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The Effect Of The Chemical And Physical Properties Of Cotton On The Retention Of Spermatozoa

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ABSTRACT

Studies have shown that spermatozoa persist on cotton fabric under various conditions after being washed multiple times. The purpose of this study was to observe the number of sperm cells removed from cotton fabric over the course of multiple wash cycles. This study also explored how various treatment methods impact the rate of removal and number of cells removed.

In this study, sperm cells were counted using a hemacytometer to estimate the average number of cells in a 20 µL semen stain. Twenty stains were prepared on cotton fabric and washed in a microfuge tube with 200 µL of reverse osmosis (RO) water. The cells removed were counted using a hemacytometer. Four stains were washed without a spin basket, and four stains were washed with a spin basket until cells were no longer removed. For samples washed with a spin basket there was an exponential decrease between the first and second wash cycle, and after 5 washes the number of cells removed was less than 1% of the cells in the stains. Twelve additional stains were washed with a spin basket for 5 washes, and the cells removed in each wash were counted. Under normal washing conditions it was determined that between 40-60% of cells were removed after 5 washes, but by the 5th wash the number of cells removed was less than 1%, indi-

cating that subsequent washes would have little to no effect. Despite this, a full genetic profile was detected from each of the washed stains through DNA analysis.

Three fabrics were washed using variable conditions—teasing the fabric, boiling water, and a cellulase solution. The cells in each wash were counted using the same hemacytometer method. Of the variable treatments, teasing the fabric showed the greatest impact in removing additional cells. Alternatively, the boiling water and cellulase treatments seemed to have no effect, and the trendlines were consistent with the exponential decrease seen in the other washed stains in the study. A full genetic profile was detected from each of the washed stains regardless of the washing condition or the estimated number of cells removed.

Keywords: spermatozoa, cotton, laundering, hemacytometer

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DNA	deoxyribonucleic acid
DTT	dithiothreitol
PSA	prostate specific antigen
RO	reverse osmosis
SAP	seminal acid phosphatase
SDS	sodium dodecyl sulfate
SEM	scanning electron microscope

CHAPTER 1

INTRODUCTION

After a sexual assault, it is imperative that biological evidence be collected as quickly as possible. Unfortunately, many survivors of these crimes do not report for a considerable amount of time ranging from days to years due to feelings of shame, guilt, or fear.[1, 2] Because of this, investigators are often required to gather evidence from other sources such as bedding, clothing, or undergarments, rather than the swabs typically collected from a victim during a medical exam. Some studies have suggested that victims who do not immediately report may store or wash these alternative evidentiary items.[3] Due to the delayed collection, the persistence of spermatozoa on laundered fabrics and the ability to recover deoxyribonucleic acid (DNA) from those fabrics has been studied at length.[3, 4, 5, 6] Recent studies have shown that under various laundering conditions and after multiple washes, cotton fabrics tend to retain spermatozoa better than other common fabrics.[4, 5, 6] Researchers have suggested that the physical or chemical properties of cotton may impact its cell retention.[3, 4, 6, 7, 8] Despite the numerous studies conducted, the point at which cells are no longer removed from clothing or bedding in the laundering process has yet to be established. To our knowledge, an estimate of the number of sperm cells remaining on the evidence after multiple washes has also not been established. The purpose of this study is to observe the removal of sperm cells from cotton during a simulated laundering process by counting the cells present in the wash fluid using a hemacytometer.

These observations may help investigators determine the point at which sperm cells are no longer removed from cotton fabric when washed. This study also explores how some of the chemical and physical properties of cotton may impact the fabric's retention of sperm cells. The findings of this study may also be relevant to investigators who are interested in removing sperm cells from cotton swabs during the differential extraction process.

Evidence Collection and Analysis

Cotton swabs have been used in forensic science to collect biological samples for decades due to their affordability and applicability to multiple surfaces.[9] However, it has been shown that at least half of the recoverable DNA collected using a swab may be retained on the swab, posing issues for forensic scientists.[10] Recovering too little DNA may result in issues such as allelic drop out and the failure to generate a full genetic profile. Because of cotton's retention of DNA, numerous alternative swabs have been tested, including nylon and foam, as a potential collection media. Although nylon swabs were shown to release more genetic material than cotton, issues were reported in the adsorption rate and the drying time. Further, cotton swabs were determined to be the "best general choice" when collecting evidence from a variety of surfaces.[9] Therefore, cotton swabs have remained one of the most common collection media for biological evidence including blood, saliva, and semen.

After a sexual assault, biological samples should be collected as quickly as possible. Sperm cells start to degrade within hours of intercourse in shape and definition.[1]

Therefore, it is recommended that medical exams be conducted within seven days of an assault for sperm cells to be detected.[11] Examinations conducted outside this window may result in a decrease in DNA recovery from any swabs.[1] However, in these instances, bedding or clothing may also be submitted for evidentiary analysis. When these evidentiary samples are submitted, they are first examined visually under natural and alternate light. If a semen stain is present, it will fluoresce under ultra-violet or blue light, specifically at wavelengths between 365—450 nm.[1]

Once a potential semen stain has been identified, the most common presumptive test used in forensic science labs is the seminal acid phosphatase (SAP) test. Acid phosphatase is a component of semen that causes naphthyl phosphate found in the SAP reagent to break down. The chemical breakdown of the naphthyl phosphate frees a phosphate group which binds with a chromogen resulting in a purple color change.[1, 11] However, this test is only considered a presumptive test for semen because it is subject to false positives. To confirm the presence of semen, a PSA (prostate-specific antigen) test or microscopic examination is necessary. Prostate-specific antigen is a protease produced by the prostate gland and considered to be a semen marker. Tests for PSA are immunochromatographic membrane kit tests that operate under antibody-antigen interactions. In the presence of PSA, an antibody-antigen complex is formed and creates a pink band in the viewing chamber. For each test, a control band will also appear, and a positive result would be indicated by the two pink bands.[12] Introduced in 1839, microscopic identification of spermatozoa is a common confirmatory technique because sperm cells are exclusively found in semen. Typically, microscopic examinations are conducted through a staining method. The Christmas tree stain is one of the more common techniques because

of its simplicity and the ability to clearly differentiate between epithelial cells and sperm cells. The nuclear fast red reagent stains sperm heads and epithelial nuclei a red/pink color while the picroindigocarmine stains the tails of sperm cells and cytoplasm of epithelial cells green.[1, 12]

This study aims to investigate what causes sperm cells to adhere better to cotton than other fabrics after numerous wash cycles. Research has shown that cotton fabrics tend to have a better retention of spermatozoa than other common fabrics including nylon, polyester, and silk despite being laundered multiple times.[3, 5, 7] Theories have been proposed that these findings may be related to the interactions between the sperm cells and the fabric having some absorbance or adsorbance effect. To explore these ideas, it is important to understand how the structure of sperm cells and cotton may allow for the molecules to interact. Further, it is important to understand how the structure of the fabric impacts its properties, such as absorption and adsorption.

The Composition of Semen and the Structure of Sperm Cells

Human semen is produced by multiple glands and contains various components. A normal semen sample can be between 1.6—5.5 mL and contains between 17—192 million sperm cells per milliliter.[12] Sperm cells are produced in the testes, but only account for a small percentage of seminal fluid. Other components of semen aid in the function and survival of sperm. Produced in the seminal vesicles, two-thirds of semen is composed of fructose, an energy source for sperm cells, amino acids, ascorbic acid, flavins,

enzymes, fibrinogen, phosphocholine, and prostaglandins. One-third of semen is produced in the prostate which produces buffers, citric acid, PSA, and acid phosphatase.

Sperm cells consist of three main regions. Encased in a plasma membrane, the sperm head contains a haploid set of DNA. The sperm head is divided into an anterior region which houses the acrosome, a cap-like structure that aids in the penetration of the ovum during fertilization.[1, 12] The posterior region is where the cytoplasm and nuclear material are located. Rather than histones, the chromatin in a sperm cell contains protamines which form disulfide bonds, protecting the DNA from degradation. The mitochondria is located in the midpiece and provides the tail with energy for mobility.[1]

The DNA of a spermatozoon is found within the nucleus in the head that is enveloped by a plasma membrane. DNA has a double helix structure formed by hydrogen bonding between the nitrogenous bases. The backbone is a five-carbon deoxyribose sugar bound to a phosphate group and one of the four nitrogenous bases: adenine, guanine, cytosine, or thymine.[13] Basyoni et al. suggested that the hydrogen atoms on the nucleic acids may form hydrogen bonds with the hydroxyl groups on the cellulose.[8] However, DNA binding to cellulose may be more relevant for other biological samples such as blood or saliva, where cells more easily open to release DNA. The structure of sperm cells protects their DNA as the head of the cell is enclosed in a plasma membrane, and the nuclear material is held within a nuclear membrane made of disulfide bonds.[14] Because of these membranes, sperm cells are resistant to lysis through sodium dodecyl sulfate (SDS) and proteinase K. To release DNA from a sperm cell, the disulfide bonds in the nuclear membrane must be weakened using dithiothreitol (DTT). Therefore, it is unlikely that the

DNA of the intact sperm cells would be able to interact with the cotton fibers. It is more plausible that some other factor impacts cotton's ability to retain spermatozoa.

The Chemical Structure of Cotton

Cotton is a naturally occurring material primarily composed of cellulose.[15] The remaining 10% of non-cellulose components include proteins, waxes, pectins, and inorganics. Cotton fibers are made through the interaction of cellulose molecules linked by 1, 4-glucodic bonds, shown in Figure 1 below.

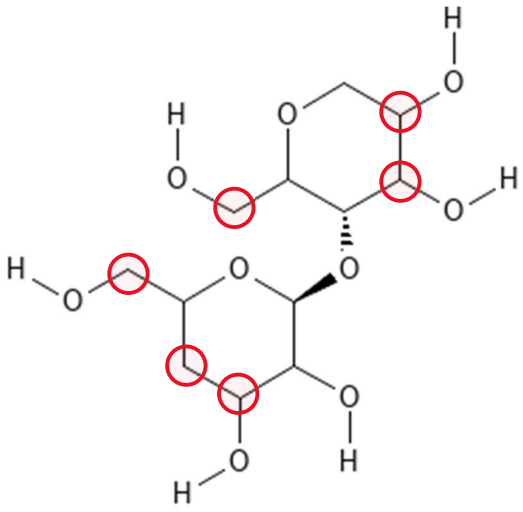

Figure 1: A cellulose molecule. The glucodic bond is shown in the middle of the structure where a singular oxygen atom binds the two ring structures together. Cellulose also contains multiple hydroxyl (-OH) groups that can be seen at carbons 2, 3, and 6, which have been denoted with red circles on each ring structure.[15]

The glucodic bonds cause steric strain resulting in cellulose having a long, rigid molecular structure. As shown in Figure 1, cellulose contains hydroxyl (-OH) groups on carbons 2, 3, and 6. These hydroxyl groups carry the potential to react with hydrogen atoms from other molecules producing strong intermolecular and intramolecular

interactions.[15] The crystalline structure caused by the intermolecular forces of hydrogen bonding make cellulose difficult to degrade due to insolubility. Further, the cellulose chains are situated close together, making accessibility difficult for enzymes.[16]

The structure of these cotton fibers may play a role in cotton's high retention of spermatozoa. A couple of theories have been suggested revolving around sperm cells being entangled, absorbed, or adsorbed to the cotton fibers. Absorbance refers to the transfer of particles into a material. Studies have shown that more absorbent materials such as cotton and terry towel had higher sperm cell retention after multiple wash cycles than their less absorbent counterparts, such as lace and silk.[5] However, in 2021, Nabi et al. refuted this theory showing that the more absorbent fabrics they studied did not necessarily retain more sperm cells than less absorbent materials. They showed that non-absorbent fabrics like chiffon and polyester had the greatest spermatozoa retention regardless of water type, indicating that factors other than a fabric's absorbent properties may impact cell retention. Adsorption is a phenomenon in which particles adhere to the surface of a material. Vorhees et al. stated that rather than being absorbed or entangled in the cotton fibers, the binding of sperm cells to cotton fibers is best described by an adsorption process, in which some type of intervention would be necessary to remove additional cells.[17] Scanning electron microscope (SEM) images showed sperm cells adhered to the much larger surface of a cotton fiber. They theorized that the use of enzymes to digest cotton fibers would lower the retention rate of sperm cells providing more DNA to forensic scientists during the extraction process.

