

2021

Assessment of DNA separation and recovery using DNA profiles from a temperature controlled differential extraction

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Thesis

**ASSESSMENT OF DNA SEPARATION AND RECOVERY USING DNA
PROFILES FROM A TEMPERATURE CONTROLLED DIFFERENTIAL
EXTRACTION**

by

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B.S., Rochester Institute of Technology, 2019

Submitted in partial fulfillment of the
requirements for the degree of
Master of Science

2021

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ACKNOWLEDGMENTS

Two and a half years ago I came from Illinois to be a part of this program almost immediately after completing my undergraduate degree. I had never been to Boston before, so I took the risk of starting something new in a place that seemed almost foreign to me at the time. I could not be more thankful that I did. I learned more than I ever thought I would, and I must begin by thanking all the faculty in the biomedical forensic science program for challenging me in ways I never imagined and for persevering through the pandemic to continue to teach us.

I also want to also thank my family, my friends, and my roommates for supporting me through graduate school; I would not be here without them today and I am forever grateful for the love they show me. I thank and congratulate my fellow peers for persevering through the pandemic and continuing to support each other regardless of the circumstances, for no one knew we would all be experiencing an extremely rare occurrence.

Finally, I would like to thank Dr. Cotton for mentoring me through the entire process of this thesis, the process of DNA analysis in general, and believing in my ability to comprehend it and extrapolate on it.

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ABSTRACT

In 2010, Bright et al. created two person mixtures to determine how effective traditional differential extraction was in determining mixtures by examining mixture proportion variation by using the peak heights from each sample. This project aims to follow that method, however, in this case using a Temperature Controlled Differential Extraction (TCDE) to analyze post coital swabs in place of a traditional differential extraction. The project also aims to determine how efficient the separation of sperm cells from epithelial cells was by comparing the mixture proportion mean of male deoxyribonucleic acid (DNA) from an Acrosolv digest that did not undergo the TCDE to the proportion of male DNA from the TCDE. The amount of DNA remaining on a swab after undergoing the TCDE was also assessed as a material fraction. Many of the material fractions generated a mixture in their profiles and thus enough DNA to generate a male profile was remaining on the swab after the TCDE in almost all cases. The sperm fractions were mostly single source male profiles or profiles with the male DNA as a major contributor and the female DNA as a minor contributor.

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LIST OF ABBREVIATIONS

CE	Capillary Electrophoresis
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EA1	Erebus Antarctica 1
EF	Epithelial Fraction
LCM	Laser Capture Microdissection
MF	Material Fraction
PC	Post-coital
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RFLP	Restriction fragment length polymorphism
RFU	Relative fluorescent unit
SDS	Sodium dodecal sulfate
SF	Sperm Fraction
SLP	Single Locus Probe
SPRED	Separation Potential Ratio of the Extraction Differential
STR	Short Tandem Repeat
TCDE	Temperature Controlled Differential Extraction

1. INTRODUCTION

1.1 Serological Procedures Prior to DNA Extraction

Prior to DNA analysis, a forensic laboratory will perform biological fluid screening on substrates that are suspected to have blood, semen, or saliva on them to determine whether the substrates do in fact contain these fluids (1). Suspected fluids may not always be visible in which case an alternative light source (ALS) or chemical mapping may be used to find the location of these stains (1).

For semen, this is commonly done by using a colorimetric test on the suspected stain that yields a color change for a positive result (1). If the result is positive i.e. yielding the appropriate color change such as purple for AP spot, a small portion of the substrate that the semen is on will be cut, spun down, stained onto a slide using either hematoxylin-eosin or nuclear fast red and picroindigocarmine (2) and analyzed under a microscope for positive identification of sperm (1) in laboratories that do microscopic confirmation of sperm. In some cases, lateral flow immunochromatographic strip tests such as RSID™-Semen may be used in place of microscopic confirmation (3). This is relevant due to the necessary presence of sperm prior to performing a differential extraction on the same substrate. Otherwise, DNA testing may not yield the expected results and resources would be wasted.

1.2 Sperm Structure

Sperm has a specific structure that protects the DNA in its nucleus from being degraded easily as compared to other general sources of DNA such as saliva, skin cells,

and white blood cells. The entire differential extraction process was born because of this specific structure. The structure of the sperm membrane also is a convenient instance of biology that allows forensic DNA analysts to separate male DNA from the DNA of other types of cells (4).

Sperm development occurs in the testes where it undergoes spermiogenesis after meiosis II, and is morphed into its differentiated state with an acrosome, flagellum, and all associated proteins (4). These mature sperm, having undergone meiosis, are haploid, only containing one set of chromosomes required for fertilization (4). During spermiogenesis, sperm histones are replaced by transition proteins 1&2 which functions are unknown other than that they are required to form fertile sperm during spermiogenesis and chromatin remodeling (5). These transition proteins are then replaced by protamines (4,5).

Unlike somatic cells, sperm cells have a nucleus that contains DNA is complexed with protamines (4,5). These protamines may aid in the condensation of chromatin where the genetic material is stored within the sperm nucleus (4,5). The structure of the chromatin after development is toroid and is more compact than somatic cells to protect it from external elements which may degrade the genetic material (5). The protamines that exist and are used in chromatin remodeling contain two cysteines that are capable of forming disulfide bonds (4). It is not known exactly how the DNA-protamine complex structure is positioned just that the disulfide bonds exist within it (4). The disulfide bonds are the reason that a dithiothreitol (DTT) solution is required to lyse sperm by reducing the disulfide bonds and obtain its genetic material in forensic investigations regarding

sexual assault (4). The disulfide bonds that exist in sperm are not found in other cell nuclei which allows for the differential extraction procedure to effectively separate the DNA from sperm from other cells (4).

1.3 Relevance of Differential Extraction in Forensics

In 1985 and prior, it was not possible to distinguish deoxyribonucleic acid (DNA) profiles in a mixture from one another (6). Gill et al. reported that a differential extraction could be used to remedy the issue by separating sperm cells from epithelial cells, and this was an important advance as it allowed the new DNA testing methods to be applied in sexual assault cases to identify the individual DNA profiles (6).

1.3.1 The Differential Extraction Method

The method consists of lysing the epithelial cells in a sodium dodecyl sulfate (SDS) /proteinase K mixture then separating the un-lysed sperm cells by centrifugation, removing the supernatant and lysing the sperm in an SDS/ proteinase K/ DTT solution for recovery of male DNA (6). The supernatant contains epithelial cell DNA and is removed (6) the sperm cells are lysed and both fractions are analyzed to link the DNA to their respective contributors, in this case the sperm to a suspect and the epithelial cells to a victim (6).

1.3.2 Differences in DNA Analysis and Differential Extraction History

DNA testing began with the paper published by Jefferys et al. on the use of multi-locus probes for human identification (7). The method of analyzing DNA profiles in mixtures since Gill et al. has since changed from use of restriction fragment length

polymorphisms (RFLPs), variable number tandem repeats, and Southern blot tests to use of short tandem repeats (STR) and polymerase chain reaction (PCR) tests which are much more sensitive and used today (6,8–10). There was also a shift from gel electrophoresis to capillary electrophoresis which granted analysts a higher resolution tool to replace gel bands (11). While the methods used to analyze the products of extraction has experienced change, the overall differential extraction method has not.

1.3.2.1 Multi-locus Probes and Southern Blot

The original method for DNA analysis was reported by Jefferys et al. for identification in the same year as the creation of a differential extraction method (7,10). ³²p radioactively labeled DNA probes that contained ‘minisatellite’ sequences hybridized to digested DNA from a restriction digest separated by gel electrophoresis and immobilized on a membrane by Southern blotting (7,10,12). The probe would then hybridize to a set of minisatellite sequences in the genomic DNA restriction fragments at varying sizes at many loci due to polymorphisms in the number of repeated sequences (7,10,12). Only after washing away excess probe and exposing the membrane to X-ray film could the profiles be compared (7,10,12). This multi-locus probe method required a high molecular weight DNA sample and resulting DNA profiles were compared by band sizes which could be hampered by the quality of samples or electrophoresis conditions (12). These resulting profiles were difficult to calculate statistically due to unknown fragment association in individual DNA profiles meaning their loci may have been inherited together (12). The resulting DNA profiles could not be easily shared with other laboratories since they were on a membrane or gel making it difficult to reliably compare

genetic profiles in the scientific community (12). The development of single locus probes attempted to resolve some of these issues (12).

1.3.2.2 Single-locus Probes

Single locus probes (SLPs) resolved some of the issues with multi locus probes by targeting specific RFLP loci (12,13). This was done by digesting human DNA at specific points in the genome using a restriction digest and cloning the sequences into bacteriophages to find specific loci in DNA recombinants that could be used as probes to hybridize at specific loci (13). Testing with single locus probes required less DNA than with multi-locus probes and allowed scientists to read allelic expression with that showed a high degree of polymorphism (12,13). The benefit of the loci that were targeted by these probes was that they had high levels of heterozygosity meaning each locus would have two alleles per contributor in most cases (12,13). SLPs did not resolve the issue of being easily able to share profiles with other laboratories since the results were still read on a membrane or gel and compared by band sizes (12,13). Though SLPs required less DNA than multi-locus probes, this method was eventually replaced by the more sensitive PCR testing and STR typing (9,12,14).

