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Analysis of M-Vac buffer flow-through for the presence of cell-free DNA in saliva

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ARAM V. CHOBANIAN & EDWARD AVEDISIAN SCHOOL OF MEDICINE

Thesis

**ANALYSIS OF M-VAC BUFFER FLOW-THROUGH FOR THE PRESENCE OF
CELL-FREE DNA IN SALIVA**

by

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ABSTRACT

Collection methods for biological forensic samples have improved over the years, including the development of the Microbial Vacuum, M-VacTM. The M-VacTM is used to collect potential biological samples that are not readily visible, particularly on large substrates or evidence items. The M-VacTM uses a wet vacuum filtration method that collects the cellular material on a filter membrane, while the buffer that flows through the filter is discarded. This study assesses whether genetic material is present in the filtrate after collection and tests different filter pore sizes and membrane materials for the retention of cell-free deoxyribonucleic acid, or cfDNA.

When pre-extracted DNA was filtered through 0.45 micron and 0.2 micron membranes, only approximately one quarter of the cfDNA was retained on the filter. In mixture samples that contained cellular material from whole saliva and cfDNA, the filters were able to retain more than 80% of the genetic material that was deposited onto the membrane. Similar DNA profiles and yields were obtained using both pore sizes. The cellulose nitrate filter membranes had a higher yield than the polyethersulfone filters and resulted in profiles with more alleles and genetic information detected on the filter.

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LIST OF ABBREVIATIONS

bp	Base Pair
cfDNA	Cell-Free DNA
CN	Cellulose Nitrate
DNA	Deoxyribonucleic acid
mL	Milliliter
M-Vac	Microbial Vacuum
ng	nanogram
nm	nanometer
PES	Polyethersulfone
qPCR	Quantitative Polymerase Chain Reaction
RFU	Relative Fluorescent Unit
SRS	Sterile Rinse Solution
μm	Micron
μL	Microliter

1. INTRODUCTION

1.1 Forensic Collection Methods

Since the development of reliable deoxyribonucleic acid (DNA) testing in 1984, DNA analysis has been one of the most prevalent developments in forensic science. DNA has been used in many criminal cases, including cold cases. It is a highly sought after type of evidence because it can individualize different people [1]. DNA is a cornerstone of forensic evidence as it is instrumental in linking people to different items of evidence, as well as to locations and to other people.

There are many ways to collect a sample in a forensic setting. Possible methods include cutting, swabbing, scraping, and tape lifting [2]. Typically, these methods are employed to collect visible stains found at a crime scene or on evidentiary items. However, if no stains consistent with blood, semen, or saliva are found, skin cells deposited by someone touching an item may be crucial to collect.

The genetic material deposited on an item that someone touched is typically referred to as “touch DNA” [3]. Each time someone touches an item or another person, some of their skin cells are deposited onto that surface. These skin cells can be collected and tested for DNA. However, these samples often contain low levels of DNA or no detectable DNA at all due to their transient nature. Touch DNA samples are more fragile than a blood sample, for example. Many factors such as weather, sunlight, water, or friction may cause the touch DNA sample to degrade or be transferred to another object [4].

Another factor that can affect the quantity of DNA obtained from a touch DNA sample is how many skin cells are shed by the donor [4]. There is significant variation in

how many skin cells are deposited from person to person [5]. Because of the variability in touch DNA samples, it is important to collect as much of the potential specimen as possible using the most effective collection method for the substrate.

One of the most common collection techniques for touch DNA is the double swab method [6]. This method is performed by using two swabs, one moistened with sterile water and one dry, and rubbing the swabs in the area of interest. Typically, only a small area of interest is swabbed in order to preserve the sample in case further testing needs to be performed [7]. Cotton swabs are a good collection tool for many substrate types but are best with a distinct area for collection rather than a large area [2].

Cuttings are only able to be used for certain types of substrates. The cutting method requires an analyst to excise a portion of the substrate from the item of evidence. Cuttings can be performed with a sterile pair of scissors or a sterile scalpel in order to remove the stain or area of interest [2]. Cuttings are typically used with fabric, or other substrates that are soft. A small cutting is able to be used directly in testing which avoids having to transfer the sample prior to testing and limits potential DNA loss.

Both taping and scraping collection methods are less popular collection methods than swabbing or cutting. Taping may inhibit downstream analysis processes due to the properties of the adhesive used [8]. Taping is better suited for large areas, such as a car trunk, when trying to avoid diluting a sample. Scraping is employed for dry stains by using a scalpel to scrape the surface of a substrate over a clean envelope or piece of paper [7]. Scraping an item to collect loosened cellular material relies on the analyst's ability to ensure that the whole sample is being caught by the collection paper or envelope beneath

the evidence item, which is sometimes difficult [2]. Taping and scraping can both be used for smooth or slightly rough surfaces, but they can be time consuming methods and not typically chosen for collection when stains are readily visible.

1.2 M-Vac™ Collection

Many of the existing collection methods are highly efficient for small, confined areas where the biological specimen is visible. For samples that are not visible to the naked eye and span a large area, an alternative collection method is the Microbial Vacuum (M-Vac Systems, Sandy, UT), or the M-Vac™. The M-Vac™ is a wet vacuum system used to rehydrate cells and collect them using a size filtration technique [9]. The device uses a nozzle that sprays a sterile surface rinse solution (SRS) onto the substrate and subsequently vacuums the SRS as it rehydrates the cells on the substrate. The solution with cells then passes through the extension tubing and into the filtration unit. The cellular material is persevered onto the filter membrane which is collected as evidence [9]. The solution that flows through the filter is collected in a waste bottle and discarded.

This method of vacuum collection can be used on various substrates. It is most applicable on large substrates or surfaces that are hard to swab. Some examples of substrates that have been processed using the M-Vac™ in forensic casework or research are different clothing items [13], cinder blocks [6], bedding [10], carpet [13], drywall [6], wet clothing [11], ropes [12], wood [6] and tile [13]. The vacuum method is effective for rough substrates because it is capable of vacuuming the surface material, including cellular

material, and getting the SRS into cracks and hard to swab places that are typically difficult to sample from.

When collecting a sample with the M-VacTM, it is recommended to run the flow through over the filter once to collect all DNA or cellular material in the sample [14]. However, the flow through is not tested to ensure that this has occurred [15]. There is also variability in the collection of cells as there are multiple different pore sizes and filter membranes that are used for the M-VacTM filtration system. The most common filter uses a polyethersulfone (PES) membrane with a 0.45 micron (μm) pore size. Another filter that is compatible with the M-VacTM uses a cellulose nitrate (CN) membrane with a 0.20 μm pore size.

1.3 Background of DNA

After collection of a DNA sample, beginning the analysis as soon as possible is crucial for the integrity of the sample [16]. DNA is an essential component for human life and is the fundamental molecule that carries all genetic information for the human body and the development of cells throughout the body. Almost every cell in the human body contains DNA and can be useful in forensic analysis.

The most common types of cells used in forensic analyses include epithelial cells, white blood cells, and semen cells. All of these cells can be trapped on the M-VacTM filter membrane. They range in size from 2 μm – 20 μm [17], which is substantially larger than the 0.2 μm or 0.45 μm filters that are used to trap the sample collected by the M-VacTM. For instance, each portion of the sperm cell - head, midpiece, and tail - all measure between

3-45 μm ; thus, they should be easily trapped on the 0.45 μm pore size membrane that is used [18].

While the DNA molecule itself stretches a great length, the packaging of the molecule allows it to take up a microscopic space. A DNA molecule that is not condensed into its packaging can be several meters in length, varying depending on the chromosome [19]. Due to the packaging of DNA around histones and into nucleosomes, the long strands of DNA are able to condense into micron size bundles. A histone is a set of complex proteins that play a role in gene expression, DNA packaging, and chromosome condensation [20]. There are four main components of the histone protein including H2A, H2B, H3, and H4 which are found in duplicate within each histone complex [21]. The complex of 8 histone proteins is able to bond with the DNA through hydrogen bonding, allowing approximate 147 base pairs (bp) to wrap around the protein complex. This creates the nucleosome complex. Several nucleosomes are packaged into a 30 nanometer (nm) chromatin fiber which can be stabilized by the H1 histone protein complex [22]. The chromatin fibers then connect to each other through protein-protein interactions called chromatin crosslinks, allowing the fibers to condense further [23]. As a result of the packaging and condensing of the DNA, it is then able to fit inside the nucleus and into the cell. The process of packaging is fluid and reversible for processes such as transcription and replication, allowing for different areas of the DNA to become exposed at different portions of the cell lifespan.

