Boston University

OpenBU **http://open.bu.edu**

Boston University Theses & Dissertations Boston University Theses & Dissertations

2025

Optimizing cell yield and fraction separation from mixed sample swabs using temperature controlled differential extraction methods

https://hdl.handle.net/2144/52247

"Downloaded from OpenBU. Boston University's institutional repository."

BOSTON UNIVERSITY

ARAM V. CHOBANIAN & EDWARD AVEDISIAN SCHOOL OF MEDICINE

Thesis

OPTIMIZING CELL YIELD AND FRACTION SEPARATION FROM MIXED SAMPLE SWABS USING TEMPERATURE CONTROLLED DIFFERENTIAL EXTRACTION METHODS

by

ANNA MARIE BERBEROGLU

B.S., Virginia Commonwealth University, 2022

Submitted in partial fulfillment of the

requirements for the degree of

Master of Science

2025

© 2025 by
ANNA MARIE BERBEROGLU
All rights reserved

Approved by

First Reader ______________________________

Robin W. Cotton, Ph.D.
Associate Professor of Anatomy & Neurobiology

Second Reader ______________________________

Mike Yakoo, M.S.
Criminalist II

Third Reader ______________________________

Ashleigh Roberts, M.S.
Forensic Scientist II

ACKNOWLEDGMENTS

I would like to thank my family for the unconditional love and for your continuous support as I find my path through my education and professional life. Thank you to my friends, near and far, for keeping me going when graduate school felt impossible to finish. Thank you to those I met in Boston for welcoming me with open arms and making this place feel like home. Thank you to my wonderful roommates for cheering me on as I finished writing section by section. Thank you to the faculty and staff of the Biomedical Forensic Sciences program for preparing me to be a forensic scientist, and for being patient with me as I completed this program part-time. Thank you to my laboratory manager and coworkers for being flexible when it came to my research.

A big thank you to past research students for developing and optimizing the TCDE method over all the years, including Emily Hoffman, Emily Nicholas, Anooja Rai, Ashleigh Roberts, Mike Yakoo, Andrew Ziegler, Faith Ramos, and Ashley Lara.

An extra special thank you to Dr. Robin Cotton and Ashley Lara, for your guidance, collaboration, and companionship while working on this project. Thank you for letting me contribute to the amazing work you have accomplished, and for sharing your love for science with me.

OPTIMIZING CELL YIELD AND FRACTION SEPARATION FROM MIXED SAMPLE SWABS USING TEMPERATURE CONTROLLED DIFFERENTIAL EXTRACTION METHODS

ANNA MARIE BERBEROGLU

ABSTRACT

The optimization of DNA extraction methods from mixed sample swabs is critical in forensic science, particularly when dealing with complex samples containing contributions from multiple individuals. This thesis investigates the use of Temperature-Controlled Differential Extraction (TCDE) methods to enhance cell yield and improve the separation of DNA fractions from mixed samples, such as vaginal, semen, and buccal swabs. The primary objective of this research was to refine TCDE protocols to achieve higher efficiency in isolating target DNA fractions (e.g., epithelial and sperm fractions) while minimizing carry-over between contributors. A series of experiments were conducted using a variety of sample types, including single-source and mixed samples, with variations in reagent preparation, enzyme treatments, and incubation conditions. Challenges were noted in the separation of DNA from mixed vaginal and semen samples, particularly in the incomplete recovery of female DNA in some mixed samples, such as mixed sample XY-J, where missing alleles were identified in the epithelial and material fractions. The findings highlight the effectiveness of the optimized TCDE protocol in extracting high-quality DNA profiles from both single-source and mixed samples, though

further refinement may be necessary to enhance consistency and address the challenges of profiling mixed contributors. This research contributes to the ongoing efforts to improve forensic DNA analysis, with potential applications in criminal justice and human identification.

Keywords: Differential extraction, mixed samples, forensic DNA analysis, temperature-controlled differential extraction (TCDE), DNA recovery, epithelial fraction, sperm fraction, forensic science

TABLE OF CONTENTS

ACKNOWLEDGMENTS……………………………………………………………. iv

ABSTRACT …………………………………………………………………… v

TABLE OF CONTENTS ……………………………………………………… vii

LIST OF TABLES ……………………………………………………………… xii

LIST OF FIGURES …………………………………………………………… xiii

LIST OF ABBREVIATIONS ………………………………………………… xiv

1. INTRODUCTION ……………………………………………………….. 1

1.1 DNA in Forensic Science ………………………………………………. 1

1.2 The Vulnerability of DNA in Forensics………………………………. 1

1.3 Sexual Assault ………………………………………………………... 3

1.3.1 Sexual Assault Collection Kits (SAECK) ……………………… 4

1.3.2 Separating Male and Female DNA ……………………………. 5

1.3.3 Recent Advancements in SAECK Processing ………………… 7

1.4 Male DNA …………………………………………………………… 8

1.4.1 Sperm Cell Structure …………………………………………… 8

1.4.2 Spermatogenesis …………………………………………….. 9

1.4.3 DNA Packaging in Sperm Cells ……………………………… 9

1.4.4 Sperm Glycocalyx …………………………………………… 9

1.5 Female DNA ………………………………………………………. 11

1.5.1 Epithelial Cells …………………………………………………. 11

1.6 Differential Extraction As We Know It………………………………………. 12
1.6.1 Chemistry……………………………………………………………….. 13
1.6.1.1 Selective Lysis ……………………………………………….. 13
1.6.1.2 Proteinase K ………………………………………………….. 15
1.6.2 Addressing The Issues ……………………………………………….. 15
1.6.3 Quantitation ……………………………………………………………….. 16
1.7 Temperature Controlled Differential Extraction (TCDE) ……………………. 17
1.7.1 Direct Lysis ...……………………………………………………………. 17
1.7.2 Enzymes…………………………………………………………………… 18
1.7.2.1 EA1……………………………………………………………… 19
1.7.2.2 Benzonase Nuclease………………………………………….. 19
1.7.2.3 AcroSolv Protease…………………………………………….. 19
1.7.3 Temperature …………………………………………………………………. 20
1.8 Cotton ……………………………………………………………………………. 21
1.8.1 Cellular Adhesion………………………………………………………. 23
1.8.2 Van der Waal’s Forces …………………………………………………. 23
1.9 Objectives ………………………………………………………………………. 24
2. MATERIALS AND METHODS ……………………………………………… 26
2.1 Sample Preparation ……………………………………………………………... 26
2.1.1 Neat Semen Preparation and Dilution ………………………………… 26
2.1.2 Semen Swabs ………………………………………………………………... 27

2.1.3 Vaginal and Buccal Swabs 27
2.1.4 Prepped Buccal Swabs 27
2.1.5 Mock Sexual-Assault Swabs 28
2.2 Quantitation 28
2.3 Incubation Modification 28
2.3.1 Various single source samples used with an enzyme cocktail 28
2.3.1.1 Sample Treatment 28
2.3.1.2 Sperm Fraction Extraction 31
2.3.1.3 Material Fraction Extraction 31
2.3.2 Various single source samples used with an enzyme cocktail: additional donors 32
2.3.2.1 Sample Treatment 32
2.3.2.2 Material Fraction Extraction 34
2.3.2.3 Microscope Slide Preparation 35
2.3.3 Various semen swabs used with enzyme cocktail 35
2.3.3.1 Sample Treatment 35
2.3.3.2 Sperm Fraction Extraction 36
2.3.3.3 Material Fraction Extraction 36
2.3.3.4 Microscope Slide Preparation 37
2.4 Modified TCDE Protocol 37
2.4.1 Master-Mix Preparation 37

2.4.2 Epithelial Cell Lysis …… 39

2.4.3 Material Fraction Lysis …… 40

2.4.4 Sperm Fraction Lysis …… 40

2.4.5 Epithelial Fraction Enzyme Inactivation …… 41

2.5 DNA Amplification and CE Separation …… 41

3. RESULTS …… 42

3.1 Various single source samples used with an enzyme cocktail …… 42

3.2 Various single source samples used with an enzyme cocktail: additional donors …… 43

3.3 Various semen swabs used with enzyme cocktail …… 45

3.4 Modified TCDE Protocol: Trial 1…… 46

3.5 Modified TCDE Protocol: Trial 2 …… 46

4. DISCUSSION …… 59

4.1 Various single source samples used with an enzyme cocktail …… 59

4.2 Various single source samples used with an enzyme cocktail: additional donors …… 60

4.3 Various semen swabs used with enzyme cocktail …… 62

4.4 Modified TCDE Protocol: Trial 1 …… 62

4.5 Modified TCDE Protocol: Trial 2 …… 63

5. CONCLUSIONS …… 67

APPENDIX …… 70

JOURNAL ABBREVIATIONS ………………………………………………………… 71

BIBLIOGRAPHY ……………………………………………………………….. 72

CURRICULUM VITAE ………………………………………………………….. 80

LIST OF TABLES

Table 1. TCDE Master-Mix Preparation …………………………..………….….... 39

Table 2. Allele Table of Mixed Samples …………………………………………… 50

Table 3. Sample Name Nomenclature ……………………………………………… 70

LIST OF FIGURES

Figure 1. TCDE Protocol 37

Figure 2. Percent recovery of the female DNA.. 42

Figure 3. Percent recovery of the male DNA 43

Figure 4. Percent recovery of the female DNA 44

Figure 5. Percent recovery of the male DNA 45

Figure 6. Percent recovery of the male DNA 46

Figure 7. DNA Recovery by Fraction... 48

Figure 8. STR Profile of Mixed Sample XY-I 51

LIST OF ABBREVIATIONS

CDC	Center for Disease Control and Prevention
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EA1	*Erebus antarctica* 1
ECM	Extracellular Matrix
EF	Epithelial Fraction
GPI	Glycophosphatidylinositol
MF	Material Fraction
NCVS	National Crime Victimization Survey
NISVS	National Intimate Partner and Sexual Violence Survey
PCR	Polymerase Chain Reaction
PK	Proteinase K
qPCR	Quantitative Polymerase Chain Reaction
RFU	Relative fluorescent unit
SAECK	Sexual Assault Evidence Collection Kit
SDS	Sodium dodecyl sulfate
SF	Sperm Fraction
STR	Short Tandem Repeat
TCDE	Temperature-Controlled Differential Extraction
TE	Tris- Ethylenediaminetetraacetic acid

1. INTRODUCTION

1.1 DNA in Forensic Science

DNA use in forensic science is a powerful tool for individual identification. Everyone's DNA is unique, excluding identical twins, which allows forensic scientists to develop identifying DNA profiles that can link biological evidence from crime scenes to individuals [1]. This high level of precision helps establish or refute connections between suspects and crime scenes, while also enabling the exclusion of innocent individuals. DNA evidence can also be revisited with advances in technology, providing opportunities to solve cold cases long after the original investigation [2]. Forensic DNA analysis is also used for paternity and genetic genealogical testing, which can be crucial in missing person's cases or inheritance disputes. By comparing DNA profiles with national and international databases, forensic experts can identify unknown victims and/or link evidence to known criminals [1]. The reliability of standardized techniques, such as PCR analysis of STR loci further underscores DNA's critical role in forensic science, supporting accurate identification and justice [2–4].

1.2 The Vulnerability of DNA in Forensics

Forensic samples are particularly susceptible to degradation due to several factors related to their nature and the conditions the samples have encountered [5–8]. Biological samples, especially those collected from crime scenes, may have been exposed to varying temperatures. High temperatures can accelerate the breakdown of DNA through processes such as hydrolysis and oxidation, while extreme cold can lead to physical damage to the samples [5,9]. Excessive moisture can facilitate the growth of

microorganisms such as bacteria and fungi, which produce enzymes that degrade proteins, lipids, and nucleic acids, contributing to the decomposition of biological material and loss of DNA quality [10–12]. Post-mortem, autolysis and putrefaction break down tissues and cells, leading to the loss of DNA and other forensic evidence [10–12]. Exposure to ultraviolet (UV) radiation can cause direct damage to DNA molecules, leading to the formation of pyrimidine dimers and other lesions that effect the integrity of the genetic material [11–13].

Forensic samples can be physically damaged during collection, handling, or transportation. Samples are often presenting miniscule quantities due to the nature of the crime scene or the difficulty in obtaining larger samples [10,14,15]. Small samples are inherently more challenging to analyze and are more susceptible to degradation. Forensic samples can be exposed to chemicals such as cleaning agents, pesticides, or other substances that can cause chemical degradation of DNA and/or interfere with the extraction and analysis processes [10].

Different types of tissues have varying levels of resilience to degradation. For example, bone and teeth preserve DNA better than soft tissues like blood or saliva, which are more prone to rapid decomposition [10,11]. The extent at which DNA is degraded depends on the time elapsed since the sample was deposited and the conditions it has been exposed to. Older or poorly preserved samples will typically be more degraded, making analysis more difficult [10,12]. Proper collection, preservation, and handling techniques are essential to minimize degradation and ensure the integrity of the forensic evidence.

1.3 Sexual Assault

In the United States, some form of sexual assault effects approximately 1 in every 3 women and 1 in every 6 men in their lifetime [16]. The National Crime Victimization Survey (NCVS 2023), which collects data on crime victimization, reports that approximately 0.7% of females aged 12 or older experienced a sexual assault in the past year [17]. The Centers for Disease Control and Prevention (CDC) National Intimate Partner and Sexual Violence Survey (NISVS) reveals that about 1 in 5 women and 1 in 71 men have been raped at some point in their lifetime [16,18]. Sexual assault is significantly underreported, with many survivors not coming forward due to fear, stigma, or distrust in the system [16]. Studies suggest that only a fraction of sexual assaults are reported to law enforcement, which means the actual prevalence is higher than reported figures [16]. Due to the increasingly high volume of cases, crime labs have built up backlogs of sexual assault kits that need examined [16].

In cases of sexual assault, forensic samples frequently comprise mixtures of DNA from several people, including the victim and the offender(s). These mixtures can hinder the analysis, as an optimal analysis would involve separating the DNA from the various individuals and interpreting it accurately [19–21]. Forensic scientists employ specialized lab techniques to segregate cell types in mixed samples, such as differential extraction, which differentiates sperm cells from other cells present in the sample [22,23]. Advanced DNA profiling techniques, like short tandem repeat (STR) analysis and probabilistic genotyping, assist in differentiating between the DNA profiles of the victim and the suspect, even when the sample contains various sources of genetic material [24,25]. The

ability to analyze and interpret these complex mixtures is crucial for establishing connections between individuals and the crime scene and may provide information to corroborate the victim's account [19].

1.3.1 Sexual Assault Evidence Collection Kits (SAECK)

Sexual assault kits, also known as forensic rape kits or sexual assault evidence collection kits (SAECK), are designed to collect and preserve evidence and are used in the investigation of sexual assault cases [26]. Contents of sexual assault kits usually include swabs, microscope slides, collection containers, documentation forms, and additional packaging for clothing items. Swabs can be collected vaginally, anally, and/or orally, as well as from locations where contact was presumed to have occurred. The swabs are used to collect biological samples from the victim's body to identify any potential semen, saliva, or other bodily fluids. Samples on microscope slides are used to examine biological samples for evidence of sperm cells. Vials and envelopes provided can be used to separately store swabs, slides, and other collected samples to prevent contamination. Medical History Forms document injuries and other pertinent details of the assault. Evidence bags are used to collect and preserve the victim's clothing and other items of evidence [26].

The collection kits are used by trained medical professionals, typically in a hospital, to collect and preserve evidence from the victim's body and clothing. The process is conducted with sensitivity and care to minimize trauma for the survivor while ensuring proper documentation and handling of evidence. Each step of the process, from

evidence collection to submission to law enforcement, is documented to maintain the chain of custody and ensure the evidence is admissible in court. The collected evidence is sent to a forensic laboratory, where it is analyzed for body fluids and any other probative information. Once the presence of body fluids is confirmed, the evidence can be sent for DNA analysis.

Sexual assault evidence often contains DNA from both the victim and the assailant, creating mixed DNA samples [26]. Techniques such as differential extraction are employed to separate male DNA from female DNA, which is crucial for most effectively resolving these mixtures and achieving optimal results [27]. Proper use and timely analysis of sexual assault kits can help address the backlog of untested kits and enhancing overall case resolution.

1.3.2 Separating Male and Female DNA

In cases of sexual assault, the forensic analyst's objective is to identify the non-victim DNA profile(s) present in the biological evidence [26]. Since these samples typically consist of mixtures of both male and female DNA, the goal is to distinguish the two. A method known as “Differential Extraction” was developed by leveraging the differences in chemical and physical structures of sperm cells and epithelial cells, which allows for the separation of the mixture into a female fraction and a male fraction [1,28]. The mixture undergoes digestion with a protease that breaks down epithelial cells while keeping the sperm cells whole [22,29,30]. This solution is centrifuged, causing the sperm cells to form a pellet, with the lysed epithelial cells remaining in the supernatant [12,29].

The supernatant is subsequently discarded, resulting in the female fraction. The sperm pellet serves as the starting point for the male fraction. At this stage, the sperm pellet can be further processed using dithiothreitol (DTT) in conjunction with a protease to lyse the sperm cells [25,28]. Ideally, after both fractions have been lysed, two completely separated DNA profiles will be obtained for examination [31,32]. The differentiation of male DNA from female DNA aids in the presentation of evidence during court proceedings.

If a mixture of DNA from two individuals is still present in the sperm fraction, the amount of female and male DNA present in the mixture has been shown to significantly impact the deconvolution process. When the male to female DNA ratio is approximately one to one, separating the two profiles is more complex. Alleles of both profiles may be shared at some loci, making it challenging to differentiate between them [25]. This complexity requires advanced analytical techniques and careful interpretation to accurately separate and identify each of the two DNA profiles [25,26,33]. Advanced methods such as probabilistic genotyping have been developed to use statistics to aid in profile deconvolution. In cases where a two-person mixture contains a single major DNA source and a minor contributor, the deconvolution process is more straightforward [26,28,33]. The dominant profile can be identified with greater confidence, and the minor profile can be extracted with less interference. This could also be considered an issue when the minor contributor is the profile in question. When a large amount of female DNA is present on evidence such as vaginal swabs from a SAECK, the male profile can

be masked by the female profile [25,26,28]. Good separation of sperm and epithelial cells ensures that each DNA profile can be attributed to the correct individual.

1.3.3 Recent Advancements in SAECK Processing

Recent advancements in DNA recovery from sexual assault kits include improvements in extraction techniques, increased sensitivity of quantification methods sensitivity of amplification kits, and standardized protocols. These developments collectively enhance the ability to recover and analyze DNA from challenging samples, leading to more accurate and reliable forensic results.

Recent refinements in differential extraction methods have targeted better separation of male and female DNA from mixed samples [26,27,36]. Enhanced protocols now include more selective lysis buffers and optimized centrifugation techniques to increase the efficiency of separating sperm from non-sperm cells. New lysis solutions with improved chemical compositions can better disrupt cell membranes and release DNA from challenging samples, such as degraded or small quantities [20,27,36–38]. These solutions often include novel detergents and enzymes that are more effective in breaking down cellular components while preserving DNA [27,36,39].