1.3.2.3 PCR- STR Analysis

In 1991, PCR- STR analysis was first offered as a possibility to replace the RFLPs and southern blotting. Edwards et al. determined that microsatellites or STRs of non-coding genes were highly polymorphic and surges of alleles could be determined at the genetic base pair level (14). Instead of exposing a membrane to X-ray film, the STRs could be detected using a fluorescent taq on one end of the PCR copy of each allele when

analyzed on an acrylamide, DNA sequencing type gel (9,14). This led to the widespread creation of STR kits that included primer sets, fluorescent dyes, ladders, and internal standards (15–17). The original kits validated for forensic uses were COfiler™ and Profiler™ (16,18). Prior to those, a PowerPlex™ kit was also developed but only for eight loci or nine if including the amelogenin locus (17). This kit also used fluorescent dyes to label the amplified DNA (17). The original kits could only detect up to nine STR loci and presently STR amplification kits detect up to 24 STR loci (19). Another benefit was that if disparate amounts of two people in a mixture were detected, due to traditional differential extraction not fully separating the DNA, peak height ratios could help to distinguish the major and the minor contributor (9). Differences in peak height ratios can be used in any mixture, not exclusively mixtures from a traditional differential extraction (9). Capillary Electrophoresis (CE) replaced gels and was validated by the FBI along with the use of these STR kits, Profiler™, Profiler Plus™, COfiler™, and PowerPlex™ in 2001 (11,20).

Very recently next generation parallel sequencing methods have been studied as a potential alternative to capillary electrophoresis. This method allows STR alleles to be recognized by sequence variation as well as repeat number (21). This may aid in mixture interpretation, as well as the analysis, degraded samples, and trace mixtures (21).

1.3.2.4 The Differential Extraction Method Today

The efficiency of the traditional differential extraction method has been tested throughout its relatively recent history while the procedure itself remains relatively unchanged.

Wiegand et al. tested the traditional method against a one-step extraction of male DNA in a proteinase K, SDS, and DTT solution in 1992 (22). They did this by testing vaginal swabs with moderate amounts of sperm on them and vaginal swabs with very few sperm on them and then comparatively performing both extraction methods (22). The authors found that the differential extraction was more effective than the one-step extraction as less female bands were present in the swabs with moderate amounts of sperm (22). In the vaginal swabs with relatively low sperm, they were not able to produce male bands from the DNA amplified in the one step extraction but did so from the DNA amplified from the differential extraction (22). The other minor difference to the differential extraction method was the change in the amount of proteinase K added to the solution based on the amount of sperm present (22) otherwise the overall method remained relatively unchanged (22).

In 1995, a study attempted to modify the differential extraction method into two steps to preferentially separate the sperm cells from a substantial number of epithelial cells (23). The modification was to directly lyse the sperm and epithelial cells in proteinase K and at a temperature of 70°C instead of 37°C (23). This temperature modification, or increased stringency, allowed more epithelial cells to be lysed while keeping the sperm cells intact as the issue is generally is not the amount of sperm recovered but the amount of epithelial cells remaining after lysis (23).

In 2003, Garvin et al. introduced a filtration method that was intended to make the process of automating the differential extraction method easier by performing practically the same method except that the separation of sperm would be done through a vacuum

filter (24). Garvin et al. found the method to be only a little better than the traditional differential extraction method obtaining a 45-fold enrichment from the amplified sperm fraction DNA (24). However, this vacuum filter could not effectively separate the sperm from old or degraded samples as these would clog or adhere to the filter membrane (21,24).

Norris et al. investigated the effects of elution detergents for recovering the most sperm cells from samples with low amounts of sperm and determined that SDS, an anionic surfactant, was the most effective in doing so (25) adding another minor change to the overall method. The study also determined that increasing the incubation temperature above 42°C lead to decreased sperm cell recovery and suggested the incubation temperature be below 56°C for sperm recovery (25). SDS was first mentioned in the original Gill et al. paper describing differential extraction (6) so this study on elution detergents confirmed that SDS is the most effective in samples with low amounts of sperm (25).

Another attempt at a one- step extraction method was published in 2009 using a nuclease protocol, enzyme solutions that degrade epithelial cell DNA but not sperm cell DNA, was successful in separating the male DNA from the female DNA (26). Though the protocol was not more efficient than the traditional differential extraction method, it did make the method more amenable to automation since it removed the necessity of multiple centrifugation steps (26).

Essentially no major changes have been made to the overall differential extraction method for practical use on sexual assault evidence samples. Even as recently as 2019, the original differential extraction method is considered the “gold standard” (27).

1.3.2.5 Automation of Differential Extraction

One approach to solve the extensive time and laborious process issues of traditional differential extraction is to automate the entire method (28). While not technically different from traditional differential extraction, it is a relatively new procedure. The robotic system developed by Timken et al. performed differential extraction in approximately 2.5 hours (28) and could more efficiently generate profiles as compared to the manual method (28). The only manual part of the automated extraction procedure was loading the tubes with the substrate and the instrument did the rest of the uncapping, pipetting, centrifuging, shaking, and aspirating on its own and stored each processed substrate in its original tube at the end (28). The authors found a difference in the yields of DNA recovered but surmised it was due to the differing amounts of DNA given to the automation system as compared to the amounts given to the analysts performing the traditional method (28). Other than the amount discrepancy, both methods yielded complete DNA profiles. The separation in samples with 0.01 microliters of semen added to them was more efficient in the manual method with this method yielding an average of 1.3ng in the manual method versus about 0.4ng in the automated method (28). The separation of male DNA was approximately equivalent in both methods with the samples with 0.02 microliters of semen added to them with about 0.33ng average male DNA recovered in the sperm fraction for the automated method and

0.34ng average male DNA recovered in the sperm fraction for the manual method (28). This study only used a small number of samples at the time of publication had not yet validated the method for practical use (28). The cost of this automation system was not discussed in the study (28). A study done by different authors utilizing an automated system with a DNase digest had similar results finding that the automated process had a 58% success rate as compared to the 52% rate for the conventional process (29). The authors explained the success rate was better for the automated method because the robot was able to process more samples in a shorter amount of time as compared to the traditional method (29). The fractions in this study also yielded minor differences between the traditional and automated methods (29). The major benefit of the automation was not necessarily better sperm recovery or cleaner fractions, but faster processing times (29).

1.4 Novel Differential Extraction Methods

There are several newer differential extraction methods invented to either reduce the amount of time it takes to undergo a full differential extraction procedure or recover more sperm cells from the substrate or both. These include laser capture microdissection, anti-sperm antibodies, alkaline digest, and acoustic trapping. There is also another novel

method that the Cotton lab at Boston University School of Medicine has developed to aid in processing times and downstream analysis.

1.4.1 Laser Capture Microdissection

The laser capture microdissection (LCM) method was devised for forensic purposes in 2003 and attempts to solve the issue of low amounts of sperm on microscope slides (30–32). The method requires the use of an infrared laser and tubes with specialized microtube lids (30–32). The underside of this lid contains a polymer transfer film which contains infrared absorbing dyes which reduces the thickness of the film when the infrared laser is focused on the film dries immediately after the laser is turned off due to the intrinsic thermodynamic properties of the transfer film (30). By placing the transfer film over the stained areas of a microscope slide and melting the transfer film in specific regions with the laser this traps the cells onto the transfer film and then can be placed directly into a tube for DNA extraction (30). A low count PCR method is two concurrent amplifications of a DNA extract and the consensus result only reported if both the runs report the same data (30). The low count PCR method was utilized in this study because the authors knew they would be using microscope slides with 300 sperm or less thus requiring them to use a method to account for the low amount of DNA recovered (30). LCM has been shown to be more effective than traditional differential extraction due to the efficiency in isolating sperm cells that cannot be achieved in the traditional method. Another study attempted to use LCM in conjunction with a manufactured buffer that would cut the extraction time from 4 hours to half an hour (32) but found it to be poor compared to the lab's in house one step extraction in separating the sperm (32).

LCM, while efficient in separating sperm, is time consuming and expensive to perform and is unlikely to replace the traditional differential extraction method.

1.4.2 Anti-sperm Antibody

This method circumvents traditional differential extraction by using anti-sperm antibodies bound to magnetic beads to capture sperm cells. A chemical species attached to the antibodies forms a covalent bond between the antibodies and the sperm after photo activation to produce a permanent adduct between the sperm and the antibody (33–35). The immunomagnetic capture of the beads with anti-sperm antibodies in a biotin-labeled avidin coupling the specific antibodies, differing in each referenced study, separates the sperm and its DNA from other potential sources of DNA since the antibodies in the beads are covalently linked to the sperm heads (33–35). Essentially, the anti-sperm antibody in the magnetic bead binds to the sperm cell antigen, usually located in the sperm head, to permanently separate the sperm cells from the epithelial cells (33–35). The issue with this method is that the anti-sperm antigen required for this method to work may not be found in degraded sperm cells which may be more common in sexual assault evidence (35). The main benefits of this method are that it is faster, more easily automated, and requires less time than traditional differential extraction (33–35). The method also may prevent unnecessary over digestion of sperm and epithelial cells from traditional lysis

(34). However, its practicality and efficacy are limited by its reliance upon specific sperm antigens that may or may not be degraded in forensic cases (33–35).