As the cell dies and degrades, these components holding the DNA together are then degraded as well. The bonds holding the DNA molecule to the histone proteins are

separated, subsequently releasing both molecules to be translocated into the extracellular space [24]. Histone release is highly coupled with DNA fragmentation as the DNA is no longer protected in the histone complex and is subjected to interactions with degradation proteins such as caspase-activated DNase and DNA fragmentation factors [24]. While DNA in the extracellular space is not harmful, histones play a role in different immune response pathways of various diseases and injuries [20].

1.4 Background of Cell-Free DNA

DNA has been a reliable source of evidence for many years. Since its incorporation into forensic casework, many of the scientific advancements in DNA analysis have been in regard to instrumentation, analysis kits, methodologies, or the number of loci utilized for analysis. In recent years, researchers have identified how to utilize another source of DNA besides that found in the nucleus and mitochondria: cell-free DNA.

Cell-free DNA, or cfDNA, are fragmented pieces of DNA that have been released from the cell and exist in the extracellular matrix. Cell-free DNA can be found naturally occurring in healthy individuals, as well as in higher concentrations in individuals who have certain diseases or something causing a heightened immune response [25]. Cell-free DNA is present in healthy individuals in concentrations of approximately 0.0139 ng/ μ L [26]. These concentrations of cfDNA will vary from person to person and within an individual depending on factors such as gender, age, physiology, and individual pathology [27]. In patients with a disease, such as cancer, the concentrations of cfDNA are much higher with an average of 0.0635 ng/ μ L due to an increased amount of cell death [26].

These concentrations of cfDNA have been able to be associated as early markers for disease and indicators that there is a heightened immune response occurring in the body [29]. Since there is a greater amount of cfDNA in these patients, that indicates higher amounts of histones being released, allowing the concentration of cfDNA to increase and subsequently play a negative role in the immune response pathway.

Since the discovery of cfDNA, researchers have been able to identify how the DNA is expelled from the cell. The most common reasons that DNA is found outside of the cell are due to cellular apoptosis, necrosis, and NETosis [27]. As the cell components are digested, the DNA is released from the cell and expelled into the extracellular matrix and repurposed into other extracellular structures or completely digested [29]. During its presence in the extracellular matrix, it can still be detected and quantified prior to digestion and being reincorporated into other extracellular components, making it valuable for forensic comparison.

An important consideration in the analysis of cfDNA is that the strands of DNA are highly fragmented. This is due to the digestion of cells and the components within the cells, including the DNA [30]. Due to the nature of DNA and the method in which it is packaged within a cell, the fragmented sizes of DNA are approximately 140-200 bp [31]. The DNA wrapping tightly around the nucleosome is represented by the cfDNA fragments that are commonly encountered during forensic analysis. Approximately 167 bp can surround the nucleosome and remain in the linking fragment, which corresponds to the most common fragment length of cfDNA [32]. Since the length of cfDNA is variable, the most common fragment lengths are in multiples of 167 bp depending on the location at which the

exonuclease cuts the DNA strand and how long the fragment has been in the extracellular matrix exposed to conditions that induce digestion. The length of the fragment can also indicate the mechanism by which the cfDNA was created [33]. For instance, apoptosis typically creates fragment lengths around 180 bp, whereas necrosis of cells typically results in fragment lengths much longer than 180 bp [33].

While cfDNA is typically found in very short fragments, literature suggests that healthier individuals have longer fragments of cfDNA [31]. Much of the literature regarding cfDNA is in relation to cancer and other diseases, and in those patients, the cfDNA fragments are much shorter due to the high immune response occurring leading to cell death. Although the fragments are short, they are still able to be analyzed and measured by forensic DNA instrumentation. Important to the quantitative PCR (qPCR) process, is the short autosomal target which detects DNA fragment ranges of 80-90 bp, while the long autosomal targets detect fragments ranging from 150-250 bp [34]. This is ideal for the cfDNA analysis because the most common fragment length falls around 167 bp.

DNA is found in practically every cell in the human body. There is DNA even in red blood cells until the cell is mature and the nucleus dies off [35]. Even with only mature red blood cells lacking DNA, cfDNA can be found in every body fluid. Epithelial cells are present in most body fluids, therefore there can be cfDNA from those skin cells. While skin cells are the most likely source of cfDNA, any cell will be able to produce cfDNA that is able to be detected. There have been amounts of cfDNA reported in bodily fluids such as semen, sweat, saliva, blood, and urine [36]. The most common bodily fluid for cfDNA identification and measurement is blood. Typically, the plasma portion of the blood is used

to measure the cfDNA concentrations in cancer patients. While cancer patients have been discovered to have high concentrations of cfDNA because of the increase in cell death, the plasma of healthy individuals can contain 10-30 ng/mL of cfDNA [37]. The use of blood can pose an issue for raw analysis methods as the serum of blood contains PCR inhibitory substances that could interfere with the polymerase or degrade the cfDNA [36]. Consequently, other bodily fluids may be used for analysis. Cell-free DNA in sweat samples may differ substantially between different individuals or even within a certain individual at different sampling times. Reportedly, 0 to 7 ng of cell-free DNA is recoverable from 150 mL of sweat [37]. This concentration of cfDNA is likely due to the cellular death of skin cells. However, the variability in the concentration of cfDNA in sweat does not make it an ideal source for testing. Along with sweat, it is possible to detect cfDNA in seminal and vaginal fluids [38]. In both seminal and vaginal fluids there may be some cfDNA due to the cell death of skin cells, but there have been cases where cfDNA is unable to be detected in vasectomized men, but it is able to be detected in fertile men [36]. This suggests that any concentration from skin cells may be minimal for males and the primary source of cfDNA in semen is from sperm cells. More reliable and consistent between healthy persons is the cfDNA concentration in saliva.

There is typically a higher concentration of cfDNA in saliva stains than in other types of stains [36]. The concentration of cfDNA in a saliva stain is approximately 6.404 ng/mL [39]. Due to the relatively consistent concentration of cfDNA between people in saliva, it is a suitable sample type for experimental testing. Along with epithelial cells

releasing cfDNA into saliva, there are also methods of passive diffusion from capillaries surrounding the mouth that add to the cfDNA concentration in saliva [40].

When using the M-VacTM for purposes of collection, the presence and amount of any DNA is typically unknown. Therefore, the preservation of any DNA present is crucial to the success of the sample analysis [41]. With the recent developments in cfDNA research and its intersection with forensics, it is important to consider the role that cfDNA plays when collecting samples with the M-VacTM.

If there are trace amounts of DNA or cfDNA in the M-VacTM flow through, they may be probative and provide information that is not present on the filter sample, including additional alleles [42]. It has been possible to generate both full and partial profiles from cfDNA fractions of a sample using forensic methodologies [42]. It has been reported that some filters used by the M-VacTM, such as the PES membrane filters, do not retain cfDNA on their surface [42]. Thus, it is crucial to determine if probative genetic information may be flowing through the membrane and discarded as waste. In this study, various conditions of DNA samples were analyzed to assess the conditions in which DNA is found in the flow through (i.e., waste buffer) of the M-VacTM filtration system.

2. MATERIALS AND METHODS

2.1 LIZ Flow Through Study

2.1.1 Sample Preparation and Collection

Based on the results from preliminary trials (data not shown), 1 μL of GeneScan™ 600 LIZ™ dye Size Standard v2.0 (Applied Biosystems, Foster City, CA) was combined with 500 μL of M-Vac™ SRS buffer (M-Vac™, Buffdale, UT). The mixture was then pipetted onto a 0.45 μm PES Nalgene™ Rapid-Flow™ Filtration Unit (Nalgene, Rochester, NY) that was connected to vacuum filtration through the M-Vac™ Extension tubing. Following deposition, the sample was allowed to dry on the membrane while connected to the vacuum for approximately 2 minutes.

A positive control sample was modeled using 1 μL of GeneScan™ 600 LIZ™ dye Size Standard v2.0 combined with 500 μL of the M-Vac™ SRS. This sample was pipetted onto a 0.45 μm PES filter membrane without connection to vacuum filtration. The sample was allowed to absorb into the filter for approximately 5 minutes.

Once the filter membranes were dry, a sterile scalpel was used to excise the portion of the filter that was saturated with the solution. The excised filters were cut into two strips and placed into separate 1.5 mL microcentrifuge tubes. The flow through solution in the collection bottle was pipetted out and transferred into a 1.5 mL microcentrifuge tube.