Advances in qPCR technology allow for precise quantification of male and female DNA from minute DNA samples, which are often seen in forensic samples [14,15,40]. This technique can assess the quality and quantity of DNA extracted from sexual assault samples, thus guiding further analysis and ensuring that sufficient DNA is available for

profiling. Automation in forensic laboratories has streamlined the processing of sexual assault kits. Robotic systems can handle multiple samples simultaneously, reducing human error and increasing throughput [41]. Automated systems also ensure consistent application of extraction protocols [41]. Additionally, advances in sample preservation and storage techniques help maintain DNA integrity over time. New storage methods and conditions have been developed to reduce the degradation of DNA in sexual assault kits, ensuring that samples remain viable for analysis [10].

New software tools have been developed to better interpret DNA mixtures. These tools use advanced algorithms and statistical models to separate mixed DNA profiles, making it easier to identify contributors and obtain reliable forensic results [25].

1.4 Male DNA

1.4.1 Sperm Cell Structure

With the primary goal of successful fertilization, the structure of sperm cells has intricate chemical and physical properties. The head contains a nucleus with highly condensed DNA, compacted by protamines, and an acrosome filled with enzymes such as hyaluronidase and acrosin, facilitating the penetration of the egg [38,44]. The midpiece is abundant in mitochondria, which produce (adenosine triphosphate) ATP to power the sperm’s movement. The tail, or flagellum, contains an axoneme with a 9+2 microtubule arrangement, pivotal for its whip-like motion, supported by a fibrous sheath [7,45,46]. The plasma membrane of the sperm cell contains specific lipids and proteins that play

key roles in egg binding and fusion [47–49]. This structural and chemical composition impacts the sperm's efficiency in reaching and fertilizing the egg.

1.4.2 Spermatogenesis

Spermatogenesis is the process by which sperm cells are produced in the seminiferous tubules of the testes [9,45,50]. It starts with spermatogonia, which are diploid germ cells found in the basal layer of the tubules. The spermatogonia undergo mitotic division to create additional spermatogonia and primary spermatocytes [9,23,45]. Each primary spermatocyte undergoes meiosis I, resulting in two secondary spermatocytes, which are haploid cells. These secondary spermatocytes then progress through meiosis II to generate spermatids [45]. Throughout spermatogenesis, spermatids experience considerable morphological changes, evolving into elongated, mature sperm cells [45,46]. This ongoing and hormonally controlled process guarantees the consistent production of sperm essential for reproduction.

1.4.3 DNA Packaging in Sperm Cells

In the last stages of spermatogenesis, DNA is tightly bound to protamines instead of histones. The DNA protamine structure results in a reduction of the overall volume of the chromatin compared to with DNA packaging in somatic cells [9,50,51]. Within the sperm head, this densely packaged chromatin not only keeps the genetic material protected, but helps optimize the overall shape of the cell, which is important for motility

and fertilization [45]. The acrosomal cap, located on the front of the sperm head, contains enzymes that function to penetrate the egg’s outer layers during fertilization [45].

1.4.4 Sperm Glycocalyx

Human sperm cells are encased in a thick layer outside of the lipid bilayer membrane called the glycocalyx, which plays a large role in how spermatozoa survive in the female reproductive tract [48,52–54].

The glycocalyx of sperm cells is a glycoprotein and glycolipid coating that covers the outer surface of the sperm cell membrane [47,48,52]. This complex, carbohydrate-rich layer plays several vital roles in sperm function and protection [54,55]. It acts as a protective barrier against environmental stresses and potential damage, helping to maintain sperm viability and function within the female reproductive tract [47,52]. Additionally, the glycocalyx is essential for successful fertilization by containing molecules that facilitate the sperm's recognition and binding to the egg's zona pellucida [47,54,55]. Furthermore, the glycocalyx helps sperm evade the female reproductive tract's immune system by masking sperm antigens, thereby reducing the likelihood of an immune response [48].

The glycocalyx also contributes to the difficulty of lysing sperm cells, which presents challenges in DNA extraction [47,52,56]. Its thick, protective layers from 20-60nm acts as a physical barrier, shielding the underlying sperm cell membrane and making it more difficult for lysis agents to penetrate and disrupt the cell [48,52]. A high cholesterol content and presence of other stabilizing components contribute to the overall

strength of the sperm cell membrane [47,48,52]. Together, these features make sperm cells particularly resilient to lysis, introducing challenges for forensic DNA extraction methods while highlighting their robustness in adverse conditions.

1.5 Female DNA

1.5.1 Epithelial Cells: Vaginal vs. Buccal

Studies have shown that vaginal epithelial cells and buccal epithelial cells are similar in structure and reaction to lysis chemistries [57]. Vaginal and buccal cells share several important similarities, despite originating from different body regions [57,58]. Both types of cells are epithelial in nature, derived from epithelial tissue that lines various surfaces and cavities within the body [57]. Vaginal cells are part of the mucosal lining of the vagina, while buccal cells are found in the oral mucosa lining the inner cheek [22,30,57]. As epithelial cells, both types serve a protective role, forming layers that cover and protect underlying tissues [57]. Structurally, vaginal and buccal cells are quite similar and exhibit characteristics typical of epithelial cells under microscopic examination [57,58]. Both types of cells are non-keratinized, contributing to their flexibility and suitability for their respective environments [57,58].

In forensic science, both vaginal and buccal cells are routinely collected using cotton swabs and are valuable for DNA analysis [22,57]. Vaginal cells are often collected in sexual assault investigations when vaginal swabs are taken to identify sperm cell DNA, while buccal cells are frequently obtained via cheek swabs for DNA profiling in

identification and genetic testing [57,58]. The process for extracting DNA from these cells involves similar techniques, highlighting their utility and comparability in forensic contexts.

1.6 Differential Extraction as We Know It

Differential extraction refers to and involves separating different cell types before lysis, making it particularly useful for forensic cases where the samples often contain cells from a victim and a perpetrator [22,26]. Differential extraction was developed in the 1980s as a specialized method for separating sperm cells from other cells in sexual assault evidence, which often contains mixed DNA from the victim and one or more sperm donors [22,26,34]. This technique was initially pioneered to address the challenge of analyzing these complex mixtures by using differential extraction methods [26,34,37]. The process involves lysing the non-sperm cells, which are less resistant, while preserving the sperm cells, which are more resistant to the lysis conditions [26,27,37,38]. This separation allows forensic scientists to isolate sperm DNA and perform accurate DNA profiling, thereby aiding in the identification of sperm donors [22,24,59].

Over time, differential extraction has evolved with advancements in technology and methodology. Modern improvements include the use of more refined and automated techniques for better separation and the integration of sophisticated DNA analysis methods, such as quantitative PCR (qPCR) and advanced STR profiling [24,60,61]. These enhancements have increased the efficiency and accuracy of extracting and analyzing mixed DNA samples, even when dealing with small or degraded samples

[24,28,37]. The evolution of differential extraction reflects ongoing efforts to improve forensic analysis and address the complexities of sexual assault evidence with greater precision and reliability.

1.6.1 Chemistry

The differential extraction process utilizes the differences in the physical and chemical properties of various cell types. For example, in sexual assault cases, the goal is to lyse non-sperm cells (such as epithelial cells) while leaving sperm cells intact [26,27].

Differential extraction uses lysis buffers that contain detergents, such as sodium dodecyl sulfate (SDS) or Triton X-100 [12,62]. These detergents disrupt cell membranes by disrupting the phospholipids and proteins that maintain the cell's structural integrity, releasing DNA into the supernatant [34,63]. Detergents used in a differential extraction are specifically chosen to lyse one type of cell while leaving another type intact, without affecting downstream processes such as PCR [12,29,62]. SDS is a common detergent used in extraction and purification methods to lyse non-sperm cells, but has been found to be a PCR inhibitor, which is not optimal in direct lysis procedures [29,64].

Enzymes such as proteases are included in lysis solutions to break down cellular proteins and membranes that might otherwise interfere with DNA extraction. They are selected to avoid excessive degradation of the DNA itself while separating the DNA from the other cellular material [65,66]. Enzymes can be targeted to break down specific cells. Proteinase K is a common protease used to lyse non-sperm cells in combination with a

detergent. DTT is commonly added to lyse the more resistant sperm cells [8,65,67]. The membrane of sperm cells is enriched with cholesterol and glycoproteins that contribute to its stability and robustness [48,52,68].

After incubation in lysis buffers, samples are typically subjected to centrifugation. This process separates the lysed cell debris from intact cells based on their size and density [27,68]. Non-sperm cells that have been lysed and broken down into smaller fragments can be separated from the intact sperm cells, which form a distinct pellet [29,65,69]. Once the cell types are separated, DNA is extracted from the intact sperm cells and is proceeded with further purification and analysis [22,38,52,70]. The isolated DNA is then quantified and analyzed using techniques such as PCR and STR profiling.

1.6.1.1 Selective Lysis

Different cell types have varying levels of resistance to enzymatic digestion due to differences in their cell wall or membrane compositions [48,52]. This variability requires careful selection and optimization of enzymes to achieve effective lysis without carry-over of DNA from other cell types [27,65,66].

Often, a combination of enzymes with other physical or chemical techniques (e.g., mechanical disruption, chemical lysis) is used to optimize the differential extraction process. This might include a mix of proteases to target both general and cell-specific proteins. The conditions (e.g., pH, temperature) under which enzymes operate are carefully controlled to maximize their effectiveness [26,63]. For example, some enzymes may be more effective at specific temperatures or pH levels, which is optimized based on

the cell types being targeted [5,7,74]. New proteases with enhanced specificity for certain cell types are being developed and tested [62,67]. These innovations aim to improve the efficiency of differential extraction by targeting specific cellular components with greater precision.

1.6.1.2 Proteinase K

Proteinase K is a protease with a broad range of activity that breaks down proteins and is commonly utilized in the process of DNA extraction [8,52,67,69,73]. It efficiently degrades proteins found in both sperm and non-sperm cells, aiding in the liberation of DNA. In differential extraction, Proteinase K is often used in the initial stages to digest proteins and lyse non-sperm cells [29,65]. Its ability to work under a range of conditions makes it versatile for extracting DNA from mixed samples. Proteinase K is activated by calcium between 20-60°C and is irreversibly inactivated when heated to temperatures between 70-95°C, though complete inactivation may not be possible with heat-inactivation [65,67,69,73]. Proteinase K can be utilized in the extraction of genomic DNA due to its capability to degrade proteins and will also deactivate DNases and RNases that could potentially destroy a targeted sample of DNA or RNA [12,13]. Proteinase K is active with or without the presence of SDS and EDTA, but functions optimally with SDS [13,29,64,65].

1.6.2 Addressing the Issues

The optimization of cell recovery and fraction separation is critical in increasing the effectiveness of forensic analyses and improving the quality of evidence in sexual assault cases. The process of DNA extraction from sexual assault samples is fraught with difficulties, including the presence of low-level or degraded DNA, the complexity of mixed samples, and the potential for contamination. Traditional methods of DNA extraction and analysis, while effective to some extent, often fall short when dealing with the unique and challenging nature of sexual assault evidence [14,75]. Recent advancements in forensic science have highlighted the need for optimized protocols that can more efficiently recover DNA from swabs and accurately separate cellular fractions to obtain clear genetic profiles.

1.6.3 Quantitation

Quantitative PCR (qPCR) is used to quantify isolated DNA from forensic samples due to its high sensitivity, specificity, and real-time measurement capabilities [14,76,77]. This technique can detect and accurately measure very small amounts of DNA, which is necessary for forensic samples that may contain degraded or limited genetic material [14,77]. On method of qPCR employs Taqman probes that bind to specific DNA sequences, allowing for precise detection even at low DNA concentrations [14,40]. qPCR provides quantitative data by measuring fluorescence during each amplification cycle, reflecting the initial amount of DNA in the sample and assessing DNA degradation to determine suitability of the sample for further analysis [77,78]. By accurately quantifying

DNA, qPCR ensures that subsequent analyses, such as STR profiling or sequencing, are performed using optimal amounts of template DNA

qPCR allows prediction of the amount of male and female DNA and the presence of degradation [14,15,40,78]. The amount of female and male DNA in a mixture directly affects the complexity of the deconvolution process [25,60]. Larger discrepancies in DNA amounts simplify the interpretation, while equal or nearly equal proportions create more complex mixtures that require advanced analytical and statistical techniques to accurately separate and interpret [59,61,79]. If there is an overwhelming amount of female DNA, the detection of male DNA in a profile may be impossible [59,61,79].

1.7 Temperature Controlled Differential Extraction (TCDE)

The current established TCDE procedure uses optimal temperatures for three enzymes to control their activity in order to release cells from cotton swab and release DNA from non-sperm cells into supernatant. Any remaining DNA in solution over sperm pellet is removed with non-specific nuclease- Benzonase. DNA is released from sperm cells using a protease, AcroSolv. This results in non-sperm and sperm fractions, which amplify and separate into two individual profiles. The most up-to-date protocol uses a 15 minute incubation at 75°C during the initial lysis step to activate the *forensic*GEM protease.

1.7.1 Direct lysis

Direct lysis methods and other DNA extraction techniques differ significantly in their approaches and applications [80]. Direct lysis involves breaking open cells and releasing DNA directly into a solution using a lysis buffer containing detergents or enzymes [36,39]. These methods are advantageous due to speed and simplicity, as they allow for rapid processing of samples with minimal handling, which can help preserve DNA quality [36,37]. However, there are limitations, such as a higher risk of contamination from unwanted materials and reduced effectiveness when dealing with mixed DNA samples from multiple sources [37,39].

In contrast, other DNA extraction methods include organic solvent extractions, silica magnetic bead-based techniques. Organic solvent methods, like phenol-chloroform extraction, use solvents to separate DNA from proteins and other contaminants but require multiple steps and careful handling. Silica and magnetic bead-based methods offer high specificity and purity by binding DNA to silica columns or magnetic beads and then washing away contaminants. While these methods provide better purity and are more suited for complex or mixed samples, they are generally more complex, time-consuming, and expensive compared to direct lysis [36,39,64].

1.7.2 Enzymes

In current DNA extraction methods, proteinase K is used. Proteinase K, a protease that can break down proteins and lyse cells requires sodium dodecyl sulfate (SDS) for optimal function. SDS is a known PCR inhibitor, which is not an issue when purification steps follow the initial extraction to dispose of it, but this is not an option when dealing

with direct lysis procedures. Direct lysis procedures are designed to prepare samples to go directly to quantitation and amplification without additional purification steps. By bypassing the use of Proteinase K, SDS, and DTT, this direct lysis procedure is a quick and effective method for extracting PCR-ready DNA without additional purification steps.

1.7.2.1 EA1 Protease (forensicGEM™)

Bacillus sp. *Erebus antarctica* 1 (EA1), is a thermophilic serine protease included in the thermolysin family. At MicroGEM, EA1 is sold commercially as *forensic*GEM™ in the *forensic*GEM Universal kit. With an optimal activity of 75°C and pH 7.5, EA1 will break down cellular proteins and nucleases. When exposed to temperatures above 90°C, EA1 becomes irreversibly inactivated. Like Proteinase K (PK), EA1 requires the presence of calcium for activity [81,82]. Though PK and EA1 share similar protease properties, PK is not thermophilic and will start to inactivate around 70°C. PK has an optimal activity temperature between 50-56°C [81].

1.7.2.2 Benzonase® Nuclease

The endonuclease Benzonase® is derived from *Serratia marcescens*, a gram-negative bacterium [83,84]. It has an optimal activity temperature of 37°C and will degrade any nucleic acids present in the solution [83,84]. Additionally, Benzonase® can be completely digested by EA1. With approximately 81 cut sites on Benzonase®, EA1 will halt any nuclease activity once added to the solution [83,84]. It is important to note

that Benzonase® lacks the ability to degrade the male DNA while it remains in the non-lysed sperm cells, making this nuclease an ideal enzyme when cleaning up the sperm fraction [83,84].

1.7.2.3 AcroSolv Protease

AcroSolv® is reagent provided in the forensicGEM™ Sperm kit. Although the specific composition of AcroSolv® is confidential, it has been demonstrated to efficiently break down sperm cells when used with the correct buffer at 52°C [86]. The enzymes in AcroSolv® are most effective at a temperature of 52°C, where they break apart various cells, including sperm cells, and release DNA into the surrounding solution [86]. The reagent is provided in the form of a lyophilized powder that can be rehydrated to form a solution. AcroSolv® is included during the SF step of the procedure, and the forensicGEM™ reagent, which is active at 75°C, neutralizes AcroSolv® when the solution is heated to this higher temperature [86]. Once the TCDE protocol is completed, all enzymes are inactivated, making it possible to proceed with quantitation without needing a separate DNA purification step.

1.7.3 Temperature

Temperature can significantly affect the recovery of DNA from swabs, influencing both the preservation and extraction processes used in forensic analysis. Low temperatures can slow down biochemical and microbial activities that lead to DNA degradation, ensuring that samples remain stable until they can be analyzed [9]. Conversely, high temperatures accelerate DNA degradation, promoting chemical reactions and increasing enzymatic activity that can denature DNA.

During the extraction process, maintaining optimal temperature conditions for each step is essential for effective DNA recovery [23]. Extraction buffers and reagents often require specific temperatures to facilitate the efficient lysis of cells and release of DNA. Enzymes used in the extraction process, such as proteases, have ideal temperature ranges for optimal activity [9]. Fluctuations in temperature during these procedures can affect the consistency and yield of DNA, making stable and appropriate temperature management critical for reliable results. This can also be helpful in utilizing more than one enzyme to enact different functions.

Temperature has a significant impact on Van der Waals forces, which are weak, non-covalent interactions between molecules or surfaces. These forces are sensitive to temperature fluctuation as the thermal motion of molecules increases with temperature. Increased kinetic energy can disrupt the balance of Van der Waals interactions by making it more difficult for molecules to remain close enough for these weak forces to be effective [66]. Consequently, higher temperatures can weaken Van der Waals forces, causing molecules to drift apart as their vibrational and rotational energy surpasses the strength of these interactions. Conversely, lower temperatures reduce molecular motion, allowing Van der Waals forces to have a greater effect [66]. With decreased kinetic energy, molecules are more likely to stay closer together, enhancing the strength and effectiveness of Van der Waals interactions [66].

1.8 Cotton Swabs

Cotton swabs are highly effective for DNA collection due to several key properties. Their fibrous structure provides a large surface area contributing to the adherence of cells and DNA [75]. Additionally, cotton's porosity allows it to efficiently absorb biological fluids, including those containing DNA. The hydrophilic nature of cotton helps in soaking up water-based fluids like saliva, blood, and semen, ensuring that cells and DNA are captured along with the fluid. The softness of cotton swabs ensures gentle collection of samples [85]. Furthermore, cotton is chemically inert and free from substances that could inhibit DNA analysis, preserving the integrity of the genetic material [66]. These properties, combined with the ease of handling and use, make cotton swabs an ideal tool for forensic DNA collection.

However, extracting DNA from these swabs involves challenges due to the physical and chemical properties of cotton and the nature of the samples collected [66,87]. Cotton swabs are designed to be absorbent, which makes them effective for collecting biological material but also presents difficulties releasing the cells and DNA during extraction [38]. This can lead to incomplete or inefficient extraction, where not all the DNA collected is recovered, potentially impacting the quality and quantity of the DNA available for analysis [66,75,85,87]. Additionally, the cellulose fibers of cotton can bind with DNA, requiring the use of more aggressive chemical treatments or longer extraction times to ensure that the DNA is fully liberated from the swab [26,38,85].

