Boston University

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

Boston University Theses & Dissertations Boston University Theses & Dissertations

2024

Optimizing an elution buffer to improve the recovery of sperm cells from cotton swabs

https://hdl.handle.net/2144/51799

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

BOSTON UNIVERSITY

ARAM V. CHOBANIAN & EDWARD AVEDISIAN SCHOOL OF MEDICINE

Thesis

OPTIMIZING AN ELUTION BUFFER TO IMPROVE THE RECOVERY OF SPERM CELLS FROM COTTON SWABS

by

ASHLEY DANIELLE LARA

B.S., University of California, Los Angeles, 2019

Submitted in partial fulfillment of the

requirements for the degree of

Master of Science

2024

© 2024 by
ASHLEY DANIELLE LARA
All rights reserved

Approved by

First Reader

Robin W. Cotton, Ph.D.
Associate Professor of Anatomy & Neurobiology
Program in Biomedical Forensic Sciences

Second Reader

Sylvia Baehman, M.S.
Criminalist II
St. Louis Metropolitan Police Department

Third Reader

Mike Yakoo, M.S.
Criminalist II

ACKNOWLEDGMENTS

I would like to thank my family for the unconditional love and support they have shown throughout every part of my life. Thank you for always encouraging me to never stop learning and to follow my dreams wherever they take me. To my best friend, thank you for being there for me through the ups and downs of graduate school, and for your endless belief in me. To my parents, I promise to continue making you proud of the daughter you raised.

Thank you to the life-long friends I made in this program. Despite the many challenges we encountered along the way, we completed this journey together, and I cannot imagine a better group of people to have done this with. I hold the memories we made together close to my heart, and I cannot wait to see the amazing things you all go on to accomplish.

To the faculty, thank you for allowing me the opportunity to learn from you. You each enriched me with vital skills and knowledge that I will carry for the rest of my life.

Lastly, I would like to especially thank Dr. Robin Cotton. Working with you for the past year has truly been the honor of my life. Thank you for trusting me with this research. Through all of the countless uncertainties this project presented, your guidance, wisdom, and infectious optimism always inspired me to keep going. You have instilled in me an even greater love for science than I thought possible, and I promise to nurture that love forever.

OPTIMIZING AN ELUTION BUFFER TO IMPROVE THE RECOVERY OF SPERM CELLS FROM COTTON SWABS

ASHLEY DANIELLE LARA

ABSTRACT

Sexual assault samples often contain a complex, disproportionate mixture of male sperm cells, male epithelial cells, and female epithelial cells, and require the precise, independent extraction of each cell type. This differential extraction (DE) procedure involves the initial lysis of male and female epithelial cells to release both male and female deoxyribonucleic acid (DNA). Male sperm cells, immune to this enzymatic treatment, remain intact and can then be extracted separately. In theory, this produces a clean female epithelial fraction (EF) and male sperm fraction (SF). In practice, however, various complications can occur that jeopardize the acquisition of a single-source male and female DNA profile. Perhaps most threatening is the well-documented poor release of sperm cells into the SF due to strong adhesion of the cells to the sample swab. While the exact mechanism for how the sperm adhere to cotton is unknown, surface glycoproteins that comprise the sperm glycocalyx are believed to play a vital role. This research sought to develop an elution solution that improves sperm cell recovery from cotton swabs and that can be easily integrated into commonly practiced DE procedures.

Cotton swabs stained with either 53 nanograms (ng) or 100 ng of male sperm DNA were incubated in one of several solutions for various times and temperatures. Solutions of sucrose, histidine, polyvinylpyrrolidone (PVP) and Tween 20, and bovine serum albumin (BSA) and Tween 20 were selected for this study based on their recovery

success demonstrated in past research, as well as to investigate if these small, soluble molecules could displace sperm cells from the swab. Solutions containing proteinase K (PK), PK and sodium dodecyl sulfate (SDS), and *forensic*GEM™ *Bacillus* sp. *Erebus antarctica* 1 (EA1) were tested to explore the activity of these proteases on the sperm glycoproteins. The eluate and material fraction (MF) from each sample were extracted and quantitated to measure the amount of sperm DNA recovered from the SF, as well as the amount of DNA left on the swab.

Of all solutions tested in this study, samples incubated *forensic*GEM™ EA1 returned the highest average recovery yields with up to 80% of total DNA extracted from the SF. Though more research is needed to understand the exact enzymatic mechanism at play, these results suggest that *forensic*GEM™ EA1 exhibits activity on the sperm glycoproteins that facilitates greater release of sperm cells into solution. Before these solutions can be incorporated into current DE protocols, validation tests using mock sexual assault samples are necessary.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Asn	Asparagine
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
Ca^{2+}	Calcium(2+)
$CaCl_2$	Calcium chloride
CAMs	Cellular adhesion molecules
DE	Differential extraction
DI	Deionized
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EA1	*Erebus antarctica* 1
ECM	Extracellular matrix
EF	Epithelial fraction
g	Grams
HSPs	Heat shock proteins
Ig	Immunoglobulin
MF	Material fraction
µL	Microliter
mg	Milligrams
mL	Milliliter
mM	Millimolar

M	Molar
ng	Nanograms
nm	Nanometers
PCR	Polymerase chain reaction
PK	Proteinase K
PVP	Polyvinylpyrrolidone
qPCR	Quantitative polymerase chain reaction
RFLP	Restriction fragment length polymorphism
rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
Ser	Serine
SF	Sperm fraction
SN	Supernatant
STR	Short tandem repeat
TE	Tris-EDTA
Thr	Threonine
U	Units

1. INTRODUCTION

1.1 The Role of DNA in Inheritance

Housed within the nuclei of cells is the molecule responsible for providing life to all organisms–deoxyribonucleic acid (DNA). DNA functions as a blueprint, carrying the biological code that makes up an organism's entire genome (1). Marked by approximately 99.9% similarity, there exists very few differences between the genome of two, unrelated humans (2). These miniscule differences, however, provide the genetic variation not only crucial to world populations but also forensic DNA analysis. In humans, genetic variation emerges from sexual reproduction when a haploid male and female gamete fuse to form a zygote (3). At this stage, the individual chromosomes inherited from each parent undergo DNA replication then recombination in which genetic information is exchanged between the male and female chromosomes resulting in a new, unique DNA sequence (4). An offspring genetically different from either parent is now produced.

1.2 Spermatozoa

1.2.1 Spermatogenesis and the Structure of Spermatozoa

Spermatogenesis is the development of mature spermatozoa that occurs within the seminiferous tubules of the testes (5,6). This process begins as unspecialized male germ cells, spermatogonia, that line the basal lamina multiply and differentiate into Dark Type A, Pale Type A, and Type B spermatogonia (5). Type B spermatogonia undergo mitosis to become primary spermatocytes, secondary spermatocytes, and spermatids (5). Meiotic

division of primary spermatocytes results in the formation of haploid spermatids, equipped with a novel genome (5). During spermiogenesis, the final stage of spermatogenesis, haploid spermatids transition into spermatozoa, and the chromatin becomes tightly packaged by protamines (5).

Another marked structural alteration of the spermatozoa that occurs during spermiogenesis is the formation of the head and the flagellum (5). Within the head resides the nucleus and the acrosome, an organelle critical to fertilization that is packed with hydrolytic enzymes (7,8). When a spermatozoon makes initial contact with the female egg, the acrosome reaction is initiated, releasing the hydrolytic enzymes via exocytosis that then digest the female zona pellucida (7). This reaction facilitates the passage of spermatozoa inside the egg (7). Absence of the acrosome is largely linked to globozoospermia, a male infertility condition that results in the production of abnormally rounded spermatozoa heads incapable of effectively tunneling through the female egg (7,9).

The flagellum is a tail-like structure that extends away from the head and gives spermatozoa their essential motility (7). The flagellum propels the spermatozoa on the journey through the male reproductive tract and assists in penetrating the female egg (7). The midpiece, located at the base of the tail, serves as storage for the mitochondria that produce the high volume of adenosine triphosphate (ATP) that fuels flagellar movement (7).

1.2.2 DNA Packaging in Sperm Cells

Throughout spermatogenesis, the packaging of sperm cell DNA undergoes dramatic changes. In the early stages, the DNA of spermatogonia and primary spermatocytes are packaged by histone proteins, in much the same manner as epithelial cells (6). Following meiosis, most of these histones are degraded and replaced by transition proteins which are then, in turn, displaced by protamines (6).

In humans, there exists two forms of protamines: P1 and P2 (6). P2 is further categorized as P2a and P2b (6). P1, P2a, and P2b are 50, 54, and 57 amino acids in length, respectively (6). Protamines are highly positively charged proteins, owed primarily to their composition of approximately 70% arginine, making them effective at binding the negatively charged DNA backbone (10). Disulfide bonds between cysteine residues connect protamines together, lending great stabilization to the chromatin during the end stages of spermatogenesis (10).

Protamines are hypothesized to have several important functions. First, protamines are crucial in compacting the sperm chromatin DNA strand (10). This tight packaging is essential in the formation of a healthy sperm head, as well as the safe transport of the genome through the male and female reproductive tracts (10,11). Secondly, protamines facilitate the cessation of transcription (10). This finding was recognized by a 1975 study performed by Kierszenbaum and Tres which found that reduced production of RNA correlated to the elevated presence of "coarse protein aggregates," increased uptake of arginine residues, and the modification of the chromatin fibers which are characteristics associated with the presence of protamines (12).

1.2.3 The Sperm Glycocalyx

On the surfaces of all cells exists the glycocalyx–a dense forest of proteins, lipids, and sugars (13,14). This complex, polar network is composed of a thicket of glycoproteins and elaborate glycan chain attachments that form associations with the cell membrane (13). The base layer of the glycocalyx consists of receptors, integral membrane proteins, and glycans, and functions as a communication interface between the cell's internal and external environment (15). The outer layer is made of proteins and polysaccharides that help maintain the overall structural integrity (15).

Approximately 92% of the proteins that comprise the glycocalyx of all cell types are glycosylated (16). This type of post-translational modification ensures proteins are in the proper configuration to form critical interactions with chaperone proteins (13,17). Size and complexity of the glycocalyx, however, vary between cell types. In sperm cells, the glycocalyx measures between 20-60 nanometers (nm) thick and is estimated to be comprised of approximately 50-150 different glycoconjugates (16).

Synthesis of the sperm glycocalyx begins in the seminiferous tubules of the testes, where new glycans are created and subsequently modified into new glycoconjugates by the sperm cell's endoplasmic reticulum/Golgi (13). Once spermatogenesis is complete, the mature sperm cells begin their journey through the epididymis where new glycans can no longer be synthesized due to the loss of the Golgi apparatus (13). Pre-existing glycans, however, can be modified via glycosylation, and novel glycans can be acquired during travel through the epididymis (13). During maturation, sperm adopt an overall

negative charge as the glycocalyx is continually modified (13,16). This is thought to be the consequence of the addition of sialic acid or acidic sperm antigens to the glycocalyx network (16). The composition of the glycocalyx continues to be altered during capacitation where glycoconjugates are modified or redistributed, and sialic acids are removed, thus priming the sperm cells for binding to the female oocyte (17).

The sperm glycocalyx plays important roles in various cell functions, including movement and fertilization. The various constituents of the glycocalyx contribute to the stickiness of all cell types and permit interactions between the cell and components in the extracellular matrix (ECM), namely collagen (18). It is hypothesized that a plethora of these nano-scale interactions fuels cell movement through the ECM (18).

Male infertility has been associated with abnormal glycoproteins in the sperm glycocalyx, suggesting that successful fertilization is dependent on the development of the glycocalyx (17). A functional glycocalyx allows sperm cells to pass through the cervical mucus and evade detection by antibodies and immune cells within the uterus (13). Once in the uterus, proper protein folding proves integral as sperm cells with abnormally folded glycocalyx proteins seldom survive the voyage through the uterotubal junction (13). If the sperm cell survives to reach the egg, the sperm-egg initial interaction occurs between the sperm glycocalyx and zona pellucida (17). Some researchers believe that proper adhesion of a sperm cell to the egg is heavily dependent on the glycan interactions between these cells (17).

1.3 Forensic DNA Analysis

1.3.1 The Past and Present

In 1985, Sir Alec Jeffreys made his "eureka" discovery that would create the field of forensic DNA analysis. Sir Jeffreys found that within certain regions of the human genome there existed variation so great that these loci could be used to individualize human beings (19). The variation of these hypervariable minisatellite regions resides in the unique number of repeating blocks of DNA that present at these genomic locations (20). Sir Jeffreys also determined that these minisatellite regions are highly conserved between somatic cell types and are passed from parent to offspring via Mendelian inheritance (19,20). Based on these criteria, Sir Jeffreys saw the potential forensic application of DNA for identification purposes (21).

Beginning in the early 1990s, forensic DNA testing underwent a transition from restriction fragment length polymorphism (RFLP) analysis of minisatellites to autosomal short tandem repeat (STR) testing of microsatellites, which remains standard practice in forensic labs in the United States (22). Microsatellites comprise approximately 1-3% of the eukaryotic genome and exist within non-coding regions (23). Like minisatellites, microsatellites owe their polymorphic nature to a variation in the number of tandem repeats at these loci; however, microsatellite repeated blocks are much smaller, totaling around 10-100 base pairs in length (24). Their small size proves to be an advantage, allowing them to be amplified and analyzed with the polymerase chain reaction (PCR) (24). With the addition of specific reagents and heat, PCR can produce billions of copies of DNA from a single molecule (25). Due to its great sensitivity, PCR has been

incorporated into STR testing, thus allowing for the analysis of low template DNA samples containing less than 1 nanogram (ng) of DNA. Furthermore, STR testing has proven to be faster than the early RFLP methods and use of PCR allows for STR multiplexing in which multiple loci can be analyzed at once (22).

1.3.2 The Steps of DNA Typing

Forensic DNA typing follows a four-step technical procedure. The first step is DNA extraction in which chemical, enzymatic, or physical methods are used to lyse the cellular and nuclear membranes in order to release DNA into solution (26). Other cellular components, including inhibitors, may be co-extracted at sufficient concentrations to interfere with downstream reactions (26). Therefore, it may be necessary to purify the sample after extraction (26). Next, the concentration of DNA in the sample is determined via quantitative PCR (qPCR). During quantitation, *Taq* polymerase, a thermally stable DNA polymerase, assembles new DNA strands complementary to the template (27). The quantity of amplicons can be measured throughout the reaction by the strength of a fluorescent signal that is produced with the construction of each new strand (28). The final concentration of the amplicons from this reaction can then be used to calculate the starting concentration of DNA in the original sample (27). After quantitation, the sample undergoes PCR amplification for multiple STR loci during which colored fluorescent labels are coupled to the newly synthesized DNA strand (29). These fluorescent labels are bound to primers designed to recognize the sequence of one of the target STR loci (29). During the final step, capillary

electrophoresis, these labeled DNA fragments are separated by size and identified by the color of their fluorescent tag. A window at the end of the capillary allows the detection of the fluorescent signal via a camera. This data is displayed using specialized software and visualized as a DNA profile (30). A thorough analysis of the generated profile is then conducted by a DNA analyst to assess the overall reliability of the data, differentiate true allele peaks from artifacts, and determine the number of contributors (31).

1.4 Differential Extraction

When performing DNA typing, some forensic evidence samples prove more challenging than others. Sexual assault samples, for example, often contain a complex, disproportionate mixture of male and female DNA. The goal of DNA extraction is to lyse all cells contained within a sample to maximize the amount of DNA recovered. The simultaneous extraction of all cell types in sexual assault samples, however, would produce a mixed DNA profile with male and female alleles and could result in the complete loss of signal from the minor contributor. Consequently, the male sperm cells, male epithelial cells, and female epithelial cells typically found on these samples must be lysed separately.

