DETERMINING OPTIMAL SWAB TYPE AND ELUTION BUFFER TO OBTAIN WHOLE CELLS FOR FUTURE DECONVOLUTION OF COMPLEX CELL MIXTURES

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ABSTRACT

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An increase of DNA testing sensitivity has led to an elevated variety of sample testing, including complex DNA mixtures, making the interpretation of DNA profiling results more complex. Currently there is no proscribed method used in laboratories to separate complex DNA mixtures by their contributors, therefore a method is needed that could reduce complex mixtures into their component parts. In this study methods of obtaining whole, intact cells from desiccated forensic samples for later cytometric sorting and downstream DNA analysis were examined. This study observed the effects of three different elution buffers (AutoMACS®, Phosphate Buffered Saline, and water) on recovery of whole, intact cells from standard cotton swabs, and then the effect of three different swab types (cotton, flocked, and dissolvable), of different composition, on recovery of whole, intact cells. This was accomplished by washing the swab containing the sample with an elution buffer that maintains the integrity of the cell membrane, resulting in a solution of intact cells. The results of this study demonstrated that the combination of AutoMACS® buffer and flocked swabs provided the highest yield of intact cells post elution. In the future, a solution of whole cells could then be grouped into categories by their cell surface proteins and sorted through cytometric sorting techniques. Once the mixtures have been separated into their component parts by the cell's surface proteins, then DNA analysis could proceed as normal, potentially resulting in single-source samples.

This thesis is dedicated to my grandfather, Delford Becke, who unfortunately passed during the time in which I was completing this thesis. He inspired the love of science and learning every day, to everyone he met.

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LIST OF ABBREVIATIONS

RFLP	Restriction Fragment Length Polymorphism
PCR	Polymerase Chain Reaction
CE	Capillary Electrophoresis
STR	Short Tandem Repeat
PGS	Probabilistic Genotyping Software
FACS	Fluorescence Activated Cell Sorting
PBS	Phosphate Buffered Saline
BSA	Bovine Serum Albumin
EDTA	Ethylenediaminetetraacetic Acid
MACS	Magnetic Activated Cell Sorting
HSD	Honest Significance Difference
SEM	Standard Error of the Mean

CHAPTER I: BACKGROUND

1.1 Statement of the Problem

In the beginning of forensic DNA profiling and analysis, the Restriction Fragment Length Polymorphism (RFLP) technique required large quantities of high-quality DNA in order to obtain a profile. With current technologies, such as the Polymerase Chain Reaction (PCR) and capillary electrophoresis (CE), DNA profiling is much more sensitive and now targets the detection of Short Tandem Repeats (STRs) [1]. Because PCR is used to make millions of copies of the DNA regions of interest, PCR technology has made it possible to obtain an autosomal DNA profile from a very small starting amount of biological material. This increase in sensitivity has led to laboratories testing a variety of sample types where processing may not have been attempted in the past, making complex DNA mixtures more prevalent and, in turn, making the interpretation of DNA profiling results more complex. A mixture of DNA is difficult to analyze because each person has two alleles at a given locus, which may be the same (homozygous) or different (heterozygous), and when DNA is mixed there is no definitive way to ascertain zygosity or if alleles are overlapping, so there is not a conclusive way to determine the exact number of contributors there are in the sample. One current method used to analyze complex mixtures of DNA is Probabilistic Genotyping Software (PGS), a computer program that uses statistical and biological models to ascertain genotypes and calculate probabilities [2]. PGS uses mathematics to account for the rarity of alleles used for analysis in the human population, and from that produces a statistic to determine the likelihood that a specific DNA profile is included in a DNA mixture. Although PGS is an incredibly useful tool in analyzing DNA mixtures, the results are not always informative with very complex mixtures, those being from more than three contributors to the mixed DNA profile. Therefore, a method is needed that could help reduce complex mixtures into their component parts. One way of doing this would be to separate the

cells from the individual contributors *prior* to the DNA analysis process. Fluorescence Activated Cell Sorting (FACS) is a flow cytometric method that could be used to do this; it is routinely used to identify cells, measure characteristics of cells, and sort cells [3]. However, flow cytometry has typically been used for analysis of live cells, in applications including immunophenotyping, cell cycle analysis, and cell sorting [4]. Few studies exist to demonstrate the use of flow cytometry on dried or aged cells, as are commonly encountered with forensic samples. In this project, I examined methods of obtaining whole, intact cells from desiccated forensic samples for later cytometric sorting and downstream DNA analysis.

1.2 The Importance of Cell-Surface Biomarkers

DNA is located inside the nucleus of a cell, protected by a cell membrane. The primary function of extraction buffers that are used in traditional DNA analysis is to lyse the cell membrane, making the cellular DNA available for further analysis [5]. However, at the point of membrane lysis, all the DNA from all of the cells in the sample get mixed together in the same solution. Because DNA is biochemically the same molecule from person to person, once it has been released into solution, the ability to separate it by contributor is lost. Even though forensic DNA analysis focuses on the small portion of DNA that is unique to each individual, in mixtures of DNA, it becomes increasingly more difficult to determine which alleles in the DNA mixture belong to which contributor. Alternatively, separating each contributor's cells prior to DNA extraction, or cell membrane lysis, would avoid creating complex DNA mixtures all together. One way to do this would be to elute cells from the sample substrate using a mild buffer solution in order to maintain the integrity of the cell membrane, ideally resulting in a solution of intact cells. A solution of whole cells could then be sorted into groups or populations cytometrically.

