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Analysis of two novel differential extraction methods

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ANALYSIS OF TWO NOVEL DIFFERENTIAL EXTRACTION METHODS

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Analysis of Two Novel Differential Extraction Methods

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ABSTRACT

Fast and effective differential extraction methods are sought to assist in facing the challenge of the backlog of sexual assault evidence in the United States. A differential extraction allows for the separation of epithelial cells from the sperm cells on sexual assault evidentiary samples. In this research, two new differential extraction methods are analyzed and compared for their robustness, accuracy, and ultimate success in the separation of the two cell types. The temperature controlled differential extraction (TCDE) method developed by the Cotton Lab at the Boston University Chobanian and Avedisian School of Medicine uses thermostable enzymes in hopes to increase male DNA recovery in the sperm fraction (SF). This method is a direct lysis approach that produces fractions that can go directly, in many cases, to amplification. The SpermX™ method developed by InnoGenomics© makes use of the different compositions of epithelial and sperm cells by incorporating a built-in sperm-trapping matrix and specialized solutions in their procedure. The membrane allows for sperm cells to be trapped while digested epithelial cells flow through the membrane into an outer tube. InnoGenomics© developed its own tubes to be used in this method so that the evidentiary sample swab is never removed, increasing the potential for DNA recovery, and decreasing the manual manipulations of the evidentiary swab by the analyst. The SpermX™ method also is designed to increase the amount of male DNA recovered in the

SF compared to other commonly used methods. In the SpermX™ procedure, DNA purification of the separated cell lysates is need in order to complete downstream DNA analysis.

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LIST OF ABBREVIATIONS

ALS	Alternative light source
bp	Base pair
CE	Capillary electrophoresis
DI	Deionized
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E-cell	Epithelial Cell
EDTA	Ethylenediaminetetraacetic acid
EF	Epithelial fraction
E-gram	Electropherogram
MF	Material fraction
µL	Microliter
mL	Milliliter
ng	Nanogram
qPCR	Quantitative polymerase chain reaction
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
pro K	Proteinase K
RFU	Relative fluorescent unit
rpm	Revolutions per minute
SANE	Sexual Assault Nurse Examiner

SDS	Sodium dodecyl sulfate
SF	Sperm fraction
STR	Short tandem repeat
TCDE	Temperature Controlled Differential Extraction

1. INTRODUCTION

1.1 Genetic Inheritance of DNA

Deoxyribonucleic acid (DNA) is the genetic material that is inherited from our parents and contains the information necessary to encode for proteins that help with specific functions in the human body. The twenty-three chromosomes found in the gametic sperm and egg cells combine to form the twenty-three pairs of chromosomes that are found in all somatic cells (1,2). The huge amount of variation and possible combinations of the genes found in the sperm and egg cells create a unique genetic code for an individual. The alleles that make up those genes at specific locations on DNA can be viewed and used for individual identification, making DNA a useful source of evidence in forensic casework (3).

1.1.1 DNA Packaging in Different Cells

In somatic cells, DNA is packaged by positively charged proteins called histones. The DNA is coiled around eight histone proteins to form a structure called the nucleosome. Then, multiple nucleosomes are compacted even closer together and folded into the chromosome structures that reside in the nucleus (4–6).

In sperm cells, the DNA is much more tightly compacted compared to that of the DNA in somatic cells (7). The amount of DNA in sperm cells is half of that found in somatic cells, but the sperm cell has about forty times less volume to store that amount of DNA (4). The small size of these gametes necessitates a mechanism to produce very tightly bound DNA. The DNA in somatic cells is coiled and looped around the histone

proteins compared to DNA in sperm cells that are tightly bound in a linear fashion (4). Whereas somatic cells are packaged by histones, spermatids contain proteins called protamines that help with DNA packaging. Due to the high percent of positively charged arginines in the protamines, they can more readily bind to DNA, and along with cysteine residues, can help form the essential disulfide bonds that are integral in stabilizing the sperm DNA (7). The breakage of these disulfide bonds is a central component in the successful lysis of sperm cells; with these bonds broken, the DNA in the sperm can be released freely into solution.

1.1.2 The Sperm Glycocalyx

The glycocalyx is a sugar coating containing molecule that is used for fertilization and surrounds the sperm cell (8). The coating can be 20-60 nanometers (nm) thick, and it acts as a protective barrier for the sperm cell, increasing its durability so that it may successfully achieve fertilization. Even the seminal fluid surrounding the sperm cell contains components that help the sperm glycocalyx remain intact until the sperm reaches the egg for fertilization (8,9). Since this sperm cell outer layer seems to be heavily connected to the cells ability to survive and move, understanding this barrier of carbohydrate residues surrounding the sperm cell can help to clarify why some sperm cells are able to remain on cotton swabs without being lysed (8,10).

1.2 Forensic DNA Analysis

The advent of sophisticated DNA technology has revolutionized the field of forensic science. Specifically, the adaptation of polymerase chain reaction (PCR)

technology to forensic casework has allowed for the accurate and sensitive analysis of evidentiary samples containing biological material. PCR allows for target DNA sequences to be amplified into a hypothetically unlimited amount of genetic material, making analysis of low amounts of DNA evidence a possibility (11). Today this technique is used in all types of forensic casework including paternity tests and wrongful conviction testing.

1.2.1 Procedure

When evidence is collected from a crime scene, the item is screened for potential biological fluids using various methods. The context of the case, in combination with an alternative light source (ALS), and presumptive tests can all be used to assist in the identification of any bodily fluids (12). For samples thought to contain semen, an acid phosphatase presumptive test is typically performed. This test makes use of the high concentration of the acid phosphatase enzyme found in semen (13). If any presumptive tests for blood, saliva, or semen, are positive, the item of evidence can be sent forward for DNA analysis (14). This screening process ensures that only the samples suspected of containing DNA are analyzed, making the DNA analysis process more efficient.

There are typically five steps used to describe forensic DNA analysis: extraction, quantitation, amplification, capillary electrophoresis (CE), and interpretation (15,16). Based on the context of the case, presumptive results observed during screening, and protocols of the laboratory, the DNA analyst can decide which extraction method is best suited for each sample. The goal of extraction is to lyse cells and remove any proteins

and other cellular components, leaving DNA from the item of evidence. Quantitation is performed to measure how much DNA is obtained from each item of evidence using quantitative polymerase chain reaction (qPCR). The concentration of DNA from the quantitative results is then used to determine the amount of DNA required to successively complete amplification. Amplification is where multiple copies of the DNA fragments will be produced. Twenty-four different, highly variable, loci are amplified during this process. The samples undergo the CE process, where the DNA fragments are detected, separated by size, and visualized using fluorescence. Finally, software is used to visualize the profile information then the analyst interprets the DNA profile. Profiles from evidence items and known individuals are compared (3).

1.3 Sexual Assault Evidence

Sexual assault is an extremely violent crime that unfortunately happens to an average of 460,000 people in the United States (US) every year (17). Even before the advancement of DNA technology, a woman named Martha Goddard knew the importance of collecting evidence after someone had experienced a sexual assault. She would go on to create a standardized kit used for the collection of sexual assault evidence; this kit is much like the sexual assault evidence collection kits, (SAECKs), examiners use today (18,19). The amount of SAECKs accumulated over the years has created a backlog of kits that have yet to be analyzed. According to the Joyful Heart Foundation, a national organization dedicated to supporting and advocating for sexual assault survivors, there are more than 250,000 untested SAECKs in the US (20,21). In

the past, some SAECKs were only processed if there was a known suspect, leaving many untested (22). Crime laboratories in the US are overwhelmed with the demand for testing backlogged kits due to the lengthy analysis times required for these cases, limited funding, and not enough analysts available for testing (23).

Sexual assault nurse examiners (SANE) are specifically trained to deal with the delicacy of collecting evidence from a sexual assault survivor in a manner that abides by the high standard of documentation and chain of custody needed in a forensic setting (24). Not only does a SANE perform the exam but they can also offer medical advice and care for the person receiving the examination. SAECKs generally include all instructions, diagrams, forms, envelopes, bags, combs, and swabs a SANE would need for evidence collection (25).

1.4 Differential Extraction Process

A differential extraction process is performed on sexual assault samples as a method to separate epithelial cells from the sperm cells. Epithelial cells contain double the amount of DNA than the haploid sperm cells and are typically in much more abundance on a sexual assault vaginal evidence swab. Therefore the overwhelming amount of DNA from the epithelial cells can mask the DNA from a male contributor, making the separation and recovery of sperm cells crucial to producing a male DNA profile (26). The different chemistries and compositions of somatic and sperm cells are used as an advantage to approach separation and lysis of the two cell types. Sperm cells have strong disulfide bonds that are resistant to lysis by sodium dodecyl sulfate (SDS),

which can denature secondary and tertiary protein structures, and Proteinase K (pro K), a protease that digests cell membranes and nucleases that degrade DNA. Both of these reagent are used in traditional differential extractions (27,28). The SDS, and pro K are added to the sample first to lyse all epithelial cells, leaving sperm cells intact. After the initial incubation, the sperm cells are pelleted to the bottom of the tube and the supernatant containing the lysed epithelial cells is removed, creating an epithelial fraction (EF). Dithiothreitol (DTT) or other reducing agent, which is capable of breaking the disulfide bonds in sperm cells, is added to the resuspended sperm pellet, along with SDS and pro K, to create a sperm fraction (SF) (27). First reported in 1985, this procedure has been the standard method for a differential extraction with little deviation over the years from the basic concept of separation (3,27).

There are three components that can define the success of a differential extraction: minimal loss of male DNA to a non-SF, overall high recovery of male DNA, and a lower proportion of female DNA than male DNA in the SF (29). If high amounts of male DNA are extracted in non-SFs, this reduces the amount of DNA present in the SF, decreasing the probability of generating a single source or primary male DNA profile. Ensuring a high recovery of male DNA from any item submitted in a SAECK is important to produce distinguishable profiles; the inability to extract male DNA from the item is detrimental to this goal. Distinguishable profiles as defined by the Scientific Working Group on DNA Analysis Methods (SWGDAM) is a profile where the peak height ratios allow for the deconvolution of a major/minor contributor (30). In addition, re-extraction of the same item is not always possible, making every piece of evidence invaluable, and

re-extraction can be more time consuming for the analyst and delay the progress of the case. Eliminating the amount of female DNA in the SF makes for easier interpretation of the SF profile when allele peaks in the electropherogram (e-gram) can be confidently attributed to only the male contributor. The genotype of the male contributor is the ultimate goal of the differential extraction process as this profile could then be compared to suspects of the sexual assault.

1.5 SpermX™ Method

The SpermX™ method was created as a new differential extraction method that would improve analysis of sexual assault evidence through a reduction of labor and time for the analyst (31). The method was compared to a traditional differential extraction approach and was determined to have a greater sperm DNA recovery in the SF than other methods. The method was determined to recover a wide range of DNA with an average recovery of 0.17 ng from a theoretical 0.3 ng sample, showing it can be very effective in analyzing samples with low amounts of male DNA (31).

The SpermX™ method uses an inner tube with a nanofiber matrix that can trap sperm cells while allowing digested epithelial cells to flow through the matrix into an outer tube. Once the epithelial DNA have washed through, the outer tube, with the epithelial cell eluate, is removed and replaced with a new outer tube. Now, pro K, the SpermX™ Sperm Digest Solution, and DTT can be added into the newly assembled device to lyse the sperm cells trapped in the membrane matrix, there-by producing the SF eluate with male DNA (31). The evidentiary swab remains in the inner tube the entire

time, decreasing the number of manipulations from the analyst and increasing the possibility for sperm recovery. The SpermX™ device can be set into a lower position when adding reagents and during incubation periods, then set in an upper position for centrifugation and collection of fractions from the outer tubes (Figure 1). With the SpermX™ kit, one has the ability to process multiple samples at a time based on laboratory supplies and capabilities. The procedure takes about 5 hours to complete, increasing with the number of samples for analysis.

Figure 1: Depiction of SpermX™ Device in Upper and Lower Positions: Example of inner and outer tubes (left) and the assembled device (right) laid flat and in upper and lower positions.

1.6 EZ1® Advanced Robot

The EZ1® Advanced (QIAGEN, Hilden Germany) robot was developed as an automated alternative for DNA extraction and purification (32,33). The robot performs three simple steps that produces a purified eluate of extracted DNA that is ready for quantitation. First, lysis is mediated by the addition of chaotropic salts that help the DNA bind to the magnetic silica beads deposited in the lysate. A magnet then helps separate the bound DNA from the lysate solution. Finally, the bounded DNA is washed off and eluted in either water or TE buffer at a volume of 50, 100, or 200 microliters (μL). The eluate is free of inhibitors, proteins, and nucleases, making for a high quality DNA extract (34,35). The instrument can process up to six samples in about twenty minutes with all the necessary salts, beads, elution buffers, and reagents provided in the EZ1&2® DNA Investigator® Kit (QIAGEN, Hilden Germany). In this research, the instrument was solely used for purification purposes for samples already extracted using the SpermX™ protocol. Since the SpermX™ protocol utilized DTT, a reagent incompatible with qPCR analysis, the EZ1® was chosen as the method for purification.

1.7 Temperature Controlled Differential Extraction Method

The Cotton Lab at the Boston University Chobanian and Avedisian School of Medicine is developing a temperature controlled differential extraction (TCDE) method for sexual assault samples. It incorporates a direct lysis differential extraction method to decrease the time typically observed in other differential extraction methods while attempting to improve sperm recovery. The enzymes used in this method have an

optimal activity at specific temperatures making the whole process temperature controlled. The method takes advantage of the activity of the enzymes at different temperatures. Thermal stability of enzymes is highly advantageous because the enzymes will not denature at high temperatures used to lyse cells (36). The incubation times for the enzymes to be effective are short and the manual steps in the method are decreased in hopes of eliminating potential human error.

1.7.1 *forensic*GEM™ Reagent

The *forensic*GEM™ reagent is manufactured by MicroGEM International PLC (Charlottesville, VA) and contains the protease Erebus Antarctica (EA1) derived from *Bacillus* sp. (36). The enzyme is active at an optimal temperature of 75°C and inactivated at 90°C. The EA1 protease can degrade proteins including the nucleases of epithelial cells (37). The *forensic*GEM™ Universal and *forensic*GEM™ Sperm kits both contain a mixture of enzymes including the EA1 protease, each dedicated to lysing epithelial and sperm cells respectively. The *forensic*GEM™ Universal enzyme is added first to the EF in the TCDE procedure to lyse any epithelial cells in the sample. The *forensic*GEM™ Sperm enzyme is added in the SF to specifically target any unlysed sperm cells in the solution.

1.7.2 Benzonase® Nuclease

One slight modification to the differential extraction process over the years has been the inclusion of enzymes that can selectively degrade epithelial DNA in solution

while leaving sperm heads untouched (27,38). One of these enzymes, the Benzonase® nuclease (Sigma-Aldrich, St. Louis, MO), is used in the TCDE procedure. Benzonase® is a genetically engineered endonuclease derived from *Serratia marcescens* and produced in *E.coli* (39). Benzonase® also has an optimal temperature of 37°C, making it active at a lower temperature than the enzyme in *forensic*GEM™. The endonuclease is capable of degrading nucleic acids in any form; thus, if any free DNA remains in the material fraction (MF) or SF after initial lysis of cells with *forensic*GEM™ during the TCDE process, the addition of Benzonase® should degrade any free epithelial cell female DNA in solution in the two fractions (40). Once the Benzonase® is done degrading the DNA in solution, the temperature is raised so that the *forensic*GEM™ can be activated. Benzonase® cannot damage the male DNA once the *forensic*GEM™ is added because the higher temperature along with the *forensic*GEM™ inactivates the Benzonase® in the solution (41). Theoretically, with the addition of Benzonase®, the SF should have little to no amount of female DNA, clearing the path for a male only SF.