In 1985, Gill et al. published a selective lysis method that allows for the independent extraction of male sperm cells and epithelial cells (21). This method, differential extraction (DE), begins with the enzymatic digestion of epithelial cell membrane proteins using a solution of proteinase K (PK), a serine protease, and sodium dodecyl sulfate (SDS), an anionic detergent (21). While effective at lysing epithelial

cells, this cocktail is unable to cleave the disulfide bonds caging the sperm cell (32). Centrifugation forces the intact sperm cells to collect at the bottom of the sample tube while epithelial DNA remains in the supernatant (21). The epithelial fraction is removed, and the male sperm cells are extracted with dithiothreitol (DTT), a compound capable of reducing disulfide bonds (33). The now separated sperm and epithelial fractions can then be carried through the rest of the standard DNA typing procedure.

Many complications threaten the successful execution of a differential extraction. First, consequential sperm cell loss, especially after long periods, may occur due to the inhospitable conditions of the anatomical sites from which these samples are commonly collected (34). Furthermore, given that these samples are typically collected from female victims, the number of female cells may greatly outnumber available male DNA (35). In these samples, inadequate separation of male and female DNA can result in the total loss of signal from the male contributor (35). Lastly, the sample collection tool, itself, functions as a major obstacle as it has been shown that large proportions of male and female cells remain adhered to the cotton swab at the conclusion of the procedure (36).

1.5 DNA Recovery

1.5.1 Forensic Significance

Despite the great progress made in improving the sensitivity and specificity of DNA detection methods, obstacles to obtaining clear, interpretable DNA profiles persist. One of the biggest challenges facing forensic DNA analysts today is how to improve

DNA recovery from substrates. Seldom do samples in ideal condition arrive in the laboratory for processing. Low availability of biological material may yield quantities of DNA unsuitable for testing; DNA can become degraded if exposed to certain environmental conditions; or, as in cases of sexual assault, samples may contain a complex mixture of multiple individuals (37). In these circumstances, lack of sufficient DNA recovery can result in allelic dropout at some loci, or complete loss of signal from a minor contributor (37). Improved DNA recovery requires ample collection of cells from the substrate as well as the adequate release of the cells from the collection tool. Therefore, there may be value in exploring both the procedural avenues that may ameliorate current elution and extractions methods, as well as the underlying mechanism by which cells cling to substrates (37).

1.5.2 Proposed Mechanisms of Cellular Adhesion

Cellular adhesion, a biological phenomenon that allows cells to adhere to neighboring cells or substrates through ionic and hydrogen bonds, plays a critical role in a variety of essential physiological functions, including the maintenance of tissue structure, cell migration, and cell communication (38,39). The strength of adhesive forces exhibits a positive relationship with the number of available, unbonded adhesion molecules on a given surface (40,41). After numerous investigations to more intimately understand the mechanism(s) behind cellular adhesion, studies point to van der Waals interactions and cellular adhesion molecules (CAMs) as potential explanations.

1.5.2.1 Van der Waals Forces

Van der Waals forces are the product of permanent or temporary dipoles induced between closely apposed charged materials, such as adjacent cells or cells and a substrate (42). The strength of van der Waals interactions depends upon the distance between interacting charges, cell size, and the roughness of the cell surface (42–44). Weak compared to other bond types, van der Waals interactions are still regarded as effective adhesion forces due to their prevalence across all molecules (44).

1.5.2.2 CAMs

Amongst the complex jungle of molecules teeming the cell membrane exists a family of specialized surface proteins that play an important role in cellular adhesion—CAMs. CAMs facilitate a range of adhesion interactions, including homotypic interactions between twin cell types, heterotypic interactions between different cell types, and interactions between cells and the ECM (45). CAMs are categorized into four distinct groups based on their molecular structure: cadherins, integrins, the immunoglobulin superfamily, and selectins (45).

Cadherins foster the development of vital biological structures, including embryos, the epithelial layer of the skin and intestines, and the axons of the nervous system (45,46). These transmembrane proteins consist of a cytoplasmic tail and an extracellular calcium-binding region that enables homotypic intercellular interactions with neighboring cadherins (45,47).

Integrins are comprised of an alpha and beta subunit tethered together by noncovalent bonds and represent the primary receptor proteins through which cells communicate with the ECM (45,48). Divalent cation binding sites on the extracellular portion of the alpha and beta subunits determine the affinity and specificity of integrin-ligand interactions and permit the reversible adhesion of cells to cells, and cells to the ECM (45,48). Integrins can be further classified by their beta subunit type which each have unique purposes in various biological processes, such as platelet aggregation and hemostasis, organ formation, and leukocyte migration to inflamed areas of tissue (45,49,50).

Members of the immunoglobulin superfamily participate in the development of the nervous system and coordinate immune and inflammatory responses (45,51). The defining feature of this diverse group of receptor proteins is the existence of at least one extracellular immunoglobulin (Ig)-like domain, characterized by two anti-parallel beta-pleated sheets anchored together by disulfide bonds (45,52). Ig-like domains mediate binding to integrins, carbohydrates or other Ig-like domains (45,52).

Selectins are a class of surface glycoproteins that have been found to perform dichotomous physiological roles as facilitators of hemostasis and the immune response, as well as cancer metastasis (53). An N-terminal lectin domain on the structure of selectins allows for calcium(2+) (Ca^{2+}) dependent binding to neighboring cell surface carbohydrates (45,53).

1.5.3 The Cotton Conundrum

Another factor which undermines DNA recovery is the tool used to collect biological samples. The use of cotton swabs for biological sample collection has long been standard practice in the field of forensics due, in part, to the affordability and success of cotton swabs in recovering cells from surfaces (54). Cotton's ability to adsorb cells is thought to be a consequence of its chemical composition (54). Cotton fibers are composed primarily of cellulose chains linked together by hydrogen bonds between neighboring hydroxyl groups (55,56). Along with maintaining the structure of the fiber, these hydroxyl groups have inter- and intramolecular hydrogen bond potential which would allow the fiber to bind to the polar components on the surface of the cell membrane (54–56).

The interactions between cells and cotton fibers, however, have proven "too effective" as attempts to remove the cells during post-collection evidence processing have demonstrated less than ideal recovery (37). A 2014 study by Adamowicz et al. found that, when following the Qiagen QIAmp DNA Investigator standard elution protocol, 50% of buccal cell DNA and 80% of blood DNA remained on the swab (37). While innocuous in high quantity, single source samples, results like these can prove problematic in complex sample types and have prompted researchers to develop improved recovery techniques. Alternative swabs have been investigated and shown to return higher DNA yields than traditional cotton swabs; however, given the ubiquity and low cost of cotton, a transition to these modern options is unlikely (57,58). Instead,

researchers have turned their attention towards designing an elution buffer that encourages the release of cells from cotton and optimizing incubation parameters.

1.5.4 Recent Strategies to Improve DNA Recovery

1.5.4.1 Sucrose and Histidine

It has been proposed that van der Waals interactions between cells and a substrate can be undermined by the presence of a sufficient concentration of small, soluble molecules with similar chemical properties as the cell surface glycoproteins (59). A 2012 study by Loomis et al. attempted to test this theory by depositing *Dictyostelium discoideum* amoeba cells onto different substrates within several microfluidic chambers then exposing the cells to glucose and amino acid solutions at increasing levels of hydrodynamic shear stress (60). Although *Dictyostelium discoideum* lack genes associated with certain cellular adhesion proteins, it has been observed that they are able to reversibly bind to substrates of various compositions (60). Loomis and his team hypothesized that, in the absence of these proteins, van der Waals forces could provide a possible explanation for adhesion (60).

Glucose is a six-carbon monosaccharide that serves as vital energy source for all organisms (61,62). It is also an essential building block of numerous carbohydrates including glycogen, glycoproteins, and proteoglycans (62). Sucrose is a highly polar disaccharide composed of one glucose and one fructose molecule (63). Eight hydroxyl groups and two anomeric carbons are part of its structure (63).

Histidine is an amino acid that plays a critical role in maintaining physiological function and chelating metallic ions (64,65). Structurally, histidine is composed of a polar hydrogen and a basic nitrogen which both confer hydrogen bond potential (65).

After incubation in the glucose and amino acid solutions, Loomis et al. found that each solution had a positive effect on dislodging the cells from most substrates after a 40-minute incubation (60). In addition to the mixture of twelve amino acids, these researchers also tested four amino acids individually—valine, arginine, histidine, and glycine (60). Histidine appeared to have the most success in displacing the highest quantity of cells (60). Loomis et al. concluded that the sugar moieties, specifically the number of hydroxyl groups present, and the peptide spines of cell surface glycoproteins play a sizeable role in van der Waals interactions and, thus, cellular adhesion (60). Lending further support to this hypothesis are the results from a 2020 study by Speidel which found that an average of 52% of epithelial cells could be recovered from a cotton swatch when incubated in a sucrose solution (66).

1.5.4.2 BSA, PVP, and Tween 20

Bovine serum albumin (BSA), polyvinylpyrrolidone (PVP), and Tween 20 have also been the focus of a DNA recovery study. In 2023, Gray et al. methodically tested, individually and in combination, several ingredients of a PCR reaction mix to determine which component(s) had a positive effect on the recovery of free and cellular DNA from cotton swabs (67). Gray and his team found that up to 80% of cellular DNA could be

recovered from swabs using an extraction buffer containing either BSA and Tween 20, or PVP and Tween 20 (67).

BSA is a scavenger protein commonly used in forensic applications due to its ability to inactivate PCR inhibitors that would otherwise interfere with the *Taq* polymerase (68). BSA has also proven valuable in prolonging *in vitro* sperm viability and reducing sperm adhesion to glass surfaces (69,70).

PVP is a polymer that readily dissolves in water (71). In addition to also being effective at neutralizing PCR inhibitors, higher DNA return has been demonstrated in Japanese larch wood samples extracted in the presence of PVP (72,73). In the Gray et al. study, BSA was replaced by PVP in their extraction buffer formulation due to the compatibility of PVP with PK (67).

Tween 20 is a non-ionic detergent comprised of hydrophobic and hydrophilic regions that can selectively solubilize lipids and proteins within and along the cell membrane (74,75). Stronger PCR signal during DNA amplification has also been shown to occur with the addition of Tween 20 to the PCR reaction mix (76).

1.5.4.3 Temperature

Past studies have also manipulated incubation temperature in an attempt to improve DNA recovery. Two separate studies by Hennekens et al. and Lounsbury et al. investigated the effect of temperature on sperm cell recovery by incubating samples in various thermal conditions (77,78). Temperatures in both studies, however, were kept under 56°C due to the belief that temperatures above that threshold would severely

compromise recovery (79). While high temperatures pose a serious threat to sperm viability *in vivo*, the disulfide bonds caging sperm DNA lend stability in extreme temperatures (80,81). Furthermore, several heat shock proteins (HSPs) have been found on the surface of ejaculated sperm (82). HSPs function to refold denatured proteins after exposure to various stressors, including intense heat (82). Both characteristics suggest that sperm cells have to potential to remain intact in warmer temperatures *in vitro*. Thus, examining the behavior of these molecules in warmer thermal conditions may reveal the possible benefits of a higher incubation temperature in sperm cell recovery.

Temperature is a measurement of the average kinetic energy of atoms and molecules (83). When a substance experiences an increase in temperature, and gains thermal energy, the kinetic energy of the molecules comprising that substance also increases (83). The molecules begin to move at higher velocities which, in turn, results in the weakening and complete breaking of hydrogen bonds between molecules (83,84). Conversely, as temperatures cool, and kinetic energy decreases, molecules begin to slow before reaching a total stop at absolute zero (83). Lowering the temperature has been shown to promote hydrogen bond formation in solutions (85).

It is well-established that van der Waals forces function independently of temperature (86). They do, however, depend on distance as stronger van der Waals interactions exist between materials in close proximity (86). Increased molecular speed in high temperatures allows the molecules to spread farther apart (83,86). Therefore, under high thermal stress, van der Waals forces become insignificant (86). Cold

temperatures should prove favorable conditions for the formation and overall strength of van der Waals interactions as molecules slow and move closer together.

If hydrogen bonds and van der Waals forces promote the adhesion between sperm cells and the cellulose network of a cotton swab, it stands to reason that a warmer incubation temperature could encourage the release of sperm cells and, thus, improve recovery.

1.5.4.4 Enzymes

Enzymes are commonly used in forensic procedures to extract DNA from various cell types, including the epithelial cells present in sexual assault samples. In traditional DE methods, these enzymes are used in the first step to lyse the epithelial cells. Following this incubation, the liquid absorbed onto the swab is then spun off to produce two fractions—epithelial DNA in the supernatant and a sperm pellet. Successful generation of a sperm and epithelial profile at the conclusion of the procedure relies on the successful separation of cell types after the first enzymatic incubation. The enzymes typically used in these extractions, *Bacillus* sp. *Erebus antarctica* 1 (EA1) and PK, are incapable of cleaving the disulfide bonds encasing the sperm cell; however, the activity of these enzymes on the glycoproteins comprising the sperm glycocalyx deserves exploration.

EA1 is a microbial, thermophilic serine proteinase capable of hydrolyzing peptide bonds (87–89). It is sold commercially by MicroGEM as *forensic*GEM™ and exhibits optimal activity at 75°C and pH 7.5 (90). The thermal stability of EA1 proves extremely

beneficial as it operates at a temperature at which nucleases are degraded (91). EA1 requires the presence of calcium and denatures when heated above 90°C (88).

Like EA1, PK is also a calcium-dependent serine peptidase capable of cleaving membrane proteins and breaking down nucleases (77,92,93). However, PK is not thermophilic and displays optimal activity between 50-56°C and pH 7.5-8.5 (94). PK is irreversibly inactivated when heated to temperatures between 70-95°C (95).

1.6 Objectives

Experiments performed as part of this research attempted to design an elution buffer that would encourage the release of sperm cells from cotton swabs. Components of the elution buffers were chosen based upon hypotheses that they would either competitively bind to the cellulose matrix or enzymatically cleave the peptide bonds within the sperm glycocalyx. In pursuit of this principal objective, this research also aims to better understand the adhesion mechanism that allows sperm cells to cling well to the cotton fibers. Lastly, this work aimed to develop a buffer compatible with currently practiced DE methods and one that could be easily implemented into these procedures.

2. MATERIALS AND METHODS

2.1 Preliminary Semen Quantitation

Spermatozoa participate in widespread agglutination which is thought to be caused by the consequent production of anti-sperm antibodies by the presence of sperm-specific antigens (96). Physiologically, the formation of spermatozoa globs is problematic for male infertility (96). However, they also complicate the even and predictable distribution of sperm cell DNA for sample preparation. Since knowledge of input DNA quantity is key for any recovery study, it became essential to develop a protocol to better homogenize the semen sample prior to sample preparation to ensure that a more consistent amount of spermatozoa, and DNA, is deposited onto the cotton swabs.

A tube of neat, liquid semen from a donor was first gently inverted and flicked ten times. The stock was then carefully mixed 15-20 times with a 1 milliliter (mL) pipette tip. One 30 microliter (µL) aliquot of the neat semen sample was placed in a new 1.5 mL tube and left to incubate for 30 minutes at 37°C and 250 revolutions per minute (rpm) on a ThermoMixer (ThermoFisher, Waltham, MA). After this initial incubation, a 1:50 dilution of the neat semen was created by adding 1470 µL of Tris-EDTA (TE) buffer to the tube containing the 30 µL aliquot. To thoroughly mix the sample, this tube was again inverted and flicked ten times then slowly mixed with a 1 mL pipette tip 15-20 times. The 1:50 sample was placed back onto the ThermoMixer for an additional 30-minute incubation.

