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Maximizing the amount of DNA recovered: a study of Mawi DNA technologies' iSWAB-ID collection device for forensic science application

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Thesis

MAXIMIZING THE AMOUNT OF DNA RECOVERED: A STUDY OF MAWI DNA TECHNOLOGIES' ISWAB-ID COLLECTION DEVICE FOR FORENSIC SCIENCE APPLICATION

by

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MAXIMIZING THE AMOUNT OF DNA RECOVERED: A STUDY OF MAWI DNA TECHNOLOGIES' ISWAB-ID COLLECTION DEVICE FOR FORENSIC SCIENCE APPLICATION

MICHELLE KRISTEN GORDON

ABSTRACT

In forensic casework, recovery of more deoxyribonucleic acid (DNA) generally leads to a better chance of obtaining a robust and reliable DNA profile. However, DNA evidence often contains a very low amount of cells, therefore, the importance of proper collection and storage to protect the DNA and ensure that maximum collection of cells is achieved cannot be over emphasized. New techniques and inventions have made the collection of DNA evidence more efficient and consistent through the development of different types of swabs, lysing buffers and various other improvements. Even with the development of these improvements, the ability to maximize the collection of cellular material from a substrate is still impeded by various issues in the extraction process along with the structural properties of swabs used for collection.

Research by Adamowicz et al. found that when extracting buccal and blood cell samples collected on cotton swabs, using the recommended protocol for swabs with the QIAamp® DNA Investigator extraction kit, over 50% of the recoverable DNA is retained on the swab or lost through the extraction process [1]. Although cotton swabs are very good at absorbing biological material, they exhibit low efficiency of DNA sample

release. Additional DNA may be lost during the extraction process. An optimal method of collection and extraction for forensic samples will maximize the collection and release of cellular material and minimize the loss of cellular DNA in the extraction process.

The design of the Mawi DNA Technologies' iSWAB^{TM} collection device allows for the release of cells captured from any type of swab into a proprietary lysis and stabilizing iSWAB^{TM} buffer. The combination of the mechanistic release of cells and the proprietary lysis buffer claims to maximize the collection of cells from single or several swabs in a pre-measured amount of buffer while eliminating the potential for bacterial growth and contamination. The iSWAB^{TM} Device is designed with three prongs and contains cell lysis buffer with DNA stabilization chemistry. As the swab is taken out of the collection device, the prongs provide resistance and essentially squeeze the excess solution and cells off of the swab. Following collection of the cellular material, cell lysis is achieved by incubation in the lysis buffer for 3 hours at room temperature. No additional reagents are necessary.

This study investigated whether the Mawi DNA Technologies' iSWAB^{TM} collection device and buffer could be considered as an alternative method to maximize the recovery of cells/DNA from swabs. Experiments were conducted to test the efficiency and forensic application of the device. The following parameters of the iSWAB^{TM} buffer and collection device were tested: 1) ability to collect dried stains; 2) ability to recover cellular material from different types and conditions of swabs; 3) ability to lyse different cell types; 4) ability to stabilize DNA over an extended period of time;

and, 5) ability to perform in downstream Polymerase Chain Reaction (PCR) testing and produce quality STR profiles.

Cumulatively, the data indicates that the iSWAB^{TM}-ID collection device is simple, fast and convenient while providing high DNA recovery. Some modifications or additional procedure developments can be done to facilitate the application for use with samples containing very small amounts of biological materials.

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LIST OF ABBREVIATIONS

˚C	Degrees Celsius
AT	Analytical threshold
CE	Capillary Electrophoresis
C_T	Threshold Cycle
dH_2O	Deionized water
DNA	Deoxyribonucleic acid
IPC	Internal Positive Control
mL	Milliliter
mm	Millimeter
ng	Nanogram
PCR	Polymerase chain reaction
PH	Peak Height
PHR	Peak Height Ratio
PPE	Personal protective equipment
ProK	Proteinase K
RFU	Relative Fluorescence Unit
rpm	Rotations per minute
SDS	Sodium dodecylsulfate
STR	Short Tandem Repeat
TE	Tris-EDTA
µL	Microliter

1. **Introduction**

1.1 Collection

Maximizing the collection and improving the quality of deoxyribonucleic acid (DNA) collected is essential in forensic casework because DNA results can be used to associate or to exclude an individual from involvement in a crime. DNA contains our unique genetic material and is found in every nucleated cell, including cells that are present in biological fluids left at crime scenes, such as saliva, blood and semen. Successful DNA profiling of forensic samples is mainly dependent on the quality and the amount of DNA that is recovered from the sample in question [2]. Generally, recovery of more DNA leads to a better chance of obtaining a robust and reliable DNA profile. Often times, DNA evidence will contain very low amounts of cells, therefore, the importance of proper collection and storage to protect the DNA and ensure that maximum collection of cells is achieved cannot be over emphasized. DNA is easily subjected to contamination, bacterial growth, and degradation that can affect the downstream processing of the sample. Common environmental factors that lead to the degradation of DNA include time, temperature, humidity (leading to the growth of microorganisms), light and exposure to various chemical substances [3]. If DNA is not handled properly or collected properly, the specimen can be unfit for analysis. New techniques and inventions have made the collection of DNA evidence more efficient and reliable through development of different types of swabs, lysing buffer and various other improvements. However, even if the cell collection is maximized, cells can be lost by the extraction process through various transfer steps [1].

A common way to collect biological evidence is swabbing. Successful recovery of DNA requires that swabs be able to absorb and release the collected biological material. The release of the cells from the swab is essential to achieve maximum collection. Most collection swabs are inefficient in releasing cells; meaning relatively substantial portions of the cells can remain entrapped in the swab [4]. Research from van Oorschot et al. [5], suggests that 20-76% of the DNA that is collected by a cotton swab is lost during the extraction phase, which may be attributed to the swab and the condition of the sample [6]. Similarly, Adamowicz et al. found that when extracting buccal and blood cell samples collected on cotton swabs, using the QIAamp® DNA Investigator extraction kit with its recommended protocol for swabs, over 50% of the recoverable DNA is retained on the swab or lost through the extraction process [1]. While there are many validated methods used to increase low input evidentiary samples, such as, concentrating samples, amplification protocol adjustments, post-polymerase chain reaction (PCR) purification and capillary injection adjustments, the most reliable method to improve the quality of a DNA profile is to maximize collection of cells and DNA initially [1]. Various alterations to extraction protocols, methods of wetting swabs for collection, lysis buffer composition, and swab material and design have been made to try to recover more cellular material off of swabs.

For the QIAamp® DNA Investigator extraction kit, altering the protocol's initial incubation time and temperature, along with adding vortexing and re-suspension of the swab increased the recovery of DNA off of the swab [1]. The study found that increased yields were observed with 3- and 18-hour incubation periods and that re-suspending the

swab yielded an average two-fold increase in recovered DNA from buccal cells and an average three-fold increase with blood cells [1]. Other methods of protocol alterations include modifying the chemical composition of buffers, such as increasing the concentration of Proteinase K (ProK) or adding DNase in differential extractions to avoid multiple rinsing steps [7]. Alternatively, other techniques have been used that do not use chemicals, such as physical manipulation techniques like laser microdissection in which cells are laser cut from a microscope slide.

Another study, discovered that wetting the tip of the swab with laboratory or commercially developed detergent solutions, like sodium dodecyl sulfate (SDS) and Triton X-100, yielded higher quantities of recovered DNA compared to the traditional method of wetting the swab with water [8]. The detergent was found to loosen and solubilize the cells to increase the cell/DNA yield due to its amphiphilic nature. Amphiphilic substances allow solubility in both water and nonpolar solvents; thus, the organic molecules that make up cells (fats, lipids and proteins) become suspended in solution. Water does not have this property and therefore it was concluded that detergents should be utilized for cellular pickup over the commonly used water [8].

Alterations in lysis buffer composition have proved to be beneficial in the recovery of sperm cells off of cotton swabs in sexual assault evidence processing. Sperm cell recovery has been enhanced to as high as 90% when using a novel one-step buffer that contains SDS and ProK, representing a 200-300% increase over conventional differential extraction buffer [7]. Additionally, alterations to the material the swab is made from and manipulations in the physical design have increased DNA yield. A novel

material, Diomics X-Swab™ has highly absorptive properties and can dissolve during certain extraction conditions to recover greater amounts of DNA than commonly used swabs [6]. The X-Swab material was also found to enhance the yield of PCR products. There are various different swab materials, such as foam, nylon flocked, polyester and rayon-tipped swabs, that are marketed to collect more DNA and recover more useable DNA depending on the cell type and cell quality.

This study determined whether or not the Mawi DNA Technologies' iSWAB™ collection device could be considered an alternative method to maximize the recovery of cells/DNA off of swabs. The design of the iSWAB™ collection device allows for the release of cells captured from any type of swab into a proprietary lysis and stabilizing iSWAB™ buffer. The combination of the mechanistic release of cells and the proprietary lysis buffer is supposed to maximum collection of cells from single or multiple swabs in a pre-measured amount of buffer while eliminating the potential for high bacterial growth and contamination. The iSWAB™ Device consists of three prongs and buffer stabilization technology contained in a leak proof tube with a screw and o-ring fitted cap. As a swab is removed from the collection device, the prongs provide resistance and essentially squeeze the excess solution and cells from the swab. Following collection of the cellular material, cell lysis is achieved by the suspension of the cellular material in the proprietary lysis buffer for 3 hours at room temperature. There are no heating steps or additional reagents necessary, which makes it a very simple and easy process. If the device can be used as an alternative collection and extraction method, the

ease of use and limited hands-on processing would allow the Criminalist to perform other tasks and increase lab efficiency [11-12].

In order for the DNA to be accessible for forensic analysis, the DNA must be released from the cells in a process called extraction. The extraction process removes inhibitors that reduce or prevent PCR amplification and lyses cells to release the DNA molecules for further analysis. There is no "universal" DNA extraction procedure, however, an optimal procedure should be non-toxic, fast, efficient, economical and should recover high purified DNA with minimal loss [9]. Commonly used extraction methods used in forensic DNA laboratories include: organic extractions, solid-phase extractions and Chelex® extractions. Organic extractions, also known as phenol-chloroform extractions, involve the serial addition of several chemicals, such as SDS and ProK to break open the cell membrane and then a phenol/chloroform mixture is used to separate contaminants into the organic phase and DNA in the aqueous phase. Organic extractions are becoming less popular due to the toxicity of the chemicals and because they are more time consuming than other methods [10]. Solid-phase extractions, such as the QIAamp® DNA Investigator extraction kit, follow a bind-wash-elute procedure where the DNA binds to a substrate, typically silica particles, while other proteins/ cellular components are washed away in a series of wash steps. Solid-phase extractions typically yield a high amount of DNA, however some DNA loss can occur throughout transfer steps, wash steps and not all DNA may elute from the silica. Chelex® extraction utilizes a chelating-resin suspension that can be added directly to the sample. Chelex® extraction involves fewer steps than the organic extraction, leading to less opportunities for

contamination, but the DNA is not removed from the other cellular components causing not as pure products. Unlike the previously mentioned extraction methods, Mawi DNA Technologies' iSWAB™-ID collection device contains a proprietary iSWAB™ buffer that directly extracts the cells without any addition of reagents or incubation periods. Compared to the commonly used extraction methods in forensics, iSWAB™ buffer extractions may be faster and easier to use without compromising DNA recovery or integrity.

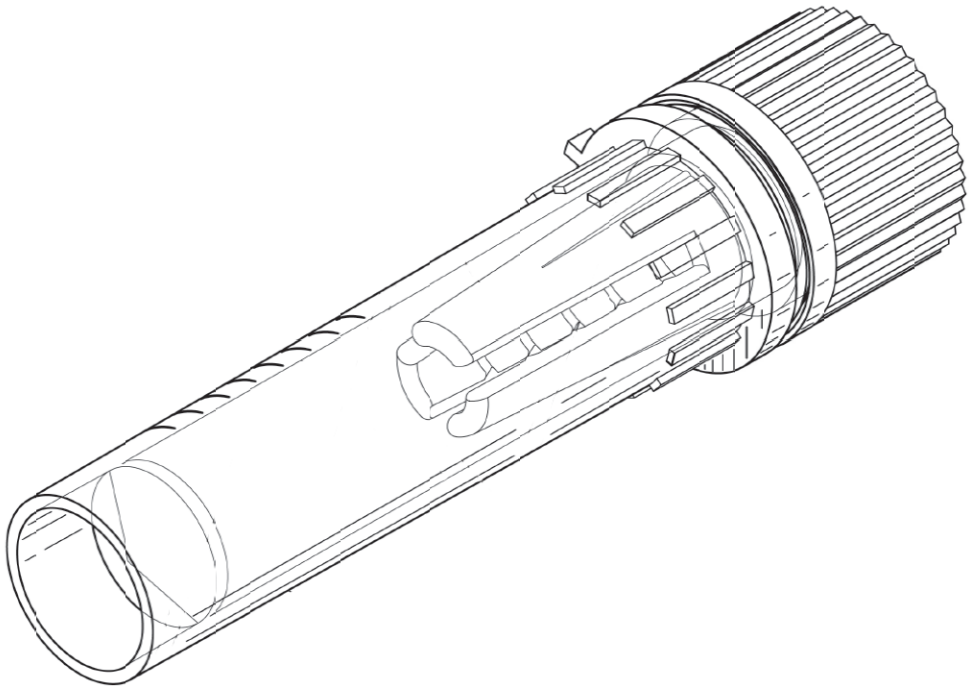

Figure 1. Sketch of iSWAB™-ID Collection Device Courtesy of Dr. Bassam El-Fahmawi

Other single tube, direct lysis buffers are on the market, such as ZyGEM's *forensic*GEM® Saliva Kit. The *forensic*GEM® Saliva Kit utilizes an enzyme and 10X

Blue buffer. The reagents do not come pre-mixed, like in the iSWAB™ collection device. The reagents must be added to the forensic sample and then heated to activate the enzyme to lyse the cells. Similar to the iSWAB™ extraction, there is less opportunity for potential contamination and loss of DNA. However, ZyGEM is a "DNA release" chemistry and does not offer assistance with cell release or long-term storage of DNA. Also, because of the heat step, *forensic*GEM® released DNA is largely single-stranded (approximately 90% according to the manufacturer) [13]. The *forensic*ZyGEM® Saliva Kit was used as a control in most of the studies in order to determine the lysis quality of the iSWAB™ buffer.

1.1 Purpose of Study

The purpose of this study was to define conditions and limitations of the use of Mawi DNA Technologies' iSWAB™-ID collection device for forensic samples and subsequent DNA testing.

The iSWAB™-ID collection protocol states that the buffer must be diluted before PCR amplification in order to prevent inhibition. Inhibitors can interfere with the PCR by interaction directly with DNA or by blocking the activity of the polymerase or other PCR mixture components, thereby preventing target amplification [14]. Experimentally, the extent of dilution of iSWAB™ buffer required for uninhibited PCR amplification was determined. The objective of the first experiment conducted was to determine the cut-off value of inhibition. This was done by using various targeted concentrations of DNA and various concentrations of iSWAB™ buffer.

