CONCENTRATION OF LARGE VOLUME BIOLOGICAL SAMPLES FOR EFFECTIVE AND EFFICIENT FORENSIC DNA ANALYSIS

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Abstract

Biological evidences such as saliva, blood, semen are often found in large volumes on stained clothing, shoes, furniture, walls, doors, sheets, tissues, fluids, or in trace amounts at crime scenes. Careful collection and storage of biological evidence are vital to prevent cross-contamination. A method designed to maximize biological collection from evidence items, particularly large evidence items, is necessary but currently not available for forensic use. Also, limited research has been conducted on identifying an optimal solution for the recovery of DNA from touched objects.

This research seeks to determine a method to concentrate large biological samples to volumes suitable for subsequent forensic DNA analyses. Optimization of this biological collection method has great potential to improve DNA and cell recovery from large evidence items. Two concentration techniques were examined in this study: evaporation and vacuum filtration to determine which method would be better for use in forensic laboratories. In addition, optimal buffer solutions were determined for the collection of biological samples.
The findings of this study indicate that vacuum filtration is a more effective method to concentrate large volume biological samples than the evaporation method. The vacuum filtration method resulted in higher DNA yields and optimal buffer solutions for the collection of biological samples (Saliva, Blood, and Semen). The optimal buffers that can be utilized in the biological collection of Saliva are DI H2O and TE. For the biological collection of Blood and Semen samples, DI H2O and Tween-80 were the optimal buffers. The Gill buffer resulted in significantly low DNA yields for all cell types (Saliva, Blood, Semen) examined in this study.

Overall, results from the vacuum filtration method revealed that DNA can be obtained from large dilute biological volumes. These results have great potential to influence the collection of large biological evidence samples from crime scenes. Dilute evidence are often encountered at crime scenes in the form of biological material on walls, furniture, bed sheets, etc. The ability to collect from large surface areas or volumes would allow maximum collection of cells and DNA recovery, resulting in effective downstream forensic DNA analyses and lead to interpretable DNA profiles that can assist in criminal investigations.
Table of Contents:

Title Page i
Reader Approval Page ii
Acknowledgments iii
Abstract iv
Table of Contents vi
List of Tables viii
List of Figures ix
Abbreviations x

1. Introduction 1
   1.1 Biological Collection Methods 6
   1.2 Purpose 10

2. Materials and Methods 11
   2.1 General Methods 11
      2.1.1 Cell Counting & Staining 11
      2.1.2 QIAGEN Extraction of Saliva, Blood and Semen Samples 12
      2.1.3 DNA Quantification 13
      2.1.4 Statistical Data Analysis 13
   2.2 Concentration Methods 13
      2.2.1 Evaporation Method 13
         2.2.1.1 Preparation of Saliva Samples 13
2.2.1.2 Fractions 1 and 2 15
2.2.1.3 Fractions 3 to 5 16
2.2.1.4 Cleaning technique 17
2.2.2 Vacuum Filtration Method 18
   2.2.2.1 Contamination study 19
   2.2.2.2 Preparation of Saliva, Blood and Semen samples 20
3. Results and Discussion 21
   3.1 Evaporation Method 21
   3.2 Vacuum Filtration Method 25
      3.2.1 Saliva 26
      3.2.2 Blood 33
      3.2.3 Semen 37
4. Conclusion 45
5. Future Directions 47
6. References 48
7. Vita 53
List of Tables:

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1</td>
<td>The number of epithelial cells counted before evaporation</td>
<td>22</td>
</tr>
<tr>
<td>Table 2</td>
<td>The number of epithelial cells counted after evaporation for fractions 1 and 3.</td>
<td>22</td>
</tr>
<tr>
<td>Table 3</td>
<td>DNA Quantities (ng/ul) obtained for fractions 1-3 and 5 after vacuum filtration</td>
<td>23</td>
</tr>
<tr>
<td>Table 4</td>
<td>DNA Quantities (ng/µl) obtained for each sample before and after vacuum filtration in the contamination study</td>
<td>26</td>
</tr>
<tr>
<td>Table 5</td>
<td>Results from ANOVA-Saliva</td>
<td>29</td>
</tr>
<tr>
<td>Table 6</td>
<td>Results from F tests conducted at a 0.05 alpha level (95% CI)</td>
<td>31</td>
</tr>
<tr>
<td>Table 7</td>
<td>Results from T tests conducted at a 0.05 alpha level (95% CI)</td>
<td>32</td>
</tr>
<tr>
<td>Table 8</td>
<td>Results from ANOVA-Blood</td>
<td>35</td>
</tr>
<tr>
<td>Table 9</td>
<td>Results from ANOVA-Semen</td>
<td>39</td>
</tr>
</tbody>
</table>
List of Figures:

Figure 1: Summary of evaporation and centrifugation steps in the evaporation method 14

Figure 2: Fractions 1 & 2 16

Figure 3: Fractions 3-5 17

Figure 4: (A) Mean DNA concentration (ng/µl) recovered from each buffer (TE, Tween-80, Gill, DI H2O) (B) Percentage of DNA recovered from whole saliva from each buffer. [whole saliva sample volumes: 30 µl, 10 µl, 5 µl, 1 µl] 27

Figure 5: Percentage of Relative Standard Deviation (RSD) derived from each buffer (TE, Tween-80, Gill, DI H2O) [whole saliva sample volumes: 30 µl, 10 µl, 5 µl, 1 µl] 29

Figure 6: (A) Mean DNA concentration (ng/µl) recovered from each buffer (TE, Tween-80, Gill, DI H2O) (B) Percentage of DNA recovered from whole blood from each buffer. [whole blood sample volumes: 30 µl, 10 µl, 5 µl, 1 µl] 34

Figure 7: Percentage of Relative Standard Deviation (RSD) derived from each buffer (TE, Tween-80, Gill, DI H2O) [whole blood sample volumes: 30 µl, 10 µl, 5 µl, 1 µl] 35

Figure 8: (A) Mean DNA concentration (ng/µl) recovered from each buffer (TE, Tween-80, Gill, DI H2O) (B) Percentage of DNA recovered from semen from each buffer. [semen sample volumes: 30 µl, 10 µl, 5 µl, 1 µl] 38

Figure 9: Percentage of Relative Standard Deviation (RSD) derived from each buffer (TE, Tween-80, Gill, DI H2O) [semen sample volumes: 30 µl, 10 µl, 5 µl, 1 µl] 39
List of Abbreviations

DNA - Deoxyribonucleic acid
TE - Tris-EDTA
RT-PCR - Real-time polymerase chain reaction
PCR - Polymerase chain reaction
STR - Short tandem repeats
m - Milli
L - Liter
RFLP - Restriction Fragment Length Polymorphism
µ - Micro
°C - Degrees Celsius
DI H₂O - Deionized water
SDS - Sodium dodecyl sulfate
M - Molar
EB - Extraction blank
CB - Cleaning blank
RNA - Ribonucleic acid
1. Introduction

According to the FBI, it was estimated that 1,318,398 violent crimes were committed in 2009 nationwide. These violent crimes include murder, rape, robbery, and aggravated assault. A significant portion of these crimes contains probative forensic evidence that is biological in nature (1). In order to facilitate technological advancements related to evidence-based analyses, the improved identification and collection of evidence in order to reconstruct events that occurred at the time of a crime is necessary.

Eyewitness testimony and physical evidence are often presented during trial and influence jury verdicts. However, research in this field of criminal justice has shown that eyewitness accounts of crimes are habitually unreliable and susceptible to memory errors, potentially leading to the erroneous identification of criminal perpetrators (2).

In 2001, Skolnick and Shaw (2) conducted two studies to determine whether eyewitness testimony or physical evidence is more influential during the jury decision process. Their studies revealed that the mock jurors’ decisions were impacted more by physical evidence. Also, there were significantly more guilty verdicts obtained from physical evidences than with eyewitness evidence.

Physical evidence may be defined as tangible items allegedly used or left behind at a crime scene which can potentially lead to the unveiling of events that occurred during the crime (3). Some common types of physical evidence encountered at crime scenes are: biological (blood, saliva, semen, DNA); trace (hair, fibers, glass, paint, soil); firearm and ballistic (handguns, rifles, shotguns); chemical (fire debris, drugs of abuse, toxins, bomb fragments); fingerprints and impression (shoe and tire marks, bite marks, tool marks,
plastic fingerprints); documents and electronic evidence (3). These items can be analyzed by forensic practitioners to acquire scientific information about each item and to corroborate or refute an association with victims, suspects, other evidence items or the crime scene. Thus, it is crucial that forensic scientists/criminalists adhere to appropriate crime scene protocols and evidence collection techniques to ensure that the quality and integrity of these items are not compromised.