After the final incubation, the sample was removed from the ThermoMixer and again homogenized following the previously used method. Two 10 µL aliquots were transferred from the 1:50 sample tube into separate 0.5 mL tubes each containing 10 µL of AcroSolv, 10 µL of 10X Red Buffer, 2 µL of *forensic*GEM™ Sperm, and 68 µL of deionized (DI) water. These two AcroSolv digests were allowed to incubate for 5 minutes at 52°C, 3 minutes at 75°C, and 3 minutes at 95°C. Each digest was then quantitated via qPCR using the Quantifiler™ Trio DNA Quantification Kit (Applied Biosystems, Foster City, CA) and the 7500 Real-Time PCR instrument (Applied Biosystems, Foster City, CA).

2.2 Preparation of 53 ng Semen Swabs

The average concentration of the 1:50 semen dilution was found to be approximately 43.9 ng/µL (SD = 0.45). To allow a workable amount of semen to be deposited onto sample swabs, the 1:50 dilution was further diluted ten-fold by transferring 35 µL of the 1:50 dilution tube into a new 1.5 mL tube with 315 µL of TE. When staining the samples, swabs were held upright and 12 µL of this new 1:500 dilution (approximately 53 ng of DNA) was carefully deposited onto the tips with a pipette. A 2016 study found that spermatozoa display very little migration away from the original area of deposition when stained on cotton substrates (97). To limit the amount of superfluous cotton in the incubation tubes, only the tips of sample swabs were removed with a scalpel and tested.

2.3 Sucrose Experiment

To investigate whether small molecules with similar chemical properties to sperm surface glycoproteins can displace sperm cells from the cotton matrix, two concentrations of sucrose and histidine were tested in separate experiments. A 500 millimolar (mM) sucrose solution was prepared by dissolving 0.855 grams (g) of molecular biology grade sucrose in 5 mL of TE buffer. A 50 mM sucrose solution was created by dissolving 0.0855 g of molecular biology grade sucrose in 5 mL of TE.

Traditional DE procedures involve multiple tube transfers and movement of swabs between tubes and spin baskets. In an attempt to minimize these manipulations, GenSpin™ Baskets (Gentueri, Verona, WI), that are designed to break at low centrifugation speeds, were used for this experiment. The tips of six dry semen swabs were removed with a sterile scalpel and placed in individual GenSpin™ spin baskets. All samples were prepared in duplicate with 300 µL of 500 mM sucrose solution added to two baskets, 300 µL of 50 mM sucrose solution added to two baskets, and 300 µL of DI water added to the remaining two baskets. The samples were placed on the ThermoMixer and allowed to incubate for 1 hour at 56°C and 400 rpm.

Once the incubation was complete, the samples were spun on a centrifuge for 5 minutes at 14000 rpm. The basket containing the now dry swab, designated the "material fraction" (MF), was transferred into a new, sterile 2.0 mL tube, and stored at -20°C for future testing. Approximately 250 µL of the eluate's supernatant was carefully removed and deposited into a new 1.5 mL tube. These "supernatant" (SN) samples were also

stored at -20°C for future testing. The original sample tubes, now designated the "sperm fraction" (SF), were then vortexed to resuspend the sperm pellet.

SF samples were extracted in accordance with the MicroGEM "DNA Extraction Using *forensic*GEM™ Sperm" procedure; however, Red Buffer was utilized in place of Orange+ Buffer due to interference known to occur between the GlobalFiler™ PCR Amplification Kit (Applied Biosystems, Foster City, CA) and the Orange+ Buffer. To separate 0.5 mL tubes, 5 µL of 10X Red Buffer, 5 µL of Acrosolv, 1 µL of *forensic*GEM™ Sperm, 34 µL of DI water, and 5 µL of resuspended pellet were added. The tubes were then vortexed, placed in a Thermal Cycler (Applied Biosystems, Foster City, CA), and left to incubate for 10 minutes at 52°C, 6 minutes at 75°C, and 3 minutes at 95°C. Incubation times were increased from standard procedure to provide additional time for the enzyme to act on the sperm cells.

Upon completion of the incubation, all samples were vortexed then quantitated via qPCR using the Quantifiler™ Trio DNA Quantification Kit and 7500 Real-Time PCR instrument.

2.4 Preparation of 100 ng Semen Swabs

After the sucrose experiment, it was decided that the mass of DNA on sample swabs be increased to 100 ng to better measure yield. The previously made 1:500 dilution was again quantitated prior to sample preparation and came to approximately 3.99 ng/µL (SD = 0.01). Based on this value, 25 µL of the 1:500 dilution was stained

onto the tips of clean cotton swabs and allowed to dry. These 100 ng swabs were used for all future experiments in this study.

2.5 Histidine Experiment

A 250 mM histidine solution was prepared by dissolving 0.387 g of L-histidine in 10 mL of TE. A 50 mM histidine solution was created by dissolving 0.0775 g of L-histidine in 10 mL of TE.

The tips of eight semen swaps were removed with a sterile scalpel and placed in individual 2.0 mL tubes. Histidine samples were prepared in triplicate by adding 300 µL of 250 mM histidine solution to three tubes and 50 mM histidine solution to three tubes. Two TE samples, each containing 300 µL of TE, were also prepared as controls. All samples were placed on the ThermoMixer and left to incubate for 1 hour at 37°C and 400 rpm. Standard spin baskets were used for these samples as well as for subsequent experiments.

2.6 PVP, BSA, and Tween 20 Experiment

Three components of the PCR reaction buffer were evaluated in this research for their efficiency to displace sperm cells from cotton. Solutions were prepared in accordance with the protocol developed by Gray et al. (67). A 1 molar (M) Tris hydrochloric acid (HCl) solution was made by first dissolving 3.0285 g of Tris in 25 mL of DI water. Small amounts of HCl were slowly pipetted into the solution until it reached

pH 8.0. A 20 mM Tris-HCl solution was subsequently created by diluting 500 µL of 1 M Tris-HCl in 24.5 mL of TE.

A 1% PVP, 1% Tween 20 solution was created by dissolving 0.1 g of PVP and 250 µL of 40% Tween 20 in 10 mL of 20 mM Tris-HCl. A 1% BSA, 1% Tween 20 solution was prepared by dissolving 200 µL of 50 milligrams (mg) per 1 mL BSA and 250 µL of 40% Tween 20 in 10 mL of 20 mM Tris-HCl.

To determine if temperature has any effect on weakening the adhesive forces between sperm cells and cotton, two samples were incubated separately at low and high temperatures. One dry semen swab was placed in the freezer at -20°C overnight. The next day, the tip of this swab was removed with a sterile scalpel and placed in a clean 2.0 mL tube with 300 µL of TE. This sample was placed on the ThermoMixer and allowed to incubate for 1 hour at 4°C and 400 rpm. To evaluate the effect of heat, the tip of a second semen swab, stored overnight at room temperature, was removed with a sterile scalpel and placed in a 2.0 mL tube. To this tube, 300 µL of TE was added. This sample was placed on the ThermoMixer and allowed to incubate for 1 hour at 75°C and 400 rpm.

The tips of seven additional dry semen swabs, stored at room temperature, were removed with a sterile scalpel and placed in individual 2.0 mL tubes. To these tubes, 300 µL of 1% PVP, 1% Tween 20 in Tris-HCl was added to three tubes, 300 µL of 1% BSA, 1% Tween 20 in Tris-HCl was added to three tubes, and 300 µL of TE was added to the last tube. These samples were subjected to the previously used incubation parameters of 1 hour at 37°C and 400 rpm.

2.7 Heat Experiment

To further elucidate if heat improves sperm cell elution, the incubation temperature for all samples in this experiment was increased. Some samples were incubated for an additional hour to evaluate whether a longer incubation benefits recovery.

The tips of ten dry semen swabs were removed with a sterile scalpel and placed in clean 2.0 mL tubes. Two two-hour time point TE samples, containing 300 µL of TE, were prepared first and placed on the ThermoMixer to incubate for 2 hours at 75°C and 400 rpm.

The one-hour time point samples were also prepared in duplicate with 300 µL of 50 mM sucrose solution added to two tubes, 300 µL of 50 mM histidine solution added to two tubes, 300 µL of 1% PVP, 1% Tween 20 in 20 mM Tris-HCl solution added to two tubes, and 300 µL of TE added to two tubes. These eight samples were placed on the ThermoMixer with the two-hour TE samples to incubate for the final hour of the incubation at 75°C and 400 rpm. All samples were vortexed periodically throughout the incubation.

2.8 First Enzyme Experiment

To investigate the effect of commonly employed DE enzymes on sperm cell recovery, PK and *forensic*GEM™ were evaluated as elution buffers.

A 2X Thermolabile PK Buffer, compatible with PK, was prepared by first dissolving 0.0221 g of calcium chloride ($CaCl_2$) in 4 mL of 1 M Tris-HCl, pH 7.5

solution. DI water was added to bring the final volume of the solution to 100 mL thus creating a 40 mM Tris-HCl, 2 mM $CaCl_2$ solution. Thermolabile PK Buffer, designed by New England Biolabs®, was chosen for this experiment in lieu of QIAGEN's G2 Buffer because the identity and concentration of the components were known whereas the recipe of G2 is proprietary.

The tips of ten dry semen swabs were removed with a sterile scalpel, gently pulled apart with clean forceps, and placed in individual 2.0 mL tubes. PK samples were prepared by adding 15 µL of 800 units (U) per 1 mL PK (QIAGEN, Hilden Germany) and 285 µL of 2X Thermolabile PK Buffer to two tubes. For the *forensic*GEM™ samples, 267 µL of DI water was initially added to two tubes. To understand the behavior of the buffer components without the presence of enzyme, buffer blanks for PK and *forensic*GEM™ were tested by adding 300 µL of 2X Thermolabile PK Buffer to two tubes and 270 µL of DI water to two tubes, respectively. Lastly, TE controls were prepared by adding 300 µL of TE to two tubes. All samples were allowed to incubate on the ThermoMixer for 1 hour at 56°C and 400 rpm. The samples were vortexed at the beginning, middle, and end of the incubation.

At the end of the incubation, 3 µL of *forensic*GEM™ and 30 µL of 10X Blue Buffer were added to the two *forensic*GEM™ samples. Additionally, 30 µL of 10X Blue Buffer was added to the two *forensic*GEM™ buffer blanks. The incubation temperature was increased to 75°C, and all samples were allowed to incubate for an additional hour at 400 rpm. Again, all samples were vortexed at the beginning, middle and end of the incubation.

After the incubation, the temperature was increased to 95°C, and all samples were left to incubate for 10 minutes with no shaking. The temperature was then lowered to 4°C for one last 10-minute incubation.

2.9 Second PK Experiment

After the previous experiment, several changes were made to the method to optimize the conditions for PK and determine the ideal incubation time needed for the enzymatic reaction. First, the concentration of the Thermolabile PK Buffer was reduced by half to more closely follow the New England Biolabs® recommended concentration. A 1X Thermolabile PK Buffer solution was made by diluting 5 mL of the previously prepared 2X Thermolabile PK Buffer in 5 mL of TE. Second, SDS was introduced into the elution buffer to determine if its presence has any influence on the ability of PK to encourage sperm cell release from the cotton swabs. PK is shown to be active in the presence of 0.2-1% SDS; however, SDS at a sufficiently high concentration is known to interfere with the qPCR chemical reaction (94). Consequently, a concentration of SDS (0.1%) below the reported optimal range was chosen for this study. Lastly, the activity of PK was measured at two time points—1 hour and 2 hours—to assess if time impacts elution success, and the incubation temperature was held constant at PK's optimal temperature of 56°C.

The tips of 17 dry semen swabs were removed with a sterile scalpel, gently pulled apart with clean forceps, and placed in individual 2.0 mL tubes. The 2-hour time point samples were prepared first. For the PK and SDS samples, 6 µL of 5% SDS, 15 µL of

PK, and 279 µL 1X Thermolabile PK Buffer were added to three tubes. Enzyme controls containing no SDS were prepared by adding 15 µL of PK and 285 µL of 1X Thermolabile PK Buffer to three tubes. Two buffer blanks each containing 300 µL of 1X Thermolabile PK Buffer and one TE control containing 300 µL of TE were also tested. All samples were placed on the ThermoMixer for 2 hours at 56°C and 400 rpm. Samples were vortexed at the beginning, middle, and end of the incubation.

The one-hour time point samples were prepared in the same way as the two-hour time point samples, except no TE control was evaluated at this time point. These eight samples were placed on the ThermoMixer and allowed to incubate for the final hour of the two-hour incubation at 56°C and 400 rpm. Like the two-hour time point samples, these samples were vortexed at the beginning, middle, and end of the incubation.

At the conclusion of the incubation, the temperature of the ThermoMixer was increased to 95°C to inactivate the PK, and all samples were left to incubate for 10 minutes with no shaking. The temperature was then reduced to 4°C for an additional 10 minutes.

2.10 Second *forensic*GEM™ Experiment

As was done in the second PK experiment, modifications to the incubation conditions were made for the second *forensic*GEM™ experiment as well. The tips of 13 dry semen swabs were removed with a sterile scalpel, gently teased apart with clean forceps, and placed in individual 2.0 mL tubes. Three samples were designed to mimic the conditions of the first *forensic*GEM™ experiment and prepared by first adding 267

µL of DI water to three tubes. These samples were placed on the ThermoMixer for 1 hour at 56°C and 400 rpm, and vortexed at the beginning, middle, and end of the incubation. At the end of the incubation, 3 µL of *forensic*GEM™ and 30 µL of 10X Blue Buffer were added to all three samples. The incubation temperature was increased to 75°C, and the samples were left to incubate for an additional hour at 400 rpm.

To determine the optimal time for the enzymatic reaction, a two-hour time point, and one-hour time point were investigated. To three tubes, 267 µL DI water, 3 µL of *forensic*GEM™, and 30 µL of 10X Blue Buffer were added. Two buffer blanks containing 270 µL of DI water and 30 µL of 10X Blue Buffer were also tested. These five samples were placed on the ThermoMixer for 2 hours at 75°C and 400 rpm, and vortexed at the beginning, middle, and end of the incubation.

The one-hour time point samples were created in the same way as the two-hour time point samples. These five samples were placed on the ThermoMixer with the two-hour samples at 75°C and 400 rpm for the final hour of the incubation, and were also vortexed at the beginning, middle, and end of the incubation.

At the conclusion of the incubation, all samples underwent a brief 10-minute incubation at 95°C with no shaking to inactivate the *forensic*GEM™. The temperature was then reduced to 4°C for 10 minutes.

2.11 Post-Incubation Sample Processing, Extraction, and Quantitation

After completion of the final incubation, the soaked swabs were removed from each tube with clean forceps and placed in a mesh spin basket. The spin basket was then

placed back in the original sample tube and spun on the centrifuge for 5 minutes at 14000 rpm. Prior to the Heat Experiment, the dry swabs, (MF), were transferred to a new 2.0 mL tube, and stored at -20°C for future testing. After, the MF began to be extracted at the same time as all other samples to improve efficiency and gain a more accurate measure of how much sperm remained on the swab. Great care was taken to not disturb the sperm pellet (SF) as 200 µL of the supernatant was removed from the eluate, leaving approximately 100 µL in the tube. The supernatant was deposited into a new 1.5 mL tube and stored at -20°C for future testing. The original sample tubes were then briefly vortexed and gently mixed with a pipette 10-15 times to resuspend the sperm pellet.