Since the buffer must be diluted to be compatible with PCR, the next experiment was performed to asses if varying the volume of iSWAB™ buffer affected the quality of cell lysis. The iSWAB™-ID collection tubes come with approximately 320 µL of buffer in the tubes, which could make low-input samples very dilute for downstream forensic processing. The concentration of DNA in each extract was determined and Short Tandem Repeat (STR) analysis was performed to assess the quality of DNA profiles from DNA produced using the iSWAB™-ID collection device.

Experiments were designed to answer questions about the efficiency and effectiveness of the iSWAB™-ID collection device for getting cells off of swabs. As previously mentioned, testing has shown that significant quantities of DNA are retained on the swab. The prong mechanism in the device was tested by comparing amounts of DNA recovered from the device to iSWAB™ buffer extractions without using the device. Various types of cells (buccal, sperm and white blood cells) and various conditions of the swabs (swabs prepared just before testing and swabs that were dried for days before testing) were tested using the device. Mawi DNA Technologies recommended Puritan®'s HydraFlock Sterile Standard Flock Swab with Polystyrene Handle (Puritan®, Guilford, Maine), so most experiments utilized the nylon flocked swabs, however, the iSWAB™-ID collection device states to be compatible with any type of swab. Therefore, Sterile Cotton Tipped Applicators (Puritan®, Guilford, Maine) were tested in addition to the nylon flocked swabs in a Dried Stain Collection Experiment because the cotton swab has long been the basic and essential tool for collection of DNA evidence for forensic casework analysis [15].

A 3-month time course study was conducted in order to test the stability of the DNA from buccal cells in the iSWAB™ buffer for long-term storage at room temperature. Sample storage, holding temperatures, and collection methods can differentially affect DNA recovery and consequently, the outcomes from downstream processing of evidence, leading to false conclusions [16]. Samplings were taken every month for 3 months. The DNA concentration in all extracts was quantified and STR profiling was conducted to compare any differences in the quality of the profiles throughout the 3-month period.

2. Materials and Methods

All experiments were carried out in compliance with the ethical standards set forth by the Institutional Review Board of Boston University School of Medicine. Proper personal protective equipment (PPE) along with cleaning measures were taken in order to prevent contamination of workspace and samples.

2.1. Dilution of iSWAB™ Buffer Solution

iSWAB™ buffer contained in the iSWAB™-ID Collection Kit (Mawi DNA Technologies, CA) was diluted with Tris-EDTA (TE) buffer (10 mM Tris, pH 8.0, and 0.1 mM EDTA) to the following concentrations: 0X, 0.05X, 0.10X, 0.15X, 0.20X, 0.25X and 0.30X to determine the limit of inhibition. A 1X concentration of iSWAB™ buffer was also tested to observe full inhibition. Samples were prepared in a final volume of 100 microliters (µL).

2.2. Dilution of QuantiFiler® Duo DNA Standard

The QuantiFiler® Duo DNA Standard (ThermoFisher Scientific, Waltham, MA) [200 nanograms (ng)/µL] was diluted with various amounts of TE buffer to target DNA concentrations of 0.5 ng/µL, 1 ng/µL and 2 ng/µL. These diluted QuantiFiler® Duo DNA Standards were used to test the inhibition of the iSWAB™ buffer.

2.3 Preparation of Saliva Samples

Neat saliva was obtained from an anonymous female donor in a 2.0 milliliter (mL) microcentrifuge tube (Eppendorf, Hamburg, Germany). From the neat saliva collected, a portion was mixed with an equal portion of TE buffer into a new 2.0 mL microcentrifuge tube to clean up the neat saliva. After gently vortexing for 10 seconds, the mixture was centrifuged at 9000 rotations per minute (rpm) for 4 minutes in an Eppendorf centrifuge 5424 (Hamburg, Germany) to allow the buccal cells in the saliva to pellet in the bottom of the tube and the mucus and other saliva components to remain in the solution. Once the supernatant was removed, the pellet was resuspended in TE buffer. The mixture was vortexed for 5 seconds and then centrifuged at 9000 rpm for 4 minutes. This process of washing and pelleting was repeated three times. Finally, the pellet was resuspended to produce a solution of buccal cells in TE buffer.

2.4 Buccal Cell Counting

A Metallized Bright-Line Hemocytometer (Hausser Scientific, Horsham, PA) was used to approximately determine the concentration of cells in the prepared buccal cell solution. First, the cell solution was vortexed for 5 seconds or until the solution appeared homogeneous. Due to the high concentration of DNA targeted, the solution was diluted 1:10. Then 7 µL of the diluted solution was loaded into the Hemocytometer slide with a cover slip. The slide was then viewed at 40X magnification on a Nikon Eclipse TE2000-S microscope using Phase Contrast Microscopy. The number of buccal cells were counted in five different 1 millimeter $(mm)^2$ sections of the Neubauer ruling on the

Hemocytometer. Cells on the perimeter of the lines were included in the count. An average of the five sections was taken to determine the average cell count in $1mm^2$, which was then used to determine the average number of cells per µL, according to the Hausser Scientific manual. The ruled surface is 0.10mm below the cover glass, so that the volume over each mm^2 is 0.10 mm^3.

One (1) microliter (µL) = One (1) cubic millimeter (mm^3)

Average number of cells/µL = Average cell count per square millimeter / volume * dilution factor (if used)

Based on the result from the calculation of the average number of cells/µL, the average concentration of ng of DNA was calculated per µL by the following calculation:

Average concentration of ng of DNA/µL = average cells/µL * 0.0066 ng/cell

2.5 Extraction

2.5.1 ZyGEM Extraction and Reagents

A *forensic*GEM™ Saliva kit containing *forensic*GEM™ (enzyme EA1) and 10X Buffer Blue was obtained from ZyGEM (Hamilton, New Zealand). All extractions were done in 0.2 mL reaction tubes (Applied Biosystems, Foster City, CA, USA). Depending on the concentration of the saliva cell suspension being used, either a 100 µL or 200 µL ZyGEM reaction was utilized. For the 100 µL reactions, 1 µL of *forensic*GEM™, 10 µL of the 10X Buffer Blue, 15 µL of saliva cell suspension and 74 µL of TE buffer was used. For the 200 µL reactions, 2 µL of *forensic*GEM™, 20 µL of the 10X Buffer Blue, 15 µL of

saliva cell suspension and 163 µL of TE buffer was used. The thermocycler procedure was followed from the *forensic*GEM™ Saliva kit manual. One cycle of a proteinase activation at 75 degrees Celsius (˚C) incubation for 15 minutes and then the proteinase inactivation at 95 ˚C for 5 minutes was performed on a thermocycler (GeneAmp PCR System 9700, Applied Biosystems, Foster City, CA).

2.5.2 Mawi DNA Technologies' iSWAB™ buffer Extraction

Mawi's iSWAB™ Collection Instructions (Mawi DNA Technologies) were followed to extract samples that were on swabs. The iSWAB™ vial was held steady in one hand while the only hand slowly twisted the swab into the iSWAB™ vial with a corkscrew motion. The swab was pushed through the resistance to the bottom of the tube. Then, the swab was moved up and down rapidly inside the tube 15 times without moving the swab out of the liquid. After, the swab was removed from the vial by slowly twisting it out of the tube in a corkscrew motion, letting the prongs in the device squeeze the excess liquid off of the swab. Once the swab was completely removed, the vial cap was tightly placed on and the vial was vortexed for 5 seconds. To extract liquid samples, 50 µL of the liquid body fluids were pipetted directly into the vial and then vortexed for 5 seconds. The samples were incubated in iSWAB™ buffer for at least 3 hours to extract.

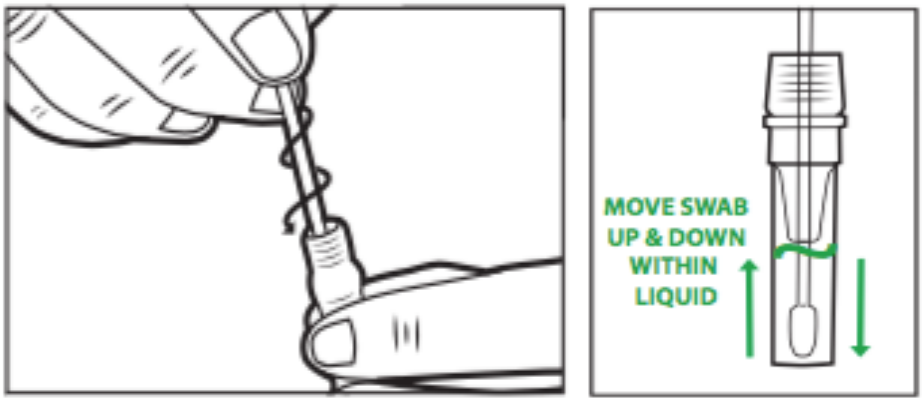

Figure 2. iSWAB™-ID Collection Technique

2.5.3 Qiagen Extraction

For the comparison study, samples were extracted using the QIAamp® Investigator extraction protocol (Qiagen, Valencia, CA). The procedure was performed according to the manufacturer's recommendations outlined in the *Isolation of Total DNA from Small Volumes of Saliva* protocol [41]. Qiagen extractions were conducted in triplicate. For each extraction, 50 µL of saliva cell suspension was used. According to the Qiagen protocol, 50 µL of Buffer ATL, along with 10 µL of ProK and 100 µL of Buffer AL were added to the tube and pulse vortexed for 15 seconds and then incubated at 56 ˚C for 10 minutes. Following incubation, tubes were briefly centrifuged and then 50 µL of pure ethanol was added and tubes were incubated at room temperature for 3 minutes. After another brief centrifugation, the lysates from each tube were transferred into QIAamp® MinElute columns that were placed into 2 mL collection tubes and centrifuged at 8000 rpm for 1 minutes. Each sample was washed with 500 µL of Buffer AW1, 700 µL of Buffer AW2 and 700 µL of pure ethanol. Following these wash steps, a

new collection tube was inserted under the columns and each tube was centrifuged at full speed (14000 rpm) for 3 minutes to dry the membrane completely. After centrifugation, the columns were placed into 1.5 mL microcentrifuge tubes (Eppendorf, Hamburg, Germany) and were allowed to sit at room temperature with the lids open for 10 minutes. Subsequently, 100 µL Buffer ATE was added to the center of the membrane and the tubes were incubated at room temperature for 1 minute. Each tube was then centrifuged at full speed for 1 minute and the end volume for each sample was assumed to be 100 µL. A 100 µL iSWAB™ buffer extraction was performed in triplicate on 50 µL of the same saliva cell suspension.

2.6 Sample Preparation and Manipulation

2.6.1 Comparison of Swabs Prepared Days Before Testing vs. Just Prior to Testing

2.6.1.1 Pre-Experiment: Cut Method vs. Scalpel Method

Two sterile dry nylon flocked swabs with no biological fluid were placed into iSWAB™ buffer to mimic the iSWAB™-ID collection protocol. One of the swabs was cut where the swab portion meets the stick and the other swab was shaved using a scalpel. The cut portion of the swab or the shavings of the swab were placed into a spin basket, which was then placed into a 1.5 mL microcentrifuge tube, and centrifuged at 9000 rpm for 4 minutes. The amount of iSWAB™ buffer liquid in the microcentrifuge tube was measured and recorded for the cut swab and shaved portions of the swab. This process was repeated 3 times. A saliva cell suspension was created and 15 µL of the saliva cell suspension was added to the iSWAB™ buffer. A positive control was created

with 15 µL of the saliva cell suspension and no iSWAB™ buffer. A 200 µL ZyGEM extraction was performed. Cut samples were diluted to a 0.1X iSWAB™ buffer concentration using TE buffer.

2.6.1.2 Wet Swab and Dry Swab Extraction

A saliva cell suspension was created using 2,000 µL of neat saliva and a resuspending volume of 800 µL of TE buffer. The saliva cell suspension was vortexed thoroughly and 50 µL of the solution was pipetted onto three swabs that were dry prior to sample application. The three swabs were left to dry for 3 days and were designated as the dry swabs for testing. Three wet swabs were created by pipetting 50 µL of the same saliva cell suspension on three swabs that were dry before sample application. The wet swabs were created moments before the experiment was conducted. A positive control was created by directly pipetting 50 µL of the saliva cell suspension into the iSWAB™ buffer. A negative control contained no saliva cells, just iSWAB™ buffer. The iSWAB™-ID collection protocol (Mawi DNA Technologies) was followed to get saliva cells off of wet and dry swabs [12].

2.6.1.3 Retention of Cells on Swab After Removal from iSWAB™ Device

Upon removal of the wet and dry swabs from the iSWAB™ device, the swabs were cut and were placed into spin baskets that sat on top of 1.5 mL microcentrifuge tubes and centrifuged at 9000 rpm for 4 minutes. After centrifugation, the amount of

iSWAB™ buffer was measured and recorded. The swab was placed in a 200 µL ZyGEM reaction to lyse any additional cells that remained on the swab.

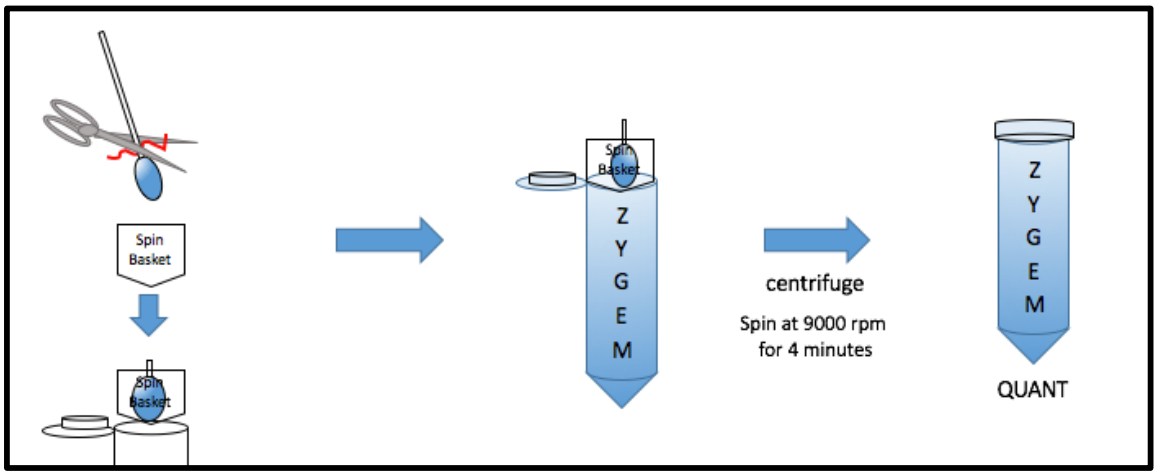

Figure 3. Visual Representation of Sample Preparation for ZyGEM Reaction

2.6.2 Prong and Non-Prong Experiment

2.6.2.1 Prong and Non-Prong Sample Preparation

To create prong-less devices for comparison purposes, approximately 320 µL of iSWAB™ buffer were transferred into each 1.5 mL microcentrifuge tubes. A saliva cell suspension was created and pipetted onto 4 swabs. Two swabs were extracted in the 1.5 mL microcentrifuge tubes with iSWAB™ buffer (non-prong samples) and the other two swabs were extracted in the iSWAB™ collection devices (prong samples). A positive control was created by pipetting 50 µL of the saliva cell suspension directly into the iSWAB device. A negative control contained 320 µL of iSWAB™ buffer without any saliva cells. Non-prong and prong samples were extracted according to the iSWAB™-ID collection protocol. For the non-prong samples, the corkscrew motion was mimicked by

moving the swab along the sides of the microcentrifuge tube. All samples extracted for 3 hours in iSWAB™ buffer.

