 μL of 10X BLUE Buffer per sample. A total of 13 μL of this dilution was added to Master-Mix B for each sample, along with 10 μL of 2.5U/ μL in 2X Benzonase[®] buffer, 49 μL of 2X Benzonase[®] buffer, and 109 μL of DI water. For better consistency between the methods, this Master-Mix B specifically for the MF was renamed Master-Mix B1.

For the Recombined MF and SF method, Master-Mix C is no longer necessary. Thus, for the Separate MF and SF method, the Master-Mix with the Benzonase[®] and *forensicGEM*[™] cocktail for nuclease treatment specifically for the SF was relabeled Master-Mix B2. This Master-Mix B2 for the SF consisted of 1 μL of *forensicGEM*[™], 10 μL of 2.5U/ μL Benzonase[®] in 2X Benzonase[®] buffer, and 4 μL of 2X Benzonase buffer.

The newly modified Master-Mixes C1 and C2 for the Separate MF and SF Method now consists of two different AcroSolv and *forensicGEM*[™] Sperm cocktails for the digestion of the MF and SF, respectively. The new Master-Mix C1 for the digestion of the MF was prepared with 22 μL 10X RED+ buffer, 26 μL AcroSolv enzyme, and 5 μL

*forensicGEM*TM Sperm (stock) enzyme per sample. The new Master-Mix C2 for the digestion of the SF was prepared with 6 μ L 10X RED+ buffer, 10 μ L AcroSolv enzyme, and 2 μ L *forensicGEM*TM Sperm enzyme per sample. Table 2 lists all the modifications made to the Master-Mixes for the Separate MF and SF Method.

Table 2. A list of the components of each Master-Mix (A, B1, B2, C1, C2) for the modified Separate MF and SF Method.

<i>Master-Mix</i>	<i>Components per Sample</i>
A	50 μ L of 10X BLUE buffer 447 μ L of DI water (3 μ L of <i>forensicGEM</i> TM added after incubation)
B1	13 μ L of 1:13 dilution of <i>forensicGEM</i> TM in 10X BLUE 10 μ L of 2.5U/ μ L Benzonase [®] in 2X Benzonase [®] buffer 49 μ L of 2X Benzonase [®] buffer 109 μ L of DI water
B2	1 μ L of <i>forensicGEM</i> TM 10 μ L of 2.5U/ μ L Benzonase [®] in 2X Benzonase [®] buffer 4 μ L of 2X Benzonase [®] buffer
C1	22 μ L of 10X RED+ buffer 26 μ L of AcroSolv 5 μ L of <i>forensicGEM</i> TM Sperm
C2	6 μ L of 10X RED+ buffer 10 μ L of AcroSolv 2 μ L of <i>forensicGEM</i> TM Sperm

2.4.2 Modifications to the Master-Mixes for Recombined MF and SF Method

For the Recombined MF and SF Method, Master-Mix B also consisted of the Benzonase[®] and *forensicGEM*TM cocktail for nuclease treatment. A 2:11 dilution of *forensicGEM*TM in 10X BLUE Buffer was prepared, with 2 μ L of *forensicGEM*TM and 9 μ L of 10X BLUE Buffer, to account for the total units of enzyme and concentration of buffer needed for a recombined MF and SF digestion. A total of 11 μ L of this 2:9 dilution

was added to Master-Mix B for each sample, 20 μL of 2.5U/ μL Benzonase[®] in 2X Benzonase[®] buffer, 38 μL of 2X Benzonase[®] buffer, and 82 μL of DI water. This essentially combined the amounts of Benzonase[®] used for the MF and SF digestions into one Master-Mix, while also keeping the other concentrations within a similar range. A similar idea was used to create the modified Master-Mix C for the Recombined MF and SF Method, which is the AcroSolv and *forensicGEM*[™] Sperm cocktail for the digestion of the recombined MF and SF. The adjusted volumes of Master-Mix C for this method were 25 μL 10X RED+ buffer, 36 μL AcroSolv enzyme, and 7 μL *forensicGEM*[™] Sperm (stock) enzyme. The table below (Table 3) lists all the modifications made to the Master-Mixes for the Recombined MF and SF Method.

Table 3. A list of the components of each Master-Mix (A, B1, B2, C1, C2) for the modified Recombined MF and SF Method.

<i>Master-Mix</i>	<i>Components per Sample</i>
A	50 μL of 10X BLUE buffer 447 μL of DI water (3 μL of <i>forensicGEM</i> [™] added after incubation)
B	11 μL of 2:9 dilution of <i>forensicGEM</i> [™] in 10X BLUE 20 μL of 2.5U/ μL Benzonase [®] in 2X Benzonase [®] buffer 38 μL of 2X Benzonase [®] buffer 82 μL of DI water
C	25 μL of 10X RED+ buffer 36 μL of AcroSolv 7 μL of <i>forensicGEM</i> [™] Sperm

2.5 Extraction Procedures

2.5.1 Modified TCDE Procedure Resulting in Separate MF and SF Extracts

Using a half swab sample, the initial incubation at room temperature was performed with the adjusted Master-Mix A that results in a 1X BLUE buffer concentration. After the incubation, 3 μL of *forensicGEM*TM was added to each tube for the E-cell lysis. The tubes were incubated at 75°C for 15 minutes and 4°C for 5 minutes. After removing the tubes from the thermal cycler, a piggyback spin was performed using spin columns and 2.0 μL microcentrifuge tubes to release the liquid from the swab. The liquid in the 2.0 μL tube was then vortexed to resuspend any sperm pellet that potentially formed before returning the liquid to the tube labeled “SF” with a micropipette. The dry swabs in the spin column were moved to new 0.5 mL tubes labeled “MF” with sterile tweezers.

The SF tube with all the liquid returned was centrifuged at 14000 rpm for 5 minutes to form a sperm pellet at the bottom of the tube. The supernatant of 470 μL was then removed, leaving approximately 30 μL over the sperm cell pellet (assuming not much liquid is still retained by swab after the piggyback spin). This liquid was placed into a new 0.5 mL tube labeled “EF,” while the sperm pellet was resuspended in the remaining liquid in the SF tube by vortexing.

For the Benzonase[®] digest of the MF, 109 μL of the newly modified Master-Mix B1 was added to the MF tubes. For the Benzonase[®] digestion of the SF, 15 μL of the newly modified Master-Mix B2 was added to the SF tubes before incubation. The MFs and SFs were incubated in the thermal cycler at 37°C for 15 minutes, 75°C for 15 minutes,

and 4°C for 5 minutes (the same as the previous method) before being vortexed and removed.

Next, the AcroSolv and *forensicGEM*TM Sperm digestion was slightly modified for this new method with two new Master-Mixes (C1 and C2) for the MF and SF digest. For this step, 53 µL of Master-Mix C1 was added to each MF tube and 18 µL of Master-Mix C2 was added to each SF tube. For sperm lysis, these fractions were incubated at 52°C for 5 minutes, 75°C for 3 minutes, 95°C for 3 minutes, and 4°C for 5 minutes. The tubes were then removed from the thermal cycler. The swab in the MF was removed from the liquid using sterile tweezers and then spun down in the spin basket in a 1.5 mL tube. The swab substrate was discarded and the liquid was returned to the original 0.5 mL tube after vortexing the sample.

Lastly, the *forensicGEM*TM enzyme in the EF tubes was inactivated by incubating the tubes at 95°C for 5 minutes and 4°C for 5 minutes. All three fractions (the EF, MF, and SF) were then stored at -20°C and were ready for downstream analysis.

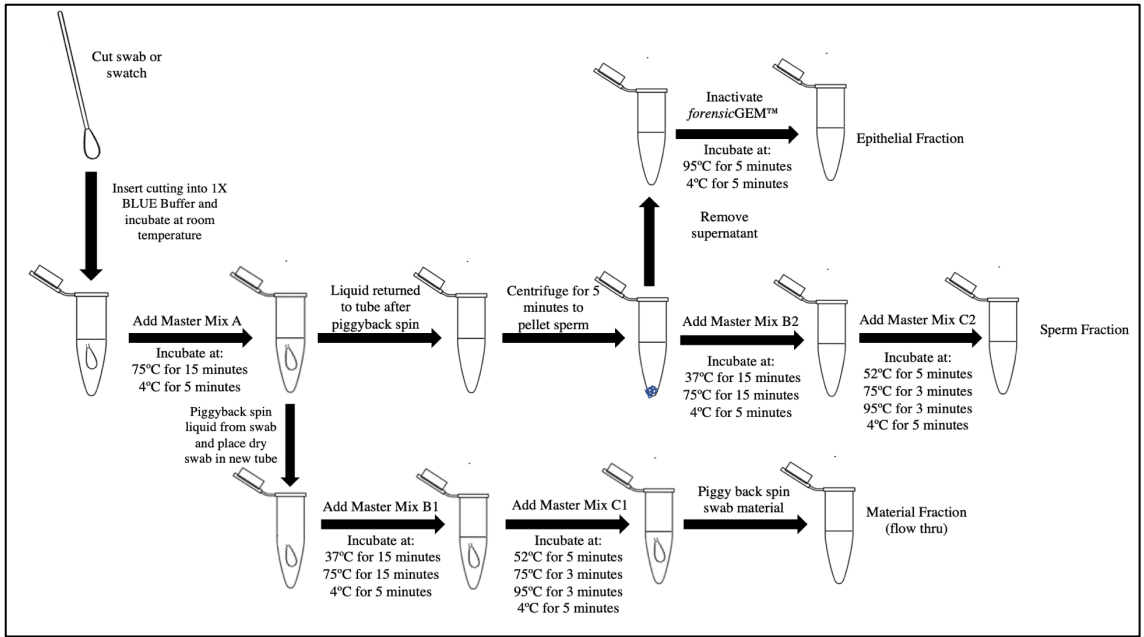


Figure 4. A diagram of the TCDE protocol with modifications for the separate MF and SF method.

2.5.2 Modifications to the Procedure Resulting in a Recombined MF and SF

For this recombination method, each Master-Mix essentially represented a combination of the Master-Mixes used in each step for the MF and the SF (as it is the recombined MF and SF being digested). The same procedure for the E-cell lysis was followed.

Prior to conducting the Benzonase® digest of either the MF or SF fraction, the dry swab that would normally be placed in a MF tube was placed in a 1.5 mL microcentrifuge tube for short-term storage. After the spin down to form the sperm pellet and the transfer of the supernatant to the EF tube, the swab was returned to the resuspended SF tube which was then labeled “MF/SF.” Proceeding with the Benzonase® digest, a total of 151 µL of the modified Master-Mix B specific to this recombination method was added to the MF/SF tube. The incubation in the thermal cycler was performed in the same manner.

For the AcroSolv and *forensicGEM*TM Sperm digest of the combined MF + SF tubes, 68 μ L of the modified Master-Mix C specific to this recombination method was added to the tubes. The rest of the procedure was performed as described.

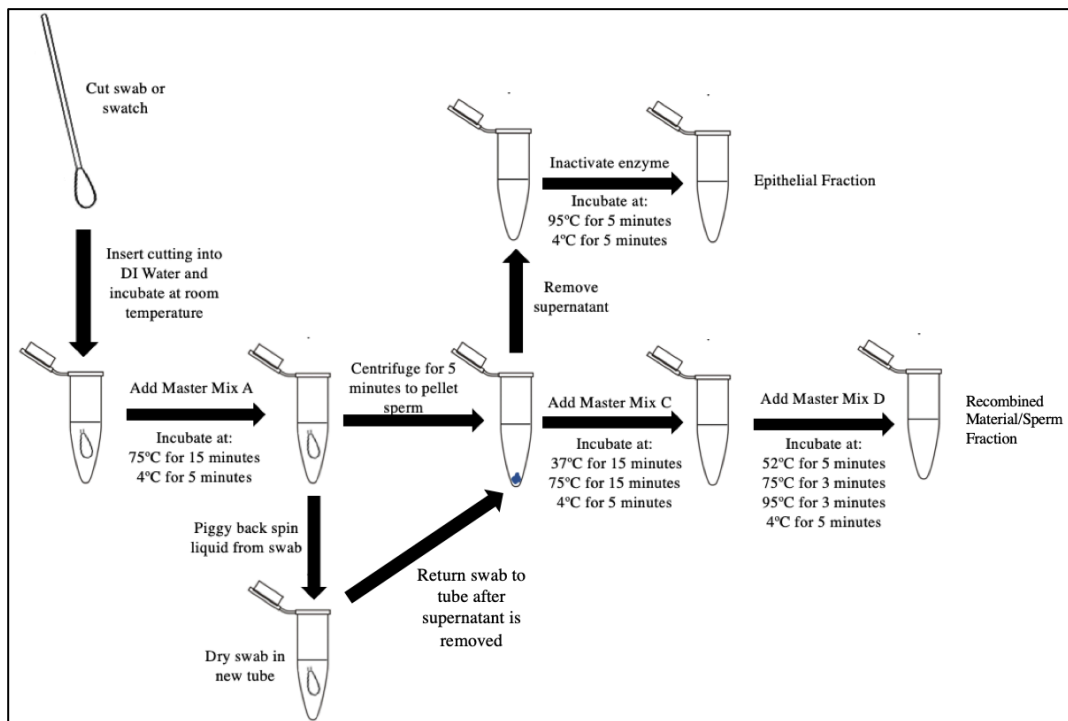


Figure 5. A diagram of the TCDE protocol with modifications for the recombined MF and SF method.

2.6 Quantitation, Amplification, and Capillary Electrophoresis

The quantitation of the extracts was performed using the qPCR technique. The 7500 Real-Time PCR instrument (Applied Biosystems, Foster City, CA) was operated according to the manufacturer's specifications after preparing samples using the Quantifiler[®] Trio Kit (Applied Biosystems, Foster City, CA). The DNA concentration values obtained (in ng/ μ L) with qPCR were determined using a set standard curve programmed into the software based on previous values from known standards. In the

preliminary experiments, the quantitation was performed in duplicate for each sample and an average was taken of the two concentration values. Because the concentration values of these replicates were highly reproducible, a single value was obtained for each sample for the mock casework. The total amount of human DNA (in nanograms (ng)) in a sample was calculated by multiplying the total volume of liquid (in μL) in the fraction by the small autosomal concentration value. Similarly, the Y concentration value and the volume of liquid was used to determine the total amount of male DNA (in ng) in a fraction. The estimated total amount of female DNA was calculated by taking the difference between the total amount of human DNA and the total amount of male DNA in the sample.

Amplifications of the samples used 0.75 ng as the target amount. Amplification was performed in a Veriti[®] ThermalCycler (Applied Biosystems, Foster City, CA) using the GlobalFiler[®] PCR Amplification Kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions.

After amplification, the separation of the DNA fragments was performed using Capillary Electrophoresis (CE) on the SeqStudio[™] Genetic Analyzer (Applied Biosystems, Foster City, CA) according to manufacturer's instructions.

The analysis of the CE results was performed using the GeneMapper[®] ID-X version 1.6 (Applied Biosystems, Foster City, CA) software. The profiles that are produced can be visualized in the form of an electropherogram. The settings used for the analysis of these mock casework samples are the standard forensic settings, with a set stochastic threshold of 250 relative fluorescence units (RFU) and a set analytical threshold of 30 RFUs.

3. RESULTS

3.1 Repeat Sperm Digestion of Material Fraction

The digestion of the MF has been proven necessary because, for many samples, a significant number of sperm cells adhere to the swab and are not collected in the sperm pellet (54). An experiment was conducted to determine if a second digestion of the MF yielded significantly greater sperm DNA recovery. A 1:10 and 1:100 dilution of two different semen donors (donor 1 and donor 2) were performed, and 10 μ L of each dilution for each donor was placed on a swab. The exact amount of male DNA on each swab was unknown. The modified TCDE protocol was performed without the use of the Benzonase[®] nuclease since no epithelial cells were present on these swabs. Once the initial MF underwent digestion by an Acrosolv and *forensicGEM*[™] Sperm cocktail, a piggyback spin was performed to release the liquid from the swab, which was placed back into the MF tube 1 (MF1). The swab was placed in a new tube, where it underwent a second Acrosolv and *forensicGEM*[™] Sperm digestion (the extract of this second digestion was labeled MF2). Using the qPCR Y concentration values, the amount of male DNA in MF1 and MF2 was calculated (Table 4).

Table 4. Calculated amounts of DNA (ng) in the First and Second Material Fraction digests using qPCR concentration values and elution volume of MFs (μL). Donor 1 is -1 and Donor 2 is -2.

Sample Name	Male DNA in MF1 of Whole Swab (ng)	Male DNA in MF2 of Whole Swab (ng)	Total Amount of Male DNA in MFs (ng)	Proportion of Male DNA in MF1	Proportion of Male DNA in MF2
1:10-1	68.1173	1.1805	69.2978	0.9830	0.0170
1:10-2	12.9684	1.6032	14.5716	0.8900	0.1100
1:100-1	8.9828	0.2263	9.2091	0.9754	0.0246
1:100-2	2.7359	1.1756	3.9115	0.6994	0.3006

These results showed that across the two different donors and different semen dilutions, a much larger amount of the male DNA being released from sperm was digested in the first MF compared to the additional MF digest. Comparing the proportion of male DNA being extracted in each MF, the average amount of DNA eluted into MF1 was approximately 70 to 100% of the total male DNA eluted in both of the fractions. Thus, it appears that a single MF is sufficient enough to recover a large number of sperm cells without an additional digest. However, the proportions of male DNA recovered were based on the total amount of male DNA released in material digests 1 and 2 and do not account for any possible male DNA that still remains on the cotton swab after both digests.

