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# Optimization of enzymatic lysis of epithelial cells for application to differential extraction of forensic sexual assault samples

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BOSTON UNIVERSITY  
SCHOOL OF MEDICINE

Thesis

**OPTIMIZATION OF ENZYMATIC LYSIS OF EPITHELIAL CELLS FOR  
APPLICATION TO DIFFERENTIAL EXTRACTION OF FORENSIC  
SEXUAL ASSAULT SAMPLES**

by

**RENA BEVERLY MONTVILLE**

B.A., Boston University, 2012

Submitted in partial fulfillment of the  
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Master of Science

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Approved by

First Reader

---

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

Second  
Reader

---

Jeffrey Hickey, M.S.  
Director of Forensics  
MicroGEM International

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ABSTRACT

The separation of sperm from female epithelial cells has been a topic of interest in forensic DNA (deoxyribonucleic acid) analysis since the origin of the field. One of the most needed applications of DNA analysis in the identification of the perpetrator of a sexual assault, as often there is little to no other evidence for identification. The largest hurdle to forensic DNA analysis in these cases is that vaginal or oral swabs from sexual assaults will have a mixture of the victim's epithelial cells and the perpetrator's sperm cells. It is well known that the analysis of complex mixtures can be difficult to impossible, especially when there is an added concern of low template DNA. Separating these cell types in the mixture evidence is the best way to avoid the need to deduce these difficult mixtures.

Sperm and Epithelial Cells are morphologically different both in cell shape and DNA packaging. Nuclear DNA in epithelial cells are more loosely packaged around histones in a structure called a nucleosome. Sperm DNA is tightly packaged around protamines rather than histones.

These DNA packaging differences can be utilized to preferentially lyse sperm and epithelial cells in order to separate them. Traditionally this is done by lysing epithelial cells with sodium dodecyl sulfate (SDS) and proteinase K (PK), separating this epithelial DNA from the sperm by centrifugations and finally lysis of the sperm using dithiothreitol (DTT) which reduces the disulfide bonds in the sperm DNA packaging. This method was developed by Peter Gill in 1985 and is still used by forensic laboratories to date.

This differential extraction is very labor intensive and time consuming. This dual-enzyme differential extraction can be performed in roughly one hour, which is highly advantageous with the large amount of backlogged sexual assault cases that forensic laboratories have. This work was undertaken to improve the separation of epithelial DNA from sperm cells in the dual-enzyme differential extraction. Here we found that the DNA carryover into the sperm fraction was due to a combination of an inability to completely separate the non-sperm fraction liquid from the sperm pellet and the decreased efficiency of ZyGEM to fully lyse epithelial cells in clumps. The solution to this problem includes the addition of a wash of the sperm pellet after initial separation of the fractions. This wash step decreased the concentration of epithelial DNA to the point that its detection may only occur with very low concentrations of sperm DNA.

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## **LIST OF ABBREVIATIONS**

ADE	Acoustic Differential Extraction
bp	Base Pairs
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
kV	Kilovolts
LCM	Laser Capture Micro-dissection
M	Molar
mL	Milliliter
N	Normal
ng	Nanogram
NSF	Non-Sperm Fraction
PCR	Polymerase Chain Reaction
PHR	Peak Height Ratio
PK	Proteinase K
qPCR	Quantitative real-time polymerase chain reaction
RFU	Relative fluorescence unit
rpm	Revolutions per minute
S.1	0.1% Sarkosyl
S.5	0.5% Sarkosyl

SDS	Sodium dodecyl sulfate
SF	Sperm Fraction
STR	Short Tandem Repeat
T	Trypsin
TE	Tris-ethylenediaminetetraacetic acid
$\mu$	Mean Average
$\mu\text{g}$	Microgram
$\mu\text{l}$	Microliter
Z	ZyGEM
W	Wash

## **1. INTRODUCTION**

### **1.1 The Original Differential Extraction Procedure**

The separation of sperm from female epithelial cells has been a topic of interest in forensic DNA (deoxyribonucleic acid) analysis since the origin of the field. In fact, the first use of DNA fingerprint analysis in forensic case work was a sexual assault case involving Colin Pitchfork.<sup>1</sup> The original differential extraction method was described in 1985 by Peter Gill et.al. and involved a modified phenol/chloroform extraction.<sup>2</sup> First, the epithelial cells were lysed using a treatment with SDS and Proteinase K (PK) overnight, and then the epithelial DNA was separated from the intact sperm by centrifugation. Finally, the sperm were preferentially lysed using dithiothreitol (DTT). This method worked because the sperm nuclei are “ramified with cross-linked thiol-rich proteins.”<sup>2</sup> This procedure was the key to prosecuting sexual assault cases, such as the Colin Pitchfork case, and is still widely used today.<sup>3,4</sup> Although this differential extraction is a strong and valuable technique, the main reason for its continued use is that a strong alternative of separation does not exist.

As recently as 2013, work by Hennekins et. al. showed that the differential extraction developed by Gill is robust enough that it is successful under most conditions, a necessary attribute given the amount of variation found in forensic casework.<sup>5</sup> In this work, they

explored the effects of varying PK concentrations, SDS concentrations and incubation temperatures, finding no significant effect or difference in effect on premature lysis of sperm.

In 2011 a study by Vulchard et. al. worked to examine the efficacy of the various differential extraction protocols employed by 9 different Swiss forensic labs. To do this, they sent samples containing mixtures of epithelial cells from buccal swabs and dilutions of semen to the labs for testing. For the four labs that still used the original differential extraction procedure, male:female ratios ranged from 1:1 to 9:1, showing large variability in the amount of female DNA carryover into the Sperm Fraction (SF).<sup>3</sup>

In this study, they also did a direct lysis of their sperm dilution and a direct lysis of the epithelial cells on swabs to determine the total ng of DNA that would be expected if the differential extraction were as efficient as direct lysis.<sup>3</sup> They determined that the sperm dilution in the samples should have yielded 188 ng of DNA and the epithelial cells should have about 7195 ng of DNA.<sup>3</sup> These amounts revealed that the differential extraction procedures for most of the labs yielded about 6% of the starting DNA from the sperm and 50-64% of the starting DNA from the buccal cells on the swabs from females.<sup>3</sup> Therefore, although the differential extraction may be consistent in not lysing sperm cells

prematurely, there is less reproducibility in the successful separation of female DNA from the sperm cells in the SF and recovery of sperm DNA.

In an effort to enhance the recovery of sperm cells and lysis of epithelial cells from sexual assault swabs with low sperm cell counts, Lounsbury et. al. developed a new buffer system to replace the original Gill Buffer.<sup>6</sup> This work was largely based on previous experiments by Norris et.al. which aimed to improve sperm recovery from cotton swabs for rape kit analysis.<sup>7</sup> From this work, it was found that the new buffer system containing MES/Tris buffer at pH 8.0, SDS (sodium dodecyl sulfate) and proteinase K had greater sperm cell recovery and epithelial lysis than the traditional Gill buffer used in Differential Extractions. Together these studies indicate that it appears that the differential extraction conditions may be stable in preventing premature sperm lysis, however, epithelial cell lysis and the separation of epithelial DNA from sperm is highly variable.

This problem leads to one of the largest potentials for sperm loss. When there are significantly less sperm cells than epithelial cells in a mixture, the concentration of epithelial cell DNA is so large that some will remain in the sperm pellet as there is not a definitive physical separation between the sperm and the epithelial cell lysate. In order to dilute this concentration of epithelial DNA, numerous wash steps are required. These wash steps can lead to loss of sperm if the pellet is disrupted.<sup>6</sup>

One alternative to wash steps that has been explored and employed is the use of DNase to degrade the epithelial DNA that is contaminating the sperm fraction.<sup>6,8-12</sup> In addition to problem, the differential extraction steps required to separate the non-sperm and sperm cells are less amenable to automation and typically require manual processes that involve more labor and time.<sup>8</sup> Thus, some studies have moved away from the original Gill differential extraction toward completely new techniques.

## **1.2 Alternatives to the Original Differential Extraction Procedure**

In recent years, various alternatives to the differential extraction procedure originally published by Gill and colleagues have been developed and referenced in the forensic literature. The goals of these new techniques include: better separation of sperm and epithelial DNA when low amounts of sperm are present, creation of an automatable differential extraction, and a decrease in the amount of processing time. In the 2009 Census of Publicly Funded Forensic Crime Laboratories, 116 labs reported that 19,000 sexual assault cases were in backlog.<sup>13</sup> This was a decrease from the 21,000 backlogged cases in 2008, but is still a substantial number, approximately 164 backlogged sexual assault cases per lab that participated.<sup>13</sup>

### **1.2.1 Laser Capture Micro-Dissection**

Laser Capture Micro-Dissection (LCM) systems combine the use of laser cutting and cell isolation technology with a microscope, allowing

the separation of cells and sub-cellular materials.<sup>14</sup> LCM has been shown to out-perform the traditional differential extraction in cleanly separating sperm from epithelial cells.<sup>15</sup> Also, the ability to selectively target sperm allows the forensic scientist to report the DNA profiling results at the source level, rather than at the sub source level.<sup>16</sup>

However, because it is solely a physical, not chemical, separation there is one downfall, which is the inability to remove epithelial DNA that may be stuck to sperm heads.<sup>15</sup> In cases where there is long exposure time of sperm to the vaginal environment, it has been shown that vaginal DNA can stick to sperm heads.<sup>17</sup> Furthermore, laser capture microdissection requires expensive equipment and specialized training for its use in the forensic laboratory.

### 1.2.2 Alkaline Extraction

Recent research into the development of a faster differential extraction procedure has led to an alkaline differential extraction. It has been demonstrated that in comparison to an alkaline differential extraction procedure, the standard DTT extraction captures less than 50% of the sperm DNA from a cotton fiber swab.<sup>8</sup> In addition to higher yield of sperm DNA, benefits of an alkaline lysis extraction method include a simple procedure with decreased extraction time and minimal reagent costs.<sup>8</sup> Furthermore, this technique is highly automatable, where the traditional differential extraction is not.

Although this method may be valuable because it is highly automatable with a capacity of 96 samples at a time, it does produce a very dilute sperm fraction. In Hudlow and Buoncristiani's procedure, a concentration step had to be used to obtain a usable concentration of sperm lysate.<sup>8</sup> This extra step may not decrease the efficiency of the procedure, however it is another transfer step, which is an opportunity for loss of DNA and decreased yield.

In 2015, Nori and McCord described another alkaline extraction procedure using Pressure Cycling Technology (PCT) for differential extraction of sexual assault samples.<sup>9</sup> PCT is a novel extraction method that involves cycles of ambient to high levels of hydrostatic pressure that causes mechanical stress on cellular structures and disrupts molecular interactions.<sup>9</sup> When the pressure is high during the cycles, the phospholipid bilayer of the cell membrane is compressed, and upon release of this pressure, the membrane is destabilized allowing release of cell components.<sup>9</sup>

In an optimized procedure by Nori and McCord, the epithelial cells are lysed in 0.1 N Sodium Hydroxide (NaOH) followed by epithelial DNA degradation by DNase; The sperm cells are then lysed in a more concentrated 1 N NaOH solution combined with pressure cycling technology(PCT).<sup>9</sup> The major drawback to this procedure is the use of DNase to eliminate epithelial DNA. There are some cases in which the

epithelial fraction may need to be analyzed, such as with inadvertent premature sperm lysis. Furthermore, in their initial work with the un-optimized alkaline differential extraction procedure, they saw significant loss of sperm DNA, which they had attributed to DNase.<sup>9</sup> An ideal differential extraction would allow the capture of both the sperm fraction and non-sperm fraction.

### 1.2.3 Differex

In 2006, a separation method was developed by Promega called Differex™. The Differex™ system uses phase separation and differential centrifugation to separate sperm and epithelial DNA.<sup>18,19</sup> This system is much faster than the traditional differential extraction developed by Gill, as it takes between 2-3 hours rather than 1-2 days.<sup>18</sup> This significant decrease in extraction time is advantageous when thinking about the need to decrease backlog in sexual assault cases. In some recent studies, Differex™ was shown to perform as well as Chelex®-100 and QIAamp® DNA mini kit.<sup>19,20</sup> Mudariki and colleagues showed Differex™ with DNA IQ® System to perform as well as QIAamp DNA mini kit but with the use of less tubes.<sup>20</sup> The use of less tubes is advantageous in any DNA extraction as it reduces the chance of DNA loss. Additionally, in a study by Tsukuda et. al., Differex™ was shown to have a higher extraction efficiency than the Gill differential extraction, regardless of being paired with DNA IQ™ or QIAamp® DNA Micro Kit.<sup>18</sup>

Although the Differex™ system has some strong advantages of requiring less time and tubes than the alternative differential extraction methods, it does have some drawbacks as well. In the Differex™ separation procedure, the sperm are separated and pelleted in a non-aqueous phase away from the epithelial DNA. The Differex™ manual calls for the removal of this non-aqueous medium before extraction of the sperm cell DNA. This removal step can lead to a significant loss of sperm.<sup>18,19</sup> In one study, this loss of sperm was avoided by the omission of the removal of the separation medium, which increased the concentration of sperm cell DNA from 0.2 ng/μl to 0.6 ng/μl to.<sup>19</sup>

Another weakness of this system is the inability to use the sperm fraction to visualize the sperm microscopically. The separation solution causes droplets that are difficult to dry and obtain an even smear with for staining.<sup>19</sup> Furthermore, clustering of sperm heads within epithelial cell debris make it difficult for visualization of sperm when using this technique.<sup>19</sup> However, the study that described these difficulties only attempted staining and did not attempt phase contrast microscopy. Some forensic laboratories require the visualization of sperm in the sperm fraction, thus making this system a difficult and non-ideal method. Regardless of these disadvantages, Differex™ is remarkably quick to perform and helps to deal with the difficulty of physically

separating solubilized DNA in solution from the sperm pellet in a differential extraction.

#### 1.2.4 Microfluidic Devices

Another novel approach to physically separating epithelial DNA from sperm is the use of microfluidic devices. Some of the advantages that microfluidic systems have over their macrofluidic counterparts include reduced reagent and sample consumption that could reduce the cost of testing as well as the potential for portability.<sup>21</sup> Some work investigating the integration of extraction and PCR amplification taking place on the same microfluidic device, it was found that microfluidics could expedite forensic DNA processes and produce at least a 5-fold reduction in analysis time.<sup>21</sup> The decreased processing time would certainly aid in decreasing the amount of backlogged sexual assault cases. Additionally, the decreased chance of contamination due to the sample being processed in a closed environment is highly beneficial to any forensic DNA analysis.

The use of microfluidic devices for differential extraction was exemplified by the acoustic differential extraction (ADE). In 2009, Norris et. al. described the use of acoustic trapping of sperm cells on a microfluidic device to separate sperm cells from epithelial DNA.<sup>22</sup> One of the greatest advantages of the ADE method is the potential to accommodate a large range of sample volumes making it ideal for

forensic applications.<sup>22</sup> These acoustic cell manipulation techniques cell utilize the forces that effect an object in an acoustic standing wave.<sup>22</sup> The ADE method described by Norris and colleagues takes advantage of the size difference between sperm cells and free DNA to generate a force strong enough to trap the sperm cells but allow free DNA to pass through.<sup>22</sup>

### **1.3 Differential Extraction Procedures Currently in use by Forensic Laboratories**

The Swedish National Laboratory of Forensic Sciences, SKL, utilizes a manual Chelex®-100 based differential extraction method.<sup>19</sup> In the study by Vulchard and associates that compared results of different forensic laboratories sexual assault evidence extractions, the differential extraction procedures ranged from Differex™ to Chelex to QIAamp DNA mini and micro kit to the traditional organic differential extraction.<sup>3</sup> As discussed previously, this study showed that the results from these different extraction procedures performed on replicate samples of sperm and epithelial cell mixtures on a swab varied greatly. The male:female mixture ratios found from these extractions ranged from 1:6, 1:1, 2:1, 5:1 and 9:1.<sup>3</sup> A modified Chelex® extraction yielded no results at all, and three of the laboratories could not produce interpretable male profiles.<sup>3</sup>

The need for standardization of procedures in forensics is well known. Standardization with an effective procedure reduces concerns

about generating high quality results as well as increases reproducibility of results. Furthermore, with the various STR amplification kits available, the ability to produce comparable extract aids in achieving comparable STR profiles from these kits. When dealing with databases such as the Combined DNA Index System (CODIS), it is imperative to produce similar profiles every time for the same person so that it can be searched against in a database.