2.6.2.2 Retention of Cells on Swabs After Swab Removal and Spin Basket

The prong samples, non-prong samples and positive control nylon flocked swabs were cut where the swab meets the stick. The swabs were all placed into spin baskets, which were in 1.5 mL microcentrifuge tubes and then centrifuged at 9000 rpm for 4 minutes. The amount of iSWAB™ buffer was measured and recorded for all samples and then left to extract for 3 hours. These liquid samples were designated as the elute samples. After being spun, the swabs were placed into new 1.5 mL centrifuge tubes and 300 µL of iSWAB™ buffer was added to each tube in order to completely cover the swabs to determine how much is left on the swab after centrifugation in the spin basket. The swabs were left to extract in the iSWAB™ buffer for 3 hours. After 3 hours, the swabs were removed and centrifuged to obtain all additional DNA extracted from the swab. These samples were designated as the swab samples.

Additionally, three nylon flocked swabs were created with 50 µL of the same saliva cell suspension used in Prong vs. Non-Prong Experiment and were placed into the iSWAB™ devices. The swabs were left in the devices for 3 hours for the entire duration of extraction. After extraction, the iSWAB™-ID collection protocol was implemented to spin out the swab then the swab was cut where the swabs meets the stick and the swab was spun out into a new 1.5 mL microcentrifuge tube. The amount of liquid was

measured and recorded and then pipetted back into the iSWAB™ device. The swabs were then extracted a second time in 300 µL of iSWAB™ buffer for 3 hours.

2.6.3 Collection of Dried Stains Experiment

2.6.3.1 Semen, Blood and Saliva Sample Preparation

Neat semen and blood samples were obtained by anonymous donors in 2 mL centrifuge tubes. Neat saliva was washed and cleaned according to the Saliva Cell Preparation protocol previously mentioned. Four petri dishes (Thermo Fisher Scientific, Waltham, MA) were obtained and each petri dish was divided into thirds using a protractor and black permanent marker, so that 50 µL of each body fluid (blood, semen and saliva) were pipetted into its own section of the petri dishes.

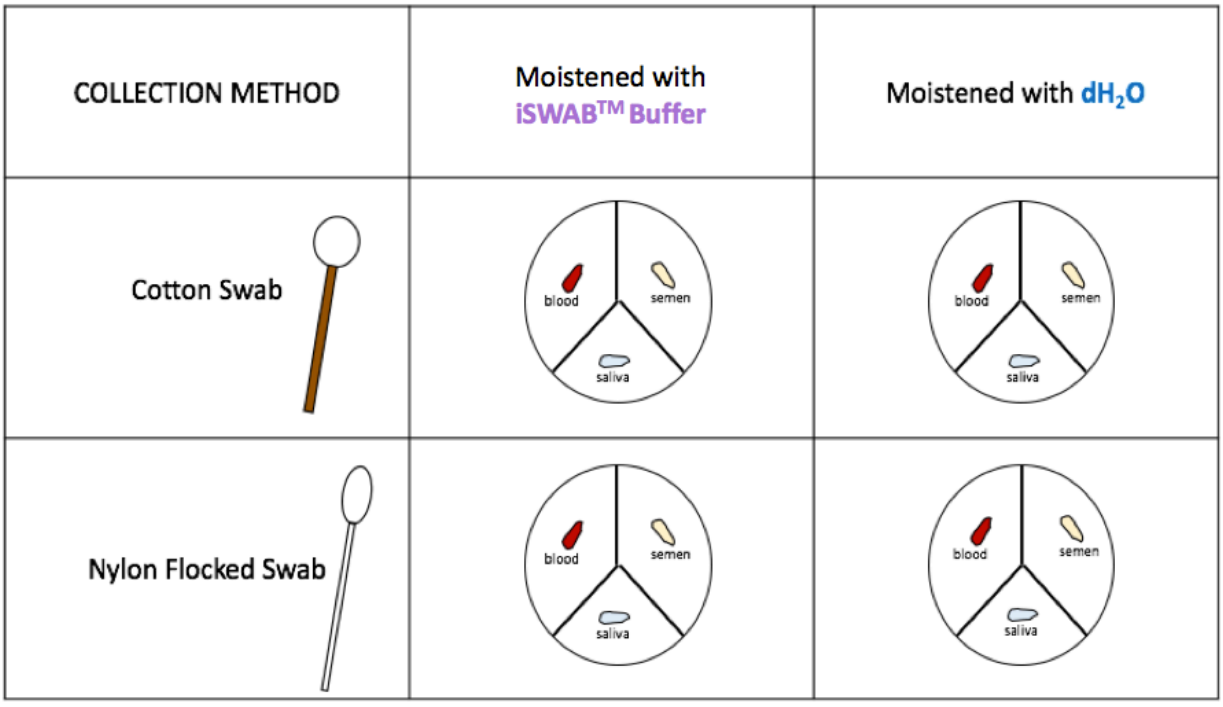

Figure 4. Visual Representation of Experimental Design for the Collection of Dried Stains Experiment

Positive and negative controls were created by pipetting 50 µL of each body fluid onto nylon flocked swabs and cotton swabs. All stains on petri dishes and control swabs were dried for 48 hours. Three nylon flocked swabs were moistened with 100 µL of iSWAB™ Buffer and used to collect the three different dried body fluids from a petri dish. Similarly, three cotton swabs were moistened with 100 µL of iSWAB™ Buffer and used to collect the dried stains out of another petri dish. This process was repeated with deionized water (dH_2O) to moisten the swabs before collection of the dried stains. Collection of the dried stains was standardized by moving the swabs over the dried stains in a circular motion 10 times while applying a similar amount of pressure. All swabs from the petri dishes and controls were extracted in iSWAB™-ID devices. The swabs were left in the buffer for 48 hours and then removed per the removal instructions from the iSWAB™-ID collection protocol. All swabs were placed in spin baskets, which were in 1.5 mL centrifuge tubes, and centrifuge at 9000 rpm for 4 minutes. The excess liquid that was eluted was pipetted back into the iSWAB™ devices.

2.6.4 Time Course Study

Three iSWAB™ device tubes were obtained and 50 µL of a saliva cell suspension was pipetted directly into each tube. As a degradation control, 50 µL of the cell suspension was also pipetted directly into a 1.5 mL microcentrifuge tube filled with 320 µL of TE buffer. Samplings of the 4 samples were taken at a 1-month, 2-month and 3-month extraction period. During each sampling period, a 20 µL ZyGEM extraction was performed on the saliva cell suspension and the TE buffer samples. The saliva cell

suspension was stored at -20 ˚C throughout the study, while the iSWAB™ devices and TE buffer with cells were stored at room temperature.

2.7 DNA Quantification

All samples were diluted to a 0.10X concentration of iSWAB™ buffer before quantification, with the exception of the samples created in the iSWAB™ Buffer Concentration study.

DNA quantification was performed on all samples using the Quantifiler® Duo Quantification Kit (Applied Biosystems, Foster City, CA) using 7500 Real-Time PCR system (Life Technologies, Carlsbad, CA) as per the manufacturer's protocol. When preparing the reactions, each sample volume totaled 25 µL, including 23 µL of the Master Mix and 2 µL of the extracted DNA sample.

The quantity of DNA within each sample was analyzed using a publicly available Microsoft Excel template. In order to account for potential differences between the various quantification values, the y-intercept and slope from runs of standard curves was used to calculate the concentration of DNA in all the samples before analysis of data, as per methods described by Grgicak et al [17].

2.8 Amplification

Amplification of all samples was performed using the AmpFℓSTR® Identifiler® Plus (Applied Biosystems, Foster City, CA) and the GlobalFiler® (Applied Biosystems, Foster City, CA) PCR Amplification Kits per the manufacturer's protocol using 28 cycles

for Identifiler® amplifications and 29 cycles for GlobalFiler® amplifications. The desired target mass was 1 ng of DNA for most Identifiler® amplifications, with the exception of targeting a mass of 0.75 ng of DNA for Identifiler® amplifications that were compared to GlobalFiler® amplifications. A target mass of 0.5 ng of DNA was used for all GlobalFiler® amplifications. When needed sample dilutions were made with TE Buffer. DNA samples were amplified on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). Positive and negative controls were run for each kit with every amplification. Amplified products were stored at -20 ˚C until they could be separated by capillary electrophoresis (CE).

2.9 Capillary Electrophoresis and STR Profile Analysis

CE was performed using a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). Samples amplified with Identifiler® Plus were prepared for CE by using a master mix of 9.5 µL of Hi-Di formamide and 0.5 µL of GeneScan™-600 Liz® Size Standard version 2.0 (Life Technologies, Carlsbad, CA) per reaction and 1 µL of amplified product. The GlobalFiler® samples were prepared using a master mix of 9.6 µL of Hi-Di formamide and 0.4 µL of GeneScan™-600 Liz® Size Standard (Life Technologies, Carlsbad, CA) per reaction. GeneMapper® ID-X Software (Applied Biosystems, Foster City, CA) was used for analysis using an analytical threshold (AT) of 30 relative fluorescence units (RFU) for Identifiler® Plus samples and an AT of 100 RFU for GlobalFiler® samples. Peak height is based on the RFU that is determined by the

fluorescence given off by an allele fragment during the data collection process. The average peak height (PH) for each profile was calculated by dividing the total PH by the total number of alleles in the profile. Peak height ratios (PHR) between sister alleles were determined by dividing the RFU from the allele with the lower PH by the RFU for the allele with the higher PH. The PHR mean was calculated across all the profiles.

2.10 Statistical Methods

Statistical analysis was performed using Microsoft® Excel for Mac 2016 (Microsoft, Redmond, WA) and JMP® Pro v. 13 (SAS Institute, Cary, NC).

3. Results and Discussion

3.1. Quantifiler® Duo Quantification Data

As previously mentioned, every sample was quantified via real-time PCR (RT-PCR) using the Quantifiler® Duo Quantification Kit and the 7500 Detection System. The Quantifiler® Duo Kit is a multiplexed TaqMan™ RT-PCR assay for DNA quantitation which amplifies a total human and a human male target simultaneously [18]. To monitor for inhibition, which occurs when compounds interfere with the reaction and cause varying levels of reduced PCR efficiency, an Internal Positive Control (IPC) is co-amplified with targets in the Quantifiler® Duo Kit. Monitoring the amplification of the IPC, the assay can provide information about the presence of PCR inhibitors and help determine the quantity of extract to use for amplification for different STR multiplex systems [19]. The Quantifiler® Duo Kit contains a known amount of exogenous DNA as the IPC that can be fortified to the sample and amplified. Monitoring the IPC amplification enables the detection of PCR failure due to inhibition when the IPC's threshold cycle (C_T) value is higher than that of an uninhibited PCR reaction.

3.1.1. Determination of Concentration of iSWAB™ buffer to Prevent Inhibition

In order to establish the concentration of iSWAB™ buffer to use for downstream PCR analysis, a gradient of iSWAB™ buffer concentrations were tested. First, a wide range of iSWAB™ buffer concentrations were used with a targeted DNA concentration of 1 ng/μL made with a dilution of the Quantifiler® Duo Positive control. The concentrations of iSWAB™ buffer were: 0X, 0.05X, 0.1X, 0.2X, 0.5X and 1X. The

samples were quantified in duplicate and an average of the values was calculated to obtain the average experimental DNA concentration in ng/uL and average IPC C_T.

Inhibition was observed at iSWAB™ buffer concentration of 0.5X and 1.0X indicating that samples extracted with iSWAB™ buffer cannot go directly to amplification without dilution (**Table 1**). No inhibition was observed in the other concentration of buffer as evident by their average IPC values falling into the acceptable normal range. The average experimental DNA concentration ranged from 1.054 ± 0.062 to 1.386 ± 0.118 ng/µL, which was very close to the 1 ng/µL that was targeted.

Table 1. Quantifiler® Duo Results for Samples with Varying Concentration of iSWAB™ Buffer Targeting 1 ng/ µL of DNA

iSWAB™ Buffer Concentration	**Expected DNA Concentration (ng/µL)**	**Average Experimental DNA Concentration (ng/µL)**	**Average IPC C_T Value**
0X	1.000	1.054 ± 0.062	29.321 ± 0.088
0.05X	1.000	1.371 ± 0.013	28.956 ± 0.080
0.1X	1.000	1.386 ± 0.118	28.817 ± 0.043
0.2X	1.000	1.070 ± 0.194	28.760 ± 0.125
0.5X	1.000	**Inhibited**	**Inhibited**
1X	1.000	**Inhibited**	**Inhibited**

This experiment was repeated with a target DNA concentration of 0.5 ng/µL, except the inhibited concentrations of the iSWAB™ buffer were excluded. The average IPC C_T values were all within the acceptable range, ranging from 28.830 ± 0.053 – 29.350 ± 0.055, giving no indications that inhibition occurred (**Table 2**). The target DNA

concentration of 0.5 ng/µL was closely met with the different iSWAB™ buffer concentrations, with exception of the 0.2X concentration. The DNA concentration for the 0.2X iSWAB™ concentration was 0.255 ± 0.107 roughly a half of what was expected. Since the IPC average C_T value for the 0.2X samples was 28.992 ± 0.149, which was within range, and the standard deviation between the samples is large, this loss of DNA could be due to poor pipetting and not due to partial inhibition of the sample.

Table 2. Quantifiler® Duo Results for Samples with Varying Concentration of iSWAB™ Buffer Targeting 0.5 ng/ µL of DNA, N=2 for each condition

iSWAB™ Buffer Concentration	Expected DNA Concentration (ng/µL)	Average Experimental DNA Concentration (ng/µL)	Average IPC C_T Value
0X	0.500	0.620 ± 0.038	29.350 ± 0.055
0.05X	0.500	0.806 ± 0.028	29.034 ± 0.075
0.1X	0.500	0.728 ± 0.047	28.830 ± 0.053
0.2X	0.500	0.255 ± 0.107	28.992 ± 0.149

In order to make sure partial inhibition was not occurring at 0.2X concentration of iSWAB™ buffer, a concentration gradient of DNA was run in duplicate at the 0.2X concentration of iSWAB™ buffer. The results in **Table 3** confirm that there is no inhibition observed at the 0.2X concentration of iSWAB™ buffer when targeting DNA concentrations of 0.1-2 ng/µL.

Table 3. Quantifiler® Duo Results for Samples with 0.2X Concentration of iSWAB™ Buffer Targeting Various Amounts of DNA, N=2 for each condition

iSWAB™ Buffer Concentration	Expected DNA Concentration (ng/µL)	Average Experimental DNA Concentration (ng/µL)	Average IPC C_T Value
0.2X	0.100	0.123 ± 0.036	28.697 ± 0.051
0.2X	0.200	0.270 ± 0.045	28.576 ± 0.033
0.2X	0.500	0.651 ± 0.084	28.563 ± 0.019
0.2X	1.000	1.364 ± 0.077	28.577 ± 0.056
0.2X	2.000	2.151 ± 0.096	28.570 ± 0.091

Finally, to determine the cut-off value for inhibition, concentration values were chosen that were a little above the 0.2X. iSWAB™ buffer concentrations of 0.1X, 0.15X, 0.2X, 0.25X and 0.3X were tested using a gradient of target amounts of DNA that included 0.1, 0.5, 1.0 and 2.0 ng/µL of DNA. As observed in **Table 4**, the IPC values indicate that partial inhibition is observed at 0.25X and complete inhibition is observed at 0.30X concentration of iSWAB™ buffer. The IPC values for the 0.35X fall above the accepted range and the targeted amounts of DNA were not observed. Full inhibition was observed at 0.3X because the IPC was inhibited and no DNA was observed. Thus, the data shows that iSWAB™ buffer should be diluted at least to a 0.2X concentration before going to PCR because above this concentration PCR inhibition will occur. For the proceeding experiments all samples were diluted to an iSWAB™ buffer concentration of 0.1X.