Another complication arises from the potential presence of contaminants on the swab. Cotton swabs can pick up extraneous materials from the environment or from the surfaces they contact, which can interfere with DNA extraction and analysis [66,87].

Contaminants such as dirt, proteins, or other substances may co-extract with the DNA, complicating the interpretation of the results [66,75,87]. Furthermore, the presence of multiple types of biological material (e.g., saliva and blood) on a single swab can create a mixed DNA profile, making it more challenging to differentiate between the contributors and accurately interpret the DNA evidence [26,75,87].

1.8.1 Mechanisms of Cellular Adhesion

Cellular adhesion primarily involves interactions between cell surface molecules and substrates, which can include extracellular matrix proteins, other cells, or surfaces like cotton swabs [88, 89]. Cells have specialized receptors on their surface, such as integrins, cadherins, and selectins. These receptors bind to specific ligands on the substrate or other cells, facilitating adhesion. Integrins, for example, mediate interactions between cells and extracellular matrix proteins, such as collagen and fibronectin [90, 91]. Adhesion molecules, like cadherins and immunoglobulin superfamily members, play a role in cell-cell adhesion. They create homophilic or heterophilic connections, aiding cells in adhering to each other or to surfaces. The extracellular matrix (ECM) offers a framework for cells, as its elements engage with cell surface receptors to facilitate adhesion [92, 93]. ECM proteins like fibronectin, laminin, and collagen attach to receptors on cell membranes, securing cells to the matrix.

1.8.2 Van der Waals Forces and Cotton Swabs

Van der Waals forces are feeble, non-covalent interactions that take place between molecules or surfaces as a result of temporary dipoles [94]. These forces also play a key role in cellular adhesion, especially in the context of adhesion to surfaces like cotton swabs [95]. Van der Waals forces arise from the interactions between fluctuating dipoles in adjacent molecules or surfaces. Though individually weak, these forces can collectively contribute to the overall adhesive strength [89]. They are important for the initial stages of adhesion, where they help cells or molecules come into proximity to the surface [88].

Cotton swabs are made of fibrous material, which presents a large surface area with numerous sites for interaction. The fibrous structure of cotton increases the surface area for Van der Waals interactions between the cell surface molecules and the cotton fibers [66]. As cells meet the cotton swab, Van der Waals forces help stabilize the initial attachment before more specific, stronger adhesion mechanisms take over. While Van der Waals forces contribute to initial adhesion, they are often complemented by other interactions, such as electrostatic forces, hydrogen bonding, and specific receptor-ligand interactions [95]. On a cotton swab, these forces work together to ensure that cells adhere firmly and are retained on the swab, which is necessary for effective sample collection. Understanding these forces is important for optimizing DNA extraction processes as they need to be broken to release the adhered DNA.

1.9. Objectives

The aims of this research were to optimize a temperature-controlled differential extraction method to yield more total DNA and a better separation between female and male DNA when extracting mixed samples from cotton swabs. The “temperature-controlled differential extraction” technique created by the Cotton research lab in the last decade takes advantage of the physical and chemical characteristics of different enzymes to extract and separate blended samples more rapidly and efficiently than the existing differential extraction protocol. These experiments explored whether a longer initial incubation time would yield more DNA from the cotton swabs in both single source and mixed samples. Samples were intended to replicate those discovered as biological evidence at a crime scene, emphasizing swabs located in sexual assault kits. With a backlog of sexual assault kits in queue to be processed across the U.S., a quicker, more efficient method is necessary to keep up with the case load and provide results in a timely manner.

2. MATERIALS AND METHODS

2.1 Sample preparation

2.1.1 Neat Semen Preparation and Dilution

One commercially acquired aliquot of semen from a single donor served as the source of male DNA for the experiments outlined below. Homogenization of the neat semen through dilutions and sample preparation was carefully performed to ensure, as much as possible, an equal quantity for each sample. The neat semen was initially gently mixed by inverting and flicking the tube 10 times. The neat semen was gradually pipetted up and down 15-20 times. Thirty microliters of the homogenized neat stock were transferred into a clean 1.5mL tube and incubated on a ThermoMixer (ThermoFisher, Waltham, MA) for 30 minutes at 37°C and at 250rpm. When the incubation was complete, 1470µL of TE (Tris-EDTA) buffer was added to the 30µL aliquot to create a dilution of 1:50. The tube was gently inverted and flicked 10 times to mix, and the solution was again carefully pipetted up and down 15-20 times. The dilution was subsequently incubated for another 30 minutes at 37°C and 250 rpm on a ThermoMixer. Before the addition to new tubes, gentle inversion of the semen tube, flicking 10 times, and slowly pipetting up and down 15-20 times was performed once more. The 1:50 solution was split into two 10 µL aliquots, which were transferred into new sterile 0.5 mL tubes containing 2 µL of forensicGEM™ Sperm, 68 µL of deionized (DI) water, 10 µL of AcroSolv, and 10 µL of 10X Red Buffer. The samples were vortexed, placed in a Thermal Cycler, and incubated at 52°C for 10 minutes, 75°C for 6 minutes, and 95°C for

3 minutes. The samples were then quantified using qPCR with the Quantifiler Trio™ DNA Quantification Kit and the 7500 Real-Time PCR instrument.

2.1.2 Semen Swabs

The concentration of the 1:50 semen dilution was determined to be 4.89 ng/µL. According to the targeted DNA mass, an exact volume of the diluted semen was applied straight to the tips of sterile cotton swabs and was permitted to dry at room temperature.

2.1.3. Vaginal and Buccal Swabs

Using a sterile scalpel, the vaginal swabs were cut into halves.

2.1.4 Prepped Buccal Swabs

One anonymous donor provided a liquid saliva sample. Using a centrifuge, the cells were pelleted and resuspended in 675µL Tris-EDTA buffer. An appropriate amount of this cell suspension was applied to sterile cotton swabs, based on the required DNA mass, and the swabs were allowed to dry at room temperature.

2.1.5. Mock Post-Coital Swab Preparation

The vaginal swabs were cut into halves using a sterile scalpel. Using 1.5mL tubes, 20.4µL of approximately 100ng of DNA in the diluted semen solution was added to the bottom. Using sterile tweezers, each half of a vaginal swab was gently twisted around the

inside the tube to absorb the semen solution. The swabs were taken out and allowed to dry at room temperature for a minimum of 24 hours before use.

2.2 Quantitation

Following the manufacturer's guidelines, each sample was quantified using quantitative polymerase chain reaction (qPCR) with the Quantifiler™ Trio DNA Quantification Kit and the 7500 Real-Time PCR instrument. Using the HID Real-Time PCR Analysis Software v1, a virtual standard curve was used. Each sample was run in duplicate, with final quantitation values being averaged. In mixed samples, the amount of female DNA was estimated by subtracting the Y-autosomal quantitation value from the small autosomal quantitation value.

2.3 Longer Incubation at a Higher Temperature

2.3.1 Various single source samples used with an enzyme cocktail

2.3.1.1 Sample Treatment

The prepared semen swabs containing approximately 100ng of DNA on their tips were handled using the protocol outlined below. Four swab tips were each positioned in a 2mL microcentrifuge tube. To each tube, 3µl of forensicGEM™, 30µl of 10X Buffer Blue, and 267µl of DI water were added. A reagent blank was initiated at this step, incorporating 270µl DI water and 30µl of 10X Buffer Blue. In a thermomixer, the samples were incubated at 75℃ for 1 hour at 400 rpm, and then 95℃ for 10 minutes and 4℃ for 10 minutes without agitation. The wet swabs were transferred into a mesh spin

basket inside a fresh 2.0 mL tube using sterile forceps. Using a centrifuge, the samples were spun down at 14,000 rpm for 2 minutes to eliminate all liquid from the swabs. The entirety of eluate from each swab was transferred back into the 0.5mL sample tube, and the dry swab was set aside in a separate 0.5mL tube designated for MF extraction. All but 100µL of the supernatant was discarded into a new microcentrifuge tube marked “YEF-#” (containing lysed epithelial cells). The 100µL and sperm pellet were subsequently resuspended and labeled “YSF-#".

The direct buccal swabs digested with forensicGEM™ were prepared in the following manner. Using a sterile scalpel, four halves from two swabs were cut and each placed in a 2mL microcentrifuge tube. To each tube, 3 µL of forensicGEM™, 267 µL of DI water, and 30 µL of 10X Buffer Blue were added. A reagent blank was created at this point using 30 µL of 10X Buffer Blue and 270 µL of DI water. In a thermomixer, the samples were incubated at 75ºC for 1 hour at 400 rpm, and then 95ºC for 10 minutes and 4ºC for 10 minutes without agitation. The wet swabs were transferred into a mesh spin basket inside a fresh 2.0 mL tube using sterile forceps. Using a centrifuge, the samples were spun down at 14,000 rpm for 2 minutes to eliminate all liquid from the swabs. The eluate from each swab was returned to the 0.5mL sample tube, and the spun-down swab was set aside in a separate 0.5 mL tube for MF extraction. The tubes holding the liquid were labeled "DEF-#" (containing lysed epithelial cells).

Prepped Buccal swabs, derived from saliva cells and containing forensicGEM™ digest, were processed using the following protocol. Four swab tips were placed into a 2mL microcentrifuge tube each. To every tube, 3µl of forensicGEM™, 267µl of DI

water, and 30µl of 10X Buffer Blue were added. A reagent blank was initiated at this stage, consisting of 270µl DI water and 30µl of 10X Buffer Blue. In a thermomixer, the samples were incubated at 75ºC for 1 hour at 400 rpm, and then 95ºC for 10 minutes and 4ºC for 10 minutes without agitation. The wet swabs were transferred into a mesh spin basket inside a fresh 2.0 mL tube using sterile forceps. A centrifuge was used to spin the samples at 14,000 rpm for 2 minutes to eliminate all fluid from the swabs. The eluate from each swab was returned to the original 0.5mL sample tube, while the spun swab was set aside in a different 0.5mL tube for MF extraction. The tubes containing the liquid were marked "PEF-#" (encompassing lysed epithelial cells).

Vaginal swabs with forensicGEM™ digest were processed using the following protocol. Using a sterile scalpel, four halves from four different donor swabs were cut and placed individually in a 2mL microcentrifuge tube. To each tube: 3µl of forensicGEM™, 267µl of DI water, and 30µl of 10X Buffer Blue were added. A reagent blank was created at this step, containing 30µl of 10X Buffer Blue and 270µl DI water (the limited availability of forensicGEM™ accounted for the absence of it in the reagent blank). In a thermomixer, the samples were incubated at 75ºC for 1 hour at 400 rpm, and then 95ºC for 10 minutes and 4ºC for 10 minutes without agitation. The wet swabs were transferred into a mesh spin basket inside a fresh 2.0 mL tube using sterile forceps. Using a centrifuge, the samples were spun-down at 14,000 rpm for 2 minutes. The eluate from each swab was transferred back into the 0.5 mL sample tube, while the spun-down swab was set aside into a separate 0.5 mL tube for MF extraction. The tubes with the liquid were labeled "XEF-#" (containing lysed epithelial cells).

2.3.1.2 Sperm Fraction Extraction

In new 0.5 mL tubes, 34 µL of DI water, 5 µL of Acrosolv, 5 µL of 10X Red Buffer, 1 µL of forensicGEM™ Sperm, and 5 µL of the resuspended pellet were added for each sample. The samples were then vortexed, placed in a Thermal Cycler, and incubated at 52°C for 10 minutes, 75°C for 6 minutes, and 95°C for 3 minutes.

2.3.1.3 Material Fraction Extraction

For each epithelial swab sample (vaginal and buccal), the spun-down swab was placed into a clean 0.5mL tube containing 178 µL of DI water, 2 µL of forensicGEM™, and 20 µL of 10X Blue Buffer. Using a Thermal Cycler, the samples were incubated at 75°C for 5 minutes, followed by 95°C for 5 minutes.

For each semen swab sample, the spun-down swab was transferred into a 0.5 mL tube containing 4 µL of forensicGEM™ Sperm , 20 µL of 10X Red Buffer, 20 µL of AcroSolv , and 156 µL of DI water. The samples were vortexed, placed in a Thermal Cycler, and incubated at 52°C for 10 minutes, 75°C for 6 minutes, and 95°C for 3 minutes. The wet swabs were transferred into a mesh spin basket inside a fresh 2.0 mL tube using sterile forceps. Using a centrifuge, the samples were spun-down at 14,000 rpm for 2 minutes. The eluate from each swab was transferred back into the original 0.5mL MF sample tube.

2.3.2 Various single source samples used with an enzyme cocktail: additional donors

2.3.2.1 Sample Treatment

Semen swabs treated with forensicGEM™ digest were processed using the protocol outlined below. Six individual swab tips from the prepared semen swabs were placed into separate 2mL microcentrifuge tubes. In each tube, 267µl of DI water, 3µl of forensicGEM™, and 30µl of 10X Buffer Blue were added. A reagent blank started at this point, consisting of 30µl of 10X Buffer Blue and 270µl DI water. In a thermomixer, the samples were incubated at 75ºC for 1 hour at 400 rpm, and then 95ºC for 10 minutes and 4ºC for 10 minutes without agitation. The wet swabs were transferred into a mesh spin basket inside a fresh 2.0 mL tube using sterile forceps. Using a centrifuge, the samples were spun-down at 14,000 rpm for 2 minutes to eliminate all liquid from the swabs. The spun-down swab was placed into a different 0.5 mL tube labeled and put aside for MF extraction. The eluate from each swab was put back into the original 0.5mL sample tube. Using a centrifuge, the samples were spun-down at 14,000 rpm for 5 minutes. The supernatant was transferred into a new microcentrifuge tube labeled "YEF-#" (containing lysed epithelial cells), leaving about 100µL of the supernatant with the pellet. The remaining 100µL and the sperm pellet were resuspended and marked as the "sperm fraction".

Buccal swabs treated with forensicGEM™ digest were processed according to the following protocol. Using a sterile scalpel, both halves of a single swab collected from three distinct donors were placed into individual 2 mL microcentrifuge tubes (six halves in total). In each tube, 3µl of forensicGEM™, 267µl of DI water, and 30µl of 10X Buffer Blue were added. A reagent blank was created at this step, consisting of 30µl of 10X

Buffer Blue and 270ul DI water. In a thermomixer, the samples were incubated at 75ºC for 1 hour at 400 rpm, and then 95ºC for 10 minutes and 4ºC for 10 minutes without agitation. The wet swabs were transferred into a mesh spin basket inside a fresh 2.0 mL tube using sterile forceps. Using a centrifuge, the samples were spun-down at 14,000 rpm for 2 minutes. The spun-down swabs were kept in separate 0.5 mL tubes designated for MF extraction, while the eluate from each swab was transferred back into the original 0.5 mL EF sample tube. The tubes containing the liquid were labeled "DEF-#" (containing lysed epithelial cells).

Vaginal swabs with forensicGEM™ digest were processed according to the following protocol. Using a sterile scalpel, one half from four different donor vaginal swabs were placed into 2mL microcentrifuge tubes (four in total). To each tube, 3µl of forensicGEM™, 267µl of DI water, and 30µl of 10X Buffer Blue were added. A reagent blank was initiated at this step, which included 30µl of 10X Buffer Blue and 270µl DI water. In a thermomixer, the samples were incubated at 75ºC for 1 hour at 400 rpm, and then 95ºC for 10 minutes and 4ºC for 10 minutes without agitation. The wet swabs were transferred into a mesh spin basket inside a fresh 2.0 mL tube using sterile forceps. Using a centrifuge, the samples were spun-down at 14,000 rpm for 2 minutes. The spun-down swabs were kept in separate 0.5 mL tubes designated for MF extraction, while the eluate from each swab was transferred back into the original 0.5 mL EF sample tube. The liquid-containing tubes were labeled "XEF-#" (which indicated the presence of lysed epithelial cells).

2.3.2.2 Material Fraction Extraction

The leftover dry swabs set aside from each 'E-Cell' extraction were put into 0.5 mL tubes containing 20 µL of 10X Blue Buffer, 2µL of forensicGEM™, and 178 µL of DI water. The samples were vortexed and then incubated in a Thermal Cycler at 75°C for 5 minutes, followed by 95°C for 5 minutes. In the same manner, the spun-down swabs set aside from each 'SF' extraction were moved into 0.5 mL tubes holding 20 µL of AcroSolv, 20µL of 10X Red Buffer, 4µL of forensicGEM™ Sperm, and 156µL of DI water. The samples were vortexed and placed into a Thermal Cycler to incubate at 52°C for 10 minutes, 75°C for 6 minutes, and 95°C for 3 minutes. The wet swabs were transferred into a mesh spin basket inside a fresh 2.0 mL tube using sterile forceps. Using a centrifuge, the samples were spun-down at 14,000 rpm for 2 minutes. The eluate was transferred back into the original 0. 5 mL MF sample tube.

2.3.2.3 Microscope Slide Preparation

About 3µL of each sample was transferred onto microscope slides and were dried on a heatblock. The slides were then stained using the 'Christmas Tree stain' (a mixture of Nuclear Fast Red and Picric Acid-Indigocarmine).

2.3.3 Various semen swabs used with enzyme cocktail

2.3.3.1 Sample Treatment

Twelve individual swab tips (two with approximately 10ng semen, two with approximately 25ng semen, two with approximately 50ng semen, two with approximately

100ng semen, two with approximately 200ng semen, and two with approximately 400ng semen) were each carefully pulled apart and placed in a 2 mL microcentrifuge tube. To each tube, 267µl of DI water, 3µl of forensicGEM™, and 30µl of 10X Buffer Blue were included. A reagent blank was initiated at this step, consisting of 270µl DI water and 30µl of 10X Buffer Blue. In a thermomixer, the samples were incubated at 75ºC for 1 hour at 400 rpm, and then 95ºC for 10 minutes and 4ºC for 10 minutes without agitation. The wet swabs were transferred into a mesh spin basket inside a fresh 2.0 mL tube using sterile forceps. Using a centrifuge, the samples were spun-down at 14,000 rpm for 2 minutes. The spun-down swabs were kept in separate 0.5 mL tubes designated for MF extraction. The eluate was transferred into a new microcentrifuge tube labeled as the "epithelial fraction", leaving about 100µL of the supernatant with the pellet. The remaining 100µL and the sperm pellet were resuspended and marked as the "sperm fraction".

2.3.3.2 Sperm Fraction Extraction

To each sample, 5µL of the resuspended pellet, 5µL of Acrosolv, 1µL of forensicGEM™ Sperm, 5µL of 10X Red Buffer, and 34µL of DI water were added to fresh 0.5 mL tubes. The samples were then vortexed, placed in a Thermal Cycler, and incubated at 52°C for 10 minutes, 75°C for 6 minutes, and 95°C for 3 minutes.

2.3.3.3 Material Fraction Extraction

The spun-down swabs that were set aside from each 'E-Cell' extraction were put into 0.5mL tubes containing 2μL of forensicGEM™, 20μL of 10X Blue Buffer, and 178μL of DI water. The samples were vortexed and subsequently incubated in a Thermal Cycler at 75°C for 5 minutes, followed by 95°C for 5 minutes. Likewise, the spun-down swabs set aside from each 'SF' extraction were moved into 0.5mL tubes containing 4μL of forensicGEM™ Sperm, 20μL of 10X Red Buffer, 156μL of DI water, and 20μL of AcroSolv. The samples were vortexed and incubated in a Thermal Cycler at 52°C for 10 minutes, 75°C for 6 minutes, and 95°C for 3 minutes. The wet swabs were transferred into a mesh spin basket inside a fresh 2.0 mL tube using sterile forceps. Using a centrifuge, the samples were spun-down at 14,000 rpm for 2 minutes. The spun-down swabs were kept in separate 0.5 mL tubes designated for MF extraction, while the eluate from each swab was transferred back into the original 0.5 mL EF sample tube.