Cellulase Breaks Down Cellulose

The insolubility of cellulose caused by the strong intermolecular forces exhibited through hydrogen bonding make the crystalline structure difficult to degrade. Because the crystalline structure is a high order, the cellulose molecules are closely packed, making enzyme accessibility difficult. However, without enzyme activity to assist in the degradation process, the half -life of cellulose in water of a neutral pH is estimated to be 100 million years.[16] Cellulase is an enzyme that speeds up the hydrolysis and break down of cellulose molecules through the breakage of glucodic bonds. The cellulase family consists of three subtypes: endocellulases, exocellulases, and processive endocellulases. Endocellulases have a more accessible active site, allowing them to bond to cellulose molecules. Alternatively, exocellulases have an active site located in a "tunnel". Cellulases operate under two primary mechanisms: hydrolysis in which the stereochemistry of the anomeric carbon is maintained and hydrolysis in which the hydroxyl group is rotated. The anomeric hydroxyl group is bound to the anomeric carbon in a cyclic monosaccharide. Molecules are only considered to be anomers if they differ in configuration at carbon 1 (aldose) or carbon 2 (ketose).[18] Currently, cellulases are commonly used in cotton processing, and they tend to have high enzymatic activity. On low molecular weight substrates, cellulases tend to operate under normal Michaelis Menten kinetics, an equation used to explain enzyme dynamics. The Michaelis Menten equation shows how the rate of a reaction is dependent on the enzyme and substrate concentrations.[19] However, in the presence of cellulose, these activities tend to be much lower due to the highly crystalline structure and insolubility making cellulose resistant to deterioration.[15, 16] Despite the lowered enzyme activity, research has shown that using enzymes to digest cotton swabs

prepared as mock evidence has resulted in a higher recovery of cellular material.[17] Of the three cellulases Vorhees et al. studied, incubating swabs with *Trichoderma viride* resulted in the highest yield of eluted cells, followed by *Trichoderma reesei* and *Aspergillus niger*. These results suggested that further research should focus on finding the optimal concentration for maximum cell elution.

Fabric Structure

Fabrics are divided into three categories—woven, non-woven, and knitted—based on the overlap of the fibers and the way they interact.[20] Woven fabrics are formed by intertwining warp yarns that run lengthwise and weft yarns that run crosswise. Most commonly, woven fabrics are used in the production of clothing. Non-woven fabrics are textile structures produced by creating some type of bond between the fibers. These bonds may be formed mechanically, chemically, thermally, or with a solvent, making non-woven fabrics great for industrial uses. Their versatility allows them to be used for a range of applications from healthcare to construction. Knitted fabrics are more comparable to woven fabrics as they are generally used in clothing production. They are made by linking loops of yarn with continuous direction change. For these yarns warp and weft strands run perpendicularly. Knitting is classified as either weft knitting or warp knitting. For weft-knitted fabrics, the loops are made along the width of the fabric. Conversely, warp-knitted fabrics are formed with loops running along the length of the fabric.

There are four basic structures for knitted fabrics: plain/jersey knit, rib, purl, and interlock. All four structures involve different interactions between loops, the basic unit of a knitted fabric. Plain knit is the simplest of the four, produced by one needle bed and

all of the loops are constructed in the same direction. These fabrics are distinguished by the V formation on the face side and the semicircle formation on the reverse side.[20, 21] For rib structure, two sets of needles are required to alternate the face and reverse stitches, causing a vertical cord appearance. Purl structures have the appearance of pearl droplets caused by rib loop transfer. These fabrics tend to be twice as thick as plain knit structures. Interlock structures appear similar to plain knit with the V formation of the stitches. However, the face and reverse side are identical for these structures, and they must be produced using specially designed knitting machines.

The structure of a fabric and the materials used to make it have been shown to have a direct effect on its physical properties including wettability, porosity, and permeability.[22, 23, 24, 25] Wettability relates to the adsorbance ability of a fabric; fabric with greater wettability will better allow molecules to adhere to their surface. Directly impacted by the hydrophilicity or hydrophobicity of a material, wettability is directly influenced by the material of which a fabric is made.[26, 27] Adhesion properties of fabrics also depend on the way particles of a stain or contaminant interact with the fibers and the fabric's porosity. The porosity of the fabric is determined by the number of interlacements between warp and weft yarns; the gaps where these interlacements occur impact the ability of a liquid contaminant to travel through the fabric via capillary action. However, the size of the molecules in the stain also plays a role and must be smaller than the pores to allow for uninterrupted travel through the fabric.[21] Knitted fabrics are porous in nature due to the open structure of the stitching.[28] However, the porosity of a knitted fabric is dependent on the tightness of the stitch used. In comparison to rib and interlock knitted structures, plain knit was shown to be the loosest, giving it the highest porosity. The thickness of the

fibers used to create a fabric also impacts its porosity, inversely, as fiber thickness decreases, porosity of the fabric increases.[23] Therefore, thinner fabrics with the same knit structure will likely have different properties related to porosity.

Porosity has also been shown to have an impact on the air and water vapor permeability of a fabric. Air and water vapor permeability refer to a fabric's ability to allow air, heat, and water vapor to be transmitted across a fabric. Fabrics with higher porosity, such as plain or jersey knit, have been shown to have a greater permeability than rib or interlock structured fabrics.[23] Related to a fabric's permeability, moisture absorbency decreases as permeability increases. This decrease in absorbance ability is likely due to the larger pore size and the decreased interaction between the fibers.[21, 26]

It has been hypothesized that fabric construction influences its ability to retain spermatozoa. Schlagetter and Glynn theorized that fabrics made with natural fibers were better at retaining sperm cells than those made with synthetic fibers.[7] Their theory was supported by Basyoni et al. who added that natural fibers have overlapping cuticles which aid in the retention of cells while synthetic fibers have a smooth outside limiting their ability to interact with the cellular material.[8] Other studies have suggested that the knit structure and absorption abilities of cotton cause the retention in spermatozoa.[5] A final theory was proposed by Vorhees et. al. and Nabi et. al. stating that the properties causing a higher sperm cell retention in cotton revolved around adsorption.[4, 17] However, no definitive conclusion has been able to be determined based on the research conducted.

Current Research

Cotton swabs are one of the most common media used for the collection of biological evidence. However, studies have shown that a substantial amount of DNA evidence is retained within the fibers of the swabs, ultimately resulting in its loss.[10] This poses issues for forensic DNA analysts as an insufficient quantity may result in unsuccessful amplification or the loss of allelic signal.

Cotton is also one of the common materials used to make clothing and bedding.[20] In recent years, many studies have been conducted to determine under which conditions spermatozoa may still be detected on fabric evidence due to the vast under reporting and delayed reporting of sexual assaults. Although these crimes are increasing in the United States, only 23% are actually reported, and 30% of those are reported after a month or more has passed.[2, 29] Because of these delays, clothing and bedding evidence may be laundered or stored before an investigation begins.

Researchers began by studying the way different fabrics retain spermatozoa under different laundering conditions. They compared common fabrics, natural and synthetic, from which most clothing items are made. In 2015, Brayley-Morris et al. observed the ability to detect spermatozoa on cotton, nylon, and polyester clothing that was stored for eight months. Their results showed that after laundering all three fabric types once, a substantial amount of DNA could be recovered. In addition, it was shown that cotton fabrics laundered three times with the non-biological detergent at 30℃ still generated a genetic profile.[3] In 2018, it was shown that spermatozoa could still be detected on cotton fabrics through microscopy after six wash cycles. It was suggested that the weave and absorption properties of cotton directly related to its ability to retain sperm cells.[5] However, a study

in 2021 refuted this hypothesis showing that less absorbent materials showed greater spermatozoa retention than more absorbent fabrics, suggesting that properties other than the absorption ability of cotton are related to its high retention rates of sperm cells.[4] Schlagetter and Glynn showed that denim, cotton, and wool had similar retention of spermatozoa based on the Christmas tree test; they theorized that this was due to the fabrics being made of natural fibers rather than synthetic ones.[7] Basyoni et. al. also supported this theory adding that natural fibers have overlapping cuticles which aid in the retention of cells while synthetic fibers tend to have a smooth outside limiting their ability to hold cellular material.[8] Although these studies demonstrate that sperm cells persist after laundering, there has yet to be a study conducted showing the point in the laundering process at which cells are no longer removed.

In one study, the impact of teasing cotton swab fibers on the removal of sperm cells was observed.[30] Their study showed that nylon swabs provided the greatest DNA recovery, followed by teased cotton swabs and regular cotton, indicating that there is some impact of the physical structure of cotton on its retention of sperm cells. Other studies suggest that the weave of cotton affects its physical properties, such as absorbance.[5] Studies on cotton fabric have shown that the tightness of the weave is directly proportional to the porosity, which creates a greater absorbance rate as discussed in the Fabric Structure section above.[21, 23]

Studies have also shown the various temperatures cotton can be washed in to still produce a DNA profile. Brayley-Morris et al. showed that after washing in 30 ℃ water three times, a complete DNA profile could still be obtained.[3] Further, they showed that after one wash cycle in 60 ℃ water a complete DNA profile could still be obtained. More

recently, in 2022, higher temperatures were explored.[6] Sapan et al. showed for the first time that using a phenol chloroform extraction method, a complete DNA profile could still be obtained on cotton washed at 90 ℃. The ability to detect sperm cells on cotton washed in temperatures higher than this for multiple wash cycles has not yet been explored. Therefore, it is of interest to observe how an increased temperature would impact sperm cell removal.