In particular, proper collection of biological evidence from a crime scene is vital for effective forensic biological screening and DNA analyses. Forensic DNA profiling has become a powerful resource in modern forensic casework and criminal investigations and allows for human individualization, potentially resulting in a linkage between suspects, victims, and/or crime scenes. Since the discovery of the structure of DNA by Watson and Crick in the 1950s, significant advancements in DNA and biological evidence-based analyses and detection have been made. One of the major advances in the field of human identity testing came about in 1985 when Alec Jeffreys established the first successful DNA typing method that enabled human individualization (4-7). The method was based on restriction fragment length polymorphisms (RFLP) found between individuals.

As time progressed, the approach to human identification techniques developed and there was a major change from the traditional RFLP analysis to the polymerase chain reaction (PCR) methodology (8). PCR, developed in 1985 by Kary Mullis, is a method that allows specific sequences of DNA to be copied exponentially, thus creating a large amount of DNA for further study. This discovery of PCR revolutionized forensic science
and DNA typing laboratories. The PCR DNA amplification technology is well suited for analysis of forensic DNA samples as it is sensitive, rapid and less limited by the quality of the DNA (9-10). It permits the analysis of minimal starting quantities of nucleic acids. Polymorphic DNA locations of interest are amplified in an exponential manner according to,

\[ C_n = C_0 \cdot 2^n \]  

(Equation 1)

where \( C_n \) is the concentration at cycle \( n \) and \( C_0 \) is the original concentration of DNA. This is particularly valuable since DNA from crime scenes is often limited in both quantity and quality.

Today, forensic DNA analysis using PCR-based methods is utilized worldwide for human identification and has made a significant impact in the criminal justice system. The sensitivity of modern DNA technology and methods has led to the reinvestigation and solving of ‘cold cases’ and exoneration of individuals unjustly accused and wrongly convicted (12-13).

In particular, as DNA technology becomes more sensitive, requiring less biological material than before, there has been an increased interest in the criminal justice community to test ‘new types’ of samples. Such samples may include touched or handled objects. Research conducted by Oorschot and Jones (14) suggested DNA profiles could be obtained from touched items such as pens, car keys, telephone handsets, leather briefcase handles, plastic knife handles, glass, mugs and vinyl gloves. Since then, a significant amount of work in the field of biological recovery of touched items has been conducted and has led to DNA testing from a variety of non-traditional items including
but not limited to, clothing, bedding, vehicles, lip cosmetics, bombs, wallets, windows, doors, firearms, jewelry, paper, tools, skin and shoes (17-27). The recovery of DNA from these items has contributed to the identification of perpetrators of a variety of crimes.

DNA typing of touched items is possible since every contact leaves its trace (3). This is the Locard Exchange Principle which predicts the trace exchange of cellular biological material after contact. Consequently, if enough cellular or biological material has been exchanged, a DNA profile from the handled surface or substrate can reveal an association between the object and the person who touched it. The quantity and quality of DNA obtained from handled surfaces or substrate is dependent upon an individual’s shedder status, handling time, the substrate surface or object and environmental conditions. According to Lowe et al. (16), a DNA profile can be obtained after hand-washing from individuals who are ‘good DNA/cell shedders’ compared to ‘poor DNA/cell shedders’ in which a DNA profile can only be obtained if an individual’s hands were not washed. This finding was corroborated when similar results were obtained from research conducted by Farmen (15) in which a DNA profile was generated from a ‘good shedder’ and barely any DNA was detected on an object from a ‘poor shedder.’ To complicate matters, Wickenheiser (17) found the amount of DNA transferred to a handled surface is not only dependent on the individual handler but also on the contacted substrate. Further, it was determined that the ability to generate a DNA profile was independent of handling time, which suggested the two variables which have the largest influence on obtaining a profile are the individual and the porosity of the surface.
Therefore, a full DNA profile could be obtained from a ‘good shedder’ who handled a porous substrate for a limited amount of time.

Although biological evidence such as saliva, blood and semen can be found in relatively high concentrations, low concentrations may also be found. That is, there may be enough biological evidence present to complete downstream PCR processing with success, but the evidence may be spread over a very large area resulting in a low concentration. To ensure PCR typing success, enough of the biological evidence must be collected in a relatively small volume. Thus, DNA extraction methods must be robust in order to ensure the quantity and quality of DNA isolated from substrates is not compromised. Some common DNA extraction methods are: organic (phenol/chloroform), Chelex® extraction, and QIAamp® DNA extraction.

Organic DNA extraction is the traditional method of DNA purification and results in the recovery of high molecular weight DNA. Although a high DNA yield may be obtained, this extraction method is time-consuming and involves manipulation of multiple tubes which may increase the risk of contamination. Also, organic extraction utilizes hazardous chemicals (10, 44).

Alternatively, an ion-exchange resin is utilized in Chelex® extraction. Briefly, biological samples are added to 5% Chelex® suspension and boiled for several minutes to lyse cells and release the DNA into solution. The DNA is denatured when exposed to 100 °C temperatures. Centrifugation pulls the Chelex® resin and cellular material to the bottom of a tube, the supernatant is removed and can be added to a PCR amplification
reaction. This extraction method often requires less time and fewer sample transfers than the traditional organic method (10).

QIAamp® DNA extraction utilizes a silica-based membrane and may be used to purify genomic DNA from samples with low amounts of DNA. Briefly, sample cells are lysed when the sample is heated in the presence of proteinase K and QIAmp® lysis buffers. The DNA is released into solution and binds to the silica support. DNA elution occurs with the addition of Elution Buffer at the end of the procedure (10, 38).

The protocols for all of these extraction methods require that substrates subjected to DNA extraction must be covered by microliter volumes of liquid. Thus, it is difficult to perform DNA extraction on large substrates due to the volume limitation. As a result, complete DNA recovery and accurate DNA analysis from a large substrate is hindered.

Currently, there are several methods used to collect biological material. Trace biological material refers to the collection of minute biological samples at crime scenes. However, currently there are no established methods to collect trace biological material from large volumes or areas. The following is a discussion of the common collection methods and the limitations of utilizing each for large volume/area samples.

1.1 Biological Collection Methods

Some of the most common collection methods are: swabbing, taping, scraping and cutting, where most biological trace samples are collected by utilizing swabs (27). The swabbing method consists of the single- and double-swab techniques. In the single-swab technique, a sterile moistened swab is rolled over a surface several times to permit collection. A disadvantage of utilizing only one swab is that the moistened swab may not
collect all of the biological material from a substrate or surface. An alternative is to utilize the double-swab technique. In doing so, the first swab is immersed in sterile distilled water and rolled on a surface. The swab is rotated with slight pressure to ensure contact between the swab and surface and to permit collection of the biological material. The second swab is kept dry and rolled over the same surface to collect any remaining biological material. Sweet et al. (27) reported a greater recovery of cells utilizing the double-swab technique. Although more biological material may be collected by using this technique, there are some drawbacks associated with this collection method for large area collection. Most notably, it is not feasible to collect a large biological stain by utilizing multiple swabs since the DNA extraction would be a multi-tube extraction, thereby increasing the probability of contamination and concentration of PCR inhibitors.

The cutting method typically involves the use of scissors, razors or a scalpel to cut a small area of the substrate of interest. The cut pieces are directly placed in tubes for downstream DNA processing. Although this is a simple method that is often employed in crime laboratories there are some drawbacks to this method. First, cutting large areas of material is problematic for the same reasons as for the swabbing method. Also, it is often challenging or impossible to cut large surface areas such as floors and ceramic tiles. Additionally, the cutting must be small enough to be processed during the DNA extraction procedures wherein substrate pieces are immersed in buffer and allowed to soak for a period of time. This can potentially lead to the co-elution of PCR inhibitors which may cause failure of downstream PCR processes. Therefore, there is a need for a
Another issue with the cutting and swabbing methods is cell-elution from swabs or substrate pieces during differential extraction. Differential extraction is a method that is often used for the retrieval of sexual assault evidence from cotton matrices. Briefly, epithelial cells are lysed, while sperm cells are kept intact with a differential lysis buffer containing Sodium Dodecyl Sulfate (SDS) and Proteinase K. The sperm cells remain intact due to the disulfide bonds which are not disrupted by SDS and Proteinase K. Next, sperm cells are pelleted by centrifugation and DNA is extracted from the sperm with a buffer containing dithiothreitol (DTT). Since complete sperm cell elution from cotton swabs or substrates does not occur, there is a need to establish a method that optimizes the quantity of cells recovered (36-37).

Another collection method is based on the use of adhesive materials. Here, tape has been routinely used to recover biological material from worn clothing and research has suggested the ‘tape-lift’ method results in higher DNA recovery than the swabbing or cutting methods (31). In the ‘tape-lift’ method, adhesive tape is pressed on a substrate such that a large area of material can be sampled. This is performed until the ability of the tape to adhere is gone. In contrast to the cutting methods, the use of tape in sampling limits the co-sampling of PCR inhibitors such as dyes (31, 32, 34). In particular, the Barash (31) study reported that DNA was obtained after utilizing the tape-lift method to collect biological material on leather, denim and soiled items which are known to contain PCR inhibitors. Furthermore, Bright’s (20) study reported DNA recovery from the
insoles of shoes and compared the tape-lift method with the double-swab method. The findings revealed that the tape-lift method resulted in higher DNA recovery.