Once all the cells have been separated by their respective contributor, DNA extraction could proceed as normal, potentially resulting in single-source samples for each contributor.

The cell must be intact for flow cytometry to separate the cells because FACS works by "tagging" the cell surface proteins on the membrane [6]. Cell sorting of biological material has been previously attempted using antibodies for several forensically relevant biomarkers. Biomarkers are cellular, biochemical, or molecular alterations that are observable or measurable inside or on the surface of cells or free-floating in other biological fluids [7]. Biomarkers that could be relevant in forensic use include Sperm Head Specific Antigen (SHA) for sperm cells [3], CD45 [3] for lymphocytes, which are a subset of white blood cells, and cytokeratin [3] for epithelial cells. Additionally, A/B/O blood group and secretor status, which have historically been used quite extensively in forensic biology, may be useful for separating mixtures of the same body fluid type by donor [8]. A/B/O blood group is determined by antigens that are attached to oligosaccharide chains protruding from the surface of red blood cells (RBC). Some people are "secretors," meaning that they express a form of A/B/O blood group antigens in bodily fluids other than blood [9]. These markers may be helpful in separating body fluids by contributor because they are known to be present on the surface of many forensically relevant cell types, such as white blood cells, sperm, and epithelial cells [3].

1.3 Forensically Relevant Sample Collection Substrates:

In order for cell sorting and eventual DNA analysis to be successful, it is critical to obtain the adequate recovery of whole cells from the collection substrate. Forensic biological samples are predominantly collected and stored on swabs, which is most commonly a standard cotton swab [10]. Cotton swabs are built by winding the fibers onto the shaft of the stick. This orientation of the cotton fibers has been shown to trap a significant portion of the cellular material within the

swab [11]. Additionally, research has also determined that different types of swabs may hold and release sample material differently [12]. For instance, another fairly common swab in the forensic community, which is often compared to cotton swabs, is the flocked swab. Flocked swabs are constructed by attaching the nylon fibers perpendicularly onto the surface through an electrostatic mechanism. This results in the fibers being more open, and these swabs, therefore, reportedly release more sample from the substrate than do the cotton swabs [12].

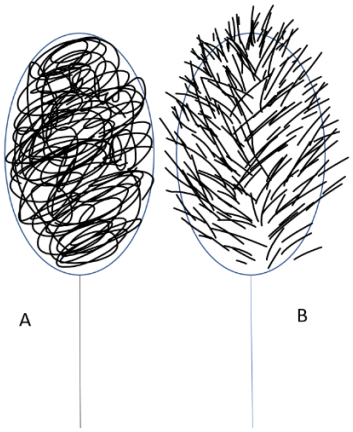

Figure 1.1: Demonstration of the difference between cotton swab fibers (A) and flocked swab fibers (B) [13].

An emerging type of forensic-use swab is the dissolvable swab. Theoretically, one should be able to recover the greatest amount of sample from a dissolvable swab, as the substrate fibers would no longer exist to retain any sample. Dissolvable swabs are insoluble in common liquids such as water and ethanol but are soluble in some DNA extraction buffers [14]. Luna is a company that makes dissolvable swabs from cellulose acetate, a durable fiber that is soluble in solutions of high chaotropic salt solutions [14]. Chaotropic agents work by disrupting the hydrogen bonds of water molecules and reduce the stability of the subject protein by weakening the hydrophobicity [15]. Chaotropic agents, in high concentrations, completely denature the natural state of proteins. These agents are typically found in solid-phase extraction buffers to lyse cells and obtain

maximum DNA recovery [14]. Unfortunately, use of chaotropic salts would pose a problem for obtaining whole-cell eluents from forensic-type samples. However, Luna dissolvable swabs may also be adequate for obtaining high cellular yields without actually dissolving the cellulose acetate fibers [16]. The Luna swabs are constructed with cellulose fibers that are 0.2 micrometers in diameter, whereas a standard cotton swab contains fibers that are 20 micrometers in diameter [16]. The smaller diameter of the fibers used in the dissolvable swabs may provide a greater surface area for sample collection and may also prevent the specimen from getting tangled within the swab fibers upon later elution [16].

1.4 Whole-Cell Elution Methods:

Biological material analysis for forensic application is usually performed on dried and/or aged samples, that later get rehydrated during the elution process. A variety of buffers may be used to rehydrate and release a desiccated sample from its substrate. Many buffers that are used for DNA extraction contain "harsh" chemicals such as detergents, enzymes, strong reducing agents, and/or chaotropic salts that lyse the outer membrane of the cell and denature proteins. As the goal in this research will be to obtain whole-cell eluents for downstream separation, finding a buffer and elution method that will not lyse the cell is of paramount importance.

Water has been shown to be effective at releasing dried biological material from substrates [18]. Phosphate Buffered Saline (PBS) is a water and salt solution containing disodium hydrogen phosphate and sodium chloride [17]. The phosphate is used to maintain constant pH that mimics the human body. Although water is an inexpensive and prevalent solute, PBS may be preferred over water because it should prevent cells from rupturing or drying out due to osmosis, i.e. the passive movement of water into and out of a cell membrane [17]. However, a search of the

scientific literature revealed no studies that demonstrate the effectiveness of water and/or PBS as elution buffers that yield intact cells from dried biological sample.