1.4.3 Alkaline Digest

First described in 1996, this procedure uses an alkaline solution to extract DNA from cells instead of using chelex, phenol-chloroform, or other traditional extraction methods (36). This method was also originally intended to extract DNA from bloodstains and other substrates, not as a replacement for differential extraction (36,37). Instead of lysing the epithelial cells and debris surrounding the sperm with SDS and proteinase K, an alkaline solution is used in this case (36–38). The separation of the sperm from the non-sperm is done by placing the substrate in a mild alkaline solution then through neutralization and enzymatic digestion, in this case with DNase, to remove epithelial cells and their DNA from the substrate (38). The sperm fraction is then generated by heating the substrate in a 1M NaOH alkaline solution and the DNA from the sperm is concentrated and purified using silica columns (38). The benefits of using this extraction method are that it is quick and cheap (36–38). In 2010, an alkaline digest study tested the efficiency of the digest on post coital swabs which are commonly tested in forensic sexual assault cases (38). The study found the 96 well alkaline digest to be sensitive and relatively quick as this method only takes an estimated four hours to complete (38). Another benefit of this method versus traditional differential extraction is the limited amount of sample handling which reduces the chances of contamination and demand on

laboratory staff (38). This study only tested samples that were frozen (38) so it is yet to be determined whether it is suitable for practical use in forensic cases.

1.4.4 Acoustic Trapping

Acoustic trapping physically separates sperm cells from other DNA by trapping the sperm cells in an acoustic wave based on the relative size of the sperm cells to free DNA and other cellular material (8,39,40). While the sperm cells are trapped, the free DNA and cellular debris are washed into another chamber thus physically separating them from possible contaminants (8,39,40). The most recent study did the whole extraction process in less than an hour (8). This method is reliant upon instruments that are not typically found in a forensic DNA lab to generate the acoustic wave (8,39,40). The instrument is a microfluidic device or a chip that also contains channels which allow washing of female DNA and excess debris to be done while the acoustic wave holds the sperm in place (8,39,40). The excess DNA is then pushed to an outlet by the wash (39) then the wave holding the sperm in place is turned off and the sperm is directed to a different outlet by inverting the flow rate from the side arm (39). This acoustic trapping method had good sperm separation from other types of cells including blood and yeast and had clean sperm fractions in each study even in samples with low amounts of sperm (8,39,40).

1.4.5 Temperature Controlled Differential Extraction

The Cotton lab at Boston University has developed its own method of differential extraction. This method is performed in a thermal cycler except for a centrifugation step. It is done by using three different enzymes at optimal temperatures instead of the

standard proteinase K/DTT solution described by Gill et al. Since the limiting factor is the temperature at which the enzymes digest, this is a Temperature Controlled Differential Extraction (TCDE).

1.4.5.1 Enzymatic Digestion

The enzymes used to digest the epithelial cells and eventually the sperm cells are thermally activated at different temperatures. These enzymes are Erebus Antarctica 1 (EA1), Benzonase[®], Nuclease, and AcroSolv (41).

EA1 is a proteolytic enzyme, and it is activated at a temperature of 75°C and deactivated at temperatures below 70°C and above 90°C which makes it ideal for releasing DNA with nucleases deactivating at higher temperatures (41). The nucleases need to be deactivated or digested at higher temperatures because they will interfere with the Taq polymerase required to replicate DNA strands if not digested or inactivated (41–43).

EA1 cannot lyse sperm and, in the protocol, used to lyse epithelial cells (41–43). Benzonase[®] is an endonuclease, an enzyme that digests both double and single stranded nucleic acids and is also used to digest residual epithelial cell DNA at its optimal temperature of 37°C. Benzonase[®] is digested by the EA1 at higher temperatures (41–43). The sperm is then digested with Acrosolv at 52°C and, following its optimal activity at 56°C, Acrosolv is digested by the EA1 protease at 75°C preventing it from interfering with the Taq polymerase (41–43).

The benefit of this method versus the traditional differential extraction method is its ability to reduce the potential for DNA loss due to the entire procedure being performed

in a thermal cycler except for a centrifugation step (41–43). This procedure reduces unnecessary handling of samples, is less time consuming and is relatively simple to perform in comparison to the other novel methods and possibly cheaper. However, it is not yet validated for forensic use currently.

1.5 Research Objective

The objective of this study was to determine the efficiency of a TCDE through statistical analysis and data comparison. The TCDE protocol has been tested with several types of mock samples and more recently with thirty post coital samples (41) but the results have not been analyzed statistically. The results from the profiles generated from the post coital samples will have contributor proportions calculated to provide a basis of comparison statistically. The formula for these contributor proportions are essentially the same ones from previous papers that have examined the traditional differential extraction method (28,29).

In 2010, Bright et al. created two person mixtures to determine how effective traditional differential extraction was in determining mixtures by examining mixture proportion variation (44). This project aims to follow that method, however, in this case using a TCDE in place of a traditional differential extraction. The project also aims to determine how efficient the separation of sperm cells from epithelial cells was by comparing the mixture proportion mean of male DNA from an Acrosolv digest that did not undergo the TCDE to the proportion of male DNA from the TCDE. The

qPCR (quantitative polymerase chain reaction) data from the Acrosolv digest will serve as the basis for the male contributor proportion in the digest.

Any of the samples from fractions that contained more than four loci which contained a mixture within their profiles was characterized as a mixture and any with four or less mixtures at a locus were characterized as a single source profile.

Generally, this is not procedure, but for this paper it is a convenient definition.

Another aim that was not part of previous studies is to compare whatever DNA was left on a swab in a material fraction to the epithelial cell and sperm fractions.

2. MATERIALS AND METHODS

2.1 GeneMapper™ ID-X and JMP

Data from thirty post coital (PC) samples extracted with TCDE were provided to me from a previous student (41). The sample data was stored in GeneMapper® *ID-X* v1.4 (Applied Biosystems™) which contained the profiles of each sample's material fraction (MF), sperm fraction (SF), and epithelial fraction (EF). The sperm fraction consisted of the DNA ideally separated from the female DNA, the epithelial fraction consisted of the DNA from epithelial cells that were lysed prior to separating the sperm pellet, and the material fraction consisted of whatever DNA was remaining on the cotton swab after the epithelial lysis step and removal of the cutting.

Each of the contributor's single source profiles in GeneMapper™ *ID-X* were determined from single source EF and SF profiles from various samples. Additionally, a genotype chart detailing the alleles for each locus and the combined alleles from each locus was created.

There were three couples represented in the data. The couple labeled A+a had 23 samples representing the mixture. The couple labeled C+c had one sample representing the mixture and the couple labeled B+d had two samples representing the mixture.

Using GeneMapper™ each supplemental profile was then cleaned of stutter peaks, bleed through, off ladder peaks, and other potential artifacts to acquire accurate peak height data on all true alleles. Bleed through was determined by checking for high rfu alleles in other dye channels in the same bin. Stutter was determined by checking base pair position and the overall genotype of the mixture which was known due to having

clean EF and SF profiles. The stutter filter was on, using manufacturer suggested stutter values and the analytical threshold was set at 30 rfu for all samples run with the Applied Biosystems® 3130 Genetic Analyzer. The LIZ markers and allelic ladders were appropriate. Samples that did not generate a profile from the student's original run were run through the Microcon® to remove potential inhibitors in each sample. The previous student had mentioned that one of the potential inhibitors may have been lubricants. Subsequently these samples were run on the Applied Biosystems® SeqStudio™ Genetic Analyzer Instrument and analyzed with GeneMapper™ *ID-X* v1.6 (Applied Biosystems™, Waltham MA) with an analytical threshold of 50 rfu. The allelic ladders and LIZ markers were appropriate.

Once the profiles were rid of artifact peaks, a custom report setting was made in GeneMapper™ *ID-X* by changing “show 2 alleles” to “show 4 alleles” and adding the allele 3 and allele 4 columns. This report was then exported into a delimited text file. The text file was opened and all the data on it was copied and pasted into an empty Excel (Microsoft Corporation, Redmond WA) workbook and all the data was sorted by loci. After sorting the data by loci, the loci determined to be suitable for statistical analysis for each sample including the peak height data, sample name, sample file, allele designation and peak height rfu were copied and pasted into separate JMP version 15.2 (SAS Institute, Cary NC) workbooks by loci. This was done to ensure the contributor proportions calculated of the loci needed the correct formula for calculation separate from each other since not each locus has the same contributor proportion calculated formula. Viable loci for calculation of contributor proportions were selected by choosing loci that

had four alleles no with shared allele, three alleles with no shared allele, two alleles with no shared allele, or three alleles with one shared allele. In this last instance, the shared allele was excluded from the calculation.

These loci for the mixture labeled a+A were D3S1358, vWA, D21S11, D18S51, D13S317, SE33, D10S1248, D12S391, and D2S1338. Contributor proportions were calculated for each viable locus. For the mixture labeled c+C the loci D1S1656, D2S441, D3S1358, D8S1179, D10S1248, D12S391, D13S317, D21S11, D22S1045, FGA, SE33, vWA were used. For the mixture labeled B+d the loci CSF1PO, D1S1656, D2S441, D7S820, D8S1179, D12S391, D13S317, D18S51, D19S443, D21S11, FGA, SE33, TH01, and vWA were used. These particular loci listed are the ones that met the criteria mentioned above. Contributor proportions were calculated accordingly.

The mixed profiles contributor proportions were estimated by taking the peak height(s) of the male allele(s) divided by the total peak heights generated and for the case of the shared allele the one male allele peak height times two over total peak heights was used (Table 2). The formulas for the contributor proportions were determined by using the formulas from Bright et al. for each respective locus (44) (Table 2). Calculations were done by adding a formula column for each locus in the JMP workbooks based on whether each locus contained four non-shared alleles, three non-shared alleles, two non-shared alleles, or three alleles but one being shared by both the male and female contributor (Table 2, Table 3). Once all the contributor proportion formulas were set up, each workbook's data was copied and pasted into a master workbook with the data from all the samples for a particular mixture. The columns were then resorted by sample name.