Studies have also shown how enzymatic digestion of cotton fibers aids in the elution of sperm cells.[17, 30] Vorhees et al. showed that using cellulase to digest cotton fibers for 1-4 hours prior to extraction would result in a higher sperm cell elution than standard extraction buffer alone.[17] Their results showed that of the three cellulases they studied, *T. viride* cellulase yielded the highest cell removal of sperm cells. However, in Corbin's study, the standard extraction provided a higher DNA concentration than extractions using cellulase.[30]

Purpose

Despite the numerous studies conducted, it has yet to be determined at what point in the laundering process cells are no longer removed from cotton fabric and how many cells remain. The present study aims to determine the point at which cell removal ceases in the laundering process by counting the cells removed after each wash. In addition to these observations, this study will measure the effect of teasing fabric, washing in high temperature water, and treating with cellulase on the retention of sperm cells.

This study differs from others by reducing the "laundering" down to the micro-level. Doing so allowed for the wash to be kept and for the sperm cells removed in each

wash to be counted. A method for counting these cells was developed throughout this study, and the results provide information about the behavior of sperm cells on cotton fabric over multiple wash cycles.

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CHAPTER 2

MATERIALS AND METHODS

Counting Sperm Cells Using a Hemacytometer

Sperm cells from semen dilutions or washings of semen stains were counted using the Hausser Scientific™ Phase Contrast Hemacytometer (Hausser Scientific, Horsham, PA). The coverslip of the Hausser Scientific™ Phase Contrast Hemacytometer was affixed to the coverslip support using water to ensure no movement occurred. Immediately after vortexing the dilution or washing, 10 µL of the sperm cell solution were pipetted onto each of the two counting chambers of the hemacytometer. The hemacytometer was placed under a Nikon Microscope ECLIPSE E200 (Nikon, Tokyo, Japan), and the center of the upper counting chamber was brought into focus under 100x magnification. The center of the counting chamber is composed of 25 squares. Inside each of the 25 squares are 16 smaller squares (see figure 2).

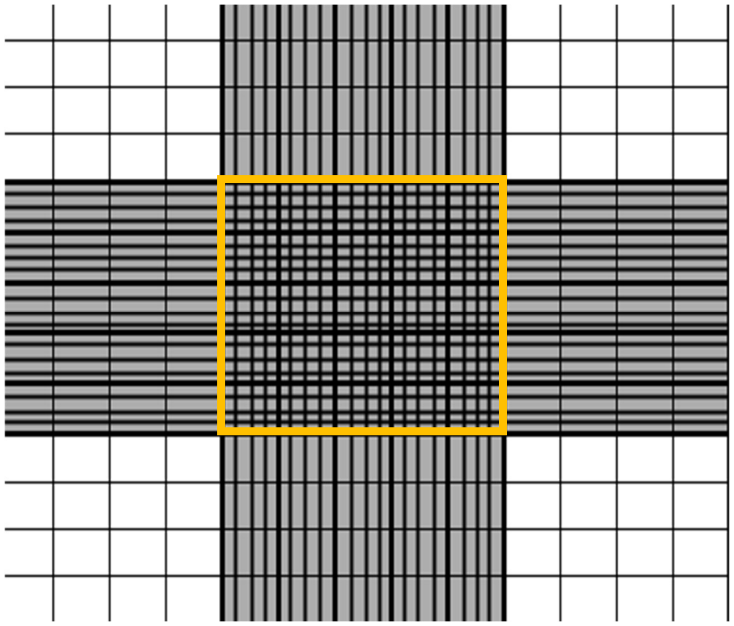


Figure 2: A schematic of a hemocytometer counting chamber. The center of the counting chamber, which is the area of interest for this study, is outlined in yellow. The center of the counting chamber is composed of 25 squares. Inside those 25 squares are 16 smaller squares which are used to count the cells present in a sample.

Once focused on the center of the counting chamber, the microscope was adjusted to 400x magnification such that only the top left square of the center chamber was visible. All the sperm cells in the 16 smaller squares within the top left square were counted and recorded; sperm heads with and without tails were counted. To avoid counting a cell twice, cells on the bottom and right line of each square were counted while cells on the top and left line of each square were omitted. If ten or more sperm cells were present within the 16 smaller squares, only 5 of the 25 squares of the center chamber were counted. The five squares counted were the top left, top right, bottom left, bottom right, and center. This counting method will be referred to as the "5-dot die method" (see figure 3). If less than ten sperm cells were present within the 16 smaller squares, then all cells in all 25 squares of the center of the chamber were counted.

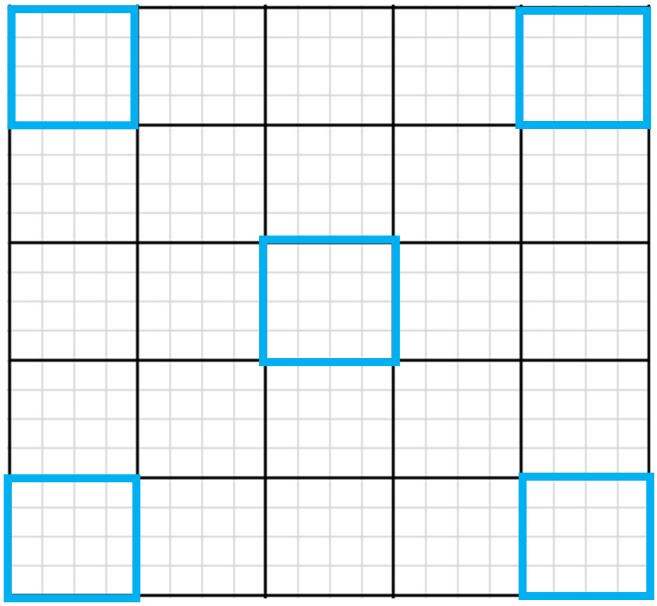


Figure 3: The 5-dot die counting method. Using the 5-dot die method, 5 of the 25 squares in the center chamber are counted. The five squares counted in this method are the top left, top right, bottom left, bottom right, and center and are outlined in blue above.

This counting process was repeated for the lower counting chamber. After both chambers were counted, the cover slip was removed. The cover slip and the counting chambers were rinsed with RO water and dried with a tissue wipe before the next sample was prepared.

Cell counts were then converted into the sperm cell concentration of the dilution or wash. When using the 5-dot die method, the total cells in the upper chamber were multiplied by 5 to estimate the number of cells present in all 25 squares of the center of the upper chamber. Next, the total cells counted in the lower chamber were multiplied by 5 to estimate the number of cells present in all 25 squares of the center of the lower chamber. The 25-square count of the upper chamber was added to the 25-square count of the lower chamber and divided by 2 to give the average number of cells present in 25 squares of a counting chamber, which represents the number of cells present in 0.1 µL of the dilution or wash. When a low sperm count required that all 25 squares be counted, the

number of sperm cells counted in the 25 squares of the upper chamber was added to the sperm cells counted in the 25 squares of the lower chamber and divided by 2. This also results in an average number of cells present in 25 squares of a counting chamber, which represents the number of cells present in 0.1 µL of the dilution or wash. Additional calculations for determining the sperm count of the original semen sample, the number of sperm present in a 20 µL semen stain, or the number of sperm present is a wash are described in the section below, "Determining the Number of Cells Present in 20 µL of Semen."

Determining the Number of Cells Present in 20 µL of Semen

Purchased human semen (BioIVT, Westbury, NY) was diluted and sperm cells were counted to assess the expected variation of sperm cells in the 20 µL semen stains prepared for this study. Fifteen 1/10 dilutions of semen to RO water were prepared in 1.5 mL DNA LoBind Microcentrifuge Tubes (Eppendorf, Hamburg, Germany) by pipetting 20 µL of the neat semen sample and 180 µL of RO water into each LoBind tube and vortexting. To provide additional information for the estimate, five 1/25 dilutions of semen to RO water were prepared in 1.5 mL LoBind tubes by adding 20 µL of semen to 480 µL of RO water. Sperm cells were counted using the method previously described in the "Counting Sperm Cells Using a Hemacytometer" section.

Once the cells counted from each dilution were recorded, the number of sperm cells present in 20 µL of the neat semen sample was calculated. Using the method previously described the average number of cells present in 25 squares of the counting chamber was estimated, representing the number of cells present in 0.1 µL. To convert this

value to the number of cells/mL of the dilution, the average was multiplied by 10,000. Multiplying by the dilution factor resulted in the number of cells/mL in the original semen sample; the dilution factor was 10 for the 1/10 dilutions and 25 for the 1/25 dilutions. To convert cells/mL to cells/µL, this value was divided by 1000. Finally, the number of cells/µL present in the original semen sample was multiplied by 20 to give the number of sperm cells in a 20 µL semen stain. These calculations were performed for all 20 dilutions. The final values of sperm cells in 20 µL of semen for each dilution were averaged to estimate how many sperm cells are present in a 20 µL semen stain.

Washing Semen Stains With a Spin Basket and Without a Spin Basket

Semen stains were prepared on cotton fabric, then washed in RO water. A Gildan (Montréal, Québec, Canada), white, ultra cotton, jersey knit shirt was stretched and taped over an empty pipette box to ensure no sample was lost by passing through the fabric to the surface below. The fabric was marked with a grid of 1.2 cm x 1.2 cm squares using a pencil. A total of eight samples were prepared by pipetting 20 µL of neat semen onto each 1.2 cm x 1.2 cm fabric square. The samples were stored in the fume hood at room temperature and dried overnight. Each square was cut, and the stained fabrics were individually placed into separate 1.5 mL DNA LoBind Microcentrifuge Tubes. Each stained fabric was folded into a smaller square such that the stain faced outward, and the folded fabric would fit comfortably inside the tube. Over the fabric, 200 µL of RO water were added to act as a wash. To simulate the laundering process, the stained fabric and water were vortexed for 45 seconds at setting 8 on a Daigger Vortex Genie 2 (Hamilton, NJ). For the 4 stains processed without a spin basket, the fabric was removed from the

tube and placed directly into the next empty LoBind tube, and the process was repeated, beginning with the addition of 200 µL of RO water. For the remaining 4 stains, after vortexing, the fabric was placed in a spin basket, the spin basket was placed back in the original tube and centrifuged for 60 seconds at 13,400 rpm to recover any excess liquid. After being centrifuged, the fabric was removed from the spin basket and placed directly into the next LoBind tube and the process was repeated, beginning with the addition of 200 µL of RO water. The cells present in each of the left-over washes were counted by pipetting 10 µL of each remaining wash solution into either chamber of the hemacytometer.

Sperm cells were counted after each wash, and each piece of stained fabric was washed until the average of the top and bottom chamber count was 2 or less of sperm cells per 0.1 µL. Sperm cells were counted as described in the "Counting Sperm Cells Using a Hemacytometer" section. For each wash, 10 µL were pipetted onto both the top and bottom counting chambers after the coverslip was situated on the support using water. The 5-dot die method was used to count cells unless less than 10 cells were counted in one of the 5 squares, in which case cells in all 25 squares were counted. For each sample, the washes were counted in order until ≤ 2 cells per 0.1 µL were recovered in the wash. For the eight samples, anywhere from 6 to 11 washes were counted for each sample. After washing was complete, DNA was extracted and analyzed from the fabric using the methods described in the "Extracting and Amplifying DNA from Washed Fabrics" section below.