3.2 Efficiency of Male DNA Recovery Using Various Extraction Buffers

The initial incubation of the half swab at room temperature for one hour was typically conducted in DI water in the previous research. To test if the use of a different extraction buffer yields different recovery rates of male DNA due to a better release of

sperm cells from the swab, three different buffers were tested using swabs with sperm only. The buffers that were used for the incubation were 1X BLUE buffer, AutoMACS® Running Buffer, and a DI water control. AutoMACS® Running Buffer was tested because previous research on obtaining intact whole cells from cotton swabs had good success with cell recovery when using this as the elution buffer (59). For this experiment, three swabs of each dilution of semen, 1:50, 1:100, and 1:500, were prepared with no female epithelial cells. The TCDE protocol was performed using the initial one hour incubation step with one whole swab per buffer condition. Because there were no epithelial cells to digest, the Benzonase® nuclease digest steps were not performed. The SF and EF both underwent digestion with the Acrosolv and *forensic*GEM™ Sperm cocktail. The total amount of male DNA in each fraction was calculated using the qPCR concentration values and the volume of liquid in each fraction after extraction (47 µL for the SF and 210 µL for the EF). By totaling the amounts of male DNA in all the fractions, the proportion of the male DNA in each fraction was calculated, assuming 100% recovery from the swabs (Table 5).

Table 5. Proportion of Male DNA in Sperm Fraction and Supernatant Fraction using qPCR concentration values and elution volumes.

Sample Name	Total Amount of DNA in SF for Whole Swab (ng)	Total Amount of DNA in EF for Whole Swab (ng)	Total Amount in Whole Swab (ng)	Proportion of DNA in SF	Proportion of DNA in EF
H2O 1:50	5.6530	1.4973	7.1503	0.7906	0.2094
H2O 1:100	2.5602	0.9635	3.5237	0.7266	0.2734
H2O 1:500	0.2052	0.1439	0.3491	0.5879	0.4121
BLUE 1:50	12.1352	1.1290	13.2642	0.9149	0.0851
BLUE 1:100	8.0181	0.8339	8.8520	0.9058	0.0942
BLUE 1:500	0.2102	0.1161	0.3263	0.6441	0.3559
AUTO 1:50	12.9517	3.0851	16.0368	0.8076	0.1924
AUTO 1:100	9.6689	1.7511	11.4200	0.8467	0.1533
AUTO 1:500	0.8170	0.3648	1.1817	0.6913	0.3087

While the three buffers performed similarly, the use of 1X BLUE Buffer as the extraction buffer produced the highest average proportion of male DNA in the SF, where it is most desired. Over 90% of the total male DNA was recovered in the SF for the 1:50 and 1:100 dilutions using 1X BLUE buffer. Ultimately, because the desired final concentration of BLUE Buffer in the initial *forensicGEM*TM digest was already 1X, it was decided that the initial swab incubation at room temperature would be performed in 1X BLUE Buffer in the modified methods. Thus, 3 μ L of *forensicGEM*TM was then added to each tube after the room temperature incubation, rather than the normal addition of Master Mix A with 3 μ L of *forensicGEM*TM in 1X BLUE Buffer.

3.3 Varying *forensicGEM*TM Concentration and Sperm Recovery

To understand if increasing the *forensicGEM*TM concentration in the initial E-cell lysis step improved downstream sperm recovery, varying *forensicGEM*TM concentrations (1X, 2X, and 3X) were tested. Swabs were prepared in the same manner with 10 μ L of a 1:50 semen dilution (without epithelial cells), and half swabs were initially incubated in solutions with different concentrations of *forensicGEM*TM (1X was 3 μ L, 2X was 6 μ L, and 3X was 9 μ L of *forensicGEM*TM). The normal TCDE protocol was performed with an Acrosolv and *forensicGEM*TM Sperm digestion, and the distribution of sperm into the various fractions (SF, EF, and left on swab (MF)) was quantified using qPCR. With the desired outcome being the majority of sperm distributed in the SF, the various concentrations were compared with regards to their effectiveness in achieving that distribution (Table 6).

Table 6. Proportion of Male DNA in Sperm Fraction, Epithelial Fraction, and Left on the Swab with Varying *forensicGEM*[™] Concentrations at the Initial Lysis

Sample Name	Proportion of DNA in SF	Proportion of DNA in EF	Proportion Left on Swab (MF)
1:50 1X-1	0.2885	0.1415	0.5701
1:50 1X-2	0.3064	0.1089	0.5847
1:50 2X-1	0.2554	0.1187	0.6259
1:50 2X-2	0.1303	0.1108	0.7589
1:50 3X-1	0.1548	0.0445	0.8007
1:50 3X-2	0.2007	0.0825	0.7168

These results do not indicate that a relationship exists between the concentration of the enzyme and the proportion of male DNA in the SF. There appears to actually be a higher proportion of DNA in the SF at the lowest concentration of the enzyme, 1X (Table 6). Because of these results, it was determined that the normal concentration of *forensicGEM*[™] in the initial digest was sufficient for further experimentation with the TCDE protocol. There was no benefit to sperm recovery with the use of higher enzyme concentrations.

3.4 Mock Casework Swab Analysis

After making modifications to improve certain aspects of the procedure with the goal of accomplishing better sperm DNA recovery, it was essential to test the modified protocol on mock casework samples. In this case, the mock casework samples were vaginal swabs from eight different female donors with a certain amount of semen, from one single male donor, applied to each swab. These swabs were extracted using either the modified Separate Method or the Recombined Method TCDE protocol. By dividing these swabs among both the Separate Method and the Recombined Method, a comparison of the

efficiency and effectiveness of these two procedures in achieving clean male sperm recovery could be accomplished. Because testing was performed on half swabs that are obtained through the manual slicing of swab material using a sterile scalpel, it was expected that there would be a different number of vaginal and sperm cells on each half. Both halves from the same swab underwent the same extraction protocol to avoid making this an added variable in the comparison. Quantification of the amount of female and male DNA in each fraction was useful in determining the separation efficiency and sperm DNA recovery. Downstream analysis, including the amplification of select samples and the generation of profiles by CE, was done in an attempt to assess the applicability of these procedures to sexual assault casework, where a clean male profile is the desired outcome.

3.4.1 Quantitation of Female and Male DNA with the Separate Method

Each of the fractions of these mock casework swabs were quantitated using qPCR for both methods. For the Separate Method, the procedure still resulted in three different fractions (the EF, MF, and SF). Because 32 half swabs were extracted for this method, the resulting number of samples quantitated using the Quantifiler[®] Trio kit was a total of 96 samples. For each of these samples, the total human DNA (in ng) was calculated using the small autosomal DNA concentration value (in ng/ μ L) for each fraction and the total volume of the fraction (in μ L), while the total male DNA (in ng) was calculated using the Y DNA concentration value (in ng/ μ L) and the total volume of the fraction (in μ L). The estimated total amount of female DNA was calculated as the difference between the total human DNA value and the total male DNA value. It is necessary to compare the total amount of

DNA in each fraction rather than the concentration because the ending volumes of the liquid in the fractions differ. The following table displays the concentration values for each fraction of these different samples where extraction was performed using the Separate Method, along with the subsequent calculations to determine the amount of DNA in ng (Table 7).

Table 7. Total amounts of male and female DNA in each fraction (EF, MF, and SF) from each half-swab extracted with the Separate Method, as calculated using the qPCR values and approximate elution volumes

Sample Name	Quantity of Total Human (ng/ul)	Quantity of Total Male (ng/ul)	Volume of Liquid (ul)	Total Human DNA in Half Swab (ng)	Total Male DNA in Half Swab (ng)	Estimated Female DNA in Half Swab (ng)
1A-1 EF	0.036	0.000	470	16.719	0.000	16.719
1A-1 MF	6.381	0.156	234	1493.255	36.571	1456.684
1A-1 SF	0.341	0.102	63	21.467	6.448	15.019
1A-2 EF	0.000	0.000	470	0.219	0.000	0.219
1A-2 MF	63.110	0.026	234	14767.726	6.045	14761.681
1A-2 SF	2.209	0.105	63	139.150	6.632	132.518
1B-1 EF	0.008	0.000	470	3.917	0.000	3.917
1B-1 MF	5.564	0.224	234	1301.882	52.377	1249.505
1B-1 SF	0.537	0.272	63	33.849	17.105	16.744
1B-2 EF	0.000	0.001	470	0.170	0.320	0.000
1B-2 MF	45.290	0.837	234	10597.751	195.812	10401.939
1B-2 SF	1.008	0.779	63	63.482	49.102	14.380
2A-1 EF	3.312	0.003	470	1556.417	1.213	1555.205
2A-1 MF	0.152	0.003	234	35.493	0.600	34.893
2A-1 SF	0.145	0.177	63	9.138	11.134	0.000
2A-2 EF	3.389	0.003	470	1592.667	1.177	1591.490
2A-2 MF	0.612	0.011	234	143.164	2.512	140.652
2A-2 SF	0.263	0.251	63	16.542	15.835	0.706
2B-1 EF	3.435	0.017	470	1614.265	8.044	1606.221
2B-1 MF	0.369	0.114	234	86.320	26.585	59.735
2B-1 SF	1.234	1.431	63	77.770	90.150	0.000
2B-2 EF	4.059	0.004	470	1907.625	1.856	1905.769
2B-2 MF	0.939	0.047	234	219.671	11.063	208.608
2B-2 SF	0.518	0.549	63	32.658	34.556	0.000
3A-1 EF	15.619	0.004	470	7340.995	1.882	7339.113
3A-1 MF	0.206	0.058	234	48.165	13.566	34.599

Sample Name	Quantity of Total Human (ng/ μ L)	Quantity of Total Male (ng/ μ L)	Volume of Liquid (μ L)	Total Human DNA in Half Swab (ng)	Total Male DNA in Half Swab (ng)	Estimated Female DNA in Half Swab (ng)
3A-1 SF	0.337	0.341	63	21.203	21.473	0.000
3A-2 EF	7.180	0.000	470	3374.627	0.066	3374.561
3A-2 MF	0.041	0.000	234	9.573	0.045	9.528
3A-2 SF	0.024	0.002	63	1.486	0.107	1.378
3B-1 EF	15.167	0.009	470	7128.553	4.201	7124.352
3B-1 MF	0.047	0.029	234	10.990	6.723	4.267
3B-1 SF	0.610	0.657	63	38.421	41.410	0.000
3B-2 EF	4.692	0.008	470	2205.442	3.565	2201.877
3B-2 MF	0.149	0.122	234	34.832	28.565	6.266
3B-2 SF	1.246	1.332	63	78.476	83.892	0.000
4A-1 EF	0.292	0.000	470	137.358	0.000	137.358
4A-1 MF	46.131	0.029	234	10794.688	6.778	10787.910
4A-1 SF	0.363	0.066	63	22.887	4.152	18.735
4A-2 EF	0.020	0.000	470	9.577	0.000	9.577
4A-2 MF	24.843	0.033	234	5813.263	7.678	5805.584
4A-2 SF	86.950	0.169	63	5477.878	10.635	5467.243
4B-1 EF	6.956	0.004	470	3269.144	1.786	3267.357
4B-1 MF	0.409	0.072	234	95.713	16.755	78.958
4B-1 SF	1.420	1.372	63	89.479	86.453	3.025
4B-2 EF	1.425	0.001	470	669.712	0.299	669.412
4B-2 MF	5.972	0.182	234	1397.514	42.566	1354.948
4B-2 SF	1.109	0.829	63	69.874	52.219	17.655
5A-1 EF	0.019	0.000	470	9.076	0.000	9.076
5A-1 MF	33.830	0.047	234	7916.224	10.964	7905.260
5A-1 SF	0.348	0.023	63	21.896	1.471	20.425
5A-2 EF	0.001	0.000	470	0.298	0.000	0.298
5A-2 MF	47.216	0.168	234	11048.501	39.238	11009.263
5A-2 SF	0.116	0.081	63	7.318	5.106	2.212
5B-1 EF	0.028	0.000	470	13.000	0.000	13.000
5B-1 MF	25.430	1.088	234	5950.662	254.552	5696.110
5B-1 SF	0.405	0.357	63	25.522	22.474	3.048
5B-2 EF	0.004	0.000	470	2.099	0.216	1.883
5B-2 MF	24.813	0.845	234	5806.222	197.797	5608.425
5B-2 SF	1.024	1.225	63	64.519	77.174	0.000
6A-1 EF	2.022	0.000	470	950.175	0.159	950.016
6A-1 MF	0.615	0.026	234	143.928	6.058	137.871
6A-1 SF	0.784	0.104	63	49.363	6.531	42.832

Sample Name	Quantity of Total Human (ng/ μL)	Quantity of Total Male (ng/ μL)	Volume of Liquid (μL)	Total Human DNA in Half Swab (ng)	Total Male DNA in Half Swab (ng)	Estimated Female DNA in Half Swab (ng)
6A-2 EF	1.657	0.000	470	778.726	0.126	778.600
6A-2 MF	1.163	0.029	234	272.084	6.844	265.240
6A-2 SF	1.228	0.337	63	77.352	21.232	56.120
6B-1 EF	1.638	0.002	470	769.898	0.875	769.022
6B-1 MF	2.676	0.214	234	626.187	50.193	575.994
6B-1 SF	0.778	0.597	63	49.037	37.584	11.453
6B-2 EF	1.086	0.010	470	510.464	4.829	505.635
6B-2 MF	3.938	0.345	234	921.413	80.702	840.711
6B-2 SF	2.014	2.205	63	126.906	138.920	0.000
7A-1 EF	0.389	0.000	470	182.771	0.000	182.771
7A-1 MF	29.191	0.194	234	6830.661	45.323	6785.338
7A-1 SF	0.504	0.138	63	31.783	8.684	23.100
7A-2 EF	0.142	0.000	470	66.585	0.000	66.585
7A-2 MF	8.367	0.044	234	1957.988	10.293	1947.695
7A-2 SF	0.947	0.082	63	59.665	5.181	54.483
7B-1 EF	2.020	0.004	470	949.390	1.904	947.485
7B-1 MF	5.580	0.661	234	1305.730	154.736	1150.994
7B-1 SF	1.068	0.958	63	67.268	60.331	6.937
7B-2 EF	0.000	0.000	470	0.208	0.000	0.208
7B-2 MF	19.216	0.673	234	4496.516	157.465	4339.051
7B-2 SF	0.832	0.830	63	52.385	52.262	0.123
8A-1 EF	2.546	0.000	470	1196.542	0.000	1196.542
8A-1 MF	7.155	0.081	234	1674.221	18.845	1655.375
8A-1 SF	2.400	0.113	63	151.200	7.101	144.099
8A-2 EF	0.585	0.001	470	274.860	0.473	274.387
8A-2 MF	0.907	0.059	234	212.197	13.695	198.502
8A-2 SF	2.046	0.307	63	128.913	19.345	109.568
8B-1 EF	0.309	0.002	470	145.206	0.723	144.483
8B-1 MF	14.004	0.359	234	3276.929	83.944	3192.985
8B-1 SF	1.718	1.163	63	108.212	73.299	34.913
8B-2 EF	2.284	0.001	470	1073.614	0.699	1072.915
8B-2 MF	4.885	0.845	234	1143.108	197.666	945.442
8B-2 SF	1.762	0.415	63	111.036	26.156	84.880

3.4.2 Female DNA Distribution for the Separate Method

3.4.2.1 Range of Female DNA

The total amount of female DNA for each sample when all the fractions are added together ranges from approximately 500 ng to 15000 ng. In general, there is variability observed in the total amount of female DNA on each half of one swab as shown in Figure 6. Additionally, there is great variation in what female donors contribute in terms of amounts of epithelial cells on to the vaginal swabs, as shown in Figure 7. This variability between half swabs and this range of total amounts of female DNA seen on these samples are similar to data from sets of vaginal swabs from other projects conducted within the Cotton Lab.