2.3.3.4 Microscope Slide Preparation

Samples were transferred onto microscope slides and left to air dry. The slides were then stained with the 'Christmas Tree stain' (a mixture of Nuclear Fast Red and Picric Acid-Indigocarmine).

2.4 Modified TCDE Protocol

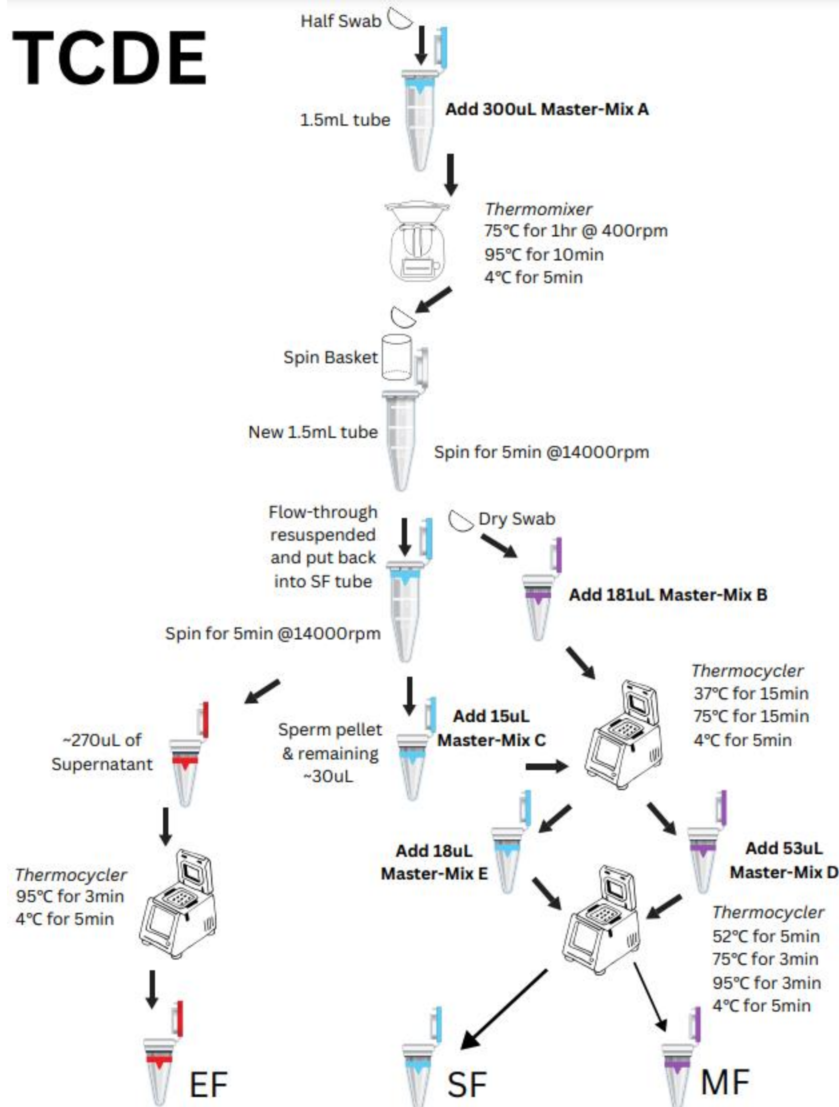


Figure 1. TCDE Protocol Flow Chart

2.4.1 Master-Mix Preparation

Table 1. Master-Mix Preparation: Per Sample Reagent Volumes.

Master-Mix A: Initial E-cell Lysis with forensicGEM™	• 3µL of forensicGEM™ • 30µL of 10X Blue Buffer • 267µL of diH2O
Master-Mix B: Nuclease treatment: Benzonase®/forensicGEM™-MF	• 109µL of diH2O • 13µL of 1:13 forensicGEM™ dilution in 10X Blue Buffer • 49µL of 2X Benzonase Buffer • 10µL of 2.5U/uL Benzonase® in 2X Benzonase Buffer
Master-Mix C: Nuclease treatment: Benzonase®/forensicGEM™-SF	• 1µL of forensicGEM™ • 4µL of 2X Benzonase Buffer • 10µL of 2.5U/µL Benzonase® in 2X Benzonase Buffer
Master-Mix D: AcroSolv/forensicGEMsperm™-MF	• 5µL forensicGEMsperm™ • 22µL of 10X Red Buffer • 26µL AcroSolv
Master-Mix E: AcroSolv/forensicGEMsperm™-SF	• 10µL of AcroSolv • 6µL of 10X Red Buffer • 2µL of forensicGEMsperm™

2.4.2 Epithelial Cell Lysis

Master-Mix A (495µL) was added to a new 1.5 mL tube for each sample. Using a sterile scalpel, about half of the swab head was cut and transferred into the tube, which was labeled with the sample name and 'SF'. In a thermomixer, the samples were incubated at 75ºC for 1 hour at 400 rpm, and then 95ºC for 10 minutes and 4ºC for 10 minutes without agitation.

2.4.3 Material Fraction Lysis

Master-Mix B (181µL) was added to a new 0.5 mL tube labeled with the sample name and 'MF’. Once the incubation was concluded, the swabs were carefully extracted from the tubes using sterile tweezers and placed into a spin basket, which was then situated in a new 1.5mL tube. The swabs were centrifuged for 3 minutes at 14,000 rpm, after which they were relocated to the corresponding 'MF' tube containing Master-Mix B. Any leftover liquid in the tube was pipetted up and down to resuspend any formed pellet, and this was then transferred to the 'SF' tube. The 'SF' tube was centrifuged for 5 minutes at 14,000 rpm to pellet the sperm cells. Most of the supernatant was transferred into a clean 0.5mL tube labeled with the sample name and 'EF,' leaving about 30 µL over the sperm pellet.

2.4.4 Sperm Fraction Lysis

The sperm pellet was resuspended in the remaining 30µL of supernatant by pipetting up and down. Master-Mix C (15µL) was added to the 'SF' tubes. Both the 'SF' and 'MF' tubes were placed in the thermocycler and incubated at 37°C for 15 minutes,

75°C for 15 minutes, and 4°C for 5 minutes. After incubation, the tubes were taken out and briefly vortexed. Then, Master-Mix E (18µL) was added to the 'SF' tubes, and Master-Mix D (53µL) was added to the 'MF' tubes. Both sets of tubes were reinserted into the thermocycler and incubated at 52°C for 5 minutes, 75°C for 3 minutes, 95°C for 3 minutes, and 4°C for 5 minutes. The tubes were vortexed briefly after incubation.

2.4.5 Epithelial Fraction Enzyme Inactivation

The 'EF' tubes were placed in the thermocycler and incubated at 95°C for 5 minutes, followed by 4°C for 5 minutes. After this, all fractions were ready for quantification.

2.5 DNA Amplification and CE Separation

Based on the quantitation values from qPCR, an amplification target concentration of 0.75ng of DNA was utilized. PCR amplification was conducted with the GlobalFiler® PCR Amplification Kit (Applied Biosystems™, Foster City, CA) utilizing a Veriti™ thermal cycler. Capillary Electrophoresis a technique for separating DNA by size, was performed using the SeqStudio Genetic Analyzer (Applied Biosystems™, Foster City, CA). Using the GeneMapper® ID-X v1.6 (Applied Biosystems™, Foster City, CA) software, the generated electropherograms data were examined using a stochastic threshold at 250 relative fluorescence units (RFU and an analytical threshold at 30 RFU.

3. RESULTS

3.1 Various single source samples used with an enzyme cocktail

The semen swabs had a high of 99.21% of the male DNA recovered in the sperm fraction, with an average of 98.4%. The prepped female buccal swabs had a high of 98.78% of the female DNA recovered in the epithelial fraction with an average of 97% recovery. The direct buccal swabs had a high of 85.73% of the female DNA recovered in the epithelial fraction with an average of 78.9% recovery. The vaginal swabs had a high of 99.62% of the female DNA recovered into the epithelial fraction with an average of 66.7% recovery.

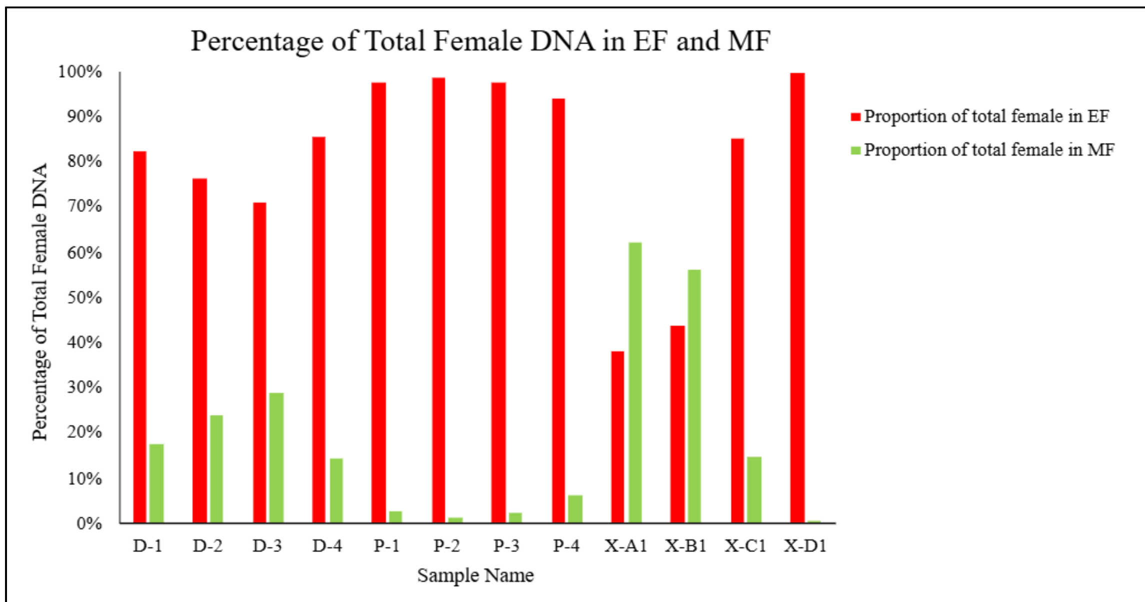


Figure 2. Percent recovery of the Female DNA: from single source direct buccal swabs (D-1, D-2, D-3 and D-4), prepped buccal swabs (P-1, P-2, P-3 and P-4), and vaginal swabs (X-A1, X-B1, X-C1 and X-D1).

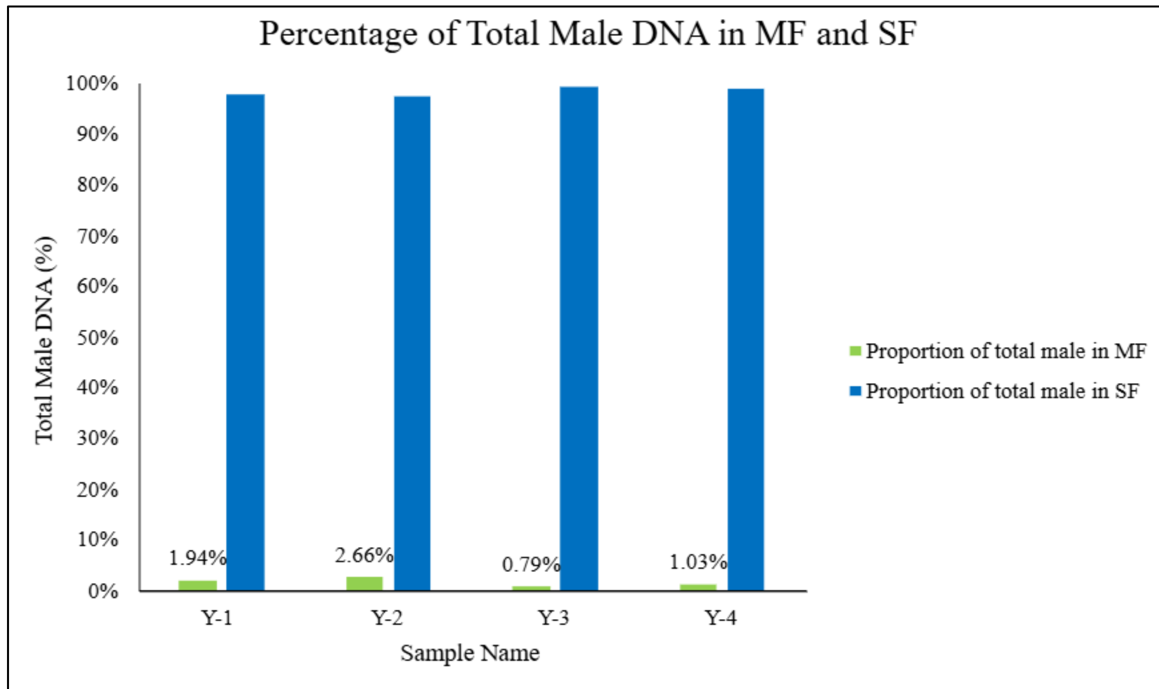


Figure 3. Percent recovery of the male DNA: from single source semen swabs (Y-1, Y-2, Y-3 and Y-4).

3.2 Various single source samples used with an enzyme cocktail: additional donors

In the second set of vaginal swabs, a high of 99.2% of the female DNA was recovered in the epithelial fraction, with an average of 86.5% recovery. In the second set of female direct buccal swabs, a high of 84.8% of the DNA was recovered in the epithelial fraction with an average of 70.4% recovery. In the second set of semen swabs, a high of 79.3% of the total male DNA was recovered in the sperm fraction with an average of 58.9% recovery.

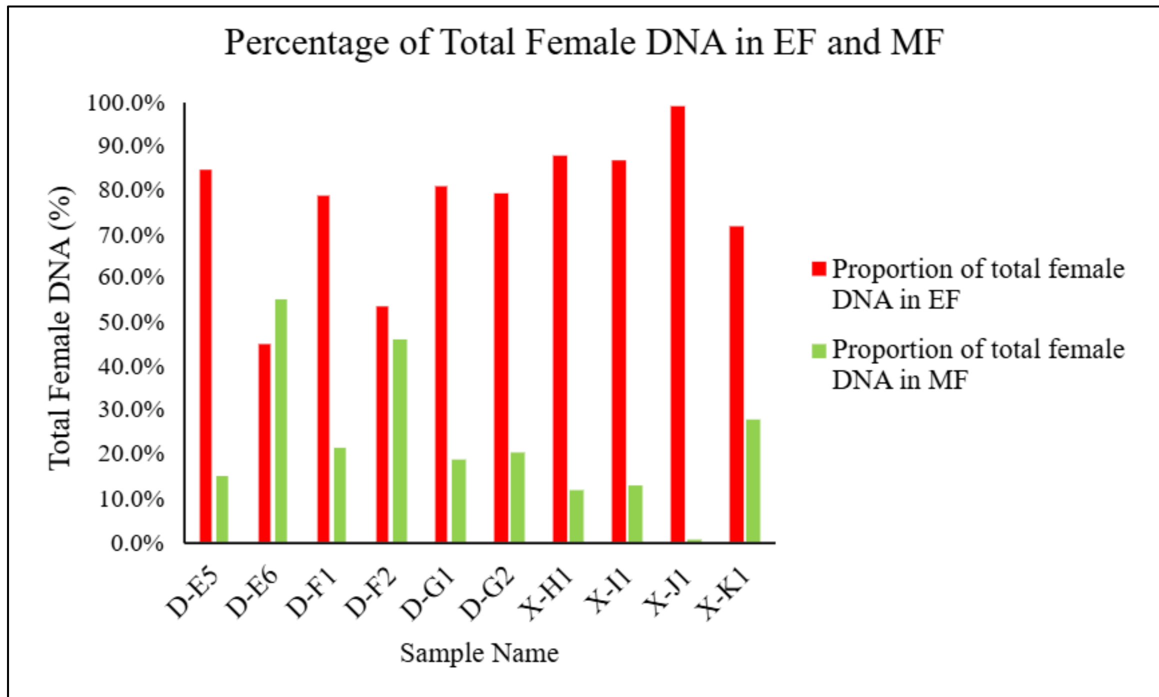


Figure 4. Percent recovery of female DNA: from second set of single source direct buccal swabs (D-E5, D-E6, D-F1, D-F2, D-G1 and D-G2) and vaginal swabs (X-H1, X-I1, X-J1 and X-K1).

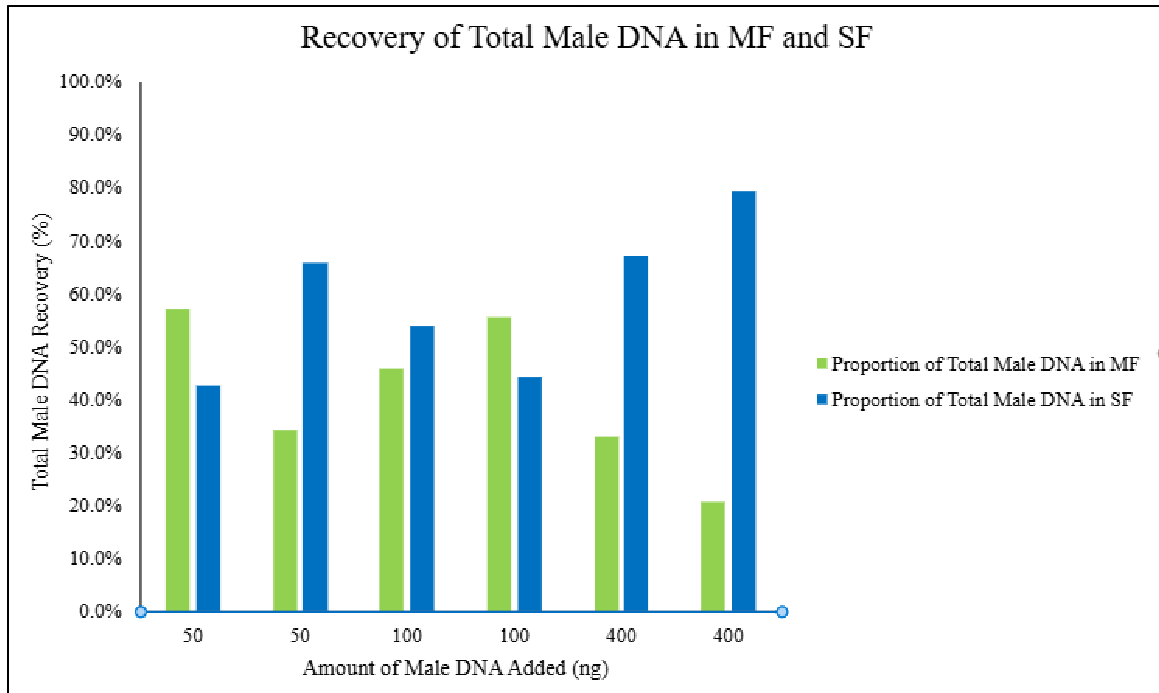


Figure 5. Percent recovery of the male DNA: from single source semen swabs of various quantities (50ng, 100ng, and 400ng).