1.7.3 AcroSolv

AcroSolv is part of the *forensic*GEM™ Sperm kit. The enzymes in AcroSolv operate at an optimal temperature of 52°C and lyse cells, including sperm cells, releasing DNA into solution. The reagent arrives in the kit as a lyophilized powder that can be made into solution. The makeup of the AcroSolv is proprietary but the kit has been shown to successfully lyse sperm cells in the appropriate buffer at 52°C. AcroSolv is added to the SF of the procedure for this reason and the *forensic*GEM™, active at 75°C,

inactivates the AcroSolv when the solution temperature is raised to 75°C. At the end of the TCDE protocol, all enzymes are deactivated, making it suitable to move on to quantitation without a DNA purification step (42,43).

1.8. Concerning Cotton

The types of swabs provided in SAECKs are typically cotton swabs, making it very important to understand how cells, specifically sperm cells, adhere and release from cotton fibers. There have been studies that show that intact sperm cells can be found adhering onto cotton months after the semen was deposited onto the sample, with a great depiction of it in Lachica et al. (44). Cells, particularly sperm cells, have a tendency to become entrapped in the cotton matrix of cotton swabs, with a recovery of about 40% of sperm from traditional differential extraction methods (45). Cotton swabs are used due to their high adsorption, low cost, and efficient drying, but as studies suggest, its ability to adhere to sperm cells might be too good (46,47). There have been other swab types that have been showed to improve the amount of eluted DNA, but until SAECKs move away from cotton swabs, these findings will not be advantageous (48). The underlying cause for the strong adhesion of cells to cotton fibers is still speculative, and finding efficient ways to elute the DNA off these swabs could drastically improve the analysis of sexual assault evidence.

1.9 Goals of Experiment

Experiments reported here investigated the new SpermX™ differential extraction procedure. The aim was to determine if the SpermX™ procedure was efficient and accurate enough to be considered as a robust method for performing differential extractions. Concerning the TCDE method, the goal was to see if a direct lysis approach with temperature-controlled enzymes could improve the release of DNA from the swab. Increasing the amount of male DNA distributed in the SF was another goal of the project. In the end, this research was able to compare the SpermX™ and TCDE methods and see which method would be more suitable for different case scenarios such as mixture interpretation and low male DNA quantity.

2. MATERIALS AND METHODS

2.1 Preliminary Sperm Only Experiments

In order to work out any experimental issues and become familiar with the SpermX™ protocol, some initial experiments were performed using swabs that only contained semen. These experiments were also performed to test if other reagents used in the TCDE protocol, such as AcroSolv and *forensic*GEM™, would be compatible with the buffers and materials provided in the SpermX™ kit. In a modified SpermX™ protocol, Red Buffer was used to perform the SpermX™ inner tube washes instead of the original Sperm Digest Solution; the solution from this wash was collected as the epithelial wash, EW. In addition, part c of the original protocol was adjusted to replace the sperm digest solution with AcroSolv and *forensic*GEM™ Sperm to produce the digested SF. The amount of DNA recovered in all the fractions using the original and modified versions of the SpermX™ protocol where then compared to see which had a higher sperm recovery. Swabs were prepared by placing 15 µL of a 1:10 semen dilution on each sterile cotton swab and letting samples dry in a fume hood overnight.

2.1.1 Preparation of Semen Dilutions

Semen dilutions were prepared by placing a stock semen tube from a donor on the thermal mixer for 30 minutes set at 37°C, 250 revolutions per minute (rpm). After heating, 10 µL of semen was placed into a new tube with 90 µL of TE, resulting in a 1:10 semen dilution. All semen dilutions were subsequently prepared in this way.

2.2 Preparation of Mock Sexual Assault Samples

Swabs were prepared to mimic sexual assault casework samples found and collected in a typical SAECK. There were seven donors total that each donated four vaginal swabs, resulting in a total of twenty-eight mock sexual assault swabs. Approximately 50 ng of male DNA from 1:10 semen dilutions were added to each swab and the swabs were allowed to dry. One prepared swab from each donor was used in the SpermX™ method and the other three swabs were used in the TCDE method.

2.2.1 Collection of Vaginal swabs

Samples were collected according to Boston University IRB protocol. Sample collection kits were prepared containing four sterile cotton swabs and instructions for collection. The instructions requested that the donors not collect swabs if they were menstruating or had had sexual intercourse within the past week; this was set in place as a measure to prevent possible contamination. Per instruction, the donors were also asked to let swabs airdry before placing the swabs back into their original packaging. Seven donors provided four vaginal swabs each from one collection kit. Upon receipt, the samples were recorded in the sample log, and the collection kits were labelled by donor (1-7). When used for analysis each swab was designated by letter (A-D). Swab A was used with the SpermX™ method while swabs B, C, and D were used with the TCDE method. Whole swabs were used for the SpermX™ procedure while half-swabs were used for the TCDE procedure.

2.2.2 Addition of Semen onto Vaginal Swabs

Semen was added to all twenty-eight vaginal swabs to produce mock sexual assault sample that would mimic casework. For the SpermX™ swabs, a total of approximately 50 ng of male DNA was targeted to be added to each swab and let dry overnight before being processed through the SpermX™ procedure. The volume of semen added to each swab was determined using the concentration of a 1:10 semen dilution. For the SpermX™ swabs, a semen dilution with an approximate DNA concentration of 0.7 ng/μL was used, therefore 71.4 μL of semen was added to each swab in order to add approximately 50 ng of male DNA to each swab. For the remaining swabs to be used in the TCDE procedure, a new 1:10 semen dilution was made using a different male donor with a concentration of 3.14 ng/μL. For every TCDE swab, 16 μL of semen was pipetted onto each swab, resulting in approximately 50 ng of male DNA added to each swab. All mock sexual assault sample swabs were prepared on the same day and allowed to dry in a fume hood until they were analyzed. Thus, the amount of semen per swab was controlled but the amount of female DNA per swab was not.

2.3 Epithelial Cell Preparation and Digestion

Tests for performances of specific enzymes became necessary following some results from the TCDE experiments on swabs B and C from all donors. These enzymes were tested on epithelial cells on fabrics and swabs. The epithelial cells were prepped by filling a 2.0 milliliter (mL) tube with saliva then splitting this in half by pipetting out 1 mL of the saliva into a new tube, resulting in two 2 mL tubes with 1 mL of saliva in each.

Then, phosphate buffered saline (PBS) was added to each tube to the 2 mL line and the tube was vortexed and centrifuged at 14000 rpm for 3 minutes. The supernatant was removed, and TE was added until the 2 mL line. The tube was again vortexed and centrifuged at 14000 rpm for 3 minutes, and the supernatant removed. This was done two more times. The cell pellets were resuspended in approximately 500 µL and were combined. 200 µL of TE was added to the empty tube to recover any remaining cell contents. The tube with 200 µL of TE was vortexed and briefly spun then pipetted into the primary tube.

When using this epithelial cell (E-cell) prep, 10 µL of this solution was used on a fabric, swab, or digested directly. Digestion of the of the E-cells was done in a total volume of 100 µL. The swab or fabric was placed in a 0.5 mL tube with 10 µL of 10X Blue Buffer, 1 µL of *forensic*GEM™, and 89 µL of deionized (DI) water then incubated at 75°C and 95°C for 5 minutes each. The substrate was then placed in a spin basket and centrifuged to release any liquid remaining on the substrate. The eluate collected was returned to the original 0.5 mL EF tube. The dry substrate was digested using 10 µL of Red Buffer, 2 µL of *forensic*GEM™, 10 µL AcroSolv, and 78 µL of DI water incubated at 52°C for 5 minutes, 75°C for 3 minutes, and 95°C for 5 minutes. At the end of the incubation, the substrate was placed in a spin basket and centrifuged to release any remaining liquid. The released liquid was then returned to the original 0.5 mL MF tube. Both the EF and MF samples were ready to be quantified.

2.4 SpermX™ Method

The SpermX™ reagents, tubes, and protocol was developed and provided by InnoGenomics© Technologies as a new approach to process sexual assault samples. An additional kit was purchased from them directly and included two solutions, inner and outer tubes, pliers, and two adapter racks required for the procedure. InnoGenomics© also provided six test swabs to be used with the SpermX™ procedure along with documentation on how much DNA was expected to be on each swab. The swabs were from the same donor and were labelled S1A-S1F. All their swabs were used in the SpermX™ protocol along with Swab A from each BU mock sexual assault sample.

2.4.1 Reagent Components

The SpermX™ kit contained two major reagents, the Epithelial Digest Solution and the Sperm Digest Solution. Proteinase K (20 mg/mL) and 1M DTT were purchased separately from ThermoFisher Scientific© to be used in this procedure. The Epithelial Digest Solution was used with pro K in the first two parts of the SpermX™ protocol to produce the Epithelial Fraction 1 (EF1) and the Epithelial Fraction 2 (EF2). The Sperm Digest Solution was used to create the Epithelial Wash (EW) and was used in conjunction with pro K and 1M DTT to produce the Sperm Fraction (SF) according to the manufacturer's instructions.

Since multiple reactions were processed at the same time, master mixes were created to limit the amount of pipetting needed. Table 1 depicts the amounts from each component needed to create each master mix. Since the EW only called for the use of the

Sperm Digest Solution, no master mix was needed, and the solution was directly pipetted from the solution bottle to the sample tubes. The sperm digest solution master mix accounts for loss of solution by adding an additional 10% to each sample.

Table 1: SpermX™ Master Mixes: A list of the components that make up each master mix used in SpermX™ protocol.

Master Mix	Component per Sample
Epithelial Digest A	Epithelial Digest Solution (585µL) Proteinase K (20 mg/mL) (30 uL)
Epithelial Digest B	Epithelial Digest Solution (600 µL) Proteinase K (20 mg/mL) (15 µL)
Sperm Digest	Sperm Digest Solution (325.6 µL) Proteinase K (20 mg/mL) (26.4 µL) 1M DTT (88 µL)

2.4.2 SpermX™ Method Adjustments

SpermX™ reagents were kindly provided by InnoGenomics© along with six swabs having approximately 1000 female and 100 male ng of DNA. The procedure used followed the manufacturer's instructions with the exceptions noted below. The SpermX™ method was adjusted to better suit the equipment available in the laboratory. For all incubations, the sample tubes were placed in a water bath adjusted to the corresponding temperature for that step. For the washes step, instead of placing the SpermX™ inner tube into a 15 mL conical tube, it was placed into a new SpermX™ outer tube and every time 500 µL was added, the tube was centrifuged, and the eluate was pipetted out into a 2.0 mL tube labelled EW. The last step in part C, where an additional 150 µL of Sperm Digest Solution was added to the sample tube, was removed since it was believed at the time that it would not

dramatically increase the yield of DNA. In addition, to be comparable to other procedures used in this lab, the swabs were removed from the wooden stick as the protocol recommends leaving the cotton swab on the stick. These adjustments were carried out for every sample analyzed through this procedure.

2.4.2.1 Substituting Reagents in the SpermX™ Method

The SpermX™ method was also modified in an attempt to substitute direct sperm lysis procedures for the DNA to be used with Red Buffer, AcroSolv, purification steps, and *forensic*GEM™ for the epithelial wash and sperm digestion steps. These changes were made to determine if replacing the Sperm Digest Solution with tested reagents that are known to adequately lyse sperm cells would still yield promising results using the SpermX™ device. The 10X Red Buffer was diluted to 1X Red Buffer in a final volume of 1500 µL to be used in the wash steps. Table 2 includes a list of Master Mixes composed of 10X Red Buffer, AcroSolv, and *forensic*GEM™ sperm that was used in the sperm digestion step to produce the SF. With the addition of these reagents, a final incubation step following the *forensic*GEM™ protocol was needed to inactivate the enzymes and for digestion to be complete. Incorporation of the AcroSolv and *forensic*GEM™ would eliminate the need for purification steps, as once sperm digestion was complete using *forensic*GEM™, the samples were ready for quantitation and amplification. These modifications were done on semen only swabs and it was determined that these adjustments would not be carried forward and used on the InnoGenomics© or BU mock sexual assault samples as the direct lysis reagents are not compatible with the SpermX™ kit and reagents.

Table 2: Modified SpermX™ Master Mixes: A list of the master mixes used in modified SpermX™ protocol.

Master Mix	Component per Sample
Epithelial Digest A	Epithelial Digest Solution (585 µL) Proteinase K (30 µL)
Epithelial Digest B	Epithelial Digest Solution (600 µL) Proteinase K (15 µL)
Wash	1X Red Buffer (500 µL each time, 1500 µL total)
Sperm Digest	10X Red Buffer (40 µL) AcroSolv (10 uL) *forensic*GEM™ Sperm (2 µL) DI Water (348 µL)

2.4.3 Final SpermX™ Extraction Procedure

The SpermX™ protocol as provided in the procedure was used with only the modifications mentioned in section 2.4.2 and the master mixes that were used are the ones listed in Table 1.

For the protocol, a SpermX™ inner tube was placed into a SpermX™ outer tube and a whole sample swab was placed inside the assembled device. Then, 615 µL of the Epithelial Digest A Master Mix was added to the tube and vortexed. The tube was incubated at 56°C for 1.5 hrs, with the tube being vortexed every half hour. After incubation, the inner tube was raised and locked in the upper position then centrifuged at 4000 rpm for 5 minutes. The eluate collected in the outer tube was transferred into a 1.5 mL tube and labelled EF1. The inner tube was then placed into a new outer tube where 615 µL of Epithelial Digest B Master Mix was added in the inner tube. The tube was vortexed and centrifuged at 4000 rpm for 1.5 minutes then incubated at 56°C for 30

minutes, where the tube was vortexed every 15 minutes. After incubation, the inner tube was raised and locked in the upper position then centrifuged at 4000 rpm for 5 minutes. The eluate was collected and transferred to a 1.5 mL tube labelled EF2. The inner tube was then placed into a new outer tube where 500 µL the Sperm Digest Solution was added to the tube, vortexed, and centrifuged at 4000 rpm for 5 minutes. The eluate was collected and transferred to a 2.0 mL tube labelled EW. This washing step was repeated two more times and the eluate was collected and transferred into the same 2.0 mL EW tube. Finally, the inner tube was placed into a new outer tube where 400 µL of the Sperm Digest Master Mix was added to the tube, vortexed, and incubated at 63°C for 45 minutes, where the tube was vortexed every 15 minutes. After incubation, the inner tube was once again raised and locked in the upper position where it was then centrifuged at 4000 rpm for 5 minutes. The eluate was collected and transferred into a 1.5 mL tube labelled SF. All four fractions were then ready for purification, quantitation, and amplification.

2.5 TCDE Method

2.5.1 Reagent Components

The TCDE protocol calls for the use of *forensic*GEM™ Universal, 10X Blue Buffer, Benzonase®, AcroSolv, 10X Red Buffer, and *forensic*GEM™ Sperm. The *forensic*GEM™ products, 10X Blue Buffer, AcroSolv, and 10X Red Buffer can be purchased together as they are part of the *forensic*GEM™ Universal and *forensic*GEM™ Sperm Lysis kits. The Benzonase® nuclease was purchased from Sigma Aldrich at a concentration of 250 units (U)/ µL.