AutoMACS® running buffer contains bovine serum albumin (BSA), Ethylenediaminetetraacetic acid (EDTA), and 0.009% azide [19]. The AutoMACS® buffer is manufactured for use in Magnetic Activated Cell Sorting (MACS), a method used to separate cells based on their surface antigens. This buffer has been used for eluting whole-cells from desiccated swabs in the DEPArray ™ method [3], which is a microchip-based digital sorter used for detection and recovery of pure homologous cell pools [20]. BSA is a protein isolated from cows that is commonly used for its stability and lack of obstruction to biological reactions [21]. BSA is a single polypeptide chain consisting of over 500 amino acids and no carbohydrates and is commonly used in molecular biology reactions to stabilize proteins and enzymatic reactions [22]. The second component to the AutoMACS® buffer is Ethylenediaminetetraacetic Acid (EDTA), which is a chelating agent that works to bind to free magnesium (Mg^{2+}). The purpose of EDTA is to prevent nucleases that may cleave DNA from binding to the magnesium, which they use as a cofactor for activation [23]. By adding EDTA to the buffer solution, it is protecting the DNA inside the nucleus from the nucleases. The final component of the AutoMACS® buffer is 0.009% azide. Azides are a class of chemical compounds that contain any three nitrogen atoms as a group, and most of these compounds are unstable and sensitive to shock. Azide is used in the AutoMACS® buffer as a preservative [24].

1.5 Specific Aims

The purpose of this study was to determine the effects of the substrate collection material and elution buffer on the ability to obtain whole, intact cells from desiccated, dried biological samples.

Aim 1: Determine an optimal elution method to recover whole cells from desiccated biological samples on a standard cotton swab.

Aim 2: Determine the optimal substrate from three forensically relevant swab types, for elution and recovery of whole cells from desiccated biological samples.

CHAPTER II: METHODS

2.1 Preliminary Studies

2.1.1 Sample Acquisition

Single source samples of human blood were obtained from Innovative Research (Novi, Michigan). Human blood was used for these studies because leucocytes, or white blood cells, are a typically encountered sample type in forensic DNA analysis, relatively abundant and easy to acquire, and fairly robust, but also more fragile and susceptible to cell lysis than sperm. Human blood is recognized as a biohazardous material because of the potential for the presence of blood-borne pathogens, and for this reason, only pre-screened blood was purchased and universal precautions for using human blood were practiced, such as wearing gloves and masks when handling the specimen. Even though human biological material was used, this research did not involve human subjects nor require Institutional Review Board (IRB) approval, as researchers were not involved in the sample collection, did not have access to the identifying information of the donors, or develop personally identifying or health-related data.

2.1.2 Cell Counting

The initial leucocyte concentration of the blood was determined microscopically using a Brightline ™ hemocytometer (Hauser Scientific, Horsham, Pennsylvania) which is a square, glass microscope slide with an etched metallic grid (**Figure 2A**) that is divided into sections for microscopic cell counting (**Figure 2B**). A 1:10 dilution of neat, human blood was prepared using 3% Acetic Acid with Methylene Blue (STEMCELL ™ Technologies, Cambridge, Massachusetts). Methylene Blue is a cationic stain, or positively charged blue dye, that is used for counting nucleated mammalian cells because it binds to negatively charged portions of the cell, such as DNA located in the cell's nucleus. This formulation with 3% acetic acid is used to

lyse erythrocytes, red blood cell, membranes so that only the leukocytes remain, and the nuclei will appear lightly stained and can be easily counted under the microscope [25]. The diluted blood solution was pipette mixed and added to each of the two chambers of the hemocytometer in 10 µL aliquots. The area of the hemocytometer's central square, which also appears as a 5x5 grid (**Figure 2B**), was observed with the aid of an Olympus BH-2 microscope (Olympus, Shinjuku, Tokyo, Japan) at 40X total magnification, and all white blood cells were counted. The central hemocytometer square has dimensions of 1.0 mm, which means the total volume of sample that is being examined at any given time is 1 x 10^{-4} mL or 0.1 mm^3 or 10^{-4} cm^3.

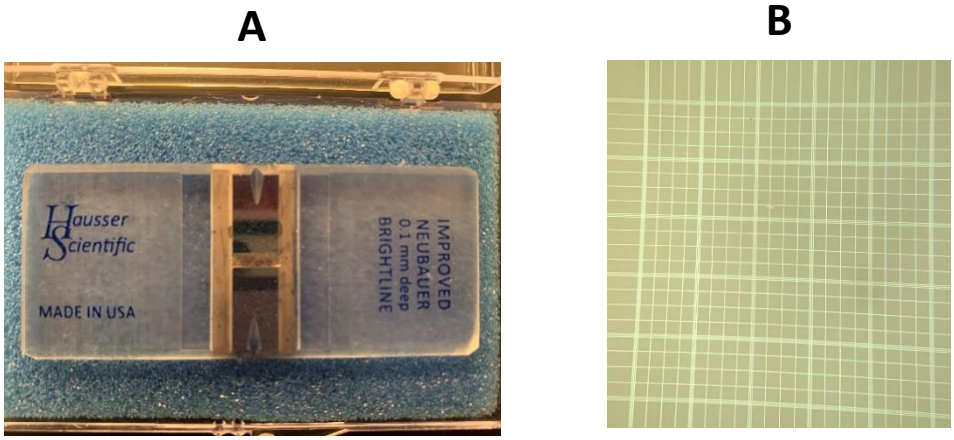

Figure 2.1: A) Hausser Scientific Brightline ™ hemocytometer, B) microscope image of the hemocytometer's etched metallic grid for cell counting viewed at 40X total magnification