3.3 Various semen swabs used with enzyme cocktail

Overall, the semen swabs containing various amounts of DNA recovered a high of 91.79% of the total male DNA in the sperm fraction, with an average of 74.45%. Amongst the different amounts deposited, there was no significant correlation between the amount of DNA added and the amount of DNA recovered in the sperm fraction.

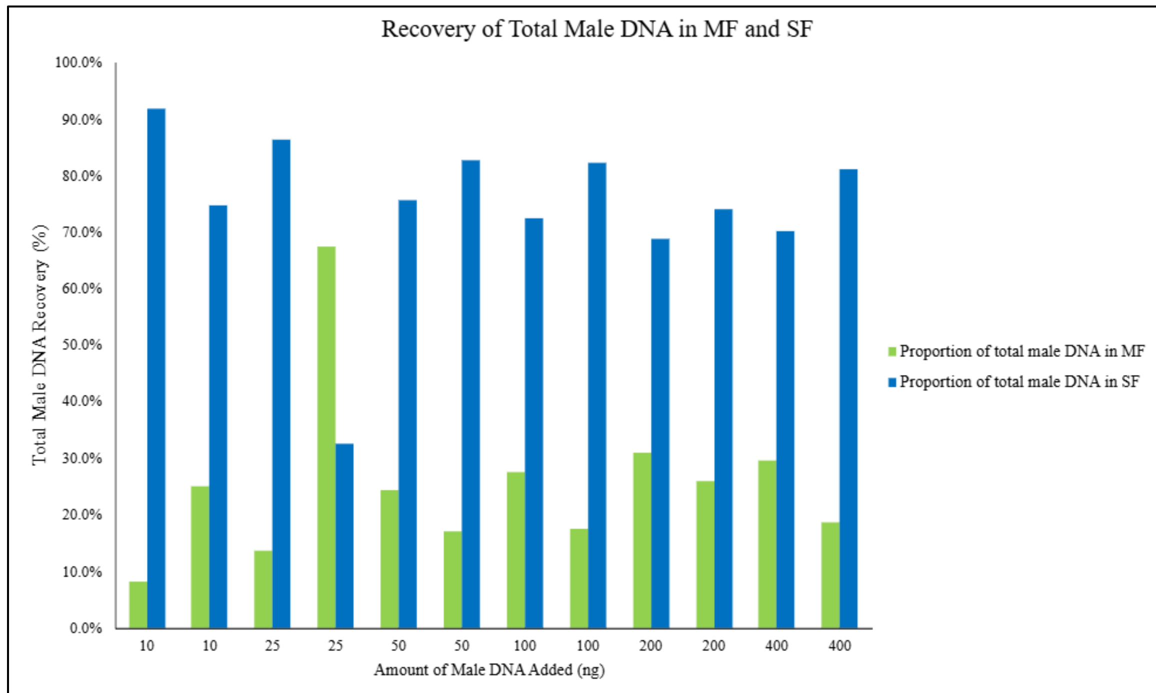


Figure 6. Percent recovery of the male DNA: from single source semen swabs of various quantities (10ng, 25ng, 50ng, 100ng, 200ng and 400ng).

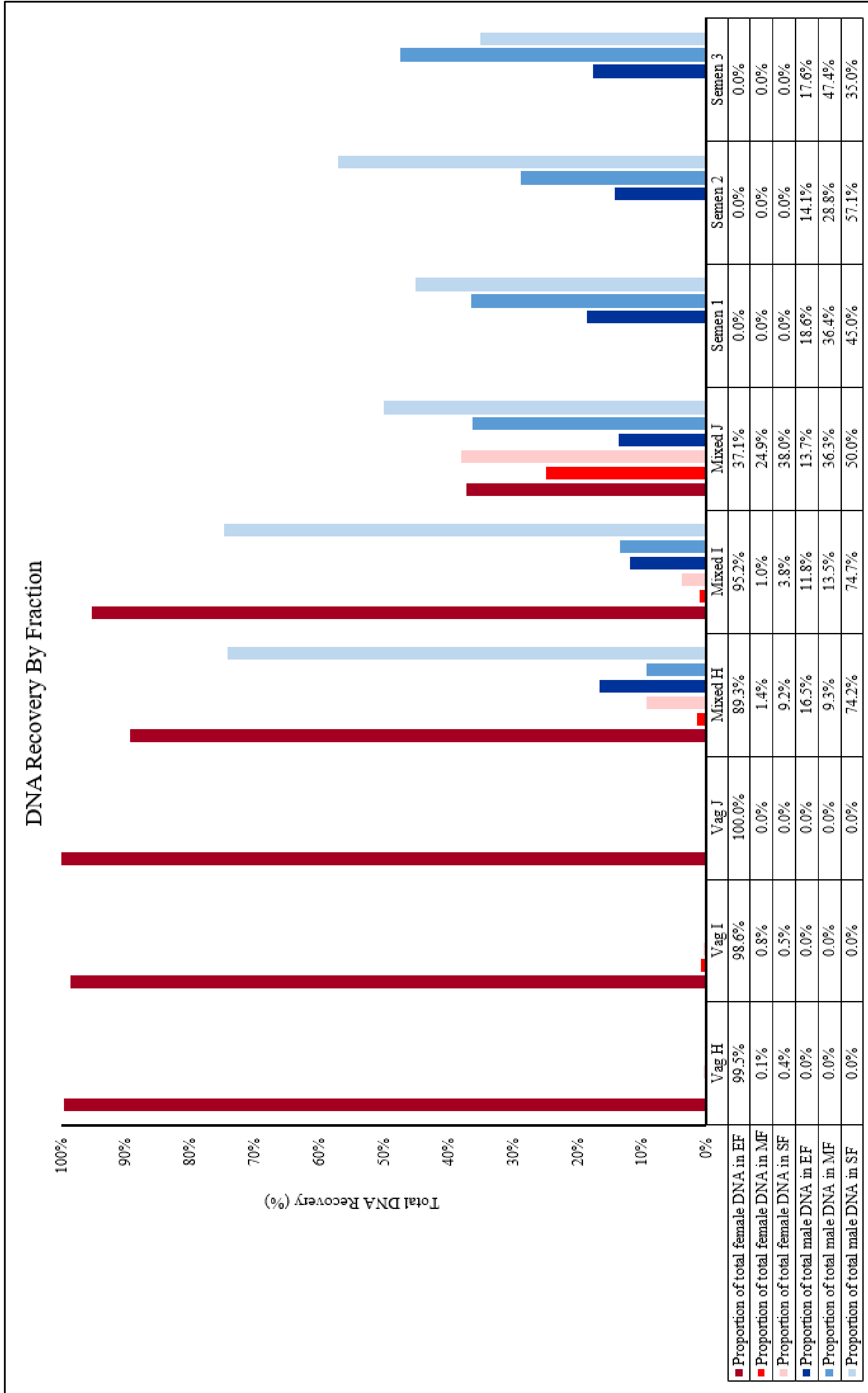
3.4 Modified TCDE Protocol: Trial 1

Due to a variety of potential factors, this experiment was not successful in recovering notable DNA yields in the sperm fraction and epithelial fraction of any the samples tested.

3.5 Modified TCDE Protocol: Trial 2

Of the vaginal only swabs, the total DNA recovered in all the fractions ranged from 27ng to 2112ng. On average, 99.3% of the DNA recovered were found in the epithelial fraction. The semen only swabs had approximately 100ng of DNA added to

them. Of these swabs, an average of 44% of the male DNA recovered was in the sperm fraction. Of the mixed vaginal and semen swabs from the same donors as the single source swabs, an average of 99.7% of the female DNA was recovered in the epithelial fraction. A high of 75% with average of 66.3% of the male DNA was recovered in the sperm fraction.



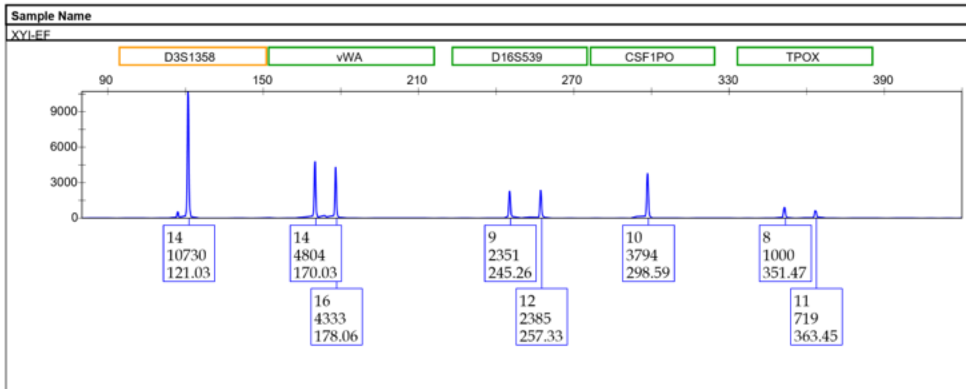
	Vag H	Vag I	Vag J	Mixed H	Mixed I	Mixed J	Semen 1	Semen 2	Semen 3
Proportion of total female DNA in EF	99.5%	98.6%	100.0%	89.3%	95.2%	37.1%	0.0%	0.0%	0.0%
Proportion of total female DNA in MF	0.1%	0.8%	0.0%	1.4%	1.0%	24.9%	0.0%	0.0%	0.0%
Proportion of total female DNA in SF	0.4%	0.5%	0.0%	9.2%	3.8%	38.0%	0.0%	0.0%	0.0%
Proportion of total male DNA in EF	0.0%	0.0%	0.0%	16.5%	11.8%	13.7%	18.6%	14.1%	17.6%
Proportion of total male DNA in MF	0.0%	0.0%	0.0%	9.3%	13.5%	36.3%	36.4%	28.8%	47.4%
Proportion of total male DNA in SF	0.0%	0.0%	0.0%	74.2%	74.7%	50.0%	45.0%	57.1%	35.0%

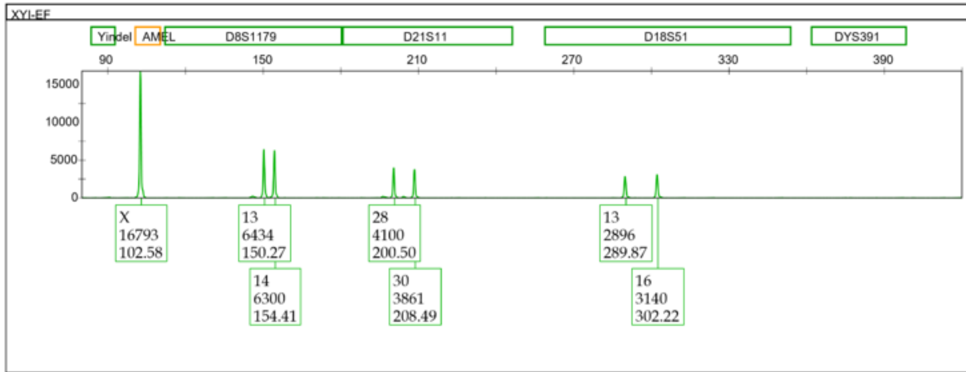
Figure 7. DNA Recovery by Fraction: including vaginal only swabs from the second set of donors (Vag H, Vag I and Vag J), mixed swabs from the second set of donors (Mixed H, Mixed I and Mixed J), and semen only swabs (Semen 1, Semen 2 and Semen 3). Small percentages can be found in the table. Analysis of all three mixed samples contained full profiles of the male donor. Samples XY-H and XY-I included full female profiles, while XY-J was missing alleles in 13 loci in the epithelial and 2 loci in the material fractions.

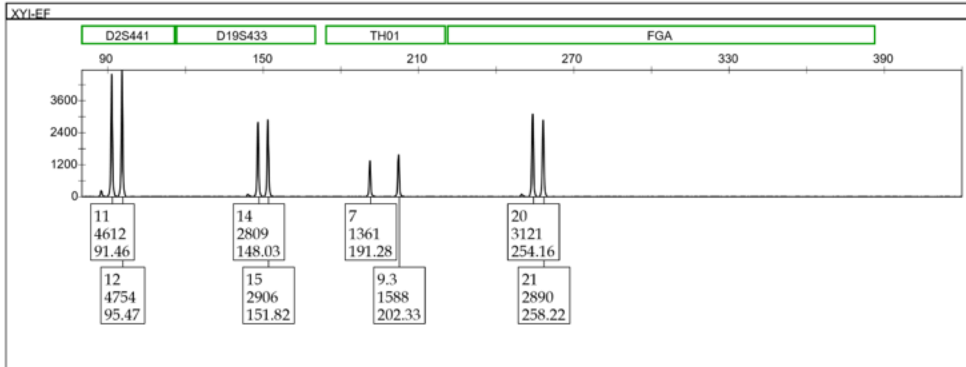
	D3S1358	vWA	D16S539	CSF1PO	TPOX	Y Indel	Amel	D8S1179	D21S11	D18S51	DYS391
XYH-EF	14,18	14,15	9,9	10,11	8,12	-	XX	14,14	29,30	13,14	-
XYH-SF	14,15	14,16	11,12	10,10	11,12	2	XY	13,14	28,29	10,16	10,10
XYH-MF	14,15	14,16	11,12	10,10	11,12	2	XY	13,14	28,29	10, 16	-
XYI-EF	14,14	14,16	9,12	10,10	8,11	-	XX	13,14	28,30	13,16	-
XYI-SF	14,15	14,16	11,12	10,10	11,12	2	XY	13,14	28,29	10,16	10,10
XYI-MF	14,15	14,16	9,12	10,10	11,12	2	XX	13,14	28,29	16*	10,10
XYJ-EF	16,18	-	-	-	-	2	XX	8,8	-	-	-
XYJ-SF	14,15	14,16	11,12	10,10	11,12	2	XY	13,14	28,29	10,16	10,10
XYJ-MF	14,15	14,16	11,12	10,10	-	2	XY	13,14	28,29	10,16	10,10

D2S441	D19S433	TH01	FGA	D22S104	D5S818	D13S317	D7S820	SE33	D10S124	D1S1656	D12S391	D2S1338
14,14	14.2,15	6,8	19,25	16,17	11,13	13,13	10,11	20.2,21	12,14	11,12	19,20	17,20
10,14	14,17	7,9.3	24,25	15,17	11,12	12,12	11,12	22.2,28.2	15,16	12,16	19,19	17,18
10,14	14,17	7,9.3	24,25	15,17	11,12	12,12	11,12	-	15,16	12,16	19,19	17,18
11,12	14,15	7,9.3	20,21	11,15	10,13	10,13	9,11	25.2,27.2	13,17	12,17	17,19	17,19
10,14*	14,17	7,9.3	24,25	15,17	11,12	12,12*	11,12	22.2,28.2	15,16*	12,16	19,19	17,18
10,14	14,17	7,9.3	-	11,15	11,12	10,12	11,12	-	15,16	12,16	19,19	17,18
11,12	-	7, 7	-	15,17	11*,13	-	-	-	14,15	17*, 17.3	-	-
10,14	14,17	7,9.3	24,25	15,17	11,12	12,12	11,12	22.2,28.2	15,16	12,16	19,19	17,18
10,14	14,17	7,9.3	24,25	15,17	11,12	12,12	11,12		15,16	12,16	19,19	17,18

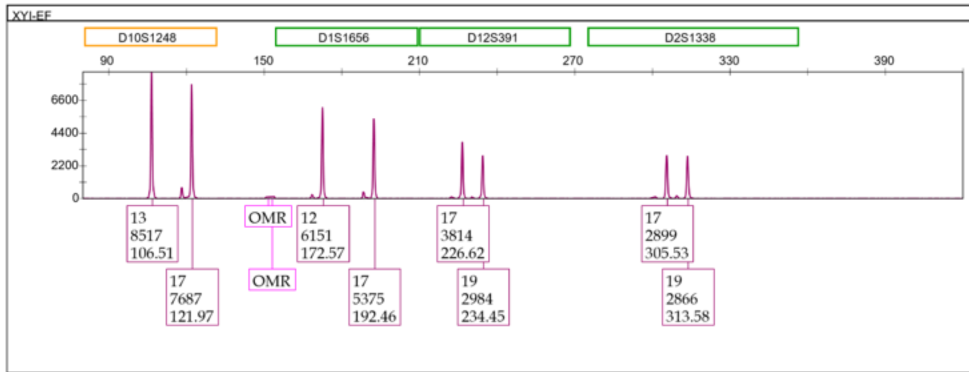
Table 2. Allele Table of Mixed Samples: the epithelial fraction (EF), sperm fraction (SF), and material fraction (MF) from three mock sexual-assault samples from vaginal swab donors H (XYH), I (XYI), and J (XYJ) Asterisks indicate possible allelic drop-out.