Washing Additional Stains for 5 Washes With a Spin Basket

Twelve additional stains were washed to further observe the removal of cells in the first washes. When spin baskets were used very few cells were recovered after the 5th wash. Therefore, the 12 additional stains were only subjected to 5 washes and all samples used a spin basket in the washing process. Semen stains were prepared and washed with a spin basket as previously described in the "Washing Semen Stains With a Spin Basket and Without a Spin Basket" section. Each stain was washed five times. The cells present in each of the 5 washes were counted using a hemacytometer. After washing was complete, DNA was extracted and analyzed from the fabric using the methods described in the "Extracting and Amplifying DNA from Washed Fabrics" section below.

Determining the Number of Cells Present in Each Wash

The cell count from each wash was used to estimate the total cells removed from the fabric with each wash. In order to make this estimate, the total volume of the wash water left in each LoBind tube had to be estimated. This value was **not** 200 µL RO water, as some water was retained by the fabric when the fabric was removed. This was true for samples washed without a spin basket and for samples washed with a spin basket. First, to establish the average weight of a LoBind tube, ten empty LoBind tubes were weighed and averaged. Each LoBind tube containing a wash was weighed after 20 µL had been removed for counting. The average weight of an empty LoBind tube was subtracted from the weight of each wash tube, resulting in the weight of the liquid in the wash tube. Using a density of 1 g/mL, the milliliters of wash present in each LoBind tube were calculated. Multiplying by 1000, the milliliters of wash were converted to

microliters. To each microliter value, 20 µL were added to account for the 20 µL used during the counting process. Once the volume of wash was determined, the number of cells removed with the wash was calculated.

Sperm cells from each wash were counted as previously described in the "Counting Sperm Cells Using a Hemacytometer" section. Using either the 5-dot die method and multiplying by 5, or by counting all 25 squares in the upper and lower counting chambers, the average number of cells per 25 squares was determined. Because the 25 squares are equal to 0.1µL, these values were multiplied by 10,000 to give the number of cells/mL of wash. Dividing by 1000 produced the number of cells/µL in the wash. The number of cells removed was determined by multiplying by the microliters of wash present in each LoBind tube. Again, this wash volume was slightly different for each wash tube.

Extracting and Amplifying DNA from Washed Fabrics

After all samples were washed, the fabrics were extracted using a Qiagen DNA Investigator Kit (Hilden, Germany) and the recommended protocol for Isolation of Total DNA from Body Fluid Stains. DNA IQ Spin Baskets (Promega Madison, WI) were used in place of QIAshedder spin columns. Samples were lysed in the 56 °C water bath for 11 hours, and the final elution volume was 50 µL. The extracted samples included the 20 washed fabric (4 no spin basket, 16 with spin basket) previously described. Also extracted were the three teased, three boiled, three cellulase-treated samples, and 9 control samples described in the next section. Each extraction set included a buccal swab, three unwashed 20 µL semen stains, and a reagent blank serving as the negative control. The

recommended protocol requires a 1M solution of DTT, that is not included with the DNeasy kit. A 1M solution of DTT was prepared by dissolving 0.154 g of DL-Dithiothreitol (Thermo Fisher Scientific, Waltham, MA) in 1 mL of RO water.

These extractions were amplified using a custom multiplex reaction. The 25 µL reaction amplified three short tandem repeat (STR) loci. Each amplification reaction contained 12.5 µL of Promega GoTaq™ Hot Start Polymerase: Colorless Master Mix (Madison, WI), 6 µL of a custom primer mix resulting in 5 pmol of each primer per reaction (Table 1), 1 or 2 µL of extracted DNA, and 4.5 or 5.5 µL of nuclease free water. The custom primer mix was made by first preparing 100 µL of a 5 pmol/µL solution for each primer, then mixing the solutions together, resulting in 600 µL where each primer was approximately 0.83 pmol/µL. Primers labeled with a fluorescent dye (Table 1) were purchased from Thermo Fisher Scientific (Waltham, MA). Unlabeled primers (Table 1) were purchased from Eurofins MWG Operon LLC (Huntsville, AL). Primer sequences for D3S1358 and D13S317 are published in Krenke et al. Primer sequences for TH01 are published in Edwards et al. The prepared samples were then amplified in an Applied Biosystem's Thermal Cycler using a program of a 95 °C hold for 11 minutes, a 96 °C hold for 1 minute, 10 cycles of a 94 °C for 30 seconds, 58 °C for 30 seconds, 70 °C for 45 seconds, then 20 cycles at 90 °C for 30 seconds, 58 °C for 30 seconds, 70 °C for 45 seconds, and a final hold at 60 °C for 30 minutes.

Table 1: Primer Sequences Used in Custom Multiplex Reaction

Locus	Dye	Sequence
D3S1358	6-FAM	5'-ATGAAATCAACAGAGGCTTGC-3'
D3S1358	None	5'-ACTGCAGTCCAATCTGGGT-3'
Th01	6-FAM	5'-GTGGGCTGAAAAGCTCCCGATTAT-3'
Th01	None	5'-ATTCAAAGGGTATCTGGGCTCTGG-3'
D13S317	VIC	5'-GGCAGCCCAAAAAGACAGA-3'
D13S317	None	5'-ATTACAGAAGTCTGGGATGTGGAGGA-3'

As with the extractions, two sets of amplifications were performed. The first amplification set included the 20 washed fabrics, one of the three unwashed semen controls, an extraction negative, previously extracted DNA as an amplification positive, and an amplification negative composed of nuclease free water. The second amplification set included the variable wash samples and their corresponding controls, a buccal swab extraction, an extraction negative, two of the unwashed semen controls, an amplification positive, and an amplification negative.

Upon completion of the amplification, the samples were prepared for capillary electrophoresis using a protocol based on the AmpFISTR Identifiler PCR Amplification Kit User Guide. Each sample contained 8.7 µL of Fisher Bioreagents formamide (Fisher Scientific, Pittsburg, PA**)** and 0.3 µL of Applied Biosystems™ GeneScan™ 500 LIZ™ Dye Size Standard (Thermo Fisher Scientific), and 1 µL of the amplification product. A custom ladder was included, prepared by our lab, by combining amplified DNA from ten individuals. Once prepared, the samples were separated on an Applied Biosystems™ SeqStudio Genetic Analyzer (Thermo Fisher Scientific). Sample analysis was completed using Genemapper 6 software (Thermo Fisher Scientific).

Effects of Varying Wash Conditions on Removal of Cells

Three different "laundering" conditions were compared: teasing fabrics down to the fibrous layer before washing, using boiling water as a wash, and washing samples in a cellulase solution. Three experimental stains and three control stains were prepared for each variable. After washing was complete, DNA was extracted and analyzed from all stains using the methods described in the "Extracting and Amplifying DNA from Washed Fabrics" section.

Preparing Teased Samples

Three control stains and three experimental stains were prepared as previously described, by pipetting 20 µL of neat semen onto 1.2 x 1.2 cm squares of jersey knit shirt and allowing the stains to dry overnight. The three control fabrics were folded into smaller squares with the stain facing outward and situated independently in 1.5 mL LoBind tubes. Using two pairs of tweezers cleaned with ethanol, the experimental fabrics were pulled apart until they were composed of individual fibers. Once completely teased, these fibers were placed in 1.5 mL LoBind tubes and washed with 200 µL of RO water. The washing and cell counting process was conducted using the methods described in the "Washing Semen Stains With a Spin Basket and Without a Spin Basket" section. Five wash cycles were conducted for each sample.

Washing in Boiling Water

Three controls and three experimental stains were prepared as previously described, by pipetting 20 µL of neat semen onto 1.2 x 1.2 cm squares of jersey knit shirt and allowing the stains to dry overnight. Using a hot plate, a beaker of 100 mL of RO water was brought to a boil. The three controls were washed in 200 µL of room temperature RO water. For the experimental stains, 200 µL of the boiling water was pipetted over the fabric in the LoBind tube, and it was immediately vortexted for 45 seconds. The washing and cell counting process was conducted in the same way as the previously described in the "Washing Semen Stains With a Spin Basket and Without a Spin Basket" section. Five wash cycles were completed for each sample.

Washing in Cellulase Solution

Three control stains and three experimental stains were prepared by pipetting 20 µL of neat semen onto 1.2 x 1.2 cm squares of jersey knit shirt and allowing the stains to dry overnight. A 50 µg/mL solution of CELLULYSIN® Cellulase, *Trichoderma viride* (MilliporeSigma, Burlington, MA) was prepared by dissolving 0.005 g of the cellulase in 100 µL of a sodium citrate buffer. The sodium citrate buffer was prepared by dissolving 0.42 g of citric acid monohydrate in 200 mL of water. The pH of the cellulase solution was adjusted to 4.5 using 1M NaOH. The three control samples were washed in 200 µL of RO water. The three experimental samples were washed in 200 µL of the 50 µg/mL cellulase solution. The washing and cell counting was conducted as described in the

"Washing Semen Stains With a Spin Basket and Without a Spin Basket" section. Five wash cycles were completed for all samples.

CHAPTER 3

RESULTS AND DISCUSSION

Determination of Cells in 20 µL of Semen

To begin this study the number of sperm cells present in 20 µL of semen was estimated. This estimate of sperm cells present in any 20 µL prepared stain allows for an estimate of how many cells remained on washed fabric throughout the rest of the study. A total of 20 dilutions were prepared: fifteen 1/10 dilutions of semen to RO water and five 1/25 dilutions. Sperm cells were counted using a hemacytometer to estimate the sperm count of the semen sample using the process described in the methods. Table 2 below shows the sperm count and estimates for each dilution. As shown in Table 2, there was some variation in the estimation of cells per 20 µL stain in each dilution. The cells counted in the 1/25 dilutions resulted in estimates that ranged from 3,240,000 to 3,750,000 while those counted in the 1/10 dilutions resulted in estimates that were generally in the 2,500,000 to 2,900,000 range. There were two 1/10 dilutions where estimates were over 3,000,000, one at 3,040,000 and one at 3,640,00 cells per 20 µL.

Table 2: Sperm Cells Counted and Sperm Cell Estimates for Each Semen Dilution

Dilution #	Dilution	Cells / 0.1 µL	Cells / mL	Cells in 20 µL
1	1/10	1490	149,000,000	2,980,000
2	1/10	1288	128,800,000	2,576,000
3	1/10	1488	148,800,000	2,976,000
4	1/10	1273	127,300,000	2,546,000
5	1/10	1220	122,000,000	2,440,000
6	1/10	1820	182,000,000	3,640,000
7	1/10	1488	148,800,000	2,976,000
8	1/10	1463	146,300,000	2,926,000
9	1/10	1325	132,500,000	2,650,000
10	1/10	1520	152,000,000	3,040,000
11	1/10	1430	143,000,000	2,860,000
12	1/10	1195	119,500,000	2,390,000
13	1/10	1263	126,300,000	2,526,000
14	1/10	1345	134,500,000	2,690,000
15	1/10	1285	128,500,000	2,570,000
16	1/25	750	187,500,000	3,750,000
17	1/25	742	185,500,000	3,710,000
18	1/25	745	186,250,000	3,725,000
19	1/25	655	163,750,000	3,275,000
20	1/25	648	162,000,000	3,240,000

There are several reasons why the cell counts varied within the same set of dilutions. Counting cells with a hemacytometer is not exactly reproducible; when using the 5 dot-die method, only five of the 25 squares present on each chamber of the hemacytometer are counted. Therefore, variation in the dispersion of the sperm cells throughout the chamber is probable. The number of cells estimated could depend on the five squares chosen to count from. Occasionally, during the migration of cells onto the hemacytometer chamber, a more concentrated area of cells could be seen clumped together. Sometimes these areas would be found on one side within the 25 square hemacytometer grid while in other instances the cells may clump together outside of the 25 square counting space. Both of these scenarios could result in an artificially high or low estimate depending on

where the clumping occurred. Although the number of sperm cells in the top and bottom chambers should be the same, this was not always the case. For example, in one dilution 258 cells were counted in the top chamber while in the bottom chamber 350 cells were counted.