The preparation of master mixes was also necessary for this procedure when dealing with multiple samples (Table 4). Before the creation of the master mixes, 2X Benzonase® Buffer, a 1:100 dilution of Benzonase® stock in 2X Benzonase® Buffer, and a 1:13 dilution of *forensic*GEM™ in 10X Blue Buffer solutions were prepared. The 2X Benzonase® Buffer was prepared at a final volume of 100 mL by mixing 4 mL of 1M Tris (pH 8.0), 0.0381grams (g) $MgCl_2$, 0.2338 g of NaCl, and DI water to a final volume of 100 mL. The Benzonase® was used to create a 1:100 dilution of Benzonase® in 2X Benzonase® Buffer, resulting in a final concentration of 2.5U/µL (Table 3).

Table 3: Solutions needed before the preparation of master mixes.

Solution	**Component**
2X Benzonase® Buffer	1M Tris (pH 8.0), (4 mL) $MgCl_2$ (0.0381 g) NaCl (0.2338 g) DI Water (to 100 mL)
1:100 Benzonase® in 2X Benzonase® Buffer	2X Benzonase® Buffer (198 µL) 250 U/µL Benzonase® (2 µL)
1:13 *forensic*GEM™ in 10X Blue Buffer	10X BLUE Buffer (12 µL) *forensic*GEM™ (1 µL)

Table 4: TCDE Master Mixes: A list of master mixes used in the TCDE method.

Master Mix	Component per Sample
A	10X Blue Buffer (50 µL) DI Water (447 µL)
B	1:13 *forensic*GEM™ in 10X Blue Buffer (13 µL) 2.5U/µL Benzonase® in 2X Benzonase Buffer (10 µL) 2X Benzonase Buffer (49 µL) DI Water (109 µL)
C	*forensic*GEM™ Universal (1 µL) 2.5U/µL Benzonase® in 2X Benzonase buffer (10 µL) 2X Benzonase buffer (4 µL)
D	10X Red Buffer (22 µL) AcroSolv (26 µL) *forensic*GEM™ Sperm (5 µL)
E	10X Red Buffer (6 µL) AcroSolv (10 µL) *forensic*GEM™ Sperm (2 µL)

2.5.2 TCDE Method Adjustments

It was noted after experiments using BU sample swabs B and C in the TCDE protocol that when 181 µL of Master Mix B was added to the tube in the MF, the swab was not completely submerged in the solution. It is important that the swab be completely covered so that the reagents can reach cells stuck on the inside of the cotton swab and ensure maximum recovery of DNA. Therefore, after this observation, the protocol was modified so that 300 µL of Master Mix B was added to the tube for the MF, ensuring that

the swab is completely submerged in the solution. Due to this increase in volume, the amount of volume per sample for each component of Master Mix B was adjusted while still maintaining its original concentrations. The component of 1:13 *forensic*GEM™ in 10X Blue Buffer was eliminated from Master Mix B. Instead, 1 µL of *forensic*GEM™ and 20 µL of 10X Blue Buffer were added directly into Master Mix B.

Other slight changes were also incorporated to improve the protocol based on experiments using an epithelial cell prep on isofabrics (Testfabrics Inc., West Pittson, PA) and swabs. Before the epithelial cell lysis, the amount of 1X Blue Buffer used in the one-hour incubation period, before the addition of *forensic*GEM™, changed from 497 µL to 495 µL. Also, during this incubation period, the tubes were placed on a thermomixer set at 20°C at 250 rpm instead of remaining idle on the benchtop. When the hour was over, 5 µL of *forensic*GEM™ was added to the tube to reach a final volume of 500 uL, instead of the 3 µL of *forensic*GEM™ added originally. After the addition of the *forensic*GEM™, the tubes were incubated in a Veriti™ 60-well Thermal Cycler at 75°C for 30 minutes instead of 15 minutes as the protocol previously stated. The increased incubation time provides more time to digest the epithelial cells in the sample and therefore decrease the amount of undigested female DNA being carried over into the MF.

2.5.3 Final TCDE Extraction Procedure

This final extraction procedure, with all the modifications mentioned above, was used for swab D from each donor from the BU mock sexual assault samples. Table 5 shows the final components used for Master Mixes A through E. The swab was cut in half and

one half was placed in a 0.5 mL tube, labelled SF, containing 495 µL of Master Mix A. The tube was incubated for one hour on a thermomixer at 20°C at 250 rpm; the tube was vortexed at the beginning, middle, and end of the hour. After the incubation, 5 µL of *forensic*GEM™ was added to the tube for E-cell lysis and the tubes were then incubated at 75°C for 30 minutes and 4°C for 5 minutes. Once finished, the swab was removed from the SF tube and placed in a 2.0 mL tube with a spin basket. This tube was centrifuged at 14000 rpm for 5 minutes to remove any liquid on the swab. After centrifugation, the swab was moved to a new 0.5 mL tube labelled MF containing 300 µL of Master Mix B. The eluate from the 2.0 mL tube was vortexed and placed back into the SF tube. The SF tube was then centrifuged at 14000 rpm for 5 minutes, the supernatant, about 470 µL, was collected and placed into a new 0.5 mL tube labelled EF. The 30 µL sperm pellet remaining in the SF tube was vortexed for resuspension and then 15 µL of Master Mix C was added to the tube. Both the SF and MF were then incubated at 37°C for 15 minutes, 75°C for 15 minutes, and 4°C for 5 minutes. After incubation, 53 µL of Master Mix D was added to the MF tube and 18 µL of Master Mix E was added to the SF tube. Both were then incubated at 52°C for 5 minutes, 75°C for 3 minutes, 95°C for 3 minutes, and 4°C for 5 minutes. Once incubation was complete, the substrate from the MF was spun down and the eluate was placed back in the MF tube. The EF tube was incubated at 95°C for 5 minutes and 4°C for 5 minutes. All three fractions were now ready for quantitation and amplification. The TCDE samples were not required to go through a purification step on the EZ1® Advanced robot. The direct lysis procedure does not require DNA purification prior to amplification.

Table 5: **Final TCDE Master Mixes:** A list of all master mixes (A-E) used in the final TCDE protocol.

Master Mix	Component per Sample
A	10X Blue Buffer (50 µL) DI Water (445 µL)
B	*forensic*GEM™ Universal (1 µL) 2.5U/µL Benzonase® in 2X Benzonase buffer (10 µL) 2X Benzonase buffer (88 µL) DI Water (181 µL) 10X Buffer Blue (20 µL)
C	*forensic*GEM™ Universal (1 µL) 2.5U/µL Benzonase® in 2X Benzonase buffer (10 µL) 2X Benzonase buffer (4 µL)
D	10X Red Buffer (22 µL) AcroSolv (26 µL) *forensic*GEM™ Sperm (5 µL)
E	10X Red Buffer (6 µL) AcroSolv (10 µL) *forensic*GEM™ Sperm (2 µL)

2.6 Purification, Quantitation, Amplification, and Capillary Electrophoresis

All fractions that were a result of the SpermX™ protocol needed to go through a DNA purification process. This was accomplished by placing the samples in the EZ1® instrument following all manufacturing guidelines for the Tip Dance protocol where a final elution volume of 200 µL was selected. After purification, all SpermX™ samples could now undergo quantitation and amplification. The quantitation of all fractions from the SpermX™ protocol and the TCDE protocol were completed using qPCR on the 7500 Real-Time PCR instrument (Applied Biosystems, Foster City, CA) operating according to the manufacturer's instructions. The samples were prepared using the Quantifiler® Trio Kit (Applied Biosystems, Foster City, CA) before being placed in the PCR instrument. The

DNA concentration values (ng/μL) were determined based on a virtual standard curve created using the PCR instrument from a known set of DNA standards. The total mass of human DNA in each sample was calculated by multiplying the small autosomal concentration value of the sample by the volume of the extract, resulting in mass in ng. Similarly, the total male human DNA was calculated by multiplying the Y autosomal concentration (ng/μL) by the volume of the sample. The total mass of female DNA was calculated by subtracting the mass of male human DNA from the mass of total human DNA.

Samples were then amplified 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. All samples were amplified using a target of 0.75 ng of DNA with samples being diluted, if needed, to reach this target number. For samples having less than 0.75 ng in total, 15 μL of sample was used for amplification. All amplifications included a positive and negative control.

After amplification, DNA fragments were separated using capillary electrophoresis on a SeqStudio™ Genetic Analyzer (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. DNA profiles could then be visualized as electropherograms (e-grams) from the results of the CE using the GeneMapper® ID-X version 1.6 (Applied Biosystems, Foster City, CA) software. The analysis method on the software used was set to an analytical threshold of 100 relative fluorescence units (RFU). DNA quantitation data was analyzed and graphs were made using the statistical software JMP.

3. RESULTS

3.1 Preliminary Sperm Only Experiments - SpermX™

Sperm only swabs were analyzed using the SpermX™ method to better understand the mechanics of the protocol. Three swabs with 15 μL of a 1:10 semen dilution were used with the standard protocol and the four resulting fractions were quantified using qPCR analysis. These results showed that the procedure was successful in recovering sperm from the swab while losing less than 1 ng to the EW. Table 6 shows a summary of the amount of DNA recovered from each swab per fraction. The total average amount of DNA recovered from all three swabs was 96 ng. The average amount of DNA recovered in the SF from the three swabs was 89 ng. Thus, an average 92% of the total DNA was recovered in the SF. It is very important for most of the DNA of the male to be recovered in the SF with little lost to the other fractions. The more male DNA that can be recovered in the SF, the more likely that a single source or distinguishable male profile will result.

Table 6: DNA Recovered in Sperm Only Experiment: The amount of DNA recovered, in ng, from three semen only swabs using the SpermX™ method.

Swab	EF1	EF2	EW	SF	TOTAL, ng
A	5.61	1.23	0.60	60.85	68.28
B	3.08	0.98	0.72	94.89	99.67
C	7.69	1.80	0.51	111.32	121.32

3.1.1 SpermX™ Procedure Modification

After the initial extraction of "sperm-only swabs" the SpermX™ protocol was modified to assess whether Red Buffer with AcroSolv and *forensic*GEM™ could be used in lieu of the Sperm Digest Solution to produce the EW and SF, respectively. Again, three swabs with 15 μL of the same 1:10 semen dilution were analyzed using this modified protocol. This time, a total average 10.5 ng male DNA was recovered from all four fractions with 2.5 ng being in the SF, making for a 23% recovery of DNA in the SF (Table 7). These modifications resulted in a decrease in the amount of DNA recovered from the entire swab and a decrease in the percentage of DNA recovered in the SF.

Table 7: DNA Recovered from Sperm Only Modified SpermX™ Protocol
Experiment: Mass, ng, of DNA recovered from three swabs using modified SpermX™ protocol.

Sample	Amount of DNA Recovered (ng)
Swab A	13.62
Swab B	9.33
Swab C	8.60

3.1.2 Are Direct Cell Lysis Reagents Compatible with the SpermX™ Device and Reagents?

Due to the low DNA recovery from the experiments on the sperm only swabs using AcroSolv instead of the Sperm Digest Solution, it was postulated that there might be some interference between the AcroSolv and the SpermX™ reagents. To assess which components of the SpermX™ kit might be incompatible with AcroSolv, experiments were done to test both the Epithelial Digest Solution and the SpermX™ device itself.

Three 1.5 mL tubes with 15 μL of a 1:10 semen liquid dilution were set up labelled A, B, and C. Tube A was used as a control and digested using the standard AcroSolv/*forensic*GEM™ Sperm procedure. The Epithelial Digest Solution was added to tubes B and C then digested with AcroSolv/*forensic*GEM™. The qPCR results showed that tube A had a total mass of 337 ng, tube B had 5 ng, and tube C showed no results for the qPCR. We know that the Epithelial Digest Solution is not a qPCR inhibitor since preliminary sperm only experiments using all the SpermX™ reagents were able to successfully produce qPCR data, therefore one can conclude that the AcroSolv does not perform well alongside the Epithelial Digest Solution, and it cannot successfully lyse the sperm cells.

Two swabs, labelled A and B, with 15 μL of the 1:10 semen dilution were prepared to be used with the SpermX™ device. Based on previous results, the Epithelial Digest Solution was replaced with TE in the first half of the SpermX™ protocol while AcroSolv and *forensic*GEM™ were used in the second half for sperm digestion. TE is known to work alongside AcroSolv and *forensic*GEM™ therefore removing another variable of inhibition of the AcroSolv. The results showed very low qPCR values for both swabs, with a recovery of 0.8 ng from both swab A and swab B (Table 8). It was then concluded that the AcroSolv could not be used in the SpermX™ device nor with the SpermX™ reagents. Based on these results, the original SpermX™ protocol with its corresponding reagents was subsequently used for the remaining SpermX™ procedures.

Table 8: **Reagent Compatibility Experiments**: DNA recovery on experiments testing AcroSolv against SpermX™ components where swab A in the left column acts as a control.

Swab	AcroSolv + Epithelial Digest Solution	TE + AcroSolv in SpermX™ Protocol
A	337.03 ng	0.82 ng
B	5.18 ng	0.82 ng
C	0 ng	NA

3.2 SpermX™ Differential Extraction with Mock Sexual Assault Evidence Swabs

InnoGenomics© provided six test swabs to be analyzed using SpermX™. These test swabs had an expected amount of 1000 ng of female DNA and 100 ng of male DNA. Additional mock sexual assault swabs from Boston University were also used with this method. To distinguish the SpermX™ test swabs and the prepared BU mock sexual assault swabs, the SpermX™ swabs were labelled S1A-S1F and the BU mock swabs were labelled M1A-M7A, with the number corresponding to the different swab donors and the letter at the end corresponding to "Swab A" in the donation kit. All swabs were run as whole swabs following the SpermX™ protocol except for S1A, which was cut in half and designated S1A-1 and -2 for the corresponding halves.

3.2.1 Quantitation of InnoGenomics© Test Swabs

All six SpermX™ test swabs had an expected total DNA amount of 1100 ng. The estimated amounts of male and female DNA in each fraction are shown in Table 9. When looking at the individual fractions, there is some male DNA carryover in the EF1 and female carryover in the SF. In the SF of swab S1B, the female is the major

contributor. Even when the male is the major contributor in the SF, the presence of female DNA can lead to profiles being mixtures instead of ideally being single sourced. There are some samples where more male DNA is released in the EF compared to that found in the SF, as seen in sample S1A-1, S1C, and S1D. Although female carryover in the SF makes interpretation harder, when performing a differential extraction, one would like for all the male DNA to be recovered in the SF since that is the profile that will most likely be utilized for comparison purposes to potential suspects. Additionally, loss of male DNA to other fractions decreases the probability of obtaining a distinguishable male profile from the SF.

Table 9: DNA Recovered from InnoGenomics© Samples: Estimated Mass of DNA recovered from InnoGenomics© samples from every fraction using SpermX™ protocol.