The master workbook retained all peak height information and proportion data from the separate workbooks for each locus.

The contributor proportions from each mixture were then compared against the quantification data from the previous student's run to create a baseline of how much male DNA may be in the MF (41). The data in JMP was also used to create the figures.

Samples in Table 1 are not included in some figures or tables due to issues with the profiles but may be included in other tables or figures describing qPCR data.

Refer to Table 1 for samples not included in the data in any later tables or figures.

Table 1. Samples Not Included. All samples not included in the analyzed data due to no data in the electropherogram or for other reasons.

Sample Name of Samples Not Included	EF	MF	SF
PC17			X
PC18	X	X	X
PC21			X
PC22			X
PC23	X	X	X
PC25	X	X	X

Table 2. List of formulas with associated loci. Formulas were determined by genotype of the mixtures and their respective peak heights.

Mixture	Locus Formula Type	Viable Loci per Genotype chart	Formula
A+a	4 alleles with none shared	D3S1358, vWA, D18S51, D13S317, SE33, D10S1248, D12S391, D2S1338	$\frac{A + B}{(A + B + C + D)}$ Where A and B are male contributor peak heights
B+d	4 alleles with none shared	CSF1PO, D1S1656, D2S441, D7S820, D8S1179, D12S391, D13S317, D18S51, D19S443, D21S11, FGA, SE33, TH01, vWA	$\frac{A + B}{(A + B + C + D)}$
C+c	4 alleles with none shared	D1S1656, D2S441, D3S1358, D8S1179, D10S1248, D12S391, D13S317, D21S11, D22S1045, FGA, SE33, vWA	$\frac{A + B}{(A + B + C + D)}$
A+a	Three alleles with none shared	D3S1358, D2S1338	$\frac{A + B}{(A + B + C)}$ Where A and B are male allele peak heights
B+d	Three alleles with none shared	CSF1PO, D21S11	$\frac{A}{(A + B + C)}$ Where A is the male allele peak height
C+c	Three alleles with none shared	D3S1358, D22S1045	$\frac{A + B}{(A + B + C)}$

			$\frac{A}{(A + B + C)}$
Mixture	Locus Formula Type	Viable Loci per Genotype chart	Formula
A+a	Three alleles with one shared allele	D16S539, TPOX, D8S1179, D2S441, D19S443, D22S1045, D5S818, D7S820, D1S1656	$\frac{2A}{(A + B + C)}$ Where A is the peak height of the known unshared male allele
B+d	Three alleles with one shared allele	D2S441, D19S443, D13S317, D1S1656	$\frac{2A}{(A + B + C)}$
C+c	Three alleles with one shared allele	D18S51, D13S317, SE33, D12S391	$\frac{2A}{(A + B + C)}$
A+a	2 alleles with none shared	D21S11	$\frac{A}{(A + B)}$ Where A is the male contributor peak height
B+d	2 alleles with none shared	D22S1045	$\frac{A}{(A + B)}$
C+c	2 alleles with none shared	TPOX	$\frac{A}{(A + B)}$

Table 3. Allele chart of contributors. Contributor allelic expression was determined by analysis of respective fractions in GeneMapper™ ID-X.

Locus Name	Male A	Male B	Male C	Female a	Female c	Female d
D3S1358	16, 17	16, 16	18, 18	18, 18	16, 17	15, 16
vWA	16, 17	15, 16	15, 18	14, 18	14, 17	18, 19
D16S539	9, 11	12, 13	12, 12	11, 12	11, 12	12, 13
CSF1PO	11, 11	12, 12	11, 11	11, 13	10, 11	10, 13
TPOX	8, 11	8, 10	11, 11	8, 12	8, 8	8, 10
D8S1179	12, 13	10, 13	13, 13	13, 14	14, 14	11, 12
D21S11	30, 30	29, 29	30.2, 32	31, 31	27, 29	26, 30
D18S51	16, 19	16, 18	13, 16	12, 14	15, 16	12, 13
DYS391	10	10	11	N/A	N/A	N/A
D2S441	11, 14	11, 14	10, 11.3	10, 14	14, 15	10, 14
D19S443	14, 18.2	13, 14	12, 14	12, 14	14, 14	11, 14
TH01	7, 8	7, 9	7, 9.3	8, 8	7, 7	6, 8
FGA	20, 23	23, 25	21, 22	23, 23	20, 27	19, 24
D22S1045	15, 16	15, 15	11, 16	11, 16	15, 15	16, 16
D5S818	11, 12	13, 13	11, 12	11, 13	11, 12	12, 13
D13S317	9, 12	9, 11	9, 11	11, 13	11, 13	11, 12
D7S820	9, 12	10, 11	10, 10	9, 11	10, 11	9, 12
SE33	14, 18	14.2, 17	17, 22.2	16, 28.2	22.2, 29.2	14, 19
D10S1248	9, 15	14, 15	14, 16	13, 14	13, 18	15, 16
D1S1656	15, 15.3	13, 15.3	16, 17.3	11, 15	11, 14	12, 15.3
D12S391	16, 19	17, 22	18, 24	18.3, 21	18, 21	15, 15
D2S1338	17, 23	20, 20	17, 24	19, 19	16, 20	20, 24

2.1.1 Acrosolv Digest and CE Run

A small portion of each swab underwent an Acrosolv digest. This digest will simultaneously lyse all cells on the sample, both epithelial cells and sperm cells. A GlobalFiler™ (Applied Biosystems®) amplification of this digest was done on a small portion of each PC swab and run on the 3130 Genetic Analyzer (Applied Biosystems®). This amplification was done to visualize the original proportions of male and female DNA that existed on the swab prior to the TCDE separation of male and female DNA into the EF, MF, and SF.

3. RESULTS

3.1 Epithelial Fractions All Mixtures and Acrosolv Results

Most of the EF samples contained single source female DNA profiles as defined in the materials and methods section. For the purposes of this paper, a single source profile defined as having a mixture at four or fewer loci with all secondary peaks being easily differentiated from the primary profile. That is, there would be no ambiguity in determining the genotypes in the major profile. Only in the samples PC23 (only B male present), PC24 and PC26 (the B+d profiles), was any male contributor DNA present in large amounts in the EF. PC23 is not included in any later figures because it does not have a conclusive mixture in any fraction. The quantitative data from the Acrosolv digest of a smaller remaining portion of the swab (less than 1/3), which provided by a previous student (41) (Table 4) shows the estimated quantities of female to male and male to human DNA after a single Acrosolv lysis step breaking open both epithelial and sperm cells. By subtracting male from human, the data gives a baseline understanding of the ratio of female to male DNA after a single lysis step of all cells in the original sample.

Table 4. Quant data for all samples after Acrosolv digest. Panel A. F/M ratio calculated by estimated nanograms female over nanograms male. Mixture observed refers to male peaks found in the profiles after the digest.

Sample	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10
Quantity	0.554	0.311	0.179	0.151	0.169	0.455	0.375	0.121	1.572	3.971
Quantity	0.033	0.014	0.016	0.006	0.014	0.017	0.007	0.000	0.069	0.458
Est.	0.522	0.297	0.164	0.146	0.155	0.438	0.369	0.122	1.503	3.513
M/H	0.059	0.044	0.088	0.037	0.085	0.038	0.018	0.000	0.044	0.115
F/M	15.878	21.740	10.318	26.367	10.819	25.254	55.020	0.000	21.754	7.663
Profile	F	F	ND	ND	ND	F	F	ND	F	M+F

Panel B

Sample	PC11	PC12	PC13	PC14	PC15	PC16	PC17	PC18	PC19	PC20
Quantity	1.018	7.640	0.845	0.867	2.120	0.319	0.638	0.187	0.183	1.210
Quantity	0.151	0.684	0.137	0.113	0.179	0.005	0.186	0.074	0.065	0.425
Est.	0.867	6.956	0.708	0.754	1.942	0.314	0.452	0.113	0.118	0.785
M/H	0.148	0.090	0.162	0.130	0.084	0.016	0.291	0.398	0.356	0.351
F/M Ratio	5.737	10.173	5.161	6.685	10.872	61.624	2.432	1.513	1.806	1.845
Profile	M+F	M+F	M+F	M+F	M+F	ND	M+F	ND	ND	M+F

Panel C

Sample	PC21	PC22	PC23	PC24	PC25	PC26	PC27	PC28	PC29	PC30
Quantity	1.150	5.131	9.304	6.534	4.037	6.065	1.335	3.196	1.428	2.311
Quantity	0.043	0.014	9.984	6.254	0.000	6.695	0.039	0.015	0.010	0.026
Est.	1.107	5.117	-0.680	0.280	4.037	-0.630	1.296	3.181	1.418	2.285
M/H	0.038	0.003	1.073	0.957	0.000	1.104	0.029	0.005	0.007	0.011
F/M	25.500	358.713	-0.068	0.045	0.000	-0.094	33.332	205.822	136.137	88.806
Profile	M+F	F	M+F	M+F	ND	M+F	M+F	F	F	F

Legend	
M+F	Mixture was observed
F	Female DNA only
ND	No data or not enough data
H (ng/uL)	Human or total DNA in nanograms per microliter
Est. Quantity F	Quantity H- Y
M/H Ratio	Male over human
F/M Ratio	Female to Male DNA ratio
uL	Microliters
Y (ng/uL)	Male DNA in nanograms per microliter

3.1.1 Contributors A+a EF Data

None of the EF fractions from the A+a were mixtures using the definition above. That is, all epithelial cell fractions were single source female. All the Acrosolv digest quantitation data calculated in this section came from Table 3 and the TCDE quantitation data is summarized in Appendix A. The range of total female DNA in the EF after the TCDE was 16.98ng to 1292ng. For the the A+a samples, male contributor peaks were observed in the Acrosolv digest (i.e., the initial sample) for samples PC11, PC12, PC13, PC14, PC15, PC17, PC20, PC21, and PC27 (Table 4). The EF profiles of these samples after the TCDE were all single source profiles.