Although a higher DNA recovery can be obtained from utilizing the tape-lift method and it allows for collection from larger surface areas, some drawbacks for large area collection are still evident. For example, taping of larger surfaces requires the use of multiple pieces of tape. This again leads to a multi-tube DNA extraction. Also, dissolving the tape or recovery of cells from the surface of the glue on the tape can be problematic. Additional tape handling steps may also increase the risk of contamination and eventually lead to failure of downstream DNA analyses.

The scraping method utilizes a razor or blade to remove biological material from the surface of evidence items. In this method, a sterile container or clean paper is placed under an evidence item to catch any biological material that was scraped from the item. Although the scrapping method permits collection from large surface areas similar to the taping method, recovery of material from a container poses a challenge. Stouder (33) conducted a study in which scrapings from T-shirts and hosiery were collected and stored in a pillbox. The pillbox was later swabbed with a moistened swab to collect the scrapings and DNA analysis was performed. Results from this study revealed that the amount of DNA recovered from pillbox scrapings was greater than the DNA recovered from the swabbing of an item alone.

Although significant improvements have been made in the sensitivity of technology to detect DNA from biological evidence samples, constraints still exist. Limitations in terms of surface area, co-elution of PCR inhibitors, and a lack of optimal
cell elution from substrates or swabs are apparent. There has been little development in technology or methods that would enable sampling from large surface areas such as bedding, clothing, furniture, walls, doors, ceilings, and floors. Research conducted by Petricevic et al. (22) revealed that DNA profiles could be obtained from bed sheets. However, the traditional biological collection method of cutting was utilized in this study which may cause the co-elution of PCR inhibitors during the incubation time period. Also, cutting a bed sheet into small pieces is not practical when a biological stain is not apparent. The cutting method employed in the Petricevic study emphasizes the need for a more effective method for optimizing biological collection from large surface areas such as bed sheets, floors, walls, etc.

Sampling from large surface areas or volumes would allow maximum collection of cells and DNA recovery that would ultimately permit effective downstream forensic DNA analyses, thereby leading to interpretable DNA profiles that can assist in criminal investigations. A method designed to maximize the collection of trace biological material, particularly from large evidence items, is necessary but not currently available for forensic use. Improvements in biological sample collection will permit robust evidence collection at crime scenes and during biological analyses, resulting in DNA profiles that can be interpreted accurately and confidently.

1.2 Purpose

This research seeks to determine a method to concentrate large biological samples to volumes more appropriate for subsequent forensic DNA analyses. Optimization of this biological collection method has great potential to improve DNA
and cell recovery from large evidence items and shed light on an area where little development has taken place in the forensic science field.

Two concentration techniques were examined in this study: evaporation and vacuum filtration. These two methods were tested to assess whether biological collection from large surface areas or volumes was possible and to determine which method would be better suited for forensic PCR analysis. Also, an optimal buffer solution for the concentration of biological samples was determined.

2. Materials and Methods

All materials were purchased from Sigma-Aldrich (St. Louis, MO) unless indicated. All aspects of this study were conducted in compliance with the ethical standards set-out by the Institutional Review Board of Boston University School of Medicine.

2.1 General Methods

2.1.1 Cell Counting and Staining

Cell counting and staining was accomplished by utilizing Kernechtrot-Picoindigocarmine staining (KPIC), also known as Christmas Tree Stain, which stains epithelial cell cytoplasm green and nuclei red. A volume of 3 µl of saliva was pipetted onto four microscope slides. The samples were heat-fixed to the microscope slides by placing it in an incubator at 70 °C for 1 hour. The Kernechtrot solution (Serological Research Institute, Richmond, CA) was added to cover the smear on the slides which were placed in a moisture chamber for 10 minutes at room temperature. Then the Kernechtrot solution was washed off with deionized water. Next, the Picoindigocarmine
solution (Serological Research Institute, Richmond, CA) was added and the slides were allowed to stain for 15 seconds. The Picoindigocarmine solution was washed off with 200-proof ethanol. The four slides were set aside to dry and then each was examined microscopically and the number of epithelial cells counted.

2.1.2 QIAGEN Extraction of Saliva, Blood and Semen Samples

The samples were extracted utilizing the QIAamp® DNA Investigator Kit. The *Isolation of Total DNA from Small Volumes of Blood or Saliva* was performed according to the manufacturer’s recommended protocol (38). The Buffer AL contained 1 µl of carrier RNA for every 100 µl of lysis buffer solution. The carrier RNA has been shown to enhance the binding of DNA to the membrane of the QIAmp® MinElute column (43-44). Generally, the protocol was as follows: (a) after the cell solution was filtered through the vacuum apparatus, the Millipore filter was cut with sterile scissors into approximately 0.5 cm squares and the filter pieces were divided into two 1.5 ml micro-centrifuge tubes. Alternatively, if a swab was tested, it was cut with a sterile razor blade and placed in a single micro-centrifuge tube. Buffer ATL, proteinase K and buffer AL were added to the tubes and saturated with the lysis buffers before incubation. (b) A volume of 50 µl of pure ethanol was added to the tubes after incubation. (c) After the addition of ethanol, the lysate from the two tubes was transferred into the QIAamp® MinElute column. (d) A volume of 700 µl of 200 proof ethanol was added to the QIAamp® MinElute column after the addition of 700 µl of Buffer AW2 and centrifugation. (e) 25 µl of Buffer ATE was applied to the center of the QIAamp® MinElute column membrane. Since the volume of eluate is up to 5 µl less than the volume of elution solution applied to the
column, the final elution volume was assumed to be 20 µl (38). For the swabbed samples, the piggyback spin technique was performed as described in section 2.2.1.3.

2.1.3 DNA Quantification

Samples were quantified utilizing the Quantifiler® Duo DNA Quantification Kit (Applied Biosystems, Foster City, CA) and the ABI 7500 Sequence Detector System (Applied Biosystems, Foster City, CA). Amplifications were performed in a 25 µl final reaction volume (39). Eight DNA standards were run in duplicate. One standard curve was utilized for all data in this study and DNA concentrations were determined by utilizing the Microsoft Excel Template publically available (49).

The Applied Biosystems 7500 Sequence Detector (Applied Biosystems, Foster City, CA) was used to determine the relative quantity of the target nucleic acid sequence in each sample by analyzing the cycle-to-cycle change in fluorescent signal as a result of amplification (39).

2.1.4 Statistical Data Analysis

Microsoft Excel Data Analysis Tool Pack (Microsoft, Redmond, WA, 2007) was utilized for descriptive statistics, ANOVA and T-Tests. Errors and error bars were reported at two standard deviations from the mean.

2.2 Concentration Methods

2.2.1 Evaporation Method

2.2.1.1 Preparation of Saliva Samples

The following three buffers were utilized in the preparation of the epithelial cell mixture solution: TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0), Gill buffer (1M
Tris-HCL, 0.5M EDTA, 1M NaCl, 0.01% SDS, pH 8.0), and deionized water (DI H2O). Different buffers were tested in order to determine the optimal buffer to be used in biological sample concentration. A volume of 250 ml of each buffer (TE, Gill, DI H2O) was aliquoted into three sterile plastic bottles and 30 µl of saliva was pipetted into each bottle, respectively. The three bottles were placed in the incubator at 70°C for approximately 3 days. The bottles were removed from the incubator once the epithelial cell mixture solution had evaporated down to approximately 50 ml. The walls of each bottle were swabbed to determine if cells had adsorbed to the walls of the container. The 50 ml mixture from each bottle was transferred into three sterile 50 ml conical tubes, gently mixed and centrifuged at 2000 rpm for 6 minutes. After centrifugation, the supernatant was removed and stored in three new sterile 50 ml tubes. Figure 1 displays a flow chart of the evaporation and centrifugation steps involved in the evaporation method to concentrate the saliva samples.