Swab (ng)	**Female DNA per Fraction (ng)**				**Male DNA per Fraction (ng)**				**TOTAL (ng)**
	EF1	**EF2**	**EW**	**SF**	**EF1**	**EF2**	**EW**	**SF**	
S1A-1	107.74	84.98	0.57	2.55	6.08	6.40	0.06	4.43	212.85
S1A-2	32.71	13.69	0.92	1.38	2.70	0.52	0.25	7.54	59.75
S1B	41.63	148.52	76.51	357.44	16.92	10.70	1.83	39.46	694.04
S1C	149.21	1.17	17.22	7.24	23.68	0	0.58	22.73	221.68
S1D	80.29	173.77	113.18	37.93	16.40	5.23	2.58	16.26	445.66
S1E	202.05	158.31	15.24	17.74	19.56	6.27	0.97	33.18	453.36
S1F	140.11	143.57	6.62	13.68	12.27	9.09	0.58	22.87	348.84

The EF2 and EW fractions are not required to be kept according to the SpermX™ protocol. However, both fractions were analyzed using qPCR in order to determine how much DNA, if any, was contained in these two fractions. The results showed that an average of 37.2% female and 2.1% male DNA of the total DNA was lost in these two fractions. Since there is an overwhelming amount of female DNA in the EF1, the loss of the female DNA is not as consequential as the loss of the male DNA to the EF2 and EW fractions.

The total observed recovery of DNA from each swab was less than the expected DNA assumed to be on the swab. An average of 406 ng of DNA was recovered from all six test swabs. That is less than half of the expected DNA of 1100 ng and an average recovery rate of about 37%. DNA remaining on the swab itself (MF), could not be analyzed using qPCR. Based on the initial experiments, the swab could not be digested with AcroSolv to produce an MF. Attempts to produce an MF from the swab by processing it through the EZ1® were unsuccessful, the swabs would repeatedly get stuck inside the tips used in the EZ1® while control swabs with only water were processed successfully using the EZ1®. Additional swabs that had undergone the SpermX™ protocol were also used on the EZ1®, but again the instrument was unable to successfully process the swabs. A possible reason for this was that the reagents in the SpermX™ protocol made the swabs too “fluffy”, and they were getting stuck in the tips in the EZ1®. It was concluded that a MF for the swabs was not feasible, therefore the amount of DNA left on the swab, if any, remained undetermined for samples analyzed using the SpermX™ protocol.

Even though the mass of DNA recovered was less than the expected amount, the proportion of female to male DNA recovered from the swab was fairly reproducible. The expected was a ratio is 90% female to 10% male. Table 10 shows the proportion of total female to total male DNA for each swab. The average proportion of female DNA to male DNA was 87% to 13%, which is very close to the expected values of 90% and 10% stated by the manufacturer.

Table 10: Female to Male ratio of total DNA observed in InnoGenomics© samples.

Swab	Total Observed DNA (ng)	Total Female DNA (ng)	Total Male DNA (ng)	Percent Female	Percent Male
S1A	272.611	244.585	28.026	89.72%	10.28%
S1B	693.041	624.114	68.927	90.05%	9.95%
S1C	221.868	174.861	47.007	78.81%	21.19%
S1D	445.666	405.183	40.483	90.92%	9.08%
S1E	453.362	393.365	59.997	86.77%	13.23%
S1F	348.841	304.005	44.836	87.15%	12.85%

3.2.2 DNA Profiles from InnoGenomics© Test Swabs

Since the qPCR results of the test swabs indicated that the EF and SF fractions would produce mixture profiles, these fractions from each swab were taken to profile for confirmation. Based on the genotypes of the EF and SF fractions from all the sample swabs, it was determined that the same donors were used for all SpermX™ test swabs.

When looking at the EF profiles, S1A-1 is a single source profile with no indication of any male DNA in the profile. Profiles S1A-2 – S1F have Y allele calls at the sex determining locus amelogenin and at the Y-indel locus, indicating some presence

of male DNA (Figure 2). Despite the indication of a male contributor, a full female distinguishable profile can be determined for the EF for all seven samples.

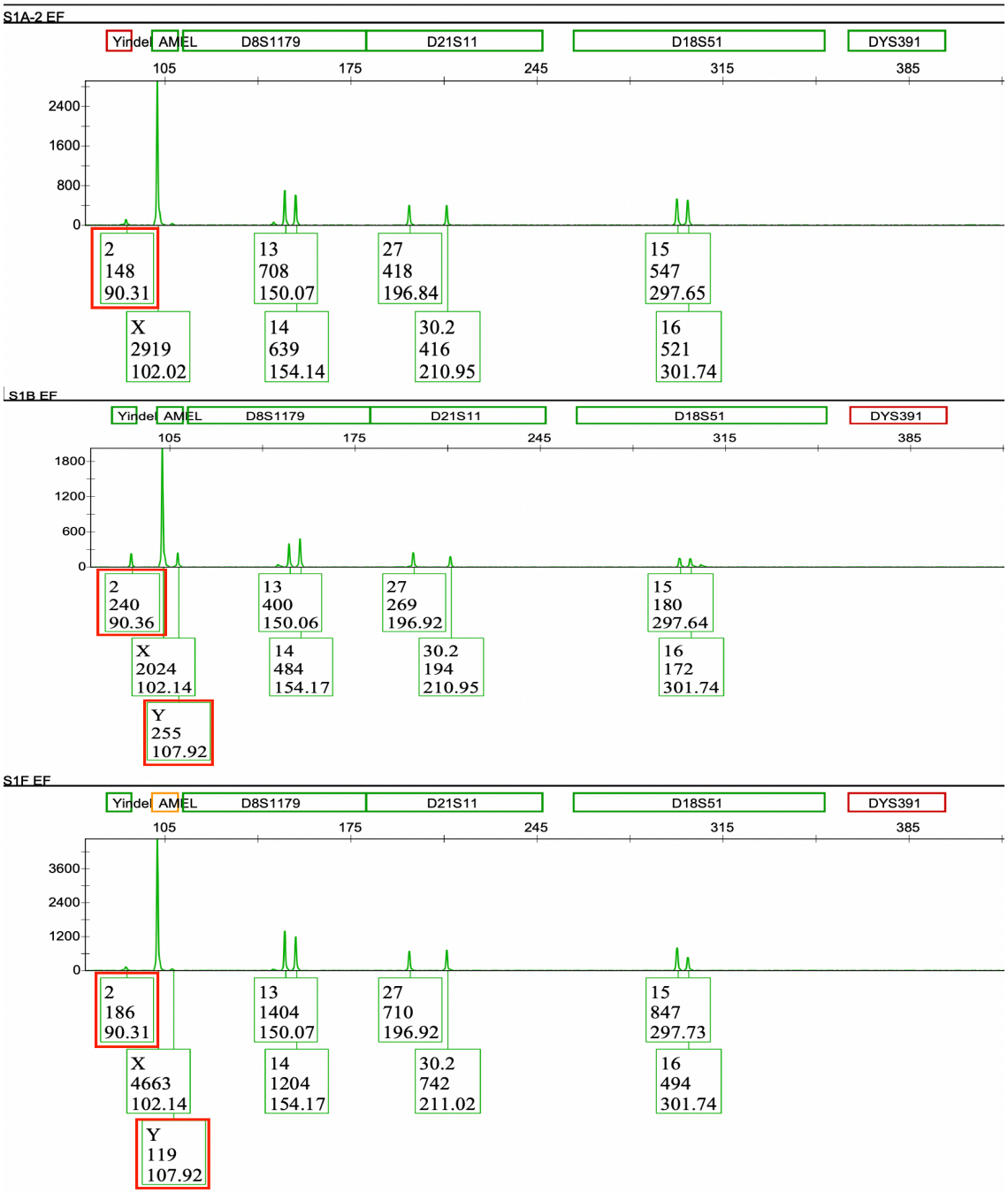

Figure 2: Male Peaks Detected in EF of InnoGenomics© samples: Allele calls in green panel from male contributor highlighted in red in EFs from S1A-2, S1B, and S1F.

The loss of male DNA in the EF was not detrimental in being able to produce an SF profile. SF profiles from the green panel for samples S1A-2, S1B, and S1D are shown in Figure 3. The SF profiles also show that for every donor swab, there are multiple loci that display three or four allele calls, indicating a minimum of at least two contributors. Even with the female reference profiles, determining the genotype of the male contributor may still be difficult and require computer assisted methods. This is seen in samples S1A-1, S1D, S1E, and S1F. Figure 4 shows examples of the blue panel of the female reference profile of S1E compared to the profile obtained from the SF. One is not able to distinguish definitively at a locus with three allele calls, the genotype of the male, especially when the peak height ratios are fairly balanced. We know that the female is a 9,12 at the D16S539 locus and it can be determined that the male is a 13 at that same locus, but it is undetermined if they are homozygous 13 or heterozygous with an undetermined allele (Figure 4). This presents challenges when considering the context of a sexual assault since the genotype of the unknown, or in this case male contributor, is of utmost importance to be able to compare the DNA profiles to reference profiles of potential suspects.

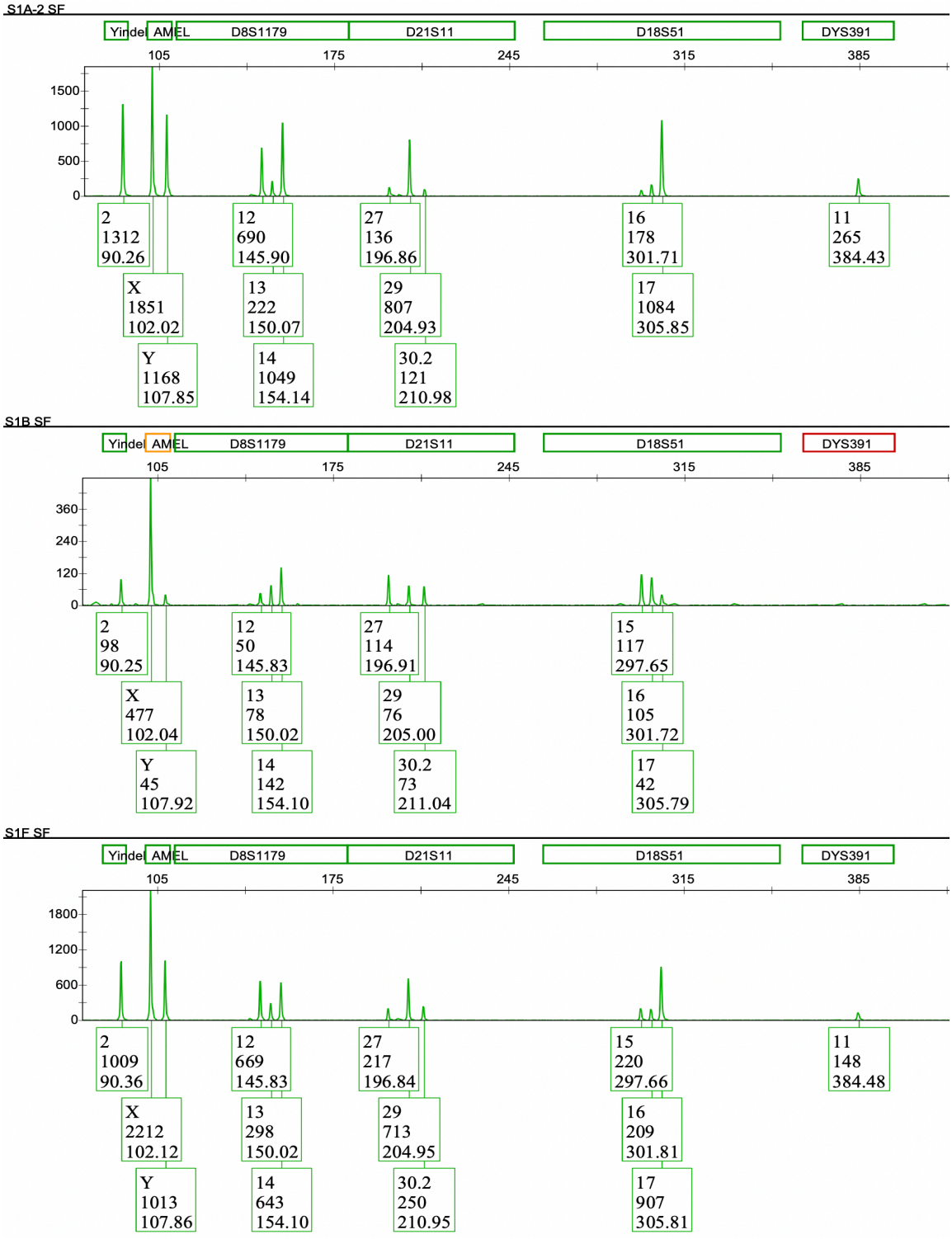

Figure 3: SF Profiles for Samples S1A-2, S1B, and S1F: Profiles from these samples show results from sperm fractions that are 84%, 10%, and 63% male, respectively.

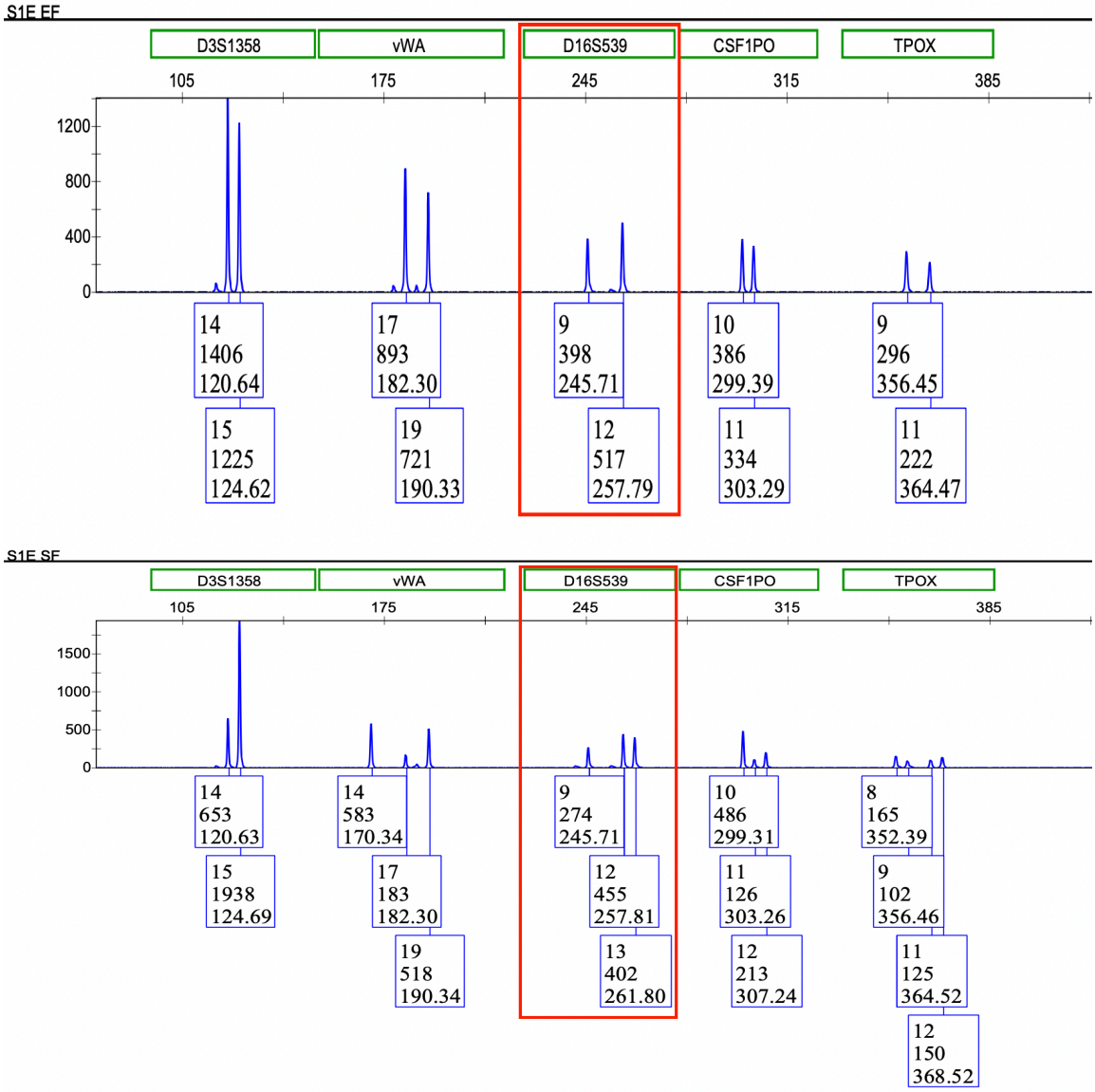

Figure 4: Comparison of Female Reference Profile to SF Mixture Profile: The blue panel of the female reference profile (above) compared to the profile obtained from the SF of the same sample (below).