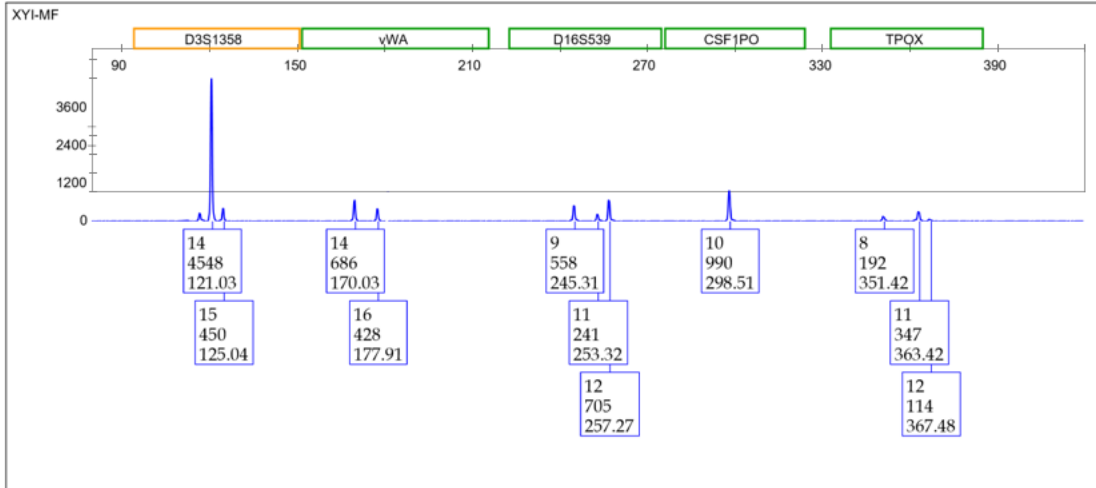

Sample Name
XYI-EF
D3S1358
vWA
D16S539
CSF1PO
TPOX
90
150
210
270
330
390
9000
6000
3000
0
14
10730
121.03
14
4804
170.03
16
4333
178.06
9
2351
245.26
12
2385
257.33
10
3794
298.59
8
1000
351.47
11
719
363.45

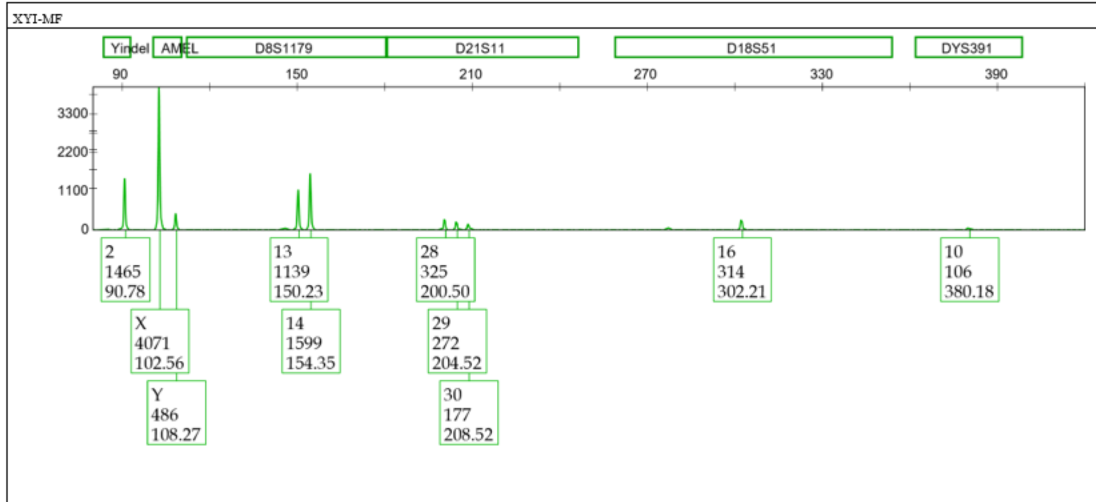

XYI-EF
Yindel
AMEL
D8S1179
D21S11
D18S51
DYS391
90
150
210
270
330
390
15000
10000
5000
0
X
16793
102.58
13
6434
150.27
14
6300
154.41
28
4100
200.50
30
3861
208.49
13
2896
289.87
16
3140
302.22

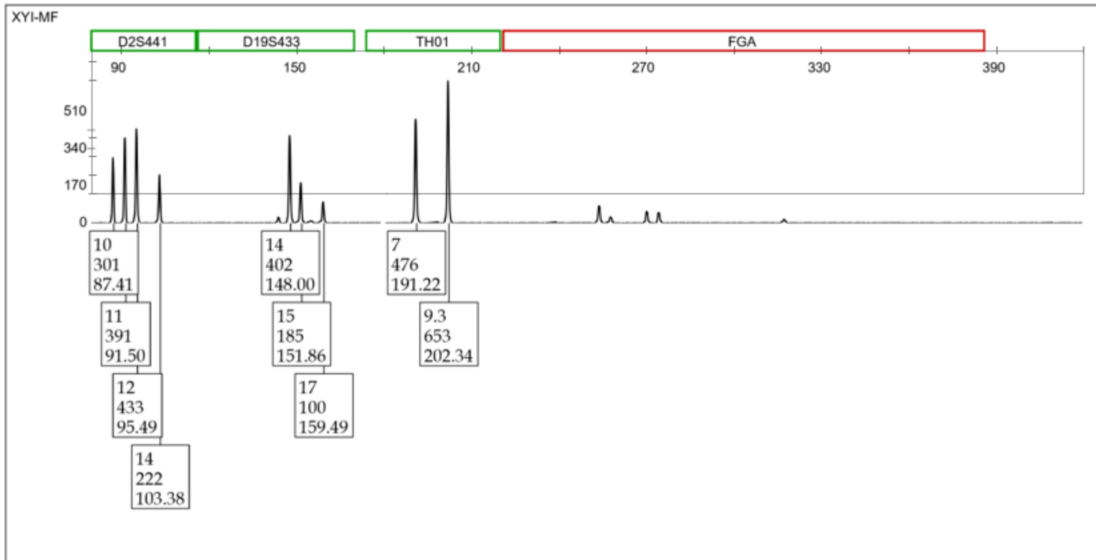

XYI-EF
D2S441
D19S433
TH01
FGA
90
150
210
270
330
390
3600
2400
1200
0
11
4612
91.46
12
4754
95.47
14
2809
148.03
15
2906
151.82
7
1361
191.28
9.3
1588
202.33
20
3121
254.16
21
2890
258.22

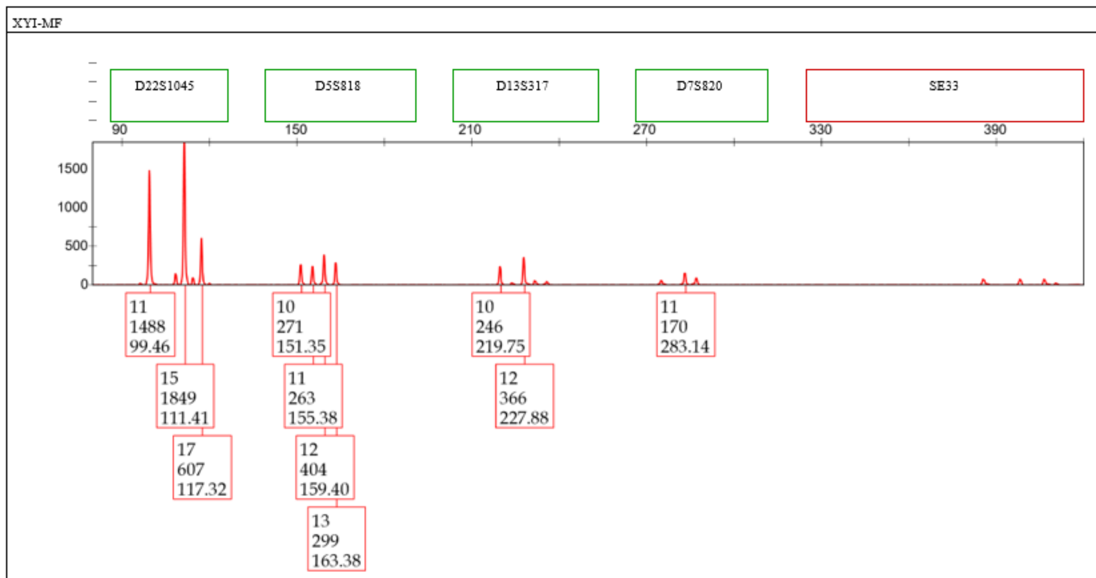
Sample Name
XYI-EF
D22S1045
D5S818
D13S317
D7S820
SE33
90
150
210
270
330
390
3900
2600
1300
0
11
3246
99.46
15
3199
111.49
10
5159
151.30
13
4977
163.41
10
5130
219.83
13
4667
231.84
9
3222
275.16
11
3441
283.15
25.2
1994
398.20
27.2
1609
406.45

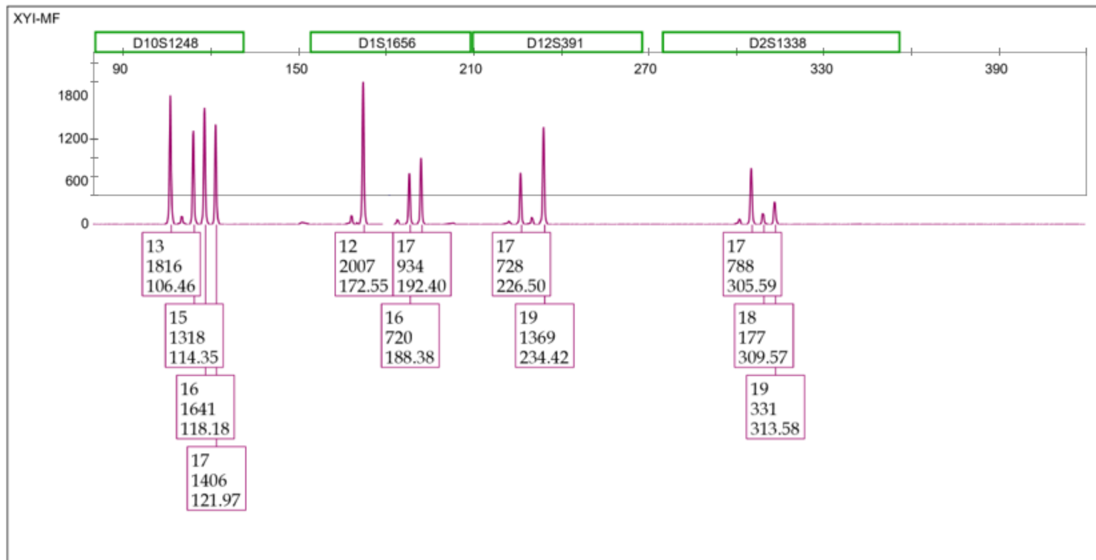

XYI-EF
D10S1248
D1S1656
D12S391
D2S1338
90
150
210
270
330
390
6600
4400
2200
0
13
8517
106.51
17
7687
121.97
OMR
OMR
12
6151
172.57
17
5375
192.46
17
3814
226.62
19
2984
234.45
17
2899
305.53
19
2866
313.58

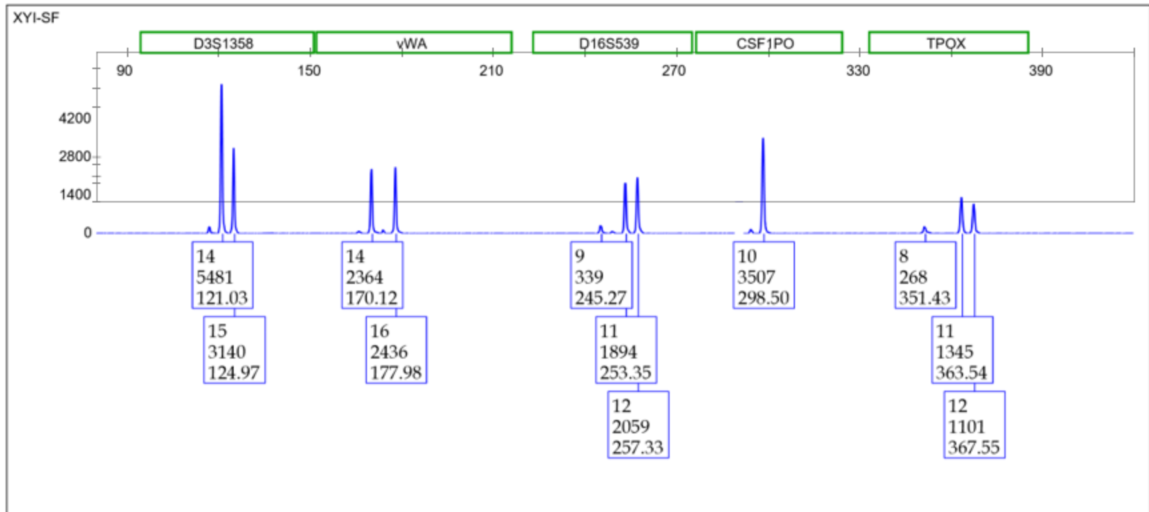

XYI-MF
D3S1358
vWA
D16S539
CSF1PO
TPOX
90
150
210
270
330
390
3600
2400
1200
0
14
4548
121.03
15
450
125.04
14
686
170.03
16
428
177.91
9
558
245.31
11
241
253.32
12
705
257.27
10
990
298.51
8
192
351.42
11
347
363.42
12
114
367.48

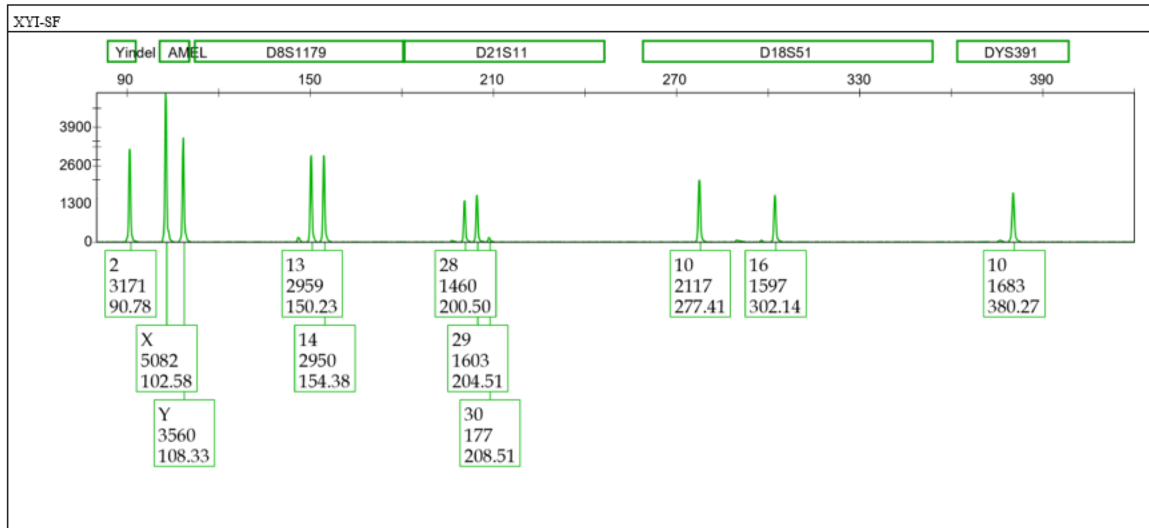

XYI-MF
Yindel
AMEL
D8S1179
D21S11
D18S51
DYS391
90
150
210
270
330
390
3300
2200
1100
0
2
1465
90.78
X
4071
102.56
Y
486
108.27
13
1139
150.23
14
1599
154.35
28
325
200.50
29
272
204.52
30
177
208.52
16
314
302.21
10
106
380.18

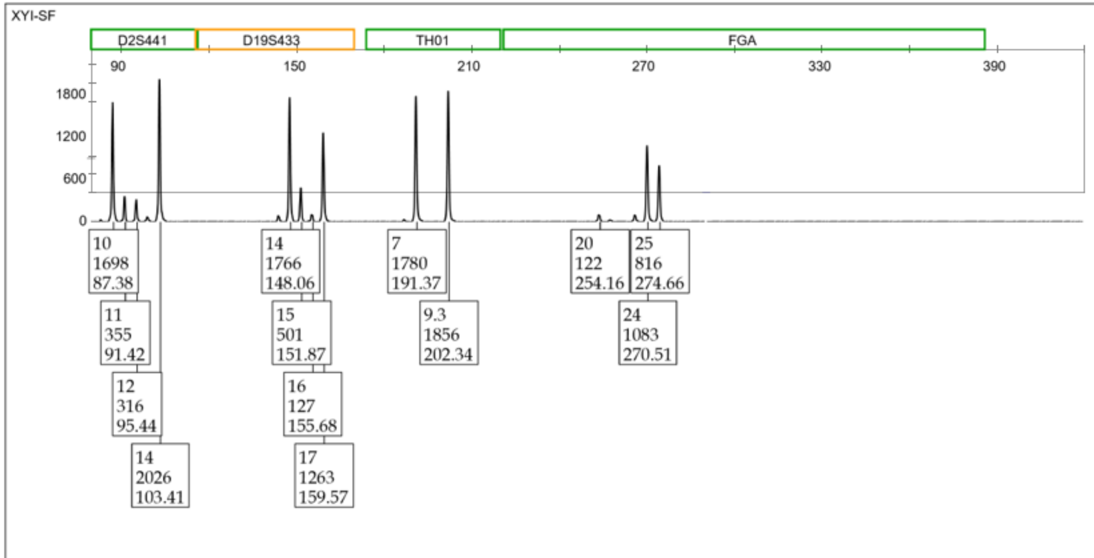

XYI-MF
D2S441
D19S433
TH01
FGA
90
150
210
270
330
390
510
340
170
0
10
301
87.41
11
391
91.50
12
433
95.49
14
222
103.38
14
402
148.00
15
185
151.86
17
100
159.49
7
476
191.22
9.3
653
202.34

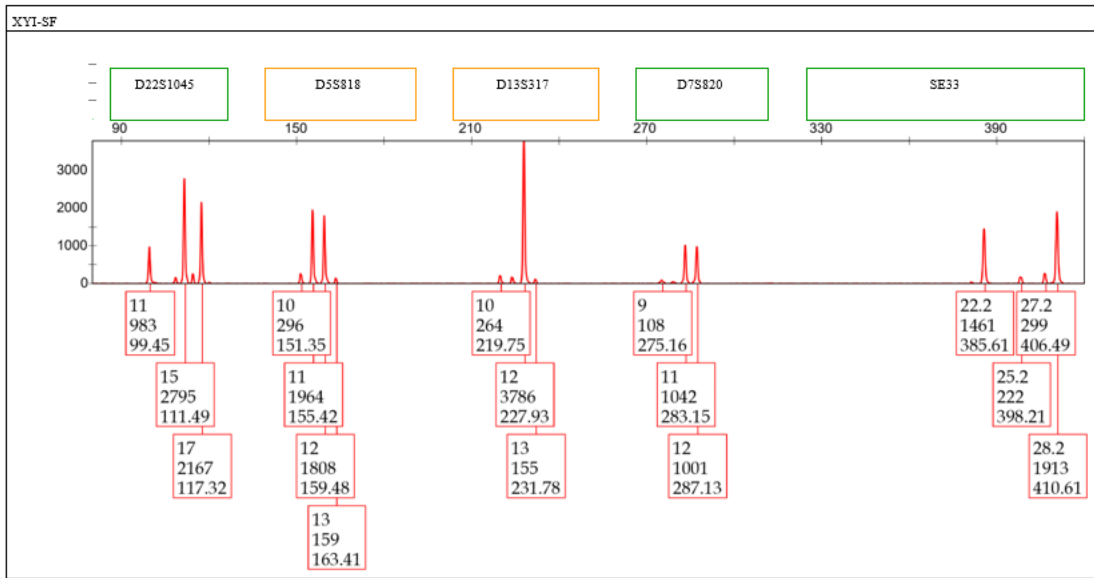

XYI-MF
D22S1045
D5S818
D13S317
D7S820
SE33
90
150
210
270
330
390
1500
1000
500
0
11
1488
99.46
15
1849
111.41
17
607
117.32
10
271
151.35
11
263
155.38
12
404
159.40
13
299
163.38
10
246
219.75
12
366
227.88
11
170
283.14


XYI-MF
D10S1248
D1S1656
D12S391
D2S1338
90
150
210
270
330
390
1800
1200
600
0
13
1816
106.46
15
1318
114.35
16
1641
118.18
17
1406
121.97
12
2007
172.55
17
934
192.40
16
720
188.38
17
728
226.50
19
1369
234.42
17
788
305.59
18
177
309.57
19
331
313.58


XYI-SF
D3S1358
vWA
D16S539
CSF1PO
TPOX
90
150
210
270
330
390
4200
2800
1400
0
14
5481
121.03
15
3140
124.97
14
2364
170.12
16
2436
177.98
9
339
245.27
11
1894
253.35
12
2059
257.33
10
3507
298.50
8
268
351.43
11
1345
363.54
12
1101
367.55


XYI-SF
Yindel
AMEL
D8S1179
D21S11
D18S51
DYS391
90
150
210
270
330
390
3900
2600
1300
0
2
3171
90.78
X
5082
102.58
Y
3560
108.33
13
2959
150.23
14
2950
154.38
28
1460
200.50
29
1603
204.51
30
177
208.51
10
2117
277.41
16
1597
302.14
10
1683
380.27


XYI-SF
D2S441
D19S433
TH01
FGA
90
150
210
270
330
390
1800
1200
600
0
10
1698
87.38
11
355
91.42
12
316
95.44
14
2026
103.41
14
1766
148.06
15
501
151.87
16
127
155.68
17
1263
159.57
7
1780
191.37
9.3
1856
202.34
20
122
254.16
25
816
274.66
24
1083
270.51


XYI-SF
D22S1045
D5S818
D13S317
D7S820
SE33
90
150
210
270
330
390
3000
2000
1000
0
11
983
99.45
15
2795
111.49
17
2167
117.32
10
296
151.35
11
1964
155.42
12
1808
159.48
13
159
163.41
10
264
219.75
12
3786
227.93
13
155
231.78
9
108
275.16
11
1042
283.15
12
1001
287.13
22.2
1461
385.61
27.2
299
406.49
25.2
222
398.21
28.2
1913
410.61

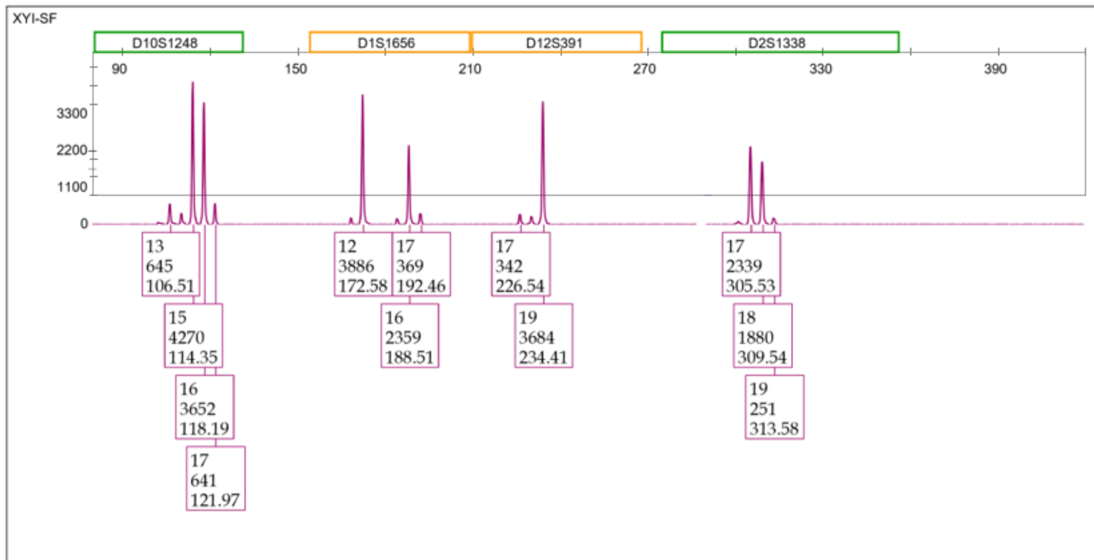


Figure 8. STR profile: of mixed sample from donor I (XY-I), including the epithelial fraction (XYI-EF), material fraction (XYI-MF), and sperm fraction (XYI-SF). The profile does not have a stutter filter on. There are only one or two true alleles detected at each loci in the EF and SF (except for locus D22S1045).