2.1.2 Sample Extraction

After the samples were collected, the filter membrane samples were extracted for DNA using Invitrogen™ Chargeswitch Forensic DNA Purification Kit (Invitrogen, Waltham, MA) using the manufacturer protocol for swabs [43].

2.1.3 Capillary Electrophoresis and Profile Analysis

Once extraction was complete, the samples were immediately run on the SeqStudio™ Genetic Analyzer System (Thermo Fischer Scientific, Waltham, MA). In each sample well, 2 µL of the sample were combined with 9.4 µL of Hi-Di™ Formamide (Applied Biosystems, Foster City, CA). The samples were run with a 10 second injection time. The data was downloaded, and profiles were generated on the Genemapper™ Software v2.0 (Thermo Fischer Scientific, Waltham, MA). The peaks of the LIZ Molecular Weight Standard were analyzed using an analytical threshold of 30 relative fluorescence units (RFUs). This experiment was conducted in duplicate.

2.2 High Molecular Weight DNA Study Using Extracted DNA

Human samples were collected from two donors, one male and one female. All aspects of this study were conducted in compliance with the ethical standards set by the Institutional Review Board of Boston University School of Medicine.

2.2.1 Sample Preparation and Collection

First, approximately 500 µL of human saliva was deposited into a 1.5 mL microcentrifuge tube. The saliva sample was then extracted using Invitrogen™ Chargeswitch Forensic DNA Purification Kit using a liquid sample protocol. The saliva sample extract of 150 µL was combined with 350 µL of M-Vac™ SRS buffer. The mixture was then pipetted onto a 0.45 µm PES Nalgene Rapid Flow Filtration Unit connected to

vacuum filtration through the M-VacTM Extension tubing. The sample was allowed dry on the membrane while connected to the vacuum for approximately 2 minutes.

A positive control sample was modeled using a saliva sample extract combined with 250 μL of the M-VacTM SRS. This sample was pipetted onto a 0.45 μm PES filter membrane without connection to vacuum filtration. The sample was allowed to absorb into the filter for approximately 5 minutes.

Once the filter membranes were dry, a sterile scalpel was used to excise the portion of the filter that was saturated with the solution. The excised filters were cut into strips and placed into separate 1.5 mL microcentrifuge tubes. The flow through solution in the collection bottle was pipetted out and into a clean 1.5 mL microcentrifuge tube.

A negative control sample was used by pipetting 200 μL of the M-VacTM SRS into a clean 1.5 mL microcentrifuge tube. This methodology was conducted in duplicate for a total of two trials.

2.2.2 Sample Extraction

After the samples were collected, in order to elute the DNA off of the filter, the filter membrane samples were extracted using InvitrogenTM Chargeswitch Forensic DNA Purification Kit using the protocol for swabs [44].

2.2.3 High Molecular Weight DNA Study – 0.2 μm Filters

The method described above was performed using a 0.2 μm PES Nalgene Rapid Flow Filtration Unit in place of the 0.45 μm PES filter described previously. A sterile swab was used to collect any flow through that was not able to be removed using a pipette. This method was conducted once.

2.3 Whole Cell Saliva Study – 0.45 μm PES Filters

2.3.1 Sample Preparation and Collection

First, approximately 500 μL of human female saliva was deposited into a 1.5 mL microcentrifuge tube. An aliquot of 100 μL of the saliva sample was combined with 400 μL of the M-VacTM SRS. The mixture was then pipetted onto a 0.45 μm PES Nalgene Rapid Flow Filtration Unit connected to vacuum filtration through the M-VacTM Extension tubing. The sample was allowed to dry on the membrane while connected to the vacuum for approximately 2 minutes.

A positive control sample was modeled using 100 μL of saliva combined with 400 μL of the M-VacTM SRS. This sample was pipetted onto a 0.45 μm PES filter membrane without connection to vacuum filtration. The sample was allowed to absorb into the filter for approximately 5 minutes.

Once the filter membranes were dry, a sterile scalpel was used to excise the portion of the filter that was saturated with the solution. The excised filters were cut into strips and placed into separate 1.5 mL microcentrifuge tubes. The flow through solution in the collection bottle was pipetted out and into a 1.5 mL microcentrifuge tube. For select samples, a sterile swab was used to collect the remaining flow through that was not collected using a pipette.

A negative control sample was used by pipetting 200 μL of the M-VacTM SRS into a 1.5 mL microcentrifuge tube. This method was conducted in duplicate for a total of two trials.

2.3.1.1 Alternative Method Preparation – Extracted and Whole Cell Sample

A sample of saliva was deposited into a 1.5 mL microcentrifuge tube. The sample was extracted using Invitrogen™ Chargeswitch Forensic DNA Purification Kit using the protocol for liquid samples. A female saliva sample was then collected in a microcentrifuge tube. Approximately 10 μ L of the male DNA extract sample was combined with 90 μ L of the whole cell female saliva. The combined saliva sample was then added to 400 μ L of the M-Vac™ SRS. The samples were then filtered as described above. A sterile swab was used to collect the remaining flow through that was not collected using a pipette. This process was conducted once with extracted female DNA and once with extracted male DNA.

2.3.2 Sample Extraction

After the samples were collected, the filter membrane samples were extracted using Invitrogen™ Chargeswitch Forensic DNA Purification Kit using the protocol for swabs.

2.4 Whole Cell Saliva and Extracted DNA Study – 0.2 μ m PES Filters

2.4.1 Sample Preparation and Collection

A sample of male saliva was deposited into a 1.5 mL microcentrifuge tube. The sample was extracted using the PrepFiler™ Forensic DNA Purification Kit (Applied Biosystems, Foster City, CA) using the protocol for liquid samples [44]. A female saliva sample was then collected in a microcentrifuge tube. Approximately 10 μ L of the male extract sample was combined with 90 μ L of the whole cell female saliva. The saliva sample was then combined with 400 μ L of the M-Vac™ SRS. The mixture was then pipetted onto a 0.2 μ m PES Nalgene Rapid Flow Filtration Unit (Nalgene, Rochester, NY) connected to

vacuum filtration through the M-VacTM Extension tubing. The sample was allowed to dry on the membrane while connected to the vacuum for approximately 2 minutes.

A positive control sample was modeled using 10 μL of extracted male saliva combined with 90 μL of whole cell female saliva and 400 μL of the M-VacTM SRS. This sample was pipetted onto a 0.2 μm PES filter membrane without connection to vacuum filtration. The sample was allowed to absorb into the filter for approximately 5 minutes.

Once the filter membranes were dry, a sterile scalpel was used to excise the portion of the filter that was saturated with the solution. The excised filters were cut into strips and placed into separate 1.5 mL microcentrifuge tubes. The flow through solution in the collection bottle was pipetted out and into a 1.5 mL microcentrifuge tube. A sterile swab was used to collect the remaining flow through that was not collected using a pipette.

A negative control sample was used by pipetting 200 μL of the M-VacTM SRS into a 1.5 mL microcentrifuge tube.

2.4.2 Sample Extraction

After the samples were collected, the filter membrane samples were extracted using PrepFilerTM Forensic DNA Purification Kit using the protocol for swabs [44]. This process was conducted in duplicate for a total of two trials.

2.5 Whole Cell Saliva and Extracted DNA Study – 0.45 μm CN Filters

2.5.1 Sample Preparation and Collection

A sample of male saliva was deposited into a 1.5 mL microcentrifuge tube. The sample was extracted using the PrepFilerTM Forensic DNA Purification Kit using the

protocol for liquid samples. A female saliva sample was then collected in a microcentrifuge tube. Approximately 46 μL of the male extract sample was combined with 154 μL of the whole cell female saliva. The saliva sample was then combined with 800 μL of the M-VacTM SRS. The mixture was then pipetted onto a 0.45 μm CN Nalgene Rapid Flow Filtration Unit connected to vacuum filtration through the M-VacTM Extension tubing. The sample was allowed to dry on the membrane connected to the vacuum for approximately 2 minutes.

A positive control sample was modeled using 10 μL of extracted male saliva combined with 90 μL of whole cell female saliva and 400 μL of the M-VacTM SRS. This sample was pipetted onto a 0.45 μm CN filter membrane without connection to vacuum filtration. The sample was allowed to absorb into the filter for approximately 5 minutes.

Once the filter membranes were dry, a sterile scalpel was used to excise the portion of the filter that was saturated with the solution. The excised filters were cut into strips and placed into separate 1.5 mL microcentrifuge tubes. The flow through solution in the collection bottle was pipetted out and into a 1.5 mL microcentrifuge tube. A sterile swab was used to collect the remaining flow through that was not collected using a pipette.