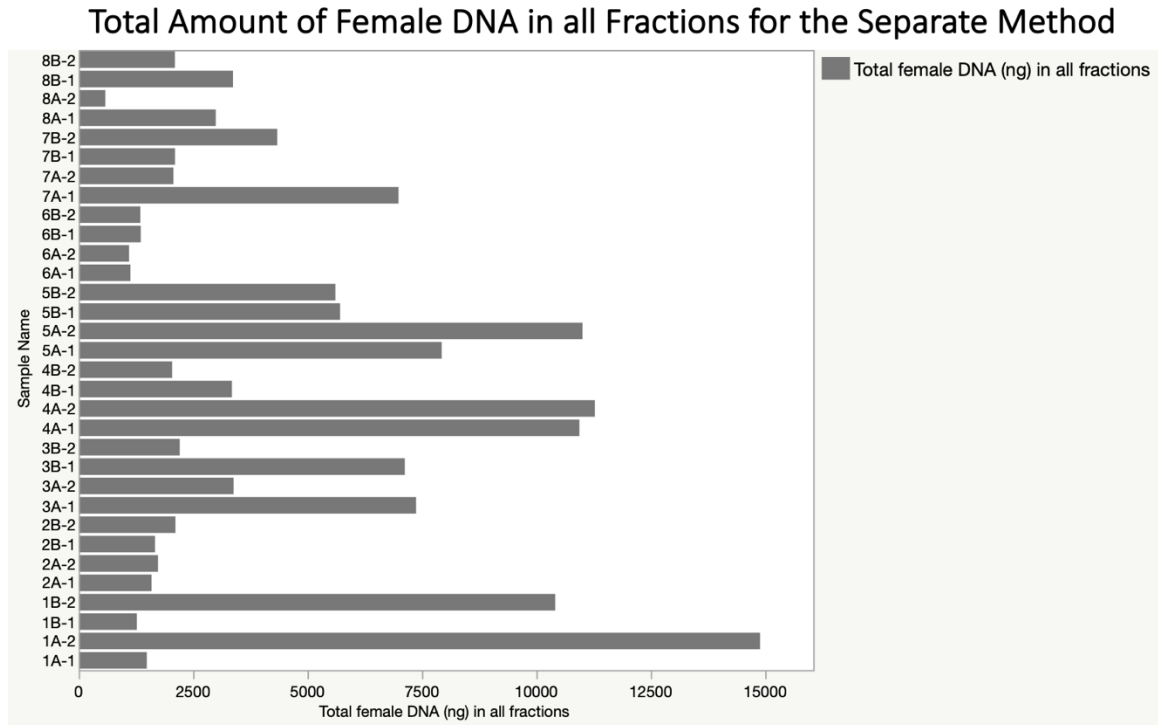


Figure 6. Total amount of female DNA in all of the fractions from one half-swab sorted by donor and swab

Total Amount of Female DNA in all Fractions for Separate Method in Ascending Order

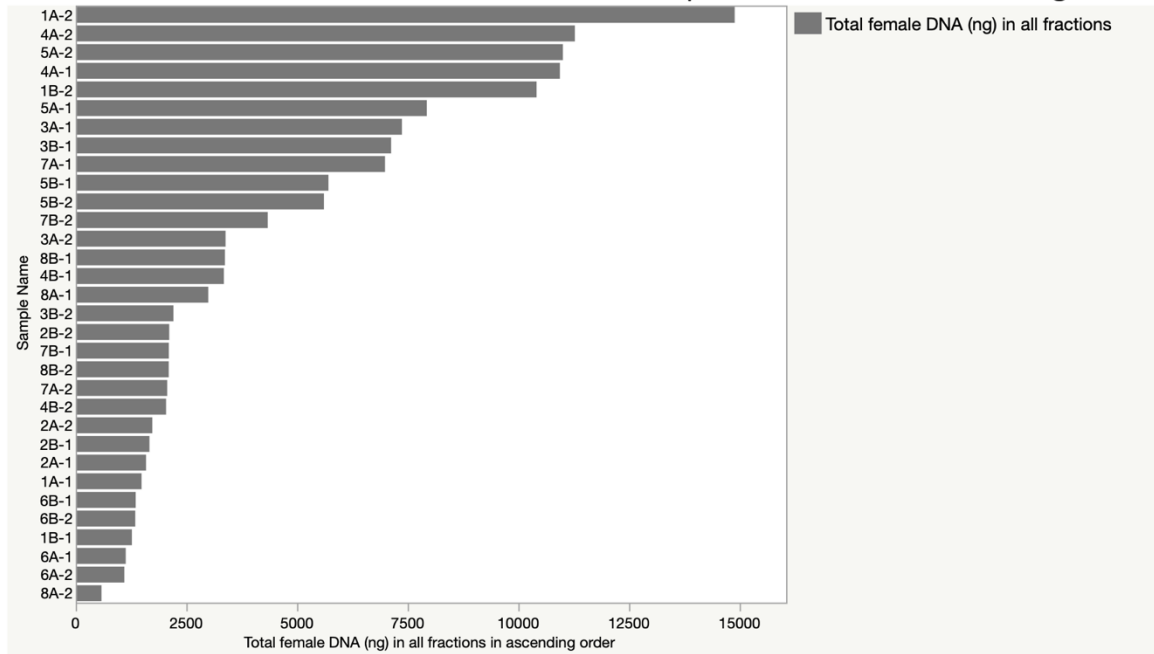


Figure 7. Total amount of female DNA in all of the fractions from one half-swab sorted in ascending order from largest amounts to smallest

3.4.2.2 Female DNA Distribution in the Fractions

Figure 8 summarizes the amounts of female DNA in the EF, MF, and SF for all 16 swabs (32 half swabs) tested with this method. Considering these results as a whole, the distribution of the female DNA among the three fractions was very unexpected for several samples. In particular, there are unusually low values of total female DNA in the EFs of samples from swabs 1A and 1B, 5A and 5B, and to some degree the samples from Donors 7 and 8. These results suggest that there was no lysis of the E-cells by the *forensicGEM*TM enzyme and no release of the female DNA into the EF solution. In the samples that have unexpected results for the EF, the female DNA was carried over into the MF, resulting in unusually high proportions of female DNA in the MFs up to 99.9%. In these MFs, the

female DNA would not have been removed by the Benzonase[®] nuclease if the cells were still intact at that step, and thus it would have been released in the last steps of the protocol due to the addition of Acrosolv and *forensicGEM*[™] Sperm. Overall, these results suggest that the *forensicGEM*[™] enzyme was not functioning properly in the initial cell lysis step. Thus, the Benzonase[®] nuclease digestion was ultimately unable to degrade the female DNA (as the female DNA had not yet been released from the cells and was not in solution at the time in which the Benzonase[®] nuclease was active). While the goal of this research was mainly focused on achieving better recovery of the male DNA and improving the distribution of the male DNA into the sperm fraction, in some samples, the analysis of that aspect of the project became complicated by the unexpected carryover of a large amount of female DNA into the MF.

It should be noted, however, that these results are observed to be acting in a manner that is highly dependent on the female vaginal swab donor. The extraction of most of the swabs from a single donor was performed with either the predicted result where an initial E-cell lysis creating an EF with the majority of the total female DNA, or the unexpected result where no initial E-cell lysis occurred and almost all of the female DNA was carried over into the MF. The following graph helps to better visualize the apparent relationship between the female donor and the distribution of the female DNA (Figure 8).

Female DNA Distribution in All Fractions Using the Separate Method

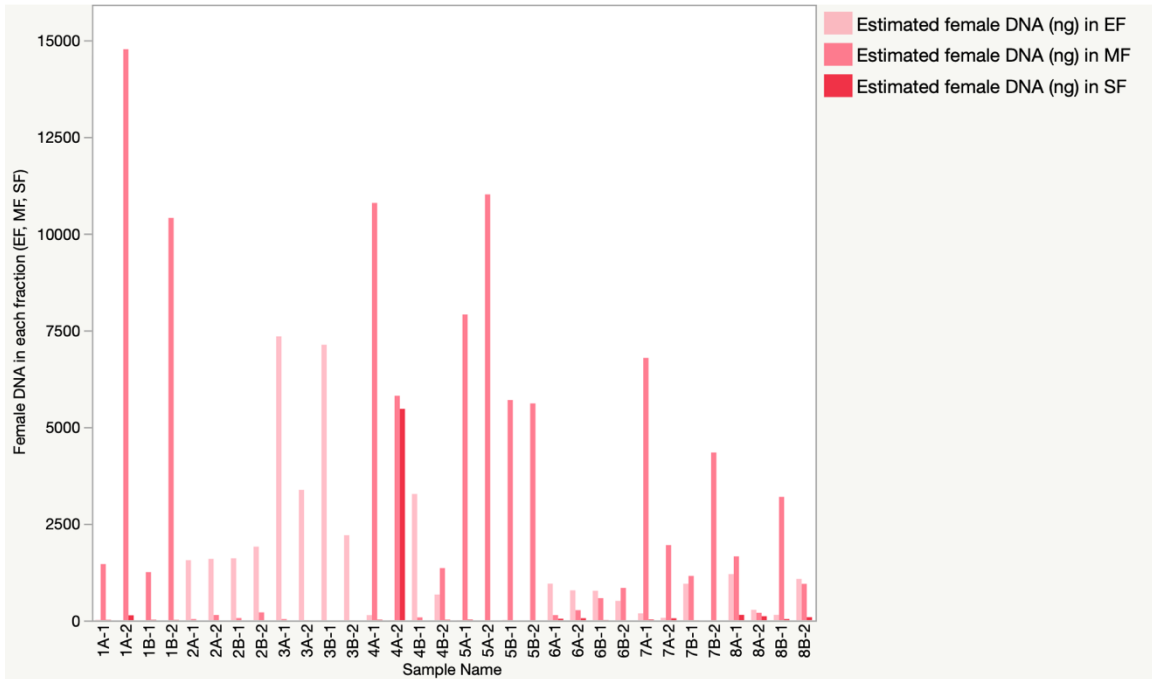


Figure 8. Bar graph depicting the female DNA distribution in each fraction (in ng) for all samples extracted with the Separate Method

The lack of E-cell lysis in certain donor groups of half swabs (for example, Donor 1 and Donor 5) is quite obvious. It is also quite obvious which donor groups have expected data, resulting in an E-cell fraction with the majority of the female DNA (for example Donors 2 and 3). However, there is also no clear correlation between the observation of no DNA in the EF and the total amount of female DNA on the swab. The following graphs highlight results from two specific donors. Donor 3 has four half swabs which fall into the “expected” results for the EF category (Figure 9). In contrast for Donor 5, the four half swabs fall into the “unexpected” results for the EF category (Figure 10).

Female DNA Distribution for Samples from Donor 3 Using the Separate Method

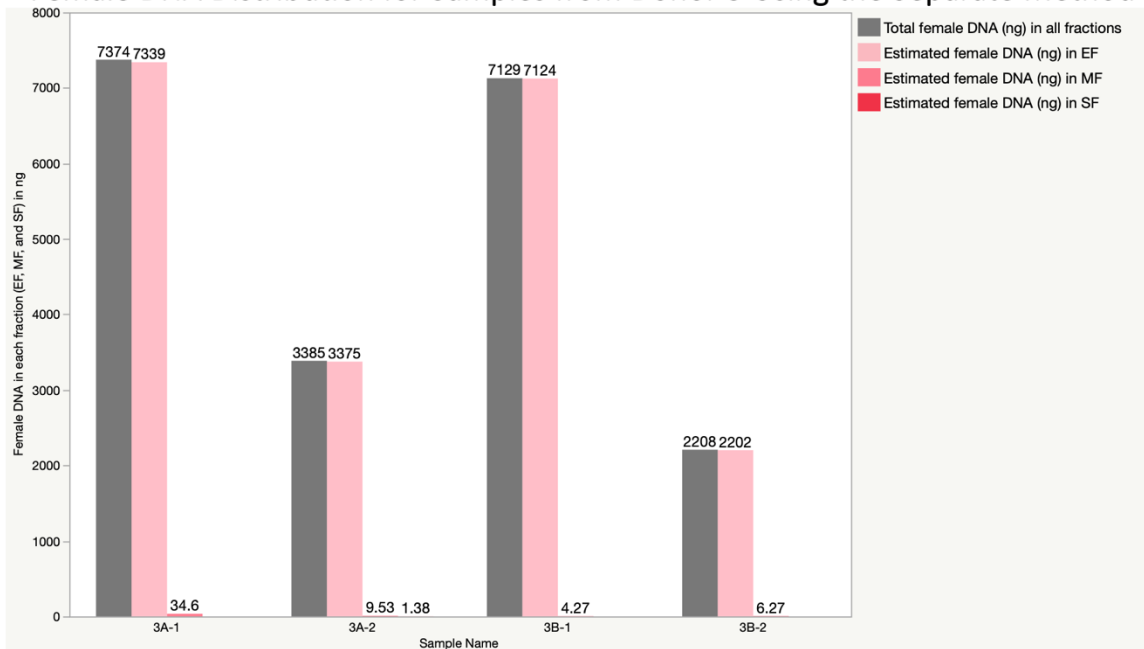


Figure 9. Bar graph depicting the female DNA distribution in each fraction (in ng) for all samples from Donor 3 extracted with the Separate Method

Female DNA Distribution for Samples from Donor 5 Using the Separate Method

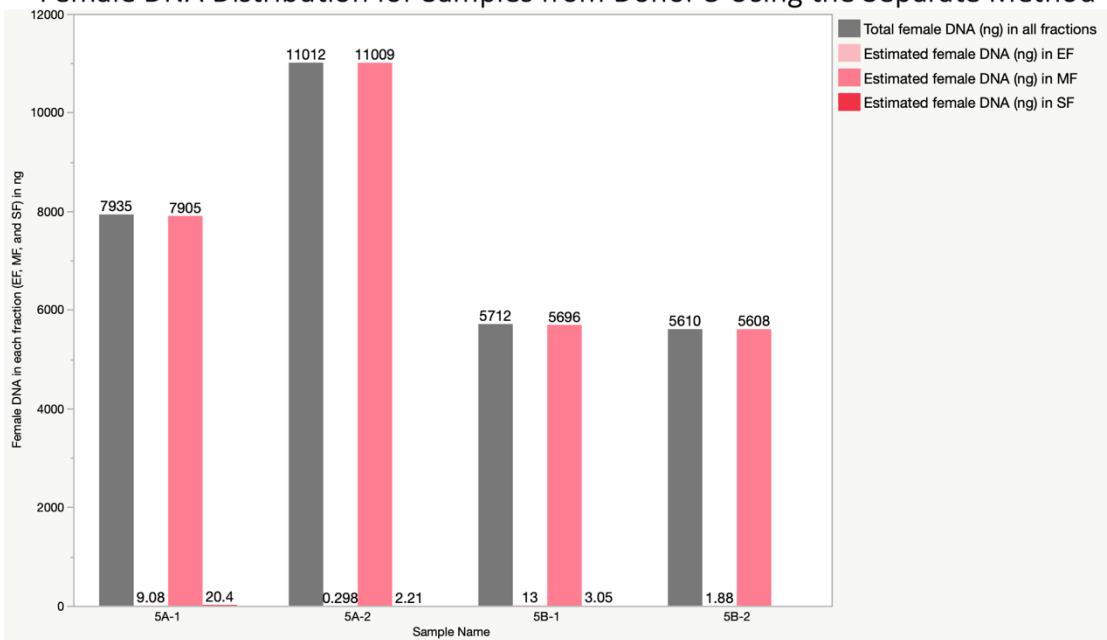


Figure 10. Bar graph depicting the female DNA distribution in each fraction (in ng) for all samples from Donor 5 extracted with the Separate Method

3.4.3 Male DNA Distribution for the Separate Method

Although it becomes more difficult to interpret the male DNA amounts when there is an overwhelming amount of female DNA in a fraction where the female DNA should not be eluting, the sperm recovery and male DNA distribution can still be examined to better understand the effectiveness of the method in recovering the sperm from the cotton swab material. In general, it was observed that for 16 of 32 samples extracted using the Separate Method, the majority of the male DNA was eluted into the SF. Based on the qPCR results, 15 of the 16 samples would likely show a single source or distinguishable male profile (Figure 11).

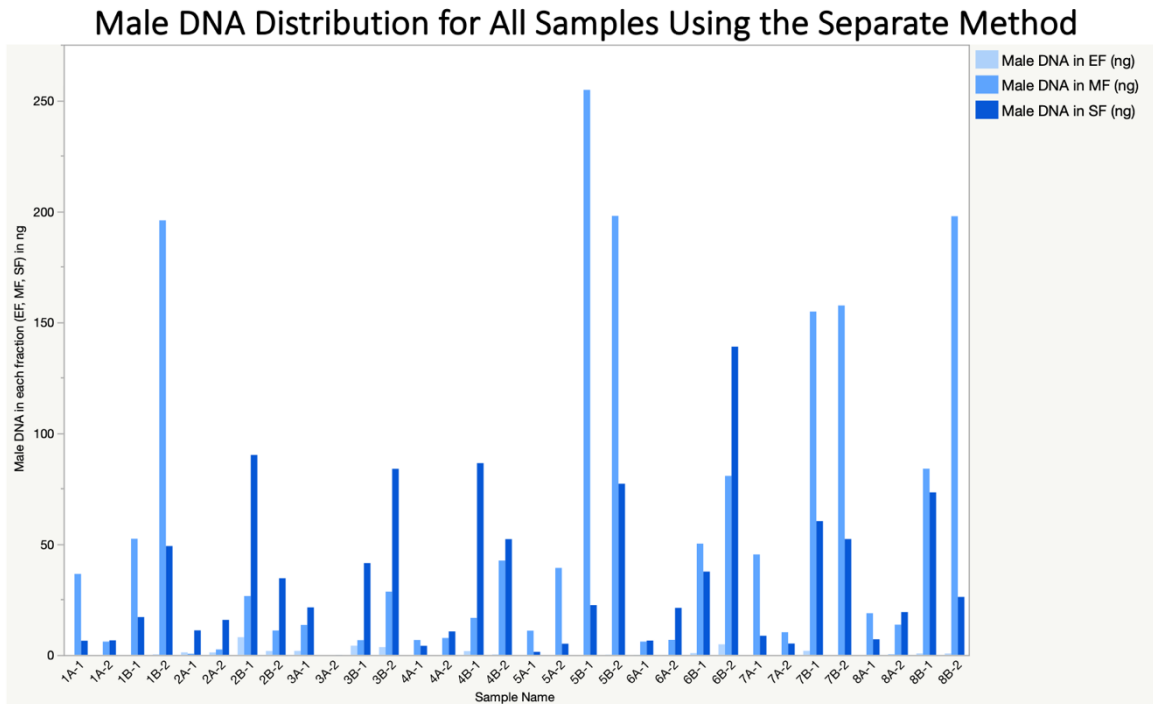


Figure 11. Bar graph depicting the male DNA distribution in each fraction (in ng) for all samples extracted with the Separate Method

The following graphs highlight the same donors as the graphs above, Donor 3 (Figure 12) and Donor 5 (Figure 13), with both the female DNA distribution and male DNA distribution displayed in a manner that assists with making a comparison of both amount for each fraction from each half swab.

