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# DNA extraction with Qiagen bio-robot EZ1 DNA investigator kit, forensicGEM Sex Crime/Universal kit and Qiagen QIAamp investigator kit: a comparison and optimization study of DNA percent recovery on body fluids for forensic applications

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BOSTON UNIVERSITY

SCHOOL OF MEDICINE

Thesis

# DNA EXTRACTION WITH QIAGEN BIO-ROBOT EZ1 DNA INVESTIGATOR KIT, FORENSICGEM SEX CRIME/UNIVERSAL KIT AND QIAGEN QIAAMP INVESTIGATOR KIT: A COMPARISON AND OPTIMIZATION STUDY OF DNA PERCENT RECOVERY ON BODY FLUIDS FOR FORENSIC APPLICATIONS

by

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B.S., Western New England University, 2016

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requirements for the degree of

Master of Science

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**DNA EXTRACTION WITH QIAGEN BIO-ROBOT EZ1 DNA INVESTIGATOR KIT, FORENSICGEM SEX CRIME/UNIVERSAL KIT AND QIAGEN QIAAMP INVESTIGATOR KIT: A COMPARISON AND OPTIMIZATION STUDY OF DNA PERCENT RECOVERY ON BODY FLUIDS FOR FORENSIC APPLICATIONS**

**ALEXANDRA ELIZABETH HANLEY**

ABSTRACT

The first step in forensic deoxyribonucleic acid (DNA) analysis process is extraction. The extraction process lyses cells and isolates the DNA from the rest of the cellular components. There are many different extraction methods that forensic laboratories can implement within their department, so it is important to determine which extraction method performs the best in regards to DNA recovery, cost, time and ease of use. The percent of DNA recovered can demonstrate how well the extraction mechanism works. Little research has been done to compare different extraction methods based on their percent DNA recovery alone. This project compares different extraction methods based on their percent DNA recovery. The three extraction methods that were investigated and compared were: the Qiagen BioRobot EZ1® DNA Investigator kit (EZ1 method) used on the Qiagen BioRobot EZ1®, the forensicGEM™ Sex Crime/Universal kit™ (ZyGEM/Acrosolv) method and the manual Qiagen QIAamp® Investigator kit. Two different percent recovery calculations were described in this study (method 1 and method 2) but only method 1 was used for analysis purposes. Only method 1 was used because it was determined to be the most reliable method for comparison. This

demonstrates how important it is to calculate and report the percent recovery consistently because the results could differ depending on how the conclusions are reported. This project demonstrated that the ZyGEM/Acrosolv extraction method outperformed the other two methods when percent recovery was being investigated with two different biological fluids (semen and saliva). The percent recovery with sperm and epithelial cells (e-cells) with the ZyGEM/Acrosolv method was 109.4% and 103.9% respectively. With the EZ1 method, the sperm and e-cell DNA percent recovery was 92.3% and 55.7% respectively and the manual Qiagen had a 39.6% recovery with e-cell DNA and a 17.3% recovery with sperm cell DNA.

A study was also performed to determine the optimum working conditions for the EZ1 method. An elution volume and incubation time study with the EZ1 method was performed and it was determined that the three elution volumes (50 µL, 100 µL and 200 µL) tested did not affect the percent recovery adversely. The different incubation times tested (3, 5 and 10 hours) did not affect the percent recovery of e-cells significantly, however, there was a downward trend in recovery as the incubation times increased. A digest volume study was also performed with the ZyGEM method which resulted in higher percent e-cell recoveries generated for the 100 µL digest volume when compared to 20 µL.

All three extraction methods generated similar results in a refined dilution study which showed that when lower concentrations of DNA were extracted, the percent recovery was higher in comparison to higher concentrations of DNA being extracted. This aspect is very important as most forensic DNA samples are low in concentration

which makes it important that these extraction methods are able to extract very low concentrations of DNA efficiently.

The cost, ease of use and analysis time was also evaluated for all three methods and it was concluded that the ZyGEM/Acrosolv method was overall the best extraction method to use in a forensic DNA laboratory. This is due to the one-tube hands-off characteristic of the ZyGEM/Acrosolv method. Because of this feature, the ZyGEM/Acrosolv method is easier to use, faster and more reliable than the other extraction methods. It also has the least amount of analyst interaction so the samples should be more consistent. During this study, the ZyGEM/Acrosolv protocol had higher and more consistent percent recoveries with both sperm and e-cells but there was a downward trend in recovery as the amount of DNA increased. This downward trend was seen more prominently with ZyGEM/Acrosolv than with the EZ1 and manual Qiagen methods. Electropherograms were also produced with selected EZ1 and ZyGEM/Acrosolv samples and both methods produced accurate and reliable profiles but the EZ1 method had less variability in peak heights in comparison to ZyGEM/Acrosolv.

Overall, both the ZyGEM/Acrosolv and the EZ1 extraction methods would be good procedures to use within a forensic DNA laboratory. The manual Qiagen extraction method generated very low percentage recoveries and had more variation when compared to the other two methods, therefore, it would not be recommended to use this method within a forensic laboratory today. The EZ1 BioRobot is currently used in many forensic laboratories and produces reliable results but this study proved that the ZyGEM/Acrosolv

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| A | Adenine |
| AT | Analytical Threshold |
| CE | Capillary electrophoresis |
| $cm^2$ | Centimeters squared |
| CCD | Charge-couple device |
| Ct | Cycle threshold |
| C | Cytosine |
| DNA | Deoxyribonucleic acid |
| DTT | Dithiothreitol |
| rpm | Eevolutions per minute |
| e-cells | Epithelial Cells |
| EDTA | Ethylenediaminetetraacetic acid |
| GuHCl | Guanidine hydrochloride |
| GuSCN | Guanidine thiocyanate |
| G | Guanine |
| IPC | Internal PCR control |
| µL | Microliter |
| mL | Milliliters |
| mM | Millimolar |
| ng | Nanograms |
| PCR | Polymerase chain reaction |

| | |
|---|---|
| pH | Potential of hydrogen |
| qPCR | Quantitative PCR, or real-time PCR |
| RFLPs | Restriction fragment length polymorphisms |
| RNA | Ribonucleic acid |
| STR | Short tandem repeats |
| T | Thymine |
| TE | Tris-buffer |
| UV | Ultra-violet |

# 1. INTRODUCTION

## 1.1 Deoxyribonucleic Acid and Forensic Science

In forensics, deoxyribonucleic acid (DNA) is known as the "gold standard" when it comes to all of the different identification methods in the world of forensic science (1). Everyone's DNA is almost identical but in forensics, analysts are looking at certain locations that are known to be different between every individual. These differences are what make everyone's DNA profile different, besides identical twins (1). Half of the DNA within an individual is inherited from the mother and the other half is inherited from the father and it is passed on randomly. The DNA molecule is found in every nucleated cell within the body (2) and therefore can be found in body fluids such as blood, saliva, and semen. The DNA molecule is made up of four bases called nucleic acids: adenine (A), guanine (G), cytosine (C), and thymine (T). These bases pair together by hydrogen bonds, (A-T and C-G) and these sequences of bases creates the unique genetic code of an individual (2, 3). These bases are connected by a sugar-phosphate backbone which is held together by a phosphodiester bond (2). The phosphodiester and hydrogen bonds within the DNA molecule make the DNA helix very stable. (3).

Investigators have been using DNA evidence to link suspects to biological evidence since the 1980's when Alec J. Jeffreys discovered multi-locus DNA patterns and coined the term DNA fingerprinting (4). Restriction fragment length polymorphisms (RFLPs), which replaced the use of multi-locus probes, are DNA regions that differ in length between individuals and can be cut by restriction enzymes at specific locations along the genome. When the sequences are cut by the enzymes, they produce certain

fragment lengths that can be analyzed and compared to those from other individuals (5, 6). It was documented that the RFLPs patterns were unique to that one individual and this gave rise to forensic DNA investigative work.

Today, forensic DNA analysts use short tandem repeats (STRs) (5, 7) for DNA analysis. STRs are repeated sequences of two to seven nucleotides in a row. These repeats are located in many locations throughout the genome but only a few are used for DNA identification and those STR positions are called loci (7, 8). Typically, 15-22 STR loci are tested and used to associate or exclude known individuals as contributors of DNA from biological evidence.

DNA profiles that are found at crime scenes can link or exonerate someone from participation in a crime. Because of this, it is extremely important to have dependable methods to generate accurate and reliable DNA profiles from the biological sample collected. Since many forensic samples have a low quantity of DNA or may be degraded (9) the DNA analysis steps must be accurate, efficient, reproducible and, most importantly, reliable.

The first step of DNA analysis in a forensic laboratory is DNA extraction, which removes the DNA from the nucleus and isolates it from the rest of the cellular components within the cell. Second is quantification, which measures how much DNA is present or the concentration of DNA within the sample. The third is amplification through the polymerase chain reaction (PCR), which amplifies or copies the DNA sequence of interest so there is plenty of DNA template for analysis. Lastly, capillary electrophoresis (CE) is performed which separates the DNA molecules from each other

based on molecular weight to determine the length of the fragments. Software is used in conjunction to the CE to generate a DNA profile (9). There are many studies published that demonstrate how different extraction methods work in regards to how consistent and accurate the DNA profile is at the end of the four steps but there are very few that study the extraction efficiency or percent recovery of the DNA. A paper by Kemp, Winters, Monroe and Barta explained that "nearly all of them (extraction methods) were initiated with no knowledge of the actual starting DNA quantity in the samples prior to extraction, so they ultimately compared the outcome of all methods relative to the best." (10) This is an extremely important issue because forensic DNA samples are usually low template or degraded so it would be important to know how well the extraction method works in regards to percent recovery before an extraction method is chosen for the laboratory to use.

### 1.2. Extraction Process

DNA extraction is the first step out of four main steps within forensic DNA analysis process. The process of solid phase extraction works by lysing the cell to release the DNA and isolating the DNA from other cellular components and substrate material (11). The solid phase extraction process itself follows five main steps. The first step is cell lysis, the second is the removal of lipids, the third is the digestion of proteins, fourth is DNA binding and lastly, elution of the DNA into a solution (figure 1) (12). There are also liquid phase extractions which do not employ a binding/elution step but follow the same principles. The goal of the extraction is to isolate the DNA from the other material present in the cell and to remove inhibitors and keep the sample stable after the extraction

process for long-term storage (5). Because the extraction step is the first step in the DNA analysis process, it is very important to make sure the method chosen recovers the nucleic acids present efficiently. If the amount of DNA on the substrate starts out in a limited quantity and then the extraction method only recovers 50% of the DNA present, this could lead to an incomplete DNA profile or no profile at all. Ideally, this process will be reliable, accurate, quick, work on different substrates, and have good extraction efficiency.

Older extraction methods were processed by hand and used organic solvents, such as phenol-chloroform. The organic solvents were hazardous and very time-consuming. For safety, these extraction methods are typically not used anymore (5). Next implemented were Chelex extractions, in which ion-exchange resins (styrene-divinylbenzene copolymers) are added to the sample. These act as chelating groups that will bind magnesium present in the sample, therefore inactivating DNA nucleases. This method is more efficient and less time-consuming than the organic extractions but can cause some PCR inhibition (5). A method that is still used today in some forensic laboratories is the Qiagen extraction using the QIAamp membrane columns. This method is also a manual technique but it uses a solid-phase extraction method in comparison to liquid-phase extractions that were used previously. The QIAamp column takes advantage of a silica membrane on which the DNA will adsorb in the presence of chaotropic salts. These salts break hydrogen bonds and keep proteins denatured so the DNA will bind to the silica membrane and then only the DNA is collected (5).

Today, extractions are largely carried out by robots which implement a solid-phase extraction rather than liquid phase. This is because solid-phase extractions are easier to automate (13). The changeover to automation has decreased the time it takes to process samples and reduces analyst error (5, 14). Another advantage is that more samples can be processed at once to increase productivity and efficiency (14). There are many different bio-robots that can be applied to forensic DNA analysis. It is up to the laboratory to choose the most efficient robot that will generate the best results. To do this, the laboratory will conduct and/or review validation studies that demonstrate the reliability and reproducibility of the robot.

But again, most studies that are performed on bio-robots do not look at the percent recoveries of the DNA extracted (10). Most studies will generate data that show the lowest concentration of DNA that can be obtained from the bio-robot procedure but not how much DNA the robot is actually picking up and eluting. One must compare the starting DNA quantity to the DNA quantity recovered after extraction to determine if the method is actually recovering as much as possible for reliable data.

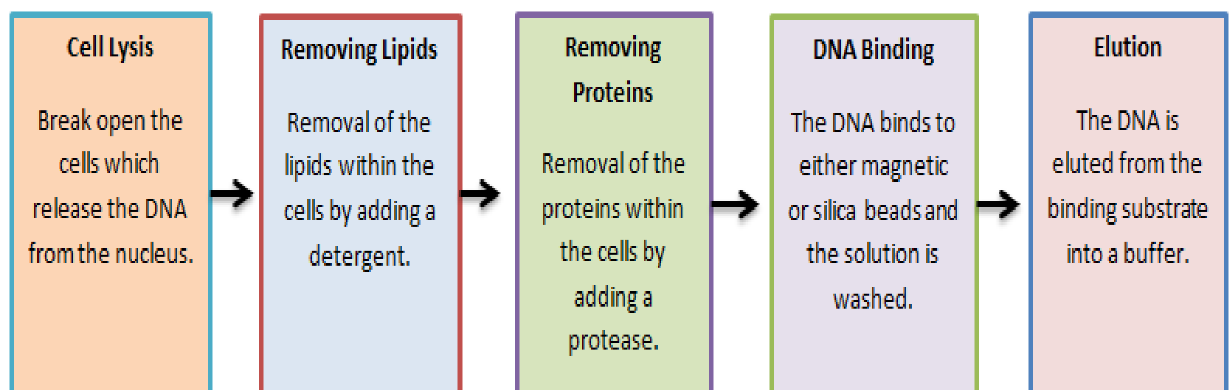



**Figure 1**: Common DNA Extraction Process (11)

## 1.3 EZ1 Extraction Robot®

**Figure 2: Overview of the QIAGEN® EZ1® Advanced Bio-Robot (QIAGEN®, Hilden, Germany)** Photo obtained from the Qiagen EZ1® manual and depicts the door, control panel, card slot and the status LEDs (16).

The QIAGEN® EZ1® (QIAGEN®, Hilden, Germany) is a commonly used bio-robot used for forensic DNA extraction. It was first made available in the mid 2000's and is still in use in many forensic DNA units across the country (15). The EZ1 workstation is compact with an easy to follow design and can extract DNA from six samples at once in less than 20 minutes. The newer EZ1 Advanced XL can extract DNA from 14 samples

per run. To operate the EZ1 workstation a protocol card is inserted into the instrument to run the correct protocol desired (15). There are many cards that have different protocols available depending on the sample type and substrate used. During this study, the QIAGEN® EZ1® DNA Investigator® Kit and the QIAGEN® EZ1® DNA Investigator® Card (QIAGEN®, Hilden, Germany), which is made for forensic-type samples, was used on all samples extracted. The chemistry behind the EZ1 instrument is magnetic bead technology (13, 15). Extraction works by DNA being isolated out from other cellular components by binding to a silica surface of the magnetic beads in the presence of a chaotropic salt (guanidine thiocyanate (GuSCN)/guanidine hydrochloride (GuHCl)) (13, 15). The chaotropic salts "lyse cells, denature proteins and inhibit nucleases as well as promote the binding of DNA to the paramagnetic-silica beads" (10, 13). The binding to the magnetic beads and the wash step occurs within the instrument pipette tip. A magnet is applied to hold the DNA and beads in place while the liquid components are removed. The DNA is then eluted and captured in a solution that has a low ionic strength, either TE buffer (Tris buffer) or water (13, 16). For genomic DNA, an incubation step with a lysis buffer (G2) and proteinase K is needed to lyse the cells prior to the robotic steps (16). Carrier ribonucleic acid (RNA) can be added to samples after the incubation period to increase the yield of DNA recovered (17). The mechanism behind this phenomenon might not be well understood but many researchers have concluded that RNA should always be added to increase recovery (16, 13, 17, and 18).

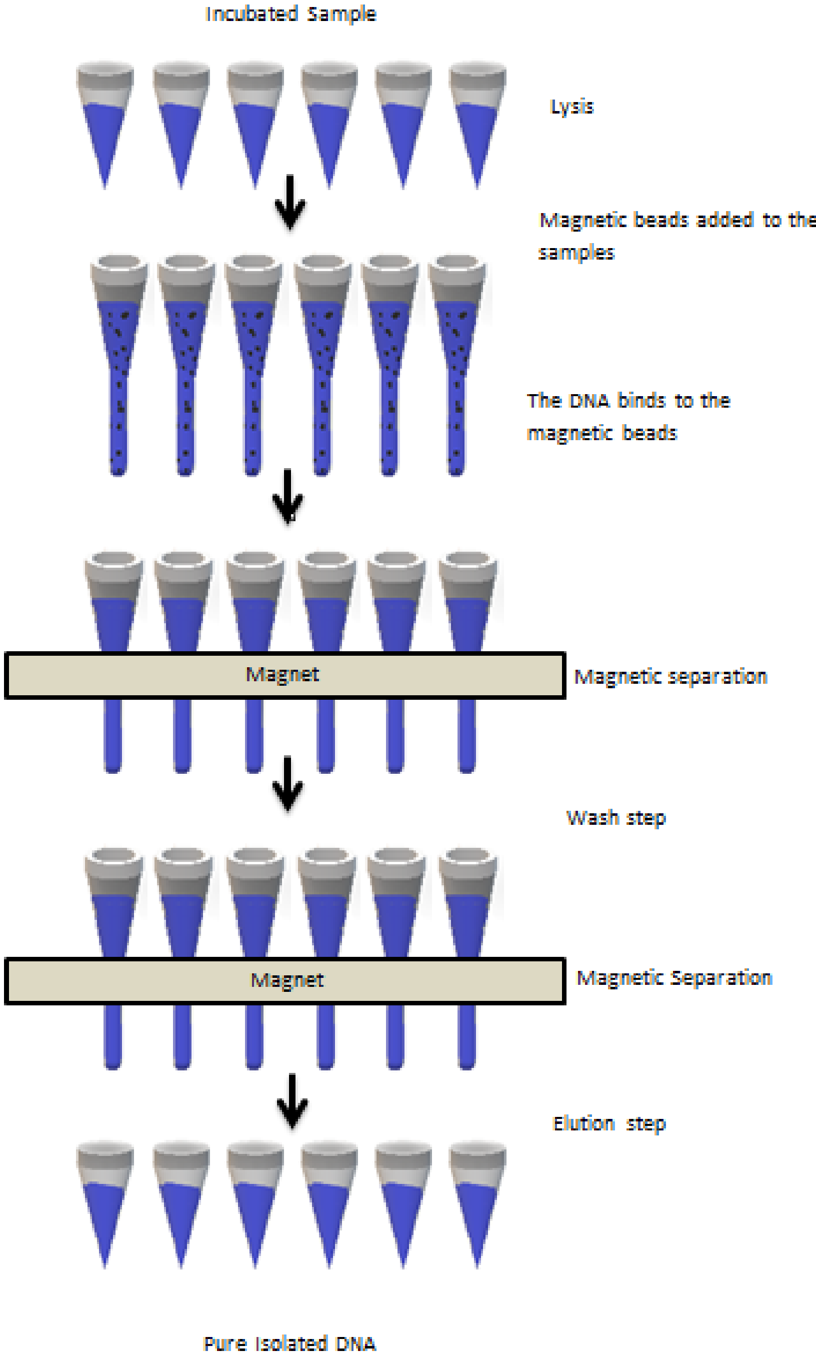



**Figure 3**: QIAGEN EZ1 Robot® Extraction Procedure (16).

**1.4 Manual Qiagen Extraction®**

Before the use of bio-robots, DNA extraction was performed manually by the forensic analyst. One manual extraction method is the Qiagen QIAamp® DNA Investigator kit. This technique is a solid-phase extraction which uses a silica-based membrane (19) in the presence of chaotropic salts (**Figure 3**). DNA can bind to the silica with the help of chaotropic salts, which break hydrogen bonds so water can bind to each other and less with DNA so DNA will be able to bind to the silica more readily (5). When the DNA is bound to the silica membrane, a solution with a pH (potential of hydrogen) <7.5 is applied to wash away cellular components from the membrane. Now that only DNA is left bound, the DNA can be eluted and collected by a low salt, slightly alkaline pH buffer (5). The biggest disadvantage of this method compared to others is that this method uses multiple tubes during the extraction process. This could lead to poor recovery because some of the DNA might be left behind in the previous tube when all of the transfer steps are being performed. Also, because this method is performed by an analyst, there could be high variation between samples when different analysts are performing the extractions. Like the EZ1 extraction method, this protocol can also be fully automated on another bio-robot, the QIACube (19, 20).