As was done in the sucrose experiment, SF samples were extracted using the MicroGEM "DNA Extraction Using *forensic*GEM™ Sperm" procedure with Orange+ Buffer again utilized in place of Red Buffer. To extract the SF of each sample, 5 µL of 10X Red Buffer, 5 µL of Acrosolv, 1 µL of *forensic*GEM™ Sperm, 34 µL of DI water, and 5 µL of resuspended pellet were added into individual 0.5 mL tubes. To extract the MF, the dry swab was transferred into a 0.5 mL tube containing 20 µL of AcroSolv, 20 µL of 10X Red Buffer, 4 µL of *forensic*GEM™ Sperm and 156 µL of DI water. The SF and MF samples were then vortexed, placed in a Thermal Cycler, and allowed to incubate for 10 minutes at 52°C, 6 minutes at 75°C, and 3 minutes at 95°C.

After removal from the Thermal Cycler, wet MF swabs were removed from their tubes with clean forceps and placed in a mesh spin basket within a new 2.0 mL tube. These samples were spun on a centrifuge for 2 minutes at 14000 rpm to remove all liquid from the swab. The eluate from each swab was then transferred back into the original 0.5

mL MF sample tube. The swab was left in the spin basket and stored at -20°C in case of any necessary future testing. All samples were vortexed again before they were quantitated via qPCR using the Quantifiler™ Trio DNA Quantification Kit and 7500 Real-Time PCR instrument.

3. RESULTS

3.1 Sucrose and Histidine Experimental Results

Given the similar chemical properties of sucrose, histidine, and the glycoproteins on the surface of sperm cells, an investigation was conducted to determine if sucrose or histidine in solution could displace the sperm cells from the cotton fibers. In these experiments, four elution buffers were tested: 500 mM sucrose, 50 mM sucrose, 250 mM histidine, and 50 mM histidine. Solutions containing only DI water or TE served as controls for the two experiments, respectively. For the sucrose experiment, approximately 53 ng of DNA from the 1:500 semen dilution was stained on each swab whereas approximately 100 ng of DNA from the 1:500 semen dilution was deposited on the swabs used in the histidine experiment. In neither experiment was the MF extracted to measure the amount of sperm cells that may have remained adhered to the swab. Therefore, the percent recovery of the SF obtained from each sample was calculated by dividing the mass of DNA obtained from the SF by the theoretical amount of starting DNA on the sample then multiplying that figure by 100. The theoretical amount of input DNA was determined by the qPCR results of the diluted semen digest. The mass of DNA recovered from the SF of each sample and the theoretical recovery percentages from the sucrose experiment are reported in Table 1.

Table 1: DNA Recovered from the Sucrose Experiment: The mass and theoretical proportion of DNA recovered from the SF of semen swabs incubated in 500 mM or 50 mM sucrose (S), or DI water (DI) for 1 hour at 56°C and 400 rpm.

Sample	SF Mass (ng)	% Recovery (SF)
500mM-S-1	0.353	0.67
500mM-S-2	0.318	0.60
50mM-S-1	0.705	1.33
50mM-S-2	0.709	1.34
DI-1	0.213	0.40
DI-2	0.071	0.13

None of the three incubation solutions demonstrated sperm cell recovery above 1.4% suggesting that these solutions were ineffective at dislodging the sperm from the cotton swabs. The 50 mM sucrose solution outperformed the other two conditions, recovering an average of 0.707 ng of DNA. However, only a maximum of 1.34% of total DNA was recovered using this elution solution.

All conditions tested in the histidine experiment showed higher DNA recovery than all elution buffers assessed in the sucrose experiment (Table 2). This result may be attributed to the switch from the GenSpin™ to the standard mesh spin baskets between experiments. Consistent with the sucrose results, the 50 mM histidine solution displayed better sperm cell elution than the more concentrated histidine solution with an average of 6.024 ng of DNA (6.02 %) recovered (Figure 1). However, the TE control gave the highest recovery with an average of 7.06 ng of DNA (7.06%) extracted from the SF.

Table 2: DNA Recovered from the Histidine Experiment: The mass and theoretical proportion of DNA recovered from the SF of semen swabs incubated in 250 mM or 50 mM histidine (H) or TE (TE) for 1 hour at 37°C and 400 rpm.

Sample	SF Mass (ng)	% Recovery (SF)
250mM-H-1	2.710	2.71
250mM-H-2	2.598	2.60
250mM-H-3	2.315	2.32
50mM-H-1	7.089	7.09
50mM-H-2	5.227	5.23
50mM-H-3	5.754	5.75
TE-1	9.418	9.42
TE-2	4.703	4.70

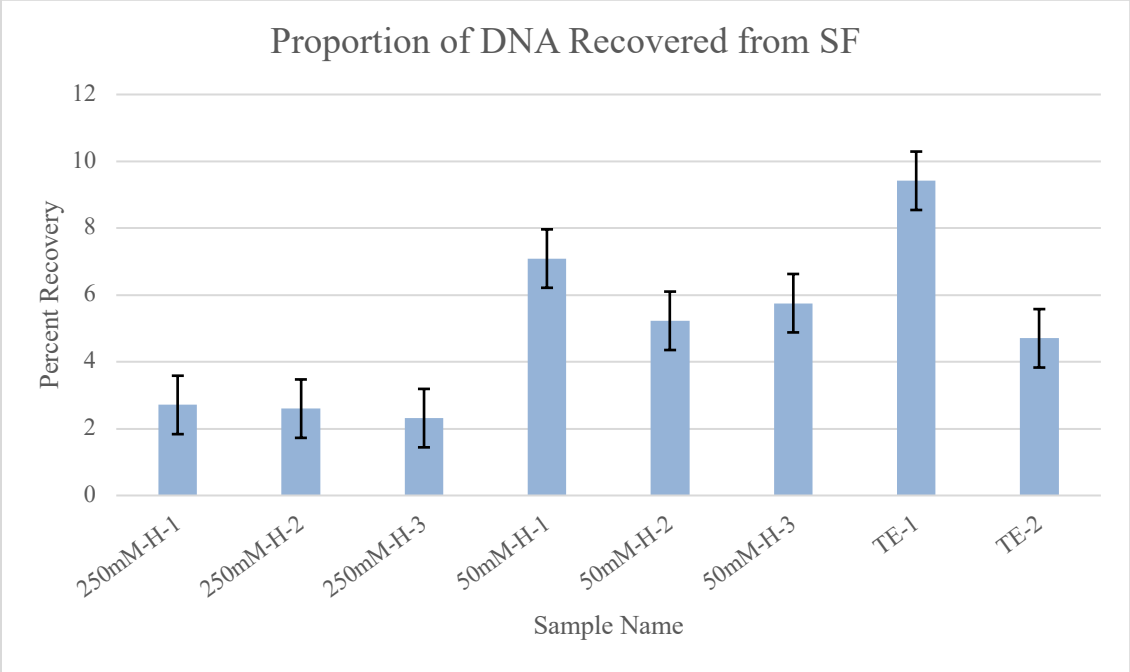

Figure 1. Proportion of sperm cell DNA recovered from the SF of semen swabs tested in the Histidine Experiment.

3.2 PVP, BSA, and Tween 20 Experimental Results

Observations by Gray et al. that extraction buffers containing PVP, BSA, and Tween 20 greatly enhanced mouse embryonic fibroblast DNA recovery prompted the application of these buffers to semen swabs in this study (67). Semen samples containing approximately 100 ng of DNA were incubated in solutions of 1% PVP and 1% Tween 20, 1% BSA and 1% Tween 20, or TE. Again, the MF of these samples was not extracted, so percent recovery was based on an estimate of input DNA. Both the BSA and Tween 20 solutions gave slightly better sperm cell recovery with an average of 6.59% of total DNA obtained in the SF compared to PVP and Tween 20 which reported an average DNA recovery of 4.72% of total DNA (Table 3).

Table 3: DNA Recovered from the PVP, BSA and Tween 20 Experiment: The mass and theoretical proportion of DNA recovered from the SF of semen swabs incubated in PVP-Tween 20 (PVP-T20), BSA-Tween 20 (BSA-T20), or TE (TE) for 1 hour at 4°C (4C), 37°C (37C), or 75°C (75C) and 400 rpm.

Sample	Incubation Temperature (°C)	SF Mass (ng)	% Recovery (SF)
PVP-T20-1	37	5.246	5.25
PVP-T20-2	37	4.765	4.77
PVP-T20-3	37	4.134	4.13
BSA-T20-1	37	5.723	5.72
BSA-T20-2	37	6.387	6.39
BSA-T20-3	37	7.672	7.67
37C-TE	37	5.473	5.47
4C-TE	4	7.963	7.96
75C-TE	75	27.101	27.10

Rather than manipulating incubation time in this experiment, samples were incubated at three different temperatures to assess whether changing the overall molecular kinetic energy encourages cell elution. Three TE samples were exposed to

either 4°C, 37°C, or 75°C during their one-hour incubation. All non-TE samples were incubated at the standard 37°C for 1 hour.

The 4°C and 37°C TE samples behaved similarly to the BSA-Tween 20 and PVP-Tween 20 samples and exhibited total DNA recoveries of 7.96% and 5.47%, respectively. The 75°C TE sample, however, recovered 27.101 ng of DNA from the SF, the highest yield observed from all experiments thus far (Figure 2). This could suggest that heating the sample, and increasing the kinetic energy of the sperm cells, weakens the van der Waals interactions between the cells and the cellulose, causing the release of sperm cells.

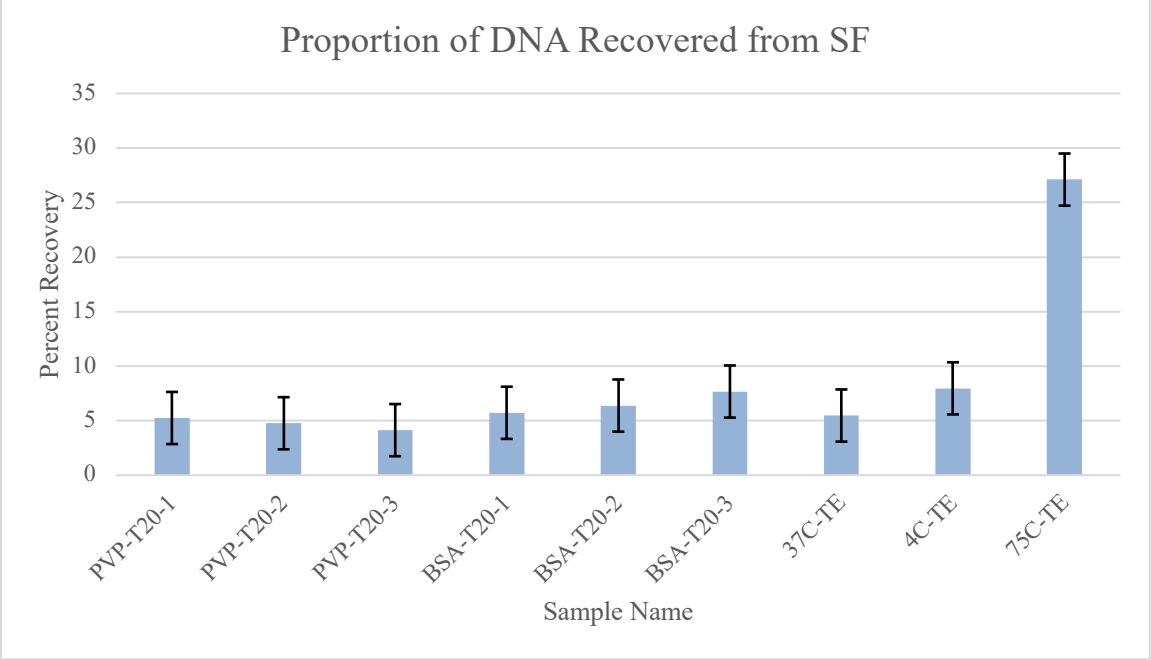

Figure 2. Proportion of sperm cell DNA recovered from the SF of semen swabs tested in the PVP, BSA, and Tween 20 (T20) Experiment.

3.3 Heat Experimental Results

The relative success of increasing the temperature seen in the last experiment guided the next experimental design of re-testing previously evaluated elution buffers at

higher incubation temperatures. The solutions showing the most promise in their respective experiments, 50 mM sucrose and 50 mM histidine, were used. The BSA and Tween 20 solution was eliminated from this testing for fear that the temperature increase would provide inhospitable conditions for the BSA. All samples were incubated at 75°C for 1 hour apart from two TE samples which underwent a two-hour incubation at 75°C.

The mass recovered from each fraction and recovery percentage are summarized in Table 4. Starting in this experiment, the MF was extracted simultaneously with the SF. Rather than using a theoretical value, a more exact total mass could now be calculated by summing the mass of the SF, the mass of the MF, and the mass of the SN, which was only extracted for this experiment. The percent recoveries were then calculated by dividing the mass obtained from each fraction by the total mass (sum of each fraction) and multiplying that figure by 100.

Table 4: DNA Recovered from the Heat Experiment: The mass and proportion of DNA recovered from the SF, MF, and SN of semen swabs incubated in 50 mM sucrose (S), 50 mM histidine (H), 1% PVP and 1% Tween 20 (PVP-T20), or TE (TE) for 1 hour (1HR) or 2 hours (2HRS) at 75°C and 400 rpm.

Sample	Incubation Time (Hours)	SF Mass (ng)	MF Mass (ng)	SN Mass (ng)	Total Mass (ng)	% Recovery (SF)	% Recovery (MF)
50mM-S-1	1	15.759	60.311	5.688	81.759	19.28	73.77
50mM-S-2	1	10.832	62.409	3.57	76.812	14.10	81.25
50mM-H-1	1	7.872	45.643	3.564	57.079	13.79	79.96
50mM-H-2	1	11.008	67.607	2.436	81.051	13.58	83.41
PVP-T20-1	1	15.385	94.662	1.6	111.648	13.78	84.79
PVP-T20-2	1	15.470	116.801	1.636	133.907	11.55	87.23
2HRS-TE-1	2	15.152	76.787	10.778	102.718	14.75	74.76
2HRS-TE-2	2	19.945	64.744	3.834	88.523	22.53	73.14
1HR-TE-1	1	11.787	76.580	6.886	95.254	12.37	80.40
1HR-TE-2	1	8.284	74.060	5.492	87.836	9.43	84.32

Recovery from the SF using the sucrose, histidine, and PVP-Tween 20 elution buffers increased by an average of over 1000%, 100%, and 150%, respectively, compared to the results obtained from their initial testing when incubated at lower temperatures. In these conditions, exposure to greater heat had a positive influence on sperm elution, suggesting that higher molecular motion might facilitate displacement of the sperm cells. The two-hour and one-hour TE samples reported average SF recovery yields of 18.64%

and 10.9%, respectively. Neither TE condition achieved as successful recovery as was observed in the previous experiment.

In all samples, more DNA was found in the MF than the SF with an average of approximately 80% of total DNA discovered to have remained on the swab across all conditions (Figure 3). This finding aligns with past studies which also found that a large proportion of male and female DNA were lost to the MF in various differential extraction procedures (36,98).