Table 4. Quantifiler® Duo Results for Varying Concentration of iSWAB™ Buffer Targeting Various Amounts of DNA to Determine Inhibition Cut-Off Value, N= 2 for each condition

iSWAB™ buffer Concentration	**Expected DNA Concentration (ng/ µL)**	**Average Actual DNA Concentration (ng/µL)**	**Average IPC Ct Value**
0.1X	0.1	**0.141 ± 0.017**	28.68 ± 0.08
0.1X	0.5	**0.661 ± 0.077**	28.76 ± 0.02
0.1X	1	**1.469 ± 0.064**	28.71 ± 0.02
0.1X	2	**2.717 ± 0.139**	28.72 ± 0.04
0.15X	0.1	**0.168 ± 0.039**	28.66 ± 0.05
0.15X	0.5	**0.647 ± 0.026**	28.63 ± 0.06
0.15X	1	**1.378 ± 0.073**	28.73 ± 0.04
0.15X	2	**2.887 ± 0.173**	28.70 ± 0.08
0.2X	0.1	**0.132 ± 0.021**	28.59 ± 0.05
0.2X	0.5	**0.509 ± 0.061**	28.72 ± 0.05
0.2X	1	**1.312 ± 0.045**	28.55 ± 0.05
0.2X	2	**2.095 ± 0.274**	28.63 ± 0.15
0.25X	0.1	**0.002 ± 0.001**	31.06 ± 0.42
0.25X	0.5	**0.228 ± 0.186**	29.09 ± 0.50
0.25X	1	**0.15 ± 0.039**	29.70 ± 0.15
0.25X	2	**0.204 ± 0.142**	30.07 ± 0.43
0.3X	0.1	**Inhibited**	**Inhibited**
0.3X	0.5	**Inhibited**	**Inhibited**
0.3X	1	**Inhibited**	**Inhibited**
0.3X	2	**Inhibited**	**Inhibited**

3.1.2. Quantification of Varying Volume of iSWAB™ buffer for Cell Lysis

Since iSWAB™ buffer must be diluted to at least a 0.2X concentration, the high volume of buffer that is contained in the device, approximately 320 µL, was a concern for low in-put samples. Therefore, an experiment was designed to test if the volume of iSWAB™ buffer affects the cell lysis. In this experiment, 300 µL, 140 µL and 60 µL of iSWAB™ buffer were pipetted into 1.5 mL microcentrifuge tubes and 20 µL of a saliva cell suspension were added to each microcentrifuge tube. After the 3-hour extraction period, all samples were brought up to a volume of 320 µL for comparison purposes. The samples were immediately diluted to a 0.1X concentration of iSWAB™ buffer and then quantified. The results in **Table 5** suggest that there is no difference in the extent of cell lysis on DNA availability for PCR when using a 1/5 of normal iSWAB™ buffer volume of a cell concentration of approximately 2-3 ng/uL.

Table 5. Quantifiler® Duo Results for Varying Volume of iSWAB™ buffer for Cell Lysis

iSWAB™ Buffer Concentration	Starting Amount of iSWAB™ buffer for Cell Lysis (µL)	Final Volume before Quantification (µL)	Average Experimental DNA Concentration (ng/µL)	Average IPC C_T Value
0.1X	300	320	2.540 ± 0.315	28.188 ± 0.006
0.1X	140	320	2.503 ± 0.040	28.431 ± 0.124
0.1X	60	320	2.895 ± 0.536	28.299 ± 0.032

3.1.3. **Comparison of Swabs Prepared Days Before Testing vs. Just Prior to Testing**

The objective of this experiment was to compare amounts of DNA obtained from wet swabs (prepared just prior to testing) and dry swabs (prepared and dried days before testing) to determine the amount of DNA retained on swabs after extraction in iSWAB^{TM} buffer. According to a study released by the National Criminal Justice Reference Service, swabbing is the preferred sample collection method over taping for recovering DNA [20]. In order to preserve the cells collected on the swab and prevent mold and other bacterial growth, the swabs need to be completely dried prior to transport and storage. Use of the iSWAB^{TM}-ID collection device eliminates the need to dry swabs, which would be beneficial during evidence collection at a scene and would also minimize contamination to prevent the previously mentioned issues. Tests were conducted to determine if there were any statistically significant differences in DNA yield when using wet swabs or dry swabs in the iSWAB^{TM} device.

3.1.3.1 Pre-Experiment Study

First, a pre-experimental study was performed to assess whether cutting the swab or scraping the swab with a scalpel was a better method to remove the swab from the stick to place it in a spin basket to be spun out. The compatibility of iSWAB^{TM} buffer with a ZyGEM reaction was also tested. The iSWAB^{TM}-ID collection protocol was mimicked using dry nylon flocked swabs with no cellular material.

Three swabs were processed using the cut method and three other swabs were processed using the scalpel method. In the cut method, the swab was cut directly where

the swab met the stick, whereas, in the scalpel method, the swab was scrapped off the stick with a scalpel. The cuts / scrapings of the swab were placed in spin baskets and the excess liquid was collected into a microcentrifuge tube. The amount of liquid was measured for each method. The cut method had an average of 89.67 ± 4.16 µL of excess liquid and the scalpel method had an average of 31 ± 20.07 µL. Due to the large deviation in the scalpel method, the cut method was used in subsequent experiments.

To determine if iSWAB™ buffer had any inhibitory effects on a ZyGEM reaction, 15 µL of a saliva cell suspension were added to the cut method excess iSWAB™ buffer liquid and TE buffer, 10X buffer and *forensic*GEM® were added to bring the final ZyGEM reaction volume to 200 µL. The initial quantitation data showed inhibition in all three cut samples. The concentration of iSWAB™ buffer in each ZyGEM reaction was calculated. The iSWAB™ buffer concentration for all three samples was higher than the 0.25X partial inhibition cut-off value as determined in the previous study. All samples were diluted to a 0.1X concentration of iSWAB™ buffer and quantified again. The results indicate that iSWAB™ buffer at 0.1X does not inhibit a ZyGEM reaction because all IPC values were within range and the expected concentration of 0.90 ng/ µL was approximately targeted for all three samples (**Table 6**). When compared to the ZyGEM control that contained no iSWAB™ Buffer, the DNA concentrations were similar. Thus, the pre-experiment data confirmed that the cut method should be utilized and a ZyGEM extraction of the cells contained in the excess iSWAB™ buffer can be successfully done without inhibition of the sample, so long as the proper concentration of iSWAB™ buffer is met to prevent buffer inhibition. An

alternative to the ZyGEM extraction is leaving the excess buffer (that contains the retained swab) for the 3-hour extraction period and then diluting to a 0.1X concentration of buffer. This alternative method was used in the subsequent experiments. However, for STR profiling comparison purposes, a ZyGEM extraction of the retained cells was performed for this experiment.

Table 6. Quantifiler® Duo Results for Pre-Experiment Using a ZyGEM Extraction

Sample Description	Average Experimental DNA Concentration (ng/μL)	Average IPC Ct Value	Concentration of iSWAB™ Buffer	Inhibition After Appropriate iSWAB™ Buffer Concentration?
Pre-Experiment, Cut Method ZyGEM Extraction, 15 μL of Cells	**0.92 ± 0.11**	28.78 ± 0.06	**0.1X**	No
	0.88 ± 0.00	28.96 ± 0.03	**0.1X**	No
	0.81 ± 0.05	28.88 ± 0.04	**0.1X**	No
ZyGEM Control, 15 μL of Cells, no iSWAB™ Buffer	**0.99 ± 0.31**	29.03 ± 0.06	**N/A**	N/A

3.1.3.2. Quantification of Wet vs. Dry Swab Study

Quantification results on the wet swabs, prepared just prior to testing, and dry swabs, prepared 3 days before testing showed that there is no statistically significant difference in the amount of DNA obtained processing a wet vs. dry swab in the iSWAB™-ID collection device (**Table 7**). All samples were diluted with TE buffer to a 0.1X concentration of iSWAB™ buffer before samples were quantified in duplicate. The total amount of DNA (ng) was calculated by multiplying the average experimental DNA concentration (ng/μL) by 320 μL, the total volume of iSWAB™ buffer contained in the

device, and then finally multiplying by 10, to take account for the dilution of the buffer for quantification of the DNA.

Table 7. Quantifiler® Duo Results for Wet and Dry Swabs Using a iSWAB™ Buffer Extraction

Sample	Average Experimental DNA Concentration (ng/ µL)	Total Amount of DNA (ng)
Dry Swabs	1.569 ± 0.056	**5,021**
	1.811 ± 0.150	**5,795**
	1.240 ± 0.027	**3,968**
Wet Swabs	1.600 ± 0.080	**5,120**
	1.650 ± 0.054	**5,280**
	1.634 ± 0.149	**5,229**
Positive Control	1.773 ± 0.046	**5,674**
Negative Control	0.0	**0**

The overall average DNA concentration for the dry swabs was 1.540 ng/µL ± 0.290 and when compared to the overall average DNA concentration of the wet swabs, which was 1.630 ng/ µL ± 0.030, using a t-test in JMP a p-value of 0.4687 was calculated, indicating that there is no statistically significant difference in the two means. In **Figure 5**, the overlapping circles indicate that there is no statistically significant difference. If there was a statistically significant difference, then the circles would not overlap at all. Therefore, either wet or dry swabs can be used in the devices and one method is not better than the other from a statistical standpoint.

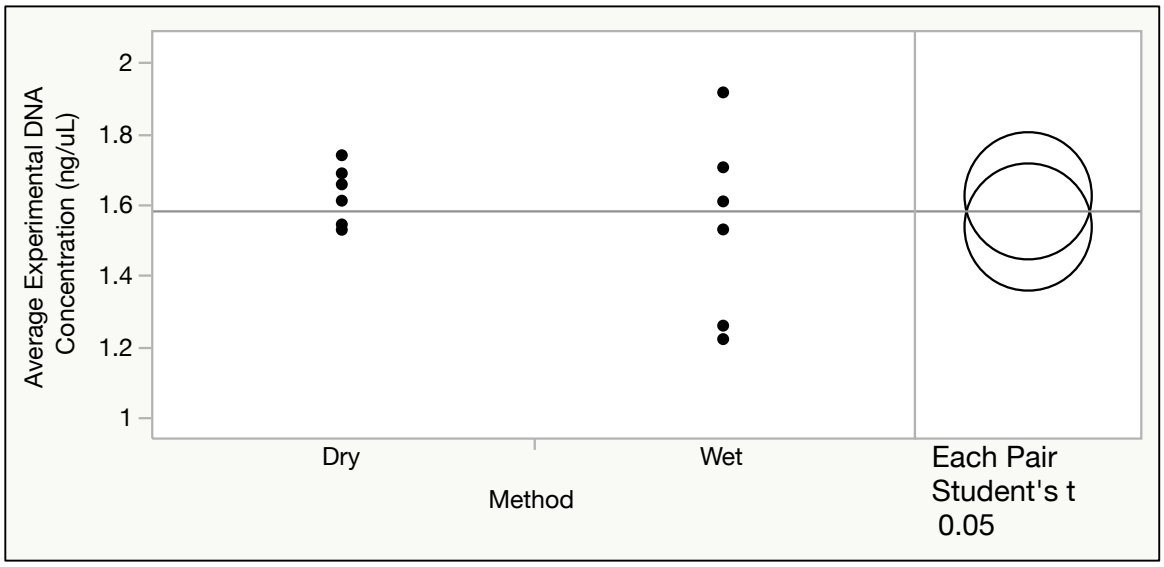

Figure 5. Comparison of Wet and Dry Swabs DNA Recovery

3.1.3.3. Quantification of Amount Retained on Swab After Using Collection Device

As previously mentioned, a ZyGEM extraction was performed on the cells in the eluted iSWAB™ buffer that was recovered after the swabs were taken out of the iSWAB™ collection device, cut and spun. All samples were diluted to a 0.1X concentration of iSWAB™ buffer and quantified in duplicate. The total ng of DNA obtained in each sample was calculated. Two methods of analyzing the data were used to obtain a percentage of the total amount of DNA that was left on the swabs after using the iSWAB™ device (**Table 9**).

The first method of analysis was to compare the amount of DNA retained on the swabs to the total amount of DNA in the positive control, which was directly pipetted in to the iSWAB™ device so it produces the total DNA applied to the swab. The total ng of DNA in the excess volume was divided by the total ng of DNA in the positive control

and then multiplied by 100 to get a percentage of approximate amount of DNA left on the swab after collection device is used. There was no statistically significant difference in the averages obtained between the approximate percent left on dry swabs vs. wet swabs. The average of the averages was calculated to be 31.8% and thus indicated that approximately 31.8% of the total DNA collected on the swab remained on the swabs. The second method of analysis, taking into account sample variation, determined that 26.3% of the total DNA remained on the swab. Instead of comparing it to the total ng of DNA in the positive control, the second method used the total ng of DNA that was on the swab for each sample individually. Therefore, for each sample, the total ng of DNA obtained in the iSWAB^{TM} buffer (**Table 7**) was added to the total ng of DNA in the excess liquid (**Table 8**) to obtain the theoretical total ng of DNA in the sample. Then, the ng of DNA in the excess liquid was divided by that total and multiplied by 100 to obtain the percent of total DNA left on the swab. Like in method 1, the averages were not statistically different between the wet and dry swabs, so an overall average was calculated to be 26.3% of the total DNA is left on the swab after using the iSWAB^{TM} collection device. These calculations assume that there are no DNA/cells remaining on the swab after it is spun.

Table 8. Comparison of the Amount of DNA Retained on the Swabs After iSWAB™ Buffer Extraction

Sample Description	Average Actual DNA Concentration (ng/µL)	Amount of DNA Left on Swab (ng)
Excess iSWAB™ buffer From Dry Swab	1.65 ± 0.04	**1,535 ng**
	2.11 ± 0.06	**1,899 ng**
	1.93 ± 0.01	**1,776 ng**
Excess iSWAB™ buffer From Wet Swab	2.17 ± 0.18	**1,931 ng**
	2.22 ± 0.00	**1,887 ng**
	2.12 ± 0.11	**1,780 ng**

Table 9. Percent of the Total Amount of DNA Retained on Swabs

Method	Overall Average Amount of DNA Left on Swabs (ng)	Method 1: Approximate average % of DNA Left on Swab (compared to Positive Control)	Method 2: Approximate average % of DNA Left on Swab (ng of DNA left on swab/total ng of DNA on sample)
Dry	1,736.4 ± 185.4	30.6	26.1
Wet	1,866.1 ± 77.8	32.9	26.4

3.1.4. Prong vs. Non-Prong

The iSWAB™-ID collection device is considered a device due to the mechanics within the collection tube. Mawi DNA Technologies' designed the mechanics of the tube to maximize the collection of DNA by placing a prong system within the tube. This patented sample recovery and collection device comprised of a tube, a cap, an insert and an opening. The insert includes an opening at the top that is adhered to the top of the tube, which is where the swab enters from. The insert then extends into three legs that

create prongs. The prongs extend inward towards each other, creating a smaller opening at the bottom of the insert. Thus, as the swab is inserted into the top opening, a little force is needed to get the swab through the smaller diameter at the bottom of the insert into the proprietary lysis buffer. Once the iSWAB^{TM}-ID collection protocol is followed, the swab is removed by pulling and twisting the swab in a corkscrew motion up through the smaller diameter of the insert, which squeezes the swab out, and then the swab is continued to be wrung out through the top of the tube [21].