Figure 1: Summary of evaporation and centrifugation steps in the evaporation method.
Five fractions were obtained for each buffer (TE, Gill, DI H$_2$O) during the evaporation method and are described below:

2.2.1.2 Fractions 1 and 2

After centrifugation in the 50 ml conical tube, 48 ml of supernatant was removed and transferred into a new tube for storage at -20°C. The remaining cell mixture (~1.5 ml) was vortexed and transferred to a 2-ml micro-centrifuge tube, gently mixed and centrifuged. This tube was centrifuged for 5 minutes at maximum angular velocity such that all but 50 µl of the supernatant was removed (Fraction 1) where half of the supernatant was retained for further testing (Fraction 2). A volume of 3 µl of Fraction 1 was pipetted onto a microscope slide after gentle mixing and stained with KPIC for microscopic examination. The DNA extraction was later performed on Fractions 1 and 2 using the QIAamp® DNA Investigator Kit and quantification was performed using Applied Biosystems Quantifiler® Duo DNA kit. DNA analyses of Fractions 1 and 2 were performed to determine whether a significant amount of DNA/cell loss occurred via cell lysis during the evaporation process. This procedure was repeated for each of the buffers (TE, Gill, DI H$_2$O). Figure 2 displays a schematic of how Fractions 1 and 2 were derived.
Fractions 3 to 5 are associated with the cotton-fiber swab that was used to recover cells that adsorbed to the bottle walls. After swabbing the bottle walls, the cotton-fiber swab was carefully cut with a scalpel and placed into a 1.5 ml micro-centrifuge tube. The swab was allowed to incubate in 500 µl of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) for 1 hour at room temperature. Then the ‘piggyback spin’ was performed in which the cotton-fiber swab was removed from the 1.5 ml micro-centrifuge tube with sterile tweezers and placed into a filterless basket (Eppendorf). The basket was placed into the tube, and the sample was centrifuged for 1 minute at maximum angular velocity. Afterwards, the basket was removed, and the swab was labeled Fraction 5 and stored in a clean 1.5 ml micro-centrifuge tube for later DNA extraction. After the piggyback spin, the pellet that formed containing the recovered epithelial cell mixture (500 µl) was labeled Fraction 3 and the tube was gently mixed and centrifuged. A volume of 3 µl of
Fraction 3 was pipetted onto a microscope slide and stained with KPIC for microscopic examination. The supernatant (450 µl) was labeled Fraction 4 and was transferred to a clean 1.5 ml microcentrifuge tube for storage. DNA extraction was later performed on Fractions 3 to 5 using QIAamp® DNA Investigator Kit. Quantification was accomplished using the Quantifiler® Duo DNA kit. Figure 3 displays a schematic of how Fractions 3 to 5 were derived.

![Diagram of Fractions 3-5]

**Figure 3: Illustration of Fractions 3-5**

### 2.2.1.4 Cleaning technique

The four plastic bottles were cleaned in the following manner: Each bottle was cleaned four times with detergent; four times with 10% (v/v) Bleach; four times with 70% (v/v) ethanol; and four times with deionized water. After cleaning, the bottles were swabbed with a cotton-fiber swab and DNA extraction and quantification was performed on this swab. Testing of this sample was performed to determine if the cleaning
procedure was sufficient to use for biological samples and did not result in sample-to-sample contamination.

2.2.2 Vacuum Filtration Method

The Millipore™ vacuum filtration system (Millipore, Billerica, MA) was the second concentration technique utilized for the concentration of biological samples. This filtration system consists of the following materials: glass filter holder assembly (47 mm) with funnel, base, stopper clamp, vacuum filtering flask (1 L), silicone No. 8 perforated stopper and silicone tubing (3/16 in). Hydrophilic polyvinylidene fluoride (PVDF) DuraPore membrane filters were utilized with a pore size of 0.45 µm and a filter diameter of 47 mm. The filter pore size was chosen after considering the average size of sperm heads (~3 µm) and epithelial cells (~50-80 µm). The filter diameter of 47 mm was chosen to ensure that the filters could fit into two 1.5 ml micro-centrifuge tubes for later DNA extraction.

The Millipore vacuum filtration system was assembled according to the manufacturer’s recommended instructions (40). After the vacuum filtration apparatus was assembled, the vacuum source was turned on and the biological sample mixture which was contained in a sterile plastic bottle was poured into the funnel to allow for filtration. The bottle walls and the sides of the funnel were rinsed with deionized water and the flushing fluid was poured into the funnel for filtration. Once all of the biological sample mixture and flushing fluid passed through the filtration system, the vacuum source was turned off. The filter was transferred with clean tweezers to a clean small weigh boat and covered with a second clean small weigh boat. This procedure was repeated in
quadruplicate for all saliva, blood, and semen samples at various concentrations. After the filtration of each biological sample, the Millipore vacuum filtration glassware and filter support screen was cleaned with 10% bleach and 70% ethanol. Once all biological samples were filtered each day, the filtration glassware and filter support screen were cleaned with detergent, 10% bleach, 70% ethanol, and deionized water, respectively. The plastic bottles were cleaned as previously described in section 2.2.1.4.

2.2.2.1 Contamination study

A contamination study was conducted before vacuum filtration was performed on the saliva, blood and semen samples to determine whether consecutive biological samples could be tested without contamination. This study entailed testing four 30 µl blood samples and four deionized water samples in which the eight samples were organized and tested in alternating sequence.

More specifically, eight samples were prepared by pouring 250 ml of deionized water into eight sterile plastic bottles. Then 30 µl of blood was pipetted into four of the bottles and these bottles were labeled as the blood samples. The remaining four bottles containing only 250 ml of deionized water were labeled as extraction blanks (EB). After the preparation of these samples, the Millipore apparatus was assembled and the vacuum method was performed as previously described in section 2.2.2. After the first blood sample was transferred to a clean weigh boat, the glassware and the filter support screen were cleaned with 10% bleach and 70% ethanol, respectively. This filtration procedure and cleaning process was repeated with each of the remaining seven samples in the following order: water, blood, water, blood, water, blood and water. Once all eight
samples were filtered, the glassware and filter support screen were swabbed after the cleaning process. The swab was labeled as the cleaning blank (CB) and was later extracted along with the eight samples using the QIAamp® DNA Extraction Investigator Kit. DNA quantification was performed on these samples utilizing Quantifiler® Duo DNA kit. Results from DNA quantification determined whether any contamination ensued during the filtration process or DNA extraction and if the cleaning procedure was sufficient to use for consecutive biological samples without sample-to-sample contamination.

2.2.2 Preparation of Saliva, Blood and Semen samples

Since results from the contamination study indicated no contamination after the vacuum filtration procedure and cleaning process were performed as described in section 2.2.2, the vacuum filtration method was conducted on the following biological samples: saliva, blood, and semen. Also, a new saliva sample was utilized in the vacuum filtration method since preliminary results obtained from the evaporation method (2.2.1) revealed a low DNA quantity for 30 µl of saliva.

The following four buffers were utilized in the preparation of each biological sample mixture solution for saliva, blood, and semen: TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0), Gill buffer (1M Tris-HCL, 0.5M EDTA, 1M NaCl, 0.01% SDS, pH 8.0), Tween-80 (0.05%, pH 7.2) and deionized water (DI H₂O). These buffers were tested in order to determine whether variations in buffer type had an influence on DNA recovery. Each of the biological samples (saliva, blood, semen) was tested in quadruplicate at the following volumes: 30, 10, 5 and 1 µl. Each day 250 ml of one buffer
(TE, Gill, Tween-80 or DI H2O) was poured into 17 sterile plastic bottles. The 16 bottles were organized into four groups each containing 4 bottles. In group one, 30 µl of the biological sample (saliva, blood or semen) was pipetted into four bottles. In group two, 10 µl of the biological sample was pipetted into four bottles. In group 3, 5 µl of the biological sample was pipetted into four bottles and 1 µl of the biological sample was pipetted into the remaining four bottles in group 4. Since, a biological sample was not added to the 17th bottle, it was labeled the extraction blank (EB).

After sample preparation, each biological sample mixture solution and the extraction blank (EB) were poured into the vacuum filtration system, and the filtration method was performed as previously discussed in section 2.2. Following the filtration of all the 30 µl biological samples, the glassware and filter screen support were swabbed after the cleaning process. The swab was labeled as the cleaning blank (CB) and went through the same downstream DNA processes as the sample filters.

3. Results and Discussion

3.1. Evaporation Method

The evaporation method consists of a technique commonly used in heterogeneous catalysis precipitation synthesis. In this method, a heterogeneous mixture consisting of a solid (cells) and liquid (buffer) is subjected to an overnight incubation at moderate i.e. 70 °C temperature. At this temperature, the water is expected to evaporate at a constant rate, consistently decreasing the volume of solution while increasing the concentration of cells.
The number of epithelial cells counted before and after the evaporation method was compared. Before evaporation, epithelial cells were counted on four slides and resulted in an average of 420±160 epithelial cells contained in 3 µl of a 1:9 dilution of whole saliva from a single source (Table 1). In contrast, only 1 to 2 epithelial cells were present in 3 µl of solution after the evaporation method was conducted with the TE buffer, Gill buffer and deionized water, suggesting a significant amount of cell loss during this concentration procedure (Table 2).