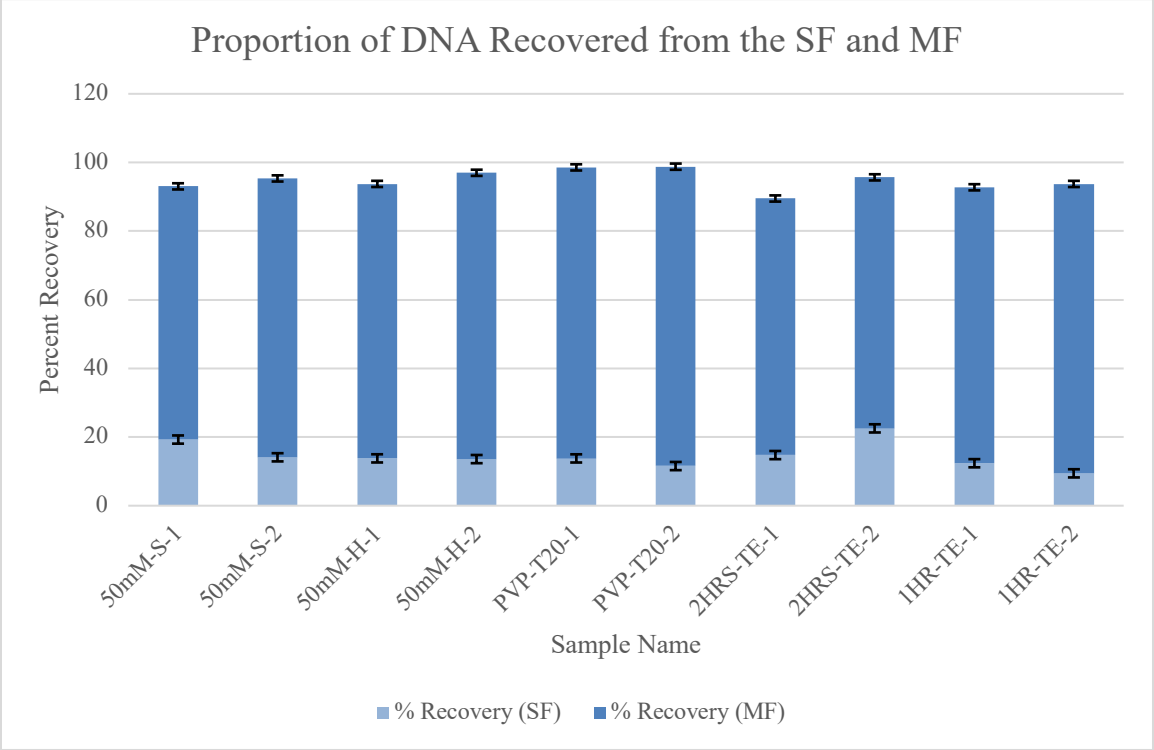

Figure 3. Proportion of sperm cell DNA recovered from the SF and MF of semen swabs tested in the Heat Experiment.

3.4 First Enzyme Experimental Results

Enzymes capable of cleaving peptide bonds are frequently employed as the first step of DE protocols to digest the cell membrane of epithelial cells. An experiment was performed to evaluate whether commonly utilized proteases, PK and *forensic*GEM™, have similar efficiency in releasing sperm cells from the cotton swab. This may involve attacking the glycoproteins coating the sperm surface. In this experiment, PK and *forensic*GEM™ were allowed to incubate for at least an hour at their optimal temperatures of 56°C and 75°C, respectively. For each enzyme, two buffer blanks were also tested to measure any effect that the enzyme buffer alone may have on sperm elution. Two TE samples were included as experimental controls.

*forensic*GEM™ proved to be the most effective condition tested thus far, exhibiting an average SF yield of 77.95% compared to an average 3.99% recovery seen in samples incubated with PK (Table 5). The *forensic*GEM™ buffer itself delivered modest recovery with an average yield of 26.31% while the PK buffer blank showed to be inefficient at eluting sperm cells, recovering an average of 0.99% of the total DNA. The TE controls returned an average recovery of 12.27%, performing on par with TE samples tested in previous experiments.

Table 5: DNA Recovered from the First Enzyme Experiment: The mass and proportion of DNA recovered from the SF and MF of semen swabs incubated in the presence of PK (PK), PK buffer only (PKB), *forensic*GEM™ (Z), *forensic*GEM™ buffer only (ZB), or TE (TE) for 1 hour at 56°C and 400 rpm followed by 1 hour at 75°C and 400 rpm.

Sample	DNA Recovered from SF (ng)	DNA Recovered from MF (ng)	Total Mass (ng)	% Recovery (SF)	% Recovery (MF)
PK-1	2.194	116.353	118.547	1.85	98.15
PK-2	6.269	95.855	102.124	6.14	93.86
Z-1	75.764	19.629	95.394	79.42	20.58
Z-2	90.717	27.909	118.627	76.47	23.53
PKB-1	1.655	121.867	123.522	1.34	98.66
PKB-2	0.833	129.975	130.808	0.64	99.36
ZB-1	40.592	88.958	129.551	31.33	68.67
ZB-2	23.544	87.052	110.597	21.29	78.71
TE-1	14.931	111.673	126.605	11.79	88.21
TE-2	15.792	108.047	123.840	12.75	87.25

Again, the MF was extracted along with the SF although the SN was not. Subsequently, the total mass was calculated by adding the mass of DNA recovered from the SF and the mass recovered from the MF. Percent recoveries for each fraction, represented in Figure 4, were then based on this total mass value. As was observed in the previous experiment, significant proportions of DNA were left on the swab for some samples (Table 5). However, an average of only 20.06% of sperm DNA remained on the swab after incubation with *forensic*GEM™ constituting a nearly 75% decrease from the previous experiment's results. For the *forensic*GEM™ buffer blanks, an average of approximately 73.69% of DNA was left on the swab, a slight improvement from the previous experiment's average MF recovery data. This could indicate that components in *forensic*GEM™ buffer may, in tandem with the enzyme, encourage sperm cell removal from the swab.

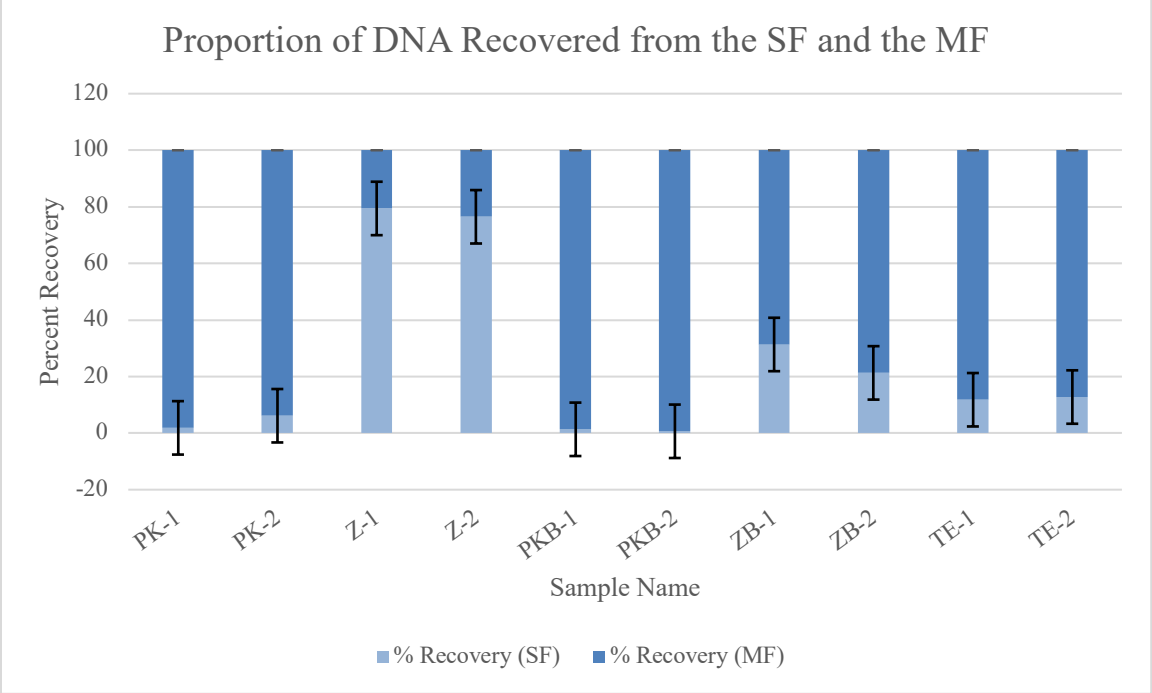

Figure 4. Proportion of sperm cell DNA recovered from the SF and MF of semen swabs tested in the first Enzyme Experiment.

3.5 Second PK Experimental Results

In this experiment, multiple changes were made to increase PK activity. The activity of PK on macromolecules has been shown to be enhanced in the presence of SDS (32). Accordingly, incubation buffers containing PK and 0.1% SDS were also tested. Secondly, in the first enzyme experiment, PK samples underwent a one-hour incubation at 75°C after the one-hour incubation at its optimal temperature of 56°C. At temperatures above 65°C, PK is irreversibly inactivated (94). Therefore, the incubation temperature in this experiment was kept at 56°C throughout the entire incubation. Data was collected at one-hour and two-hour time points to determine the optimal incubation time for the

enzymatic reaction. Two buffer blanks were assessed at each time point, and one TE sample was tested at the two-hour time point as a control.

The mass and proportion of DNA recovered from each fraction are summarized in Table 6. When in the presence of SDS, sperm cell elution greatly improved with an average DNA recovery of 60.14% seen in the two-hour samples and 59.87% observed in the one-hour samples. These values represent an approximately 912% and 1260% increase over the two-hour and one-hour PK without SDS samples, respectively, which returned average DNA recovery yields of 5.94% and 4.40%. The additional one-hour of incubation time at 56°C had no significant impact on the activity of the enzyme as minimal recovery differences were observed between the two time points.

Table 6: DNA Recovered from the Second PK Experiment: The mass and proportion of DNA recovered from the SF and MF of semen swabs incubated in the presence of PK (PK), PK and 0.1% SDS (PK-SDS), PK buffer only (PKB), or TE (TE) at 56°C and 400 rpm for 1 hour (1HR) or 2 hours (2HRS).

Sample	DNA Recovered from SF (ng)	DNA Recovered from MF (ng)	Total Mass (ng)	% Recovery (SF)	% Recovery (MF)
2HRS-PK-SDS-1	37.681	35.179	72.860	51.72	48.28
2HRS-PK-SDS-2	32.881	21.205	54.087	60.79	39.21
2HRS-PK-SDS-3	42.476	20.078	62.554	67.90	32.10
2HRS-PK-1	1.632	59.832	61.464	2.66	97.34
2HRS-PK-2	8.540	72.346	80.887	10.56	89.44
2HRS-PK-3	2.734	56.638	59.373	4.61	95.39
2HRS-PKB-1	0.830	52.469	53.300	1.56	98.44
2HRS-PKB-2	0.698	61.193	61.892	1.13	98.87
2HRS-TE-1	14.485	41.523	56.008	25.86	74.14
1HR-PK-SDS-1	45.202	46.768	91.971	49.15	50.85
1HR-PK-SDS-2	61.096	31.802	92.898	65.77	34.23
1HR-PK-SDS-3	39.056	21.310	60.367	64.70	35.30
1HR-PK-1	1.363	58.532	59.896	2.28	97.72
1HR-PK-2	4.038	54.444	58.483	6.91	93.09
1HR-PK-3	2.661	63.531	66.193	4.02	95.98
1HR-PKB-1	0.500	97.840	98.340	0.51	99.49
1HR-PKB-2	0.363	75.176	75.539	0.48	99.52

The buffer blanks in this experiment performed similarly to the PK buffer blanks evaluated in the first enzyme experiment, showing an average SF recovery of 1.35% for the two-hour time point samples and 0.5% for the one-hour time point samples. Additionally, an extra incubation hour for the PK buffer blanks had no statistically significant difference on elution. Compared to the PK only samples and PK buffer blanks, the TE control demonstrated better recovery with 25.86% of total DNA extracted from the SF.

A comparison of the recovery yields from the SF and the MF can be seen in Figure 5. Samples incubated in PK and SDS represent the only condition in which less DNA was found in the MF than the SF. An average of 39.86% and 40.13% of the total input DNA were extracted from the swabs of the two-hour and one-hour samples, respectively, reflecting a significant improvement over the PK MF data from the previous experiment.

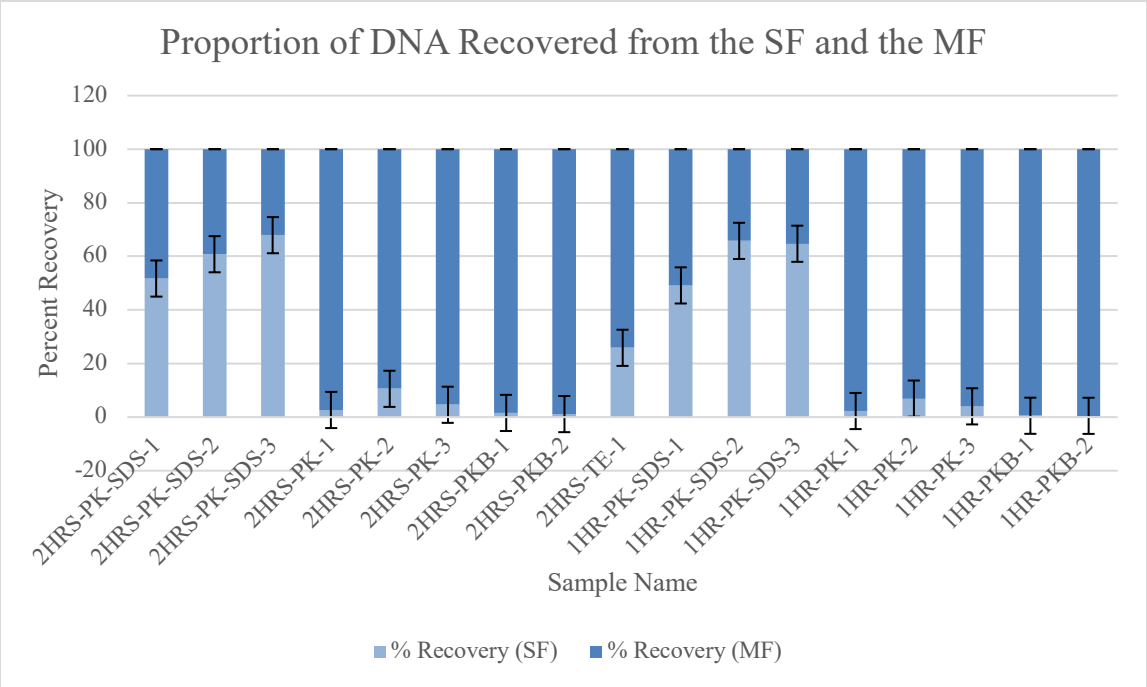

Figure 5. Proportion of sperm cell DNA recovered from the SF and MF of semen swabs tested in the second PK Experiment.

3.6 Second *forensic*GEM™ Experimental Results

To determine if the *forensic*GEM™ results seen in the first enzyme experiment were repeatable, a second *forensic*GEM™ experiment was conducted. As was done for the second PK experiment, incubation parameters were re-configured to provide more

optimal conditions for the enzyme. One set of sperm swabs served as controls and was subjected to identical experimental conditions as used in the first enzyme experiment—an hour incubation at 56°C followed by a one-hour incubation at 75°C. All other samples underwent an incubation at 75°C, EA1's optimal temperature, for either one or two hours to measure the enzymatic activity. Two buffer blanks, containing only 10X Blue Buffer and DI water, were tested at each time point to investigate the role of the buffer components in cell elution.