3.2.3 Quantitation of Mock Sexual Assault Swabs from BU

All swabs marked "A" from the seven BU donor kits were processed using the SpermX™ protocol. For these swabs, the amounts of female DNA deposited onto each swab was unknown. Approximately 50 ng of male DNA was placed onto each swab.

The qPCR results for each fraction of the SpermX™ extraction are shown in Table 11. While there was detectable male DNA in the EF1, it was small compared to the amount of female DNA present. For example, in sample M1A, there was approximately 709 ng of female DNA compared to 4 ng of male DNA in the EF1 (Table 11). The large mass of female DNA present in each EF1 fraction masked the male DNA, resulting in clear single source female profiles.

Table 11: Mass of female and male DNA recovered per fraction from BU mock sexual assault samples using SpermX™ protocol.

Swab (ng)	Female DNA per Fraction (ng)				Male DNA per Fraction (ng)				TOTAL (ng)
	EF1	**EF2**	**EW**	**SF**	**EF1**	**EF2**	**EW**	**SF**	
M1A	708.72	574.32	8.86	35.37	4.00	0.60	0.09	29.84	1361.81
M2A	987.95	616.17	10.69	35.05	2.73	1.83	0.06	27.61	1682.09
M3A	1412.22	741.08	29.55	0.00	4.28	1.10	0.90	30.90	2220.03
M4A	1427.14	645.34	18.10	0.00	3.57	0.68	0.41	28.35	2123.60
M5A	367.10	380.95	36.54	2.87	0.80	0.64	0.09	23.31	812.31
M6A	802.12	363.34	17.44	3.42	1.76	0.65	0.29	28.01	1217.04
M7A	1226.72	178.44	16.55	69.04	0.91	0.18	0.32	15.54	1507.71

Based on the qPCR results, there were three samples, M1A, M2A, and M7A, where the female was the major contributor to the SF. In samples M1A and M2A, the ratio of female to male is approximately: 54% female to 46% male and 56% female to 44% male, respectively. For these two samples, it is possible that the peak height ratios

of the alleles will be very balanced making the mixture interpretation more difficult. Sample M7A has approximately four times more female DNA than male DNA in the SF. In samples M3A and M4A, there was impressively no female carryover in the SF, which would indicate that a single source male profile would be produced for this fraction. For samples M5A and M6A the clear major male contributor with a female contributor is observed using both qPCR and DNA profile data.

In terms of male DNA recovery in the SF, each sample had more than 80% of male DNA recovered in the SF. Thus, the procedure has excellent and reproducible recovery of sperm DNA in the SF. According to the qPCR results, four out of the seven mock sexual assault samples separated following the SpermX™ protocol produced distinguishable or close to single source male profiles and three had male to female ratios close to 50:50.

Once again, the EF2 and EW fractions were quantified to determine how much, if any, loss of DNA there was in these two fractions. Since the total female DNA in the EF was so high, loss of this in the EF2 and EW would not be significant. However, it should still be noted that a large mass of female DNA was in the EF2, indicating that this step is necessary to remove additional female DNA prior to sperm cell lysis. The total loss of female DNA in the EF2 and EW was an average of 35% and the loss of male DNA in these two fractions was calculated to be about 4% of the total amount of male DNA. The 4% loss reaffirms the good recovery rate of male DNA in the SF using the SpermX™ protocol.

3.2.4 Profiles of Mock Sexual Assault Evidence Donor Swabs from BU

The profiles for the EF1 for all donors confirmed the expected results of the qPCR, seven single source female profiles were able to be generated using the EF1, all of which had well balanced peak heights.

For samples M1A, M2A, M7A, two-person mixture profiles were observed in the SF fraction as expected. Interestingly, the two samples where qPCR data indicated there was no female carryover in the SF (M3A, M4A) also produced mixture profiles with a minor female contributor. That is, there were three or four allele calls at some loci not attributed to stutter, indicating more than one contributor (Figure 5). In both cases, when comparing the female reference to the SF profile along with examining peak height ratios, one can still determine the major and minor contributors, with the female being the latter. Samples M5A and M6A produced two person mixtures as expected. Both profiles made for a more difficult mixture interpretation, even with a clear female reference sample and with the male DNA being in much more abundance in the SF. This shows the importance of trying to limit the amount of female carryover in the SF as much as possible. The "blue" panel from each SF profile from the BU samples is shown in Figure 6. The "blue" panel from the SF profiles of the InnoGenomics© samples are shown in Figure 7 for comparison.

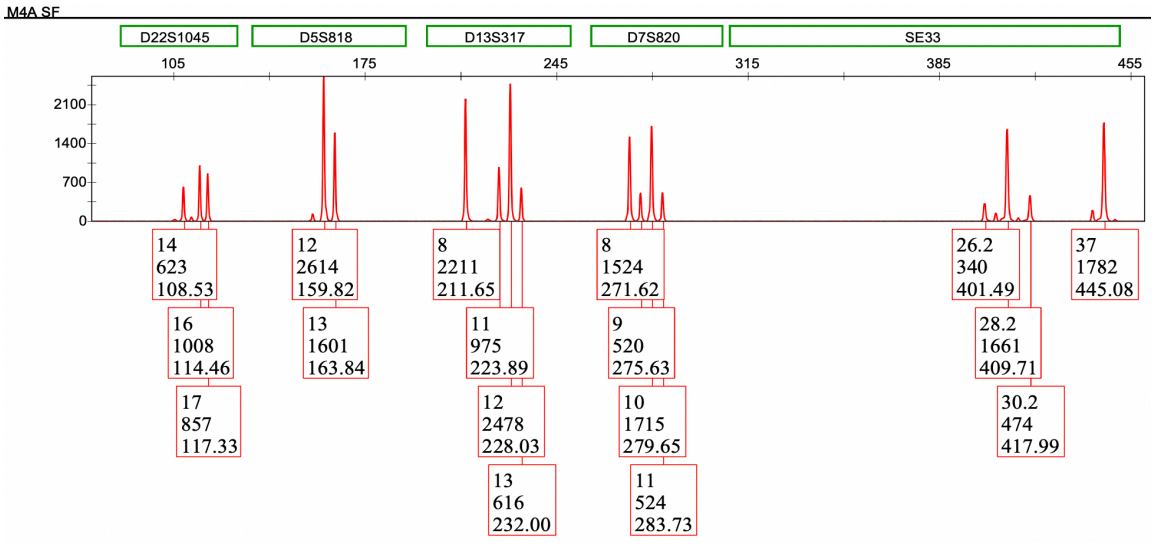

Figure 5: Mixture Profile from SpermX™ Sample that had Zero Female Carryover from qPCR Results: An example in the red panel of an SF profile at the SE33 locus showing allele calls not attributed to stutter in a sample that according to qPCR data only had one contributor**.**

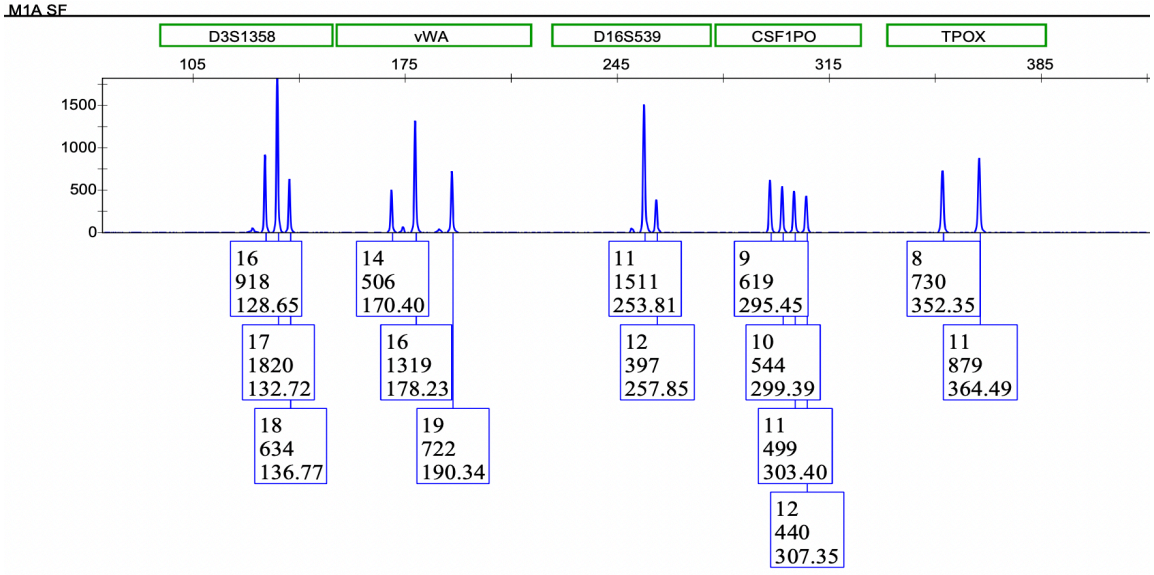

Figure 6: SF Profiles from Blue Panel from Each BU Donor Swab from SpermX™ Method.

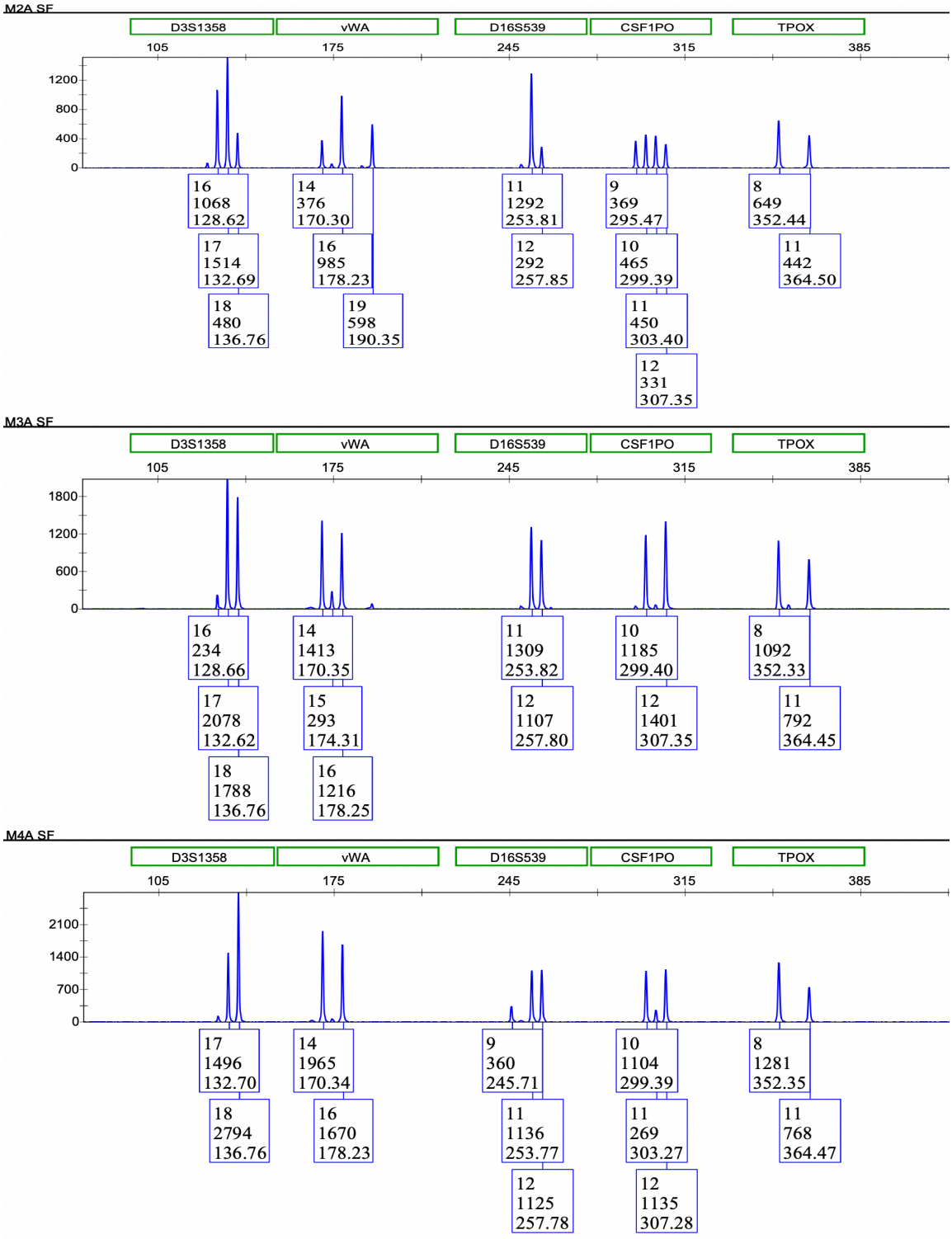

Figure 6: SF Profiles from Blue Panel from Each BU Donor Swab from SpermX™ Method.