4. DISCUSSION

4.1 Various single source samples used with an enzyme cocktail

The results from using the enzyme cocktail to extract DNA from various single-source samples demonstrate highly efficient DNA recovery across all sample types, with some notable differences in the distribution of DNA into specific fractions. Using the semen dried onto swabs, up to 99.21% of the male DNA was recovered in the sperm fraction, with an average of 98.4%. The near-complete recovery of male DNA in the sperm fraction supports the idea that the enzyme cocktail is releasing sperm cells from the swab.

In the case of the buccal swabs, the results show a distinction between cotton swabs with a buccal cell preparation added to the swab then buccal cells swabbed directly from inside the mouth. The prepped female buccal swabs yielded up to 98.78% of female DNA in the epithelial fraction, suggesting that the preparation step may have optimized the collection and extraction process, possibly by removing other components of saliva. This high recovery rate supports the effectiveness of the enzyme cocktail in isolating epithelial DNA from the oral cavity, which typically contains a rich mixture of cells from the buccal mucosa. In contrast, the direct buccal swabs had a lower recovery, with a high of 85.73% of the female DNA recovered in the epithelial fraction. This reduction in efficiency may be due to the direct collection process, which may include a greater amount of non-epithelial DNA, leading to a less efficient extraction. Nonetheless, the direct buccal swabs still demonstrate a relatively high recovery rate, which suggests that the enzyme cocktail is effective even without additional processing steps.

The vaginal swabs showed the highest recovery rate among the female DNA sources, with a high of 99.62% of the female DNA being released into the epithelial fraction. This is consistent with the fact that vaginal samples are typically rich in epithelial cells, which are easily collected and lysed. The high recovery rate also highlights the enzyme cocktail's efficacy in extracting DNA from vaginal swabs, where epithelial DNA predominates. This finding reinforces the importance of vaginal swabs in forensic DNA analysis, particularly in cases where clear differentiation between female and male DNA is critical. Overall, these results demonstrate the versatility and efficiency of the enzyme cocktail combined with the hour-long 75°C incubation in extracting high-quality DNA from a variety of sample types, though some variability in recovery rates, particularly with direct buccal swabs, suggests that protocol adjustments or optimizations could further enhance consistency across different sample collection methods.

4.2 Various single source samples using an enzyme cocktail: additional donors

To see if the success of extraction was donor specific, the previous experiment of single source samples was repeated with new buccal swab donors and new vaginal swab donors. Among the 8 vaginal and 4 buccal swabs donors used, there was a variation of quantities of DNA released but no significant different in how well the DNA separated into the epithelial and material fractions.

The results from the second set of vaginal swabs indicate a high recovery of female found in the epithelial fraction. This suggests that the majority of the recovered DNA is derived from epithelial cells, which are typically sloughed off from the vaginal

lining. The high percentage of female DNA recovery in this fraction supports the idea that epithelial cells, which are abundant and easily collected and can be released from the swab at 75°C enzyme incubation temperature. However, the variation in recovery rates between individuals highlights the potential for biological variability in DNA collection efficiency and release efficiency, which warranted the further investigation with different donors.

In comparison, the results from the second set of female direct buccal swabs show a lower recovery of DNA in the epithelial fraction, with a range of 44.9% to 84.8% of DNA recovered with an average of 70.4% of the female DNA. While this is still a substantial recovery, it is noticeably lower than the vaginal swab results. This could be due to differences in the types of epithelial cells collected from the oral cavity. The buccal swabs may have a different cell membrane composition, influencing the efficiency of DNA recovery. Additionally, individual variations in saliva or tissue characteristics might contribute to the lower recovery percentage observed.

An average of 59.9% of the total male DNA was recovered from the semen swabs in the sperm fraction. The range of recovery was 42.7% to 79.3%. Semen samples are a complex mixture containing sperm cells, epithelial cells, and seminal fluid, making it more challenging to selectively recover sperm DNA. The high of 79.3% recovery rate suggests that while sperm DNA can be efficiently isolated, there is likely competition between different cellular components within the sample, which may dilute the sperm DNA in the overall recovery. Further refinement in collection techniques or separation methods may be needed to increase the efficiency of sperm DNA recovery, especially in

cases where male DNA needs to be isolated from mixed or contaminated samples. The differences in DNA recovery rates across sample types (vaginal, buccal, and semen) underline the importance of considering the cellular composition and biological context of each type of sample when evaluating forensic or diagnostic DNA recovery techniques.

4.3 Various semen swabs used with an enzyme cocktail

Since vaginal swabs are a direct source of female DNA, miniscule quantities of male DNA can be overwhelmed or completely muted in the profiles if the sperm DNA cannot be fully separated. By extracting various quantities of male DNA from cotton swabs, it was presumptively determined that this method is effective even when very little male DNA is present on vaginal swabs. The longer incubation at 75°C was initially optimized on single source semen swabs by Ashley Lara, and the results in this experiment were consistent with Lara’s study [20].

4.4 Modified TCDE Protocol: Trial 1

The first experiment using the modified TCDE protocol did not yield notable DNA recovery in the sperm and epithelial fractions, which raises important concerns about the factors influencing the success of the extraction process. The low DNA yield observed across all samples suggests that several procedural or technical issues may have contributed to the failure. One potential source of error is the improper storage of enzymes, which can compromise their effectiveness and lead to incomplete cell lysis or inefficient DNA release. Enzymes are highly sensitive to storage conditions, and

deviations from protocols could render them less effective. Additionally, incorrect reagent preparation, inaccurate calculations or improper mixing may have led to suboptimal enzyme concentrations, further impairing the extraction process.

Another potential issue that likely impacted the experiment was the decision to attempt such a large sample size in a single session. When the experiment was later repeated with a smaller sample size, it was easier to maintain control over the process, leading to more consistent results. This highlights the importance of scaling experiments appropriately, especially when dealing with intricate protocols and multiple reagents. The lessons learned from this first trial will inform future optimizations of the TCDE protocol, particularly in terms of reagent preparation, sample management, and the careful handling of enzymes to ensure reliable DNA recovery.

4.5 Modified TCDE Protocol: Trial 2

The single source experiments were successful in foreshadowing how the mixed samples would extract. The addition of the longer incubation time at 75°C during the initial lysis released more of the total female DNA into the epithelial fraction, providing less of a risk of female DNA adhering to the cotton swab or lingering in the sperm fraction. The addition of the Benzonase nuclease at a temperature too low for EA1 effectively degraded any remaining epithelial cells left in the supernatant. The amount of male DNA recovered in the sperm fraction suggests that the Benzonase only degraded the epithelial cells while leaving the sperm cells intact. Looking at the profiles produced from the mixed samples, full male profiles were produced. This suggests that the starting

quantities of female DNA on the swabs does not correlate to separation success when using the TCDE method, since the samples had varying differences in female and male DNA quantity.

The results from the second experiment using the modified TCDE protocol show a marked improvement in DNA recovery compared to the first experiment, although some variability remains, particularly in the semen-only and mixed swabs. The high percentage of recovery in the epithelial fraction is consistent with previous findings, where vaginal swabs typically contain a predominance of epithelial cells. The wide range of total DNA recovered (from 27ng to 2112ng) likely reflects individual biological variability, with factors such as the amount of cellular material present on the swab and the efficiency of the DNA extraction also contributing to these differences. The overall high recovery rate suggests that the modified TCDE protocol is effective in isolating and extracting female DNA from vaginal swabs.

In contrast, the semen-only swabs, which had approximately 100ng of male DNA, exhibited a lower recovery rate, with an average of 44% of the male DNA recovered in the sperm fraction. This lower recovery percentage could be attributed to the complex nature of semen samples, which contain not only sperm cells but also seminal fluid and other cellular material. The presence of these additional components may hinder the selective recovery of sperm DNA, making it more challenging to isolate male DNA from the mixed cellular environment. Despite this, the sperm fraction did recover on average, 56.1% of the male DNA, and the results suggest that the protocol is effective in isolating sperm DNA.

The mixed vaginal and semen swabs, which included samples from the same donors as the single-source swabs, demonstrated high recovery rates for female DNA, with an average of 99.7% of the female DNA recovered in the epithelial fraction. This result is consistent with the finding from the vaginal-only swabs, indicating that the female DNA recovery is not significantly impacted by the presence of semen. However, the male DNA recovery was more variable, with a high of 75% and an average of 66.3% of the male DNA being recovered in the sperm fraction. This suggests that while the modified TCDE protocol is effective in extracting sperm DNA, the presence of female DNA in the mixed swabs still exists. The variability in male DNA recovery across mixed samples indicates that the protocol may need further optimization to improve the efficiency of male DNA extraction in the presence of significant female DNA. Overall, these results demonstrate that the modified TCDE protocol is a promising approach for recovering DNA from both single-source and mixed samples, although further refinements may be needed for more consistent recovery.

The analysis of the three mixed samples provided valuable insights into the DNA recovery and profile completeness across different sample types. Notably, both samples XY-H and XY-I yielded full male and female profiles, indicating that the DNA extraction process was successful in recovering complete profiles from both contributors in these samples. However, sample XY-J presented a different outcome, where some alleles were missing from the female profile in both the epithelial and material fractions. This partial loss of female alleles could indicate that the DNA recovery in XY-J was less efficient, possibly due to an error in the amplification or capillary electrophoresis processes. The

missing alleles could also be due to factors such as incomplete cell lysis, sample degradation, or insufficient DNA recovery from the epithelial cells, which are often the primary source of female DNA in mixed samples. The target mass for amplification of 0.75ng per sample was reached for all samples. Further investigation into the causes of incomplete recovery in XY-J could help optimize protocols for handling complex mixtures and ensure more reliable results in forensic and diagnostic applications.

5. CONCLUSIONS

This research successfully evaluated the efficiency of a modified temperature controlled differential extraction procedure for releasing DNA on cotton swabs from single-source samples, achieving DNA recovery ranging from 42% to 98% from the respective sperm and epithelial fractions. The success of this protocol was consistent across various sample types, including semen, vaginal, and buccal cells, supporting its robustness across different biological materials. This high yield indicated that the protocol would likely be effective at isolating DNA from distinct cell types, such as sperm and epithelial cells, in mixed DNA samples. Mike Yakoo developed and optimized extraction using the TCDE method with mixed liquid samples [80]. By experimenting with mixed samples on cotton swabs specifically, the results in this study were consistent with Yakoo's results using types of samples often found in SAECK [80].

The incorporation of an extended incubation period at a higher temperature in the initial lysis step, based on success from Lara's trial on semen swabs, resulted in significant improvements in the quantity of DNA recovered [20]. Specifically, when applied to mixed samples (vaginal and semen) and single-source samples (vaginal, semen, and buccal), the modified protocol led to greater overall DNA release in both the sperm and epithelial fractions. This is also consistent with results from Ashleigh Roberts, who determined when conducting the TCDE method on various substrates, incubation prior to and/or in the beginning of extraction helps facilitate cell release and lysis [96]. The hour-long incubation time at 75°C facilitated more efficient lysis of cells, which, in turn, increased the yield of DNA. Importantly, the resulting fractions were visibly cleaner

compared to previous iterations of the protocol, suggesting that the extended incubation aided in separating the unwanted cell types.

Microscopic examination of the separated fractions provided additional evidence of the protocol's specificity. When the sperm and epithelial fractions were stained and observed under a microscope, no sperm cells were detected in the epithelial fraction, and conversely, very few epithelial cells were present in the sperm fraction. This helped confirm that the TCDE protocol effectively separated the sperm and epithelial cells, ensuring that the DNA profiles from each fraction would likely be distinct once analyzed.

DNA profiling from the mock sexual assault samples was successful. Despite the female-dominant nature of the sample, a complete male DNA profile was successfully retrieved from the sperm fraction. This result highlights the sensitivity and specificity of the modified TCDE protocol, as it was able to isolate a full male DNA profile from the sperm fraction, even in the presence of a large amount of female DNA. The ability to obtain a full and accurate male profile in this context is of particular importance in forensic casework, where distinguishing male DNA in sexual assault cases is often critical for identification and prosecution.

The clean separation of sperm and epithelial cells, along with the retrieval of complete and clean DNA profiles, suggests that this protocol could significantly improve the efficiency of forensic DNA analysis, particularly in cases involving sexual assault or other mixed-source samples. Future studies should continue to optimize the TCDE protocol in volumes and equipment typically found in forensic laboratories. The most

common thermocycler capacity is typically for 0.2mL to 0.5mL tubes. The use of 1.5mL tubes are necessary for drying the wet swabs in spin baskets. Two ideal solutions would be a thermocycler with a 1.5mL tube capacity or a spin basket that fits in a 0.5mL tube, neither of which currently exist. This updated protocol should be further validated using a larger sample set of mixed samples.

APPENDIX:

Table 3. Sample Name Nomenclature: Breakdown that correlates to all samples presented in the text, figures, and other tables.

<table>
<tr><td colspan="4">Sample Nomenclature</td></tr>
<tr><td colspan="4">Example: X-A1, first vaginal swab from Donor A</td></tr>
<tr><td>Sample Type</td><td>Type Identifier</td><td>Donor Identifier</td><td rowspan="6">-Swab/ Swab Half Used (e.g. -2 would be the second half of the first swab used)</td></tr>
<tr><td>Vaginal Swab</td><td>X</td><td>A-J</td></tr>
<tr><td>Direct Buccal Swab</td><td>D</td><td>E-G</td></tr>
<tr><td>Prepped Buccal Swab</td><td>P</td><td>-</td></tr>
<tr><td>Semen Swab</td><td>Y</td><td>-</td></tr>
<tr><td>Mixed Swab</td><td>XY</td><td>A-J</td></tr>
</table>

LIST OF JOURNAL ABBREVIATIONS

Anal Chim Acta	Analytica Chimica Acta
Biomed Res Int	BioMed Research International
Eur J Biochem	European Journal of Biochemistry
Forensic Sci Int	Forensic Science International
Forensic Sci Int Genet	Forensic Science International: Genetics
Forensic Sci Res	Forensic Sciences Research
Genome Res	Genome Research
Int J Mol Sci	International Journal of Molecular Sciences
J Biol Chem	Journal of Biological Chemistry
J Forensic Identific	Journal of Forensic Identification
J Forensic Sci	Journal of Forensic Sciences
J Proteome Res	Journal of Proteome Research
Mol Biol Rep	Molecular Biology Reports
Mol Reprod Dev	Molecular Reproduction and Development
Proc Nat Acad Sci USA	Proceedings of the National Academy of Sciences of the United States of America
Sci Justice	Science and Justice

BIBLIOGRAPHY

1. Gill P, Jeffreys AJ, Werrett DJ. Forensic application of DNA "fingerprints." *Nature*. 1985;318(6046):577–9. https://doi.org/10.1038/318577A0.

2. Ziegle JS, Su Y, Corcoran KP, Nie L, Eric Mayrand P, Hoff LB, et al. Application of automated DNA sizing technology for genotyping microsatellite loci. *Genomics*. 1992;14(4):1026–31. https://doi.org/10.1016/S0888-7543(05)80126-0.

3. A beginner's guide to RT-PCR, qPCR and RT-qPCR. 2020.

4. McDonald C, Taylor D, Linacre A. PCR in Forensic Science: A Critical Review. Genes (Basel). 2024;15(4):438. https://doi.org/10.3390/genes15040438.

5. Jiang MX, Zhu Y, Zhu ZY, Sun QY, Chen DY. Effects of cooling, cryopreservation and heating on sperm proteins, nuclear DNA, and fertilization capability in mouse. *Mol Reprod Dev*. 2005;72(1):129–34. https://doi.org/10.1002/mrd.20328.

6. DNA Fragment Analysis by Capillary Electrophoresis. 2014.

7. Mann SL, Patton WC, King A, Chan PJ. Comparative Genomic Hybridization Analysis of Sperm DNA Apoptosis After Exposure to Heat Shock. 2002.

8. HILZ H, WIEGERS U, ADAMIETZ P. Stimulation of Proteinase K Action by Denaturing Agents: Application to the Isolation of Nucleic Acids and the Degradation of 'Masked' Proteins. *Eur J Biochem*. 1975;56(1):103–8. https://doi.org/10.1111/j.1432-1033.1975.tb02211.x.

9. Jorban A, Lunenfeld E, Huleihel M. Effect of Temperature on the Development of Stages of Spermatogenesis and the Functionality of Sertoli Cells In Vitro. *Int J Mol Sci*. 2024;25(4):2160. https://doi.org/10.3390/ijms25042160.