An experiment was designed to assess the effectiveness of the prong mechanics of the device. Two saliva cell swabs in the iSWAB^{TM}-ID collection device were compared to two saliva cell swabs prepared in the same amount of iSWAB^{TM} buffer in microcentrifuge tubes. Mawi DNA Technologies did invent a tool to easily remove the prongs out of the device, but it was not used in this experiment. The collection protocol was mimicked while pulling the swabs out of the microcentrifuge tubes. Similar to the previous experiment, the amount of cells that were retained on the swabs was assessed by spinning the swabs in spin baskets into microcentrifuge tubes. This study went further and determined the amount of cellular material left on the swab after the spin basket step. All swabs, after being spun in the spin baskets, were placed in an iSWAB^{TM} buffer volume of 300 µL (volume necessary to fully submerge the swab). All samples (prong/non-prong samples, eluted samples and swabs) were extracted for 3 hours in the iSWAB^{TM} buffer. After the extraction, samples were diluted to a 0.1X concentration of iSWAB^{TM} buffer and quantified. The average total ng of DNA was calculated for all samples by multiplying the concentration of DNA in ng/µL by the volume of the sample

and then by 10 to take into account the dilution factor. A calculation of the total ng of DNA per sample was calculated by taking a sum of the ng of DNA in the prong/non-prong sample, the eluted sample and the swab sample. These calculations assume that no DNA remains on the swab after the swab was fully extracted.

Table 10. Comparison of the Amount of Total ng of DNA Recovered for the Prong and Non-Prong Samples

Sample Description	Average Experimental DNA Concentration (ng/μL)	Total ng of DNA	Average Total ng of DNA	% of total ng of DNA recovered
Prong Samples	1.264 ± 0.083	3,488.60	3,326.10 ± 229.81	**78.6**
	1.138 ± 0.052	3,163.60		
Non-Prong Samples	1.231 ± 0.064	2,597.40	2,833.35 ± 333.68	**56.5**
	1.441 ± 0.013	3,069.30		

The average total ng of DNA on the swabs that were processed using the prong device was approximately 4,320 ng and for the swabs in the non-prong microcentrifuge tubes the total was approximately 5,013 ng. The percent of total ng of DNA recovered from the prong and non-prong samples was calculated by dividing the total ng of DNA in the prong/non-prong sample by the total ng of DNA on the swab used for the sample and then multiplying by 100 to get a percentage.

$$\%\ of\ total\ ng\ of\ DNA\ recovered = \frac{total\ ng\ of\ DNA\ recovered\ from\ sample}{total\ ng\ of\ DNA\ on\ the\ Swab\ before\ testing} \times 100$$

$$total\ ng\ of\ DNA\ on\ the\ swab\ before\ testing = ng\ of\ DNA\ in\ sample + ng\ of\ DNA\ in\ eluted\ sample + ng\ of\ DNA\ in\ swab\ sample$$

For prong samples, the average percent recovery was 78.6% of the total ng of DNA, which was significantly higher than the 56.5% average recovery of the total ng of DNA for the non-prong samples (**Table 10**). A p-value of 0.0409 when running a t-test on the data was obtained, which signifies that there is a statistically significant difference in the two different methods. As for the amount of DNA that was eluted from the swab after being spun, the prong samples had approximately 20.4% of the total DNA retained on the swab, whereas the non-prong samples had approximately 31.5% of total ng of DNA retained on the swab. Thus, leaving only 1% of the total ng of DNA on the swab, after the swab is spun out, for the prong samples and 12% of the total ng of DNA on the non-prong samples (**Figure 6**). It is evident that the prongs squeeze excess buffer that contains cellular material, off of the swabs and as expected would have a smaller eluted volume of buffer/cellular material. However, the prongs do an exceptional job of getting more cellular material off of the swabs, as evident in the 1% that remains on the swab in the prong samples versus the 12% of cellular material that remains on the swabs in the non-prong samples after elution. Therefore, use of the iSWAB^{TM}-ID collection device results in a significant increase in the collection of cellular material.

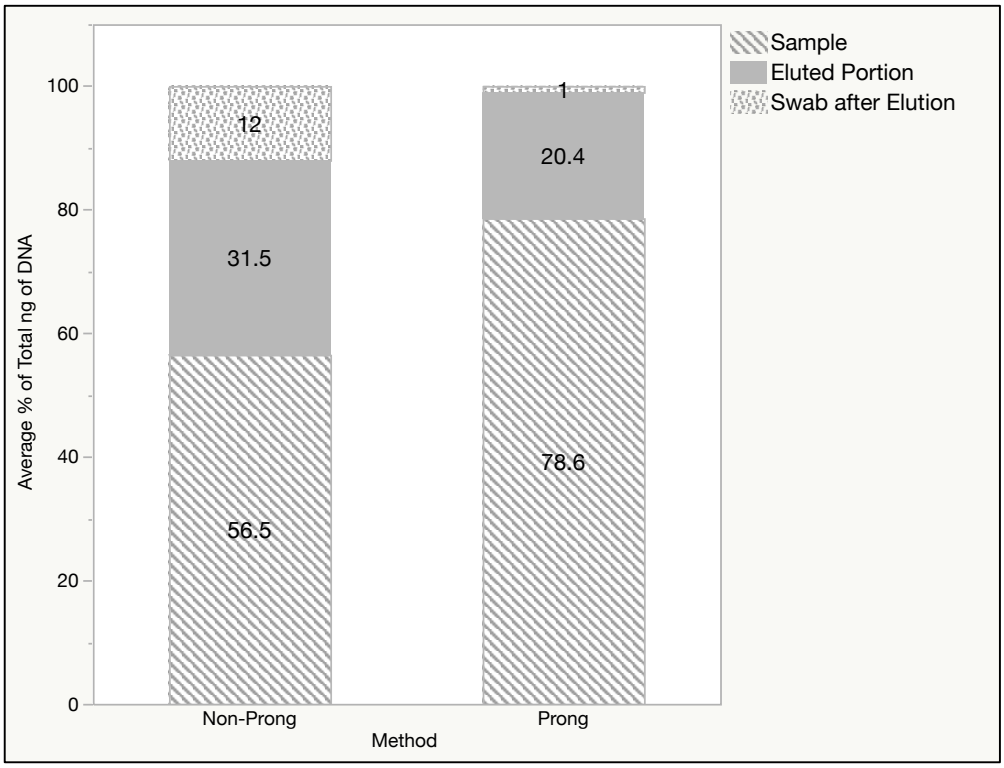

Figure 6. Percent of DNA that is Recovered and Retained for the Prong and Non-Prong Samples

Due to the Results for the experiment, a sub-experiment was designed that left the swab in the device for the full 3-hour extraction and then pulled the swab through the prongs of the device using the iSWAB^{TM}-ID collection protocol for removing the swab. Saliva cell swabs, prepared using the same method as the swabs in the previous study, were placed into the iSWAB^{TM}-ID collection devices and removed after 3 hours. The swab material was cut as described previously, spun in spin baskets and the recovered liquid was returned to the original device. The spun swab was placed into 300 µL iSWAB^{TM} buffer to determine the amount of cellular material retained on the swab after elution. An average of approximately 0.5% of the total ng of DNA was retained on the

swab after the 3-hour extraction period. Thus, for forensic samples, when using the iSWAB^{TM}-ID collection device, to maximize release of cells and DNA, swabs can be kept in the device for the 3-hour extraction period, removed and spun and then liquid recovered can be placed back into the device.

3.1.5. Collection of Dried Stains

The previous studies were conducted on buccal cells using nylon flocked swabs. An additional study was designed that incorporated semen and whole blood, along with another type of swab—the classic cotton swab. The objective of this study was to determine the most effective method of collection for dried body fluid stains. In forensics, blood, semen, body tissue, hair, saliva and urine can be transferred to victims, suspects, witnesses, objects or scene directly or indirectly. Once liquid biological specimens have been deposited, they adhere to the surface or substrate and become stains. The fluids will dry onto the surface and remain there until disrupted or collected. Once the stain is dried onto the surface, full collection of the stain becomes challenging.

Typically, dH_2O is used to moisten a sterile cotton swab to collect the dried stained area [22]. It has recently been suggested that cotton swabs may not be particularly effective at retrieving and later releasing cellular material [22] and thus a flocked swab design made from nylon was designed. In the previous experiments, nylon flocked swabs were used because they were provided by Mawi DNA Technologies. The nylon flocked design enables rapid absorption by capillary action and minimizes entrapment of collected samples by holding the sample close to the swab surface. Due to

the increased absorption, only a single wet swab is required when sampling, instead of the wet and dry swab taken when using cotton swabs. However, since the iSWAB^{TM}-ID collection device is compatible with many different types of swabs and the use of cotton swabs is still common, cotton swabs were incorporated into this experiment for comparison.

Semen, saliva and blood stains were deposited onto sterilized petri dishes. Care was taken to guarantee that all petri dishes were created in an equal fashion. After several days of drying the stains, cotton and nylon flocked swabs were used for collection. Swabs were moistened with 100 µL of dH_2O or iSWAB^{TM} buffer before collecting the stain. A standardized swabbing technique was employed to ensure that all swab stain combinations were treated equally. All swabs of samples were left in iSWAB^{TM}-ID collection devices for approximately 3 days to extract, due to prior information that sperm cells would lyse in iSWAB^{TM} buffer if left for a longer extraction period. All samples were spun in spin baskets, the eluted liquid was returned to the original device tube, and the samples were diluted to a 0.1X concentration of iSWAB^{TM} buffer and quantified in duplicate.

The total amount of DNA in each sample was obtained by multiplying the concentration of DNA in ng/µL times 320 µL, the volume of iSWAB^{TM} buffer, and then times 10 to account for the dilution factor. The average percent recovery was calculated by comparing the average total ng of DNA of the sample to either the average total ng of DNA on the control cotton swab or the control nylon flocked swab, depending on the sample.

iSWAB™ buffer was able to lyse all cells types—results were obtained with expected concentrations for sperm cells, buccal cells and white blood cells. As observed in **Figure 7**, moistening the swabs with iSWAB™ buffer proved to be an equally as good and often better collection method across all body fluids. Contrary to what was excepted, cotton swabs outperformed nylon flocked swabs for the collection of dried blood and saliva stains. It is important to note that the dried blood stains were very flaky and hard to collect in a fair and equal manner. It did appear that a significant amount of the flakes was caught in the nylon flocked swabs after extraction in the iSWAB™ buffer, which could explain the variation. However, it is also important to mention that moistening the cotton swab was a very simple task due to its absorbent nature, but moistening the nylon flocked swab required a lot more care and attention to ensure that the swab adsorbed the specific amount of either dH_2O or iSWAB™ buffer. This could have affected the results as well. For sperm collection, the Results for this experiment are consistent with current research in the field that nylon flocked swabs are more efficient than cotton swabs for picking up sperm cells [23]. Interestingly, dried saliva stains recovered the least amount of total ng of DNA.

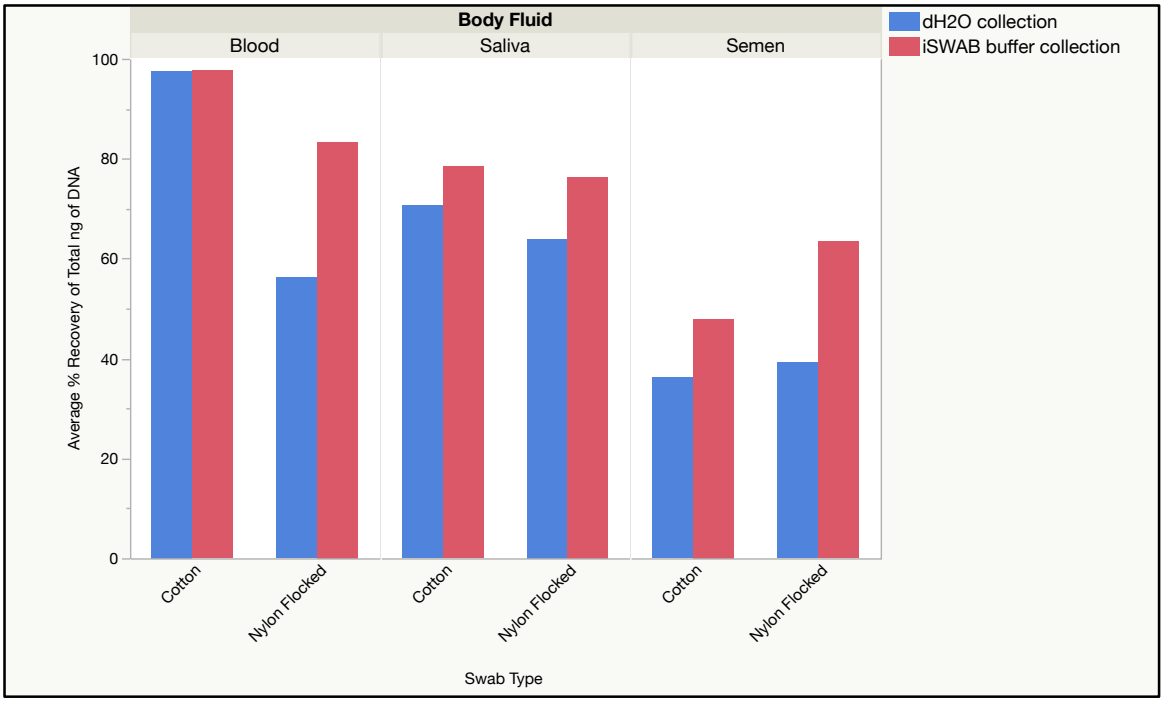

Figure 7. The Average Percent Recovery of Total ng of DNA from the Collection of Dried Stains Experiment. The blue represents the average percent of total ng of DNA recovered for samples that were collected with swabs moistened with dH_2O. The red represents the average percent of total ng of DNA recovered for samples that were collected with swabs moistened with iSWAB™ buffer.

3.1.6. Comparison of Qiagen Extracted Samples Study

The Qiagen QIAamp® DNA Investigator Kit is one of the most commonly used extraction method to process forensic samples. A Qiagen extraction is comprised of four main steps: disruption of cellular membranes using a combination of enzymatic detergent activity and mechanical lysis (heating and shaking); binding of DNA to the silica-based membrane of the spin column; washing of contaminants through the column using buffers; and DNA elution [24]. In forensics, it is essential to obtain optimal quantities of DNA that are free of contamination, however, as previously mentioned Qiagen is known

to have DNA loss throughout the extraction process [1]. Unlike a Qiagen extraction procedure, an iSWAB™ extraction does not remove any contaminants. A comparison study was designed to assess the recovery of DNA and the quality of the amplification / STR profiles between Qiagen and iSWAB™ Buffer. For each extraction, 50 µL of a saliva cell suspension was pipetted directly into the buffers. No swabs were utilized in this experiment.