### Table 1: Number of epithelial cells counted before evaporation.

<table>
<thead>
<tr>
<th>Slide</th>
<th># of epithelial cells counted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slide 1</td>
<td>402</td>
</tr>
<tr>
<td>Slide 2</td>
<td>524</td>
</tr>
<tr>
<td>Slide 3</td>
<td>432</td>
</tr>
<tr>
<td>Slide 4</td>
<td>329</td>
</tr>
</tbody>
</table>

### Table 2: Number of epithelial cells counted after evaporation for fractions 1 and 3.

<table>
<thead>
<tr>
<th>Buffers</th>
<th>Fraction 1 Recovered cells from bottle</th>
<th>Fraction 3 Recovered cells from bottle walls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gill</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>TE</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>DI H₂O</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

Since there is a significant loss of biological material, examinations into the source/reasons for the loss were evaluated. Two potential reasons were considered: (1) cells adsorbing to the vial walls and/or (2) cell lysis. To analyze the propensity of cell
adsorption to the walls of the container, a swab of the container walls was taken and incubated for 1 hour at room temperature. After incubation the cells were removed from the swab. The procedure is described in the Materials and Methods section and labeled Fraction 3.

The percentage of cells recovered from Fraction 3 for all solution types was ≤ 0.5%. This indicates that epithelial cell loss was partially induced by cell adsorption to the vial walls during evaporation. Although cell recovery was affected by adsorption of the cells to the wall of the container, premature cell lysis cannot be discounted as an additional source of the decrease in numbers. However, cell lysis is not expected to have a significant impact on DNA recovery of Fraction 1. If Fraction 1 contains a significant amount of DNA, adsorption of the cells to the wall - although contributing to sample loss - may not be significant. To test this, the quantity of DNA recovered was determined.

The concentration and quantity of DNA obtained from recovered cells from Fractions 1, 2, 3 and, 5 in buffers TE, Gill and deionized water were compared (Table 3).

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Before Filtration</th>
<th>After Filtration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30µl whole saliva (ng/µl)</td>
<td>Fraction 1 recovered cells from bottle (ng/µl)</td>
</tr>
<tr>
<td>TE</td>
<td>1.37</td>
<td>0.178</td>
</tr>
<tr>
<td>DI H₂O</td>
<td>0.135</td>
<td>0.135</td>
</tr>
<tr>
<td>Gill</td>
<td>0.161</td>
<td>0.001</td>
</tr>
</tbody>
</table>

The percent recovery of Fraction 1 from the TE, DI H₂O and Gill buffers was 12.9%, 9.85% and 11.75%, respectively, indicating a significant level of DNA loss. Analysis of
the swabs from the vial walls corroborate the microscopic evaluation of this fraction, suggesting a portion of biological material is lost due to cell adsorption. However quantitative analysis of the portion of cells adsorbed versus pre-lysed is not possible, since surface swabbing is expected to capture only a fraction of the total number of cells. Further, although cell adsorption has an impact on loss, 90% of the total DNA quantity is lost during this process, indicating that cell pre-lysis may have an influence in this process.

It should be noted that no DNA was obtained after cleaning of the plastic bottles for all buffers, indicating the cleaning technique utilized was adequate. The Tween-80 buffer and Fraction 4 were not tested further due to the low percent recovery obtained in these preliminary results.

Moreover, it was also observed that the DNA quantity obtained from whole saliva was 1.37 ng/ul for 30 µl. This DNA concentration is considerably low compared to results obtained for similar volumes in this laboratory (44) and may be due to the fact that the saliva sample utilized may have come from an individual who is a poor shedder (15-17) or who has a mouth containing high amounts of bacteria or an unusually high/low pH.

Overall, preliminary results acquired from this method indicate that using evaporation as a concentration method is not practical, since it involves long incubation times and DNA/cell loss by cell lysis or cells adsorption is apparent. Thus, the vacuum filtration method was subsequently tested on saliva, blood, and semen samples.
3.2 Vacuum Filtration Method

The first test was used to distinguish whether the same vacuum filtration device could be used for multiple samples with no sample-to-sample carry-over. This contamination study revealed that biological samples can be tested consecutively when utilizing the vacuum filtration method. Results from DNA quantification are displayed in Table 4. No DNA was detected in the four extraction blanks following a cleaning procedure which consisted of cleaning the glassware and the filter support screen with 10% bleach and 70% ethanol, respectively. This method can be repeated consecutively with biological samples without sample-to-sample contamination. Furthermore, no DNA was obtained in the cleaning blank (CB) indicating the cleaning technique utilized on the filtration glassware and filter support screen was adequate.

A closer look at the results obtained from the four blood samples after vacuum filtration reveals the DNA quantities for 30 µl of whole blood were: 5.52, 9.22, 6.41, and 5.55 ng/µl, respectively (Table 4), resulting in an average concentration of 7 ng/µl. The standard deviation was 2 ng/µl, representing a relative standard deviation of 28%, suggesting this method results in quantities which are as reproducible as other extraction studies performed previously (44, 50).
Table 4: DNA Quantities (ng/ul) obtained for each sample before and after vacuum filtration in the contamination study.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Before Filtration DNA Qty (ng/ul)</th>
<th>After Filtration DNA Qty (ng/ul)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30µl Whole Blood 1</td>
<td>samp</td>
<td>5.52</td>
</tr>
<tr>
<td>Blank 1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>30µl Whole Blood 2</td>
<td>9.22</td>
<td>0</td>
</tr>
<tr>
<td>Blank 2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>30µl Whole Blood 3</td>
<td>6.41</td>
<td>0</td>
</tr>
<tr>
<td>Blank 3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>30µl Whole Blood 4</td>
<td>5.55</td>
<td>0</td>
</tr>
<tr>
<td>Blank 4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cleaning swab</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

3.2.1 Saliva

To evaluate the effectiveness of vacuum filtration in DNA recovery for the saliva sample, the mean DNA concentrations obtained for 30, 10, 5, and 1 µl of saliva were compared for each buffer (TE, Gill, Tween-80, DI H₂O) and are displayed in Figure 4A. After filtration, the highest mean DNA concentrations were obtained in the DI H₂O and TE buffer and reported as 6 ± 2 ng/µl and 3.2 ± 0.7 ng/µl, respectively for 30 µl of Saliva (Figure 4A). For 10 µl of saliva, the highest mean DNA concentration between buffers was 1 ± 2 ng/µl and 1 ± 2 ng/µl for both the DI H₂O and Tween-80 buffer. The highest mean DNA concentration for 5 µl of saliva was 3 ± 3 ng/µl for the TE buffer. For 1 µl of
saliva, the highest mean DNA concentrations were $0.3 \pm 0.2$ ng/µl and $0.3 \pm 0.1$ ng/µl for the Tween-80 buffer and DI H$_2$O, respectively.

Qualitatively, it was observed that Gill buffer resulted in the lowest mean DNA concentrations for all volumes of whole saliva indicating this buffer may not be suitable for the biological collection of saliva when utilizing vacuum filtration as the concentration technique.

Figure 4: (A) Mean DNA concentration (ng/µl) recovered from each buffer (TE, Tween-80, Gill, DI H$_2$O) (B) Percentage of DNA recovered from whole saliva from each buffer. [Whole saliva sample volumes: 30 µl, 10 µl, 5µl, 1 µl]
Figure 4B depicts the percentage of DNA recovered from each buffer for all volumes of whole saliva tested. The highest percentages of DNA recovered from the vacuum concentration method for 30 µl of saliva were 36 ± 21% and 8 ± 5% for DI H$_2$O and Tween-80 buffers, respectively. For 10 µl of saliva, the highest percentages of DNA recovered were obtained from the DI H$_2$O and Tween-80 buffers and reported as 40 ± 60% and 38 ± 66%, respectively (Figure 4B). A high percentage of DNA was recovered from the TE buffer for 5 µl of saliva and was reported as 98 ± 126%. For 1 µl of saliva, the highest percentages of DNA recovered from the vacuum filtration method were obtained from the Tween-80 and DI H$_2$O buffers and reported as 57 ± 48% and 56 ± 34%, respectively. As suggested by Figure 4A, the lowest recovery for all volumes was observed when Gill buffer was used, where the percent recoveries ranged from 0.15±0.17% to 0.29 ± 0.44%, representing a significant loss of biological material. Since percent recovery was calculated by dividing the recovered DNA concentration by the initial concentration at a given volume, a means to calculate the errors associated with the percent recovery was necessary. To accomplish this, errors were calculated by utilizing the propagation of error equation (41):

$$\frac{\sigma_{\% \text{recovered}}}{\% \text{recovered}} = \sqrt{\left(\frac{\sigma_a}{a}\right)^2 + \left(\frac{\sigma_b}{b}\right)^2}$$  \hspace{1cm} \text{(Equation 2)}
Figure 5: Percentage of Relative Standard Deviation (RSD) derived from each buffer (TE, Tween-80, Gill, DI H2O) [whole saliva sample volumes: 30 µl, 10 µl, 5 µl, 1 µl]

Figure 5 depicts the percent relative standard deviation (RSD) of the DNA concentration for each sample and is defined as the standard deviation divided by the average \( \times 100\% \). The highest percent RSD was obtained from sample concentrations with Gill buffer containing 0.01% (v/v) SDS and were 94%, 72%, 50% for 30, 10, and 5µl of saliva, respectively, and indicates that not only is the percent of DNA recovered low when Gill buffer is used, but the reproducibility is also low.