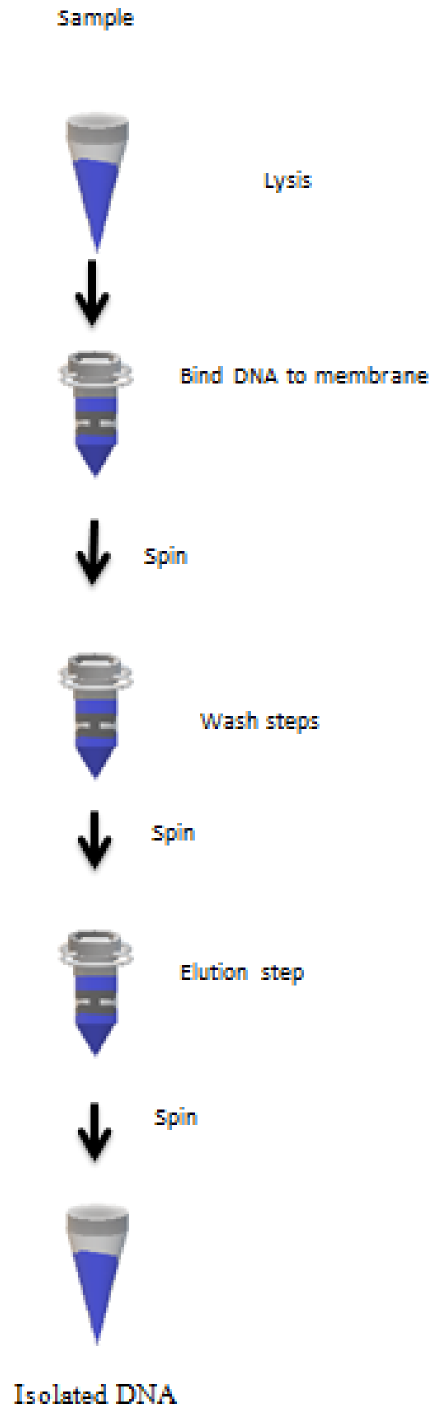



**Figure 4**: Qiagen QIAamp® DNA Investigator Extraction Procedure (20).

### 1.5 ZyGEM/Acrosolv Extraction®

The forensicGEM kits® use a "proprietary extremophile protease whose activity is highly temperature-dependent, and capable of rapidly digesting non-nucleic acid components in a sample" (21). This extraction method uses a mixture of thermophilic and mesophilic enzymes and uses temperature variation to activate those different enzymes. At temperatures around $75^0$C, mesophilic cell wall degrading enzymes (22) activate then thermophilic proteinases are activated and lyse the cells, inactivate the nucleases and remove the DNA from its nucleosomes. The final temperature stage around $95^0$C deactivates the thermophilic proteinase (22). For samples containing semen/sperm, the reagent Acrosolv must be used which is contained within the forensicGEM® Sex Crime kit. Acrosolv is a mixture of proprietary reagents that weaken tissue cell walls, like sperm, and release the DNA (22). The ZyGEM extraction method is a single tube extraction process. This means that in theory, no nucleic acids should be lost because there are no transfer steps and no solid phase extractions. This is the main advantage of this extraction method. Therefore, the ZyGEM extraction method was used as a control to compare to the other extraction methods in this study.

### 1.6 Quantification qPCR Process

The main purpose of this study is to compare the yield from extraction methods, so the DNA quantification step is just as important to take into consideration as the extraction step. The quantification step measures how well the extraction method performed. The main instrument used for the quantification of DNA for forensic purposes is the qPCR (quantitative PCR, or real-time PCR) instrument. The qPCR kit that was

used during these experiments was the Quantifiler® Duo kit. The Quantifiler® Duo kit is comprised of a multiplexed TaqMan® real-time PCR assay (23). The assay can identify human DNA, human male DNA and contains an internal PCR control (IPC) in a single reaction. The IPC shows whether or not the sample has any PCR inhibition (23). The qPCR works by the TaqMan® probe annealing to a sequence between the forward and reverse primers (23). This probe consists of a reporter dye, a quencher and the AmpliTaq Gold® DNA polymerase. When the probe is intact there is no fluorescence from the reporter molecule. When DNA polymerase starts amplifying, the reporter will be released from the quencher and fluorescence will accumulate. This accumulation in fluorescence will be detected by a charge-coupled device (CCD) camera. There is a fluorescent threshold within the software that tells the instrument how much fluorescence is accumulating during each cycle. The software will generate a cycle threshold (Ct) value which shows how many PCR cycles it took for the fluorescent accumulation to cross this threshold (5, 23). This value is inversely proportional to the amount of DNA present within the sample. If the Ct value is low that means that more DNA is present in the sample. The Ct value is used to calculate the concentration of human DNA or human male DNA present in the sample. By comparison to a standard curve, the Y-intercept and slope are used in the formula to calculate the concentration (24).

**1.7 Purpose of Study**

The purpose of this experiment was to determine if the EZ1 method outperformed other DNA extraction methods chosen in regards to percent recovery, to determine optimum working parameters for the methods and to determine which extraction method

would be better to implement into a forensic DNA laboratory based on recovery, ease of use, cost and analysis time.

There are many extraction methods on the market today, so it is important to ensure that the extraction method chosen will produce the best results (DNA recovery/DNA purity) in comparison to the other extraction methods. A study by Montpetit et al. compared aspects such as elution volume and sample type differences with the EZ1 bio-robot and the EZ1 Qiagen tissue kit (13). They found that elution volumes of 200 μL and 50 μL generated better results in comparison to 100 μL. They also reported that the BioRobot EZ1 has the ability to extract DNA from small amounts of saliva found on a variety of substrates (13) so it is important to determine what working parameters are necessary for the EZ1 Qiagen Investigator Kit. There are many research articles comparing the BioRobot EZ1 itself to other extraction methods but not many used the Investigator Kit which is produced for forensic casework samples, or to compare these three methods specifically. No studies have been performed comparing the ZyGEM and EZ1 methods, so it is important to determine what method out-performs the other and will be best implemented into a forensic laboratory.

Aspects such as elution and digest volumes, incubation time, sample volume, and cell type (sperm and e-cells) could all play a role in how well DNA is recovered. If the extraction method performs poorly then the next steps in the forensic DNA analysis process could be hindered and therefore ideal results might not be produced. The methodology for this paper was modeled after Kemp et al., which discussed how important it is to determine the starting concentration of DNA in the sample to determine

the percent recovery of the extraction method correctly (10). This method was followed to determine the percent efficiency of each extraction method chosen to determine which one is the more efficient. Electropherograms were also produced for selected EZ1 and ZyGEM/Acrosolv samples to compare the peak heights and to determine if any drop out was seen (5).

## 2. MATERIALS AND METHODS

All benches, pipettes and equipment were cleaned with 10% Sodium Hypochlorate (bleach) followed by 70% Ethanol. The QIAGEN EZ1 extraction robot® was cleaned with 70% Ethanol and 20 minutes of ultra-violet (UV) decontamination.

### 2.1 Cell Suspension Preparation

2.1.1 Buccal Cell Preparation

Saliva samples were collected from a female donor into a 1.5 mL (milliliter) microcentrifuge tube (Eppendorf, UK). The epithelial cells that are contained in the saliva needed to be cleaned up to minimize debris present in the sample. To do this, 300 µL of the fresh neat saliva was pipetted into a new 1.5 mL microcentrifuge tube with 300 µL of TE buffer (10mM (millimolar) Tris, pH 8.0, and 0.1 mM EDTA (Ethylenediaminetetraacetic acid)). The 1.5 mL tube was then placed into an Eppendorf centrifuge 5424 (Hamburg, Germany) and spun by centrifugation at 500 revolutions per minute (rpm) for 5 minutes. The supernatant was removed and discarded. The remaining pellet was re-suspended in 400 µL of TE buffer. The centrifuge and pellet re-suspended step was repeated three times to eventually have an end product of 200 µL solution of buccal cells in TE buffer.

2.1.2 Epithelial Cell Counting

A Hemacytometer with a coverslip was used to determine the concentration of the buccal cell solution. The 200 µL solution of buccal cells in TE buffer was vortexed at high speed for 10 seconds to ensure the cells were not settled to the bottom of the tube. Then 8 µL of the buccal cell solution was pipetted into the wells on each side of the Hemacytometer and the coverslip was added. The Hemacytometer slide was then placed on a Nikon Eclipse TE200-S microscope using Phase Contrast Microscopy at 40x magnification. The cells within both wells were observed by use of a MAXDATA computer set up with MMI Cell Cut (Molecular Machines & Industries, Eching, Germany) equipment and software. Both sides were viewed and the epithelial cells within each grid were counted. The number of cells within each grid was totaled and divided by the number of grids counted to determine the average number of cells on each side of the Hemacytometer. The average number of cells on each side was then totaled together and averaged to generate one average number of cells. That number was multiplied by 10 to determine the average cells/ µL or average cell count in solution and then multiplied by 0.0066 to determine the concentration of cells in solution in ng/ µL.

2.1.3 Saliva Stains Preparation

Saliva samples were collected from a female donor (Dilution A) into a 1.5 mL microcentrifuge tube (Eppendorf, UK). A 1:2 dilution was created in the same manner as previously stated from the neat saliva and TE buffer. Nine 0.5cm$^2$ (centimeter squared) sized cotton fabric cuttings were obtained. One microliter of the 1:2 saliva dilution was pipetted onto four pieces of fabric, 5 µL of the 1:2 saliva dilution was pipetted onto four

pieces of fabric and 10 µL of the 1:2 saliva dilution was pipetted onto one of the pieces of fabric. The pieces of fabric were left to dry for four days under a laboratory hood.

2.1.4 Epithelial Cell Quantification using real time PCR

To determine the actual starting concentration of the epithelial cell solution, the solution was quantified using a 7500 Real Time PCR or qPCR (Applied Biosystems®, Foster City, CA) with the Quantifiler® Duo Quantification Kit (Applied Biosystems® Foster City, CA). The reaction was prepared based on the Quantifiler® Duo DNA Quantification manufacturer's instructions (23). Positive and negative controls that were included in the kit were run with the sample.

**2.2 Semen Preparation**

2.2.1 Liquid Semen Preparation

Semen samples from an anonymous male donor (PO59- BR1171316) were transferred into a 1.5 mL microcentrifuge tube (Eppendorf ©, UK). A portion of the neat sample was then pipetted into a 0.2 mL individual PCR tube with attached flat cap (AB-0337 Thermo Fisher Scientific Inc ©, UK) for individual use. A 1:10 dilution was created from the neat semen and TE buffer.

2.2.2 Semen Stains Preparation

A 1:10 dilution (Dilution A) was created from the neat semen and TE buffer. Nine 0.5cm$^2$ sized cotton fabric cuttings were obtained. One microliter of the 1:10 semen dilution was pipetted onto four pieces of fabric, 5 µL of the 1:10 semen dilution was pipetted onto four pieces of fabric and 10 µL of the 1:10 semen dilution was pipetted

onto one of the pieces of fabric. The pieces of fabric were left to dry for four days under a laboratory hood.

2.2.3 Sperm Cell Quantification using real time PCR

To determine the actual starting concentration of the sperm cell solution, the solution was quantified using a 7500 Real-Time PCR system (qPCR) with the Quantifiler® Duo Quantification Kit (Applied Biosystems® Foster City, CA). The reaction was prepared based on the Quantifiler® Duo DNA Quantification manufacturer's instructions (23). Positive and negative controls that were included in the kit were run with the sample.

**2.3 QIAGEN EZ1® Extraction**

2.3.1 DNA Investigator Kit Purification Protocol for Liquid Samples-"Trace

The "Trace Protocol" is recommended for liquid samples and directly pipettes the solution without the need of any secondary movement or assistance from the EZ1 robot (4). The extraction protocol was followed according to the "EZ1® DNA Investigator® Handbook" which accompanied the "EZ1 DNA Investigator® Kit" purchased from QIAGEN© (Hilden, Germany). The EZ1® DNA Investigator® card was inserted into the card slot and the EZ1 was turned on. A UV decontamination process was performed for 20 minutes to ensure no contamination from previous extractions. For liquid saliva samples, proteinase K and G2 buffer were vortexed and 10 µL of proteinase K and 140-190 µL of G2 buffer was added into each of the 2 mL sample tubes. Then, the appropriate amount of sample (up to 50 µL) was added to the corresponding tube, to equal a volume of 200 µL. The tubes were placed in the $56^0$C water bath for 1 hour. After incubation, 1

µL of RNA was added. For liquid semen samples, proteinase K and G2 buffer were vortexed and 10 µL of Proteinase K, the appropriate amount of G2 buffer and 20 µL DTT (Dithiothreitol) was added into each of the 2 mL sample tubes. Then, the appropriate amount of sample (up to 50 µL) was added to the corresponding tube, to equal a volume of 300 µL. The tubes were placed in the $56^0$C water bath for 2 hours. After incubation, 1 µL of RNA was added to each sample (16).

At the EZ1 instrument, on the display screen, "START" was pressed to start the extraction worktable set-up, "1" was pressed for the Trace Protocol, the elution volume was chosen (3 were tested) and TE as the elution solution. The reagents cartridges were removed from their box and were inverted to ensure that the magnetic particles were thoroughly distributed throughout the solution. The reagent cartridges were loaded into the cartridge rack and the rack placed on the instrument. Elution tubes, filter tips and tip holders, and sample tubes were placed onto the tip rack.. The instrument door was closed and the extraction method was started. After the process, the samples were recapped and either stored at $-20^0$C or analyzed by real-time PCR (16).

2.3.2 DNA Investigator Kit Purification Protocol for Dried Cotton Swatch Samples "Tip Dance Protocol"

The "Tip-Dance Protocol" on the EZ1 instrument is recommended for the processing of solid materials such as fabric, swabs or cigarette butts. For this experiment, cotton fabric swatches were chosen as a substrate for liquid semen and saliva samples. This protocol works by moving the filter-tip back and forth relative to the platform while pipetting the solutions to pipet around the substrate. This allows for the solid materials to

be kept in the sample tube during the extraction process (16). The extraction protocol was followed according to the “EZ1® DNA Investigator® Handbook” which accompanied the “EZ1 DNA Investigator® Kit” purchased from QIAGEN©. The EZ1® DNA Investigator® card was inserted into the card slot and the EZ1 was turned on. A UV decontamination process was performed for 20 minutes to ensure no contamination from previous extractions. For dried saliva samples, proteinase K and G2 buffer were vortexed and 10 µL of proteinase K and 140-190 µL of G2 buffer was added into each of the 2 mL sample tubes. Then, the appropriate amount of sample (up to 50 µL) was added to the corresponding tube, to equal a volume of 200 µL. The tubes were placed in the $56^0$C water bath for 1 hour. After incubation, the tubes were taken out and 1 µL of RNA was added to each sample. For dried semen samples, proteinase K and G2 buffer were vortexed and 10 µL of proteinase K, the appropriate amount of G2 buffer and 20 µL DTT (Dithiothreitol) was added into each of the 2 mL sample tubes. Then, the appropriate amount of sample (up to 50 µL) was added to the corresponding tube, to equal a volume of 300 µL. The tubes were placed in the $56^0$C water bath for 2 hours. After incubation, 1 µL of RNA was added to each sample. The tubes were then briefly to remove any drops from inside the lid (17).

At the EZ1 instrument, on the display screen, “START” was pressed to start the extraction worktable set-up, “2” was pressed for the Tip-Dance Protocol, the elution volume was chosen and TE or water was chosen as the elution solution. The reagents cartridges were removed from their box and were inverted to ensure that the magnetic particles are thoroughly distributed throughout the solution. The reagent cartridges were

loaded into the cartridge rack and were placed into the EZ1 instrument. Elution tubes, filter tips and tip holders, and sample tubes were placed onto the tip rack. The instrument door was closed and the extraction method was started. After the process, the samples were recapped and either stored at -20$^0$C or analyzed by real-time PCR (16).

**2.4 EZ1 Incubation Time Study**

The EZ1 protocol states that the incubation time in the 56$^0$C water bath should be at least 15 minutes and up to 24 hours (16). To understand if altering the incubation time will affect the percent recovery, a study was performed on cotton fabric with the Tip Dance Protocol and different amounts of saliva and semen were deposited onto the fabric, according to **Table 1**. The time points that were chosen were 3 hours, 5 hours and 10 hours.

**Table 1:** Components of the EZ1 extraction protocol of liquid saliva and semen samples and incubation times chosen.

| EZ1 | Sample 1- 1ul of Saliva 3 Hours | Sample 1 - 5ul of Semen 3 Hours | Sample 2- 1ul of Saliva 5 Hours | Sample 2- 5ul of Semen 5 Hours | Sample 3- 1ul of Saliva 10 Hours | Sample 3- 5ul of Semen 10 Hours |
|---|---|---|---|---|---|---|
| Sample Swatch | $0.5cm^2$ | $0.5cm^2$ | $0.5cm^2$ | $0.5cm^2$ | $0.5cm^2$ | $0.5cm^2$ |
| PK (ul) | 10ul | 10ul | 10ul | 10ul | 10ul | 10ul |
| Buffer (ul) | 190ul | 270ul | 190ul | 270ul | 190ul | 270ul |
| DTT (ul) | - | 20ul | - | 20ul | - | 20ul |
| RNA (u) | 1ul | 1ul | 1ul | 1ul | 1ul | 1ul |
| **TOTAL VOLUME (ul)** | **200ul** | **300ul** | **200ul** | **300ul** | **200ul** | **300ul** |

Each sample was run in duplicate for a total of 6 samples for each elution volume.

## 2.5 EZ1 Elution Volume Study

There are three different elution volumes the analyst can choose in conjunction with the extraction protocol (50 µL, 100 µL, and 200 µL) on the BioRobot EZ1. To determine whether or not the elution volume chosen would affect the DNA recovery, a study was performed with liquid saliva (Dilution B) with the Trace protocol according to **Table 2**.

**Table 2:** Components of the EZ1 extraction protocol of liquid saliva and elution volumes chosen.

| EZ1 | Sample 1 50uL | Sample 2 50uL | Sample 3 50uL | Sample 1 100uL | Sample 2 100uL | Sample 3 100uL | Sample 1 200uL | Sample 2 200uL | Sample 3 200uL |
|---|---|---|---|---|---|---|---|---|---|
| Cells (ul) | 10ul | 5ul | 1ul | 10ul | 5ul | 1ul | 10ul | 5ul | 1ul |
| PK (ul) | 10ul | 10ul | 10ul | 10ul | 10ul | 10ul | 10ul | 10ul | 10ul |
| Buffer (ul) | 179ul | 184ul | 188ul | 179ul | 184ul | 188ul | 179ul | 184ul | 188ul |
| RNA (u) | 1ul | 1ul | 1ul | 1ul | 1ul | 1ul | 1ul | 1ul | 1ul |
| **TOTAL VOLUME (ul)** | **200ul** | **200ul** | **200ul** | **200ul** | **200ul** | **200ul** | **200ul** | **200ul** | **200ul** |

Each sample was run in duplicate for a total of 6 samples for each elution volume.

## 2.6 EZ1 Recovery Study

A refined dilution study of volumes of liquid semen dilutions “A” (1:10) and “B” (1:10 of A) and saliva dilutions “C” (1:2) and “D” (1:10 of C) extracted using the EZ1 Trace protocol to determine if there was a trend in the DNA recovery based on the amount of DNA deposited. The protocols for saliva and semen were followed according to **Tables 3 and 4.**

**Table 3:** Components of the EZ1 extraction protocol of liquid saliva and dilution volumes chosen.

| Dilution | D 1:10C | D 1:10C | D 1:10C | D 1:10C | D 1:10C | D 1:10C | D 1:10C | D 1:10C | C 1:2 | C 1:2 | C 1:2 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Sample Amount (ul)** | 10ul | 1ul | 5ul | 10ul | 15ul | 30ul | 40ul | 60ul | 6ul | 12ul | 10ul |
| **Buffer(ul)** | 79ul | 88ul | 84ul | 79ul | 74ul | 59ul | 49ul | 29ul | 83ul | 77ul | 79ul |
| **PK(ul)** | 10ul | 10ul | 10ul | 10ul | 10ul | 10ul | 10ul | 10ul | 10ul | 10ul | 10ul |
| **RNA (ul)** | 1ul | 1ul | 1ul | 1ul | 1ul | 1ul | 1ul | 1ul | 1ul | 1ul | 1ul |
| **Total Volume(ul)** | 100ul | 100ul | 100ul | 100ul | 100ul | 100ul | 100ul | 100ul | 100ul | 100ul | 100ul |

Table for the saliva protocol. Dilution C (1:2) and dilution D (1:10 of dilution C) were used. Each sample was quantifed in triplicate.

**Table 4:** Components of the EZ1 extraction protocol of liquid semen samples and incubation times 3, 5 and 10 hours.

| Dilution | B 1:10A | B 1:10A | B 1:10A | B 1:10A | B 1:10A | B 1:10A | B 1:10A | B 1:10A | A 1:10 | A 1:10 | A 1:10 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Sample Amount (ul)** | 10ul | 1ul | 5ul | 10ul | 15ul | 30ul | 40ul | 60ul | 6ul | 12ul | 10ul |
| **DTT (ul)** | 20ul | 20ul | 20ul | 20ul | 20ul | 20ul | 20ul | 20ul | 20ul | 20ul | 20ul |
| **PK (ul)** | 10ul | 10ul | 10ul | 10ul | 10ul | 10ul | 10ul | 10ul | 10ul | 10ul | 10ul |
| **RNA (ul)** | 1ul | 1ul | 1ul | 1ul | 1ul | 1ul | 1ul | 1ul | 1ul | 1ul | 1ul |
| **Buffer (ul)** | 259ul | 268ul | 264ul | 259ul | 254ul | 239ul | 229ul | 209ul | 263ul | 257ul | 259ul |
| **Total Volume(ul)** | 300ul | 300ul | 300ul | 300ul | 300ul | 300ul | 300ul | 300ul | 300ul | 300ul | 300ul |

Table for the semen protocol. Dilution A (1:10) and dilution B (1:10 of dilution A) were used. Each sample was quantifed in triplicate.