3.1.2 Contributors B+d and C+c EF Data

The C+c PC16 profile did not contain any male peaks in the Acrosolv digest or in the EF. The DNA profile in the EF was single source female for PC16. The samples PC24 and PC26 contained male peaks in the DNA profiles resulting in a mixture in the EF.

3.2 Material Fraction Data and Comparison with qPCR MF Data

All samples showed that there is remaining female and male DNA on the swab even after the cutting is used for differential extraction; in this study through the TCDE method (Figures 1 and 2). The male contributor proportion is generally much smaller in this fraction than the SF (41) and Figure 1. The range of values for the male contributor proportion in the EF (samples PC24 and PC26 only), the MF, and the SF (for all samples) is shown in Figure 2. The single source profiles for both fractions were included as a

contributor proportion of one, meaning that there is no detectable female DNA present. Any MFs that contained single source female profiles were added as a proportion of zero meaning no detectable male DNA. The average male contributor proportion across all MFs from the A+a data was 0.226 (Figure 1) and the standard deviation for the proportion calculated from male contributor proportion data calculated from the MF in the A+a is 0.209. The entirety of the male contributor proportion data gathered is displayed in the overlay of fractions in Figure 2. Figure 3 allows comparison between the male contributor proportion in the EF, MF, and SF fractions of each sample, and is very similar to Figure 1 in that the MF male contributor proportions are close to zero and the SF male contributor proportions are close to one or above one in some cases. The box plot of the total male contributor proportions in each fraction also depicts the sperm fraction as being much closer to one compared to both the MFs and EFs (Figure 3). The C+c mixture did not have any male contributor peaks in either its material fraction or its sperm fraction. Proportions of male DNA in the loci of the SF used for calculation are generally above 80% and thus would likely allow deduction of the male contributor or the male profile and, depending on the laboratory protocol, would meet the criteria for being the major contributor.

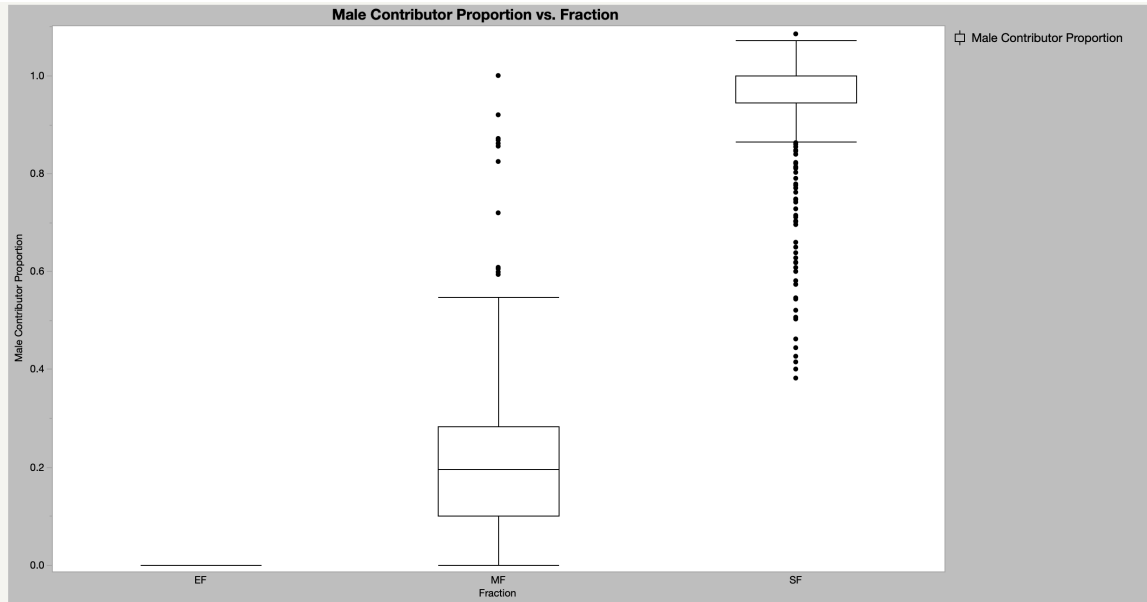


Figure 1. The box plots show the distribution of all male proportion data from all 23 samples that contained the DNA from the couple labeled A+a. The mixture of A+a had 17 loci that were able to be used for the calculation of the mixture proportion.

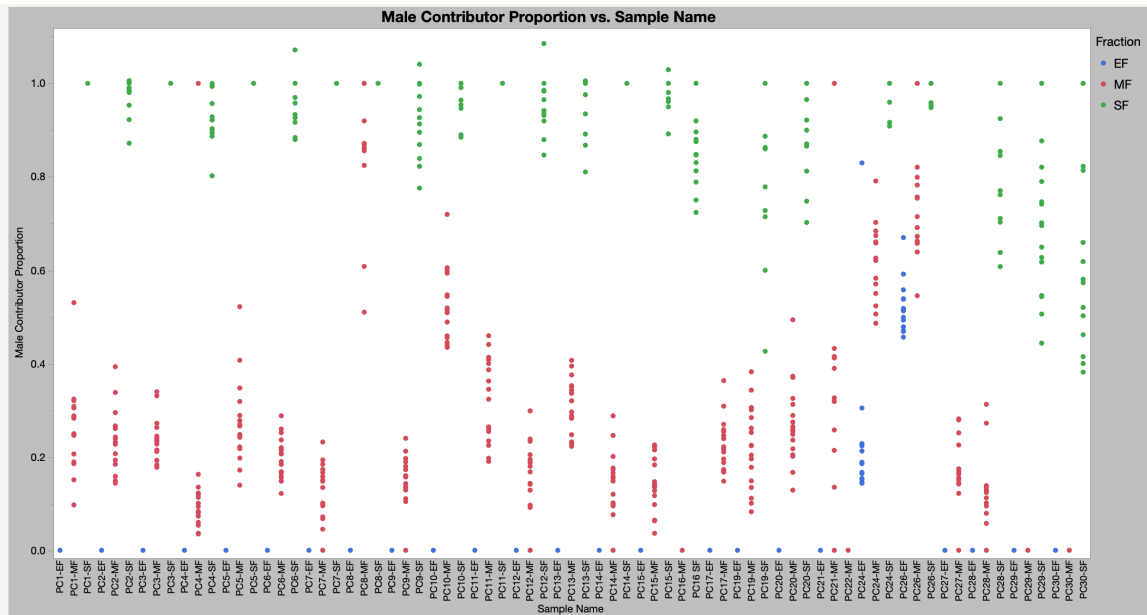


Figure 2. Overlaid EF, SF, and MF fraction male contributor proportions. Some samples are missing due to containing no data in their profile.

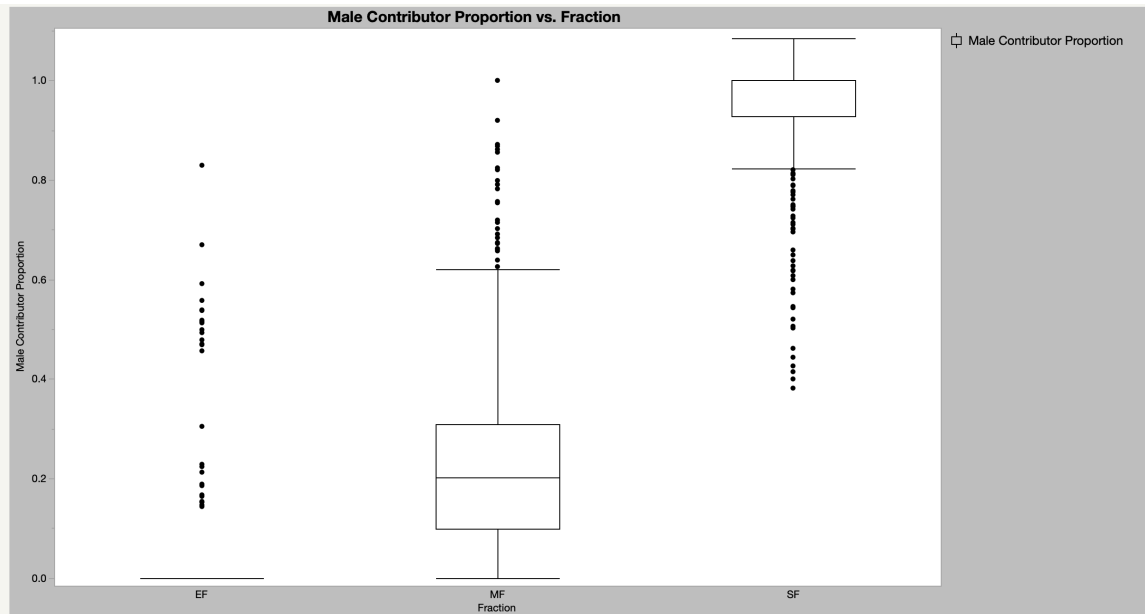


Figure 3. Box plots of each fraction’s total male contributor proportion for all samples. The EF contains some outliers from samples PC24 and PC26.