A negative control sample was used by pipetting 200 μL of the M-VacTM SRS into a 1.5 mL microcentrifuge tube.

2.5.2 Sample Extraction

After the samples were collected, the filter membrane samples were extracted using PrepFilerTM Forensic DNA Purification Kit using the protocol for swabs. This process was conducted in duplicate for a total of two trials.

2.6 Instrumental Analysis Methods

2.6.1 Sample Quantitation

The saliva samples were quantified in triplicate using the Quantifiler® Trio DNA Quantification Kit and the ABI Prism 7500, following the manufacturer's protocol [45]. Analysis was performed using the small autosomal target concentrations. A virtual standard curve was used to determine the concentration values.

2.6.2 Amplification and Capillary Electrophoresis

After quantitation, the saliva samples were amplified using the VeritiPro™ Thermal Cycler and the GlobalFiler™ PCR Amplification Kit using manufacturer's protocols for 29 cycles [46]. The samples were run on the SeqStudio™ Genetic Analyzer System using a 10 second injection time. In the case of low levels of DNA, i.e., samples that did not reach the target 0.75 ng of DNA, the maximum amount of DNA was amplified. The data was downloaded, and profiles were generated on the Genemapper™ Software v2.0, analyzed at an analytical threshold of 50 RFUs, and compared to the reference samples previously generated.

3. RESULTS AND DISCUSSION

3.1 Presence of LIZ on Filter versus Flowthrough

The goal of this experiment was to determine the bp sizes of DNA that flowed through the filter membrane with a known fragmented sample using the DNA LIZ size standard as a sample.

The profiles obtained by capillary electrophoresis were compared between the DNA dried onto the filter samples and flow through samples. A positive control filter sample, which did not undergo vacuuming, was analyzed to ensure that the LIZ was able to elute off the filter if present. After analysis with Genemapper software, all peaks (60-460 bp) were found in the flow through samples (Figure 1).

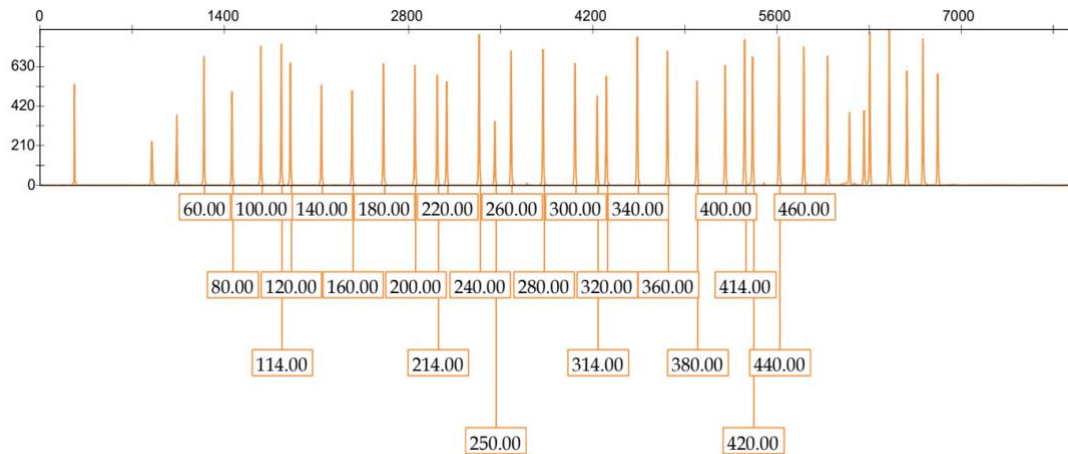


Figure 1. Electropherogram from LIZ Flow Through Sample. Orange Dye panel of electropherograms produced by LIZ and SRS vacuumed through a 0.45 μm PES Filter membrane and recovered from the flow through sample.

The positive control filter itself also yielded a profile consistent with the LIZ markers. All of the peaks on the positive filter were above 30 RFU, while the peaks resulting from the flow through sample were above 200 RFU.

In both replicates of the LIZ experiment, the filter sample did not result in the detection of any LIZ markers. Due to the lack of LIZ Molecular Size Standard, the electropherogram produced a profile that did not have any sizing data (Figure 2).



Figure 2. Electropherogram from LIZ Filter Sample. Orange Dye panel of electropherograms produced by LIZ and SRS vacuumed through a 0.45 μm PES Filter membrane and recovered from the filter sample.

3.2 Comparison of Flow Through and Filter Extracts

3.2.1 Analysis of 0.45 μm PES Filter Samples - High Molecular Weight DNA

The amount of extracted DNA put onto the filter was determined in nanograms (ng). The percent yield for each sample was calculated and compared.

In trial 1, 4095.36 ng of extracted DNA was deposited onto the filter membrane that was connected to the vacuum. Overall, about 38% of the total DNA deposited onto the

filter was recovered. Of the total recovery between the filter and flow through samples in trial 1, 85% of the recovered DNA was found in the flow through and 15% was present in the filter. There was 2721.6 ng of extracted DNA deposited onto the positive control filter. From the positive membrane filter, which did not undergo vacuuming, 49% of the deposited DNA was recovered from the membrane extraction. The filter membrane samples and flow through sample all resulted in full profiles.

In trial 2, there was 2525.76 ng of extracted DNA deposited onto the filter membrane that was connected to the vacuum. Overall, about 34% of the total DNA deposited onto the filter was recovered. Of the total recovery between the filter and flow through samples in trial 2, 88% of the recovered DNA was found in the flow through and 12% was present in the filter. There was 2609 ng of extracted DNA deposited onto the positive control filter. On the positive membrane filter, 15.78% of the deposited DNA was recovered from the DNA extraction of the membrane. The negative controls resulted in low quantities of DNA present. This was likely due to contamination of the buffer. A different package of buffer was utilized in further experiments and resulted in no quantity

of DNA present in the samples. The filter membrane samples and flow through sample all resulted in full profiles. The results from trials 1 and 2 are outlined in Table 1.

Table 1. Comparison of Average Quantities and Masses from High Molecular Weight DNA Experiment Samples of extracted female saliva deposited onto 0.45 μm PES filter membranes. Two trials of the experiment were conducted, designated by “Sample name-1” or “Sample name-2”. Average DNA quantity was measured after extraction and correlates to the total mass and percent yield.

Sample	Mass Input (ng)	Average Concentration Recovered (ng/ μL)	Total Mass Recovered (ng)	Mass Percent Yield (%)
Filter-1	4095.36	1.59	238.5	5.8
Flowthrough-1		8.71	1306.5	32
Positive Filter-1	2721.6	4.48	1344	49
Negative-1	SRS Only	0.00016	0.024	N/A
Filter-2	2525.76	0.66	99.72	3.94
Flowthrough-2		5.12	767.25	30
Positive Filter-2	2609	2.75	411.99	15.78
Negative-2	SRS Only	0.00019	0.029	N/A

3.2.2 Analysis of 0.2 μm PES Filter Samples - High Molecular Weight DNA

Results are outlined in Table 2. There was 293.57 ng of extracted DNA put onto the filter membrane that was connected to the vacuum. Overall, approximately 5.2% of the total DNA deposited onto the filter was recovered. Of the total recovery between the filter and flow through samples, 17.57% of the recovered DNA was found in the flow through and 82.43% was present in the filter. There was 190.51 ng of extracted DNA deposited onto the positive control filter. From the positive membrane filter, 0.58% of the deposited

DNA was recovered from the membrane extraction. The filter membrane samples and flow through samples all resulted in full profiles. Due to the low yield, no further replicates of this experiment were conducted.

Table 2. Comparison of Average Quantities and Masses from High Molecular Weight DNA Deposited onto a 0.2 μm Filter. Extracted female DNA was deposited onto a 0.2 μm PES filter. Average DNA quantity was measured after extraction and correlates to the total mass and percent yield.

Sample	Mass Input (ng)	Average Concentration Recovered (ng/ μL)	Total Mass Recovered (ng)	Mass Percent Yield (%)
Filter	293.57	0.26	12.98	4.42
Flow Through		0.017	0.85	0.29
Flow Through Swab		0.038	1.92	0.65
Positive Filter	190.51	0.022	1.11	0.58
Negative	SRS Only	0.00	0.00	N/A

3.2.3 Analysis of 0.45 μm PES Filter Samples - Whole Cell Saliva

In trial 1, there was 1689 ng of DNA put onto the filter membrane that was connected to the vacuum. Of the total recovery between the filter and flow through samples from trial 1, 0.1% of the recovered DNA was found in the flow through and 99.9% was present in the filter. Overall, approximately 42% of the total DNA deposited onto the filter was recovered. There was 1689 ng of DNA deposited onto the positive control filter. From the positive membrane filter, 55% of the deposited DNA was recovered from the membrane extraction. The filter membrane sample resulted in a full profile (Figure 3).