## 2.7 ZyGEM Extraction

### 2.7.1 ZyGEM Extraction for Liquid Saliva

The appropriate saliva dilution was taken out of the $-20^0$C freezer along with the *forensic*GEM™ Universal kit which contains *forensic*GEM™ solution and 10X BLUE buffer (Hamilton, New Zealand). Digests were set up according to the appropriate table in the corresponding protocol in 0.2 mL individual PCR tube (Applied Biosystems, Foster City, CA, USA). The samples were placed in the SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific, Waltham MA). On the SimpliAmp™ Thermal work table displays the “General ZyGEM Protocol” was chosen (25):

1. $75^0$C for 15 minutes.
2. $95^0$C for 5 minutes.
3. $4^0$C for infinity

### 2.7.2 ZyGEM Acrosolv Extraction for Liquid Semen

The appropriate semen dilution was taken out of the $-20^0$C freezer along with the *forensic*GEM™ Sex Crime kit reagents which contain 10X Orange buffer, *forensic*GEM™ solution and Acrosolv. Digests were set up according to the appropriate table in the corresponding protocol in 0.2 mL individual PCR tube. The samples were placed in the SimpliAmp™ Thermal Cycler. On the SimpliAmp™ Thermal Cycler work table display the “General ZyGEM Protocol” was chosen and the three incubation times were changed manually to (22):

1. $52^0$C for 5 minutes.

2. $75^0$C for 3 minutes.
3. $95^0$C for 3 minutes.

2.7.3 ZyGEM Extraction for Dried Saliva

The dried saliva samples on cotton fabric were obtained after the drying period. The pieces of fabric were incubated in 20 μL TE buffer for 1 hour to allow the cells to rehydrate into solution in a 0.2 mL individual PCR tube with attached flat cap. After 1 hour, the PCR tube was opened and the appropriate reagents according to **Table 5** were added to the corresponding tubes. The *forensic*GEM™ Universal kit protocol was followed (18).

**Table 5:** Components of the ZyGEM extraction protocol of liquid saliva to compare to the EZ1 liquid saliva incubation experiment.

| ZyGEM | Sample 1- 1ul of Saliva | Sample 2- 5ul of Saliva | Sample 3- 10ul of Saliva |
|---|---|---|---|
| Sample Swatch | $0.5cm^2$ | $0.5cm^2$ | $0.5cm^2$ |
| Zygem (ul) | 2ul | 2ul | 2ul |
| BLUE Buffer for Saliva (ul) | 10ul | 10ul | 10ul |
| Acrosolv (ul) | - | - | - |
| TE Buffer (ul) | 68ul | 68ul | 68ul |
| **TOTAL VOLUME (ul)** | **100ul** | **100ul** | **100ul** |

### 2.7.4 .ZyGEM Acrosolv Extraction for Dried Semen

The dried semen samples on cotton fabric were obtained after the drying period. The pieces of fabric were incubated in 20 μL TE buffer for 1 hour to allow the cells to rehydrate into solution in a 0.2 mL individual PCR tube with attached flat cap. After 1 hour, PCR tube was opened and the appropriate reagents according to **Table 6** were added to the corresponding tubes. The *forensic*GEM™ Sex Crime kit protocol was followed (22).

**Table 6:** Components of the Acrosolv extraction protocol of liquid semen to compare to the EZ1 liquid semen incubation experiment.

| **Acrosolv** | **Sample 1- 1ul Semen** | **Sample 2- 5ul Semen** | **Sample 3- 10ul Semen** |
|---|---|---|---|
| Sample Swatch | $0.5cm^2$ | $0.5cm^2$ | $0.5cm^2$ |
| Zygem (ul) | 2ul | 2ul | 2ul |
| ORANGE Buffer for Semen(ul) | 10ul | 10ul | 10ul |
| Acrosolv (ul) | 10ul | 10ul | 10ul |
| TE Buffer (ul) | 58ul | 58ul | 58ul |
| **TOTAL VOLUME (ul)** | **100ul** | **100ul** | **100ul** |

## 2.8 ZyGEM Digest Volume Study

There are different digest volumes the analyst can choose in conjunction with their protocol. In the past, students have used 20 µL and 100 µL as digest volumes with the ZyGEM/Acrosolv extraction method. To determine whether or not the elution volume chosen would affect the DNA recovery, a study was performed with liquid saliva (Dilution B) according to **Table 7**.

**Table 7:** Components of the ZyGEM extraction protocol of liquid saliva and digestion volumes chosen.

| ZyGEM | A1 | A2 | A3 | A4 | B1 | B2 | B3 | B4 |
|---|---|---|---|---|---|---|---|---|
| Cells (ul) | 10ul | 5ul | 1ul | 1ul of a 1:10 Dilution (1ul of cells + 9ul of TE) | 10ul | 5ul | 1ul | 1ul of a 1:10 Dilution (1ul of cells + 9ul of TE) |
| ZyGEM (ul) | 2ul | 2ul | 2ul | 2ul | 2ul | 2ul | 2ul | 2ul |
| BLUE Buffer (ul) | 2ul | 2ul | 2ul | 2ul | 10ul | 10ul | 10ul | 10ul |
| TE Buffer (u) | 6ul | 11ul | 15ul | 15ul | 78ul | 83ul | 87ul | 87ul |
| **TOTAL VOLUME (ul)** | **20ul** | **20ul** | **20ul** | **20ul** | **100ul** | **100ul** | **100ul** | **100ul** |

Samples labeled A are samples in a digest volume of 20 µL and samples labeled B are samples in a digest volume of 100 µL.

## 2.9 ZyGEM/Acrosolv Recovery Study

A refined dilution study of volumes of liquid semen dilutions “A” (1:10) and “B” (1:10 of A) and saliva dilutions “C” (1:2) and “D” (1:10 of C) were extracted using the ZyGEM/Acrosolv protocol (19) to determine if there was a trend in the DNA recovery based on the amount of DNA deposited. This experiment was also used to compare the extracted DNA amounts recovered to the same experiment conducted with the EZ1 and manual Qiagen extraction protocols. The protocols for saliva and semen were followed according to **Tables 8 and 9.**

**Table 8:** Components of the ZyGEM extraction protocol of liquid saliva and dilution volumes chosen.

| Dilution | D<br>1:10C | D<br>1:10C | D<br>1:10C | D<br>1:10C | D<br>1:10C | D<br>1:10C | D<br>1:10C | C<br>1:2 | C<br>1:2 |
|---|---|---|---|---|---|---|---|---|---|
| **Sample Amount (ul)** | 1ul | 5ul | 10ul | 15ul | 30ul | 40ul | 60ul | 6ul | 12ul |
| **ZyGEM (ul)** | 2ul | 2ul | 2ul | 2ul | 2ul | 2ul | 2ul | 2ul | 2ul |
| **BLUE Buffer (ul)** | 10ul | 10ul | 10ul | 10ul | 10ul | 10ul | 10ul | 10ul | 10ul |
| **TE Buffer (ul)** | 87ul | 83ul | 78ul | 73ul | 58ul | 48ul | 28ul | 82ul | 76ul |
| **Total Volume (ul)** | 100ul | 100ul | 100ul | 100ul | 100ul | 100ul | 100ul | 100ul | 100ul |

Table for the saliva protocol. Dilution C (1:2) and dilution D (1:10 of dilution C) were used. Each sample was quantifed in triplicate

**Table 9:** Components of the Acrosolv extraction protocol of liquid semen and dilution volumes chosen.

| Dilution | B<br>1:10A | B<br>1:10A | B<br>1:10A | B<br>1:10A | B<br>1:10A | B<br>1:10A | B<br>1:10A | B<br>1:10A | B<br>1:10A | A<br>1:10 | A<br>1:10 | A<br>1:10 | A<br>1:10 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Sample Amount (ul)** | 10ul | 10ul | 1ul | 5ul | 10ul | 15ul | 30ul | 40ul | 60ul | 6ul | 12ul | 10ul | 10ul |
| **ZyGEM (ul)** | 2ul | 2ul | 2ul | 2ul | 2ul | 2ul | 2ul | 2ul | 2ul | 2ul | 2ul | 2ul | 2ul |
| **ORANGE Buffer (ul)** | 10ul | 10ul | 10ul | 10ul | 10ul | 10ul | 10ul | 10ul | 10ul | 10ul | 10ul | 10ul | 10ul |
| **Acrosolv (ul)** | 10ul | 10ul | 10ul | 10ul | 10ul | 10ul | 10ul | 10ul | 10ul | 10ul | 10ul | 10ul | 10ul |
| **TE Buffer (ul)** | 68ul | 68ul | 77ul | 73ul | 68ul | 63ul | 48ul | 38ul | 18ul | 72ul | 66ul | 68ul | 68ul |
| **Total Volume (ul)** | 100 ul | 100 ul | 100 ul | 100 ul | 100 ul | 100 ul | 100 ul | 100 ul | 100 ul | 100 ul | 100 ul | 100 ul | 100 ul |

Table for the semen protocol. Dilution A (1:10) and dilution B (1:10 of dilution A) were used. Each sample was quantifed in triplicate.

## 2.10 Manual Qiagen- QIAamp Investigator Extraction

### 2.10.1 Manual Qiagen- QIAamp Investigator Extraction of Liquid Saliva

The appropriate amount of liquid saliva dilution “C” (1:2) and “D” (1:10 of C) was added to a 2 mL microcentrifuge tube. Buffer ATL, Proteinase K, Buffer AL, ethanol, Buffer AW1, and Buffer AW2 were added to the samples according to the QIAamp® DNA Investigator Handbook (20). The lysate was transferred into a QIAamp MinElute column and eluted with Buffer ATE according to protocol (20).

### 2.10.2 Manual Qiagen- QIAamp Investigator Extraction of Liquid Semen

The appropriate amount of liquid semen dilution “A” (1:10) and “B” (1:10 of A) was added to a 2 mL microcentrifuge tube. Buffer ATL, Proteinase K, DTT, Buffer AL, ethanol, Buffer AW1, and Buffer AW2 were added to the samples according to the QIAamp® DNA Investigator Handbook (20). The lysate was transferred into a QIAamp MinElute column and eluted with Buffer ATE according to protocol (20).

## 2.11 Manual Qiagen- QIAamp Investigator Extraction Recovery Study

A refined dilution study of volumes of liquid semen dilutions “A” (1:10) and “B” (1:10 of A) and saliva dilutions “C” (1:2) and “D” (1:10 of C) were extracted using the manual Qiagen QIAamp® DNA Investigator protocol to determine how the manual Qiagen protocol compared to the EZ1 and ZyGEM/Acrosolv extraction methods in regards to DNA recovery. The same samples used in the previous EZ1 and ZyGEM/Acrosolv experiments were set up and extracted using the manual Qiagen protocol to compare to the same volumes extracted with EZ1 and ZyGEM/Acrosolv.

**2.12 DNA Quantification with Quantifier® Duo**

The samples were quantified with Quantifier® Duo Quantification Kit (Applied Biosystems® Foster City, CA). The Quantifier® Duo Kit quantifies total human DNA and total male DNA (23). All Quantifier® Duo reactions were prepared according to the manufacturer's protocol in the Quantifier® Duo DNA Quantification Kit User's Manual (23). All samples were quantified using a 7500 Real- Time PCR System (Applied Biosystems®, Foster City, CA). Positive and negative controls were carried out for each experiment using the positive and negative control samples included in the Quantifier® Duo DNA Quantification Kit (23).

2.12.1 Use of Quantification Data to Calculate Percent DNA Recovery

The main goal was to determine how much DNA was present in the sample prior to the extraction procedure and how much DNA was recovered after that process. To do this, first, the concentration of the sample determined from the qPCR instrument was used to calculate how many nanograms (ng) of DNA were present in that sample after the extraction process. This value is what the analyst would use to determine if there is enough DNA present to go on to further testing (9). To calculate how many ng of DNA are present within the sample, which is the total mass, the concentration value from the qPCR was multiplied by the elution volume in microliters.

$$\textbf{(1)} \quad \boldsymbol{ng\ of\ DNA\ within\ sample\ (Total\ Mass)}$$

$$Concentration\ value\ from\ qPCR\ \left(\frac{ng}{uL}\right) X\ Elution\ (EZ1) or\ Digest\ Volume (ZyGEM)\ (\mu L)$$

For example: If the concentration generated from the qPCR was 0.987ng/ µL and the elution volume was 100 µL, the calculation would be:

$$\textbf{(2)} \quad \boldsymbol{ng\ of\ DNA\ within\ sample\ (Total\ Mass)} = 0.987 \left(\frac{ng}{uL}\right) X\ 100\ (\mu L)$$

$$\boldsymbol{Total\ Mass} = \mathbf{98.7ng}$$

That value would serve as the numerator in the percent recovery calculation.

To determine the total mass of DNA in the sample before the extraction process is not a perfect method. This is because the only way to determine how much mass is being added to a sample before the extraction process is to use the concentration generated from the qPCR after the extraction process. It was concluded by this research team that the best way to estimate the amount of DNA before the extraction process would be to back-calculate or normalize each of the ZyGEM/Acrosolv sample's actual total mass generated from the qPCR to 1 ng/µL (**Method 1**) (Shown below). In other words, what would be the theoretical total mass of the sample in question if only 1 µL was deposited? This value would serve as the "known" or "standard" starting amount of DNA present. The ZyGEM/Acrosolv samples were chosen to use as the "standard" because it was assumed that the ZyGEM/Acrosolv extraction method would generate results close to or 100% recovery in comparison to the BioRobot EZ1 or manual Qiagen. This was done to generate many samples that were normalized back to the same value so there could be multiple samples that now can be averaged together to generate accurate results. An

average total mass would give the best assumption on how much DNA was present before the extraction process. To calculate this average, all of the samples produced from the same cell preparation or same donor with the ZyGEM/Acrosolv method were normalized to the total mass of DNA in 1 µL starting cell prep solution. To do this, the actual total mass from each sample (~50 samples total) using the concentration from the qPCR was calculated. Then, to back-calculate, the total mass of each sample was divided by its corresponding microliters of sample deposited. The resulting number would the total mass within that sample if only 1 µL was deposited.

$$\textbf{(3)}\quad \boldsymbol{ng\ of\ DNA\ within\ sample\ (Total\ Mass)} =$$

$$Concentration\ value\ from\ qPCR\ \left(\frac{ng}{uL}\right) X\ Elution\ or\ Digest\ Volume\ (\mu L)$$

**Method 1:**

$$\textbf{(4)}\quad \boldsymbol{Normalization\ to\ 1\ \mu L} = \frac{Total\ Mass(ng)}{Sample\ Deposited(\mu L)}$$

Once all of the samples were normalized, then the average was calculated and that value of ng/ µL was used to estimate the total mass for a given volume of sample deposited. For example: If the average of the normalized values resulted in 3.3ng then that number would be multiplied by the amount deposited for each volume tested and this would be the estimated mass in each of the volumes of cell prep before the extraction process.

$$\textbf{(5)}\quad \boldsymbol{Estimated\ Starting\ Mass\ for\ Corresponding\ Amount\ Deposited} =$$

$$\text{Average Normalized Values X Amount Deposited}$$

$$\textbf{(6)}\quad \boldsymbol{Estimated\ Starting\ Mass\ for\ 10\ \mu L} = 3.3ng/\mu L\ X\ 10\mu L$$

= **33ng** for sample where 10 µL was deposited.

This value would serve as the denominator in the percent recovery calculation. Thus, the estimated starting mass and recovered mass is used to calculate the percent recovery for each experimental sample.

$$\textbf{(7)} \quad \boldsymbol{Percent\ Recovery}\ (\%) = \frac{Recovered\ Mass\ (ng)}{Estimated\ Staring\ Mass\ (ng)} X\ 100$$

For example:

$$\textbf{(8)} \quad \boldsymbol{Percent\ Recovery}\ (\%) = \frac{9.87\ (ng)}{33.0\ (ng)} X\ 100$$

Percent Recovery = **29.9%**

It was previously stated that this research team determined that using the ZyGEM/Acrosolv samples would be best to serve as a comparison to the other methods because it was assumed that the ZyGEM/Acrosolv extraction method would generate results close or equal to 100% recovery. The other way to calculate percent recovery is to assume that the ZyGEM/Acrosolv extractions actually generate 100% recovery every time (**Method 2**). Percent recoveries were also calculated in the same fashion by determining the numerator as previously stated and dividing it by the individual ZyGEM/Acrosolv sample's total mass value for the same corresponding EZ1 or manual Qiagen sample. For example, to calculate the percent recovery for the EZ1 30 μL sample, the total mass would be calculated in the same manner as before but the denominator would be the total mass calculated from the individual 30 μL ZyGEM extraction. If the calculated total mass in the EZ1 extraction sample was 35.6ng and the total mass for the same ZyGEM extraction sample was 56.1ng then the calculation would be set up in this manner:

**Method 2:**

$$\textbf{(9)} \quad \textbf{\textit{Percent Recovery for EZ1 Extraction}}(\%) = \frac{35.6\ (ng)}{56.1\ (ng)} X\ 100$$

Percent Recovery = **63.5%**

To calculate the percent recovery for the ZyGEM extraction this way, the equation would be the same but the numerator and denominator would be the same value and the percent recovery would be 100% every time. This is because we are assuming that the ZyGEM/Acrosolv method results in 100% recovery every time.

$$\textbf{(10)} \quad \textbf{\textit{Percent Recovery for EZ1 Extraction}}(\%) = \frac{56.1\ (ng)}{56.1\ (ng)} X\ 100$$

Percent Recovery = **100%**

Both methods of calculating percent recovery were calculated for each sample and are shown in the data tables. Only method 1 was used to generate the graphs to compare data.

**2.13 Amplification**

Selected samples were chosen to be amplified with the GlobalFiler™ (Applied Biosystems, Foster City, CA) PCR amplification kit. This kit amplifies 21 autosomal STR loci and amelogenin. Once the plate set up was complete, the samples were denatured in a thermocycler for 95$^0$C for 1 minute, 94$^0$C for 10 seconds, 59$^0$C for 90 seconds, and finally 60$^0$C for 10 minutes (26). The protocol requires 29 cycles to be performed for the target amplification of 1ng of DNA. Depending on what mass was being targeted, various amounts of sample were added to make the total volume of sample added to the PCR reaction 15 µL. The samples were amplified using the

GeneAmp ® PCR System 9700 (Applied Biosystems, Foster City, CA) with negative and positive controls (26).

**2.14 Capillary Electrophoresis/ STR Profiles**

Capillary electrophoresis was performed on the 3130 Genetic Analyzer ® (Applied Biosystems, Foster City, CA) with selected samples. The master mix was created and added to a 96 well plate according to manufacturer's protocol. The allelic ladder was added in the designated wells and 1 µL of sample DNA was added into the corresponding wells. Once the plate set up was complete, the samples were denatured at $95^0$C for 3 and then placed on ice or $4^0$C for 3 minutes (27). The samples were then analyzed by GeneMapper ID-X® (version 1.4) software (Applied Biosystems, Foster City, CA) the profiles were analyzed with an analytical threshold (AT) of 30 relative fluorescence units (RFUs) (27). The stutter peals were edited out before peak height ratios were generated from the sister alleles.

**2.15 Statistical Data Methods**

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

# 3. RESULTS

## 3.1 Percent Recovery Experiments

As previously mentioned, two ways to calculate percent recovery were described (**Method 1 and Method 2**). For all of the experiments, method 1 and method 2 were calculated and reported in the data tables. For the analysis of the data produced, only method 1 was chosen for comparison purposes. This is because using an average of reactions is more accurate than using one data point for an evaluation of the extraction efficiency.

### 3.1.1 ZyGEM Digest Volume Study

The purpose of this experiment was to determine if the digest volume of the ZyGEM extraction could be significant in regards to how much DNA could be recovered by comparing the two digest volumes tested. According to a study done by Graziano, El-Mogy and Haj-Ahmad, they found that an extraction method should provide a wide range of elution volume options to allow the analyst to decide what elution volume is best for the DNA concentration (28). They found that the concentration was inversely proportional to the elution volume but the DNA integrity/stability was consistent regardless of elution volume (28). Therefore, it is important to determine the optimum elution volume and digest volume for each extraction method and concentration. This experiment determined that a digest volume of 100 μL had a better and more consistent DNA recovery in comparison to a 20 μL digest volume for saliva samples of different concentrations (**Tables 10 and 11 and Figure 5**). This was also the only experiment performed where there was a higher percent recovery seen with higher amounts of DNA

deposited in both elution volumes. This could be because the sample size (2 samples per volume tested) was not large enough to see this trend or the ZyGEM mechanism produces a higher recovery with higher mass until a certain exhaustion point of the enzyme activity is reached and this study did not reach that point (**Figure 5**).

**Table 10:** Digest Volume Recovery with ZyGEM 20 µL Saliva Liquid- Cell Prep A-1.

| Volume of Sample Used (ul) | Conc. (ng/ul) | Elution Volume (ul) | Total Mass Recovered (ng/ul) | Average Mass Recovered (ng) | Percent Recovery – Method 1 (%) | Percent Recovery – Method 2 (%) | Normalization (ng) |
|---|---|---|---|---|---|---|---|
| 10ul | 7.204ng/ul | 20ul | 144.08ng | **138.91ng±5.17** | **138.91/173.3= 80.2%** | **138.91/138.9= 100%** | **13.89ng** |
| 10ul | 6.687ng/ul | 20ul | 133.74ng | | | | |
| 5ul | 3.359ng/ul | 20ul | 67.18ng | **67.25ng±0.07** | **67.35/86.65= 77.7%** | **67.25/67.25= 100%** | **13.45ng** |
| 5ul | 3.366ng/ul | 20ul | 67.32ng | | | | |
| 1ul | 0.404ng/ul | 20ul | 8.08ng | **8.59ng±0.51** | **8.59/17.33= 49.6%** | **8.59/8.59= 100%** | **8.59ng** |
| 1ul | 0.455ng/ul | 20ul | 9.10ng | | | | |
| 0.1ul | 0.041ng/ul | 20ul | 0.82ng | **0.71ng±0.11** | **0.71/1.78= 39.9%** | **0.71/0.71= 100%** | **7.1ng** |
| 0.1ul | 0.030ng/ul | 20ul | 0.60ng | | | | |

The denominator in the average *percent* recovery calculations are from an *average* of the qPCR results from a back calculation of the Cell Prep A-1 and B-1 ZyGEM extractions to determine what the mass and concentration of 1ul would be to calculate the expected mass and concentrations of 5ul and 10ul. The *individual* percent recovery calculation was determined from the *individual* qPCR concentration for each amount.