Male and Female DNA Distribution for Samples from Donor 3 Using the Separate Method

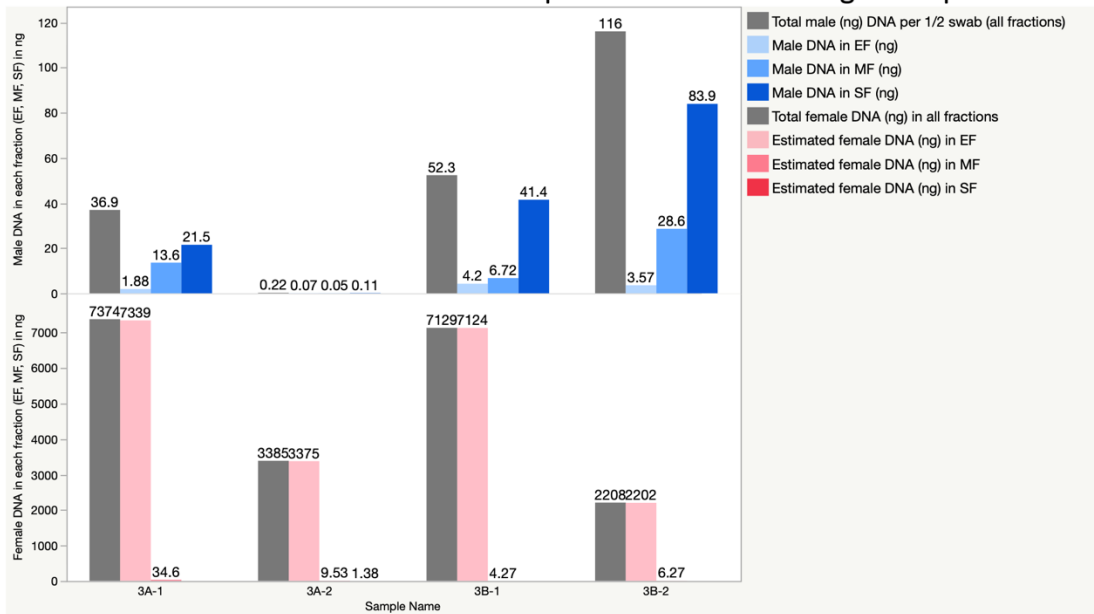


Figure 12. Bar graph depicting the female and male DNA distribution in each fraction (in ng) for the samples from Donor 3 extracted with the Separate Method

As seen in Figure 12, the amounts of male DNA in the SFs of Donor 3 range from approximately 50-70% of the total amount of male DNA in all fractions. In most cases, there is no female DNA detected in the SF, but for sample 3A-2, there is actually more female DNA than male DNA in the SF (1.36 ng vs. 0.11 ng), even though the proportion of female DNA in the SF is so low compared to the total amount of female DNA.

Male and Female DNA Distribution for Samples from Donor 5 Using the Separate Method

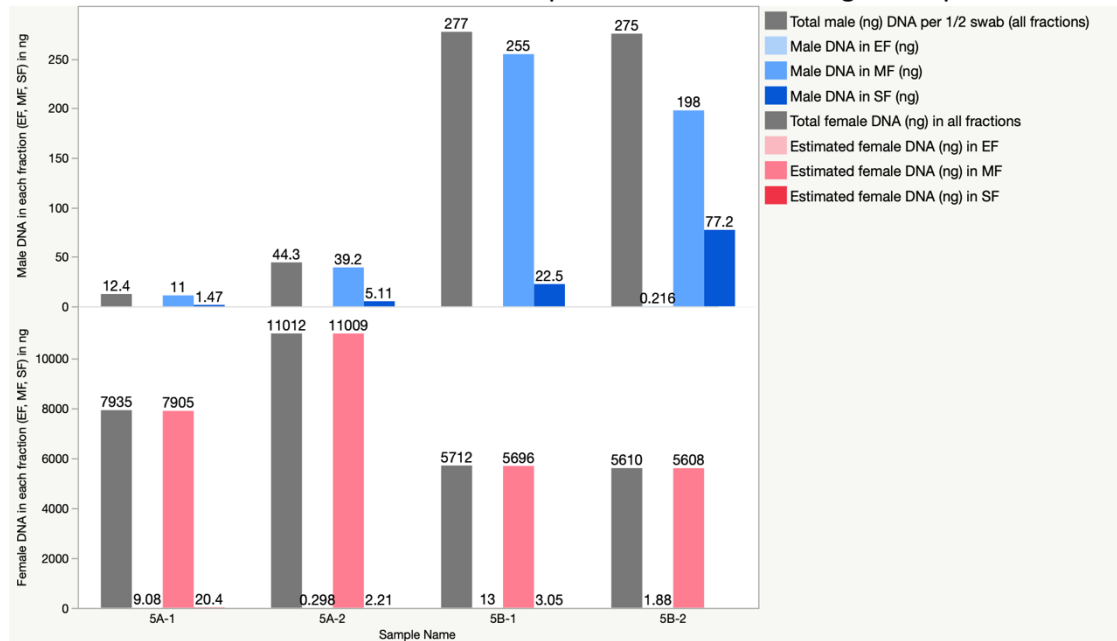


Figure 13. Bar graph depicting the female and male DNA distribution in each fraction (in ng) for the samples from Donor 5 extracted with the Separate Method

For Donor 5, the amounts of male DNA in the SF ranged only from about 10-30% of the total amount of male DNA in all fractions (Figure 13). In the MFs that contained the majority of the female DNA, the amounts of female DNA were approximately 5600 to 11000 ng, which was much greater than the approximately 10 to 255 ng range seen for the male DNA. For any sample like this, the female DNA is bound to overwhelm the male profile. Even though some male DNA eluted into the SF, the female to male DNA ratios are still greater for some of these samples or so close that the male and female profiles would not be discernable.

3.4.4 Profiles from the Separate Method

A number of samples were selected for amplification and separation by capillary electrophoresis in order to assess the resulting DNA profiles. The selected samples were from the same donors, 3 and 5, where the female and male DNA distributions were previously highlighted. The electropherograms were analyzed for the number of alleles at each locus, as greater than two alleles means a mixture of two contributors. A profile was generated for each fraction from sample 3B-2, a half-swab from Donor 3 which had an expected E-cell lysis result, as well as from sample 5B-1, a half-swab from Donor 5 which had an unexpected result of no E-cell lysis in the EF. These samples were both extracted using the Separate Method, so three different profiles were generated for the EF, MF, and SF. Only the blue and green dye channels of the profiles were included in the figures for these samples.

Figure 14 below shows the profile of the EF from sample 3B-2. This profile displays a clear single source female profile. This profile is a good example of what is typically expected to be generated for the EFs produced during differential extraction (when the E-cell lysis works properly).

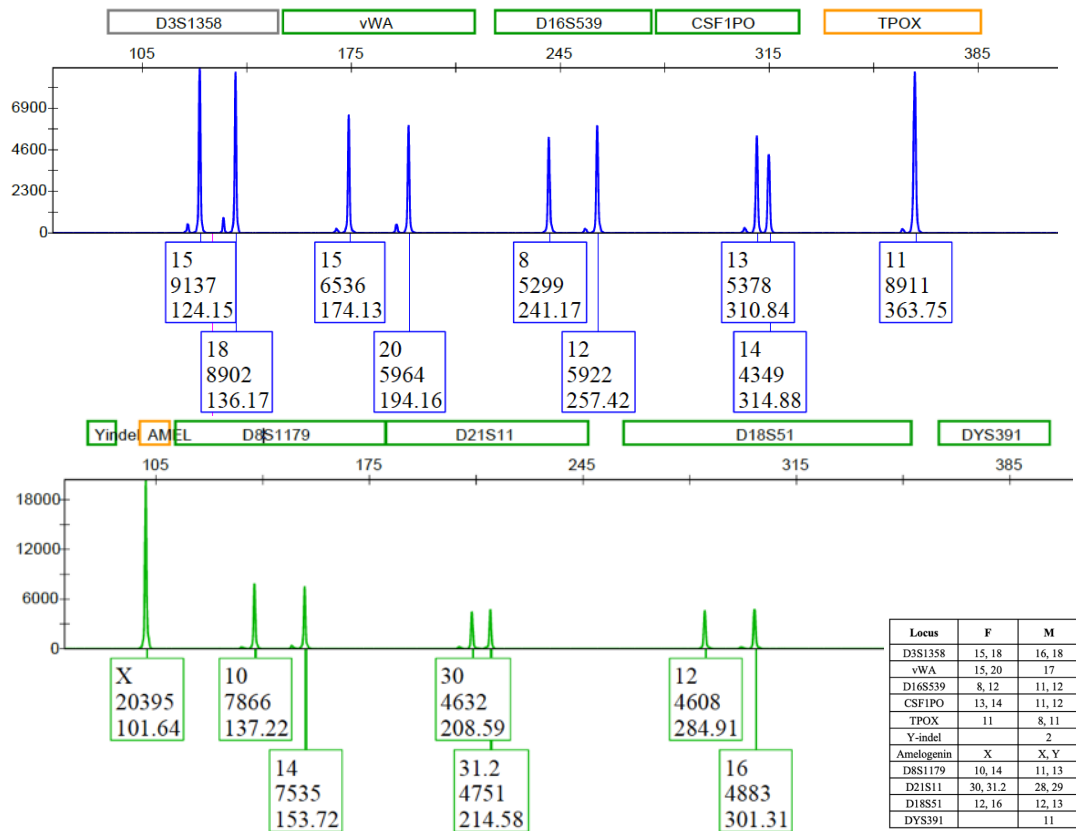


Figure 14. Profile from the green and blue dye channels of EPG for the EF from an example half-swab, 3B-2

The profile for the MF of sample 3B-2 (Figure 15) is a mixture of a male and a female contributor. The female contributor proportion is approximately 0.24 using peak heights at locus vWA, which was consistent with the previously determined female to male proportion from qPCR of approximately 0.22. There is sloping with this profile, where the peak heights of the alleles drop as the length of the fragment increases along the dye channel, indicating degradation or inhibition in the fraction.

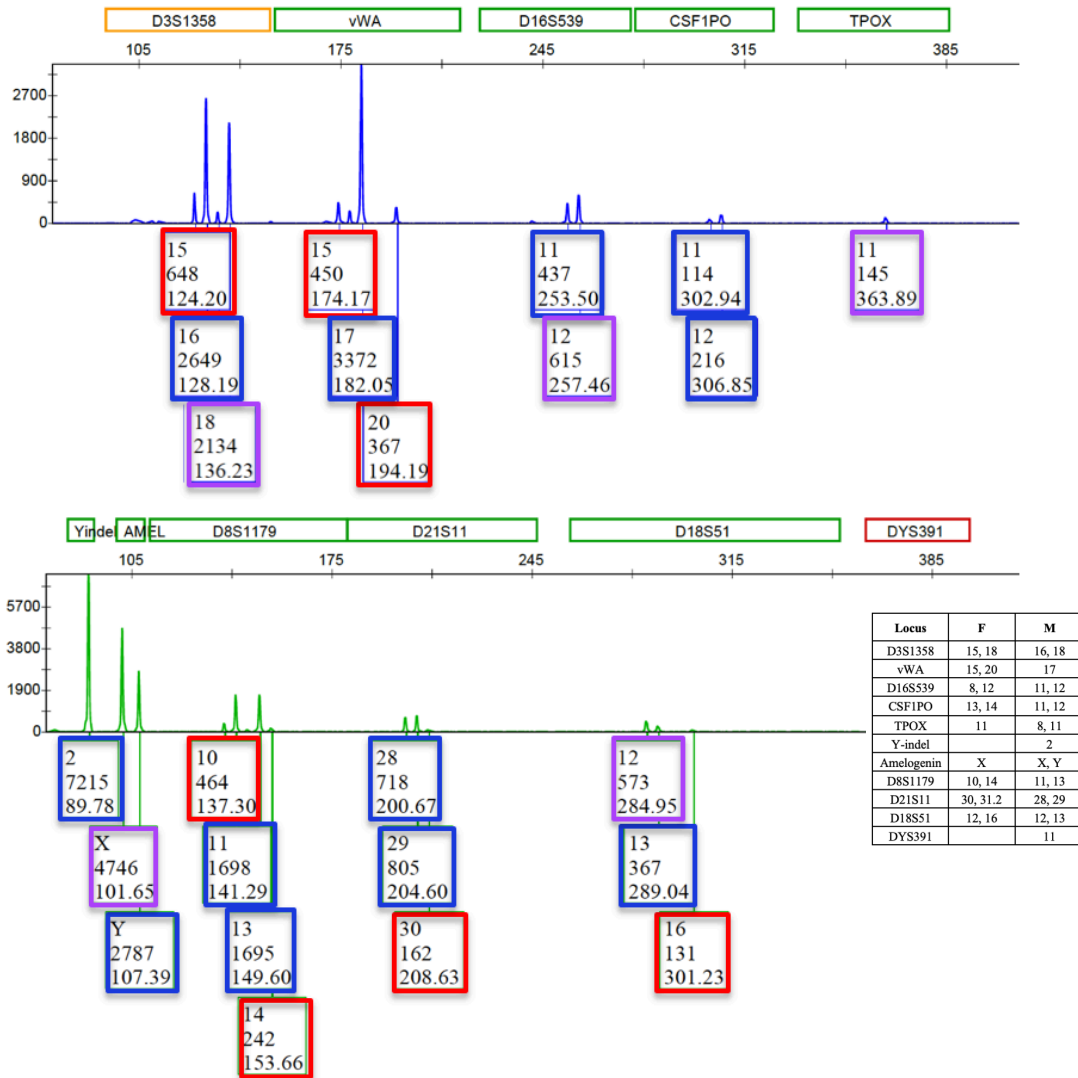


Figure 15. Profile from the green and blue dye channels of EPG for the MF from an example half-swab, 3B-2, with the alleles from the female profile boxed in red, those from the male profile boxed in blue, and the shared alleles in purple.

The EPG from the SF from this sample, 3B-2, is shown below (Figure 16). Similar to the MF from this sample, the SF consists of a mixture with both a male and female contributor. Although the quantitation values suggested that there was no female DNA present in this fraction, the female donor does appear to be contributing three alleles at

some loci at very low peak heights (this is likely due to the slight inaccuracy in estimating female DNA as the difference of the total DNA and the male DNA). Nonetheless, the major contributor is male, and thus a clear male profile can also be determined from this fraction.