Since 1 cm^3 is equivalent to 1 mL, the sample's cellular concentration (cells/mL) can be calculated from this information and the count of the cells observed, using **Equation 1.**

Eq. 1: Cells per mL = Cell Count x 10^4 x dilution factor

To arrive at the total number of cells in the blood sample, one would simply multiple the concentration of cells obtained from **Equation 1** by the sample's original volume, in this case 10

mL. As the optimal starting concentration of cells or blood volume to be spotted on the swabs for later elutions was unknown, aliquots of 25 µL (containing ~110,000 cells), 50 µL (containing ~220,000 cells), 75 µL (containing ~330,000 cells), and 100 µL (containing ~440,000 cells) of neat blood were spotted onto cotton swabs (SKU#:806-WC, Puritan, Guilford, Maine) and allowed to dry for approximately 24 hours. These dried blood samples on the cotton swabs were then used as "test swabs" to determine which starting sample volume was optimal for recovery and observation post-elution.

When the test swabs were eluted with AutoMACS® buffer (Miltenyl Biotec, Waltham, Massachusetts), few to no cells were observed from 25 µL (containing ~110,000 cells) and 50 µL (containing ~220,000 cells) of neat blood. Cell counts obtained from the 75 µL (containing ~330,000 cells) neat blood swabs were slightly higher on average, than the swabs containing 100 µL (containing ~440,000 cells) of neat blood. The 100 µL neat blood swabs appeared to retain blood following the elution and centrifugation process. This data suggested that spotting approximately 75 µL of neat blood, or approximately 330,000 cells, on swabs would be optimal.

In these preliminary studies, both Methylene Blue (STEMCELL ™ Technologies, British Columbia, Canada) and Trypan Blue (BioWhittaker®, Walkersville, Maryland) dyes were used to provide contrast during the microscopic cell counting. Trypan Blue is a membrane-impermeable dye that binds with intracellular proteins and DNA, meaning that it can aid in the identification of nucleated cells, only once the membrane is already permeabilized, either by chemical agents or during cell death [26]. Trypan Blue is often used as a live/dead stain, based on the principle that live cells have intact membranes that can exclude the dye from the cell, but dead cells do not. Dead cells, therefore, appear blue when examined microscopically. In comparing both Methylene Blue and Trypan Blue in these preliminary studies, Methylene Blue

was better to visualize cells from fresh/liquid blood samples, whereas Trypan Blue was better for the visualization of cells that were previously dried and eluted from swabs.

2.2 Study Sample Preparation

For preparation of samples to be used in both Aim 1 and Aim 2, a fresh (~1-2 days post draw) vial of single donor, human whole blood containing Anticoagulant Citrate Dextrose (ACD) was acquired from Innovative Research (Novi, Michigan). The cell counting procedure described above was followed. A 1:10 dilution of the blood sample was counted four times (**Table 1**). Using **Equation 1**, it was determined that there were approximately 4.1 x 10^6 white blood cells per mL. From the preliminary studies, it was determined that ~330,000 cells in the 75 µL volume was optimal for spotting on the swabs. Therefore, it was calculated that approximately 328,000 cells would in in 80 µL of neat blood, and this is the volume of blood spotted onto swabs used for both the Aim 1 and Aim 2 studies.

Table 2.1: Replicate counts of intact white blood cells in whole blood diluted 1:10 in 3% Acetic Acid with Methylene Blue. This average cell count was used to

Replicate	**Whole Cell Count**
1	54
2	38
3	33
4	39
Average	**41**

.

A power analysis was performed for a balanced one-way ANOVA using R statistical software (r-project.org, version 4.0.3), to determine the minimum number of samples needed per test group in this study. With a significance factor (α) of 0.05, a power (1-β) of 0.8, a large effect size of 0.8, and a total of 6 groups, a minimum of 4.36 samples would be needed for each group in Aim 1. For aim 2, with the same analysis parameters, but with a total of four groups instead of

six, the power analysis showed that a minimum of 5.34 samples per group was needed. To be conservative, in both aims, it was determined that analysis of eight samples for each group would be conducted. To prepare all the samples that would be needed to complete both Aim 1 and Aim 2, 80 µL of neat blood (containing ~328,000 cells) was spotted onto 32 cotton swabs (Puritan), 8 FLOQswabs® (Copan Diagnostics, Inc., Murrieta, California), and 8 dissolvable swabs (Luna Innovations Incorporated, Roanoke, Virginia). At the same time, positive controls were also prepared by adding 80 µL of neat liquid blood directly to 24 Sorenson ™ Dolphin microcentrifuge tubes (2 mL) (Sigma-Aldrich, St. Louis, Missouri), without the addition of a swab. All of the swabs were allowed to air-dry at room temperature for approximately 24 hours before being packaged and stored at approximately 4°C, and the positive control samples were stored at approximately 4°C until processing.