**Table 11:** Digest Volume Recovery with ZyGEM 100 µL Saliva Liquid- Cell Prep B-1.

| Volume of Sample Used (ul) | Conc. (ng/ul) | Elution Volume (ul) | Total Mass Recovered (ng/ul) | Average Mass Recovered (ng) | Percent Recovery – Method 1 (%) | Percent Recovery – Method 2 (%) | Normalization (ng) |
|---|---|---|---|---|---|---|---|
| 10ul | 2.418ng/ul | 100ul | 241.8ng | **250.3ng±8.45** | **250.3/173.3= <u>144.4%</u>** | **250.3/250.3= <u>100%</u>** | **25.03ng** |
| 10ul | 2.587ng/ul | 100ul | 258.7ng | | | | |
| 5ul | 0.928ng/ul | 100ul | 92.8ng | **97.8ng±4.95** | **97.8/86.65= <u>112.9%</u>** | **97.8/97.8= <u>100%</u>** | **19.56ng** |
| 5ul | 1.027ng/ul | 100ul | 102.7ng | | | | |
| 1ul | 0.140ng/ul | 100ul | 14.0ng | **17.8ng±3.8** | **17.8/17.33= <u>102.9%</u>** | **17.8/17.8= <u>100%</u>** | **17.80ng** |
| 1ul | 0.216ng/ul | 100ul | 21.6ng | | | | |
| 0.1ul | 0.024ng/ul | 100ul | 2.4ng | **2.2ng±0.25** | **2.2/1.78= <u>123.6%</u>** | **2.2/2.2= <u>100%</u>** | **22.00ng** |
| 0.1ul | 0.019ng/ul | 100ul | 1.9ng | | | | |

The denominator in the average *percent* recovery calculations are from an *average* of the qPCR results from a back calculation of the Cell Prep A-1 and B-1 ZyGEM extractions to determine what the mass and concentration of 1ul would be to calculate the expected mass and concentrations of 5ul and 10ul. The *individual* percent recovery calculation was determined from the *individual* qPCR concentration for each amount.

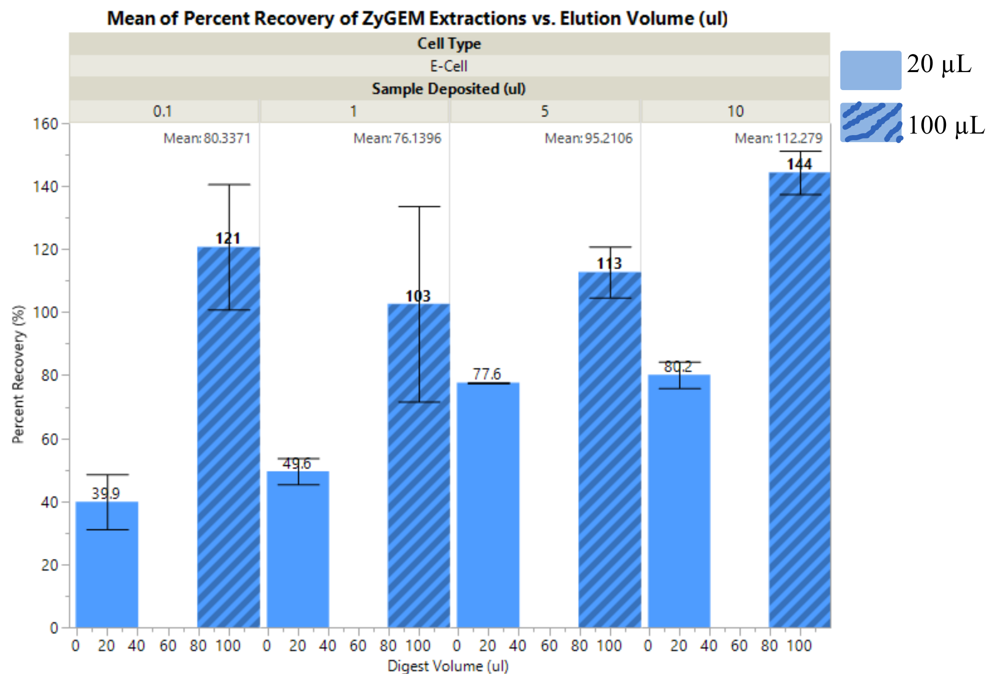



**Figure 5:** The Average Percent Recovery of DNA from Average of ZyGEM Extractions with Liquid Saliva and Different Digest Volumes. The solid blue bars represents the samples that had a digest volume of 20 µL and the striped blue bars represent the samples that had a digest volume of 100 µL. The numbers above each bar represents the average of percent recovery for each sample type. The standard deviations for each sample type are also shown.

### 3.1.2 EZ1 Elution Volume Study

As previously mentioned, other research has determined that elution volume can affect the DNA recovery (28). This experiment was designed to determine whether using different elution volumes had any effect on recovery with varying DNA masses with the tested extraction methods to support previous research. The experiment conducted during this research established that the elution volume chosen did not affect the DNA percent recovery. The average recoveries of all three volumes tested were 55.9% with 50 µL, 43.3% with 100 µL, and 57.2% with 200 µL. It did also show that the average percent recovery of 1 µL of sample deposited was higher (64.1%) in comparison to 5 µL of sample deposited (37.6%) across all elution volumes (Table 12 and Figure 6).

**Table 12:** Elution Recovery with Trace Protocol Saliva Liquid- Cell Prep B. The elution volumes chosen were 50 µL, 100 µL and 200 µL.

| Elution Volume= **50ul- Liquid** | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Volume of Sample Used (ul)** | **Conc. (ng/ul)** | **Elution Volume (ul)** | **Total Mass Recovered (ng/ul)** | **Average Mass Recovered (ng)** | **Percent Recovery –Method 1 (%)** | **Percent Recovery –Method 2 (%)** | **Normalization (ng)** |
| 10ul | 0.381ng/ul | 50ul | 19.05ng | **NA** | **19.05/43= 44.3%** | **19.05/28.3= 67.3%** | **1.91ng** |
| 5ul | 0.175ng/ul | 50ul | 8.75ng | **8.1ng±0.70** | **8.1/21.5= 37.7%** | **8.1/17.7= 45.8%** | **1.62ng** |
| 5ul | 0.147ng/ul | 50ul | 7.35ng | | | | |
| 1ul | 0.072ng/ul | 50ul | 3.6ng | **3.2ng±0.40** | **3.2/4.3= 74.4%** | **3.2/5.3= 60.4%** | **3.2ng** |
| 1ul | 0.056ng/ul | 50ul | 2.8ng | | | | |

| Elution Volume= **100ul-Liquid** | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Volume of Sample Used (ul)** | **Conc. (ng/ul)** | **Elution Volume (ul)** | **Total Mass Recovered (ng/ul)** | **Average Mass Recovered (ng)** | **Percent Recovery –Method 1 (%)** | **Percent Recovery –Method 2 (%)** | **Normalization (ng)** |
| 5ul | 0.052ng/ul | 100ul | 5.2ng | **7.4ng±2.02** | **7.4/21.5= 34.4%** | **7.4/17.7= 41.8%** | **1.48ng** |
| 5ul | 0.07ng/ul | 100ul | 7ng | | | | |
| 5ul | 0.101ng/ul | 100ul | 10.1ng | | | | |
| 1ul | 0.016ng/ul | 100ul | 1.6ng | **2.2ng±0.63** | **2.2/4.3= 51.2%** | **2.2/5.3= 41.5%** | **2.2ng** |
| 1ul | 0.031ng/ul | 100ul | 3.1ng | | | | |
| 1ul | 0.020ng/ul | 100ul | 2.0ng | | | | |

| Elution Volume=**200ul- Liquid** | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Volume of Sample Used (ul)** | **Conc. (ng/ul)** | **Elution Volume (ul)** | **Total Mass Recovered (ng/ul)** | **Average Mass Recovered (ng)** | **Percent Recovery –Method 1 (%)** | **Percent Recovery –Method 2 (%)** | **Normalization (ng)** |
| 10ul | 0.059ng/ul | 200ul | 11.8ng | **11.8ng** | **11.8/43= 27.4%** | **11.8/28.3= 41.7%** | **1.18ng** |
| 5ul | 0.054ng/ul | 200ul | 10.8ng | **9.1ng±1.70** | **9.1/21.5= 42.3%** | **9.1/17.7= 51.4%** | **1.82ng** |
| 5ul | 0.037ng/ul | 200ul | 7.4ng | | | | |
| 1ul | 0.012ng/ul | 200ul | 2.4ng | **3.1ng±0.70** | **3.1/4.3= 72.1%** | **3.1/5.3= 58.5%** | **3.1ng** |
| 1ul | 0.019ng/ul | 200ul | 3.8ng | | | | |

The denominator in the average *percent* recovery calculations are from an *average* of the qPCR results from a back calculation of the Cell Prep B ZyGEM extractions to determine what the mass and concentration of 1ul would be to calculate the expected mass and concentrations of 5ul and 10ul. The *individual* percent recovery calculation was determined from the *individual* qPCR concentration for each amount.

**Figure 6**: The Average Percent Recovery of DNA from EZ1 Extraction with Liquid Saliva and Different Elution Volumes. The orange bars represent the percent recovery of DNA for each elution volume (50 µL, 100 µL and 200 µL). The mean percent recovery for 1 µL and 5 µL of liquid saliva deposited are shown and also the standard deviations for each sample type are shown.

### 3.1.3 EZ1 Incubation Time Study on Fabric with Dried Semen or Saliva

Most biological samples in forensic laboratories are dried fluids on fabrics found at crime scenes (13). The research team asked whether or not the percent recovery differs when the fluid is dried on fabric in comparison to samples in liquid form. The EZ1 instrument with the Tip-Dance protocol and the ZyGEM/Acrosolv method were used in this experiment. The biological samples that were chosen were dried semen and dried saliva on cotton fabric and equivalent liquid samples. Two different volumes of sample were used for each biological fluid type. It was shown that regardless of biological fluid,

the average DNA recovery with ZyGEM was higher (106.9%) in comparison to the EZ1 extraction method (61.9%). Because there is no opportunity for DNA loss with ZyGEM, we have compared the recovery values from ZyGEM to the EZ1 DNA recovery amounts. It was also determined that the ZyGEM and Acrosolv extraction methods produced about the same percent recoveries with both e-cells (105.3%) and sperm cells (108.5%). But, with the EZ1 method, the e-cells produced low recoveries (32.6%) in comparison to the sperm cell recoveries (91.2%). Lastly, it was concluded that with both extraction methods and with both biological fluids that the lower amount of sample deposited resulted in higher recovery (**Table 13 and 14**).

The second question asked during this experiment was whether altering the time for the incubation step had any effect on the percent recovery. Results show that there was not any apparent trend but it did show that e-cells might need a shorter incubation time than sperm cells to be lysed. Additionally, an extended incubation time (>10 hours) could have a degradation effect. Like previous experiments, we discovered that the smaller amounts deposited resulted in higher percent recoveries regardless of the incubation time and biological sample tested (**Table 13 and 14**). There was no change in “incubation time” for the ZyGEM/Acrosolv samples. They were used as a control for comparison and only one sample was extracted for each volume of sample deposited at each time point.

**Table 13:** Mass Percent Recovery with ZyGEM Extraction and Saliva Stains.

| Saliva Stains ZyGEM Control | | | | | | |
|---|---|---|---|---|---|---|
| **Volume of Sample Used (ul)** | **Conc. (ng/ul)** | **Elution Volume (ul)** | **Total Mass Recovered (ng/ul)** | **Average Mass Recovered (ng)** | **Percent Recovery – Method 1 (%)** | **Percent Recovery – Method 2 (%)** |
| 10ul | 2.638 ng/ul | 100ul | 263.8ng | **263.8/294= 89.7%** | **263.8/263.8= 100%** | **26.38ng** |
| 5ul | 1.176ng/ul | 100ul | 117.6ng | **117.6/147= 80.0%** | **117.6/117.6= 100%** | **23.52ng** |
| 1ul | 0.384ng/ul | 100ul | 38.4ng | **38.4/29.4= 130.6%** | **38.4/38.4= 100%** | **38.40ng** |

The denominator in the *average* percent recovery calculations are from an *average* of the qPCR results from a back calculation of the Cell Prep A ZyGEM extractions to determine what the mass and concentration of 1ul would be to calculate the expected mass and concentrations of 5ul and 10ul. The *individual* percent recovery calculation was determined from the *individual* qPCR concentration for each amount. There was no change in “incubation time” for the ZyGEM/Acrosolv samples. They were used as a control for comparison.

**Table 14:** Incubation Time Recovery with EZ1 Tip Dance Protocol and Saliva Stains.

| Saliva Fabric Tip Dance EZ1- Stains | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Incubation Time** | **Volume of Sample Used (ul)** | **Conc (ng/ul)** | **Elution Volume (ul)** | **Mass Recovered (ng)** | **Percent Recovery –Method 1 (%)** | **Percent Recovery –Method 2 (%)** | **Normalization (ng)** |
| 3 Hours | 5ul | 0.308ng /ul | 100ul | 30.8ng | **30.8/147= 21.0%** | **30.8/117.6 = 26.2%** | **6.16ng** |
| | 1ul | 0.155ng /ul | 100ul | 15.5ng | **15.5/29.4= 52.7%** | **15.5/38.4= 40.4%** | **15.50ng** |
| 5 Hours | 5ul | 0.366ng /ul | 100ul | 36.6ng | **36.6/147= 24.9%** | **36.6/117.6 = 31.2%** | **7.32ng** |
| | 1ul | 0.164ng /ul | 100ul | 16.4ng | **16.4/29.4= 55.9%** | **16.4/38.4= 42.7%** | **16.40ng** |
| 10 Hours | 5ul | 0.162ng /ul | 100ul | 16.2ng | **16.2/147= 11.0%** | **16.2/117.6 = 13.8%** | **3.24ng** |
| | 1ul | 0.088ng /ul | 100ul | 8.8ng | **8.8/29.4= 29.9%** | **8.8/38.4= 22.9%** | **8.80ng** |

The denominator in the *average* percent recovery calculations are from an *average* of the qPCR results from a back calculation of the Cell Prep A ZyGEM extractions to determine what the mass and concentration of 1ul would be to calculate the expected mass and concentrations of 5ul and 10ul. The *individual* percent recovery calculation was determined from the *individual* qPCR concentration for each amount.

**Table 15:** Mass Percent Recovery with ZyGEM/Acrosolv Extraction and Semen Stains.

| Semen Stains ZyGEM | | | | | | |
|---|---|---|---|---|---|---|
| **Volume of Sample Used (ul)** | **Conc. (ng/ul)** | **Elution Volume (ul)** | **Total Mass Recovered (ng/ul)** | **Percent Recovery – Method 1 (%)** | **Percent Recovery – Method 2 (%)** | **Normalization** |
| 10ul | 0.763 ng/ul | 100ul | 76.3ng | 76.3/92= **82.9%** | 76.3/76.3= **100%** | **7.63ng** |
| 5ul | 0.443ng/ul | 100ul | 44.3ng | 44.3/46= **96.3%** | 44.3/44.3= **100%** | **8.86ng** |
| 1ul | 0.111ng/ul | 100ul | 11.1ng | 11.1/9.2 **120.7%** | 11.1/11.1= **100%** | **11.10ng** |

The denominator in the *average* percent recovery calculations are from an average of the qPCR results from a back calculation of the Dilution A ZyGEM/Acosolv extractions to determine what the mass and concentration of 1ul would be to calculate the expected mass and concentrations of 5ul and 10ul. The *individual* percent recovery calculation was determined from the individual qPCR concentration for each amount. There was no change in "incubation time" for the ZyGEM/Acrosolv samples. They were used as a control for comparison.

**Table 16:** Incubation Time Recovery with EZ1 Tip Dance Protocol on Semen Stains.

| Semen Fabric Tip Dance EZ1- Stains | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Incubation Time** | **Volume of Sample Used (ul)** | **Conc (ng/ul)** | **Elution Volume (ul)** | **Mass Recovered (ng)** | **Percent Recovery –Method 1 (%)** | **Percent Recovery –Method 2 (%)** | **Normalization (ng)** |
| 3 Hours | 5ul | 0.320ng/ul | 100ul | 32ng | 32/46= **69.6%** | 32/44.3= **72.2%** | **6.40ng** |
| | 1ul | 0.104ng/ul | 100ul | 10.4ng | 10.4/9.2= **113.0%** | 10.4/11.1= **93.7%** | **10.40ng** |
| 5 Hours | 5ul | 0.381ng/ul | 100ul | 38.1ng | 38.1/46= **82.8%** | 38.1/44.3= **86.0%** | **7.62ng** |
| | 1ul | 0.127ng/ul | 100ul | 12.7ng | 12.7/9.2= **138.0%** | 12.7/11.1= **114.4%** | **12.70ng** |
| 10 Hours | 5ul | 0.360ng/ul | 100ul | 36.0ng | 36/46= **78.3%** | 36/44.3= **81.3%** | **7.20ng** |
| | 1ul | 0.060ng/ul | 100ul | 6.0ng | 6/9.2= **65.2%** | 6/11.1= **54.1%** | **6.00ng** |

The denominator in the *average* percent recovery calculations are from an average of the qPCR results from a back calculation of the Dilution A ZyGEM/Acosolv extractions to determine what the mass and concentration of 1ul would be to calculate the expected mass and concentrations of 5ul and 10ul. The *individual* percent recovery calculation was determined from the individual qPCR concentration for each amount.

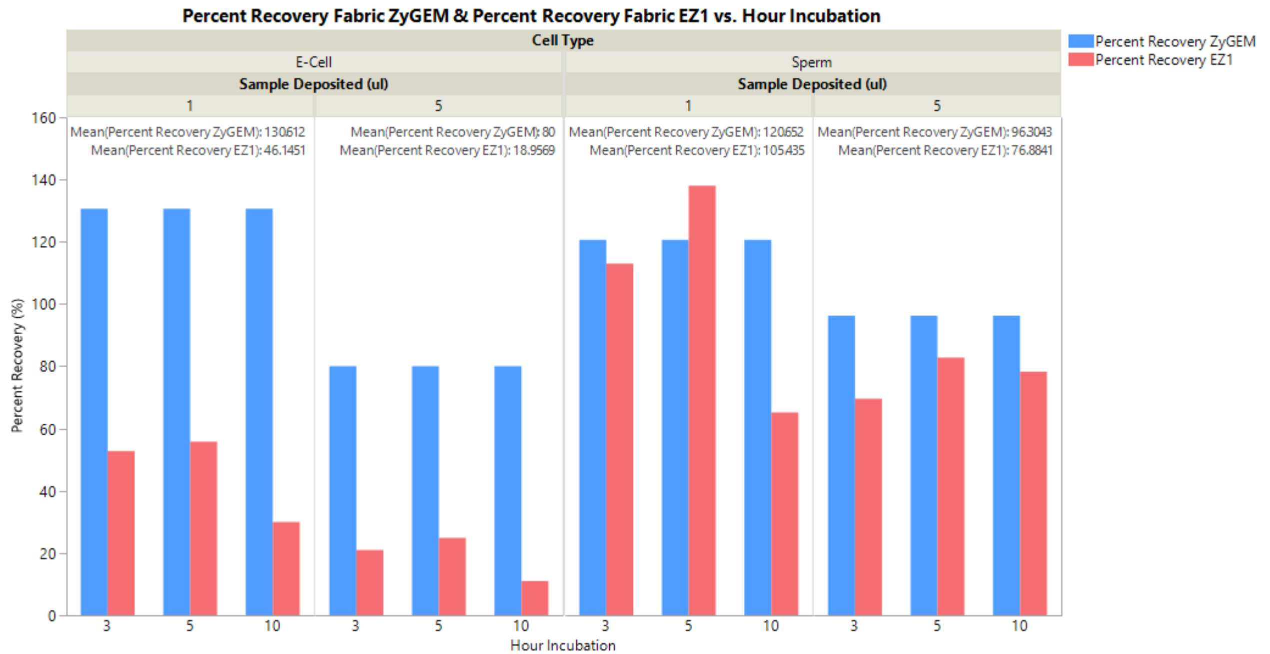



**Figure 7:** The Percent Recovery of DNA from EZ1 Extraction with Dried Saliva and Dried Semen different volumes deposited and The Percent Recovery of DNA from ZyGEM/Acrosolv Extraction with Dried Saliva and Dried Semen different volumes deposited. The red bars represent the EZ1 percent recovery for each sample type and the blue bars represent the ZyGEM/Acrosolv percent recovery for each sample type. ZyGEM/Acrosolv samples are the same for each time point depending on how much sample was deposited because they were used as a standard for comparison and no incubation time was changed.

### 3.1.4 Percent Recovery of Liquid Saliva and Liquid Semen- EZ1 Extraction

One of the main questions posed in this research was if the EZ1 recovered desirable amounts of DNA. To determine this, dilution series of both liquid semen and liquid saliva were produced and extracted on the EZ1 robot. This protocol would allow us to determine if low and high amounts of sample deposited gave different percent recoveries. Additionally, recovery of e-cell DNA was compared to recovery of sperm cell DNA. It was determined that there was no apparent trend in the amount of sample deposited and percent recovery for both biological fluids tested (**Table 17 and 18**). However, there was a large difference in percent recovery when the two biological fluids

were compared to each other. The average percent recovery of DNA from sperm cells was 92.3% while e-cell DNA recovery was 55.7% (**Figure 8**).