#### **1.4 The ZyGEM-Trypsin Differential Extraction**

##### 1.4.1 Sperm Morphology

The main difference that any chemical differential lysis of mixed sexual assault samples is taking advantage of is the morphological differences between sperm and epithelial cells. Epithelial cells are large, consisting of a phospholipid bilayer membrane containing the cytosolic components and the nucleus, housed in the nuclear envelope. Sperm cells consist of a head, mid-piece and tail, as depicted in figure 1. All of the Sperm DNA is contained in the nucleus. The tail is a flagellum that is used for motility and the mid-piece contains many mitochondria that provide energy to the tail for movement. The acrosome is a cap in the head of the sperm that contains proteases that are released upon fertilization so that the sperm can penetrate the ovum.

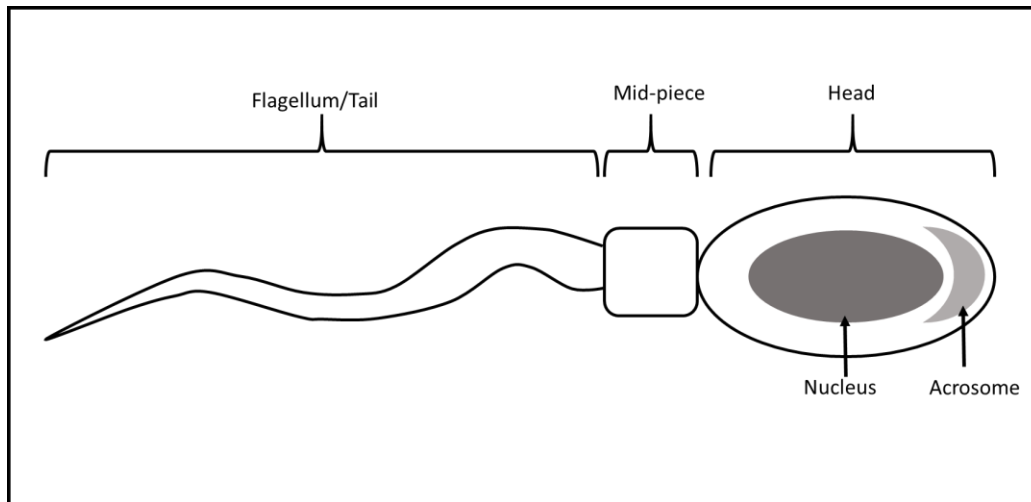


Figure 1: Morphologic appearance of an intact sperm cell.

The DNA contained in the sperm nucleus is condensed and the DNA is tightly bound to protamines, which contrasts greatly from the nucleosome packaging of DNA to histones found in epithelial cells.<sup>10</sup> Protamines are known to have a large percent of positively charged arginine and lysine residues as well as cysteine residues that are capable of forming disulfide bonds.<sup>10,23,24</sup> Two structural elements of protamines have been identified in all vertebrates.<sup>24</sup> One is an anchoring region that contains the positively charged lysine and arginine residues that are used to bind the protamine to DNA, and the other is the presence of threonine and serine residues that could be used as phosphorylation sites.<sup>24</sup> Additionally, the protamines of placental mammals have been shown to contain cysteine residues that form disulfide bonds that link the protamines together.<sup>24</sup> Furthermore, the structural importance of positively charged arginine residues in the protamine amino acid



With respect to this proposed structure, the Gill differential extraction most likely reduces the disulfide bonds between the cysteine residues in protamines by the use of the thiol reducing agent DTT.

The disruption of the disulfide bonds most likely releases the DNA protamine structure in such a way that it becomes more accessible to degradation by PK. If the Biegeleisin model is a close representation of the structure of the protamine-DNA complex, then the most effective way to free sperm DNA from protamines would be to disrupt the many arginine residues that form salt bridges between the DNA and the protamines.

#### 1.4.2 ZyGEM Extraction of Epithelial Cells

ZyGEM utilizes a neutral protease from Antarctic *Bacillus* sp.EA1.<sup>26</sup> The EA1 protease cleaves proteins wherever there is a leucine or phenylalanine in the amino acid sequence.<sup>27</sup> Through research on the protein sequence of protamines 1, 2a and 2b, Matt Fisher, M.S. found that there are only two EA1 cleavage sites within the amino acid sequence of protamines, indicating that ZyGEM would not release sperm DNA.<sup>27</sup> Extractions with EA1 are closed-tube and easily automatable DNA extraction methods.<sup>28</sup> A closed tube extraction greatly decreases the risk of sample contamination, and the ability to automate certainly helps to decrease analyst time and backlogged cases. The ZyGEM DNA extraction is also significantly faster than other commonly used

extraction kits, having an extraction time of 22 minutes with only 2 minutes of actual hands on work.<sup>26</sup>

In previous work described by Rachel Martinez, M.S., ZyGEM had been employed in the epithelial cell lysis portion of a novel enzymatic differential extraction.<sup>29</sup> ZyGEM was chosen as an enzyme for the epithelial cell lysis due to its inability to cleave the disulfide bonds that are present in sperm DNA packaging, but not epithelial cell DNA packaging.<sup>29</sup> The ZyGEM extraction time is significantly shorter than Gill's original differential extraction, thus making it more advantageous for forensic laboratories with large amounts of backlogged sexual assault cases.<sup>26,29,30</sup> Furthermore, ZyGEM is a closed tube reaction that requires no tube transfers, thus limiting the opportunities for sample contamination and loss of sperm cells.<sup>26,28,29</sup>

#### 1.4.3 Trypsin Extraction of Sperm Cells

Trypsin is a serine protease, a protease with a serine at its active site at which hydrolysis of peptide bonds occurs.<sup>27</sup> Trypsin cleaves proteins at lysine and arginine in the amino acid sequence.<sup>27</sup> This specificity to arginine is fortuitous for separation of protamines from sperm DNA, as over half of the amino acid residues in protamines are arginine residues.<sup>23</sup> Removal of the lysine and arginine residues would also effectively remove the salt bridges that hold the sperm DNA to the protamine structure as suggested by the model developed by Biegelson in

2006.<sup>23</sup> Furthermore, Trypsin has been previously shown to be effective at extracting DNA from sperm.<sup>27,29</sup>

In the work by Matt Fisher, M.S., the Trypsin extraction of sperm was shown to have higher yields of DNA than Qiagen.<sup>27</sup> The trypsin extraction, much like the ZyGEM extraction, is considerably shorter than Qiagen extractions<sup>27</sup> and is certainly shorter than the organic extractions typically used with differential extraction. The combination of ZyGEM and trypsin for a differential extraction would be advantageous in its short processing time alone.

## **2. MATERIALS AND METHODS**

All benches, pipettes and equipment with the exception of the PrepFiler™ Magnetic Stand were cleaned with 10% Sodium Hypochlorate (bleach) followed by 70% Ethanol. The PrepFiler™ Magnetic Stand was cleaned with 70% Ethanol only.

### **2.1 Cell Suspension Preparation**

#### 2.1.1 Buccal Cell Suspension Preparation

Buccal epithelial cells in saliva collected from an anonymous female donor were used to simulate vaginal cells. Buccal epithelial cells and vaginal epithelial cells are morphologically indistinguishable from each other<sup>31</sup>; furthermore, the histological and permeability features of human vaginal and buccal mucosa are similar.<sup>32</sup> Fresh saliva samples were used in order to avoid damage to the epithelial cells by freezing and thawing the saliva samples and to avoid the loss of cells on a substrate, such as a cotton swab.

To begin, fresh, neat saliva was collected in a 1.5 mL microcentrifuge tube (Eppendorf, UK, Ltd). From this neat saliva, 300uL were pipetted into a new 1.5 mL microcentrifuge tube with 300 µL of TE buffer.(10mM Tris, pH 8.0, and 0.1 mM EDTA) The buccal cells in the saliva were then pelleted in the bottom of the tube by centrifugation at 6000 rpm for 5 minutes in an Eppendorf centrifuge 5424 (Hamburg, Germany). The supernatant was removed from the tube, leaving only the

pellet containing the buccal cells. The pellet was re-suspended in 200 $\mu$ L of TE buffer. This pelleting and washing procedure was repeated three times, ultimately producing a 200 $\mu$ L solution of buccal cells in TE buffer.

#### 2.1.2 Epithelial Cell Counting

A disposable Hemacytometer (Cell-Vu®, Millennium Sciences and Engineering, Chantilly, VA, USA) was used in order to determine the concentration of cells in the prepared cell solutions. For epithelial cells, the solution was vortexed at high speed until the solution appeared homogeneous. Then 4 $\mu$ L was placed on the hemacytometer slide with a cover slip, following the Cell-Vu manual. The slide was then placed on a Nikon Eclipse TE200-S microscope using Phase Contrast Microscopy at 40x magnification. The cells were viewed by use of a MAXDATA computer set up with MMI Cell Cut (Molecular Machines & Industries, Eching, Germany) equipment and software. The number of epithelial cells counted in each subsection of the hemacytometer grid was counted and recorded. The total number of cells in the grid, sum of the cells counted in each zone, was divided by the volume of the grid to determine the concentration of cells in the epithelial cell solution.

#### 2.1.3 Semen Sample Preparation

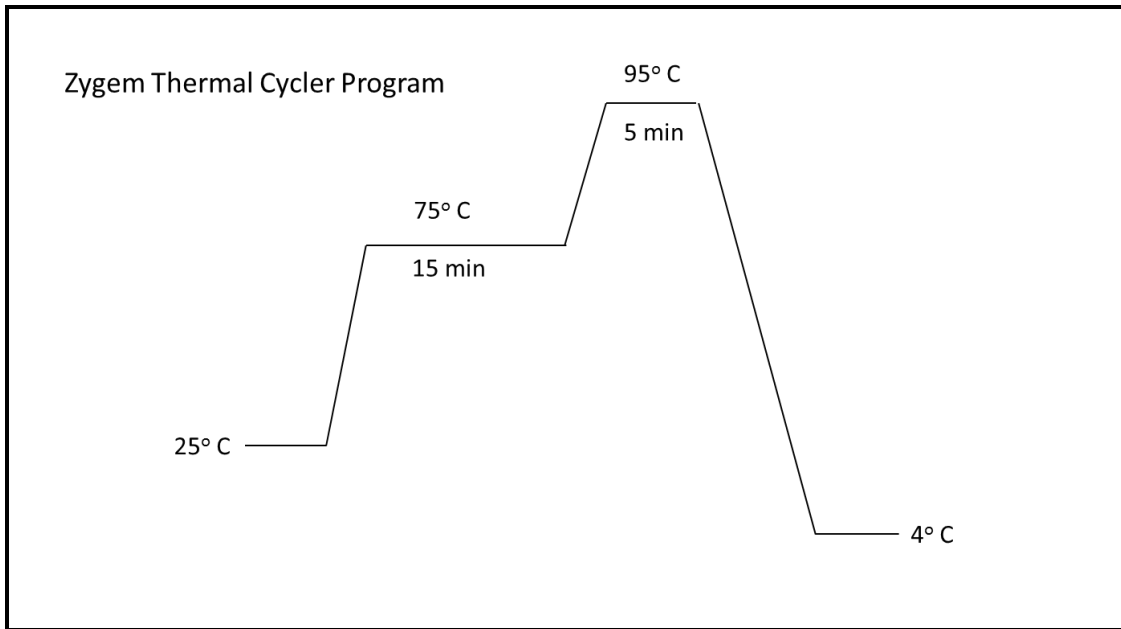
Single source semen samples were acquired from Bioreclamation IVT (Hicksville, New York). These samples were counted on disposable sperm cell counting chamber (Cell-Vu®, Millennium Sciences and Engineering,

Chantilly, VA, USA). The number of sperm counted in the grid were multiplied by 50 to obtain the amount of sperm per microliter. Dilutions were made in deionized water in order to achieve the desired amount of sperm cells per reaction.

## **2.2 ZyGEM Extraction and Reagents**

### **2.2.1 ForensicGEM Saliva™ Kit Components**

A forensicGEM™ Saliva kit was obtained from ZyGEM (Hamilton, New Zealand). The kit included the enzyme EA1 and 10x Buffer Blue. The ForensicGEM™ Saliva kit manual procedure calls for the use of 20µL of eluate from a buccal swab in DNA-free water with 10µL of 10x Buffer Blue, 69µL of DNA free water and 1ul of forensicGEM (enzyme EA1).<sup>30</sup> This 100ul reaction is then incubated at 75 degrees Celsius for 15 minutes for enzyme EA1 to lyse the epithelial cells followed by an incubation at 95 degrees Celsius for 5 minutes to inactivate enzyme EA1 by denaturation (figure 1).<sup>30</sup> All extractions were done in 0.2mL reactions tubes (Applied Biosystems, Foster City, CA, USA) and the incubations were performed using a thermal cycler (ABI GeneAmp® PCR System 9700).



*Figure 3: Thermal cycler Settings for ZyGEM Extraction using forensicGEM Saliva. For the separation of NSF and SF fractions, this program was paused after the 75 degrees Celsius incubation for the separation, and then resumed after the separation.*

### 2.2.2 Modifications to ForensicGEM Saliva™ extraction from previous work

The previous work done in developing this differential extraction showed that the use of 4ul of *forensicGEM* (enzyme EA1) produced the best epithelial lysis by comparing lysis with 1ul, 2ul, 4ul and 10ul of *forensicGEM* (enzyme EA1).<sup>29</sup> This procedural change were adopted into the *forensicGEM* procedure that was used. Also, the volume of cell solution used was determined based on the desired amount of buccal cells and the concentration of buccal cells in solution was determined using cell counting. The final reaction mix consisted of 10μL of Buffer Blue, 4μL of *forensicGEM* Saliva, a volume of cell suspension to achieve the desired number of buccal cells and enough DNA-free water to bring the reaction volume up to 100μL.

### 2.2.3 Trypsin Extraction of Sperm Cells

First, a ZyGEM Master Mix of 1x ZyGEM Buffer Blue and ZyGEM (EA1).

**Table 1:** Example of ZyGEM Master Mix for Trypsin Extraction

<b>Reagent</b>	n+2 Samples	Volume Per Reaction	Master Mix Volume
<b>1x ZyGEM Buffer Blue</b>	6	9μL	54μL
<b>ZyGEM</b>	6	1μL	6μL

To each SF tube, the following was added: 16μL of Deionized Water, 4μL of ZyGEM 10x Buffer Blue, 10μL of ZyGEM Master Mix, and 10μL of Stock Gibco Trypsin (Thermo Fisher Scientific Inc., Waltham, MA). The final concentration of Trypsin in this reaction is 6.25 mg/mL. These

reactions were then vortexed and centrifuged briefly, then placed in the thermal cycler. The thermal cycler was then run using the program detailed in figure 3. All samples were stored at -20 degrees Celsius.

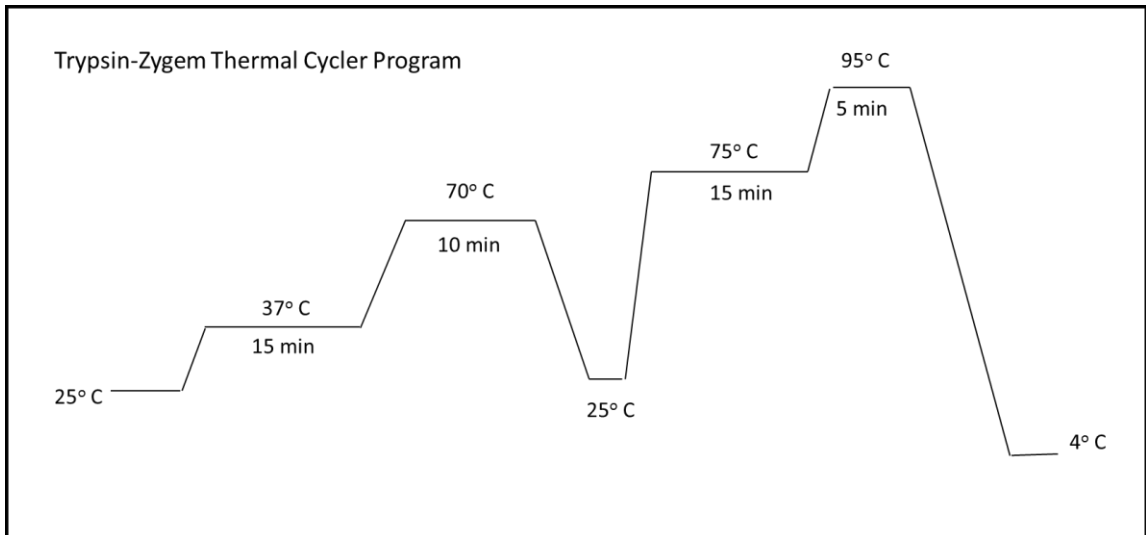


Figure 4: Thermal cycler program for Trypsin-ZyGEM extraction of sperm.