Results from ANOVA tests revealed a significant difference between the DNA concentrations of 30, 10, 5, and 1 µl of filtered saliva samples.

Table 5: Results from ANOVA for filtered saliva samples conducted at a 0.05 alpha level (95% CI)

<table>
<thead>
<tr>
<th>whole saliva sample volume (µl)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>1.13E-06</td>
</tr>
<tr>
<td>10</td>
<td>0.0002</td>
</tr>
<tr>
<td>5</td>
<td>0.0005</td>
</tr>
<tr>
<td>1</td>
<td>5.83E-05</td>
</tr>
</tbody>
</table>
Table 5 displays the p-values obtained for 30, 10, 5 and 1µl of whole saliva. These p-values are less than the alpha level of 0.05 at the 95% confidence interval. Thus, the null hypothesis that there is no significant difference between the DNA concentrations of filtered and unfiltered saliva samples (30, 10, 5, and 1µl) was rejected.

Results from F-tests revealed significant differences between all pairs of saliva samples and sample volumes (30, 10, 5, and 1 µl) and are presented in Table 6. To determine specifically which pairs of buffers showed significant differences between each other, T-tests were performed on pairs of saliva samples and results are displayed in Table 7.

In Table 6, F-tests reveal that there is a significant difference in variance between all pairs of saliva samples and at all volumes for Gill buffer. Additionally, Table 7 shows the results obtained from a T-test using a significance level of 0.05. For all saliva samples at all volumes, the percent of DNA recovered when using Gill Buffer is significantly different than all of the others, suggesting Gill buffer is not suitable to be used in the biological concentration of saliva samples since the amount of DNA recovered is low.

In general, results from the F-test indicate that there is no significant difference between TE, DI H₂O and Tween-80 buffers. For the DI H₂O and TE buffer pair, no significant difference was observed at 1/4 of the sample volumes. The DI H₂O and Tween-80 buffer pair resulted in no significant difference at 3/4 of the sample volumes. The TE and Tween-80 buffer pair resulted in no significant difference at 2/4 of the sample volumes. In comparison, T-tests specifically identified that no significant differences were observed in 3/4, 3/4 and 2/4 of the sample volumes for the following
pairs of buffers DI H$_2$O and TE; DI H$_2$O and Tween-80; TE and Tween-80, respectively. These results suggest that either TE, DI H$_2$O and Tween-80 buffers can be utilized in the concentration of saliva, and show no indication that one buffer is more reliable than the other two.

Table 6: Results from F tests conducted at a 0.05 alpha level (95% CI)

<table>
<thead>
<tr>
<th>Saliva</th>
<th>30ul</th>
<th>10ul</th>
<th>5ul</th>
<th>1ul</th>
</tr>
</thead>
<tbody>
<tr>
<td>DI H$_2$O &amp; TE</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>X</td>
</tr>
<tr>
<td>DI H$_2$O &amp; Tween</td>
<td>*</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>DI H$_2$O &amp; Gill</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>TE &amp; Tween</td>
<td>X</td>
<td>*</td>
<td>*</td>
<td>X</td>
</tr>
<tr>
<td>TE &amp; Gill</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Tween &amp; Gill</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Blood</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>DI H$_2$O &amp; TE</td>
<td>X</td>
<td>*</td>
<td>*</td>
<td>X</td>
</tr>
<tr>
<td>DI H$_2$O &amp; Tween</td>
<td>X</td>
<td>X</td>
<td>*</td>
<td>X</td>
</tr>
<tr>
<td>DI H$_2$O &amp; Gill</td>
<td>X</td>
<td>X</td>
<td>*</td>
<td>X</td>
</tr>
<tr>
<td>TE &amp; Tween</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>TE &amp; Gill</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Tween &amp; Gill</td>
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<table>
<thead>
<tr>
<th>Semen</th>
<th></th>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>DI H$_2$O &amp; TE</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>DI H$_2$O &amp; Tween</td>
<td>*</td>
<td>X</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>DI H$_2$O &amp; Gill</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>X</td>
</tr>
<tr>
<td>TE &amp; Tween</td>
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<td>X</td>
<td>X</td>
<td>*</td>
</tr>
<tr>
<td>TE &amp; Gill</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>X</td>
</tr>
<tr>
<td>Tween &amp; Gill</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>X</td>
</tr>
</tbody>
</table>

*= indicates a significant difference
x= indicates no significant difference
Table 7: Results from T tests conducted at a 0.05 alpha level (95% CI)

<table>
<thead>
<tr>
<th></th>
<th>30ul</th>
<th>10ul</th>
<th>5ul</th>
<th>1ul</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saliva</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DI H₂O &amp; TE</td>
<td>*</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>DI H₂O &amp; Tween</td>
<td>*</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>DI H₂O &amp; Gill</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>TE &amp; Tween</td>
<td>*</td>
<td>X</td>
<td>*</td>
<td>X</td>
</tr>
<tr>
<td>TE &amp; Gill</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Tween &amp; Gill</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td><strong>Blood</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DI H₂O &amp; TE</td>
<td>*</td>
<td>*</td>
<td>X</td>
<td>*</td>
</tr>
<tr>
<td>DI H₂O &amp; Tween</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>DI H₂O &amp; Gill</td>
<td>*</td>
<td>*</td>
<td>X</td>
<td>*</td>
</tr>
<tr>
<td>TE &amp; Tween</td>
<td>*</td>
<td>*</td>
<td>X</td>
<td>*</td>
</tr>
<tr>
<td>TE &amp; Gill</td>
<td>X</td>
<td>*</td>
<td>*</td>
<td>X</td>
</tr>
<tr>
<td>Tween &amp; Gill</td>
<td>X</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td><strong>Semen</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DI H₂O &amp; TE</td>
<td>*</td>
<td>*</td>
<td>X</td>
<td>*</td>
</tr>
<tr>
<td>DI H₂O &amp; Tween</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>DI H₂O &amp; Gill</td>
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<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>TE &amp; Tween</td>
<td>*</td>
<td>*</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>TE &amp; Gill</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Tween &amp; Gill</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

*= indicates a significant difference
x= indicates no significant difference
3.2.2 Blood

To evaluate the effectiveness of the vacuum filtration method in DNA recovery for the blood sample, the mean DNA concentrations obtained for 30, 10, 5, and 1µl of blood were compared for each buffer (TE, Gill, Tween-80, DI H₂O) and are displayed in Figure 6A. Results indicate that the DI H₂O and Tween-80 buffers yielded the greatest DNA concentrations and are the preferred buffers for the biological collection of blood at all sample volumes. The highest mean DNA concentrations were obtained from DI H₂O and Tween-80 buffers and reported as 17 ± 4 ng/µl and 14 ± 8 ng/µl, respectively for 30 µl of blood. The TE and Gill buffers resulted in the lowest mean DNA concentrations for blood, indicating that these buffers may not be suitable for the biological collection of blood when utilizing the vacuum filtration method. The lowest mean DNA concentrations were obtained from TE and Gill buffers as 0.4 ± 3 ng/µl and 0.4 ± 0.3 ng/µl, respectively for 1 µl of blood.
Figure 6: (A) Mean DNA concentration (ng/µl) recovered from each buffer (TE, Tween-80, Gill, DI H$_2$O) (B) Percentage of DNA recovered from whole blood from each buffer. [whole blood sample volumes: □ 30 µl, □ 10 µl, □ 5µl, □ 1 µl]

Figure 6B depicts the percentage of DNA recovered from each buffer for all volumes of whole blood tested. The highest percentages of DNA recovered from the vacuum concentration method were reported as 75 ± 18% from DI H$_2$O for 30 µl and 71 ± 35% from Tween-80 for 1 µl of whole blood. The Gill buffer resulted in the lowest DNA recovery at 15 ± 16% for 10 µl of whole blood. In Figure 7, the highest percent RSD was obtained from DI H$_2$O at 5µl and was reported as 96%. This high percentage indicates the lack of reproducibility of DNA recovery for this particular buffer and sample volume.
Also, the high percentage may be due to potential errors introduced during the filtration and DNA extraction procedures.

Figure 7: Percentage of Relative Standard Deviation (RSD) derived from each buffer (TE, Tween-80, Gill, DI H2O) [whole blood sample volumes: 30 µl, 10 µl, 5 µl, 1 µl]

Results from ANOVA tests revealed a significant difference between the DNA concentrations of 30, 10, 5, and 1 µl of filtered blood samples.