Table 11. Comparison of the Total Amount of DNA (ng) Recovered from Samples Extracted with iSWAB™ Buffer vs. Samples Extracted with Qiagen

Sample	Experimental DNA Concentration (ng/µL)	Total Amount of DNA (ng)	Average Total Amount of DNA (ng)
iSWAB™ Buffer Extracted Samples	0.414	621	**552 ± 62**
	0.334	501	
	0.356	534	
Qiagen Extracted Samples	0.129	129	**162 ± 40**
	0.150	150	
	0.207	207	

All samples were diluted with TE buffer to a 0.1X concentration before samples were quantified in duplicate. For the iSWAB™ Buffer extracted samples, the total amount of DNA (ng) was calculated by multiplying the experimental DNA concentration (ng/µL) by 150 µL, the total volume of the iSWAB™ buffer (100 µL) and of the cell suspension (50 µL) mixture contained in the device, and then finally multiplying by 10, to take account for the dilution of the buffer for quantification. The total amount of DNA (ng) obtained in the Qiagen extracted samples was calculated similarly, with the exception of multiplying by 100 µL, the total volume of elution. As observed in **Table**

11, the iSWAB™ buffer extracted samples obtained an average of 552 ± 62 ng of total DNA, approximately 2 times more DNA than the Qiagen extracted samples that obtained an average total amount of DNA of 162 ± 64 ng in 100 µL. This result is an example of the loss of DNA that can be observed using a Qiagen extraction compared to a direct lysis method.

This experiment was done under very controlled settings using washed saliva samples, which were added directly to iSWAB™ buffer or Qiagen's ATL buffer, which prevented most contaminates. Additional experiments are needed to address the impact of contaminant concentrations on the two methods.

3.1.7. Three-Month Time Course Evaluation

Once extracted, DNA is a very stable molecule. However, the time from cell collection at a crime scene to lysis and analysis in the laboratory can vary from hours to days to months to even years. The stability of different cell types can vary and often depends on the conditions of storage. Liquid semen and saliva, if stored at -20 ˚C can be stable up to a year later. Most cells require refrigeration or freezing or drying to maintain their integrity. The iSWAB™-ID collection device stores samples at room temperature and keeps the DNA stable for over a year, according to the manufactures [12]. Preliminary evaluation of storage time was done to assess the effects of storage of saliva samples at room temperature in the iSWAB™ buffer. A saliva cell suspension was pipetted into the iSWAB™-ID collection device and left for 1 to 3 months. Additionally, the same amount of saliva cell suspension was put into a 2.0 µL microcentrifuge tube

containing 320 µL of TE Buffer to assess the degradation effects of storage of saliva cells at room temperature. The cell suspension was stored in the -20 ˚C and sampled every month as a control. During each month of sampling, a ZyGEM extraction was performed on the control saliva cell suspension sample and the TE buffer sample.

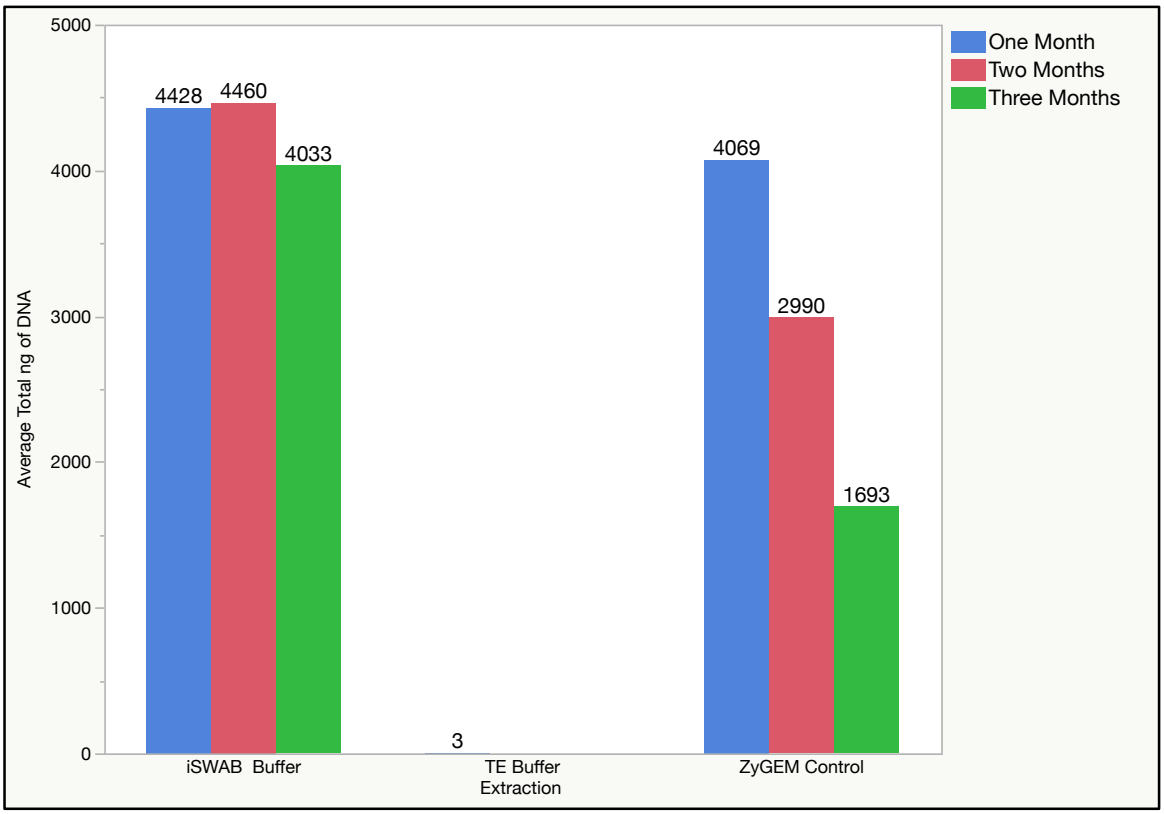

Figure 8. Bar graph Showing Average Total Amount of DNA (ng) Observed Across the Three-Month Time Course Study. The iSWAB™ Buffer and TE Buffer cells were stored at room temperature. The ZyGEM control was stored at -20 ˚C.

Figure 8 displays the Results for the time course study. There was no statistically significant difference in the average total amount of DNA (ng) between the 1st and 2nd month samplings, nor the 1st and 3rd month samplings, however, there was a statistically significant difference between the 2nd and 3rd month samplings, with a p-value = 0.0359.

Since there was no statistically significant difference between the 1st and 3rd samplings, it can be concluded that there was no statistically significant change in the total ng of DNA after 3-months. The statistically significant difference between the 2nd and 3rd month could be due to pipetting error or variability / difference in quantitation sensitivity because the samples were not run together on the same quant.

There was a statistically significant difference among the total amount of DNA obtained each month using a ZyGEM extraction on the saliva cell suspension which had been stored in -20 ˚C. Suggesting that long term storage of an un-extracted cell suspension in the freezer, along with freezing and thawing multiple times, can degrade the cellular material. However, unlike the monthly samplings from the iSWAB™ devices, only one sampling was taken from the saliva cell suspension. Perhaps the differences in the three sampling of the saliva cell suspension would have been less significantly different and more accurate had multiple samplings been taken each time.

3.2. STR Profiles

A total DNA amount of 1 ng was amplified using the AmpFℓSTR® Identifiler® Plus kit for the samples in the Wet vs. Dry Swabs and the Prong vs. Non- Prong experiments. For samples of the Collection of Dried Body Fluid, Qiagen Comparison and Time Course Study, a total DNA amount of 0.75 ng was amplified using the Identifiler® Plus kit and 0.5 ng was amplified using the GlobalFiler® kit. These amounts were chosen based on current research that GlobalFiler® yields profiles with significantly higher peak heights than other kits [39]. The profiles generated were analyzed using

GeneMapper IDX software using manufacturer provided allelic ladders, bins and panels. The quality of the profiles was compared among samples and between the PCR Amplification kits. Full STR profiles were successfully obtained from all samples. The quality of the DNA profiles was evaluated based upon the total number of reportable alleles, peak heights and peak height ratios. The reportable number of alleles is defined by the alleles within the loci analyzed where the peak heights were above a detection threshold and all stutter and other artifacts have been removed [27].

The overall average PHR across the samples extracted with the iSWAB™ buffer and amplified with Identifiler Plus targeting 1.0 ng was 0.88, which is above the 0.60 peak height ratio threshold cutoff that is commonly used [25]. In fact, all loci produced PHR that were above 0.6. **Figure 9** shows the PHR of all loci amplified with Identifiler® Plus targeting 1.0 ng. The boxplot depicts the range of peak height ratios of the samples (N=24).

Figure 10 compares the PHR and average PH for the samples extracted with ZyGEM compared to the samples extracted with iSWAB™ buffer. The comparison was only done on samples that targeted 1.0 ng of DNA. Since more of the samples were extracted with iSWAB™ buffer, there is a bigger range for the values of iSWAB™ buffer, however, the average of the PHR for iSWAB™ buffer samples and ZyGEM samples are very similar. The average PHR for iSWAB™ extracted samples was 0.86, which was close to the PHR of 0.90 for ZyGEM samples, which again had a smaller sample size, so an equal comparison cannot be made.

An equal comparison can be made to the samples that were extracted with iSWAB^{TM} buffer and collected with either cotton swabs or nylon flocked swabs. There was absolutely no difference in the quality of the profiles for either method of collection. The overall peak height averages were the same, averaging 0.84 (**Figure 11**).

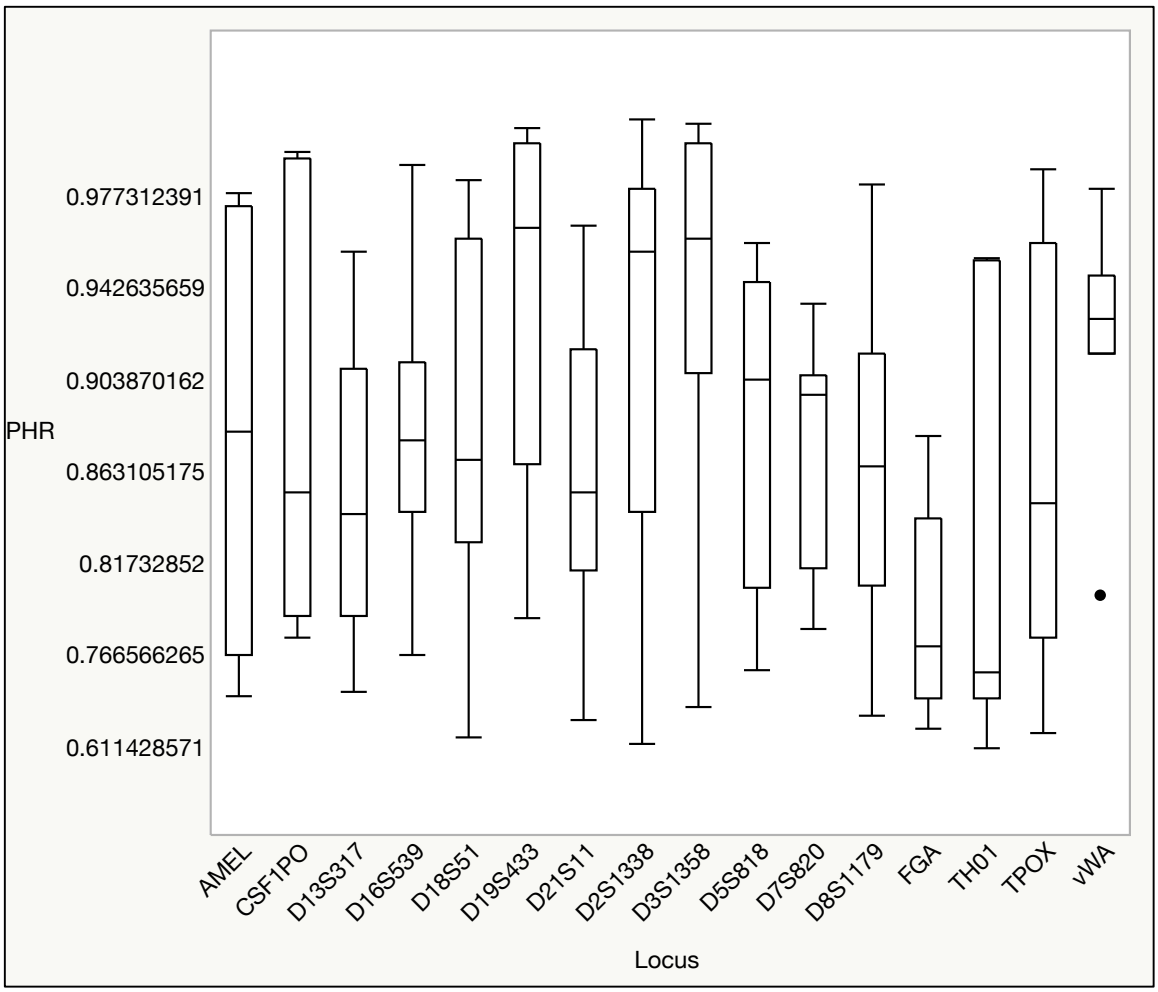

Figure 9. Peak Height Ratios across 16 loci of the Identifiler® Plus Kit. The boxes represent the 25th to the 75th percentile of the data, the whiskers show the range of the data and the point outside the whisker observed at the vWA loci is a suspected outlier (data point is different than the mean by more than twice the pooled standard deviation). The horizontal line within the box represents the mean value for each locus.