**Table 17:** Mass Percent Recovery with EZ1 Extraction and Liquid Saliva.

| Liquid Saliva EZ1 -Liquid Saliva 1:2 C and 1:10 D | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Volume of Sample Used (ul)** | **Conc. (ng/ul)** | **Elution Volume (ul)** | **Total Mass Recovered (ng/ul)** | **Average Mass Recovered (ng)** | **Percent Recovery –Method 1 (%)** | **Percent Recovery –Method 2 (%)** | **Normalization (ng)** |
| 1ul D | 0.001 | 100ul | 0.1ng | **2.2ng±1.00** | **2.2/2.1= 104.8%** | **2.2/2.9= 75.9%** | **2.2ng** |
| 1ul D | 0.025 | 100ul | 2.5ng | | | | |
| 1ul D | 0.018 | 100ul | 1.8ng | | | | |
| 5ul D | 0.085 | 100ul | 8.5ng | **6.3ng±1.62** | **6.3/10.5= 60.0%** | **6.3/10.7= 58.9%** | **1.26ng** |
| 5ul D | 0.059 | 100ul | 5.9ng | | | | |
| 5ul D | 0.046 | 100ul | 4.6ng | | | | |
| 10ul D | 0.097 | 100ul | 9.7ng | **9.5ng±0.36** | **9.5/21= 45.2%** | **9.5/21.6= 44.0%** | **0.95ng** |
| 10ul D | 0.098 | 100ul | 9.8ng | | | | |
| 10ul D | 0.090 | 100ul | 9.0ng | | | | |
| 15ul D | 0.151 | 100ul | 15.1ng | **14.8ng±0.57** | **14.8/32= 46.5%** | **14.8/34.3= 43.1%** | **0.99ng** |
| 15ul D | 0.140 | 100ul | 14.0ng | | | | |
| 15ul D | 0.153 | 100ul | 15.3ng | | | | |
| 30ul D | 0.336 | 100ul | 33.6ng | **36.1ng±1.80** | **36.1/63= 57.3%** | **36.1/67.7= 53.5%** | **1.20ng** |
| 30ul D | 0.372 | 100ul | 37.2ng | | | | |
| 30ul D | 0.376 | 100ul | 37.6ng | | | | |
| 40ul D | 0.429 | 100ul | 42.9ng | **45.1ng±1.84** | **45.1/84= 53.7%** | **45.1/72.5= 62.2%** | **1.13ng** |
| 40ul D | 0.474 | 100ul | 47.4ng | | | | |
| 40ul D | 0.451 | 100ul | 45.1ng | | | | |
| 60ul D | 0.704 | 100ul | 70.4ng | **69.8ng±0.60** | **69.8/126= 55.4%** | **69.8/116.6= 60.0%** | **1.16ng** |
| 60ul D | 0.690 | 100ul | 69.0ng | | | | |
| 60ul D | 0.701 | 100ul | 70.1ng | | | | |
| 6ul C | 0.640 | 100ul | 64.0ng | **61.2ng±4.00** | **61.2/115.5= 53.0%** | **61.2/117.4= 52.1%** | **10.20ng/10 (1.02ng)** |
| 6ul C | 0.566 | 100ul | 55.6ng | | | | |
| 6ul C | 0.642 | 100ul | 64.2ng | | | | |
| 12ul C | 1.206 | 100ul | 120.6ng | **137.7ng±12.76** | **137.7/230.4= 59.8%** | **137.7/241.7= 57.0%** | **11.48ng/10 (1.15ng)** |
| 12ul C | 1.512 | 100ul | 151.2ng | | | | |
| 12ul C | 1.414 | 100ul | 141.4ng | | | | |

The denominator in the *average* percent recovery calculations are from an *average* of the qPCR results from a back calculation of the Dilution C (1:2) / D (1:10 of C) ZyGEM extractions to determine what the mass and concentration of 1ul would be to calculate the expected mass and concentrations of 5ul and 10ul. The *individual* percent recovery calculation was determined from the *individual* qPCR concentration for each amount.

**Table 18:** Mass Percent Recovery with EZ1 Extraction and Liquid Semen.

| Liquid Semen EZ1 -Liquid Semen 1:10 A and 1:10 of 1:10 B | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Volume of Sample Used (ul)** | **Conc. (ng/ul)** | **Elution Volume (ul)** | **Total Mass Recovered (ng/ul)** | **Average Mass Recovered (ng)** | **Percent Recovery –Method 1 (%)** | **Percent Recovery – Method 2 (%)** | **Normalization (ng)** |
| 1ul B | 0.010ng/ul | 100ul | 1.0ng | **1.0ng±0.12** | **1.03/1.1= 93.9%** | **1.0/2.2= 45.5%** | **1.00ng** |
| 1ul B | 0.009ng/ul | 100ul | 0.9ng | | | | |
| 1ul B | 0.012ng/ul | 100ul | 1.2ng | | | | |
| 5ul B | 0.054ng/ul | 100ul | 5.4ng | **5.6ng±0.24** | **5.6/5.3= 105.7%** | **5.6/5.5= 101.8%** | **1.12ng** |
| 5ul B | 0.059ng/ul | 100ul | 5.9ng | | | | |
| 5ul B | 0.054ng/ul | 100ul | 5.4ng | | | | |
| 10ul B | 0.086ng/ul | 100ul | 8.6ng | **9.9ng±1.33** | **9.9/10.6= 93.4%** | **9.9/12.4= 79.8%** | **0.99ng** |
| 10ul B | 0.117ng/ul | 100ul | 11.7ng | | | | |
| 10ul B | 0.093ng/ul | 100ul | 9.3ng | | | | |
| 15ul B | 0.153ng/ul | 100ul | 15.3ng | **14.4ng±1.99** | **14.4/15.9= 90.7%** | **14.4/17.7= 81.4%** | **0.96ng** |
| 15ul B | 0.162ng/ul | 100ul | 16.2ng | | | | |
| 15ul B | 0.116ng/ul | 100ul | 11.6ng | | | | |
| 30ul B | 0.316ng/ul | 100ul | 31.6ng | **29.8ng±2.52** | **29.8/31.8= 93.7%** | **29.8/33.9= 87.9%** | **0.99ng** |
| 30ul B | 0.315ng/ul | 100ul | 31.5ng | | | | |
| 30ul B | 0.262ng/ul | 100ul | 26.2ng | | | | |
| 40ul B | 0.414ng/ul | 100ul | 41.4ng | **42.3ng±0.64** | **42.3/42.4= 99.8%** | **42.3/39.2= 107.9%** | **1.06ng** |
| 40ul B | 0.427ng/ul | 100ul | 42.7ng | | | | |
| 40ul B | 0.428ng/ul | 100ul | 42.8ng | | | | |
| 60ul B | 0.554ng/ul | 100ul | 55.4ng | **54.8ng±3.26** | **54.8/63.6= 86.2%** | **54.8/51.8= 105.8%** | **0.91ng** |
| 60ul B | 0.584ng/ul | 100ul | 58.4ng | | | | |
| 60ul B | 0.505ng/ul | 100ul | 50.5ng | | | | |
| 6ul A | 0.609ng/ul | 100ul | 60.9ng | **64.0ng±2.22** | **64.0/69.0= 92.8%** | **64.0/64.5= 99.2%** | **10.67ng/10 (1.07ng)** |
| 6ul A | 0.656ng/ul | 100ul | 65.6ng | | | | |
| 6ul A | 0.656ng/ul | 100ul | 65.6ng | | | | |
| 12ul A | 1.065ng/ul | 100ul | 106.5ng | **105.2ng±4.00** | **105.2/138= 76.2%** | **105.2/107.7= 97.7%** | **8.77ng/10 (0.88ng)** |
| 12ul A | 0.998ng/ul | 100ul | 99.8ng | | | | |
| 12ul A | 1.093ng/ul | 100ul | 109.3ng | | | | |

The denominator in the *average* percent recovery calculations are from an average of the qPCR results from a back calculation of the Dilution A (1:10) / Dilution B (1:10 of A) ZyGEM/Acosolv extractions to determine what the mass and concentration of 1ul would be to calculate the expected mass and concentrations of 5ul and 10ul. The *individual* percent recovery calculation was determined from the individual qPCR concentration for each amount.

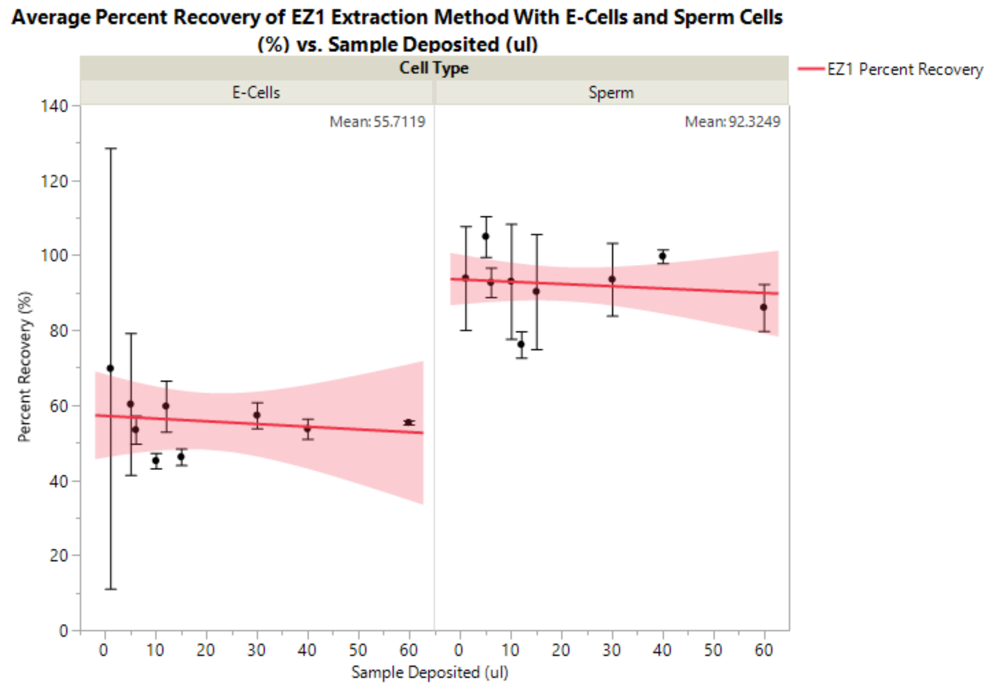



**Figure 8:** Average Percent Recovery of the EZ1 extraction Robot with liquid saliva and liquid semen. The average percent recovery of E-cells with the EZ1 extraction was 55.7% and sperm cells was 92.3%.

### 3.1.5 Percent Recovery of Liquid Saliva in Comparison to Liquid Semen-ZyGem/Acrosolv Extraction

Another core question that this research was asking was how the ZyGEM/Acrosolv compared to the EZ1 and QIAamp manual extraction. To determine this, dilution series of liquid semen and liquid saliva were produced and extracted using ZyGEM/Acrosolv standard protocols (22, 25). This was to determine if low and high amounts of sample deposited resulted in different percent recoveries. Whether better recoveries occurred with saliva that was extracted in comparison to semen was also calculated. It was determined that there was a slight downward trend in the percent recovery as the amount of sample increased. The higher amount of sample deposited resulted in slighter lower percent recoveries and this was apparent in both biological fluid

samples (**Table 19 and 20**). The average percent recovery with sperm cells was 109.5% while e-cell recovery was 104.8% (**Figure 9**). So, the ZyGEM/Acrosolv extraction method seems to have a more consistent recovery when different biological fluids are extracted.

**Table 19:** Mass Percent Recovery with ZyGEM Extraction and Liquid Saliva.

| Liquid Saliva ZyGEM -Liquid Saliva 1:2 C and 1:10 D | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Volume of Sample Used (ul)** | **Conc. (ng/ul)** | **Elution Volume (ul)** | **Total Mass Recovered (ng/ul)** | **Average Mass Recovered (ng)** | **Percent Recovery – Method 1 (%)** | **Percent Recovery – Method 2 (%)** | **Normalization (ng)** |
| 1ul D | 0.039 | 100ul | 3.9ng | **2.9ng±0.50** | **2.9/2.1= 138.1%** | **2.9/2.9= 100%** | **2.9ng** |
| 1ul D | 0.025 | 100ul | 2.5ng | | | | |
| 1ul D | 0.023 | 100ul | 2.3ng | | | | |
| 5ul D | 0.108 | 100ul | 10.8ng | **10.7ng±0.26** | **10.7/10.5= 101.9%** | **10.7/10.7= 100%** | **2.14ng** |
| 5ul D | 0.109 | 100ul | 10.9ng | | | | |
| 5ul D | 0.103 | 100ul | 10.3ng | | | | |
| 10ul D | 0.209 | 100ul | 20.9ng | **21.6ng±1.71** | **21.6/21= 102.9%** | **21.6/21.6= 100%** | **2.16ng** |
| 10ul D | 0.240 | 100ul | 24.0ng | | | | |
| 10ul D | 0.200 | 100ul | 20.0ng | | | | |
| 15ul D | 0.367 | 100ul | 36.7ng | **34.3ng±1.96** | **34.3/32= 107.2%** | **34.3/34.3= 100%** | **2.29ng** |
| 15ul D | 0.344 | 100ul | 34.4ng | | | | |
| 15ul D | 0.319 | 100ul | 31.9ng | | | | |
| 30ul D | 0.669 | 100ul | 66.9ng | **67.7ng±2.12** | **67.7/63= 107.5%** | **67.7/67.7= 100%** | **2.26ng** |
| 30ul D | 0.706 | 100ul | 70.6ng | | | | |
| 30ul D | 0.655 | 100ul | 65.6ng | | | | |
| 40ul D | 0.730 | 100ul | 73.0ng | **72.5ng±0.81** | **72.5/84= 86.3%** | **72.5/72.5= 100%** | **1.81ng** |
| 40ul D | 0.714 | 100ul | 71.4ng | | | | |
| 40ul D | 0.732 | 100ul | 73.2ng | | | | |
| 60ul D | 1.218 | 100ul | 121.8ng | **116.5ng±4.08** | **116.6/126= 92.5%** | **116.6/116.6= 100%** | **1.94ng** |
| 60ul D | 1.119 | 100ul | 111.9ng | | | | |
| 60ul D | 1.157 | 100ul | 115.7ng | | | | |
| 6ul C | 1.176 | 100ul | 117.6ng | **117.4ng±5.35** | **117.4/115.2= 101.9%** | **117.4/117.4= 100%** | **19.57ng/10 (1.96ng)** |
| 6ul C | 1.108 | 100ul | 110.8ng | | | | |
| 6ul C | 1.239 | 100ul | 123.9ng | | | | |
| 12ul C | 2.564 | 100ul | 256.4ng | **241.7ng±10.6** | **241.7/230.4= 104.9%** | **241.7/241.7= 100%** | **20.14ng/10 (2.01ng)** |
| 12ul C | 2.341 | 100ul | 234.1ng | | | | |
| 12ul C | 2.336 | 100ul | 233.6ng | | | | |

The denominator in the average percent recovery calculations are from an average of the qPCR results from a back calculation of the Dilution C (1:2) / D (1:10 of C) ZyGEM extractions to determine what the mass and concentration of 1ul would be to calculate the expected mass and concentrations of 5ul and 10ul. The individual percent recovery calculation was determined from the individual qPCR concentration for each amount.

**Table 20:** Mass Percent Recovery with Acrosolv Extraction and Liquid Semen.

| Liquid Semen Acrosolv -<u>Liquid Semen 1:10 A and 1:10 of 1:10 B</u> | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Volume of Sample Used (ul)** | **Conc. (ng/ul)** | **Elution Volume (ul)** | **Total Mass Recovered (ng/ul)** | **Average Mass Recovered (ng)** | **Percent Recovery – Method 1 (%)** | **Percent Recovery – Method 2 (%)** | **Normalization (ng)** |
| 1ul B | 0.023ng/ul | 100ul | 2.3ng | **2.2ng±0.41** | **2.2/1.1= <u>200.0%</u>** | **2.2/2.2= <u>100%</u>** | **2.2ng** |
| 1ul B | 0.027ng/ul | 100ul | 2.7ng | | | | |
| 1ul B | 0.017ng/ul | 100ul | 1.7ng | | | | |
| 5ul B | 0.052ng/ul | 100ul | 5.2ng | **5.5ng±0.25** | **5.5/5.3= <u>103.8%</u>** | **5.5/5.5= <u>100%</u>** | **1.10ng** |
| 5ul B | 0.057ng/ul | 100ul | 5.7ng | | | | |
| ~~5ul B~~ | ~~0.033ng/ul~~ | ~~100ul~~ | ~~3.3ng~~ | | | | |
| 10ul B | 0.156ng/ul | 100ul | 15.6ng | **12.4ng±2.26** | **12.4/10.6= <u>117.0%</u>** | **12.4/12.4= <u>100%</u>** | **1.24ng** |
| 10ul B | 0.108ng/ul | 100ul | 10.8ng | | | | |
| 10ul B | 0.108ng/ul | 100ul | 10.8ng | | | | |
| 15ul B | 0.125ng/ul | 100ul | 12.5ng | **17.7ng±4.09** | **17.7/15.9= <u>111.3%</u>** | **17.7/17.7= <u>100%</u>** | **1.18ng** |
| 15ul B | 0.225ng/ul | 100ul | 22.5ng | | | | |
| 15ul B | 0.18ng/ul | 100ul | 18ng | | | | |
| 30ul B | 0.371ng/ul | 100ul | 37.1ng | **33.1ng±3.12** | **33.9/31.8= <u>106.6%</u>** | **33.9/33.9= <u>100%</u>** | **1.10ng** |
| 30ul B | 0.326ng/ul | 100ul | 32.6ng | | | | |
| 30ul B | 0.295ng/ul | 100ul | 29.5ng | | | | |
| 40ul B | 0.399ng/ul | 100ul | 39.9ng | **39.2ng±2.58** | **39.2/42.4= <u>92.5%</u>** | **39.2/39.2= <u>100%</u>** | **0.98ng** |
| 40ul B | 0.419ng/ul | 100ul | 41.9ng | | | | |
| 40ul B | 0.357ng/u | 100ul | 35.7ng | | | | |
| 60ul B | 0.43ng/ul | 100ul | 43ng | **51.8ng±7.23** | **51.8/63.6= <u>81.4%</u>** | **51.8/51.8= <u>100%</u>** | **0.86ng** |
| 60ul B | 0.607ng/ul | 100ul | 60.7ng | | | | |
| 60ul B | 0.518ng/ul | 100ul | 51.8ng | | | | |
| 6ul A | 0.606ng/ul | 100ul | 60.6ng | **64.5ng±4.18** | **64.5/69.0= <u>93.4%</u>** | **64.5/64.5= <u>100%</u>** | **10.75ng/10 (1.08ng)** |
| 6ul A | 0.703ng/ul | 100ul | 70.3ng | | | | |
| 6ul A | 0.626ng/ul | 100ul | 62.6ng | | | | |
| 12ul A | 1.021ng/ul | 100ul | 102.1ng | **107.7ng±4.16** | **107.7/138.0= <u>78.0%</u>** | **107.7/107.7= <u>100%</u>** | **8.98ng/10 (0.90ng)** |
| 12ul A | 1.121ng/ul | 100ul | 112.1ng | | | | |
| 12ul A | 1.088ng/ul | 100ul | 108.8ng | | | | |

The denominator in the *average* percent recovery calculations are from an average of the qPCR results from a back calculation of the Dilution A (1:10)/ B (1:10 of A) ZyGEM/Acosolv extractions to determine what the mass and concentration of 1ul would be to calculate the expected mass and concentrations of 5ul and 10ul. The *individual* percent recovery calculation was determined from the individual qPCR concentration for each amount. One digest (5ul) was removed as an outlier.

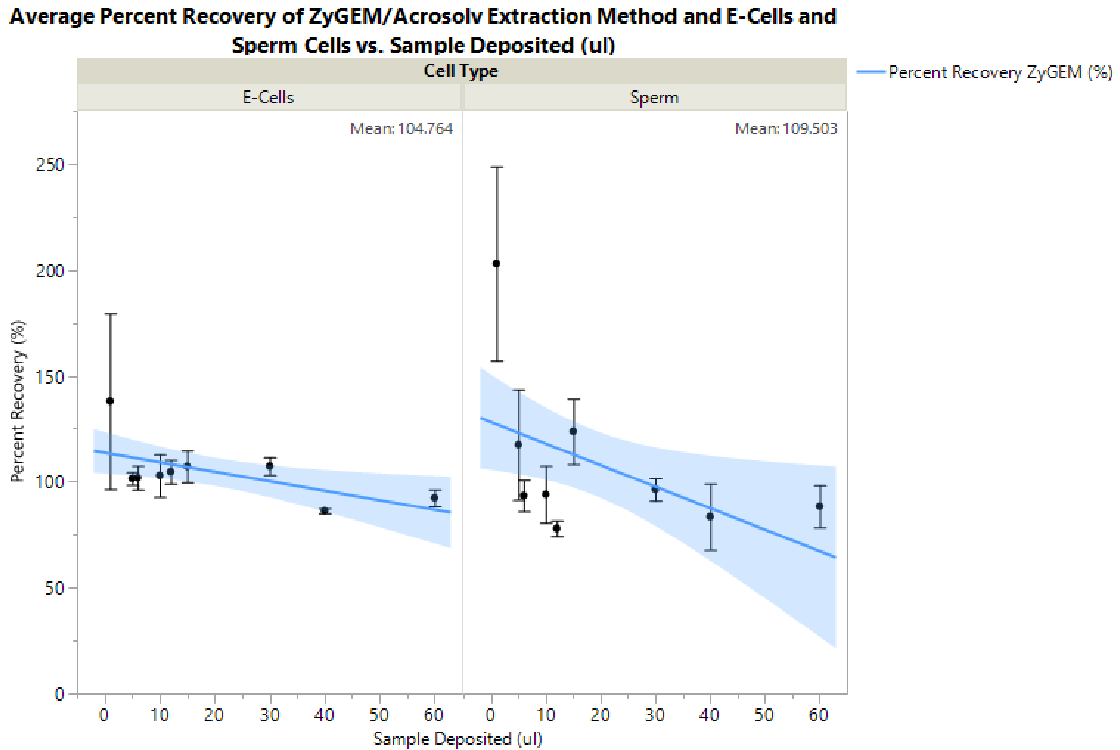



**Figure 9:** Average Percent Recovery of the ZyGEM/Acrosolv extraction with liquid saliva and liquid semen. The average percent recovery of E-cells with the ZyGEM extraction was 104.8% and Acrosolv with sperm cells was 109.5%.