Table 8: Results from ANOVA for filtered blood samples conducted at a 0.05 alpha level (95% CI)

<table>
<thead>
<tr>
<th>whole blood sample volume (µl)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>0.0003</td>
</tr>
<tr>
<td>10</td>
<td>8.33E-07</td>
</tr>
<tr>
<td>5</td>
<td>0.0009</td>
</tr>
<tr>
<td>1</td>
<td>0.006</td>
</tr>
</tbody>
</table>

Table 8 displays the p-values obtained for 30, 10, 5 and 1 µl of whole blood. These p-values are less than the alpha level of 0.05 at the 95% confidence interval. Thus, the null hypothesis that there is no significant difference between the DNA concentrations of filtered and unfiltered blood samples (30, 10, 5, and 1 µl) was rejected.
Results from F-tests (Table 6) indicate that significant differences were observed in 2/4, 1/4 and 1/4 of the sample volumes for the following pairs of buffers: DI H2O and TE; DI H2O and Tween-80; and DI H2O and Gill, respectively. It is not clear from the F-test results alone which pairs of buffers showed significant differences in variances between each other. T-test results (Table 7) reveal that there is a difference between concentrations of all pairs of blood samples and at all volumes for Gill buffer. Significant differences were observed in 3/4, 2/4 and 3/4 of the sample volumes for the following pairs of buffers: Tween-80 and Gill, TE and Gill, and DI H2O and Gill, respectively. These results indicate that the Gill buffer is not a suitable buffer to be used in the biological concentration of blood samples. Similar to results obtained from saliva samples, significantly less DNA was recovered from the Gill buffer. No significant difference was observed between DI H2O and Tween-80 buffers suggesting these can be utilized in the biological concentration of blood.
3.2.3 Semen

The means of DNA concentrations obtained for 30, 10, 5, and 1 µl of semen were compared for each buffer (TE, Gill, Tween-80, DI H$_2$O) to evaluate the effectiveness of the vacuum filtration concentration method in DNA recovery of the semen sample. Results are displayed in Figure 8A and reveal that the DI H$_2$O and Tween-80 buffers yielded the greatest DNA concentrations and are the preferred buffers for the biological collection of semen at all sample volumes. After filtration, the highest mean DNA concentrations were obtained from the DI H$_2$O and Tween-80 buffers and reported as 110 ± 50 ng/µl and 100 ±10 ng/µl, respectively for 30 µl of semen. The lowest mean DNA concentrations were obtained from the Gill buffer and were reported as 0.2 ± 0.3 ng/µl for 10 µl of semen and 0.4 ± 0.5 ng/µl for 30 µl of semen. As a whole, the Gill buffer again resulted in the lowest DNA yield indicating that this buffer may not be suitable for the biological concentration of semen when vacuum filtration is utilized. The results obtained in Figures 8A and 8B are similar in that the overall highest DNA yield was acquired from both the DI H$_2$O and Tween-80 buffers. The highest DNA recoveries were obtained from 10 µl of semen and reported as 90 ± 74% and 89 ± 79% for DI H$_2$O and Tween-80 buffers, respectively.
Figure 8: (A) Mean DNA concentration (ng/µl) recovered from each buffer (TE, Tween-80, Gill, DI H₂O) (B) Percentage of DNA recovered from semen from each buffer. [semen sample volumes: 30 µl, 10 µl, 5 µl, 1 µl]

As is observed in Figure 9, the Gill buffer has the highest relative standard deviation percentage at all sample volumes tested indicating the poor reproducibility of this buffer in comparison to the other buffers.
Results from ANOVA tests revealed a significant difference between the DNA concentrations of 30, 10, 5, and 1 µl of filtered semen samples.

Table 9: Results from ANOVA for filtered semen samples conducted at a 0.05 alpha level

<table>
<thead>
<tr>
<th>Semen sample volume (µl)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>0.0003</td>
</tr>
<tr>
<td>10</td>
<td>0.002</td>
</tr>
<tr>
<td>5</td>
<td>0.003</td>
</tr>
<tr>
<td>1</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

Table 9 displays the p-values obtained for 30, 10, 5 and 1 µl of semen. These p-values are less than the alpha level of 0.05 at the 95% confidence interval. Thus, the null hypothesis that there is no significant difference between the DNA concentrations of filtered and unfiltered semen samples (30, 10, 5, and 1 µl) was rejected.

Results from F-tests (Table 6) indicate that significant differences were
observed in 3 out of the four sample volumes for the following pairs of buffers: DI Tween-80 and Gill; TE and Gill; and DI H$_2$0 and Gill, respectively. These results exhibit a similar trend that was observed in both the saliva and blood cell types in which significant differences between all pairs, volumes and cell types was obtained for Gill buffer. Additionally, for T-test results, no significant difference was observed between DI H$_2$0 and Tween-80 buffers suggesting that these buffers can be utilized in the biological collection of semen.

Moreover, no DNA was detected in the extraction blanks, cleaning blanks, and reagent blanks that were tested along with each sample type (saliva, blood, semen). These results indicate that the vacuum filtration method can be repeated consecutively with biological samples without contamination, and the cleaning technique utilized in the vacuum filtration method was adequate.

In summary, the average % recovery for saliva, blood and semen were 40%, 54% and 64% respectively (excluding recoveries obtained with Gill buffer). These recoveries are consistent with other values found in the literature. In a study conducted by Millipore (45), RNA samples were concentrated with Microcon (Microcon 100K NMWL and 30K NMWL) and Centricon (Centricon 100K NMWL and Centricon 30K NMWL) concentrators. RNA recovery was above 85% for all RNA concentrations evaluated which ranged from 0.025 µg/ml to 10 µg/ml. Also, RNA loss due to adsorption to each device was minimal and was reduced to 2% when each device was passivated.

Another study conducted by Millipore (46) evaluated and compared ethanol precipitation methods for DNA with ultrafiltration in Centricon and Microcon centrifugal
filter devices. DNA recovery from these devices was close to 100% with concentrations as low as 10 ng/ml. High DNA recoveries were obtained from these devices similar to results obtained in the previous Millipore study discussed (45). In contrast, DNA recoveries from the ethanol precipitation methods were significantly lower and varied depending on the temperature and time of incubation. DNA incubated at –70 °C for 15 minutes resulted in 14, 15, 23, 52 and 55% DNA recoveries for DNA concentrations of 10, 25, 50, 250 and 1000 ng/ml, respectively. Overnight precipitation at –20 °C resulted in greater DNA recoveries of 31, 45, 45, 76 and 67% for all DNA concentrations tested. However, the high recoveries obtained from the Microcon and Centricon devices in both Millipore studies (45-46) indicate these devices are more effective in concentrating nucleic acids than ethanol precipitation. Additionally, a trend was observed in both Millipore studies for each concentration method evaluated–as DNA/RNA concentrations increased the percent DNA recovered also increased.

Shapiro et al. observed this same trend when traditional ethanol precipitation methods were compared to a microtube ultracentrifugation method that was developed. The ultracentrifugation method allowed for efficient DNA recoveries that ranged from 70 to 100% when it was applied to DNA concentrations ranging from 10 to 10,000 ng/ml. Similar to the Millipore studies discussed earlier, as DNA concentrations increased, the percent DNA recovered also increased in this study (47).

This, however, was not the case in the current study in which the percent DNA recovered from all buffers and cell types did not show a correlation with initial volume of sample and may be due to the fact that the samples were concentrated before DNA
extraction. Also, the RSD’s, although comparable to previous studies, show a lack of reproducibility regardless of sample type or buffer. However, it should be noted that the errors encompass all errors that have occurred from all of the techniques performed during experimentation and include but are not limited to errors inherent in the aliquoting of each biological specimen, vacuum filtration, DNA extraction and quantification.

A study by Sweet et al. investigated an improved Chelex® method of extracting DNA from saliva stains and compared it to the traditional Chelex® extraction method and organic (phenol-chloroform) extraction. The modified Chelex® method entailed the collection of 100 µl of whole saliva from individual forearms with the double-swab method. The swabs were washed with proteinase K and incubated at 56°C and 100°C. Micro-concentration of the solution was performed using Microcon-100 (Amicon) tubes and DNA was quantified utilizing the slot-blot procedure. For 100 µl of whole saliva, 31.9 ± 4.22% of the DNA was recovered from the traditional Chelex® method and 47.7 ± 6.90% of the DNA was recovered from the modified Chelex® extraction method, again indicating the potential for high relative standard deviations and low DNA recoveries during extraction (48).