2.3 Processing of Positive Controls

Since the positive controls were liquid blood, and even with the ACD preservative whole cells are known to degrade quickly in liquid blood, the positive controls were processed first. The same elution procedure was followed for these positive control samples as was used for the swab samples, the only difference being the absence of the swab. There were a total of 24 positive controls prepared, eight for each of the three respective buffers being tested: AutoMACS® (Miltenyl Biotec), PBS buffer (Sigma-Aldrich, Milwaukee, Wisconsin), and UltraPure ™ distilled water (Invitrogen, Grand Island, New York). To begin, 1 mL of the respective buffer was added to each positive control tube. The tubes were allowed to incubate on a ThermoMixer F1.5 (Eppendorf AG, Germany) with constant agitation at a low speed (~500 rpm) for one hour at room temperature. Following agitation, samples were centrifuged at 200 xg for 10 minutes. The supernatant was then removed and discarded so that only the cell pellet and

approximately 50 µL remained in the nose of each tube. To wash the cell pellet, 500 µL of TE^{-4} buffer (TEKnova, Mansfield, Massachusetts) was added to each tube, then samples were again centrifuged at 200 xg for 10 minutes. Once again, the supernatant was removed and discarded, this time leaving only the cell pellet and as little liquid as possible in the nose of the tube. The cell pellet was then reconstituted to a total volume of 50 µL using Trypan Blue. The solution was mixed and added to the hemocytometer chambers in 10 µL aliquots. Each sample was counted five times, until the entire 50 µL was counted.

Throughout this study, a negative control was also processed, which consisted of cleaning the hemocytometer grid with alcohol in between samples. This cleaned hemocytometer grid as observed under the microscope to ensure the grid was clean and no cells were being left behind.

2.4 Processing of Aim 1 Samples

The first aim of the project was to compare the efficacy of the three different buffers – PBS, water, and AutoMACS®. Cotton swabs were used for this aim because cotton swabs are most commonly used in a forensic context. On eight separate days, three cotton swabs, that were previously spotted with 80 µL of human blood (containing ~328,000 cells), were eluted with each of the three buffers, one buffer per swab. The swab samples were processed in a similar manner as the positive controls. Briefly, the heads of the swabs were removed and placed in separate 2 mL Sorenson ™ dolphin-nosed microcentrifuge tubes; the respective test buffer was added to each tube; samples were agitated at room temperature. Following which, the swab heads were transferred to DNA IQ ™ Spin Baskets (Promega Corporation, Madison, Wisconsin), which were returned to their respective dolphin-nosed tubes and centrifuged for 15 minutes at 200 xg. The increase in the centrifugation time from the positive control was to ensure sufficient release of cellular material and drying of the swab. After the initial centrifugation

period, the spin baskets with swabs and all but ~50 µL of supernatant were removed and discarded. The cell pellets were washed in the manner described above, and then the cell pellet was reconstituted to a total volume of 50 µL with Trypan Blue. The entire volume of each sample was counted in 10 µL increments, for a total of five replicate counts per sample.

2.5 Processing of Aim 2 Samples

The second aim of the project was to compare the effect of swab composition on the recovery of whole cells. The swabs compared were Puritan ™ 6" standard cotton swab with wooden stick, Copan FLOQswab®, and Luna dissolvable swabs. The dissolvable swabs were kindly donated by Luna Innovations Incorporated for the purposes of this research. Eight of each swab type, for a total of twenty-four samples, were previously prepared by spotting each with 80 µL of neat human blood (containing ~328,000 cells), allowed to dry overnight, and then stored at ~4°C until processing. As Aim 1 demonstrated that the AutoMACS® buffer was most successful for obtaining whole cells from standard cotton swabs, this buffer was used in aim 2 for processing all of the Aim 2 samples.

On eight separate days, one of each of the three swab types was eluted with the AutoMACS® buffer following the same procedure described above for the Aim 1 samples.

CAHPTER III: RESULTS AND DISCUSSION

3.1 Positive Control Analysis

For this study, the quantitative data collected were numerical counts of intact leucocytes which were eluted from substrates of various composition with various liquid buffers. Positive control data was collected from liquid blood without the presence of a swab substrate, using only the three liquid buffers tested in Aim 1. The positive control data was compared with buffer data collected from Aim 1 and swab data collected from Aim 2 in order to determine what affect, if any, elution buffer and substrate composition had on whole-cell recovery. From the methods section, each of the eluted samples was resuspended in a total volume of 50 µL, which was counted five times, in 10 µL aliquots. The total cell count is the summation of those five replicate counts for each sample, and the average count is the total count divided by five. All numerical averages were rounded down to the nearest whole number since what was being measured, was whole, intact cells.

Positive control data was collected prior to testing swabs in Aim 1 and 2 because the controls were samples of neat, liquid blood, without a preservative. **Table 3.1** lists the total cell counts and average cell counts for each of the eight positive control samples eluted with AutoMACS® buffer. Similarly, **Table 3.2** lists the total and average counts for each of the eight positive control samples eluted with PBS, and **Table 3.3** lists the total and average counts for each of the positive control samples eluted with water.

Table 3.1: Total and average counts for liquid blood positive controls eluted with AutoMACS® buffer.