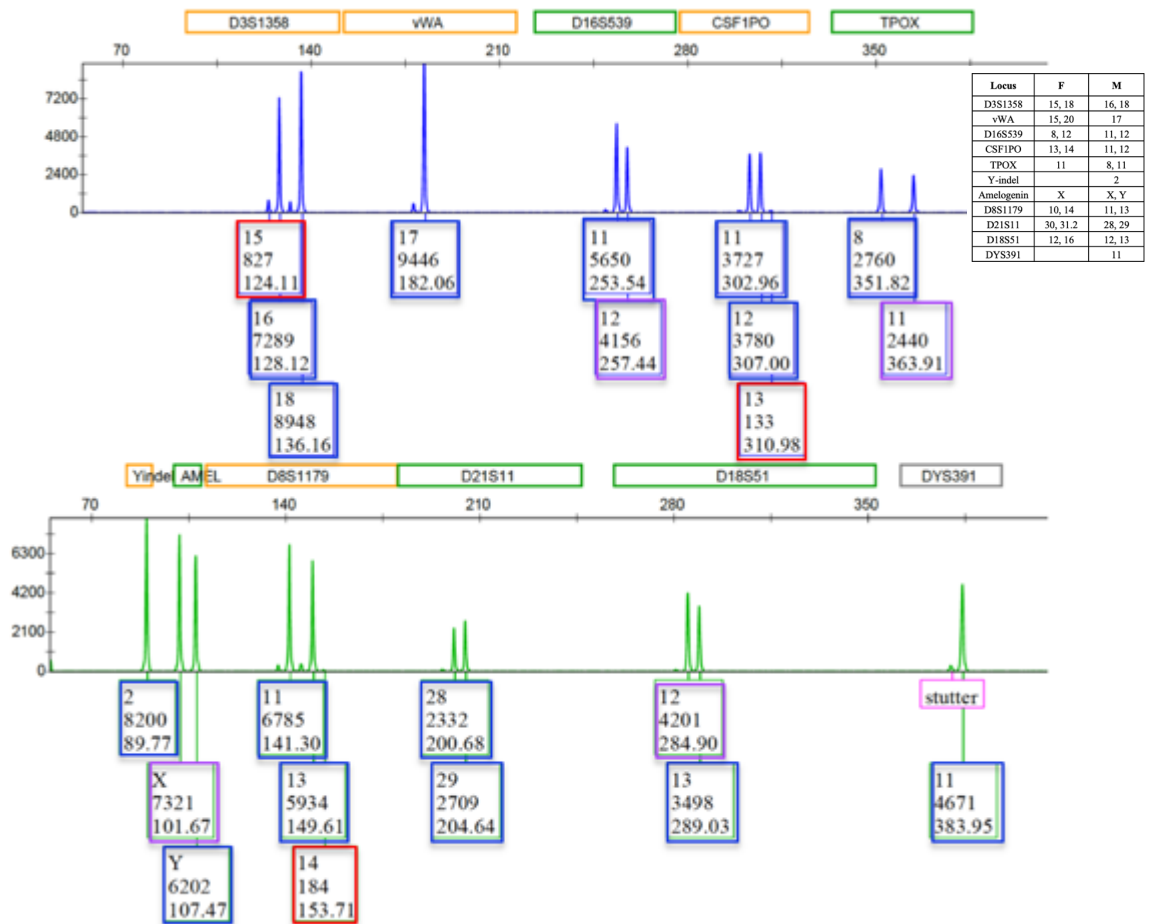


Figure 16. Profile from the green and blue dye channels of EPG for the SF from an example half-swab, 3B-2, with the alleles from the female profile boxed in red, those from the male profile boxed in blue, and the shared alleles in purple.

For the example sample that had very little E-cell lysis in the EF, the three fractions looked vastly different from what is ideally seen with differential extraction. The EF of

sample 5B-1 (Figure 17) had a small amount of female DNA (only about 13 ng) and no male DNA, resulting in a single source female profile with sloping and allelic dropout at loci of larger molecular weight fragments along the dye channels (the TPOX locus, for example). While it is normal to see a single source female profile, the low allelic peak heights and dropout in the EF, as seen in this profile, is not usually of concern.

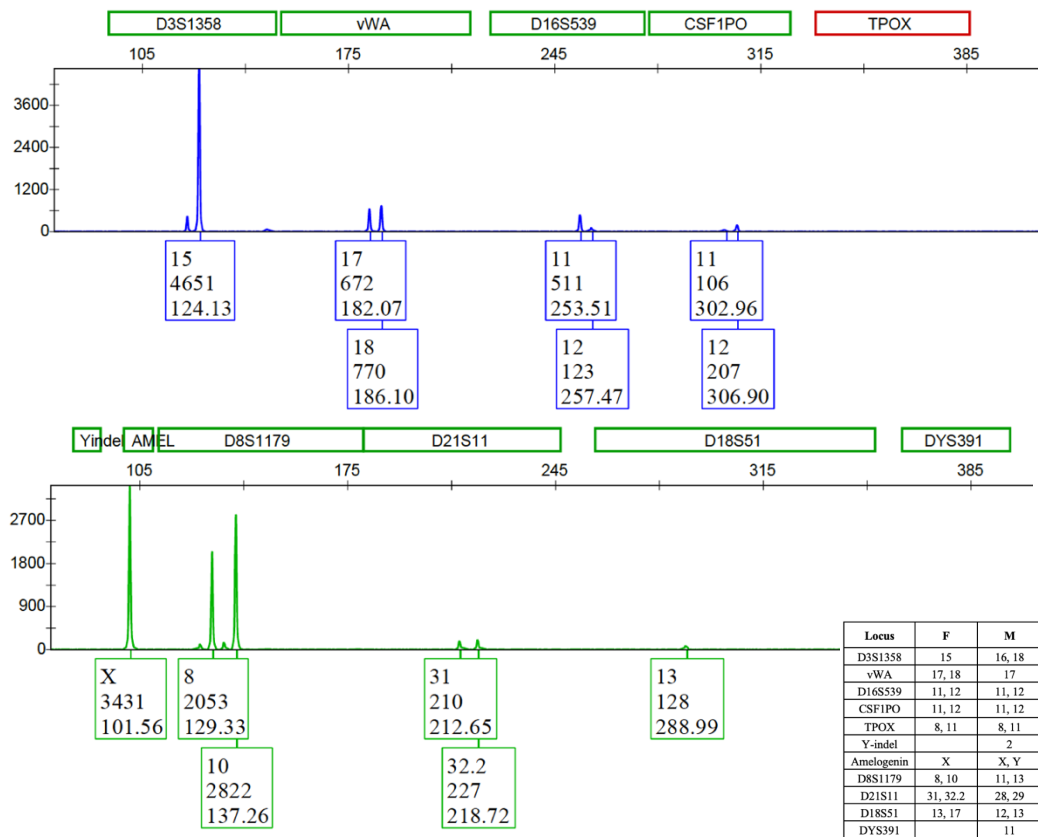


Figure 17. Profile from the green and blue dye channels of EPG for the EF from an example half-swab, 5B-1

The MF profile for sample 5B-1 (Figure 18) was determined to be a mixture just as in sample 3B-2. The difference here, however, was that all the female DNA was present in this MF in abundance. Because the female was the major contributor with a much

greater amount of DNA, the male minor contributor only a few alleles at some loci. Using the qPCR data, the proportion of male DNA in this MF is only approximately 0.043.

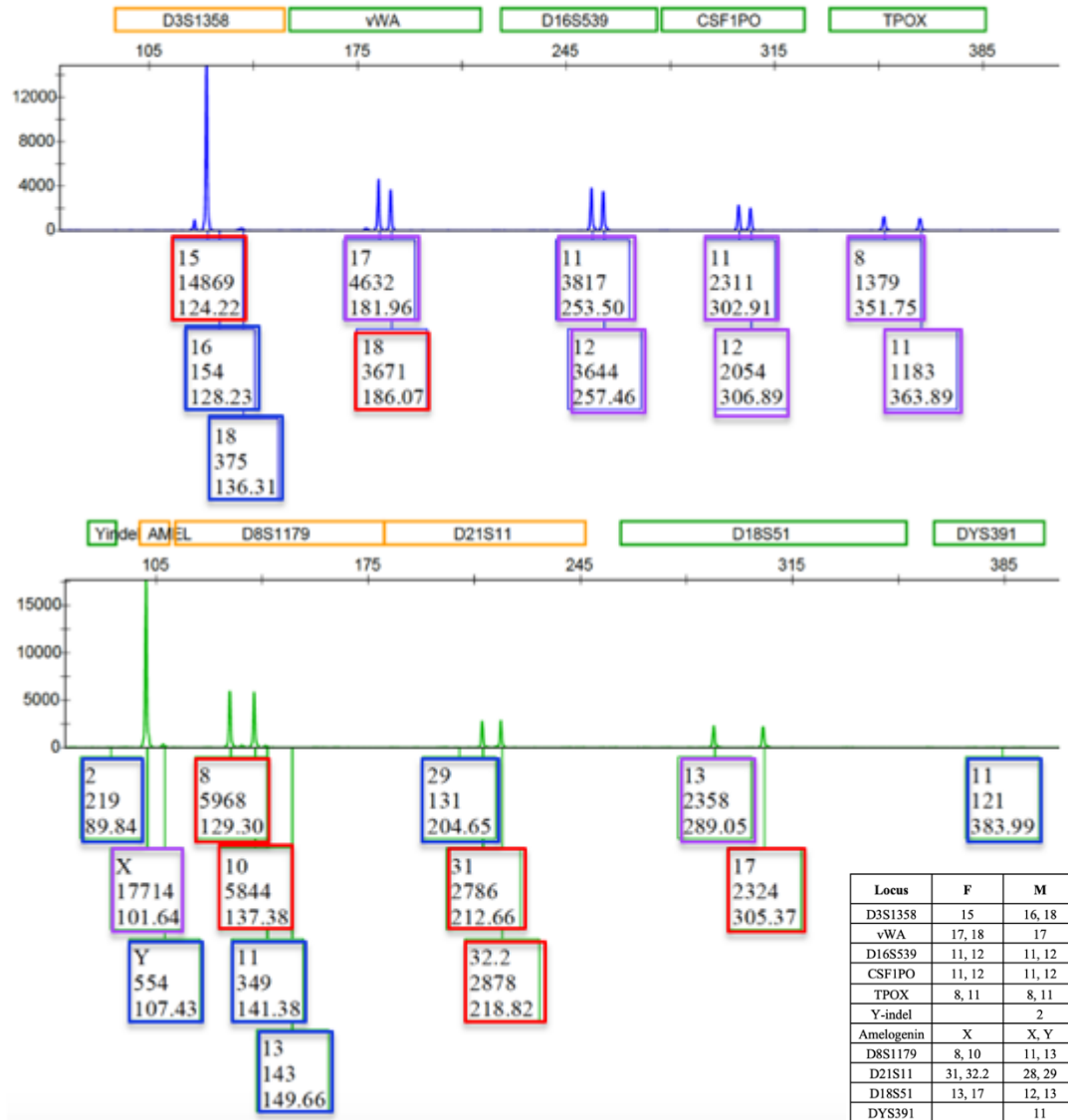


Figure 18. Profile from the green and blue dye channels of EPG for the MF from an example half-swab, 5B-1, with the alleles from the female profile boxed in red, those from the male profile boxed in blue, and the shared alleles in purple.

The SF of this sample (Figure 19) had a discernable male major profile at most loci; however, the male contributor proportion is approximately 0.70, as shown by the peak

heights. The proportion of male DNA in the fraction by qPCR is 0.88. While this sample produced unusual EF and MF results for the EPGs, the SF results are still good because there is a distinguishable male profile.

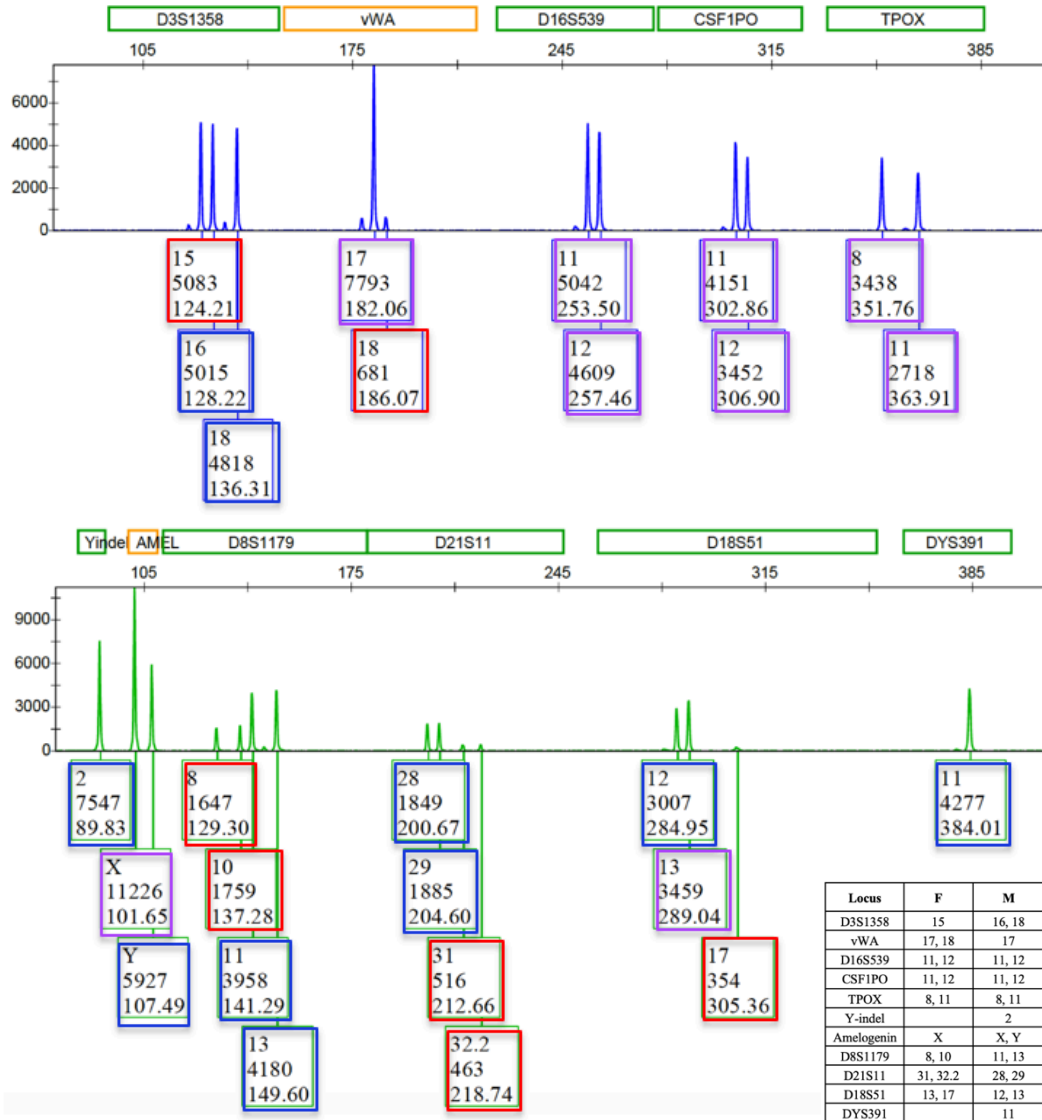


Figure 19. Profile from the green and blue dye channels of EPG for the SF from an example half-swab, 5B-1, with the alleles from the female profile boxed in red, those from the male profile boxed in blue, and the shared alleles in purple.

3.4.5 Quantitation of Female and Male DNA with the Recombined Method

For the Recombined Method, the procedure was modified to result in only two fractions (the EF and a recombined MF/SF). Thus, when quantitated using qPCR, there were only a total of 64 samples from the 32 half swabs of the same eight donors. The final volumes of liquid of these different fractions were now 470 μ L for the EF and 249 μ L for the new recombined MF/SF. The same calculations as above were performed using these concentration values to determine both the female and male DNA amounts in each of these fractions using this method (Table 8).

Table 8. Total amounts of male and female DNA in each fraction (EF and MF/SF) from each half-swab extracted with the Recombined Method, as calculated using the qPCR values and approximate elution volumes

Sample Name	Quantity of Total Human (ng/ul)	Quantity of Total Male (ng/ul)	Volume of Liquid (ul)	Total Human DNA in Half Swab (ng)	Total Male DNA in Half Swab (ng)	Estimated Female DNA in Half Swab (ng)
1C-1 EF	0.001	0.000	470	0.673	0.000	0.673
1C-1 MF/SF	2.683	0.004	249	668.137	0.949	667.188
1C-2 EF	0.004	0.000	470	1.938	0.000	1.938
1C-2 MF/SF	17.160	0.125	249	4272.913	31.005	4241.908
1D-1 EF	0.001	0.000	470	0.662	0.000	0.662
1D-1 MF/SF	5.355	0.168	249	1333.485	41.719	1291.766
1D-2 EF	0.015	0.000	470	7.263	0.000	7.263
1D-2 MF/SF	32.338	0.449	249	8052.162	111.717	7940.445
2C-1 EF	1.906	0.001	470	895.769	0.422	895.347
2C-1 MF/SF	3.814	0.002	249	949.597	0.469	949.129
2C-2 EF	0.010	0.000	470	4.708	0.000	4.708
2C-2 MF/SF	18.784	0.023	249	4677.128	5.644	4671.485
2D-1 EF	0.828	0.000	470	389.104	0.000	389.104
2D-1 MF/SF	4.528	0.023	249	1127.406	5.713	1121.694
2D-2 EF	2.396	0.000	470	1126.004	0.063	1125.941
2D-2 MF/SF	9.273	0.482	249	2308.897	120.105	2188.792
3C-1 EF	19.675	0.000	470	9247.383	0.160	9247.223
3C-1 MF/SF	0.046	0.003	249	11.425	0.761	10.665
3C-2 EF	13.953	0.001	470	6558.025	0.398	6557.627