### 3.1.6 Percent Recovery of Liquid Saliva in Comparison to Liquid Semen- Manual Qiagen Extraction

The last extraction method used for comparison was the manual Qiagen extraction method. Dilution series of liquid semen and liquid saliva were produced and extracted in the same manner according to the Qiagen QIAamp Investigator kit standard protocols (20). The dilution series was used to determine if the manual Qiagen extraction method produced higher percent recoveries with lower amounts of DNA or the opposite. It was determined that this extraction method produced higher percent recoveries with lower amounts of DNA present in both saliva and semen biological fluids. It was also concluded that when saliva was used as the biological fluid, the percent recoveries were

higher in comparison to semen (**Table 21 and 22**). The average percent recovery of e-cells was 39.6% and sperm was 17.3% (**Figure 10**). Regardless of the fact that e-cells had a better recovery, both of the average percent recoveries are relatively low.

**Table 21:** Mass Percent Recovery with Manual Qiagen QIAamp and Liquid Saliva.

| Liquid Saliva Manual -Liquid Saliva 1:2 C and 1:10 D | | | | | |
|---|---|---|---|---|---|
| **Volume of Sample Used (ul)** | **Conc. (ng/ul)** | **Elution Volume (ul)** | **Total Mass Recovered (ng/ul)** | **Average Mass Recovered (ng)** | **Percent Recovery – Method 1 (%)** |
| 1ul C | 0.191 ng/ul | 50ul | 9.55ng | **9.30ng±0.25** | **9.30/19.26= 48.3%** |
| 1ul C | 0.179 ng/ul | 50ul | 8.95ng | | |
| 1ul C | 0.188 ng/ul | 50ul | 9.40ng | | |
| 5ul C | 0.745 ng/ul | 50ul | 37.35ng | **36.1ng±0.86** | **36.1/96.3= 37.5%** |
| 5ul C | 0.715 ng/ul | 50ul | 35.75ng | | |
| 5ul C | 0.707 ng/ul | 50ul | 35.35ng | | |
| 10ul C | 1.619 ng/ul | 50ul | 80.95ng | **76.8ng±4.40** | **76.8/192.6= 39.9%** |
| 10ul C | 1.421 ng/ul | 50ul | 70.60ng | | |
| 10ul C | 1.568 ng/ul | 50ul | 78.40ng | | |
| 15ul C | 2.195 ng/ul | 50ul | 109.75ng | **126ng±5.18** | **126/288.9 = 43.6%** |
| 15ul C | 2.434 ng/ul | 50ul | 121.70ng | | |
| 15ul C | 2.241 ng/ul | 50ul | 112.05ng | | |
| 30ul C | 3.441 ng/ul | 50ul | 172.05ng | **180.8ng±6.16** | **180.8/577.8= 31.3%** |
| 30ul C | 3.675 ng/ul | 50ul | 183.75ng | | |
| 30ul C | 3.723 ng/ul | 50ul | 186.15ng | | |
| 40ul C | 6.607 ng/ul | 50ul | 330.35ng | **312.9ng±12.66** | **312.9/770.4= 40.6%** |
| 40ul C | 6.151 ng/ul | 50ul | 307.55ng | | |
| 40ul C | 6.015 ng/ul | 50ul | 300.75ng | | |
| 60ul C | 8.340 ng/ul | 50ul | 417.00ng | **412.9ng±16.69** | **412.9/1155.6= 35.7%** |
| 60ul C | 7.813 ng/ul | 50ul | 390.65ng | | |
| 60ul C | 8.618 ng/ul | 50ul | 430.90ng | | |
| 6ul D | 0.109 ng/ul | 50ul | 5.45ng | **4.15ng±0.94** | **4.15/12.6= 32.9%** |
| 6ul D | 0.065 ng/ul | 50ul | 3.25ng | | |
| 6ul D | 0.075 ng/ul | 50ul | 3.75ng | | |
| 12ul C | 2.348 ng/ul | 50ul | 117.40ng | **134ng±11.74** | **134/230.4= 58.2%** |
| 12ul C | 2.838 ng/ul | 50ul | 141.90ng | | |
| 12ul C | 2.854 ng/ul | 50ul | 142.70ng | | |

The denominator in the average percent recovery calculations are from an average of the qPCR results from a back calculation of the Dilution C (1:2) / D (1:10 of C) ZyGEM extractions to determine what the mass and concentration of 1ul would be to calculate the expected mass and concentrations of 5ul and 10ul. The individual percent recovery calculation was determined from the individual qPCR concentration for each amount.

**Table 22:** Mass Percent Recovery with Manual Qiagen QIAamp and Liquid Semen.

| Liquid Semen Manual -Liquid Semen 1:10 A and 1:10 of 1:10 B | | | | | | |
|---|---|---|---|---|---|---|
| **Volume of Sample Used (ul)** | **Conc. (ng/ul)** | **Elution Volume (ul)** | **Total Mass Recovered (ng/ul)** | **Average Mass Recovered (ng)** | **Percent Recovery – Method 1 (%)** | **Percent Recovery – Method 2 (%)** |
| 1ul B | 0.008 ng/ul | 40ul | 0.32ng | **0.3ng±0.07** | **0.3/1.1= 27.3%** | **0.3/2.2= 13.6%** |
| 1ul B | 0.004 ng/ul | 40ul | 0.16ng | | | |
| 1ul B | 0.007 ng/ul | 40ul | 0.28ng | | | |
| 5ul B | 0.017 ng/ul | 40ul | 0.68ng | **0.71ng±0.02** | **0.71/5.3= 13.3%** | **0.71/5.5= 12.9%** |
| 5ul B | 0.018 ng/ul | 40ul | 0.72ng | | | |
| 5ul B | 0.018 ng/ul | 40ul | 0.72ng | | | |
| 10ul B | 0.06 ng/ul | 40ul | 2.4ng | **2.0ng±0.33** | **2.0/10.6= 19.0%** | **2.0/12.4= 16.1%** |
| 10ul B | 0.051 ng/ul | 40ul | 2.04ng | | | |
| 10ul B | 0.04 ng/ul | 40ul | 1.6ng | | | |
| 15ul B | 0.052 ng/ul | 40ul | 2.08ng | **1.8ng±0.32** | **1.8/15.9= 11.4%** | **1.8/17.7= 10.1%** |
| 15ul B | 0.05 ng/ul | 40ul | 2ng | | | |
| 15ul B | 0.034 ng/ul | 40ul | 1.36ng | | | |
| 30ul B | 0.158 ng/ul | 40ul | 6.32ng | **5.6ng±0.55** | **5.6/31.8= 17.7%** | **5.6/33.9= 16.5%** |
| 30ul B | 0.124 ng/ul | 40ul | 4.96ng | | | |
| 30ul B | 0.139 ng/ul | 40ul | 5.56ng | | | |
| 40ul B | 0.14 ng/ul | 40ul | 5.6ng | **6.24ng±1.23** | **6.24/42.4= 14.7%** | **6.24/39.2= 15.9%** |
| 40ul B | 0.199 ng/ul | 40ul | 7.96ng | | | |
| 40ul B | 0.129 ng/ul | 40ul | 5.16ng | | | |
| 60ul B | 0.253 ng/ul | 40ul | 10.12ng | **9.5ng±1.73** | **9.5/63.6= 14.9%** | **9.5/51.8= 18.3%** |
| 60ul B | 0.235 ng/ul | 40ul | 9.4ng | | | |
| 60ul B | 0.224 ng/ul | 40ul | 8.96ng | | | |
| 6ul A | 0.32 ng/ul | 40ul | 12.8ng | **13.4ng±1.73** | **13.4/69.0= 19.4%** | **13.4/64.5= 20.8%** |
| 6ul A | 0.394 ng/ul | 40ul | 15.76ng | | | |
| 6ul A | 0.291 ng/ul | 40ul | 11.64ng | | | |
| 12ul A | 0.821 ng/ul | 40ul | 32.84ng | **33.9ng±0.90** | **33.9/138= 24.6%** | **33.9/107.7= 31.5%** |
| 12ul A | 0.848 ng/ul | 40ul | 33.92ng | | | |
| 12ul A | 0.876 ng/ul | 40ul | 35.04ng | | | |

*The denominator in the *average* percent recovery calculations are from an average of the qPCR results from a back calculation of the Dilution A (1:10)/ Dilution B (1:10 of A) ZyGEM/Acosolv extractions to determine what the mass and concentration of 1ul would be to calculate the expected mass and concentrations of 5ul and 10ul. The *individual* percent recovery calculation was determined from the individual qPCR concentration for each amount.

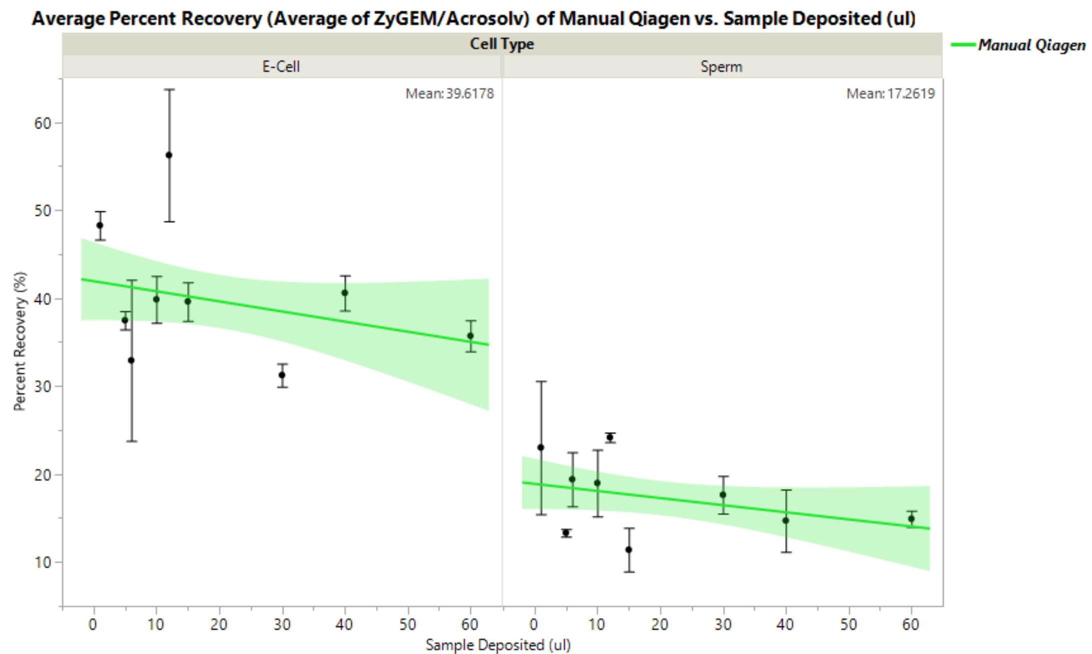



**Figure 10:** Average Percent Recovery of the Manual Qiagen QIAamp Extraction with liquid saliva and liquid semen. The average percent recovery of E-cells with the QIAamp extraction was 39.6% and sperm cells was 17.3%.

3.1.7 EZ1 vs ZyGEM vs Manual Qiagen Percent Recovery with Liquid Saliva and Semen Comparison

To determine which extraction method had the highest percent recovery, the three extraction methods were plotted against each other based on cell type extracted (**Figure 11).** It was determined that the ZyGEM/Acrosolv protocol outperformed both EZ1 and manual Qiagen in both cell types. Using the normalized denominator (Method 1), the ZyGEM/Acrosolv extraction had a mean percent recovery of 103.9% with e-cells and 109.4% with sperm cells while EZ1 had 55.7% e-cells and 92.3% sperm cells and manual Qiagen had 39.6% and 17.3%, respectively. Overall, the ZyGEM/Acrosolv protocol not only generates higher amount of DNA recoveries than other methods but is also more consistent with the amount recovered for each body fluid. It should also be noted that even though ZyGEM/Acrosolv outperformed the other methods, there is a downward

trend where the more DNA present in the sample, the lower the percent recovery. This is not observed in the other two extraction methods (**Figure 11**). The average IPC values from the semen dilution series for each method were plotted against each other to see if there was any variation between the extraction methods. It was determined that while the EZ1 method produced the lowest IPC values, all methods performed well which shows the DNA is pure in solution (**Figure 12**).

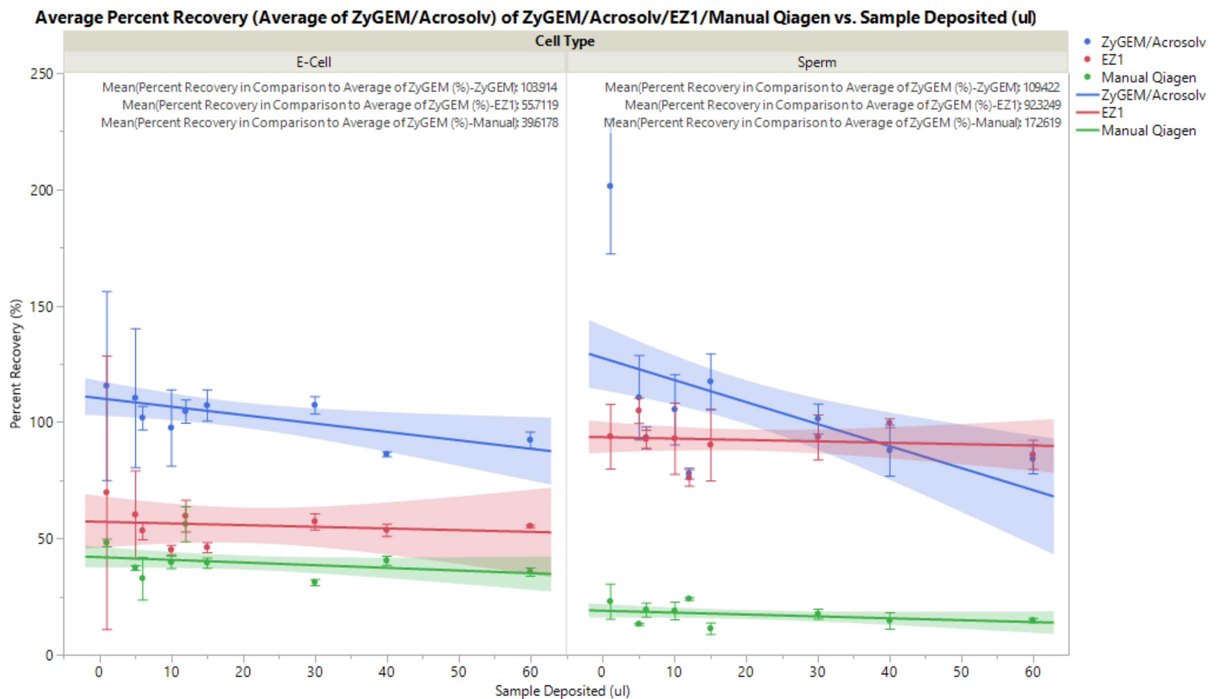



**Figure 11:** Average Percent Recovery Comparison of EZ1, ZyGEM/Acrosolv and Manual Qiagen Extraction procedures with liquid semen and saliva.

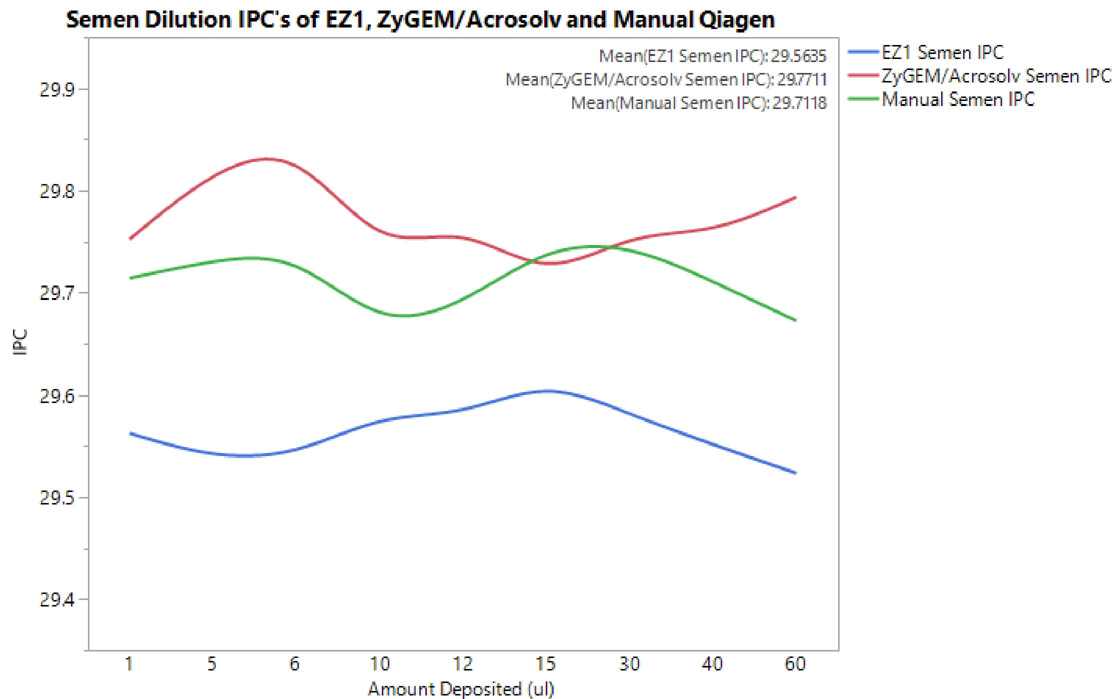



**Figure 12:** Average of the dilution semen series IPC values of the EZ1, ZyGEM/Acrosolv and manual Qiagen extraction.

3.1.8 Comparison of Data Using both Percent Recovery Calculation Methods.

As previously mentioned, there are two ways to calculate the denominator of the equation when calculating percent recovery. The graph below (**Figure 13**) represents how the percent recoveries differ in regards to the EZ1 extraction when the different methods of calculations are performed. The graph depicts that with lower concentrations of DNA there is more variation or a higher standard deviation. The trend lines demonstrates that with method 2 (Individual of ZyGEM) the line (percent recovery) increases while the concentration increases while with method 1 (Average of ZyGEM) the line (percent recovery) decreases. This further shows how important it is to be consistent with how percent recovery is reported and how different studies can report differing results with the same type of samples.

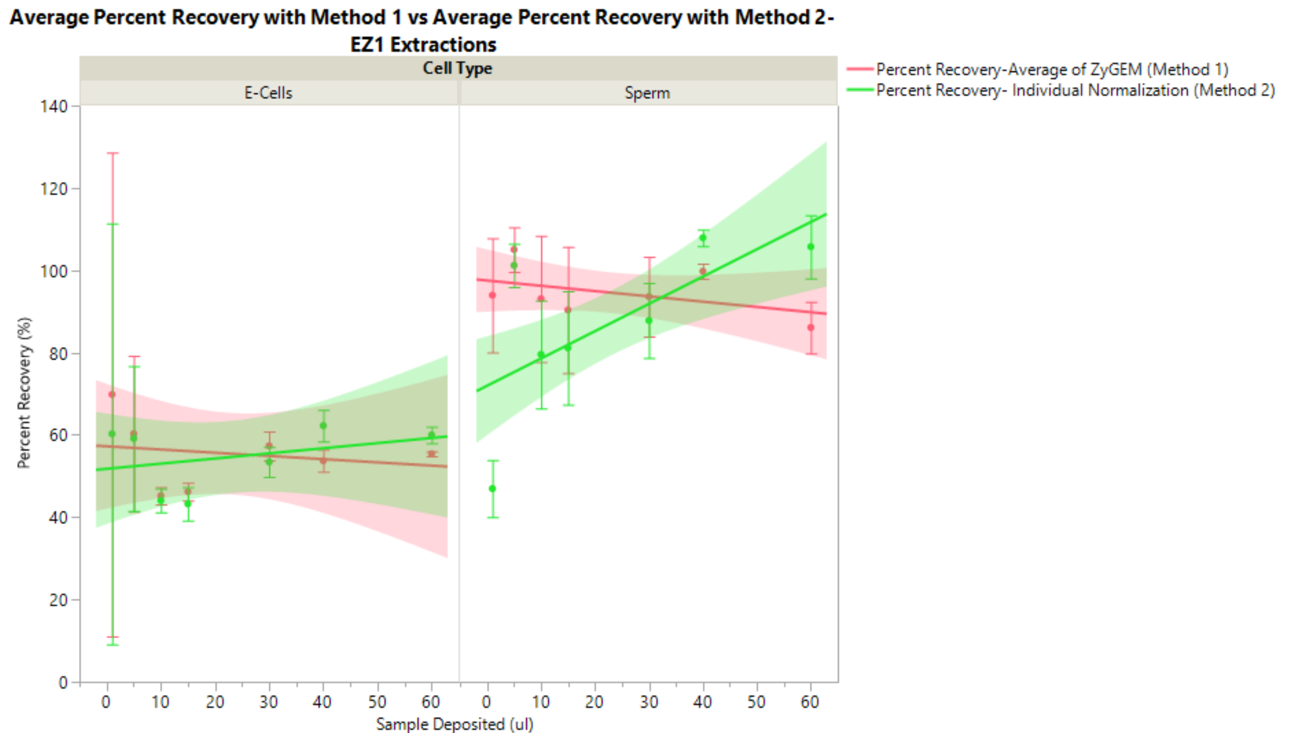



**Figure 13:** Average Percent Recovery Average of ZyGEM/Acrosolv samples (Method 1) calculation and Average Percent Recovery Individual Normalization of ZyGEM/Acrosolv samples (Method 2) Comparison.