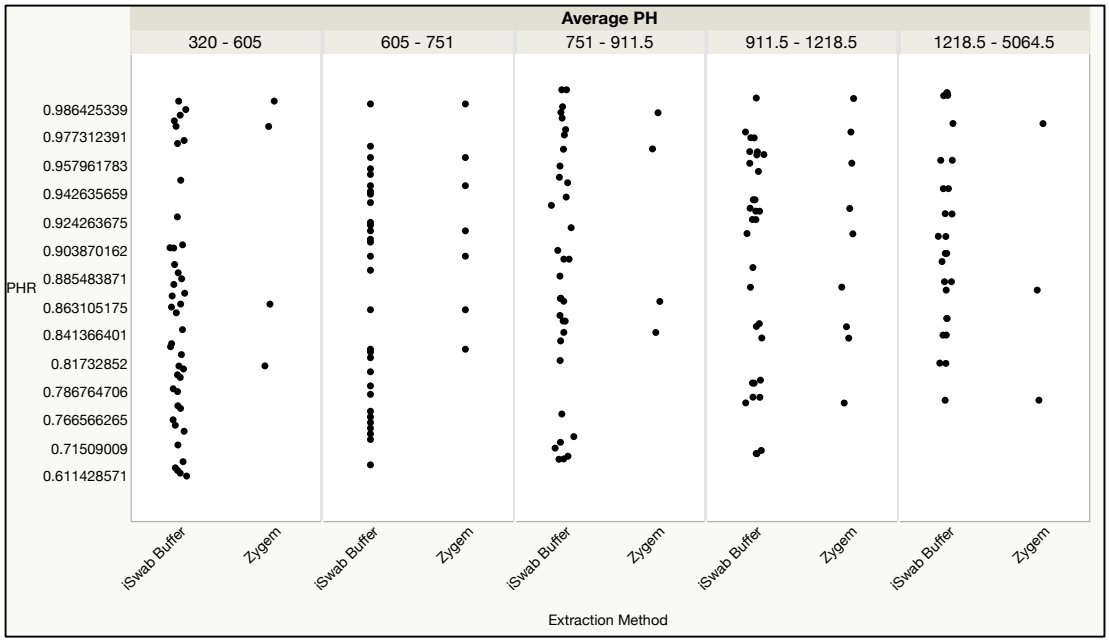

Figure 10. A Comparison of Peak Height Ratios and Average Peak Heights between samples extraction with the iSWAB^{TM} Buffer and ZyGEM

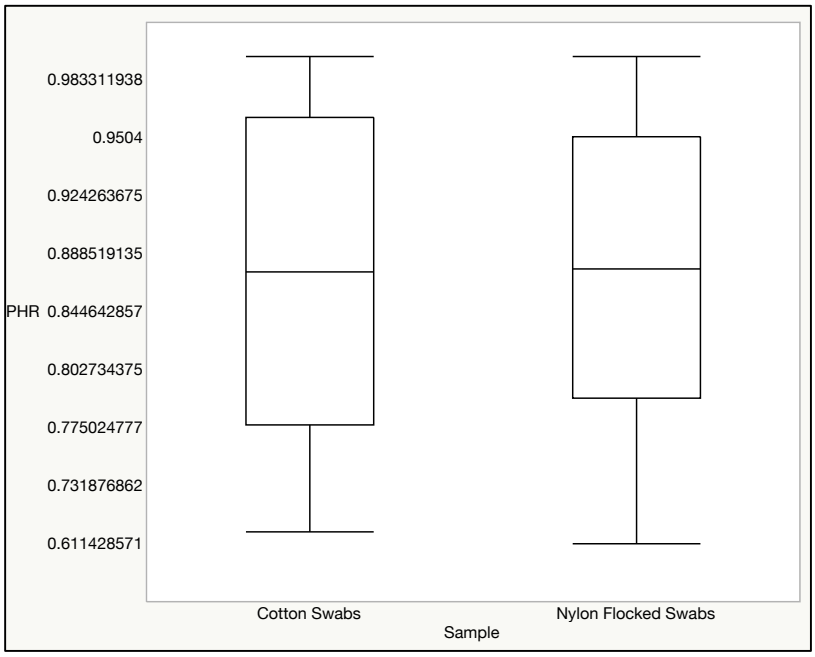

Figure 11. Comparison of the Peak Height Ratios for Cotton Swabs and Nylon Flocked Swabs

More than one amplification kit was used to assess the quality of the STR profiles from an iSWAB™ buffer extraction. Samples included in the Qiagen comparison experiment, Collection of Dried Body Fluid experiment and Time Course experiment were amplified using both the Identifiler® Plus Kit and GlobalFiler® Kit and a comparison was made between the samples and between the kits. The overall average of PH for the samples amplified with GlobalFiler® was 1202 RFU, which is roughly twice the overall average PH of 632 RFU for the samples amplified with Identifiler® Plus. This result is consistent with the findings of current research comparing the two amplification kits [39].

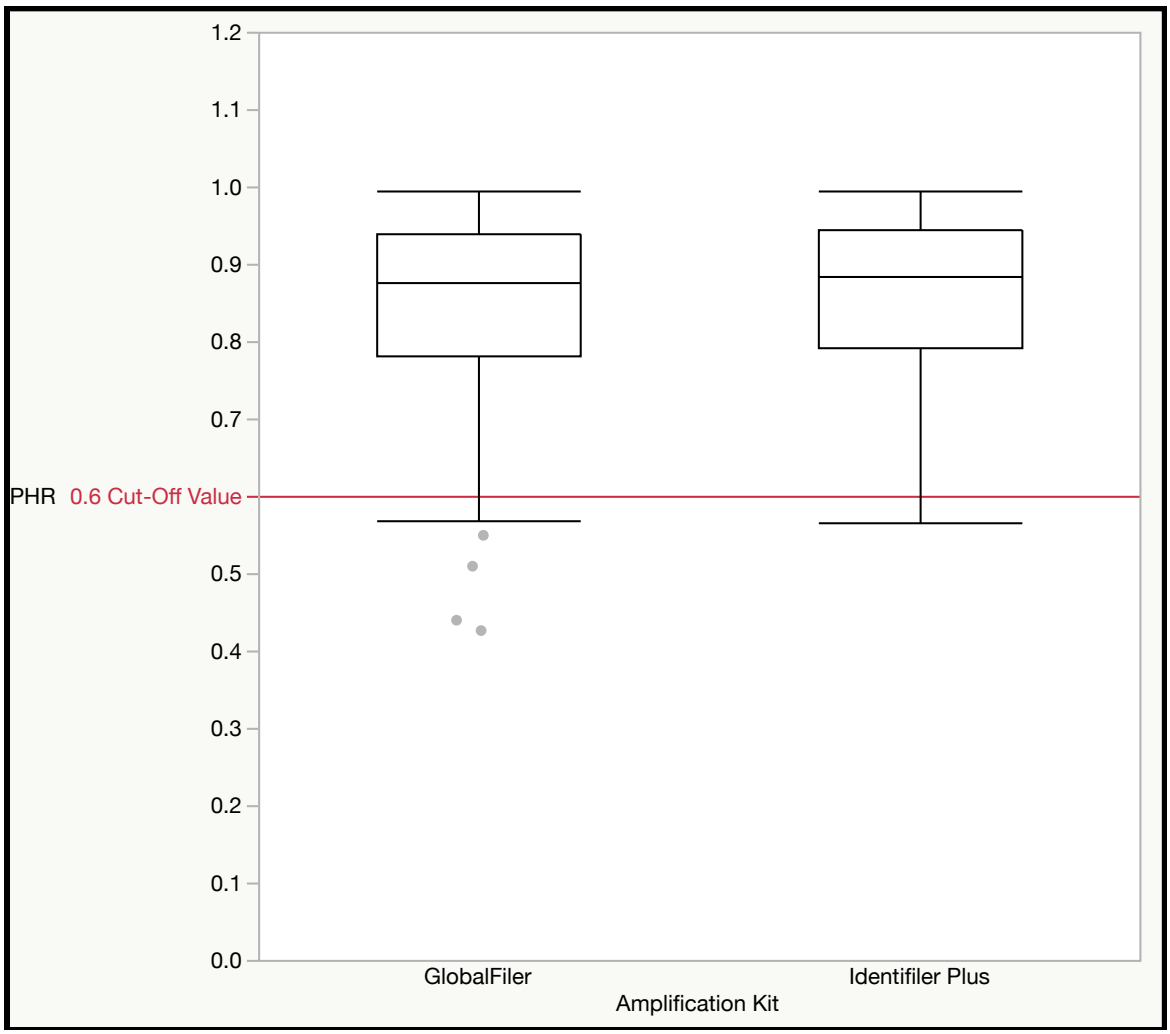

Figure 12. PHR Comparison Between the Samples Amplified using the GlobalFiler® and the Identifiler® Plus Amplification Kits

The overall PHR for samples amplified with both kits was approximately 0.87, which was above the acceptable PHR cut-off value of 0.6 designated in red in **Figure 12**. Thus, the data shows that iSWAB™ buffer extractions are compatible with 5-dye and 6-

dye kits and that the kits perform similarly with respect to PHR. There were a few loci in 3 profiles that had PHR that fell below the acceptable PHR. When processing a sample, if there is concern about the amount of DNA recovered, especially once diluted when extracted with iSWAB™ buffer, GlobalFiler® may prove to be more advantageous to use as an amplification kit. Since the average PH of GlobalFiler® profiles was approximately 2X the average PH of the Identifiler® Plus profiles, when targeting a lower amount of DNA for the GlobalFiler® reactions, there would be a higher probability of obtaining a full profile with the GlobalFiler® kit.

Interestingly, when comparing a subsample of that data, specifically looking at the Qiagen vs. iSWAB™ buffer extraction samples, the Identifiler® Plus amplifications had a higher PHR for both types of extractions. The average PHR for the samples amplified with Identifiler® Plus was 0.87, which is statistically significantly higher than the average PHR of 0.82 for the GlobalFiler® reactions. This difference could be due to the fact that this subset contained multiple GlobalFiler® profiles that had loci with PHR values that fell below the 0.6 cut-off value, marked in red in **Figure 13**. The samples extracted with Qiagen tended to have higher PHR than the samples extracted with the iSWAB™ buffer, with a total average PHRs of 0.85 and 0.83 respectively, however, this difference is not statistically significant. Thus, the two different extraction methods produced similar quality profiles.

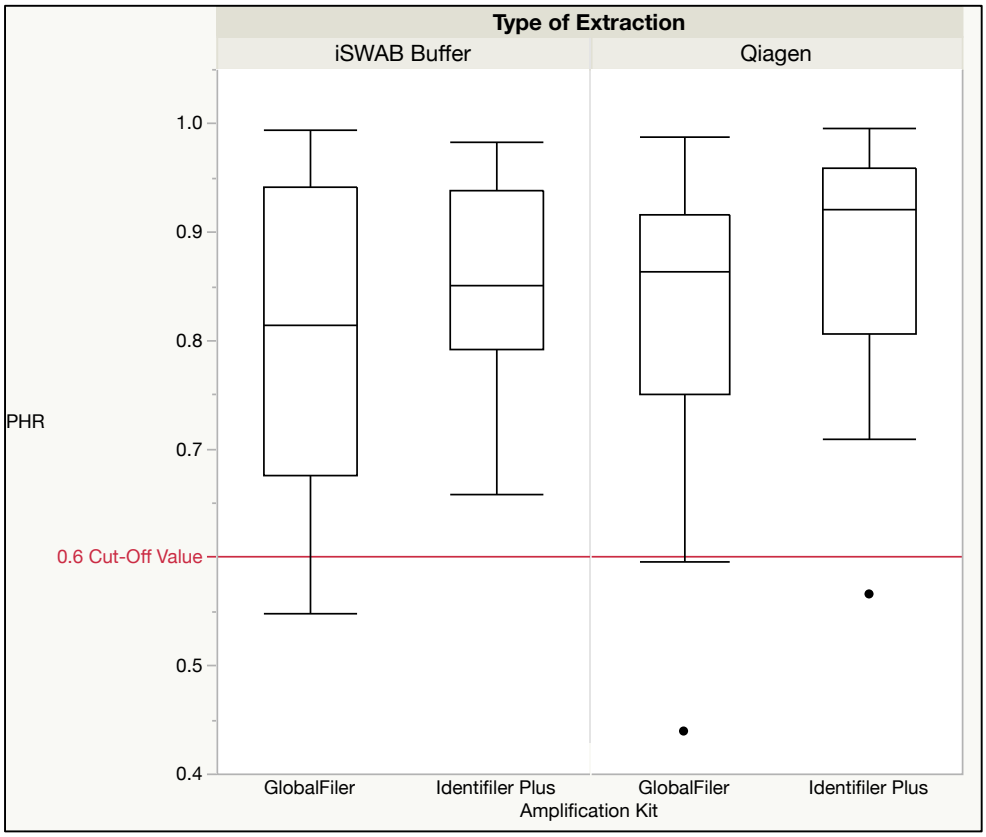

Figure 13. Comparison of the PHR of Samples Using Different Extraction Methods and Different Amplification Kits

The Time Course Study showed no degradation in the profiles throughout the 3-month period. As seen in **Figure 14**, the average PH increased throughout the study. This increase was observed using both the GlobalFiler® and Identifiler® Plus amplification kits. The average PHR of the samples for each month were all above the 0.6 cut-off value, designated by the red line in **Figure 15**. For the samples amplified with Identifiler® Plus kit, the average PHR increased each month. Whereas, the average PHR for the samples amplified with the GlobalFiler® was highest in the second month

sampling. However, the difference in PHR across all of the samples from the study, samples amplified with both kits, is not statistically significant, indicating that there is no change in the quality of the profile when storing samples in iSWAB™ buffer for an extended period of time.

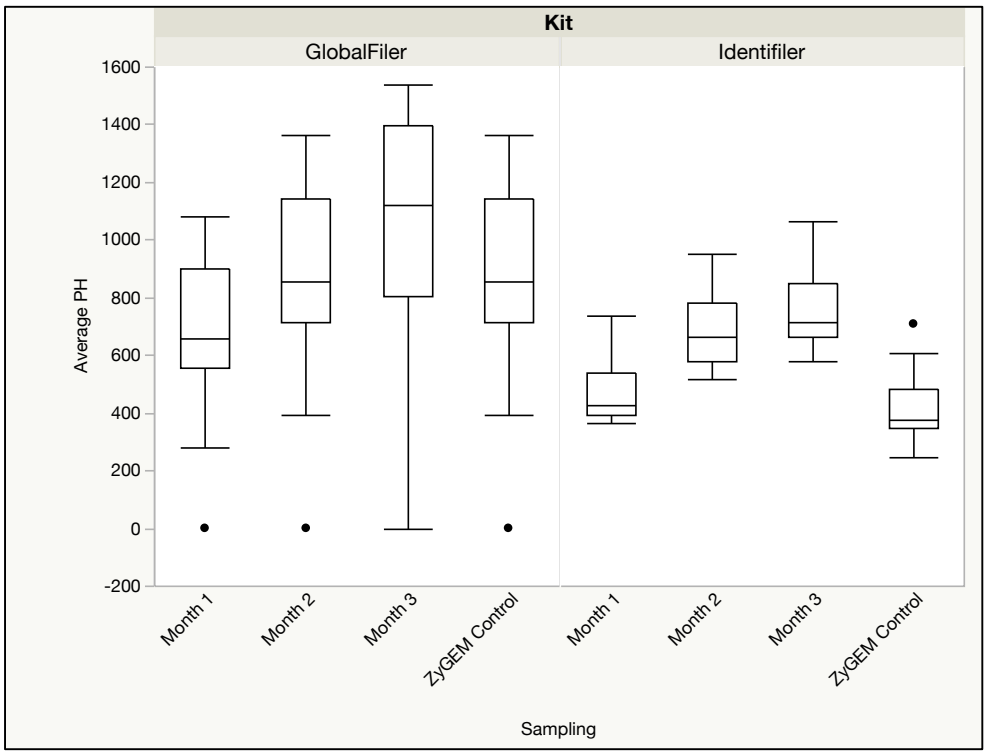

Figure 14. Comparison of average PH across the Time Course Study using GlobalFiler® vs. Identifiler® Amplification Kits