### **2.3 Simulated Differential Extraction using ForensicGEM Saliva™ and Modifications Attempted**

Since the goal of these experiments was to elucidate the reason why the ZyGEM-Trypsin differential extraction was not successfully separating the female epithelial DNA from the sperm, experiments without sperm were carried out to examine how much and where epithelial DNA was at the end of the procedure. First, we replicated the differential extraction developed by Martinez that showed the best separation with only buccal epithelial cells. For all experiments, samples were stored at -20 degrees Celsius after extraction.

A series of modifications were made to the epithelial cell lysis procedure in an attempt to improve the lysis and separation of epithelial cells from sperm. The first modification was the addition of Proteinase K to determine if it would improve the separation by increasing the lysis of the epithelial cells. The second experiment used the addition of Sarkosyl to determine if the destabilization of the epithelial cell membrane would improve the cell lysis by ZyGEM. Next, we attempted to improve the separation by physically pulling the epithelial DNA from the SF using PrepFiler® Forensic DNA Extraction Kit. Finally, we attempted to decrease the amount of epithelial DNA carryover in the SF by using a wash step. The wash step was proposed as it would decrease the

concentration of epithelial DNA in the solution of the SF, ultimately decreasing the amount leftover in the SF.

### 2.3.1 Determining number of buccal cells to be used

To determine the number of buccal cells that should be used in subsequent experiments, the simulated differential extraction using forensicGem Saliva™ was performed using 600 buccal cells, 1200 buccal cells and 1800 buccal cells per reaction. To achieve this, the appropriate volume of buccal cell solution, 10µL of Buffer Blue, 4µL of EA 1 and enough DNA-free water to bring the reaction volume to 100µL was added to each tube. The reaction was mixed by vortexing and quickly centrifuged (Eppendorf centrifuge 5424, Hamburg, Germany) then placed in the thermal cycler at 75 degrees Celsius for 15 minutes. The reaction was then cooled, vigorously vortexed and centrifuged at 13950 x g (Earth's gravitational force). All but 10ul were removed after centrifugation, simulating the step where the epithelial DNA would be separated from the pelleted sperm in differential extraction. All tubes were then returned to the thermal cycler for the 5 minute 95 degree Celsius EA1 inactivation step. The mock sperm fraction was re-suspended in 20µL of TE Buffer.

## **2.4 Addition of Proteinase K to ForensicGEM Saliva™ Differential Extraction**

The first modification attempted was the use of Proteinase K (PK) in an attempt to increase the epithelial lysis. Proteinase K from the

QIAamp Investigator Kit (Qiagen, Redwood City, CA, USA). was used for these experiments. Because of the results seen in our initial forensicGEM experiment, 1200 cells were added to each reaction in all of the proteinase K experiments. For all experiments, samples were stored at -20 degrees Celsius after extraction.

#### 2.4.1 ForensicGEM followed by Proteinase K Extraction

First, the *forensicGEM* extraction were prepared as above, except with 10ul less water so that the ultimate reaction volume after addition of PK would still be 100ul. The reaction was incubated as above through the EA1 inactivation step but without separating the two fractions. Then the reactions were allowed to cool to room temperature. Ten microliters of PK was added to the reactions, and they were returned to the thermal cycler for a 10 minute incubation at 56 degrees Celsius followed by a 10 minute incubation at 75 degrees Celsius to denature the PK. Finally, the reactions were vortexed, centrifuged and separated into simulated non-sperm and sperm fractions.

#### 2.4.2 Proteinase K followed by forensicGEM Extraction

The second PK experiment involved lysis by PK first and then by *forensicGEM*. For these reactions, 10 $\mu$ L of Proteinase K, 10 $\mu$ L of Buffer Blue, 1200 cells and DNA-free water were added to a final volume of 96 $\mu$ L per 100 $\mu$ L reaction. To improve reproducibility and mitigate variation in lysis due to cell concentration variation, a master mix was

made in a 1.5 mL microcentrifuge tube as depicted in table . This master mix was vortexed and spun, and then 96 $\mu$ L was aliquotted into each individual 0.2 mL reaction tube. These tubes were then placed in the thermal cycler to incubate at 56 degrees Celsius for 10 minutes. Next, the reaction was incubated at 75 degrees Celsius for 10 minutes to denature the PK, so it would not digest EA1 in the following lysis step. The reactions were allowed to cool to room temperature, then 4 $\mu$ L of *forensicGEM* was added to each reaction. From here, the *forensicGEM* differential extraction procedure from above was followed exactly.

**Table 2:** PK-ZyGEM Differential Extraction Master Mix

<b>Reagent</b>	<b>100ul Reaction</b>	<b>Master Mix ( n+1) n = 4</b>
<b>Deionized Water</b>	100 $\mu$ L -(24 $\mu$ L + volume of cells added)  Example = 67 $\mu$ L	335 $\mu$ L
<b>Buccal Cell Preparation</b>	Volume of cells required to obtain 1200 cells in reaction  Example = 9 $\mu$ L	45 $\mu$ L
<b>ZyGEM 10x Buffer Blue</b>	10 $\mu$ L	50 $\mu$ L
<b>Proteinase K</b>	10 $\mu$ L	50 $\mu$ L
<b>Total Volume</b>	96 $\mu$ L	480 $\mu$ L

Four Control reactions were run with the same buccal cell preparation, but with only the ZyGEM Differential Extraction procedure. For these controls, the same procedure was used as in section 2.4.2, however this time a master mix was used to decrease the variability in

results from cell clumping. This master mix procedure is shown in table 3.

**Table 3:** ZyGEM Differential Extraction Master Mix

<b>Reagent</b>	<b>100ul Reaction</b>	<b>Master Mix (n+1) N = 4</b>
<b>DI Water</b>	100 $\mu$ L -(14 $\mu$ L + volume of cells added)  Example = 77 $\mu$ L	385 $\mu$ L
<b>Saliva Cell Preparation</b>	Volume of cells required to obtain 1200 cells in reaction  Example = 9 $\mu$ L	45 $\mu$ L
<b>ZyGEM 10x Buffer Blue</b>	10 $\mu$ L	50 $\mu$ L
<b>ZyGEM</b>	4 $\mu$ L	20 $\mu$ L
<b>Total Volume</b>	100 $\mu$ L	500 $\mu$ L

## **2.5 Addition of Sarkosyl to ForensicGEM Saliva™ Differential Extraction**

A 20% Sarkosyl Solution, N-Lauroylsarcosine sodium salt solution, was obtained from Sigma Aldrich (St. Louis Missouri). For all experiments using Sarkosyl, a 5% solution was made by making a 1:4 dilution of the 20% Sarkosyl Stock. After each experiment, all samples were stored at -20 degrees Celsius.

### **2.5.1 0.1% and 0.5% Sarkosyl-PK-ZyGEM Differential Extraction without sperm**

In order to obtain a final Sarkosyl concentration of 0.5%, 10 $\mu$ L of the 5% Sarkosyl solution was added to each 100 $\mu$ L reaction. To obtain a

0.1% final concentration of Sarkosyl, 2 $\mu$ L of the 5 % Sarkosyl solution was added to each 100 $\mu$ L reaction. First, a master mix of all of the reagents and epithelial cell solution was combined as depicted in table . This master mix was vortexed and spun, then 96 $\mu$ L was aliquotted into each individual 0.2 mL microcentrifuge tube. These reactions were then placed in the thermal cycler for 10minutes at 56 degrees Celsius, followed by 10 minutes at 75 degrees Celsius as was done in the PK-ZyGEM extraction procedure. Next, 4 $\mu$ L of *forensicGEM* Saliva was added to each reaction. These tubes were returned to the thermal cycler and carried through the ZyGEM Differential Extraction protocol in section 2.3.

**Table 4:** 0.5% Sarkosyl-PK-ZyGEM Differential Extraction Master Mix

<b>Reagent</b>	<b>100ul Reaction</b>	<b>Master Mix ( n+1) n = 4</b>
<b>Deionized Water</b>	100 $\mu$ L -(24 $\mu$ L + volume of cells added)  Example = 57 $\mu$ L	285 $\mu$ L
<b>5% Sarkosyl Solution</b>	10 $\mu$ L	50 $\mu$ L
<b>Buccal Cell Preparation</b>	Volume of cells required to obtain 1200 cells in reaction  Example = 9 $\mu$ L	45 $\mu$ L
<b>ZyGEM 10x Buffer Blue</b>	10 $\mu$ L	50 $\mu$ L
<b>Proteinase K</b>	10 $\mu$ L	50 $\mu$ L
<b>Total Volume</b>	100 $\mu$ L	500 $\mu$ L

**Table 5:** 0.1% Sarkosyl-PK-ZyGEM Differential Extraction Master Mix

<b>Reagent</b>	<b>100ul Reaction</b>	<b>Master Mix ( n+1) n = 4</b>
<b>Deionized Water</b>	100 $\mu$ L -(24 $\mu$ L + volume of cells added)  Example = 65 $\mu$ L	325 $\mu$ L
<b>5% Sarkosyl Solution</b>	2 $\mu$ L	10 $\mu$ L
<b>Buccal Cell Preparation</b>	Volume of cells required to obtain 1200 cells in reaction  Example = 9 $\mu$ L	45 $\mu$ L
<b>ZyGEM 10x Buffer Blue</b>	10 $\mu$ L	50 $\mu$ L
<b>Proteinase K</b>	10 $\mu$ L	50 $\mu$ L
<b>Total Volume</b>	96 $\mu$ L	480 $\mu$ L

### 2.5.2 0.1% Sarkosyl -ZyGEM Differential Extraction without sperm

To obtain a 0.1% concentration of Sarkosyl, 2 $\mu$ L of the 5% Sarkosyl solution was added to each 100 $\mu$ L reaction as in the 0.1% Sarkosyl-PK-ZyGEM extraction. A master mix was used and was made by combining the reagents in table 5. After making, vortexing and centrifuging the master mix, 100 $\mu$ L was added to each individual 0.2 mL microcentrifuge tube. These reactions were then carried through the ZyGEM differential extraction procedure explained in section 2.3 but using the Master Mix in table 5.

**Table 6:** 0.1% Sarkosyl- ZyGEM Differential Extraction Master Mix

<b>Reagent</b>	<b>100ul Reaction</b>	<b>Master Mix (n+1) n = 4</b>
<b>Deionized Water</b>	100ul -(24ul +volume of cells added)  Example = 65μL	325μL
<b>5% Sarkosyl Solution</b>	2μL	10μL
<b>Buccal Cell Preparation</b>	Volume of cells required to obtain 1200 cells in reaction  Example = 9ul	45μL
<b>ZyGEM 10x Buffer Blue</b>	10μL	50μL
<b>ZyGEM</b>	4μL	20μL
<b>Total Volume</b>	100μL	500μL

## **2.6 ForensicGEM Saliva™ Differential Extraction Combined with PrepFiler™**

A PrepFiler® Forensic DNA Extraction Kit was obtained from Applied Biosystems (Carlsbad, CA). Wash Buffer A was prepared by diluting the Wash Buffer A concentrate with 93 mL of 95% ethanol (PHARMCO-AAPER). Wash Buffer B was prepared by diluting the Wash Buffer B concentrate with 19.5 mL of 95% ethanol (PHARMCO-AAPER). Before each PrepFiler™ experiment, the Magnetic Particles were incubated at 37 degrees Celsius for 30 minutes. For both of the following experiments, one ZyGEM master mix was made for all of the samples as in table 2 in section 2.4.2.

### **2.6.1 ZyGEM Differential Extraction without Sperm with a PrepFiler™ Clean-up Step**

For this experiment, the ZyGEM Differential Extraction without Sperm from section 2.3 was followed through completely. Then PrepFiler™ was performed on the Simulated Sperm Fraction to identify how much epithelial cell DNA was being carried over.

First all of the SF tubes were allowed to come to room temperature. Then 20µL of PrepFiler™ Lysis Buffer was added to the SF. All of the SF was then transferred to a new 1.5 mL microcentrifuge tube (Fisher Brand MCT Graduated Natural). Next, 130µL of PrepFiler™ Lysis Buffer was added, the tube was vortexed and centrifuged briefly. The PrepFiler™

Magnetic Particles were then vortexed for 5 seconds and 10ul were added to the tube containing the SF. This was vortexed to 10 seconds at low speed and centrifuged. To bind the DNA to the PrepFiler™ Magnetic Particle, 100ul of isopropanol (Sigma-Aldrich, St. Louis, Missouri) was added to the tube, which was then vortexed at low speed and centrifuged briefly. Next the tube was vortexed at low speed for 10 minutes. The tube was then vortexed at maximum speed for 10 seconds, centrifuged briefly and then placed into the PrepFiler™ 16 Position Magnetic Stand (Applied Biosystems, Carlsbad, CA).

Once the pellet of magnetic particles stopped growing in size, approximately 2 minutes, all of the liquid was removed from the tube and added to a new 1.5 mL microcentrifuge tube and designated the SF. The DNA bound to the PrepFiler™ Magnetic Particles was then washed, first using 300µL of Wash Buffer A then using 300µL of Wash Buffer B. The tube was then left open in the magnetic stand for 8 minutes to dry the magnetic particles with bound DNA.

Next, 50µL of PrepFiler™ Elution Buffer was added to the tube with the magnetic particle-bound DNA, then vortexed at maximum speed to re-suspend the pellet. This tube was then placed in a water bath in an oven (Thermo Fisher, Waltham, MA) at 70 degrees Celsius for 5 minutes. In the middle of this 5 minute incubation, the tube was removed and vortexed briefly. After the 70 degree Celsius incubation, the sample was

vortexed at high speed until no magnetic particle pellet was visible. The sample was then returned to the 16-Position Magnetic stand and left there for 2 minutes. Finally, all of the liquid containing the epithelial cell carry-over was removed and placed in a new 0.2 mL microcentrifuge tube. All samples were stored at -20 degrees Celsius.

#### 2.6.2 ZyGEM Extraction with PrepFiler™ Separation without Sperm

For this experiment, the ZyGEM Differential Extraction procedure was carried out without separating the NSF and SF fractions. The samples were allowed to return to room temperature, then underwent the PrepFiler™ extraction process. This was done using the same procedure as above, but with the following volumes: 500µL PrepFiler™ Lysis Buffer, 360µL of Isopropanol, 300µL of Wash Buffer A, 300µL of Wash Buffer B, and 50µL of PrepFiler™ Elution Buffer. These volumes were used to compensate for the volume of the ZyGEM extract being twice the volume of extract in the PrepFiler™ Kit Manual Protocol.<sup>33</sup> All samples were stored at -20 degrees Celsius.

#### **2.7 Addition of a Wash Step after ForensicGEM Saliva™ Lysis**

A wash step was attempted on the simulated Sperm Fraction using TE buffer. For this the ZyGEM differential extraction procedure without sperm from section 2.3 was followed through the separation of the NSF from the SF. Then the SF was re-suspended in 90µL of TE buffer, vortexed and centrifuged for 5 minutes at 13950 g. Ninety microliters was then removed

from the SF and discarded. All NSF and SF samples were returned to the thermal cycler for 5 minutes at 95 degrees Celsius to inactivate the *forensicGEM* EA1 enzyme. All samples were stored at -20 degrees Celsius.

## **2.8 ForensicGEM Saliva™-Trypsin Differential Extraction with Sperm**

For all of these experiments, both an epithelial cell solution and a separate sperm cell control were extracted at the same time as the mixture samples. This was done to be able to isolate any effects from lysis of non-sperm male cells in the semen.<sup>10</sup> For this procedure, the ZyGEM protocol from section 2.3 was used with the Master Mix in table 2.4.2 for the ZyGEM lysis of the epithelial cells and separation of NSF and SF. Following the ZyGEM digestion and separation of the NSF, the SF underwent an extraction using Trypsin as explained in section 2.2.3. This extraction was performed with and without a wash after the removal of the NSF.