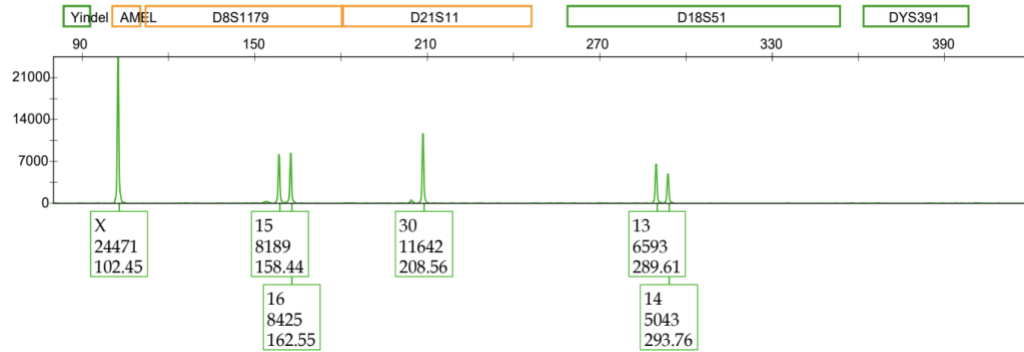


Figure 3. Electropherogram from Whole Cell 0.45 µm Filter Sample. Green Dye panel of electropherogram produced by whole cell saliva from a female sample and SRS vacuumed through a 0.45 µm PES Filter membrane and recovered from the trial 1 filter membrane sample.

The flow through sample from trial 1 resulted in a partial profile with 11 total alleles present in the profile (Figure 4). The flow through sample showed lower RFU values compared to the filter sample.

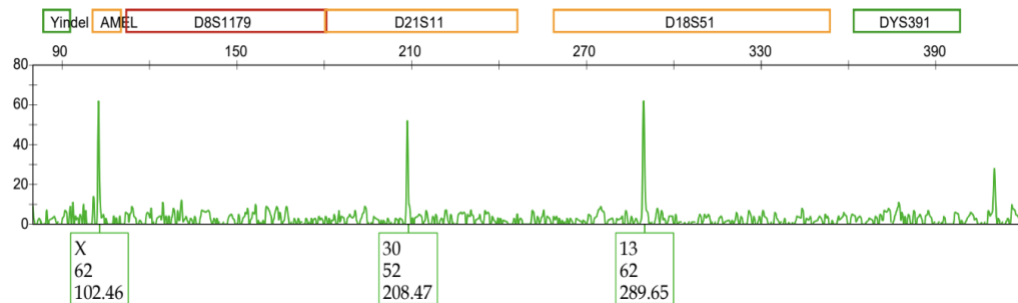


Figure 4. Electropherogram from Whole Cell 0.45 µm Flow Through Sample. Green Dye panel of electropherogram produced by whole cell saliva from a female sample and SRS vacuumed through a 0.45 µm PES Filter membrane and recovered from the flow through sample during trial 1.

In trial 2, there was 540 ng of extracted DNA put onto the filter membrane that was connected to the vacuum. Of the total recovery between the filter and flow through samples in trial 2, 0.1% of the recovered DNA was found in the flow through and 99.9% was present in the filter. Approximately, 41.023% of DNA that was initially deposited onto the filter was able to be recovered. There was 540 ng of extracted DNA deposited onto the positive control filter. From the positive membrane filter, 29% of the deposited DNA was recovered from the membrane extraction. The filter membrane sample resulted in a full profile (Figure 5).

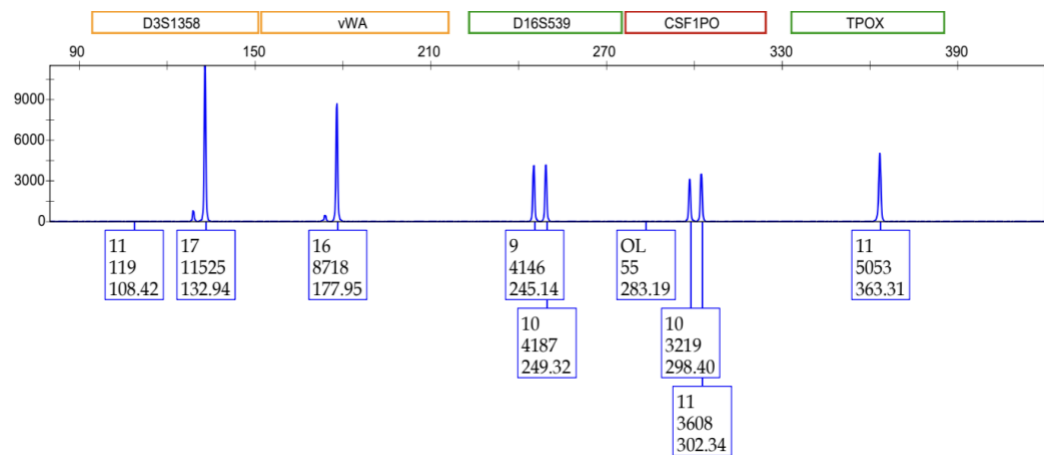


Figure 5. Electropherogram from Whole Cell 0.45 μ m Filter Sample - Trial 2. Blue Dye panel of electropherogram produced by whole cell saliva from a female sample and SRS vacuumed through a 0.45 μ m PES Filter membrane and recovered from the filter membrane.

The flow through sample from trial 2 resulted in a partial profile with 8 total alleles present in the profile (Figure 6). The swab used to collect the remainder of the flow through liquid resulted in a partial profile with 23 total alleles (Figure 7). Both the flow through

and flow through swab samples had lower RFU values, consistent with a low quantity DNA sample. The results from trials 1 and 2 are outlined in Table 3.

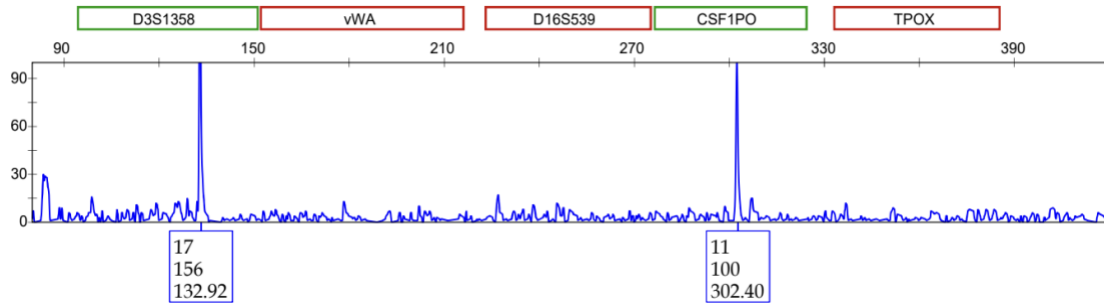


Figure 6. Electropherogram from Whole Cell 0.45 µm Flow Through Sample - Trial 2. Blue Dye panel of electropherogram produced by whole cell saliva from a female sample and SRS vacuumed through a 0.45 µm PES Filter membrane and recovered from the flow through.

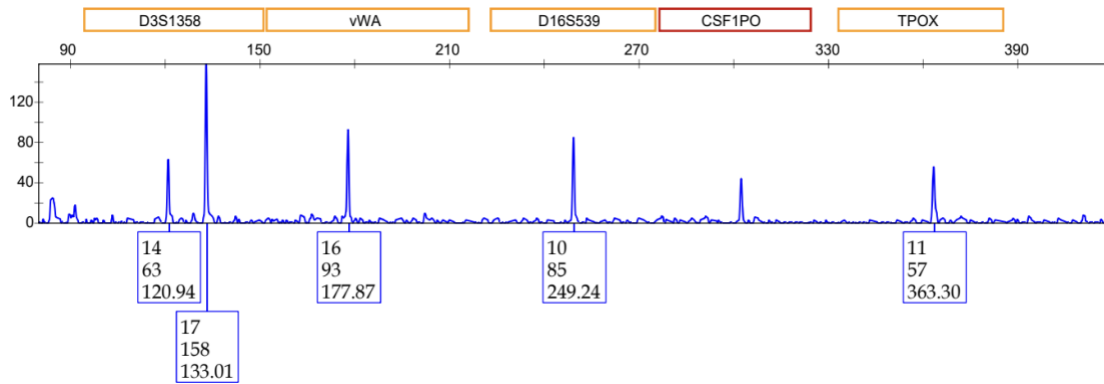


Figure 7. Electropherogram from Whole Cell 0.45 µm Flow Through Swab Sample - Trial 2. Blue Dye panel of electropherogram produced by whole cell saliva from a female sample and SRS vacuumed through a 0.45 µm PES Filter membrane and recovered from the flow through swab.