Significant proportions of DNA were, again, recovered from the SF of all samples incubated with *forensic*GEM™ (Table 7). The *forensic*GEM™ controls, designed to mimic the conditions of the initial *forensic*GEM™ experiment, helped to support the repeatability of these conditions by returning a nearly similar recovery yield as was observed in the first enzyme experiment with 74.29% of the input DNA retrieved from the SF. As has been observed in previous experiments, elution does not depend on time. In fact, *forensic*GEM™ samples incubated for one hour at 75°C recovered an average of 11.02% more DNA than the two-hour samples. However, the buffer blanks appeared somewhat reliant on the additional incubation hour as an average of 35.94% and 27.55% of DNA was extracted from the two-hour samples and one-hour samples, respectively. The continued relative success of these enzyme-less samples solidifies that the buffer components, in some way, contribute to sperm cell elution.

Table 7: DNA Recovered from the Second *forensic*GEM™ Experiment: The mass and proportion of DNA recovered from the SF and MF of semen swabs incubated in the presence of *forensic*GEM™ (Z) or *forensic*GEM™ buffer only (ZB) for 1 hour (1HR) or 2 hours (2HRS) at 75°C (75) and 400 rpm. Three samples (56Z-1/2/3) underwent a 1-hour incubation at 56°C followed by a 1-hour incubation at 75°C and 400 rpm.

Sample	DNA Recovered from SF (ng)	DNA Recovered from MF (ng)	Total Mass (ng)	% Recovery (SF)	% Recovery (MF)
56Z-1	58.855	19.443	78.298	75.17	24.83
56Z-2	50.018	16.723	66.741	74.94	25.06
56Z-3	55.907	20.915	76.823	72.77	27.23
2HRS-75Z-1	41.474	29.021	70.495	58.83	41.17
2HRS-75Z-2	52.969	22.143	75.112	70.52	29.48
2HRS-75Z-3	52.608	15.299	67.908	77.47	22.53
2HRS-ZB-1	25.572	47.558	73.131	34.97	65.03
2HRS-ZB-2	30.154	51.571	81.726	36.90	63.10
1HR-75Z-1	51.994	17.170	69.164	75.17	24.83
1HR-75Z-2	67.013	14.286	81.299	82.43	17.57
1HR-75Z-3	66.324	14.273	80.597	82.29	17.71
1HR-ZB-1	27.583	42.539	70.122	39.34	60.66
1HR-ZB-2	14.052	75.169	89.222	15.75	84.25

Across all time and temperature combinations, more DNA was found in the SF than the MF for all samples incubated with *forensic*GEM™ in its elution buffer (Figure 6). Since the one-hour *forensic*GEM™ samples exhibited the highest SF recovery, the lowest amount of DNA, approximately 20.04%, was obtained from the MF of these samples. The highest amount of DNA was left on the buffer blank swabs with an average 64.06% and 72.46% extracted from the two-hour and one-hour samples, respectively.

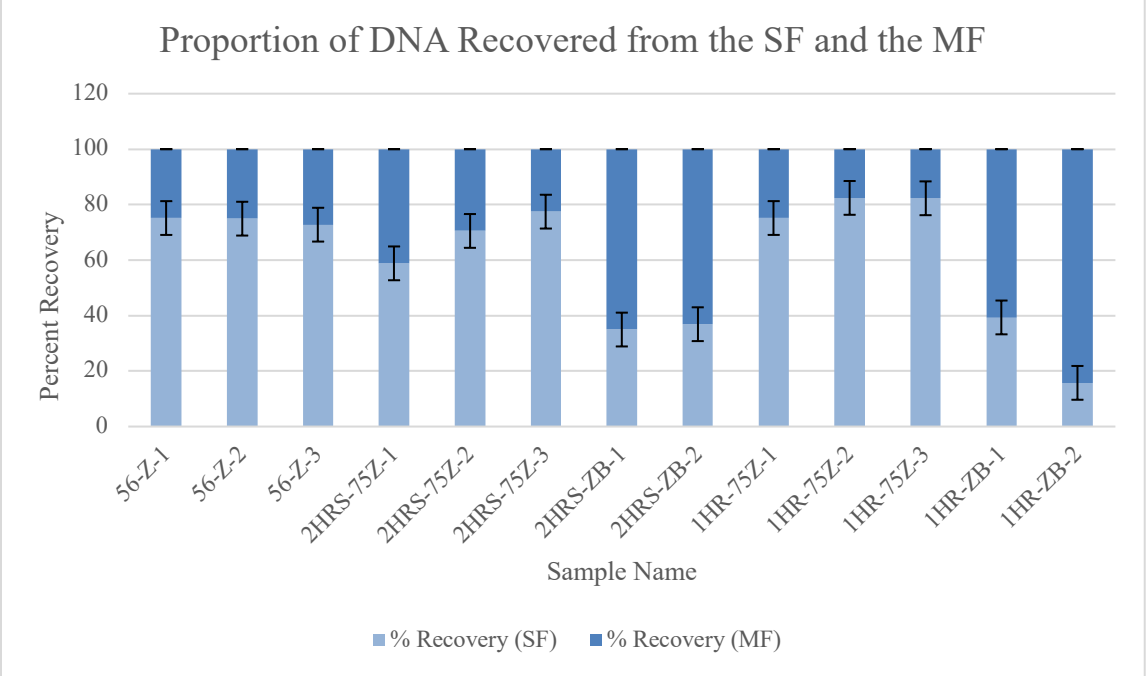

Figure 6. Proportion of sperm cell DNA recovered from the SF and MF of semen swabs tested in the second *forensic*GEM™ Experiment.

3.7 A Comparison of All Elution Conditions

In this study, 73 semen samples and 33 conditions were evaluated in seven experiments. To more concisely summarize all of the data, the average recovery yield of each condition was calculated and graphed (Figure 7). The most successful sperm elution was observed in samples that were incubated in the presence of *forensic*GEM™. Across all *forensic*GEM™ samples, an average of 75.04% of DNA was extracted from the SF.

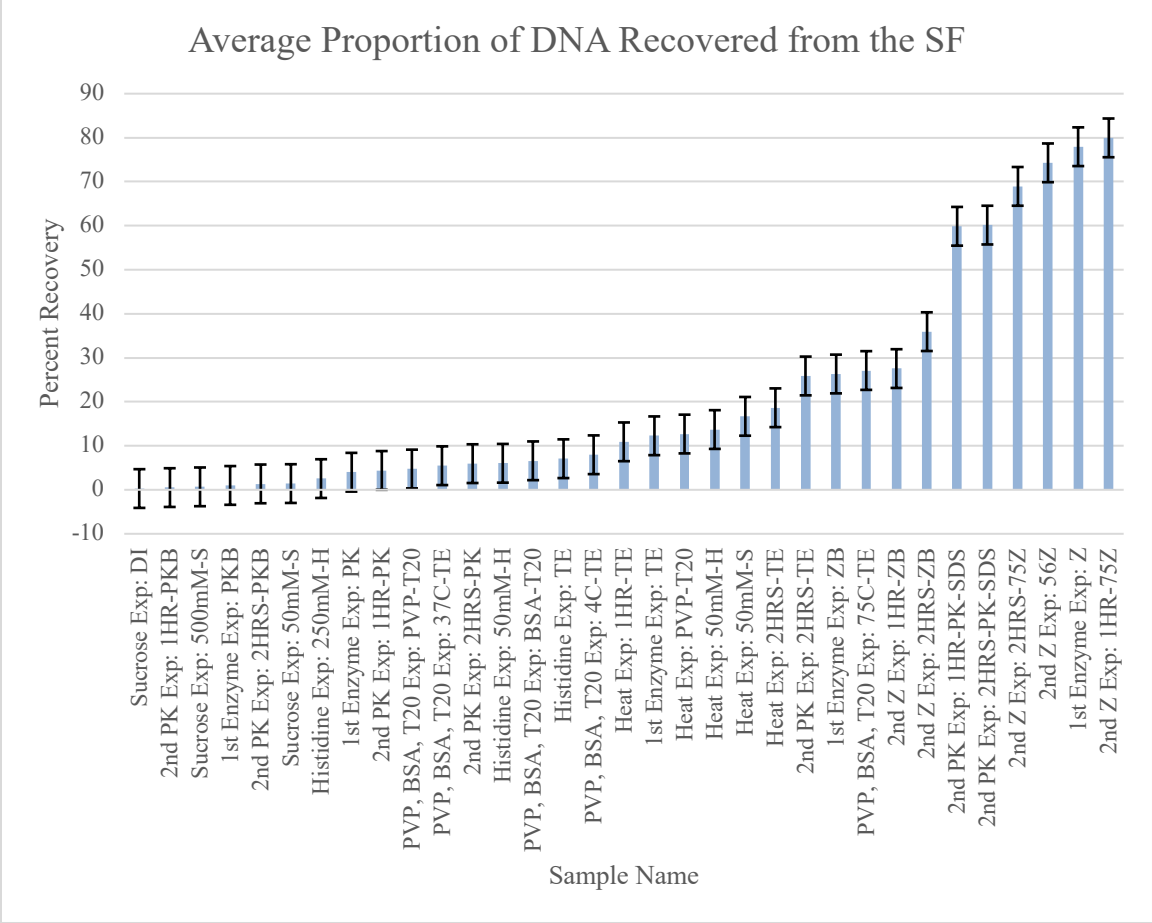

Figure 7. Average Percent Recovery of the SF from all Experimental Conditions. The experimental condition is preceded by its experiment (Exp) name in the chart.

Of the experiments in which the MF was extracted, the least amount of DNA was recovered from the MF of *forensic*GEM™ samples incubated for 1 hour at 75°C (Figure 8). It was found that, on average, 20.04% of the total mass of DNA was left stuck to the swab after the one-hour incubation. In contrast, semen swabs incubated in PK buffer with no SDS for 1 hour at 56°C demonstrated the poorest elution with an average of 99.5% of total DNA found on the cotton swab.

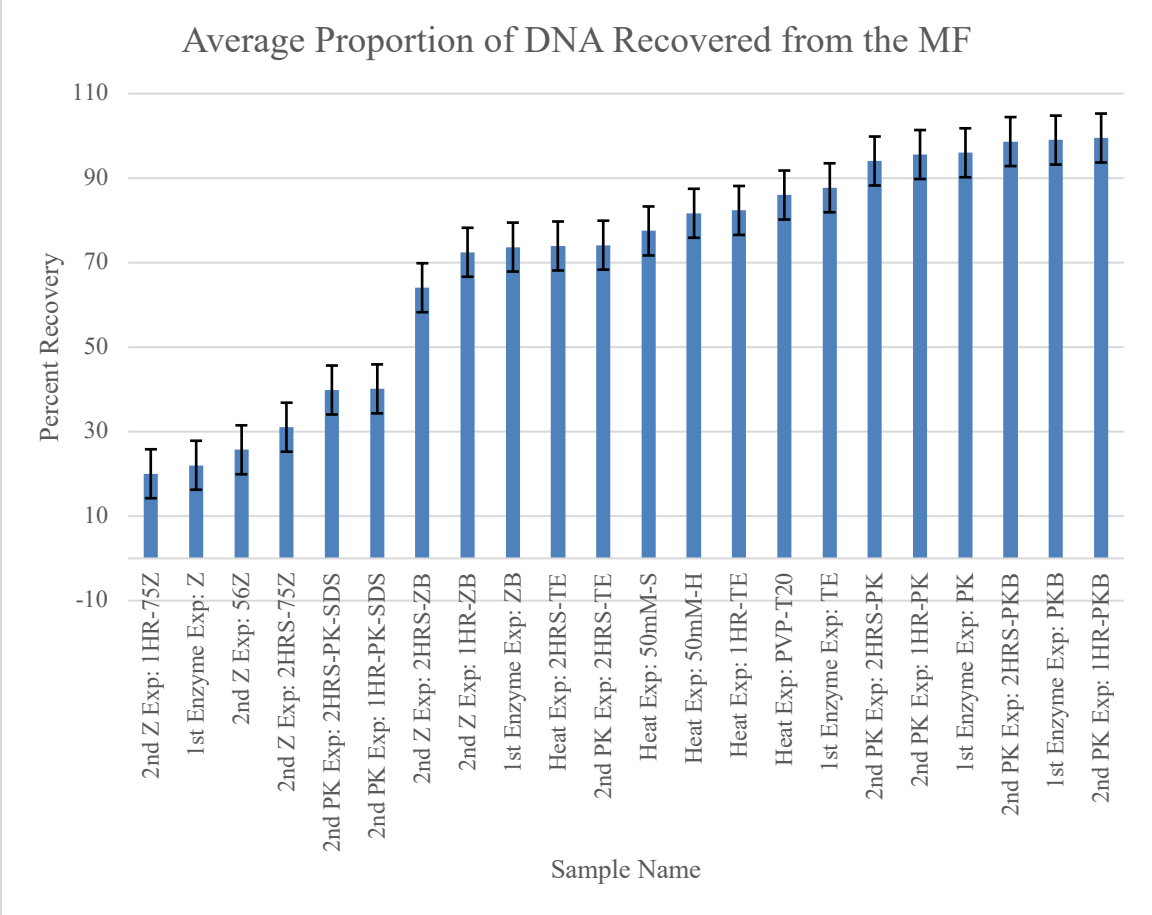

Figure 8. Average Percent Recovery of the MF from all Experimental Conditions. The experimental condition is preceded by its experiment (Exp) name in the chart.

4. DISCUSSION

4.1 Sucrose and Histidine

Despite the promising recovery results obtained from Loomis et al. using *Dictyostelium discoideum* amoeba cells and Speidel using epithelial cells, neither sucrose nor histidine improved the release of sperm cells from cotton swabs. An essential principle upon which the Loomis et al. study was designed is that these small, soluble molecules must be in solution at concentrations similar to the total amount of cellular glycoproteins to overcome the van der Waals attractive forces between the cell and the substrate (60). Accordingly, Loomis et al. deposited a known number of cells (~500) onto their test substrates, estimated the total number of cellular glycoproteins that would be present on this amount of cells, and selected concentrations of sucrose and histidine based on this estimation (60). In this study, between 14,000-28,000 sperm cells were deposited on each swab, greatly outnumbering the 500 used in Loomis's study. Thus, it is possible that solutions were prepared at insufficient concentrations of sucrose or histidine needed to undermine the extensive van der Waals forces between the sperm cells and cotton swab. To better assess the efficacy of sucrose and histidine elution buffers, an approximation of the number of glycoproteins surrounding all sperm cells in a sample must first be determined then solutions of sucrose and histidine at comparable concentrations must be tested.

The sucrose and histidine experiments were also two of three experiments in this study in which the MF of each sample was not extracted along with the SF. Instead, percent recovery was calculated based on the theoretical amount of DNA deposited on

the swab during sample preparation. Later experiments demonstrated that, despite depositing equal volumes of the same semen dilution onto each swab, the amount of DNA on each swab was highly variable. For samples in which the MF was extracted, the target mass of sperm DNA deposited onto each swab was 100 ng. In these samples, however, the average total mass of DNA deposited on swabs and the standard deviation were found to be approximately 84.859 ng of DNA and 22.86, respectively. Thus, the use of a theoretical starting amount of DNA will yield inexact recovery data when all DNA in each sample is not accounted for. A more accurate measure of recovery using sucrose and histidine solutions would be obtained by extracting the MF of each sample.

4.2 PVP, BSA, and Tween 20

Although incubation solutions containing PVP, BSA, and Tween 20 delivered moderate recovery, the results obtained in this study did not reach the same level of success as seen in the Gray et al. study. Gray's research set out to design an elution buffer compatible with qPCR (67). However, their final design was optimized using cultured mouse embryonic fibroblasts in solution and incorporated components capable of lysing epithelial cells (67). Therefore, it is likely that the elution recovery results reported in the study reflected cells that were both removed and extracted from the swab during the incubation rather than cells that were eluted from the cotton swab exclusively.

While Gray's buffer may perform efficiently with epithelial cells, it may not as well be suited in use with sperm cells.