### 2.8.1 Dilution Series of Epithelial Cells with the ZyGEM-Trypsin Differential Extraction

Because our data appeared to behave as if the ZyGEM enzyme was being overloaded with an excess of cells, a dilution series of epithelial cells was attempted. A saliva cell preparation was made as in previous experiments and cells were counted. The volume of this cell solution required to achieve 1200 cells per 100  $\mu$ l ZyGEM reaction was calculated

and added to the stock cell concentration reaction. Dilutions of 1:10, 1:50 and 1:250 were made from the stock saliva cell suspension in duplicate. The same volume of cell suspension that was used for the stock cell concentration reaction was used for all of the dilution reactions as well. A master mix of all reagents without epithelial cells was used. The determined amount of cell solution was added to a 100  $\mu$ l ZyGEM reaction for each dilution. This was also repeated with the addition of a sperm cells to a ZyGEM master mix that was aliquotted to individual 0.2 ml microcentrifuge tubes, such that a target of 1600 sperm would be in each 100  $\mu$ l ZyGEM reaction. Both dilution series experiments, with and without sperm cells, were done in duplicate. Two saliva cell controls were extracted with ZyGEM, and two sperm controls were extracted with the trypsin sperm extraction procedure.

## **2.9 DNA Quantification with Quantifiler Duo®**

The DNA from all of the sample extracts were quantified with the Quantifiler® Duo Quantification Kit (Applied Biosystems® Foster City, CA). Quantifiler® Duo quantifies both total human DNA and total male DNA.<sup>34</sup> All Quantifiler® Duo reactions were prepared based on the manufacturer's instructions in the Quantifiler® Duo DNA Quantification Kit User Manual.<sup>34</sup> Samples were quantified using a 7500 Real Time PCR System (Applied Biosystems®, Foster City, CA). Deionized water was used as a negative control, which was used for every quantification run.

### 2.9.1 Use of Quantification Data to Determine Effectiveness of the Differential Extraction

From the Quantifiler® Duo, the concentration of female DNA was found by subtracting the concentration of male DNA from the total human DNA concentration as shown below, in equation [1].

$$[1] \quad [female\ DNA] (ng/\mu l) = [Total\ Human\ DNA](ng/\mu l) - [Male\ DNA](ng/\mu l)$$

The mass of DNA could then be calculated by multiplying these concentrations by the volumes of their respective fractions as shown in equation [2].

$$[2] \quad Mass\ of\ DNA\ in\ NSF = [DNA]_{NSF} (ng/\mu l) * 90\ \mu l$$

These calculated DNA masses could then be used to determine what percentage of each fraction came from male and what percentage came from female. These calculations were done using equations [3-6].

$$[3] \quad \% \ of\ SF\ from\ male = \frac{Male\ DNA\ in\ SF\ (ng)}{Total\ Human\ DNA\ in\ SF\ (ng)}$$

$$[4] \quad \% \ of\ SF\ from\ female = \frac{(Total\ Human\ DNA\ in\ SF\ (ng) - Male\ DNA\ in\ SF\ (ng))}{Total\ Human\ DNA\ in\ SF\ (ng)}$$

$$[5] \quad \% \ of\ NSF\ from\ male = \frac{Male\ DNA\ in\ NSF\ (ng)}{Total\ Human\ DNA\ in\ NSF\ (ng)}$$

$$[6] \quad \% \ of\ NSF\ from\ female = \frac{(Total\ Human\ DNA\ in\ NSF\ (ng) - Male\ DNA\ in\ NSF\ (ng))}{Total\ Human\ DNA\ in\ NSF\ (ng)}$$

Male:Female mixture ratios could also be calculated by dividing the amount of male DNA (ng) in the SF by the amount of female DNA (ng) in the SF as shown in equation [7].

$$[7] \quad \text{Male:Female ratio} = \frac{\text{Male DNA in SF (ng)}}{\text{Female DNA in SF (ng)}}$$

## **2.10 DNA Amplification**

The DNA extracts from selected samples were amplified using the AmpFISTR® Identifiler® Plus PCR Amplification Kit (Applied Biosystems®, Foster City, CA).<sup>35</sup> All Identifiler® Plus reactions were made according to the instructions from the AmpFISTR® Identifiler® Plus PCR Amplification Kit Manual.<sup>35</sup> The desired target mass for these reactions was 1 ng of DNA. This target was achieved by dilution with TE Buffer. If 1 ng of DNA could not be achieved within the required sample maximum of 10µL, 10µL of sample was added, resulting in less than 1ng. DNA samples were amplified on a GeneAmp® PCR System 9700 (Applied Biosystems®, Foster City, CA), using the manufacturer specified Identifiler® Plus program consisting of 28 cycles. With each amplification, a positive and negative control was run. Amplified samples were stored at -20 degrees Celsius until they could be separated by capillary electrophoresis and analyzed.

### **2.11 Capillary Electrophoresis and STR Profile Analysis**

The amplified DNA from samples were separated using Capillary Electrophoresis, using a 3130 Genetic Analyzer (Applied Biosystems®, Foster City, CA) using POP-4 polymer and with a 5 second injection at 3 kV unless otherwise stated. Amplified samples were prepared using Hi-Di™ Formamide and GeneScan 600 Liz Size Standard (Applied Biosystems®, Foster City, CA) following manufacturer's protocol.<sup>35</sup>

Electropherograms generated from the 3130 Genetic Analyzer were analyzed using GeneMapper® ID-X v1.1.1 software (Applied Biosystems®, Foster City, CA). The sample-generated profiles were analyzed with an analytical threshold of 30 relative fluorescence units (RFU). The stutter threshold was turned off and artifacts were manually removed from the profiles.

### **2.12 Statistical Methods**

All statistics were calculated using either Microsoft® Excel® 2013 (Microsoft Office Corporation, Santa Rosa, CA) or JMP Pro v. 11.2 (SAS Institute, Cary, NC).

### **3. RESULTS AND DISCUSSION**

Although the same fresh buccal cell preparation was used for each experiment, there was variation in quantity of DNA obtained within each experiment. The initial experiments done to determine the amount of cells to use in these experiments showed optimal results at 1200 cells. A target amount of 1200 buccal cells per 100 $\mu$ L reaction target amount was used for all experiments except the experiments looking at the full differential extraction on a mixture with sperm and buccal cells. Clumping of cells was noticed when counting, which may have contributed to the variation in starting cell amounts. Master mixes including the epithelial cells with reagents were used for each experiment in an attempt to decrease this variability in cell amounts within experiments.

#### **3.1 Results of Epithelial Cell Experiments with ZyGEM and ZyGEM with a Wash Step**

For this comparison, a single wash step was added as detailed in materials and methods section 2.7. The wash step reduced the amount of epithelial DNA in the sperm fraction by approximately 9.56 %. A summary of the data is shown in figure 5 and tabulated in tables 7-8.

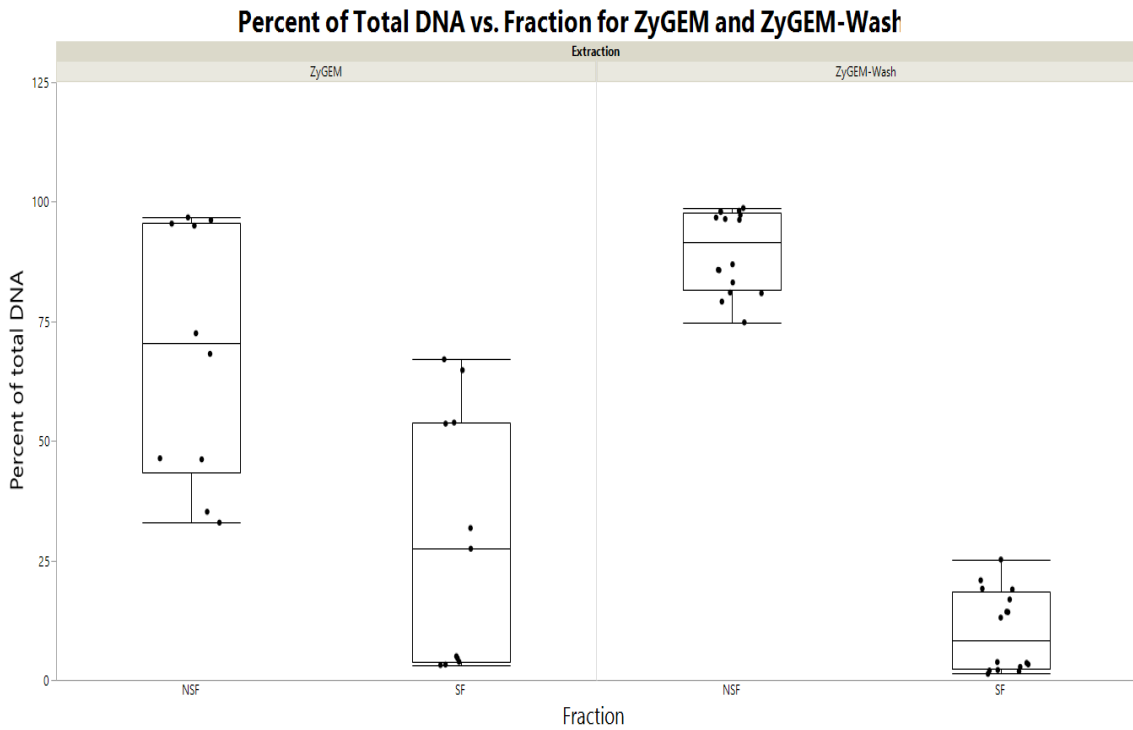


Figure 5: This boxplot compares the percent of epithelial DNA in each fraction of the ZyGEM extraction to that of the ZyGEM extraction with a wash step.

### 3.1.1 Results of ZyGEM Alone

The mean percentage of epithelial DNA in the SF for the extraction with ZyGEM alone was 19.79%. The mean percentage of epithelial DNA in the NSF was 80.21%.

**Table 7:** Results of ZyGEM Differential Extraction Alone on Epithelial Cells.  
 \*Outlier not included in averages.

Date	Fraction	Concentration (ng/ul)	Mass (ng)	Total DNA (ng)	% of Total DNA	Mean% SF	Mean% NSF	Mean Total ng DNA
6/10/2016	<b>SF</b>	0.70	34.77	64.83	<b>53.63</b>	<b>59.84</b>	<b>40.16</b>	<b>68.96</b>
6/10/2016	<b>NSF</b>	0.33	30.07		<b>46.37</b>			
6/10/2016	<b>SF</b>	0.52	26.21	48.67	<b>53.85</b>			
6/10/2016	<b>NSF</b>	0.25	22.46		<b>46.15</b>			
6/10/2016	<b>SF</b>	1.01	50.58	75.41	<b>67.07</b>			
6/10/2016	<b>NSF</b>	0.28	24.83		<b>32.93</b>			
6/10/2016	<b>SF</b>	1.13	56.32	86.91	<b>64.80</b>			
6/10/2016	<b>NSF</b>	0.34	30.59		<b>35.20</b>			
6/7/2016	<b>SF</b>	0.20	1.98	43.53	<b>4.56</b>	<b>17.20</b>	<b>82.80</b>	<b>43.55</b>
6/7/2016	<b>NSF</b>	0.46	41.55		<b>95.44</b>			
6/7/2016	<b>SF</b>	0.19	1.93	38.67	<b>4.98</b>			
6/7/2016	<b>NSF</b>	0.41	36.75		<b>95.02</b>			
6/7/2016	<b>SF</b>	1.49	14.93	54.33	<b>27.48</b>			
6/7/2016	<b>NSF</b>	0.44	39.40		<b>72.52</b>			
6/7/2016	<b>SF</b>	1.20	11.97	37.64	<b>31.79</b>			
6/7/2016	<b>NSF</b>	0.29	25.68		<b>68.21</b>			
5/19/2016	<b>SF</b>	0.04	0.41	12.49	<b>3.26</b>	<b>3.93</b>	<b>96.07</b>	<b>13.91</b>
5/19/2016	<b>NSF</b>	0.13	12.09		<b>96.74</b>			
5/19/2016	<b>SF</b>	0.06	0.63	16.38	<b>3.84</b>			
5/19/2016	<b>NSF</b>	0.18	15.75		<b>96.16</b>			
5/19/2016	<b>SF</b>	0.04	0.44	13.89	<b>3.15</b>			
5/19/2016	<b>NSF</b>	0.15	13.45		<b>96.85</b>			
5/19/2016	<b>SF</b>	0.07	0.70	12.87	<b>5.47</b>			
5/19/2016	<b>NSF</b>	0.14	12.17		<b>94.53</b>			
3/26/2016	<b>SF</b>	0.06	1.72	34.77	<b>4.96</b>	<b>4.15</b>	<b>95.85</b>	<b>40.32</b>
3/26/2016	<b>NSF</b>	0.37	33.05		<b>95.04</b>			
3/26/2016	<b>SF</b>	0.14	4.05	81.64	<b>4.96</b>			
3/26/2016	<b>NSF</b>	0.86	77.59		<b>95.04</b>			
3/26/2016	<b>SF</b>	0.66	19.74	19.74	<b>100.00*</b>			
3/26/2016	<b>NSF</b>	0.00	0.00		<b>0.00*</b>			
3/26/2016	<b>SF</b>	0.02	0.63	25.12	<b>2.52</b>			
3/26/2016	<b>NSF</b>	0.27	24.48		<b>97.48</b>			

**Table 7 (continued):** Results of ZyGEM Differential Extraction Alone on Epithelial Cells

Date	Fraction	Concentration (ng/ul)	Mass (ng)	Total DNA (ng)	% of Total DNA	Mean% SF	Mean% NSF	Mean Total DNA (ng)
3/16/2016	<b>SF</b>	2.03	60.99	124.08	<b>49.15</b>	<b>22.56</b>	<b>77.44</b>	<b>85.67</b>
3/16/2016	<b>NSF</b>	0.70	63.09		<b>50.85</b>			
3/16/2016	<b>SF</b>	0.22	6.63	63.24	<b>10.48</b>			
3/16/2016	<b>NSF</b>	0.63	56.61		<b>89.52</b>			
3/16/2016	<b>SF</b>	0.46	13.86	80.91	<b>17.13</b>			
3/16/2016	<b>NSF</b>	0.75	67.05		<b>82.87</b>			
3/16/2016	<b>SF</b>	0.33	10.02	74.46	<b>13.46</b>			
3/16/2016	<b>NSF</b>	0.72	64.44		<b>86.54</b>			
2/3/2016	<b>SF</b>	0.44	13.08	118.75	<b>11.02</b>	<b>11.10</b>	<b>88.90</b>	<b>113.26</b>
2/3/2016	<b>NSF</b>	1.17	105.66		<b>88.98</b>			
2/3/2016	<b>SF</b>	0.56	16.85	97.47	<b>17.29</b>			
2/3/2016	<b>NSF</b>	0.90	80.62		<b>82.71</b>			
2/3/2016	<b>SF</b>	0.27	8.05	116.97	<b>6.88</b>			
2/3/2016	<b>NSF</b>	1.21	108.93		<b>93.12</b>			
2/3/2016	<b>SF</b>	0.37	11.04	119.84	<b>9.21</b>			
2/3/2016	<b>NSF</b>	1.21	108.80		<b>90.79</b>			

### 3.1.2 Results of ZyGEM with a Wash Step

The mean percentage of epithelial DNA in the SF for ZyGEM with a wash step was 10.2%. The mean percentage of epithelial DNA in the NSF for ZyGEM with a wash step was 89.8%.