Table 3. Comparison of Average Quantities from 0.45 μm PES Filter Whole Cell Saliva Samples. Two trials were conducted, designated by “Sample name-1” and “Sample Name-2”, consisting of whole cell saliva samples. Average DNA quantity was measured after extraction and correlates to the total mass and percent yield.

Sample	Mass Input (ng)	Average Concentration Recovered (ng/ μL)	Total Mass Recovered (ng)	Mass Percent Yield (%)
Filter-1	1689	4.68	702	42
Flowthrough-1		0.00025	0.038	0.0022
Positive Filter-1	1689	6.20	930	55
Negative-1	SRS Only	0.00	0.00	N/A
Filter-2	540	1.49	223.5	41
Flow through-2		0.00025	0.038	0.007
Flow Through Swab-2		0.00055	0.082	0.015
Positive Filter-2	540	1.057	158.55	29
Negative-2	SRS Only	0.00	0.00	N/A

3.2.3.1 Extracted DNA Combined with Whole Cell Sample 0.45 μm PES Filter

For the samples with extracted female DNA and female whole cell saliva from two different donors, there was 708 ng of female DNA deposited onto the filter membrane that was connected to the vacuum. Of the total recovery between the filter and flow through samples, 1.44% of the recovered DNA was found in the flow through and 98.56% was present in the filter. Approximately 33.95% of the total DNA deposited onto the filter was recovered between the flow through and filter samples. The filter membrane sample resulted in a full profile. The flow through sample resulted in a profile with alleles present

at 23 loci. The swab used to collect the remainder of the flow through liquid resulted in a profile with alleles at 23 loci. There was 708 ng of DNA deposited onto the positive control filter. From the positive membrane filter, 34.1% of the deposited DNA was recovered from the membrane extraction. The positive membrane filter resulted in a full profile.

A similar experiment used samples consisting of extracted male DNA and female whole cell saliva, and 88.17 ng of DNA was deposited onto the filter membrane that was connected to the vacuum. Overall, about 38.24% of the total DNA deposited onto the filter was recovered. Of the total recovery between the filter and flow through samples, 1.93% of the recovered DNA was found in the flow through and 98.07% was present in the filter. The filter membrane sample resulted in a mixture profile from two contributors, with the female donor being the major contributor and male donor being the minor contributor (Figure 8). The positive control filter also had 88.17 ng of DNA deposited onto the filter. From the positive membrane filter, 18.68% of the deposited DNA was recovered from the membrane extraction.

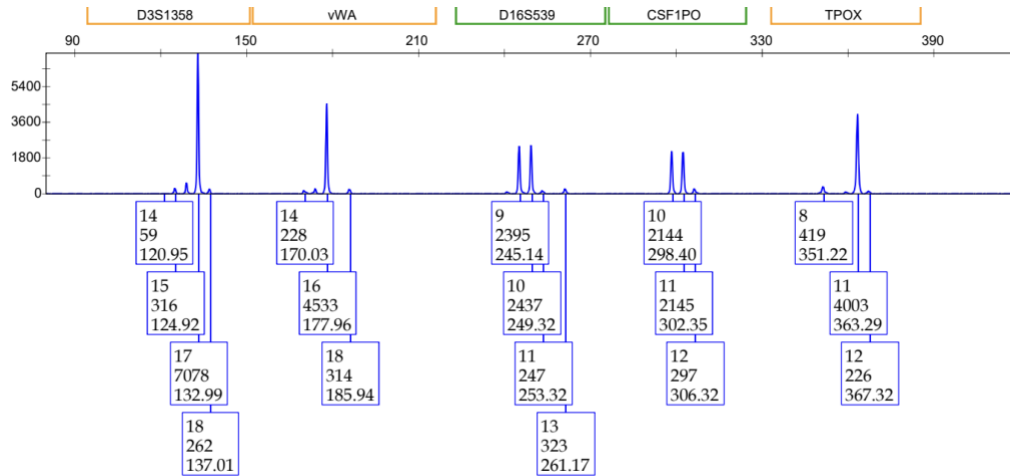


Figure 8. Electropherogram from Female Whole Cell and Male Extracted DNA 0.45 μm Filter. Blue Dye panel of electropherogram produced by whole cell saliva from a female sample, extracted DNA from a male donor, and SRS vacuumed through a 0.45 μm PES filter membrane and recovered from the filter membrane sample.

For the samples consisting of extracted male DNA with female whole cell saliva, the flow through sample resulted in a partial profile with 20 alleles present across 17 loci. The swab used to collect the remainder of the flow through liquid resulted in a mixture profile with alleles at 21 loci. The profile generated from the flow through swab indicated a low quality sample due to the ski slope pattern found within the profile, leading to drop out of alleles at the TPOX locus (Figure 9).

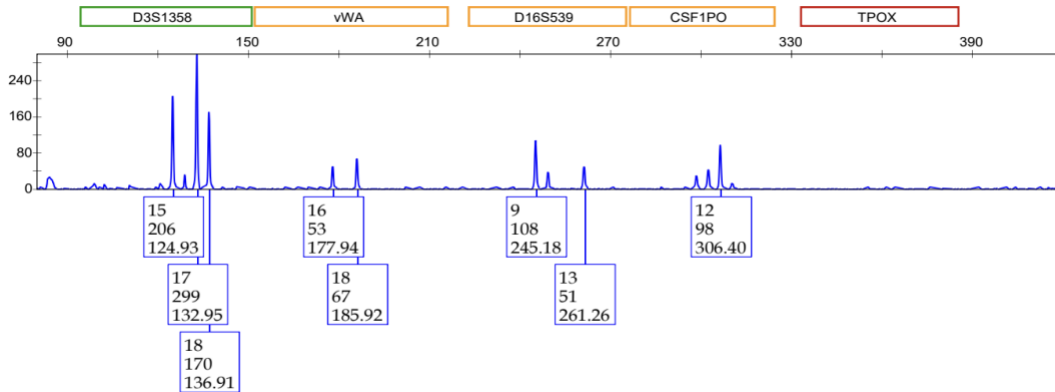


Figure 9. Electropherogram from Whole Cell 0.45 μ m Flow Through Swab Sample - Male Extracted DNA. Blue Dye panel of electropherogram produced by whole cell saliva from a female sample, extracted DNA from a male donor, and SRS vacuumed through a 0.45 μ m PES Filter membrane and recovered from the flow through swab sample.

The male DNA was used to simulate cell free DNA and was the extracted portion of the sample. The male to female ratios are indicative of extracted DNA from the male and female whole cell DNA being present in the sample. Male DNA was present in both the filter membrane sample and the flow through sample, indicating cfDNA is both retained on the filter and flows through the filter. The data from the whole cell saliva and extracted DNA samples are outlined in Table 4.

Table 4. Comparison of Average Quantities from 0.45 μm PES Filter Whole Cell Saliva and Extracted DNA Samples - The samples from two different filters are designated by “Sample name -F” for samples with female extracted DNA and female whole cell saliva and “Sample Name- M” for samples with male extracted DNA and female whole cell saliva. Average quantity was measured after extraction and correlates to the total mass and percent yield.

Sample	Mass Input (ng)	Average Concentration Recovered (ng/ μL)	Average Male Concentration Recovered (ng/ μL)	Total Mass Recovered (ng)	Mass Percent Yield (%)
Filter-F	708	1.58	N/A	236.99	33.4
Flow Through-F		0.023	N/A	3.48	0.49
Flow Through Swab-F		0.0031	N/A	0.46	0.065
Positive Filter-F	708	1.61	N/A	241.22	34.1
Negative-F	SRS Only	0.00	N/A	0.00	N/A
Filter-M	88.17	0.66	0.0024	33.1	37.54
Flow Through-M		0.0012	Und	0.59	0.68
Flow Through Swab-M		0.0011	0.0005	0.054	0.061
Positive Filter-M	88.17	0.33	0.017	16.47	18.68
Negative-M	SRS Only	0.00	N/A	0.00	N/A

3.2.4 Analysis of 0.2 µm PES Filter Samples - Whole Cell Saliva and Extracted DNA

In trial 1, 129.47 ng of DNA was deposited onto the filter membrane that was connected to the vacuum. Of the total recovery between the filter and flow through samples from trial 1, 6.58% of the recovered DNA was found in the flow through and 93.42% was present in the filter. Approximately 7.42% of the total DNA deposited onto the filter was able to be recovered. The filter membrane sample resulted in a mixture profile from two contributors, with the female donor being the major contributor and male donor being the minor contributor. The flow through sample resulted in a mixture profile with alleles present across all 24 loci. The swab used to collect the remainder of the flow through liquid resulted in a mixture profile with alleles at 16 loci. There was 129.47 ng of DNA deposited onto the positive control filter. From the positive membrane filter, 3.33% of the total deposited DNA was recovered from the membrane extraction.