The EF does have a more outliers in its data in Figure 3, but it also contains two samples with a mixture in the EF.

3.2.1 Calculation of Mixture Proportion in MF Using Peak Height vs qPCR Data

The MF mixture proportion data from the A+a profiles was compared to its qPCR data (shown in Appendix A). This comparison does not include any single source profiles and only full MF profiles that are not degraded (Figure 4). The MFs of PC22, PC29, and PC30 material fractions were not included in this comparison or in other figures as their material fractions contained single source female profiles, again defined as having a mixture at four or less loci. PC18 had no peak height data across any fractions so it is not included in any figures. The comparison did not show a linear correlation (Figure 4). For the samples PC1, PC2, PC4, PC5, PC6, PC7, PC8, PC9, PC10, PC13, PC17, PC19, PC20, PC21, PC24, PC26, PC27, and PC28 the MF male

proportion calculated from the qPCR data was greater than the male proportion data calculated from the peak height ratios. This disparity does not mean that the TCDE did not recover all male DNA as in many cases the values are close to the values calculated from the peak heights. In the STR profile, data is sampled from many loci with the PCR product available at the end of 29 cycles of amplification whereas the qPCR data is collected from one locus for male and one locus for total DNA and the extrapolation of the DNA quantity relies on a standard. The values may be consistent but not identical. For most samples the qPCR data is a reasonable predictor of actual mixture proportions. This is possible in qPCR because the mixture contains two contributors of opposite gender.

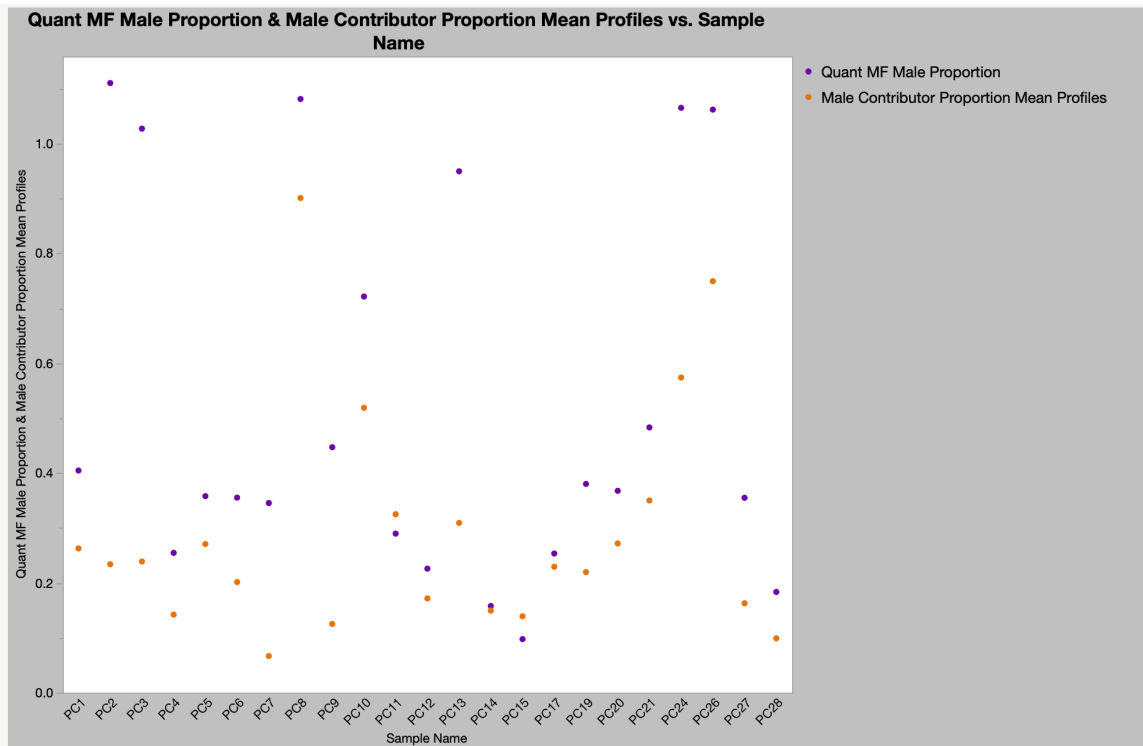


Figure 4. Data points from the quantitative male contributor proportions and from the male contributor proportions from peak heights in the MFs with mixtures. This shows no direct correlation between the two.

3.3 Sperm Fraction Data

The average male contributor proportion for the SF was close to one in most cases in the A+a mixture since the fraction consisted mainly of the sperm cells with very little remaining epithelial cell DNA. Figure 3 shows each fraction's individual male contributor proportions by sample and many of the sperm fraction proportions are, near, or above one. Single source profiles for the SF were included in the data as a contributor proportion of one to accurately reflect the effectiveness of the overall method across all samples that generated a profile. A male contributor proportion of one does not mean that there is no epithelial cell DNA just that it is not detectable. Since most SF male contributor proportions were near, at, or above a proportion of one, the TCDE did effectively produce either a single source male profile or a male profile with a minor female contributor in almost all A+a samples' SFs with peaks.

In the B+d and the C+c samples, (Sample numbers PC16, 24, and 26) the SFs produced profiles with mixtures. The values for the B+d SF average male contributor proportion is still near, or at one and can be seen in Figure 3. The C+c SF average male contributor proportion data also was near one. Because the TCDE does not purify the DNA, there is a greater potential for observing the effects of inhibitors. Once corrected by the microcon step, they are consistent with proportions of male DNA in the MF and SF as seen in other samples. It is difficult to appropriately assess the impact that potential inhibitors may have on the TCDE without including the other samples that did not produce peaks in these experiments and use of a larger sample set.

4. DISCUSSION

4.1 TCDE Overall Effectiveness

The TCDE worked mostly as expected with the box plot in Figure 3 showing the majority of the EFs averaging at or near 0 male contributor proportion and most of the SFs averaging at or near one with a median of one. The MFs show that DNA is being left on the swab even after the swab has gone through the entire extraction procedure with a median male contributor proportion of 0.2. Readers need to look closely at Figure 3 to avoid being misled by the outliers in the EF. The outliers are easy to see but the average is depicted by a line at zero. Additionally, in Figure 2, the EF mixtures are clearly a very small number in comparison to the MF mixtures. The amount of male and female DNA remaining on the cotton also varies from sample to sample as shown by the proportion of total male in fraction in Appendix A. This discrepancy is concerning since ideally the procedure should completely lyse and separate all the DNA on the swab for analysis. The failure to completely recover all the DNA is ineffable. It's possible that the procedure is not effectively removing the DNA from the cotton, or the DNA was still trapped in the cotton after the first extraction and then was eased out in the second which would be a substrate/cell interaction issue. In any case, the method is effective in recovering sperm and reducing DNA carryover, but the issue of the missing DNA, particularly the male DNA, needs to be resolved.

4.2 TCDE and the SPRED Method

Klein and Buoncristiani developed a method known as the Separation Potential Ratio of the Extraction Differential or SPRED (45). This ratio is the percent sperm DNA recover divided by the percent female DNA carryover in the sperm fraction (45). This calculation was used for all the sperm fractions of the post-coital samples. The female carryover in several cases was a negative number due to the qPCR estimation of slightly more male than human DNA in the sperm fraction which Klein and Buoncristiani also had issues with (45). Klein and Buoncristiani remedied this by back calculating how much sperm they originally had (45), but in this paper the starting amount of sperm is unknown. So, for the purposes of this paper if the sample was single source male and there was more male DNA than human DNA in the sperm fraction, the female carryover was defined as a proportion equal to .05 which would be a very small female carryover in most DNA profiles. If the sample was a mixture, the female carryover was 1 minus the average male contributor proportion in the sperm fraction of the sample. For the samples with no SF data, 'No Data' was entered. The average SPRED value was 1541 with a range of values from 24 to 10967. The SPRED values suggest that this protocol performed better in reducing female DNA carryover than the two protocols in Klein and Buoncristiani which had a SPRED value of 896 for their Erase method and 217 for the traditional method (45). The 1 lysis step in Klein and Buoncristiani did recover sperm effectively but it also did not fully lyse most of the epithelial cells (45) which was an issue this method also had as shown by the proportion of male in each sample in

Appendix A. The 2-lysis step was more effective and had a SPRED value of 1273 (45) which is closer to the average from the TCDE.

Table 5. SPRED Values. The SPRED values calculated from Appendix A and overall mixture proportions.

Sample Name	SPRED Value
PC1	1237.517
PC2	1722.7394
PC3	1897.6490
PC4	1397.9161
PC5	1141.6132
PC6	1274.6536
PC7	1129.1147
PC8	1275.4409
PC9	10967.4994
PC10	1520.5325
PC11	923.6617
PC12	1118.3810
PC13	1156.1434
PC14	666.6977
PC15	533.0731
PC16	24.2819
PC17	No Data
PC18	No Data
PC19	889.0042
PC20	1368.8793
PC21	No Data

Sample Name	SPRED Value
PC22	No Data
PC23	89.6313
PC24	110.3282
PC25	No Data
PC26	6337.2978
PC27	936.1093
PC28	556.7933
PC29	84.0068
PC30	190.9826

5. CONCLUSIONS

5.1 TCDE Effectiveness

It is difficult to assess the overall effectiveness of the TCDE compared to other methods as a material fraction was assessed in this study and is generally not assessed in practical casework and no standard differential extraction method was incorporated in this analysis. While the resulting SF profiles are mostly single source male, the loss of sperm DNA which remains in the MF is concerning. The issue of DNA loss was also noted in a former student's thesis and has some similarities to this one (43).