**Table 8:** Results of ZyGEM Differential Extraction with a Wash Step

Date	Fraction	Concentration (ng/ul)	Mass (ng)	Total DNA (ng)	% of Total DNA	Mean % SF	Mean % NSF	Mean Total DNA (ng)
6/15/2016	SF	0.12	5.83	30.74	<b>18.96</b>	<b>21.03</b>	<b>78.97</b>	<b>26.78</b>
6/15/2016	NSF	0.28	24.91		<b>81.04</b>			
6/15/2016	SF	0.13	6.42	25.47	<b>25.20</b>			
6/15/2016	NSF	0.21	19.05		<b>74.80</b>			
6/15/2016	SF	0.12	6.25	29.95	<b>20.86</b>			
6/15/2016	NSF	0.26	23.70		<b>79.14</b>			
6/15/2016	SF	0.08	4.00	20.95	<b>19.11</b>			
6/15/2016	NSF	0.19	16.95		<b>80.89</b>			
6/10/2016	SF	0.10	4.87	34.25	<b>14.22</b>	<b>14.61</b>	<b>85.39</b>	<b>38.69</b>
6/10/2016	NSF	0.33	29.38		<b>85.78</b>			
6/10/2016	SF	0.10	4.98	34.84	<b>14.29</b>			
6/10/2016	NSF	0.33	29.86		<b>85.71</b>			
6/10/2016	SF	0.10	5.08	38.86	<b>13.07</b>			
6/10/2016	NSF	0.38	33.78		<b>86.93</b>			
6/10/2016	SF	0.16	7.89	46.82	<b>16.86</b>			
6/10/2016	NSF	0.43	38.92		<b>83.14</b>			
6/7/2016	SF	0.13	1.35	48.82	<b>2.76</b>	<b>2.37</b>	<b>97.63</b>	<b>51.48</b>
6/7/2016	NSF	0.53	47.47		<b>97.24</b>			
6/7/2016	SF	0.16	1.65	50.11	<b>3.29</b>			
6/7/2016	NSF	0.54	48.46		<b>96.71</b>			
6/7/2016	SF	0.12	1.18	55.58	<b>2.12</b>			
6/7/2016	NSF	0.60	54.40		<b>97.88</b>			
6/7/2016	SF	0.07	0.67	51.40	<b>1.31</b>			
6/7/2016	NSF	0.56	50.73		<b>98.69</b>			
5/19/2016	SF	0.12	1.17	32.51	<b>3.59</b>	<b>2.80</b>	<b>97.20</b>	<b>27.26</b>
5/19/2016	NSF	0.35	31.34		<b>96.41</b>			
5/19/2016	SF	0.03	0.33	17.55	<b>1.86</b>			
5/19/2016	NSF	0.19	17.22		<b>98.14</b>			
5/19/2016	SF	0.04	0.35	17.82	<b>1.99</b>			
5/19/2016	NSF	0.19	17.47		<b>98.01</b>			
5/19/2016	SF	0.16	1.55	41.16	<b>3.77</b>			
5/19/2016	NSF	0.44	39.61		<b>96.23</b>			

### **3.2 Additions made to the ZyGEM Differential Extraction**

In an attempt to enhance the cell lysis of epithelial cells in the ZyGEM step of the differential extraction, experiments with the addition of PK, Sarkosyl and or PrepFiler™ were performed as described in materials and methods sections 2.5-2.6.

In the first experiment, PK was added to ZyGEM in an attempt to further reduce the amount of epithelial DNA carried over into the SF by aiding the EA1 enzyme in digesting the protein components of the epithelial cells.

#### **3.2.1 Extraction with ZyGEM followed by PK**

The experiment adding an incubation with PK after lysis with ZyGEM gave no quantification values for the samples and the Internal PCR Controls. This is likely due to incomplete denaturation of the PK, which would damage Taq Polymerase since no purification step is employed post ZyGEM extraction.

#### **3.2.2 Extraction with PK followed by ZyGEM**

The mean percentage of epithelial DNA in the SF for PK-ZyGEM was 20.56%, comparison to the mean percentage of 19.79% that was seen when using Zygem alone. The mean percentage of epithelial DNA in the NSF for PK-ZyGEM was 79.44%, where ZyGEM alone produced a mean of 80.21% of the epithelial DNA in the NSF. Therefore, the addition

of PK produced no improvement over ZyGEM alone. Results are shown in figure 6 and tabulated in table 10.

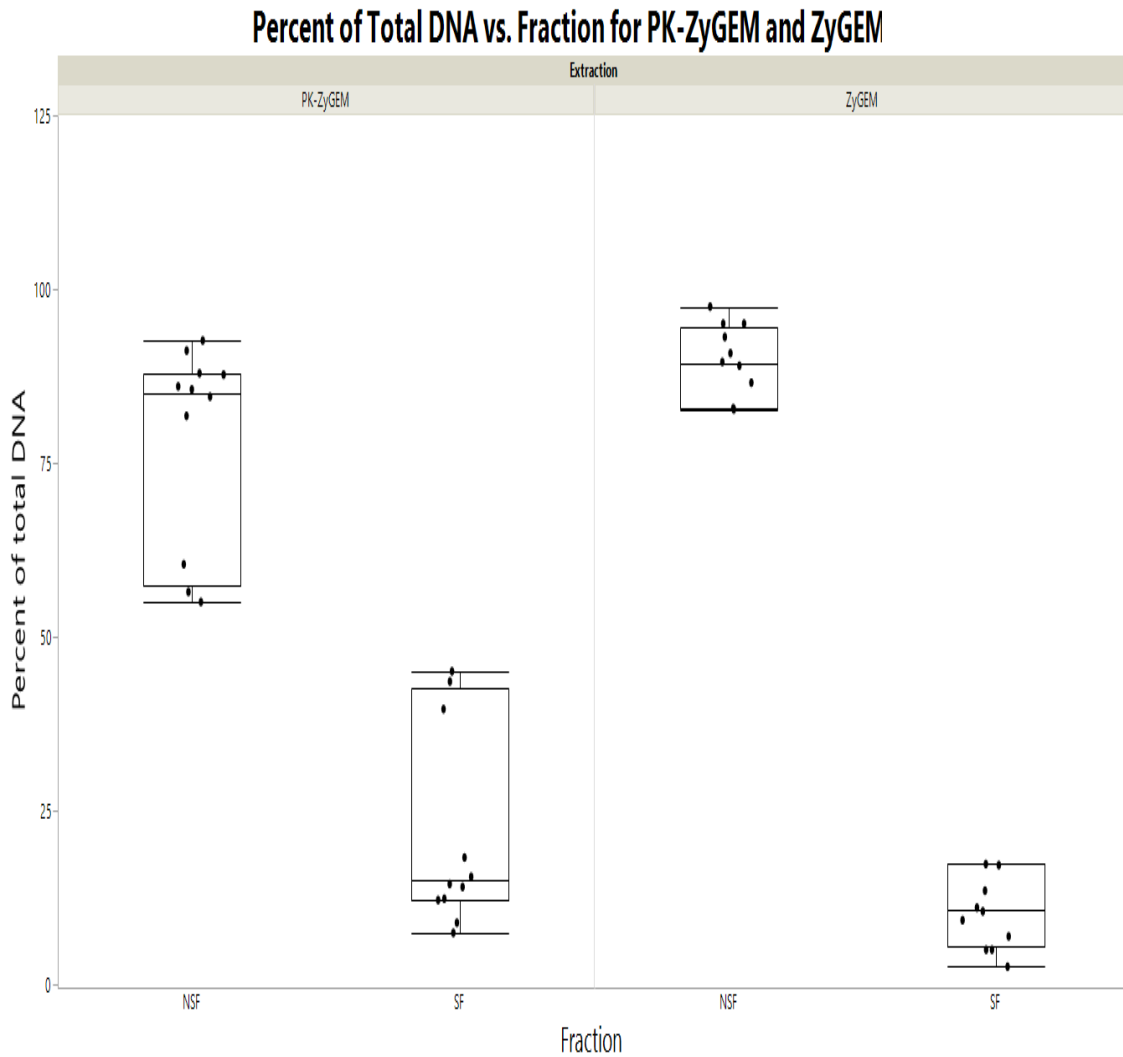


Figure 6: This boxplot shows the percent of epithelial DNA in each fraction for both the ZyGEM extraction and the PK-ZyGEM extraction. There is not a significant increase in removal of epithelial DNA from the SF for the PK-ZyGEM in comparison to the ZyGEM extraction.

**Table 9:** Results of the PK-ZyGEM Differential Extraction on Epithelial Cells

Date	Fraction	Concentration (ng/ul)	Mass (ng)	Total DNA (ng)	% of Total DNA	Mean% SF	Mean% NSF	Mean Total ng DNA
3/26/2016	<b>SF</b>	0.14	4.06	29.04	<b>13.99</b>	<b>15.89</b>	<b>84.11</b>	<b>17.98</b>
3/26/2016	<b>NSF</b>	0.28	24.97		<b>86.01</b>			
3/26/2016	<b>SF</b>	0.18	5.29	29.04	<b>18.23</b>			
3/26/2016	<b>NSF</b>	0.26	23.74		<b>81.77</b>			
3/26/2016	<b>SF</b>	0.03	0.93	0.93	<b>100.00</b>			
3/26/2016	<b>NSF</b>	0.00	0.00		<b>0.00</b>			
3/26/2016	<b>SF</b>	0.07	2.00	12.92	<b>15.46</b>			
3/26/2016	<b>NSF</b>	0.12	10.92		<b>84.54</b>			
3/16/2016	<b>SF</b>	1.52	45.69	115.44	<b>39.58</b>	<b>35.06</b>	<b>64.94</b>	<b>99.97</b>
3/16/2016	<b>NSF</b>	0.78	69.75		<b>60.42</b>			
3/16/2016	<b>SF</b>	1.73	51.93	115.38	<b>45.01</b>			
3/16/2016	<b>NSF</b>	0.71	63.45		<b>54.99</b>			
3/16/2016	<b>SF</b>	1.58	47.49	109.05	<b>43.55</b>			
3/16/2016	<b>NSF</b>	0.68	61.56		<b>56.45</b>			
3/16/2016	<b>SF</b>	0.24	7.26	60.00	<b>12.10</b>			
3/16/2016	<b>NSF</b>	0.59	52.74		<b>87.90</b>			
2/3/2016	<b>SF</b>	0.35	10.56	119.49	<b>8.84</b>	<b>10.73</b>	<b>89.27</b>	<b>138.18</b>
2/3/2016	<b>NSF</b>	1.21	108.93		<b>91.16</b>			
2/3/2016	<b>SF</b>	0.55	16.62	135.26	<b>12.29</b>			
2/3/2016	<b>NSF</b>	1.32	118.64		<b>87.71</b>			
2/3/2016	<b>SF</b>	0.75	22.61	156.76	<b>14.42</b>			
2/3/2016	<b>NSF</b>	1.49	134.15		<b>85.58</b>			
2/3/2016	<b>SF</b>	0.35	10.43	141.23	<b>7.39</b>			
2/3/2016	<b>NSF</b>	1.45	130.79		<b>92.61</b>			

### 3.2.3 Addition of Sarkosyl to the ZyGEM Differential Extractions

A higher Sarkosyl concentration of 0.5% Sarkosyl showed low amounts of DNA in the SF and showed complete inhibition of amplification during qPCR quantitation of the NSF. Because there was inhibition in the NSF, the percent of total DNA could not be obtained for either fraction. No other investigation into the use of 0.5% Sarkosyl was done.

With a lower Sarkosyl concentration of 0.1%, the mean percentage of epithelial DNA in the SF using was 23.98%. The mean percentage of epithelial DNA in the NSF using 0.1% Sarkosyl-ZyGEM was 76.02%. Control Samples using Zygem only yielded a mean percent of epithelial DNA of 10.68% of in the SF and 89.33% in the NSF. Although there is less variability in the results with 0.1% Sarkosyl, the separation of epithelial DNA from the SF is worse than that of ZyGEM alone. The data for this experiment is shown in figure 7 and table 10.

### Percent of Total DNA vs. Fraction for 0.1% Sarkosyl-ZyGEM and ZyGEM

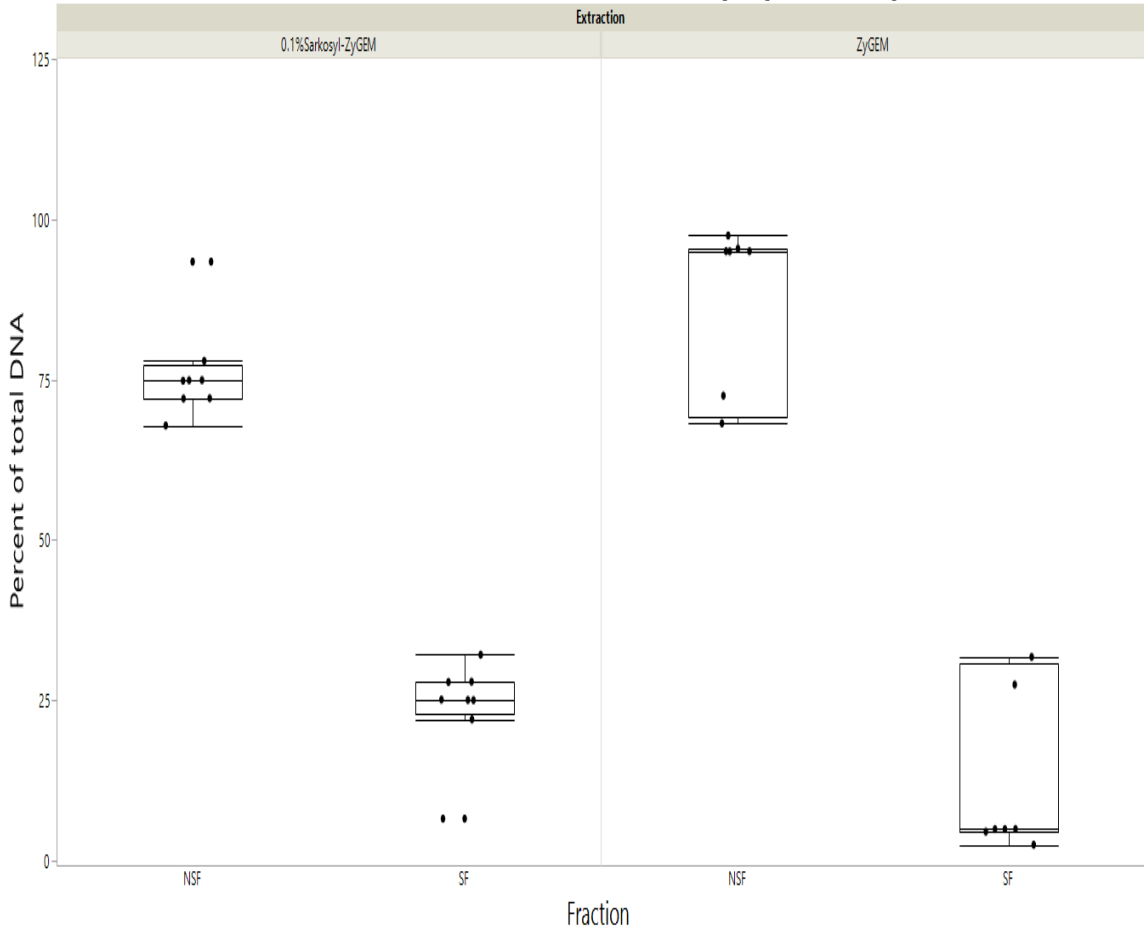


Figure 7: This boxplot shows the percent of epithelial DNA in each fraction for 0.1% Sarkosyl-ZyGEM compared to ZyGEM alone.

**Table 10:** Results of ZyGEM Differential Extraction with the addition of 0.1% Sarkosyl on Epithelial Cells.

Date	Fraction	Concentration (ng/ul)	Mass (ng)	Total DNA (ng)	% of Total DNA	Mean % SF	Mean % NSF	Mean Total DNA (ng)
6/7/2016	<b>SF</b>	1.76	17.58	54.72	<b>32.13</b>	<b>28.27</b>	<b>71.73</b>	<b>53.08</b>
6/7/2016	<b>NSF</b>	0.41	37.14		<b>67.87</b>			
6/7/2016	<b>SF</b>	1.63	16.30	58.37	<b>27.93</b>			
6/7/2016	<b>NSF</b>	0.47	42.07		<b>72.07</b>			
6/7/2016	<b>SF</b>	1.39	13.93	49.95	<b>27.89</b>			
6/7/2016	<b>NSF</b>	0.40	36.02		<b>72.11</b>			
6/7/2016	<b>SF</b>	1.24	12.40	49.29	<b>25.15</b>			
6/7/2016	<b>NSF</b>	0.41	36.89		<b>74.85</b>			
3/21/2016	<b>SF</b>	0.17	5.14	20.50	<b>25.08</b>	<b>19.70</b>	<b>80.30</b>	<b>20.51</b>
3/21/2016	<b>NSF</b>	0.17	15.36		<b>74.92</b>			
3/21/2016	<b>SF</b>	0.03	0.85	12.94	<b>6.59</b>			
3/21/2016	<b>NSF</b>	0.13	12.08		<b>93.41</b>			
3/21/2016	<b>SF</b>	0.21	6.17	24.61	<b>25.06</b>			
3/21/2016	<b>NSF</b>	0.20	18.44		<b>74.94</b>			
3/21/2016	<b>SF</b>	0.18	5.29	23.98	<b>22.06</b>			
3/21/2016	<b>NSF</b>	0.21	18.69		<b>77.94</b>			

### 3.2.4 Addition of Sarkosyl and PK to ZyGEM Differential Extraction

A combination of 0.1% Sarkosyl, PK and ZyGEM digestion of the epithelial cells was also performed as described in materials and methods section 2.5.1. The mean percentage of epithelial DNA in the SF for 0.1% Sarkosyl-PK-ZyGEM with was 38.76%. The mean percentage of epithelial DNA in the NSF for 0.1% Sarkosyl-PK-ZyGEM was 61.24%. Comparisons of these conditions demonstrated that the combination of 0.1% Sarkosyl and PK with ZyGEM performs worse than ZyGEM alone (19.79% in SF, 80.21% in NSF), PK with ZyGEM (20.56% in SF, 79.44% in NSF) and 0.1% Sarkosyl with ZyGEM (23.98% in SF, 76.02% in NSF). Data from this experiment is shown in figure 8 and table 11.