Additionally, there is likely some variation in the number of cells present in every 20 µL sample of semen. There are likely differences in the number of cells taken up into the pipette from the original, viscous semen sample. There also could be some differences in the number of cells retained in the pipette when dispensing the 20 µL of semen originally pipetted into each dilution. The reason for a lower estimate of cells in the 1/10 dilutions compared to the 1/25 dilutions is unclear. One possible explanation is that more clumping occurred in the 1/10 dilutions outside the counting area. Despite the variations, all estimates obtained align with expected values. There are between 17—192 million sperm cells per milliliter of semen.[1] As shown in Table 2, the lowest number of cells estimated for 20 µL of semen was 2,440,000 and the highest number of cells was 3,750,000. The number of cells in each dilution were averaged, resulting in the estimate that approximately 3,100,000 sperm cells were present in 20 µL of semen. This number was used as a baseline to which all other acquired data was compared.

Washing Method: Spin Basket vs. No Spin Basket

Overall, using a spin basket during the washes results in more cells removed from the fabric in the first wash, and less washes were required before only ≤ 2 cells per 0.1

µL were observed. The number of cells removed in each wash was calculated as described in the "Washing Semen Stains With a Spin Basket and Without a Spin Basket" section of the methods. This number is reported in the following tables, along with the percentage of cells removed based on the estimate of 3,100,000 sperm cells in each 20 µL stain. Specifically, the number of cells removed was divided by 3,100,000, resulting in the percentage. For the first four samples, a spin basket was not used. Table 3 below shows the percentage of cells removed after each wash when a spin basket was not used.

Table 3: The Percentage of Cells Removed After Each Wash Without a Spin Basket

	Fabric A		Fabric B		Fabric C		Fabric D	
Wash #	Cells Removed	%	Cells Removed	%	Cells Removed	%	Cells Removed	%
1	641,350	20.7%	646,425	20.9%	557,600	18.0%	47,600	1.5%
2	592,900	19.1%	760,000	24.5%	641,250	20.7%	864,275	27.9%
3	148,740	4.8%	615,950	19.9%	538,200	17.4%	634,500	20.5%
4	186,430	6.0%	202,500	6.5%	283,975	9.2%	199,820	6.4%
5	116,765	3.8%	114,345	3.7%	161,805	5.2%	81,900	2.6%
6	53,070	1.7%	44,415	1.4%	50,400	1.6%	35,295	1.1%
7	20,520	0.7%	50,630	1.6%	169,200	5.5%	51,750	1.7%
8	25,350	0.8%	58,590	1.9%	89,040	2.9%	25,885	0.8%
9	17,575	0.6%	23,230	0.7%	26,055	0.8%	2,910	0.1%
10	3,740	0.1%	19,780	0.6%	8,145	0.3%	0	0.0%
11	3,030	0.1%	2,820	0.1%	3,380	0.1%	n/a	n/a
TOTAL	1,809,470	58.4%	2,538,685	81.9%	2,529,050	81.6%	1,943,935	62.7%

After the 5th wash, most washes resulted in less than 2% of the cells originally present on each fabric being removed in each subsequent wash. The two exceptions to this were the 7th wash (5.5%) and 8th wash (2.9%) of Fabric C. For Fabric A, of the 58% of cells removed, 54% were removed in the first five washes. For Fabric B, of the 82% of the cells removed, 76% were removed in the first five washes. For Fabric C, of the 82% of cells removed, 71% were removed in the first five washes. For Fabric D, of the 63% of cells removed, 59% were removed in the first five wash cycles. As shown in Table 3,

even though a low number of cells were counted in wash 9, wash 10 was attempted for Fabric D but yielded no cell removed. Wash 11 for Fabric D was not performed.

The low number of cells recovered from the first wash of Fabric D (1.5%) could be attributed to several reasons. For starters, the washing process did not account for variation in the agitation that seemed to occur. During the vortexing of each fabric, some fabrics were seen to spin inside the LoBind tube, while others would remain still, and the wash would not form a spinning vortex. The lack of spinning fabric in combination with the wash could have resulted in the removal of less cells due to less interactions of all sides of the fabric and stain with the wash. It is possible that this first wash, though not resulting in the removal of many cells, loosened the cells remaining on the fabric, resulting in a much higher number of cells recovered in the second wash. As shown in Table 3, wash 2 of Fabric D had the greatest cell recovery in comparison to the second wash cycles for the other fabrics. This pattern of having a lower-than-average cell count wash followed by a higher-than-average cell count wash was not normal, but was seen for other washes in this study, including washes beyond the first wash and washes where a spin basket was used.

Another unexpected challenge for estimating cells removed was determining the volume of liquid remaining after each wash. Without using a spin basket, the fabric absorbed some of the wash solution after each cycle, which was transferred to the next LoBind tube and utilized in the subsequent wash. To achieve the most accurate estimation of cells removed, it was necessary to determine the volume of the wash solution remaining after the fabric was removed. For fabrics washed without a spin basket, the

volume ranged from 85 µL (for a first wash) to 207 µL. A larger amount of liquid remaining on the fabric could have resulted in a larger number of cells remaining on the fabric and available to be removed in the following wash. Without wicking away additional liquid from the fabric, the water retained on the fabric could have contained cells that were transferred to the next wash. Therefore, in the first wash, when a spin basket was not used, a larger number of cells may have been loosened from the fabric, but not removed in the wash to be counted.

Originally, the plan was to wash all the samples without using a spin basket. However, due to a substantial amount of water retained in the fabric when a spin basket was not used, the method was adapted to include a spin basket to remove any excess water. Using a spin basket allowed for the excess water to be removed, making the left-over wash and cell estimates more uniform. Further, with the addition of the spin basket, cells were removed quicker in the washing process; fewer wash cycles were required to achieve a negligible number of cells removed. A negligible number of cells removed was considered to be less than 2 cells per 25 squares on the hemacytometer.

A spin basket was introduced for the next set of samples to determine if removing the excess wash solution from the fabric would have any effect on the number of washes required to cease cell removal. As previously stated, the spin basket also resulted in the left-over wash being more uniform. Additionally, adding the spin basket provided a more accurate simulation of the "laundering" process. The spin basket and centrifuging step simulated a spinning process conducted by conventional washing machines to wick

away excess liquid before drying. Table 4 below shows the number of cells and percentages of the original 3,100,000 cells removed after each wash with a spin basket.

Table 4: The Percentage of Cells Removed After Each Wash With a Spin Basket

	Fabric A		Fabric B		Fabric C		Fabric D	
Wash #	Cells Removed	%	Cells Removed	%	Cells Removed	%	Cells Removed	%
1	1,880,000	60.6%	1,630,850	52.6%	525,825	17.0%	1,443,200	46.6%
2	214,800	6.9%	119,595	3.9%	85,095	2.7%	56,700	1.8%
3	28,520	0.9%	24,700	0.8%	77,280	2.5%	30,360	1.0%
4	26,190	0.8%	21,160	0.7%	10,120	0.3%	28,055	0.9%
5	7,320	0.2%	9,845	0.3%	11,100	0.4%	8,460	0.3%
6	14,175	0.5%	3,560	0.1%	925	<0.1%	8,775	0.3%
7	6,510	0.2%	n/a	n/a	n/a	n/a	3,860	0.1%
8	1,840	0.1%	n/a	n/a	n/a	n/a	n/a	n/a
Total	2,179,355	70%	1,809,710	58%	710,345	23%	1,579,410	51%

For samples washed with a spin basket, a majority of the cells were removed within the first two washes. After wash 3, less than 1% of cells were removed in all supplemental washes. As shown in Table 4, for 3 of the 4 fabrics, at least 50% of cells were removed. However, for Fabric C only 23% of cells were removed. The low percentage of cells removed may be attributed to less cells being present in the original 20 µL semen deposit on the fabric. As shown in Table 1, the estimated number of cells in 20 µL of semen varied from 2,390,000 to 3,750,000. Also, pipetting semen onto fabric may result in more variation when compared to pipetting semen into water for dilutions. When pipetting into water for dilution preparation, the pipette tip was rinsed with water by dipping the tip into the water, then repeatedly moving the pipette knob from the first stop to ready position. When pipetting onto fabric, there is not an opportunity to rinse the tip, so more of the viscous semen and sperm cells may be left in the pipette tip. It is possible that there were less cells present in the 20 µL sample pipetted onto Fabric C, causing lower

results when the percentage removed was determined based on the 3,100,000 sperm per 20 µL average.

A comparison of the number of cells removed after each wash with and without a spin basket can be seen in Figure 4. For samples washed with a spin basket, Figure 4 shows an exponential decrease in the number of cells removed with each wash. For samples washed without a spin basket, there is a more gradual decrease in the number of cells being removed in each wash. Without a spin basket, less cells are removed in the first wash due to wash solution being retained in the fabric that may contain cells. The fabric containing the excess wash is transferred to the next wash cycle, making the cell removal more gradual.

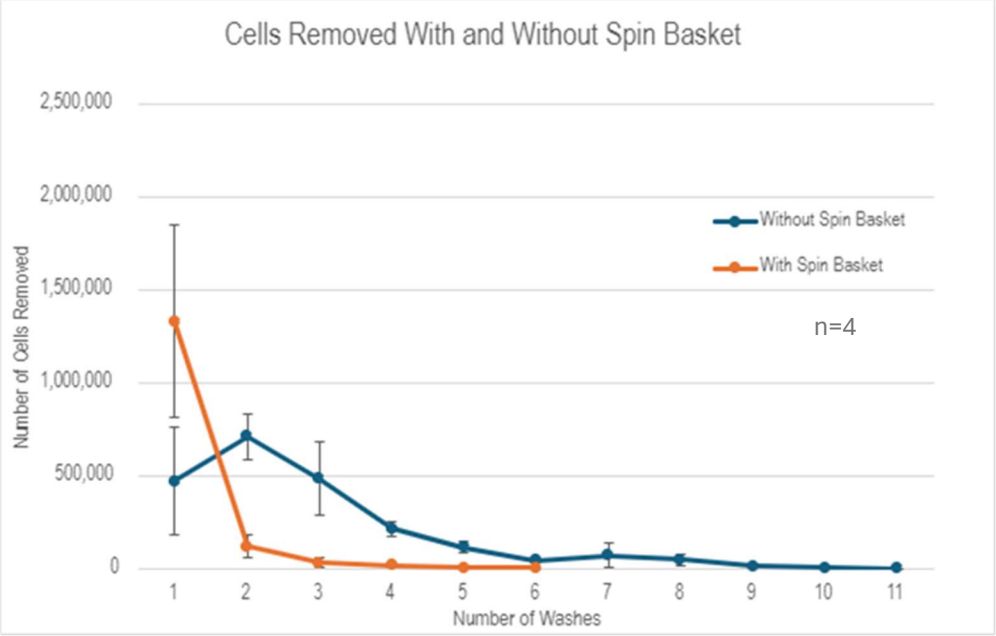


Figure 4: A graph comparing the number of cells removed with and without a spin basket. The number of cells removed when using a spin basket is much higher after the first wash and drops exponentially. Using a spin basket, more washes are required to remove cells. For both conditions, after the 5th wash cycle the number of cells removed is negligible for any subsequent washes.