	Total Cell Count	Average Cell Count
AutoMACS_1	479	96
AutoMACS_2	604	121
AutoMACS_3	267	53
AutoMACS_4	286	27
AutoMACS_5	1468	294
AutoMACS_6	418	84
AutoMACS_7	317	63
AutoMACS_8	394	79

Table 3.2: Total and average cell counts for liquid blood positive controls eluted with PBS buffer.

	Total Cell Count	Average Cell Count
PBS_1	187	37
PBS_2	182	36
PBS_3	177	35
PBS_4	181	36
PBS_5	251	50
PBS_6	194	39
PBS_7	204	41
PBS_8	97	19

Table 3.3: Total and average counts for liquid blood positive controls eluted with Water.

	Total Cell Count	Average Cell Count
Water_1	0	0
Water_2	0	0
Water_3	0	0
Water_4	5	1
Water_5	1	0
Water_6	3	1
Water_7	26	5
Water_8	0	0

So as to not adversely affect sample variances, the total counts rather than the average counts were used for statistical analysis. A Grubb's test for outliers was performed on the positive control data, which revealed that the samples AutoMACS_5, PBS_5, PBS_8, and Water_7 were outliers within their respective groups. A one-way ANOVA, significance set to

0.05, was performed on the positive control dataset to determine if there was a significant difference between the groups eluted using the three different buffers. The p-value for the ANOVA was 0.000595, meaning that there was a statistically significant difference between the buffers used to elute the positive controls. Following a post-hoc analysis with a Tukey's Honest Significant Difference (HSD) test, it was determined that each of the buffers were significantly different in the total number of intact cells that they eluted from the positive liquid blood controls, with AutoMACS® being the best and water being the worst (**Figure 3.1**).

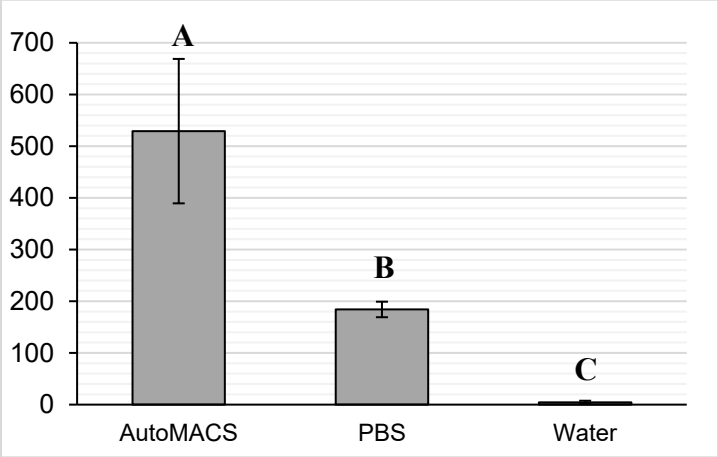

Figure 3.1: Total whole cell counts eluted from liquid blood samples with each of the three buffers. Results displayed represent the average of the eight trials +/- the Standard Error of the Mean (SEM). Letter designations represent Tukey's HSD comparisons: the same letter designation means results are not statistically different; when letter designations differ between groups, the p-value is less than 0.05.

3.2 Specific Aim 1 – Buffers

The data collected for the completion of Aim 1 was the counts of whole cells eluted from cotton swabs with each of the three test buffers (AutoMACS®, PBS, and water). From the methods section, each of the eluted samples was resuspended in a total volume of 50 µL, which was counted five times, in 10 µL aliquots. The total cell count is the summation of those five replicate counts for each sample. **Table 3.4** displays the data collected for the total whole cell

counts for each buffer type, as well as the average, standard deviation, and standard error of the mean (SEM).

Table 3.4 Total whole cell counts, overall averages, standard deviations, and standard error of the mean for cotton swabs eluted using one of the three test buffers.

	AutoMACS®	PBS	Water
Replicate 1	88	61	26
Replicate 2	58	21	19
Replicate 3	22	17	13
Replicate 4	56	41	21
Replicate 5	100	45	26
Replicate 6	122	32	43
Replicate 7	125	36	41
Replicate 8	68	54	23
Average	**79.875**	**38.375**	**26.500**
Standard Deviation	**35.498**	**15.175**	**10.447**
SEM	**12.551**	**5.365**	**3.694**

No outliers were observed in the elution buffer data using a Grubb's test on the total cell counts. A one-way ANOVA demonstrated that there was significant difference between the buffer elution groups ($p = 0.00037$). A Tukey's HSD demonstrated that the number of intact cells eluted from standard cotton swabs using PBS and water were not statistically different from each other, while significantly more intact cells were recovered using the AutoMACS® buffer (**Figure 3.2**). Because of this, the AutoMACS® buffer was used to complete Aim 2, in which the composition of different swabs were compared.