The male DNA was used to simulate cell free DNA and represents the extracted portion of the sample. The male quantity is indicative of how much extracted male DNA was recovered in the sample. Male DNA was present in all of the samples of trial 1. Approximately half of the DNA recovered from the flow through samples was from a male donor, and approximately $\frac{1}{4}$ of the DNA recovered from the filter during trial 1 was male DNA.

In trial 2, 74.75 ng of DNA was deposited onto the positive control filter. From the positive membrane filter, 310% of the deposited DNA was recovered from the membrane extraction. The results being over 100% is likely because the original quantity of the sample

was not determined from a homogenous solution and therefore was not accurate of the sample as a whole. The positive control filter membrane resulted in a full profile.

There was 74.75 ng of DNA put onto the filter membrane that was connected to the vacuum. Together, the filter membrane and flow through samples recovered over 100% of the total theoretical DNA deposited onto the filter, again likely due to a non-homogenous sample resulting in a misrepresentation of what was deposited onto the filter. Of the total recovery between the filter and flow through samples in trial 2, 0.075% of the recovered DNA was found in the flow through and 99.9% was present in the filter. The filter membrane sample resulted in a mixture profile from two contributors, with the female donor being the major contributor and male donor being the minor contributor. The swab used to collect the remainder of the flow through liquid resulted in a mixture profile with alleles across all 24 loci. The flow through sample resulted in a mixture profile with alleles present across all 17 loci. The profile resulted in dropout at several loci, specifically D7S820 and SE33 (Figure 10).

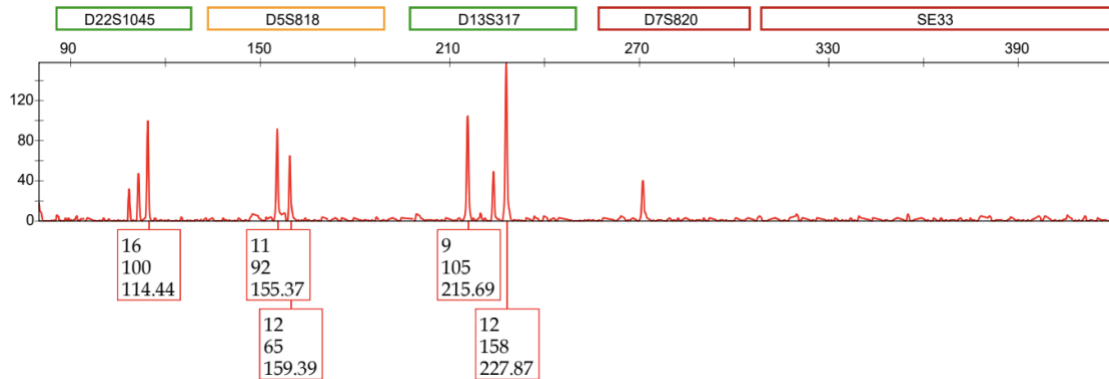


Figure 10. Electropherogram from Whole Cell 0.2 μ m Flow Through Sample - Trial 2. Red Dye panel of electropherogram produced by whole cell saliva from a female sample, extracted DNA from a male donor, and SRS vacuumed through a 0.2 μ m PES Filter membrane and recovered from the flow through sample.

The male DNA was used to simulate cell free DNA and was the extracted portion of the sample. The male quantity is indicative of extracted DNA from the male present in the sample. Male DNA was present in all of the samples from trial 2. Only male DNA was recovered from the flow through and flow through swab samples. The data from trials 1 and 2 are outlined in Table 5.

Table 5. Comparison of Average Quantities from 0.2 μm PES Filter Whole Cell Saliva and Extracted DNA Samples. Trials 1 and 2, designated by “Sample name -1” or “Sample Name-2”. Whole cell saliva samples (female) with added extracted DNA (male). Average quantity was measured after extraction and correlates to the total mass and percent yield.

Sample	Mass Input (ng)	Average Concentration Recovered (ng/ μL)	Average Male Concentration Recovered (ng/ μL)	Total Mass Recovered (ng)	Mass Percent Yield (%)
Filter-1	129.47	0.18	0.044	8.99	6.95
Flow Through-1		0.0021	0.0011	0.106	0.082
Flow Through Swab-1		0.011	0.00061	0.53	0.41
Positive Filter-1	129.47	0.086	0.029	4.32	3.33
Negative-1	SRS Only	0.00	N/A	0.00	N/A
Filter-2	74.75	19.84	0.79	991.94	1327
Flow Through-2		0.00082	0.001	0.041	0.055
Flow Through Swab-2		0.014	0.016	0.71	0.95
Positive Filter-2	74.75	4.64	0.29	231.94	310
Negative-2	SRS Only	0.00	N/A	0.00	N/A

3.2.5 Analysis of 0.45 µm CN Filter Samples - Whole Cell Saliva and Extracted DNA

In trial 1, there was 235.43 ng of DNA deposited onto the positive control filter. From the positive membrane filter, 2.24% of the deposited DNA was recovered from the membrane extraction. The positive control filter resulted in a full profile. For trial 1, there was 173.8891 ng of DNA put onto the filter membrane that was connected to the vacuum. Between the filter membrane and flow through samples, approximately 291.12% of the total DNA deposited onto the filter was recovered. The results being over 100% is likely because the original quantity of the sample was not determined from a homogenous solution and therefore was not accurate of the sample as a whole. Of the total recovery between the filter and flow through samples from trial 1, 0.042% of the recovered DNA was found in the flow through and 99.95% was present in the filter. The filter membrane sample resulted in a mixture profile from two contributors, with the female donor being the major contributor and male donor being the minor contributor. The swab used to collect the remainder of the flow through liquid resulted in a partial profile with 14 alleles across 13 loci. The flow through sample resulted in a profile with one allele present at the D2S441 locus (Figure 11).

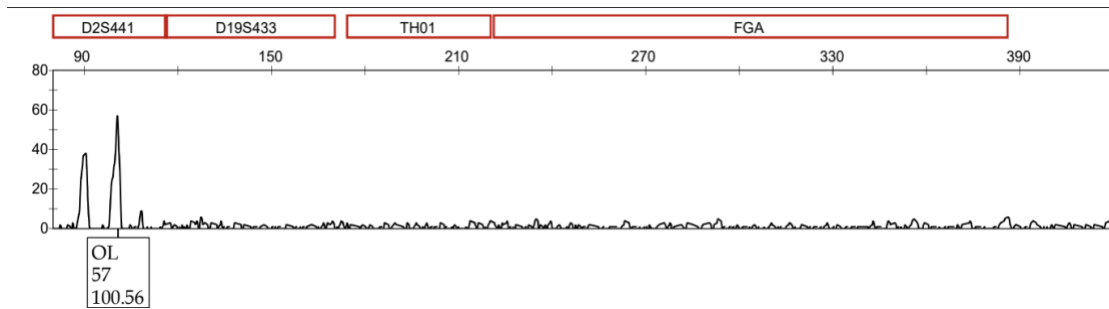


Figure 11. Electropherogram from Whole Cell 0.45 μm CN Flow Through Sample - Trial 1. Yellow Dye panel of electropherogram produced by whole cell saliva from a female sample, extracted DNA from a male donor, and SRS vacuumed through a 0.45 μm CN Filter membrane and recovered from the flow through sample. The off-ladder allele was confirmed to be a true allele miscalled by the software.

The extracted portion of the sample was from a male donor and was used to simulate cell-free DNA. The male quantity is indicative of how much extracted male DNA was in the sample. Male DNA was present in all of the samples. Approximately all of the DNA recovered from the flow through samples was from a male donor, and less than 10% of the DNA recovered from the filter was male DNA.