Gray hypothesized that elution success depends upon displacement of macromolecules (67). Following this hypothesis, if the molecular forces between the cell and the substrate are not disrupted enough to allow PVP and BSA to form replacement hydrogen bonds with the cotton, the cells will not elute. It must be considered that a lack of adequate displacement, as well as the improper concentration of PVP, BSA, and Tween 20, might have been responsible for the modest recovery observed in this study.

Samples incubated in TE at 75°C outperformed all other temperature conditions by as much as 22.97%. This result indicates that increasing the temperature of the sample sufficiently weakens cell and substrate interactions allowing more sperm cells to release from the cotton swabs. This could support the idea that van der Waals forces may may affect sperm cell adhesion.

Notably, the PVP, BSA, and Tween 20 experiment represents the last experiment in which the amount of DNA left on the MF was not measured. Therefore, recovery was based on a theoretical amount of input DNA which, as previously discussed, is not exact. To better assess the elution efficacy of PVP, BSA, and Tween 20 solutions, the MF must be extracted.

4.3 Heat

Rather than calculating recovery yield with a theoretical mass of starting DNA, the total mass of DNA was determined by summing the mass of DNA extracted from

each fraction, thus allowing for the calculation of a more accurate percent recovery. Compared to the results obtained from incubations at lower temperatures, results presented show that sucrose, histidine, and PVP and Tween 20 solutions all demonstrated higher DNA recovery from the SF when incubated at higher temperatures. This provides further evidence that heat disrupts the binding of sperm cells to cotton.

Neither the 1 hour nor 2-hour TE time point performed as well as the previous experiment. Had the MF been extracted in the previous experiment, it is possible that the recovery data of the 75°C TE sample would be on par with what was observed in this experiment. Higher recovery was observed with TE samples incubated for an additional hour at high heat. In theory, a longer incubation would allow for maximum cell displacement, however, it must be determined any effects on cell integrity that long incubations at high temperatures may have.

4.4 Initial Enzyme Experiment Findings

The incubation of sperm swabs in the presence of commonly used DE enzymes yielded the best results obtained in this study. Swabs incubated in the presence of *forensic*GEM™ returned an average recovery of nearly 80% of total DNA in the SF. Without SDS, PK demonstrated low recovery with less than 4% of total DNA in the SF. When 0.1% SDS was incorporated into the PK incubation solution, the average recovery increased to approximately 60% of total DNA in the SF. This difference between PK

without SDS and PK with SDS demonstrates the efficiency of low levels of SDS in unfolding sperm glycoproteins and presumably exposing PK cleavage sites.

To investigate the efficacy of these enzymes on sperm glycoproteins, 50 human seminal plasma N-linked glycoproteins characterized by Saraswat et al. were run through PeptideCutter (SIB Swiss Institute of Bioinformatics), a website that identifies the enzyme cut sites on a particular protein (99). This work revealed that PK cleaves nearly twice as many sites as EA1 for most glycoproteins evaluated. Notably, however, EA1 has cut sites closest to the N-terminal end of the peptide relative to PK. During spermatogenesis, sperm glycoproteins undergo either N-glycosylation, the addition of an oligosaccharide to an Asparagine (Asn) amide, or O-glycosylation, the addition of a glycan via covalent bonds to the hydroxyl groups of Serine (Ser) or Threonine (Thr) (100,101). It is known that PK effectively cuts peptide bonds at the C-terminal end of aromatic, aliphatic and hydrophobic amino acid residues while EA1 can cleave bonds of various amino acids, including Asn, Ser, and Thr (92,102). This suggests that EA1 is capable of acting on both N- and O-glycosites while PK can only cleave the sites of O-linked glycoproteins. More effective removal of the sperm's glycoprotein coat may aid in the release of sperm cells into solution; however further research is needed to verify the effect of each enzyme's activity on the sperm glycoproteins.

Sperm cell elution also proved to be more successful with incubation in only the *forensic*GEM™ buffer in contrast to the PK buffer. The constituents of *forensic*GEM™ 10X Blue Buffer are proprietary, preventing the formation of an informed hypothesis for

how these results occur. However, it is clear that some effect of the buffer itself facilitates the removal of sperm cells from the cotton.

4.5 PK Re-Testing

Low DNA recovery observed in PK samples from the initial enzyme test prompted the addition of 0.1% SDS to the PK elution buffer in the second PK experiment which outperformed the non-SDS PK controls by as much as 1000%. It is widely established that the proteolytic activity of PK is enhanced by the presence of SDS; however, these results suggest that the detergent activity may also improve sperm elution (32).

SDS is an effective chemical lysis method because it interferes with hydrophobic-hydrophilic interactions (103). At sufficient concentration and temperature, SDS molecules will aggregate in solution to form amphipathic micelles (104,105). Upon contact with the micelles, proteins are imparted with a negative charge and begin to extend around the micelle's hydrophilic shell (105). As the proteins extend, they unfold from their native conformation and denature (105). It has been postulated that PK exhibits better activity on unfolded proteins due to the inherent exposure of more cut sites (95). Therefore, if SDS induces the same conformational changes to the sperm

glycoproteins, it may allow PK to better access to the cleavage sites, resulting in a greater release of sperm cells from the cotton swab.

4.6 *forensic*GEM™ Re-Testing

A repeat *forensic*GEM™ experiment confirmed the efficacy of the enzyme to recover sperm cells from cotton swabs. Although substantial proportions of DNA were again recovered from the SF of all samples incubated in the presence of the *forensic*GEM™ enzyme, extending the incubation time to 2 hours at 75°C appeared to have a slightly negative effect. *forensic*GEM™ buffer blanks incubated for the same time and temperature performed better than the 1-hour samples which may suggest that the additional time or temperature has an adverse effect on EA1 activity. The T_{50} of EA1, the temperature at which EA1 loses 50% of its activity during a 30-minute incubation, has been identified as 85°C (106). Given the short incubations typically applied to this enzyme, it remains unclear how the enzyme may be affected by exposure to high temperature for longer periods of time.

5. CONCLUSIONS

5.1 Main Findings

In this study, several incubation solutions were designed and tested throughout seven experiments. The components of each were selected from previously reported research due to their promise in recovering cells from different substrates. Solutions containing sucrose or histidine did not enhance sperm release suggesting that these solutions may be better suited for their original purpose of eluting non-sperm cell types. Attempted elution using PVP-Tween 20 and BSA-Tween 20 solutions also exhibited low DNA recovery in the SF, indicating that a significant amount of sperm cells remained behind on the swab.

Results from this study show that cell displacement can be encouraged by the application of heat. When incubated at higher temperature, the cell release and sperm DNA recovery from all previously tested solutions improved. This result is consistent with the premise that higher kinetic energy produces an environment unfavorable to the integrity of van der Waals forces, thus promoting cell detachment from its substrate.

Sperm elution using enzymatic solutions proved to be the most successful strategy with *forensic*GEM™ solutions and PK with 0.1% SDS recovering up to 80% and 60% of total DNA in the SF, respectively.

5.2 Future Directions

An important purpose of this work was to design a solution that would maximize the amount of DNA recovered from the SF while minimizing DNA left on the swab, and

that could be easily implemented into current, commonly practiced DE procedures. While the *forensic*GEM™ incubation solution demonstrated extraordinary recovery in this study, it must be tested on mock sexual assault samples containing both male sperm cells and epithelial cells to truly establish its efficacy. In theory, a longer incubation at optimal temperature in an enzymatic solution would have the advantage of removing more sperm cells from the swab, as well as extracting more epithelial cells, thus reducing the amount of all DNA in the MF. More research is needed, however, to better understand the activity of these enzymes at longer incubation times, and the potential effects this extended incubation would incur on the different cell types.

APPENDIX A

Table A: Sample key summarizing the components of the incubation solutions, incubation temperature, and incubation time of all experimental conditions.

Sample Key				
Experiment Name	**Sample Name**	**Incubation Solution Component(s)**	**Incubation Temperature (°C)**	**Incubation Time (Hours)**
Sucrose Experiment	500mM-S-1 500mM-S-2	500 mM Sucrose	56	1
	50mM-S-1 50mM-S-2	50 mM Sucrose	56	1
	DI-1 DI-2	DI Water	56	1
Histidine Experiment	250mM-H-1 250mM-H-2	250 mM Histidine	37	1
	50mM-H-1 50mM-H-2	50 mM Histidine	37	1
	TE-1 TE-2	TE	37	1
PVP, BSA, Tween 20 Experiment	PVP-T20-1 PVP-T20-2 PVP-T20-3	1% PVP and 1% Tween 20 in 20 mM Tris-HCl	37	1
	BSA-T20-1 BSA-T20-2 BSA-T20-3	1% BSA and 1% Tween 20 in 20 mM Tris-HCl	37	1
	37C-TE	TE	37	1
	4C-TE	TE	4	1
	75C-TE	TE	75	1
Heat Experiment	50mM-S-1 50mM-S-2	50 mM Sucrose	75	1
	50mM-H-1 50mM-H-2	50 mM Histidine	75	1
	PVP-T20-1 PVP-T20-2	1% PVP and 1% Tween 20 in 20 mM Tris-HCl	75	1
	2HRS-TE-1 2HRS-TE-2	TE	75	2
	1HR-TE-1 1HR-TE-2	TE	75	1

Sample Key				
Experiment Name	**Sample Name**	**Incubation Solution Component(s)**	**Incubation Temperature (°C)**	**Incubation Time (Hours)**
First Enzyme Experiment	PK-1 PK-2	Proteinase K, 2X Thermolabile PK Buffer	56 (1st hour) 75 (2nd hour)	2
	Z-1 Z-2	*forensic*GEM™, 10X Blue Buffer, DI Water	56 (1st hour) 75 (2nd hour)	2
	PKB-1 PKB-2	2X Thermolabile PK Buffer	56 (1st hour) 75 (2nd hour)	2
	ZB-1 ZB-2	10X Blue Buffer, DI Water	56 (1st hour) 75 (2nd hour)	2
	TE-1 TE-2	TE	56 (1st hour) 75 (2nd hour)	2
Second PK Experiment	2HRS-PK-SDS-1 2HRS-PK-SDS-2 2HRS-PK-SDS-3	Proteinase K, 2X Thermolabile PK Buffer, 0.1% SDS	56	2
	2HRS-PK-1 2HRS-PK-2 2HRS-PK-3	Proteinase K, 2X Thermolabile PK Buffer	56	2
	2HRS-PKB-1 2HRS-PKB-2	2X Thermolabile PK Buffer	56	2
	2HRS-TE-1	TE	56	2
	1HR-PK-SDS-1 1HR-PK-SDS-2 1HR-PK-SDS-3	Proteinase K, 2X Thermolabile PK Buffer, 0.1% SDS	56	1
	1HR-PK-1 1HR-PK-2 1HR-PK-3	Proteinase K, 2X Thermolabile PK Buffer	56	1
	1HR-PKB-1 1HR-PKB-2	2X Thermolabile PK Buffer	56	1
Second *forensic*GEM™ Experiment	56Z-1 56Z-2 56Z-3	*forensic*GEM™, 10X Blue Buffer, DI Water	56 (1st hour) 75 (2nd hour)	2
	2HRS-75Z-1 2HRS-75Z-2 2HRS-75Z-3	*forensic*GEM™, 10X Blue Buffer, DI Water	75	2

Sample Key				
Experiment Name	**Sample Name**	**Incubation Solution Component(s)**	**Incubation Temperature (°C)**	**Incubation Time (Hours)**
Second *forensic*GEM™ Experiment	2HRS-ZB-1 2HRS-ZB-2	10X Blue Buffer, DI Water	75	2
	1HR-75Z-1 1HR-75Z-2 1HR-75Z-3	*forensic*GEM™, 10X Blue Buffer, DI Water	75	1
	1HR-ZB-1 1HR-ZB-2	10X Blue Buffer, DI Water	75	1

LIST OF JOURNAL ABBREVIATIONS

Adv Colloid Interface Sci	Advances in Colloid and Interface Science
Am J Med	The American Journal of Medicine
Annu Rev Biochem	Annual Review of Biochemistry
Annu Rev Cell Dev Biol	Annual Review of Cell and Developmental Biology
Appl Environ Microbiol	Applied and Environmental Microbiology
Biochim Biophys Acta	Biochimica et Biophysica Acta
Biomed Res Int	BioMed Research International
Chem Biol Drug Des	Chemical Biology & Drug Design
Chem Cent J	Chemistry Central Journal
Curr Opin Cell Biol	Current Opinion in Cell Biology
Eur J Biochem	European Journal of Biochemistry
Fertil Steril	Fertility and Sterility
Forensic Sci Int	Forensic Science International
Forensic Sci Int Genet	International Society for Forensic Genetics
Genome Biol	Genome Biology
Genome Biol Evol	Genome Biology and Evolution
Hum Reprod Update	Human Reproduction Update
Int J Legal Med	International Journal of Legal Medicine
Int J Mol Sci	International Journal of Molecular Sciences
Investig Genet	Investigative Genetics

J Agric Food Chem	Journal of Agricultural and Food Chemistry
J Cell Biol	Journal of Cell Biology
J Clin Pathol	Journal of Clinical Pathology
J Forensic Sci	Journal of Forensic Sciences
J Proteome Res	Journal of Proteome Research
J Reprod Immunol	Journal of Reproductive Immunology
Meas Sci Technol	Measurement Science & Technology
Mol Reprod Dev	Molecular Reproduction and Development
Nat Phys	Nature Physics
Nucleic Acids Res	Nucleic Acids Research
Proc Natl Acad Sci U S A	Proceedings of the National Academy of Sciences of the United States of America
Sci Rep	Scientific Reports

BIBLIOGRAPHY

1. Bruce Alberts, Alexander Johnson, Julian Lewis, Martin Raff, Keith Roberts, Peter Walter. The Structure and Function of DNA. Molecular Biology of the Cell. New York: Garland Science, 2002.

2. Understanding Human Genetic Variation. National Institutes of Health (US). 2007. https://www.ncbi.nlm.nih.gov/books/NBK20363/#:~:text=Between%20any%20two%20humans%2C%20the,different%20between%20any%20two%20individuals. (accessed June 1, 2024).

3. Yadav V, Sun S, Heitman J. On the evolution of variation in sexual reproduction through the prism of eukaryotic microbes. Proc Natl Acad Sci U S A 2023;120(10). https://doi.org/10.1073/pnas.2219120120.

4. Bruce Alberts, Alexander Johnson, Julian Lewis, Martin Raff, Keith Roberts, Peter Walter. General Recombination. Molecular Biology of the Cell. New York: Garland Science, 2002.

5. Zini A, Agarwal A, editors. Sperm Chromatin. 1st ed. Springer, 2011.

6. 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.

7. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. Sperm. Molecular Biology of the Cell. New York: Garland Science, 2002.

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

9. Crafa A, Condorelli RA, Vignera S La, Calogero AE, Cannarella R. Globozoospermia: A Case Report and Systematic Review of Literature. World Journal of Men's Health 2022;41(1). https://doi.org/10.5534/wjmh.220020.

10. Balhorn R. The protamine family of sperm nuclear proteins. Genome Biol 2007;8(9). https://doi.org/10.1186/gb-2007-8-9-227.

11. Sheng K, Liang X, Huang S, Xu W. The role of histone ubiquitination during spermatogenesis. Biomed Res Int 2014;2014. https://doi.org/10.1155/2014/870695.

12. Kierszenbaum AL, Tres LL. STRUCTURAL AND TRANSCRIPTIONAL FEATURES OF THE MOUSE SPERMATID GENOME. J Cell Biol 1975;65(2):258–70.