Additionally, the average number of cells removed in the first wash without a spin basket is lower than that with a spin basket. Figure 4 shows that, for fabrics washed without a spin basket, more cells were removed in the second wash than the first wash, likely because of the transference of excess water and loosened cells from the first wash cycle. Also, the first wash average without a spin basket is lowered by Fabric D, which only removed an estimated 47,600 cells. As previously discussed, this may have been due to an incomplete vortexing of a fabric affecting the ability to remove cells in the wash. When using a spin basket, all wash solution remaining on the fabric was wicked away through the centrifuging process, resulting in any loosened cells retained in the wash solution on the fabric being filtered back into the LoBind tube. Removing the excess water caused a higher average in the cells removed in the first wash with a spin basket. Under both conditions, after the 5th wash the number of cells removed becomes negligible. Despite the percentage of cells removed after the 5th wash being less than 1%, the total cells removed does not approach 100%, suggesting that there are still hundreds of thousands of cells present on each fabric. For example, the maximum number of cells removed after 11 washes without a spin basket for sample Fabric C was 2,538,685 (Table 3). Therefore, based on the average 3,100,000 cells in 20 µL of semen, there would theoretically be approximately 600,000 cells still retained on the fabric after 11 washes. The lowest number of cells removed was 710,345 after 5 washes with a spin basket for sample Fabric C (Table 4). Based on this, there would theoretically be approximately 1.4 million cells remaining on the fabric. These findings align with current research showing that after multiple wash cycles spermatozoa could still be detected. Brayley-Morris et al. showed that after three washes a complete DNA profile could still be obtained from cotton fabrics.[2] Nolan

et al. showed that after six washes sperm cells could still be observed through microscopy.[3]

For the remaining washes in this study, 5 wash cycles were conducted with a spin basket. The information obtained by these early samples suggests few cells are removed after the 5th wash, and enough cells remain on the fabric for successful DNA analysis. This trend continued for all remaining samples in this study, where few cells were seen in the 5th wash, and DNA analysis of the fabric was successful.

Number of Washes Required to Cease Cell Removal

One of the fundamental research questions of this study was to determine at which point in the laundering process sperm cells are no longer removed from cotton. Without the spin basket, cells were able to be removed out to the 11th wash; however, after the 5th wash the number of cells removed only exceeded 100,000 cells one time for wash 7 of sample Fabric C (Table 3) and remained below 20,000 cells after the 9th wash. When using a spin basket, cells were removed at a much quicker rate and remained below 20,000 cells after the 4th wash. By the 5th wash using a spin basket, the number of cells removed was negligible, when compared to the estimated number of cells remaining on the fabric.

To get a better idea of the number of cells being removed in each wash, the estimated number of cells remaining on the fabric is reported with the percentage of remaining cells removed in each wash. Table 5 uses a sample washed without a spin basket to

show how the cells remaining and the percent of remaining cells removed is calculated. As a reminder, the cells removed is an estimate of the cells removed in a wash based on the cell count. The "% Removed of Total" is the cells removed divided by 3,100,000. The "Cells Remaining" is calculated by subtracting the cells removed from the cells remaining after the previous wash. For example, the cells remaining after wash 2 are 1,865,750 which is equal to 2,458,650 – 592,900. The "percent removed of remaining" is the cells removed divided by the cells remaining after the previous wash. For example, the % removed of remaining for wash 2 is 24.1% which is equal to 592,900 / 2,458,650.

Table 5: The Estimated Number of Cells Remaining from Fabric A Without a Spin Basket

Wash #	Cells Removed	% Removed of Total	Cells Remaining	% Removed of Remaining
1	641,350	20.7%	2,458,650	20.7%
2	592,900	19.1%	1,865,750	24.1%
3	148,740	4.8%	1,717,010	8.0%
4	186,430	6.0%	1,530,580	10.9%
5	116,765	3.8%	1,413,815	7.6%
6	53,070	1.7%	1,360,745	3.8%
7	20,520	0.7%	1,340,225	1.5%
8	25,350	0.8%	1,314,875	1.9%
9	17,575	0.6%	1,297,300	1.3%
10	3,740	0.1%	1,293,560	0.3%
11	3,030	0.1%	1,290,530	0.2%

Despite millions of cells being present on a fabric after multiple washes, only a small percentage are removed with each wash. As shown in Table 5, in the 3rd wash, only 8% of the 1.8 million cells on the fabric are removed. By wash 10, less than 1% of the remaining 1.3 million cells on the fabric are able to be removed with additional washing. This data suggests that additional wash cycles beyond 11 washes would have minimal

effect on removing additional cellular material from the cotton. This trend was consistent for samples with and without a spin basket, as shown in Tables 6 and 7.

Table 6: The Estimated Number of Cells Remaining, and the Percentage of Cells Removed for Washes Without a Spin Basket

	Fabric A		Fabric B		Fabric C		Fabric D	
Wash	Cells Remaining	% Removed of Remaining	Cells Remaining	% Removed of Remaining	Cells Remaining	% Removed of Remaining	Cells Remaining	% Removed of Remaining
1	2,458,650	20.7%	2,453,575	20.9%	2,542,400	18.0%	3,052,400	1.5%
2	1,865,750	24.1%	1,693,575	31.0%	1,901,150	25.2%	2,188,125	28.3%
3	1,717,010	8.0%	1,077,625	36.4%	1,362,950	28.3%	1,553,625	29.0%
4	1,530,580	10.9%	875,125	18.8%	1,078,975	20.8%	1,353,805	12.9%
5	1,413,815	7.6%	760,780	13.1%	917,170	15.0%	1,271,905	6.0%
6	1,360,745	3.8%	716,365	5.8%	866,770	5.5%	1,236,610	2.8%
7	1,340,225	1.5%	665,735	7.1%	697,570	19.5%	1,184,860	4.2%
8	1,314,875	1.9%	607,145	8.8%	608,530	12.8%	1,158,975	2.2%
9	1,297,300	1.3%	583,915	3.8%	582,475	4.3%	1,156,065	0.3%
10	1,293,560	0.3%	564,135	3.4%	574,330	1.4%		
11	1,290,530	0.2%	561,315	0.5%	570,950	0.6%		

Table 7: The Estimated Number of Cells Remaining, and the Percentage of Cells Removed for Washes With a Spin Basket

	Fabric A		Fabric B		Fabric C		Fabric D	
Wash	Cells Remaining	% Removed of Remaining	Cells Remaining	% Removed of Remaining	Cells Remaining	% Removed of Remaining	Cells Remaining	% Removed of Remaining
1	1,220,000	20.7%	1,469,150	52.6%	2,574,175	17.0%	1,656,800	46.6%
2	1,005,200	17.6%	1,349,555	8.1%	2,489,080	3.3%	1,600,100	3.4%
3	976,680	2.8%	1,324,855	1.8%	2,411,800	3.1%	1,569,740	1.9%
4	950,490	2.7%	1,303,695	1.6%	2,401,680	0.4%	1,541,685	1.8%
5	943,170	0.8%	1,293,850	0.8%	2,390,580	0.5%	1,533,225	0.5%
6	928,995	1.5%	1,290,290	0.3%	2,389,655	0.0%	1,524,450	0.6%
7	922,485	0.7%					1,520,590	0.3%
8	920,645	0.2%						

For samples washed with a spin basket, after 5 washes the number of cells removed was less than 1% of the estimated cells remaining on the fabric. Therefore, it is unlikely that any additional washing would have an impact on the number of cells removed. Although millions of cells are still theoretically present on the fabric, additional

washes are not shown to have much effect due to the properties of cotton that cause a high sperm cell retention. Whether performing 5 or 50 washes, there ultimately becomes a point, seemingly early in the laundering process, that sperm cells cannot be washed off cotton fabric at a significant rate. This supports the suggestion by Voorhees et al. that the sperm cells are not simply becoming entangled among the cotton fibers through an absorption process, but the smaller sperm cells are adsorbed to the much larger cotton fiber.[4]

After their final wash, all fabric samples were subject to DNA extraction and analyzed via capillary electrophoresis. Each DNA extraction yielded a complete allelic profile supporting the idea that hundreds of thousands of cells are left on the fabric after numerous wash cycles. These findings align with the current literature. Brayley-Morris et al. showed that after three wash cycles, a DNA profile could still be obtained.[2] Nolan et al. found that sperm cells could still be detected on cotton fabric after six wash cycles.[3]

Cells Removed During the First 5 Washes

In order to gain more information about the variability of cells in the original stain, the trend of cells removed in each wash, and the cells remaining after washing, twelve more 20 μL semen stains were washed 5 times using a spin basket with each wash. The cells remaining for each sample, including the original 4 spin basket samples previously described are shown in Table 8.

Table 8: The Number of Cells Removed from Each Fabric After 5 Washes

Stain	Cells Removed	% of Total	Cells Remaining
10/08 A	2,156,830	69.6%	943,170
10/08 B	1,806,150	58.3%	1,293,850
10/08 C	709,420	22.9%	2,390,580
10/08 D	1,566,775	50.5%	1,533,225
10/08 E	1,332,910	43.0%	1,767,090
10/10 A	1,351,025	43.6%	1,748,975
10/10 B	1,518,955	49.0%	1,581,045
10/16 A	1,197,440	38.6%	1,902,560
10/16 B	1,668,160	53.8%	1,431,840
10/16 C	316,205	10.2%	2,783,795
10/16 D	1,503,670	48.5%	1,596,330
10/19 A	1,265,090	40.8%	1,834,910
10/19 B	1,839,165	59.3%	1,260,835
10/19 C	358,965	11.6%	2,741,035
10/19 D	609,555	19.7%	2,490,445
10/19 E	269,025	8.7%	2,830,975

In general, somewhere between 40-60% of the cells deposited on the fabric were removed after 5 washes. For some stains, a much lower number of cells seem to have been removed. Assuming 3,100,000 cells were in the original stain, the percentage of cells removed was below 30% for 5 of the 16 stains: 10/08 C, 10/16 C, 10/19 C, 10/19 D, and 10/19 E from which only 22.9%, 10.2% 11.6%, 19.7%, and 8.7% of cells deposited were removed, respectively. Some potential explanations for these numbers have previously been discussed. If a fabric did not spin uniformly, or did not spin at all, the lack of agitation caused by a failure to vortex could have resulted in a fewer number of cells being removed from some fabrics. However, these percentages are for the total number of cells removed in all five washes. It is unlikely that a fabric failed to vortex throughout all five of its wash cycles. Another explanation is that fewer cells were present in the original 20 µL deposited on these fabrics. When observing the number of alleles amplified from all samples, all produced a full profile of 6 alleles, giving no indication that there

was a lower number of cells in the five samples mentioned above. Peak heights may be an indicator of the quality and quantity of DNA in the extraction; however, these five samples did not have unusually low peak height values (data not shown).