Sample Name	Quantity of Total Human (ng/ μ L)	Quantity of Total Male (ng/ μ L)	Volume of Liquid (μ L)	Total Human DNA in Half Swab (ng)	Total Male DNA in Half Swab (ng)	Estimated Female DNA in Half Swab (ng)
3C-2 MF/SF	0.396	0.051	249	98.528	12.711	85.817
3D-1 EF	7.477	0.002	470	3514.367	0.948	3513.420
3D-1 MF/SF	0.046	0.020	249	11.520	4.934	6.586
3D-2 EF	6.519	0.010	470	3063.857	4.604	3059.253
3D-2 MF/SF	0.061	0.004	249	15.179	1.029	14.150
4C-1 EF	0.200	0.000	470	93.936	0.000	93.936
4C-1 MF/SF	2.712	0.002	249	675.338	0.445	674.893
4C-2 EF	0.230	0.000	470	108.079	0.000	108.079
4C-2 MF/SF	8.233	0.034	249	2050.001	8.353	2041.648
4D-1 EF	0.163	0.000	470	76.444	0.108	76.336
4D-1 MF/SF	5.455	0.070	249	1358.304	17.464	1340.840
4D-2 EF	0.348	0.000	470	163.776	0.000	163.776
4D-2 MF/SF	1.368	0.060	249	340.529	15.019	325.510
5C-1 EF	0.048	0.000	470	22.592	0.000	22.592
5C-1 MF/SF	1.170	0.006	249	291.364	1.541	289.823
5C-2 EF	0.002	0.000	470	0.785	0.105	0.680
5C-2 MF/SF	3.884	0.004	249	967.181	0.880	966.301
5D-1 EF	0.080	0.000	470	37.745	0.113	37.632
5D-1 MF/SF	0.903	0.008	249	224.867	1.871	222.997
5D-2 EF	0.118	0.000	470	55.490	0.000	55.490
5D-2 MF/SF	14.129	0.302	249	3518.100	75.085	3443.015
6C-1 EF	3.224	0.000	470	1515.392	0.000	1515.392
6C-1 MF/SF	0.385	0.007	249	95.934	1.761	94.173
6C-2 EF	0.799	0.001	470	375.326	0.244	375.082
6C-2 MF/SF	2.904	0.047	249	723.131	11.645	711.486
6D-1 EF	2.380	0.001	470	1118.561	0.667	1117.894
6D-1 MF/SF	0.194	0.012	249	48.278	3.026	45.252
6D-2 EF	1.459	0.008	470	685.703	3.848	681.855
6D-2 MF/SF	2.993	0.125	249	745.192	31.144	714.049
7C-1 EF	0.007	0.000	470	3.070	0.000	3.070
7C-1 MF/SF	19.235	0.005	249	4789.514	1.275	4788.239
7C-2 EF	0.011	0.000	470	5.196	0.000	5.196
7C-2 MF/SF	31.214	0.178	249	7772.214	44.431	7727.783
7D-1 EF	0.335	0.000	470	157.560	0.159	157.401
7D-1 MF/SF	7.082	0.175	249	1763.445	43.645	1719.801
7D-2 EF	0.686	0.002	470	322.458	0.753	321.705
7D-2 MF/SF	20.347	0.417	249	5066.516	103.880	4962.636
8C-1 EF	0.599	0.000	470	281.405	0.000	281.405
8C-1 MF/SF	6.454	0.037	249	1607.000	9.198	1597.802

Sample Name	Quantity of Total Human (ng/ μL)	Quantity of Total Male (ng/ μL)	Volume of Liquid (μL)	Total Human DNA in Half Swab (ng)	Total Male DNA in Half Swab (ng)	Estimated Female DNA in Half Swab (ng)
8C-2 EF	0.249	0.000	470	117.195	0.000	117.195
8C-2 MF/SF	6.915	0.060	249	1721.775	14.857	1706.917
8D-1 EF	0.728	0.000	470	342.188	0.000	342.188
8D-1 MF/SF	1.467	0.049	249	365.316	12.156	353.160
8D-2 EF	0.272	0.000	470	127.639	0.000	127.639
8D-2 MF/SF	5.000	0.049	249	1245.024	12.300	1232.725

In many ways, these values are comparable to those that were seen when extracting using the Separate Method, with the same issues that were seen in terms of the lack of E-cell lysis also being observed with these samples. With this method, however, the interpretation becomes even more complex with the fact that all the male DNA is being eluted into the one fraction, the recombined MF/SF. With the lack of E-cell lysis in the EF of some of the donors again observed, many of the samples have an overabundance of female DNA that was eventually released in the recombined MF/SF (up to 8,000 ng or 99.9% of the total female DNA). This female DNA overabundance is likely to disguise any male DNA (which ranges from around 1 ng to 120 ng), as preferential amplification of the female DNA will occur, causing potential dropout of certain alleles from the male profile. Even if the male DNA in the fraction was to be discernable as a full profile within the MF/SF, it would only be recognized as a minor contributor to the profile in a very low contributor proportion. This is clearly not a desired result when the goal is to develop a major male profile in the MF/SF that can be clearly distinguished and there is assurance of no dropout. Additionally, the unexpected results obtained also complicate the ability to compare the effectiveness of the Recombined Method to the modified Separate Method in

achieving a better sperm recovery and generating a clear male profile (due to the abnormally large amount of female DNA).

3.4.6 Female and Male DNA Distribution with the Recombined Method

The data shown in Figures 20 and 21 illustrates the female and male DNA distribution for the swabs from each donor extracted using the Recombined Method. Similar trends were observed in that the lack of E-cell lysis in the EF was generally donor-dependent. As observed with the Separate Method, Donors 2, 3, and 6 had the greatest proportions of female DNA in the EF (closer to 100%) of all the donors (Figure 20). Significantly, all of the swabs from both Donors 1 and 5 again saw close to no E-cell lysis in the EF.

Furthermore, Figure 21 shows that the male DNA was fully recovered in the recombined MF/SF. However, even in the samples from Donor 3 where the E-cell lysis was close to complete, the amounts of female DNA remaining on the swab is approximately one to 13 times the amount of male DNA. Thus, the best outcome would be a 1:1 male to female mixture ratio and the worst outcome would be a minor male DNA profile with much lower peak heights than the female major contributor.

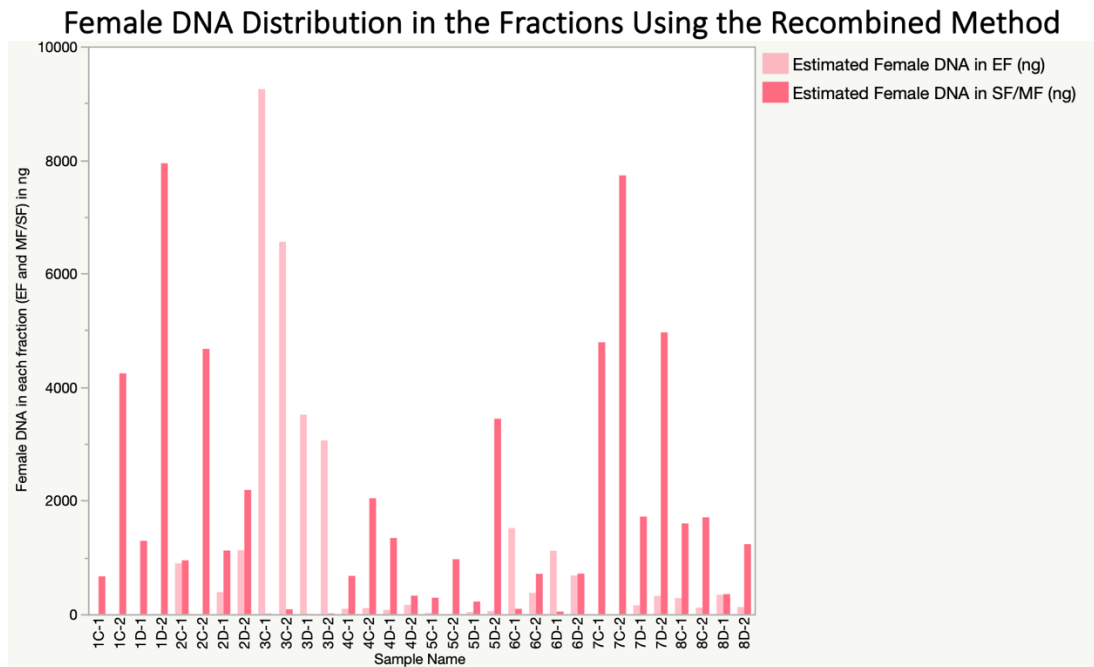


Figure 20. Bar graph depicting the female DNA distribution in each fraction (in ng) for all samples extracted with the Recombined Method

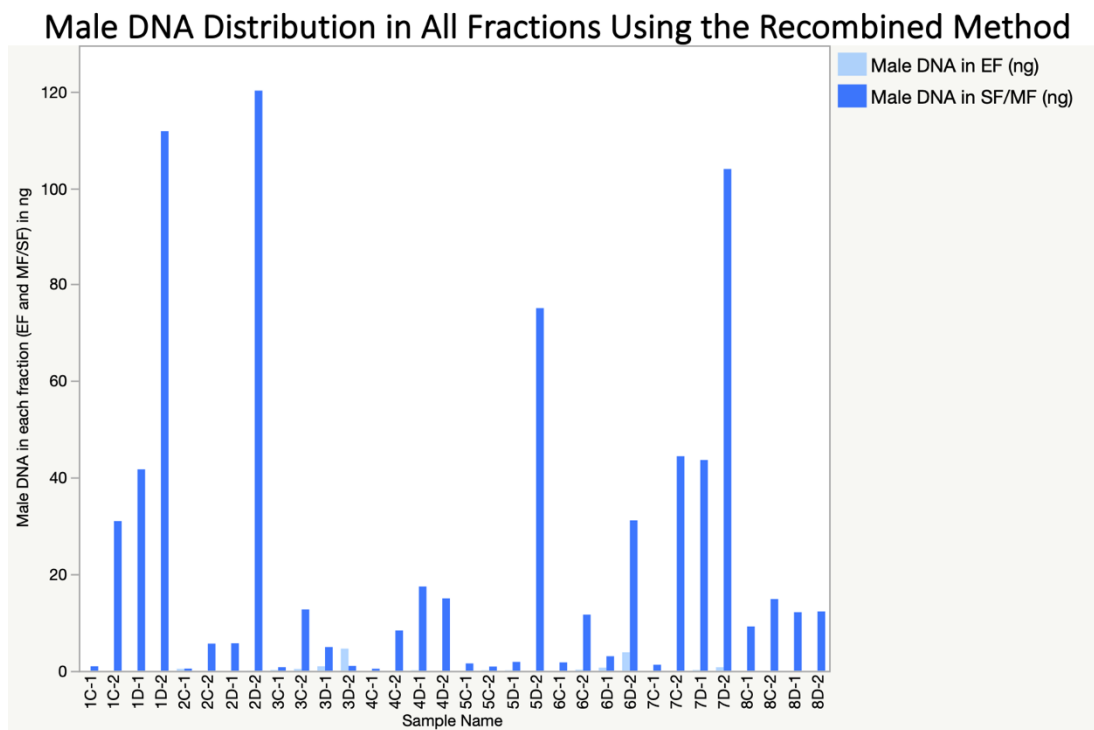


Figure 21. Bar graph depicting the male DNA distribution in each fraction (in ng) for all samples extracted with the Recombined Method

Figures 22 and 23 detail both the male and female DNA distribution for the samples from Donor 3, with the expected result of E-cell lysis in the EF, and from Donor 5, with the unexpected result of no E-cell lysis in the EF. These are the same samples that were highlighted with the Separate Method, which helps to further visualize the similarities seen between samples from the same donors despite the different extraction methods being performed on different days.

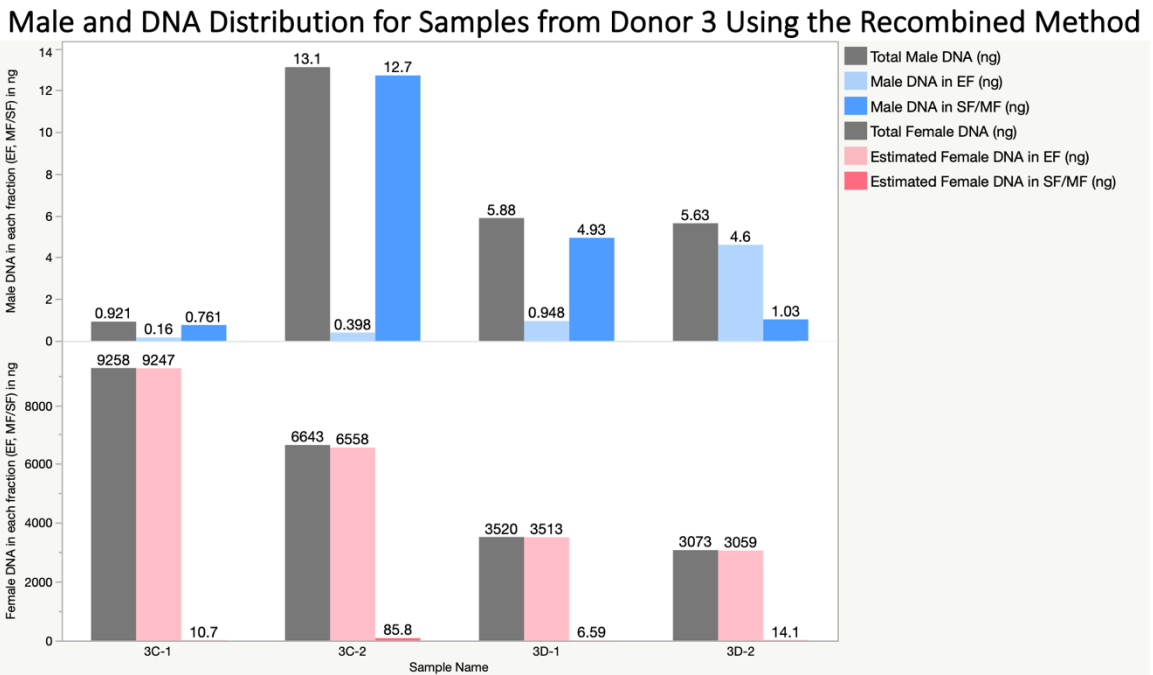


Figure 22. Bar graph depicting the female and male DNA distribution in each fraction (in ng) for Donor 3 extracted with the Recombined Method

Male and DNA Distribution for Samples from Donor 5 Using the Recombined Method

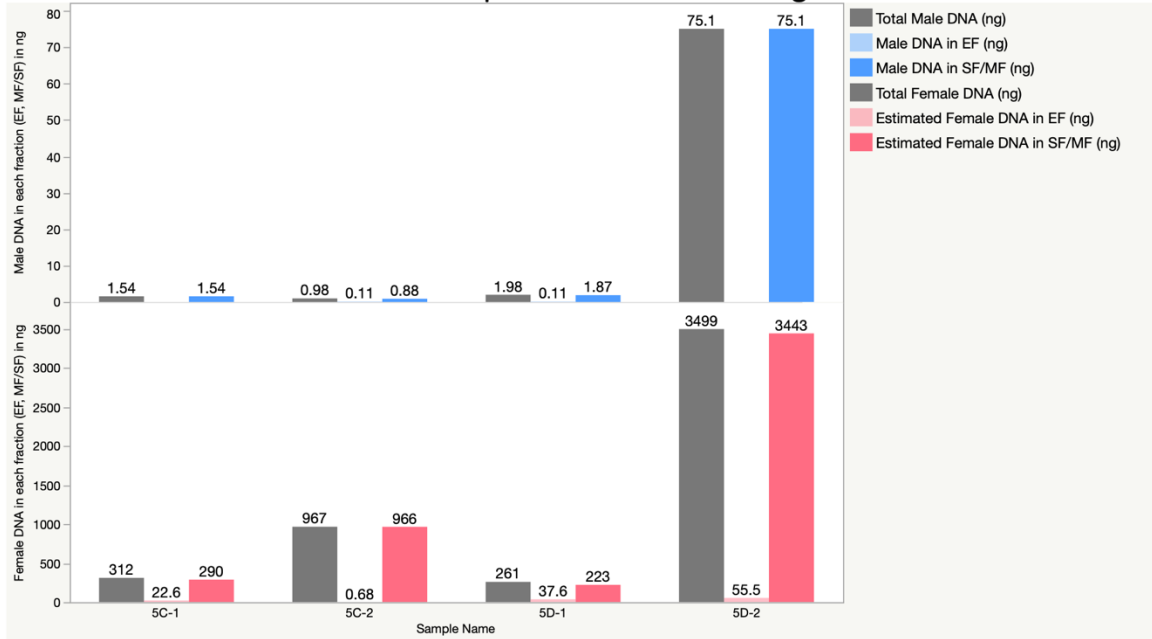


Figure 23. Bar graph depicting the female and male DNA distribution in each fraction (in ng) for Donor 3 extracted with the Recombined Method

3.4.7 Profiles from the Recombined Method

Samples from the same donors, 3 and 5, which were extracted using the Recombined Method underwent amplification to generate profiles of the EF and MF/SF for direct comparison. As mentioned above, these donors performed similarly when extracted with both of the different methods in terms of E-cell lysis. For this method, only two EPGs were generated (one of the EF and one of the MF/SF). Because of this, the ideal end product would have been a single source female profile in the EF and a single source or at least easily distinguishable major male profile in the MF/SF. This method focused on recovering all of the sperm male DNA in one fraction to generate an even clearer full male profile of higher allelic peak heights.

The EF of the sample from Donor 3 (3D-2) produced a single source female profile (Figure 24) with no apparent dropout. This was consistent with the lack of quantifiable male DNA in the EF and the abundance of female DNA in the desired fraction when E-cell lysis functions as expected.