The peak height proportion data from the material fraction was compared to its corresponding qPCR data and appeared to be mostly consistent in value. This comparison is not directly related to the effectiveness of the TCDE, but it does show that the female to male ratios were accurate in predicting the outcome of the mixtures in the STR profiles in most cases.

5.2 Future Directions

The data suggests that the protocol as it is now may have some issues in fully recovering the DNA from the swab after undergoing the procedure. The separation data does suggest that it is effective at separating male from female DNA as evidenced by the number of male profiles with little to no female DNA in the sperm fractions. Further testing to confirm the results of this study is necessary since there were only thirty samples used in this study which may not be enough to account for other variations in the DNA recovery and separation data.

The issue of incomplete recovery of sperm from the collection device, which may have affected other types of differential extraction methods as well, is important to address. The use of a two-lysis protocol published by Klein and Buoncristiani suggests this issue may be present in other protocols as well (45). Therefore, better understanding, and quantitation of the extent of, DNA loss to substrate is needed.

APPENDIX A: PROPORTION DATA FROM QPCR DATA

SAMPLE	MF DNA, proportion male	Proportion of total female (in sample) in MF	Proportion of total male (in sample) in each fraction
PC1 EF		0.8140	0.0000
PC1 MF	0.4047	0.1860	0.3812
PC1 SF		0.0000	0.6188
PC2 EF		1.0000	0.0566
PC2 MF	1.1105	0.0000	0.0820
PC2 SF		0.0000	0.8614
PC3 EF		1.0000	0.0000
PC3 MF	1.0274	0.0000	0.0512
PC3 SF		0.0000	0.9488
PC4 EF		0.8923	0.0315
PC4 MF	0.2547	0.1077	0.2695
PC4 SF		0.0000	0.6990
PC5 EF		0.8835	0.0244
PC5 MF	0.3580	0.1165	0.4048
PC5 SF		0.0000	0.5708
PC6 EF		0.7678	0.0192
PC6 MF	0.3553	0.2322	0.3434
PC6 SF		0.0000	0.6373
PC7 EF		0.7672	0.0000
PC7 MF	0.3455	0.2328	0.4354
PC7 SF		0.0000	0.5646
PC8 EF		1.0000	0.0285
PC8 MF	1.0814	0.0000	0.3337
PC8 SF		0.0000	0.6377
PC9 EF		0.9201	0.1657
PC9 MF	0.4472	0.0793	0.7642
PC9 SF		0.0006	0.0701
PC10 EF		0.9785	0.0241
PC10 MF	0.7216	0.0215	0.2157
PC10 SF		0.0000	0.7603

SAMPLE	MF DNA, proportion male	Proportion of total female (in sample) in MF	Proportion of total male (in sample) in each fraction
PC11 EF		0.6559	0.0300
PC11 MF	0.2897	0.3441	0.5082
PC11 SF		0.0000	0.4618
PC12 EF		0.5369	0.0142
PC12 MF	0.2259	0.4631	0.4266
PC12 SF		0.0000	0.5592
PC13 EF		0.9853	0.0294
PC13 MF	0.9497	0.0147	0.3925
PC13 SF		0.0000	0.5781
PC14 EF		0.2777	0.0243
PC14 MF	0.1579	0.7223	0.6424
PC14 SF		0.0000	0.3333
<u>PC15 EF</u>		0.1757	0.0174
<u>PC15 MF</u>	0.0976	0.8243	0.7161
<u>PC15 SF</u>		0.0000	0.2665
PC16 EF		0.0769	0.0000
PC16 MF	0.0471	0.9224	0.9623
PC16 SF		0.0007	0.0377
PC17 EF		0.4434	0.0063
PC17 MF	0.2535	0.5566	0.3241
PC17 SF		0.0000	0.6696
PC18 EF		0.5767	0.0000
PC18 MF	0.2270	0.4233	0.4563
PC18 SF		0.0000	0.5437
PC19 EF		0.4883	0.0182
PC19 MF	0.3803	0.5117	0.5373
PC19 SF		0.0000	0.4445
PC20 EF		0.1170	0.0011
PC20 MF	0.3677	0.8830	0.3145
PC20 SF		0.0000	0.6844

SAMPLE	MF DNA, proportion male	Proportion of total female (in sample) in MF	Proportion of total male (in sample) in each fraction
PC21 EF		0.8140	0.5277
PC21 MF	0.4832	0.1860	0.2891
PC21 SF		0.0000	0.1833
PC22 EF		1.0000	0.3109
PC22 MF	0.0401	0.0000	0.1908
PC22 SF		0.0000	0.4983
PC23 EF		1.0000	0.0407
PC23 MF	1.3741	0.0000	0.1286
PC23 SF		0.0000	0.8307
PC24 EF		0.8923	0.0582
PC24 MF	1.0654	0.1077	0.0895
PC24 SF		0.0000	0.8523
PC25 EF		0.8835	na
PC25 MF	0.0000	0.1165	na
PC25 SF		0.0000	0.0150
PC26 EF		0.7678	0.0317
PC26 MF	1.0621	0.2322	0.3345
PC26 SF		0.0000	0.6337
PC27 EF		0.7672	0.0000
PC27 MF	0.3550	0.2328	0.5319
PC27 SF		0.0000	0.4681
PC28 EF		1.0000	0.0000
PC28 MF	0.1835	0.0000	0.7216
PC28 SF		0.0000	0.2784
PC29 EF		0.9201	0.3320
PC29 MF	0.0710	0.0793	0.4359
PC29 SF		0.0006	0.2321
PC30 EF		0.9785	0.0000
PC30 MF	0.0109	0.0215	0.3199
PC30 SF		0.0000	0.6801

LIST OF JOURNAL ABBREVIATIONS

<i>Anal Chem</i>	<i>Analytical Chemistry</i>
<i>Ann Hum Genet</i>	<i>Annals of Human Genetics</i>
<i>Am J Hum Genet</i>	<i>American Journal of Human Genetics</i>
<i>Forensic Sci Int</i>	<i>Forensic Science International</i>
<i>Forensic Sci Int Genet</i>	<i>Forensic Science International: Genetics</i>
<i>Forensic Sci Int Genet Suppl ser</i>	<i>Forensic Science International: Genetics Supplementary Series</i>
<i>Int J Legal Med</i>	<i>International Journal of Legal Medicine</i>
<i>J Biol Chem</i>	<i>Journal of Biological Chemistry</i>
<i>J Forensic Sci</i>	<i>Journal of Forensic Sciences</i>
<i>Sci Justice</i>	<i>Science and Justice</i>

BIBLIOGRAPHY

1. Blood & Bodily Fluids. Forensic Resources. <https://forensicresources.org/forensic-disciplines/blood-bodily-fluids/>. (accessed May 13, 2021).
2. Allery JP, Telmon N, Mieusset R, Blanc A, Rougé D. Cytological detection of spermatozoa: comparison of three staining methods. *J Forensic Sci* 2001;46(2):349–51.
3. Old J, Schweers BA, Boonlayangoor PW, Fischer B, Miller KWP, Reich K. Developmental Validation of RSIDTM-Semen: A Lateral Flow Immunochromatographic Strip Test for the Forensic Detection of Human Semen*: Immunochromatographic Strip Test for the Forensic Detection of Human Semen. *J Forensic Sci* 2012;57(2):489–99. <https://doi.org/10.1111/j.1556-4029.2011.01968.x>.
4. Cotton RW, Fisher MB. Review: Properties of sperm and seminal fluid, informed by research on reproduction and contraception. *Forensic Sci Int Genet* 2015;18:66–77. <https://doi.org/10.1016/j.fsigen.2015.03.009>.
5. Hao S-L, Ni F-D, Yang W-X. The dynamics and regulation of chromatin remodeling during spermiogenesis. *Gene* 2019;706:201–10. <https://doi.org/10.1016/j.gene.2019.05.027>.
6. Gill P, Jeffreys AJ, Werrett DJ. Forensic application of DNA ‘fingerprints.’ *Nature* 1985;318(6046):577–9. <https://doi.org/10.1038/318577a0>.
7. Jeffreys AJ, Wilson V, Thein SL. Individual-specific ‘fingerprints’ of human DNA. *Nature* 1985;316(6023):76–9. <https://doi.org/10.1038/316076a0>.
8. Clark CP, Xu K, Scott O, Hickey J, Tsuei A-C, Jackson K, et al. Acoustic trapping of sperm cells from mock sexual assault samples. *Forensic Sci Int Genet* 2019;41:42–9. <https://doi.org/10.1016/j.fsigen.2019.03.012>.
9. Clayton TM, Whitaker JP, Sparkes R, Gill P. Analysis and interpretation of mixed forensic stains using DNA STR profiling. *Forensic Sci Int* 1998;91(1):55–70. [https://doi.org/10.1016/S0379-0738\(97\)00175-8](https://doi.org/10.1016/S0379-0738(97)00175-8).
10. Jeffreys AJ, Wilson V, Thein SL. Hypervariable ‘minisatellite’ regions in human DNA. *Nature* 1985;314(6006):67–73. <https://doi.org/10.1038/314067a0>.
11. Moretti TR, Baumstark AL, Defenbaugh DA, Keys KM, Brown AL, Budowle B. Validation of STR typing by capillary electrophoresis. *J Forensic Sci* 2001;46(3):661–76.