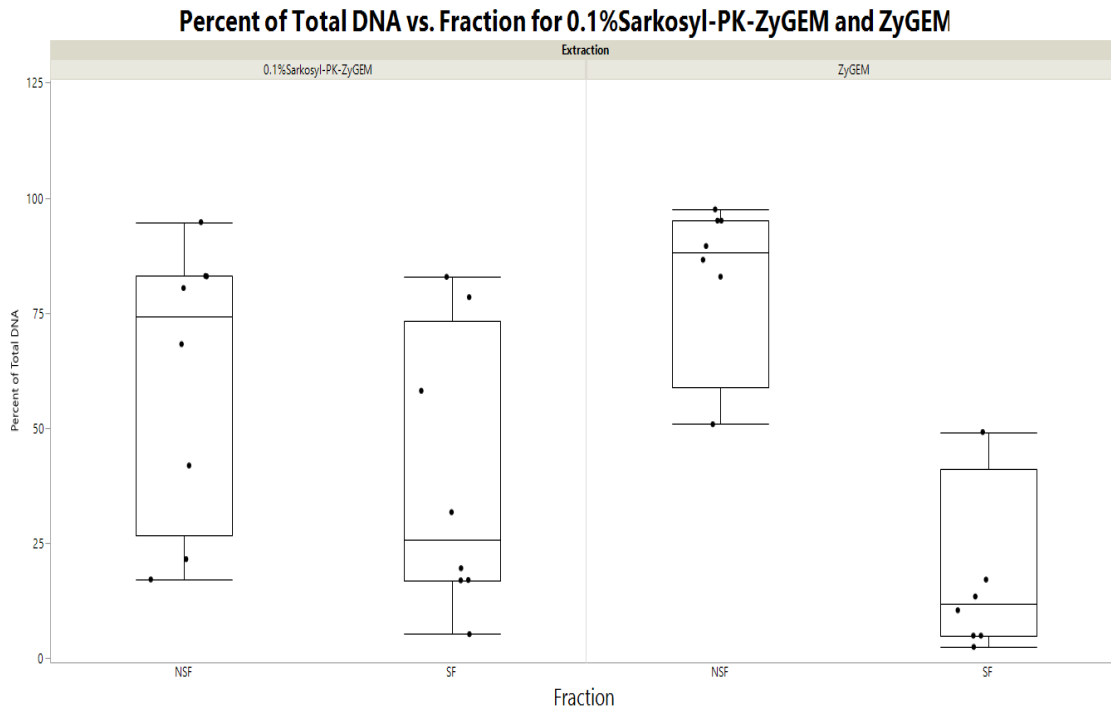


Figure 8: This boxplot shows the percent of epithelial DNA in each fraction for 0.1% Sarkosyl-PK-ZyGEM compared to ZyGEM alone. The separation of epithelial DNA from the SF is worse than that of ZyGEM and 0.1% Sarkosyl-ZyGEM., with the majority of the epithelial DNA being found in the SF.

**Table 11:** Results of Differential Extraction with of 0.1% Sarkosyl-PK-ZyGEM Procedure.

3/21/2016	Fraction	Concentraion (ng/ul)	Mass (ng)	Total DNA (ng)	% of Total DNA	Mean % SF	Mean % NSF	Mean Total DNA (ng)
3/21/2016	SF	0.08	2.51	12.81	19.60	14.72	85.28	33.72
3/21/2016	NSF	0.11	10.30		80.40			
3/21/2016	SF	0.51	15.19	89.19	17.04			
3/21/2016	NSF	0.82	74.00		82.96			
3/21/2016	SF	0.07	2.17	12.80	16.97			
3/21/2016	NSF	0.12	10.63		83.03			
3/21/2016	SF	0.04	1.06	20.07	5.29			
3/21/2016	NSF	0.21	19.00		94.71			
3/16/2016	SF	3.51	105.18	126.96	82.84	62.79	37.21	78.53
3/16/2016	NSF	0.24	21.78		17.16			
3/16/2016	SF	2.75	82.47	105.15	78.43			
3/16/2016	NSF	0.25	22.68		21.57			
3/16/2016	SF	0.96	28.71	49.41	58.11			
3/16/2016	NSF	0.23	20.70		41.89			
3/16/2016	SF	0.35	10.35	32.58	31.77			
3/16/2016	NSF	0.25	22.23		68.23			

### 3.2.5 Addition of 0.1% Sarkosyl to the ZyGEM Extraction with a Wash Step

Because of the decreased variation in epithelial DNA yield observed with the addition of 0.1% Sarkosyl to the ZyGEM lysis, a variation of the Sarkosyl treatment was performed where the detergent was used as a pre-treatment on the cells before the lysis step rather than as another component of the ZyGEM reaction master mix. By doing this, the epithelial cell membranes should have been weakened by the higher concentration of Sarkosyl, which was then diluted to the 0.1% Sarkosyl concentration by the addition of the rest of the ZyGEM reaction reagents. This process was done both with and without a wash step.

The Sarkosyl pre-treatment showed no improvement on the separation or the variability in epithelial DNA yield in comparison to the previous version of the 0.1% Sarkosyl-ZyGEM extraction. The mean percentage of epithelial DNA in the SF for 0.1% Sarkosyl-ZyGEM with a wash step was 23.98%. The mean percentage of epithelial DNA in the NSF for 0.1% Sarkosyl-ZyGEM with a wash step was 76.02%. This combination appears to be an improvement over ZyGEM alone, however when compared to the ZyGEM alone with a wash, the addition of 0.1% Sarkosyl made the separation worse than that of the ZyGEM-Wash procedure.

One possible explanation for this increase of epithelial DNA in the SF with the addition of Sarkosyl may be the decrease in viscosity in the reaction mixture during centrifugation. Centrifugation separates cell components based on shape and molecular mass of the component.<sup>36</sup> The density of the DNA is not changing and the density difference of the Sarkosyl is not large enough to effect the separation based on density. However, the Sarkosyl may be decreasing the viscosity and decreasing the resistance against which the long strands of DNA are moving through, allowing more of the DNA to accumulate in the bottom region of the tube than without the Sarkosyl in the reaction solution. Data is depicted in figure 9 and table 12.

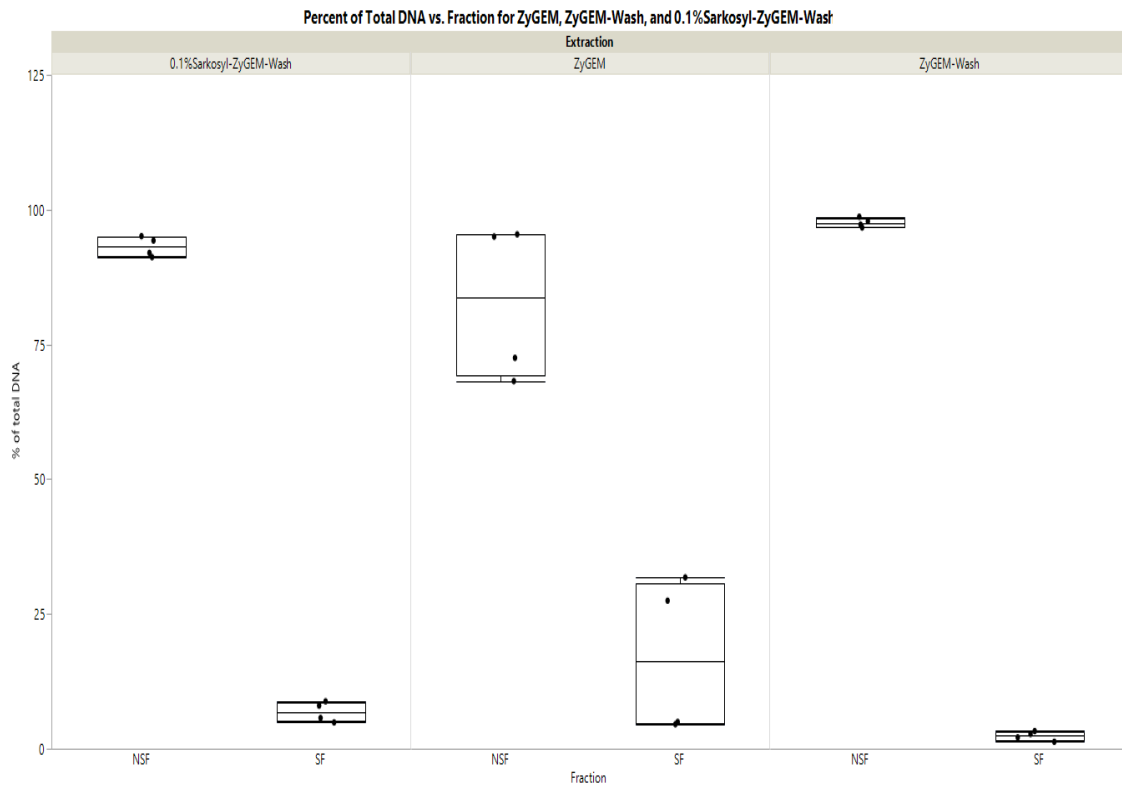


Figure 9: This boxplot compares the percent of epithelial DNA found in each fraction with the modified 0.1% Sarkosyl-ZyGEM-Wash extraction to ZyGEM alone and ZyGEM alone with a wash step..

**Table 12:** Results of 0.1% Sarkosyl-ZyGEM Differential Extraction with and without a Wash Step. All of these extractions were performed on the same day using the same epithelial cell preparation.

Extraction	Average % of DNA	SD (% of DNA)	Average Total DNA	SD (Total DNA)
Z, SF	17.20	14.46	43.55	7.63
Z, NSF	82.80			
Z-W, SF	2.37	0.85	51.48	2.93
Z-W, NSF	97.63			
S.1-Z, SF	28.27	2.88	53.08	4.28
S.1-Z, NSF	71.73			
S.1-Z-W, SF	6.86	1.85	42.48	4.11
S.1-Z-W, NSF	93.14			

### 3.2.6 PrepFiler® Separation and Clean-up Experiments

The use of PrepFiler™ as a method of separating the epithelial cell DNA from the sperm or cleaning up the SF after separation via centrifugation is not suggested. The addition of isopropanol to the SF would interfere with extraction and analysis of the DNA from the sperm. However, quantitation of the DNA in the Clean-up step fraction does show the variability in amount of epithelial cell DNA carry-over into the sperm fraction (See Appendix Table 18).

### **3.3 Comparison of All Modifications Made to the ZyGEM lysis with Epithelial Cells Only**

All of the modifications described above were made to the ZyGEM lysis of epithelial cells in an attempt to enhance the epithelial cell lysis and separation. To determine if the extraction modifications were aiding in solving the suspected cell clumping problems, we compared both the percent of epithelial DNA found in the SF as well as the difference in concentration of epithelial DNA in the NSF and SF for each experiment. A decrease in percent of epithelial DNA found in the SF indicated improvement with the modification made to the ZyGEM lysis as it indicates that more of the epithelial DNA was removed into the NSF fraction and the male to female ratio would be closer to a single source male DNA sample in the full differential extraction. Similarly, the

concentration of epithelial DNA obtained for the SF could be used to determine the efficacy of epithelial DNA separation.

Since we cannot physically remove all of the liquid NSF from the SF by pipetting, there will always be some of the epithelial DNA solution that remains in the SF. This solution in the SF is expected to have the same concentration of epithelial DNA as the solution in the NSF. This is why we expect to see a concentration of epithelial DNA in the SF equal to the concentration of epithelial DNA in the NSF. If the mean difference in concentration between the NSF and SF is calculated, we would expect it to be zero. A positive mean difference between the NSF and SF indicates that there is a lower concentration of epithelial DNA in the SF than in the NSF. A negative mean difference between the NSF and SF would indicate that there is more epithelial DNA carryover than expected in the SF, most likely from incomplete removal of the epithelial DNA in solution. Furthermore, if a 95% confidence interval of this mean difference is taken and does not contain zero, the difference is considered to be significantly different.

To compare the concentration of epithelial DNA in the SF to the concentration of epithelial DNA in the NSF the mean difference in concentration and confidence intervals for each extraction was calculated and can be found in table 13. The concentration of epithelial DNA in the SF appeared to decrease with the addition of 0.1% Sarkosyl or the

addition of PK, but not the 0.1% Sarkosyl-PK-ZyGEM combination. The mean difference in concentration of epithelial DNA between the NSF and SF for 0.1% Sarkosyl-ZyGEM was 0.13 ng/ $\mu$ l, indicating a decrease in epithelial DNA concentration in the SF as compared to ZyGEM alone; however, the 95% Confidence Interval for this mean difference included zero, which suggests that the difference is not statistically significant. There was a statistically significant mean difference in epithelial DNA concentration between the NSF and SF of 0.17 ng/ $\mu$ l for ZyGEM with a wash, and the percent of epithelial DNA found in the SF decreased in comparison to the ZyGEM extraction without a wash step (19.79 % for ZyGEM and 10.2% for ZyGEM-Wash). This wash step is most likely decreasing the concentration of epithelial DNA in the solution that remains in the SF, thus producing results indicative of better removal of epithelial DNA in the SF.

**Table 13:** Mean difference in concentration (NSF-SF) for all extractions on epithelial cells

<b>Extraction</b>	<b>Mean Difference in DNA Concentration in ng/<math>\mu</math>l (NSF-SF)</b>	<b>SD</b>	<b>95 % Confidence Interval</b>
<b>ZyGEM-PrepFiler™ Clean-up</b>	<b>-0.30</b>	<b>0.50</b>	<b>-0.3,1.3</b>
<b>PK-ZyGEM</b>	<b>0.20</b>	<b>0.15</b>	<b>0.03,0.28</b>
<b>0.1% Sarkosyl -PK-ZyGEM</b>	<b>-0.07</b>	<b>0.50</b>	<b>0.09,0.91</b>
<b>0.1% Sarkosyl-ZyGEM</b>	<b>0.13</b>	<b>0.02</b>	<b>-0.01,0.05</b>
<b>ZyGEM</b>	<b>0.00</b>	<b>0.87</b>	<b>0.41,1.33</b>
<b>ZyGEM-Wash</b>	<b>0.17</b>	<b>0.15</b>	<b>0.22-0.52</b>

### **3.4 Experiments with a Mixture of Sperm and Epithelial Cells**

The final two experiments replicated the full enzymatic differential extraction by using a mixture of epithelial cells and sperm cells as the starting cell material. The combination of cells that was prepared as described in materials and methods contained approximately 1600 sperm cells and 800 epithelial cells, except where a dilution series of epithelial cells were used.

#### **3.3.1 ZyGEM with a Wash and without a Wash Step with Mixture**

##### **Samples**

Because the addition of a wash step after the separation of the NSF from the SF had demonstrated an improvement in removal of epithelial DNA from the SF in the experiment with only epithelial cells, the procedure comparing ZyGEM with and without a wash step was repeated using a mixture of female epithelial cells and sperm cells as described in materials and methods section 2.7-2.8. Unexpectedly, the majority of all DNA, both from sperm and epithelial cells, was found in the SF with very little DNA in the NSF. The amounts of total DNA in the NSF was very low, containing only 3-10% of the mean total DNA per reaction for both ZyGEM with and without a wash step (Table 14). In these initial experiments that had a high total starting cell amount per reaction, there appeared to be little to no improvement on the male:female mixture ratio obtained in SF for the ZyGEM-Trypsin extraction with a wash step in

comparison to the ZyGEM-Trypsin extraction without a wash step (Table 14). For both procedures, the results mimicked what would be expected if no ZyGEM had been used at all based on the preliminary experiment that was performed to determine optimal cell number for this project. This procedure was repeated twice and the data is shown in figure 10 and table 14.