10. Ng DPK, Koh D, Choo SGL, Ng V, Fu Q. Effect of storage conditions on the extraction of PCR-quality genomic DNA from saliva. *Clinica Chimica Acta.* 2004;343(1–2):191–4. https://doi.org/10.1016/j.cccn.2004.01.013.

11. Chacon-Cortes D, Haupt LM, Lea RA, Griffiths LR. Comparison of genomic DNA extraction techniques from whole blood samples: A time, cost and quality evaluation study. *Mol Biol Rep*. 2012;39(5):5961–6. https://doi.org/10.1007/S11033-011-1408-8.

12. Qamar W, Khan MR, Arafah A. Optimization of conditions to extract high quality DNA for PCR analysis from whole blood using SDS-proteinase K method. *Saudi J Biol Sci*. 2017;24(7):1465–9. https://doi.org/10.1016/J.SJBS.2016.09.016.

13. Santos ALF, Oliveira CQP, Arruda GNPN, Martins JK. Comparison of DNA extraction using proteinase K and extraction kit: Analysis of the quality of the genetic material. *J Bras Patol Med Lab*. 2018;54(2):70–5. https://doi.org/10.5935/1676-2444.20180013.

14. Lee SB, Mccord B, Buel E. Advances in forensic DNA quantification: A review. Electrophoresis. 2014.

15. Gill P, Bleka Ø, Fonneløp AE. Limitations of qPCR to estimate DNA quantity: An RFU method to facilitate inter-laboratory comparisons for activity level, and general applicability. *Forensic Sci Int Genet.* 2022;61. https://doi.org/10.1016/j.fsigen.2022.102777.

16. Centers for Disease Control and Prevention. CDC twenty four seven. Saving Lives, Protecting People Sexual Violence Prevention https://www.cdc.gov/sexual-violence/about/index.html.

17. National Crime Victimization Survey (NCVS) https://bjs.ojp.gov/data-collection/ncvs.

18. National Intimate Partner and Sexual Violence Survey (NISVS) https://www.cdc.gov/nisvs/about/index.html.

19. Wiegand P, Schiirenkamp M, Schiitte U. Legal Medicine DNA extraction from mixtures of body fluid using mild preferential lysis. 1992.

20. Lara AD. SPERM CELLS FROM COTTON SWABS. 2024.

21. Norris J V., Manning K, Linke SJ, Ferrance JP, Landers JP. Expedited, Chemically Enhanced Sperm Cell Recovery from Cotton Swabs for Rape Kit Analysis*. *J Forensic Sci*. 2007;52(4):800–5. https://doi.org/10.1111/j.1556-4029.2007.00453.x.

22. 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.

23. Hennekens CM, Cooper ES, Cotton RW, Grgicak CM. The Effects of Differential Extraction Conditions on the Premature Lysis of Spermatozoa ·. *J Forensic Sci*. 2013;58(3):744–52. https://doi.org/10.1111/1556-4029.12098.

24. 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.

25. Haned H, Benschop CCG, Gill PD, Sijen T. Complex DNA mixture analysis in a forensic context: Evaluating the probative value using a likelihood ratio model. *Forensic Sci Int Genet*. 2015;16:17–25. https://doi.org/10.1016/j.fsigen.2014.11.014.

26. Clark C, Turiello R, Cotton R, Landers JP. Analytical approaches to differential extraction for sexual assault evidence. *Anal Chim Acta.* 2021;1141:230–45. https://doi.org/10.1016/J.ACA.2020.07.059.

27. Hudson BC, Dawson Green T. Modified differential lysis for sexual assault samples using a combined enzymatic and alkaline approach. *Forensic Sci Res*. 2024;9(2). https://doi.org/10.1093/fsr/owae022.

28. 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.

29. Goldenberger D, Perschil I, Ritzler M, Altwegg M. A simple "universal" DNA extraction procedure using SDS and proteinase K is compatible with direct PCR amplification. *Genome Res*. 1995;4(6):368–70. https://doi.org/10.1101/GR.4.6.368.

30. Lounsbury JA, Nambiar SM, Karlsson A, Cunniffe H, Norris J V., Ferrance JP, et al. Enhanced recovery of spermatozoa and comprehensive lysis of epithelial cells from sexual assault samples having a low cell counts or aged up to one year. *Forensic Sci Int Genet*. 2014;8(1):84–9. https://doi.org/10.1016/j.fsigen.2013.06.015.

31. Kimpton CP, Oldroyd NJ, Watson SK, Frazier RRE, Johnson PE, Millican ES, et al. Validation of highly discriminating multiplex short tandem repeat amplification systems for individual identification. *Electrophoresis.* 1996;17(8):1283–93. https://doi.org/10.1002/ELPS.1150170802.

32. Lygo JE, Johnson RE, Holdaway • D J, Woodroffe • S, Whitaker JR, Clayton • T M, et al. The validation of short tandem repeat (STR) loci for use in forensic casework. 1994.

33. Butler JM, Buel E, Crivellente F, McCord BR. Forensic DNA typing by capillary electrophoresis using the ABI Prism 310 and 3100 genetic analyzers for STR analysis. *Electrophoresis.* 2004.

34. Bruijns BB, Tiggelaar RM, Gardeniers H. The Extraction and Recovery Efficiency of Pure DNA for Different Types of Swabs. *J Forensic Sci*. 2018;63(5):1492–9. https://doi.org/10.1111/1556-4029.13837.

35. Gill P, Sparkes R, Pinchin R, Clayton T, Whitaker J, J. Buckleton. Interpreting simple STR mixtures using allele peak areas. *Forensic Sci Int.* 1998;91(1):41–53. https://doi.org/10.1016/S0379-0738(97)00174-6.

36. Schellhammer SK, Hudson BC, Cox JO, Dawson Green T. Alternative direct-to-amplification sperm cell lysis techniques for sexual assault sample processing. *J Forensic Sci. 2022*;67(4):1668–78. https://doi.org/10.1111/1556-4029.15027.

37. Optimization and validation of a novel direct-lysis differential extraction procedure. 2018.

38. Hennekens CM, Cooper ES, Cotton RW, Grgicak CM. The Effects of Differential Extraction Conditions on the Premature Lysis of Spermatozoa,. *J Forensic Sci*. 2013;58(3):744–52. https://doi.org/10.1111/1556-4029.12098.

39. Arya R, Hudson BC, Green TD. Alternative direct-to-amplification cell lysis techniques for forensically relevant non-sperm cells. *Journal of Forensic Sciences*. 2023.

40. Liu JY. Direct qPCR quantification using the Quantifiler® Trio DNA quantification kit. *Forensic Sci Int Genet*. 2014;13:10–9. https://doi.org/10.1016/J.FSIGEN.2014.06.016.

41. Sample to Insight__ EZ1&2 ® DNA Investigator ® Kit Handbook For automated purification of DNA from forensic and human ID samples using EZ1 ® instruments 2. 2021.

42. Andersen JF, Greenhalgh MJ, Butler HR, Kilpatrick SR, Piercy RC, Way KA, et al. Further validation of a multiplex STR system for use in routine forensic identity testing. *Forensic Sci Int*. 1996;78(1):47–64. https://doi.org/10.1016/0379-0738(95)01861-1.

43. Ensenberger MG, Thompson J, Hill B, Homick K, Kearney V, Mayntz-Press KA, et al. Developmental validation of the PowerPlex® 16 HS System: An improved 16-locus fluorescent STR multiplex. *Forensic Sci Int Genet*. 2010;4(4):257–64. https://doi.org/10.1016/j.fsigen.2009.10.007.

44. Gadella BM, Luna C. Cell biology and functional dynamics of the mammalian sperm surface. *Theriogenology.* 2014;81(1):74–84. https://doi.org/10.1016/J.THERIOGENOLOGY.2013.09.005.

45. Berruti G, Paiardi C. Acrosome biogenesis. *Spermatogenesis.* 2011;1(2):95–8. https://doi.org/10.4161/spmg.1.2.16820.

46. 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.

47. Tecle E, Gagneux P. Sugar-coated sperm: Unraveling the functions of the mammalian sperm glycocalyx. *Molecular Reproduction and Development*. 2015.

48. Schröter S, Osterhoff C, Mcardle W, Ivell R. The glycocalyx of the sperm surface. 1999.

49. Young LG, Goodman SA. Characterization of Human Sperm Cell Surface Components.

50. Sheng K, Liang X, Huang S, Xu W. The Role of Histone Ubiquitination during Spermatogenesis. *Biomed Res Int*. 2014;2014:1–8. https://doi.org/10.1155/2014/870695.

51. Wykes SM, Krawetz SA. The Structural Organization of Sperm Chromatin. *Journal of Biological Chemistry.* 2003;278(32):29471–7. https://doi.org/10.1074/jbc.M304545200.

52. Sun S, Zhang X, Lan R, Xin M, Hao Z, You S, et al. Biological functions and large-scale profiling of protein glycosylation in human semen. *Journal of Proteome Research*. 2020.

53. Saraswat M, Joenväärä S, Tomar AK, Singh S, Yadav S, Renkonen R. N-Glycoproteomics of Human Seminal Plasma Glycoproteins. *J Proteome Res.* 2016;15(3):991–1001. https://doi.org/10.1021/acs.jproteome.5b01069.

54. Schmidt S, Weigelin B, Riet J te, Boekhorst V te, Lindert M te, Wijers-Rouw M, et al. Glycocalyx-mediated Cell Adhesion and Migration. 2020.

55. Schroter S. The glycocalyx of the sperm surface. *Hum Reprod Update*. 1999;5(4):302–13. https://doi.org/10.1093/humupd/5.4.302.

56. Lan R, Xin M, Hao Z, You S, Xu Y, Wu J, et al. Biological Functions and Large-Scale Profiling of Protein Glycosylation in Human Semen. *J Proteome Res.* 2020;19(10):3877–89. https://doi.org/10.1021/acs.jproteome.9b00795.

57. Thompson IOC, Van der Bijl P, Van Wyk CW, Van Eyk AD. A comparative light-microscopic, electron-microscopic and chemical study of human vaginal and buccal epithelium. *Arch Oral Biol.* 2001;46(12):1091–8. https://doi.org/10.1016/S0003-9969(01)00082-6.

58. Van Der Bijl P, Thompson IOC, Squier CA. Comparative permeability of human vaginal and buccal mucosa to water. *Eur J Oral Sci*. 1997;105(6):571–5. https://doi.org/10.1111/J.1600-0722.1997.TB00219.X.

59. Udogadi NS, Abdullahi MK, Bukola AT, Imose OP, Esewi AD. Forensic dna profiling: Autosomal short tandem repeat as a prominent marker in crime investigation. *Malaysian Journal of Medical Sciences.* 2020.

60. Analysis and interpretation of mixed forensic stains using DNA STR profiling - ScienceDirect. https://www.sciencedirect.com/science/article/abs/pii/S0379073897001758?via%3Dihub (accessed September 14, 2024).

61. Udogadi NS, Abdullahi MK, Bukola AT, Imose OP, Esewi AD. Forensic dna profiling: Autosomal short tandem repeat as a prominent marker in crime investigation. *Malaysian Journal of Medical Sciences*. 2020.

62. Özçelik B, Aytar P, Gedikli S, Yardimci E, Çalişkan F, Çabuk A. Production of an alkaline protease using Bacillus pumilus D3 without inactivation by SDS, its characterization and purification. *J Enzyme Inhib Med Chem.* 2014;29(3):388–96. https://doi.org/10.3109/14756366.2013.788503.

63. Ramos F. Analysis of Two Novel Differential Extraction Methods. 2023.

64. Goldenberger D, Perschil I, Ritzler M, Altwegg M. Technical A Simple "Universal" DNA Extraction Procedure Using SDS and Proteinase K Is Compatible with Direct PCR Amplification.

65. BAJORATH J, HINRICHS W, SAENGER W. The enzymatic activity of proteinase K is controlled by calcium. *Eur J Biochem*. 1988;176(2):441–7. https://doi.org/10.1111/j.1432-1033.1988.tb14301.x.

66. Voorhees JC, Ferrance JP, Landers JP. Enhanced elution of sperm from cotton swabs via enzymatic digestion for rape kit analysis. *Journal of Forensic Sciences*. 2006. p. 574–9.

67. Shahriar M, Haque MR, Kabir S, Dewan I, Bhuyian MA. Effect of proteinase-K on Genomic DNA extraction from gram-positive strains. *Stamford Journal of Pharmaceutical Sciences.* 2011;4(1):53–7. https://doi.org/10.3329/sjps.v4i1.8867.

68. Frazer Z, Yoo C, Sroya M, Bellora C, DeWitt BL, Sanchez I, et al. Effect of Different Proteinase K Digest Protocols and Deparaffinization Methods on Yield and Integrity of DNA Extracted From Formalin-fixed, Paraffin-embedded Tissue. *Journal of Histochemistry and Cytochemistry*. 2020;68(3):171–84. https://doi.org/10.1369/0022155420906234.

69. - Interchim U. Proteinase K.

70. Muiño-Blanco T, Pérez-Pé R, Cebrián-Pérez JA. Seminal plasma proteins and sperm resistance to stress. *Reproduction in Domestic Animals*. 2008;43(SUPPL.4):18–31. https://doi.org/10.1111/J.1439-0531.2008.01228.X.

71. Mótyán J, Tóth F, Tőzsér J. Research Applications of Proteolytic Enzymes in Molecular Biology. *Biomolecules.* 2013;3(4):923–42. https://doi.org/10.3390/biom3040923.

72. Adekoya OA, Sylte I. The Thermolysin Family (M4) of Enzymes: Therapeutic and Biotechnological Potential. *Chemical Biology & Drug Design.* 2009;73(1):7–16. https://doi.org/10.1111/j.1747-0285.2008.00757.x.

73. EBELING W, HENNRICH N, KLOCKOW M, METZ H, ORTH HD, LANG H. Proteinase K from Tritirachium album Limber. *Eur J Biochem.* 1974;47(1):91–7. https://doi.org/10.1111/j.1432-1033.1974.tb03671.x.

74. Naaby-Hansen S, Herr JC. Heat shock proteins on the human sperm surface. *J Reprod Immunol*. 2010;84(1):32–40. https://doi.org/10.1016/J.JRI.2009.09.006.

75. Gray A, Kuffel A, Daeid NN. An improved rapid method for DNA recovery from cotton swabs. *Forensic Sci Int Genet*. 2023;64:102848. https://doi.org/10.1016/J.FSIGEN.2023.102848.

76. Horsman KM, Hickey JA, Cotton RW, Landers JP, Maddox LO. Development of a human-specific real-time PCR assay for the simultaneous quantitation of total genomic and male DNA. *J Forensic Sci.* 2006;51(4):758–65. https://doi.org/10.1111/J.1556-4029.2006.00183.X.

77. Timken M, Swango K, Orrego C, Date Chong M, Buoncristiani M. Quantitation of DNA for Forensic DNA Typing by qPCR. 2005.

78. Dinis V, Bento AM, Teixeira AL, Gouveia N, Bogas V, Porto MJ, et al. Comparative study between a direct DNA quantification methodology and the standardized methodology in the forensic workflow. *Forensic Sci Int Genet Suppl Ser*. 2017;6:e216–7. https://doi.org/10.1016/j.fsigss.2017.09.076.

79. Bille T, Weitz S, Buckleton JS, Bright JA. Interpreting a major component from a mixed DNA profile with an unknown number of minor contributors. *Forensic Sci Int Genet*. 2019;40:150–9. https://doi.org/10.1016/j.fsigen.2019.02.017.

80. Yakoo M. The development and optimization of a direct lysis differential extraction method. 2017.

81. Saul DJ, Williams LC, Toogood HS, Daniel RM, Bergquist PL. Sequence of the gene encoding a highly thermostable neutral proteinase from Bacillus sp. strain EA1: expression in Escherichia coli and characterisation. Biochimica et Biophysica Acta (BBA) - *Gene Structure and Expression*. 1996;1308(1):74–80. https://doi.org/10.1016/0167-4781(96)00074-7.

82. Moss D, Harbison S-A, Saul DJ. An easily automated, closed-tube forensic DNA extraction procedure using a thermostable proteinase. *Int J Legal Med*. 2003;117(6):340–9. https://doi.org/10.1007/s00414-003-0400-9.

83. Biedermann K, Jepsen PK, Riise E, Svendsen I. Purification and characterization of a Serratia marcescens nuclease produced by Escherichia coli. *Carlsberg Res Commun*. 1989;54(1):17–27. https://doi.org/10.1007/BF02910469.

84. Eaves GN, Jeffries CD. ISOLATION AND PROPERTIES OF AN EXOCELLULAR NUCLEASE OF *SERRATIA MARCESCENS*. *J Bacteriol*. 1963;85(2):273–8. https://doi.org/10.1128/jb.85.2.273-278.1963.

85. forensicGEM Universal: Extract DNA for forensic applications. MicroGEM.

https://microgembio.com/product/forensicgem-universal-dna-extraction-kit/

(accessed July 16, 2024).

86. Adamowicz MS, Stasulli DM, Sobestanovich EM, Bille TW. Evaluation of methods to improve the extraction and recovery of DNA from cotton swabs for forensic analysis. *PLoS One*. 2014;9(12). https://doi.org/10.1371/JOURNAL.PONE.0116351.

87. Norris J V., Manning K, Linke SJ, Ferrance JP, Landers JP. Expedited, chemically enhanced sperm cell recovery from cotton swabs for rape kit analysis. *J Forensic Sci*. 2007;52(4):800–5. https://doi.org/10.1111/j.1556-4029.2007.00453.x.

88. Grinnell F. Cellular Adhesiveness and Extracellular Substrata. *Int Rev Cytol*. 1978;53(C):65–144. https://doi.org/10.1016/S0074-7696(08)62241-X.

89. Pethica BA. The physical chemistry of cell adhesion. *Exp Cell Res*. 1961;8(SUPPL.):123–40. https://doi.org/10.1016/0014-4827(61)90344-5.

90. Loomis WF, Fuller D, Gutierrez E, Groisman A, Rappel W-J. Innate Non-Specific Cell Substratum Adhesion. *PLoS One*. 2012;7(8):e42033. https://doi.org/10.1371/journal.pone.0042033.

91. Takeichi M. CADHERINS: A MOLECULAR FAMILY IMPORTANT IN SELECTIVE CELL-CELL ADHESION. *Annu Rev Biochem*. 1990; 59(1):237–52. https://doi.org/10.1146/annurev.bi.59.070190.001321.

92. Hammer D, Saxman K, Torney D, Dembo M. The reaction-limited kinetics of membrane-to-surface adhesion and detachment. . . 1988.

93. Khalili A, Ahmad M. A Review of cell adhesion studies for biomedical and biological applications. *International Journal of Molecular Sciences*. 2015.

94. Hajizadeh K, Mehdian H, Hajisharifi K, Robert E. A van der Waals force-based adhesion study of stem cells exposed to cold atmospheric plasma jets. *Sci Rep*. 2022;12(1):12069. https://doi.org/10.1038/s41598-022-16277-1.

95. Kendall K, Roberts AD. van der Waals forces influencing adhesion of cells. *Philosophical Transactions of the Royal Society B: Biological Sciences*. 2015;370(1661):20140078. https://doi.org/10.1098/rstb.2014.0078.

96. Roberts, A. Optimization of a novel temperature controlled differential extraction procedure for aged sample analysis. 2019.

CURRICULUM VITAE