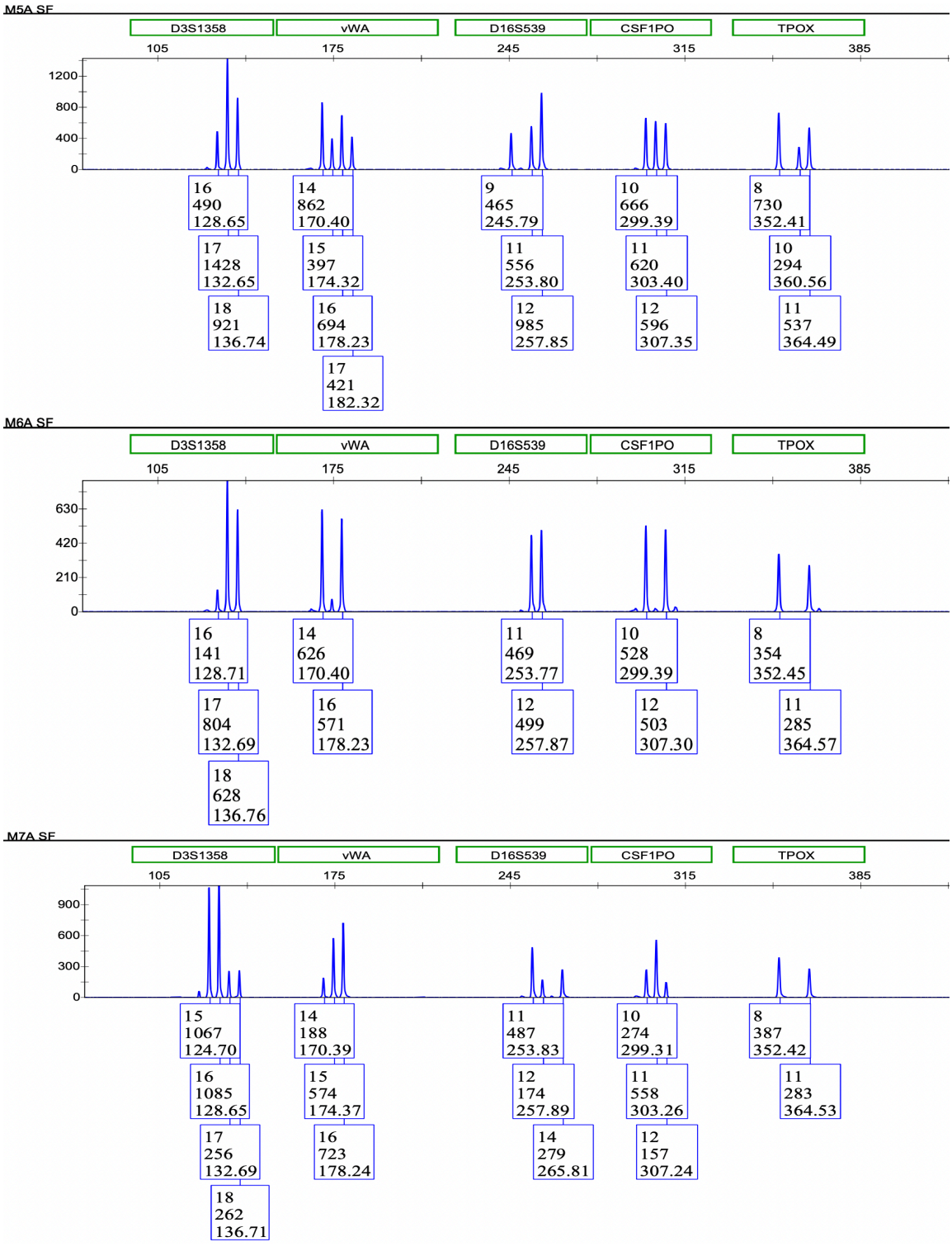

Figure 6: SF Profiles from Blue Panel from Each BU Donor Swab from SpermX™ Method.

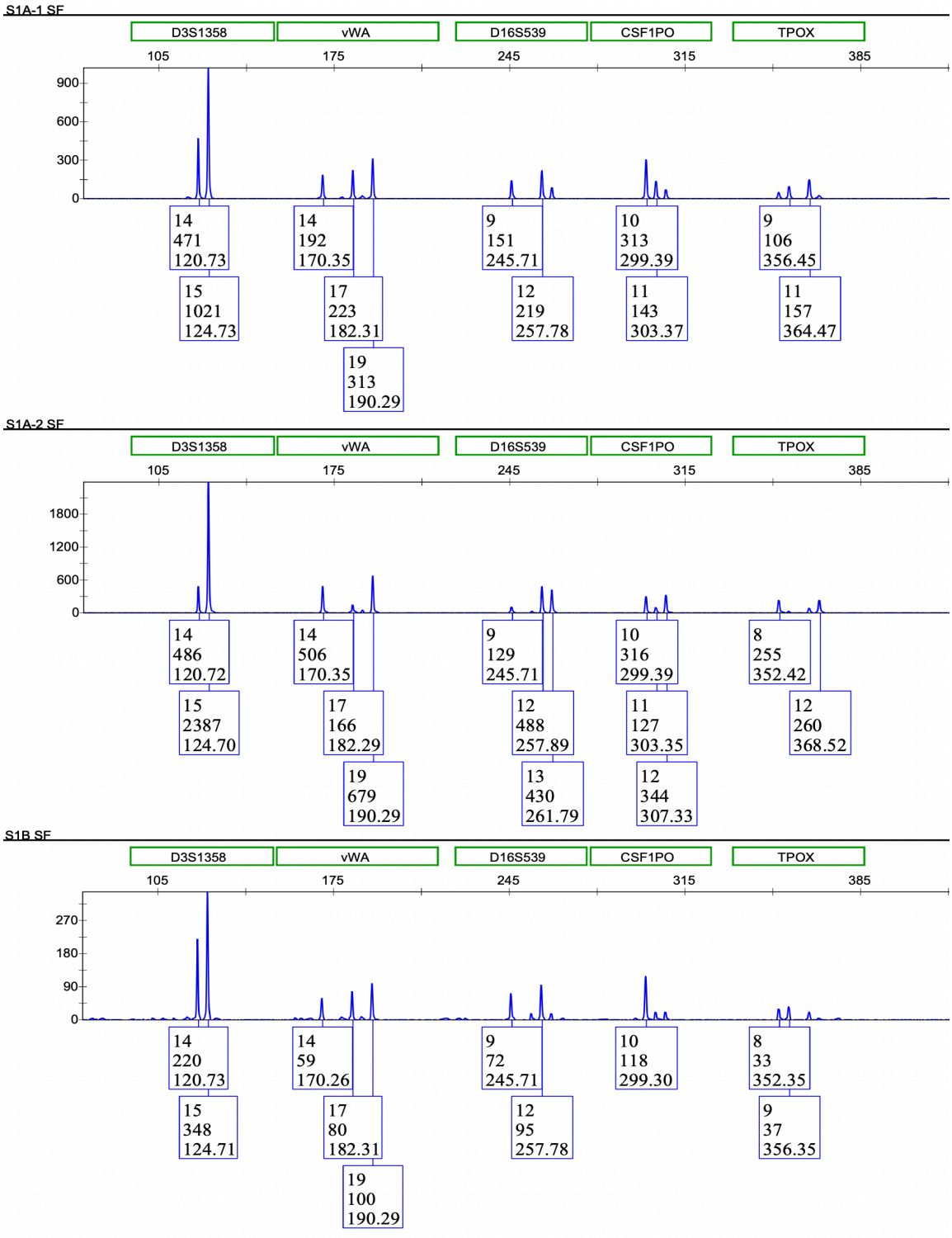

Figure 7: SF Profiles from Blue Panel from Each InnoGenomics© Swab from SpermX™ Method.

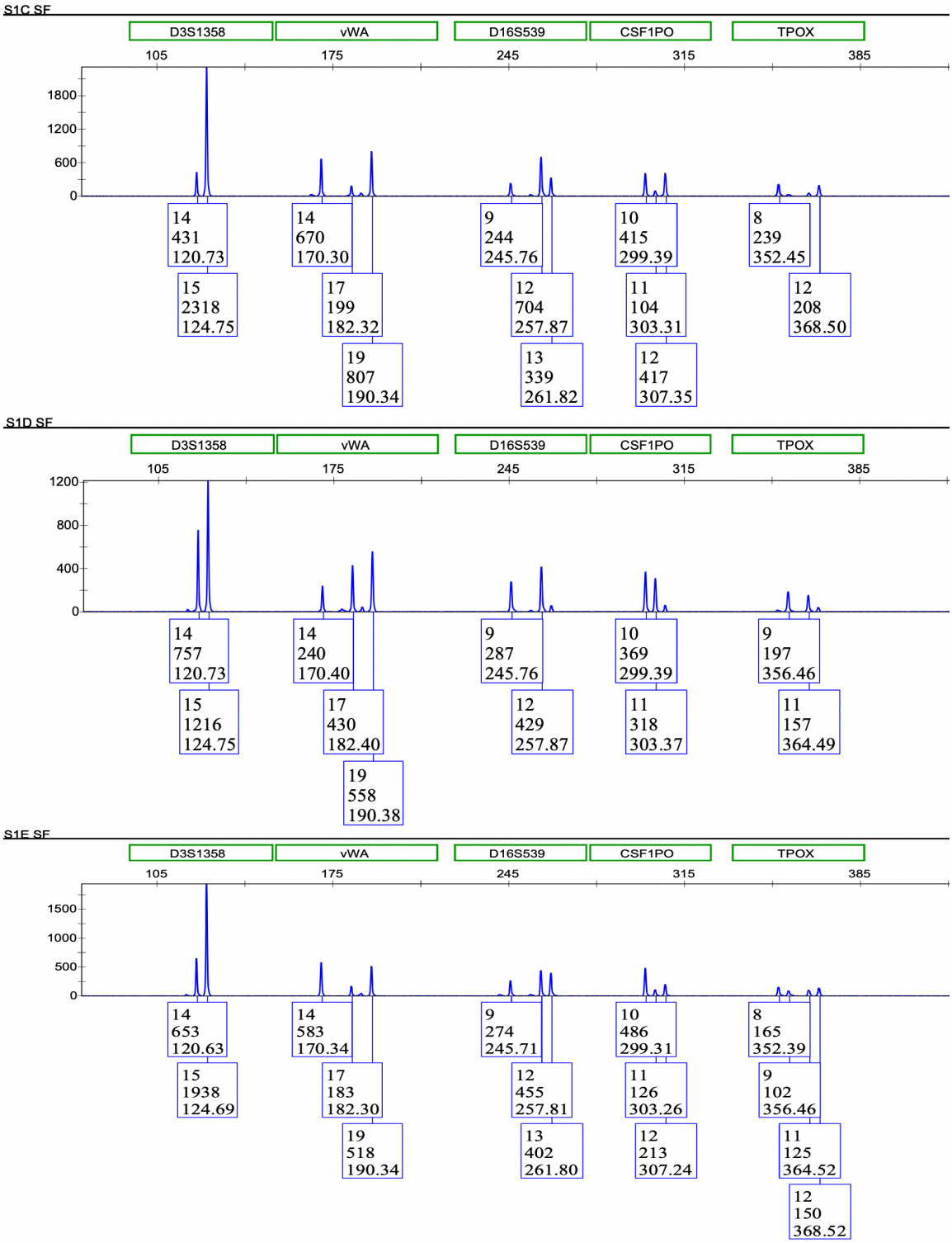

Figure 7: SF Profiles of Blue Panel from Each InnoGenomics© Swab from SpermX™ Method.

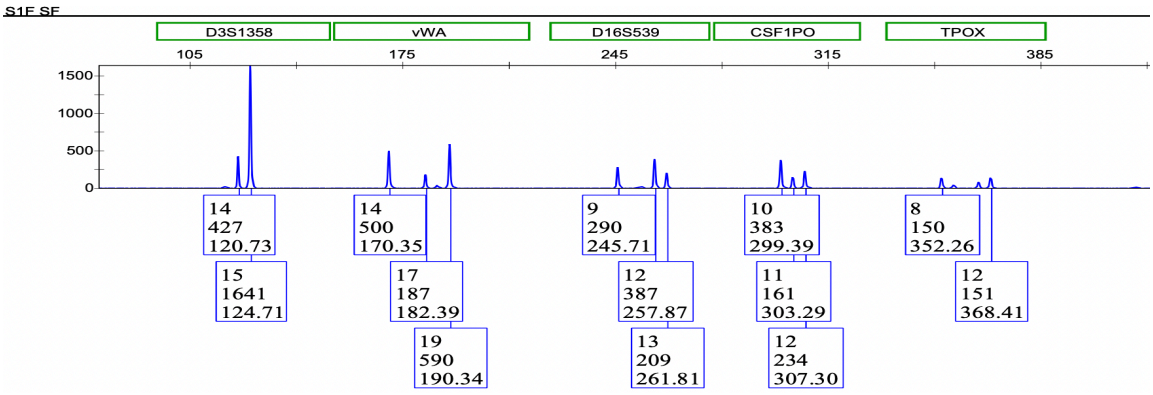

Figure 7: SF Profiles from Blue Panel from Each InnoGenomics© Swab from SpermX™ Method.

3.3 TCDE with Mock Sexual Assault Evidence Swabs from BU

Mock sexual assault case swabs B, C, and D from the seven donors were analyzed using the TCDE method. All swabs had approximately 50 ng of male DNA from a 1:10 semen dilution deposited on the swab, with an undetermined amount of female DNA from the seven female donors. All swabs were cut in half and tested as half swabs.

3.3.1 Quantitation of TCDE Swabs

The unmodified version of the TCDE protocol was used for swabs B and C from each of the seven donors of the BU mock sexual assault samples. The data from the qPCR analysis showed an increased amount of female and male DNA in the MF and more surprisingly very low values of female DNA in the EF fractions (Figure 8). For swab B, there were five different sample swabs that had more female DNA in the MF than in the EF. For swab C, all swabs except those from donor five had this same pattern. These results suggest there was little to no lysis of epithelial cells after the initial addition

of *forensic*GEM™, therefore limited female DNA was released in the EF solution. The low amount of DNA in the EF would explain the unusually high amount of female DNA in the MF. If the *forensic*GEM™ failed to lyse the epithelial cells, then the intact cells would be left adhering to the swab. The addition of the AcroSolv and *forensic*GEM™ Sperm in the steps to produce the MF would then lyse any intact cells, leading to an increased amount of female DNA observed in the MF. As a result of these observations, subsequent experiments were conducted using an E-cell prep on isofabrics and the same swab type used in the donation kits to assist with understanding the result. Modifications were subsequently made to the TCDE protocol to improve the amount of female recovery in the EF and decrease the amount of female DNA observed in the MF. These modifications were implemented using half of each swab D from each donor.