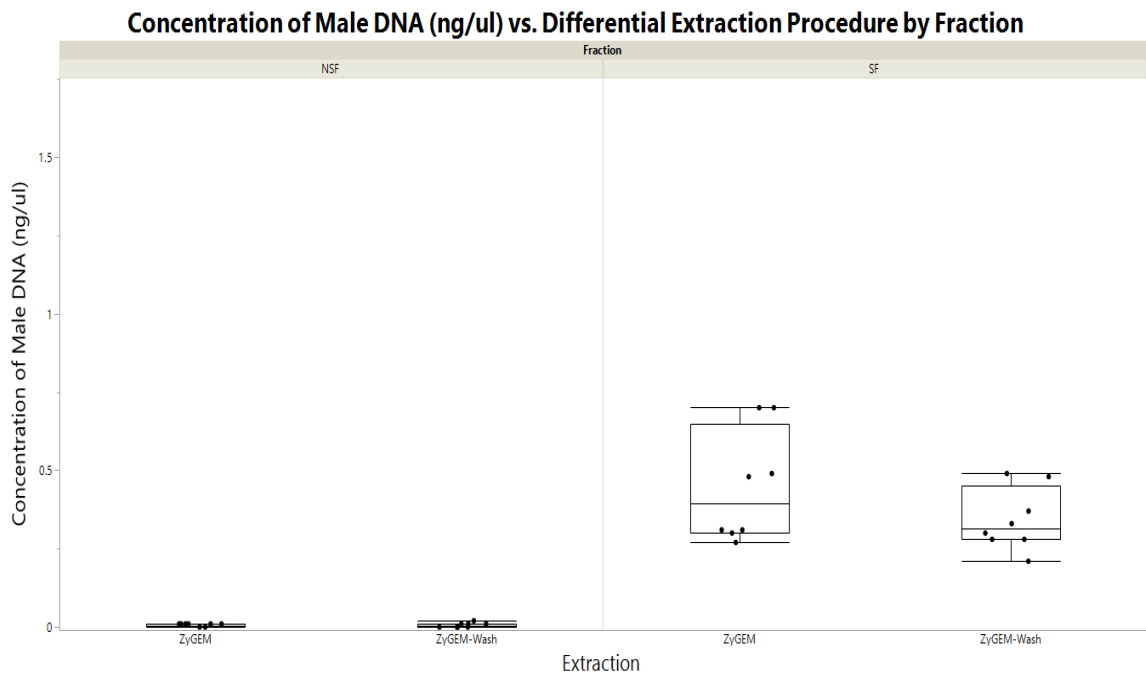
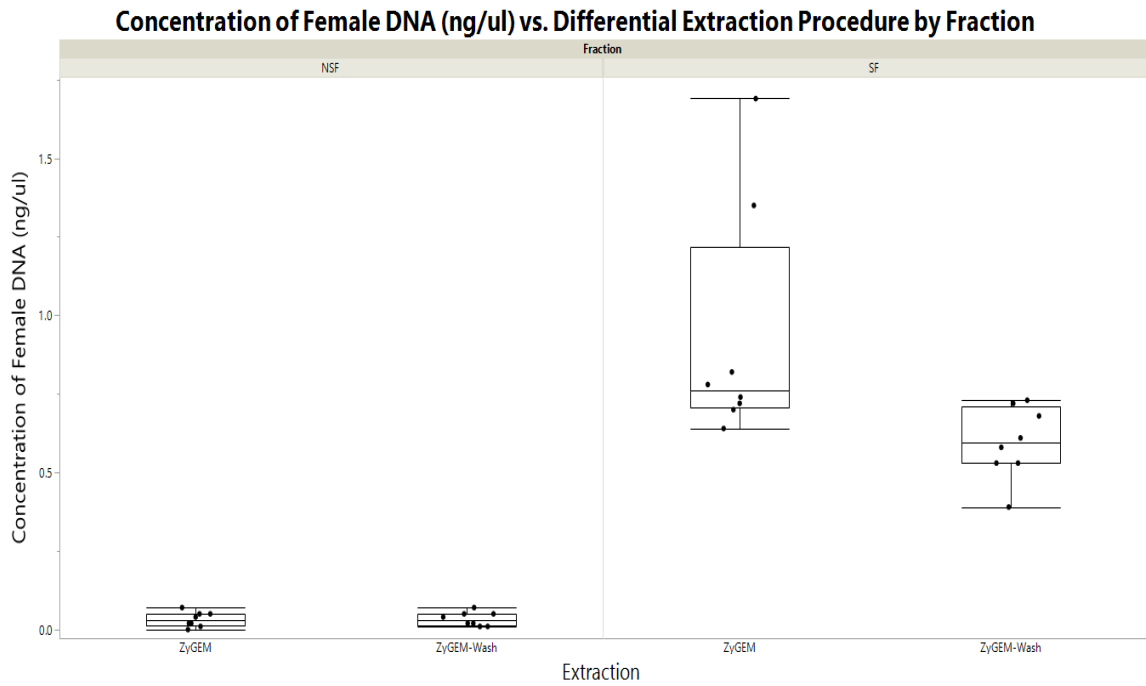


Figure 10: These boxplots compare the concentration of male DNA (ng/ul) found in each fraction to the concentration of female DNA (ng/ul) found in each fraction for ZyGEM and the ZyGEM-Wash procedure.

**Table 14:** Results of Experiments comparing the effects of ZyGEM and ZyGEM with a Wash Step on mixtures of Sperm and Epithelial Cells. A mixture ratio above 1 indicates that there is more male than female DNA. A mixture ratio below 1 indicates that there is less male DNA than female DNA.

Date	Extraction	Average Female DNA SF (ng)	Average Male DNA SF (ng)	Average Female DNA NSF (ng)	Average Male DNA NSF (ng)	Average Mixture Ratio Male:Female SF	
6/15/2016	Zygem-Trypsin	38.21	14.96	1.07	0.56	0.39	
6/10/2016	Zygem-Trypsin	29.66	54.74	0.71	4.79	1.85	
6/15/2016	Zygem-Wash-Trypsin	31.52	13.34	1.39	0.38	0.42	
6/10/2016	Zygem-Wash_Trypsin	20.91	28.05	0.91	4.75	1.34	
Date	Extraction	Total DNA in NSF	Total DNA in SF	% of Total DNA in NSF	% of Total DNA in SF	% of Female DNA in NSF	% of Female DNA in SF
6/10/2016	Zygem-Trypsin	1.63	53.17	2.97	97.03	2.72	97.28
6/15/2016	Zygem-Trypsin	5.50	84.41	6.12	93.88	2.35	97.65
6/10/2016	Zygem-Wash_Trypsin	1.77	44.87	3.80	96.20	4.24	95.76
6/15/2016	Zygem-Wash_Trypsin	5.66	48.96	10.36	89.64	4.17	95.83

### 3.3.2 Epithelial Cell Dilutions to Determine Digestion Limits of ZyGEM

Because the ZyGEM with and without a wash step experiment performed on mixtures produced results similar to what would be expected if no EA1 enzyme had been added, we considered that there may be too many cells and cellular proteins in these mixtures, which could have been decreasing the efficiency of cell lysis by EA1. Since the EA1 enzyme is a protease that will lyse at any lysine or phenylalanine, it will not distinguish those amino acids in the epithelial cells from those amino acids in the sperm cells.<sup>27</sup> Although EA1 may try to cleave proteins in the sperm cells at any lysine or phenylalanine, it cannot fully lyse the sperm and release the DNA into solution; this is confirmed by

finding little to no sperm DNA in the NSF in these experiments. If the enzyme is losing efficiency because of the high amounts of cells in the mixture reactions, then increasing the amount of enzyme and length of digestion should aid the enzyme in lysing all of the epithelial cells. However, previous work by Rachel Martinez, M.S. revealed that there is no increase in epithelial cell lysis or epithelial DNA separation when the amount of enzyme and length of incubation are increased beyond the parameters used in these experiments.<sup>29</sup>

To explore the possibility that there may be more cellular proteins than the EA1 enzyme can digest, an experiment using serial dilutions of epithelial cells with and without a 1600 sperm cells was performed using the ZyGEM-Wash procedure as described in materials and methods section 2.8.1. These dilutions were made in duplicate.

In the experiment without sperm, a steady decrease in percent of epithelial DNA in the SF was observed as the amount of epithelial cells in the total reaction decreased. The separation of epithelial DNA from the SF was best at when approximately 2 ng of total DNA in the entire reaction was observed. Unfortunately, this range of optimal separation would not work for difficult sexual assault mixtures, as a very low male:female ratio at 2ng of total sample DNA would have less than 1 ng of male DNA in the whole sample. A profile generated by less than 1 ng of male DNA by amplification would increase the difficulty of

interpretation, and there would be a larger probability for dropout of alleles with such low amounts of template DNA.

In the middle range of epithelial cell amount per reaction, the percentage of DNA in the SF, NSF and wash was close to equal. This indicates that the wash step is improving the removal of epithelial DNA from the SF because if that amount of DNA had remained in the SF, over half of the epithelial DNA would have been found in the SF. The improvement of removal of epithelial DNA of the wash step at this middle range of epithelial cell amounts may suggest that additional wash steps could aid in better removal of epithelial DNA from the SF. At the high and low ends of epithelial cell amounts, there was a lower percent of epithelial DNA found in the SF than in the NSF or wash. However, there was a larger amount of epithelial DNA in the SF at higher starting amounts of epithelial cells. Once again, although the percentage of DNA in the SF is smaller, the amount of epithelial DNA in the SF could overwhelm a small amount of sperm DNA in a low mixture ratio sample. Data from these experiments are contained in table 15.

**Table 15:** Results of the ZyGEM with a wash extraction performed on a dilution series of epithelial cells. The stock epithelial cell solution was counted and a target of 1200 cells was used for the reactions with the stock solution.

<b>E-cell Dilutions without Sperm</b>	<b>Concentration of DNA (ng/ul)</b>	<b>Mass of DNA (ng)</b>	<b>DNA in Wash (ng)</b>	<b>Total DNA (ng)</b>	<b>% of Total DNA</b>
<b>Replicate Series A</b>					
No Dilution,SF	0.55	27.42	24.98	137.86	19.89
No Dilution,NSF	0.95	85.46			61.99
1: 10 dilution,SF	0.04	2.13	2.84	6.98	30.57
1: 10 dilution,NSF	0.02	2.01			28.76
1:50 dilution, SF	0.00	0.09	0.63	2.71	3.38
1:50 dilution, NSF	0.02	1.99			73.37
1:250 dilution, SF	0.00	0.01	0.00	0.01	55.56
1:250 dilution, NSF	0.00	0.00			44.44
<b>Replicate Series B</b>					
No Dilution,SF	0.03	1.32	8.52	961.55	0.14
No Dilution,NSF	10.57	951.72			98.98
1: 10 dilution,SF	0.05	2.25	1.62	11.59	19.43
1: 10 dilution,NSF	0.09	7.72			66.60
1:50 dilution, SF	0.00	0.00	0.00	1.83	0.00
1:50 dilution, NSF	0.02	1.83			100.00
1:250 dilution, SF	0.00	0.00	0.00	0.22	0.00
1:250 dilution, NSF	0.00	0.22			100.00

In the epithelial cell dilution series with a constant amount of sperm, more female DNA was found in the SF than in the NSF for all of the dilutions. However, this was not observed for the reaction with no epithelial cell dilution, which contained the highest amount of epithelial cells with a mean total DNA amount of 155.8 ng. These larger epithelial cell samples also showed the highest amount of DNA in the wash ( $\mu = 27.09$  ng). This indicates that the wash step is more helpful with higher

starting amounts of epithelial cells than with lower starting amounts for mixture samples. This trend may indicate that at higher starting amounts of cells the high concentration of epithelial DNA in solution is a larger contributor to the female DNA carryover into the SF than the incomplete lysis of epithelial cells; whereas, at the lower starting cell amounts the incomplete lysis is most likely the major cause of epithelial DNA carryover.

A comparison of mixture ratios in the SF to starting mixture ratios was performed by calculating the sum of female and male DNA amounts per reaction and dividing the total male DNA per reaction by the total female DNA per reaction. This analysis showed an increase in the male:female mixture ratio in the SF as the starting epithelial cell amounts decreased, showing improvement in removal of epithelial DNA from the SF at lower starting cell amounts. Additionally, at the lower concentrations of epithelial cells, the mixture ratios were higher for the SF of the samples lysed with the ZyGEM-Wash-Trypsin procedure than the calculated starting mixture ratio for those samples. This indicates that the separation was improving with decreased starting cell amounts, and the increased mixture ratio was not solely a product of a larger starting male:female ratio. Results of these experiments are tabulated in table 16.

**Table 16:** Results of the ZyGEM with a wash extraction performed on a dilution series of epithelial cells with a constant amount of 1600 sperm cells per reaction. Any negative value for female DNA (ng) was treated as zero for calculations of mixture ratios.

<b>E-cell Dilutions with Sperm</b>	<b>Male DNA (ng)</b>	<b>Female DNA (ng)</b>	<b>Total DNA (ng)</b>	<b>% of SF from Male DNA</b>	<b>% of SF from Female DNA</b>	<b>Sample Mixture Ratio</b>
<b>Replicate Series C</b>						
No Dilution,SF	17.41	46.59	157.56	27.20	72.80	0.17
No Dilution,NSF	1.69	66.87				
1: 10 dilution,SF	12.52	-0.59	24.07	104.98	-4.98	2.56
1: 10 dilution,NSF	1.99	6.21				
1:50 dilution, SF	16.13	6.93	27.18	69.94	30.06	2.79
1:50 dilution, NSF	1.15	-0.78				
1:250 dilution, SF	15.42	1.63	19.45	90.44	9.56	5.43
1:250 dilution, NSF	1.00	1.40				
<b>Replicate Series D</b>						
No Dilution,SF	17.90	35.64	154.05	33.44	66.56	0.20
No Dilution,NSF	2.73	68.59				
1: 10 dilution,SF	19.34	9.09	33.08	68.04	31.96	1.99
1: 10 dilution,NSF	0.46	0.85				
1:50 dilution, SF	16.91	6.69	26.73	71.65	28.35	3.43
1:50 dilution, NSF	2.25	-1.12				
1:250 dilution, SF	17.68	2.64	23.48	87.01	12.99	5.98
1:250 dilution, NSF	1.64	0.58				

The serial dilutions of epithelial cells with sperm were carried through to amplification, separation and analysis as described in materials and methods sections 2.10-2.11. The profiles generated showed mixtures that became more distinguishable as a major and minor contributor as the amount of starting epithelial cells decreased. Some of this must be attributed to the increase in starting male:female

cell ratio. However, as discussed previously, the qPCR data showed the mixture in the SF had a larger male:female ratio than the total reaction mixture ratio (table 17), and thus some of the improvement in the ability to distinguish male profile may be attributed to the ZyGEM-Wash-Trypsin procedure. Electropherograms showing the red color channel for comparison of these dilutions are provided in figure 11.