13. Tecle E, Gagneux P. Sugar-coated sperm: Unraveling the functions of the mammalian sperm glycocalyx. Mol Reprod Dev 2015;82(9):635–50. https://doi.org/10.1002/mrd.22500.

14. Kuo JC-H, Paszek MJ. Glycocalyx Curving the Membrane: Forces Emerging from the Cell Exterior. Annu Rev Cell Dev Biol 2021;37:257–83. https://doi.org/10.1146/annurev-cellbio-120219.

15. Kuo JC-H, Gandhi JG, Zia RN, Paszek MJ. Physical biology of the cancer cell glycocalyx. Nat Phys 2018;14(7):658–69. https://doi.org/10.1038/s41567-018-0186-9.

16. Schröter S, Osterhoff C, McArdle W, Ivell R. The glycocalyx of the sperm surface. Hum Reprod Update 1999;5(4):302–13. https://doi.org/10.1093/humupd/5.4.302.

17. 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.

18. Schmidt S, Weigelin B, Te Riet J, Te Boekhorst V, Te Lindert M, Wijers-Rouw M, et al. Glycocalyx-mediated Cell Adhesion and Migration. 2020. https://doi.org/10.1101/2020.06.12.149096.

19. Jeffreys AJ, Wilson V, Thein SL. Individual-specific “fingerprints” of human DNA. Nature 1985;316:76–9.

20. Jeffreys AJ, Wilson V, Thein SL. Hypervariable “minisatellite” regions in human DNA. Nature 1985;314:67–73.

21. Gill P, Jeffreys AJ, Werrett DJ. Forensic Application of DNA “fingerprints.” Nature 1985;318:577–9.

22. Thomson JA, Pilotti V, Stevens P, Ayres KL, Debenham PG. Validation of short tandem repeat analysis for the investigation of cases of disputed paternity. Forensic Sci Int 1999;100:1–16.

23. Bagshaw ATM. Functional Mechanisms of Microsatellite DNA in Eukaryotic Genomes. Genome Biol Evol 2017;9(9):2428–43. https://doi.org/10.1093/gbe/evx164.

24. Bennett P. Demystified... Microsatellites. J Clin Pathol 2000;53:177–83.

25. Mullis KB. The Unusual Origin of the Polymerase Chain Reaction. 1990.

26. Lee SB, Shewale JG. DNA Extraction Methods in Forensic Analysis. Encyclopedia of Analytical Chemistry. Wiley, 2017;1–18.

27. Artika IM, Dewi YP, Nainggolan IM, Siregar JE, Antonjaya U. Real-Time Polymerase Chain Reaction: Current Techniques, Applications, and Role in COVID-19 Diagnosis. Genes (Basel) 2022;13(12). https://doi.org/10.3390/genes13122387.

28. Timken MD, Swango KL, Orrego C, Date Chong M, Buoncristiani MR. Quantitation of DNA for Forensic DNA Typing by qPCR. 2005.

29. McDonald C, Taylor D, Linacre A. PCR in Forensic Science: A Critical Review. Genes (Basel) 2024;15(4):1–22. https://doi.org/10.3390/genes15040438.

30. DNA Fragment Analysis by Capillary Electrophoresis. 2014.

31. Butler, John M. Data Interpretation Overview. Advanced Topics in Forensic DNA Typing: Interpretation. Elsevier, 2015;3–24.

32. 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.

33. Denaturation and Hydrolysis of Proteins. National Institute of Justice. 2023. https://nij.ojp.gov/nij-hosted-online-training-courses/dna-extraction-and-quantitation-forensic-analysts/organic-extraction/organic-extraction-process/denaturation-and-hydrolysis-proteins (accessed May 10, 2024).

34. Theunissen GMG, Gibb A, Lin PKT, Rolf B, Forat S, Jäger R. DNA profiling of single sperm cells after whole genome amplification. Forensic Science International: Reports 2021;4. https://doi.org/10.1016/j.fsir.2021.100240.

35. Vuichard S, Borer U, Bottinelli M, Cossu C, Malik N, Meier V, et al. Differential DNA extraction of challenging simulated sexual-assault samples: A Swiss collaborative study. Investig Genet 2011;2(1). https://doi.org/10.1186/2041-2223-2-11.

36. Ramos FK. Analysis of Two Novel Differential Extraction Methods [Master of Science Thesis]. Boston, MA: Boston University Chobanian & Avedisian School of Medicine, 2023.

37. 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.

38. Maître JL, Heisenberg CP. The role of adhesion energy in controlling cell-cell contacts. Current Opinion in Cell Biology. 2011.

39. Khalili AA, Ahmad MR. A Review of cell adhesion studies for biomedical and biological applications. International Journal of Molecular Sciences. 2015.

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

41. Shen Y, Nakajima M, Kojima S, Homma M, Kojima M, Fukuda T. Single cell adhesion force measurement for cell viability identification using an AFM cantilever-based micro putter. Meas Sci Technol 2011;22(11). https://doi.org/10.1088/0957-0233/22/11/115802.

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

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

44. 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). https://doi.org/10.1038/s41598-022-16277-1.

45. Petruzzelli L, Takami M, Humes HD. Structure and Function of Cell Adhesion Molecules. Am J Med 1999;106(4):467–76.

46. Takeichi M. Cadherins: a molecular family essential for selective cell-cell adhesion and animal morphogenesis. Annu Rev Biochem 1990;59:237–52. https://doi.org/10.1146/annurev.bi.59.070190.001321.

47. Ranscht B. Cadherins and catenins: interactions and functions in embryonic development. Curr Opin Cell Biol 1994;6:740–6.

48. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. Integrins. Molecular Biology of the Cell. New York: Garland Science, 2002.

49. Shattil SJ, Kashiwagi H, Pampori N. Integrin signaling: The platelet paradigm. Blood. 1998.

50. Springer TA. Traffic Signals for Lymphocyte Recirculation and Leukocyte Emigration: The Multistep Paradigm Review. 1994.

51. Springer TA. Adhesion receptors of the immune system. 1990.

52. Wai Wong C, Dye DE, Coombe DR. The role of immunoglobulin superfamily cell adhesion molecules in cancer metastasis. International Journal of Cell Biology. 2012.

53. Tvaroška I, Selvaraj C, Koča J. Selectins-The two Dr. Jekyll and Mr. Hyde faces of adhesion molecules-A review. Molecules. 2020.

54. Mulligan CM, Kaufman SR, Quarino L. The Utility of Polyester and Cotton as Swabbing Substrates for the Removal of Cellular Material from Surfaces. J Forensic Sci 2011;56(2):485–90. https://doi.org/10.1111/j.1556-4029.2010.01659.x.

55. Hsieh YL. Chemical structure and properties of cotton. Cotton: Science and Technology. Elsevier Inc., 2007;3–34.

56. Cotton Morphology and Chemistry. Cotton Incorporated. 2020. https://www.cottoninc.com/quality-products/nonwovens/cotton-fiber-tech-guide/cotton-morphology-and-chemistry/#:~:text=After%20scouring%20and%20bleaching%2C%20cotton,of%20water%20(glycoside%20bonds). (accessed March 22, 2024).

57. 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.

58. Canfield JR, Jollie M, Worst T, Oechsle C. Comparison of swab types & elution buffers for collection and analysis of intact cells to aid in deconvolution of complex DNA mixtures. Forensic Sci Int 2022;340:1–4. https://doi.org/10.1016/j.forsciint.2022.111448.

59. Nir S, Andersen M. Van der Waals Interactions between Cell Surfaces. Journal of Membrane Biology 1977;31:1–18.

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

61. Hantzidiamantis PJ, Awosika AO, Lappin SL. Physiology, Glucose. StatPearls Publishing. 2004. https://www.ncbi.nlm.nih.gov/books/NBK545201/ (accessed May 3, 2024).

62. Nakrani MN, Wineland RH, Anjum F. Physiology, Glucose Metabolism. StatPearls Publishing. 2024. https://www.ncbi.nlm.nih.gov/books/NBK560599/ (accessed May 3, 2024).

63. Queneau Y, Jarosz S, Lewandowski B, Fitremann J. Sucrose Chemistry and Applications of Sucrochemicals. Advances in Carbohydrate Chemistry and Biochemistry. 2007.

64. Kessler AT, Raja A. Biochemistry, Histidine. StatPearls Publishing. 2024.

65. Liao S-M, Du Q-S, Meng J-Z, Pang Z-W, Huang R-B. The multiple roles of histidine in protein interactions. Chem Cent J 2013;7(1). https://doi.org/10.1186/1752-153X-7-44.

66. Speidel SG. The Quest to Improve DNA Extraction Efficiency: Cellular Adhesion to Cotton Fabric [Master of Science Thesis]. Boston, MA: Boston University School of Medicine, 2020.

67. Gray A, Kuffel A, Daeid NN. An improved rapid method for DNA recovery from cotton swabs. Forensic Sci Int Genet 2023;64. https://doi.org/10.1016/j.fsigen.2023.102848.

68. Kreader CA. Relief of Amplification Inhibition in PCR with Bovine Serum Albumin or T4 Gene 32 Protein. Appl Environ Microbiol 1996;62(3):1102–6.

69. Rachmawati A, Ismaya, Widyobroto BP, Bintara S, Susilawati T. Effect of Different Bovine Serum Albumin (BSA) Levels on the Sperm Viability of Ongole Cross Bred Bull during 5°C Storage. IOP Conference Series: Earth and Environmental Science. Institute of Physics Publishing, 2020.

70. Harrison RAP, Dott HM, Foster GC. Bovine Serum Albumin, Sperm Motility, and the “Dilution Effect.” Journal of Experimental Zoology 1982;222(1):81–8. https://doi.org/10.1002/jez.1402220111.

71. Kariduraganavar MY, Kittur AA, Kamble RR. Polymer Synthesis and Processing. In: Kumbar SG, Laurencin CT, Deng M, editors. Natural Synthetic and Biomedical Polymers. Elsevier Science, 2014;1–31.

72. Rezadoost MH, Kordrostami M, Kumleh HH. An efficient protocol for isolation of inhibitor-free nucleic acids even from recalcitrant plants. 3 Biotech 2016;6(1):1–7. https://doi.org/10.1007/s13205-016-0375-0.

73. Lee BJ, Kim S, Lee J-W, Lee H-M, Eo SH. Technical note: Polyvinylpyrrolidone (PVP) and proteinase-K improve the efficiency of DNA extraction from Japanese larch wood and PCR success rate. Forensic Sci Int 2021;328. https://doi.org/10.1016/j.forsciint.2021.111005.

74. Tween 20. Sigma. https://www.sigmaaldrich.com/US/en/product/sial/p1379 (accessed April 26, 2024).

75. Dresser L, Graham SP, Miller LM, Schaefer C, Conteduca D, Johnson S, et al. Tween-20 Induces the Structural Remodeling of Single Lipid Vesicles. Journal of Physical Chemistry Letters 2022;13(23):5341–50. https://doi.org/10.1021/acs.jpclett.2c00704.

76. Bachmann B, Luke W, Hunsmann G. Improvement of PCR amplified DNA sequencing with the aid of detergents. Nucleic Acids Res 1990;18(5).

77. 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.

78. 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.

79. 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.

80. 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). https://doi.org/10.3390/ijms25042160.

81. Weiss K, Racho J, Riemer J. Compartmentalized disulfide bond formation pathways. Redox Chemistry and Biology of Thiols. Elsevier, 2022;321–40.

82. 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.

83. TEMPERATURE AND MOLECULAR MOTION. 2021. https://www.upliftparent.org/cms/lib01/TX01001293/Centricity/Domain/273/HEAT-INTERNET-HANDOUT.pdf (accessed April 25, 2024).

84. Li Y. Study on the Properties of Water Molecule at Different Temperatures. Journal of Physics: Conference Series. IOP Publishing Ltd, 2022.

85. Bakó I, Pusztai L, Temleitner L. Decreasing temperature enhances the formation of sixfold hydrogen bonded rings in water-rich water-methanol mixtures. Sci Rep 2017;7(1). https://doi.org/10.1038/s41598-017-01095-7.

86. Sethi MS, Satake M. Intermolecular Forces and Crystal Structures. In: Mido Y, Iqbal SA, editors. Chemical Bonding. New Delhi: Discovery Publishing House, 2016.

87. Saul DJ, Williams LC, Toogood HS, Daniel RM, Bergquist PL. Sequence of the gene encoding a highly thermostable neutral proteinase from Bacillus sp. strain EAI: expression in Escherichia coli and characterisation. Biochim Biophys Acta 1996;1308:74–80.

88. Daniel RM, Saul DJ. THERMOSTABLE PROTEINASES FROM THERMOPHILIC BACTERIA. 2009.

89. Patel S. A critical review on serine protease: Key immune manipulator and pathology mediator. Allergologia et Immunopathologia. 2017.

90. Miao H, Zhe Y, Xiang X, Cao Y, Han N, Wu Q, et al. Enhanced Extracellular Expression of a Ca2+- and Mg2+-Dependent Hyperthermostable Protease EA1 in Bacillus subtilis via Systematic Screening of Optimal Signal Peptides. J Agric Food Chem 2022;70(50):15830–9. https://doi.org/10.1021/acs.jafc.2c06741.

91. Moss D, Harbison SA, 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.

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

93. Barrett AJ, Rawlings ND, Woessner JF. Proteinase K. In: Barrett AJ, Rawlings ND, Woessner JF, editors. Handbook of Proteolytic Enzymes. Elsevier Science & Technology, 2012;3240–2.

94. Qiagen. Proteinase K - Solution. 2023.

95. interchim. Proteinase K.

96. Yakirevich E, Naot Y. Characterization of a potent sperm-agglutinating monoclonal antibody and its cognate antigens. Fertil Steril 1999;71:502–10.

97. Brown LT. Migration Patterns of Seminal Fluid Components and Spermatozoa in Semen Stains Exposed to Water and Blood [Master of Science Thesis]. Boston, MA: Boston University School of Medicine, 2016.

98. Nicholas EL. Comparison of Results Using Temperature Controlled Differential Extraction and Differential Extraction Using the QIAGEN EZ1® Advanced [Master of Science Thesis]. Boston, MA: Boston University School of Medicine, 2021.

99. 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.

100. Glycosylation. UniProt. 2022. https://www.uniprot.org/help/carbohyd (accessed May 27, 2024).

101. Wang S, Li Y, Xin A, Yang Y, Tao S-C, Gu Y, et al. Glycosylation on Spermatozoa, a Promise for the Journey to the Oocyte. Modifications in Biomacromolecules. 2024;1–29.

102. Heinrikson RL. Applications of Thermolysin in Protein Structural Analysis. In: Hirs CHW, Timasheff SN, editors. Methods in Enzymology. Academic Press, 1977;175–89.

103. Islam MS, Aryasomayajula A, Selvaganapathy PR. A Review on Macroscale and Microscale Cell Lysis Methods. Micromachines (Basel) 2017;8(3):1–27. https://doi.org/10.3390/mi8030083.

104. Johnson M. Detergents: Triton X-100, Tween-20, and More. Labome. 2023. https://www.labome.com/method/Detergents-Triton-X-100-Tween-20-and-More.html (accessed May 28, 2024).

105. Otzen DE, Pedersen JN, Rasmussen HØ, Pedersen JS. How do surfactants unfold and refold proteins? Adv Colloid Interface Sci 2022;308:1–13. https://doi.org/10.1016/j.cis.2022.102754.

106. Adekoya OA, Sylte I. The Thermolysin Family (M4) of Enzymes: Therapeutic and Biotechnological Potential. Chem Biol Drug Des 2009;73(1):7–16. https://doi.org/10.1111/j.1747-0285.2008.00757.x.

CURRICULUM VITAE