### 3.1.9 Normalization to the Mass of 1 µL of Liquid Saliva or Liquid Semen

The last aspect investigated was how consistent were all the extractions when they were back calculated or normalized to 1ng/ µL. They should be all around the same value if the extraction method performed efficiently no matter how much DNA was present on the item. The graph below (**Figure 14**) shows that with the EZ1 method, the average was 1.1ng with e-cells and 1.00ng with sperm cells while the ZyGEM/Acrosolv method had 2.2ng with e-cells and 1.30ng with sperm cells. Both of these methods performed quite well but as the graph shows, the EZ1 method is more consistent in regards to extraction uniformity than the ZyGEM/Acrosolv method. The ZyGEM/Acrosolv method again has

a downward trend meaning that it is not recovering everything when larger amounts of cells are deposited (**Figure 14**).

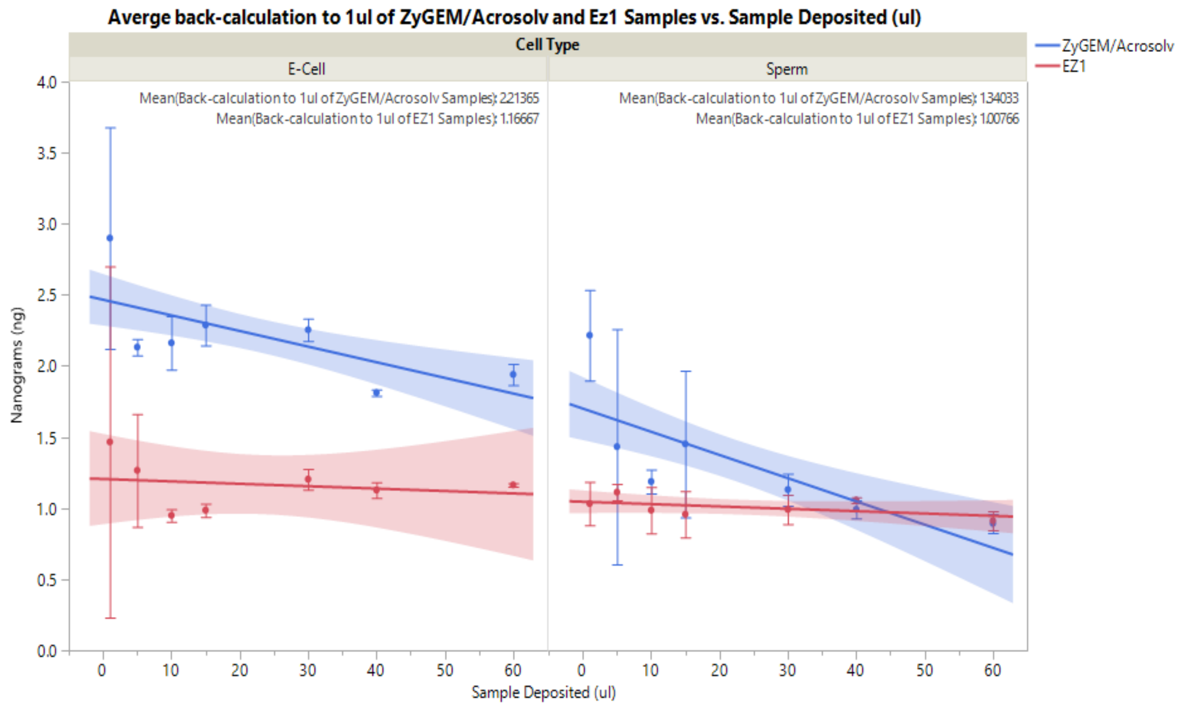



**Figure 14:** The average normalization or back-calculations of all quantification sample values to 1 ng/µL to find consistency in the extraction method.

### 3.2 Capillary Electrophoresis STR Profile Analysis

Selected samples were chosen to generate STR profiles using the GlobalFiler™ amplification kit on the 3130 genetic analyzer and the GeneMapper ID-X® software (25). Different concentrations of DNA from EZ1 and ZyGEM/Acrosolv samples were chosen to compare the average peak height ratios generated from the profiles. Equal known concentrations of DNA from the different extraction methods were amplified. The results showed that the EZ1 and ZyGEM/Acrosolv samples generated profiles with good peak height ratios (**Appendix**). The peak height ratios ranged from 75% to 91% among the extraction methods so it can be concluded that they amplify in a similar manner (data not

shown). Only one ZyGEM sample resulted in a highly degraded profile which was not included in the peak height average calculations. Select samples of EZ1 and ZyGEM/Acrosolv extractions were chosen and plotted against each other depending on fluid tested. Either 1ng or 0.1ng amplified mass was used to compare for each method. The sperm cell DNA graph (**Figure 15**) showed that with both extraction methods the peak heights were comparable. Additionally, the EZ1 sample peak heights were more clustered in comparison to the Acrosolv extraction peak heights. Therefore, there is less variation between the two peak heights with the EZ1 extraction method. When comparing 1ng and 0.1ng mass, there is no significant difference but as expected there were higher peak heights with 1ng on the Acrosolv method. This was not the case with the EZ1 method. It showed that there were higher peak heights with 0.1ng but less variation with 1ng.

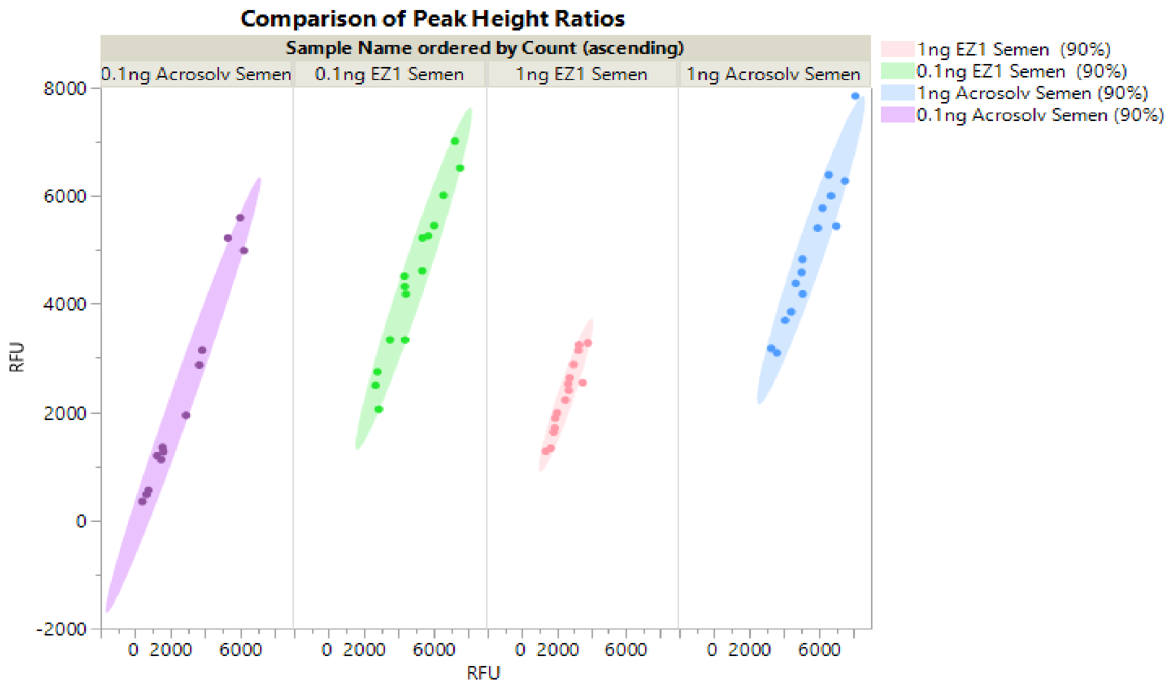



**Figure 15:** The Comparison of Peak Heights of 1ng and 0.1ng Amplification EZ1 and Acrosolv Semen Samples.

The same experiment as mentioned above was performed with e-cell DNA. This graph shows that again, both extraction methods are comparable. But, there is a slight variation between the two peak heights with the ZyGEM extraction method in comparison to the EZ1 extraction method (**Figure 16**). When comparing 1ng and 0.1ng mass, there is no significant difference with both extraction methods but again, the EZ1 amplified at 1ng showed lower peak heights when compared to 0.1ng EZ1.

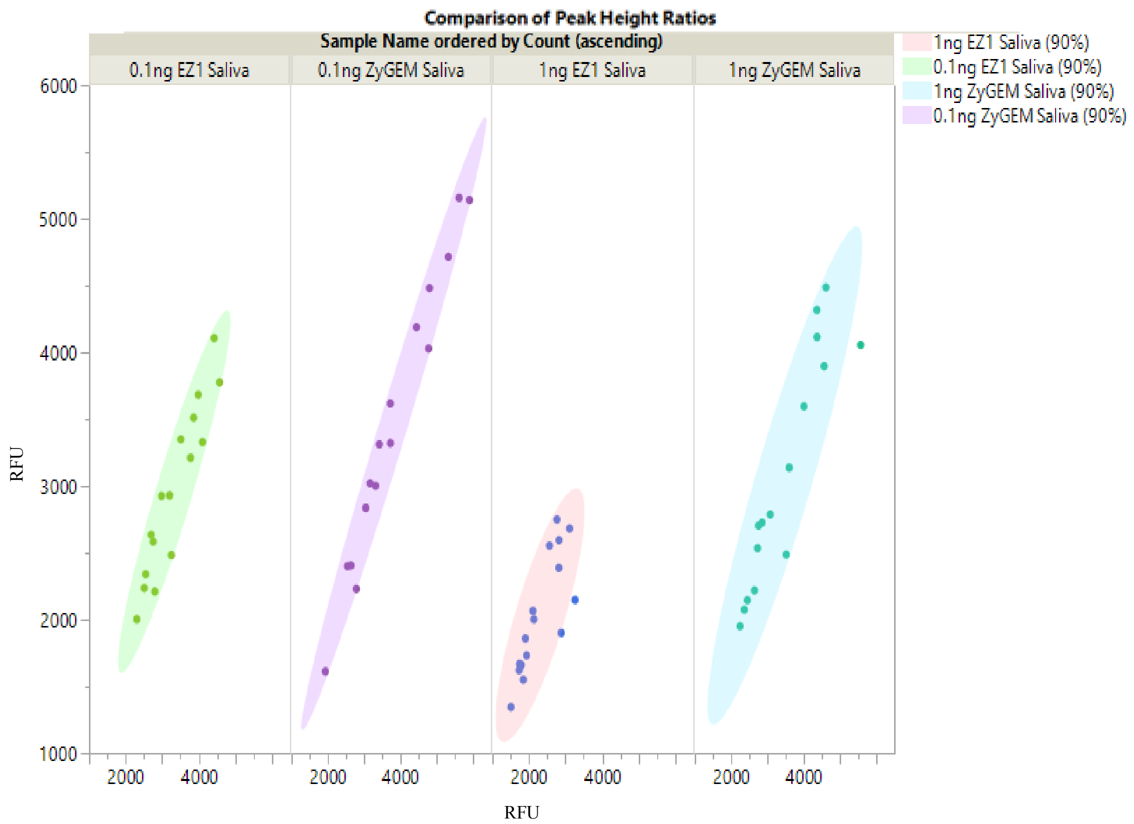



**Figure 16:** The Comparison of Peak Heights of 1ng and 0.1ng Amplification EZ1 and Acrosolv Saliva Samples.

# 4. CONCLUSIONS AND FUTURE RESEARCH

## 4.1 Conclusions

The first step of forensic DNA analysis is extraction. The extraction step isolates DNA present on a piece of evidence. There are many different extraction methods that a forensic DNA crime laboratory can choose but the method must be reliable, reproducible, fast and most importantly, generate a high percent DNA recovery. The DNA must also be clean enough to be able to amplify using PCR. Some extraction methods like the phenol/chloroform method recover DNA well but are known to also amplify PCR inhibitors. This may lead to having to dilute the samples to obtain more "clean" or profiles that have fewer artifacts present. Therefore, extraction method that extracts less DNA may be actually more favorable. But, when an extraction method is validated, the laboratory might not calculate the percent recovery but rather just generate an electopherogram to see if the profile generated has no degradation, no dropout and has good peak height ratio. The percent recovery of an extraction method should always be taken into consideration because the piece of evidence might contain more DNA than if reflected in the quantification results. During validation, forensic laboratories should study how much DNA is lost when the extraction step is performed.

The three extraction methods that were tested during this study were the Qiagen BioRobot EZ1, ZyGEM/Acrosolv, and the manual Qiagen extraction method. The main condition being tested was how well the extraction method recovered the DNA and reduced loss. It was determined that the ZyGEM/Acrosolv extraction method extracted the most DNA and reduced loss when compared to the other extraction methods, in all

areas tested. The ZyGEM/Acrosolv method outperformed the other methods especially when different biological fluids were tested. The EZ1 and the manual Qiagen extractions seemed to have one biological fluid that outperformed the other. The EZ1 has better recovery with semen while manual Qiagen generated higher recoveries with saliva. This should not be the case because the nucleic acids are the same after extraction no matter what biological fluid has been extracted. The chemistry behind both Qiagen methods might not be optimal in regards to any biological fluid. With the ZyGEM/Acrosolv extraction, this was not that case. The only inadequate trend with the ZyGEM/Acrosolv method was that when a higher amount of DNA was present, the recovery decreased. This shows that the ZyGEM/Acrosolv recovery might not be consistent regardless of how much DNA is present. This phenomenon might be caused by an exhaustion of the enzyme activity and if this point is met, no more DNA would be able to be recovered.

A surprising result was the fact that the manual Qiagen extraction had very low percent recoveries in comparison to the EZ1. This could be due to analyst pipetting errors or because the methodology behind the manual Qiagen is not as good as the magnetic bead mechanism of the EZ1. These large variations in the two methods show how inconsistent manual extractions are.

The other aspects of extraction methods that were investigated were the elution and digest volumes. The elution volume is the amount of reagent the DNA is eluted in after extraction and the digest volume is the volume of reagents used for the DNA extraction. Different elution/digest volumes could change the percent recovery so it is important to choose the optimal elution/digest volume for each extraction method. This is

because it is known that using larger elution volumes will dilute the DNA and will generate lower mass recoveries during qPCR. When two digest volumes were tested for the ZyGEM/Acrosolv method, it was determined that 100 µL generated a higher percent recovery in comparison to 20 µL. Three elution volumes were tested for the EZ1 method and it was determined that each volume had about the same percent recovery. The theory behind different elution volumes affecting recovery did not apply to the EZ1 elution study and had an opposite result for the ZyGEM digests. The hypothesis for the ZyGEM/Acrosolv results might be because more enzymes are required to efficiently break open the cells and recover the DNA. Therefore, for the EZ1 method, the elution volume might not affect the recovery significantly while digest volume affects the ZyGEM/Acrosolv method greatly.

One step that the EZ1 method contains but the ZyGEM/Acrosolv method does not is an incubation period. The incubation period for the EZ1 method occurs in a water-bath to activate the enzymes to break open the cells. The ZyGEM/Acrosolv method does not contain a water bath incubation step before extraction because every step occurs in a thermocycler. Three incubation times (3, 5 and 10 hours) were tested with the EZ1 method to determine if altering the incubation time affects the percent recovery on fabric stains. It was determined that with both biological fluids, lower amounts of fluid generated higher percent recoveries and between 3-5 hours seemed to produce better recoveries with both fluids and after 5 hours the recoveries decreased. This could be due to DNA degradation due to the exhaustion of the enzymes. The semen samples had a greater percent recovery in comparison to saliva, which suggests that the EZ1 mechanism

might favor sperm cells. This experiment also showed that when ZyGEM/Acrosolv was used on fabric in the same manner as the EZ1 method, the ZyGEM/Acrosolv method outperformed the EZ1 in percent recoveries at any incubation time and in both biological fluids.

The normalization to 1 ng/µL demonstrated how consistent the extraction method is regardless of how much DNA is deposited on an item. It was determined that the EZ1 method extracted the DNA more uniformly compared to the ZyGEM/Acrosolv method. As more DNA is present in the sample, the percent recovery with ZyGEM/Acrosolv decreased while the EZ1 method was more consistent. This could be more evidence that supports the exhaustion of the enzyme activity of the ZyGEM/Acrosolv method being reached while the EZ1 method does not have this issue. The IPC values from the qPCR were compared for each extraction method and demonstrated that while the EZ1 method had the lowest IPC values, they were all very close to each other. Therefore, each method extracts DNA cleanly and has high purity.

It is also important how the percent recovery is calculated. There were two ways the percent recovery could have been calculated in this research (method 1 and method 2). The reason why both methods were shown was that it is extremely important to calculate the percent recovery correctly when presenting how well an extraction method performs. When investigating validation studies on an instrument or protocol, the analyst must understand the way percent recovery is calculated and recognize the differences when comparing to other validation studies. As previously discussed, it was determined for this study that the average percent recovery of the ZyGEM/Acrosolv samples was

used as the "standard" or "known" starting amount of DNA present. But, it was also important to show that other methods of comparison could be chosen because different ways of reporting percent recovery could lead to different conclusions.

The profiles generated from selected EZ1 and ZyGEM/Acrosolv samples produced good peaks heights with good peak height ratios, excluding the one degraded ZyGEM sample. It was also concluded that the EZ1 method had slightly less variability between sister alleles (peak height ratios) across all loci for each sample tested. This verifies that both extraction methods are effective for forensic purposes. Another factor investigated was to determine if higher sample concentrations was amplified would it produce more artifacts. This turned out to be true in both extraction methods tested. It was shown that when higher concentrations were deposited it did result in more robust peaks and better peak height ratios, but it also lead to more artifacts being produced. Artifacts could be misidentified as a second contributor therefore it is important to find a balance of how much sample should be deposited into the amplification reaction.

Lastly, the cost, analysis time, and ease of use were evaluated for each method. These aspects are extremely important in forensic laboratories because they are usually government funded, and have many samples that need to be processed in a timely manner. The ZyGEM/Acrosolv method was the front-runner in each of these categories. The time for the ZyGEM/Acrosolv method to be completed is between 12-20 minutes for liquid samples in comparison to 75-135 minutes for the EZ1 method and 100-160 minutes for the manual Qiagen extraction (depending on the analyst). For ease of use, it was clear that the ZyGEM/Acrosolv protocol was the easiest to follow and perform when

compared to the other two methods. The one tube aspect of the protocol is the most efficient and most important part of the ZyGEM/Acrosolv method when it comes to ease of use. The ZyGEM/Acrosolv method also has the least amount of analyst interaction so the reproducibly should be the best. The cost of one EZ1 investigator kit (not including the one-time cost of the investigator card) is $495.00 for 48 samples (16). The ZyGEM SexCrime kit costs $600.00 for 100 reactions and the ZyGEM Universal Kit costs $400.00 for 100 reactions (21). The manual QIAamp DNA Investigator Kit cost is $255.00 for 50 samples (19). But this method requires most of the analyst time so Qiagen kit may be less expensive in cost per sample compared to the other kits but the analyst must be present at the extraction at all times so sample efficiently decreases. So, again the ZyGEM/Acrosolv outperforms the other two methods when it comes to cost per sample.

After performing each method and understanding how each one works, it can be concluded that the ZyGEM/Acrosolv extraction method outperforms the EZ1 and manual Qiagen extraction methods in almost every aspect tested in this research. Even though the EZ1 did not outperform the ZyGEM/Acrosolv method, it does not mean that the EZ1 bio-robot does not perform well. The EZ1 still produces good, reliable data however; the overall data obtained from the ZyGEM/Acrosolv method shows it is a robust extraction method and should be implemented in more DNA forensic laboratories in the United States because it will increase productivity and reliability.

**4.2 Future Research**

More research should be done in the future to gather more data for this research topic. One of the main aspects that should be investigated further is if these extraction

methods perform in the same manner when blood is the biological fluid being extracted and if other sample types such as cigarettes butts or swabs extract well regardless of the substrate. Blood and other biological samples were not tested in this study based on time restrictions. A larger pool of samples for each method should also be tested to fully ensure the starting mass is as accurate as possible and to determine the percent recovery precisely. A larger sample size will also ensure confidence in the data that proves what extraction method actually recovers better. If more or higher mass samples are tested then the exhaustion of the enzyme point of the ZyGEM/Acrosolv method could be found.