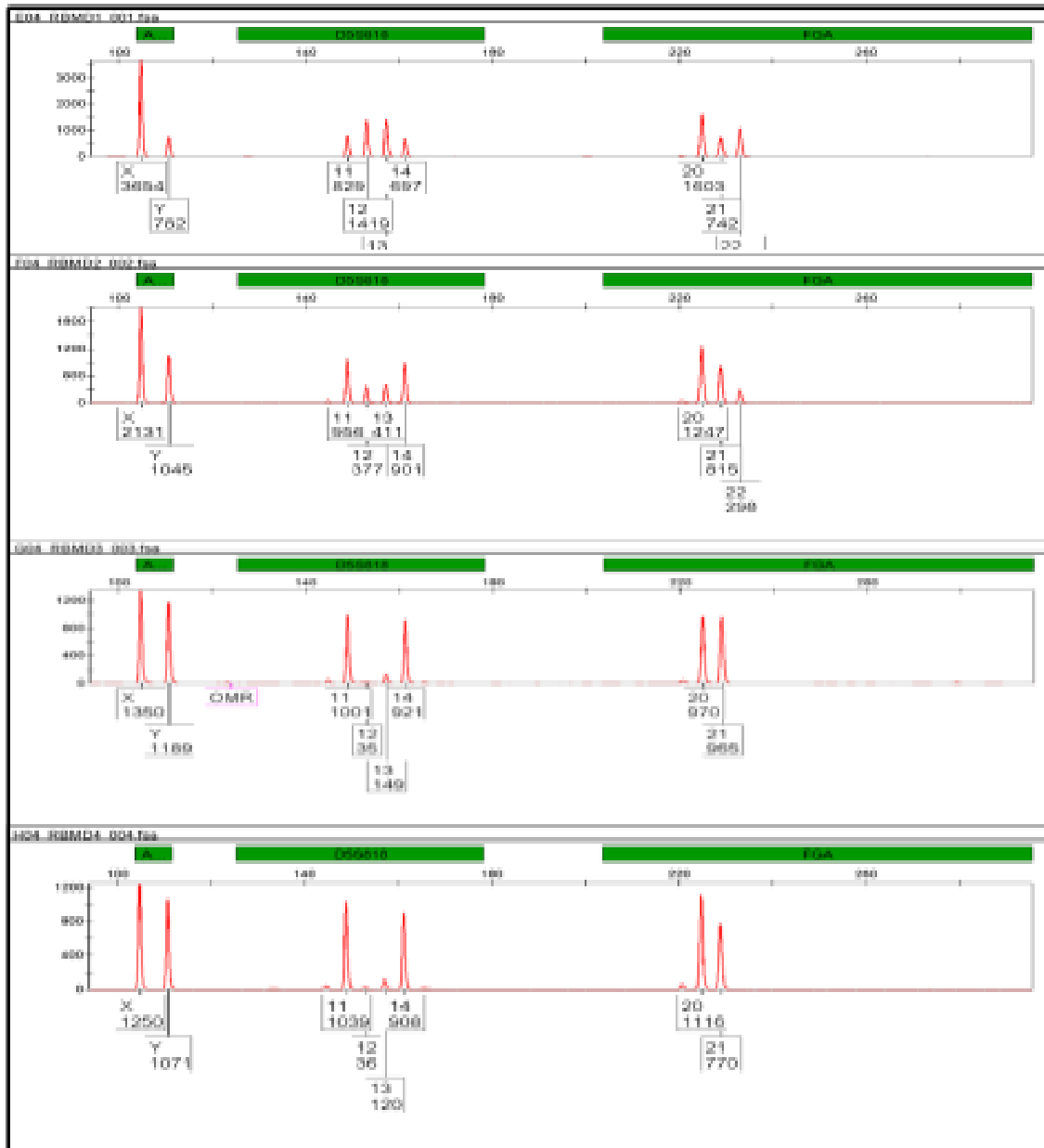


Figure 11: Electropherograms of the epithelial cell dilution series with constant amounts of sperm. As the starting amount of epithelial cells decreases, the male profile becomes more distinguishable.

#### **4. CONCLUSIONS**

The difficulties of developing any differential extraction relies on both chemically and physically separating epithelial cell DNA from sperm cell DNA. The various techniques discussed here show the difficulties of attempting to only use one or the other. The most effective differential extraction techniques to date involve a combination of both cell preferential lysis and physical manipulation of the different cell types. The original differential extraction designed by Gill and colleagues suffered from an inefficient physical separation, thus requiring many wash steps to achieve a clear, interpretable male DNA profile.

The dual enzyme differential extraction procedure that was examined is significantly faster, easier to use and requires less reagents to perform. However, it still suffers from the same lack of physical separation of epithelial DNA from the sperm in the sperm fraction due to the free epithelial DNA in solution. In the experiments where additions were made to the ZyGEM procedure with epithelial cells, the extractions with 0.1% Sarkosyl-PK-ZyGEM showed large variability in total DNA yield and separation of epithelial DNA from the SF. The extraction with 0.1% Sarkosyl-ZyGEM showed less variation in separation than the 0.1% Sarkosyl-PK-ZyGEM and less variation in total epithelial DNA yield than both ZyGEM alone and 0.1% Sarkosyl-PK-ZyGEM. ZyGEM alone and ZyGEM followed by a wash step showed the least variability in separation

and greatest separation of epithelial DNA. Thus, with the exceptions of the addition of a wash step following the ZyGEM lysis, all other attempts to optimize the dual enzyme differential extraction were unsuccessful.

The addition of a wash step decreased the concentration of female DNA in the SF and improved the male:female ratio of DNA in the SF of mixture samples. Although the wash step showed significant improvement upon the amount of epithelial DNA carry-over in the SF in epithelial cell only experiments, it could not solve the problem of inefficient epithelial cell lysis and separation that was observed with mixtures. Ultimately, a method for effectively separating sperm and non-sperm cells for this simple direct lysis that releases the DNA in each fraction is needed. Regardless of its weaknesses, this procedure is far easier to perform and much faster than a traditional differential extraction and many other alternative methods.

#### 4.1 Future Directions

The primary future direction for this technique is to develop a better way to physically separate the epithelial DNA from the sperm cells. The potential for use of magnetic bead based kits like PrepFiler™ to pull the epithelial DNA away from the sperm pellet may be a strong choice for this. However, PrepFiler™ would not be ideal for this as it fills the SF with Isopropanol from the DNA binding step.<sup>33</sup> Lounsbury et. al. described the benefits of using an enzyme-based extraction on a

microfluidic device in comparison to the combination of a solid phase extraction based technique with a microfluidic device.<sup>26</sup> This would be a strong candidate because it would eliminate centrifugation and maintain a closed extraction environment even during the separation of the SF and NSF.

Although solving the limitation of physical separation in this procedure would aid in decreasing the amount of sperm DNA recovered in the SF, it is not the only cause of poor epithelial and sperm DNA separation. Samples containing more than 300 cells (2 ng of DNA) have decreased ZyGEM lysis and separation. When analyzing forensic evidence samples, there is no control over how many cells are obtained from a sample. Furthermore, existing differential extraction procedures are capable of extracting larger cell amounts.<sup>3</sup> Investigation into how modifications made to this procedure on epithelial cells only would affect separation of epithelial DNA from sperm in mixture samples should be explored. Although they showed no improvement on the simulated differential extraction with epithelial cells only, they could help with the problem that was observed with the sperm and epithelial cell mixture experiments.

The majority of these experiments were performed on epithelial cell solutions made from saliva and mixtures with liquid semen. All sexual assault evidence consists of dried stains either on a substrate or a swab.

The evidence in which most sperm and epithelial cell mixtures are present are post-coital swabs. Some work has been done to examine the use of the Trypsin Extraction on swabs containing sperm.<sup>37</sup> Further studies need to be done to examine how this ZyGEM-Trypsin differential extraction performs on post-coital or simulated post-coital swabs.

Another area of interest for future research, is determining the range of mixture ratios on which this procedure works. The range of mixture ratios explored during the epithelial cell dilution series was limited. Male:female mixture ratios can be as low as or even lower than 1:100, especially if the victim has waited many hours to report the assault and have a vaginal swab collected.<sup>11</sup> A comparison of performance from high to low male:female ratios should be made for both liquid and swab extractions so that any loss of sperm due to elution from the swab could be characterized. This range would help to further determine the limit of separation of epithelial DNA and sperm for this procedure.

## Appendix A: Additional Data Tables

**Table 17:** Difference in concentration (NSF-SF) for all extractions

Extraction	Concentration of DNA in SF (ng/μl)	Concentration of DNA in NSF (ng/μl)	Difference (NSF-SF)	Mean Difference (NSF-SF)	SD	95% Confidence Interval
Z-PC	0.042	0.09	0.05	<b>-0.30</b>	<b>0.50</b>	<b>-0.3,1.3</b>
Z-PC	0.109	0.17	0.06			
Z-PC	1.748	1.45	-0.30			
Z-PC	1.671	0.67	-1.00			
PK-Z	0.05	0.28	0.23	<b>0.20</b>	<b>0.15</b>	<b>0.03,0.28</b>
PK-Z	0.06	0.26	0.20			
PK-Z	0.01	0	-0.01			
PK-Z	0.02	0.12	0.10			
PK-Z	0.51	0.78	0.27			
PK-Z	0.58	0.71	0.13			
PK-Z	0.53	0.68	0.15			
PK-Z	0.08	0.59	0.51			
S.1-PK-Z	0.03	0.11	0.08	<b>-0.07</b>	<b>0.50</b>	<b>0.09,0.91</b>
S.1-PK-Z	0.17	0.82	0.65			
S.1-PK-Z	0.02	0.12	0.10			
S.1-PK-Z	0.01	0.21	0.20			
S.1-PK-Z	1.17	0.24	-0.93			
S.1-PK-Z	0.92	0.25	-0.67			
S.1-PK-Z	0.32	0.23	-0.09			
S.1-PK-Z	0.12	0.25	0.13			
S.1-Z	0.06	0.17	0.11	<b>0.13</b>	<b>0.02</b>	<b>-0.01,0.05</b>
S.1-Z	0.01	0.13	0.12			
S.1-Z	0.07	0.2	0.13			
S.1-Z	0.06	0.21	0.15			
Z	0.02	0.37	0.35	<b>0.00</b>	<b>0.87</b>	<b>0.41,1.33</b>
Z	0.05	0.86	0.81			
Z	0.22	0	-0.22			
Z	0.01	0.27	0.26			
Z	0.68	0.7	0.02			
Z	0.07	0.63	0.56			
Z	0.15	0.75	0.60			
Z	0.11	0.72	0.61			
Z	0.02	0.04	0.02			
Z	0.02	0.13	0.11			
Z	0.06	0.15	0.09			
Z	0.05	0.12	0.07			
Z	0.10	0.11	0.01			
Z	0.28	0.14	-0.14			
Z	0.10	0.07	-0.03			
Z	3.53	0.46	-3.07			

**Table 18:** Results of the PrepFiler™ Experiments. The variation in amount of epithelial DNA found in the PrepFiler™ clean-up step highlights the variation in epithelial cell lysis efficiency that is seen in the ZyGEM extraction.

Extraction	Fraction	Number of E-cells Added	Concentration (ng/μL)	DNA (ng)	Average DNA (ng)
ZyGEM-Prepfiler Clean-up	NSF	1224	0.09	8.12	<b>53.585</b>
ZyGEM-Prepfiler Clean-up	NSF	1224	0.17	14.85	
ZyGEM-Prepfiler Clean-up	NSF	1224	1.45	130.87	
ZyGEM-Prepfiler Clean-up	NSF	1224	0.67	60.5	
ZyGEM-Prepfiler Clean-up	SF2	1224	0.01	0.42	<b>8.925</b>
ZyGEM-Prepfiler Clean-up	SF2	1224	0.02	1.09	
ZyGEM-Prepfiler Clean-up	SF2	1224	0.35	17.48	
ZyGEM-Prepfiler Clean-up	SF2	1224	0.33	16.71	
ZyGEM-Prepfiler	NSF	1224	0.13	6.5	<b>28.025</b>
ZyGEM-Prepfiler	NSF	1224	1.24	62.15	
ZyGEM-Prepfiler	NSF	1224	0.59	29.58	
ZyGEM-Prepfiler	NSF	1224	0.28	13.87	

**Table 19:** Comparison of ZyGEM to ZyGEM with wash on separation of epithelial cells from SF

<b>Extraction, Fraction</b>	<b>Date</b>	<b>Concentration Total Human DNA (ng/ul)</b>	<b>Concentration Male DNA (ng/ul)</b>	<b>Concentration Female DNA (ng/ul)</b>	<b>Male DNA (ng)</b>	<b>Female DNA (ng)</b>
ZyGEM, SF	6/15/2016	1.04	0.30	0.74	15.11	36.92
ZyGEM, NSF	6/15/2016	0.02	0.01	0.01	0.66	1.05
ZyGEM, SF	6/15/2016	1.13	0.31	0.82	15.65	40.84
ZyGEM, NSF	6/15/2016	0.01	0.01	0.00	0.66	0.35
ZyGEM, SF	6/15/2016	1.03	0.31	0.72	15.72	35.91
ZyGEM, NSF	6/15/2016	0.03	0.01	0.02	0.79	1.48
ZyGEM, SF	6/15/2016	1.05	0.27	0.78	13.38	39.16
ZyGEM, NSF	6/15/2016	0.02	0.00	0.02	0.13	1.39
ZyGEM-Wash, SF	6/15/2016	1.01	0.28	0.72	14.22	36.20
ZyGEM-Wash, NSF	6/15/2016	0.02	0.01	0.01	0.56	1.14
ZyGEM-Wash, SF	6/15/2016	0.96	0.28	0.68	14.00	33.92
ZyGEM-Wash, NSF	6/15/2016	0.02	0.00	0.01	0.45	1.20
ZyGEM-Wash, SF	6/15/2016	1.02	0.30	0.73	14.80	36.41
ZyGEM-Wash, NSF	6/15/2016	0.02	0.00	0.02	0.29	1.59
ZyGEM-Wash, SF	6/15/2016	0.60	0.21	0.39	10.35	19.58
ZyGEM-Wash, NSF	6/15/2016	0.02	0.00	0.02	0.20	1.66
ZyGEM-Wash, SF	6/10/2016	1.18	0.48	0.70	34.99	24.05
ZyGEM-Wash, NSF	6/10/2016	0.06	0.01	0.05	4.37	0.67
ZyGEM, SF	6/10/2016	1.13	0.49	0.64	32.13	24.55
ZyGEM, NSF	6/10/2016	0.05	0.01	0.04	3.54	0.71
ZyGEM, SF	6/10/2016	2.04	0.70	1.35	67.31	34.83
ZyGEM, NSF	6/10/2016	0.07	0.01	0.05	4.94	1.18
ZyGEM, SF	6/10/2016	2.40	0.70	1.69	84.54	35.22
ZyGEM, NSF	6/10/2016	0.07	0.00	0.07	6.32	0.28
ZyGEM, SF	6/10/2016	0.90	0.37	0.53	26.47	18.38
ZyGEM, NSF	6/10/2016	0.06	0.01	0.05	4.70	0.65
ZyGEM-Wash, SF	6/10/2016	1.09	0.48	0.61	30.59	24.04
ZyGEM-Wash, NSF	6/10/2016	0.05	0.00	0.04	3.97	0.24
ZyGEM-Wash, SF	6/10/2016	0.91	0.33	0.58	28.88	16.69
ZyGEM-Wash, NSF	6/10/2016	0.08	0.01	0.07	6.26	0.75
ZyGEM-Wash, SF	6/10/2016	1.02	0.49	0.53	26.26	24.54
ZyGEM-Wash, NSF	6/10/2016	0.07	0.02	0.05	4.07	2.00

## **LIST OF JOURNAL ABBREVIATIONS**

Anal Bioanal Chem	Analytical and Bioanalytical Chemistry
Anal Chem	Analytical Chemistry
Forensic Sci Int Genet	Forensic Science International Genetics
Investig Genet	Investigative Genetics
J Biol Chem	Journal of Biological Chemistry
J Forensic Identific	Journal of Forensic Identification
J Forensic Sci	Journal of Forensic Sciences
J Teor Biochem	Journal of Theoretical Biochemistry
Sci Justice	Science and Justice

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## **CURRICULUM VITAE**

Rena B. Montville

b.1990

9 Brookfield Circle, Framingham, MA, 01701

PH: 508-816-5071. E-mail: rbmontville@gmail.com

Education:

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Currently working towards a Masters in Biomedical Forensic Sciences at Boston University.

Courses Completed:

- Crime Scene Investigation
- Criminal Law and Ethics
- Criminal Law with Mock Trial
- Forensic Chemistry
- Trace Evidence Analysis
- Molecular Biology of Forensic DNA Analysis
- Forensic DNA Analysis Laboratory
- Instrumental Analysis Laboratory
- Digital Forensics and Investigations
- Anatomy and Osteology
- Forensic Biology Laboratory
- Forensic Biology
- Forensic Pathology and Medico-legal Death Investigation
- Elementary Biostatistics

Bachelor of Arts in Biochemistry and Molecular Biology, May 2012.  
Boston University.

Courses:

- General Chemistry with lab
- Organic Chemistry with lab
- Physical Biochemistry
- Biochemistry with lab
- Molecular Biology
- Genetics lab
- Microbiology with lab
- Systems Physiology with lab
- Human Anatomy with lab

Minor in Dance, May 2012 Boston University.

Honors:

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Member of the Delta Delta Epsilon Forensic Sciences Honor Society, September 2015-current.

Memberships:

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Boston University Forensic Sciences Society, September 2014-August 2016.

Employment:

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Teaching Assistant, Forensic Chemistry, Boston University, January-May 2016.

Teaching Assistant, Forensic Toxicology, Boston University, May-June 2015.

Teaching Assistant, Analysis of Controlled Substances, Boston University, September-December 2015.

Introductory Biology Lab Undergraduate Assistant, Boston University, July 2009.