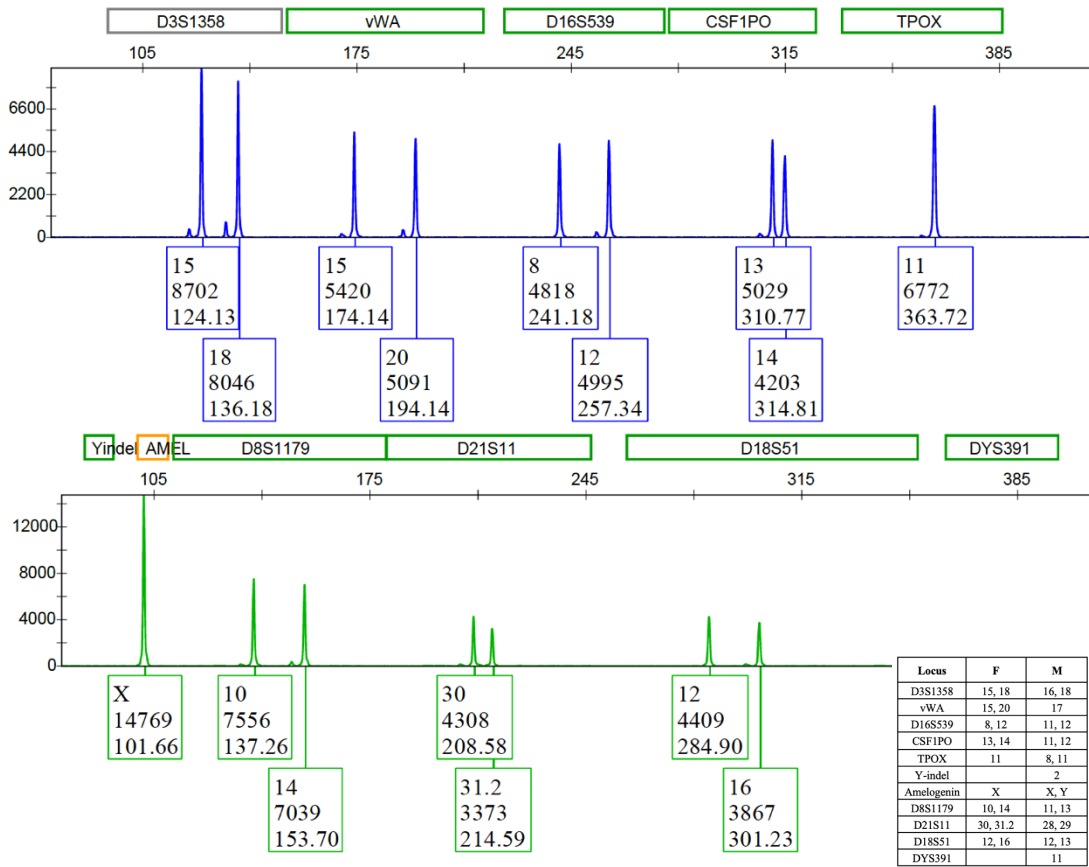


Figure 24. Profile from the green and blue dye channels of EPG for the EF from an example half-swab, 3D-2

Although there was approximately 1 ng of in the MF/SF, it was present with the much larger amount of female DNA (approximately 14 ng) still remaining in this fraction (carried over on the swab material into this fraction). Ultimately, the female donor was the

major contributor to this profile, while the male contributed a few alleles at some loci (Figure 25). Additionally, there is a great amount of sloping in this profile, suggesting that there is an inhibitor within this sample preventing the amplification of these longer fragments.

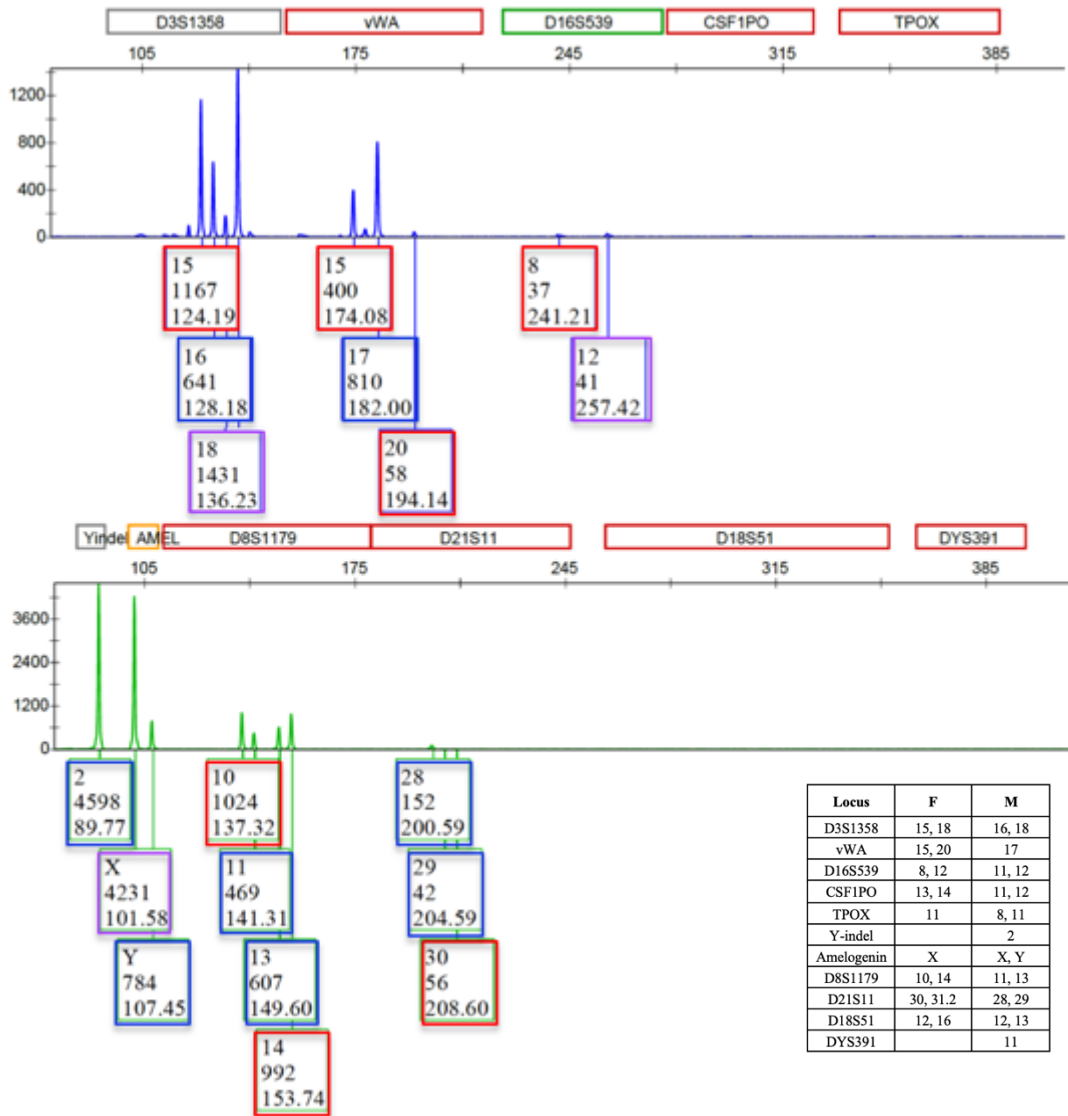


Figure 25. Profile from the green and blue dye channels of EPG for the MF/SF from an example half-swab, 3D-2, with the alleles from the female profile boxed in red, those from the male profile boxed in blue, and the shared alleles in purple.

For sample 5D-1 extracted using the Recombined Method that exhibited an unexpected lack of E-cell lysis in the EF, there is still small amount of female DNA that was still amplifiable into a full single source female profile (Figure 26). However, because of all the carryover of female DNA that wasn't lysed in the EF into the recombined MF/SF, there is an excess of female DNA to the point where the profile is only of a single source female contributor (Figure 27). The male contributor does not show up at all in this profile of the fraction where the male DNA should be present in excess. Both profiles show drop-off of signal at loci of higher molecular weights indicating possible inhibition here as well.

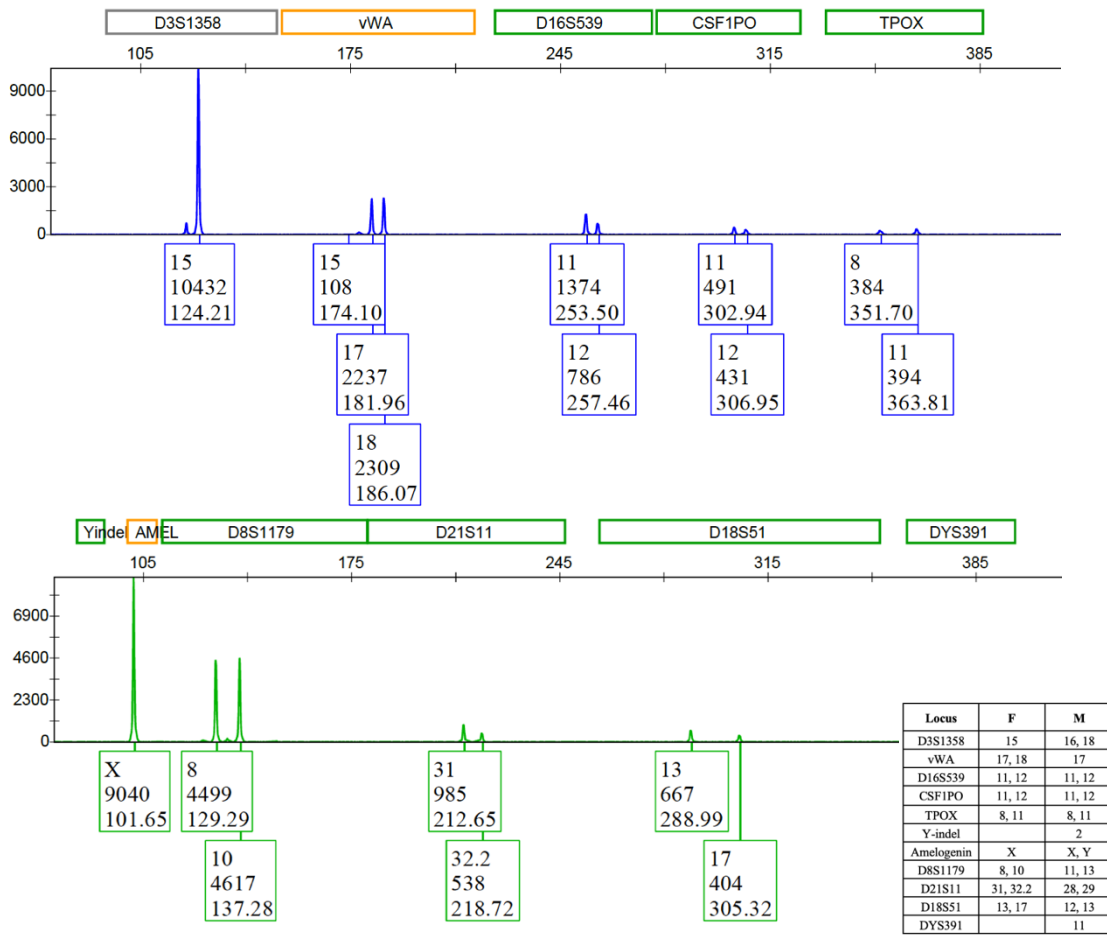


Figure 26. Profile from the green and blue dye channels of EPG for the EF from an example half-swab, 5D-1

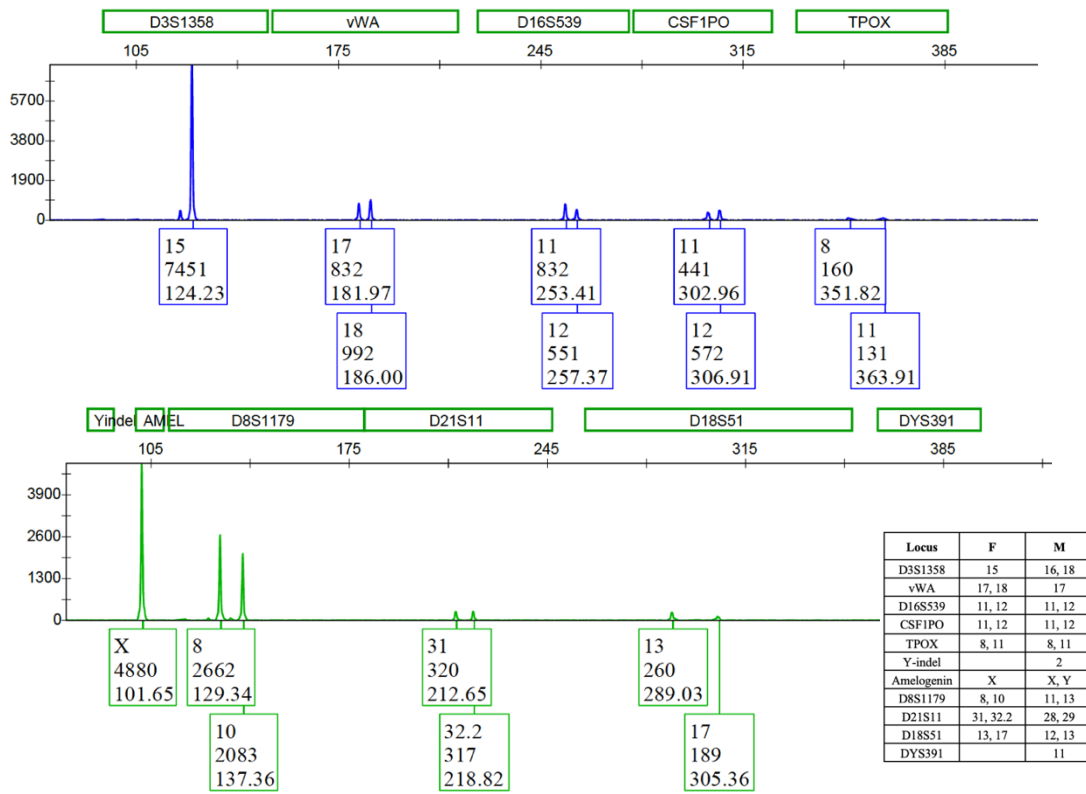


Figure 27. Profile from the green and blue dye channels of EPG for the MF/SF from an example half-swab, 5D-1

4. DISCUSSION

4.1 Lack of Female DNA in the Epithelial Fractions

As discussed previously, the result observed regarding the lack of female DNA in some of the EFs in this data set is unusual and has not been seen in any of the previous research. Possible technical errors were considered to explain the unusual manner in which these specific samples acted in extraction, resulting in the ineffectiveness of the *forensicGEM*TM enzyme in the EFs of these donors.

In the end, technical errors as an explanation of these results are mainly disproved by the fact that the results observed are donor dependent and are observed in both the separate and recombined methods. For example, the swabs from one donor that had poor female epithelial cell digestion in the EF for one method also had poor female E-cell digestion for the other method. It seems unlikely that the same error, such as no addition of *forensicGEM*TM to those samples, would occur with the same samples during entirely different extractions performed on different days. Another possible explanation is an issue with the thermal cycler, where some well positions did not reach the desired 75°C temperature essential for enzyme function. However, a quality control check using the temperature verification kit showed that the thermal cycler passed all verification tests. The samples were also placed in different positions in the thermal cycler on different days due to the difference in the total number of samples each of the two methods yielded.

It seems that other explanations of the unusual results should be considered. One explanation for the results observed could be differences between the components of the vaginal samples from specific donors. The inhibition of the *forensicGEM*TM enzyme for

particular donors could be due to a number of factors, including inhibitors in the vaginal fluid, the pH of the final solution, the stage of the menstrual cycle, or any potential vaginal products used by the female donor. A potential explanation as to why the female DNA was then detected in the MF despite potential inhibitors would be that the intact epithelial cells were carried into the MF on the cotton swab while the inhibitors stayed in the liquid of the EF. Furthermore, the Acrosolv and *forensicGEM*TM Sperm enzyme cocktail would have been capable of digesting these cells and releasing the female DNA into the MF, probably without being disrupted by any inhibitors as they would now be present at substantially lower concentrations.

4.2 Female DNA Distribution

As seen with the Donor 3 example, when the E-cell lysis occurs as predicted with the use of the *forensicGEM*TM enzyme, the female DNA is largely found in the EF. However, due to the large range of female DNA (in ng) compared to the smaller range of male DNA experimentally chosen, even a very low proportion of the total female DNA in the MF or SF would have drastic effects on the amplification of a male profile, since it may be less than or similar to the total female DNA also present in the SF. Additionally, because the Benzonase[®] nuclease steps occur before sperm lysis with Acrosolv and *forensicGEM*TM Sperm, female epithelial cells that are not lysed in the EF or MF would be lysed with the sperm lysis enzymes. Thus, epithelial cells carried over to the MF or SF will release female DNA during sperm lysis with Acrosolv and *forensicGEM*TM Sperm, and the female DNA would not have been in solution during the Benzonase[®] steps.