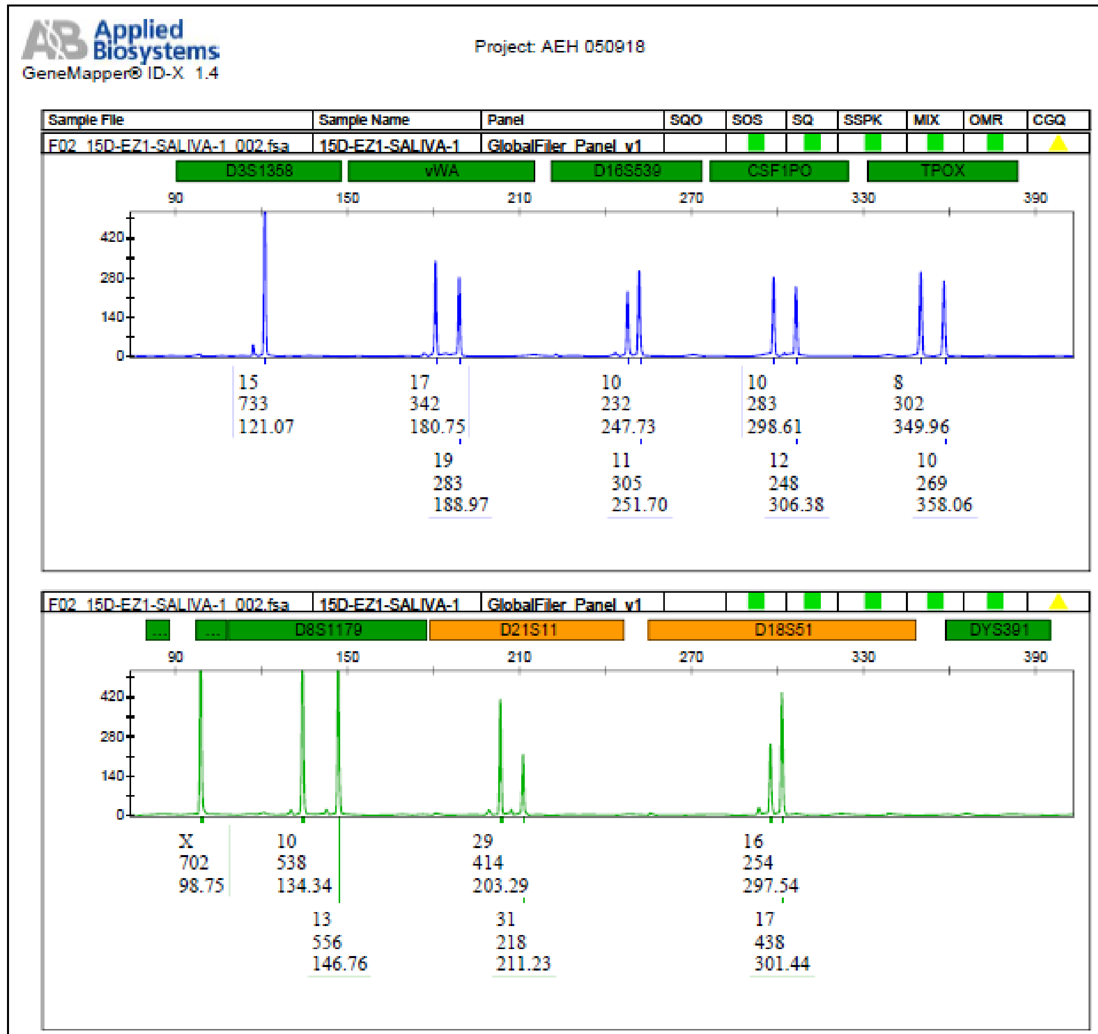
Other ways to calculate the starting mass should also be investigated in the future to demonstrate how different the percent recoveries could be depending on how the starting mass is calculated. Other ways of quantification could also be looked at in the future like using the Nanodrop® to determine what the A260 value is in comparison to the qPCR value. All of the information gathered with different quantification methods would give a better insight on the starting total mass or concentration of DNA in a sample. This will also highlight how differences in calculation methods will determine different conclusions even if all the other variables of the study were kept constant.

For the incubation and elution/digest volume experiments, more time points and volume amounts should be tested to determine the optimal time and volume for each extraction method. This would allow for less variation between analysts or laboratories if one digest/elution volume and incubation time was chosen per biological fluid being tested.

The QIAamp Investigator kit could be tested more with various body fluids to determine if that method truly results in recoveries consistently that low. This would also explain how much variation that protocol is susceptible to when multiple analysts are performing the extractions and further illustrates how important bio-robots are to implement in a laboratory.

Lastly, there are many other extraction methods available for use in DNA forensic laboratories. To really understand the best extraction method in regards to percent recovery, cost, time and ease of use, more extraction methods should be tested for comparison. Other extraction methods that should be tested against the manual Qiagen are the automated version of that protocol on the QIACube® and the manual ThermoFisher PrepFiler® Forensic DNA Extraction Kit, which uses magnetic bead technology like the EZ1. This will give more insight as to why the manual Qiagen extraction recoveries were so low in comparison to the EZ1. Using the QIACube and PrepFiler might show if it is the chemistry behind the silica membrane or if operator error is producing the low recoveries, but this could be a limitation based on the cost of the extraction kits to conduct these studies. In the future, if more students work on different extraction methods, all of the research could be compiled and analyzed in the same manner to find which extraction method performs the best.

## APPENDIX:



Applied Biosystems
GeneMapper® ID-X 1.4

Project: AEH 050918

| Sample File | Sample Name | Panel | SQO | SOS | SQ | SSPK | MIX | OMR | CGQ |
|---|---|---|---|---|---|---|---|---|---|
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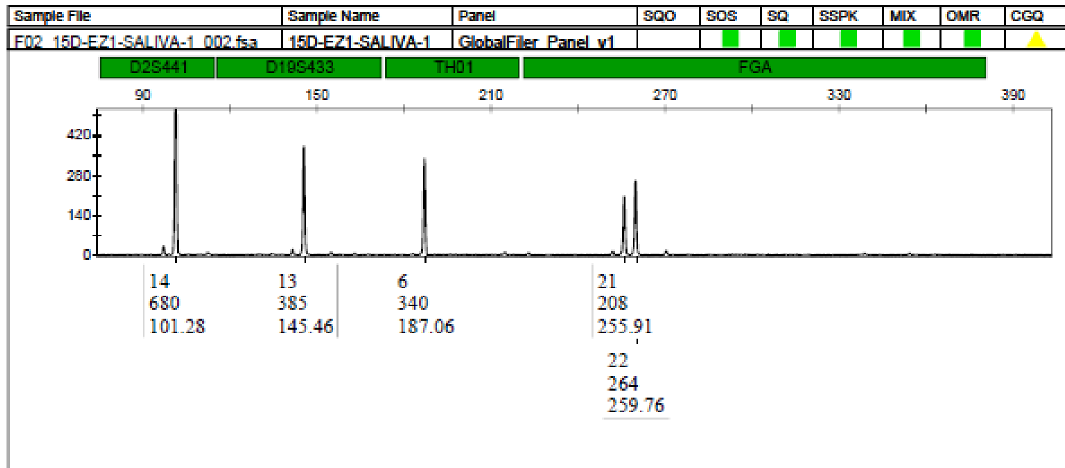


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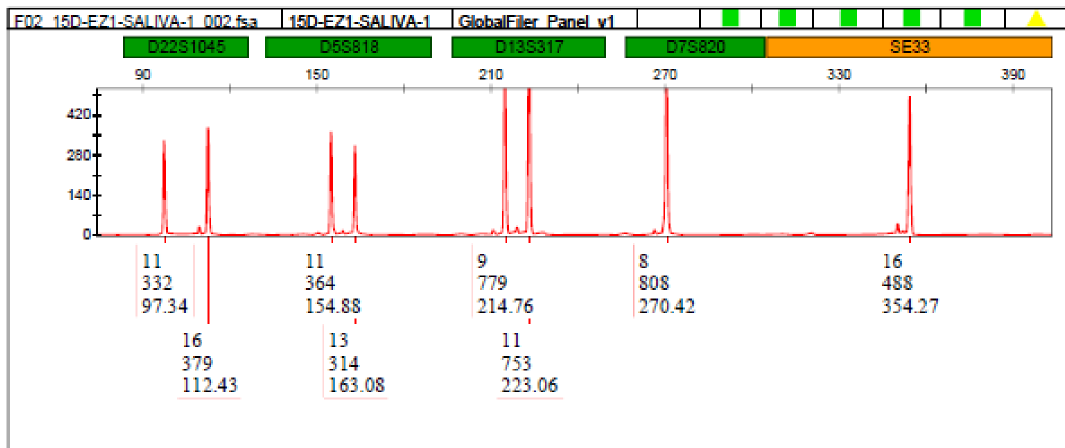

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GeneMapper® ID-X 1.4

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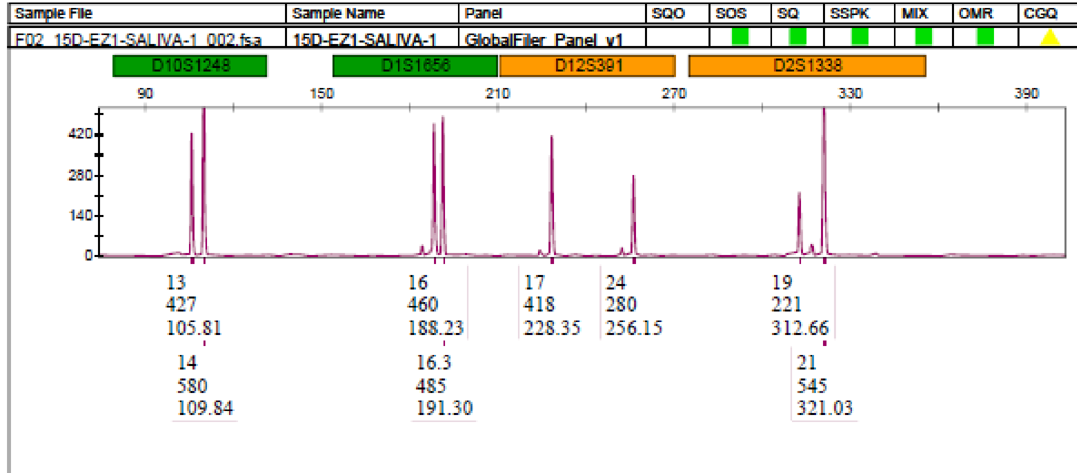


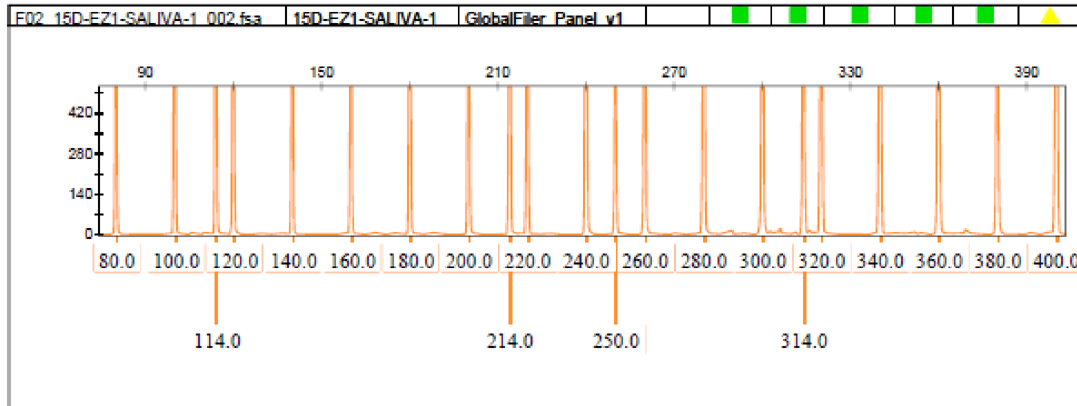


**Figure A**: EZ1 Saliva 15D sample GeneMapper ID-X 1.4 electopherogram. The sample concentration is 0.1ng/ul and the amplification mass is 0.1ng.

Applied Biosystems
GeneMapper® ID-X 1.4

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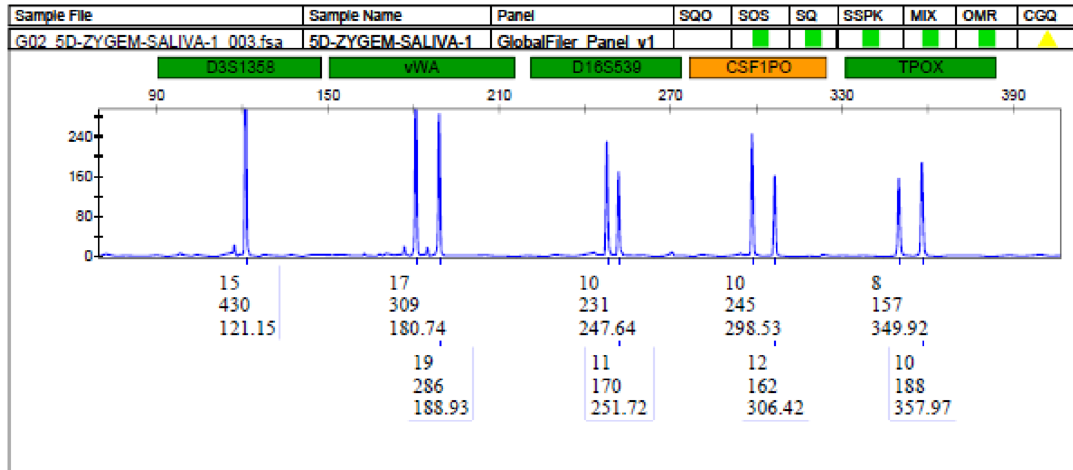


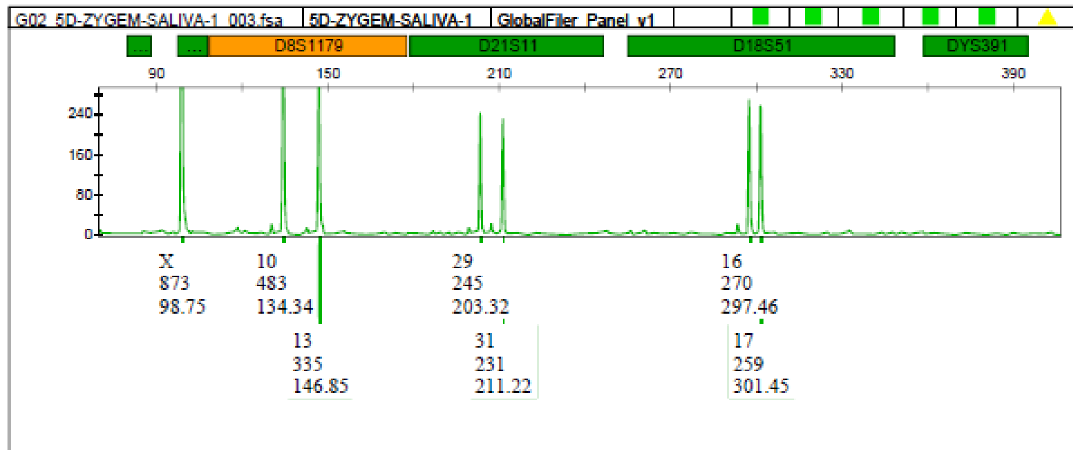

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GeneMapper® ID-X 1.4

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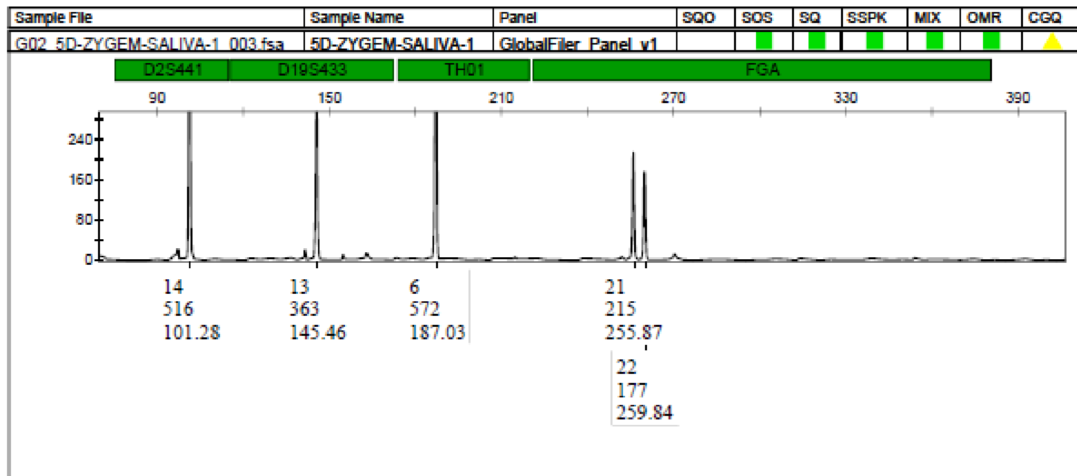


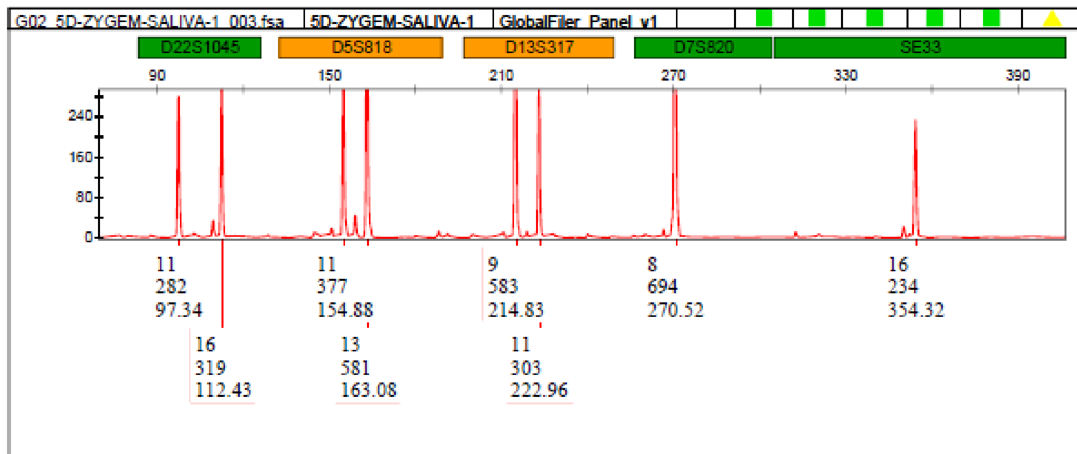

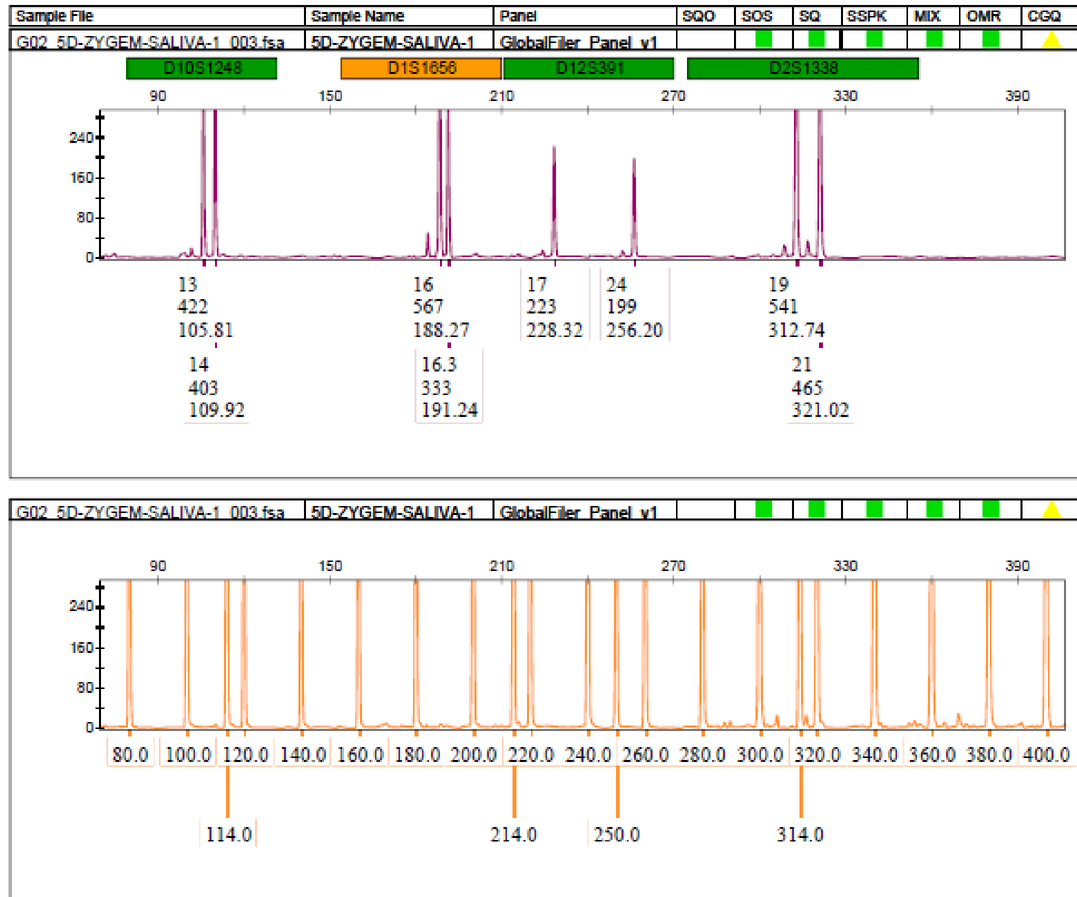


**Figure B**: ZyGEM Saliva 5D sample GeneMapper ID-X 1.4 electopherogram. The sample concentration is 0.1ng/ul and the amplification mass is 0.1ng.

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