12. Roewer L. DNA fingerprinting in forensics: past, present, future. *Investig Genet* 2013;4(1):22. <https://doi.org/10.1186/2041-2223-4-22>.
13. Wong Z, Wilson V, Patel I, Povey S, Jeffreys AJ. Characterization of a panel of highly variable minisatellites cloned from human DNA. *Ann Hum Genet* 1987;51(4):269–88. <https://doi.org/10.1111/j.1469-1809.1987.tb01062.x>.
14. Edwards A, Civitello A, Hammond HA, Caskey CT. DNA typing and genetic mapping with trimeric and tetrameric tandem repeats. *Am J Hum Genet* 1991;49(4):746–56.
15. Wallin JM, Buoncristiani MR, Lazaruk KD, Fildes N, Holt CL, Walsh PS. TWGDAM validation of the AmpFISTR blue PCR amplification kit for forensic casework analysis. *J Forensic Sci* 1998;43(4):854–70.
16. LaFountain MJ, Schwartz MB, Svete PA, Walkinshaw MA, Buel E. TWGDAM validation of the AmpFISTR Profiler Plus and AmpFISTR COfiler STR multiplex systems using capillary electrophoresis. *J Forensic Sci* 2001;46(5):1191–8.
17. Lins AM, Micka KA, Sprecher CJ, Taylor JA, Bacher JW, Rabbach DR, et al. Development and population study of an eight-locus short tandem repeat (STR) multiplex system. *J Forensic Sci* 1998;43(6):1168–80.
18. Frank, WE, Llewellyn, BE, Fish, PA, Riech, AK, Marcacci, TL, Gandor, DW, et al. Validation of the AmpFISTR™ Profiler Plus PCR Amplification Kit for Use in Forensic Casework. *J Forensic Sci* 2001;46(3):642–6.
19. Ludeman MJ, Zhong C, Mulero JJ, Lagacé RE, Hennessy LK, Short ML, et al. Developmental validation of GlobalFiler™ PCR amplification kit: a 6-dye multiplex assay designed for amplification of casework samples. *Int J Legal Med* 2018;132(6):1555–73. <https://doi.org/10.1007/s00414-018-1817-5>.
20. Moretti TR, Baumstark AL, Defenbaugh DA, Keys KM, Smerick JB, Budowle B. Validation of short tandem repeats (STRs) for forensic usage: performance testing of fluorescent multiplex STR systems and analysis of authentic and simulated forensic samples. *J Forensic Sci* 2001;46(3):647–60.
21. Tao R, Wang S, Zhang J, Zhang J, Yang Z, Sheng X, et al. Separation/extraction, detection, and interpretation of DNA mixtures in forensic science (review). *Int J Legal Med* 2018;132(5):1247–61. <https://doi.org/10.1007/s00414-018-1862-0>.
22. Wiegand P, Schurenkamp 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>.

23. Yoshida K, Sekiguchi K, Mizuno N, Kasai K, Sakai I, Sato H, et al. The modified method of two-step differential extraction of sperm and vaginal epithelial cell DNA from vaginal fluid mixed with semen. *Forensic Sci Int* 1995;72(1):25–33. [https://doi.org/10.1016/0379-0738\(94\)01668-U](https://doi.org/10.1016/0379-0738(94)01668-U).
24. Garvin AM. Filtration based DNA preparation for sexual assault cases. *J Forensic Sci* 2003;48(5):1084–7.
25. Norris JV, 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>.
26. Garvin AM, Bottinelli M, Gola M, Conti A, Soldati G. DNA Preparation from Sexual Assault Cases by Selective Degradation of Contaminating DNA from the Victim. *J Forensic Sci* 2009;54(6):1297–303. <https://doi.org/10.1111/j.1556-4029.2009.01180.x>.
27. Jankova R, Jakovski Z, Janevski R. Differential extraction method as a golden standard in analyzing semen stains in sexual-assault cases. *Forensic Sci Int Genet Suppl Ser* 2019;7(1):838–40. <https://doi.org/10.1016/j.fsigss.2019.10.196>.
28. Timken MD, Klein SB, Kubala S, Scharnhorst G, Buoncristiani MR, Miller KWP. Automation of the standard DNA differential extraction on the Hamilton AutoLys STAR system: A proof-of-concept study. *Forensic Sci Int Genet* 2019;40:96–104. <https://doi.org/10.1016/j.fsigen.2019.02.011>.
29. Wong H, Mihalovich J. Automation of the Differential Digestion Process of Sexual Assault Evidence,. *J Forensic Sci* 2019;64(2):539–50. <https://doi.org/10.1111/1556-4029.13877>.
30. Elliott K, Hill DS, Lambert C, Burroughes TR, Gill P. Use of laser microdissection greatly improves the recovery of DNA from sperm on microscope slides. *Forensic Sci Int* 2003;137(1):28–36. [https://doi.org/10.1016/S0379-0738\(03\)00267-6](https://doi.org/10.1016/S0379-0738(03)00267-6).
31. Di Martino D, Giuffrè G, Staiti N, Simone A, Le Donne M, Saravo L. Single sperm cell isolation by laser microdissection. *Forensic Sci Int* 2004;146:S151–3. <https://doi.org/10.1016/j.forsciint.2004.09.046>.
32. Meredith M, Bright J-A, Cockerton S, Vintiner S. Development of a one-tube extraction and amplification method for DNA analysis of sperm and epithelial cells recovered from forensic samples by laser microdissection. *Forensic Sci Int Genet* 2012;6(1):91–6. <https://doi.org/10.1016/j.fsigen.2011.02.007>.
33. Marshall, Pamela. Optimization of Spermatozoa Capture During the Differential Extraction Process for STR Typing with the Potential for Automation.

34. Li X-B, Wang Q-S, Feng Y, Ning S-H, Miao Y-Y, Wang Y-Q, et al. Magnetic bead-based separation of sperm from buccal epithelial cells using a monoclonal antibody against MOSPD3. *Int J Legal Med* 2014;128(6):905–11. <https://doi.org/10.1007/s00414-014-0983-3>.
35. Alsalafi D, Goodwin W. Capturing spermatozoa for STR analysis of sexual assault cases using anti-sperm antibodies. *Forensic Sci Int Genet Suppl Ser* 2019;7(1):707–10. <https://doi.org/10.1016/j.fsigss.2019.10.146>.
36. Dissing J, Rudbeck L, Marcher H. A Five Minute Procedure for Extraction of Genomic DNA from Whole Blood, Semen and Forensic Stains for PCR. In: Carracedo A, Brinkmann B, Bär W, editors. 16th Congress of the International Society for Forensic Haemogenetics (Internationale Gesellschaft für forensische Hämogenetik e.V.), Santiago de Compostela, 12–16 September 1995. Berlin, Heidelberg: Springer Berlin Heidelberg, 1996;269–71.
37. Klintschar M, Neuhuber F. Evaluation of an alkaline lysis method for the extraction of DNA from whole blood and forensic stains for STR analysis. *J Forensic Sci* 2000;45(3):669–73.
38. Hudlow WR, Buoncristiani MR. Development of a rapid, 96-well alkaline based differential DNA extraction method for sexual assault evidence. *Forensic Sci Int Genet* 2012;6(1):1–16. <https://doi.org/10.1016/j.fsigen.2010.12.015>.
39. 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>.
40. Xu K, Clark CP, Poe BL, Lounsbury JA, Nilsson J, Laurell T, et al. Isolation of a Low Number of Sperm Cells from Female DNA in a Glass–PDMS–Glass Microchip via Bead-Assisted Acoustic Differential Extraction. *Anal Chem* 2019;91(3):2186–91. <https://doi.org/10.1021/acs.analchem.8b04752>.
41. Emily Elizabeth Hoffman. Optimization of the Temperature Controlled Differential Extraction for Casework-Type Samples. 2020.
42. Andrew David Ziegler. Modification of a Novel Temperature Controlled Differential Extraction Procedure for Better Application In Forensic Casework. 2019.
43. Ashleigh Roberts. Optimization of a Novel Temperature Controlled Differential Extraction Procedure for Aged Sample Analysis. 2019.
44. Bright J-A, Turkington J, Buckleton J. Examination of the variability in mixed DNA profile parameters for the Identifiler™ multiplex. *Forensic Sci Int Genet* 2010;4(2):111–4. <https://doi.org/10.1016/j.fsigen.2009.07.002>.

45. Klein SB, Buoncristiani MR. Evaluating the efficacy of DNA differential extraction methods for sexual assault evidence. *Forensic Sci Int Genet* 2017;29:109–17.
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- Infrared (IR) Spectroscopy
- Analytical Balances
- Vertical and Horizontal Gel Electrophoresis Apparatus
- NMR
- Analyte-Specific (pH and ISE) Meters/ Probes
- UV-Vis Single and Double Beam Absorption Spectrophotometer
- 3130 Genetic Analyzer

Volumetric/Gravimetric Analysis

- Calibration curves
- Preparing standards
- Pipetting
- Filtration
- Concentration
- Distillation
- Separation Techniques

Laboratory Applications

- Chemical storage/ labeling
- Quality control
- Safety practices
- Precision/accuracy of results
- Data collection
- Lab notebook organization
- Interpretation and reporting of data
- GeneMapper ID-X[®]
- Material Safety Data Sheets
- Spread sheets and Graphs
- PowerPoint presentation of data

Microscope Slide Techniques

- Aseptic Technique
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PUBLICATIONS AND PRESENTATIONS

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