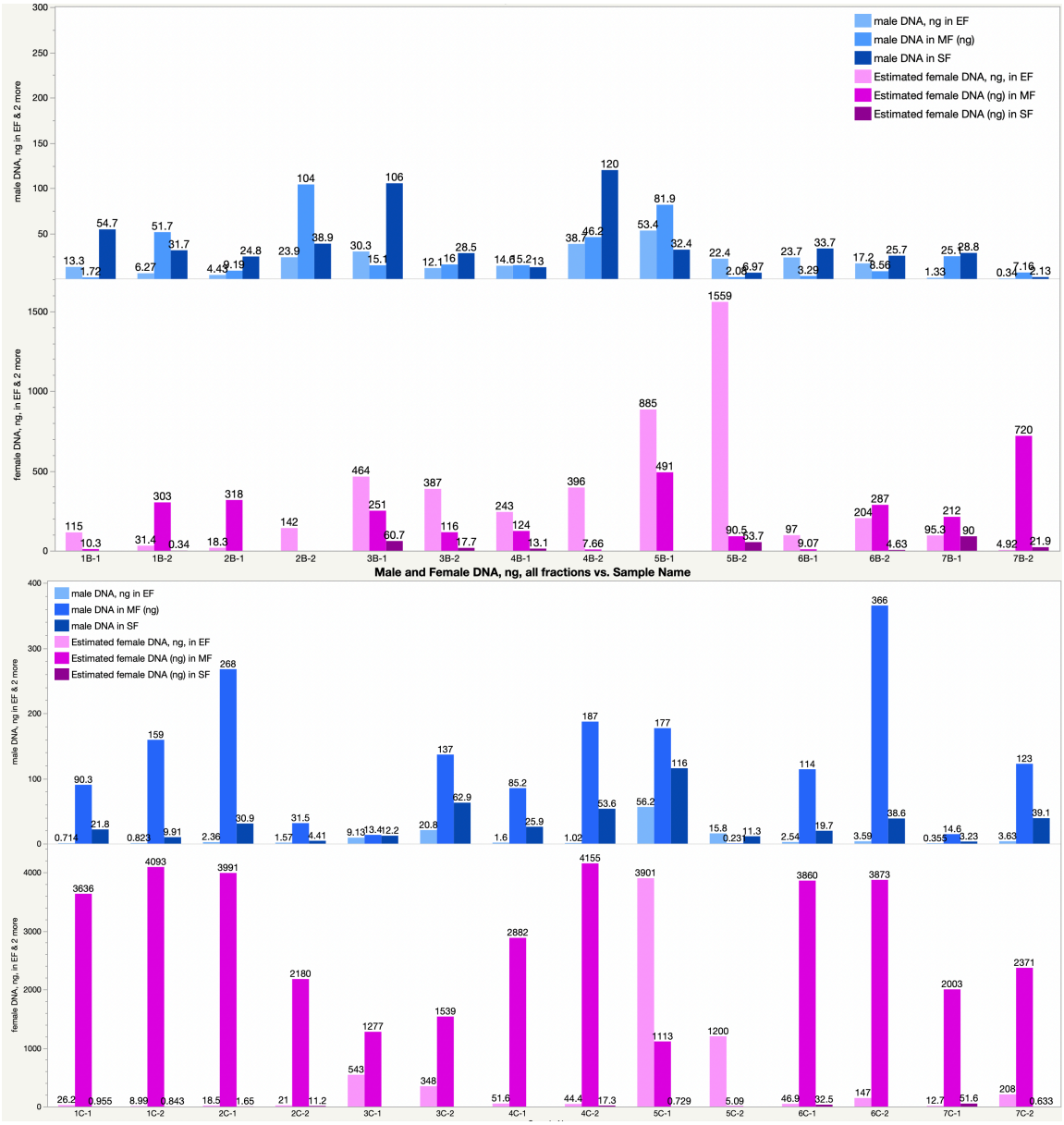

Figure 8: Male and Female DNA Recovered from Swab B and C from TCDE Samples: The amount of female and male DNA, ng, recovered in each fraction from swabs B (above) and C (below)

The qPCR data for the one half of the D swabs revealed that the EF for samples M1D-1 – M6D-1 had a substantial amount of female DNA; sample M7D-1 was an

outlier, with only about 12 ng of DNA appearing in the EF (Table 12). All samples from the EF would presumably result in a distinguishable female profile. There was some quantifiable male DNA in the EF, as shown in Table 12 but the percent of the total DNA in the EF ranged from 0.3% to 37.4%. Even the sample with the lowest amount of DNA in the EF, sample M7D-1, had no male carryover in the SF, indicating it should produce a single sourced female profile.

Table 12: DNA recovered in all fractions from half of swab D using final TCDE method.

Swab	Female DNA, ng, per fraction			Male DNA, ng, per fraction			Total, ng, All Fractions
	EF	MF	SF	EF	MF	SF	
M1D-1	528.37	2568.95	0.00	6.76	142.27	11.77	3258.11
M2D-1	259.33	1590.76	2.45	3.31	89.67	19.06	1964.58
M3D-1	1152.22	8318.11	5.10	8.91	152.32	26.60	9663.26
M4D-1	807.79	1252.71	0.63	4.46	98.00	9.87	2173.45
M5D-1	2739.09	5115.43	0.00	16.23	84.71	7.06	7962.51
M6D-1	664.18	2371.53	0.00	6.48	116.51	22.47	3181.16
M7D-1	12.02	3544.64	44.58	0.00	321.53	15.21	3937.98

Even though there was a large quantity of female DNA in the EF, there was still an even larger amount of DNA in the MF (Figure 9). For each half swab the amount of female and male DNA in the MF was greater than the female and male DNA in the EF and the SF respectively. This means that most of the DNA remained on the swab after initial digestion and most of the sperm cells were not released from the cotton substrate.

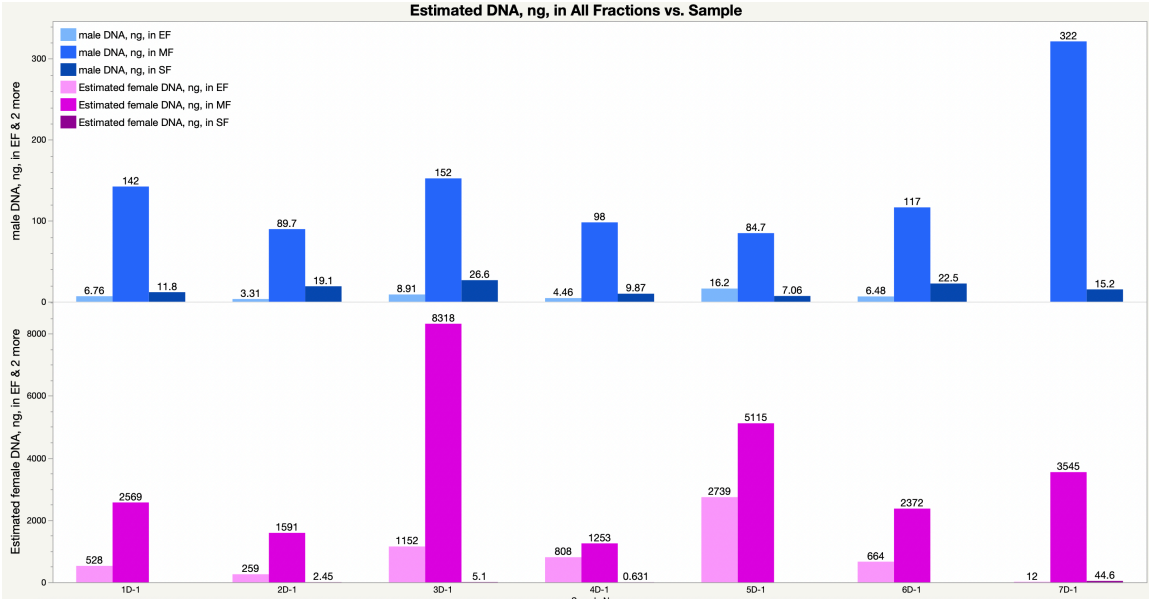

Figure 9: High Quantity of DNA found in MF for Swab D-1 from TCDE Samples: For every sample, more female DNA was found in MF than EF and more male DNA was in MF than SF.

For the SF, the qPCR results showed that samples M1D-1, M5D-1, and M6D-1 had no female carryover and would theoretically produce clean, single source male profiles. The other four samples have some female DNA carryover in the SF. Three of them have less than 20% of the total amount of DNA in the SF be female. The male in these three samples should be distinguished as a major contributor. Sample 7D-1 had a significant amount of female DNA in the SF accounting for about 74% of the total DNA in this fraction.

3.3.2 Comparison of Isofabrics and Cotton Swabs

Due to the high amount of DNA observed on the MF on the B and C cotton swabs and the TCDE procedure, the amount of epithelial DNA released from isofabrics and

cotton swabs was compared. There are documented instances where sperm cells have been known to stick onto the cotton fibers of the swab, making lysis of them more difficult. In this instance, the high amount of female and male DNA remaining on the swab was of concern. For the test swabs and cotton fabric, 10 µL of E-cell prep was deposited onto the substrates and allowed to dry. These samples were treated with *forensic*GEM™ according to the manufacturer's guidelines. A control of 10 µL of liquid E-cell prep was also digested with *forensic*GEM™ along with the prepared swabs and fabrics. This control value of ng of DNA was used as a baseline for comparison as the "expected" amount of DNA.

The qPCR results showed that more DNA was released in the EF than the MF from the isofabrics than from the swabs. The liquid E-cell prep digestion provided a baseline of 73 ng of DNA expected from 10 µL of the E-cell prep. Figure 10 shows the amounts of DNA, in ng, released from the isofabrics and swabs. The isofabrics released 45 ng on average in the EF whereas the swab released 27 ng on average in the EF. When looking at the respective total amount of DNA for each sample, the isofabrics released an average proportion of 76% of DNA in the EF compared to an average of 46% from the swabs. It was determined that the isofabrics were more efficient at releasing the DNA in the EF and that the swabs used in the donor kits could be a contributing factor to the high amount of DNA seen in the MF. This experiment was also testing the *forensic*GEM™ enzyme. The results showed that the enzyme was working properly, and the high amount of DNA in the MF was not due to low activity or inactivity of the *forensic*GEM™.

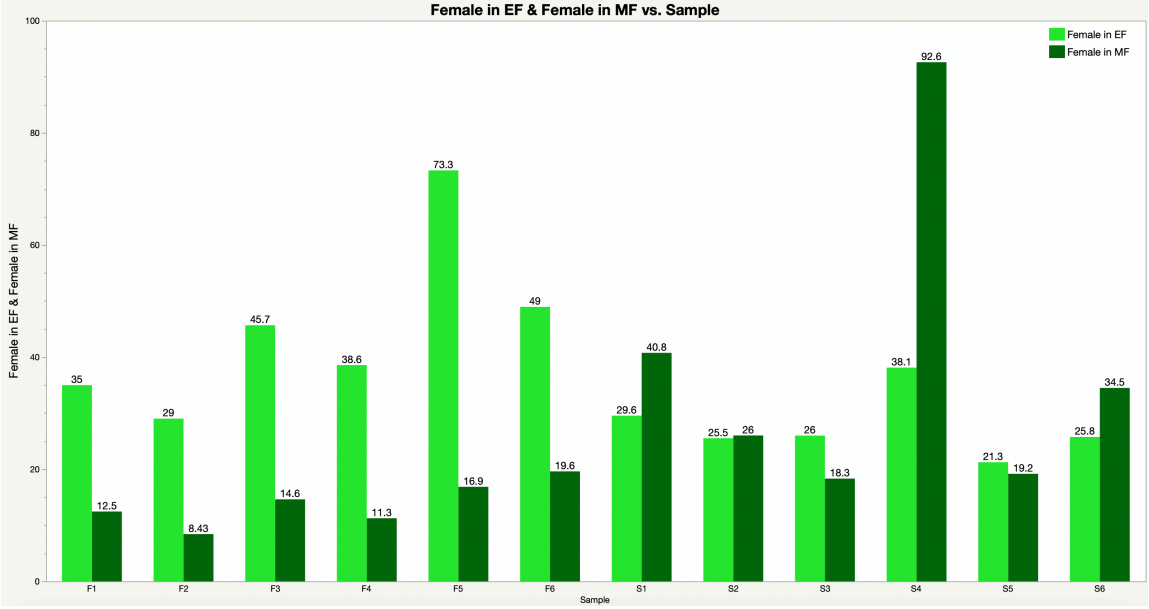

Figure 10: Mass of DNA Recovered from Isofabrics and Swab Substrates: Diagram depicting mass, ng, of DNA recovered in EF and MF from Isofabrics (F1-F6) and cotton swabs (S1-S6).

3.3.3 Profiles of Mock Sexual Assault Donor Swabs

The decision was made to only amplify and analyze the samples from swab D and not swabs B and C. This was due to the fact that the results from swab D were deemed to be more representative of the TCDE procedure with its new improvements. Therefore, swab D was used as the main source of interpretation of the TCDE method and was compared to swab A analyzed using the SpermX™ method. The profiles of the "blue" channel for every donor of swab D are shown in Figure 11.

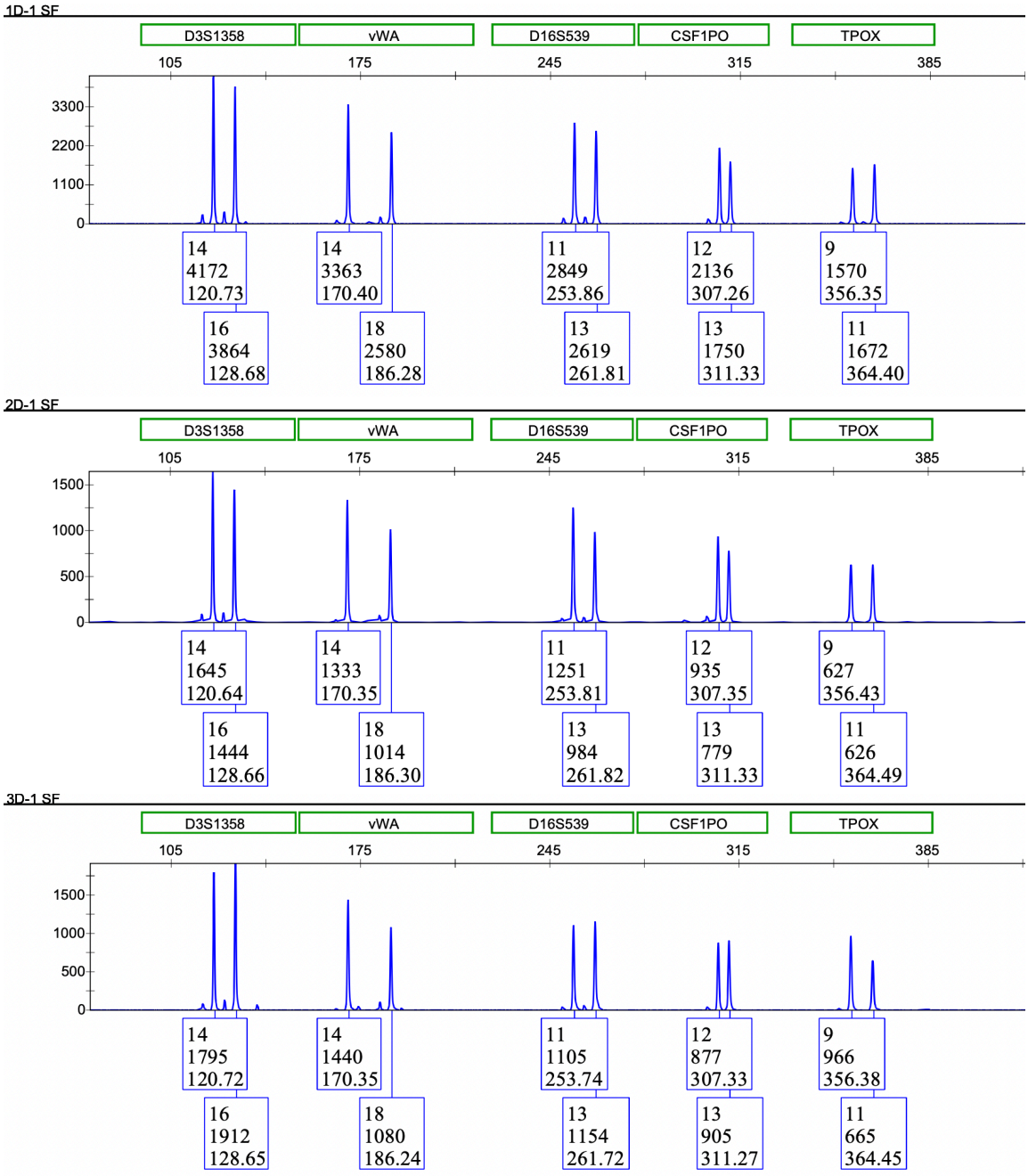

Figure 11: SF Profiles of Blue Panel for Each BU Donor from TCDE Method.