One final variation causing these differing results could lie in the error in the counting method. As previously described, for the 5-dot-die method, choosing a different 5 squares to count could result in a different estimate of cells removed from a particular fabric. However, similar to incomplete vortexing, this inaccurate counting may explain a single high or low count, but would not explain the low percentages across the five washes seen in samples 10/08 C, 10/16 C, 10/19 C, 10/19 D, and 10/19 E.

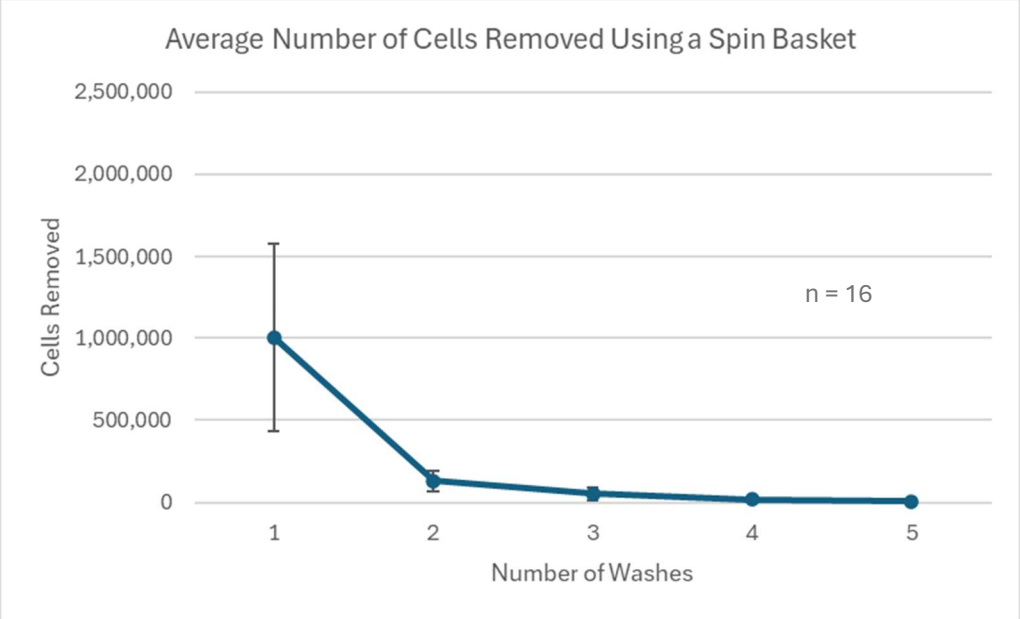


Figure 5: A trend line for the number of cells removed after each wash cycle. The overall trend shows that the majority of the cells removed come off in the first wash cycle and decrease exponentially with the second. The number of cells removed continues to decrease through the 5th cycle, but at a much slower rate.

The overall trend for all 16 spin basket samples (Figure 5) aligns with that of the cells removed using a spin basket for the first 4 samples (see Figure 4). Most of the cells removed come off after the first wash and decrease exponentially with the second. The remaining washes yield substantially less cell removal than the first and at a much slower rate. As shown in Figure 6, by the 5th wash, the estimated number of cells removed was low, ranging from 890 to 11,100 cells. Despite the variation seen in the total number of cells removed, the trendlines are uniform, and only deviate from the exponential curve for two samples. One sample increased from the second wash (121,770 cells removed) to the third wash (174,525 cells removed). Another sample had only a slight decrease from the first wash (276,900 cells removed) to the second wash (249,795 cells removed). These deviations could be explained by incomplete vortexing or the clumping of sperm cells skewing the cell count.

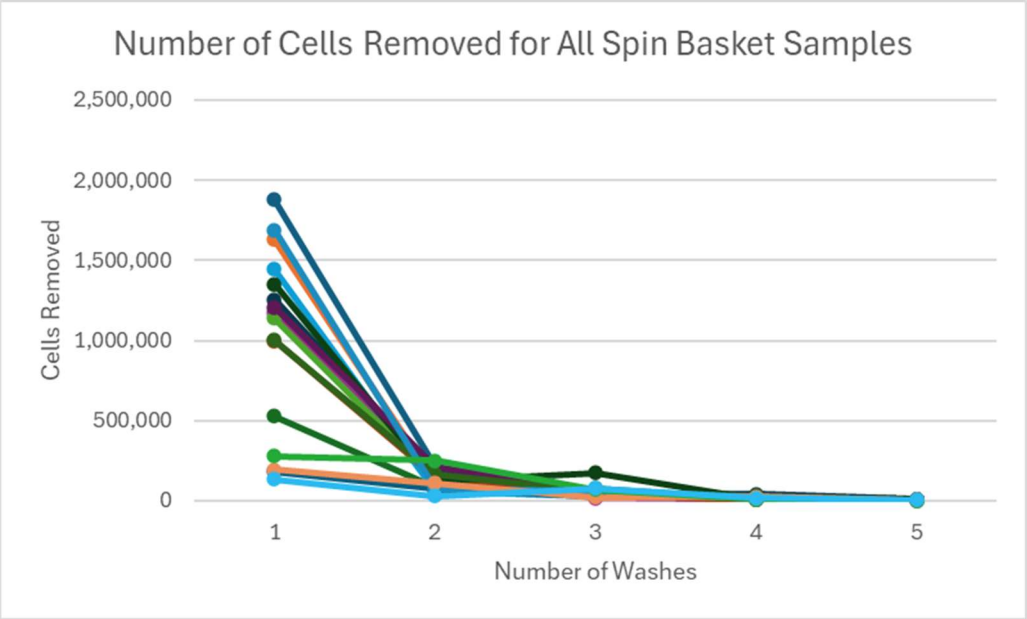


Figure 6: A graph showing the number of cells removed after each wash cycle for all spin basket samples. For most samples, the majority of the cells removed come off in the first wash cycle and decrease exponentially. Some samples start with a lower number of cells removed in the first wash.

Effect of Variable Washes on Cell Removal

Three variable washing conditions were conducted: teasing fabrics down to their fibrous layer, washing in boiling water, and washing in a cellulase solution. Each condition was evaluated based on the number of cells removed from a 20 µL stain of semen in 5 washes by counting the cells removed in each wash. Table 9 below shows the totals after 5 washes of cells removed, percentage of cells removed, and the cells remaining for each variable.

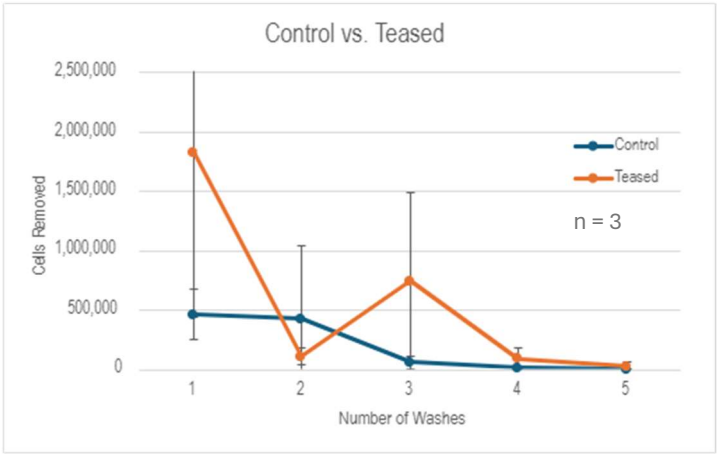
Table 9: The Percentage of Cells Removed After 5 Wash Cycles Under Variable Conditions

Treatment	Cells Removed	% of Total	Cells Remaining
11/10 Control 1	741,725	23.9%	2,358,275
11/10 Control 2	1,512,285	48.8%	1,587,715
11/10 Control 3	701,195	22.6%	2,398,805
11/10 TEASED 1	3,370,245	108.7%	-270,245
11/10 TEASED 2	3,547,650	114.4%	-447,650
11/10 TEASED 3	1,526,270	49.2%	1,573,730
11/20 Control 1	1,170,295	37.8%	1,929,705
11/20 Control 2	737,595	23.8%	2,362,405
11/20 Control 3	894,280	28.8%	2,205,720
11/20 BOILED 1	857,560	27.7%	2,242,440
11/20 BOILED 2	1,152,255	37.2%	1,947,745
11/20 BOILED 3	779,440	25.1%	2,320,560
12/08 Control 1	1,803,275	58.2%	1,296,725
12/08 Control 2	1,427,040	46.0%	1,672,960
12/08 Control 3	702,510	22.7%	2,397,490
12/08 ENZYME 1	1,676,035	54.1%	1,423,965
12/08 ENZYME 2	251,095	8.1%	2,848,905
12/08 ENZYME 3	214,630	6.9%	2,885,370
Average Teased	2,814,722	90.8%	285,278
Average Boiled	929,752	30.0%	2,170,248
Average Enzyme	713,920	23.0%	2,386,080
Average Control	1,076,689	34.7%	2,023,311

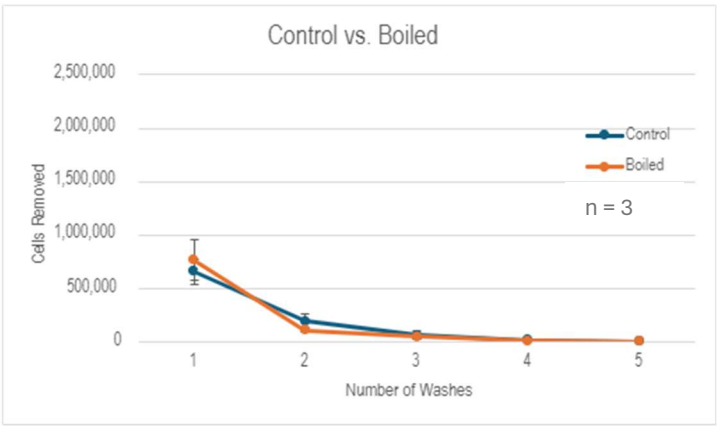
As shown in Table 9, the teased samples resulted in the highest number of cells removed while the cellulase had the lowest yield of cells removed. However, there was variation among the three samples in each treatment set. For teased sample 3, only 49.2% of cells were removed, less than half of that removed from teased samples 1 and 2. Similarly, for cellulase sample 1, 54.1% of cells were removed. However, for cellulase samples 2 and 3, less than 10% of cells were removed. The samples washed in boiling water did show more uniformity in the number of cells removed, between 24-37%; however, the efficiency of this method was the same as the controls that were washed in room temperature water. Three control samples per variable were washed alongside the treatment

samples. These control samples were compared to the variable samples to determine an overall trend. Figure 7 below shows the three graphs comparing controls to each respective variable.