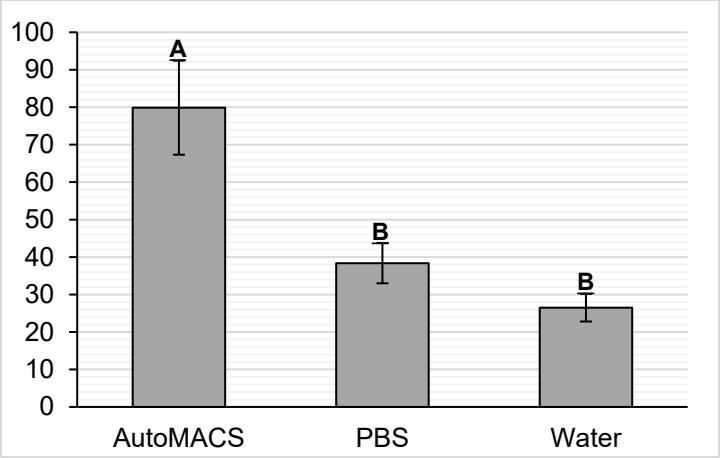

Figure 3.2: Total whole cell counts eluted from cotton swabs with each of the three buffers. Results displayed represent the average of the eight trials +/- the SEM. Letter designations represent Tukey's HSD comparisons: the same letter designation means results are not statistically different, when letter designations differ between groups, the p-value is less than 0.05.

In comparing the buffer data from the liquid blood positive controls and the cotton swabs, an interesting feature is present. With the positive controls, none to few cells were observed for each sample when eluted with water. However, after the introduction of a Puritan ™ 6" standard cotton swab and allowing the blood to dry, a higher quantity of cells (26.5 cells on average) were observed after elution with water. A possible explanation for this is cytolysis, or the cells rupturing due to an excess of water present. Osmosis is the passive movement of water, into and out of the cell, across the cell membrane. When a cell has too much water present inside the cell membrane, it can become hypotonic, and cause the cell membrane to burst. The cells in the liquid blood likely exist in an isotonic state, meaning that there is an equal concentration of water inside and outside the cell. When water was added to the liquid blood from the elution buffer, the cells likely became hypotonic, meaning there was an influx of water through the cell membrane. The cotton swab samples eluted with water were dried, meaning the cells desiccated, becoming

hypertonic. Consequently, when water was added as an elution buffer to the dried sample, the cells returned to their isotonic state and did not suffer rupturing.

3.3 Specific Aim 2 – Swabs

The data collected for the completion of Aim 2 was the counts of whole cells eluted from eight Puritan ™ 6" standard cotton swabs, eight Copan FLOQswab®, and eight Luna dissolvable swabs with AutoMACS® buffer. From the methods sections, each of the eluted samples was resuspended in a total volume of 50 µL, which was counted five times in 10 µL aliquots. The total cell count is the summation of those five replicate counts for each sample. **Table 3.5** displays the data for the total whole cell counts for each swab type, as well as the average, standard deviation, and standard error of the mean.

Table 3.5: Total whole-cell counts, overall averages, standard deviations, and standard error of the mean for cotton, flocked, and dissolvable swabs eluted using AutoMACS® buffer.

	Cotton Swab	Flocked Swab	Dissolvable Swab
Replicate 1	59	108	11
Replicate 2	28	133	5
Replicate 3	35	78	16
Replicate 4	70	139	12
Replicate 5	133	191	8
Replicate 6	88	198	9
Replicate 7	113	160	12
Replicate 8	58	296	18
Average	**73.000**	**162.875**	**11.375**
Standard Deviation	**36.497**	**67.011**	**4.207**
SEM	**12.903**	**23.692**	**1.487**

A Grubb's test for outliers revealed one potential outlier in the dataset, the 296 summation from the eighth flocked swab replicate. A one-way ANOVA demonstrated that there was significant difference between the swab groups ($p= 0.3933 \times 10^{-6}$). The Tukey HSD test demonstrated that all groups were statistically different from each other, with the most intact

cells being recovered from flocked swabs, followed by cotton swabs, then dissolvable swabs (**Figure 3.3**).

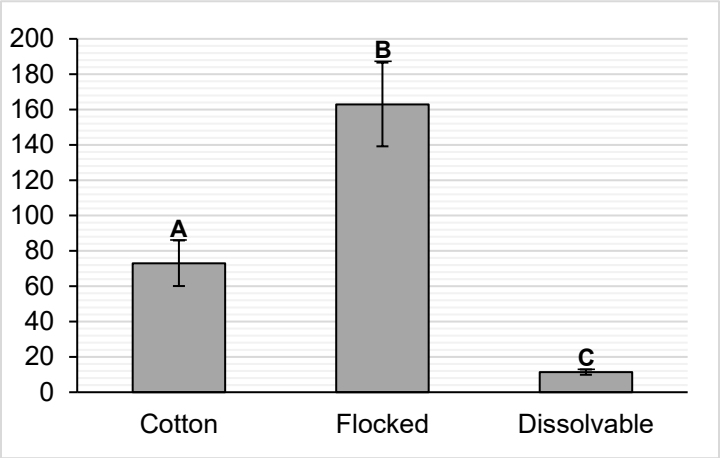

Figure 3.3: Total whole-cell counts eluted from cotton, flocked, and dissolvable swabs with AutoMACS® buffer. Results displayed represent the average of the eight trials +/- the SEM. Letter designations represent Tukey's HSD comparisons: the same letter designation means results are not statistically different, when letter designations differ between groups, the p-value is less than 0.05.