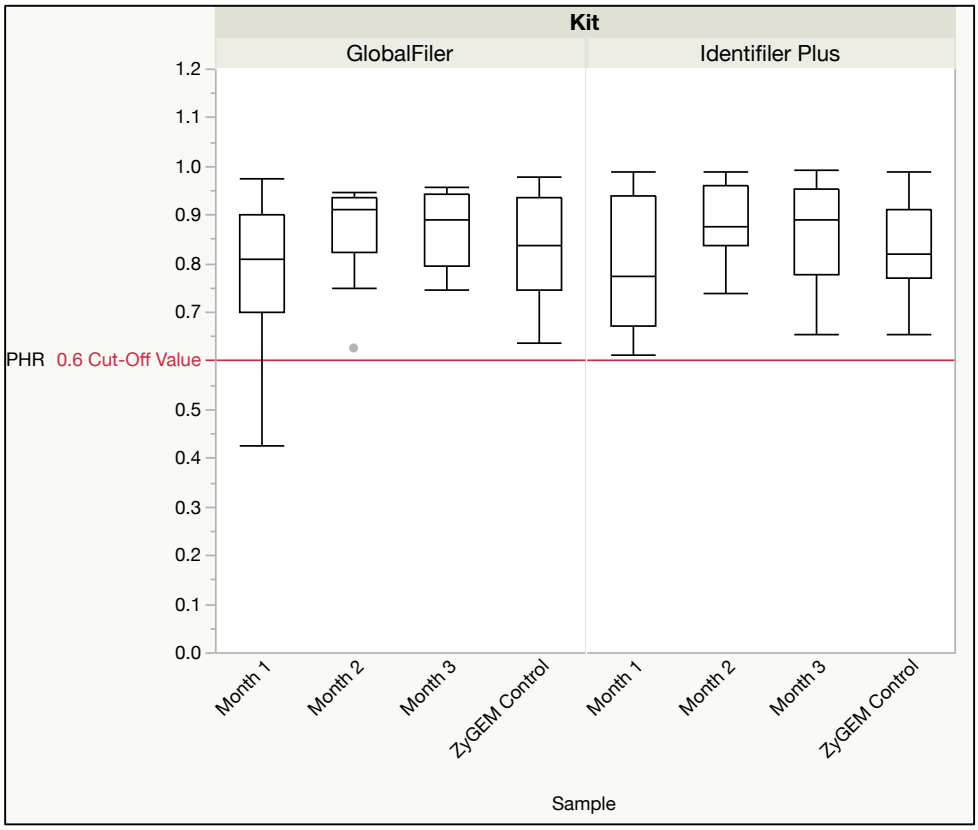

Figure 15 Comparison of the PHRs across the Time Course Study Using GlobalFiler® and Identifiler® Plus

Figure 16 and **Figure 17** are examples of STR Profiles produced by a sample that was extracted in iSWAB™ buffer. This sample was the third month sampling from the Time Course study. These profiles demonstrate the relatively even peak heights at each loci. The Identifiler Plus profile in **Figure 17** contains one peak that is labeled OL, which stands for off-ladder. This means that the peak is not found on the allelic ladder and is not a true allele, which categorizes the peak as an artifact. In this profile, the OL peak in the blue channel is pull-up, otherwise known as bleed-through, from the green

channel. Less of the sample can be injected into the CE or the sample can be re-amplified with less input DNA to avoid pull-up. Pull-up was a common artifact found throughout the samples. No additional types of profile artifacts were observed.

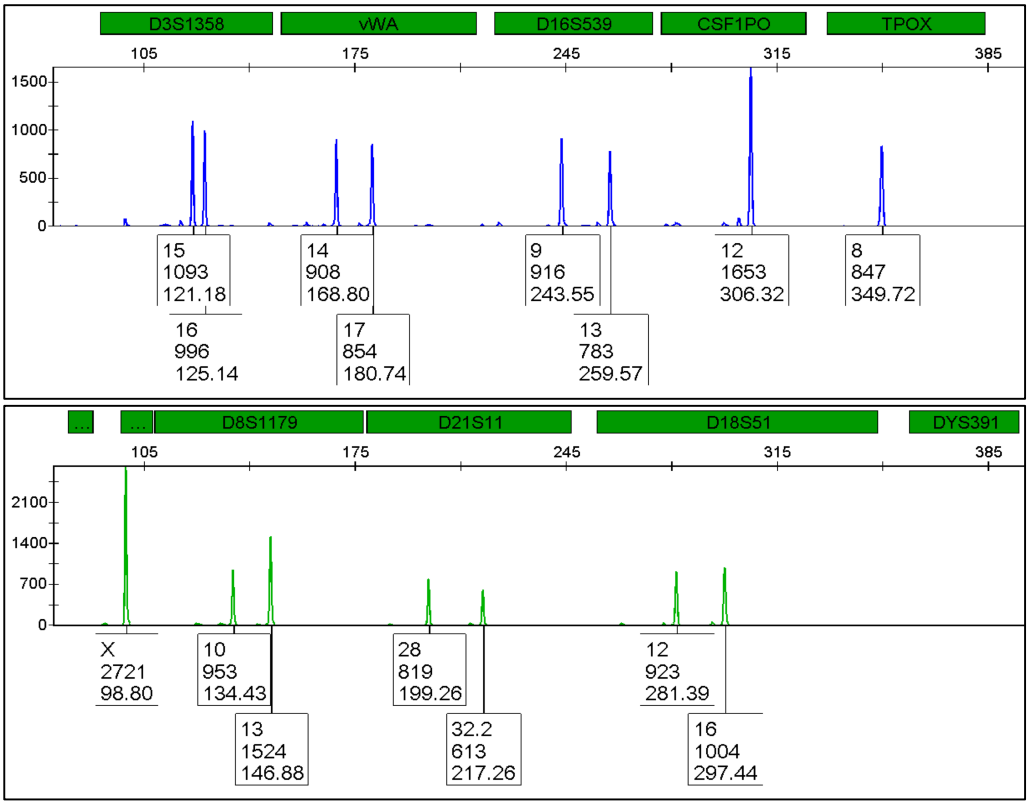

Figure 16. Blue and Green Dye Channels of a Sample Extracted in iSWAB™ Buffer and Amplified Using the GlobalFiler® Kit (0.5 ng of DNA)

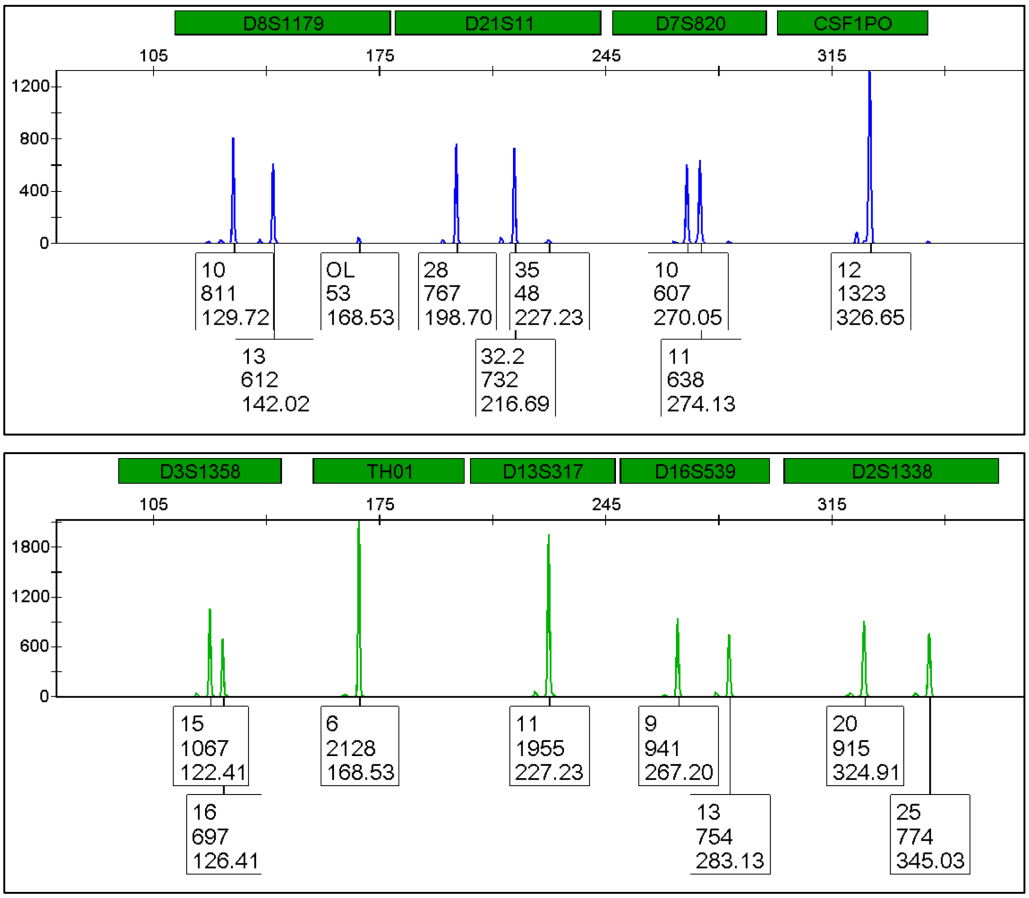

Figure 17. Blue and Green Dye Channels of a Sample Extracted in iSWAB™ Buffer and Amplified Using the Identifiler® Plus Kit (0.75 ng of DNA)

4. Considerations and Conclusion

Mawi DNA Technologies' slogan "Collect-Stabilize-Transport-Store: All in a Single Tube" truly describes the ease of their product. The development of the iSWAB^{TM}-ID collection device not only provides benefits for bio-sampling in the medical field, but also serves as an extremely efficient way to collect, stabilize, transport and store forensic DNA samples [6-7]. Some important parameters were determined in this study. First, a dilution of at least a 0.20X concentration of the iSWAB^{TM} buffer must be made to be compatible with downstream forensic PCR based processes. Second, the volume of iSWAB^{TM} buffer used for lysis does not affect the quality of cell lysis. Third, the iSWAB^{TM}-ID collection device is compatible with various swabs and the collected samples do not have to be dried before processing in the device. The mechanism within the iSWAB^{TM}-ID collection device significantly improves the recovery of cells off of swabs, especially when the swab is spun in a spin-basket and the excess liquid containing cells is returned to the collection device. In terms of forensic application, this study demonstrated that the iSWAB^{TM} buffer can lyse various types of cells and can be used as a wetting agent on the swab to improve cell pick-up and recovery of dried stains. Also, this study demonstrated that the buffer can keep DNA stable at room temperature for an extended period of time and that full profiles can be obtained using different STR kits targeting as low as 0.5 ng of iSWAB^{TM} buffer extracted DNA. While initial research in this study suggests that the iSWAB^{TM}-ID collection kit is beneficial to the forensic community, there are some limitations to the device and this study that should be mentioned.

The volume of iSWAB buffer that comes in the device is potentially too large for some types of forensic samples. iSWAB™ buffer extracted samples cannot go directly to amplification because of the inhibitory properties of the 1X concentration of the chemistry in the proprietary buffer. Thus, the buffer must be diluted at least to 0.2X concentration before PCR can occur. The current volume that comes in the iSWAB™-ID collection device is perfect for processing known standards of suspects or victims, however, not very practical for most evidence samples. At crime scenes, the amount of DNA collected is never actually known and can vary in sample size. If a low in-put DNA sample is collected and placed into the 320 uL of iSWAB™ buffer, the buffer itself will dilute the sample and then the sample will be diluted even further in order to prevent inhibition in PCR. This could leave the sample in too low of a concentration for analysis. Since this study demonstrates that the volume of buffer does not affect the quality of the cell lysis, Mawi DNA Technologies should consider an additional design of the iSWAB™-ID collection device for low input samples.

A study should be conducted using low input samples. Using the Hemocytometer, a small number of cells can be counted and isolated for extraction with a very low volume of iSWAB™ buffer to determine the lysis efficiency of the buffer. Also, a compatible concentration method or other solution should be determined to address potential problems of low input samples that would be too dilute for further processing.

An alternative collection protocol for maximizing the recovery of DNA should be added to Mawi DNA Technologies' repertoire. According to the results and conclusions

of this study, to maximize the collection and recovery of DNA, first the swab should be moistened with the iSWAB™ buffer. Then the stain / area of interest should be collected. A cotton swab should be used if the fluid is a suspected blood or saliva stain. If it is thought that a stain is semen, a nylon flocked swab should be used to maximize the collection of cells. Once the bodily fluid is collected, the swab should be placed into the iSWAB™-ID collection device. The swab should be left in the iSWAB™ buffer at room temperature for at least 3 hours for suspected saliva stains or more than 48 hours if other bodily fluid is suspected. After the incubation period, the swab should be moved up and down within the buffer 15 times and then the swab should be removed from the device using a corkscrew motion to pull the swab through the prongs and out of the tube. The swab should be spun in a spin basket back for 4 minutes at 9,000 rpm and the eluted portion should be placed back into the device to maximize recovery of the DNA.

Throughout this study, the samples were all diluted after extraction of cellular material in the iSWAB™ buffer. Another test should be designed to determine if the buffer would still lyse at the same efficiency if a 0.2X concentration of buffer or lower is created before addition of cellular material. Assuming that the buffer would be marketed at a diluted concentration to allow direct PCR, it is hypothesized that the quality of lysis would decrease if a dilution was created prior to the extraction of cellular material. However, it would be beneficial to confirm that hypothesis. In addition, the stability of the diluted samples should be analyzed. The samples taken for the 3-month time course were diluted upon sampling every month. It would be interesting to know if the stability of the samples would stay as consistent over a 3-month room temperature storage period,

if after the cellular material was initially extracted for 3 hours, it was diluted to at least a 0.2X concentration of iSWAB™ buffer and then stored.

As shown in this study, the device can be used with various bodily fluids, such as semen, saliva and blood and is compatible with different types of swabs. Further testing of the device should be done using other biological evidence (hairs, nails, teeth, etc.) and using mixtures of body fluids. A preliminary iSWAB™ buffer extraction experiment performed on cleaned nail clippings determined that the iSWAB buffer extraction recovered the highest amount DNA compared to other extraction methods [40]. An additional experiment was performed that utilized nails that were spiked with various body fluids (blood, semen and saliva) extracted with iSWAB™ Buffer. The findings suggest that the iSWAB™ buffer recovered the foreign profile (the profile of the fluid the nail was spiked with) and limited recovery of the nail profile [40].

Perhaps, since the sperm cells take longer to lyse in the buffer, the iSWAB™ buffer could benefit sexual assault evidence and provide something similar to a differential extraction. It would be interesting to see the quality of the profiles produced in a mixture analysis, including different ratios of mixtures.

Mawi DNA Technologies decided to challenge the paradigm by transforming a well-established, but inefficient sample collection tool, swabs, into a tool that leverages the ease and convenience of a swab collection without the disadvantages. The device produces high yields of double stranded, long fragmented DNA. Mawi DNA Technologies' method overcomes the common forensic DNA collection problems of low recovery, high bacterial content and degraded DNA. This system allows for maximizing

sample recovery and obtaining human DNA compatible with ID profiling assays emitting the transport, dry time and storage problems of typical swab processing in forensics.

LIST OF JOURNAL ABBREVIATIONS

Am J Forensic Med Pathol	The American Journal of Forensic Medicine and Pathology
Appl Environ Microbiol	Applied and Environmental Microbiology
BioMed Res Int.	Biomedical Research International
Forensic Sci Int Genet	Forensic Science International: Genetics
Front Oncol	Frontiers in Oncology
Investig Genet	Investigative Genetics
J Clin Microbiol	Journal of Clinical Microbiology
J Forensic Sci	Journal of Forensic Science
J Virol Methods	Journal of Virological Methods
JFDS	Journal of Forensic Dental Sciences
Methods Mol Bio	Methods in Molecular Biology

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