A



B



C

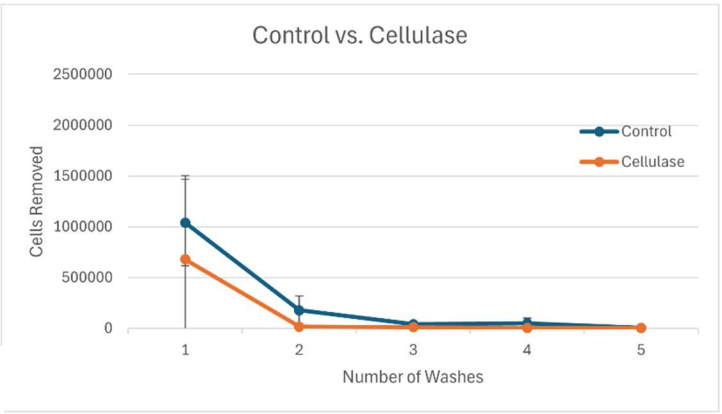


Figure 7: Graphs depicting the number of cells removed using variable laundering conditions and regular RO water laundering conditions. Graph A(top) shows the comparison of the teased samples to the respective control samples. The teased samples yielded more cell removal overall, but the wash in which the cells were removed was sporadic. Graph B (middle) shows a comparison of laundering in boiling water compared to controls. The overall trend is the same with the boiling water having a slightly higher cell yield after the first wash. Graph C (bottom) shows the trend for "laundering" in cellulase compared to control cycles. Although the trendlines are similar, the cellulase had a lower cell yield than standard washing conditions.

As shown in Figure 7, the teased samples had the greatest number of cells removed in comparison to their respective controls. However, as seen throughout this study, the number of cells removed from these washes were sporadic. A large portion of the cells were removed by the first cycle, but there was a dip in the number of cells removed by the second cycle. After the teased fibers were centrifuged following the first wash, the teased fibers clumped together and were not re-teased for the following washes. This may explain the low number of cells removed in the second wash. However, in wash 3, there is a spike in cell removal before another drop in wash 4. This spike in wash 3 occurred in 2 of the 3 teased samples. The reason for this spike is unknown. Despite the irregular removal patterns, significantly more cells were removed from the teased samples than the controls. For teased samples 1 and 2, over 100% of the cells deposited, based on the average number of cells present in 20 µL, were removed. This could be due to variation in the number of cells present in each 20 µL semen stain. As shown in table 2, the number of cells estimated for a 20 µL stain ranged from 2.4 million to 3.7 million. Thus, it is possible that the semen deposits on teased samples 1 and 2 had cell concentrations on the higher end of the spectrum. These findings support the theory that spermatozoa are retained on cotton through absorbance properties or entanglement rather than adsorption.[2, 3] Teasing the cotton fabric down to the fibrous layer resulted in the loosening of sperm cells from the fibers, and the higher rates of cell removal. However, the reason behind the retention being absorbance or entanglement would need to be further explored.

Regarding the samples washed in boiling water, there did not seem to be much effect. Studies have shown that sperm cells persisted when washing cotton in temperatures varying from 30 °C to 90 °C.[2, 3, 5, 6] In a majority of these studies, a full DNA profile was

obtained, so these results were not surprising. The percentage of cells removed and the trendline are relatively consistent with that of the original washes and the controls. As shown in Figure 7, graph B, the trendlines are similar.

The trendline for the cellulase samples aligned with the trendline for their respective controls (see Figure 7, graph C). However, the samples "laundered" using standard conditions recovered more cells than those using the cellulase, refuting other studies. In 2006, Vorhees et al. showed that allowing cellulase, specifically *Trichoderma viride*, to digest cotton fibers resulted in a greater sperm cell removal.[4] The results in this study may differ from the literature due to the time the cellulase in this study was in contact with the fabric. In the Vorhees study, samples were allowed to incubate in a cellulase solution for 1-4 hours. Conversely, in the present study, the cellulase was pipetted onto the cotton fabric, and it was vortexed for 45 seconds. Therefore, it is likely that the cellulase solution in this study did not have enough time and contact with the cotton to cause any significant digestion of the fibers. The findings were not consistent for all samples washed in cellulase. As shown in table 5, 54.1% of cells were removed from cellulase sample 1, but less than 10% of cells were removed from cellulase samples 2 and 3 when compared to the average.

DNA Amplification

Once all wash cycles were completed, the fabric samples were subjected to a DNA extraction and amplification to determine if a full genetic profile could be collected.

For every sample, a complete profile was produced indicating that a substantial number of cells were left on the fabrics despite the cessation of cell removal. The peak heights for each allele detected in each sample were recorded to give a general idea of which samples contained the most DNA. In theory, the samples that showed the greatest cell removal would have the smallest peak heights. Additionally, for samples that were theorized to have a lower number of cells deposited in the original stain, those samples would be expected to have lower peak heights. There was not a correlation between peak height and the number of cells remaining on the fabric. Figure 8 below shows an example electropherogram obtained in this study.

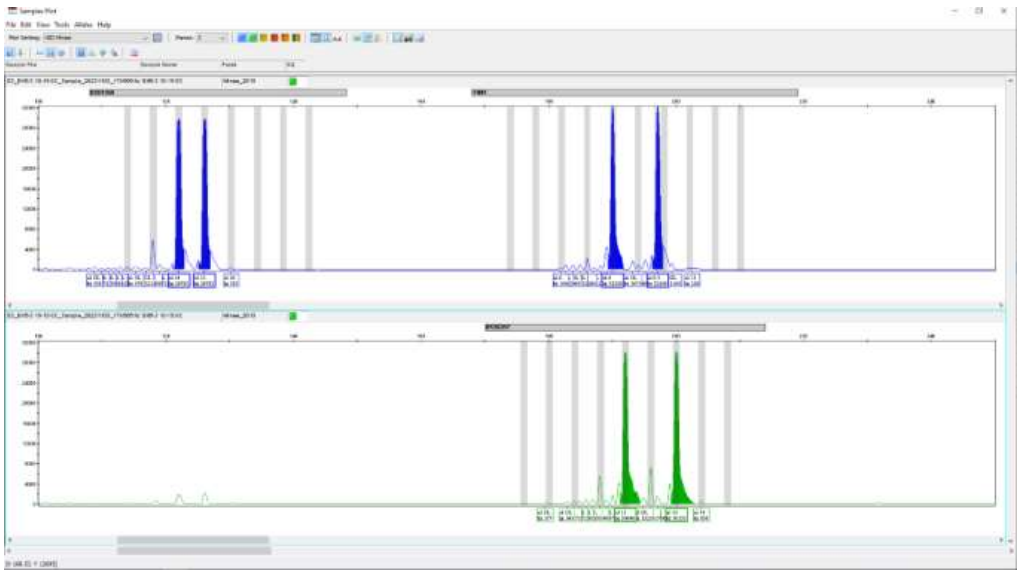


Figure 8: An example electropherogram. A complete allelic profile was obtained for the three loci observed: D3S1358, Th01, and D13S317.

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CHAPTER 4

CONCLUSION

One purpose of this study was to determine the number of washes necessary to cease sperm cell removal from cotton fabrics and estimate the number of cells remaining on the fabric. When a 20 µL semen stain on cotton fabric was washed in 200 µL of RO water, the number of cells removed with each wash decreased and were no longer removed after 6-11 washes, depending on whether a spin basket was used during the wash. Without a spin basket, there was a gradual decrease in the number of cells removed over 11 washes, whereas when a spin basket was used to help recover more liquid from the washed fabric, a more exponential decrease was seen between the first and second wash. With a spin basket, after five wash cycles, the number of cells was negligible and most often less than 1% indicating that with any subsequent wash, there would be a small removal of cells. Over 900,000 sperm cells were estimated to remain on every fabric sample after 5 washes. A complete genetic profile was generated from all washed fabrics due to the high number of cells left on the fabric.

In general, 40-60% of cells were removed at the completion of five wash cycles under standard conditions and the pattern of cell removal followed an exponential curve. Of the estimated 3,100,000 cells present in each 20 µL stain, most samples had over 500,000 removed in the first wash. However, there were some samples that deviated from

this trend of a high number removed in the first wash followed by an exponential decrease. These deviations could have been caused by numerous factors. One explanation is that some of the cell counts were inaccurate. The cause for a miscount lies in the way the cells were distributed in the hemacytometer. Because the cells present in only 5 squares were counted to provide an estimate of cells removed, it is possible that a different estimate would occur if a different five squares were chosen other than those in the 5-dot-die method. Clumps of cells that fell outside the center 25 square grid could also lead to an artificially low count. The washing mechanics may also play a role in the way cells were removed. Some fabrics did not actually spin while being vortexed, which could have led to less interaction between the water and the fabric, causing fewer cells to be removed in one wash and more cells available to be removed in the following wash. Finally, there could have been variation in the number of cells present in 20 µL of semen. Although the average, 3.1 million, was used to perform all calculations, the number of cells present in 20 µL of semen varied from 2.3 million to 3.7 million when dilutions were used to make the estimate. When stains were made on fabric this amount could have varied due to the inability to rinse the pipette tip. However, despite the possible variations, the trendline remained the same and showed an exponential decrease in the number of cells removed where the majority were removed within the first wash, and by the 5^{th} wash the number of cells removed was negligible.

The other goal of this study was to determine if different washing conditions would have any effect on the rate of or the number of cells removed. The wash treatments included teasing the cotton fabric down to its fibrous layer, washing in boiling water, and washing in a cellulase solution. Teasing the fabric proved to have the greatest effect as

more cells were removed from these samples when compared to unteased control fabric. These findings support the idea that some kind of entanglement or absorbance of the sperm cells occurs within the cotton fabric which is relieved when the fabric is separated. The boiling water did not seem to have an effect on the number of cells removed. The trendline did remain consistent with the controls showing an exponential decrease in the cells removed. The cellulase solution also did not seem to have any impact on the number of cells removed. This was likely caused by the cellulase not having enough time to digest any of the cellulose molecules in the 45 second wash cycle.

APPENDIX A

IRB APPROVAL LETTER

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Office of the Institutional Review Board for Human Use

470 Administration Building
701 20th Street South
Birmingham, AL 35294-0104
205.934.3789 | Fax 205.934.1301 |
irb@uab.edu

NHSR DETERMINATION

TO: Linville, Jason G

FROM: University of Alabama at Birmingham Institutional Review Board
Federalwide Assurance # FWA00005960
IORG Registration # IRB00000196 (IRB 01)
IORG Registration # IRB00000726 (IRB 02)
IORG Registration # IRB00012550 (IRB 03)

DATE: 02-May-2023

RE: IRB-300011101
The Effect of the Chemical and Physical Properties of Cotton on the Retention of Spermatozoa

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The Office of the IRB has reviewed your Application for Not Human Subjects Research Designation for the above referenced project.

The reviewer has determined this project is not subject to FDA regulations and is not Human Subjects Research. Note that any changes to the project should be resubmitted to the Office of the IRB for determination.

if you have questions or concerns, please contact the Office of the IRB at 205-934-3789.

Additional Comments:

Purchased specimens from BioIVT (Hicksville, NY) repository