For trial 2, there was 258.4 ng of DNA deposited onto the positive control filter, of which 3.15% was recovered from the membrane extraction. There was a full profile generated by the positive filter membrane. In trial 2, there was 497.42 ng of DNA put onto the filter membrane that was connected to the vacuum. Approximately 7.25% of the total DNA deposited onto the filter was recovered between the filter and flow through samples. Of the total recovery between the filter and flow through samples from trial 2, 0.11% of the recovered DNA was found in the flow through and 99.9% was present in the filter. The filter membrane sample resulted in a mixture profile from two contributors, with the female donor being the major contributor and male donor being the minor contributor. The flow

through sample resulted in a partial profile with 4 alleles present across 4 loci. The swab used to collect the remainder of the flow through liquid resulted in a mixture profile with 7 alleles across 5 loci. Although there was male DNA detected on the flow through swab sample of trial 2, there was significant dropout at the sex-determining loci, Y-Indel, Amelogenin, and DYS391 (Figure 12).

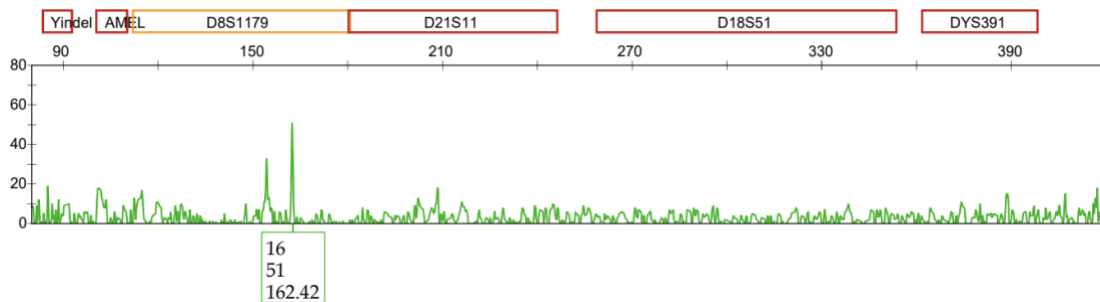


Figure 12. Electropherogram from Whole Cell 0.45 μm CN Flow Through Swab Sample - Trial 2. Green Dye panel of electropherogram produced by whole cell saliva from a female sample, extracted DNA from a male donor, and SRS vacuumed through a 0.45 μm CN Filter membrane and recovered from the flow through swab sample.

The extracted portion of the sample was from a male donor and was used to simulate cell-free DNA. The male quantity is indicative of how much extracted male DNA was in the sample. Male DNA was present in all of the samples of trial 2. Approximately all of the DNA recovered from the flow through samples was from a male donor, and less than 25% of the DNA recovered from the filter was male DNA. The results from trials 1 and 2 are outlined in Table 6.

Table 6. Comparison of Average Quantities from 0.45 μm CN Filter Whole Cell Saliva and Extracted DNA Samples. Trials 1 and 2, designated by “Sample name - 1” or “Sample Name-2”. Whole cell saliva samples (female) with added extracted DNA (male). Average quantity was measured after extraction and correlates to the total mass and percent yield.

Sample	Mass Input (ng)	Average Concentration Recovered (ng/ μL)	Average Male Concentration Recovered (ng/ μL)	Total Mass Recovered (ng)	Mass Percent Yield (%)
Filter-1	173.89	10.14	0.59	506.87	291
Flow Through-1		0.000098	Und	0.0049	0.0028
Flow Through Swab-1		0.00042	0.0011	0.21	0.12
Positive Filter-1	235.43	0.11	0.012	5.27	2.24
Negative-1	SRS Only	0.00	N/A	0.00	N/A
Filter-2	497.42	0.72	0.15	36.01	7.24
Flow Through-2		0.00011	Und	0.0055	0.0011
Flow Through Swab-2		0.00067	0.00074	0.034	0.0067
Positive Filter-2	258.4	0.16	0.0055	8.15	3.15
Negative-2	SRS Only	0.00	N/A	0.00	N/A

4. CONCLUSIONS

Contrary to expectations, the data did not consistently support the hypothesis that because cell-free DNA is smaller in size than the pores of the filter membrane it will always flow through the membrane and be present in the filtrate. When the sample exclusively contained extracted DNA consistent with the size of cfDNA (167 bp), the occurrence of DNA present in the flow through greatly increased [31]. The LIZ experiment, in which only DNA fragments of known size were added to a 0.45 μm PES filter, demonstrated that all DNA pieces between 60 bp and 460 bp flowed through the 0.45 μm membrane and were recovered in the filtrate. The most common size fragments of cfDNA falls within this range, indicating that it would be expected for cfDNA to flow through the membrane while intact cellular material is caught on the membrane [31]. When high molecular weight DNA samples alone were deposited onto a 0.45 μm PES filter, the extracted DNA was more likely to be found in the flow through when compared to samples that had both extracted DNA and whole cells from saliva. When high molecular weight DNA alone was deposited onto a filter membrane, 12% of the recovered DNA was found on the membrane, demonstrating that the membrane material may play a role in retaining some DNA even when the fragments are smaller than the pore size. When examining the sample that contained a mixture of extracted DNA and whole cells from saliva, the amount of DNA recovered from the flow through greatly decreased. In other words, when cfDNA is present in a mixture with intact epithelial cells, there was a greater likelihood that the cfDNA was

retained on the filter. This was not true for the 0.2 μm filters, however. In the case of the smaller pore size, the majority of the DNA was consistently found on the membrane.

Both the 0.45 μm and 0.2 μm filters retained more than 93% of the total DNA that was recovered between the filter and flow through when whole cell saliva was combined with extracted DNA. This indicates that the cellular material could be blocking the pores of the filter, therefore limiting the amount of cfDNA able to flow through. Consistently throughout the experiments consisting of mixtures of male extracted DNA and female whole cell saliva, the flow through was from the male donor. There was also male DNA found on the filters throughout the whole cell saliva experiments. The cfDNA partially flows through the filters, but the cellular material may help trap some of the cfDNA.

When comparing the PES and CN filter membranes, the CN filters appeared to be better at retaining all of the DNA on the membrane. The data suggested that only the extracted DNA from the male donor was able to flow through the membrane while the cells from saliva sample belonging to the female donor remained trapped on the filter. The DNA profiles developed from the CN filtrate ranged from 1 allele to 14 alleles, whereas the profiles from the PES flow through ranged from 8 alleles to 23 alleles. This indicates that the CN membrane may be more suitable when degraded or extracellular DNA is present as there is less loss to the filtrate.

Some of the variation in the yield between the 0.45 μm PES and 0.45 μm CN membranes may result from the different extraction kits used. Due to supply limitations, the CN filter and 0.2 μm PES filter experiments were extracted with the PrepFiler™ extraction kit. The PrepFiler kit resulted in an overall less yield than the 0.45 μm PES

experiments that used Chargeswitch Extraction kit. This may be due to the compatibility between the filter membrane and the solutions used in each kit, leading to different extraction efficiencies. Overall, the magnetic bead extraction method resulted in a generally low yield. This is likely due to the loss experienced by extracting DNA that is out of a cell and exposed to digestive enzymes.

It is especially important to consider the presence of cell-free DNA in the waste buffer because when using an M-VacTM in forensic casework the relative amounts of intact cells and degraded/cell-free DNA are not known. The data suggests that when only cfDNA is present in a sample, the flow through rate is greater, therefore, preserving the filtrate for analysis may be desirable in some instances. While some genetic material on a substrate may be extracellular during original deposition of the sample, other samples may become cell-free between deposition and collection as the sample degrades. Furthermore, the M-VacTM is typically used to collect trace DNA samples that usually have low quantities of DNA present. Identifying the presence of cfDNA is important in these circumstances as it could improve the quality of the DNA profiles that are developed.

4.1 Future Directions

There are several areas in which this research can be expanded. Crucial to the field of forensic science would be testing for the presence of cell-free DNA on simulated evidence items that may have potential PCR inhibitors. It would also be important to evaluate the persistence of cell-free DNA on an item of evidence at different time periods to determine the effects of degradation on cell-free DNA. The present study could also be

improved by including more sample replicates and trials. It would also be beneficial to expand upon the current study by utilizing different extraction methods in attempt to increase the DNA yield.

LIST OF JOURNAL ABBREVIATIONS

Cancer Res	Cancer Research
Exp Eye Res	Experimental Eye Research
Forensic Sci Int	Forensic Science International
Forensic Sci Int Genet	Forensic Science International Genetics
J Clin Oncol	Journal of Clinical Oncology
J Forensic Sci	Journal of Forensic Sciences
J Oral Pathol Med	Journal of Oral Pathology & Medicine
Neurooncol Adv	Neuro-Oncology Advances
Phys Biol	Physical Biology
Sci Rep	Scientific Reports

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