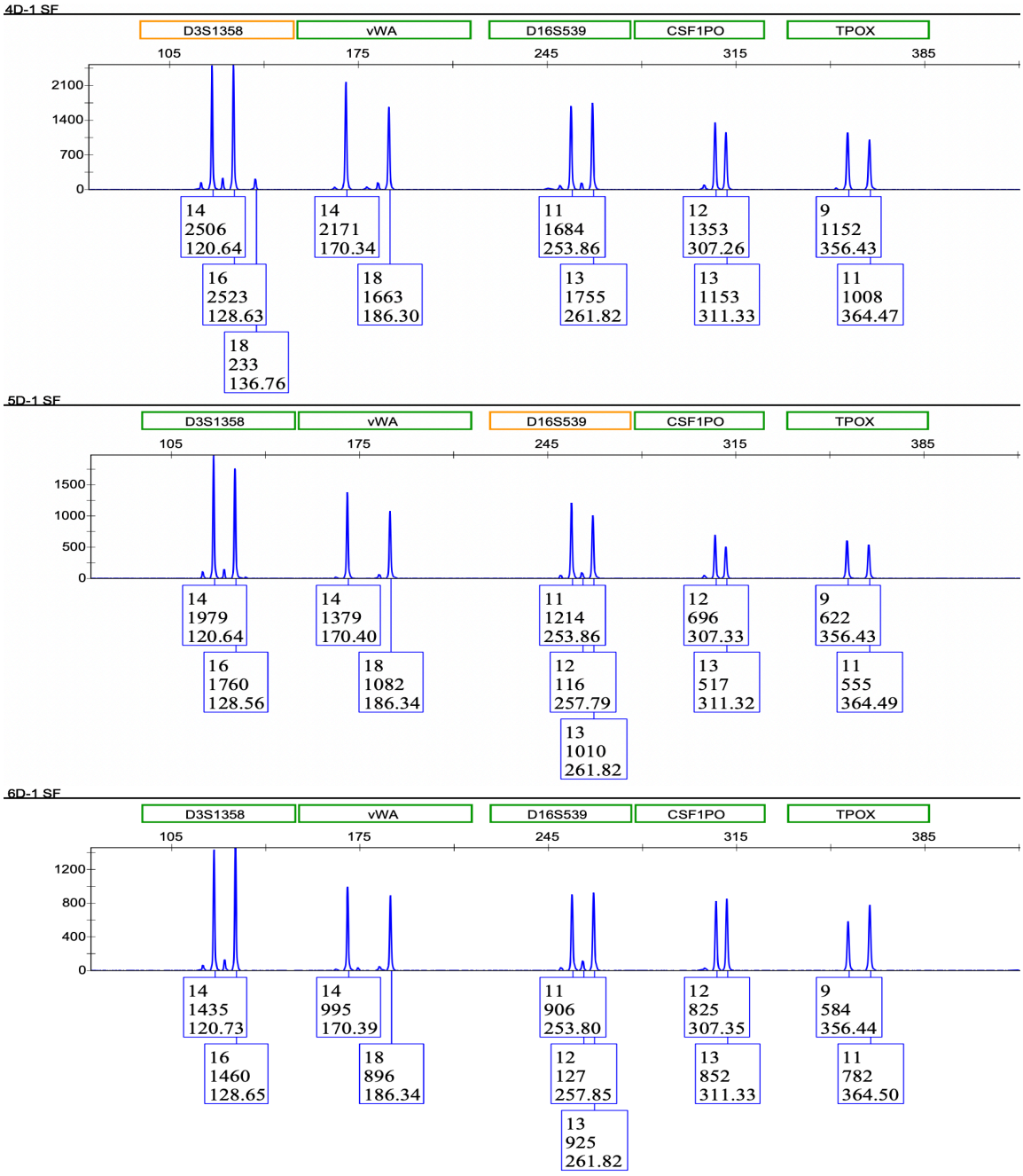

Figure 11: SF Profiles of Blue Panel for Each BU Donor from TCDE Method.

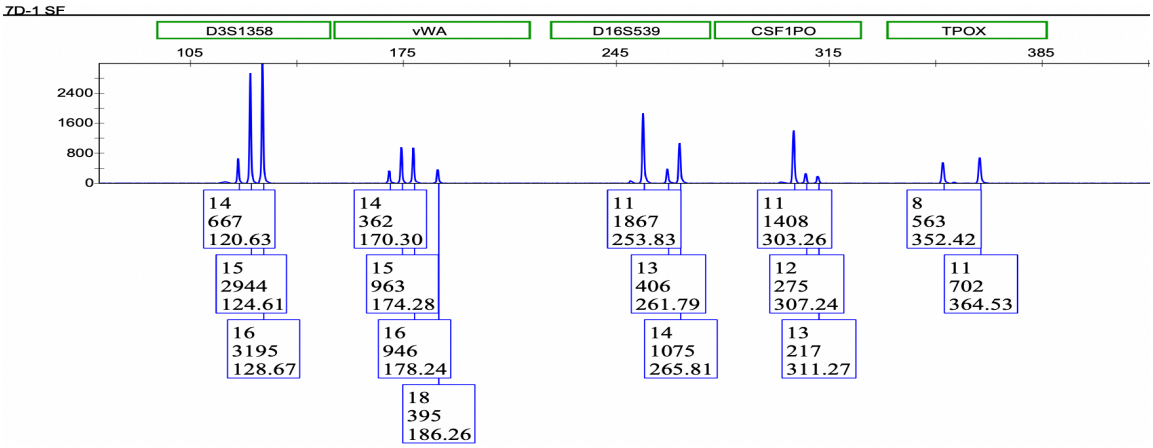

Figure 11: SF Profiles of Blue Panel from Each BU Donor from TCDE Method.

The EF fractions for all donor samples were not amplified since female reference profiles were already produced from the M1A-M7A samples. If any mixtures were seen in the SF e-grams from the TCDE swab D samples, those female profiles were used to distinguish any male alleles.

For the SF, apart from sample M7D-1, the profiles obtained were much easier to interpret. All samples were either single source or a clear distinguishable male profile was able to be determined from a mixture. For sample M1D-1, the qPCR results suggested that a single source profile would be produced, however, there was some loci that had more than two allele calls that did not stem from stutter (Figure 12). These peak heights were very low and could be designated as originating from female donor 1. The other two samples that were also believed to be single sourced according to the qPCR data were confirmed as so in the e-grams.

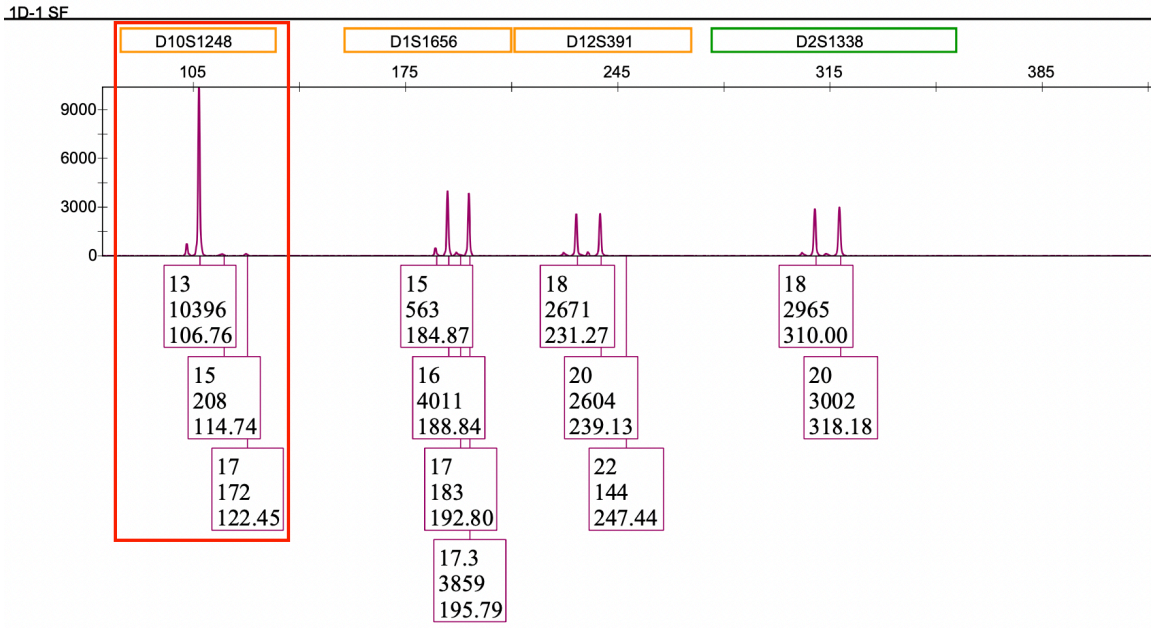

Figure 12: Example of Mixture Profile in Sample Expected to be Single Sourced: At the D10S1248 locus, there are two alleles from the female donor even though the qPCR results indicated the sample would be single sourced.

As stated previously, there were four samples with some quantifiable female DNA in the SF. Sample M2D-1 only had one locus where the female contributor was observed and could be distinguished from the male. Analysis of sample M3D-1 was able to generate a single source male profile despite it having female carryover in the SF. Sample M4D-1 exhibited three loci where alleles from the female donor were apparent. Once again, the peak heights for these alleles were very low and the male could be distinguished as the major contributor. Sample M7D-1 proved to be the most difficult to interpret, as expected from the very high quantity of female DNA present in the SF. Since the female was the major contributor according to the qPCR results, the alleles with the lower peak heights were determined to be from the male source. This was

accomplished in conjunction with using the reference profile for female donor 7. It is important to mention however that alleles with low peak heights can sometimes be interpreted as stutter from the larger peaks. One must be very careful of this and be aware of this possibility when interpreting the e-grams and determining the genotype of the male.

4. DISCUSSION

4.1 Female DNA in Epithelial vs. Material Fractions in TCDE Method

As previously mentioned, in most samples analyzed in the TCDE method, there was often more DNA, either male and female, or both observed in the MF than in the EF or SF. User error was eliminated as a possibility due to these results occurring consistently in samples from swabs B, C, and D; all of which were analyzed on different days. Problems with instruments were also eliminated as a possibility when quality control checks were performed on all thermocyclers and on the 7500 qPCR instrument. Previous research has mentioned this pattern, but it was concluded that the results were likely donor dependent. Based on the results presented here, there is no indication of the abundance of DNA in the MF being donor dependent and other explanations need to be explored.

Low enzyme activity is also unlikely to be a factor for this result since the experiments on the isofabrics and swabs showed that the *forensic*GEM™ was digesting the epithelial cells and releasing DNA into the solution. However, it was shown that the swabs were not as efficient in releasing the DNA compared to the cotton fabric. The type of swab used could be a factor in why so much E-cell DNA remained in the MF versus the EF, but based on the substrate comparison, the magnitude of DNA left on the swab seems to be impacted by more than just the swab itself. Other factors that could possibly affect the digestion of the epithelial cells could all stem from vaginal chemistry. Many variables such as vaginal pH, hygiene products, and stage of the menstrual cycle, that were not controlled could be causes for the inhibition of the *forensic*GEM™. It is clear

that whatever was inhibiting the *forensic*GEM™ did not inhibit the AcroSolv and *forensic*GEM™ Sperm enzymatic activity, resulting in more DNA displayed in the MF than the EF.

4.2 Sperm Recovery

High sperm recovery, especially in the SF, is an essential component of a differential extraction. For all samples processed through the SpermX™ protocol, eight out of fourteen samples were able to produce SFs with majority of the DNA being male. Of those samples, six were able to produce full single sourced or distinguishable male profiles. In the TCDE method this was true for six of the seven samples, with those six samples showing single source or distinguishable male profiles.

For the SpermX™ protocol, the average recovery of male DNA in the SF was 65.2% for all fourteen samples analyzed. In the TCDE method, the average sperm recovery rate in the SF was 10.4% for the seven samples; with the remainder of the male DNA in the MF.

4.3 SpermX™ vs TCDE

Both methods did not drastically succeed in shortening the length of time needed to complete a differential extraction. However, the use of robotics with the SpermX™ method lessens the possibility of human error. In addition, the SpermX™ method also

limited the possibility of human error with its design of inner and outer tube as the evidence swab was never removed after being placed inside the inner tube.
The time needed to complete each method was comparable, with both procedures taking about 6-7 hours to completely process seven samples at once by hand.

The SpermX™ method had excellent sperm recovery, but the female carryover in the SF profile made interpretation more difficult. The use of proprietary materials makes it difficult to adjust the protocol for any additional improvement.

Based on the set of experiments completed, the TCDE method was able to produce more profiles from the male contributor free of female DNA carryover. Less mixture interpretation was necessary for the SF profiles from the TCDE method. However, the loss of DNA to the MF could mean that this method is not suitable for low quantity DNA samples. The TCDE has the added advantage of not requiring a DNA a purification step, decreasing the time needed for analysis.

5. CONCLUSIONS

5.1 Conclusions from Data

The SpermX™ protocol should be followed using the indicated solutions outlined in the procedure as other sperm lysis reagents tested to be unsuccessful when used with the SpermX™ device. The method was able to successfully produce profiles with six of the fourteen samples analyzed using the SpermX™ method resulting in distinguishable male profiles.

Typically, the MF is not analyzed during casework, but the results in these TCDE experiments show that the DNA remaining on the substrate can be used to explain otherwise confusing low recovery in the EF and SF. The data shows that low amounts of DNA in the EF and SF does not necessarily mean that the sample itself is of low DNA quality or quantity, but that possible lysis failure could be the cause. This is essential information a DNA analyst must consider when trying to generate distinguishable DNA profiles. Single source or distinguishable male profiles were achieved in six out of seven representative TCDE swabs. However, the TCDE method has a high loss of DNA in the MF.

5.2 Considerations for Future Research

Further considerations for this research could be applied concerning the high amount of DNA found in the MF of samples that underwent the TCDE protocol. Modifications to the protocol including changes in incubation times or changes in concentration of enzymes could be investigated further to strive for a decrease of DNA

lost in the MF. Using a different brand of cotton swabs or different substrates altogether could also be researched further to determine if the protocol is better suited for other substrate types. One must consider the implications of the loss of DNA, if this problem cannot be solved, when dealing with low amounts of male DNA; the recovery from this MF could be extremely important in these cases. Using a larger sample size of donors could also shed light on whether the high presence of DNA in the MF is donor dependent. The results could be donor dependent and could have simply not been an explanation in this small sample size with these specific donors. Controlling the variables surrounding donor collection could also help narrow down an explanation for the high amount of DNA in the MF.

For the SpermX™ method, the original protocol asks to leave the wooden stick attached to the swab during the procedure. One can compare the DNA recovery from samples with the wooden stick attached versus removed. In addition, other ways to determine the amount of DNA left on the swab processed through the SpermX™ protocol can be investigated to produce a true MF. This additional fraction could give a more accurate insight on DNA recovery using this method. Additional investigation on how to minimize female DNA carryover in the SF could make the high recovery rate of male DNA in the SF more valuable and eliminate the need for mixture interpretation. Also,

using lower level DNA samples and calculating their sperm recovery could further establish if this method is suitable for samples containing low quantities of DNA.

Finally, as we know, it is possible sexual assaults could involve more than two people. Conducting research to determine if the two methods are still reliable when the number of contributors increases could be very important.

LIST OF JOURNAL ABBREVIATIONS

Forensic Sci Int Genet	Forensic Science International: Genetics
Gene Expr	Gene Expression
Glob Med Genet	Global Medical Genetics
Int J Legal Med	International Journal of Legal Medicine

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