Another study by Phillips et al. (44) shed light on the performance and efficiency of the QIAamp® spin columns. This study reported that an average of 20% of DNA remains on the spin column after the first elution and this DNA was only recovered when a second elution was performed. In this current study, only one elution was performed which indicates that some DNA loss may have occurred during DNA extraction.
Although there is no method currently available that would maximize biological collection from large evidence items, an unpublished study conducted by Sorensen Forensics with Microbial-Vacuum Systems, Inc (MSI) examined the use of a hand-held wet vacuum surface sampling device for forensic science evidence collection (42). The Microbial-Vacuum system (M-Vac) allows for sampling of large areas. The Sorensen study compared traditional collection methods for swabbing and cutting to the M-Vac system and the amount of DNA extracted and quantified from blood and saliva stains on blue denim, polyester, cotton and nylon. The M-Vac system resulted in a much higher rate of DNA recovery than the swabbing and cutting methods. However, the concentration method utilized in conjunction with the M-Vac collection method was not effective and underscores the need for a procedure that can concentrate large biological samples to volumes more appropriate for forensic DNA analyses. The Amicon filter and Vivacon DNA concentration devices were utilized and results indicated that half of the DNA added to the filter was lost during Amicon concentration.

Specifically in the Sorenson study, a biological mixture solution was created when 500 µl of a 1:2 dilution of saliva was spotted on white cotton material and collected with 50 ml of buffer (TE) using the Microbial-Vacuum. After collection, the sample was concentrated using the Amicon filter and Vivacon DNA concentration devices. The average DNA concentration obtained for the biological mixture solution (500 µl of 1:2 saliva + 50 ml TE) was 1.43 ng/µl. Although the M-Vac collection method resulted in a higher DNA yield (ng) than the swab method, the M-Vac method also resulted in a higher
DNA yield than the neat saliva samples. Raw data was not provided, so the reason for this result is yet unanswered.

In comparison to 500 µl of 1:2 saliva used in the Sorenson study (42), the current study used 30 µl as the largest sample volume tested for whole saliva. A large dilute biological mixture solution was created when 30 µl of whole saliva was added to 250 ml of TE buffer. After the filtration concentration method, the average DNA concentration obtained was 3.2 ± 0.6 ng/µl for the dilute biological mixture solution (30 µl saliva + 250 ml TE). The filtration concentration method described in this current study resulted in a higher DNA concentration than the Amicon filter and Vivacon DNA concentration methods previously discussed (42).

It is significant to note that even though a large dilute biological mixture (30 µl + 250 ml TE) was utilized in the filtration concentration method, DNA was still recovered and the amount obtained was greater than the more concentrated biological solution mixture consisting of 500 µl of saliva and 50 ml of TE utilized in the Amicon and Vivacon DNA concentration methods for the Sorenson study. These results indicate that vacuum filtration is an effective concentration method and TE buffer is an optimal buffer that can be utilized in the biological collection of saliva samples. Moreover, results from the Sorensen study suggest that the M-Vac system has potential for use in forensic science biological evidence collection. However, Amicon filter or Vivacon DNA concentration devices are not sufficient for concentrating large dilute biological mixture solutions. Instead, vacuum filtration as a concentration technique is suggested.
4. Conclusion

Results obtained in this study indicate that vacuum filtration is a more effective method to concentrate large volume biological samples than the evaporation method. Several drawbacks associated with the evaporation method were discovered. These disadvantages did not allow for the maximum collection of cells and the DNA yields obtained were significantly low. The evaporation method involved long procedures, and required careful attention to several fractions and extra handling of biological samples and tubes that may have increase the risk of contamination. Furthermore, the potential for DNA/cell loss by cell lysis or cells adsorbing to bottle walls was evident.

In contrast, the vacuum filtration method resulted in higher DNA yields and is a more effective method for optimizing biological collection from large volumes or surface areas. Optimal buffer solutions were determined for the collection of each biological sample (saliva, blood, semen). The results from the vacuum filtration method revealed the importance of understanding how a buffer interacts with cells and can maximize or hinder the collection of biological samples. According to the findings of this study, the optimal buffers that can be utilized in the biological collection of saliva are DI H$_2$O and TE. For the biological collection of blood and semen samples, DI H$_2$O and Tween-80 were the optimal buffers. The Gill buffer resulted in significantly low DNA yields for all the cell types (saliva, blood, semen) examined in this study suggesting DI H$_2$O, TE or Tween-80 are appropriate buffers to concentrate cellular material using this method, while Gill buffer should be avoided.
Overall, results from the vacuum filtration concentration method reveal that DNA can be obtained from large volumes containing dilute biological material. These results have great potential to influence the collection of large biological evidence samples or samples from large substrates at crime scenes and in the laboratory. Large dilute biological stains are often encountered at crime scenes on items such as walls, furniture or bed sheets. The ability to collect from large surface areas or volumes would allow maximum collection of cells and DNA recovery. Doing so permits effective downstream forensic DNA analysis which may lead to interpretable DNA profiles that can assist in criminal investigations.

The forensic science community is often inundated with demanding samples, increasing caseloads and is pressed for accurate identification of evidence. The use of an effective collection method for large biological samples would improve the overall efficiency of modern forensic science laboratories and accurate quantifications and successful STR genotyping would ensue.
5. Future Directions

Future experimentation may seek to determine the effect time has on filter storage after vacuum filtration and the effect of time on the DNA integrity of the biological material in solution. Additionally, it would be useful to test different types of filters and determine if the amount of DNA recovered varies and the amount of DNA lost due to the specific filter utilized. Further research should be conducted with the filtration concentration method and the M-Vac system to determine if that method can be utilized to retrieve DNA from large evidence samples such as bed sheets, walls, floors, and couches.
6. References


8. Vita

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Education:

2011 M.S.  Biomedical Forensic Sciences, Boston University School of Medicine-Boston, MA  
**Master's Thesis:** Concentration of Large Volume Biological Samples for Effective and Efficient Forensic DNA Analysis

2009 M.A  Medical Science-Mental Health & Behavioral Medicine  
Boston University School of Medicine-Boston, MA  
**Master's Thesis:** Methadone Dose, Take-Home Status and Healthcare Utilization among Methadone-Maintenance Patients

2005 B.S  Rensselaer Polytechnic Institute-Troy, NY  
Major: Biology; Minor: Community & Health Psychology

Relevant Coursework

1. Forensic DNA Analysis Lecture/Lab  
2. Forensic Biology Lecture/Lab  
3. Advanced Forensic DNA Analysis Lecture/Lab  
4. Molecular Biology  
5. Biochemistry  
6. Cell Biology  
7. Genetics  
8. Histology  
9. Statistics

Laboratory Training and Experience:  
**Graduate Level:**

1. DNA Extraction with Qaigen® DNA Investigator Kit  
2. Real-time quantification with Quantifiler® Duo DNA quantification using ABI 7500 Sequence Detector and SDS software  
3. KPIC Cell Staining and Microscopy with Nikon Eclipse TE2000-S  
4. Statistical Analysis using Microsoft Excel Data Analysis Tool Pack  
5. Medical Records Analyses
Professional Experience:

2011 Boston University: Trained graduate students in Forensic DNA Analysis, Molecular Biology techniques: Vacuum Filtration, DNA extraction, Real-time DNA quantification, KPIC Cell staining and Microscopy.

2009 Center for Urban Research, Education and Training, Inc., Hartford, CT Evaluated the literacy level of adults enrolled in the Adult Literacy Program, and determined their class placements in the Literacy to Employment Program.

2008 Boston Medical Center, Boston, MA: Research Associate in Addiction Medicine, gained clinical exposure in harm reduction, screening, diagnosing and testing patients with drug and alcohol addictions. Also, conducted retrospective data collection, reviewed and assessed the quality of patient care, inpatient and outpatient medical records, physician notes and addressed compliance issues.

2007 Teacher/ Tutor for Hartford public school students in science, mathematics and language arts; organized educational enrichment field trips for the students.

2004 Internship Project: Obesity studies and effects of Estradiol Valerate on female rats. Acquired animal handling training and experimentation. Research conducted with Dr. Reid at Rensselaer Polytechnic Institute, Troy, NY- Siena College, Loudonville, NY.

2000 UCONN Research Apprentice Program: “Constructing a MBP-GFP Recombinant DNA Sequence.” Advisor, Dr. John Carson, professor in the Department of Biochemistry, UCONN Health Center, Farmington, CT. Acquired training in DNA gel electrophoresis, PCR and Recombinant DNA techniques.

Awards & Honors:

2005 RPI Academic Merit Award 2005-Academic Excellence/4.0 Semester GPA
2003-2005 Rensselaer Polytechnic Institute (RPI) Dean’s List
2001 Rensselaer Polytechnic Institute Mathematics and Science Medal
2001 Third-Place Award, Junior Humanities & Science Symposium, UCONN-Storrs, CT
Student Committees & Leadership:

2010-2011  Secretary, Boston University Forensic Science Society at Boston University, organized monthly meetings, regional and national conferences.

To Present  Classically trained violinist with 20+ years performances as a soloist, in orchestras and ensembles; founded string ensemble with ongoing performances at weddings, special events programs and concerts.

Professional Associations

Affiliate of American Academy of Forensic Sciences

Presentations

Boston University, Boston, MA- Gretchen Johnson. Awareness on Hearing Loss. 2008