4.3 Male DNA Distribution and Sperm Recovery

The male DNA distribution was as expected in many samples, where the SF had the majority of the male DNA. The sperm recovery was high enough to generate single source or distinguishable male profiles in the SF for 15 of the 32 samples. This was particularly true with the samples where the E-cell lysis performed as expected, as any leftover female DNA was in solution to be degraded by Benzonase[®], resulting in a majority of male DNA in the SF. However, even in some of these samples, the female DNA outweighed the male DNA.

In samples where all or a large proportion of female DNA was not released in the EF, but remained on the swab, a large proportion of the male DNA also remained on the swab. While some of the male DNA was eluted in the SF for these samples, most of the SF profiles would be expected to be mixtures based on the qPCR data. It is important to note that in the comparisons made between the amounts of male DNA and female DNA in each fraction, it is clear that for samples with large amounts of female DNA present on the swab, even large amounts of male DNA in the SF (or high proportions of the total male DNA) can still be compromised by a small amount proportion of the total female DNA remaining in the SF.

4.4 Comparison of the Two Methods

This data showed that with this new Recombined Method, the male DNA has to compete for amplification with the higher amounts of female DNA in the recombined fraction. The female DNA typically carried over into the MF is now in the MF/SF, where

the majority of the male DNA is found. With the amounts of female DNA in excess of the male DNA in many of these samples, the improved male recovery can be disguised in the data. On the other hand, with the Separate Method, a single source or distinguishable male profile can still be determined from the SF for approximately half of the samples because there is a smaller amount of female DNA being carried over into this fraction.

5. CONCLUSIONS

5.1 Conclusions from Data

First, it is important to recognize that there is the potential for normal E-cell lysis to fail in some samples. While no underlying cause was determined based on the available data, it is important for forensic DNA analysts to understand that this could be observed in forensic casework. While analysis of the material fraction is not done in casework, analysis of this fraction in an experimental setting shows the great variation in male and female DNA distributions between the fractions.

Buffer modifications made to protocol in this research did not negatively affect the results. Successful sperm DNA recovery in the SF, which would generate a single source or distinguishable male profile, was achieved in approximately half of the samples.

The implementation of this TCDE protocol has the potential to alter how sexual assault evidence is processed within forensic laboratories and could assist in reducing the backlog of unanalyzed sexual assault samples. This research has confirmed that the procedure can be faster and less labor intensive while still producing clean DNA profiles in downstream analysis.

5.2 Future Work and Considerations

A more thorough consideration of the problem presented by the presence of large amounts of female DNA should be explored. This is especially important because the results presented here that even a very small proportion of the total female DNA in those

fractions can greatly overwhelm the male DNA present when the initial amounts of sperm are small.

In addition, testing with a larger sample size of mock post-coital swabs in the future will be necessary to confirm the effectiveness of this method. Because of the large variability seen in the amount of female DNA on vaginal swabs, it is essential to obtain data from a larger sample set of vaginal swabs to replicate all the different amounts of female DNA that may be seen in real casework from SAECKs. It could be beneficial to study the qPCR data of the fractions obtained from actual casework samples to understand the female and male DNA distributions in these samples. This would also allow for the study of samples collected from trained Sexual Assault Nurse Examiners (SANE) that represent the variation in samples that are of particular interest to forensic laboratories.

LIST OF JOURNAL ABBREVIATIONS

Anal Chem	Analytical Chemistry
Anal Chim Acta	Analytica chimica acta
Biochim Biophys Acta	Biochimica et biophysica acta
Biol Reprod	Biology of Reproduction
Chem. Eng. News	Chemical and Engineering News
Chem Soc Rev	Chemical Society Reviews
Forensic Sci	Forensic Science
Forensic Sci Int	Forensic Science International
Forensic Sci Int Genet	Forensic Science International: Genetics
Genome Biol	Genome Biology
Hum Reprod Update	Human Reproduction Update
Immunol Today	Immunology Today
Int J Legal Med	International Journal of Legal Medicine
Investig Genet	Investigative Genetics
J Biol Chem	Journal of Biological Chemistry
J Can Soc Forensic Sci	Canadian Society of Forensic Science Journal
J Forensic Identific	Journal of Forensic Identification
J Forensic Sci	Journal of Forensic Sciences
Mol Reprod Dev	Molecular Reproduction and Development
Nat Educ	Nature Education
Natl. Hum. Genome Res. Inst	National Human Genome Research Institute

Proc Nat Acad Sci USA

Proceedings of the National Academy of Sciences
of the United States of America

Sci Justice

Science and Justice

Trends Genet

Trends in Genetics (TIG)

BIBLIOGRAPHY

1. Watson, J.D., Crick FHC. Genetical Implications of the Structure of Deoxyribonucleic Acid. *Nature* 1953;140(3):214–5. <https://doi.org/10.1007/BF00434693>.
2. Cooper G. *The Cell: A Molecular Approach*. Hered. Genes, DNA. 2000. <https://www.ncbi.nlm.nih.gov/books/NBK9944/>.
3. Green ED. Chromosomes. *Natl. Hum. Genome Res. Inst.* 2022. <https://www.genome.gov/genetics-glossary/Chromosome>.
4. Genes and Genetics Explained. BetterHealth Channel. 2021. <https://www.betterhealth.vic.gov.au/health/conditionsandtreatments/genes-and-genetics>.
5. Annunziato A. DNA Packaging: Nucleosomes and Chromatin. *Nat Educ* 2008;1(1):26.
6. Holde KE van. *Chromatin*. 1989.
7. Alberts B, Johnson A, Lewis J, Al. E. *Molecular Biology of the Cell*. 4th editio. New York: Garland Science, 2002.
8. Ward WS, Coffey DS. DNA Packaging and Organization in Mammalian Spermatozoa: Comparison with Somatic Cells. *Biol Reprod* 1991;44:569–74.
9. Biegeleisen K. The probable structure of the protamine-DNA complex. *Journal of Theoretical Biology* 2006;241(3):533–40. <https://doi.org/10.1016/j.jtbi.2005.12.015>.
10. Balhorn R. The protamine family of sperm nuclear proteins. *Genome Biol* 2007;8(9). <https://doi.org/10.1186/gb-2007-8-9-227>.
11. Rathke C, Baarends WM, Awe S, Renkawitz-Pohl R. Chromatin dynamics during spermiogenesis. *Biochim Biophys Acta - Gene Regul Mech* 2014;1839(3):155–68. <https://doi.org/10.1016/j.bbagr.2013.08.004>.
12. McConville MJ, Ferguson MAJ. The structure, biosynthesis and function of glycosylated phosphatidylinositols in the parasitic protozoa and higher eukaryotes. *Biochemical Journal* 1993;294(2):305–24. <https://doi.org/10.1042/bj2940305>.
13. Tecle E, Gagneux P. Sugar-coated sperm: Unraveling the functions of the mammalian sperm glycocalyx. *Mol Reprod Dev* 2015;82(9):635–50. <https://doi.org/10.1002/mrd.22500>.

14. Schröter S, Osterhoff C, McArdle W, Ivell R. The glycocalyx of the sperm surface. *Hum Reprod Update* 1999;5(4):302–13.
<https://doi.org/10.1093/humupd/5.4.302>.
15. Drisdell RC, Mack SR, Anderson RA, Zaneveld LJD. Purification and partial characterization of acrosome reaction inhibiting glycoprotein from human seminal plasma. *Biol Reprod* 1995;53(1):201–8.
<https://doi.org/10.1095/biolreprod53.1.201>.
16. Gill P, Jeffreys AJ, Werrett DJ. Forensic application of DNA “fingerprints.” *Nature* 1985;:8–10.
17. Arnaud CH. Thirty years of DNA forensics: How DNA has revolutionized criminal investigations. *Chem. Eng. News*. 2017. <https://cen.acs.org/analytical-chemistry/Thirty-years-DNA-forensics-DNA/95/i37> (accessed May 16, 2022).
18. Bell GE. Erratum: The highly polymorphic region near the human insulin gene is composed of simple andemly repeating sequences (*Nature* (1982) 295, (31-35)). *Nature* 1982;296(5857):585. <https://doi.org/10.1038/296585c0>.
19. Moody MD. DNA Analysis in Forensic Science: Genetic tools for the solution of violent crimes. *Forensic Sci* 2010;39(1):31–6.
20. Bell J. The polymerase chain reaction. *Immunol Today* 1989;10(10):351–5.
[https://doi.org/10.1016/0167-5699\(89\)90193-X](https://doi.org/10.1016/0167-5699(89)90193-X).
21. The Evaluation of Forensic DNA Evidence. Natl. Res. Council. Comm. DNA Forensic Sci. An Updat. 1996;:Chapter 6 DNA Evidence in the Legal System. <https://www.ncbi.nlm.nih.gov/books/NBK232607/> (accessed May 16, 2022).
22. Desroches AN, Buckle JL, Fournay RM. Forensic biology evidence screening past and present. *J Can Soc Forensic Sci* 2009;42(2):101–20.
<https://doi.org/10.1080/00085030.2009.10757600>.
23. Body Fluid Identification. Natl. Inst. Justice. 2017.
<https://nij.ojp.gov/topics/articles/body-fluid-identification> (accessed May 16, 2022).
24. DNA Evidence: Basics of Analyzing. Natl. Inst. Justice. 2012;:Overview of Steps in Analyzing DNA Evidence. <https://nij.ojp.gov/topics/articles/dna-evidence-basics-analyzing> (accessed May 16, 2022).
25. Victims of Sexual Violence: Statistics. RAINN. .
<https://www.rainn.org/statistics/victims-sexual-violence> (accessed May 24, 2022).

26. Vuichard S, Borer U, Bottinelli M, Cossu C, Malik N, Meier V, et al. Differential DNA extraction of challenging simulated sexual-assault samples: A Swiss collaborative study. *Investig Genet* 2011;2(1):1–7. <https://doi.org/10.1186/2041-2223-2-11>.
27. Clark C, Turiello R, Cotton R, Landers JP. Analytical approaches to differential extraction for sexual assault evidence. *Anal Chim Acta* 2021;1141:230–45. <https://doi.org/10.1016/j.aca.2020.07.059>.
28. Scalzo TP. Sexual Assault Medical Forensic Examinations: A National Protocol. 2005;19(4):2–6.
29. Ladd M, Seda J. Sexual Assault Evidence Collection. Treasure Island, FL: StatPearls Publishing, 2022.
30. Strom K, Hickman M. Untested Sexual Assault Kits: Searching for an Empirical Foundation to Guide Forensic Case Processing Decisions. *Criminolog. American Society of Criminology*, 2016.
31. Peterson J, Johnson D, Herz D, Graziano L, Oehler T. Sexual Assault Kit Backlog Study. Final grant Rep ... 2012.
32. Chong KWY, Thong Z, Syn CK. Recent trends and developments in forensic DNA extraction . *WIREs Forensic Science* 2021;3(2):1–23. <https://doi.org/10.1002/wfs2.1395>.
33. Goodwin W, Linacre A, Hadi S. An Introduction to Forensic Genetics. John Wiley and Sons, Ltd., 2007.
34. Hoff-Olsen P, Mevåg B, Staalstrøm E, Hovde B, Egeland T, Olaisen B. Extraction of DNA from decomposed human tissue: An evaluation of five extraction methods for short tandem repeat typing. *Forensic Sci Int* 1999;105(3):171–83. [https://doi.org/10.1016/S0379-0738\(99\)00128-0](https://doi.org/10.1016/S0379-0738(99)00128-0).
35. Lounsbury JA, Coult N, Miranian DC, Cronk SM, Haverstick DM, Kinnon P, et al. An enzyme-based DNA preparation method for application to forensic biological samples and degraded stains. *Forensic Sci Int Genet* 2012;6(5):607–15. <https://doi.org/10.1016/j.fsigen.2012.01.011>.
36. Saul DJ, Williams LC, Toogood HS, Daniel RM, Bergquist PL. Sequence of the gene encoding a highly thermostable neutral proteinase from *Bacillus* sp. strain EA1: Expression in *Escherichia coli* and characterisation. *Biochim Biophys Acta - Gene Struct Expr* 1996;1308(1):74–80. [https://doi.org/10.1016/0167-4781\(96\)00074-7](https://doi.org/10.1016/0167-4781(96)00074-7).

37. EA1 Protease. CustomScience. 2015.
<http://www.customscience.co.nz/brands/zygem/16-products/zygem/29-eal>.
(accessed May 21, 2022).
38. MicroGEM. forensicGEM Universal. 2022.
<https://microgembio.com/product/forensicgem-universal-dna-extraction-kit/>
(accessed May 22, 2022).
39. Wiegand P, Schürenkamp M, Schütte U. DNA extraction from mixtures of body fluid using mild preferential lysis. *Int J Legal Med* 1992;104(6):359–60.
<https://doi.org/10.1007/BF01369558>.
40. Simone NL, Bonner RF, Gillespie JW, Emmert-Buck MR, Liotta LA. Laser-capture microdissection: Opening the microscopic frontier to molecular analysis. *Trends Genet* 1998;14(7):272–6. [https://doi.org/10.1016/S0168-9525\(98\)01489-9](https://doi.org/10.1016/S0168-9525(98)01489-9).
41. Vandewoestyne M, Van Nieuwerburgh F, Van Hoofstat D, Deforce D. Evaluation of three DNA extraction protocols for forensic STR typing after laser capture microdissection. *Forensic Sci Int Genet* 2012;6(2):258–62.
<https://doi.org/10.1016/j.fsigen.2011.06.002>.
42. Williamson VR, Laris TM, Romano R, Marciano MA. Enhanced DNA mixture deconvolution of sexual offense samples using the DEPArray™ system. *Forensic Sci Int Genet* 2018;34(June 2017):265–76.
<https://doi.org/10.1016/j.fsigen.2018.03.001>.
43. Laurell T, Petersson F, Nilsson A. Chip integrated strategies for acoustic separation and manipulation of cells and particles. *Chem Soc Rev* 2007;36(3):492–506. <https://doi.org/10.1039/b601326k>.
44. Norris JV, Evander M, Horsman-Hall KM, Nilsson J, Laurell T, Landers JP. Acoustic differential extraction for forensic analysis of sexual assault evidence. *Anal Chem* 2009;81(15):6089–95. <https://doi.org/10.1021/ac900439b>.
45. Clark CP, Xu K, Scott O, Hickey J, Tsuei AC, Jackson K, et al. Acoustic trapping of sperm cells from mock sexual assault samples. *Forensic Sci Int Genet* 2019;41(March):42–9. <https://doi.org/10.1016/j.fsigen.2019.03.012>.
46. Adamowicz MS, Stasulli DM, Sobestanovich EM, Bille TW. Evaluation of methods to improve the extraction and recovery of DNA from cotton swabs for forensic analysis. *PLoS One* 2014;9(12):1–18.
<https://doi.org/10.1371/journal.pone.0116351>.

47. Norris J V., Manning K, Linke SJ, Ferrance JP, Landers JP. Expedited, chemically enhanced sperm cell recovery from cotton swabs for rape kit analysis. *J Forensic Sci* 2007;52(4):800–5. <https://doi.org/10.1111/j.1556-4029.2007.00453.x>.
48. Voorhees JC, Ferrance JP, Landers JP. Enhanced elution of sperm from cotton swabs via enzymatic digestion for rape kit analysis. *J Forensic Sci* 2006;51(3):574–9. <https://doi.org/10.1111/j.1556-4029.2006.00112.x>.
49. Ito S, Kobayashi T, Ara K, Ozaki K, Kawai S, Hatada Y. Alkaline detergent enzymes from alkaliphiles: Enzymatic properties, genetics, and structures. *Extremophiles* 1998;2(3):185–90. <https://doi.org/10.1007/s007920050059>.
50. Yakoo M. The development and optimization of a direct lysis differential extraction method. 2017. <https://open.bu.edu/handle/2144/26954>
51. Rai A. Optimization and validation of a novel direct-lysis differential extraction procedure. 2018. <https://open.bu.edu/handle/2144/33006>
52. Roberts A. Optimization of a novel temperature controlled differential extraction procedure for aged sample analysis. 2019. <https://open.bu.edu/handle/2144/38690>
53. Hoffman E. Optimization of the Temperature Controlled Differential Extraction for Casework-Type Samples. 2020. <https://open.bu.edu/handle/2144/41310>
54. Nicholas E. Comparison of Results Using Temperature Controlled Differential Extraction Using the Qiagen EZ1 Advanced. 2021.
55. forensicGEM Sperm. MicroGEM Int. PLC. 2022.
56. Anonymous. Benzonase endonuclease. EMD Millipore 2013;:23.
57. Montville R. Optimization of enzymatic lysis of epithelial cells for application to differential extraction of forensic sexual assault samples. 2016. <https://open.bu.edu/handle/2144/19192>
58. Sinha SK, Brown H, Holt H, Khan M ro, Brown R, Sgueglia JB, et al. Development and validation of a novel method “SpermX™” for high throughput differential extraction processing of sexual assault kits (SAKs) for DNA analysis. *Forensic Sci Int Genet* 2022;59(March):102690. <https://doi.org/10.1016/j.fsigen.2022.102690>.
59. Jollie M. Determining Optimal Swab Type and Elution Buffer to Obtain Whole Swabs for Future Deconvolution of Complex Cell Mixtures. 2021. http://rave.ohiolink.edu/etdc/view?acc_num=bgsu161667366449865

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