One notable feature from the Aim 2 data is that the dissolvable swabs yielded the lowest amount of whole intact cells post elution. From the unique features of the dissolvable swabs, it was originally believed that they would preform the best. However, without the intention of actually dissolving them, it is possible that their full potential was not observed in this study. When spotting the liquid blood onto the dissolvable swabs, the fibers of the swab did not absorb the blood as easily as the cotton or flocked swabs did. Rather, on the dissolvable swabs, the liquid blood remained in droplets on the surface of the swabs. This caused the blood to flake during the drying process. Because the blood was flaking and not being held within the fibers of the dissolvable swabs, it is not certain that the full ~328,000 cells spotted on the swabs were still present at the start of elution.

Additionally, when the elution data from the various swab types was compared to the positive control data for the AutoMACS® buffer, it demonstrates that the introduction of a swab, regardless of the type, causes a decrease in cell recovery post elution (**Figure 3.4**). This phenomenon could be caused by either the drying and rehydrating of the blood cells or the introduction of a swab resulting in cells are becoming trapped within the fibers of the substrate [12]. Additionally, not only could the cells be being retained within the swab fibers, but it is also possible that the swab fibers were too abrasive on the cells and causes shearing or rupturing. Since only whole, intact cells were being observed, any cells that were potentially damaged from the swab fibers would not have been observed.

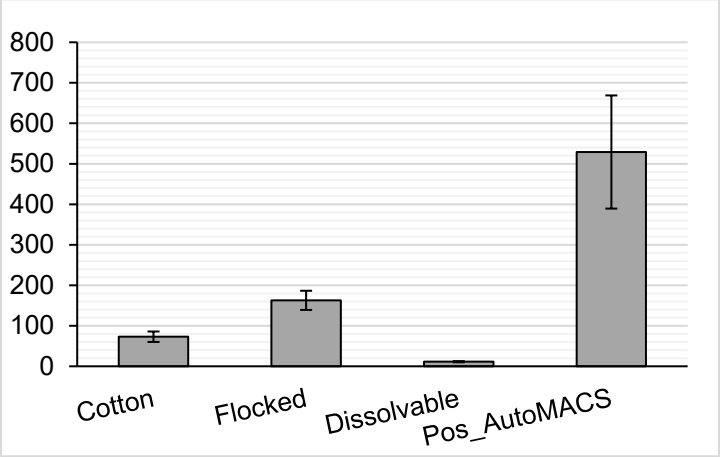

Figure 3.4: Total whole-cell counts eluted from cotton, flocked, and dissolvable swabs with AutoMACS® buffer compared to liquid blood. Results displayed represent the average of the eight trials +/- the SEM

CHAPTER IV: CONCLUSION

The purpose of these studies was to determine an optimal method of obtaining whole, intact cells from swabs containing biological material. The idea being that once whole cells are obtained, they could then be separated by cell type or contributor prior to downstream DNA analysis to alleviate interpretation issues in complex mixture analysis. In furtherance of this goal, three elution buffers (AutoMACS®, PBS, and water) were compared, as well as three swab substrates (cotton, flocked, and dissolvable).

Through literature research, AutoMACS® buffer seemed to be a reasonable option to maintain the integrity of the cells' membranes, keeping them intact. It was also apparent through a search of the literature that the material and composition of the swab substrate would have a major impact on quantity and quality of cell recovery. The dissolvable swabs seemed as if they would be a good option to avoid cells being trapped within the fibers of the substrate, because theoretically the swab would no longer exist after elution. However, due to the nature of how the swabs dissolve and that complicating the recovery of intact cells, it was decided to not actually dissolve these swabs, which in turn meant that they did not perform as well as initially hoped. Regardless, the outcome of these studies demonstrate that the combination of AutoMACS® buffer and flocked swabs was the best for recovery of whole cells post elution. The AutoMACS® buffer outperformed both Phosphate Buffered Saline (PBS) and water, while FLOQswab® outperformed both standard cotton swabs and dissolvable swabs. Due to the unique chemical composition of AutoMACS®, and the orientation of swab fibers on the FLOQswabs®, the integrity of the cells' membranes were preserved and whole, intact cells were observed post elution.

In the future, this information could be used for cell sorting purposes. Once whole cells are obtained, the proteins and cell surface characteristics of the cell membranes could be utilized.

Flow cytometry is a cell sorting technique that could be used to sort cells in a mixture by individual contributor. Once the cells of a complex mixture have been separated into component parts, DNA extraction could proceed as normal. Future avenues for research in this area include attempting elution of alternate cell types and actually implementing the cell sorting method. These studies were performed on blood samples because nucleated white blood cells are relatively abundant in whole blood, which is easily obtained and worked with, and the cells are somewhat robust compared to skin epithelial cells. Other cells types, such as sperm or epithelial cells, have features that could behave differently with the elution buffer or substrate type. Sperm cells have a long tail that results in a higher potential to become trapped within substrate fibers, and epithelial cells are more fragile and may be more susceptible to cell lysis. Additionally, the future goal is for this data collected from this study to be utilized in separating complex cell mixtures, but this study only focused on a single cell type from a single human donor.

This study could also be expanded to be more forensically relevant by swabbing already dried biological materials from surfaces with pre-moistened swabs. This study focused on the quality of the swabs and their retention of cells. However, in forensic context, it is typical to swab surfaces to pick up already dried biological material, like blood spatter at a crime scene. Future studies could test how swabbing already dried material would alter the results, rather than allowing the material to dry after being deposited on the swabs.

The separation of complex cell mixtures is relatively new area of research and the information discovered from these studies will help future studies, by acting as preliminary knowledge.

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